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## Calcium-induced aggregation of bovine lens alpha crystallins

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*The theory of light scattering indicates that aggregation of the crystalline lens proteins can produce opacity. Furthermore, it is known that calcium increases in the cataractous lens. We have, therefore, investigated the effectiveness of calcium ion in producing aggregates of the soluble proteins of the bovine lens. The experimental results show: (1) CaCl<sub>2</sub> produces aggregates of the soluble protein of the lens; (2) the pre- $\alpha$  and  $\alpha_1$  fraction of the lens protein are the specific targets of calcium ion even in the presence of sulfhydryl reducing reagent; (3) the aggregation can be reversed and transparency restored when calcium is removed either by dialysis or chelation; and (4) the molecular weight distribution and index of refraction of the aggregates have been measured.*

**Key words:** light scattering, opacity, calcium, aggregation, pre- $\alpha$  crystallin,  $\alpha_1$  crystallin, diffusion coefficient, Svedberg constants, index of refraction, reversibility.

**I**t is known that nuclear cataractous lenses are characterized by a reduction in content of low molecular weight proteins and an increase in insoluble protein.<sup>1</sup> On this basis, a number of authors<sup>1-5</sup> have hypothesized that cataract formation is,

in fact, associated with the conversion of low molecular weight proteins to higher molecular weight aggregates, the heaviest of which correspond to the insoluble or albuminoid fraction.

In addition, several authors<sup>6, 7</sup> have suggested that the presence of high molecular weight protein aggregates in the lens can lead to increased scattering of light by the lens and a consequent loss of transparency. Indeed, a review by Waley<sup>8</sup> includes the suggestion that "the increase in light scattering with age may also be due to aggregation, a stage on the way towards the formation of insoluble proteins."

Quite recently these biophysical and biochemical speculations have become considerably more specific and quantitative. On the biophysical side, there have been theoretic advances in our understanding of the microstructural basis for the trans-

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parency or opacity of the ocular media.<sup>9, 10</sup> In the case of the lens, this theory has been applied to calculate quantitatively the turbidity produced in the lens by a spatially random distribution of protein aggregates of molecular weight  $M_n$ .<sup>9</sup> The calculation shows that the lens turbidity is proportional to the product of three quantities: (1) the molecular weight of the aggregate ( $M_n$ ); (2) the mass fraction of protein in the form of aggregates  $\xi$ ; and (3) a quantity

$$\xi = (n_a^2 - n_i^2)/(n_a^2 + 2n_i^2)$$

which depends on the difference between the index of refraction of the aggregate,  $n_a$ , and that of the normal lens,  $n_i$ . By estimating roughly the magnitude of  $n_a$  and  $n_i$ , one can compute theoretically the molecular weight of the protein aggregates responsible for producing opacity. This computation indicates the molecular weight of such aggregates in human cataractous lenses as being  $50 \times 10^6$  Gm. per mole or larger.<sup>9</sup>

On the biochemical side, recent investigations by Spector and co-workers<sup>11-13</sup> have demonstrated that a high molecular weight fraction of  $\alpha$  crystallin does in fact occur among the soluble proteins of the bovine lenses. These studies show that the molecular weight of these aggregates does in fact exceed  $50 \times 10^6$  Gm. per mole as predicted in the theory of Benedek.<sup>9</sup> Also, Spector and his co-workers<sup>12</sup> showed a dramatic increase in the relative proportion of this high molecular weight fraction in the nuclear portion of the lens as the age of the animal increases. In fact, as the animal ages from the calf (three months) to the bull (eight years), the fraction of protein having a molecular weight greater than  $15 \times 10^6$  Daltons was found to increase from 15 to 80 per cent, i.e., a factor of approximately 5. It is important to realize that these aggregates are found in the *soluble* fraction of the lens homogenate.

Spector and co-workers<sup>13</sup> have also investigated the biochemical mechanism by which aggregation occurs. By breaking native  $\alpha$  crystallin into its monomeric and

dimeric subunits, they were able to induce re-aggregation into the very heavy aggregated form by recombining the subunits in the presence of glucose. This suggests that one possible form of aggregation of lens proteins may involve glucose.

Another factor which suggests itself for consideration in connection with aggregation is calcium. Previous studies<sup>14, 15</sup> have shown that there is a marked increase in calcium ion concentration concomitant with the development of lens opacity. Accordingly we have undertaken the present investigation with the following objectives: First, to investigate the role of calcium ion in producing aggregation of the native lens proteins; second, to investigate the molecular weight distribution of calcium-induced aggregates; and, third, to determine whether the index of refraction of these aggregates is sufficient to lead to lens opacity.

#### Materials and methods

Calf eyes were obtained from a local abattoir soon after slaughtering and the lenses were removed. The lenses were used fresh or were frozen until needed. Homogenates were prepared from decapsulated lenses by grinding the lenses with 5 times their volume  $2 \times 10^{-3}$  M phosphate buffer, pH 6.8, in a Tenbroeck homogenizer. The homogenates were centrifuged at 13,400 r.p.m. for 30 minutes.

