STUDIES ON THE CARBOXYL TERMINAL AMINO ACID RESIDUES OF RABBIT LENS PROTEINS

by

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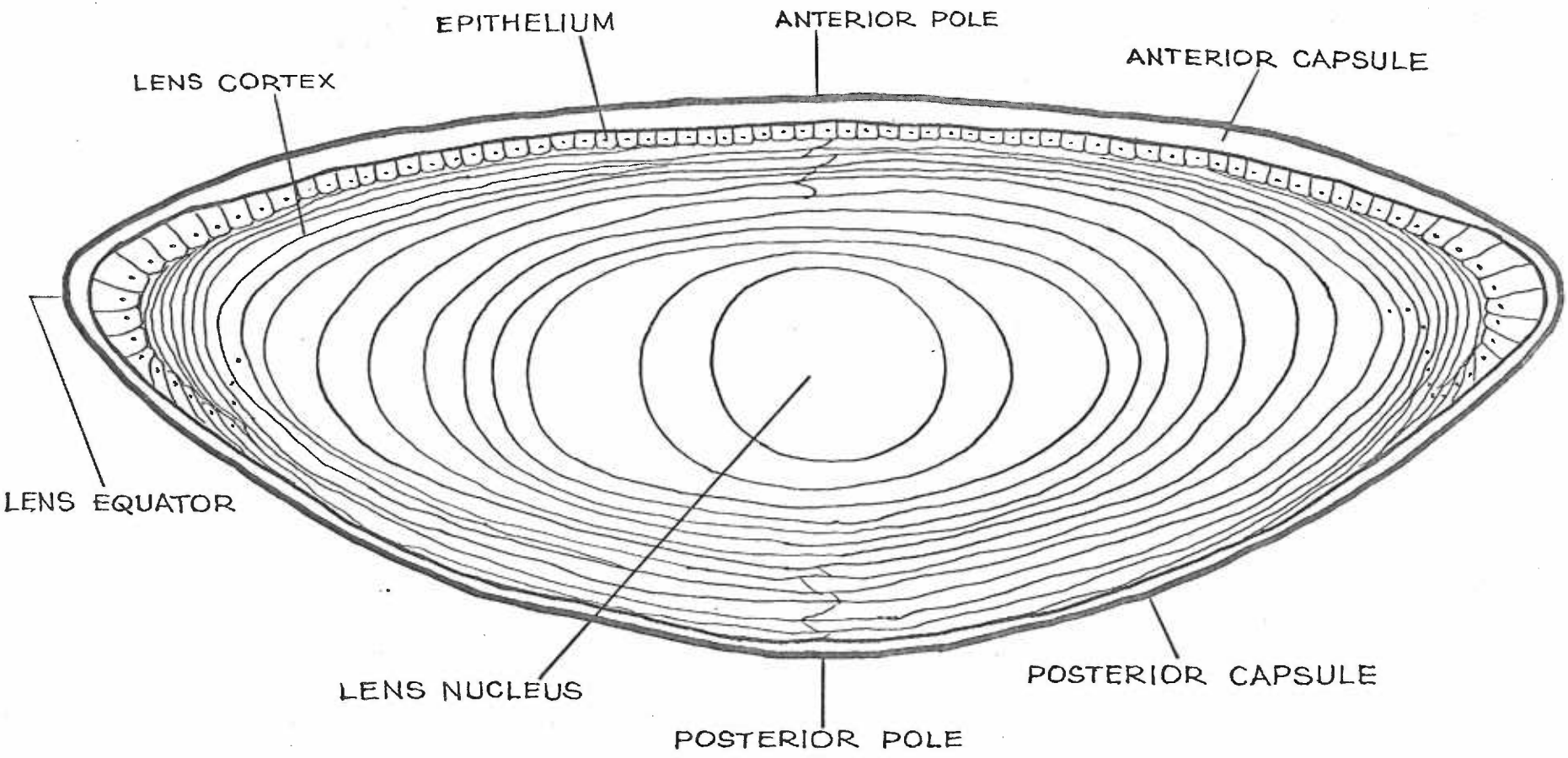
INTRODUCTION I

The lens 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 up 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 single 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 continues to grow 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 this case the cells are packed into the nucleus instead of

Figure 1. Cross section diagram of the lens


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

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 above-described general mode of development of the lens, there are three distinct features about it (1).

1. It is cytologically isolated from its surroundings at an early embryonic stage. Thereafter, there is no interchange of cells between the lens and its surroundings.
2. It contains only epithelial cells at all stages of development.
3. Its growth never ceases and its weight increases throughout the life span of the organism.

In 1830, Berzelius found a protein fraction in the lens which he termed crystallin (2). Mörner described the separation of the lens proteins into two main fractions in 1894 (3).

1. The soluble proteins which he further separated by isoelectric precipitation into the alpha and the beta crystallin fractions.
2. The insoluble protein which was termed albuminoid.

Subsequent investigations in the field of lens proteins can be grouped into two main categories.

1. Physicochemical studies.
2. Physiological studies.

A general description of the typical investigations being carried out in each of these categories is presented here.

Physicochemical Studies

Unless otherwise specified, bovine lenses were used in the studies reviewed here. 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 (4). They noted that at physiological pH ranges, beta crystallin by itself tended to precipitate out of solution. However, when a solution of the alpha and beta crystallin fractions was maintained at physiological pH ranges, no spontaneous precipitation was observed. A year later the same investigators isolated gamma crystallin from the beta crystallin fraction (5). Alpha crystallin was isolated by precipitation at pH 5.0. Beta crystallin was defined as the protein precipitating 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 $(NH_4)_2SO_4$ to a concentration of 50% saturation.

Krause modified Mörner's method for the preparation of alpha crystallin, beta crystallin and albuminoid (6). In subsequent work he performed a general quantitative analysis on the alpha, beta and gamma crystallin fractions, albuminoid and the lens capsule (7). On the basis of his results he estimated the molecular weights of alpha, beta and gamma crystallin to be 73000, 76000 and 48000 respectively.

In 1939 Hesselvik electrophoretically separated whole lens extracts and obtained two main components (8). The faster moving component had a sulfhydryl (SH) content similar to alpha crystallin and the slower moving component had an SH content similar to beta

crystallin. The faster moving and the slower moving components had isoelectric points of 5.1 and 6.1, respectively.

Francois and his group presented a new method for the fractionation of the soluble lens proteins (9). Extracts were made of the nuclear and cortical lens proteins and electrophoresed on paper. In both cases, three fractions were observed, designated as Ic, IIc and IIIc for the cortical extracts and In, IIn and IIIn for the nuclear extracts. When the cortical lens extracts were put through the pH 5.0 isoelectric precipitation procedure for the removal of the alpha crystallin fraction, only a portion of fraction Ic was removed. Addition of ethanol to a concentration of 13% caused almost complete removal of fraction Ic from the supernatant electropherogram. The protein removed by isoelectric precipitation at pH 5.0 was termed alpha-1-c crystallin and that removed by the addition of ethanol to a concentration of 13% was termed alpha-2-c crystallin.

Adjusting the solution to pH 8.0 and adding ethanol to a concentration of 26% resulted in the disappearance of fractions IIc and IIIc. This protein was termed beta-c crystallin. This latter fraction was found to be very resistant to separation by zone electrophoresis. The fractions from the nuclear extracts behaved differently, indicating a difference between the cortical and nuclear proteins. Adjusting the pH to 5.0 caused almost complete removal of fraction In from the electropherogram. This fraction was termed alpha-n crystallin. The beta-n crystallin fraction was obtained by adjusting the pH to 8.0 and adding ethanol to a concentration of 13%. Using light scattering methods, the estimated molecular weights for alpha-2-c

and beta-c crystallin were 1.2×10^6 and 2.0×10^5, respectively.

Two years later Resnik studied the alpha crystallin from calf lens using the isolation procedures described by Francois (10). Using the ultracentrifuge, he could not demonstrate any difference between the alpha-1-c and the alpha-2-c crystallin fractions. Both crystallins had sedimentation coefficients of 18.8 S corresponding to a molecular weight of 1×10^6. The value for the same material analyzed by light scattering was 1.5×10^6. Resnik concluded that the addition of ethanol in the procedure of Francois was necessary only to obtain complete precipitation of the alpha crystallin fraction. Francois and Rabaey showed by paper and agar electrophoresis the presence of up to at least four fractions in extracts of the human lens (11). The general trend observed was that the lens extracts from children were much more amenable to resolution by electrophoresis than were the lens extracts from adults. In most cases, electropherograms from the adult lenses yielded only a large, ill-defined polydisperse peak.

Bloemendal and Ten Cate reported another method for the isolation of the alpha, beta and gamma crystallins (12). Alpha crystallin was isolated by starch block electrophoresis. The fraction thus isolated showed a single homogeneous peak by paper and free boundary electrophoresis. Beta and gamma crystallin were obtained by salting-out procedures using $(NH_4)_2SO_4$. Amino terminal (N-terminal) analyses were performed on all three crystallin fractions using the FDNB method of Sanger (13). For alpha crystallin the following dinitrophenyl (DNP) amino acid derivatives were found in decreasing concentration;

glutamate, serine, glycine, alanine and threonine. Experiments with beta and gamma crystallin yielded similar results.

A year later the same group reported further studies on the lens proteins (14). As previously, alpha crystallin was prepared by starch block electrophoresis. Comparison studies showed alpha, beta and gamma crystallin to have respectively decreasing mobilities in an electrical field. Ultracentrifugation of cortical lens extracts revealed three components. Isoelectric precipitation of alpha crystallin at pH 5.0 caused the disappearance of the fastest sedimenting component. It was also noted that alpha crystallin prepared by starch block electrophoresis gave a single component in the ultracentrifuge, but isoelectric precipitation and subsequent ultracentrifugation of this alpha crystallin resulted in the appearance of two components.

The effect of pH on alpha crystallin was studied by Resnik and Kenton (15). Lowered sedimentation coefficients and increased intrinsic viscosities indicated that alpha crystallin dissociated into two subunits at pH 3.0 under conditions of low ionic strength. The possibility that these two subunits could be similar to the alpha-1-c and alpha-2-c crystallin fractions of Francois was ruled out when similar results were obtained using either of the latter two fractions. This dissociation was not observed when the ionic strength of the solution was raised. The explanation offered was that electrostatic repulsion at low ionic strength caused the observed dissociation. Sedimentation coefficients were also determined and no concentration dependence was observed.

Björk reported another method for fractionating the soluble lens proteins (16). Vertical-column electrophoresis with cellulose as the support was the method employed and three distinct fractions, designated I, II and III were obtained. Fraction II was shown to be heterogeneous by ultracentrifugal and free electrophoresis criteria, but fraction I was shown to be homogeneous by the same criteria. Fraction III was homogeneous in the ultracentrifuge, but no electrophoretic data was obtained due to solubility problems. From these observations it was concluded that fractions I, II and III corresponded to the alpha, beta and gamma crystallin fractions, respectively.

Spector separated the whole lens proteins into ten fractions using diethylaminoethyl (DEAE) cellulose, eluting the fractions off with a stepwise increase in the buffer concentration at constant pH (17). The group of Santamaria studied some of the physicochemical properties of alpha-n crystallin (18). The protein was obtained by isoelectric precipitation of the nuclear extracts of the lens. Electrophoretic homogeneity from pH 3.5 - 8.6 was demonstrated. They also determined the apparent partial specific volume, intrinsic viscosity, ultraviolet absorption spectrum and extinction coefficient of alpha-n crystallin.

Perry and Koenig used a combination of isoelectric precipitation and salt fractionation of the whole lens proteins to prepare alpha, beta and gamma crystallin (19). Alpha crystallin was removed by the isoelectric precipitation procedure while the beta and gamma crystallins were separated by a salt fractionation scheme which is presented in their paper. Electrophoretic and sedimentation studies were

performed and from the data obtained, molecular weights of 857000, 545900 and 46930 were calculated for alpha, beta and gamma crystallin, respectively.

Bon reported other physicochemical studies on the lens proteins in which the sedimentation and electrophoretic characteristics of alpha and beta crystallin were observed (20). The whole lens extract revealed two components by electrophoresis while the ultracentrifugal studies showed three components. Isoelectric precipitation to remove alpha crystallin and subsequent study of the precipitate revealed a single component by electrophoresis but two components by ultracentrifugal analysis. The supernatant showed one component by both electrophoresis and ultracentrifugation. It was concluded that the pH 5.0 precipitate was alpha crystallin and the protein in the supernatant was beta crystallin. The two components of alpha crystallin seen in the ultracentrifuge were termed alpha-A and alpha-B crystallin. Serological studies showed similar antigenic groups in both fractions. It was postulated that the appearance of alpha-B crystallin was due to denaturation of alpha-A crystallin, the latter being considered to be the native globular form and the former a denatured, elongated form of the native alpha crystallin.

Björk separated gamma crystallin from alpha and beta crystallin by the use of Sephadex G-75 gel filtration (21). Alpha and beta crystallin appeared first in the effluent of the column and the gamma crystallin fraction emerged last. A comparative study of the gamma crystallin in the lens cortex and nucleus showed that this protein is

present in a higher concentration in the nucleus than in the cortex. Gamma crystallin was shown to be homogeneous by sedimentation and diffusion analysis but free boundary electrophoresis at pH 5.0 showed four components and immunoelectrophoretic studies at pH 8.2 showed two bands. Since the different components could not be detected by ultracentrifugation and eluted together from a gel filtration column, it was concluded that the components observed, if indeed they were different, had similar molecular weights in the range of 20000 to 25000. Analysis of the SH content in the three classical lens protein fractions showed gamma crystallin to have a higher concentration of SH groups than either alpha or beta crystallin, though beta crystallin did have a higher SH content than alpha crystallin.

Bloemendal et al showed that alpha crystallin is the slowest moving component in starch gel electrophoresis (22). This is opposite to the behavior of alpha crystallin in other supportive media. This observation was attributed to the large size of alpha crystallin. The mobility of alpha crystallin was increased upon denaturation. Apparently, denaturation involved a change in the tertiary and quaternary structure of the molecule. Treatment of the beta and gamma crystallin fractions with urea resulted in an increase in the number of bands seen in starch block electrophoresis. Also, some of the bands obtained by this method from beta and gamma crystallin corresponded to bands obtained by a similar treatment of alpha crystallin. This was taken to indicate the possibility of the three protein fractions having at least one subunit in common. Homogeneity of the alpha crystallin obtained by the starch block electrophoresis technique

was concluded from electrophoretic (free boundary and zone) and ultracentrifugal studies which both showed one homogeneous component, N-terminal determinations according to the FDNB method of Sanger which revealed only glutamate as the N-terminal amino acid and the observation of only a single band in immunological studies using the Ouchterlony precipitin technique.

Bont and co-workers observed that alpha crystallin, shown to be homogeneous by vertical starch block electrophoresis, dissociates into subunits when treated with 7.0 M urea, as evidenced by the appearance of 12 - 13 bands in vertical starch block electrophoresis (23). Experiments with gamma crystallin and beta crystallin yielded the same results. Treatment of alpha crystallin with 3.5 M urea and subsequent ultracentrifugal analysis showed the presence of three components but similar treatment with 7.0 M urea and analysis in the ultracentrifuge revealed only one component. This was taken to indicate a partial deagregation of the molecule with 3.5 M urea and a breakdown of the molecule into subunits of similar size, shape and molecular weight with 7.0 M urea. The sedimentation coefficient of the subunit was determined to be 1.8 S. Applying the approach to equilibrium method, this value corresponded to an approximate molecular weight of 26000. Analysis of native alpha crystallin in the ultracentrifuge revealed a symmetrical peak and a sedimentation coefficient of 19.0 S.

As mentioned above, treatment with 7.0 M urea resulted in a breakdown of the molecule into subunits having a sedimentation coefficient of 1.8 S. Dialysis of the solution against phosphate

buffer to remove the urea resulted in a recovery of the symmetrical peak and 19.0 S sedimentation coefficient value of the native alpha crystallin. Ultracentrifugal analysis of the whole lens extracts showed three peaks. Treatment of the whole lens extracts with 7.0 M urea resulted in the appearance of only one peak. Removal of urea by dialysis resulted in the appearance of two peaks and a third rapidly sedimenting component. From these experiments it was concluded that the effect of urea on the individual crystallin fractions and on the soluble lens proteins as a whole was at least partially reversible. The results of the experiment with whole lens extracts were taken to indicate a hybridization between subunits of the different crystallin fractions upon removal of urea, resulting in the modified ultracentrifugal pattern observed.

Using preparative column electrophoresis, Firfarova and Levdikova separated whole lens extracts into the alpha, beta and gamma crystallin fractions (24). The beta crystallin prepared by this method was homogeneous by free boundary electrophoresis but revealed two components in the ultracentrifuge. Electrophoresis of the gamma crystallin fraction also showed at least two components to be present. It was noted that some of the properties of the beta and gamma crystallin fractions were changed during the separation procedure and this was attributed to the oxidation of SH groups in the molecule.

Using DEAE cellulose fractionation procedures, Papaconstantinou et al showed that the cortical lens fibers have several proteins not found in the nuclear lens fibers (25). Electrophoretic studies on the protein peaks from the DEAE cellulose column confirmed the former

reports of heterogeneity of the alpha, beta and gamma crystallin fractions. Alpha crystallin purified by isoelectric precipitation showed four components when put through a DEAE cellulose column. This latter observation was confirmed by immunochemical experiments. Two of the four components were observed to undergo an irreversible transformation at alkaline pH.

Bloemendal and his group prepared alpha crystallin by vertical starch block electrophoresis and showed the material thus obtained to have a sedimentation coefficient of 19.0 S corresponding to an approximate molecular weight of 810000 (26). Treatment of the material with 7.0 M urea caused a breakdown of the protein into subunits having a sedimentation coefficient of 1.8 S. Removal of the urea by dialysis again resulted in the recovery of the 19.0 S component. This confirmed the observations of Bont et al (23). Starch gel and polyacrylamide gel electrophoresis of 7.0 M urea-treated alpha crystallin resulted in the appearance of 12 - 13 bands if the run was carried out in gels containing 7.0 M urea.

Niyogi and Koenig reported on a study of the relative effects of pH and ionic strength on the structural organization of alpha crystallin (27). Alpha crystallin was isolated by isoelectric precipitation from cortical lens extracts. The protein obtained in this manner was shown to be homogeneous by electrophoresis at various acid and alkaline pH values and ionic strengths. Above pH 3.0, sedimentation studies showed essentially the same results as did the electrophoretic studies. However, below pH 3.0, two or three components were observed. Raising the pH above 3.0 resulted in the disappearance of

the other peaks and resolution into one peak. From sedimentation, electrophoretic, viscosity and light scattering data, it was postulated that at pH 2.0, unfolding of the molecule occurs leading to dissociation of the molecule into subunits. Dissociation at pH 2.0 was found to be related to the ionic strength, three components being observed when the ionic strength was 0.05 and two components when the ionic strength was 0.2. The pH of the solution was also found to be important, the protein being dissociated at a pH of 2.0 even when the ionic strength was raised to 0.2. Reassociation of the dissociated protein was observed when the pH was raised to 3.5 even if the ionic strength was dropped back down to 0.05. Studies on the protein before dissociation and after reassociation led to the conclusion that reassociation, though it apparently occurs, is incomplete.

Björk isolated alpha crystallin from whole lens extracts by use of vertical column zone electrophoresis (28). The alpha crystallin isolated by this method was further separated into two fractions by DEAE cellulose chromatography namely, alpha' and alpha''. The two alpha crystallin fractions obtained had markedly different free electrophoretic mobilities at pH 8.0 but small differences in the observed sedimentation coefficients. These fractions were shown to have essentially the same amino acid composition, amide group content, intrinsic viscosity and optical rotatory dispersion. Molecular weight estimations by light scattering revealed essentially the same results for both. Double diffusion tests showed an immunological identity between the two alpha crystallin fractions and it was postulated on the basis of the above observations that the two alpha

crystallin fractions have either slightly different secondary or tertiary structures, or that one of the two has more of an electrically charged group bound to it than the other.

Using gamma crystallin isolated by Sephadex G-75, it was shown that four components could be obtained by free boundary electrophoresis (29). When the same material was applied to a sulfoethyl (SE) Sephadex column four fractions were again detected upon elution. When the pooled material from the SE Sephadex column was chromatographed on a phosphocellulose (PC) column, six fractions termed Ia, Ib, II, III, IVa and IVb were obtained. Rechromatography of fraction III on the same type of column (PC) but at a different pH caused separation into two fractions designated IIIa and IIIb. Four of the above described fractions; namely, II, IIIa, IIIb and IVb were shown to be individually homogeneous and crystallizable. These four fractions were shown to have differences in SH content and amino acid composition. The differences in amino acid composition were small but definite. All four fractions had the same N-terminal amino acids and exhibited no differences in antigenic identity sites. The N-terminal amino acid was glycine. No ultracentrifugal studies on any of the four were performed since even the unfractionated gamma crystallin revealed only a single peak in the ultracentrifuge. The above observations suggested that each of the four fractions were composed of one polypeptide chain only.

A method for the separation of alpha crystallin from cortical lens extracts was presented by Bloemendal et al (30). The solution containing the alpha, beta and gamma crystallin fractions was layered

over a continuous sucrose density gradient (8 - 22%) and subjected to high speed centrifugation. Subsequent analysis of the centrifuged material showed two protein peaks. By determination of the sedimentation coefficient (19.0 S) the heavier component was shown to be alpha crystallin and the lighter component was shown to be composed of beta and gamma crystallin. Alpha crystallin prepared by this gradient density centrifugation technique did not have the contaminating band in acrylamide gel electrophoresis as did the alpha crystallin fractions prepared by isoelectric precipitation and vertical starch block electrophoresis methods.

