A BIOCHEMICAL MECHANISM OF CATARACT FORMATION

by

Larry Leroy David

A DISSERTATION

Presented to the Department of Biochemistry
and the Oregon Health Sciences University
School of Medicine
in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy

May 1986

APPROVED: [redacted]

(Professor in Charge of Thesis)

[redacted]

(Chairman, Graduate Council)

TABLE OF CONTENTS

Acknowledgements	iv
Abstract	v-vi
Introduction	1- 38
Manuscript 1: State of Sulfhydryl in Selenite Cataract	39- 61
Manuscript 2: Calcium-Activated Proteolysis in the Lens Nucleus During Selenite Cataractogenesis	62- 99
Manuscript 3: Purification of Calpain II from Rat Lens and Determination of Endogenous Substrates.	100-130
Manuscript 4: Origin of the Insoluble Protein in the Selenite Cataractous Lens	131-166
Manuscript 5 - A Report: Demonstration of Calpain in Monkey and Human Lenses	167-184
Conclusions	185-191

ACKNOWLEDGEMENTS

It is difficult to list all the people that have helped me get to this final phase of formal education. I have had so many superb teachers through the years. Two that come to mind are Bud Kasper my high school chemistry teacher, and Dr. Thomas Conover who first got me into the research lab.

I wish to thank the many reseachers at OHSU who have been missing equipment ever since I arrived, some of who are: Ted Acott, Westley Bullock, Howard Creamer, Jack Fellman, Walter Gabler, Richard Jones, Michael Litt, and Howard Mason.

I wish to thank the high school apprentices Na Lee, Loan Nguyen, and Mai Huong Tran for their help.

A special thanks to the members of my research advisory committee: Ted Acott, Walter Gabler, Jack Fellman, and Demetrios Regas. They took the time to listen, and made many useful comments.

I wish to acknowledge the generous support of the Tarter Trust Fellowship and the Marquam Hill Society for rent, food and travel.

I also wish to thank Dr. Jones who went out of his way to provide department funds to support me my first year. He also helped in other ways.

A special acknowledgement to my dear friend Ruth Anderson who I share a lab and many thoughts with, thank-you for always being there.

To all the members of my family who have always given me all the love and support possible, I dedicate this thesis.

Finally I want to thank my thesis advisor Thomas Shearer. He was never too busy to put away everything and talk, even if he had a class in 5 min. Dr. Shearer always made me feel that I was his colleague. He was always there to proof-read my 20th draft, drive to the Primate Center, turn off a gel when I was skiing, and to be a friend. I would do it all over again.

ABSTRACT

A single overdose of the trace mineral selenium, in the form of sodium selenite, caused dense nuclear cataract in young rats in 4 days time. Selenite is a potent sulfhydryl oxidant *in vitro*, but caused no detectable increase in lens protein disulfide following cataract formation. This suggested that selenite cataract was caused by a mechanism other than formation of disulfide linked protein aggregates.

During selenite cataract formation there was a 17 fold increase in the lens dry weight concentration of calcium occurring in the lens nucleus. This elevation of calcium was associated with proteolysis of soluble α and β-crystallin polypeptides, insoluble proteins, and membrane proteins in the lens nucleus. Purified calcium-dependant neutral protease (calpain) from rat lenses degraded lens proteins *in vitro* in a manner similar to lens proteins degraded *in vivo* during selenite cataract formation. This suggested that calpain may be responsible for increased protein degradation in selenite cataract.

Protein degradation in selenite cataract may cause formation of light scattering insoluble protein. Insoluble protein was elevated 5 fold in the cataractous lens nucleus. Concentrations of soluble β-crystallin aggregates decreased in the lens nucleus during cataract formation. Insoluble protein from the nucleus of cataractous lens contained mostly β and γ-crystallin polypeptides. Two polypeptides isolated from the cataractous insoluble protein were identified as partially degraded β-crystallin polypeptides. This suggested that proteolysis may have caused insolubilization of β-crystallin aggregates, resulting in formation of light scattering insoluble protein.

Calpain was also detected in young human lenses. These results suggested that proteolysis by calpain could also be a cause of cataracts in young human lenses.

Introduction

A single injection of selenite in young rats causes cataracts in 4 days. The goal of this thesis is to elucidate the biochemical mechanism of selenite cataract formation. The hypothesis tested is that cataract formation may be caused by proteolysis of lens proteins. In order to develop this hypothesis, the introduction is organized into the sections: normal lens, cataractous lens, calcium-dependant neutral protease (calpain), and the selenite cataract.

Normal Lens

The lens is a transparent nonvascularized tissue located directly behind the iris. The lens separates the aqueous humor of the anterior chamber from the vitreous humor in the posterior chamber. The functions of the lens are to focus incoming light on the retina, and protect the retina from UV radiation. The lens is encapsulated by a collagenous basement membrane. Attached to this lens capsule are the zonule ligaments suspending the lens. The ciliary body provides tension to the zonule ligaments, and changes the thickness and shape of the lens in response to autonomic control. This change in lens shape allows the eye to focus the image of both close and far objects by a process known as accommodation.

Structurally, the lens is composed of a single layer of mitotically active epithelial cells on the anterior surface, and differentiated fiber cells making up the bulk of the lens. Epithelial cells in the equatorial region continually elongate and differentiate into fiber cells. These newly differentiated fiber cells grow towards the lens bow. At the lens bow the fiber cells rotate and elongate towards the two poles of the lens, where they meet and form the Y sutures on the posterior and anterior poles. Throughout life the lens continues to grow by forming new layers of fiber

cells which cover over and compress the underlying older fibers.

The lens has been arbitrarily divided into the outer cortex and the nucleus in the center of the lens. The nuclear fibers are the oldest, and the outer cortical fibers the youngest. There is no loss of inner lens fibers with age. The fiber cells in the central nuclear region of the lens were formed *in utero* (1). The survival of these cells is unusual given the fact that fiber cells lose their nuclei and other organelles during differentiation, and the rate of protein synthesis in the nuclear region of the lens is low (2, 3). Interestingly, functional polyribosomes were recently found in the nuclear region of bovine and human lenses (4). Cells in the lens nucleus contain no nuclei, and such long term survival of RNA is remarkable.

Lenses contain one of the highest protein concentrations of any tissue. Lens protein concentrations increase with aging, but are approximately 35% of lens wet weight (5). This high protein concentration results in the large index of refraction necessary for focusing divergent light rays. Lenses are normally transparent and do not scatter incident light. Lack of light scattering by the lens is interesting because 1) the lens is composed of regions of differing refractive index: lens fiber cell membranes, and cytoplasm, and 2) the cytoplasm contains proteins which range in size from 10^5 to 10^8 daltons, which would be expected to cause both small and large particle scattering (6). Several hypotheses attempt to explain the lack of light scattering by the normal lens. Scattering does not occur possibly because the lens proteins adopt a spatial ordering and a paracrystallin state in the fiber cells. The summation of the phases of the scattered waves from proteins in this paracrystallin state would be essentially zero (6). Another hypothesis for lens transparency does not

require lens proteins to exist in a paracrystallin state. In this theory lens proteins packed in a high density would result in the fluctuation in the number of protein molecules over a dimension comparable to the wavelength of light to be small (7). As in the paracrystallin state, the end result would be a summation of scattered light waves equal to zero. Another hypothesis explains the low scatter from lens membranes. The thickness of phospholipid membranes may be such that the reflected waves at the top and bottom surfaces of the adjacent fiber cell membranes are almost 180° out of phase and cancel (6).

Approximately 90% of the dry weight of lenses is made up of the lens structural proteins called crystallins (8). These crystallins have been classified as α-, β_H-, β_L-, and γ-crystallins based on their elution during molecular sieve chromatography (5). α-crystallin is a protein aggregate with a molecular weight of 6 to 9 x 10^5 (9). This aggregate is composed of 4 major polypeptide subunits. These polypeptides are isolated by deaggregation in 6 M urea and separation by either ion exchange chromatography (10) or chromatofocusing (9). The four major polypeptides of α-crystallin all have a molecular weight close to 20,000. They are designated αA_1, αA_2, αB_1, and αB_2. The A and B refer to their pI's being either acid or basic. αA_2 and αB_2 are primary gene products, while the αA_1 and αB_1 are derived from αA_2 and αB_2 by post-translational changes such as deamination (9). The αA_2 and αB_2 polypeptides are very similar in primary structure, and probably were derived by gene duplication (9). During aging these α-crystallin polypeptides undergo slow truncation from the C-terminal ends of the molecules, forming a complex mixture of polypeptides in the α-crystallin aggregate (9). The truncation of α-crystallin polypeptides is believed to occur non-enzymatically. During aging lens crystallins show

greater heterogeneity due to post-translational modifications such as these.

The β-crystallins are a complex group of polypeptide aggregates. They consist of β_H-crystallin (approximately 150,000 molecular weight) and β_L-crystallin (approximately 50,000 molecular weight). Six different β-crystallin polypeptides have been identified as primary gene products (9). Many of these polypeptides are contained in both β_H and β_L-crystallin. The individual polypeptides of β-crystallins range in molecular weight from 10,000 to 60,000 depending on the species (9). Herbrink isolated 6 individual β-crystallin polypeptides by DEAE chromatography in 6 M urea (11). These polypeptides were named βB_1-βB_5 on their order of elution from the DEAE column. The sixth polypeptide eluted was named βA because of its acidic pI. The βB_3 polypeptide is also known as βB_p, because it is one of the principle soluble polypeptides of the lens (molecular weight = 27,000) (11). Determination of the amino acid sequence of βB_p has indicated that this polypeptide has close homology to a γ-crystallin polypeptide (9).

The γ-crystallins are isolated during molecular sieve chromatography of lens soluble protein as one or two peaks of approximately 20,000 molecular weight. γ-crystallins are unique because unlike other crystallins they exists as monomers. These low molecular weight crystallins are composed of at least seven homologous proteins with molecular weights of approximately 20,000, and isoelectric points between pH 7.1 and 8.1 (9). The structure of γ-crystallins are known in greater detail than the other crystallins, because γ-crystallin is easily crystallized. X-ray analysis has shown that bovine γ-II-crystallin has the highest internal symmetry of any protein yet studied (12). γ-II-crystallin is organized into C and N-terminal

globular domains in close proximity to one another interconnected by a short linear polypeptide chain. Each globular domain contains two motifs. The four motifs within the entire molecule show remarkable similarity. Each is folded in a pattern similar to a Greek key pattern found on ancient pottery (12). Some γ-crystallin polypeptides undergo phase transitions and decreases in solubility when the lens is cooled below body temperature. This leads to the cold cataract phenomenon, where the nuclear region of young lenses containing the highest concentration of γ-crystallin becomes cloudy when cooled (13). The phase transition of γ-crystallin is reversible, and these cataracts dissappear upon warming the lens back to body temperature.

In addition to the soluble crystallins, the lens also contains water insoluble proteins. These are isolated by centrifugation, and are divided into those proteins which will dissolve in 6 M urea (cytoskeletal proteins and insoluble crystallins), and the urea insoluble membrane proteins which will dissolve in sodium dodecyl sulfate (SDS) (5). Young lenses contain few insoluble crystallin polypeptides. However, during aging and cataract formation the number of insoluble crystallin polypeptides dramatically increases (14). Insoluble crystallin polypeptides will be described below in the section on cataract formation.

Intermediate sized filaments (vimentin), microfilaments (actin), and microtubules (tubulin) are found in the lens cytoskeletal fraction (15-17). Vimentin is contained in the cytoplasmic space, while actin is associated with lens fiber cell membranes (15). The function of the lens cytoskeleton remains unknown, however there are several hypotheses. The cytoskeleton may play a role in differentiation during lens fiber cell elongation, or it may confer flexibility to the fiber cells during accommodation (16). Lens

cytoskeleton may also be involved in protein synthesis (8). Interestingly, lens cytoskeletal proteins are lost during aging of lens fiber cells. This results in little or no actin or vimentin remaining in the nuclear region of human lenses (16). Whatever function the cytoskeleton plays in the lens, it is apparently most important in young lens cells.

Approximately 2% of total lens proteins are urea insoluble, SDS soluble proteins from plasma membranes (5). The best characterized of these membrane proteins is the 26,000 molecular weight main intrinsic protein (MIP). MIP comprizes 80 % of the intregral membrane protein of lenses, and is important because it may form the lens gap junctions (18). While the role of MIP in formation of the lens gap junction has not been positively proven (18), recent experiments in which MIP was reconstituted in liposomes suggests that MIP does act to form the intercellular channals of gap junctions (19). Morphological analysis of the lens fiber cell suggests that 30 - 60% of its plasma membrane may be involved in intercellular junctions. Cells from most other tissues contain fewer intercellular junctions. For instance, only 1-2% of the hapatacyte membrane is involved in intercellular junctions (18). Lens transparency may depend on the maintenance of these gap junctions so that nutrient, waste, and ion fluxes can take place in the avascular lens. Damage to MIP may lead to isolation of the lens fiber cell, and could contribute to cataractogenesis.

Energy metabolism in the lens exhibits many similarities to energy metabolism of the erythrocyte. It has been estimated that only 5 % of glucose metabolized by the lens passes through the citric acid cycle (20). The low activity of oxidative metabolism is due to the loss of mitocondria from lens fiber cells during maturation. Glycolysis alone appears capable of providing sufficient ATP production in the lens.

Lenses can be cultured in the presence of fluoroacetate or N_2 and maintain ATP levels, protein synthesis, and normal ion homeostasis (20). Reducing potential in the lens is generated primarily by the hexose monophosphate shunt (20). While some of the NADPH produced in the lens is used for the synthesis of new compounds, much of it is utilized to maintain compounds such as glutathione (GSH) in a reduced state (20).

Lenses contain a remarkably high concentration of endogenously synthesized GSH (20). This GSH is maintained almost entirely in a reduced state by the enzyme GSH reductase which utilizes NADPH for reducing equivalents (20). GSH prevents oxidative damage and maintains the lens in a reduced state by several mechanisms. GSH may directly reduce intermolecular protein disulfide bonds or mixed disulfide bonds formed between GSH and protein sulfhydryl (21). The selenium containing enzyme GSH peroxidase breaks down hydrogen peroxide and membrane damaging lipid hydroperoxides utilizing GSH as an electron doner (22). GSH may also serve as a detoxifying agent in the lens by conjugating with electrophilic toxins by action of the enzyme glutathione-S-transferase (22). Other antioxidative enzymes in the lens include catalase and superoxide dismutase. However, catalase is not as important as GSH peroxidase in breaking down hydrogen peroxide in the lens because it is in lower activity, and has a higher K_m for hydrogen peroxide than GSH peroxidase (20).

Cataractous Lenses

Cataract is defined as any opacity in the lens of the eye interfering with vision. About 60 percent of people between ages of 65 and 74 show some signs of cataract. Cataracts are the third leading cause of legal blindness

in the U.S., with approximately 3.3 million people in the U.S. alone showing visual impairment due to cataracts (23). At the present time, the only effective method of treating cataracts is removal of the lens. This is an extremely common operation, with the cost of cataract extractions in the United States per year totaling 1.2 billion dollars (23). While a majority of these operations are successful in restoring useful vision, there are a small percentage of patients that do not have their sight restored by these operations. If it were possible to slow the progression of cataract formation by 10 years, the number of lens extractions could be decreased by 45%. This would result in a estimated savings of 530 million per year in medical cost in the United States alone (23).

A variety of agents alter the homeostasis of the lens, but the end result is the same - cataract formation. Cataracts may be caused by aging, diabetes, light, radiation, toxins, and inherited disorders.

Senile Cataract. The most common form of human cataract is senile cataract formed in aged lenses. The majority of lens research has been directed at finding the cause of the senile cataract. Accumulation of oxidative damage in the lens appears to be the most likely cause.

Senile cataractous lenses contain high molecular weight (HMW) protein aggregates and insoluble protein linked by intermolecular disulfide bonds (24). These disulfide bonded aggregates are large enough to scatter light and cause opacity (25). The aggregates from cataractous human lens are a complex mixture of proteins containing all three classes of lens crystallins (26). In addition to the crystallin polypeptides, the HMW protein contains a 43,000 molecular weight extrinsic membrane protein component (25). This protein may serve as a nucleation site on the fiber cell membrane for

attachment of lens crystallins by disulfide linkage.

Several mechanisms may cause increased disulfide formation in senile cataract. Cataractous lenses invariably contain less GSH than normal lenses (27). Loss of GSH could be caused by a decreased rate of synthesis, increased breakdown, net efflux, or decrease of lens GSH reducing capability. Aged human lenses exhibit a decrease in hexokinase activity (28). This is believed to limit the lens hexose monophosphate shunt activity, and lead to a decreased rate of lenticular NADPH production (28). Lower NADPH production in turn leads to a decreased ability to reduce oxidized GSH. Without high levels of GSH the lens looses its capability to reduce protein disulfides, and break down oxidants. Oxidants present in the lens which may cause disulfide formation in the compromized aged lens include hydrogen peroxide, hydroxyl radical, superoxide anion, and singlet oxygen (29). However, the relative role these oxidants play in causing disulfide formation is unknown (20).

Sugar Cataract. Another mechanism causing cataract formation is a change in the osmotic pressure of the lens. Diabetic patients have an increased risk of developing cataracts (30). Streptozotocin-induced diabetic rats (30), and rats given diets high in galactose also develop cataract (31). In these cataracts, glucose or galactose taken up by the lens is reduced by aldose reductase to the respective polyol and osmotic swelling of lens fibers occurs. This is followed by permeability changes in the lens fiber membrane leading to uptake of sodium and release of potassium, decrease in protein synthesis, and formation of opacity (32, 33). Experimental sugar cataracts can be prevented by inhibitors of aldose reductase (30). Inhibitors of aldose reductase are being examined as possible theraputic agents in man (23).

Other Cataracts. Doses of approximately 200 rads of x-rays or γ-rays, and approximately 75 rads of neutron radiation may lead to cataract formation in man (34). X-rays are commonly used to induce experimental cataracts in animals. A single 1000-1800 rad dose to the eye of rats or rabbits will cause severe cataract involving the entire lens in 8-16 weeks (35, 36). The site of initial insult by radiation appears in the lens epithelium. By 4.5 hours following X-ray exposure, abnormalities appear in the lens epithelium (37). In the following weeks, lenses then exhibit changes similar to the senile and sugar cataracts. GSH is decreased, high molecular weight disulfide linked protein is formed (38), Na/K ratios are altered and protein synthesis is decreased (39). While the initial site of damage is in the epithelium, how this ultimately leads to the other lenticular changes and opacity is unknown.

X-rays are not the only form of radiation known to induce cataracts. Cataracts can also be induced by ultraviolet light and infrared radiation. Exposure of experimental animals to UV radiation near 300 nm will cause cataracts (40). Tryptophan residues absorb the most UV radiation in the lens. Excited tryptophan residues undergo photolysis to produce various kynurenine derivatives which may act as photosensitizers for the production of singlet oxygen (40). Singlet oxygen may then react to cross-link proteins. Infrared radiation is believed to cause cataracts by inducing heating in the lens (41). Glassblowers cataract has been recognized in individuals after many years of exposure to intense heat.

Many toxic agents or drugs also induce cataracts. Agents can induce cataracts by acting either as photosensitizers or interfering with the normal metabolism of the lens. Examples are: naphthalene, heptachor, iodoacetate, 2,4-dinitrophenol, and the cholesterol synthesis inhibitor

triparanol (42). Systemic and topical treatment with glucocorticoids may also induce cataract in man (43).

Cataracts are also caused by congenital defects. Hereditary cataracts are usually transmitted as an autosomal dominant trait and represent a major cause of childhood blindness (23). One genetically transmited disease associated with cataract formation is retinitis pigmentosa (44). Many animal models of hereditary cataracts are known (45). Rodent models most closely examined include the Fraser, Nakano, and Philly mouse cataracts, and the RCS rat cataract. Rodent hereditary cataracts are good models to study human cataract formation because these lenses undergo changes similar to those occurring during human cataract formation (46, 47). While the specific cause of most rodent hereditary cataracts is unknown, recent evidence suggests that the Philly mouse cataract may be caused by the absence of a 27,000 molecular weight β-crystallin polypeptide (48).

Role of Calcium and Proteolysis in Cataract Formation

As in other tissues, the lens epithelium and fiber cells contain an ATP driven calcium pump activated by calmodulin (49). This pump maintains a low intracellular concentration of free calcium in fiber cells at approximately 30 μM (50). This is a much higher free intracellular calcium concentration than found in most cells, but does demonstrate that calcium is extruded from the lens against a concentration gradient.

Extensive evidence from human and experimental animal cataracts shows that lens calcium is elevated during cataract formation. The mean calcium concentration of cataractous human lenses was 2-13 times higher than the mean calcium concentration of normal lenses (51). Cataractous human lenses also contained increased calcium bound to membranes and insoluble proteins

(52). In rabbit lenses with x-ray induced cataract, lens calcium concentrations were elevated over 100 times normal values (35). Lens calcium was elevated by 100 % one week prior to maturation of the x-ray cataract, indicating that calcium elevation may be a cause rather than a result of cataract. Lenses with x-ray cataracts also exhibited elevated calcium specifically in opaque regions of the lens, while clear regions contained normal concentrations of calcium. Two hereditary mouse cataracts were also shown to contain elevated calcium (46,53). Incubation of whole rabbit lenses in medium containing elevated calcium caused cataract formation, further implicating lens calcium elevation as a cause of cataract (54).

Elevated calcium may contribute to cataract formation by several different mechanisms. Calcium may diminish lens protein synthesis, inhibit Na/K-ATPase activity, and disrupt lens membrane integrity (55). Calcium may bind directly to lens proteins and cause formation of HMW aggregates (56,57). Elevated calcium may also indirectly cause protein aggregation by inducing proteolysis of lens crystallins. Proteolyzed lens crystallins may then lose their native conformation and form abnormal light scattering aggregates.

Extensive evidence supports the idea that increased proteolysis is associated with cataract. The hereditary Nakano mouse cataract showed an increase in degraded lens polypeptides below 22,000 molecular weight, a total loss of the main intrinsic membrane polypeptide, and a rise in a 22,000 M.W. membrane polypeptide (47). The Fraser mouse cataract showed a loss of β_H crystallin, and new polypeptides were present in the lens, which may have been formed by proteolysis (58). During formation of the galactose cataract in rats, crystallin polypeptides were degraded in the soluble and

insoluble protein fractions (59). Low molecular weight polypeptides (4,000-8,000 M.W.) were also found in human nuclear cataract, and these fragments were absent in transparent lenses (60). A major fraction of the disulfide linked HMW protein from cataractous human lenses was composed of low molecular weight polypeptides of approximately 10,000 molecular weight (61). The 10,000 M.W. polypeptides were likely derived from degradation of crystallin polypeptides and 43,000 M.W. polypeptide (62,63). The 10,000 molecular weight polypeptides comprize a large portion of the HMW protein of cataractous lenses. Presence of such large amounts of degraded lens proteins in cataractous aggregates suggests that proteolysis could be as important in formation of light scattering aggregates as formation of disulfide cross links.

Calcium Dependent Neutral Protease (Calpain)

Evidence suggests that the protease calpain may contribute to proteolysis observed during cataract formation. Calpains are calcium-dependent proteolytic enzymes found ubiquitously in mammalian and avian cells (64). Calpain is unique from other proteases because it requires both calcium and a sulfhydryl reducing agent for activity. Two types of calpain have been found: type 1 requires low (μM) calcium for activation, and type II requires high (mM) calcium for activation. Most tissues examined also contain calpastatin, an endogenous inhibitor of calpain of varying molecular weight, depending on the tissue (64). Calpastatin modulates calpain activity *in vivo*, and its concentration varies between different tissues (64). The relative concentration of calpastatin in different tissues is believed to directly determine the extent of calpain

activation (64).

Calpains may act physiologically to accomplish specific functions rather than by acting as general proteases (65). Proteins degraded by calpain are usually degraded only to large fragments, and in most tissues, calpain has a limited number of substrates. Calpain can degrade neurofilaments, C-protein, desmin, filamin, vimentin, epidermal growth factor receptor, and steroid hormone receptor (65,66). Calpain may play a pathological role during disease, possibly leading to pathological proteolytic changes in muscular dystrophy and myocardial infarction (67,68).

Calpains have the following properties: M.W. 100,000-120,000, pH optimum = 7.5, not activated by magnesium, require a SH reducing agent for full activity during isolation, inhibited by sulfhydryl alkylating reagents and protease inhibitors of microbial origin (luepeptin and E 64), and not inhibited by the serine protease inhibitor phenylmethylsulfonyl fluoride (65, 69). Calpain contains a domain similar in primary structure to papain and other sulfhydryl proteases, and a calcium binding domain similar to the primary structure of calmodulin and other calcium binding proteins (70). This suggests that calpain may have evolved by fusion of genes for a thiol protease and a calcium binding protein.

Both our laboratory and others have shown calpain to be present in lens tissue. Proteolysis could be induced by incubating whole lenses or lens homogenates in elevated calcium (71-74). Whole rat and rabbit lenses incubated with elevated calcium showed proteolysis which could be inhibited by leupeptin (71,72). The proteolysis in the incubated rat lenses was similar to proteolysis observed in opaque mouse lenses cultured in low glucose medium (71). The cytoskeletal protein vimentin was degraded in bovine lens homogenates incubated with calcium (73, 74). Calpain was also

postulated to be responsible for the loss of vimentin occurring in lens fiber maturation.

Lens calpain has also been purified. Purified bovine lens calpain partially degraded α-crystallin (75). Subsequent research showed that purified bovine lens calpain degraded lens actin and vimentin, showed regional distribution in the lens, and was apparently modulated by the presence of lens calpastatin (76,77). Calpain was also demonstrated in mouse lens, and the enzyme was inactivated following development of Nakano mouse hereditary cataract (78). Importantly, calpain inactivation occurred following initiation of lens proteolysis, not before. Results also suggested that mouse lens calpain may degrade α- and β-crystallin polypeptides during cataractogenesis. Thus, calpain is present in lenses and may be reponsible for normal proteolysis occurring with maturation, and increased proteolysis during cataract formation.

Review of Selenium Chemistry and Selenite Cataract.

Cataract induced by the trace mineral selenium has become a useful model for studies on the role of calcium, proteolysis, and calpain in cataract formation.

Chemistry of Selenium. Selenium (Se) is located between sulfur and tellurium in the Periodic Table, and has been classified both as a metal and a nonmetal (79). In many respects selenium mimics the chemistry of sulfur. For instance, selenium exists in the same oxidation states as sulfur and forms oxides analogous to those of sulfur: selenide (Se^{2+}), selenite (SeO_3^{2+}), and selenate (SeO_4^{2+}). The reaction of selenite with thiol groups is the most important path by which inorganic selenium can be

incorporated into living systems (80). Through a complex series of reactions selenite forms a compound known as a selenotrisulfide (RS-Se-SR) (I):

$$4\ RSH + H_2SeO_3 \longrightarrow RS\text{-}Se\text{-}SR + RSSR + 3\ H_2O \qquad (I)$$

While selenotrisulfides can form by reaction with sulfhydryl groups of proteins (81), the most common intracellular selenotrisulfide is likely formed by reaction with 4 GSH molecules (82). Selenotrisulfide undergoes several different possible reactions. GSH reductase can reduce selenotrisulfide to selenopersulfide (GSSeH) (II), or excess GSH can non-enzymatically form selenopersulfide (III):

$$GSSeSG + NADPH + H^+ \xrightarrow{\text{GSH reductase}} GSH + GSSeH + NADP^+ \qquad (II)$$

$$GSSeSG + GSH\ (\text{excess}) \longrightarrow GSSG + GSSeH \qquad (III)$$

The selenopersulfide can then decompose giving elemental selenium and GSH (IV), or it can form the selenide ion which is methylated by the liver to dimethyl selenide or trimethylselenonium ion by transfer of methyl groups from S-adenosyl-methionine (V):

$$GSSeH \longrightarrow Se^0 + GSH \qquad (IV)$$

$$GSSeH \longrightarrow H_2Se \xrightarrow{SAM} CH_3\text{-}SeH \xrightarrow{SAM} CH_3\text{-}Se\text{-}CH_3 \xrightarrow{SAM} CH_3\text{-}\overset{\displaystyle +}{\underset{\displaystyle CH_3}{Se}}\text{-}CH_3 \qquad (V)$$

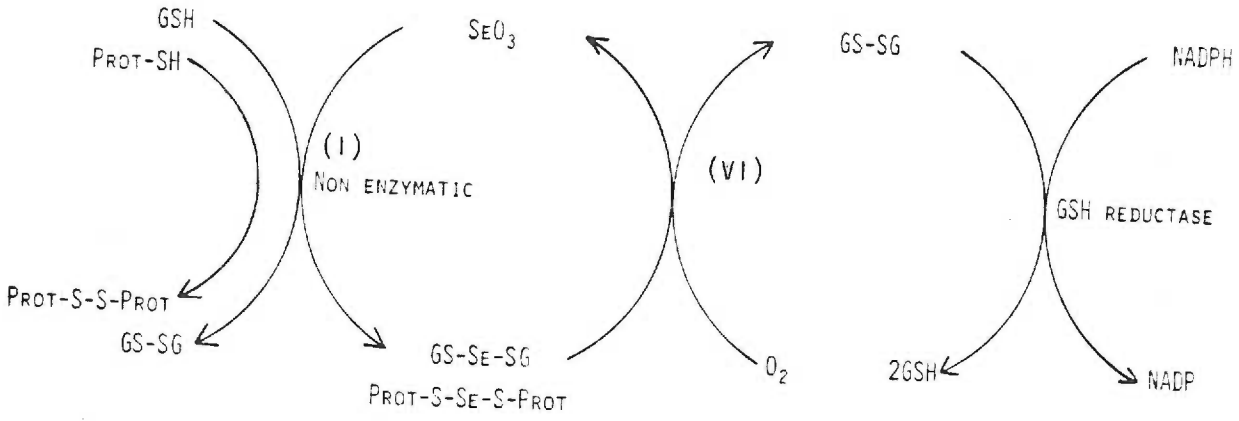
Dimethyl selenide is volatile and is excreted by the lungs, while trimethylselenonium is excreated in the urine (83).

In 1958 Tsen and Tappel found that selenite catalytically oxidizes the sulfhydryl groups of glutathione and cysteine to disulfides in the presence

of oxygen and proposed the following reaction (VI) (84):

$$2\ OH^- + GS\text{-}Se\text{-}SG + O_2 \longrightarrow GSSG + SeO_3^{-2} + H_2O \quad (VI)$$

Coupling reactions I and VI with the reaction catalyzed by glutathione reductase gives the following set of reactions:



In this scheme selenite causes the catalytic oxidation of cellular NADPH and formation of protein disulfide bonds. This catalytic oxidation of NADPH, depletion of GSH, and formation of protein disulfide may at least partially explain the high toxicity of selenium.

Selenium is also an essential trace mineral for man and a variety of mammals and birds. The basis of selenium as an essential mammalian micronutrient was discovered when selenium was shown to be a component of glutathione peroxidase. Glutathione peroxidase contains an essential selenocysteine residue (79). Because of the role of glutathione peroxidase, selenium is widely viewed as an antioxidant. This antioxidant property of selenium has been used to explain the anticarcinogenic effect of selenium on experimentally induced tumors in animals (85).

Several disease syndromes of domestic and laboratory animals (white muscle disease, exudative diathesis, and necrotic liver degeneration) were shown to be prevented by administration of selenium (79). Because Oregon,

as well as other geographical areas, contain low selenium concentrations in the soil, selenium is routinely added to animal feeds. The daily dietary requirement of adult humans is about 60 µg selenium (79). The average North American diet (including Oregon) supplies a total of 80-240 µg selenium per day. This is slightly above the estimated daily requirement, and much lower than the estimated toxic daily dose which is 10 times higher. In man selenium deficiency has been linked to cardiomyopathy (86).

Selenite Cataract.

1. Conditions for cataract. In 1977 Ostadalova et al. described a new symptom of selenium toxicity (87). A single subcutaneous injection of 20µmoles sodium selenite/kg body weight to 10 day old suckling rats caused nuclear opacities to develop in only four days. The treated animals appeared normal, except for the appearence of bilateral nuclear cataracts. Injection of 2 month old rats with 20µmole/kg body weight sodium selenite induced mortality in 11 out of 15 rats, but caused no cataracts to form in the surviving rats. Thus, older rats are more suseptible to selenite toxicity, but do not develop cataracts. The cataractogenic action was limited to selenite, because injection of selenomethionine did not induce cataracts (88).

Rats under 5 days of age, and over 14 days of age exhibited a decrease in the incidence of selenite cataract (89). This sensitivity of the young rats to selenite cataractogenesis may be due to differential uptake of selenite into the lenses of young and old rats. Two week and 10 week old rats were given 30 µmole/kg injections of sodium selenite labeled with ^{75}Se

(90). At 4 hours post-injection the 2 week old rat lenses took up 500 times the amount of radioactivity per gram lens tissue than did the 10 week old rats. Therefore, older rats may not be suseptible to selenite cataractogenesis, because they do not take up enough selenite into their lenses following selenite injection. The increased uptake of selenium into young rat lenses was explained both by a greater uptake of selenite from surrounding solutions (90), and a decreased ability of young rats to methylate and excrete selenium (83).

It must be emphasized that selenite induced cataracts serve only as a model to study the mechanisms of cataract formation. There is no evidence that selenium toxicity in humans causes cataracts. The 20 μmole/kg injection of sodium selenite is over 1000 times the amount of selenium contained in the daily North American diet.

2. Mechanism of selenite induced cataract.

Results summarized below have lead to the following hypothesis. Selenite enters the lens and damages the lens epithelial cells. Because of the lower metabolic activity of the inner regions of the lens, the nuclear lens fibers are dependant upon intact epithelium to maintain homeostasis. The nuclear fibers either become leaky to calcium ion, or the activity of the calcium ATPase pump decreases. Nuclear calcium concentrations increase and calcium dependant neutral protease (calpain) is activated. Calpain degrades lens crystallins, membrane proteins, and cytoskeleton. Degraded crystallins undergo aggregation/insolubilization and scatter light leading to opacity.

Several pieces of evidence suggests that lens epithelial cells are the initial site of attack by selenium. The rate and distribution of

selenite uptake following a cataractous dose of selenite was examined (91). Ten day old Sprague-Dawley rat pups were injected with 20μmole/kg sodium selenite tagged with ^{75}Se. Lens selenite uptake peaked at 12-24 hours following injection, and the majority of selenite was associated with lens proteins. When the regional distribution of selenium uptake in the lenses were determined, very little selenium was found in the lens nucleus where the opacity formed. Autoradiography also showed that most Se^{75} was in the outer cortex (unpublished results). Therefore, it was postulated that binding of selenium to the proteins of the epithelial-cortex region of the lens may have lead to the formation of the central nuclear cataract.

The hypothesis that the initial site of selenite damage is in lens epithelial-cortical region was also supported by slit lamp and histological examinations. Rat pups open their eyes on day 14 of age. Injection of 20 μmole/kg sodium selenite on day 14 of age allows both cataract formation, and detailed slit lamp examination of lenses during cataractogenesis (93). Selenite treated rat lenses went through 4 distinct stages during cataract formation. During stage 1 (13-24 hours post-injection), a posterior subcapsular cataract (PSC) formed. This PSC appeared as a thin film of haziness on the posterior side of the lens, and was composed of swollen lens fibers which sometimes contained vacuoles. Since these affected fibers were located on the outer surface of the lens, these data support the hypothesis that selenite first damages the outer regions of the lens.

Further slit lamp observations suggested that following damage to the outer region of the lens, successively deeper regions of the lens become altered. Beginning at stage 2 (24-72 hours post-injection), the PSC declined, and a refractile shell and swollen fibers formed in the cortex surrounding the nuclear region. During stage 3 (3-5 days), the

characteristic dense nuclear cataract formed, and the swollen fibers became more extensive. In the last stage of the cataract (5-10 days), some of the nuclear cataracts became more opaque and angular. It must be emphasized that most alterations of the inner regions of the lens occurred after lens selenium concentrations started to return to normal. This again suggested that selenium was not directly effecting the inner lens regions, but that damage to the outer regions caused alterations deeper in the lens.

Further detailed histological studies using the light microscope were carried out on selenite cataractous lenses (93). These experiments were an attempt to relate histologic changes to the slit lamp changes described above. During stage 1 extensive vacuolization was evident in the lens bow that spread throughout the posterior subcapsular region. This confirmed the hypothesis made during slit lamp observations that the PSC was caused by vacuole formation (92). Thin sections of lenses during stage 2 stained more densely with basophilic dyes in the region corresponding to the refractile shell. This increase in staining, and the appearence of a refractile shell may have been due to a change in the protein concentration in this region. Changes in the distribution of water are known to occur during selenite cataract formation (94). During stage 3, when the dense nuclear cataract formed, extensive histological alterations were present in the lens nucleus. The peripheral nucleus contained well defined fiber cell membranes, but these membranes contained clumped cytoplasm. The cell damage in the central nuclear region containing the opacity was more extensive. The central nuclear region contained dense particles, but no outlines of fiber cells could be seen. These dense particles were postulated to contain fiber cell membrane fragments and light scattering insoluble protein aggregates.

Selenite cataractous lenses were also examined by transmission electron microscopy (95). There were few changes seen in the cortex during the formation of the selenite cataract, but the opaque nucleus was strikingly different. Control nucleus contained fiber cells closely apposed and filled with featureless cytoplasm, whereas peripheral nucleus of cataractous lenses contained enlarged irregularly shaped fibers. These irregular fibers contained electron opaque cytoplasm lying along the cell membranes. This electron opaque cytoplasm was also postulated to be aggregated insoluble protein responsible for light scatter. The central nuclear region was not characterized because this region was difficult to section.

Recently, the hypothesis that selenite causes nuclear cataracts by damaging the outer regions of the lens was further supported by examination of lens epithelial cells (96). Cataracts were induced in 14 day old rats and lens capsule epithelium was removed and placed flat on microscope slides. Only 5-6 hours following selenite injection, mitosis was decreased by 72% in the germinative zone of epithelial cells compared to control lenses. The percentage of germinative epithelial cells in prophase were also decreased 59% by selenite injection. This suppression of cell mitosis was taken as evidence that selenite damages lens epithelium.

In addition to causing changes in the deeper regions of the lens, it was postulated that damaged epithelial cells formed abnormal lens fibers. These damaged lens fibers may be responsible for a secondary cortical cataract that forms 15-30 days following selenite injection (96).

Early work on the biochemical mechanism of selenite cataract examined the hypothesis that oxidation was the cause of cataract. Selenite was postulated to catalytically oxidize lens sulfhydryl and lead to the formation of light scattering aggregates. Bhuyan et al. (97) induced

selenite cataracts in 10 day old Sprague-Dawley rat pups by single or twice weekly injections of selenite. Lenses of rats receiving a single selenite injection underwent a rapid increase in lens insoluble protein, loss of water soluble protein, decrease in GSH, and decrease in soluble protein sulfhydryl. Twice weekly injections of selenite for 3-6 weeks caused hypermature cataracts to form with both nuclear and cortical involvement. Hypermature cataracts were similar to those seen following a single selenite injection, except the changes listed above were more extensive. These results suggested that selenite was oxidizing lens sulfhydryl and forming disulfide linked aggregates (97).

Lens catalase, superoxide dismutase, glutathione peroxidase, malondialdehyde, and concentrations of hydrogen peroxide in the aqueous humor were measured following selenite injection (98). Catalase and superoxide dismutase were decreased in selenite cataract, while glutathione peroxidase remained unchanged. Both the concentration of hydrogen peroxide, and the amount of lipid peroxidation, as measured by malondialdehyde formation, also increased. These results also suggested that increased oxidation may be the cause of selenite cataract.

Bunce and Hess (99) induced selenite cataracts in rats and measured a decreased lens growth rate, rapid increase in lens insoluble protein, and decreases in GSH, NADPH, and soluble protein sulfhydryl. They also concluded that selenite was catalytically oxidizing lens sulfhydryl and forming disulfide linked aggregates. It was suggested that the decrease in lens NADPH was caused by utilization of NADPH to reduce disulfide formed by selenite. Another interesting finding was that unlike many other cataracts, the concentrations of sodium and potassium was not altered in selenite nuclear cataract.

The simultaneous injection of other trace minerals with selenite also suggested that selenite may interact with lens sulfhydryl (100). Injection of mercury, silver, and arsenic with selenite prevented cataract formation. Since these other trace metals are known to bind to sulfhydryl groups, it was postulated that they protected lens sulfhydryl from selenite catalyzed oxidation (100).

These early reports concluded that disulfide linked aggregates may be the cause of selenite cataract (97, 99). These conclusions were based on loss of soluble protein sulfhydryl per gram lens wet weight. However, decreases in lens sulfhydryl may have been due to loss of soluble protein from lenses, and disulfide was not measured. Subsequent measurement of lens protein sulfhydryl and disulfide per gram lens protein detected no increase in protein disulfide in selenite cataract (101). It was concluded that if selenite caused oxidation in selenite cataract, it was restricted to a small portion of lens proteins, and that disulfide formation was not responsible for formation of light scattering aggregates.

