

## CONNECTIVE TISSUE MECHANICS OF *METRIDIUM SENILE*

### II. VISCO-ELASTIC PROPERTIES AND MACROMOLECULAR MODEL

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The visco-elastic properties of the body-wall connective tissue (mesogloea) of the sea anemone *Metridium senile* were previously studied by Alexander (1962). He carried out creep tests (extension under constant stress) over long periods of time and observed that the tissue could be extended reversibly to over three times its initial length with very small forces. The material behaved very nearly like a single Voigt element with a retardation time of about 3500 sec. Although Alexander did not propose any specific molecular mechanism to account for the extensibility and apparent slow elasticity, he did note that collagen is thought to be a major component of mesogloea and suggested that the collagen must play a major role in determining the mechanical properties of the tissue.

Gosline (1971) investigated the structural and compositional aspects of *Metridium* mesogloea in more detail. Polarized light and electron-microscopic evidence showed that mesogloea is a two-phase system of collagen fibres embedded in a matrix. On the basis of structural similarities between mesogloea collagen and vertebrate collagen it was inferred that mesogloea collagen is mechanically similar to other collagens. That is, it is a relatively inextensible fibrous protein with a high modulus of elasticity. As such a rigid, inextensible fibre cannot alone account for the mechanical properties observed by Alexander (1962) for mesogloea, it can be concluded that the matrix plays a major, if not dominant, role in determining the mechanical properties of this tissue. In this study dynamic mechanical tests on isolated mesogloea, both native and chemically treated, have been used to investigate the nature of the interaction between collagen and matrix in mesogloea and the role the matrix plays in determining the mechanical properties of the tissue. On the basis of this information a macromolecular model for the visco-elastic properties of *Metridium* mesogloea is proposed.

#### *Some principles of polymer physics*

The experimental approach used in this study is based on the theory and techniques developed by physical chemists to understand the molecular basis for the mechanical properties of synthetic polymers. The mechanical behaviour of macromolecular materials can be analysed theoretically in terms of conformational changes in ideal, flexible, long-chain molecules. A convenient measure of the dimensions of a polymer

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molecule is the root-mean-square end-to-end distance ( $\bar{R}^2$ ). An ideal polymer made up of  $N$  small segments each of length  $a$  will take up what is known as a random-coil conformation provided that (1) the junctions between segments allow completely free rotation, (2) there is sufficient thermal energy available to keep all segments of the molecule in motion, and (3) the molecule is unrestrained. The random-coil has the dimensions,  $\bar{R}^2 = Na^2$  (Bueche, 1962). Thus when  $N$  is large, the extended length of the molecule,  $Na$ , is very much larger than the end-to-end distance,  $N^{1/2}a$ .

When considering real polymer molecules in terms of ideal random coils it is tempting to relate the spacing between atoms in the polymer chain to the segment length. As chemical bonds impose considerable limitations on the rotational freedom between atoms, the correlation between bond length and segment length is unrealistic. It is possible, however, to imagine a number of bond lengths which together act as a freely orientating segment, where the effective segment length will be determined by the flexibility of the polymer chain.

The rubber-like or configurational entropy elasticity which is characteristic of some polymeric materials is based on the random-coil nature of polymer molecules. An unrestrained random coil is in a state of high entropy owing to the disorder in the system. An applied force which tends to move the chain ends apart (i.e. to increase  $\bar{R}^2$ ) restricts the thermal motion of the polymer chain and therefore decreases the entropy of the system. The work done in moving the chain ends apart, or the energy input to the system, is stored in this entropy change and is recoverable elastically when the applied force is removed (Treloar, 1958). A polymeric material made up of a large number of random-coil molecules will show rubber-like elasticity only if the individual molecules are linked together so that a force applied to the sample will alter the conformation of the molecules (i.e. increase  $\bar{R}^2$ ) and not just cause the molecules to flow past one another. If the number of links between molecules is great enough to tie all of the polymer chains together into a single unit the system is described as a network. Thus, cross-linked rubbers are networks and show entropy elasticity; while non-cross-linked polymers behave much like viscous liquids.

If a tensile load (stress;  $\sigma = \text{force/area}$ ) is applied to an object, the object will be deformed (strain;  $\epsilon = \Delta L/L_0$ ). A measure of the rigidity of the object is the elastic modulus ( $E = \sigma/\epsilon$ ). A low molecular weight, non-cross-linked, amorphous (i.e. the molecules can be described as random coils) polymer is mechanically similar to a viscous liquid. Such a material will deform under a stress as long as the stress is applied. Thus, the modulus of the material will decrease quickly with time (Fig. 1, curve D). If a stress is applied to a polymer network, the material will deform asymptotically over a period of time to new dimensions and then maintain these dimensions (figure 1, curve A). In other words, there is an equilibrium modulus characteristic of the network. The term 'cross-link' as applied to networks generally implies permanent bridges, such as covalent bonds, between polymer chains. But high molecular weight, non-cross-linked polymers respond to mechanical tests in a manner that suggests they contain temporary cross-bridges. There is a pseudo-equilibrium modulus or plateau (Fig. 1, curves B and C) where the modulus decreases very gradually with time, but if the stress is applied for long enough the material will begin to behave like a viscous liquid and the modulus will drop quickly. The pseudo-equilibrium is due to entanglements and other temporary interactions between polymer molecules which act as

Time-dependent cross-bridges and retard the viscous flow of molecules past one another. Given sufficient time the chains will disentangle and the cross-bridges break down. Increased molecular weight increases both the number and stability of these cross-bridges, and thus extends the plateau or pseudo-equilibrium to longer times.

Another important aspect is the effect of temperature on the random-coil molecule. In a homogeneous system when the thermal energy available to segments decreases, intersegmental attractive forces become important and segmental motion is greatly reduced until finally the polymer chains become 'frozen' into a fixed position. This transition, known as the glass transition, from kinetically free chains to essentially

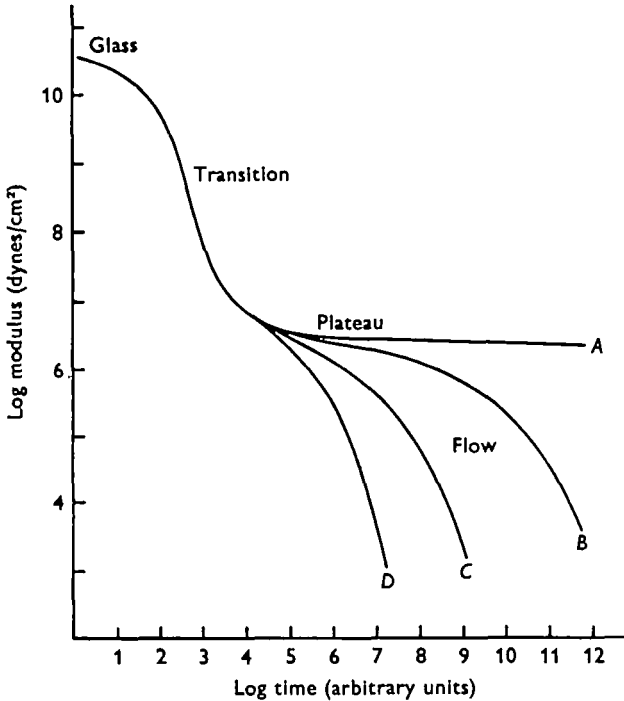


Fig. 1. The mechanical properties of typical amorphous (random-coil) polymers. (A) Cross-linked network polymer; (B) high-molecular-weight non-cross-linked polymer; (C) non-cross-linked polymer of intermediate molecular weight; (D) low-molecular-weight non-cross-linked polymer.

fixed ones should not be confused with crystal phase changes which may also occur in polymeric systems. A polymer glass is a non-crystalline solid. The change in modulus associated with the glass transition is usually several orders of magnitude.

