Phase Separation of a Protein-Water Mixture in Cold Cataract in the Young Rat Lens

Abstract. *A remarkable resemblance between the appearance of opacity in lysozyme–salt water mixtures and the development of opacity in cold cataract in the young rat lens is strong evidence that cold cataract is fundamentally a phase separation of the "protein-water binary mixture" in the lens.*

We report a striking resemblance between the development of opacity in a lysozyme–salt water mixture near phase separation and the development of opacity in cold cataract in the lens of a young rat, as revealed by light-scattering techniques. In cold cataract the opacity appears reversibly on lowering the temperature of the excised lens of certain young animals such as the calf and the young rat (*1*). Much cataract research has been devoted to this phenomenon (*2*). It has been known that the opacity associated with cold cataract develops as a result of changes in the physicochemical state of the lens proteins (*3*), but the precise nature of such changes has not been clearly understood. Observations made with light-scattering techniques are consistent with the description of these changes as the phase separation of a "protein-water binary mixture" (*4*). The observations reported here constitute strong evidence that this description is correct.

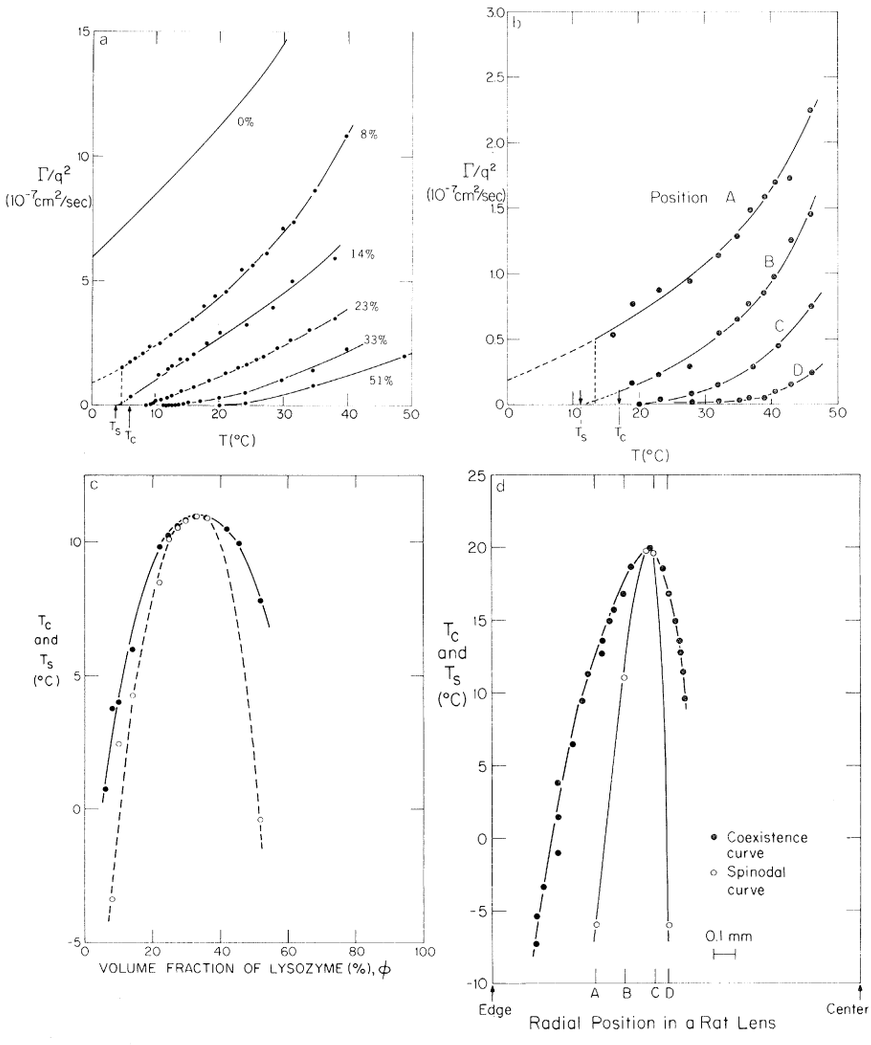

Fig. 1. (a and b) Temperature dependence of Γ/q^2 for (a) a lysozyme–salt water mixture at several different volume fractions, and (b) a protein-water mixture in a fresh young rat lens at several different radial positions in the equatorial plane of the lens. The radius is 1.75 mm. The curve labeled 0 percent in (a) was calculated for an infinitely dilute lysozyme–salt water mixture. (c and d) Coexistence curve (●) and spinodal curve (○) for (c) lysozyme–salt water mixtures, and (d) protein-water mixtures in a young rat lens, determined by measurements of the intensity and the decay rate of fluctuations of laser light scattered by the mixtures. Some points in the coexistence curve for the lens were measured by observing the appearance of opacity.

