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The Conformation of Native and Denatured Tropomyosin B*

Alfred Holtzer, Robert Clark, and Susan Lowey

ABSTRACT: Light-scattering, viscosity, and sedimentation experiments on solutions of rabbit tropomyosin B in several aqueous media show that the molecule is a rod 490 A long and 20 A in diameter and has a mass of 74,000 amu. The hypothesis that the molecule is a double-stranded, α-helical coiled coil is consistent with the diameter and mass per unit length found. The molecular structure of the native molecule is thus analogous to that found previously for paramyosin and light meromyosin fraction 1.

Similar experiments on solutions containing 5 M guanidine hydrochloride give the same molecular weight, indicating that the tropomyosin molecule consists of one covalently bonded unit. Comparison of the measured root mean square radius (110 A) in guanidine solutions with theories of chain statistics and experiments on rotational barriers in polypeptides suggests that the tropomyosin chain is not a simple, linear, random coil, but may contain cross linkages or loops.

Up to the present time, three fibrous proteins have been isolated that are believed to play a major role in the contraction of all muscles: myosin, actin, and tropomyosin; certain specialized muscles contain, in addition to these, large quantities of a fourth protein, paramyosin. Myosin, under suitable circumstances, can be broken into two fragments, one rodlike (light meromyosin fraction 1) and the other partially globular (heavy meromyosin) (Gergely, 1950, 1953; Mihalyi and Szent-Györgyi, 1953; Szent-Györgyi, 1953; Lowey and Holtzer, 1959; Szent-Györgyi *et al.*, 1960).

Actin and heavy meromyosin have relatively low α-helix content, which limits any further discussion about the folding of their polypeptide chains. This is not the case with light meromyosin fraction 1 (LMM),[1] tropomyosin, and paramyosin. All three of these proteins have an α-helix content greater than 90% (Cohen and Szent-Györgyi, 1957), which makes it possible to reach some conclusions about their tertiary structure, meaning the arrangement of the α-helices in the molecule. Using hydrodynamic and light-scattering techniques we have obtained a mass per unit length for both paramyosin (Lowey *et al.*, 1963) and LMM (Holtzer *et al.*, 1962; Lowey and Cohen, 1962) which is consistent with a molecular model of two α-helical chains in a cross section.

Since tropomyosin possesses about the same helix content as paramyosin and LMM, that is, has essentially the same secondary structure, it is reasonable to suppose, at least as a first hypothesis, that its tertiary structure is also the same. Yet, the earlier data on tropomyosin do not support this conclusion. One need only examine the sedimentation coefficients for the α-helical fibrous proteins to see the incongruities; paramyosin, LMM, and tropomyosin all reportedly have sedimentation coefficients of 3.0 S, and yet the molecular weights range from 53,000 for tropomyosin (Tsao *et al.*, 1951; Kay and Bailey, 1960) to 220,000 for paramyosin (Lowey *et al.*, 1963). The sedimentation coefficient of a rodlike macromolecule is, of course, more sensitive to its diameter than to its weight, but, nevertheless, some variation would be apparent if the tertiary structures were the same. Closer analysis of the earlier data on the molecular properties of tropomyosin shows, indeed, that tropomyosin, unlike its fellows, conforms to no simple, molecular model (Noelken, 1962).

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[1] Abbreviations used: LMM, light meromyosin fraction 1; Gu, guanidine; rms, root mean square.

Because of our reluctance to give up this attractive postulate of a simple structural relationship among these three fibrous proteins, and because our earlier investigations of myosin, the meromyosins, and paramyosin did differ significantly from previous findings, we decided to reinvestigate the molecular parameters of rabbit tropomyosin.

We find that tropomyosin in aqueous salt solutions has a rodlike shape, a mass of 74,000 ± 5000 amu, a length of 490 ± 50 A, a diameter of 20 A, and consists of two adjacent α-helices. The structure of native tropomyosin is thus analogous to paramyosin and LMM.

In addition, we have characterized the molecule in a strongly denaturing medium where it apparently is randomly coiled. The molecular weight is unchanged under these circumstances, indicating that the molecule consists of one covalently bonded entity. Furthermore, there is some evidence from the measurements of the root-mean-square (rms) radius of the denatured molecule that it is not a simple, linear, random chain but may be cross-linked or looped; it will be seen below that evidence on the latter point is not conclusive, but could be made more so if suitable measurements are made on certain model systems.

Experimental Methods and Treatment of Data

Reagents. Water was deionized before use. Guanidine hydrochloride was prepared from the carbonate (Eastman Highest Purity) by the method of Anson (1941). All other chemicals were reagent grade.

Protein Preparation. Tropomyosin was prepared from rabbit muscle by the method of Bailey (1948) with slight modifications (Noelken, 1962). Two isoelectric precipitations and three cycles of salting out with $(NH_4)_2SO_4$ were employed. The protein was lyophilized from a salt-free water solution and was stored at −20°.

Concentration. All protein concentration determinations in this study are based on micro-Kjeldahl analysis; the nitrogen content was taken as 16.7% (Bailey, 1948). In most cases, nitrogen analyses were carried out directly either on the stock solution from which the sample solutions were made or on the samples themselves.

In the light-scattering experiments, however, it is not feasible to perform the measurements on solutions prepared by quantitative dilution of a stock solution (see below). Yet, preparation of individual samples would require a prohibitively large number of nitrogen analyses. Therefore, a stock solution of the particular protein preparation in the medium of interest was used to determine the extinction coefficient at 277 mμ, and the individual sample concentrations were determined by measurement of the optical density at this wavelength. The extinction coefficients in the two media most commonly used in this study (1.00 M KCl, 0.1 M K[PO$_4$],[2] pH 7.4, and 0.39 M KCl, 0.01 M HCl, pH 2.0) were found to be the same; the optical density of a 1% solution of 1-cm path length at 277 mμ is 3.14.

The determination of protein concentration of solutions containing large amounts of guanidine hydrochloride presents a special problem. The high nitrogen content of the solvent makes direct Kjeldahl analysis impractical. The following procedure, typical of the method used, was found to be highly reproducible.

A tropomyosin stock solution was prepared and thoroughly dialyzed *vs.* 1.00 M KCl, 0.100 M K[PO$_4$], pH 7.4.[3] The protein concentration of this solution was determined by micro-Kjeldahl analysis. Solid guanidine hydrochloride was dried *in vacuo* over P_2O_5, and a 47.77-g (0.5 mole) sample was weighed into a 100-ml volumetric flask; this flask and the erlenmeyer containing the protein stock solution were suspended in a water bath kept at 20.0 ± 0.1°, which was also, roughly, the temperature of the room. Three successive 20.00-ml samples of the protein stock solution were then pipeted into the volumetric flask, each transfer being followed by swirling the volumetric flask. Finally, the solution was diluted to the 100-ml mark with dialysate, and final mixing was effected by successive slow inversions of the stoppered flask. The pH of the final solution was found to be 6.1.

