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# Equilibrium Mechanochemistry of Collagen Fibers*

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ABSTRACT: The isothermal shrinkage of a fiber under constant tensile force by varying the concentration of a component of the surrounding medium has been demonstrated previously. The thermodynamic corollary of this process states that the extent of chemical interaction between the fiber and such a component should be altered by applying force to the fiber. The interaction between collagen fibers and the denaturant LiBr, its variation with tensile force or relative length of the fiber, and the analysis of the mechanochemical properties of this system in terms of a two-state model are the subjects of this report. The experimental procedure included (1) preliminary exposure of the fibers to extremes of denaturant concentration and force to enhance reversibility of measured effects, (2) mercurimetric titration of LiBr, (3) use of a mechanochemically inert tracer [$^{14}$C]glycerol in evaluating excess LiBr in the fiber phase relative to the external solution (enrichment), and (4) correlation of length and enrichment measurements for individual fibers. The variations of fiber length with tensile force and denaturant activity were semiquantitatively consistent with computations according to the two-state model of Hill. In solutions of constant denaturant activity, the measured enrichment decreased linearly with fiber length; in 5 M LiBr the molar ratio of excess LiBr:amino acid residue varied from 1:4 in relaxed fibers to 1:7 in fully stretched fibers. The relationship between the sigmoidal force dependence of the enrichment and the sigmoidal dependence of fiber length upon denaturant activity fulfilled the thermodynamic predictions.

Conformational changes of fibrous macromolecules, accompanying the reversible interaction with specific reagents, provide a general mechanism for the isothermal conversion of chemical energy into mechanical work (Katchalsky *et al.*, 1960). A well-documented example for such conversion is the contractility of collagen fibers, which shrink appreciably by reversible combination with several neutral salts, and which develop concomitantly large mechanical forces (Flory and Spurr, 1961; Yonath *et al.*, 1965; Puett *et al.*, 1965; von Hippel and Schleich, 1969). It was found that LiBr in particular causes a rapid chemical melting of collagen molecules, and that the forces developing during the process could be utilized for the operation of a mechanochemical engine (Steinberg *et al.*, 1966). The interaction with LiBr will serve as the basic example of the equilibrium studies of this paper.

On *a priori* grounds the isothermal contractile behavior of collagen fibers may be characterized by four parameters: by the length, $l$, and the force, $f$, by the chemical potential, $\mu$, of the reagent, and by the extent of reagent combination with the protein molecules. A full set of quantitative relations among these four parameters, whether empirical or theoretical, would constitute an "equation of state" for the collagen–salt system. Although for different fibrous proteins and salts, different numerical parameters would enter the equation of state, it is hoped that the basic form of the equation might hold for many systems; and hence the interest in evaluating the behavior of a reproducible, and well-defined example.

While the mechanical measurements of the dependence of force upon length are easily carried out, and may be readily interpreted by existing theories of rubberlike behavior, it has proven difficult to determine the binding of salt to collagen and to evaluate its dependence upon mechanical force. Ciferri *et al.* (1967) studied the selective interaction of [$^{14}$C]KCNS with collagen at different temperatures but did not correlate the binding with conformational changes under variable mechanical stresses. Oplatka and Yonath (1968) measured total KCNS and water uptake during the first denaturation cycle of strips of natural collagen. Since our aim was to obtain reversible data suitable for thermodynamic analysis, we used reconstituted collagen fibers which had undergone several contraction–expansion cycles, and developed a procedure to measure LiBr combination with the fiber under variable stresses. The method will be described in more detail in the experimental part.

A quantitative correlation between force and length in media of different salt concentrations was carried out by Flory and

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Spurr (1961). Recently Reich *et al.* (1968) showed that the stress–strain relations in collagen obey the Langevinian statistics of rubberlike molecules with finite macromolecular chains. The basic molecular parameter which characterizes the length of the statistical segments, however, changes with the concentration of the salt. An additional theoretical analysis is therefore required to correlate the extent of chemical reaction with mechanical behavior.

It was found that a useful approximation to a more complete equation of state is provided by a composite of the "autone" model as developed by Hill (1952) for fibrous proteins and the model for allosteric interactions in regulatory proteins, by Monod *et al.* (1965). In the model the real distribution of segments in the fiber, ranging from random coils to aligned collagen helices (von Hippel, 1967), is replaced by a linear array of inelastic units which may exist in only two states of different length. Interaction with a mechanochemically potent reagent, as well as the application of mechanical stress, changes the ratio of short to long units and thereby changes the macroscopic length of the fiber. Despite its simplicity, the model comprises both chemical reaction and mechanical response, and when combined with a thermodynamic treatment, is a useful guide in the correlation of the results presented below.

## Thermodynamic Considerations

*Reversible Contraction in a Binary Solution.* For the isolation of pertinent parameters and the evaluation of suitable criteria for the reversibility of the experimental data, it is advantageous to introduce several thermodynamic relations for a system composed of a contractile fiber and a bath of reagent. Several of the relations used below were derived previously by Katchalsky and Oplatka (1965).

The integrated equation of Gibbs for the contractile collagen fiber may be written as

$$U = TS - pV + fl + n_s\mu_s + n_w\mu_w + n_c\mu_c \qquad (1)$$

where the total energy, $U$, is given as a function of the regular parameters of state (absolute temperature, $T$, entropy, $S$, pressure, $p$, and volume, $V$). It is worth noting that for a very long and thin fiber, the stress–strain relations are represented by the compression term $-pV$ and the work of stretching, $fl$, due to the application of a force, $f$, to a fiber of length, $l$. It is assumed that only salt and water interact with the fiber and hence the corresponding chemical terms in the equation are the number of moles of salt, $n_s$, multiplied by the molar chemical potential of salt in the fiber, $\mu_s$, and an equivalent term, $n_w\mu_w$, representing the total energy of water in the fiber. The last term, $n_c\mu_c$, is contributed by the energy of the collagen matrix composed of $n_c$ moles with chemical potential, $\mu_c$. It will be noted that the differential equation of Gibbs

$$dU = TdS - pdV + fdl + \mu_s dn_s + \mu_w dn_w \qquad (2)$$

does not include a term for the collagen matrix, since $n_c$ is assumed to be constant.

Equation 1 may be used for the construction of several thermodynamic potentials from which a wealth of cross relations may be derived. We shall consider here two potentials $\psi_1$ and $\psi_2$ of the following structure

$$\psi_1 = U + pV - TS - n_s\mu_s - n_w\mu_w \qquad (3)$$

and

$$\psi_2 = U + pV - TS - fl - n_s\mu_s - n_w\mu_w \qquad (4)$$

Upon differentiating eq 3 and 4 and inserting $dU$ from eq 2 we obtain

$$d\psi_1 = -SdT + Vdp + fdl - n_s d\mu_s - n_w d\mu_w \qquad (5)$$

$$d\psi_2 = -SdT + Vdp - ldf - n_s d\mu_s - n_w d\mu_w \qquad (6)$$

It will be noted that for a reversible process, such as the equilibrium processes considered in this paper, $d\psi_1$ and $d\psi_2$ are total differentials. This conclusion will be important in the derivation of cross relations for experimental testing.

