Interactions of Protein-Denaturing Salts with Model Amides*

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ABSTRACT: The interactions of *N*-methylacetamide (NMA) and *N,N*-dimethylacetamide (DMA) with denaturants were studied by viscometry, calorimetry, and crystallography. Lithium and calcium chlorides show strong interactions by all three criteria; these cations probably interact with peptide groups.

Alkali thiocyanate, nitrate, and perchlorate salts show interactions with amide groups in crystalline complexes, but show little interaction by viscometry and calorimetry. Guanidinium chloride forms a weak crystalline complex with *N,N*-dimethylacetamide and produces intermediate viscosity and heat effects. A crystalline complex of urea with cysteine ethyl ester was prepared.

There are two general mechanisms by which salts may denature proteins: (1) interaction with the protein, probably at peptide or other polar groups (possibly including the π electrons of the aromatic side chains); (2) alteration of the solvent structure. We shall not consider here salts with large apolar chains which can be bound to apolar regions of the protein. Geschwind (1960) reported that aqueous mixtures of urea and lithium bromide showed high viscosities; similar viscosity increases were found by Bello and Bello (1961, 1962) for aqueous solutions of lithium chloride or bromide with *N*-methylacetamide (NMA)[1] or *N,N*-dimethylacetamide (DMA). DMA was investigated as an analog of proline and hydroxyproline which contain no NH group when present in peptide linkage. These observations led to the suggestion that lithium halides interact with the amide groups, and that they denature proteins by interaction with peptide or other polar groups. Crystalline complexes of NMA with lithium chloride and bromide were obtained (Bello and Bello, 1962) from an aqueous environment and their structures have been determined by X-ray diffraction (Haas, 1964). In this communication, we report viscometric, calorimetric, and crystallographic data on the interactions of NMA and DMA with a number of denaturing salts.

Experimental Procedures

Calorimetry. The calorimeter was a simple dewar flask of 125-ml capacity. Temperature changes were measured with a thermistor (Yellow Springs Instrument Co.) of 0.1° sensitivity and pen recorder. The thermistor probe was fastened to a Teflon stirring paddle consisting of a perforated disk at the end of a rod. The temperature-sensitive end of the thermistor was positioned in one of the holes in the perforated disk so that liquid flowed over the probe tip during mixing. The reactants were brought to room temperature, the one to be used in larger volume was placed in the calorimeter, the paddle-thermistor assembly inserted, and the second reactant added rapidly with stirring. The highest steady temperature was read. Occasionally, a small sharp pike of higher temperature was recorded very shortly after addition of the second reactant. This was the result of poor mixing at the beginning. The rate of heat loss was negligible in relation to the other errors of the method. The calorimeter constant and specific heats of salt–amide solutions were determined from the temperature rise resulting from passing a known current through a resistor. Densities of solutions were either measured or taken from the "International Critical Tables." The total volume of reactants was 50 or 100 ml.

Viscometry. Viscosities were measured at 39° in Ubbelohde-type viscometers (Cannon Instrument Co., State College, Pa.). Aqueous salt solutions were mixed with NMA in various proportions.

Crystalline Complexes of Amides. Complexes of NMA or DMA were prepared by dissolving the salt in warm amide to saturation and cooling. The crystals were separated from mother liquor by rapid filtration and blotting with filter paper. The composition of the crystals containing bromide or chloride were inferred from titration with silver nitrate. The titration data are subject to error arising from incomplete blotting, moisture pickup, and trapped liquid. The titration data were required only as a guide for crystallographic work. The exact stoichiometry was obtained from the crystal structure analysis. The complex of urea with cysteine

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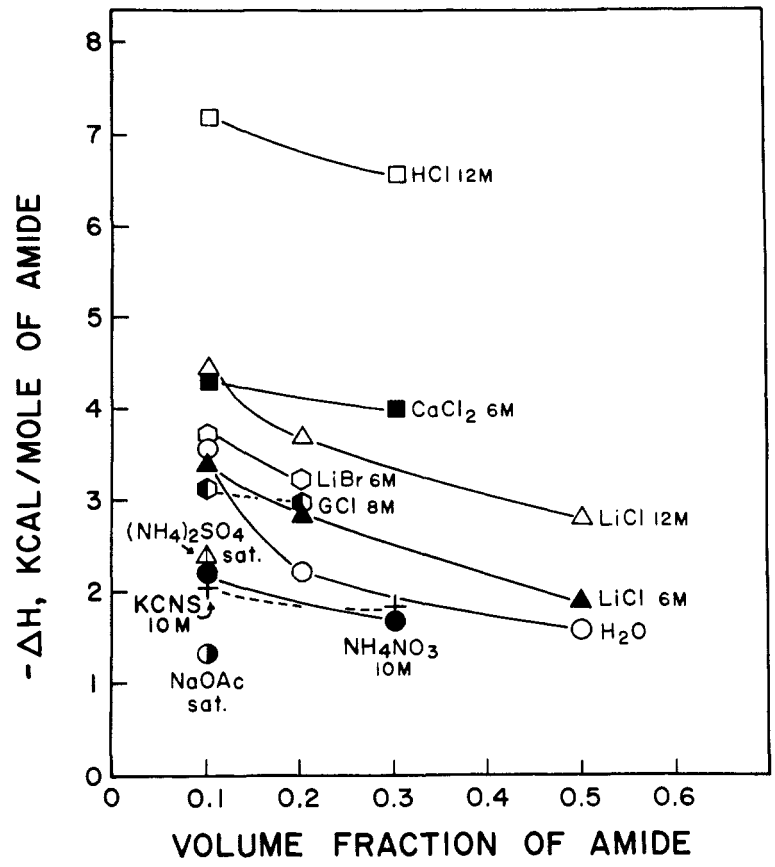
[1] Abbreviations: NMA, *N*-methylacetamide; DMA, *N,N*-dimethylacetamide; GCl, guanidinium chloride; ATGEE, acetyltetraglycine ethyl ester.