The protein concentration of the initial homogenate and of each fraction collected by diethylaminoethyl (DEAE) chromatography was measured by the method of Warburg and Christian.<sup>16</sup> Protein concentrations of the samples used for index of refraction measurements were determined from the dry weight of the samples studied. Absorption at  $280 \mu$  was used to estimate both the initial and final concentrations of pre- $\alpha$  and  $\alpha$  crystallins in the absence and presence of calcium chloride ( $\text{CaCl}_2$ ) following chromatography on Sepharose 2B.

DEAE-cellulose type 20 was obtained from the Brown Company, Berlin, N. H., and was prepared and used according to the method of Spector.<sup>17</sup> One modification of his procedure was as follows: The final elution step of  $\alpha$  crystallin was performed at room temperature with 0.5 M phosphate buffer, pH 6.8. In this text, that fraction of protein eluted with 0.05 M phosphate buffer is referred to as pre- $\alpha$  crystallin. Protein fractions eluted with 0.08 M, 0.1 M, and 0.5 M phosphate buffers are re-

ferred to as  $\alpha_2$ ,  $\alpha_3$ , and  $\alpha_4$  crystallins, respectively. By this modified method, the  $\alpha_4$  fraction used corresponds to a combination of the proteins eluted by Spector with 0.4 M phosphate buffer and 0.2 M phosphate buffer + 0.2 M sodium chloride. Phosphate buffer was removed from all of the fractions obtained from DEAE by dialyzing each fraction against two 4 L. changes of Tris buffer (0.1 M, pH 7.4) for 24 hours at 5° C.

Sephacrose 2B was obtained from Pharmacia (Piscataway, N. J.) and was packed in Pharmacia columns. The gel beds used routinely measured 2.5 by 37 cm. and were equilibrated and eluted with Tris buffer (0.1 M, pH 7.4).

Extensive measurements of diffusion coefficient (D) are used in these studies. Measurements of D were carried out using the newly developed technique of optical mixing spectroscopy.<sup>18, 19</sup> This method permits a rapid (approximately 15 minutes) and convenient means of measuring D even for relatively low concentration solutions. The measurement of D was used in two different ways. First, it was used qualitatively to detect the macromolecular aggregation induced by calcium. Since D, at low concentration, is inversely proportional to the effective molecular diameter, aggregation of proteins will result in marked changes in D. The second use of D was in the quantitative analysis of the molecular weight of the aggregates. This analysis is based on the fact that for globular proteins, the hydrodynamic frictional ratio is a constant<sup>20</sup> and, therefore, the use of the Stokes-Einstein relation and the Svedberg equation implies that the molecular weight (M) can be related to D by an equation of the form:

$$M = C/D^3,$$

where C is a constant for all globular proteins. We constructed a graph of M versus D for a range of globular proteins varying in mass from  $1.4 \times 10^3$  to  $1.9 \times 10^8$ . Daltons and found the equation above to be an accurate representation using

$$C = 1.4 \times 10^{14} \text{ (Gm. per mole)} \times (\text{sec./cm.}^2)^3.$$

Thus, by measuring D we could immediately deduce M. Fig. 1 illustrates the graph used for M versus D.

A second procedure for the determination of molecular weight was used and proved particularly useful in examining the properties of a molecular weight distribution. In this procedure, use of an analytic ultracentrifuge (Spinco Model E) permitted the determination of the distribution of sedimentation velocities of aggregates present in solution. Once again, on the assumption that the aggregates produced are globular, one can use the balance between centripetal force (including buoyancy) and the viscous frictional force to obtain an expression for M in terms of the sedimentation velocity, i.e.,  $M = \text{constant} (S^{3/2})$ . Here,

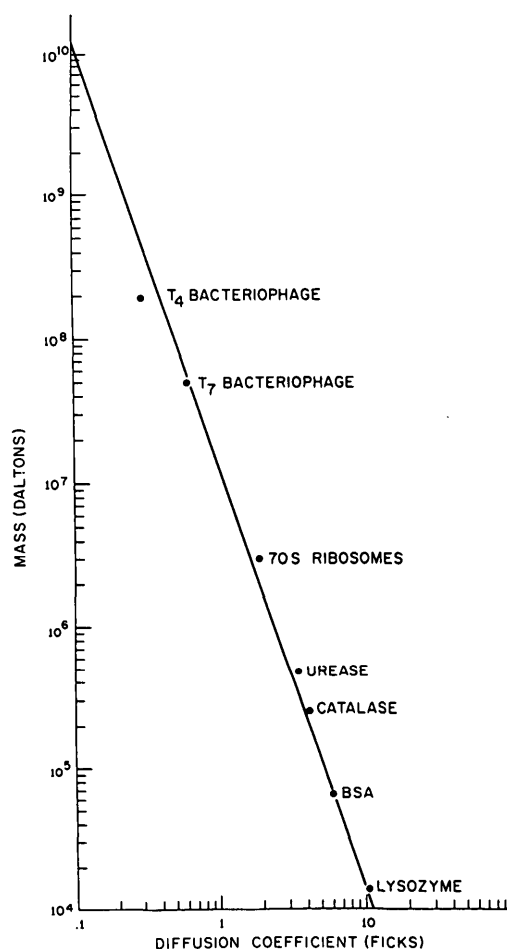


Fig. 1. Mass vs. diffusion coefficient for globular particles.

