ALKYLATION OF RAT LENS CRYSTALLINS
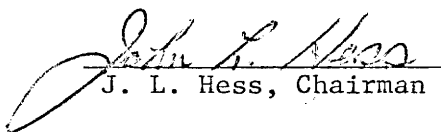
WITH IODOACETAMIDE

by

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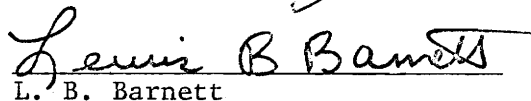
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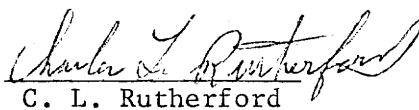
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EDTA: ethylenediaminetetraacetic acid

DTNB: 5,5'-dithiobis (2-nitrobenzoic acid)

SDS: sodium dodecyl sulfate

INTRODUCTION

The water soluble structural proteins of the mammalian lens can be divided into three main classes, the α-, β-, and γ-crystallins. Different in molecular weight and electrophoretic mobility, the crystallins are aggregates of polypeptides of differing molecular weight in the case of α- and β-crystallin, and of the same molecular weight in the case of γ-crystallin. Properties of α- and β-crystallins have been studied extensively, but little is known about the three-dimensional structure of the crystallins or how they are arranged and/or associated in vivo.

During cataractogenesis, regardless of cause, tremendous changes in the internal structure of the lens result in an opacity. It is reasonable to expect that some of these changes might be reflected in the relative proportions and types of crystallins present. Thus, by comparing crystallin populations of normal and cataractous lens proteins, it might be possible to gain some insight into the mechanism of cataract formation. Similarly, in vitro modification of the lens, followed by analysis of the crystallins, could provide information about their structure and interrelationships. Secondarily, if an in vitro modification of protein results in a crystallin population characteristic of cataractous lens, the mechanism of this modification might resemble the in vivo mechanism of cataractogenesis.

In attempts to protect against autoxidation of the lens sulfhydryl, soluble protein of rat lenses ground in the presence of iodoacetamide and separated by gel filtration showed complete loss or severe

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depletion of the high molecular weight β-crystallin. This same phenomenon has been observed in the elution profile of lens protein obtained from cataractous lenses (1).

By studying the characteristics of crystallins isolated in the presence and absence of iodoacetamide, it should be possible to determine whether a crystallin class has been changed, degraded, or become insoluble, or whether β-crystallin is a normal component of the lens or an artifact of isolation due to autoxidation of the lens sulfhydryl.

This phenomenon has been examined by quantifying protein and sulfhydryl content of the lens proteins and by comparing electrophoretic and molecular size properties of individual crystallin populations before and after alkylation.

LITERATURE REVIEW

The Lens

Function — Basically a hollow sphere, the eye is divided into two chambers, the aqueous and vitreous humors, by the lens. The outer wall of the eye consists of three layers: the sclera, or tough outer coat of connective tissue; the heavily vascularized, darkly pigmented choroid, which provides a blood supply to the rest of the eye; and the retina, the multi-layered membrane containing the photoreceptor cells and connected by the optic nerve to the brain. The lens is suspended just behind the pupil by suspensory ligaments extending from the iris.

The function of the lens is to focus light on the retina. In order to project a clear image onto the retina, all rays of light originating from a single point source must be brought together at a single point on the retina. This process requires that incident light be bent and is accomplished by the focusing system of the eye, the lens, and the cornea.

Although the cornea refracts most of the incident light, the light rays striking it must be nearly parallel, which occurs only when the subject is far away. If the subject is near, the light rays striking the cornea are at much smaller angles to each other and must be severely bent to focus properly on the retina. The lens accomplishes this refraction by changing from a fully stretched position, in which it does not bend light, to a convex position, in which light rays are strongly bent, in response to the relaxing or tightening of the suspensory ligaments.

The ability of the lens to accommodate for the distance from the subject is governed by two factors: 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. These two factors are likewise regulated by the relative amounts of protein and water present. An increase in the relative protein concentration will increase the refractive index difference, but an increase in water content will enable the lens to change more easily its degree of curvature during accommodation (2).

Structure — The lens is a transparent tissue, bounded by an elastic capsule. It receives all of its oxygen and nutrients through the capsule from the aqueous and vitreous humors. Although the capsule is relatively impermeable to large macromolecules such as globulins and albumin, it is freely permeable to water and electrolytes and there is abundant evidence for active transport of glucose and amino acids (3).

Immediately beneath the anterior capsule is a layer of metabolically-active epithelial cells. In this region the cells are actively dividing and moving towards the equator of the lens where they elongate, with typical flattening of the nucleus, to form the ribbon-like lens fiber cell. As new cells are formed and elongate, mature cells are pushed toward the core of the lens, forming the tightly packed nucleus. Cells in this region are denucleated and have lost all internal organelles. Because there is no turnover of the lens proteins (4), those cells at the very core of the nucleus are the same cells that

formed the nucleus at birth. For this reason, the lens is a unique tissue for studying the aging process.

Composition — Water and protein comprise 99% of the lens (water 66%, protein 33%). When decapsultated lenses are ground in distilled water and centrifuged, the protein can be divided into two classes, water-soluble and water-insoluble. The insoluble fraction can be further divided into urea-soluble and urea-insoluble by treatment with a concentrated urea solution. The water-soluble fraction is composed mainly of the lens crystallins. Three classes of crystallins, α-, β-, and γ-crystallin are distinguishable by size, electrophoretic, and immunologic characteristics.

Inorganic analysis of rat lenses (5) yielded 0.617% ash that consisted of 0.222% potassium, 0.078% sodium, 0.024% sulfate, 0.005% calcium, 0.029% chloride, 0.240% phosphate, and 0.019% CO_2. Trace elements found in the rat lens include aluminum, calcium, copper, cobalt, chromium, iron, magnesium, molybdenum, nickel, selenium, and zinc (6).

Lipids comprise 1% of the wet weight of the human lens. Distribution of various lipid classes in human lenses is 50% neutral lipids, 45% phospholipids, and 5% gangliosides (7).

Glutathione is manufactured in the lens and decreases in human lens from about 3.5 μmole/g of lens at 20 years of age to about 1.8 μmole/g of lens at age 65 years (8).

Cataractogenesis in Mammals

Dietary Cataract — The most common lesion of the lens is the cataract, a general term describing any opacity in the lens caused by any one or a combination of a number of factors. Three major causes of cataract are: physical injury or trauma, diet, and radiation.

The best studied dietary cataract is the so-called sugar cataract. In the mammalian lens, galactose, glucose, and xylose are absorbed through the aqueous humor into the lens where they are converted into the corresponding sugar alcohol by aldose reductase. Increase in blood galactose or xylose by dietary intake, or glucose as occurs in diabetes mellitus, results in a high concentration of sugar alcohol in the lens. Because sugar alcohols are not readily transported through the lens capsule, the increase in sugar alcohol concentration creates an osmotic pressure difference alleviated by the influx of water. The first noticeable damage brought about by this hydration of the lens is the appearance of large water vacuoles. As the vacuoles enlarge, they coalesce. Gradually, the lens becomes uniformly opalescent and, finally, the lens opacifies (9).

Cataracts have also been produced by feeding experimental animals diets deficient in some nutrient. The only experimentally reproducible cataract of this type is that produced by a tryptophan deficiency (10). Deficiencies in vitamin A and riboflavin have also been reported to cause experimental cataract (11).

Toxic Cataract — Although cataracts can be caused by abnormal levels of ordinary metabolites, ingestion of toxic substances can also

cause cataract. Napthalene, mimosene, dinitrophenol, and triparanol are a few examples of these cataractogenic substances (12).

Radiation Cataract — Microwave, infrared, ultraviolet, x-ray, and gamma radiation are all absorbed by the lens. High doses of these types of radiant energy have been documented to raise the incidence of cataract in humans (13).

Senile Cataract — A senile cataract is one which cannot be attributed to any of the above factors, and it occurs more frequently with old age. This broad category can be divided into nuclear and cortical cataract. Nuclear cataract is characterized by an increase in both water and urea insoluble protein. Accumulation of a brown pigment in the lens commonly results in a brunescent cataract. Wet weight, water content, and total protein remain unchanged, and absorbtion of light by the brown pigment, plus light scattering by clusters of insoluble protein, are the factors thought to be responsible for lens opacity. Unlike the nuclear cataract, in cortical cataract there is a marked decrease in total lens protein--in particular, low molecular weight protein--and a marked increase in the water content of the lens, especially at advanced stages of opacification, and light scattering by water vacuoles is considered to be responsible for opacity (14).

Lens Crystallins

General Properties — In order to observe changes at the molecular level during cataractogenesis, much research has been done to elucidate

the characteristics of the crystallin population in normal and cataractous lenses. In the mammalian lens, populations of four non-identical subunits of about 20,000 daltons aggregate to form the largest of the crystallins, α-crystallin, with a molecular weight of approximately 1,000,000 daltons. Although the isoelectric points of the subunits range from pH 5.6 to pH 7.4, the α-crystallin aggregate has a more acidic isoelectric point, around pH 5 (16). Compared to the other crystallins, α-crystallin is relatively low in thiol content compared to the other crystallins, containing about 3 half-cystine residues/1000 amino acid residues (17).

In contrast, γ-crystallin has the highest thiol content of the crystallins (34 half-cystine residues/1000 amino acid residues) (18), and unlike the other crystallins which are heterogeneous aggregates, it is a mixture of four different polypeptide chains of around 20,000 daltons each. Gamma-crystallin has the highest isoelectric point of the crystallins, approximately 7.5-8.0 (16).

Beta-crystallins are the least studied of the crystallins. There are two classes of these crystallins, designated β_H (high molecular weight) and β_L (low molecular weight) by Bloemendal (20), with molecular weight ranges of 100,000-200,000 and 40,000-90,000, respectively. Both types of crystallins are complex aggregates consisting of seven or more subunits as determined by urea polyacrylamide gel electrophoresis (20) and having isoelectric points ranging from pH 5.7 to pH 7.0 (16). In bovine lens, β_H- and β_L-crystallins have one major polypeptide with a molecular weight of 27,500 daltons, and a number of minor polypeptides in common. Sulfhydryl content of the

β-crystallins is high (17 half-cystine residues/1000 amino acid residues and 9 half-cystine residues/1000 amino acid residues, respectively, for β_H- and β_L-crystallins), but it is not as high as in the γ-crystallin fraction (20).

As the lens ages, generally the relative percent of α-crystallin increases and there occurs a corresponding decrease in the relative percent of γ-crystallins. According to Harding (4), synthesis of γ-crystallins decreases with age resulting in a higher proportion of γ-crystallins in the nucleus than in the cortex.

In cataractous lenses, the story is more complex. According to Dilley et al. (21), high molecular weight aggregates represent 3.1% of the total soluble protein in normal human lenses, while in cataractous lenses they constitute 8.5% of the total soluble protein, 5.1% of which appears to be held together by disulfide bonds. A number of authors have reported diminished amounts of γ-crystallin in cataractous lenses (22, 33). Fillnow (1) has shown that in tryptophan-deficient cataracts, β_H-crystallin fraction is lost completely (Figure 1). Three major hypotheses for the loss of γ-crystallin which can be applied to the loss of β_H-crystallin are the following: polypeptides have leaked out of a damaged capsule (24), low molecular weight protein has become insoluble (25), or it has become cross-linked with itself or other crystallins to form a high molecular weight aggregate (26).

