

Suppression of phase separation in solutions of bovine γ IV-crystallin by polar modification of the sulfur-containing amino acids

(lens proteins/cold cataract/cysteine/methionine/oxidation)

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ABSTRACT The calf lens protein γ IV-crystallin, a strong determinant of the net phase-separation temperature of the lens, was chemically modified with *N*-bromoacetyethanolamine phosphate. The phase-separation temperatures of solutions of the modified protein were measured and found to be dramatically reduced with respect to those of the native protein. At neutral pH the reagent alkylates only the cysteine and methionine residues and introduces a doubly charged phosphate anion at a maximum distance of 10–12 Å from the sulfur atoms. At a protein concentration of 38 g/liter, and with 30% of the cysteines and 40% of the methionines alkylated, the phase-separation temperature is lowered from $\approx 25 \pm 2^\circ\text{C}$ to $\approx 12 \pm 2^\circ\text{C}$. The ascending limbs of the coexistence curves for the native and modified proteins were determined at two different degrees of modification. The coexistence curve of the protein with 35% of the cysteines and 40% of the methionines modified shows that as protein concentration approaches the critical concentration of 289 g/liter, there is a much larger suppression of the critical temperature, from $\approx 38 \pm 2^\circ\text{C}$ in the native protein to $\approx 16 \pm 2^\circ\text{C}$. Incubation of intact calf lenses *in vitro* with the reagent results in the suppression of the phase-separation temperature by 3–9°C. These results are consistent with the view that the observed suppression in the critical temperature is due to an increase in the hydrophilicity of the protein in the vicinity of the sulfur-containing residues.

The opacification of the ocular lens results from alterations in the short-range order in the spatial distribution of the lens proteins (1–3). These alterations are produced by two distinct types of molecular changes. The first is the formation of high molecular weight protein aggregates (4, 5). The second is the separation of the cytoplasmic proteins into small, spatially dispersed domains consisting of protein-rich and protein-poor phases in thermodynamic equilibrium with each other (6). This latter mechanism, known as liquid–liquid phase separation, is responsible for the phenomenon of “cold cataract” observed in normal young mammalian lenses of many species. It also plays a central role in cataract formation in a variety of young animal model systems (7–11).

The γ -crystallins, a family of monomeric lens proteins, are principally responsible for the phenomenon of phase separation in the lens (12). They are distinguished by the presence of a large number (typically five to seven) of cysteine and methionine residues in their amino acid sequences (13–16). A variety of posttranslational modifications of the lens proteins have been known to occur in aging and cataractous lenses (17). One such modification that occurs in some forms of cataract, particularly maturity-onset nuclear cataract, is the oxidation of the cysteine and methionine residues of the γ -crystallins in these lenses (18–20). Furthermore, cysteine

oxidation has been linked to the formation of high molecular weight protein aggregates (20, 21). However, the role played by cysteine and methionine modifications in facilitating or inhibiting protein phase separation has not been investigated.

In view of these findings, we have undertaken a series of studies in which we induced a variety of specific chemical modifications of the cysteines and methionines of calf lens γ IV-crystallin[‡] and measured the effect on the critical temperature, T_c . In the present communication, we report the results of one such study in which γ IV-crystallin was reacted with a well-documented, strongly polar alkylating agent, *N*-bromoacetyethanolamine phosphate (NBAEP) (24–26). This reagent introduces a doubly charged phosphate anion at a maximum distance of 10–12 Å from the sulfur atom of cysteine or methionine.

While all members of the γ -crystallin family are rich in cysteines and methionines, we chose to study the high- T_c protein γ IV-crystallin, since it is a strong determinant of the net T_c of the lens (12). The parameter kT_c , where k is Boltzmann's constant and T_c is in kelvins, is a sensitive measure of the net intermolecular interaction energy that controls the separation of the protein solutions into distinct coexisting phases. We believe that this interaction energy, kT_c , is a useful index of the net interprotein association energy, which may also govern the formation of high molecular weight protein aggregates.

In general, modifications of cysteine and methionine, oxidative or otherwise, essentially (a) alter or maintain the oxidation state of the sulfur atom and (b) alter the hydrophilicity or hydrophobicity of the environment around the sulfur. We expect both these effects to influence the T_c of the lens proteins.