S is the sedimentation velocity per unit of centrifugal acceleration as measured in units of the Svedberg, i.e.,  $10^{-13}$  second. A plot of M versus S for globular proteins is shown in Fig. 2 and shows good agreement with the formula when

$$C = 6.6 \times 10^3 \text{ (Gm. per mole)} (S\text{vedbergs})^{-3/2}.$$

Finally, it is possible to deduce M quite independently of assumptions as to the molecular shape by measuring both S and D and using the Svedberg equation to determine M.

Measurements of the index of refraction ( $n_p$ ) of calcium-induced aggregates of  $\alpha$  crystallin were made by determining the index of refraction ( $n_s$ ) of solutions of the aggregates as a function of the mass concentration C (gram/cubic centimeter) of the solution. It can be shown that  $n_p$ ,  $n_s$ , and  $n_0$  are related by the equation:

$$(n_s^2 - n_0^2) = (n_p^2 - n_0^2) (3n_0^2 / (n_p^2 + 2n_0^2)) \nu C$$

for dilute solutions. Here,  $\nu$  is the partial molal

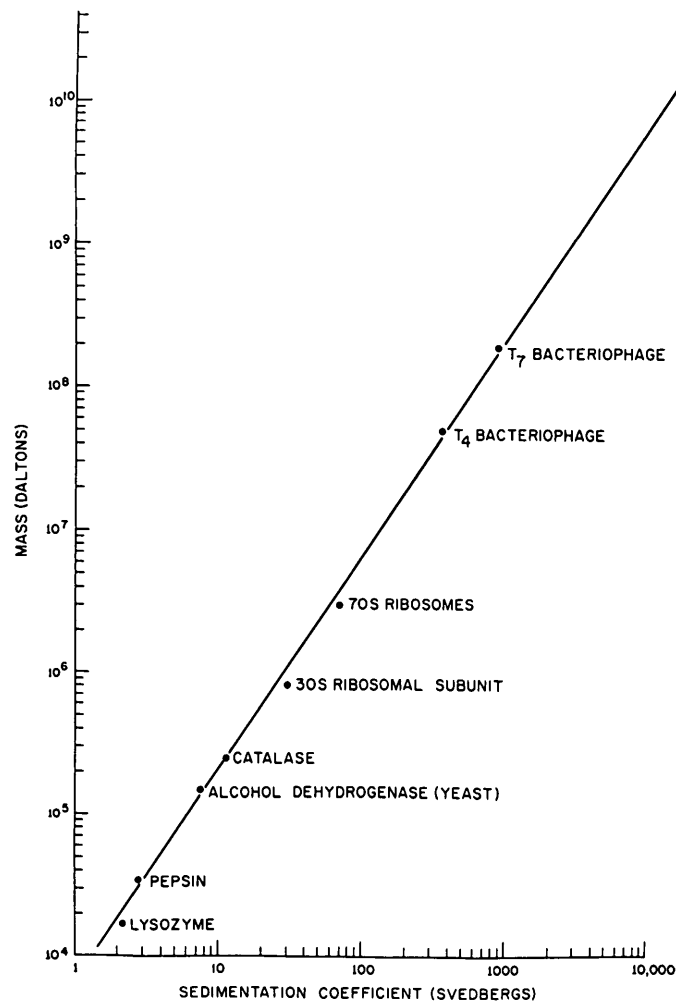


Fig. 2. Mass vs. sedimentation coefficient for globular particles.

volume, i.e., the volume of water displaced per gram of protein;  $n_s$  and  $n_n$  were measured using a Bausch and Lomb Abbe 3L refractometer.

## Results

**Effect of  $\text{CaCl}_2$  on lens extract.** In an exploratory effort, the effectiveness of calcium in inducing protein aggregation in the supernatant fraction of the lens homogenate was examined. First, a solution of the soluble lens proteins from fresh decapsulated lenses was prepared by homogenizing the lens in Tris buffer [0.1 per cent beta-mercaptoethanol ( $\beta$ -mETOH) + 0.01 M Tris, pH 7.4]. Separate aliquots of the supernatant fluid obtained after centrifugation were then dialyzed against the same

Tris buffer and against Tris buffer containing 0.01 M  $\text{CaCl}_2$  for 24 hours. Dialysis against the buffer containing calcium ions produced a tenfold increase in turbidity over the control sample as measured at 500  $\mu$ . After centrifugation of the turbid solution, a precipitate of insoluble protein representing approximately 37 per cent of the initial soluble protein was separated and retained. This converted protein was resuspended in Tris buffer and excess  $\text{CaCl}_2$  was removed by dialysis. Following dialysis, this sample was recentrifuged. The final supernatant contained soluble proteins whose weight was equal to 12 per cent of the weight of the converted protein

