Light Scattering in the Eye Lens Near the Spinodal

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Scattered light intensities measurements of the nuclear part of adult bovine lenses are reported. In the investigated samples the existence of a binary liquid phase separation from a metastable state is demonstrated by the phenomenon of hysteresis. Fluctuations near spinodal are studied by means of a tentative theoretical approach. Accordingly the scattered light intensities were analyzed reduced by the intensity obtained for the stable state of the system. Fluctuations in the stable state are ascribed to protein aggregates and are analyzed by means of random density fluctuation theory. To evaluate the correlation length of the fluctuations near spinodal the Ornstein-Zernike theory is adopted. Temperature dependence of the correlation length ξ of the fluctuations near spinodal can be described by equation for critical fluctuations $\xi = \xi_0 \left(\frac{T - T_s}{T_s}\right)^{-\nu}$. For the investigated lenses the exponent ν varies from 0.65 to 0.74 and the parameter ξ_0 varies from 1.6 nm to 3.6 nm. The spinodal temperature T_s for the investigated samples is evaluated.

Introduction

Cell cytoplasm of eye lens contains both structural proteins called α-, β- and γ-crystallins and high molecular weight aggregates (Bours *et al.,* 1991a; Bours *et al.,* 1991b; Harding, 1997). The high transparency of eye lens is a result of the spatial organization of crystallin proteins (Benedek, 1971; Delay and Tardieu, 1983). Aggregation and phase separation are processes which lowers the transparency of eye lens. Aggregation is an irreversible process and one of the cataract- formation mechanisms (Tanaka and Benedek, 1975; Benedek *et al.,* 1987). Protein aggregates stimulate density fluctuations in normal and cataractous lenses (Bettelheim and Paunovic, 1979; Siew *et al.,* 1981; Bettelheim *et al.,* 1995). Phase separation of eye lens cytoplasm arises from attractive interactions between γ-crystallins. The characteristic temperature for the phase separation is related to protein-water and protein-protein interactions energies (Pande *et al.,* 1991). At temperature about 15 °C in calf lenses this phenomenon is visible as an opacification called cold cataract (Zigman and Lerman, 1964). In adult bovine lenses a decrease of temperature causes decrease of transparency, however, the opacification characteristic for the cold cataract is not observed.

Purified γ-crystallin solutions can be treated as an idealized model of eye lens cytoplasm undergoing phase separation. This solutions have a stable dispersion at higher temperatures. At lower temperatures a coexistence curve and a spinodal line is observed (Siezen *et al.,* 1985; Thomson et al., 1987; Broide *et al.,* 1991). Critical phenomena in γ-crystallin solution can be analyzed by means of the rigorous theory of critical fluctuations (Schurtenberger *et al.,* 1989). The lens cytoplasm has a more complex composition than a purified crystallin solution. Biochemical studies as well as investigations of light scattering have shown that the lens cytoplasm in its stable state is an inhomogeneous medium (Tanaka *et al.,* 1977; Delay *et al.,* 1982; Ansari *et al.,* 1998). This fact does not affect significantly the phase diagram of young lenses (Clark and Benedek, 1980; Tanaka *et al.,* 1983). Effects associated with phase separation are clearly visible in purified solutions. In adult lenses the proteine aggregates blurres the effects. For example, to study the nucleation process of adult lenses the technique of standardized residuals was adopted to reduce the scattered light intensity responsible for structural elements (Grzegorzewski,

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1999). For calf lens the fluctuations near spinodal and structural elements were modeled with the use of a bimodal distribution of spherical scatterers (Delay *et al.*, 1982).

In this study static light scattering measurements for the nuclear part of adult bovine lenses are reported. The fluctuations near the spinodal in the lenses are identified and correlation lengths responsible for the fluctuations are evaluated. A simplified theoretical approach to the problem is proposed due to lack of a rigorous theory of light scattering near the spinodal line in the lens. It is assumed that the scattered light intensity is a sum of processes responsible for both protein aggregates and fluctuations near the spinodal line. To determine the correlation length responsible for fluctuations near spinodal the Ornstein-Zernike theory is adopted.

