The Swelling of Bovine Ligamentum Nuchae as a Function of pH

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1. The swelling and solubilization of foetal and adult bovine ligament over a range of pH values has been investigated. 2. Maximum swelling occurs at pH 2.7 and pH 8.5 for both types of ligament. Above pH 13 complete solubilization occurs. 3. Considerably greater swelling and solubilization occurs with foetal ligament below pH 12. 4. The presence of 2M-sodium chloride markedly suppresses both swelling and solubilization. Above 1N-sodium hydroxide actual dehydration occurs and little or no protein is extracted. 5. It is suggested that the intermolecular cross-links in a considerable proportion of foetal elastin are weak and that extraction with aqueous salt solution at pH 8.5 would provide optimum conditions for extracting soluble proteins, including soluble elastin if this is present.

It seems likely, in the light of current concepts of protein synthesis, that a soluble protein is a necessary precursor of insoluble elastin. This would be analogous to the position of soluble collagen as a precursor of insoluble collagen. As part of an effort to isolate and characterize an elastin precursor, a study has been made of the swelling properties of bovine ligamentum nuchae, an elastin-rich tissue, to find optimum conditions of extraction.

Two opposing factors are responsible for the swelling of fibrous proteins when placed in acid or alkaline solutions: (1) the swelling pressure due to the Donnan membrane effect; (2) cohesive forces present in the fibrous proteins themselves, either between fibrils and fibres, or intramolecularly (Procter & Wilson, 1916; Lloyd, 1938; Bowes & Kenten, 1950). The degree of swelling will depend on the balance of these two forces, complete solution occurring when the swelling pressure completely overcomes the cohesive forces.

A number of studies have been made of the swelling properties of collagen and gelatin (reviewed by Gustavson, 1956). In their detailed study, Bowes & Kenten (1950) demonstrated the marked difference in swelling of collagen at low pOH as compared with that at low pH (Fig. 3), and also that a decrease in cohesive forces is the most important factor in determining swelling of collagen in alkaline solutions.

The present study was carried out to determine the swelling properties of a tissue rich in elastin. Very few such studies have been previously reported. Roddy & O'Flaherty (1938, 1939) observed the swelling of elastin that occurs in hides during the chrome tanning process, pointing out that elastin swelled less than collagen in both acid and alkaline solutions; Lloyd & Garrod (1948) reported briefly on the swelling of elastin in various solvents.

Three objectives were in mind at the start of the experiment: (1) to determine maximal swelling with regard to pH; (2) to determine if maximum solubilization occurs when there is maximum swelling; (3) to ascertain the effect of ionic strength on swelling and solubilization. Bovine ligamentum nuchae was selected, as it has been shown to contain a high proportion of elastin (Bowes & Kenten, 1949). It was considered likely that foetal ligament, since it is derived from a rapidly growing system, would contain a higher concentration of soluble elastin. Therefore both adult and foetal elastin were studied.

EXPERIMENTAL

Materials. Ligamentum nuchae was obtained from freshly killed adult cows and from the foetuses of cows killed at term pregnancy. After careful cleaning of all extraneous material, including surrounding fascia, the specimens were cut into 2-3 in. segments and frozen, until such times as they were used for the swelling studies. Table 1 gives the composition of the materials used in the experiment and also of 6-month-old calf ligament. Agreement was within 2% on individual determinations, even though specimens were obtained from several animals of the same age.

Solutions. Swelling studies were carried out in unbuffered solutions of acid and alkali, ranging from 1N- to 1 μ N-HCl

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Table 1. *Composition of bovine ligamentum nuchae*

Nitrogen was determined by micro-Kjeldahl analysis (Ma & Zuazaga, 1942). Moisture content was determined gravimetrically after drying for 24 hr. at 110°. Total collagen was determined by hydroxyproline determination on the gelatin hydrolysate by the method of Woessner (1961). Hydroxyproline content of collagen was assumed to be 14.4% (w/w) (Jackson, Leach & Jacobs, 1958). Elastin content was determined gravimetrically (Partridge, Davis & Adair, 1955).

