THE EFFECTS OF SALTS ON THE STABILITY OF THE COLLAGEN HELIX UNDER ACIDIC CONDITIONS

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Summary

In the acid pH region, the relative effects of various salts on the thermal stability of the collagen helix are quite different from their effects at neutral pH. The magnitude of the decrease in thermal stability brought about by the salts studied depends mainly on the nature and concentration of the anion and very little on the nature of the cation, whereas at neutral pH the nature of both anions and cations affects the collagen helix stability, the effects of the two ions being roughly additive. The magnitude of the effect of salts at acid pH is much greater than that at neutral pH whereas for a non-ionized denaturant, urea, the magnitudes at both neutral and acid pH are similar. The data are discussed in terms of possible interactions between salts and the positively charged protein with particular consideration of the effects of salts on the pK_a of protein carboxyl groups.

I. Introduction

In recent years, the effects of a wide range of neutral salts on the stability of the collagen-fold have been studied by various workers (reviewed by von Hippel 1967). Extensive studies have been carried out by von Hippel and Wong (1962, 1963), who have determined the relative effectiveness of various ions in stabilizing or destabilizing the native form of collagen against thermal denaturation at neutral pH. In these investigations it was shown that both cations and anions affect the stability, their effects being roughly additive. Although this work was carried out at neutral pH values, there has been a tendency to extend the results of these experiments to the acid pH region. For example, it has been assumed (Dick and Nordwig 1966) that because sodium chloride has little effect on the stability of collagen at neutral pH values, the same applies under acidic conditions. This report demonstrates that, in the acid pH range, the relative effectiveness of various salts in stabilizing the collagen helix is quite different from that in neutral solution. The salts with which this report is concerned are potassium, sodium, barium, and calcium chlorides, lithium, sodium, magnesium, and calcium bromides, sodium, magnesium, and ammonium sulphates, and potassium thiocyanate. For the purpose of comparison, the effects of the more conventional protein denaturants, urea and guanidine hydrochloride, have also been studied.

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II. Experimental

The soluble collagen used in these experiments was extracted from rat tail tendon according to the method of Dimitru and Garrett (1957). The collagen solution so obtained was centrifuged at 23,000 *g* for 1 hr at 4°C to remove suspended impurities, and the collagen was subsequently precipitated by adding sodium chloride to a final concentration of 18% (w/v) (Bensusan and Hoyt 1958; Jackson, Leach, and Jacobs 1958). After exhaustive dialysis of the precipitate against deionized water, the collagen was lyophilized and stored at 4°C.

Optical rotation measurements on collagen in various ionic environments were made with a Perkin–Elmer model 141 spectropolarimeter. The solutions were thermostated by means of jacketed tubes, 1 dm in length, connected to a Colora circulating water-bath, and heating curves were constructed by raising the temperature of the solutions at a rate of 1 degC per 5 min. The cell temperature was measured to $\pm 0 \cdot 1$ degC, and all optical rotation measurements were made at the 365 mμ Hg line.

The transition temperature, T_m, was arbitrarily taken as the average value of the temperatures at which the helix to random coil transition was one-quarter and three-quarters completed. Values of T_m were reproducible to $\pm 0 \cdot 1$ degC. A collagen solution whose T_m was to be determined in the presence of a salt was dialysed for 24 hr at 3°C against a solution of this salt adjusted to pH 3·0 with 0·1M hydrochloric acid. In the cases reported in the text where no salt was present, the pH ($\pm 0 \cdot 1$) of the collagen solution was adjusted directly with 0·1M hydrochloric acid, using a Beckman Zeromatic pH-meter.

All reagents used were of analytical reagent grade, except magnesium bromide, which was the best quality available, and the guanidine hydrochloride, which was prepared from guanidine carbonate and recrystallized from water.