The significance of the GSH decrease during selenite cataract formation was also examined. Buthionine sulfoximine (BSO), an inhibitor of glutathione synthetase, was given to young rats (101). BSO caused a greater loss of lens GSH than did selenite treatment, but no cataracts were observed. This suggested that GSH loss was not exclusively the cause of the selenite cataract (101).

Since above results suggested that disulfide formation was not a cause of opacity in the selenite cataract, the possible role of calcium was examined. Selenite caused lens calcium concentrations to increase 5 fold at 4 days post-injection (102, 103). Calcium levels were also elevated before cataract formation, suggesting that calcium elevation could be a cause, and

not a result of selenite cataract. The greatest concentrations of calcium were also found in the lens nucleus containing the opacity (104).

Lens proteins were examined during selenite cataract formation (104). Selenite caused proteolysis specifically in the lens nucleus containing the greatest calcium elevation (104). We posulated that calcium may induce lens proteolysis by activating calpain. This hypothesis was tested by incubating rat lens homogenates with calcium (104). Incubation with calcium induced proteolysis similar to proteolysis observed in selenite cataract. Further evidence that calpain may have caused proteolysis in selenite cataract was provided by purification of calpain from rat lenses (105). Lens proteins degraded by purified calpain showed some similarities to lens proteins degraded in selenite cataract (105). This also suggested that calpain was activated during selenite cataract formation.

Proteolysis may be the cause of opacity in the selenite cataract. No soluble HMW protein aggregates were found in selenite cataractous lenses (105). This suggested that the light scattering material may be in the insoluble fraction. Soluble β-crystallins were decreased in the lens nucleus following cataract formation (104). These β-crystallins may have become insolubilized, explaining the large increase in the amount of insoluble protein of cataractous lenses (97, 99, 101, 104). Characterization of the insoluble protein supported this hypothesis, because a major portion was found to be composed of β-crystallin polypeptides (106). When these insoluble β-crystallin polypeptides were examined, two of them were identified as proteolytic products of larger soluble β-crystallin polypeptides (106). Finding proteolytic fragments in the cataractous insoluble protein supported the hypothesis that proteolysis could have lead to its production.

In conclusion, selenite may cause cataracts not by forming disulfide linked aggregates as first believed, but by inducing proteolysis. This proteolysis may then cause lens crystallin polypeptides to non-covalently aggregate and become insoluble. This insoluble protein may then scatter light and cause opacity.

References for Introduction

1. Marshall, J., Beaconsfield, M., and Rothery, S. (1982). The anatomy and development of the human lens and zonules. Trans. Ophthal. Soc. U.K., 102,423-440.
2. Young, R.W. and Fulhorst, H.W. (1966). Regional differences in protein synthesis within the lens of the rat. Invest. Ophthalmol. Vis. Sci. 5, 288-297.
3. Wannemacher, C.F. and Spector, A. (1968). Protein synthesis in the core of calf lenses. Exp. Eye Res. 7, 623-625.
4. Ozaki, L., Jap, P., and Bloemendal, H. (1986). Protein Synthesis in bovine and human nuclear fiber cells. Exp. Eye Res. 41, 569-575.
5. Bloemendal, H. (1982). Lens Proteins. CRC Crit. Rev. Biochem. 12, 1-39.
6. Duncan, G. and Jacob, T.J.C. (1984). The lens as a physicochemical system. in The Eye, 3rd edition (Ed. Davson, H.), pp. 159-206. Academic Press, New York.
7. Benedek, G.B. (1971). Theory of transparency of the eye. Applied Optics. 10, 459-473.
8. Bloemendal, H. (1985). Lens research: from protein to gene. Exp. Eye Res. 41, 429-448.
9. Harding, J.J. and Crabbe, M.J.C. (1984). The lens: development,

proteins, metabolism and cataract. in The Eye, 3rd edition (ed. Davson, H.), pp.207-492. Academic Press, New York.

10. van Kleef, F.S.M., Nijzink-Maas, M.J.C.M., and Hoenders, H.J. (1974). Intracellular degradation of α-crystallin. Fractionation and characterization of degraded αA-chains. Eur. J. Biochem. 48, 563-570.

11. Herbrink, P., van Westreenen, and H. Bloemendal. (1975). Further studies on the polypeptide chains of β-crystallin. Exp. Eye Res. 20, 541-548.

12. Lindley, P.F., Narebor, M.E., Summers, L.J., and Wistow, G.J. (1985). The structure of lens proteins. in The Ocular Lens. Strucure, Function and Pathology. (ed. Maisel, H.) pp. 123-167. Marcel Dekker, Inc. New York.

13. Benedek, G.B., and Clark, J.I. (1980). Light scattering in the lens: Phase transition and opacification. In Red Blood Cell and Lens Metabolism, (ed. Srivastava, S.K.), pp.431-434. Elsevier North Holland, Inc., New York.

14. Li, L-L, Roy, D., and Spector, A. (1986). Changes in lens protein in concentric fractions from individual normal human lenses. Curr. Eye Res. 5, 127-135.

15. Ramaekaekers, F.C.S., Poels, L.G., Jap, P.H.K., and Bloemendal, H. (1982). Simulaneous demonstration of microfilaments and intermediate-sized filaments in the lens by double immunofluorescence. Exp. Eye Res. 35, 363-369.

16. Maisel, H. and Ellis, M. (1984). Cytoskeletal proteins of the aging human lens. Curr. Eye Res. 3, 369-381.

17. Piatigorsky, J. (1975). Lens cell elongation in vitro and microtubules. Ann. N.Y. Acad. Sci. 253, 333-347.

18. FitzGerald, P.G., Bok, D., and Horwitz, J. (1985). The distribution of the main intrinsic membrane polypeptides in ocular lens. Curr. Eye Res. 4, 1203-1218.

19. Gooden, M.M., Takemoto, L.J., and Rintoul, D.A. (1985). Reconstitution of MIP26 from single human lenses into artificial membranes. I. Differences in pH sensitivity of cataractous vs. normal human lens fiber cell proteins. Curr. Eye Res. 4, 1107-1115.

20. Spector, A. (1985). Aspects of the biochemsitry of cataract. in The Ocular Lens. Structure, Function, and Pathology. (ed. Maisel, H.) pp. 405-438. Marcel Dekker, Inc. New York.

21. Augusteyn, R.C. (1979). On the possible role of glutathione in maintaining human lens protein sulfhydryls. Exp. Eye Res. 28, 665-671.

22. Rathbun, W.B. (1981). Glutathione: biosynthesis and functions in the lens. In Symposium on the lens. (ed. Regnault, F.) pp. 38-61. Excerpta Medica, Amsterdam.

23. Report of the cataract panel. (1982). Executive Summary. Vision Research. A National Plan. 1983-1987. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health. Publ. No. 82-2469.

24. Spector, A. and Roy, D. (1978). Disulfide-linked high molecular weight protein associated with human cataract. Proc. Natl. Acad. Sci. U.S.A. 75,3244-3248.

25. Spector, A. (1984). The search for a solution to senile cataracts. Proctor Lecture. Invest. Ophthalmol. Vis. Sci. 25, 130-146.

26. Takemoto, T.J., Hansen, J.S., and Horwitz, J. (1984). High molecular weight aggregates from human cataracts: characterization by Western blot analysis. Biochem. Biophys. Res. Comm. 122, 1028-1033.

27. Rathbun, W.B. (1986). Activity of glutathione synthesis enzymes in the rhesus monkey lens related to age: a model for the human lens. Curr. Eye Res. 5, 161-166.22.

28. Cheng, H-M. and Chylack, L.T., Jr. (1980). Thiol oxidation in the crystallin lens. 1. The rate-limiting role of hexokinase in aging rat and human lenses. Invest. Ophthalmol. Vis. Sci. 19, 522-528.

29. Bhuyan, K.C. and Bhuyan, D.K. (1984). Molecular mechanism of cataractogenesis: III. Toxic metabolites of oxygen as initiators of lipid peroxidation and cataract. Curr. Eye Res. 3, 67-81.

30. Beyer-Mears, A. and Cruz, E. (1985). Reversal of diabetic cataract by sorbinil, an aldose reductase inhibitor. Diabetes. 34, 15-21.

31. Hu, T-S., Merola, L.O., Kuwabara, T., and Kinoshita, J.H. (1984). Prevention and reversal of galactose cataract in rats with topical sorbinil. Invest. Ophthalmol. Vis. Sci. 25, 603-605.

32. Kinoshita, J.K. (1974). Mechanisms initiating cataract formation. Invest. Ophthalmol. Vis. Sci. 13, 713-724.

33. Kador, P.F., Zigler, J.S., Kinoshida, J.H. (1979). Alterations of lens protein synthesis in galactosemic rats. Invest. Ophthalmol. Vis. Sci. 18, 696-702.

34. Lerman, S. (1980). Radient energy and the eye. Macmillan, New York.

35. Hightower, K., Giblin, F.J., and Reddy, V.N. (1983). Changes in the distribution of of lens calcium during development of x-ray cataract. Invest. Ophthalmol. Vis. Sci. 24, 1188-1193.

36. Kodama, T., Reddy, V.N., Giblin, F., Kinoshita, J.H., and Harding, C. (1983). Scanning electron microscopy of x-ray-induced cataract in mice on normal and galactose diet. Ophthmic. Res. 15,324-333.

37. Broglio, T.M., and Worgul, B.V. (1982). The lens epithelium and radiation cataract. IV. Ultrastructural studies of interphase dealth in the meridional rows. Virchows Arch (Cell Pathol) 39,49-57.

38. Giblin, F.J., Chakrapani, B. Reddy, V.N. (1978). High molecular weight protein aggregates in x-ray-induced cataract. Exp. Eye Res. 26, 507-519.

39. Garadi, R., Foltyn, A.R., Giblin, F.J., and Reddy, V.N. (1984). Protein synthesis in x-irradiated rabbit lens. Invest. Ophthamol. Vis. Sci. 25,147-152.

40. Bonkman, R.F. (1984). Cataracts and photochemical damage in the lens. in Human Cataract Formation (eds. Nugent, J. and Whelan, J.) pp. 88-109. Ciba Foundation Symposium 106, Pitman, London.

41. Van Heyningen, R. (1969). The lens: metabolism and cataract. in The Eye, second edition, Vol. 1. (ed. Davson, H.) pp.444-450. Academic Press, New York.

42. Van Heyningen, R. (1969). The lens: metabolism and cataract. in The Eye, second edition, Vol. 1. (ed. Davson, H.) pp.450-464. Academic Press, New York.

43. Koksma, J.M.A., Hoyng, F.J., Willekens, B.W. (1984). Lens deviations in rabbit offspring after short-term prenatal exposure to dexamethasone: a light-microscope study. Exp. Eye Res. 39, 195-206.

44. Clayton, R.M. (1985). Developmental genetics of the lens. in The Ocular Lens. Structure, Function, and Pathology (ed. Maisel, H.) pp. 61-92. Marcel Dekker, Inc. New York.

45. Gelatt, K.N. and Das, N.D. (1984). Animal models for inherited cataracts: a review, Curr. Eye Res. 3, 765-778.

46. Kuck, J.F.R. and Kuck, K.D. (1983). The Emory mouse cataract: loss of soluble protein, glutathione, protein sulfhydryl and other changes. Exp. Eye Res. 36, 351-362.

47. Roy, D., Garner, M.H., Spector, A., Carper, D. and Russell, P. (1982). Investigation of Nakano lens proteins. Exp. Eye Res. 34, 909-920.

48. Carper, D., Smith-Gill, S.J., and Kinoshita, J.H. (1986). Immunocytochemical localization of the 27K β-crystallin polypeptide in the mouse lens during development using a specific monoclonal antibody: implications for cataract formation in the Philly mouse. Develop. Biol. 113,104-109.

49. Iwata, S. (1985). Calcium-pump and its modulator in the lens: a review. Curr. Eye Res. 4, 299-305.

50. Hightower, K.R., Duncan, G., and Harrison, S.E. (1985). Intracellular calcium concentration and calcium transport in the rabbit lens. Invest. Ophthalmol. Vis. Sci. 26, 1032-1034.

51. Jedziniak, J.A., Nicoli, D. F., Yates E.M., and Benedek, G.B. (1976). On the calcium concentration of cataractous and normal lenses and protein fractions of cataractous lenses. Exp. Eye Res. 23, 325-332.

52. Hightower, K.R. and Reddy, V.N. (1982). Calcium content and distribution in human cataract. Exp. Eye Res. 34, 413-421.

53. Kador, P.F., Fukui, H.N., Fukushi, S., Jernigan, H.M., Jr., and Kinoshita, J.H. (1980). Philly mouse: a new model of hereditary cataracts. Exp. Eye Res. 30, 59-68.

54. Hightower, K.R., and Reddy, V.N. (1982). Calcium-induced cataract. Invest. Ophthalmol. Vis. Sci. 22, 263-267.

55. Hightower, K.R., and Reddy, V.N. (1985). Cytotoxic effects of internal calcium on lens physiology: a review. Curr. Eye Res. 4, 453-459.

56. Giblin, F.J., Hightower, K.R., Ragatzki, P.A., and Reddy, V.N. (1984). Calcium-induced high molecular weight proteins in the intact rabbit lens. Exp. Eye Res. 39, 9-17.

57. Fein, M., Pande, A., and Spector, A. (1979). Further investigation of the role of calcium in human lens protein aggregation. Invest. Ophthamol. Vis. Sci. 18, 761-765.

58. Garber, T., Srirk, L., and Gold, R.J.M. (1983). Abnormalities of crystallins in the lens of the Cat Fraser mouse. Exp. Eye Res. 36, 165-169.

59. Williams, E.H., Chaplain, T.L., and Meaken, T. (1985). A temporal and spatial study of the synthesis and degradation of water-soluble and insoluble proteins in galactosemic rat lenses. Exp. Eye Res. 41, 475-486.

60. Horwitz, J., Hansen, J.S., Cheung, C.C., Ding, L.L., Straatsma, B.R., Lightfoot, D.O., and Takemoto, L.J. (1983). Presence of low molecular weight polypeptides in human cataracts. Biochem. Biophy. Res. Comm. 113, 65-71.

61. Roy, D. and Spector, A. (1978). High molecular weight protein from human lenses. Exp. Eye Res. 22, 273-279.

62. Spector, A., and Roy, D. (1978). Human insoluble lens protein II. Isolation and characterization of a 9600 dalton polypeptide. Exp. Eye Res. 26, 445-459.

63. Garner, W.H., Garner, M.H., and Spector, A. (1979). Comparison of the 10,000 and 43,000 dalton polypeptide populations isolated from the water soluble and insoluble fractions of human lenses. Exp. Eye Res. 29, 257-276.

64. Marachi, T. Intracellular Ca^{2+} proteases and its inhibitor protein: calpain and calpastatin. In: Calcium and Cell Function. Vol. IV, (Cheung, W.Y., ed.), pp. 387-410. Academic Press, New York.

65. Kay, J. (1982). Ca^{2+}-activated proteinases, protein degradation and muscular dystrophy. In Proteases: Potential Role In Health and Disease. (eds. Horl, W.H. and Heidland, A.) pp. 519-531. Plenum Press, New York.

66. Yeaton, R. W., Lipari, M.T., and Fox, F. (1983). Calcium-mediated degradation of epidermal growth factor receptor in dislodged A431 cells and membrane preparations. J. Biol. Chem. 258, 9254-9261.

67. Tanaka, W. (1983). Absorption, distribution, metabolism, and excretion of leupeptin. in Protease Inhibitors: Medical and Biological Aspects. (eds. Katunuma, N., Umezawa, H., and Holzer, H.) pp.17-24. Springer-Verlag, Berlin.

68. Hanada, K., Tamai, M., Adachi, T., Oguma, K., Kashiwagi, K., Ohmura, S., Kominami, E., Towatari, T., and Katunuma, N. (1983). Characterization of the three new analogs of E-64 and their therapeutic application. in Protease Inhibitors: Medical and Biological Aspects. (eds. Katunuma, N., Umezawa, H., and Holzer, H.) pp.25-36. Springer-Verlag, Berlin.

69. Waxman, L. (1981). Calcium-activated proteases in mammalial tissues. in Methods in Enzymology, Vol. 80, (ed. Lorand, L.) pp. 664-680. Academic press, New York.

70. Ohno, S., Emori, Y., Imajoh, S., Kawasaki, H., Kisaragi, M. and Suzuki, K. (1984). Evolutionalry origin of a calcium-dependent protease by fusion of genes for a thiol protease and a calcium-binding protein? Nature, 312, 566-570.

71. Russell, P. (1984). In vitro alterations similar to posttranslational modification of lens proteins. Invest. Ophthalmol. Vis. Sci. 25, 209-212.

72. Lorand, L., Conrad, S.M., and Velasco, P.T. (1985). Formation of a 55 000-weight cross-linked β crystallin dimer in the Ca^{2+}-treated lens. A model for cataract. Biochemistry, 24, 1525-1531.

73. Ireland, M. and Maisel, H. (1984). Evidence for a calcium activated protease specific for lens intermediate filaments. Curr. Eye Res. 3, 423-429.

74. Roy, D., Chiesa, R., and Spector, A. (1983). Lens calcium activated proteinase: degradation of vimentin. Biochem. Biophy. Res. Comm. 116, 204-209.

75. Yoshida, H., Murachi, T., and Tsukahara, I. (1984). Limited proteolysis of bovine lens α-crystallin by calpain, a Ca^{2+}-dependent cysteine proteinase, isolated from the same tissue. Biochem. Biophys. Acta., 798, 252-259.

76. Yoshida, H., Murachi, T., and Tsukahara, I. (1984). Degradation of actin and vimentin by calpain II, a Ca^{2+}-dependent cysteine protease, in bovine lens. FEBS Letters, 170, 259-262.

77. Yoshida, H., Murachi, T., and Tsukahara, I. (1985). Distribution of calpain I, calpain II, and calpastatin in bovine lens. Invest. Ophthalmol. Vis. Sci. 26, 953-956.

78. Yoshida, H., Murachi, T., and Tsukahara, I. (1985). Age-related changes of calpain II and α-crystallin in the lens of hereditary cataract (Nakano) mouse. Curr. Eye Res. 4, 983-988.

79. Stadtman, T.C. (1979). Some selenium-dependent biochemical processes. Adv. Enzymol. 48, 1-28.

80. Kice, J.L. (1980). The mechanism of the reaction of thiols with selenite and other Se (IV) species. in Selenium in Biology and Medicine (eds. J.E. Spallholz, J.L. Martin, and H.E. Ganther) pp. 17-32. AVI Publishing Company, Westport, Connecticut.

81. Ganther, H.E., and Corcoran, C. (1969). Selenotrisulfides. II. Crosslinking of reduced pancreatic ribonuclease with selenium. Biochemistry 8, 2557-2563.

82. Ganther, H.E. (1971). Reduction of the selenotrisulfide derivative of glutathione to a persulfide analog by glutathione reductase. Biochemistry 10,4089-4098.

83. Ostadalova, I., Babicky, A., and Kopoldova, J. (1982). Ontogenic changes in selenite metabolism in rats. Arch. Toxicol., 49, 247-252.

84. Tsen, C.C. and Tappel, A.L. (1958). Catalytic oxidation of glutathione and other sulfhydryl compounds by selenite. J. Biol. Chem. 233, 1230-1232.

85. Shamberger, R.J. (1983). Selenium in health and disease. in Biochemistry of Selenium, pp.207-271. Plenum Press, New York.

86. Chen, X., Yang, G. Chen, J., Chen, X, Wen, Z., and Ge, K. (1980). Studies on relations of selenium and Keshan disease. Biol. Tr. El. Res. 2, 91-107.

87. Ostadalova, I., Babicky, A., and Obenberger, J. (1977). Cataract induced by the administration of a single dose of sodium selenite to suckling rats. Experientia, 34, 222-223.

88. Shearer, T.R., McCormack, D.J., Desart, D.J., J.L. Britton, and Lopez, M.T. (1980). Histological evaluation of selenium induced cataracts. Exp. Eye Res. 31,327-333.

89. Ostadalova, I. and Babicky, A. (1983). Delimitation of the period of sensitivity to the cataractogenic action of selenite in rats. Physiologia Bohemoslovaca, 32, 324-328.

90. Babicky, A. Rychter, Z., Kopoldova, J. and Ostadalova, I. (1985). Age dependence of selenite uptake in rat eye lenses. Exp. Eye Res. 40, 101-103.

91. Shearer, T.R., Anderson, R.S., and Britton, J.L. (1983). Uptake and distribution of radioactive selenium in cataractous rat lens. Curr. Eye Res. 2, 561-564.

92. Shearer, T.R., Anderson, R.S., Britton, J.L. and Palmer, E.A. (1983). Early development of selenium-induced cataract: slit lamp evaluation. Exp. Eye Res. 36, 781-788.

93. Shearer, T.R. and Anderson, R.S. (1985). Histological changes during selenite cataractogenesis: a light microscopy study. Exp. Eye Res. 40, 557-565.

94. Palmquist, B.-M., Fagerholm, P., and Landau, I. (1986). Selenium-induced cataract - a coorelation of dry mass content and light scattering. Exp.Eye Res. 42, 35-42.

95. Russell, N.J., Royland, J.E., McCawley, E.L., and Shearer, T.R. (1984). Ultrastructural study of selenite-induced nuclear cataracts. Invest. Ophthalmol. Vis. Sci. 25, 751-757.

96. Anderson, R.S., Shearer, T.R., and Claycomb, C.K. (1986). Selenite-induced epithelial damage and cortical cataract. Curr. Eye Res. 53-61.

97. Bhuyan, K.C., Bhuyan, D.K., and Podos, S.M. (1981). Cataract induced by selenium in rat: I. Effect on the lenticular protein and thiols. IRCS Med. Sci. 9, 194.

98. Bhuyan, K.C., Bhuyan, D.K., and Podos, S.M. (1981). Cataract induced by the selenium in rat: II. Increased lipid peroxidation and impairment of enzymatic defense against oxidative damage. IRCS Med. Sci. 9 195-196.

99. Bunce, G.E. and Hess J.L. (1981). Biochemical changes associated with selenite-induced cataracts in the rat. Exp. Eye Res. 33, 505-514.

100. Shearer, T.R,, Anderson, R.S., and Britton, J.L. (1983). Influence of selenite and fourteen trace elements on cataractogenesis in the rat. Invest. Ophthalmol. Vis. Sci. 24, 417-423.

101. David, L.L., and Shearer, T.R. (1984). State of sulfhydryl in selenite cataract. Toxic. Appl. Pharmacol. 74, 109-115.

102. Shearer, T.R. and David, L.L. (1983). Role of calcium in selenium cataract. Curr. Eye Res. 2, 777-784.

103. Bunce, G.E., Hess, J.L. and Batra, R. (1984). Lens calcium and selenite-induced cataract. Curr. Eye Res. 3, 315-320.

104. David, L.L. and Shearer, T.R. (1984). Calcium-activated proteolysis in the lens nucleus during selenite cataractogenesis. Invest. Ophthalmol Vis. Sci. 25, 1275-1283.

105. David, L.L. and Shearer, T.R. (1986). Purification of calpain II from rat lens and determination of endogenous substrates. Exp. Eye Res. (in press).

106. David, L.L. and Shearer, T.R. (1986). Origin of insoluble protein in selenite cataract. Invest. Ophthalmol. Vis. Sci. (in preparation).

Manuscript 1

STATE OF SUFHYDRYL IN SELENITE CATARACT[1,2]

L.L.David and T.R. Shearer[3]

Departments of Biochemistry and Ophthalmology

Oregon Health Sciences University

Portland, Oregon 97201

Corresponding author:
L. David
Dept. of Biochemistry
School of Dentistry
Oregon Health Sciences University
611 S.W. Campus Drive
Portland, Oregon 97201

STATE OF SULFHYDRYL IN SELENITE CATARACT

L. L. David and T.F. Shearer

Toxicology and Applied Pharmacology **74**, 109-115 (1984)

ABSTRACT

A dose of 20 μmoles selenite/kg b.w. is a potent and very rapid inducer of cataracts in young rats. We investigated the rate at which physiological concentrations of selenite would catalyze the oxidation of glutathione *in vitro* and found that selenite was a strong sulfhydryl oxidant. To test if selenite had the same effect *in vivo*, the oxidation state of five kinds of lenticular sulfur were measured in suckling rats following a cataractous dose of selenite. The measurements included reduced glutathione (GSH), oxidized glutathione (GSSG), protein-bound glutathione (PSSG), reduced protein sulfhydryl (PSH), and oxidized protein sulfhydryl (PSSP). While selenite caused a 44% decrease in lens GSH by 6 days postinjection, there was no concurrent increase in either GSSG or PSSG. Likewise, there was no evidence for increased oxidation of PSH to PSSP.

To determine if GSH loss were the cause of the selenite cataracts, we injected normal rats with the glutathione synthesis inhibitor buthionine sulfoximine (BSO). Lens GSH dropped more than 96% by 4 days post-BSO injection, however, no cataracts formed.

Thus, selenite cataract does not appear to be caused by extensive sulfhydryl oxidation and cannot be attributed exclusively to GSH loss.

INTRODUCTION

Studies on the biochemical causes of various experimental animal cataracts are being used to investigate the underlying mechanisms of cataract formation in man. Experimental cataracts can be induced by administration of excess selenium to young rats (Ostadalova et al., 1978). The mechanism responsible for the formation of the selenium overdose cataract is unknown. By giving an injection of 20 µmol sodium selenite/kg body weight to suckling rats, bilateral nuclear cataracts form in approximately 3 days (Bunce and Hess, 1981). The formation of the cataract is seen only in young animals, with the frequency of the cataract formation falling dramatically if rats are injected after 15 days of age (Ostadalova et al., 1979).

A theory concerning the formation of cataracts is that the aggregation of lens proteins forms a species which will scatter light (Benedek, 1971). This aggregation of lens proteins in cataracts may be caused by the formation of disulfide crosslinks (Spector and Roy, 1978). Additionally, in many experimental cataracts there is a drop in lens glutathione prior to opacification (Rathbun, 1981).

Sulfhydryl groups in reduced glutathione (GSH) are catalytically oxidized by selenite in the presence of oxygen to form selenotrisulfides (GSSeSG) and oxidized glutathione (GSSG) (Tsen and Tappel, 1958; Kice, 1980):

$$H_2SeO_3 \longrightarrow SeO_2 + H_2O$$

$$4\ GSH + SeO_2 \longrightarrow GSSG + GSSeSG + 2\ H_2O$$

$$GSSeSG + O_2 \longrightarrow GSSG + SeO_2$$

Selenite incubated *in vitro* with sulfhydryl-containing proteins also caused the formation of selenotrisulfides and disulfide bonds (Ganther and Corcoran, 1969). Therefore, a possible mode of action of selenite in cataractogenesis may be the oxidation of lens GSH and the formation of proteinaceous disulfide crosslinks, causing protein aggregation and light scatter.

The purpose of the present study was to test the above hypothesis by (1) investigating the rate of GSH oxidation by selenite and other trace metals *in vitro* at a physiological concentration of selenite, (2) determining if the oxidation state of various sulfhydryl groups in the lens was altered in selenite cataract, and (3) determining if the loss of lens GSH is the initiating factor in the development of the cataract.

METHODS

State of Sulfhydryl Following Selenite Injection.

Sprague-Dawley albino female rats and their litters of 10 pups (Simonsen Laboratories, Gilroy, Calif.) were individually housed in plastic cages and fed standard rat chow (Wayne Lab Blox, F-6) and distilled water *ad libitum*. At 10 days of age, five pups from each litter were injected sc with 0.05 ml of a solution containing 20 μmoles sodium selenite/kg body weight. The remaining five pups in each litter were not injected and served as controls. At various intervals following the injection, ten pups were killed from each group, and the lens were removed from the eyes by a posterior approach. Each group of four lenses were blotted dry, weighted, and homogenized in 2.5 ml of 0.02 M sodium phosphate buffer containing 0.02 M EDTA (pH 7.3) under a nitrogen atmosphere. All subsequent manipulations of lens protein were carried out under a nitrogen atmosphere with solutions

flushed with nitrogen. The homogenates were spun at 100,000g for 15 min to separate the soluble and insoluble lens fractions. The insoluble pellet was washed once, dissolved in 0.5% sodium dodecyl sulfate (SDS), and spun again for 15 min at 10,000g to spin down a minimal amount of material which was SDS insoluble. The supernatant fraction was used for assays of the lens insoluble protein.

Five separate sulfhydryl derminations were then carried out on the lens homogenates. Protein sulfhydryl measurements were based on a modified procedure of Sedlek and Lindsay (1968). Our assay used a final reaction mixture containing: 0.2 M Tris (pH 8.5), 0.02 M EDTA, 0.5% SDS as a denaturant, and 0.3 mM dithionitrobenzoic acid (DTNB). After 30 min the absorbance was read at 410 nm/570 nm with an Microelisa Auto Reader[4]. The sulfhydryl reacting with DTNB in the soluble fraction is called assay A (Table 1), and that reacting with DTNB in the insoluble fraction, assay B.

The reduction of the oxidized sulfhydryl groups in the soluble and insoluble lens protein was carried out according to the method of Brown (1960). Approximately 2 to 5 mg $NaBH_4$/mg lens protein was used during the reduction. After 30 min at 37°C, the excess $NaBH_4$ was destroyed by addition of acetone followed by HCl. The DTNB reaction was then performed as above. The sulfhydryl reacting with DTNB in the soluble fraction after reduction is assay C, that reacting in the insoluble fraction after reduction is assay D.

Oxidized glutathione (GSSG) was measured in the soluble protein fraction by modification of the procedure of Srivastava and Beutler (1968). Free sulfhydryls were alkylated with N-ethylmaleimide (NEM), followed by precipitation of protein with TCA and extraction of TCA and NEM from the supernatant fraction with ether. The extracted supernatant fraction was

then assayed for oxidized glutathione (assay E) by the spectrophotometric enzymatic cycling assay described by Brehe and Burch (1976).

Total free lens glutathione, both oxidized and reduced, was assayed with the TCA extract of the soluble protein fraction by the glutathione cycling assay (assay F). A third glutathione assay was performed after subjecting the soluble protein to a $NaBH_4$ reduction similar to the reduction described in assay C and D. This reaction released the protein-bound glutathione, and after precipitation of all of the protein and destruction of excess $NaBH_4$ by TCA, the extract was diluted and assayed for glutathione content by the glutathione cycling assay (assay G).

Reduced glutathione[5] was the sulfhydryl standard in all assays. Protein was assayed according to the method of Lowry et al. (1951), with bovine serum albumin as a standard. Table 1 shows the calculations used to derive the values of the various lens sulfhydryl fractions, based on the results of the previously described assays A-G.

Inhibition of Lens Glutathione Synthesis by Buthionine Sulfoximine.

At 10 days of age, pups received an ip injection of 0.2 ml saline containing 4 mmol buthionine sulfoximine[6]/kg body weight. Lenses were analysed for total free glutathione by assay F described above. Also, on days 4 and 5 postinjection, eyes were observed with a slit lamp to detect the presence of lens opacities.

In Vitro Oxidation of GSH by Trace Metals.

The *in vitro* rate of GSH oxidation caused by various trace metals was measured enzymatically by incubating 5 mM GSH at 25° C in the presence of 50 mM sodium phosphate (pH 7.0), 0.30 mM NADPH, 2 U/ml glutathione reductase[7],

and various trace metals. The oxidation of NADPH was followed spectrophotometrically at 340 nm. The reaction was initiated by adding the trace metal.

RESULTS

In Vitro Oxidation of GSH by Trace Metals.

In vitro, selenite was found most effective of all ions studied in oxidizing GSH (Table 2). The 10 μM concentration of selenite used is similar to the increase in concentration of selenium found in the lens following a cataractogenic dose of selenite (Bunce and Hess, 1981). The rate of GSH oxidation by selenite was linear and continued until all of the NADPH was utilized. This finding indicated that the oxidation was catalytic because the stochiometry of the reaction was much greater than 1, with a molar excess of NADPH becoming oxidized compared to selenite added. The effectiveness of selenium compounds in oxidizing GSH at these concentrations appears to be limited to selenite (SeO_3^{2-}) since selenate (SeO_4^{2-}) was not active.

State of Lens Glutathione following Selenite Injection.

One day following selenite injection, lens GSH decreased by 25% (Fig. 1A, upper curves). This decrease was most pronounced by 6 days postinjection. By 22 days postinjection, the reduced glutathione returned toward normal, even in the presence of severe nuclear opacity which develops at approximately 3 days post-selenite injection. Although lens GSH decreases in selenite cataract, there was no concurrent rise in lens GSSG

(Fig. 1A, lower curves). The lens GSSG remains at approximately 60 nmol/g lens wet weight in both control and selenite-treated animals, indicating that the lens maintains GSH/GSSG at a very high ratio.

The amount of glutathione which is bound to the lens soluble protein (PSSG) did not significantly change following selenite injection (except for an anomalous decrease at day 3) (Fig. 1B).

State of Lens Proteinaceous Sulfhydryl Following Selenite Injection.

Figure 2 shows the quantity of lens soluble and insoluble protein sulfhydryl (PSH) and protein disulfide (PSSP) following selenite injection. No significant differences in the amounts of lens PSH or PSSP were found between the selenite and control rats either during of after cataract development. This finding was also true when the amount of lens PSH and PSSP was expressed per gram lens total protein (data not shown).

Lens Protein Composition Following Selenite Injection.

Selenite caused a significant decrease in the amount of lens soluble protein by 3 days postinjection (Fig. 3A). The amount of lens insoluble protein was also affected by selenite, with a 2.5-fold increase occurring over control lenses by 14 days postinjection (Fig. 3B). We also found that selenite had a suppressing effect on the growth rate (wet weight) of the lens (data not shown).

Effects of an Inhibitor of Glutathione Synthesis.

To investigate if the formation of the selenite-induced cataract could be explained solely on the decrease in GSH which occurs following a single injection of selenite, we injected a specific inhibitor of GSH synthesis,

buthionine sulfoximine (BSO) into normal 10-day-old rats. Although the animals continued to nurse and appeared normal, lens glutathione decreased to nondetectable levels by 4 days postinjection of BSO (Table 3). This decrease in lens glutathione after BSO injection was more severe than after selenite injection. However, there was no evidence of cataract formation as determined by careful slit lamp examination on days 4 and 7 postinjection. The body weight and lens weight of the BSO-treated rats did not significantly change from controls (data not shown).

DISCUSSION

The two major findings of the present study were (1) lack of evidence for sulfhydryl oxidation in selenite cataract, and (2) lens glutathione levels could be dramatically decreased in normal lenses, yet no cataracts formed. The lack of protein sulfhydryl oxidation in selenite cataract is unlike many other experimental cataracts where extensive lens protein sulfhydryl oxidation was reported (Takemoto and Azari, 1975: Giblin et al., 1979: Kuck and Kuck, 1983). Our findings are also in contrast to earlier reports showing that selenite caused a loss of lens soluble protein sulfhydryl (Bunce and Hess, 1981: Bhuyan et al., 1981). These earlier reports expressed the soluble protein sulfhydryl content per lens wet weight and not per mass lens soluble protein. When our data were expressed as soluble protein sulfhydryl per gram lens wet weight, we also found a similar decrease in lens soluble protein sulfhydryl. This decrease is likely due to a decrease in the percentage lens wet weight as soluble protein and does not indicate actual sulfhydryl oxidation.

Tsen and Tappel (1958) investigated the rate various metal ions oxidized GSH and also found that selenite was most effective. Our measurements of selenite catalyzed GSH oxidation confirmed that selenite could oxidize GSH *in vitro* at an appreciable rate and at concentrations of selenium and GSH found in the lens. The lack of sulfhydryl oxidation in the selenite cataracts was surprising: we expected to observe the same catalytic oxidation of sulfhydryls to occur *in vivo*. However, no increase in lens GSSG or PSSP occurred following selenite injection. While it is possible that no increased GSSG was found because it was either leaving the lens or being converted to another form, it seems likely that no GSSG accumulated because lens-reducing systems were capable of adequately responding to the oxidative insult caused by selenite. One of the functions of the high level of GSH found in the lens is thought to be the protection of lens protein sulfhydryls from oxidation (Augusteyn, 1979). To fulfill this role, lens GSH must remain in a reduced state, and this state is accomplished by the enzyme glutathione reductase, which utilizes NADPH. The rate of glucose utilization through lens hexokinase and the hexose monophosphate (HMP) shunt has been postulated to be the limiting factor in the ability of the lens to maintain an adequate supply of NADPH to reduce GSSG to GSH and maintain lens clarity (Cheng and Chylack, 1980). Hess et al. (1983) recently found stimulation of lens HMP shunt activity following selenite exposure in a lens culture system. When selenite-treated lenses were simultaneously treated with a second GSH oxidant, t-butylhydroperoxide, additional stimulation of HMP shunt activity occurred. This result indicated that the lens HMP shunt activity was not maximally stimulated by selenite. Our data also indicated that lens sulfhydryl reducing systems are not overstressed by selenite, since no increase in lens disulfide occurred.

After selenite injection, we found decreases in lens GSH which were similar in magnitude to the results of Bunce and Hess (1981). This loss of GSH may be due to a change in the rates of lens GSH synthesis or degradation, or to efflux of glutathione from the lens. Since the half-life of GSH is relatively short (28 hr) (Rathbun, 1981), the rapid drop in GSH could be explained by an inhibition of GSH synthesis by selenite. Therefore, BSO was used in the present study to determine if cataracts could be caused by inhibiting GSH synthesis. BSO was a highly effective inhibitor of lens γ-glutamylcysteine synthetase (Griffith and Meister, 1979), since lens GSH was reduced to non-detectable levels. However, no cataracts appeared even with the severe depression of glutathione. This lack of cataractogenesis could be due to the absence of an oxidant (i.e., selenite), but the data do exclude glutathione loss as the exclusive cause of the selenite cataract. Future experiments could test if BSO and subcataractogenic doses of selenite cause cataracts, and also if cataracts could be caused by extended glutathione depletion from BSO administration.

Our results indicated that no sulfhydryl oxidation occurred in the selenite cataract and suggested that the selenite cataract is not caused by disulfide linked aggregates. However, the possibility still exists that sulfhydryl oxidation was restricted to a small fraction of lens proteins or to a specific site within the lens not detectable by analyzing whole lens homogenates. Further studies characterizing other biochemical changes occurring during selenite cataractogenesis may find that this cataract and the initiating event causing other types of cataracts have a common cause, which initially may not involve disulfide linked aggregates.

REFERENCES

Augusteyn, R.C. (1979). On the possible role of glutathione in maintaining human lens protein sulfhydryls. Exp. Eye Res. 28, 665-671.

Benedek, G.B. (1971). Theory of transparency of the eye. Appl. Optics 10, 459-473.

Bhuyan, K.C., Bhuyan, D.K., and Podos, S.M. (1981). Selenium-induced cataract: biochemical mechanism. In Selenium in Biology and Medicine (J.E. Spalholz, J. L. Martin, and H.E. Ganther, eds.), pp. 403-412. AVI Publishing Company, Westport, Connecticut.

Brehe, J.E., and Burch, H.B. (1976). Enzymatic assay for glutathione. Anal. Biochm. 74,189-197.

Brown, W.D. (1960). Reduction of protein disulfide bonds by sodium borohydride. Biochim. Biophys. Acta 44, 365-367.

Bunce, G.E., and Hess, J.L. (1981). Biochemical changes associated with selenite-induced cataract in the rat. Exp. Eye Res. 33, 505-514.

Cheng, H.M., and Chylack, L.T., Jr. (1980). Thiol oxidation in the crystalline lens. I. The rate-limiting role of hexokinase in aging rat and human lenses. Invest. Ophthalmol. Vis. Sci. 19, 522-528.

Ganther, H.E., and Corcoran, C. (1969). Selenotrisulfides. II. Crosslinking of reduced pancreatic ribonuclease with selenium. Biochemistry 8, 2557-2563.

Giblin, F.J., Chakrapani, B., and Reddy, V.N. (1979). The effects of X-irradiation on lens reducing systems. Invest. Ophthalmol. Vis. Sci. 18, 468-475.

Griffith, O.W., and Meister, A. (1979). Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (S-n-butyl homocysteine sulfoximine). J. Biol. Chem. 254, 7558-7560.

Hess, J.L., Wisthoff, F., and Bunce, G. E. (1983). Impact of selenite on rat lens metabolism. ARVO, 1983. Invest. Ophthalmol. Vis. Sci. (Suppl) 24, 31 (abstr.).

Kice, J.L. (1980). The mechanism of the reaction of thiols with selenite and other Se (IV) species. In Selenium in Biology and Medicine (J.E. Spallholz, J.L. Martin, and H. E. Ganther, eds.), pp. 17-32. AVI Publishing Company, Westport, Connecticut.

Kuck, J.F.R., and Kuck, K.D. (1983). The Emory mouse cataract: loss of soluble protein, glutathione, protein sulfhydryl and other changes. Exp Eye Res. 36, 351-362.

Lowry, O.H., Rosebrough, N. J., Farr, A.L., and Randell, R. J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

Ostadalova, I., Babicky, A. and Obenberger, J. (1978). Cataract induced by administration of a single dose of sodium selenite to suckling rats. Experientia 34, 222-223.

Ostadalova, I., Babicky, A., and Obenberger, J. (1979). Cataractogenic and lethal effect of selenite in rats during postnatal ontogenesis. Physiol. Bohemoslovaca 28, 393-397.