**Table I.** Effect of  $\text{CaCl}_2$  on the diffusion coefficients of soluble lens proteins

Sample	$D$ ( $10^{-7}$ cm. <sup>2</sup> /sec.)			$\frac{D \text{ (control)}}{D \text{ (CaCl}_2\text{-treated)}}$
	Dialysis treatment			
	0.01M Tris (24 hrs.)	0.01M Tris (48 hrs.)	0.01M Tris + $\text{CaCl}_2$ (24 hrs.)	
$\gamma$	4	4	4	1
$\beta$	2.5	1.7	1.0	1.7
Pre- $\alpha$	2.3	2.0	0.2	10.0
$\alpha_2$	2.4	2.2	0.47	4.7
$\alpha_3$	2.3	2.0	1.4	1.4
$\alpha_4$	1.9	2.0	0.25	8.0

Protein (14.4 mg.) in a volume of 12.5 ml. were added and eluted from a 2.5 by 37 cm. column of DEAE-cellulose. The samples shown represent protein eluted with the following buffers:  $\gamma$ , 0.002M phosphate;  $\beta$ , 0.03M phosphate; pre- $\alpha$  0.05M phosphate;  $\alpha_2$ , 0.08M phosphate;  $\alpha_3$ , 0.1M phosphate; and  $\alpha_4$ , 0.5M phosphate. Diffusion coefficients were measured at 3 mg. per milliliter  $\gamma$  crystallin. The remaining crystallins were adjusted to contain 1.5 mg. per milliliter. In Column 5, the ratio of the diffusion coefficient of control to test sample is tabulated. The cube of the entries in Column 5 is an indication of the ratio of the molecular weight of the  $\text{CaCl}_2$ -treated protein to that of the control sample.

suspension. When analyzed by Sepharose 2B chromatography, all of the protein in the supernatant solution appeared in the void volume. A pool of the void volume peak was made and measured to find the value of the diffusion constant,  $D$ . By the method of optical mixing spectroscopy,  $D$  was found to be  $0.16 \times 10^{-7}$  cm.<sup>2</sup> per second. By using the relationship for globular proteins (see Methods), it was deduced that the molecular weight of the aggregated protein measured approximately  $3 \times 10^9$  Daltons.

This effort demonstrated clearly that treating the soluble lens proteins with calcium ions leads to a wide molecular weight distribution of aggregates. After removing the excess calcium ions and centrifuging, part of this distribution was separated and found to consist of aggregates having molecular weights as large as  $3 \times 10^9$  Daltons.

It should be recognized that even the supernatant part analyzed will contain a distribution of molecular sizes. The method employed for measuring  $D^{18, 19}$  weights the molecular aggregates in proportion to their light-scattering power. Therefore, the molecular weight given above represents the heavy end of the molecular weight distribution present in the supernatant.

**Effect of  $\text{CaCl}_2$  on individual lens crystallins.** Since the experiment described above showed that a solution of  $\text{CaCl}_2$

was capable of aggregating the native soluble lens proteins, the next objective was to identify which particular crystallins were participating in aggregate formation. For this purpose  $\alpha$ ,  $\beta$ , and  $\gamma$  fractions of the soluble lens proteins were separated by DEAE chromatography. The individual fractions were then dialyzed against 8 L. of 0.01 M Tris buffer, pH 7.4, for 24 hours to remove phosphate buffer. Each fraction was separated into two parts. One part, a control, was dialyzed against Tris for an additional 24 hours while the second part, a test sample, was dialyzed for 24 hours against Tris containing 0.01 M  $\text{CaCl}_2$ . The effectiveness of  $\text{CaCl}_2$  solution in aggregating each of the fractions selected was studied by measuring the value for  $D$  of each control sample and the corresponding test sample. Since  $D$  is inversely proportional to molecular dimension, the ratio of the cube of  $D$  of the control sample to that of the test sample can be expected to be roughly equal to the ratio of the molecular weight of the protein in the test sample to that of the control sample.

The results of the experiments are given in Table I. As shown, the  $\text{CaCl}_2$  solution had no appreciable effect on  $\gamma$  crystallin. In the case of  $\beta$  crystallin, while prolonged dialysis against Tris does seem to affect the sample, there is little significant aggregation of the treated sample relative to the control.