We have also investigated the effect of NBAEP on intact calf lenses. The results reveal that the introduction of charged groups in the vicinity of the sulfur atoms leads to a large suppression of T_c not only in γ IV-crystallin solutions but also in the intact calf lens.

MATERIALS AND METHODS

Preparation of γ IV-Crystallin. Lenses from calves under 6 weeks of age were obtained from Antech, Tyler, TX, by overnight express and stored at 2°C before further processing. The total monomeric γ -crystallin fraction was prepared

Abbreviations: NBAEP, *N*-bromoacetyethanolamine phosphate; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); IEF, isoelectric focusing; T_c , critical temperature for phase separation; T_{ph} , temperature for phase separation at a given protein concentration other than the critical concentration.

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‡According to the currently accepted nomenclature for mammalian γ -crystallins, γ IVa = γ E, γ IVb = γ A, γ IIIa = γ D, γ IIIb = γ C, and γ II = γ B (22, 23).

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from lens homogenate (27). γ IV-Crystallin was isolated from the γ fraction by ion-exchange chromatography on sulfopropyl (SP) Sephadex C-50 essentially by the method of Björk (13). The γ IV fraction so obtained was exhaustively dialyzed against 0.1 M sodium phosphate buffer (pH 7.1) at room temperature. This was our native γ IV-crystallin solution. All experiments were performed in 0.1 M sodium phosphate buffer (pH 7.1).

Preparation of NBAEP and Reaction with γ IV-Crystallin. Bromoacetyl bromide was obtained from Aldrich. Ethanolamine phosphate and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma. These were used without further purification. All other chemicals and solvents were reagent grade. NBAEP was initially a gift from Fred C. Hartman, Oak Ridge National Labs, and subsequently synthesized by us according to his published procedure (28). Reagent solutions at the required concentrations were incubated with protein solutions at a concentration of 1–2 g/liter, at room temperature. In parallel with each native γ IV-crystallin solution being modified, a corresponding protein solution was incubated with an equivalent volume of buffer not containing reagent; this was our control sample. Following incubation for 6 hr, the reaction was stopped by exhaustive dialysis of the modified proteins against 0.1 M sodium phosphate buffer (pH 7.1). The control samples were dialyzed separately for the same duration. Several experiments were performed in this manner, for a fixed reaction time of 6 hr and increasing reagent/protein mole ratios. To assess the possible effect of oxidation, initial experiments were performed in degassed solutions with and without 2-mercaptoethanol. These precautions had no effect on T_c and were eliminated in subsequent experiments.

Measurement of the Phase-Separation Temperature (T_{ph}) of Protein Solutions. Protein solutions at a concentration of 1–2 g/liter were filtered through 0.22- μ m filters and concentrated to the required value by ultrafiltration. Samples (80–200 μ l) were placed in a test tube immersed in a circulating water bath. The T_{ph} at a given protein concentration was measured as described (27). Coexistence curves were obtained by plotting T_{ph} versus protein concentration for native, control, and two modified γ IV-crystallin samples.

Characterization of Protein Samples. Extent of modification of cysteine residues. The number of moles of unreacted thiol groups was estimated by the DTNB assay according to the procedure of Truscott and Martin (29). All solutions were thoroughly degassed before use and kept under nitrogen during measurement. Free thiol concentrations were calculated using a molar extinction coefficient of 13.6×10^3 liter/(mol \cdot cm) for the 2-nitro-5-mercaptobenzoic acid produced in the reaction (30). Protein concentrations were calculated using a specific absorbance coefficient ($A_{280}^{1\%,1\text{cm}}$) of 21 for both native (13) and modified proteins, since no significant change in A_{280} was observed during the reaction. The number of modified thiol groups was given by $[\text{SH}]_{\text{mod}} = 6 - [\text{SH}]_{\text{unmod}}$, where 6 is the reported number (23) of cysteines in native γ IV-crystallin. One of the six cysteines, Cys-53, is a tentative assignment, based on the sequence homology with rat γ E-crystallin (23).

