Under the assumptions made, the derived equations indicate several kinetically different cases corresponding to different possible mechanisms in terms of the pH dependence of the kinetic parameters, the maximum velocity and Michaelis constant. These differences may be summarized as follows: if both ionized forms of substrate are utilizable, V_F and the ratio $V_F(1 + (H^+)/K_{SH})/K_F$ would be expected to vary with pH in a manner more complex than would be expected from the ionization of one or two groups in the active site of the enzyme molecule. On the other hand, when only one ionized form of substrate is utilizable, the two possibilities that the non-utilizable form of substrate be either a "good" or "poor" competitive inhibitor are kinetically distinguishable. For both these possibilities, $V_F(1 + (H^+)/K_{SH})/K_F$ *vs.* pH is a function only of the ionizable groups in the enzymatically active site which are responsible for activity and the pH dependence may be described easily by an equation similar to equation II,3. For the case of poor competitive inhibition, the maximum velocity represents only the ionization of groups of the active site of enzyme to which substrate is bound. However, for the case of good competitive inhibition, the maximum velocity will be influenced by the degree of ionization of the substrate and will therefore be more complex than would be expected.

In order that the equations derived in this paper be applicable, a large amount of kinetic data must be obtained. These data must be uncomplicated by interference due to substrate inhibition, substrate activation, irreversible enzyme denaturation, buffer effects due to different concentrations of anions or cations at different pH values and so forth. Thus, care must be exercised in the reinterpretation of data already presented in the literature. For example, the two enzymes arginase and enolase which have been discussed both involve metal ions for enzymatic activity. Although constant metal ion concentration was used, the mechanism of interaction of enzyme with the metal ions is not completely understood.

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The Elastic Properties of Elastin[1,2]

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Force–temperature measurements have been carried out on elastin (ox *ligamentum nuchae*) held at fixed elongation and immersed in glycol–water (3:7) mixture. The equilibrium degree of swelling of elastin in this mixture is independent of temperature, and the retractive force is directly proportional to the absolute temperature. It follows that $(\partial E/\partial L)_{TV} = 0$ for elastin and hence that the internal energy of the elastin chain is independent of its conformation. Contrary to previous studies on elastin, in which the influence of changes of swelling with temperature were overlooked, the thermoelastic behavior offers no indication whatever for crystallization on stretching at any elongation. The shape of the stress–strain curve is explained in terms of the morphology of native elastin; the abrupt rise in stress at high elongations is attributed to straightening out of the initially curled fibers of collagen which are associated with the native elastin.

Introduction

Elastin is an important constituent of various elastic tissues including ligaments, blood vessel walls and skin. It possesses a high extensibility combined with a low modulus not unlike that of rubber. Moreover the stress–strain curve for elastin, like that of rubber, swings upward sharply at high extensions[5]; the rise of the stress–strain curve is however more abrupt, and it occurs at somewhat lower elongations, as compared with vulcanized rubber. Thermoelastic studies[4–6] on elastin have yielded large positive stress–temperature coefficients even at low extensions. It has been inferred from this alleged deviation from *ideal* rubber elasticity that crystallization occurs on stretching. On the contrary, however, Astbury[7] found only an amorphous halo in the X-ray diffraction pattern of stretched elastin in which the collagen component had been destroyed.

The principles underlying rubber-like elasticity of amorphous polymers are of course well known. Progress recently has been made in the analysis of the thermoelastic behavior of partly crystalline polymers.[8] In extension of studies in this area, and especially those relating to fibrous proteins, it became of interest to examine elastin, and in particular to endeavor to resolve the apparent contradiction between the thermoelastic results and those of X-ray diffraction.

Theoretical

Insight into the nature of the molecular processes involved in elastic deformation may be gained by analysis of experimentally determined stress–strain–temperature results according to the thermodynamic equation of state for elastic deformation. This equation may be expressed as follows for a system subject to elongation at constant pressure

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

(1) Support of the National Science Foundation is gratefully acknowledged.

(2) Presented in September 1957 at the 132nd American Chemical Society Meeting, New York, N. Y.

(3) Mellon Institute, Pittsburgh, Pennsylvania.

(4) K. H. Meyer and C. Ferri, *Pflüger's Arch. ges. Phys.*, **238**, 78 (1936).

(5) E. Wöhlisch, *Kolloid Z.*, **89**, 239 (1939).

