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# The Effect of Guanidinium, Carbamoylguanidinium, and Guanylguanidinium Salts on the Solubility of Benzoyl-L-tyrosine Ethyl Ester and Acetyltetraglycine Ethyl Ester in Water*

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ABSTRACT: The effects of the chloride, bromide, iodide, and thiocyanate salts of guanidinium, carbamoylguanidinium, and guanylguanidinium cations on the water solubility of benzoyl-L-tyrosine ethyl ester, a model hydrophobic compound, and acetyltetraglycine ethyl ester, a model peptide and amide compound, have been examined. Regardless of the cation used, the solubility of both model compounds increases progressively through the series chloride < bromide < iodide < thiocyanate. This anion series parallels the effectiveness of these anions as denaturants of several proteins. When the anion is held constant, the cation effect on the solubility of benzoyl-L-tyrosine ethyl ester is guanidinium < guanylguanidinium < carbamoylguanidinium and the cation effect on the solubility of acetyltetraglycine ethyl ester is guanidinium < carbamoylguanidinium < guanylguanidinium.

It is concluded that the protein denaturing effectiveness of these guanidinium, carbamoylguanidinium, and guanylguanidinium salts is due to their ability to increase the solubility of protein hydrophobic and peptide amide groups.

The denaturing effectiveness of salts of guanidinium, carbamoylguanidinium, and guanylguanidinium cations toward rabbit muscle aldolase, ovalbumin, and bovine serum albumin increases through the series: $Cl^- < Br^- < I^- < CNS^-$ (Castellino and Barker, 1968a). This anion series parallels that obtained by Hofmeister (1888) for a large number of ionic phenomena including precipitation of proteins. When combined with neutral inorganic salts, essentially the same anion series exists for denaturation of collagen (Bello *et al.*, 1962; von Hippel and Wong, 1962), ribonuclease (von Hippel and Wong, 1964), DNA (Hamaguchi and Geiduschek, 1962), and fumarase (Massey, 1953). When the anion is held constant the cation effect on the denaturation of rabbit muscle aldolase, ovalbumin, and bovine serum albumin increases through the series guanidinium < carbamoylguanidinium < guanylguanidinium (Castellino and Barker, 1968a).

This report deals with the effect of the chloride, bromide, iodide, and thiocyanate salts of guanidine, carbamoylguanidine, and guanylguanidine on the solubility of models for the hydrophobic and amide groups which are exposed to the solvent in the denatured state of a protein. A model hydrophobic compound (BTEE, I)[1] and a model peptide (ATGEE, II) were examined and the effectiveness of the salts in solubilizing the compounds was compared with the effect of urea.

## Materials and Methods

*BTEE* was purchased from Cyclo Chemical Co. and used without further purification.

*Acetyltetraglycine Ethyl Ester-$^{14}C$.* Tetraglycine ethyl ester was prepared according to the method of Fischer (1904) and twice recrystallized from ethanol. Acetylation was performed by dissolving 1.5 g of tetraglycine ethyl ester in 150 ml of 40% aqueous pyridine and slowly adding a total of 2.5 ml of acetic anhydride-1-$^{14}C$ (New England Nuclear Corp.) at 4°. Crystals appeared upon concentration in a rotary evaporator under water-aspirator pressure. The product was recrystallized from water to constant specific activity, and a final yield of 60% was obtained. The product melted at 267° with decomposition, in good agreement with the mp of 264° reported for this compound by Robinson and Jencks (1965a). The specific activity of the ATGEE-$^{14}C$ was 22,215 cpm/mg at approximately 70%

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[1] Abbreviations used are: BTEE, benzoyltyrosine ethyl ester; ATGEE, acetyltetraglycine ethyl ester; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

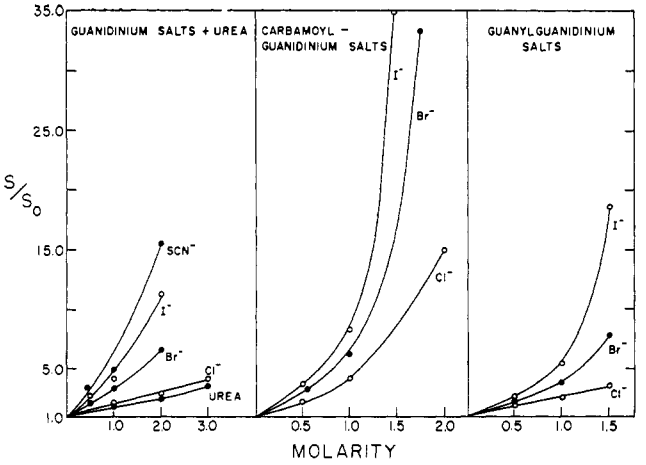



FIGURE 1: The effect of urea, guanidinium salts, and substituted guanidinium salts on the solubility of BTEE, showing the effectiveness of the anion series. Temperature 27 ± 0.3°.