FGIRE 1: Heats of reaction of NMA with aqueous salts.

ethyl ester hydrochloride was prepared by evaporation of an aqueous solution of the two reactants and visual selection of crystals.

Urea Adducts. Twice-redistilled dodecanethiol (0.52 g, 0.0028 mole) was added to 25 ml of 8 M urea (containing 0.20 mole of urea) in a glass vial, which was rotated at about 10 rpm for 3 days. The white solid was filtered and dried over P_2O_5; yield 2.89 g. Nitrogen was analyzed by the Nessler method of Miller and Miller (1948). The adduct contained 35.4% nitrogen, equivalent to 76% urea; or 10.7 urea molecules/thiol.

Results

Viscometry of Amide–Salt Solutions. Following the work on urea–LiBr and on NMA or DMA with LiCl or LiBr, we extended viscometric measurements to other salts. Viscosities were measured over a range of compositions as described earlier for NMA–LiBr (Bello and Bello, 1961). The viscosities shown in Table I are those for the compositions of maximum viscosity. The effects are in the order: $CaCl_2 > LiBr \approx LiCl > LiNO_3 > NaBr \approx GCl > KCNS$, where GCl is guanidinium chloride. The order $LiNO_3 > NaBr$ is based on an observed linear relation between viscosity and salt concentration, and interpolation of the 7.4 M point for $LiNO_3$.

Calorimetry. The heats of mixing of NMA with aqueous salts are shown in Figure 1. The NMA contained 10% by volume of DMA to depress the melting point of NMA below room temperature. The discussion below is based on data at an amide volume fraction of 0.1. Data for more dilute solutions are probably not reliable. The error is probably about ±0.3 kcal/mole at 0.1-volume fraction, and less at higher mole fractions.

Crystalline Amide–Salt Complexes. During the course of this study, we prepared seven crystalline complexes, including one containing urea; the crystal structures of five of these were determined by three-dimensional X-ray crystallographic analyses. A crystalline complex of DMA with $CaCl_2$ was also isolated; no crystallographic data are yet available for this crystal. The detailed structural investigations are being reported elsewhere. Some of the most significant features of the five crystal structures solved to date are the following: (a) In the 1:1 L-cysteine ethyl ester hydrochloride–urea complex (Figure 2), urea is hydrogen bonded to the carbonyl oxygen and ammonium nitrogen of the amino acid derivative. (b) In the 4:1 NMA-lithium chloride complex (Figure 3), each lithium ion is coordinated by four carbonyl oxygens while the halide ion is hydrogen bonded by four NH groups (Haas, 1964). The NMA–LiBr crystal is isomorphous with the

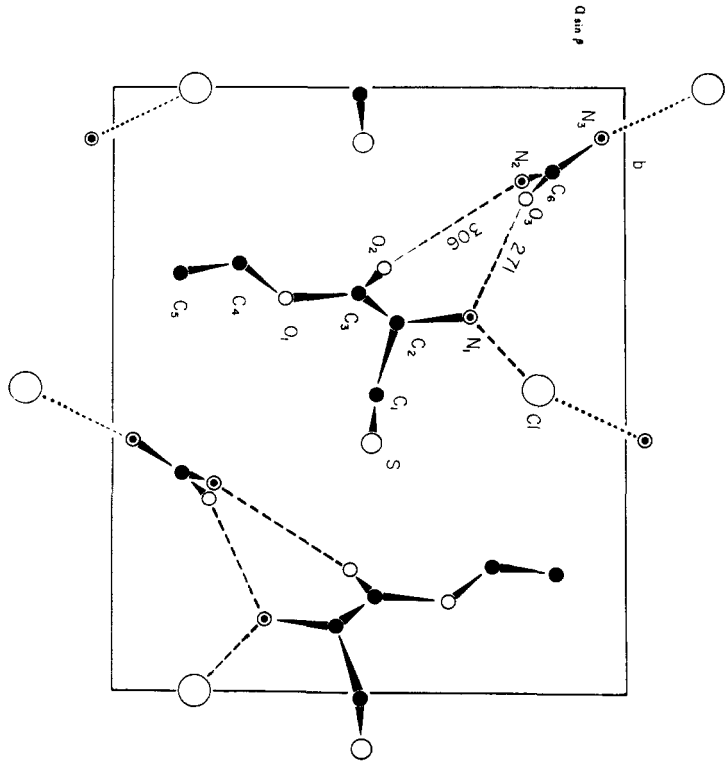


FIGURE 2: L-Cysteine ethyl ester hydrochloride–urea (1:1). The four-atom structure at the upper left is a urea molecule; in the cysteine ethyl ester · HCl molecule, C_4 and C_5 are the ethyl carbons, O_2 and O_1 are the ester oxygens. The structure can be described as alternating layers of chloride ions and hydrogen-bonded urea–cysteine ethyl ester molecules. O_3–N_1 and N_2–O_2 form the hydrogen bonds between the two independent molecules while N_1 and N_3 coordinate the chloride ions.

TABLE I: Viscosities[a] of Aqueous Salt–NMA Solutions.

Salt (M)	Vol Fraction NMA[b]	Viscosity
None (H_2O only)	0.70	1.5
KCNS (10)	0.70	2.3
NaBr (7.4)	0.64	2.3
GCl (9)	0.70	2.6
$LiNO_3$ (13)	0.64	5.3
LiCl[c] (6)	0.64	6.3
LiBr (10)	0.64	7.8
LiCl (10)	0.64	8.3
$CaCl_2$[c] (6)	0.65	17.5
LiBr (13)	0.81	Crystals formed

[a] Viscosity relative to that calculated on the assumption that the viscosity of the mixture is the sum of the viscosities of the amide and water–salt, each multiplied by its volume fraction in the mixture. [b] Volume fraction of NMA at which the maximum viscosity is obtained. [c] Data for DMA–salt solutions.

