

---

# Observation of protein diffusivity in intact human and bovine lenses with application to cataract

*Toyoichi Tanaka and George B. Benedek*

*Using the technique of optical mixing spectroscopy, we have observed the Brownian movement and measured the diffusivity of proteins within whole, intact human and bovine lenses. The magnitude and the temperature dependence of the protein diffusivity in bovine and normal human lenses implies that cold cataract is the result of a first-order phase separation of the protein-water mixture in the lens. The magnitude of the diffusivity of proteins within cataractous human lenses indicates the presence of large aggregates of proteins.*

**Key words:** cataract, protein diffusivity, aggregation, phase separation, opacity, light scattering, correlation range, correlation time.

On the basis of the theory of light scattering, it has been proposed<sup>1</sup> that opacification of the lens in cataract is produced by the spatial fluctuations in the index of refraction of the protein-water system in the lens. In particular, these spatial fluctuations are proposed to be in the form of heavy molecular weight aggregates of lens proteins. By biochemically separating the various lens constituents, such heavy molecular weight aggregates have indeed been

observed in *solution* for both bovine and human lens homogenates.<sup>2-4</sup> It is natural to inquire whether the aggregates observed in solution actually exist in the intact lens itself, or whether the observed aggregates are, at least in part, produced in the process of biochemical separation.

In this paper we report the detection and quantitative characterization of protein aggregates (or concentration fluctuations) *in the intact lens*. The experiments measure the quasi-elastic spectrum (or the intensity autocorrelation function) of laser light scattered from the whole lens. The techniques and applications of these types of experiments are fully described in a number of reviews.<sup>5-8</sup> From the width of the scattered light spectrum (or from the correlation time of the scattered light intensity fluctuation), one can observe the Brownian movement<sup>8</sup> of the scattering elements, and determine their diffusivity,  $D$ . If the system

---

From the Department of Physics and Center for Material Science and Engineering, Massachusetts Institute of Technology, Cambridge, Mass.

This work was supported by the National Institutes of Health Interdisciplinary Program in Biomaterials Science under Grant IPO1-HL-14322-01, by the General Electric Foundation, and also by the National Institutes of Health Eye Institute (Grant No. EY-54465) and the NIH Biomedical Sciences Support Grant to MIT.

Submitted for publication Sept. 9, 1974.

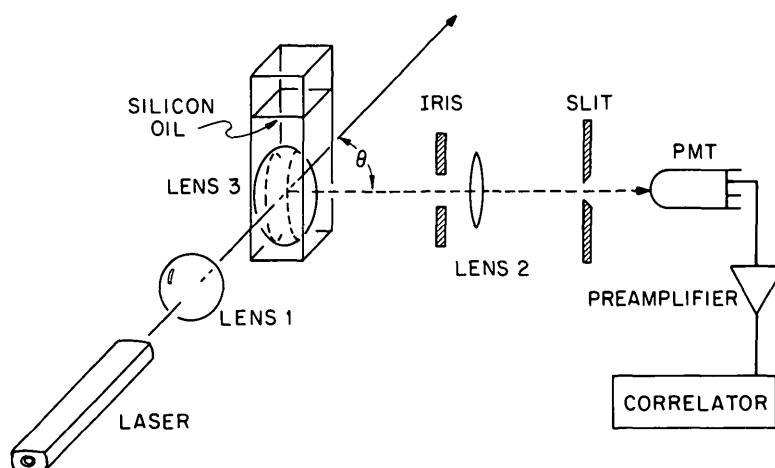


Fig. 1. Experimental setup of laser light scattering and optical mixing spectroscopy. Lens 3 shows a human or calf lens.

under study is a solution far from the phase separation, the diffusivity can be used to determine the size of the solute molecules. On the other hand, if the solution is near the separation into co-existing phases, the scattering fluctuations consist of correlated groups of molecules of size  $\xi$  called the correlation range. The spectral width of the scattered light characterizes the Brownian movement of these correlated groups of molecules. It is well known<sup>7</sup> that as one approaches the critical point on the co-existence curves of binary mixtures, the correlation range  $\xi$  grows very large, and at the same time the solution becomes turbid because the long-range concentration fluctuations scatter light very effectively. In fact, it is known that in such cases the diffusivity,  $D$ , can be related to the correlation range as

$$D = kT/6\pi\eta\xi,$$

and that the scattered light intensity is proportional to  $\xi^2$ .<sup>7</sup> Here  $k$  is the Boltzmann constant,  $T$  the absolute temperature, and  $\eta$  the viscosity of the solvent.