Extent of modification of methionine residues. The number of unreacted methionines in the protein samples was determined by amino acid analysis after hydrolysis in 6 M HCl at 110°C for 22 hr, at the Harvard Microchemistry Facility. The extent of methionine modification was given by $[\text{Met}]_{\text{mod}} = 5 - [\text{Met}]_{\text{unmod}}$, where 5 is the number of methionines in the native protein (23). No other amino acids besides cysteine and methionine were found to be alkylated by NBAEP. We note that under the conditions of acid hydrolysis, up to 20% of methionines are typically regenerated from modified samples (31). Hence the actual extent of methionine modification in our samples could be up to 20% higher. A more reliable

estimate of the degree of methionine modification can be obtained by performic acid oxidation of the protein samples, followed by amino acid analysis.

Cation-exchange chromatography and isoelectric focusing (IEF). To demonstrate that modification with NBAEP introduces negative charges into the protein, we performed cation-exchange chromatography and IEF of the native and modified samples. Cation-exchange chromatography was performed on a SP Sephadex C-50 column as detailed above for native γ IV-crystallin (13, 27). Flat-bed IEF was carried out on an LKB Multiphor system using Ampholine-PAG plates pH 3.5–9.5 (Pharmacia), without urea (32). The pI values were determined with standard pI markers.

Intact Lens Experiments. Several calf lenses were incubated in a 0.1 M solution of NBAEP in 0.1 M sodium phosphate buffer (pH 7.1) for 3–48 hr, and the T_{ph} values of these lenses were compared with those incubated in the same buffer without reagent. T_c was measured as follows. The encapsulated control and treated lenses were placed in a cuvette containing buffer, and cold cataract was induced by rapidly cooling the lens to 0°C. The transmitted beam intensity of a He-Ne laser beam through the opaque lens was measured and found to be close to zero. Each lens was then warmed slowly in increments of 2°C to about 28°C, a value well above the T_c of the normal lens (33, 34). Temperature equilibrium was maintained at each step and the increase in the transmitted beam intensity was recorded simultaneously. Shifts in the T_c of the modified lenses were obtained from plots of percent relative transmittance versus temperature, for control and treated lenses.

RESULTS AND DISCUSSION

Reaction of γ IV-Crystallin with NBAEP and Effect on T_{ph} . γ IV-Crystallin solutions at a concentration of 1–2 g/liter were reacted with increasing mole ratios of NBAEP for a fixed reaction time of 6 hr. The modified proteins were dialyzed exhaustively against 0.1 M sodium phosphate buffer (pH 7.1) to remove excess reagent. The reagent-free proteins were concentrated to 38 g/liter and the T_{ph} measured. Fig. 1 shows the T_{ph} values corresponding to each of the reagent/thiol ratios employed in the reaction. The results indicate a monotonic decrease in the T_{ph} of the protein with increasing concentration of NBAEP. A sharp drop in T_{ph} by 5–6°C at 18-fold molar excess of reagent is followed by a more moderate decrease at higher reagent concentrations. As the mole ratio of NBAEP to thiol groups approaches 200, T_{ph} is

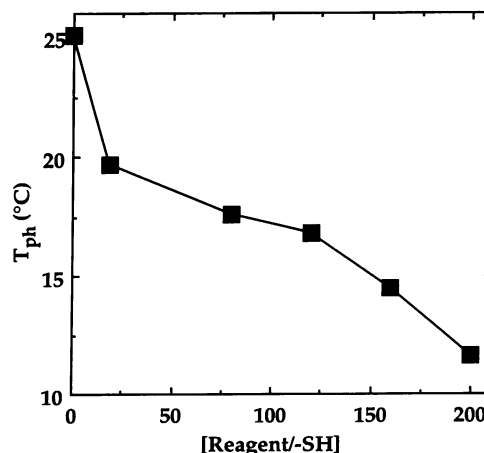


FIG. 1. Change in T_{ph} of γ IV-crystallin solutions as a function of NBAEP concentration (shown as a reagent/thiol mole ratio). Protein concentration was 38 g/liter. Reaction time was 6 hr. Solutions were in 0.1 M sodium phosphate buffer (pH 7.1).

again lowered sharply, to 11.8°C. Therefore, under these reaction conditions NBAEP dramatically lowers the T_{ph} of γ IV-crystallin solutions from 25.1°C to 11.8°C.

