STUDIES ON THE SUBUNIT STRUCTURE OF RABBIT LENS ALPHA CRYSTALLIN

by

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LIST OF ABBREVIATIONS

CM	Carboxymethyl
C-terminal	Carboxyl terminal
D_a	Apparent diffusion coefficient of a substance under conditions described in the text
DEAE	Diethylaminoethyl
DTT	Dithiothreitol
$D_{20,w}$	Diffusion coefficient of a substance corrected to standard conditions (water at 20°C). The concentration used is indicated at the appropriate place in the text. Units are in cm^2/sec.
$E_{280}^{1\%}$	Extinction coefficient of a 1% solution of the material at a wavelength of 280 nm.

$$E_{280}^{1\%} = \frac{(A_{280})}{(C)(L)}$$

where

A_{280} = optical density units

C = concentration = 1%

L = length of light path through the solution

GCl	Guanidine chloride
N-terminal	Amino terminal
S	Svedberg unit describing the sedimentation rate of a substance with reference to a unit centrifugal field. The unit has the physical dimensions of time, each unit representing 1×10^{-13} sec.

SE	Sulfoethyl
s_{obs}	Observed sedimentation coefficient of a substance under conditions described in the text.
$s_{20,w}$	Sedimentation coefficient of a substance corrected to standard conditions (water at 20°C). The concentration used is indicated at the appropriate place in the text.
$s^{o}_{20,w}$	Sedimentation coefficient of a substance corrected to standard conditions (water at 20°C) and extrapolated to zero concentration.

INTRODUCTION

The lens of the eye has the function of providing a flexible, transparent medium through which light is transmitted and focused from outside the eye, into the eye. Knowledge of the structure of components in the lens both at the cellular and molecular level is essential to the understanding of how the lens performs its function. At the molecular level, it is well accepted by now that maintenance of the overall structure of a molecule is essential for maintenance of biological function.

The lens of the eye consists of about 98% protein and water (1). The total amount of protein in the lens varies with species and age. In adult mammals the concentration of protein is between 35-45%. The protein concentration in the lens nucleus is higher than in the cortex. In addition to water and protein, the lens is about 1% lipid and has less than 1% inorganic ions, the latter being mainly potassium ions.

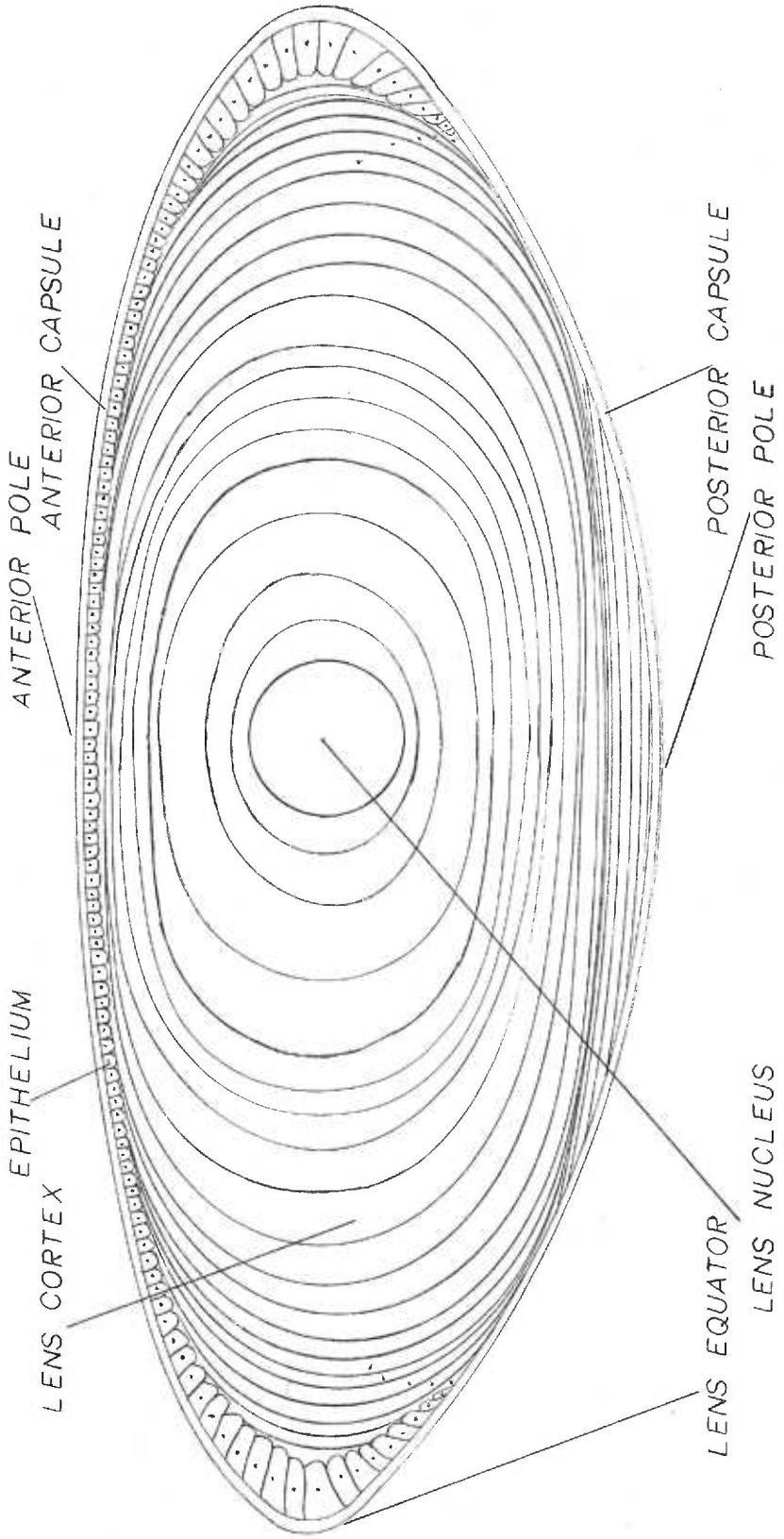
Because of the high protein content of the lens, the lens proteins have been implicated in the functional role of the lens. Thus it has been assumed that whatever changes occur when the lens becomes nonfunctional (e. g., cataract formation), should be accompanied by changes in the structure of the lens proteins. In order to

recognize and understand the changes in structure that occur when the lens proteins cease to be functional, a knowledge of the structure that obtains in the normal functional state is essential.

The lens of the eye (figure 1) is formed in the embryonic stage of development by a process of invagination of the surface ectoderm which leads to formation of a hollow capsule of epithelial cells in the optic cup. The cells in the posterior wall of the capsule continue to grow in an elongated fashion until the internal cavity is filled with elongated cells. These are called the primary lens fibers. A single layer of epithelial cells remains at the anterior of the lens and cell division in this layer is responsible for all future growth of the lens. The daughter cells from the epithelial layer are largely formed at or very near to the lens equator and once formed, start elongating and migrating inward, simultaneously losing their nuclei. The elongated cells are referred to as the lens fibers. As each fiber elongates, it curves over the older cells beneath it, the ends of the elongating cell moving in the general direction of the anterior and posterior poles of the lens.

The lens grows throughout the life span of the organism at a continuously decreasing rate. Since growth of the newly formed lens fibers forces the older fibers toward the nucleus, the nucleus becomes more dense with increasing age of the organism. The process is comparable to the renewal of the cells of the skin, though in

Figure 1. A diagram of the cross section of the lens.


ANTERIOR POLE
ANTERIOR CAPSULE
POSTERIOR CAPSULE
POSTERIOR POLE
EPITHELIUM
LENS CORTEX
LENS NUCLEUS
LENS EQUATOR

this case the cells are packed into the nucleus instead of being keratinized and sloughed off as are the cells of the skin.

The lens is covered by an elastic capsule believed to be secreted by the single layer of epithelial cells. Due to the mode of development of the lens, it has three distinct features (2).

1. It is cytologically isolated from its surroundings at an early embryonic stage.
2. It contains only epithelial cells at all stages of development, thus the proteins in the lens all originate from one type of cell, the epithelial cell.
3. Its growth never ceases and its weight increases throughout the life span of the organism.

The interest in lens proteins dates back to 1830 when Berzelius isolated a protein fraction of the lens which he termed crystallin (3). It was not until 1894 however, that a systematic study of the lens proteins was reported when Mörner described the separation of the lens proteins into two main fractions (4).

1. The soluble proteins which he further separated by isoelectric precipitation into the alpha and beta crystallin fractions (19.5% and 32% respectively of total protein).
2. The insoluble protein termed albuminoid (48% of total protein).

In 1927 Woods and Burky reviewed the preparative methods of

Mörner and studied the immunological and chemical properties of the unfractionated lens proteins and the alpha and beta crystallins (5). A year later the same investigators isolated gamma crystallin from the beta crystallin fraction (6). Alpha crystallin was isolated by precipitation at pH 5. 0. Beta crystallin was defined as the protein precipitating out at pH 6. 0. Gamma crystallin was defined as the protein remaining in the supernatant of the pH 6. 0 precipitation and removable by addition of ammonium sulfate to a concentration of 50% saturation. Since that time a variety of methods have been devised for the separation of the soluble lens proteins into the three classical fractions of alpha, beta and gamma crystallin. These methods employ procedures such as isoelectric precipitation, molecular sieve chromatography, gel electrophoresis, continuous flow electrophoresis and ultracentrifugation. A brief review of these methods has been made (7).

Of the three major lens proteins, alpha crystallin has been chosen by many investigators in the field of lens proteins because of its ready solubility in aqueous solvents. Alpha crystallin sediments fastest in an ultracentrifugal field and also migrates fastest toward the anode in an electrical field. The $s^{o}_{20,w}$ for alpha crystallin was calculated to be 19. 0 S (8). Calculations based on ultracentrifugal data indicate that the native alpha crystallin molecule has a molecular weight of about 1×10^{6} (9).

The cell has at least three general methods available for the synthesis of a protein such as alpha crystallin. These are:

1. Synthesis of the protein as one long continuous polypeptide chain.
2. Synthesis of identical subunits followed by assembly of the macromolecule.
3. Synthesis of a variety of subunits followed by assembly of the macromolecule.

In the case of alpha crystallin, evidence for the dissociation of the macromolecule has been reported (10, 11, 12). Thus it is likely that one of the latter two methods mentioned above obtains.

Work on the nature of the alpha crystallin molecule and more specifically, the type of subunits that comprise it has been going on in other laboratories where bovine lenses are used as the starting material. For example: Bon reported ultracentrifugal studies which indicated that alpha crystallin may consist of two subunits differing in shape but of equal molecular weight (13); Spector and Katz cited references which indicated that by free electrophoresis and sedimentation data, alpha crystallin appears homogeneous (9, 10, 14, 15, 16); however, their sedimentation equilibrium studies showed that independent species coexist under dissociating conditions pointing to an overall heterogeneity (15). Using DEAE-cellulose to fractionate alpha crystallin, several peaks were obtained. Amino

acid analysis of the material in these peaks showed the amino acid composition to be the same or very similar (17); Waley reported one thiol group per 20000 gm protein, no disulfide bonds and only one unique amino acid sequence around the thiol group (18). Later work indicated that at least two classes of proteins were present with the possibility existing that one of the classes was either an impurity or actually a different alpha crystallin (17). Wisse et al. found that the number of bands obtained by polyacrylamide gel electrophoresis of alpha crystallin in urea varied with the urea concentration (19). Palmer and Papaconstantinou obtained five alpha crystallin fractions from DEAE-cellulose. All five, however, had similar physicochemical properties and exhibited immunochemical identity (20).

Since the work described in this thesis was initiated, other reports on the subunit structure of bovine alpha crystallin have appeared. Chromatography of alpha crystallin on SE-sephadex at pH 3.2 resulted in the separation of the protein into two fractions labeled I and II (21). Both fractions were later shown to be composed of two polypeptide chains (22). Fraction I was termed acidic (A_1 and A_2), fraction II, basic (B_1 and B_2). Tests for SH showed all SH to be in fraction I to the extent of one mole SH per 21500 gm protein. The polypeptides in fraction I could be separated by SE-sephadex chromatography at pH 5.5. Subsequent analysis for SH showed that both A_1 and A_2 had one mole SH per 21500 gm protein.

This value, however, did not agree with the amount of cysteic acid found after performic acid oxidation. A_1 had 1.8 moles and A_2 had 1.6 moles of cysteic acid per 21500 gm protein. The suggested reason for this discrepancy was that the alkylation of the protein was not preceded by a reduction step. The alkylation reaction itself was not carried out under reducing conditions, and thus, oxidation of the SH groups during or prior to the alkylation reaction was possible.

Separation of fractions I and II was also achieved by isoelectric focusing of each fraction (22, 23). Thus, fraction I could be separated into A_1 and A_2 by isoelectric focusing in 6 M urea over the pH range 4-6. Isoelectric points were 5.60 and 5.92 respectively. Fraction II could be separated into B_1 and B_2 by isoelectric focusing in 6 M urea over the pH range 6-8. Isoelectric points observed were 7.42 and 7.07 respectively. Peptide maps of chymotryptic digests performed on A_1 and A_2, together with the amino acid analysis of both fractions, indicated that the only difference between the two components was most likely an amide group replacing the corresponding acid (22). Such a replacement may account for the slight difference in the isoelectric points.

Investigations on the molecular weight of dissociated bovine alpha crystallin calculated on the basis of ultracentrifugal experiments showed that the calculated molecular weights varied with the length of time that the protein was exposed to the denaturing

agent (24). The reported molecular weights for various subunits of alpha crystallin ranged from 11000 to 21300. The value of 21300 was for a fraction described as an acidic (A) fraction obtained by chromatography of S-carboxymethylated alpha crystallin on SE-sephadex in 6 M urea, pH 3.0. During the ultracentrifuge run the protein was in 6.0 M urea, 0.9 M NaCl, pH 5.6. The value of 11000 was for a component obtained by chromatography of the acidic (A) fraction on SE-sephadex in 6 M urea, pH 5.0. This was termed A_2 and during the ultracentrifuge run it was also in 6 M urea, 0.9 M NaCl, pH 5.6. No explanation was offered for the almost two-fold difference in the molecular weights obtained. In both cases the protein being examined in the ultracentrifuge had been in the 6 M urea solution for three days.

The reports on bovine alpha crystallin bring out the following points. The native molecule is large, having a molecular weight of about 1×10^6. The alkylated protein may be separated into two acidic components and two basic components by either of two methods; chromatography on SE-sephadex, or isoelectric focusing over the pH range 4-8. The total SH content appears in the fraction containing the two acidic components to the extent of one mole SH per 21500 gm protein. Separation of the acidic components into A_1 and A_2 and analysis for SH showed that both A_1 and A_2 had one mole SH per 21500 gm protein. These values were derived from

CM-cysteine determinations and were not in agreement with those obtained by determination of the cysteic acid content in performic acid oxidized alpha crystallin. The reason given for the difference was that the alkylation procedure employed was performed under conditions which would allow oxidation of SH. Determinations of the molecular weight by ultracentrifugation showed a range of values between 11000 and 21300, which varied with the length of time that the protein was exposed to the denaturing agent. Estimates of the molecular weight have been made on the basis of SH determinations. These estimates are based on the assumption that each polypeptide chain has only one SH. Values obtained from such estimates center around 20000. Since it has been shown by Shoenmakers et al. (21, 22, 23) that there are polypeptides of bovine alpha crystallin that do not contain SH, such estimates are undoubtedly erroneous.

Schoenmakers et al. who performed much of the recent work explained the discrepancy between the amount of CM-cysteine found and the amount of cysteic acid detected by postulating incomplete alkylation of the SH groups. This, in fact, is a main point of difference between their work and the work done in our laboratory. In the method used by Schoenmakers et al. (22), the alkylation step was not preceded by a reduction step. Furthermore, the alkylation procedure itself was not performed under reducing conditions.

When doing structural studies on proteins which serve similar

functions in several species, comparison of data from the different species is needed to allow deductions on the relation of observed structure of the protein to the function it serves. Such a comparison indicates which part or parts of the protein structure may or may not be varied without affecting the overall function. Thus it is useful to compare the structure of lens proteins from different species. In this way, an understanding is gained of the basic structure of the proteins that are required for performing the function of the lens.

Alpha crystallin was prepared in our laboratory from rabbit lenses according to the method of Mason and Hines (25), in which the alpha crystallin was separated from beta and gamma crystallin by continuous flow paper electrophoresis. The protein prepared by this method was compared with alpha crystallin from bovine lenses. The bovine alpha crystallin used in this comparison was also prepared by continuous flow paper electrophoresis. Criteria such as sedimentation coefficient, SH content and N-terminal studies showed that the material prepared in this manner from rabbit lenses has the classical characteristics of the protein designated as alpha crystallin from bovine lenses.

Ultracentrifuge experiments with the native and succinylated alpha crystallin showed apparent homogeneity of both in spite of the more than ten-fold larger S value for the native molecule (7). However, analysis of the C-terminal region of the succinylated alpha

crystallin by means of carboxypeptidase-A hydrolysis indicated that there was more than one unique C-terminal sequence. In general, these investigations and those conducted by others on bovine material could not unambiguously determine whether alpha crystallin subunits were different or similar (19, 20).

