Influence of Denaturing Agents on Solvent Structure*

Gordon G. Hammes and John C. Swann

ABSTRACT: Ultrasonic attenuation has been measured in aqueous solutions of guanidinium chloride, acetamide, sodium chloride, and ammonium chloride. All of these compounds decrease the attenuation of water indicating a breakdown of the solvent structure. The effect of the above reagents on the ultrasonic relaxation spectrum of polyethylene glycol has also been studied. Guanidinium chloride closely resembles urea (Hammes and Schimmel, *J. Am. Chem. Soc. 89*, 442 (1967)) in that at a concentration of approximately 2.5 *m* it induces a sudden decrease in the relaxation time characteristic of polymer–solvent interactions. Intrinsic viscosity measurements indicate a slight increase in solvation of the polymer as the guanidinium chloride concentration increases, but no sharp transition occurs. Sodium chloride and ammonium chloride appear to reduce the solvation of the polymer and create new relaxation processes which largely mask the original polymer–solvent interaction. Acetamide, which has less effect on the attenuation of water than the other reagents, causes very small changes in the relaxation time and intrinsic viscosity of the polymer. The mode of breakdown of solvent structure by urea and guanidinium chloride, therefore, is distinctive. The sharp transition in the relaxation time is probably due to alteration of the local water structure around the polymer. This sharp transition is reminiscent of protein and nucleic acid denaturation induced by these reagents in aqueous solution and suggests the importance of such processes in the mechanism of denaturation.

Two general types of mechanism have been suggested for the denaturation of proteins and nucleic acids by compounds such as urea and guanidinium chloride. The first is the specific binding of the denaturant to groups in the macromolecule, with a resultant weakening of noncovalent bonds responsible for maintaining the native configuration. Gordon and Jencks (1963) investigated the structural requirements for effectiveness of denaturing agents toward bovine serum albumin, and concluded that specific interactions were of primary importance in this case. A second possible mode of action of denaturants is through their effect on the solvent structure. Denaturants often possess possibilities for strong hydrogen bonding to surrounding water molecules, and might be expected to affect hydrophobic interactions involved in the stabilization of protein and nucleic acid structures (Kauzmann, 1959; Scheraga, 1963). The increased solubility in aqueous solution of hydrocarbons (Wetlaufer *et al.*, 1964) and of amino acids (Nozaki and Tanford, 1963) on increasing the concentration of urea suggests that urea increases the hydrophobic character of the solvent. Levine *et al.* (1963) proposed that this effect was important in the action of denaturants on DNA. However little direct experimental evidence exists to suggest the disruption of water structure by denaturants. The near ideality of concentrated urea solutions (Scatchard *et al.*, 1938) often has been taken as an indication that urea has little effect on the structure of water, but a more likely interpretation (Abu-Hamdiyyah, 1965) is that the urea and water molecules form structures similar to, but quite distinct from, those existing in pure water.

Recent use of ultrasonic attenuation measurements as a probe of microscopic solvent structure has provided strong evidence for the breakdown of the hydrogen-bonded structure of water by urea (Hammes and Schimmel, 1967). Furthermore, measurements made in the presence of the synthetic polymer polyethylene glycol showed that on increasing the urea concentration a sharp change could be induced in the ultrasonic relaxation time. This change implied a sudden increase in the rate of a process involving the interaction between solvent and polymer at a urea concentration of about 2.5 *m*. The apparently cooperative nature of this transition simulates denaturation processes which have also been found to take place over narrow ranges in urea concentration (*cf.* Martin and Bhatnagar, 1966); moreover, the transition observed with polyethylene glycol solutions is almost certainly caused by the effect of the urea on the solvent structure rather than by specific binding.

To further assess the relation of this polymer–solvent interaction to denaturation, we have studied the ultrasonic attenuation of aqueous solutions of guanidinium chloride, acetamide, sodium chloride, and ammonium chloride both in the presence and absence of polyethylene glycol. The results show that guanidinium chloride closely resembles urea both in breaking down water structure and in its effect on polyethylene glycol. Acetamide was found to be a much weaker structure breaker of water, and to have little effect (in a 6 *m* solution) on the relaxation time of the poly-