In a comparative study of calf and ox lenses, Niyogi and Koenig found that there were slight differences between the alpha crystallins from the two different sources (31). In both cases, the alpha crystallin was isolated by isoelectric precipitation. Electrophoretic and sedimentation studies indicated that the alpha crystallin from these two sources had similar degrees of homogeneity, though by the former criterion, the protein from the ox lenses showed a greater degree of homogeneity. Alpha crystallin was more difficult to obtain by isoelectric precipitation from calf lenses than from ox lenses and on the whole, calf lens alpha crystallin showed slightly higher intrinsic viscosities, densities and sedimentation coefficient values than did ox lens alpha crystallin. These results led the investigators to conclude that though there were physicochemical differences between the alpha crystallin fractions from the two sources, these differences were not large.

Spector and Katz studied the effect of known dissociating agents

on the behavior of alpha crystallin in the ultracentrifuge (32). Sedimentation equilibrium studies at neutral pH indicated the presence of a number of different sized molecular species. A progressive deaggregation of alpha crystallin was observed with increasing pH. Other sedimentation equilibrium studies were performed on the protein at pH 12.8 as well as in the presence of various dissociating agents such as 7.0 M urea and guanidine hydrochloride. These studies and others performed on the succinylated protein showed a molecular heterogeneity.

Van Dam and Ten Cate applied soluble lens extracts to a Sephadex G-200 column and observed six peaks in the effluent, designated peaks a through f (33). Immunological and electrophoretic analyses of these fractions showed peak a to be alpha crystallin, peaks b, c and d to be beta crystallin and peak e to be a fraction termed pre-alpha crystallin, possibly contaminated with some beta and gamma crystallin. Peak f was composed of low molecular weight compounds, possibly polypeptides. Carboxyl terminal (C-terminal) analysis of the alpha crystallin fraction showed serine to be present to an extent of 0.8 - 1.0 mole per 48000 grams of anhydrous protein. Assuming a molecular weight of 1×10^6 for alpha crystallin these results were taken to indicate that alpha crystallin consisted of 20 - 25 subunits per molecule, each subunit having the same C-terminal amino acid.

Wisse et al prepared alpha crystallin by a combination of zone electrophoresis, gradient density centrifugation and Sephadex gel filtration (34). The molecular weight of the material obtained was determined by three different methods; namely, the approach to equilibrium method, sedimentation diffusion equilibrium method and

determination of cysteine SH groups which gave molecular weights of 24000, 21000 and 23000, respectively. These investigators showed that the alpha crystallin fraction used was dissociated into subunits by treatment with urea, but an attempt to correlate the conditions of urea treatment with the number of bands obtained by polyacrylamide gel electrophoresis was not successful. Dissociation of the molecule by urea and recombination of the separated subunits by removal of urea, both procedures evaluated by polyacrylamide gel electrophoresis, did not result in clear-cut, reproducible results. From this and other data, it was theorized that the varied results obtained upon recombination could be due to the slow isomerization, after dissociation, of the subunits into one or more final products which may or may not be the same as the original molecule. The term isomerization as used here is taken to indicate differences in secondary and/or tertiary structure of the subunits, not a difference in the primary structure.

Another method for the separation of the lens proteins into their three classical fractions was presented by Spector (35). In contrast to other methods which generally remove alpha crystallin as a precipitate, leaving beta and gamma crystallin in solution, precipitation by zinc glycinate was used to remove beta and gamma crystallin from the solution leaving the alpha crystallin fraction in the supernatant. The precipitate was then resolubilized and separated by Sephadex G-75 chromatography. Paper electrophoresis of these fractions showed all three to be relatively homogeneous, especially the alpha and gamma crystallin fractions. The beta crystallin fraction had a trace component migrating in a manner similar to alpha

crystallin. Alpha, beta and gamma crystallin were observed to have a decreasing order of mobility in the electrophoretic analysis. Ultracentrifugal analysis of the fractions as prepared above showed alpha and gamma crystallin to have only a single component each whereas the beta crystallin fraction revealed three components.

The three crystallin fractions were also shown to be separable by DEAE cellulose chromatography using an increasing ionic strength gradient to elute them off the column. The gamma crystallin was first to emerge from the column followed by the beta and then the alpha crystallin. However, when each of these individual fractions separated by DEAE cellulose chromatography was reapplied to the DEAE cellulose column, more than one peak was observed in the effluent. Comparison of the alpha, beta and gamma crystallin fractions prepared by the DEAE cellulose method and the zinc glycinate method showed these three fractions to be similar by the criterion of electrophoresis. However, ultracentrifugal studies indicated that the beta crystallin obtained by DEAE cellulose fractionation had only two components in contrast to the three components of the beta crystallin prepared by the zinc glycinate-Sephadex G-75 method. The alpha and gamma crystallin fractions prepared by the two methods appeared to be similar by the criterion of ultracentrifugation.

Physiological Studies

Nordmann noted that in senile sclerosis of the lens, one of the chemical changes observed is an accumulation of insoluble proteins (36). This observation was attributed to the slow decrease in

solubility of the beta crystallin fraction of the lens proteins. Dische et al studied the changes in total lens proteins of rats during aging (37). The general observation was that the total amount of protein in individual rat lenses increased throughout the life span of the animal. The soluble lens proteins increased at a rapid rate during and up to the first twenty weeks of life after which the increase proceeded at a continuously declining rate. The albuminoid likewise showed a slowly declining rate of increase throughout the life span of the animal.

Rupe, et al noted that before cataract formation, free electrophoresis of the soluble lens proteins revealed eight components, numbered 1 through 8 (38). Cataracts were induced by x-irradiation and the electrophoretic pattern of the soluble lens proteins was monitored with time. Components 1 - 3 were shown to disappear shortly after the formation of the cataract and components 6 - 8 likewise disappeared within 195 days of x-irradiation. It was further shown that with loss of the total soluble lens proteins, there was no concomitant increase in insoluble proteins during the process of cataract formation. From this, it was concluded that the actual amount of soluble protein present diminishes during x-irradiation-induced cataract formation.

Kinoshita and Merola showed that the protein sulfhydryl (PSH) concentration was higher in the nucleus than in the cortex (39). On the other hand, glutathione sulfhydryl (GSH) concentration in the cortex was much higher than in the nucleus, but the total SH content of cortex and nucleus (GSH+PSH) was found to be almost identical. It

appears that as the proteins of the cortex are converted into the proteins of the nucleus there occurs a drop in GSH concentration and an increase in PSH concentration; i.e., there appears to be a conversion of non-protein SH to protein SH with age.

In the normal lens of the adult newt the lens fibers have gamma crystallin but the epithelial cells do not (40). A protein fraction corresponding to gamma crystallin appears only when the epithelial cells differentiate into fiber cells. It was concluded that gamma crystallin synthesis characterizes epithelial cell differentiation. This agrees with the findings of Papaconstantinou who showed that the epithelial cells of adult bovine and calf lenses do not have any appreciable amount of gamma crystallin but that the lens fibers do (41). Gamma crystallin was run through a DEAE cellulose column and four fractions were detected in the effluent. This agrees with the results of Björk who used a SE Sephadex column to fractionate gamma crystallin.

A comparison of the elution pattern of gamma crystallin from different regions in the lens and from different age groups showed the following results. The elution pattern from embryonic lens is the same as that from calf and adult nucleus but different from the elution pattern from adult cortex. Calf cortical gamma crystallin has similarities with embryonic and adult nuclear gamma crystallin. An increasing complexity of the gamma crystallin elution pattern with advancing embryonic age was observed. All these results indicated that gamma crystallin synthesis is characteristic of epithelial cell

differentiation into lens fibers. Furthermore, similarity of the gamma crystallin from the adult and calf nucleus with that from the embryonic lens indicated that the differences in gamma crystallin elution patterns observed were due to a type of protein being synthesized at a certain age and not to a breakdown of the protein. This is probably similar to the case of adult and fetal hemoglobin.

Studies on cold induced cataracts by Lerman and his group showed that when whole lens extracts were brought to zero degrees centigrade, a precipitate formed (42). Subsequent analysis showed the precipitate to be composed mainly of gamma crystallin. Two general observations indicate involvement of gamma crystallin in the formation of cold-induced cataracts.

1. With respect to the concentration of alpha and beta crystallin, gamma crystallin concentration in the lens decreases with age.
2. Cold cataracts become increasingly difficult to induce as the animal gets older.

Amino acid analysis of gamma crystallin showed this fraction to be a rather basic protein with many hydrophobic side chains. Since very low concentrations of urea prevents formation of cold cataracts, it has been suggested that apolar interactions are of importance in keeping the gamma crystallin in solution. It is possible that the ability of alpha and beta crystallin to keep the gamma crystallin in solution is due to their formation of hydrophobic interactions with it.

INTRODUCTION II

From the foregoing section it is evident that the results of studies on the lens proteins do not all agree. However, there are several things that can be said about the lens proteins based on the material presented above.

There are two major types of proteins in the lens, namely, the insoluble protein termed albuminoid and the water soluble lens proteins which can be separated into three fractions designated as alpha, beta and gamma crystallin. Fractionation of the soluble lens proteins is based on differences in isoelectric points, solubilities in salt solutions, electrophoretic mobilities, retention by molecular sieves and rates of sedimentation in an ultracentrifugal field. The isoelectric points of alpha and beta crystallin were shown by several workers to be 5.0 - 5.1 and 6.0 - 6.1, respectively. In an electrical field, alpha, beta and gamma crystallin are arranged in an order of decreasing mobility towards the anode.

When percolated through a gel filtration column, alpha and beta crystallin, with alpha crystallin leading beta crystallin, are separated from gamma crystallin. In the ultracentrifuge, alpha crystallin is the fastest sedimenting component followed by beta crystallin and then gamma crystallin. There appear to be differences between the soluble proteins found in the nuclear lens fibers and those in the cortical lens fibers. Gamma crystallin is found only in the lens

fibers (nuclear and cortical) and not in the epithelial cells. Each of the three classical fractions, alpha, beta and gamma crystallin have been further separated into subfractions. Reports on the number of subunits per fraction, unique or otherwise, are not in agreement.

Almost all the techniques used so far for the detection of these subunits are based on physicochemical characteristics of proteins which are equivocal such as sedimentation rates, electrophoretic mobilities, viscosity changes, immunological specificities and ion exchange chromatography. These characteristics are equivocal in the sense that any of them may be sufficiently modified by a simple shift in the tertiary structure of the polypeptide chain or subunit. Thus, a technique was needed which would yield results which were not appreciably influenced by the tertiary structure of the polypeptide chain.

It was decided to approach the question of whether or not the classical lens protein fractions have more than one type of subunit by determining the C-terminal amino acid residues of the dissociated alpha, beta and gamma crystallins by the method of carboxypeptidase hydrolysis, assuming the following to be true.

1. Two or more polypeptide chains comprising a protein would not have the same C-terminal amino acid residue.
2. A denatured polypeptide chain having a C-terminal amino acid residue would have this C-terminal amino acid available to enzymic attack.

It is true that N-terminal studies on the lens proteins have not

yielded very good results due to the problem of blocked N-terminal amino acid residues. Also, the possibility of the C-terminal amino acid residues of the lens proteins being unavailable to enzymic attack is very real. However, the value of the experiments and results to be presented here is not diminished since there is, as yet, no proof or evidence available indicating that the C-terminal amino acid residues of the lens proteins are unavailable to carboxypeptidase hydrolysis. Moreover, there are numerous references to the successful use of the method on other proteins (43 - 45).

METHODS AND MATERIALS

Laboratory Apparatus

Spectrophotometric determinations were carried out with a Beckman Model DU spectrophotometer. All determinations were performed at room temperature.

The pH of solutions was determined electrometrically using either a Beckman Expanded Scale pH meter or a Beckman Zeromatic pH meter.

Water bath temperature control units used were capable of maintaining the selected temperature within 0.01°C. One unit was acquired from the Bronwill Scientific Division, Will Corp., Rochester, New York and the other was from Neslab Instruments Inc., Durham, New Hampshire.

Fractions were collected with a Gilson Medical Electronics Model V^2 fraction collector capable of handling a maximum of 225 tubes in a rectangular tray. Fractions were collected on a constant volume basis.

Preparative centrifugations were performed with a Lourdes Model A refrigerated centrifuge using either the 9RA rotor at 17000 rpm (36900 x g) or the VRA rotor at 13000 rpm (27500 x g).

Thin layer chromatography (TLC) was carried out on silica gel-G using apparatus acquired from Brinkmann Instrument Co. Samples were spotted in 10% isopropanol solution with air drying. The solvent system was 95% C_2H_5OH and 34% NH_4OH in a ratio of 70:30 (v/v) respectively, defining concentrated NH_4OH solution to be 100% NH_4OH. The

color reagent was a solution of 0.25% ninhydrin in acetone. The plates were placed in a developing tank with the solvent system level slightly below the level of the samples and allowed to develop for approximately 2.5 hours after which they were removed, air dried, oven dried for 5 min at 114° C and then sprayed with the color reagent.

Where accurate weight determinations were necessary, a Mettler automatic balance which could accurately measure 0.1 mg was used.

Amino acid analyses were performed on a modified Beckman Spinco Model 120 Amino Acid Analyzer. For the most part, peak areas were determined by the automatic integrator method. In certain cases, the peak areas were determined by the height times width method.

Sedimentation velocity experiments were carried out in a Spinco Model E Analytical Ultracentrifuge using the AN-D rotor at 59780 rpm (259610 x g) with the temperature automatically maintained at 20° C. Sedimentation patterns were observed using schlieren optics and recorded on Kodak metallographic plates.

Continuous flow paper electrophoresis was carried out in a Beckman Spinco Model CP Continuous Flow Electrophoresis Cell. Buffer used was 0.04 M Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.1 (Tris buffer).

Determination Of Protein Concentrations

Concentrations of the protein solutions were determined by measuring their absorbance at 280 millimicrons (mμ) against appropriate

solvent blanks. The extinction coefficient of a 1% solution of the protein at 280 mμ was used for obtaining the actual concentrations. The native lens protein fractions had the following extinction coefficient values; alpha crystallin = 8.3, beta crystallin = 21.5 and gamma crystallin = 17.6 (46). The succinylated lens protein fractions had the following values; alpha crystallin = 6.9, beta crystallin = 19.9 and gamma crystallin = 18.5. The dry weight method was used in calculating the amount of protein for the extinction coefficient determinations.

Enzymes

Carboxypeptidase-A (CPA) was obtained from Sigma Chemical Company and Worthington Biochemical Corporation. Specific activity was assayed at appropriate intervals using N-Carbobenzoxy-Glycyl-L-Phenylalanine (N-CBZGP) from Sigma Chemical Company as the substrate. A solution of the substrate was prepared which was 0.01 M with respect to N-CBZGP, 0.025 M with respect to Tris-HCl and 0.1 M with respect to NaCl. The pH was adjusted to 7.65. Two and nine-tenths ml of this solution was placed in a cuvette and the reaction was started by the addition of 0.1 ml of a 10% LiCl solution, pH 7.4 containing 25 micrograms (μg) of the enzyme suspension per ml. Optical density (OD) was determined at 233 mμ every 5 min for 20 min against a blank made up of 1.5 ml of the substrate solution and 1.5 ml of distilled water. Specific activity was defined as $\frac{\text{ave change in OD/min}}{\text{mg enzyme/ml assay solution}}$.

Preparation Of Soluble Lens Proteins

Rabbit eyes were obtained from a local slaughterhouse. 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 then placed in distilled water. After a few minutes in the distilled water the lens was decapsulated and placed in Tris buffer. The mixture was mechanically stirred until the cortical portion of the lens had dissolved away leaving the nucleus which was then manually removed. The volume was finally adjusted with Tris buffer to have approximately 1 ml of extract per lens.

After the nuclei had been removed the extract was homogenized for about 1 min in a Potter-Elvejhem homogenizer and centrifuged to remove the remaining particulate material. The supernatant was dialyzed overnight against a large volume of Tris buffer to remove low molecular weight contaminants. A small amount of precipitate formed during the dialysis procedure and this was removed by centrifugation. The protein solution was then applied to a 7.8 x 100 cm column of Sephadex G-75 and eluted with Tris buffer at the rate of about 3.3 ml per min. The protein was applied to the column in volumes of 50 - 70 ml at a concentration of 30 - 50 mg per ml. Twenty-five ml fractions were collected.

The protein peaks were located by measuring the OD at 280 mμ and

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

and transferred to a tube containing 2.25 ml of 0.2 M sodium citrate buffer, pH 2.2. This latter procedure stopped the reaction and also precipitated some protein if succinylated protein was used. Similar aliquots were withdrawn and treated in the same way at specified time intervals. The precipitates, if any, were removed by centrifugation and 1.0 ml aliquots of the supernatants were analyzed in the amino acid analyzer.

Sedimentation Velocity Experiments

Sedimentation velocity experiments were performed on native and succinylated alpha and beta crystallin. The protein was dissolved in 0.1 M NaCl at concentrations ranging from 5.0-18.0 mg per ml. The protein solution was placed in a standard sector cell and centrifuged at 59780 rpm the temperature being maintained at 20°C. Movement of the concentration boundary was observed using schlieren optics and recorded on Kodak metallographic plates. Measurements of the distance travelled by the component peak were made using the Gaertner microcomparator and recorded on printed calculation sheets, an example of which is given in figure 2.

From the printed calculation sheets, H, X_1', X_2', X_1'', X_2'', X and log X were calculated. The factors F, f_1 and f_2 were defined as follows.

1. F = the magnification factor of the analytical ultracentrifuge optical system = 2.106.
2. f_1 and f_2 = the factors used to compensate for the stretching

Figure 2. Calculation sheet for ultracentrifugal analysis data.

						TIME OF DAY	1
						TIME INTERVAL IN SECONDS	2
						TIME ELAPSED IN SECONDS	3
						MICROCOMPARATOR RDG. IN CM. — OUTSIDE OF INNER HOLE A	4
						MICROCOMPARATOR RDG. IN CM. — OUTSIDE OF MINISCUS B	5
						MICROCOMPARATOR RDG. IN CM. — MAXIMUM OF COMPONENT C	6
						MICROCOMPARATOR RDG. IN CM. — INSIDE OF OUTER HOLE D	7
						REFERENCE DISTANCE H = D-A cm.	8
						MINISCUS DISTANCE FROM — INNER HOLE E_1 = B-A cm.	9
						MINISCUS DISTANCE FROM — OUTER HOLE E_0 = D-B cm.	10
						COMPONENT DISTANCE FROM — INNER HOLE X_1' = C-A cm.	11
						COMPONENT DISTANCE FROM — OUTER HOLE X_2' = D-C cm.	12
						$X_1'' = \frac{X_1'}{F}$ cm.	13
						$X_2'' = \frac{X_2'}{F}$ cm.	14
						$X_1 = 5.7 + f_1 + X_1''$ cm.	15
						$X_2 = 7.3 + f_2 - X_2''$ cm.	16
						$X = \frac{X_1 + X_2}{2}$ cm.	17
						Log X	18

of the sample cell= 0.02 cm and 0.025614 cm, respectively. Finally, log X was plotted versus time in min. The observed sedimentation velocity (s_{obs}) was calculated using the equation

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

Since in the above equation the only variable between experiments is the slope, a factor (y) was computed and multiplied by the slope to obtain the s_{obs} where

$$y = \frac{(2.303/60)}{[(2\pi)(59780/60)]^2} = 9.799 \times 10^{-10}.$$

From the s_{obs}, the rate of sedimentation of the protein in water at 20°C ($s_{20,w}$) was calculated using the following equation (47).

$$s_{20,w} = (s_{obs})(n_t/n_{20})(n/n_o)(1-\bar{V}_{p20,w}/1-\bar{V}_{pt})$$

where n_t = viscosity of water at the temperature of the experiment

n_{20} = viscosity of water at 20°C

n/n_o = viscosity of the solvent relative to the viscosity of water = 1.009

$\bar{V}$ = partial specific volume of the protein, in this case assumed to be 0.7

$P_{20,w}$ = density of water at 20°C = 0.998203

P_t = density of solvent at the temperature of the experiment = 1.002303

The $s_{20,w}$ values were then plotted versus the percent protein concentration and extrapolated to zero concentration.

Phenylmethyl Sulfonylfluoride (PMSF) Treatment Of Enzymes

Phenylmethyl Sulfonylfluoride obtained from Calbiochem was used to treat both CPA and alpha chymotrypsin. Fahrney and Gold report that PMSF is as effective as diisopropylfluorophosphate (DFP) in the inhibition of chymotryptic activity but unlike DFP, does not inhibit acetylcholinesterase activity (48). At the concentration of 0.001 M PMSF chymotryptic activity was completely inhibited.

