

INVESTIGATION OF THE GAMMA CRYSTALLIN OF  
RABBIT LENS PROTEINS

by

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## INTRODUCTION

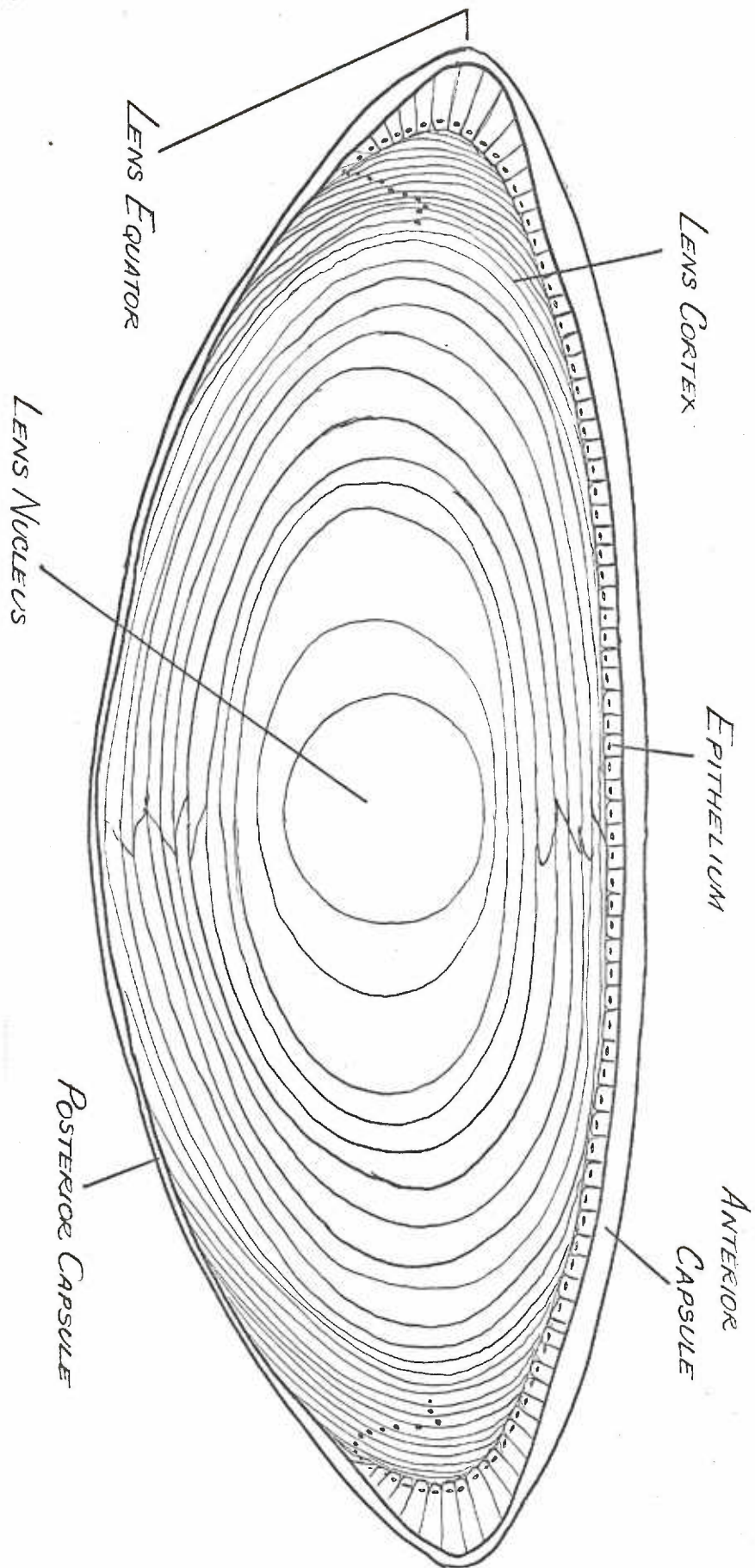
### Part I

In order to understand the lens proteins it is necessary to examine not only the historical development of the current concepts of the protein's structure, but also the environment in which these particular proteins exist.

The lens is formed from the surface ectoderm of the embryo and grows until, at maturity, it looks akin to the lens in Figure 1. From this stage, all further growth is produced by cell division of the single layer of epithelium and subsequent elongation of these daughter cells. As the cells elongate they curve around the deeper and older formations towards the anterior and posterior surfaces. These elongated cells are referred to as lens fibers, and as they are displaced towards the nucleus by newer cells, the older fibers lose their nuclei. Thus the fibers appear to "migrate" into the lens nucleus. The division between cortex and nucleus is not anatomical, for there is no separating membrane or cell layer. Rather, the division is operational, as the two are usually separated by differences in solubility.

The lens is distinguished from other organs by three characteristic features (44). Since it is fed by no blood vessels, it is well isolated. All chemical substances necessary for its existence and maintenance must reach it by

Figure 1. Diagrammatic representation of the lens  
in cross-section.





diffusion or "active transport" through the lens capsule from the surrounding aqueous or vitreous humor. Secondly, the embryonic origin of the lens is solely epithelial tissue, thus the lens represents a culture of epithelial cells. The final distinguishing feature is that the lens never ceases to grow, since none of the older cells are discarded. As was previously described, the older cells "migrate" towards the nucleus as the new cells are produced, with a resulting increase in density of the lens. While growth does continue throughout the lifetime of the organism, the growth rate actually decreases with age. The significance of continued growth and embryonic homogeneity is yet unknown, but a theory has been proposed as to the effect of isolation of the lens on the lens proteins. This theory will be discussed later.

Although most credit is given to C.T. Mörner for the discovery of proteins in the lens, it was actually Berzelius who, in 1830, found a protein fraction which he designated as crystallin (1). It was up to Mörner, however, to isolate various fractions of the lens crystallin (29), and from his work have come the classical definitions. He found two soluble protein groups and an insoluble one, the latter being designated albuminoid. The first of the soluble fractions, which he called alpha crystallin, was defined as that protein which precipitated at approximately pH 4.8.

when the resulting supernatant was raised to a pH of 6.0, a second protein fraction precipitated; it was designated beta crystallin.

There was then a lapse of thirty-three years before Burky and Woods, in 1928, defined a third crystallin fraction (14,15). They, too, isolated the alpha and beta fractions by isoelectric precipitation; but when the final supernatant was treated with ammonium sulfate, a third protein fraction was found to precipitate. This fraction was designated by them as gamma crystallin. It is these three classifications; alpha, beta, and gamma crystallin, which are in use today.

Then for a number of years interest in the problem slacked with but few publications (16, 23, 26, 31). It was not until 1955 that Francois, Rabaey, and Wieme started the inquiry anew with a paper electrophoretic analysis of the lens crystallin of oxen (21). These investigators divided the lens into two parts, nucleus and cortex, and analysed each separately. They found alpha crystallin to be the fastest moving, gamma the slowest, and beta crystallin intermediate. The isolation of nuclear alpha crystallin was at pH 5, as per Woods and Burky's technique, but Francois found that after precipitating cortical alpha crystallin at pH 5 (alpha<sub>1</sub> cortex), the addition of ethanol to the supernatant resulted in another precipitate, which he designated alpha<sub>2</sub> crystallin. Upon electrophoresis

both alpha crystallins migrated together. The electrophoretic pattern of the beta crystallin from both nucleus and cortex showed two components. Thus the first indication, albeit poorly substantiated, of any difference between nuclear and cortical proteins. Molecular weights of the combined alpha crystallins and of the heavier component of beta crystallin were determined by light scattering to be  $1.2 \times 10^6$  and 200,000 respectively.

The conclusion that Resnik reached after doing sedimentation analyses on both  $\alpha_1$  and  $\alpha_2$  as prepared by the above method, was that the addition of alcohol did not precipitate a different protein fraction, but rather was needed for complete precipitation in the first place (35). He found that both alpha crystallins from calves' lens had identical sedimentation constants, and that the reciprocal of the sedimentation coefficient of either crystallin did not vary with the concentration of protein. In addition, the molecular weight was calculated from sedimentation and diffusion and from light scattering data. Values of  $1 \times 10^6$  and  $1.5 \times 10^6$  were obtained respectively, which is in close agreement to the values obtained by Francois. About a year later a new method was used to isolate alpha crystallin from bovine lenses. Bloemendal and Ten Cate employed starch-zone electrophoresis to isolate proteins on which N-terminal amino acid studies were subsequently done (6).

Using Sanger's FDNB method they found glutamic acid to be the major N-terminal residue of alpha, beta, and gamma crystallins; thus indicating a similarity or possible relationship between the three fractions. In a work published a year later these same two investigators confirmed their previous results that glutamic acid was indeed the predominant N-terminal residue in all fractions (7). In an experiment designed to determine the effect of iso-electric precipitation upon alpha crystallin, ultracentrifugal studies were done on protein prepared by isoelectric precipitation and on protein isolated by zone electrophoresis. It was found that isoelectric precipitation did indeed change the ultracentrifugal pattern, so any further isolation would have to be done by other methods. The zone electrophoresis also indicated that the protein was heterogeneous, possibly being composed of different aggregations of the same protein subunit. In addition they studied the effect of pH upon alpha crystallin employing the Tiselius electrophoresis apparatus. The conclusion reached was that between pH's 4.2 and 8.9 there is no change in the molecule, but at more extreme pH values the protein becomes labile.

In another study on the effect of pH upon alpha crystallin, Resnik and Kenton, as had Bloemendal, found that at pH's below 4.5 some change in the protein is achieved (36). They studied the intrinsic viscosity and sedimentation patterns and were lead to the conclusion that the

molecule may split into two subunits at low pH's and small ionic strengths.

Bjork, in 1960, used vertical column zone electrophoresis employing cellulose as the supporting medium to separate the fractions from whole lens proteins (2). The pH used was 8.0 and under such conditions three fractions are isolated. When examining these fractions by free electrophoresis under the same conditions, the slowest moving fraction (III) precipitates, thus the mobility of this fraction remained unknown. Ultracentrifugal analysis of the other two fractions showed one to be homogeneous with a sedimentation coefficient of 18.3S (I), while the other (II) was heterogeneous. The sedimentation coefficient of the fraction with highest electrophoretic mobility (I) indicates that it is alpha crystallin. Precipitation of fraction III at pH 8.0 would argue for the fact that it is gamma crystallin, for the isoelectric point of gamma crystallin is close to pH 8. The fraction with intermediate electrophoretic mobility was assumed to be beta crystallin since the ultracentrifugal pattern indicates multiple sedimentation coefficients of the order of the magnitude predicted for molecules of approximately 200,000 molecular weight.

One of the puzzling results of some investigators is the ability to resolve as many as thirteen different components from the lens proteins. Work has been done by

Spector (39) using ion exchange chromatography, by Perry and Koenig (33) and Rupe (37) using electrophoresis, and by Manski (28) using immunoelectrophoresis, in which ten, thirteen, eight, and ten components were found respectively in the whole lens proteins. The significance of the great number of fractions, if they are indeed true fractions, is as yet unexplained.