Using this technique, we have studied the magnitude and temperature dependence of the spectral width (or the correlation time) of light scattered from (1) calf lenses, (2) normal human lenses, and (3) cataractous human lenses. These

measurements indicate that: (1) the phenomenon of cold cataract in the calf lens is the manifestation of a first-order phase separation of the protein-water mixture in the lens into separate co-existing phases. The cold cataract appears at nearly the same temperature as that at which the mean diffusivity,  $\bar{D}$ , of the solution reaches zero. (2) In normal human lenses the temperature dependence of the mean diffusivity,  $\bar{D}$ , is similar to that in the calf lens. However, the temperature at which phase separation would occur is about  $-5^\circ\text{C}$ ., and cold cataract therefore does not occur above the freezing point of water. (3) In cataractous human lenses the average diffusivity of the protein in the lens is roughly a factor 5.5 smaller than that of the normal lens. This indicates a marked aggregation of proteins in situ within the cataractous lens. The temperature corresponding to phase separation in human cataractous lenses is about the same as that for the normal lens (i.e.,  $\sim -5^\circ\text{C}$ .).

#### Experimental method

In Fig. 1, we show schematically how we determined the autocorrelation function of the light scattered from the intact lens.<sup>5-8</sup> The incident light is provided by a Spectra Physics Model 124 laser with a power output of 15 mW. The optics were arranged so that the laser beam could be positioned anywhere in the medial plane of the

lens. The collection optics permitted us to pick up the scattered light at any radial position from the center of the lens. Light scattered 90° from the incident direction was collected by the system of iris, lens 2, and slit, and imaged onto the photomultiplier tube (PMT). The photocurrent pulses in the output of the PMT were amplified, shaped, and discriminated by a preamplifier, and the temporal correlations in the pulse train were analyzed using a 19-channel digital autocorrelator.

The diffusivity of the protein-water system in the lens was determined from the mean decay rate of the autocorrelation function. Polydispersity of the protein-water system in the lens produced a correlation function which was not a simple exponential decay. Therefore, we measured the mean decay rate  $\bar{\Gamma}$  by always adjusting the clock time of the correlator so that the correlation function decays to (1/e) of its initial value at the fourteenth clock channel. This procedure gives us a consistent means of comparing the correlation functions found in different experimental conditions. The correlation function was then analyzed using the method of cumulants,<sup>9</sup> and the mean decay rate was found. The mean decay rate,  $\bar{\Gamma}$ , is related to the mean diffusivity,  $\bar{D}$ , of the concentration fluctuation by the equation

$$\bar{\Gamma} = 2\bar{D}K^2.$$

Here  $K$  is the scattering vector,

$$K = (4\pi/\lambda) \sin(\theta/2),$$

where  $\lambda$  is the wavelength of the incident light in the solution, and  $\theta$  is the scattering angle. In the case of 90° scattering ( $\theta = 90^\circ$ ),  $K$  is given by

$$K = 2\sqrt{2}\pi/\lambda.$$

If the solution is far from a phase separation, and the concentration of solute is low, the diffusivity is equal to the diffusion constant, which in turn is related to the molecular dimensions of the solute particles through the friction factor  $f$  by the Einstein relation  $D = kT/f$ . In the special case of spherical molecules  $f$  is related to the molecular radius,  $a$ , by

$$f = 6\pi\eta a.$$

Here  $\eta$  is the viscosity of the solvent.

If the temperature and composition of the mixture is such that the system is in the vicinity of the critical point of the co-existence curve separating different phases of the mixture, the diffusivity has been shown<sup>11</sup> to be related to the correlation range  $\xi$  of the concentration fluctuations by the relation

$$D = \frac{kT}{6\pi\eta\xi} \quad (1)$$

It is well known that as one approaches the co-existence curve in the vicinity of the critical point of a binary mixture, the correlation range  $\xi$  grows very large, and the solution becomes quite turbid.<sup>10</sup>

It should be mentioned that in our experiments we are in essence observing the random walk of the solute proteins over dimensions comparable to the light wavelength. Since the smallest dimensions of the individual cells in the lens are large compared to the wavelength of light, we are detecting the diffusive motion of the protein within each cell.

