The Effect of Compounds of the Urea–Guanidinium Class on the Activity Coefficient of Acetyltetraglycine Ethyl Ester and Related Compounds[1]

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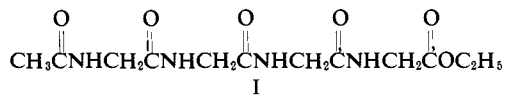
The effects of several protein denaturing agents on the activity coefficients of acetyltetraglycine ethyl ester (ATGEE), three carbobenzoxyglycine derivatives, toluene, and benzyl alcohol in aqueous solution have been determined by solubility measurements. Urea (8 M) and 7 M guanidine hydrochloride cause a 3.2- and 7.5-fold increase in solubility, respectively, of ATGEE, a compound composed principally of amide groups. This is not a "hydrophobic" effect because (i) alkyl substitution of urea and guanidine hydrochloride eliminates their ability to solubilize ATGEE, but increases their ability to solubilize nonpolar compounds; (ii) solvents for nonpolar compounds, such as ethanol and dioxane, have little or no effect on ATGEE; (iii) the activity coefficient effect of urea on ATGEE, in contrast to that on hydrocarbons, decreases with increasing temperature from 0 to 40°. The activity coefficients of carbobenzoxyglycineamides are decreased by both unsubstituted and alkyl-substituted ureas and guanidine hydrochlorides, which suggests that both "hydrophobic" and "nonhydrophobic" effects are significant with these compounds. The activity coefficient effect of urea displays little sensitivity to the number of glycine units in a series of short carbobenzoxyglycine peptides, i.e., a simple additivity relation, which might be expected to result from nonspecific solvent effects, does not hold in this case. The relative effectiveness of different denaturing agents in decreasing the activity coefficient of ATGEE generally parallels their effectiveness in denaturing bovine serum albumin and some other proteins. It is concluded that a "nonhydrophobic" effect of denaturing agents of the urea–guanidinium class makes a major contribution to their denaturing effectiveness toward proteins by decreasing the activity coefficients of exposed amide and peptide groups in the denatured protein. Similar conclusions are drawn for the effects of these compounds on the solubility and dissociation into subunits of proteins. The mechanism of the "nonhydrophobic" effect has not been established, but the results are consistent with a direct interaction of the denaturing agent with ATGEE to form a complex, with an association constant of 0.29 for urea and 0.90 for guanidinium chloride at 25° and a heat of association on the order of −2800 cal./mole for the urea complex.

Introduction

Although urea and related compounds have long been known to denature proteins, the mechanism of this effect remains unclear. It was once widely assumed that urea denatures proteins by its ability to break hydrogen bonds.[2] Subsequent work, however, has suggested that monofunctional hydrogen bonds between amide groups have little or no stability in aqueous media[3] and that the denaturing effectiveness of different compounds toward proteins does not follow the order expected for simple, monofunctional hydrogen bond formation.[4] Urea has been shown to increase the solubility of nonpolar compounds in aqueous solution, and it has recently been suggested that this hydrophobic effect contributes to the denaturation of proteins by urea.[5–8] However, other evidence indicates that with some proteins the denaturing ability of urea cannot be attributed solely to its hydrophobic effects.[4,9]

The experiments reported here were carried out in an attempt to determine whether urea and related compounds interact with the peptide and amide groups of proteins in a manner which would contribute to the denaturing action of these compounds. To this end, the effect of a series of denaturing agents on the solubility and activity coefficient of acetyltetraglycine ethyl ester (ATGEE, I), an uncharged compound composed



I

principally of amide groups and a model for the peptide and amide groups of proteins, was examined. It has been shown previously that urea causes a moderate increase in the solubility of diketopiperazine and that this effect is larger at lower temperatures.[10,11] In a preliminary communication, Whitney and Tanford reported unpublished experiments which showed that the solubility of the side chain of asparagine is increased in urea (but not ethanol) solutions and suggested that polar groups of proteins may to some extent be hydrophobic.[7] The experiments with ATGEE, which have been reported in preliminary communications,[12] demonstrate a large effect of compounds of the

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urea–guanidinium class on the activity coefficient of the peptide group, which is not a hydrophobic effect, and lead to the conclusion that this nonhydrophobic effect makes a major contribution to the denaturing action of such compounds toward proteins. More recently, Nozaki and Tanford have presented evidence that urea decreases the activity coefficient of peptide and amide groups[8] and Tanford has attempted to account quantitatively for the denaturing effect of urea on proteins from the results of experiments on model compounds.[13] Heat capacity and enthalpy measurements on peptides suggest that the enthalpy of interaction of urea and the peptide bond is negative and that the interaction is accompanied by a decrease in heat capacity, which may result from the freeing of water molecules.[14]

Experimental

Preparation of Acetyltetraglycine Ethyl Ester (ATGEE). To 1.0 g. of recrystallized tetraglycine ethyl ester hydrochloride[15] in 100 ml. of 40% aqueous pyridine, 1.9 ml. of redistilled acetic anhydride was added slowly at 0° with stirring. The volume of the solution was reduced to about 50 ml. under vacuum and the product began to crystallize. After storage overnight at 2°, the product was obtained in about 70% yield. It was recrystallized from water and dried over phosphorus pentoxide under vacuum, m.p. 264° dec. (uncor.) *Anal.* Calcd. for $C_{12}H_{20}N_4O_6$: C, 45.56; H, 6.37; N, 17.71. Found: C, 45.67; H, 6.37; N, 17.87. The infrared spectrum shows strong bands at 3270, 3070, 1753, 1670, 1635, 1558, 1203, and 1027 cm.$^{-1}$.

Other compounds were obtained commercially and were recrystallized or redistilled at least once before use. Ethanol and acetone were reagent grade materials and were used without further purification. Glass-distilled water was used throughout.

Solubility Determinations. An excess of the solid or liquid material was placed with solvent in screw-capped tubes, which were sealed with a Teflon liner and dipped in paraffin. Mixing was accomplished by turning the tubes end over end in a rotating rack at 25.00 ± 0.05, 40.00 ± 0.10, or 0 ± 0.05°.

