Biochemistry of Bovine Lens Proteins. III. Chemical and Physical Properties of α-Crystallin Subunits*

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ABSTRACT: The α-crystallins of the adult bovine lens can be resolved into five components by DEAE-cellulose chromatography. These fractions have similar physicochemical properties and are immunochemically identical. They can be progressively disaggregated into their component subunits by increasing concentrations of sodium dodecyl sulfate (SDS). All five α-crystallins respond identically with this SDS treatment. Chromatography of 7 M urea treated α-crystallins on 7 M urea equilibrated DEAE-cellulose revealed that the five α-crystallins are composed of the same subunits, accounting for their immunochemical identity. However, the quantity of the different subunits in each α-crystallin varies, thus accounting for the differences in their chromatographic properties. Differences between the isolated subunits have been revealed by peptide mapping.

The α-crystallins, which are structural proteins of the vertebrate lens, have been shown to have a molecular weight of about 1×10^6 (Resnik, 1957) and are known to be composed of subunits whose molecular weights are about 25,000 (Bloemendal *et al.*, 1962; Spector and Katz, 1965). It has been shown by acrylamide electrophoresis (Bloemendal *et al.*, 1962) and by ion exchange chromatography (Björk, 1964) that this protein contains several different types of subunits. The seven subunits isolated by the latter technique were shown to differ in both their amino acid compositions and their electrophoretic mobilities on polyacrylamide gels (Björk, 1964).

There has been a great deal of controversy as to whether α-crystallin is a single distinct protein or whether it actually represents a group of closely related proteins. For example, some investigators believe that there is only one α-crystallin (Bloemendal *et al.*, 1964; Spector, 1965), while others have fractionated them into two to four components (Björk, 1963; Papaconstantinou *et al.*, 1962; Manski *et al.*, 1961; François *et al.*, 1955). In this paper, the resolution of the α-crystallins into five distinct fractions by DEAE-cellulose chromatography is discussed.

We have found that there is a great deal of similarity between the five α-crystallin fractions. They have similar sedimentation coefficients, show identity reactions with each other on agar diffusion plates, and respond similarly to treatment with sodium dodecyl sulfate (SDS).[1] Therefore, some of the following experiments were designed to determine how these protein fractions differ from each other. On the basis of our results, we have proposed an explanation for the discrepancies between the number of α-crystallins reported by various investigators. A preliminary report of this work has appeared elsewhere (Palmer and Papaconstantinou, 1967).

Materials and Methods

Preparation of α-Crystallins. Adult bovine eyes were obtained from freshly slaughtered cows and were immediately placed on ice. After removal of the lens, the epithelial layer of cells was discarded and the remainder of the lens (fiber cells) was stored at −10° until needed.

Approximately 10–15 lenses were thawed and stirred in 0.005 M sodium phosphate buffer (pH 7.0) (5 ml/lens) until most of the cortical fiber cells were removed. These fiber cells were separated from the fiber cells of the nucleus region by decanting. The cells were homogenized, and the homogenate was cleared by centrifugation at 12,000*g* for 10 min.

Purification of the α-crystallins was carried out as follows. (a) *Isoelectric precipitation.* The cleared homogenate was adjusted to pH 5.0 with 0.1 N HCl, the volume was brought to 13% ethanol, and the solution was stirred for 30 min at 5°. The precipitated α-crystallins were centrifuged and redissolved in 0.005 M sodium phosphate buffer (pH 7.0). The pH was adjusted to 7 with 0.1 N NaOH. The precipitation procedure was repeated to further purify the α-crystallins. The precipitate was again dissolved in 0.005 M sodium phosphate buffer (pH 7.0) and was dialyzed against the same buffer. (b) *DEAE-cellulose fractionation.* The isoelectric precipitated α-crystallins were further purified and resolved into five distinct fractions by column chromatography. DEAE-cellulose (type 20) was obtained from the Schleicher & Schuell Co.,

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[1] Abbreviation used: SDS, sodium dodecyl sulfate.

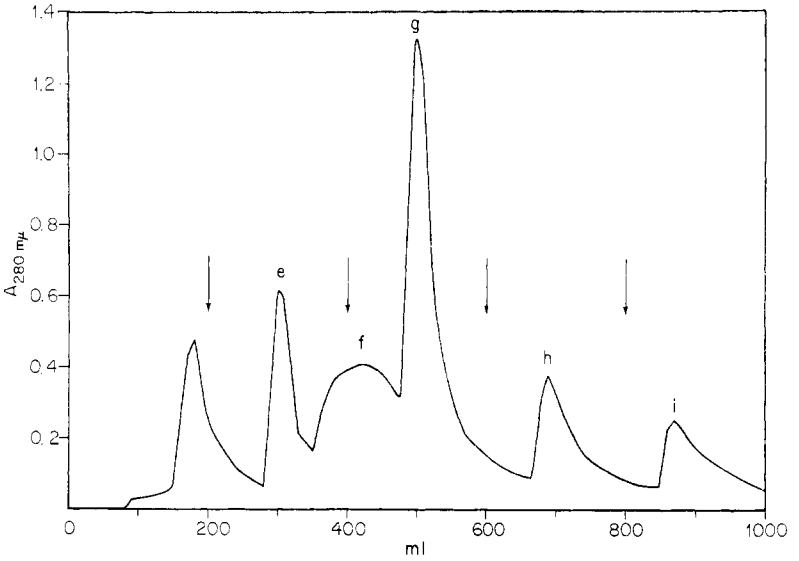

FIGURE 1: Fractionation of α-crystallins on a DEAE-cellulose column. The proteins were eluted from the column by the stepwise addition of the following buffers: (I) 0.02 M sodium phosphate (pH 5.7) (200 ml, to eliminate any contaminating β- and γ-crystallins from the isoelectric precipitated proteins), (II) 0.02 M sodium phosphate (pH 5.7)–0.1 M NaCl (200 ml), (III) 0.06 M sodium phosphate (pH 5.7)–0.1 M NaCl (200 ml), (IV) 0.1 M sodium phosphate (pH 5.7)–0.1 M NaCl (200 ml), and (V) 0.1 M sodium phosphate (pH 5.7)–0.2 M NaCl (200 ml). The buffer changes are indicated by arrows.

