Alpha, beta, and gamma crystallins in the ocular lens of rabbits: Preparation and partial characterization

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Alpha, beta, and gamma crystallins from rabbit eye lens have been prepared by continuous-flow paper electrophoresis and gel filtration. These methods yielded well-defined fractions in a highly reproducible manner with essentially quantitative recovery of material. The behavior of each of the crystallins in diethylaminoethyl cellulose has been examined. Sulfhydryl contents of 2.9, 6.1, and 26.0 moles per 10^5 Gm. of protein were found for alpha, beta, and gamma crystallin, respectively. These values were altered to 5.9 and 8.5 for alpha and beta crystallin in 8 M urea, but that for gamma crystallin was unchanged. Extinction coefficients, E^25^0 of 8.3, 21.5, and 17.8 were found for alpha, beta, and gamma crystallin, respectively. Sedimentation constants of 18.9 S and 2.5 S were calculated for alpha and gamma crystallin, respectively. Glycine was found to be the N-terminal amino acid of gamma crystallin. The presence of free N-terminal amino acids for alpha and beta crystallins could not be demonstrated. For these parameters, the characteristics of the rabbit lens crystallins are similar to those of bovine origin.

The soluble eye lens proteins were operationally defined into three fractions which Mörner termed alpha, beta, and gamma crystallins. The crystallins were prepared by methods dependent upon their characteristic solubilities. Recently, several different methods for the fractionation of the soluble lens proteins have been developed. Some of these methods have pertained to only one of the crystallins; other methods have concerned the preparation of all three crystallins. In addition, each crystallin has been shown to consist of a number of closely related proteins. However, precise information concerning the number of structurally distinct proteins in each crystallin, their stoichiometry, and the nature of their interactions has not been obtained.

Because of the complex nature of the crystallins, some investigators have deemed it desirable to secure initial fractionation of the soluble lens proteins into the three classical fractions before undertaking detailed physicochemical studies of these crystallins.
proteins.\textsuperscript{6, 8} To be meaningful, such studies require preparative procedures providing clear separation and quantitative recovery of each crystallin. Several of the recently reported methods for the preparation of soluble lens proteins have been evaluated by us for this purpose. The ability of these methods to fulfill the required criteria successfully is quite variable. Therefore, we have developed a scheme for achieving the desired fractionation utilizing continuous-flow paper electrophoresis and gel filtration. These methods yield well-defined fractions in a highly reproducible manner with essentially quantitative recovery of material.

The fractions obtained by this combination of methods are not affected by the individual methods as judged by submitting each crystallin to refractionation. This consideration is pertinent because of the complex and somewhat unstable nature of isolated alpha and beta crystallins. Thus, Spector\textsuperscript{7} reported the "transformation" of beta crystallin to alpha crystallin and vice versa upon rechromatography on diethylaminoethyl cellulose. Unfortunately, other preparative procedures have not been evaluated for this effect.

**Experimental and results**

Eyes from rabbits 8 to 12 weeks old were obtained from a local abattoir. The eyes were enucleated within one hour following death and kept in normal saline until excision of the lenses. The excised lenses were rinsed in distilled water, decapsulated, placed in 0.1 M phosphate buffer at pH 6.8, and slowly stirred mechanically. Approximately 1 ml of buffer per lens was used for this procedure. The stirring was continued until only intact nuclei remained. The nuclei were then removed and discarded. The cortical suspension was homogenized in an all-glass Potter-Elvejhem homogenizer to complete disruption of fibers. The debris was removed by centrifugation at 12,000 \( \times \) g for 30 minutes and the supernatant fluid collected. All procedures prior to centrifugation were carried out at room temperature; subsequent manipulations were performed in the cold.

Protein concentrations were determined by measuring the optical density at 280 m\( \mu \) except where otherwise indicated.

**Sephadex G-75 Chromatography.** The gamma-crystallin fraction of the soluble rabbit lens proteins was obtained in the same manner as that used by Björk for calf lens.\textsuperscript{4} Volumes of 20 to 60 ml of lens extract were percolated through a 7.7 by 100 cm column of medium-grade Sephadex G-75, with 0.1 M phosphate at pH 6.8 as eluant. Buffer flow rates of about 4 ml per minute were employed. Twenty milliliter fractions were collected and analyzed for protein content. Recoveries were 95 per cent or better in all runs made. The elution profile for the rabbit lens proteins was in very good agreement with that for calf lens.

**Continuous-flow Electrophoresis.** The contents of the tubes found to contain the unsieved component from the Sephadex G-75 chromatography were pooled and the volume reduced either by ultrafiltration or ammonium sulfate precipitation. The solvent of the concentrated protein solution was changed by dialysis to 0.02 ionic strength Tris-HCl, pH 8.1. After thorough dialysis, the solution was subjected to Beckman/Spinco Model CP Continuous-Flow Paper Electrophoresis with a constant current of 60 Ma at 640 volts. This procedure yielded satisfactory separation of the alpha and beta fractions (Fig. 1) with approximately 95 per cent recovery of material.

