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EFFECTS OF IONIC ENVIRONMENT ON VISCOSITY OF CATCH CONNECTIVE TISSUE IN HOLOTHURIAN BODY WALL

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SUMMARY

1. The effects of cations (H^+, Na^+, K^+, Ca^{2+}, Mg^{2+}) on the mechanical properties of the catch connective tissue in the dermis of a sea cucumber, *Holothuria leucospilota* Brandt, were studied.

2. The manipulation of the ionic environment caused rapid (apparent in minutes) and reversible viscosity change.

3. Artificial sea water (ASW) with a high concentration of Ca^{2+} (50 mmol l^{-1}) increased the normal viscosity 9 times; Ca^{2+}-free ASW decreased it to one-tenth. Ca^{2+} was the only ion which caused a viscosity change whose range was as large as the range of the viscosity distribution of the dermis in ASW.

4. Manipulation of the concentration of other ions caused an increase in viscosity. An increase of more than five-fold was observed in the following solutions: ASW with acidic (pH 4) or basic (pH 9, 10) pH; Na^+-free ASW whose Na^+ was replaced by either sucrose or choline; ASW with high (100 mmol l^{-1}) concentration of K^+; ASW with low (0 mmol l^{-1}) or high (250 mmol l^{-1}) concentrations of Mg^{2+}.

5. The dermis contracted in distilled water, although this tissue contained no muscle cells.

INTRODUCTION

Echinoderms have catch connective tissues which can exhibit rapid changes in mechanical properties under nervous control. They are composed of collagen fibres, ground substance, and neurones with secretory granules (Motokawa, 1982*a*, 1984*a*, 1985; Wilkie, 1984). Pioneering work of Wilkie (1978, 1979) showed that the viscosity of the intervertebral ligament of a brittlestar was Ca^{2+} dependent. Wilkie suggested that the removal of Ca^{2+} from the extracellular matrix by the activity of the neurosecretory cells caused the viscosity decrease of the matrix, and thus made the ligament soft enough to break at arm autotomy (Wilkie, 1979).

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Key words: catch connective tissue, viscosity, Ca^{2+}, ionic environment, sea cucumber.

Cations other than calcium also affect the mechanical properties of the isolated catch connective tissues. Elevation of potassium concentration in artificial sea water (ASW) bathing the tissues increases viscosity of sea urchin catch apparatus (Takahashi, 1967) and of sea cucumber dermis (Motokawa, 1981, 1982*b*, 1984*c*). However, potassium-rich solutions cause a decrease in the viscosity of the tendon of the pharyngeal retractor muscle of a sea cucumber (Smith & Greenberg, 1973) and the intervertebral ligament of a brittlestar (Wilkie, 1978). These contradictory results have been interpreted to mean that K^+-rich ASW may stimulate nerves which cause stiffening in some tissues and cause softening in other tissues (Motokawa, 1984*a*; Wilkie, 1984).

The effects of ionic conditions upon the mechanical properties of catch connective tissues could involve direct effects upon the extracellular materials, effects upon the nerves controlling the mechanical properties of these materials, or effects upon muscle cells when these are present.

In the present report, we have studied the effects of the ionic environment on the viscosity of the dermis of a holothurian. This tissue contains no muscle cells (Motokawa, 1982*a*). Such effects have been studied previously (Eylers, 1976, 1982; Greenberg & Eylers, 1984) but the experimental solutions contained only one or two cations. In the present study the ionic manipulations were carried out with the tissue bathed in ASW, which is likely to be similar to the ionic environment of the dermis (Koizumi, 1935).

MATERIALS AND METHODS

Dermis sample

Sea cucumbers, *Holothuria leucospilota* Brandt, were kept in the aquarium at the Marine Science Centre of the University of the Ryukyus, Sesoko Island, after collection in the lagoon in front of the island. A square column of dermis (1 mm thick, 1 mm wide and 7–9 mm long) was dissected from the body wall of a sea cucumber with a razor blade. The dermis of the lateral interambulacrum was used because the ambulacral dermis contains water vascular canals with muscular walls (Motokawa, 1981, 1982*a*). The dissection was performed so that the long axis of the column was parallel to the long axis of the animal. The tissue contained no muscle, no large nerve trunks and no major component of the water vascular systems.