(6) E. Wöhlisch, H. Weitnauer, W. Grüning and R. Rohrbach, *ibid.*, **104**, 14 (1943).

(7) W. T. Astbury, *J. Intern. Soc. Leather Trades' Chem.*, **24**, 69 (1940).

(8) J. F. M. Oth, E. T. Dumitru, O. K. Spurr and P. J. Flory, THIS JOURNAL, **79**, 3288 (1957); J. F. M. Oth and P. J. Flory, *ibid.*, **80**, 1297 (1958).

where f is the refractive force, E the internal energy, L the length, T the absolute temperature and P the pressure on the sample. For systems at constant volume V

$$f = (\partial E/\partial L)_{VT} + T(\partial f/\partial T)_{VL} \qquad (2)$$

The advantages of experimentation at constant pressure weigh heavily in favor of eq. 1, by means of which $(\partial E/\partial L)_{PT}$ may be evaluated from observations on the temperature coefficient of the force at constant pressure. Unfortunately, however, it is the constant volume coefficient $(\partial E/\partial L)_{VT}$ that is representative of the energy change due solely to changes in molecular conformation brought about by the deformation. The constant pressure coefficient includes a contribution from dilation of the sample.

Direct evaluation of the force–temperature coefficient at constant volume (eq. 2) being generally impractical, it is customary to determine $(\partial f/\partial T)_{PL}$ from measurements at constant pressure and subsequently to apply suitable corrections for volume changes with temperature.[9] Through use of eq. 2 it is then possible to deduce the desired coefficient $(\partial E/\partial L)_{VT}$, which affords an index of the molecular processes accompanying deformation.

Manifestation of high elasticity in fibrous proteins generally requires the presence of a swelling agent, usually water. If the sample is in equilibrium with the swelling agent available in large excess, as for example if the sample is immersed in the swelling medium, then the quantity of diluent in the sample will, in general, depend on L and T. Equations 1 and 2 are readily shown to apply to such an open thermodynamic system comprising the swollen polymer in equilibrium with the external diluent phase, provided however that the partial derivatives are understood to be restricted to states in which swelling equilibrium prevails. Then E may be considered to represent the sum of the internal energies of both phases; P, V and L refer to the polymer phase specifically.

Direct evaluation of $(\partial f/\partial T)_{VL}$ in general would require the experimentally awkward feat of applying pressures to the polymer phase in order to maintain its volume constant. This difficulty may be avoided by choice of a diluent in which the volume V of the swollen polymer is independent of temperature, *i.e.*, for which $(\partial V/\partial T)_{P,L} = 0$. If this condition holds for the unstrained network, then it may be assumed to hold also for extended states, to a close approximation at least (*i.e.*, neglecting the effects of anisotropy, which should be trivial). It follows from well-known thermodynamic relations that

$$(\partial E/\partial V)_{TL} = T(\partial P/\partial T)_{VL} - P = -P$$

under the foregoing conditions, and hence that

$$(\partial E/\partial L)_{TP} = (\partial E/\partial L)_{TV} + (\partial E/\partial V)_{TL}(\partial V/\partial L)_{TP} = (\partial E/\partial L)_{TV} - P(\partial V/\partial L)_{TP}$$

Except for high pressures, we have to a very good approximation that

$$(\partial E/\partial L)_{TP} = (\partial E/\partial L)_{TV} \qquad (3)$$

Hence we may use the experimentally convenient eq. 1 to evaluate the theoretically desired derivative $(\partial E/\partial L)_{VT}$ of eq. 2, provided, of course, that the condition $(\partial V/\partial T)_{PL} = 0$. It is obvious also that $(\partial f/\partial T)_{PL}$ is then identical with $(\partial f/\partial T)_{VL}$.

The procedure indicated has been adopted in the present study. A mixed diluent was used; possible disproportionation of the two components between the phases is of no consequence, provided only that the diluent phase is present in large excess.

