Elasticity of the Fibrous Muscle Proteins*

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Laki and Bowen observed that glycerinated muscle contracts in potassium iodide solutions. We have investigated the underlying mechanism of this process by thermoelastic analysis. Reversible stress-temperature measurements were performed on muscle samples crosslinked with *p*-benzoquinone. After maximum contraction in potassium iodide solutions to about 60% of their resting lengths these samples are rubber-like, representative of the amorphous state. After partial contraction in the same medium, however, the samples are partly crystalline and partly amorphous. The strongly negative enthalpy-length coefficient is a consequence of oriented crystallization accompanying stretching. The mechanism of contraction of these samples is a phase transition from oriented, crystalline to randomly coiled, amorphous protein.

Although muscle action has been investigated extensively, the molecular basis of its mechanism is still unknown. A great obstacle in studying the underlying principle is the complexity of processes involved. It is known that contraction is triggered by electrical impulses from nerve endings. Furthermore, enzyme reactions occur whereby adenosine triphosphate (ATP) is dephosphorylated to adenosine diphosphate (ADP), which in its turn may be rephosphorylated in the reverse reaction. This cycle is supposedly coupled with contraction and relaxation. However, not even the molecular mechanism of any of these events is understood, let alone their interrelations. Under these circumstances it seems advisable to focus attention on one aspect at a time, rather than to attempt grasping muscle action in its entirety.

In this paper it is our object to study the elastic properties of the fibrous proteins of muscle, unattended by enzyme reactions. Of course, the molecular mechanism of elastic deformation, and in particular contraction, may then differ from that *in vivo*. However, a knowledge of muscle contractility, even when not under physiologic conditions, may provide a clue to better understanding of the basic processes occurring *in vivo*.

Thermodynamic analysis of elasticity measurements has been fruitful for a number of polymers, including fibrous proteins. If reversible tension-temperature measurements are performed at constant length, the tension may be resolved into its entropy and energy contributions, given by $T(\partial f/\partial T)_L$ and $(\partial E/\partial L)_T$ respectively. It follows from straightforward thermodynamics (see for example Flory, 1953) that

$$f = (\partial E/\partial L)_{V,T} + T(\partial f/\partial T)_{V,L} \qquad (1)$$

and

$$f = (\partial H/\partial L)_{p,T} + T(\partial f/\partial T)_{p,L} \qquad (2)$$

where f is the retractive force and E, H, V, L, p, and T are, respectively, the internal energy, enthalpy, volume, length, pressure, and absolute temperature of the sample. Since the term $p(\partial V/\partial L)_{p,T}$ is usually small at atmospheric pressure, we may write to a good approximation $(\partial H/\partial L)_{p,T} \simeq (\partial L/\partial L)_{p,T}$. In general, different results are obtained when the force is decomposed into its components according to equations (1) and (2), corresponding to different constraints.

Since the deformation mechanism for rubber-like polymers is now rather well understood, it is desirable to investigate first whether the elastic properties of the sample are rubber-like, characteristic of amorphous polymers. It is then of primary importance to obtain the value of $(\partial E/\partial L)_{V,T}$, abbreviated as f_e. If the magnitude of f_e/f is small, rubber elasticity is virtually assured and hence the molecular mechanism of deformation is at once clear. On application of tension the distribution of conformations along the chains shifts, resulting in elongation of the sample. Entropy changes accompanying this redistribution are considerable; if, however, the energy differences between conformations are small, as is true in most cases, redistribution involves only small energy changes.