Source of ligament	Nitrogen (% dry wt.)	Moisture (%)	Total collagen (% dry wt.)	Insoluble elastin (% dry wt.)
Term foetal calf	15.51	75.0	15.2	47.6
6-month-old calf	16.71	56.2	12.3	74.0
Adult cow	16.70	56.5	16.3	71.3

and 2N- to 1M-NaOH and in distilled water. To study the effect of salt concentration, NaCl was added to a final concentration of 2M.

Swelling procedure. Weighed samples (500–550 mg.) of freshly thawed ligament were soaked in 200 ml. of the various solutions in beakers at room temperature, without agitation. A crystal of thymol was added to each solution to retard the growth of bacteria and moulds. The beakers were sealed with Parafilm. After 3 days, the specimens were removed from solution, carefully blotted on filter paper with frequent turning for 5 min. and reweighed. Determination of final wet weights proved difficult only at the very high pH range (> pH12.5) of the non-saline solutions, where the final material was very gelatinous and fragile. Final weights in this range were thus not very reliable and represent a rough estimation.

This problem did not exist with the solutions containing NaCl (2M) even at high pH values, since the soaked ligament in all instances was a hard rubber-like substance that became progressively smaller as pH increased. All determinations of swelling were repeated at least twice for each type of tissue and solution.

Determination of pH. This was carried out immediately on removal of the ligament from solution, with a Beckman Zeromatic pH-meter with an Ag-AgCl reference electrode and a type E-2 Beckman glass electrode, to give an accurate coverage of almost the entire pH range. The meter was standardized at intervals of 2 pH units with prepared buffers as supplied by the manufacturer. Salt was added to the standard buffers to give a concentration of 2M-NaCl when determining the final pH of the saline solutions. At and above pH13, meter readings were not considered accurate, so titrations were carried out on a sample of the solutions, after removal of the ligament remnants, with standard 0.100N-HCl and phenolphthalein as indicator. No change from the original molarity was observed at these high pH values. High alkaline concentrations are expressed as normality rather than pH on all the graphs (Figs. 1, 2 and 3).

Determination of solubilization. Nitrogen determinations were carried out on a sample of each of the solutions after

removal of the ligament, by using the micro-Kjeldahl method of Ma & Zuazaga (1942). The amount of protein in solution was calculated on the basis of 16% nitrogen content.

Calculation of swelling. Swelling was calculated for each sample as follows:

$$\text{Percentage swelling} = \frac{w - (a - b)}{a - b} \times 100$$

where *a* is the dry weight of ligament, *b* is the amount of protein solubilized and *w* is the wet weight of swollen sample. Where complete solubilization of the sample occurred, swelling was assumed to be infinite, since in these cases it may be regarded that swelling has been sufficient to disperse the entire sample evenly throughout the solution. No correction for the osmotic effects exerted by solubilized protein against the unsolubilized portion was made. However, this was not considered to be important since the introduction of such factors would not alter the overall shape of the curves and would contribute little to the information we were seeking.

RESULTS

Swelling and solubilization at acid pH (pH 0–6)

Without salt. Both adult and foetal ligament have a maximal swelling (Figs. 1*a* and 1*b*) and solubilization (Figs. 2*a* and 2*b*) in the pH range 2–3. The solubilization curve of foetal ligament shows two humps in this area, but because of their small amplitude they are probably not of significance. The hydrolytic effect of H⁺ ions no doubt plays some part in keeping solubilization relatively high at the very low pH values. The low degree of swelling observed at pH 4–5 corresponds to the isoelectric point of acid-solubilized elastin, which has been reported in the pH range 3.9–4.8, depending on the ionic strength of the solution (Partridge, Davis & Adair, 1955).

