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PHASE DIAGRAM FOR CELL CYTOPLASM FROM THE CALF LENS

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SUMMARY: We have determined the phase diagram for cytoplasmic homogenate isolated from the cells of calf lenses. In the intact lens, this material is associated with the reversible opacification known as cold cataract. The coexistence curve we find resembles that found in concentrated high polymer solutions. Two coexisting phases are found below the transition temperature. Both these phases have similar protein compositions but different net protein concentrations. The constituent proteins have the following molecular weights: 17,000, 20,000, 22,000, 26,000, 28,000, 31,000, 36,000, 52,000 and 55,000 daltons. Scanning electron microscopy shows that the phase separation in the cytoplasmic homogenate is accompanied by a subtle structural reorganization which consists of increased spatial density fluctuations of dimensions comparable to the wavelength of light.

INTRODUCTION: Reversible opacification can be produced in calf lenses by lowering the temperature to 5°C or by increasing the ionic strength in the medium surrounding the calf lens (1,2,3,4). Tanaka and Benedek and coworkers studied the effect of temperature on quasielastic laser light scattering during the opacification of the calf lens and their findings suggest that reversible opacification in intact lenses may be due to a first order phase transition of the constituents of the lens cell cytoplasm (2,5,6,7). In principle, it should be possible to experimentally demonstrate the existence of two phases by separating them from the cytoplasm. In practice, this experiment is complicated by the fact that the physical properties of lens cell cytoplasm are easily disturbed by normal biochemical methods. In this study, we report procedures for the successful preparation of purified lens cell cytoplasm, the isolation of the separate phases and the analysis of the protein composition of the separated phases. In addition, we have used the isolated cytoplasm to determine a phase diagram for lens cell cytoplasm.

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The phase diagram is a graphical representation of the state of the cytoplasm under various conditions of temperature and protein concentration. To experimentally determine the phase diagram for the cytoplasm, the phases which are in equilibrium with one another at a given temperature must be physically identified. Next the concentration of each phase is plotted as a function of temperature. The plot of these protein concentrations, which correspond to the condensed (protein rich) and dilute (protein poor) phases, define a line called the coexistence curve. The coexistence curve is the boundary between the region in the phase diagram where the cytoplasm exists as a single transparent phase and the region where the opaque separated phases in the cytoplasm "coexist" as distinct phases which are dispersed through the cytoplasm. The coexistence curve is especially important for our studies because it describes the conditions under which the cytoplasm goes through a phase transition and becomes opaque. Our experimental results show the conditions for opacification of the lens cell cytoplasm and provide direct experimental evidence for the view that lens cell cytoplasm behaves as a binary polymer solution which undergoes a phase separation (5,6,7).

METHODS: In order to produce lens cell cytoplasm which undergoes a phase separation near room temperature, we first dissected 40 lenses from fresh calf eyes. Then the lenses were placed in 100ml of 0.6M phosphate buffered saline (P.B.S.) containing 0.05% sodium azide to prevent bacterial growth. The lenses were incubated 36 hours at room temperature (22°C) and the solution was replaced twice with fresh P.B.S. to permit complete diffusion of saline into the lenses. Under these conditions the lens nucleus becomes very opaque. This opacity is reversible in the sense that it disappears when the salt is diffused out of the lens or when the lens is warmed above 31°C, which is the phase transition temperature for lenses incubated in 0.6M P.B.S.(1,2,8). At the end of the 36 hour period, the opaque lenses were chopped into small pieces and placed in a 15ml homogenizer. The homogenizer was maintained at 35°C while the sample was homogenized for 3-6 strokes using a stainless steel pestle. The warm homogenate was poured into a 15ml corex tube and centrifuged at 30,000g for 4 hours at 35°C to remove the cell fragments and membranous debris. This procedure results in 8ml of lens cytoplasm having a phase separation temperature of approximately 17°C. It is interesting to note that this procedure, which removes membrane associated constituents from the cytoplasm results in a decrease in the phase transition temperature from 31°C in the intact lens to 17°C in the membrane free cytoplasm. To determine the phase transition temperature of the cytoplasm obtained by the preceeding method, we plotted temperature versus relative transmittance, which is defined as I_t/I_{max} (Figure 1). This is simply the ratio of the intensity, I_t, of laser light which was transmitted through a cuvette containing a sample of homogenate at an experimental temperature, t, to the maximum intensity, I_{max}, of light transmitted through the same sample at 25°C. Since 25°C is well above