III. Results

(*a*) *Effects of pH*

The effect of pH on the transition temperature of collagen in the absence of added salt is shown in Figure 1(*a*), curve *A*. The thermal stability of the collagen helix was markedly dependent on pH at pH values $< 3 \cdot 0$ but, after passing through a maximum, this dependence was greatly reduced in the pH region above 3·0. When the experimental conditions were altered so that the ionic strength was kept constant at 0·6 using a sodium chloride–hydrochloric acid system the data shown in Figure 1(*a*), curve *B* were obtained. The region above pH 3·0 could not be studied because of the insolubility of the collagen under these conditions but it is clear that there was a considerable shift in the curve as a whole in the direction of higher pH.

(*b*) *Effects of Concentration of Various Salts at pH 3·0*

The decrease in T_m by more than 7 degC at pH 3·0 as a result of the addition of sodium chloride [shown in Fig. 1(*a*)] led us to investigate the effect of salt concentration on T_m at this pH for a range of added salts. The data shown in Figure 1(*b*) indicate that if T_m is plotted against the molar concentration of the anion smooth curves are obtained which are concave upward. These may be contrasted with the linear relationship obtained at neutral pH (von Hippel and Wong 1962, 1963). The upper limit of concentration of salt in each series was determined by the solubility of collagen in the particular system.

(*c*) *Effects of Urea and Guanidine Hydrochloride*

The influence of the more conventional protein denaturants, urea and guanidine hydrochloride, on the transition temperature at pH 3·0 has been studied over the

same concentration range as the salts listed in Figure 1(*b*), giving the data shown in Figure 2. The relationship between T_m and urea concentration (curve *A*) is linear, whereas in the case of guanidine hydrochloride (curve *B*) the curve is similar in shape to those of the other chlorides shown in Figure 1(*b*) but is displaced in the direction of lower T_m values.

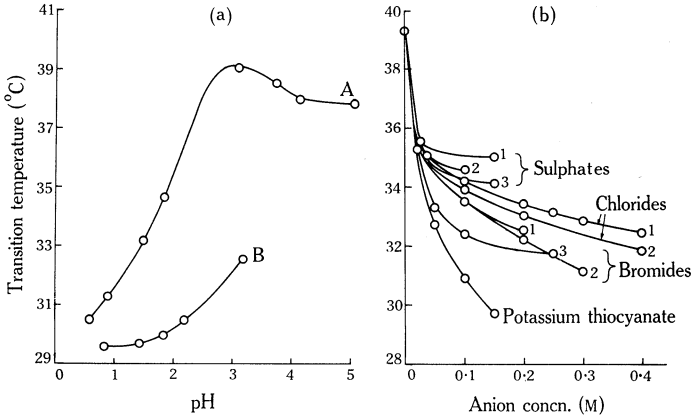

Fig. 1.—(*a*) Transition temperature (T_m) of rat tail tendon collagen as a function of pH of solution in the absence of added salt (*A*), and at a constant ionic strength of 0·6 (*B*). (*b*) Transition temperature (T_m) of rat tail tendon collagen as a function of anion concentration of the following added salts at pH 3·0:

Sulphates *1*, $MgSO_4$; *2*, Na_2SO_4; *3*, $(NH_4)_2SO_4$.
Chlorides *1*, KCl and $BaCl_2$; *2*, $CaCl_2$.
Bromides *1*, $MgBr_2$; *2*, $CaBr_2$; *3*, NaBr and LiBr.

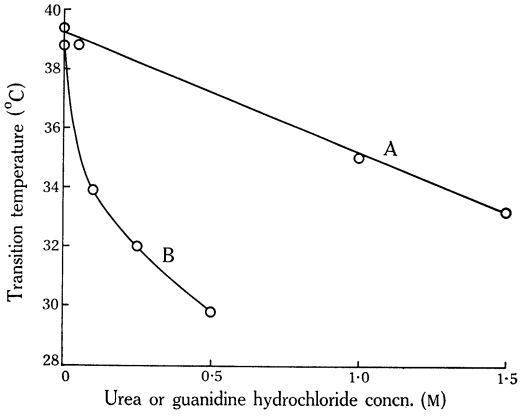

Fig. 2.—Transition temperature (T_m) of rat tail tendon collagen as a function of urea concentration at pH 3·0 (*A*), and of guanidine hydrochloride concentration at pH 3·0 (*B*).

