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Random Coil Behaviour of Proteins in Concentrated Urea Solutions

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Measurements have been made of the intrinsic viscosities and osmotic pressures of protein polypeptide chains in concentrated urea solutions, in the presence of β-mercaptoethanol. The results show that both properties depend on molecular weight exactly as predicted for randomly coiled linear polymer chains. It can therefore be assumed that protein polypeptide chains, in the solvent medium employed, are random coils, retaining practically no elements of their native conformation. In addition, from the osmotic pressure data, second virial coefficients have been calculated. By combining the intrinsic viscosities and second virial coefficients the unperturbed dimensions of protein polypeptide chains have been obtained. Their values are in good agreement with those determined from the viscosity data alone.

INTRODUCTION

In a series of papers Tanford and co-workers reported the results of measurements of intrinsic viscosity[1,2] sedimentation[2], potentiometric titration[3], optical rotatory dispersion[4], and osmotic pressure[5] of proteins in concentrated aqueous solutions of guanidine hydrochloride (GuHCl), a very potent denaturant, containing a moderate amount of a reducing agent, β-mercaptoethanol (RSH), in order to rupture disulfide bonds and to prevent their formation by oxidation of thiol groups when they are not present in the native protein. They found that protein polypeptide chains, in the solvent medium employed, are true random coils, retaining no parts of their original native conformation. On the basis of viscosity measurements alone, by using the procedure of Kurata and Stockmayer[6] or the equivalent method of Stockmayer and Fixman[7], they obtained the relation

$$\langle L^2 \rangle_0 = 70\ n \tag{1}$$

where $\langle L^2 \rangle_0$ is the mean-square unperturbed end-to-end distance in Å^2, and n is the number of residues per chain.

The same parameter was also determined with the aid of second virial coefficients obtained from the osmotic pressure data and the result was[5]

$$\langle L^2 \rangle_0\ (60 \pm 10)\ n \tag{2}$$

Owing to the approximate character of some of the relations used in both calculations the agreement may be considered as satisfactory and the values of $\langle L^2 \rangle_0$ as being close to their real values.

In this paper a similar study of proteins in another potent denaturant, urea, is reported. Urea has been known as being equally effective as GuHCl for

many proteins. For the present study we have chosen only such proteins for which there is little doubt that they are completely denatured by urea. Most solutions contained also 0.1 *M* RSH. The properties studied were intrinsic viscosity and osmotic pressure since we intended to find out whether Eqs. (1) and (2) hold true, as they should if the proteins are random coils, in concentrated urea solutions.

Most viscosity data of this paper have been reported as unpublished data in Tanford's review article on the denaturation of proteins[8].

EXPERIMENTAL

Proteins

Ribonuclease used was lot 95 B-0330 from the Sigma Chemical Corp. It was designated Type I-A, protease free. β-Lactoglobulin (Type B) was donated by Dr. R. Townend, of the Eastern Utilization Research and Development Division, U. S. Department of Agriculture. It was recrystallized before use. α-Chymotrypsinogen A was a 3 × crystallized sample from Worthington Biochemical Corp. and pepsinogen a chromatographically pure sample from the same company. Bovine serum albumin was a crystalline product from International and Nuclear Corp.

Other Reagents

The urea used in this study was a Calbiochem product, Grade A. Before use, it was recrystallized twice from 80% ethanol. Other reagents used were the best available commercial products.

Preparation of Solutions

For all the proteins, relatively concentrated stock solutions were prepared, the solvent being water (ribonuclease, α-chymotrypsinogen, bovine serum albumin) or 0.1 *M* solution of NaCl (β-lactoglobulin). Suspended impurities were removed by centrifugation or filtration. The stock solutions were dialyzed against the solvents and, if necessary, NaOH was added to obtain a *p*H higher than 6.0. For pepsinogen, three different solvents were used: two of them contained 0.005 *M* Tris buffers having *p*H 7.6 and 8.1, respectively. The third was a phosphate buffer with the following composition: 0.032 *M* Na_2HPO_4, 0.004 *M* NaH_2PO_4, and 0.05 *M* NaCl. Its *p*H was 8.1. Protein concentrations were obtained from dry weight content. Solutions for measurements were prepared by weighing out appropriate amounts of protein stock solutions, stock solutions of urea, and RSH. Reference solvents were prepared by using the dialyzates in place of the protein solutions. The densities of all solutions were calculated from the composition using the density data of Kawahara and Tanford[9]. For components other than urea, additivity was assumed. The protein contribution was calculated by assuming an approximate value for the partial specific volume.