**Table II.** Effect of sulfhydryl reducing reagent on CaCl<sub>2</sub>-induced aggregation

Sample	D (10 <sup>-7</sup> cm. <sup>2</sup> /sec.)	
	Reduced protein (control)	Reduced protein and CaCl <sub>2</sub> (test)
γ	4.8	4.8
β <sub>1</sub>	2.8	2.9
β <sub>2</sub>	2.6	2.6
β <sub>3</sub>	1.3	1.3
Pre-α	2.1	0.2
α <sub>2</sub>	2.4	2.4
α <sub>3</sub>	2.2	2.3
α <sub>4</sub>	1.5	0.26

Protein concentrations used for measuring D were the same as in Table I. 0.1 per cent β-mETOH was added to each fraction after a 48-hour dialysis against Tris buffer (0.01 M, pH 7.4). After 15 minutes D values were measured. CaCl<sub>2</sub> (0.01 M) was added to the remainder of the reduced protein. D values were again measured after a 15 minute interval following treatment. The samples shown were eluted with the following buffers: γ, 0.002 M phosphate; β<sub>1</sub>, 0.015 M phosphate; β<sub>2</sub> and β<sub>3</sub>, 0.03 M phosphate; Pre-α, 0.05 M phosphate; α<sub>2</sub>, 0.08 M phosphate; α<sub>3</sub>, 0.1 M phosphate; and α<sub>4</sub>, 0.5 M phosphate.

Calcium solution has quite a different effect on the α fractions. The results show clearly that the pre-α fraction experiences a tenfold decrease in D. The α<sub>2</sub> fraction suffers a decrease in D by a factor of approximately 5. On the other hand, the α<sub>3</sub> component is essentially unaffected by CaCl<sub>2</sub>. The α<sub>4</sub> fraction though is quite markedly affected by treatment with CaCl<sub>2</sub>. These findings clearly show that pre-α, α<sub>2</sub>, and α<sub>4</sub> are the only crystallins that are significantly aggregated by CaCl<sub>2</sub> solution. It should be pointed out that the measurement of D was conducted on that fraction of each sample which passed through a 3μ Millipore filter. Thus, if the CaCl<sub>2</sub> treatment produced aggregates of D less than that listed in the Table, they would be excluded by the filter and not be observed.

**Effect of sulfhydryl reducing reagent on CaCl<sub>2</sub>-induced aggregation.** Since it is recognized that sulfhydryl oxidation of lens protein may lead to insolubilization,<sup>3, 21, 22</sup> the possibility that the aggregates produced in the presence of CaCl<sub>2</sub> are due to the formation of disulfide bonds was next investigated. For this study, each crystallin fraction was dialyzed for 48 hours against 0.01 M Tris, pH 7.4. Each fraction was treated with 0.1 per cent β-mETOH and was di-

vided into two parts. The first was the control and the second was treated with 0.01 M CaCl<sub>2</sub>. After 15 minutes, D values of both the control and test sample were measured as a test for aggregation. The results of this experiment are shown in Table II and are quite definitive. The Table shows that D values of the γ and β crystallins are the same in the presence and absence of CaCl<sub>2</sub>. This was expected in the case of γ crystallin and further implies that the slight change in D for β crystallin shown in Table I was most likely due to disulfide bond formation. In the α crystallin group, D values for pre-α and α<sub>4</sub> were markedly affected by 0.01 M CaCl<sub>2</sub>, indicative of extensive aggregation. Alpha<sub>1</sub> and α<sub>3</sub> in these studies were unaffected. Therefore, the aggregation of α<sub>2</sub> recorded in Table I may have been due to sulfhydryl oxidation.

A quantitative estimate of the extent of aggregation of both pre-α crystallin and α<sub>4</sub> crystallin induced by CaCl<sub>2</sub> was obtained by using Sepharose 2B chromatography. In the case of pre-α crystallin, it was found that 98.9 per cent of native crystallin could be recovered from the column while only 67.5 per cent CaCl<sub>2</sub>-treated pre-α crystallin was recovered after elution. The difference between these values, 31.4 per cent, was the amount of CaCl<sub>2</sub>-treated protein excluded from the column and, thus, converted to large molecular size. These findings are illustrated in Fig. 3 which gives a comparison of the elution profile of native pre-α crystallin (closed circles) and the CaCl<sub>2</sub>-treated protein (open circles). In the case of the CaCl<sub>2</sub>-treated material, a marked reduction in the concentration of pre-α crystallin clearly occurs at the elution points corresponding to the native pre-α crystallin.

In the case of α<sub>4</sub> crystallin, 71.3 per cent of the native crystallin was recovered by chromatography elution while only 49.2 per cent of CaCl<sub>2</sub>-treated α<sub>4</sub> crystallin was recovered. The difference between these values showed that 22.1 per cent of the native material was aggregated by CaCl<sub>2</sub>.