In Fig. 1 the glass transition is shown as a function of time and not of temperature. This implies that if measurements are made at very short times the material will behave like a glass. The process through which a polymer molecule responds to an applied force can be thought of as a reaction proceeding at a rate determined by a characteristic activation energy. It follows from kinetics that as the temperature is decreased this rate will decrease until, at some low temperature, the rate will be so low that the polymer chains will not respond at all and the material is a glass. At constant temperature, the shorter the time the less chance a

polymer chain will have to respond to a stress, and at very short times the chains will not respond at all. They will appear to be 'frozen', and the material will behave like a glass. This equivalence of time and temperature, known as the 'time-temperature-superposition principle', is commonly used to determine mechanical properties over a much broader range of times than can be measured directly (Nielsen, 1965). By lowering the temperature, the rate at which a polymer chain responds to a stress is reduced. Thus, a process which would normally take place in a very short time will be spread out over a longer, more useful time range. At higher temperatures, processes which normally take a long time can be observed over much shorter time intervals. The usefulness of this principle for accurately predicting mechanical properties over an extended time scale is dependent on constant activation energy, or in other words, on the response mechanism being the same over the temperature range used. This assumption appears to be true for a number of relatively simple polymer materials, but even when it is not possible to make accurate predictions the general equivalence of time and temperature holds.

The response curves drawn in Fig. 1 are typical of ones which would be obtained from stress-relaxation experiments with random-coil polymers. In this type of experiment the sample is stretched at the start of the test, and then, with the strain held constant, the change in the stress is followed with time. As the time scale in Fig. 2 is much broader than could be measured directly, the results from a number of tests run at different temperatures would have to be combined to give these total response curves. There are four zones which are characteristic of random-coil polymers in these response curves. The glass and transition zones are common to all random-coil polymers. The plateau zone of cross-linked, network polymers will extend to very long times and no flow zone will be observed. A low-molecular-weight non-cross-linked polymer will go almost directly from the transition zone to the flow zone showing little, if any, plateau. Increased molecular weight will extend the plateau zone, but in the absence of a cross-linked network there will always be a flow zone.

Another technique for measuring the mechanical properties of polymer materials is periodic stress-strain testing or dynamic testing. This technique involves applying a sinusoidally varying stress to a test sample and observing the resulting strain. In dynamic testing the inverse of frequency is similar to time in a stress-relaxation test. The frequency-dependent storage modulus ( $E'$ ) derived from dynamic tests is similar, but not precisely the same, as the time-dependent modulus derived from stress-relaxation tests. The storage modulus follows the same pattern in the four characteristic zones of the response curve of random-coil polymers as does the stress-relaxation modulus. It is high in the glass region, drops quickly in the transition, drops slowly through the plateau, and then drops rapidly again in the flow zone. Dynamic testing also provides information about mechanical hysteresis or viscous energy loss per cycle in the form of damping term ( $\tan \delta$ ). The damping provides another useful indicator identifying the four zones. In the glass region damping is very low. It reaches a maximum in the transition zone, goes through a minimum in the plateau, and then increases again in the flow zone. Damping is generally high in any region of the response curve where there is a rapid change in modulus.

## MATERIALS AND METHODS

*Metridium senile* of at least 10 cm diameter were maintained and the tissue prepared as previously described (Gosline, 1971). Because traces of muscle were unavoidably left on the prepared samples of mesogloea, all experiments were carried out in a mixture of 20%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  and sea water, unless otherwise specified.

Periodic stress-strain measurements were carried out on a non-resonance, forced-vibration instrument which I constructed. It was driven by a variable-speed motor through a gear system and provided a frequency range of  $10^{-3}$  to 3 Hz. Force and deformation were recorded simultaneously on a two-channel oscillographic recorder (Hewlett-Packard, model 7702B). The maximum sensitivity of the force transducer was about 10 dyn and of the deformation transducer about 0.005 mm.

Fig. 2 shows a diagram of the apparatus. The specimen (*S*) was attached to two stainless-steel clamps (*C*) with Eastman 910 contact cement (Eastman Kodak, Rochester, N.Y.). The bottom clamp, attached to a solid base (*B*), could be adjusted vertically with an adjustment screw (*A*). The top clamp was attached to a stainless-steel proving ring (*R*) which in turn was attached to the variable speed motor drive through a system of levers (*L*). An offset shaft on the motor drive caused the proving ring to move vertically and deform the sample in tension. A linear variable differential transformer ( $T_1$ ) (model 100-HR, Schaevitz Engineering, New Jersey) positioned inside the proving ring provided a signal proportional to the force on the sample being tested. Another linear transformer ( $T_2$ ) located with its movable core attached to the upper clamp and its transformer windings fixed to the apparatus frame provided a signal proportional to the deformation of the sample. The carrier system (model CAS-2500, Schaevitz Engineering, New Jersey) powering each linear variable differential transformer provided a direct current signal to the recorder. The force transducer was calibrated by adding known weights; the deformation transducer was calibrated with a Prior micromanipulator. Both gave linear response over the range used.

Data from the recorder were in the form of parallel sine waves. The amplitude of each sine wave was determined and converted into force or displacement. Specimen dimensions were measured with a millimeter rule and used to calculate stress from force ( $\sigma = \text{force/area}$ ) and strain from displacement ( $\epsilon = \Delta L/L_0$ ). The maximum strain used in any of the periodic stress-strain experiments was 1.5%. Slight differences occurred in values from different experimental series because of small errors in measurements of sample dimensions, but in any experimental series a single sample was used a number of times to make comparison within the series valid. Any errors due to sample measurements are at most 20–25% whereas the changes which are of importance in this study are orders of magnitude.

The ratio of maximum stress to maximum strain yields a complex Young's modulus ( $|E|$ ) which can be resolved as follows;  $|E|^2 = (E')^2 + (E'')^2$ , where  $E''$  (loss modulus is a measure of the mechanical hysteresis or viscous energy loss per cycle, and  $E'$  (storage modulus) is associated with energy storage in cyclic deformations. The loss modulus and the storage modulus are determined by measuring the time lag (expressed as an angular phase shift,  $\delta$ ) between stress and strain from the recorder output and substituting the phase angle into the following equations:  $E'' = |E| \sin \delta$ , and  $E' = |E| \cos \delta$ . The ratio  $E''/E'$  is defined as the damping and is equal to  $\tan \delta$ .

Stress-relaxation tests were run on the same apparatus, but the variable speed motor drive was not used. The specimen was attached with Eastman 910 contact cement to a set of clamps modified to hold wide pieces of tissue. The material was deformed at the start of the experiment by lowering the bottom clamp a known distance with the adjustment screw (Fig. 2A). The deformation was held constant and the force was recorded with time. Stress and strain were calculated and the Young's modulus was determined directly from the ratio of the stress to the strain. The strains used in stress-relaxation experiments were on the order of 20–30%.