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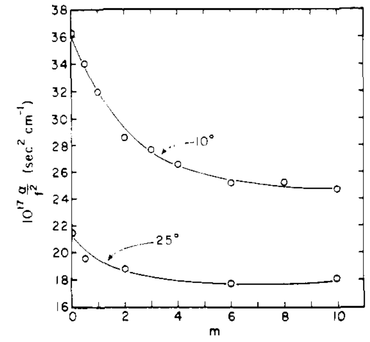

FIGURE 1: α/f^2 *vs.* guanidinium chloride molality at 10 and 25°.

mer. The salts NaCl and NH_4Cl were found to be just as effective as guanidinium chloride in disrupting the hydrogen-bonded structure of water. However, their effects on the polymer were different from those of urea and guanidinium chloride in that new relaxation processes occur, and the polymer apparently becomes more compact, as contrasted to the looser structure which appears to exist in solutions of the denaturants. The effects of urea and guanidinium chloride on the local solvent structure around the hydrophobic groups of the polymer thus seem to be distinctive and may be related to their denaturing ability.

Experimental Section

Polyethylene glycol E-20,000 (av mol wt 20,000) was generously supplied by Dow Chemical Co., Midland, Mich. Guanidine monohydrochloride was a Baker grade reagent (J. T. Baker Co., Phillipsburg, N. J.). This compound was stored in a desiccator when not in use. Acetamide, sodium chloride, and ammonium chloride were reagent grade compounds and all solutions were prepared with distilled water. Procedures for the measurement and calculation of ultrasonic attenuation coefficients, ultrasonic velocities, densities, and solvent viscosities have been previously described (Hammes and Lewis, 1966; Hammes and Schimmel, 1967). Intrinsic viscosities were determined with a Cannon–Ubbelohde viscometer.

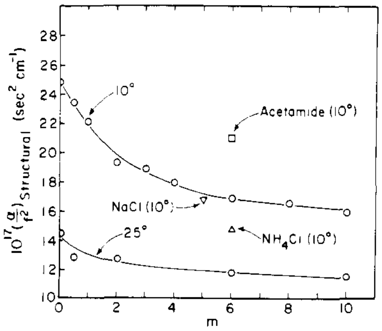

FIGURE 2: $(\alpha/f^2)_{\text{structural}}$ *vs.* solute molality. Points not marked refer to guanidinium chloride.

Results

Solutions of guanidinium chloride, acetamide, NaCl, and NH_4Cl showed no relaxation processes in the frequency range 10–175 Mcycles/sec. The value of α/f^2 (where α is the ultrasonic pressure amplitude attenuation coefficient and f is the frequency) decreases with increasing guanidinium chloride concentration (Figure 1) in a manner similar to that observed by Hammes and Schimmel (1967) with urea. The experimental quantity α/f^2 contains contributions from the "classical absorption" due to viscosity and heat conduction effects (*cf.* Eigen and de Maeyer, 1963) as well as the absorption due to structural relaxations with shorter relaxation times. The classical absorptions due to the shear viscosity of the solutions are calculated in Table I from the measured densities (ρ), viscosities (η), and sound velocities (v), using the equation (Eigen

TABLE I: Densities, Viscosities, Ultrasonic Velocities, and Classical Absorption.

m	ρ (g/cc)	$10^2\eta$ (poise)	10^{-5} v (cm/sec)	$10^{17} \times \alpha_{\text{classical}}/f^2$ (sec^2/cm)
	Guanidinium chloride (10°)			
0	0.9997[a]	1.3077[a]	1.44	11.5
0.5	1.013	1.312	1.47	10.7
1.0	1.025	1.324	1.51	9.9
2.0	1.046	1.356	1.54	9.3
3.0	1.064	1.399	1.58	8.8
4.0	1.077	1.447	1.60	8.6
6.0	1.100	1.562	1.65	8.3
8.0	1.122	1.704	1.67	8.6
10.0	1.143	1.887	1.70	8.8
	Guanidinium chloride (25°)			
0	0.9971[a]	0.8937[a]	1.50	7.0
0.5	1.010	0.905	1.51	6.8
2.0	1.041	0.963	1.57	6.2
6.0	1.098	1.154	1.67	5.9
10.0	1.136	1.401	1.70	6.6
	Acetamide (10°)			
6.0	1.021	2.357	1.60	14.9
	NaCl (10°)			
5.0	1.174[a]	2.213[a]	1.74	10.4
	NH_4Cl (10°)			
6.0	1.071[a]	1.208[a]	1.64	6.8

[a] Obtained from International Critical Tables (1928).