In order to obtain f_e, corrections have to be applied to values of $(\partial E/\partial L)_{p,T}$ obtainable from stress-temperature measurements at constant pressure and application of equation (2). In general these corrections cannot easily be evaluated. For amorphous polymers, however, they may be obtained with the equations for rubber elasticity. When amorphous polymers are immersed in diluents and swelling equilibrium is established, it is advantageous to use a solvent or solvent mixture in which the volume of the unstrained sample remains constant with temperature. As has been shown previously (Hoeve and Flory, 1958, 1962) f_e/f values may then be obtained without correction according to equations (1) and (2). It is true that this procedure is valid for amorphous polymers only; for other samples errors may result. We may conclude, however, that if f_e/f values obtained in this manner are found to be small in magnitude, the procedure is consistent and rubber elasticity is indicated. With this method we found (Hoeve and Flory, 1958) that $f_e/f = 0$ for elastin, indicating that the retractive force is of purely entropic origin; in view of the above considerations the molecular mechanism of deformation must then be the same as that for cross-linked natural rubber. In contradistinction, the deformation mechanism may be completely different for partly crystalline polymers, as explained by Flory (1956a,b, 1957). In accordance with this mechanism the values found for $(\partial E/\partial L)_{p,T}$ are strongly negative for partly shrunken collagen (Oth *et al.*, 1957). Even when corrections are applied for alterations in degree of swelling, large negative values are obtained for the change in energy associated with elongation of the sample. In this case oriented crystallization of amorphous chains occurs on extension, accompanied by a decrease in energy; alternatively, the transition from an oriented, crystalline state to an amorphous state underlies contraction (Flory, 1956a,b, 1957). In view of the successful interpretation of these mechanisms we have applied a similar analysis to muscle samples.

We used rabbit muscle (*psoas*) extracted with glycerol-water mixtures in our experiments (Szent-Györgyi, 1951). The activation mechanism is destroyed by this treatment, and salts, globular proteins, and nucleotides are extracted, leaving intact the fibrous proteins. Un-

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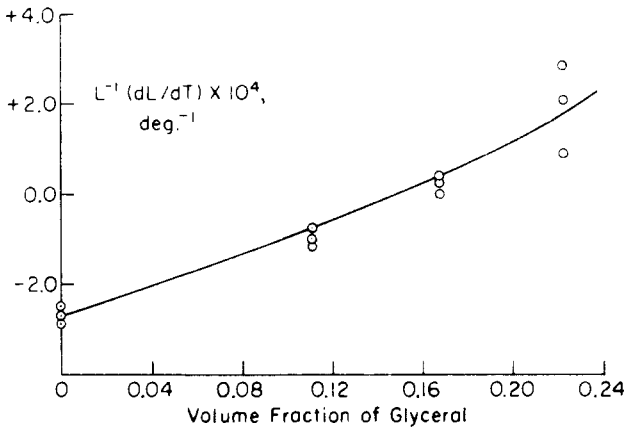

FIG. 1.—Length-temperature coefficients for muscle samples in glycerol-water mixtures of various compositions; the concentration of potassium iodide is 2 M throughout.

fortunately, such samples do not display the characteristic elasticity of the native state. They may be plasticized and contracted, however, by immersion in potassium iodide solutions as described by Laki and Bowen (1955) and Bowen and Laki (1956). Just as in collagen samples, relatively stable crosslinks are required, since otherwise the fiber bundles are unable to sustain an equilibrium force on extension. Whereas mercuric ions aid contraction, presumably by introduction of crosslinks, for reversible thermoelastic measurements more stable crosslinks are required; they were provided by reaction with *p*-benzoquinone. Stress-temperature measurements were performed on the cross-linked samples immersed in potassium iodide solutions in a mixture of glycerol and water, so chosen that the volume of swelling was independent of temperature.

Experimental

Rabbit muscle (*psoas*), glycerinated at its resting length according to the method of Szent-Györgyi (1951), was cross-linked by immersion in a 0.1% solution of *p*-benzoquinone in water at room temperature for about 4 hours. Bundles of approximately 6 cm length and 0.3–0.5 cm width were contracted in a 2 M solution of potassium iodide in water at 80°. Maximum shrinkage, reached after 2 days, was to about 60% of the original length. Length-temperature coefficients of the shrunken samples were determined in 2 M potassium iodide solutions in water-glycerol mixtures of various compositions from 20° to 85°. Length readings performed with a cathetometer were reversible.

In order to perform the stress-temperature measurements, the samples were clamped at each end between two stainless steel plates held together by screws. The lower clamp was fixed, whereas the upper clamp was suspended from a strain gauge which could be adjusted vertically. The strain gauge (Statham Instrument Company, Transducer Model G-1) had a capacity of 750 g and a linear response of 0.05 mv per g under a supplied e.m.f. of 12 v. The output of the strain gauge was displayed on a Leeds and Northrup recorder giving a full scale deflection of 10 mv. The instrument was calibrated by addition of known weights.

