# The Denaturing Effectiveness of Guanidinium, Carbamoylguanidinium, and Guanylguanidinium Salts*

F. J. Castellino and R. Barker

ABSTRACT: A series of guanidinium and substituted guanidinium salts have been compared with urea as denaturants of rabbit muscle aldolase, bovine serum albumin, and ovalbumin using the increase in levorotation of these proteins at 330 mμ as a criterion of denaturation. Guanidinium salts were increasingly effective through the series chloride < bromide < iodide < thiocyanate.

Carbamoylguanidinium and guanylguanidinium salts were more effective denaturants than the corresponding guanidinium salts. The anion series remained unchanged regardless of the cation used.

The number of subunits in proteins is usually calculated from a comparison of the molecular weight of the native protein to the molecular weight of the protein measured in a solvent capable of causing complete denaturation. It has been amply demonstrated (Gordon and Jencks, 1963; Tanford *et al.*, 1966; Nozaki and Tanford, 1967; Lapanje and Tanford, 1967; Castellino and Barker, 1968) that 6.0 M guanidine hydrochloride containing mercaptoethanol is one of the most effective protein denaturants. Concentrated solutions of neutral inorganic salts also denature proteins. In general, the denaturing effectiveness of these salts has been found to parallel their position in the Hofmeister series (Wolff, 1962; von Hippel and Wong, 1964), that is, for anions $Cl^- < Br^- < I^- < SCN^-$. It seemed probable that guanidinium salts of anions situated above $Cl^-$ in the Hofmeister series would be more effective denaturants than the $Cl^-$. It also seemed possible that substitution on the guanidinium cation of urea (carbamoylguanidinium) or another guanidinium (guanylguanidinium) would lead to increased protein denaturing effectiveness. Results have been obtained which verify these predictions.

## Materials and Methods

*Aldolase* was prepared from rabbit muscle according to the method of Taylor *et al.* (1948) and twice recrystallized from $(NH_4)_2SO_4$. The specific activity was 12–13 units/mg. Bovine serum albumin and ovalbumin were gifts of Professor H. B. Bull.

*Salts.* Guanidinium chloride and guanidinium thiocyanate were purchased from Eastman Organic Chemicals and recrystallized from methanol.

Salts of carbamoylguanidine and other salts of guanidine were prepared from the corresponding free bases by titration with the aqueous acid to neutrality. The bases were generated by treating aqueous solutions of guanidinium chloride or carbamoylguanidinium sulfate with Rexyn 201 ($OH^-$). The complete removal of $Cl^-$ and $SO_4^{2-}$ was established by qualitative tests with $Ag^+$ and $Ba^{2+}$, respectively, and by titration of the free bases with the various acids. The salts were isolated by removal of $H_2O$ at 50° under aspirator pressure and recrystallization from a minimal volume of absolute ethyl alcohol. In all cases, yields of 60–70% were obtained. The salts were dried *in vacuo* over Drierite before use.

Thiocyanic acid was generated from 0.05 to 0.1 M $NH_4SCN$ by the batchwise addition of a twofold excess of Rexyn 101 ($H^+$). The use of more concentrated solutions of $NH_4SCN$ led to polymer formation of the acid produced.

Guanylguanidinium salts were prepared from the free base (biguanide) purchased from the Aldrich Chemical Co., by rapidly adjusting aqueous solutions to pH 6.0 with the desired acid. In all cases, 1 mole of acid was required/mole of free base. The salts were purified by recrystallization from ethyl alcohol.

Urea was purchased from Fisher Scientific Co. and recrystallized from absolute ethyl alcohol.

*Optical rotations* were measured using a Shimadzu spectropolarimeter Model QV-50 at 25.5–26.0°. All values reported here were obtained at 330 mμ using protein concentrations of 7–8 mg/ml. The results are reported in terms of the specific rotation, $[\alpha]$, $[\alpha] = 3[\alpha']/(n^2 + 2)$, where $[\alpha']$ is the observed specific rotation and $n$ is the refractive index of the medium.

