

## EFFECTS OF CERTAIN PHYSICO-CHEMICAL AGENTS ON THE MECHANICAL PROPERTIES OF THE CATCH APPARATUS OF THE SEA-URCHIN SPINE

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### SUMMARY

The catch apparatus (CA), a collagenous connective tissue which connects the sea-urchin spine to the test, is known to undergo a remarkable change in its mechanical properties. Effects of various physico-chemical factors on the mechanical properties of the CA have been investigated in order to characterize the linkages which are responsible for determining the mechanical properties of the CA. The stress-strain relations obtained by stretching the specimen at a slow constant speed,  $7 \mu\text{m s}^{-1}$ , were taken as a measure of the viscous resistance of the CA. The viscous resistance of the CA depended largely on the pH and the ionic strength of the medium. It increased with increasing pH and with decreasing ionic strength. The viscous resistance was markedly reduced in a Ca-free medium containing 5 mM-EGTA. It is suggested that linkages sensitive to low pH and those mediated by  $\text{Ca}^{2+}$  play a significant role in determining the mechanical properties of the CA.

### INTRODUCTION

The echinoid spine is connected to the test by the catch apparatus (CA), a cylindrical sheath of collagenous tissue which surrounds the joint at the base of the spine. Though the CA is mainly composed of extracellular collagen fibres, it is known to undergo rapid and reversible changes in its mechanical properties (Takahashi, 1966, 1967*a,b*). The CA serves to hold the spine in a given posture for some time by increasing its stretch resistance. On the other hand, when the CA is in an extensible state, the spine can be moved freely by contraction of the spine muscle. The isolated preparation of the CA also responds to various kinds of stimuli by changes in its extensibility under a constant load (Takahashi, 1966, 1967*b*).

Though the CA contains a small number of muscle cells, it has been shown that these muscle cells alone cannot account for the large changes in the mechanical properties of the CA (Smith, Wainwright, Baker & Cayer, 1981; Hidaka & Takahashi, 1983). Since an elongation of the CA causes sliding of adjacent collagen fibrils, it has been suggested that the mechanical properties of the CA depend mainly on the cohesive force between the collagen fibrils (Hidaka & Takahashi, 1983). Changes in the mechanical properties of the CA may reflect changes in the number

and/or stability of physico-chemical linkages in the collagen fibre system. However it is not yet known what kind of physico-chemical linkages are responsible for the cohesive force between the collagen fibrils.

The CA shows a creep phenomenon and responds to various kinds of stimuli with changes in the rate of isotonic extension. The stretch resistance of the CA in an extensible state is largely dependent on the rate of stretch (Hidaka & Takahashi, 1983). These observations show that changes in the mechanical properties of the CA are mainly due to changes in viscosity and that weak secondary bonds play a significant role in determining the mechanical properties of the CA. Labile linkages such as electrostatic interactions, hydrogen bonds, hydrophobic bonds, disulphide bonds, and Schiff base linkages are likely to be involved in maintaining the stretch resistance of the CA.

In the present study, the effects of various physico-chemical factors on the mechanical properties of the CA were examined in order to characterize the linkages which provide the stretch resistance of the CA. On the basis of this fundamental study, possible mechanisms by which the mechanical properties of the CA are modified will be discussed.

#### MATERIALS AND METHODS

##### *Tensile testing apparatus*

A diagram of the tensile testing apparatus especially constructed for tensile tests of the CA is shown in Fig. 1. The basic mechanical design was similar to the tensometer used by Joffe & Hepburn (1974), but a servo-system was adopted for length control to avoid possible error caused by compliance in the testing apparatus. A rigid brass frame (F) could be moved vertically by the rotation of the screw (S) to produce longitudinal deformation of the specimen. The specimen was firmly fixed by clamping the spine at one end and a piece of the test at another. The upper clamp holding the spine was connected to a steel plate which was in turn attached to the movable frame. On this steel plate was mounted a pair of semiconductor strain gauges which served as a force transducer. The displacement of this steel plate was measured by a linear variable differential transformer (LVDT) (Shinko Denshi Ltd) and the displacement signal was fed back to a servo-amplifier for length control. A d.c. servomotor (Sawamura Electric Industry Ltd) drove the screw so that the signal from the displacement transducer should follow the ramp voltage generated by the ramp generator. With this arrangement the specimen could be stretched at a predetermined constant velocity up to a desired strain. Since the deformation of the specimen clamp could not be compensated by this length control system, the compliance of the clamp was made very low: less than  $1 \mu\text{m}/100 \text{ g}$ .

The signals from the force and displacement transducers were displayed on an Iwatsu DS-5016 oscilloscope to obtain the stress-strain curve and were photographed. Simultaneously they were recorded as a function of time on a pen-recorder (Multicorder, Watanabe).

##### *Preparation and experimental procedures*

Specimens of *Anthocidaris crassispina* measuring 5–7 cm in test diameter were collected near the Misaki Marine Biological Station, University of Tokyo. They w

