It is now possible to separate alpha, beta, and gamma crystallins by a relatively simple procedure combining precipitation with zinc glycinate, and isoelectric precipitation followed by sephadex 75 chromatography. Paper electrophoresis and ultracentrifugal analyses indicate that the isolated crystallins are similar to material isolated by other procedures and contain only minor contaminants.

Calf lens protein components separated by DEAE chromatography have been related to the crystallins isolated by the new method. Gamma crystallin is the first fraction eluted from the column, followed by the beta crystallin components, and then the alpha crystallin subgroup. Since the DEAE separable components could thus be identified as members of the alpha, beta, or gamma crystallin subgroups, another method is provided for isolating the crystallins.

The properties of the crystallins obtained by the two methods were studied. Electrophoretic and ultracentrifugal analyses indicate that the alpha and gamma fractions isolated by DEAE chromatography are similar to crystallins isolated by the zinc glycinate procedure. However, the beta crystallin fractions isolated by the two procedures appear to have similar electrophoretic mobilities but differ in their sedimentation properties.

In 1894, Mörner demonstrated that the lens was composed of three soluble proteins: alpha, beta, and gamma crystallins. Up to the present time this classification together with the insoluble protein fraction, the albuminoid, has been used to describe the major groups of lens protein. Originally, alpha crystallin was isolated by isoelectric precipitation, beta, by salting out procedures, and gamma comprised the remaining soluble protein. The validity of this classification was later confirmed by electrophoresis at pHi 8.6. With this system, alpha crystallin was the fastest moving component, gamma crystallin the slowest, and beta crystallin of intermediate mobility. Ultracentrifugal studies appeared to confirm this simple classification. However, the application of newer methods of separating proteins has shown that lens protein may be composed of ten or more components.

It is important to establish the relationship between the newly discovered components and the classical crystallin fractions. A serious obstacle to the clarification of this problem was the lack of a simple preparative method for isolating relatively pure fractions of the three crystallins. For example, the isolation of pure alpha and...
beta crystallins requires numerous reprecipitations during which considerable denaturation occurs. Two new methods have aided in overcoming former difficulties. Björk found that gamma crystallin could be separated from the remaining soluble protein by passage through a sephadex 75 column, and, in this laboratory, preliminary experiments by Kinoshita suggested that beta crystallin could be selectively precipitated from cattle cortex with zinc glycinate. The combination of these two methods in conjunction with the isoelectric precipitation of alpha crystallin provides a simple scheme for isolating the three crystallins from calf lens in a relatively pure state. With this new method it is now possible to correlate the relationship between the crystallins and the multiple protein components obtained by DEAE chromatography.

Methods

Calf eyes were obtained from a local abattoir. The lenses were removed within a few hours after slaughter and were used immediately or frozen until needed. After homogenizing the lenses in cold water (one gram of lens, wet weight, in a total volume of 5 ml.), the homogenate was centrifuged in a 4° C. cold room at 15,400 x G for 10 minutes and the insoluble material discarded.

Aliquots of 5 to 10 ml. were treated in the following manner. To each 5 ml. of the cold supernatant, 1 ml. of 0.1M Tris, pH 7.3, was added. This was followed by the addition, with vigorous stirring of 120 µl. of a pH 7.4 solution containing 0.173M zinc glycinate and 0.1M glycine. After cooling the solution for 5 minutes in a 10° C. water bath, 0.5 ml. of cold ethanol was added. Following an additional 5 minutes in the 10° C. water bath, the solution was centrifuged in the cold room at 15,400 x G.

The supernatant fluid containing the alpha crystallin was rapidly brought to pH 5.0 with 2N HCl, while stirring vigorously. After 5 minutes in a 10° C. bath, the solution was centrifuged at 15,400 x G for 5 minutes. Following decantation of the supernatant fluid, the alpha precipitate was suspended in 2 ml. of cold 0.1M KCl and brought into solution by the slow addition of 0.1M NaOH, until the pH was 7.4. The turbid solution was then stirred in a 4° C. cold room for 20 minutes to facilitate solubilization. The slight sediment remaining was removed by centrifugation.