Theoretical Background

Let us consider that eye lens cytoplasm undergoes phase separation. At higher temperatures, well above spinodal temperature, the cytoplasm is an inhomogeneous medium. Density fluctuations in the cytoplasm are responsible for protein aggregates (Bettelheim and Paunovic, 1979; Siew *et al.*, 1981; Bettelheim, 1985). The correlation function $\gamma(r)$ of the fluctuations has the form

$$\gamma(r) = \sum_{i=1}^{2} A_i \exp\left\{-\left(\frac{r}{a_i}\right)^2\right\}. \tag{1}$$

The correlation length a_1 corresponds to the size of protein aggregates. The second correlation length a_2 is usually larger compared to a_1 and describes the size of the spacing between aggregates. The correlation function is normalized by $\sum_{i=1}^{2} A_i = 1$. According to the Debye-Bueche theory (Kerker, 1969; Bettelheim and Paunovic, 1979) the intensity of light scattered by density fluctuations is given by

$$I_a = C\bar{\eta}^2 \int \gamma(r) \frac{\sin Kr}{Kr} r^2 dr, \tag{2}$$

where $\bar{\eta}^2$ is the average deviation in density fluctuation, C is a constant, $\gamma(r)$ is the correlation function of the density fluctuations, K is the wave vector defined by

$$K = \frac{4\pi n}{\lambda} \sin\frac{\Theta}{2}, \tag{3}$$

where λ is the wavelength of the incident radiation, n is the refractive index in the medium and Θ is the scattering angle. The algebraic inversion of static light scattering data permits to find the correlation function $\gamma(r)$ (Kerker, 1969). The correlation function $\gamma(r)$ can be obtained even if absolute light scattered intensities are not determined. Due to the fact that the correlation function (1) is a sum of two elements we can define the intensity contribution responsible for the correlation length a_1 by

$$I_1 = C\bar{\eta}^2 \int A_1 \exp\left\{-\left(\frac{r}{a_1}\right)^2\right\} \frac{\sin Kr}{Kr} r^2 dr \tag{4}$$

and the contribution responsible for a_2 by

$$I_2 = C\bar{\eta}^2 \int (1-A_1) \exp\left\{-\left(\frac{r}{a_2}\right)^2\right\} \frac{\sin Kr}{Kr} r^2 dr. \tag{5}$$

The intensity of light scattered by cytoplasm and the intensity contributions I_1 and I_2 can be calculated using parameters of the correlation function $\gamma(r)$ (For example, in a human lens $A_1 = 0.3$; $a_1 = 0.3$ μm; $a_2 = 0.6$ μm; Bettelheim and Paunovic, 1979; Siew *et al.*, 1981). With $\lambda = 0.63$ μm the scattered light intensity at wave vectors higher than ~ 8 μm^{-1} is determined by the size of protein aggregates. On the other hand, at wave vectors higher than ~ 8 μm^{-1} the influence of the contribution I_2 on the scattered light intensity can be neglected. At small values of the wave vector the intensity is dependent on a_1 and a_2.

At high temperature the system is stable but inhomogeneous. Lowering the temperature the system reaches spinodal. Phase separation in eye lens arises due to attractive interactions between γ-crystallins. It is rather clear that protein aggregates can not participate in propagation of the interaction. Thus close to the spinodal interactions can propagate between protein aggregates from one γ-crystallin to the other. For this case of scattering we propose a simplified theoretical approach and assume that in the region of the wave vectors where the influence of protein aggregates on the scattering is dominant the intensity of scattered light is a sum of the component I_1 described by the density fluctuation theory (Kerker, 1969; Bettelheim and Paunovic, 1979) and the intensity responsible for critical fluc-

tuations described by the Ornstein-Zernike theory (Dhont, 1996)

$$I = I_1 + \frac{C_1}{\xi^{-2}+K^2} \cdot \tag{6}$$

In equation (6) ξ is the correlation length which describes the range of interactions and C_1 is a constant. We assume that the formula can be used in case the cytoplasm concentration is less then the critical one. The correlation length ξ can be determined by reduced intensity defined by

$$I(K,T) - I(K,T \gg T_s) = \frac{C_1}{\xi^{-2}+K^2} \cdot \tag{7}$$

where T is temperature and T_s represents the spinodal temperature for the investigated sample. The correlation length ξ can be obtained even if absolute light scattered intensities are not determined. For a purified solution the intensity of light scattered at higher temperature $I(K,T \gg T_s) \rightarrow 0$. The intensity for zero wave vector diverges as the system reaches the spinodal. The divergence is most evident at critical concentration (Dhont, 1996).