Through the use of statistical mechanical procedures, the following equations have been derived[10] for an amorphous polymer under a tensile force while in swelling equilibrium

$$(\nu V_1/N_a V_0)[<\alpha>_0^2(L_{io}/L) - v_2/2] = -[\ln(1 - v_2) + v_2 + \chi_1 v_2^2] \qquad (4)$$

If the retractive force is zero, eq. 4 becomes

$$(\nu V_1/N_a V_0)[<\alpha>_0^2 v_2^{1/3} - v_2/2] = -[\ln(1 - v_2) + v_2 + \chi_1 v_2^2] \qquad (5)$$

where

ν is the total number of chains in the network structure
V_1 is the molar volume of the diluent
N_a is Avogadro's number
L_{io} and V_0 are, respectively, the isotropic length and the volume of the polymeric sample in the absence of a diluent
v_2 is the volume fraction of polymer in the swollen network
$<\alpha>_0$ is the dilatation factor in the absence of diluent and is given by

$$<\alpha>_0^2 = \overline{r_{io}^2}/\overline{r_0^2}$$

where $\overline{r_{io}^2}$ is the mean square end-to-end distance of the polymer chains in the isotropic amorphous sample in the absence of diluent and $\overline{r_0^2}$ is the corresponding quantity for unconstrained free chains. χ_1 is the interaction parameter for solvent–polymer interaction.

In general the degree of swelling v_2^{-1} depends on the temperature coefficients of $<\alpha>_0^2$ and χ_1. The temperature coefficient of χ_1 is proportional to the heat of swelling which, if positive, causes the swelling to increase on raising the temperature, $<\alpha>_0^2$ being considered constant. On the other hand, depending on the nature of the energy barrier restricting bond rotation, $\overline{r_0^2}$, and hence $<\alpha>_0^2$, may change with temperature. (We neglect for the moment the relatively small effects of the bulk thermal expansion.) For amorphous polyethylene $d<\alpha>_0^2/dT$ has been found[11] to be positive, hence $d\overline{r_0^2}/dT$ is negative, as a result, no doubt, of rotational hindrance about the C—C bonds of the chain. Consequently measurement of the temperature coefficient of swelling alone is not sufficient for determining $d<\alpha>_0^2/dT$. However, by combining swelling–temperature measurements with force–temperature

(9) P. J. Flory, "Principles of Polymer Chemistry," Cornell University Press, Ithaca, N. Y., 1953, pp. 440–444.

(10) P. J. Flory, THIS JOURNAL, **78**, 5222 (1956).

(11) P. J. Flory, C. A. J. Hoeve and A. Ciferri, paper presented before the IUPAC Conference on Macromolecular Chemistry, Nottingham, 21–24 July, 1958.

measurements at constant length separate evaluation of $d\chi_1/dT$ and of $d<\alpha>_0^2/dT$ is in principle possible through use of eq. 4 and 5.

The relation for the force as a function of the length, temperature and degree of swelling is given by[10]

$$f = \nu kT(<\alpha>_0^2/L_{io}^2)L(1 - L_{io}^3/v_2L^3) \qquad (6)$$

where v_2 is in general a function of T and L as determined by eq. 4. At large extensions, the second term of eq. 6 is small and therefore the force–temperature coefficient at constant length can be related directly to changes in $<\alpha>_0^2/L_{io}^2$ and hence in $\overline{r_0^2}$. For small L the changes in v_2 with temperature will in general contribute to the force–temperature coefficient. But, if a solvent is chosen in which the volume of the swollen polymer (proportional to L^3_{io}/v_2) at zero force remains constant with temperature, the increase in volume from thermal expansion is exactly counterbalanced by de-swelling, and the force–temperature coefficient at constant length can be related without appreciable error to changes in $\overline{r_0^2}$ over the whole range of extensions.

This deduction from eq. 6 is in accord with the foregoing thermodynamic considerations. Thus, at constant length and at swelling equilibrium in a medium in which the condition $(\partial V/\partial T)_{LP} = 0$ holds, we have

$$\left[\frac{\partial \ln(f/T)}{\partial T}\right]_{VL} = -\mathrm{d} \ln \overline{r_0^2}/dT = -(\partial E/\partial L)_{TV}/Tf \quad (7)$$

as has been shown for single phase polymer systems.[11]

Experimental

Fiber bundles of *ca.* 0.05 cm.^{2} in cross section and 4 cm. in length were obtained from unpurified ox *ligamentum nuchae*. Length and diameter were measured with a cathetometer. Measurements in water showed that the degree of swelling decreased in water on elevation of the temperature. However, the dimensions remained essentially constant from 0 to 50° in a 30% by volume glycol–water mixture. Force measurements were performed in this medium.

