Effect of Compounds of the Urea-Guanidinium Class on Renaturation and Thermal Stability of Acid-Soluble Collagen

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The effects of guanidinium salts in decreasing the renaturation rate and lowering the thermal stability of acid-soluble calf-skin collagen have been compared with those of formamide and urea. With the exception of guanidinium sulphate at higher concentrations, no qualitative differences were apparent in the effects of these perturbants, which thus differed only in molar activity. Activity variation in the guanidinium salts reflected a net effect resulting from additivity of cation and anion contributions. As observed in other protein systems, lyotropic activity increased in the series formamide < urea < guanidinium ion, and in the guanidinium salts in the anion order fluoride < sulphate < chloride < bromide < nitrate < iodide. Low activities of guanidinium fluoride and sulphate were attributable to counter-effects of the anions, which acted as structural stabilizers. Changes in renaturation kinetics induced by either temperature or added perturbants appeared to conform with the Flory-Weaver model for the collagen transition. Additivity and non-specificity of the observed effects are discussed with particular reference to a common mechanism involving weak, non-saturated binding of perturbants at protein peptide groups.

Although lyotropic effects of compounds of the urea-guanidinium salt class on proteins generally are well documented, the underlying mechanisms remain controversial (reviewed by von Hippel & Schleich, 1969; Tanford, 1968, 1970). Reagents of this type are generally characterized as strong hydrogen-bond formers and hence might be expected competitively to disrupt structural hydrogen bonding in proteins. However, the observation that urea increases the solubility in aqueous solution of hydrocarbons (Wetlaufer *et al.*, 1964) and amino acids (Nozaki & Tanford, 1963) suggests that urea denatures proteins by increasing the hydrophobic character of the solvent, thereby weakening interactions between non-polar amino acid residues that contribute to structural stability in the native protein (Kauzman, 1959).

In recent studies we have adopted an approach to the elucidation of lyotropic mechanisms in which the effects of related perturbants in a standard collagen-buffer system have been compared (Russell & Cooper, 1969*a,b*, 1970; Hart *et al.*, 1971; Cooper *et al.*, 1971). Compared with globular proteins, structural regularity and the relatively well-defined stabilizing factors in individual molecules of soluble collagen confer some advantages in interpretation of protein-environment interactions. Structural stabilization is attributed to co-operative hydrogen bonding between the three helical polypeptide chains of the molecule and chain rigidity conferred by rotational restrictions at pyrrolidine residues and peptide bonds (reviewed by Ramachandran, 1967). In contrast with globular proteins, external location of the side chains in collagen, including those with non-polar structure,

suggests that these are likely to be equally accessible to the solvent environment in both native and denatured states.

The results of formamide and urea addition to a renaturing acid-soluble collagen system and effects on thermal stability have been described in previous reports (Russell & Cooper, 1969*b*, 1970). In the present study, this approach has been extended to compare the effects of a range of guanidinium salts with those of formamide and urea.

Experimental

Materials

Acid-soluble calf-skin collagen was prepared by the method previously described after removal of the neutral salt-soluble fraction (Cooper & Davidson, 1965). Reagents were A.R. grade or laboratory-grade materials. Guanidinium bromide, iodide and fluoride were prepared by addition of guanidinium carbonate to a slight excess of the appropriate halogen acid in dilute solution, followed by removal of water in a rotary evaporator and desiccator. With guanidinium iodide, free iodine was removed by extraction with chloroform and sublimation at 110°C. Analysis of the various products for halide content indicated purities greater than 99%.

Renaturation

Renaturation at $15 \pm 0.05^\circ\text{C}$ was monitored by polarimetry (at 365 nm in 10 cm water-jacketed tubes in a Perkin-Elmer 141 polarimeter) and viscometry

(with Canon-Fenske flow viscometers, sizes 50 and 100, B.S. 188). Acid-soluble collagen in 0.15M-potassium acetate buffer, pH4.8, was denatured at 45°C for 15 min before addition to a weighed amount of the compound under investigation dissolved in buffer. Further buffer was added to give a final volume of 10ml and a protein concentration of 0.86mg dry wt. of protein/ml. The solution was then transferred rapidly to polarimeter and viscometer tubes at $15 \pm 0.05^\circ\text{C}$ and the extent of reversion measured at various time-intervals. With guanidinium and potassium iodide a trace of sodium thio-sulphate was added to the buffer solution to inhibit the liberation of free iodine.

