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Viscosity analysis of the temperature dependence of the solution conformation of ovalbumin

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Abstract

The viscosity of ovalbumin aqueous solutions was studied as a function of temperature and of protein concentration. Viscosity–temperature dependence was discussed on the basis of the modified Arrhenius formula at temperatures ranging from 5 to 55°C. The activation energy of viscous flow for hydrated and unhydrated ovalbumin was calculated. Viscosity–concentration dependence, in turn, was discussed on the basis of Mooney equation. It has been shown that the shape parameter S decreases with increasing temperature, and self-crowding factor K does not depend on temperature. At low concentration limit the numerical values of the intrinsic viscosity and of Huggins coefficient were calculated. A master curve relating the specific viscosity η_{sp} to the reduced concentration $c[\eta]$, over the whole range of temperature, was obtained and the three ranges of concentrations: diluted, semi-diluted and concentrated, are discussed. It has been proved that the Mark–Houvink–Kuhn–Sakurada (MHKS) exponent for ovalbumin does not depend on temperature.

Keywords: Ovalbumin; Activation energy; Intrinsic viscosity; Huggins coefficient; Mark–Houvink–Kuhn–Sakurada exponent

1. Introduction

Ovalbumin is the major globular protein of chicken egg white. It is a member of the serpin superfamily and is classified as a non-inhibitory serpin [1]. Ovalbumin consists of a single polypeptide chain of 385 amino acid residues that folds into a globular conformation with three β-sheets, nine α-helices and three short helical segments of three to four residues [2–4]. This globular protein contains electrophoretically three distinguishable fractions with, respectively, two, one and zero phosphate groups per molecule. However, they possess the same overall native protein conformation [5]. The crystal structure of the protein, as revealed by X-ray crystallography, indicates that the ovalbumin molecule is approximately a tri-

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axial ellipsoid with overall dimensions $7 \times 4.5 \times 5$ nm [2].

Ovalbumin, both native and denatured, has been the object of physicochemical studies for many years. The studies have based on the experimental techniques such as viscometry [6–9], ^{1}H-NMR spectroscopy [10], dielectric spectroscopy [11], densimetric and ultrasonic velosimetric titration [12], fluorescence and circular dichroism [13,14], differential scanning calorimetry [15] and Fourier transform infrared spectroscopy [16]. The results of the investigations give information about the functional properties, protein denaturation, hydration and structure of ovalbumin. However, little attention has been devoted to the hydrodynamic properties of ovalbumin. This is especially the case for the viscosity of ovalbumin solutions, where the results are still fragmentary and limited to one temperature.

This work presents the results of viscosity measurements for ovalbumin aqueous solutions at temperatures ranging from 5 to 55°C and at a wide range of concentrations. On the basis of these results the viscosity–temperature and viscosity–concentration relationships are discussed. Such rheological quantities as activation energy of viscous flow, Simha parameter and self-crowding factor are calculated. At low concentrations, the temperature dependence of the intrinsic viscosity and of Huggins coefficient is presented. Using the dimensionless parameter $[\eta]c$, the existence of three characteristic ranges of concentrations is shown. By applying Lefebvre's equation for the relative viscosity in the semi-dilute regime, the MHKS exponent for ovalbumin is evaluated.

2. Materials and methods

2.1. Materials

Crystallized hen ovalbumin (grade V) was obtained from Sigma Chemical Co. and was used without further purification for all the measurements. Aqueous solutions of the ovalbumin were prepared by dissolving the material in distilled water. Such solutions were then filtered by means of filter papers in order to remove possible undissolved fragments. The samples were stored at 4°C until just prior to viscometry measurements, when they were warmed from 5 to 55°C. The pH values of such prepared samples were approximately 6.4 and changed only insignificantly during the dilution of the solutions.

2.2. Viscometry

Viscometry is still extensively used in many investigations of biological macromolecules in solution because of its extreme sensitivity and technical simplicity ([17] and references therein). We have used an Ubbelohde-type capillary microviscometer with a flow time for water of 28.5 s at 25°C. The microviscometer was immersed in a thermostated water bath at 5–55°C $\pm$ 0.05°C. The same viscometer was used for all measurements and was mounted so that it always occupied the same position in the bath. Sample solution was temperature-equilibrated and passed once through the viscometer before any measurements were made. For most concentrations the viscosity measurements were done from 5 to 55°C in 5°C intervals. At the temperatures higher than 55°C the thermal denaturation of ovalbumin occurs and the lower protein concentration the higher denaturation temperature. The viscosities of the ovalbumin solutions were measured for concentrations from 6.16 kg/m^3 up to 429.8 kg/m^3. Solutions densities and protein concentrations were determined as described earlier [18,19].