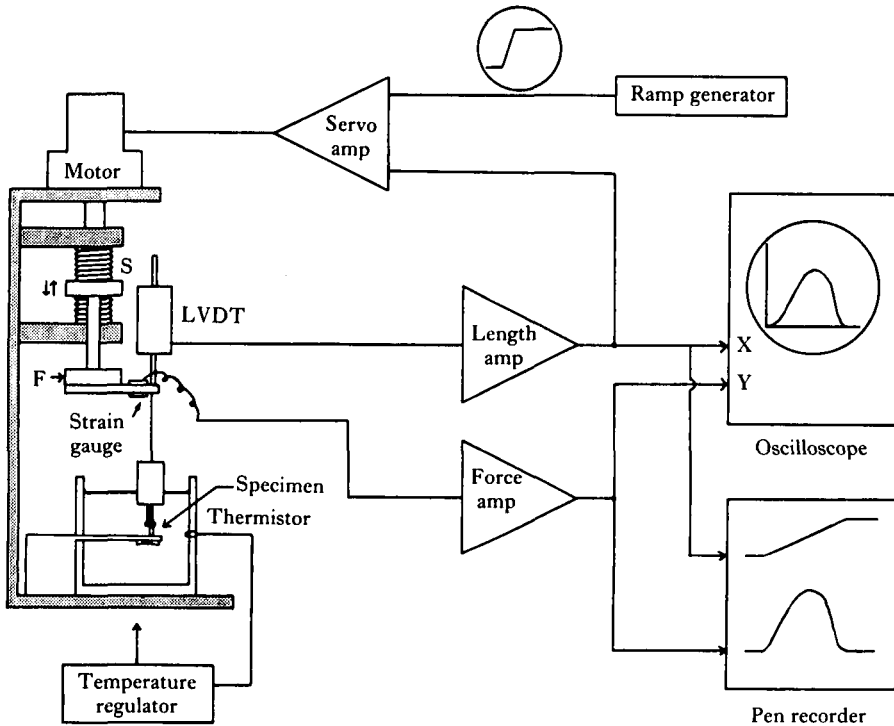


Fig. 1. A diagram of the testing apparatus. The specimen was attached to the apparatus firmly by clamping the spine at one end and a piece of test at another. A linear variable differential transformer (LVDT) produced a length signal. A pair of semiconductor strain gauges mounted on a steel plate served as a force transducer. This steel plate was in turn firmly attached to a movable frame (F). A d.c. motor drove the screw (S) to produce the longitudinal movement of the specimen so that the length signal should follow the command voltage from the ramp generator. Length and force signals were simultaneously recorded on an oscilloscope to obtain a stress-strain curve and also on a pen-recorder as a function of time.

used immediately or after being maintained in a basket hung in the sea at the pier. Interambulacral primary spines were used throughout this experiment. The CA was exposed by removing the epithelium and the spine muscle with forceps. A strip of the CA 0.5 mm wide was prepared by cutting away the rest of it. The original length and thickness of the strip were measured under a dissecting microscope provided with an ocular micrometer. Then the specimen was attached to the testing apparatus and was equilibrated in artificial sea water (ASW) for 5 min. During this time a slight stretch was applied to the specimen until a small tension (5–10 g) was generated so that the slack in the specimen was removed. Then the initial length of the specimen was measured, since the length of the strip usually changed during these procedures. The initial cross-sectional area of the specimen was calculated by dividing the original volume of the specimen by the initial length. After 5 min of adaptation, the medium was exchanged for the test solution after rinsing the chamber twice with the test solution. The specimen was immersed in the test solution for 20 min before testing. The solution was continuously stirred with a magnetic stirrer and the temperature of the solution was held at 20°C, approximately

the average temperature of the sea water at the site of collection of the sea urchins.

The ASW for adapting the specimen contained (in mM) NaCl, 433.7; KCl, 10.0; CaCl<sub>2</sub>, 10.1; MgCl<sub>2</sub>, 52.5; NaHCO<sub>3</sub>, 2.5. The pH was adjusted to 8.0 with HCl or NaOH.

In the experiment to study the effect of pH, the following buffers were used instead of NaHCO<sub>3</sub>, other ionic compositions being the same as that of the adapting ASW: glycine-hydrochloride-glycine (ionic strength,  $\mu = 0.01$ ) pH 3.0; acetic acid-sodium acetate ( $\mu = 0.01$ ) pH 4.0, 5.0; MES-NaOH (0.01 M) pH 6.0; HEPES-NaOH (0.01 M) pH 7.0; Tris-HCl ( $\mu = 0.01$ ) pH 8.0, 9.0; glycine-sodium glycinate ( $\mu = 0.01$ ) pH 10.0. The pH of all solutions was adjusted to within  $\pm 0.05$  pH unit at 20 °C.

Two series of experiments were carried out to determine the effect of the ionic strength of the medium on the mechanical properties of the CA. First, the ionic strength of the medium was varied by varying the concentration of NaCl in ASW. Since ASW of low ionic strength is hypotonic, isotonicity of the solution was retained by adding sucrose to it. Though ASW of high ionic strength is hypertonic, it is impossible to compensate tonicities of such solutions. The effect of hypertonic ASW prepared by adding sucrose to the standard ASW was examined as a control. Second, various concentrations of NaCl solutions were used to examine the effect of ionic strength. All the solutions were buffered to pH 8.0 with Tris-HCl ( $\mu = 0.01$ ).

In the experiment to investigate the effect of divalent cations, the specimens were immersed in ASW which contained various concentrations of CaCl<sub>2</sub> or MgCl<sub>2</sub>. The isotonicity of the solution was retained by adjusting the concentration of NaCl.

Sodium borohydride (NaBH<sub>4</sub>) is known to stabilize Schiff base links. The effect of NaBH<sub>4</sub> was examined by treating the specimens with 0.1 M-NaBH<sub>4</sub> in non-buffered ASW for 20 min. Then the specimens were rinsed in ASW buffered to pH 5.0 for 3 min and were immersed in ASW buffered to a different pH for 20 min before stretch. Effects of other chemical reagents were examined by treating the specimens with the reagents dissolved in the standard ASW for 20 min before stretch.

To examine the dependency of the mechanical properties of the CA on the ambient temperature, the tensile tests were made at different temperatures, 10, 20 and 30 °C. The temperature of the medium was regulated by a thermistor-controlled thermomodule. The specimens were first immersed in adapting ASW at 20 °C for 5 min, and then the medium was replaced by the test solution which had been kept at a predetermined temperature. Because of the thermal capacity of the chamber and of the heat leakage, the temperature of the solution could not reach the desired value immediately after the solution exchange. But the temperature gradually approached the desired value and reached it within 20 min. The test took place 20 min after the solution exchange.

Changes in the mechanical properties of the CA are due to changes in the viscosity and can be detected only by a tensile test with sufficiently slow stretches (Hidaka & Takahashi, 1983). In this experiment, the rate of stretch was  $7 \mu\text{m s}^{-1}$ , which has been shown to be appropriate for detecting changes in the mechanical properties of the CA. The specimens were stretched until they were completely broken and the passive tension fell to zero. To describe the stress-strain relations of the CA quantitatively, the maximum slope of the stress-strain curve (apparent stiffness) and the maximum

Force per unit initial cross-sectional area of the specimen (tensile strength) were measured (Fig. 2).