Viscosity Measurements

Viscosity measurements were made in Cannon-Fenske capillary viscometers. The procedure was the same as described previously[2]. All measurements were made at (25.00 ± 0.01)° C.

Osmotic Pressure Measurements

Osmotic pressure measurements were made with the Melabs recording osmometer, Model CSM-2. Membranfiltergesellschaft, Göttingen, membranes UFF »fein« and »feinst« (corresponding to Schleicher and Schuell's B 18 and B 19, respectively) were used. The former was used for serum albumin, the latter for other proteins. Before use, the membranes were equilibrated with 2, 4, and 6 *M* urea over periods of four hours and finally over night with 8 *M* urea + 0.1 *M* RSH. Once installed, a membrane could be used for days or even weeks. During nights, the instrument was switched off and an outer thermostat was used to maintain the temperature. This is especially convenient since the time necessary for reaching equilibrium was

between one and two hours which is much longer than in the case of concentrated GuHCl solutions[5]. An important criterion for the accuracy of each set of measurements is the reproducibility of the solvent and solution readings. As in the previous case[5], those sets of measurements were judged as satisfactory in which the fluctuations were less than 0.15 cm. For each protein at least two independent sets of measurements, *i. e.*, measurements with freshly prepared solutions, were performed. The estimated error for each protein is given in the table containing experimental data. All measurements were made at 25° C. Since we did not have enough sample, osmotic measurements were not performed with pepsinogen.

RESULTS

The concentration of urea in all experiments was 8 *M*. For all the proteins studied, this concentration is assumed to be high enough to produce a more or less complete transition to the denatured state[8]. The concentration of added β-mercaptoethanol (RSH) was 0.1 *M*.

Intrinsic Viscosity

The viscosities of ribonuclease, β-lactoglobulin, and α-chymotrypsinogen in 8 *M* urea without and with RSH were found to be practically constant over the period of a few hours during which measurements were made. The measurements started appr. 60 minutes after solutions had been prepared. Solutions of pepsinogen and serum albumin, however, behaved differently. In the solutions of pepsinogen containing the Tris buffer and having *p*H 7.6, the reduced viscosities were found to be extremely time dependent. The viscosity of the solutions, after rising sharply during the unfolding process, decreased with time and after more than 90 hours became more or less constant. The values observed are much less than those for a random coil with the same characteristics and a molecular weight of about 40,000. We do not have an explanation for this behaviour. The solutions containing the Tris buffer and having *p*H 8.1 behaved normally, *i. e.*, their viscosities did not change with time and their values were as expected. Therefore, we decided to use also a phosphate buffer, the composition of which has been given above, having *p*H 8.1. In this case again, no anomaly was observed. The viscosities of serum albumin increased slightly with time.

The experimental data used in the determination of intrinsic viscosities are presented in Fig. 1. The figure includes data measured in the absence as well as in the presence of RSH. As previously[2], the data in the presence of RSH were fitted by least squares to the equation

$$\eta_{sp}/c = [\eta] + k\,[\eta]^2\,c \qquad (3)$$

in which $[\eta]$ is the intrinsic viscosity and k a dimensionless constant known as the Huggins constant. The concentration units are g. cm.$^{-3}$. The values of $[\eta]$ are given in Table I.

In Fig. 2 a logarithmic plot of the viscosities of Table I *vs.* the number of monomer units (n) per polypeptide chain is given. As in the case of 6 *M* GuHCl/RSH, an essentially linear relationship is observed. The straight line shown in the figure was obtained by the method of least squares and corresponds to the equation