*Refractive Indices.* For refractive index measurements the crystalline salt was weighed and dissolved in approximately 1 ml of $H_2O$. Several solutions were prepared. A small quantity was taken for the refractive index measurement, and the concentration of each solution was determined by evaporating 0.5 ml of the remaining solution to dryness in a vacuum oven at 100° and weighing the residue.

* From the Department of Biochemistry, The University of Iowa, Iowa City, Iowa 52240. *Received July 17, 1968.* Supported in part by a U. S. Public Health Service Grant (HD02618) from The Institute of Child Health and Human Development, a U. S. Public Health Service fellowship award to F. J. C. (GM 34,008), and a U. S. Public Health Service research career development award to R. B. (GM 24,808) from the Institute of General Medical Science.

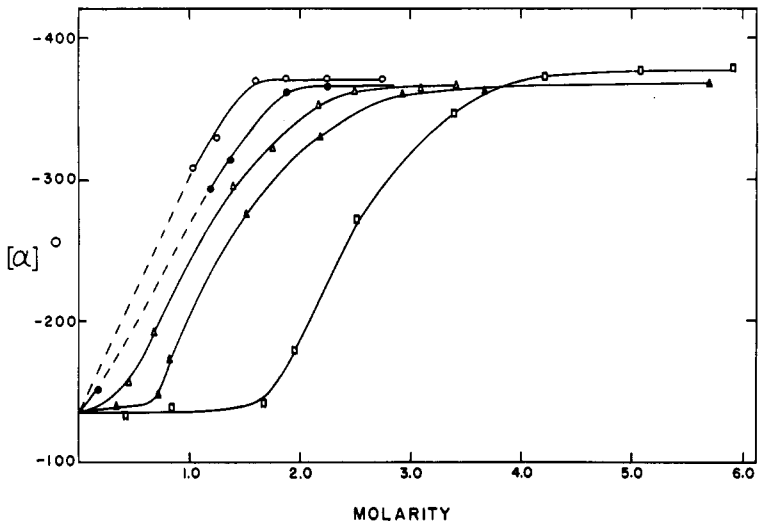



FIGURE 1: The effect of guanidinium salts and urea on the specific rotation of aldolase. (□) Urea, (▲) guanidinium chloride, (△) guanidinium bromide, (●) guanidinium iodide, (○) guanidinium thiocyanate, and (- - -) protein insoluble.

Aldolase, stored in 52% $(NH_4)_2SO_4$, was centrifuged, and the pellet was dissolved in a small volume of $H_2O$. The protein solution was dialyzed against several changes of $H_2O$ during 2 days. The protein concentration of the stock solution was determined spectrophotometrically using $\epsilon^{1\%}_{1\,cm}$ 0.938 (Donovan, 1964). Stock solutions of bovine serum albumin and ovalbumin were prepared by weighing salt-free lyophilized samples and dissolving in a known amount of $H_2O$. Protein concentrations were corrected for the $H_2O$ content of each protein.

Stock solutions of salts were prepared by dissolving a weighed sample in $H_2O$, adjusting the pH to 6.0 and a known final volume.

For optical rotation measurements, approximately ten separate samples were prepared for each salt by adding in succession 0.2 ml of the protein stock solution, a measured volume of $H_2O$, 10 $\mu$l of mercaptoethanol, and a measured volume of salt stock solution. The final volumes were 1 ml. Readings were taken approximately 10 min after mixing. No changes in rotation after 10 min were observed.