3. Results and discussion

3.1. Viscosity–temperature dependence

Very recently we have proved, for aqueous solutions of bovine serum albumin [20] and hen egg-white lysozyme [19], that the most useful relation connecting the viscosity with temperature is a somewhat modified Arrhenius formula. It has the form:

$$\eta = \exp\left(-B + DT + \frac{E_s}{RT}\right) \quad (1)$$

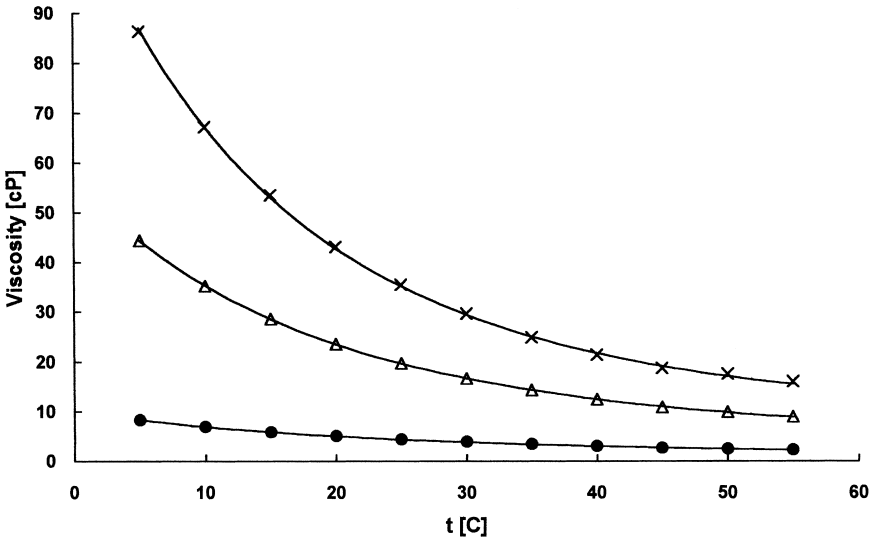

Fig. 1. Temperature dependence of the viscosity of ovalbumin aqueous solutions for concentrations $c = 246.89$ (●), 370.6 (Δ) and 397.59 (×) kg/m^3. The curves show the fit obtained by using Eq. (1) with the parameters: $B = 35.166$, $D = 3.632 \times 10^{-2}$ K^{-1} and $E_s = 46.889$ kJ/mol for $c = 246.89$ kg/m^3; $B = 46.342$, $D = 5.392 \times 10^{-2}$ K^{-1} and $E_s = 65.288$ kJ/mol for $c = 370.6$ kg/m^3; $B = 57.853$, $D = 7.273 \times 10^{-2}$ K^{-1} and $E_s = 81.367$ kJ/mol for $c = 397.59$ kg/m^3.

where B and D are parameters, E_s is the activation energy of viscous flow of solution and R, T are gas constant and absolute temperature, respectively. Fig. 1 shows the results of viscosity measurements at three various concentrations of ovalbumin. As seen, curves obtained by using the function from the above equation give a good fit to the experimental points over the whole range of temperatures.

