PHYSICAL AND CHEMICAL PROPERTIES OF RUBBER-LIKE ELASTIC FIBRES FROM THE OCTOPUS AORTA

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SUMMARY

We investigated the physical and chemical properties of highly extensible elastic fibres from the octopus aorta. These fibres are composed of an insoluble rubber-like protein which we call the octopus arterial elastomer. The amino acid composition of this protein is different from that of other known protein rubbers, being relatively low in glycine and high in acidic and basic residues. Up to extensions of 50 %, mechanical data from native elastic fibres fit a theoretical curve for an ideal Gaussian rubber with elastic modulus $G = 4\cdot65 \times 10^5 \, \text{N m}^{-2}$, and this is unchanged by prolonged exposure to formic acid. Thermoelastic tests on this protein indicate that the elastic force arises primarily from changes in conformational entropy, as predicted by the kinetic theory of rubber elasticity. Analysis of the non-Gaussian behaviour of the elastic fibres at extensions greater than 50 % suggests that the molecular chains in this octopus protein are somewhat less flexible than those in resilin or elastin. Some speculations on the molecular design of these protein rubbers are made.

INTRODUCTION

Compliance and elastic recoil in the walls of major arteries provide an important pulse smoothing mechanism for the high pressure closed circulatory systems of vertebrates. The elasticity of vertebrate arteries at physiological pressures is based primarily on elastin, a fibrous connective tissue protein with rubber-like properties (McDonald, 1974). Although elastin is the only arterial elastomer which has been described in detail, histological reports of 'elastic' fibres in the blood vessels of some molluscs, crustaceans and annelid worms (Elder & Owen, 1967; Elder, 1973) suggest that at least some of the more advanced invertebrate circulatory systems contain an elastic component. However, these invertebrate 'elastic' fibres cannot be elastin because elastin occurs exclusively among the vertebrates (Sage & Gray, 1979, 1980). Thus, elasticity of invertebrate blood vessels may be based on rubber-like proteins which have not yet been described.

To determine if any elastin analogues are present in invertebrate blood vessels, we began to investigate the connective tissue architecture and mechanical properties of

the arteries of cephalopod molluscs. In our initial study we found that the octopus aorta is a compliant and elastic tube, and that the vessel wall contains connective tissue fibres that are highly extensible (Shadwick & Gosline, 1981). These fibres stain with aldehyde fuchsin (an 'elastic' fibre stain) and are present throughout the artery wall and also as a layer that lines the vessel lumen (Shadwick & Gosline, 1983). Preliminary experiments with fibres isolated from the luminal layer of the octopus aorta provided evidence that this material is an insoluble protein with rubber-like properties (Shadwick & Gosline, 1981). The current study extends these observations, and presents a quantitative analysis of the physical and chemical properties of the octopus elastic fibre protein, based on the theory of rubber elasticity.

THEORETICAL BACKGROUND

The kinetic theory of rubber elasticity is a useful mathematical description of the mechanical properties of highly extensible elastic polymers (see Treloar, 1975, for a complete description). Briefly, this theory assumes that: (1) the material is composed of flexible, linear polymer molecules, (2) these molecules are randomly coiled, so that the end-to-end separation of each molecular chain is much smaller than its fully extended length, (3) the molecular chains are kinetically free and in constant motion due to thermal agitation and (4) the chains are joined together by permanent crosslinks to form an isotropic three-dimensional network.

The kinetic theory treats the individual chains as 'ideal' random walks. Each polymer chain is considered to be a series of random links, where each link has complete rotational freedom with respect to its neighbours. This allows the whole chain to take on the statistically most probable conformations. When an ideal network is mechanically deformed the chains are forced to adopt new conformations of lower probability. In other words, the conformational entropy of the network is decreased with increasing deformation, and an elastic force arises from the tendency of these random-coil chains to return spontaneously to the state of highest entropy (i.e. the undeformed state).

If the polymer chains in a real rubber network are able to change their conformation with little or no distortion of the chemical bonds in the polymer backbone, then elastic energy will be stored in the network as a decrease in conformational entropy, with little or no change in internal energy. However, there are some important differences between real polymer molecules and ideal random chains. Because there are physical restrictions to rotation around the covalent bonds in the backbone of a polymer, it is not possible for an individual bond to exhibit the complete rotational freedom assumed to occur between the links in an ideal random chain. Thus, in the application of the kinetic theory to real molecules it is necessary to replace the concept of an ideal random link with a 'functional' random link. This functional link may be defined as a length of molecule which contains enough bonds with partial rotation so that the segment has essentially the same kinetic freedom as an ideal random link.

The entropy change and the resulting elastic force which accompanies deformation of an ideal rubber network can be predicted from Gaussian statistics of

random walks. Protein rubbers are rubbery only when swollen in a polar solvent like water (Gosline, 1980). For this case we may use the following relationship for the force and uniaxial extension of a swollen rubber (Treloar, 1975):

$$f = NkTv^{1/3}(\lambda - \lambda^{-2}),\tag{1}$$

where f is the nominal stress, defined as tensile force per unit of unstrained cross-sectional area, N is the number of chains per unit volume of material, an indication of cross-link density (note that a 'chain' is the segment of molecule between adjacent crosslinks), k is Boltzman's constant ($1\cdot38\times10^{-23}$ J °K^{-1}), T is the absolute temperature, v is the volume fraction of polymer in the swollen network, and λ is the extension ratio, defined as the extended length divided by the unstrained length.

A rubber can be characterized further by a single material constant called the elastic modulus, G, given as:

$$G = NkT = \varrho RT/M_c,\tag{2}$$

where G is a measure of the stiffness of the material, in Newtons/metre^2 (N m^{-2}), and ϱ is the density of the dry polymer (for protein $\varrho = 1\cdot33\times10^3$ kg m^{-3}). R is the gas constant ($8\cdot31$ J mol^{-1}°K^{-1}), and M_c is the average molecular weight of the chains between adjacent cross-links.

