

High-molecular-weight (HMW) protein from human cataractous lenses, isolated by differential centrifugation, was deaggregated in 7M urea and then reaggregated in either the presence or absence of 10 mM CaCl₂. Over 90% of the material reaggregated in the presence of calcium appears to have a size greater than 50 × 10⁶ daltons. By contrast, only 20% to 25% of the material calcium appears to have a size greater than 50 × 10⁶ daltons. Disulfide formation during reaggregation is unlikely in the latter experiment, since the addition of 50 mM mercaptoethanol caused no change in results. About 60% to 70% of the low-molecular-weight (LMW) protein fraction deaggregated in 7M urea buffer can be converted to HMW species if the deaggregating agent is removed. However, only 5% to 10% of this protein is converted to HMW species if the deaggregation step is eliminated. Experiments with ⁴⁰Ca indicate that whereas calcium is necessary for the formation of the HMW aggregates, only one calcium per approximately 5 × 10⁶ daltons remains bound in the reaggregated material. The data suggest that although calcium may be required to induce aggregation to HMW species, it is not required to stabilize such macromolecules. SDS-polyacrylamide gel electrophoresis of the HMW species formed upon reaggregation of the dissociated HMW species with calcium indicates the presence of all the major polypeptide subunits of the original HMW species present in the lens; however, reaggregation in the absence of calcium yields HMW species lacking in the 9600 dalton component.

The experimental studies of Spector et al.¹ and the theoretical work of Benedek² clearly suggest that the generation of high-molecular-weight (HMW) aggregates in the lens may lead to lenticular opacity (see Harding and Dilley³ for additional references). It was shown by Jedzinski et al.⁴ that calcium can induce the aggregation of bovine lens alpha-crystallin to HMW aggregates. Spector et al.⁵ demonstrated that deaggregated bovine HMW alpha-crystallin reaggregates to HMW species upon removal of the deaggregating agent. However, if calcium is omitted, only low-molecular-weight (LMW) species are observed. Calcium is not effective in reaggregating dissociated LMW alpha-crystallin to HMW aggregates. It is probable that a cooperative reaction involving modified polypeptides and calcium is required to reconstitute the HMW alpha-crystallin.

Unlike the bovine lens, in which alpha-crystallin is the primary HMW species, in the human lens, polypeptides from all major proteins appear to be present in the HMW species.⁶ The deaggregated human HMW species have also been shown to reaggregate in the presence of calcium⁷ to HMW aggregates. In conjunction with these studies of the in vitro effects of calcium on the lens proteins, there also have been reports of the presence of higher levels of calcium in some cataractous lenses than in normal lenses. The results obtained from these studies have been summarized by Jedzinski et al.⁸ In a recent investigation of normal and cataractous human lenses, Jedzinski et al.⁹ concluded that the mean calcium concentration of cataractous lenses is two to 13 times higher than is normally found.

How calcium brings about this transformation of the deaggregated proteins to HMW aggregated forms and whether it is required for the stabilization of these giant macromolecules has never been clear. The present studies strongly suggest that although calcium induces aggregation of the deaggregated human lens proteins to giant aggregates, it is not required to stabilize the HMW aggregate structure.