## Results

Plots of [$\alpha$] of aldolase solutions against the concentration of denaturant (molarity) for guanidinium, carbamoylguanidinium, and guanylguanidinium salts are shown in Figures 1, 2, and 3, respectively. Each point represents the average of two experimental values obtained on separate samples. Observed rotations ranged from approximately $-0.150°$ for the native protein to approximately $-0.420°$ for the denatured protein. Each observed rotation could be repeated within $\pm 0.005°$. An error of $\pm 7°$ is estimated for each point on the graph. The broken lines present in some of the graphs represent those concentrations of salt in which aldolase was insoluble, and for which optical rotation values were not obtained. Titration curves of aldolase in carbamoylguanidinium thiocyanate and guanylguanidinium thiocyanate were not obtained since aldolase was insoluble in these salts at concentrations below 1.3 M, and at this concentration denaturation was complete.

The minimum concentration of salt required to pro-

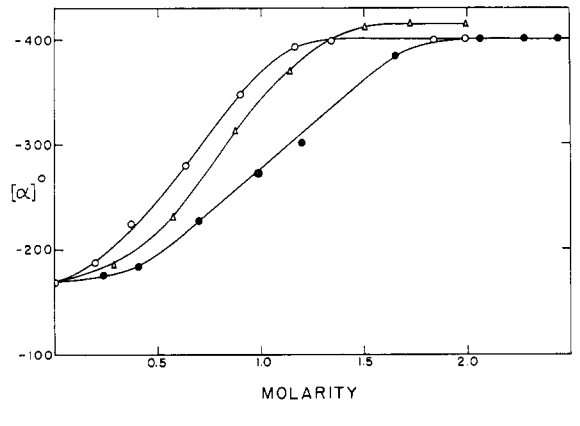



FIGURE 2: The effect of carbamoylguanidinium salts on the specific rotation of aldolase. (●) Carbamoylguanidinium chloride, (△) carbamoylguanidinium bromide, and (○) carbamoylguanidinium iodide.

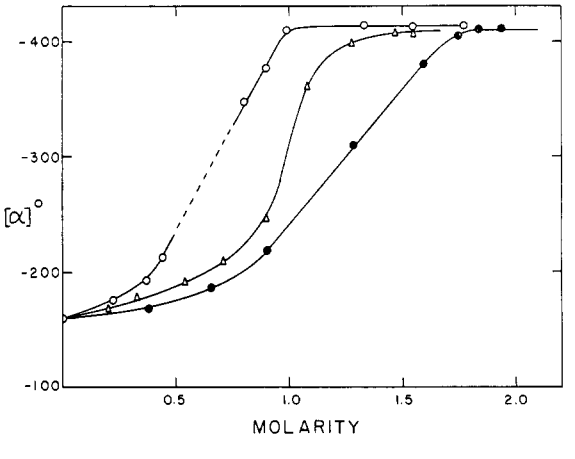



FIGURE 3: The effect of guanylguanidinium salts on the specific rotation of aldolase. (●) Guanylguanidinium chloride, (△) guanylguanidinium bromide, (○) guanylguanidinium iodide, and (- - -) protein insoluble.

TABLE I: Effectiveness of Various Salts as Denaturants of Aldolase.

| Denaturing Agent | Minimum Concn Required to Produce Complete Denaturation (M) |
|---|---|
| Urea | 4.48 |
| Guanidinium chloride | 3.35 |
| Guanidinium bromide | 2.88 |
| Guanidinium iodide | 2.34 |
| Guanidinium thiocyanate | 1.82 |
| Carbamoylguanidinium chloride | 2.03 |
| Carbamoylguanidinium bromide | 1.55 |
| Carbamoylguanidinium iodide | 1.23 |
| Guanylguanidinium chloride | 1.90 |
| Guanylguanidinium bromide | 1.46 |
| Guanylguanidinium iodide | 1.15 |

TABLE II: Effectiveness of Various Salts as Denaturants of Ovalbumin and Serum Albumin.