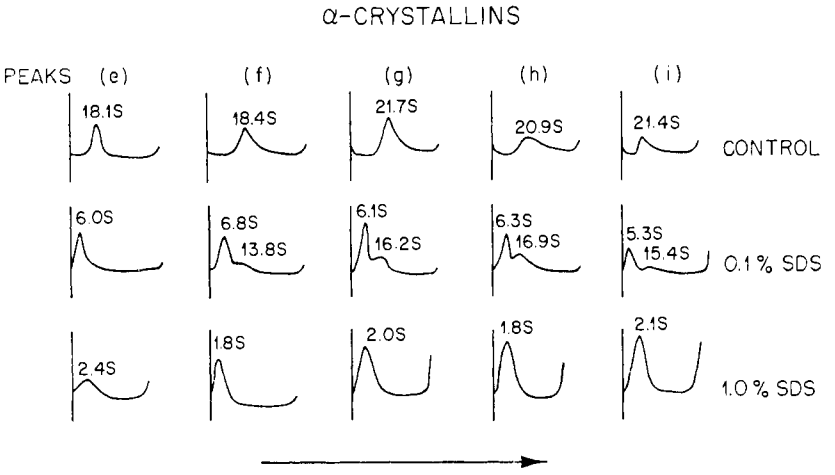

FIGURE 2: An ultracentrifugation analysis of SDS-treated α-crystallins eluted from a DEAE-cellulose column. Native and 0.1% SDS-treated α-crystallins were run simultaneously in a double-sectored cell at 39,460 rpm for 32 min at 25° and with a bar angle of 60°. α-Crystallins treated with 0.1% SDS and 1.0% SDS were run in a double-sectored cell at 39,460 rpm for 40 min followed by 40 min at 59,780 rpm. Pictures were taken 24 min after reaching speed. The temperature was kept constant at 25° with a bar angle of 65°.

Keene, N. H. Initial activation or reactivation of the DEAE-cellulose was carried out by the procedure of Sober *et al.* (1956) with the following modifications. DEAE-cellulose (10 g) was suspended in 500 ml of 0.2 M NaH_2PO_4 and permitted to stand for 1 hr. The NaH_2PO_4 was decanted and the DEAE-cellulose was washed with 0.005 M sodium phosphate buffer (pH 7.0) and then suspended in 1 N NaOH for 1 hr. The supernatant was decanted and the DEAE-cellulose was washed with 0.005 M sodium phosphate buffer (pH 7.0) until it was equilibrated.

A DEAE-cellulose column (2.5 × 14 cm) was prepared and was charged with 400 mg of an α-crystallin solution in 0.005 M sodium phosphate (pH 7.0). A sufficient amount of the first buffer was added to form a 4–5-cm liquid head over the top of the DEAE-

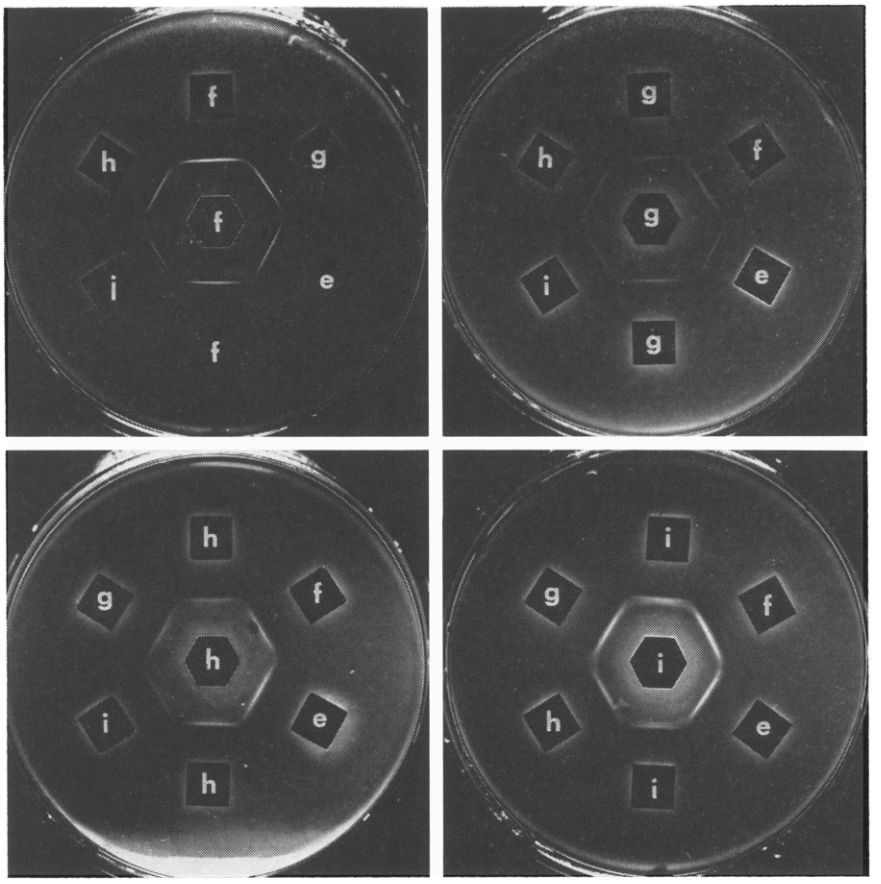

FIGURE 3: An agar diffusion analysis of the native α-crystallins and their respective antibodies. In each case the proteins were placed in the peripheral well. Each well is labeled for the specific α-crystallin it contains. In each case the antiserum is in the central well and is labeled according to the α-crystallin used to make the antiserum. All agar diffusion plates were developed at 4° for 1 week.

cellulose column. The Na_2HPO_4–NaH_2PO_4–NaCl stepwise elution system described previously (Papaconstantinou *et al.*, 1962) was used to elute four distinct α-crystallin peaks from the column. A fifth α-crystallin was eluted by the addition of 0.1 M sodium phosphate buffer (pH 5.7)–0.2 M NaCl. The protein fractions from each peak were pooled and dialyzed against two changes of 60% ammonium sulfate and stored in this form at 4°.

DEAE-cellulose was also utilized in the fractionation of the purified α-crystallins into their subunits. For this purpose, the DEAE-cellulose was equilibrated with 7 M urea–0.005 M sodium phosphate (pH 7.0) and packed by gravity into a 2.5 × 32 cm column. The stepwise elution system used was described by Björk (1964).

Protein Determinations. The protein concentration of the starting homogenate and of each fraction collected from the column was determined spectrophotometrically (Warburg and Christian, 1941).