Electrophoresis was carried out on a sample of the original lens protein extract of both rabbit and bovine lenses in order to compare results with those of Wood and associates\textsuperscript{13} and Manski and associates.\textsuperscript{11} The results as presented in Fig. 2 showed three components to be present in the original extracts with curtain electrophoresis. By definition, these components represented alpha, beta, and gamma crystallins proceeding from the fastest migrating to the slowest, respectively.\textsuperscript{15} Some variation...
Alpha, beta, and gamma crystallins in rabbit lens

Fig. 1. Continuous-flow paper electrophoresis of alpha and beta crystallin. Electrophoretic conditions were: 0.02 ionic strength Tris-HCl; pH, 8.1; wick and overflow settings, 9.0; sample feed rate, 0.7 ml. per hour; constant current of 60 Ma. at 640 volts; protein concentration, 3 per cent. The arrows indicate the portions of each peak collected for subsequent study. The fraction of intermediate mobility may be further resolved by a second electrophoresis. The cathode is at the right.

Fig. 2. Continuous-flow paper electrophoresis of soluble rabbit lens proteins. The electrophoretic conditions were the same as those in Fig. 1 except for a sample feed rate of 0.2 ml. per hour. Very similar results were obtained with soluble bovine lens proteins. The cathode is at the right.

in the electrophoretic profile was obtained from one run to another, but the same general features were always present. Failure to dialyze the extract thoroughly, for example, caused an altered profile, presumably due to the presence of low molecular weight components.

Wood and associates, using rabbit lens proteins, found five essentially homogenous components. The discrepancy with our results may be due to differences in experimental conditions; e.g., Wood and associates used 0.02 ionic strength Veronal buffer, pH 8.9, and a sample feed rate of 3.2 ml. per hour, which is extremely high according to our experience. Manski and associates, investigating bovine lens proteins, obtained two components with indications of the presence of a third component. A shift in the resolution pattern with time was encountered. We have not found this phenomenon, and we suggest that the previously reported instability may be due either to the use of phosphate buffer at pH 8.6, which is considerably removed from the pK for this buffer, or to the use of constant voltage rather than constant current during the electrophoresis.

Since our results did not agree with previously reported findings, we investigated the conditions of electrophoresis to find those giving the optimum reproducible resolution of components. Initially, satisfactory matching of the flow rate through the wicks and curtain could not be obtained. This difficulty was overcome by wrapping the wicks in cellophane to make the flow rates essentially independent of each other. The overflow tube, which controlled the rate of flow of solvent down the curtain, was set at a reading of 9.0 on the scale provided. This reading represented a compromise between the rate of buffer flow (and therefore the time during which the protein solution is subjected to electrophoresis) and the rate at which the protein solution may be fed onto the curtain, which may affect the resolution if too high. A setting of 9.0 resulted in an excursion time down the curtain of 3.5 ± 0.25 hr. and a maximum sample feed rate of 0.8 ml. per hour.

Investigation of the effect of pH showed similar results at pH 6.95 (0.02 M potas-
sium phosphate, 70 Ma. at 540 volts) and pH 8.9 (0.02 ionic strength Tris-HCl, 60 Ma. at 630 volts) to those at pH 8.1. The latter pH was chosen because it represents the pK for Tris buffer and therefore its region of greatest stability. Electrophoresis at pH 8.1 with Tris-HCl buffer of 0.01 ionic strength showed considerably less separation of components than at 0.02 ionic strength. Electrophoresis at 80 Ma. and 770 volts gave the same results as 60 Ma. and 640 volts, but was discarded because of increased heating at the higher current.

Protein solutions up to about 5 per cent concentration were subjected to electrophoresis without any indication that this parameter influenced the separation.

**Ion-exchange chromatography of alpha, beta, and gamma crystallins.** Papaconstantinou and associates and Spector have shown that chromatography of bovine lens proteins on diethylaminoethyl cellulose yields more fractions than the three classical crystallins. Spector, moreover, has correlated the chromatographic elution position of the various components with their respective classical fractions and has described a chromatographic procedure for the isolation of each of the crystallins. We used this procedure with rabbit lens proteins and obtained the results presented in Fig. 3. Comparison of the elution profiles for rabbit and bovine lens protein fractions showed a general agreement with respect to the ionic strength necessary to elute each major fraction; e.g., gamma crystallin was largely unretained at pH 7.0, 0.002 M phosphate. In the case of rabbit lens proteins, there is sufficient overlap of the conditions necessary for elution of alpha and beta crystallins to preclude complete separation of these two fractions.