Potassium iodide solutions, 2 M, in a mixture of 85% water and 15% glycerol by volume were used throughout the stress-temperature measurements. The shrunken samples were extended at 80° to a maximum extension ratio of 1.5 relative to the shrunken length. Subsequently the length was fixed and stress relaxation was permitted to occur for about one day; thereafter the force was essentially constant. After the force was recorded, the temperature was lowered to 20° and the force was measured again without further waiting. When the temperature was raised to 80° the same value was obtained for the force as at the first recording, thus assuring reversibility.

In a similar set of experiments cross-linked muscle samples were allowed to shrink partially to predetermined, fixed lengths. After one day at 70° the tension was constant and reversible measurements were performed as described above between 20° and 70°.

Electron micrographs were obtained by M. D. Maser at Mellon Institute. Cross-linked muscle was blended and stained with a 1% solution of phosphotungstic acid in water before examination under the electron microscope. Samples contracted in potassium iodide solutions were blended and stained in this solution. Subsequently the potassium iodide was removed by washing before electron micrographs were obtained.

Results and Discussion

It is well known that elevation of temperature and treatment with strong salt solutions promote melting in originally crystalline, fibrous proteins. In accordance with expectation, the double refraction of the cross-linked fiber bundles diminishes markedly on immersion in 2 M potassium iodide solutions at 80° and has practically disappeared at maximum shrinkage. Rubber elasticity of the samples is then anticipated, and accordingly we used the procedure described in the introduction to obtain values of f_e/f characteristic for the elastic properties of the protein chains.

It was first necessary to find a solvent or solvent mixture in which the volume of the protein fibers remains constant with temperature (Hoeve and Flory, 1958, 1962). For this purpose length-temperature measurements were performed on the unstrained, shrunken samples in aqueous glycerol solutions; the results are given in Figure 1. We see that length-temperature coefficients for 2 M potassium iodide solutions in water were negative; however, positive values were observed for mixtures rich in glycerol. For a mixture of 15% glycerol in water by volume the length-temperature coefficient was approximately zero, indicating that the volume of the protein fibers remained approximately constant with temperature in this solution. Consequently all tension-temperature measurements were performed in a mixture of 15% glycerol and 85% water by volume.

Results of stress-temperature measurements are given in Table I; they refer to samples which were first contracted without application of a force in 2 M potassium iodide solutions and then extended to elongation ratios α, given in the first column. α is measured relative to the length reached after contraction, which is roughly

TABLE I

STRESS-TEMPERATURE RESULTS FOR MUSCLE SAMPLES AFTER MAXIMUM CONTRACTION

Protein mass per unit length of uncontracted muscle samples is approximately 10^{-2} g cm.$^{-1}$

α	$<f>$, g	$(\partial f/\partial T)_{L,p}$, g deg.$^{-1}$	$f^{-1}(\partial E/\partial L)_{p,T}$
1.35	37.8	0.100	0.13
1.24	13.4	0.0284	0.31
1.44	39.1	0.080	0.33
1.65	59.5	0.133	0.27
1.64	50.5	0.124	0.18
1.64	40.1	0.109	0.12

60% of the resting length (see under Experimental). $< f >$, given in the second column, denotes the average force measured over the temperature range investigated. Values of the force-temperature coefficients are given in the third column, and, finally, the values of $f^{-1}\ (\partial E/\partial L)_{p,T}$, calculated according to equation (2), are given in the last column. After extension, the bundles were up to 10% longer than before extension. A similar effect is observed for cross-linked natural rubber, and is referred to as "set," but the elongation was more pronounced in the present case. Possibly some decomposition of proteins or irreversible changes in morphology occurred in the course of these prolonged experiments. Whatever the cause, the force-temperature measurements at constant length were reversible under the conditions of our experiments. The values of $f^{-1}\ (\partial E/\partial L)_{p,T}$ in the last column of Table I range from 0.1 to 0.3, indicating that the retractive force is largely of entropic origin. The small magnitude of $f^{-1}\ (\partial E/\partial L)_{p,T}$ for this glycerol-water mixture indicates rubber-like elasticity of the samples, in accordance with expectation. In this case $f^{-1}\ (\partial E/\partial L)_{p,T}$ may be equated to f_e/f. There can be little doubt that the proteins in the shrunken muscle samples are amorphous and that deformation occurs according to the same mechanism as that for cross-linked natural rubber. Although deviations of f_e/f values from zero may occur for amorphous networks, as has been shown before (Flory *et al.*, 1960), the relatively small values found here could be due to experimental errors in the length-temperature measurements (Fig. 1) resulting in slight volume changes with temperature in the solvent mixture chosen.