Sedimentation Studies. Ultracentrifugation analyses were performed in the Spinco Model E analytical ultracentrifuge, employing the schlierien optics system. In these studies the ammonium sulfate precipitated α-crystallins were dissolved in 0.05 M sodium phosphate buffer (pH 6.8) and dialyzed exhaustively against this same buffer. All protein solutions were adjusted to a final concentration of 0.8%, and sedimentations were carried out at 25°. Sedimentation constants reported have been corrected to water at 20° (Svedberg and Pederson, 1940). A value of 0.74 ml/g was used for $\bar{v}$ (Resnik, 1957).

Immunochemical Procedures. Purified α-crystallin fractions were dissolved in 0.9% NaCl, and the protein concentration was adjusted to 10 mg/ml. Antibodies to these α-crystallins were prepared by subcutaneous injection of 2 cc of a 1:1 mixture of the protein solution in Freund's complete adjuvant. Rabbits were injected with this mixture once a week for 3 consecutive weeks. The rabbits were bled 1 month after the last injection.

Peptide Analyses. Ammonium sulfate precipitated α-crystallins were dissolved and dialyzed exhaustively against 0.1 M $(NH_4)_2CO_3$ buffer (pH 8.0) and then adjusted to 5-mg/ml $(NH_4)_2CO_3$. Proteolytic digestions were carried out with trypsin and chymotrypsin (Calbiochem, grade A). Stock solutions containing 2 mg of enzyme/ml of buffer were prepared just prior to use. Trypsin (0.025 ml/ml of protein solution) was

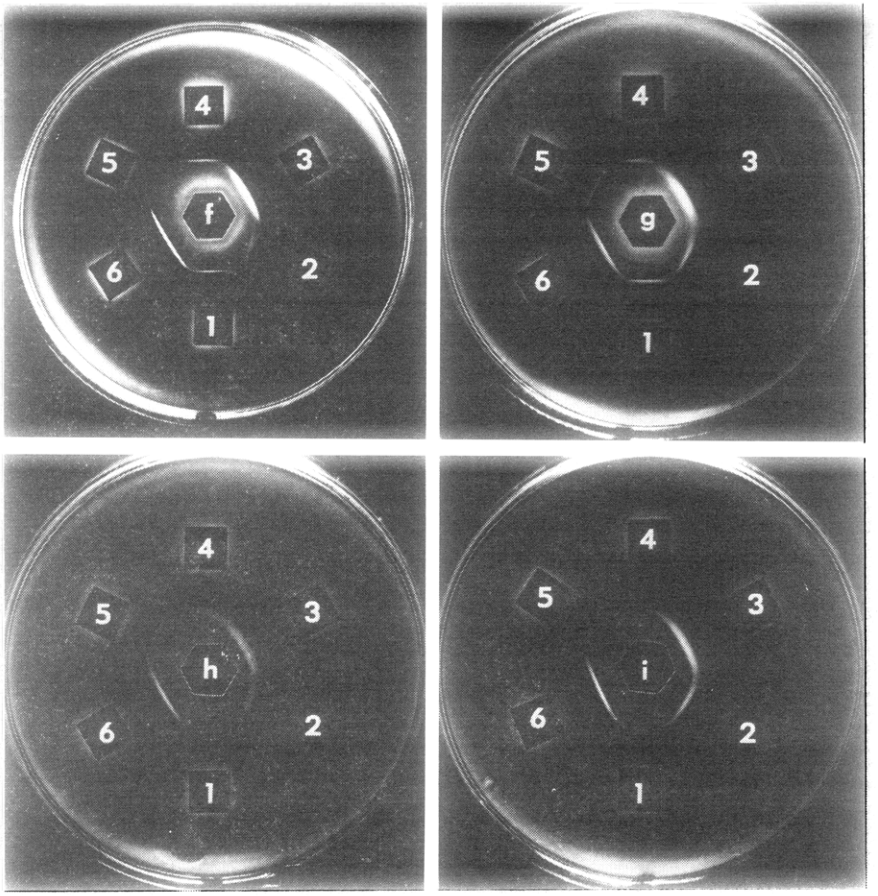

FIGURE 4: An agar diffusion analysis of the immunochemical properties of α-crystallins dissociated with SDS and partially reassociated by dialysis against 0.9% NaCl. The antiserum to native α-crystallins (peaks f–i) is in the central well, and each central well is labeled according to the specific antiserum. The peripheral wells contain the homologous antigen to the antiserum in the central well. The protein placed in the wells was treated as follows: wells 1 and 4 contain native α-crystallin; wells 2 and 3 contain 0.1% SDS-treated and 1.0% SDS-treated α-crystallin, respectively; wells 5 and 6 contain 0.1% SDS-treated and 1.0% SDS-treated protein, respectively, followed by exhaustive dialysis.

added to the protein solution and the digestion was allowed to proceed for 2 hr at 37°. Chymotrypsin (in the same concentration as trypsin) was then added and the digestion was continued for 2 more hr. At the end of this period, the digestion mixture was immediately frozen in an acetone–Dry Ice bath and lyophilized. The peptides were dissolved in distilled water and relyophilized twice to remove the volatile buffer salt. Dried material was placed over P_2O_5 and NaOH pellets in a desiccator for 3 days to remove any remaining $(NH_4)_2CO_3$. The desalted peptide mixture was dissolved in water (50 mg/ml). Insoluble material (less than 1% of the total weight of the peptide digest) was removed by centrifugation.

A small spot containing 50 μl of peptide mixture was placed in the center of a Whatman 3MM filter paper sheet (18 × 22 in.). Following chromatography with butanol–acetic acid–water (4:1:5) the paper was rotated through 90° and high-voltage electrophoresis at 2500 v for 45 min (45 v/cm) was performed in a Gilson high-voltage electrophorator. The electrophoresis buffer used in all experiments was pyridine–water–acetic acid (10:90:0.4, pH 6.4).

Polyacrylamide Gel Electrophoresis. Disc electrophoresis was performed as described by Davis (1964). A 7% separating gel was used. Both the gels and the trough buffer (Tris–glycine, pH 8.3) were made up with 7 M urea.