Denaturation

'Melting' curves were obtained by polarimetry by using the '30min method' (von Hippel & Wong, 1965), the temperature at the mid-point of the transition being taken as the melting point.

Measurement of initial reversion rates

Initial reaction rates corresponding to the zero-time gradients of the kinetic plots were obtained by least-squares fitting of initial-rate data to a two-parameter second-order equation by using a Hewlett-Packard 9100 B programmable calculator. For a second-order process at equivalent reactant concentrations, it can be shown that:

$$1/(y_\infty - y_1) = 1/y_\infty + kt_1 \quad (1)$$

and hence:

$$y_1 = y_\infty \{1 - [1/(ky_\infty t_1 + 1)]\} \quad (2)$$

where y_1 and y_∞ represent the reaction amplitude at time t_1 and infinite time respectively, and k is the second-order rate constant. Reaction profiles are described explicitly by eqn. (2), from which initial rates may be calculated by differentiation and setting t_1 equal to zero; i.e.:

$$\left(\frac{dy_1}{dt_1}\right)_{t_1=0} = y_\infty^2 k \quad (3)$$

For collagen mutarotation reversion, $y_1 = [\alpha]_t - [\alpha]_0$, where $[\alpha]_0$ is the zero-time specific rotation corresponding to the denatured state. Use of experimental y_∞ values obtained after extended reaction times, as required for the linear transform (eqn. 1), was unsatisfactory, however, since deviation from second-order kinetics occurred after two to three reaction half-lives.

This difficulty could be obviated by treating both y_∞ and k as parameters and applying linear least-squares regression to the alternative double-reciprocal

transform of eqn. (4):

$$1/y_1 = 1/y_\infty + 1/y_\infty^2 kt \quad (4)$$

which has the same mathematical formalism as in enzyme kinetics (Wilkinson, 1961; Cleland, 1963). In addition, to avoid bias, weighting of the experimental y_1 values implicit in eqn. (2) was preserved by multiplying transformed y_1 values ($y_1' = 1/y_1$) by the appropriate transformed weights (w_1') calculated from eqn. (5) (Jurs, 1970):

$$w_1' = \left(\frac{\partial y_1'}{\partial y_1}\right)^{-2} = \left(\frac{\partial [1/y_1]}{\partial y_1}\right)^{-2} = y_1^4 \quad (5)$$

before these were used in normal linear regression analysis.

Results

Effect of guanidinium salts on renaturation profiles

Typical optical-rotation and reduced-viscosity-reversion profiles with various amounts of guanidinium bromide are shown in Fig. 1. As in previous renaturation studies (Russell & Cooper, 1969a,b; Cooper *et al.*, 1971), exponential-type reaction curves were obtained, which appeared to approach various limiting values asymptotically. Initial mutarotation and, to a lesser extent, viscosity-reversion rates decreased progressively with increasing guanidinium bromide concentration. Examination of the experimental curves after extended reaction times, however, indicated complex reversion kinetics in which the initial rapid reversion process was followed by a slow reversion stage that was incomplete after 48 h. This complex situation was also reflected in a tendency of the experimental curves to cross after the initial reaction step at intermediate guanidinium bromide concentrations. Thus in the viscosity-reversion profiles, which were particularly sensitive to the slow reversion process, recoveries after 1 h at 0.25M- and 0.5M-guanidinium bromide exceeded that of the control. Similar effects were observed previously in collagen renaturation profiles obtained at various urea concentrations (Russell & Cooper, 1969b). Changes in initial kinetic characteristics were confirmed in the present study by deviation of the experimental curves from second-order kinetics after periods corresponding to two to three reaction half-lives. Similar non-conformity with simple kinetic expressions, indicative of the occurrence of two or more contributory processes of differing intrinsic rates, has been reported in other collagens (Harrington & Karr, 1970; Hauschka & Harrington, 1970).