Since our experiments were carried out under isothermal and isobaric conditions, *i.e.*, at $dT$ and $dp = 0$

$$d\psi_1 = fdl - n_s d\mu_s - n_w d\mu_w \qquad (7)$$

and

$$d\psi_2 = -ldf - n_s d\mu_s - n_w d\mu_w \qquad (8)$$

We further require that our contractile fiber maintains an equilibrium with an external bath containing $n_s^0$ moles of salt and $n_w^0$ moles of water. The Gibbs–Duhem expression for the external bath for constant $p$ and $T$ is

$$n_s^0 d\mu_s + n_w^0 d\mu_w = 0 \qquad (9)$$

It should be noted that the maintenance of equilibrium under variable conditions implies that $d\mu_s$ and $d\mu_w$ in eq 7–9 have the same values. We may therefore evaluate $d\mu_w$ from eq 9 and insert it into eq 7 and 8 to obtain

$$\begin{aligned} d\psi_1 &= fdl - \epsilon_{s,w} d\mu_s \\ d\psi_2 &= -ldf - \epsilon_{s,w} d\mu_s \end{aligned} \qquad (10)$$

where

$$\epsilon_{s,w} = n_s - \frac{n_s^0}{n_w^0} n_w \qquad (11)$$

The term $\epsilon_{s,w}$ was denoted previously as the "salt enrichment" in the fiber (Yonath *et al.*, 1965). To clarify the meaning of the enrichment, we arbitrarily divide the total salt within the fiber into two categories: $n_s^b$ moles of "bound" salt and $n_s^f$ moles of "free" salt ($n_s = n_s^b + n_s^f$) and similarly the water content of the collagen fiber is written as $n_w = n_w^b + n_w^f$. It is further assumed that the ratio of free salt to free water within the fiber is the same as in the external solution, *i.e.*, $n_s^f/n_w^f = n_s^0/n_w^0$. Inserting these expressions into eq 11 we find that

$$\epsilon_{s,w} = (n_s^f + n_s^b) - \frac{n_s^f}{n_w^f}(n_w^f + n_w^b)$$

$$= n_s^b - \frac{n_s^0}{n_w^0} n_w^b$$

Thus, the enrichment factor measures the relative binding of salt *vs.* water. If no water is bound to the protein matrix ($n_w^b = 0$) or if the external ratio, $n_s^0/n_w^0$, is very small, $\epsilon_{s,w} \simeq n_s^b$. Whatever be the interpretation for $\epsilon_{s,w}$, it is a measurable quantity which may be used to test whether our experiments are reversible and obey equilibrium conditions.

The fact that $d\psi_1$ and $d\psi_2$ are total differentials leads to the following cross relations

$$\left(\frac{\partial \epsilon_{s,w}}{\partial l}\right)_{\mu_s} = -\left(\frac{\partial f}{\partial \mu_s}\right)_l$$
$$\left(\frac{\partial \epsilon_{s,w}}{\partial f}\right)_{\mu_s} = \left(\frac{\partial l}{\partial \mu_s}\right)_f \tag{12}$$

If eq 12 is rigorously obeyed the system is at equilibrium; these equations may therefore serve as criteria of reversibility. The application of these equations to collagen fibers will be discussed in more detail in the experimental part.

*Enrichment Measurements with an Inert Third Component.* While the determination of $\epsilon_{s,w}$ and its dependence upon either $l$ or $f$ is sufficient for the study of reversibility, it is desirable to determine in more direct manner the amount of bound salt, $n_s^b$, which is a parameter in the mechanochemical equation of state. For this purpose the following approximate procedure was adopted.

The collagen fibers were equilibrated with an aqueous salt solution which contained a very small number, $n_g^0$, of moles of radioactively labeled glycerol. Let the number of moles of glycerol within the fiber be $n_g$, we then construct a thermodynamic potential, $\psi_3$

$$\psi_3 = U + pV - TS - n_s\mu_s - n_w\mu_w - n_g\mu_g \tag{13}$$

of which the differential, at constant $p$ and $T$, is

$$d\psi_3 = f dl - n_s d\mu_s - n_w d\mu_w - n_g d\mu_g \tag{14}$$

The Gibbs–Duhem equation for the outer solution, for $p$, $T$ constant, is

$$n_s^0 d\mu_s + n_w^0 d\mu_w + n_g^0 d\mu_g = 0 \tag{15}$$

where the $d\mu_i$'s are the same in eq 14 and 15. Substituting $d\mu_g$ from eq 15 into eq 14 we obtain

$$d\psi_3 = f dl - \left(n_s - \frac{n_s^0}{n_g^0} n_g\right) d\mu_s - \left(n_w - \frac{n_w^0}{n_g^0} n_g\right) d\mu_w \tag{16}$$
$$= f dl - \epsilon_{s,g} d\mu_s - \epsilon_{w,g} d\mu_w$$

where

$$\epsilon_{s,g} = n_s - \frac{n_s^0}{n_g^0} n_g$$

and

$$\epsilon_{w,g} = n_w - \frac{n_w^0}{n_g^0} n_g \tag{17}$$

With the same approximations as considered above, the salt and water content of the fiber may each be described as the sum of bound and free components. Since the glycerol is highly diluted in these experiments, and much higher concentrations of glycerol do not shift the melting point of these fibers (see Experimental Section), the effect of bound glycerol on the contractile cycle may be neglected. Under these conditions the concentration of glycerol in the free water of the fiber should be the same as in the external water, so that $n_g/n_w^f = n_g^0/n_w^0$. Inserting these assumptions into eq 17, we find

$$\epsilon_{w,g} = n_w^b \tag{18}$$

Thus, the water enrichment relative to glycerol, $\epsilon_{w,g}$, measures the amount of bound water. Further, with the previous assumption that $n_s^f/n_w^f = n_s^0/n_w^0$, we find immediately that

$$\epsilon_{s,g} = n_s - \frac{n_s^0}{n_g^0} n_g = n_s^b \tag{19}$$

and hence the determination of salt enrichment, *vs.* glycerol, provides the amount of bound salt.

## Application of the Autone Model to the Collagen–Salt Interaction

*Description of the Model.* The autone model was proposed by Gergely and Laki in 1950 and expressed in quantitative form by Hill in 1952. In adapting this model to the molecular structure of collagen (see von Hippel, 1967), the real fiber is represented as a system of parallel triple-stranded molecular chains extending along the entire length of the fiber. The number of macromolecular chains is $N$, and each chain comprises $B$ statistical elements, as defined by Kuhn (1936, 1939). The elements may be in either a long state, corresponding to collagen helices or a short state, corresponding to molten, random coils. We assign an average length, $l_1$, to a long segment and $l_2$ to a short segment. Each statistical element is required to contain the minimum number of amino acid residues capable of forming a stable triple-stranded helical unit, independent of the state of other units along the same or adjacent molecular chains. The entire statistical unit of the model is assumed to lie between covalent cross-linking points within the real fiber. Each segment carries the same number, $n$, of sites which can combine with the ligand (here LiBr), with an association constant of $k_1$ or $k_2$ in the long or short state, respectively. The sites within a segment are independent, except that they undergo coordinated changes between the two states and hence also in the intrinsic affinity for the ligand (see Figure 1).