To show that equilibrium had been reached the following two methods were used. In the first method one of two identical tubes was supersaturated with solute by warming. It was then equilibrated at the desired temperature, so that equilibrium was approached from a supersaturated solution. The duplicate sample was equilibrated directly at the desired temperature. In the second method, the concentration of solute was redetermined after a second period of equilibration of 24–48 hr. It was found that in all cases equilibrium was reached within 20 hr. at 40°, 72 hr. at 25°, and 7 days at 0°. Each reported solubility, except for toluene, is the average of two determinations which agree to within 5%. In every case the attainment of equilibrium was demonstrated by one of these two methods. Toluene values represent single determinations after incubation for 72 hr.

After equilibration the phases separated readily upon standing in the thermostat for periods up to 24 hr. In early experiments, rapid filtration or centrifugation was done, but was found to be unnecessary. Equilibration of carbobenzoxytriglycineamide (Cbz-gly$_3$-NH$_2$) in water and urea solutions resulted in a fine suspension which did not separate from the solution on standing. This was prevented by the addition of 0.02 *M* acetate buffer, pH 4.8, to solutions of Cbz-gly$_3$-NH$_2$. Solutions of other solutes were carefully examined for turbidity and none was found.

Assay of Solutions. The concentration of ATGEE was determined by the biuret method.[16] The absorbance at 540 mμ was found to be proportional to the concentration of ATGEE over the range of concentration used in these experiments A standard solution of tetraglycine ethyl ester hydrochloride was assayed in the presence of each solvent. The expected absorbance at 540 mμ due to this peptide was obtained by subtraction of the absorbance due to the solvent alone, except in the case of guanidine hydrochloride solutions, which reduced the expected absorbance of the peptide up to 25%. Therefore, the absorbance of standard solutions of ATGEE was determined in the presence of each concentration of guanidine hydrochloride.

The solubility of ATGEE in water at 25.0° is 0.78 g./l. This value is the average of 20 determinations done over the course of these experiments, with a range of 0.77–0.79 g./l. The solubility was found to remain constant when the amount of solid phase remaining at equilibrium ranged from 20 to 200% of that required to reach saturation.

The concentrations of carbobenzoxy peptides, benzyl alcohol, and toluene were determined from the difference between the absorbance at 263 and 275 or between that at 257 and 275 mμ. The differences in absorbance at the same wave lengths for the solvents alone were subtracted, and accounted for <20% of the total difference in absorbance of the solute plus solvent, except in the case of 3 *M* guanidine hydrochloride, which accounted for approximately 50% of the total difference in absorbance. The benzyl alcohol solutions were diluted 1:250 prior to the readings, so that solvent contributions were negligible. The absorbance in water of a standard solution of Cbz-gly$_3$-NH$_2$ at 257, 266, and 275 mμ was not significantly affected by 4 *M* urea or 3.5 *M* guanidine hydrochloride. Cbz-gly$_3$-NH$_2$, Cbz-gly$_2$-NH$_2$, and benzyl alcohol were found to have values of 200, 200, and 186 M^{-1} cm.$^{-1}$, respectively, for the difference in absorbance $A_{257} - A_{275}$.

The concentrations of ethyl acetate solutions were determined by the hydroxamic acid method.[17]

Results

Presentation of Results. Solubilities are expressed directly as the solubility in g./l., *S*, or as the solubility ratio S/S^0, in which *S* is the solubility in a given solvent and S^0 the solubility in water. Activity coefficients are

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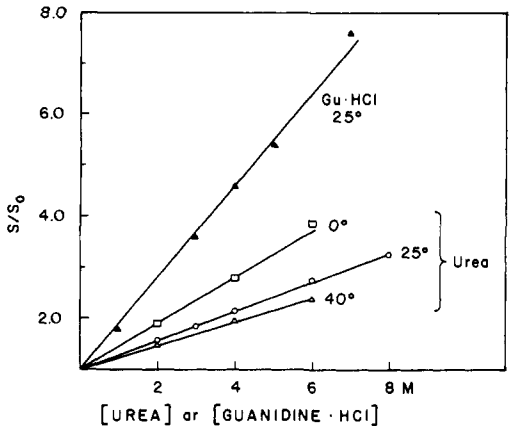

Figure 1. Solubility of ATGEE in urea solutions at 0, 25, and 40°, and in guanidine hydrochloride solutions at 25°.

taken from the relation

$$\gamma = \frac{S^0}{S} \qquad (1)$$

based upon the convention that the activity coefficient of the solute in pure water is 1.0.[18] This is different from the convention adopted by Nozaki and Tanford,[8] but leads to the same conclusions regarding free energies of transfer, for a given concentration scale. Self-interaction effects of the peptides are assumed to be negligible because the final concentration of the uncharged peptides was always less than 0.02 *M*, except for Cbz-gly-NH_2, which was less than 0.06 *M*.

ATGEE. Urea and guanidine hydrochloride cause a large increase in the solubility of ATGEE (Table I). Urea (8 *M*) and 7 *M* guanidine hydrochloride cause a 3.2- and 7.5-fold increase in solubility, respectively. The increase in solubility (or decrease in activity coefficient) is approximately linear with respect to the concentrations of these compounds (Figure 1). The effect of urea on ATGEE decreases with increasing temperature over the range 0–40°.

Table I. Solubility of Peptides in Urea and Guanidine Hydrochloride Solutions[a]

Solvent	*M*	ATGEE at 0°	ATGEE at 25°	ATGEE at 40°	Cbz-gly_2-NH_2 at 25°
Water	–	0.26	0.78	1.65	1.18
Urea	2	0.49	1.21	2.46	1.9
	3		1.43		2.6
	4	0.72	1.68	3.22	3.2
	6	1.00	2.15	3.94	4.5
	8		2.54		5.9
Guanidine hydrochloride	1		1.4		
	3		2.8		
	4		3.6		
	5		4.2[b]		
	7		5.9		

[a] Solubility expressed in g./l. of solution. Each number is the average of two determinations. [b] Single determination.