As observed previously for neutral salts (von Hippel & Wong, 1962; reviewed by von Hippel & Schleich, 1969), substantially linear relationships were apparent when the logarithm of the initial

Fig. 1. Effect of guanidinium bromide at various concentrations on (a) specific-rotation and (b) reduced-viscosity recoveries of heat-denatured acid-soluble collagen at 15°C

Acid-soluble calf-skin collagen [heat-denatured (45°C; 15 min); (0.85 mg/ml)] was in 0.15 M-potassium acetate buffer, pH 4.8. Upper and lower limits (broken lines) denote specific-rotation and reduced-viscosity values for collagen (15°C) and gelatin (43°C) respectively.

mutarotation rate was plotted against concentration for formamide, urea and the various guanidinium salts with the exception of guanidinium sulphate (Fig. 2). The latter showed positive curvature indicative of a progressive decrease in molar effect with increasing concentration. Thus, with the exception of guanidinium sulphate, the guanidinium salts conformed to

Fig. 2. Logarithm of initial mutarotation reversion rate of heat-denatured acid-soluble calf-skin collagen in 0.15M-potassium acetate buffer at 15°C as a function of amide and guanidinium salt concentration

The intermediate plot (lower broken line) denotes values for guanidinium ion obtained by difference (for details see the text.) The solubility limit for guanidinium nitrate restricted the range of data. ○, Formamide; ●, urea; △, guanidinium fluoride; ▲, guanidinium chloride; ▽, guanidinium bromide; ▼, guanidinium iodide; □, guanidinium sulphate; ■, guanidinium nitrate.

the linear expression previously proposed for neutral salts (von Hippel & Wong, 1962):

$$\ln \left\{ - \left(\frac{d[\alpha]}{dt} \right)_T \right\} = \ln \left\{ \left(\frac{d[\alpha]}{dt} \right)_T^0 + k_0 c_s \right\} \quad (6)$$

where $-(d[\alpha]/dt)_T$ is the initial rate of helix formation in a given salt at concentration c_s (mol/litre) and temperature T (°C), $-(d[\alpha]/dt)_T^0$ is the corresponding rate in the absence of added salt and k_0 is the slope of the $\ln-(d[\alpha]/dt)$ vs. c_s plot. The various values of k_0 were calculated by linear least-squares fitting to the experimental data of Fig. 2, subject to the constraint that $\ln-(d[\alpha]/dt)_T^0$ found from the mean of a number of replicate controls was a common intercept.

A comparable k_0 value for guanidinium sulphate corresponding to molar activity at infinite dilution was found by fitting a least-squares parabola to the data and determining the gradient at zero concentration. Molar activities of the various compounds are compared on the basis of k_0 values in Table 1, in which related compounds are ranked in order of effectiveness in decreasing the renaturation rate. As noted previously (Russell & Cooper, 1970), urea was twice as effective as formamide on a molar basis in retarding helix formation. Activities in the guanidinium halides increased with anionic size and, together with guanidinium sulphate and nitrate, followed the general Hofmeister series for increasing anion activity observed in a number of protein and non-protein systems (reviewed by von Hippel, 1967).

Effects of guanidinium salts on 48h mutarotation and reduced-viscosity recoveries are shown in Fig. 3 together with comparable recoveries in formamide and urea (from Russell & Cooper, 1970). As with these uncharged amides, recovery maxima of similar height were observed at intermediate guanidinium

Table 1. Molar activities of amides, guanidinium and potassium salts in renaturation

Values for formamide and urea were calculated from data reported by Russell & Cooper (1969b, 1970).