IV. Discussion

Perhaps the most important aspect of the present results is the marked difference between both the relative and absolute effects of different salts on the value of T_m for collagen at pH 3·0 and their effects at neutral pH. This can be demonstrated clearly by a comparison of the effects of potassium chloride and calcium chloride. Von Hippel and Wong (1963) have shown that the negative slope of the curve relating

salt concentration with T_m for calcium chloride is about five times that for sodium chloride. At an anion concentration of 0·4M the respective lowerings of T_m at neutral pH are about 3 and 1 degC whereas at pH 3·0 the corresponding values are 7·0 and 6·6 degC. Thus at pH 3·0 not only are the magnitudes of the depressions in T_m for the two salts very similar but they are also much greater than at neutral pH.

On the other hand, salts with different anions but with a common cation such as potassium chloride and potassium thiocyanate show large differences in their effect on T_m. Thus the curves tend to fall into groups defined by the particular anions so that the value of T_m is dependent mainly on the nature and concentration of the anion, the effect of cation being reflected more in its contribution to the overall ionic strength than in any specific effect. This may be contrasted with the data at neutral pH of von Hippel and Wong (1962, 1963) who found that the nature of both cation and anion determined the relative effects of various salts on the value of T_m. The relative order of different anions in their effect on T_m at pH 3·0, viz.

$$CNS^- > Br^- > Cl^- > SO_4^{2-}$$

is the same as that at neutral pH (von Hippel and Wong 1963) but the magnitude of the effect at pH 3·0 is much greater. It should be pointed out that the effect of salt on T_m is apparently not governed by a mass action effect since the curve in Figure 1(*b*) for potassium chloride is independent of collagen concentration over a threefold range from 0·06 to 0·18%.

Bianchi *et al.* (1967) have also observed that an increase in ionic strength under acidic pH conditions leads to a depression in T_m for the helix–coil transition in collagen but the range of salts they examined was not sufficient to enable them to recognize the effect of anion specificity.

Why does the addition of neutral salt cause a depression of T_m at low pH values? Since the collagen molecule carries a net positive charge at pH 3·0 one might expect *a priori* that the principal effect of an increase in ionic strength would be an electrostatic screening of intramolecular repulsive interactions. On these grounds one would predict an increase in the stability of the helix with increasing ionic strength and no anion specificity would be expected. In a study of another fibrous protein, tropomyosin, E. F. Woods (personal communication) has shown that the thermal stability of this protein at acid pH values is in fact increased by increasing ionic strength and he is able to account for this in terms of an electrostatic screening effect.

It is apparent that if intramolecular repulsive interactions are screened by the ions this effect is of minor importance and we must therefore consider other possible interactions between the positively charged collagen and neutral salts. One might predict that the concentration of cations would be very low in the neighbourhood of the similarly charged protein molecule and hence the nature of the cation would have little effect on the stability of the helix. Binding of anions to specific sites on the protein molecule seems unlikely to be an overriding factor since this would decrease its net charge and thus increase its thermal stability. If, as Harrington and von Hippel (1961) have suggested, structurally bound water plays a special role in the stabilization of the collagen helix it is possible that interaction of the anion with this might be a factor in the lowering of T_m by salts at acid pH. More recently Erlander