With 2M-sodium chloride. Complete suppression of the swelling curve occurs for adult ligament (Fig. 1*a*). Foetal ligament undergoes a gradual increase of swelling with increase of pH, so that at the isoelectric pH there is more swelling in the presence of salt than in its absence (Fig. 1*b*). This reversal of swelling curves at isoelectric pH is also manifest by some other proteins (Page & Page, 1927), and has been shown to occur with collagen, as seen in Fig. 3 (after Bowes & Kenten, 1950).

Swelling and solubilization at neutral and mildly alkaline pH (pH 6–12)

Without salt. Very marked swelling of foetal ligament occurs in this range, reaching a maximum in the pH range 8–8.5 (Fig. 1*b*). Solubilization correlates closely with swelling, both dropping off above pH 8.5, but at different rates (Figs. 1*b* and 2*b*). Adult ligament (Fig. 1*a*) shows swelling properties

Fig. 1. Swelling of adult (a) and foetal (b) bovine ligamentum nuchae in non-saline (●) and in 2M-NaCl (▲) solutions at various pH values at 20° for 3 days.

Fig. 2. Solubilization of adult (a) and foetal (b) bovine ligamentum nuchae in non-saline (●) and in 2M-NaCl (▲) solutions at various pH values at 20° for 3 days.

similar to foetal ligament, but to a much smaller degree. In this range, solubilization of adult ligament (Fig. 2a) is markedly different from that of

foetal ligament (Fig. 2b) in that there is virtually no increase above that observed at the isoelectric pH, whereas with foetal tissue there is twice as

Fig. 3. Swelling of dried ox hide in HCl-NaCl and NaOH-NaCl systems at 20° for 3 days: ●, non-saline solutions; ▲, 2M-NaCl solutions. From Bowes & Kenten (1950).

much solubilized protein at pH 8.4 as compared with that at pH 5.5.

With 2M-sodium chloride. With foetal ligament, there is complete suppression of the marked swelling that was observed in the absence of salt (Fig. 1b). A slight trough occurs in the swelling curves for foetal and adult ligament between pH 11 and 12, corresponding to the valleys that occur at this pH range in swelling in non-saline medium. In general, swelling and solubilization are markedly suppressed in this range for both tissues, but the degree of depression is much greater with the foetal ligament.

Swelling and solubilization at very alkaline pH (above pH 12)

Without salt. A marked upward deflexion occurs in this range in both the swelling and solubilization curves for both ligaments (Figs. 1 and 2). As with collagen, this may indicate weakening of the cohesive forces and probably some hydrolysis of peptide bonds, resulting in the dissolution of the elastin fibres. At approx. 0.5N-sodium hydroxide both tissues are completely solubilized. However, solubilization of foetal ligament increases steadily from pH 9 upward, even though swelling is actually decreasing through part of this range.

With 2M-sodium chloride. Unusual phenomena are also manifest in this portion of the curves. There is a definite tendency towards increased swelling and solubilization of both tissues above pH 12, but apparently the osmotic effect of the increased concentration of sodium hydroxide together with the presence of 2M-sodium chloride

is stronger than its ability to solubilize the ligament, so that at concentrations greater than 1N-sodium hydroxide, in the presence of salt, there is complete 'protection' against dissolution. Ligament taken from the 2N-sodium hydroxide solution, washed and placed in a lightly buffered solution at pH 8.4 without salt, swells in the usual fashion and is not completely solubilized, as might have been expected if the presence of high salt concentration was preventing release of fragments of elastin resulting from hydrolysis at the high pH values.

DISCUSSION

Swelling and solubilization. With collagen (Fig. 3) swelling on the alkaline side of the isoelectric point deviates markedly from that predicted by the Procter & Wilson (1916) application of the Donnan effect. Bowes & Kenten (1950) pointed out that this is probably due to the destructive effect of high alkaline pH on the cohesive forces that hold together the polypeptide chains of the collagen molecule and similar intramolecular cross-links. A similar deviation occurs with elastin, above pH 13. However, the only covalent cross-links so far identified in elastin are those involving the pyridinium derivatives desmosine and isodesmosine (Thomas, Elsdon & Partridge, 1963). These, however, would not be susceptible to alkaline conditions. Only a few peptide bonds are hydrolysed in collagen under these conditions (Bowes & Kenten, 1950). If this is also true of elastin then it is possible that important cross-links other than the desmosines occur in elastin and that these are susceptible to alkaline conditions.