NMA–LiCl crystal, and must have the same structure. (c) In the 2:1 NMA–sodium perchlorate complex (Figure 4), amide nitrogen is hydrogen bonded to perchlorate ion and sodium is associated with carbonyl oxygen. (d) In the 1:3 DMA–guanidinium chloride complex, the DMA molecules are disordered and are located in continuous sinuous cavities, about 6 A in diameter, passing through the crystalline framework of guanidinium chloride (Figures 5 and 6). The crystallographic analysis of this structure has been described (Haas *et al.*, 1965). This structure is an inclusion compound in which the guest molecule is held, not only by dispersion forces, but also by a hydrogen bond to a guanidinium ion. The latter is inferred from the 2.8- to 3.1-A distances between oxygens and the nearest NH_2 groups. The guanidinium–carbonyl interaction appears to be very weak. In measuring the density of the crystal in a kerosene–carbon tetrachloride density gradient, the crystal rested only briefly at its density of 1.2 g/cm^3, then settled slowly to the bottom of the tube, without loss of crystalline appearance. The density of the crystals increased beyond that of guanidinium chloride itself. This behavior is probably the result of exchange

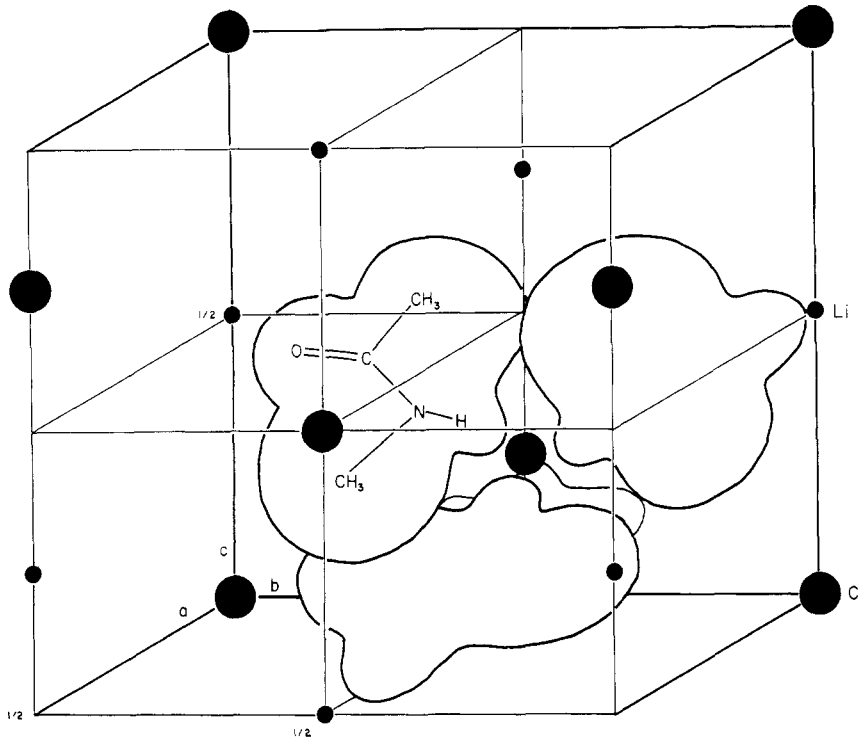


FIGURE 3: NMA–lithium chloride (4:1). A chloride ion is shown surrounded by the NH groups of four NMA molecules. A Li ion (right rear edge) is shown in proximity to the bulge of a carbonyl oxygen. The symmetry of the crystals places three more oxygens (not shown) tetrahedrally around the Li.

of carbon tetrachloride from the gradient for DMA in the cavities.

Discussion

We shall discuss the question of salt–amide interactions largely within the context of the experiments reported here. Robinson and Jencks (1965a,b) have recently reviewed in detail the evidence relating to salt–peptide and salt–solvent interactions, as well as similar data for urea; they conclude that the preponderance of the evidence points to peptide–salt interactions.

High viscosity can be interpreted as a manifestation of the presence of aggregates, held together by salt–amide interactions. The viscometric data suggest that the interaction of an amide group with KCNS, GCl, $LiNO_3$, or NaBr is weaker than with LiCl, LiBr, or $CaCl_2$; that lithium and calcium denature by mechanisms different from those of other ions. The small effect of NaBr indicates that both of these ions have little interaction with amides. However, bromide (as lithium bromide) was shown by X-ray diffraction to interact with NMA. The small effect of $LiNO_3$ compared with LiBr suggests that bromide interacts with amides more strongly than does nitrate. The large effect of $CaCl_2$, 2.8 times as great as that of LiCl, is in remarkable agreement with the observation that $CaBl_2$ lowers the melting point of a gelatin gel 2.7 times as much as does LiCl (Bello *et al.*, 1956). This similarity suggests that the mechanism of denaturation of the collagen-type helix by these two salts is similar to the mechanism of interaction with the model amide. Whether or not this is so, it appears likely that lithium and calcium operate by similar mechanisms, calcium being more effective as a result of its greater charge density. While high viscosity suggests the presence of salt–amide interactions, low viscosity does not necessarily demonstrate the contrary. Viscosity depends not only on the strength of the interactions, but also on the structure of the solution. Other types of evidence would be desirable. Therefore, we investigated the heats of reaction of aqueous salts with NMA, and the structures of crystalline amide–salt complexes.

