

STUDIES OF A LOW MOLECULAR WEIGHT ZN-CONTAINING
PROTEIN POPULATION OF LENS TISSUE

by

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LIST OF ABBREVIATIONS

A:	Absorbance
EDTA:	ethylenediaminetetraacetic acid
GSH:	reduced glutathione
GSSG:	oxidized glutathione
PAGE:	polyacrylamide gel electrophoresis
SDS:	sodium dodecyl sulfate
TCA:	trichloroacetic acid

INTRODUCTION

Maintenance of transparency in the lens depends on the native configuration of lens proteins. Sulfhydryl metabolism is an important factor in the regulation of lens protein conformation. Interruptions of normal sulfhydryl metabolism may result in cataract formation.

The tripeptide glutathione (L- γ -glutamyl-L-cysteinyl-glycine) has a key role in lens sulfhydryl metabolism. Depletion of glutathione levels is correlated with cataract formation, since glutathione maintains proteins in a reduced state and is a scavenger of toxic electrophiles and peroxides(1). A model for the study of changes in cataractous vs. normal lens metabolism is selenite-induced cataract in which there are reproducible, abrupt and sustained depletions of glutathione levels beginning prior to cataract formation(2).

Low molecular weight proteins (less than 20,000 daltons) are known to exist in the lens. Much recent interest has focused on the cysteine-rich low molecular weight (6,500 daltons), metal-binding protein metallothionein which has been isolated from various mammalian tissues. Like glutathione, the important chemical moiety of metallothionein is the sulfhydryl group of cysteine. The relatively high cysteine content dictates the function of this protein. Glutathione and metallothionein have been proposed to have similar physiological roles, including detoxification, maintaining proteins in a reduced state and functioning as a cellular sulfhydryl source(3). Thus it is of interest to establish the presence and metabolism of metallothionein in the lens.

Trace metals are important in the lens and altered levels are

associated with cataract formation. Elevated metal levels are typically observed with cataract and can significantly impair glucose metabolism in the lens. Transparency of the lens depends on an intact energy metabolism(4). Thus, the interaction between trace metals and protein may be important in maintaining transparency.

Lens Zn levels were quantified at different ages in various species, and as a function of dietary changes and selenite-induced cataract. A Zn-enriched low molecular weight population of proteins was isolated from bovine and rat lens tissue by protein purification techniques based on the parameters of molecular size and charge. SDS-polyacrylamide gel electrophoresis, isoelectric focusing and amino acid determination were used to aid the further characterization of this low molecular weight Zn-containing protein population of lens tissue.

LITERATURE REVIEW

The Lens

The eye lens is considered to be an ideal tissue for the study of various fundamental biological processes. Since no cells are ever sloughed from it, there exists a single cell type where the cells are concentrically arranged by age. The lens is a transparent, highly refractive structure which functions to focus light on the photosensitive retina. Since it is completely avascular, nutrients and oxygen are received by diffusion from the surrounding fluids.

The bulk of the lens consists of fiber cells which arise from differentiation and elongation of the single layer of metabolically active epithelial cells. As the fiber cells mature, they lose their nucleus and most of their cellular organelles, thereby reducing light scattering. The entire lens surface is covered by a mucopolysaccharide capsule produced by the epithelial cells(5).

The lens has the highest protein concentration of any tissue; approximately 35% of the wet weight and nearly all the dry weight is protein. Lens specific, water-soluble structural proteins called crystallins comprise about 90% of this protein. Three major classes of crystallins, the α -, β - and γ -crystallins, are present in the lenses of mammals. Crystallins have been highly conserved during species evolution suggesting that there are strong selective constraints operating against changes in the primary structure. It is inferred then that the functional integrity of the crystallins demands strict maintenance of structure(6).

The insoluble protein fraction is defined as the portion of lens protein which does not dissolve when the lens is homogenized in aqueous buffer. The water-insoluble fraction of lenses is partly the lens membranes but when examined after solubilization in a denaturant (e.g. guanidine, urea), consists mostly of similar proteins as are found in the soluble fraction. The partitioning of the protein between the insoluble and soluble fractions depends on species, age and method of preparation. The state of the insoluble protein in vivo is not known nor is it clear what effect it has on the functioning of the lens(7).

Water content is of great importance to the function of the lens. Accommodation, the automatic adjustment by which the eye adapts itself to distinct vision at different distances, is regulated by the relative amounts of water and protein present. The efficiency of accommodation depends on the ease with which the lens can change its degree of curvature and the difference in refractive index between the lens and its surrounding humors. Water is the medium for all chemical reactions in the lens and participates in most of them. It is essential for the maintenance of pH, osmotic pressure and viscosity. Protein conformation is clearly influenced by the degree of hydration(8).

Crystallins

Lens crystallins are the only proteins known to be organ specific and have a role in the stabilization of membrane and cytoskeletal components. They are extremely heterogeneous with respect to both molecular size and charge.

α -crystallins have the highest molecular weight of the lens proteins and exist as a mixture of different sized macromolecular aggregates of

greater than 500,000 daltons. They are also distinguished by their low isoelectric points; values are in the range of 4.8-5.0. All α -crystallins basically are made up of the four subunits A_1 , A_2 , B_1 and B_2 , each with a molecular weight of 20,000 daltons. A portion of these subunits undergoes post-translational modification which contributes to the heterogeneity and polymerization of native α -crystallin. Compared to the other crystallins, α -crystallin is relatively low in thiol content, containing about 3 half-cysteine residues/1000 amino acid residues(7).

β -crystallins have been the least well defined structural proteins of the lens. The content of β -crystallins generally increases gradually throughout life and they are the major protein in all except very young lenses. Like the α -crystallins, they are a heterogenous class of proteins which exist as oligomers of several different sizes in the range of 28,000-210,000 daltons(7). β -crystallins can be separated into two fractions of differing molecular size by gel filtration chromatography. The two fractions are termed β_H (high) and β_L (low). Both types are complex aggregates consisting of seven or more subunits, as determined by UREA-PAGE¹, and have pI values ranging from pH 5.7 to pH 7.0(9).

The lowest molecular weight crystallin species, the γ -crystallins, are homogeneous in terms of size but electrophoretically heterogeneous. They are monomers of molecular weight 20,000 daltons with highly homologous sequences. The isoelectric points for the γ -crystallins are the most basic of the crystallins, falling in the range of pH 7.1-8.1(9). The three-dimensional structure of bovine γ -crystallin has been characterized by Blundell et al.(10) and possesses the highest degree of

¹Abbreviations appear in the List of Abbreviations, p. vi.

intrachain symmetry of any protein thus far studied in this manner. Blundell proposes that this high degree of symmetry contributes towards the stability of lens proteins since they must exist throughout the life of the animal without being denatured(10). γ -crystallins have a much higher thiol content than the other crystallins with 34 half-cysteine residues/1000 amino acid residues. γ -crystallins constitute 25% of the total soluble protein of the calf lens and 60% in the weanling rat lens(11). The relative proportions of water-soluble lens proteins are altered with increasing age; the proportion of the γ -crystallin component decreases while the proportion of the α -crystallin increases(11).

Lens Sulfhydryl Metabolism

Human senile cataract development is associated with several perturbations of lens sulfhydryl metabolism. One of these is the increased protein disulfides caused by progressive loss of protein sulfhydryl through the formation of protein-protein disulfides and mixed disulfides of protein and glutathione(12). Also, senile nuclear cataract formation is accompanied by a progressive oxidation of cysteine and methionine(13).

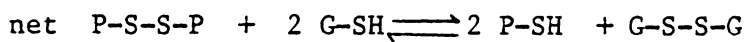
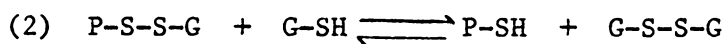
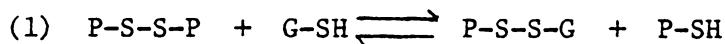
Harding(14) showed that during cataract development lens proteins change their native conformation. He assessed the rate of carboxymethylation of protein thiol groups with iodoacetate of normal vs. cataractous lenses. The thiol groups of the cataractous tissue were more reactive, suggesting that they are more accessible. Since most protein thiol groups in the normal lens are unreactive, he proposed that they were buried within the native protein structure. Protein chains of the

cataractous tissue had at least partly unfolded, exposing previously buried thiol groups to reagents in the aqueous environment(14).

Thiol oxidation occurs in the aging and cataractous human lens and is not counteracted effectively(15). There is an age dependent reduction in glutathione content; levels decrease from about 3.5 $\mu\text{mole/g}$ of lens at 20 years of age to about 1.8 $\mu\text{mole/g}$ of lens at age 65 years. An increase in protein-glutathione mixed disulfide is also found. The disulfided reducing activity is diminished(16).

The tripeptide glutathione (L- γ -glutamyl-L-cysteinyl-glycine) has a physiological role in the lens of maintaining the protein sulfhydryl of cellular proteins in a reduced state and in detoxification of peroxides and electrophiles(17). Glutathione is synthesized in the lens. Virtually no catalase and very little superoxide dismutase are found in the lens as barriers against free oxygen radicals; thus glutathione is very significant in inhibiting oxidative reactions. It is the most abundant low molecular weight thiol-containing molecule with a lens physiological concentration of 3mM(11).

The mechanism whereby glutathione in the lens maintains protein thiol in a reduced state has been proposed by Augusteyn as:



The protein disulfide is represented as P-S-S-P. Glutathione reacts with the protein disulfide to form a mixed disulfide, P-S-S-G, which is then further reduced by the addition of another GSH molecule. Glutathione can thus protect protein sulfhydryl groups from oxidation provided

that the glutathione reductase system is functioning(17).

Glutathione reductase catalyzes the reaction: $\text{GSSG} + \text{NADPH} \rightleftharpoons \text{GSH} + \text{NADP}^+$. The NADP^+ generated by this reaction is then reduced by glucose-6-phosphate dehydrogenase of the hexose monophosphate shunt. Endogenous glutathione therefore protects lens protein sulfhydryl groups from oxidation provided NADPH and glucose-6-phosphate dehydrogenase are present to maintain a reduced pool of glutathione(2).

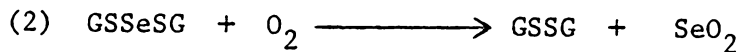
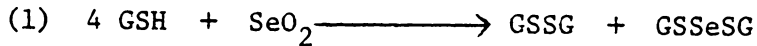
Selenite-Induced Cataract

Selenite administration to young rats can be used as an experimental model for inducing changes in lens sulfhydryl metabolism. Ostadalova and colleagues originally reported that cataracts could be generated in suckling rats by subcutaneous injection of 20 μmole sodium selenite/kg body weight(18). There were no other gross signs of injury or toxicity. Cataracts appeared within 72 hours following injection and were preceded by a 60% decrease in lens glutathione levels which remained depressed to 30% of normal through 12 days post-injection. Lens selenium increased from 0.25 to 0.75 ppm in the 24 hours following injection and thereafter slowly declined. NADPH levels were also perturbed as a decrease of 31% was observed beginning 24 hours post-injection. However, the lens NADPH pool was recovered to normal levels by 72 hours following injection. Longer term biochemical changes accompanying cataract development include a decrease in lens weight and protein content(2).

Selenite exposure, by virtue of its ability to influence lens glutathione levels, can be used to investigate the importance of oxidative stress to cataract development. The mechanism of the effect of

selenite is catalytic and not stoichiometric since the accumulated selenium amounts to only 6 pmoles/mg lens but the glutathione loss is over 3 nmoles/mg lens(2).

Selenite can stimulate a non-enzymatic oxidation of glutathione according to the following equations(19):



The occurrence of these reactions in the lens of the selenite-injected rat would help explain the reduction in glutathione levels. Other possible mechanisms whereby selenium can cause cataract formation and biochemical changes in the lens would be through alterations in the activities of some of the enzymes of glucose metabolism necessary for energy production(2).

Low Molecular Weight Lens Polypeptides

In the normal lens, age-related changes in α -, β - and γ -crystallin populations occur. With cataractous tissue, high molecular weight protein increases and the content of low molecular weight protein decreases. The diminished levels of low molecular weight protein could result from protein leakage through the capsule, conversion to insoluble protein or protein cross-linking to higher molecular weight species(7). Proteins of molecular weight less than the γ -crystallins have been isolated and characterized from lens tissue. Although some of these species are proposed to be degradation products of lens crystallins, there is some support that low molecular weight polypeptides are normal components of the lens soluble protein.

In a study of the γ -crystallins of the human lens, Zigler et al. isolated a 10,000 molecular weight species which was shown to increase in amount with age(20). It was not a γ -crystallin since it did not cross react with antiserum specific for γ -crystallin and contained the most highly acidic components present in the lens proteins. The 10,000 dalton polypeptide was isolated by gel filtration chromatography and had very low A_{280} associated. In 9-month old human lenses there was no detectable 10,000 dalton species. Zigler et al. suggested that the 10,000 dalton polypeptide was a degradation product(20).

Roy and Spector(21) isolated a polypeptide of molecular weight 9,600 that was very heterogeneous with respect to molecular charge. Analysis of tryptic peptides revealed that this 9,600 dalton polypeptide was generated at least partly as a result of post-translational C-terminal shortening of the α -crystallin A chain. They further reported that the α -A chains are subject to preferential modification and are the source of this low molecular weight polypeptide(21).

An 11,000 dalton protein was isolated from human lenses in a study of the molecular weights of the native protein constituents of the water-soluble protein(22). With aging and cataract development, the amount of this 11,000 dalton species decreased. The authors proposed that this component might represent a building block which could aggregate with either proteins of the same molecular weight or with other polypeptides(22).

A protein has been isolated from soluble extracts of bovine lens tissue which inhibits both trypsin and the endogenous lens proteinases(23). The inhibitor was purified from α -crystallins extracted with 2.5% TCA at 70°C. Gel filtration chromatography separated the inhibitor species

which had a molecular weight of 10,000-11,000 as determined by SDS-PAGE. The pI of this proteinase inhibitor was 4.8. With aging, an increase in proteinase activity was observed along with a concomitant decrease in inhibitor activity. Enhanced inhibitor activity was found in the water-insoluble fraction of older lenses. Insolubilization of the inhibitor was proposed to lead to the increased proteinase activity seen during aging(23).

Metallothionein and Trace Metals

A noteworthy protein of low molecular weight isolated from many tissues but not yet reported to be in the lens is metallothionein. Metallothionein is a metal-containing protein with a molecular weight of 6,500 daltons that is ubiquitous in nature. It is an unusual protein since 20 of its 61 amino acids are cysteine. The cysteinyl side chains are bound to metal ions in mercaptide complexes and the stoichiometry of bound metal depends on the specific metal ion bound. The synthesis of metallothionein can be induced by metal ions. Other characteristics of this protein are that it contains no aromatic amino acids and that its primary structure is highly conserved with respect to cysteine, serine and basic amino acid positions(24). Metallothionein possesses high electrophoretic mobility; reported pI values are tissue and species dependent and fall in the range of 3.5-5.5(25).

The roles of metallothionein are not clear, although it is known to be important in metal detoxification. Its synthesis is induced by Cd, Zn and Hg. Other proposed physiological roles of metallothionein include that it could serve as an intracellular reservoir for essential

elements such as Zn and Cu, it could function in the maintenance of redox potentials (analogous to the role of glutathione), or in ion transport (25).

The sulfhydryl groups in metallothionein are involved in detoxification as is the sulfhydryl of glutathione. An inter-relationship between glutathione and metallothionein has been tested by Wong and Klaassen. They found that alkylating agents such as diethyl maleate, can increase the concentration of hepatic metallothionein and decrease the level of glutathione in rats(3). This opposing change in hepatic levels of these two sulfhydryl compounds after treatment with alkylating agents suggested a relationship between metallothionein and glutathione. However, the induction of metallothionein caused by metals was not preceded by any significant change in glutathione levels, although cadmium injection did decrease the glutathione levels slightly(3).

