The Swelling of Collagen in Alkaline Solutions

2. SWELLING IN SOLUTIONS OF UNIVALENT BASES

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In Part 1 (Bowes & Kenten, 1950) it was shown that changes in cohesion as well as osmotic effects must play an important part in determining the swelling of collagen in solutions of sodium hydroxide. It seemed probable from these results that the increased swelling at high pH values was due to a decrease in the cohesion of the collagen, and that this decrease in cohesion was associated with the presence of a high concentration of hydroxyl ions rather than with any specific effect of the sodium ion. It has been suggested, however, that bases differ in their effect on collagen; e.g., ammonia is reported to cause more splitting up of the fibres (an indication of decreased cohesion) than sodium hydroxide (Kaye & Jordan Lloyd, 1924).

The swelling of collagen in solutions of a number of strong and weak bases has, therefore, been studied to gain information regarding these effects, and, in particular, with respect to the nature of the decrease in cohesion occurring at high pH values in solutions of sodium hydroxide. In a brief account of the preliminary work (Bowes & Kenten, 1947) it was shown that, in contrast to strong bases, the swelling of collagen in solutions of weak bases decreased at high pH values in apparent accordance with the Donnan theory. It was suggested that the undissociated base in some way inhibited the action of the hydroxyl ions on cohesion. By comparison with swelling curves obtained in the presence of organic solvents it has now been shown that the decrease is related to the progressive departure from an aqueous medium as the concentration of base increases.

EXPERIMENTAL

Materials

Collagen. The alkali-treated sheepskin collagen D and E described in Part 1 (Bowes & Kenten, 1950) was used.

Guanidine. The free base was prepared from the sulphate by the addition of a slight excess of $\text{Ba}(\text{OH})_2$ followed by removal of excess Ba^{++} by the addition of H_2SO_4 . The solution so prepared gave no test for SO_4^{--} , and the ash content was 0.0012 g./100 ml.

Tetramethylammonium hydroxide. An aqueous solution of tetramethylammonium bromide prepared from trimethylamine and methyl bromide (Hickinbottom, 1948) was treated with Ag_2O , and the solution filtered. The resulting solution was 0.66 N.

Diethylamine. Technical diethylamine was shaken with charcoal, dried over solid NaOH and the free base distilled off.

The other bases and organic solvents were either of A.R. or technical grade as supplied by British Drug Houses Ltd.

Determination of swelling curves

The technique adopted was the same as that used in Part 1 (Bowes & Kenten, 1950). The weight of water or solution held by the collagen, expressed in terms of percentage of the moisture- and ash-free weight, was again taken as a measure of swelling. No special precautions were taken to avoid contamination with CO_2 or loss of base by volatilization, but the time during which the solutions were exposed to the atmosphere was kept to a minimum.

With the weak bases, changes in pH with concentration were small, and, in addition to pH measurements, the final concentration of base was determined by titration. In some cases it was obvious that appreciable amounts of collagen were dissolved, but except when using acetone and dioxan, it was not possible to check this by N determinations owing to the high N content of the bases. Where appreciable solution of the collagen was suspected, the samples were air-dried, heated in an air oven at 106° for 6 hr. and the percentage loss in weight, based on final weight, taken as a measure of the swelling. This method of calculating the results is not ideal, since appreciable amounts of base may be retained by the collagen after drying and heating under alkaline conditions may lead to some decomposition of the collagen.

All pH values were determined at 20° and pH values quoted refer to the values at 20° . The Cambridge glass electrode standardized with phthalate buffer (pH 4.00) and borate buffer (pH 9.22) was used for pH values below 9.5, and the special Beckman electrode standardized with $\text{Na}_2\text{CO}_3\text{-Na}_2\text{B}_4\text{O}_7$ buffer (pH 10.00) for the pH values above 9.5. There is some doubt regarding the pH values of the solutions containing high concentrations of weak bases or organic solvents. The pH values may be in error owing to the limitations of the glass electrode in alkaline solutions (Dole, 1941*a*). This error is probably small in solutions of organic bases (Dole, 1941*a*; Hill, 1929; Hubbard, Hamilton & Finn, 1939) and will cause the observed pH to be lower than the true value. McInnes & Dole (1930) found little error with 0.1 N solutions of tetramethylammonium hydroxide. Deviation of the activity of water from unity may also introduce errors, but by analogy with similar effects in acid solutions and solvent water mixtures these are also likely to be small (Dole, 1941*b*).