Heats of Reaction. The observed heat of reaction is the resultant of the heats of several association and dissociation reactions. For the case of lithium chloride these are shown in the following equations with the expected or observed sign of ΔH. Analogous reactions may be written for other salts.

$$(NMA)_n \longrightarrow n\text{NMA} \qquad \Delta H = (+) \quad (1)$$

$$LiCl \cdot mH_2O \longrightarrow LiCl \cdot (m-q)H_2O + qH_2O \qquad \Delta H = (+) \quad (2)$$

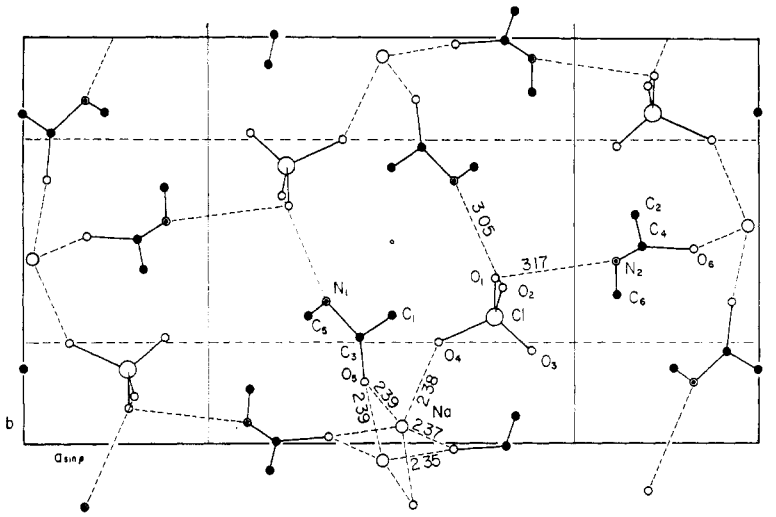


FIGURE 4: NMA–sodium perchloriate (2:1). In the lower center is a sodium ion coordinated to the carbonyl oxygens of three NMA molecules as well as to two ClO_4 ions. The N_1 of NMA is hydrogen bonded to ClO_4 (distance 3.05 A at right center also applies to distance N_1–O at left center, by symmetry). O_3 of the perchlorate ion is in contact with N_2 and lies along a line perpendicular to the plane of the molecule.

$$\text{NMA} + \text{LiCl}\cdot(m\text{–}q)\text{H}_2\text{O} \longrightarrow \text{NMA}\cdot\text{LiCl}\cdot(m\text{–}q)\text{H}_2\text{O} \qquad \Delta H = (-) \quad (3)$$

$$\text{NMA} + \text{H}_2\text{O} \longrightarrow \text{NMA}\cdot\text{H}_2\text{O} \qquad \Delta H = (-) \quad (4)$$

$$\text{NMA} + \text{LiCl}\cdot m\text{H}_2\text{O} \longrightarrow \text{NMA}\cdot m\text{H}_2\text{O}\cdot\text{LiCl} \qquad \Delta H = (-) \quad (5)$$

$$\text{LiCl}\cdot m\text{H}_2\text{O} + \text{H}_2\text{O} \longrightarrow \text{LiCl}\cdot(m+1)\text{H}_2\text{O} \qquad \Delta H = (-) \quad (6)$$

$$(\text{H}_2\text{O})_p \longrightarrow p\text{H}_2\text{O} \qquad \Delta H = (+) \quad (7)$$

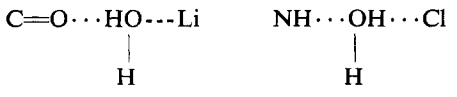
$$(\text{LiCl})_x \longrightarrow (\text{LiCl})_{x-y} + y\text{LiCl} \qquad \Delta H = (+) \quad (8)$$

$$|\Delta H_{(3)}| > |\Delta H_{(5)}| \qquad (9)$$

$$|\Delta H_{(2)}| > |\Delta H_{(6)}| \qquad (10)$$

$$|\Delta H_{(4)}| > |\Delta H_{(1)}| + |\Delta H_{(7)}| \qquad (11)$$

$\text{LiCl}\cdot m\text{H}_2\text{O}$ in (5) represents a more highly hydrated salt than $\text{LiCl}\cdot(m\text{–}q)\text{H}_2\text{O}$ in (3). The coefficients of (3–5) may differ from unity; the coefficient of H_2O in (6) is unlikely to be greater than 1. In reaction 3 direct amide–ion interactions are meant. Reactions 2 plus 3 represent the displacement of water by NMA to bring NMA into the first solvation shell. In reaction 5 structures such



are indicated. (A dotted line represents a hydrogen bond, and a dashed line an ion-dipole interaction.) Peptide–water–ion interactions are expected, and are suggested by enhanced viscosity effects in amide–D_2O–LiBr solutions (Bello and Bello, 1962). In (8) ion clusters undergo partial dissociation; subsequent solvation of dissociated LiCl would be exothermic. Inequality 11 is evident from Figure 1, and (9) is expected on the ground that interaction of NMA with Li ion will be stronger the smaller the hydration number of the latter. Similar reasoning applies to (10). No distinction is made between LiCl and its constituent ions. The above list does not exhaust all possible subreactions.

The most negative ΔH is shown by hydrochloric acid which is known to protonate amides. Calcium chloride (6 M), lithium chloride (12 M), and bromide (6 M) are next. Lithium chloride (6 M) produces a smaller effect than 12 M, but 3 M (not shown) has about the same effect as 6 M. In 12 M LiCl, the ratio of water:LiCl is 3.5:1; at 6 and 3 M the ratios are 8:1 and 17:1, respectively. Dehydration of lithium chloride is endothermic, but less so in 3 or 6 M solution than in 12 M solution. Dilution of 12 M LiCl with water was observed to be exothermic. The smaller $|\Delta H|$ in 6 M LiCl solution compared to 12 M may result from a larger ratio of reactions 5 to 3 at 6 M. At 3 M, reaction 5 should be still more important relative to reaction 3; (2) and (6) should be less important, and reactions 4 and 7 more important. $|\Delta H|$ of reaction 4 is smaller than that of reaction 3. These compensatory effects may account for the similarity of the results at 6 and 3 M. At salt concentration of about 1 M, the heat of reaction is to a large extent that of the amide–water interaction. This suggests that the equilibrium constants for the direct amide–salt interactions are small. This is discussed below in greater detail. With such a diversity of reactions, an unambiguous interpretation of the calorimetric data is difficult.