Numerical values of the parameters B, D and

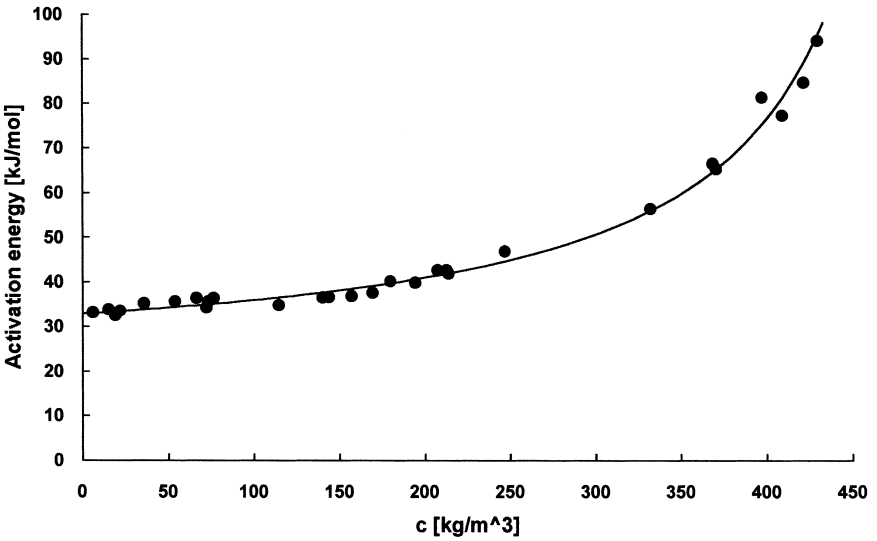

Fig. 2. Plot of the solution activation energy E_s vs. concentration. (●) experimental points were obtained by using the least squares method; the curve shows the fit according to Eq. (2) with the parameters given in the text.

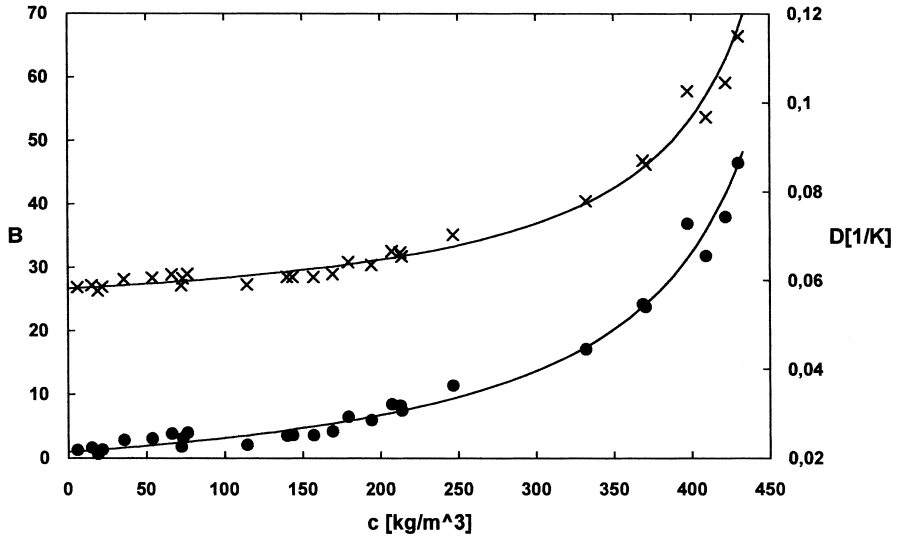

Fig. 3. Plot of the coefficients B (×) and D (●) vs. concentration. Experimental points were obtained by using the least squares method; the curves show the fit according to Eqs. (3) and (4), respectively.

E_s for all measured concentrations were calculated by using the least squares method [20]. The results are shown in Figs. 2 and 3. As for bovine serum albumin and lysozyme, both activation energy E_s and the parameters B and D monotonically increase with increasing concentration. The explanation of this fact is given in our earlier paper [20]. At the same time, it has been shown that the following relations have to be fulfilled:

$$E_s = \frac{c}{\alpha - \beta c}(E_p - E_w) + E_w \quad (2)$$

$$B = \frac{c}{\alpha - \beta c}(B_p - B_w) + B_w \quad (3)$$

$$D = \frac{c}{\alpha - \beta c}(D_p - D_w) + D_w \quad (4)$$

where $\alpha = \rho_w M_p / M_w$ and $\beta = \alpha\xi - 1$. The quantities c, ρ_w, ξ, M_p and M_w denote the solute concentration and water density in kg/m^3, the effective specific volume of a protein and the molecular masses of the dissolved proteins and water, respectively.