Sixty-five mg of PMSF (molecular weight = 174.2) was dissolved in 5 ml of absolute isopropanol yielding a solution of 13 mg per ml PMSF. The substrate solution used to assay for chymotryptic activity was 0.00107 M Benzoyl-L-Tyrosine Ethyl Ester in 50% methanol (w/w) obtained in powder form from Sigma Chemical Co. The buffer was ammounium acetate buffer. "Test" and "blank" reaction mixtures were made up in cuvettes as follows.

	test	blank
Buffer	1.5 ml	1.5 ml
Substrate	1.5 ml	1.5 ml
Inhibitor	40 µl	40 µl
Enzyme	5 µl	-----

Forty µl of the inhibitor solution per 3.0 ml of reaction mixture gives an inhibitor concentration of (0.040)(13)/3 - 0.1733 mg per ml or 0.1733/174.2 = 0.000995 or 0.001 M. In the assay procedure, the

"blank" and "test" cuvettes were filled as indicated on the previous page with the omission of the substrate. The mixture was stirred and allowed to stand for 1 hour. The substrate was then added to the "blank" cuvette with mixing and the spectrophotometer was zeroed at 256 mμ. Immediately, substrate was added to the "test" cuvette with mixing and the OD at 256 mμ was read at appropriate time intervals. The spectrophotometer was re-zeroed against the blank before any subsequent readings were made. A control experiment was performed in which all conditions and manipulations were exactly as described above except for the omission of the inhibitor. The "test" and "blank" cuvettes for the control experiment were made up as shown below.

	test	blank
Buffer	1.5 ml	1.5 ml
Substrate	1.5 ml	1.5 ml
Enzyme	5 μl	-----

Succinylation Of Proteins

The experiments of Mr. L. Jackson of our laboratory indicated that for complete succinylation of the lens proteins, at least a 40-fold molar excess of succinic anhydride over the lysine content of the protein is needed. Completeness of succinylation was indicated by the observation that addition of succinic anhydride beyond a 40-fold molar excess did not increase the number of bands seen when the succinylated

protein was subjected to polyacrylamide gel disc electrophoresis. It was decided to use at least a 50-fold molar excess in the experiments presented here. In each case the lysine content of the protein was calculated from available information in the literature and the corresponding amount of succinic anhydride was used.

The protein was dissolved in distilled water, the range of concentrations used being 10-30 mg per ml. The pH of the solution was adjusted to 8.0 with 1.0 N NaOH and the succinic anhydride was added in small portions with continuous magnetic stirring. The pH of the reaction mixture was maintained at 8.0 ± 0.3 by the addition of 1.0 N NaOH. Habeeb et al (49) report that at pH values of 7.0 and above there is hardly any difference in the extent of succinylation; however, the efficiency of the reaction decreases markedly below pH 7.0. After completion of the reaction the solution was dialyzed against the appropriate buffer.

Reagents

All other reagents employed were reagent grade and were used without further treatment.

EXPERIMENTAL AND RESULTS

Phenylmethyl Sulfonylfluoride Treatment Of Carboxypeptidase-A

Carboxypeptidase-A was treated with PMSF and then assayed for chymotryptic activity. Figure 3 is a plot of the OD at 256 mμ versus time for the system with PMSF and figure 4 is a similar plot for the system without PMSF.

Phenylmethyl Sulfonylfluoride Treatment Of Alpha Chymotrypsin

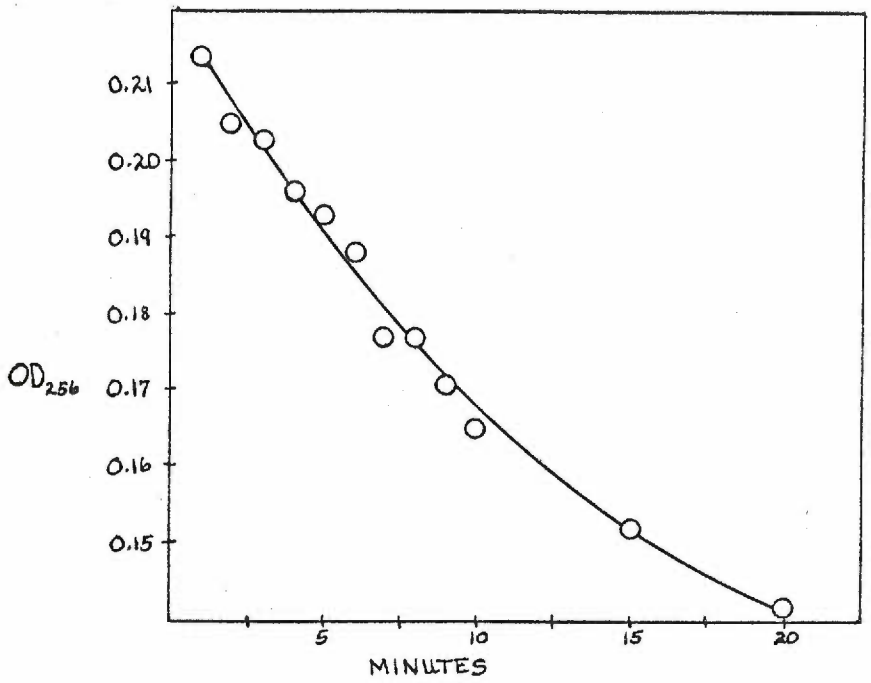
A solution of alpha chymotrypsin (1 mg per ml) in ammonium acetate buffer was prepared, treated with PMSF and then assayed for chymotryptic activity. Figure 5 is a plot of the OD at 256 mμ versus time for the system with PMSF and figure 6 is a similar plot for the system without PMSF.

Carboxypeptidase-A Hydrolysis Of Bovine Serum Albumin (BSA)

A solution of 620 mg BSA in 31.0 ml ammonium acetate buffer was hydrolyzed with 720 units of CPA. A unit of enzyme as used here refers to the amount of enzyme which causes a change in OD at 256 mμ of 0.01 per min under standard conditions. Three ml aliquots were taken at 0, 0.5, 1, 3, 6, 15 and 24 hours and deproteinized by addition of 9.0 ml of absolute ethanol. The precipitates were removed by centrifugation and the supernatants taken to dryness in an oven at 114°C. The residues were taken up in 25 μl of 10% isopropanol and spotted on a TLC plate.

Figure 3. Assay for chymotryptic activity in PMSF treated CPA

Figure 4. Assay for chymotryptic activity in untreated CPA


0.21
0.20
0.19
0.18
OD256
0.17
0.16
0.15
5
10
15
20
MINUTES

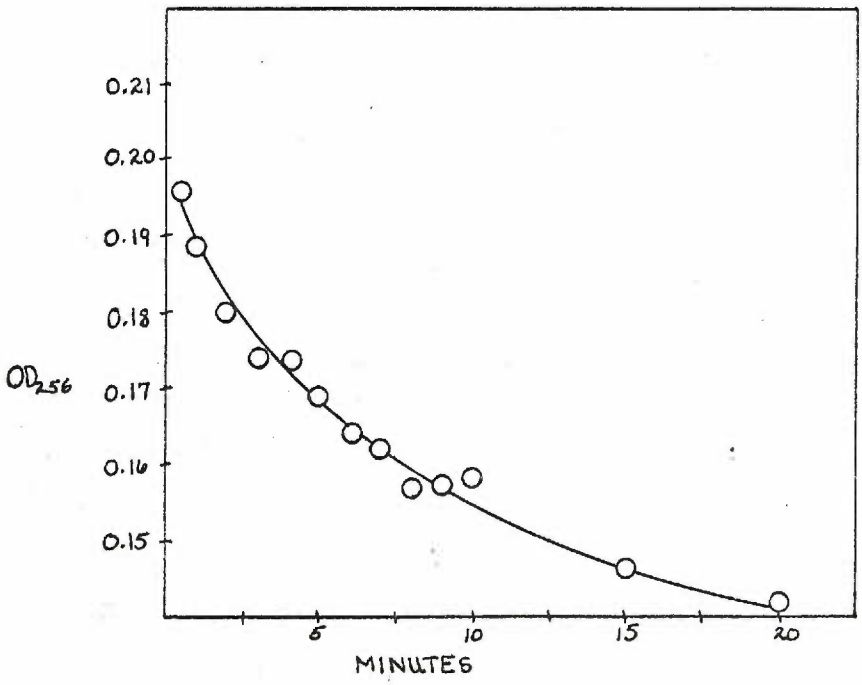
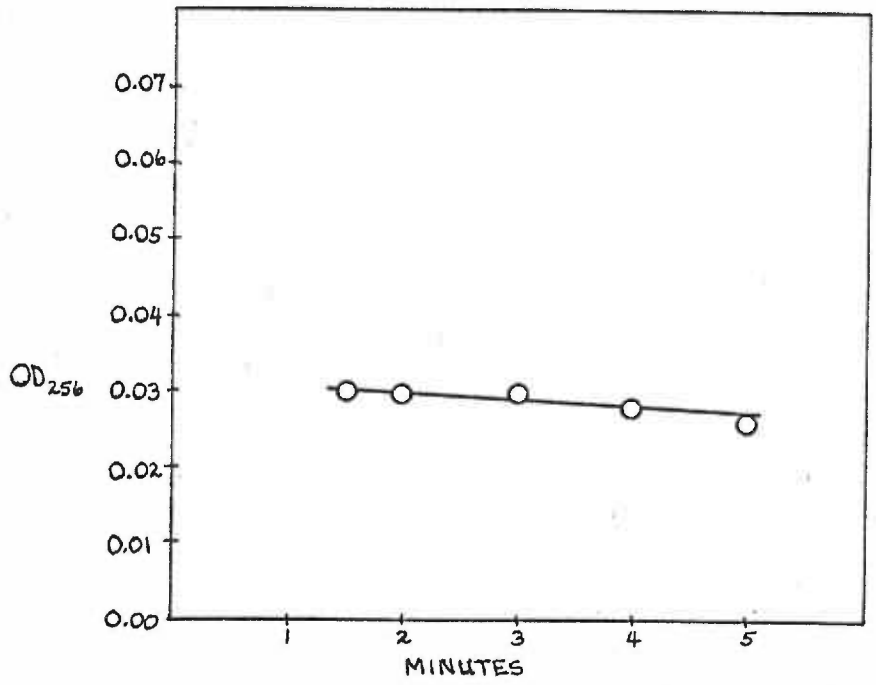
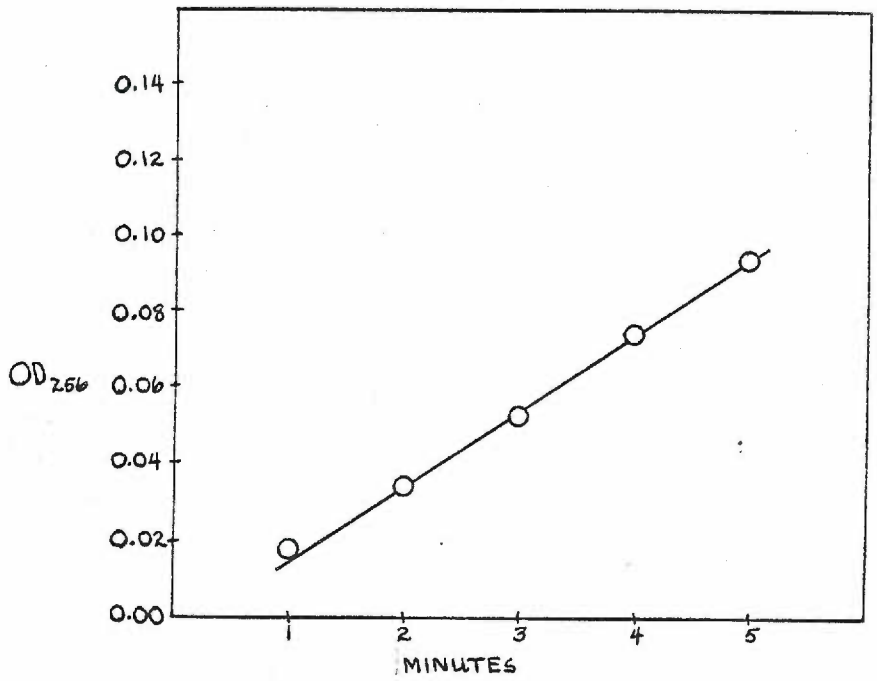

0.21
0.20
0.19
0.18
OD256
0.17
0.16
0.15
5
10
15
20
MINUTES

Figure 5. Assay for chymotryptic activity in PMSF treated alpha chymotrypsin

Figure 6. Assay for chymotryptic activity in untreated alpha chymotrypsin


0.07
0.06
0.05
0.04
OD_{256} 0.03
0.02
0.01
0.00
1
2
3
4
5
MINUTES


0.14
0.12
0.10
0.08
OD_{256} 0.06
0.04
0.02
0.00
1
2
3
4
5
MINUTES

The plate was developed for 2.25 hours, dried and sprayed with the color reagent. Figure 7 shows that there is an increase in amino acid release with respect to time.

Essentially the same experiment was performed again, this time the rate and specific amino acid released were observed by use of the amino acid analyzer. Due to the smaller sample size required for analysis by this method, slight modifications were made in the procedure. Sixty mg of BSA was dissolved in 3.0 ml of ammonium acetate buffer and the pH adjusted to 7.5. This brought the volume to 3.5 ml resulting in a protein concentration of 17.05 mg per ml. Sixty units of the enzyme was added and aliquots were taken at 0.5, 1, 2, 4, 11, 16 and 24 hours. Figure 8 shows the amino acid release versus time for this experiment and table 1 gives the tabulated data.

Succinylation Of Bovine Serum Albumin

Bovine serum albumin has a molecular weight of 70000 and a lysine content of 60 micromoles (μmole) per umole of BSA. Using a 50-fold molar excess of succinic anhydride over lysine, 70 mg of BSA was succinylated with (50)(60) = 3000 μmole of succinic anhydride (300.2 mg).

Carboxypeptidase-A Hydrolysis Of Native Alpha Crystallin

Hydrolysis of native alpha crystallin by CPA was carried out using a protein concentration of 10 mg per ml and 16.8 units of enzyme. Aliquots were taken at 9, 8, 16 and 24 hours. Figure 9 and table 2

Figure 7. Thin layer chromatogram of hydrolysates from the CPA hydrolysis of native BSA

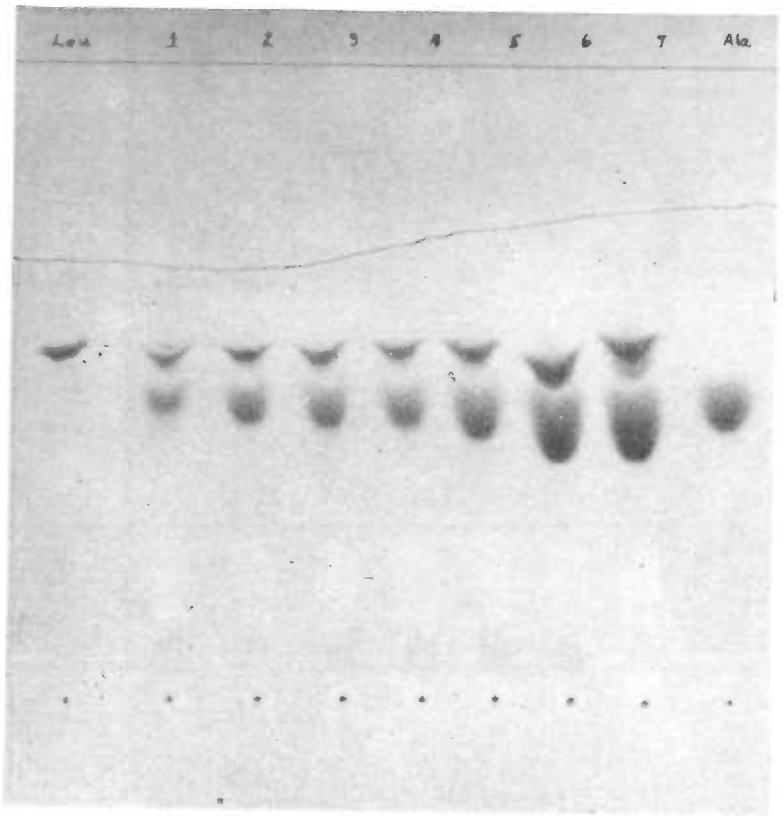

Leu
1
2
3
4
5
6
7
Ala

Figure 8. Plot of μmole of amino acid released versus time for the CPA hydrolysis of native BSA.

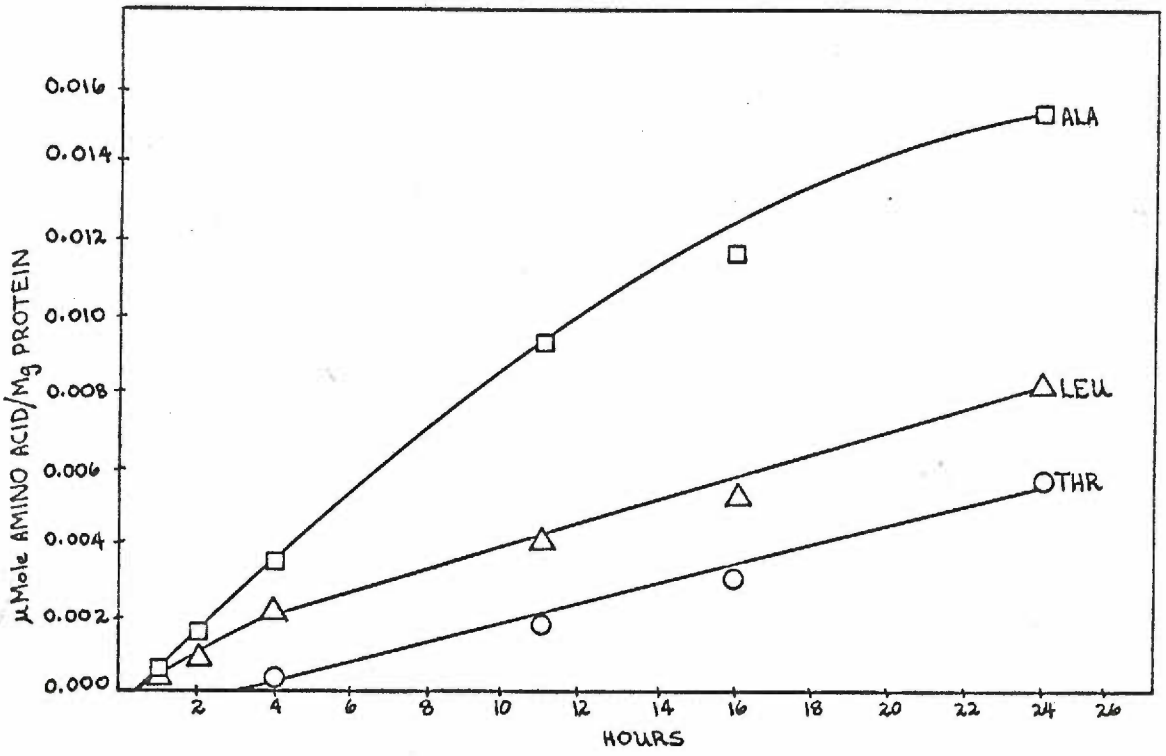


Table 1

Amino acids released by the CPA hydrolysis of native BSA

µmole Amino Acid/mg Protein

Hours	Thr	Ala	Val	Leu
1	---	.0005	---	.0004
2	---	.0015	---	.0009
4	.0002	.0035	---	.0021
11	.0018	.0092	---	.0040
16	.0030	.0116	---	.0051
24	.0056	.0153	.0003	.0081

Figure 9. Plot of μmole of amino acid released versus time for the CPA hydrolysis of native alpha crystallin.

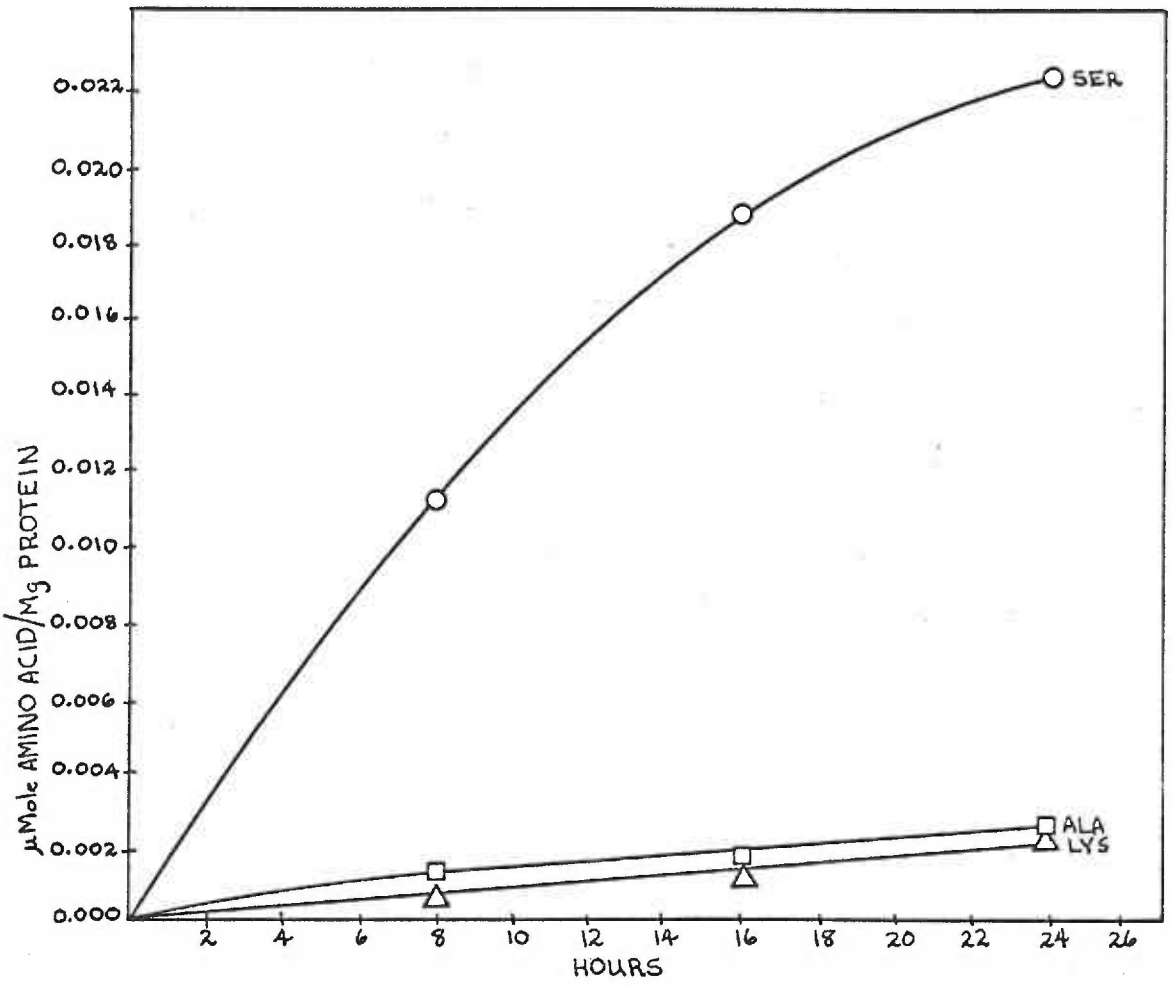


Table 2

Amino acids released by the CPA hydrolysis of native alpha crystallin

µmole Amino Acid/mg Protein

Hours	Lys	His	Asp	Ser	Glu	Gly	Ala	Val	Ileu	Leu	Tyr
8	.0008	---	.0007	.0111	---	---	.0014	---	---	.0013	.0012
16	.0015	---	.0006	.0186	---	.0006	.0018	.0004	.0007	.0015	.0013
24	.0023	.0009	.0008	.0222	.0007	.0007	.0026	.0010	.0011	.0016	.0012

present the results obtained from this experiment. A repeat of this experiment yielded comparable results.