## Experimental results and discussion

**Calf lenses.** In Fig. 2 we plot the mean diffusivity,  $\bar{D}$ , as a function of the temperature,  $T$ , of the calf lens for different positions along the diameter of the lens.

We studied  $\bar{D}$  versus temperature for a total of 10 calf lenses. The results obtained were very consistent with the data presented in Fig. 2. In fact, there seemed to be little variation in  $\bar{D}$  at fixed temperatures and positions for different lenses. It is interesting to note that at body temperature (37° C.) the diffusivity,  $\bar{D}$ , in the intact lens ( $1.0 \sim 2.5 \times 10^{-7}$  cm.<sup>2</sup> per second) is roughly the same as that of  $\alpha$ -crystallin protein of the calf lens in solution (again at 37° C.) ( $D \sim 2.8 \times 10^{-7}$  cm.<sup>2</sup> per second).<sup>12</sup>

Fig. 2 also shows the striking decrease of the diffusivity,  $\bar{D}$ , as the temperature is lowered. As the temperature is lowered, and  $\bar{D}$  falls, one also observes that a certain temperature,  $T_c$ , is reached at which the lens scatters light so intensely that the medium in the lens effectively becomes opaque. It becomes difficult to take data below the temperature  $T_c$ . The values of  $T_c$  for each position on the lens diameter are shown in Fig. 2. The actual measured values of  $T_c$  are, in fact, the temperatures at which cold cataract appears. The decrease of  $\bar{D}$  and the occurrence of opacity corresponds to the behavior of a solution as one approaches the co-existence curve (or "cloud line") along a line of constant concentration.<sup>7</sup> The value of  $T_c$  corresponds to the temperature at which phase separation occurs in the mixture. It is interesting to observe that the temperature,  $T_s$ , at which  $\bar{D}$  extrapolates to zero is lower than  $T_c$ . This, too, is quite similar to the behavior of mixtures undergoing phase separation. The diffusivity approaches zero at

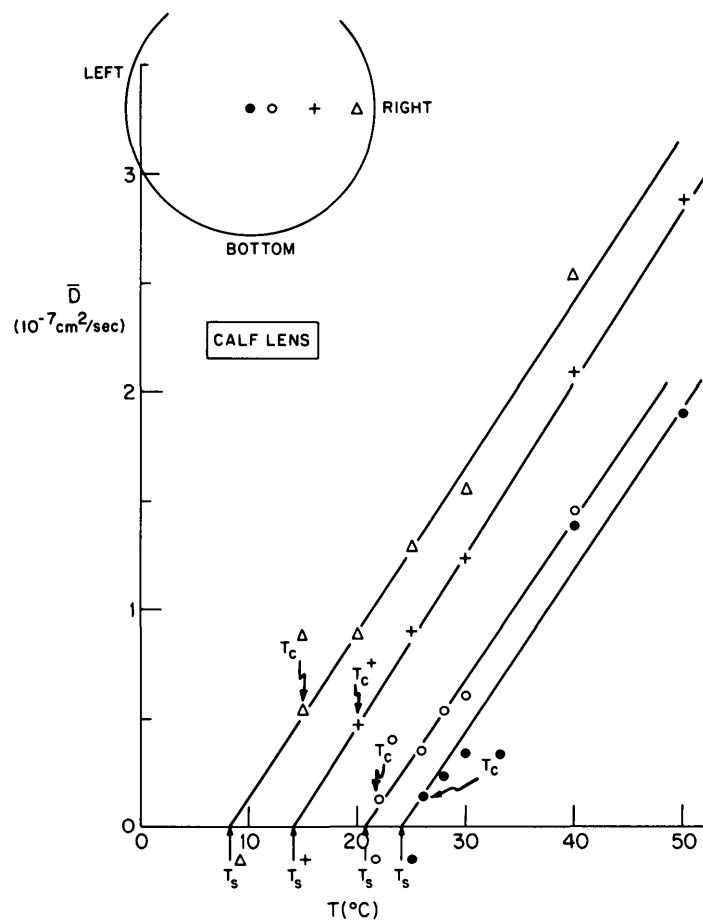


Fig. 2. Mean diffusivity,  $\bar{D}$ , in a calf lens as a function of temperature and position. At the lowest point on each line, the phase separation occurs and the cold cataract appears.

a point along the so-called spinodal line<sup>10</sup> inside the co-existence curve. This line denotes the limit of supercooling of the homogeneous phase of the mixture. At a temperature,  $T$ , below this line the mixture cannot be further supercooled without spontaneous separation into two phases whose concentrations are denoted by two intersections of the line,  $T = \text{const}$ , and the co-existence curve. We therefore denote this temperature as  $T_s$ , "s" for spinodal.