This molecular interpretation of the autone model differs in some fundamental aspects from the model for allosteric interactions in regulatory proteins, proposed by Monod *et al.* (1965). In the allosteric model each of the identical ligand binding sites is envisioned as a highly specific constellation of binding groups, contributed by a single protein subunit or protomer. The cooperative interactions of a molecule contain-

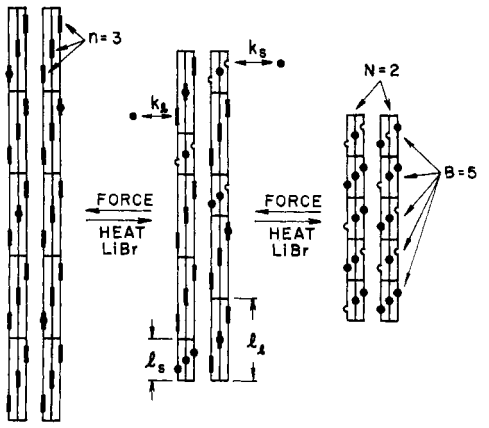


FIGURE 1: The autone model as applied to the shrinkage of collagen fibers. Each conformational unit can exist in a long state of length, $l_l$, where all the binding sites for LiBr (●) are in one form (bars), or in a short state of length, $l_s$, where the binding sites are in a different form (semicircles). The intrinsic association constants are $k_l$ and $k_s$, respectively, with $k_s > k_l$. The equilibrium can be shifted by force, heat, or LiBr concentration as shown. $N$ is the number of triple-stranded chains, $B$ is the number of conformational units per chain, and $n$ is the number of binding sites per conformational unit. The parameter values shown were chosen for simplicity and are unrealistic.

ing several subunits (an oligomer) are attributed to the conformational changes in all the subunits as a consequence of their packing in the macromolecule as a whole. On the other hand, in the autone model the interaction sites may consist simply of one or more peptide bonds, which may have a low but measurable affinity for a wide range of ligands. The number of such binding sites within a conformational unit is not necessarily equal to the number of subunits or polypeptide chains (three, for segments of a collagen helix) but is determined by the number of residues required to stabilize the native structure.

*The Role of Water.* A basic premise for the application of the autone model to these data is that the salt is the component of the solution which effects the transition from the long to short state of collagen. The assumption of a direct interaction between LiBr and collagen is supported by the demonstration of crystalline complexes of lithium halides with model amides (Bello *et al.*, 1966). There is no doubt that the combination with water plays an important role in the process as well, and no contraction of collagen fibers in anhydrous salt solutions has been detected (Sherebrin and Oplatka, 1968). It is likely that LiBr interacts with water to form hydrated species which are then bound by the collagen. Such interactions could distort the results for salt enrichment, $\epsilon_{s,g}$, if the tracer glycerol is excluded from the hydrates. It is also assumed in this simple treatment that any interaction of collagen with water makes no energetic contribution to the contractile cycle, *i.e.*, that the association constants of the long and short states with water are identical, and that the binding of water does not compete with the binding of salt.

*Length as a Function of Force and Salt Activity.* Hill's equation for the average length of a statistical unit may be written as

$$l = \frac{l_s(1 + k_s a)^n + l_l K e^{\lambda f}(1 + k_l a)^n}{(1 + k_s a)^n + K e^{\lambda f}(1 + k_l a)^n} \tag{20}$$

where $a$ is the activity of the ligand

$$a = e^{\mu_s/\kappa T}$$

$$\lambda = \frac{l_l - l_s}{\kappa T} \tag{21}$$

$\kappa$ is the Boltzmann constant and $K$ is the equilibrium constant for the conversion of short into long segments in the absence of salt ($a = 0$) and force ($f = 0$). Equation 20 may be cast in the simple form

$$ln \frac{l - l_s}{l_l - l} = lnK + \lambda f + n\, ln \frac{1 + k_l a}{1 + k_s a} \tag{22}$$

which is amenable to a straightforward experimental test.

In the data, the values of $l$ refer to the relative length of the fiber ($B$ times the length of a statistical unit, divided by the reference length, $l_0$) while the value of $f$ represents the force on the whole fiber ($N$ times the force per molecular chain). The empirical expression for $\lambda$ in $dyn^{-1}$ is therefore

$$\lambda = \frac{l_0}{NB} \frac{l_l - l_s}{\kappa T}$$

*Enrichment as a Function of Force (or Length) and Salt Activity.* The outcome of the thermodynamic consideration is taken for granted in this section: that measurements of salt enrichment relative to an inert tracer, $\epsilon_{s,g}$, permit the determination of bound salt as a function of mechanical stress and salt concentration in the external bath. Hill's (1952) derivations may then be extended to evaluate $\bar{Y}$ the fraction of association sites occupied by salt under given force and salt activity, analogous to the saturation function of Monod *et al.* (1965)

$$\bar{Y} = \frac{\epsilon_{s,g}}{\epsilon_{max}} = \frac{k_s a(1 + k_s a)^{n-1} + K e^{\lambda f} k_l a(1 + k_l a)^{n-1}}{(1 + k_s a)^n + K e^{\lambda f}(1 + k_l a)^n} \tag{23}$$

where $\epsilon_{max}$ denotes the maximal enrichment, when all salt binding sites are occupied

$$\epsilon_{max} = nNB \tag{24}$$

Combining eq 20 and 23 we obtain an alternative form for the saturation function

$$\bar{Y} = \frac{\epsilon_{s,g}}{\epsilon_{max}} = \frac{l}{l_l - l_s}\left(\frac{k_l a}{1 + k_l a} - \frac{k_s a}{1 + k_s a}\right) + \frac{l_l}{l_l - l_s}\left(\frac{k_s a}{1 + k_s a}\right) - \frac{l_s}{l_l - l_s}\left(\frac{k_l a}{1 + k_l a}\right) \tag{25}$$

This equation indicates that for a given activity of salt ($a$ = constant) the enrichment, $\epsilon_{s,g}$, should depend linearly upon the relative fiber length, $l$.