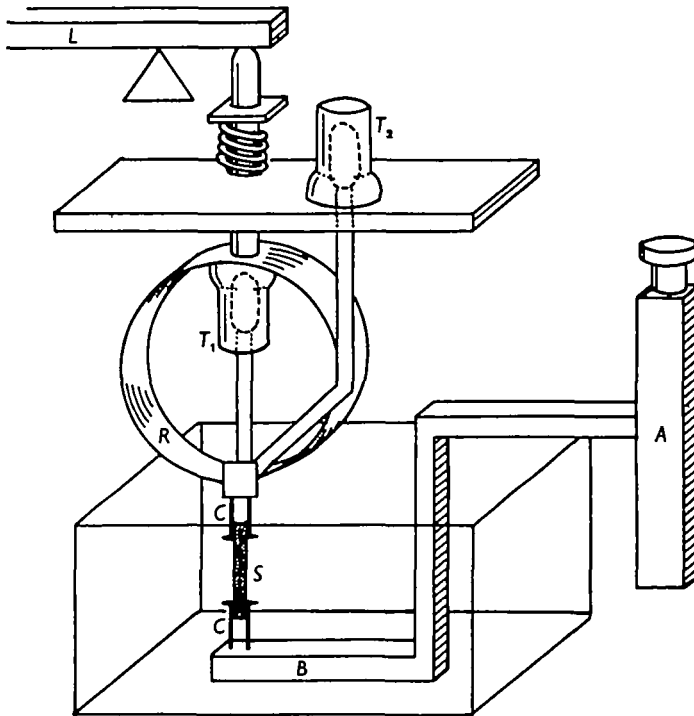


Fig. 2. A diagram of the dynamic testing apparatus. The sample (*S*) was placed in the clamps (*C*) attached at the top to a proving ring (*R*) and on the bottom to a fixed base (*B*). The material was deformed in tension by a system of levers (*L*) which moved the proving ring vertically. Linear variable differential transformer  $T_1$  provided a force signal, and transformer  $T_2$  provided a deformation signal.

In all of the periodic stress-strain experiments and some of the stress-relaxation experiments the deformation was parallel to the longitudinal axis of the animal. This type of experiment will be described as one in which the mesogloea was stretched longitudinally. In some of the stress-relaxation experiments the initial deformation was in a horizontal plane with respect to the animal, tending to increase the circumference of the cylindrical body wall. This type of experiment will be described as one in which the mesogloea was stretched circumferentially.

In all but the low temperature experiments the test sample was immersed in sea water or another aqueous medium in a small aluminum tank. Water circulated into a larger outer tank from a thermostatically controlled water bath maintained the temperature of the test solution over the range 3–50 °C ( $\pm 0.25$  °C). In low-temperature

Experiments the sample was tested in air. The small inner tank was kept empty and sealed on top with a sheet of Styrofoam. The outer tank contained a dry ice-ethanol bath which could lower the temperature of the sample to about  $-35^{\circ}\text{C}$ . Temperatures were measured with an alcohol thermometer with its bulb placed next to the sample and are accurate to about  $\pm 2^{\circ}\text{C}$ . The dry ice-ethanol bath was not thermostatically controlled; thus, it was not possible to hold any given temperature. Dry ice was added gradually to provide a cooling rate of about  $0.5^{\circ}\text{C}/\text{min}$ .

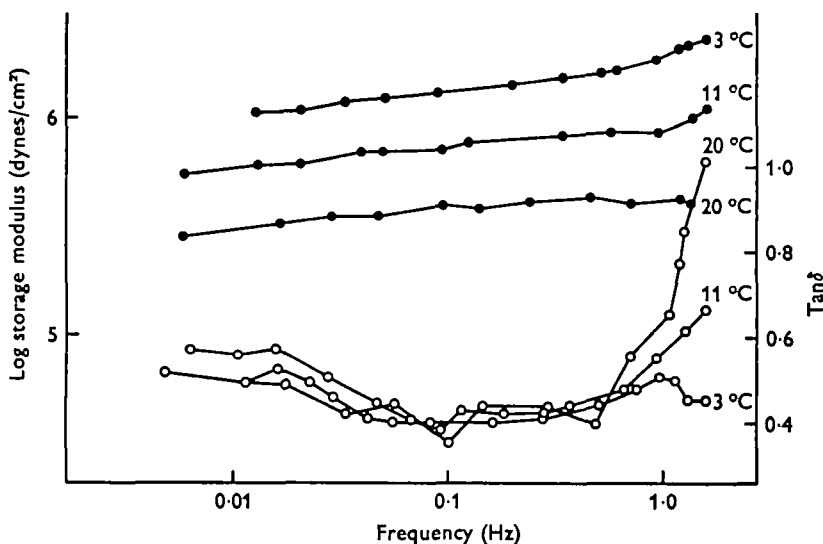


Fig. 3. Periodic stress-strain measurements on native mesogloea. ●, Storage modulus; ○, damping.

## RESULTS AND DISCUSSION

### *Periodic stress-strain measurements*

The first attempts to characterize the molecular structure of mesogloea involved making dynamic measurements over a range of frequencies at fixed temperatures. Experiments were run at temperatures in the range of  $2-50^{\circ}\text{C}$ . The results of two experimental series with native mesogloea are shown in Figs. 3 and 4. The data presented are representative of five such experimental series carried out with mesogloea from five different animals. In each series a single piece of mesogloea was used in a number of experiments at different temperatures. The testing procedure was as follows. The sample was attached to the testing apparatus, cooled to the lowest temperature used, and allowed to equilibrate for 30 min. The material was tested starting at low frequencies working to high frequencies. After testing, the temperature was increased, the material was allowed to equilibrate for 30 min and then tested again. The temperature increment was usually  $4-5^{\circ}\text{C}$ . In Fig. 3 data from  $7^{\circ}$ ,  $14^{\circ}$ ,  $17^{\circ}$  and  $25^{\circ}$  were omitted to simplify the graph. In these figures log storage modulus ( $E'$ ) and damping ( $\tan \delta$ ) are plotted against frequency at a number of temperatures.

At temperatures which approximate to the environmental temperature range of the animal (those in Fig. 3) several characteristic features were observed in all five

experimental series: (1) at constant temperature the modulus decreased gradually with decreased frequency, (2) at a single frequency, the modulus decreased gradually with increased temperature, (3) damping at all temperatures went through a minimum at about 0.1 Hz, and (4) the damping rise at the high-frequency end was much greater at higher temperatures. The gradual decrease in storage modulus with frequency and temperature and the damping minimum at 0.1 Hz seen for mesogloea in this experimental series resemble the properties of a high-molecular-weight, non-cross-linked polymer in the plateau or pseudo-equilibrium region of its response curve. It is not possible from this evidence alone to specify the molecular structure of the material involved, but the results indicate the presence of some high-molecular-weight entanglement-like interaction which is retarding viscous flow. The breadth of the plateau suggests that the molecular weight of the compound involved is very high indeed. The damping rise at the high frequency end of the 20 °C isotherm is very suggestive of a temperature-dependent transition, which will probably occur at a slightly higher temperature.

The results of another such experimental series carried out in the manner previously specified but at higher temperatures is shown in Fig. 4. The mechanical properties at 27 °C are much the same as those observed at 20 °C (see Fig. 3). Modulus varies with frequency; damping is minimum at about 0.1 Hz. At temperatures above 27 °C properties changed dramatically. Modulus increased between 27 and 32 °C instead of the expected decrease, but at 37 and 42 °C the modulus again decreased with increased temperature. More striking was the sudden drop-off of the damping which occurred first at high frequencies and then along the entire frequency range. These changes, which appear to start at about 28 °C, must be due to a major conformational change of at least one of the components, and the collagen is a likely candidate. The thermal shrinkage characteristic of collagens could account for the increased modulus, and the transition from a rod-like, helical structure to a random-coil conformation that causes the shrinkage could account for the decrease in the damping. I have taken X-ray diffraction patterns of mesogloea held in sea water at 35 °C for 1 h, cooled, washed in distilled water and dried. These patterns are identical with patterns taken of native mesogloea, indicating that a major transition is unlikely or that the transition is reversible. Alexander (1962) also noted a change in properties at 29 °C. He suspected that collagen was involved but observed considerable decrease in the birefringence of mesogloea collagen at 50 °C in sea water and not at lower temperatures. This suggests that the thermal denaturation of mesogloea collagen takes place at about 50 °C. Rigby (1961) reports that vertebrate collagen in 3 M urea contracts in two stages: a low-temperature reversible contraction at 20 °C followed by an irreversible contraction at 38 °C. If this transition in mesogloea involves the collagen it must be similar to the low-temperature reversible contraction observed by Rigby in vertebrate collagen. If the collagen is not involved, any component which has an extended conformation stabilized by weak bonds which break at elevated temperatures could account for this transition. On the other hand, an increase in the number of cross-links in the system, either between the collagen and the matrix or within the matrix, would result in the observed increase in modulus. At the moment all that can be said for certain is that the processes which account for this transition are not clear and should be investigated further.