The solubility of ATGEE in a number of other solvents at 25° is shown in Table II. Alkyl substitution progressively decreases the solubilizing effectiveness of urea, and tetramethylurea actually causes a *decrease* in the solubility of ATGEE. Similarly, 1,1,3,3-tetramethylguanidine hydrochloride causes a decrease in ATGEE solubility. The small solubilizing effect of simple amides is also reversed by substitution of methyl groups for hydrogen on the amide nitrogen atom.

Simple organic solvents which do not contain aromatic or N–H groups, such as dioxane, ethanol, acetone, and tetrahydrofuran, have little effect on ATGEE solubility. Ethylamine hydrochloride causes a decrease and acetic acid causes an increase in solubility.

Carbobenzoxyglycine Peptides. The effects of several solvents on the solubilities of carbobenzoxydiglycineamide (Cbz-gly_2-NH_2), toluene, and benzyl alcohol are shown in Table III. Urea, guanidine hydrochloride, and formamide cause moderate increases in the solubility of toluene and benzyl alcohol. Alkyl substitution of these compounds markedly *increases* their solubilizing effectiveness toward toluene and benzyl alcohol.

The solubility of Cbz-gly_2-NH_2 is markedly increased by urea, guanidine hydrochloride, and formamide (Tables I and III). In contrast to the situation with ATGEE, alkyl substitution of these reagents either has little effect or increases their solubilizing effectiveness. Furthermore, dioxane and ethanol also increase the solubility of Cbz-gly_2-NH_2.

The effect of 8 *M* urea on the solubility of a series of carbobenzoxyglycine derivatives is shown in Table IV. An increase in the number of glycyl groups in the molecule from one to three has very little influence on the solubilizing effect of 8 *M* urea. Substitution of a free carboxylic acid for an amide group results in a larger solubilizing effect of urea. This suggests that the carboxylic acid group may undergo the same type of interaction as an amide group with aqueous urea, but that the effect is somewhat larger.

Contribution of the Ester Group of ATGEE. The effects of urea and guanidine hydrochloride on the solubility of ethyl acetate at 25° (Table V) are much smaller than on the solubility of ATGEE. This suggests that the ester group of ATGEE makes a relatively small contribution to the effects of these compounds on ATGEE.

Composition of the Pure Solid Phase. It is unlikely that any significant change in the solid phase of the peptides occurred during equilibration with solvents, for the following reasons. (1) The solubilities of ATGEE and Cbz-gly_2-NH_2 increase steadily with increasing urea or guanidine hydrochloride concentrations, with no indication of the leveling off which would be expected upon saturation with respect to a new solid phase. In contrast, the solubility of diketopiperazine approaches a limit at high urea concentrations, associated with the precipitation of a urea–diketopiperazine complex.[11] (2) There was no change in the appearance of crystals of ATGEE or the carbobenzoxy peptides during equilibration with any of the solvents. Crystals of ATGEE were examined microscopically in many cases. (3) The formation of a solid solution is rare with crystalline organic compounds, and usually occurs when the two compounds are isomorphous.[19]

The quantitative interpretation of experiments with benzyl alcohol and toluene is complicated by the appre-

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(19) J. H. Hildebrand and R. L. Scott, "Solubility of Non-electrolytes," 3rd Ed., Reinhold Publishing Corp., New York, N. Y., 1950, p. 303.

Table II. Solubility of ATGEE at 25°

Solvent, 3 M[a]	g./l.	S/S^0[b]	Solvent, 3 M	g./l.	S/S^0[b]
Water	0.78	...	N-Dimethylformamide	0.69	0.89
Urea	1.43	1.85	Acetamide	0.92	1.20
Ethylurea	1.29	1.68	N-Dimethylacetamide	0.62	0.80
Ethyleneurea	1.13	1.46	Propionamide	1.03	1.33
1,3-Dimethylurea	1.09	1.41	2-Pyrrolidone (γ-butyrolactam)	0.92	1.18
Tetramethylurea	0.57	0.74	Ethylamine hydrochloride	0.49	0.63
Thiourea (1.5 M)	~2.0[c]	~2.6	Acetic acid[d]	1.32	1.7
Guanidine hydrochloride	2.7	3.5	p-Dioxane	0.75	0.98
1,1,3,3-Tetramethylguanidine hydrochloride	0.49	0.63	Ethanol	0.65	0.85
Formamide	0.96	1.25	Tetrahydrofuran	0.87	1.13
			Acetone	0.82	1.05

[a] All solvents are 3 M aqueous solutions except thiourea. [b] S^0 = solubility in water; S = solubility in solvent indicated. [c] Solubility determined by adding increasing amounts of ATGEE to 1.5 M thiourea solutions and observing the presence of excess ATGEE at equilibrium. The value reported is approximate to ±10%. [d] Solvent consisted of 3 M acetic acid and 0.3 M potassium acetate and gave an observed S/S^0 = 1.54. This is corrected for the salting out of ATGEE caused by 0.3 M potassium acetate, estimated from the solubility of ATGEE in sodium acetate solutions reported by D. R. Robinson and W. P. Jencks, *J. Am. Chem. Soc.*, **87**, 2470 (1965).

Table III. Solubility of Solutes Containing Nonpolar Groups in Aqueous Solvents at 25°

Solvent, 3 M[a]	Solubility, S/S^0: Toluene	Benzyl alcohol	Cbz-gly$_2$-NH$_2$
Urea	1.48[b]	1.35	2.2
Urea (8 M)	2.38	2.03	5.0
1,3-Dimethylurea	3.45	1.72[b]	3.1
Guanidine hydrochloride	1.70	1.69	4.1
1,1,3,3-Tetramethylguanidine hydrochloride	2.5[c]	*d*	4.3[e]
Formamide	1.72	1.51	1.9
N-Dimethylformamide	3.78	3.55	3.6
p-Dioxane	3.15	2.04	4.2
Ethanol	1.77	1.25	1.4

[a] Solvents are 3 M unless otherwise specified. [b] Single determination. [c] Based on $A_{270} - A_{275}$. [d] Miscible in 2.2 M 1,1,3,3-tetramethylguanidine. [e] Concentration determined by the biuret method.