The protein concentration of the final solution is, then, accurately known (60% of the concentration of the stock solution), as is the concentration of guanidine hydrochloride (5.00 M). The final concentrations of KCl and K[PO$_4$], of course, depend upon the volume of dialysate that has to be added in the last step in order to reach the 100-ml mark. In one experiment, this volume was determined by dispensing the solution from a buret; it was found to be 4.15 ml. Thus, the KCl concentration in the final solution is very close to 0.64 M and the K[PO$_4$] very close to 0.064 M. In any case, the properties of interest are insensitive to the exact concentrations of KCl and K[PO$_4$] so long as they are near this level. Solutions of tropomyosin in guanidine hydrochloride solutions containing no KCl, however, display anomalously high second virial coefficients and are therefore difficult to work with.

Solvent for blanks, or for use as diluent, may be prepared in exactly the same way. Furthermore, since the protein concentration is accurately known, determination of the optical density provides an extinction coefficient, so that the concentration of any derived sample solution in this medium can be checked. In particular, for solutions made in the foregoing manner for two different tropomyosin preparations, we find OD 3.00 ± 1% for a 1% solution in a 1.00-cm cell at 277 mμ.

Viscosity. Viscosity measurements were made with Ostwald-Fenske viscometers at 20.0 ± 0.02°. Flow

[2] K[PO$_4$] is a mixture of K_2HPO_4 and KH_2PO_4.

[3] Since it will be necessary to make frequent reference to solvent media that are rather complex, some sort of shorthand notation is desirable. A convenient method involves writing the chemical formula of each component (other than water) with its molarity as a subscript, followed by the pH. The solvent mentioned then becomes $(KCl)_{1.0}(K[PO_4])_{0.1}(7.4)$. This method will be used throughout.

times for solvents were in the range of 280–350 sec. Flow times for tropomyosin solutions ranged from 50 to 200 sec over solvent flow time. These long efflux times make kinetic energy corrections negligible. Solutions were routinely centrifuged for 2 hr at 40,000*g* prior to measurement.

Ultracentrifugation. Sedimentation constants were calculated from plots of log (distance) *vs.* time. The usual corrections were made for solvent viscosity and density.

Archibald molecular weights were obtained essentially as described in previous publicat.ons (Lowey and Holtzer, 1959; Lowey and Cohen, 1962). As before, the over-all accuracy of the procedure was tested by a determination of the molecular weight of bovine serum mercaptalbumin. In the present investigation, the reciprocals of the apparent molecular weights were plotted as a function of the concentration existing at the meniscus. This latter quantity is more meaningful than bulk concentration in that it correctly represents the concentration of the material at the location and time for which the calculation is made. Two procedures were used to vary the concentration: (1) the stock solution was quantitatively diluted in successive runs, or (2) a 1% sample was run at increasing speeds, from 8225 to 20,000 rpm, thereby lowering the protein concentration at the meniscus (Mueller, 1964). All runs were performed at 20°. Two different tropomyosin preparations were used.

In order to obtain the molecular weight of isoionic protein from ultracentrifugation experiments on multicomponent systems, the apparent partial specific volume should be evaluated using dialysate as solvent and expressing protein concentration in grams of isoionic protein per milliliter of solution (Casassa and Eisenberg, 1960, 1961). Using this procedure, Kay (1960) obtained a value of 0.739 ml/g for rabbit tropomyosin in $(KCl)_{1.0}$-$(K[PO_4])_{0.05}(7.0)$. We have assumed that this quantity has this same value for all solutions employed. Support for this assumption is provided by a study of the apparent partial specific volume of myosin in 5 M guanidine hydrochloride (Gu·HCl) (Kielley and Harrington, 1960), wherein it was found to differ from that previously obtained for 0.5 M KCl solutions (Parrish and Mommaerts, 1954) by only 1%.

Refractive Index Increment. The quantity $\Delta n/c$ (Δn = difference in refractive index between tropomyosin solution and dialysate, c = protein concentration in grams of isoionic protein per milliliter of solution) was determined in the same manner as previously described (Lowey *et al.*, 1963). All measurements were made with light of wavelength 436 mμ. The region of protein concentration used was the same as in the light-scattering experiments. Determinations were made on tropomyosin dissolved in three different aqueous media: (1) $(KCl)_{0.39}$-$(HCl)_{0.01}(2.0)$; (2) $(KCl)_{1.0}(K[PO_4])_{0.1}(7.4)$; (3) $(Gu{\cdot}HCl)$-$_{5.0}(KCl)_{0.64}(K[PO_4])_{0.064}(6.1)$. For measurements of protein dissolved in the third medium, difficulties encountered previously (Lowey *et al.*, 1963) are compounded by the very large concentration of added electrolyte and by the diminished magnitude of the protein refractive increment. For these solutions, therefore, special precautions are necessary. The following procedure was found to be effective.

First, the cell holder of the Brice-Phoenix differential refractometer was modified so that the cell can readily be removed and reproducibly replaced. This was accomplished by placing a permanent stop on the floor of the holder in front of the cell and a spring-loaded ball bearing in a hole in one of the side plates. The cell is then held in proper horizontal position perpendicular to the light beam by the pressure of the ball bearing on one side of the cell which causes the other side to press against the two adjustable positioning screws already present in the instrument. Horizontal alignment parallel to the light beam is accomplished by pushing the cell flush against the floor stop. Reproducible vertical alignment is assured by pressing down on the cell so that the cell bottom is flush with the floor of the holder. Next, a large drybox with a transfer air lock, glove ports, etc., was converted to a "wetbox" by placing on the inside several large beakers filled with solvent. After several days, the inside of the box became, in essence, a laboratory (at room temperature) whose atmosphere was in equilibrium with the solvent to be used. Tropomyosin solutions and the guanidine hydrochloride solvent were prepared in the manner described above and introduced into the wetbox along with the refractometer cell and other necessary equipment. The solution was then dialyzed *vs.* solvent for 2 days. Through the glove ports, the dialysis bag was opened, and the cell was loaded with solution and dialysate. The cell was sealed with a lightly greased ground-glass cover, removed from the box, and immediately placed in the instrument. After less than 10 min, thermal equilibrium was attained and the measurement of Δn could be reliably made. Afterwards, the optical density of the same solution was measured, and the concentration was calculated from it as described above.