This thesis presents studies which were conducted on the subunit structure of rabbit alpha crystallin. The main point of these studies was to determine if the subunits of alpha crystallin were identical or nonidentical. It was realized that with a protein such as alpha crystallin, dissociation of aggregates was a prime consideration. Since the most common ways in which polypeptide chains aggregate are via secondary bonds and disulfide bridging, the dissociating conditions chosen were aimed at preventing both secondary bonding and disulfide bridging from occurring. Fractionation procedures were applied and appropriate methods were used to monitor the presence or absence of real fractionation. The fractions obtained were then analyzed to learn how they differed from each other.

MATERIALS AND METHODS

Reagents

DTT was obtained from Calbiochem, iodoacetamide came from Sigma Chemical Company, trypsin used was the 2 x crystallized preparation from Worthington Biochemicals. GCl was the ultra pure grade from Mann Research Laboratories and pyridine was reagent grade material from Merck & Company which had been redistilled from ninhydrin. All other reagents used were at least reagent grade.

Determination of Protein Concentration

Concentrations of protein solutions were determined by measuring their absorbance at 280 nm against appropriate solvent blanks with a Beckman DU spectrophotometer. The $E_{280}^{1\%}$ was used for obtaining actual concentrations. Native rabbit alpha crystallin has an $E_{280}^{1\%} = 8.3$. The above described method of determining protein concentrations was used only when direct weighing was not performed.

Preparation of Alpha Crystallin

Rabbit eyes were obtained from a local slaughterhouse. The genetic background of the rabbits was not known. Shortly after

death the eyeballs were removed, placed in 0.9% NaCl and transported to the laboratory. The back of the eyeball was slit from the point of the optic nerve down the side and the vitreous humor was pushed aside. The lens was then released from its attachment to the ciliary body with a pair of iris scissors and placed in distilled water. After 20 min the lens was transferred to another beaker containing distilled water where it was allowed to sit for another 20 min. Two 20 min periods in distilled water were included in the procedure to allow rabbit hair and other debris that had adhered to float away. The entire 20 min was not absolutely essential; it was merely a convenient interval. The lens was decapsulated by slitting the capsule and pushing the lens out, and then placed in 0.04 M Tris-HCl, pH 8.0, 10^{-3} M DTT. The mixture was mechanically stirred until the cortical portion of the lens dissolved away leaving the nucleus. After the nuclei were removed, the volume was adjusted with the same Tris buffer to have approximately 1 ml of extract per lens. The extract was homogenized for 1.5 min in a Potter-Elvejhem homogenizer and centrifuged to remove the remaining particulate material, then stored in the freezer overnight. The next morning the thawed out extract was again centrifuged to remove additional particulate material if any (a small amount of precipitate sometimes forms upon freezing) and the alpha crystallin fraction was obtained by continuous flow paper electrophoresis using the Beckman Spinco Model

CP Continuous Flow Electrophoresis Cell. Details of the continuous flow paper electrophoresis procedure are described by Mason and Hines (25). The appropriate material was pooled and precipitated by addition of ammonium sulfate (50 gm per 100 ml extract). The precipitate was dialyzed against four changes of water with at least 12 hrs between changes. The resulting aqueous solution was lyophilized. The material thus prepared was subjected to electrophoresis in a Spinco Durrum Paper Electrophoresis Cell. The latter procedure was merely a check on the results obtained with the continuous flow paper electrophoresis method.

Reduction and Alkylation

Earlier reduction and alkylation procedures employed 7 M urea as the denaturing agent instead of 6 M GCl. For reasons explained in the "Results" section, the use of 6 M GCl was preferred. The protein was dissolved in a minimum amount of 0.5 M Tris-HCl, pH 8.5, 6 M GCl. A 40-fold molar excess of reducing agent (DTT) over the calculated SH content was then added and the solution was magnetically stirred for 24 hrs at room temperature. Then a 10-fold molar excess of alkylating agent (iodoacetamide) over the reducing agent was added and the solution was magnetically stirred for 24 hrs at room temperature. All operations from the dissolving of the protein to this point were done under positive nitrogen pressure.

The excess reagents were dialyzed away against water and the resulting precipitate was brought back into solution by addition of solid urea with stirring. The precipitate which formed when the GCl was dialyzed away was insoluble even when the pH was raised to 12. Addition of urea solubilized the precipitate. Subsequent removal of the protein from urea solution by the method described below yielded a precipitate which was soluble in aqueous media.

Performic Acid Oxidation

A modification of the Sanger procedure was used (26). Eleven mg of unmodified alpha crystallin was dissolved in 1 ml of 88% formic acid. Then 0.06 ml of 30% hydrogen peroxide was added and the reaction was allowed to proceed at room temperature for 1 hr. Then 1 ml of water was added to the solution and mixed well. The protein was removed from solution by the method for removal of proteins from urea solutions described below. Yield was 7.6 mg oxidized protein (69%).

Determination of Extent of Alkylation

The amino acid content of unfractionated reduced, alkylated alpha crystallin was determined according to the amino acid analysis procedure described below. Values for CM-cysteine in alpha crystallin that had been reduced and alkylated in the presence of 7 M

urea were compared with those for alpha crystallin that had been reduced and alkylated in the presence of 6 M GCl. The results showed the latter procedure to be more effective than the former. Unless otherwise indicated, reduced, alkylated alpha crystallin used in the experiments described in this thesis was obtained by the latter procedure.

Removal of Protein from Urea Solutions

The precipitating agent consisted of acetone/1.0 N HCl, in a ratio of 39 ml/1 ml. The ratio of precipitating agent to protein solution was 16 ml/1 ml. After the initial addition of the precipitating agent, the mixture was ultrasonicated (Biosonik II) to ensure complete mixing and then centrifuged. The precipitate was washed three times with the precipitating agent, followed by washing (two times) with cold anhydrous ether. Drying was done in an incubator at 40°C overnight. This method was not used to remove proteins from GCl solutions because it also precipitated some of the GCl and a false high yield was obtained.

Determination of Sedimentation Coefficient

A 1% solution of reduced, alkylated alpha crystallin was made up in 0.1 M Millers buffer in 6 M GCl, pH 7.7. The buffer was prepared by mixing 467.6 mg NaCl, 16.53 µl concentrated HCl,

412.4 mg sodium barbital and 100 ml 6 M GCl. This buffer was selected because the physical characteristics had already been determined in the laboratory of Dr. D. A. Rigas. The protein solution was dialyzed against 0.1 M Millers buffer overnight to remove salts other than those in the buffer, that may have been present. The analysis was carried out using a double sector epon centerpiece, AN-D rotor, 59780 rpm (259610 x g) at 20°C. Schlieren optics was used to monitor movement of the boundary and the resultant patterns were recorded on Kodak metallographic plates. Calculations were performed according to the following equations (7, 27).

$$s_{obs} = \frac{1}{w^2} \quad \frac{d\ln X}{dt}$$

where

w^2 = angular velocity of rotor in radians/sec

= $(2\pi)(59780/60)$

X = distance in cm of component boundary from the center of rotation

t = time in sec (measured in min, therefore multiply by 60)

A plot was made of log X vs t in min and the slope was determined.

$$s_{obs} = \frac{(2.303)(d \log X)}{(w^2)(dt)(60)}$$

$$= \frac{(2.303/60)(d \log X/dt)}{[(2\pi)(59780/60)]^2}$$

$$= \frac{(2.303/60)(\text{slope})}{[(2\pi)(59780/60)]^2}$$

Under similar conditions, the only variable in a series of experiments is the slope; thus, a factor, y, was calculated where:

$$y = \frac{(2.303/60)}{[(2\pi)(59780)]^2} = 9.794 \times 10^{-10}$$

and

$$s_{obs} = (\text{slope})(y)$$

$$s_{20,w} = (s_{obs})(n_t/n_{20})(n/n_o)(1 - \bar{v}\rho_{,20,w}/1 - \bar{v}\rho_{,t})$$

where

(n_t/n_{20}) = ratio of viscosity of water at temperature of the experiment to the viscosity of water at 20°C

(n/n_o) = ratio of viscosity of the solvent to the viscosity of water

$\bar{v}$ = partial specific volume of bovine alpha crystallin
= 0.74 ml/gm (19, 24). The value of bovine alpha crystallin was used because no corresponding value for rabbit alpha crystallin has been determined.

$\rho_{20,w}$ = density of water at 20°C = 0.9982 gm/ml

ρ_t = density of solvent at temperature of the experiment

Determination of Diffusion Coefficient

The protein solution was prepared as described for the determination of the sedimentation coefficient. A double sector synthetic boundary epon centerpiece was used. The experiment was carried out with the AN-J rotor at 5227 rpm (1986 x g) at 20°C. Schlieren optics was used to observe the boundary and the patterns were recorded on Kodak metallographic plates. Calculations were performed according to the following equations (27).

$$D_a = \frac{(A_{sch})^2}{(Hm)^2} \quad \frac{1}{4\pi (t' + \Delta t)}$$

where

A_{sch} = area of schlieren peak in sq cm

Hm = height in cm of schlieren peak at peak maximum, value is proportional to dc/dx (concentration gradient) at boundary

t' = measured time in sec

Δt = zero time correction to compensate for imperfect boundary formation

A plot of $\frac{(A_{sch})^2}{(Hm)^2}$ vs t in sec was made and the slope was determined.

$$D_a (t' + \Delta t) = \frac{(A_{sch})^2}{(Hm)^2 (4\pi)}$$

$$D_a = \frac{\text{slope}}{4\pi}$$

At $t' = 0$,

$$\Delta t = \frac{(A_{sch})^2}{(Hm)^2 (4\pi)(D_a)} = \frac{\text{y intercept}}{\text{slope}}$$

$$D_{20,w} = D_a \frac{(293.2)}{(273.2 + t)} (n_t/n_{20})(n/n_o)$$

where

t = temperature in centigrade

(n_t/n_{20}) = ratio of viscosity of water at temperature of the experiment to the viscosity of water at 20°C

(n/n_o) = ratio of the viscosity of the solvent to the viscosity of water

Ion Exchange Chromatography

SE-sephadex was swelled in water over a steam bath for 3 hrs with occasional stirring. Fines were aspirated off and the SE-sephadex was equilibrated with 0.2 M sodium formate, pH 3.2, 10^{-3} M DTT batchwise. A slurry was prepared and the material was packed under gravity flow into precision bore air jacketed glass columns. Flow was regulated with a peristaltic pump (Harvard Apparatus Company). The effluent was monitored at 280 nm with a column monitor (Instrumentation Specialties Co., Model 222) connected to a recorder. Fractions were collected volumetrically (Gilson Medical Electronics Model V15[2]). The desired fractions were pooled and concentrated by ultrafiltration. The protein was

removed from urea solution by the method described previously. When a column was re-used, it was regenerated by pumping 5 bed volumes of 0.2 M sodium formate, pH 3.2, 10^{-3} M DTT, 7 M urea, 4 M NaCl through the column. This was followed by pumping 0.2 M sodium formate, pH 3.2, 10^{-3} M DTT, 7 M urea through the column until the effluent conductivity reading matched that of the influent.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed with the Canalco Model C apparatus. Basic gels (pH 8.9) were made up according to the directions in the Canalco manual. All solutions including buffers were made 7 M with respect to urea. A current of 5 milliamps per tube was used. Stain was 1% Buffalo Black in 7% acetic acid. Photographs were made with a studio camera.

Amino Acid Analysis

Samples were hydrolyzed with 5.7 N constant boiling HCl in sealed, evacuated ampules at 115°C for 17 hrs, then dried in a vacuum dessicator over sodium hydroxide pellets and silica gel. Amino acid analyses were performed with a Beckman Spinco Model 120 C amino acid analyzer (28). Peak areas were calculated by the height times width method. An integrator was available but in our experience, reproducibility was not reliable. The peak areas were

converted into μmoles by the use of color values obtained by analysis of an aliquot of the Beckman standard amino acid mixture. Standards for CM-cysteine and cysteic acid were obtained from Dr. N. R. M. Buist and diluted accordingly.

Peptide Mapping

Tryptic Hydrolysis

Five mg of the protein was dissolved in water at room temperature and the pH was adjusted to 8. 0 with 0. 1 M NH_4OH. Fifty μg of trypsin was added and the reaction mixture was automatically maintained at pH 8. 0 with a pH stat (Metrohm Combititrator 3D) using 0. 1 M NH_4OH. When a plateau level in the hydrolysis rate was reached (about 2 hrs) the reaction tube was immersed in boiling water for 5 min and then cooled in ice water. Another 50 μg of trypsin was added to ensure completeness of hydrolysis. When hydrolysis was complete (a total of about 4 hrs) the mixture was cooled to a point just above the freezing point and then evaporated to dryness in a vacuum dessicator over silica gel. The residue was taken up in 125 μl of 0. 1 M NH_4OH. In later experiments, a 50% solution of pyridine in water was used in place of 0. 1 M NH_4OH as a solvent for the dried peptides.

Paper Chromatography

Twenty-five μl of the tryptic hydrolysate (containing 1 mg of protein) was spotted on a strip of Whatman #3 MM chromatography paper and chromatographed (descending) with the solvent system, pyridine/water/n-propanol in the ratio of 15 ml/35 ml/50 ml for 20 hrs. The paper strip was air dried.

High Voltage Electrophoresis

High voltage electrophoresis was performed at a 90° angle to the direction of the previous chromatographic procedure. The air dried paper strip was sewn into appropriately sized sheets of the same paper. The whole sheet was wet as evenly as possible with a solution of pyridine/acetic acid/water in the ratio of 10 ml/0.4 ml/90 ml, pH 6.5. The sheet was placed in a Gilson Medical Electronics High Voltage Electrophorator Model DW and electrophoresed at 3000 volts for 30 min at an average of 400 milliamps. Buffer was the pH 6.5 pyridine/acetic acid/water solution described earlier and the coolant was Varsol. When the electrophoresis was over, the paper was air dried and dipped in a 2% solution of ninhydrin in acetone and air dried again. The spots which developed within 1 hr were encircled and the sheet was placed in an oven at 115°C for 5 min and any spots which were developed by this method were also noted. Peptides containing histidine were detected by spraying the

sheet with the Pauly reagent which was prepared by reacting 10 ml of 1% sulfanilic acid and 9 ml of 5% sodium nitrite for 5 min in an ice bath and neutralizing the solution by addition of 20 ml of 10% sodium carbonate. Peptides containing histidine stain red with the Pauly stain, whereas those with tyrosine stain blue with the Pauly stain. In our experiments, no accurate method was available for detecting the peptides that stained blue with the Pauly stain. This was due to the previous ninhydrin staining (purple) which had not completely faded.

The peptide maps were transferred to graph paper by drawing a grid over each peptide map and positioning the spots on the graph paper according to the coordinates they had on the peptide map. Spots which were very faint were not graphed. Photography of the peptide maps was also used to record the results. In the latter case, a 5 x 7 studio camera with high contrast pan film and an "A" filter was used.

Isolation and Amino Acid Analysis of Individual Peptides

The method of peptide mapping previously described was used with the following modifications.

1. Instead of spotting 25 µl of the tryptic hydrolysate, 75 µl was used.
2. Instead of dipping the dried paper, after electrophoresis,

in 2% ninhydrin in acetone, the paper was sprayed with a 0.2% solution of ninhydrin in acetone. The desired spots were encircled, cut out and washed thoroughly with acetone to remove the excess ninhydrin. The spots were sewn into paper strips and eluted with 5.7 N HCl. The eluates were sealed in ampules under vacuum and hydrolyzed at 115°C for 17 hrs. The hydrolysates were dried in a vacuum desiccator over silica gel and sodium hydroxide pellets. Amino acid analyses were performed as previously described.

RESULTS

Preparation of Alpha Crystallin

Alpha crystallin was prepared from 100 lenses. The total amount obtained was 1.978 gm. A sample was analyzed for homogeneity by paper electrophoresis in a Spinco Durrum electrophoresis cell. The results are shown in figure 2.