and Tobin (1967) have proposed that interaction with negatively hydrated domains of anions may cause the disruption of structures stabilized by hydrogen bonds. The anion concentrations in Figure 1(*b*) are much lower than the minimum concentrations necessary for hydrogen bond disruption in the systems discussed by Erlander and Tobin. However, the effective concentration of anions in the immediate vicinity of the positively charged collagen molecule at pH 3·0 will be higher than in the solution as a whole and thus the proposals of Erlander and Tobin cannot be discounted as a possible contributing factor to the destabilization of the collagen helix by salts at pH 3·0. A further factor to be considered is the influence of anion interaction on the apparent pK_a values of the carboxylate groups of the protein. It is well known (e.g. Cannan, Palmer, and Kibrick 1942; Cohn and Edsall 1943) that an increase in ionic strength leads to an increase in the pK_a values of protein carboxylate groups. Since the protein has a net positive charge at pH 3·0 an increase in the apparent pK_a of the carboxylate groups would result in an increased deviation of the molecule from isoelectric conditions and hence tend to decrease its stability to thermal denaturation.* There is very little data concerning specific effects of anions on the apparent pK_a of carboxylate groups in proteins other than those of Steinhardt and co-workers (Steinhardt 1941) on keratin and egg albumin. Their data are consistent with the proposal that this is a contributing factor to the effects shown in Figure 1(*b*).

The depression in T_m caused by the presence of urea (Fig. 2) differs from that caused by the salts not only in being linear with respect to concentration but also in being much smaller in magnitude. The depression in T_m may be expressed as $T_m = -4$°C/mole/l which may be compared with the value of $-2{\cdot}5$°C/mole/l found by von Hippel and Wong (1963) for cooled calfskin gelatin at neutral pH. Allowing for the fact that our data were obtained with native tropocollagen whereas the latter workers used cooled gelatin, the similarity in the values suggests that the destabilizing effect of urea is similar both at neutral and acidic pH. The curve of T_m *v.* concentration for guanidine hydrochloride (Fig. 2) lies significantly below the main group of chlorides and suggests that in this case the guanidinium ion itself is exerting an additional influence, possibly similar to that caused by urea.

Turning to the effect of pH on the T_m value for collagen in the absence of salt [Fig. 1(*a*)] we may compare the present data with those previously obtained by Burge and Hynes (1959) and by Bianchi *et al.* (1967). A significant difference is the maximum at about pH 3 shown in Figure 1(*a*) which was not observed by the previous workers. Burge and Hynes used a different species of collagen and an acetic acid–hydrochloric acid mixture to adjust the pH but Bianchi *et al.* used similar conditions to ours. However, both groups estimated T_m from the variation of viscosity with temperature and this may not be comparable under all conditions with estimates from optical rotation. The latter parameter probably gives a more direct measure

* The effect of ionic strength on the apparent pK_a of carboxylate groups was considered by Bianchi *et al.* (1967) as an explanation for their data on the depression of T_m by added salt at acid pH values. However, this explanation was discarded because they came to the conclusion [based on the effect of salts on the pK_a of carboxylate groups in polyglutamic acid rather than in proteins (Ciferri, personal communication)] that the deviations from isoelectric conditions would be reduced rather than increased.

of the transition from the helical to the random-coil form. We have considered the possibility that binding of phosphate ion during extraction (Dimitru and Garrett 1957) is a factor causing the observed maximum but the same effect was observed with collagen extracted only with 1M sodium chloride. Unfortunately, it is not possible to completely deionize collagen by the use of mixed-bed ion-exchange resins because of gelation and precipitation of the protein. The reason for the maximum in thermal stability in the vicinity of pH 3 is not clear. It would be expected that some protonation of carboxylate groups would occur on lowering the pH from 4·0 to 3·0. As a result the net charge on the molecule would be increased and a lowered stability would be expected. Clearly there must be some type of stabilizing influence in this pH region which outweighs the electrostatic effect.

The sharp decrease in thermal stability of the molecule in the range pH 3–1 has been attributed both by Burge and Hynes (1959) and by Bianchi *et al.* (1967) as primarily due to the electrostatic effect of the increased net charge on the molecule as the pH is lowered. However, the concomitant increase in chloride ion concentration from 0·001M to 0·1M in the range of pH 3·0–1·0 and its contribution to the decreased stability must also be considered. Reference to Figure 1(*b*) indicates that such an increase in chloride ion concentration at pH 3·0 decreases T_m by about 5 degC. Since this represents a substantial proportion of the decrease in T_m (8 degC) between pH 3·0 and 1·0 in the absence of added salt the effect of counter-ion concentration in this pH range cannot be ignored.

V. Acknowledgment

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