At $c = 0$ the parameters $E_s = E_w = 32.013$ kJ/mol, $B = B_w = 25.936$, $D = D_w = 2.014 \times 10^{-2}$ K^{-1} and the Eq. (1) gives the viscosity–temperature relationship for water, where E_w denotes an activation energy of viscous flow of water molecules. The parameters E_p, B_p and D_p are connected with dissolved proteins and, in particular, E_p is an activation energy of ovalbumin. In Eqs. (2)–(4) the quantities E_p, ξ; B_p, ξ and D_p, ξ, respectively, must be taken into account as two adjustable parameters. To establish their values, the molecular mass of ovalbumin is needed. By using the least squares method, for $M_p = 45$ kDa [2], the following values were obtained: $E_p = 6.241 \times 10^4$ kJ/mol and $\xi = 1.928 \times 10^{-3}$ m^3/kg; $B_p = 3.45 \times 10^4$ and $\xi = 1.991 \times 10^{-3}$ m^3/kg; $D_p = 63.56$ K^{-1} and $\xi = 1.934 \times 10^{-3}$ m^3/kg. As seen in Figs. 2 and 3, the functions from Eqs. (2)–(4), with the parameters values presented above, give good approximation to the experimental values. The three values of the effective specific volume obtained above differ each other only slightly and give the average value $\langle\xi\rangle = 1.951 \times 10^{-3}$ m^3/kg.

However, as has been shown by various techniques [12,21–24], there exists a hydration shell of water surrounding the protein molecules in solution, which is distinct from bulk water. On the basis of microwave dielectric measurements, it

was shown for bovine serum albumin, that it does not change with temperature [23]. This hydration shell must be taken into account when the mass and volume of the hydrodynamic particle are computed, because they influence on the values of some experimental parameters. The molecular mass of hydrated protein is $M_h = M_p(1 + \delta)$ [25], where δ denotes the amount of grams of water associated with the protein per gram of protein. For globular proteins a value of 0.3–0.4 has been obtained from experiments and computer simulations [17] and, in particular, for ovalbumin $\delta = 0.36$ [26]. This value is within the range (0.42 ± 0.09), which was quite recently found by Harding et al. [27] on the basis of covolume measurements. It gives the molecular mass of hydrated ovalbumin $M_h = 61.2$ kDa. To calculate the parameters E_p, B_p, D_p and ξ in Eqs. (2)–(4) for hydrated ovalbumin the M_p should be replaced by M_h. By using once more the least squares method the following values one can obtain: $E_p = 8.487 \times 10^4$ kJ/mol, $B_p = 4.691 \times 10^4$ and $D_p = 86.44$ K^{-1}. It is interesting that the effective specific volume of a protein ξ, obtained in this case, is exactly the same as obtained earlier. The curves in Figs. 2 and 3 are identical for parameters obtained for both hydrated and unhydrated ovalbumin.

3.2. Viscosity–concentration dependence

The most useful relation describing the dependence of relative viscosity of aqueous solutions of globular proteins on concentration is that of Mooney [28]:

$$\eta_r = \exp\left[\frac{S\Phi}{1 - K\Phi}\right] \tag{5}$$

where $\eta_r = \eta/\eta_0$ and η_0 is the viscosity of the solvent. Φ is the volume fraction of the dissolved particles, S denotes the shape parameter and K is a self-crowding factor. The volume fraction $\Phi = N_A V c/M_h$, where N_A and V are Avogadro's number and the hydrodynamic volume of one dissolved particle, respectively. The solute concentration c is in kg/m^3. As has been shown in our earlier works [19,20], the shape parameter S and a self-crowding factor K can be written by the following equations:

$$S = \frac{M_w}{\rho_w N_A V}\left[-(B_p - B_w) + (D_p - D_w)T + \frac{E_p - E_w}{RT}\right] \tag{6}$$

$$K = \left(\xi - \frac{M_w}{\rho_w M_h}\right)\frac{M_h}{N_A V} \tag{7}$$

Both coefficients can be calculated when the hydrodynamic volume and mass of the dissolved proteins is known. The volume of hydrodynamic particle may be calculated from two terms [29]: $V = V_0 + M_p\delta/N_A\rho_w$, where V_0 is a volume of the unhydrated molecule and the other term denotes the volume of the hydration shell.