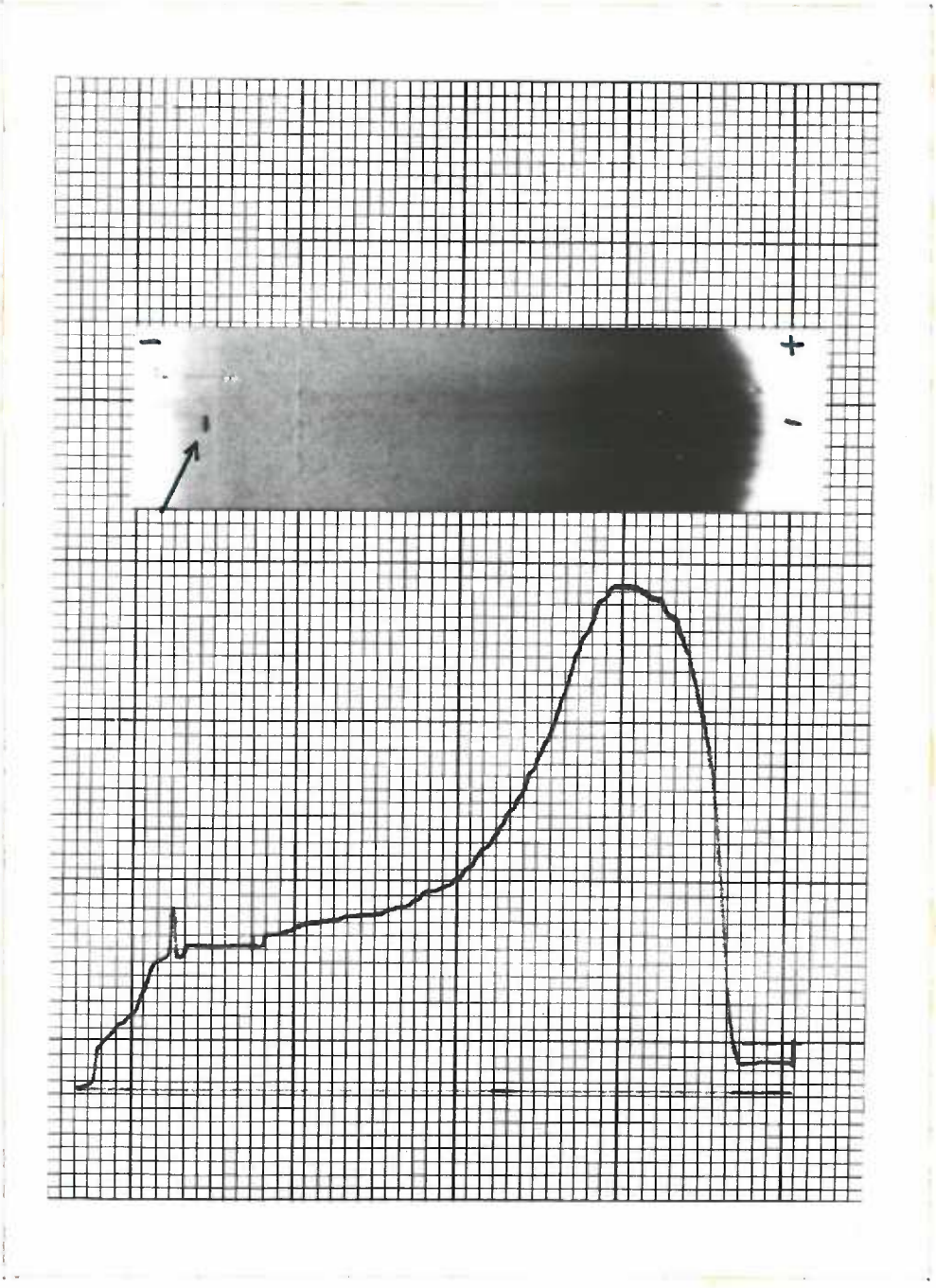
Performic Acid Oxidation

Unmodified alpha crystallin was oxidized with performic acid. The cysteic acid formed was determined after acid hydrolysis by means of the amino acid analyzer. The value obtained was 0.48 mole cysteic acid per 9737 gm protein.

Reduction and Alkylation

Alpha crystallin was reduced and alkylated using 2 denaturing agents, 7 M urea and 6 M GCl. Table 1 shows the amino acid composition for alpha crystallin reduced and alkylated by the two methods. Comparison of the amounts of CM-cysteine present shows that alkylation in the presence of 6 M GCl was essentially complete whereas that in the presence of 7 M urea was incomplete. For this

Figure 2. Paper electrophoresis of native alpha crystallin in a Spinco Durrum electrophoresis cell. Sample size = 0.6 mg; buffer = 0.075 ionic strength sodium barbital pH 8.6; current = 2 mA per cell; origin at arrow. The tracing shown is from a densitometer scan of the paper strip.



plotting these values versus the tube number. The gamma crystallin fraction was dialyzed against distilled water and lyophilized. The alpha plus beta crystallin fraction was pooled and concentrated either by ultrafiltration or precipitation by addition of enough $(NH_4)_2SO_4$ to obtain 73% saturation followed by dialysis of the precipitate against distilled water to remove the salt. The alpha plus beta crystallin fraction was then separated by continuous flow electrophoresis using the method of Mason and Hines (46). The protein peaks were again located by measuring the OD at 280 mμ and plotting the values versus tube number. The separated protein fractions were precipitated by addition of $(NH_4)_2SO_4$ (73% saturation) and dialyzed against distilled water. The resulting aqueous solutions of the proteins were lyophilized.

Carboxypeptidase-A Hydrolysis

Twenty to fifty mg of the protein to be analyzed was dissolved in 2.0 ml of 0.1 M CH_3COONH_4, pH 7.5 (ammonium acetate buffer) and the pH of the solution was adjusted back to 7.5. In cases where the starting material was in solution, the solution was dialyzed against the ammonium acetate buffer and the volume adjusted by dilution or ultrafiltration to give a protein concentration of 10-25 mg per ml. Two and one-half ml of the protein solution was then placed in a tube suspended in a water bath maintained at 27°C and allowed to stand for 5 min. The enzyme suspension was added and the solution quickly mixed by magnetic stirring. Within 10 sec 250 microliters (μl) was withdrawn

reason, the former method was used in the preparation of reduced alkylated alpha crystallin for the studies of subunit structure which follow.

Ion Exchange Chromatography

Three forms of alpha crystallin were chromatographed on SE-sephadex C-50 under similar conditions. The results obtained with unmodified alpha crystallin and alpha crystallin reduced and alkylated in 7 M urea are shown in figures 3 and 4 respectively. Figures 5 and 6 show the results obtained with alpha crystallin reduced and alkylated in 6 M GCl. As figure 5 shows, the separation of fraction I from fraction II was not complete. Thus, when the separation procedure was repeated, a longer column and shallower NaCl gradient were used. The longer column allowed the protein to go through more theoretical plates and the shallower gradient allowed a higher ratio of buffer volume to the change in ionic strength. Figure 6 shows that the separation was improved. Conditions pertaining to each run are in the captions opposite each figure. No analytical work was done on material in the peak before fraction I because it is believed to be a dimer of alpha crystallin subunits. Estimation of the extent of reduction and alkylation of alpha crystallin in different denaturing media showed that the material used in figures 5 and 6 was most efficiently reduced and alkylated (table 1). Material used

Figure 3. SE-sephadex C-50 chromatography of unmodified alpha crystallin. Sample size = 101 mg; column size = 1.0 x 18.0 cm; buffer = 0.2 M sodium formate, pH 3.2, 7 M urea, 10^{-3} M DTT; elution gradient = 0.0-0.2 M NaCl linear gradient in sodium formate buffer; flow rate = 10 ml per hr; fraction volume = 10 ml; volume per cylinder in gradient former = 200 ml.

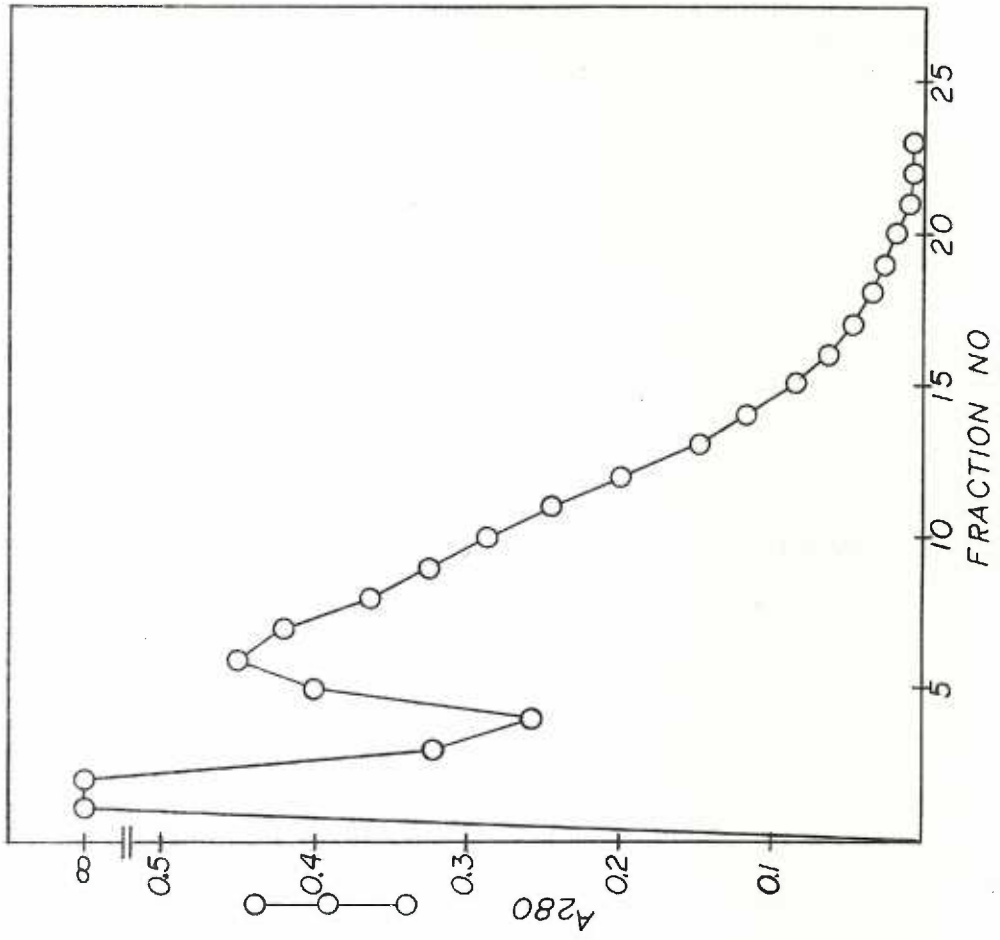

A280
∞
0.5
0.4
0.3
0.2
0.1
5
10
15
20
25
FRACTION NO

Figure 4. SE-sephadex chromatography of alpha crystallin reduced and alkylated in the presence of 7 M urea. Sample size = 51 mg; column size = 1.0 x 17.0 cm; buffer = 0.2 M sodium formate, pH 3.2, 7 M urea, 10^{-3} M DTT; elution gradient = 0.0-0.2 M NaCl linear gradient in sodium formate buffer; flow rate = 10 ml per hr; fraction volume = 10 ml; volume per cylinder in gradient maker = 200 ml.

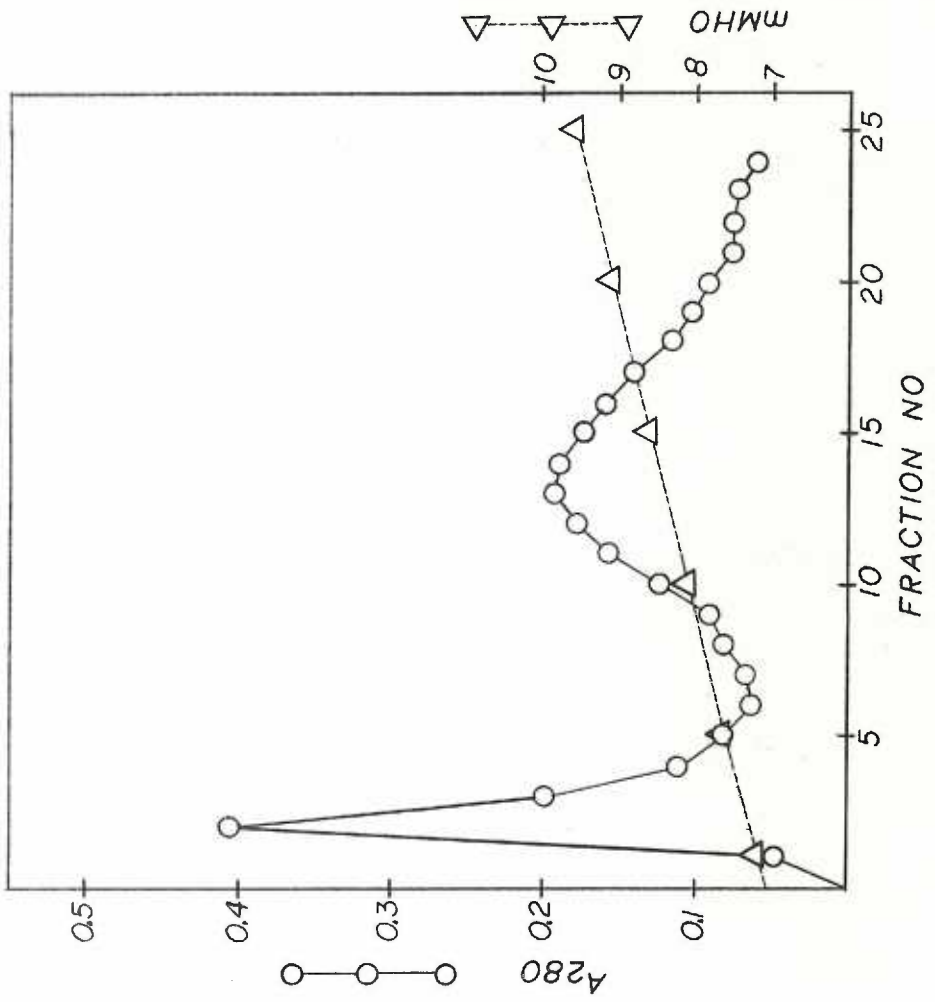

mMHO
10
9
8
7
A280
0.5
0.4
0.3
0.2
0.1
5
10
15
20
25
FRACTION NO

Figure 5. SE-sephadex chromatography of alpha crystallin reduced and alkylated in 6 M GCl. Sample size = 150 mg; column size = 1.0 x 30.0 cm; buffer = 0.2 M sodium formate, pH 3.2, 7 M urea, 10^{-3} M DTT; elution gradient = 0.0-0.2 M NaCl linear gradient in sodium formate buffer; flow rate = 10 ml per hr; fraction volume = 10 ml; volume per cylinder in gradient maker = 200 ml.