Succinylation Of Alpha Crystallin

Alpha crystallin has a lysine content of approximately 5 mg% (50). Since the molecular weight of lysine is 146.19 this means that there is 5/146.19 = 0.034 millimole (mmole) of lysine per 100 mg of alpha crystallin or 0.00034 mmole of lysine per mg of alpha crystallin. A 50-fold molar excess of succinic anhydride over lysine = (50)(0.00034) = 0.017 mmole of succinic anhydride (1.7 mg) per mg of alpha crystallin. In the experiment, 142.8 mg of alpha crystallin was succinylated with (1.7)(142.8) = 242.8 mg of succinic anhydride.

Carboxypeptidase-A Hydrolysis Of Succinylated Alpha Crystallin

The succinylated protein was dialyzed against 3 changes of ammonium acetate buffer and the volume was reduced by ultrafiltration resulting in a concentration of 11.9 mg per ml. Two and one-half ml of this solution was hydrolyzed with 39 units of CPA and aliquots were taken at 0,0.5, 1, 2, 4.6, 8, 17 and 24 hours. The results are given in table 3. In another experiment, a different preparation of alpha crystallin was succinylated and similarly treated. The protein concentration was 10 mg per ml and aliquots were taken at 0.25, 0.5, 1, 2, 4.5, 8, 16 and 24 hours. Table 4 presents the data obtained from this experiment.

Table 3

Amino acids released by CPA hydrolysis of succinylated alpha crystallin

μmole Amino Acid/mg Protein

Hours	His	Asp	Thr	Ser	Glu	Gly	Ala	Val	Ileu	Leu	Tyr	Phe
0	---	---	---	---	---	---	---	---	---	---	---	---
0.5	---	---	.0015	---	---	---	.0032	.0006	---	.0019	.0008	.0018
1	---	---	.0026	.0005	---	---	.0045	.0011	.0004	.0027	.0008	.0021
2	---	---	.0044	.0008	---	.0003	.0057	.0026	.0007	.0035	.0013	.0027
4.6	---	---	.0062	.0019	.0006	.0006	.0066	.0040	.0008	.0038	.0014	.0039
8	---	---	.0066	.0031	.0015	.0008	.0072	.0050	.0015	.0050	.0018	.0058
17	---	---	.0084	.0058	.0020	.0014	.0087	.0064	.0028	.0062	.0030	.0088
24	.0025	.0013	.0088	.0060	.0022	.0017	.0095	.0067	.0035	.0076	.0029	.0107

Table 4

Amino acids released by CPA hydrolysis of succinylated alpha crystallin

µmole Amino Acid/mg Protein

Hours	Asp	Thr	Ser	Glu	Gly	Ala	Val	Ileu	Leu	Tyr	Phe
1	---	.0021	.0022	---	.0012	.0023	.0015	.0008	.0024	.0014	.0030
2	---	.0028	.0034	---	.0015	.0033	.0024	.0012	.0030	.0017	.0039
4.5	.0010	.0044	.0050	.0008	.0015	.0038	.0036	.0022	.0041	.0020	.0059
8	.0012	.0061	.0071	.0012	.0014	.0041	.0053	.0033	.0060	.0048	.0097
16	.0021	.0083	.0094	.0023	.0015	.0057	.0082	.0057	.0092	.0045	.0157
24	---	.0064	.0096	---	.0019	.0059	.01000	---	---	---	---

Carboxypeptidase-A Hydrolysis Of Native Beta Crystallin

A solution of 25 mg per ml beta crystallin in ammonium acetate buffer was prepared and hydrolyzed using 42 units of CPA. Aliquots were taken at 0, 3, 8, 12 and 24 hours. The results of this experiment are shown in figure 10 and table 5.

Succinylation Of Beta Crystallin

The assumed lysine content of beta crystallin was 5 mg% (50). Since this is similar to that for alpha crystallin, the same ratio of succinic anhydride to protein was used. In this experiment, 282.2 mg of succinic anhydride was used to succinylate 166 mg of beta crystallin.

Carboxypeptidase-A Hydrolysis Of Succinylated Beta Crystallin

The succinylated beta crystallin was extensively dialyzed against ammonium acetate buffer and the volume was reduced by ultrafiltration. The resulting solution had a protein concentration of 19.3 mg per ml. Two and one-half ml of this solution was hydrolyzed with 40 units of CPA and aliquots were taken at 0, 0.25, 0.5, 1, 2, 4, 8, 18 and 25 hours. Table 6 shows the results obtained from this experiment. A. repeat of this experiment was performed using a different preparation of beta crystallin. The beta crystallin was succinylated under conditions similar to those described above. The protein concentration of the solution was 19.8 mg per ml and 2.5 ml of this was hydrolyzed with 39 units of CPA. The results are seen in table 7.

Figure 10. Plot of μmole of amino acid released versus time for the CPA hydrolysis of native beta crystallin.

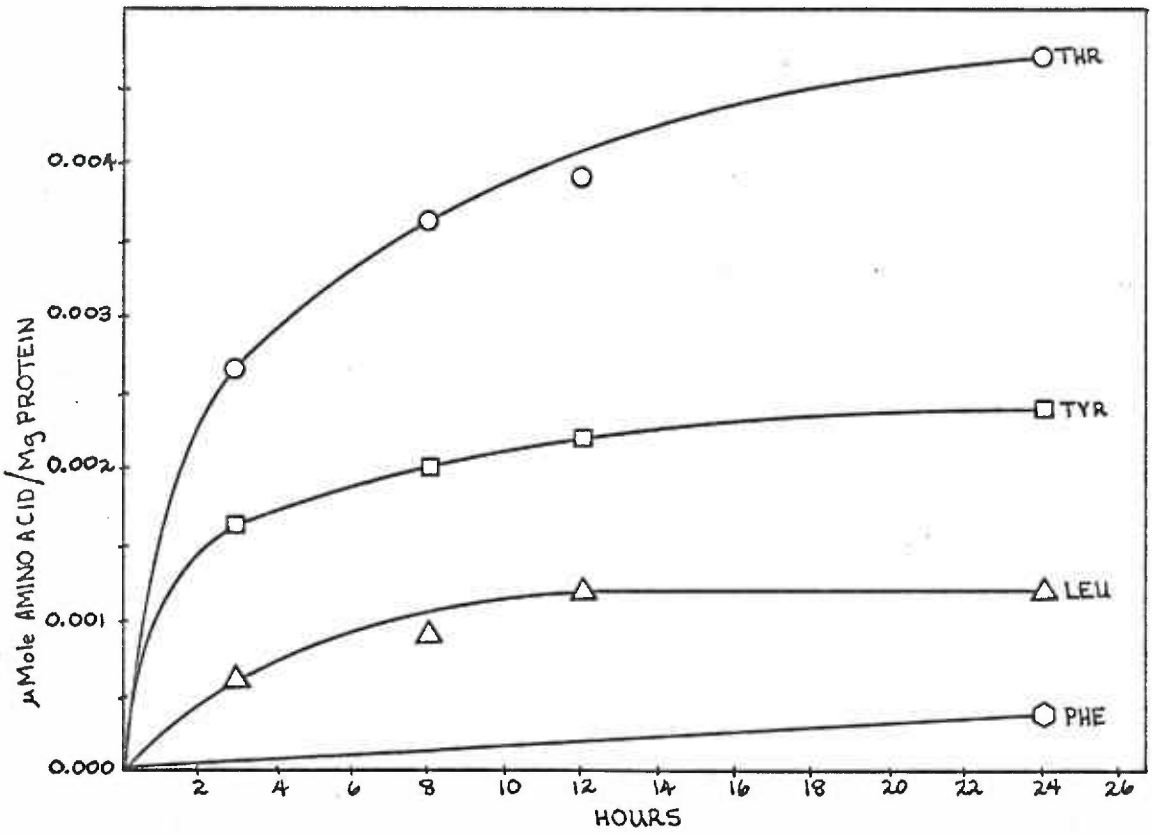


Table 5

Amino acids released by the CPA hydrolysis of native beta crystallin

µmole Amino Acid/mg Protein

Hours	Thr	Leu	Tyr	Phe
3	.0026	.0006	.0016	---
8	.0036	.0009	.0020	---
12	.0039	.0012	.0022	---
24	.0047	.0012	.0024	.0004

Table 6

Amino acids released by CPA hydrolysis of succinylated beta crystallin

μmole Amino Acid/mg Protein

Hours	Lys	His	Asp	Thr	Ser	Glu	Gly	Ala	Val	Met	Ileu	Leu	Tyr	Phe
0	---	---	---	---	---	---	---	---	---	---	---	---	---	---
0.25	---	---	---	---	.0002	---	---	---	---	---	---	.0006	.0031	.0011
0.5	---	---	---	.0003	.0006	---	---	.0002	---	---	---	.0012	.0032	.0012
1	---	---	.0002	.0007	.0009	---	.0002	.0004	.0004	---	.0002	.0021	.0032	.0014
2	---	.0004	.0008	.0020	.0011	---	.0006	.0006	.0011	.0003	.0005	.0026	.0032	.0012
4	---	.0008	.0015	.0045	.0012	---	.0011	.0008	.0023	.0007	.0011	.0031	.0032	.0016
8	---	.0014	.0018	.0088	.0012	---	.0016	.0012	.0038	.0007	.0025	.0037	.0035	.0020
18	.0002	.0018	.0021	.0130	.0018	.0003	.0020	.0012	.0055	.0008	.0052	.0046	.0036	.0026
25	.0002	.0021	.0025	.0149	.0018	.0004	.0022	.0012	.0067	.0008	.0059	.0047	.0040	.0032

Table 7

Amino acids released by CPA hydrolysis of succinylated beta crystallin

µmole Amino Acid/mg Protein

Hours	His	Thr	Ser	Glu	Gly	Ala	Val	Met	Ileu	Leu	Tyr	Phe
0	---	---	---	---	---	---	---	---	---	---	---	---
0.25	---	.0003	.0010	---	.0005	.0007	---	---	---	.0014	.0005	.0015
0.5	---	.0008	.0017	---	.0007	.0016	.0008	---	---	.0024	.0008	.0023
1	---	.0017	.0028	---	.0007	.0020	.0013	---	.0005	.0031	.0009	.0025
2	.0008	.0034	.0038	---	.0008	.0026	.0017	---	.0009	.0039	.0011	.0028
4	.0015	.0066	.0057	---	.0012	.0033	.0027	---	.0015	.0046	.0012	.0035
10	.0030	.0149	.0041	.0002	.0015	.0042	.0042	.0005	.0038	.0064	.0011	.0045
16	.0037	.0199	.0045	.0004	.0017	.0050	.0053	.0008	.0060	.0079	.0013	.0054
25	.0034	.0240	.0040	.0017	.0022	.0057	.0062	.0009	.0079	.0092	.0016	.0060

Carboxypeptidase-A Hydrolysis Of Native Gamma Crystallin

A 10 mg per ml solution of gamma crystallin in ammonium acetate buffer was prepared and 2.3 ml of this solution was hydrolyzed with 42 units of CPA. Aliquots were taken at 4, 8 and 16 hours. Figure 11 and table 8 show the results obtained from this experiment.

Succinylation Of Gamma Crystallin

Gamma crystallin was assumed to have a lysine content of 2.4 mg% (50). Taking the molecular weight of lysine as 146.19, this yields a value of 2.4/146.19 = 0.016 mmole of lysine per 100 mg of gamma crystallin. A 50-fold molar excess of succinic anhydride over lysine is equal to (50)(0.016) = 0.8 mmole of succinic anhydride (80 mg) per 100 mg of gamma crystallin. In the experiment, 166.5 mg of gamma crystallin was succinylated with 133.2 mg of succinic anhydride.

Carboxypeptidase-A Hydrolysis Of Succinylated Gamma Crystallin

A 10.4 mg per ml solution of the succinylated gamma crystallin in ammonium acetate buffer was prepared and 2.5 ml of this solution was hydrolyzed with 40 units of CPA. Aliquots were taken at 0, 0.5, 1, 2, 4, 8, 16 and 24 hours. Table 9 shows the results obtained. A repeat of this experiment was performed on a different preparation of gamma crystallin which was succinylated in the same way as described above. The protein concentration was 12.36 mg per ml and 2.2 ml of the solution was hydrolyzed with 39 units of CPA. The results are shown in table 10.

Figure 11. Plot of μmole of amino acid released versus time for the CPA hydrolysis of native gamma crystallin.

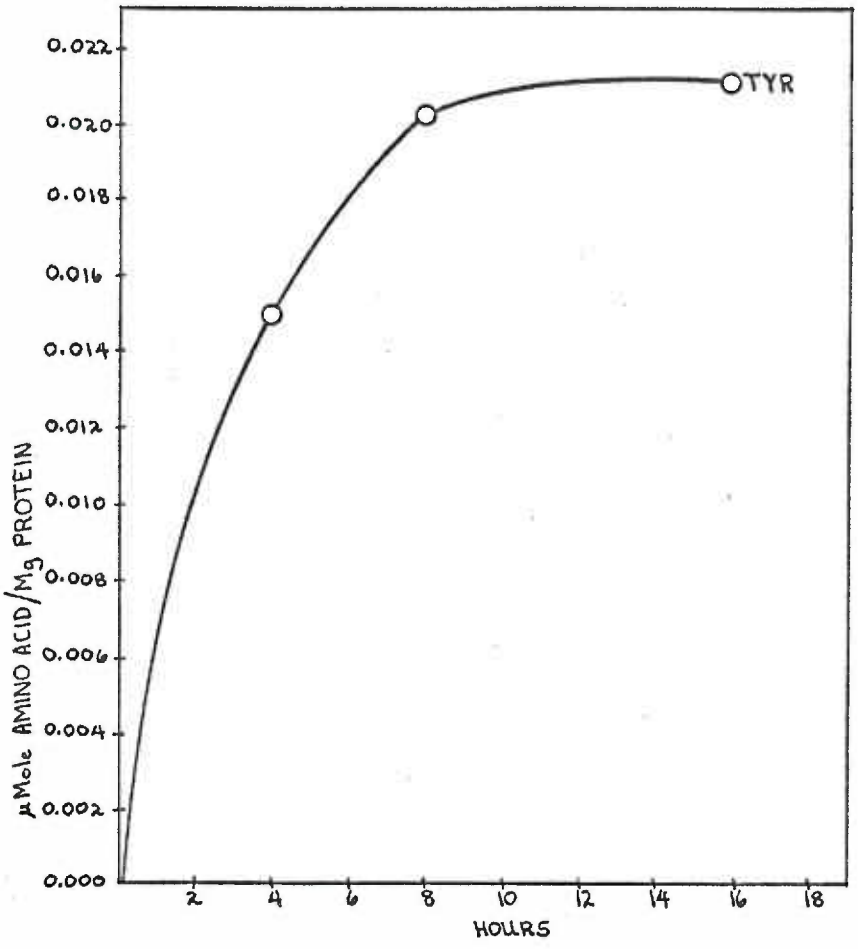


Table 8

µmole tyrosine/mg Protein released by the CPA hydrolysis of native gamma crystallin.

Hours	µmole
4	.0149
8	.0202
16	.0210

Table 9

Amino acids released by CPA hydrolysis of succinylated gamma crystallin

µmole Amino Acid/mg Protein

Hours	Asp	Thr	Ser	Gly	Ala	Val	Met	Ileu	Leu	Tyr	Phe
0	---	---	---	---	---	---	---	---	---	---	---
0.5	---	---	---	---	---	---	---	---	---	.0005	---
1	.0123	---	.0004	.0009	.0016	.0130	.0081	---	.0065	.0348	.0189
2	.0169	---	---	.0008	.0022	.0198	.0117	.0005	.0079	.0343	.0211
4	.0207	---	.0002	.0014	.0024	.0252	.0118	.0005	.0078	.0353	.0224
8	.0231	.0005	.0015	.0025	.0032	.0282	.0121	.0011	.0083	.0352	.0231
16	.0236	---	.0010	.0024	.0029	.0298	.0122	.0012	.0086	.0361	.0238
24	.0244	.0005	.0007	.0025	.0032	.0308	.0127	.0014	.0090	.0364	.0246

Table 10

Amino acids released by CPA hydrolysis of succinylated gamma crystallin

µmole Amino Acid/mg Protein

Hours	Asp	Gly	Ala	Val	Met	Ileu	Leu	Tyr	Phe
0	---	---	---	---	---	---	---	---	---
0.5	.0019	---	---	.0019	.0017	---	.0068	.0323	.0097
1	.0054	---	---	.0061	.0041	---	.0093	.0325	.0112
2	.0100	---	---	.0133	.0064	---	.0103	.0334	.0158
4	.0141	.0004	---	.0192	.0070	---	.0098	.0336	.0180
8	.0163	.0008	.0004	.0232	.0072	.0003	.0107	.0337	.0194
16	.0178	.0011	.0006	.0252	.0073	.0006	.0111	.0346	.0211
24	.0186	.0013	.0007	.0262	.0077	.0007	.0114	.0353	.0217

Ultracentrifugal Analysis Of Native Alpha Crystallin

Twenty mg of native alpha crystallin was dissolved in 2.0 ml of 0.1 M NaCl and analyzed in the ultracentrifuge. Figure 12 shows the results from this experiment.

Ultracentrifugal Analysis Of Native Beta Crystallin

A 10 mg per ml solution of native beta crystallin in 0.1 M NaCl was prepared and analyzed in the ultracentrifuge. Figure 13 shows the sedimentation pattern obtained.

Ultracentrifugal Analysis Of Succinylated Alpha Crystallin

Four different concentrations of succinylated alpha crystallin in 0.1 M NaCl were prepared; namely, 5.91, 10.1, 11.7 and 18.1 mg per ml. These solutions were analyzed in the ultracentrifuge to determine if the sedimentation velocity is concentration dependent. Figure 14 shows the sedimentation pattern for the 11.7 mg per ml concentration. The nature of the component was the same for the other concentrations although the sedimentation velocity values were different, indicating a negative concentration dependence.

Ultracentrifugal Analysis Of Succinylated Beta Crystallin

As with succinylated alpha crystallin, four different concentrations of succinylated beta crystallin in 0.1 M NaCl were prepared (5.4, 8.3, 10.6 and 16.4 mg per ml). The sedimentation velocity was

Figure 12. Sedimentation pattern of 1.0% native alpha crystallin in 0.1 M NaCl, at 59780 rpm.

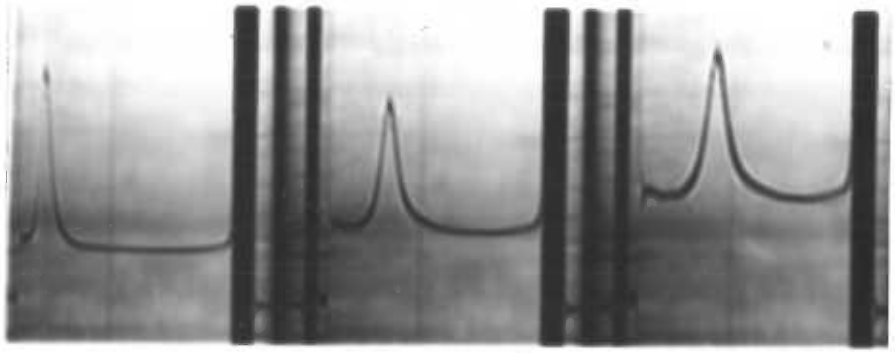
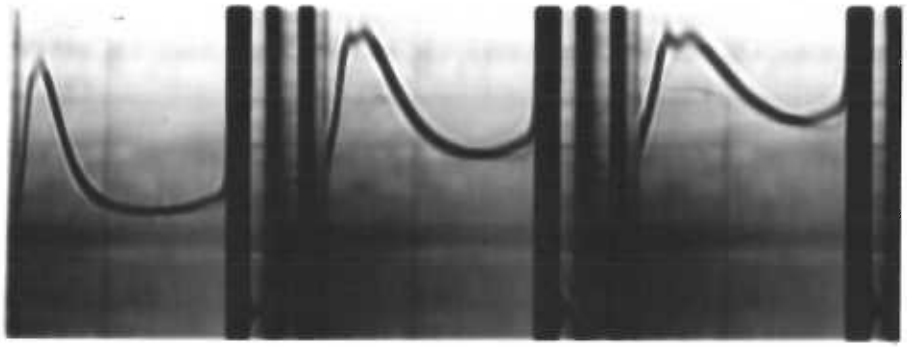


Figure 13. Sedimentation pattern of 1.0% native beta crystallin in 0.1 M NaCl, at 59780 rpm.