## RESULTS

### *Hydrogen ion concentration*

The pH of the bathing medium had a large effect on the mechanical properties of the CA. Fig. 2A and B show the stress-strain curves of the CA obtained at pH 9 and pH 5, respectively. When stretched at  $7 \mu\text{m s}^{-1}$ , the CA developed a large tension at pH 9, but it developed only a small tension at pH 5. Fig. 3 shows the effect of pH on the apparent stiffness and the tensile strength of the CA. Both the apparent stiffness and the tensile strength showed a large dependence on the ambient pH when specimens were stretched at  $7 \mu\text{m s}^{-1}$ . But they were almost constant when the rate of stretch was  $740 \mu\text{m s}^{-1}$ . Thus, the changes in the mechanical properties of the CA induced by changes in the ambient pH could be detected only when the stretch was sufficiently slow. This indicates that the changes in the mechanical properties of the CA are mainly due to changes in viscosity, as is the case with changes induced by acetylcholine (ACh) and adrenaline (Adr) (Hidaka & Takahashi, 1983). Since both the apparent stiffness and the tensile strength measured at  $7 \mu\text{m s}^{-1}$  represent the viscous resistance of the CA to stretch, they are expressed simply as viscous resistance in the following description of the results.

The viscous resistance of the CA increased as the ambient pH was increased in the range pH 5–10. There was a sharp increase in the viscous resistance above pH 8. The viscous resistance also increased at very low pH, pH 4 or less. But the specimens immersed in such a low pH solution underwent an irreversible change; their viscous resistance decreased markedly when the medium was exchanged for ASW buffered to pH 8 or 9.

### *Ionic strength*

The effect of ionic strength on the mechanical properties of the CA is shown in

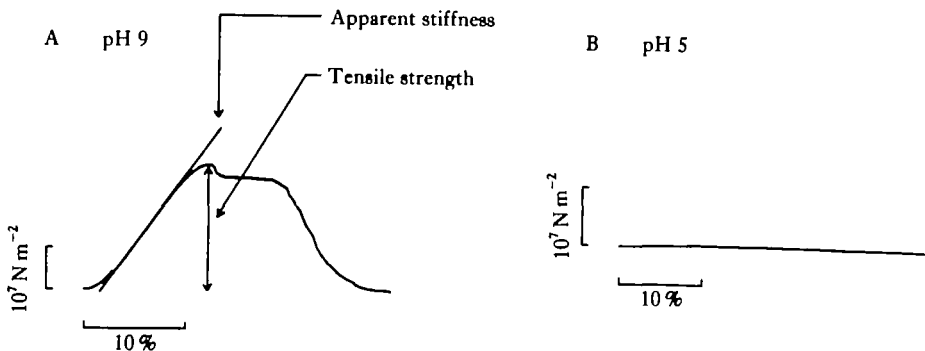


Fig. 2. Stress-strain curves of the CA immersed in ASW buffered to pH 9 (A) and to pH 5 (B). Horizontal bars represent 10% strain. Rate of extension,  $7 \mu\text{m s}^{-1}$ . The apparent stiffness and the tensile strength were defined as the maximum slope of the stress-strain curve and the maximum force per unit initial cross-sectional area of the specimen, respectively.

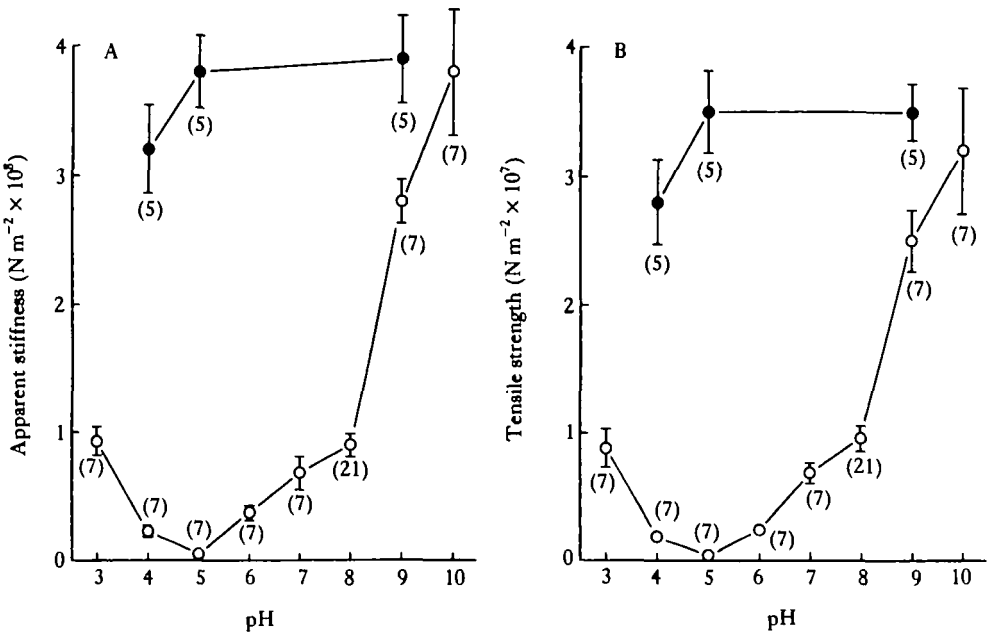


Fig. 3. Effect of pH on the apparent stiffness (A) and the tensile strength (B) of the CA. The specimens were immersed in ASW buffered to various pH at  $20^\circ\text{C}$  for 20 min before testing. Open circles and filled circles represent the data obtained by stretching the specimen at  $7 \mu\text{m s}^{-1}$  and at  $740 \mu\text{m s}^{-1}$ , respectively. The number of measurements is shown in parentheses. Means  $\pm$  s.e.m.