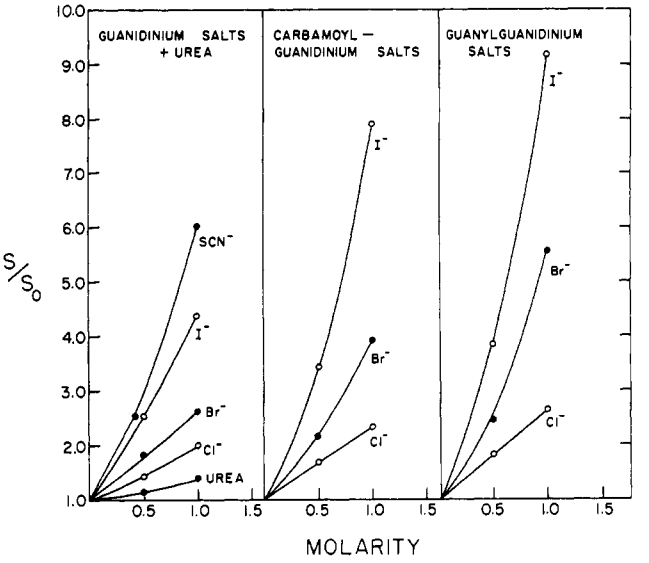
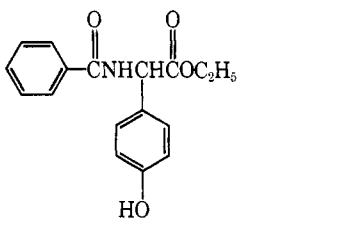



FIGURE 2: The effect of urea, guanidinium salts, and substituted guanidinium salts on the solubility of ATGEE, showing the effectiveness of the anion series. Temperature 27 ± 0.3°.



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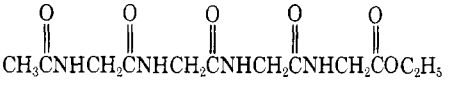

II

efficiency. The solubility of ATGEE-$^{14}$C in water at 27°, as determined by radioactive content, was 0.83 mg/ml, in agreement with the solubility of 0.78 mg/ml at 25° determined by the biuret method (Robinson and Jencks, 1965a).

*Salts.* Guanidinium hydrochloride was purchased from Eastman Organic Chemicals and purified as previously described (Castellino and Barker, 1968b). Urea was purchased from Eastman Organic Chemicals and recrystallized from absolute ethanol at 50° immediately prior to use.

Guanidinium thiocyanate was purchased from Eastman Organic Chemicals and used without further purification.

Carbamoylguanidinium, guanylguanidinium, and all other guanidinium salts used were prepared as previously reported (Castellino and Barker, 1968a).

*Solubility Determinations.* An excess of BTEE or ATGEE was placed in a 2-ml ampoule, approximately 0.5–1.0 ml of the appropriate solvent was added, and the vial was sealed. The vial was placed in a rocking water bath maintained at 26.8–27.3° for 4 days. Approximately 72 hr was required to reach equilibrium. At the end of this period the seal was broken, the solution was filtered rapidly through a Millipore filter, and the filtrate was assayed for ATGEE-$^{14}$C or BTEE.

The concentration of ATGEE was determined by counting the $^{14}$C present in 50 μl of the filtrate using a Packard Model 2002 scintillation counter. All standards and samples were counted in 15 ml of the scintillation fluid consisting of 3 g of PPO, 0.12 g of POPOP, 500 ml of toluene, and 12.5 ml of methanol. None of the salts used an solvents in this study at their highest concentrations had an effect on the counting rate of standard samples of ATGEE-$^{14}$C.

The concentration of BTEE was determined by measuring the absorbance at 272–273 mμ of a dilution of the filtrate with $H_2O$ using a Cary 15 spectrophotometer. A blank was used consisting of the solvent diluted in the same fashion. Dilutions of 3–20-fold were made.

The solubility of BTEE in all solvents was very small and its molar extinction coefficient could not be ascertained. For this reason concentrations of BTEE are reported in terms of absorbancy. In water at 27° a saturated solution of BTEE has an absorbance of 1.50.