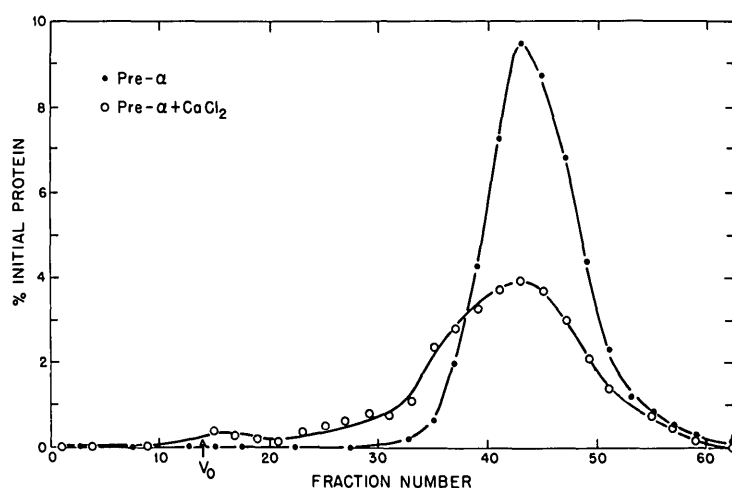


Fig. 3. Elution profile of native and  $\text{CaCl}_2$ -treated pre- $\alpha$  crystalline on Sepharose 2B. Pre- $\alpha$  crystallin ( $280 \mu$ ) (14.2 mg.) in a volume of 5 ml. were eluted from a Sepharose 2B column equilibrated with Tris buffer (0.01 M, pH 7.4), closed circles. An equivalent amount of protein dialyzed against Tris buffer (0.01 M, pH 7.4) and  $\text{CaCl}_2$  (0.01 M) was eluted from the same column, open circles. The ordinate represents per cent initial absorption of  $280 \mu$  while the abscissa represents the fraction number. Each fraction was 3.5 ml. and the eluant in both cases was the same as the equilibration buffer.

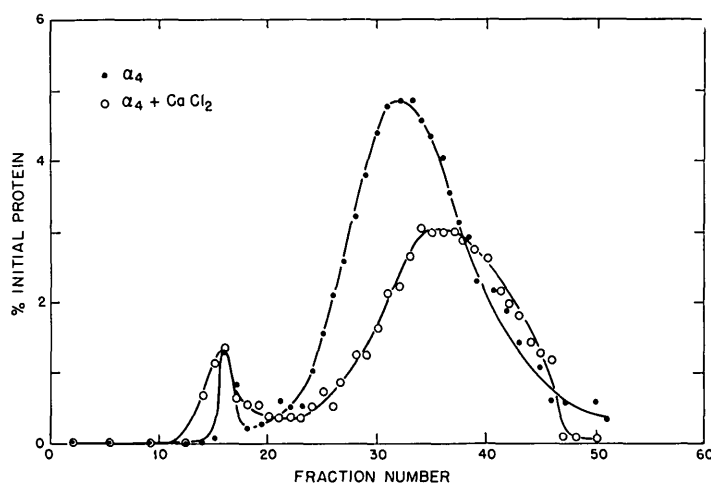


Fig. 4. Elution profile of native and  $\text{CaCl}_2$ -treated  $\alpha_4$  crystallin on Sepharose 2B.  $\alpha_4$  crystallin ( $280 \mu$ ) (5 mg.) in a volume of 5 ml. were added to a Sepharose 2B column as described in Fig. 3. The elution profile of nature  $\alpha_4$  crystallin is shown as closed circles. The open circles illustrate the elution profile of 8.1 mg.  $\alpha_4$  after a 24 hour dialysis against 4 L. Tris buffer (0.1 M, pH 7.4) containing  $\text{CaCl}_2$  (0.01 M). The coordinates are the same as in Fig. 3.

and was excluded from the column. The elution profiles of both native  $\alpha_4$  crystallin and  $\text{CaCl}_2$ -treated  $\alpha_4$  are shown in Fig. 4. As was the case with pre- $\alpha$  crystallin, there is a significant fall in the amount of  $\text{CaCl}_2$ -treated  $\alpha_4$  that appears in the elution profile as compared with the control sample.

It can be concluded from these findings that the pre- $\alpha$  and  $\alpha_4$  fractions are specifically induced to aggregate by  $\text{CaCl}_2$  and that this aggregation is not associated with the formation of disulfide bonds.

*Molecular weight distribution of  $\text{CaCl}_2$ -induced aggregates.* Using the methods

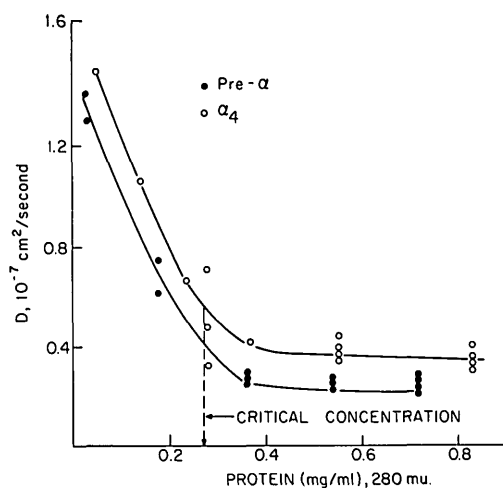


Fig. 5. The effect of protein concentration on  $\text{CaCl}_2$ -induced aggregation. The diffusion coefficient of increased concentrations of pre- $\alpha$  and  $\alpha_4$  crystallins (0.05 to 0.85 mg. per milliliter) were measured at a fixed concentration of  $\text{CaCl}_2$  (0.01M) after 15 minutes pre-incubation with calcium. Pre- $\alpha$  is shown as closed circles; and,  $\alpha_4$  as open circles.

