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COEXISTENCE CURVES FOR PHASE SEPARATION IN THE CALF LENS CYTOPLASM

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SUMMARY: We isolate native cytoplasm of calf lens nucleus by centrifuging the lens nuclear homogenate without preliminary dilution. We then dilute the concentrated nuclear cytoplasm using physiological buffer as a solvent to give various solutions ranging from 16 to 42% by dry weight. At each concentration we measure the temperature at which the mixture opacifies due to the effect of a phase separation and directly construct the coexistence curve of the cytoplasm-solvent system. We show that this coexistence curve is depressed when cortical cytoplasm is mixed with the nuclear cytoplasm.

INTRODUCTION: A reversible "cold cataract" is produced in the calf lens when its temperature is lowered.[1,2] The opacity first appears at 19°C in the center of the nucleus, where the protein concentration is the largest and as the temperature is decreased, the opacity spreads radially into the cortex where the protein concentration is lower than the nucleus.[2] The lens cortex never opacifies under these conditions. The "cold cataract" represents an interesting model for reversible stages of other cataracts including human nuclear cataract.[3,4,5] A number of experiments suggest that this reversible cataract is due to a phase separation of the protein-solvent mixture of the cytoplasm.[2-6] Droplets of a separate protein phase form in the cell and these droplets constitute strong light scatterers.[6,7] Recently, Clark and Benedek succeeded in separating two coexisting phases by centrifugation of the opaque lens homogenate at low temperatures.[6] In this paper we isolate concentrated

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nuclear cytoplasm in its native state. We show that the native cytoplasm can be diluted and used to construct the coexistence curve of cytoplasm. This coexistence curve is depressed when constituents of the cortical cytoplasm are mixed with the nuclear cytoplasm.

METHODS:

Isolation of cell cytoplasm: We isolate the cell cytoplasm from 60 lenses of fresh calf eyes using the following procedure. The lenses are cooled to 4°C which is a temperature where the entire nucleus appears opaque while the cortex remains transparent. The opaque nuclei are separated from the cortical tissue and both are chopped into small pieces and homogenized separately. The homogenizations are performed in 15ml homogenizers at 35°C to reduce the viscosity and 0.01% sodium azide is added to prevent bacterial growth. The warm homogenates are poured into transparent tubes and centrifuged at 35°C, for 20 to 40 hours at 50,000 RPM in a SW 50.1 rotor using the Beckmann ultracentrifuge L5-50B. We thus prepare separate homogenates which have the physical and chemical properties of the nucleus and of the cortex.

The nuclear homogenate separates into four distinct fractions:

Fraction	Description
I	a watery layer which is found at the top
II	a membrane fraction, which is turbid even at high temperatures
III	a cytoplasmic fraction which is transparent at room temperature and has the same phase behavior as the intact nucleus
IV	a very concentrated pellet of 60% weight concentration which does not phase separate at low temperature

We found that no protein denaturation occurred during the preparation and that the protein composition was the same in all fractions. The procedure described above provides us with a fraction of nuclear cell cytoplasm (Fraction III) of concentration very similar to the native lens. Fraction III was diluted with physiological buffer to determine the coexistence curve of native cytoplasm and with cortical cytoplasm to determine the effect of cortical cytoplasm on the coexistence curve.

The cortical homogenate separates into three distinct fractions:

Fraction	Description
I'	a watery fraction
II'	a membrane fraction
III'	a cytoplasmic fraction which remains transparent from -10°C to 40°C

The fractions (I') which was 19% dry weight fraction, and (III') of average dry weight fraction 32%, were used to dilute the nuclear cytoplasm in later experiments to test the effect of cortical cytoplasm on nuclear phase separation.

Dilution of the nuclear cytoplasm: By diluting the nuclear cytoplasm, we produce a series of samples each of which has a different dry weight concentration. The concentration, C, shall be defined as the fractional dry weight concentrati C = dry weight/total wet weight of the sample.[6] This quantity is an accurate representation of the protein dry weight fraction since the proteins account for 98% of the weight of the non-water constituents of the cytoplasm. After homogenization, the nuclear fraction III has a dry weight fraction of 42%. To determine the coexistence curve of this material, we prepared samples of fraction III having various protein concentrations by direct dilution with 0.154M phosphate buffer, which has the composition given in reference 5, and by careful mixing using a vortex agitator. The dry weight composition of each sample was obtained by weighing the sample before and after lyophilization. The concentrations of the diluted samples fall in a range between $15\% < C < 42\%$. To test the effect of cortical homogenate on the coexistence curve of the nuclear homogenate, we substituted cortical fraction I' or cortical fraction III' for the buffer in the preparation of the diluted samples of nuclear fraction III. Please note that the concentration of each sample was determined after the opacification temperature, T_{cx}, was obtained using the procedure described below.