Preparation of Radioactive α-Crystallins. Eyes were removed from calves within 10 min after death and placed on ice for transportation to the laboratory. Ten lenses were removed with their capsules intact and incubated in 10 cc of Hank's salts solution containing 20 μc of [^{14}C]amino acids (New England Nuclear) for 4 hr at 37°. At the end of the incubation period, the lenses were rinsed with ice-cold Hank's solution and the epithelial cells and fiber cells were separated. Radioactive α-crystallin (peak e) from these fiber cells was prepared as described in Materials and Methods of this paper.

Analysis of Radioactive Fractions. Protein fractions were analyzed for radioactivity in a Nuclear-Chicago

Mark II liquid scintillation counter. In each case 0.3 ml of the fraction was dissolved in a Hyamine–toluene–fluors mixture composed of 2 ml of a 1.5 M solution of Hyamine 10X (diisobutylcresoxyethoxydimethylbenzylammonium chloride monohydrate) in methanol plus 13 ml of a toluene solution of 2,5-diphenyloxazole (4 g/l.) and *p*-bis(5-phenyloxazolyl-2)benzene (50 mg/l.). All radioactivity is reported as disintegrations per minute.

Results

Column Chromatography of Isoelectric Precipitated α-Crystallins. The α-crystallins are separated from the other lens proteins by isoelectric precipitation (François *et al.*, 1955) and then resolved into five distinct fractions by DEAE-cellulose chromatography. A typical elution pattern is shown in Figure 1. Generally, a significant quantity of other proteins remains with the α-crystallin preparation during isoelectric precipitation. The function of the first elution buffer is to remove these non-α-crystallin impurities. The α-crystallins are referred to as peaks e–i because the letters a–d have been used to designate the β- and γ-crystallins (Papaconstantinou *et al.*, 1962).

Ultracentrifuge Analyses of SDS-Treated α-Crystallins. Sedimentation profiles of the α-crystallin fractions eluted from DEAE-cellulose are shown in Figure 2. The patterns for these protein fractions appear to be monodisperse. The $s_{20,w}$ for peaks e–i were found to range from 18.1 for peak e to 21.7 for peak g (Table I). These values agree well with sedimentation constants reported previously (Papaconstantinou *et al.*, 1962).

Each α-crystallin fraction was treated with 0.1 and 1.0% SDS. Treatment of the protein with 0.1% SDS generally gave rise to the formation of two peaks (Figure 2). The major peak has a sedimentation constant which varies from 5.3 to 6.8, whereas the sedimentation constant of the minor peak varies from 13.8 to 16.9 (Table I). When α-crystallins are treated with 1% SDS, they are converted to species having sedimentation constants which vary from 1.8 to 2.4. Similar sedimentation values have been observed for subunits produced by treatment of α-crystallins with 7 M urea (Bloemendal, 1962) and guanidine hydrochloride (Spector, 1965).

TABLE I: Effects of SDS on the Sedimentation Coefficients ($s_{20,w}$) of the α-Crystallin Fractions Eluted from DEAE-cellulose.

	Peaks				
% SDS[a]	e	f	g	h	i
0	18.1	18.4	21.7	20.9	21.4
0.1					
Main peak	6.0	6.8	6.1	6.3	5.3
Shoulder		13.8	16.2	16.9	15.4
1.0	2.4	1.8	2.0	1.8	2.1

[a] The % SDS was obtained by adding appropriate amounts of SDS from a 5% stock solution to a 0.8% solution of protein.

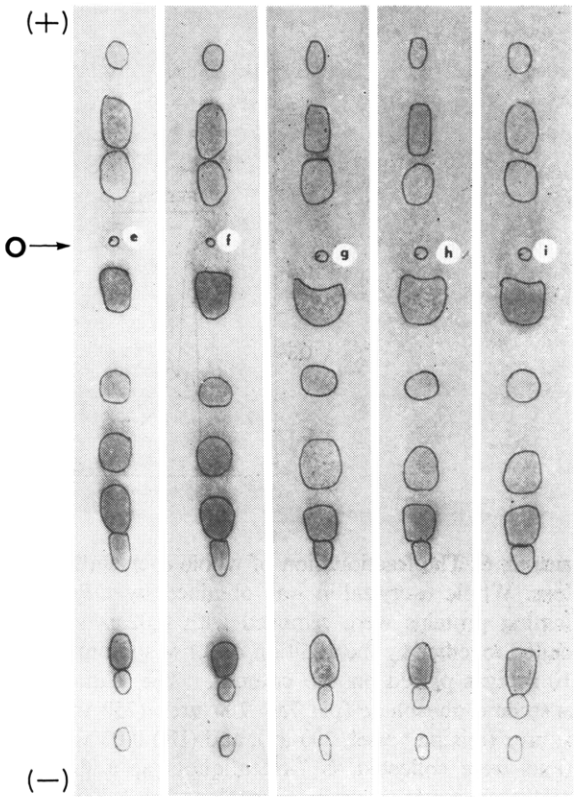

FIGURE 5: High-voltage electrophoresis patterns of the five α-crystallin peaks after proteolytic digestion.

In an attempt to determine whether the disaggregated subunits could reaggregate, some of the SDS-treated samples were dialyzed against 0.05 M sodium phosphate buffer (pH 6.8) to remove the detergent. After dialysis, $s_{20,w}$ values for both the 0.1 and 1% SDS-treated proteins were observed to increase. The 0.1% SDS fractions have sedimentation constants of 13.7–14.2, whereas the 1% SDS fractions have sedimentation constants of 3.3. These observations suggest that the SDS-treated proteins undergo a slight degree of recombination upon removal of the detergent. A more complete reaggregation may be blocked by residual, tightly bound SDS which is not removable by dialysis.