The proposed approach is insufficient to predict quantitatively the intensity alterations in the region of small wave vectors. As the fluctuations near the spinodal develop the cytoplasm between the aggregates undergoes a rearrangement leading to alterations of the intensity component I_2. One can expect that the development of the fluctuations can be associated with a decrease of the intensity component I_2. By definition (5) it is allowed that the reduced intensity can be either positive or negative.

For a purified solution the optical turbidity can be used to determine the correlation length ξ. The above considerations show that for lenses the optical turbidity can not be a base to determine the correlation length. An integral (8) of the reduced intensity (7) can be used to evaluate the temperature dependence of the correlation length ξ.

$$Int\{I(K_1; K_2, T)\} = \int_{K_1^2}^{K_2^2} [I(K^2, T) - I(K^2, T \gg T_s]d(K^2) = C_1 \, In\left(\frac{\xi^{-2}+K_2^2}{\xi^{-2}+K_1^2}\right), \tag{8}$$

where K_1 is the smallest wave vector at which the intensity component I_2 can be neglected and K_2 is determined by the largest scattering angle used in the experiment.

Materials and Methods

The bovine eyes were obtained from a local slaughterhouse. The fresh lenses were dissected from the eyes and their wet weight was determined and the nuclear part of the lens was separated. Then the nuclear part was placed between two plane parallel glass plates and squashed. The 70 μ-m spacer was placed between the glass plates to fix the final thickness of the layer. The samples were performed at room temperature. The refractive index of the nuclear part was estimated with the use of an Abbe refractometer. By this procedure the cellular structure of the lenses was destroyed and therefore the experimental results may not provide an accurate representation of intact lens cytoplasm.

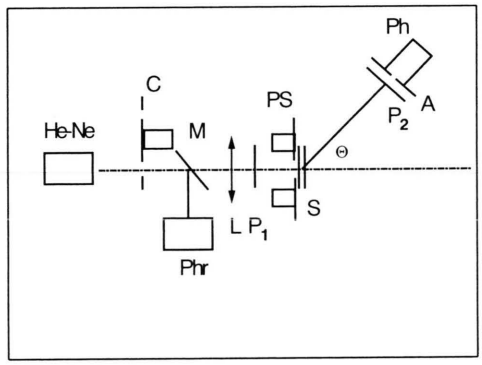

Fig. 1. Schematic diagram of the experimental set up for the measurements of the intensity of scattered light. He-Ne, helium-neon laser; C, chopper; M., half-passing mirror; Phr, reference photomultiplier, L, optical system; P_1, polarizer; PS, Peltier system; S, sample; P_2, analyzer; A, aperture; Ph, photomultiplier.

The experimental set up for the light scattering experiment is shown in Fig. 1. Light was provided by a He-Ne laser operating at the wavelength 632.8 nm. The temperature of the sample was fixed with an accuracy of 0.1 °C by a Peltier cooling system. The measurements were performed within the temperature range from 20 °C to 35 °C. At a fixed temperature the $I_{||}$ mode of the scattered light was detected as a function of the scattering angle. The measurements of the intensity of the scattered light show that the intensity in the I_+ mode is much smaller than in the $I_{||}$ mode.

Results

Fig. 2 shows the intensity of scattered light as a function of temperature for the nuclear part of bo-

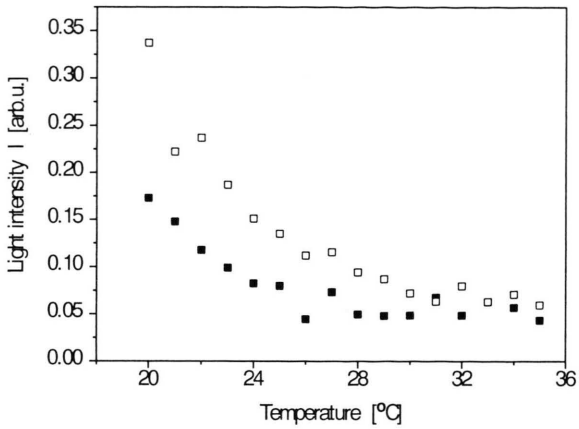

Fig. 2. Intensity of light scattered by the nuclear part of the bovine lens with a wet weight of 2.75 g versus temperature at scattering angle $\Theta = 55°$. Open symbols denote intensity measured as a function of increasing temperature and filled symbols denote intensity measured as a function of decreasing temperature.

vine lens. Increasing the temperature the intensity shows higher values compared to the intensity values obtained for decreasing temperature. For the sample at about 30 °C the intensities reach comparable minimum values. Fig. 2 illustrates the phenomenon of hysteresis associated with phase separation from metestable state.