The zinc glycinate precipitate containing the beta-gamma fraction was redissolved with 200 µl of 0.1M EDTA, pH 7.3, and 1 ml. of cold 0.1M Tris, pH 7.3, after which 4 ml. of cold 0.1M KCl was added. The pH was then adjusted to 7.40, and 120 µl. of the zinc glycinate solution was added while stirring. After 10 minutes in a 10° C. bath, the solution was centrifuged in the usual manner. Upon the complete removal of the supernatant fluid, the beta-gamma precipitate was dissolved with 0.4 ml. of 0.1M EDTA, pH 7.3. A 1.3 ml. solution containing 0.05M KCl and 0.005M Tris, pH 7.3, was then added and the small remaining precipitate was centrifuged off. The beta and gamma crystallins were then separated in the cold room with a sephadex 75 column. A modification of Björk's procedure for the separation of gamma crystallin from soluble lens protein was used. Up to 14 ml. of the redissolved beta-gamma fraction was added to a sephadex 75 column (3.2 x 90) prepared in the usual manner. The material was eluted with 0.05M KCl, 0.005M Tris, pH 7.3. The separated crystallins were then dialyzed for 8 hours against a fiftyfold excess of cold water and finally lyophilized. The fractionation procedure is summarized in Fig. 1.

The chromatography was followed by automatically scanning the eluted fluid at 280 nm. A DB spectrophotometer was simply modified by drilling holes in the sample cover and through the lower right side of the sample compartment. The column was connected to the spectrophotometer with narrow gauge Teflon tubing, PF No. 16,* which passed through the side of the instrument. The tubing was connected to a 4.5 cm. piece of silica tubing (O.D. 5 mm., I.D. 3.3 mm.) mounted in an aluminum housing containing a slit 2 mm. wide and 6 mm. long. The slit was centered to allow the 280 nm light to pass through the tubing to the photo cell. Another piece of Teflon tubing was used to lead the effluent fluid from the silica cuvette to the fraction collector. The spectrophotometer and fraction collector were connected to a recorder which continuously monitored the absorption and indicated the fraction being collected.

Paper electrophoresis was performed at pH 6.6 with 0.05M Veronal buffer. The paper strips were developed according to the procedure of Block. A Spinco model E analytical ultracentrifuge was used to obtain the sedimentation data. All sedimentation coefficients were corrected to 20° C. Slight modification of a previously reported method was used to fractionate lens protein on DEAE cellulose. See text for further details.

*Obtained from the Pennsylvania Fluorocarbon Company, Clifton Heights, Pa.
Results

The results obtained from the fractionation of soluble lens protein with zinc glycinate are shown in Table I. About 23 per cent of the original material was isolated in the alpha fraction by precipitation at pH 5.0, and 50 per cent was recovered in the beta-gamma fraction from the second zinc glycinate precipitation. Thus, the total recovery was 73 per cent. The effectiveness of the separation of alpha crystallin from the beta-gamma fraction becomes apparent when the sulfhydryl values of these fractions are compared. Beta and gamma crystallins are known to have about three times as many sulfhydryl groups as alpha crystallin. The sulfhydryl values shown in Table I suggest that this procedure gives the expected results. Possibly, this distribution of sulfhydryl groups is the basis of the zinc glycinate fractionation, that is, the zinc reacts with the sulfhydryl groups, reducing the solubility of the sulfhydryl-rich fractions.

The crystallins were identified by electrophoresis at pH 8.6, as shown in Fig. 2.

![Diagram of the fractionation procedure of lens protein.](image-url)
Table I. Fractionation of lens protein with zinc glycinate

<table>
<thead>
<tr>
<th>Material</th>
<th>Milligrams</th>
<th>Per cent recovery</th>
<th>P-moles SH/Gm.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original homogenate</td>
<td>1,092</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 5 precipitate</td>
<td>260</td>
<td>23</td>
<td>30</td>
</tr>
<tr>
<td>First zinc glycinate precipitate</td>
<td>688</td>
<td>64</td>
<td>86</td>
</tr>
<tr>
<td>Second zinc glycinate precipitate</td>
<td>552</td>
<td>50</td>
<td>95</td>
</tr>
</tbody>
</table>

The method of Grunert and Phillips was used to determine the sulfhydryl groups.

*Based on a glutathione standard.

The upper picture illustrates the electrophoresis of the soluble protein of a dialyzed calf lens homogenate. The most rapidly moving component is the alpha crystallin followed by the beta and gamma crystallins, respectively. The next electrophoretograms, B and C, illustrate the results obtained with the zinc glycinate precipitates. An apparent alpha contaminant which remains after the first precipitation has been greatly reduced in the course of the second precipitation. The following electrophoretogram represents the material obtained from the redissolved pH 5.0 precipitate. This material appears to be a pure alpha crystallin.