Cu and Zn ions are involved in lens metabolism and alterations in their levels occur on aging and with cataract formation. Elevated metal levels have been shown to impair glucose metabolism in human and bovine lens homogenates(4). Copper is a component of essential metalloenzymes. Metabolically, the copper in the lens is probably required to maintain transparency and to assure the proper functioning of the cytochrome oxidase and coenzyme A dehydrogenase systems. If this were the function, then Cu content would be elevated in the epithelial layer since it is the only site in the lens which possesses mitochondria. Copper content of lens tissue decreases progressively with age, but as cataract develops the levels increase. Fully developed cataractous lenses have

been reported to contain ten times more copper than normal(4). Zinc in the lens could be important in the growth of the epithelial layer and contribute to the maintenance of the optimal configuration of soluble proteins and the plasma membrane, thus contributing to the maintenance of transparency. In a study using the amphibian lens, it was observed that Zn was bound tightly, in an unexchangeable form for the most part(26). The relationship between lens zinc concentration and cataract is unclear as reports of elevated, depressed and unchanged levels exist(4).

In summary, it is apparent that the uniqueness of lens tissue requires that the native protein conformation of the specific protein population be strictly maintained. It is therefore necessary to describe, identify and relate how sulfhydryl metabolism, crystallins, low molecular weight peptides, and trace metals interact to maintain lens transparency.

EXPERIMENTAL PROCEDURES

Materials

Sprague-Dawley strain rats and Purina Rat Lab Chow were obtained from Flow Laboratories, Inc. (Dublin, Virginia).

Bovine lenses were obtained from the VPI & SU Food Science Department from recently slaughtered animals.

Sephacryl S-200 (superfine), Sephadex G-25 (superfine), Sephadex G-75 (fine), Blue Dextran, Polybuffer Exchanger 94 and polybuffers for chromatafocusing were purchased from Pharmacia Fine Chemicals (Piscataway, New Jersey).

Ultrafiltration membranes, UM2 (2,000 MW cut-off) and centriflo CF25 (25,000 MW cut-off), cone plastic supports and graduated collection tubes were obtained from Amicon Corporation (Lexington, Massachusetts).

Bovine Serum Albumin, Fluorescamine, Coomassie Brilliant Blue R-250, Tris (Sigma 7-9 and Trizma base) and Glycine were obtained from Sigma Chemical Company (St. Louis, Missouri).

High purity acrylamide, high purity N,N'-methylene bis acrylamide, N,N,N',N'-tetramethylethylenediamine, sodium dodecyl sulfate, electrophoretic grade ammonium persulfate and the silver stain kit were obtained from Bio-Rad Laboratories (Richmond, California).

Ampholyte Polyacrylamide Gel Electrophoresis plates for isoelectric focusing were purchased from LKB (Upsala, Sweden).

Ultrex HCl for atomic absorption spectrophotometry was purchased from J.T. Baker (Phillipsburg, New Jersey). Certified Zn atomic absorption standard was purchased from Fisher (Fairlawn, New Jersey).

^{203}Hg and ^{65}Zn were obtained from New England Nuclear (Boston, Massachusetts). L-15 culture medium was from Flow Laboratories (Rockville, Maryland).

Methods

Care and Feeding of Rats - Dams with litters were housed in plastic cages in a temperature-controlled room (21°C) which had a 12 hour day/night cycle. They were fed commercial lab chow and deionized water ad libitum. Weaned animals were housed in hanging stainless steel cages and allowed free access to chow and water.

Preparation of Lens Homogenates - Rats were decapitated or suffocated in CO_2 and the lenses removed. Bovine lenses were dissected from the eye immediately after death. When used, the epithelial layer was dissected from the cortex and nucleus. Samples were homogenized at 4°C in a 1:10 ratio of weight to buffer volume of a Tris buffer using a teflon pestle-glass homogenizer. After centrifugation at $35,000 \times g$ for 30 minutes, the soluble fraction was removed and either used directly or further fractionated with Amicon membranes.

Gel Filtration Chromatography - The soluble protein was separated into α -, β_{H} , β_{L} and γ -crystallin fractions by Sephacryl S-200 chromatography at room temperature. Resin was contained in a 1.5×90 cm column and equilibrated with either 0.065M Tris-Cl (pH 7.6), 0.05M NaCl and 0.001M EDTA or 0.10M Tris-Cl (pH 7.6). A flow rate of 1.1 ml/min was used and 2 ml fractions collected. A_{280} content of fractions was monitored by manual absorbance measurements on a Gilford spectrophoto-

meter (Model 250), while the Zn content of fractions was determined by atomic absorption spectrophotometry.

Samples analyzed by Sephadex G-75 were applied to a 1.0 x 50 cm column of resin equilibrated with 10mM Tris-Cl, pH 8.6 at 4°C. A 1.0 x 30 cm Sephadex G-25 column was used with an elution buffer of 100mM Tris-Cl, 50mM NaCl, pH 7.6 at 4°C. Fractions were monitored for absorbance at 280nm and for Zn content.

For all gel filtration experiments the column void volume was calculated from the elution peak of 5 mg of blue dextran dissolved in an appropriate sample volume of buffer. The total column volume was determined by the peak elution volume of either standard potassium dichromate or bromophenol blue solutions.

Ultrafiltration- The cone-shaped CF25 membrane was inserted into a plastic support which had a small hole at the bottom. This was placed in a graduated collection tube. The supernatant sample was applied and centrifuged at 1,000 x g for 90 minutes at 4°C. Filtrates were analyzed directly by gel filtration chromatography.

Zinc containing protein peaks were pooled and concentrated under nitrogen pressure (45 psi) at 4°C using an Amicon UM-2 membrane and ultrafiltration cell.

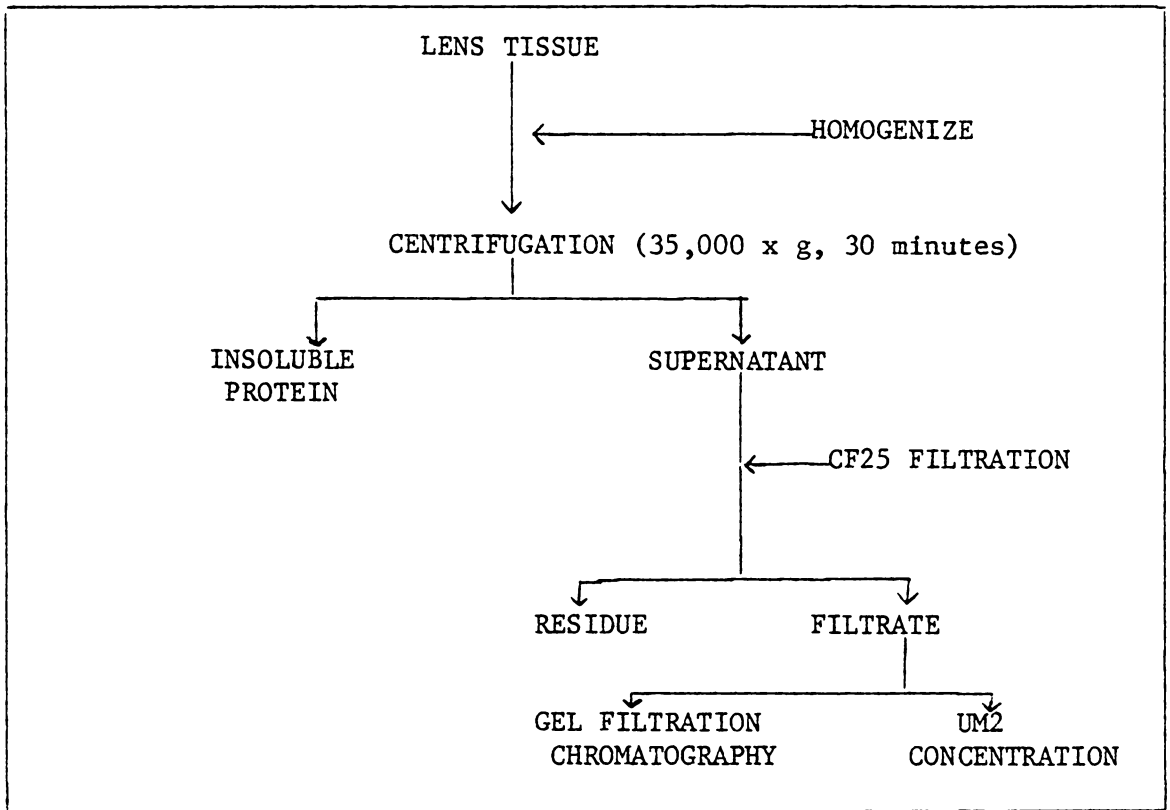
A summary of a typical sequence of steps for separation of low molecular weight protein is shown in Figure 1.

To evaluate the potential binding/absorption of Zn to CF25 membranes, L-15 culture medium containing ^{65}Zn was applied either directly or diluted 1:4 in 100mM Tris-Cl, 50mM NaCl, pH 7.6 to new CF25 membranes and centrifuged at 1,000 x g for 90 minutes. The ^{65}Zn recovered in the

FIGURE 1

Isolation Protocol for a Molecular Weight Defined Protein Preparation

Lens tissue was homogenized in a Tris buffer and then centrifuged to obtain a soluble protein or supernatant sample. This was applied to an Amicon CF25 ultrafiltration cone (exclusion limit 25,000 daltons) and centrifuged at 1,000 x g for 90 minutes to resolve the supernatant into high molecular weight, residue, and low molecular weight, filtrate fractions. The filtrate sample was then further characterized by gel filtration chromatography or concentrated on UM2 membranes to remove very low molecular weight moieties.



residue and filtrate fractions was measured in a gamma counter (Beckman Gamma 4000). Also, the CF25 membrane was dry-ashed (540°C, 24 hours), the ash solubilized in 1% Ultrex HCl and the Zn content measured by atomic absorption spectrophotometry.

Chromatofocusing - The ion-exchange resin PBE-94 was equilibrated in the appropriate start buffer (27-29). The column dimensions were 1.0 x 30 cm and the bed height after packing was 15 cm. Focusing was begun by initially loading 5 ml of elution buffer which was followed immediately by the sample. Thereafter, elution was carried out at a flow rate of approximately 0.7 ml/minute. All chromatofocusing was done at 4°C. Fractions were examined for pH, absorbance at 280nm and Zn content.

Zinc Analysis - All work was performed on a Perkin Elmer Model 560 Atomic Absorption Spectrophotometer. Column fractions were analyzed directly. Lens samples were dried overnight at 100°C prior to ashing at 550°C for 24 hours. Ash was dissolved in 1% HCl, diluted if necessary, and analyzed at 214nm according to standard procedure recommended by the manufacturer.

Protein Determination - Protein was determined either by the method of Lowry(30) or with the fluorescamine reagent(31). Bovine serum albumin was used as a reference protein for both assays.

SDS-Polyacrylamide Gel Electrophoresis - Stock solutions of SDS, acrylamide and buffers were prepared according to the method of Laemmli(32). Either 11 or 15% acrylamide slab gels of 1.5mm thickness were poured and

stacked with a 4% gel. The samples were prepared by diluting in an appropriate volume of the sample buffer which contained 2% SDS, 10% glycerol, 0.7M mercaptoethanol in 0.05M Tris, pH 6.8. The solution was then kept in a water bath at 100°C for 3 minutes to dissociate proteins into their polypeptide constituents. After addition of a drop of 0.05% bromophenol blue solution, 50 μ l of each sample was underlaid on each slot of the gel. Constant current of 14.4 mamps/1.5mm gel was applied. Electrophoresis was terminated when the indicator dye migrated to approximately 3cm from the end of the gel. Gels were stained for at least 2 hours in 0.07% Coomassie Brilliant blue R-250 in H₂O:methanol:acetic acid (45:45:10). Destaining was accomplished by repeated changes of 7.5% acetic acid-5% methanol solution. Staining was also performed using the Bio-Rad Silver Stain(33) as outlined by Switzer(34).

Isoelectric Focusing - The LKB Ampholine PAG-plate was placed on the cooling plate of the Savant electrophoresis unit (cooled to 10°C). Two Desaga/Brinkman platinum ribbon electrodes were placed on appropriately sized wicks of solvent saturation paper at either end of the gel; the wicks having been soaked in the anode and cathode electrolyte solutions. Samples of 20 μ l were applied to 5 x 10 mm pieces of filter paper which were placed on the gel approximately 2-3 cm from the anode end of the gel and 1 cm apart. The applicator pieces were removed after one hour to prevent tailing. The current was initially set at 15 mamps and the voltage allowed to increase to 1500 volts, usually taking one hour. At 1500 volts, the gel was run for 2-2½ hours and then the power

switched off and the pH gradient read. Staining was performed either with Coomassie R-250 or the Bio-Rad Silver Stain Kit(33).

Amino Acid Analysis - Amino acid analysis was performed using the 2-column system of Spackman et al. (35) on a Beckman 121 Automatic Amino Acid Analyzer. Samples used in standard amino acid analysis were hydrolyzed for 24 hours in 6N HCl.

Hg-Binding Assay - The method of Piotrowski et al. was used to assay mercury binding(36). Tissues were homogenized in cold 1.15% KCl to a final ratio of 7ml/g tissue. Mercury was added to displace other bound metals. A tracer amount of ^{203}Hg was also added. After 10 minutes, the mixture was precipitated with TCA to a final concentration of 2% and then centrifuged. The amount of ^{203}Hg in the TCA supernatant was determined with a Beckman gamma counter. The specific activity of the ^{203}Hg used was $220\ \mu\text{Ci}/\mu\text{mole}$.

RESULTS

Total Lens Zinc Experiments

The influence of age, species, dietary zinc status and selenite-induced cataractogenesis on total lens Zn concentrations are reported in Tables I-III. The assay of two different ages of rat lenses (Table I) indicated a 30% decrease in the concentration of rat lens Zn from day 10 to day 35 postpartum. The total lens Zn increased 2.4 fold in this time interval whereas the lens weight increased 3.6 fold.

Rat lens Zn status was also affected by nutritional depletions (Table II). Lenses were analyzed from animals that were fed a diet containing either less than 1 ppm Zn or approximately 50 ppm Zn. Animals fed the low-Zn diet for 6 weeks had approximately 25% less Zn present in the lens than control groups. This effect was not manifested after two weeks, perhaps because body Zn stores had not been significantly depleted in that short interval. Since consumption of Zn deficient diets results in loss of appetite(37), a group of pair-fed control rats was also included in the study. The Zn level of lenses from animals consuming Zn adequate diets ad libitum or pair fed to the low dietary Zn group were similar, indicating that decreased lens Zn content of the latter was not due to reduced diet consumption.

It was of interest to know if selenite-induced cataract influenced lens Zn. Animals were injected with 20 μ mole/kg body weight sodium selenite at day 9 postpartum and killed on day 28. Lenses were assayed for Zn content and compared with control animals of the same litter

TABLE I

Lens Zinc from Different Animal Species

Lenses were dried (100°C, 12 hours) and a dry weight obtained. Then the lenses were dry-ashed (540°C, 24 hours), the ash solubilized in 1% Ultrex HCl and the Zn content assayed by atomic absorption spectrophotometry. The concentration of lens Zn is expressed as ppm dry weight \pm S.D. Each sample, n, contained at least two lenses for the rat samples or one lens for the other species.

<u>Lens Source</u>	<u>Lens Zn (ppm dry wt.)</u>	<u>n</u>	<u>Lens Dry Wt. (mg)</u>	<u>Total Zn/Lens (ug)</u>
Rat-10 days	26.8 \pm 2.8	7	2.6 \pm 0.2	0.072
Rat-35 days	19.7 \pm 1.7	11	8.8 \pm 1.3	0.172
Bovine	28.3 \pm 3.0	3	617 \pm 35	17.5
Rabbit	22.8 \pm 4.6	4	138 \pm 12	3.14
Sheep	49.8 \pm 7.6	6	521 \pm 68	30.0

TABLE II

Dietary Zinc Deficiencies Effect on Lens Zinc

Animals on a low-Zn diet (less than 1 ppm Zn) were compared with control groups (50 ppm Zn). Lenses were dried (100°C, 12 hours) and a dry weight obtained. The samples were then dry-ashed (540°C, 24 hours), the ash solubilized in 1% Ultrex HCl and the Zn content assayed by atomic absorption spectrophotometry. The concentration of lens Zn is expressed as ppm dry weight \pm S.D. Each sample, n, contained two rat lenses. Statistical tests were done with the standard one-way ANOVA analysis of variance.