and de Maeyer, 1963)

$$\left(\frac{\alpha}{f^2}\right)_{\text{classical}} = \frac{8\pi^2\eta}{3v^3\rho} \qquad (1)$$

(This form of the equation neglects absorption due to heat conduction; this effect is usually negligible in aqueous solutions.) The structural part of α/f^2, calculated by subtraction of the classical value from the measured value, is plotted in Figure 2. Results for 6 *m* solutions of acetamide and NH_4Cl, and 5 *m* NaCl are also shown in this figure. Since the guanidinium chloride solutions contained small amounts of free HCl (the pH of the 10 *m* solution was 1.4), the ultrasonic absorption coefficient of 1 M HCl was measured. The α/f^2 value for this solution at 10° was the same as that for water within experimental error (±2%). Adiabatic compressibilities for the solutions were calculated from the measured velocities and densities using the relation (Eigen and de Maeyer, 1963)

$$\kappa_S = \frac{1}{\rho v^2} \qquad (2)$$

where κ_S is the adiabatic compressibility. Figure 3 shows the variation of κ_S with solute concentration.

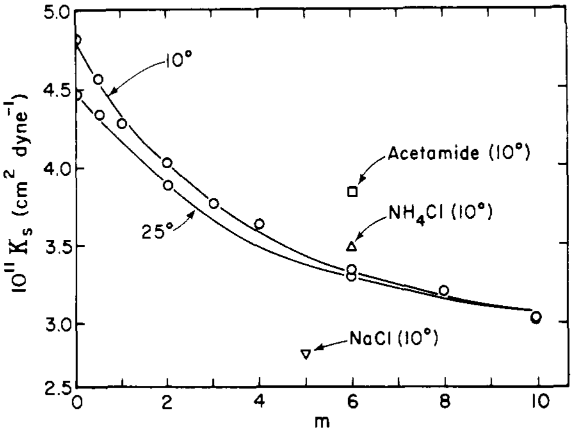

FIGURE 3: Adiabatic compressibility, κ_S, *vs.* solute molality. Points not marked refer to guanidinium chloride.

Relaxation spectra of polyethylene glycol in some of the aqueous solutions studied are shown in Figure 4. The mole fraction of polymer was the same in all cases (0.036 calculated as mole fraction of ethylene oxide monomer). Hammes and Lewis (1966) showed that polyethylene glycol in water undergoes a single relaxation in the frequency range 10–175 Mcycles/sec; *i.e.*, the frequency dependence of α/f^2 could be described by an equation of the form

$$\frac{\alpha}{f^2} = \frac{A\tau}{1 + (\omega\tau)^2} + B \qquad (3)$$

where $\omega = 2\pi f$, *A* and *B* are amplitude parameters, and τ is the relaxation time. Hammes and Schimmel (1967) also found a single relaxation process for polyethylene glycol in aqueous urea solutions. In the present study it was found that the curves obtained for polyethylene glycol in solutions of guanidinium chloride and acetamide could also be described by a single relaxation time. The relaxation parameters *A*, *B*, and τ were found by the method described by Piercy and Subrahmanyam (1965) and are given in Table II. The estimated errors are ±15% in *A*, ±3% in *B*, and ±10% in τ. The data obtained for polyethylene glycol in NH_4Cl and NaCl solutions could not be described by eq 3.

TABLE II: Ultrasonic Parameters and Intrinsic Viscosities for Polyethylene Glycol in Solutions of Various Reagents at 10°.

Reagent Concn (*m*)	10^8A (sec cm^{-1})	$10^{17}B$ (sec^2 cm^{-1})	$10^9\tau$ (sec)	[η] (dl g^{-1})
		Guanidinium chloride		
0	3.3	41.5	6.1	0.39_6
2.0	2.8	40.6	6.5	0.41_5
2.5	2.8	39.6	5.4	—
3.0	3.2	38.4	4.1	0.42_1
4.0	3.1	37.2	4.3	—
6.0	3.0	35.3	4.0	0.43_2
10.0	2.8	35.5	3.6	0.44_0
		Acetamide		
6.0	2.0	46.7	5.7	0.41_6
		NaCl		
5.0	—	—	—	0.23_7
		NH_4Cl		
6.0	—	—	—	0.28_1

In Figure 5 the relaxation time is plotted against the concentration of guanidinium chloride. Intrinsic viscosities for the polymer in the same solutions are plotted on the same figure, and in Table II are compared with those found for polyethylene glycol in the acetamide, NaCl, and NH_4Cl solutions.