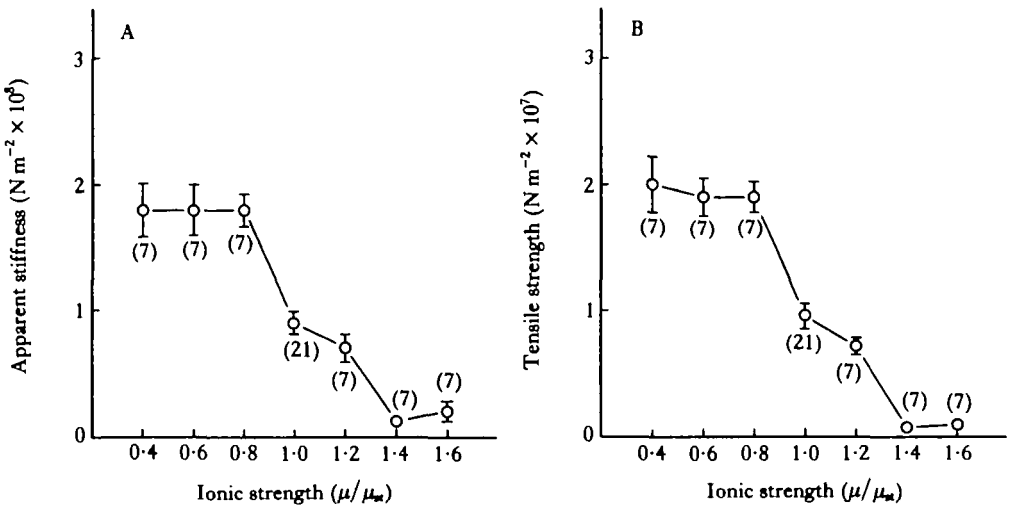


Fig. 4. Effect of ionic strength on the apparent stiffness (A) and the tensile strength (B) of the CA. The specimens were immersed in ASW of various ionic strength for 20 min before testing. The ionic strength was varied by varying the concentration of NaCl in the ASW and is expressed relative to the ionic strength of the standard ASW ( $\mu_{st}$ ). ASW of low ionic strength was made isotonic by adding sucrose to it. All solutions were buffered to pH 8 with Tris-HCl and were held at  $20^\circ\text{C}$ . Rate of extension,  $7 \mu\text{m s}^{-1}$ . The number of measurements is shown in parentheses. Means  $\pm$  s.e.m.

Figs 4 and 5. In Fig. 4, the ionic strength was varied by varying the concentration of NaCl in ASW with other ionic compositions being the same as the standard ASW. The viscous resistance of the CA increased about twofold as the ionic strength of the medium was decreased from the standard to 0.8 times the standard. The viscous resistance was constant at this relatively high level over the range of ionic strength 0.4–0.8 times the standard as long as isotonicity of the solution was maintained. If the tonicity was not compensated, the viscous resistance decreased as the ionic strength was further lowered to below 0.8 times the standard. The viscous resistance decreased with increasing ionic strength and it became very low at ionic strengths above 1.4 times the standard. A hypertonic ASW prepared by adding sucrose to the standard ASW slightly increased the viscous resistance in the range of tonicity 1.2–1.5 times the standard, but it reduced the viscous resistance to almost the same extent as a NaCl hypertonic ASW at a tonicity 1.7 times the standard.

Fig. 5 shows the result of an experiment in which the specimens were immersed in various concentrations of NaCl solutions for 20 min before stretch. In this experiment too, the increased ionic strength decreased the viscous resistance. The viscous resistance was very high in the buffer solution without NaCl. But it decreased sharply as the ionic strength was increased to 0.2 times the standard. No detectable tension was developed in the specimen immersed in NaCl solution whose ionic strength was 1.4 times the standard. While both the apparent stiffness and the tensile strength measured in the buffer solution without salts were high, the increase in the apparent stiffness was larger than the increase in the tensile strength. This may imply that, in a buffer solution, the bonds responsible for the viscous resistance of the CA become more stable than in a NaCl solution but that they cannot easily be re-formed once they are broken. It is likely that inorganic ions are necessary for the linkage formation.

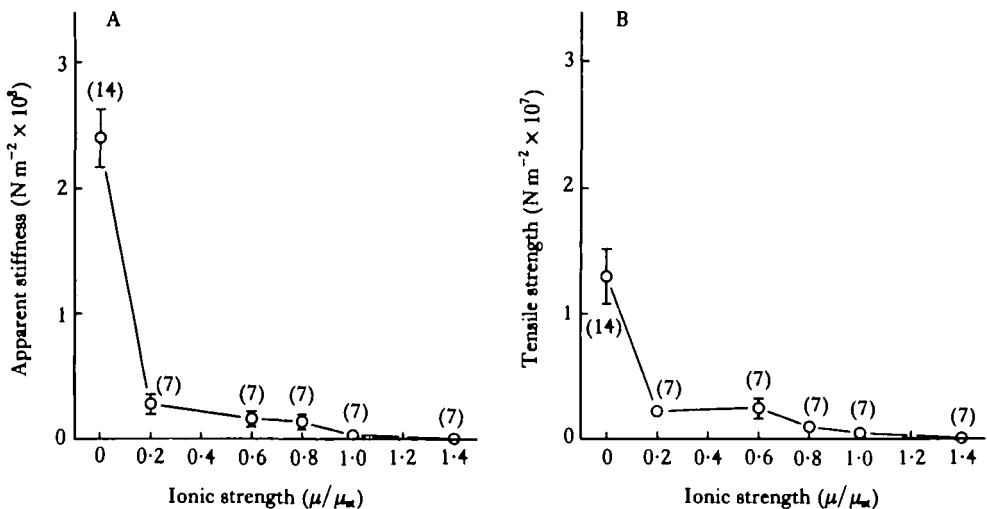


Fig. 5. Effect of ionic strength on the apparent stiffness (A) and the tensile strength (B) of the CA. The specimens were immersed in various concentrations of NaCl solutions for 20 min before testing. The ionic strength is expressed relative to the standard ASW. All solutions were buffered to pH 8 with Tris-HCl and were held at 20 °C. Rate of extension,  $7\ \mu\text{m}\ \text{s}^{-1}$ . The number of measurements is shown in parentheses. Means  $\pm$  s.e.m.