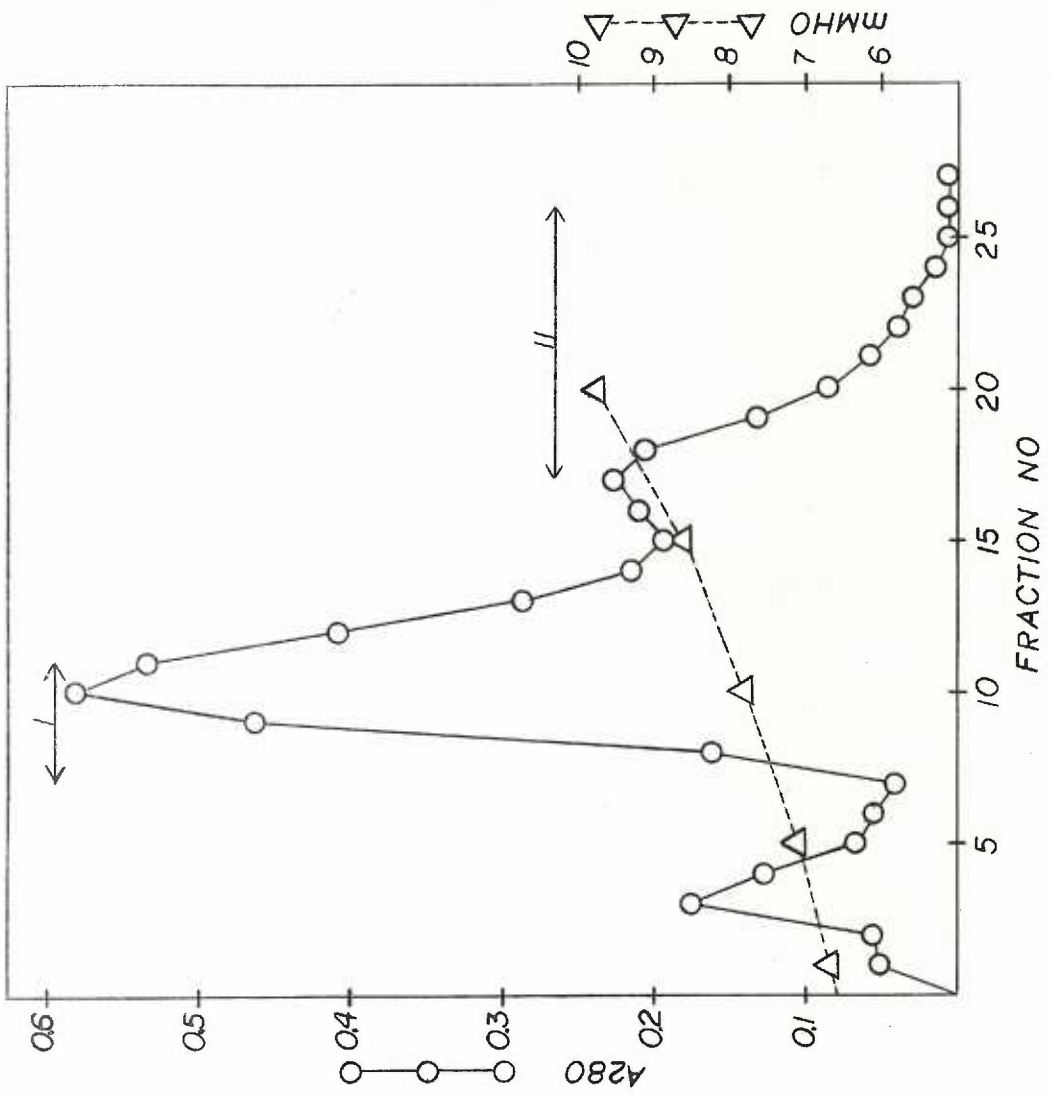
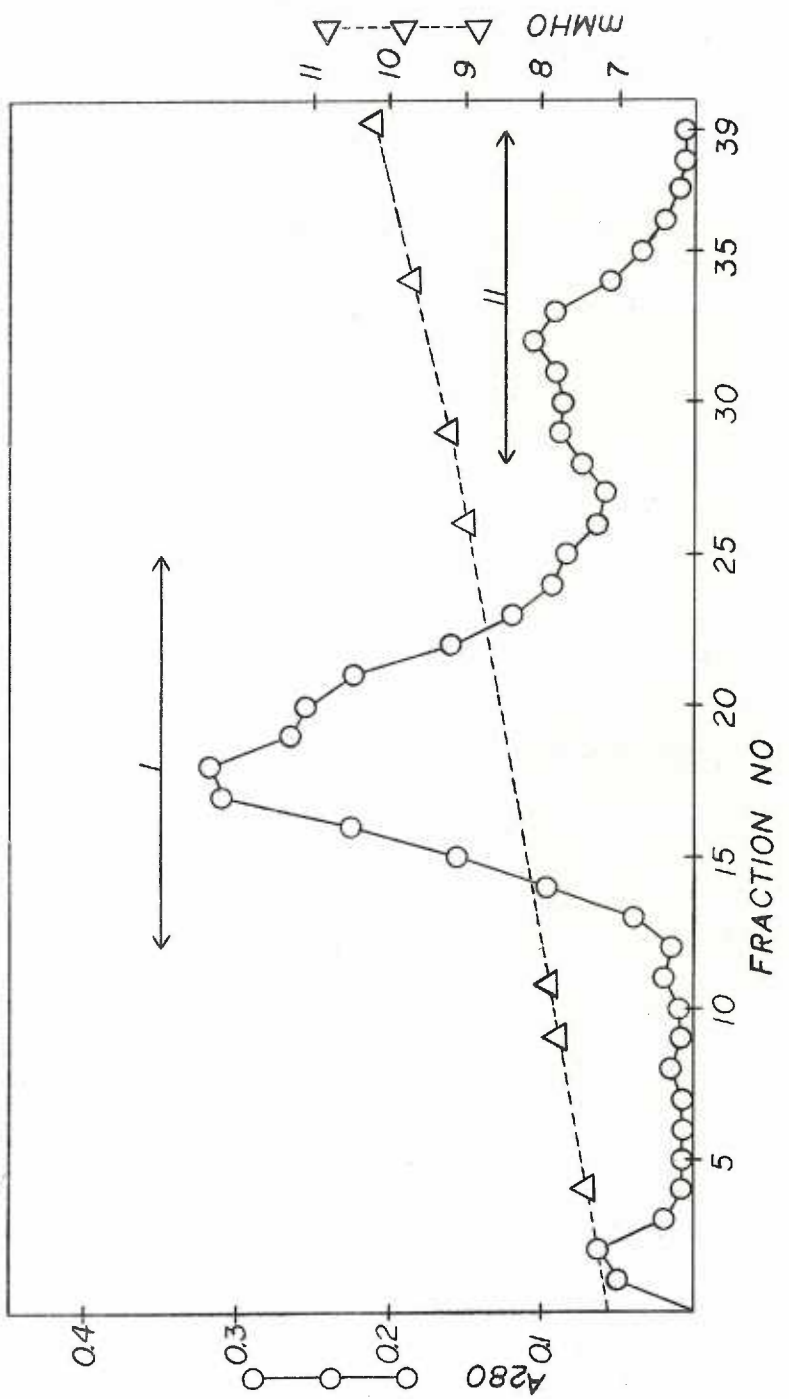

A280
0.6
0.5
0.4
0.3
0.2
0.1
mMHO
10
9
8
7
6
FRACTION NO
5
10
15
20
25
I
II

Figure 6. SE-sephadex chromatography of alpha crystallin reduced and alkylated in the presence of 6 M GCl. Sample size = 172 mg; column size = 1.0 x 70.0 cm; buffer = 0.2 M sodium formate, pH 3.2, 7 M urea, 10^{-3} M DTT; elution gradient = 0.0-0.2 M NaCl linear gradient in sodium formate buffer; flow rate = 10 ml per hr; fraction volume = 10 ml; volume per cylinder in gradient maker = 500 ml.


mMHO
11
10
9
8
7
$10 A_{280}$
0.4
0.3
0.2
FRACTION NO
5
10
15
20
25
30
35
39
I
II

in figure 3 was not reduced and alkylated. Comparison of figures 3, 4, 5, and 6 shows that with increasing extent of reduction and alkylation, the amount of material in the first peak is diminished. Total recovery from column for experiment in figure 6 = 88.2 mg (51%) as follows: fraction I = 48.6 mg; fraction II = 39.6 mg.

Polyacrylamide Gel Electrophoresis

Fractions I and II from the separations in figures 5 and 6 were subjected to polyacrylamide gel electrophoresis in 7 M urea. Figure 7 shows the results obtained. Conditions of the experiment are given in the caption. Figure 8 is a diagram of the results in figure 7.

Peptide Mapping

Peptide maps were prepared of fractions I and II from the separations in figures 5 and 6, and the unfractionated reduced, alkylated alpha crystallin. The results are shown in figures 9-18 as follows:

Figure 9 Photograph of peptide map "U"

Figure 10 Diagram of peptide map "U"

Figure 11 Photograph of peptide map I/5

Figure 12 Diagram of peptide map I/5

Figure 13 Photograph of peptide map II/5

Figure 7. Polyacrylamide gel electrophoresis of fractions I and II of reduced and alkylated alpha crystallin from a SE-sephadex column. Unfractionated reduced, alkylated alpha crystallin and unmodified alpha crystallin were also electrophoresed for purposes of comparison. Gel = 10%, 7 M urea, pH 8.9; current = 5 mA/tube; electrophoresis time = 1 hr, 40 min for I/5 and II/5; 2 hrs for other samples. Samples are identified below.

N	50 μg unmodified alpha crystallin
U	50 μg unfractionated reduced, alkylated alpha crystallin
II/6	70 μg fraction II from figure 6
I/6	70 μg fraction I from figure 6
II/5	50 μg fraction II from figure 5
I/5	50 μg fraction I from figure 5

The absolute distance traveled by components in I/5 and II/5 is different from the other samples because the gels were run at a different time. The gels used were also prepared from a different batch of starting materials and there may be differences in percentage of cross linking achieved.

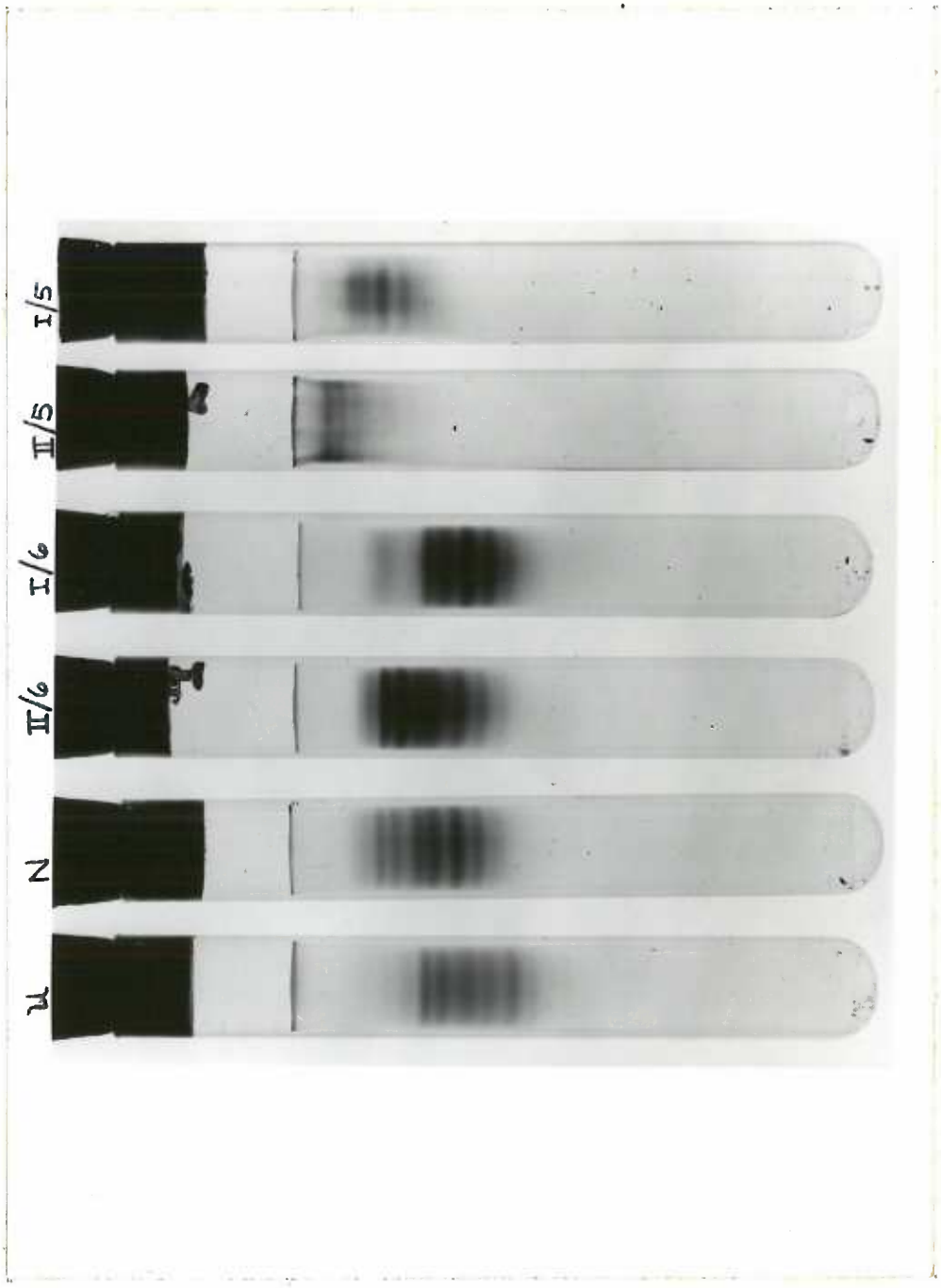

I/5
II/5
I/6
II/6
N
U

Figure 8. Diagram of the results in figure 7. The numbering of the bands is arbitrary and was done only for identification.

N	50 μg unmodified alpha crystallin
U	50 μg unfractionated reduced, alkylated alpha crystallin
II/6	70 μg fraction II from figure 6
I/6	70 μg fraction I from figure 6
II/5	50 μg fraction II from figure 5
I/5	50 μg fraction I from figure 5

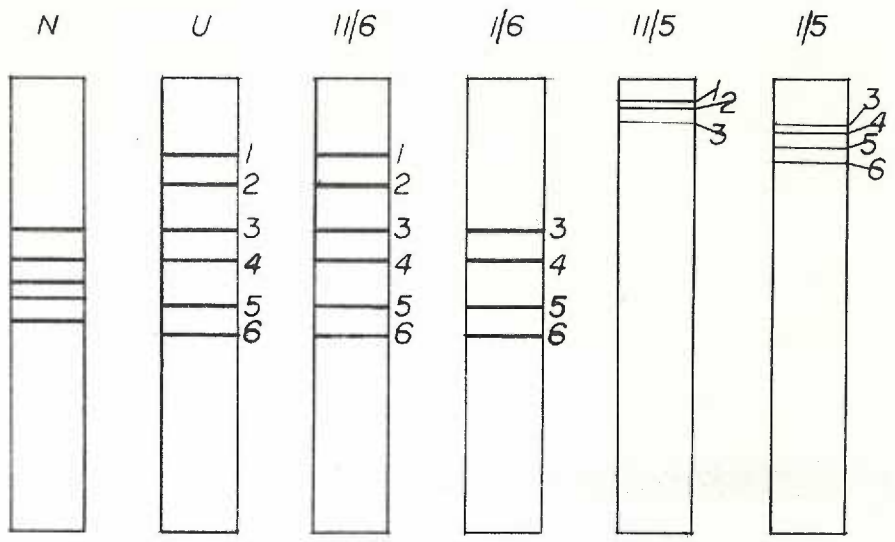

N
U
II/6
I/6
II/5
I/5
1
2
3
4
5
6

Figure 9. Peptide map of tryptic hydrolysates of unfractionated reduced, alkylated alpha crystallin. Chromatography is from top to bottom with pyridine/water/n-propanol solvent system described in text. Electrophoresis was done with the positive electrode on the left, negative electrode on the right. Sample size = 1 mg; current = about 400 mA; voltage = 3000 volts; electrophoresis time = 35 min; buffer = pyridine/acetic acid/water pH 6.5, composition described in text. These general conditions also apply to figures 11, 13, 15, and 17. Arrow indicates origin (top).

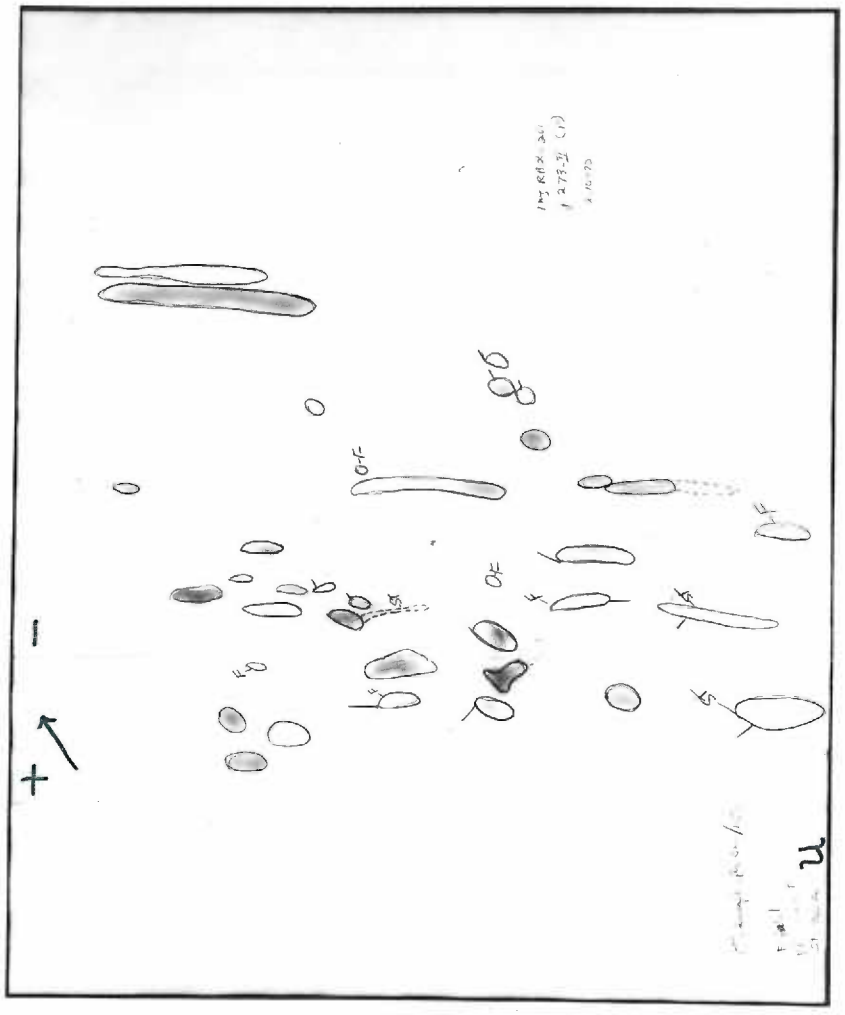
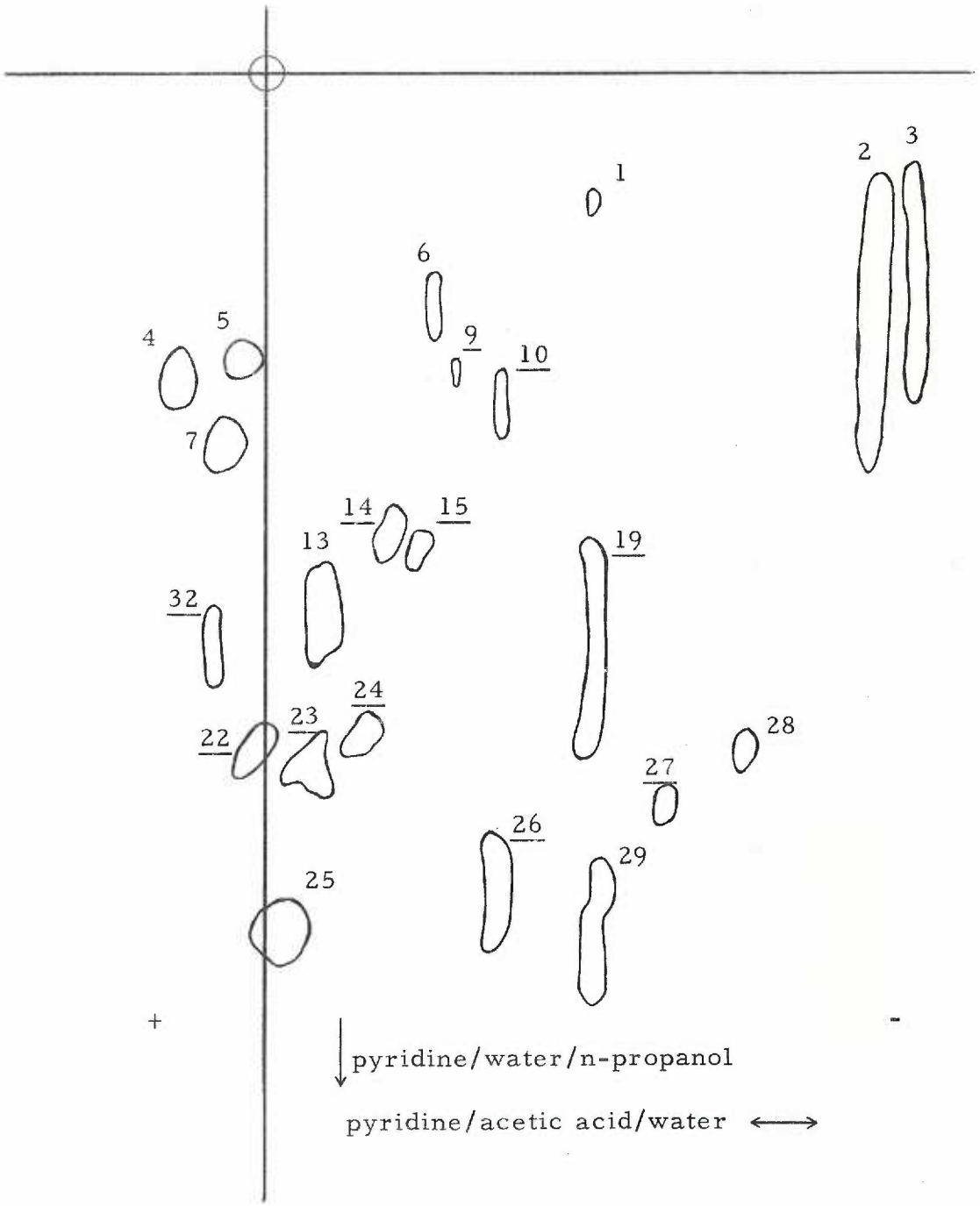


