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ABSTRACT

The shear viscosity of aqueous collagen solutions was experimentally investigated over the temperature range of 303–353 K and collagen concentrations of 1–7 wt. %. A structural phase transition was observed at ~315 K, corresponding to the onset of protofibril formation. It is shown that below this temperature, protofibrils containing both ordered and disordered segments are formed, with the proportion of ordered segments increasing as the temperature decreases, reaching ~30% at 303 K. An analysis of the temperature dependence of the order parameter for the structural transition in the water–collagen system suggests that this transition exhibits characteristics of a second-order phase transition.

I. INTRODUCTION

Collagen is a biopolymer composed of chains containing 12 types of amino acid residues.[1–4] In its lowest potential energy state, the collagen chain adopts a helical conformation.

Over the past few decades, numerous studies have been focused on the physical, chemical, and biological properties of collagen (see Refs. 5–9 and references therein), which is unsurprising given that collagen fibers are a fundamental component of the human body. Each fiber consists of fibrils, which are composed of protofibrils. A protofibril, in turn, is made up of three single helices twisted into a common triple helix and is also referred to as a tropocollagen molecule.[10–13] This highlights the importance of understanding the mechanism of protofibril formation in any study related to collagen. A common approach to investigating this mechanism involves using aqueous collagen solutions as a model system, facilitating experimental exploration of protofibril formation.

There is one prevailing hypothesis regarding protofibril formation in aqueous collagen solutions outlined in the literature.[13–16] According to this hypothesis, collagen chains in aqueous solutions exist as random coils at temperatures above 303 K.[17] As the temperature decreases, these chains gradually adopt a helical conformation. The final step involves the assembly of individual helices into a triple helix. The objective of this paper is to test this hypothesis.

The spatial transformations of collagen macromolecules in water can be viewed as a structural transition, with one of the key indicators being change in the size of the macromolecule. This aspect has been studied using various physicochemical methods, including dynamic light scattering,[18,19] small-angle neutron scattering,[20,21] atomic force microscopy,[22,23] small-angle x-ray scattering,[24,25] pulsed-field gradient NMR spectroscopy,[26,27] gel permeation chromatography,[28,29] and capillary viscometry.[30,31]

In this study, we focused on a method that allows for the determination of macromolecular size as an indicator of structural transitions via the measurements of the shear viscosity of aqueous collagen solutions. To justify this choice, one can mention that the viscometric approach enables us to simultaneously assess two factors in a single experiment: (1) the variation in macromolecular size as a function of temperature and protein concentration and (2) the dynamics of solvent molecules interacting with the macromolecule through intermolecular forces. Given these advantages,

viscometry was selected as the primary experimental method for this investigation.

II. EXPERIMENTAL PART

A. Materials and methods

Freshly prepared distilled water of purity class 2, in accordance with the ISO 22519:2023 standard,[32] was obtained using an Adrona Crystal EX Double Flow water purification system (Adrona SIA, Latvia). The samples were prepared by dissolving type I collagen (purity grade ≥95%, CAS 9007-34-5, Sigma-Aldrich) in the purified water. No further purification of the samples was performed, and given the nature of viscometric experiments, it was assumed that the presence of low-concentration impurities would not affect the trends or dependencies studied in this work.

The experimental measurements of density and kinematic viscosity were conducted over a temperature range of 303–353 K and collagen concentrations of 1–7 wt. % using standard methods.[33] The density ρ was determined using the pycnometric method with an error of 0.05%. The kinematic viscosity ν was measured via capillary viscometry, with a measurement error not exceeding 1.0%. A UTU-10 ultrathermostat (Krakow, Poland) with a precision of ±0.1 K was used to maintain the desired temperatures.

B. Results

The temperature dependence of the shear viscosity $\eta = \rho\nu$ for aqueous collagen solutions at various concentrations C is shown in Fig. 1.

The results obtained can also be used to plot isotherms in the shear viscosity–concentration plane.