Chemical Modification of Crystallins — Much information has been obtained about crystallin characteristics by studying the results of

FIGURE 1

Gel permeation chromatography of soluble lens proteins

     A sample of lens homogenate was applied to a 90 ×
2.5 cm column of Sephadex G-200 (medium). Proteins
were eluted with a 0.65 Tris-CL (pH 7.6), 0.05 M NaCl,
1 mM EDTA buffer with a flow rate of between 15-18 ml/hr.
Quantification was by means of a Lowry determination
and triangulation. Protein recovered per peak for the
control was: total protein, 48.5 mg; α, 8.6 mg; β_H
5.8 mg; β_L 14.7 mg; γ, 19.4 mg. Protein recovered per
peak for the 9-week tryptophan deficit group was:
total protein, 12.6 mg; α, 3.5 mg; β_H, 0; β_L, 3.5 mg;
γ, 5.6 mg.
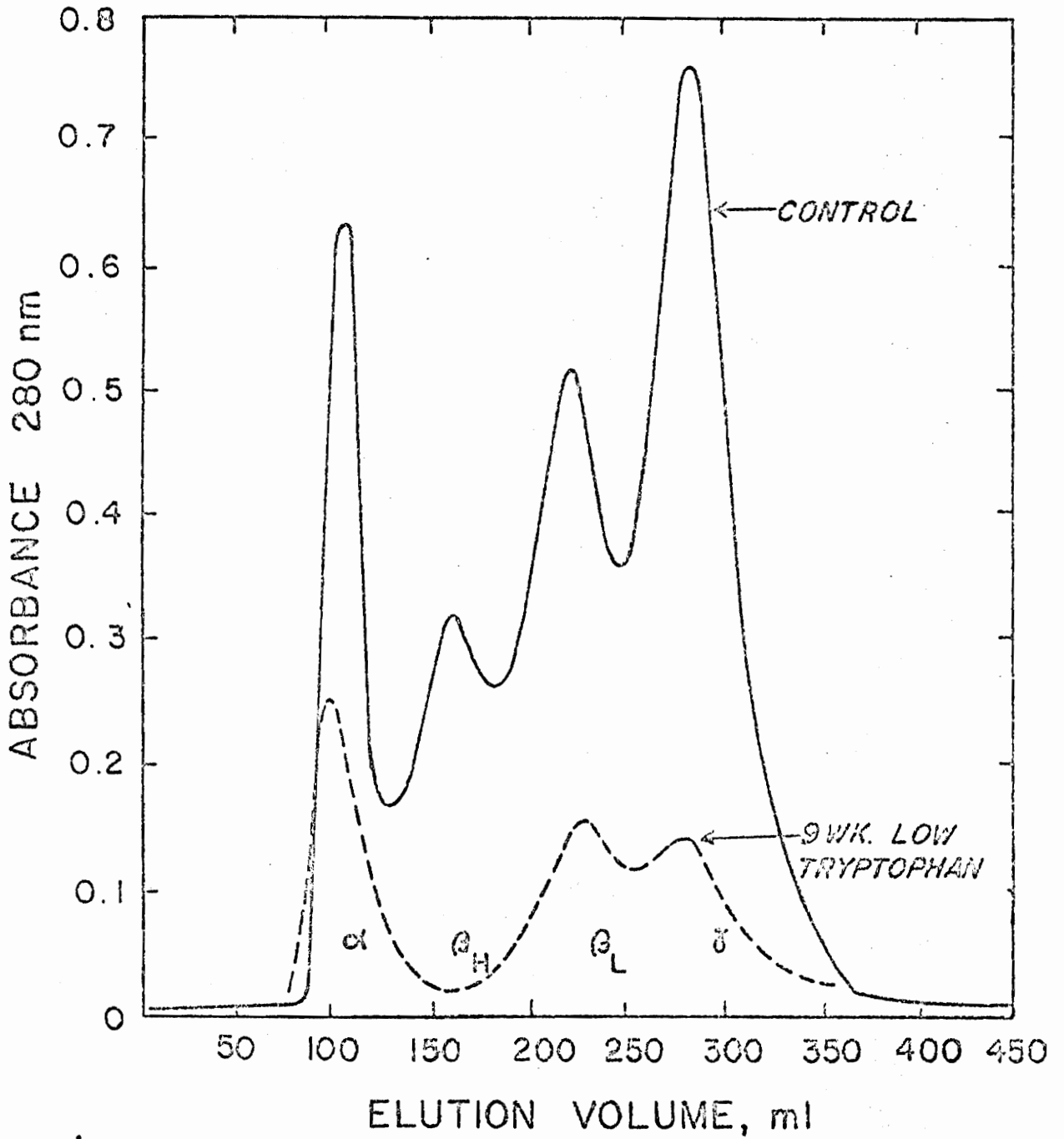
chemical modification of the crystallins. Harding (27) tested the susceptibility of thiols to carboxymethylation in both cataractous and normal human lenses. Using ^{14}C-iodoacetate, he demonstrated that a greater proportion of the total thiol reacted in the cataractous lenses and that the rate of reaction had increased. Since the reaction occurred under non-denaturing conditions, the author suggested that some unfolding had taken place in the cataractous lenses exposing previously buried sulfhydryl groups.

The high molecular weight β-crystallin appears to be particularly susceptible to in vitro modification. For example, when lens crystallins were dissociated with 7 M urea and then allowed to reassociate by dialyzing away the urea, the β_H-crystallin fraction was severely depleted (28). Correct reassociation occurred only if the protein was diluted with 7 M urea to a concentration of 2 mg protein/ml before dialysis. Polyacrylamide gels of the reaggregated fractions showed that both α- and β_L-crystallin peaks had become contaminated with β_H-crystallin polypeptides during reaggregation.

A similar discrepancy appeared with the β_H-crystallin when crystallins were synthesized in a cell-free synthesizing system (29). Using radioactively-labeled amino acids, it was discovered that in vitro synthesis of β_H-crystallin was almost nonexistent, and it was thus suggested that β_H-crystallin formation either takes place at a much slower rate than the other crystallins or that β_H-crystallin arises from β_L-crystallin by an age-related aggregation process.

Harding (30) has carried out chemical modification studies of the insoluble protein of the lens. He showed that most of the urea-insoluble

protein is held together by disulfide bonds. As there was no urea-
insoluble fraction when lenses were isolated anaerobically, Harding
indicated that the urea-insoluble protein was probably formed by
oxidation of cysteine residues with atmospheric oxygen. If this is
true, it is not surprising that γ-crystallin is the major component
of the urea-insoluble fraction since it contains the highest amount
of cysteine.

In efforts to protect thiols from oxidation, Harding tried
alkylating the sulfhydryl with iodoacetate, but anaerobic conditions
were still necessary in order to completely eliminate the urea-insoluble
fraction. After alkylation under anaerobic conditions, the proteins
remained protected even when exposed to aerobic conditions. These data
indicated that formation of disulfide bonds is extremely rapid during
isolation of crystallins but that once alkylated, the thiol is protected
from autoxidation.

Alkylation of Proteins with Haloacids

Characteristics of the Alkylation Reaction — Although the
alkylation reaction done by Harding (31) was meant to protect free
sulfhydryl groups only, alkylation with haloacids is quite general,
depending on the conditions used.

Thunberg (31) first suggested that toxicity of certain haloacids
resulted from their reaction with tissue sulfhydryl groups. Iodo-,
bromo-, and chloroacetic acids and the corresponding amides are used
most often as alkylating agents. Under various conditions they can
react with sulfhydryl, imidazole, thioether, and amino groups of

proteins (Figure 2). Rates of carboxymethylation can be determined either from the rate of halide ion release (32) or by the release of protons (33).

Of the mentioned groups, thiols are the most reactive. Reactivity increases with pH since the anion is the reactive species, but to prevent reaction with other molecules a neutral pH is normally used. On amino acid analysis, carboxymethylcysteine elutes very rapidly, before aspartic acid (32).

Reaction with imidazole groups is considerably slower. Reaction occurs above pH 5.5 and is the principal reaction in proteins not containing thiol groups. Three derivatives can result: the 1- and 3-carboxymethylhistidine, and the 1,3-carboxymethylhistidine. 1-carboxymethylhistidine elutes near proline, while 3-carboxymethyl-histidine elutes nearer cystine (32, 33).

Haloacetates react with methionine over a wide pH range. At low pH where other reactive groups are protonated, the alkylation reaction is relatively specific for methionine. Since methionine residues are often located in the hydrophobic interior of proteins, denaturation with urea, guanidine, and low pH are needed to obtain a satisfactory reaction rate. The product of the reaction, an S-carboxymethylmethionyl-sulfonium cation, is destroyed during acid hydrolysis, but its three decomposition products, S-carboxymethyl homocysteine, homoserine lactone, and methionine, are all measurable by amino acid analysis (33).

Unprotonated amino groups on proteins, e.g., on arginine or lysine, will be alkylated at high pHs, but at a rate about 1/100 as fast as

FIGURE 2

<u>Alkylation of different functional groups with haloacetates</u>

Typical alkylation reactions of haloacetates. (1) alkylation of
free sulfhydryl (cysteine); (2) alkylation of imidazole (histidine);
(3) alkylation of thioether (methionine); (4) alkylation of amino
groups (arginine and lysine).

1   (P)$-S^- + ICH_2CONH_3^+$ $\xrightarrow{pH \geq 7}$ (P)$-S-CH_2CONH_3^+ + I^-$

2   (P)$\overset{\displaystyle N}{\underset{\displaystyle \underset{H}{N}}{\diagup\diagdown}} + ICH_2CONH_3^+$ $\xrightarrow{pH > 5.5}$ (P)$\overset{\displaystyle N-CH_2CONH_3^+}{\underset{\displaystyle N}{\diagup\diagdown}}$ $+ I^- + H^+$

3   (P)$-\underset{\displaystyle CH_3}{S} + ICH_2CONH_3^+$ $\xrightarrow{pH\ 2-8.5}$ (P)$-\overset{\displaystyle CH_2CONH_3^+}{\underset{\displaystyle CH_3}{S^+}} + I^-$

4   (P)$-NH_2 + ICH_2CONH_3^+$ $\xrightarrow{pH > 8.5}$ (P)$-NH-CH_2CONH_3^+ + I^- + H^+$
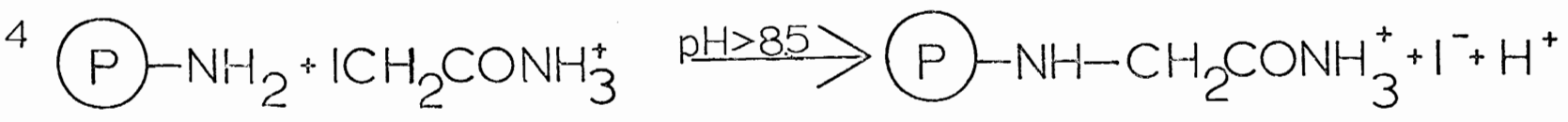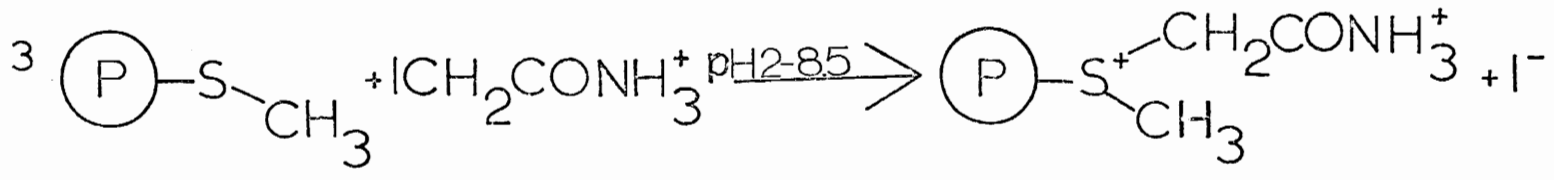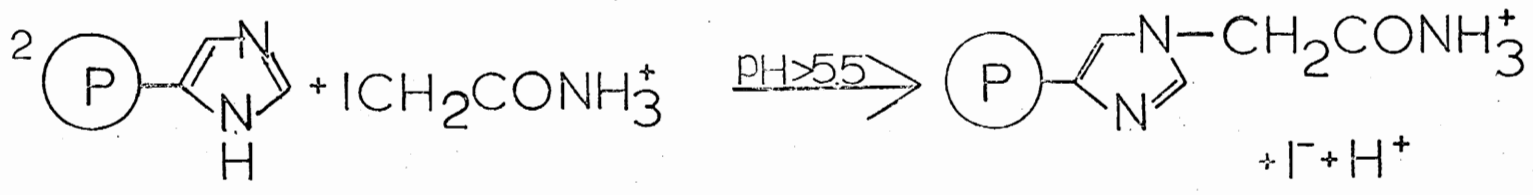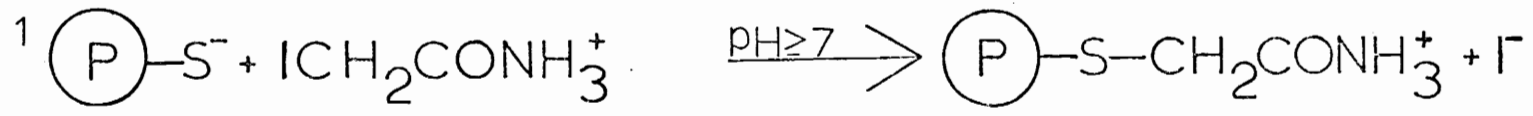
sulfhydryl groups. Like histidine, the alkylation reaction can proceed in two steps to dialkylated derivatives. Both mono- and dialkyl derivatives are stable during, and can be isolated and quantified after, acid hydrolysis. Thus, alkylation with haloacetates is a broad-spectrum reaction with the possibility of many different products and chemical modifications as the result.

EXPERIMENTAL PROCEDURES

Materials

Male Sprague-Dawley rats, 90-100 g, were obtained from Flow Laboratories, Inc. (Dublin, Virginia).