<u>Time on Diet</u>	<u>Group</u>	<u>Lens Zn ppm Dry Wt.</u>	<u>n</u>	<u>Total Zn/ Lens (ng)</u>
Two weeks	Ad Libitum	15.4 \pm 2.6	6	62 \pm 13
	-Zn Diet	15.6 \pm 4.2	5	66 \pm 16
Six weeks	Ad Libitum	16.3 \pm 2.5	6	234 \pm 22
	Pair Fed	16.1 \pm 2.4	4	223 \pm 23
	-Zn Diet	11.6 \pm 2.3 ¹	5	171 \pm 18 ²

¹Determined to be significant as statistically different from control groups at the 0.001 level.

²Determined to be significant as statistically different from control groups at the 0.025 level.

TABLE III

Lens Zn from Selenite-Injected and Control Rats

Animals were injected with 20 μ mole/kg body weight sodium selenite on day 9 postpartum and killed on day 28. Control animals of the same litter were also killed on day 28. Lenses were dried (100°C, 12 hours) and a dry weight obtained. Then the lenses were dry-ashed (540°C, 24 hours), the ash solubilized in 1% Ultrex HCl and the Zn content assayed by atomic absorption spectrophotometry. Each sample, n, contained at least two lenses.

<u>Group</u>	<u>Zn ppm</u> <u>Dry Wt.</u>	<u>n</u>	<u>Lens Dry</u> <u>Wt. (mg)</u>	<u>Lens Wet</u> <u>Wt. (mg)</u>	<u>Total Zn/</u> <u>Lens (ng)</u>
Control	23.7 \pm 4.1	7	13.6 \pm 0.3	33.5 \pm 1.8	332 \pm 53
+Selenite	27.2 \pm 1.7	5	11.7 \pm 0.7	29.3 \pm 1.9	307 \pm 18

(Table III). There was no significant difference between the lens Zn content of selenite-injected and control animals. Furthermore, rats injected with selenite on day 9 postpartum and killed on day 11 also showed no change in lens Zn as compared with control animals.

Mercury-Binding Assay

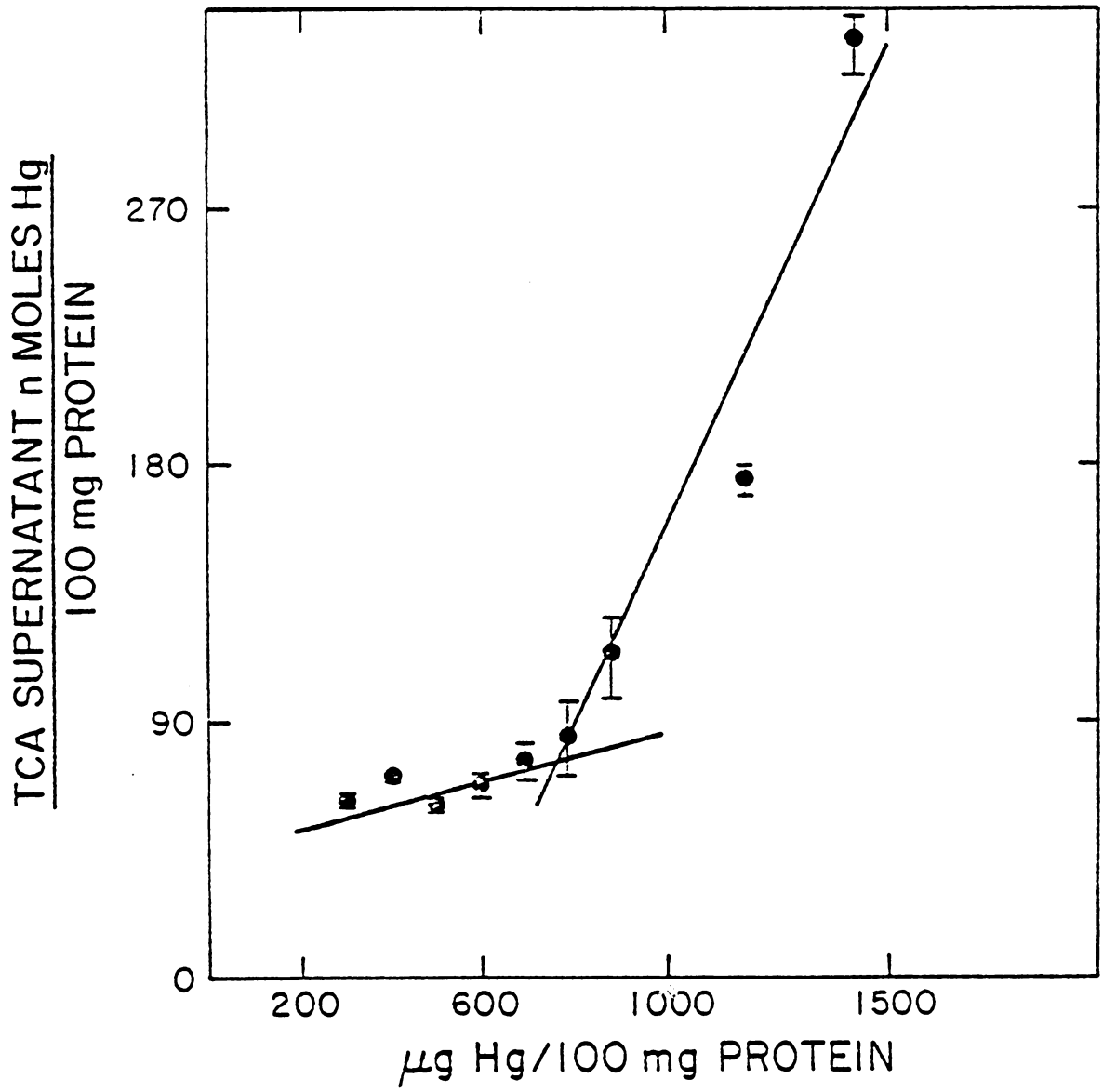
Metallothionein content of tissues can be estimated by the competitive mercury-binding assay(36). Displacement of bound cations from a metalloprotein is accomplished by addition of mercury in an acidic solution. The Hg-binding assay includes first the addition of HgCl_2 to homogenized tissue, followed by deproteinization of the homogenate with TCA. TCA precipitates high molecular weight proteins and facilitates the exchange of Hg for Zn or other metals. Gel filtration chromatography of the TCA supernatant can separate the Hg bound to metallothionein from the Hg either free or complexed to smaller molecular weight moieties, such as amino acids and peptides.

To determine the appropriate amount of Hg required to saturate all potential binding sites on lens proteins, the quantity of Hg in the TCA soluble fraction vs. added Hg is measured(38). Rat lens soluble protein fraction exhibits a characteristic saturation curve (Figure 2) of samples which contain low molecular weight acid-soluble substances with affinity for binding metal. The plateau region of the curve indicates the quantity of Hg required to achieve saturation of high affinity metal-binding species. For the rat lens protein, between 700-800 μg Hg/100 mg protein are required for saturation, and represents $5 \pm 2\%$ of the added Hg bound to TCA-soluble components. With the addition of Hg at

FIGURE 2

Mercury Saturation Curve of Rat Lens Soluble Protein

Indicated quantities of HgCl_2 containing ^{203}Hg were added to 20 mg of protein from the 35,000 x g supernatant from rat lens homogenate. TCA was next added to a final concentration of 2% and precipitated materials removed by centrifugation. The level of Hg in solution was determined. Each test point was analyzed in duplicate.



this level, it can be assumed that all metal-binding species of the TCA supernatant are complexed with Hg. Once the protein population is fully saturated, the linear response reflects the excess free Hg. The rat lens saturation response was compared with the Hg-binding of rat liver homogenate, using an equivalent amount of protein and Hg. For the rat liver, $12 \pm 2\%$ of the added Hg remained in the TCA supernatant. Additionally, Sephadex G-75 chromatography of the lens TCA supernatant showed a peak eluting near the total column volume whereas with liver supernatant the elution peak is earlier, at twice the column void volume. This suggests that the Hg-binding species in the lens samples are of lower molecular weight than those found in rat liver.

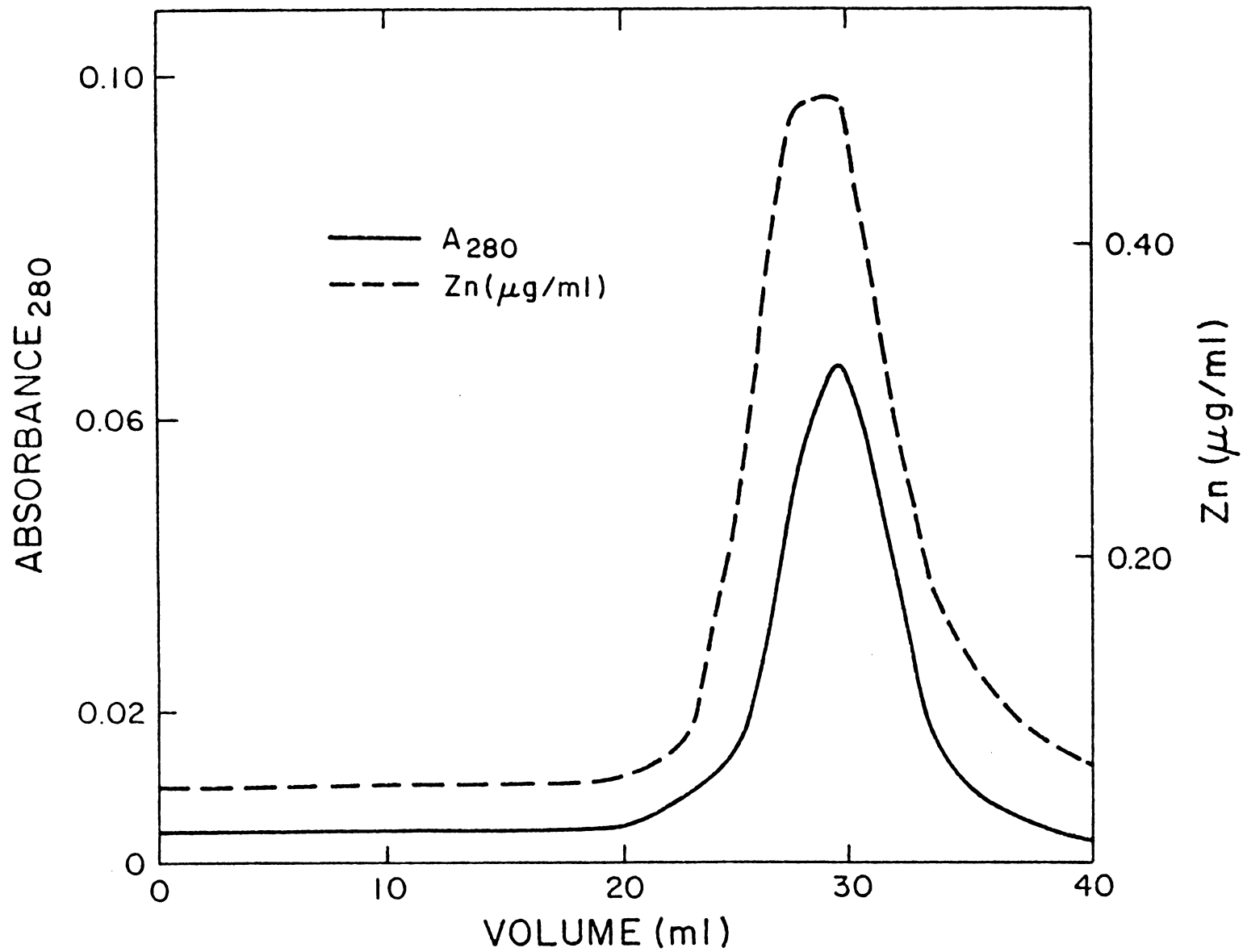
Ultrafiltration Preparation of a Low Molecular Weight Protein Population

A defined lens protein preparation with respect to molecular weight was obtained by the use of ultrafiltration membranes (Figure 1). Soluble protein was fractionated to yield low molecular weight proteins by passage through CF25 (exclusion limit 25,000 daltons) membranes. The CF25 filtrate thus contains proteins of molecular weight less than 25,000 daltons. Analysis of a rat lens filtrate sample by Sephadex G-75 chromatography (Figure 3) yields one peak of low A_{280} which co-elutes with Zn. Metallothionein exhibits this type of Sephadex G-75 elution behavior(25) so it seemed that a metallothionein-like protein in the lens could be isolated by this molecular weight dependent ultrafiltration protocol. It should be noted that the addition of 5mM mercaptoethanol to the homogenization buffer yielded lower values for Zn complexed with the lens soluble protein.

FIGURE 3

Sephadex G-75 Chromatography of Rat Lens CF25 Filtrate

Two ml of a rat CF25 filtrate sample from 21 day old rat lenses (containing 5 mg of protein) homogenized in 0.010M Tris-Cl, pH 8.6 was applied to a 1.0 x 50 cm column of Sephadex G-75. One ml fractions were collected at a flow rate of 0.5 ml/minute. The column void volume was 13 ml and the total column volume was 32 ml.



The proteins isolated in the CF25 filtrates were different from γ -crystallins, although γ -crystallins have a molecular weight range of 20,000-25,000 daltons which is at or near the CF25 membrane exclusion limit. Sephadex G-75 chromatography of isolated γ -crystallin protein pools showed that the γ -crystallins eluted earlier than material in the CF25 filtrate. Also, there was no distinct peak of Zn associated with the A_{280} peak. Isoelectric focusing of filtrate samples from bovine and rat lenses (Figure 4) indicates that the low molecular weight fraction is composed of a variety of polypeptides with differing isoelectric points. The majority of the bands observed were in the pH range of 6.0-7.0, although some faint bands were present around pH 5.0.

The possibility of nonspecific binding of Zn to CF25 membranes was ascertained. Application of a sample containing ^{65}Zn in L-15 culture medium (to stimulate endogenous low affinity Zn-binding ligands present in the cytosol) showed that only 80% of the Zn was recovered. When the membrane cone was counted, 10% of the applied ^{65}Zn was accounted for as being bound to it. The membrane cone was also ashed in the muffle furnace and analyzed for Zn. A value of 17 $\mu\text{g/g}$ cone was determined. Since the cone weighs approximately 0.5g, each cone contains 8 μg of Zn.

Data concerning the levels of protein and Zn in the CF25 residue and filtrate fractions (Table IV) show that the filtrate was enriched in the Zn/protein content. For both rat and bovine lens samples, approximately a ten-fold increase was observed. Although the total protein content of the filtrate sample is small in comparison with the larger

FIGURE 4

Isoelectric Focusing of Bovine and Rat Lens CF25 Filtrate Samples

A pH range 9.5-3.5 LKB multiphor isoelectric focusing gel was run at 10°C until focusing was completed. Power was increased from 300 to 1500 volts over a one hour period and then kept at 1500 volts for an additional two hours. The anode electrolyte solution was 1M H₃PO₄ and the cathode electrolyte solution was 1M NaOH. The pH gradient was measured with a surface electrode and the gel was then stained with silver staining. Lanes X and Y are 15 µg of two different bovine lens filtrate samples. Lane Z is 15 µg of a rat lens filtrate sample.