Samples of investigated lenses were first incubated in the stage of the experimental set up at temperature 35 °C for 0.5 h to avoid non-equilibrium states. Then at a fixed temperatures the angular dependence of the scattered intensity was measured. Fig. 3 shows the intensity of scattered light as a function of wave vector at different temperatures for the nuclear part of the bovine lens. In general, intensity of the scattered light decreases as a function of the wave vector however fluctuations of the intensity can be observed. Up to about 32 °C the intensity at larger values of the wave vector has a minimum and next increases with decreasing temperature. Thus we can assume that at 35 °C the system is stable. At this temperature fluctuations arising near spinodal can be neglected. On the other hand at 35 °C light is scattered only by protein aggregates of the lens. Fig. 4 shows the experimental data of the scattered intensity at temperature 35 °C and the calculated intensity contributions responsible for the correlation length a_1 and a_2 respectively. Density fluctuation theory gives parameters of the correlation function (1) for the particular sample. At wave vectors higher than ~ 6 μm^{-1} the scattered light intensity is determined by the correlation length a_1. At small values of the wave vector the intensity is determined both by a_1 and a_2 however the contribution I_2 is much higher compared to I_1. The reduced intensity $I(K,T) - I(K,T = 35\ °C)$ for various temperatures is shown in Fig. 5. At wave vectors larger then ~ 6 μm^{-1} the reduced intensity is positive and increases with decreasing temper-

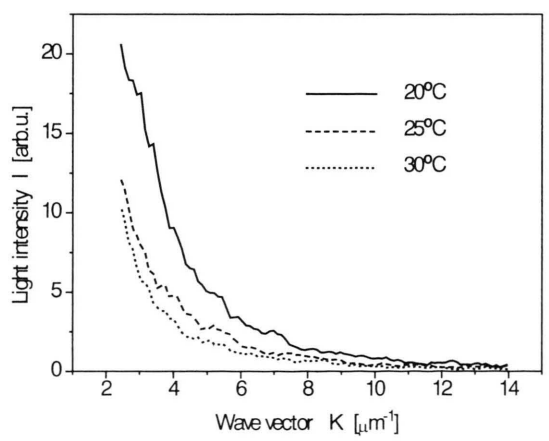

Fig. 3. Intensity of light scattered by the nuclear part of the bovine lens with a wet weight of 2.73 g versus wave vector for various temperatures.

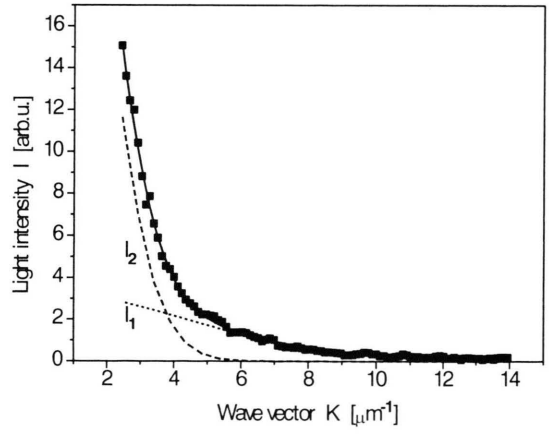

Fig. 4. Intensity of light scattered by the nuclear part of the bovine lens with a wet weight of 2.73 g as function of wave vector at temperature 35 °C (■). The solid line represents calculated intensity at $A_1 = 0.64$; $a_1 = 0.32$ μm; $a_2 = 0.88$ μm. The dotted line shows the protein aggregates contribution and the dashed line shows the aggregate separations contribution.

ature. At wave vectors less then ~ 6 μm^{-1} the reduced intensity decreases, at about 29 °C reaches a minimum and at lower temperatures increases. According to Fig. 4 and Fig. 5 the negative values of the reduced intensity arise in the region of wave vectors where the correlation length a_2 has a significant influence on the scattered intensity.