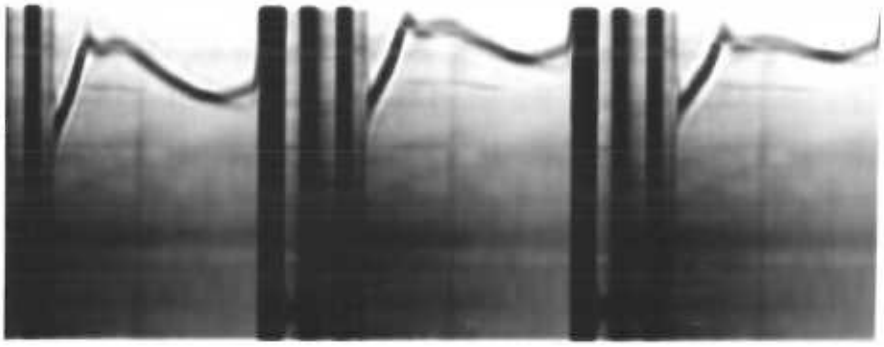
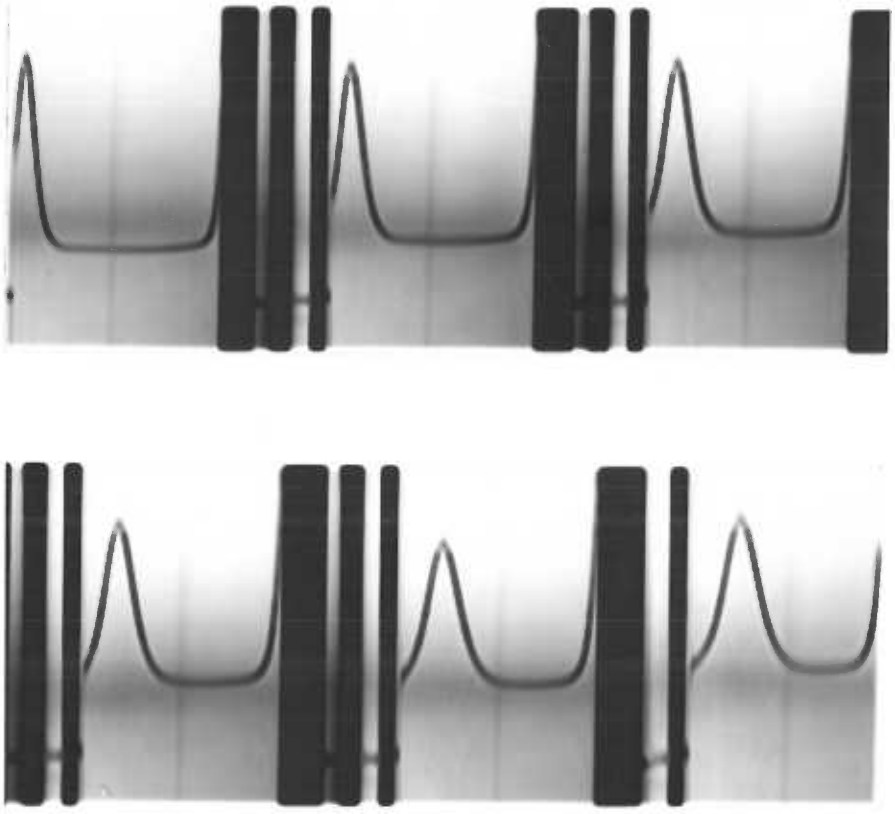


Figure 14. Sedimentation pattern of 1.17% succinylated alpha crystallin in 0.1 M NaCl, at 59780 rpm.



determined for each concentration. Again, a negative concentration dependence was observed. Figure 15 shows the sedimentation pattern for the 16.4 mg per ml concentration.

The $s_{20,w}$ values were calculated for each of the different concentrations of succinylated alpha and beta crystallin. Figure 16 is a plot of $s_{20,w}$ versus percent protein concentration for both succinylated alpha and beta crystallin. Table 11 presents the data used to obtain the plots in figure 16.

Figure 15. Sedimentation pattern of 1.64% succinylated beta crystallin in 0.1 M NaCl, at 59780 rpm.

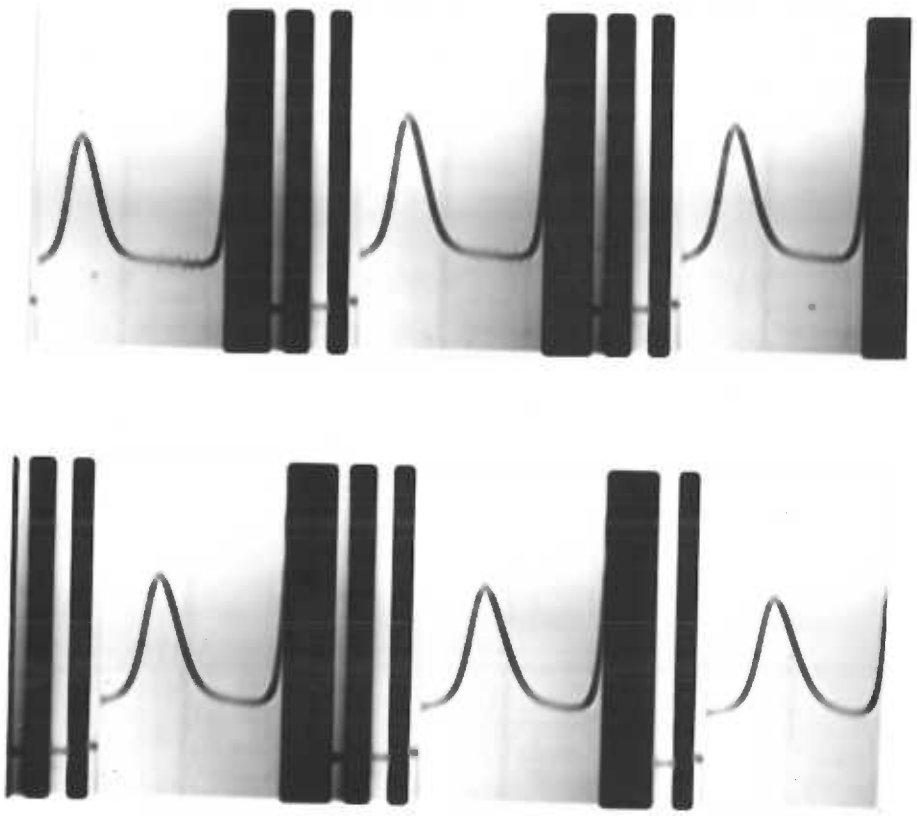


Figure 16. Plot of s_{20w} values versus protein concentration (%) for succinylated alpha and beta crystallin. Solvent was 0.1 M NaCl. The protein was centrifuged at 59780 rpm, temperature was maintained at 20°C.

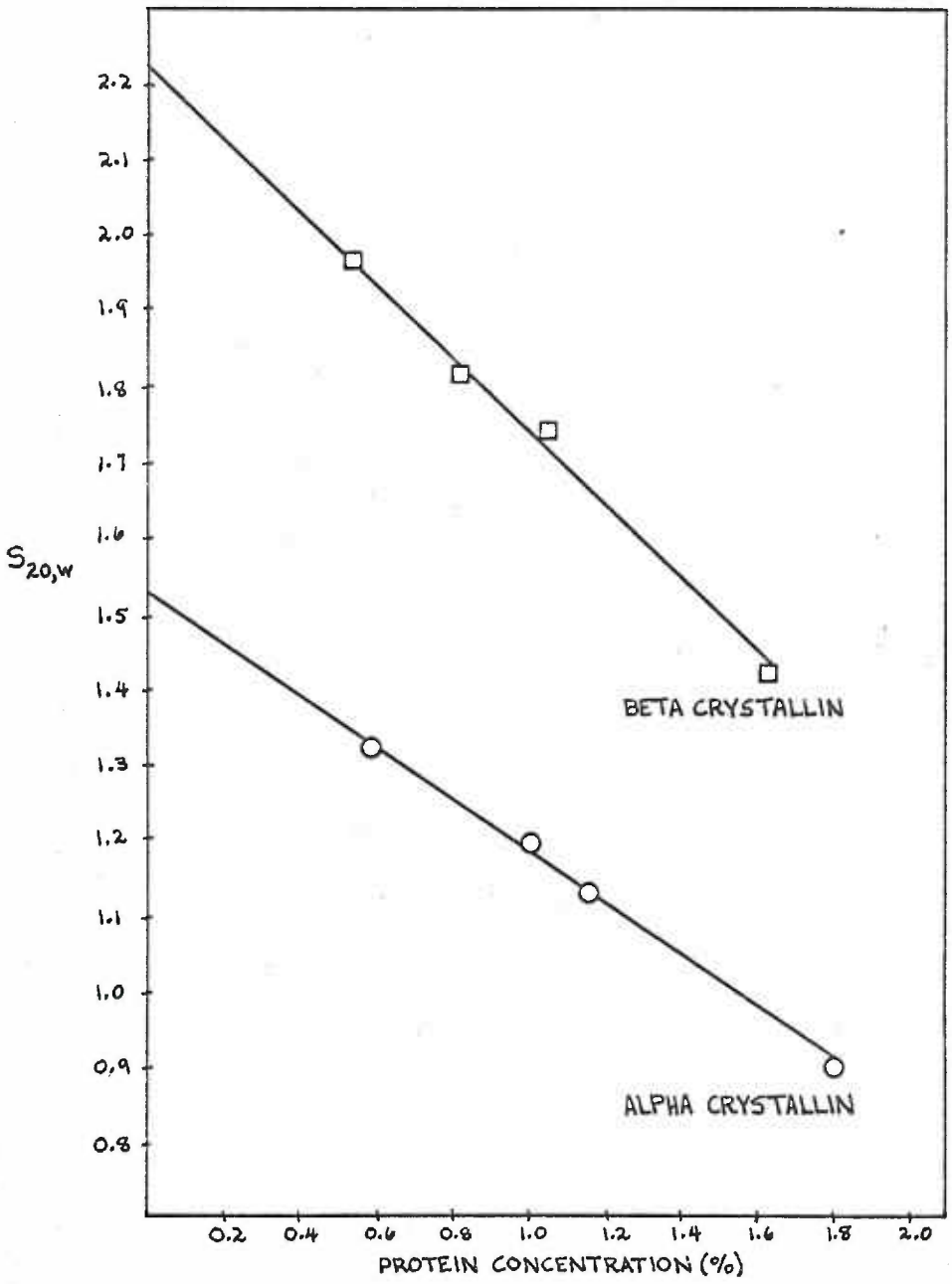


Table 11

Concentration dependence of the $s_{20,w}$ values for succinylated alpha and beta crystallin.

Succinylated alpha crystallin				
Concentration (%)	0.59	1.01	1.17	1.81
$s_{20,w}$	1.320	1.194	1.128	0.895
Succinylated beta crystallin				
Concentration (%)	0.54	0.83	1.06	1.64
$s_{20,w}$	1.966	1.811	1.737	1.420

DISCUSSION

From the introduction it is seen that the lens protein fractions can be dissociated into subunits by various procedures such as variation of ionic strength and pH of the medium, urea treatment and succinylation. The results of these dissociations were observed by other investigators via techniques which depend mainly on the secondary, tertiary and quaternary structure of the protein. As a result of this, a population of polypeptide chains having the same primary structure but two or more secondary or tertiary structural conformations of similar free energy could appear to be heterogeneous.

A means of resolving this problem was to apply a method of identifying polypeptide chains which was dependent on the primary structure. Assuming that each polypeptide or subunit would have a unique C-terminal amino acid, it was decided to conduct C-terminal analyses before and after dissociation procedures. Of the several methods available for C-terminal analysis, carboxypeptidase hydrolysis promised to be most favorable.

The preferred substrates of CPA are peptides having aromatic amino acids or branched chain aliphatic amino acids at the carboxyl terminus. Peptides having basic C-terminal amino acids are not readily hydrolyzed; however, these are the preferred substrates for carboxypeptidase-B (51). In the experiments presented here, only CPA was used, mainly because of

its much greater stability as compared with carboxypeptidase-B. Also, it was observed from preliminary experiments that the CPA preparations on hand released basic C-terminal amino acids, indicating possible contaminating carboxypeptidase-B activity. Thus, peptides having basic C-terminal amino acids presumably did not go undetected.

The CPA preparations used were purchased as DFP-treated enzymes. However, it was considered desirable to check on possible contaminating chymotryptic activity since any such contamination could result in false C-terminal data. Incubation of a substrate of chymotrypsin with CPA revealed what appeared to be chymotryptic activity (figure 3). Treatment of the enzyme with PMSF did not result in loss of this apparent chymotryptic activity (figure 4). Results of experiments conducted with alpha chymotrypsin are illustrated in figures 5 and 6. These indicate that PMSF does inhibit chymotryptic activity. Furthermore, the observed OD changes in figures 3 and 4 are in a direction opposite to those seen in figure 6. For these reasons, the CPA preparations were considered to be free of contaminating chymotryptic activity.

To obtain assurance that the CPA procedure was valid as performed, experiments were conducted using BSA which has a known C-terminal sequence; namely, -val,thr,leu,ala. (52). Table 1 and figures 7 and 8 show this to be true.

Results of CPA hydrolyses of native alpha, beta and gamma crystallin are presented in tables 2, 5 and 8 and figures 9, 10 and 11. Serine is the only amino acid released in substantial amounts by the CPA

hydrolysis of native alpha crystallin. The CPA hydrolysis of native beta crystallin yielded the following amino acids arranged in order of decreasing amounts; threonine, tyrosine, leucine and phenylalanine. Tyrosine was the only amino acid released by the CPA hydrolysis of native gamma crystallin.

These results per se do not indicate the presence of more than one unique polypeptide subunit per lens protein fraction. However, since masking of other C-terminal residues is very possible, it was decided to dissociate the individual fractions and then analyze the C-terminal sequence of the dissociated material.

Of several dissociation methods available, succinylation was chosen for three reasons.

1. No covalent bonds are broken
2. Unlike other methods such as urea denaturation, the dissociation is not reversible.
3. Simultaneous denaturation of the enzyme is avoided since free succinic anhydride need not be present in the medium to maintain the dissociated state, unlike such procedures as acid or base dissociation or urea denaturation.

Other workers have shown succinylation to be an effective dissociating procedure (32, 49, 53). Also, in our laboratory, experiments performed by Mr. L. Jackson in which succinylated lens proteins were subjected to polyacrylamide gel disc electrophoresis indicate that the procedure does cause the dissociation of the individual lens protein

fractions as evidenced by the appearance of several bands in the electropherogram.

To further confirm the effect of succinylation on lens proteins, ultracentrifugal analyses were performed on native alpha and beta crystallin and on their succinylated derivatives. Figure 12 shows the sedimentation pattern obtained from native alpha crystallin. The calculated $s_{20,w}$ value from these data is 18.2 S which is not too much in variance with the $s_{20,w}$ value at zero concentration ($s^{\circ}_{20,w}$) of 19.0 S obtained by other investigators for the same protein fraction of bovine origin. Mason and Hines have determined an $s^{\circ}_{20,w}$ of 18.9 S for rabbit lens alpha crystallin (46). In fact the value of 18.2 S is almost certainly low since the succinylated protein showed a negative concentration dependence (figure 16) and extrapolation to zero concentration was not performed.

Figure 14 shows the sedimentation pattern of succinylated alpha crystallin. Apparent homogeneity was observed with both native and succinylated alpha crystallin. However, the succinylated alpha crystallin was calculated to have an $s^{\circ}_{20,w}$ value of 1.5 S. Thus, it is seen that the succinylation procedure causes more than a 10-fold decrease in the $s^{\circ}_{20,w}$ value of alpha crystallin. The results of similar experiments with beta crystallin are seen in figures 13 and 15. Figure 13 shows the sedimentation pattern of native beta crystallin while figure 15 is that for succinylated beta crystallin. No $s_{20,w}$ value was calculated for the native beta crystallin since it was obviously

heterogeneous. The succinylated beta crystallin however, was calculated to have an $s^{\circ}_{20,w}$ value of 2.2 S. Thus, it was shown that the succinylation procedure dissociates alpha and beta crystallin into subunits.

The results of CPA hydrolysis of succinylated alpha crystallin are given in tables 3 and 4. Comparison of these results with data in table 2 shows that there is a very marked difference in the pattern of amino acid release for the CPA hydrolysis of native and succinylated alpha crystallin. In fact, the patterns of amino acid release for succinylated alpha crystallin are so complex that at first glance they appear to be the result of endopeptidase hydrolysis. Similar experiments with beta and gamma crystallin showed the same type of results; i.e., the amino acid release patterns for the succinylated protein are very different from those for the native protein.

Tables 6 and 7 show the results of CPA hydrolysis of succinylated beta crystallin. Comparing these results with the data in table 5 again points out the effect of succinylation on the availability of C-terminal amino acids. Tables 9 and 10 contain data obtained from the CPA hydrolysis of gamma crystallin. Although no ultracentrifugal analyses of native and succinylated gamma crystallin were performed, a comparison of the data in tables 9 and 10 with that in table 8 shows that as in the case of alpha and beta crystallin, succinylation unmasks C-terminal amino acids in gamma crystallin.

To verify the assumption that succinylation does not break any

covalent bonds, BSA was succinylated and a CPA hydrolysis was performed. The results were compared with those obtained by the CPA hydrolysis of native BSA. The only difference noted was that the rate of amino acid release from the succinylated BSA was much faster than the rate of amino acid release from native BSA.

The data from the CPA hydrolysis of the succinylated protein fractions was used to make plots of μmole of amino acid released versus time. From these plots, amino acids were chosen which, from their pattern of release, appeared to be C-terminal. These were then plotted separately using similar coordinates. The original plot was again referred to and this time, amino acids were chosen which appeared to be possible penultimate residues to those chosen as C-terminals. These penultimate amino acids were then plotted with their probable C-terminal pairs. This procedure was used to obtain the amino acids which appeared to be the third, fourth, etc., from the C-terminus.

Since at least two hydrolyses per succinylated protein fraction were performed, the apparent C-terminal amino acids and the amino acids which appeared to be penultimate, etc., from one run were compared with those from another run. Although from one experiment to the next, absolute amounts of amino acid released were not exactly similar, emphasis was placed on the comparison of relative amounts released. For example, if in both experiments, leucine and phenylalanine appeared to be the C-terminal and penultimate amino acids, respectively of a polypeptide, the ratio of μmole phe/μmole leu x 100 from one experiment was compared

with that from another. Arbitrarily, 15% was set as the maximum limit of variability between results from the two experiments, although most results varied only within 10%.

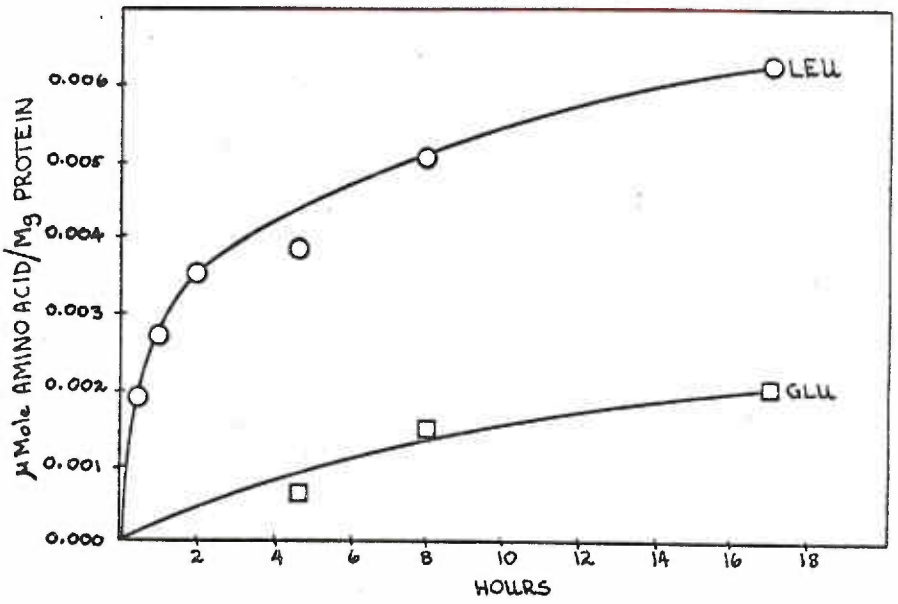
The results of this treatment of the data from succinylated alpha crystallin (tables 3 and 4) are presented in figures 17 - 19. Figures in the subclass A were calculated from data in table 3 and those in the subclass B, from table 4. From these data and figures it is apparent that alpha crystallin is composed of at least three different subunits with valine, leucine and phenylalanine as the C-terminal amino acids. Serine and alanine were released in substantial amounts but neither could be paired with a consistent penultimate or C-terminal amino acid. The significance of this is explained in a later section of the discussion.

The data obtained from the CPA hydrolysis of succinylated beta crystallin (tables 6 and 7) was analyzed in the same way as was the succinylated alpha crystallin data. The results are seen in figures 20 - 22. Figures in the subclass A were plotted from data in table 6 while those in the subclass B were plotted from data in table 7. It is seen that there are at least three possible subunits in beta crystallin with leucine, valine and threonine as the C-terminal amino acids.

Treatment of the data from the CPA hydrolysis of succinylated gamma crystallin (tables 9 and 10) in the manner described for the two previous fractions yielded the results presented in figures 23 and 24. Figures in the subclass A were plotted from data in table 9 while those in the

Figure 17-A. Plot of μmole of leucine and glutamate released versus time for the CPA hydrolysis of succinylated alpha crystallin. Relative concentrations are; leucine = 100%, glutamate = 32%.