The Gaussian model holds only for relatively small extensions. As a real network is deformed the chains are pulled out from their coiled conformations, and eventually the network becomes stiffer than predicted by the theory. Treloar (1975) developed a non-Gaussian model which accounts for the finite extensibility of chains in real networks. The shape of the non-Gaussian force-extension curve is highly dependent on the number of random links (n) in each network chain. Decreasing n causes a greater deviation from the Gaussian curve, for which n is infinite. By comparing the force-extension curve for a real network with the non-Gaussian model (see Treloar, 1975, equation 6.22) we can estimate the number of functional random links in the network chains.

Thermodynamic relationships

The fundamental feature of the theory of rubber elasticity is that deformation of an ideal rubber gives rise to an elastic restoring force which is due solely to a decrease in the conformational entropy of the molecular network. Equation 1 shows that the elastic force in a stretched rubber is proportional to the temperature. If an elastic material is stretched and held at constant length, the elastic force will vary with temperature according to the following equation (Flory, 1953):

$$f = (\partial H/\partial L)_{T,P} - T(\partial S/\partial L)_{T,P},\tag{3}$$

where f is the elastic force, H is the enthalpy, L is the length of the sample, T is the absolute temperature, S is the entropy and P is the pressure. This relationship shows that, in general, the elastic force can be composed of both enthalpy and entropy

components. Flory (1953) gives another equation which allows the entropy component to be measured experimentally:

$$-(\partial S/\partial L)_{T,P} = (\partial f/\partial T)_{L,P}. \tag{4}$$

That is, the slope of a force vs temperature curve, obtained at constant length, gives the entropy component of the elastic force at that length. Combining equations 3 and 4 yields:

$$f = (\partial H/\partial L)_{T,P} + T(\partial f/\partial T)_{L,P}. \tag{5}$$

The enthalpy component can be calculated from equation 5 as the force at $T = 0\,^\circ K$.

These relationships provide a simple method (known as the thermoelastic experiment) for determining the entropy and enthalpy components of the elastic force for any material, under conditions of constant pressure. However, the kinetic theory of rubber elasticity predicts specifically that the change in internal energy will be zero when the network is deformed at constant volume. Thus, only under conditions of constant volume will the enthalpy change be equal to the internal energy change, and the entropy component will be due solely to changes in the conformational entropy of the polymer chains. For water-swollen protein rubbers the distinction between testing at constant pressure and constant volume may be important if the volume of the protein network varies with temperature. If so, then $(\partial H/\partial L)_{T,P}$ will not be just the internal energy change associated with the conformation of the molecule, but it will also have an energy component associated with the mixing of water and the protein; similarly $(\partial S/\partial L)_{T,P}$ will also have a component due to this mixing process (Gosline, 1980).

The condition of constant volume has been obtained experimentally for protein rubbers by eliminating swelling with the use of appropriate solvent mixtures (Hoeve & Flory, 1958), pH (Weis-Fogh, 1961a) or by testing the hydrated sample as a closed system, e.g. immersed in oil (Gosline, 1980). An alternative method is to test the sample as an open system in water, and then correct the data to account for the swelling changes (Dorrington & McCrum, 1977). This involves transforming the force-temperature plots from constant length to constant extension ratio, λ. Assuming that swelling is isotropic, this procedure corrects to constant volume. Under these conditions the following equations are applicable (Flory, 1953):

$$f = (\partial U/\partial L)_{T,V} - T(\partial S/\partial L)_{T,V} \tag{6}$$

and

$$-(\partial S/\partial L)_{T,V} \simeq (\partial f/\partial T)_{\lambda,P}. \tag{7}$$

Together these yield:

$$f \simeq (\partial U/\partial L)_{T,V} + T(\partial f/\partial T)_{\lambda,P}, \tag{8}$$

which is analogous to equation 5. Now, the slope of the force-temperature plot at constant λ gives the entropy change $-(\partial S/\partial L)_{T,V}$, and the intercept at $T = 0\,^\circ K$ is the internal energy change $(\partial U/\partial L)_{T,V}$ associated solely with the orientation of the molecular chains.

METHODS

Composition of the artery wall

The dorsal aorta was dissected from specimens of *Octopus dofleini* that had been cold-anaesthetized and killed by decerebration. Tissues were minced, blotted and weighed before and after drying at 60 °C to determine the water content. Extraction and purification of elastic connective tissue from the artery wall was based on methods for elastin (Steven, Minns & Thomas, 1974; Rasmussen, Bruenger & Sandberg, 1975; Sage & Gray, 1977) which assume that the material is covalently crosslinked into an insoluble network. Dry aortae were ground in a glass tissue homogenizer and then extracted with continuous agitation in 98 % formic acid at room temperature for periods of either 6, 18, 24, 48 or 72 h. After centrifugation at 12 000 g for 20 min the pellets were resuspended and washed with distilled water several times. The residues were dried at 60 °C to a constant weight. As an alternative method aortae were extracted in 6 mol l^{-1} guanidine HCl + 1 % mer-captoethanol for 48 h with continuous agitation, followed by centrifugation, washing in water, and autoclaving for 4 h at 1 atmosphere pressure and 120 °C.

Other samples of aorta were minced, weighed and then washed for 24 h in several volumes of distilled water. This was to remove soluble salts, proteins and carbohydrates. The insoluble material was separated by centrifugation (15 min at 5000 g) and autoclaved in distilled water for 6 h to solubilize collagen and precipitate other proteins. After centrifugation for 20 min at 12 000 g the supernatant was passed through a 1-μm Millipore filter, and made up to a known volume with water. This autoclave-soluble fraction was assumed to be a crude collagen extract. The total dry weight of this fraction was determined by drying aliquots of the solution. The amount of collagen in the artery wall was then estimated by comparing the amino acid composition of the collagen preparation with published data for purified octopus collagen.