$$[\eta] = 0.85\,n^{0.61_4} \qquad (4)$$

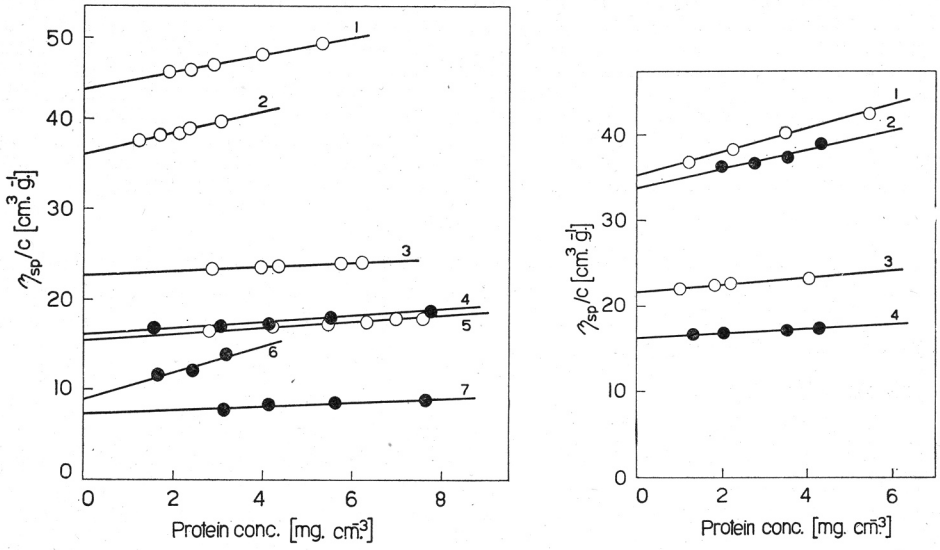

Fig. 1. Viscosity data in 8 *M* urea/0.1 *M* RSH at 25° C.

(a) 1, serum albumin; 2, pepsinogen in phosphate buffer; 3, chymotrypsinogen; 4, serum albumin RSH omitted, 5, ribonuclease; 6, chymotrypsinogen, RSH omitted; 7, ribonuclease, RSH omitted.

(b) 1, pepsinogen in Tris buffer; 2, pepsinogen in Tris buffer, RSH omitted; 3, β-lactoglobulin; 4, β-lactoglobulin, RSH omitted.

TABLE I

Intrinsic Viscosities of Polypeptide Chains in 8 M Urea, 0.1 M RSH, at 25° C

Protein	*M*	Res. per chain	pH	[η] [cm.³ g.⁻¹]		$\langle L^2\rangle^{1/2}$ [Å]
				urea	u./RSH	
Ribonuclease	13,680	124	8.1	7.6	15.6	101
β-Lactoglobulin	18,400	162	6.5	16.2	21.6	122
Chymotrypsinogen	25,700	245	6.3	10.8	22.6	144
Pepsinogen	40,000	365	8.1[a]	33.9	35.2	189
			8.1[b]		36.3	192
Serum albumin	69,000	627	6.8	16.6	43.2[c]	242

[a] In Tris buffer. [b] In phosphate buffer. [c] The value after 43 hours.

Since Eq. (4) shows that the viscosity data for the proteins studied obey the equation for randon coils[1,2], they may be used to calculate root-mean-square end-to-end distances, $\langle L^2\rangle^{1/2}$, from the relation[10]

$$\langle L^2\rangle^{3/2} = nM_0[\eta]/\Phi \qquad (5)$$

where M_0 is the average molecular weight per monomer and Φ the Flory constant. As previously[1], M_0 was put equal to 110 and Φ to 2.1×10^{23} c. g. s. u.

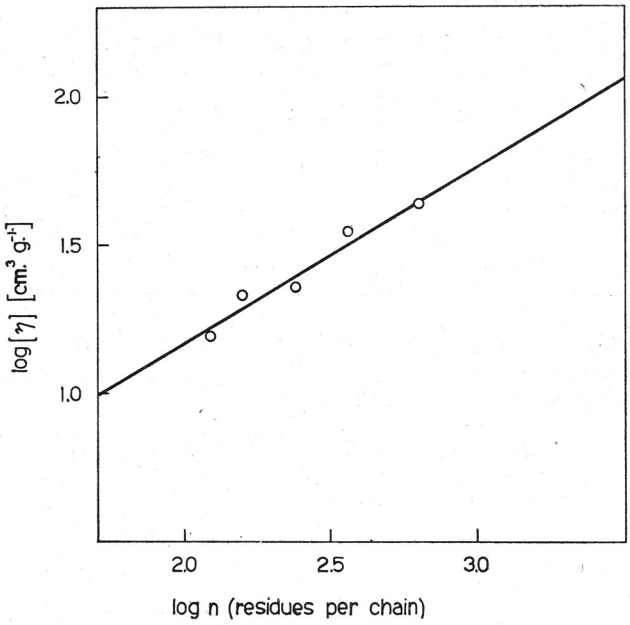

Fig. 2. Intrinsic viscosity as a function of chain length. The straight line represents Eq. (4).