The fiber bundle was suspended between clamps at both ends. The lower clamp was fixed, while 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 connected to a Leeds and Northrup recorder giving a full scale deflection of 10 mv. The instrument was calibrated by addition of known weights before and after each experiment.

Reversible measurements were made in the following way. The fiber bundle was extended at the highest temperature (50.5°) and allowed to remain in this condition for half an hour for dissipation of stress relaxation. The temperature then was lowered to 0.5°, and again relaxation was allowed to occur for 30 minutes, whereupon the force was recorded. After returning the sample to 50.5°, the previous value for the force was restored; hence attainment of equilibrium forces was assured. Thereafter the elongation of the sample was changed and the cycle repeated.

Fiber bundles were decomposed enzymatically with an impure preparation of elastase extracted from pancreas according to the procedure of Baló and Banga.[12]

Results and Discussion

As observed in the microscope[13] the elastin samples consisted of many separate fibers with irregularly curled thinner fibers between them. Staining techniques[13] showed the thicker fibers to consist of elastin and the thinner ones of collagen. Decomposition of the elastin component with elastase left fibers which, though coherent, accounted for only about 20% of the initial (dry) specimen. The lengths of these residual fibers, when straightened out under tension, were about twice those of the relaxed native sample. Identification of the residual fibers with the irregularly curled component seen under the microscope is thus indicated. These fibers displayed low elongation and high modulus; they underwent shrinkage (in water) at 65° to about one-fourth of their length. These observations leave little doubt that the residual fibers are collagen. The fact that macroscopic collagen fibers survive the enzymatic treatment is clear proof of the co-existence in ox *ligamentum nuchae* of separate fabrics comprising elastin and collagen fibers.

The results of the force–temperature measurements are given in Fig. 1. The points were taken in random order. Deviations from the lines are small, in further support of the conclusion that equilibrium was closely approached. The coefficients $(\partial f/\partial T)_{LV}$ were obtained from the slopes, and $(\partial E/\partial L)_{VT}$ values were calculated according to eq. 2. For extensions up to 50% and temperatures ranging from 0 to 50.5°, $(\partial E/\partial L)_{VT}$ does not deviate from zero by more than 1% of the total force, which is well within the experimental error, estimated to be about 2%. Compelling evidence is thus afforded for the conclusion that elastin does not crystallize on stretching. The failure of elastin to crystallize is rather unexpected, since most proteins are at least partly crystalline in their natural environment even without the assistance of elongation.

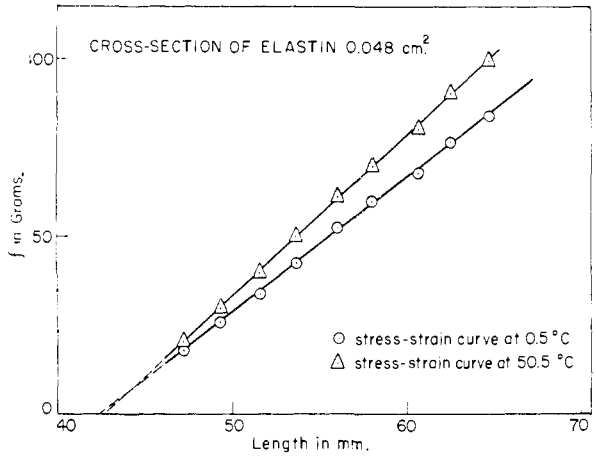

Fig. 1.—Stress–strain curves of an elastin bundle at different temperatures in 30% glycol–water mixture.

On application of eq. 6 to these results it is seen that $<\alpha>^2/L_{io}^2$, and therefore $\overline{r_0^2}$, is independent of temperature. Thus the different conformations of the protein chain must possess equal energy. This result is by no means at variance with current views[14,15] on impedance of rotation about the C—N

(12) J. Baló and I. Banga, *Biochem. J.*, **46**, 384 (1950).

(13) A. A. Maximow and W. Bloom, "A Textbook of Histology," W. B. Saunders Co., Philadelphia, 1952, p. 70.

(14) R. B. Corey and L. Pauling, *Proc. Roy. Soc.* (*London*), **B141**, 10 (1953).

(15) S. Mizushima, "The Structure of Molecule and Internal Rotation," Academic Press, Inc., New York, N. Y., 1954.