Discussion

The structural contribution to the ultrasonic attenuation in pure water can be attributed to energy losses occurring when the ultrasonic wave perturbs the liquid structure. In aqueous solutions equilibria presumably exist between water molecules which are hydrogen bonded in different structures and energy states. No relaxation processes are observed in the frequency range investigated because structural relaxation times of most liquids are considerably shorter than 5×10^{-10} sec which is the shortest time constant

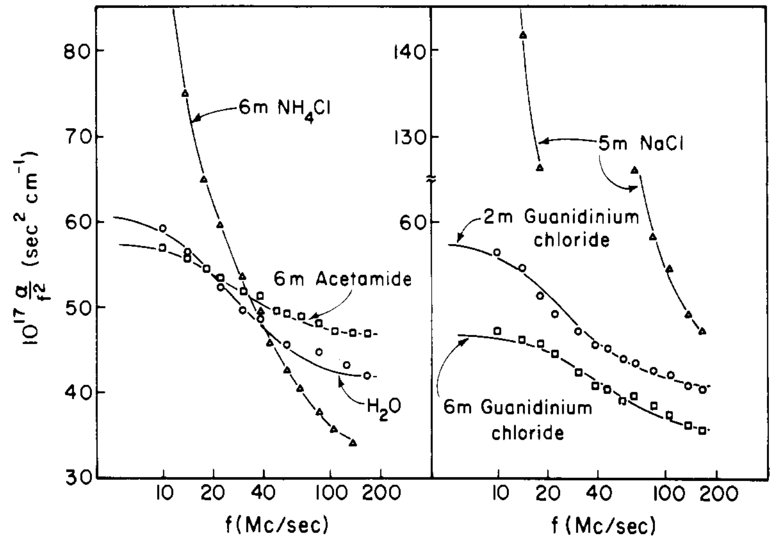

FIGURE 4: α/f^2 *vs.* f for polyethylene glycol in various solutions at 10°. For the NaCl and NH$_4$Cl solutions the curves are arbitrarily drawn to best fit the experimental points. For the other solutions the curves are theoretical relaxation curves plotted according to eq 3 using the parameters in Table II.

accessible with the techniques employed (*cf.* Herzfeld and Litovitz, 1959). Addition of each of the compounds studied to water decreased the value of $(\alpha/f^2)_{\text{structural}}$ (Figure 2). This implies a change in solvent structure and presumably represents a structure-breaking effect of the solute, *i.e.*, a breakdown of the open networks of hydrogen-bonded water molecules. Of course simultaneous formation of new structures must occur which involves noncovalent interactions of water with the solute, but the new liquid structures apparently undergo less perturbations of the type which contribute to ultrasonic absorption. Although such changes would also account for the observed decrease in adiabatic compressibility (Figure 3), the adiabatic compressibility does not appear to be a good index of solvent structure since even compounds which are believed to increase structural ordering of the solvent, such as polyethylene glycol (Hammes and Schimmel, 1967), increase both the density and the velocity of sound propagation in the solution and must, therefore, lower the adiabatic compressibility. The values of $(\alpha/f^2)_{\text{structural}}$ for guanidinium chloride solutions of a given concentration are less at 25 than at 10°, but the effect of guanidinium chloride in lowering $(\alpha/f^2)_{\text{structural}}$ is much more pronounced at 10° (Figure 2). A similar result was found by Hammes and Schimmel (1967) for urea. Since it is generally accepted that water contains less hydrogen-bonded clusters or ice structures at 25 than at 10°, these results strongly suggest that the ultrasonic attenuation of an aqueous solution provides a measure of the amount of such structures and decreases according to the extent to which they are broken down by a solute.

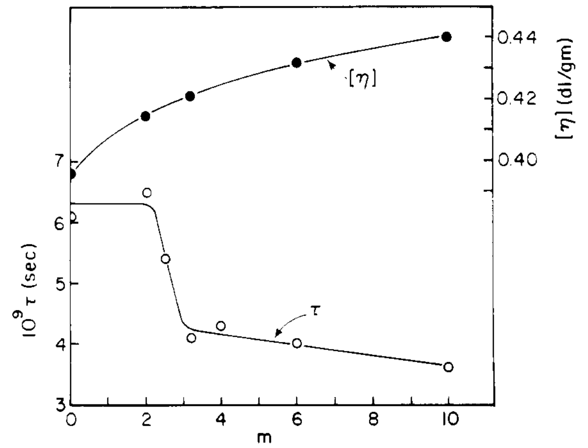

FIGURE 5: Relaxation time (τ) and intrinsic viscosity ([η]) for polyethylene glycol at 10° *vs.* concentration of guanidinium chloride.