It is customary<sup>11</sup> in the study of binary mixtures near their critical point to describe the temperature dependence of  $\bar{D}$  in terms of the critical exponent  $\gamma^*$  and the spinodal temperature,  $T_s$ , by the formula

$$\bar{D} = \alpha(T - T_s)^{\gamma^*} \quad (2)$$

In the case of the calf lens  $\alpha = 7.5 \times 10^{-9}$  ( $\text{cm}^2$  per second  $^\circ\text{K}$ ) and  $\gamma^* = 1.0$ .  $T_s$ , the spinodal temperature, depends on the position in the lens.

In the case of simple solutions of low molecular weight molecules, such as the isobutyric acid and water mixture,  $\gamma^* = 0.67$ . On the other hand, for polymer solutions, such as the polystyrene-cyclohexane system,  $\gamma^*$  is found to be equal to unity.<sup>7</sup> This latter result is in agreement with our findings for the calf lens.

We may further strengthen our interpretation of  $T_c$  as a phase-separation temperature by examining the dependence of  $T_c$  on position in the lens. This is shown in Fig. 3. The triangles represent the temperature,  $T_c$ , at which cold cataract appears as a

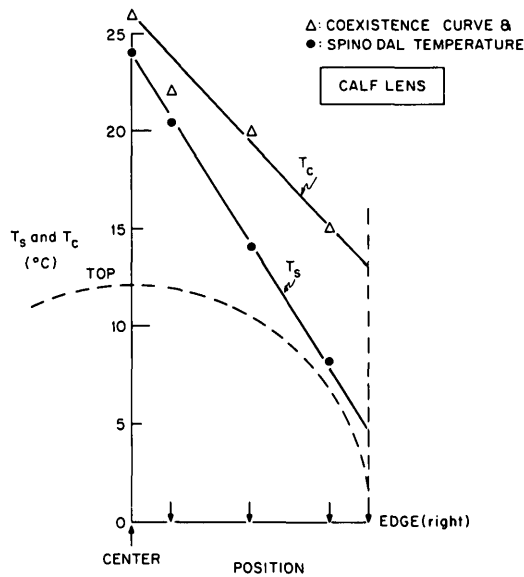


Fig. 3. Cold cataract temperature,  $T_c$ , and spinodal temperature,  $T_s$ , as a function of the position in a calf lens.

function of the radial distance from the lens center. As the temperature is lowered, the center of the lens first becomes opaque at about  $26^\circ\text{C}$ . With further lowering of the temperature, the boundary of the opaque region moves toward the periphery. Only when the temperature falls to  $\sim 13^\circ\text{C}$  does the opacification reach the full radius of the lens.

We also plot in Fig. 3,  $T_s$  as a function of position along the radius of the lens.  $T_s$  is closest to  $T_c$  at the lens center, and the difference ( $T_c - T_s$ ) increases with distance from the center.

The variation of  $T_c$  and  $T_s$  with position in the calf lens can be understood if we remember that the protein concentration in the calf lens varies as a function of position.<sup>12</sup> The protein concentration at the lens center is  $\sim 40$  per cent, while at the periphery it is  $\sim 27$  per cent. Thus, by changing radial position within the lens, we are in effect altering the concentration of the solution under study. Thus, if we take protein concentration as decreasing in proportion to radial distance from the lens center, Fig. 3 represents a portion of the well-known co-existence curve and the spinodal line for the phase separation of