| Denaturing Agent | Minimum Concn Required to Produce Complete Denaturation (M) | |
|---|---|---|
| | Ovalbumin | Serum Albumin |
| Urea | 8.48 | |
| Guanidinium chloride | 5.30 | 3.36 |
| Guanidinium bromide | 4.70 | 2.94 |
| Guanidinium iodide | 3.10 | 2.33 |
| Guanidinium thiocyanate | 2.21 | 2.00 |
| Carbamoylguanidinium chloride | | 2.28 |
| Carbamoylguanidinium bromide | | 1.51 |
| Guanylguanidinium chloride | | 2.21 |
| Guanylguanidinium bromide | | 1.50 |

duce complete denaturation of aldolase, as judged from the magnitude of $[\alpha]$, is given in Table I. Table II gives the minimum concentration of salt required to denature fully bovine serum albumin and ovalbumin as measured by changes of $[\alpha]$.

The refractive index of each salt, which is required to correct values of $[\alpha']$ at several salt concentrations at 589 m$\mu$, is given in Table III. A standard curve of $n$ *vs.* concentration of salt was constructed and used to extrapolate for required concentrations of salt. The refractive indices at the wavelength used to determine optical rotations (330 m$\mu$) should be used to calculate values of $[\alpha]$. However, no suitable refractometer was available, and the absolute values of $[\alpha]$ reported are, therefore, somewhat in error. Since the shapes of the curves are probably not affected as a result of the use of values of $n$ determined at 589 m$\mu$, the conclusions drawn from these results are also unaffected.

## Discussion

The effectiveness of the anions in the denaturation of the proteins used in this study increased through the series $Cl^- < Br^- < I^- < SCN^-$ regardless of the cation used. This is in general agreement with other findings (Wolff, 1962; von Hippel and Wong, 1964; Brandts, 1964; Nagy and Jencks, 1965).

With regard to the cation series the order of protein denaturing effectiveness appears to be guanidinium < carbamoylguanidinium = guanylguanidinium.

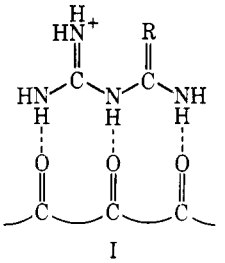
The most effective denaturant for aldolase is guanylguanidinium iodide and concentrations of approximately 1.2 M produced complete denaturation. That denaturation is complete is inferred from the observation that the value of $[\alpha]$ was the same, within experimental error, as that observed in 6.0 M guanidinium chloride plus mercaptoethanol which has been clearly shown to produce complete denaturation (Gordon and Jencks, 1963; Tanford *et al.*, 1966; Nozaki and Tanford, 1967; Castellino and Barker, 1968). Further evidence that a fully unfolded and fully dissociated aldolase molecule is produced in this low concentration of guanidylguanidinium iodide is the observation that $s_{20,w}$ for aldolase in 1.3 M guanylguanidinium iodide is 1.41 S, in excellent agreement with the value of 1.35 S which was previously obtained for aldolase in guanidinium chloride plus mercaptoethanol and which was shown to be fully dissociated and unfolded (Castellino and Barker, 1968).

It is probable that there are several mechanisms involved in the unfolding of proteins by concentrated salt solutions and the cation and anion may function in quite different ways. However, the increased effectiveness as denaturants in the anion series $Cl^- < Br^- < I^- < SCN^-$ parallels their effectiveness in the solubilization of acetyltetraglycine ethyl ester (Robinson and Jencks, 1965), their affinities for cation-exchange resins (Peterson, 1954), and their ability to decrease the viscosity of water (Gurney, 1953; Frank and Evans, 1945).