It is the hydroxyl-ion activity which governs swelling, however, and although the observed pH values may not be greatly in error, alterations in K_w and in the activity of water

will affect the pH-pOH relationship, and hence the position of the swelling curves relative to one another. For true comparison with the NaOH curves, those of the weak bases should be progressively shifted towards the left as the concentration of base increases. Judging from the values of K_w in dioxan and water mixtures (K_w decreases from 0.6809×10^{-14} to 1.234×10^{-16} as the concentration of dioxan increases from 0 to 45%; Harned & Fallon, 1939), and from the change in pH of 0.01 N-NaOH solutions on the addition of acetone (Table 1), this shift should be of the order of one pH unit when the concentration of base is increased from zero to between 30 and 40%.

RESULTS

Swelling curves in solutions of strong and weak univalent bases

The swelling curve of collagen in tetramethylammonium hydroxide solutions was almost identical with that in sodium hydroxide solutions (Fig. 1);

Fig. 1. Swelling curves of collagen in solutions of NaOH, $\circ-\circ$; tetramethylammonium hydroxide, $+---+$; diethylamine, $\square-\square$; NH_3 , $\triangle-\triangle$; NH_3 with the addition of 0.5M-NaCl, $\times-\times$.

swelling increased rapidly with pH up to 11.5, increased more slowly in the range 11.5–13.0, and then increased rapidly with further increases in pH. The curve for guanidine (Fig. 2) was of the same shape, but swelling was less than in sodium hydroxide solutions at pH values up to 13.5. The smaller amount of swelling at pH values below 13.5 is probably due to combination of guanidinium ions with the collagen so that they make no contribution to the swelling pressure.

The swelling curves of collagen in solutions of the weak bases (Figs. 1 and 2) varied only slightly from that in sodium hydroxide solutions up to about pH 12.5, but at higher pH values they began to show considerable divergencies, the swelling decreasing with pH in apparent agreement with the Donnan Theory, until at about pH 13.5 it approached that found in the isoelectric range. The decreased swelling is not due to disintegration of the collagen, since the pieces in the most concentrated solutions were obviously unswollen. Also on drying there was no appreciable loss in weight of the collagen. Although variations in the pH-pOH relationship with increasing concentration of base give rise to some doubt regarding the exact position of the curves for the weak bases in relation to the curve for sodium hydroxide (p. 524), such variations do not alter the fact that the swelling decreased with increasing concentration of base. The addition of 0.5M-sodium chloride decreased swelling in solutions of ammonia at all pH values, but the shape of the curve was

Fig. 2. Swelling curves of collagen in solutions of NaOH, $\circ-\circ$; guanidine, $\square-\square$; piperidine, $+---+$.

unchanged. At pH 12.0 the decrease in swelling was of the same order as that in sodium hydroxide solutions at equivalent sodium chloride concentrations.

These findings rule out the possibility that the decrease in cohesion observed at high pH values in sodium hydroxide solutions is due to a specific effect of the sodium ion and tend to confirm the view that it is associated with high concentrations of hydroxyl ions which by some mechanism, probably breaking of hydrogen bonds, reduce the cohesion of the collagen. The reason why this effect is apparently

not operative in solutions of weak bases was not at first clear. The most likely explanation appeared to be that the undissociated base inhibited the effect of the hydroxyl ions on cohesion. If this were so, then it might be expected that the molar concentration of the base would be a major factor in determining the effect.