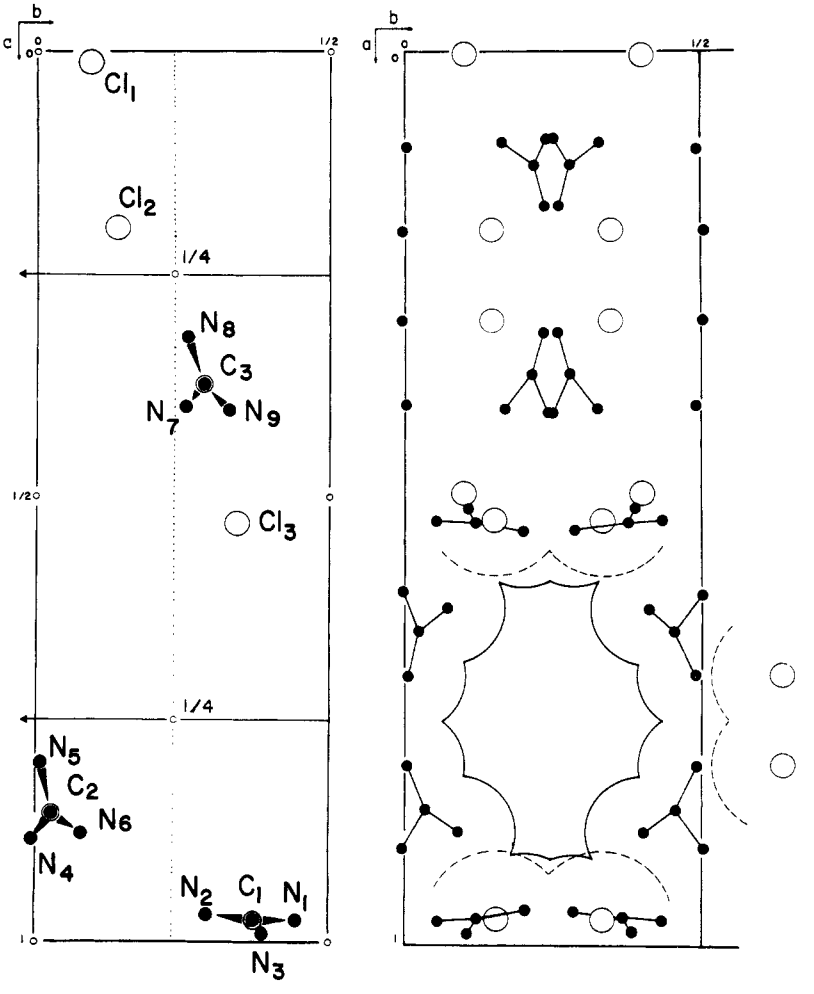


FIGURE 5: DMA–GCl (1:3). Left side: atoms in the asymmetric unit and the symmetry elements that give one-half of the atoms in the unit cell. The four-atom structures are G ions, the open circles chloride ions. Right side: projection along *c* axis of one-half of unit cell with cavity outlined by van der Waals radii of NH_2 groups of G ion (solid arcs) and crystal radii of Cl ion (dashed arcs). The DMA molecules fit into the cavity.

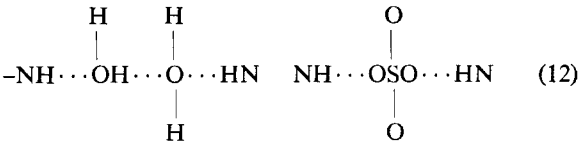
The free energy and entropy changes are not known at present.

The reaction of 6 M $CaCl_2$ (nearly saturated) with NMA is as exothermic as that of 12 M LiCl. The calorimetric data, like the viscometric, indicate that calcium and lithium ions denature by similar mechanisms. $|\Delta H|$ for 6 M LiBr is greater than for 6 M LiCl, in agreement with their relative effects as denaturants. These results suggest that bromide interacts with amides more strongly than does chloride. But reaction 2 may be less endothermic for LiBr than for LiCl. However, chloride appears to have little denaturing effect as judged by the action of NaCl. This is based on the assumption that sodium also has little effect. The same total effect could arise from assigning to sodium a large denaturing effect and to chloride a similar antidenaturing effect, or *vice versa*. Any arbitrary assignment of effects to sodium and chloride ions would lead to a self-consistent, if unrealistic, set of values for the entire lyotropic series.

GCl produces a heat of reaction similar to those of LiBr and LiCl while its effect as a denaturant is equal to that of the former and greater than that of the latter (Bello *et al.*, 1956; von Hippel and Wong, 1964). The effect of GCl on the viscosity of NMA is smaller than those of lithium and calcium chlorides. The viscosity data suggest that GCl may act by a different mechanism from LiBr and LiCl; but the heat data point to similar mechanisms. However, guanidinium does not belong in the same series as the other cations, as it is larger and has hydrogen-bonding potentialities. A saturated solution of GCl in anhydrous DNA is very viscous, a manifestation of the interaction in the crystalline complex.

Smaller heat effects are produced by potassium thiocyanate and ammonium nitrate, both effective denaturants. Thiocyanate is a more effective denaturant of the collagen helix than either lithium chloride or GCl (von Hippel and Wong, 1964). The viscosity and heat data are not in support of a peptide–anion interaction for these salts, although a contribution from protein–ion interactions is suggested by the crystal data. For these salts endothermic reactions analogous to (2) may be more important than for LiCl and $CaCl_2$. Robinson and Jencks (1965a,b) have proposed that denaturing anions lose some water in order to interact with peptide groups. The fact that these denaturants show heats of reaction similar to those of the antidenaturants sodium acetate and ammonium sulfate makes it still more difficult to decide on the mechanism of denaturation.