Table IV. Solubility of Carbobenzoxyglycine Peptides in Water and 8 M Urea at 25°

Peptide	H_2O, g./l.	8 M urea: g./l.	8 M urea: S/S^0[b]	ΔF_{tr},[c] cal./mole
Cbz-gly-NH$_2$	2.75	12.1	4.4	−870
Cbz-gly$_2$-NH$_2$	1.18	5.9	5.0	−960
Cbz-gly$_3$-NH$_2$	0.38[d]	1.9[d]	5.0	−960
Cbz-gly-OH[a]	4.56	40	8.8	−1280
Cbz-gly$_2$-OH[a]	0.75	6.6	8.8	−1280

[a] From the data of Nozaki and Tanford,[8] originally expressed in g./100 g. of solvent. Approximate values for the solubility in g./l. were calculated assuming densities of 1.00 and 1.11 for water and 8 M urea solutions, respectively. [b] S = solubility in 8 M urea; S^0 = solubility in water. [c] $\Delta F_{tr} = -RT \ln S/S^0$, based on molar concentration scale. [d] In the presence of 0.02 M acetate buffer.

ciable solubility in the nonaqueous phase of one component of the aqueous phase. Toluene and benzyl alcohol are both miscible with dioxane, ethanol, tetrahydrofuran, and dimethylformamide. Crystals of 1,3-dimethylurea and 1,1,3,3-tetramethylguanidine hydrochloride were found to be slightly soluble in benzyl alcohol. However, urea and guanidine hydrochloride have little tendency to dissolve in benzyl alcohol and

Table V. Effect of Urea and Guanidine Hydrochloride on the Solubility of Ethyl Acetate at 25°

Concn., M	S/S^0[a]: Urea	Guanidine hydrochloride
2	1.11	1.06
4	1.20	1.15
6	1.24	
7		1.35
8	1.35	

[a] S^0 = solubility in water; S = solubility in solvent indicated. Solubilities measured by conversion to the hydroxamic acid; see ref. 17.

none of the urea–guanidine compounds show significant solubility in toluene. In the cases in which the added organic compound has a significant solubility in benzyl alcohol or toluene, the decreases in activity coefficients estimated from solubility determinations will be smaller than the actual decreases because the activity of the reference phase of the solute will be decreased by addition of solvent.[20] However, this effect appears to be very small or negligible for compounds of the urea–guanidine class.

Discussion

Our interpretation of the results described in this and the following two papers is based upon the assumptions that certain changes in the physical state of proteins are accompanied by changes in the exposure of some parts of the protein to the solvent and that qualitative or semiquantitative conclusions regarding the effects of solvents on the different classes of groups which become exposed to solvent may be drawn from the effects of solvents on model compounds.[6,8,13,21,22] For reversible denaturation, the equilibrium between native and a given species of denatured protein is given by eq. 2, in which K is the equilibrium constant, C, a, and γ refer

(20) J. H. Hildebrand and R. L. Scott, ref. 19, p. 296.
(21) J. F. Brandts, *J. Am. Chem. Soc.*, **86**, 4302 (1964).
(22) (a) M. L. Anson and A. E. Mirsky, *J. Gen. Physiol.*, **17**, 399 (1933–1934); (b) J. A. Schellman, *Compt. Rend. Trav. Lab. Carlsberg*, **29**, 230 (1955); (c) J. A. Schellman, *J. Phys. Chem.*, **62**, 1485 (1958); (d) L. Peller, *ibid.*, **63**, 1199 (1959); (e) C. Tanford, *J. Am. Chem. Soc.*, **84**, 4240 (1962); (f) H. A. Scheraga in "Polyamino Acids, Polypeptides and Proteins," M. A. Stahmann, Ed., University of Wisconsin Press, Madison, Wis., 1962, p. 241.

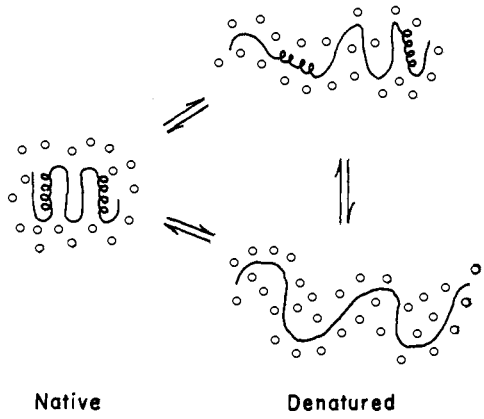

Figure 2. Schematic diagram of the equilibria between a native globular protein in aqueous solution and two denatured states of the protein.

to concentration, activity, and activity coefficient in the molar scale, and the subscripts N and D refer to native and denatured protein, respectively. Activity

$$K = \frac{a_D}{a_N} = \frac{C_D\gamma_D}{C_N\gamma_N} \qquad (2)$$

coefficients are taken as 1.0 in dilute solutions in water. The increase in the concentration of denatured protein in a denaturing solvent may, in most instances, be ascribed to a decrease in the activity coefficient of the denatured protein in such a solvent, relative to water, which is caused by an energetically more favorable interaction of the solvent with those portions of the protein which become exposed to solvent upon denaturation (Figure 2). Estimates of the nature of this interaction may be made from measurements of the effects of solvents on the activity coefficients of compounds which are models for the hydrophobic, peptide, or other groups of the protein which become exposed to solvent upon denaturation. An observed decrease in an activity coefficient may be caused either by a nonspecific solvent effect which stabilizes a group in a particular solvent or by binding of one of the components of the solvent to the solute. Qualitatively, denaturation according to this model may be thought of as a "dissolving out" of the interior of the protein by the denaturing solvent.