Light Scattering. The instrumentation and techniques employed are essentially as described previously (Schuster, 1963; Holtzer *et al.*, 1962; Holtzer and Lowey, 1959).

Recently, the influence of back-reflection in light scattering has been reassessed (Tomimatsu and Palmer, 1963). In view of this development, it is worth emphasizing that the cells used in this study are of the erlenmeyer type, which show no back-reflection. To ensure that this requirement is satisfied, the dissymmetry of the same protein solution was measured in the erlenmeyer cell and in a semioctagonal cell; the latter have been shown to be free of back-reflection difficulties (Tomimatsu and Palmer, 1963). An erlenmeyer was rejected for use if the dissymmetries did not agree to better than 1%.

Immediately after measurement of the scattering, the optical density of the protein solution was determined and the concentration calculated from it. Attempts to perform successive dilutions in the scattering cell always failed because of formation of large, visible aggregates on stirring. The practical, accessible protein concentration region lies between 0.20 and 0.59% for 1 M KCl and 0.30 and 1.8% for 5 M guanidine solutions. The lower concentration limit is set by the magnitude of excess

scattering, which is less in guanidine because of the diminished refractive increment; the upper limit is set by difficulties in removing dust and large aggregates, a problem less serious in a good solvent like concentrated guanidine hydrochloride.

Treatment of Light Scattering Data. Measurements of protein solutions in $(KCl)_{1.0}(K[PO_4])_{0.1}(7.4)$ or in $(KCl)_{0.39}(HCl)_{0.01}(2.0)$ present no special problems of interpretation; details may be found in earlier papers (Holtzer *et al.*, 1962; Lowey *et al.*, 1963). Briefly, the Zimm equation (Zimm, 1948) was used

$$Kc/R_\theta = 1/MP(\theta) + 2Bc + F(B, C, \theta)c^2 + \ldots \quad (1)$$

where θ is the scattering angle; R_θ is the difference in Rayleigh ratio between solution and dialysate; $P(\theta)$ is the particle-scattering factor; B is the second virial coefficient; $F(B, C, \theta)$ indicates a rather complex function of the second (B) and third (C) virial coefficients and of θ; and K is equal to $2\pi^2 n_0^2 (\Delta n/c)^2/N_0\lambda^4$, where n_0 is the dialysate refractive index, N_0 is Avogadro's number, and λ is the wavelength, *in vacuo*, of incident light. With these definitions, M becomes, to good approximation, the molecular weight of the isoionic protein (Casassa and Eisenberg, 1960, 1961).

Plots of Kc/R_θ *vs.* $\sin^2(\theta/2)$ were always found to be linear for tropomyosin solutions in these two media; the best line was determined by least squares, using an electronic computer; the "apparent" molecular weight (*i.e.*, R_0/Kc) was obtained from the intercept at $\theta = 0$ for each solution, and the mean molecular radius from the slope and intercept in the usual way (Geiduschek and Holtzer 1958). The scattering is sufficiently great in these solutions that it is feasible to measure solutions of such low concentration that terms in eq 1 beyond the second (and, to good approximation, even the second) may be neglected.

Solutions containing high concentrations of guanidine hydrochloride, on the other hand, do present special difficulties. In 5 M guanidine, the protein refractive increment, and consequently the scattering, is low and the second virial coefficient is high. It is impossible, therefore, to measure the scattering accurately in a region of concentration sufficiently low that the contribution of the third virial coefficient may be neglected. The data were consequently treated using, essentially, a method described by Flory (1953).

Flory's method was used in the following form. Since plots of Kc/R_θ *vs.* $\sin^2(\theta/2)$ are accurately linear for all of the solutions measured, there is no difficulty in fitting the angular scattering envelopes to straight lines by the method of least squares and in extrapolating to zero angle. For the scattering at zero angle, Zimm's equation (eq 1) reduces to (Zimm, 1948)

$$Kc/R_0 = 1/M + 2Bc + 3Cc^2 + \ldots \quad (2)$$

Flory points out that the theory of dilute polymer solutions leads to the conclusion that the third virial coefficient is proportional to the square of the second, a relationship which can be written as

$$C = gMB^2 \quad (3)$$

where g is the constant of proportionality. If eq 3 is introduced into (2) and, for convenience, a quantity Γ_2, defined by

$$\Gamma_2 \equiv MB \quad (4)$$

is also inserted, we find

$$Kc/R_0 = (1/M)(1 + 2\Gamma_2 c + 3g\Gamma_2^2 c^2) \quad (5)$$

assuming that terms beyond the third are negligible.

Extensive studies on solutions of synthetic polymers show that, to good approximation, the numerical value of g may be taken as 0.25 (Flory, 1953). Equation 5 then shows that the concentration dependence of the experimental quantity Kc/R_0 is dictated by the two quantities M and Γ_2. Flory recommends evaluating these by the following means. First, a theoretical curve is drawn of $\log(1 + 2\Gamma_2 c + 0.75\Gamma_2^2 c^2)$ *vs.* $\log(\Gamma_2 c)$. The data are then plotted, to the same scale and on a transparent sheet, as $\log(Kc/R_0)$ *vs.* $\log c$. The graph of the experimental points is superimposed on the theoretical one so that both coordinate axes are in congruence, and the former curve is translated until the data best fit the theoretical curve beneath. As eq 5 makes clear, the ordinate shift provides the molecular weight and the abscissal shift yields the value of Γ_2. These two quantities, inserted into (4), give B, and then eq 3 provides C. Thus, the molecular weight and the second and third virial coefficients can be determined.

In more conventional light-scattering investigations, where terms beyond the second are negligible, eq 1 is used as the basis for (linear) extrapolation of Kc/R_θ to zero concentration for scattering angles other than zero. The limiting ($\theta \to 0$) slope of the resulting ($c = 0$) curve is then employed to obtain the mean-square radius. Unfortunately, in the present instance the third term is not negligible and, furthermore, at scattering angles other than zero, eq 1 must be used rather than 2; thus, the third term is not only appreciable, but dependent on B as well as C, and also on the scattering angle; the extrapolation method of Flory, so helpful in the zero-angle case, is therefore not strictly valid.

Nevertheless, Flory's approach does provide a systematic, objective method of extrapolation, and was simply used as such for data at angles other than zero. The ordinate shift was interpreted as $1/MP(\theta)$; the abscissal shift, of course, has no physical meaning except as an empirical characterization of the concentration dependence of Kc/R_θ for the given angle. By this means, $P(\theta)^{-1}$ was obtained as a function of angle, and from it the mean molecular radius was obtained.