Compound	k_0 (lm·ol ⁻¹)		
Formamide	-0.6		
Urea	-1.3		
Anion	Guanidinium	Potassium	Δk_0
Fluoride	-0.3	+2.4	-2.7
Chloride	-3.3	-1.0	-2.3
Bromide	-4.8	-2.4	-2.4
Iodide	-8.5	-6.0	-2.5
Sulphate	-1.2*	+1.4	-2.6
Nitrate	-5.3	-2.6	-2.7
		Mean	-2.5

* Activity per equivalent/at infinite dilution.

Fig. 3. Variation in (a) 48h specific-rotation and (b) 48h reduced-viscosity recoveries of heat-denatured acid-soluble collagen at 15°C

Values for the collagen in 0.15M-potassium acetate buffer are given as a function of amide and guanidinium salt concentration. Symbols and explanation are as given for Figs. 1 and 2.

salt concentrations, the effect being particularly pronounced in the viscosity-reversion plots. Both the optical-rotation and reduced-viscosity maxima shifted to lower perturbant concentrations with increasing molar activity. Optical-rotation maxima, however, occurred at a consistently lower concentration than the corresponding viscosity maxima, suggesting that to a degree the two processes were independent. At high perturbant concentrations, both optical-rotation and viscosity recoveries tended to decrease to limiting values approaching those of the denatured state ($[\alpha]_{365}^{15} = -360^\circ$; $\eta_{red.} = 0.5 \text{ dl/g}$), indicating complete suppression of renaturation. With the exception of guanidinium sulphate, which showed anomalous effects with increasing concentration (cf. Fig. 2), the various optical-rotation and viscosity-recovery plots could be superimposed by rescaling the concentration axis in each case by a suitable factor corresponding to the relative molar activity of the perturbant, as observed previously for formamide and urea (Russell & Cooper, 1970). Relative activities determined on this basis were identical with those found for perturbant effects on initial reversion rates (Fig. 2).

Effect of amides and guanidinium salts on thermal stability

The perturbants in general reduced the 'melting' temperature of native soluble collagen, resulting in linear or slightly curved (increasing molar effect) temperature vs. concentration plots as measured polarimetrically (Fig. 4). Guanidinium sulphate was again anomalous, showing negative curvature (de-

creasing molar effect) with stabilization apparent at higher concentrations. Similar findings have been reported for the effects of guanidinium sulphate and other guanidinium salts on ribonuclease (von Hippel & Wong, 1965). Thus, with the exception of guanidinium sulphate, the perturbants at moderate concentrations conformed closely with the linear expressions proposed for neutral salt effects (von Hippel & Wong, 1962; Mandelkern & Stewart, 1964) of the form:

$$T_m = T_m^0 + Kc_s \quad (7)$$

where T_m is the 'melting' temperature at salt concentration c_s (in mol or equiv./litre), T_m^0 is the 'melting' temperature in the absence of added salt and K represents the molar activity of the particular perturbant. K values were calculated by fitting least-squares lines or parabolas to the experimental points to obtain overall or initial gradients (Table 2). Relative molar activity was again identical with that noted for perturbant effects on initial renaturation rates and 48h recoveries.

Molar activities in denaturation relative to that of formamide are compared with similar activities from renaturation rates and 48h recoveries in Table 3 and average values calculated.

Additivity of guanidinium cation and anion effects

Additivity of anion effects in accounting for activity differences in the guanidinium salts was examined by measuring initial optical-rotation reversion rates in the corresponding potassium salts. As with amides

Fig. 4. Variation in 'melting' temperature of acid-soluble, calf-skin collagen in 0.15M-potassium acetate buffer with amide and guanidinium salt concentration

Symbols and explanation are as given for Figs. 1 and 2.

and guanidinium salts, closely linear relationships were observed when the logarithm of the initial rate was plotted against salt concentration. Least-square k_0 values are shown in Table 1. Activities of the potassium halides increased with anionic size as in the guanidinium compounds. Both potassium fluoride and potassium sulphate increased the renaturation rate relative to the control (positive k_0 values), consistent with the stabilizing effects on protein structure reported for sulphate ions in particular (von Hippel & Schleich, 1969). Substantially constant activity differences (Δk_0) were obtained for all the guanidinium-potassium salt pairs examined, irrespective of whether the potassium salts accelerated or retarded renaturation rates, confirming that the various ionic activities were mutually independent and additive to a high degree. If zero activity is assigned to the potassium ion, the average difference value (-2.5 litre mol^{-1}) is a measure of the guanidinium ion activity, which was approximately twice that of urea on this basis.