If the data in Fig. 3 do indeed indicate the plateau region of an amorphous polymer one would expect to observe a glass transition at shorter times (i.e. at high frequencies) or at lower temperatures, and a flow zone at longer times. Because the dynamic testing apparatus could not be used at frequencies above 3 Hz, short-term phenomena were investigated by lowering the temperature. Fig. 5 shows the results of a single periodic stress-strain experiment run at constant frequency with temperature varying from  $-35$  to  $20$  °C. The data are representative of four such experiments on native mesogloea from different animals. Fig. 5 is a plot of log storage moduli ( $E'$ ) and damping ( $\tan \delta$ ) against temperature. The material was tested at 0.3 Hz. In all four experiments the following changes were observed. Between  $20$  and  $-10$  °C the modulus

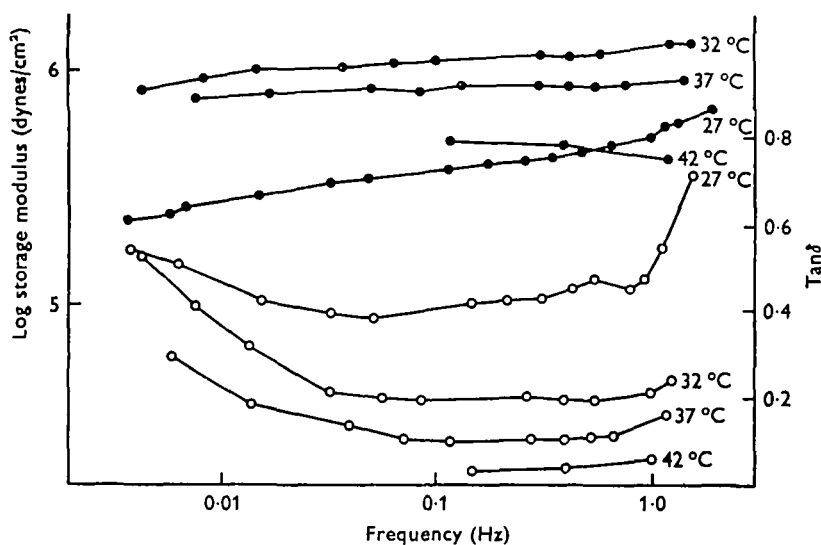


Fig. 4. Periodic stress-strain measurements on native mesogloea. Note the shift in properties which occurs between  $27$  and  $32$  °C. ●, Storage modulus; ○, damping.

increased gradually with decreased temperature. This is the plateau seen in Fig. 3. Then, over a short temperature range the modulus increased by a factor of about 500 and leveled off to a value of about  $4 \times 10^8$  dyn/cm<sup>2</sup> at  $-30$  °C. Damping decreased gradually to a minimum at about  $-5$  to  $-10$  °C, was maximum at  $-14$  °C, and then dropped off to very low values at temperatures below  $-22$  °C.

The mechanical properties of mesogloea at low temperatures closely resemble the properties of an amorphous polymer in the glass-transition region, but it is not possible to specify that this abrupt shift indicates a glass transition. It is quite likely that ice crystals forming in the highly hydrated matrix gel act as rigid filler particles and play a major role in this transition. In a single experiment with mesogloea which had been held in 6 M urea for several days a similar transition was observed, but at a slightly higher temperature ( $-7$  °C). If this transition in mesogloea is due primarily to the formation of ice, one would expect high urea concentrations to decrease rather than increase the transition temperature. Thus, the changes which occur at  $-15$  °C in native mesogloea are probably not entirely due to ice formation. The phase transition of water to ice is a thermodynamically first-order transition while the changes that

occur at the glass transition of amorphous polymers conforms to the pattern expected for a second-order phase transition (Ritchie, 1965). Thus, it should be possible to measure the appropriate parameters and to determine the order of the transition observed in native mesogloea. For example, a plot of the specific volume against temperature for a material which undergoes a first-order transition would show a 'jump' at the transition; while a similar plot for a material which undergoes a second-order transition (i.e. glass transition) would show a change in the slope of the plot or a

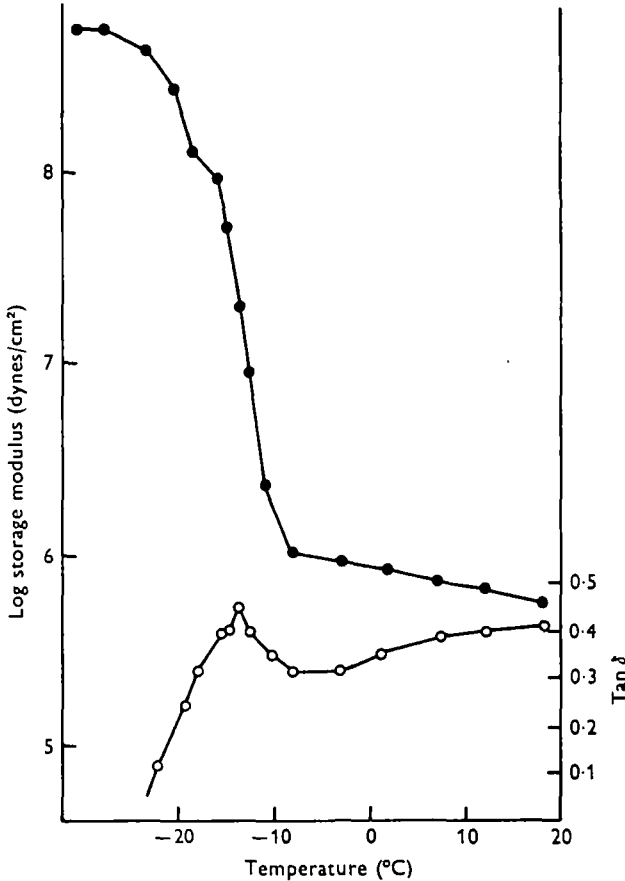


Fig. 5. The dynamic properties of mesogloea at low temperatures. Storage modulus (●) and damping (○) were measured at 0.3 Hz.

'fold' at the transition, not a 'jump'. Clearly, information of this type is needed before the observed shift in the properties of mesogloea can rigorously be defined as a glass transition, and until this information is available this shift will be referred to simply as a transition. In the four experiments run with native mesogloea the transition temperature, defined as the temperature at which damping is maximum, was within the range of  $-14$  to  $-16$  °C. Regardless of the thermodynamic nature of this transition in mesogloea, the presence of a transition provides one piece of valuable information. At temperatures above  $-15$  °C the material which plays the major role in determining the mechanical properties of mesogloea is a non-crystalline, amorphous polymer.