Determination of the opacification temperature: To measure the opacification temperature, T_{cx}, each preparation is poured into a small glass tube. This tube is inserted in a silicon oil bath matching the refractive index of the tube. The temperature of the bath is regulated with an accuracy of 0.1°C. A laser beam is focused on the sample and the transmitted light I_{tr} is measured by a photodiode. I_{tr} was measured as a function of temperature upon increasing the temperature (T) through the phase separation. After each change in temperature the system is allowed to equilibrate so that I_{tr} comes to a final steady state value. The optical system used is described in detail in Reference 7. Using the graph of I_{tr} versus T, the temperature of opacification, T_{cx}, was defined as the temperature at which the I_{tr} falls to a value that is 10% less than its maximum value. T_{cx} was evaluated with an accuracy of ± 0.3°C and was very reproducible when measured as a function of increasing temperature. If T_{cx} was measured as a function of decreasing temperature, we found some hysteresis in the I_{tr} versus T curve and T_{cx} in the decreasing T direction was slightly higher by an amount $(\Delta T)_{HYST}$ than the T_{cx} found on increasing the temperature. It is interesting to note that $(\Delta T)_{HYST}$ is equal to zero at the concentration of 37%. However, at any concentration, C, other than 37%, ΔT increased as $|37\%-C|$ increases.

RESULTS: Figure 1 shows the characteristic plots of transmitted intensity, I_{tr}, versus temperature, T, for various concentrations (curves a,b,c and d) of the nuclear cytoplasm. Curves a,b,c, and d were measured at protein concentrations of 37%, 29%, 21% and 16% respectively. Each curve shows a plateau of I_{tr} at high T and a sharp decrease in I_{tr} at lower T. T_{cx} for curves a,b,c and d are determined to be 15.3°C, 12.6°C, 8.8°C and 7.4°C respectively. We also noticed a decrease of approximately 2.0 units in the maximum transparency of the samples as the samples were diluted from 37% to 16%.

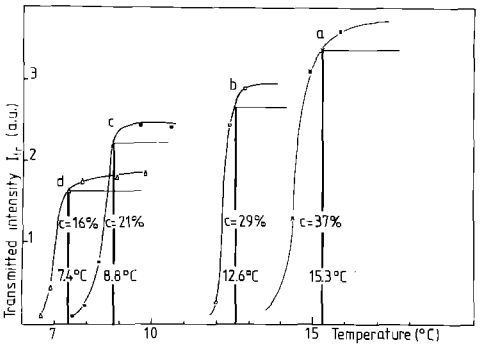

FIGURE 1: Characteristic behavior of intensity with temperature in isolated nuclear cytoplasm. Curves a, b, c and d show transmitted intensity, I_{tr}, measured as a function of temperature, T, in isolated nuclear cytoplasm having concentration of 37%, 29%, 21% and 16% respectively. The characteristic temperature of the transition, T_{cx}, decreases with decreasing dry weight concentration of the samples.

We use the data in Figure 1 to plot the change in T_{cx} with dry weight fraction. In figure 2, T_{cx} is plotted as a function of the dry weight fraction, C, of the nuclear cytoplasm. Curve a is the coexistence curve that was obtained by diluting the nuclear fraction with physiological buffer. Curve a shows that T_{cx} increases with C to a maximum value at C=37% and then falls to 12.5°C at C=42%. Samples having 42% < C < 60% have the consistency of a gel and are very difficult to prepare so the right side of the coexistence curve is formed by a dotted line. The dotted line represents the decrease in T_{cx} as C is increased to 60%, which is the concentration of nuclear fraction IV. Fraction IV does not phase separate even at temperatures as low as -17°C so the right side of the coexistence curve is shown to fall off steeply to this value. The maximum value of T_{cx}= 15.3°C occurs at 37% which is defined as the critical concentration, C_c. At concentrations above or below C_c the value of T_{cx} is less than 15.3°C.