For each reagent/thiol mole ratio shown in Fig. 1, the fractional degree of modification of the cysteine and methionine residues of the protein was determined (Table 1). These data show that modification profiles of the cysteines and methionines follow distinctly different functional relationships to reagent concentration. Modification of the cysteines is essentially linear up to about 30%, while that of the methionines is only weakly dependent on reagent/thiol mole ratios between 18 and 200. Amino acid analysis revealed that no other residues were modified by the reagent. Therefore the change in T_{ph} shown in Fig. 1 is entirely due to the alkylation of the sulfur-containing amino acids of γ IV-crystallin. The most likely products of the reaction are shown in Fig. 2. Product I results from cysteine alkylation, and product II, a sulfonium salt, from methionine alkylation.

Nature of the Modified Proteins. Modification with NBAEP is expected to introduce several negative charges into the protein. A preliminary acid–base titration shows that the pK_a of the phosphate group of NBAEP lies between 2 and 3. At the reaction pH of 7.1, the phosphate group is in the dianionic form and is introduced at a maximum distance of 10–12 Å from the sulfur atom of cysteine or methionine. Thus the protein becomes increasingly polar as alkylation proceeds. To confirm this premise, we performed cation-exchange chromatography of the native and modified protein samples. Fig. 3 shows the SP Sephadex C-50 elution profile of a sample with an average of 15% of the cysteines and 35% of the methionines alkylated. Several peaks that were eluted prior to the control protein are evident. This profile is consistent with the formation of modified protein derivatives that are negatively charged with respect to the unmodified protein.

IEF patterns (not shown) of samples with increasing degrees of modification support the data from cation-exchange chromatography. We found that the control, unmodified sample migrated as two bands, an intense band with a pI of about 7.9 (35), and a weak band with a pI near 8.6. This pattern is consistent with the finding that γ IV-crystallin is composed of a major component, γ IVa; a minor, more basic component, γ IVb; and a small percentage of unidentified fractions (36, 37). For the modified sample whose chromatographic profile is shown in Fig. 3, with about 15% of the cysteines and 35% of the methionines alkylated, we observed several bands with progressively decreasing pI values ranging from 7.4 to 5.9. For a sample with a higher degree of modification (30% of cysteines and 40% of methionines), protein bands with pI values as low as 5.9–5.2 were observed. These results confirm that the protein acquires many negative charges due to alkylation at several of the modifiable sites.

Coexistence Curves of Native and Modified Proteins. The results above clearly indicate that the incorporation of polar

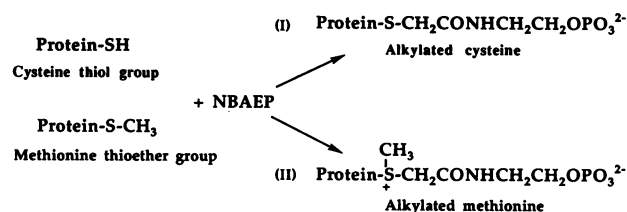


FIG. 2. Products of the alkylation of thiol and thioether groups of γ IV-crystallin with NBAEP.

groups in the vicinity of the sulfur-containing amino acids dramatically suppresses T_{ph} of the high- T_c protein γ IV-crystallin. However, these data were obtained at a single protein concentration. To determine the shift in the maximum of T_{ph} (T_c), we measured T_{ph} values for a series of protein concentrations, at two different extents of reaction. To prepare a sample with a slightly higher degree of cysteine modification than that shown in Table 1, the protein was alkylated at a fixed reagent concentration but with increased reaction times. A solution of γ IV-crystallin was reacted with an 80-fold molar excess of NBAEP relative to thiol groups, and two samples were withdrawn, at 6 and 18 hr of reaction, and processed as described in *Materials and Methods*. The ascending limbs of the coexistence curves were mapped by plotting T_{ph} against protein concentration for the native and control proteins and the two modified samples (Fig. 4).

In Fig. 4, the uppermost coexistence curve (0 hr) represents data from control (Δ) and native (∇) γ IV-crystallin solutions. The two data sets are indistinguishable, which confirms that the control samples are identical to the native protein solutions. The curve directly below this corresponds to the modified product obtained at 6 hr of reaction, with a modification of about 15% of the cysteines and 35% of the methionines. The bottom curve in Fig. 4 corresponds to 18 hr of reaction. Here, the modification increases to 35% of the cysteines and 40% of the methionines. A comparison of the coexistence curve at 0 hr with that at 18 hr shows clearly that there is a very large suppression of T_c , from $38 \pm 2^\circ\text{C}$ to about $16 \pm 2^\circ\text{C}$ at 289 g/liter. These data demonstrate that as a result of the polar modification of the sulfur-containing groups, the coexistence curve of the high- T_c protein γ IV-crystallin (12), approaches that of a typically low- T_c protein such as γ II-crystallin (27).