Yet another paper indicating the numerous possible fractions or subunits of alpha crystallin is that of Papaconstantinou, Resnik, and Saito (32). By eluting the soluble lens proteins through a diethylaminoethyl--cellulose (DEAE) column they obtained four fractions of alpha crystallin. In support of this result they also obtained four bands of alpha crystallin using the agar diffusion techniques of immunochemistry. DEAE chromatography also showed a difference between nuclear and cortical lens proteins. This difference was not in the number of fractions, but rather in the proportion of the total that each fraction represented. In addition, they found by sedimentation analysis that alpha crystallin was split irreversibly in half at low pH.

The idea that alpha crystallin was composed of some sort of subunit was furthered by the work of Bon in 1961 (10). He analysed the cortical lens extract by ultracentrifugation and electrophoresis and found three sedimentation and two electrophoretic boundaries. By precipitation at

pH 5, the alpha crystallin was isolated and found to contain one electrophoretic boundary and two sedimentation boundaries (15 and 7.5 S). The supernatant from above was analysed and resulted in one electrophoretic and one sedimentation boundary (3 S). The treatment of alpha crystallin with acid at pH 2.5 resulted in molecules whose sedimentation coefficient was 1 S. He states that these data indicate that there are two species of alpha crystallin, one being relatively globular and the other fibrous, but of similar molecular weights. An alternative hypothesis is that acid causes disaggregation of the 15 S molecule, and that this phenomenon is dependent upon the actual concentration of the hydrogen ion. Thus at pH 5 a 7.5 S unit is observed, while at pH 2.5 a 1 S unit is seen. Unfortunately for these hypotheses, however, Bon states in a later paper that alpha and beta crystallin in solution together prevent sedimentation and electrophoretic analyses from being definitive (11). Thus the previous data could be interpreted as coming from mixtures of alpha and beta crystallins, rather than from just alpha crystallin.

In the first work on the effects of urea on alpha crystallin, Bloemendal treated the protein with 3.5 M urea and found that the large 19.5 S band in native protein had disappeared and that the 10 S band had become more predominant (8). Other components seen in the ultracentrifugal pattern had sedimentation coefficients of 5.3, 3.6, and

1.6 S. The authors interject the idea that the heterogeneity observed in alpha crystallin by other workers was not heterogeneity of the native protein, but denaturation resulting in seeming polydispersity.

In further work done by the same group, 7M urea was found to dissociate the alpha crystallin to a species of 1.8 S having a molecular weight of approximately 26,000 (12). After removal of urea by dialysis the sedimentation pattern showed two peaks, one of 11S and the other 4 S. Thus the action of urea on alpha crystallin, whatever it may be, is at least partially reversible.

In 1963 Bjork further explored the idea of two alpha crystallins (4). Upon elution of alpha crystallin through DEAE-cellulose at pH 8, two species were separated. In attempting to differentiate the two, free electrophoretic and ultracentrifugal analyses were done. Both techniques yielded significant differences. However, additional studies by light scattering, rotatory dispersion, intrinsic viscosity, and amino acid content yielded no significant difference between the two, so Bjork concluded that alpha crystallin was not composed of two species.

Niyogi and Koenig present some data from ox lens proteins which appears to be in direct conflict with the data of other workers (30). Previously, no concentration dependence of the sedimentation coefficient of alpha crystallin had been exhibited, but these two investigators do find



such a concentration dependence and interpret their results as indication of association-dissociation phenomena.

Then in 1964 Spector isolated the various lens protein fractions from calves in a new manner (40). He found that the addition of zinc glycinate and ethanol to the whole lens extract caused the beta and gamma fractions to precipitate, while the alpha crystallin remained soluble. The former two proteins were then separated using gel filtration. The method did give good separation as tested by paper electrophoresis, ultracentrifugation, and ion exchange chromatography. The alpha and gamma crystallins had sedimentation coefficients of 19.7 and 2.4 S respectively; and appeared as single, symmetrical peaks at neutral pH. The beta crystallin exhibited three peaks with sedimentation coefficients of 5.0, 9.2, and 14.5 S. Preliminary observations by the author indicates that all components eluted through the ion exchange column, except gamma crystallin, can be broken down by urea to 1.8 S units. These findings agree with the previous results of Bloemendal and further substantiate the hypothesis that at least alpha and beta crystallins of the lens proteins are complicated aggregations. The findings here indicated that gamma crystallin is not composed of subunits which can be detected after treatment with urea.

In experiments performed by Bloemendal's group on the

splitting and recombination of alpha crystallin, the protein size was found to vary with the concentration of urea (9). As before, the native alpha crystallin had a sedimentation coefficient of 19 S, while in 7 M urea the species present was of such a size to have a sedimentation coefficient of 1.8 S. Removal of urea by dialysis resulted in a protein whose sedimentation coefficient was again 19 S. These data indicate that the effect of urea on the size of the protein is reversible.

The first definitive work done on gamma crystallin was by Bjork in 1961 (3). He used the principle of molecular sieving and found that if the whole lens extract was eluted through a Sephadex G-75 column, the alpha and beta fractions were eluted together in the void volume, while the gamma crystallin was small enough to be retained and eluted later. The ultracentrifugal pattern of the gamma crystallin protein thus isolated showed a single, symmetrical peak with a sedimentation coefficient which was independent of concentration. The molecular weight was determined to be 20-25,000 assuming a partial specific volume of 0.70. When the sedimentation patterns of protein at pH's 5.0 and 9.0 were examined, a single boundary was seen, and studies done with synthetic boundary cell indicated that the apparent diffusion coefficient was constant. Thus all the data from sedimentation analyses indicated that gamma crystallin was a homogeneous protein. The results of free electrophoresis

did not justify the same conclusion, however. The protein was analysed at pH 5.0 and found to contain four different components, while immunoelectrophoresis at pH 8.2 resulted in the detection of two different components, and a total of nine in the whole lens extract. The conclusion reached by Bjork was that the homogeneity was more apparent than real. That the fractions of gamma crystallin were not separated in a centrifugal field and were eluted together in gel filtration probably means that they possess similar molecular weights. The experimental conditions under which the immunoelectrophoresis was carried out, ie pH 8.2, was the probable explanation for the appearance of only two components. Upon elemental analysis 307  $\mu$  moles of sulfhydryl per gram of protein were found. Assuming a molecular weight of 25,000, this figure approximately corresponds to 7.5 moles of sulfhydryl per mole of protein. The significance of the high sulfur content in this fraction is as yet unknown, but it is believed to play a role in cataract formation (17).

As a sidelight, it is interesting to note that the first appearance of gamma crystallin seems to be associated with the process of cell differentiation described earlier. Takata, Albright, and Yamada studied the lens cells during growth and subsequent elongation into lens fibers (42). Employing immunoelectrophoresis and immunofluorescence they found that gamma crystallin did not appear in the cell

until elongation had started.

In summary, then, the general hypothesis evolving from these works is that the lens proteins are complicated aggregations of smaller units. Alpha and beta crystallins are comprised of these aggregations, but native gamma crystallin appears to be present in its smallest possible unit. Although a relatively large quantity of work has been done on alpha crystallin, both beta and gamma crystallins have been meagerly investigated. It would indeed be interesting to elucidate the relationship of each of these proteins fractions to one another, and the relationship between their subunits, if any exists. Before these relationships can be worked out, it seems necessary to more fully characterize all three fractions. Thus the goal of the research presented in this paper is to isolate and characterize, both physically and chemically, the gamma crystallin fraction of lens proteins.

## INTRODUCTION

## Part II

There are a few aspects of lens proteins that were not examined in the previous section which deserve some comment. The problem of the effect of age on the proteins, as well as the species specificity and involvement of the proteins in cataract formation will be discussed.

In order to understand some of the changes in the lens composition, it is important to know that the level of glutathione and ascorbic acid in the lens is higher than in most other organs of the body. In comparing a number of workers' results, Pirie and Van Heyningen point out that the rat lens contains between 172 and 320 mg of glutathione per 100 g of tissue (34). The organ with the next highest level is the liver, which contains 173 mg of glutathione per 100 g of tissue; and in contrast, the blood contains only 19 mg of glutathione per 100 g of tissue. The authors add that the ascorbic acid level in lenses is also high in comparison to other organs. The observed phenomena in this case is not as marked as in the case of glutathione, for in most animals examined the hypophysis and adrenal glands had a level of ascorbic acid much higher than the lens. The significance of the presence of these compounds in such high concentrations in the lens has not been firmly estab-

lished.

In work done by Dische, both the protein and sulfhydryl content of the rat lens were examined in animals of different ages (18). The levels of glutathione and ascorbic acid were found to increase until the animal was about 150 days old, and to decrease thereafter. The total amount of soluble lens proteins increased with age until the animal was one year old, at which time it started to decline. The author points out the fact that the time of synthesis of soluble protein roughly corresponds to the time of increasing levels of glutathione and ascorbic acid, and suggests that these compounds may play a role in protein synthesis. Another phenomenon observed by Dische was that the insoluble lens protein, albuminoid, increases slowly with age. The albuminoid contains approximately equal amounts of cysteine and cystine, while the soluble protein contains no cysteine. Thus, aging of the lens is associated not only with net oxidation of cysteine to cystine; but also with increasing amounts of albuminoid and decreasing amounts of soluble protein, as well as decreasing levels of glutathione and ascorbic acid. The implication that the sulfhydryl group plays a role in the formation of albuminoid through aggregation of soluble proteins is interesting to consider.

In an experiment designed to isolate the location of various sulfhydryl containing compounds, Kinoshita and Merola found that the sulfhydryl in the cortex of bovine

lenses was mainly present as glutathione (25). On the other hand, the sulfhydryl in the nucleus was predominantly protein bound. Although the total levels of sulfhydryl in both nucleus and cortex were approximately equal, there was noticed a difference between nuclear protein sulfhydryl content and cortical glutathione sulfhydryl content. This difference was found to be constant, while the total amount of sulfhydryl in both nucleus and cortex increased with age. If these results are examined in the light of Dische's experiments, we can conclude that increasing age is accompanied by increasing protein sulfur and albuminoid in the nucleus. Unfortunately, it appears that not a sufficient amount of work has been done in relating the role of sulfhydryl groups to the solubility of the protein, so that any conclusion as to the fate of the sulfhydryl moiety are quite tentative.

The theory that the lens proteins are most probably involved in cataract formation has been expounded for a number of years. Among the researchers who have found evidence in favor of this theory is Stephen Trokel, who, in 1962, measured the light scattering of the lens (43). He found that any aggregation of the soluble proteins or any change tending to decrease the order of the fibers will cause a marked increase in the scattering intensity. Thus the opacity observed in a cataractous lens may be due to the changes in the lens proteins. In addition, the amount



of back scattering produced by the lens increased with age, thus hinting at a possible similarity in the changes occurring to the proteins in both aging and cataract formation. It has been shown that both processes involve an increase in the albuminoid content and a decrease in some portion of the soluble protein (34). The difference between the two phenomena seems to lie in the fact that a general decrease in total amount of protein is associated with cataracts, while the opposite is the case in aging.