(16) S. M. Partridge and H. F. Davis, *Biochem. J.*, **61**, 21 (1955).

bond of the amide group, $\begin{matrix} H \\ | \\ C{-}{-}N \\ \| \\ O \end{matrix}$, owing to its partial double bond character. In fact, this planarity of the amide group should be expected to suppress steric hindrance to rotation about the other bonds in the protein chain, thereby reducing the change of energy with chain conformation. Furthermore, elastin is known[16] to contain few polar amino acids and the electrical forces should not therefore be expected to contribute appreciably to the conformational character of the chain.

Although the elastin bundle conforms to the condition $(\partial E/\partial L)_{VT} = 0$ for ideal rubber elasticity, the linear stress–strain relation presents a striking contrast to the isotherm reported for rubber. This feature finds qualitative explanation in the fact that elastin consists of separate fibers, curled to different degrees. Consequently some of the fibers do not contribute at all to the force at low extensions. As the extension increases the proportion of the fibers subjected to extension increases; hence more of them contribute to the force. The stress–strain relation which would obtain for a homogeneous polymer is therefore modified to the extent that the initial negative curvature which would otherwise be observed is eliminated. The linearity noted within experimental error over the range of extensions given in Fig. 1 is regarded as fortuitous.

At extensions higher than *ca.* 70% the collagen fibers evidently become taut and hence cause the stress to rise abruptly with further extension. Collagen, being a crystalline polymer where elastic modulus and tensile strength are much higher than those of elastin, prevents rupture of the fiber bundle at higher stresses. In contrast with rubber, the steep rise in stress at elongations near the maximum attainable length is not precipitated by crystallization, but by a permanently crystalline component (collagen) interwoven with the deformable (elastin) component.

The conclusions we have reached depart from the interpretations of Meyer and Ferri[4] and of Wöhlisch and co-workers.[5,6] These investigators concluded from stress–temperature measurements that elastin crystallizes on stretching. Wöhlisch went even so far as to calculate the heat of crystallization of elastin from the stress–temperature relation. The value found was surprisingly low. Failure to take account of de-swelling of elastin in water with elevation of temperature led these investigators to the erroneous inferences. The internal energy changes with length as deduced by them are to be attributed rather to the energy change associated with the aforementioned de-swelling with rise in temperature.

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[CONTRIBUTION NO. 1507 FROM THE STERLING CHEMISTRY LABORATORY, YALE UNIVERSITY]

Hemoglobin Studies. III. The Effect of Pyridine on the Combination of Heme with Carbon Monoxide[1]

BY AKITSUGU NAKAHARA[2] AND JUI H. WANG

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The chemical equilibrium between heme and carbon monoxide was studied in the presence of pyridine. It was shown that small amounts of added pyridine markedly increase the affinity of the heme solution for carbon monoxide in accordance with the ligand-field theory of metal complexes.

It was reported in Paper I of this series that the saturation curve of heme by carbon monoxide corresponds to a simple chemical equilibrium which may be represented by

$$H_2O\text{–heme–}OH_2 + CO \rightleftharpoons H_2O\text{–heme–}CO + H_2O$$

The half-saturation pressure of this reaction was found to be 0.24 cm. which is about 40 times larger than the corresponding value for hemoglobin at room temperature and *p*H 7.4. Since the hemes in hemoglobin are believed to be attached to the imidazole groups of the globin, it seems to be of interest to study the above equilibrium in solutions containing a tertiary amine. Pyridine was chosen in this work instead of imidazole because of the greater solubility of the pyridine–heme complexes.

In general when carbon monoxide is equilibrated with an aqueous solution of heme and pyridine, the equilibrium mixture may contain many molecular species such as H_2O–heme–OH_2, H_2O–heme–CO, py–heme–OH_2, py–heme–py, py–heme–CO, etc., where the abbreviation "py" represents pyridine. Thus a quantitative treatment of the equilibrium mixture would include the evaluation of the equilibrium constants for the interconversion of the various pairs of different heme complexes. Fortunately in case of the dilute solutions studied in this work, the treatment of experimental data can be effectively simplified so that the major physical significance of these results can be seen readily without delving in the tedious procedure for evaluating the individual equilibrium constants.

The following solutions at *p*H 11 were prepared and studied: solution A, 0.5×10^{-4} *M* heme/l.; solution B, 0.5×10^{-4} *M* heme + 0.5×10^{-4} *M* pyridine/l.; solution C, 0.5×10^{-4} *M* heme × 1.0

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