Immunochemical Analysis of SDS-Treated α-Crystallins. Antibodies to α-crystallin peaks f–i were prepared for use in our studies on the nature of the α-crystallin subunits. (Antibodies to protein in peak e were not prepared because of the ease with which this protein denatures.) To determine the degree of specificity of the antibodies, each preparation was treated with all five α-crystallins by the double-diffusion technique (Ouchterlony, 1948). It was found that all five α-

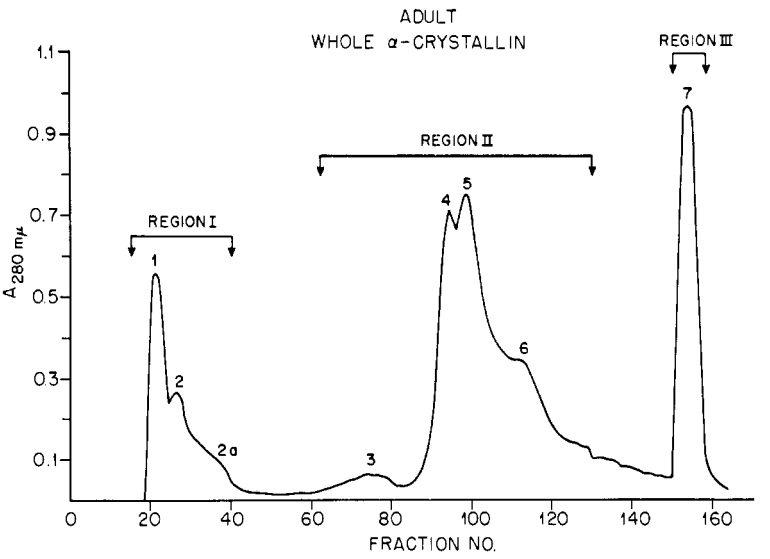

FIGURE 6: The fractionation of whole α-crystallin from adult bovine lenses on 7 M urea equilibrated DEAE-cellulose. Whole α-crystallin was obtained by DEAE chromatography of isoelectric precipitated proteins. (Contaminating proteins were removed with 0.02 M sodium phosphate (pH 7.0) and then all of the α-crystallins were eluted together by the addition of 0.1 M sodium phosphate (pH 5.7)–0.2 M NaCl.) Protein (230 mg) in a volume of 10 ml was placed on the column. The elution system used in these experiments was as follows: (I) 0.005 M sodium phosphate (pH 7.0)–7 M urea (250 ml), convex gradient from (I) to 0.03 M sodium phosphate (pH 5.7)–7 M urea (mixing vessel, 300 ml), and (III) 0.03 M sodium phosphate (pH 5.7)–0.1 M NaCl–7 M urea (200 ml). The fractions were collected as 7-ml aliquots at a flow rate of 6 min/fraction. The individual peaks are labeled with arabic numerals, the subunit regions are labeled with roman numerals.

crystallins give a reaction of identity with the individual antibodies (Figure 3).

Agar diffusion analyses were also carried out to determine whether new antigenic sites are exposed when the α-crystallins are dissociated by SDS (Figure 4). A solution of each α-crystallin was adjusted to 0.1 and 1% SDS, and part of this solution was subsequently dialyzed exhaustively against 0.9% NaCl. The rates of diffusion of the SDS-treated proteins, as indicated by the relative positions of the precipitin bands, parallel the changes observed by sedimentation analysis. With increasing concentrations of SDS, the precipitin bands appear closer to the antibody well. This change in the position of the precipitin band reflects the decrease in sedimentation constant. Also, as the concentration of SDS is increased, the bands become denser and more diffuse. This observation indicates that more reactive sites are exposed as the protein is dissociated. When the SDS is removed by dialysis, the precipitin bands become sharper and are further removed from the central well. A small spur was occasionally observed between the native and the 1.0% SDS-treated α-crystallin (Figure 4, peak g). It appears, therefore, that new antigenic sites, or altered sites, may be exposed when the protein is broken down into its subunits.

Peptide Analysis. From the data described above, it becomes apparent that there is a great deal of structural and immunochemical similarity between the five α-crystallin fractions. Therefore, the following experiments were designed to determine if differences in their primary protein structure were responsible for their elution properties on DEAE-cellulose. Each of the five α-crystallin fractions was digested with trypsin and chymotrypsin and the resultant peptide mixture was analyzed by high-voltage electrophoresis. The results are shown in Figure 5. The electrophoresis of the digests produced 11 spots for each α-crystallin. All of the electrophorograms appear to be identical. These experiments were repeated several times with consistent results. These observations indicate that the five α-crystallins are composed of subunits with the same or similar amino acid sequences. However, they do not rule out the possibility that a major difference between the α-crystallins may lie in the relative proportions of their subunits.

Urea–DEAE Chromatography of α-Crystallin Subunits. It has been established that α-crystallin is completely dissociated into 2S subunits with 7 M urea (Bloemendal *et al.*, 1962). These subunits can be fractionated by urea-equilibrated DEAE-cellulose (Björk, 1964). This technique was adopted to determine whether there are quantitative differences in the subunit content of the individual α-crystallin fractions prepared by DEAE-cellulose chromatography. Each α-crystallin fraction was dissociated in 7 M urea and the subunits were fractionated on urea–DEAE-cellulose columns. The elution pattern obtained for a whole, unresolved α-crystallin preparation is shown in Figure 6. There are seven clearly discernible peaks (labeled

with arabic numerals). For the sake of discussion, the column profiles have been divided into three groups (regions I, II, and III) according to the buffers with which they were eluted. Peaks 1 and 2 fall in region I, peaks 3–6 in region II, and subunit peak 7 comprises region III. In several of the following experiments, peak 2 was not resolved from peak 1 but instead appeared as a shoulder to peak 1. Peak 3 was not clearly discernible in the columns run with the individual α-crystallins because a much smaller amount of protein was used for these experiments.

The column profiles of the individual α-crystallin fractions are shown in Figure 7. The relative percentage of protein found in regions I–III can be seen in Table II.

TABLE II: The Subunit Composition of the Various α-Crystallin Fractions.

α-Crystallin Fractions	Percentage of Each Subunit Region in Total Protein			Ratio of Regions I:II
	I	II	III	
Whole α	18.2	63.5	18.3	0.29
Peak e	17.4	68.1	14.5	0.26
Peak f	16.4	68.5	15.1	0.24
Peak g	14.6	57.9	27.5	0.25
Peak h	9.7	47.9	42.3	0.20
Peak i	9.4	36.7	53.9	0.26