Creep test

A creep test was used to measure the viscosity of the dermis. A dermis sample was attached to two stainless steel holders with cyanoacrylate glue. One holder was fixed to a platform in a trough, and the other holder was connected to a beam *via* a fine connecting rod made of bamboo. The trough was filled with 12·5 ml ASW. The temperature of the medium was maintained at $25{\cdot}0 \pm 0{\cdot}5$°C by circulating temperature-controlled water around the trough. Before the mechanical test was performed, the sample was left in the trough for at least 15 min to equilibrate to the water temperature. A weight (approx. $1{\cdot}3\ g\ mm^{-2}$) was applied to the sample *via* the

beam so as to stretch the sample along the longitudinal axis of the sea cucumber. The lengthening of the dermis was detected with a linear variable differential transformer (DCP 2.5, Shinko Denshi) and was recorded with a pen recorder (Type 3047, Yokogawa).

At the application of the load, the dermis extended rapidly, and then after 5–10 min the speed of lengthening (strain rate) became constant (Motokawa, 1984*b*). When constant phase was attained, ASW was drained from the trough and a test solution was introduced.

The normal viscosity of the dermis (η, in Pa·s) is given by the following formula (Motokawa, 1984*b*):

$$\eta = \sigma/\dot{\epsilon},$$

where σ is the stress (in Pa) and $\dot{\epsilon}$ is the strain rate (in s^{-1}). The effect of manipulation of the ionic environment was expressed by the relative normal viscosity: the normal viscosity of the dermis, measured 10 min after the application of a test solution, was expressed as a ratio of the normal viscosity just before the application of the test solution. In this paper, we call the relative normal viscosity simply the 'viscosity'.

Because the large reduction in viscosity caused rapid elongation of the sample and thus reduction in cross-sectional area, the measurement of reliable values of relative viscosity after 10 min immersion in Ca^{2+}-free ASW was impossible. Therefore the viscosity values in this solution were determined as follows: the specimen was rested in Ca^{2+}-free solution for 15 min and then the load was applied; 10 min after loading, the solution was changed to ASW; the viscosity value at the change of the solution was normalized by the value after 10 min immersion in ASW.

Test solutions

The artificial sea water (ASW) had the following composition (in mmol l^{-1}): NaCl, 433·7; KCl, 10·0; $CaCl_2$, 10·1; $MgCl_2$, 52·5; $NaHCO_3$, 2·5. The pH was adjusted to 7·0, which was around the pH of the coelomic fluid of this sea cucumber.

Two series of solutions with different pH values were used in the study of the effect of pH. One series consisted of ASW without buffers: the pH was adjusted by adding either HCl or NaOH. During the experiment with non-buffered solutions, the pH values of the solution in the experimental trough was monitored with a pH electrode and was kept constant by adding either HCl or NaOH. The other series was ASW containing the following buffers instead of $NaHCO_3$: acetic acid–sodium acetate ($I = 0{\cdot}01$) for pH 4·0 and 5; 2-(*N*-morpholino)ethanesulphonic acid (Mes, 0·01 mol l^{-1}) for pH 5·5 and 6; *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (Hepes, 0·01 mol l^{-1}) for pH 7·0; tris(hydroxymethyl)-aminomethane (Tris, $I = 0{\cdot}01$) for pH 8·0 and 9·0; 3-(cyclohexylamino)-1-propanesulphonic acid (Caps, 0·01 mol l^{-1}) for pH 10·0. The pH of all solutions was adjusted to within 0·05 pH unit at 25 °C. In Ca^{2+}-free-EGTA ASW, 5 mmol l^{-1} EGTA (ethyleneglycol bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid) was added to Ca^{2+}-free ASW.