Rathbun, W.B. (1981). Glutathione: biosynthesis and functions in the lens. In Symposium on the Lens (F. Renault, ed.), p. 39. Excerpta Medica, Amsterdam.

Sedlak, J., and Lindsay, R.H. (1968). Estimation of total, protein-bound and nonprotein sulfhydryl proups in tissue with Ellmann's reagent. Anal. Biochem. 25, 193-205.

Spector, A., and Roy, D. (1978). Disulfide-linked high molecular weight protein associated with human cataract. Proc. Natl. Acad. Sci. USA 75, 3244-3248.

Srivastava, S.K., and Beutler, E. (1968). Accurate measurement of oxidized glutathione content of human, rabbit, and rat red blood cells and tissues. Anal. Biochem. 25, 70-76.

Takemoto, L. J., and Azari, P. (1975). Role of sulfhydryl groups in the formation of a hereditary cataract in the rat. Exp. Eye Res. 20, 1-13.

Tsen, C.C., and Tappel, A.L. (1958). Catalytic oxidation of glutathione and other sulfhydryl compounds by selenite. J. Biol. Chem. 233, 1230-1232.

FOOTNOTES

1) Supported in part by NIH grant EY-3600 form the National Eye Institute.

2) Presented in part on May 2, 1983, at the Annual Meeting of the Association for Research in Vision and Ophthalmology, Sarasota, Florida.

3) To Whom correspondence should be adressed.

4) Dynatech Laboratories, Inc., Alexamdria, Va. 22314.

5) No. G-4251, Sigma Chemical Co., Saint Louis, MO 63178.

6) Chemical Dynamics Corp., South Plainfield, N.J. 07080.

7) No. G-4759, Sigma Chemical Co., Saint Louis, MO 63178.

Table 1

Lens Sulfhydryl Calculations[a]

Content	Calculation
Soluble protein sulfhydryl	= A - F + E/ grams soluble protein
Soluble protein disulfide (expressed as 1/2 cystine)	= C - A - E - 2(G - F)/grams soluble protein
Insoluble protein sulfhydryl	= B/grams insoluble protein
Insoluble protein disulfide (expressed as 1/2 cytine)	= D - B/grams insoluble protein
Reduced glutathione	= F - E/grams lens wet weight
Oxidized glutathione (expressed as 1/2 GSSG)	= E/grams lens wet weight
Protein bound glutathione	= G - F/grams lens wet weight

[a] Letters used in calculations refer to assays described in text.

TABLE 2

Oxidation Rate of Reduced Glutathione by Various Trace Metal Compounds[a]

Trace Metal Compound	Ionic Form	Oxidation Rate[b] (µmolar/min)	
Na_2SeO_3	Selenite	13.2	0.2[c]
Na_2SeO_4	Selenate	0.0	0.0
$Cu(CH_3COO)_2$	Cupric	6.5	0.2
K_2TeO_3	Tellurite	1.4	0.0
$FeCl_3$	Ferric	0.0	0.0

[a] Oxidation was initiated by addition of the trace metal compound at 10 µM concentration.
[b] The oxidation rate is given as µmolar/min decrease in NADPH.
[c] Mean ± SD (n=4).

TABLE 3

Lens Glutathione Levels Following a Single Injection of Buthionine Sulfoximine (BSO)[a]

Days post injection	GSH + 1/2 GSSG (µmoles/g lens wet wt)					Incidence of cataracts (%)[b]
	Control		+BSO		% decrease	
1	5.06	0.16[c]	2.39	0.20	53	-
2	4.31	0.45	0.66	0.14	85	-
4	5.82	0.22	N.D.[d]		> 96	0
7	5.86	0.25	0.37	0.11	94	0

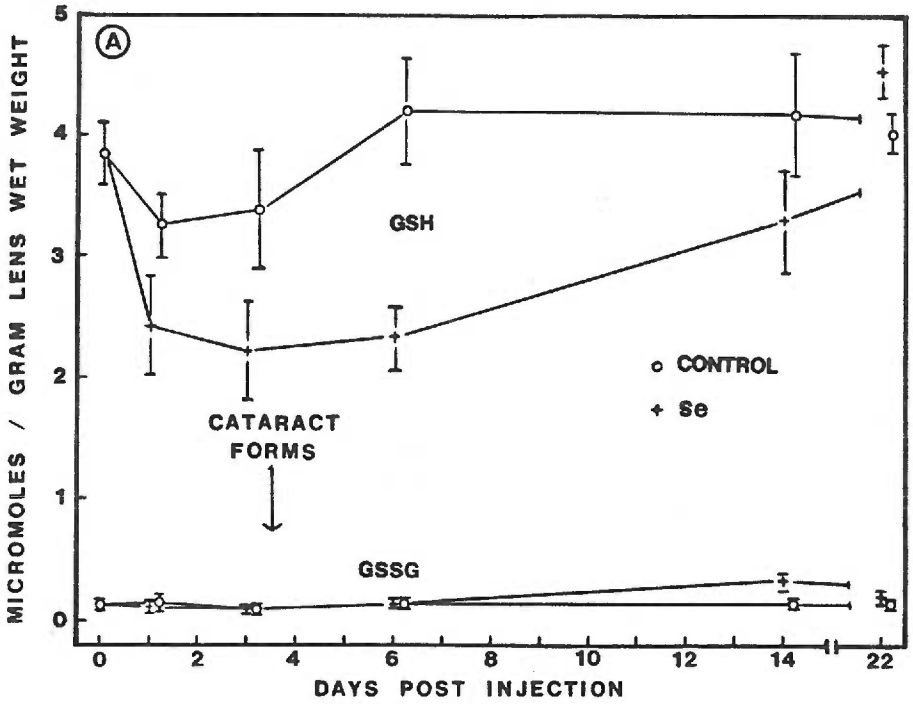
[a] Ten-day-old rats were either noninjected (controls) or given a single injection of 4 mmol BSO/kg body weight, and 2 lenses from each animal were pooled and analyzed for glutathione.

[b] On days 4 and 7, separate litters containing 5 controls and 5 BSO-injected rats were examined with a slit lamp, and no opacities were observed. Litters were not examined by slit lamp on days 1 and 2 postinjection since eyes did not open until 14 days of age.

[c] Mean ± SD (n=5). The means of each time period were statistically significant at $p < 0.05$.

[d] N.D., Not detectable.

Figure 1: State of lens glutathione in rat pups following a single injection of 20 μmol sodium selenite/kg body weight at 10 days of age. Four lenses were pooled for each sample, and each point represents the mean of 4-10 samples ± SD. (A) Lens reduced glutathione (GSH) (upper curves). The means at all time periods were significantly different ($p < 0.05$). Lower curves show lens oxidized glutathione (GSSG) expressed as 1/2 GSSG. Only means at day 14 postinjection were significantly different ($p < 0.05$). (B) Lens protein bound glutathione. Only means at day 3 postinjection were significantly different ($p < 0.05$).


A
MICROMOLES / GRAM LENS WET WEIGHT
GSH
CONTROL
Se
CATARACT
FORMS
GSSG
DAYS POST INJECTION

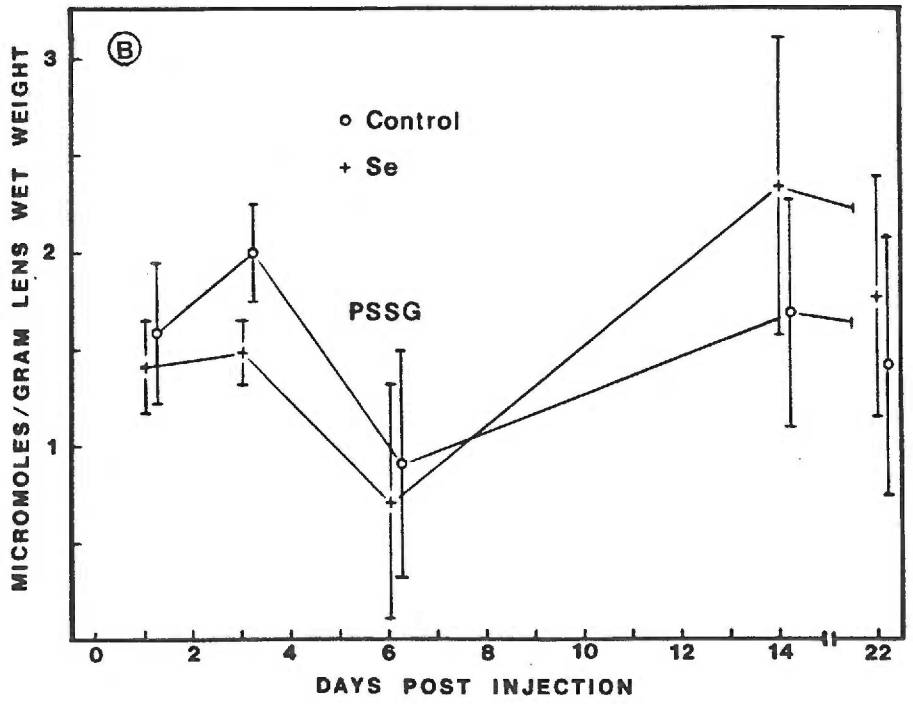
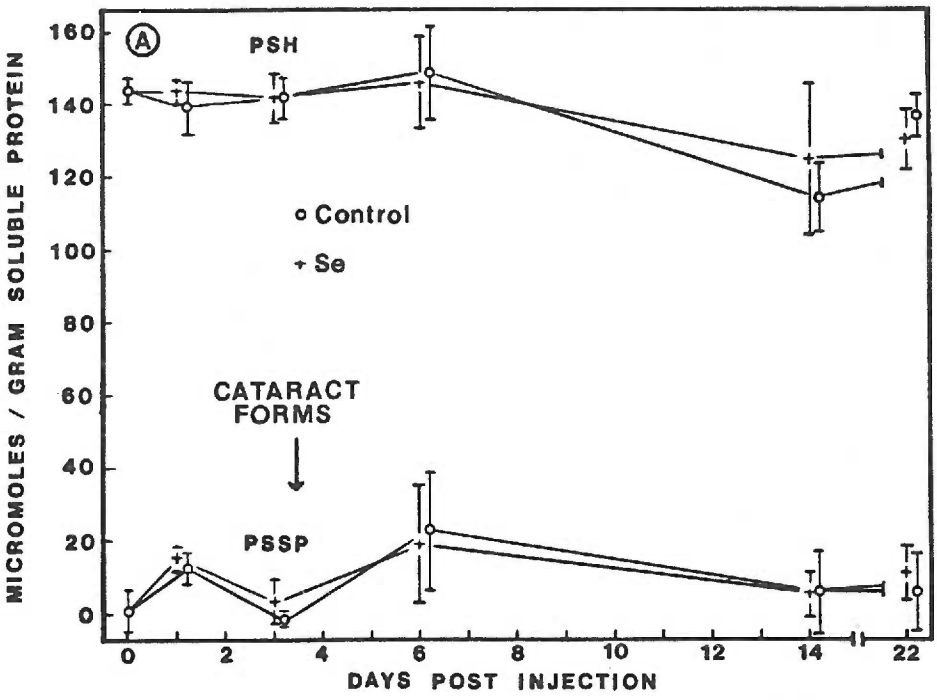

B
MICROMOLES/GRAM LENS WET WEIGHT
Control
Se
PSSG
DAYS POST INJECTION

Figure 2: State of lens protein sulfhydryl in rat pups following selenite injection at 10 days of age. Four lenses were pooled for each sample, and each point represents the mean of 4-10 samples ± SD. (A) Lens soluble protein (B) lens insoluble protein. Upper curves indicate the protein sulfhydryl (PSH), and lower curves the protein disulfide (PSSP) expressed as 1/2 PSSP. No significant differences were found between the means at any time interval ($p < 0.05$).


A
PSH
o Control
+ Se
CATARACT FORMS
PSSP
MICROMOLES / GRAM SOLUBLE PROTEIN
160
140
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
22
DAYS POST INJECTION

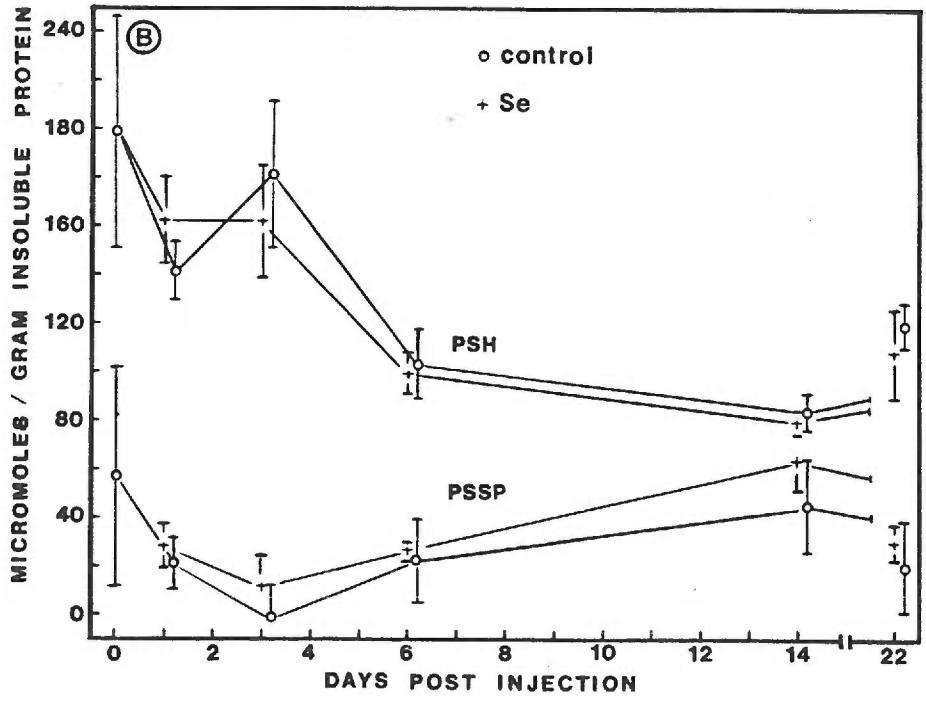
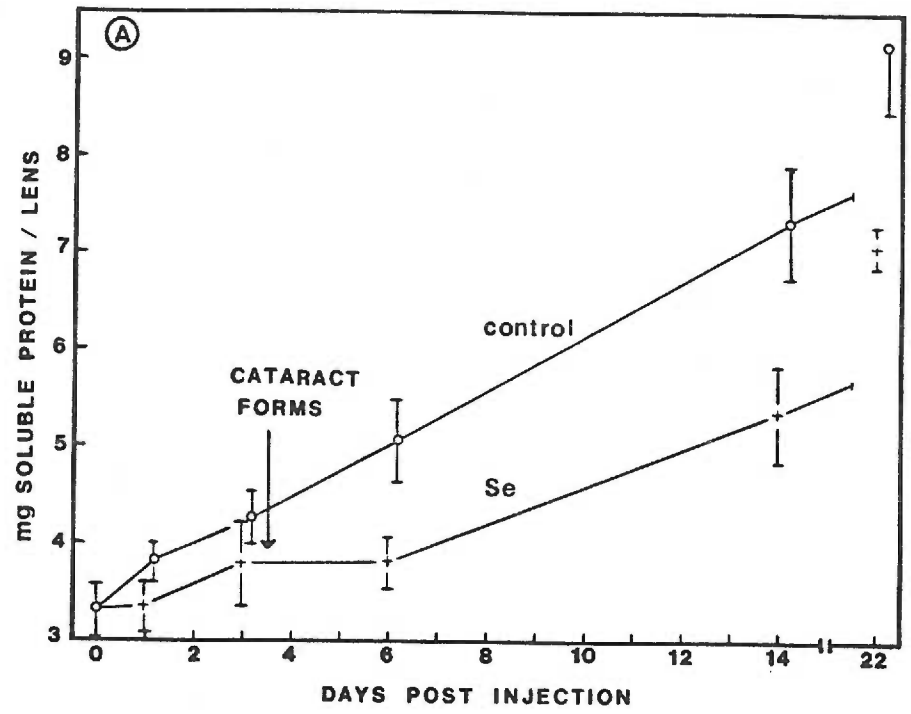
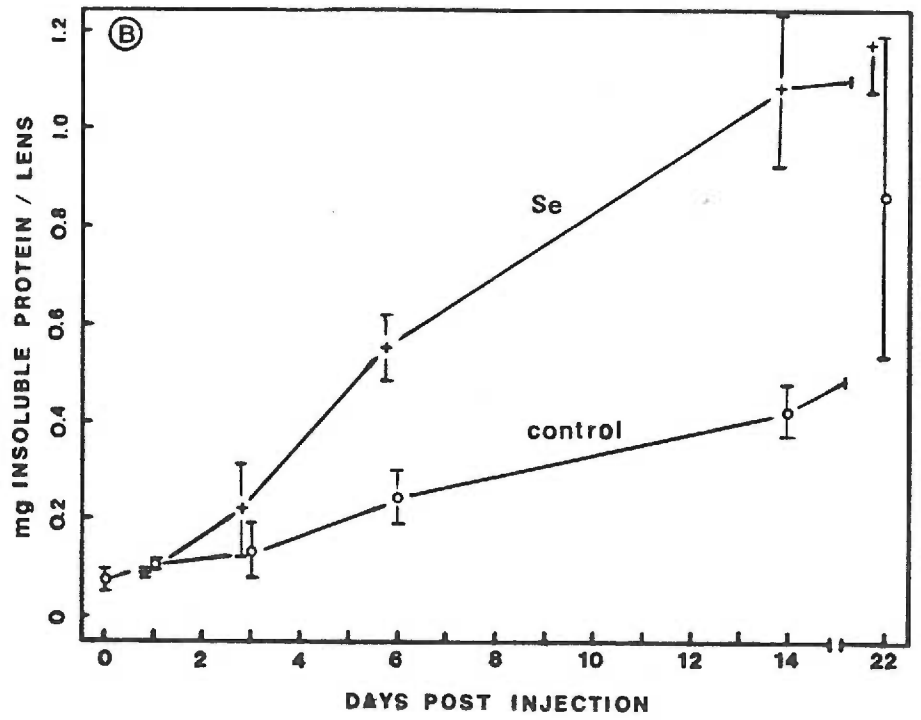

B
o control
+ Se
PSH
PSSP
MICROMOLES / GRAM INSOLUBLE PROTEIN
240
180
160
120
80
40
0
0
2
4
6
8
10
12
14
22
DAYS POST INJECTION

Figure 3: Amount of soluble (A) and insoluble (B) protein in lenses of rat pups following selenite injection at 10 days of age. Four lenses were pooled for each sample, and each point represents the mean of 5 to 10 samples $\pm$ SD. All means at each time period were significantly different ($p < 0.05$) except for the amount of insoluble protein at days 1 and 22 postinjection.


A
mg SOLUBLE PROTEIN / LENS
9
8
7
6
5
4
3
control
CATARACT
FORMS
Se
0
2
4
6
8
10
12
14
22
DAYS POST INJECTION


B
mg INSOLUBLE PROTEIN / LENS
1.2
1.0
0.8
0.6
0.4
0.2
0
Se
control
0
2
4
6
8
10
12
14
22
DAYS POST INJECTION

Manuscript 2

Calcium-Activated Proteolysis in the Lens Nucleus during Selenite Cataractogenesis

L.L. David* and T.R. Shearer*+

Departments of Biochemistry and Ophthalmology
Oregon Health Sciences University
Portland, Oregon 97201

Short Title: Proteolysis In Selenite Cataract
Key Words: Selenite Cataract, Rat Lens, Calcium Activated Protease, Proteolysis, Lens Proteins.

From the Departments of Biochemistry* and Ophthalmology+, Oregon Health Sciences University, Portland Oregon.

Supported in part by grant no. EY03600 from the National Eye Institute, National Institutes of Health, Bethesda, Maryland.

Submitted for publication: April 9, 1984.

Reprint requests: T.R. Shearer, 611 S.W. Campus Drive, Portland, OR 97201.
611 S.W. Campus Drive
Portland, Oregon 97201

Abstract

A single injection of 20 μmol sodium selenite/kg body weight in 10-day-old rats caused severe nuclear cataract within 4 days. By 4 days postselenite injection, nuclear calcium levels increased from 0.4 to 6.8 mmol/kg lens dry weight. The purpose of these experiments was to determine if this calcium increase was associated with proteolysis specifically in the lens nuclear region. Sodium dodecyl sulfate polyacrylamide electrophoresis of lens nuclear proteins following selenite injection showed: loss of 30, 27, and 26 K molecular weight polypeptides in the soluble fraction, loss of 83, 52, 30, 27, and 26 K polypeptides in the insoluble fraction, and loss of the major 26 K membrane protein. Gel chromotography of nuclear soluble proteins indicated a decrease in β_H and β_L crystallins following selenite injection. Two-hour in vitro incubation of nuclear lens homogenates with calcium duplicated many of the proteolytic changes occurring in lenses in vivo following selenite injection. Calcium induced proteolysis in vitro was inhibited by EGTA, leupeptin, and iodoacetate but was not inhibited by phenylmethylsulfonyl fluoride. These properties are similar to calcium activated protease (CAP) from other tissues. Activation of CAP, and subsequent degradation of nuclear proteins, may be causes of selenite cataract. Invest Ophthalmol Vis Sci 25: 1275-1283, 1984

A single injection of 20 μmol sodium selenite/kg body weight in young rats causes the appearance of severe nuclear cataract within 4 days (1,2,3). Selenite cataract provides a useful model for the study of the in vivo effect of elevated lenticular calcium. During selenite cataractogenesis, a fivefold increase in whole lens calcium was localized in the lens nucleus (4,5). Selenite cataractous lenses also exhibit a rapid rise in lens insoluble protein and a decrease in reduced glutathione (1,2,6). Despite these changes, there were no increases in total lens protein disulfide (6).

Elevated lens calcium (7-9) and proteolysis (10) have been associated with other cataracts. Bovine and rat lenses contain calcium-activated neutral proteases (11-14). We postulated that calcium-activated proteolysis may occur specifically in the lens nucleus during selenite cataractogenesis. This theory was tested in the following report by: (1) measuring the time relationship between elevated nuclear calcium and proteolytic changes in vivo following selenite injection and (2) observing the effect of incubating lens homogenates with calcium. We showed that calcium-activated proteolysis may be involved in the development of the selenite cataract.

Materials and Methods

Isolation of lens protein

Ten-day-old Sprague-Dawley suckling rat pups (Simonsen Laboratories: Gilroy, CA) were given a single 0.05-ml subcutaneous injection of a solution containing 20 μmol sodium selenite/kg body weight. Pups were killed by decapitation at various times after injection, and the lenses were removed from the enucleated eyes by a posterior approach. The capsules were removed and the lenses dissected with tweezers under a microscope into cortical and nuclear regions in buffer 1 containing 20 mM sodium phosphate (pH = 7.3), 20 mM EDTA, and 10 mM iodoacetate at approximately 20°C. Development of cold cataract facilitated this separation, since cold cataract was localized in the nuclear region. The dissection also was aided by a tendency of the cortex to separate from the nucleus along an interface, which appeared when the cortex was removed. The cortex and nuclear regions, each consisting of approximately 1/2 the total lens protein, were homogenized separately in buffer 1 at a ratio of approximately 15 lens regions/ml buffer. The homogenates were contrifuged at 10,000 g for 15 min at 4°C to separate the soluble and insoluble lens protein. The insoluble pellet was washed once and dissolved in buffer 2, containing 20 mM sodium phosphate (pH = 7.3), 20 mM EDTA, 2 mM iodoacetate, and 2% sodium dodecyl sulfate (SDS). During the isolation of the intrinsic membrane proteins, the insoluble pellet was not dissolved in buffer 2, but instead resuspended in buffer 3, containing 50 mM Tris (pH = 7.4), 5 mM EDTA, and 10 mM 2-mercaptoethanol, and the method of Russell et al (15) was used to obtain the intrinsic membrane proteins.

Determination of Lens Calcium

Lens capsules were removed in 1 mM EGTA/0.9% saline solution and the dissection of cortex and nuclear regions carried out in 0.9% saline. The dissected regions from four lenses were placed in tared acid washed glass vials, dried at 60°C for 12 hr and weighed to ± 0.01 mg. The lens regions were digested as previously described (4), dissolved in 1.0 ml 0.2% lanthanum chloride/1% HCl solution, and analyzed for total calcium content by atomic absorption spectroscopy.

In Vitro Incubation of Homogenates of Normal Lenses with Calcium

Lens dissection and homogenizations were carried out in buffer 4, containing 20 mM Tris (pH = 7.4), 1.0 mM EGTA, 2.0 mM 2-mercaptoethanol, and 0.01 % sodium azide. The protein concentrations were adjusted to 15 mg/ml in the homogenates and the incubation was initiated by addition of calcium chloride in 2.0 or 3.0 mM excess over EGTA. In those samples incubated with protease inhibitors, the inhibitor was added 30 min prior to incubation. After 1 or 2 hr at 37°C, the incubation was terminated by the addition of EGTA at a final 5.0 mM concentration. The incubated homogenates then were centrifuged at 10,000 g for 15 min at 4°C to separate the soluble and insoluble fractions and the insoluble pellet washed once and dissolved in buffer 2, or resuspended in buffer 3 to isolate the intrinsic membrane protein as before. Synthetic leupeptin and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Company (St. Louis, MO).

Column Chromatography and Electrophoresis

Lens soluble, insoluble, and intrinsic membrane proteins were separated on Laemmli (16) 12% SDS-polyacrylamide gels following reduction using

2-mercaptoethanol, and stained with 0.1% Coomassie Blue. Apparent molecular weights were determined by comparison with a mixture of 7 proteins of known molecular weights (Dalton Mark VII-L, Sigma Chemical Co.: St. Louis, MO). Soluble lens crystallins were isolated using a 2.5 cm x 96 cm column of Sephadex G-200 (Pharmacia Fine Chemicals: Piscataway, NJ). After chromatography, the various crystallins were concentrated by ultrafiltration using a YM5 filter (Amicon Corporation: Lexington, MA). The α-crystallin fraction appearing at the void volume of Sephadex G-200 was applied to a 2.5 cm X 96 cm column of agarose A5M (Bio-Rad Laboratories: Richmond, CA) to separate high molecular weight protein from α-crystallin. The elution buffer of both columns used a flow rate of 15 ml/hr and contained 50 mM Tris (pH = 7.4), 100 mM sodium chloride, and 0.02 % sodium azide. Protein was assayed by the method of Lowry et al. (17), using bovine serum albumin as a standard. Rats in this study were treated in accordance with the ARVO Resolution on the Use of Animals in Research.

RESULTS

Calcium Concentrations in Lens Cortex and Nucleus

Nuclear cataract appeared in the selenite treated rats in approximately 4 days following injection. In the lens cortex, selenite injection caused calcium to increase from 0.34 to 1.71 mmol calcium/kg lens dry weight by 4 days postinjection (Fig. 1A). The lens nucleus (Fig. 1B) was, however the major site of calcium increase since by 4 days postinjection, the total calcium concentraction increased from 0.40 to 6.81 mmol/kg lens dry weight. Following formation to the nuclear cataract, the lens calcium levels began to decrease.

Lens Protein Time Study

Figure 2A shows the results of SDS polyacrylamide electrophoresis (PAGE) of the total soluble nuclear protein from lenses of control and selenite treated pups. From 10 to 21 days of age, nuclear proteins from control animals (lanes 1, 2, and 3) showed few changes, except for a decrease in the apparent molecular weight M_r = 30 K band. However, by 2 days postinjection in the selenite treated animals (lane 5), a major decrease in 30 K and 26 K bands was noted. An additional loss of the 27 K band occurred by 4 days postinjection (lane 7). The intensity of bands in the 25 K region also increased following selenite injection, and then decreased with time, and were nearly absent by 11 days postinjection (lane 9). Over this time period, new low molecular weight bands also increased in the 18 K region. In contrast, the soluble protein from cortex showed no changes during selenite cataractogenesis when analyzed by SDS-PAGE (data not shown).

The nuclear insoluble protein from control lenses (Fig. 2B, lanes 1,

2, and 3) underwent age related changes from 10 to 21 days. These changes included a decrease in polypeptides above 25 K and an increase in polypeptides in the 21-25 K range. The band at 20 K also decreased in controls during aging. The nuclear insoluble protein from selenite-injected rats underwent similar changes as the control lenses, except the changes were more pronounced and occurred more rapidly. By 2 days postinjection (lane 5), a loss of 83, 52, 30, and 26 K polypeptides occurred. By 3 days postselenite injection (lane 6), the 27 and 20 K polypeptides were also lost. The age related increase in the 21-25 K bands occurring in the control lenses, proceeded more rapidly and to a greater extent in the selenite treated rats. By 4 days postinjection, insoluble protein was limited almost entirely to the 21-25 K bands. These changes in insoluble protein were localized in the nucleus since selenite injection did not cause significant changes in the polypeptides in insoluble protein from cortex (data not shown). Furthermore, a fivefold increase in the proportion of insoluble protein was found to be localized in the nucleus and not the cortex (Table 1).

Isolation of Lens Crystallins

Major loss of the β_H, and a partial loss of the β_L-crystallin fraction, occurred in the nucleus from cataractous selenite lenses 4 days postinjection (Fig. 3A). No changes were observed in the relative proportions of the crystallins from the cortex of cataractous selenite lenses. Thus, the loss of β-crystallins was localized in the lens nucleus, which contained the opacity.

The α-crystallin fractions were concentrated and applied to an agarose A5-M column (Fig. 3B) to isolate any high molecular weight species, which

eluted together with α-crystallin on the Sephadex G-200 column. The amount of protein eluting at the void volume was very minimal in both the control and cataractous selenite lens nucleus, indicating that high molecular weight aggregates were not a significant proportion of the nuclear soluble protein in selenite cataract. The α-crystallin isolated from cortical regions also contained no significant amount of high molecular weight protein (data not shown). In addition to the α-crystallin peak, another peak eluted at approximately fraction number 85 in equal amounts in both control and cataractous lenses. This protein coeluted with α-crystallin during the G-200 separation, and its identity was unknown.

The various crystallins from control and cataractous lenses were further analyzed by SDS-PAGE (Fig. 4). In selenite lenses, dramatic changes took place in the α (lane 5), β_H (lane 6), and β_L (lane 7) fractions, while the γ (lane 8) fraction appeared to be unaffected. The α-crystallin fraction from the cataractous selenite nucleus contained three new polypeptides below 20 K. New 25 and 23 K bands were also present in α-crystallin, which corresponded to the size of several β_H and β_L polypeptides. The appearence of these putative β-polypeptides in α-crystallin was not due to poor resolution during column chromatography steps, since the α-crystallin was passed through 2 columns with different exclusion limits (Fig. 3) before SDS-PAGE. It is worth noting that these changes in α-crystallin polypeptides occurred without a major change in the concentration or molecular weight of α-crystallin (Fig. 3).

Figure 4, lanes 6 and 7 also show that changes in the 30, 27, 26, and 25 K polypeptides seen in cataractous total soluble proteins (Fig. 2A) were caused by changes in β_H and β_L-crystallin aggregates. The reduction in 30, 27, and 26 K polypeptides during selenite cataractogenesis can be explained

both by the relative decrease of these individual polypeptides, and by the overall decrease in the concentrations of β_H and β_L-aggregates (Fig. 3A). A major proportion of the β_H and β_L-aggregates from the selenite cataractous nucleus was composed of a new 25 K polypeptide.

Unlike nuclear crystallins, isolated cortical crystallins showed no significant changes in polypeptide composition following selenite cataract formation (data not shown).

Cortical and nuclear soluble crystallins and insoluble protein from control and selenite cataractous rat lenses, at 4 days postselenite injection, also were separated by SDS-PAGE without prior reduction with 2-mercaptoethanol (data not shown). Although there was an appearence of a putative 40 K dimer of α-crystallin in the cortex of cataractous lenses, these gels did not reveal any increase in disulfide-linked high molecular weight aggregates. This finding was also in agreement with the chromatography data shown in Figure 3, where no high molecular weight aggregates were found following selenite cataract formation.

Intrinsic Membrane Proteins during Selenite Cataractogenesis

Intrinsic membrane proteins from the lens nucleus were isolated and analyzed by SDS-PAGE (Fig. 5). In control rats, the major lens intrinsic membrane protein (MP 26) appeared at 26 K (lane 1). Heating at 100°C for 5 min caused the characteristic coagulation of MP 26, and resulted in the loss of MP 26, and appearance of protein that would not enter the gel (lane 2). Two days postselenite injection, a decrease of MP 26 and an increase in a smaller polypeptide at approximately 25 K occurred (lane 3). Both the remaining MP 26 and the 25 K polypeptide were coagulated by heating at 100°C (lane 4). By 4 days postinjection, MP 26 was decreased dramatically and 24

and 22 K polypeptides appeared (lane 5), which also were coagulated by heating (lane 6). Similar analysis of intrinsic membrane proteins from lens cortex following selenite injection indicated no loss of cortex MP 26 (data not shown).

Incubation of Normal Rat Lens Homogenates with Calcium

Incubation of cortical and nuclear homogenates of normal lens from 10-day-old rat pups with 3.0 mM calcium produced many of the polypeptide changes observed after selenite administration in vivo. That is, in the soluble proteins, calcium incubation caused a decrease in the 30 K polypeptide and a disappearance of the 26 K polypeptide (Fig. 6A, lane 2). New bands also appeared in the calcium incubated sample at 25, 19, and 18 K. EGTA prevented these changes (lane 1). Calcium also activated proteolysis during incubation at 4° C, except the new bands at 19 and 18 K did not appear (lane 3). Heating the nuclear homogenates to 70° C for 1 min prior to addition of calcium inhibited the calcium-induced changes (lane 4). Addition of PMSF (a serine protease inhibitor) at 1 mM concentration did not inhibit the calcium-induced changes (lane 5), while addition of 0.2 mM leupeptin (an inhibitor of calcium activated protease in other tissues) did (lane 6). Five mM iodoacetate was also effective in inhibiting the calcium-induced changes (lane 7). The activation was specific for calcium, since addition of 3.0 mM magnesium did not activate the proteolysis (lane 8).

The lens nuclear insoluble protein also was isolated from the homogenates incubated with calcium (Fig. 6B). Calcium incubation caused the decrease or disappearance of bands at 83, 52, 30, and 26 K and the appearance of a new band at 19 K (lane 2). As was found in the soluble

protein, 4° C caused little inhibition of the calcium affect (lane 3). However, unlike the results with the soluble protein, heating the homogenates to 70° C prior to incubation did not completely abolish the calcium induced loss of insoluble polypeptide bands (lane 4). Heating to 70° C also caused insolubilization of various crystallins in the homogenates, which likely accounts for the increase in the 20K band in the insoluble fraction. As was found in the soluble lens fraction, PMSF did not inhibit the polypeptide loss (lane 5), while leupeptin and iodoacetate inhibited polypeptide loss (lanes 6 and 7). Also, substitution of magnesium for calcium did not induce polypeptide changes (lane 8).

When lens cortical homogenates were incubated with 3.0 mM calcium, similar results were obtained, indicating that the lens cortex as well as the nucleus exhibits calcium activated proteolysis (data not shown).

Intrinsic Membrane Proteins Following Incubation of Lens Homogenates with Calcium

Homogenates of normal 10-day-old rat lens nucleus and cortex were incubated with 2.0 mM calcium, and the intrinsic membrane proteins isolated (Fig. 7). Calcium incubation caused a new band to appear at approximately 25 K (lane 3), which like MP 26 was coagulated by heating (lane 4). Similar results were obtained in cortical homogenates (lanes 5-8). The appearance of the 25 K polypeptide in the intrinsic membrane fraction caused by calcium also could be inhibited by 0.2 mM leupeptin (data not shown).

DISCUSSION

The major findings of this study were that the selenite cataract was associated with proteolysis occurring specifically in the lens nucleus, and that this proteolysis appeared to be activated by increased lenticular calcium.

Proteolysis during selenite cataractogenesis was indicated because a rapid loss of numerous polypeptides in the lens nucleus was observed. Polypeptides were lost in all three soluble, insoluble, and intrinsic membrane fractions. In the insoluble protein of cataractous lenses, a rapid loss of all polypeptides above 25 K occurred, so that by 3 days postinjection, only 25-21 K polypeptides remained. In the nuclear soluble proteins, 30, 27, and 26 K β-crystallin polypeptides decreased. The nuclear intrinsic membrane protein MP 26 also was reduced drastically by 4 days post-injection. It should be noted that losses of higher molecular weight polypeptides also occurred in the insoluble nuclear fraction of normal control lenses during maturation from 10 to 21 days of age. Proteolysis in the nuclear insoluble protein, which required 11 days in control rats took place in only 2 days following selenite injection. The polypeptide pattern in the nuclear insoluble protein from 21-day-old control rats, and 12-day-old, 2 days post-injection rats was remarkably similar. Thus, selenite appeared to accelerate maturational proteolysis of the insoluble protein in the rat lens. We propose that the protease responsible for the maturational changes was also responsible for the proteolysis occurring in the nuclear soluble and membrane protein fractions following selenite injection.

Several pieces of evidence suggested that calcium activated the proteolysis observed in the selenite cataract. First, increased lens calcium and proteolytic changes occurred simultaneously during selenite cataractogenesis. At 1 day postinjection, only minor elevation of lens nuclear calcium and no nuclear proteolysis were observed. By 2 days postinjection, both calcium elevation and proteolysis occurred in the nucleus. This proteolysis continued during the time of calcium elevation, and essentially ended when the lens calcium levels began to decrease.

A second piece of evidence indicated that calcium-activated proteolysis may be involved in selenite cataractogenesis. This was the finding that in vitro incubation of normal lens homogenates with calcium reproduced some of the proteolytic changes caused by selenite in vivo. Changes that could be duplicated were: (1) degradation of soluble 30 and 26 K polypeptides and increase in 25 K polypeptide, (2) loss of insoluble 83, 52, 30, and 26 K polypeptides, and (3) partial degradation of MP 26. Calcium incubation did not cause as extensive degradation of lens polypeptides as observed following selenite injection. However, those polypeptides first decreased after selenite injection were also the polypeptides decreased by calcium incubation. Russell (11) recently described remarkably similar soluble protein and membrane changes when rat lens homogenates were incubated with calcium. Our findings also are supported by Hess et al (18). Using the whole lens, these workers found a loss of insoluble polypeptides following selenite injection, which could be duplicated by incubation of lens homogenates with calcium. Several workers using bovine lenses also have demonstrated that calcium-activated proteolysis caused the degradation of the 57-58 K protein vimentin (12,13). In the present study we found a 57 K polypeptide in the cortex insoluble protein (data not shown) that was absent

from the nucleus of both control and cataractous lenses. However, insoluble polypeptides with molecular weights both above and below vimentin were lost in the nucleus following selenite injection. Parenthetically, it also should be noted that incubation of cortex homogenates with 3.0 mM calcium activated proteolysis in vitro, but proteolysis was not observed in the cortex in vivo after selenite injection. We postulate that the calcium-activated proteolysis observed in cortical homogenates did not occur in vivo because calcium levels in the cortex of the selenite injected animals were not elevated sufficiently.

The third piece of evidence that supported the involvement of calcium-activated proteolysis in selenite cataract was the finding that the protease(s) responsible for the proteolysis in lens homogenates incubated with calcium exhibited properties similar to those of calcium activated protease. This enzyme has been extensively characterized in other tissues (19-21) and recently purified in bovine (14) and rabbit lenses (personal communication with B.-S. Hong, Dept. of Ophthalmology and Visual Sciences and Biochemistry, Texas Tech University Health Sciences Center, Lubblock, TX). Some of the similar properties were: activation by mM calcium, inhibition by EGTA, leupeptin, and iodoacetate, lack of inhibition by PMSF, and little or no activation by magnesium.

One of the consequences of the extensive proteolysis observed in selenite-treated lenses may be the insolubilization of lens proteins. The nuclear region of the selenite cataractous lens showed major increses in insoluble protein. High molecular weight aggregates, that scatter light are thought to be a cause of lens opacity (22). Since no high molecular weight aggregates were found in the soluble nuclear proteins 4 days following selenite injection, the increased nuclear insoluble protein fraction may be

responsible for the opacity. Nonreducing SDS-PAGE indicated that this insoluble protein did not contain intermolecular disulfide linkages. Thus, if the insoluble protein in selenite cataract does exist in light-scattering aggregates, its aggregation may be hydrophobic in nature instead of covalent.

Insoluble protein in selenite cataractous lenses reacted with antibody to α-crystallin (personal communication with J.L. Hess, Dept. of Biochemistry and Nutrition, Virginia Tech., Blacksburg, Va.). However, our data suggested that the majority of the insoluble protein in the selenite cataract nucleus may be derived from β_H and β_L-crystallin fractions. During selenite cataractogenesis, there were major losses of β_H and β_L-crystallins. Loss of β_H-crystallin has been reported in other experimental cataracts (23-35). Soluble 30, 27, and 26 K β-crystallin polypeptides were lost following selenite injection. These partially degraded polypeptides and the remaining β-crystallin polypeptides may be the source of the increased insoluble protein. The 25-21 K molecular weight range of the insoluble protein found in the cataractous nucleus supports this conclusion, however this cannot be proven until the insoluble protein and β-crystallin polypeptides are compared by peptide mapping or immunologic tests.