Temperature-perturbant effects on renaturation

In terms of the Flory & Weaver (1960) model for reversion kinetics, the reversion rate constant for a particular collagen varies inversely with temperature, arising from the following functional dependence on the degree of undercooling:

$$k = B e^{-A/RT\Delta T} \quad (8)$$

where A and B are constants and ΔT is the degree of undercooling ($T_m - T$). In accordance with this relationship, it follows that:

$$\ln(-d[\alpha]/dt) = \ln(-d[\alpha]/dt)_T^0 + A/RT(1/\Delta T^0 - 1/\Delta T) \quad (9)$$

Table 2. Molar activities of amides and guanidinium salts in denaturation

Compound	K ($^{\circ}\text{l} \cdot \text{mol}^{-1}$)
Formamide	-1.6
Urea	-3.1
Guanidinium fluoride	-0.5
Guanidinium chloride	-7.0
Guanidinium bromide	-12.6
Guanidinium iodide	Pptd.
Guanidinium sulphate	-2.0
Guanidinium nitrate	-14.0

where $\ln(-d[\alpha]/dt)_T^0$ is the mutarotation reversion rate at temperature T in the absence of perturbant, corresponding to a degree of undercooling ΔT^0 . In terms of this model the influence of temperature or perturbant concentration, through non-specific effects on ΔT , should be similar over the restricted range of experimentally accessible conditions and linear plots of $\ln(-d[\alpha]/dt)$ vs. $1/RT\Delta T$ can be expected in either case with values of A obtainable from the slopes (Mandelkern & Stewart, 1964; Russell & Cooper, 1969a, 1970).

Accordingly, the effect of urea concentration on mutarotation reversion rates at various temperatures was examined. Results are plotted in terms of the Flory-Weaver model in Fig. 5. Kinetic data obtained for other perturbants at 15°C are also shown. At fixed urea concentration and varying temperature, a series of substantially linear plots with similar gradients was obtained ($10.8 < A < 16.7 \text{ kcal} \cdot \text{degree} \cdot \text{mol}^{-1}$) in accord with the Flory-Weaver model. Similar linear dependence on varying renaturation temperatures at constant solution composition has been reported for renaturation in a variety of collagens (Flory & Weaver, 1960; von Hippel & Wong, 1963a; Harrington & Rao, 1970).

At fixed renaturation temperature and various urea concentrations a second series of parallel plots was distinguishable, in which the average slope was significantly greater than that obtained by temperature variation ($25.5 < A < 55.4 \text{ kcal} \cdot \text{degree} \cdot \text{mol}^{-1}$). Thus, for a given change in the degree of undercooling, changes in perturbant concentration had a greater influence on renaturation rate than temperature variation, reflected in a higher A value. However, non-specificity of perturbant effects suggested in the rate-concentration and 'melting' temperature-concentration studies (Figs. 2 and 4) was supported by the observation that Flory-Weaver plots of the rate data for the various other perturbants at 15°C conformed to the linear trend for urea at the same temperature.

Table 3. *Relative molar activities of amides and guanidinium salts*

Activities are relative to the unit activity for formamide.