As was mentioned earlier the X-ray crystallography revealed that the ovalbumin molecule is approximately a tri-axial ellipsoid with the main semiaxes $\hat{a} = 3.5$ nm, $\hat{b} = 2.25$ nm and $\hat{c} = 2.5$ nm. It gives a volume of unhydrated molecule $V_0 = 82.467$ nm^3. For $\delta = 0.36$, the volume of the hydration shell is 26.897 nm^3 and $V = 109.36$ nm^3. The hydrodynamic volume of ovalbumin may be obtained experimentally, too. As has been shown by using high-performance size-exclusion chromatography and intrinsic viscosity measurement, the Stokes radius of ovalbumin is 3 nm [8]. It corresponds to the hydrodynamic volume $V = 113$ nm^3 and is in a good agreement with the value given above.

The numerical values of the shape parameter S obtained from Eq. (6) are presented in Table 1. As is seen this parameter decreases with increasing temperature from $S = 3.782$ (at $t = 5$°C) up to $S = 3.435$ (at $t = 55$°C). Simha [30] proved for hard ellipsoids of revolution ($\hat{a} \neq \hat{b} = \hat{c}$) immersed in a solution, that in the high temperature limit i.e. in the case when the orientation of particles is completely at random, the factor S depends on the axial ratio $p = \hat{a}/\hat{b}$ of the dissolved particles. For ellipsoids of revolution for which $1 < p < 15$, it can be calculated from the asymptotic formula [31]:

Table 1
The numerical values of the shape parameter S, the intrinsic viscosity $[\eta]$ and the Huggins coefficient k_1 for ovalbumin calculated from Eq. (6), Eq. (10) and Eq. (11), respectively

$t[C]$	5	10	15	20	25	30	35	40	45	50	55
S	3.782	3.723	3.669	3.623	3.582	3.545	3.514	3.488	3.466	3.448	3.435
$[\eta]\times 10^3$ $[m^3/kg]$	4.070	4.007	3.949	3.899	3.855	3.816	3.782	3.754	3.730	3.712	3.697
k_1	0.9792	0.9868	0.9938	1.0002	1.0059	1.0112	1.0157	1.0196	1.0229	1.0255	1.0276

$$S = 2.5 + 0.4075(p-1)^{1.508} \tag{8}$$

One can easily calculate from the above relation that the high temperature value of S ($S = 3.435$) corresponds to the ellipsoid of revolution with $p = 2.735$. The shape parameter S can be obtained for tri-axial ellipsoids also [17]. However, in this case, the calculations are very troublesome. For unhydrated ovalbumin $\hat{a}/\hat{b} = 1.56$ and $\hat{a}/\hat{c} = 1.4$. To assess the theoretical value of S we take their mean value $p = 1.48$ and it gives, from Eq. (8), $S = 2.635$. Comparison of the experimental and theoretical value of S shows that hydrated ovalbumin is more elongated than the unhydrated form. This conclusion is consistent with the recent results of Harding et al. [27]. It means that the hydration shell of water is not a uniform monolayer but a patchwork of water clusters, covering some atoms in charged groups by water layers while leaving some part of the protein surface uncovered.

As is seen from Eq. (7), the self-crowding factor K does not depend on temperature. Substitution of the hydrodynamic volume $V_h = 109.36\ nm^3$ into Eq. (7) gives the numerical value $K = 1.81$. This value lies within the range $(1.35 \div 1.91)$ which was obtained, on the basis of purely geometric calculations, for rigid spherical particles by Mooney [28]. However, the measurements for bovine serum albumin [20] ($K = 1.25$) and for hen egg-white lysozyme [19] ($K = 2.91$) showed that for aspherical particles, the values of K may lie outside of this range.

The Mooney formula [Eq. (5)] describes (for a given temperature) the viscosity–concentration dependence from very diluted up to very concentrated solutions. At low concentrations, an expansion of Eq. (5) in the power series of concentration yields to the following polynomial:

$$\frac{\eta_{sp}}{c} = [\eta]\left\{1 + k_1[\eta]c + k_2[\eta]^2c^2 + \ldots\right\} \tag{9}$$

where $[\eta] = \lim_{c\to 0}\frac{\eta_{sp}}{c}$ is the intrinsic viscosity and $\eta_{sp} = \eta_r - 1$ is the specific viscosity. The intrinsic viscosity $[\eta]$ and the Huggins coefficient k_1 can be calculated from the following expressions [20]:

$$[\eta] = \frac{1}{\alpha} \times\left[-(B_p - B_w) + (D_p - D_w)T + \frac{E_p - E_w}{RT}\right] \tag{10}$$

$$k_1 = \frac{1}{2}\left[\frac{2\beta}{-(B_p - B_w) + (D_p - D_w)T + \frac{E_p - E_w}{RT}} + 1\right] \tag{11}$$