For the sake of completeness we shall evaluate from the

equations of the autone model the thermodynamic cross relations, eq 12, in explicit form. Differentiation of $\epsilon_{s,g}$ with respect to length in centimeters and insertion of eq 24 give

$$\left(\frac{\partial \epsilon_{s,g}}{l_0 \partial l}\right)_{\mu_s} = \left(\frac{\partial \epsilon_{s,g}}{l_0 \partial l}\right)_{a} = l\frac{nNB}{l_0(l_1 - l_s)}\left(\frac{k_1 a}{1 + k_1 a} - \frac{k_s a}{1 + k_s a}\right) \quad (26)$$

On the other hand, we can obtain from eq 22

$$\left(\frac{\partial f}{\partial \mu_s}\right)_l = \frac{a}{\kappa T}\left(\frac{\partial f}{\partial a}\right)_l = \frac{-n}{\lambda \kappa T}\left(\frac{k_1 a}{1 + k_1 a} - \frac{k_s a}{1 + k_s a}\right)$$

which upon insertion of $\lambda$ in $dyn^{-1}$ gives

$$\left(\frac{\partial f}{\partial \mu_s}\right)_l = \frac{-nNB}{l_0(l_1 - l_s)}\left(\frac{k_1 a}{1 + k_1 a} - \frac{k_s a}{1 + k_s a}\right) \quad (27)$$

so that

$$\left(\frac{\partial \epsilon_{s,g}}{l_0 \partial l}\right)_{\mu_s} = -\left(\frac{\partial f}{\partial \mu_s}\right)_l$$

as required by eq 12. Evidently the equality of eq 26 and 27 is only a test of the consistency of the equations for the autone model and *not* a new relation. The explicit expressions eq 25 and 27 nevertheless provide additional relationships which will be found useful in the correlation of experimental data.

## Experimental Methods

*Preparation and Analysis of Solutions.* Concentrated aqueous solutions of LiBr (Matheson Coleman and Bell) were decolorized with activated charcoal. Densities of stock and incubation solutions were determined in triplicate and compared with published values (International Critical Tables, 1928); all dilutions were made gravimetrically and in duplicate. Bromide analyses were performed by mercurimetric titration (Macdonald, 1960; White, 1961). The titrant was 0.005 M $Hg(ClO_4)_2$, standardized against dried analytical grade KCl and NaBr, and Primary Standard NaCl (Anachemia). To 5 ml of aqueous solution containing weighed aliquots of the sample were added 20 ml of absolute ethanol, 0.5 ml of 0.5 N $HNO_3$, and 5 drops of diphenylcarbazone (10 mg/ml of absolute ethanol). Titrations to the bright magenta end point were producible with average deviations of 0.15% for 4 ml of 0.01 M standard halide solution and 0.5% for smaller volumes of fiber extracts from binding experiments. This degree of precision was necessitated by the large correction factors (see below). The activity of LiBr in each experimental solution was determined by calculating the salt molality from the observed concentration and density, and obtaining the corresponding molal activity coefficient by graphical interpolation between published values (Harned and Owen, 1958).

[U-$^{14}$C]Glycerol (New England Nuclear Corp.) was added to the LiBr solutions as an inert tracer for residual solution on fibers after removal from the incubation media. Unlabeled glycerol was added to a final concentration of 0.5% (v/v) to preclude artifacts due to binding of traces of the radioactive material by the fibers or glassware. This concentration of glycerol was shown to be mechanochemically inert by its failure to shift the shrinkage temperature of the fibers from 67.2° in water or 16.4° in 5.0 M LiBr. Weighed 1-ml aliquots of labeled extracts or diluted media were mixed with 10 ml of scintillation fluid (667 ml of toluene–333 ml of Triton X-100 detergent (Packard)–42 ml of Liquiflour (New England Nuclear Corp.)) and counted in a Packard Tri-Carb scintillation spectrometer. Averages of four 5-min counting cycles, corrected for average background during each counting period, were used in all calculations. Samples contained 300–800 cpm/ml. For incubation media containing both LiBr and [$^{14}$C]glycerol, suitable dilutions were selected so that bromide concentrations and radioactivity could be determined on the same dilution, and quenching of counts by the salt would be negligible.

*Preliminary Treatment of Collagen Fibers.* Collagen tape, prepared by extrusion into acetone of ficin-treated steer tendon collagen, was a generous gift of the Ethicon Corp., Somerville, N. J. Untreated tape (size %, cross-section $2 \times 10^{-4}$ $cm^2$) was wound on a glass frame and covalent intermolecular cross-links were introduced by soaking in 0.25% formaldehyde in 0.05 M potassium phosphate (pH 8.5) for 15 hr at room temperature, followed by thorough washing and drying on the frame (see Veis and Drake, 1963). The thermolability of cross-links derived from formaldehyde (Flory and Spurr, 1961) was inconsequential here, since all mechanical and salt-binding experiments were performed at 5 or 20°.

Fibers approximately 30 cm long, cut from a single batch of treated tape, were moistened and tied at both ends to black nylon thread. A short thread at one end was clamped to the bottom of a stainless steel bar (50 × 1.2 × 0.4 cm); a long thread at the other end was strung vertically through the open upper clamp, over a low-friction plastic pulley. The fibers were incubated in solutions stirred by slow nitrogen bubbling in glass cylinders (2.4-cm i.d.) in a water bath thermostated to ±0.1°. Fiber lengths were measured between the fiber-thread knots using a 1-m Gaertner cathetometer accurate to ±0.002 cm, kindly loaned by Dr. J. M. Cassel of the National Bureau of Standards. A counterweight of 0.7 g on the pulley was routinely used to straighten water-soaked fibers to the "unstretched" reference length, $l_0$.

Since the reversible or equilibrium mechanochemical interactions were to be studied, all fibers were subjected to a preliminary treatment comprising a complete cycle of chemical denaturation and renaturation and several cycles of stretching and relaxation. The initial denaturation consisted of exposure of unstressed fibers to 10 M LiBr for 4 hr at 5°, and resulted in an average relative fiber length, $l$, of 0.41. Four cycles of stretching to the initial length were then carried out in the cold denaturant by progressively increasing the counterweight to 120 g (17,000 times the dry fiber weight), then removing the weights. About 5% of the fibers were broken during this treatment. Renaturation to a stable, unstressed relative length, $>0.99\ l_0$, was accomplished by exhaustive washing and continuous stretching under 30-g force in water at 5° for 8 hr.

*Length–Force Experiments at Constant Salt Activity.* The preliminary treatment described above was applied simultaneously to twelve fibers in six clamp and cylinder setups with independent pulleys. The cold water used for renaturation was removed by siphoning and was replaced in the respective cylinders by cold LiBr of the following concentrations: 4.1, 5.0, 5.2, 5.5, 6.1, and 6.7 M. After 3-hr incubation at 5° the

temperature of the water bath was raised from 5 to 20° (5°/hr), and maintained at 20° overnight before the reported mechanical data at this temperature were obtained. Each increment or decrement of stretching force was maintained until no further length change could be detected. The equilibration time required after a given fractional length change varied inversely with denaturant concentration from less than 1 hr in 9 M LiBr to several hours in 5 M LiBr. The coincidence of lengths observed during extension and relaxation cycles provided the ultimate criterion for mechanical equilibrium.