*Stress-relaxation experiments*

Stress-relaxation experiments with native mesogloea were used to investigate the response of this amorphous polymer system at long times. The results of several experiments in which the mesogloea was stretched longitudinally are plotted in Figs. 6 and 7. Each line represents a single experiment; thus, Fig. 6 is a plot of three experiments at 4 °C and two at 13 °C. In these figures log modulus is plotted against time on a logarithmic scale. At short times the stress-relaxation modulus of mesogloea at 4 °C is comparable to the storage modulus observed under similar conditions with periodic stress-strain measurements. At 3 °C and 0.1 Hz (Fig. 3) the storage modulus is slightly greater than  $10^8$  dyn/cm<sup>2</sup>. This is quite close to the stress-relaxation modulus at 4 °C and 10 sec. In the 4 °C experiments the modulus decreased gradually for about

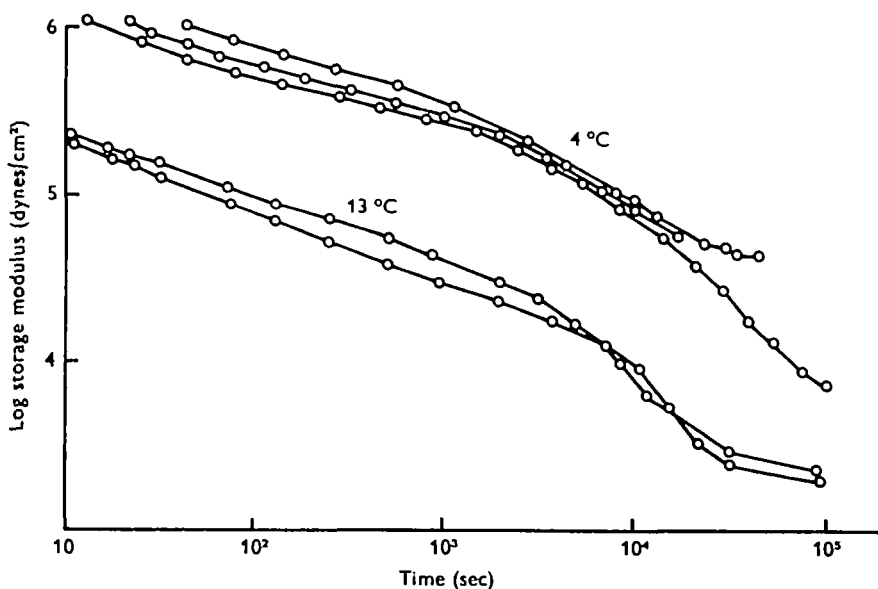


Fig. 6. Stress-relaxation of longitudinally stretched mesogloea at 4 and 13 °C. Note the levelling off of modulus at long times in experiments carried out at 13 °C. Each line is the result of a single experiment.

$10^3$  sec and then dropped off more rapidly. In the one experiment that was followed to long times ( $10^5$  sec) there was no indication of levelling off to a constant modulus. At 13 °C the whole curve appears to be shifted downward, but the general shape of the curve is the same. At long times there was a definite levelling at a value of about  $3 \times 10^3$  dyn/cm<sup>2</sup>. The modulus of mesogloea at 23 °C (Fig. 7) dropped almost linearly for about  $2 \times 10^4$  sec and then levelled off at a value of about  $3 \times 10^3$  dyn/cm<sup>2</sup>.

The gradual decrease in modulus at short times in 4 °C experiments is an indication of the plateau seen in the previous experiments. After about  $10^3$  sec the modulus dropped off more rapidly, suggesting the start of the flow region. Although there is no indication of a decrease in the slope at long times in 4 °C experiments, there is an obvious decrease in experiments at higher temperatures. According to the time-temperature superposition principle higher temperature is equivalent to longer time.

Thus, if the experiments at 4 °C were followed for longer times, say to  $10^6$  sec (c. 280 h), a levelling similar to that seen at higher temperature should be observed. The levelling to constant modulus in experiments at 13 and 23 °C is a clear indication of an equilibrium modulus and therefore the amorphous polymer in mesogloea can be described as a network. If the polymer system in mesogloea was not a network, the modulus would drop off with increasing slope and rapidly go to zero. The equilibrium compliance and creep recovery which Alexander (1962) observed in creep tests with mesogloea provide further evidence for a polymer network.

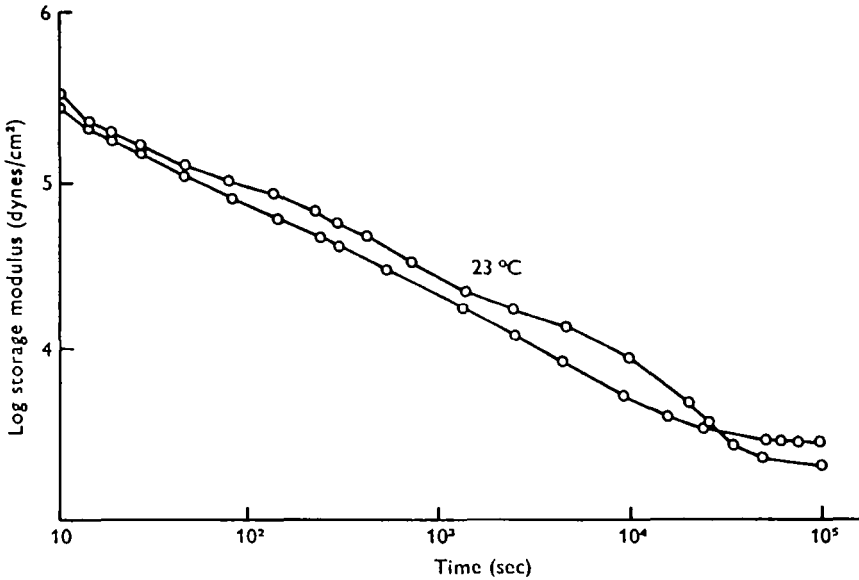


Fig. 7. Stress-relaxation of longitudinally stretched mesogloea at 23 °C. Again, note the levelling off of modulus at long times.

The results of the previously described mechanical tests have provided the following information about the molecular organization of mesogloea. (1) An amorphous polymer plays a major role in determining the mechanical properties of mesogloea. (2) This amorphous polymer system is tied together into some kind of a network. (3) There is a high molecular weight, entanglement-like mechanism that retards the deformation of mesogloea at short times.

Clearly, the matrix must provide the amorphous polymeric component. The collagen fibres, which are 'crystalline' aggregates of rod-like molecules, could not possibly account for the observed 'glass' transition. Quantitative analyses of *Metridium* mesogloea (Gosline, 1971) indicate that the matrix is a highly hydrated protein-polysaccharide complex with a concentration of about 2.4% (i.e. 2.4 g dry weight of matrix material per 100 g water). Part, if not all, of the matrix material must be long-chain random-coil molecules. Mechanical tests have indicated that these matrix molecules are linked together in some manner to form a network. Ferry (1961, p. 391) defines a polymer gel as 'a moderately dilute, cross-linked solution, whether linked by chemical bonds or crystallites or some other kind of junction... which exhibits no steady-state flow'. The amorphous polymer system of mesogloea clearly falls within

This definition of a polymer gel. Although no information is available to indicate the nature of the cross-links in this gel, crystalline interactions between polymer chains such as the double helical regions described by Anderson *et al.* (1969) in gel-forming, sulphated polysaccharides, might well provide these cross-links. The polymer network in mesogloea can account for both the extensibility and elasticity of the tissue. The viscous retardation or slow elasticity of this network is due either to interactions of the network molecules with other high molecular weight components or to stiffness of the network polymer chains or to both of these factors.

The collagen must play a relatively passive role in this system. Rat-tail tendon collagen tested at 20 °C under conditions similar to those used to test mesogloea in Fig. 5 (i.e. at short times) has a modulus of  $8 \times 10^9$  dyn/cm<sup>2</sup>, and the stress-relaxation modulus of rat-tail tendon collagen after 24 h is  $10^9$  dyn/cm<sup>2</sup> (Rigby *et al.* 1959). The modulus of mesogloea at 20 °C is about  $8 \times 10^5$  dyn/cm<sup>2</sup> at short times (Fig. 5) and  $3 \times 10^3$  dyn/cm<sup>2</sup> after 24 h (Fig. 7). There is a difference of four to five orders of magnitude between rat-tail tendon collagen and mesogloea at all times. Even allowing for the difference in the amount of collagen in the two tissues, it is clear that collagen fibres in mesogloea are essentially undeformed, even when the mesogloea is stretched up to 200%. What then is the role of the collagen?