The zinc glycinate precipitation was followed by column chromatography on sephadex 75 to separate beta and gamma crystallins (Fig. 3). Beta was the first component eluted followed by the gamma fraction. The recovery of material from the column was 94 per cent. To eliminate the possibility of overlapping of the separated materials, fractions 51 to 61, which contained little material, were discarded. After separation, each pooled fraction was dialyzed and lyophilized, and then examined by paper electrophoresis. The two lower electrophoretograms in Fig. 2 show that while the beta fraction still has a slight alpha contaminant, the gamma component appears to be pure.

A more critical evaluation of the purity of the three fractions was achieved by ultracentrifugal analysis. Conditions similar to those already reported in the literature were used for the studies on the alpha and gamma crystallins. The ultracentrifugal patterns shown in Fig. 4 indicate that the gamma component appears to be free of contaminants and has an $S_{w0}$ of 2.4, which is in excellent agreement with results obtained by Björk.

The alpha and beta fractions were analyzed in a wedge cell with the alpha component in the upper section (Fig. 4).
While the alpha material appears free of any significant beta contaminant, it does contain an apparent impurity in the gamma region of approximately 10 per cent. Studies reported further on in this paper indicate that this material is not a gamma crystallin fraction but is probably a true alpha crystallin component. An S_{20w} of 19.7 was calculated for the major alpha fraction. This is in agreement with observations made by Resnik.\textsuperscript{7} Further analyses of the alpha peak indicate that it is asymmetrical and may contain a heavier component which possibly represents as much as 15 per cent of the total peak.

The beta fraction is composed of a number of components. The three major peaks have S_{20w} values of 5.0, 9.2, and 14.5. Although there is no evidence of a gamma impurity, the asymmetry of the fastest peak indicates a trace of a heavier contaminant.

Having obtained the crystallins in a reasonably pure state, we attempted to correlate them with the lens protein fractions isolated from DEAE chromatography. Fig. 5 shows the many components which are obtained from DEAE fractionation of lens protein. Since the soluble protein of one calf lens had been previously used, the amount of each crystallin added to the column was equal to that found in a single lens. The results are shown in Fig. 6. After addition of the isolated gamma crystallin to the DEAE column, 96 per cent was recovered in the breakthrough peak and 4 per cent in the 0.015M peak. The over-all recovery was 97 per cent.

Chromatography of the isolated beta crystallin indicated that no material could be removed with the starting buffer. Thirty-three per cent of the recovered material was eluted with 0.015M buffer, 42 per cent with the 0.03M buffer, and 11 per cent at 0.05M. Thus, 86 per cent of the recovered beta crystallin was eluted with the three buffers. The over-all recovery of the beta crystallin from the column was 82 per cent. With alpha crystallin, 88 per cent of the material recovered from the DEAE column was eluted with buffers of 0.08M or higher salt concentration. In all, 94 per cent of the material added to the column was recovered in this case.

![Fig. 3. Separation of the beta-gamma fraction on sephadex 75. The figure illustrates the data obtained from analysis of the eluant solution with the scanning system described in Methods.](image-url)
Fig. 4. Ultracentrifuge diagrams of the crystallins isolated by the zinc glycinate procedure. Alpha and beta crystallins were examined in a wedge cell at a speed of 56,100 r.p.m. and 20° C. The picture was taken 29 minutes after attaining operating speed. Alpha was in a 0.2M KCl, 0.01M Tris, pH 7.45 solution. Beta was in a 0.1M PO₄ buffer, pH 7.08. The gamma material was centrifuged at 56,100 r.p.m. in a 0.1M PO₄ solution, pH 7.10, at 14° C. The picture was taken 37 minutes after attaining operating speed.