Curves b and c of Figure 2 were obtained by plotting T_{cx} versus dry weight concentration of cytoplasm for nuclear samples that were diluted with cortical fraction I' or III'. We see that dilution of nuclear cytoplasm over the range 40% < C < 20% with cortical fraction I' produces the coexistence curve b which is similar to coexistence curve a, except that each T_{cx} is significantly lower in curve b than curve a. Curve c of Figure 2 was obtained by plotting T_{cx} versus

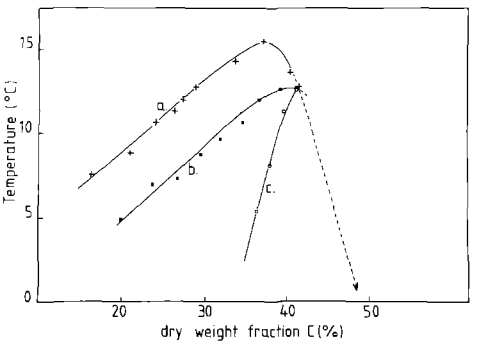

FIGURE 2: Coexistence curves for isolated nuclear cytoplasm. The curves were determined by plotting T_{cx} versus the concentration of samples that were diluted with physiological buffer, a; cortical fraction I', b; or cortical fraction III', c.

dry weight concentration of cytoplasm for nuclear samples that were diluted with cortical fraction III' over a range of 40% < C < 35%. The coexistence curve of nuclear cytoplasm is shifted even more markedly when cortical fraction III' is used to dilute the nuclear samples.

DISCUSSION: Our results characterize the behavior of cytoplasm as a function of temperature and protein concentration. The sharp transition from the transparent to the opaque state with temperature (Figure 1) is similar to the behavior of cytoplasm in intact lens cells and also similar to polymer solutions which phase separate at temperatures below some characteristic temperature T_{cx}.[2, 6, 8, 9] Below T_{cx} the phase separated solution opacifies because droplets of a new phase are nucleated in the cytoplasm.[6,7] The index of refraction of the droplets is different from the bulk cytoplasm so light scattering increases and transmitted intensity decreases as the temperature is lowered below T_{cx}.[7]

The curves of T_{cx} versus concentration (Figure 2) are very similar to coexistence curves for phase separations in a binary mixture.[2,6,8,9] It is characteristic of phase separation phenomena that a maximum value of T_{cx} occurs at a critical concentration, C_c, and lower values of T_{cx} are found for concentrations that are higher or lower than C_c. Our experiments also show phenomena of supercooling and hysteresis which are associated with non-equilibrium states of

phase separating solutions.[8,9,10,11] In particular, we found no hysteresis in samples whose concentration, C, is equal to C_c while at $C \neq C_c$, we observed hysteresis in the plots of I_{tr} versus T. This is because at $C = C_c$ the transition is second order but for $C \neq C_c$, the transition is first order and a metastable transparent state can exist at a temperature, T_s, less than T_{cx}. T_s is known as the spinodal temperature.[8,9,10] Our finding that ΔT_{HYST} increased as C_c-C increased is consistent with the behavior of T_s in the metastable state. Taken together, these results are direct support for the suggestion that native cytoplasm behaves as a binary polymer solution which phase separates at the opacification temperature, T_{cx}.

In addition, our findings in Figures 2b and 2c imply that the cortical fractions I' and III' contain molecular components that are capable of lowering the coexistence curve and T_{cx} of nuclear cytoplasm. In fact, the existence of these components may be responsible for the prevention of the phase separation cataract in the cortex.[2,5] Previous investigations suggest that the identification of these cortical constituents may be important in developing methods for inhibiting cataracts in an early stage of their development.[5]

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REFERENCES:

1. Zigman, S., Lerman, S. (1965) Exp. Eye Res. 4, 2430.
2. Tanaka, T., Benedek, G.B. (1975) Invest. Ophthal. 14, 449-456.
3. Tanaka, T., Ishimoto, C., Chylack, L.T. (1977) Science 197, 1010-1012.
4. Clark, J.I., Mengel, L., Benedek, G.B. (1980) Ophthalmic Res. 12, 16-33.
5. Benedek, G.B., Clark, J.I., Serralach, E.N., Young, C.Y., Mengel, L., Sauke, T., Bagg, A., Benedek, K. (1979) Phil. Trans. R. Soc. Lond. A293, 329-340.
6. Clark, J.I., Benedek, G.B. (1980) Biochem. Biophys. Res. Comm. 95, 482-489.
7. Delaye, M., Clark, J.I., Benedek, G.B., Biophysical Journal (in press).

8. Landau, L., Lifshitz, E.N. (1955) Statistical Physics, Vol. 5, Pergamon Press, pp. 300-314.

9. Stanley, H.E., (1971) Introduction to Phase Transitions and Critical Phenomena, Clarendon Press, Oxford.

10. Langer, J.S., (1974) Physica 73, 61-72.

11. Goldburg, W.I., (1980) The Dynamics of Phase Separation Near the Critical Point: Spinodal Decomposition and Nucleation. Proceedings of NATO Summer School: Scattering Technique Applied to Supramolecular and Non-equilibrium Systems. Ed. S.H. Chen, B. Chu and R. Nossal. NATO ASI Series A, Plenum Press, New York (In Press).