It is worth noting that this procedure is, in reality, no more suspect than the usual use of a linear extrapolation based on eq 1 when the third term is small. It must be kept in mind that eq 1 itself is valid only in the "single

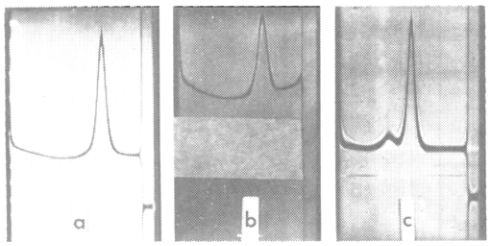

FIGURE 1: Sedimentation patterns of tropomyosin. All runs at 20°, 59,780 rpm, and 0.7% protein. a, $(KCl)_{0.39}(HCl)_{0.01}(2.0)$, bar angle 50°; b, $(KCl)_{1.0}$-$(K[PO_4])_{0.1}(7.4)$, bar angle 60°; c, $(KCl)_{0.5}(K[PO_4])_{0.1}$-(7.4), bar angle 50°. See footnote 3 for shorthand notation.

contact" approximation (Zimm, 1948), and that, otherwise, the coefficient of c in the second term depends on angle and concentration. It has become customary to ignore the possible effect on the second term of failure of the single contact approximation, and, therefore, extension of this custom to the third term is hardly cause for alarm. In fact, because of its objectivity, the Flory method has some advantages over the usual graphical procedure.

Results

Refractive Index Increment. Results for the three solvent media of interest are: (1) $(KCl)_{0.39}(HCl)_{0.01}(2.0)$, three tropomyosin solutions ranging between 0.46 and 0.71% protein gave an average value of $\Delta n/c = 0.1917$ ml/g, the total spread of the three determinations being 0.0010 ml/g. (2) $(KCl)_{1.0}(K[PO_4])_{0.1}(7.4)$, three protein solutions in the range 0.45–0.94% gave $\Delta n/c = 0.1870$ ml/g with a spread of 0.0052 ml/g. (3) $(Gu \cdot HCl)_{5.0}$-$(KCl)_{0.64}(K[PO_4])_{0.064}(6.1)$, three solutions (0.4–1.0%) gave $\Delta n/c = 0.147$ ml/g with a spread of 0.0010 ml/g.

Sedimentation Velocity. The homogeneity of a protein preparation may be assessed, in part, by examination of its sedimentation pattern; a single peak in the ultracentrifuge is a necessary, though not a sufficient condition for monodispersity. Tropomyosin samples used in this study were found to satisfy this condition in acid and alkaline media, in 1 M KCl at pH 7, and in 5 M guanidine hydrochloride; however, the well-known tendency of tropomyosin toward aggregation at pH 7 (Tsao *et al.*, 1951) is evidenced by the small leading peak in the pattern for 0.5 M KCl (Figure 1). Thus, the aggregation is less sharply dependent on ionic strength for tropomyosin than for myosin or paramyosin, the latter proteins being insoluble at low ionic strengths, but completely molecularly dispersed at 0.5 M.

Results of some of the sedimentation-velocity measurements for various solvent media are displayed in Figure 2. The data points shown for $(KCl)_{1.0}(K[PO_4])_{0.1}(7.4)$ and for $(KCl)_{0.39}(HCl)_{0.01}(2.1)$ fall on the same line, given by

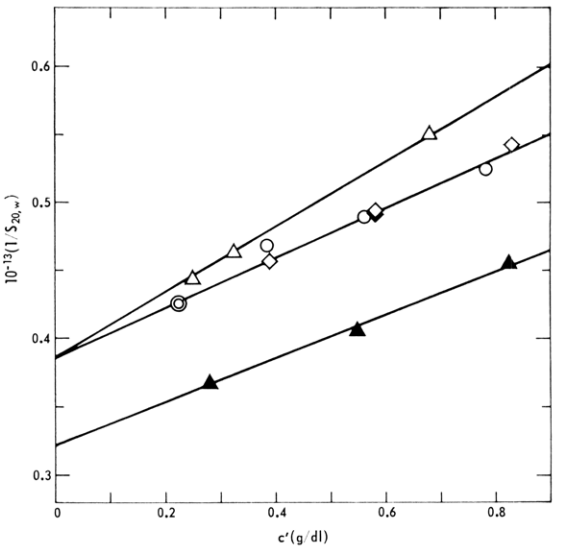

FIGURE 2: Sedimentation coefficients of tropomyosin in various solvents. Filled triangles, $(KCl)_{0.09}$-$(K[CO_3])_{0.005}(9.7)$; open triangles, $(KCl)_{0.09}(HCl)_{0.01}$-(2.1); open squares, $(KCl)_{0.39}(HCl)_{0.01}(2.3)$; open circles, $(KCl)_{1.0}(K[PO_4])_{0.1}(7.4)$. See footnote 3 for shorthand notation.

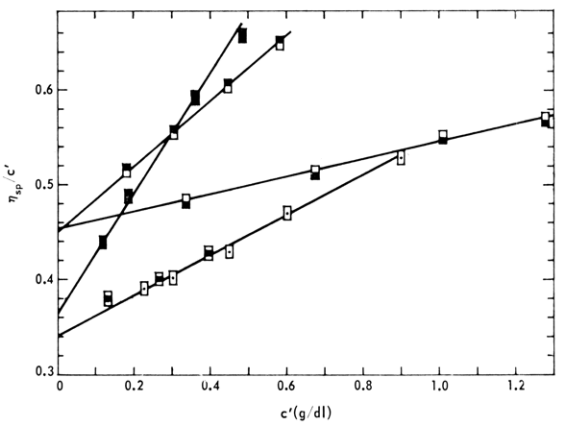

FIGURE 3: Viscosity data for tropomyosin in various solvents. Top-filled rectangles, $(KCl)_{0.5}(K[PO_4])_{0.1}(7.2)$; open rectangles, $(KCl)_{1.0}(K[PO_4])_{0.1}(7.2)$; center-filled rectangles, $(KCl)_{2.0}(K[PO_4])_{0.07}(7.2)$; bottom-filled rectangles, $(Gu \cdot HCl)_{5.0}(KCl)_{0.64}(K[PO_4])_{0.064}(6.1)$; filled rectangles, $(KCl)_{0.39}(HCl)_{0.01}(2.0)$. See footnote 3 for shorthand notation.

$$10^{-13}(1/s_{20,w}) = (1/2.59) + 0.182c' \qquad (6)$$

where c' is protein concentration in g/100 ml of solution. The agreement of results in these solvents suggests that tropomyosin is monomeric under these conditions. Furthermore, since optical rotatory dispersion studies in these same solvents (Noelken, 1962) indicate that almost all of the molecule is in the α-helical configura-