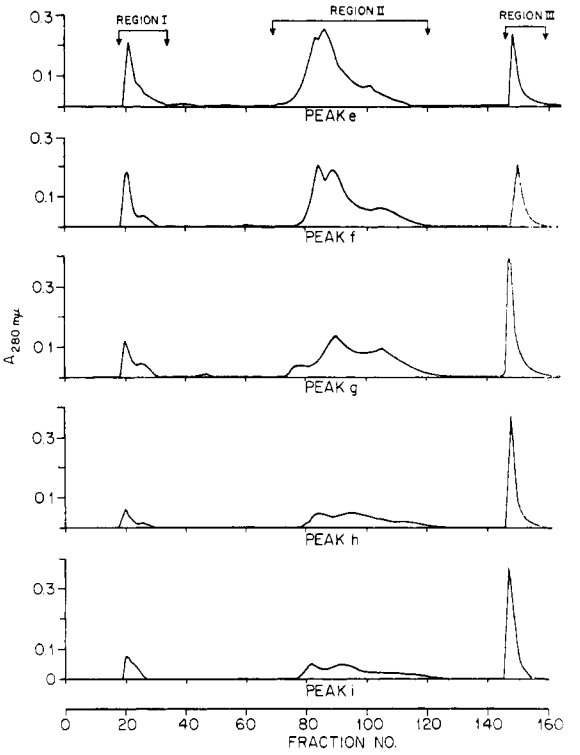

FIGURE 7: The fractionation of the five α-crystallin fractions on 7 M urea equilibrated DEAE-cellulose. The following amounts of protein were placed on the columns: peak e, 58 mg; peak f, 50 mg; peak g, 55 mg; peak h, 40 mg; and peak i, 42 mg.

The same types of subunits appear to be present in each of the α-crystallins thus indicating that qualitatively the five α-crystallins are the same. However, the quantity of the different subunits in each α-crystallin varies. An exception to this is that the percentages of the three subunits regions are almost identical for peaks e and f. This observation falls in line with earlier observations which indicate that peaks e and f are very closely related (Palmer, 1967). The subunits of regions I and II are most concentrated in peaks e and f and decrease progressively from peak f to peak i. Conversely, the concentration of region III increases progressively from peak e to peak i. Thus, region III makes up about 50% of the total subunit content of peak i whereas it is as low as 15% in peaks e and f. These data, combined with the peptide analyses, indicate that the heterogeneity of the α-crystallins is due to quantitative differences in their subunit content.

Acrylamide Analysis. The subunits eluted from the 7 M urea columns for all five α-crystallins were then examined by polyacrylamide electrophoresis in 7 M urea in order to establish that the five α-crystallins indeed contain the same subunits. The results are shown in Figure 8. The subunits eluted in region I give rise to three major bands, whereas the subunits of regions II and III give rise to one major band each. Several minor bands are also eluted in each region. The salient fact is not the number of bands appearing upon electrophoresis of each subunit region but rather that the bands in each region have the same electrophoretic mobility for all of the native α-crystallin fractions. It may be seen that within any one region there is some variation in the density of the bands between the different α-crystallin fractions. This is a further indication that these regions are heterogeneous and that the proportions of subunits within the individual α-crystallins vary.

Peptide Mapping of the α-Crystallin Subunits. High-voltage electrophoresis was carried out on the tryptic-chymotryptic digests of the three subunit regions isolated from whole α-crystallins to show that they represent polypeptides within different primary amino acid sequences. The electrophoresis revealed an identical pattern for regions II and III. Region I, however, produced a markedly different pattern from the other subunits (Figure 9). Region I contains two unique peptide spots and six spots in common with the other subunit regions.

To determine whether differences in primary structure do exist between subunit regions II and III, the high-voltage electrophoresis patterns were further resolved into a more complex peptide map by chroma-

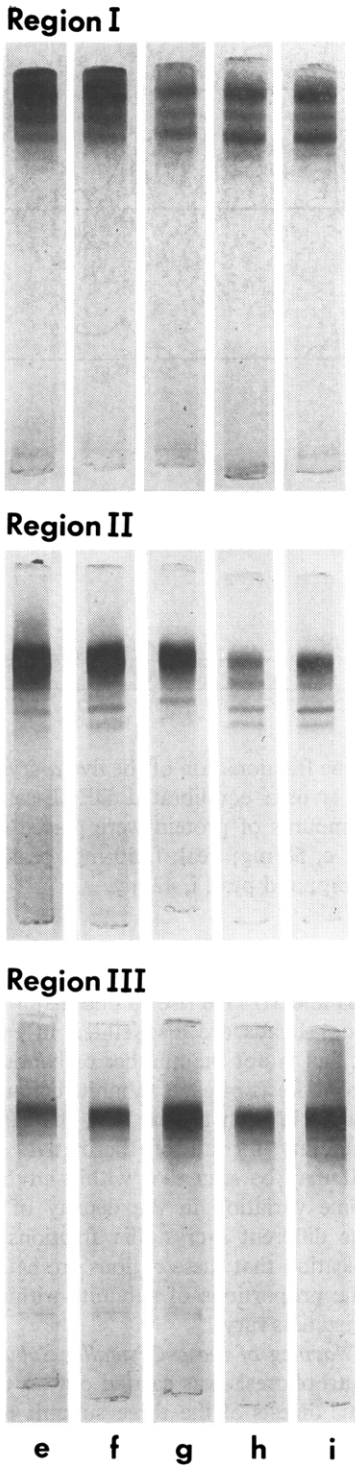

FIGURE 8: Polyacrylamide gel electrophoresis comparing the subunit regions for each α-crystallin.

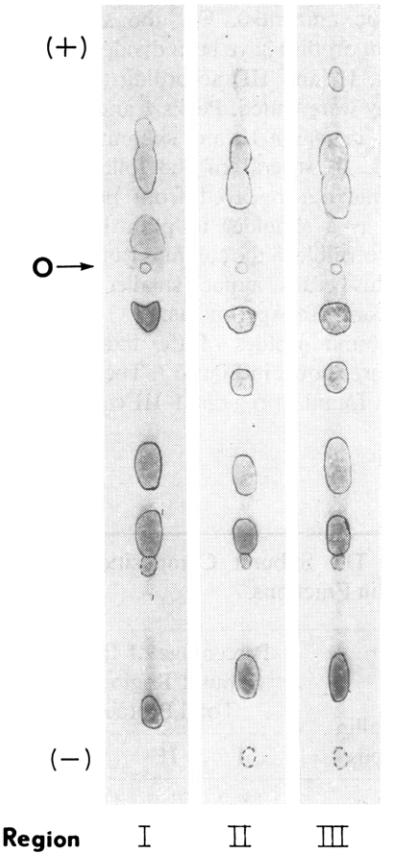

FIGURE 9: High-voltage electrophoresis patterns of the three subunit regions after proteolytic digestion.

tography on the same sheet of paper (Figure 10). In this case, several peptides were found to be unique for each region (II and III) while they still had 30 peptide spots in common.