When the concentration of calcium, potassium or magnesium was changed, the sodium concentration was adjusted so as to keep the osmotic concentration constant. When the concentration of sodium was reduced, the isotonicity of the solution was maintained by adding either choline or sucrose or both.

RESULTS

Effect of Ca^{2+}

The viscosity of the dermis decreased in Ca^{2+}-free ASW whereas it increased in ASW with a high concentration of Ca^{2+} (Figs 1, 2A). Ca^{2+}-free ASW, both with and without EGTA, reduced the viscosity to less than one-fifth of that in ASW. The viscosity change was reversible (Fig. 1A,B): when the sample was washed with ASW, the viscosity increased again to the value before application of the test solution (baseline value) in 3–17 min.

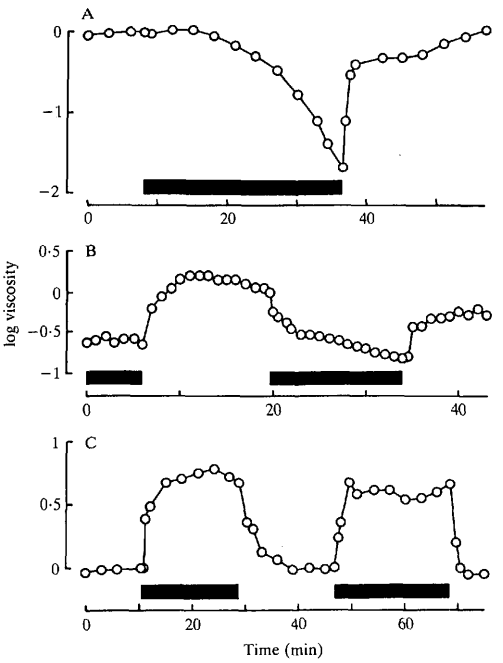

Fig. 1. Effects of Ca^{2+} on viscosity. (A) Ca^{2+}-free artificial sea water (ASW) with 5 mmol l^{-1} EGTA; (B) Ca^{2+}-free ASW without EGTA; (C) ASW with 100 mmol l^{-1} Ca^{2+}. In this and the following figures note that viscosity is plotted on a logarithmic scale. Therefore the log viscosity value is 0 at the application of a test solution, which is indicated by a thick horizontal bar.

ASW with a high concentration of Ca^{2+} (50–100 mmol l^{-1}) increased the viscosity of the dermis in 1 min (Fig. 1C). When the high-Ca^{2+} ASW was washed out, the viscosity decreased to half the value at the beginning of the wash in 1–2 min (Fig. 11B). The viscosity decreased again to the baseline value in 5–25 min.

Effect of Mg^{2+}

In contrast to Ca^{2+}-free ASW, Mg^{2+}-free ASW increased the viscosity by eight-fold in 3–10 min (Figs 2B, 3A). The viscosity recovered completely when the dermis was washed in ASW for 15–77 min.

ASW with a high (250 mmol l^{-1}) Mg^{2+} concentration caused a slight decrease in viscosity, lasting less than 3 min. The lowest viscosity value observed was one-third of the baseline value. The viscosity then began to increase, reaching a level of about five times the baseline value (Figs 2B, 3B). The increase could be reversed by washing with ASW, but in four preparations out of eight, the viscosity did not decrease to the baseline value even after a 60-min wash.

The response of the dermis to ASW with 100 mmol l^{-1} Mg^{2+} showed variations: initially there was a slight viscosity decrease; then in four preparations out of seven the viscosity gradually increased to a value which was a little larger than the baseline value; in the other preparations, the viscosity remained decreased.