An important consequence of proteolysis in selenite cataract was the loss of membrane protein. By 2 days postselenite injection, the lens nuclear MP 26 protein began to decrease and was replaced by new 24 and 22 K polypeptides at day 4 postinjection. The loss of MP 26 is significant because it is the major protein associated with lens gap junctions (26). Its degradation in the selenite cataracts lens may cause isolation of the nuclear region and contribute to the opacification.

Thus, the present investigation has led to the hypothesis that elevated calcium may initiate a series of extensive proteolytic changes following selenite treatment. Proteolysis of nuclear β-crystallins may lead to production of insoluble aggregates, which scatter light. Proteolysis of membranes also may contribute to cataract formation. We suggest that calcium-activated protease also may play a role in other catararcts containing elevated calcium.

REFERENCES

1. Bunce GE, and Hess JL: Biochemical changes associated with selenite-induced cataract in the rat. Exp Eye Res 33:505,1981.
2. Bhuyan KC, Bhuyan DK, and Podos SM: Selenium-induced cataract: biochemical mechanism. In Proceedings of Second International Symposium on Selenium in Biology and Medicine, Spallholz, JE, Martin, JL, and Ganther, HE, editors. Westport, CT, AVI Publishing Co., 1981, pp. 403-412.
3. Shearer TR, Anderson RS, Britton JL, and Palmer EA: Early development of selenium-induced cataract: slit lamp evaluation. Exp Eye Res 36:781, 1983.
4. Shearer TR, and David LL: Role of calcium in selenium cataract. Curr Eye Res 2:777, 1983.
5. Bunce GE, Hess JL, and Batra R: Lens calcium and selenite-induced cataract. Curr Eye Res 3:315, 1984.
6. David LL, and Shearer TR: State of sulfhydryl in selenite cataract. Toxicol Appl Pharmacol 74:109, 1984.
7. Hightower KR, and Reddy VN: Calcium content and distribution in human cataract. Exp Eye Res 34:413, 1982.
8. Duncan G, and Bushell AR: Ion analyses of human cataractous lenses. Exp Eye Res 20:223, 1975.
9. Hightower KR, Giblin FJ, and Reddy VN: Changes in the distribution of lens calcium during development of x-ray cataract. Invest Ophthalmol Vis Sci 24:1188, 1983.
10. Horwitz J, Hansen JS, Cheung C-C, Ding L-1, Straatsma BR, Lightfoot DO, and Takemoto LJ: Presence of low molecular weight polypeptides in human brunescent cataracts. Biochem Biophys Res Commun 113:65, 1983.

11. Russell P: In vitro alterations similar to posttranslational modification of lens proteins. Invest Ophthalmol Vis Sci 25:209, 1984.

12. Ireland M, and Maisel H: Evidence for a calcium activated protease specific for lens intermediate filaments. Curr Eye Res 3:423, 1984.

13. Roy D, Chiesa R, and Spector A: Lens calcium activated proteinase: degradation of vimentin. Biochem Biophys Res Commun 116:204, 1983.

14. Yoshida H, Murachi T, and Tsikahara I: Limited proteolysis of bovine lens α-crystallin by calpain, a Ca_2^+-dependent cysteine proteinase, isolated from the same tissue. Biochim Biophy Acta 798:252, 1984.

15. Russell P, Robison WG, and Kinoshita JH: A new method for rapid isolation of the intrinsic membrane proteins from lens. Exp Eye Res 32:511, 1981.

16. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680, 1970.

17. Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ: Protein measurement with the Folin phenol reagent. J Biol Chem 193:265, 1951.

18. Hess JL, Bunce GE, and Wisthoff FE: Changes in lenticular insoluble proteins in selenite induced cataract in rats. ARVO Abstracts. Invest Ophthalmol Vis Sci 25(Suppl):134, 1984.

19. Murachi T, Tanaka K, Hatanaka M, and Murakami T: Intracellular Ca^{2+}-dependent protease (calpain) and its high-molecular-weight endogenous inhibitor (calpastatin). Adv Enzyme Regul 19:407:1981.

20. Siman R, Baudry M, and Lynch G: Purification from synaptosomal plasma membranes of calpain I, a thiol protease activated by micromolar calcium concentrations. J Neurochem 41:950, 1983.

21. Kajikawa N, Kishimoto A, Shiota M, and Nishizuka Y: Ca^{2+}-dependent neutral protease and proteolytic activation of Ca^{2+}-activated, phospholipid-dependent protein kinase. In Methods in Enzymology, Vol 102, Means AR and O'Malley BW, editors. New York, Academic Press, 1983, pp. 279-290.

22. Benedek GB: Theory of transparency of the eye. Appl Optics 10:459, 1971.

23. Bunce GE, Hess JL, and Fillnow GM: Investigation of low tryptophan induced cataract in weanling rats. Exp Eye Res 26:399, 1978.

24. Hu T-S, Russell P, and Kinoshita JH: In vitro incubation paralleling changes occurring during mouse cataract formation. Exp Eye Res 35:521, 1982.

25. Garber AT, Stirk L, and Gold RJM: Abnormalities of crystallins in the lens of the Cat FRASER mouse. Exp Eye Res 36:165, 1983.

26. Takemoto LJ, and Hansen JS: Gap junctions from the lens: purification and characterization by chemical crosslinking reagent. Biochem Biophys Res Commun 99:324, 1981.

Table 1. Localization of Lens Insoluble Protein %

Region	% Total lens protein	
	Control	Cataractous[1]
Cortex	1.8 ± 1.1[2]	3.6 ± 1.3
Nucleus	2.8 ± 0.4	17.8 ± 12.8[3]

[1] Four days after selenite injection on day 10.
[2] Mean ± standard deviation (n = 5).
[3] Mean significantly different from controls at $P < 0.05$.

Figure 1

Concentrations of total calcium in the lens cortex (A) and nucleus (B) of control rats (open circles) and selenite-injected rats (crosses). Treated and control rats were both 10 days old at time of injection. Each data point is an average of four to six pools of four lens regions. Error bars represent $\pm$ 1 SD. The means on days 2 and 4 postinjection in A, and 1, 2, 4, and 8 days postinjection in B, are significantly different at $P < 0.05$

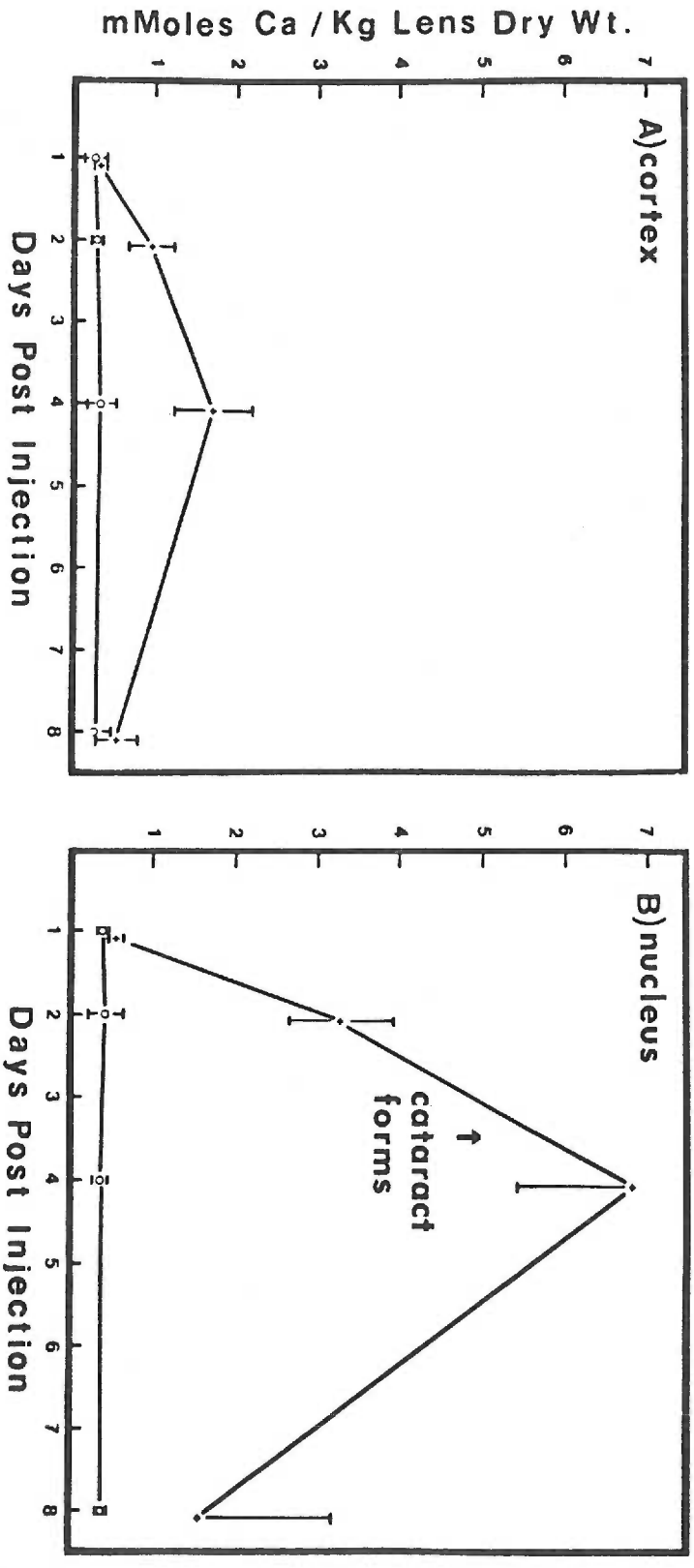
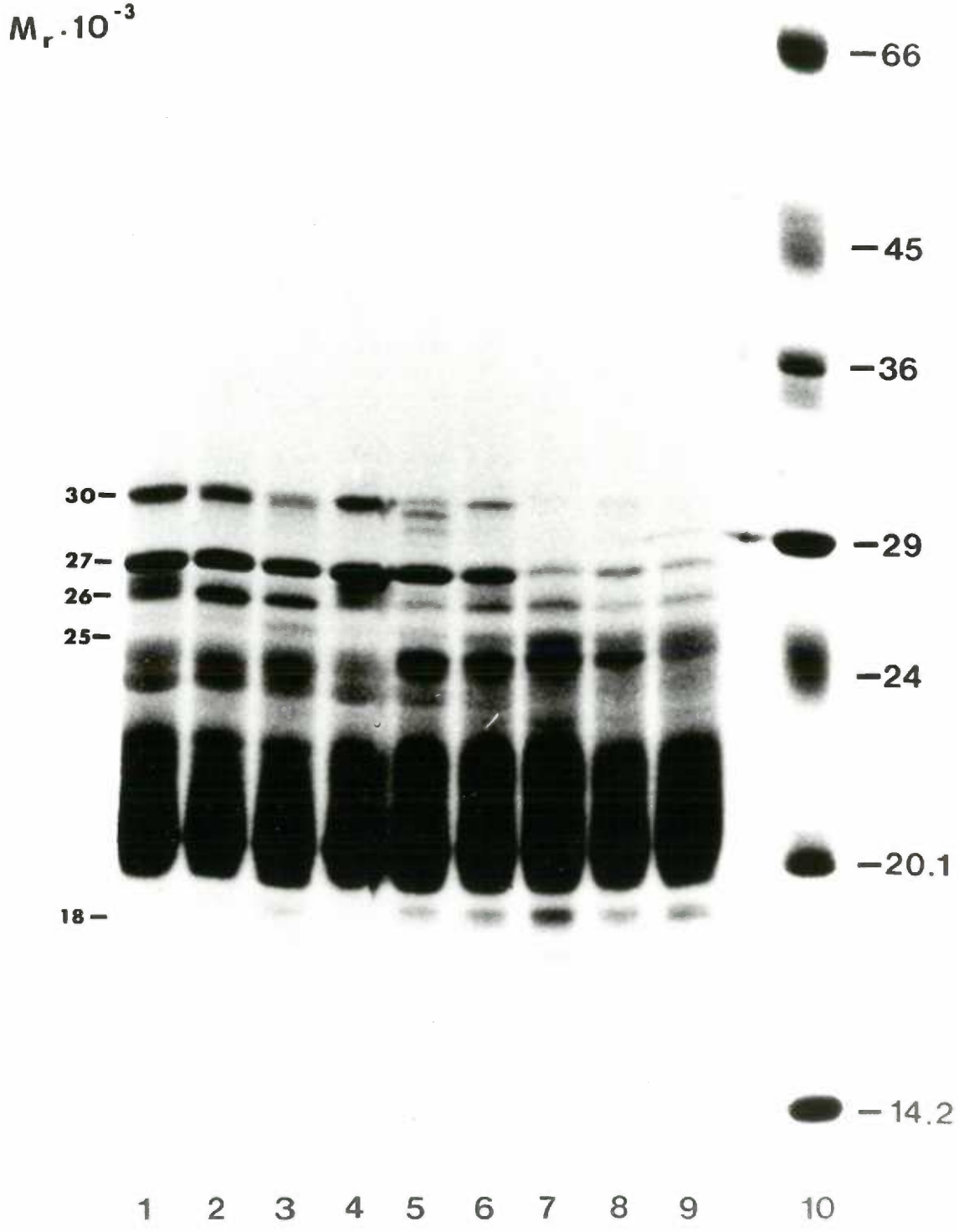

mMoles Ca / Kg Lens Dry Wt.
A) cortex
Days Post Injection
B) nucleus
cataract forms
Days Post Injection

Figure 2

SDS-PAGE of rat lens nuclear soluble (A) and insoluble (B) proteins. Lanes 1, 2, and 3 were from control rats 10, 17, and 21 days of age, respectively. Lanes 4, 5, 6, 7, 8, and 9 were from selenite-injected rats injected on day 10 of age and killed on 1, 2, 3, 4, 7, and 11 days postinjection, respectively. Lane 10 contains 7 molecular weight markers with corresponding molecular weights indicated to the right. Numbers to the left show positions and apparent molecular weights (M_r) of polypeptides referred to in text.

A



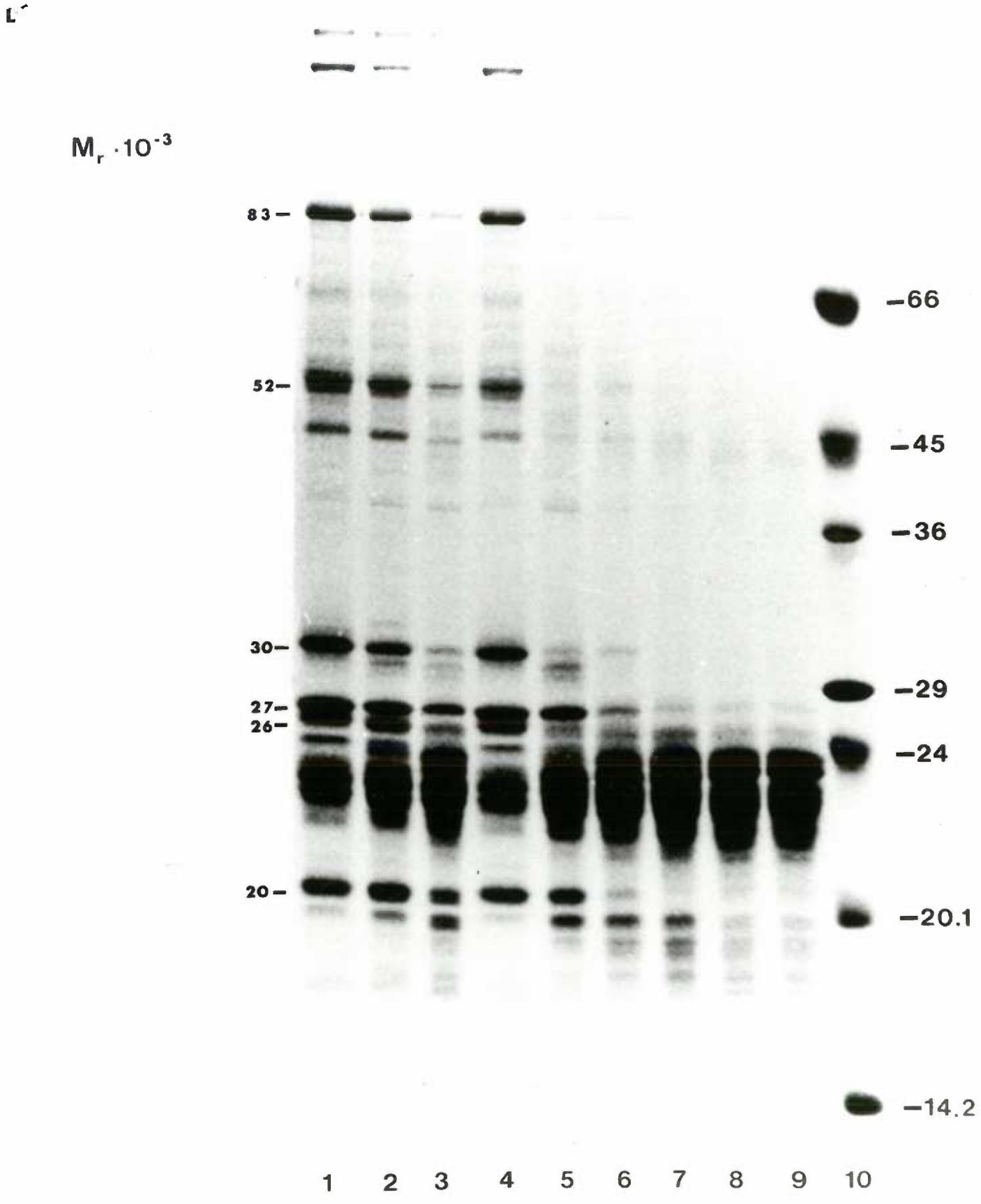

$M_r \cdot 10^{-3}$
83
52
30
27
26
20
66
45
36
29
24
20.1
14.2
1 2 3 4 5 6 7 8 9 10

Figure 3

Gel filtration profiles of lens nuclear soluble protein from 14-day-old control rats (____), and 14-day-old, 4-day postselenite injection rats (---). (A) Sephadex G-200 profile of 60 mg of control and selenite-treated rat soluble nuclear protein. The % of the total area under the peaks of control nuclear soluble protein was: α-15.2, β_H-13.2, β_L-17.0, and γ-54.7. The % total area under the peaks of selenite cataractous nuclear soluble protein was: α-18.9, β_H-4.7, β_L-11.7, and γ-64.9. (B) A5M profile of 3.5 mg of control and selenite-treated rat α crystallin fraction pooled from part A, V_o indicates position of void volume. Vertical bars in A and B indicate which which fractions were pooled for subsequent SDS-PAGE and integration of peak areas.

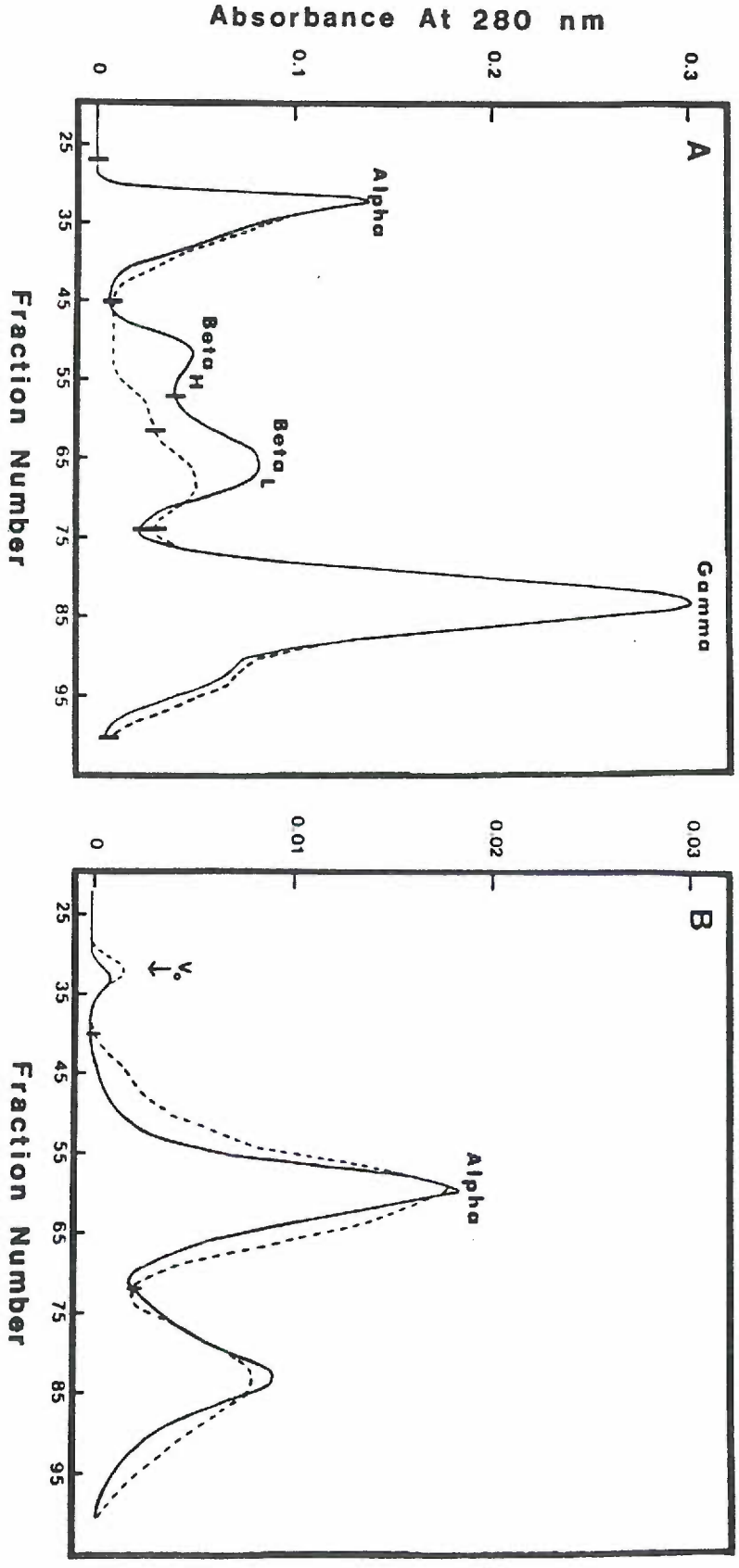

Absorbance At 280 nm
A
Alpha
Beta H
Beta L
Gamma
Fraction Number
B
Alpha
Fraction Number

Figure 4

SDS-PAGE of the crystallins from the nuclear region of rat lenses. Lanes 1, 2, 3, and 4 were α, β_H, β_L, and γ-crystallin fractions from 14-day-old control rats, respectively. Lanes 5, 6, 7, and 8 were α, β_H, β_L, and γ-crystallin fractions from 14-day-old, 4-day postselenite-injected rats, respectively. Lane 9 contains molecular weight markers.

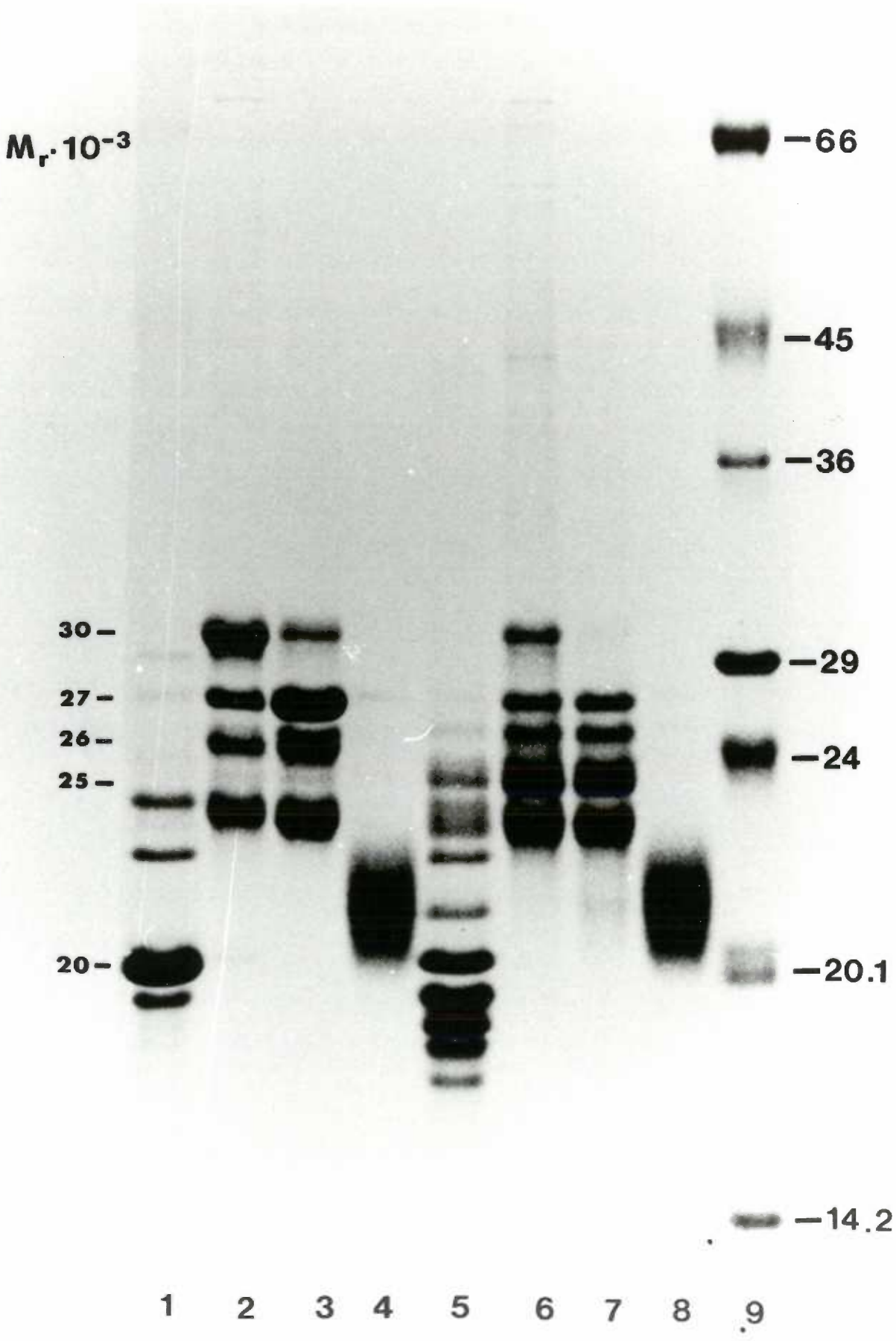

$M_r \cdot 10^{-3}$
66
45
36
30
27
26
25
29
24
20
20.1
14.2
1
2
3
4
5
6
7
8
9

Figure 5

SDS-PAGE of rat lens nuclear intrinsic membrane proteins. Lane 1, 14-day-old control rats; lane 2, same as 1, except heated at 100°C for 5 min; lane 3, 12-day-old, 2-day postselenite injection rats; lane 4, same as 3 except heated; lane 5, 14-day-old, 4-day postselenite injection rats; lane 6, same as 5 except heated. Lane 7 contains molecular weight markers.

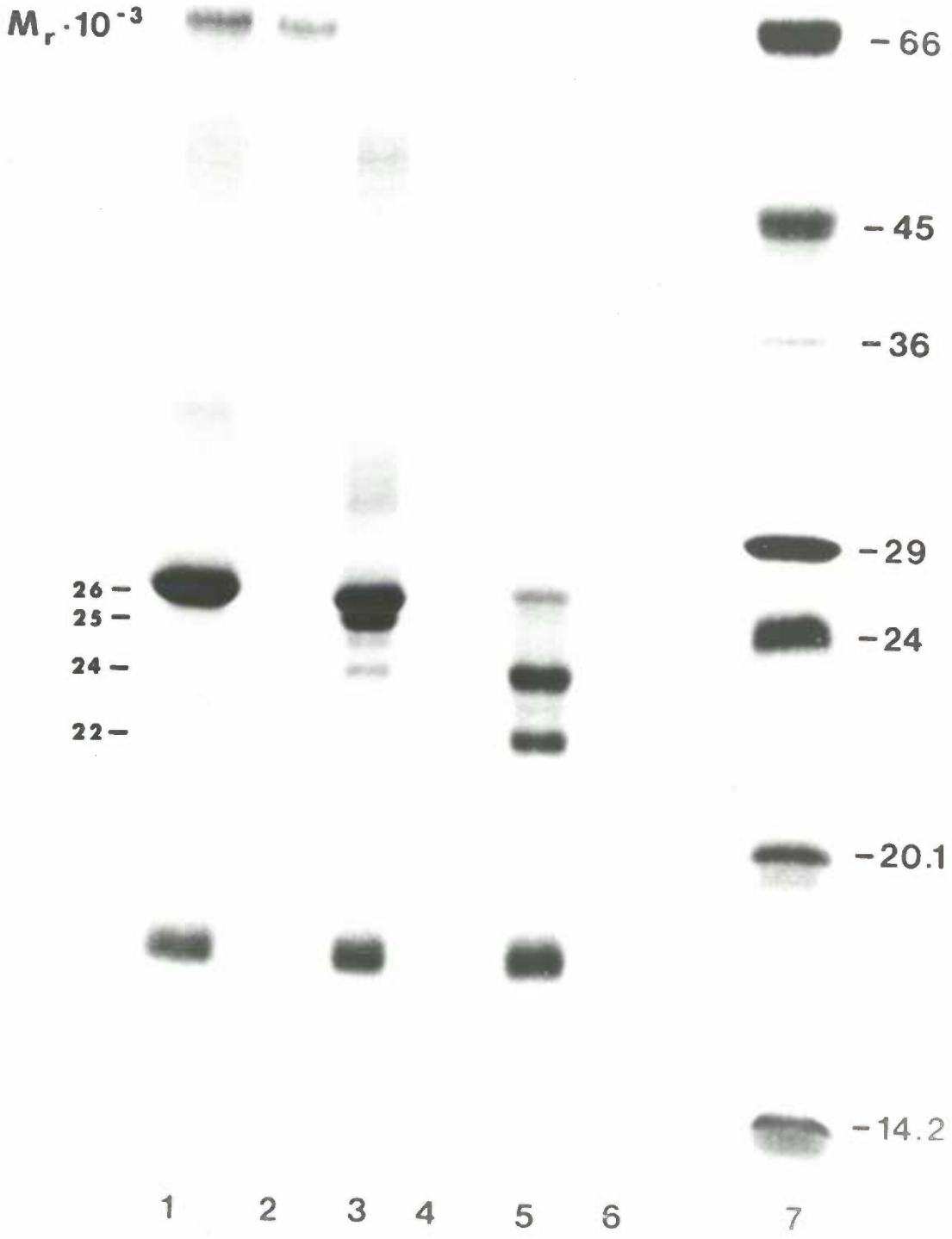
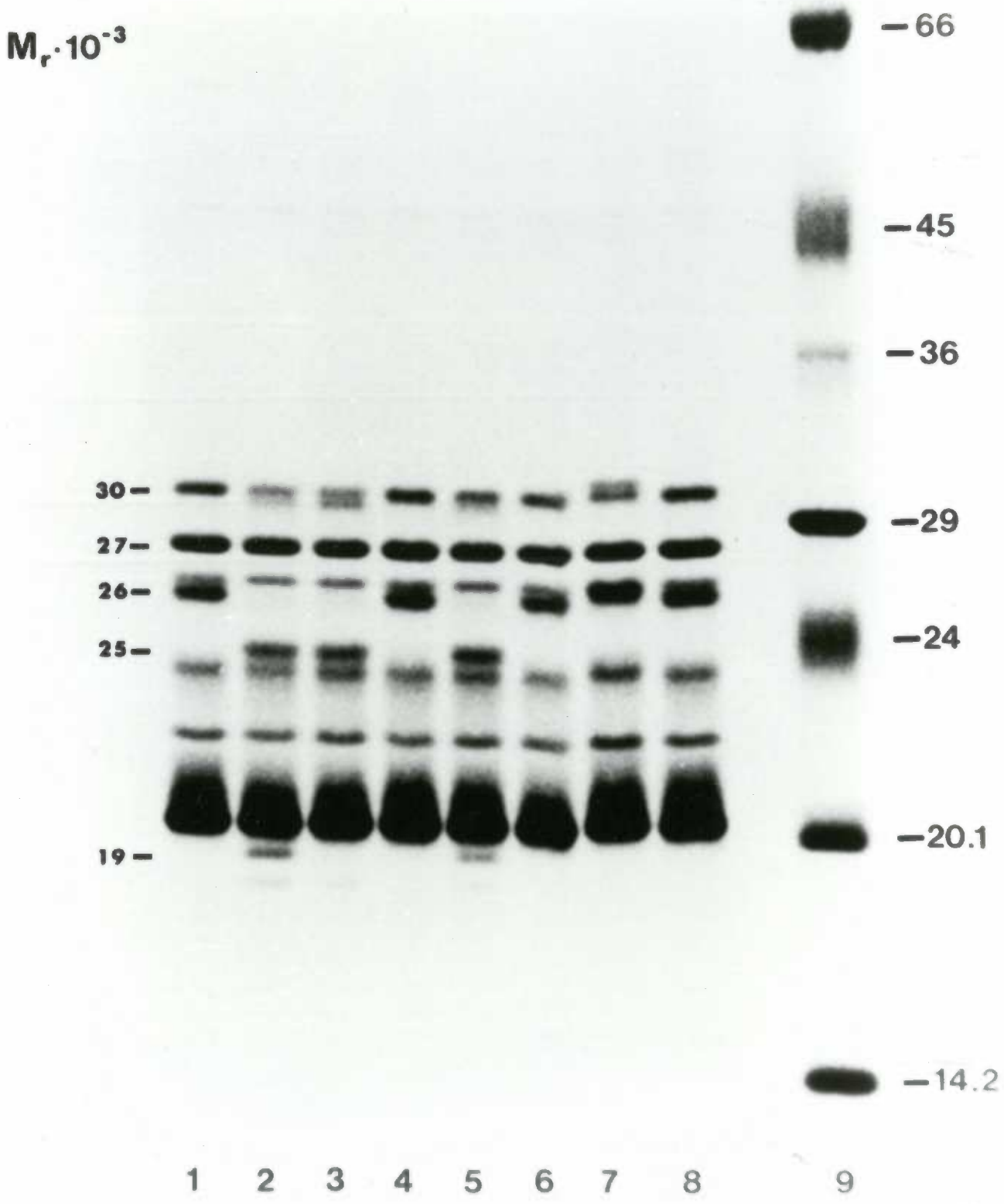

$M_r \cdot 10^{-3}$
66
45
36
29
24
20.1
14.2
26
25
24
22
1
2
3
4
5
6
7

Figure 6

SDS-PAGE of rat lens soluble (A) and insoluble (B) proteins isolated from nuclear homogenates incubated for 2 hr at 37°C with; lane 1, 1.0 mM EGTA: lane 2, 3.0 mM Ca^{++}; lane 3, 3.0 mM Ca^{++} -homogenate incubated at 4°C instead of 37°C; lane 4, 3.0 mM Ca^{++} -homogenate first heated to 70°C for 1 min before incubation; lane 5, 3.0 mM Ca^{++} + 1.0 mM PMSF: lane 6, 3.0 mM Ca^{++} + 0.2 mM leupeptin; lane 7, 3.0 mM Ca^{++} + 5.0 mM iodoacetate; lane 8, 3.0 mM Mg^{++}. Lane 9 contains molecular weight markers.

A



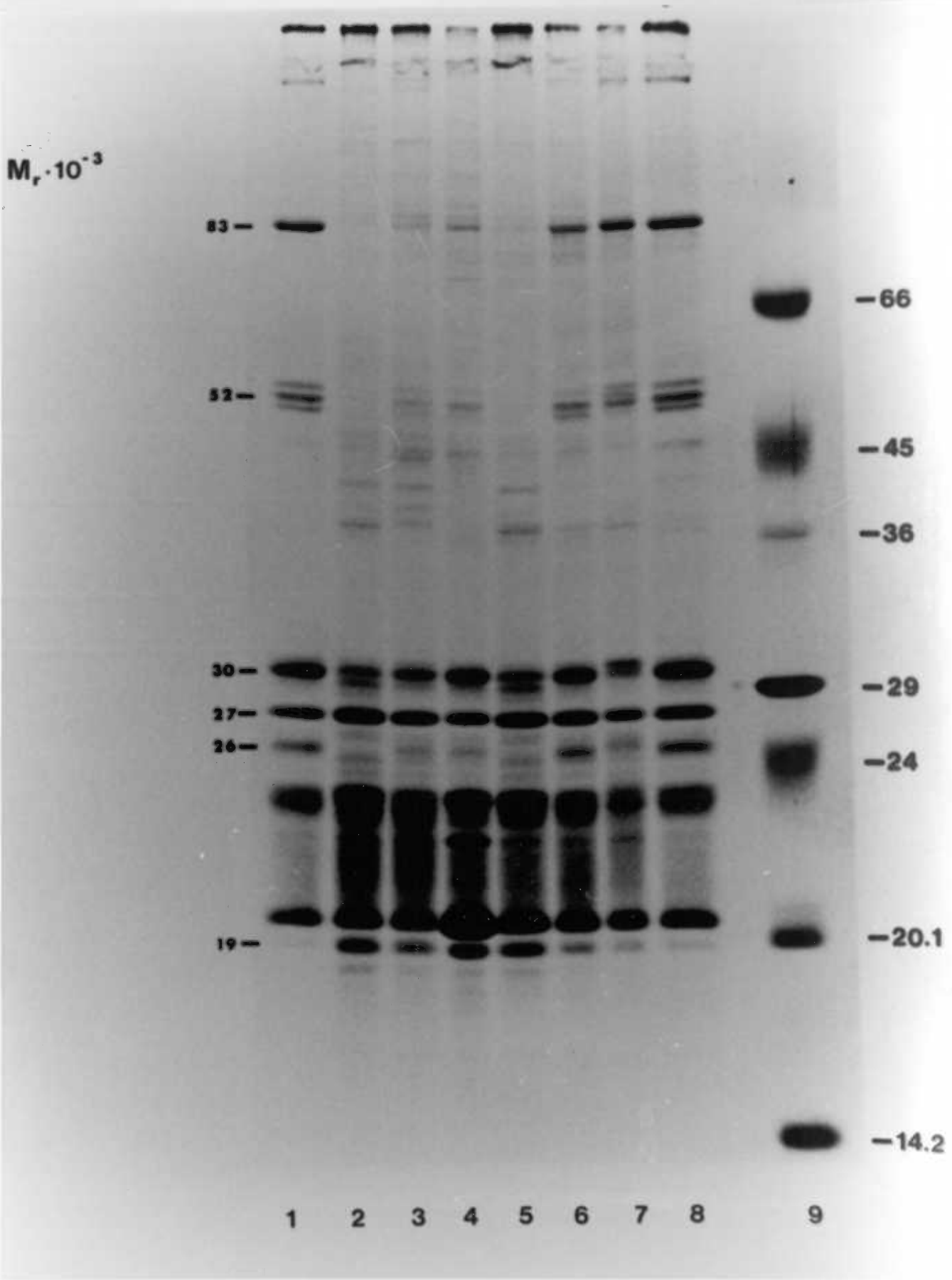
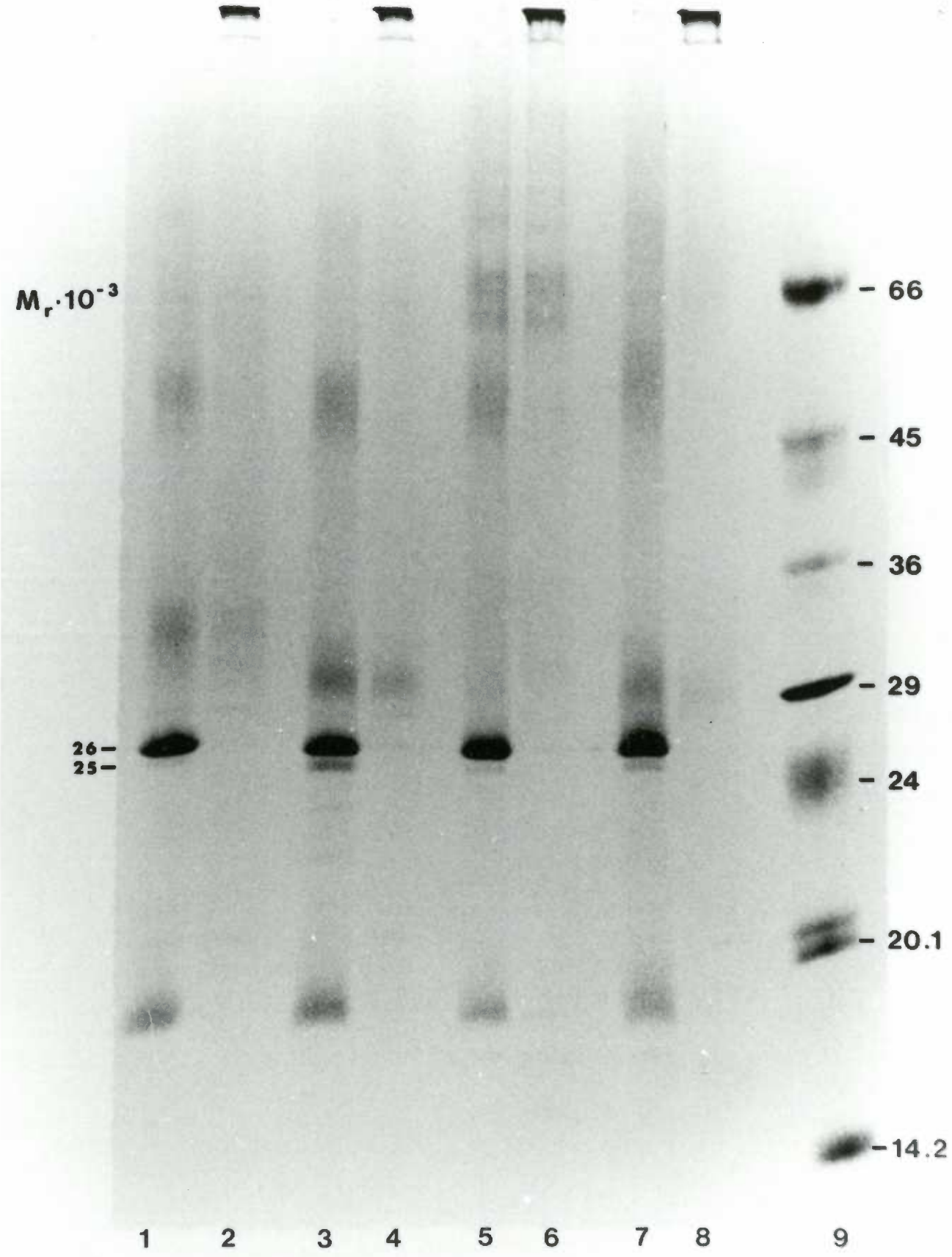

B
$M_r \cdot 10^{-3}$
83
52
30
27
26
19
66
45
36
29
24
20.1
14.2
1
2
3
4
5
6
7
8
9

Figure 7

SDS-PAGE of rat lens intrinsic membrane proteins isolated from nuclear (lanes 1-4) and cortical (lanes 5-8) homogenates incubated for 1 hr at 37°C with: lanes 1 and 5, 1.0 mM EGTA: lanes 2 and 6, same as 1 and 5, except heated to 100°C for 5 min: lanes 3 and 7, 2.0 mM Ca^{++}: lanes 4 and 8, same as 3 and 7, except heated. Lane 9 contains molecular weight markers.