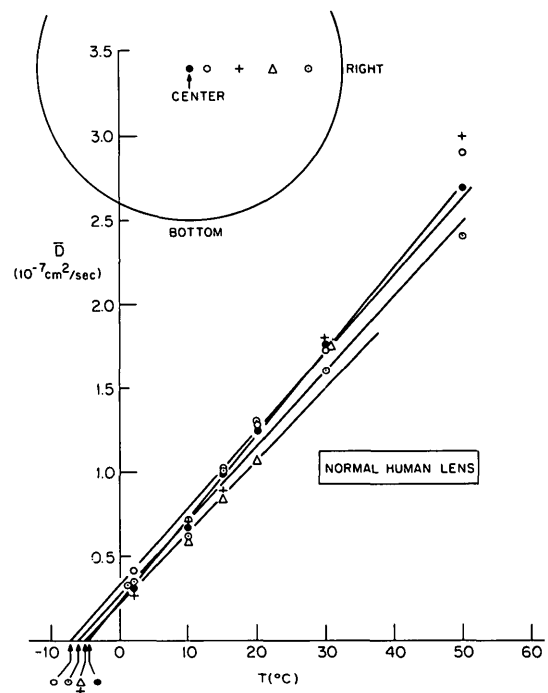


Fig. 4. Mean diffusivity,  $\bar{D}$ , in human lenses as a function of temperature and position in the lenses: normal lens.

this mixture.<sup>7</sup> In fact, the magnitude of decrease of  $T_c$  that we observe, i.e.,  $\Delta T_c \sim 10^\circ\text{C}$  for  $\Delta C \sim 13$  per cent, is consistent with that found along the co-existence curve of the isobutyric acid-water system.<sup>10</sup> Of course, it is also possible that the variation of  $\bar{D}$  with position may be due to a change in the size distribution of the solute proteins as a function of position. In principle one can investigate the size distribution of proteins by their effect on the precise shape of the correlation function of the scattered light.<sup>9</sup> Such further investigations are certainly desirable.

Another element of support for the view that the cold cataract is a manifestation of a first-order phase separation is the experimental fact that there is no hysteresis in the variation of  $\bar{D}$  with the temperature. The cold cataract formation and dissociation are strictly reversible phenomena, as one expects for phase separation.

**Normal human lens.** In Fig. 4, we plot  $\bar{D}$  as a function of temperature at different

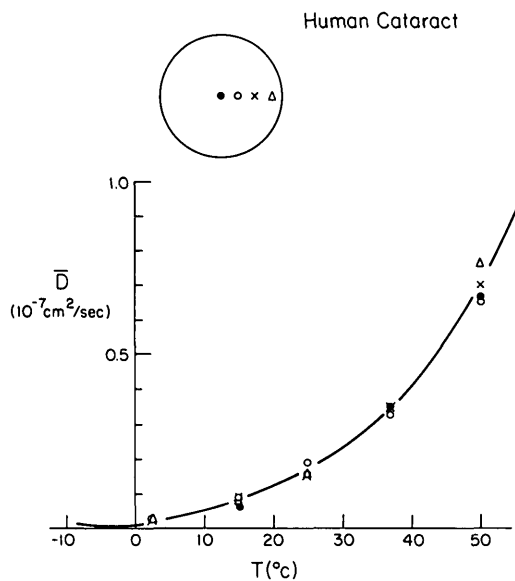


Fig. 5. Mean diffusivity  $\bar{D}$  in cataractous human lenses as a function of temperature and position in the lens.

positions along the lens radius for a transparent normal human lens. The donor of this lens was a 43-year-old male; these measurements were carried out 20 hours after lens removal. We observe from Fig. 4 that  $T_s$  is essentially independent of position in the lens. These data imply spatial uniformity in the average protein concentration and the size distribution of proteins in this normal human lens. Also the value of  $T_s$  is  $\sim -5^\circ \text{C}$ ., which is much lower than the value of  $26^\circ \text{C}$ . which is obtained for the center of the calf lens. In fact these data indicate that a cold cataract would occur in the human lens at a temperature  $\sim -5^\circ \text{C}$ . below the freezing point of water. Our measurements did not extend below  $0^\circ \text{C}$ . because of limitations on our present temperature control system.

If we fit the data on  $\bar{D}$  versus temperature,  $T$ , to an equation of the form of Equation 2, we find  $\gamma^* = 1.0$  and  $\alpha = 4.5 \times 10^{-9} \text{ cm}^2 \text{ per second } ^\circ \text{C}$ .  $\bar{D}$  varied reversibly with  $T$  as the temperature was raised and lowered.