The effect of the concentration of organic base at high pH values

To test the suggestions made above, the swelling of collagen in sodium hydroxide solutions containing varying amounts of ammonia was determined. The ammonia concentration was kept constant at 3.8, 7.6 and 10.6*M* and the pH was varied by the addition of sodium hydroxide (Fig. 3). There was a considerable scatter of points, but the results indicated that at any given ammonia concentration, the amount of swelling was at first unaffected by rise of pH, but

Fig. 3. The effect of additions of NH_3 on the swelling curve of collagen in NaOH solutions. NaOH, $\circ-\circ$; NH_3 , $\triangle-\triangle$; NaOH + 3.8*M*- NH_3 , $+ - +$; NaOH + 7.6*M*- NH_3 , $\times - \times$; NaOH + 10.6*M*- NH_3 , $\nabla - \nabla$.

finally increased in the same way as with sodium hydroxide alone. Apparently, the higher the pH, the greater the concentration of weak base necessary to suppress swelling. Consideration of the curves in Figs. 1-3 indicates that at equivalent molar concentrations diethylamine was much more effective than ammonia, and slightly more effective than piperidine in suppressing swelling. The effect of a number of different bases at the same molar concentration over a range of pH values was, therefore, determined, using 5*M*-solutions of the bases, adjusted to the

required pH's with sodium hydroxide or hydrochloric acid. With all the bases swelling increased with pH, but the relative effects varied greatly (Fig. 4). There was an indication that the effectiveness of the bases in suppressing swelling was related to their concentration in g., but some specific effect was also apparent; e.g. diethylamine had a much greater effect than other bases at the same or greater concentrations. It was also found that the swelling curve in 36% (v/v) acetone solutions was not very different from that found with pyridine and some of the other weak bases (Fig. 4).

Fig. 4. The swelling of collagen in 5*M*-solutions of NH_3 , $\triangle-\triangle$; monomethylamine, $\circ-\circ$; dimethylamine, $\nabla-\nabla$; diethylamine, $\square-\square$; pyridine, $\times - \times$; piperidine, $+ - +$; acetone, $\oplus - \oplus$.

The effect of acetone and dioxan on swelling

To obtain further information on the influence of the medium on swelling, the effect of increasing concentrations of acetone and dioxan on the pH and the swelling properties of 0.01 and 0.1*N*-sodium hydroxide solutions was determined. The addition of acetone progressively increased the pH (Table 1) due to alterations in K_w (p. 525), the shift in pH being of the order of 1.1-1.5 units in 50% (v/v) acetone. The shift in pH with increasing dioxan concentration was much less, about 0.7 pH unit; this may have

Table 1. The effect of acetone and dioxan on the pH values of sodium hydroxide solutions and the effect of acetone on the soluble nitrogen derived from collagen after soaking for 3 days in sodium hydroxide solutions at 20°

(The time elapsing between the addition of the dioxan and the determination of pH varied from 0.5 to 1 hr.)

Concentration of acetone or dioxan (ml./100 ml.)	Acetone in 0.01 N-NaOH			Acetone in 0.1 N-NaOH			Dioxan in 0.01 N-NaOH	
	pH		Soluble N (mg./g. collagen)	pH		Soluble N (mg./g. collagen)	pH	
	Initial	After 3 days' contact with collagen		Initial	After 3 days' contact with collagen		Initial	After 3 days' contact with collagen
0	12.05	11.98	3.8	12.97	12.99	9.2	12.06	12.02
5	—	12.10	2.3	13.09	13.09	9.4	12.08	12.05
10	12.28	12.21	6.1	13.18	13.19	10.5	12.22	12.08
15	12.38	12.34	4.2	13.36	13.33	7.8	12.12	12.10
20	—	12.45	3.8	13.44	13.47	9.1	12.28	12.12
25	12.68	12.58	5.1	13.56	13.59	9.6	12.38	12.13
30	12.75	12.72	12.2	13.67	13.67	13.0	12.46	12.10
35	12.99	12.86	9.0	13.78	13.81	14.3	12.50	12.06
40	13.12	13.02	9.3	13.97	13.92	16.5	12.60	11.93
45	13.31	13.20	5.4	14.06	14.01	15.8	12.72	11.62
50	13.48	13.36	7.1	14.13	14.15	16.4	12.74	11.12

been due to reaction with the dioxan since after 3 days the pH of the concentrated dioxan solutions had fallen below that of the original sodium hydroxide (Table 1).

isoelectric range. With acetone in 0.01N-sodium hydroxide the net result of these two effects, rise in pH and decrease in swelling, was to give a swelling curve similar in shape to those found with the weak bases (Fig. 5) except that it descended more sharply with pH, since there was no increase in hydroxyl ion concentration due to dissociation of base. With 0.1 N-sodium hydroxide the curve was of the same shape,

Fig. 5. The effect of increasing additions of acetone on the swelling of collagen in 0.01 (□—□) and 0.1M-NaOH (○—○) solutions. The swelling curve obtained with NH₃ (△—△) is included for comparison.