The heat of reaction of ammonium sulfate includes the heat of precipitation of a portion of the salt; ammonium sulfate is much less soluble in NMA than are the denaturants. This points to a much weaker interaction for this salt and suggests that antidenaturation is unlikely to involve salt–peptide interactions. It is difficult to see how general salt–peptide interactions could protect a protein against denaturation. Such interactions are more likely to destablize a protein by preventing normal intramolecular associations. If antidenaturing salts exert their effect, in part, by direct action on protein, it may be by binding to special sites, probably cationic. Sulfate in crystalline myoglobin occupies a cationic site (Kendrew, 1962). If the native protein contains stabilizing water bridges, substitution of an ion for water to form an ion bridge might increase stability, as in (12)



(12)

The sums of the bond lengths are nearly the same; two NHO and one OHO bridges total 8.2–8.7 A, and two NHO bridges and one OSO unit total about 8.4–8.8 A (Pauling, 1960). The difference in conformation may be small if some departure from linear hydrogen bonds is permitted.

The antidenaturing salts are also used for crystallizing proteins. It is widely accepted that many proteins exist in solution in a multiplicity of conformations of nearly the same energy. This may make it difficult to fit into a crystal lattice. Relative stabilization of one conformation by binding of antidenaturing ions may lead to crystallization. Generally, some salt is required for crystallization. For example, salt-free ribonuclease does not crystallize from 2-methylpentane-2,4-diol; addition of a small stoichiometric quantity of phosphate, 2 moles/mole of ribonuclease, affords crystals.

Amide–Water Interaction. The reaction of NMA with water is exothermic by about 3.5 kcal/mole of amide for a water:amide molar ratio of 35. Since this exothermic reaction includes the two endothermic reactions 1 and

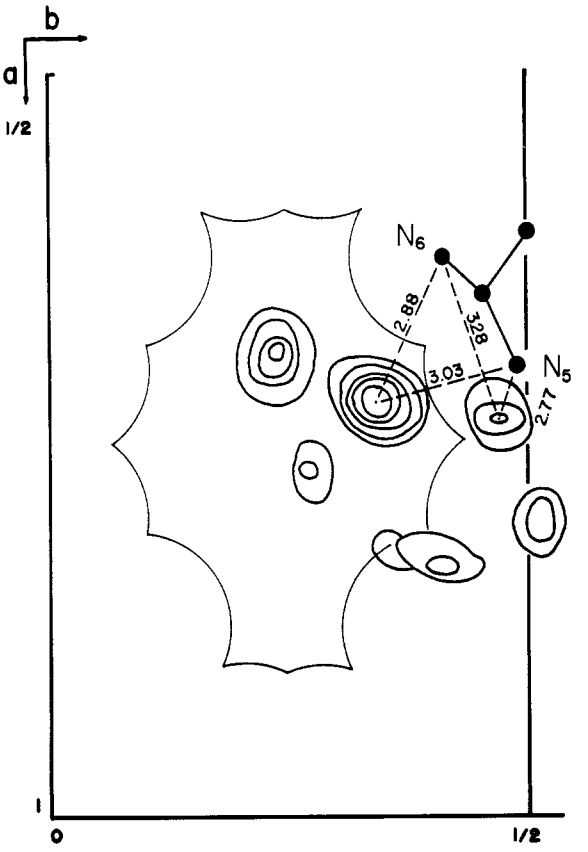
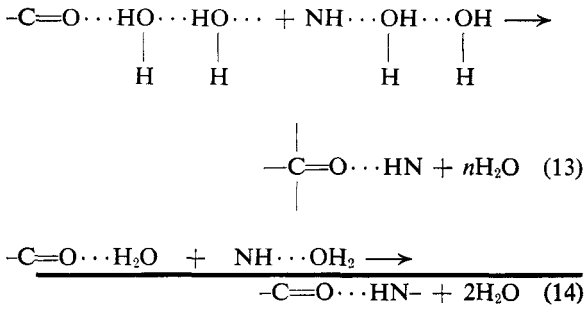


FIGURE 6: DMA–GCl. The cavity with electron density contours of the relatively stationary atoms of the DMA, probably oxygens, showing distances to nearest nitrogen atoms of a G ion.

7, it is probable that most of the NMA is hydrogen bonded to water rather than to NMA. This is in agreement with the observation of Klotz and Franzen (1962) that there is very little interamide hydrogen bonding in dilute solutions of NMA in water. By extrapolation to low NMA concentrations, Klotz and Franzen were able to measure the extent of reaction for dimerization of NMA and found $\Delta H^\circ = 0.0$ kcal/mole. At high amide concentration (10–12 M) Klotz and Franzen found that 50–80% of the NH groups were hydrogen bonded to carbonyl. Much of this increase must arise from there being an insufficient amount of water present to bind to all of the NMA, some from the less negative ΔS for interamide hydrogen bonding resulting from the close proximity of amides. Némethy *et al.* (1963) and Schellman (1955) have presented evidence that formation of interpeptide H bonds in water has a negative ΔH. Some of the greater degree of interamide hydrogen bonding at high amide concentration may arise from a more negative ΔH than in dilute amide solution. Frank (1958) has pointed out that on forming a hydrogen bond between two water molecules, the two become more polar and can form stronger hydrogen bonds with other water molecules. The same principle applies to the formation

of peptide–water hydrogen bonds. In dilute solution, amide probably is hydrated as in the left side of eq 13, while in concentrated amide solution the situation is more like that of (14)



In (13), the effect of binding a second, or third, water to the one bonded to C=O is to make the hydrogen of C=O···H more positive, and to make the C=O···H bond stronger than if only one water were involved. Similarly, the NH···O bond is strengthened because the oxygen is more negative than it would be if only one water were involved. Thus, interamide hydrogen bonding in reaction 14 should be more exothermic than in reaction 13. In passing from a fairly compact, almost native protein conformation, stabilized by hydrophobic forces, to a fully native conformation, the hydrogen bonding process may resemble (14) rather than (13), resulting in a favorable ΔH. Thus, the stability of interamide hydrogen bonds within a compact protein may be considerably greater than expected from the results of the dimerization of dilute NMA.