Concentration fluctuations on the scale of the wavelength of visible light in a solution result in corresponding fluctuations of the refractive index. The rate of decay, Γ, of the concentration fluctuations can be determined by measuring the autocorrelation function of the intensity fluctuation of laser light scattered by the solution (*5*). According to the widely accepted dynamical theory of Kawasaki (*6*), this is related to the spatial correlation length, ξ, of the concentration fluctuations (*7*) by

$$\Gamma = (kT/6\pi\eta\xi)q^2 \qquad (1)$$

where k is Boltzmann's constant, T is the absolute temperature, η is the viscosity of the solution (*8*), and q^2 is the square of the magnitude of the scattering vector $\mathbf{q}$ (*9*). In our experiments incident light of wavelength 514.5 nm was provided by a Spectra-Physics model 164 argon ion laser at a power output of about 100 mw. The beam was focused on the scattering region and the light scattered at 90° was collected and imaged on a photomultiplier tube. The photocurrent pulses were amplified and then analyzed using a 19-channel digital autocorrelator and a minicomputer.

Lysozyme in the form of crystal flakes was gently dissolved, with precautions taken to avoid denaturation, in an aqueous solution of 0.5*M* NaCl; several different volume fractions (percentages of the solution volume occupied by lysozyme molecules) were prepared. The dissolved mixture was centrifuged for 5 minutes at 1500*g* to remove air bubbles and remaining undissolved material. The volume fraction was determined by using the measured absorbance at 280 nm (A_{280}) after a 1000-fold dilution in water; the extinction coefficient of lysozyme $E^{1\%}_{1\,cm} = 26$; and the partial specific volume of lysozyme, $\bar{\nu} = 0.703$. The volume fraction was then multiplied by 0.74, so that 100 percent corresponded to the close-packed arrangement in which the shape of the lysozyme molecule is approximated by a sphere. The scaled volume fraction was given by

$$(1000/0.74)\,A_{280}\bar{\nu}/E^{1\%}_{1\,cm} = 37\,A_{280}\,\text{percent}$$

Since crystals slowly formed in all the mixtures, measurements were performed repeatedly to make certain that the results were not changed by the crystallization process. No significant changes due to crystallization were noted over the initial period of 2 to 3 hours after sample preparation. The crystals represent the true equilibrium condition; the mixtures measured were in metastable equilibrium.

Figure 1a shows the dependence of Γ/q^2 on temperature for a mixture of lysozyme (Worthington; specific activity, 11,770 units) and an aqueous solution of 0.5*M* NaCl at several different volume fractions. As the temperature is lowered Γ/q^2 diminishes, until at a certain temperature, T_c, it suddenly goes to zero. According to Eq. 1, this behavior indicates that the correlation length, ξ, and the scattering intensity, I, which is proportional to ξ^2, increase as the temperature is lowered and that at T_c there is a discontinuous increase in the scattered light intensity and the correlation time of its fluctuation to effectively infinity. The mixture in this condition appears completely opaque (*4*). The extrapolated curve of Γ/q^2 or I plotted against T intercepts the horizontal axis at the spinodal temperature, T_s. Figure 1c shows the dependence of both T_c and T_s on the volume composition of the mixture. The curves are, respectively, the coexistence curve and the spinodal curve (*4*). These two curves have maxima at the same position in the diagram, the critical mixing point. At this point, the volume fraction of lysozyme, the critical volume fraction, is 33 percent. This set of observations is entirely consistent with the characteristics of critical binary mixtures (*10*). These phenomena have been investigated in many binary mixtures of small molecules and in binary mixtures of flexible polymers and solvents (*11*). In all these cases, below T_c the mixture is partitioned into two bulk phases. In the lysozyme–salt water mixtures, however, below T_c the domains of the separated phases remain dispersed and do not grow to visible dimensions, so that the mixtures remain homogeneously opaque.

The lysozyme–salt water mixture should strictly be described as a many-component mixture rather than as a binary mixture. However, in view of the nature of the lattice model on which the theory of the phase separation of binary mixtures is based (*7*), it is reasonable to treat it as a binary mixture in which one component is lysozyme and the other component is the aqueous solution of 0.5*M* NaCl. The positions of the coexistence curve and the spinodal curve are greatly affected by the *p*H and the ionic strength of the solution. The *p*H of these samples (~5.5), measured on dissolving the lysozyme crystals in an aqueous solution of 0.5*M* NaCl, is essentially constant throughout the concentration range studied. The aqueous solution of 0.5*M* NaCl was used in order to investigate a set of lysozyme–salt water mixtures with the critical point at a conveniently studied temperature. Without salt, the critical point is below −10°C.