The results for fiber bundles allowed to shrink to predetermined fixed lengths in the same solvent mixture are given in Table II. The ratio, R, of the length of the partially shrunken fibers to the rest length, is given in the first column. The values of $f^{-1}\ (\partial E/\partial L)_{p,T}$ are strongly negative, ranging from -3 to -7, in sharp contrast with the values for fibers shrunken in the absence of constraints, given in Table I. These results are in harmony with the assumption that the sample is partly crystalline. According to Flory (1956a,b, 1957), preventing the sample from shrinking by fixation of the length restricts melting, and therefore the sample remains partly crystalline. Increase and decrease in temperature at constant length induce, respectively, melting and crystallization. Alternatively, elongation of the sample at constant temperature is accompanied by crystallization, resulting in large negative values of the energy contribution to the force, $f^{-1}\ (\partial E/\partial L)_{p,T}$. Of course, the entropy contribution, $f^{-1}T(\partial f/\partial T)_{p,L}$, must then be positive and even larger in magnitude in order to account for a positive retractive force. Since the volume of the shrunken fibers is approximately constant with temperature, the heat of swelling should be small. The values found for $f^{-1}\ (\partial E/\partial L)_{p,T}$ in Table II are therefore directly related to the heat of fusion. Unfortunately, the amount of crystallinity at the beginning of the experiment is unknown, precluding meaningful quantitative conclusions.

The muscle fibers contracted without constraints showed no tendency to return to the oriented, crystalline state upon cooling. This result might appear to be at variance with the capacity of the partially contracted fibers to recrystallize as indicated by the thermoelastic measurements. However, it is to be recognized that residual crystalline regions in the partly shrunken fibers provide interfaces for growth of the crystalline phase upon cooling. Crystalline nuclei with the original orientation are required for observable *oriented* recrystallization. When random crystallization occurs in unoriented fashion the length of the macroscopic sample does not change appreciably. These facts are in keeping with observations in other fibrous polymer systems.

TABLE II

STRESS-TEMPERATURE RESULTS FOR MUSCLE SAMPLES ALLOWED TO CONTRACT TO THE RATIO R, RELATIVE TO THE ORIGINAL LENGTH

Protein mass per unit length of uncontracted muscle samples is approximately 10^{-2} g cm.$^{-1}$

R	$< f >$, g	$(\partial f/\partial T)_{L,p}$, g deg.$^{-1}$	$f^{-1}\ (\partial E/\partial L)_{p,T}$
0.978	44.2	0.725	−4.2
0.903	36.8	0.468	−3.2
0.802	32.1	0.436	−3.3
0.800	23.6	0.362	−3.9
0.700	26.1	0.636	−6.9
0.700	24.4	0.540	−6.1

The results given above demonstrate that the muscle proteins participating in contraction under the conditions of our experiments occur in the crystalline state prior to shrinkage, that melting is responsible for shrinkage, and that the resulting shrunken sample exhibits typical rubber-like elasticity.

Thermoelastic measurements performed by Woods (1946) on reconstituted, uncontracted muscle threads are not easily interpreted, owing to unknown contributions of changes in degree of swelling with temperature. The same difficulty exists in interpreting the results of Botts and Morales (1951) and Morales and Botts (1953), obtained for similar samples. It is true that Botts *et al.* (1951) have shown that $(\partial H/\partial L)_{p,T} \simeq (\partial E/\partial L)_{p,T}$ to a good approximation. But for investigating the internal energy changes of protein chains, the value of $f_e = (\partial E/\partial L)_{V,T}$ is required and hence their values of $(\partial E/\partial L)_{p,T}$ are in need of further correction. As a result of thermal expansion and changes in degree of swelling the volume of the polymer usually changes. The effects of these changes on the decomposition of the force into its components are most pronounced at small elongations. Botts and Morales (1951) found that the large (positive) entropy and (negative) energy contributions to the force at small elongations decreased in absolute value at higher elongations. This result is consistent with a negative value of $(\partial L/\partial T)_p$ at zero force. As in the cases of elastin (Hoeve and Flory, 1958), collagen (Oth *et al.*, 1957), and our muscle samples in aqueous potassium iodide solutions, the negative value is probably due to a negative swelling-temperature coefficient resulting from a negative energy of polymer-solvent interaction. Botts and Morales suggested that electrical charges attached to the chain would repel each other. On extension the energy would decrease as a result of the decreasing repulsion energy. As we see now, such an explanation is not required. The *negative* entropy and *positive* energy components reported by Woods (1946), supposedly for samples containing less water, may be understood if it is assumed that for these samples thermal expansion dominates the swelling contribution of opposite sign and that consequently the value of $(\partial L/\partial T)_p$ at zero force would be positive. The positive energy component found by Woods for these uncontracted samples is in keeping with expectation for crystalline muscle proteins.