$M_r \cdot 10^{-3}$
66
45
36
29
24
20.1
14.2
26
25
1
2
3
4
5
6
7
8
9

Manuscript 3

Purification of Calpain II from Rat Lens and Determination of Endogenous Substrates.

Larry L. David* and Thomas R. Shearer+

*Department of Biochemistry, School of Medicine, +Departments of Biochemistry and Ophthalmology, Schools of Dentistry and Medicine, Oregon Health Sciences University, Portland, Oregon 97201, U.S.A.

Running title: Rat Lens Calpain II

Key Words: rat lens, protease, calpain II, protein degradation, cataract.

Address reprint requests to: Dr. T. R. Shearer, Oregon Health Sciences University, Department of Biochemistry, 611 S.W. Campus Drive, Portland, Oregon 97201.

Summary

Calpain II (EC 3.4.22.17), a calcium-dependent neutral protease, was purified approximately 7000 fold from the soluble fraction of rat lens. The estimated molecular weight of rat lens calpain II was 120,000, and the enzyme was composed of 80 and 28 K subunits. Calpain II required 400 μM calcium, a reducing agent, and pH = 7.5 for maximal activity. The enzyme could not be activated by magnesium, and was inhibited by leupeptin and iodoacetate, but not phenylmethylsulfonyl fluoride. Purified calpain II degraded rat α-, β_H-, and β_L-crystallins, insoluble proteins, and intrinsic membrane proteins. γ-crystallin was not degraded. The proteolysis caused by purified calpain II was similar to proteolysis occurring during the formation of several experimental cataracts in rodents: this suggested that the enzyme may play a role in cataract formation.

1. Introduction

Evidence from both experimental cataract models and human cataracts suggests that increased degradation of lens proteins may be involved in cataract formation (Piatigorsky, Kador, and Kinoshita, 1980: Roy, Garner, Spector, Carper, and Russell, 1982: Hu, Russell, and Kinoshita, 1982: Garber, Goring, and Gold, 1984: David and Shearer, 1984: Roy and Spector, 1978: Takemoto, Hansen, and Horwitz, 1983). Lenses contain both endopeptidases (Srivastava and Ortwerth, 1983a,b: Fleshman and Wagner 1984: Wagner and Margolis, 1985) and exopeptidases (Swanson, Davis, Albers-Jackson, and McDonald, 1981) which may cause proteolysis during cataract formation.

A newly characterized lens protease, calpain (EC 3.4.22.17), may be responsible for proteolysis occurring in an experimental rodent cataract (David and Shearer, 1984). Calpains are sulfhydryl, neutral endopeptidases found ubiquitously in a variety of mammalian tissues (Ohno, Emori, Imajoh, Kawasaki, Kisaragi, and Suzuki, 1984). They exist in two forms: calpain I requires approximately 10 μM calcium for full activation, and calpain II requires nearly 1 mM calcium for full activation (Yoshida, Murachi, and Tsukahara, 1985).

Several lines of evidence indicate that calpain may be active in lens tissue. The presence of calpain in lenses was first suggested when proteolysis was induced in whole lenses and crude lens homogenates by incubation with calcium (Roy, Chiesa, and Spector, 1983: Russell, 1984: Ireland and Maisel, 1984: David and Shearer, 1984: Lorand, Conrad, and Velasco, 1985). Recently calpains I and II and the endogenous inhibitor, calpastatin, were assayed in bovine lenses and showed regional distributions (Yoshida et al., 1985). Bovine lens calpain II was also purified to

homogeneity, and the enzyme hydrolyzed α-crystallin, vimentin, and actin (Yoshida et al., 1984a,b).

Although cataracts in rodents have been used extensively for models of cataractogenesis, calpain in rat lens has never been purified or characterized. The goal of the present study was to purify calpain II from rat lens and to determine its properties and endogenous substrates. The substrate specificity of partially purified calpain II provided evidence that calpain may be responsible for the proteolytic changes observed in rodent cataracts.

2. Materials and Methods

Purification of Rat Lens Calpain II

Lenses were obtained from 1-6 month old Sprague-Dawley rats (Simonsen Laboratories, Gilroy, CA). Decapsulated lenses were stored frozen at -70° C in buffer A containing 20 mM Tris-HCl (pH=7.5), 1.0 mM EGTA, 1.0 mM EDTA, and 10 mM 2-mercaptoethanol. One hundred and twenty thawed lenses were homogenized on ice in 12 ml of buffer A. The lens homogenate was centrifuged at 10,000 g for 30 min at 4° C. Calpain II was purified by 3 sequential chromatography steps. Initial attempts using the procedure for purification of bovine lens calpain (Yoshida et al., 1984a) were not successful with rat lens because of poor recovery of rat calpain during blue agarose chromatography. In our procedure, the supernatant was first applied to a 2.5 x 10 cm column of DEAE Bio-Gel A (Bio Rad Laboratories, Richmond, CA) at 4° C. The column was washed for 5 hours with buffer A at a flow rate of 50 ml/hour. Bound proteins were then eluted with 500 ml linear gradient of 0-300 mM NaCl in buffer A at a flow rate of 50 ml/hour. Fractions showing calpain II activity were pooled and concentrated by ultrafiltration (YM5 filter, Amicon Corporation, Lexington, MA) and applied to a 7.5 x 300 mm TSK 3000 SW high pressure liquid chromatography (HPLC) gel filtration column equilibrated with buffer containing 20 mM imidazole (pH = 7.0), 1.0 mM EGTA, 1.0 mM EDTA, 10 mM 2-mercaptoethanol, and 100 mM Na_2SO_4. Proteins were eluted at 0.5 ml/min, and active fractions were pooled and diluted 1:1 with buffer A containing 2 M $(NH_4)_2SO_4$. The final chromatography step utilized a 7.5 X 75 mm TSK Phenyl 5-PW HPLC hydrophobic interaction column equilibrated with buffer A containing 1.0 M $(NH_4)_2SO_4$. Following injection and a 10 min wash, proteins were eluted by a 30 min

linearily decreasing gradient of 1.0 - 0 M $(NH_4)_2SO_4$ in buffer A at 1.0 ml/min.

In order to obtain larger quantities of calpain II for substrate specificity experiments, another purification procedure utilizing only standard open columns was used. The active DEAE Bio-Gel A fractions were concentrated and applied to a 2.5 X 95 cm column of Bio-Gel A1.5m (Bio-Rad) equilibrated with buffer A containing 100 mM NaCl. Proteins were eluted at a flow rate of 25 ml/hour. Active fractions were applied directly to second 1.5 X 5.0 cm column of DEAE Bio-Gel A. This column was washed with buffer A containing 100 mM NaCl for 2 hours at 25 ml/hour. Proteins were then eluted with 100 ml linear gradient of 100-300 mM NaCl in buffer A at 25 ml/hour. This procedure resulted in a lower overall purification (approximately 1600 fold) compared to the HPLC procedure described above, but a typical recovery of 40% was obtained.

The composition of calpain II preparations following each chromatography step was monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). This and all other SDS-PAGE in the investigation was carried out in 15 x 20 cm 12% gels using the method of Laemmli (1970). Gels were stained by Coomassie blue. Molecular weights of subunit polypeptides were determined by comparison to a mixture of 8 protein standards containing: phosphorylase b, bovine serum albumin, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen, trypsin inhibitor, and α-lactalbumin (Sigma Chemical Company, St. Louis, MO). The molecular weight of non-denatured calpain II was estimated during gel filtration steps by comparison to Bio-Rad gel filtration standards.

Calpain II Assay

Calpain II activity was measured using a modification of the procedure of Twining (1983). A 0.1 ml reaction mixture contained 0.06 ml of suitably diluted chromatography column eluent, 0.02 ml 5 mg/ml fluorescein isothiocyanate (FITC)-labeled casein, and 0.02 ml of a solution containing 15 mM $CaCl_2$, 20 mM Tris-HCl (pH = 7.5), and 10 mM 2-mercaptoethanol. FITC-casein was prepared from α -casein (No. C 7891, Sigma) according to the method of Twining (1983), and following dialysis and lyophilization it was dissolved in buffer containing 20 mM Tris-HCl (pH=7.5) and 10 mM 2-mercaptoethanol. Blanks were simultaneously run containing buffer, substrate, and calcium but no calpain II. Since no non-calcium activated caseinolysis was detected in any of the column fractions, it was not necessary to run blanks containing column eluent and no calcium. After incubation at 30° C for 30 min, the reaction was stopped by placing the samples on ice, 0.1 ml 6 mg/ml bovine serum albumin was added, and this was followed by addition of 0.2 ml 10% trichloroacetic acid. After centrifugation at 1000 g for 30 min, 0.3 ml supernatant was removed and added to 2.5 ml 0.5 M Tris-HCl (pH=8.6). Fluorescence of the samples was determined at 525 nm after excitation at 365 nm. The amount of FITC-labeled casein released as acid soluble fragments was determined by comparison to standards of 0-4 µg undegraded FITC-labeled casein dissolved in 2.5 ml of 0.5 M Tris-HCl (pH = 7.5) (This assumed the casein was uniformly labeled by FITC). One unit of calpain activity was defined as one µg FITC-labeled casein released as acid soluble fragments/min. Assay of calpain II was linear up to the release of at least 6 µg of acid soluble FITC-casein fragments during the 30 min incubation. This assay was found to work well

for measuring calpain II activity in all preparations of the enzyme after the initial DEAE Bio-Gel purification step. Accurate estimations of calpain II activity were more difficult in the crude supernate fractions of the lens because the assay exhibited loss of linearity at increasing amounts of supernate, possibly due to the presence of endogenous calpain inhibitor.

Calcium Activation, pH optimum, and Inhibitors

Calcium activation, pH optimum, and action of inhibitors were determined for calpain II isolated following the initial DEAE Bio-Gel purification step. For determination of calcium activation, EGTA and EDTA were removed from partially purified calpain II by extensive ultrafiltration, and then the enzyme preparation and 10 to 1000 μM $CaCl_2$ were added to the standard calpain assay. The pH optimum of rat lens calpain II was determined in 100 mM Tris-imidazole buffer at various pH's ranging from 5.5-8.5. Possible exogenous inhibitors of calpain II, including synthetic leupeptin, phenylmethylsulfonyl fluoride (PMSF), and iodoacetate (Sigma) were also tested by adding these compounds to the standard calpain assay.

Proteolysis of Isolated Lens Proteins by Calpain II

Lenses from 1-2 week old Sprague-Dawley rat pups were used as a source of proteins to test the substrate specificity of rat lens calpain II. One hundred decapsulated lenses were homogenized in buffer B containing 20 mM Tris-HCl (pH=7.5), 1.0 mM EGTA, 1.0 mM EDTA, 100 mM NaCl, and 10 mM iodoacetate at a ratio of 20 lenses/ml buffer. The homogenate was then centrifuged at 10,000 g for 30 min at 4°C, and the supernatant was applied to a 2.5 x 95 cm column of Bio-Gel A1.5m (Bio Rad) equilibrated with buffer

B containing no iodoacetate. The proteins were eluted at 25 ml/hour, and the various lens crystallins were concentrated by ultrafiltration. The insoluble pellet obtained from the 10,000 g spin of the 1-2 week old rat lenses was washed twice and resuspended by vortexing in buffer A without 2-mercaptoethanol. The insoluble lens protein remained in suspension and no solubilizing agents were used.

Proteolysis of each isolated lens protein was tested in a 0.2 ml reaction volume containing 200 µg of protein, 1.4 units (7 µg) of partially (1600 fold) purified lens calpain II (second DEAE fraction), 20 mM Tris HCl (pH=7.5), 5 mM 2-mercaptoethanol, and $CaCl_2$ in a 1.2 mM excess over EGTA and EDTA. The reaction mixture was incubated at 30 ^{o}C. At various times following the addition of calcium, samples were removed and placed in Laemmli (1970) sample buffer containing SDS which stopped proteolysis. The extent of proteolysis in each lens protein fraction was then determined by SDS-PAGE. To detect proteolysis of intrinsic membrane proteins, EGTA in 5 mM excess over calcium (instead of SDS) was added to the incubation mixture containing suspended lens insoluble protein. This terminated proteolysis, and the intinsic membrane proteins were isolated by the method of Russell, Robison and Kinoshita (1981) prior to SDS-PAGE.

Protein concentrations in this study were determined using the Bio-Rad dye binding assay with bovine serum albumin as a standard.

3. Results

Purification and Characterization of Rat Lens Calpain II

The sequential purification of calpain II from the soluble protein of rat lenses is shown in Fig. 1. During chromatography on DEAE Bio-Gel A, one peak of calcium dependent proteolysis was eluted at 180 mM NaCl (Fig. 1A). Active fractions applied to a TSK 3000 SW HPLC gel filtration column again yielded a single peak of activity (Fig. 1B). During final purification on a TSK phenyl 5-PW HPLC column, calpain II activity eluted near the end of the $(NH_4)_2SO_4$ gradient (Fig. 1C). The final enzyme preparation was purified nearly 7000 fold with a yield of 9.7 % (Table 1). Active fractions from each of the purification steps were analyzed by SDS-PAGE (Fig. 2). The final purification step shown in lane 5 revealed two major bands at 80 and 28 K and several minor bands. The relative molecular weight of the intact, partially purified enzyme was 120,000, as determined by gel filtration chromatography on Bio-Gel A 1.5m and TSK 3000 SW (graphic data not shown).

Partially purified calpain II after the DEAE Bio-Gel step required approximately 125 μM calcium for 1/2 maximal activity and 400 μM calcium for full activation (Fig. 3). The activation was specific for calcium since 20 mM magnesium would not substitute for calcium for activation. Enzymatic activity was greatest at pH=7.5 and required a reducing agent, 2-mercapto-ethanol (graphic data not shown). The enzyme was inhibited by 5.0 mM iodoacetate and 10 μM leupeptin, but not by 1.0 mM PMSF. These properties were evidence that the activity purified was calpain II.

Using an HPLC TSK DEAE 5-PW column, we were also able to quantitate calpain II in single pairs of human lenses (data to be published elsewhere).

Substrate Specificity of Rat Lens Calpain II

High molecular weight (HMW) protein, which appeared at the void volume of the Bio-Gel A1.5m column during isolation of rat lens crystallins, and α-crystallin were proteolyzed by calpain II *in vitro*. Action of calpain II on HMW protein caused the appearance of a new low molecular polypeptide band at 18 K (Fig. 4A). Action of calpain II on α-crystallin caused low molecular weight polypeptide bands at 19 and 18 K to increase with time (Fig. 4B). β_H and β_L-crystallins were also proteolyzed. The 30 K polypeptide of β_H-crystallin decreased, and was replaced with a 29 K polypeptide (Fig. 4C). In β_L-crystallin (Fig. 4D), the polypeptide at 25.5 K was rapidly lost, and a new band appeared at 24 K. Unlike the other cystallins, γ-crystallins did not undergo any observable proteolysis (data not shown). Similar to rat lens crystallins, α and β-crystallins from human lenses were partially hydrolyzed by rat lens calpain II (data not shown).

Insoluble proteins from lens were tested as substrates for purified lens calpain II (Fig. 4E). The 90, 57, 50, and 44 K polypeptides were completely proteolyzed in only 5 min. While the 57 and 44 K polypeptides were not positively identified , their apparent molecular weights suggested that they may have been vimentin and actin. Following exposure to purified calpain II, a 66 K intrinsic membrane polypeptide was also lost, and new polypeptides appeared at 29 and 25 K (Fig 4F).

4. Discussion

The present study is the first report on purification of calpain II from rat lens. Purification of rat lens calpain II and determination of its endogenous substrates was essential because a large body of information regarding the biochemical mechanism of cataract formation has been published using rodent models (Harding and Crabbe, 1984). Because of possible differences in substrate specificity and biological control, valid conclusions concerning the possible involvement of calpain in rodent cataracts could only be made by examining the enzyme from lenses of the same species.

Rat lens calpain II did show some similarities to calpain II purified from bovine lenses (Yoshida et al., 1984a,b). Bovine and rat calpain II had similar relative molecular weights of 110,000-120,000. While rat lens calpain II was not purified to apparent homogeniety, evidence suggested that like the bovine enzyme, it was composed of two subunits of approximately 80 and 29 K. This is consistent with the subunit composition of calpain II from other tissues (Kay, 1982). Both bovine and rat calpain II also degraded α-crystallin and insoluble lens polypeptides.

The major difference between bovine and rat calpain II was the finding that rat lens calpain II degraded β-crystallins, while bovine calpain II did not. Hong (1985) also recently reported that partially purified rabbit lens calpain II degraded rabbit β-crystallin. It is not known if the resistance of bovine β-crystallin to proteolysis is due to differences in β-crystallin substrates, or due to an actual difference in substrate specificity of the enzymes. The finding that β-crystallin was a substrate for rat lens calpain II was important because evidence presented below suggests that

β-crystallins, as well as other lens proteins, may undergo degradation during rodent cataract formation.

In the present study, purified rat lens calpain II produced 19 and 18 K polypeptides when reacted with α-crystallin. The 30 and 25.5 K polypeptides in β-crystallin, and the higher molecular weight polypeptides in the insoluble proteins were lost after incubation with calpain II. The 66 K intrinsic membrane was also lost, and a new 25 K intrinsic membrane polypeptide was produced. Similar changes, indicative of proteolysis, were observed in an in vivo rodent model of cataractogenesis (David and Shearer, 1984). In the suckling rat given a single overdose of selenite, the following changes were observed in the lens nucleus within 4 days: severe cataract, 17 fold increase in concentration of dry lens weight calcium, proteolysis of α-crystallin leading to the production of 19, 18, and 17.5 K polypeptides, proteolysis of β-crystallin leading to the decrease of 30, 27, and 25.5 K polypeptides and production of intensely stained bands at 25 and 24 K, proteolysis of insoluble proteins leading to the loss of polypeptides above 26 K, initial production of a 25 K intrinsic membrane polypeptide, loss of intrinsic membrane polypeptides at 66 and 26 K ending with the appearance of new intrinsic membrane polypeptides at 24 and 22 K (David and Shearer, 1984). These data indicated that calpain might induce proteolysis in selenite cataract.

Barber, Rosenberg, Nikuni, Obazawa, and Kinoshita (1979) demonstrated that cultured rat lens deprived of glucose underwent calcium uptake and rapid proteolysis, as measured by the release of free amino acids. Proteolysis also occurred in mouse lenses incubated in glucose free medium (Hu et al., 1982). The incubation resulted in: opacity,

disappearance of a 31 K β-crystallin polypeptide, increase in a soluble 25 K polypeptide, appearance of soluble polypeptides below 19 K, and degradation of the main intrinsic membrane protein. Incubation of rat lens homogenates in the presence of calcium reproduced many of the proteolytic changes found in lens incubated in low glucose medium (Russell, 1984). Such data implicate calpain in the proteolytic changes observed in the in vitro culture of lens material.

Proteolysis was also observed during the development of three hereditary mouse cataracts. In the Nakano mouse cataract, a soluble 30 K β-crystallin polypeptide decreased, soluble polypeptides below 22 K increased, and the main intrinsic membrane protein was degraded (Roy et al., 1982). The development of the Philly mouse cataract was characterized by an increase in lens calcium (Kador, Fukui, Fukushi, Jernigan, and Kinoshita, 1980), absence of higher molecular weight β_H-crystallin polypeptides, and appearence of cleavage products of α-crystallin (Piatigorsky et al., 1980). Similarily, Cat^{FRASER} mouse cataractous lenses exhibited increased degradation of α-crystallin (Garber et al. 1984). While proteases other than calpain II could also be activated during the formation of these rodent cataracts, the similarity of proteolysis observed in a number of experimental rodent cataracts to calpain II-induced proteolysis is noteworthy. Definitive proof of the activation of calpain in cataractogenesis will be available when the proteolytic fragments from the in vitro and in vivo situations are compared by such techniques as peptide mapping and amino acid sequencing.

Rat lens calpain II required a lower calcium concentration for activation than bovine lens calpain II. Bovine lens calpain II was not appreciably active until approximately 200 μM calcium (Murachi et

al., 1985), while rat lens calpain II showed some activation at approximately 50 μM calcium. Using an HPLC TSK DEAE 5-PW column, which provided better resolution than the DEAE Bio-Gel A column used in the present study, we have detected an unusual endogenous inhibitor of calpain. Preliminary data suggest this inhibitor has an approximate 9,600 MW (data to be published elsewhere). However, this calpain inhibitor may be present in rat lens in low enough concentration to allow in vivo calpain activity, since we could measure calpain activity in crude rat lens homogenates. These findings are significant because Hightower, Duncan, and Harrison (1985) recently determined that the free calcium concentration in normal rabbit lens fibers was approximately 30 μM, and this increased with age to 50 μM. The low concentrations of endogenous inhibitor and presence of calpain II which may be activated by normal levels of calcium, indicate that lens calpain II may play a normal physiological role in rat lenses. One postulated role for calpain under normal calcium concentrations is the degradation of cytoskeletal proteins during in aging lens fibers (Ireland and Maisel, 1984: Ringens, Hoenders, Bloemendal, 1982). Perhapes these normal substrates are degraded by calpain in the presence of physiological calcium, while gross calcium elevation causes more widespead pathological proteolysis during cataractogenesis.

The evidence in this report clearly demonstrated that calpain was present in rat lens. Substrate specificity was compatible with possible roles for this enzyme in cataractogenesis and/or lens fiber maturation in rodent models. Furthermore, since we were also able to measure calpain in single pairs of human lenses, techniques are now availible to evaluate the role of this enzyme in human lenses.

Acknowledgments:

This work was partially supported by grant number EY-03600 from the National Eye Institute, National Institutes of Health, Bethesda, Maryland.

References

Barber, W.G., Rosenberg, S.B., Mikuni, I., Obazawa, H., and Kinoshita, J.H. (1979). Net proteolysis in glucose-deprived rat lenses incubated in amino acid-free medium. Exp. Eye Res. 29, 663-669.

David, L.L. and Shearer, T.R. (1984). Calcium-activated proteolysis in the lens nucleus during selenite cataractogenesis. Invest. Ophthalmol. Vis. Sci. 25, 1275-1283.

David, L.L. and Shearer, T.R. (1985). Characteristics of calcium-activated protease from rat lens. Invest. Ophthalmol. Vis. Sci. 26, (Suppl.) 160.

Fleshman, K.R., and Wagner, B.J. (1984). Changes during aging in rat lens endopeptidase activity. Exp. Eye Res. 39, 543-551.

Garber, A.T., Goring, D., and Gold, R.J.M. (1984). Characterization of abnormal proteins in the soluble lens proteins of Cat^{Fraser} mice. J. Biol. Chem. 259, 10376-10379.

Harding, J.J., and Crabbe, M.J.C. (1984). The lens: development, proteins, metabolism, and cataract. In: The Eye (Davson, H., ed.). Volume 1b, Academic Press, New York, Chapter 3, pp. 207-492.

Hightower, K.R., Duncan, G., and Harrison, S.E. (1985). Intracellular calcium concentration and calcium transport in the rabbit lens. Invest. Ophthalmol. Vis. Sci. 26, 1032-1034.

Hong, B.-S. (1985). Isolation and purification of a calcium-dependent neutral protease from rabbit eye lens. Invest. Ophthalmol. Vis. Sci. 26, (Suppl.) 300.

Hu, T., Russell, P., and Kinoshita, J.H. (1982). In vitro incubation paralleling changes occurring during mouse cataract formation. Exp. Eye Res. 35, 521-533.

Ireland, M. and Maisel, H. (1984). Evidence for a calcium activated protease specific for lens intermediate filaments. Curr. Eye Res. 3, 423-429.

Kador, P.F., Fukui, H.N., Fukushi, S., Jernigan, H.M., and Kinoshita, J.H. (1980). Philly mouse: a new model of hereditary cataract. Exp. Eye Res. 30, 59-68.

Kay, J. (1982). Ca^{2+}-activated proteinases, protein degradation and muscular dystrophy. in Proteases: Potential Role in Health and Disease (Horl, W.H., and Heidland, A., Eds.) pp 519-531, Plenum Press, New York.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Lorand, L., Conrad, S.M., and Velasco, P.T. (1985). Formation of a 55 000-weight cross-linked β crystallin dimer in the Ca^{2+}-treated lens. A model for cataract. Biochemistry, 24, 1525-1531.

Ohno, S, Emori, Y., Imajoh, S., Kawasaki, H., Kisaragi, M., and Suzuki, K. (1984). Evolutionary origin of a calcium-dependent protease by fusion of genes for a thiol protease and a calcium-binding protein? Nature 312, 566-570.

Piatigorsky, J., Kador, P.F., and Kinoshita, J.H. (1980). Differential synthesis and degradation of protein in the hereditary philly mouse cataract. Exp. Eye Res. 30, 69-78.

Ringens, P.J., Hoenders, H.J., and Bloemendal, H. (1982). Effect of aging on the water-soluble and water-insoluble protein pattern in normal human lenses. Exp. Eye Res. 34, 201-207.

Roy, D., Chiesa, R., and Spector, A. (1983). Lens calcium activated proteinase: Degradation of vimentin. Biochem. Biophys. Res. Commun. 116, 204-209.

Roy, D., Garner, M.H., Spector, A., Carper, D., and Russell, P. (1982). Investigation of Nakano lens proteins. Exp. Eye Res. 34, 909-920.

Roy, D. and Spector, A. (1978). Human insoluble lens protein II. Isolation and characterization of a 9600 dalton polypeptide. Exp. Eye Res. 26, 445-459.

Russell, P. (1984). In vitro alterations similar to posttranslational modification of lens proteins. Invest. Ophthalmol. Vis. Sci. 25, 209-212.

Russell, P., Robison, W.G. Jr., Kinoshita, J.H. (1981). A new method for rapid isolation of the intrinsic membrane proteins from lens. Exp. Eye Res. 32, 511-516.

Srivastava, O.P., and Ortwerth, B.J. (1983a). Age-related and distributional changes in trypsin inhibitor activity of bovine lens. Exp. Eye Res. 36, 695-709.

Srivastava, O.P., and Ortwerth, B.J. (1983b) Isolation and characterization of a 25K serine proteinase from bovine lens cortex. Exp. Eye Res. 37, 597-612.

Swanson, A.A., Davis, R.M., Albers-Jackson, B., and McDonald, J.K. (1981). Lens exopeptidases. Exp. Eye Res. 32, 163-173.

Takemoto, L.J., Hansen, J.S., and Horwitz, J. (1983). Comparison of in vitro proteolysis products of the normal lens with putative in vivo proteolysis products of the cataractous lens. Curr. Eye Res. 2, 849-854.

Twining, S.S. (1983). Fluorescein isothiocyanate-casein assay for proteolytic enzymes. Fed. Proc. (abstract) 42, 1951.

Wagner, B.J. and Margolis, J.W. (1985). Neutral protease activity in human lens autolysis. Invest. Ophthalmol. Vis. Sci. 26, (Suppl.) 160.

Yoshida, H., Murachi, T., and Tsukahara, I. (1984a). Limited proteolysis of bovine lens α-crystallin by calpain, a Ca^{2+}-dependent cysteine proteinase, isolated from the same tissue. Biochim. Biophys. Acta 798, 252-259.

Yoshida, H., Murachi, T., and Tsukahara, I. (1984b). Degradation of actin and vimentin by calpain II, a Ca^{2+}-dependent cysteine proteinase, in bovine lens. FEBS Lett. 170, 259-262.

Yoshida, H., Murachi, T., and Tsukahara, I. (1985). Distribution of calpain I, calpain II, and calpastatin in bovine lens. Invest. Ophthalmol. Vis. Sci. 26, 953-956.

TABLE I

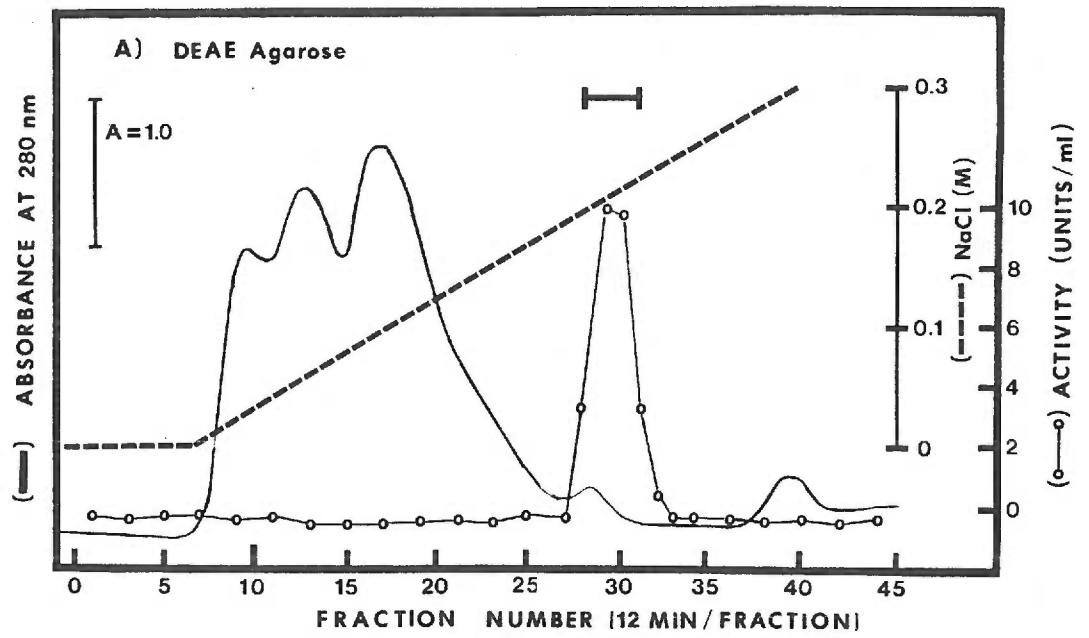
Purification of Calpain II from Rat Lens

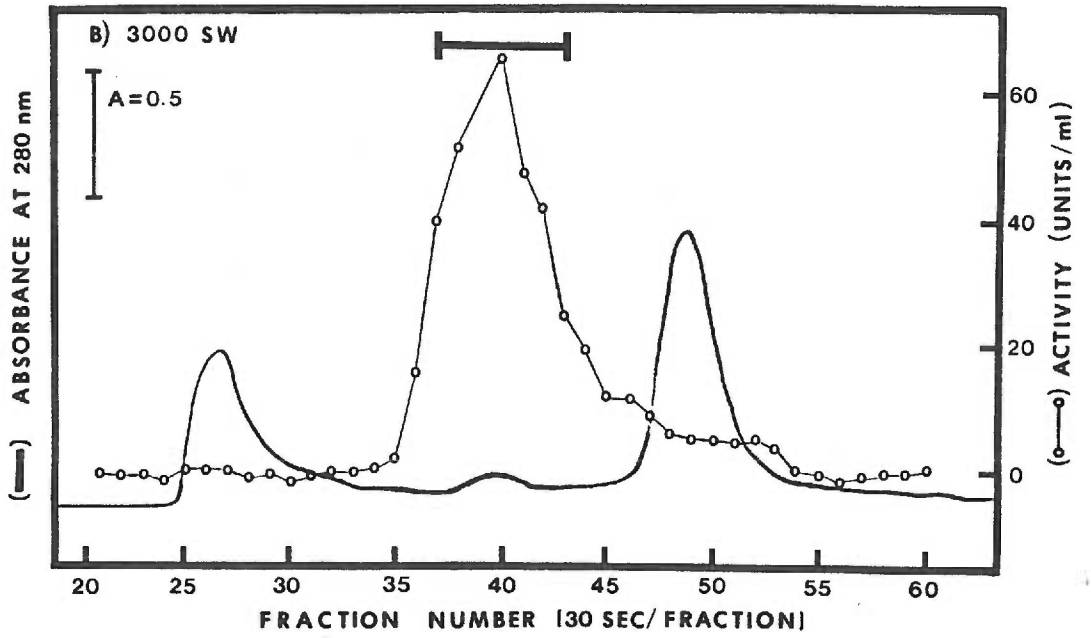
Step	Total protein (mg)	Total activity		Specific activity	
		(units)	(% yield)	(units/mg protein)	(fold)
Supernatant*	619	140	-	0.226	1
DEAE Bio-Gel A	3.38	140	100	41.4	183
TSK 3000 SW	0.137	48.9	35	356	1570
TSK Phenyl 5-PW	0.009	13.6	9.7	1580	6990

*Supernatant obtained from 120 rat lenses. Unit defined as one µg FITC-casein degraded to acid soluble fragments/min at 30°C. Activity in the supernatant fraction was estimated by assuming 100 % recovery during the DEAE Bio-Gel step.

Figure 1

Sequential chromatography steps used for the purification of calpain II from the soluble protein of 120 rat lenses. Solid horizonal bars indicate calpain II fractions pooled for subsequent analyses. A) Ion exchange on DEAE Bio-Gel A, B) HPLC Gel filtration on TSK 3000 SW. C) HPLC hydrophobic interaction chromatography on TSK Phenyl 5-PW.


A) DEAE Agarose
A=1.0
(—) ABSORBANCE AT 280 nm
(----) NaCl (M)
(—o—) ACTIVITY (UNITS/ml)
0.3
0.2
0.1
0
10
8
6
4
2
0
0 5 10 15 20 25 30 35 40 45
FRACTION NUMBER (12 MIN/FRACTION)


B) 3000 SW
A=0.5
(—) ABSORBANCE AT 280 nm
(—o—) ACTIVITY (UNITS/ml)
60
40
20
0
20 25 30 35 40 45 50 55 60
FRACTION NUMBER (30 SEC/FRACTION)

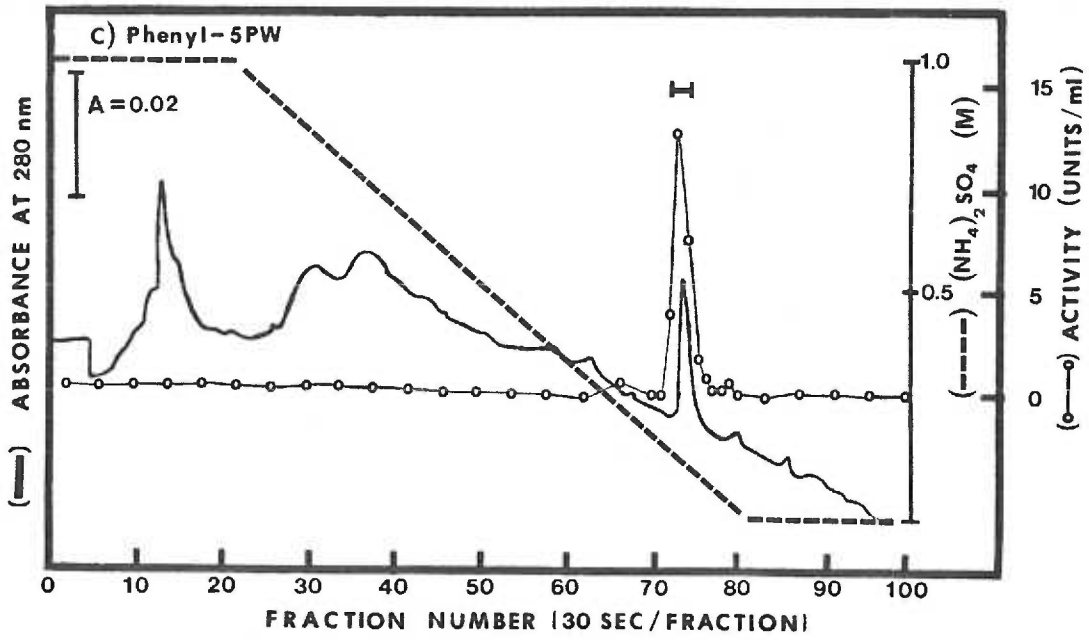

C) Phenyl-5PW
A=0.02
(—) ABSORBANCE AT 280 nm
(----) $(NH_4)_2SO_4$ (M)
(—o—) ACTIVITY (UNITS/ml)
1.0
0.5
15
10
5
0
0 10 20 30 40 50 60 70 80 90 100
FRACTION NUMBER (30 SEC/FRACTION)

Figure 2

SDS-PAGE of calpain II showing increasing purification following each sequential chromatography step. Lane 1, molecular weight markers with their corresponding molecular weights indicated to the left; lane 2, 20 µg total lens soluble protein; lane 3, 15 µg protein after DEAE Bio-Gel A; lane 4, 10 µg protein after TSK 3000 SW; lane 5, 5 µg protein after TSK 5-PW. Numbers to the right show positions and apparent molecular weights (M_r) of major calpain II polypeptides.

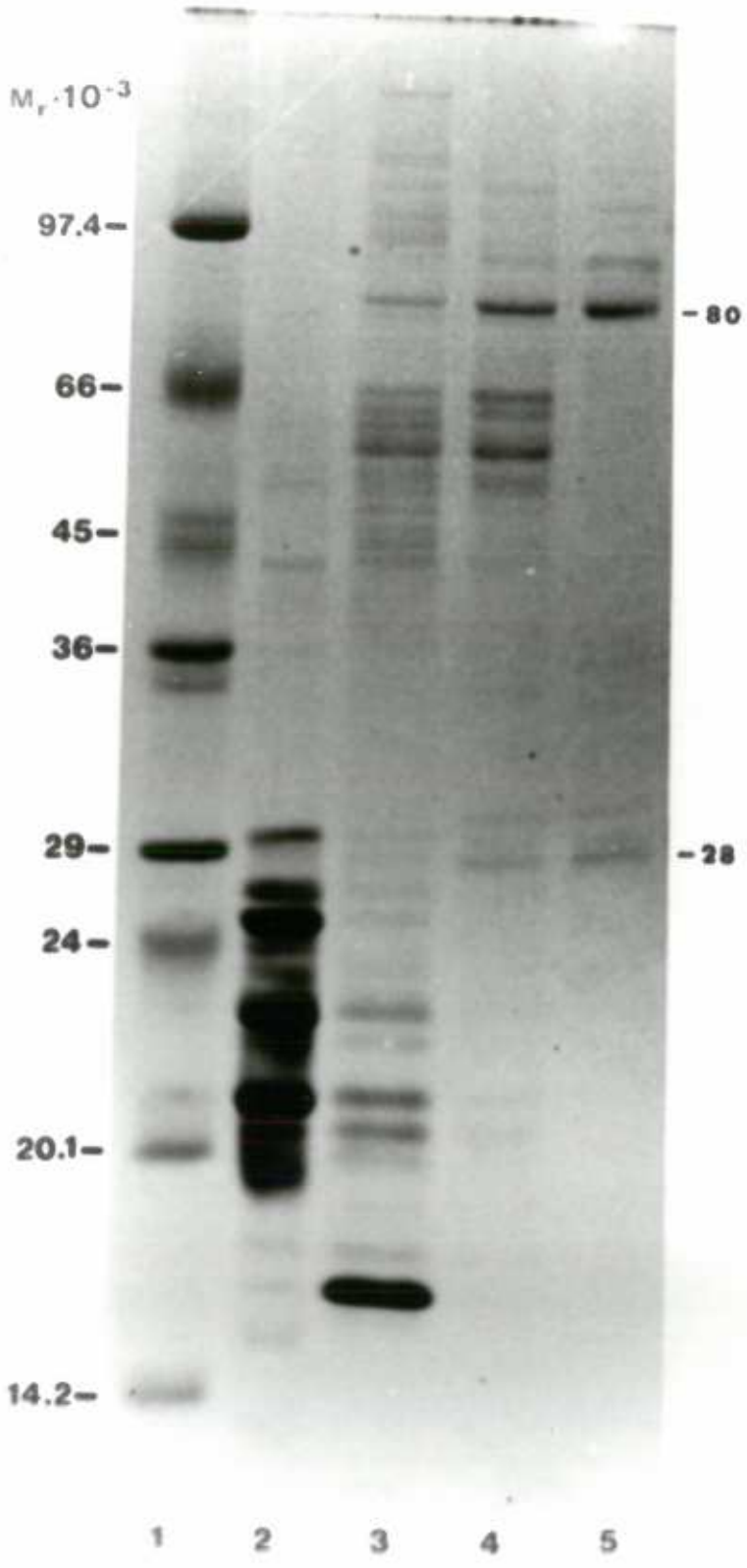

$M_r \cdot 10^{-3}$
97.4
66
45
36
29
24
20.1
14.2
80
28
1
2
3
4
5

Figure 3

Activation of partially purified calpain II by calcium.