Irreversible denaturation may be interpreted in the same manner as reversible denaturation if it is assumed that the irreversible step is preceded by reversible partial unfolding of the protein (Figure 3).[22c,23] A denaturing solvent may then increase the rate of denaturation by increasing the concentration of partially unfolded intermediates without exerting any effect on the specific rate constant of the irreversible step, k_D.

The solubility and dissociation into subunits of a protein may be described in similar terms (Figure 4). Upon solution or dissociation of a protein, groups will become exposed which previously were not exposed to the solvent, and the solubilizing or dissociating effectiveness of different solvents may be evaluated in terms of their effects upon the activity coefficients of these groups.[24] To the extent to which the same class of

(23) (a) F. G. Hopkins, *Nature*, **126**, 328, 383 (1930); (b) M. L. Laskowski, Jr., and H. A. Scheraga, *J. Am. Chem. Soc.*, **83**, 266 (1961); (c) R. B. Simpson and W. Kauzmann, *ibid.*, **75**, 5139 (1953).

(24) The activity of a solid protein precipitated from different solvents is not necessarily constant, because components of the solvent may be included in the solid phase and the protein may precipitate in different conformations. In particular, precipitation by dilute solutions of well known protein precipitants, such as trichloroacetic acid, presumably involves binding of the precipitating agent to the insoluble protein. Nevertheless, solution of a protein certainly involves an increase in the exposure of some groups on a protein to the solvent and for many purposes it is useful to consider solvent effects on protein solubility in terms of effects on the activity coefficients of these groups.

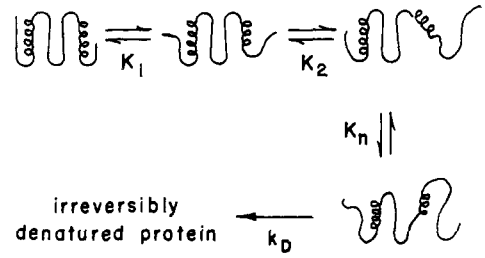

Figure 3. Diagram illustrating the successive stages of partial denaturation which precede the rate-determining step of irreversible denaturation of a protein.

groups becomes exposed to the solvent in denaturation, solution, and dissociation, solvents which favor denaturation will also increase the solubility and tend to cause dissociation of a protein and solvents which protect against denaturation will tend to decrease protein solubility and cause aggregation or association. Urea and lithium bromide are well known examples of the former and ammonium sulfate of the latter class of compound. Proteins often show a decrease in solubility and aggregation upon denaturation, but this is thought to be a secondary phenomenon caused by intermolecular interaction of portions of the protein, such as disulfide and hydrophobic groups, which are not available for such interactions in the native protein.[25]

The "Nonhydrophobic" Nature of the Effects on the Activity Coefficient of ATGEE. Urea and guanidine hydrochloride increase the solubility (and decrease the activity coefficient) of hydrocarbons, the nonpolar residues of amino acids, and nucleic acid bases (Table III and ref. 5–8, 12, and 26); there is other, less direct, evidence for a similar effect.[14,27] By favoring the exposure of such groups to the solvent, this property of urea and guanidine hydrochloride will tend to break hydrophobic bonds and will favor the denaturation of DNA[26a,28] and some proteins.[4–8,29] Substitution of alkyl groups for hydrogen or an increase in the chain length of alkyl substituents *increases* the effectiveness of denaturing agents in solubilizing these model compounds and in denaturing macromolecules by this mechanism (Table III and ref. 26a, 28, and 29). An interaction of the aromatic system with the amide groups of urea[19] may contribute to the solubilizing effect of urea on aromatic compounds and substituents.

The results reported here show conclusively that the effect of urea and related compounds on ATGEE is not a "hydrophobic" effect of the kind observed with non-

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polar solutes for the following reasons. (1) Substitution of alkyl groups for hydrogen atoms on urea, guanidine hydrochloride, and simple amides *decreases* the effectiveness of these compounds in decreasing the activity coefficient of ATGEE (Table II). Complete substitution of alkyl groups for hydrogen actually causes a reversal of the effect, so that an increase in the activity coefficient of the peptide is observed in the presence of tetramethylurea, N-dialkylamides, or tetramethylguanidine hydrochloride. In contrast, alkyl substitution increases the effectiveness of these compounds in increasing the solubility of such relatively nonpolar compounds as toluene and benzyl alcohol (Table III). (2) Addition to the solvent of such nonaromatic compounds as ethanol, dioxane, acetone, and tetrahydrofuran, which is well known to increase the solubility of nonpolar compounds, has little or no effect on the solubility of ATGEE (Table II). (3) The activity coefficient effect of urea on ATGEE decreases with increasing temperature over the range 0–40° (Table I); in contrast, the effect of urea on hydrocarbons increases with increasing temperature between 5 and 50°.[6] (4) The ratio of the solubility of a peptide in alcohol, compared to water, is unaffected by the addition of an unsubstituted amide group and is decreased by an increase in the number of internal peptide bonds, *i.e.*, the free energy of transfer of an amide or peptide group from water to alcohol is near zero or is positive.[30] In contrast, the results reported here and those of Nozaki and Tanford[8] show that the free energy of transfer of amide or internal peptide groups from water to aqueous urea is negative.

Effects of Solvents on Carbobenzoxydiglycineamide. The solubility of carbobenzoxydiglycineamide, which contains a large hydrophobic group as well as several amide groups, is increased by alkyl-substituted ureas and guanidine hydrochlorides, as well as by the unsubstituted compounds and by ethanol and dioxane. The latter solvents, as well as substituted ureas and tetramethylguanidine hydrochloride, increase the solubility of toluene and benzyl alcohol, models for the carbobenzoxy group, and the effectiveness of the different reagents toward carbobenzoxydiglycineamide may be reasonably accounted for as a summation of the effects on the hydrophobic and amide parts of the molecule. The effect of unsubstituted urea and guanidine hydrochloride on the peptide is considerably larger than the effect on toluene, and this increase presumably represents an effect on the peptide portion of the molecule.