*Correlation of Salt Enrichment, $\epsilon_{s,g}$, with Mechanical Change.* Following the preliminary treatment, each fiber to be used for the determination of salt enrichment and corresponding relative length was incubated for 8 hr under the final conditions of force and salt concentration at 5°. The temperature was then increased to 20° and maintained for several hours until no further length change was detectable. To maintain the equilibrium force on the fibers during removal of excess medium, the upper clamp was firmly secured on the threads through which the tensile force was transmitted. The clamp apparatus, holding two or three fibers incubated in the same solution but under different forces, was then removed from the cylinder. Many direct effects of either evaporation or water adsorption by the solution clinging to the fibers were avoided by studying the interaction of the collagen with the denaturant relative to [$^{14}$C]glycerol $\epsilon_{s,g}$ rather than to water $\epsilon_{s,w}$ as in previous investigations (Ciferri *et al.*, 1967; Oplatka and Yonath, 1968). However, indirect effects of local changes in salt concentration during the exposure of the fibers to the ambient temperature and vapor pressure could be reduced only by performing all subsequent operations as rapidly as possible.

Each fiber was wiped gently with filter paper, grasped with haemostats, cut from the threads, and transferred to a weighing bottle. After determination of the wet weight (as an approximate indication of the amount of occluded medium) each fiber was extracted with about 9 ml of water for 20 hr. The precise total extract weight was obtained immediately before removal of aliquots for titration and radioassay. The fiber was washed to remove remaining extract before being transferred to a VirTis vacuum vial for overnight lyophilization. Dry fiber weights obtained on a Cahn gram electrobalance immediately after removal from the vacuum were reproducible to ±0.01 mg.

The primary data from mercurimetric titrations and radioassays of weighed aliquots of fiber extracts and diluted media were combined in the calculation of the amount of LiBr on each fiber above that attributable to retention of some concentrated denaturant solution as measured by the tracer [$^{14}$C]-glycerol

$$\epsilon_{s,g} = \frac{86.86}{\text{mg of dry collagen}} \times \frac{\text{mmoles of standard halide}}{\text{ml of titrant}} \times \text{total g of extract} \times \left[ \frac{\text{ml of titrant}}{\text{g of extract}} - \frac{^{14}\text{C cpm}}{\text{g of extract}} \times \left( \frac{\text{ml of titrant}}{\text{g of diluted medium}} \Big/ \frac{^{14}\text{C cpm}}{\text{g of diluted medium}} \right)_{av} \right] \quad (28)$$

The av in this formulation refers to the average of at least four dilutions of each medium for the ratio of bromide to tracer in terms of the measurable parameters.

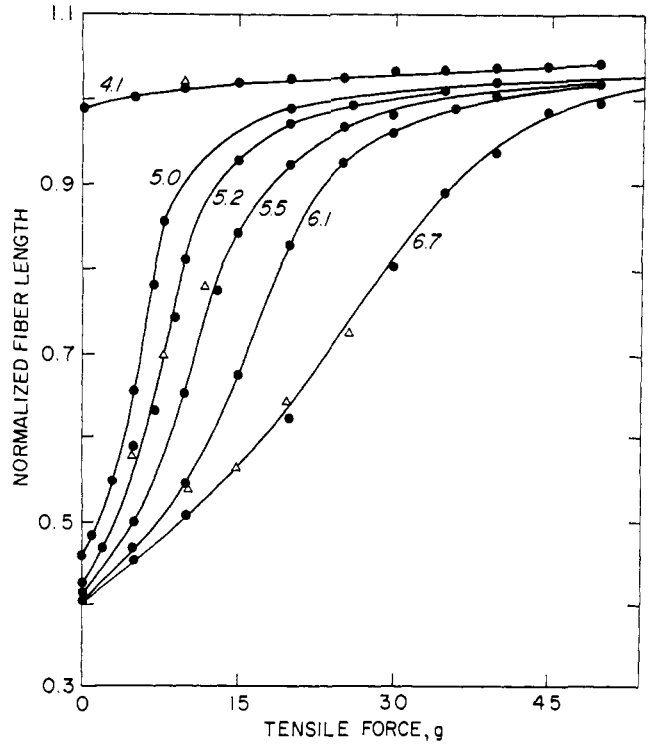


FIGURE 2: Length–force dependence of pretreated collagen fibers in various LiBr concentrations, showing reversibility. Average length, relative to unstretched fiber in water, of duplicate fibers carried through complete cycle of extension (●) and relaxation (△) at 20° in solutions of indicated molarities.

As controls for experiments on salt enrichment in solutions causing cooperative shrinkage of the fibers at 20°, parallel experiments were conducted in 4.1 M LiBr in which no force-dependent interaction was expected below the shrinkage temperature of about 37°.

## Results and Discussion

*Equilibrium Length as a Function of Force and LiBr Activity.* Although the dependence of the length of collagen fibers upon force and salt concentration has been studied previously (Flory and Spurr, 1961; Puett *et al.*, 1965; Yonath and Oplatka 1968; Oplatka and Yonath, 1968), we repeated the measurements under conditions which ensured reversibility and which would provide data suitable for thermodynamic treatment. Collagen fibers which had undergone the preliminary treatment described above were subjected to variable force in baths of different concentration. In each experiment the force was raised by small increments and then decreased. As shown in Figure 2 the stretching and relaxation points coincide, indicating that the process is reversible, as required. The equilibrium length–force curves at 20° are characterized by a high degree of sigmoidicity, of the same type as observed in the first contraction cycle of submucosal collagen in KCNS solutions (Yonath and Oplatka, 1968).

An alternative representation of these experimental results is provided by the plot of length *vs.* LiBr activity, at different forces (Figure 3). The striking cooperativity of the length changes at the "melting activity" of the salt resembles closely

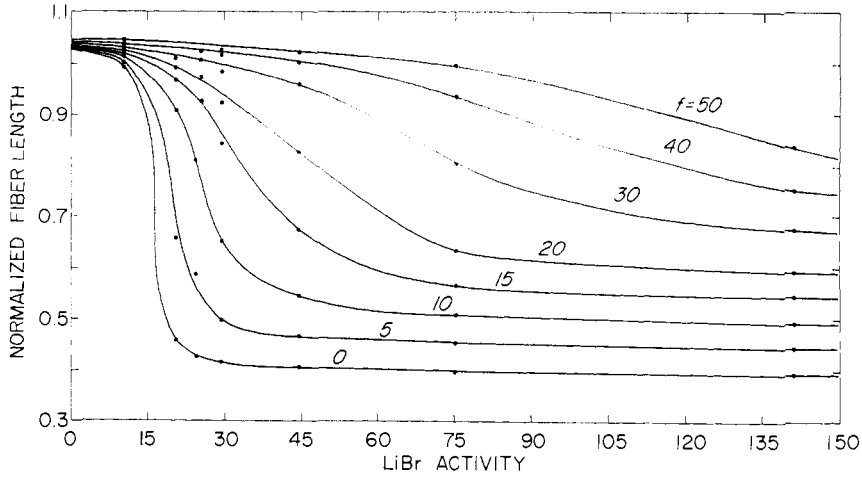


Figure 3: Cooperative effect of LiBr activity on shrinkage of fibers under constant tensile force. Data from experiments of Figure 2; force, $f$, expressed as grams on pulley (see Experimental Methods).

the chemical melting during the first cycle (Oplatka and Yonath, 1968). It will be noted that, as required thermodynamically, the melting activity increases with the stretching force. Moreover the change in length becomes smaller and more gradual with $f$, and at very high forces contraction cannot be induced by the highest salt concentrations.