Crystal Complexes. The existence of hydrogen bonding in the urea–cysteine ester crystal is of interest in connection with hydrogen bonding of urea to peptides. Evidence for such hydrogen bonding has been discussed by numerous workers, recently, in particular, by Robinson and Jencks (1963). A crystalline complex of urea with diketopiperazine was reported by Gill *et al.* (1961). On the other hand, the work of Levy and Magoulas (1962) has often been cited to show that urea–peptide bonds are unimportant. These authors showed that 8 M urea has little effect on the presumed intramolecular hydrogen bonds of maleic acid and the acid maleate ion. This result is, of course, strongly influenced by the entropy change of the reaction. In the case of a protein, the entropy effect is quite different from that of a small molecule. The possibility of ordered urea structures around the hydrocarbon chain has been proposed (Bruning and Holtzer, 1961), but Frank (1964) has considered it unlikely in the presence of water, say in 8 M urea. We have found that 1-dodecanethiol and tridecane give solid inclusion compounds from aqueous 8 M urea, but a short chain such as hexane does not. In the case of short side chains of proteins, which are attached to the bulky backbone, a well-ordered urea structure is unlikely. Abu-Hamdiyyah (1965) has suggested that aqueous urea forms clusters with cavities that can better accommodate apolar groups than can the cavities of water. Essentially, this is an argument for a partially ordered urea–water clathrate cage. Perhaps these mixed clusters have an excess of donatable hydrogens for bonding to the polar groups of proteins, resulting in denaturing by a combined mechanism.

Hibberd and Alexander (1962) have studied the effect of urea on hydrogen bonding in monolayers of octadecylacetamide. Urea decreased the temperature of transition of the hydrogen-bonded two-dimensional crystal to, presumably, a nonbonded conformation of lower order. This latter was proposed to be a *cis* conformation. A *cis*-amide conformation is very unlikely in the absence of special influences.[2] It is more likely that the "melting" of the film arises from some cause other than conversion from *trans* to *cis*. It could arise from breaking of the hydrogen bonds as a result of hydration of the amide groups and accompanying rotation of CC bonds in the octadecyl chain. In the case of urea, interamide hydrogen bonds may be broken by formation of amide–urea bonds, giving a result opposite to the conclusion of Levy and Magoulas, as noted by Scheraga (1963). It is also possible that in the monolayer case amide–urea hydrogen bonding is not the primary cause of melting, but rather that urea provides an environment more hospitable to the apolar group than does water. The entropy change in the case of the "melting" of an extensively hydrogen-bonded and hydrophobically bonded monolayer is more like that of protein denaturation than is the entropy change in the maleic acid experiments.

The interaction of DMA with GCl in the crystalline complex suggests interaction in denaturation by guanidinium ion. This very weak amide–guanidinium interaction, which can be disrupted even by the nonpolar liquid carbon tetrachloride, may not be strong enough to exist in water to an appreciable extent, although Robinson and Jencks (1965a) have estimated a binding constant of 0.92 for GCl with ATGEE.

Obviously, it is difficult to extrapolate from crystal data to aqueous protein solutions. While some of the interactions shown, *e.g.*, Li–carbonyl, ClO_4–HN, etc., are attractive in explaining the effectiveness of some ions as denaturants, the Na–carbonyl and Cl–HN interactions would lead to the prediction that sodium chloride should be an effective denaturant, which is not the case. Secondly, can interactions such as those in the crystal occur in the presence of high concentrations of water; *i.e.*, what are the association constants, k, for salt–amide interactions? For the case of gelatin–$CaCl_2$ an estimate of 0.1–0.4 was proposed (Bello, 1963, 1965) on the basis of a comparison of the relative effects of $CaCl_2$ and biuret complex formation on the collagen-like helix of gelatin. An estimate of 0.1, or 0.2, if corrected for the activity coefficient of $CaCl_2$ (Robinson and Stokes, 1959), was calculated for gelatin–$CaCl_2$ by Mandelkern and Stewart (1964) based on the thermodynamics of polymer melting. For ATGEE–$CaCl_2$, Robinson and Jencks (1965b) calculated $k = 0.2$. The agreement among these three estimates is remarkable.

[2] We are grateful to Dr. David Harker for valuable discussion on *cis*- and *trans*-amide bonds in crystalline amides.

Robinson and Jencks also made estimates of k for several other salts, *e.g.*, 0.41 for LiBr, 0.72 for KSCN, and 0.92 for guanidinium chloride. These values, especially the last, appear rather high in the light of our viscosity and ΔH data and of the relative denaturing effects of $CaCl_2$ and of GCl on gelatin and collagen. These values of k were calculated from the solubilities of ATGEE in the salt solutions on the basis that the increase of solubility arises from a peptide–salt interaction. Possibly, ATGEE does bind these salts more strongly than does NMA or gelatin. Another difference is that LiCl melts the collagen-like helix of cold gelatin solutions, but salts out ATGEE; generally, denaturants salt in ATGEE.