Isolation and composition of elastic fibres

Fresh aortae were cut open longitudinally and pinned out flat on wax under filtered sea water. With the use of a stereo microscope and fine forceps native elastic fibres were stripped from the internal elastic layer which lines the vessel lumen (Shadwick & Gosline, 1983). Some fibres were used for mechanical tests immediately, while others were stored either frozen or at 4 °C in a 0·01 % solution of penicillin + streptomycin. Some fibres were exposed to 98 % formic acid at room temperature for up to 72 h and also used for mechanical tests and amino acid analyses.

Water content of the elastic fibres was determined at 20 °C in the following way. Small (1 ml) polyethylene capsules with snap caps were weighed empty and dry.

Samples of native elastic fibres (about 300 mg) were put into open capsules and equilibrated over a solution of $0.08 \, mol \, l^{-1}$ NaCl in distilled water (relative humidity $= 99.7 \%$) in a vacuum chamber. This allowed the fibres to become fully hydrated without surface condensation. The chamber was opened and the capsules were immediately capped and weighed. Then the capsules were opened and the contents were dessicated in a vacuum chamber with phosphorus pentoxide for 48 h. The chamber was opened and the capsules were covered immediately and weighed. An empty capsule served as control and showed no weight change throughout the hydration and drying procedure.

To determine protein content, samples of native elastic fibres were dried, weighed, dissolved in hot alkali ($0.1 \, mol \, l^{-1}$ NaOH, 100 °C for 5 min) and assayed for protein by the method of Dorsey, McDonald & Roels (1977), using bovine serum albumin and gelatin as standards. This is a heated biuret-folin technique which is equally chromogenic for different proteins.

Carbohydrate content of native elastic fibres was determined by the phenol-sulphuric method of Lo, Russel & Taylor (1970). In this case the samples and standards were heated to 100 °C for 5 min in the acid reagent to dissolve the elastic fibres. The assay is sensitive to any carbohydrate with free or potential reducing groups, and includes hexoses, pentoses and uronic acid but not amino sugars. The results are expressed as glucose equivalents.

Samples for amino acid analyses were hydrolysed in $6 \, mol \, l^{-1}$ HCl at 105 °C for 24 h in evacuated glass tubes. The tubes were opened and the acid was diluted and evaporated under vacuum. The samples were chromatographed with a Beckman 118C automatic amino acid analyser and peak areas were integrated manually.

Mechanical tests

Force-extension tests were conducted by stretching elastic fibres on a micro-force transducer stage fitted to a Wild M21 compound microscope, as previously described (Aaron & Gosline, 1981; Shadwick & Gosline, 1981). A bundle of around 10 to 15 µm diameter, consisting of 2 to 5 fibres, was mounted onto the transducer by attaching one end to a slender glass cantilever and the other end to a moveable glass plate with rubber cement. The two ends of the fibre bundle were allowed to dry in order to be glued to the glass, while the middle section of the sample remained in a drop of water. After gluing, the transducer stage was flooded with water. The sample was stretched by sliding the glass plate over a thin film of high vacuum grease. The grease kept the plate stationary at each extension. Tensile force in the sample was calculated from the deflection of the glass cantilever using simple beam theory. Extension of the sample was determined by measuring changes in the spacing of surface features on the fibres. For each sample, the length at which the minimum tensile force was detectable was defined as the unstrained length. All measurements were made optically under the microscope at 300× with a Wild travelling crosshair micrometer eyepiece. The error in these measurements was estimated to be not greater than 5 % (Aaron & Gosline, 1981). These experiments were done at 20 °C.

Force-temperature measurements were also made by using the micro-force transducer. A fibre sample was stretched and held at a constant length at 35 °C for

2 h. The sample was cooled, in steps of a few degrees, down to 5 °C. After 2 min at each temperature the new force was measured. Force measurements were also made as the sample was heated back to 35 °C.

The effect of temperature on swelling of native elastic fibres in water was investigated. A fibre sample was mounted on the transducer stage by attaching one end while leaving the other end free to move. A 1-mm diameter probe from an electronic thermometer was placed immediately adjacent to the sample to monitor temperature. The preparation was covered with water and viewed under the microscope. A temperature controlled microscope stage was used to heat and cool the sample. Length measurements were made optically, with the filar micrometer, over the temperature range 3 to 60 °C.

RESULTS

Composition of the artery wall

The water content of the artery wall was 70 % by weight. The autoclave-soluble fraction was 30 % of the tissue dry weight. Hydroxyproline was present in this fraction as 42 residues per 1000. In purified octopus collagen, hydroxyproline accounts for 81 residues per 1000 (Kimura, Takema & Kubota, 1981). Therefore collagen must make up about 15 % of the dry weight of the artery wall.

The results of formic acid extractions on pieces of whole artery wall are given in Fig. 1. This showed that extraction for at least 24 h was required to remove soluble
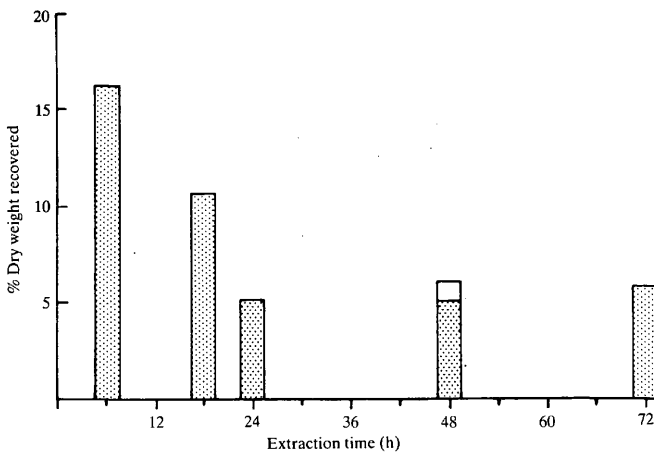

Fig. 1. Yields of insoluble residue from whole octopus aortae after extraction in 98 % formic acid (shaded bars), or in $6 \, mol \, l^{-1}$ guanidine + mercaptoethanol, followed by autoclaving (unshaded bar). Each result is the average of three determinations.

contaminants, and that longer exposure to formic acid does not reduce the amount of insoluble residue. Assuming that the formic acid insoluble fraction is indeed the elastic fibre component then these fibres comprise about 5 % of the dry weight of the artery wall. This assumption is consistent with the amino acid data presented below. A 48-h extraction on an aorta by the guanidine HCl/mercaptoethanol and autoclave procedure yielded an insoluble residue that was about 6 % of the dry weight (Fig. 1).