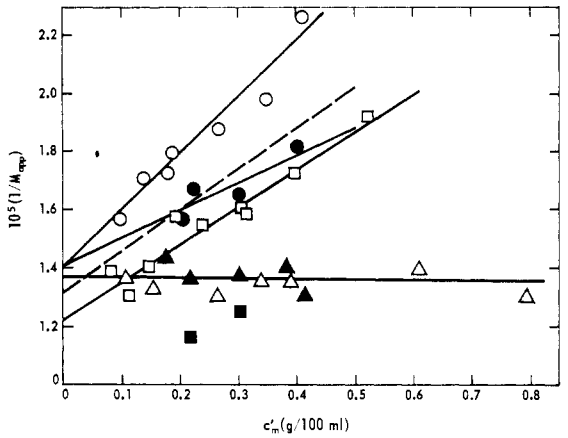

FIGURE 4: Data from Archibald runs on tropomyosin in various solvents. Filled squares, $(KCl)_{0.5}(K[PO_4])_{0.05}$-(7.2); open triangles, $(KCl)_{1.0}(K[PO_4])_{0.1}(7.2)$; open squares, $(KCl)_{0.09}(HCl)_{0.01}(2.1)$; filled triangles, $(KCl)_{0.39}(HCl)_{0.01}(2.3)$; open circles, $(KCl)_{0.19}(KOH)_{0.01}$-(12); filled circles, $(Gu\cdot HCl)_{5.0}(KCl)_{0.64}(K[PO_4])_{0.064}$-(6.1). Dashed line was calculated as predicted from light-scattering results on guanidine solutions (*i.e.*, from eq 8). See footnote 3 for shorthand notation.

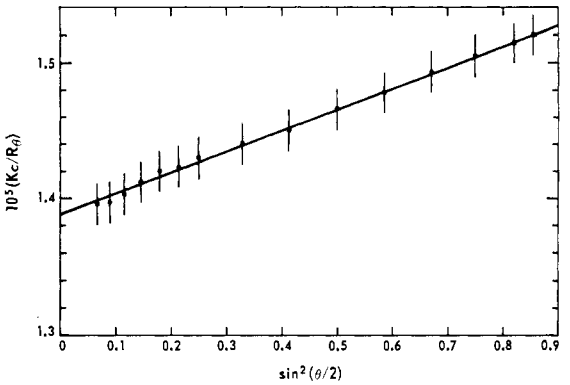

FIGURE 5: Experimental angular scattering envelope of tropymyosin. Data for 0.585% protein solution in $(KCl)_{1.0}(K[PO_4])_{0.1}(7.4)$. See footnote 3 for shorthand notation.

tion ($b_0 \sim 600$), these media must assume some prominence in any characterization of the tropomyosin molecule. There is evidence from optical rotatory dispersion that tropomyosin is somewhat unfolded at high pH (Lowey, 1965); this may account for the higher value of the intrinsic sedimentation coefficient ($[s_{20,w}]$.

Viscosity. Because of its importance in macromolecular characterization and its sensitivity to the presence of aggregates, the intrinsic viscosity of tropomyosin was measured in a variety of solvent media. Some of these results are displayed in Figure 3. The reduced, specific viscosity is linear in concentration for all cases and the data were fit to the well-known relation

$$\eta_{sp}/c' = [\eta] + k'[\eta]^2c' \qquad (7)$$

The values of $[\eta]$ and of k' are recorded in Table I. Since optical rotatory dispersion measurements indicate essentially the same b_0 for the first six entries in Table I, the variation in viscosity must be due to aggregation and not to a conformation change. Furthermore, guanidine solutions excepted, the values of k' are rather high even in those solvents displaying the lowest intrinsic viscosities. Ordinarily, in work on synthetic polymers, aggregation is suspected if $k' > 1.0$. However, this shibboleth is without theoretical foundation, and we believe that, in this instance, it must be discounted. The evidence against the existence of such aggregation in solutions showing the minimum viscosity is rather strong: (1) the intrinsic viscosity at pH 7.4 decreases with increasing ionic strength, becoming constant after 1.0 M; (2) the intrinsic viscosity at pH 2.0 also falls with increasing salt concentration, reaching the same minimum value as at the higher pH; (3) inhomogeneity, as indicated by the sedimentation patterns, disappears under solvent conditions consistent with expectations from the viscosity results; (4) molecular weight measurements (see below) in such solvents are completely self-consistent and show no anomolous concentration dependence. We conclude, therefore, that the intrinsic viscosity of "native," monomeric tropomyosin is 0.35 dl/g.

TABLE I: Viscosity of Tropomyosin Solutions.

Medium[a]	$[\eta]$ (dl/g)	k'
$(KCl)_{0.5}(K[PO_4])_{0.1}(7.2)$	0.45	1.7
$(KCl)_{1.0}(K[PO_4])_{0.1}(7.2)$	0.34	1.9
$(KCl)_{2.0}(K[PO_4])_{0.07}(7.2)$	0.34	1.9
$(KCl)_{0.09}(HCl)_{0.01}(2.0)$	0.53	2.1
$(KCl)_{0.19}(HCl)_{0.01}(2.0)$	0.47	2.2
$(KCl)_{0.39}(HCl)_{0.01}(2.0)$	0.36	4.9
$(Gu\cdot HCl)_{5.0}(KCl)_{0.64}$-$(K[PO_4])_{0.064}(6.1)$	0.45	0.46

[a] Shorthand notation for solvents described in footnote 3.

There is no such difficulty with the data from guanidine solutions; the value of k' found is in keeping with expectations for a molecularly dispersed polymer. The intrinsic viscosity is 0.45 dl/g.

Archibald Determination of Molecular Weights. Data were collected largely for systems indicated to be molecularly dispersed by the criteria of sedimentation velocity and viscosity. A few measurements were also made on systems in which evidence of aggregation exists. Some of the results are plotted in Figure 4.