Sephadex G-200 (40-120 μ mesh), Sephacryl S-200 superfine (40-105 μ mesh) for gel permeation chromatography, and Blue Dextran were obtained from Pharmacia Fine Chemicals (Piscataway, New Jersey).

High purity acrylamide, high purity N,N'-methylenebisacrylamide, and N,N,N',N'-tetramethylethylenediamine were obtained from Ames Company (Elkhart, Indiana). Electrophoresis-grade ammonium persulfate was obtained from BioRad Laboratories (Richmond, California).

For pressure dialysis, PM 10 (10,000 molecular weight cut-off) membranes were obtained from the Amicon Corporation (Lexington, Massachusetts).

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

Ultra-pure Guanidine Hydrochloride was obtained from Schwarz/ Mann (Orangeburg, New York). Reagent grade guanidine hydrochloride and Coomassie Brilliant Blue G-250 were obtained from Eastman Kodak Company (Rochester, New York).

Liquifluor and 2,5-dipheynyoxazole (PPO) were obtained from New England Nuclear (Boston, Massachusetts). Iodoacetamide-1-^{14}C was

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obtained from ICN Company, Chemical and Radioisotope Division (Irvine, California).

Methods

Care and Feeding of Rats — Rats were housed in stainless steel cages in a temperature-controlled room (21°C) having a 12 hour day/ night cycle and were maintained on Charles River commercial lab ration until the time they were sacrificed; animal weight usually did not exceed 110 g.

Preparation of Lens Homogenates — Rats were sacrificed by decapitation or cervical dislocation, lenses were removed and placed in a tared 10 ml beaker on ice. After obtaining lens wet weight, control samples were homogenized in a 0.01 M Tris-Cl, pH 8 using a teflon pestle-glass homogenizer; alkylated samples were homogenized in the same manner in 0.01 M Tris-Cl buffer containing appropriate levels of iodoacetamide. All samples were centrifuged at 24,000 × g for 30 min, the soluble fraction removed, and the insoluble fraction resuspended in 0.01 M Tris-Cl buffer, pH 8.

Alkylation of Lens Crystallins — Lenses were homogenized in 0.01 M Tris-Cl, pH 8 containing quantities of iodoacetamide necessary to give a mg iodoacetamide/mg protein ratio of 1, pH was adjusted to 8 and samples reacted at room temperature (22-25°C) for 1 hr. Whole lens homogenates were centrifuged and immediately subjected to gel filtration chromatography to remove excess iodoacetamide.

Isolated crystallin samples in 0.065 M Tris-Cl buffer, pH 7.6, 0.05 M NaCl, 0.001 M EDTA[1] were alkylated by the addition of solid iodoacetamide to avoid dilution of the samples, pH was adjusted to 8 and samples reacted at room temperature (22-25°C) for 1 hr. Samples were then dialyzed against 5 volumes of elution buffer to eliminate excess iodoacetamide.

Gel Filtration Experiments — Separation of the water-soluble fraction into α-, β_H-, β_L-, and γ-crystallins was achieved using Sephadex G-200 or Sephacryl S-200. Either 90 cm × 2.5 cm or 90 cm × 1.6 cm columns were used, depending upon the amount of protein in the sample. Proteins were eluted with a buffer containing 0.065 M Tris-Cl, pH 7.6, 0.05 M NaCl, and 0.001 M EDTA. With Sephadex G-200, flow rates of 6-10 ml/hr were realized; Sephacryl S-200 gave flow rates of 1 ml/min with the 1.5 cm column, 2 ml/min with the 2.5 cm column. Eluates from the 1.5 cm × 90 cm column were collected in 2 ml fractions, while 3 ml fractions were collected from the 2.5 cm × 90 cm column.

To obtain radioactive whole lens preparation, lenses were homogenized in an iodoacetamide solution containing 5 mg/ml ^{14}C-labeled iodoacetamide (specific activity 1.97 µCi/mg iodoacetamide), the soluble fraction was isolated, and 2 ml were layered on the 1.5 cm × 90 cm Sephacryl S-200 column. Aliquots (250 µl) from each 2 ml fraction were added to 10 ml of a triton-toluene scintillation fluor (34) and counted in a Beckman LS-133 scintillation counter. Relative efficiency was monitored by use of the external standard ratio.

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[1]The abbreviations used in this text are defined in the List of Abbreviations, page vi.

For co-chromatography experiments, concentrated β_H- and β_L-crystallins were alkylated with a 4 mg/ml ^{14}C-iodoacetamide (specific activity 1.23 μCi/mg iodoacetamide) and chromatographed on the 1.5 cm × 90 cm Sephacryl S-200 column, eluting with 0.065 Tris-Cl buffer, pH 7.6, 0.05 M NaCl, 0.001 M EDTA. One ml aliquots from β_H- and β_L-crystallin peak fractions were pooled and concentrated using an Amicon 50 ml ultrafiltration unit with a PM 10 membrane, then combined with a portion of a non-alkylated soluble fraction. Each solution was chromatographed on the 1.5 cm × 90 cm Sephacryl S-200 column, eluting with 0.065 M Tris-Cl buffer, pH 7.6, 0.05 M NaCl, 0.001 M EDTA; 280 nm absorbance was monitored, and 250 μl aliquots were counted in 10 ml of Bray's scintillation cocktail (35) using a Beckman LS-133 scintillation counter. Counting efficiencies were monitored by the external standard ratio.

Protein Determination — Protein determination was routinely performed using a modified method of Lowry et al. (36) with bovine serum albumin as a reference protein. The Kalckar equation (37) was sometimes used to obtain an approximate protein concentration so that purified crystallin fractions could be alkylated as quickly as possible, but protein was later determined by the Lowry method.

Sulfhydryl Determinations — For non-denatured samples, 0.1 ml sample aliquots were diluted to 2.9 ml with 0.1 M Tris-Cl buffer, pH 8.1. Sulfhydryl was measured by adding 0.1 ml of Ellman's reagent, DTNB (38), and reading samples at 405 nm after 5 min.

For denatured samples, protein samples were precipitated by adding aliquots of 50% TCA so that the final concentration was 5% TCA, centrifuging at approximately 3,000 × $\underline{g}$ in a clinical centrifuge, and resuspending the pellet in 6 N guanidine-HCl, pH 8.1. One-tenth ml samples were dilted to 2.9 ml with 6 N guanidine-HCl, pH 8.1, and analysis was continued as with the non-denatured samples.

The extinction coefficient of the thio-anion of DTNB, 3-carboxylato-4-nitro-thiophenolate, was determined by making a standard curve using aliquots of 1.43 mM mercaptoethanol standard (10 μl mercaptoethanol in 100 ml Tris-Cl) and determining thiol content with DTNB. The extinction coefficient was determined each day sulfhydryl determinations were performed to compensate for any differences in assay solutions that might affect the extinction coefficient.

In experiments requiring various concentrations of alkylating agent, lenses were homogenized in 0.01 M Tris-Cl, pH 8 containing 0.1 mg/ml iodoacetamide. To 250 μl of this solution were added 250 μl of iodoacetamide solutions in increasing concentrations. After 1 hr at 25°C, pH 8, samples were centrifuged at 12,000 × $\underline{g}$ for 30 min, and the supernates were refrigerated until analysis.

Isoelectric Focusing — Procedures used for isoelectric focusing were modified from previously reported techniques (39, 40, 41, 16). Slab gels (7.5% acrylamide) containing 2% Biolyte 3-10 ampholytes were polymerized overnight in a Bio-Rad Model 220 Slab Cell apparatus under a 15 watt fluorescent light, then refrigerated until use (not more than 5 days).

When polymerized, the gel was sandwiched between 2 glass plates. After the glass plates were pried apart, the gel adhered to one of the plates. This plate was placed on the cooling plate of the Savant electrophoresis unit which had previously been cooled to 4°C. Two Desaga/Brinkman platinum ribbon electrodes were placed on 10 mm × 60 mm wicks of solvent saturation paper at either end of the gel; the wicks had been soaked in anode and cathode electrolyte solutions, respectively (anode electrolyte: 0.06 N H_2SO_4; cathode electrolyte: 0.02 N $Ca(OH)_2$ and 0.04 N NaOH). Samples of 10-40 µl were applied to 5 × 10 mm pieces of Whatman 3 mm filter paper which had been placed on the gel, ≃ 2-3 cm from the anode end of the gel and 1 cm apart. Power was initially set at 4000 v and gradually increased to 1,000 v, never exceeding 10 milliamps. At 1,000 v, the gel was run for 1-1/2 hr and then the power switched off. Staining was performed using the R-250 staining procedure for isoelectric focused gels distributed by LKB Instrument Inc. (42).

SDS Gels — Gels containing 15% acrylamide were prepared according to Neville (43) and cast in the Bio-Rad 220 Slab Cell. Protein samples that were 1% SDS + 20% sucrose were incubated for 1 hr at 37°C; then 10-40 µl samples were layered into the sample wells of the stacking gel. The gel was run in the slab unit at 20°C at 0.6 ma/cm^2 until the tracking dye entered the separator gel, and then at 0.3 ma/cm^2 until the run was completed (total running time ≃ 3 hours). Staining was performed by the method of Fairbanks (44).

Amino Acid Analysis — Amino acid analysis was performed using the 2 column system of Spackman et al. (45) on a Beckman 121 Automatic Amino Acid Analyzer. Samples used in standard amino acid analysis were hydrolyzed for 24 hr in 6N HCl. Samples analyzed for cysteine and methionine were oxidized with performic acid and lyophilized before undergoing acid hydrolysis.

Carboxymethylhistidine and carboxymethylcysteine standards were prepared by adding 1 mg of iodoacetamide to solutions containing 1 mg/ml histidine or cysteine in 0.01 M Tris-Cl, pH 8, and reacting for 1 hr at 25°C pH8.

RESULTS

Gel Filtration Studies

Since isolation of the soluble crystallins from rat lens was performed aerobically, artifacts might occur due to autoxidation of the lens free-sulfhydryl. Autoxidation was minimized by alkylating free-sulfhydryl with iodoacetamide.

When lenses were homogenized in the presence of 50 mg/ml iodoacetamide, a major change in the Sephadex G-200 elution profile resulted, as seen in Figure 3. In the control column, where lenses were homogenized in 0.01 M Tris, pH 8,0, four peaks result, corresponding to α-, β_H-, β_L-, and γ-crystallins, which is consistent with the literature (4). However, when lenses were ground in the presence of iodoacetamide, no β_H-crystallin was present in the elution pattern.

This striking omission is especially interesting in that an elution profile of cataractous lenses obtained by feeding rats a tryptophan-deficient diet shows the same loss of β_H-crystallin (Figure 1).

With preliminary results indicating the possibility that the in vitro modification with iodoacetamide might be related to the in vivo modification occurring in the deficient diet, further studies to determine the nature of the iodoacetamide modification were made.

Although the original gel filtration studies were done using lenses from various age rats, in subsequent studies only male rats weighing approximately 100 gm were used. Using rats of similar age

FIGURE 3

Gel solution of alkylated and non-alkylated
water soluble lens crystallins on
Sephadex G-200