The values of $\langle L^2 \rangle^{1/2}$ obtained in this way are also given in Table I. As in the case of solutions of GuHCl/RSH[2], they are of course much larger than the dimensions expected for globular particles of the same molecular weight. When RSH is not added to the solvent medium, disulfide bonds, in proteins containing cystine, remain intact. This prevents complete unfolding, and the intrinsic viscosity is correspondingly lower, cf. Table I.

As has been mentioned above, it is possible to obtain from viscosity data alone by use of the procedure of Kurata and Stockmayer[6] or Stockmayer and Fixman[7] the unperturbed dimensions which reflect only the geometry of the monomer units and of the bonds joining them together. The result obtained (with L in Ångströms) is

$$\langle L^2 \rangle_0 = 69\, n \tag{6}$$

with an uncertainty of about 15%.

Osmotic Pressure

Typical experimental data are shown in Fig. 3. Molecular weights and second virial coefficients obtained from the data are given in Table II. In Fig. 4 the product of the second virial coefficients and molecular weights, $A_2 M^{1/2}$, is plotted as a function of molecular weight. As in the case of GuHCl/RSH[5], it is observed that the results fall on a relatively smooth curve, and that $A_2 M^{1/2}$ is increasing with increasing molecular weight, which is typical for polymers composed of identical subunits[11].

In order to obtain from the second virial coefficient the unperturbed dimensions, we will, as previously[5], make use of the relation

$$\alpha^2 = \langle L^2 \rangle / \langle L^2 \rangle_0 \tag{7}$$

TABLE II

Results of Osmotic Pressure Measurements

Protein	M exptl.	$A_2 \times 10^3$ [cm.^{3} mole g.$^{-2}$]
Ribonuclease	13,500	0.93 ± 6%
β-Lactoglobulin	16,700	0.87 ± 8%
Chymotrypsinogen	25,000	0.87 ± 8%
Serum albumin	64,000	0.56 ± 15%

where α is the molecular expansion factor. Since a detailed survey of the possible relations between A_2 and α has been given previously[5], we need only remember that Berry's equation[11]

$$A_2 M^{1/2} = \frac{4 N_A (\pi/6)^{3/2} (\langle L^2 \rangle_0 / M)^{3/2}}{a_1} (\alpha^2 - 1) \qquad (8)$$

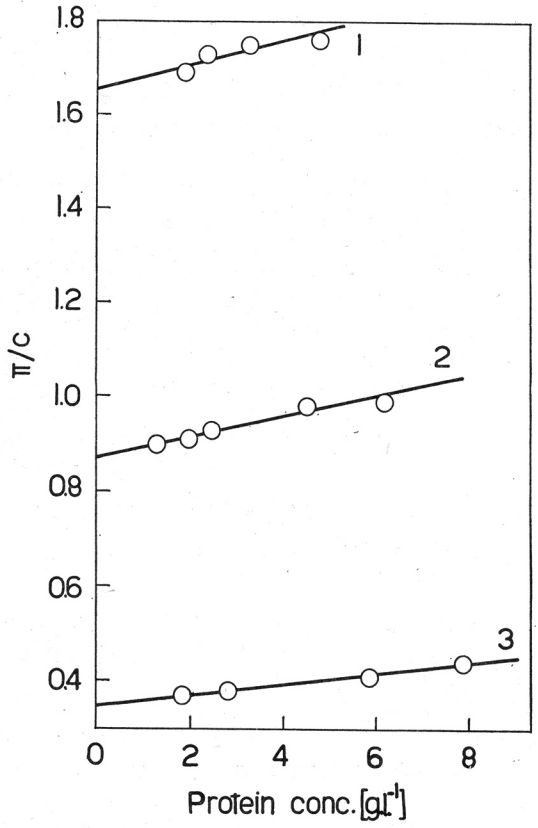

Fig. 3. Representative osmotic data. The units for osmotic pressure are centimetres of solvent (density 1.12 g. cm^{-3}). The value of RT in the units employed at 25° C is 2.24×10^4. Curves 1, 2. and 3 are representative runs for ribonuclease, chymotrypsinogen, and serum albumin, respectively.