The higher coefficients of expansion k_2, k_3 and so on, are connected with the Huggins coefficient k_1 [19] and are omitted here. As shown earlier, the parameters α, B_p, D_p and E_p are different for hydrated and unhydrated ovalbumin. However, as calculations showed, in both cases the values of $[\eta]$ and k_1 are identical and they are presented in Table 1. It is worth noting that the numerical value of the intrinsic viscosity calculated from Eq. (10) at $t = 25°C$ ($[\eta] = 3.855\times 10^{-3}\ m^3/kg$) agrees very well with the value given in

Table 2
Mark–Houvink–Kuhn–Sakurada exponent, critical concentrations, reduced critical concentrations and slopes of the regression lines log η_{sp} vs. log[η]c for ovalbumin, obtained from the fit of the curves in Figs. 4 and 5 and from Eq. (12)

t[C]	5	10	15	20	25	30	35	40	45	50	55
a	0.323	0.323	0.322	0.322	0.323	0.323	0.325	0.324	0.321	0.325	0.323
c^* [kg/m^3]	41.2	41.8	42	42.6	43.2	43.5	43.7	44	44.4	44.4	44.6
c^{**} [kg/m^3]	349	349	350	353	352	350	349	355	345	350	350
c^* [η]	0.168	0.168	0.166	0.166	0.167	0.166	0.165	0.165	0.166	0.165	0.165
c^{**} [η]	1.42	1.398	1.382	1.376	1.357	1.336	1.32	1.333	1.287	1.299	1.294
Slopes											
$c < c^*$	1.09	1.09	1.09	1.09	1.09	1.09	1.09	1.08	1.08	1.08	1.08
$c > c^{**}$	8.41	8.17	7.90	7.75	7.58	7.38	7.28	7.14	7.04	7	7.06

the literature at the same temperature ([η] = 3.9 $\times 10^{-3}$ m^3/kg) [7]. As pointed out by Tanford [25], the intrinsic viscosity of rigid macromolecules should be essentially independent of temperature. In our case, [η] slowly decreases with increasing temperature and this indicates that ovalbumin is not a perfectly stiff molecule in the considered range of temperatures. The problem will be discussed below also.