Chemical composition of isolated elastic fibres

The biuret-folin assay showed that the elastic fibres are 98–100 % protein. The carbohydrate content of the fibres was 0·4–0·5 %. Each gram of native fibres contained 0·53 g of water at 20 °C. This gives a volume fraction v = 0·40, assuming a value of $1·33 \times 10^3 \, kg \, m^{-3}$ for the density of dry protein (v is the ratio of the unswollen protein volume to the water-swollen protein volume).

The elastic fibre protein could not be dissolved and retained its elasticity after 72 h in 98 % formic acid. The protein was also resistant to prolonged autoclaving. Elastin and other protein rubbers are similarly insoluble. However, unlike elastin, the octopus elastic protein was dissolved within 10 min in $0·1 \, mol \, l^{-1}$ NaOH at 100 °C.

The amino acid composition of purified elastic fibres from the internal elastic layer of the octopus aorta is distinctly different from that of the protein rubbers elastin, resilin and abductin; the octopus protein has a relatively low glycine content and a high proportion of large amino acids (Table 1). The average amino acid weight is 110.

Analyses of the insoluble residues from whole aortae extracted for 6, 18, 24 and 72 h in formic acid and for 48 h in guanidine HCl with mercaptoethanol followed by autoclaving are given in Table 1. Residues after 6 and 18 h in formic acid contain some collagen, as indicated by the high hydroxyproline and glycine content. Extraction of whole aortae for 24 h or longer gave a constant composition as well as constant weight recovery (Fig. 1), and this composition is virtually identical to the elastic protein of the isolated fibres. These results suggest that the residues after prolonged extraction in formic acid or guanidine represent only the elastic protein, and that this protein comprises about 5 % of the dry weight of the artery wall. If the elastic fibres make up 5 % of the dry weight of the artery wall, and these fibres contain 53 % water while the whole wall is hydrated to an average of 70 %, then the hydrated elastic fibres will occupy about 3 % of the hydrated volume of the artery wall.

Force-extension tests

Fibres stripped from the internal elastic layer of fresh aortae were highly extensible and elastic. However, when allowed to dry out, the fibres lost their elasticity and became stiff and brittle. Hydrated fibres mounted on the test apparatus could be extended by over 100 % without breaking. At each extension the elastic fibres reached equilibrium quickly; there was no appreciable hysteresis or change in properties with consecutive cycles of extension. We found no mechanical differences between elastic fibres tested fresh and those tested after cold or frozen storage. Fig. 2 shows force-extension data for native fibres and also for fibres which were exposed to formic acid for 72 h, rehydrated in water, and then tested.

Table 1. *Comparison of amino acid profiles*

Amino acid	A	B	C	D	E	F	G	H	I
Hyp	40	29	5	0	5	0	9	—	—
Asp	86	94	107	101	107	91	6	94	23
Thr	42	42	39	59	59	64	15	20	10
Ser	57	71	75	81	63	72	12	128	61
Glu	106	114	126	132	118	121	19	42	14
Pro	73	68	72	41	51	55	113	75	10
Gly	212	200	162	88	72	85	313	422	627
Ala	69	80	54	74	67	72	244	70	50
Cys	3	1	2	10	4	7	—	—	1
Val	41	36	39	52	66	62	128	12	5
Met	16	14	7	13	21	21	—	—	92
Ile	40	39	36	47	61	60	18	9	6
Leu	59	57	48	81	83	74	54	30	3
Tyr	23	20	21	19	43	36	19	12	1
Phe	25	21	21	42	47	42	33	12	83
His	10	7	12	28	14	21	1	12	2
Lys	44	43	28	75	60	68	5	8	8
Arg	60	65	66	55	59	46	8	46	4
Small amino acids (Gly+Ala+Ser)						229	569	620	738
Average residue weight						110	85	89	79

Amino acid compositions (number of residues per 1000): insoluble fractions from whole octopus aortae after extraction in formic acid for (A) 6 h, (B) 18 h, (C) 24 h, (D) 72 h; (E) insoluble fraction from a whole octopus aorta after extraction in guanidine + mercaptoethanol for 48 h followed by autoclaving; (F) elastic fibres isolated from the octopus aorta internal elastic layer and purified by 24 h in formic acid; (G) elastin (Sage & Gray, 1977); (H) resilin (Andersen, 1971); (I) abductin (Kelly & Rice, 1967).

According to theory, a graph of f vs $(\lambda - \lambda^{-2})$, for a Gaussian network, yields a straight line with a slope equal to $Gv^{1/3}$. Up to $\lambda = 1.5$ (i.e. $\lambda - \lambda^{-2} = 1.05$) the data fit the Gaussian relationship, while at higher extension ratios the points deviate upwards from the straight line. The elastic modulus, G, was calculated from the slope of the regression lines, using $v = 0.40$. For the native fibres $G = 4.65 \times 10^5$ N m^{-2} and for the formic acid fibres $G = 4.62 \times 10^5$ N m^{-2}. This is almost identical to the modulus of elastin (Aaron & Gosline, 1981) and means that the elastic fibres have about the same stiffness as a common rubber band. These results indicate that long exposure to formic acid had no effect on the elastic properties of the fibres, at least up to extensions of $\lambda = 1.5$. Comparison of the two curves in Fig. 3 confirms that the formic acid treated fibres were mechanically identical to native fibres, even in the non-Gaussian range of extensions $(\lambda > 1.5)$.