The lack of species specificity of lens proteins raises interesting questions. While serum proteins have changed with evolution, Maisel and Langmen have examined the lens proteins of various vertebrates and have found that alpha crystallin is present in these animals in identical or partially identical forms (27). The study was done employing agar diffusion of antigen and antibody and noting the reaction. The beta crystallin fraction was found to be identical or partially identical only in those animals relatively close in the phylogenetic scale, while the gamma crystallin seemed to take an intermediate position between alpha and beta crystallins. Two possible explanations have been proposed by Halbert for this phenomenon (22). The constancy of the lens proteins in the vertebrates may be due to the fact that the lens is relatively well isolated, or that the protein structures are so well suited to use that no change has been possible without damage to the system. Both explanations are necessarily speculative.



## ADDENDUM

In a paper just recently published, Bjork further studied the gamma crystallin fraction of calf lens proteins (5). He used sulfoethyl (SE) - Sephadex and Phosphocellulose columns and obtained four and six fractions respectively. The protein was eluted from the SE-Sephadex by using an ionic strength

linear gradient of 0.2 to 0.5 of acetate buffer at pH 5.0. The chromatography of each of these four fractions on a PC column yielded results that indicate two of the four fractions were homogeneous and two were not. The eluant used in this case was a linear gradient of phosphate buffer from 0.02 M, pH 6.0, to 0.1 M, pH 7.0. Molecular weight determinations of the four major fractions by the approach to sedimentation equilibrium method showed that all fractions had essentially the same molecular weight, i.e. about 19,000. Amino acid content studies indicated some degree of difference between the fractions, but the N-terminal amino acid sequences gly, glu, leu or ileu were found to be identical. In addition, the sulfhydryl content was determined by a spectrophotometric method based on the reaction of 5,5'-dithio-bis-(2-nitrobenzoic acid) with the protein. The results show that three of the four fractions have different sulfhydryl content, but that all fractions contain approximately half the sulfhydryl content as determined by Bjork in an earlier paper (3).

Again the conclusion reached was that there are four major fractions of gamma crystallin. These fractions appear very similar and probably differ only in some portion of their primary structure. That each fraction had only one N-terminal amino acid probably means that there is only one peptide chain, or that the N-terminal amino acid of any other chain present cannot be found.

## METHODS AND MATERIALS

### 1. Laboratory Apparatus

A Beckman Model DU spectrophotometer was used in all spectrophotometric measurements. When constant temperature was desired, the cell holder housing with appropriate modifications was employed. Unless otherwise specified measurements were made at room temperature. The concentration of protein solutions was determined by measuring their absorbance at 280 m $\mu$ . The blanks used were the respective protein solvents.

The pH of solutions was measured electrometrically, except when very approximate pH was desired, in which case pHyrion paper was employed.

Centrifugations were carried out with a Lourdes Model A refrigerated centrifuge at 10,000 rpm and either 12,800 or 16,300 X G, depending upon the rotor utilized.

The fraction collector employed was the Gilson Medical Electronics model equipped to collect a maximum of 225 samples in a rectangular tray. The volume collected was variable and was accomplished by pipettes calibrated to the desired volume with a light sensitive device.

### 2. Special Apparatus

Glass columns employing glass wool stoppers were utilized in all column chromatographic procedures. The packed

column material was covered with a circular Whatman No. 1 filter paper to prevent disturbance of the surface when solutions were added. The Sephadex used was supplied by the Pharmacia Company of Sweden in both medium and coarse grades with relative pore sizes of G-50 and G-75. The procedure employed in packing the Sephadex columns was as follows; the dry Sephadex material was suspended in a large quantity of water, and allowed to swell at least overnight. The fines were then removed by decantation, the material washed thrice with distilled water, and finally thrice with the appropriate buffer. The column was packed by first filling it with the solvent, then placing a glass wool plug at the bottom, and finally allowing the material to flow in from a continuously stirred reservoir. For the first few minutes no buffer was allowed to flow, so the material could start to form a bed on top of the plug. After this brief waiting period, the buffer was allowed to flow at its maximum rate. When all the material was packed, a circular Whatman No. 1 filter paper was placed on top.

The packing of the phosphocellulose (PC) column differed in only two ways. First, the material was washed with 1 M KCL, or 0.5 M KCL-0.5 M KOH, or 1 M KCL and then 1 M KOH. It was then rinsed at least 4 times with distilled water and finally three times with the correct buffer. The actual packing was done in the same manner as above, except that 2-6 lbs per sq. inch pressure was used. Without pressure

the flow rate of the PC columns was too slow. The PC material was originally from the Bio-Rad company, and had an exchange capacity of 1.08 milliequivalents per gram dry weight.

The ultracentrifugal analyses were performed on Dr. D. A. Rigas's Spinco Model E ultracentrifuge with the kind assistance of Mr. Grayson Capp. All sedimentation velocity runs were done at 20° and employed the AN-D rotor at a speed of 59,780 rpm. Schlieren optics were used and recorded on Kodak Metallographic plates. Complete instructions on the operation of the ultracentrifuge and development of the photographic plates are available in the manuals, and hence shall not be covered here.

A Brice-Phoenix Model 2000-D light scattering spectrometer was used in the molecular weight determinations. All solutions used in these experiments were prepared from the doubly glass distilled water made available to us by Dr. V. Weimar, and were filtered through a 0.22  $\mu$  millipore filter immediately prior to analysis. A Brice-Phoenix differential refractometer was employed for the measurement of the refractive index increment. Once again, detailed instructions are available in the manuals, so that the reader is referred to them for all operational information.

### 3. Isolation

The rabbit eyes were excised from 8 to 12 week old

animals slaughtered at the Crane abbatoir in Beaverton. Within half an hour after the death of the animal the globes were immersed in 0.9% saline and maintained there until further work was ready to be done. This time usually amounted to a few hours, but when the globes remained in saline overnight the solution was cooled to 4° C.

In order to expose the lens, a slit was made across the back of the eye and the vitreous removed. The zonular fibers were then severed and the lens and lens capsule washed in distilled water for a few minutes. The capsule was then slit with a scalpel, while applying a small amount of pressure to the mass by squeezing, thus forcing the lens to emerge from its capsule. The capsule was discarded and the whole lens stirred into a solution of 0.1 M phosphate buffer, pH 6.8, whose volume was approximately 1 ml per lens. Stirring was continued until all cortices were suspended and only the nuclei remained whole. These nuclei were then removed and discarded, and the cortical lens protein mixture was homogenized in a Potter-Elvehjem glass homogenizer. The solution was then centrifuged at 12,800 x G for 30 minutes at 15°, and the supernatant dialyzed against three changes of 500 mls of 0.1 M phosphate, pH 6.8 for at least 6 hours. In some cases no dialysis was done. When dialysis was done a slight amount of precipitate appeared, which was removed by centrifugation at 12,800 x G for 30 minutes at 15°. The resulting protein solution was then eluted

through a 5 x 60 cm Sephadex G-75 (medium grade) column at rates between 0.5 and 1 ml per minute and at 4° C. The protein was applied to the columns in 10-20 ml volumes and at concentrations close to 40 mg per ml. In one experiment a volume of 30 mls with a concentration of 25 mg per ml was applied. The fractions collected were usually 14ml volumes, but 15 ml collecting tubes were used occasionally. Approximately 1 L of 0.1 M phosphate, pH 6.8 or 0.05 M phosphate-0.05 M KCL, was used as the eluant. The desired protein effluent, which was located by determining the absorbance of the samples at 280 mμ, was pooled and concentrated either by precipitation with 70 g ammonium sulfate per 100 mls of solution, or by ultrafiltration. The protein was then stored at 4° as the ammonium sulfate precipitate or in solution under toluene vapors. When protein was needed for further analysis, the precipitate was dialyzed at 4° C against the appropriate buffer. If the protein had been stored under toluene vapors, a simple dilution was made with the correct solvent to give a solution of the desired concentration.

## EXPERIMENTAL AND RESULTS

## 1. Sephadex Chromatography

A medium grade Sephadex G-75 column was prepared in 0.05 M phosphate - 0.05 M KCl in the usual manner. The elution of the whole lens proteins as described above resulted in repeatable results as depicted in Figure 2. The alpha and beta crystallins were eluted together in the void volume, while the gamma crystallin was retained and eluted later. If the cortical lens extract was not dialyzed prior to gel filtration, a third component was eluted, which consisted of small molecular weight peptides. The two fractions were collected and stored as the ammonium sulfate precipitate or under toluene vapors.

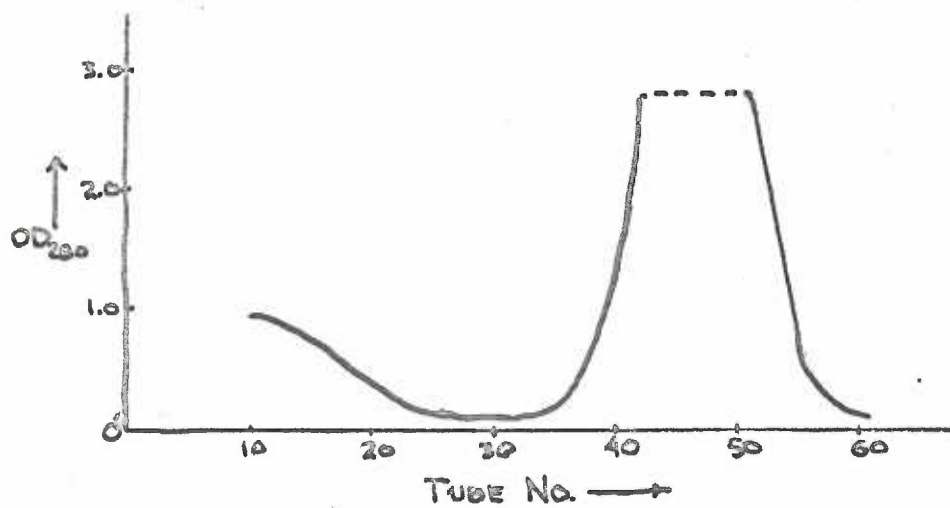
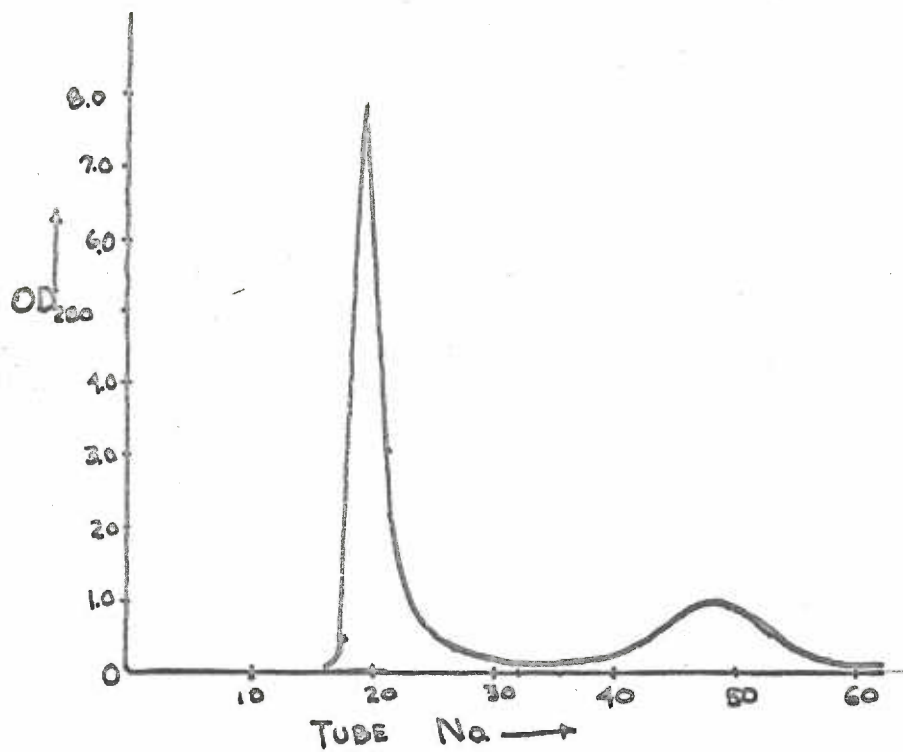
Upon dialysis of the gamma crystallin precipitate against 0.1 M phosphate, pH 6.8, a small amount of light-brown precipitate is formed after the majority of the protein has dissolved. In all instances this precipitate was removed by centrifugation at 12,800 x G before further analyses were done on the protein.