Spector noted that rechromatography resulted in transformation of some characteristics of beta crystallin to those of alpha crystallin, and vice versa. We have verified this finding with respect to rabbit lens beta crystallin. On the other hand, Spector reported a rather uniform 10 to 15 per cent transformation per passage, whereas we found the percentage of transformation to be quite variable. Rabbit lens alpha and beta crystallins were examined regarding this tendency toward instability of the isolated fractions by subjecting previously fractionated material to a second electro-
phoresis. The results showed no evidence of transformation of one isolated fraction to the other.

**Ultracentrifugation of alpha and gamma crystallins.** Alpha and gamma crystallins were examined in the Spinco Model E analytical ultracentrifuge and their sedimentation coefficients determined. Examples of the schlieren patterns are presented in Fig. 4. The alpha crystallin fraction consisted of a major heavy component and a very minor light component. It is difficult at the present time to exclude the possibility of the minor component being a constituent of the alpha crystallin fraction, since there is substantial evidence that this fraction consists of aggregates of protein molecules.\textsuperscript{5, 9, 17} Gamma crystallin appeared to be homogeneous in the ultracentrifuge, in agreement with the findings for bovine gamma crystallin.\textsuperscript{6, 7} However, other criteria indicate heterogeneity.\textsuperscript{4, 11}

Sedimentation coefficients ($s_{20, w}$) were determined by making the appropriate measurements from a series of photographic plates using a microcomparator. Calculations of the sedimentation constants from these measurements were made according to Schachman.\textsuperscript{14} Sedimentation constants of $18.9$ S and $2.5$ S were found for alpha and gamma crystallin, respectively. The sedimentation coefficient for alpha crystallin was characterized by an inverse dependence on protein concentration while that for gamma crystallin was independent of protein concentration.

Beta crystallin was polydisperse in the ultracentrifuge.

**Extinction coefficients of the lens protein fractions.** The extinction coefficient

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**Fig. 4.** Ultracentrifuge patterns of alpha (A) and gamma (B) crystallins. The conditions used were: $0.1$ M phosphate, pH $6.8$, $20^\circ$ C., and $59,780$ r.p.m. Alpha crystallin picture, taken 11 min. after attaining operating speed with protein concentration, $6$ mg. per milliliter (upper trace) and $15$ mg. per milliliter. Gamma crystallin picture, taken 143 min. after attaining operating speed with protein concentration, $13.3$ mg. per milliliter. Direction of sedimentation is to the left.
for a 1 per cent protein solution at 280 m\(\mu\), \(E_{280}^{1%}\), was determined for each of the crystallins. The absorption at 280 m\(\mu\) for selected dilutions of concentrated stock solutions of each of the crystallins was measured using a Beckman Model DU spectrophotometer and 1 cm. cells. Beer’s law was obeyed up to at least 2.0 optical density in each case. Alpha crystallin concentrations were determined with a Brice-Phoenix differential refractometer at 546 m\(\mu\). The coefficient of refractive index increment, \(dn/dc\), for alpha crystallin was assumed to be the same as that for bovine serum albumin (19). The concentrations of the beta and gamma crystallin stock solutions were determined by the dry weight method. Extinction coefficients of 8.3, 21.5, and 17.6 were found for alpha, beta, and gamma crystallins, respectively. These values may be compared with 8.0 and 8.3\(^{29}\) and approximately 21\(^{11}\) for bovine alpha and gamma crystallins, respectively.

**Sulfhydryl contents of the protein fractions.** Sulfhydryl contents of the three fractions were measured spectrophotometrically\(^{21}\). The results, expressed as moles of —SH/10\(^6\) Gm. of protein, were 2.9, 6.1, and 26.0 for alpha, beta, and gamma crystallins, respectively. These measurements were repeated for the urea-denatured proteins in order to ascertain the influence of structure upon the availability of reactive —SH groups. Sufficient solid urea was added to the initial reaction mixtures to yield a final urea concentration of 8 M. This procedure gave values of 5.9 and 8.5 for alpha and beta crystallins. Urea denaturation did not alter the number of available —SH groups of gamma crystallin.

**Amino-terminal Amino Acids.** Each of the crystallins was subjected to N-terminal amino acid analysis. The dinitrofluorobenzene and phenylisothiocyanate methods were used for these investigations\(^{22}\). Bovine serum albumin was included in these experiments as a control. Glycine was found to be the only N-terminal amino acid of gamma crystallin by both methods. Alpha and beta crystallins, however, yielded only trace amounts of the dinitrophenyl and phenylthiohydantoin derivatives of various amino acids, predominantly glutamic acid and glycine. Variation of experimental conditions; e.g., prolonged reaction with dinitrofluorobenzene, changes in hydrolysis time, and so forth, did not appreciably alter these results.