Peptide groups are highly polar and should be able to compete, to some extent, with water for ions. The question of competitive hydration of peptide groups and ions has been discussed by Robinson and Jencks (1965b). Also, for some of the common denaturants effective denaturation does not take place until the denaturant concentration has reached 3–6 M. (However, for some proteins denaturation takes place at lower salt concentration.) In these solutions the water:salt ratio is typically 20:1 to 10:1 or less; at the lower ratio the charges on the ions are not as well saturated as in dilute solution, and salt–peptide association constant should increase after an initial lag at low salt concentration. This may explain, in part, the sigmoid shapes of the isothermal curves of nativeness (as expressed, *e.g.*, by optical rotation or viscosity) *vs.* salt concentration. This may be mimicked for a curve of salt–amide association *vs.* salt concentration by suitable assignment of association constants at various salt concentrations. The effectiveness of salts at high concentrations arising from incomplete hydration is reasonable in that the ion–peptide interaction should be stronger for less hydrated ions. Ions such as calcium or lithium which have strong affinities for water may act in the hydrated form (Robinson and Jencks, 1965b; Bello and Bello, 1961, 1962). As the salt concentration becomes high, direct peptide–ion interactions probably become important even for ions with a strong affinity for water. Also, as the salt concentration increases, the proportion of ion pairs and higher aggregates increase; these may be better denaturants than are the individual ions. The effectiveness of a denaturant is not directly related to its activity; *e.g.*, for $CaCl_2$, the rise in activity coefficient lags behind the denaturing power. However, the activity coefficients available are the mean values for both ions; it is possible that the activity of the more effective ion increases faster than the mean and is parallel to the denaturing effectiveness. An alternate explanation for the sigmoid curve of nativeness *vs.* salt concentration is that over a considerable range of salt concentration, a large destabilization of native structure can take place with little change in the ratio of native to denatured protein. Then, over a small increment of salt, a change from a slightly stabilized native conformation to a slightly stabilized denatured conformation can produce a large amount of denatured protein.

In addition to direct ion–peptide interactions, salts may act by increasing the denaturing power of water. This could arise by disruption of the water structure with liberation of hydrogen-bonding groups, or by interaction with ions to change the hydrogen-bonding ability of water (Bello and Bello, 1962). Thus, in the hydrated lithium ion the hydrogens are more positively charged than in ordinary water and are, therefore, better able to compete for a receptor site in the protein. (The oxygen is less negative and, therefore, a poorer hydrogen acceptor.) The reverse is the case for anions. The result would be that the solution would contain better hydrogen-bond donors and acceptors to react with the protein. Robinson and Jencks (1965b) have proposed that a hydrated cation may be hydrogen bonded to two peptide groups, but that hydrated anions are not effective. Binding of a cation to two peptide groups may give rise to intramolecular cross-links.

The data presented here suggest that there are ions that operate directly on proteins, *e.g.*, lithium and calcium; for these ions all three criteria, viscosity, heats of reaction, and crystal complexes, are in agreement. For other salts, action on both protein and solvent may be involved, although the latter is not directly supported by the data, but only inferred from the small viscosity and heat effects. One or the other mechanism may be dominant depending on the concentration of salt, the nature of the protein, and the stage of denaturation.

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Intramolecular Interactions in Flavin Nucleotides*

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ABSTRACT: The intramolecular interactions of flavin–adenine dinucleotide (FAD) have been investigated using optical rotatory dispersion techniques. A Cotton effect with a trough at 395 mμ and a peak at 355 mμ was observed for flavin–adenine dinucleotide in aqueous solution. An equimolar mixture of its constituent mononucleotides, riboflavin 5′-phosphate and adenosine 5′-phosphate, exhibited a curve with no maxima or minima in these wavelength regions. Moreover, the shapes of this curve and that of FAD between 280 and 220 mμ were dissimilar, and the rotational magnitude near 260 mμ in the case of the dinucleotide was almost 10-fold greater than that observed for the combined mononucleotide components. These divergences were attributed to intramolecular interactions occurring in FAD. Environmental conditions that disrupt such interactions were then studied in relation to specific changes in optical rotatory dispersion properties. Several solvents of low dielectric constant were shown to diminish the amplitude of the Cotton effect of FAD in the region of 400–350 mμ and, in the case of riboflavin 5′-phosphate, induce a Cotton effect in this spectral region. In contrast to results obtained with water as solvent, FAD in certain nonaqueous solvents exhibited optical rotations equal to the added rotations of its component mononucleotides.

It is well established that flavin–adenine dinucleotide (FAD)[1] and nicotinamide–adenine dinucleotide (NADH) exist in folded conformations, with the two component ring systems of either compound occurring in close spatial juxtaposition. The internal complex formation, *i.e.*, interaction, between the adenylyl and isoalloxazine moieties of FAD results in marked quenching of the flavin fluorescence (Weber, 1950; Bessey *et al.*, 1949). Furthermore, the absorption spectra of the constituent mononucleotides of FAD are not additive in the dinucleotide spectrum (Warburg and Christian, 1933; Whitby, 1953). Some specific structural requirements for formation of intramolecular complexes of this type have been elucidated (Tsibris *et al.*, 1965; Chassey and McCormick, 1965). Following the action of nucleotide pyrophosphatase on NADH, increased absorption of light at 260 mμ was observed (Seigel *et al.*, 1959) together with disappearance of the fluorescence spectrum due to activation at 260 mμ (Kornberg and Pricer, 1950). One should note that the constituent mononucleotides of NADH are not fluorescent; adjacent to one another in the dinucleotide, however, they allow transfer of excitation energy from adenine to pyridine moieties (Weber, 1957, 1958). When the nucleotide coenzymes are bound to specific proteins, a wide variety of properties due to interactions occurs (Beinert, 1960; Kaplan, 1960).

Ultraviolet optical rotatory dispersion techniques have been applied to the study of the ordered stacking of the bases in nucleic acids (Samejima and Yang, 1964, 1965) and in polynucleotides (Sarker and Yang, 1965), and to the thermal disruption of these structures.

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[1] Abbreviations used in text: FAD, flavin-adenine dinucleotide; FMN, riboflavin 5′-phosphate; NAD^+ and NADH, oxidized and reduced nicotinamide-adenine dinucleotide; AMP, adenosine 5′-phosphate.