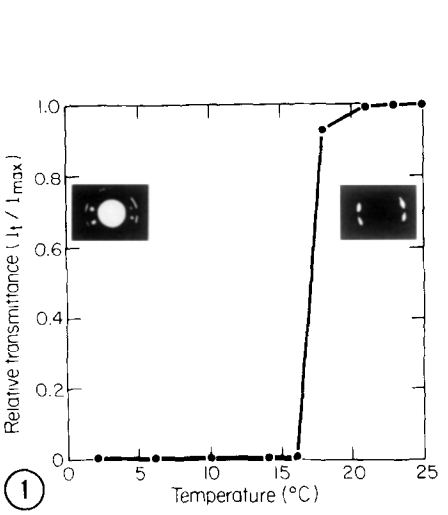
the transition temperature for this material, the homogenate is completely clear at this temperature. The phase transition temperature was arbitrarily chosen to be that for which the relative transmittance decreases to the value $(I_t/I_{max}) = 0.5$. The experimental apparatus which was used to measure transmittance is described elsewhere (2).

The homogenate was used in the following procedure to obtain the phase diagram for lens cell cytoplasm (Figure 2). The temperature of the cytoplasmic homogenate was lowered to 15°C at which temperature the homogenate becomes opaque. This temperature was carefully maintained by keeping the samples in a Lauda waterbath (Brinkman Instruments) which controls the temperature to within 0.1°C. At this temperature and at all temperatures below 17°C the cytoplasm is quite viscous (1). To physically separate the phases in the viscous sample, we found that it was necessary to centrifuge the sample at 6000g for 30 minutes in a Beckman J21B temperature controlled centrifuge. After centrifugation, the sample contains two physically distinct layers which correspond to the phases we wish to analyse.

We measured the concentration of each phase using a spectrophotometric assay for the dry weight concentration $(\frac{\text{dry weight}}{\text{total weight}})$ of each phase. As a control experiment, we showed that the dry weight concentration is directly proportional to the U.V. absorbance at 280 nanometers of a diluted solution of the cytoplasm. To determine the concentration in each phase, we took an aliquot of each phase after centrifugation, diluted the aliquot by a known dilution factor and measured the absorbance at a wavelength of 280nm. The value of the absorbance was converted to dry weight corrected by the dilution factor and plotted on the X-axis of Figure 2. After the measurement of the concentration of the separated phases, the remaining sample was rewarmed to about 35°C and remixed to form a single trans parent phase. The remixed sample was then cooled to 10°C and permitted once again to phase separate. As before the 2 phases in the sample were spatially separated by centrifugation and the concentration of each phase was measured again. This procedure was repeated at 5°C and 0°C and the protein concentrations which were found in each phase were plotted versus temperature in Figure 2.

Next we analysed the protein composition of the clear cytoplasm at a temperature well above 17°C, and in the condensed and dilute phases, at a temperature below 17°C (Figure 3). We used a modification of the polyacrylamide gel electrophoresis method of Laemmli (9). In our method, the separating gel contained 12% polyacrylamide (acrylamide:bis-acrylamide ratios 30:0.8) in 0.375M Tris-HCL, pH 8.8. The stacking gel contained 3% polyacrylamide in 0.125 M Tris-HCL, pH 6.8. The gels were polymerized by the addition of 0.025% ammonium persulfate and 0.05% tetramethyl ethylene diamine (TEMED). The electrode buffer was 380mM glycine, 50mM Tris base at pH 8.3, and the entire gel system contained 0.1% SDS. An 1µℓ sample of each phase was added to 250µℓ of sample buffer which contained 1% 2 mercaptoethanol, 1% SDS, 0.001% bromphenol blue, 10% glycerol, and 50mM Tris-HCL, pH 6.8. The sample was boiled for 2 minutes and a volume of sample buffer containing approximately 5µg protein was loaded in each well of the slab gel. Electrophoresis was carried out at 10m Amps for 8 hours. Gels were fixed in methanol/water/acetic acid (5:4:1) for 6 hours, stained in 1% Coomassie Blue in the methanol/H_2O/acetic acid solution and destained in 10% acetic acid.

The cytoplasmic homogenate is a viscous fluid which can be prepared for scanning electron microscopy using routine fixation and dehydration methods. Small drops (about 2mm in diameter) of the lens cytoplasm were fixed in 4% glutaraldehyde in 0.15M phosphate buffer, pH 7.4, post-fixed in 1% osmium tetroxide and critical point dried. The dried material was cracked to expose the inner structure of the drop. The samples were coated with 300Å of gold for observation using a JEOL JSM 35 scanning electron microscope. The transparent cytoplasm was fixed at 35°C and the phase separated cytoplasm was fixed at 4°C (Figure 4). Our preparation procedure has been shown to preserve the optical properties of lens tissue (2).