Compound	$k_0/k_{0(\text{form.})}$	$[\alpha]_{365}^{15}/[\alpha]_{365}^{15(\text{form.})}$	$\eta_{\text{red.}}/\eta_{\text{red.}(\text{form.})}$	$T_m/T_{m(\text{form.})}$	Mean
Formamide	1.0	1.0	1.0	1.0	1.0
Urea	2.1	2.1	2.0	1.9	2.0
(Guanidinium ion)	—	—	—	—	(3.9)*
Guanidinium fluoride	0.5	0.7	—	0.3	0.5
Guanidinium chloride	5.2	5.1	4.7	4.3	4.8
Guanidinium bromide	7.6	7.9	7.1	7.7	7.6
Guanidinium iodide	13.5	14.9	15.0	Pptd.	14.5
Guanidinium sulphate	1.8	1.6	1.6	1.2	1.5
Guanidinium nitrate	8.8	9.1	8.3	8.7	8.7

* Calculated by difference from renaturation data, assuming zero activity for K^+ . Fig. 5. *Flory-Weaver plots of initial mutarotation reversion rates of heat-denatured acid-soluble collagen*

Rates were measured in 0.15M-potassium acetate buffer at various temperatures and perturbant concentrations as a function of the extent of undercooling (ΔT). Symbols are identified in Fig. 2.

Discussion

The results of the present study indicate that perturbant effects of compounds of the urea-guanidinium class in collagen renaturation and denaturation are characterized by a high degree of non-specificity and, for the guanidinium salts, by mutually independent additivity of individual ion effects. With the exception of guanidinium sulphate at higher concentrations, no qualitative differences were apparent in the reversion profiles for the guanidinium salts compared with those of the uncharged amides, in spite of the complex kinetics prevailing during re-

naturation. Similar conclusions apply irrespective of whether changes in short-range (optical-rotation monitoring) or long-range (reduced-viscosity monitoring) structure are examined in renaturation.

Non-specificity of effects within this perturbant group is also apparent from consideration of perturbant effects on 48 h optical-rotation and viscosity recoveries. As reported previously for formamide and urea (Russell & Cooper, 1969b, 1970), pronounced maxima, particularly in viscosity recovery, occurred at intermediate concentrations of guanidinium salt. Analogous with temperature effects (Kühn *et al.*, 1964; Beier & Engel, 1966), this recovery optimum has been attributed to the influence of direct perturbant hydrogen bonding on the slow 'annealing' reaction in collagen renaturation, whereby less-ordered structures produced during the initial rapid reaction phase gradually re-form to more collagen-like structures (Russell & Cooper, 1969b, 1970).

Detailed examination of the quantitative additivity of cation and anion activities for the guanidinium salts in renaturation indicated that the apparent molar activity of the various salts represents the net structure-altering influence of the particular ion combination (Table 1). At the same time, qualitative similarity in the effects of the amides and guanidinium salts indicates that charge-specific effects are absent, confirming that electrostatic interactions between the component ions and charged protein groups are not essential for lyotropic effects. In addition, pH adjustment by addition of alkali to the control systems in the region pH 4.8–5.2 corresponding to the range of experimental variation had no apparent effect, indicating that changes in charge profile on the polypeptide chains were not significant over this range. Similar conclusions regarding the absence of charge effects in the lyotropic activity of neutral salts have been reported previously (Bello *et al.*, 1956, 1962). Low activities for guanidinium fluoride and sulphate

are attributed to counter-effects of the anions, which accelerated renaturation and acted as structural stabilizers.

Consistent relative activities (based on unit activity for formamide) derived from renaturation and denaturation data have been demonstrated for the various perturbants (Table 3). Examination of activity-structure relationships in the series formamide, urea, guanidinium ion indicates that activity increases with hydrogen-bonding capacity as reflected by the number of potential donor hydrogen atoms present. In the guanidinium halides activity increased with anionic size. A similar trend to increasing activity has been reported for lyotropic effects on globular proteins in the series urea < guanidinium ion < carbamoylguanidinium ion, whereas anion effectiveness increased through the series chloride < bromide < iodide regardless of the cation present (Castellino & Barker, 1968).