In order to test the effectiveness of the separation of gamma crystallin by gel filtration, a solution of 12 ml of the gamma crystallin was rechromatographed in the 5 x 60 cm Sephadex column under the same conditions as were previously used. Approximately 1 L of 0.1 M phosphate, pH 6.8, was used as the eluant and 14 ml fractions were collected.



Figure 2. Elution of whole lens extract through a Sephadex G-75 column. The buffer used was 0.1 M phosphate, PH 6.8. Column dimensions were 5 x 60 cm.

Figure 3. Rechromatography of gamma crystallin through a Sephadex G-75 column using 0.1 M phosphate, pH 6.8 as the eluant.



As can be seen in Figure 3, the results show that the gamma crystallin is well separated from the rest of the lens proteins in the first gel filtration procedure. The recovery of protein from this column was found to be 95% or better in all experiments.

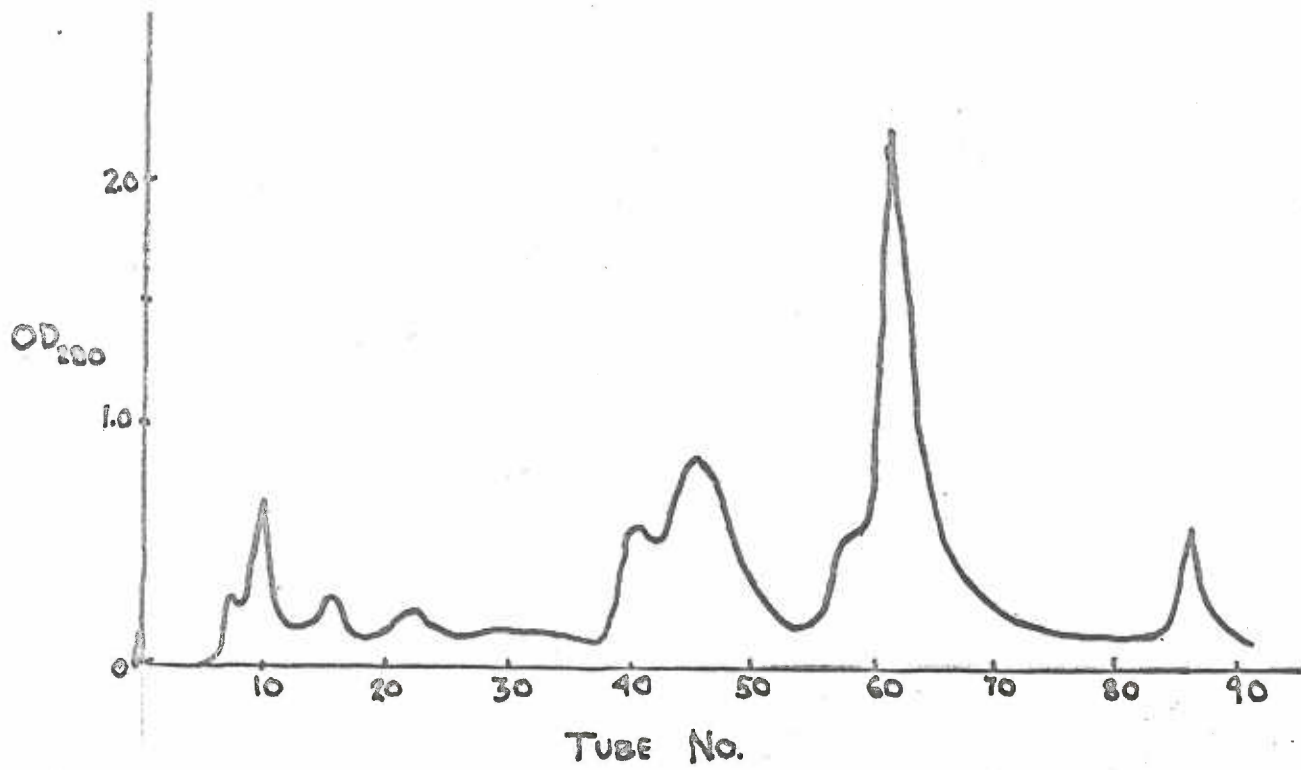
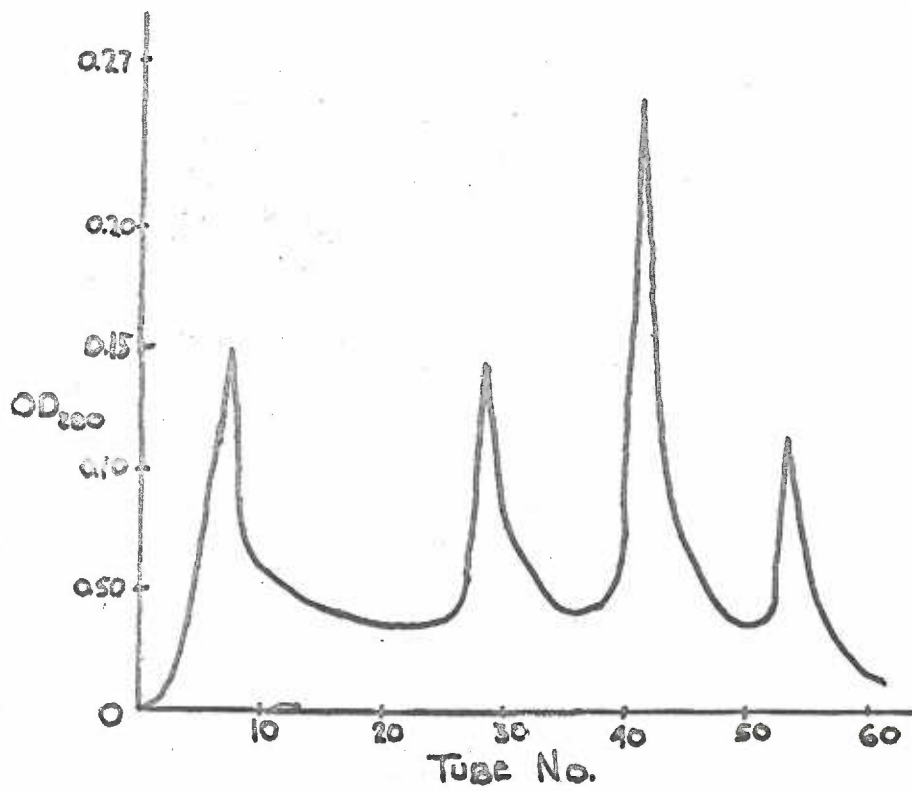
## 2. Phosphocellulose Chromatography

The preparation of the gamma crystallin protein for ion exchange chromatography included dialysis of the ammonium sulfate precipitate against 0.1 M phosphate, pH 6.0. A brown precipitate resulted, which was removed by centrifugation and then discarded. A PC column was prepared as described above in 0.1 M phosphate buffer, pH 6.0. The column size was 1.6 x 30 cm and was run under 2-3 lbs per sq. inch pressure. A protein solution containing 18 mg was applied to the column and eluted with 100 mls of the following 0.1 M phosphate buffers; pH 6.0, 6.2, 6.5, and 6.8. Fractions collected were 10 mls each and protein recovery was between 85 and 90%. Four fractions are eluted under these conditions as seen in Figure 4.

Using a larger PC column (2.5 x 29 cm) and approximately 10 times as much protein, the elution pattern is somewhat changed. In this experiment 200 mls of each of the afore mentioned buffers were used to elute a total of 88% of the protein applied. Once again, 10 ml fractions were collected, and the elution done at 4°. The pattern

Figure 4. Chromatography of gamma crystallin through a Phosphocellulose column. The protein was eluted with a pH gradient as described in the text.

Figure 5. Chromatography of gamma crystallin through a PC column. The concentration of protein in this experiment was approximately 10 times that shown in Figure 4.



obtained is depicted in Figure 5 and, as can be seen, more than 4 components are eluted. The results shown are repeatable with minor differences between experiments.

After collection of fraction III of Figure 5, the protein was precipitated with ammonium sulfate, dialyzed against 0.1 M phosphate, pH 6.0, and rechromatographed on a PC column employing the afore mentioned buffers. The elution pattern showed heterogeneity of that fraction, but the pattern was not reproducible between experiments.

### 3. Determination of the Extinction Coefficients and

#### Observance of the Lambert-Beer Law

A solution of gamma crystallin isolated in the usual manner and concentrated by ultrafiltration was dialyzed free of buffer salts. The final protein solution had an absorbance at 280 m $\mu$  of 2.46 and 10 ml aliquots of it were placed in each of three previously tared weighing bottles. In addition, 10 ml portions of the final dialyzate were placed in three other tared bottles. The samples were then dried at 110° for 48 hours in a desiccator, cooled for 30 minutes, and the weights recorded. The procedure was repeated, only 2 hours of drying time were used. Successive weighings varied by  $\pm$  0.1 mg. From these data the extinction coefficient of a 1% gamma crystallin solution at 280 m $\mu$  was calculated to be 17.6.