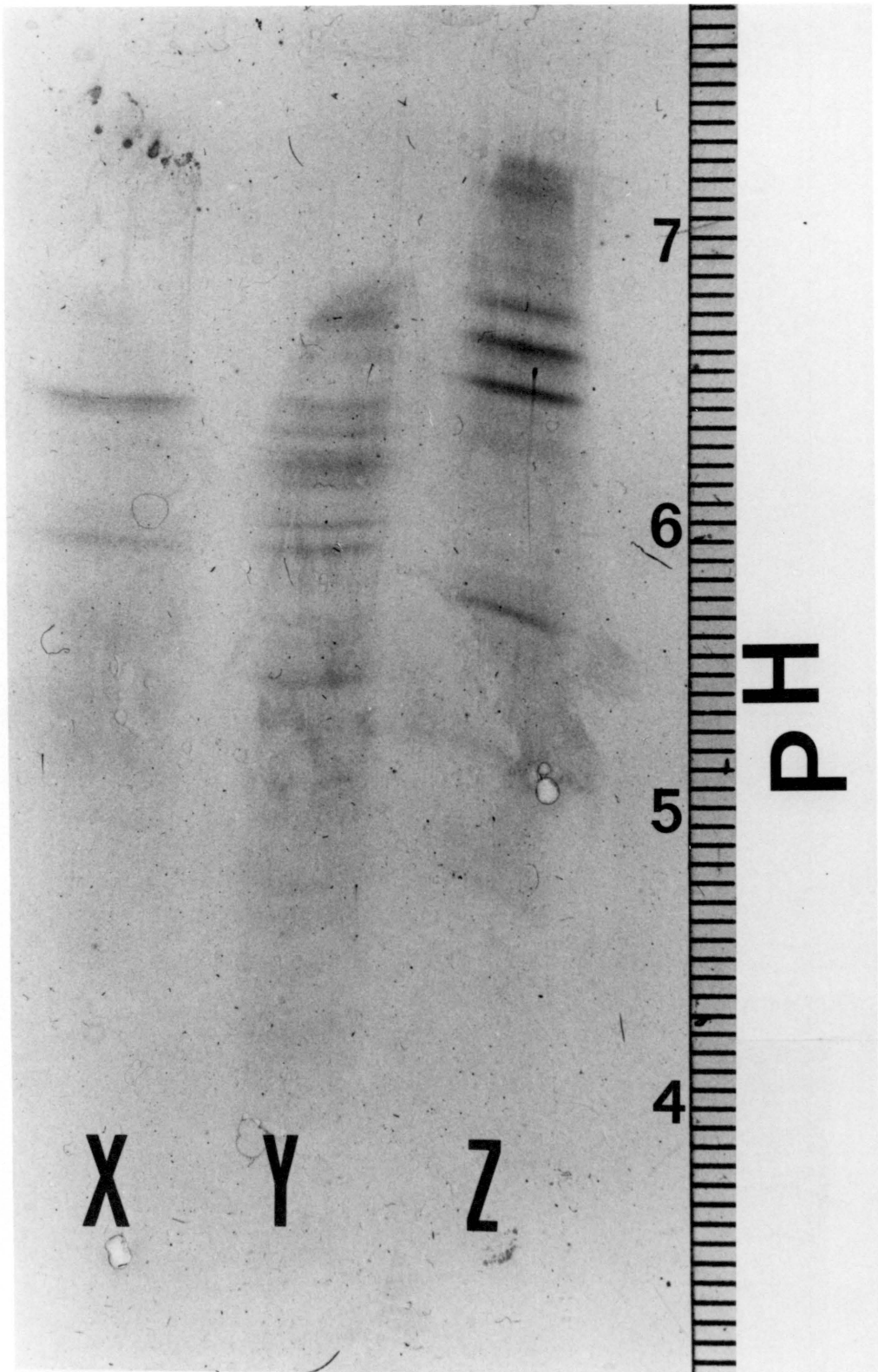


TABLE IV

Protein and Zinc Content of CF25 Fractions from Bovine and Rat Lenses

Lowry(30) protein and Zn content of the 35,000 x g supernatant, CF25 residue and filtrate fractions of bovine and rat lens tissue. Different amounts of protein were fractionated for each sample according to the size or quantity of lenses used. Protein results are reported as an average of triplicate samples; Zn reported as an average of triplicate samples of two different dilutions. Standard deviation values were within 10%. Protein recovery was from 90-100%. Zn recovery was 90% for rat samples and 60% for bovine samples. For the rat samples, 8 lenses were used from 50 g animals and 5 lenses were used from the 240 g animals. One bovine lens was used per sample.

<u>Lens Source</u>	<u>Supernatant</u>			<u>CF25 Residue</u>			<u>CF25 Filtrate</u>		
	<u>mg protein</u>	<u>μg Zn</u>	<u>μg/mg</u>	<u>mg protein</u>	<u>μg Zn</u>	<u>μg/mg</u>	<u>mg protein</u>	<u>μg Zn</u>	<u>μg/mg</u>
Rat-50 g	41.0	1.7	0.042	34.2	1.3	0.038	0.75	0.35	0.46
Rat-240 g	43.2	1.6	0.038	39.4	0.85	0.022	0.93	0.64	0.69
Bovine - 1.	455	31.8	0.070	439	14.7	0.032	8.0	3.10	0.39
- 2.	510	22.4	0.044	469	11.7	0.025	6.6	2.24	0.34

molecular weight protein which is retained on the residue, approximately 25% of the Zn was recovered there. Furthermore, dialysis of filtrate samples on UM2 membranes showed 60% of the Zn remained with species larger than 2,000 molecular weight.

The quantity of protein present in the filtrate is greater when determined by the fluorescamine reagent as compared to the Lowry (Table V). (The Coomassie dye-binding assay(39) yields similar results as the Lowry). The opposite effect is seen with the CF25 residue and supernatant fractions as the amount of total protein assessed by fluorescamine is about half of that calculated from Lowry measurements. Since fluorescamine reacts quantitatively with primary amino groups, including the epsilon amino group of lysine and free N-terminal amino groups of protein(31), these data indicate that there is a greater concentration of lysine or free amino groups in the filtrate. When the filtrate samples were concentrated by UM2 ultrafiltration (exclusion limit 2,000 daltons), 50-80% of the fluorescamine protein was retained implying that the filtrate contained larger molecular weight species and not just low molecular weight primary amines.

Sephadex G-25 Chromatographic Separations

The Zn/protein peak of the lens CF25 filtrate samples elutes at or near the total column volume using Sephadex G-75(Figure 3). Sephadex G-25 was used to improve the resolution of low molecular weight components (Figure 5). Zinc eluted slightly earlier than materials absorbing at 280nm. Moreover, the elution pattern suggests that Zn is associated with a protein smaller than insulin. A similar elution

TABLE V

Comparison of Lowry and Fluorescamine Protein Determination for the CF25 Ultrafiltration Fractions

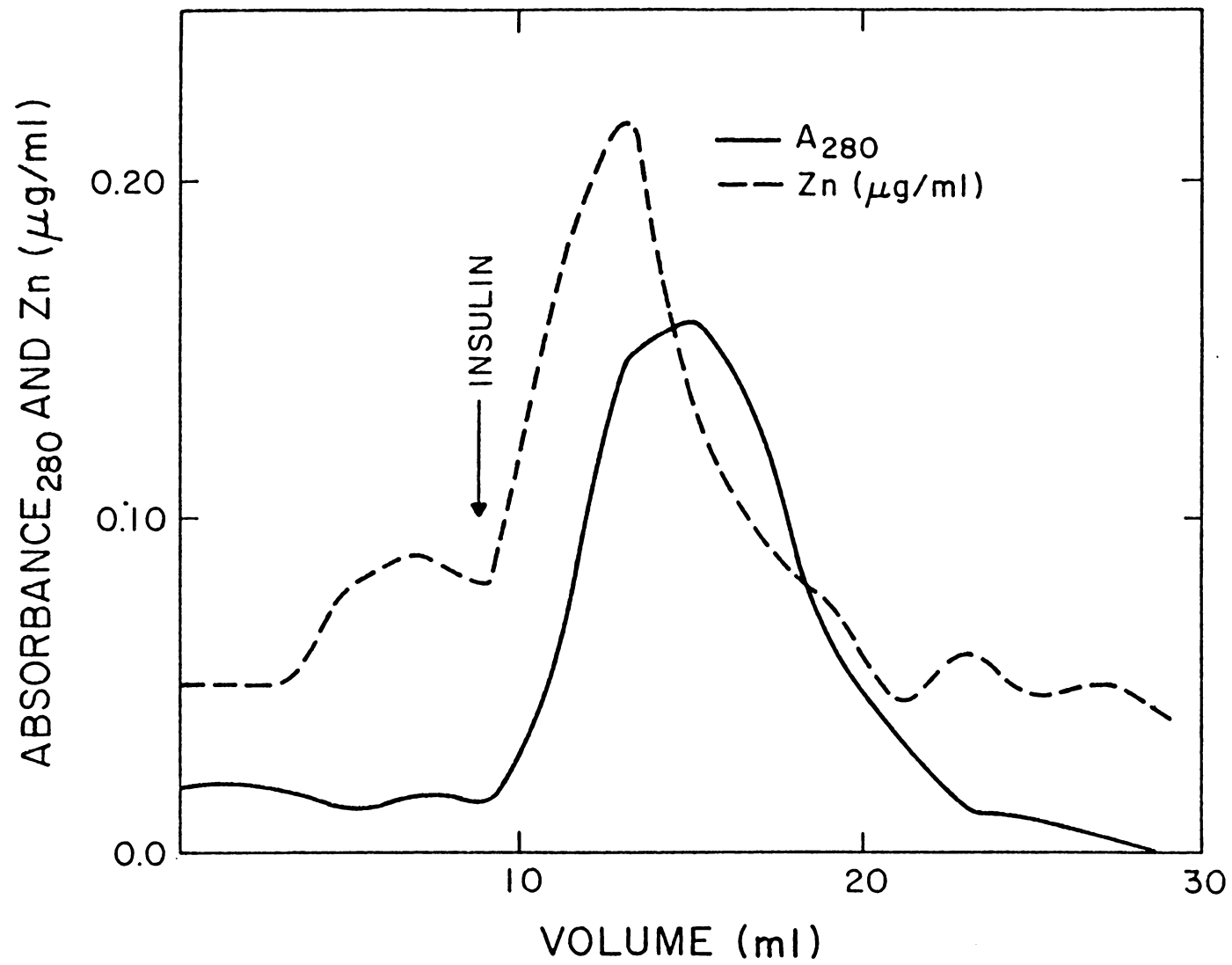
Lowry(30) total protein (L mg) and Fluorescamine total protein (F mg) were determined for the 35,000 x g supernatant, CF25 residue and filtrate fractions of bovine and rat lens tissue. Bovine serum albumin was used as a reference protein. Different amounts of protein were applied in each sample due to the size or quantity of lenses used. Results are reported as an average of four assayed samples of two different dilutions, except for the Lowry filtrate values where the filtrate was assayed directly in triplicate. The relative percentages contained in the filtrate and residue with these protein assays are indicated in parentheses. F/L is the ratio of Fluorescamine to Lowry protein. Overall protein recovery was 90% and standard deviation was \pm 5-8%. For the rat samples, 18 lenses were used from 35 g animals and 14 lenses were used from 50 g animals. Two bovine lenses were used for each sample.

<u>Lens Source</u>	Supernatant			CF25 Residue			CF25 Filtrate		
	<u>L mg</u>	<u>F mg</u>	<u>F/L</u>	<u>L mg</u>	<u>F mg</u>	<u>F/L</u>	<u>L mg</u>	<u>F mg</u>	<u>F/L</u>
Rat-35g	193	118	0.61	180 (99)	85 (91)	0.47	2 (1)	8.5 (9)	4.3
Rat-50g	127	81	0.64	105 (99)	68 (91)	0.65	0.7 (.6)	6.4 (9)	9.1
Bovine - 1.	1054	802	0.76	944 (99)	657 (88)	0.70	7.9 (.8)	86 (12)	10.9
- 2.	1299	652	0.50	1321 (99)	610 (91)	0.46	11.1 (.8)	59 (9)	5.3

FIGURE 5

Sephadex G-25 Chromatography of CF25 Filtrate with Marker Proteins

Six lenses from 150g rats were homogenized in a 1:10 ratio of 0.1M Tris-Cl, 0.05M NaCl, pH 7.6. After centrifugation at 35,000 x g for 30 minutes, the supernatant was applied to a CF25 ultrafiltration cone and centrifuged at 1,000 x g for 90 minutes to yield a low molecular weight filtrate fraction; 0.5 ml of the filtrate (containing 2 mg protein) was applied to a 1.0 x 30 cm column of Sephadex G-25 equilibrated in the homogenization buffer. A flow rate of 30 ml/hr was used and 1 ml fractions collected. The column void volume was 8 ml and the total column volume was 21 ml. Insulin was used as a marker protein. 1 mg was applied in 0.5 ml and it eluted with a peak maximum at 9 ml as indicated by the arrow.



pattern was observed with bovine lens filtrate. These results indicate that the putative Zn-binding protein is smaller than metallothionein.

The components of the G-25 protein/Zn peak were further characterized by SDS-PAGE using 15% gels (Figure 6). Samples were run of the pooled peak and of rat and bovine filtrate samples. Two different rat filtrate samples (lanes 1) contained two distinct molecular weight components, migrating close to or at the same position as the lysozyme standard. An additional component of slightly higher molecular weight was observed with three different bovine filtrate samples (lanes 2). Lane 3 is the concentrated pool of fractions 11-13 of the Sephadex G-25 column of rat filtrate (Figure 5). This sample contained a large amount of this low molecular weight protein, with a minor higher molecular weight band also detectable. Since lysozyme has a molecular weight of 14,400, these results suggest aggregation or polymerization of pooled materials prior to or during electrophoretic analysis.

Sephacryl S-200 Chromatography

Molecular exclusion chromatography is classically used to separate the α , β_H , β_L and γ -crystallin components. The total soluble lens protein from bovine and rat samples (i.e. without any molecular size dependent fractionations) was analyzed by Sephacryl S-200 chromatography (Figures 7 & 8).

EDTA is often a component of the elution buffer for Sephacryl S-200 chromatography of lens proteins since it acts as a metal scavenger(40). The Zn associated with the various crystallin complexes is markedly

FIGURE 6

SDS-Polyacrylamide Gel Electrophoresis of Bovine and Rat Filtrate
Samples and the Zn/Protein Peak of the Sephadex G-25 Column

Samples were in 2% SDS, 10% glycerol, 0.7M mercaptoethanol in 0.05M Tris, pH 6.8 and were heated at 100°C for 3 minutes. A drop of 0.05% bromophenol blue was added and 75 µl of each sample was underlaid on each gel slot. Constant current of 14.4 mamps/1.5 mm gel was applied. Electrophoresis was terminated when the indicator dye reached to 3 cm from the bottom of the gel. Staining was performed using Coomassie Brilliant blue R-250. The stacking gel was 4% acrylamide; separating gel was 15% acrylamide. Lanes 1 are rat filtrate samples. Lanes 2 are bovine filtrate samples. Lane 3 is the Sephadex G-25 Protein/Zn pool (fractions 11-13 concentrated on UM2 membrane (Figure 5)). S represents molecular weight standards; L is lysozyme (14,400), C is carbonic anhydrase (31,000), O is ovalbumin (45,000), A is bovine serum albumin (66,200) and P is phosphorylase B (92,500).