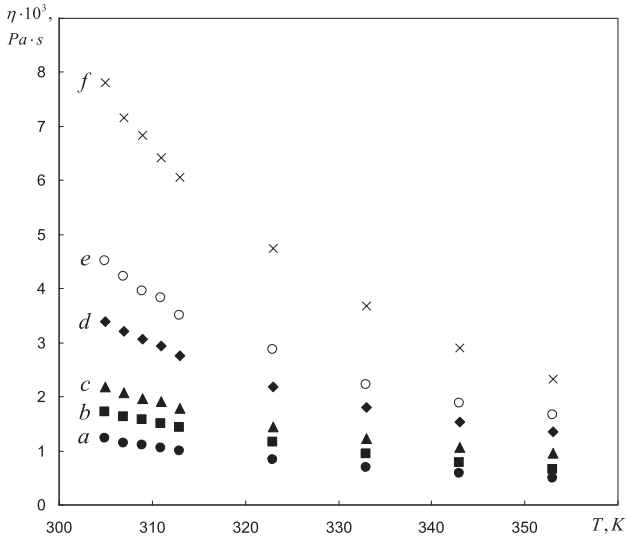

FIG. 1. Temperature dependences of the shear viscosity for aqueous collagen solutions at various concentrations: 1% (a), 2% (b), 3% (c), 4% (d), 5% (e), and 7% (f).

III. DISCUSSION

A. Interpretation of the experimental results using a continuum solution model: Determination of the shape of inclusions

In the model tested, each solution is considered to be a two-component system consisting of a liquid matrix and solid inclusions (e.g., see Ref. 34). The solvent acts as the matrix, while the molecules of various substances, along with their hydrate layers, are considered to be inclusions. The shear viscosity, η, is taken in a form of power series,

$$\eta = \eta_0\left(1 + b_1\varphi + b_2\varphi^2 + \cdots\right), \tag{1}$$

where η_0 is the shear viscosity of the solvent (Pa · s) and φ is the relative volume occupied by the solute. The coefficient b_1 depends on the shape of the inclusions. For spherical inclusions,

$$b_1 = 2.5. \tag{2}$$

For inclusions in the shape of an ellipsoid of revolution,

$$b_1 = 2.5 + 0.4075(p+1)^{1.508} \qquad (1 < p < 15), \tag{3}$$

$$b_1 = 1.6 + \frac{p^2}{5}\left[\frac{1}{3(\ln 2p - 1.5)} + \frac{1}{\ln 2p - 0.5}\right] \qquad (p > 15), \tag{4}$$

where $p = a_1/a_2$ is the anisotropy parameter, defined as the ratio of the ellipsoid axes a_1/a_2 with a_2 being the minor axis length.[35] In order to link the employed model with the experimental results, it is useful to express the relationship between φ and p.

The mass concentration C can be written as

$$C = \frac{\rho_C V_C}{\rho_C V_C + \rho_W V_W}, \tag{5}$$

where ρ_C and ρ_W are the mass densities (kg/m^3) of collagen and water, respectively, and V_C and V_W are the volumes (m^3) occupied by collagen and water, respectively. Considering the equation for the total system volume,

$$\frac{V_C}{V} + \frac{V_W}{V} = 1, \tag{6}$$

the following expression for φ is obtained:

$$\varphi = \frac{V_C}{V} = \frac{C\rho_W}{\rho_C(1-C) + \rho_W C}. \tag{7}$$

The density of collagen, ρ_C, was estimated using literature data on the tropocollagen molecule (e.g., Ref. 1). The molecule's length and width are 300 and 1.5 nm, respectively, with a molecular weight of 300 000 Da (300 kg/mol). These data allow the approximation of the tropocollagen molecule as an ellipsoid of revolution with axes $a = a_1 = 150$ nm and $b = c = a_2 = 0.75$ nm, making it effectively a spheroid (a_1, a_2, a_2).

When considering this molecule as a cylinder of 300 nm long and 1.5 nm diameter, the estimated density ρ_C is ~10^3 kg/m^3, equivalent to the density of water. This result allows transforming Eq. (6) into an equality,