Comparison with Effects on Proteins. The denaturing effectiveness of a series of ureas, amides, and guanidine hydrochlorides toward bovine serum albumin, as measured by optical rotation, parallels the effect of these solvents on the activity coefficient of ATGEE in that there is a progressive decrease and finally an elimination of activity upon substitution of alkyl groups for hydrogen atoms.[4] The order of activity in both systems is guanidine hydrochloride ~ thiourea > urea > formamide. Furthermore, this type of denaturation of albumin is, like the solubility of ATGEE, insensitive to aliphatic organic solvents, such as ethanol and dioxane. Other examples are known of a decrease in the denaturing effectiveness of ureas and guanidine hydrochlorides upon alkyl substitution[31] and there is other evidence that the denaturing action of urea toward certain proteins[9,29,32] and a synthetic polypeptide[33] cannot be accounted for by a hydrophobic mechanism. We conclude that a major part of the denaturing activity of urea and guanidine hydrochloride toward some proteins can be accounted for by an interaction of the solvent with the peptide and amide groups of the protein by a "nonhydrophobic" mechanism.

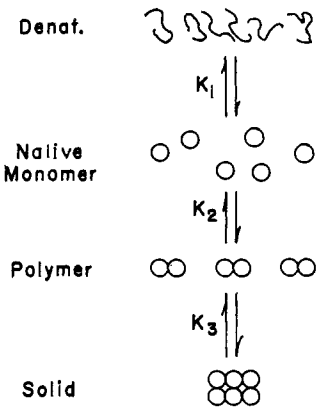

Figure 4. Schematic diagram illustrating the equilibria between a protein in the solid state, a native protein composed of subunits or in a polymeric state, native monomeric protein, and denatured protein.

The denaturation of many proteins by urea proceeds to a greater extent or more rapidly as the temperature is decreased.[34] Following Hopkins,[23a] this has frequently been ascribed to a more favorable equilibrium constant for binding of urea to the protein at lower temperature. It cannot readily be accounted for by effects of urea on the activity coefficients of nonpolar groups, because such effects increase with increasing temperature.[6] It is, however, consistent with an effect of urea on amide and peptide groups, because the effect of urea on ATGEE increases with decreasing temperature. These opposite effects of temperature indicate that the "hydrophobic" effect of urea will make a relatively larger contribution to urea denaturation at elevated temperatures, while effects on amide and peptide groups are more important at lower temperatures.

The denaturation of some proteins, such as ovalbumin and the lobster pigment, crustacyanin, is facilitated by organic solvents and alkyl-substituted denaturing agents, as well as by the unsubstituted compounds.[4,23c,29] Such denaturation is similar to the solubilization of carbobenzoxydiglycineamide in that interactions of the solvent with both hydrophobic and amide groups contribute to the observed effect. This type of denaturation is frequently irreversible, as might be expected if the breaking of hydrophobic bonds leads

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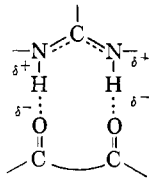
to the disruption of tertiary structure and to the exposure of groups which can undergo irreversible changes.

The results obtained with ATGEE suggest that the well known solubilizing action of urea and guanidine hydrochloride on proteins and peptides[35,36] may be, in large part, explained by the solubilizing effects of these reagents on amide and peptide groups. This conclusion is supported by the fact that the solubilizing effect of urea on polyglutamate derivatives increases with increasing glutamine content, at a corresponding degree of ionization.[36]

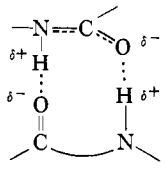
The dissociating effect of urea and related compounds on polymeric proteins will be discussed in a subsequent paper.[37]

Mechanism of the Activity Coefficient Effects. The following evidence, although not conclusive, is more easily interpreted in terms of a direct interaction between ATGEE and active solubilizing agents than in terms of a nonspecific solvent effect.

1. The specific structural requirements for effects of denaturing agents on the model peptide and on bovine serum albumin[4] suggest the existence of a specific interaction. Inspection of the data of Table II, for example, indicates that there is no correlation of the effects on ATGEE with the dipole moment of the denaturing agent. Active compounds have a hydrogen atom bound to a nitrogen atom with a partial positive charge, which is connected by conjugated bonds to either a similar NH group or a basic group, and could undergo polyfunctional hydrogen bonding to peptide groups as shown in II and III. The much greater solubilizing



II



III

effect of guanidinium chloride than simple amides on ATGEE suggests that structure II has greater stability than III. Polyfunctional hydrogen bonds, which have a more favorable entropy of formation, are more stable than monofunctional hydrogen bonds, and the rigidity of urea and guanidinium ion will facilitate the formation of such cyclic structures; formation of stable hydrogen-bonded complexes with rings containing up to 16 atoms has been described.[38]

There is evidence in the literature that monofunctional hydrogen bonds or complexes of structure III have, at best, a small stability in water. Activity coefficient data are consistent with the formation of weak complexes of urea with itself[39] and with diketopiperazine,[10,11] but Klotz and Franzen were unable to find infrared evidence for the self-association of urea or N-methylacetamide in the presence of excess water.[3] Susi, *et al.*, have recently reported infrared evidence for the formation in aqueous solution of an unstable dimer of δ-valerolactam.[40] Activity coefficient data are consistent with an interaction between the peptide portions of 2 molecules of triglycine.[41] There is conflicting evidence as to whether urea and guanidinium chloride bind to proteins and polypeptides,[42] but the fact that urea *can* bind tightly to properly oriented components of a protein is shown by the facts that the ureido group of biotin contributes a factor of 10^7 to 10^8 to the binding of biotin to avidin and that dilute solutions of urea inhibit this binding.[43]

The fact that cationic dyes do not bind to the uncharged polymer polyvinylpyrrolidone if they contain $-NR_2^+$ groups, but do bind if one or both of the R groups is replaced by hydrogen, has been taken as evidence for significant stabilization of complex formation by $>NH\cdots O=C<$ hydrogen bonding in this system.[44] The oxygen atoms of a peptide bear a negative charge of approximately 0.4, because of the large resonance contribution of the dipole structure, $-C=N^+<$.[45] The observed binding of guanidinium ion to tetraphosphate,[46] the oxygen atoms of which also carry a partial negative charge, may serve as a precedent for the postulated binding of guanidinium ion to ATGEE.