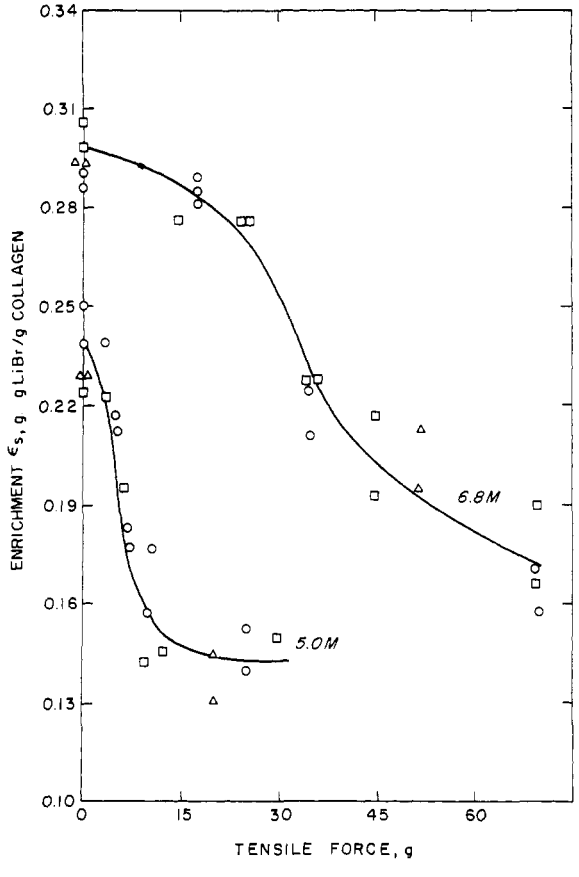


FIGURE 4: Effect of mechanical force on a chemical interaction, the enrichment of LiBr in the fiber relative to [$^{14}$C]glycerol. The symbols △, ○, and □ indicate data obtained in the same experiment; each point represents analysis of a separate fiber.

*Enrichment of Salt, Relative to [$^{14}$C]Glycerol* $\epsilon_{s,g}$. Utilizing the glycerol technique described in the Experimental Methods section, a set of values of $\epsilon_{s,g}$ was obtained in solutions of 5.0 and 6.8 M LiBr under different stretching forces. In this range of salt concentrations, there is considerable scatter in determinations of enrichment since it represents the small difference between the total salt on the wiped fiber and the amount attributable to retention of external solution, as measured by the tracer (eq 28). The technique was not sufficiently sensitive to detect enrichment at lower concentrations or to obtain reproducible values at higher concentrations, *e.g.*, 9.8 M LiBr. The results in Figure 4 nevertheless give a clear picture of the general trends and may be used for semiquantitative analysis.

It will be observed that for a given force, $\epsilon_{s,g}$ increases with $\mu_s$ so that

$$\left(\frac{\partial \epsilon_{s,g}}{\partial \mu_s}\right)_f > 0 \tag{29}$$

On the other hand, $\epsilon_{s,g}$ decreases with $f$ at constant $\mu_s$, *i.e.*

$$\left(\frac{\partial \epsilon_{s,g}}{\partial f}\right)_{\mu_s} < 0 \tag{30}$$

According to eq 12, $(\partial \epsilon_{s,g}/\partial f)_{\mu_s} = (\partial l/\partial \mu_s)_f$ and it is apparent from Figure 3 that $(\partial l/\partial \mu_s)_f < 0$. The observed decrease in enrichment with increasing force is thus an expected result.

It is instructive to plot $\epsilon_{s,g}$ *vs.* $f$ and $l$ *vs.* $f$ on the same diagram (Figure 5). This representation shows that at constant $\mu_s$, the dependence of $l$ upon $f$ is approximately the mirror image of $\epsilon_{s,g}$ *vs.* $f$. Moreover, the direct correlation of $\epsilon_{s,g}$ with $l$ (at given salt concentration) is approximately linear with a negative slope, *i.e.*

$$\left(\frac{\partial \epsilon_{s,g}}{\partial l}\right)_{\mu_s} < 0 \tag{31}$$

and is approximately constant (Figure 6). The inverse dependence of enrichment upon length is again a consequence of

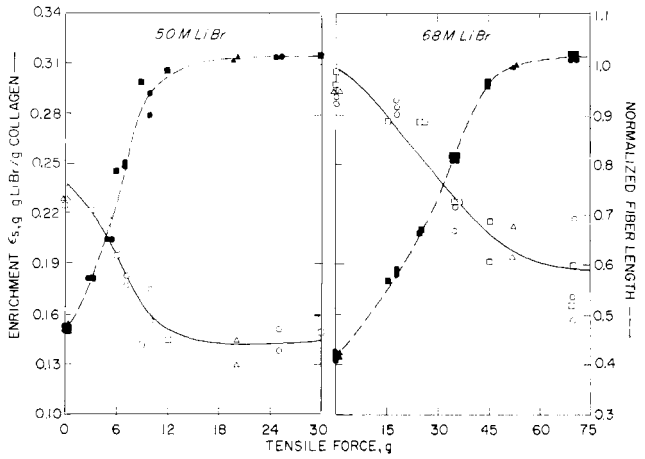


FIGURE 5: Correlation of force dependence of salt enrichment $\epsilon_{s,g}$ and of relative fiber length, $l$. Each pair of values for $\epsilon_{s,g}$ (open symbols) and $l$ (filled symbols) refers to a single fiber equilibrated under indicated force. Solid curves through enrichment data correspond to the linear variation with length shown in Figure 6.

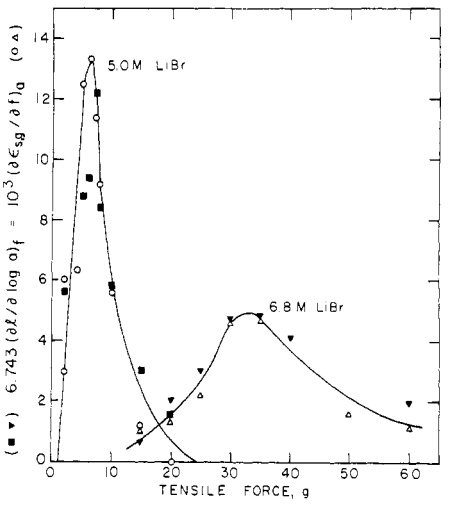


FIGURE 7: Comparison of partial differentials of enrichment with respect to force at constant salt activity $(\partial\epsilon_{s,g}/\partial f)_a$ and of length with respect to chemical potential under constant force $(\partial l/\partial\mu_s)_f = (\partial l/\partial \log a)_f \times (2.303\ RT)^{-1}$ (see eq 12). Values of $(\partial l/\partial \log a)_f$ (filled symbols) were obtained from data of Figure 3. Factor $6.743 \times 10^{-3}$ converts units of $(\partial l/\partial \log a)_f$ into those of $(\partial\epsilon_{s,g}/\partial f)_a$ (open symbols) obtained from data of Figure 4.

eq 12, since from Figure 3 we deduce that $(\partial f/\partial\mu_s)_l > 0$ and hence $(\partial\epsilon_{s,g}/\partial l)_{\mu_s}$ must be negative.