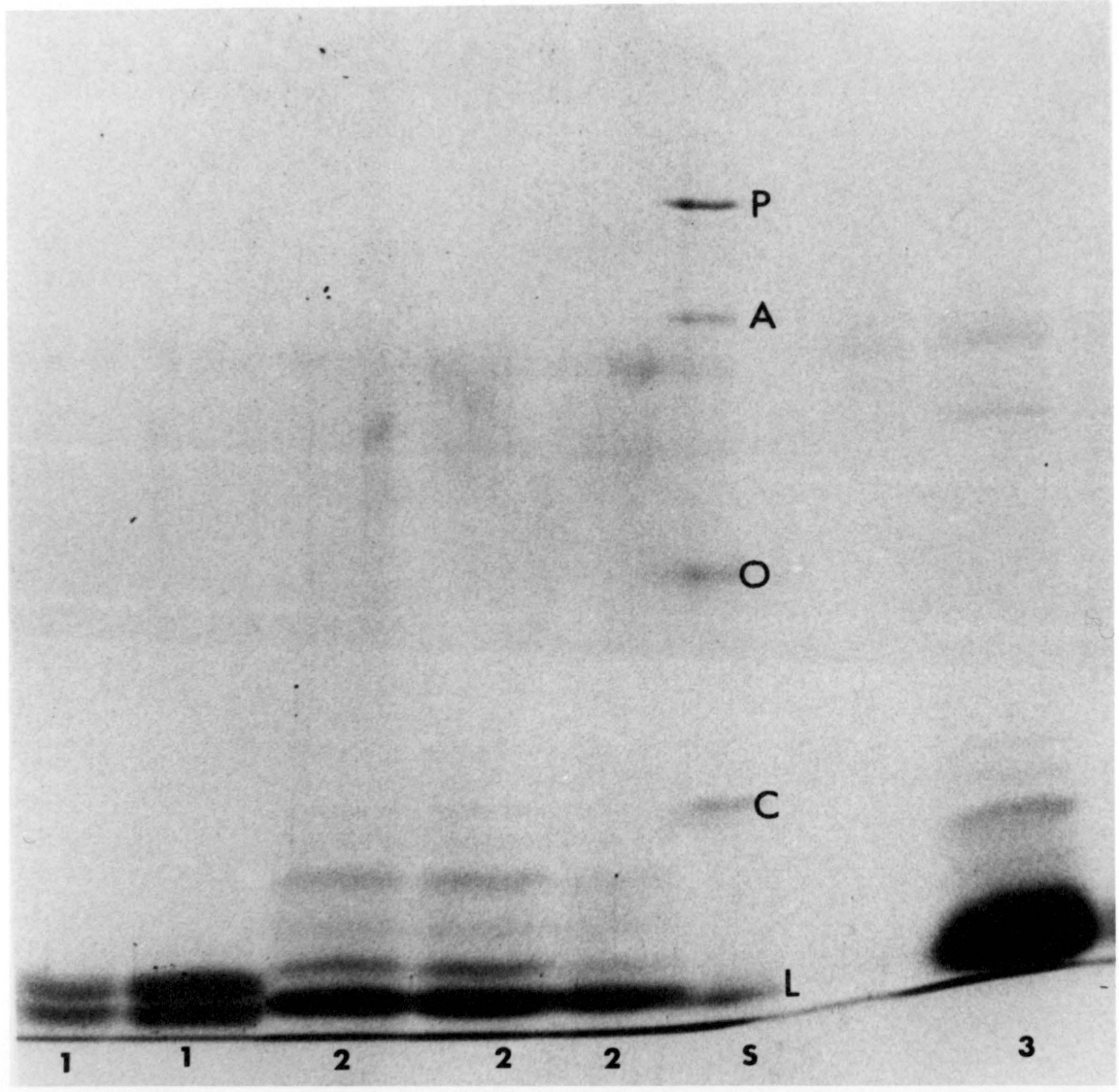


FIGURE 7

Sephacryl S-200 Chromatography of Bovine Lens Soluble Protein
with 100 mM Tris-Cl as Eluent

One bovine lens was homogenized in a 1:10 ratio of weight to volume of 100mM Tris-Cl, pH 7.6. The homogenate was centrifuged at 35,000 x g for 30 minutes and then a 3 ml sample of supernatant (containing 100 mg protein) was applied to a 1.5 x 90 cm column of Sephacryl S-200 at room temperature. Proteins were eluted with 100mM Tris-Cl, pH 7.6 with a flow rate of 1.1 ml/min; 2 ml fractions were collected. The column void volume was 42 ml. The solid line represents A_{280} and the dashed line represents Zn ($\mu\text{g/ml}$).

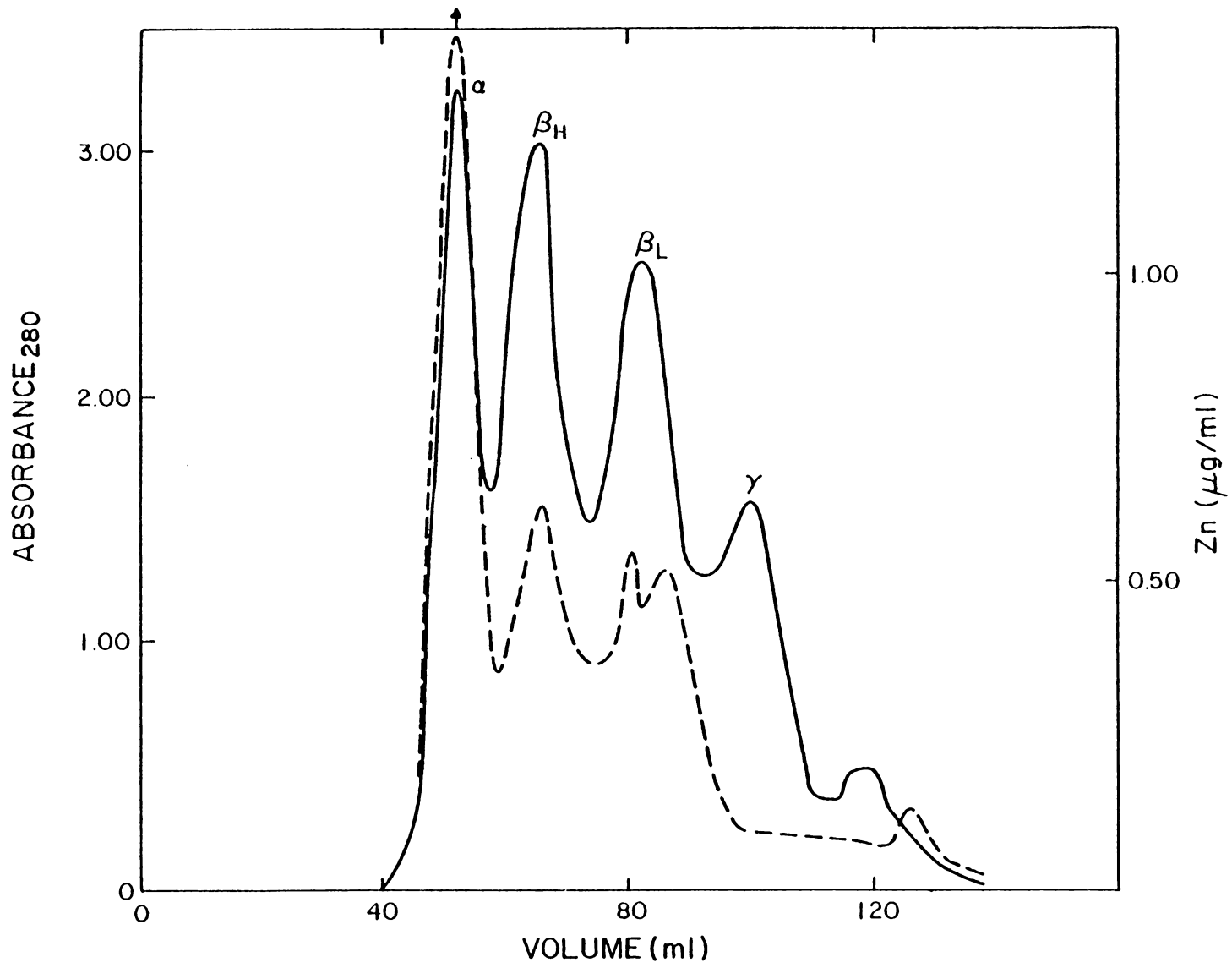
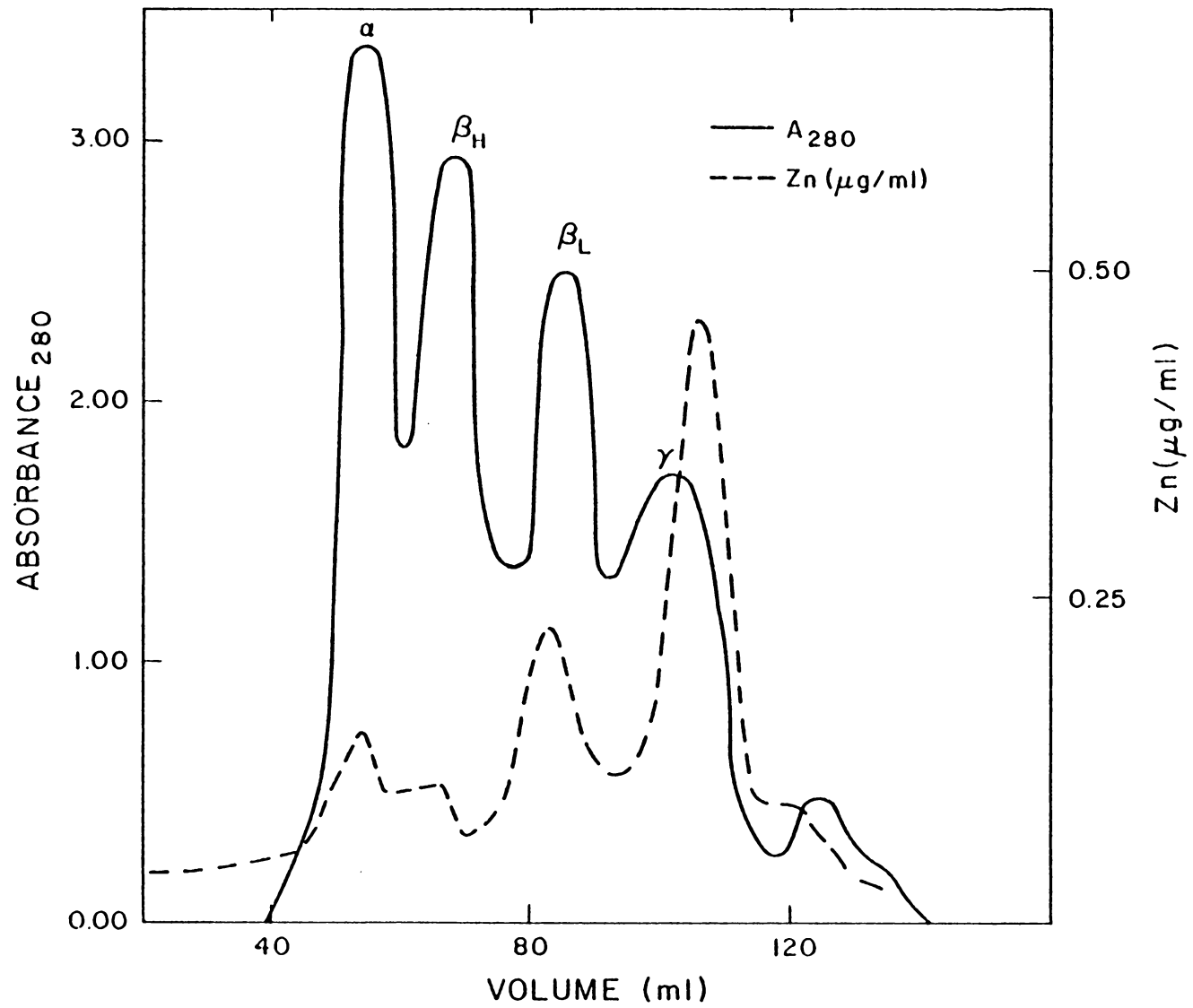


FIGURE 8

Sephacryl S-200 Chromatography of Bovine Lens Soluble Protein
with an EDTA-Containing Buffer

One bovine lens was homogenized in a 1:10 ratio of weight to volume of 0.065M Tris-Cl, 0.05M NaCl, 0.001M EDTA, pH 7.6. The homogenate was centrifuged at 35,000 x g for 30 minutes and then a 3 ml sample of the supernatant (containing 100 mg protein) was applied to a 1.5 x 90 cm column of Sephacryl S-200 at room temperature. Proteins were eluted with the homogenization buffer with a flow rate of 1.1 ml/min; 2 ml fractions were collected. The column void volume was 42 ml.



affected by the presence of EDTA. In the absence of EDTA (Figure 7), the majority of the lens Zn elutes at the void volume with the α -crystallin peak. Some Zn was also associated with the various β -fractions, particularly β_L . With the addition of 1mM EDTA (Figure 8), a Zn peak elutes immediately after the γ -crystallins and there is very little Zn associated with other crystallin fractions. The elution patterns shown are from bovine lenses but similar Zn-elution profiles are observed when rat lens tissue was chromatographed with and without EDTA. However, the γ -crystallin peak for rat lens was much greater, in comparison with the bovine lens and the α -crystallin population was less for rat lens.

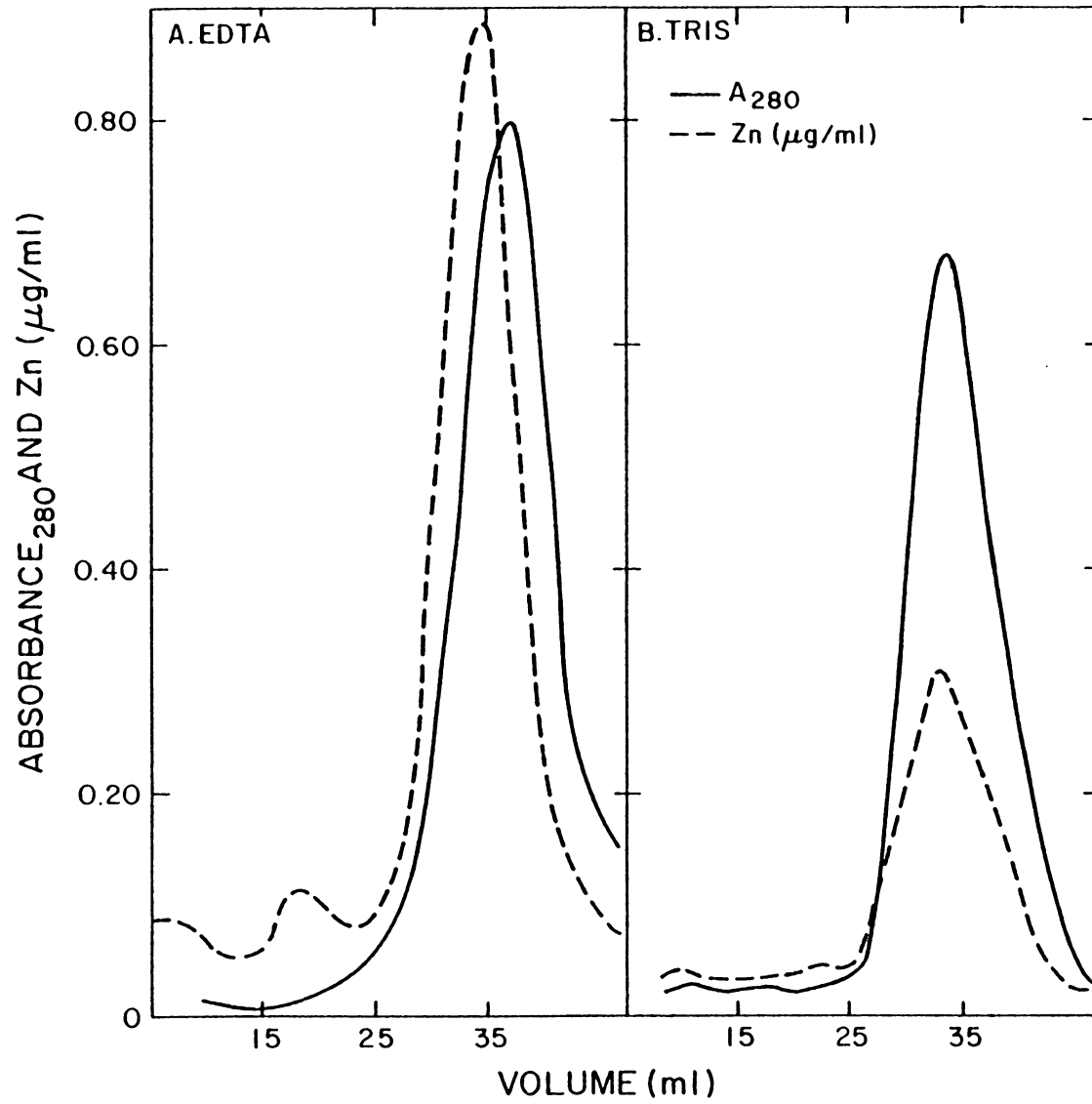
The fractions which contained Zn were concentrated and compared. On native gel electrophoresis (11% gels), the EDTA-derived fractions showed several more bands than those identical fractions obtained in the absence of EDTA. SDS-PAGE (11% gels), verified this finding but the major protein component from either type of buffer comigrated with the carbonic anhydrase standard which had a subunit molecular weight of 31,000 daltons. From the position where the protein/Zn peak eluted on Sephacryl S-200 chromatography, it is unlikely that it could weigh 30,000. Apparently aggregation or anomolous behavior occured.