Robinson and Jencks (1965) have proposed that guanidinium ions and urea interact with the peptide backbone of a protein by hydrogen bonding with the carbonyl groups. The substitution of carbamoyl or guanyl residue for a hydrogen in the guanidinium ion increases the length of the molecule and may allow it to accommodate better to the steric requirements of the

TABLE III: Refractive Indices (589 mμ) of Various Concentrations of Denaturing Agents.

| Urea | | Guanidinium Iodide | | Carbamoylguanidinium Bromide | |
|---|---|---|---|---|---|
| *n* | *M* | *n* | *M* | *n* | *M* |
| 1.3502 | 2.020 | 1.3610 | 0.964 | 1.3481 | 0.543 |
| 1.3662 | 3.976 | 1.3851 | 1.818 | 1.3528 | 0.705 |
| 1.3830 | 5.853 | 1.4119 | 2.778 | 1.3757 | 1.487 |
| 1.3989 | 7.820 | 1.4608 | 4.546 | 1.3810 | 1.658 |
| Guanidinium Chloride | | Guanidinium Thiocyanate | | Carbamoylguanidinium Iodide | |
| 1.3546 | 1.036 | 1.3669 | 1.415 | 1.3671 | 1.029 |
| 1.3672 | 2.072 | 1.3977 | 2.733 | 1.3715 | 1.144 |
| 1.3840 | 3.108 | 1.4200 | 4.094 | 1.3780 | 1.348 |
| 1.4008 | 4.144 | 1.4589 | 5.485 | 1.3801 | 1.422 |
| Guanidinium Bromide | | Carbamoylguanidinium Chloride | | Guanylguanidinium Chloride | |
| 1.3611 | 1.3228 | 1.3638 | 1.183 | 1.3448 | 0.413 |
| 1.3729 | 1.897 | 1.3710 | 1.463 | 1.3520 | 0.650 |
| 1.3889 | 2.710 | 1.3889 | 2.165 | 1.3629 | 1.034 |
| 1.4120 | 3.872 | 1.4019 | 2.669 | 1.3700 | 1.283 |
| | | Guanylguanidinium Iodide | | Guanylguanidinium Bromide | |
| | | 1.3459 | 0.340 | 1.3468 | 0.452 |
| | | 1.3490 | 0.406 | 1.3630 | 0.900 |
| | | 1.3628 | 0.798 | 1.3701 | 1.129 |
| | | 1.3821 | 1.200 | 1.3881 | 1.668 |



I

carbamoylguanidinium, R = O
guanylguanidinium, R = NH

peptide backbone. It is also possible that a tridentate hydrogen-bonded complex (I) can form with carbamoylguanidine or guanidylguanidine. Such a complex would be considerably more stable than the bidentate complex which could form with urea or guanidium ion.

## References


Brandts, J. F. (1964), *J. Am. Chem. Soc. 86*, 4291, 4302.

Castellino, F. J., and Barker, R. (1968), *Biochemistry 7*, 2207.

Donovan, J. W. (1964), *Biochemistry 3*, 67.

Frank, H. S., and Evans, M. W. (1945), *J. Chem. Phys. 13*, 507.

Gordon, J. A., and Jencks, W. P. (1963), *Biochemistry 2*, 47.

Gurney, R. W. (1953), Ionic Processes in Solution, New York, N. Y., Dover.

Lapanje, S., and Tanford, C. (1967), *J. Am. Chem. Soc. 89*, 5030.

Nagy, B., and Jencks, W. P. (1965), *J. Am. Chem. Soc. 87*, 2480.

Nozaki, Y., and Tanford, C. (1967), *J. Am. Chem. Soc. 89*, 742.

Peterson, S. (1954), *Ann. N. Y. Acad. Sci. 57*, 144.

Robinson, D. R., and Jencks, W. P. (1965), *J. Am. Chem. Soc. 87*, 2462, 2470.

Tanford, C., Kawahara, K., and Lapanje, S. (1966), *J. Biol. Chem. 241*, 1921.

Taylor, J. F., Green, A. A., and Cori, J. T. (1948), *J. Biol. Chem. 173*, 591.

von Hippel, P. H., and Wong, K.-Y. (1964), *Science 145*, 577.

Wolff, J. (1962), *J. Biol. Chem. 237*, 230.