3.3. Three ranges of concentrations and determination of the MHKS exponent

One of the commonly accepted method of ex-

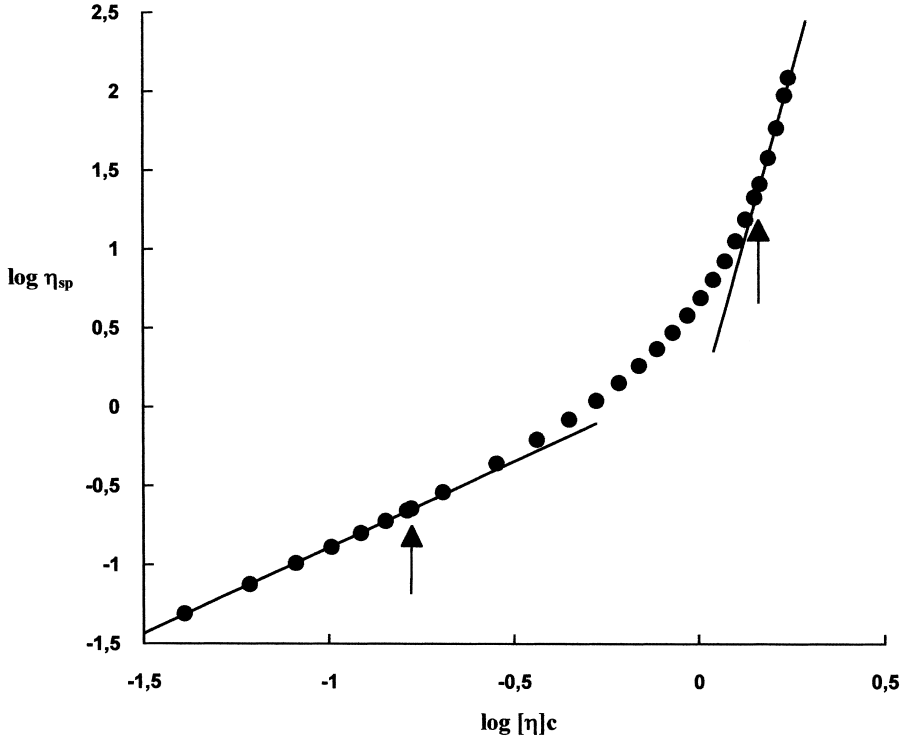

Fig. 4. Specific viscosity as a function of c[η] in a log–log plot for ovalbumin at t = 5°C; straight lines show different slopes in dilute ($c < c^*$) and concentrated ($c > c^{**}$) regions. The arrows show the boundary concentrations c^* (left arrow) and c^{**} (right arrow).

perimental data presentation for different polymer systems consists of using reduced variables. In the case of the viscosity–concentration relation, this variable is a dimensionless parameter $c[\eta]$ [32]. The viscosity measurements for some polysaccharides showed that the dependence of the specific viscosity on $c[\eta]$ in a log–log plot exhibits a transition from the dilute to concentrated region at some concentration c^* [33–36]. In both regions the dependence is linear with different slopes. However, for cellulose derivatives [37], citrus pectins [38], randomly coiled globular proteins [7], native mammalian hemoglobins [39] and some other biopolymers [40], it has been shown that two critical concentrations c^* and c^{**}, instead of one, exist on log η_{sp}–log $[\eta]c$ plots. The concentrations c^* and c^{**} reveal transitions from dilute to semi-dilute solution, and from semi-dilute to concentrated solution, respectively. In Fig. 4, the master curve for ovalbumin at 5°C is shown. As is seen, the three regions exist with the boundary concentrations c^* and c^{**}. The master curves have the same form up to 55°C. The parameters describing the curves are gathered in Table 2.

In the dilute region ($c[\eta] < c^*[\eta]$), the molecular dimension is not perturbed by the other molecules. As is seen in Table 2, the values of c^* slowly increase with increasing temperature and the product $c^*[\eta]$ is nearly the same over the whole range of temperatures. The slopes are nearly identical too. It is worth noting that the slopes in the dilute region, for quite different sorts of molecules, are in the range of 1.1–1.4 [33,37–40].

In the semi-dilute domain ($c^*[\eta] < c[\eta] < c^{**}[\eta]$), a non-linear dependence of log η_{sp}–log $[\eta]c$, for randomly coiled globular proteins [7], citrus pectins [38] and mammalian hemoglobins [39] was observed. This is the case for ovalbumin over the whole range of temperatures also. As was proved by Lefebvre, in the semi-dilute region, the following equation for the relative viscosity is fulfilled [7]:

$$\ln \eta_r = 2a[\eta]c^* \left(\frac{c}{c^*}\right)^{1/2a} - (2a-1)[\eta]c^* \qquad (12)$$

where a is the MHKS exponent. In many cases, this quantity is used as a conformation indicator of molecules in solution. The values of the exponent a are: 0 for hard spherical particles, 0.3–0.35 for hard quasi-spherical mammalian hemoglobins [39], 0.5–1 for random coils [7,34,35,38,40–42] and