Materials and methods. Cataractous human lenses ranging from color 1 to 5 and opacification 2 to 4 on the basis of the classification of Anderson and Spector⁴ were homogenized at a concentration of 0.7 to 1.0 gm of lens wet weight to 10 ml of 0.01M Tris, 0.1M KCl at pH 7.6. The HMW and LMW fractions were separated by the method of differential centrifugation.⁶ According to this procedure, the supernatant obtained after the first centrifugation step of the lens homogenate at 12,000 rpm for 15 min at the average centrifugal force of 9900 × g contains a mixture of HMW and LMW proteins. Further centrifugation of this su-
pernatant at 30,000 rpm for 30 min at an average centrifugal force of 59,400 × g yields the HMW protein as a pellet, and LMW proteins separate out in the supernatant. The pellet from the 59,400 × g centrifugation was suspended in deaggregation buffer (0.01 M Tris, 0.001 M 2-mercaptoethanol, 7 M urea at pH 7.6) at a protein concentration of 5 to 10 mg/ml and dialyzed in an 8000 dalton cutoff dialysis tubing (Arthur H. Thomas Co.) against the same buffer at a ratio of 1:500 (v/v) for 2 hr at room temperature, followed by overnight dialysis in fresh deaggregation buffer at 4° C. The dialysate was then divided into two parts. In the first part the effect of CaCl₂ was tested by dialyzing against a buffer containing 10 mM CaCl₂ in addition to 10 mM Tris and 1 mM 2-mercaptoethanol; the other part was used as a control and was dialyzed against 30 mM KCl, the concentration of KCl yielding the same ionic strength as 10 mM CaCl₂. After a 2 hr dialysis at 4° C the dialysis solutions were changed to salt-free Tris-mercaptoethanol buffer, pH 7.6. After removing the salts, the protein solution was fractionated on a Bio-Gel A-50m (100 to 200 mesh) column (1.5 cm by 36 cm), and an aliquot was centrifuged at 59,400 × g. The fractions were dialyzed against water at 4° C and lyophilized for subsequent analysis. The supernatant obtained from the 59,400 × g spin was divided into two parts. One part was subjected to the same deaggregation protocol as the pellet, whereas in the other part the effect of calcium without deaggregation was examined.

Radioactive ⁴⁵CaCl₂ was obtained from New England Nuclear Corp. and diluted to a specific activity in the range of 5 to 7 mCi/mM with non-radioactive CaCl₂. A 1 ml aliquot of deaggregated protein was placed in a dialysis casing to which 10 mM radioactive calcium was added. This preparation was dialyzed for 2 hr in the cold against 10 ml of 0.01 M Tris, 10 mM CaCl₂, 1 mM 2-mercaptoethanol at pH 7.6, which also contained labeled calcium with the same specific activity as in the preparation. In another series of experiments, the deaggregation protocol was modified by including 10 mM EGTA in the deaggregation buffer to more effectively remove any calcium from the protein preparation. This was followed by a further dialysis to remove the EGTA. Then the original reaggregation protocol using labeled calcium was followed. After a 2 hr reaggregation in the presence of labeled calcium, the preparation was usually dialyzed for 24 hr against several changes of the Tris buffer to remove nonassociated label.

The protein was then fractionated in the cold on the A-50m column. Aliquots of the eluted material were taken up in Aquasol II and analyzed with a Packard Tricarb liquid scintillation counter with a counting efficiency of 60% for ⁴⁵Ca. All results are corrected for background radioactivity. Aliquots of

![Fig. 1](https://example.com/fig1.png)

**Fig. 1.** Effect of calcium upon reaggregation of deaggregated human HMW lens protein. Profiles represent the elution of aliquots of protein from a Bio-Gel A-50m column. Vo, Void volume of the column; Ve, elution position of the LMW alpha-crystallin.
Molecular weight  $\times 10^4$

> 100

43

27

22

20

9.6

A  B  C

Fig. 2. SDS-polyacrylamide gel-electrophoresis. A, HMW protein isolated after reaggregation of the deaggregated HMW protein in the presence of calcium. B, HMW protein obtained upon reaggregation in the absence of calcium and isolated as the void volume fraction of the A-50m column (i.e., peak I). C, LMW protein obtained upon reaggregation of dissociated HMW polypeptides in the absence of calcium (i.e., peak II).

The percent recovery of various protein and polypeptide fractions is based on amino acid analyses. Amino acid analyses of the proteins and SDS-polyacrylamide gel electrophorises were performed as described previously. All water used in the experiments was deionized and distilled, and all urea solutions were passed through Amberlite MB-3 (Mallinckrodt Inc., Jersey City, N. J.).