Polarized-light micrographs (Gosline, 1971) showed that mesogloea has two characteristic layers: an outer crossed-fibrillar layer and an inner layer of circular and radial fibres. Extension of the outer layer, either longitudinally or circumferentially, will tend to align the fibres in the direction of the stress. Extension of the inner layer will tend to move the fibres in a manner dependent on the direction of the extension. In a longitudinally stretched sample fibres will be moved laterally apart, and in a circumferentially stretched sample the fibres will slide along one another. On the basis of this structural anisotropy one would expect a mechanical anisotropy as well, and the nature of the mechanical anisotropy should provide some information about the role of the collagen in mesogloea.

Fig. 8 shows the results of three stress-relaxation experiments at 23 °C in which the mesogloea was stretched circumferentially. The results appear to be different in each of the three experiments. In Expt. 1 the modulus was nearly constant for  $10^3$  sec and then dropped off rapidly to an equilibrium value of about  $10^4$  dyn/cm<sup>2</sup>. In Expt. 2 the modulus dropped off fairly gradually and then held an equilibrium value of slightly lower than  $10^4$  dyn/cm<sup>2</sup>. The modulus of the mesogloea tested in Expt. 3 dropped off with increasing slope and appeared to approach zero at around  $10^4$  sec.

These differences can be understood by correlating the initial strain in each experiment to the observed result. The initial strains are as follows; Expt. 1,  $\epsilon = 0.19$ ; Expt. 2,  $\epsilon = 0.23$ ; Expt. 3,  $\epsilon = 0.29$ . The material in Expt. 3 received the greatest initial strain and apparently yielded. In Expt. 2, with slightly lower initial strain, the material was probably partly disrupted, but did not yield completely. Expt. 1, which was run with the lowest initial strain, is assumed to most nearly show the behaviour of intact mesogloea. Creep curves of circumferentially stretched mesogloea (Alexander, 1962) are very similar to the results of Expt. 1, suggesting that this is a reasonable assumption.

Comparison of stress-relaxation data from longitudinally and circumferentially stretched mesogloea at 23 °C reveals the following differences: (1) the plateau region of

circumferentially stretched mesogloea extends to longer times, (2) the modulus of circumferentially stretched mesogloea in the plateau is greater by a factor of two to three, and (3) the equilibrium modulus of circumferentially stretched mesogloea is greater by a factor of about three. The differences in the plateau region indicate that a deformation which tends to slide the collagen fibres along one another (i.e. circumferential stretching) will experience greater viscous retardation than a deformation which moves the fibres laterally apart. This is reasonable considering the shear gradient that must exist in the matrix when the closely spaced, circumferential collagen fibres

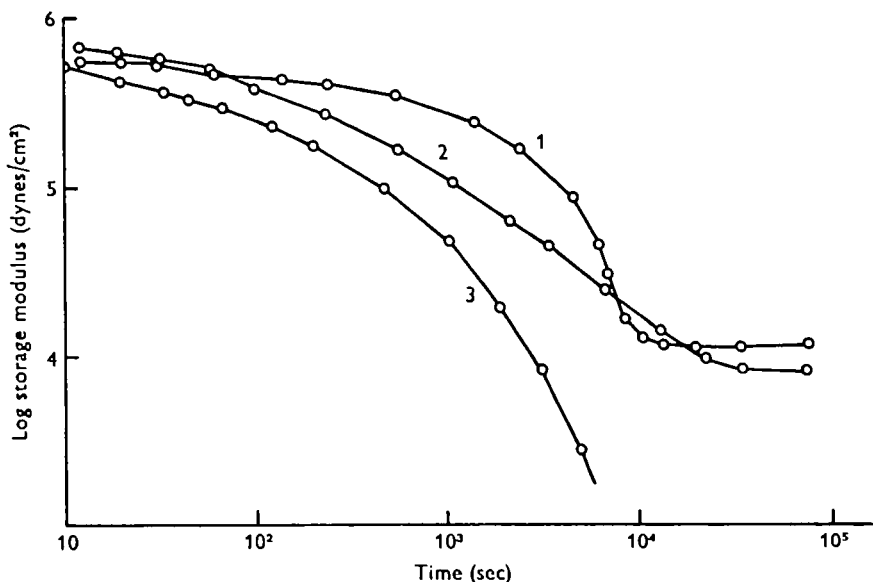


Fig. 8. Stress-relaxation of circumferentially stretched mesogloea at 23 °C. The initial strains for each experiment are: Expt. 1,  $\epsilon = 0.19$ ; Expt. 2,  $\epsilon = 0.23$ ; Expt. 3,  $\epsilon = 0.29$ .

of the inner layer slide along one another. The difference in the equilibrium modulus can also be explained by high shear strains in the matrix during circumferential stretching due to the close spacing of collagen fibres. In these localized areas between fibres the shear strain is very much greater than the net strain in the material as a whole. This results in an apparent modulus which is greater in circumferential stretching than in longitudinal stretching. The fact that the collagen appears to affect the equilibrium properties of the network strongly suggests that the collagen fibres are not just floating freely in the matrix but are directly linked to the matrix molecules. These observations indicate that the collagen fibres play a major role in the high molecular weight, entanglement-like interaction which results in the plateau region and also affect the equilibrium properties of the network. The interaction of collagen and matrix in the plateau provides short-term stability to an otherwise very flimsy network, thus the role of the collagen in mesogloea can be described as that of a reinforcing filler.

#### *A macromolecular model*

On the basis of the results presented in this paper and in a previous paper (Gosline, 1971) the following macro-molecular model is proposed for the visco-elastic properties

Of *Metridium* mesogloea. Collagen fibres arranged in a crossed-fibrillar array are embedded in a 'rubbery' matrix made of a high-molecular-weight, amorphous polymer. The matrix molecules, probably hydrated protein-polysaccharide complexes, form a dilute gel which is linked together to form a permanent network. This network provides an elastic mechanism based on configurational entropy changes ('rubber elasticity') of the folded matrix molecules. The properties of the matrix alone account for both the extensibility and elasticity of the tissue. The collagen, which acts as a reinforcing filler, provides short-term rigidity to the flimsy polymer network. The collagen fibres are not directly cross-linked to one another, but are tied together through the amorphous matrix. If the collagen fibres were linked directly together the 40° angle between the helically arranged fibres and the long axis of the resting anemone would limit the extensibility of the tissue to about 30% longitudinally and 55% circumferentially (Gosline, 1971). Alexander (1962) recorded extensions of over 200% in creep tests, indicating that the collagen fibres cannot be directly linked together. When the tissue is stretched the collagen fibres tend to align with the stress axis (Gosline, 1971) and also flow relative to one another along this axis.

#### *Collagen matrix interactions*

Several attempts were made to further characterize the matrix and to understand the interactions between the collagen and the matrix. The theory of rubber elasticity predicts that a network polymer will have an equilibrium modulus equal to  $E_{\text{equilibrium}} = 3\rho RT/M_c$ , where  $M_c$  is the molecular weight between cross-links and  $\rho$  is the weight concentration of the polymer in grams per cm<sup>3</sup> (Treloar, 1958). The equilibrium modulus observed from mesogloea is extremely low ( $3 \times 10^3$  dyn/cm<sup>2</sup>), indicating either a low concentration of the polymer material or a low degree of cross-linking and hence a high  $M_c$ . The low equilibrium modulus of mesogloea is probably due to both of these factors. Quantitative analysis of mesogloea (Gosline, 1971) show that the concentration of the matrix polymer is indeed quite low. The degree of cross-linking in the matrix network is more difficult to assess, but it appears to be unusually low. If the degree of cross-linking were high the equilibrium modulus would be greater and the extensibility of the tissue would be greatly reduced.