One ml of a 24,000 × g supernate of lenses
from 400 g male rats ground in 0.01 M Tris-Cl
buffer, pH 8,0, containing 42.3 mg of soluble
lens protein (————). One ml of a 24,000 × g
supernate of lenses from 200 g male rats ground
in a solution of 50 mg/ml iodoacetamide in 0.01 M
Tris-Cl buffer, pH 8,0, and reacted for 1 hour at
25°C, pH 8.0 containing 9.8 mg of soluble lens protein
(— ·—). The 1.5 × 90 cm column was eluted with
0.065 M Tris-Cl buffer, 0.05 M NaCl, 0.001 M EDTA,
pH 7.6 at a flow rate of 0.12 ml/min; 2 ml fractions
were collected.
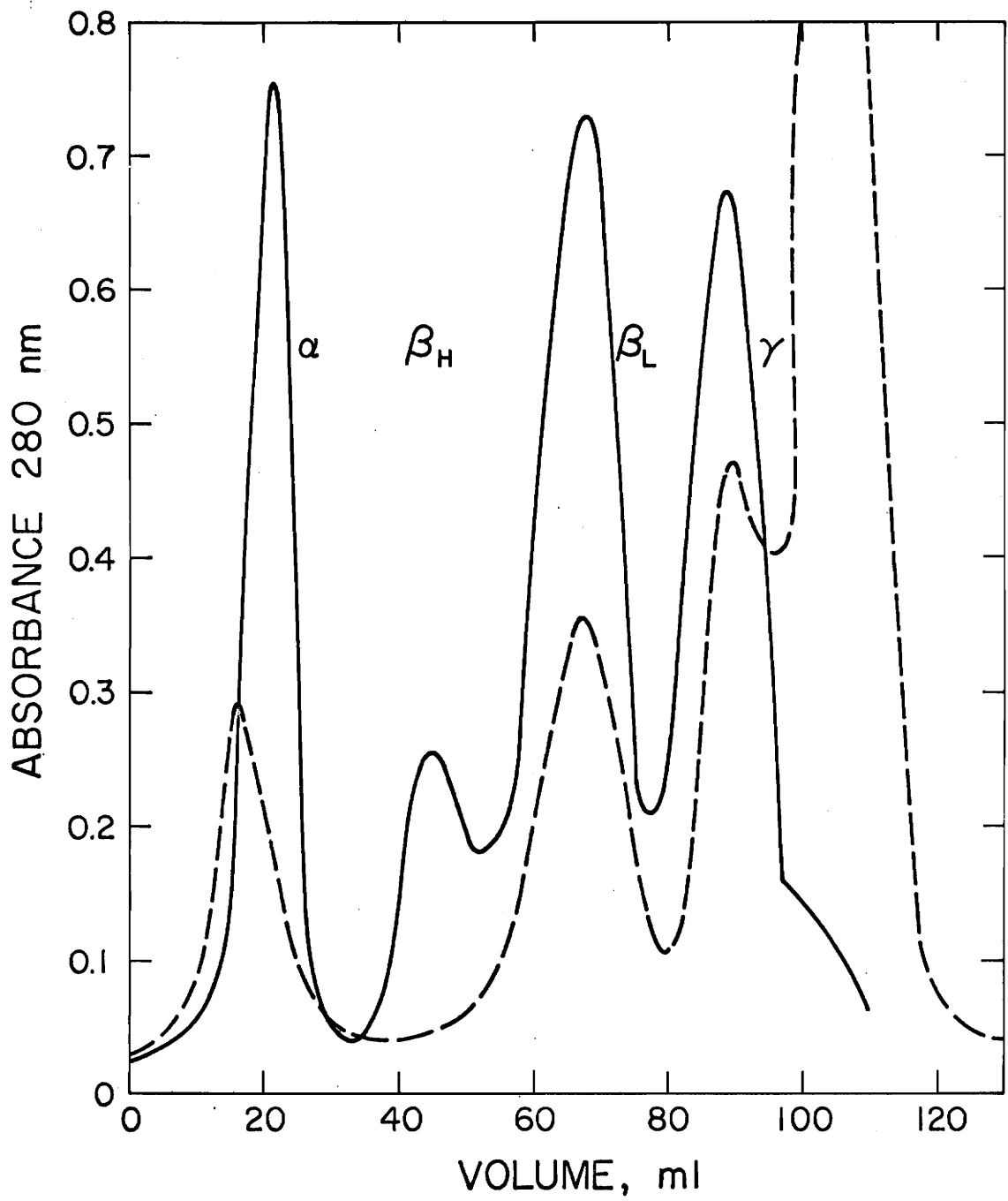
was necessary because changes in the relative proportions of the crystallins occur at different ages.

Sephacryl S-200 was used instead of Sephadex G-200 in most of the gel filtration experiments because of the large increase in flow rate obtainable with a small loss in resolution.

Conditions Necessary for Alkylation and Separation of Lens Crystallin — As research progressed, it became evident that specific conditions had to be met to achieve (1) good separation of the crystallins, and (2) loss of β_H-crystallin with alkylation.

By testing many variables, it was determined that fresh lens homogenates were needed for satisfactory resolution of the crystallins. If frozen homogenates, to a lesser extent frozen lenses, or even samples refrigerated for more than a few days were chromatographed, resolution decreased, particularly in the β-crystallin region.

Results of alkylation experiments at different concentrations of iodoacetamide indicate that in order for β_H-crystallin to disappear, a one to one ratio of mg iodoacetamide to mg protein was needed. This ratio is equivalent to a 50 mmoles of iodoacetamide for every mmole of free-sulfhydryl. Application of this rule is seen in the Sephacryl S-200 elution profile of alkylated and non-alkylated lens crystallins from 100 g rats (Figure 4). Here, β_H-crystallin peak is gone at a ratio of 10 mg iodoacetamide/10 mg protein.

Soluble vs. Insoluble Protein

Since an obvious possibility for the loss of a major class of proteins was that they had become insoluble, soluble/insoluble ratios

FIGURE 4

Gel filtration of alkylated and non-alkylated water soluble lens crystallins from 100 g male rats on Sephacryl S-200

Two ml of a 24,000 × g supernate from lenses ground in 0.01 M Tris-Cl buffer, pH 8.0 containing 30.7 mg of soluble lens protein (————). Two ml of a 24,000 × g supernate from lenses ground in a solution of 10 mg/ml iodoacetamide in 0.01 M Tris-Cl buffer, pH 8.0, and reacted for 1 hour at 25°C, pH 8.0, containing 21.0 mg soluble lens protein (— — — —). The 2.5 × 90 cm column was eluted with 0.065 M Tris-Cl buffer, 0.05 M NaCl, 0.001 M EDTA, pH 7.6 at a flow rate of 2 ml/min; 3 ml fractions were collected.
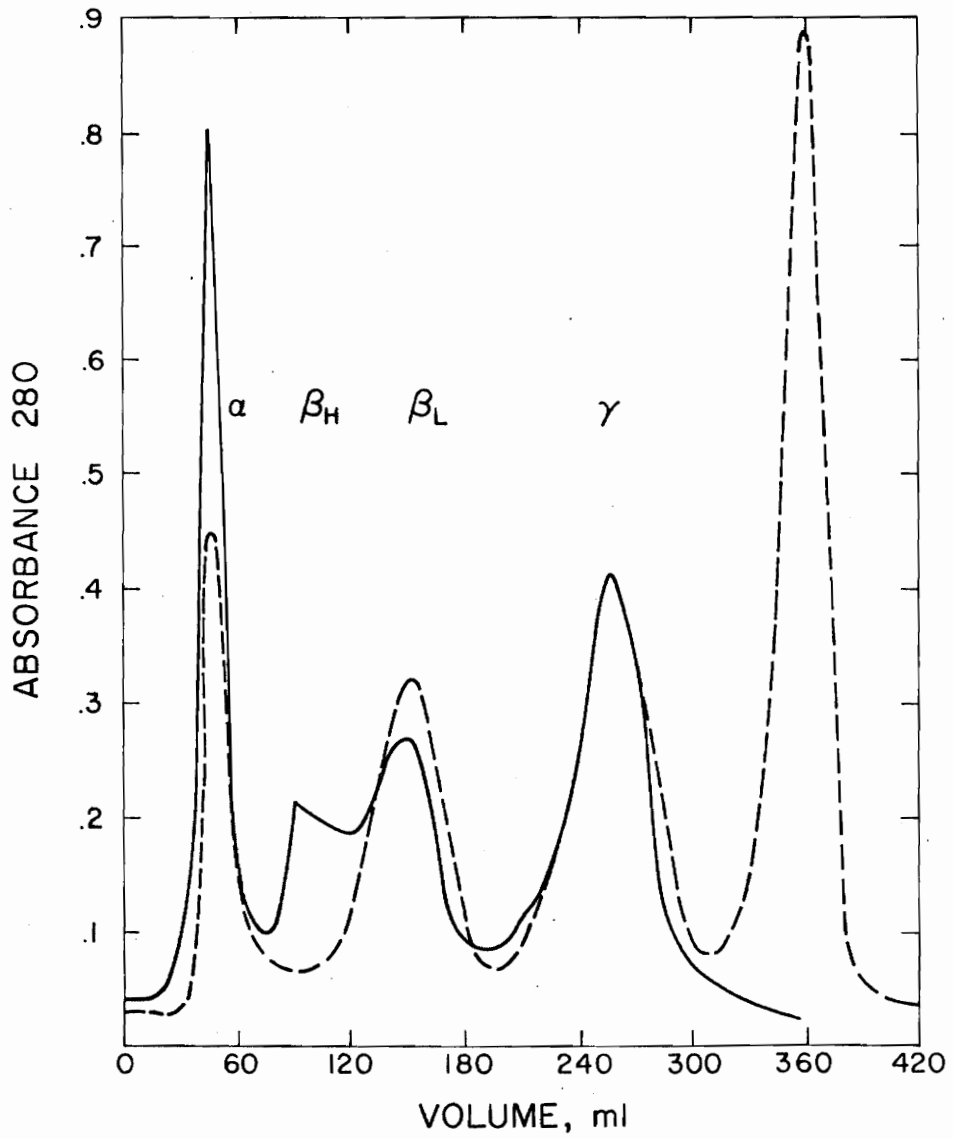
were measured in a number of preparations as seen in Table I. Within

experimental error, no significant differences could be seen in the

soluble/insoluble ratios of alkylated vs. control preparations. As

β_H-crystallin represents approximately 10% of the lens crystallin

population in 100 gm rats, a total conversion of this fraction to

insoluble material should have been sufficient to alter the soluble/

insoluble ratios. Therefore, from these results, a conversion of β_H-

crystallin to insoluble material with alkylation was not indicated.

Sulfhydryl Studies

It became important to examine the effectiveness of the alkylation

reaction at various levels of reagent, since apparent loss of β_H-

crystallin occurred only with concentrations of at least 1 mg iodoaceta-

mide/mg protein.

The extent of the alkylation reaction was indirectly determined

by measuring remaining free-sulfhydryl at various concentrations of

iodoacetamide, in denaturing and non-denaturing solvent (Figure 5).

Measurement of free-sulfhydryl in 0.1 M Tris-Cl, pH 8.1 indicated that

exposed sulfhydryl was alkylated at very low levels of iodoacetamide,

and that virtually all of the exposed sulfhydryl was alkylated at a

ratio of 0.16 mg iodoacetamide/mg protein. Recall that β_H-crystallin

did not disappear until the mg iodoacetamide/mg protein ratio was one.

Measurement of free-sulfhydryl in denaturing solvent (Figure 5)

reflected alkylation of the exposed sulfhydryl; the additional amount

(approximately 6 μm-SH/mg protein) of sulfhydryl at each point

represented buried sulfhydryl that was not readily exposed to alkylation.

TABLE I

Soluble and insoluble protein of alkylated
and non-alkylated lens homogenates

Lowry (36) protein measurements of the 24,000 × g supernate (soluble fraction) and the resuspended pellet (insoluble fraction) of alkylated and non-alkylated lens homogenates. (Non-alkylated homogenates were obtained by grinding lenses in 0.01 M Tris-Cl buffer pH 8.0. Alkylated homogenates were obtained by grinding lenses in a solution of 10 mg/ml iodoacetamide in 0.01 M Tris-Cl buffer, pH 8.0 and reacting for 1 hour at 25°C, pH 8.0.) Percent reported as averages of 3 different samples; protein determinations on each sample were done in triplicate.

Sample	Protein[a] (mg/lens)		Percent ± s.d.	
	Soluble	Insoluble	Soluble	Insoluble
Control	14.2	2.4	84.1 ± 1.62	15.9 ± 1.62
Alkylated	14.5	2.2	84.0 ± 2.37	16.0 ± 2.37

[a]Protein data from one typical lens preparation.