Since it now appears that the α-crystallins differ from each other quantitatively with respect to their subunit composition, the possibility exists that the five α-crystallins are produced by a dissociation and reassociation of the proteins during their extraction and fractionation. Experiments were carried out to determine whether this dissociation–reassociation can occur during the fractionation of the α-crystallins. Highly purified, radioactive α-crystallin (peak e) was stirred for 12 hr with a nonradioactive mixture of all five α-crystallins obtained by isoelectric precipitation. The mixture was then fractionated by DEAE-cellulose into peaks e–i (Figure 11). The majority of the radioactivity remained with peak e, a considerable amount was transferred to peak f, and a very small amount passed to peak g. Peaks h and i were completely unlabeled. (Note also that the non-α-crystallin protein, which is eluted prior to peak e, does not contain radioactive material.) Peaks e and f are known to be highly related and interconvertible (Palmer, 1967). Therefore, it was not surprising to find the transfer of label from peak e to peak f. However, the fact that no significant amount of label enters the other proteins indicates that the α-crystallins remain essentially intact during DEAE-cellulose fractionation and are not an artefact

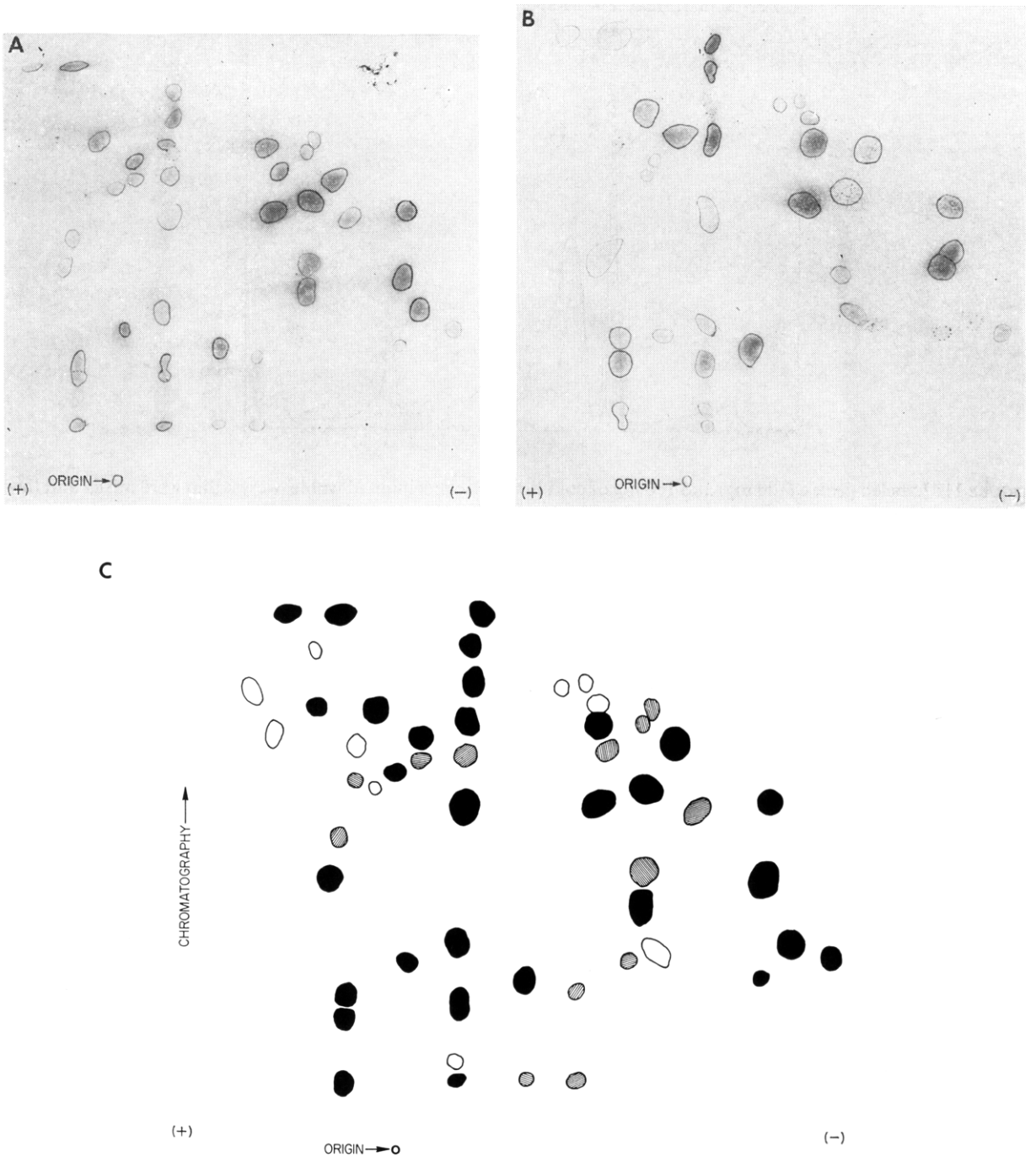

FIGURE 10: Peptide maps. (A) Region II; (B) region III. (C) A composite study comparing the spots on the two peptide maps. The solid circles are spots common to regions II and III, the hatched spots are unique to region II, and the open circles are unique to region III.

of our procedure. Furthermore, it has been found that isoelectric precipitation of the α-crystallins prior to column chromatography has no effect upon the elution pattern of these proteins. Whole lens homogenates and α-crystallin preparations obtained by isoelectric precipitation give identical elution profiles in the α-crystallin region.