*Divalent cations*

The effect of varying the  $\text{Ca}^{2+}$  concentration in the bathing medium on the viscous resistance of the CA is shown in Fig. 6. When the specimen immersed in Ca-free ASW containing 5 mM-EGTA was stretched, no detectable tension was generated. Though CA-free ASW without EGTA reduced the viscous resistance, the extent of reduction was variable, probably depending on the amount of  $\text{Ca}^{2+}$  remaining within the tissue. Low Ca-ASW (1 mM) had little effect on the viscous resistance of the CA. High Ca-ASW (20 mM) increased the viscous resistance of the CA nearly twofold. But a further increase in  $\text{Ca}^{2+}$  concentration did not lead to a further increase in the viscous resistance. Instead the viscous resistance decreased gradually at  $\text{Ca}^{2+}$  concentrations above 50 mM.

When the concentration of  $\text{Mg}^{2+}$ , instead of  $\text{Ca}^{2+}$ , was increased, the viscous resistance was lowered and was almost constant over the range of  $\text{Mg}^{2+}$  concentration 62–142 mM (Fig. 7). It is clear that  $\text{Mg}^{2+}$  has no capacity to stabilize or augment the bonds responsible for the viscous resistance of the CA. Thus the effect of  $\text{Ca}^{2+}$  on the mechanical properties of the CA is specific and is not a general effect of divalent cations.

*Other chemical reagents*

Table 1 shows the effects of certain chemical reagents which are known to affect weak secondary bonds such as the hydrogen bond, hydrophobic bond, and disulphide bond. High concentrations of guanidine hydrochloride have a disrupting effect on the hydrogen bonds. Chaotropic anions such as thiocyanate weaken hydrophobic interactions. Guanidine hydrochloride (1 M) and sodium thiocyanate (0.1 M) in ASW

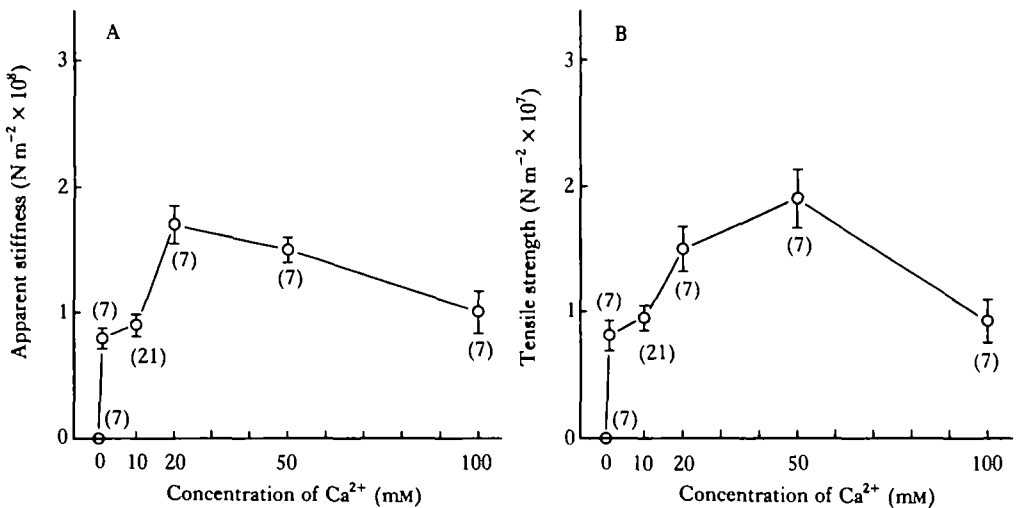


Fig. 6. Effect of  $\text{Ca}^{2+}$  on the apparent stiffness (A) and the tensile strength (B) of the CA. The specimens were immersed in ASW which contained various concentrations of  $\text{CaCl}_2$  for 20 min before testing. The solutions were made isotonic by adjusting the concentration of  $\text{NaCl}$ . All solutions were buffered to pH 8 with Tris-HCl and were held at 20°C. Rate of extension,  $7 \mu\text{m s}^{-1}$ . The number of measurements is shown in parentheses. Means  $\pm$  s.e.m.



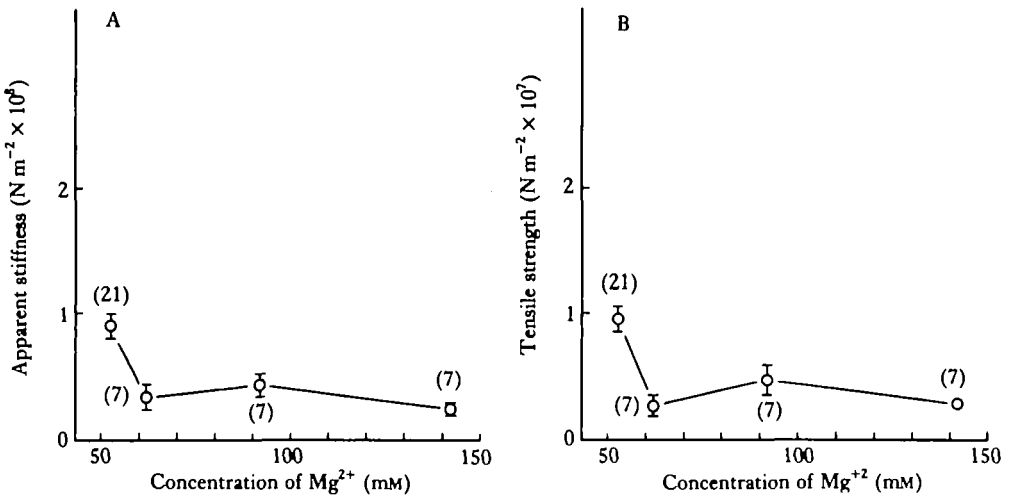


Fig. 7. Effect of Mg<sup>2+</sup> on the apparent stiffness (A) and the tensile strength (B) of the CA. The specimens were immersed in ASW which contained various concentrations of MgCl<sub>2</sub> for 20 min before testing. The solutions were made isotonic by adjusting the concentration of NaCl. All solutions were buffered to pH 8 with Tris-HCl and were held at 20°C. Rate of extension, 7 μm s<sup>-1</sup>. The number of measurements is shown in parentheses. Means ± s.e.m.