Figure 10. Diagram of the peptide map of a tryptic hydrolysate of unfractionated reduced, alkylated alpha crystallin shown in figure 9. Not all the spots which were encircled in figure 9 were graphed because it could not be accurately determined if the faint spots were actual spots or just streaks from other spots. Spot #32 was ninhydrin negative, but stained red with the Pauly stain. Underlined numbers indicate spots which stained red with the Pauly stain.



Reduced, alkylated alpha crystallin (U)

Figure 11. Peptide map of tryptic hydrolysate of fraction I/5. Conditions are the same as those listed for figure 9.

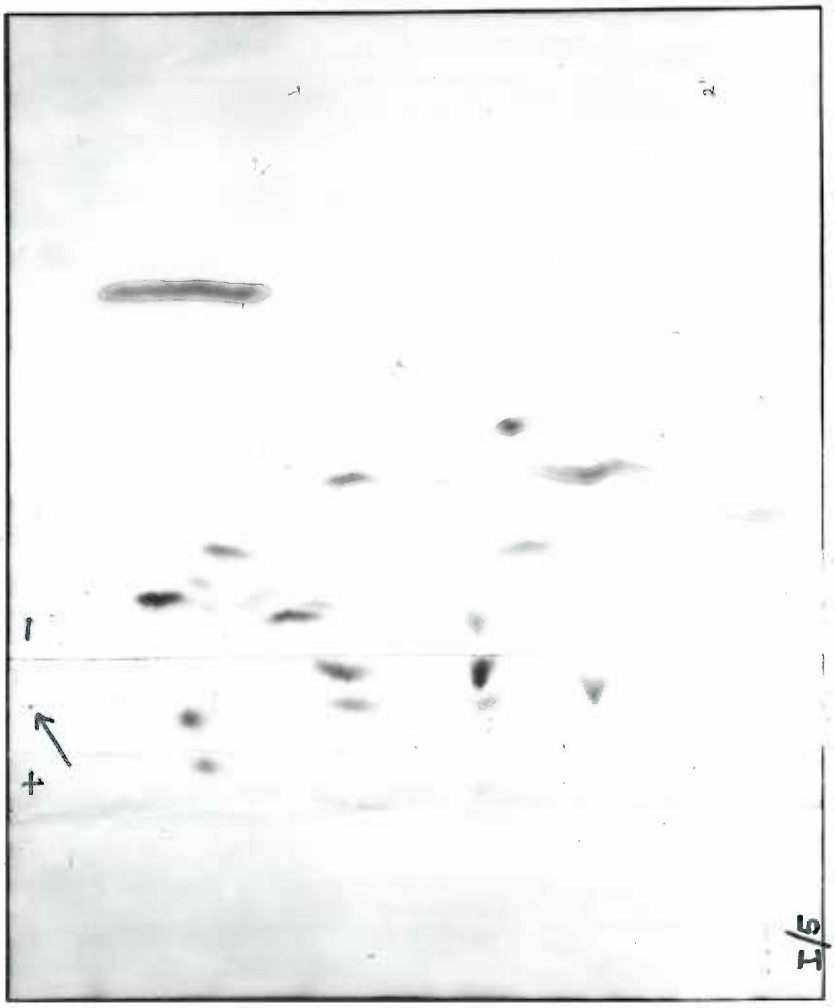

1
+
H/5

Figure 12. Diagram of the peptide map of a tryptic hydrolysate of fraction I/5 shown in figure 11. Spot #32 was ninhydrin negative but stained red with the Pauly stain. Underlined numbers indicate spots which stained red with the Pauly stain.

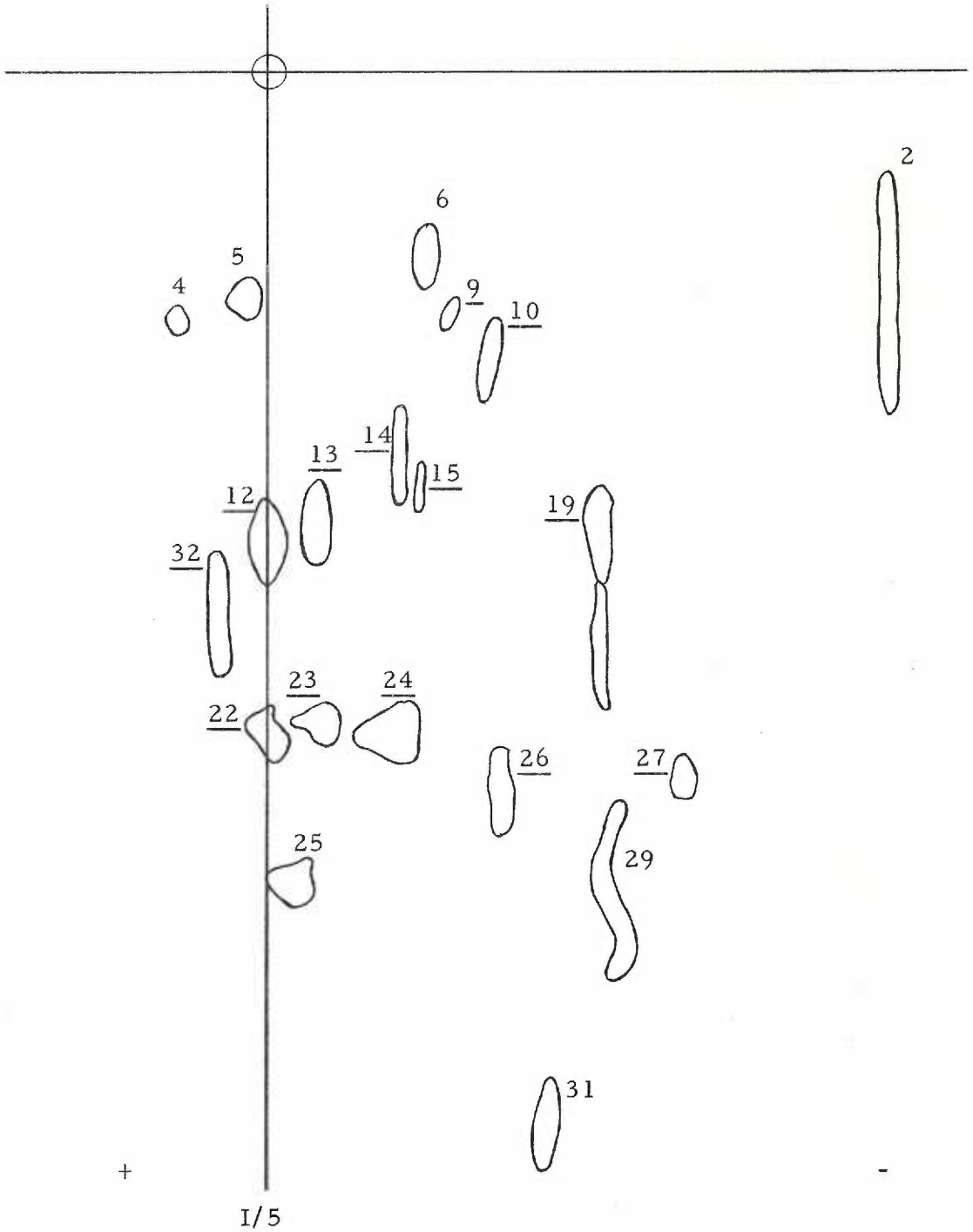
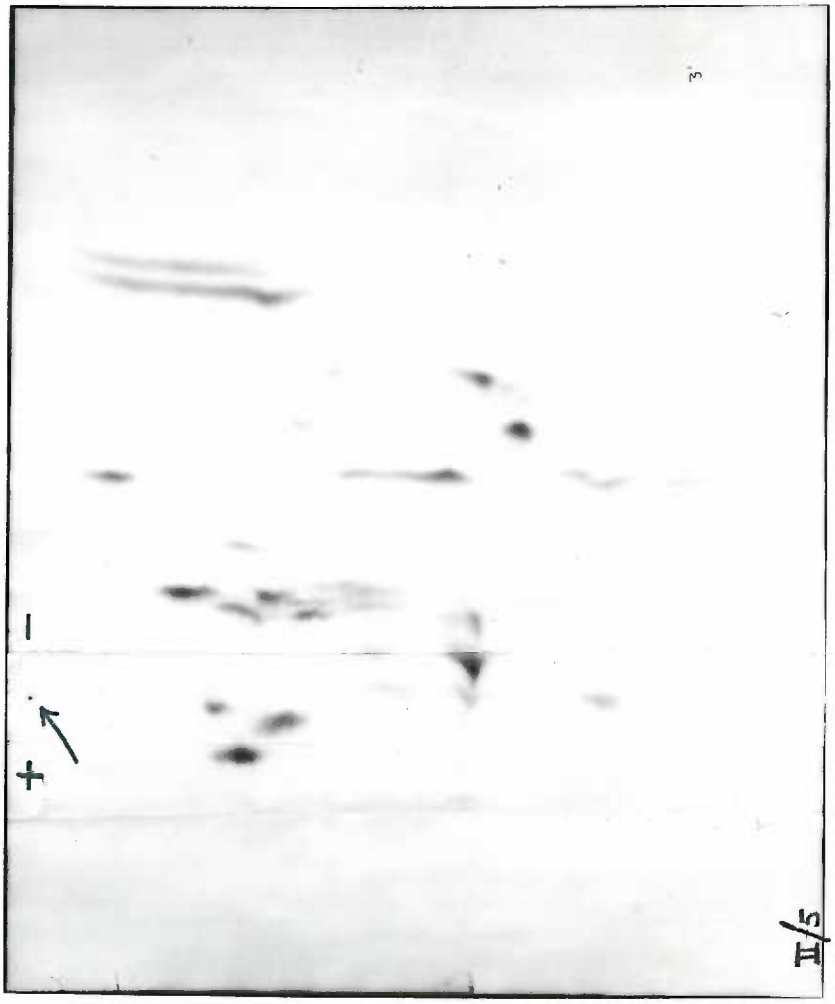

2
6
5
4
9
10
14
13
15
12
19
32
23
24
22
26
27
25
29
31
+
-
I/5
pyridine/water/n-propanol
pyridine/acetic acid/water

Figure 13. Peptide map of a tryptic hydrolysate of fraction II/5. Conditions are the same as those listed for figure 9.


I
+
II/5

In the diagrams of the peptide maps, numbers were arbitrarily assigned to the spots. Underlined numbers indicate spots which stained red with the Pauly stain. Peptide #32 was ninhydrin negative, but stained red with the Pauly stain.

Determination of Sedimentation Coefficient

The sedimentation coefficient of the unfractionated reduced, alkylated alpha crystallin was determined. An example of the schlieren patterns obtained is shown in figure 19. The s_{obs} = 0.44 S. Applying the appropriate corrections, $s_{20,w}$ = 1.26 S.

Determination of Diffusion Coefficient

The diffusion coefficient of unfractionated reduced, alkylated alpha crystallin was determined. The schlieren patterns obtained are shown in figure 20. The $D_a = 7.321 \times 10^{-7}$. Applying the necessary corrections, $D_{20,w} = 1.207 \times 10^{-6}$. The units are in cm^2/sec.

Figure 14. Diagram of the peptide map of a tryptic hydrolysate of fraction II/5 shown in figure 13. Spot #32 was ninhydrin negative but stained red with the Pauly stain. Underlined numbers indicate spots which stained red with the Pauly stain.

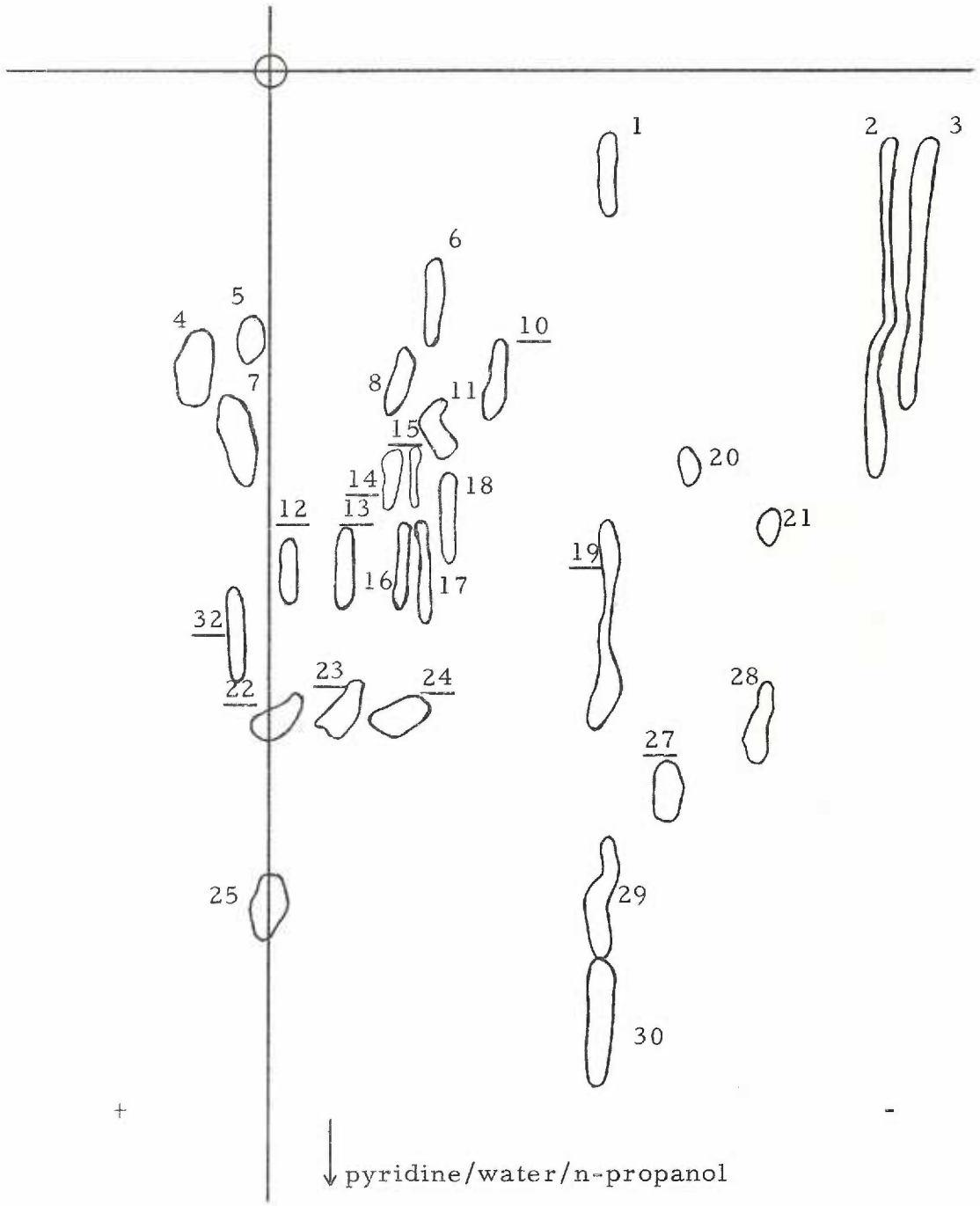

1
2
3
4
5
6
7
8
10
11
15
14
13
18
12
20
21
19
16
17
32
22
23
24
28
27
25
29
30
+
-
pyridine/water/n-propanol
II/5
pyridine/acetic acid/water

Figure 15. Peptide map of a tryptic hydrolysate of fraction I/6. Conditions are the same as those listed for figure 9.