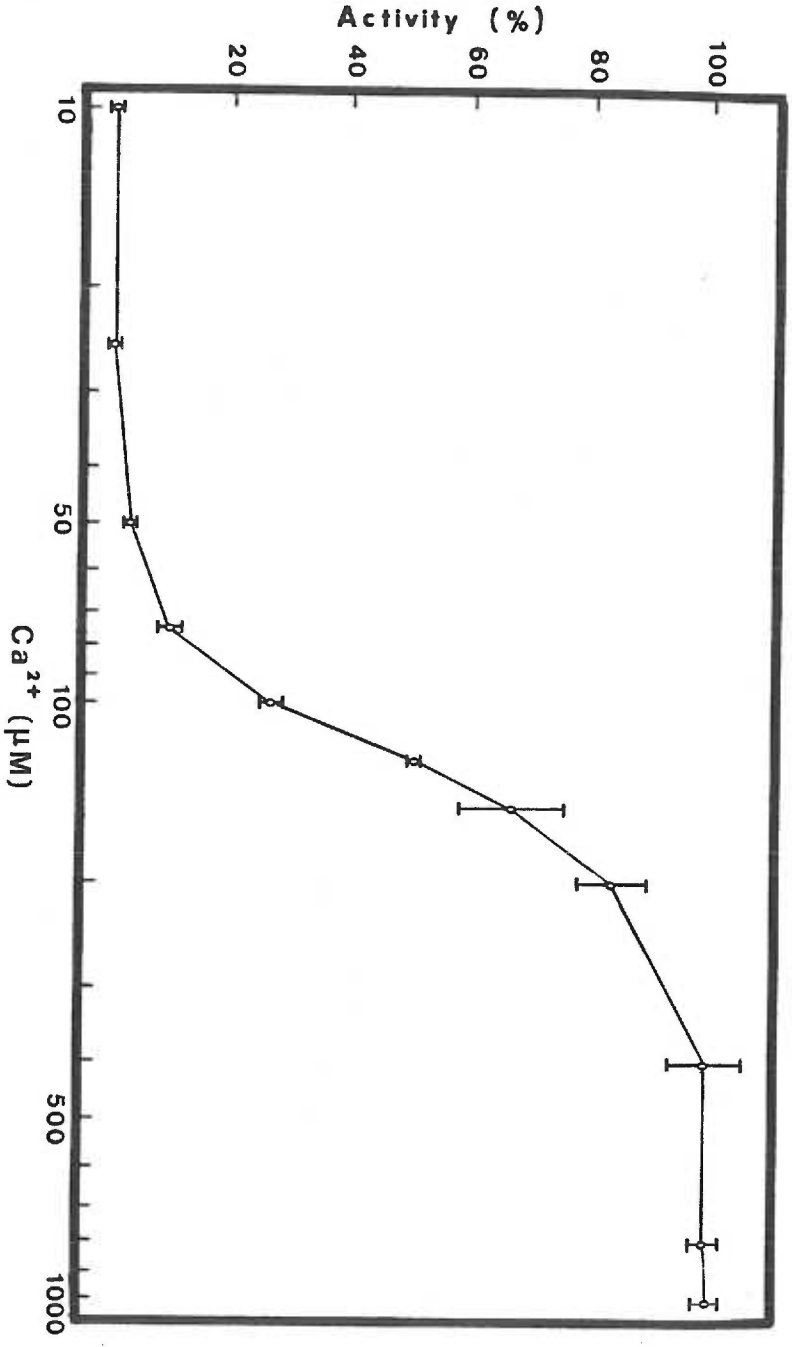
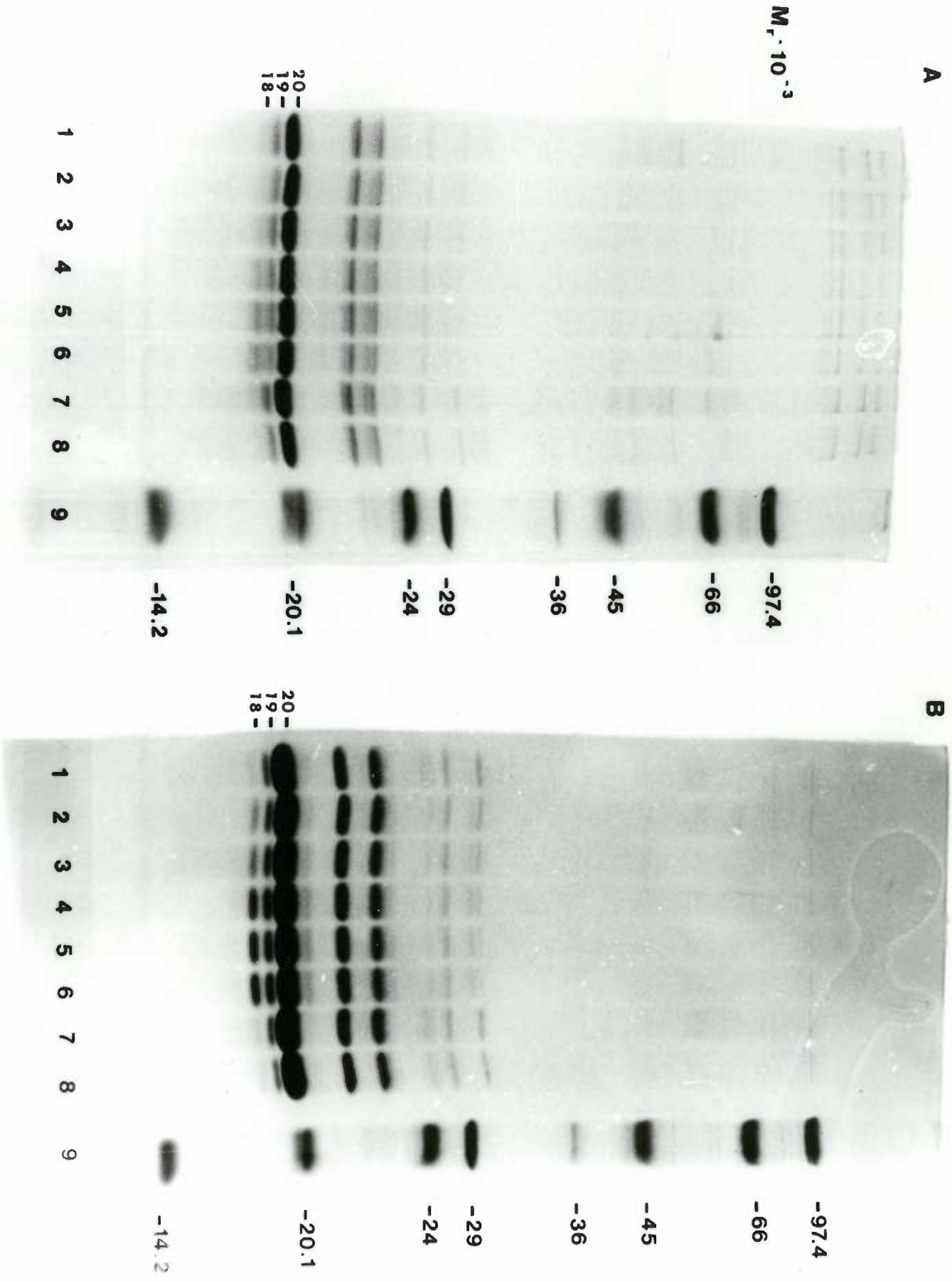
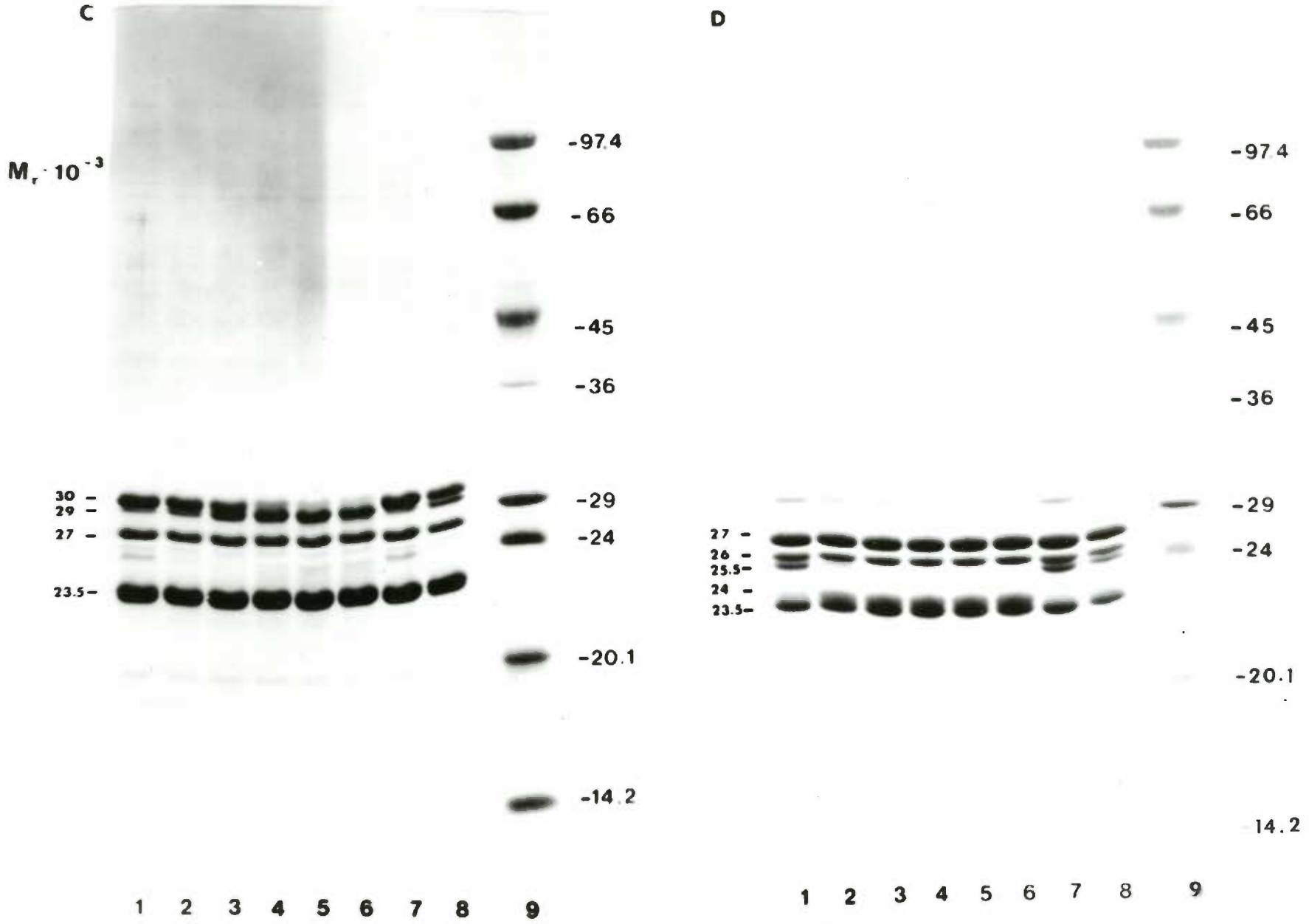
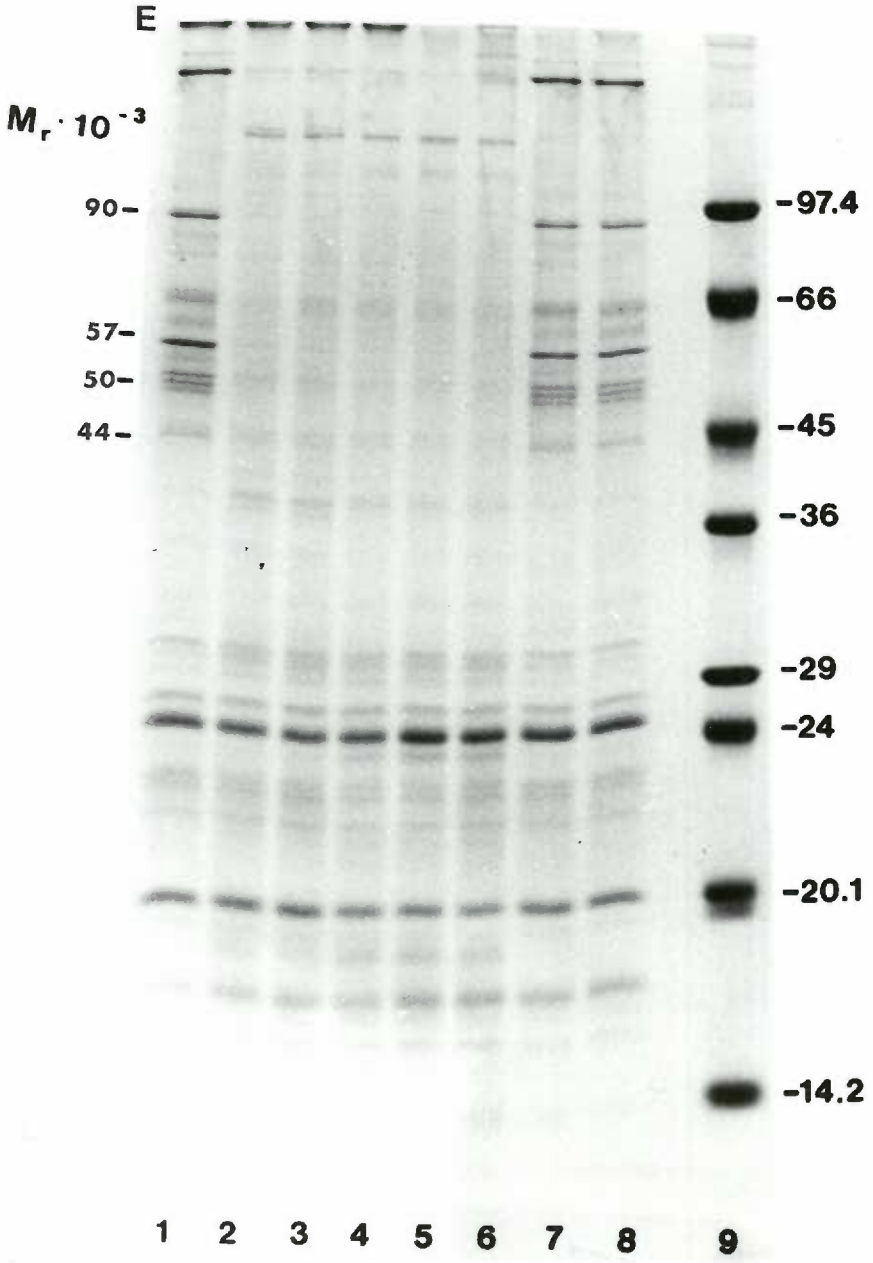

Activity (%)
20
40
60
80
100
10
50
100
500
1000
Ca2+ (μM)

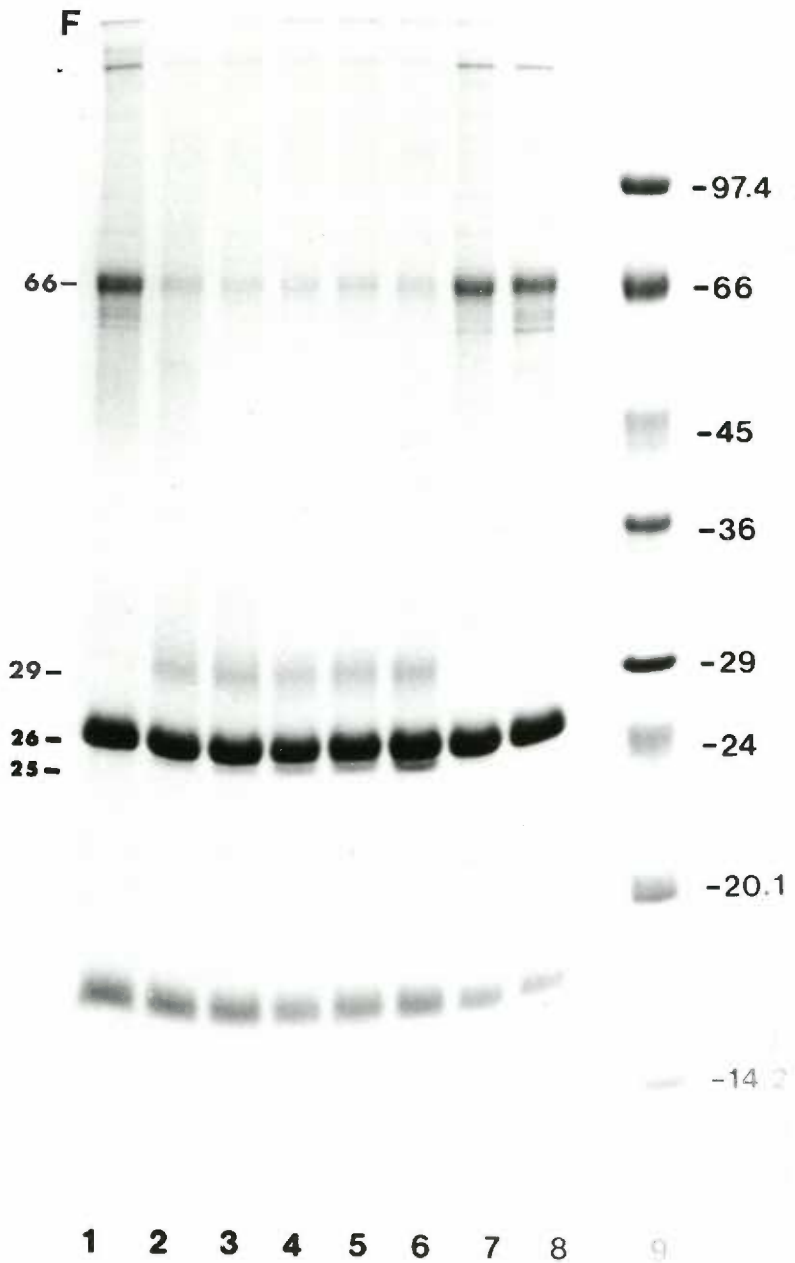
Figure 4

Proteolysis of isolated rat lens proteins by lens calpain II. A) High molecular weight lens protein, B) α-crystallin, C) β_H-crystallin, D) β_L-crystallin, E) insoluble protein, F) intrinsic membrane proteins. Lanes 1 - 6 on each gel indicate: 0, 2.5, 5, 10, 30, and 60 min incubation times in the presence of 1.2 mM $CaCl_2$. The proteins in lane 7 sample were incubated 60 min with no calcium. The proteins in lane 8 were incubated for 60 min with calcium, but no calpain II. Lane 9 contained molecular weight markers.


A
$M_r \cdot 10^{-3}$
-97.4
-66
-45
-36
-29
-24
-20.1
-14.2
20
19
18
1 2 3 4 5 6 7 8 9
B
-97.4
-66
-45
-36
-29
-24
-20.1
-14.2
20
19
18
1 2 3 4 5 6 7 8 9


C
D
$M_r \cdot 10^{-3}$
-97.4
-66
-45
-36
-29
-24
-20.1
-14.2
30
29
27
23.5
26
25.5
24
1 2 3 4 5 6 7 8 9


E
$M_r \cdot 10^{-3}$
90–
57–
50–
44–
–97.4
–66
–45
–36
–29
–24
–20.1
–14.2
1 2 3 4 5 6 7 8 9


F
66–
29–
26–
25–
–97.4
–66
–45
–36
–29
–24
–20.1
–14.2
1 2 3 4 5 6 7 8 9

Manuscript 4

Origin of the Insoluble Protein in the Selenite Cataractous Lens

L.L. David* and T.R. Shearer*+

Departments of Biochemistry* and Ophthalmology,+
Oregon Health Sciences University, Portland, Oregon

Short Title: Insoluble Protein in Selenite Cataract

Key Words: Lens, cataract, selenium, selenite, insoluble protein, lens proteins, insoluble protein, crystallins.

Supported in part by grant no. EY03600 from the National Eye Institute, National Institutes of Health, Bethesda, Maryland.

Reprint request: T.R. Shearer, 611 S.W. Campus Drive, Portland, OR 97201.

Abstract

Nuclear cataracts were induced in 10 day old rats by a single injection of 30 μmoles sodium selenite/kg body weight. During cataract formation there was a 5 fold increase in the amount of insoluble protein in the lens nucleus. The origin of this insoluble protein was examined by separating the cataractous insoluble protein of lens nuclei by 2-dimensional electrophoresis and immunoblotting with monospecific antisera against rat lens crystallins. Formation of cataractous insoluble protein was due to increased insolubilization of β and γ-crystallin polypeptides.

Polypeptides isolated from cataractous insoluble protein, and normal β_L-crystallin aggregates were compared by tryptic mapping. Approximately 20 % of the insoluble protein from opaque nuclei was composed of 24,700 and 24,200 molecular weight polypeptides derived by limited proteolysis of a 26,500 molecular weight β_L-crystallin polypeptide. This suggested that proteolysis may contribute to the formation of insoluble protein in the selenite induced cataract.

During aging and cataract formation, insoluble protein in the human lens is increased primarily due to crystallin insolubilization (1, 2, 3). Many post-translational alterations of lens proteins occur during aging and cataract formation. These include disulfide formation, proteolysis, amino acid racemization, and glycosylation, but the primary cause of insolubilization is unknown (4). Elucidation of the mechanisms causing the formation of insoluble protein is important because insoluble proteins contribute to light scatter in cataractous lenses (5).

Selenite induced cataract is a useful model to study the insolubilization of lens protein during cataractogenesis. Injection of 20 μmoles sodium selenite/kg body weight in young rats caused the appearance of severe nuclear cataract within 4 days (6). Insoluble protein in the cataractous lens nucleus increased nearly 5 fold (7). Neither high molecular weight soluble protein or increased disulfide formation were detected in the selenite cataractous lens (7,8).

We hypothesized that proteolysis of lens proteins may contribute to the formation of insoluble protein in the selenite cataract by interrupting normal interactions between lens proteins, thus leading to non-covalent association and insolubilization of lens crystallins. During selenite cataract formation, α and β-crystallins, high molecular weight insoluble proteins, and membrane proteins in the lens nucleus were proteolyzed (7). Since a decrease in total concentrations of soluble β_H and β_L-crystallins was noted, we further postulated that proteolyzed β-crystallin polypeptides were selectively insolubilized during cataract formation (7).

The purposes of the present study were: (1) to immunologically characterize the composition of the insoluble protein in the selenite cataractous lens nucleus, and (2) to isolate polypeptides from the lens

insoluble protein and determine if the insoluble protein contained proteolyzed β-crystallin polypeptides. Results supported the hypothesis that proteolysis may contribute to crystallin insolublization during cataractogenesis.

Materials and Methods

Production of Crystallin Antibodies

Lenses from 10 day old rat pups (Simonson Laboratory, Gilroy, CA) were dissected and separated into cortical and nuclear regions as previously described (7). The lens nuclei were homogenized in 20 mM Tris (pH 7.5), 1.0 mM EDTA, 1.0 mM EGTA, 100 mM NaCl at a ratio of 20 nuclei/ml buffer. Following centrifugation at 10,000 g at 4°C for 20 min, supernatant was applied to a 2.5 x 95 cm Bio-Gel A1.5m column (Bio-Rad, Richmond, CA). Proteins were eluted at 25 ml/hr, and absorbance monitored at 280 nm. The various crystallins were pooled and concentrated by ultrafiltration (YM5 membrane, Amicon Corp. Lexington, MA). Crystallins were mixed with Freunds complete and incomplete adjuvant and injected subcutaneously into New Zealand White rabbits. Serum was collected and tested for specificity against purified crystallins by dot-blotting α, β, and γ-crystallin onto nitrocellulose membranes and detecting primary antibody binding using the Bio-Rad Immun-Blot goat anti-rabbit IgG horseradish peroxidase conjugate assay kit (Bio-Rad, Richmond, CA).

To remove cross-reacting antibodies, anti-α and anti-β serum were affinity purified. α and β_L-crystallins purified as described above were coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) using the procedure recommended by the manufacturer. Anti-β antibody was purified by diluting 2.0 ml of anti-β_L serum 1:1 with 0.1 M borate buffer (pH 8.3) in 0.5 M NaCl. Diluted serum was then applied to a 0.8 x 3.0 cm β_L-crystallin sepharose 4B column. The column was washed with 10 volumes of 0.1 M borate (pH 8.3) in 0.5 M NaCl, and the bound proteins eluted with 0.1 M glycine (pH 2.5). Fractions were collected in 1.0 M Tris

(pH 8.0) to prevent antibody denaturation. Anti-α antibody purification required two affinity chromatography steps. First, anti-α serum was applied as above to a β_L-crystallin sepharose 4B column, and contaminating anti-β antibodies removed by collecting the unbound anti-α antibody. This partially purified α-antibody was further purified by applying to a α-crystallin Sepharose 4B column, and collecting the protein eluted by the low pH wash. The final preparations of α, β, and γ-antibodies were monospecific.

Isolation and Immunological Characterization of Cataractous Nuclear Insoluble Protein

Ten-day old Spraque-Dawley rat pups were given a single subcutaneous injection of 30 μmoles sodium selenite/kg body weight. Dense nuclear opacities developed 4 days following injection, and the pups were sacrificed 7 days postinjection. Nuclear insoluble protein was isolated by homogenizing dissected lens nuclei in buffer containing 20 mM Tris (pH 7.5), 1.0 mM EGTA, 1.0 mM EDTA, and 100 mM NaCl at a ratio of 20 lenses/ml buffer. The insoluble pellet was washed once, and dissolved in buffer containing 10 mM Tris (pH 8.5), 2.0 mM dithioerythritol, and 6 M urea. Membranes were then removed from the solubilized proteins by centrifugation at 10,000 g for 30 min. Lenses from 10 control and selenite treated rats yielded approximately 1.0 and 5.0 mg of nuclear insoluble protein respectively.

The nuclear insoluble protein from selenite cataractous lenses was comprised of a heterogeneous mixture of polypeptides with most molecular weights ranging from 26.5-22.5 K . Two-dimensional electrophoresis was required to separate these polypeptides before immunoblotting. Proteins

were separated in the first dimension by the nonequilibrium pH gradient electrophoresis (NEPHGE) method of O'Farrell et al. (9), using a 1.5 mm x 14 cm first-dimension gel, pH 3.5-10 ampholines, and a Protean II electrophoresis apparatus (Bio-Rad). The NEPHGE method allowed simulataneous separation of both basic and acidic polypeptides. Electrophoresis in the second dimension was by the method of Laemmli (10) using 12% acrylamide gels.

Following two-dimensional electrophoresis, proteins were electrophoretically transfered to nitrocellulose membranes by the method of Towbin et al. (11). Nitrocellulose membranes were incubated in diluted anti-crystallin primary antibody, and antigens localized by the Immun-Blot (GAR-HRP) assay kit (Bio-Rad). Gels were stained with Coomassie blue and photographed. Molecular weights of crystallin polypeptides were estimated by comparison to standards of glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsin inhibitor, and α-lactalbumin (Sigma Chemical Co., St. Louis, MO). Protein concentrations were determined by the Bio-Rad dye binding assay.

Isolation of Polypeptides from Insoluble Protein

Insoluble protein from nuclei was isolated from cataractous rat lenses 7 days post-selenite injection as described above. Insoluble protein was dissolved in buffer containing 10 mM Tris (pH 8.5), 2.0 mM dithioerythritol, and 6 M urea. Urea was deionized using AG 501-X8 mixed bed ion exchange resin (Bio-Rad). The insoluble protein was dissolved at approximately 5.0 mg protein/ml. Following centrifugation as before, 4-6 mg of urea solubilized protein was applied to a 7.5 X 75 mm TSK DEAE 5-PW high performance liquid chromatography column equilibrated with buffer containing

10 mM Tris (pH 8.5), 2.0 mM dithioerythritol, and 6 M deionized urea. The column was washed for 10 min, and bound polypeptides were eluted with a linear 0-100 mM NaCl gradient over 45 min. Fractions of 30 sec were collected, and absorbance at 280 nm monitored. Data acquisition and integration of peaks was carried out by Adalab and Chromatochart (Interactive Microware, State College, PA). Major peaks were collected, extensively dialyzed against distilled water, and lyophilized. The composition of each peak was determined by 2-dimensional electrophoresis as described above.

Isolation of Polypeptides from β_L-Crystallin

Crystallins were isolated from the nuclei of 17 day old rat lenses by gel filtration as described above. β_L-crystallins were isolated, concentrated and desalted by ultrafiltration, lyophilized, and then dissolved in buffer containing 20 mM Tris (pH 7.5), 2.0 mM dithioerythritol, and 6 M deionized urea. The deaggregated β_L-crystallin polypeptides were then separated on DEAE as described above, except a 0-150 mM NaCl gradient was used. β_L-crystallin polypeptide peaks were collected, dialyzed extensively against distilled water, and lyophilized. Composition of each β_L-crystallin peak was determined by 2-dimensional electrophoresis.

Tryptic Mapping of Purified Insoluble and Crystallin Polypeptides

Lyophilized insoluble and β_L-crystallin polypeptides were suspended/dissolved in 20 mM ammonium bicarbonate buffer (pH 8.5) at 1.0 mg protein/ml. TPCK-treated trypsin (Cooper Biomedical, Alvern, PA), dissolved in 1.0 mM HCl, was added at a final concentration of 0.02 mg trypsin/mg substrate. Trypsinization proceeded at 25°C for 4 hours in a

rocking water bath. The reaction was terminated by adjusting the pH to 2-3, and the solution was lyophilized. Lyophilized tryptic fragments were then dissolved in 0.05 % trifluoroacetic acid (TFA), and 0.3-0.5 mg of trypsinized polypeptides were injected onto a 3.9 mm x 15 cm Nova-Pak C_{18} HPLC column (Waters Associates, Milford, MA). Following a 10 min wash with 0.05 % TFA, tryptic polypeptides were eluted with a 0-40 % acetonitrile gradient in 0.05 % TFA over 2 hr. Absorbance was detected at 217 nm.

Rats in this study were treated in accordance with the ARVO Resolution on the Use of Animals in Research.

Results

Two-Dimensional Electrophoresis of Nuclear Insoluble Protein

The insoluble protein from control and selenite cataractous lenses was compared by two-dimensional electrophoresis (Fig. 1, A and B). The 5 fold increase in insoluble protein in the cataractous lens was the result of marked insolubilization of at least 10 different polypeptides between 24.7 and 22.5 K molecular weight. The control and cataractous insoluble protein contained many polypeptides in common (Fig. 2). However, selenite caused a loss or decrease of polypeptides at 29.0, 26.5, 25.9, 22.4, 21.3, and 20.6 K, and appearance or increase in intensity of many polypeptides. Of greatest significance for the present report was the increased intensity of insoluble polypeptides at 24.7 and 24.2 K in cataractous lenses (Fig. 1). This indicated that the process of crystallin insolubilization in cataractous lenses was significantly altered.

As described previously (7), selenite also caused the disappearence of all insoluble polypeptides with molecular weights above the largest 29 K crystallin polypeptide.

Immunological Identification of the Polypeptides in Insoluble Protein

The origin of most polypeptides in the insoluble protein from control and selenite cataractous lenses was determined by antibody binding after protein blotting. This allowed assignment of polypeptides to a particular class of crystallins (Fig. 3). α, β, and γ-crystallins were present in both control and selenite insoluble protein. The identity of each immunologically reactive polypeptide, which corresponded in position to major insoluble polypeptides visible by Coomassie blue staining in figure 1,

is summarized in table 1.

Two striking differences were noted in the immunoblots of figure 3. First, a majority of the β-crystallin polypeptides from cataractous insoluble protein showed heavier staining with anti-β antibody compared with controls (figure 3C and D). This is consistant with the observation that several of these β-crystallin polypeptides were in greater concentration in cataractous insoluble protein (figure 1). Second, all insoluble α-crystallin polypeptides in the nucleus of cataractous lenses were degraded to a number of lower molecular weight fragments (figure 3B). These degraded α-crystallin polypeptides did not significantly contribute to the bulk of the insoluble protein, because they were in low concentration (figure 1). Therefore, the majority of control and cataractous lens insoluble protein was composed of insolubilized β and γ-crystallin polypeptides.

Characterization of Polypeptides from Insoluble Protein and β_L-Crystallin

Insoluble protein from the cataractous lens nucleus contained 7 major peaks of protein (figure 4). The polypeptide composition of each DEAE peak is summarized in table II. γ-crystallin polypeptides did not bind to the DEAE column, and eluted as single large peak 1 comprising an estimated 21% of the cataractous insoluble protein. The remaining peaks 2-7 contained an estimated 66 % of the cataractous lens insoluble protein, and were composed of mainly polypeptides from β-crystallin.

β_L-crystallin from the soluble fraction of lens nuclei contained 5 major peaks (figure 5). Figure 6A shows the 2-dimensional electrophoresis of β_L-crystallin, while figure 6B shows the composition of only peak 1 from β_L-crystallin. These data showed that peak 1 of soluble β_L-crystallin contained two major polypeptides with apparent molecular weights of 26.5 K.

Peptide Mapping

Peaks 2 and 3 from cataractous insoluble protein (figure 4, arrows), and peak 1 from soluble β_L-crystallin (figure 5, arrow) were compared by peptide mapping. Peaks 2 and 3 from insoluble protein were almost identical (Figure 7B and C), and closely resembled the tryptic map of peak 1 from β_L-crystallin (Figure 7A). These results strongly suggested that the 26.5 K peak 1 from soluble β_L-crystallin gave rise to the 24.7 and 24.2 K polypeptides found in peaks 2 and 3 of the cataractous insoluble protein. Further examination of the other insoluble β-crystallin polypeptides was not carried out because they were not sufficiently purified by DEAE chromatography to perform peptide mapping. While insoluble peak 7 was a single β-crystallin polypeptide, it did not match any of the tryptic maps of β_L-crystallin peaks 2-5.

Discussion

The major goal of the present research was to determine if the insoluble protein of selenite cataractous lenses contained proteolytic fragments derived from soluble lens crystallins. We found α, β, and γ-crystallins in the insoluble protein of selenite cataractous lenses. This was also true in normal lenses, and the insoluble protein form both control and selenite lenses shared many common polypeptides. However, the proportions of certain polypeptides in the insoluble protein of selenite cataract differed significantly from normal lenses. At least 7 β-crystallin polypeptides were enhanced in the insoluble protein of cataractous lenses. Two of these enhanced polypeptides were the 24.7 and 24.2 K molecular weight β-crystallin polypeptides. Tryptic mapping showed that these polypeptides were derived from the limited proteolysis of a 26.5 K nuclear β_L-crystallin polypeptide.

Partially degraded fragments of α-crystallin were also present in cataractous lens insoluble protein. However, because these polypeptides were low in concentration, there was no evidence that degraded α-crystallin polypeptides were insolubilized as extensively as β and γ-crystallins. γ-crystallin made a significant contribution to both control and cataractous insoluble protein, but no proteolysis was observed.

Several additional pieces of evidence support the hypothesis that proteolysis may lead to insolubilization of lens crystallins. Proteolysis was observed before formation of selenite cataract (7). The 30 and 27 K polypeptides in the soluble β-crystallins decreased, while a 25 K β-crystallin polypeptide dramatically increased (7). This previously reported 25 K soluble β-crystallin polypeptide was previously resolved only with 1 dimensional electrophoresis, and may be identical to the 24.7 and 24.2 K insoluble β-crystallin polypeptides of the present study.

Evidence that proteolysis of the β-crystallin polypeptides occurred before insolubilization supports the hypothesis that proteolysis could be a cause of insolubilization. Increased proteolysis of α and β-crystallins as well as loss of soluble β-crystallin have also been well documented in several other types of experimental rodent cataracts (12-19).

Lenses contain several proteases that may contribute to proteolysis observed above (20-23). Calcium dependent neutral protease (calpain) has been demonstrated in rat, mouse, rabbit, bovine, and human lenses (15, 24-26), and lens calcium is increased before selenite cataract is observed (7). Purified calpain from rat lens degraded the 30 and 25.5 K polypeptides of β-crystallin in vitro, but no degradation of the 26.5 K β-crystallin was detected (26). Future experiments will determine if longer incubation of purified rat lens calpain with β-crystallin will produce the partially degraded 24.7 and 24.2 K β-crystallins found in the insoluble protein of selenite cataractous lenses.

We propose the following working hypothesis for the development of the selenite cataract. Selenite enters the lens and first damages the epithelial cells (27). Damage of epithelial cells leads to accumulation of lens calcium in the nuclear region. Lens calpain is activated and causes proteolysis of lens proteins. Proteolyzed β-crystallin and non-proteolyzed γ-crystallin polypeptides associate non-covalently to form light-scattering insoluble protein.

References

1. Li L-K, Roy D, and Spector A: Changes in lens protein in concentric fractions from individual normal human lenses. Curr Eye Res 5:127, 1986.

2. Spector A: The search for a solution to senile cataracts: Proctor Lecture. Invest Ophthalmol Vis Sci 25:130, 1984.

3. Roy D, and Spector A: High molecular weight protein from human lenses. Exp Eye Res 22:273, 1976.

4. Spector A: Aspects of the biochemistry of cataract. In The Ocular Lens. Structure, Function, and Pathology, Maisel H, editor. New York, Marcel Dekker, Inc. 1985, pp. 405-438.

5. Bettelheim FA: Physical basis of lens transparency. In The Ocular Lens. Structure, Function, and Pathology, Maisel H, editor. New York, Marcel Dekker, Inc. 1985, pp. 265-300.

6. Shearer TR, Anderson RS, Britton JL, and Palmer EA: Early development of selenium-induced cataract: slit lamp evaluation. Exp Eye Res 36:781, 1983.

7. David LL and Shearer TR: Calcium-activated protolysis in the lens nucleus during selenite cataractogenesis. Invest Ophthalmol Vis Sci 25: 1275, 1984.

8. David LL and Shearer TR: State of sulfhydryl in selenite cataract. Toxicol Appl Pharmacol 74:109, 1984.

9. O'Farrell PZ, Goodman, HM, O'Farrell, PH: High resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell 12:1133, 1977.

10. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680, 1970.

11. Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose: Procudure and some applications. Proc Natl Acad Sci USA 76:4350, 1979.

12. Roy D, Garner MH, Spector A, Carper D, and Russell P: Investigation of Nakano lens proteins. Exp Eye Res 34:909, 1982.

13. Piatigorsky J, Kador PF, and Kinoshita, JH: Differential synthesis and degradation of protein in the hereditary Philly mouse cataract. Exp Eye Res 30:69, 1980.

14. Garber, AT, Goring D, and Gold RJM: Characterization of abnormal proteins in the soluble lens proteins of Cat^{Fraser} mice. J Biol Chem 259:10376, 1984.

15. Yoshida, H, Murachi T, and Tsukahara, I: Age-related changes of calpain II and α-crystallin in the lens of hereditary cataract. Curr Eye Res 4:983, 1985.

16. Williams, EH, Chaplain, TL, and Meakem, T: A temporal and spacial study of the synthesis and degradation of water-soluble and insoluble proteins in galactosemic rat lenses. Exp Eye Res 41:475, 1985.

17. Bunce GE, Hess JL, and Fillnow GM: Investigation of low tryptophan induced cataract in weanling rats. Exp Eye Res 26:399, 1978.

18. Hu T-S, Russell P, and Kinoshita JH: In vitro incubation paralleling changes occurring during mouse cataract formation. Exp Eye Res 35:512, 1982.

19. Garber AT, Stirk L, and Gold RJM: Abnormalities of crystallins in the lens of the Cat^{Fraser} mouse. Exp Eye Res 36:165, 1983.

20. Ray K, and Harris H: Purification of neutral lens endopeptidase: Close similarity to a neutral proteinase in pituitary. Proc Natl Acad Sci 82:7545, 1985.

21. Wagner BJ, Margolis JW, Abramovitz, AS, and Fu S-CJ: Differential inhibition of two proteolytic activities in bovine lens neutral-proteinase preparations. Biochem J 228:517, 1985.