Table 1. Effects of some reagents which are known to disrupt hydrogen bonds, hydrophobic bonds and disulphide bonds

The specimens were immersed in ASW containing the reagent for 20 min before testing. All solutions were buffered to pH 8 with Tris-HCl and were held at 20°C. Rate of stretch, 7 μm s<sup>-1</sup>. The number of measurements is shown in parentheses. Means ± s.e.m.

	Apparent stiffness (× 10 <sup>7</sup> N m <sup>-2</sup> )	Tensile strength (× 10 <sup>7</sup> N m <sup>-2</sup> )
Control	9.0 ± 0.87 (21)	0.95 ± 0.10 (21)
0.1 M-guanidine HCl	6.8 ± 2.0 (4)	1.0 ± 0.30 (4)
1 M-guanidine HCl	0.0 (4)	0.0 (4)
0.01 M-NaSCN	10 ± 1.3 (4)	0.58 ± 0.20 (4)
0.1 M-NaSCN	0.0 (4)	0.0 (4)
0.2 M-β-mercaptoethanol	9.6 ± 3.3 (5)	0.88 ± 0.34 (5)

eliminated the viscous resistance of the CA almost completely. This may indicate that hydrogen bonds and hydrophobic bonds play a significant role in maintaining the viscous resistance of the CA. It is, however, difficult to identify the site of action of these reagents, since hydrogen bonds and hydrophobic bonds might be involved in almost all levels of structural organization of the CA.

Though treatment with 0.2 M-β-mercaptoethanol had no significant effect on the viscous resistance of the CA, sulphhydryl reagents increased the rate of isotonic extension in a creep experiment (Fig. 8). Dithiothreitol (0.03 M) increased the extensibility of the specimen considerably. The specimen lengthened up to the final rupture within 5–10 min after the application of the reagent under a small load (5 g). Also, 0.2 M-β-mercaptoethanol appeared to increase the rate of isotonic extension slightly and the specimen ruptured about 20 min after the application of the reagent.

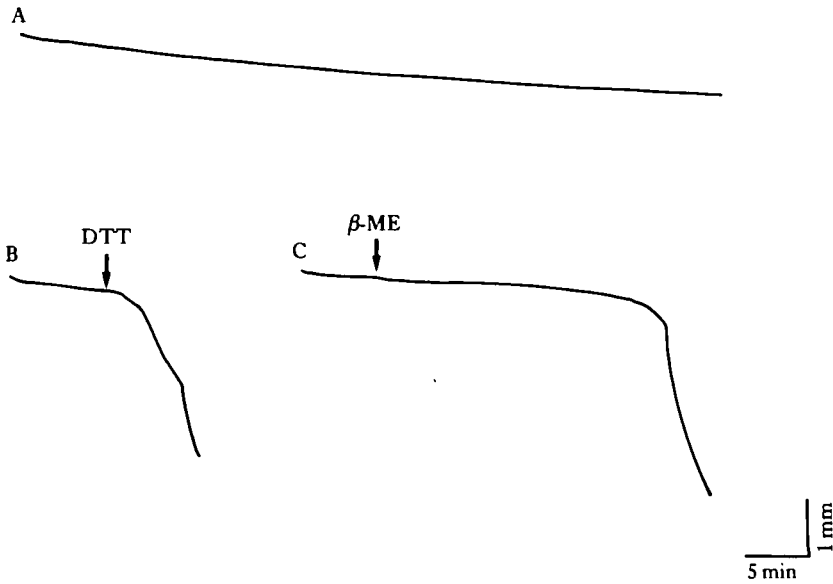


Fig. 8. Effects of sulphhydryl reagents on the rate of isotonic extension of the CA. (A) Control curve of an untreated CA showing a low and more or less constant rate of extension that continued for about 160 min before rupture took place. Application of 0.03 M-dithiothreitol, DTT (B) or 0.2 M- $\beta$ -mercaptoethanol,  $\beta$ -ME (C) to a CA whose rate of extension had been similar to that of (A) markedly increased the rate of extension. Load, 5 g.

Table 2. Effect of  $\text{NaBH}_4$  on the apparent stiffness and the tensile strength of the CA and on their pH dependence

The specimens were immersed in 0.1 M- $\text{NaBH}_4$  in non-buffered ASW for 20 min, rinsed in ASW at pH 5 for 3 min, and then immersed in ASW buffered to a different pH for 20 min before stretch. Rate of stretch,  $7 \mu\text{m s}^{-1}$ . The number of measurements is shown in parentheses. Means  $\pm$  s.e.m.

	Apparent stiffness ( $\times 10^7 \text{ N m}^{-2}$ )	Tensile strength ( $\times 10^7 \text{ N m}^{-2}$ )
pH 5	$1.3 \pm 0.69$ (5)	$0.12 \pm 0.07$ (5)
pH 8	$1.2 \pm 0.70$ (5)	$0.07 \pm 0.04$ (5)
pH 9	$1.5 \pm 1.0$ (5)	$0.07 \pm 0.03$ (5)
Control (pH 8)	$9.0 \pm 0.87$ (21)	$0.95 \pm 0.10$ (21)

Sodium borohydride ( $\text{NaBH}_4$ ) has been known to stabilize Schiff base linkages, which are involved in cross-linking between collagen molecules (Bailey, 1968). Pretreatment with 0.1 M- $\text{NaBH}_4$  did not increase the viscous resistance of the CA. On the contrary it decreased the viscous resistance measured at pH 5, 8 and 9 to almost the same low level (Table 2). This indicates that Schiff base linkages are not responsible for providing the viscous resistance of the CA.