A series of dilutions of gamma crystallin solution

of concentration 1.6 mg/ml was performed with 0.1 M phosphate, pH 6.8, and with 0.1 M NaCl to determine the linearity of the relationship between OD 280 and concentration of protein. The results indicate that a linear relationship does indeed exist in both solvents, and thus the Lambert-Beer Law,  $OD = \epsilon lc$ , is obeyed in the concentration range investigated.

#### 4. Spectrum

The ultraviolet spectrum of gamma crystallin was determined by measuring the absorbance at 5 mμ intervals of a solution of the protein in 0.1 M phosphate, pH 6.8. A plot of wavelength v. optical density is shown in Figure 6.

#### 5. Solubility in ammonium sulfate

A solution of gamma crystallin was thoroughly dialyzed against 0.2 M phosphate, pH 6.8 in the cold. All other procedures of this experiment were done at room temperature. A saturated ammonium sulfate solution was prepared by suspending an excess of 75 g of the salt in each 100 ml of 0.2 M phosphate and adjusting the pH with 0.2 M KOH to 6.8. This solution was allowed to equilibrate for a number of days before use. Aliquots of the gamma crystallin solution, whose OD 280 was 2.18, were diluted with specified volumes of saturated ammonium sulfate such that the resulting solutions varied in percent saturation (of ammonium sulfate) between 20 and 80 at 10% intervals. In each case a blank was prepared with solvent substituted for protein solution.

The solutions were mixed, allowed to equilibrate for 30 minutes, and then centrifuged for 20 minutes at  $12,800 \times G$  at  $25^{\circ}$  (room temperature). The absorbance at 280 m $\mu$  of the resulting supernatants was determined, corrected for dilution, and plotted versus percent saturation. In the region of the break of the curve depicted in Figure 7, the procedure was repeated to give solutions of 45, 55, and 65 % saturation. The final graph of solubility (OD 280) versus percent ammonium sulfate saturation shows a single, sharp decrease in gamma crystallin solubility at 47.5% saturation.

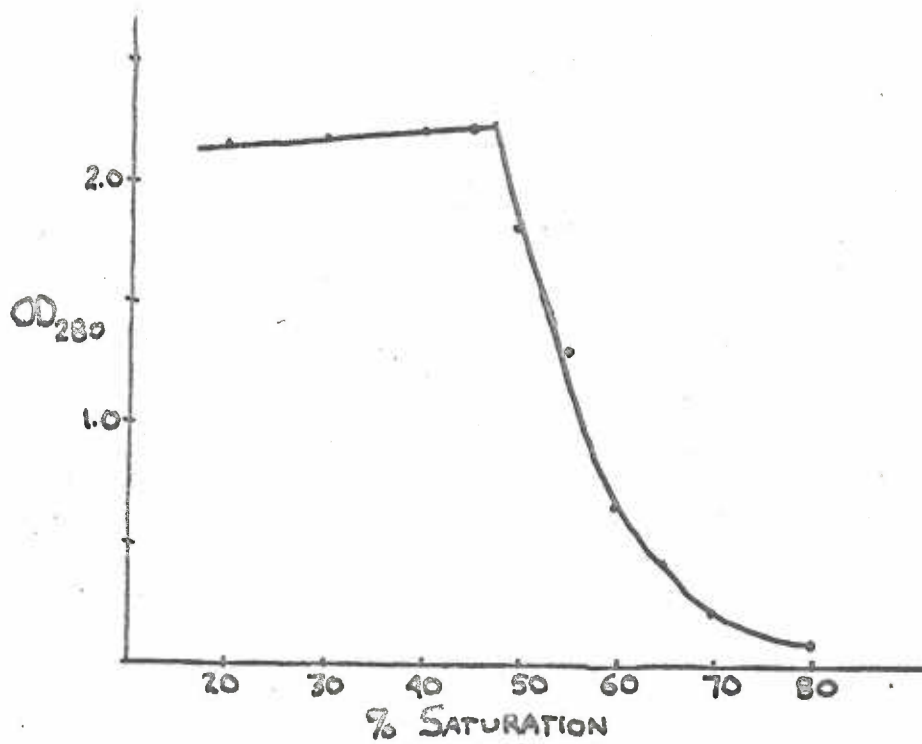
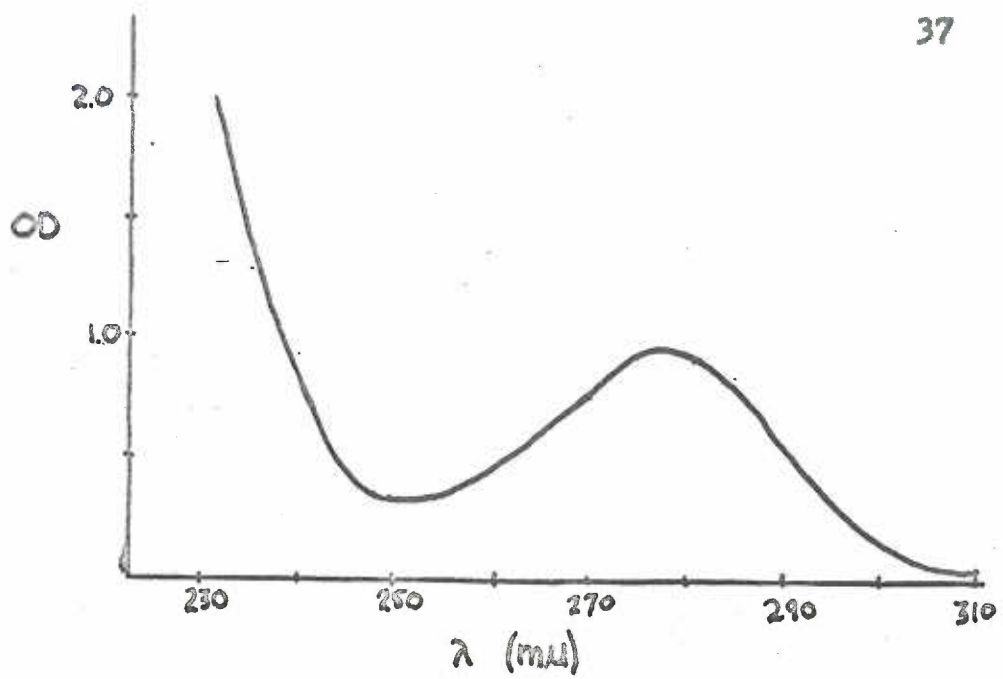
#### 6. Ultracentrifugation

Sedimentation velocity runs were made on the Model E at gamma crystallin concentrations of 2.41, 3.60, 4.43, 4.82, 6.03, 8.04, and 13.31 mg per ml in a solvent of 0.1 M phosphate buffer, pH 6.8. The series of photographs shown in Figure 8 is from the ultracentrifugation run of protein concentration at 13.31 mg per ml. These photographs show a single, symmetrical peak, thus indicating homogeneity. These results were reproducible over the concentration range used. Measurements were made from photographs with a two dimensional microcomparator, and calculations from these data were made according to the method of Schachman (38). The resulting  $S_{20,w}$  values are shown in Figure 9 and were calculated assuming the density of the solvent was the same as water and that the relative viscosity was approximately 1.029. Extrapolation of these data to zero



- Figure 6. Absorption Spectrum of gamma crystallin in 0.1 M phosphate buffer, pH 6.8.

Figure 7. Solubility of gamma crystallin in varying concentrations of ammonium sulfate solutions.



concentration gives a  $S_{20,w}$  value of 2.48. As can be seen, the sedimentation constant of gamma crystallin at pH 6.8 is independent of the concentration.

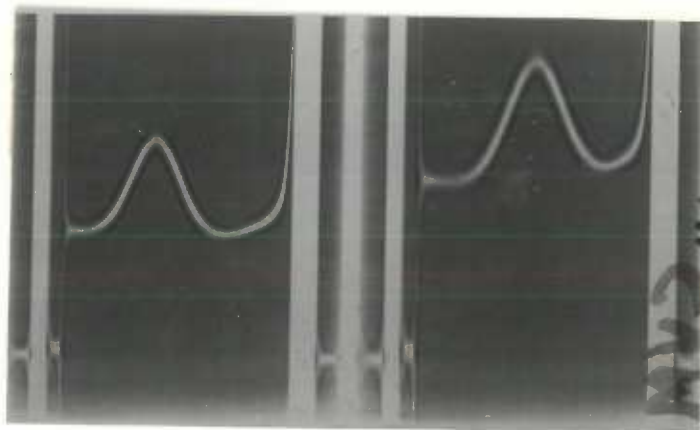
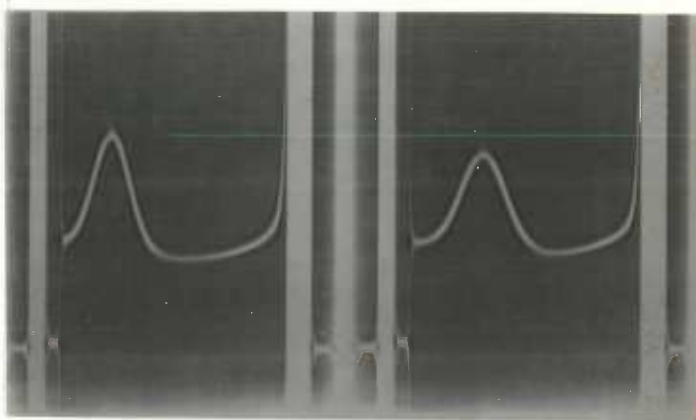
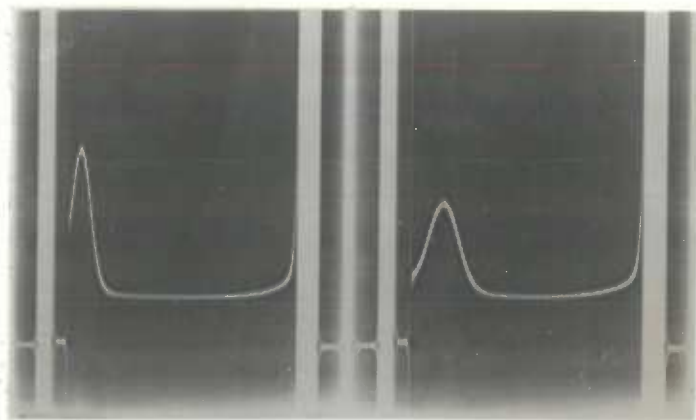
## 7. Light Scattering

Light scattering analyses were done on gamma crystallin solutions of 4.69, 3.39, 2.35, and 1.15 mg per ml in 0.1 M phosphate buffer, pH 6.8, at 546 mμ. In each case the dissymmetry (s), turbidity (t), depolarization (P), and relative refractive index were measured. The molecular weight dependence upon concentration is described by the equation  $Hc/r = 1/M + 2Bc$  where H is a quantity depending upon wavelength of light used and relative refractive index of the solution, c is the concentration, M the molecular weight, and B is a constant. The observed turbidity was corrected for depolarization as suggested by Stacey (41), but no correction was necessary for dissymmetry since the gamma crystallin molecule was considered small in comparison to the wavelength of light used. The calculated values of  $Hc/t$  were plotted versus concentration, and extrapolation of the data to zero concentration gave a molecular weight of 32,000 (see Figure 1 O).