**Results.** Human lens HMW material was isolated by differential centrifugation and passed through a Bio-Gel A-50m column. The material eluted in the void volume, indicating its size to be at least $50 \times 10^6$ daltons (Fig. 1, a). Deaggregation of this material followed by reaggregation in the presence of 10 mM calcium chloride, resulted in 80% of the protein re-forming HMW species (Fig. 1, b). When the reaggregation was carried out in the absence of calcium, only 20% to 25% of the material returned to HMW aggregates (Fig. 1, c). In order to determine whether HMW species obtained in the absence of calcium contained disulfide linkage, the deaggregation-reaggregation experiments were repeated in buffers containing 50 mM 2-mercaptoethanol instead of the usual 1 mM concentration. This increase in the concentration of 2-mercaptoethanol did not produce any change in the results. Therefore the possibility of these HMW species being disulfide-bound is highly unlikely.

When the LMW species were deaggregated and then reaggregated in the presence of 10 mM calcium, 60% to 70% could be pelleted at 59,400 $\times g$ indicative of the formation of HMW components. The remaining material eluted in the LMW range of the A-50m column. Deaggregation-reaggregation of the LMW species in the absence of calcium resulted in the reformation of only LMW species with the same elution profile as the original LMW material. Under nondeaggregating conditions, dialysis of the LMW protein (i.e., the 59,400 $\times g$ supernatant) for 2 to 48 hr against a buffer containing 10 mM calcium at 4°C, resulted in only 5% to 10% of the material pelleting at 59,400 $\times g$. When the remaining material was passed through a Bio-Gel A-50m column, it eluted in the LMW range. The percentage of the protein converted to the HMW material remained the same when dialysis was performed at room temperature.

SDS-polyacrylamide gel electrophoresis of the calcium-induced HMW aggregates (Fig. 2, A) show the presence of all the major polypeptide components present in the native HMW species. The noncalcium-reaggregated HMW species, on the other hand, did not appear to contain the 9600...
Table I. Distribution of $^{45}$Ca in fractions of reaggregated HMW material passed through a Bio-Gel A-50m column

<table>
<thead>
<tr>
<th>Fraction</th>
<th>cpm per fraction</th>
<th>Polypeptide per fraction (nmol)*</th>
<th>$^{45}$Ca per fraction (nmol)</th>
<th>Polypeptide $^{45}$Ca</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>4647</td>
<td>21</td>
<td>0.69</td>
<td>30</td>
</tr>
<tr>
<td>18</td>
<td>25,440</td>
<td>100</td>
<td>3.80</td>
<td>26</td>
</tr>
<tr>
<td>19</td>
<td>34,600</td>
<td>120</td>
<td>5.16</td>
<td>23</td>
</tr>
<tr>
<td>20</td>
<td>17,200</td>
<td>54</td>
<td>2.57</td>
<td>21</td>
</tr>
<tr>
<td>21</td>
<td>6200</td>
<td>22</td>
<td>0.93</td>
<td>24</td>
</tr>
<tr>
<td>22</td>
<td>2760</td>
<td>11</td>
<td>0.41</td>
<td>27</td>
</tr>
<tr>
<td>23</td>
<td>1800</td>
<td>9</td>
<td>0.25</td>
<td>36</td>
</tr>
</tbody>
</table>

*Based on amino acid analysis, assuming an average molecular weight of 20,000 d. for each polypeptide subunit.

Table II. Effect of EGTA-pretreatment upon $^{45}$Ca binding during aggregation

<table>
<thead>
<tr>
<th>EGTA pretreatment*</th>
<th>Protein (mg)</th>
<th>Polypeptide (nmol)</th>
<th>$^{45}$Ca (nmol)</th>
<th>Polypeptide $^{45}$Ca</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>4.5</td>
<td>225</td>
<td>8</td>
<td>28</td>
</tr>
<tr>
<td>+</td>
<td>6.7</td>
<td>336</td>
<td>14</td>
<td>24</td>
</tr>
</tbody>
</table>

*Dialyzed against a buffer containing 10 mM EGTA, prior to reaggregation (see text for details).