It is interesting to note that the diffusivity,  $\bar{D}$ , obtained for the intact normal lens protein ( $1.2 \times 10^{-7} \text{ cm}^2 \text{ per second at } 25^\circ \text{C}$ .) is roughly the same as that

obtained for the  $\alpha$ -crystallin protein in the solution of the lens homogenate ( $1.3 \times 10^{-7} \text{ cm}^2 \text{ per second at } 25^\circ \text{C}$ .)<sup>4</sup>

We also measured  $\bar{D}$  versus  $T$  as a function of position for a second, different normal human lens, and obtained results which were within 10 per cent of the values shown in Fig. 4. Clearly it is desirable to study the effect on  $\bar{D}$  of age and also its possible variation from lens to lens.

**Cataractous human lens.** In Fig. 5, we show a plot of the diffusivity,  $\bar{D}$ , versus  $T$  for a pale, yellowish cataractous human lens. The diffusivity is essentially independent of position along the lens radius, as is  $T_s$ . These findings imply, for this particular cataractous lens, that there is no marked spatial variation either in the size distribution of proteins, or the protein concentration. The value of  $T_s$  is  $\sim -5^\circ \text{C}$ . which is the same as for the normal human lens. In other respects, however, the results for the cataractous lens are markedly different from the normal lens. First of all, the numerical magnitude of  $\bar{D}$  is markedly smaller in the cataractous lens. For example, at body temperature ( $37^\circ \text{C}$ .),  $\bar{D}_{\text{cataract}}$  is 5.5 times smaller than  $\bar{D}_{\text{normal}}$ . Second, the relationship between  $\bar{D}$  and  $(T - T_s)$  is not linear but rather quadratic. The data can be fit by an equation of the form of Equation 2 with  $\gamma^* = 2$ , and  $\alpha = 2.2 \times 10^{-11} \text{ cm}^2 \text{ per second } (^\circ \text{C})$ .<sup>2</sup> We also found that the results for  $\bar{D}$  versus  $T$  were quite reversible; the same results were found on raising or lowering the temperature.

We also investigated a second pale yellowish cataract from a different patient. The results for  $\bar{D}$  versus  $T$  were in excellent agreement with that shown in Fig. 5. In further experiments it would clearly be desirable to investigate the  $\bar{D}$  versus  $T$  relationship for various types of cataractous lenses.

These results can be interpreted as follows. The small value of  $\bar{D}$  signifies that the diffusing proteins consist, at least in part, of large units of protein aggregates. The effective size, or correlation length of

these units, are (at 37° C.) about 5.5 times larger in radius than the average size of the proteins in the normal lens. The molecular weight of these units thus can be estimated as 170 times larger than in the normal lens, and is about  $5 \times 10^8$  Dalton at 37° C. It is very interesting to note that this estimate of molecular weight gives a value near that of the soluble heavy molecular weight protein aggregates (molecular weight  $\sim 2 \times 10^8$  Dalton) which have been found by biochemical separation of the constituents of lens homogenate.<sup>4</sup> The small value of  $\bar{D}$  in the cataractous lens thus is consistent with the presence of large molecular weight aggregates.

The decreasing  $\bar{D}$  with temperature implies that these large aggregates take part in diffusive movements which become correlated over larger and larger distance  $\xi$  (see Equation 1) as the temperature is lowered toward that at which a phase separation would occur. The curvature of the  $\bar{D}$  versus  $T$  plot suggests that the interaction between protein aggregates in the cataractous lens is of a different nature than for the proteins in the normal lens. Despite this, it is interesting that the value of  $T_s$  is nearly the same for both normal and cataractous lenses.

### Conclusion

The magnitude of the mean diffusivity  $\bar{D}$  in the intact normal human and bovine lens is roughly the same as that of the  $\alpha$ -crystallin protein found in solution of lens homogenates. Also, the magnitude of the diffusivity in the cataractous lens is roughly the same as that of the soluble heavy molecular weight component reported as obtained from solutions of human lens homogenates. This encourages us to believe that our technique does in fact give information on the Brownian movement of proteins within the intact lens.

In the calf lens the temperature dependence of the diffusivity at different points in the lens suggests that the phenomenon of cold cataract in bovine lenses is a manifestation of the phase separation of the

protein-water mixture in the lens. The monotonic decrease in concentration of the protein-water solution as one approaches the lens periphery may be responsible for the corresponding decrease of cold cataract temperature.