of analytic ultracentrifugation and measurements of  $D$ , estimates were formed of the molecular weight distribution of calcium-induced protein aggregates. Initial experiments were carried out on crystallins prepared from frozen lenses. Alpha<sub>4</sub> crystallin was again separated on DEAE. After phosphate buffer was removed, the protein was dialyzed against 0.01 M Tris, pH 7.4, containing 0.01 M  $\text{CaCl}_2$  for 3 hours. The aggregates formed were passed through a 14  $\mu$  Millipore filter and  $D$  was measured. The values of  $D$  obtained ranged between  $0.18 \times 10^{-7}$  cm.<sup>2</sup> per second and  $0.018 \times 10^{-7}$  cm.<sup>2</sup> per second. It should be remembered that the method for measuring  $D$  used weights the high end of the mass distribution of aggregates. The molecular weights indicated in this measurement are in the range from  $2 \times 10^9$  to  $2 \times 10^{12}$  Daltons. To obtain a more accurate estimate of the mass distribution, the sedimentation velocity distribution function was measured and relatively broad peaks in this distribution were found to occur at sedimentation velocities primarily at  $V_s$  of approximately 1,300, 2,800, and 4,500 Svedbergs. These

peaks in turn indicate proteins with molecular weights of approximately  $300 \times 10^6$ ,  $1 \times 10^9$ , and  $2 \times 10^9$  Daltons, respectively. The sedimentation velocity profile indicates that  $\text{CaCl}_2$  produces a hierarchy of aggregates which range in weight from approximately  $300 \times 10^6$  to beyond  $2 \times 10^9$  Daltons.

**Index of refraction.** Since the amount of light scattered by protein aggregates is proportional to the difference between their refractive index and the index of the surrounding material, it is of great importance that this difference be accurately determined. For this reason index of refraction measurements were made for native  $\alpha$ -crystallin and calcium-induced  $\alpha$ -crystallin aggregates.

Using 1.71 per cent protein solutions, we have determined that the index of refraction of both the native  $\alpha$  crystallin and the calcium-aggregated  $\alpha$  crystallin is the same within 1 per cent and the absolute value of these refractive indices is  $1.595 \pm 0.03$ . This number is in close agreement with the refractive index of 1.609 that one can deduce from the refractive index increment measurements that were made by Perry and Koenig<sup>23</sup> on  $\alpha$  crystallin. This value is substantially larger than the index of refraction of the lens itself,  $n = 1.38 - 1.4$ ,<sup>24</sup> indicating that such aggregates would scatter light effectively in the lens.

**Active ion responsible for  $\text{CaCl}_2$ -induced aggregation.** The experiments so far described show that a solution of  $\text{CaCl}_2$  aggregates  $\alpha$  crystallin. To identify the active ion in this salt,  $\alpha_4$  crystallin was dialyzed against Tris-buffered solutions containing either 0.01 M  $\text{CaCl}_2$  or 0.01 M calcium acetate or 0.01 M  $\text{MgCl}_2$  or a solution containing 0.01 M  $\text{CaCl}_2$  and 0.01 M ethylenediaminetetraacetate (EDTA). All of the dialyses were carried out for 12 hours at 5° C. By measuring the  $D$  values of the  $\alpha_4$  fraction before and after treatment with each of these salts, it was found that both  $\text{CaCl}_2$  and calcium acetate did produce marked aggregation. On the other hand,  $\text{MgCl}_2$  had no effect in aggregating  $\alpha_4$



crystallin. Also, EDTA, chelating agent, completely inhibited the aggregation effect of  $\text{CaCl}_2$ . It must therefore be concluded from these findings that the calcium ion is the responsible agent involved in these protein aggregation studies.

**Conditions required for calcium-induced aggregation and deaggregation.** The concentration of calcium ions necessary to aggregate pre- $\alpha$  and  $\alpha_4$  crystallins was also studied. The minimum concentration of pre- $\alpha$  and  $\alpha_4$  required to produce aggregation in the presence of a fixed amount of calcium (0.01 M) was established and is shown in Fig. 5. The criterion used for aggregation was to detect a change in the value of  $D$  of the solution after 15 minutes preincubation of protein with calcium. In this experiment the marked onset of the aggregation occurred when the  $\alpha$  crystallin concentration exceeded approximately 0.3 mg. per milliliter. As shown in Fig. 6, at a fixed concentration of either pre- $\alpha$  or  $\alpha_4$  (0.30 mg. per milliliter), the calcium ion concentration necessary to produce aggregation was found to be  $\sim 4 \times 10^{-3}$  M. At this critical concentration the values for  $D$  of both  $\alpha$  crystallin fractions were markedly reduced. Further increments in the concentration of calcium up to 0.01 M produced no observable reduction in the  $D$  values found for  $\sim 4 \times 10^{-3}$  M calcium. The first of these findings suggests that a certain critical concentration of  $\alpha$  protein is necessary for aggregation to occur. The second result suggests that even when the critical concentration of both  $\alpha$  crystallins is exceeded, a definite number of calcium ions are needed to initiate the aggregation. Calcium present in concentration lower than  $\sim 4 \times 10^{-3}$  M will not trigger the reaction.