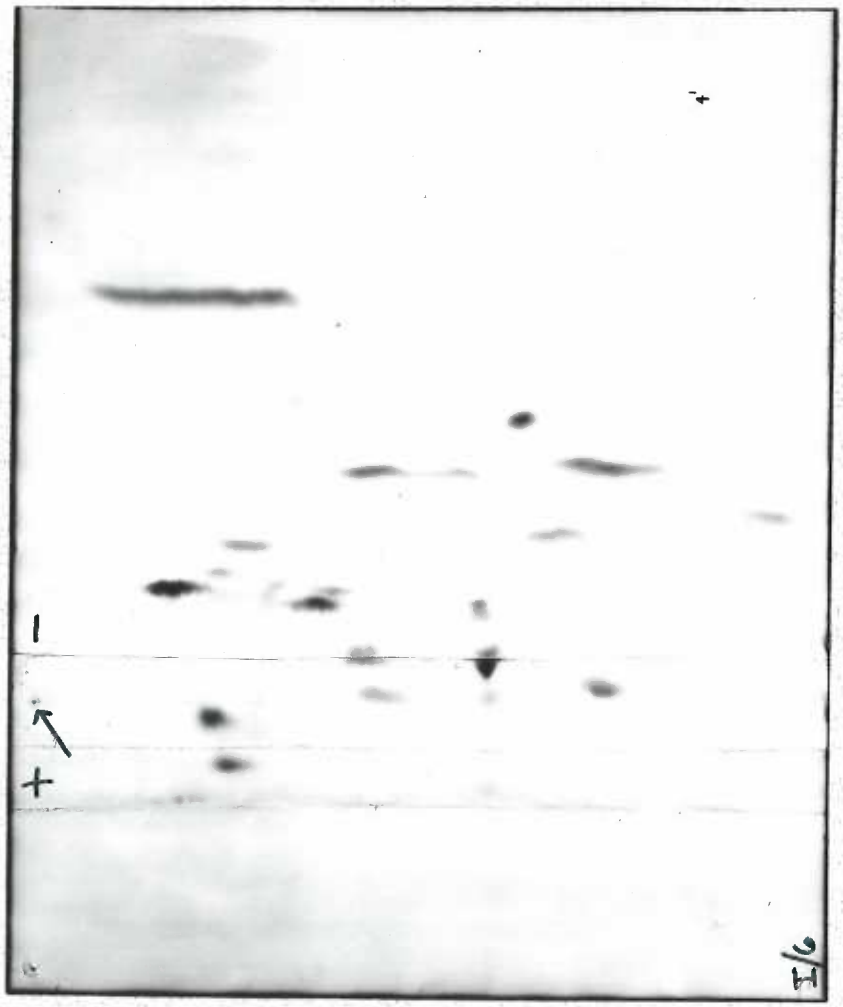

1
+
9/4

Figure 16. Diagram of the peptide map of a tryptic hydrolysate of fraction I/6 shown in figure 15. Spot #32 was ninhydrin negative but stained red with the Pauly stain. Underlined numbers indicate spots which stained red with the Pauly stain.

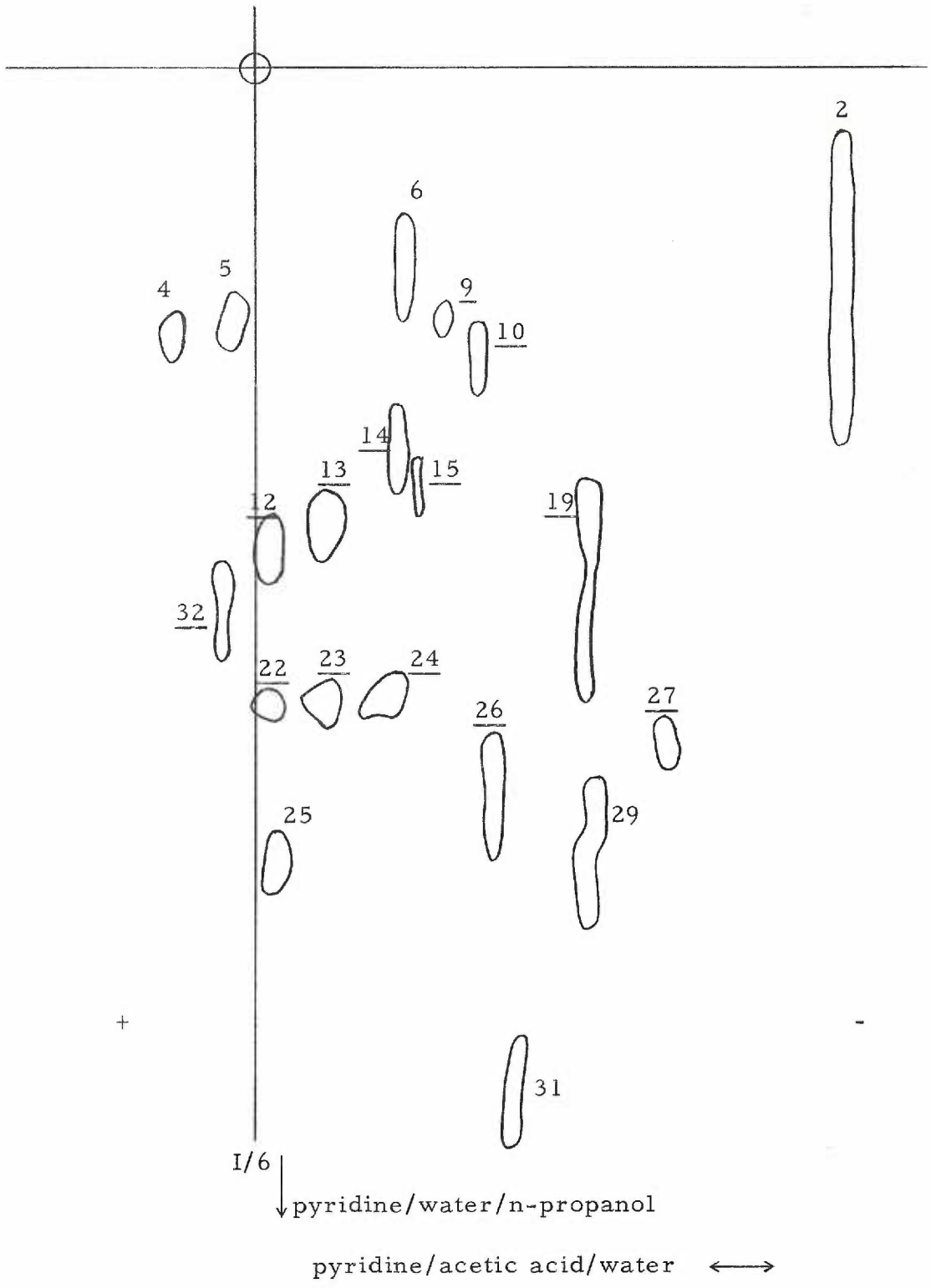

2
6
4
5
9
10
14
15
13
12
19
32
22
23
24
26
27
25
29
+
-
31
I/6
pyridine/water/n-propanol
pyridine/acetic acid/water

Figure 17. Peptide map of a tryptic hydrolysate of fraction II/6. Conditions are the same as those listed for figure 9.

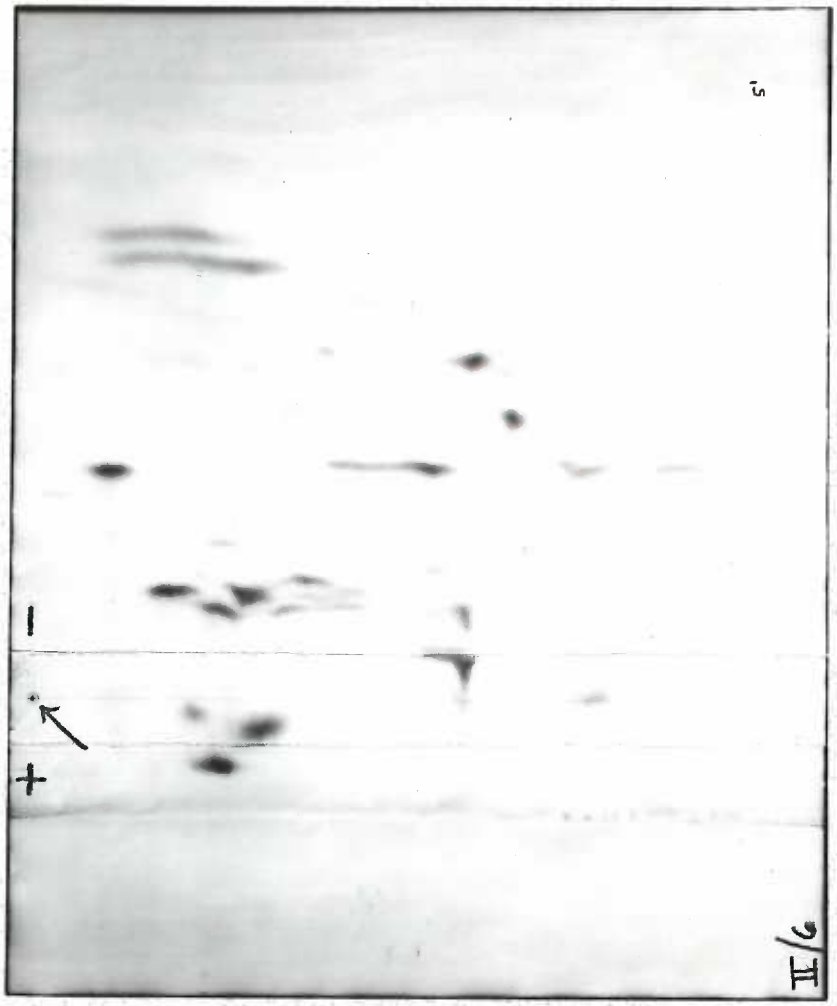

II/6
+
1

Figure 18. Diagram of the peptide map of a tryptic hydrolysate of fraction II/6 shown in figure 17. Spot #32 was ninhydrin negative but stained red with the Pauly stain. Underlined numbers indicate spots which stained red with the Pauly stain.

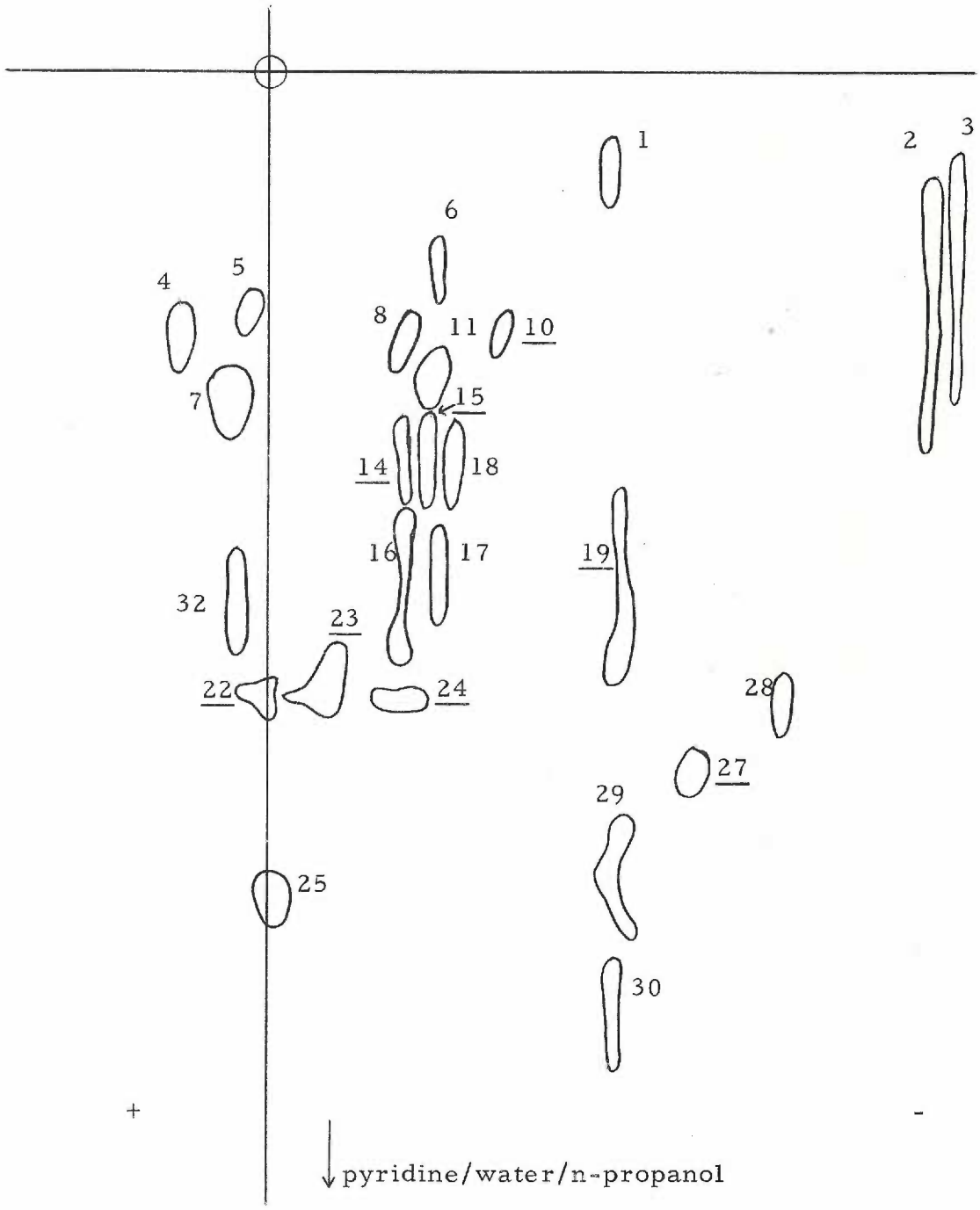
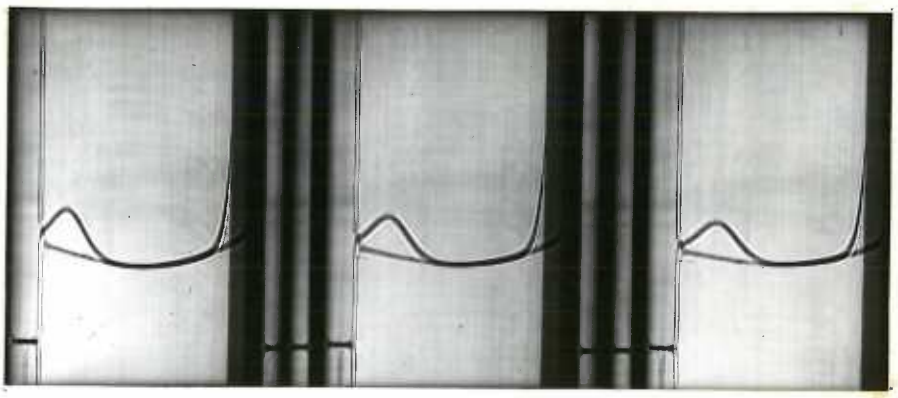

1
2
3
4
5
6
7
8
10
11
14
15
16
17
18
19
22
23
24
25
27
28
29
30
32
+
-
pyridine/water/n-propanol
II/6
pyridine/acetic acid/water

Figure 19. Schlieren patterns obtained in the determination of the sedimentation coefficient of a 1% solution of unfractionated reduced, alkylated alpha crystallin in 0.1 M Millers buffer, 6 M GCl, pH 7.7. Sedimentation is from left to right. The first frame was taken 224 min after attainment of speed; others were taken at intervals of 64 min. Speed = 59780 rpm (259610 x g); rotor = AN-D; temperature = 20°C; $s_{20,w}$ = 1.26 S.