#### Effect of temperature

Fig. 9 shows the viscous resistance of the CA measured at three different temperatures. The viscous resistance decreased when the temperature was lowered from  $20^\circ\text{C}$  to  $10^\circ\text{C}$  ( $P < 0.05$ ,  $t$ -test for the apparent stiffness;  $P < 0.005$ ,  $t$ -test for the tensile strength). It is interesting that the viscous resistance was lower at  $10^\circ\text{C}$  than

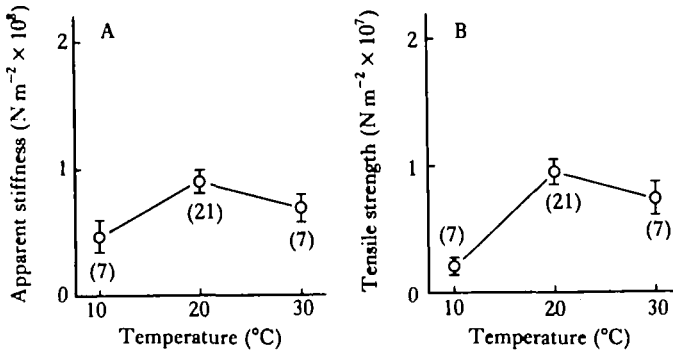


Fig. 9. Effect of temperature on the apparent stiffness (A) and the tensile strength (B) of the CA. Solutions were buffered to pH 8 with Tris-HCl at each temperature. Rate of extension,  $7 \mu\text{m s}^{-1}$ . The number of measurements is shown in parentheses. Means  $\pm$  s.e.m.

at 20 °C, as the viscosity of a fluid usually increases as the temperature is lowered. The decrease in the tensile strength at low temperature was more marked than the decrease in the apparent stiffness. There was no significant difference between the viscous resistance of the CA measured at 20 °C and 30 °C, although the average value slightly decreased as the temperature was raised to 30 °C.

#### DISCUSSION

The present study has shown that both the pH and the ionic strength of the bathing medium have a large effect on the mechanical properties of the CA. The primary effect of changing the pH and the ionic strength of the medium may be to alter the number and the distribution of charged groups on the surface of tissue components and to change the extent of electrostatic interactions between them. Therefore, electrostatic interactions seem to play a significant role in determining the mechanical properties of the CA.

The viscous resistance of the CA increased as the pH of the medium increased within the range of pH 5–10. Generally, the number of surface negative charges of polyelectrolytes increases as the ambient pH is raised. Since the increased number of surface negative charges leads to increased viscous resistance of the CA, it is likely that either one or both of the following interactions are involved in maintaining the viscous resistance of the CA. (1) Electrostatic attractive forces between fixed negative charges that are cross-linked by divalent cations such as  $\text{Ca}^{2+}$ . (2) Steric interactions caused by entanglement of the macromolecules which may assume more extended conformations as a result of electrostatic repulsive forces between negatively charged sites on them.

Since the collagen fibrils slide past one another on elongation of the CA, interactions between the collagen fibrils and interfibrillar substances may play an important role in providing the viscous resistance of the CA as well as interactions between the interfibrillar matrix molecules (Hidaka & Takahashi, 1983). The viscous resistance of the CA was lowest at pH 5, and it increased at both higher and lower pH's. The isoelectric point of soluble collagen and of collagen gel is reported to be about

pH 5 (Öbrink & Wasteson, 1971). It is interesting that the viscous resistance of the CA is lowest at the isoelectric point of collagen. It is likely that, at pH above 5, electrostatic attractive forces between negatively charged groups on the collagen and those on the interfibrillar matrix molecules cross-linked by divalent cations such as  $\text{Ca}^{2+}$  provide the resistive force against sliding of collagen fibrils. It can be speculated that, at pH below 5, electrostatic interactions between positively charged groups on the collagen and negatively charged groups on the interfibrillar matrix molecules might provide the viscous resistance of the CA.

Harkness (1970) has reported that the breaking load of rat tail skin decreased as the ambient pH was lowered over the range of pH 5–11. Reduction in the breaking load at pH below 7 has also been observed using the rat tail tendon, though in this case the breaking load also decreased at high pH (Bailey, 1968). It has been suggested that reduction in the breaking load of tendon fibres at low pH is attributable to dissociation of the Schiff base links between aldehyde and amino groups in the collagen molecules (Bailey, 1968). However, it is difficult to account for the large increase in the viscous resistance of the CA at pH 8–9 by Schiff base links alone. Furthermore  $\text{NaBH}_4$  which is known to stabilize the Schiff base links did not increase the viscous resistance of the CA, while it increased the breaking load of the rat tail skin in less than 30 min (Harkness & Harkness, 1968). So it is unlikely that Schiff base links play a major role in maintaining the viscous resistance of the CA.

It is known that high concentrations of inorganic ions reduce electrostatic interactions by masking the charged groups on the surface of polyelectrolyte molecules. The fact that the lower ionic strengths of the medium increased the viscous resistance of the CA indicates that the viscous resistance increased with an increasing extent of electrostatic interactions. This is consistent with both models described above.

The tonicity of the medium also had a large effect on the viscous resistance of the CA. But the effect of tonicity is difficult to interpret, since changes in tonicity cause changes in both the extent of hydration and the ionic strength of the tissue. The effect of sucrose-hypertonic ASW was biphasic, increasing the viscous resistance at a moderately high tonicity (1.2–1.5 times the standard) and decreasing it at a very high tonicity (1.7 times the standard). It is possible that sucrose-hypertonic ASW increases the viscous resistance by lowering the extent of hydration at a moderately high tonicity and decreases it by raising the ionic strength in the tissue at a very high tonicity.