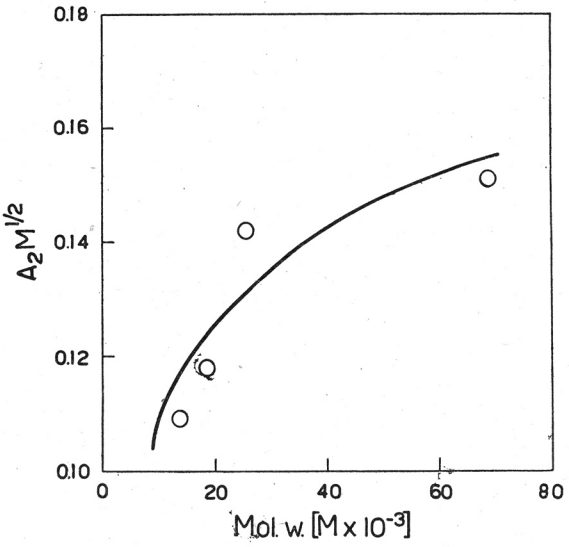

Fig. 4. Plot of $A_2M^{1/2}$ as function of molecular weight.

in which N_A is Avogadro's number and a_1 a universal constant equal to 134/105, has proved useful for solutions of GuHCl/RSH though it requires previous knowledge of the very parameter, $\langle L^2\rangle_0/M$, which we wish to calculate. However, we need only an approximate value of $\langle L^2\rangle_0$ to start with and then by using Eqs. (5) and (7) we obtain a new value of $\langle L^2\rangle_0$. From this, by the method of iteration, another value of $\langle L^2\rangle_0$ is calculated, *etc.* After a few iterations the limiting value of $\langle L^2\rangle_0$ is obtained. The most natural choice for the initial value of $\langle L^2\rangle_0$ is surely the one given by Eq. (6). Also, the two values should be relatively close to each other, and the number of iterations may differ from protein to protein. This has been actually observed. Table III gives the results

TABLE III

Expansion Factor α and Unperturbed End-to-End Distances, $\langle L^2\rangle_0^{1/2}$ in Å.

Protein	α	$\langle L^2\rangle_0^{1/2}$	
		Eq. (6)	Eq. (8)
Ribonuclease	1.17	93	86
β-Lactoglobulin	1.15	106	106
Chymotrypsinogen	1.24	130	117
Serum albumin	1.22	208	199

of calculation of α from Eqs. (7) and (8). It also contains the values of $\langle L^2\rangle_0$ calculated from Eq. (6) as well as those obtained from Eq. (8) using the above described iterative procedure. In Fig. 5 the values of $\langle L^2\rangle^{1/2}_0$ from Eq. (8) are plotted *vs.* $M^{1/2}$ and a reasonable fit to the expected linear relation may be

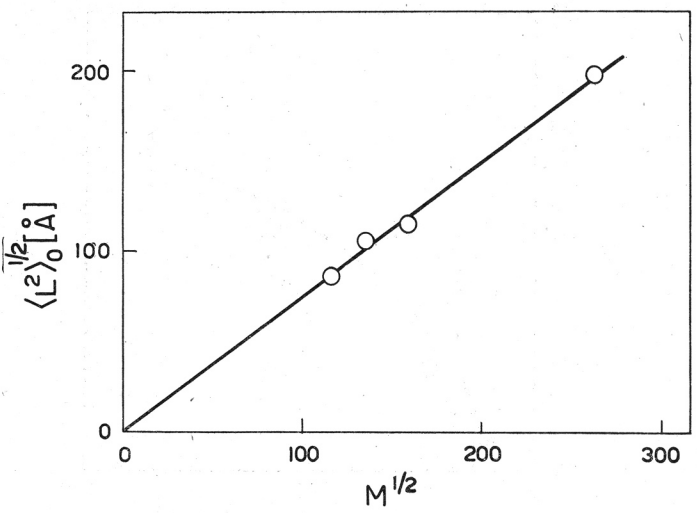

Fig. 5. Unperturbed end-to-end distances, from Table III, as a function of $M^{1/2}$.

observed. The least square equation for the straight line in Fig. 5 is $\langle L^2 \rangle^{1/2}_0 = 0.75\ M^{1/2}$. In terms of the number of residues per chain this becomes

$$\langle L^2 \rangle_0 = 62\ n \tag{9}$$

with an uncertainty of about 15%. The result is in good agreement with Eq. (6) which was determined from viscosity data alone.