*Quantitative Test of Eq 52 and Confirmation of Reversibility.* Equation 12 states that $(\partial\epsilon_{s,g}/\partial f)_{\mu_s}$ determined from enrichment measurements should be equal to $(\partial l/\partial\mu_s)_f$ determined from the length–force–activity data. Agreement between the empirical values of these differentials would confirm that the mechanochemical system operates reversibly. It should be noted that $(\partial l/\partial\mu_s)_f$ is itself a function of $f$, while $(\partial\epsilon_{s,g}/\partial f)_{\mu_s}$ is a function of $\mu_s$, so that the comparison must be made at the same values of $f$ and $\mu_s$.

To evaluate $(\partial\epsilon_{s,g}/\partial f)_{\mu_s}$, a smooth curve was drawn through the enrichment data for each LiBr concentration (Figure 4), and the slopes of tangents to these curves at various forces were calculated. The results for the differential of enrichment (in $g_{LiBr}\ g_{collagen}^{-1}$) with respect to force (in g on the pulley) are indicated by the open symbols of Figure 7. To evaluate $(\partial l/\partial\mu_s)_f$, the data of Figures 2 and 3 for relative fiber length was plotted as a function of log $a$ for experimental and interpolated values of the stretching force. Tangents were drawn to these curves at values of the salt activity used in the binding experiments ($a$ = 20.6 for 4.95 M LiBr, $a$ = 85.0 for 6.82 M LiBr). The unitless slopes of these tangents $(\partial l/\partial \log a)_f$ are divided by $2.303RT = 5.611 \times 10^{10}$ erg mol$^{-1}$ to obtain $(\partial l/\partial\mu_s)_f$, where $l$ denotes fiber length relative to $l_0$. Introducing the average mass per unit length for this batch of fibers, $2.25 \times 10^{-4}$ g cm$^{-1}$, the data for $(\partial l/\partial \log a)_f$ may be converted into the empirical units of $(\partial\epsilon_{s,g}/\partial f)_{\mu_s}$ by multiplication by

$$\frac{(980\ \text{cm sec}^{-2})(86.86\ g_{LiBr}\ \text{mol}^{-1})}{(2.25 \times 10^{-4}\ g_{collagen}\ \text{cm}^{-1})(5.611 \times 10^{10}\ \text{erg mol}^{-1})} = 6.743 \times 10^{-3}\ g_{LiBr}\ g_{collagen}^{-1}\ \text{g on pulley}^{-1}$$

The results in this form are shown by filled symbols in Figure 7.

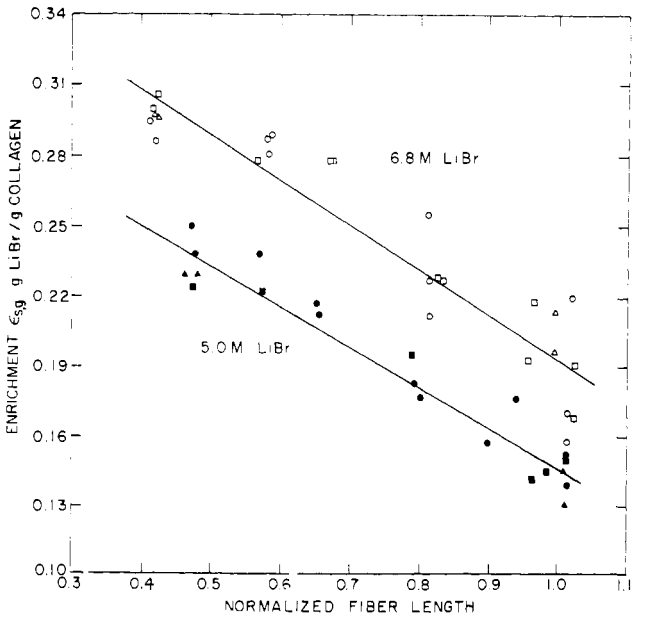


FIGURE 6: Best fit of enrichment data to the linear dependence upon fiber length, predicted by the two-state model (eq 25).

The variation of the differentials with force is shown by the solid curve through the data for each salt concentration. A comparison of filled versus open symbols at each force and concentration demonstrates that $(\partial l/\partial\mu_s)_f$ and $(\partial\epsilon_{s,g}/\partial f)_{\mu_s}$ agree in both magnitude and the shape of their force dependence. Within the limitations imposed by the scatter of the enrichment measurements and the approximate methods used to evaluate the differentials, these data fulfill thermodynamic requirements of a reversible mechanochemical system.

*Evaluation of Parameters for the Model.* For detailed comparison of the observed effects of LiBr and force on collagen fibers with computations according to the two-state model, the following parameters had to be evaluated: $l_s$, the relative

length of the fiber when completely denatured or compressed (with all conformational units in the short state); $k_s$, the intrinsic association constant of a site within a short unit for LiBr; $l_l$ and $k_l$, the corresponding parameters for the crystalline (long) state ; $n$, the number of LiBr binding sites per conformational unit; $K$, the equilibrium constant for conversion of a short into a long unit in the absence of force or salt; $\lambda$, the coefficient of force dependence of the transition; and $\epsilon_{max}$, the value of the enrichment corresponding to saturation of LiBr binding sites.

The first approximation to $l_l$ and $l_s$ was made by inspection of the length–force curves in Figure 2. Refinement of these values, as well as adjustment of several other parameters, was based on a normalization of the length measurements through eq 22 in which the intrinsic equilibrium constant, the force dependence and the activity dependence are represented by separate terms.

In the refined approximation the limiting lengths were obtained from the fit of the least-square deviation of the length–force dependence at each salt concentration according to eq 22. The best-fitting values of $l_l$ and $l_s$ for the whole set of data were then used in the estimation of intrinsic binding constants and $\epsilon_{max}$. Least-square deviation "plots" for the enrichment as a linear function of length at each activity were computed from the data in Figure 6. Next, use was made of an assumption of the two-state model that the saturation function of *each state* follows classical (hyperbolic) activity dependence (Monod *et al.*, 1965). Linear extrapolations of the values of $\epsilon_{s,g}$ at each activity to the limiting lengths could therefore be analyzed according to

$$\left(\frac{1}{\epsilon_{s,g}}\right)_i = \frac{1}{\epsilon_{max}} + \frac{1}{\epsilon_{max} k_i}\left(\frac{1}{a}\right) \qquad (32)$$

where $i$ refers to either short or long state. Linear plots of $(\epsilon_{s,g})_i^{-1}$ *vs.* $a^{-1}$ for both states permitted estimation of $\epsilon_{max}$ from the common intercept and the intrinsic binding constants from the respective slopes.