While our data indicate that a third ($\approx 35\%$) of the six cysteines of γ IV have reacted with NBAEP, we do not know the relative reactivities of the cysteine residues. When ranked according to solvent accessibilities obtained from the x-ray crystal structure of γ IV-crystallin, it would seem that Cys-53

Table 1. Percent modification of the cysteine and methionine residues of calf γ IV-crystallin with increasing concentration of NBAEP

Reagent/-SH, mol/mol	% Cys modified*	% Met modified†	T_{ph} ,‡ °C
0	0	0	25.1
18	11.9	8	19.7
80	15.4	34	18.0
120	17.5	34	17.2
160	28.4	34	15.0
200	28.7	38	11.8

*Determined by the DTNB assay, according to Truscott and Martin (29).

†Determined by amino acid analysis.

‡Measured at a protein concentration of 38 g/liter.

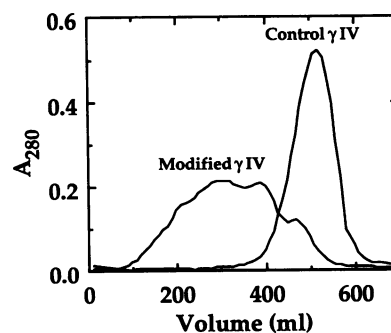


FIG. 3. Cation-exchange chromatography of control and NBAEP-modified γ IV-crystallin solutions on a SP Sephadex C-50 column (1.5 × 40 cm); elution was with a linear gradient from 0 to 0.2 M NaCl in 0.275 M sodium acetate buffer (pH 4.8) at a flow rate of 36 ml/hr. The modified sample had 15% of the cysteines and 35% of the methionines alkylated.

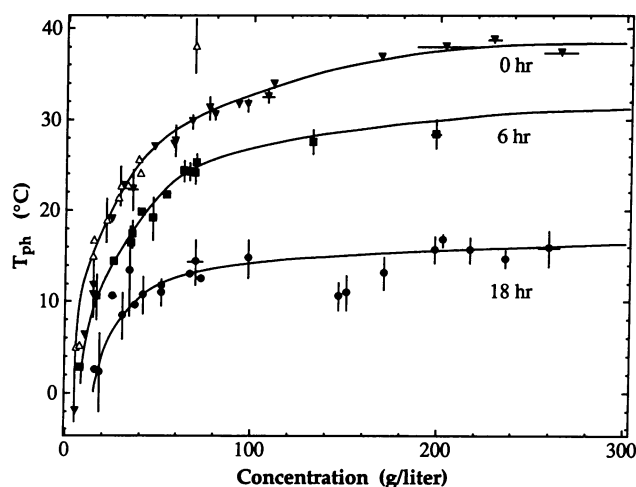


FIG. 4. Coexistence curves for native and NBAEP-modified γ IV-crystallin solutions in 0.1 M sodium phosphate buffer (pH 7.1). Top curve, control (Δ) and native (∇) γ IV-crystallin; middle curve, 6-hr sample with 15% of the cysteines and 35% of the methionines alkylated (\blacksquare); bottom curve, 18-hr sample with 35% of the cysteines and 40% of the methionines alkylated (\bullet).

(21 \AA^2) reacts first, followed by Cys-41 (10 \AA^2) and Cys-18 (6 \AA^2). (Accessibility data are from C. Slingsby, personal communication.) It must be understood, however, that the reactivity of a functional group may be determined by factors other than solvent accessibility alone. Four of the five methionines are accessible to solvent: Met-136 (86 \AA^2), Met-102 (75 \AA^2), Met-127 (57 \AA^2), and Met-69 (57 \AA^2). This may explain, in part, the relative ease of alkylation of these residues.