2. An increase in the number of amide bonds in a peptide has no appreciable influence on the activity coefficient effect of urea on the peptides (Table IV). Such behavior may be more readily interpreted in terms of complex formation, in which the addition of 1 molecule of denaturing agent to the peptide restricts the addition of additional molecules because of blocking of available sites, than in terms of a nonspecific bulk solvent effect. In contrast, the solubilizing action of both ethanol[47] and urea[6,8] on compounds containing hydrocarbon groups, which is most readily interpreted as a bulk solvent effect, increases with increasing size and appears to be an approximately additive property of the size of the hydrocarbon group.

3. It is difficult to account for the effect of urea and related compounds on ATGEE in terms of currently popular hypotheses based on alterations in water structure. The solubilizing effect is not caused by an increase in water structure, because alkyl-substituted ureas, guanidinium ions, and amides tend to increase water structure compared to the unsubstituted compounds, as estimated by the large increases in viscosity caused by the alkyl-substituted compounds,[48] but show a decreased effectiveness toward ATGEE.

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(47) Reference 41, Chapter IX.

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Rupley has recently suggested that protein denaturation by urea may be caused by a structure-breaking effect.[49] Although the small changes in viscosity which are observed upon addition of urea to water have been interpreted as evidence for a structure-breaking effect of urea,[49] heat capacity measurements do not support such an effect of urea in pure water; the conclusion that solution of amide and nonpolar groups of peptides requires less organization of the solvent in aqueous urea than in pure water may be simply a reflection of the large size of the urea molecule, which would require the immobilization of fewer urea than water molecules around a solute.[14] Both the enthalpy and entropy of the urea–ATGEE interaction are negative (see below), whereas a positive entropy and a small or positive enthalpy would be expected for a structure-breaking mechanism. A number of salts, such as potassium chloride, have a structure-breaking effect, but *increase* the activity coefficient of ATGEE.[19] Furthermore, if the solubility of ATGEE were dependent only on the amount of "unstructured" water, aliphatic organic compounds, such as dioxane and ethanol, which are believed to increase water structure,[50] should decrease ATGEE solubility, but such compounds have little or no effect on ATGEE. Finally, the effect of urea on elastoidin in ethylene glycol is as large as the effect in water solution; clearly this effect cannot be ascribed to "water-structure."[51]

4. The linear increase in ATGEE solubility with increasing urea or guanidine hydrochloride concentration is more easily explained in terms of complex formation (eq. 3), in which the total peptide in solu-

$$\text{ATGEE (A)} + \text{urea (D)} \rightleftharpoons \text{ATGEE}\cdot\text{urea (AD)} \qquad K = [\text{AD}]/[\text{A}][\text{D}] \quad (3)$$

tion is equal to [A] + [AD] and increases with increasing denaturant concentration with slope K, than in terms of nonspecific solvent effects, which often follow the Setschenow equation,[18] $\log \gamma = K_s[\text{D}]$, with a *logarithmic* increase of solubility with increasing concentration of denaturing agent. The solubility of butane in these solvents, which is probably more accurately described as a bulk solvent effect, shows a nonlinear increase with increasing denaturant concentration.[6]

Values of K, based on the assumption of complex formation, are shown in Table VI. The equilibrium constant for interaction with urea decreases with increasing temperature. The results do not give a constant value for the apparent enthalpy of complex formation (−2970 cal./mole at 0–25° and −2240 cal./mole at 25–40°), but they do indicate that the heat of complex formation is negative. The value over the range 0–40° is approximately −2800 cal./mole. The entropy of complex formation is negative and has a value of roughly −11.8 e.u. at 25°. The ΔH of this reaction may be compared to the value of −3.4 ± 0.5 kcal./mole for the postulated interaction of urea with diketopiperazine.[11]

Quantitative Application to Peptides and Proteins. The free energies of transfer of ATGEE from water to solutions of denaturing agents are given in Table VII; the values for transfer to 8 M urea and 7 M guanidine hydrochloride are −710 and −1190 cal./mole, respectively, based on the molarity scale.[52] If corrections are made for the free energies of transfer of ethyl acetate to these solvents, to correct for the contribution of the corresponding portions of the ATGEE molecule, the contributions of the peptide portion of ATGEE to the free energy of transfer are −530 and −1010 cal./mole for 8 M urea and 7 M guanidine hydrochloride, respectively. The corresponding value for transfer to 8 M urea of the peptide portion of the carbobenzoxyglycine peptides with a terminal amide group, obtained by subtracting the free energy of transfer of benzyl alcohol or toluene from that of the peptide, is approximately −500 cal./mole, similar to that obtained with ATGEE. In view of (a) the absence of a large increase in the effect of urea with an increase from two to four in the number of amide groups in the carbobenzoxyglycine peptides (Table IV) and (b) the fact that the increase in solubility of ATGEE is proportional to the first power of the concentration of urea or guanidine hydrochloride (Figure 1), these values may tentatively be taken as the free energies of interaction of these denaturing agents with a single site on the peptide, which probably is composed of at least two amide groups. A requirement for two peptide groups (or a peptide and a free carboxylic acid group) is also suggested by the much larger effect of urea on triglycine than on diglycine, which corresponds to a difference in free energy of transfer of 310 cal./mole for 8 M urea.[8] The value of approximately −500 cal./mole for the contribution of a peptide site to the free energy of transfer to 8 M urea is considerably

Table VI. Apparent Equilibrium Constants for Complex Formation between ATGEE and Urea or Guanidine Hydrochloride

Solvent	Temp., °C.	K',[a] M^{-1}
Urea	0	0.46
Urea	25	0.29
Urea	40	0.24
Guanidine hydrochloride	25	0.90

[a] K = [DA]/[D][A], where D = denaturant and A = ATGEE. Based on the concentration of each species in moles/l.