In order to determine the abundance of calcium in the deaggregated and then reaggregated HMW protein, experiments were performed with $^{45}$Ca. Following reaggregation, the protein was dialyzed to remove extraneous unbound $^{45}$Ca. In the final dialysate only 50 to 100 cpm/ml, above the background, were detected. Little $^{45}$Ca was found in the dialyzed reaggregated protein material. Furthermore, most of this label represented nonbinding $^{45}$Ca and eluted in the LMW region (Fig. 3) after fractionation on Bio-Gel A-50m. More extensive dialysis before column fractionations resulted in the detection of less nonassociated label. However, there was little alteration in the amount of label bound to the protein. A comparison of the specific activity of each fraction across the protein peak indicated some variation in the calcium associated with the HMW protein (Table I). Assuming an average subunit molecular weight of 20,000 daltons, one calcium per 21 to 36 subunits was derived, with an average of one calcium per 24 subunits, i.e., one calcium per approximately $5 \times 10^4$ daltons based on a subunit molecular weight of $2 \times 10^4$. If EGTA was utilized to remove bound calcium from the deaggregated protein before reaggregation in the presence of $^{45}$Ca, similar results were obtained, indicating that nonradioactive calcium is not blocking the binding of $^{45}$Ca (Table II).

Discussion. It appears clear from the experiments reported in this communication that little calcium (average approximately one calcium per 24 subunits) is associated with the reaggregated HMW protein. It is difficult to propose a system where such small quantities of calcium can be involved in stabilizing the aggregate structure. Furthermore, the variability in binding observed in this investigation suggests a nonspecific trapping of the calcium. Jedziniak et al. 8 on analysis of HMW protein isolated from cataractous lenses, also found a marked variation in calcium associated with the protein with minimal values in the order of 0.2 µg/mg or approximately one calcium per 10 subunits. Their results also suggest a wide variation in the degree of calcium trapped in the aggregate. The data are consistent with the viewpoint that calcium is probably not required for stabilization of the aggregate molecule although it appears to be necessary for inducing aggregation.

In the absence of calcium, only 20% to 25% of the dissociated HMW material reaggregates to a HMVV species. This HMW material is found lacking in the 9600 dalton polypeptide component (Fig. 2, B). The gel profile of the LMW protein fraction obtained after reaggregation in the absence of calcium also showed the presence of all the major polypeptide components, as shown in Fig. 2, C.

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In the absence of calcium, only 20% to 25% of the dissociated HMW material reaggregates to a HMVV species. This HMW material is found lacking in the 9600 dalton polypeptide component, present in the other HMW and LMW species reported in this paper (Fig. 2). It is possible that this abnormal HMW species represents a fraction of the native HMW species that may have remained undissociated in the deaggregation buffer used in this study.

Native or the calcium-reaggregated HMW material can be dialyzed against 10 mM EGTA without any significant change in size. However, Spector et al. 7 had reported earlier that some reversal of HMW to LMW species is possible. Of the various calcium-chelating agents tried by these inves-
tigators, penicillamine was the most effective and EGTA had almost no effect. It is quite likely that the role of penicillamine in this transformation may involve some other mechanism, not directly related to the removal of calcium.

Essentially the only effect of calcium on the LMW fraction of the soluble human lens proteins is observed after it is dissociated with urea. This would suggest that the LMW protein is constrained from aggregating either because the calcium nucleation sites are masked or because the overall structure of the material prevents a calcium-catalyzed reaction. Such conclusions suggest that a modification in tertiary and quaternary structure is required for a cooperative aggregation induced by calcium.

From the Biochemistry and Molecular Biology Laboratory, Department of Ophthalmology, College of Physicians and Surgeons, Columbia University, New York, N. Y. Supported by grants from the National Eye Institute, National Institutes of Health, Department of Health, Education and Welfare. Martin Fein is a recipient of a National Research Service Award, National Institutes of Health. Ajay Pande is a Fight for Sight Fellow.


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REFERENCES