## 8. N-terminal Amino Acid Analysis

The fluorodinitrobenzene (FDNB) method as described by Frarnkel-Conrat, Harris, and Levy was used with a few alterations (20). A solution of gamma crystallin was

Figure 8. Ultracentrifugal pattern of a sedimentation velocity run of gamma crystallin at 13.3 mg/ml in 0.1 M phosphate buffer, pH 6.8.



dialyzed free of salts and then lyophilized. Twenty-five mg of protein were suspended in 0.25 mls of water, to which was added 0.5 mls of a 10% (w/v) solution of FDNB in 95% ethanol. The mixture was shaken at room temperature over night in a stoppered tube. After reaction, the mixture was extracted 4 times with 10 ml portions of ether (or until the ether does not extract any additional FDNB, and is thus colorless), twice with 10 ml portions of  $10^{-3}$  M HCl, and a final time with 10 mls of ether. Then 1 ml of 6 N HCl was added to the aqueous DNP-protein, and the tube sealed. Acid hydrolysis was achieved by allowing the tube to heat at  $105^{\circ}$  for 8 hours. The hydrolysis tube was then opened and the solution diluted with water to make the acid approximately 1 N. The mixture was then extracted with four 5 ml portions of ether, and the ether extracts combined and evaporated over a steam bath employing negative pressure to prevent condensation. The resulting yellow residue was presumably the DNP-amino acid derivatives.

A two dimensional chromatography system was employed in this analysis. The apparatus for the first dimension consisted of a cylindrical glass container 12" in diameter and 24" high. Placed in the bottom of this cylinder were two crystallizing dishes, one 150 x 75 mm and the other 190 x 100 mm. The larger dish was supported by a ring of rubber tubing, while the smaller dish was placed inside the larger one. The chromatography paper used was Whatman No. 1 filter

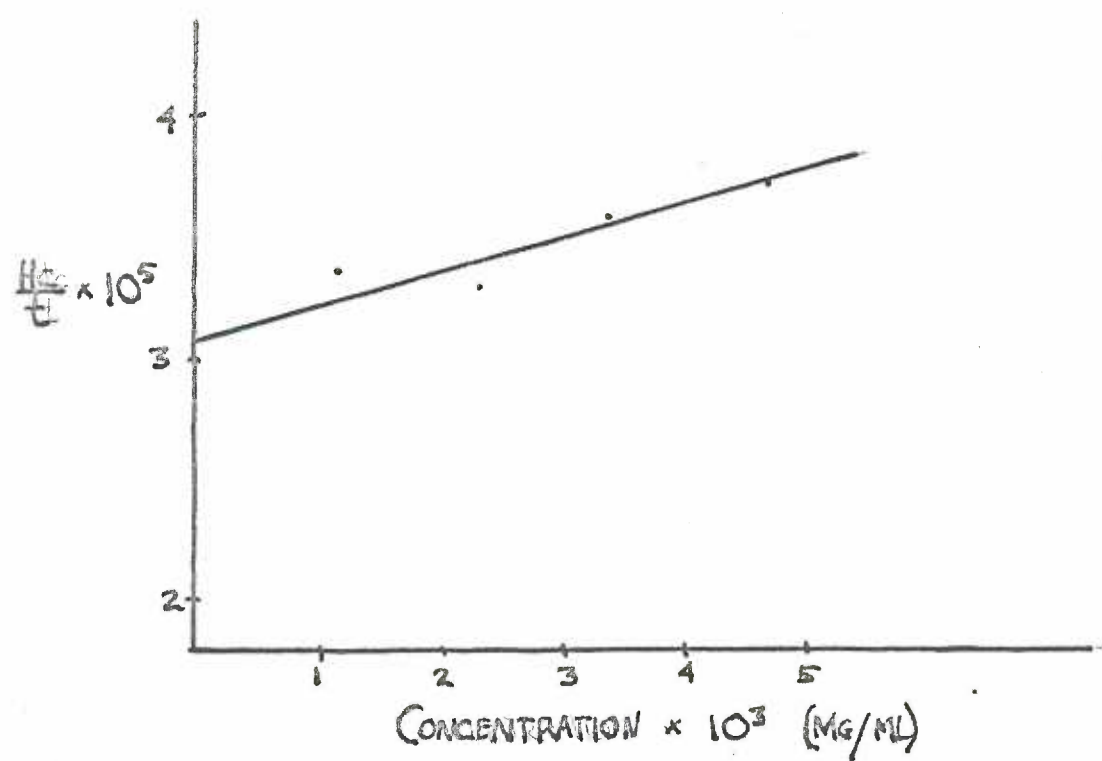
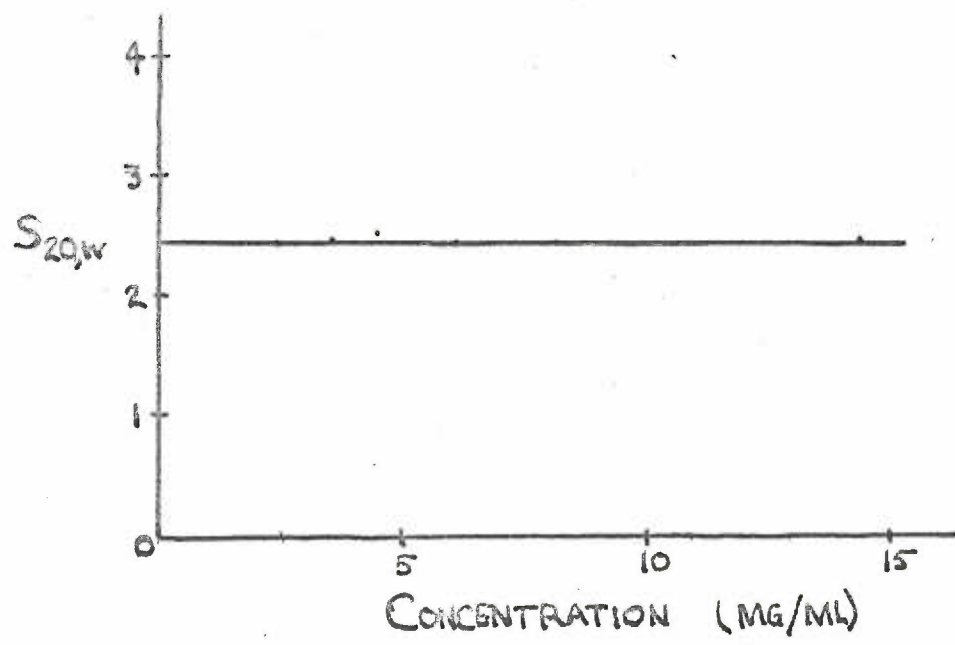
paper,  $18\frac{1}{4}$ " x  $22\frac{1}{2}$ ", which was rolled into a cylinder  $18\frac{1}{4}$ " high with a 1" overlap. The paper was fastened by staples, and a rectangle 2" high and 1" long was removed from the bottom edge in such a manner so as to prevent the overlap from contacting the solvent. The solvent was prepared by mixing 30 mls of toluene, 9 mls of pyridine, and 18 mls of 2-chloroethanol, and adding 18 mls of 0.8 M  $\text{NH}_3$  in such a manner as to prevent further mixing. This solution was allowed to sit approximately 2 hours before use. The DNP-amino acid was dissolved in a few drops of acetone and then applied to the paper 2" from the long edge and 5" from the joint. The paper was then positioned in the large crystallizing dish and 200 mls of 0.8 M  $\text{NH}_3$  added to the small dish. The aqueous layer of the "toluene" solvent prepared above was discarded, and the organic layer introduced into the large dish by means of a long funnel. The cylinder was then closed, and the solvent allowed to ascend for 24 hours. At the end of that time the paper was removed, the solvent front marked, and the paper dried overnight at room temperature.

The second dimension consisted of 1.5 M sodium phosphate, pH 6.0, utilized in a descending manner. DNP-amino acid markers were run simultaneously with the unknown amino acid derivatives. Descending chromatography required 24 hours, at the end of which time the paper was removed, the solvent front marked, and the chromatograph dried.  $R_f$  values were

Figure 9. The dependence of the sedimentation coefficient of gamma crystallin upon concentration.

Figure 10. Graph of  $Hc/t$  versus concentration of gamma crystallin





then calculated and the probable identity of the DNP-amino acids established. Figure 11 shows a drawing of the chromatograph after descending chromatography, while Figure 12 shows the calculated  $R_f$  values of the markers and the unknown derivatives. As can be seen, the N-terminal amino acid of gamma crystallin is glycine. To more firmly establish the identity of the N-terminal amino acid, some of the gamma crystallin DNP-amino acid was spotted on a 19 x 57 cm Whatman No. 1 filter paper. A solvent of benzene: 1% acetic acid (1:1) was employed in a descending manner and allowed to run for 8 hours. The DNP derivatives of glycine, glutamic acid, and aspartic acid did not move in this solvent, but DNP-OH had an  $R_f$  of about 0.95. The chromatogram was then dried and the second solvent, 1.5 M phosphate, pH 6.0, was then run in a descending system. The  $R_f$  of the gamma crystallin DNP-amino acid corresponded to the  $R_f$  of the DNP-glycine marker.