Urea and guanidinium chloride cause quite similar changes in $(\alpha/f^2)_{\text{structural}}$. On the other hand, acetamide, which was chosen for investigation because, like urea it contains a CONH$_2$ grouping, appears to be less than one-half as effective as urea as a structure breaker. (The $(\alpha/f^2)_{\text{structural}}$ value for 6 *m* acetamide corresponds to that obtained for about 2 *m* urea or guanidinium chloride.) This is not too surprising since the CH$_3$ group of acetamide would be expected to have some compensating structure-forming effect (*i.e.*, increasing the extent of hydrogen bonding between water molecules). Neutral salts such as NaCl and NH$_4$Cl are just as effective as guanidinium chloride in lowering the structural contribution to the ultrasonic absorption of water (Figure 2). It has been previously observed (Kurtze and Tamm, 1953) that some alkali metal halides reduce the ultrasonic attenuation of water; this result was attributed to the structure-changing influence of the salt on water. Metal cations in aqueous solution tend to solvate, with a consequent disruption of hydrogen-bonded structures in surrounding layers of water (Frank and Wen, 1957). In a 5 or 6 *m* salt solution relatively few water molecules would be bound in the normal structures. However, since NaCl and NH_4Cl are not generally considered to be denaturants it appears that the ability to break hydrogen bonds in water is not in itself sufficient to cause denaturation.

The relaxation process observed with polyethylene glycol in water is affected by guanidinium chloride and urea in a very similar manner. With either denaturant at a concentration around 2.5 *m* a sudden shortening of the relaxation time is observed (see Figure 5 of this paper and Figure 7 of Hammes and Schimmel, 1967). The decrease in τ is slightly greater with urea than with guanidinium chloride. In both cases the intrinsic viscosity of the polymer shows no corresponding sudden change; hence the change in τ does not seem to be due to a change in polymer structure. The change in τ is probably due to the breakdown of local solvent structure, which markedly affects the solvent–polymer interactions responsible for the observed relaxation process. This process, which is apparently cooperative, involves the interrelated phenomena of hydrocarbon–hydrocarbon interactions, hydrogen bonding of water to the polymer oxygen, and the presence of water clusters around hydrophobic groups. The smooth increase in the instrinic viscosity of polyethylene glycol on raising the concentration of urea or guanidinium chloride indicates a slight expansion of the polymer coil and is probably caused by increased solvation. Bailey and Callard (1959) have previously postulated that in aqueous solutions the polyethylene glycol coil is appreciably expanded by hydration. The apparently greater solvation which we find in solutions of denaturants is consistent with the belief that these reagents reduce the unfavorable entropy of hydration of hydrocarbon-like groups, and thereby weaken hydrophobic bonding.

The parameter B in eq 3 is a measure of the residual attenuation at frequencies higher than that which characterizes the observed relaxation process. In some systems the B value is that of the pure solvent. When it is higher, as is the case for polyethylene glycol solutions, the excess absorption may be due to the altered solvent structure in the presence of the solute or to additional unrelaxed reactions or to both. However the main contribution of the polymer to the viscosity of the medium has probably relaxed at lower frequencies (Hammes and Lewis, 1966). The excess absorption for polyethylene glycol is probably due to increased ordering of the solvent around the hydrocarbon groups. The parameter B decreases only from 41.5 to 35.5 between 0 and 10 *m* guanidinium chloride (Table II). The decrease in α/f^2 for water over the same concentration range is almost twice as large (Figure 1). Again similar behavior was observed with urea (Hammes and Schimmel, 1967). The local water structure around the hydrophobic groups of the polymer is apparently broken down less by denaturing agents than is the bulk water structure.