FIGURE 5

Free-sulfhydryl content of soluble lens protein

Samples were alkylated at increasing levels of iodoacetamide;
sulfhydryl content was determined in the presence and absence of a
denaturing solvent. 250 µl samples of lens homogenate, ground in the
presence of 0.4 mg/ml iodoacetamide in 0.01 M Tris, pH 8.0, were
combined with 250 µl samples of increasing concentrations of
iodoacetamide in 0.01 M Tris, pH 8.0; reacted at 25°C, pH 8.0 for 1
hour; and centrifuged at 12,000 × g for 30 min. Supernates were
assayed for sulfhydryl by bringing 0.1 ml sample aliquots to 2.9 ml
with 0.1 M Tris pH 8.1 (—△—△—△—△—) or 6 N guanidine hydrochloride
pH 8.1 (—o—o—o—o—), adding 0.1 ml DTNB and reading absorbance at
412 nm after 5 minutes. Data in presence of guanidine hydrochloride
represent a single determination, and in the presence of buffer, data
points are the average of triplicate determinations.
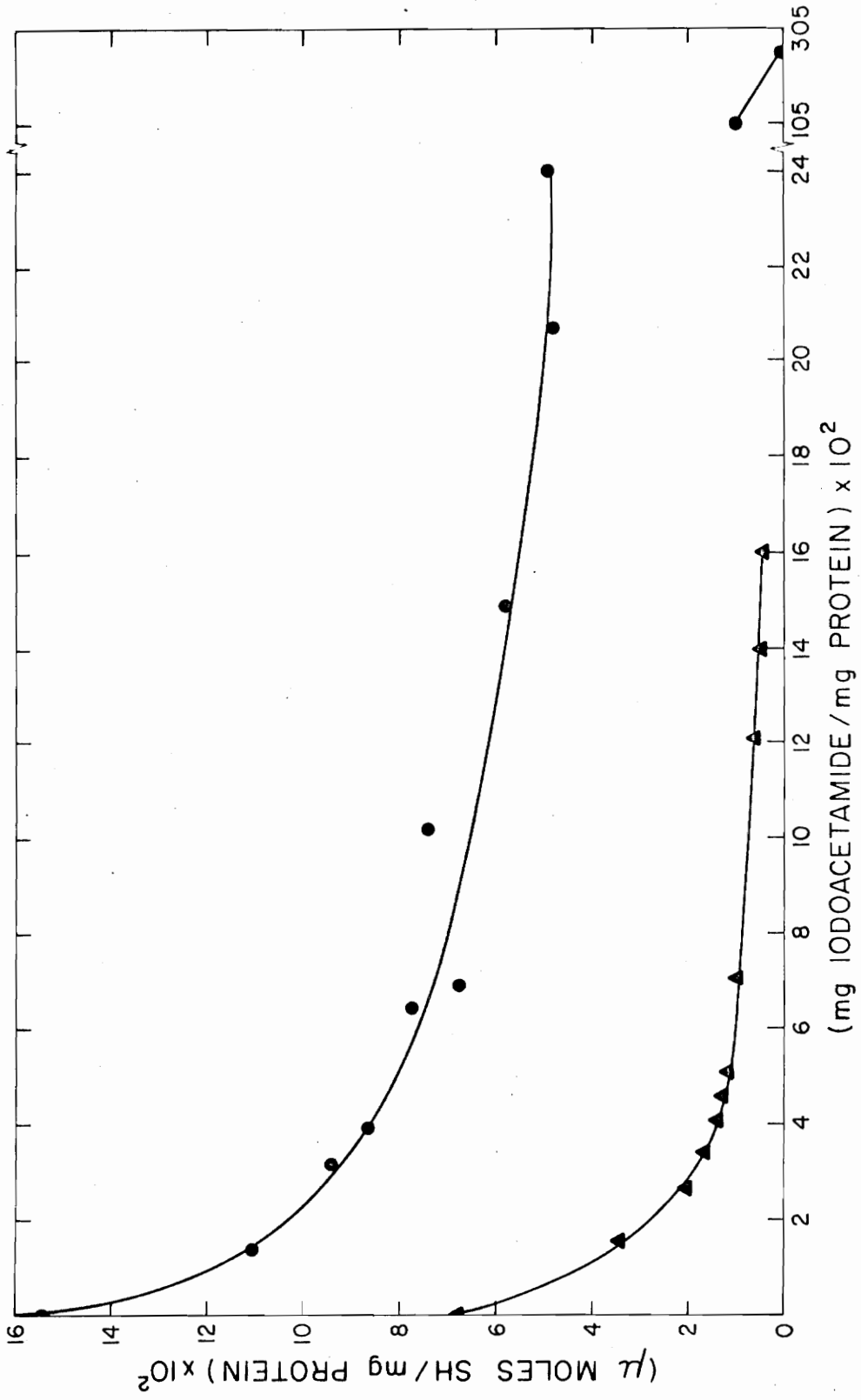
However, when the weight of iodoacetamide equaled the weight of protein, even this buried sulfhydryl had been alkylated, indicating that at high levels of iodoacetamide the lens protein unfolded, exposing previously buried sulfhydryl.

Sulfhydryl data of Figure 5, plotted on a log linear scale, are shown in Figure 6. Total sulfhydryl gives a linear response, but the exposed sulfhydryl plot is a biphasic curve, indicating that two types of exposed sulfhydryl exist, differing in their reactivity with iodoacetamide.

To determine whether the trends seen in the sulfhydryl data on the total soluble fraction were due to specific changes in the sulfhydryl of any one of the crystallins, lenses were homogenized in the presence of ^{14}C-iodoacetamide (specific activity 1.97 μCi/mg iodoacetamide) and the soluble protein separated on Sephacryl S-200, monitoring both absorbance at 280 nm and radioactivity. Results are shown in Figure 7. Radioactivity in general follows the relative thiol content of each crystallin type, i.e., with α-crystallin containing the least amount of sulfhydryl, the β-crystallin an intermediate amount, and γ-crystallin containing the highest amount of sulfhydryl. It should be noted that the alkylation was not at the high levels needed to completely eliminate β_H-crystallin (mg iodoacetamide/mg protein = 0.42) and that, in this intermediate stage, there is a shift of the β_H-crystallin peak to an apparent lower molecular weight.

FIGURE 6

Log transformation of free-sulfhydryl content of soluble lens protein
samples alkylated with increasing levels of iodoacetamide in
denaturing and non-denaturing solvent

Data of Figure 4 were plotted as μm-SH/mg protein versus log mg
iodoacetamide/mg protein ratio. (-○-○-○-○-○-) free-sulfhydryl in presence
of 6N guanidine-HCl (-△-△-△-△-) 0.1 M Tris-Cl. (-●-●-○-○-) right scale;
(-△-△-△-△-) left scale.
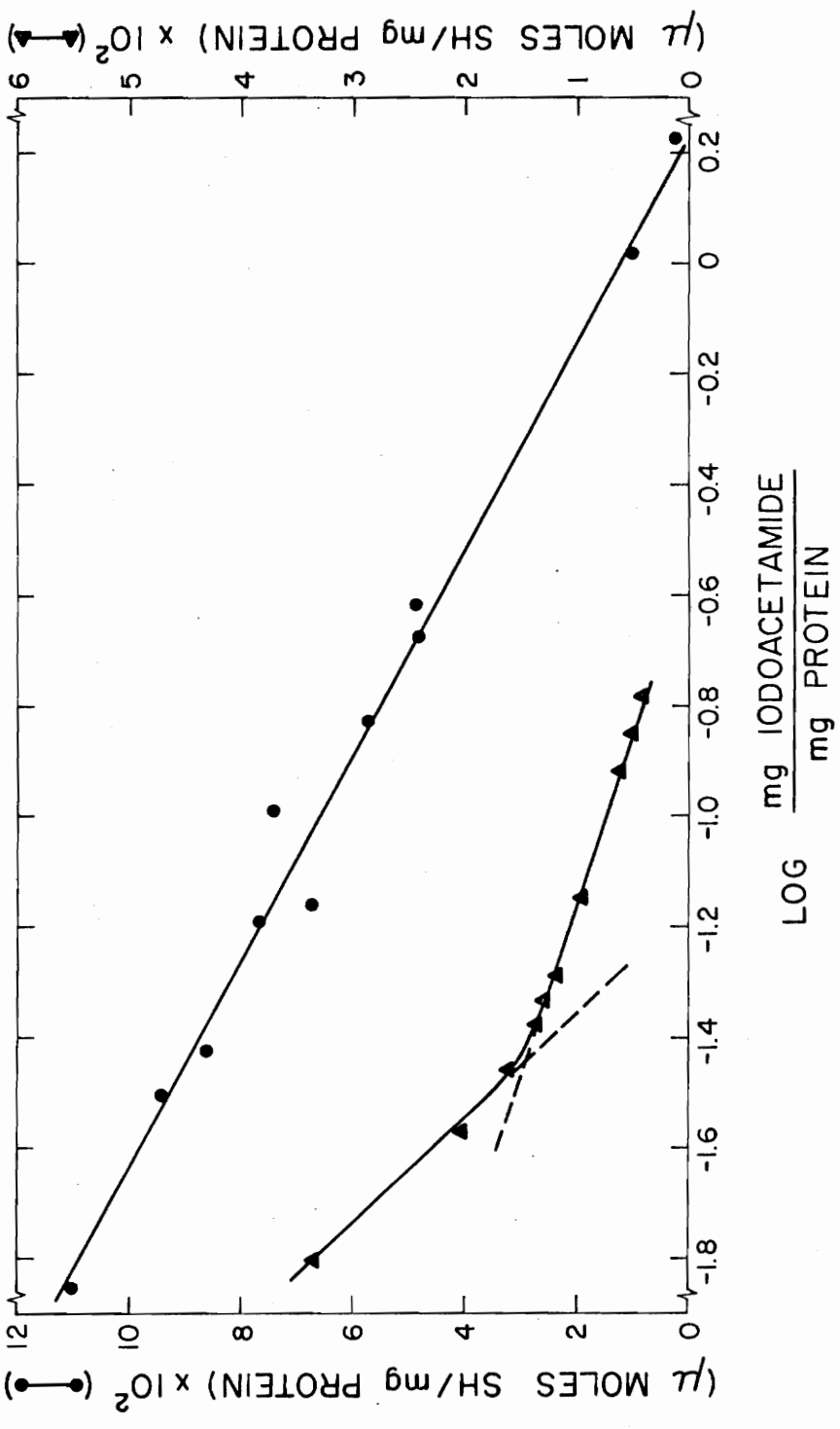
FIGURE 7

Incorporation of ^{14}C–iodoacetamide into lens crystallin fractions

Two ml (containing 23.6 mg soluble lens protein) of a 24,000 × g supernate of lenses homogenized in a 5 mg/ml ^{14}C–iodoacetamide solution (specific activity 1.95 μCi/mg iodoacetamide) and reacted for 1 hr at 25°C pH 8 was layered on the 1.5 × 90 cm Sephacryl S–200 column and eluted with 0.065 M Tris–Cl, 0.05 M NaCl, 0.001 M EDTA, pH 7.6, at a flow rate of 0.8 ml/min. Radioactivity (–o–o–o–o–o–o–); absorbance at 280 nm (————————).
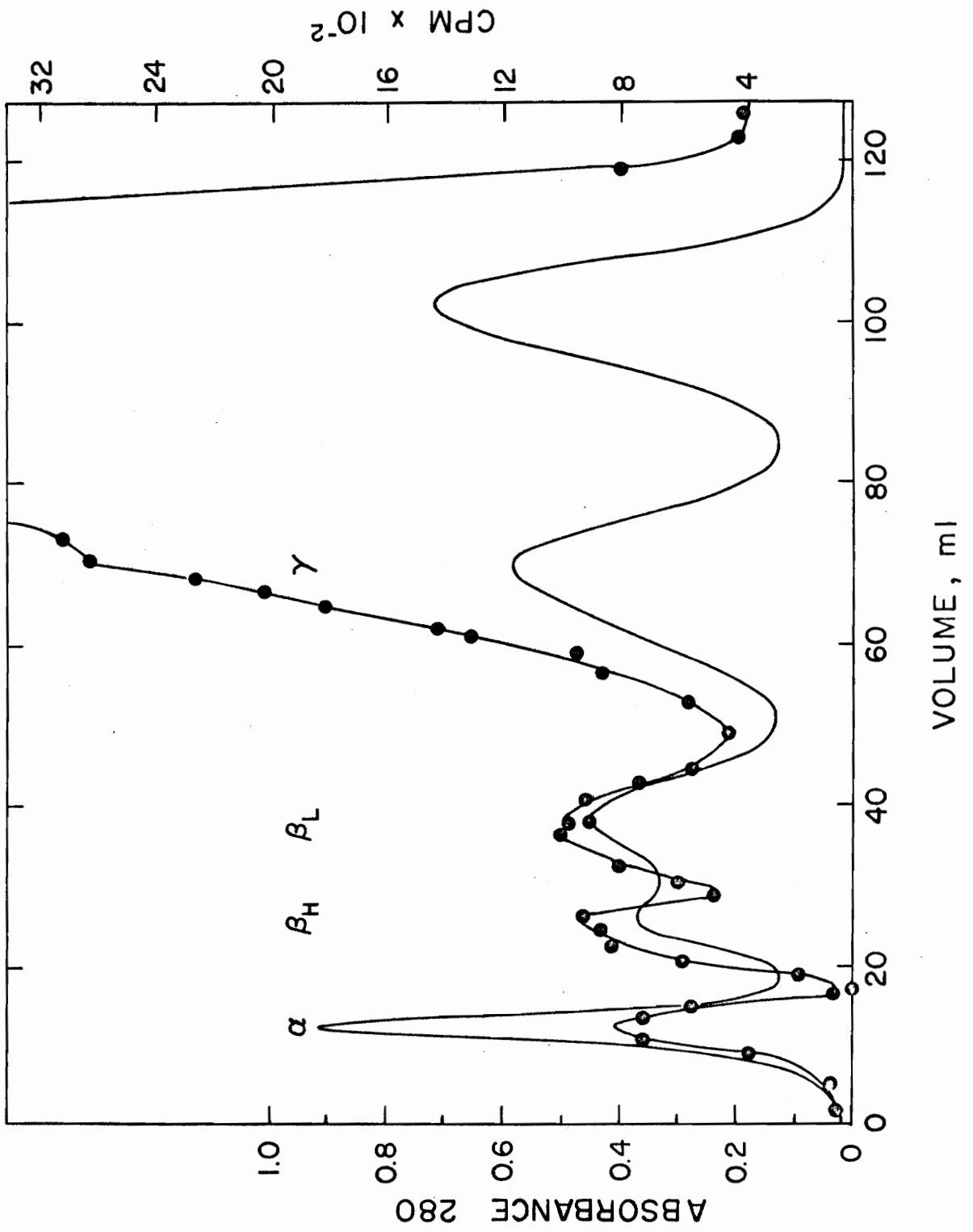
Electrophoretic and Molecular Size Properties
of Alkylated and Non-Alkylated
Crystallin Classes

Co-chromatography Experiments — At this point in the research, it
was evident that in order to clearly define what was happening to
individual crystallin fractions (particularly β_H-crystallin) during
alkylation, it was necessary to obtain each crystallin aggregate
uncontaminated by the others. This purification was accomplished by
selectively pooling fractions from a Sephacryl S-200 column of a lens
homogenate from 21 lenses.