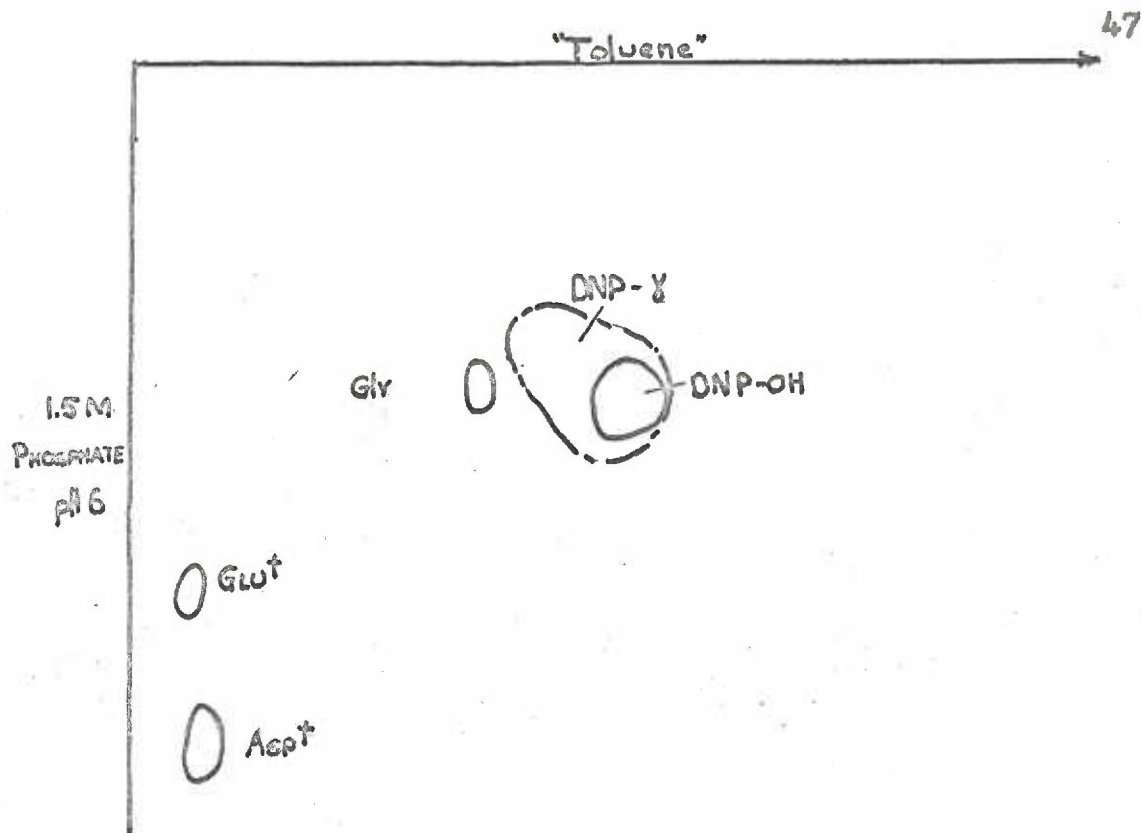
#### 9. Determination of Sulfhydryl Content

The spectrophotometric determination of sulfhydryl content of gamma crystallin was done according to the method of Boyer (13). The method is based on the increase in a absorbance at 250 mμ caused by the formation of a mercaptide from para-hydroxymercuribenzoate (p-HMB) and the reactive protein sulfhydryl group.

A relatively concentrated solution of gamma crystallin in 0.1 M phosphate, pH 6.8, was prepared and various dil-

Figure 11. Two-dimensional paper chromatogram of DNP-amino acids and DNP-amino acids from gamma crystallin.

Figure 12. Table showing the relative  $R_f$  values of the DNP-amino acids.



† CHROMATOGRAPHED ONLY IN PHOSPHATE BUFFER

MATERIAL	SOLVENT		
	1	2	3
DNP - OH	0.35	0.21	0.92
DNP - NH <sub>2</sub>	0.95	0.03	
DNP - GLU		0.34	0
DNP - ASP		0.42	0
DNP - GLY	0.24	0.21	0
DNP - $\gamma$	0.27	0.19	0

1. "TOLUENE"

3. BENZENE - ACETIC ACID

2. 1.5 M PHOSPHATE

pH 6.0

utions made. The resulting solutions were reacted with a constant and known amount of p-HMB in a 3 ml cuvette. After reaction had gone to completion in an hour, the OD 250 was read, and from this value was subtracted the absorbance due to p-HMB and protein. The resulting change in OD 250 was due only to the formation of the mercaptide, and was plotted versus concentration of gamma crystallin as seen in Figure 13. From the point of sharp change in slope, the number of sulfhydryl groups was determined to be 8.34 per molecule of gamma crystallin, assuming a molecular weight of 32,000.

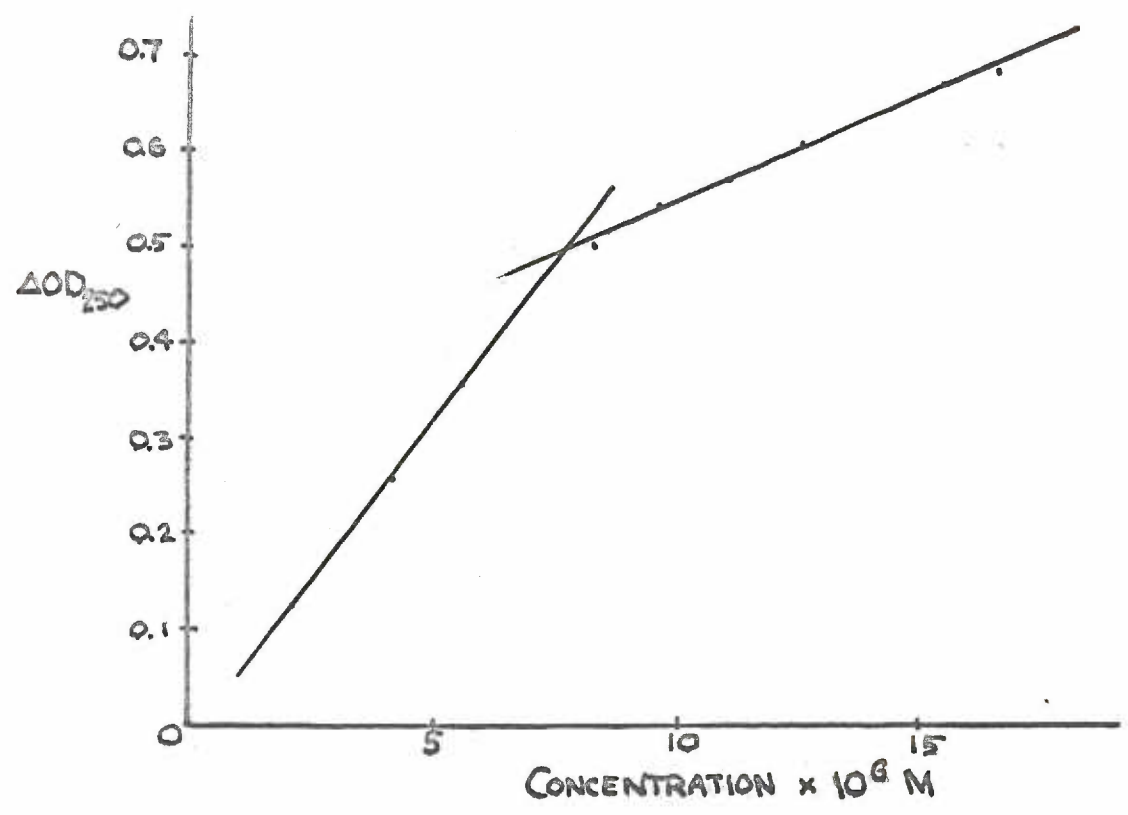
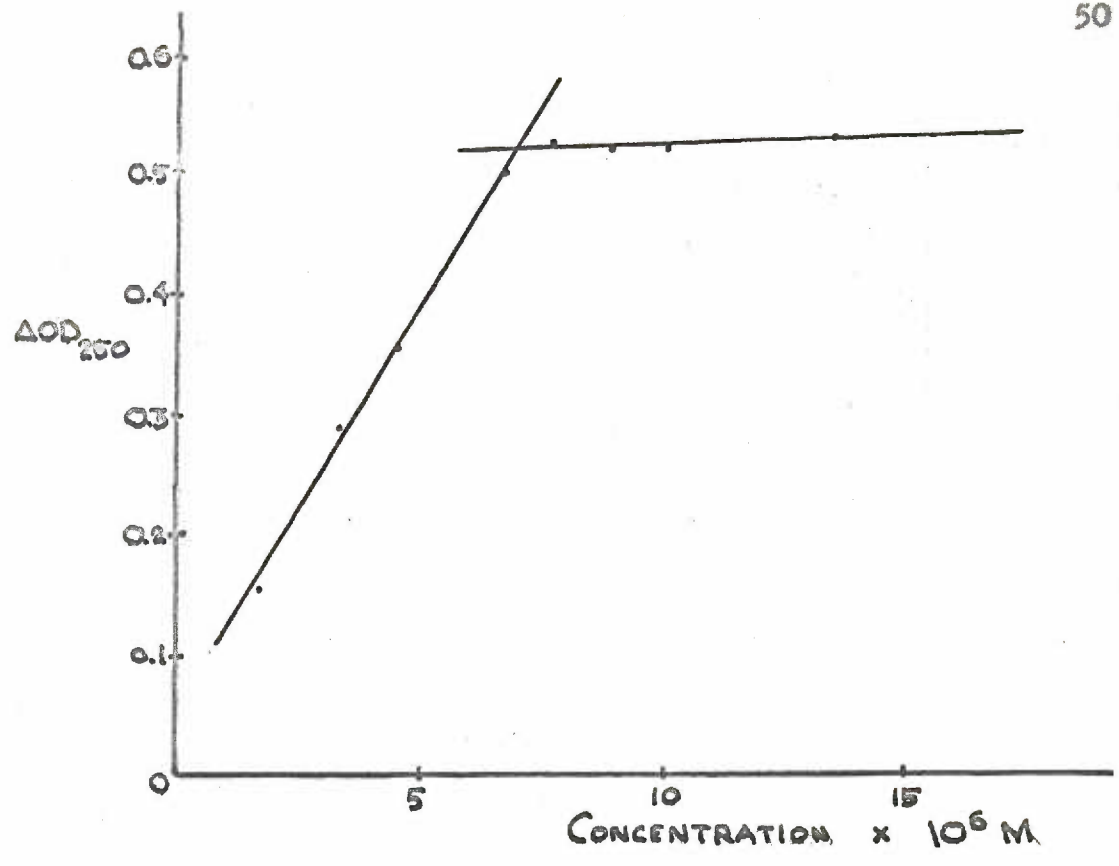
In order to determine the number of reactive sulfhydryl groups in denatured gamma crystallin the experiment was repeated, but with urea added. Sufficient urea was added to both the reaction mixtures and the blanks to make them 8 M. Thus the results obtained indicate the total number of sulfhydryl groups in the native protein plus those made available in the denatured state, or in the transition to this state. The results as seen in Figure 14, show that there are no additional sulfhydryl groups made reactive to p-HMB when protein is denatured by 8 M urea. Calculation of the data indicates that there are 8.28 sulfhydryl groups per molecule of urea-treated gamma crystallin.

#### 10. Bound Carbohydrate Content

The bound carbohydrate content was determined by the Minnesota method as described by Hodge (24). The method utilizes the reaction of any carbohydrate with phenol and

Figure 13. The increase in absorbance at 250 mμ versus concentration of native gamma crystallin.

Figure 14. The increase in absorbance at 250 mμ versus concentration of urea-treated gamma crystallin.



sulfuric acid, and the formation of an unknown compound which absorbs at 480 mμ.