Figure 17-B. Plot of μmole of leucine and glutamate released versus time for the CPA hydrolysis of succinylated alpha crystallin. Relative concentrations are; leucine = 100%, glutamate = 25%.


LEU
GLU
μMole AMINO ACID/Mg PROTEIN
0.000
0.001
0.002
0.003
0.004
0.005
0.006
2
4
6
8
10
12
14
16
18
HOURS

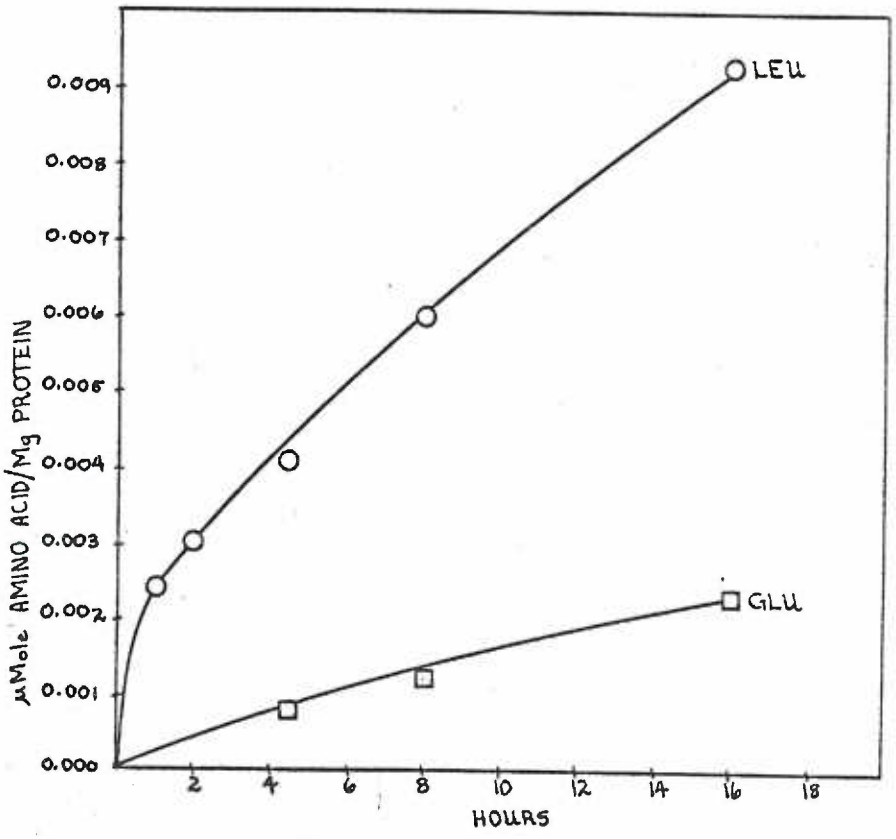
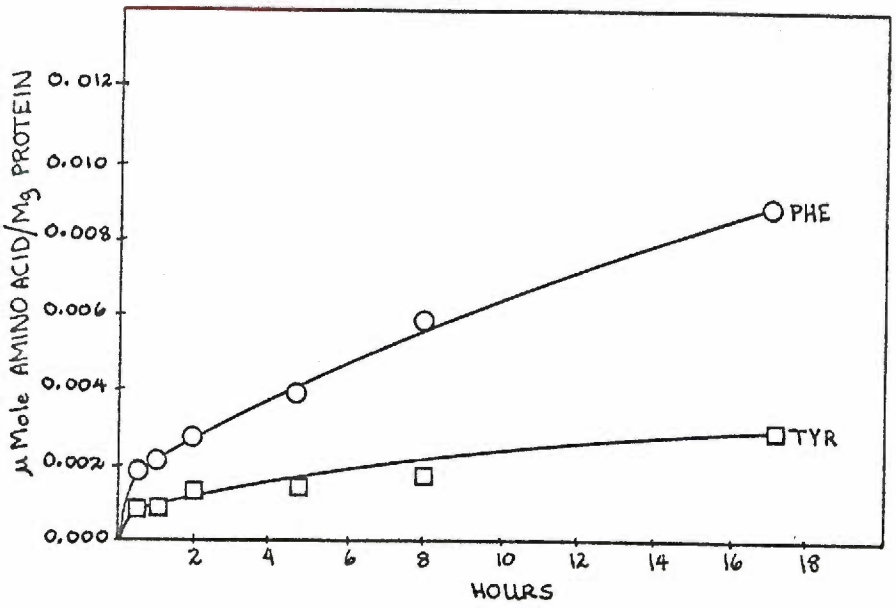

LEU
GLU
μMole AMINO ACID/Mg PROTEIN
0.000
0.001
0.002
0.003
0.004
0.005
0.006
0.007
0.008
0.009
2
4
6
8
10
12
14
16
18
HOURS

Figure 18-A. Plot of μmole of phenylalanine and tyrosine released versus time for the CPA hydrolysis of succinylated alpha crystallin. Relative concentrations are; phenylalanine = 100%, tyrosine = 34%.

Figure 18-B. Plot of μmole of phenylalanine and tyrosine released versus time for the CPA hydrolysis of succinylated alpha crystallin. Relative concentrations are; phenylalanine = 100%, tyrosine = 29%.


µ Mole AMINO ACID/Mg PROTEIN
0.012
0.010
0.008
0.006
0.004
0.002
0.000
2
4
6
8
10
12
14
16
18
HOURS
PHE
TYR

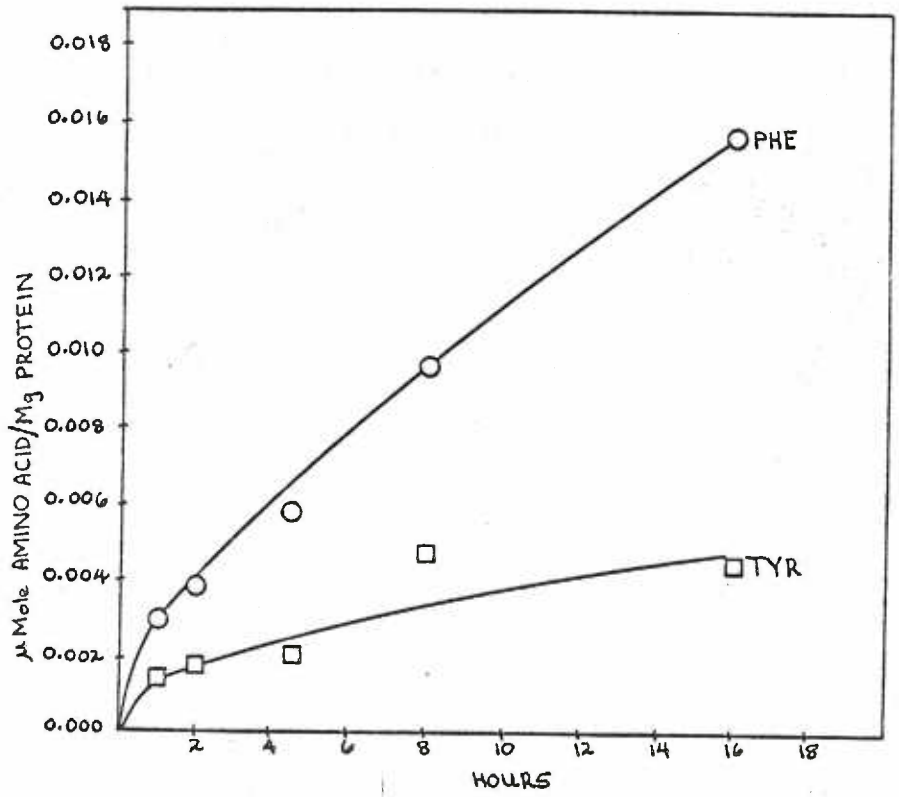
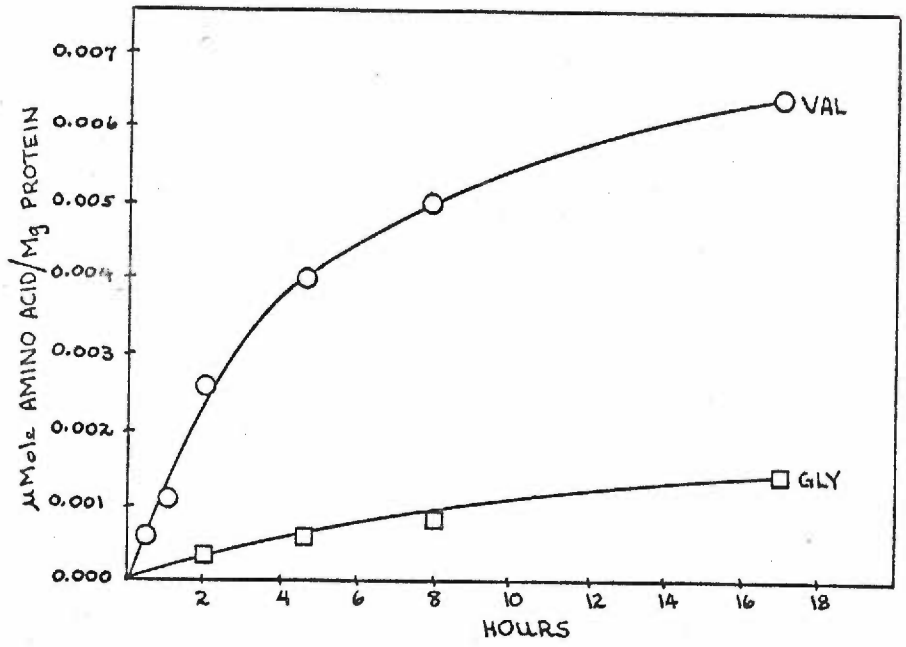

µ Mole AMINO ACID/Mg PROTEIN
0.018
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
2
4
6
8
10
12
14
16
18
HOURS
PHE
TYR

Figure 19-A. Plot of μmole of valine and glycine released versus time for the CPA hydrolysis of succinylated alpha crystallin. Relative concentrations are; valine = 100%, glycine = 34%.

Figure 19-B. Plot of μmole of valine and glycine released versus time for the CPA hydrolysis of succinylated alpha crystallin. Relative concentrations are; valine = 100%, glycine = 28%.


0.007
0.006
0.005
0.004
0.003
0.002
0.001
0.000
µMole AMINO ACID/Mg PROTEIN
VAL
GLY
2
4
6
8
10
12
14
16
18
HOURS

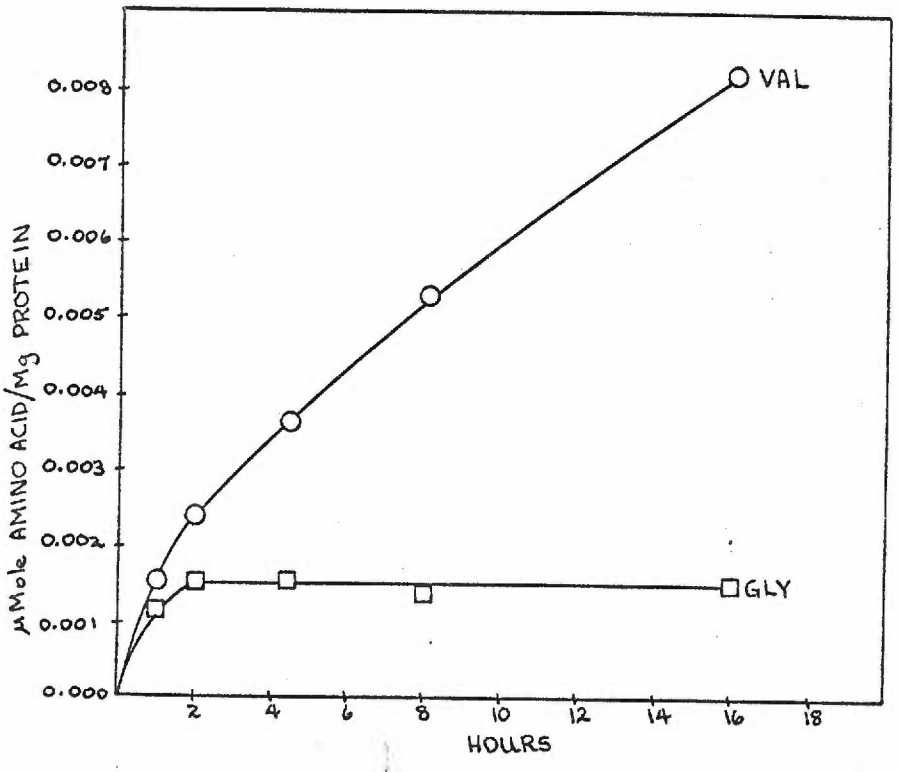
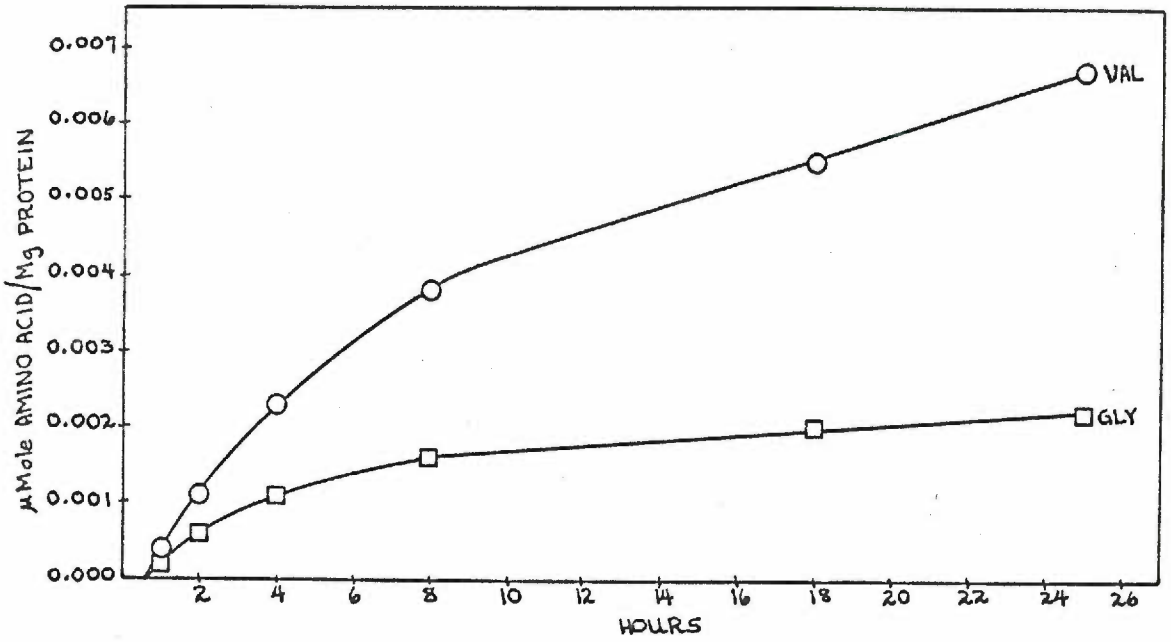

0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0.000
µMole AMINO ACID/Mg PROTEIN
VAL
GLY
2
4
6
8
10
12
14
16
18
HOURS

Figure 20-A. Plot of μmole of valine and glycine released versus time for the CPA hydrolysis of succinylated beta crystallin. Relative concentrations are; valine = 100%, glycine = 33%.

Figure 20-B. Plot of μmole of valine and glycine released versus time for the CPA hydrolysis of succinylated beta crystallin. Relative concentrations are; valine = 100%, glycine = 36%.


0.007
0.006
0.005
0.004
0.003
0.002
0.001
0.000
µMole AMINO ACID/Mg PROTEIN
VAL
GLY
2
4
6
8
10
12
14
16
18
20
22
24
26
HOURS

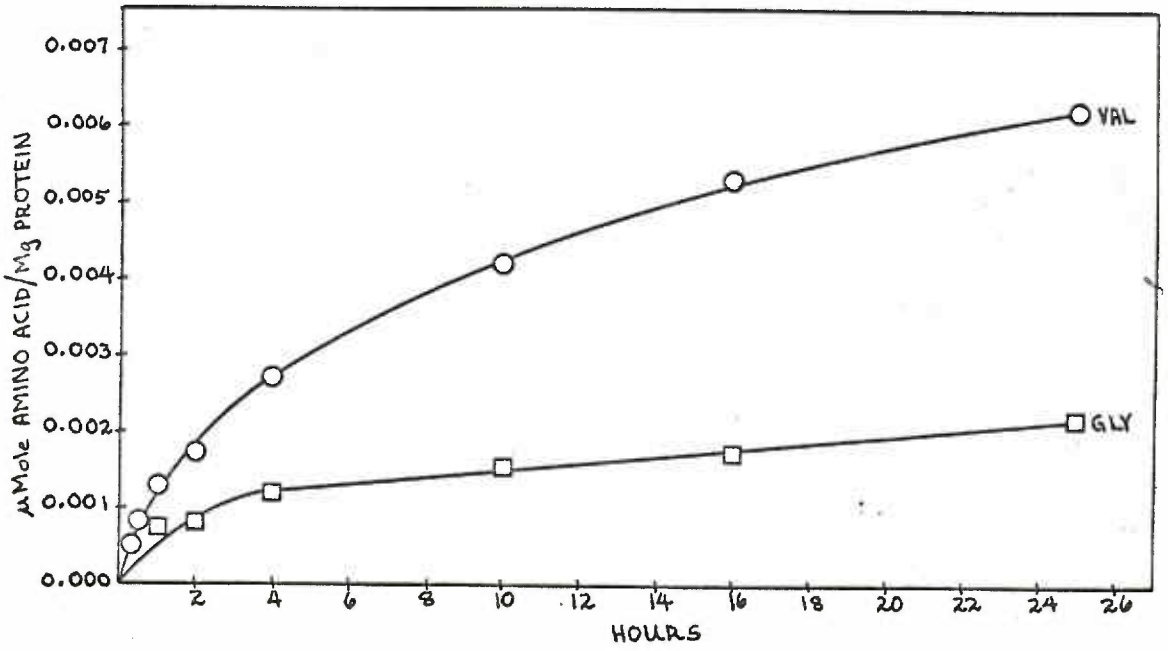

0.007
0.006
0.005
0.004
0.003
0.002
0.001
0.000
µMole AMINO ACID/Mg PROTEIN
VAL
GLY
2
4
6
8
10
12
14
16
18
20
22
24
26
HOURS

Figure 21-A. Plot of μmole of threonine, isoleucine, histidine and methionine released versus time for the CPA hydrolysis of succinylated beta crystallin. Relative concentrations are; threonine = 100%, isoleucine = 39%, histidine = 14%, methionine = 6%.

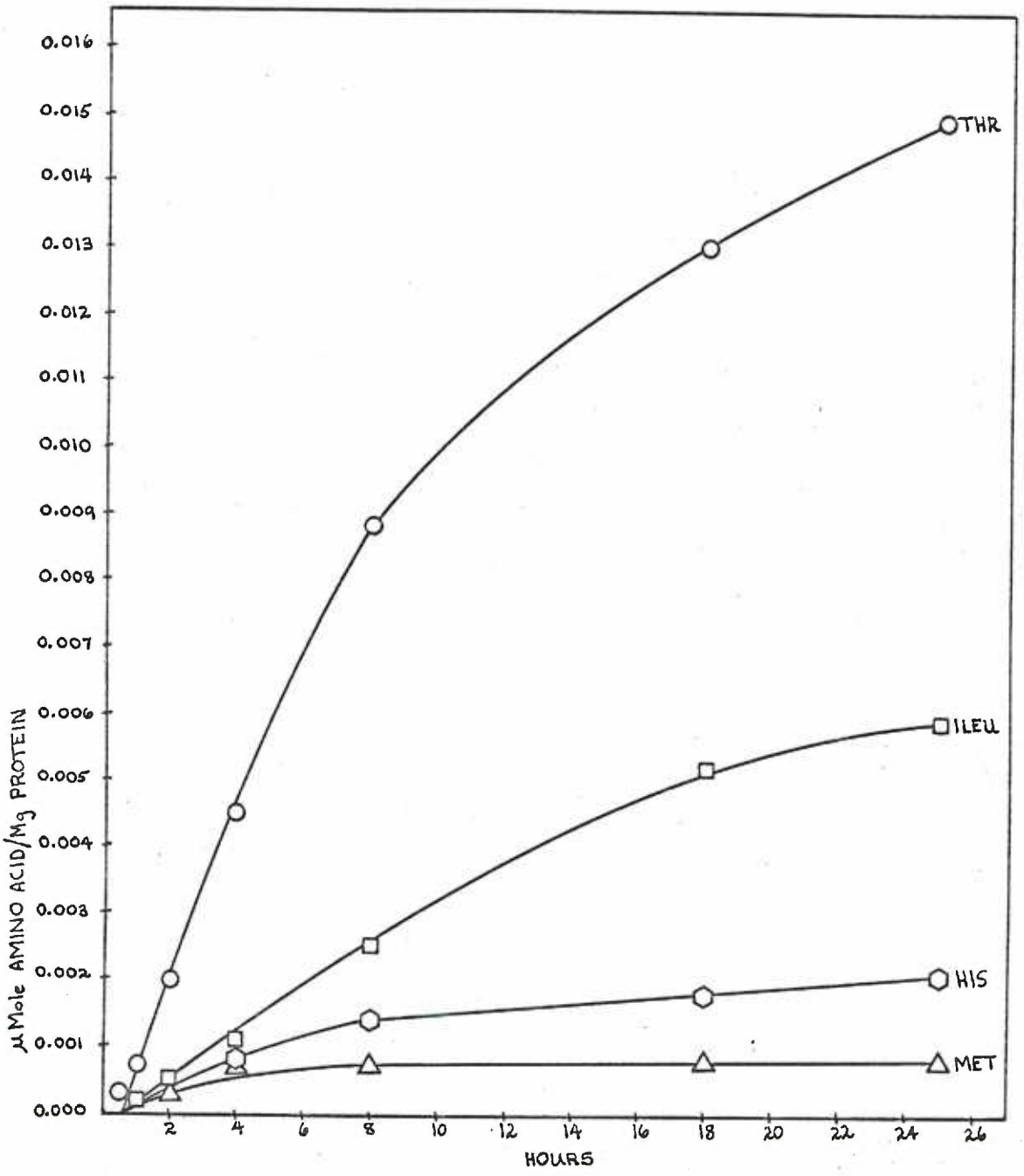

0.016
0.015
0.014
0.013
0.012
0.011
0.010
0.009
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0.000
μMole AMINO ACID/Mg PROTEIN
2
4
6
8
10
12
14
16
18
20
22
24
26
HOURS
THR
ILEU
HIS
MET

Figure 21-B. Plot of μmole of threonine, isoleucine, histidine and methionine released versus time for the CPA hydrolysis of succinylated beta crystallin. Relative concentrations are; threonine = 100%, isoleucine = 33%, histidine = 14%, methionine = 4%.