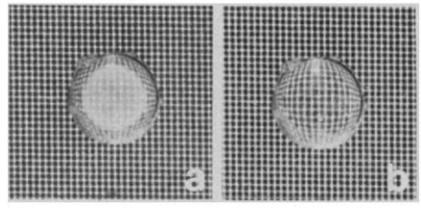

Fig. 2. Excised lens of a 100-g albino rat (a) at 12°C, showing the opacity of cold cataract in the form of a shell, and (b) at 25°C, showing the absence of the opacity. The radius of the lens is 1.75 mm.

Figure 1b shows the dependence on temperature of Γ/q^2, and Fig. 1d shows T_c and T_s, at several radial positions in the equatorial plane inside the excised lens of a young albino rat (body weight, 100 g). The radius of the lens was 1.75 mm. The values of T_c and T_s were determined in the same way as for the lysozyme–salt water mixtures. Some determinations of T_c were also made by observing the appearance of opacity. The long, spindle-shaped fiber cells of the lens are packed in such a fashion that each fiber cell lies approximately along a concentric shell. Since there is no extensive compartmentalization of the cytoplasm of an individual fiber cell, the protein volume fraction can be considered internally homogeneous. The volume fraction may, however, vary from cell to cell. Scattered light was collected from a scattering region with an approximate linear dimension of 20 μm, which is several times the diameter of a lens fiber cell. There is a remarkable similarity between the curves in Fig. 1, a and b, and those in Fig. 1, c and d. The general character of the dependence of the features of the curves in Fig. 1b and of the values of T_c and T_s (Fig. 1d) on radial position may be attributable in large part to the monotonic increase of the protein volume fraction along the radius from the periphery to the center of the lens (*12*). As the temperature of the lens is lowered, a concentric shell of opacity appears at 20°C (Fig. 2). The shell of opacity grows both toward the center and toward the periphery as the temperature is lowered further. This corresponds to the widening distance between the two intersections of the isotherm with the coexistence curve as the temperature is lowered. At a given temperature, opacity due to phase separation is produced at all volume fractions between the two intersections. The presence of opacity at lower temperatures below the coexistence curve is similar to the behavior of the lysozyme–salt water mixture rather than of binary mixtures of small molecules, which undergo bulk phase separation. It is interesting that in cold cataract of the calf lens the region of opacity appears as a concentric solid spheroid rather than a shell (*4*). In this case, T_c is a monotonically decreasing function of the radial distance from the center. This is presumably because the protein volume fraction does not increase to the critical volume fraction even at the center (*4*).

It has been suggested by Lerman and Zigman (*3*) that the change of the state of the protein-water mixture in cold cataract reflects a change in the conformation of the lens protein, perhaps to expose more hydrophobic regions at low temperatures. The analysis presented here shows that such conformational changes are not needed to explain the experimental findings. The critical behavior of a binary mixture can be understood theoretically by considering the free energy to include a constant mixing enthalpy term and a temperature-dependent mixing entropy term (*7*). The lens fiber cell contains many different species of soluble proteins. The effect of this heterogeneity on our interpretation requires further investigation. It is possible that the presence of many protein components requires analysis in terms of higher-order mixtures. However, the general features of the critical behavior of binary mixtures can be seen in Fig. 1, b and d. Reversible cold precipitation of the isolated protein of the young rat lens has been observed (*3*). One component, γ-crystallin, is able to undergo reversible cold precipitation both with and without the other components. The concentration gradient of γ-crystallin along the radius of the young rat lens is larger than that of the total soluble protein (*3*). It would clearly be desirable to investigate the critical behavior of salt water mixtures of individual components of lens protein and of their combinations.

The concept of critical binary mixtures, heretofore used to describe mixtures of small molecules, can also be used to describe protein-water mixtures. The phenomenon of cold cataract is entirely consistent with such a description of protein-water phase separation, which may provide a useful language with which to work in investigating cold cataract.

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8. The so-called high-frequency shear viscosity (6), η, is significantly different from the macroscopic viscosity in the case of lysozyme–salt water mixtures (C. Ishimoto and T. Tanaka, in preparation).
9. The scattering vector, $\mathbf{q}$, is defined as $\mathbf{q} = \mathbf{k}_s - \mathbf{k}_i$, where $\mathbf{k}_s$ and $\mathbf{k}_i$ are the wave vectors of the scattered light and the incident light, respectively; q^2 is a constant for a fixed scattering angle.
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13. We thank G. B. Benedek, P. Crooker, and J. D. Litster for helpful discussions. Supported by NIH grant EY01696 and the Health Sciences Fund, Massachusetts Institute of Technology.

16 March 1977

Immunological Resolution of a Diploid-Tetraploid Species Complex of Tree Frogs

Abstract. *Micro–complement fixation studies of eastern and western populations of the North American tree frog* Hyla chrysoscelis *reveal they have been genetically isolated for about 4 million years. Immunological comparisons of populations of the cryptic tetraploid* Hyla versicolor *indicate a recent origin, from hybridization between eastern and western* H. chrysoscelis.