Three samples of approximately 10 mg each of lyophilized gamma crystallin were weighed and dissolved in 1 ml of water. Two blanks and two standards containing 50 mg of galactose were also prepared. To each of the samples was added 1 ml of a 5% phenol solution and 5 ml of 95% sulfuric acid. The tubes were then shaken and allowed to stand in a water bath at 25-30° for 20 minutes. At the end of this period the absorbances at 480 mμ were read and compared to a previously prepared standard curve. Calculations from these data indicate that the amount of bound carbohydrate in gamma crystallin is 0.343% by weight, which corresponds to 0.612 moles of carbohydrate per mole of protein, assuming an average molecular weight of carbohydrate of 180. Thus there is little loosely bound carbohydrate present in gamma crystallin.



## DISCUSSION AND CONCLUSION

The examination of the gamma crystallin fraction of rabbit lens proteins can be divided into three categories. Some of the data obtained indicates that this fraction is a homogeneous protein, some that it is heterogeneous, and some gives no evidence for either hypothesis.

In the research presented in this paper, the analysis of cortical gamma crystallin was undertaken in order to avoid any possible difference between cortical and nuclear proteins. In addition, the cortical gamma crystallin was chosen because it is the most recently formed gamma crystallin.

The significance of the brown precipitate, which appears only upon dialysis of the ammonium sulfate precipitate of gamma crystallin, is unknown. No other researcher has yet performed this type of concentration procedure on gamma crystallin, and thus not recorded this phenomena. The precipitation can and should be avoided by concentrating the protein using ultrafiltration.

The extinction coefficient of 17.6 determined for gamma crystallin is in the range of other proteins, and is close to the value of approximately 21 found by Bjork for his four fractions of gamma crystallin (5). The ultraviolet spectrum is a normal protein spectrum due to the absorption of the amino acids tryptophan, tyrosine, and phenylalanine.

The extrapolation of light scattering data to zero concentration of gamma crystallin gives a value for the molecular weight of 32,000. The accuracy of this method is probably no better than  $\pm 10\%$  (41), but the value obtained is close to the value of 20-25,000 determined by Bjork on calf lens gamma crystallin in 1961 (3), although it is higher than his value of 19,000 determined later(5) .

Possible sources of error in light scattering determination are dissymmetry of the molecule, depolarization of the incident beam, and anomalously high results due to dust particles in the solution. Dissymmetry of the molecule was not corrected for because it was assumed that gamma crystallin behaved as a point scatterer. The correction for depolarization was approximately 0.1, but did decrease with decreasing concentration, as expected. Before each solution was analysed, it was filtered through a 0.22  $\mu$  millipore filter and visually examined for dust particles. The data obtained, as seen in Figure 10, falls within the molecular weight range of 26,600 to 31,500. Although there appears to be a decreasing value of  $H_c/t$  (and thus increasing molecular weight) with decreasing concentration, the amount of data accumulated and the degree of difference between values obtained is insufficient to give a definite indication of any dependence of molecular weight upon protein concentration.

The sulfhydryl determination yields results which indicate that the denaturation of gamma crystallin exposes no additional sulfhydryl groups for reaction with para-hydroxymercuribenzoate. The number of sulfhydryl groups found per mole of protein was approximately 8.3, while Bjork found 9.8 moles of sulfhydryl per mole of gamma crystallin (3). These figures closely correspond to the average value of the cysteine contents of the gamma crystallin fractions found by Bjork in 1964 (5). He found 8.7 moles of cysteine per mole of protein, assuming a molecular weight of 32,000.

The question of heterogeneity of the gamma crystallin was first raised by Bjork (3). The preponderance of evidence presented in this paper suggests that gamma crystallin is in fact a single, homogeneous protein. The sephadex chromatography and rechromatography of the protein at pH 6.8 as seen in Figures 2 and 3 shows only one symmetrical peak. Essentially identical results were obtained by Bjork both in 1961 and 1964 (3,5). The ultracentrifugal pattern of gamma crystallin at various concentrations, as typified in Figure 8, shows a single, symmetrical sedimentation boundary. The  $S_{20,w}$  values obtained from various runs were calculated as described previously. The plot of  $S_{20,w}$  versus concentration of protein shows that there is no dependence of the sedimentation coefficient upon concentration. Bjork also observed a concentration independence of  $S_{20,w}$  between concentrations of 2 and 12 mg/ml (3). His value of

$S_{20,w}$  at zero concentration was 2.36 S, close to the 2.4 S value obtained in this research. The concentration independence of the sedimentation coefficient can be explained by either of two reasons. Firstly, there may be no interaction between the protein molecules, thus no particular form, i.e. monomer or polymer, is favored by low concentration. The second possibility is that there are negating interactions. The effects due to protein-protein interaction, protein-ion interaction, and protein-water interaction may all be operative, but may be of such magnitude that the sum of them is zero. At this point the actual reason for these results is speculative, and the elucidation of the explanation is beyond the scope of this paper.

The solubility of gamma crystallin in ammonium sulfate solutions of varying percent saturation, as seen in Figure 7, shows a single, sharp decrease beginning at 47.5% saturation. This experiment is another piece of evidence indicating that gamma crystallin is a homogeneous protein.

The fact that the N-terminal studies show only one amino acid, glycine, favors the hypothesis that gamma crystallin is a single protein. Again, Bjork obtained identical results, but, in addition, determined the N-terminal amino acid sequence (3). He found only one sequence of gly, glu, leu or ileu in gamma crystallin of bovine lens proteins. Thus the indications from these experiments are that a single gamma crystallin protein exists.



The case for homogeneity of the gamma crystallin protein is by no means complete, for there is evidence to the contrary. Experiments done on the ion exchange chromatography of the protein show that there are four major fractions. Phosphocellulose chromatography conducted in this laboratory (see Figures 4 and 5) yields four components. The elution was accomplished by a step-wise pH gradient between pH's 6.0 and 6.8 as described earlier. Although four major fractions were observed in each experiment, the percentage of the total that each fraction represented was not constant. In the first experiment (depicted in Figure 4) the percentages were 24, 29, 29, and 17 from left to right, whereas in the latter experiment they were 26, 19, 44, and 11 respectively. In addition, the rechromatography of fraction III on phosphocellulose under identical conditions yields results which are not repeatable between experiments. The reasons for this apparent change in composition of the total protein and of fraction III are unclear. Possibly the results are due to the inability to control the pH stringently enough, and consequently the protein changes in some manner.

In comparison, Bjork obtains four major components of gamma crystallin when a combination of sulfoethyl (SE)-sephadex and Phosphocellulose (PC) chromatography are employed (5). When the protein is eluted through a SE-Sephadex column at pH 5.0 using a linear gradient of ionic strength, four fractions are observed, one being a minor

component. The chromatography of the third fraction through a PC column at pH 3.0 yields two components, and of the fourth fraction at pH 6 to 7 also yields two components, although one is very minor.

The observed heterogeneity of gamma crystallin discussed above has been found on the basis of only one criteria, that of charge of the protein at various pH's. This heterogeneity might be a result of the protein's sensitivity of secondary or tertiary structure to environment, in particular pH and ionic strength. The change in structure caused by a change in environment could expose or mask different ionizing groups, thus changing the relative charge on the protein with which the ion-exchange group reacts. Hence, the gamma crystallin protein may exist as four or five differently charged species, all of which would have the same molecular weight, solubility in concentrated salt solutions, sedimentation coefficient, and N-terminal amino acid sequence.

That this theory is at least feasible can be substantiated by examining the changes of the lens during cataract formation. The chemical changes within the lens must indeed be small, for the lens is constantly in contact with the normal aqueous and vitreous humor. Yet the transmittance characteristics of the lens changes drastically during cataract formation. Thus either the order of the fibers or the size of the proteins are changing. Hence, a small environmental change causes a large change in protein size

or orderliness.

Another possible explanation of the data obtained is that gamma crystallin is composed of subunits which interact to form the various fractions. The particular combination of these subunits could determine the structure and thus the relative charge on the resulting molecule. The major objection to this theory is that no such subunit has been isolated. Both native gamma crystallin and all fractions of it isolated thus far have essentially the same molecular weight.

Yet another interpretation of the data is that gamma crystallin is heterogeneous and is composed of four different proteins. All four proteins would have the same N-terminal amino acid sequence, solubility, molecular weight, and sedimentation coefficient; but would have different sulfhydryl contents and small differences in amino acid composition. Again, an objection arises to this theory. The rechromatography of one of these proteins isolated by PC chromatography yields more than one component, but the results are not reproducible. Thus it would appear that the protein is either unstable or that it is not a different protein, but rather a differently charged state of the parent protein.

The tentativeness of any conclusions drawn from the work presented here or from the work done by other investigators cannot be over emphasized, for the data accumulated

to date is not complete enough to warrant the acceptance of one theory in favor of another. Thus, much work remains to be done before any firm theory of the structure of gamma crystallin can emerge.



## SUMMARY

The soluble rabbit lens proteins can be divided into three groups; alpha, beta, and gamma crystallin. In the work presented here the gamma crystallin fraction isolated by gel filtration was physically and chemically characterized. Some of the techniques used were also criteria of purity, from which conclusions were drawn as to the homogeneity of gamma crystallin.

The whole lens protein extract was eluted through a Sephadex G-75 column from which two peaks emerged. One of these was the alpha and beta fractions, and the other the gamma crystallin fraction. The protein prepared in this manner was subsequently concentrated either by ammonium sulfate precipitation or by ultrafiltration. Upon dialysis of the ammonium sulfate precipitate an insoluble brown material was observed. No meaningful significance could be attached to this observation, except that it was better avoided by using ultrafiltration of the gamma crystallin solution.

Phosphocellulose chromatography of the gamma crystallin protein showed four major components when eluted with a step-wise pH gradient of 0.1 M phosphate buffer, pH 6.0 to 6.8. Upon rechromatography of one of the fractions thus isolated, irreproducible results were obtained.

The extinction coefficient was determined to be 17.6

and the ultraviolet spectrum was a normal protein spectrum.

Light scattering data extrapolated to zero protein concentration showed the molecular weight of gamma crystallin to be approximately 32,000, while ultracentrifugal analyses at varying protein concentrations gave an  $S_{20,w}$  value at infinite dilution of 2.48 S. The ultracentrifugal patterns at all concentrations showed a single, symmetrical peak.

The N-terminal amino acid of gamma crystallin was determined by the F<sup>D</sup>NB procedure to be glycine.

The sulfhydryl content, as determined by the spectrophotometric method based on the formation of mercaptide between the protein sulfhydryl and para-hydroxymercuribenzoate, showed that gamma crystallin possesses approximately 8.3 sulfhydryl groups per molecule. The denaturation of the protein with 8 M urea did not expose any additional sulfhydryl groups.

The bound carbohydrate was determined by reacting the protein with phenol and sulfuric acid. Calculations from the data indicated the amount of bound carbohydrate in gamma crystallin was 0.343 % by weight.

The results obtained above were compared to the work of Bjork (3,5), and theories as to the explanation of the apparent homogeneity and heterogeneity discussed. The conclusion reached was that the acceptance of one theory over another was premature at this time, for there is still

insufficient evidence available.

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