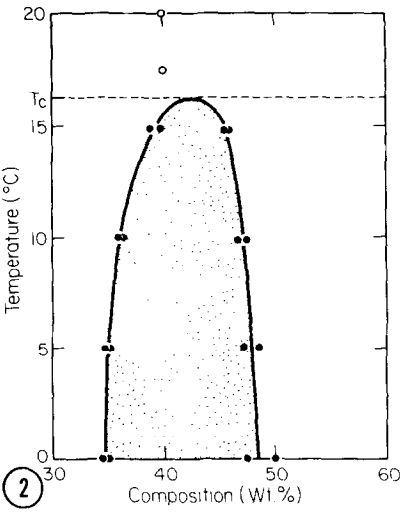


Figure 1. The effect of temperature on the transparency of lens cytoplasm. A sharp transition from the transparent to the opaque state occurs in the cytoplasm at approximately 17°C. The appearance of opaque lens is shown in the inset on the left of the figure and the appearance of a transparent lens is shown in the inset on the right.

Figure 2. Phase diagram of lens cytoplasm. The coexistence curve for the isolated cytoplasmic homogenate is similar to that of a phase separation in a binary polymer solution. The shaded area represents the conditions of temperature and composition where phase separation occurs and the cytoplasm is opaque. The area outside the curve represents the conditions for transparency of lens cell cytoplasm. The concentration of protein in the homogeneous transparent material was approximately 40% dry weight (open circles). The concentrations measured in the separated phases of the opaque cytoplasm are plotted as solid circles.

RESULTS: In Figure 1, the relative transmittance of the cytoplasmic homogenate is plotted as a function of the temperature. The homogenate is transparent at temperatures above 17°C and the relative transmittance is 1.0. At temperatures below 17°C, the homogenate is so opaque that the relative transmittance is only 0.002. The steep decrease in transmittance at 17°C demonstrates the sharpness of the transition from the transparent to the opaque state. The insets show the appearance of the lens at temperatures above the transition, where the nucleus is transparent, and below the transition where the nucleus is completely opaque.

In Figure 2 we show the phase diagram for the lens cell cytoplasm. Using the phase diagram, we can determine the conditions which will produce opacification due to a phase separation and those which will produce transparency. The cytoplasm exists as a single transparent phase at temperature above 17°C. The

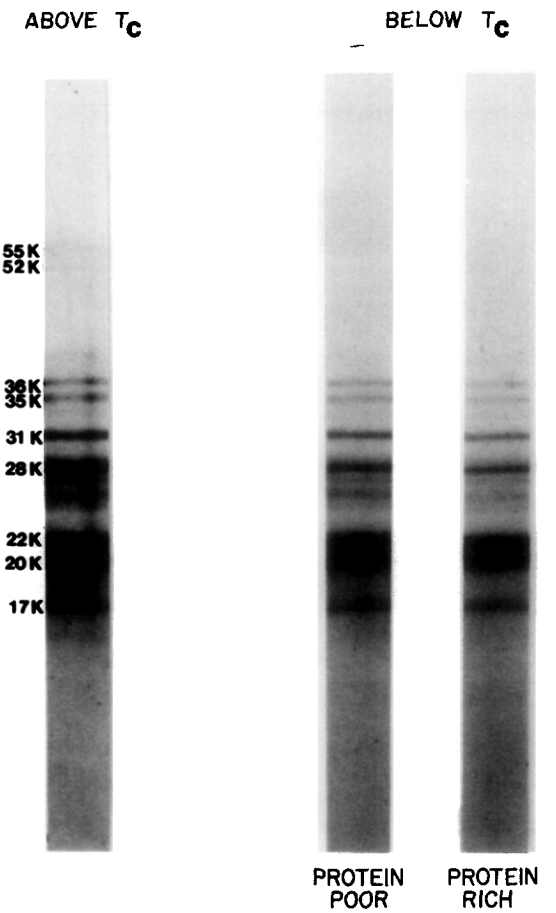


Figure 3. SDS polyacrylamide gel electrophoresis of lens cytoplasm. The protein composition in the cytoplasm when it exists as a single phase (above T_c) is the same as the protein composition in the protein poor and protein rich states of the phase separated cytoplasm (below T_c).

protein concentration in the single phase region is approximately 40% by dry weight. This value was measured at 18°C and 20°C (Figure 2, open circles). At each temperature below the transition temperature, two phases were separated and the plot of the concentration of the separated phases at each temperature defines the coexistence curve. From the coexistence curve we see that the concentration of the protein-poor phase varies from 35% to 41% and the concentration of the protein rich phase varies from 41 to 49% over the range of temperatures from 0°C to 15°C. The steepness of the coexistence curve shows that the phase separation temperature is very sensitive to changes in the protein concentration of the cyto-