The foregoing results for $l_l$, $l_s$, $k_l$, and $k_s$ provided the initial values for solution of a matrix of the entire set of length–force–activity data according to eq 22. This process was reiterated with systematic variation of the fixed parameters to solve for the set of coefficients producing the minimum average deviation of computed from observed lengths. In the final iteration, the coefficient of the activity term was rounded, to obtain from the combination of chemical and mechanical data the two nearest integral values for the number of binding sites and the corresponding values of $K$ and $\lambda$.

Computations were performed on the Golem computer at the Weizmann Institute of Science and the IBM 360 at the National Institutes of Health and were converted into graphical form by the Calcomp plotter.

*Comparison of Experimental and Theoretical Results.* For the sample of collagen under investigation, an adequate set of parameters is the following. If the length of the fully stretched fiber is equal to the reference length, or $l_l = 1.00$, then the fully contracted length is $l_s = 0.38$. The association constant of salt to the amorphous collagen is $k_s = 0.2$ and the constant for the stretched macromolecules is about seven times smaller $k_l = 0.03$. The equilibrium constant long/short at $f = 0$ and $a = 0$ is 24,700 and the parameter $\lambda = 0.2468$ g$^{-1}$. The number of binding sites per segment is $n = 10$. Figure 8 represents the computed variation of $l$ with $f$ at different LiBr concentrations for these values of the parameters.

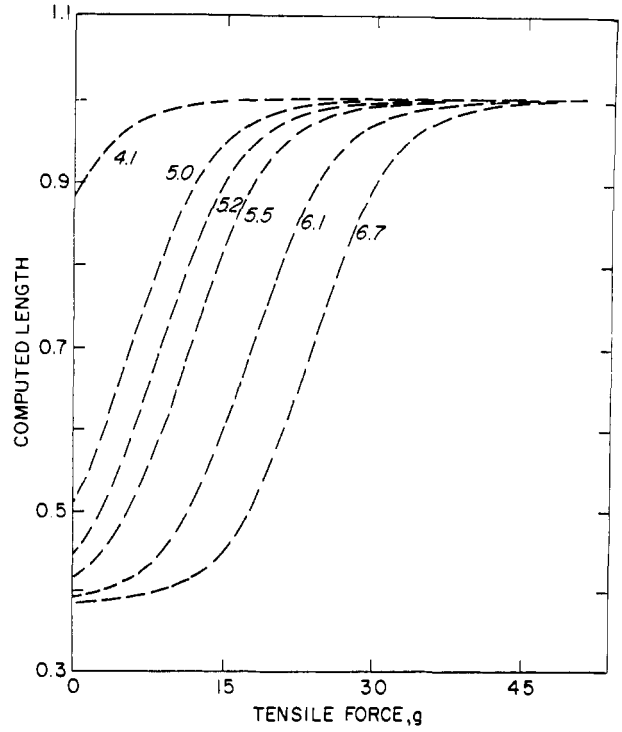


FIGURE 8: Length–force dependence predicted by the two-state model for experimental conditions of Figure 2. Computations were made according to eq 20 for salt activities corresponding to the indicated molarities of LiBr and the following values of model parameters: $n = 10$, $K = 24{,}770$, $\lambda = 0.2468$ g$^{-1}$, $k_s = 0.2$, $k_l = 0.03$, $l_s = 0.38$, and $l_l = 1.00$.

The comparison of Figure 8 with Figure 2 shows that semiquantitatively the two-state model describes correctly the run of the experimental curves. A small systematic discrepancy is apparent, however. The length–force curves computed from eq 20 for different concentrations are parallel but shifted along the force axis, while the empirical curves decrease in steepness with increasing salt concentration. This behavior of the real fibers would correspond to an inverse dependence of the value of the parameter $\lambda$ upon salt concentration, which is not accounted for in the model.

A similar evaluation of length *vs.* LiBr activity at different forces is given in Figure 9, which should be compared with the experimental data in Figure 3. Here the agreement of the model with the experiments is more impressive and there is little doubt that all the essential features of the observed behavior are adequately described.

The final comparison of the two-state model with the data is the quantitative variation of $\epsilon_{s,g}$ with $f$ or $l$. The solid lines of Figure 6 represent the best fit of the data to the linear dependence of $\epsilon_{s,g}$ upon $l$ predicted by eq 25. The corresponding dependence of $\epsilon_{s,g}$ upon $f$ is shown by the solid curves of Figure 5. The agreement is as good as the precision of the experiments.

*Concluding Remarks.* The range of values for the equilibrium constant of the short to long transition in the absence of force or salt corresponds to a free energy change of 6.0–6.6

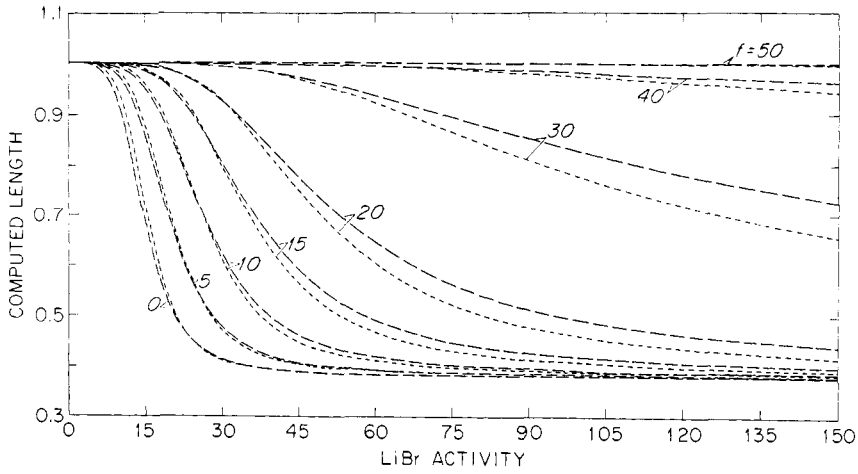


FIGURE 9: Activity dependence of fiber length computed for two estimates of the number of salt-binding sites, $n$, involved in cooperative transitions between the two states of the model. Computations according to eq 20 for same parameter values as Figure 8, with $n = 10$ (—), and for $n = 11$, $K = 82{,}060$, and $\lambda = 0.2494$ g$^{-1}$ (– –). Compare experimental results of Figure 3.

kcal/mole of conformational unit (Figure 9). The physical extent of such a unit may be estimated from a combination of results from mechanical and chemical studies. The highest observed excess of LiBr in the fiber phase corresponds to approximately 1 mole of salt/3 moles of amino acid residue or 1 mole of salt/mole of glycine residue (Figure 4). The cooperativity of the observed length–activity curves can be simulated by assuming ten interaction sites per conformational unit of the model (compare Figures 3 and 9). Together, these results imply the participation of about 30 amino acids in coordinated transitions between the helical and amorphous states.

The simplicity of the model and the lack of quantitative agreement with the data, however, must temper any conclusions based on the values of the parameters selected for the illustrative computations. The numerical results describe the behavior of a single batch of cross-linked fibers at one temperature, 20°. The two-state model nevertheless provides a framework for the characterization of the mechanochemical transitions under these conditions, and their comparison with the effects of force and ligands on transconformational reactions in other macromolecular systems.

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