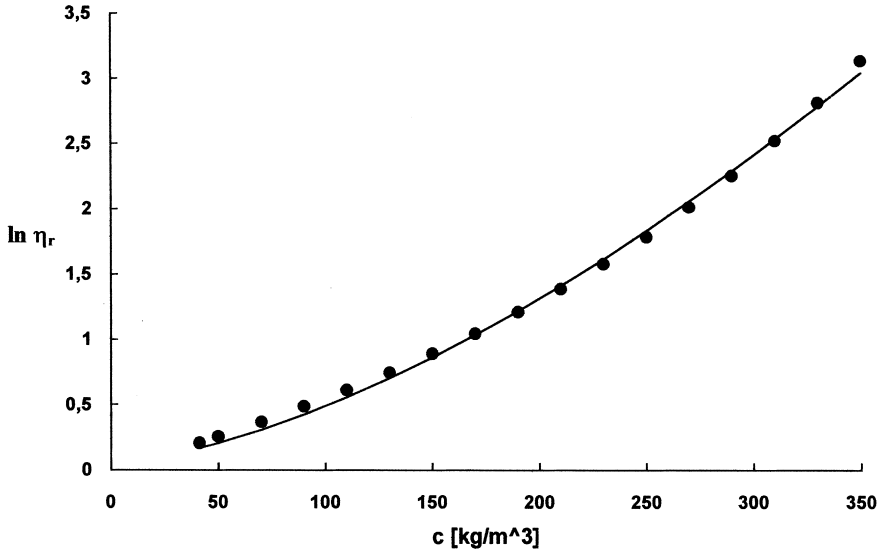

Fig. 5. Plot of the relative viscosity vs. concentration in a log-normal plot in a semi-dilute region for ovalbumin at $t = 5$°C. The curve shows the fit obtained by using Eq. (12).

approximately 1.8 for hard long rods [17]. Fig. 5 shows a plot of the relative viscosity vs. concentration in a log-normal plot, for ovalbumin at 5°C, in a semi-dilute region. The curve shows the fit to the experimental points obtained using Eq. (12). The parameters c^* and a can be established by two methods: (i) taking c^* from the master curve (Fig. 4) and the exponent a is the only parameter in Eq. (12) then; or (ii) taking c^* and a as adjustable parameters in Eq. (12). In both cases the values of c^* and a are nearly identical and the curve obtained on the basis of Eq. (12) gives a good fit to the experimental values. The exponent a (Table 2) does not depend on temperature (within the experimental errors) and its average value is 0.323. The effect of the solution temperature on the MHKS exponent is insignificant for stiff polymers. For relatively flexible chains the values of a increase with increasing solution temperature [42].

As is seen in Table 2, the second boundary concentration c^{**} is (within the experimental errors) the same over the whole range of temperatures. In the concentrated region ($c[\eta] > c^{**}[\eta]$), the effects of intermolecular interactions or entanglements become important. In the case of rigid molecules the action of one molecule encountering a neighboring molecule and limiting the number of paths available to this molecule may serve as an entanglement [43]. As pointed out by some authors, the slope in the log η_{sp}–log $[\eta]c$ plot may be an indicator of stiffness of the polymer. For relatively flexible polymers like citrus pectins [38] the slope in this region is 3.4. A higher value (up to 5) was obtained for semi-rigid cellulose derivatives [37] and even higher value of 6.5 was obtained for a galactomannan [35]. The measurements for some mammalian hemoglobins (which are stiff chained molecules) showed that the slope is in the range of 6.05–7.57. The slope in the concentrated region for ovalbumin (Table 2) indicates that ovalbumin molecules are stiff. Moreover, because the slope changes with a temperature, it suggests that the stiffness of ovalbumin molecules also change. As the slope decreases with increasing temperature, so the lower temperature the more stiff ovalbumin molecules are, as could be expected.

4. Conclusions

The viscosity of ovalbumin aqueous solutions at temperatures ranging from 5 to 55°C may be quantitatively described by a modified Arrhenius formula Eq. (1). The parameters in this formula depend on concentration and they allow, among others, calculation of the activation energy of viscous flow ($E_p = 8.487 \times 10^4$ kJ/mol for hydrated ovalbumin). The shape parameter S in Mooney's approximation decreases with increasing temperature. A comparison of the high temperature value of S with the theoretical value suggests, that the hydrated ovalbumin is more elongated than unhydrated one. The self-crowding factor K, in turn, does not depend on temperature and for ovalbumin has the numerical value $K = 1.81$. Both the intrinsic viscosity and the Huggins coefficient depend on temperature: $[\eta]$ decreases and k_1 increases with increasing temperature. The plot of log η_{sp} vs. log $[\eta]c$ showed that the three regions of concentrations exist: diluted, semi-diluted and concentrated. The MHKS exponent, calculated on the basis of Lefebvre's relation in the semi-dilute region, has the value $a = 0.323$ and does not depend on temperature. Ovalbumin molecules in aqueous solution behave as hard quasi-spherical particles. However, their stiffness changes with temperature: the lower temperature the more stiff ovalbumin molecules are.

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