22. Tse SS, and Ortwerth, BJ: Activation of proteinases with trypsin-like specificity from bovine and human lenses. Exp Eye Res 32:605, 1981.

23. Oettgen HC, Taylor, A: Purification, preliminary characterization, and immunological comparison of hog lens leucine aminopeptidase (EC 3.4.11.1) with hog kidney and beef lens aminopeptidases. Anal Biochem 146:238, 1985.

24. Yoshida, H, Murachi, T, and Tsukahara, I: Distribution of calpain I, calpain II, and calpastatin in bovine lens. Invest Ophthalmol Vis Sci 26:953, 1985.

25. Hong, B-S: Isolation and purification of a calcium-dependant neutral protease from rabbit eye lens. Invest Ophthalmol Vis Sci (Suppl.) 26:300, 1985.

26. David, LL, and Shearer, TR: Purification of calpain II from rat lens and determination of endogenous substrates. Exp Eye Res (in press), 1986.

27. Anderson RS, Shearer TR, Claycomb CK: Selenite-induced epithelial damage and cortical cataract. Curr Eye Res 5:53, 1986.

Table I.

Comparison of Immunological Identity of Polypeptides in Insoluble Proteins[1]

Reactivity to anti-	Control	Cataractous
	MW X 10^{-3}	
α	24.4, 21.3, 20.6	20.1, 19.4, 18.3, 17.3
β	26.5, 23.5A	26.5, 24.7, 24.2, 24.0, 23.8, 23.5B, 23.5N, 23.5A
γ	23.1, 22.5B	23.1, 22.5B, 22.5A

[1] Data from figure 3. Immunoreactive polypeptides which correlated with positions of major insoluble polypeptides in figure 1 were identified by assigning corresponding estimated molecular weights.

Table II.

Composition of the Major Peaks in the Insoluble Protein from Cataractous lenses

Peak[1]	Polypeptide Composition[2]	% of total area[3]	Immunological Identity of Polypeptides[4]
	M.W. x 10^{-3}		
1	23.1, 22.5B, 22.5N	21.3 ± 5.0	γ
2	24.7	4.8 ± 0.8	β
3	24.2	14.6 ± 1.6	β
4	24.0, 23.8	15.7 ± 1.5	β
5	24.0, 23.8	14.7 ± 1.8	β
6	17.3, 23.5N, 24.5	8.8 ± 1.4	α and β
7	23.5A	7.5 ± 1.3	β
	Total	87.4	

[1] Peak numbers refer to peaks in figure 4.

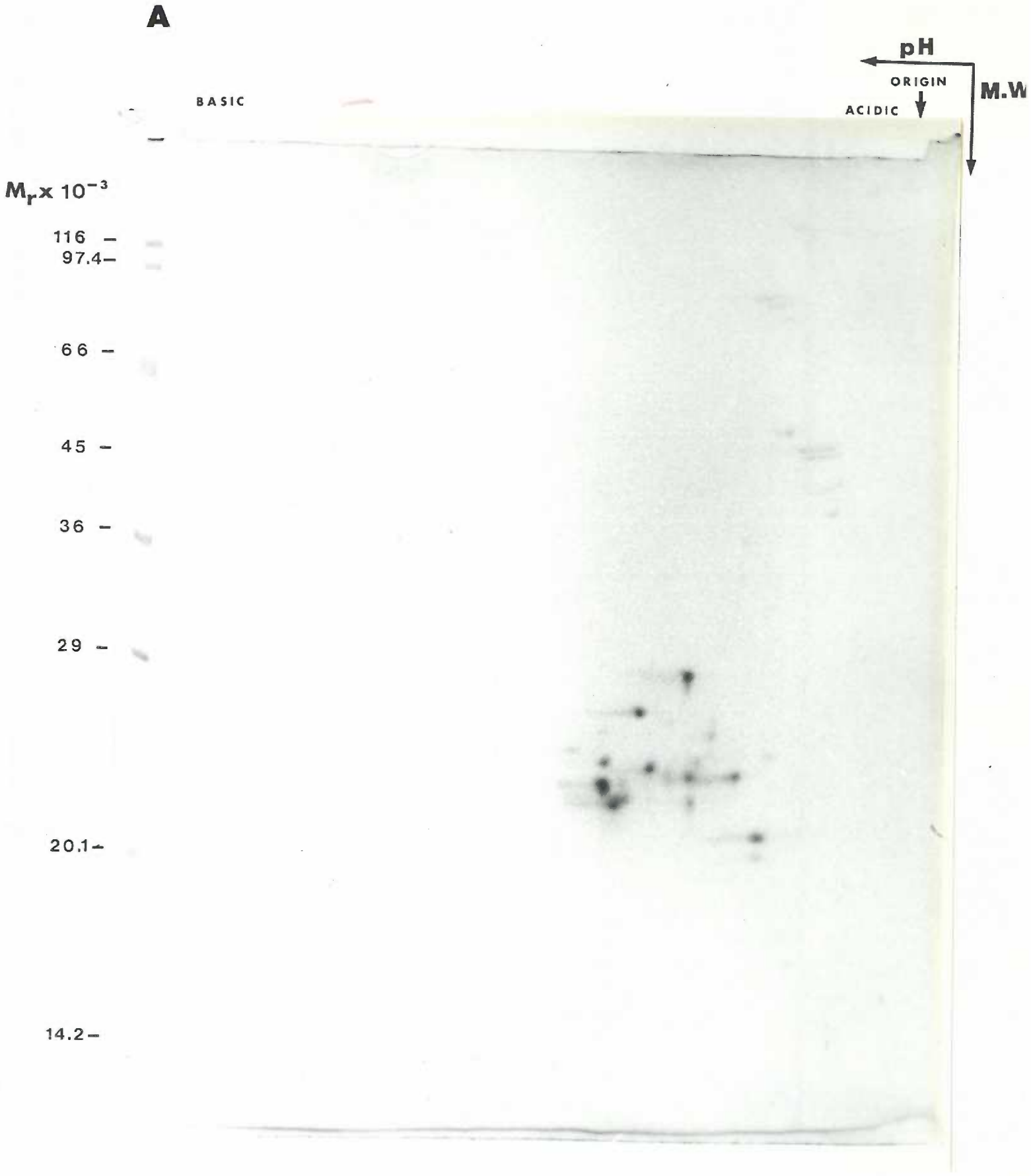
[2] Polypeptide composition determined by 2-dimensional electrophoresis of isolated peaks (gels not shown). Polypeptide identification was carried out by comparison to figure 1B, and polypeptides were assigned the estimated molecular weights given in figure 2.

[3] % total area at 280 nm ± S.D. (n = 4).

[4] Crystallin assignments were based on comparison of 2-dimensional electrophoresis of isolated peaks to immunoblots in figure 3.

Figure 1

Two dimensional electrophoresis of nuclear insoluble protein from (A) 17 day old control lenses, and (B) 17 day old, 7 day postselenite injection cataractous lenses. Both gels were stained with Coomassie blue. Bands on the left of each gel are molecular weight markers.


A
pH
ORIGIN
M.W
BASIC
ACIDIC
$M_r \times 10^{-3}$
116
97.4
66
45
36
29
20.1
14.2

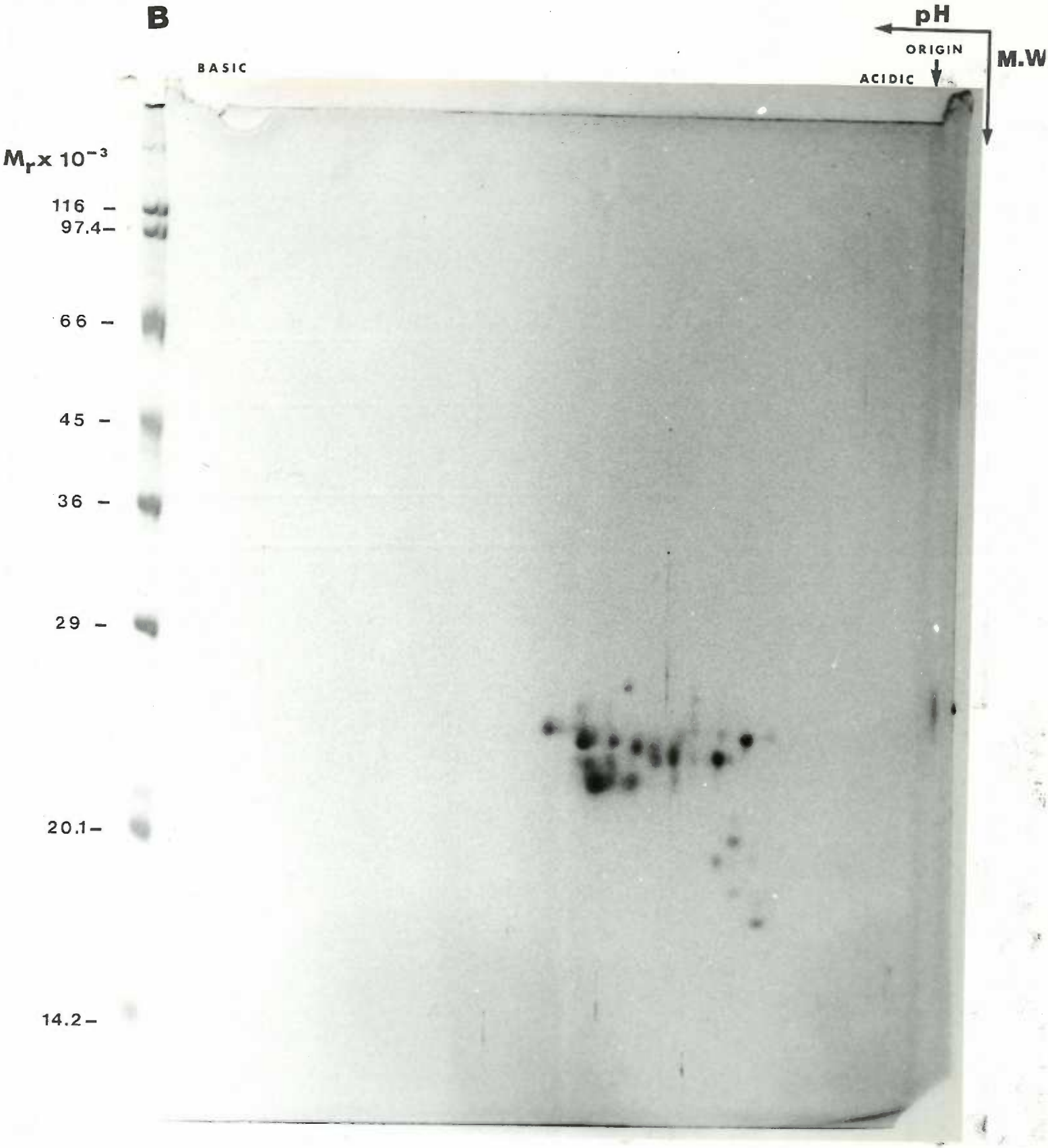

B
pH
ORIGIN
M.W
BASIC
ACIDIC
$M_r \times 10^{-3}$
116 –
97.4–
66 –
45 –
36 –
29 –
20.1–
14.2–

Figure 2

Diagram of middle portion of figure 1 showing 2-dimensional electrophoresis of insoluble crystallin polypeptides. Polypeptides are identified with an estimated molecular weight x 10^{-3}. Polypeptides given the same estimated molecular weight in control and cataractous are assumed to be the same polypeptide, based on similar positions in the 2-dimensional electrophoresis gel. Polypeptides with the same estimated molecular weight were assigned letters according to the relative positions to one another in the first dimension pH gradient (A = acidic, B = basic, N = neutral, in-between A and B).

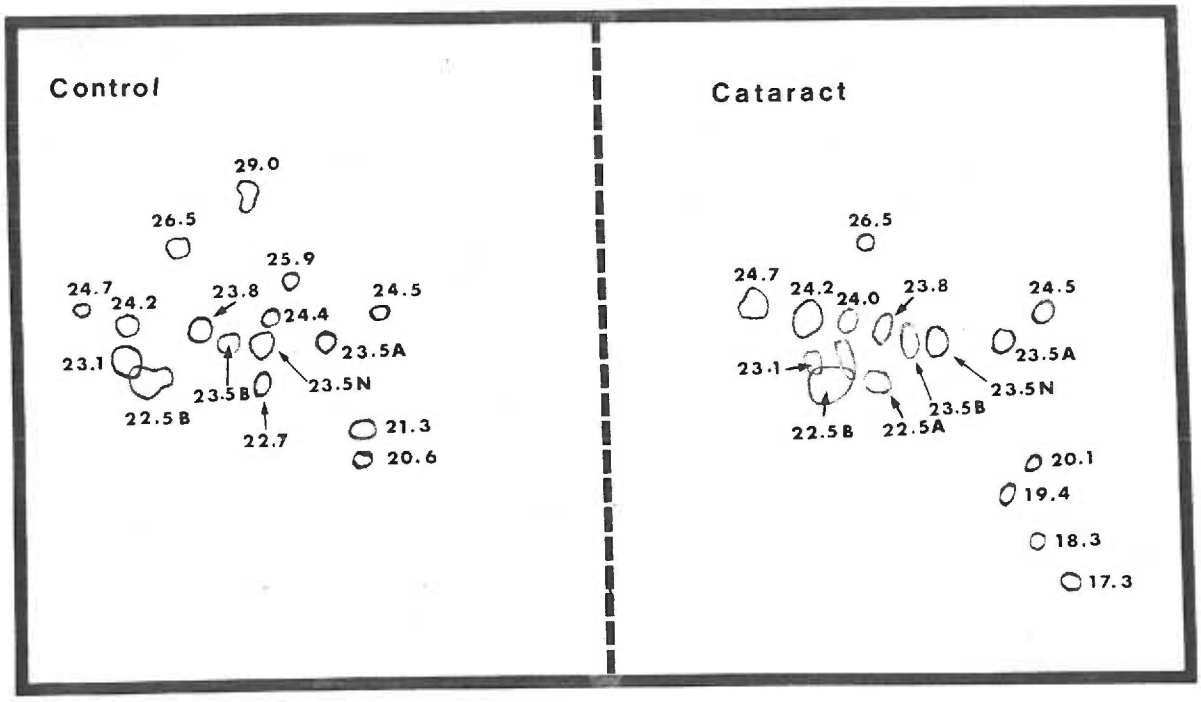
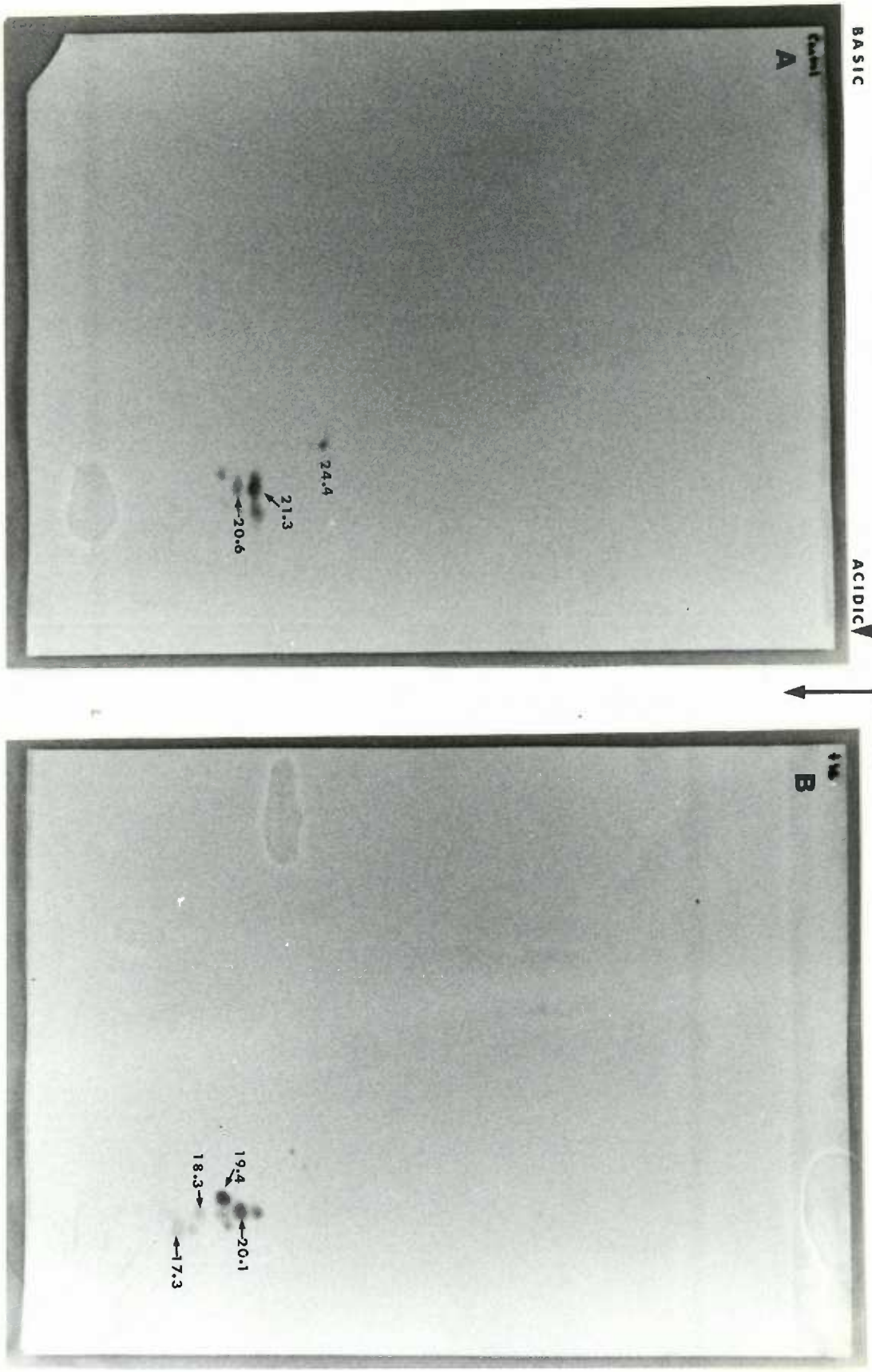
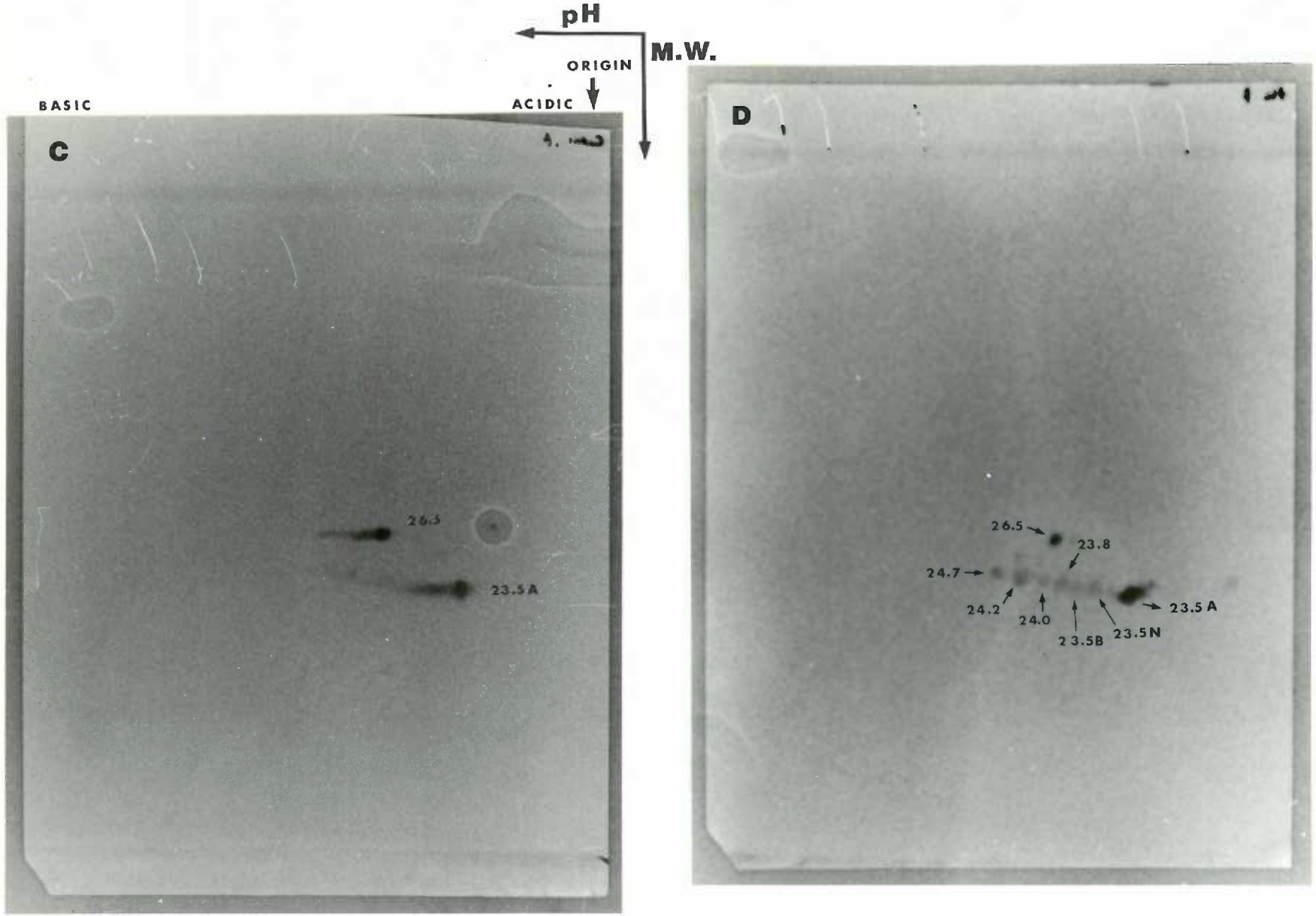

Control
29.0
26.5
25.9
24.7
24.2
23.8
24.4
24.5
23.5A
23.1
23.5B
23.5N
22.5B
22.7
21.3
20.6
Cataract
26.5
24.7
24.2
24.0
23.8
24.5
23.5A
23.1
23.5N
23.5B
22.5B
22.5A
20.1
19.4
18.3
17.3

Figure 3

Immunoblots of insoluble protein from rat lens nucleus. Control proteins were from 17 day old rats, and cataractous proteins were from 17 day old, 7 day postselenite injected rats. (A) control probed with anti-α antibody, (B) cataractous probed with anti-α antibody, (C) control probed with anti-β antibody, (D) cataractous probed with anti-β antibody, (E) control probed with anti-γ antibody, (F) cataractous probed with anti-γ antibody. Polypeptides with positions similar to those diagramed in figure 2 were tentively identified with molecular weights x 10^{-3}.


Control
A
24.4
21.3
20.6
BASIC
ORIGIN
ACIDIC
pH
M.W.
B
19.4
20.1
18.3
17.3


pH
M.W.
ORIGIN
BASIC
ACIDIC
C
26.3
23.5A
D
26.5
23.8
24.7
24.2
24.0
23.5B
23.5N
23.5A

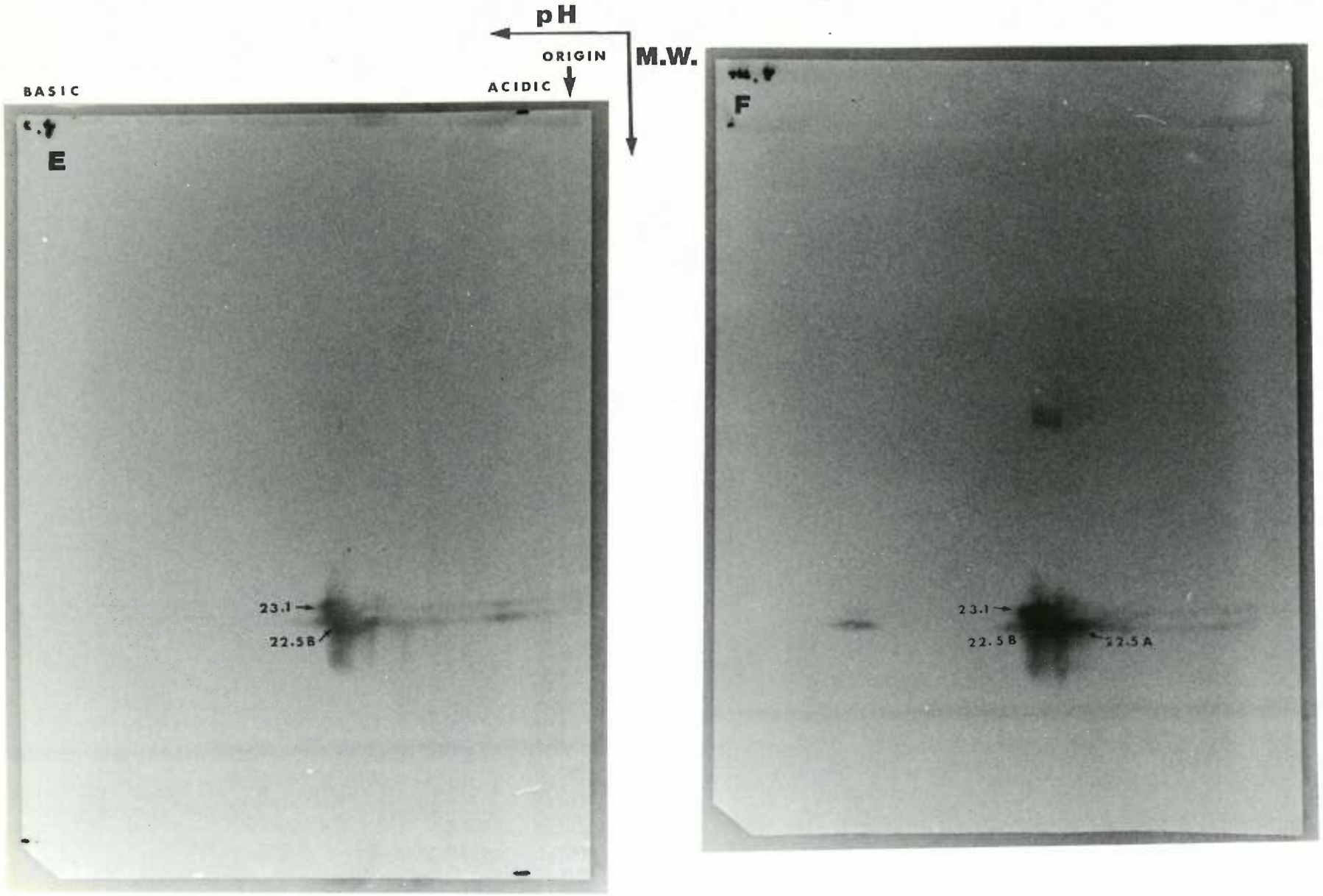

pH
ORIGIN
M.W.
BASIC
ACIDIC
E
23.1
22.5B
F
23.1
22.5B
22.5A

Figure 4

HPLC DEAE 5-PW chromatography of urea solubilized insoluble protein from the nuclei of 17 day old (7 day postselenite) cataractous rat lenses. Numbered peaks were collected. Vertical bars indicate fractions pooled. Arrows indicate peaks which were trypsinized and mapped in figure 7.

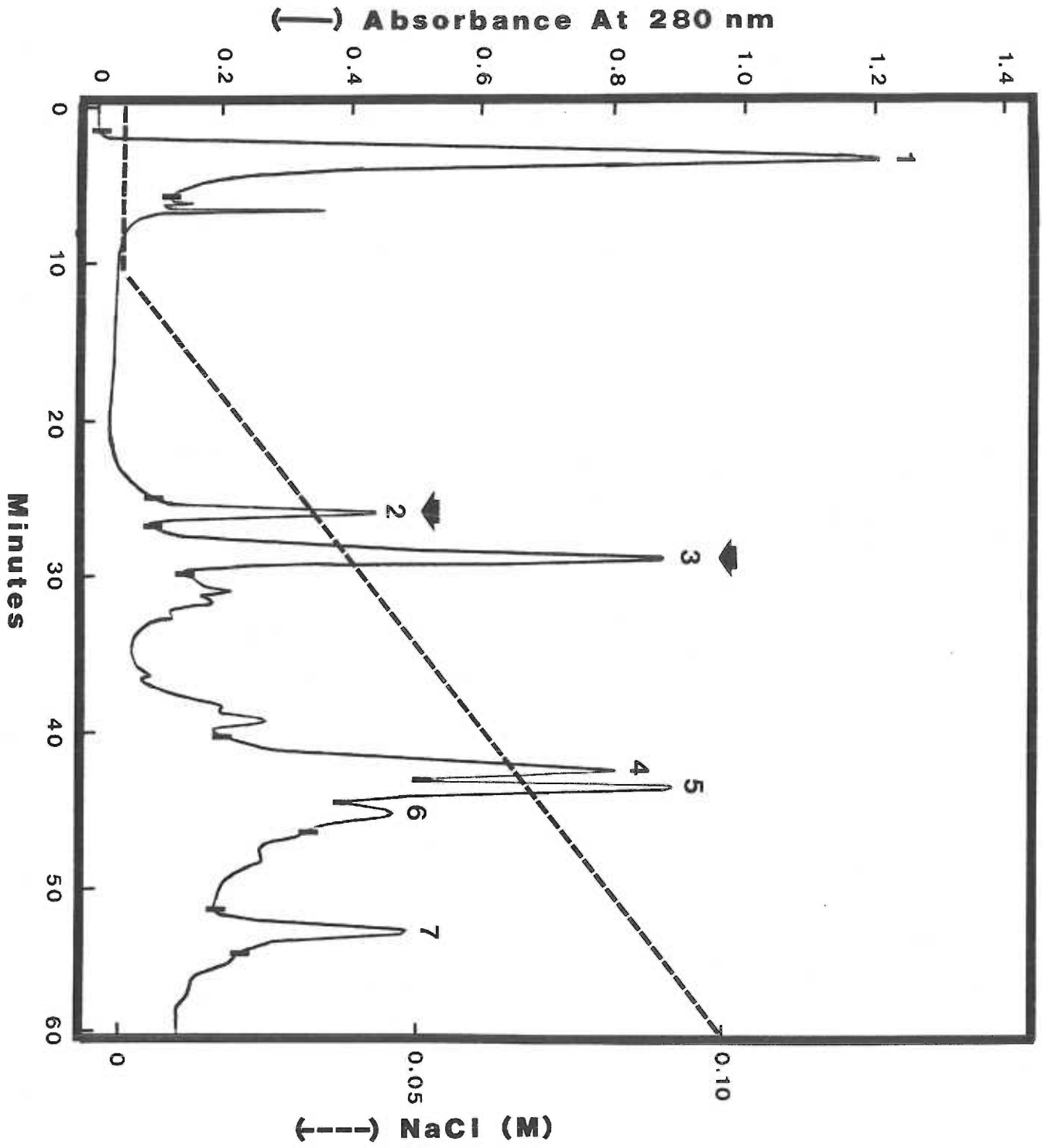

(——) Absorbance At 280 nm
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Minutes
0
10
20
30
40
50
60
1
2
3
4
5
6
7
0
0.05
0.10
(- - -) NaCl (M)

Figure 5

HPLC DEAE 5-PW chromatography of β_L-crystallin from the nuclei of 17 day old control rat lens in 6 M urea. Numbered peaks were collected. Vertical bars indicate fractions pooled. Peak 1 represented 27 % of the total area under the curve.

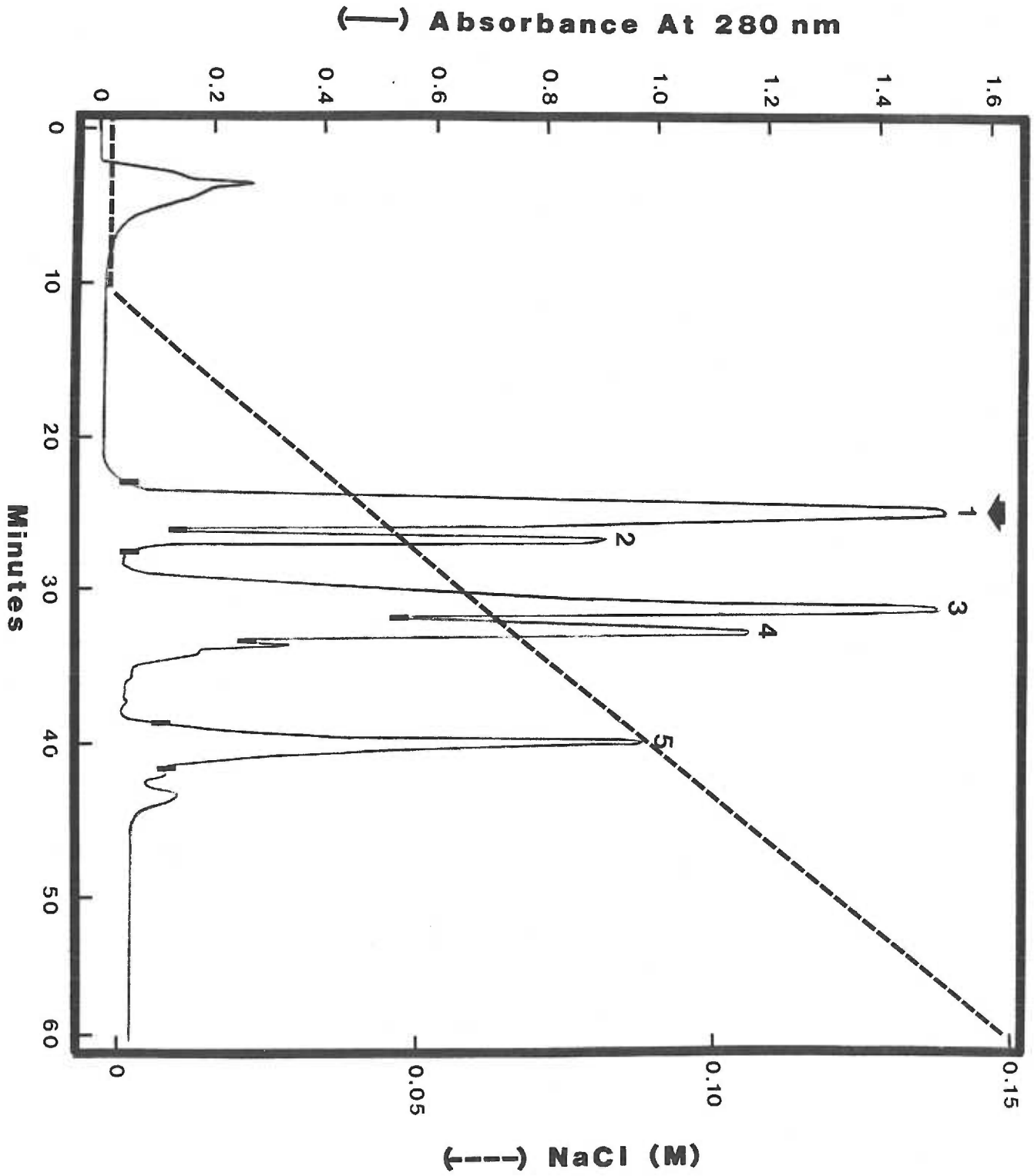

(—) Absorbance At 280 nm
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
Minutes
0
10
20
30
40
50
60
1
2
3
4
5
0
0.05
0.10
0.15
(---) NaCl (M)

Figure 6

Two-dimensional electrophoresis of (A) whole β_L-crystallin, and (B) peak 1 of β_L-crystallin from figure 5. The estimated molecular weights x 10^{-3} are indicated. Bands to the left are molecular weight markers.

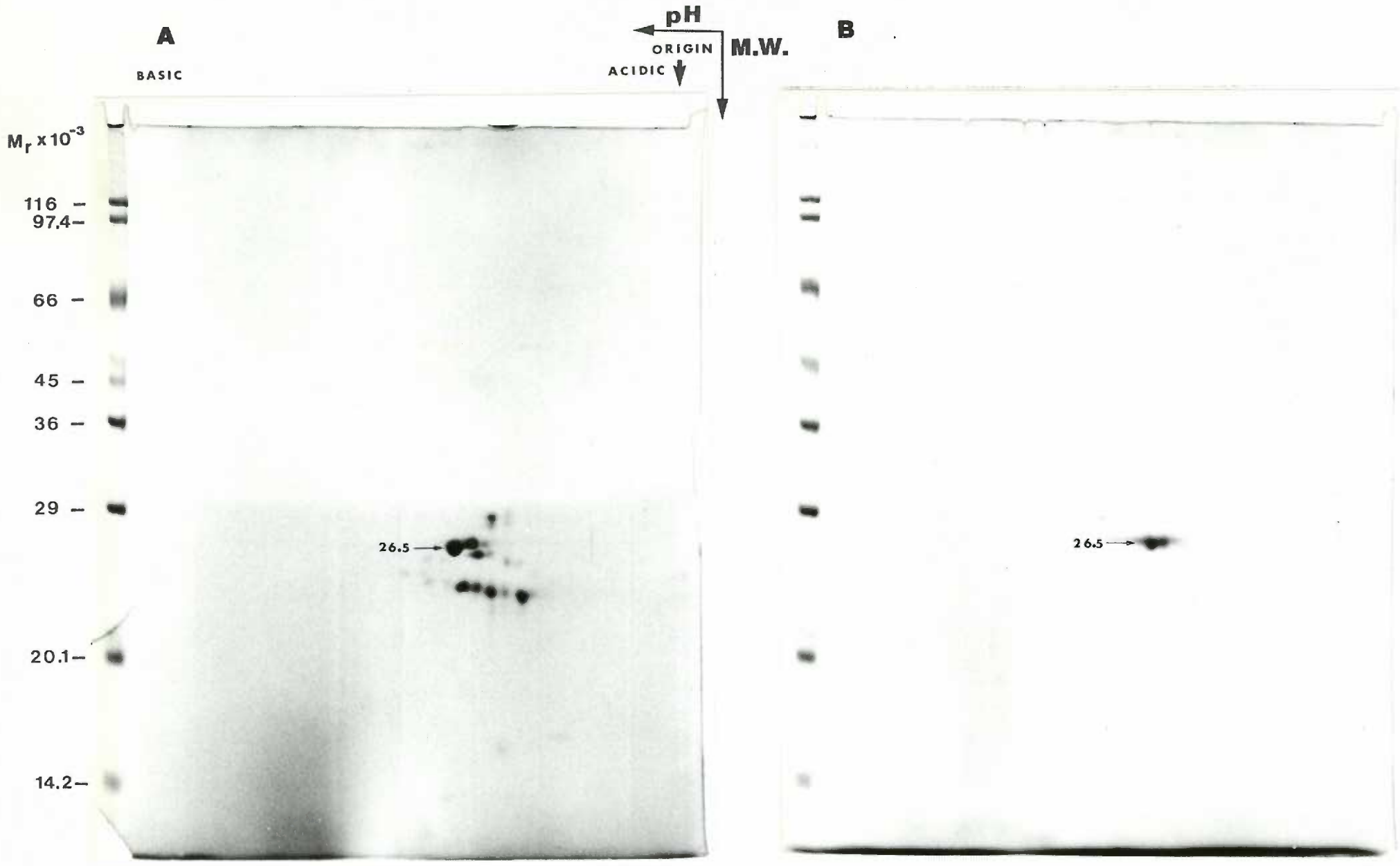
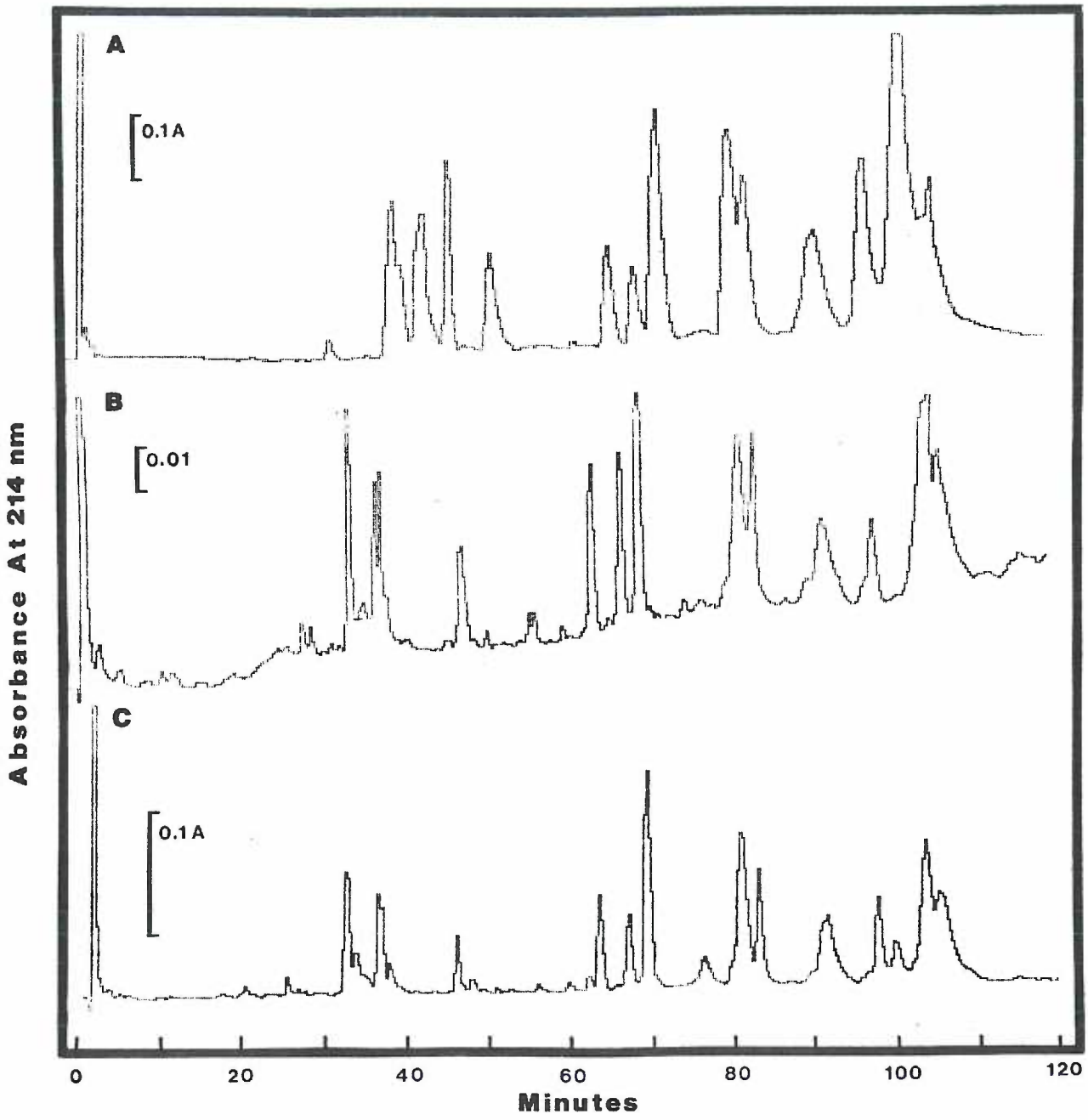

A
BASIC
pH
ORIGIN
ACIDIC
M.W.
$M_r \times 10^{-3}$
116
97.4
66
45
36
29
20.1
14.2
26.5
B
26.5

Figure 7

Tryptic maps of (A) peak 1 from β_L-crystallin, (B) peak 2 from insoluble cataractous protein, and (C) peak 3 from insoluble cataractous protein.