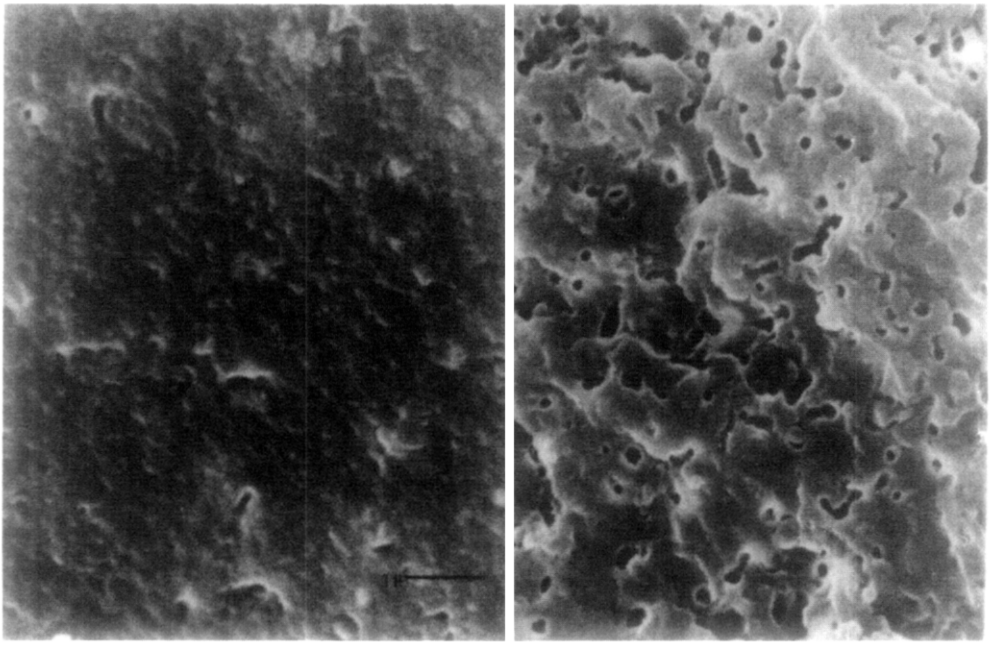


Figure 4. Scanning electron micrographs of a transparent single phase and the opaque phase-separated cytoplasm. The cytoplasm in the single phase condition (left figure) contains small density fluctuations while the phase separated cytoplasm (right figure) contains large density fluctuations. Bar = 1 micron.

plasm. At protein concentrations which are less than 35% or greater than 49%, phase separation does not occur and the cytoplasm is homogeneous and transparent.

The SDS polyacrylamide gels of the transparent and phase separated cytoplasm are shown in Figure 3. Above the transition temperature, where the cytoplasm exists as a single transparent phase, the most prominent proteins migrate at positions which correspond to molecular weights of 17,000, 20,000, 22,000, 26,000, 28,000, 31,000, 35,000, 36,000 daltons with minor constituents at 52,000 and 55,000. Below the transition temperature, the proteins in both the protein poor and the protein rich phases have approximately the same distribution of molecular weights as were found in the single phase of the cytoplasm. The molecular weight determinations were based on the migration of the protein standards: lysozyme, ribonuclease A, chymotrypsin, ovalbumin, BSA.

Electronmicrographs of the transparent single-phase cytoplasm and the opaque cytoplasm are seen in Figure 4. The transparent cytoplasm is a smooth

homogeneous material while the opaque material which has undergone separation into coexisting phases is rough and contains inhomogenities which are as large as one micron in size. Density fluctuations of this scale would be expected to cause light scattering, since the wavelength of light is 0.5 to 0.6 microns.

DISCUSSION: From these results we conclude that reversible opacification in the calf lens is accompanied by a phase transition within the cell cytoplasm. Under the conditions of this transition the lens cell cytoplasm behaves in a manner which is analogous to a polymer solution which exists as a single homogeneous phase above the transition temperature and separates into two phases below the transition temperature (10, 11). Since both phases have similar protein constituents, we suggest that the cytoplasm separates into two bulk phases which differ in their protein concentration. At the microstructural level, these bulk phases occupy separate volumes of space in the cytoplasm and produce the density fluctuations which are seen in the electron micrographs of the phase separated cytoplasm. When the fluctuations in density are of dimensions which are comparable to the wavelength of light, scattering occurs (12). When the fluctuations are small, scattering is decreased so that the cytoplasm appears transparent. It should be noted that this is the first physico-chemical determination of a phase diagram for cytoplasm separated from any cell system. The phenomenon of separation into coexisting phases may be an important stage in the development of cataracts as has already been demonstrated in cataracts of galactosemic rats (5). Unpublished results demonstrate that X-ray induced cataracts in rabbits also are associated with a phase separation in the intact lens. In addition, chemical reagents which improve transparency in the phase transition cataract also improve transparency in human pathological cataracts (1,8,13). It appears that the phase separation of lens cell cytoplasm is intimately associated with the mechanism for the formation of scattering centers in the reversible stages of lens opacification.

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