The viscous resistance of the CA soaked in an isotonic NaCl solution was much smaller than that of the CA immersed in the standard ASW. The reduction in the viscous resistance of the CA in a NaCl solution might be due to the absence of other cations such as  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . Recently, Smith *et al.* (1981) have shown by creep experiments that divalent cations are necessary for the catch in the CA of *Arbacia punctulata*. The present study has shown that the viscous resistance of the CA was markedly dependent on the concentration of  $\text{Ca}^{2+}$ . If  $\text{Ca}^{2+}$  were removed from the medium by a Ca-chelating agent, the viscous resistance was greatly reduced. High concentrations of  $\text{Ca}^{2+}$  (20–50 mM) had an increasing effect on the viscous resistance of the CA. These observations indicate that the linkages mediated by  $\text{Ca}^{2+}$  play a major role in providing the viscous resistance of the CA. This supports the idea that  $\text{Ca}^{2+}$  ions act as ionic cross-links between the fixed negative charges on the surface of tissue components. For this role,  $\text{Mg}^{2+}$  cannot substitute for  $\text{Ca}^{2+}$  as Ca-free ASW

duced the viscous resistance even in the presence of  $Mg^{2+}$ . Excess  $Mg^{2+}$  (62–142  $\mu M$ ) decreased the viscous resistance of the CA (Fig. 7). Thus the effect of  $Ca^{2+}$  is highly specific.

The CA was made extensible by sulphhydryl reagents such as dithiothreitol and, less markedly,  $\beta$ -mercaptoethanol. It has been reported that the body wall tissue of holothuroids can be disaggregated into collagen fibrils in a neutral salt solution containing  $\beta$ -mercaptoethanol (Matsumura, Shinmei & Nagai, 1973). It is likely that linkages sensitive to sulphhydryl reagents are also important for the cohesive force between collagen fibrils in the CA. At present, however, the site of action of these reagents is not identified.

The viscous resistance of the CA was reduced as the ambient temperature was lowered from 20°C to 10°C. The decrease in the tensile strength was more marked than the decrease in the apparent stiffness. This might be accounted for by assuming that the probability of linkage re-formation is reduced at low temperature. It is likely that thermal vibration of molecules increases the probability of linkage re-formation, though a further increase in the temperature may act to augment breaking of the linkages. The apparent viscosity decrease in this temperature range might also indicate the importance of hydrophobic interactions within the CA. Hydrophobic interactions are very temperature dependent in this temperature range and their strength decreases markedly over this temperature range (Némethy & Scheraga, 1962).

The present study has shown that the linkages sensitive to low pH and those sensitive to a Ca-chelating agent are important for determining the mechanical properties of the CA. It has been reported that ACh renders the CA highly inextensible and Adr makes the CA highly extensible (Takahashi, 1967*b*). Changes in the viscous resistance of the CA induced by ACh and Adr have been measured (Hidaka & Takahashi, 1983). It is supposed that ACh and Adr excite some effector cells which mediate changes in the physico-chemical environment in the tissue thus resulting in changes in the mechanical properties. If this is the case, such changes in the physico-chemical environment would modify the mechanical properties of the CA to an extent comparable to the changes induced by ACh and Adr.

At present, ASW buffered to pH 9 is the only test solution that has been known to increase the viscous resistance of the CA to the same extent as  $10^{-4}$  M-ACh. It is likely that ACh increases the viscous resistance of the CA by raising the pH in the tissue up to 9. Solutions which decreased the viscous resistance of the CA to the extent comparable to  $10^{-4}$  M-Adr are Ca-free ASW containing 5 mM-EGTA, ASW buffered to pH 5, ASW of high ionic strength (above 1.4 times the standard), and 1 M-guanidine hydrochloride or 0.1 M-sodium thiocyanate in ASW. But it is unlikely that a large change in the extracellular inorganic ion concentration occurs *in vivo*. It is also unlikely that such reagents as guanidine hydrochloride or sodium thiocyanate are present at a high concentration in the CA. So it is a tempting idea that Adr decreases the viscous resistance of the CA by lowering the pH or the concentration of  $Ca^{2+}$  in the tissue.

The ophiuroid intervertebral ligament, which is also a collagenous tissue, is known to alter its mechanical properties drastically at autotomy or in response to certain stimuli (Wilkie, 1978*a,b*). Wilkie (1978*b*) has found that excess  $Ca^{2+}$  renders the

intervertebral ligament inextensible. The Ca-chelating agent EGTA increases the extensibility of the ligament, although Ca-free ASW has no discernible effect on the extensibility in the absence of the chelating agent. It has been suggested that changes in mechanical properties may be produced by changes in the availability of  $\text{Ca}^{2+}$  within the interfibrillar matrix of the connective tissue (Wilkie, 1978b).

At present a large effect of  $\text{Ca}^{2+}$  on the mechanical properties of the collagenous tissue is found only in echinoderms. It has been reported that treatment with EDTA produces no significant difference in the breaking strength of the rat tail skin (Harkness & Harkness, 1966) and only a slight decrease in the breaking strength of the rat tail tendon fibres (Bailey, 1968). Divalent cations seem to have no apparent effect on the mechanical properties of mesogloea of sea anemone (Gosline, 1971). It is, however, suggested that  $\text{Ca}^{2+}$  or other metal ions are implicated in the association of the collagen fibrils with the interfibrillar matrix in mammalian collagenous tissues (Steven, 1967). It is likely that linkages mediated by  $\text{Ca}^{2+}$  play a major role in the mechanical properties of the CA and other collagenous tissues in echinoderms, and that a unique system which controls these linkages had evolved in echinoderms.

It has been reported that the abdominal cuticle of *Rhodnius* becomes more extensible when the insect feeds (Bennet-Clark, 1962) and also when 5-hydroxytryptamine is injected into the haemocoel (Reynolds, 1974). Reynolds (1975) has shown that a change in pH occurs within the cuticle on plasticization and that this change in pH is probably large enough to account for the increased extensibility shown by a plasticized cuticle. He proposed that cells may actually produce a change in the extracellular pH to control the mechanical properties of the cuticle. The viscous resistance of the CA increases greatly as the ambient pH increases by one unit from pH 8 to pH 9. It seems not unreasonable to assume that such a change in pH occurs in the CA.

The present study suggests that changes in the extracellular pH and the concentration of  $\text{Ca}^{2+}$  are most likely to be involved in the *in vivo* mechanism of change of the mechanical properties of the CA.

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