A
0.1A
B
0.01
C
0.1A
Absorbance At 214 nm
0
20
40
60
80
100
120
Minutes

Manuscript 5

A Report

Demonstration of Calpain in Monkey and Human Lenses

L.L. David[*] and T.R. Shearer[*+]

[*]Department of Biochemistry, School of Medicine. [+]Departments of Biochemistry and Ophthalmology, Schools of Dentistry and Medicine, Oregon Health Sciences University, Portland, Oregon 97201.

Running Title: Primate Lens Calpain

Key Words: monkey lens, human lens, calpain, protease, proteolysis, cataract.

Address reprint request to: Dr. T.R. Shearer, Oregon Health Sciences University, Department of Biochemistry, School of Dentistry, 611 S.W. Campus Drive, Portland, Oregon, 97201.

Abstract

This is the first report on the direct assay and detection of calcium-dependant neutral protease (calpain) and its inhibitor in lenses from monkey and man. Monkey lens calpain had an estimated molecular weight of 110,000 during gel filtration. Human lens calpain was detected only in lenses of donors 18 years or younger. Human calpain activity was also localized in the lens cortex, since no activity could be measued in the nucleus. Two peaks of calpain inhibitor were detected in the DEAE eluents of human lens cortex. The presence of calpain in human lenses suggested that this protease may be partly responsible for proteolysis occurring during lens aging and cataract formation.

Introduction

Lenses contain a variety of proteolytic activities (1-3). Evidence for increased proteolysis in human lenses during aging and cataract formation has been presented (4, 5). However, the proteases responsible for these proteolytic changes remain unknown. Recently calpain, a calcium-dependent neutral protease, was purified from lenses of several species (6-8). Calpain may be responsible for increased proteolysis in several rodent experimental cataracts (8). In spite of the evidence implicating calpain in cataract formation, calpain has never been demonstrated in primate lenses. The purpose of the present study was to determine if primate lenses contain calpain.

Meterials and Methods

Monkey Lens Calpain

Lenses were obtained at autopsy from 3 rhesus and 1 cynomologus monkeys. Lenses were decapsulated and homogenized in 8.0 ml buffer A containing 20 mM Tris (pH = 7.5), 1.0 mM EDTA, 1.0 mM EGTA, and 10 mM 2-mercaptoethanol. Following centrifugation at 12,000 g for 60 min at 4° C, the supernatant containing 173 mg protein was applied to a 1.5 x 5.0 cm column of DEAE Bio-Gel A (Bio-Rad, Richmond, CA) equilibrated with buffer A. Following a 4 hour wash with buffer A at a flow rate of 15 ml/hour, proteins were eluted with a linear gradient of 0-0.3 M NaCl in buffer A. Fractions were assayed for calpain activity using FITC-labeled casein as substrate as previously described (8). One unit of calpain activity was defined as the amount of enzyme producing 1 µg acid soluble casein

fragments/min. Active fractions were concentrated by ultrafiltration (YM5 membrane, Amicon, Lexington, MA) and applied to a 7.5 x 300 mm high performance liquid chromatography (HPLC) gel filtration column (TSK G 3000 SW) equilibrated with buffer B containing 20 mM imidizole (pH = 7.0), 1.0 mM EGTA, 1.0 mM EDTA, and 10 mM 2-mercaptoethanol. Proteins were eluted at 0.5 ml/min, and calpain was assayed as described above. The molecular weight of calpain was estimated by comparison to gel filtration standards (Bio-Rad, Richmond, CA). Protein was assayed by the dye binding method (Bio-Rad).

Human Lens Calpain

Pairs of decapsulated lenses from human donors were homogenized in 2.0 ml buffer A and centrifuged at 100,000 g for 60 min at 4° C. Supernatant was applied to a 7.5 x 150 mm HPLC DEAE 5-PW column equilibrated with buffer A. Following a 20 min wash with buffer A at 1.0 ml/min, proteins were eluted with a linear gradient of 0-0.7 M NaCl over 45 min. Calpain in each fraction was assayed as above. Distribution of lens calpain activity within regions of the lens was determined by dissecting a pair of decapsulated human lenses with forcepts under a dissecting scope. A region approximating the lens nucleus (inner 1/3 of the lens) containing 28% of the total lens protein was removed. The remaining region was considered lens cortex. Each region was homogenized and centrifuged as above, and 29 mg of cortical and 9 mg of nuclear protein was applied to an HPLC DEAE column as above.

To detect calpain inhibitor, fractions from DEAE showing calpain activity were pooled and 0.03 ml pooled calpain fraction was mixed with 0.03 ml DEAE fractions exhibiting no calpain activity. After a 10 min incubation at 25° C, the mixture was assayed for calpain activity as before. Synthetic leupeptin, iodoacetate, and phenylmethylsulfonyl fluoride were purchased from Sigma Chemical Company (St. Louis, MO).

Results

Monkey Lens Calpain

During chromatography of protein from monkey lenses on DEAE, a single peak of calpain activity eluted at approximately 180 mM NaCl (Fig. 1). Subsequent gel filtration chromatography showed monkey lens calpain to elute as a single peak of activity with an estimated molecular weight of 110,000 (Fig. 2). Based on a 100 % recovery from the DEAE column, monkey lens capain was purified 530 fold by the two chromatography steps.

Human Lens Calpain

Initial testing showed that no calpain activity could be detected in pools of human lenses when the donors were of adult age. Since calpain may have been present only in young human lenses, single pairs of human lenses of different ages were assayed after DEAE chomatography. A peak of calpain activity was detected in the protein from single pairs of lenses from 1 and 18 year old donors, but not in 27 year old lenses (Table 1).

Several protease inhibitors and activators were tested against human lens calpain (Table 2). Human lens calpain was inhibited by leupeptin and iodoacetate, but calpain was not appreciably inhibited by the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF). Activity was dependent on the presence of calcium, and magnesium would not substitute for calcium in activation.

The distribution of calpain was determined in a pair of 18 month old human lenses. Cortex contained a single peak of calpain activity (Fig. 3A). Based on 100% recovery from this column, the specific activity of calpain in lens cortex was 0.021 units/mg protein. Nucleus contained no detectable calpain activity.

Calpain activity could not be detected in either human or monkey lenses until after DEAE chromatography. This suggested that primate lenses may contain an endogenous inhibitor of calpain. Indeed, 2 peaks of calpain inhibitor activity were detected in lenses from an 18 month old human donor (Fig. 3B).

Discussion

The present report represents the first direct assay of calpain and its inhibitor in primate lenses. This was important because up to now calpain had only been detected in non-primate lenses (6, 7, 8). This opens the possibility that calpain may be important in human lenses either during maturation, or during the pathology of cataract. The suceptability of human lens calpain to activators and inhibitors also confirmed that the activity measured in the present report was due to calpain (8). The 110,000 estimated molecular weight of monkey lens calpain was similar to the estimated molecular weight of bovine and rat lens calpain (6, 8). Elution from DEAE suggested that monkey and human lenses contained high calcium requiring type II calpain (9).

Primate lenses contained lower amounts of calpain activity than rat lenses. Whole rat lenses contained 0.226 U calpain/mg lens protein (8). This was approximately 10 times higher than the calpain activity in 18 month human lens cortex and whole monkey lenses. Detection of calpain in monkey lenses suggested that monkeys may be a good model to study the role of calpain in human lenses.

Although the number of lenses analyzed was small, another important finding from the present data was that calpain may be inactivated during aging in human lens. This inactivation occurred at relatively young age.

Calpain activity was detected in lenses from 1 and 18 year old donors. Calpain activity was not detected in 27 year old lenses or in lenses pooled from donors > 50 years of age. Further studies are needed to determin if calpain is totally inactivated with age, or if it becomes restricted to certain regions such as newly synthesized lens fibers. Calpain activity was found in the cortex, but not the nucleus of 18 month old human lenses. The function of calpain in cortical cells has been postulated to be for degradation of cytoskeletal proteins during maturation (10-12).

Inhibitors of calpain were detected in human lenses. The activity of calpain inhibitor in lens seems to vary with animal species. Bovine lenses, as human and monkey lenses, contained enough inhibitor activity to prevent measurement of calpain in crude whole lens homogenate prior to DEAE chromatography (13). Rats lenses contain a unique low molecular weight calpain inhibitor, yet calpain activity was still easily dectected in crude lens homogenates exposed to elevated calcium (14). In contrast,, mouse lenses contained no detectable calpain inhibitor (7). The relative concentration of calpain inhibitor may determine the extent of calpain activation during cataract formation.

In rodent lenses, calpain may contribute to cataract formation (7, 8). Furthermore, some human cataractous lenses contain increased calcium (15, 16), and evidence for increased proteolysis has been provided (5). Further studies on the function of calpain in human cataract are justified.

References

1. Wagner BJ, Margolis JW, Abramovitz, AS, and Fu S-CJ: Differential inhibition of two proteolytic activities in bovine lens neutral-proteinase preparations. Biochem J 228:517, 1985.
2. Tse SS, and Ortwerth BJ: Activation of proteinases with trypsin-like specificity from bovine and human lenses. Exp Eye Res 32:605, 1981.
3. Oettgen HC, Taylor A: Purification, preliminary characterization, and immunological comparison of hog lens leucine aminopeptidase (EC 3.4.11.1) with hog kidney and beef lens aminopeptidases. Anal Biochem 146:238, 1985.
4. Spector A: Aspects of the biochemistry of cataract. in The Ocular Lens. Structure, Function, and Pathology. (ed. Maisel H) pp.405-438. Marcell Dekker, Inc. New York, 1985.
5. Horwitz J, Hansen JS, and Cheung CC, Ding LL, Straatsma BR, Lightfoot DO, and Takemoto LJ: Presence of low molecular weight polypeptides in human cataracts. Biochem Biophy Res Comm 113:65, 1983.
6. Yoshida H, Murachi T, and Tsukahara I: Limited proteolysis of bovine lens α-crystallin by calpain, a Ca^{2+}-dependant cysteine proteinase, isolated from the same tissue. Biochem Biophys Acta 798:252, 1984.
7. Yoshida H, Murachi T, and Tsukahara I: Age-related changes of calpain II and α-crystallin in the lens of hereditary cataract (Nakano) mouse. Curr Eye Res 4:983, 1985.
8. David LL, and Shearer TR: Purification of calpain II from rat lens and determination of endogenous substrates. Exp Eye Res (in press)

9. Marachi T: Intracellular Ca^{2+} protease and its inhibitor protein: calpain and calpastatin. In: Calcium and Cell Function. Vol. IV, (ed. Cheung, W.Y.), pp. 387-410. Academic Press, New York.

10. Yoshida H, Murachi T, and Tsudahara I: Degradation of actin and vimentin by calpain II, a Ca^{2+}-dependent cysteine protease, in bovine lens. FEBS Letters, 170:259, 1984.

11. Ireland M, and Maisel H: Evidence for a calcium activated protease specific for lens intermediate filaments. Curr Eye Res, 3:423, 1984.

12. Maisel H, and Ellis M: Cytoskeletal proteins of the aging human lens. Curr Eye Res, 3:369, 1984.

13. Yoshida H, Murachi T, and Tsukahara I: Distribution of calpain I, calpain II, and calpastatin in bovine lens. Invest Ophthalmol Vis Sci, 26:953, 1985.

14. David LL, Shearer TR, and Palmer EA: Characterization of a novel inhibitor of calcium dependent neutral protease from rat lens. Invest. Ophthalmol. Vis. Sci. (Supplement). 27:274, 1986.

15. Jedziniak JA, Nicoli DF, Yates EM, and Benedek GB: On the calcium concentration of cataractous and normal lenses and protein fractions of cataractous lenses. Exp Eye Res, 23:325, 1976.

16. Hightower KR, and Reddy VN: Calcium content and distribution in human cataract. Exp Eye Res 34:413, 1982.

Table I

Calpain Activity in Whole Human Lens*

Age of Donor yrs.	Calpain Activity Units/mg lens prot.+
1	9.8×10^{-3}
18	7.8×10^{-3}
27	0

* After HPLC DEAE chromatography of single pairs of lenses.

\+ Calpain specific activity was determined by assuming 100 % recovery during DEAE chromatography.

Table II

Inhibition and Activation of Calpain from Human Lenses*

Factor	% Activity Remaining
Ca^{++} (1.8 mM)	100
Ca^{++} and 1.0 mM PMSF	87
Ca^{++} and 0.2 mM leupeptin	19
Ca^{++} and 5.0 mM iodoacetate	12
EGTA (1.2 mM)	19
Mg^{++} (1.8 mM)	19

* HPLC DEAE purified calpain from a pair of 1 year old human lenses.

Figure 1

DEAE Bio-Gel A chromatography of the soluble protein from 8 monkey lenses.

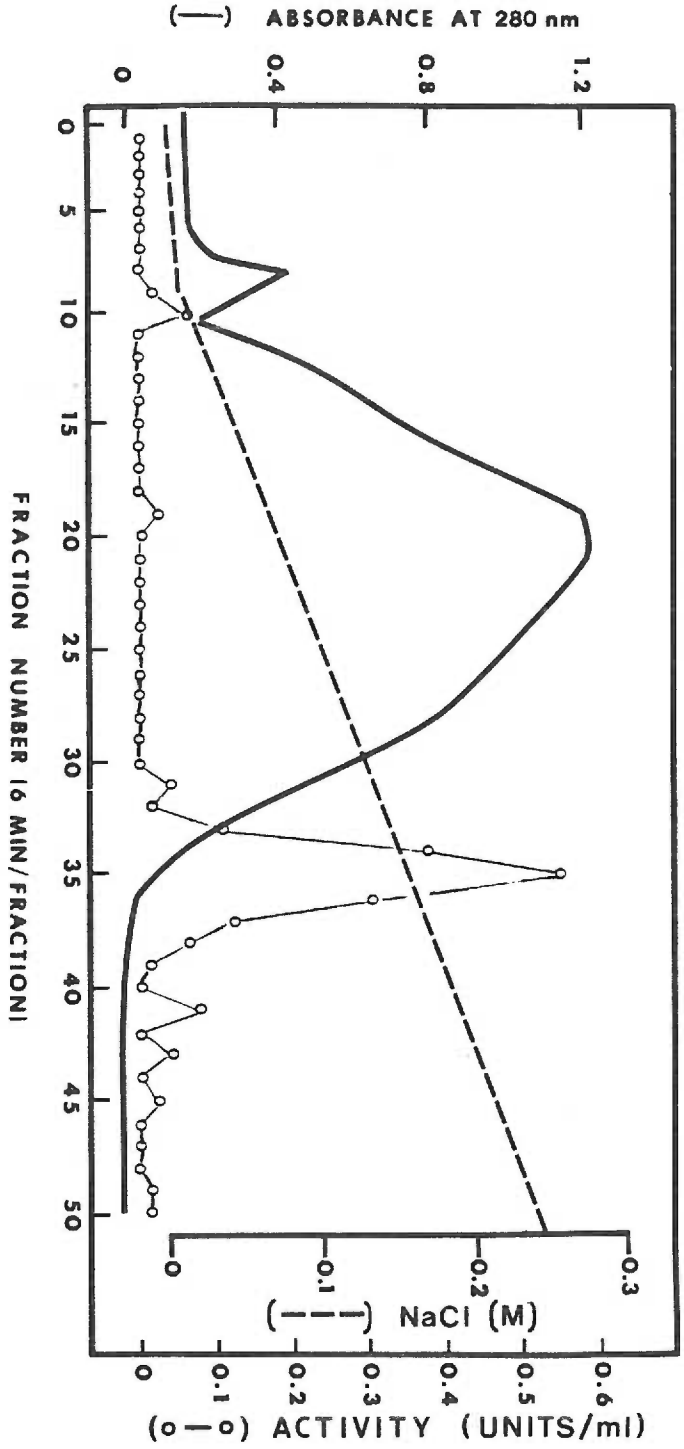

(—) ABSORBANCE AT 280 nm
0
0.4
0.8
1.2
FRACTION NUMBER (6 MIN/FRACTION)
0
5
10
15
20
25
30
35
40
45
50
0
0.1
0.2
0.3
(— — —) NaCl (M)
0
0.1
0.2
0.3
0.4
0.5
0.6
(o — o) ACTIVITY (UNITS/ml)

Figure 2

HPLC gel filtration of DEAE purified monkey lens calpain.

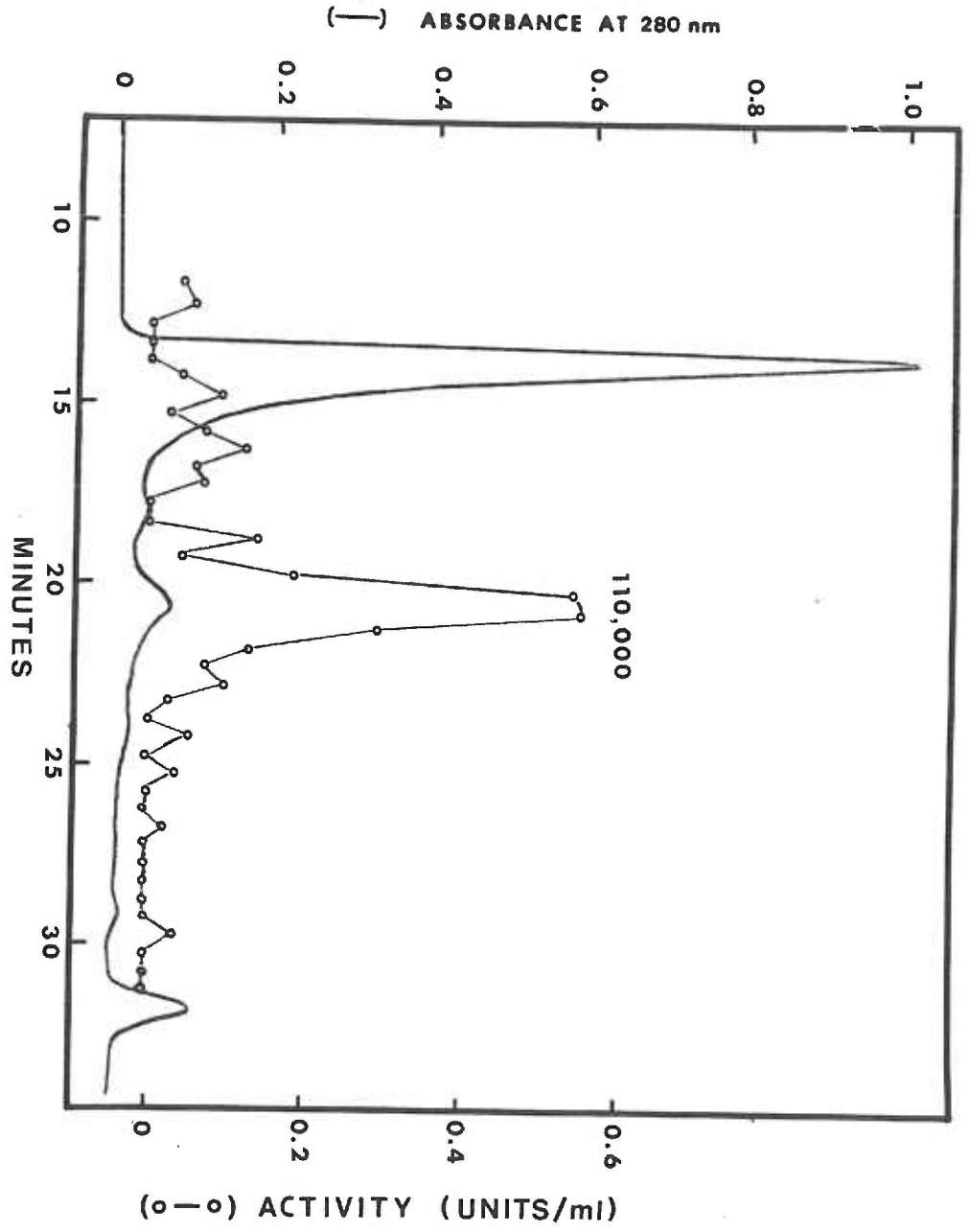
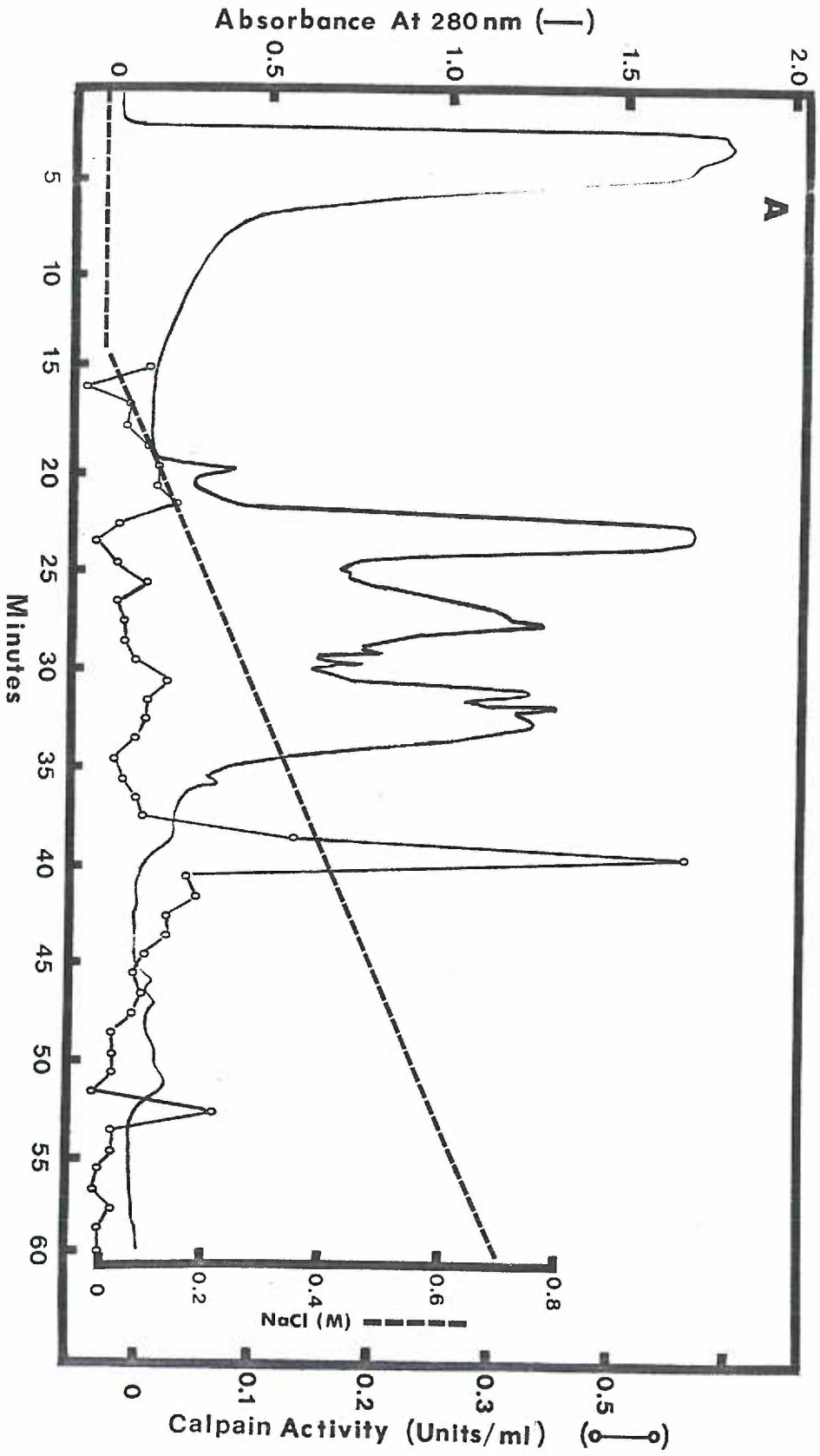
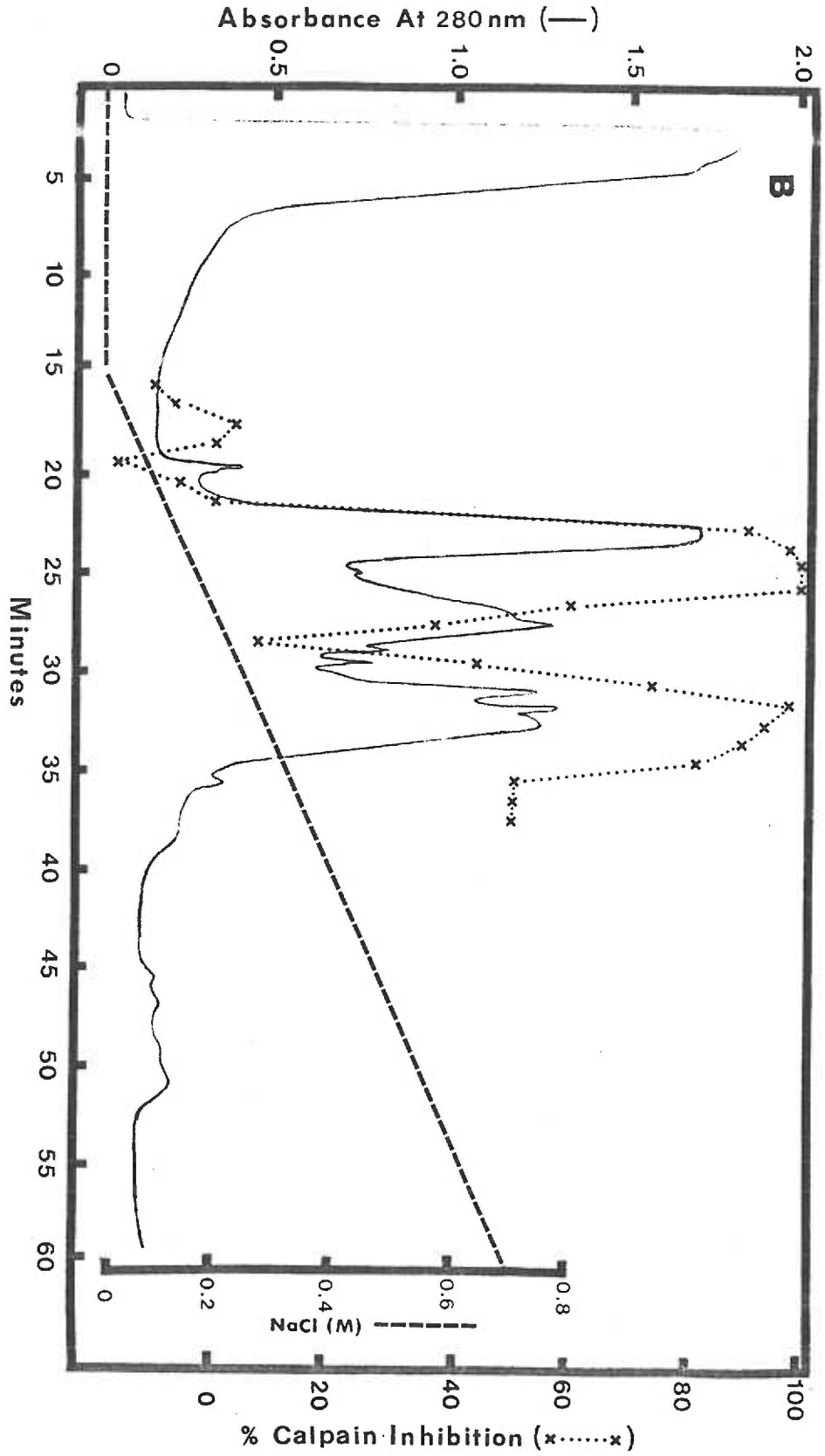

(—) ABSORBANCE AT 280 nm
0
0.2
0.4
0.6
0.8
1.0
10
15
20
25
30
MINUTES
110,000
0
0.2
0.4
0.6
(o—o) ACTIVITY (UNITS/ml)

Figure 3

HPLC DEAE 5-PW chromatography of the soluble protein from the cortex of a single pair of 18 month old human lenses. (A) calpain activity, (B) calpain inhibitor activity.


Absorbance At 280nm (—)
0
0.5
1.0
1.5
2.0
A
5
10
15
20
25
30
35
40
45
50
55
60
Minutes
0
0.2
0.4
0.6
0.8
NaCl (M)
0
0.1
0.2
0.3
0.5
Calpain Activity (Units/ml) (o—o)


Absorbance At 280nm (—)
0
0.5
1.0
1.5
2.0
B
Minutes
5
10
15
20
25
30
35
40
45
50
55
60
0
0.2
0.4
0.6
0.8
NaCl (M)
0
20
40
60
80
100
% Calpain Inhibition (×·····×)

Conclusions

The overall contribution of these studies to our understanding of the biochemistry of the lens was that proteolysis may be an important factor in cataract formation. This idea was supported by 6 major findings: (1) selenite cataract was not the result of extensive disulfide linked aggregate formation, (2) gross elevations of calcium occurred in the lens nucleus during selenite cataract formation, (3), increases in calcium were associated with increased proteolysis in the lens nucleus, (4) proteolysis in the selenite cataract may be caused by activation of the protease calpain, (5) activation of calpain may cause formation of insoluble protein leading to light scatter and opacity in the selenite cataract, and (6) human lenses also contained calpain, suggesting that some forms human cataract could be caused by calpain activation.

Finding no evidence of protein disulfide formation in selenite cataract was surprising, given the potent ability of selenite to oxidize sulfhydryl groups (Manuscript 1). Cataractous lenses from selenite animals were capable of maintaining the majority of protein sulfhydryl in a reduced state inspite of oxidative insult by selenite. Formation of light scattering aggregates in selenite cataracts must have been caused by a different mechanism.

The significance of GSH loss during selenite cataract formation was also examined. Manuscript 1 was the first report on the use of BSO to inhibit GSH synthesis and deplete lens GSH. Formation of selenite cataract could not be attributed entirely to GSH loss since BSO caused lens GSH concentrations to decrease to non-detectable levels, yet no cataracts formed. BSO should be a useful tool to study the importance of GSH in maintaining lens clearity.

Finding elevated calcium in the selenite cataract provided a new direction for study of the mechanism of selenite cataract (manuscript 2). Little is known about how selenite caused increased calcium in the lens nucleus. However, damage to lens epithelial cells may be the underlying cause. The lens epithelium is responsible for the majority of calcium pumping in the lens (1). Oxidants were shown to cause disulfide formation in proteins from lens membranes, leading to increased membrane permeability to calcium (2). Similarily, selenite may oxidize epithelial cell membranes and lead to increased leakiness of these cells to calcium.

Extensive proteolysis occurred in the lens nucleus during selenite cataract formation (Manuscript 2). Soluble α and β-crystallin polypeptides, insoluble proteins, and the main intrinsic membrane protein were degraded. The simultaneous elevation of calcium and initiation of proteolysis in selenite cataract suggested that the calcium-dependant neutral protease calpain was involved. Incubation of lens homogenates with calcium also produced similar proteolysis and again suggested that calpain was active in rat lens.

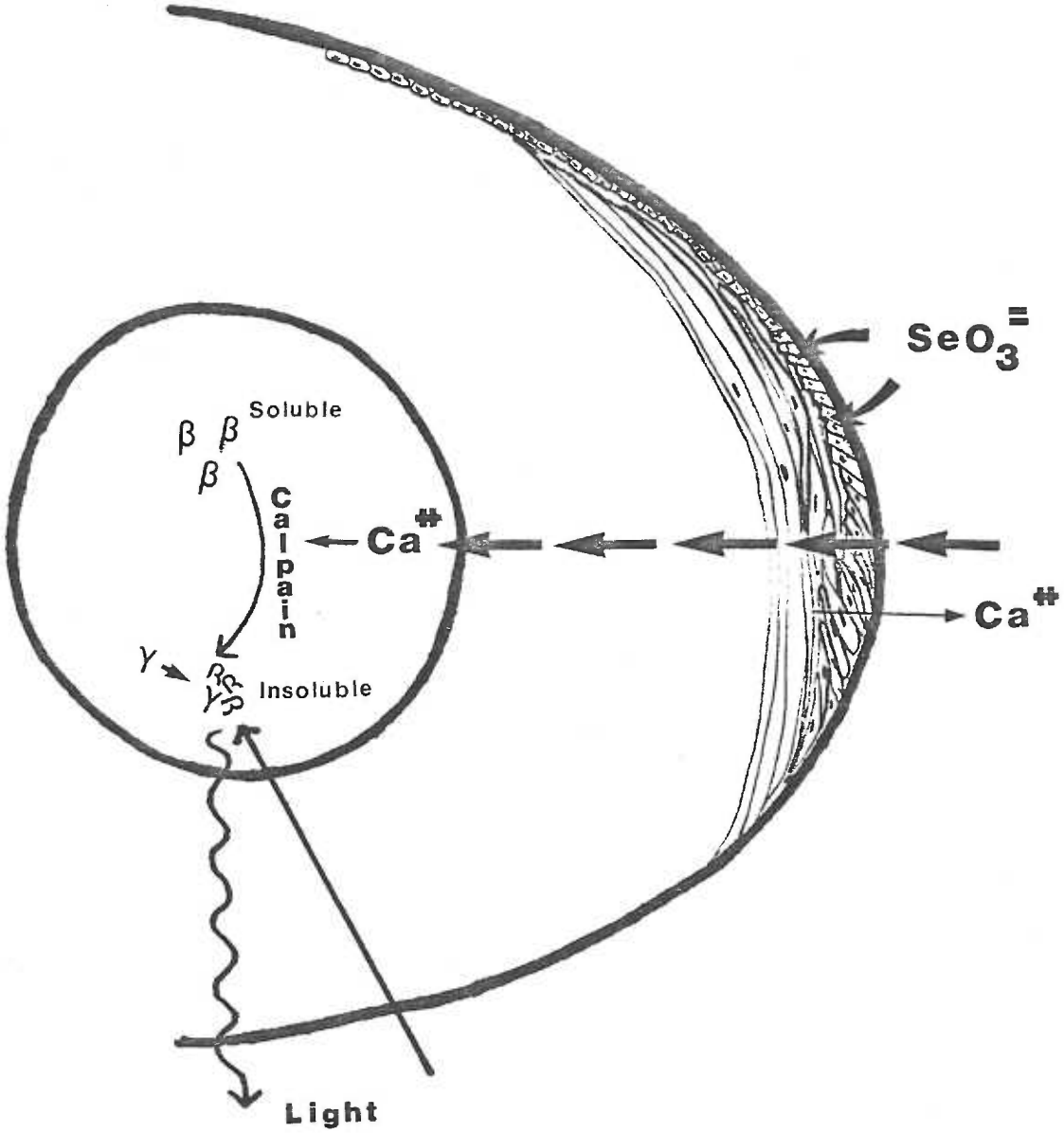
Rat lens calpain was purified and its endogenous substrates were determined (manuscript 3). Purified calpain degraded lens α and β-crystallin polypeptides, insoluble protein, and membrane proteins. The proteolysis caused by purified calpain was similar, but not precisely the same as that occurring during selenite cataract formation. For example, purified calpain did hydrolyze α, and several β-crystallin polypeptides, and insoluble polypeptides as in selenite cataract. However, purified calpain did not extensively degrade a 27K β-crystallin polypeptide, but this polypeptide showed extensive degradation in selenite cataract. The lack of 27K β-crystallin polypeptide degradation by purified calpain may have been

due to the short incubation time, or low concentration of calpain in the incubation mixture. Longer incubation with a higher concentration of purified calpain will determine if calpain can more closely mimic proteolysis during cataract formation. Alternatively, the lens contains proteases other than calpain (3, 4, 5), and these might also be involved during cataract formation.

The composition of the insoluble protein in selenite cataract was determined (manuscript 4). The insoluble protein fraction increased in selenite cataract. Since no HMW aggregates were found in the soluble protein of opaque nuclei, the insoluble protein fraction was postulated to be the cause of increased light scatter. Insoluble protein in selenite cataract was composed mainly of β and γ-crystallin polypeptides. These results explained the loss of soluble β-crystallin described in manuscript 2. Many of the lower molecular weight β-crystallin polypeptides were enhanced in selenite cataract insoluble protein. Peptide mapping data suggested that 2 of the lower molecular weight insolubilized β-crystallin polypeptides were derived from a soluble 26.5K β-crystallin polypeptide. These findings supported the hypothesis that proteolysis may be a cause of crystallin insolubilization and cataract.

The figure below is a summery of our hypothesis of selenite cataract formation. Selenite damages lens epithelial cells allowing entry of extracellular calcium and decreased extrusion of calcium from the nuclear fibers. Calpain is activated by the increased nuclear calcium concentration and soluble β-crystallins and other lens proteins are proteolyzed. These partially proteolyzed β-crystallin polypeptides have altered physical characteristics, such as exposed internal hydrophobic residues, which cause them to become insoluble. During insolubilization they may interact with

non-proteolyzed γ-crystallin polypeptides. The insoluble β-γ-crystallin polypeptides then cause light scatter, leading to formation of nuclear cataract.



The purpose of elucidating the biochemical mechanism of selenite cataract and determining the properties of rat lens calpain was to understand human cataract formation. We provided the first direct evidence

for calpain activity in human lenses (manuscript 5). Since calcium elevations occur during human cataract formation (6, 7), calpain could be partly responsible for increased proteolysis, and formation of light scattering aggregates in human cataract (8, 9). Evidence in manuscript 5 also suggested that human calpain may be inactivated during lens fiber cell aging, and modulated by endogenous inhibitors. These data may indicate that in man calpain may play a role in cataract formation only in young lenses.

References for Conclusion Section

1. Cheng, H.-M. and Chylack, L.T. (1985). Lens metabolism. in The Ocular Lens. Stucture Function, and Pathology. (ed. Maisel, H.) pp. 223-264. Marcel Dekker, Inc. New York.
2. Hightower, K. (1986). Membrane -SH groups and permeability. Invest. Ophthalmol. Vis. Sci. (Supplement) 27, 8.
3. Fleshman, K.R. and Wagner, B.J. (1984). Changes during aging in rat lens endopeptidase activity. Exp. Eye Res. 39, 543-551.
4. Srivastava, Om.P. and Ortwerth, B.J. (1983). Isolation and characterization of a 25 K serine proteinase from bovine lens cortex. Exp. Eye Res. 37, 597-612.
5. Taylor, A., Surgenor, T. Thomson, D.K.R., Graham, R. ., and Oettgen, H. (1984). Comparison of leucine aminopeptidase from human lens, beef lens and kidney, and hog lens and kidney. Exp. Eye Res. 38, 217-229.
6. Jedziniak, J.A., Nicoli, D.F., Yates, E.M., and Benedek, G.B. (1976). On the calcium concentration of cataractous and normal lenses and protein fractions of cataractous lenses. Exp. Eye Res., 23, 325-332.

7. Hightower, K. R. and Reddy, V.N. (1982). Calcium content and distribution in human cataract. Exp Eye Res 34, 413-421.

8. Horwitz, J., Hansen, H.S., Cheung, C.-C., Ding, L.-L., Straatsma, B.R., Lightfoot, D.O., and Takemoto, L.J. (1983). Presence of low molecular weight polypeptides in human brunescent cataracts. Biochem. Biophys. Res. Comm. 113, 65-71.

9. Spector, A. (1985). Aspects of the biochemistry of Cataract. in The Ocular Lens. Structure, Function, and Pathology. (ed. Maisel, H.) pp.405-438. Marcel Dekker, Inc. New York.