It is interesting to study the morphologic changes occurring on muscle contraction in potassium iodide solutions. Electron micrographs of cross-linked and un-cross-linked glycerinated muscle were undistinguish-

able and showed clearly the Z-lines and A- and I-bands. The distance between Z-lines was about 2.5 μ, whereas the length of the A-band was approximately 1.5 μ. After contraction in potassium iodide solutions the bands were more vague; both the A-band and the Z-Z distance contracted to approximately half their original length. This result contrasts with the microscopic results (Huxley and Hanson, 1954; Huxley and Niedergerke, 1954) obtained with light optics for contraction under physiologic conditions, for which the A-band has been reported to remain constant in length, whereas the Z-Z distance decreases. However, the results of Knappeis and Carlsen (1956), obtained by electron microscopy of muscle contracted under physiologic conditions and staining techniques similar to ours, indicate contraction of the A-bands also. Of course, this is not to be construed as proof that the mechanism of contraction under our conditions is identical to that under physiologic conditions. However, in view of the demonstrated ability of muscle to contract as a result of the transition from an oriented, crystalline state to an amorphous phase, this mechanism deserves serious consideration for contraction under physiologic conditions (see also the following paper [Hoeve *et al.*, 1963]).

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Evidence for a Phase Transition in Muscle Contraction*

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The length of glycerinated muscle fibers in solutions of ATP in aqueous glycerol and ethylene glycol is measured as a function of water content and temperature. Abrupt contraction is observed with small changes in one of the variables while the other remains fixed; therefore, a phase change underlies contraction. Since such phase changes are not inherent in the mechanisms proposed by Riseman and Kirkwood, Morales and Botts, Kuhn *et al.*, and Huxley, these mechanisms, unless modified, are unsatisfactory. Among the models proposed by Hill, Pauling and Corey, and Flory, all assuming phase transitions, the last appears the most likely; in it a phase change from oriented, crystalline to random, amorphous protein is the basis for contraction. In accordance with Bowen's conclusions, our results show no correlation between ATP splitting and contraction.

It is well known that glycerinated muscle fibers can be made to contract in solutions of adenosine triphosphate (ATP). Furthermore, this substrate is enzymatically hydrolyzed into adenosine diphosphate or orthophosphate. The opinion is widespread that the free energy change in this reaction must drive muscle contraction *in vivo* as well as *in vitro*, although no clear *molecular* mechanism has been advanced for this mechanochemical coupling and experimental evidence in its favor is lacking. Several suggestions have been made for the molecular mechanism underlying contraction. To facilitate discussion the most important models are here summarized.

Astbury (1947) supposed, in analogy to supercontraction in wool, that muscle contraction is accompanied by a change from a crystalline α-protein, oriented parallel to the fiber axis, to the cross-β state, partly oriented perpendicular to this direction. On the other hand, Pauling and Corey (1951) proposed a phase transition between two crystalline states: the extended pleated sheet form and the shorter α-helical form.

Riseman and Kirkwood (1948) supposed that the high charge of adsorbed ATP keeps the amorphous muscle proteins in the extended state. As a consequence of ATP splitting the charge would be reduced, resulting in contraction of the proteins; however, contraction should not in this case be accompanied by a phase change. These ideas were modified and elaborated by Morales and Botts (1953) and later by Morales *et al.* (1955). Kuhn *et al.* (1960) showed the feasibility of these models by inducing contractions and elongations in polyacrylic acid samples by changing the *p*H. In this case the electrical interactions are short-range and may be treated as Donnan effects.

Hill (1953) described several possible contractile systems, including models in which separation into two liquid phases occurs. By imposing geometrical restrictions on the polymer phases, he constructed theoretical

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