DISCUSSION

The results of viscometric and osmotic measurements in concentrated urea solutions may be interpreted along similar lines as those for solutions of GuHCl/RSH[2,5]. Thus the value of the exponent in Eq. (4) is equal to 0.61_4 which is typical for random coils. By inclusion of data for other globular proteins which are denatured by 8 *M* urea a slightly different value of the exponent would probably be obtained. This would reflect the fact that the proteins are not composed of identical residues. Furthermore, comparison of the value 0.61_4 with that obtained for the same proteins in 6 *M* GuHCl, 0.66, clearly shows that urea is a »weaker« denaturant than GuHCl, or in terms of the polymer chemist, that the latter is a better solvent than urea. This has of course been known for some time. At present it is impossible to explain quantitatively why GuHCl is a better solvent. However, a qualitative explanation is fairly obvious. Smaller dimensions are due, above all, to two factors: First, electrostatic interaction between charges on polypeptide chains (neutral *p*H's) which in concentrated solutions of GuHCl is practically eliminated, and, second, more intensive binding of GuHCl than urea to the proteins.

The value of $\langle L^2 \rangle_0$ for urea solutions in terms of n, according to Eq. (6) is practically identical to that for GuHCl solutions where the relation $\langle L^2 \rangle_0 = 70\ n$ has been found[1]. This, in the author's opinion, is a striking confirmation of the fact that in concentrated urea solutions, as in GuHCl solutions, true

random coil behaviour is observed. If this were not the case, the unperturbed dimensions in urea would be different from those in GuHCl.

The results of osmotic measurements also support the idea that the behaviour of the proteins studied is that of random coils. First of all, we have noticed, cf. Fig. 4, the increase of $A_2M^{1/2}$ with M in a manner typical for flexible polymers. Though the amino acid content varies from protein to protein, the average hydrodynamic as well as thermodynamic interaction parameters which characterize each polypeptide chain apparently do not vary significantly. The values of A_2 are also such as expected for flexible polymers of comparable molecular weights. They are smaller than in solutions of GuHCl, which again reflects the fact that urea is a weaker solvent. Application of Berry's equation leads to values of $\langle L^2 \rangle_0$ which are in good agreement with those obtained from viscosity data using the Kurata-Stockmayer-Fixman procedure. This proves that the combination of the results of hydrodynamic and thermodynamic measurements gives values of $\langle L^2 \rangle_0$ which are, within the limits of approximations involved, identical with those obtained from hydrodynamic measurements alone. This agreement lends further support to the validity of Eqs. (6) and (9). When discussing the values of $\langle L^2 \rangle_0$ as well as of $\langle L^2 \rangle$ we naturally must not forget that Eq. (7) is also an approximation, since the value of Φ depends on the chain length, and the value we have used is not firmly established. For a detailed review, the reader is referred to the papers of Berry[11]. Recently, Miller and Goebel[12] have published the results of theoretical calculations of unperturbed dimensions based on an appropriate model for the polypeptide chain. The values obtained are in reasonable agreement with experimental values in 6 M GuHCl and, consequently, in 8 M urea. Concluding we can say that the results obtained suggest that unfolded protein polypeptide chains in 8 M urea solutions behave as random coils. Since the methods used yield average values of measured properties, it is quite possible that small structured parts, if they exist, have been overlooked, and that by other, more subtle methods they would be detected. However, ORD data for the same proteins in 8 M urea[8] are also very similar to those in 6 M GuHCl which gives further support to the views expressed above.

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IZVLEČEK

Beljakovine kot naključni klobčiči v koncentriranih raztopinah sečnine

S. Lapanje

Izmerjene so bile lastne viskoznosti in osmozni tlaki beljakovinskih polipeptidnih verig v koncentriranih raztopinah sečnine v prisotnosti β-merkaptoetanola. Obe lastnosti zavisita od molske mase natanko tako kot upogljivi linearni polimeri. Zato je upravičena domneva, da so beljakovinske polipeptidne verige v tem topilu naključni klobčiči, ki ne vsebujejo praktično nobenih ostankov prvotne, nativne konformacije. Iz osmoznih meritev so bili izračunani tudi drugi virialni koeficienti. Iz teh in iz lastnih viskoznosti pa so bile dobljene nemotene dimenzije beljakovinskih polipeptidnih verig. Njihove vrednosti se zadovoljivo ujemajo s tistimi, ki so bile dobljene samo iz viskoznosti.

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