Effect of Na^{+}

The sodium concentration was lowered to either 200, 20 or 0 mmol l^{-1}, keeping the osmotic concentration constant by adding sucrose. All the solutions increased the viscosity in 1 min (Figs 4A, 5A, 11A). All the preparations in Na^{+}-free ASW and one preparation out of 14 in 20 mmol l^{-1} Na^{+} ASW hardly elongated in these media; the

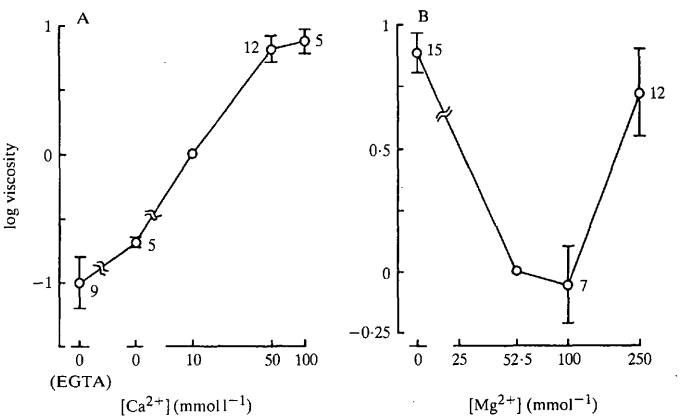

Fig. 2. Effects of divalent cations on viscosity: dose–response curves of Ca^{2+} (A) and Mg^{2+} (B). Circle, mean; bar, 2 S.E.; numeral by the circle, number of experiments.

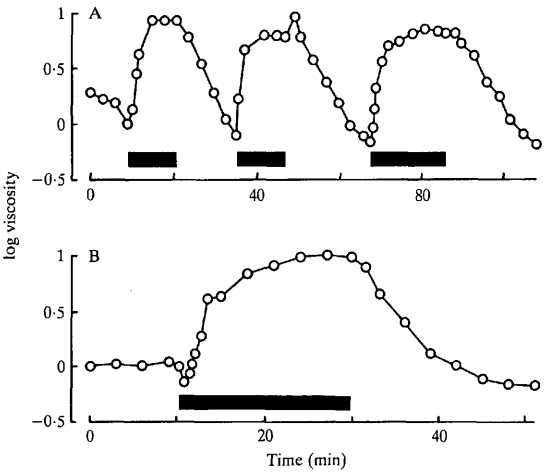

Fig. 3. Effects of Mg^{2+} on viscosity. (A) Mg^{2+}-free artificial sea water (ASW); (B) ASW with 250 mmol l^{-1} Mg^{2+}. The thick horizontal bars indicate the period of application of test solutions.

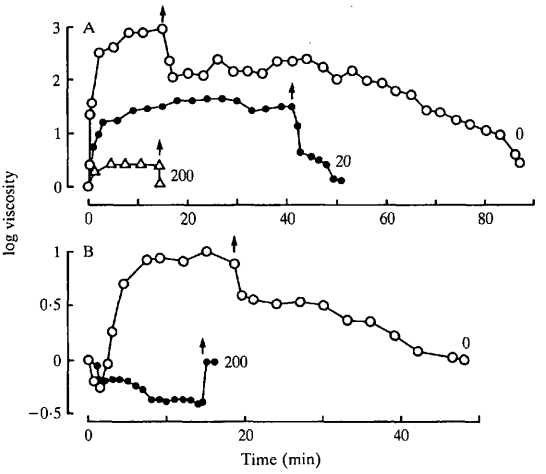

Fig. 4. Effects of Na^+ on viscosity. Na^+ in artificial sea water (ASW) was replaced with either sucrose (A) or choline (B). The numerals by the curves refer to Na^+ concentration (in mmol l^{-1}). The test solutions were introduced at time 0, and they were washed out at times indicated by arrows.

viscosity increased by more than 1000 times, but accurate measurement of the viscosity was impossible. For the preparations exposed to 20 or 200 mmol l^{-1} Na^+, a subsequent wash in ASW resulted in complete recovery of viscosity in 5 min. In the case of immersion in Na^+-free ASW, washing in ASW resulted in a decrease in viscosity but recovery was incomplete after 60 min observation.