$$\varphi \approx C. \tag{8}$$

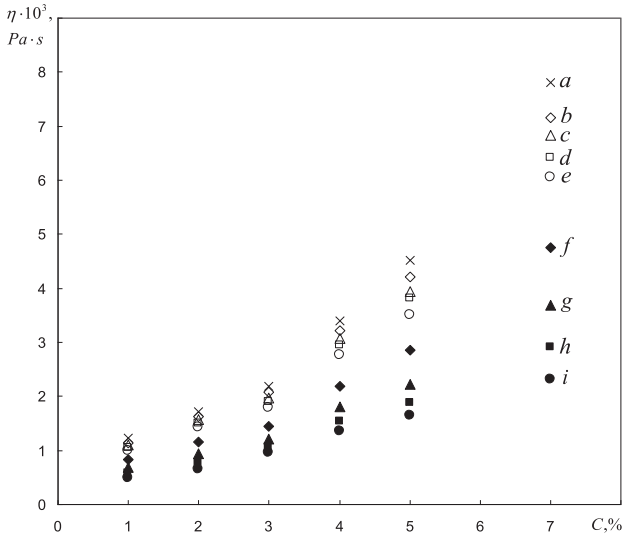

FIG. 2. Isotherms of the concentration dependence of shear viscosity for aqueous collagen solutions at temperatures: 305 K (a), 307 K (b), 309 K (c), 311 K (d), 313 K (e), 323 K (f), 333 K (g), 343 K (h), and 353 K (i).

Therefore, isotherms shown in Fig. 2 in the shear viscosity–concentration plane are in fact the same in the viscosity–relative volume plane [$\eta = \eta(\varphi) = \eta(C)$].

Fitting the data from Fig. 2 with the series in Eq. (1) up to the first order, and incorporating Eq. (8), provides the temperature dependence of $b_1 = b_1(T)$, which is presented in Fig. 3.

As previously noted, on average, a random coil can be approximated as a sphere. In this case, Eq. (1) suggests that the coefficient b_1 should equal 2.5. However, as can be seen in Fig. 3, the experimentally determined values of b_1 are significantly higher than 2.5. This indicates that the collagen inclusions dissolved in water do not have a spherical shape. Based on this result, it is assumed from this point onward that the inclusions take the shape of the ellipsoids of revolution. The temperature dependence of the anisotropy parameter p of the ellipsoids modeling the collagen solutions in water, calculated using Eqs. (3) and (4) based on the data from Fig. 3, is presented in Fig. 4.

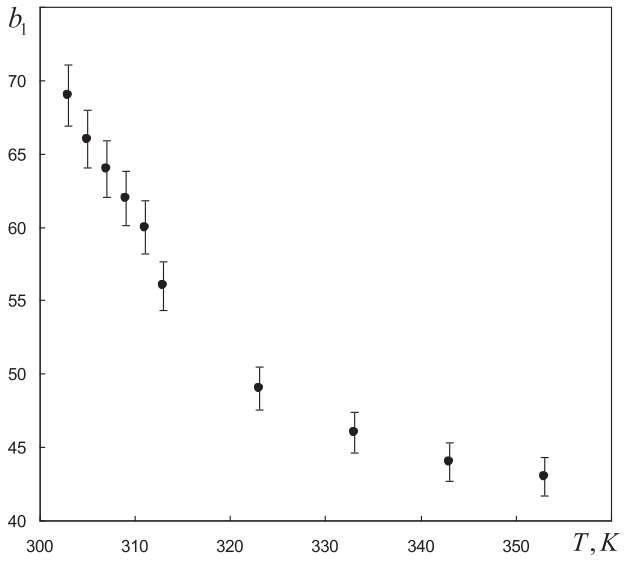

FIG. 3. Temperature dependence of the coefficient b_1 from the shear viscosity expression (1).

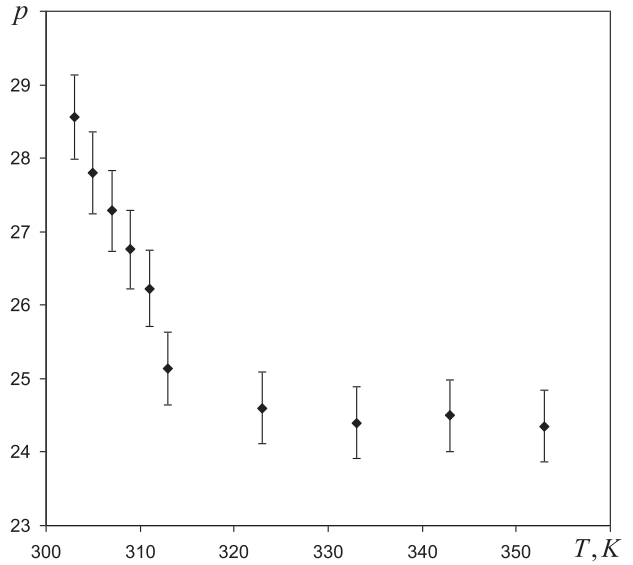

FIG. 4. Temperature dependence of the anisotropy parameter p of an ellipsoid modeled by a dissolved collagen particle in water.