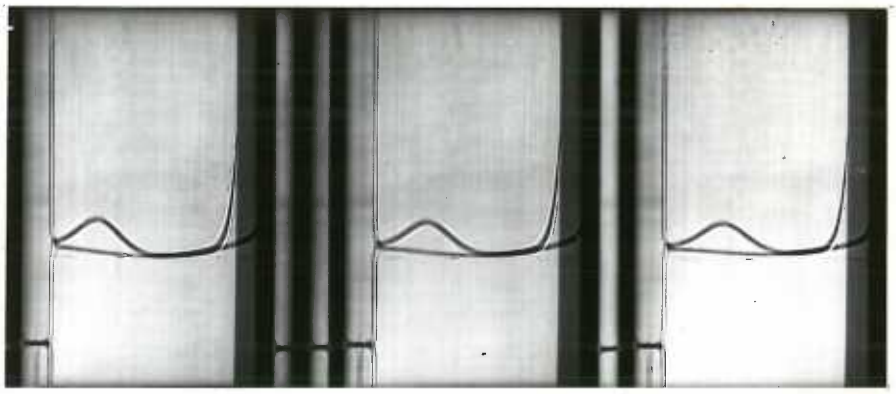
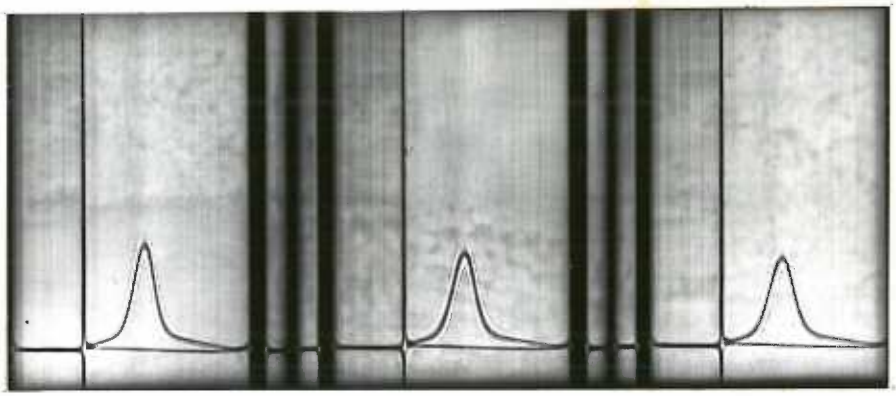


Figure 20. Schlieren patterns obtained in the diffusion coefficient determination of a 1% solution of unfractionated reduced, alkylated alpha crystallin in 0.1 M Millers buffer, 6 M GCl, pH 7.7. Rotor = AN-J; speed = 5227 rpm (1986 x g), temperature = 20°C; interval between frames = 16 min; $D_{20,w} = 1.207 \times 10^{-6}$ cm^2/sec; $(D_a)(\Delta t) = 6.687 \times 10^{-4}$.



Calculation of Molecular Weight

Using the $s_{20,w}$ and $D_{20,w}$, the molecular weight was estimated according to the following equation (29).

$$M = \frac{RTS}{D(1 - \bar{v}\rho)}$$

where

M = molecular weight

R = gas constant = 8.314×10^7 ergs/mole/degree

T = absolute temperature

S = $s_{20,w}$

D = $D_{20,w}$

$\bar{v}$ = partial specific volume of bovine alpha crystallin
= 0.74 ml/gm (19, 24)

ρ = density of water at 20°C = 0.9982 gm/ml

The value obtained was 9737.

Amino Acid Analysis

Table 2 shows the results of amino acid analysis of fractions I/5, II/5, I/6, and II/6. The values are expressed as moles of amino acid per 9737 gm protein.

Table 2. Amino Acid Composition of Alpha Crystallin Fractions* (moles per 9737 gm protein)

	I/5	II/5	I/6	II/6	U
Lysine	2.54	2.95	2.99	3.30	2.62
Histidine	2.90	3.32	2.93	3.40	2.77
Arginine	4.97	5.81	5.37	6.11	5.03
CM-cysteine	0.61	0.47	0.54	0.37	0.49
Aspartic Acid	7.80	7.13	7.62	6.87	7.21
Threonine	2.71	3.09	3.29	3.30	2.74
Serine	7.92	7.26	7.85	7.19	7.51
Glutamic Acid	9.30	9.79	9.86	10.22	9.20
Glycine	4.64	4.32	4.60	4.32	4.48
Alanine	2.18	2.34	2.11	2.47	2.33
Valine	4.22	4.05	4.67	4.43	4.31
Methionine	0.22	0.25	0.21	0.21	0.20
Isoleucine	2.52	3.20	2.49	3.17	3.01
Leucine	7.06	6.85	6.58	6.56	7.74
Tyrosine	3.10	2.22	2.61	1.81	2.81
Phenylalanine	6.16	5.80	5.13	5.10	6.43

*First symbol in the column designations refers to the fraction number. Second symbol refers to the figure in the text which shows the experiment from which the fraction was taken. "U" refers to unfractionated reduced, alkylated alpha crystallin (denatured with 6 M GCl).

Isolation and Amino Acid Analysis of Individual Peptides

Several peptides in both fractions I and II were isolated and put through the amino acid analysis procedure. The results obtained from the analysis of peptides 3/14, 3/18, 9/12, and 9/16 are presented in table 3. The first number in the peptide designation refers to the arbitrarily assigned number of the peptide spot. The second number refers to the figure in the text where the peptide is described. Thus, peptide 3/14 is peptide number 3 in the diagram shown in figure 14. The results for peptides 3/14 and 3/18 show that the peptide contained only lysine. The results for peptide 9/16 indicate a peptide, 9 amino acids long with the composition: His, Arg, Asp_2, Ser, Glu_2, Gly, Ile. In the case of peptide 9/12, no results were available for histidine and arginine, but the results that were available indicate a peptide having the composition: Asp_2, Ser, Glu_2, Gly, Ile. It is likely that if the basic amino acid analysis for peptide 9/12 were available it would also show the presence of histidine and arginine in the same proportions as they appear in peptide 9/16. In either case, it is not possible to tell whether the two aspartic acid residues and two glutamic acid residues are actually present in the protein as the acid form or the amide form, or a combination of both. Other peptides were isolated and analyzed; however, the results indicated that they were not pure, therefore, the data are not presented here.

Table 3. μmole Amino Acid per Tryptic Peptide of Alpha Crystallin*

	3/14	3/18	9/12	9/16
Lysine	0.0129	0.0120		----
Histidine	----	----		0.0149
Arginine	----	----		0.0164
CM-cysteine	----	----	----	----
Aspartic Acid	0.0008	0.0012	0.0188	0.0357
Threonine	----	----	----	----
Serine	0.0019	0.0017	0.0069	0.0114
Glutamic Acid	0.0018	0.0018	0.0167	0.0301
Glycine	0.0046	0.0047	0.0090	0.0147
Alanine	----	----	----	----
Valine	----	----	----	----
Methionine	----	----	----	----
Isoleucine	----	----	0.0062	0.0118
Leucine	----	----	----	----
Tyrosine	----	----	0.0024	0.0041
Phenylalanine	----	----	----	----

*First symbol in the column designations refers to the arbitrarily assigned peptide spot number. The second symbol refers to the figure in the text which shows the peptide map that the peptide spot appears in. Peptide 9/12 does not have any values listed for the basic amino acids because the short column run was faulty. Glycine is commonly found in hydrolysates of material eluted from paper, and is believed to come from the paper itself. Of the 16 peptides analyzed (results of 4 are presented here) 3/14 contained the lowest amount of glycine.

DISCUSSION

The soluble proteins of the lens can be separated into three major classes--alpha, beta and gamma crystallin. Of these three, alpha crystallin sediments the fastest in an ultracentrifugal field and migrates the fastest toward the anode in an electrical field. The $s^{o}_{20,w}$ for rabbit alpha crystallin was calculated to be 18.17 S (7). The N-terminal was found to be blocked and the C-terminal analysis of the native material revealed the presence of serine only.

Alpha crystallin was obtained in our laboratory by continuous flow paper electrophoresis of an aqueous extract of rabbit lenses according to the method of Mason and Hines (25). Paper strip electrophoresis of the protein showed it to be homogeneous electrophoretically. Ultracentrifugal analysis also indicated homogeneity (7, 25). N-terminal analysis showed that the N-terminal was blocked. C-terminal analysis of the native material revealed only serine present.

The SH and disulfide content of unmodified alpha crystallin was determined as cysteic acid in an acid hydrolysate of performic acid oxidized alpha crystallin. The results of that determination (table 1) show a content of 0.48 mole cysteic acid per 9737 gm protein. Reference to table 1 shows that the alpha crystallin reduced and alkylated in the presence of 7 M urea had an SH content (as

CM-cysteine) of 0.33 mole SH per 9737 gm protein. Alpha crystallin reduced and alkylated in the presence of 6 M GCl had 0.49 mole SH per 9737 gm protein. These data indicate that the 6 M GCl sufficiently denatured alpha crystallin such that most of the potential SH was alkylated, whereas 7 M urea had not accomplished this. From these results, it can also be inferred that the subunits that comprise alpha crystallin are tightly aggregated in the native state. Treatment of a protein molecule with 7 M urea is generally sufficient to break down the tertiary and/or quaternary structure. As the results indicate, 7 M urea treatment was not drastic enough to completely expose all the SH in alpha crystallin to the reducing agent. Both urea and GCl are thought to denature proteins by similar mechanisms, though in this instance, GCl appears to be more effective. The observation that GCl is a better denaturing agent on a molar basis has also been reported for other systems (30).

Ultracentrifugal analysis of the unfractionated reduced, alkylated alpha crystallin in 6 M GCl showed that the material was apparently homogeneous. The $s_{20,w}$ was calculated to be 1.26 S (figure 19). The $D_{20,w}$ of the unfractionated reduced, alkylated alpha crystallin was also determined (1.207×10^{-6}, figure 20). From these data, the molecular weight of the unfractionated reduced, alkylated alpha crystallin was estimated to be 9737.

Polyacrylamide gel electrophoresis of the unfractionated

reduced, alkylated alpha crystallin in 7 M urea, pH 8.9 showed that the apparently ultracentrifugally homogeneous material had components with different electrophoretic mobilities (figure 7). This indication of apparent heterogeneity of alpha crystallin subunits agrees with the C-terminal analysis of succinylated alpha crystallin which also indicated subunit heterogeneity (7).

Reduced, alkylated alpha crystallin was chromatographed on SE-sephadex, pH 3.2, 7 M urea, 10^{-3} M DTT and two fractions were obtained, I and II. The elution patterns are shown in figures 5 and 6. It will be noted that there is a peak which precedes the peak labeled as fraction I. This material is considered to be an aggregate of disulfide bridged alpha crystallin subunits on the basis of the following. Figures 3, 4, 5 and 6 show that the elution pattern of alpha crystallin from SE-sephadex changed with the extent of reduction and alkylation of the protein. The pattern in figure 3 (unmodified alpha crystallin) shows essentially two peaks, the first being a large spike eluting with the void volume. The alpha crystallin reduced and alkylated in the presence of urea (figure 4) shows essentially the same general pattern, but the first peak is smaller and a shoulder appears in the trailing edge of the second peak. Alpha crystallin reduced and alkylated in the presence of 6 M GCl (figures 5 and 6) shows a further decrease in the size of the first peak and the appearance of the third peak. Thus, it is seen that with an

increase in the extent of the alkylation of alpha crystallin, the pattern of elution from SE-sephadex shifted from that seen in figure 3 to that seen in figures 5 and 6.

The possibility that the first peak is a contaminant of alpha crystallin has been discounted on the basis of the following:

1. The unmodified alpha crystallin was shown to be apparently homogeneous by ultracentrifugal criteria (7, 25).
2. The unfractionated reduced, alkylated alpha crystallin appears to be homogeneous (figure 19).

In both instances the homogeneity of the alpha crystallin was judged by the nature of the schlieren patterns obtained. Admittedly, this method cannot detect a contaminant if it has physical dimensions similar to those of alpha crystallin in the native and in the reduced, alkylated state.

The preparation procedure employed for obtaining alpha crystallin depends on differences in electrophoretic mobilities. Of the three soluble lens proteins, alpha crystallin has the fastest electrophoretic mobility, followed by beta crystallin, then by gamma crystallin (7, 25). Thus, the most likely contaminant of alpha crystallin in this case would be beta crystallin. Native alpha crystallin has an $s_{20,w} = 18.17$ S, native beta crystallin is polydisperse, showing two unsymmetrical maxima (7, 25). Succinylated alpha crystallin has an $s_{20,w} = 1.19$ S, succinylated beta crystallin has an $s_{20,w} =$

1.75 S. These values were determined on 1% solutions of the proteins in 0.1 M NaCl (7). Native gamma crystallin has an $s_{20,w}$ = 2.5 S (25). The $s_{20,w}$ values for succinylated alpha and beta crystallin are referred to here because the succinylated form of the protein is believed to be a dissociated form. For example, the $s_{20,w}$ for succinylated alpha crystallin is 1.19 S. The $s_{20,w}$ for bovine alpha crystallin in 7 M urea is 1.8 S (31).

Considering the different $s_{20,w}$ values for the lens proteins, contamination of one protein by another should be detectable by ultracentrifugal means. Since apparent homogeneity was observed for both the native and for the reduced, alkylated alpha crystallin, contamination of the alpha crystallin preparation is considered unlikely. It should be noted, however, that if a contaminant is present in a sufficiently low concentration, its presence may not be detected unless the schlieren patterns obtained are subjected to a rigorous check for conformity to the gaussian shape. Such a check has not been performed on the schlieren patterns obtained in our experiments.

The amount of fractions I and II recovered in the experiments described in figure 6 represents 51% of the amount of protein applied to the column. At the time of this experiment an attempt was made to elute off more protein by pumping 0.2 M sodium formate, pH 3.2, 7 M urea, 10^{-3} M DTT, 4 M NaCl through the column. No

detectable 280 nm absorbing material was eluted. There are three probable explanations for this low recovery.

1. In pooling the material in fractions I and II, the material between the peaks (two tubes) was discarded. Material prior to the peak representing fraction I was also discarded.
2. The procedure used to recover proteins from urea solutions is not 100% efficient. In our experience, a range of 69-80% recovery has been encountered.
3. There may be material which is bound to the SE-sephadex.

Polyacrylamide gel electrophoresis was performed on the unmodified alpha crystallin; unfractionated reduced, alkylated alpha crystallin; and material from fractions I and II. The results presented in figures 7 and 8 indicate that fractions I and II contained different polypeptide populations. Unfractionated reduced, alkylated alpha crystallin (labeled "U" in figures 7 and 8) had six readily distinguishable bands. Fraction I had four bands corresponding to the last four bands in the unfractionated material. Fraction II had two heavy bands corresponding to the first two bands in the unfractionated material and four bands corresponding to bands 3, 4, 5 and 6 in both the unfractionated material and in fraction I. All samples had a faintly visible seventh band. Though they did have electrophoretic bands in common, fractions I and II from rabbit lenses

appeared to have different polypeptide populations.

The peptide maps shown in figures 9-18 also indicate that the polypeptide population of fraction I was different from that of fraction II. The maps show similarities as well as differences. Table 4 is a compilation of the peptide spots which were absent from the indicated peptide maps. One of the first seemingly inconsistent observations is that the peptide map of unfractionated reduced, alkylated alpha crystallin (figure 10) did not have the full complement of peptide spots. This apparent inconsistency can be explained by considering the dilution involved. For example, peptide spots 16 and 17 were absent from the peptide map of unfractionated reduced, alkylated alpha crystallin (figure 10). Reference to table 4 shows that these spots were present in the peptide maps of fraction II (figures 14 and 18) but were absent from the peptide maps of fraction I (figures 12 and 16). This indicates that the polypeptide which contained peptides 16 and 17 occurred in sufficiently high concentration in fraction II to be detected. The apparent absence from the unfractionated material can be explained by postulating that the concentration of the polypeptide containing peptides 16 and 17 in the unfractionated material was below the limits of detection of the system employed.

The peptide mapping results agree with the results shown in figures 5 and 6. Figures 5 and 6 are elution patterns of reduced,

Table 4. Peptide Spots Absent from Peptide Maps

U	8	11	12	16	17	18	20	21	30	31		
I/5	1	3	7	8	11	16	17	18	20	21	28	30
II/5	9	12	13	26	31							
I/6	1	3	7	8	11	16	17	18	20	21	28	30
II/6	9	12	13	26	31							

alkylated alpha crystallin from SE-sephadex. Figure 6 shows a better separation of fraction II from fraction I than does figure 5. Comparison of the peptide maps in figures 13 and 17 lead to the same observation. Peptide spots 12 and 13 appeared faintly in the peptide map of fraction II/5 (figure 13). These peptide spots were not diagrammed because they were very faint. The peptide map of fraction II/6 (figure 17) shows an absence of even faint traces of peptide spots 12 and 13. Figures 11 and 15 show that peptide spots 12 and 13 were definitely present in fraction I. Presumably, the concentration of peptide spots 12 and 13 was greater in the peptide map shown in figure 13 than it was in the peptide map shown in figure 17 since the spots were faintly visible in figure 13 but not in figure 17. On the basis of the results in figures 5 and 6, and the peptide mapping observations mentioned above, the separation of fraction II from fraction I is considered to be better in the experiment represented in figure 6 than in the experiment represented in figure 5.