When the sodium concentration was lowered to 200 mmol l^{-1}, keeping both the osmotic concentration and the ionic strength constant by adding choline chloride instead of sucrose, the solution produced a decrease in viscosity to two-thirds of the baseline value (Figs 4B, 5A). When the sodium concentration was further lowered to 0 mmol l^{-1}, the viscosity increased to a level of 12 times the baseline value (Figs 4B, 5A). In four samples out of seven, a decrease to half the baseline value preceded the increase (Fig. 4B). These decreases and increases caused by the choline-substituted solutions were reversible in ASW.

When sodium was replaced partly by choline and partly by sucrose, the viscosity value was between that in the totally sucrose-substituted solution and that in the totally choline-substituted one (Fig. 5A).

Effect of K^+

During immersion in K^+-free ASW there was no change in viscosity, but when ASW was restored, the viscosity decreased a little in four preparations out of 10. ASW with 100 mmol l^{-1} K^+ reversibly increased the viscosity by 16-fold (Figs 5B, 6), as reported previously (Motokawa, 1981, 1984*b*).

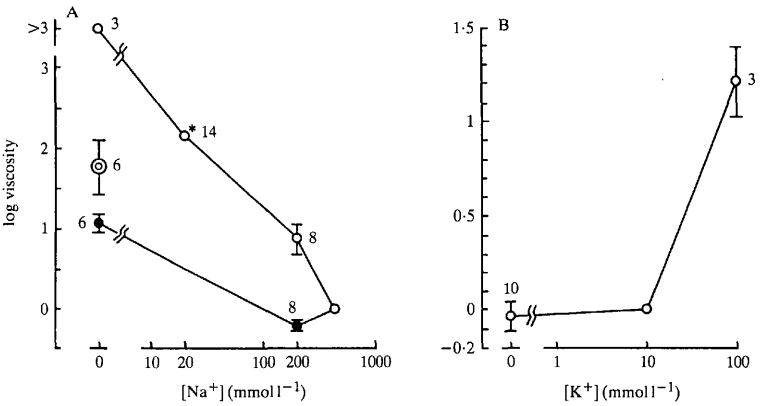

Fig. 5. Effects of monovalent cations on viscosity: dose–response curves of Na^+ (A) and K^+ (B). Circle, mean; bar, 2 S.E.; numeral by the circle, number of experiments. In A, open circles represent the results of sucrose substitution, filled circles those of choline substitution and the concentric circle that of choline and sucrose substitution in which 200 mmol l^{-1} Na^+ was replaced with the same amount of choline and the remaining Na^+ was replaced with sucrose. * Median was given instead of mean, because one preparation showed too high a viscosity value to be measured accurately.

Effect of distilled water

Distilled water caused contraction of the dermis (Fig. 7), which was reversible in ASW.

Effect of pH

The viscosity of the dermis increased in media with both basic pH (pH 9,10) and acidic pH (pH 4, 5) (Figs 8, 9). In buffered ASW, the viscosity increased 51 times at pH 4 and 26 times at pH 10. There was recovery at neutral pH.

Fig. 9 compares the effects of buffered ASW with those of non-buffered ASW. The mean values in the two ASWs showed no significant difference at the same pH by t-test ($P = 0{\cdot}05$) except at pH 5. In the pH 5 medium with buffer, the viscosity increased by five-fold. In ASW without buffer, however, all the samples showed a little viscosity decrease at the application of the solution; then the viscosity began to increase a little in three preparations out of nine; the viscosity remained decreased in other preparations. The minimum viscosity observed was one-third the baseline value. No viscosity decrease was observed in media with other pH values.

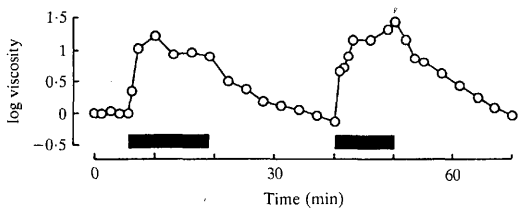

Fig. 6. Effect of artificial sea water with 100 mmol l^{-1} K^+ on viscosity. Thick horizontal bars indicate the periods of application of the test solution.