The swelling of the collagen decreased as the concentration of the acetone increased and in 50 % (v/v) acetone the swelling was of the same order as in the

Fig. 6. The effect of the addition of 50 % (v/v) acetone on the swelling of collagen in HCl and NaOH. No acetone, ○—○; 50 % acetone, +—+, and 50 % acetone, allowance made for effect on K_w , × — ×.

but was shifted to correspondingly higher pH values. Acetone also decreased swelling in the isoelectric range and in acid solutions (Fig. 6).

The effect of organic solvents on solution of collagen

Although acetone and dioxan decreased swelling in alkaline solutions, they also tended to favour solution of the collagen (Tables 1 and 2). Other

organic solvents miscible with water, e.g. *isopropanol* and *pyridine*, also had a similar effect (Table 2). If the hydroxyl ion concentration exceeded about 0.5M the above solvents at 5M concentrations and 20° caused almost complete solution of the collagen in 3 days.

Table 2. *The effect of organic solvents on the amount of collagen dissolved during 3 days' soaking in sodium hydroxide solutions at 20°*

Solvent	Normality NaOH	pH at 20°	Percentage loss in weight
Dioxan (40%, v/v)	0.79	Approx. 14.1	Nearly all dissolved
	0.53	Approx. 14.1	Nearly all dissolved
	0.26	Approx. 14.1	All dissolved except grain
<i>n</i> -Propanol (37%, v/v)	0.63	13.9	Dissolved in 1 day
	0.30	13.7	Dissolved in 2 days
Pyridine (40%, v/v)	0.10	13.8	80
	0.08	13.7	56
	0.05	13.7	14
	0.04	13.6	8
	0.02	13.4	2
Diethylamine (40%, v/v)	0.002	13.5	2
Acetone (36%, v/v)	0.63	14.1	Dissolved in 1 day
	0.30	14.0	Dissolved in 2 days
Acetone (50%, v/v)	0.05	13.9	3.5
	0.03	13.8	2.2
	0.02	13.7	2.7
	0.015	13.6	2.5
	0.01	13.4	2.4
	0.007	13.3	2.0
	0.002	12.6	0.9

DISCUSSION

The results of this investigation confirm the suggestion that the increased swelling at high pH values in sodium hydroxide solutions is associated with the presence of a high concentration of hydroxyl ions rather than with any specific effect of the sodium ion. The other strong bases studied gave swelling curves of the same shape as those obtained with sodium hydroxide and the addition of 0.5M-sodium chloride did not alter the shape of the ammonia curve, but reduced swelling at all pH values. Variations in swelling caused by the different bases, both strong and weak, at pH values up to 12.5 can be attributed to variations in the extent to which the cations combine with collagen, thus reducing their contribution to the swelling pressure. On this basis the guanidinium cation has a much greater, and the ammonium ion a slightly greater, affinity for collagen than the cations of the other bases studied. The power of guanidine salts to denature proteins may be related to the high affinity of the guanidinium ions for proteins. The decrease in swelling at high pH values in solutions of weak bases, in apparent

agreement with the Donnan theory, is less readily explained. It was thought at first that the presence of the undissociated base in some way inhibited the effect of the hydroxyl ions on cohesion (Bowes & Kenten, 1947), but since depression of the swelling was found to be dependent on the percentage rather than on the molar concentration of the base it seemed more likely that it was related to changes in the medium. The fact that swelling curves of similar shape are obtained by progressively increasing the concentration of acetone in 0.01 and 0.1N-sodium hydroxide solutions lends support to this view. However, there are also specific differences between the bases, for the extent to which swelling is decreased is not entirely dependent on the percentage composition of their solutions; e.g. swelling in diethylamine solutions is much less than that in solutions of other bases at similar or higher percentage concentrations.