The molecular weights obtained for molecularly dispersed solutions are all in satisfactory agreement

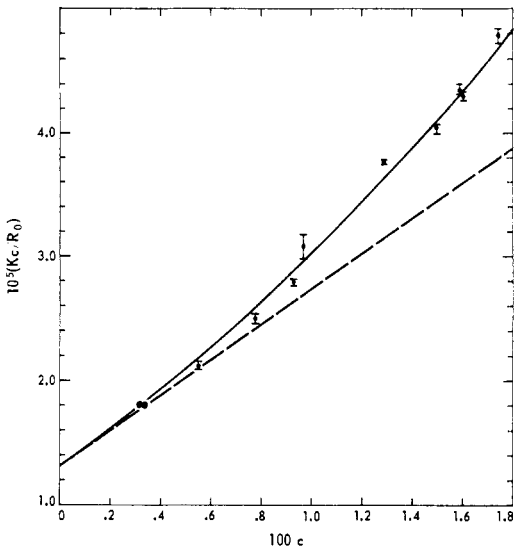

FIGURE 6: Concentration dependence of the zero-angle scattering of tropomyosin in $(Gu \cdot HCl)_{5.0}(KCl)_{0.64}(K[PO_4])_{0.064}(6.1)$. The plotted points at the two lowest concentrations represent one measured solution each. All other plotted points represent the average of intensity measurements of at least two and at most four separate solutions at the given protein concentration; the error bars represent the total spread of the results at that concentration. Solid line is eq 8. Dashed line is for the first two terms of eq 8. See footnote 3 for shorthand notation.

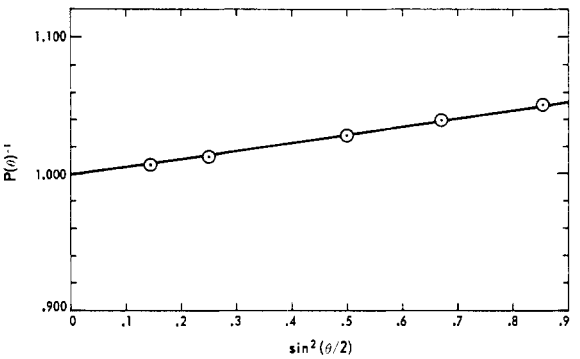

FIGURE 7: Angular dependence of the reciprocal particle-scattering factor of tropomyosin in $(Gu \cdot HCl)_{5.0}(KCl)_{0.64}(K[PO_4])_{0.064}(6.1)$. Points were obtained by the procedure described in the text. The straight line gives a root-mean-square radius of 110 A. See footnote 3 for shorthand notation.

with one another; these include data for solutions of high ionic strengths at pH 7.2, 2.1, and 12.0, and for solutions containing guanidine hydrochloride; the average molecular weight found in these four media is 72,000 with a total spread of 2000.

The molecular weights found in media where viscosity and sedimentation rate measurements gave evidence of aggregation are considerably higher. Results for two such solutions are shown in Figure 4 for comparison.

Light Scattering. A large number of measurements were made on solutions of "native," monomeric tropomyosin in $(KCl)_{1.0}(K[PO_4])_{0.1}(7.4)$ (nine solutions measured) and in $(KCl)_{0.39}(HCl)_{0.01}(2.0)$ (six solutions). In both cases, individual measurements showed no systematic variation of Kc/R_0 with concentration (*i.e.*, the second virial coefficient is apparently close to zero, in agreement with the results from Archibald runs). The average of all the molecular weight values in the near-neutral medium is 77,000 (with a total spread of 10,000) and in acid medium 81,900 (with a spread of 10,200). Two separate protein preparations were represented. A sample angular envelope is displayed in Figure 5.

As is evident from Figure 5, the root-mean-square molecular radius ($\langle \rho^2 \rangle^{1/2}$) is rather difficult to measure accurately in this system since the dissymmetries are so low ($R_{45}/R_{135} \sim 1.1$); the result obtained in this study is 142 A with a total spread of 20 A.

The angular scattering envelopes of 23 solutions of tropomyosin in $(Gu \cdot HCl)_{5.0}(KCl)_{0.64}(K[PO_4])_{0.064}(6.1)$ were measured. After linear extrapolation to zero angle, the data are as shown in Figure 6; the solid curve was obtained as described in the experimental section and is given by

$$Kc/R_0 = (1/76{,}200) + 2(7.13 \times 10^{-4})c + 3(9.68 \times 10^{-3})c^2 \quad (8)$$

The evaluation of $P(\theta)^{-1}$ was carried out as described in the experimental section and provided the results shown in Figure 7. Accurate estimation of the rms radius is again difficult because of the low dissymmetry; the result here is 110 A.

The molecular parameters deduced from several different kinds of measurements are summarized in Table II.

Discussion

Molecular Weight. The molecular weights found in this study by light scattering measurements of tropomyosin dissolved in $(KCl)_{1.0}(K[PO_4])_{0.1}(7.4)$ and $(KCl)_{0.39}(HCl)_{0.01}(2.0)$ and by the Archibald measurements of protein in the same media and in $(KCl)_{0.2}(KOH)_{0.01}(12)$ solutions are quite self-consistent, yielding an average value of 74,000.

These results are, however, quite inconsistent with previous determinations on solutions in essentially the same media, using measurements of osmotic pressure (64,500; Tsao *et al.*, 1951) and light scattering (53,000; Kay and Bailey, 1960; Sanders, 1952). We can offer no definitive or even cogent reasons for the discrepancy. The disagreement is reminiscent of a similar one that has been discussed in detail (Lowey *et al.*, 1963), and we refer the reader to the earlier paper for those parts of the discussion that are applicable.

Of course, one could ascribe the discrepancy to ag-

TABLE II: Molecular Parameters of Tropomyosin.

Medium[a]	$[\eta]$ (dl/g)	$10^{13}s_{20,w}$ (sec.$^{-1}$)	$10^{-3}M$	$10^{4}B$ (mole ml/g^2)	$[\rho^2]^{1/2}$ (A)
$(KCl)_{0.39}(HCl)_{0.01}(2.0)$	0.36	2.59	76.7	~0	153
$(KCl)_{1.0}(K[PO_4])_{0.1}(7.4)$	0.34	2.59	74.2	~0	142
$(KCl)_{0.19}(KOH)_{0.01}(12)$	...	...	71.5	9.8	...
$(Gu\cdot HCl)_{5.0}(KCl)_{0.64}$-$(K[PO_4])_{0.064}(6.1)$	0.45	...	73.8	7.13	110

[a] Shorthand notation for solvents described in footnote 3.

gregation, which is a problem ever-present with fibrous proteins and particularly troublesome with tropomyosin. Indeed, in the early days of this investigation we were much inclined to this view. However, this explanation is evidently as incorrect as it is facile. In the first place, the observed agreement of the minimum molecular weights found for a variety of solvent media would require for its explanation a combination of circumstances coincidental beyond belief. Second, the trend of viscosities and the appearance of the sedimentation patterns (as discussed above) as the solvent is altered argues strongly against aggregation. Third, it is very difficult to explain how an aggregated solution (ours) could display a lower intrinsic viscosity (0.35 dl/g) than the presumably unaggregated one (0.57 dl/g, Tsao *et al.*, 1951); the possible rationalization that the aggregation is side-to-side (lowering the axial ratio and hence the intrinsic viscosity) is at variance with studies showing the aggregation of tropomyosin to be end-to-end (Kay and Bailey, 1960). Finally, a side-to-side aggregate would have a larger sedimentation rate than the monomer; instead, we observe one peak in the ultracentrifuge with a lower intrinsic sedimentation coefficient (2.6 S) than that found by earlier workers (3.0 S; Kay and Bailey, 1960).