CF25 filtrate samples were generated using the identical buffers as for Sephacryl S-200 chromatography. The elution profiles (Figure 9) are consistent with the Sephacryl S-200 data in that the Zn peak isolated in the presence of EDTA is much greater in magnitude than that isolated without EDTA. These results indicate that EDTA causes a preferential

FIGURE 9

Sephadex G-75 Chromatography of Bovine CF25 Filtrate Samples
Run in the Presence and Absence of EDTA

CF25 filtrate samples were generated from two different bovine lenses using (A) 0.065M Tris-Cl, 0.05M NaCl, 1mM EDTA, pH 7.6 or (B) 0.10M Tris-Cl, pH 7.6. Sephadex G-75 chromatography was done using the identical buffers for each sample, on a 1 x 50 cm column. A flow rate of 0.5 ml/min was used and 1 ml fractions were collected. Approximately 5 mg of protein (in 1.5 ml of sample) were applied to each column run. The column void volume was 13 ml and the total column volume was 32 ml.



partitioning of metal to low molecular weight proteins or Zn-EDTA complexes.

Chromatafocusing Experiments

Chromatafocusing separates proteins according to their isoelectric points. A pH gradient is generated on an ion-exchange resin and proteins bind and elute in order of their isoelectric points. Also, focusing effects result in sample concentration and high resolution(27).

Bovine lens soluble protein was fractionated into several peaks via chromatafocusing, using a pH gradient of 9-6 (Figure 10). Rat lens soluble protein exhibited chromatafocusing elution patterns similar to bovine protein. The γ -crystallins have the highest isoelectric points of the lens proteins and are the first to elute. The various β -crystallin fractions then follow. The lens α -crystallins have pI values in the range of 5.5-6.0, so would not elute with a pH gradient of 9-6. A 1M NaCl wash was used at the end of the gradient to remove the remaining bound proteins, including α -crystallins. Also, a protein of metallothionein-like character, with an acidic pI, would be in the salt wash.

The proteins which eluted with the salt wash can be separated by Sephadex G-75 chromatography (Figure 11). Two protein peaks were observed. The first elutes at the column void volume and are the α -crystallins. A high Zn containing protein peak elutes at or near the total column volume and would be expected to contain a low molecular weight metallothionein-like protein.

FIGURE 10

Chromatofocusing of Bovine Lens Soluble Protein, pH Gradient 9-6

One bovine lens was homogenized in 10mM Tris-Cl, pH 8.6 which contained 5mM mercaptoethanol. After centrifugation at 35,000 x g for 30 minutes, 5 ml of supernatant, containing 150 mg of protein, were applied and the gradient begun. The column was initially equilibrated in the start buffer, 0.025M ethanolamine, pH 9.4, and the eluent was a 1:10 dilution of Polybuffer 96, pH 6.0. The column was run at 4°C at a flow rate of 35 ml/hr. One ml fractions were collected. At the end of the gradient run, 1M NaCl was applied to the column and twenty one ml salt wash fractions collected.

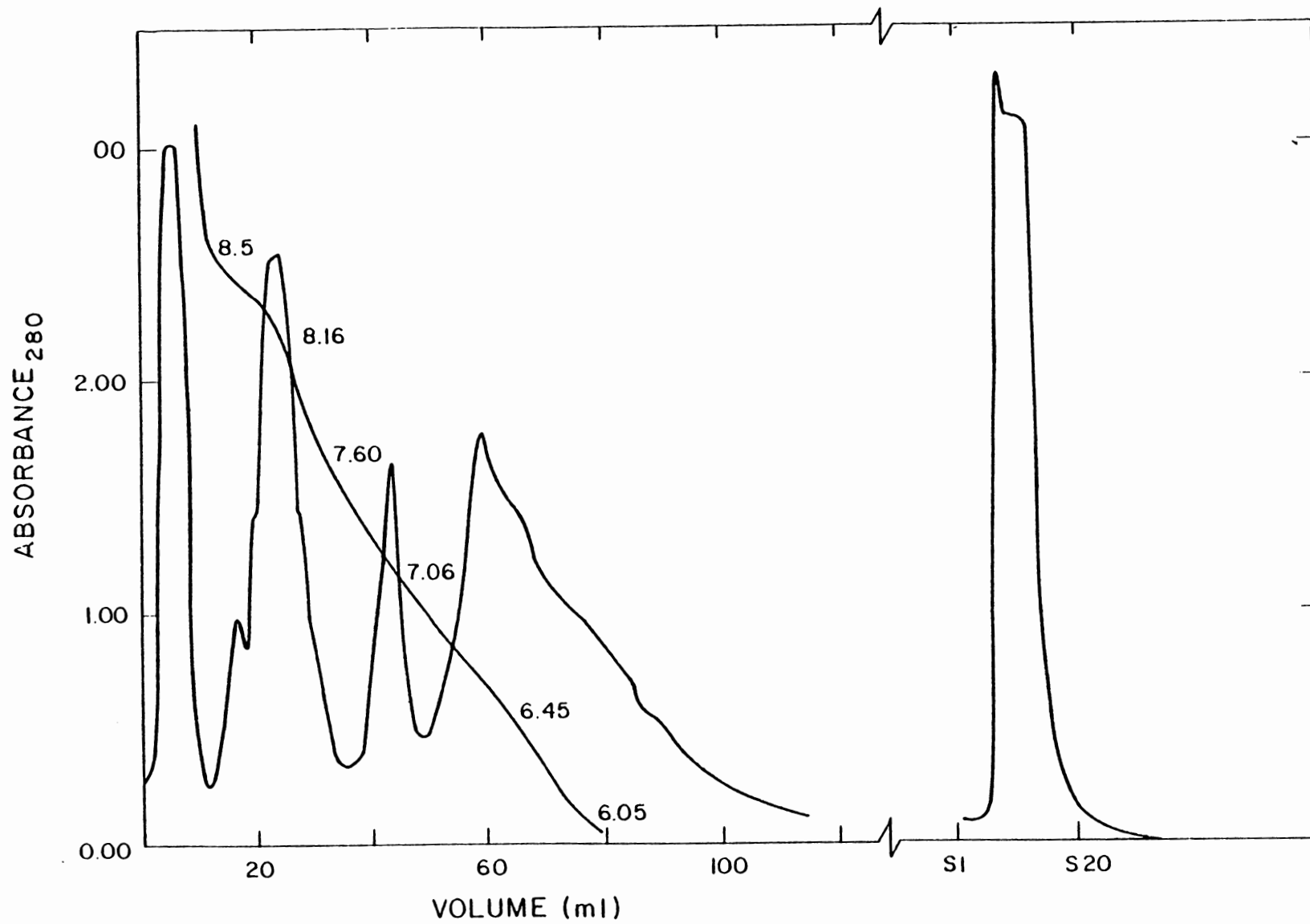
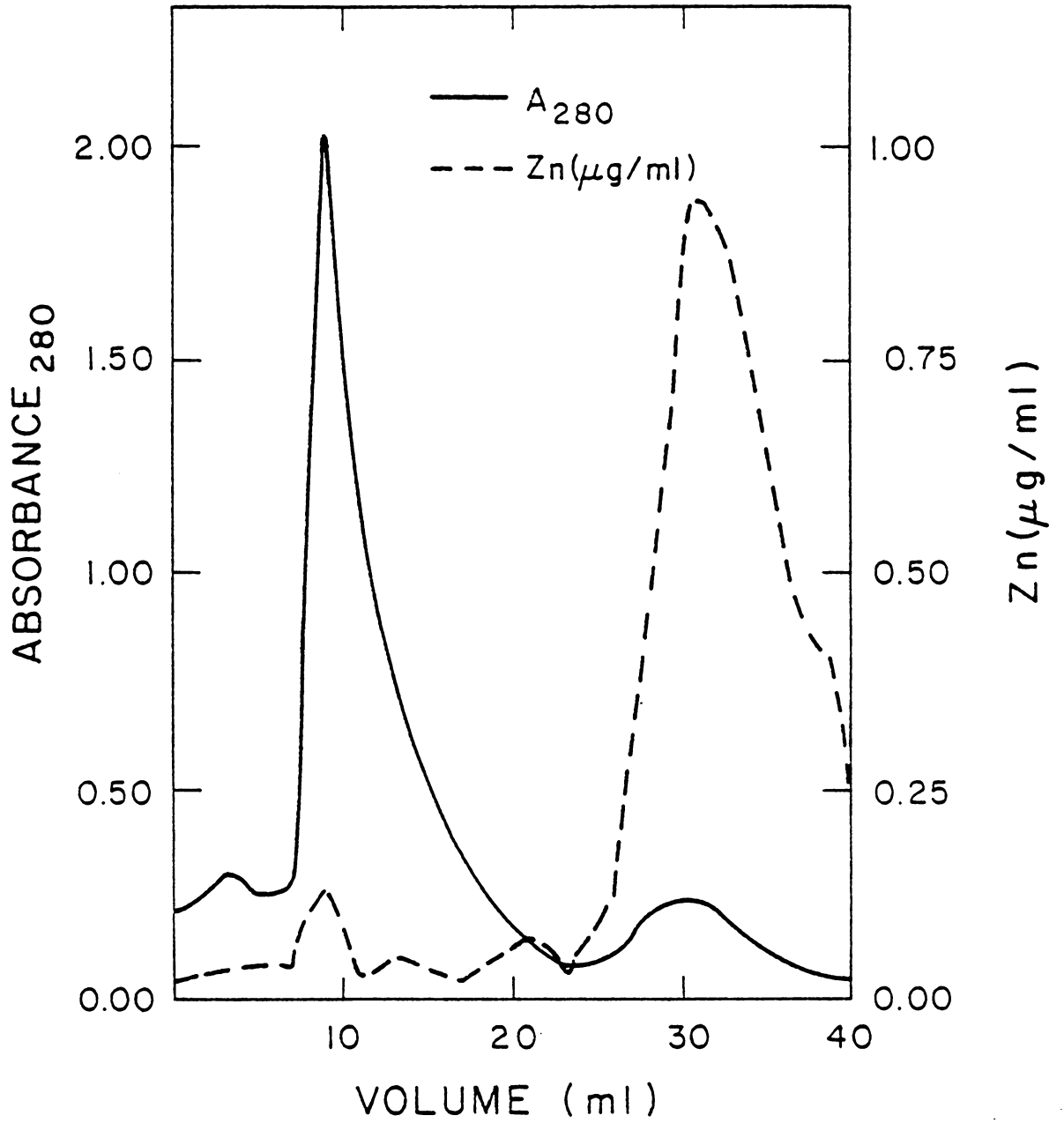


FIGURE 11

Sephadex G-75 Chromatography of Bovine Lens 9-6
Chromatafocusing Salt Wash

1.5 ml of the salt wash, containing 10 mg of protein, from the chromatafocusing pH 9-6 column were applied to a 1.0 x 50 cm column of Sephadex G-75 at 4°C and eluted with 0.010M Tris-Cl, pH 8.6, containing 5mM mercaptoethanol. A flow rate of 0.5 ml/min was used; 1 ml fractions were collected. The column void volume was 13 ml and the total column volume was 32 ml.



Chromatofocusing can be done using a variety of possible pH gradients. When bovine lens soluble protein is chromatofocused with a pH gradient of 7-4 (Figure 12), the protein of low pI can be directly collected from the column eluent. This protein peak elutes in the pH range of 5.2-4.3, has a peak of Zn associated with it, and has greater A_{250} than A_{280} . Absorbance at 250nm is indicative of the sulfhydryl-metal bond and this property is characteristic of metallothionein.

Peaks I and II in Figure 12 were further characterized by isoelectric focusing. A densitometer scan (Figure 13) shows a major protein peak for both of these pooled fractions at pH 5.5 with several other minor components in the range of pH 5.5-5.8.

The amino acid composition of Peak I (Table VI) reveals these peptides are distinct from metallothionein. Metallothionein is easily identified by high cysteine content and this fraction contains no cysteine. The content of aspartic acid and glutamic acid residues is consistent with the pI of these peptides, although no evaluation of amide content was made.

Epithelial Layer Studies

The lens epithelial layer is the most metabolically active part of the lens(40). A distribution to the epithelial layer of a low molecular weight metalloprotein which could have an active physiological was therefore investigated. Fifteen ppm Zn were associated with the epithelial layer while the whole bovine lens had 28 ppm Zn (Table 1).

FIGURE 12

Chromatofocusing of Bovine Lens Soluble Protein, pH Gradient 7-4

One bovine lens was homogenized in 10mM Tris-Cl, pH 8.6 which contained 5mM mercaptoethanol. After centrifugation at 35,000 x g for 30 minutes, 10 ml of supernatant containing 300 mg of protein were applied and the gradient begun. The column was initially equilibrated in the 0.025M imidazole-HCl start buffer, pH 7.4. The eluent was a 1:8 dilution of Polybuffer 74, pH 4.0. A flow rate of 35 ml/hr was used and 1 ml fractions were collected. The peaks labeled I and II were the fractions pooled and further analyzed.