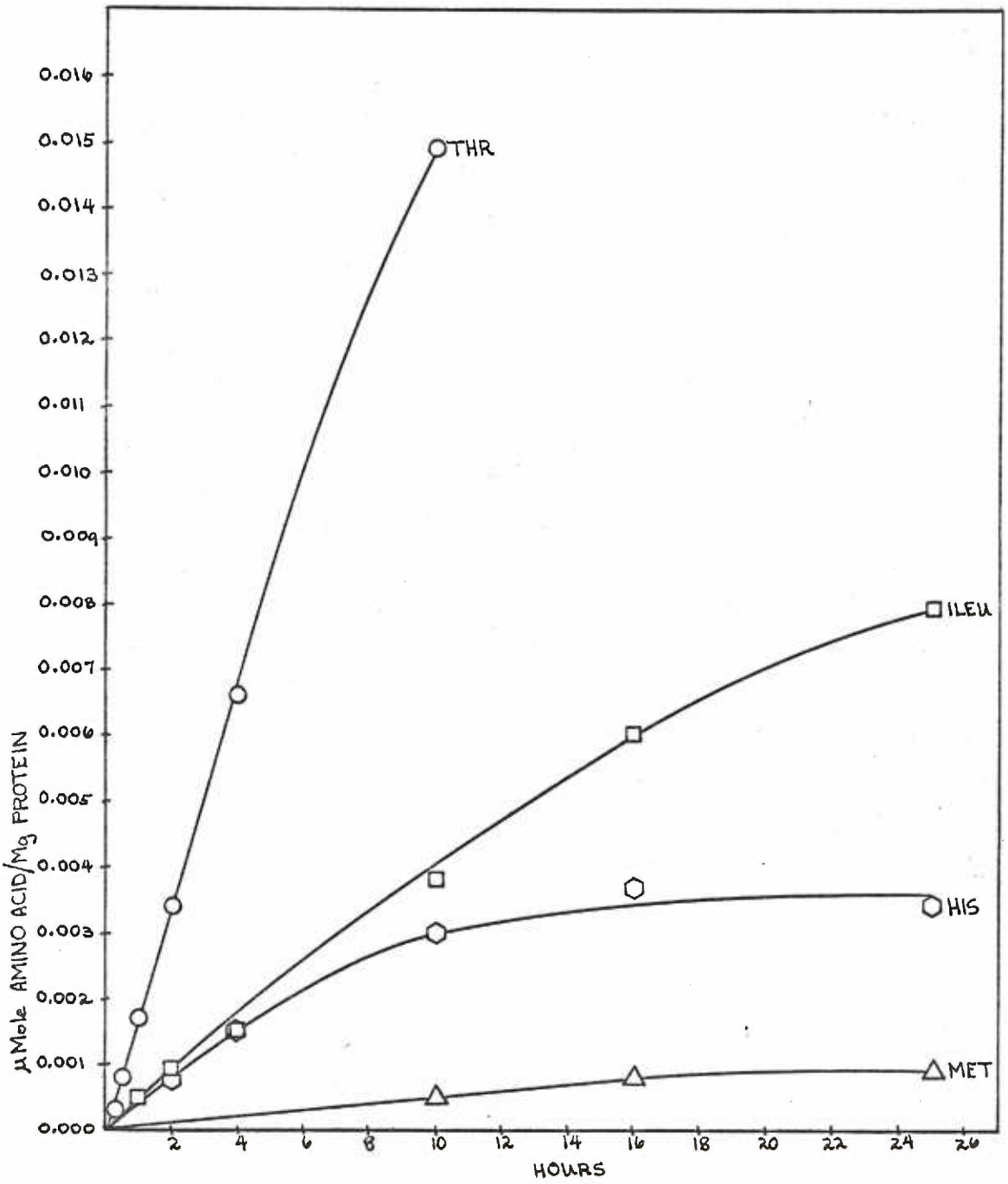
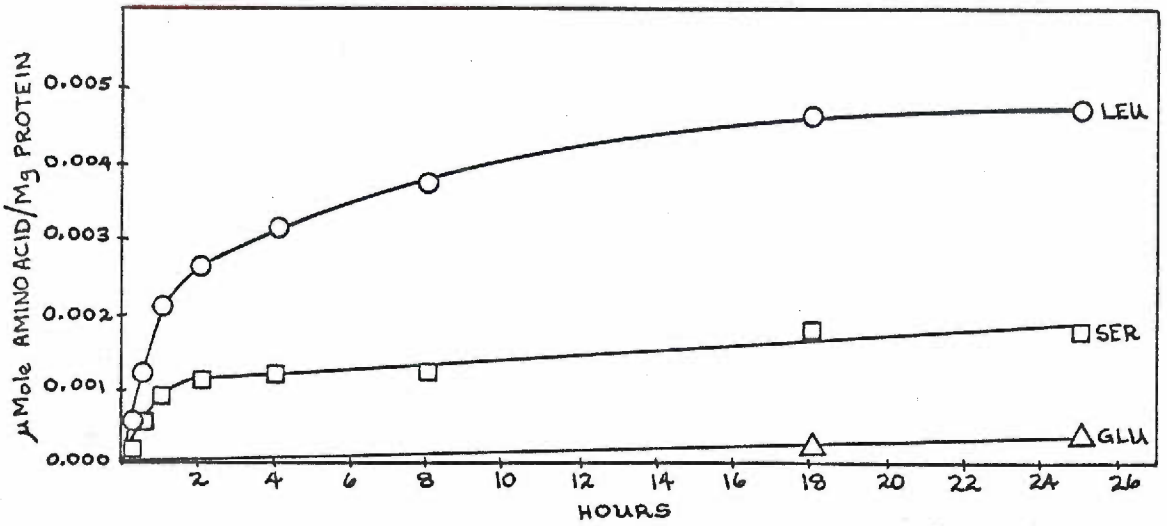

0.016
0.015
0.014
0.013
0.012
0.011
0.010
0.009
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0.000
µMole AMINO ACID/Mg PROTEIN
2
4
6
8
10
12
14
16
18
20
22
24
26
HOURS
THR
ILEU
HIS
MET

Figure 22-A. Plot of μmole of leucine, serine and glutamate released versus time for the CPA hydrolysis of succinylated beta crystallin. Relative concentrations are; leucine = 100%, serine = 39%, glutamate = 9%.

Figure 22-B. Plot of μmole of leucine, serine and glutamate released versus time for the CPA hydrolysis of succinylated beta crystallin. Relative concentrations are; leucine = 100%, serine = 43%, glutamate = 19%.


µMole AMINO ACID/Mg PROTEIN
0.000
0.001
0.002
0.003
0.004
0.005
2
4
6
8
10
12
14
16
18
20
22
24
26
HOURS
LEU
SER
GLU

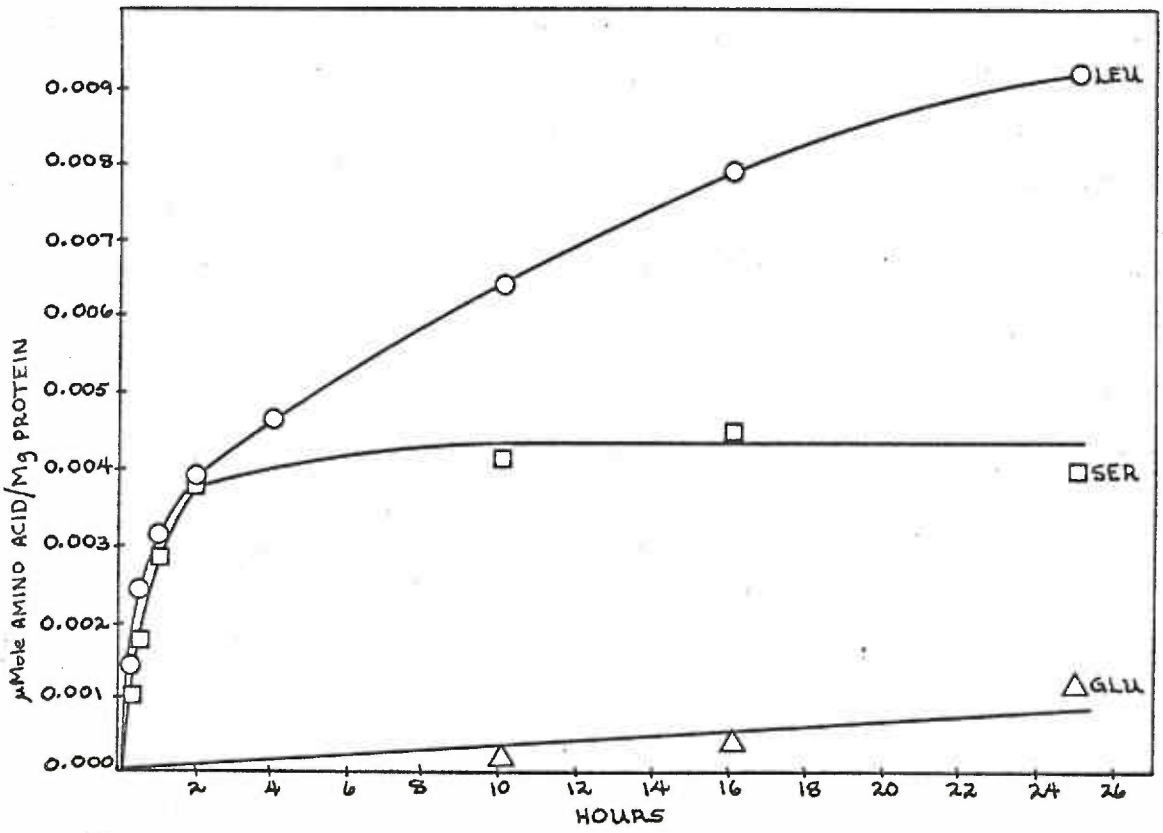
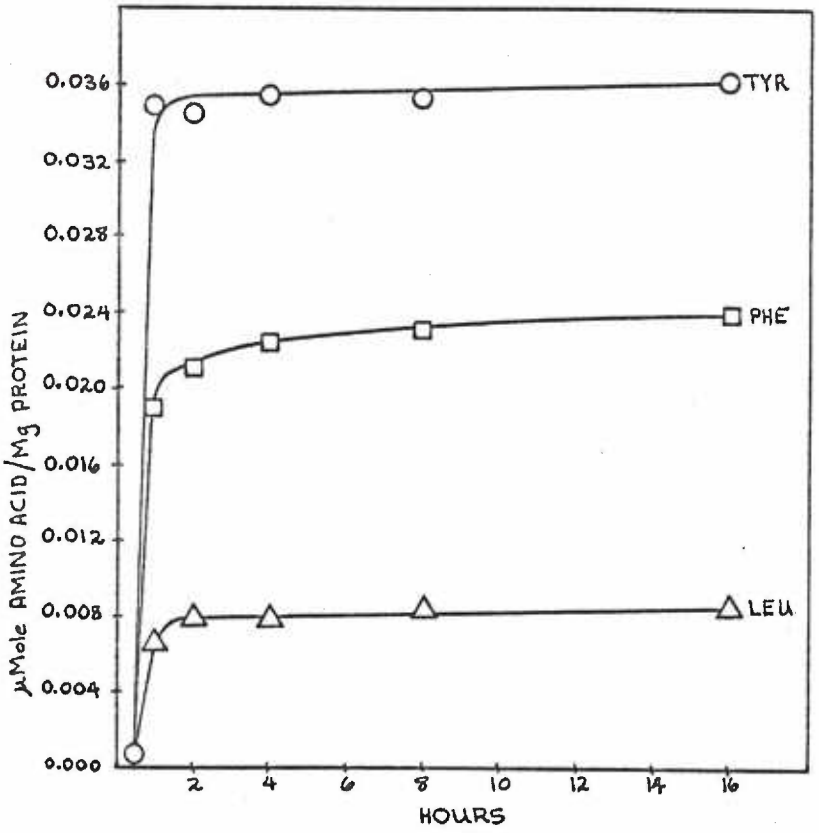

µMole AMINO ACID/Mg PROTEIN
0.000
0.001
0.002
0.003
0.004
0.005
0.006
0.007
0.008
0.009
2
4
6
8
10
12
14
16
18
20
22
24
26
HOURS
LEU
SER
GLU

Figure 23-A. Plot of μmole of tyrosine, phenylalanine and leucine released versus time for the CPA hydrolysis of succinylated gamma crystallin. Relative concetrations are tyrosine = 100%, phenylalanine = 68%, leucine = 25%.

Figure 23-B. Plot of μmole of tyrosine, phenylalanine and leucine released versus time for the CPA hydrolysis of succinylated gamma crystallin. Relative concentrations are; tyrosine = 100%, pheylalanine = 62%, leucine = 32%.


0.036
0.032
0.028
0.024
0.020
0.016
0.012
0.008
0.004
0.000
µMole AMINO ACID/Mg PROTEIN
TYR
PHE
LEU
2
4
6
8
10
12
14
16
HOURS

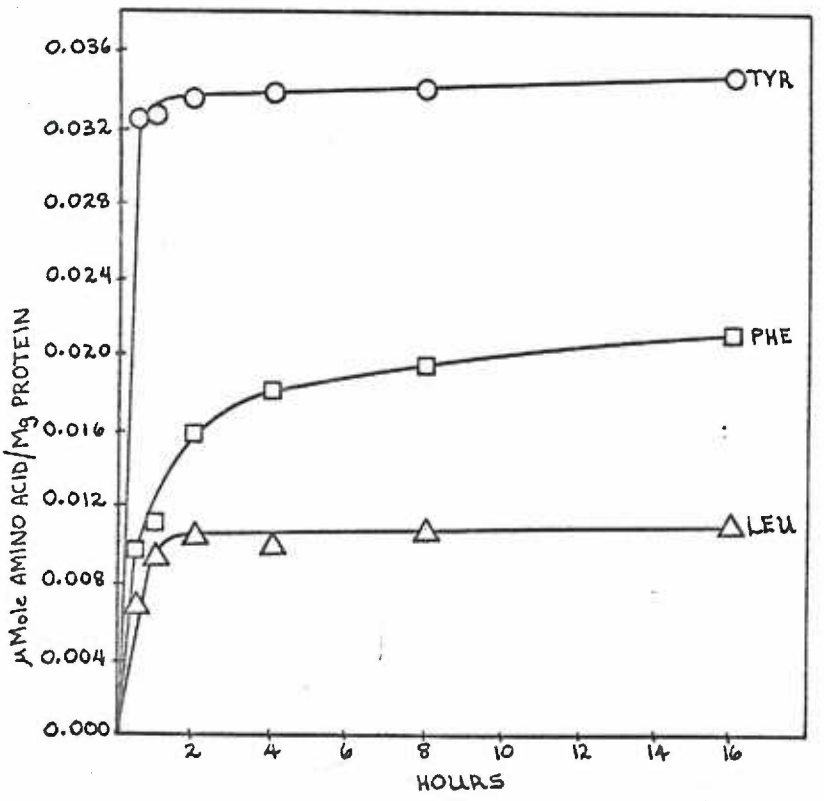

0.036
0.032
0.028
0.024
0.020
0.016
0.012
0.008
0.004
0.000
µMole AMINO ACID/Mg PROTEIN
TYR
PHE
LEU
2
4
6
8
10
12
14
16
HOURS

Figure 24-A. Plot of μmole of valine, aspartate, methionine, glycine and isoleucine released versus time for the CPA hydrolysis of succinylated gamma crystallin. Relative concentrations are; valine = 100%, aspartate = 79%, methionine = 41%, glycine = 8%, isoleucine = 4%.

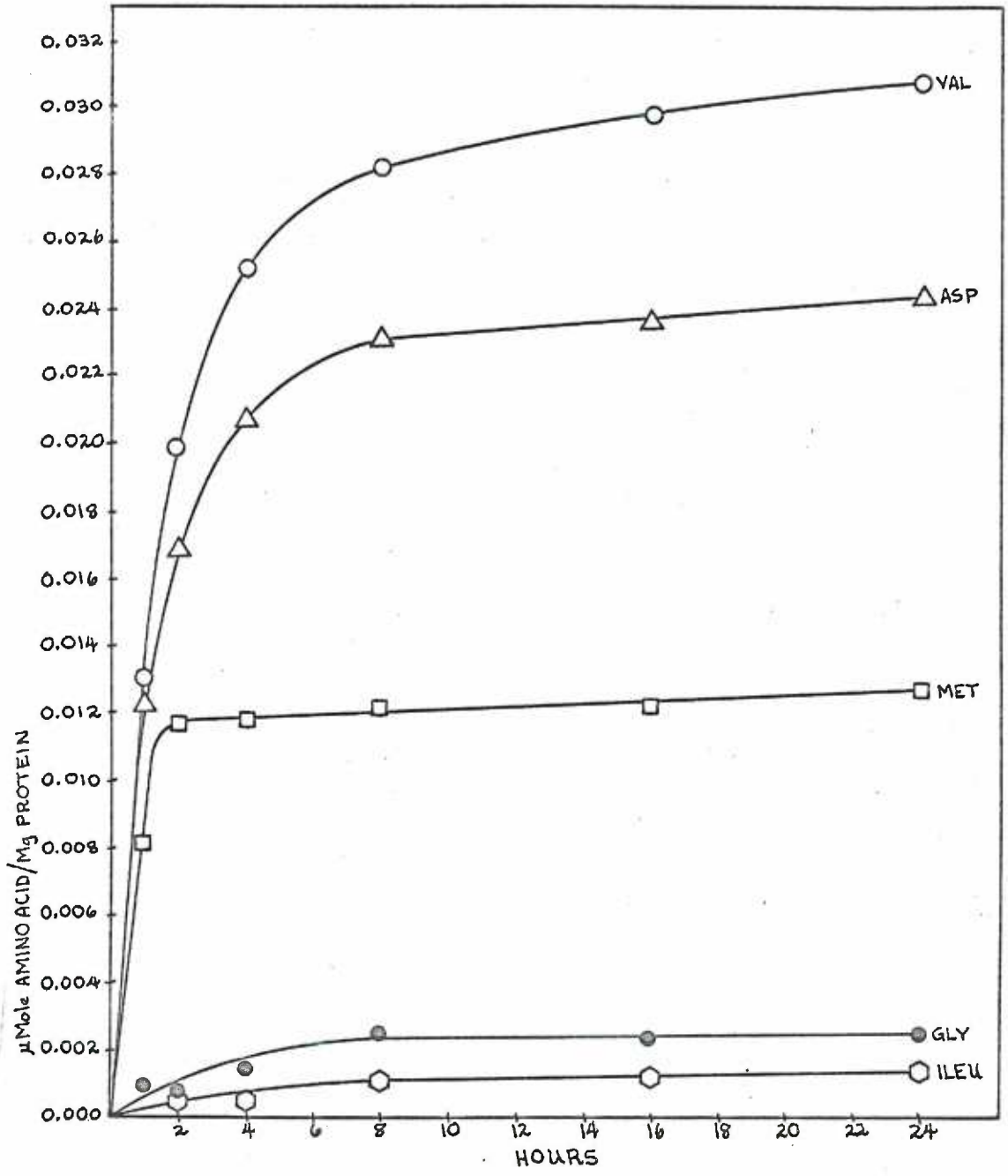
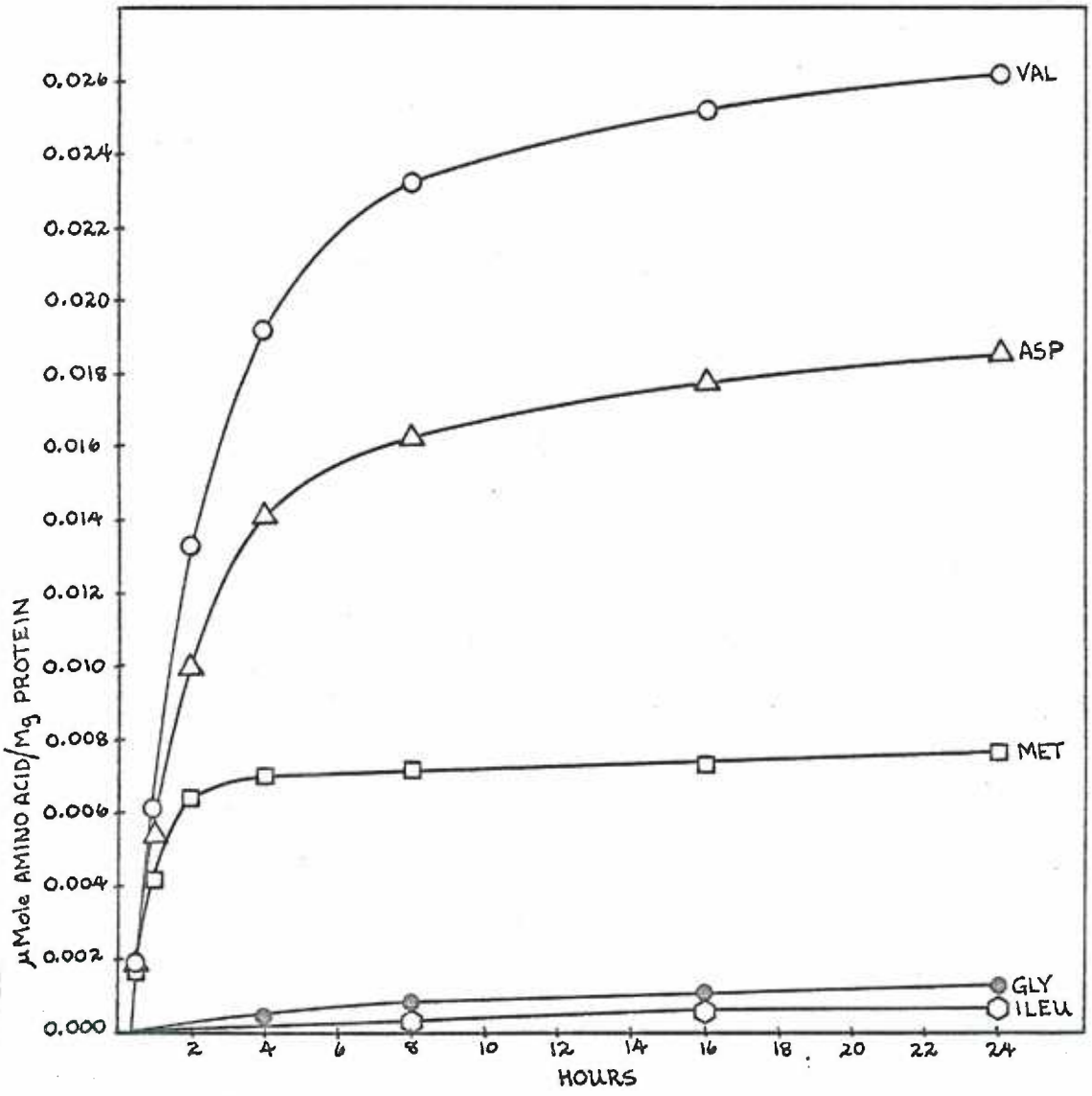

0.032
0.030
0.028
0.026
0.024
0.022
0.020
0.018
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
µMole AMINO ACID/Mg PROTEIN
2
4
6
8
10
12
14
16
18
20
22
24
HOURS
VAL
ASP
MET
GLY
ILEU

Figure 24- B. Plot of μmole of valine, aspartate, methionine, glycine and isoleucine released versus time for the CPA hydrolysis of succinylated gamma crystallin. Relative concentrations are; valine = 100%, aspartate = 71%, methionine = 29%, glycine = 5%, isoleucine = 3%.