Fig. 6 presents the reciprocal reduced intensity versus K^2 for two temperatures. At temperatures below 23 °C the reciprocal reduced intensity shows a nonlinear dependence versus K^2. The linear dependence of the reciprocal reduced intensity as a function of K^2 at temperature 23 °C was observed. This fact confirms the theoretical prediction given by formula (7) at $\xi \to \infty$. This formula predicts the linear dependence of the reciprocal reduced intensity at arbitrary values of correlation length ξ. Small values of the reduced intensity at higher temperatures and especially fluctuations of the intensity stimulate large fluctuations of the reciprocal intensity. This increases errors in analysis of the data. A fitting procedure applied to the reduced intensity permits to find values of the correlation length ξ as a function of temperature. The data presented in Fig. 7 show that the temperature dependence of the correlation length can be described by equation (9) with the assumption that C_1 is temperature-independent.

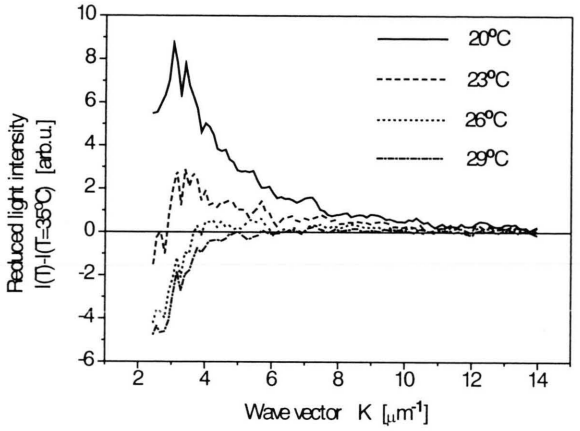

Fig. 5. Reduced intensity of light scattered by the nuclear part of the bovine lens with a wet weight of 2.73 g as a function of wave vector for various temperatures.

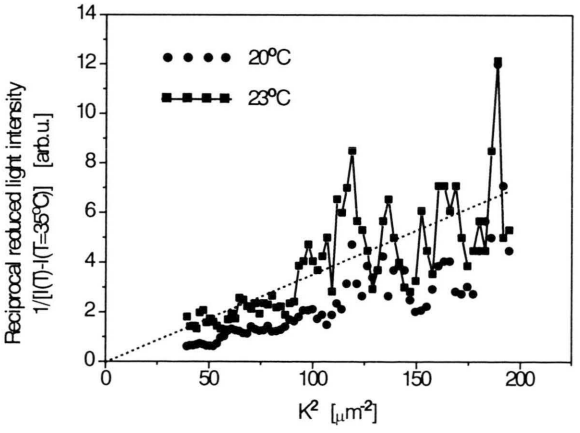

Fig. 6. Reciprocal reduced intensity of light scattered by the nuclear part of bovine lens with a wet weight of 2.73 g versus K^2 for various temperatures. The dotted line represents the theoretical values of the reciprocal reduced intensity at $\xi \to \infty$.

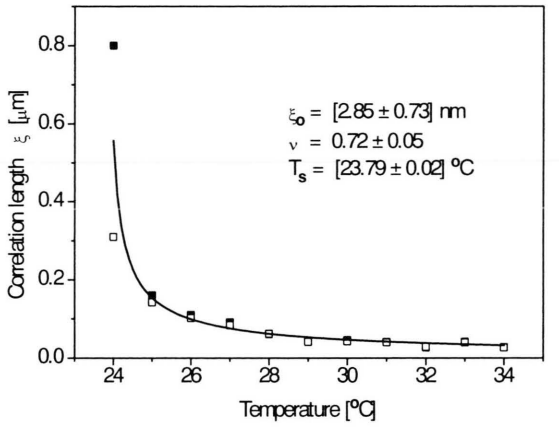

Fig. 7. Correlation length of the fluctuations near spinodal vs. temperature for the nuclear part of bovine lens with a wet weight of 2.73 g. Filled symbols denote correlation length calculated from the reduced intensity and open symbols represent correlation length calculated from the integral of the reduced intensity. The line represents the best fit using equation (9).