Another point of correlation between the peptide mapping results and the SE-sephadex chromatography results is in the number of tryptic peptide spots observed for each fraction. From table 4, it is seen that fraction I lacked 12 peptides out of 32. Fraction II lacked 5 peptides out of 32. This means that fraction I had 20 peptides and fraction II had 27 peptides as detected by the system

employed. Since the specificity of trypsin causes hydrolysis at a lysine or arginine carboxyl group, it follows that the fraction with the most peptides also has the greater number of trypsin hydrolysis points. Since the trypsin hydrolysis points are associated with positive charges, the fraction with the greater number of peptides would be expected to have the greater number of positive charges. In this case, fraction II with 27 peptides is expected to have a greater number of positive charges than fraction I. SE-sephadex is a negatively charged ion exchanger which is fully dissociated at a pH of 3.5. It is expected that the first component of a mixture to emerge from such a column at pH 3.2 would be that component which has the least positive charge. In this case, fraction I does appear to have fewer positive charges than does fraction II and it (fraction I) did elute off before fraction II.

The polyacrylamide gel patterns in figures 7 and 8 show that fraction II/6 had more components of fraction I/6 than II/5 did of I/5. One possible explanation for this observation is that the gels of II/6 and I/6 had about 17% more sample than did the gels of II/5 and I/5. Thus, it could be expected that II/6 would have a greater absolute amount of "contaminating" electrophoretic bands. Another explanation is that the bands observed represent components that did "belong" to fraction II and were not merely contaminants from fraction I. Since the results from the SE-sephadex chromatography and peptide

mapping experiments both indicate that fraction II/6 is less contaminated with fraction I/6 than fraction II/5 is with fraction I/5, the latter explanation may be correct. That is, bands 3, 4, 5 and 6 (figure 8) do represent components of fraction II.

Table 2 shows the results obtained when the fractions from SE-sephadex and the unfractionated reduced, alkylated alpha crystallin were hydrolyzed and analyzed for amino acids present. The results as presented indicate that whatever differences exist between fractions I and II are likely to be minor. This idea is advanced in view of the apparent similarity of the amino acid contents of the fractions. Some of the values presented in table 2 related to the results of the peptide mapping experiments.

Thus, it is seen that fractions I and II had equal amounts of lysine but fraction II had one more arginine residue than did fraction I. This extra arginine could be one source of the greater net positive charge on fraction II. Another possible source of charge differences may be the amino acids, glutamine or asparagine. Both of these amino acids were detected as glutamic acid and aspartic acid respectively following acid hydrolysis of the polypeptide. Thus, if one fraction had a certain number of glutamic acid residues and another fraction had a similar number of glutamine residues, there would be a charge difference. This charge difference would not be evident from the amino acid analysis of an acid hydrolysate because

both fractions would appear to have equal amounts of glutamic acid.

The actual nature of this charge difference is not known as the condition of the amide group of glutamine or asparagine under the conditions used is not known. However, it would be expected that a polypeptide containing asparagine or glutamine would have fewer negative charges than a similar polypeptide containing an equivalent number of aspartic acid or glutamic acid residues.

Another point to consider in view of the results presented in table 2 is the number of lysine and arginine residues in relation to the number of peptides obtained in peptide maps of tryptic hydrolysates of the protein. From the results in figures 9-18, fraction I had 20 peptide spots and fraction II, 27. This observation is not in accord with the known specificity of tryptic hydrolysis. If the trypsin used was free of other enzyme activities, and if it is assumed that each fraction contained a homogeneous polypeptide population, the theoretical number of peptides expected would be 9 for fraction I and 10 for fraction II. These values are obviously at variance with the experimental findings. One probable explanation for the greater number of peptides seen is that the trypsin preparation may have been contaminated with chymotrypsin. If this is indeed the case, the potential number of peptides based on the results in table 2, would be at least 24 for both fraction I and II.

Another possibility to consider is that each fraction did not

contain a homogeneous polypeptide population. If this was the case, the theoretical number of tryptic peptides expected would be the sum of the arginine and lysine residues plus one multiplied by the number of unique polypeptide chains in each fraction.

The amino acid content of several peptides was determined in an attempt to find out how fractions I and II differed from each other. From table 2, it is seen that the amino acid composition of the fractions is similar, with only minor differences. Thus, the differences that exist between fractions I and II are likely to be minor. Certain peptides were selected for amino acid analysis in an attempt to find peptides which had different electrophoretic mobilities, but identical amino acid compositions. If such peptides were found, it would indicate that at least one of the amino acid residues (aspartic acid or glutamic acid) existed in the amide form in one of the peptides. The peptides chosen for isolation and amino acid analysis were those which appeared to be unique to either fraction I or II. This was undertaken on the reasoning that if a particular peptide appeared in the peptide maps of both fractions I and II in the same position, obviously a difference in electrophoretic mobility did not exist. On this basis, an amide difference would not be expected.

As the results presented in table 3 indicate, no such peptides were found. However, this does not mean they were not present. Before such a conclusion can be drawn, the amino acid content of all

the peptides in the peptide maps would have to be determined. The results presented in table 3 are for peptides which appeared to be reasonably pure. Other peptides were also analyzed in a similar manner. The results from these are not presented because they appeared to be impure. For example, peptides 1/14 and 1/18 appeared to have equal amounts of lysine and arginine. These peptides were obtained by tryptic hydrolysis and it is not likely that a tryptic peptide would have both lysine and arginine residues. Thus, it is presumed that such a peptide is actually a mixture of at least two peptides. The results presented for peptides 3/14 and 3/18 indicate that there is a segment along the chain of fraction II where lysine is preceded by arginine or another lysine residue. This argument of course will not hold true if the trypsin preparation used was contaminated with chymotrypsin.

The fractions of alpha crystallin were found to contain approximately 0.5 mole SH per 9737 gm protein (measured as CM-cysteine). Cysteic acid determination of the acid hydrolysate of performic acid oxidized protein showed a cysteic acid content of 0.48 mole cysteic acid per 9737 gm protein. These data indicate that the SH values determined represent the actual amount of SH present. This statement of course is made on the assumption that performic acid oxidation converts all the SH and potential SH to cysteic acid.

From the foregoing, the following general statement can be

made about rabbit lens alpha crystallin. The native alpha crystallin molecule is a large, apparently homogeneous molecule of about 1 x 10^6 molecular weight. Reduction and alkylation in the presence of 6 M GCl causes the molecule to dissociate into subunits of approximately 10000 molecular weight. At this stage, ultracentrifugal experiments in 6 M GCl indicate apparent homogeneity whereas polyacrylamide gel electrophoresis experiments in 7 M urea indicate heterogeneity.

The subunits can be separated into two fractions by chromatography on SE-sephadex in 7 M urea, pH 3.2. Each of the two fractions thus obtained contains a different polypeptide population. The components in fractions I and II differ on the basis of polyacrylamide gel electrophoresis experiments in 7 M urea, pH 8.9 and peptide mapping of tryptic hydrolysates. The differences between fractions I and II are not thought to be great in view of the results of amino acid analyses (table 2). Both fractions I and II contain approximately 0.5 mole SH per 10000 gm protein. The fractional value can be explained if it is postulated that both fractions I and II contain at least two components of approximately 10000 molecular weight each, one of the two components in each fraction having one SH, the other having no SH. In a situation such as this, analysis of the SH content in fractions I and II would yield 0.5 mole SH per 10000 gm protein.

The polyacrylamide gel electrophoresis results indicate that

the two fractions contain components having both similar and different electrophoretic mobilities. As indicated in an earlier section, the existence of bands common to both fractions I and II may indicate contamination. It may also mean that fraction I actually has components which have electrophoretic mobilities similar to components belonging to fraction II. The results indicate that each fraction contains components with heterogeneous electrophoretic mobilities. This heterogeneity can be due to an actual heterogeneous population of alpha crystallin subunits in the lens. On the other hand, genetic reasons may be responsible for the observed heterogeneity. The protein was obtained from animals for which no genetic background was available. The material used in these experiments was material pooled from 50 animals. If the DNA coding for the lens proteins is readily subject to nonlethal mutations, minor differences in the subunits of the lens proteins are to be expected. This question can be resolved only by looking at material obtained from a single animal. It should be possible to extract sufficient material from the lenses of a single animal for reduction and alkylation and subsequent polyacrylamide gel electrophoresis in 7 M urea to be performed.

Future experiments along these lines should include improvement of the separation procedure. Several methods may be attempted such as use of another type of ion exchange resin, isoelectric focusing, preparative polyacrylamide gel electrophoresis, and

others. Once homogeneous polypeptide populations are obtained, more critical work can be done to determine how they differ from each other. For example, the polypeptides may be hydrolyzed with trypsin or a suitable proteolytic enzyme, and the composition of the peptides may be elucidated by use of techniques perfected for polypeptide sequencing.

When comparing the results obtained in our laboratory on rabbit lenses with those from other laboratories on bovine lenses, both similarities and dissimilarities were found. Table 5 is a comparison of certain parameters of alpha crystallin from bovine lenses and from rabbit lenses. Table 6 compares the amino acid composition of bovine and rabbit alpha crystallin.

The results obtained from polyacrylamide gel electrophoresis experiments on rabbit alpha crystallin are similar but not identical to those obtained by other laboratories working on bovine alpha crystallin (21, 22). Schoenmakers et al. (21, 22, 23) obtained two fractions (I and II) of alpha crystallin from an SE-sephadex column, pH 3.2, 6 M urea. Polyacrylamide gel electrophoresis at pH 8.9, 6 M urea was performed. The overall band pattern could be numbered 1-4 from top to bottom. Fraction I had two bands corresponding to the last two positions (bands 3 and 4). Fraction II had two bands corresponding to the first two positions. The difference between these results and those obtained in our laboratory could be due either

Table 5. Comparison of Some Properties of Bovine and Rabbit Alpha Crystallin

	Bovine	Rabbit
$s^{o}_{20,w}$ of native aggregate	19.0 S	18.9 S
$s_{20,w}$ of 1% reduced, alkylated alpha crystallin in 6 M GCl		1.26 S
$s^{o}_{20,w}$ of alpha crystallin in 7 M urea	1.8 S	
N-terminal	Blocked	Blocked
C-terminal	Serine	Serine
Apparent subunit molecular weight	11000-23000	9737
Fractions from SE-sephadex	4	2
SH content (CM-cysteine)	1 mole/21500 gm	0.5 mole/9737 gm
Cysteic acid content	2 moles/21500 gm	0.5 mole/9737 gm

Table 6. Amino Acid Composition of Bovine and Rabbit Alpha Crystallin (moles per 100 moles) and SE-sephadex Fractions*

	IB	IR	IIB	IIR	UB	UR
Lysine	4.2	4.4	5.9	4.8	4.8	3.8
Histidine	4.0	4.2	5.2	4.9	4.4	4.1
Arginine	7.6	7.8	8.6	8.9	8.0	7.3
CM-cysteine		0.7		0.6		0.7
Cysteic Acid	1.0		1.0		0.7	
Aspartic Acid	9.7	11.0	7.8	10.0	9.1	10.5
Threonine	3.1	4.8	4.0	4.8	3.3	3.9
Serine	11.9	11.5	8.9	10.5	10.8	10.9
Glutamic Acid	10.5	14.4	10.5	14.8	10.5	13.8
Glycine	6.3	6.7	5.0	6.2	5.8	6.6
Alanine	3.8	3.1	5.5	3.6	4.2	3.4
Valine	5.7	6.8	5.5	6.4	5.6	6.3
Methionine	1.0	0.3	1.0	0.3	1.0	0.3
Isoleucine	5.0	3.6	5.3	4.6	5.2	4.4
Leucine	8.2	9.6	8.7	9.6	8.4	11.2
Tyrosine	3.3	3.8	1.2	2.6	2.7	4.1
Phenylalanine	8.0	7.4	7.5	7.4	7.8	9.3

*Column headings have two symbols, the first referring to the fraction analyzed (U is unfractionated), the second referring to the source of the protein, B for bovine, R for rabbit. Values for bovine material were obtained from reference 21. Values for rabbit material were from table 2 adjusted to moles per 100 moles.

to a better fractionation of the bovine material or an actual difference in electrophoretic mobility of the polypeptide population of fraction II from bovine material and fraction II from rabbit material.

Fraction I from bovine material was termed the acidic fraction and could be separated by rechromatography or isoelectric focusing into fractions A_1 and A_2. Peptide maps were performed on chymotryptic digests of fractions A_1 and A_2 from bovine alpha crystallin (22). A peptide in fraction A_1 was found, which had a similar amino acid content to a peptide in fraction A_2. The electrophoretic mobilities were different. The amino acids present were aspartic acid, serine, glutamic acid, alanine and leucine. It was postulated that either glutamic acid or aspartic acid occur as the corresponding amide in one of the peptides. It is likely that such a situation also exists for rabbit alpha crystallin, but this still has to be shown. Table 6 presents a comparison of the amino acid contents of the fractions obtained when bovine and rabbit alpha crystallin is fractionated on a SE-sephadex column. It will be noted that rabbit material contains more aspartic acid and glutamic acid than does the bovine material. On the other hand, bovine material contains more methionine than does the rabbit material.

Ultracentrifugal experiments on various fractions of bovine alpha crystallin were reported (19, 24). Evidence was presented indicating that the molecular weights of bovine alpha crystallin

calculated on the basis of ultracentrifugal criteria vary with the length of time that the protein is exposed to the denaturing agent. The values reported range from 11000 to 23000. The low value of 11000 is not too different from our calculated value of 9737 for rabbit alpha crystallin.

Reports on SH determination of bovine alpha crystallin indicate that the two acidic subunits of bovine alpha crystallin each have one mole SH per 21500 gm protein. The two basic subunits did not contain any SH (22). It should be noted, however, that the same investigators did not get good correlation between the cysteic acid values and the CM-cysteine values. The acidic fractions (A_1 and A_2) had 1.8 and 1.6 moles respectively of cysteic acid per 21500 gm protein. Determination of the CM-cysteine content yielded 1.03 and 1.05 moles CM-cysteine per 21500 gm protein for A_1 and A_2 respectively. The values for the cysteic acid determinations may be rounded off to two moles cysteic acid per 21500 gm protein. If this is done, and if it is also assumed that the subunits which do contain SH contain it (SH) to the extent of one SH per subunit, the estimated molecular weight would turn out to be 10750. This value is in the same range as the 9737 obtained in our laboratory on rabbit material and the 11000 reported elsewhere for bovine material (24).

The results presented in this thesis indicate that alpha crystallin, in the reduced and alkylated state appears to be homogeneous

by ultracentrifugal criteria. When subjected to polyacrylamide gel electrophoresis, heterogeneity is noted. The material can be separated into two fractions by chromatography on SE-sephadex and each fraction has been shown to contain polypeptide populations that are chemically different by peptide mapping experiments. The amino acid analyses of the fractions show that the amino acid contents of the fractions are similar; thus, the differences which exist are considered to be minor. A subunit molecular weight was calculated to be 9737 on the basis of ultracentrifugal criteria.

SUMMARY AND CONCLUSION

Alpha crystallin was isolated from rabbit lenses and shown to be essentially homogeneous by electrophoresis and ultracentrifugation. The protein was reduced and alkylated in 6 M GCl. Ultracentrifugal analysis in 6 M GCl of the reduced, alkylated material resulted in a calculated molecular weight of 9737. The reduced, alkylated protein was passed through a SE-sephadex column, pH 3.2, 7 M urea, 10^{-3} M DTT and two fractions were obtained, I and II. Polyacrylamide gel electrophoresis at pH 8.9, 7 M urea, showed that fractions I and II contained components with different electrophoretic mobilities. Peptide maps of tryptic hydrolysates also showed that fractions I and II contained different polypeptide populations. Amino acid analysis of acid hydrolysates of the fractions showed that the amino acid composition of fractions I and II was similar, with minor differences. Cysteic acid content of performic acid oxidized alpha crystallin was 0.48 mole cysteic acid per 9737 gm protein. This correlated with the amount of CM-cysteine found in the unfractionated reduced, alkylated alpha crystallin (0.49 mole CM-cysteine per 9737 gm protein).

These results indicate that the native alpha crystallin molecule can be dissociated by reduction in 6 M GCl. Random dimerisation

can be prevented by reducing and alkylating the SH groups. The subunits of alpha crystallin are not identical as shown by the fractionation (I and II) achieved when the dissociated material was passed through a SE-sephadex column. That these fractions were actually composed of different polypeptide species was shown by the polyacrylamide gel electrophoresis and peptide mapping experiments. The amino acid analyses of the fractions and the ultracentrifugal analysis of the unfractionated reduced, alkylated material indicate that the differences between the fractions are minor. The fractional value obtained for the SH content has been interpreted as an indication of the possible existence of at least two different polypeptide chains per fraction. The fact that the native molecule is completely dissociated (as measured by SH determinations) by 6 M GCl but not by 7 M urea indicates that the subunits are tightly aggregated in the native state.

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