To estimate the number of functional links per random chain between crosslinks in the elastic protein, the experimental curve for native fibres was compared to theoretical plots for random networks with different values of n, where n is the number of ideal links per random chain (Fig. 4). Although the curve for the elastic fibres does not have the exact shape of any one theoretical curve it does fall between the plots for n = 4 and n = 5 at extension ratios greater than 1.6. On the basis of this analysis it is concluded that there are, on average, 4 or 5 functional links in the random chains of the octopus elastic protein.
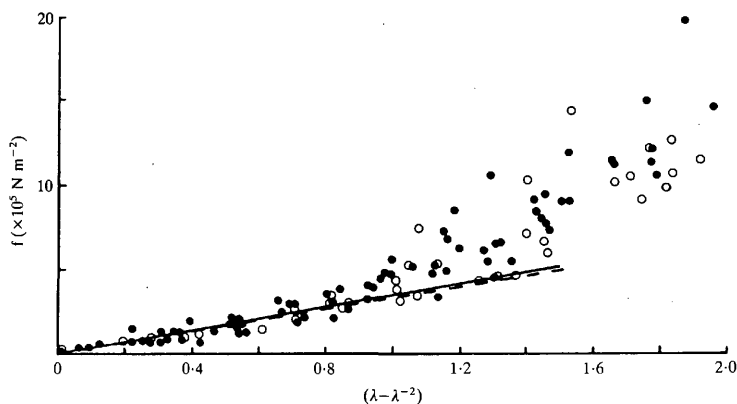
Fig. 2. Force-extension data from tests on eight samples of native elastic fibres (filled circles), and from tests on three samples of elastic fibres which had previously been exposed to formic acid for 72 h (open circles), both plotted according to equation 1. Linear regression analyses of data up to $(\lambda - \lambda^{-2}) = 1\cdot05$ gave slopes of $3\cdot44 \times 10^5\,\mathrm{N\,m^{-2}}$ for the native fibres (solid line), and $3\cdot42 \times 10^5\,\mathrm{N\,m^{-2}}$ for formic acid treated fibres (broken line). Correlation coefficients were $r = 0\cdot86$ for the former and $r = 0\cdot98$ for the latter.

Temperature-dependent swelling

The effect of temperature on the swollen length of unstrained elastic fibres is shown in Fig. 5. The data were normalized to the specimen length at 20 °C, the temperature at which the volume fraction was determined. The length of the fibre sample varied linearly with temperature between 0 °C and 35 °C, with a coefficient of linear expansion of $-10^{-3}/°C$. The thermoelastic measurements were made over this temperature range. Above 35 °C there appeared to be a greater rate of decrease in sample length. The reason for this shift in the swelling curve above 35 °C is not yet understood.

Thermoelastic tests

Fig. 6 shows that the force required to hold a fibre sample at constant length increased linearly with temperature, and that the slopes of the force-length plots (curves A, B, C) increased with length. In addition, Fig. 6 gives regression lines for the data when corrected from constant length to constant extension ratio (curves A', B', C'). The difference between these two sets of curves is explained below.

Fig. 5 shows that with increasing temperature the unstrained fibre length decreases. Therefore, when a fibre sample is held at a constant extended length while the temperature is increased, λ will increase due to decreased swelling. To obtain force-temperature curves at constant λ the following procedure was used.
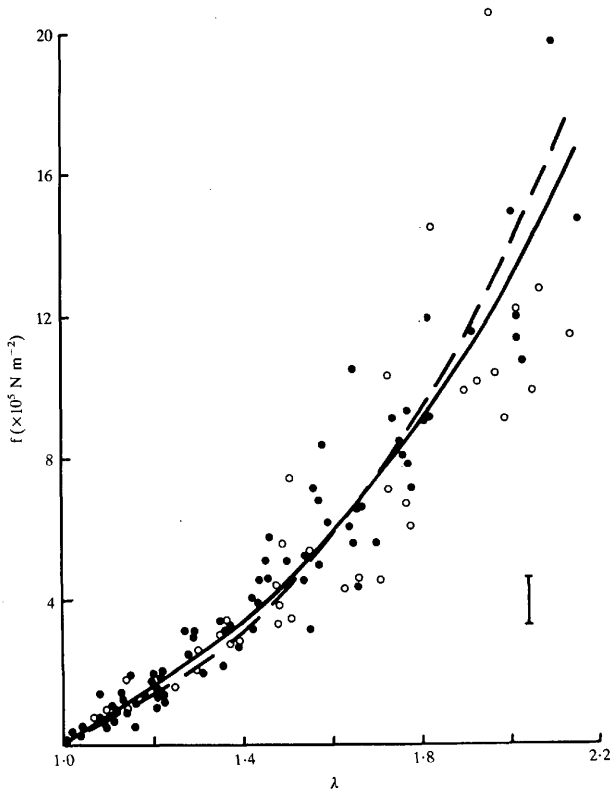
Fig. 3. Data from Fig. 2 plotted as force (f) *vs* extension ratio (λ), along with best fit polynomial regression curves. Filled circles and solid line are for native elastic fibres; open circles and broken line are for formic acid treated fibres. The vertical bar is one standard deviation for the polynomial function (solid curve).