Interpretation of Results. We can interpret the results of these experiments in terms of a lattice model for binary liquid phase separation (38). By adapting this model to protein/water solutions (39), it can be shown that T_c (expressed in kelvins) is related to the protein-protein, water-water, and protein-water interaction energies (E_{pp} , E_{ww} , and E_{pw}) by the equation

$$kT_c = A[E_{pw} - (E_{ww} + E_{pp})/2].$$

Here A is a positive numerical constant whose magnitude is determined by the detailed form of the protein-water entropy of mixing and the number of nearest neighbors around each type of molecule in the mixture. For protein/water solutions we expect that the value of each of the interaction energies E_{ww} , E_{pp} , and E_{pw} will be a negative number; i.e., the interactions are attractive. For phase separation to occur it is necessary that kT_c be positive. This will occur if the mean negative, like-like attractive interaction energy term $(E_{ww} + E_{pp})/2$, is larger in magnitude than the negative, like-unlike attractive energy E_{pw} . From this equation it is clear that protein modifications that increase the magnitude of the protein-water attractive interaction energy relative to the mean, like-like interaction energy will result in a decrease of T_c . Conversely, a decrease in the magnitude of E_{pw} relative to $(E_{ww} + E_{pp})/2$ will produce an increase in T_c .

For the reaction reported here, the strongly polar PO_3^{2-} group is expected to increase the net hydrophilicity of the protein and therefore increase the magnitude of the E_{pw} term, thereby lowering T_c . This is consistent with the experimental observations. The alkyl group of NBAEP introduced at the sulfur atom is a composite of both polar and nonpolar moieties, as shown in Fig. 2. The nonpolar groups could produce a hydrophobic contribution to E_{pw} , but the strongly hydrophilic effect of the PO_3^{2-} group appears to be dominant. This suggests experiments involving modifiers analogous to

NBAEP but with longer and shorter alkyl chains or other nonpolar groups. Studies of the change in T_c as a function of alkyl chain length could establish the relative roles of nonpolar and polar groups in fixing the overall hydrophilicity of this type of modification of the sulfur-containing residues.

Oxidation State of the Sulfur Atoms. When cysteine is alkylated with NBAEP, the oxidation state of the sulfur atom (-2) remains unaltered (40). This is therefore a nonoxidative modification, unlike the oxidation of thiol sulfur (-2) to the disulfide form (-1). For methionine, however, the sulfur atom undergoes a change in oxidation state from -2 to 0 following alkylation with NBAEP, similar to the oxidation of methionine (-2) to methionine sulfoxide (0). For methionine, alkylation apparently results in a change in the electronic configuration of the sulfur atom identical to that of an oxidative modification. However, both residues experience a net increase in polarity near the sulfur atoms, which, we believe, is responsible for the reduction in T_c .

Effect in Intact Calf Lenses *in Vitro*. Covalent modification with NBAEP results in stable products under our experimental conditions. This is indicated by the reproducibility of the T_{ph} values and IEF profiles of samples stored for several weeks at 4°C. Therefore NBAEP may be important as a potential inhibitor of phase-separation cataracts. To test this hypothesis, we studied the effect of the reagent on the intact calf lens by diffusing solutions of this reagent into encapsulated calf lenses for times ranging from 3 to 48 hr (see *Materials and Methods*). These preliminary results show that the net T_c of the NBAEP treated lenses was 3–9°C lower than that of lenses incubated with buffer alone.

CONCLUSIONS

(i) Alkylation of calf γ IV-crystallin with NBAEP leads to a marked reduction in the T_c of the protein solutions.

(ii) The reaction is specific to the sulfur-containing amino acids of the protein. Several negatively charged groups are bound to the protein as reaction progresses.

(iii) Modification of increasing numbers of cysteine and methionine residues leads to increased suppression of phase separation. This effect is irreversible under our experimental conditions. When about 35% of the cysteines and 40% of the methionines are modified the T_c of γ IV-crystallin, a high- T_c protein, approaches that of the low- T_c γ II-crystallin.

(iv) The overall hydrophilic-hydrophobic alterations in the vicinity of the sulfur atom, rather than its oxidation state alone, appear to play a dominant role in governing T_c .

(v) The reagent is also effective in reducing the temperature for opacification in the intact calf lens *in vitro* by 3–9°C and, in principle, could serve as an inhibitor of phase-separation cataract.

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