Purified crystallins were alkylated and rechromatographed, but
such small amounts of purified protein were available to put on the
column that the resultant profiles were not clear. Limited protein
made peaks small and midpoints difficult to locate, particularly in
the β-crystallin region. Results indicated that α-crystallin did
not change in molecular weight with alkylation and that β_H-crystallin
did shift towards β_L-crystallin; however, in these experiments β_L-
crystallin appeared to shift slightly towards a lower molecular
weight. It was not possible to evaluate alkylated γ-crystallin, as it
precipitated out of solution at pH 8.0, probably due to an isoelectric
precipitation.

To clarify the situation, β_H- and β_L-crystallin fractions were
alkylated with radioactive iodoacetamide at a 1 mg iodoacetamide/mg
protein ratio, and co-chromatographed with non-alkylated lens homogenate.
The results of these experiments are presented in Figures 8 and 9.
Alkylated β_L-crystallin co-chromatographed exactly with β_L-crystallin

FIGURE 8

Co-chromatography of non-alkylated soluble lens
crystallins with ^{14}C-alkylated β_H-crystallin

A radioactive, alkylated β_H-crystallin sample
($\simeq$ 1 mg) was combined with 1.9 ml of a 24,000 × g
supernate from lenses ground in 0.01 M Tris-Cl
buffer, pH 8.0 (containing 25.4 mg soluble lens
protein); loaded on a 1.5 × 90 cm Sephacryl S-200
column, and eluted with 0.65 M Tris-Cl, 0.050 NaCl,
0.001 M EDTA, pH 7.6 at a flow rate of 0.8 ml/min.
(————) Absorbance at 280 nm. (-o-o-o-o-) radioac-
tivity. Total sample volume was 3.4 ml.

FIGURE 9

Co-chromatography of non-alkylated soluble lens
crystallins with ^{14}C-alkylated β_L-crystallin

A radioactive, alkylated β_L-crystallin sample
($\simeq$ 1 mg) was combined with 1.9 ml of a 24,000 × g
supernate from lenses ground in 0.01 M Tris-Cl
buffer, pH 8.0 (containing 25.4 mg soluble lens
protein); loaded on a 1.5 × 90 cm Sephacryl S-200
column, and eluted with 0.65 M Tris-Cl, 0.050 NaCl,
0.001 M EDTA, pH 7.6 at a flow rate of 0.8 ml/min.
(————) Absorbance at 280 nm. (-•-○-○-•-○)
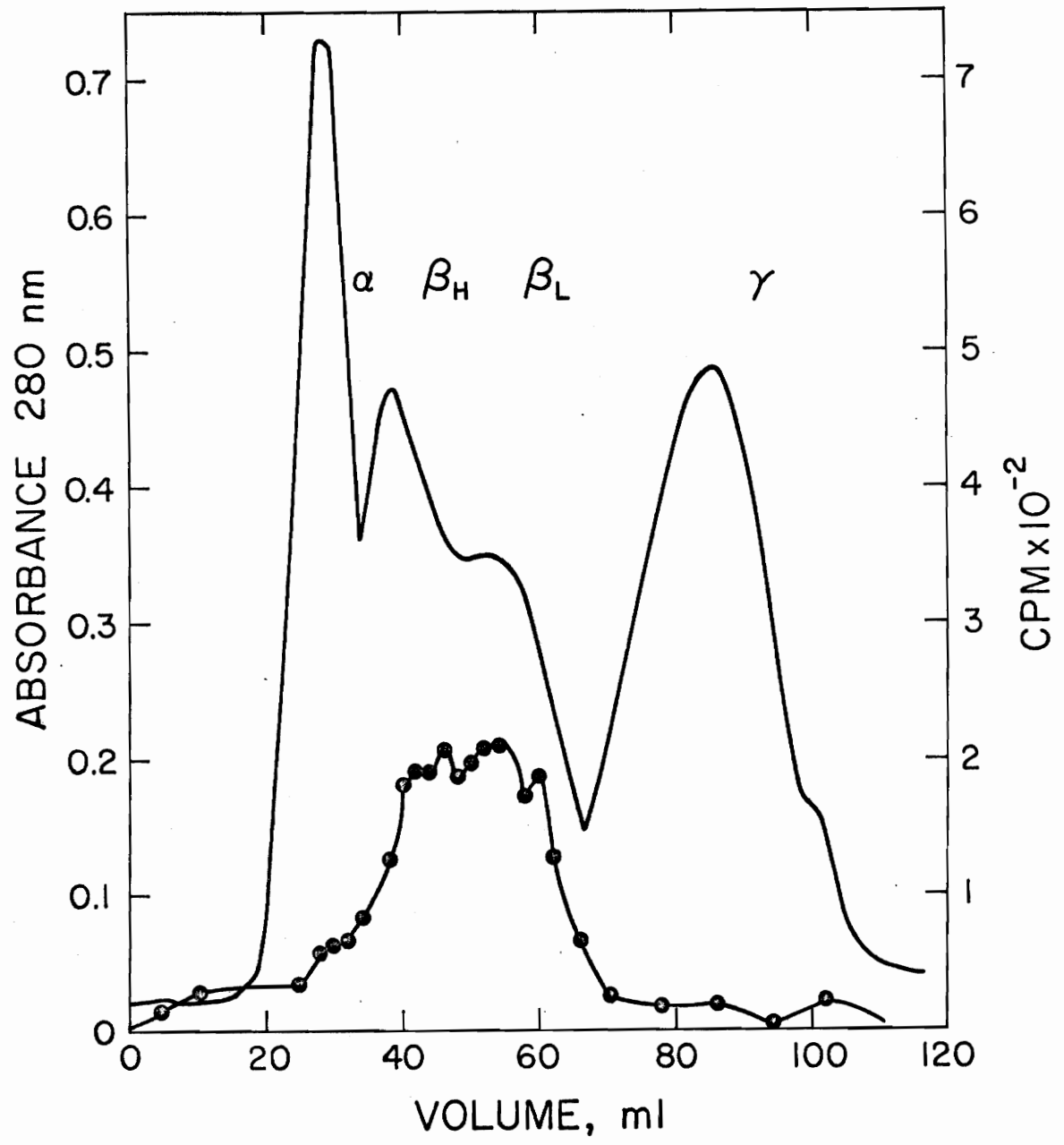radioactivity. Total sample volume was 3.6 ml.
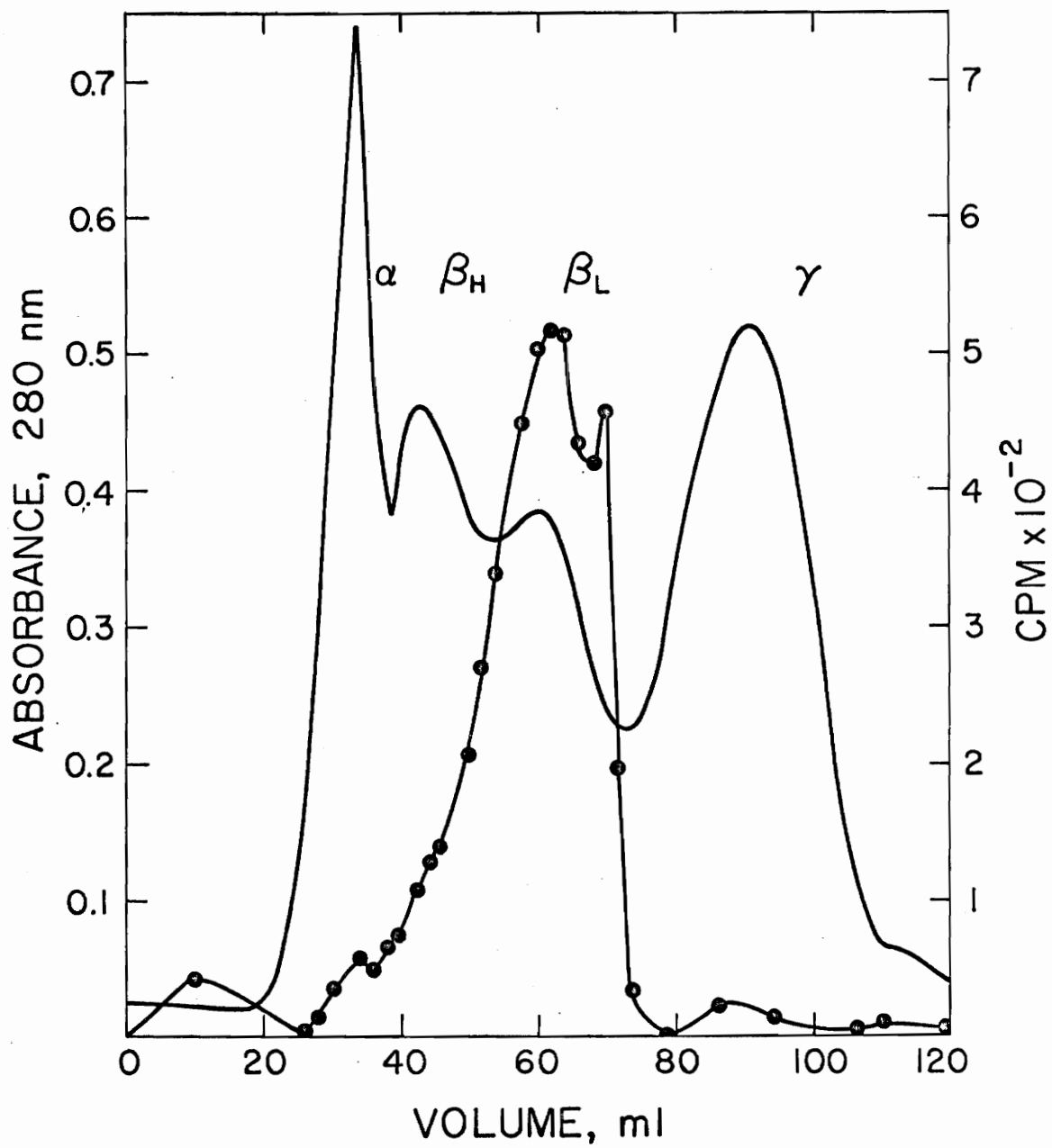
peak. However, alkylated β_H-crystallin has shifted with respect to β_H-crystallin, also co-chromatographing with β_L-crystallin.

Isoelectric Focusing — To detect any differences in charge of the lens proteins with alkylation, isoelectric focusing was performed on total lens preparations and separated crystallin fractions. A typical linear pH gradient of a focused slab gel is shown in Figure 10. Isoelectric focusing on total crystallin preparations at various concentrations of iodoacetamide can be seen in Figure 11. Observed in the alkylated samples were two new bands with isoelectric points higher than those of any polypeptides in the control that became more prevalent with increased degree of alkylation.

Isoelectric focusing of individual crystallins (Figure 12) revealed no major changes in isoelectric point with alkylation of α- and β_L-crystallin (Figure 12, B and F) but a slight pattern change in β_H-crystallin and the appearance of one, possibly two, bands with higher isoelectric point than any of control β_H-polypeptides (Figure 12, D).

SDS Gels — To determine whether any molecular weight changes had occurred with alkylation, a 15% acrylamide slab gel was prepared for which protein samples had been incubated at 37°C for 1 hr in 1% SDS. The resultant pattern can be seen in Figure 13. No large molecular weight changes are seen with aklylation, although subtle shifts in molecular weight with alkylation are observed, resulting in clearer and more numerous bands.