Force-length isotherms in Fig. 7 were plotted from the uncorrected regression lines in Fig. 6; the abscissa is normalized to the unstrained length at 20 °C. The zero-force lengths at other temperatures were determined from the swelling data (Fig. 5). Based on these starting dimensions, lines of constant λ were plotted across the isotherms in Fig. 7. Now the force-temperature curves for constant λ can be drawn, and these are shown in Fig. 6. In principle, this correction method reduces the slope of the force-temperature curve by eliminating the force increase with temperature that arises due to the decrease in swollen dimensions of the protein.
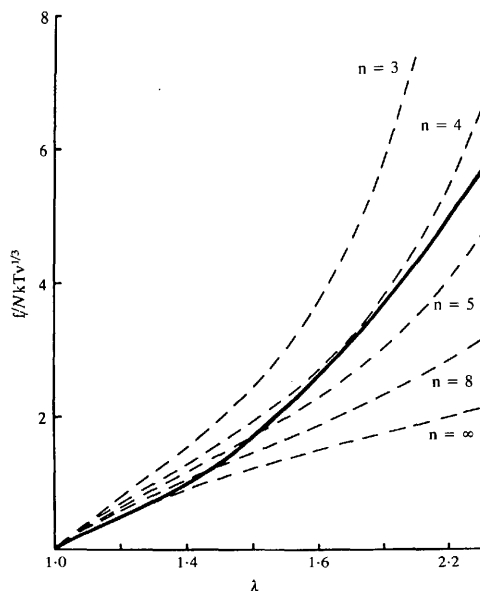
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Fig. 4. Non-Gaussian properties of the octopus elastic protein. The force-extension curve for native fibres from Fig. 3 (solid line) is compared to plots of the non-Gaussian model for rubber networks with different values of n, the number of random links per molecular chain between crosslinks (Treloar, 1975). When $n = \infty$, the curve describes the Gaussian model (equation 1). The ordinate is normalized to the force per chain ($f/NkTv^{1/3}$).

The linearity of the force-temperature curves allows us to proceed with the thermodynamic analysis of the constant length data according to equation 5, and of the constant λ data according to equation 8. The results are given in Table 2 and plotted in Fig. 8 for $T = 20\,°C$. When uncorrected for swelling changes, the extension of the elastic fibres appears to be associated with a large negative enthalpy change, $(\partial H/\partial L)_{T,P}$, and at the same time a large decrease in entropy, $-T(\partial S/\partial L)_{T,P}$. However, when the force-temperature data are analysed in terms of constant λ (swelling correction), the internal energy change, $(\partial U/\partial L)_{T,V}$, is much less than the enthalpy change. In fact $(\partial U/\partial L)_{T,V}$ is close to zero at low extensions, as predicted by the kinetic theory for an ideal rubber. The slope of the constant λ force-temperature curve represents the decrease in conformational entropy that results from deformation of the molecular network at constant volume. This change in entropy accounts for almost all of the elastic force at extensions up to $\lambda = 1·42$, and for 77 % of the force at $\lambda = 1·62$ (Table 2).

DISCUSSION

This study presents evidence that the elastic fibres in the wall of the octopus aorta are composed of a rubber-like protein. This protein, which we have called the
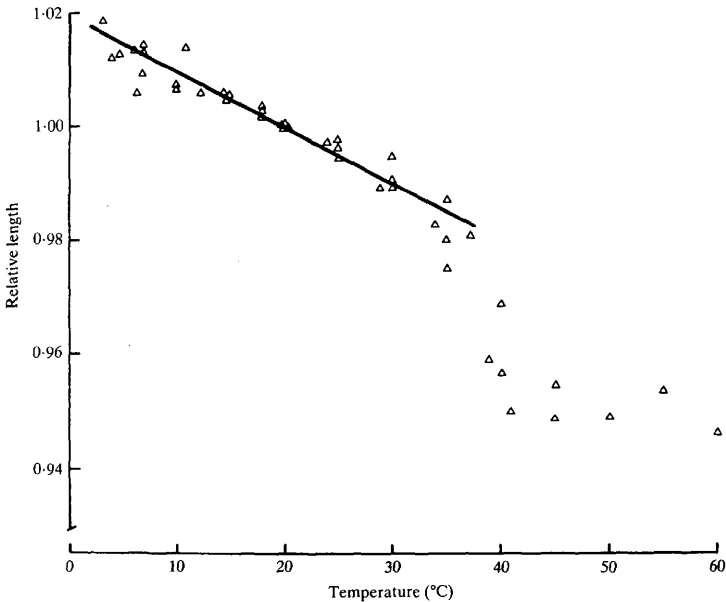
Fig. 5. The effect of temperature on the water-swollen length of unstretched elastic fibres, relative to the length at 20 °C. Data from three samples. Regression line calculated for temperatures between 0 and 35 °C is shown.

octopus arterial elastomer (OAE), appears to have physical and chemical properties that can be described by the kinetic theory of rubber elasticity. Like other protein rubbers and lightly crosslinked synthetic rubbers the OAE protein has a relatively low elastic modulus and high extensibility. These properties, along with the high degree of insolubility, are consistent with the model of a crosslinked network of thermally agitated, flexible, random-coil molecules. The network is hydrated under physiological conditions, with the volume fraction of protein in a swollen sample, $v = 0.40$ at 20 °C. For elastin, which is more hydrophobic, $v = 0.55$ at 20 °C (Gosline, 1978b). Resilin and abductin are also water-swollen *in vivo*, with v equal to about 0.45 and 0.50 respectively (Weis-Fogh, 1961b; Alexander, 1966). The OAE fibres become brittle and glassy when dehydrated. To have rubber-like properties the network chains must be kinetically free and have random configurations. Without water, or some other polar solvent, protein chains will fold into a fixed conformation, held by intra-molecular hydrogen bonds. Presumably, water disrupts these hydrogen bonds and imparts kinetic freedom to the network (Gosline, 1976, 1978a).