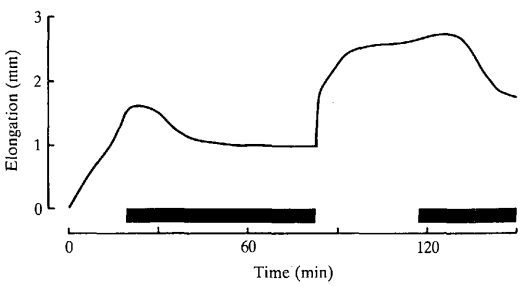

Fig. 7. Effect of distilled water on the creep curve (elongation curve). The application of distilled water (thick horizontal bars) caused shortening of the dermis.

DISCUSSION

Manipulation of the ionic environment produced large, rapid and reversible changes in viscosity. These changes were superimposed upon a variation of more

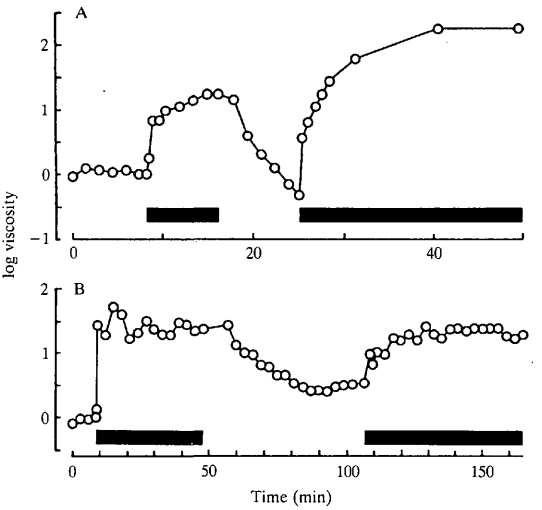

Fig. 8. Effects of pH on viscosity. Artificial sea water buffered to pH 4 (A) and to pH 10 (B) was applied during the period indicated with thick horizontal bars.

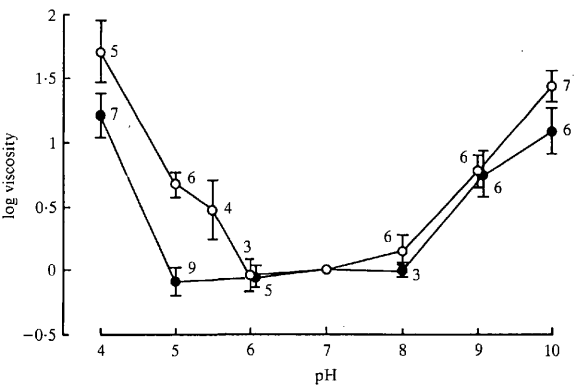

Fig. 9. Effect of pH on viscosity. Open circles give the results of buffered media and filled circles those of non-buffered ones. Circles, mean; bar, 2S.E.; numeral by the circles, number of experiments.

than two orders in the value of the viscosity in ASW (upper histogram, Fig. 10). Similar large variations are observed in the dermis of other sea cucumbers (Motokawa, 1984*b*,*c*) as well as in other catch connective tissues (Smith, Wainwright, Baker & Cayer, 1981; Motokawa, 1983). These variations are believed to be due to the catch connective tissues being in a relaxed, intermediate or catch state in ASW.

The large changes in viscosity observed under the different ionic conditions are displayed in the lower part of Fig. 10. The largest changes in viscosity were observed following alteration of the Ca^{2+} concentration, and covered the whole of the range observed in ASW, shown in the histogram. The changes produced by manipulation of other cations covered the upper ranges of viscosity in the histogram.

The rapidity of the response to ionic change is summarized in Fig. 11A. Doubling of the viscosity was usually obtained in less than 1 min, not much greater than the value of 10–20 s observed in response to optical stimulation, which is likely to stimulate the control mechanism *in vivo* (Motokawa, 1984*d*).