To ascertain whether the removal of calcium from the aggregates results in deaggregation, pre- $\alpha$  and  $\alpha_4$  calcium-induced aggregates were treated with EDTA (0.01 M) or EGTA (0.005 M). In both instances the turbidity of both solutions disappeared. Also, the values for  $D$  measured for both proteins in the solution returned to values

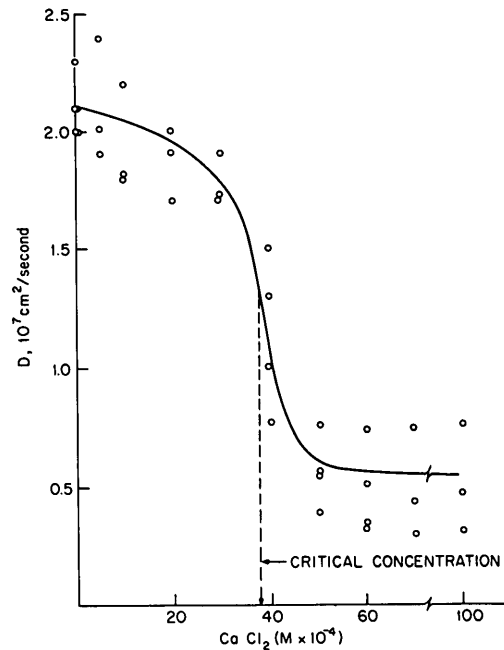


Fig. 6. The effect of increased calcium ion concentration on the diffusion coefficient of  $\alpha_4$  crystallin. The diffusion coefficients of fixed amounts of  $\alpha_4$  crystallin (0.3 mg. per milliliter) were measured 15 minutes after calcium was added at increased concentrations (0 to 100 M  $\times 10^{-4}$ ).

characteristic of the native pre- $\alpha$  and  $\alpha_4$  crystallins.

Alternatively, calcium could be dialyzed out of both pre- $\alpha$  and  $\alpha_4$  calcium-induced aggregates. Again, with the removal of calcium, it was found that both solutions became clarified and the original  $D$  values were restored.

These findings imply that calcium is only weakly bound or acts indirectly to produce aggregation and that the calcium-induced aggregation can be reversed (at least in solution) by removal of the calcium ions.

## Discussion

The experimental findings enable us to draw a number of conclusions regarding the role of calcium aggregation of bovine crystallin lens proteins.

We have found that a concentration of calcium in excess of  $4 \times 10^{-3}$  M is capable of producing marked aggregation of soluble lens proteins. By treating solutions of each

of the lens protein fractions with calcium, we have determined that the soluble pre- $\alpha$  and  $\alpha_4$  crystallins are the only components specifically aggregated by calcium ions. The  $\beta$  and  $\gamma$  crystallins are substantially unaffected and aggregation of  $\alpha_2$  crystallin is apparently associated with disulfide bond formation and could be suppressed using  $\beta$ -mETOH.

The molecular weight of the aggregated  $\alpha$  crystallins is quite large and distributed over a wide range. Analytic ultracentrifugation and diffusion coefficient measurements indicate that the calcium-induced aggregates in solution range in molecular weight from approximately  $300 \times 10^6$  to greater than  $2 \times 10^9$  Daltons.

The index of refraction of these aggregates was measured and found to be  $n = 1.6$ . This is substantially different from the average index of refraction of the lens itself ( $n \cong 1.4$ ), thereby demonstrating that these aggregates would scatter light effectively if present in the lens.

It was found that the aggregation process could be reversed and the solutions of the aggregates made transparent by removal of calcium. The calcium was removed by treatment with either EDTA or EGTA. The latter is a more specific chelating agent for calcium. Alternatively, the calcium ions were moved by dialysis. In either process, measurement of the diffusion constant demonstrated that the protein aggregates dissociated and returned to the size of the native  $\alpha$  crystallins.

It is to be particularly noted that in distinction to the glucose-induced aggregates described by Spector and co-workers,<sup>12</sup> the aggregates in these experiments were produced in the absence of glucose and with the native  $\alpha$  crystallins. Furthermore, the levels of calcium needed for aggregation are within the range of calcium concentration known to occur in cataractous lenses.

It must, of course, be stressed that the increase of molecular weight found in these experiments is associated with calcium-induced aggregation of  $\alpha$  crystallin in

aqueous solution. The size of calcium-induced aggregates *in situ* may well be different and depend on a number of factors.

These findings give reason to believe that calcium-induced aggregates may play a role in reducing the transparency of the intact lens itself. Accordingly, control of the calcium level may permit inhibition of the aggregation process with the consequent maintenance of the transparency of the lens.

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