The measurements of guanidine hydrochloride solutions of tropomyosin, under conditions where denaturation is essentially complete (*i.e.*, where $b_0 \cong 0$; Noelken, 1962), give almost exactly the same molecular weight as obtained in the aforementioned media. It is, of course, highly unlikely that aggregates, as postulated above, could continue to exist in such a good solvent ($B \cong 7 \times 10^{-4}$ mole ml/g^2).

The agreement of the molecular weights in benign and in denaturing media implies that the tropomyosin molecule is a single, covalently bonded entity. Since the tropomyosin molecule may contain one disulfide linkage (Szent-Györgyi *et al.*, 1959), however, this result does not necessarily imply that this molecule consists of only one polypeptide chain.

Molecular Configuration in Benign Media. Since optical rotatory dispersion experiments indicate that tropomyosin is almost completely α-helical in these media, the rod model seems the most reasonable to use. A method of testing the self-consistency of this model has been adequately described previously (Holtzer and Lowey, 1959). Briefly, the method involves three separate computations of the rod (*i.e.*, rigid string of beads) diameter from the experimental data and the equations for, respectively, the intrinsic viscosity, intrinsic sedimentation coefficient, and the partial specific volume.

Using this model, the experimental radius (142 A) may be converted to a rod length of 490 A. The measured intrinsic viscosity (0.35 dl/g) corresponds, as is seen from the viscosity equation, to an axial ratio of 24.5, which, in conjunction with the rod length, provides a diameter of 20 A. The measured sedimentation coefficient, used with the axial ratio (from viscosity) in the sedimentation equation, gives a diameter of 20 A. The partial specific volume, used in *its* equation along with the measured molecular weight and length, gives a value for the diameter of 19 A. These results are thus completely self-consistent and are reminiscent of the results for paramyosin (Lowey *et al.*, 1963) and light meromyosin fraction I (Lowey and Cohen, 1962; Holtzer *et al.*, 1962), showing the same diameter.

Another way of demonstrating the internal consistency of the rod model involves combining the theoretical expressions for intrinsic viscosity and sedimentation coefficient and using the resulting expression, with the experimental values of $[\eta]$ and $[s]$, to calculate the molecular weight (Holtzer and Lowey, 1963). The value obtained is 78,000, in excellent accord with the absolute values from light scattering and ultracentrifugation.

The number of α-helical chains in tropomyosin may also be computed. The amino acid analysis of this protein indicates 834 residues/10^5 g (Kominz *et al.*, 1957) or 634 residues/molecule; this implies a length of (coiled-coil) α-helix of $1.48 \times 634 = 937$ A. The average number of chains packed side-to-side is thus $937/490 = 1.91$, or, within experimental error, two chains. In this respect, as in the others noted, tropomyosin is very much like paramyosin and LMM. All three proteins seem to be related by a similar principle of over-all design.

Since the molecular weight measurements of solutions in guanidine have already led us to conclude that the tropomyosin molecule consists of one covalently bonded entity, the question remains as to the relationship of the two adjacent helical chains; *i.e.*, do they form, when unwound, one cyclic chain or one linear

chain or two separate, but disulfide-linked, chains, etc.?

The authors hardly need point out the utility of chemical methods of end-group analysis in deciding questions of this kind. Although a review of the work in this field would be out of place here, it is worth noting that, in spite of some disagreement as to details, it appears clear that there is at least one free end group per molecule (Kominz *et al.*, 1957; Jen *et al.*, 1958). One should bear in mind, however, that the word "cyclic" as operationally defined in an end-group study of a polypeptide chain has not quite the same meaning as it would in a study of physical properties. A single polypeptide chain could be cross-linked at a residue near (but not at) each end. This cross-link could be a disulfide bridge or perhaps any of the "atypical" linkages sometimes found in certain proteins and polypeptide hormones. Such a chain would reveal two typical terminal groups in a chemical assay, and yet be a cyclic chain from the, unfortunately rather coarse-grained, macromolecular point of view. For this reason, the physical and chemical methods of analysis must be viewed as complementary. In the discussion below, we will employ the word "cyclic" in the gross, physical sense noted.

Molecular Configuration in Guanidine Solutions. Since optical rotatory dispersion measurements indicate little or no α-helix content for tropomyosin in solutions containing 5 M guanidine hydrochloride, the most reasonable model to adopt is that of a random coil. Almost all of the detailed experimental and theoretical work on random coils refers to linear (noncyclic) chains; consequently, it is convenient to examine the suitability of this model before considering other possibilities.

Viewed as a linear random coil, a molecule of the given measured mean radius (110 A) would have a root-mean-square end-to-end separation of 269 A. We now inquire whether this value is a reasonable one by comparing its magnitude, after suitable corrections, first with expectations from the theory of polymer chain statistics and second with expectations from the theory of viscosity of polymer solutions.

The observed value of the rms end-to-end length of a random coil depends on several properties: (1) the number of links in the chain, *i.e.*, the degree of polymerization; (2) the chemical nature of the chain, *i.e.*, bond angles and lengths; (3) barriers to rotation about the chemical bonds; (4) thermodynamic interaction of polymer segments with solvent and with other polymer segments. Of these, the first is readily calculated from the polymer and monomer molecular weights, the second can usually be calculated by vector analysis if the characteristic bond distances and angles are known, the third is unknown, and the fourth can be estimated in several ways, one being, for example, from the second virial coefficient. If all of these estimates are carried out, the experimental end-to-end distance provides a measure of (3), which then, by comparison with other known systems, may be judged as "reasonable" or "unreasonable," thus providing support for (or casting doubt on) the validity of the model.

Proceding with this program, we write the general relationship between the mean-square end-to-end distance and the degree of polymerization (in this case, n, the number of amino acid residues/molecule) in the form (Flory, 1953, Chapter X; Volkenstein, 1963, Chapter IV):

$$\langle r^2 \rangle = n b_{fo}^2 z^2 \alpha^2 = \langle r^2 \rangle_0 \alpha^2 \qquad (9)$$

$$\langle r^2 \rangle_{fo} = n b_{fo}$$

where b_{fo}, the effective bond length of the corresponding chain with free rotation about all single bonds, is a function of bond angles and distances; z is a parameter introduced to characterize rotational barriers; α is the Flory expansion factor, which accounts for the thermodynamic interactions mentioned above (Flory, 1953; Chapters XIII and XIV); $\langle r^2 \rangle_0$ represents the mean-square end-to-end separation unperturbed by the latter thermodynamic effects; and $\langle r^2 \rangle_{fo}$ is the unperturbed mean-square end-to-end separation for the corresponding freely rotating (about all single bonds) chain.