Reversibility of the response was observed during wash-out of all the test solutions (Fig. 11B). The recovery time following changes in Ca^{2+} or Na^{+} was similar to the

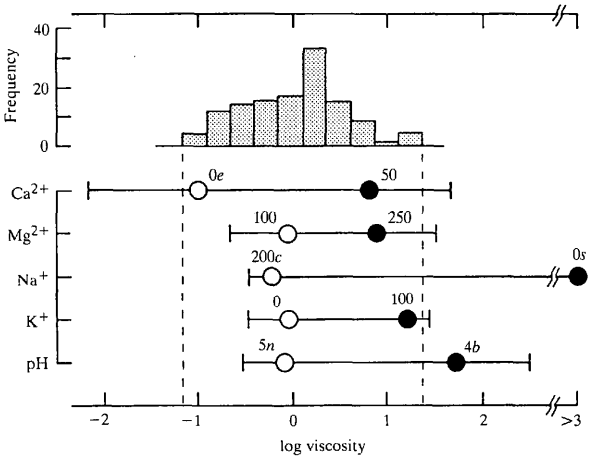

Fig. 10. Frequency distribution of the viscosity in artificial sea water (ASW) (upper histogram, $N = 123$), and the viscosity range which was observed by the manipulation of cation concentrations (lower figure). Values are expressed relative to the value observed in ASW (log value 0). In the lower figure, the maximum viscosity value and the minimum value, which were observed for a particular ion species are connected with a horizontal line. The filled circle gives the mean value in the medium in which the maximum value was observed. The empty circle gives the mean value in the medium in which the minimum value was observed. The numerals by the circles refer to the concentration (in mmol l^{-1}) of the media. *e*, with EGTA; *c*, choline substitution; *s*, sucrose substitution; *n*, no buffer; *b*, with buffer.

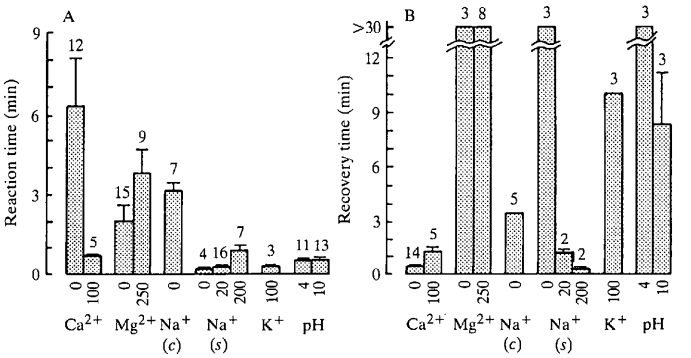

Fig. 11. Reaction time (A) and recovery time (B) in artificial sea water (ASW) with various cation concentrations. The concentration was given in mmol l^{-1} (except that of H^+). The reaction time was defined as the time for the viscosity to increase to twice the value at the application of the test solutions. In the case of Ca^{2+}-free ASW, it was defined as the time needed to be a half instead of twice the value at the application of the test solution. The recovery time was defined as the time for the viscosity to decrease to a half of the value at the beginning of washing by ASW. In the case of Ca^{2+}-free ASW, it was defined as the time needed to be twice instead of half the value at the beginning of washing by ASW. The bar refers to mean + S.E. When the samples with recovery time greater than 30 min were observed, median values were given instead of means. The numerals on the bars denote the number of samples. *c*, choline substitution; *s*, sucrose substitution.

value of 1–2 min following photic stimulation (Motokawa, 1984*d*). The much larger recovery times observed following some ionic manipulations may imply that those conditions do not occur *in vivo*.

Our data thus form a basis for the understanding of the mechanochemistry of the extracellular materials and of the ionic dependence of the neural control mechanisms, although the ionic changes *in vivo* have not been characterized.