The problem then arises, how to keep the degree of cross-linking low? A polymeric material, such as mesogloea, made of proteins and polysaccharides will have a large number of ionized sites which can interact electrostatically to cross-link the system. One solution to this problem is to mask charged groups, and thus block potential electrostatic interactions. *Metridium* is a marine organism, and the inorganic ion concentration of mesogloea is probably very similar to that of sea water. It seems probable that these inorganic ions provide a suitable agent to block electrostatic interactions. In order to evaluate this possibility a series of experiments was carried out to determine the effect of inorganic ion concentration on the properties of mesogloea. The results are plotted in Fig. 9. The experiments were carried out at 15 °C in various concentrations of NaCl adjusted to pH 8.0 with Sørensen's borate buffer. The testing procedure was as follows. The material was tested at sea-water salinity (34‰), the medium was changed and the material was left to equilibrate for 2 h, and the material was then tested at the new salinity. Fig. 9 is a plot of log storage modulus ( $E'$ ) and damping ( $\tan \delta$ ) against salinity (expressed as parts per thousand NaCl). The

values plotted are from periodic stress-strain measurements at 0.3 Hz. Each point is the result of a single measurement.

The modulus remained fairly constant at about  $5 \times 10^5$  dyn/cm<sup>2</sup> from salinities of 8‰ to 78‰ NaCl, with only a slight increase in modulus at high salinities. Damping was also virtually constant over this range. At low salinities there was a dramatic increase in modulus and a sharp decrease in damping. The modulus of mesogloea in distilled water is roughly 30 times greater than the modulus in 8‰ NaCl. Although the values given at each salinity are not equilibrium values, the relationship shown here for short times probably holds for long times as well. The increase in modulus at

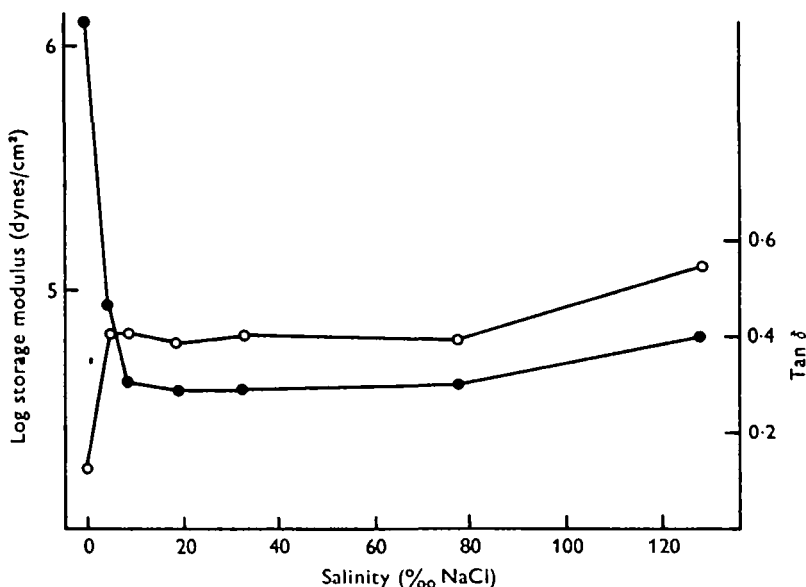


Fig. 9. The effect of inorganic ion concentration on the mechanical properties of mesogloea. Modulus (●) and damping (○) were measured at 0.3 Hz in various concentrations of NaCl (expressed as ‰ NaCl). Test solutions were buffered to pH 8 and held at 15 °C.

low salinities is a good indication of an increase in the degree of cross-linking. Thus, inorganic ions in the material mask ionized sites on both the matrix polymer chains and the collagen fibres and prevent electrostatic interactions.

This role of inorganic ions in mesogloea suggests that the presence of high concentrations of Mg<sup>2+</sup> ion in the test medium may affect the mechanical properties of mesogloea. Divalent cations such as Mg<sup>2+</sup> and Ca<sup>2+</sup> are known to provide electrostatic cross-bridges in a number of systems, but there does not seem to be any detectable effect on mesogloea. The mechanical properties of mesogloea tested in Mg<sup>2+</sup>-free NaCl solutions and of mesogloea tested in sea water with magnesium sulphate appear to be essentially the same. The polymer gel in mesogloea is probably so dilute that the Mg<sup>2+</sup> ions do not make any important contributions to the cross-linking.

If ionized groups on the polymer chains are masked by inorganic ions, one would expect that the titration of these groups would have little or no effect on the mechanical properties of the material. Fig. 10 shows the results of a single experiment carried out to determine the effect of pH on the mechanical properties of mesogloea. The material



Was tested at 15 °C at 0.3 Hz in NaCl solutions (34‰ NaCl salinity) buffered to known pH. Sørensen's borate buffer was used for pH 7.5 and above, and phosphate-citric acid buffer was used for pH 7 and below. The material was allowed to equilibrate at each pH for 2 h before testing. The results indicate that neither the storage modulus nor the damping of native mesogloea vary significantly over the range of pH 3.8–9.2.

A number of chemical techniques are known for cross-linking collagenous systems Bowes & Cater (1968) studied the effects of various aldehydes on collagen and found formaldehyde to be a suitable cross-linking agent. Fig. 11 shows the results of a series of experiments in which the mesogloea system was cross-linked with formaldehyde.

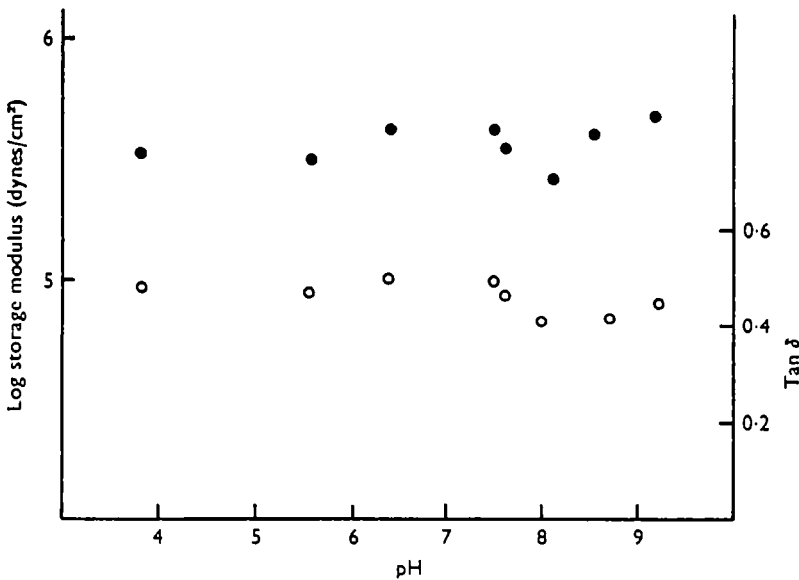


Fig. 10. The effect of pH on the mechanical properties of mesogloea. Modulus (●) and damping (○) were measured at 0.3 Hz in NaCl solutions (34‰) buffered to known pH at 15 °C.

The degree of additional cross-linking was controlled by varying the length of time the material was kept in a 1% solution of formaldehyde in sea water. Log storage modulus ( $E'$ ) and damping ( $\tan \delta$ ) measured at 0.5 Hz are plotted as a function of temperature. This figure shows the properties of native mesogloea, mesogloea kept in 1% formaldehyde in sea water for 1 h, 4 h, and 5 days, and mesogloea washed in distilled water. Each point is the result of a single measurement. The following changes were observed: (1) as the degree of cross-linking was increased the modulus increased, but the temperature-dependence of the modulus decreased; (2) damping decreased with increased cross-linking; (3) the modulus and damping of mesogloea held in 1% formaldehyde in sea water for 5 days are virtually identical to that of mesogloea washed in distilled water. Again the values measured in this experimental series are not equilibrium values, but the similarity between mesogloea cross-linked with formaldehyde and mesogloea washed in distilled water indicates that the changes observed in Fig. 9 for mesogloea at low salinities may well be due to an increase in the degree of cross-linking.