**Discussion**

The soluble lens proteins have been shown conclusively to consist of aggregates of subunits in the case of alpha and beta crystallins\(^{5, 7, 9, 10, 17, 20, 23, 21}\) and a family of closely related proteins in the case of gamma crystallin\(^{11}\). The subunits of alpha crystallin, particularly, have been shown to interact under a variety of conditions; e.g., upon the removal of urea after dissociation\(^{24}\). Moreover, the crystallins, as usually defined, also interact and are mutually influential. Thus, Woods and Burky\(^{25}\) reported for the first time the observation that beta crystallin is unstable in the absence of alpha crystallin. In view of these considerations, investigators examining the soluble lens proteins have considered a preliminary fractionation of these proteins to be a desirable first step\(^{6, 8}\).

The combination of gel filtration and continuous-flow paper electrophoresis provides a procedure for obtaining the lens crystallins as operationally well-defined fractions suitable for further physicochemical analysis. This procedure offers an acceptable alternative to other procedures for obtaining the lens crystallins and has certain desirable features: (1) there is high recovery of fractionated material; (2) there is reproducible fractionation of gram quantities of material (typically 2 to 4 grams); (3) the separated proteins are obtained as their classically defined fractions; (4) extremes of pH are avoided; and (5) artificial changes in the separated components due to the fractionation methods are apparently absent.

Our results are in general agreement with previously published results concerning
lens proteins. A comparison with the findings of Wood and associates is difficult to make except in the case of their fraction 5 (the fastest migrating). They reported a sedimentation coefficient of 19.20 S, which compares closely to 18.9 S for the fastest moving component found in the present work. The ultracentrifugal data for rabbit lens alpha and gamma crystallins agree rather closely with those found for bovine lens alpha and gamma crystallins. The relatively wide range of values for the sedimentation constant of alpha crystallin could be due to the variety of ways in which it has been prepared.

Previous results concerning the sulfhydryl contents of the soluble lens proteins show that alpha crystallin contains less sulfhydryl than beta or gamma crystallins. Kinoshita and Merola, using the amperometric method for —SH determinations and lens protein fractions prepared by precipitation methods, found 6.9 and 15.5 moles of —SH/100 Gm. of protein for undenatured cortical alpha and beta crystallins, respectively. Firfarova and Lev-dikova, using the same technique, found values of 0.83 and 5.8 for alpha and beta crystallins, respectively. The present findings of 2.9 and 6.1 for rabbit alpha and beta crystallins is in agreement with this general feature.

The finding of a dependence of sulfhydryl content of alpha and beta crystallins upon the state of folding of the proteins may be anticipated, since this is a somewhat general aspect of proteins and since these proteins are quite complex. An increase in titratable —SH groups upon urea denaturation has been reported by Merola and Kinoshita for the case of total lens proteins. The present work shows this effect to be due to the alpha and beta fractions. It is of interest in this connection that Waley found that ox lens alpha crystallin would react with iodoacetate only in the presence of urea.

The molecular weight of an alpha crystallin subunit containing a single thiol group may be calculated from the present data to be about 33,000 for the native protein and 17,000 for the denatured state. Waley reported a molecular weight of 20,000 for the subunb and the data of Kinoshita and Merola yield a value of 14,500 for the subunit of native alpha crystallin. The differences in results may be a reflection of the varying degrees of specificity of the several techniques for sulfhydryl determination employed by the various investigators. Oxidation of some of the sulfhydryl groups may have occurred during isolation of the crystallins and therefore could also account for these disparities. However, other investigators have not used anaerobic conditions for the isolation of the crystallins either. Consequently, the magnitude of this factor is very difficult to evaluate. In any event, the values for the molecular weight of the alpha crystallin subunit(s) determined by sulfhydryl determination, assuming one sulfhydryl per subunit, agree reasonably well with values obtained by other methods.

Björk originally reported an —SH content for calf lens gamma crystallin of 30.7 moles of —SH per 100 Gm. as determined with the amperometric titration method, but later revised this figure to 16.8 using a spectrophotometric method. Our value of 26.0 compares favorably with Björk’s earlier finding. A value of 2.0 moles of —SH has also been reported.

The finding of N-terminal glycine for rabbit lens gamma crystallin agrees with results reported by Björk for four separate fractions of calf lens gamma crystallin, and points up once again the close interspecies similarities of the soluble lens proteins. The inability to demonstrate the presence of a free N-terminal amino acid for alpha and beta crystallin leads us to suggest that these amino acids are blocked to reaction with dinitrofluorobenzene or phenylisothiocyanate by linkage to acetyl radicals or other groups. This suggestion is not unique, and many examples are available to support such a proposal. Perhaps even more pertinent are the results reported by Bloemendal and associates, who found glu-
tamic acid to be the N-terminal amino acid of alpha crystallin, but quantitatively glutamic acid accounted for less than 2 percent of theoretical. We are investigating the validity of this interpretation at the present time.

REFERENCES