The presence of Ca^{2+} is necessary for the change in the mechanical properties of catch connective tissues in response to stimulation. Thus, removal of Ca^{2+} blocks the stiffening caused by mechanical stimulation in the catch apparatus of sea urchins (Smith *et al.* 1981; Diab & Gilly, 1984) and inhibits the softening caused by potassium in the cirral ligament of a featherstar (Wilkie, 1983). The effect of high potassium concentration upon the cirral ligament is inhibited by chloroform, so the effect of Ca^{2+} may be mediated by an effect upon receptors or nerves (Wilkie, 1983). Action potentials of some echinoderm nerves are dependent on Ca^{2+} rather than Na^+ (Brehm, 1977; Berrios, Brink & del Castillo, 1985).

Calcium also affected the baseline viscosity of the body wall, as has been observed for the catch apparatus (Hidaka, 1983) and the intervertebral ligament of a brittlestar (Wilkie, 1978). These effects probably reflect a direct action of calcium upon the extracellular materials, for it has been shown that the holothurian dermis has a higher viscosity in isotonic $CaCl_2$ than in isotonic NaCl (Greenberg & Eylers, 1984). Also,

the collagen fibres in the holothurian dermis disintegrate to collagen fibrils in Ca^{2+}-free medium (Matsumura, Shinmei & Nagai, 1973). The extracellular matrix of the dermis is composed of negatively charged glycosaminoglycans (Motohiro, 1960; Katzman & Jeanloz, 1973). Ca^{2+} may crossbridge the glycosaminoglycan molecules through electrostatic interaction (Comper & Laurent, 1978) and thus provide the baseline viscosity of the dermis.

Viscosity appeared to be dependent on Na^+ concentration, since an increase in viscosity was produced by substitution of choline for sodium. Viscosity was also affected by ionic strength, since a profound increase was observed with sucrose substitution of Na^+, and the viscosity increase observed with substitution of Na^+ half by choline and half by sucrose was greater than that observed with substitution by choline alone. We have also observed that the effect of choline substitution is Ca^{2+} dependent, whereas the effect of sucrose substitution is not (unpublished data). This may imply that the change in osmotic strength has a direct effect upon the extracellular material, because the nerves seem to be inactivated in the Ca^{2+}-free solution.

Contraction of the body wall was observed at low ionic strength, i.e. in distilled water. This contraction is achieved without muscle cells and can be explained by a rise in osmotic pressure inside the tissue because of the fixed charges of the matrix. Water molecules flow into the tissue causing lateral swelling and shortening in the axial direction (direction of loading), as has been suggested to explain the contraction of vertebrate cartilage (Myers, Armstrong & Mow, 1984). Osmotic swelling may explain the viscosity increase in sucrose-substituted low-Na^+ solutions, because the swelling pressure, which is lower than that in distilled water, may decrease the elongation rate rather than cause contraction. Another possible mechanism for the viscosity increase is based on the shielding effect of Na^+ (Greenberg & Eylers, 1984). Monovalent cations, such as Na^+, inhibit the electrostatic interaction of extracellular materials (Mathews, 1970). Therefore the removal of monovalent cations may cause the viscosity increase of the dermis.

Viscosity was raised by a change to more acid pH and to more basic pH. This may result partly from an effect upon the nervous control mechanisms, since pH is known to affect nerve function in other phyla (Woodhull, 1973), and partly from an effect upon the number of charged groups on the extracellular macromolecules, which would change their electrostatic interactions (Lindahl & Höök, 1978). Increase in viscosity has been observed to be related to an increase in pH value in the catch apparatus of a sea urchin (Hidaka, 1983), and to a decrease in pH in the intervertebral ligament of a brittlestar (Wilkie, 1978). These differences may result partly from the use of buffer solutions for the study upon the brittlestar, in contrast to the use of buffered ASW in the present study and the study upon the sea urchin. Otherwise, the differences could reflect differences in the chemistry of the extracellular macromolecules or in the nervous control mechanisms.

The complicated effects of magnesium concentration upon viscosity may result from the combined effects of Mg^{2+} on the extracellular materials (Greenberg & Eylers, 1984) and on the nerves (Motokawa, 1981).

In conclusion, the ionic environment greatly affected the mechanical properties of the holothurian dermis. It is now necessary to study the effects of ions upon the extracellular materials independently of the effects upon the cells.

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