0.026
0.024
0.022
0.020
0.018
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
µMole AMINO ACID/Mg PROTEIN
2
4
6
8
10
12
14
16
18
20
22
24
HOURS
VAL
ASP
MET
GLY
ILEU

subclass B were plotted from data in table 10. It is seen that gamma crystallin apparently has two subunits, with valine and tyrosine as the C-terminal amino acids.

A review of the C-terminal data presented above shows that the number of amino acids actually released is greater than the number of amino acids accounted for by the analysis of the data discussed here. For example, table 3 shows that CPA hydrolysis of succinylated alpha crystallin in one experiment releases 12 amino acids but analysis of the data as presented above accounts for only 6 amino acids. The same general observation is made with respect to the data from the CPA hydrolysis of succinylated beta and gamma crystallin. An explanation of this apparent disparity in the results is presented below.

The data in table 3 is from preparation A of alpha crystallin. Previous analysis of this data does not account for the presence of histidine, aspartate, threonine, serine, alanine and isoleucine. From figure 21 it is seen that beta crystallin has a peptide with the C-terminal sequence, -met,his,ileu,thr. The average relative amounts of methionine, histidine and isoleucine as compared to threonine are 5%, 14% and 36%, respectively. From table 3 it is seen that the relative amounts of histidine and isoleucine with respect to threonine are 28% and 40%, respectively. This observation suggests that the histidine, isoleucine and threonine are released from a peptide which is a contaminant from the beta crystallin fraction.

Figure 9 shows that when native alpha crystallin was hydrolyzed

with CPA, serine was the only amino acid released in substantial amounts. There was no apparent penultimate amino acid to serine, an observation which may be explained by postulating that the penultimate amino acid does not comply with the specificity requirements of CPA. Analysis of the data in table 3 gave the same results; i.e., there was no apparent penultimate amino acid to serine. These observations indicate that serine is the C-terminal amino acid of a polypeptide in the alpha crystallin fraction which does not have a CPA-hydrolyzable penultimate amino acid.

Attempts to find a penultimate or C-terminal amino acid pair for aspartate were fruitless. However, reference to figure 24 shows that gamma crystallin has a polypeptide with the C-terminal sequence, -ileu, gly,met,asp,val. The average relative amounts of isoleucine, glycine, methionine and aspartate with respect to valine are 4%, 7%, 35% and 75%, respectively. If it is theorized that the aspartate in preparation A of alpha crystallin is due to contamination by a polypeptide from the gamma crystallin fraction, it follows that the presence of valine in preparation A of alpha crystallin is partly due to contamination also. To evaluate this possibility, aspartate was equated to 75% and the amount of valine corresponding to 100% was calculated. The value turned out to be 0.0017 µmole. This 0.0017 µmole was then subtracted from the total amount of valine in table 3 (0.0067 µmole) leaving 0.0050 µmole of valine. This 0.0050 µmole of valine was then presumed to be from a polypeptide in the alpha crystallin fraction. A search

for a penultimate amino acid pair for valine showed that glycine release (0.0017 μmole) was 34% of the valine release (0.0050 μmole). This is in agreement with the data in figure 20 which shows that beta crystallin has a polypeptide which has valine as a C-terminal and glycine as a penultimate amino acid, glycine being released in amounts 35% with respect to valine. Thus, it was concluded that the aspartate released from the alpha crystallin fraction was from a contaminating polypeptide of the gamma crystallin fraction. Furthermore, it appears that alpha crystallin and beta crystallin have a polypeptide in common. It is not thought that the valine in beta crystallin is due to contamination from alpha crystallin, or vice versa because in both fractions valine and glycine were released in approximately equal amounts.

Reference again to table 3 shows that CPA hydrolysis of succinylated alpha crystallin released alanine in substantial amounts (0.0095 μmole) but no penultimate or C-terminal amino acid could be matched with alanine. Since alanine was released by the CPA hydrolysis of native alpha crystallin but not by the CPA hydrolysis of either native beta or gamma crystallin, it was concluded that alanine is the C-terminal amino acid of a polypeptide in the alpha crystallin fraction which does not have a CPA-hydrolyzable penultimate amino acid. This extended manipulation of data accounts for all the amino acids released when preparation A of succinylated alpha crystallin was hydrolyzed with CPA.

The data in table 4 is from the CPA hydrolysis of preparation B of alpha crystallin. When the data was analyzed as described above,

essentially the same results were obtained and the same conclusions made. There was a minor difference though in the explanation of the presence of isoleucine in preparation B of succinylated alpha crystallin. As above, it is thought that the isoleucine and threonine are due to contamination of the alpha crystallin fraction by a polypeptide from the beta crystallin fraction. Calculations show, however, that the total amount of isoleucine present (0.0057 µmole) is 69% of the total amount of threonine present (0.0083 µmole). Subtraction of 0.0027 µmole of isoleucine from 0.0057 µmole yields 0.0030 µmole which is 36% of 0.0083 µmole. This is in agreement with the average value of 36% in figure 21. The presence of the extra 0.0027 µmole of isoleucine cannot be explained. It is possible that the 0.0027 µmole of isoleucine is due to contamination but no proof or evidence of this can be offered.

No data for the release of histidine in this experiment is presented because there was not enough material to make a basic amino acid analysis on the amino acid analyzer.

The general conclusion that can be drawn from the analysis of the data presented above is that alpha crystallin is composed of at least five unique subunits, the following amino acids being those at the C-terminus; phenylalanine, leucine, valine, serine and alanine. It will be noted that the preliminary analysis of the alpha crystallin data led to the conclusion that alpha crystallin was composed of three subunits. This ambiguity will be re-examined in a later section of

the discussion.

Reference to table 6 shows that when preparation A of beta crystallin was succinylated and hydrolyzed with CPA, 14 amino acids were released. A preliminary analysis of the data presented previously accounted for only 9 of the 14 amino acids. Alanine, aspartate, tyrosine, phenylalanine and lysine were unaccounted for. As in alpha crystallin, alanine could not be paired with any C-terminal or penultimate amino acid. It is thought that no penultimate amino acid was apparent due to enzyme specificity reasons. The presence of alanine in the beta crystallin fraction may or may not be due to contamination by a polypeptide chain from the alpha crystallin fraction. The reasons for making this statement are given in a later section of the discussion.

The amount of phenylalanine released with respect to tyrosine in preparation A of beta crystallin was 80%. Reference to figure 23 shows that gamma crystallin has a polypeptide with the C-terminal sequence, -leu,phe,tyr. The average relative amounts of leucine and phenylalanine released with respect to tyrosine were 29% and 65%, respectively. From these results it was concluded that the presence of phenylalanine and tyrosine in preparation A of beta crystallin was due to contamination of the beta crystallin fraction by a polypeptide from the gamma crystallin fraction. Aspartate was released to the extent of 0.0025 µmole and lysine to the extent of 0.0002 µmole when preparation A of succinylated beta crystallin was hydrolyzed with CPA. Attempts to

explain the presence of these amino acids were unsuccessful. Neither were present in preparation B of beta crystallin. At present the only statement that can be ventured regarding their presence is that they are contaminants.

The data in table 7 is from the CPA hydrolysis of succinylated beta crystallin (preparation B). The previous analysis of the data did not account for the presence of phenylalanine, alanine and tyrosine. The explanation for the presence of alanine in this preparation of beta crystallin is the same as that offered above for preparation A of beta crystallin. However, it is not thought that the presence of phenylalanine and tyrosine in preparation B of beta crystallin was due to contamination by a polypeptide from the gamma crystallin fraction. The amount of tyrosine released with respect to phenylalanine in preparation B of beta crystallin was 27%. Reference to figure 18 shows that alpha crystallin has a polypeptide with the C-terminal sequence, -tyr,phe. The average relative amounts of tyrosine released with respect to phenylalanine in alpha crystallin was 32%. From these observations it was concluded that the presence of tyrosine and phenylalanine in preparation B of beta crystallin was due to contamination by a polypeptide from the alpha crystallin fraction.

Two reasons are given for attributing the presence of phenylalanine and tyrosine in preparation A of beta crystallin to contamination by a polypeptide from the gamma crystallin fraction and at the same time, attributing the presence of these same amino acids in preparation B of

beta crystallin to contamination by a polypeptide from alpha crystallin.

1. In preparation A of beta crystallin more tyrosine than phenylalanine was released while the converse is true in preparation B.
2. The kinetics of release of tyrosine in preparation A were very different from the kinetics of release of tyrosine from preparation B.

Comparison of the kinetics of release of tyrosine in preparation A with those of tyrosine in gamma crystallin showed that the two were very similar. A similar comparison of the kinetics of release of tyrosine in preparation B with those of tyrosine in alpha crystallin likewise showed that the two were very similar.

From the analysis of the data presented above, the general conclusion is that beta crystallin is composed of at least three unique subunits, with leucine, valine and threonine as the C-terminal amino acids.

The data in table 9 is from the CPA hydrolysis of succinylated gamma crystallin (preparation A). It shows that alanine, serine and threonine were unaccounted for in the previous analysis of data. As in the alpha and beta crystallin fractions, no penultimate or C-terminal amino acids could be matched with the alanine in preparation A of gamma crystallin. Serine in preparation A of gamma crystallin could not be matched with any penultimate or C-terminal amino acids. The significance of the presence of these two amino acids (alanine and serine) in

preparation A of gamma crystallin is discussed in a later section.

Reference to figure 21 shows that beta crystallin has a polypeptide with the C-terminal sequence, -met,his,ileu,thr. The average relative amounts of methionine, histidine and isoleucine released with respect to threonine were 5%, 14% and 36%, respectively. In preparation A of gamma crystallin, threonine was released to the extent of 0.0005 µmole. Thirty-six per cent of this is 0.0002 µmole. Isoleucine was released to an extent of 0.0014 µmole in preparation A of gamma crystallin. Subtraction of 0.0002 µmole from 0.0014 µmole yields 0.0012 µmole. Comparison of the relative amounts of isoleucine released with respect to valine in both preparations of gamma crystallin shows that isoleucine release was 4% and 3% of valine release in preparations A and B, respectively. It was thus concluded that the presence of 0.0005 µmole of threonine and 0.0002 µmole of isoleucine in preparation A of gamma crystallin was due to contamination of preparation A by a polypeptide from the beta crystallin fraction. The remaining 0.0012 µmole of isoleucine was from the polypeptide in gamma crystallin of which valine is the C-terminal amino acid.

From table 10 it is seen that alanine and isoleucine were not accounted for in the preliminary analysis of the data obtained from the CPA hydrolysis of succinylated gamma crystallin (preparation B). As indicated in the paragraph immediately preceeding this, the isoleucine can be from the polypeptide in gamma crystallin of which valine is the C-terminal. Since there were no amino acids which could be penultimate

or C-terminal to alanine, it was concluded that the alanine was from a polypeptide similar to the one observed in beta and alpha crystallin, i.e., a contaminant. The data presented above indicate that gamma crystallin is composed of at least two unique subunits with valine and tyrosine as the C-terminal amino acids.

The term contaminant as used in this discussion is defined as a component present in low amounts. Table 12 gives the amounts of some amino acids which were found to be present in all three lens protein fractions. As was mentioned previously, the polypeptide with the C-terminal sequence, -gly,val. is thought of as being common to both the alpha and beta crystallin fractions. This is because valine is present to about the same extent in both alpha and beta crystallin. The valine in the gamma crystallin fraction is from a different polypeptide having the C-terminal sequence, -ileu,gly,met,asp,val. The polypeptide with the C-terminal sequence, -met,his,ileu,thr. is thought to be present in the alpha and gamma crystallin fractions as a contaminant from the beta crystallin fraction because the amounts of threonine in alpha and gamma crystallin are low in comparison to the amounts of threonine in the beta crystallin fraction. The behavior of serine and alanine in the three lens protein fractions is not very readily explained. The two amino acids have four points in common.

1. Neither can be paired with a C-terminal or penultimate amino acid.
2. Both are present in approximately similar amounts in alpha crystallin.

Table 12

Comparison of the amounts (μmole) of valine, threonine, alanine and serine in the different preparations of the crystallin fractions.

Amino Acid	Alpha A	Alpha B	Beta A	Beta B	Gamma A	Gamma B
Serine	.0060	.0094	.0018	.0040	.0010	-------
Threonine	.0088	.0083	.0149	.0240	.0005	-------
Valine	.0050	.0054	.0067	.0062	.0308	.0262
Alanine	.0095	.0059	.0012	.0057	.0032	.0007

3. Both behave erratically from one preparation to another within any of the three crystallin fractions.
4. Neither can be definitely designated as contaminants in the beta and gamma crystallin fractions from the alpha crystallin fraction though it is possible that they are.

There are several reasons for making the last point. In the first place it is possible that the polypeptides of which alanine and serine are presumed to be the C-terminal acids are from the alpha crystallin fraction because in the CPA hydrolysis of the native lens protein fractions, it is only from native alpha crystallin that these amino acids are released. The uncertainty of their being contaminants in beta and gamma crystallin is due to their erratic behavior. For example, the amount of alanine in preparation A of beta crystallin is low enough to be considered as a contaminant from the alpha crystallin fraction. However, the amount of alanine in preparation B of beta crystallin is not low enough to be considered as a contaminant. In fact, the correlation of alanine content between preparation B of beta crystallin and preparation B of alpha crystallin is better than that between preparation A and B of alpha crystallin.

Similarly, though the amount of alanine in preparation B of gamma crystallin is low enough to be considered as a contaminant from the alpha crystallin fraction, the same statement cannot be made with regard to the alanine in preparation A of gamma crystallin. Consideration of the behavior of serine presents a similarly confusing situation.

An explanation is not readily available although two possibilities may be presented.

1. Serine and alanine are the C-terminal amino acids of two polypeptides which are not very definitely affected one way or another by the procedures used to separate the lens protein fractions. Thus though they may belong to one definite lens protein fraction, the extraction and separation procedures employed cause a distribution of the polypeptides among the three lens protein fractions.
2. The separation of the lens proteins into the three classical fractions is artificial; i.e., the three classical fractions are artifacts of the separation procedures. It is very possible that the lens proteins do not exist in the lens as defined groups of polypeptides but rather as a conglomeration of polypeptides which can be partitioned into the alpha, beta and gamma crystallin fractions by the extraction procedures used.

If the latter were true it would be in agreement with the findings presented above of cross-contamination from one fraction to another. This concept would also be in agreement with the findings of other workers. For example, Bon reported that the three lens protein fractions have some antigenic determinants in common (20). Bloemendal and his group also noted that when the individual lens protein fractions are treated with urea and subjected to starch block electrophoresis, there are

bands from each of the three fractions that migrate similarly (22).

It should be pointed out that if the lens proteins exist in the lens as a conglomeration of polypeptides, it is not probable that these polypeptides are just randomly located. This is inferred from the observed specificity of their interactions, as indicated by the reversibility of such procedures as urea denaturation (23, 26).

One important factor that should be kept in mind when considering the C-terminal data presented here is the possibility that some C-terminal amino acids may have gone undetected due to enzyme specificity reasons. If this is the case, the conclusions regarding the number of unique subunits per fraction will be modified. The correlations drawn however, would still hold because it is not possible that the amino acids released could belong to a polypeptide with a non-hydrolyzable C-terminal amino acid.

Another important point that should be mentioned concerns the levels of significance of the data presented. At the level of 0.005 μmole, the amino acid analyzer has an accuracy of 95%. This accuracy falls off at lower concentrations. Thus, the data presented should be considered in this light, i.e., the correlations made, especially those involving amino acids in low concentrations could be in error. It is interesting to note that the deductions and correlations made in the foregoing section of the discussion could not have been possible if only one of the lens protein fractions had been examined.

Attempts were made to confirm the C-terminal data obtained by the

carboxypeptidase method. Hydrazinolyses of BSA were performed but no meaningful results could be obtained. Operational problems were encountered in the separation of the amino acid hydrazides from the free amino acids.

Reviewing the tables and graphs of amino acid release with time which have been presented, it should be noted that no single amino acid or amino acid type was predominantly released in all three lens protein fractions. This observation further supports the contention that no contaminating endopeptidase activity was present in the CPA preparations used. Although no assay for trypsin activity was performed, the fact that the basic amino acids were not predominantly released consistently in all three fractions infers that no contaminating tryptic activity was present. Similarly, it can be pointed out that the preferred substrates of chymotrypsin such as leucine and phenylalanine were not predominantly released consistently in all three protein fractions. This adds to the evidence presented earlier for the absence of contaminating chymotryptic activity.

Thus, it can be said with reasonable confidence that the data presented here represent actual C-terminal entities.

SUMMARY AND CONCLUSION

The proteins of the lens can be divided operationally into two main classes.

1. Insoluble proteins, also termed albuminoid.
2. Soluble proteins which can be separated into three fractions; namely, alpha, beta and gamma crystallin.

Investigations into the physicochemical properties of the soluble lens proteins have shown that each of the three soluble lens protein fractions can be dissociated into subfractions or subunits. Studies on the number of subunits per soluble protein fraction have not yielded consistent results.

In all previous investigations, the effects of various dissociating agents were monitored by techniques which depend mainly on the secondary, tertiary and quaternary structure of the polypeptide chains. Thus, a population of polypeptide chains having the same primary structure but two or more secondary, tertiary, or quaternary structures of equal free energy could appear to be heterogeneous. Therefore, the problem of determining the number of subunits per soluble lens protein fraction could be resolved only by the use of monitoring methods which depend on the primary structure of the polypeptide.

Assuming that two or more subunits of a protein would have different C-terminal amino acids, it was decided to conduct C-terminal

analyses of the individual lens protein fractions before and after dissociation procedures. The carboxypeptidase hydrolysis method for C-terminal analysis was used. Dissociation of the protein samples was accomplished by succinylation.

At first glance, the results obtained presented a confusing picture and indicated that there was cross contamination among the three soluble lens protein fractions; i.e., the fractionation procedures used did not effect a clean separation of the soluble lens protein fractions from each other. However, a detailed analysis of the data permitted the following deductions to be made.

Alpha crystallin has at least five different subunits. The C-terminal amino acids are serine, valine, phenylalanine, leucine and alanine.

Beta crystallin has at least three different subunits. The C-terminal amino acids are leucine, valine and threonine.

Gamma crystallin has at least two different subunits. The C-terminal amino acids are tyrosine and valine.

Thus it may be concluded that each of the classical lens protein fractions is an aggregate of more than one type of subunit. That each of these fractions is an aggregate of subunits has previously been known. However, the results obtained in this work are the first unambiguous indications that within each fraction, these subunits are different.

Furthermore, the results obtained do not contradict the possibility that the lens proteins do not exist in vivo as physically distinct

fractions and that the three classical fractions are artifacts of the extraction procedure.

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