In the normal human lens the linear decrease of diffusivity with temperature implies that this system as well is approaching a phase separation point at which cold cataract would occur. The temperature for this cold cataract is expected at about  $-5^\circ$  C. The uniformity of  $\bar{D}$  at different points in the lens suggests that the protein concentration and size distribution in the human lens is spatially much more uniform than in the calf lens.

In the intact human cataractous lens the small magnitude of the diffusivity is consistent with the existence of heavy molecular weight aggregates. This is also consistent with the prediction of the theory of transparency and opacity of the lens.<sup>1</sup> The temperature dependence of the diffusivity in the cataractous lens implies a different interaction between proteins in cataractous lenses as compared with normal lenses. Nevertheless, a phase separation  $\sim -5^\circ$  C. is also indicated by the variation of  $\bar{D}$ .

We are now investigating whether this technique can be safely extended to the study of lens proteins of human lenses in vivo. Using a laser power below the permissible level for retinal irradiation, we have already succeeded in measuring the correlation function of light scattered from the lens of a living rabbit. The lens of this rabbit remains healthy even now, one month following the experiments. Experiments in vivo in the human lens should permit a quantitative characterization of cataract development during its early stages even before visible opacification appears. Also, since we can study cataract development in the intact lens as a whole, it should be possible to evaluate promptly and accurately the effects of various reagents on the formation or blockage of the protein aggregation associated with lens opacification.

The authors would like to express their sincere gratitude to Dr. J. Jedziniak for her helpful suggestions and discussions.

#### REFERENCES

1. Benedek, G. B.: Theory of transparency of the eye, *Appl. Optics* **10**: 459, 1971.
2. Spector, A., Li, L. K., Augusteyn, R. C., et al.:  $\alpha$ -Crystallin, the isolation and characterization of distinct macromolecular fractions, *Biochem. J.* **124**: 337, 1971.
3. Jedziniak, J. A., Kinoshita, J. H., Yates, E. M., et al.: Calcium-induced aggregation of bovine lens alpha crystallins, *INVEST. OPHTHALMOL.* **11**: 905, 1972.
4. Jedziniak, J. A., Kinoshita, J. H., Yates, E. M., et al.: On the presence and mechanism of formation of heavy molecular weight aggregates in human normal and cataractous lenses, *Exp. Eye Res.* **15**: 185, 1973.
5. Dubin, S. B.: Measurements of translational and rotational diffusion coefficients by laser light scattering, *in: Methods in Enzymology*, Hirs and Timasheff, editors. New York, 1972, Academic Press, vol. 26C, pp. 119-174.
6. Benedek, G. B.: Optical mixing spectroscopy with applications in physics, chemistry, biology, and engineering, Jubilee Volume in honour of Professor A. Kasler, *in, Polarization, Matter and Radiation*, Paris, 1969, Presses Universitaires, pp. 49-84.
7. Chu, B.: Laser light scattering, *Ann. Rev. Phys. Chem.* **21**: 145, 1970.
8. Clark, N. A., Lunacek, J. H., and Benedek, G. B.: Study of Brownian motion using light scattering, *Am. J. Phys.* **38**: 575, 1970.
9. Koppel, D. E.: Analysis of macromolecular polydispersity in intensity correlation spectroscopy: the method of cumulants, *J. Chem. Phys.* **57**: 4814, 1972; Mazer, N. A.: Data analysis for light scattering studies of macromolecular polydispersity, B.S. thesis, Massachusetts Institute of Technology, 1973.
10. Chu, B., Schoenes, F. J., and Fisher, M. E.: Light scattering and pseudospinodal curves: the isobutyric acid-water system in the critical region, *J. Chem. Phys.* **47**: 3816, 1967.
11. Kadanoff, L. P., and Swift, J.: Transport co-efficients near the liquid-gas critical point, *Phys. Rev.* **166**: 89, 1968; Kawasaki, R.: Kinetic equations and time correlation functions of critical fluctuations, *Ann. Phys.* **61**: 1, 1970.
12. Spector, A., Stauffer, J., and Sigelman, J.: Preliminary observations upon the proteins of the human lens, *The Human Lens—in Relation to Cataract*, Ciba Foundation Symposium 19, Amsterdam, 1973; Waley, S. G.: The lens: function and macromolecular composition, *in: The Eye*, Davson, H., editor. New York, 1969, Academic Press.