The decrease in swelling with increasing concentration of weak base or organic solvent must be due either to a lowering of the swelling pressure or to an increase in cohesion. Since the presence of organic solvents tended to disperse collagen, which is in itself an indication of decreased cohesion, the former suggestion seems the more likely. Some effect analogous to the 'salting out' of soluble proteins may also play a part as the effects are apparent even when the swelling pressure is very low, e.g. at very high or low pH values and in the isoelectric range.

The mechanism by which the swelling pressure is decreased is not at present clear. It is unlikely, judging from the titration curves of proteins in organic solvents, and in particular of gelatin (Lichtenstein, 1940), that it can be attributed to alterations in the ionization of the reactive groups of the collagen. It is possible that it may be connected with the effect of the solvents on the activities of the ions. The distribution of the ions between the internal and the external phases in the Donnan equilibria is more properly represented by activities than by concentrations. Thus

$$e' = 2a_y y + a_z z - 2a_x x,$$

where e' , x , y and z have their usual significance (Bowes & Kenten, 1950; Bolam, 1932) and a_x , a_y and a_z are their respective activity coefficients. In aqueous sodium hydroxide solutions the activity coefficients will not differ greatly from unity, but in the presence of organic substances the activity coefficients will be appreciably lower, and e' , and hence the swelling pressure, will be proportionately smaller. To what extent the swelling may be affected by differences between the activity of the components of the solvent phase inside and outside the fibres is not evident.

Although giving no positive evidence regarding the possible reasons for the decrease in cohesion of

the collagen at high pH values, the results are not inconsistent with the view that the main cause is breaking of intermolecular links between the polypeptide molecules. The breaking of hydrogen bonds between polypeptide groups will almost certainly be involved to some extent, but in view of recent work by Lindley (1947), who finds that the specific removal of amide groups greatly increases the solubility of wool in alkaline solutions, links involving these groups may also be concerned. Treatment of collagen in sodium hydroxide solution at pH 13.7 for 3 days decreased the amide nitrogen from 0.36 to 0.12 mmol./g. and caused over 700 % swelling, whereas treatment in ammonia or diethylamine solutions at the same observed pH values had no appreciable effect on the amide nitrogen and caused no swelling. In this connexion it is of interest that Ames (1944) states that loss of amide nitrogen is essential for the production of gelatin from collagen. Another possibility is that the decrease in cohesion is related to removal of mucopolysaccharides. Partridge (1948), as a result of work on the state of combination of chondroitin sulphate in cartilage, suggests that the chondroitin sulphate, acting as a multivalent anion, cements together the protein molecules to form fibrous macromolecules. He finds that breakdown of the gelatin-chondroitin sulphate complex causes a large decrease in the viscosity of gelatin. Treatment with alkali or calcium chloride are common methods of extracting chondroitin sulphate from cartilage and other tissues, and since both these treatments lead to a decrease in cohesion (for information on the effect of calcium chloride see Bowes, 1950) it seems possible that its removal may at least be a contributory cause of this decrease.

SUMMARY

1. The swelling of ox-hide collagen in solutions of strong and weak bases has been studied. Differences in swelling at pH values up to pH 12.5 are ascribed to variations in the affinity of the cations for the collagen.

2. The swelling curves obtained with tetramethylammonium hydroxide and guanidine were of the same general shape as the curve obtained with sodium hydroxide, suggesting that the increased swelling at high pH values is due to increase in the hydroxyl ion concentration rather than to any specific effect of the sodium ion.

3. With weak bases swelling decreased at pH values above 12.5, in apparent accordance with the Donnan theory. Curves of similar shape were obtained by the addition of increasing amounts of neutral organic solvent, and it is concluded that the decrease in swelling is due to the progressive departure from an aqueous medium. There were also specific effects due to the different bases.

4. The effect of the organic solvents was presumably due to decrease in the swelling pressure rather than to increase in cohesion, since their presence was found to favour solution of the collagen.

5. The results are consistent with the view that decrease in the cohesion of collagen at high pH values is due to breaking of intermolecular linkages. The possible nature of these linkages is discussed.

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