B. Constructing a molecular model of protofibril formation using the data on the shape of inclusions

The results of the viscometric experiments, interpreted within the framework of the continuum solution model, allow for an evaluation of the prevailing hypotheses regarding the structure of aqueous collagen solutions outlined in the literature.[8–11] One key assumption—that random coils are present in the collagen solution—should be rejected.

The proposed in the literature scheme of protofibril formation suggests that the collagen chain successively changes its conformation as follows: coil, single helix, and triple helix.[36–39] As mentioned previously, a straightened single helix would be ~300 nm in length and about 1 nm in thickness. If we approximate the shape of a single helix as an ellipsoid, the resulting anisotropy parameter p would be on the order of hundreds. However, the data presented in Fig. 4 unequivocally reject this possibility, indicating that the presence of single helices in aqueous collagen solutions is virtually excluded.

Furthermore, Fig. 4 provides insights into the actual configuration of collagen chains in aqueous solutions. The dependence $p = p(T)$ shown in Fig. 4 exhibits a kink at a temperature of

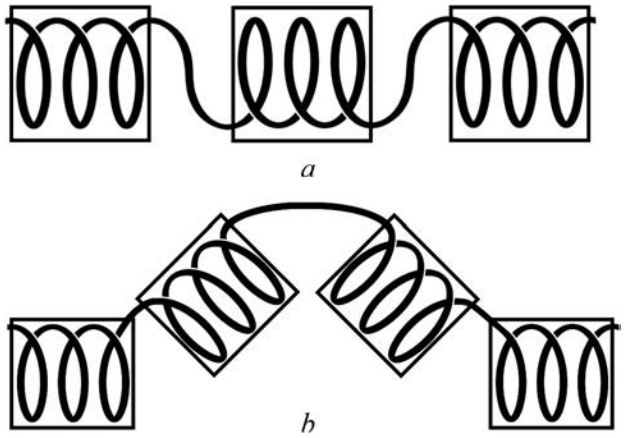

FIG. 5. Configuration of the collagen chain in the fiber: (a) $T < 315$ K—the chain axis in the fiber is parallel to the fiber axis, and (b) $T \geq 315$ K—the axis of the chain becomes bent.

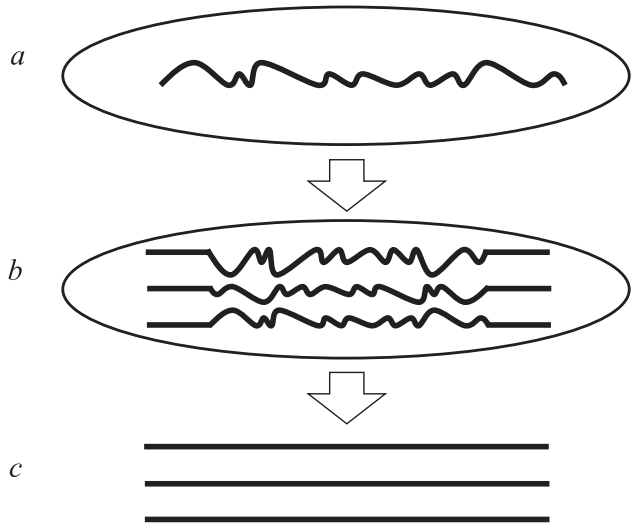

FIG. 6. Configurations of collagen chains in solution: (a) $T > 315$ K—isolated chain, (b) $T < 315$ K—protofibril preparation, and (c) $T < 315$ K—formed protofibril.

315 K, which suggests the existence of a structural transition in the aqueous collagen solution. Notably, the temperature of this structural transition coincides with the singular temperature of water, T = 315 K.[40–44]

Following the concepts outlined in Ref. 45, we consider the collagen chain as a sequence of rigid helical blocks connected by flexible bridges. For simplicity, we assume that the axis of the chain in the fiber is parallel to the fiber axis [Fig. 5(a)]. The contraction of the fiber that occurs at $T \geq 315$ K implies that the chain axis becomes bent, as illustrated in Fig. 5(b).