Discussion

Most physicochemical and immunochemical studies on the properties of α-crystallins have been performed with protein samples composed of a mixture of α-crystallins. The α-crystallins can be fractionated by chromatography (Björk, 1963; Papaconstantinou *et al.*,

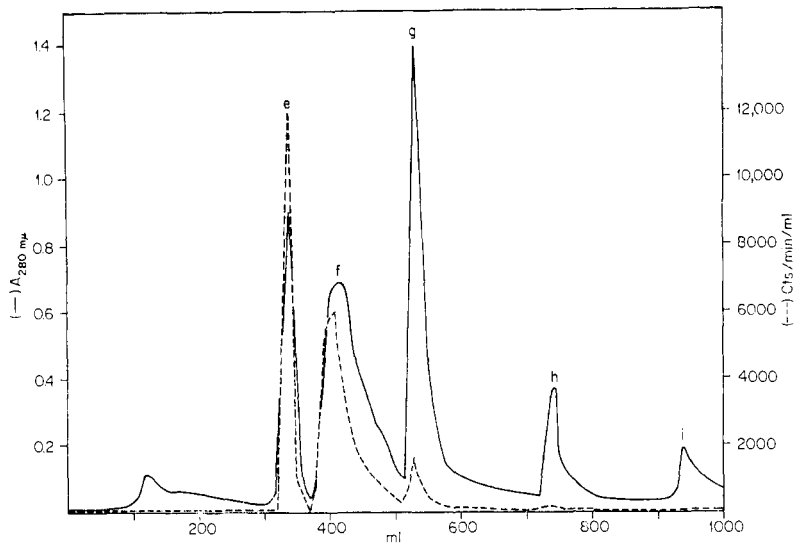

FIGURE 11: Labeled peak e (100 mg) and 100 mg of cold isoelectric precipitated whole α-crystallin were mixed and then fractionated by DEAE-cellulose. (---------) cpm/ml; A_{280} mμ (———).

1962) or by immunoelectrophoresis (Manski *et al.*, 1961) into several components. However, until now no significant chemical or physical difference other than chromatographic properties could be attributed to the individual α-crystallins. It is because of this that it was widely believed that the apparent heterogeneity of α-crystallin preparations was due to chromatographic artefacts. The data presented in this paper indicate that the five α-crystallins isolated by DEAE-cellulose chromatography are structurally very similar. They are progressively broken down by increasing concentrations of SDS into small subunits, they are immunochemically identical, and the native proteins have similar sedimentation constants.

In this study, we have shown that the differences between the various α-crystallins lie in the proportions of individual subunits rather than in the chemical nature of the subunits. Fractionation of these individual α-crystallins on urea-equilibrated DEAE-cellulose shows that they all contain the same subunits; however, the amount of each subunit varies from one α-crystallin to the next. For example, the concentration of the last subunit eluted from urea–DEAE-cellulose (subunit 7) varies progressively from 15% of the total protein in peaks e and f to 54% of the total protein in peak i. Subunit 7 requires the greatest ionic strength to elute it from DEAE, and this subunit is found in its greatest concentration in the native α-crystallin eluted by the highest ionic strength buffer. Therefore, the ionic strengths of the buffers required to elute the native α-crystallins from DEAE-cellulose can be correlated to their relative concentrations of subunit 7.

We now believe that the results of the experiments presented in this study offer an explanation for the existence of multiple forms of α-crystallins. The degree of resolution of the mixture of α-crystallins into individual proteins depends largely upon the sensitivity of the fractionation procedure. For example, Björk resolved the α-crystallins into two fractions with DEAE-cellulose chromatography using a convex gradient elution system (Björk, 1963). The ionic strength gradient in this buffer system was, however, quite steep and hence, in all probability, was not sensitive enough to resolve proteins which have very similar net negative charges. François *et al.* (1955) fractionated the α-crystallins into two components: α_1 which precipitated at pH 5 and α_2 which precipitated upon the addition of ethanol. We have found that his first component contains those proteins which we have designated as peaks g–i and that peaks e and f are those precipitated by the addition of ethanol. As we have shown, the only difference between these proteins is their quantitative subunit composition, and yet this variation may be the cause for the variation in solubility shown by François *et al.*

It is not certain at this time whether this multiplicity of α-crystallins exists naturally within the fiber cell. Our experiments indicate that these multiple forms are not a product of the fractionation procedures used. These experiments do show, however, that peaks e and f may be interconvertible. The urea–DEAE-cellulose columns for these two α-crystallins also show a close relationship in subunit content. The fact that peaks g–i remain essentially unlabeled, however, in the mixing experiment shown in Figure 11 indicates that there is no rearrangement of the subunits upon contact with DEAE-cellulose to produce multiple forms of α-crystallins. The production, therefore, of mutiple forms of α-crystallins must occur *in vivo* or must be attributed to some other phenomenon occurring prior to the fractionation steps.

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Enzymic Hydrolysis of Synthetic Polypeptides under High Helical Content*

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ABSTRACT: The effect of ethanol on the hydrolysis of polyglutamic acid and polylysine by papain was investigated. Under conditions favoring the helical conformation in aqueous solutions addition of ethanol to a concentration of 20 vol % lowered the rate by a factor of 10^2; in 40% ethanol the rate is lowered by a factor of at least 10^4. In the hydrolysis of polyglutamic acid by ficin and subtilisin, lowering the ionic strength shifts the alkaline limb of the rate–pH curve toward higher pH, while not affecting its slope. On the low pH (helix favoring) side of the rate–pH curve an appreciable rate of hydrolysis is observed at all ionic strengths. The rate data are considered in terms of the previously proposed model (Miller, W. G. (1964a), *J. Am. Chem. Soc. 86*, 3913) involving conformation and charge-state restrictions.

All data are consistent with the proposal that the helix is not amenable to enzymic hydrolysis. The high rate at low pH in aqueous solutions of polyglutamic acid is shown to be a result of residual random coil residues which are always present. The model assuming hydrolysis occurs only in random coil peptide bonds with adjacent side chains uncharged is found to be in qualitative agreement with all the experimental data, and in semiquantitative agreement with the ionic strength effects.

We have previously investigated the enzymic hydrolysis of high molecular weight PGA[1] (Miller, 1961, 1964a) and poly-α-L-lysine (Miller, 1964b) in 0.2 M NaCl by a variety of proteolytic enzymes. In most cases the rate of hydrolysis exhibited an unusually large pH dependence. The charge state and conformation of the substrate appeared to be of predominant importance. The pH dependence of the action of chymotrypsin, elastase, ficin, papain, and subtilisin on both polyglutamic acid and polylysine was accounted for in terms of a single mechanism. This mechanism assumed peptide bonds in helical regions of the polypeptide were not hydrolyzed, and that in random coil regions only those peptide bonds were hydrolyzed in which the adjacent amino acid side chains were uncharged. Several other model mechanisms were considered, but their predicted pH dependence was in poor agreement with the experimental data. Incorporation of some type of substrate charge dependence in the proposed mechanism had precedence in the work of Kimmel and Smith (1954), and was strongly supported by our data. The necessity for invoking a substrate conformational dependence was not as great, principally because low

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[1] Abbreviation used: PGA, poly-α-L-glutamic acid.