Acetamide at a concentration of 6 *m* has little effect on the relaxation time for the polymer–solvent interaction (Table II). The intrinsic viscosities indicate that solvation of polyethylene glycol in 6 *m* acetamide is only about as much as is attained in 2 *m* urea or guanidinium chloride. These results indicate that acetamide has less pronounced effects on the local solvent structure around the polymer than do urea and guanidinium chloride. This may partly account for the fact that acetamide does not generally behave as a denaturant toward proteins. Although the parameter B in eq 3 is somewhat higher for polyethylene glycol in 6 *m* acetamide than in water (Table II), this can largely be accounted for in terms of the greater classical absorption shown by 6 *m* acetamide (Table I). The structure-breaking effect of acetamide is suppressed by the presence of the polymer just as with urea and guanidinium chloride. The amplitude parameter A is lower in acetamide solution than in water or any of the guanidinium chloride solutions. Since, however, A is a complex function of thermodynamic variables, it is difficult to ascertain the cause of this change.

The effects of NaCl and NH_4Cl on the ultrasonic relaxation spectrum of polyethylene glycol are quite different from those of the organic compounds studied. In both solutions α/f^2 becomes very large at low frequencies (Figure 4) and the data are no longer consistent with the occurrence of a single relaxation process. Apparently new reactions with longer relaxation times are occurring as well as the original polymer–solvent interaction. The present data do not provide much information about the nature of these reactions. Association of the polymer with the salt may be of importance. Viscosity measurements (Bailey and Callard, 1959; Lundberg *et al.*, 1966) showed no evidence for association of alkali metal halides with polyethylene glycol, although association processes could not be ruled out on the basis of these results. Slight evidence for association in the case of ammonium halides was found. Our viscosity results, although at much higher salt concentrations than were used by the above authors, are in agreement with their work in showing that the intrinsic viscosity of the polymer is decreased by added salts. The salts appear to reduce the solvation of polyethylene glycol and produce a more compact molecule. As noted by Lundberg *et al.* (1966) this phenomenon resembles the salting out of nonelectrolytes from aqueous solution.

The conclusion to be drawn from this work on the relaxation of polyethylene glycol in binary solvents is that denaturing agents appear to disrupt water structure in a distinctive manner. Whereas hydrogen bonding of water is also diminished in neutral salt solutions it appears that in these solutions hydrophobic bonding is strengthened, *i.e.*, less solvation of hydrocarbon-like groups occurs. However, in urea and guanidinium chloride solutions hydrophobic bonding is apparently weaker since the solvation of polyethylene glycol increases. Guanidinium chloride, although a salt, behaves toward polyethylene glycol in a manner analogous to urea, rather than like neutral salts. Nevertheless guanidinium chloride does appear to have some salting out effect on the polymer. The polymer solution in 10 *m* guanidinium chloride at 10° was supersaturated and tended to form a precipitate when kept at this temperature for more than a few hours. The striking similarity of urea and guanidinium chloride in producing a sharp change in the relaxation time characteristic of the solvent–polymer interactions is of interest because it may be related to the mechanism of protein denaturation. Specific interactions cannot be ruled out as the cause of the sharp transition in relaxation time. However, the occurrence of specific binding is unlikely since no contribution from other relaxation processes was evident in the ultrasonic relaxation spectrum of polyethylene glycol in solutions of denaturants. Since weak interactions between charged species or dipoles would not be expected to have relaxation times too long to be observed in the frequency range studied, any specific binding would have to be associated with relaxation times shorter than 5×10^{-10} sec. To term this specific binding hardly seems appropriate since the relaxation time for an association–dissociation reaction involving diffusion processes only, *i.e.*, with no chemical binding, can be estimated to be about 10^{-10} sec for the system under discussion. Both denaturants studied cause the sharp transition in relaxation time at almost the same concentration, *viz.*, about 2.5 *m*, whereas guanidinium chloride usually acts at a lower concentration than urea in denaturation of proteins (Gordon and Jencks, 1963) and in increasing the solubility in water of model amides (Robinson and Jencks, 1963). Hence, other factors must contribute to these processes and it seems reasonable to suppose that the mechanism of denaturation of a protein or nucleic acid by reagents of the urea–guanidinium chloride class may involve both specific binding and the breakdown of solvent structure. Certain other reagents such as detergents or alkyl-substituted ureas, although probably water structure formers by virtue of their hydrocarbon content, can presumably cause denaturation by hydrophobic interactions between groups on the protein and the denaturant. Our results suggest that a breakdown of both the bulk and local water structure probably plays a role in the action of certain denaturants but do not imply that the ability to break down water structure is either a sufficient or necessary requisite for a denaturant.

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