The fact that the structural transition in the studied solutions occurs at the same temperature as the transition in the fiber allows for the assumption that a situation analogous to that depicted in Fig. 5 might be observed near the temperature of 315 K. Following this analogy, the possible configurations of the collagen chains in the studied solutions are illustrated in Fig. 6, where the bold line represents the axis of the chain.

Several arguments can be presented to justify the validity of the proposed model for collagen behavior. As previously mentioned, the viscometric experiments unequivocally reject the formation of random coils. This possibility is excluded due to the interactions between collagen chains and water molecules, which additionally promote the unfolding of the chains. However, entropic forces prevent the collagen chain from fully unfolding into a single helix. Instead, at $T > 315$ K, the chain adopts a configuration that can be enclosed within an ellipsoid with an axis ratio of 1:20. This configuration can be represented as a broken line composed of segments, each with a length equal to the block length l. Denoting the angle between the axis of the j-th block and the axis of the ellipse by α_j, we obtain

$$2a_1 = \sum_j l \cos\alpha_j, \tag{9}$$

$$2a_2 = \sum_j l \sin\alpha_j . \tag{10}$$

Thus, the anisotropy parameter p can be written as

$$p = \frac{\sum_j l \cos\alpha_j}{\sum_j l \sin\alpha_j}. \tag{11}$$

The smallest value of the anisotropy parameter, denoted as p_0, corresponds to temperatures $T > 315$ K.

Continuing the analogy with fibers, which undergo significant contraction at $T > 315$ K—resulting in a negligible amount of chain segments parallel to the fiber axis—it is assumed that collagen chains in aqueous solutions at $T > 315$ K do not contain any blocks with axes parallel to the axis of the ellipsoid. Furthermore, it is assumed that all angles α have the same value α_0, leading to the following formula:

$$tg\alpha_0 = \frac{1}{p_0}. \tag{12}$$

C. Analysis of the introduced molecular model

Since we previously assumed that the transition in the studied solutions is of the same nature as that in fibers, it follows that collagen chains in aqueous solutions at $T > 315$ K should have blocks with axes parallel to the axis of the model ellipsoids. However, it is unlikely that this tendency will lead to the formation of single helices, as the absence of such helices was confirmed by the viscometric experiments. Instead, it is thermodynamically more favorable for the chains to associate with two neighboring chains, forming a single triple block (or multiple blocks) with an axis aligned with that of the fiber. Such an aggregate of three chains [see Fig. 6(b)] can be regarded as a prephase for the future protofibril [see Fig. 6(c)].

Let ζ and ζ' be the relative numbers of blocks that are parallel and non-parallel to the axis of the prephase, respectively. The quantity ζ can be considered an order parameter. It is evident that

$$\zeta + \zeta' = 1. \tag{13}$$

Denoting the width of the triple block as h and approximately accepting that the axes of non-parallel blocks are inclined to the axis of the prephase at the same angle α_0, one can write

$$2a_1 = \zeta l + (1 - \zeta) l \cos\alpha_0, \tag{14}$$

$$2a_2 = \zeta l + (1 - \zeta) l \sin\alpha_0. \tag{15}$$

An equation can then be formulated to relate the anisotropy parameter to the unknown relative numbers of blocks as follows:

$$p = \frac{\zeta l(1 - \langle\cos\alpha_0\rangle) + l\langle\cos\alpha_0\rangle}{\zeta(h - \langle l \sin\alpha_0\rangle) + l\langle\sin\alpha_0\rangle}. \tag{16}$$

Taking Eq. (13) into account, its solution is given by

$$\varsigma = \frac{pl\langle\sin\alpha_0\rangle - l\langle\cos\alpha_0\rangle}{l(1 - \langle\cos\alpha_0\rangle - p(h - l\langle\sin\alpha_0\rangle))}. \tag{17}$$

Neglecting the thickness of the block in comparison with its length, Eq. (17) can be rewritten in the following form:

$$\varsigma = \frac{p\langle\sin\alpha_0\rangle - \langle\cos\alpha_0\rangle}{1 - \langle\cos\alpha_0\rangle - p\langle\sin\alpha_0\rangle}. \tag{18}$$

Substituting into Eq. (12) instead of p_0 its value equal to 20 allows assuring the inequality

$$\alpha_0 \ll 1. \tag{19}$$

Taking it into account, Eq. (19) can be rewritten as

$$\zeta = 1 - \frac{p_0}{p}. \tag{20}$$

The dependence $\zeta = \zeta(T)$ obtained based on the data presented in Fig. 4 is presented in Fig. 7.