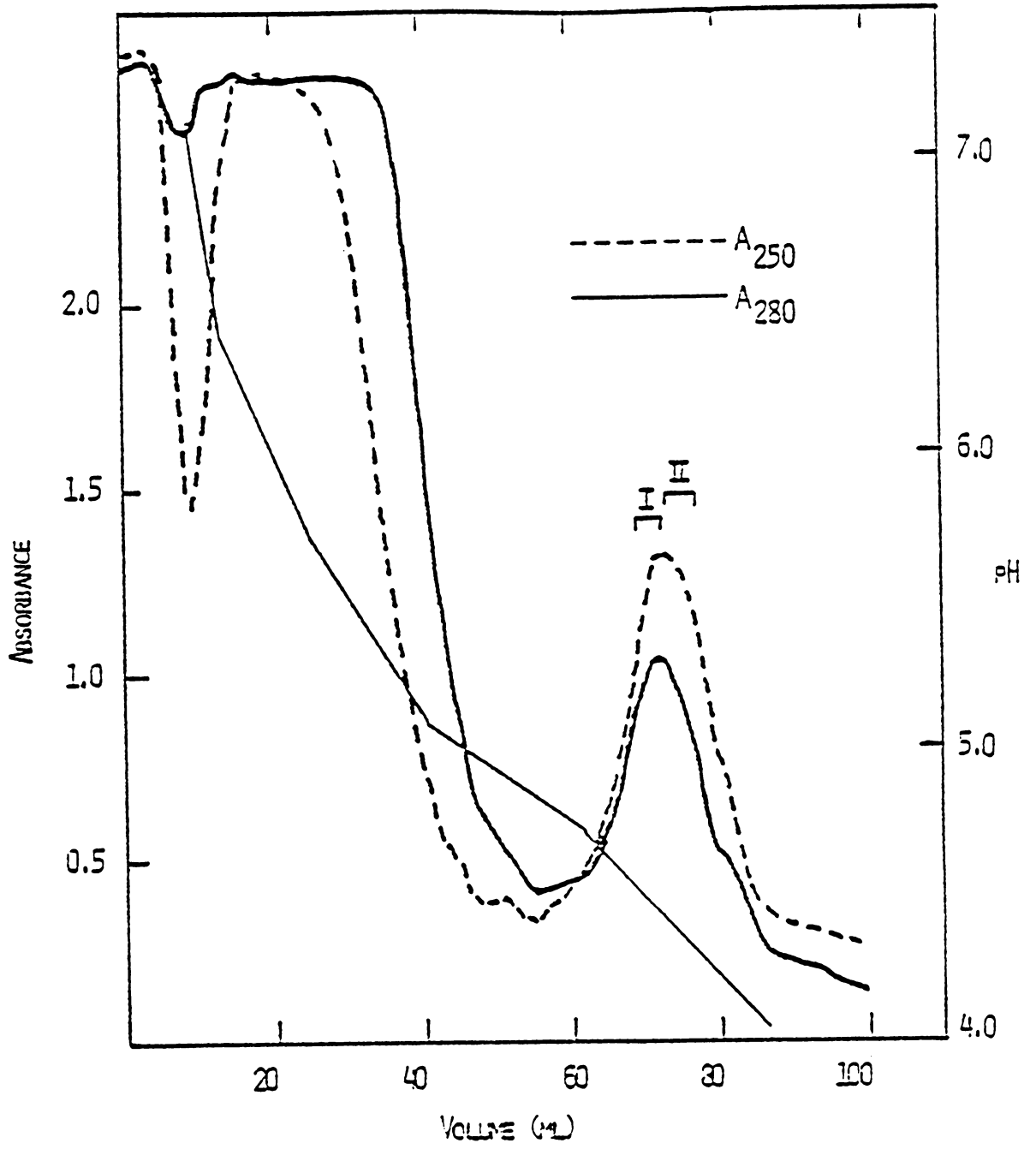


FIGURE 13

Densitometer Scan of Isoelectric Focusing Gel of Chromatofocusing
Pooled Fractions I and II from pH Gradient 7-4 Column

An LKB Multiphor pH range 9.5-3.5 isoelectric focusing gel was run at 10°C until focusing was complete. The anode-electrolyte solution was 1M H_3PO_4 and the cathode-electrolyte solution was 1M NaOH. The gel was run at 15 mamps until a voltage of 1500 volts was reached (approximately one hour). Then the voltage was kept constant at 1500 volts for two hours. The gel was stained with Coomassie R-250. Densitometer scanning was done at 650nm and only those bands significantly above background are shown. Pool I represents fractions 69-72 and Pool II fractions 73-77 of the chromatofocusing pH 7-4 column. Both pools were concentrated on UM2 membranes before analysis. Approximately 5 μ g of protein were applied for each sample.

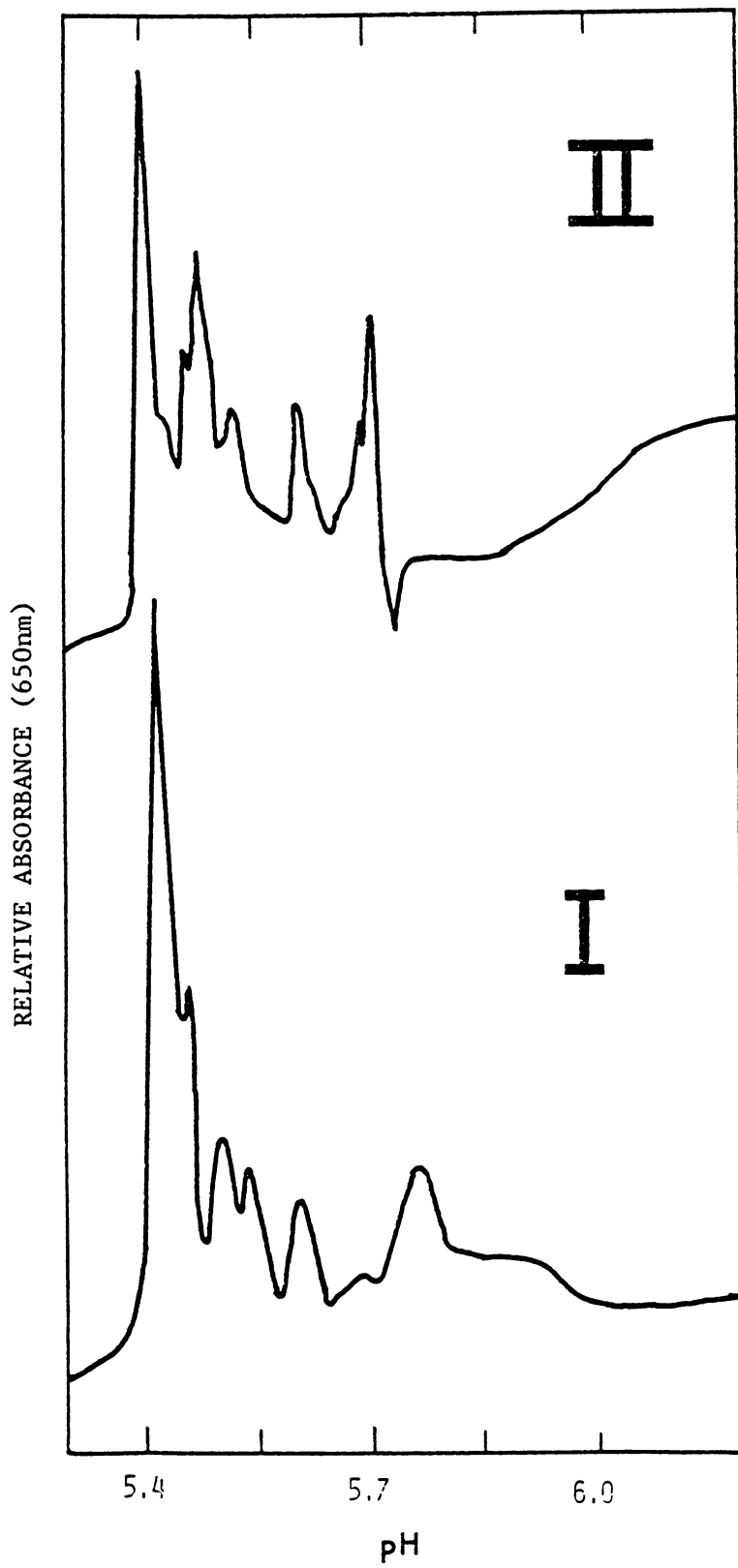


TABLE VI

Amino Acid Composition of Pool I from Chromatofocusing pH 7-4 Column

Fractions 69-72 from the chromatofocusing, pH 7-4 column (Figure 12) labeled I, were pooled and concentrated on a UM2 membrane. Approximately 200 μ g of protein were acid hydrolyzed (6N HCl, 24 hours) and analyzed for amino acid content.

Amino Acid	Residue Percent
Lysine	5.9
Histidine	3.3
Arginine	3.2
Aspartic Acid	10.5
Threonine	3.6
Serine	12.4
Glutamic Acid	14.7
Proline	0.0
Glycine	11.5
Alanine	6.6
Half Cysteine	0.0
Valine	7.0
Methionine	0.0
Isoleucine	5.6
Leucine	8.7
Tyrosine	0.7
Phenylalanine	6.9

The lens epithelial layer, after separation from the cortex and nucleus, was homogenized and a soluble protein fraction obtained. Analysis of the epithelial layer supernatant on Sephadex G-75 chromatography (Figure 14) yielded two peaks of high Zn and low A_{280} . The first Zn-containing peak, which eluted at twice void (fractions 17-25) showed a unique peptide of pI 5.2 on isoelectric focusing using a pH 6.5-4.0 gel. (This protein of pI 5.2 was also present in the total column volume second Zn peak, along with several other components). Since this protein peak appeared sufficiently pure, an amino acid analysis (Table VII) was done. The absence of cysteine residues indicated that this low molecular weight protein of pI 5.2 was not metallothionein. The high concentration of negatively charged amino acids account for this low pI.

FIGURE 14

Sephadex G-75 Chromatography of Epithelial Layer Soluble Protein

The epithelial layer was extracted from two bovine lenses and homogenized in a 1:10 ratio of weight to volume with 10mM Tris-Cl, pH 8.6. After centrifugation at 35,000 x g for 30 minutes, 0.75 ml of the supernatant, containing 25 mg of protein, was applied to a 1.0 x 50 cm Sephadex G-75 column. Elution was carried out using the homogenization buffer at a flow rate of 0.45 ml/min; 1 ml fractions were collected and monitored for A_{280} and Zn content. The column void volume was 13 ml. The total column volume was 32 ml.

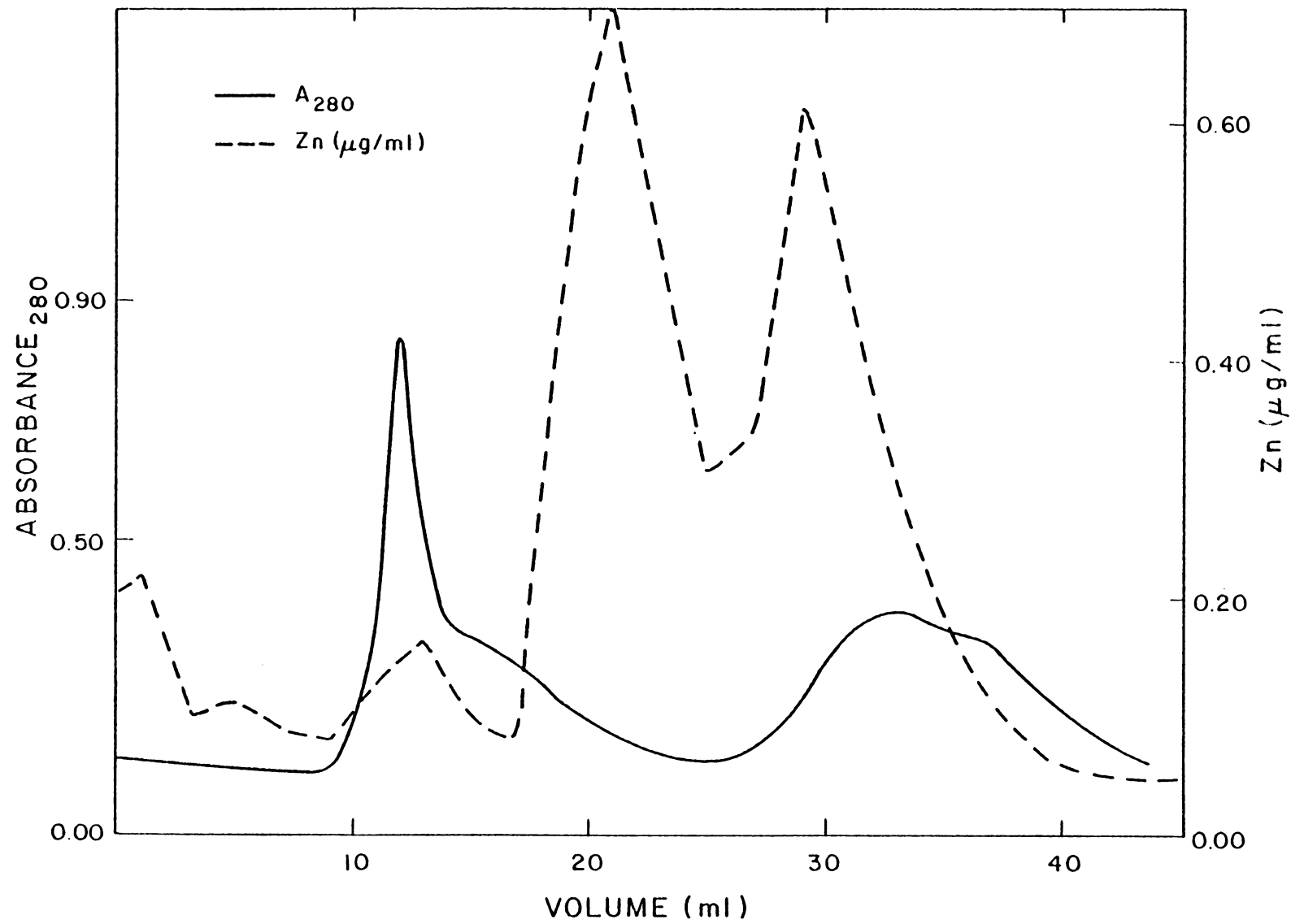


TABLE VII

Amino Acid Composition of Epithelial Layer Polypeptide of pI 5.2

The twice void Zn peak (fractions 17-25) from Sephadex G-75 chromatography of the epithelial supernatant was pooled and concentrated on a UM2 membrane. The UM2 residue was then further concentrated by lyophilization. One protein band of pI 5.2 was observed on isoelectric focusing. Approximately 200 μ g of protein were acid hydrolyzed (6N HCl, 24 hours) and analyzed for amino acid content.

Amino Acid	Residue Percent
Lysine	7.7
Histidine	3.2
Arginine	7.1
Aspartic Acid	9.2
Threonine	4.1
Serine	8.1
Glutamic Acid	10.4
Proline	4.6
Glycine	9.3
Alanine	5.2
Half Cysteine	0.0
Valine	5.5
Methionine	4.1
Isoleucine	5.2
Leucine	8.5
Tyrosine	2.9
Phenylalanine	4.9

DISCUSSION

Initial evidence that a metallothionein-like protein was present in lens tissue was obtained from these results; (1) influence of age on lens Zn, (2) Hg-binding properties and (3) gel filtration chromatography of isolated low molecular weight lens proteins. The observed decrease in rat lens Zn concentration which occurs with maturation (Table I) may correlate with alterations in lens metalloprotein levels. The concentration of hepatic Zn and metallothionein content is higher in rat neonates than in older animals(25). The Hg saturation response observed for rat lens soluble protein (Figure 2) also indicated that a low molecular weight metal-binding protein was present in the lens. The Sephadex G-75 elution behavior of the low molecular weight filtrate from lens showed a distinct peak of Zn eluting with the protein peak (Figure 3), again a property consistent with metallothionein.

Total lens Zn demonstrated that changes in the lens as a result of maturation influence lens Zn concentration. The 30% decrease in rat lens Zn levels that occurs from day 10 to day 35 postpartum (Table I) may correlate with alterations in lens metalloprotein levels. However, if a metalloprotein is present in low concentrations, total metal levels may not be that useful in assessing real differences.

Dietary Zn deficiencies can lower total lens Zn. In the chronically deficient animal, Zn levels in many tissues probably become depleted since body Zn stores must be used. The observation that it takes apparently six weeks to alter lens Zn pools would indicate that the bioavailability of the lens Zn is low. A study done with trout

which had cataracts due to presumed dietary depletions of Zn showed that supplementation of the diet with 150 ppm Zn reversed the cataractogenic effect(41).

Selenite-induced cataractogenesis did not alter lens Zn. Although cataract persists, some of the biochemical changes in the lens tissue following selenite-induced cataract recover with development(2). The similar study done with rats injected at day 9 and killed on day 11 also revealed no change in total lens Zn levels. If there is a time-dependent response of lens Zn to selenium, then the proper time point has not been established.

Lens tissue contains 25ppm Zn, which is four-fold lower than rat liver where Zn is 100 ppm of the total dry weight(37). It was necessary to know how Zn partitions among proteins. The Hg-binding assay revealed a relatively small percentage (4-8%) of the added Hg of the lens saturation curve (Figure 2) recovered in the TCA supernatant. In the plateau region of the curve a sufficient amount of Hg had been added to react with all metalloproteins so excess Hg binds to other proteins which are subsequently precipitated by TCA. The amount of protein remaining in the TCA supernatant, as well as its metal-binding affinity, influence the percentage of Hg bound. The three-fold lower percentage of Hg in the TCA soluble fraction of lens samples compared to the noninduced liver homogenates is consistent with the high concentration of lens structural proteins (crystallins).