Equation 9, of course, holds only in the limit of large n. We will refer to a coiled molecule as "Gaussian" if, in solvents in which $\alpha = 1$, the quantity $\langle r^2 \rangle / n$ is a constant, independent of n, as required by eq 9. A coil, to be Gaussian, must be sufficiently long, as well as sufficiently flexible (Benoit and Doty, 1953). That the tropomyosin molecule *is* sufficiently long to be Gaussian may be seen in two ways: (1) A helpful rule of thumb is that, for a Gaussian coil, $\langle r^2 \rangle^{1/2} / L < 0.33$, where L is the stretched-out length of the chain (Treloar, 1943). Since the extended length of a peptide unit is 3.61 A, we find for this ratio 269/[(634) (3.61)] = 0.118, well below 0.33. (2) It has been found that, for the stiffest random chain yet investigated (cellulose trinitrate), the Gaussian range is attained for $n > 500$ (Holtzer *et al.*, 1954; Hunt *et al.*, 1956). We therefore expect a polypeptide chain of 634 units to be Gaussian, and eq 9 to be adequate.

The expansion factor α may be estimated from the theory of Orofino and Flory (1957), which provides the relation:

$$\ln [1 + (\pi^{1/2}/2)(\alpha^2 - 1)] = [3^3 \Phi / \pi N_0 2^{5/2}](BM/[\eta]) \qquad (10)$$

in which N_0 is Avogadro's number, and Φ is a constant with the numerical value 2.2×10^{21} if $[\eta]$ is expressed in dl/g.

This equation gives $\alpha = 1.44$. The unperturbed rms end-to-end distance $\langle r^2 \rangle_0^{1/2}$ is thus 269/1.44 = 187 A. There is good evidence from a variety of sources that the peptide groups in polypeptide chains have the *trans* conformation in solution as well as in the solid state (Schellman, 1964). Straightforward application of vector analysis to the problem of an all-*trans*, but otherwise freely rotating, polypeptide chain provides the result (Flory, 1960)

$$b_{fo}^2 = 27.9 \text{ A}^2 \qquad (11)$$

Putting this into (9) along with the numerical value of $\langle r^2\rangle_0^{1/2}$ (187 A) and n (634), we find $z = 1.40$, thus characterizing numerically the rotational barriers in the molecule.

Unfortunately, we have been unable to locate any exact determinations of the extension of well-characterized polypeptide random coils in solution with which this result might be compared. However, evidence appears to be accumulating (Doty *et al.*, 1956; Brant and Flory, 1965) that is self-consistent and that indicates that z is independent of side chain or solvent and has a numerical value very close to 2.0. Thus, while we cannot say anything conclusive from our molecular radius data, it is certainly true that they do not gibe very well, if the chain is linear, with what is known about barriers to rotation in other peptide polymers. As more such data accumulate for well-characterized polypeptides, perhaps synthetic ones, this same approach can be employed with more certainty.

We turn next to an examination of the viscosity results for guanidine solutions. The equation governing the viscosity of a randomly coiled high polymer is (Flory, 1953, Chapter XIV)

$$[\eta] = \Phi'\langle\rho^2\rangle^{3/2}/M \qquad (12)$$

where Φ' is a constant with the numerical value 3.23×10^{22} dl/mole cm^3. Using (12) with the experimental values of M and $[\eta]$, we find: $\langle\rho^2\rangle^{1/2} = 102$ A, which compares favorably with the light-scattering value of 110 A.

We now ask whether the data are consistent with the cyclic chain model. We note first that the viscosity eq 12 applies equally well to cyclic or linear chains. Consequently, the satisfactory agreement of the value of $\langle\rho^2\rangle^{1/2}$ from light scattering and from (12) argues equally convincingly for both models. We must turn to a consideration of the chain statistics to find a basis of choice.

It has been shown (Zimm and Stockmayer, 1949) that the mean-square radius of a cyclic chain $\langle\rho_c^2\rangle$ is just half that of the corresponding linear chain, *i.e.*, that

$$2\langle\rho_c^2\rangle = \langle\rho_l^2\rangle \qquad (13)$$

Thus, if the tropomyosin molecule is actually a cyclic chain, the rms radius of the corresponding linear chain in the same solvent would be $110\sqrt{2} = 156$ A. This fictitious linear chain would thus have a rms end-to-end separation of about $156\sqrt{6} = 382$ A. Interpreting this result according to eq 9, 10, and 11, we find $z = 2.0$, in agreement with the values found for the few polypeptides that have thus far been studied.

Thus, the cyclic chain model produces results in accord both with the theory of viscosity of random polymers and with the molecular extension estimated from chain statistics and data on rotational barriers in other polypeptides.

Unfortunately, it is at present impossible to draw any firm conclusions about the chain structure in the native molecule from this analysis because of the possible presence of cross-linkages, covalent or otherwise. These, of course, would have the effect of reducing the spatial extension of the chain and would thus result in a calculated value of z that is too small. The agreement obtained with the cyclic chain model may thus be fortuitious. Indeed, there are three obvious possibilities for such linkages in tropomyosin: first, as mentioned above, it is likely that there is one disulfide bridge in the molecule (Szent-Györgyi *et al.*, 1959); second, there are approximately three cysteine residues/molecule and it is possible that, in the denaturing medium, some random oxidation takes place, thus forming "nonnative," intramolecular disulfide bridges; third, it is possible that some of the secondary forces that stabilize the helical structure in nondenaturing media may persist, in less ordered form, even in solutions containing large quantities of guanidine.

The possible presence of native or "artificial" disulfide bridges is, of course, not a fundamental difficulty in applying this method, since it should be possible to perform similar measurements and analysis of either fully oxidized or fully reduced samples. These should provide less equivocal results. Similarly, the possibility of persistence of secondary forces could be investigated by increasing the guanidine concentration or the temperature.

A further difficulty in this kind of analysis of the data lies in the paucity of precise information on the spatial extension of linear polypeptide chains; as noted above, results on only a few synthetic polymers are available. However, it should be possible to collect more such information and thus characterize z unambiguously for linear chains. This value would then serve as a firmer basis for comparison.

To our knowledge, this approach has not hitherto been used to answer questions of this kind; it might well be employed in many cases to complement end-group studies.

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