FIGURE 10

pH gradient of a typical isoelectric focused slab gel

pH was plotted as a function of the distance from the anode by
eluting successive 5 × 10 mm pieces of the gel with carbon dioxide-free
distilled water, and measuring the pH.
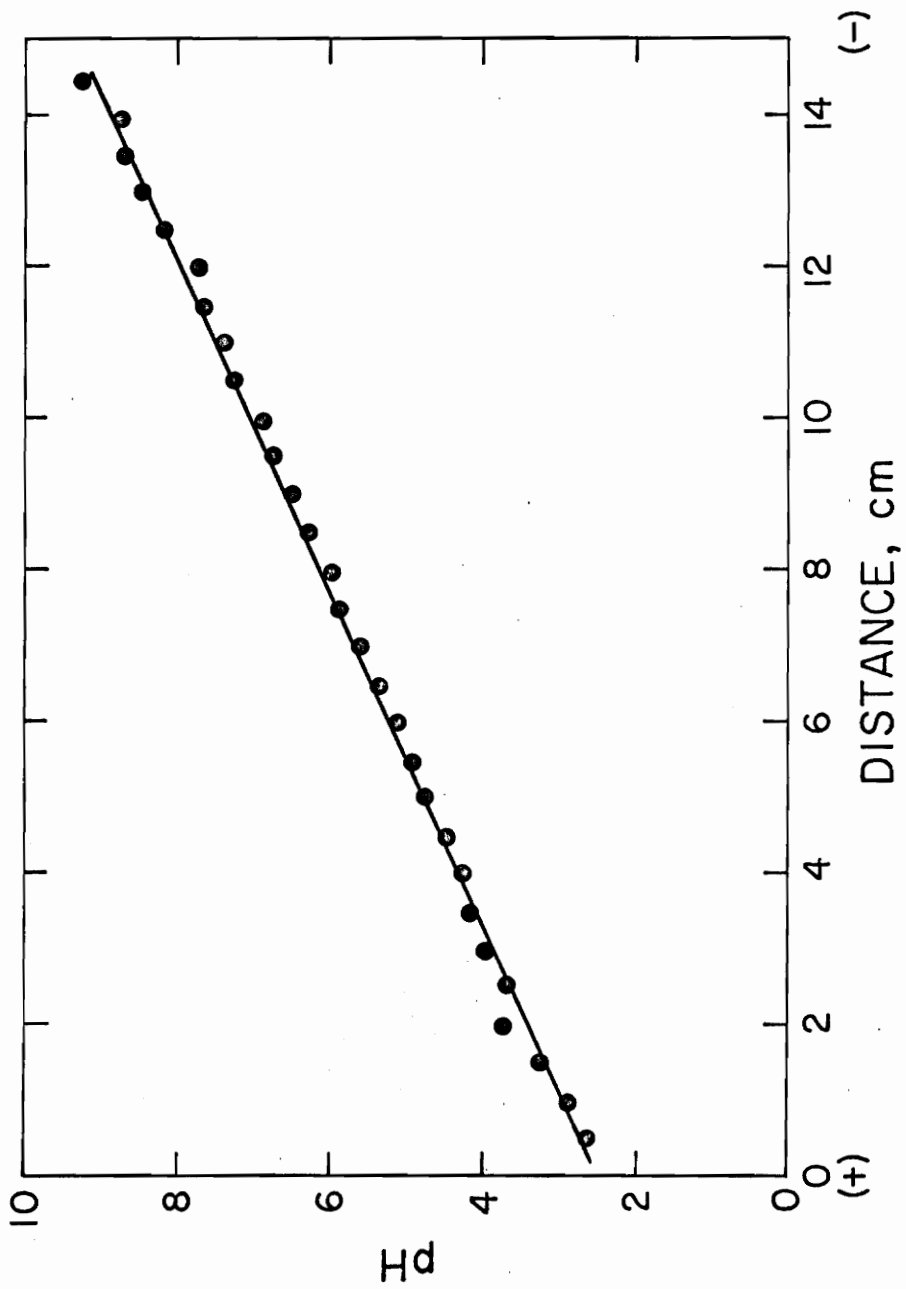
FIGURE 11

Isoelectric focusing of total lens homogenates
alkylated with increasing levels
of iodoacetamide

    Lenses were homogenized in 3 solutions containing
0, 1, or 10 mg iodoacetamide/ml 0.01 M Tris-Cl, pH 8.0.
15 µl samples containing 205, 173, and 169 µg of protein,
respectively, were applied at the anode end of the 7.5%
polyacrylamide gel (1.5 × 80 × 160 mm) containing 2%
Biolyte 3-10 ampholytes. Power was increased from 300
to 1,000 volts over a 1-1/2 hr period and run at 1,000
volts for an additional 1-1/2 hr. The gel was stained
with a Coomassie R-250 solution. Anode electrolyte,
0.06H H_2SO_4; cathode electrolyte, 0.02N $Ca(OH_2)$ and
0.04N NaOH.

mg Iodoacetamide/mg Protein
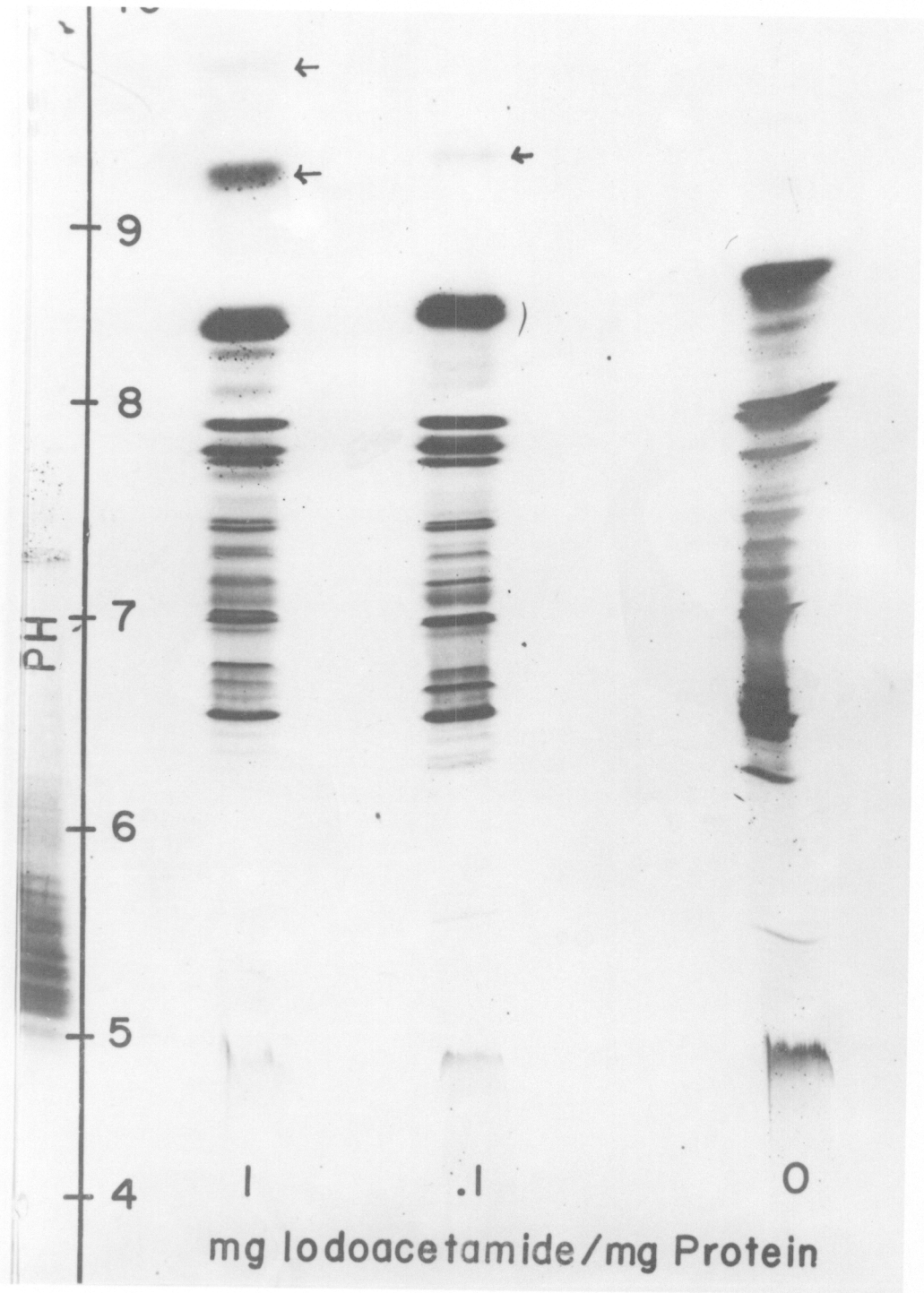
FIGURE 12

Isoelectric focusing of purified lens
crystallin fractions

7.5% Polyacrylamide slab gel (7.5%, 1.5 × 80 × 160 mm) containing 2% Biolyte 3-10 ampholytes. Samples were applied 3 cm from the anode end. Power was increased from 300 to 1,000 volts over a 1-1/2 hr period, and run at 1,000 volts for an additional 1-1/2 hr. The gel was stained using a Coomassie R-250 solution. Anode electrolyte, 0.06N H_2SO_4. Cathode electrolyte, 0.02N $Ca(OH_2)$ and 0.04N NaOH. (A) 75 μg of α-crystallin; (B) 60 μg of alkylated α-crystallin; (C) 78 μg of $β_H$-crystallin; (D) 84 μg of alkylated $β_H$-crystallin; (E) 66 μg of $β_L$-crystallin; (F) 66 μg of alkylated $β_L$-crystallin; (G) 72 μg of γ-crystallin.
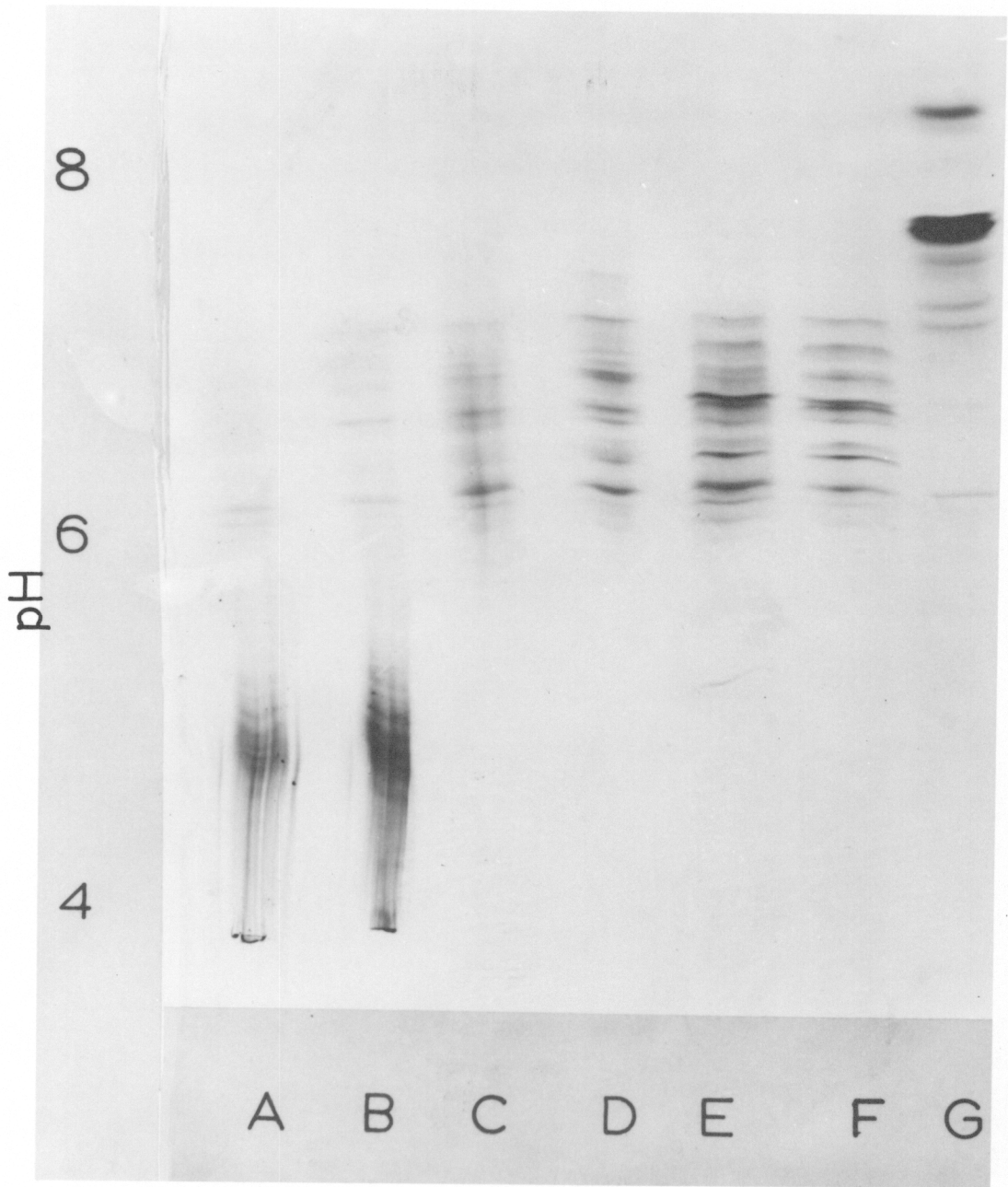
FIGURE 13