$$\xi = \xi_0 \left(\frac{T - T_s}{T_s} \right)^{-\nu}, \tag{9}$$

where ξ_0 is a constant, ν an exponent and T_s the spinodal temperature. For critical concentration the spinodal temperature T_s becomes the critical temperature T_c and ν represents the critical exponent. The solid line in Fig. 7 represents the best fit calculated by formula (9). Fig. 8 shows the integral of the reduced intensity as a function of temperature. The correlation lengths ξ calculated from the integrals of the reduced intensity are shown in Fig. 7. The

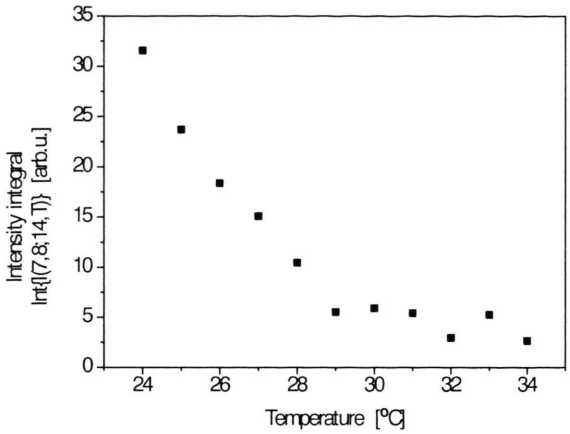

Fig. 8. Integral of the reduced intensity of light scattered by the nuclear part of the bovine lens with a wet weight of 2.73 g as a function of temperature.

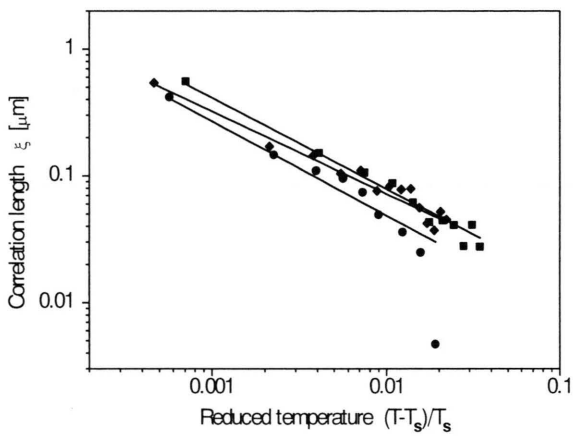

Fig. 9. Correlation length versus reduced temperature for bovine lenses with a wet weight of 2.03 g (◆), 2.57 g (●) and 2.73 g (■). Lines were obtained from the best fit on the base of equation (9).

temperature dependence of the correlation length ξ obtained for three various lenses is shown in Fig. 9. For the lens with a wet weight of 2.03 g we have obtained $\xi_0 = [3.6 \pm 1.1]$ nm, $\nu = [0.65 \pm 0.05]$ and $T_s = 27.9$ °C while for the lens with a wet weight of 2.57 g $\xi_0 = [1.6 \pm 1.1]$ nm, $\nu = [0.74 \pm 0.13]$ and $T_s = 24.6$ °C has been found. The parameters for the lens 2.73 g wet weight are shown in Fig. 7.

Discussion

Phase separation may occur in a solution where interactions of molecules have an attractive component. In eye lenses attractive interactions are present among γ-crystallins (Tardieu and Delaye, 1988). The nuclear part of adult bovine lenses has a complex composition. Membranes, protein aggregates as well as the presence of a whole family of α, β and γ-crystallins makes the system difficult to define (Harding, 1997). Especially, the concentration of lens cytoplasm is unknown. In calf lenses the concentration of the nuclear cytoplasm is close to the critical one (Delay *et al.*, 1981). With lens aging the concentration of γ-crystallins decreases. The thin-layer isoelectric focusing of the nucleus of the bovine lens demonstrates a significant presence of γ-crystallins up to 34 years (Bours *et al.*, 1991a). For human lenses γ-crystallins are observed up to 25 years (Bours *et al.*, 1991b). In this paper lenses 2–15 years old were investigated (Hockwin *et al.*, 1963). Thus one can expect that the cytoplasm concentration of the investigated lenses is less then the critical one. The phenomenon of hysteresis illustrated in Fig. 2 is characteristic for phase separation from metastable state. This result is an indicator of the phase separation process and confirms that the concentration of the nuclear cytoplasm of adult bovine lenses is less then the critical one. The phenomenon of hysteresis was already observed for isolated calf nuclear cytoplasm at concentration less than the critical one (Delay *et al.*, 1981).