## Results

*Benzoyltyrosine Ethyl Ester.* The absorbances at 272 mμ of saturated solutions of BTEE are given in Table I; each is an average of two determinations made on separate samples. The maximum variation observed was 6%. Appropriate blanks were used in all cases since some of the solvents absorbed at 272 mμ. The effect of all salts on the $\lambda_{max}$ and the absorbance of a saturated solution of BTEE in water was examined by adding an equal volume of concentrated salt solution to bring the concentration of salt up to the highest concentration used in any experiment. In all cases the recorded absorbance was reduced to one-half its value in $H_2O$ and the $\lambda_{max}$ was unchanged. Therefore, it was concluded that the only effect of added salts (or urea) was to increase the solubility of the solute (BTEE).

Plots of the solubility ratio $S/S_0$ of BTEE *vs.* molarity of added salt (or urea) are presented in Figure 1. $S_0$ and $S$ are the molar solubilities (absorbances) of BTEE in water and the solvent indicated.

Free energies of transfer, $\Delta F_t$, of BTEE from water to each solvent used in this study are given in Table I. The $\Delta F_t$ was

TABLE I: The Effect of Urea and Guanidinium Salts on the Solubility of BTEE and ATGEE in Water at 27 ± 0.3° and the Free Energies of Transfer for the Process.

| | | BTEE | | ATGEE | |
|---|---|---|---|---|---|
| Solvent | M | $A$ at 272 mμ ± 0.03 | $-\Delta F_t$ (cal/mole) | Solubility (mg/ml ± 0.04) | $-\Delta F_t$ (cal/mole) |
| $H_2O$ | | 1.50 | | 0.83 | |
| Urea | 0.5 | | | 0.96 | 88 |
| | 1.0 | 1.83 | 119 | 1.15 | 188 |
| | 2.0 | 2.43 | 286 | | |
| | 3.0 | 3.53 | 512 | | |
| Guanidinium chloride | 0.5 | | | 1.19 | 213 |
| | 1.0 | 2.09 | 191 | 1.67 | 411 |
| | 2.0 | 2.93 | 399 | | |
| | 3.0 | 4.17 | 608 | | |
| Guanidinium bromide | 0.5 | 2.16 | 215 | 1.54 | 367 |
| | 1.0 | 3.25 | 460 | 2.18 | 574 |
| | 2.0 | 6.61 | 882 | | |
| Guanidinium iodide | 0.5 | 2.77 | 370 | 2.14 | 565 |
| | 1.0 | 4.17 | 773 | 3.64 | 888 |
| | 2.0 | 11.36 | 1204 | | |
| Guanidinium thiocyanate | 0.42 | 3.41 | 465 | 2.09 | 569 |
| | 1.0 | 4.80 | 692 | 5.03 | 1073 |
| | 2.0 | 15.50 | 1389 | | |
| Carbamoylguanidinium chloride | 0.5 | 2.38 | 268 | 1.40 | 309 |
| | 1.0 | 4.05 | 596 | 1.94 | 499 |
| | 2.0 | 14.98 | 1371 | | |
| Carbamoylguanidinium bromide | 0.54 | 3.23 | 459 | 1.78 | 447 |
| | 1.0 | 6.25 | 852 | 3.22 | 811 |
| | 1.75 | 33.33 | 1848 | | |
| Carbamoylguanidinium iodide | 0.5 | 3.68 | 537 | 2.85 | 733 |
| | 1.0 | 8.33 | 1025 | 6.59 | 1234 |
| | 1.5 | 35.71 | 1890 | | |
| Guanylguanidinium chloride | 0.5 | 2.00 | 173 | 1.52 | 360 |
| | 1.0 | 2.53 | 310 | 2.21 | 583 |
| | 1.5 | 3.38 | 483 | | |
| Guanylguanidinium bromide | 0.5 | 2.27 | 244 | 2.04 | 537 |
| | 1.0 | 3.84 | 560 | 4.61 | 1019 |
| | 1.5 | 7.81 | 984 | | |
| Guanylguanidinium iodide | 0.5 | 2.66 | 340 | 3.21 | 805 |
| | 1.0 | 5.36 | 757 | 7.68 | 1323 |
| | 1.5 | 18.75 | 1508 | | |

calculated from the equation $\Delta F_t = -RT \ln S/S_0$, and represents the free energy of transfer on the molar scale.

*Acetyltetraglycine Ethyl Ester.* The concentrations of ATGEE required to saturate the various solvents are given in Table I. Each value represents an average of two separate determinations which in all cases differed by less than 4%. The actual cpm recorded for 50 μl of sample ranged from 923 cpm in water to 8529 cpm in guanylguanidinium iodide. Counting errors were less than 2%. That the salts had no effect on the counting efficiency of ATGEE-$^{14}$C was demonstrated by counting a mixture of 50 μl of a saturated aqueous solution of ATGEE-$^{14}$C and 50 μl of a concentrated solution of each salt used.