Random conformation in the OAE molecular network is suggested by the observation that the fibres are non-birefringent when unstressed (Shadwick &
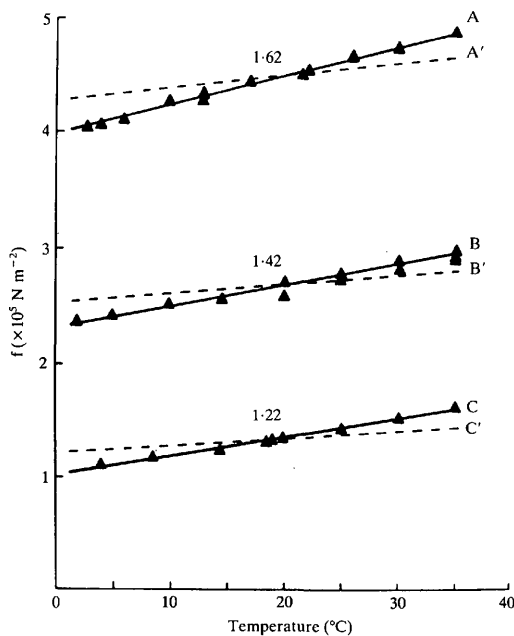
Fig. 6. Force-temperature plots. Force was measured in an elastic fibre sample held at constant length while the temperature was varied. Regression lines are drawn through data points for experiments at lengths of (A) 1·62, (B) 1·42, (C) 1·22 units relative to the unstretched length at 20°C (solid lines). Regression lines are shown for the same data after correction to constant extension ratios of (A') 1·62, (B') 1·42, (C') 1·22 (broken lines).

Gosline, 1981), in contrast to the large intrinsic birefringence of highly ordered protein molecules such as collagen fibres (Aaron & Gosline, 1980). When stretched to large extensions the OAE fibres become highly birefringent, as do other protein rubbers, presumably because deformation imposes a degree of order upon the network chains, and this gives rise to optical anisotropy (Gosline, 1980). We observed that the birefringence increased with extension of the OAE fibres, but a more detailed study is needed to determine the exact stress-birefringence relation in this protein. That the molecules of the OAE protein are held together by stable covalent crosslinks is shown by the long-range reversible elastic properties (i.e. there was virtually no creep in extended samples) and by the insolubility of the fibres in protein solvents such as formic acid and guanidine HCl.

From the elastic modulus G, it is possible to estimate the average molecular weight of the chains between crosslinks, using equation 2. For the OAE protein M_c is 6900. In theory, this equation relates to a network in which four chains meet at each junction point (Treloar, 1975). Imperfections such as loose ends will
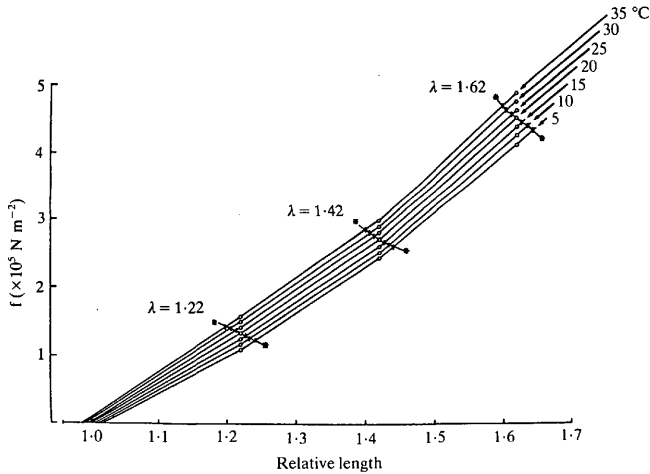
Fig. 7. Force-length isotherms for elastic fibres. Force values were taken from the regression lines A, B, C of Fig. 6 at temperatures of 5, 10, 15, 20, 25, 30, 35 °C and plotted (open circles) as a function of length relative to the unstretched length at 20 °C. Based on swelling data from Fig. 5, lines of constant extension ratio λ were drawn across the isotherms (*—*). Force-extension data were taken from the points of intersection of the isotherms and the lines of constant λ, and plotted as regression lines A', B', C' in Fig. 6.

contribute to an overestimate of M_c. A correction for equation 2 has been developed (Treloar, 1975):

$$G = (\varrho RT/M_c)(1-2M_c/M), \qquad (9)$$

where M is the molecular weight of the uncrosslinked precursor molecule. Nothing is known about the precursor of the OAE protein, but based on evidence for elastin and resilin (Sandberg, 1976; Weis-Fogh, 1961*b*) it is reasonable, as a first approximation, to estimate M at about 10^5. The 'corrected' value of M_c from equation 9 would be 6100. We may expect, therefore, that the molecular weight between crosslinks in OAE is approximately 6100 to 6900. Since the average amino acid residue weight for the protein is 110 this means that each chain between

Table 2. *Thermoelastic data at 20 °C for three different specimen lengths*

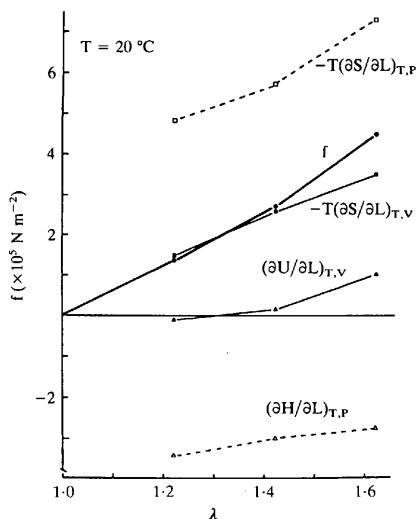| Extension (L or λ)	Force ($\times 10^5$ N m^{-2})	Enthalpy $(\partial H/\partial L)_{T,P}$	Entropy $-T(\partial S/\partial L)_{T,P}$	Energy $(\partial U/\partial L)_{T,V}$	Entropy $-T(\partial S/\partial L)_{T,V}$	Energy/Force
1·22	1·34	−3·47	4·81	−0·12	1·46	−0·09
1·42	2·69	−3·02	5·71	0·12	2·57	0·05
1·62	4·49	−2·84	7·33	1·02	3·47	0·23

Fig. 8. A force-extension plot for elastic fibres at 20 °C, resolved into entropy and enthalpy components according to equation 3 (broken lines). Calculated changes in entropy and internal energy for deformation at constant volume according to equation 6 are also shown (solid lines).