Fig. 5. Chromatographic pattern obtained when 250 mg. of soluble calf lens protein was added to a 16 g. DEAE cellulose column. The flow system described in Methods was used. The per cent transmission read-out was changed to absorbancy units to facilitate comparison of different peaks. Stepwise elution was performed by successive application of buffers prepared from 0.500M phosphate, pH 6.80, as indicated in the figure. The final eluting solution contained 0.20M NaH₂PO₄ and 0.2M NaCl.
Thus it is clear that only gamma crystallin was eluted with the starting buffer. The four per cent of the gamma crystallin eluted with the 0.015M buffer is probably a contaminant which was not observed in the ultracentrifugal and electrophoretic systems. Although the beta crystallin does not seem to have any gamma contaminant, 15 per cent of the beta material appeared in the region where the bulk of the alpha crystallin was eluted. Paper electrophoresis and ultracentrifugal analysis of the isolated beta crystallin material also indicated a minor component which was similar to

![Chromatographic Pattern](image1.png)

**Fig. 6.** Chromatographic pattern obtained from alpha, beta, and gamma crystallins previously isolated by the zinc glycinate procedure. The initial buffer was 0.002M PO₄, pH 7.0. See text and Fig. 1 for further details.

![Bar Graph](image2.png)

**Fig. 7.** Bar graph depicting the distribution of lens protein components obtained from DEAE chromatography. The per cent of the material added to the column isolated with each buffer is indicated on the ordinate axis.
alpha. As for alpha crystallin, although 12 per cent of the isolated protein was eluted with the 0.05M buffer, electrophoretic and ultracentrifugal analyses of the starting material produced no evidence of a beta contaminant. Moreover, while ultracentrifugal analysis of the isolated alpha protein indicated a component which sedimented in the gamma region, no alpha material was removed from the column before elution with the 0.05M buffer. It therefore does not appear likely that the isolated alpha crystallin is contaminated with gamma crystallin.

The chromatographic analyses are summarized in Fig. 7. With the exception of the 0.05M component, all DEAE fractions can be reasonably assigned to one of the crystallins. Furthermore, the components arising from each of the crystallins are grouped in an orderly sequence with the gamma crystallin leading, followed by the beta subgroup, and finally the alpha components. It should therefore be possible to fractionate soluble lens protein into the classical alpha, beta, and gamma crystallins by DEAE chromatography.

The results of such a fractionation are shown in Fig. 8. The 0.05M material has been assigned to the beta group, since ultracentrifugation gave an $S_{20w}$ of 14.1, a value which falls in the beta class. Alpha crystallin was eluted from the column with 0.4M buffer rather than the $\text{PO}_4$-NaCl buffer, since the latter buffer causes irreversible insolubilization. With this procedure approximately 30 per cent of the alpha crystallin remains on the column.

Thus, by fractionation with DEAE cellulose, 20 per cent of the starting material was isolated as gamma crystallin, 52 per cent as beta crystallin, and 13 per cent as alpha crystallin. The material was eluted with the aid of nitrogen pressure at the rate of approximately 240 ml. per hour. The entire fractionation procedure required only 5 hours.

After the pooled fractions were dialyzed and lyophilized, they were examined by paper electrophoresis and ultracentrifugal analysis, as shown in Figs. 9 and 10. Paper electrophoresis revealed that the alpha and gamma fractions contained no impurities, and gave the mobilities associated with the classical crystallins. The beta fraction also had the expected mobility, but, as in the zinc glycinate procedure, had a component which migrated to the alpha position. It should be noted that paper electrophoresis of the 0.05M fraction under similar conditions gave a mobility between the major beta and alpha components. Thus on the basis of paper electrophoresis at pH 8.6, no differences can be detected between the beta crystallin isolated by the zinc glycinate procedure and by DEAE chromatography.
The results obtained from ultracentrifugal analyses of these column fractions are shown in Fig. 10. Examinations of the gamma and alpha components under conditions similar to those used in the zinc glycinate studies gave essentially the same results. The gamma fraction had an $S_{20,w}$ of 2.7 and appeared homogeneous. A $S_{20,w}$ of 19.7 was again found for the alpha fraction. A very light component and asymmetry on the frontal boundary of the major alpha peak were again observed.

However, analyses of the ultracentrifugal patterns of the beta fraction gave only two major components with $S_{20,w}$ values of 4.8 and 18.6. The major component obtained from the zinc glycinate beta preparation, $S_{20,w}$ of 9.2, was no longer observable. Thus the alpha and gamma fractions obtained from zinc glycinate or DEAE procedures are similar by electrophoresis and sedimentation analyses while the beta fraction appears identical to only paper electrophoresis.