Plots of the solubility ratio ($S/S_0$) of ATGEE in each solvent are presented in Figure 2.

$\Delta F_t$ of ATGEE from water to each solvent employed in this study are given in Table I and were calculated in the same manner as those for BTEE.

## Discussion[2]

The two compounds used in this study were chosen as mod-

[2] No attempt is made to review the extensive literature on the interactions of proteins with urea and ionic denaturants. Reference is made only to immediately relevant material.

els for the peptide bond (ATGEE) and for a typical hydrophobic group (BTEE). They are not perfect models since both are contaminated with substituents which are not found in proteins. For example, there are contributions to $\Delta F_t$ from ethyl and methyl groups in ATGEE which are not present in a simple peptide. The contribution is probably not large since the value of $S/S_0$ for ethyl acetate in 4.0 M guanidine hydrochloride is only 1.15 (Robinson and Jencks, 1965a) and the highest concentration of salt used in this study was 1.0 M. The $\Delta F_t$ values obtained for AGTEE should therefore reflect a property of the peptide groups.

In the case of BTEE the values of $\Delta F_t$ include transfer of a single peptide bond as well as the transfer of the hydrophobic residues. However, it is unlikely that this peptide bond is responsible for the effect observed.

The increased solubility of BTEE and ATGEE in the various guanidinium, carbamoylguanidinium, and guanylguanidinium salts may be due to a direct interaction between the salt and BTEE or ATGEE or to an indirect effect of the salt on the structure of water. The data presented do not allow any conclusions to be drawn regarding the mechanism of the solubilization process. However, the following findings argue against, but do not disprove, a direct interaction of the salts with BTEE. (1) When the ultraviolet spectrum of BTEE in the various solvents is compared with that in water it is found that neither the shape of the curve nor the absorption maximum is altered. (2) The magnitude of the absorption at the $\lambda_{max}$ in $H_2O$ is not affected by addition of any salt. (3) After equilibration of BTEE with each of the solvents, the solutions were filtered and the crystals ground in a mortar and dried. The melting point was then checked to determine whether a new solid phase had been deposited. In all cases the melting point was identical with that of authentic BTEE.

A similar effect of anions on the solubility of ATGEE as observed here was reported by Robinson and Jencks (1965b) for neutral, inorganic salts. These authors concluded that this effect was due to a direct interaction of the anions with the polarizable amide group.

If such a direct interaction occurs, then the anions of the guanidinium, carbamoylguanidinium, and guanylguanidinium salts should interact with ATGEE in a manner similar to those of the neutral, inorganic salts.

The effect of cations on the solubility of ATGEE increases through the series guanidinium < carbamoylguanidinium < guanylguanidinium. In this case a direct interaction of the cation with the peptide backbone of ATGEE stabilized by polyfunctional hydrogen bonds is possible (Robinson and Jencks, 1965b; Castellino and Barker, 1968a). The effectiveness of guanylguanidinium and carbamoylguanidinium ions suggests that they might form more stable hydrogen-bonded structures than guanidinium ion. These cations should have fairly rigid planar structures because of resonance which will facilitate formation of cyclic hydrogen-bonded structures. Stable cyclic hydrogen-bonded structures with as many as 16 atoms have been described (Brown *et al.*, 1963).

In an earlier study it was shown that carbamoyl- and guanylguanidinium salts are more effective than the corresponding guanidinium salts in the denaturation of rabbit muscle aldolase, bovine serum albumin, and ovalbumin (Castellino and Barker, 1968a). In addition, it has been demonstrated that guanidinium chloride interacts preferentially with denatured protiens (H. B. Bull, personal cummunication, 1969; Hade and Tanford, 1967; Noelken and Timasheff, 1967) although the mechanism(s) of this (these) interaction has (have) not been established. It is clear that the cation as well as the anion plays a role in the denaturation reaction. If, as proposed by Tanford (1962), the protein in the native state has its hydrophobic regions and peptide bonds buried and in the denatured state has these parts exposed to the solvent, then agents which complex with the peptide bond would favor the denatured state. However, Klotz (1962) and H. B. Bull (personal communication, 1969) have suggested that a large portion of the protein surface is hydrophobic, and that structured water surrounds these regions (Klotz, 1962). Therefore, agents which disrupt water structure should favor denaturation by releasing the hydrophobic regions from the restraints of the structured water enclosing them. Thus a model for denaturation by guanidiniumlike species can be proposed which involves two processes, one the disruption of water structure and the loosening of hydrophobic interactions, and the other the solubilization of the interior of the protein due to specific interactions with the peptide bonds and solubilization of the hydrophobic regions.

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