Fig. 2 as well as Fig. 3 and 5 show that nuclear parts of adult bovine lenses are stable at 35 °C. Especially Fig. 4 shows that in the stable state the sample is inhomogeneous. The correlation lengths a_1 and a_2 have values comparable to the values obtained for thin sections of human lenses while the parameter A_1 has higher values (Bettelheim and Paunovic, 1979; Siew *et al.*, 1981). That optical parameters obtained for the investigated samples in the stable state may not be fully representative for *in vivo* lenses, since the cellular structure was partially destroyed during the sample preparation.

Lowering the temperature of a solution in a stable state the system can cross coexistence curve and reach spinodal. According to the theoretical predictions the intensity of scattered light in the region of relatively large wave vectors is a sum of contribution responsible for protein aggregates and contribution responsible for fluctuations near spinodal. We have assumed that the fluctuations near spinodal can be described by the Ornstein - Zernike theory. To verify the assumption is neces-

sary to show that the reciprocal of the reduced intensity as a function of K^2 can be described by linear function at arbitrary value of the correlation length ξ. The experimental data demonstrate the linear dependence at $\xi \to \infty$. It permits to estimate the spinodal temperature. This case can be simply recognized because at lower temperatures the reciprocal of the reduced intensity is a nonlinear function of K^2. The intensity contribution responsible for fluctuations near spinodal is relatively small. The cellular structure of the lens is not completely destroyed and fragments of the structure are large scattering units. The intensity fluctuations caused by the units make difficult evaluation of the correlation length ξ at arbitrary temperature. However, under the condition that the constant C_1 is temperature-independent the reduced intensity data permit to find the correlation lengths ξ. Analysis of the integral of the scattered intensity gives values of the correlation length ξ close to that obtained from the previous method. It is a confirmation of the theoretical assumptions. The data presented in Fig. 7 and Fig. 9 show that the temperature dependence of the correlation length ξ can be expressed by the formula that at critical concentration describes critical fluctuations. Due to the fact that the system reaches spinodal from metastable state the exponent ν can not be treated as the critical exponent; its values are close to that for the critical exponent for liquids. For a γII-crystallin solution the critical exponent is $\nu = 0.68$ (Schurtenberger *et al.,* 1989). The values of the exponent evaluated from our measurements varies from 0.65 to 0.74. The parameter ξ_0 varies from 1.6 nm to 3.6 nm. For a γII-crystallin solution at critical concentration the parameter ξ_0 is 0.6 nm (Schurtenberger *et al.,* 1989).

At small wave vectors the reduced intensity is negative. Figs 5 and 6 show that the values of the reduced intensity correspond to a decrease of the intensity contribution I_2 responsible for aggregate separations. The decrease of the intensity contribution responsible for aggregate separations is connected with an increase of the contribution responsible for fluctuations near spinodal. Thus alterations of the reduced intensity illustrate a reorganization of the cytoplasm within protein aggregates. By means of the proposed model one can explain the small alterations of transmission of adult bovine lens lowering the temperature (Grzegorzewski, 1996; Grzegorzewski *et al.,* 1998;). The restricted space for propagation of interactions between γ-crystallins implies that the magnitude of light scattered by fluctuations near spinodal is relatively small. Also the concentration of the cytoplasm being less than a critical results in relatively small alterations of the intensity.

In the center of a calf lens the cytoplasm becomes opaque at 19 °C (Delay *et al.,* 1982). The change of the heat capacity of a calf lens nucleus occurs at 16 °C (Bettelheim and Christian, 1983). The decrease of the γ-crystallin concentration in adult lenses should be associated with a decrease of the spinodal temperature. Indeed it was shown that for young lenses the characteristic temperature decreases with aging (Clark *et al.,* 1983). Investigations of adult bovine lenses show that the spinodal temperature is well above 20 °C (Grzegorzewski, 1996, 1999; Grzegorzewski *et al.,* 1998). In the X-irradiated eye an increase of the temperature was observed (Clark *et al.,* 1982). The characteristic temperature for the phase separation of lenses from galactosemic young rats is much higher compared to the temperature for the normal lens (Ishimoto *et al.,* 1979). Due to the fact that the lenses investigated in this paper were normal it seems that the high spinodal temperatures of adult bowine lenses are caused by processes connected with aging.

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