The North American cryptic species pair of hylid frogs, *Hyla chrysoscelis* and *H. versicolor*, are respectively diploid and tetraploid (*1, 2*). Despite their remarkable morphological resemblance, these two species can be distinguished by karyotypic analysis, the nonoverlapping pulse rates of the male mating calls, and their incompatibility in hybridization tests (*3, 4*).

Electrophoretic studies have demonstrated that populations of the diploid *H. chrysoscelis* from South Carolina, Georgia, Ohio, and Mississippi are genetically different from diploid populations of central Texas (*5*). The two population groups, "eastern" and "western" *H. chrysoscelis*, can be readily distinguished because they are monomorphic for different alleles at the *LDH-B* [heart LDH (lactate dehydrogenase)] locus. The two groups of populations also have significantly different, but overlapping, mating call pulse rates and durations (*5*).

We were interested in determining the time of separation of *H. chrysoscelis* into eastern and western gene pools, and estimating the age of origin of the tetraploid, *H. versicolor*. To approach these questions, we used the quantitative immunological technique of micro–complement fixation. This approach is useful in estimating divergence times of related species (*6*), in reconstructing phylogenetic histories (*7*), and in detecting cases of convergent evolution (*8*). At the time of collection, the species of all frogs used in our study were identified by mating call (*9*). Subsequent karyotypic analyses of samples from each population confirmed the specific designations (*2, 10*).

Antiserums were produced to purified serum albumin of *H. chrysoscelis* from Oktibbeha County, Mississippi, and of *H. chrysoscelis* from an allopatric locality in Bastrop County, central Texas, by techniques described in Champion *et al.* (*11*). The two antiserums were tested for cross reaction with plasma samples of *H. chrysoscelis* from the same populations, from other eastern and western localities, and with plasma samples of *H. versicolor* from Texas and northeastern populations. Genetic distances between sample pairs are reported in immunological distance units (IDU). For hylid albumins, we have shown that a single IDU corresponds roughly to a single amino acid substitution (*8*), and the mean evolutionary rate of albumin is 1 IDU per 0.6 million years (*6*).

Thirty-three *H. chrysoscelis* were tested against both the eastern and western antiserums (Table 1). Serum albumins of 15 *H. chrysoscelis*, from four eastern localities, were identical to the Mississippi *H. chrysoscelis* antiserum (IDU = 0.4 ± 0.7), but an average distance of 4.5 ± 1.7 IDU from the central Texas, or western, *H. chrysoscelis* antiserum (*12*). Conversely, 18 frogs from five western populations were essentially identical to the antiserum against central Texas *H. chrysoscelis* (IDU=0.9 ± 1.3), but an average distance of 9.3 ± 1.5 IDU from the eastern *H. chrysoscelis* antiserum (*13*). The populations tested include one population sympatric with *H. versicolor*, three allopatric populations within 50 km to the west of sympatry and one population at the western extremity of the range in central Texas. In all of these cases, *H. chrysoscelis* individuals tested against both antiserums could be unequivocally assigned to either the eastern or western population group (*14*). Such magnitudes of immunological distance are unknown among populations of other species of hylid frogs. For example, immunological studies of geographically widely separated populations of *H. crucifer* show no albumin differentiation (*15*), and extensive studies of geographically separated and morphologically diverse *H. regilla* populations, many considered subspecies (*16*), differ

Table 1. Albumin comparisons involving populations of eastern and western *Hyla chrysoscelis*. The numbers in parentheses indicate the number of individuals for which immunological distance was measured.

Individual plasma from	Immunological distance to *Hyla chrysoscelis*	
	Eastern*	Western†
Eastern populations:		
Mississippi (homologous)	0.0	5.0
Mississippi (4)	0.5	5.2
Ohio (4)	0.5	6.5
Georgia (3)	0.3	4.0
South Carolina (4)	0.2	2.2
Average (15)	0.4 ± 0.7	4.5 ± 1.7
Western (Central Texas) populations:		
Bastrop County (homologous)	9.0	0.0
Bastrop County (4) (Alum Creek, sympatric)	8.5	1.0
Bastrop County (3) (Colorado River, allopatric)	8.0	0.0
Bastrop County (3) (Elgin, allopatric)	9.7	0.0
Gonzales County (5) (Highway 90, allopatric)	10.8	2.2
Gillespie County (3) (Fredriksburg, allopatric)	9.0	0.3
Average (18)	9.3 ± 1.5	0.9 ± 0.9

*Antiserum produced to *H. chrysoscelis* from Oktibbeha County, Mississippi. †Antiserum produced to *H. chrysoscelis* from Bastrop County, Texas.