crosslinks will average 55 to 63 amino acids in length. Now, from the non-Gaussian analysis (Fig. 4), we estimate that about five random links make up each chain and, therefore, that 11 to 12 amino acids are necessary to give one functional random link, and since each amino acid provides two single bonds of rotation, the random link in the octopus protein requires about 23 rotational bonds. This suggests that the flexibility of the protein molecules is fairly restricted, but does not imply that the conformation is non-random. Rather, as shown by Fig. 2, ideal deformation of the network is limited to small extensions. This network property probably results from the high proportion of amino acids with large side groups (e.g. phenylalanine, tyrosine, aspartic acid, glutamic acid) which cause steric hindrances in bond rotation (Dickerson & Geis, 1969). In contrast to OAE, natural polyisoprene rubber, with $M = 8310$ has about 75 functional links per chain, and each link corresponds to only 1·6 isoprene residues, or 4·8 bonds with rotation (Treloar, 1975). Other protein-rubber networks are also much less flexible than isoprene rubber, but slightly more flexible than the OAE molecule. This characteristic is reflected in differences in the average amino acid residue weight, the number of functional links per chain, and the number of amino acids per link. For elastin the average residue weight is 85; there are approximately 10 functional links per chain and 8 amino acids per link (Aaron & Gosline, 1981). For resilin the average residue weight is 89; there are about 7 links per chain and 9 amino acids per link (Weis-Fogh, 1961b). Differences in network flexibility of these proteins can be seen by comparing their

force-extension curves (Fig. 9). The OAE shows the greatest deviation from the Gaussian curve, while the more flexible elastin molecule becomes non-Gaussian at slightly higher extension than the other protein rubbers.

The thermodynamic analysis of the OAE protein, tested in water as an open system, shows that the elastic force has a large negative enthalpy component (Fig. 8). In fact, at low extensions the enthalpy change is more than twice as great as the total force (Table 2). Consequently, the decrease in entropy is also much larger than the force, in order to satisfy equation 3. A similar result was obtained for hydrated elastin tested as an open system (Meyer & Ferri, 1936). This result was incorrectly attributed to strain-induced crystallization, a phenomenon which occurs in natural rubber at very high extensions (Treloar, 1975). It is now known that the very large enthalpy change is a direct result of swelling changes that occur in the elastin molecule. Specifically, this involves changes in enthalpy associated with mixing of water and non-polar side groups in the protein (Gosline, 1976). The large negative enthalpy term observed for the OAE fibres in this study may be attributed, for the most part, to changes in temperature-dependent network swelling.

When the thermoelastic data are corrected to constant volume, the internal energy change accompanying extension is reduced to nearly zero and the elastic force is predominantly due to changes in the conformational entropy of the system, as predicted by the kinetic theory (Fig. 8 and Table 2). However, $(\partial U/\partial L)_{T,V}$ at $\lambda = 1\cdot62$ is not zero, but actually represents 23 % of the total force. At high extensions there may actually be a small positive internal energy contribution to the elastic force due to interactions between chains. On the other hand, $(\partial U/\partial L)_{T,V}$ may be essentially zero, and the result for $\lambda = 1\cdot62$ may represent an over-correction of the constant length data. The temperature swelling relationship for the elastic fibres has been studied only for samples under no tensile load (Fig. 5). The degree of
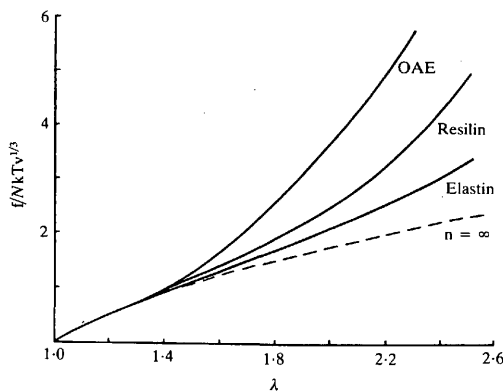

Fig. 9. Force-extension curves for rubber-like proteins, plotted as force per chain ($f/NkTv^{1/3}$) against extension ratio (λ). The theoretical curve for a Gaussian network is shown by the broken line. (Data for elastin from Aaron & Gosline, 1981; data for resilin from Weis-Fogh, 1961b.)

swelling of a polymeric molecule is inversely proportional to the network stiffness (Flory, 1953). Since the OAE protein is in the non-Gaussian region at $\lambda = 1\cdot62$ the network stiffness has effectively increased, and therefore one might expect the magnitude of the swelling change with temperature to be less. The swelling correction that was applied is probably quite valid for extensions in the Gaussian region but may be too large at extensions where the network becomes stiffer and non-Gaussian; thus $(\partial U/\partial L)_{T,V}$ may have been overestimated at the high extension.

The isolation and characterization of the OAE in this study is a significant addition to our fairly limited knowledge of an unusual group of molecules, the protein rubbers. The tremendous variation in amino acid composition among the four known protein rubbers (Table 1) suggests to us that each protein arose independently during evolution in response to selection for similar mechanical design. The intriguing question of what type of sequence patterns allow these special proteins to exist as random, kinetically-free chains is still unanswered. In contrast, most proteins have stable conformations which arise uniquely from each amino acid sequence. Based on the compositions of elastin, resilin and abductin it was suggested that a large proportion of the small amino acids (glycine, alanine and serine) may be necessary in order to minimize steric hindrances and permit a kinetically-free network to exist under normal physiological conditions (Gosline, 1980). Table 1 shows that this is not the case for the OAE protein, where glycine, alanine and serine together make up only 23 % of the composition, compared with 57–74 % in the other protein rubbers.

An alternative strategy in 'designing' a protein rubber might be to have a high proportion of charged amino acids (aspartic and glutamic acids, arginine and lysine) which prevent the formation of stable secondary structures. The charged amino acids account for 33 % of the OAE protein. The large side groups on the charged amino acids might also hinder the flexibility of the molecular chains, as mentioned above. However, a relatively inflexible network which becomes non-Gaussian at low extensions may be a useful characteristic for a protein rubber that provides elastic stability to the wall of a blood vessel. The reasons for this are discussed in the following paper, a detailed analysis of the mechanical properties of the octopus aorta.

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