It has been reported (Robert & Poullain, 1963) that complete dissolution of elastin occurs more readily under alkaline conditions in the presence of ethanol. Since elastin contains a very high proportion of non-polar amino acids, it is probable that the presence of ethanol renders hydrophobic bonds more susceptible to alkaline conditions. The degree of peptide hydrolysis and the size of the structures present in solution after alkali treatment are not known.

The Donnan effect would be expected to be the same for both foetal and adult ligaments. Thus the markedly greater swelling and solubilization of foetal ligament, at all pH values (Figs. 1 and 2), suggest that the cohesive forces are weaker in this tissue than in adult ligament. This is compatible with the finding that the concentration of desmosines is lower in foetal elastin than in adult elastin (Miller, Martin & Piez, 1964).

Foetal ligament contains only 47.6% of insoluble elastin as compared with 71.3% for adult elastin (Table 1). This also suggests that the difference in swelling and solubilization may be due to the

presence of a higher concentration of poorly cross-linked elastin in the foetal ligament.

The maximum swelling observed at pH 8.5 is not explainable by the Donnan equilibrium. If the cohesive forces remained unaffected the maximum should occur at pH 11–12. The observation is puzzling, and at this time we can offer no explanation.

Effect of 2M-sodium chloride. The swelling and dissolution of both foetal and adult ligament in 1.5N- and 2N-sodium hydroxide is completely suppressed by the presence of 2M-sodium chloride. This absence of swelling is due to the suppression of the osmotic pressure differences, the osmotic pressure inside and outside the ligament due to salt being the major donator to the Donnan equilibrium. Thus the reduced solubilization in the presence of salt indicates that the cohesive forces weakened by alkaline conditions are still sufficient to resist the much reduced swelling pressure and maintain the normal structure of the ligament. This also suggests that the degree of peptide hydrolysis may be small.

The overall suppression of swelling in the presence of 2M-sodium chloride is predictable for the Donnan equilibrium. However, the actual dehydration, which occurs above pH 12, is not predictable on this basis and is in marked contrast with the failure of salt to affect the swelling of collagen in this pH range. However, it is possible that the effect may be similar to the so-called 'pickling' effect seen with collagen at pH 1 and salt concentrations greater than 1M (Gustavson, 1956), which also causes a dehydrating effect.

Gross (1957) and Wirtschafter & Bentley (1962) reported that the extractability of collagen paralleled the rate of growth of the rat, with the period of maximal growth at 35 days giving maximal neutral-salt-soluble collagen concentration in the skin. It was thought that 6-month-old calf ligamentum nuchae might show maximum swelling and solubilization also, since this is the period of maximal growth for this animal (Altman & Dittmer, 1962). A complete set of swellings over the entire pH range was thus carried out with and without salt. These curves were identical with those obtained for adult ligament and are therefore not included. The results in Table 1 indicate that the chemical properties of 6-month-old ligament are very similar to those of adult ligament.

The swelling curves previously obtained for collagen, of which Fig. 3 is a typical example, are markedly different from those we have obtained for ligament. Thus the presence of collagen in the ligaments is not likely to have had much effect on the behaviour of the elastin in this tissue.

The main object of this study was to obtain information that would enable us to choose optimum

swelling conditions for obtaining soluble elastin under mild non-hydrolytic conditions, which might then lead to the isolation of an elastin molecule analogous to tropocollagen. The results we have obtained suggest that aqueous solution at pH 8.4 would be suitable for this purpose. Under these conditions, it would be expected that soluble elastin, as well as soluble collagen and globular proteins, such as are found in other connective tissues (Humphrey, Neuberger & Perkins, 1957), would be extracted.

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