The analysis of the temperature dependence of the order parameter $\zeta = \zeta(T)$ during a structural transition in an aqueous collagen solution at a temperature of 315 K indicates that the transition we studied is analogous to a second-order phase transition. The analogy between structural transitions involving biopolymer macromolecules and second-order phase transitions in magnetic materials was first noted by O'Keefe, Moser, and Moser.[46,47] Our results are consistent with their conclusions.

As shown in Fig. 7, the proportion of the ordered segments increases with decreasing temperature reaching ~30% at 303 K. Notably, even at this relatively low temperature (for collagen), the phase transition—the formation of protofibrils from isolated collagen chains—is still not complete, as completion corresponds to a value $\zeta = 1$ for a fully formed protofibril. This suggests that the formation of fibers initiates with protofibrils containing disordered segments, and the protofibril structure becomes fully ordered during the fiber formation process.[48,49] However,

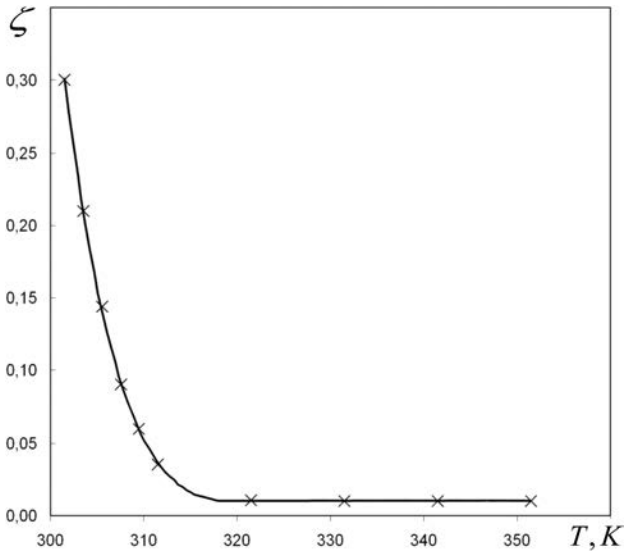

FIG. 7. Temperature dependence of the order parameter during protofibril formation in an aqueous collagen solution. The solid line is provided as a guide to the eye.

the relatively low concentration of the studied solutions did not allow us to observe the mentioned fiber formation process in this experiment.

IV. CONCLUSIONS

Protofibril formation in aqueous collagen solutions occurs within the temperature range of 303 K < T < 355 K. At T > 315 K, the collagen chains are isolated from each other. They do not exist as random coils but rather adopt elongated configurations, which can be approximated by an ellipsoid of revolution with an axis ratio of 1:20. At T = 315 K, a structural transition is observed, during which a protofibril prephase forms from the isolated collagen chains. This prephase consists of three chains and contains both disordered and ordered segments, the latter corresponding to the structure of an ideal protofibril. The transition exhibits characteristics similar to a second-order phase transition.

AUTHOR DECLARATIONS

Conflict of Interest

The authors have no conflicts to disclose.

Author Contributions

Leonid A. Bulavin: Conceptualization (equal); Methodology (equal); Supervision (lead); Writing – original draft (supporting). **Kostyantyn V. Cherevko**: Validation (equal); Writing – original draft (supporting); Writing – review & editing (equal). **Oleksii V. Khorolskyi**: Data curation (equal); Investigation (lead); Resources (lead); Validation (equal); Writing – original draft (lead);

Writing – review & editing (equal). **Oksana S. Svechnikova**: Data curation (equal); Investigation (supporting); Visualization (lead); Writing – original draft (equal). **Yurii F. Zabashta**: Conceptualization (equal); Methodology (lead); Validation (equal); Writing – review & editing (supporting).

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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