It is at present not certain that the matrix molecules are covalently linked to the

collagen, but the stress-relaxation experiments with longitudinally and circumferentially stretched mesogloea suggest that some very stable linkage is present. Katzman & Jeanloz (1970) have isolated glucosyl-galactosyl-hydroxylysine from the gelatin of *Metridium dianthus*. They suggest that the hydroxylysine provides an attachment site on the collagen for the matrix polysaccharide. The sugars found in assays of *Metridium* mesogloea are predominantly neutral sugars but a small amount of amino sugar is present as well. There are no acidic sugars (Gross, Dumsha & Glazier 1958; Katzman & Jeanloz, 1970; Gosline 1971). Katzman & Jeanloz (1970) state that the

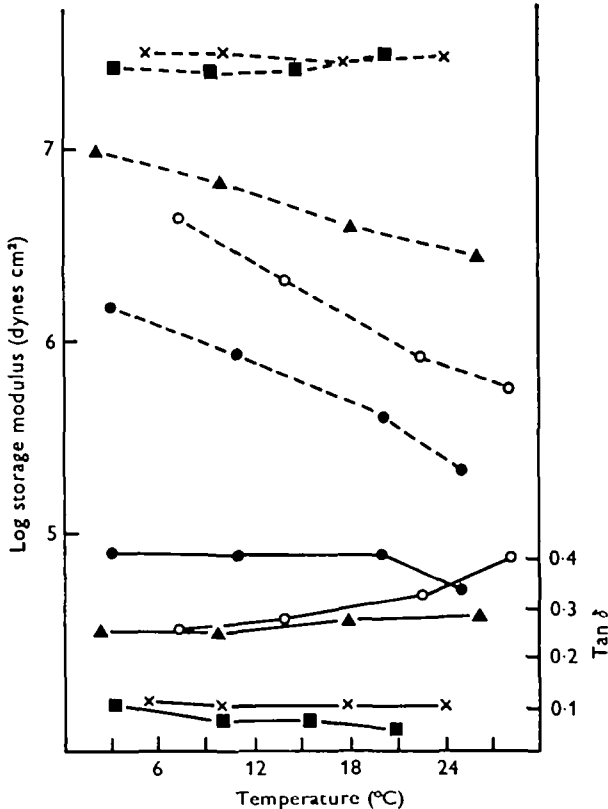


Fig. 11. The effect of formaldehyde cross-linking on the mechanical properties of mesogloea. Modulus (dashed lines) and damping (full lines) are plotted against temperature at 0.5 Hz for native mesogloea (●), mesogloea treated with 1% formaldehyde in sea water for 1 h (○), for 4 h (▲), and for 5 days (■), and mesogloea washed with distilled water (×).

mesogloea of *Metridium dianthus* is the only collagenous tissue known which does not contain acid glycosaminoglycan associated with the collagen. This striking lack of acid polysaccharide in mesogloea must have some significance, and the possibility that this lack is tied up with the mechanical properties of the tissue must be considered.

It is possible that neutral sugars enhance the elastic properties of the material. The elastic mechanism proposed for mesogloea depends upon a random-coil conformation for the polymer molecules involved. Acidic polysaccharides are poly-electrolytes which at pH 8 contain a high density of negative charges. This charge density causes segments of such a polymer chain to repel each other, thus causing the molecule to

like on an extended conformation which differs significantly from a true random coil (Tanford, 1961). Although this effect is moderated to a large extent at high ionic strength, it is possible that the presence of neutral polysaccharides instead of acidic polysaccharides allows the polymer chains in the matrix network to take on a more nearly random-coil conformation.

It is also possible that neutral sugars provide a means of reducing electrostatic interactions within the material. The extensibility and unusually low modulus of mesogloea are due to the low degree of cross-linking of the matrix network and to the fact that the collagen fibres are not directly linked together. The presence of neutral polysaccharides instead of acidic ones would greatly reduce the number of charged sites and therefore limit electrostatic binding within the matrix network and between the matrix and the collagen. Or perhaps the neutral polysaccharides along with inorganic ions mask ionic binding sites on the collagen fibres and prevent them from linking together.

This study has provided an understanding of the molecular interactions which account for the functional properties of *Metridium* mesogloea observed by Alexander (1962). The most important features of the mesogloea system to the animal are the elasticity and extensibility of the tissue under very small stresses, and the behaviour of the animal appears to be closely tied to these properties. They allow the animal to deform its body-wall mesogloea with the low pressure, ciliary pumping mechanism and to undergo the various slow postural changes which are characteristic of its normal behavioural patterns (Batham & Pantin, 1950*a, b*).

The presence of an inner layer of densely packed circumferential fibres in mesogloea along with the outer layer of fibres in crossed helics (Gosline, 1971) was found to result in a mechanical anisotropy. The densely packed circumferential fibres appear to reinforce the material to the extent that the circumferential, tensile modulus is roughly three times greater than the longitudinal, tensile modulus over the entire time range studied (10 to 10<sup>6</sup> sec). Chapman (1953) also observed a mechanical anisotropy in tensile strength tests with *Metridium* mesogloea. It is possible to understand the value of this anisotropy to the animal by considering the forces which arise in the body wall under normal conditions (i.e. when the animal is alive and not when it is cut up and stretched on some machine). If the body form is modelled by a closed cylinder with an internal hydrostatic pressure it is possible to calculate the stress in the cylinder wall due to the internal pressure. The general solution to this problem, known to engineers (Faupel, 1964) as well as biologists (Wells, 1950) for many years, is that the 'hoop' stress or circumferential stress is twice that of the longitudinal stress. Thus, in a closed cylinder made of a mechanically isotropic material which expands under a hydrostatic pressure, the diameter will increase twice as much as the length. The mechanical anisotropy in *Metridium* mesogloea allows the animal to extend itself longitudinally as much or a bit more than it can circumferentially without having to depend on muscles to restrict circumferential expansion. The more one studies mesogloea the more obvious it becomes that the mechanical properties of this tissue play a central role in governing the behaviour of the animal.

This study is important in another more general sense. Mesogloea is one of a wide variety of collagen-containing connective tissues. Most of the work to date on the mechanical properties of collagenous tissues has dealt with isolated components such

as collagen (see Elden, 1968; Bailey, 1968) and elastin (see Balazs, 1970). Little is known about the mechanical properties of the ground substance or matrix which is present to varying degrees in all collagenous tissues, or about the mechanics of whole connective tissues. The wide variation in the properties of collagenous tissues (Harkness, 1968) and the apparent constancy of collagen and elastin leads me to believe that the matrix plays an extremely important role in determining the mechanical properties of most collagenous tissues. This work with mesogloea describes for the first time the molecular interactions which account for the mechanical properties of a complex, collagenous tissue. The properties of this tissue were found to depend almost completely on the properties of the matrix. Thus, mesogloea provides a unique system for more clearly understanding the nature of matrix materials in collagenous tissues and the role these materials play in determining the mechanical properties of connective tissues.

#### SUMMARY

1. The mechanical properties of the mesogloea of the sea anemone *Metridium senile* were investigated. An amorphous polymer network in the matrix was found to play a major role in determining the mechanical properties of the tissue.
2. The matrix network provides an elastic mechanism based on 'rubber elasticity' of the folded matrix molecules. The properties of the matrix network alone account for the extensibility and elasticity of mesogloea.
3. The collagen acts as a reinforcing filler providing short-term rigidity to the flimsy polymer network.
4. The collagen fibres are not directly cross-linked to one another but are tied together through the amorphous matrix.
5. The extensibility and elasticity of the tissue appear to be dependent on a very low degree of cross-linking in the mesogloea system. Inorganic ions mask ionized groups on the collagen and matrix polymer chains and block electrostatic interactions which could cross-link the system.

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