Examination of temperature changes in conjunction with perturbant addition in terms of the Flory-Weaver model yielded approximately linear plots irrespective of the nature of the perturbation, indicating that both factors influence the nucleation step in renaturation. A single linear trend indicating complete non-specificity of temperature and perturbant effects (through their effect on ΔT only) was not obtained, however. Instead, the increase in apparent activation energy ($A/\Delta T$) was significantly greater in addition of perturbant. In terms of the Flory-Weaver model, this observation implies that at a given level of undercooling, added perturbants produce a more rapid increase in the minimum size of stable nuclei required for structural growth, resulting in a correspondingly rapid reduction in renaturation rate

(von Hippel & Wong, 1963b; Hauschka & Harrington, 1970).

Non-specificity of perturbant effects, indicated in the rate-concentration and 'melting' temperature-concentration studies, was consistent with the observation that Flory-Weaver plots for the various other perturbants at 15°C conformed closely to the linear trend for urea at the same temperature. Parallel effects of the perturbants on renaturation rates and 'melting' temperatures, as predicted by the Flory-Weaver model, serve to emphasize the essential independence of the interaction mechanism of the conformational state of the polypeptide chains.

The difficulty of explaining effects of neutral salts on proteins in terms of electrostatic or non-polar interactions has led to the conclusion that changes in denaturation, solubility and dissociation are due to salt effects on protein peptide groups, which undergo a change in their degree of exposure to the solvent environment (Robinson & Jencks, 1965). As with hydrogen-bonding reagents such as the amides and guanidinium cation, interaction of anions at peptide groups is also feasible through van der Waals-London ion-dipole association, which increases with polarizability and, hence, anionic size as observed in the halides. Non-specific binding of this type would serve to account for the qualitatively similar effects of the various perturbants on the collagen system. In terms of current views of collagen structural stabilization, polar binding at peptide groups would exert a twofold effect by increasing rotational freedom about these bonds due to electronic shifts (von Hippel & Schleich, 1969; Mattice & Mandelkern, 1971) as well as competitively disrupting interchain hydrogen bonding. On this basis, interaction at peptide bonds can simultaneously influence mutarotation reflecting changes in polypeptide type II helical structure within polypeptide chains, as well as directly affecting structural hydrogen bonding between chains in collagen denaturation and renaturation, consistent with the annealing effects observed.

As pointed out by von Hippel & Schleich (1969), a consistent mechanism for both stabilizers and destabilizers is feasible if competitive interactions involving all three components of the system (macromolecule, perturbant, water) are considered. On this basis, stabilizers are regarded as perturbants that decrease the overall level of interaction between a macromolecule and its predominantly aqueous environment by decreasing the availability of free water. Structure-making effects of stabilizers in aqueous solution are supported by the effects of potassium fluoride and sulphate, both of which increased the viscosity of the 0.15M-acetate buffer medium used (Fig. 6), consistent with an increase in the level of hydrogen bonding in solution (von Hippel & Schleich, 1969). In contrast, potassium salts of de-

Fig. 6. Effect of potassium salts on viscosity of 0.15M-potassium acetate buffer, pH 4.8, at 15°C as a function of salt concentration

Δ, Potassium fluoride; ▲, potassium chloride; ▽, potassium bromide; ▼, potassium iodide; □, potassium sulphate; ■, potassium nitrate.

stabilizing anions such as chloride, nitrate, bromide and iodide decreased viscosity progressively in the order cited, consistent with a structure-breaking effect in solution.

In spite of structural differences between collagen and globular proteins, previous studies indicate similar relative activities for compounds of the urea-guanidinium type on bovine serum albumin and ovalbumin (Schellman *et al.*, 1953; Castellino & Barker, 1968), lysozyme (Hamaguchi & Sakai, 1965) and ribonuclease (von Hippel & Wong, 1965). Anomalous concentration-dependence of guanidinium sulphate effects on ribonuclease stability, paralleling those observed on collagen, are particularly striking in this respect. Generality and non-specificity of lyotropic effects in these compounds suggests that a common interaction mechanism is operative, which is independent of compositional or conformational details. As opposed to hydrophobic interaction, weak, non-saturated, polar binding of perturbants at peptide groups would appear to account for the high degree of additivity and non-specificity of perturbant effects on collagen in particular and on other proteins.

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