**Discussion**

The fractionation of soluble calf lens protein by zinc glycinate and sephadex 75 offers a number of advantages. Gram quantities of relatively pure alpha, beta, and gamma crystallins can be obtained within 24 hours. Furthermore, sedimentation analysis and paper electrophoresis indicate that no significant alterations have occurred during the purification. Recoveries of 70 per cent or better are uniformly obtained.

The elution of soluble lens proteins from DEAE cellulose has been found to give the following pattern: Components with low molecular weight and low electrophoretic mobilities at alkaline pH are eluted first followed by larger components with higher electrophoretic mobilities. Not only
do the alpha, beta, and gamma crystallins follow this pattern but the reported experiments suggest that the components comprising the beta and alpha groups are also eluted in this manner. It should be noted that Papaconstantinou and co-workers\textsuperscript{16} have also observed an increase in electrophoretic mobility with succeeding fractions eluted from DEAE columns. Thus, with the appropriate eluting buffer, any of the three crystallins or components of the crystallins can be isolated in 3 hours from a dialyzed lens homogenate.

The alpha and gamma crystallins isolated in this manner appear similar to material isolated by the zinc glycinate procedure. However, the beta crystallins isolated by the two methods differ in their ultracen-

Fig. 10. Ultracentrifuge diagrams of the crystallins isolated from DEAE chromatography. The fractions were studied in solutions similar to those indicated in Fig. 4. The conditions used were as follows: alpha, 52,640 r.p.m., 20° C, picture taken 9.5 minutes after attaining operating speed; beta, 56,100 r.p.m., 20° C, picture taken 16 minutes after attaining operating speed; gamma, 56,100 r.p.m., 20° C, picture taken 59 minutes after attaining operating speed.
trifugal profiles. In the chromatographic procedure the beta components are pulled apart by the exchanger and then recombined during the concentration procedure. Apparently, the reformed aggregates are not similar to the original beta crystallin. However, the same beta DEAE components can be obtained from chromatography of material arising from either isolation procedure.

It is difficult to assess the chromatographic homogeneity of the alpha, beta, and gamma crystallins obtained by the zinc glycinate procedure or by DEAE fractionation. A relatively symmetrical elution pattern of a particular DEAE component is not an absolute indication of purity. More than one protein may have the same chromatographic characteristics. Occasionally, rechromatography of isolated material was used to verify the validity of the original results. While this approach did not reveal any unexpected contamination of the DEAE isolated components, an interesting transformation was noted. When the beta group is rechromatographed, 80 to 85 per cent is eluted in its original position and 10 to 15 per cent of the material is shifted toward the alpha region. A second rechromatography of the major beta material again gives the same results. Preliminary ultracentrifugal results suggest an increase in the sedimentation constant of the altered material. Thus it seems clear that there is an instability in the components of the beta crystallin which may result in the transformation of some of their characteristics to those of alpha crystallin components. A similar kind of instability can also be observed with the alpha crystallin group.

Instability of isolated lens protein fractions has been observed before. However, transformations such as those noted above have not been previously observed. Similar changes may slowly occur in the intact lens. Now another change observed with the beta and alpha components during the procedures noted in this paper is that of insolubilization. Possibly this insoluble material may be related to the insoluble lens albuminoid fraction which has been shown to increase slowly with age.

Preliminary observations obtained in this laboratory and experiments reported by Bloemendal clearly indicate that lens proteins are complicated aggregates which can be broken down into small subunits ($S_{20w}$ 1.8) in the presence of 6M urea. Experiments to be reported at a later date suggest that all DEAE components other than the gamma fraction can be broken down in this manner. The relationship of these subunits to the alpha, beta, and gamma crystallins is not clearly understood. It cannot be ascertained at this time whether the classical crystallin fractions are simply an operational delineation of lens protein or are in fact physiologically meaningful protein aggregates. However, it is now clear that beta and alpha crystallins can be fractionated into a number of components, each of which is an aggregate of smaller subunits. As for gamma crystallin, Björk has shown that this is also a heterogeneous material, although experiments in this laboratory suggest that it is not composed of subunits dissociable by urea.

We are indebted to Dr. Elton Katz for his assistance with the ultracentrifugal experiments. We also wish to thank Dr. Jin Kinoshita for discussions regarding this study. The technical assistance of Mary Pruyn is gratefully acknowledged.

REFERENCES
Separation of alpha, beta, and gamma crystallins