Analysis of the lens TCA supernatant by Sephadex G-75 chromatography revealed that the Hg-containing species eluted near the total column

volume. By comparison, the Hg-containing species in the liver homogenate eluted at twice the void volume on a Sephadex G-75 column(38), inferring that the liver Hg-binding proteins are of greater molecular weight.

Determination of the size of the Zn-proteins from the filtrate by Sephadex G-75 and Sephadex G-25 gel filtration chromatography clarified that the Zn-containing protein components that were being examined were of molecular weight less than 5,000 daltons. It was also assumed that free amino acids or glutathione, both normal lens components, were not complexed with the Zn peak observed since the peak eluted substantially before the total column volume (Figure 5). Furthermore, the combination of Sephadex G-25 chromatography and ultrafiltration data assert that the size of the Zn/protein peak is between 2,000-5,000. The elution behavior indicates that the lens low molecular weight proteins are smaller than previously studied mammalian metallothioneins. However, a metallothionein like protein of molecular weight 2,200 has recently been isolated from *Neurospora*(42).

It became apparent that additional characterization of this low molecular weight fraction was required. An important criterion for identification of a protein as a metallothionein is its characteristic high cysteine content. Thus rat and bovine lens homogenates were fractionated by ultrafiltration, gel filtration and chromatofocusing techniques in order to obtain sufficiently pure fractions for characterizing isoelectric points, peptide and amino acid composition. The further characterization of lens low molecular weight proteins discussed

below revealed low levels or the total absence of cysteine residues. Therefore, it was recognized that metallothionein was not a component of the isolated lens low molecular weight proteins. They were further examined on their own merit as being unique Zn-containing low molecular weight proteins. It should be pointed out that metal-binding proteins do not necessarily have as high a cysteine content as metallothionein. For example, a Cd-binding protein has been isolated from oyster which contains a high percentage of dicarboxylic amino acids and only 8% cysteine(43).

The CF25 ultrafiltration filtrate was characterized as being distinct from γ -crystallins, of high amine content and possessed 25% of the total Zn present in the 35,000 x g supernatant. γ -crystallin fractions exhibited different chromatographic behavior and on isoelectric focusing focus in the pH range of 7.1-8.1(7) which is higher than the pH range where the majority of the filtrate proteins focused. The filtrate had high fluorescamine protein values, indicating that its primary amine content is high in comparison with the residue or supernatant samples. Moreover, the high amine content was not just associated with very small molecular weight species, such as amino acids, since concentration of the filtrate by ultrafiltration with a UM2 membrane resulted in retention of 50-80% of the applied protein. The protein in the filtrate samples perhaps cannot be quantitatively assessed with the Lowry assay since observed protein values were very low. The Zn in the filtrate is also bound with molecular weight moieties of greater than 2,000 daltons since at least 60% of the Zn remained associated with the

UM2 residue from concentration or dialysis experiments.

The molecular weight of the proteins in the filtrate as separated by Sephadex G-25 chromatography (Figure 5) were determined to be 12,000-15,000 by SDS-PAGE (Figure 6) which is inconsistent with the elution position. There are reports in the literature of anomolous behavior of low molecular weight proteins on SDS-PAGE. For example, Nakamura reported that the molecular weight of a metallothionein-like protein was estimated to be larger than 35,000 by SDS-PAGE(44). He observed that metallothionein or metallothionein-like proteins may behave as larger molecules on SDS-PAGE owing to their characteristic amino acid composition or polymerization. Also, Jedziniak noted that low molecular weight lens proteins are extremely unstable when concentrated and aggregation can occur(22).

Chromatafocusing, separation based on protein charge, was used as a different approach to isolating low molecular weight, acidic lens proteins. Chromatafocusing of bovine lens soluble protein on a pH gradient of 9-6 followed by a salt wash demonstrated that a protein peak with a large peak of Zn associated (Figure 11) could be isolated from the low pI salt wash. This fraction eluted near the total column volume on Sephadex G-75 so a molecular weight of 5,000 or less is implicated. The low pI protein peak can be separated directly from other lens proteins with a pH 7-4 gradient run (Figure 12). The peak which eluted in the pH range 5.2-4.5 was unique with its higher absorbance at 250nm than at 280nm. It should be noted that when total soluble lens crystallins are separated by gel filtration chromatography,

there is a final peak eluting after the γ -crystallins which usually has higher A_{260} than A_{280} and is classified as being nucleotides(22). However, since nucleotides would have a much lower pI, this chromatofocusing peak is probably not nucleotides. Although A_{280} is not affected, the Polybuffer composition of this pH range may contribute to absorption.

The proteins in the pH 5.2-4.5 peak from the chromatofocusing pH 7-4 column exhibited higher pI values on isoelectric focusing (Figure 13). Although a major protein peak from both the pooled fractions focuses at pH 5.5, there are a few other bands distinguishable in the pH range 5.4-5.8, thus indicating that the peak is composed of a mixture of proteins. Amino acid composition of Peak I from the pH 5.2-4.5 chromatofocusing fraction (Table VI) showed the absence of cysteine residues.

The lens grows throughout life by building up layer after layer of fiber cells arising from differentiation of epithelial cells. Proteins in the nucleus of the lens either turn over very slowly or not at all. Therefore, the proteins located closer to the outer surface of the lens, namely the components of the epithelial layer, are most metabolically active(5). Hence, the localization of a metalloprotein to the epithelial layer was investigated since this metalloprotein would probably be involved in protein metabolism or detoxification reactions.

The Sephadex G-75 chromatographic elution profile of the epithelial layer supernatant (Figure 14) has three distinct Zn-containing peaks. The peak which had high Zn content and very low A_{280} eluted at twice the column void volume, implying a molecular weight of roughly 5,000

daltons. Isoelectric focusing showed that it contained a unique peptide of pI 5.2. Amino acid determination again indicated an absence of cysteine residues (Table VII).

Sephacryl S-200 chromatography of lens soluble protein in the presence of 1mM EDTA reveals a low molecular weight Zn-containing protein peak eluting following the γ -crystallins. Assuming that the molecular weight of the proteins in this peak are 5,000 daltons and that there is one mole of Zn bound for each mole of protein, the quantity of protein calculated that should be in this peak as arising from the Zn is much lower (at least 100-fold) than the actual amount observed by Lowry determination. Since Zn is associated with such a small amount of protein in this peak, the Zn-metalloprotein is a very minor component of the total lens proteins. In the absence of EDTA, the majority of the Zn elutes with the α -crystallin peak (Figures 7 & 8). The protein distribution, as monitored by A_{280} elution behavior, is not influenced by the components of the buffer used. Sephadex G-75 chromatography of bovine CF25 filtrate (Figure 9) indicates again that EDTA addition facilitated transfer of Zn to the low molecular weight protein fraction. Therefore it became questionable whether the Zn/protein peak which elutes following the α -crystallin peak on Sephadex G-75 chromatography with EDTA present is a unique metalloprotein fraction. Fractions concentrated on UM2 membranes retained 60-80% of the total Zn in the residue which implies a molecular weight greater than 2,000 daltons, supporting that it is a true protein fraction. Electrophoretic examination of the Zn-containing peptides isolated with the different homogenization buffers indicate that unique peptides are observed with the

presence of EDTA.

Possibly, the low molecular weight species is a proteinase. Literature exists which points to a low molecular weight (11,500 daltons) protein which is affected by EDTA. In the normal lens there is little evidence of protein turnover and in the lens nucleus proteins may remain throughout life. However, a neutral proteinase has been partially purified from bovine lens which is capable of hydrolyzing lens proteins but the native structure of α -crystallin must be disrupted somewhat before it can be efficiently hydrolyzed by the released proteinase. Activation could be prevented by EDTA, suggesting that divalent metal ions bound to endogenous lens proteins are required for activity. Partially digested proteins resulting from lens proteinase activity could serve as a mechanism for aggregate formation(45). Indeed, aggregates from human cataractous lenses contain a 9,600 dalton polypeptide which appears to be a cleavage product of lens proteins(46). Also, specific C-terminal degradation or chain shortening of α -crystallin does occur and the number of degraded chains is greatest with older lenses(8).

SUMMARY

A population of low molecular weight proteins that comprise a small portion of the total lens soluble protein was defined by various purification techniques. The different procedures used demonstrated the presence of low molecular weight Zn-containing proteins with acidic isoelectric points. These proteins were further characterized as being different from metallothionein since they contained low levels of cysteine. Rat and bovine lens tissue yielded similar results with regard to isolation of low molecular weight proteins. Additional properties of the proteins examined are given according to the protocol used.

(1) CF25 Ultrafiltration - The filtrate contained proteins of molecular weight less than 5,000 which eluted as a single peak of A_{280} with a distinct Zn peak on Sephadex G-25 or Sephadex G-75 chromatography. Aggregation on SDS-PAGE yielded apparent higher molecular weights of the filtrate components. By isoelectric focusing and molecular exclusion chromatography, the filtrate's proteins were recognized as being distinct from γ -crystallins. The filtrate had a high amine content as detected by Fluorescamine protein determination. Of this amine content, 60-80% was associated with proteins of molecular weight greater than 2,000 as characterized by ultrafiltration. Only 1% of the total soluble protein, but 25% of the total lens Zn was recovered in the filtrate.

(2) Sephacryl S-200 Chromatography - A distinct protein/Zn peak eluted after the γ -crystallins with Sephacryl S-200 chromatography of the soluble lens protein done in the presence of 1mM EDTA. Electrophoresis of these fractions verified that the presence of EDTA resulted in different

peptides in the low molecular weight protein population.

(3) Chromatafocusing - A Zn/protein peak could be isolated from the salt wash of a pH 9-6 chromatafocusing column. This low pI fraction could be separated directly by chromatafocusing using a pH gradient of 7-4 and was unique in having a higher absorbance at 250nm than at 280nm. The major component of this peak had a pI of 5.5, but several other components focused in the pH range of 5.5-5.8.

(4) Epithelial Layer Studies - The epithelial layer soluble protein contains a unique polypeptide of pI 5.2 which elutes at approximately 5,000 daltons on Sephadex G-75 with very low A_{280} and high Zn. The protein is abundant in negatively charged amino acids and contains no cysteine.

The low molecular weight Zn-containing lens proteins isolated by different procedures had similar properties. The epithelial layer polypeptide of pI 5.2 must be a component of the CF25 filtrate. It is evident that this polypeptide is a minor component of the total filtrate protein since isoelectric focusing of the total lens filtrate shows most protein components focusing in the pH range of 6-7. Perhaps the filtrate's total Zn is associated with proteins of lower pI. Chromatafocused low molecular weight fractions must also be present in the CF25 filtrate since the Sephadex G-75 elution behavior of the salt wash proteins of pI less than 6 is identical to the filtrate's elution behavior. The exact molecular weight of the Zn-containing protein peaks from the chromatafocusing pH 7-4 column were not determined so a direct correlation with the filtrate's components cannot be made.

EPILOGUE

The physiological significance of the low molecular weight proteins could be judged by their inducibility by metal ions as metalloprotein synthesis, e.g. metallothionein and ferritin, responds to elevated metal levels. Subcutaneous injection of ^{65}Zn to rats followed by isolation of the low molecular weight fraction and quantification of Zn or protein recovered, would test the inducibility of this protein fraction.

The lens epithelial layer is enriched with regard to Zn content. The Zn could have a structural role or perhaps be associated with a metalloprotein. The Zn-containing protein isolated from the epithelial layer that had a pI of 5.2 should be further examined to assess if it is a unique metalloprotein and the only component of this peak.

The low molecular weight proteins comprise a very small proportion of the total soluble lens protein. It is therefore necessary to use large quantities of lenses to obtain significant quantities of this protein fraction. For this reason, rat lenses are not as useful for isolation protocols and larger lenses, e.g. bovine or sheep, should be used in order to obtain a significant amount of low molecular weight protein. During isolation, extreme dilution of protein samples may alter their stability in solution. The low molecular weight protein should perhaps be lyophilized immediately after preparation or use to avoid instability.

The lens CF25 filtrate contained four or more distinct peptides as characterized by isoelectric focusing. It would be of interest to determine which of the proteins contained Zn. Since these peptides

have different isoelectric points, it should be possible to separate them on a preparative scale using ion-exchange chromatography. However, it would be necessary to start with a large quantity of protein since it would be extensively diluted and separated into several protein peaks. The proteins isolated had isoelectric points of approximately 5.0 and glutamic acid and aspartic acid were prevalent. Since the pI of the proteins is acidic, this indicates that the aspartic and glutamic acids are primarily present in the carboxylic acid form. However, the amide content of these proteins needs to be evaluated.

An extensive quantification of the molecular weight as estimated by gel filtration or ultrafiltration and the elution behavior of Zn bound to low molecular weight moieties should be analyzed. Possible experiments include Sephadex G-25 chromatography of cysteine and reduced glutathione complexed with Zn. If the elution of the Zn peak is not near the total column volume, then this would indicate that the presumed proteins detected may just be low molecular weight moieties complexed with Zn. Bacitracin, a cyclic peptide of molecular weight 1,500 daltons, could be used as an additional molecular weight marker.

Zn deficiency is accompanied by cataract development in trout. Whether this effect is also true for rats is not reported since Zn deficient diets cause animals to become very sick and they are probably not maintained for a long enough period of time to observe lens abnormalities. The observation that dietary Zn deficiency can affect lens Zn levels should be confirmed.

The influence of EDTA on the partitioning of lens Zn is worth

further examination. Whether or not a real protein population associated with the α -crystallins and complexed with Zn is dissociated by EDTA could be tested. ^{65}Zn could be used as a marker to check if, when added to the lens homogenate, it exchanges with the endogenous Zn in the low molecular weight fraction.

Zn was the only metal ion monitored in the study of low molecular weight metal-containing proteins. The existence of other metal ions, e.g. Cu or Cd, complexed with this low molecular weight fraction would assess its specificity of metal ion binding.

The affinity of Zn-binding to the low molecular weight proteins has only been evaluated by chromatography and ultrafiltration association. The ability of the protein fraction to bind metal can be assayed by removal of the metal by lowering the pH to approximately 2.0 and then raising the pH and allowing the protein solution to equilibrate with added Zn. The gel filtration elution profile could be examined before and after to see if Zn is associated with the A_{280} peak.

There is merit in the further characterization of lens low molecular weight Zn-containing proteins. Even though they are such a minor component of total lens proteins, they may have an important role in maintaining the optimal configuration of the lens proteins.

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STUDIES OF A LOW MOLECULAR WEIGHT ZN-CONTAINING
PROTEIN POPULATION OF LENS TISSUE

by

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(ABSTRACT)

Maintenance of reduced protein sulfhydryl groups is an important function of lens metabolism. In an attempt to inter-relate how lens sulfhydryl metabolism, low molecular weight peptides and trace metals interact in the lens, total lens Zn was quantified and a low molecular weight Zn-containing protein population was studied. The concentration of rat lens Zn decreased 30% from day 10 to day 35 postpartum. After 6 weeks on a low-Zn diet, rats had 25% less lens Zn than control groups. Selenite-induced cataract did not affect lens Zn concentration. Of the total lens Zn, 25% was recovered in the ultrafiltration fraction of less than 20,000 molecular weight which contained 1% of the total soluble protein. Lens tissue has low molecular weight TCA-soluble components that can bind Hg. Isoelectric points of low molecular weight protein fractions were between pI 5.2-5.5. Polypeptide molecular weight was determined to be less than 5,000 daltons by gel filtration chromatography. Aggregation on SDS-polyacrylamide gel electrophoresis yielded apparent higher molecular weights of these proteins. Although the proteins isolated had metallothionein-like character in that they were of low molecular weight, contained Zn and had acidic pI values, the paucity of cysteine residues indicates that metallothionein is not a component of low molecular weight Zn-containing lens proteins.