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(51) L. Mandelkern, G. Canty, and A. F. Diorio, *J. Phys. Chem.*, **67**, 2882 (1963).

(52) Somewhat larger values are obtained (Table VII) if the calculations are based on the mole fraction scale. We are not certain which of these scales is the more appropriate for the utilization of data obtained with model compounds for the prediction of the behavior of a polymer. While the mole fraction scale is preferable for many physical chemical calculations, particularly for calculations of deviations from ideal solution behavior, groups on a protein which become exposed to solvent will "see" a certain volume, not a certain number of moles of solvent, and a volume fraction or molarity scale may be more appropriate for an *empirical* comparison of the behavior of model compounds and proteins. Fortunately, the effects observed with ATGEE in urea and guanidine hydrochloride solutions are large enough that the conclusions will not be sensitive to the choice of scale. Some of the data obtained with hydrocarbons in urea are more sensitive to the choice of concentration scale; ethane is more soluble in 7 M urea than in water on the mole fraction scale and less soluble on the molarity scale,[6] so that free energies of transfer and conclusions regarding the effect of urea on nonpolar groups may be different, depending on the concentration scale chosen. Tanford and Nozaki have avoided this problem by comparing the free energies of transfer of two different molecules and taking the difference as the contribution of the structural difference between the two molecules to the free energy of transfer[8,13]; however this approach requires that the contributions to the free energy of transfer of different parts of a molecule be additive.

Table VII. Comparison of Solubilities and ΔF_{tr} for ATGEE at 25°, Based on the Molarity and Mole Fraction Scales

Solvent	M	Molarity scale S/S^0	Molarity scale $\Delta F_{tr,S}$,[a] cal./mole	Mole fraction scale N/N^0	Mole fraction scale $\Delta F_{tr,N}$,[a] cal./mole
Urea	3	1.85	−366	2.0	−410
Urea	8	3.3	−710	4.15	−850
1,3-Dimethylurea	3	1.41	−206	1.7	−315
Guanidine hydrochloride	3	3.5	−745	4.7	−920
Guanidine hydrochloride	7	7.4	−1190	12	−1480
Tetramethylguanidine hydrochloride	3	0.63	+266	0.96	+27
Formamide	3	1.25	−150	1.32	−165
N,N-Dimethylformamide	3	0.89	−70	1.06	−35
Ethanol	3	0.85	+96	0.96	+27
Dioxane	3	0.98	+14	1.17	−96

[a] $\Delta F_{tr,S} = RT \ln S^0/S$; $\Delta F_{tr,N} = RT \ln N^0/N$; ΔF_{tr} represents the free energy of transfer of solute from water to another solvent at the same solute concentration.

larger than the value of −145 cal./mole, which was calculated from our data by Nozaki and Tanford for the contribution of a glycyl residue in this process.[8] However, the calculation of the latter value was based upon the assumptions that the acetyl and ester groups of ATGEE have the same effect on the free energy of transfer as butane and that additivity of free energy contributions holds for individual adjacent peptide units.

The same values, −710 and −1190 cal./mole, respectively, are obtained if the interaction of ATGEE with urea or guanidine hydrochloride is treated as a direct binding and the free energies of transfer are calculated from eq. 4[22b] and the equilibrium constants of Table V.

$$\Delta F = -nR + \ln (1 + K[\text{denaturant}]) \qquad (4)$$

This treatment differs from that of Schellman in that a single binding site[5] is assumed for each peptide molecule, rather than separate sites for each NH and CO group.[22b]

In our opinion, more data is required before numbers of this kind can be used for a detailed calculation of the free energy of protein denaturation in different solvents. The principal problems are the absence of additivity of the free energy contributions of adjacent glycyl units and the absence of data on the effects of side chains on the denaturant–peptide interaction. Studies of the effects of urea and guanidine hydrochloride on more complex, short, uncharged peptides may provide further information on these questions.

The Effect of Concentrated Salt Solutions on the Activity Coefficient of Acetyltetraglycine Ethyl Ester

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Contribution No. 354 from the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts. *Received December 31, 1964*

The activity coefficient of the uncharged model peptide, acetyltetraglycine ethyl ester (ATGEE), has been determined in the presence of concentrated salt solutions by solubility measurements. The results suggest that the effects of concentrated salt solutions on the denaturation, dissociation, and solubility of proteins may be accounted for, in large part, by effects of the salts on the peptide and amide groups which become exposed to the solvent during these processes. The results cannot easily be explained by effects of the salt solutions on the availability of solvating water, by effects on water "structure," by electrostatic treatments of the Debye–Kirkwood type, or by effects on the internal pressure of the solvent. The results are consistent with a summation of two effects: (1) an ordinary "salting-out" effect, which may be described in terms of the average cohesive energy or internal pressure of the solvent, and (2) a direct interaction between certain large anions and the amide dipole. The second of these interpretations is supported by the close correlation between the order of effectiveness of anions toward ATGEE and their order of binding to anion-exchange resins and other charged groups. In addition, the results provide evidence for the existence, in aqueous solution, of an interaction between aromatic and other highly polarizable compounds and the amide group, which may be of importance in maintaining protein structure and in protein–solvent and protein–solute interactions.

Introduction

The Hofmeister or lyotropic series of ions, which describes the order of effectiveness of ions in influencing a very large number of chemical and physical phenomena, was determined by Hofmeister by measurements of the relative effectiveness of ions in causing the precipitation of proteins.[1,2] With minor exceptions, the same order of activity of ions is found for denaturation, depolymerization, and dissociation of proteins and for the inhibition or activation of a number of enzymes. In general, those ions which are most effective in causing protein precipitation also are most effective in preventing denaturation and dissociation into subunits, and those ions which increase the solubility of proteins

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