SDS polyacrylamide gel electrophoresis of purified lens crystallin fractions

Purified lens crystallin fractions that were 1% SDS and 20% sucrose were incubated for 1 hr at 37°C, and samples were layered into the sample wells of the stacking gel. The gel was run at 0.6 ma/cm^2 until the tracking dye entered the separating gel, and then at 1.3 ma/cm^2 for the remainder of the run (Total running time, 3 hr). Staining was performed using Coomassie blue R-250. Stacking gel 3% acrylamide; separating gel 15% acrylamide. (A) 72 μg of α-crystallin; (B) 60 μg of alkylated α-crystallin; (C) 78 μg of β_H-crystallin; (D) 84 μg of alkylated β_H-crystallin; (E) 66 μg of β_L-crystallin; (F) 66 μg of alkylated β_L-crystallin; (G) 72 μg of γ-crystallin. Molecular weights were estimated from α-crystallin subunit and ovalbumin standard (1) 20,000 daltons; (2) 37,000 daltons; (3) 43,000 daltons.
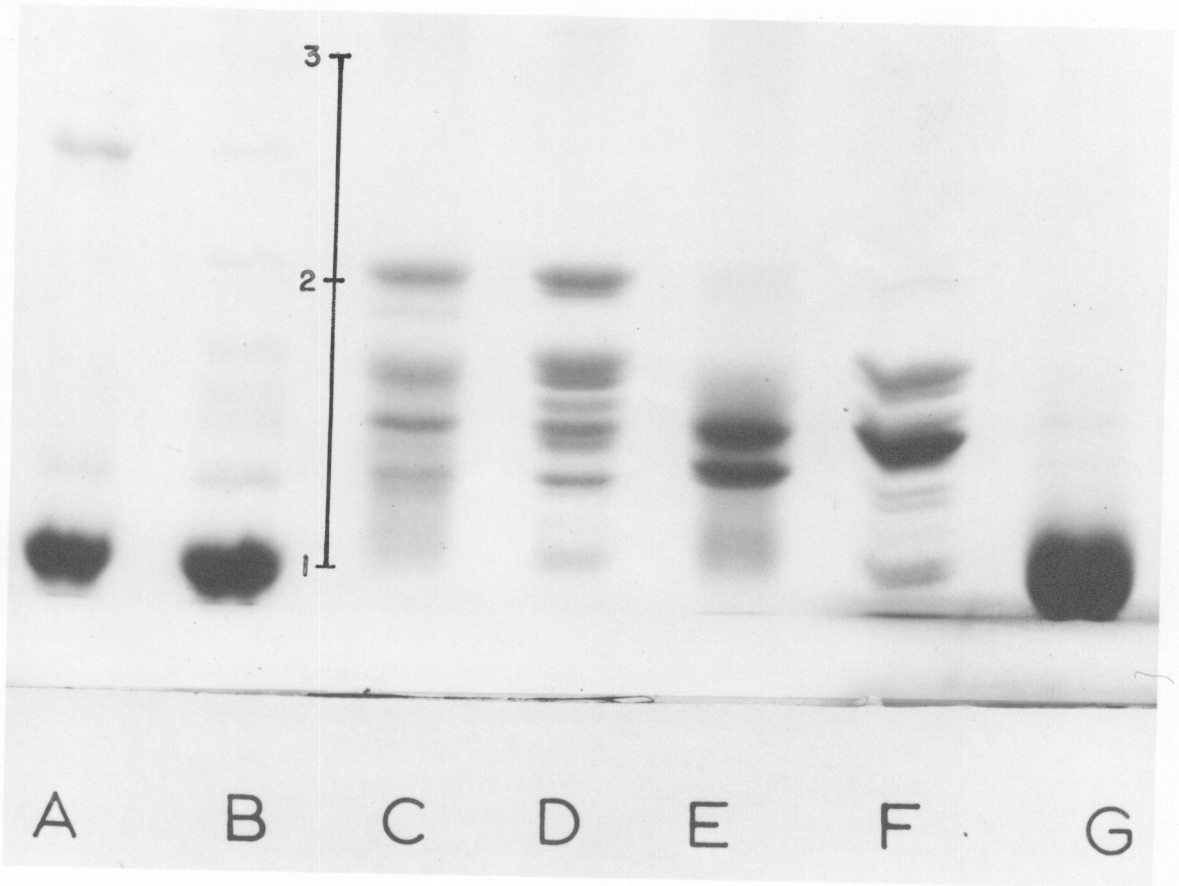
Amino Acid Analysis

Amino acid analysis of alkylated and non-alkylated crystallins
showed a total loss of histidine in alkylated α- and β_H-crystallin
samples and the appearnace of a number of new peaks, eluting just
before aspartate (Tables II and III). This loss only occurred in
samples left in the presence of iodoacetamide for about a week
before analysis. Analysis of a carboxymethylhistidine standard (see
methods) resulted in three peaks with elution times of 51.9, 54.3,
and 57.4 that corresponded to unidentified peaks seen in α- and
β_H-crystallin. These first three peaks probably correspond to
1,3-carboxymethylhistidine, although the exact elution time of this
derivative has not been determined. Two other major peaks occurred
in the carboxymethylhistidine standard; one peak co-chromatographing
with glycine and one peak corresponding to 3-carboxymethylhistidine
coinciding with cystine.

Unfortunately, in samples containing carboxymethylhistidine, it
was impossible to detect the presence of carboxymethylcysteine, as the
carboxymethylcysteine standard also has a major peak eluting at 51.9 min
and a minor peak at 55.3 min. Like carboxymethylhistidine, carboxy-
methylcysteine has a major peak co-chromatographing with glycine and a
minor peak co-chromatographing with serine. The large peaks
co-chromatographing with glycine are probably due to excess iodoacetamide
for the following reasons. (1) The literature reports only one peak for
carboxymethylated cysteine, eluting before aspartate; (2) carboxymethyl-
cysteine and carboxymethylhistidine share a very large unidentified
peak; and (3) excess iodoacetamide was not removed for these determinations.

TABLE II

Amino acid composition of α-, α(alkylated)-,
and γ-crystallin

Amino Acid	α	α(alkylated)[a]	γ
Lysine	4.7	2.3	1.8
Histidine	4.2	0.0	4.1
Arginine	8.5	8.5	14.0
Unidentified	<1.0[b]	4.0[c]	1.2[b]
Aspartic Acid	10.5	10.2	13.0
Threonine	3.5	3.6	2.9
Serine	9.8	10.0	6.6
Glutamic Acid	11.0	11.3	11.7
Proline	6.5	6.6	3.9
Glycine	7.2	7.1	8.8
Alanine	4.3	4.3	2.4
Half cystine	0.0	<1[d]	4.8
Valine	6.5	6.8	4.8
Methionine	0.7	0.9	1.3
Isoteucine	3.7	3.9	3.0
Leucine	8.5	8.9	6.4
Tyrosine	2.9	3.2	8.5
Phenylalanine	7.4	7.7	5.8

NOTE: Values are expressed as mole percent.

[a]Sample left in the presence of iodoacetamide for about 1 week before analysis.

[b]Probably cysteic acid at elution time 51.9 (min).

[c]Five unidentified peaks with elution times (min)of 51.2, 55.2, 53.7, 58.3, and 63.9.

[d]Probably corresponds to 3-carboxymethylhistidine, not half cystine.

TABLE III

Amino acid composition of β_H-, β_H(alkylated)-, β_L-, and β_L(alkylated)-crystallin

Amino Acid	β_H	β_H(alkylated)[a]	β_H(alkylated)	β_L	β_L(alkylated)
Lysine	4.1	3.7	4.7	5.5	5.3
Histidine	2.7	0.0	3.0	3.8	3.7
Arginine	7.3	7.8	8.7	6.3	7.7
Unidentified	0.9[b]	7.2[c]	1.9[d]	1.3[e]	1.3[f]
Asparatic Acid	10.0	9.0	9.0	10.6	10.0
Threonine	4.0	4.3	3.9	3.7	3.8
Serine	6.6	7.2	6.8	7.2	7.7
Glutamic Acid	14.7	13.7	14.4	15.9	14.5
Proline	6.4	6.1	5.0	6.7	5.3
Glycine	10.1	11.5	10.2	9.7	10.3
Alanine	6.3	6.0	6.0	5.4	5.4
Half Cystine	0.0	<1.0[c]	0.0	0.0	0.0
Valine	6.2	6.0	6.1	5.5	5.6
Methionine	0.9	0.9	0.9	0.8	0.6
Isoleucine	4.4	3.9	3.8	4.2	4.4
Leucine	6.8	6.3	6.0	5.3	5.5
Tyrosine	3.2	1.3	2.8	3.8	4.0
Phenylalanine	5.2	5.3	5.2	4.2	4.8

NOTE: Values are expressed as mole percent.

[a]Sample left in the presence of iodoacetamide for about 1 week before analysis.

[b]Three unidentified peaks with elution times (min) of 51.2, 54.9, and 61.4.

[c]Probably 3-carboxymethyl histidine, not half-cystine.

[d]Four unidentified peaks with elution times (min) of 51.5, 54.5, 58.0, and 73.3.

[e]Probably cysteic acid at elution time 51.8 (min).

[f]Two unidentified peaks with elution times (min) of 51.9 and 71.9.

Alkylated samples that had excess iodoacetamide removed by chromatography or dialysis did not show a decrease in histidine (β_H-(alkylated), β_L-(alkylated) crystallins, Table III), but they did have peaks corresponding to carboxymethylcysteine confirming that alkylation of cysteine residues had taken place with alkylation.

Alkylated and non-alkylated β_H- and β_L-crystallin fractions were analyzed for cysteic acid to determine if the alkylated samples decreased in cysteic acid due to the conversion of cysteine to carboxymethylcysteine. However, it was not possible to distinguish between the two because carboxymethylcysteine and cysteic acid have the same or very similar elution times.

DISCUSSION

Gel Filtration Studies

Elution profiles of alkylated vs. control preparations of soluble

lens protein (Figures 3 and 4) reveal complete loss of the β_H-crystallin

peak. Results obtained with Sephadex G-200 on control and alkylated

preparations were reproducible using Sephacryl S-200, indicating that

loss of β_H-crystallin is not due to a preferential binding with the

Sephadex gel.

Controls used for comparison of alkylated and non-alkylated

crystallins were always freshly prepared lens homogenates immediately

applied to columns for separation. If aged or stored at 5°C or less

than 0°C, although all four crystallins are present, resolution of

peaks is poor.

Loss of β_H-crystallin appears to be age-independent as lenses

from both young (Figure 4) and old (Figure 3) rats produced the same

result. However, experience proved that in order to completely

eliminate the β_H-crystallin peak, approximately a 50 millimolar excess

of iodoacetamide had to be used. This excess was achieved when the

mg iodoacetamide/mg protein ratio equaled one. Levels below this ratio

resulted in an intermediate loss of β_H-crystallin, with remaining

β_H-crystallin appearing to shift towards β_L-crystallin, often forming

a shoulder on the β_L-crystallin peak (Figure 7). This shift towards

an apparent lower molecular weight could be explained in two ways: that

the β_H-crystallin aggergate had broken down to form a new aggregate

of lower molecular weight, or that the β_H-crystallin is eluting

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later because of a change in shape or charge, retarding its progress through the gel.

Soluble and Insoluble Protein

With the loss of a major class of soluble proteins, one may hypothesize that this class has become insoluble. Two experiments rule out this possibility. First, percent insoluble protein does not increase with alkylation (Table I). Although there is a standard deviation of 2%, β_H-crystallin represents approximately 10% of the total soluble protein and a transfer from soluble to insoluble protein would be detected within experimental error. Second, one would expect that if β_H-crystallin were being converted to insoluble material that it would precipitate out of solution at a threshold level of iodoacetamide rather than being converted in stages as is indicated by samples with only partial loss of β_H-crystallin (Figure 7). Also, no precipitation is observed when purified samples of β_H-crystallin are analyzed.

Sulfhydryl Measurements

Measurement of exposed sulfhydryl in non-denaturing solvent (Figure 5) revealed that most of the sulfhydryl had been alkylated at a ratio of 0.16 mg iodoacetamide/mg protein. However, this ratio was not sufficient to cause disappearance of β_H-crystallin, indicating that alkylation of exposed sulfhydryl was not the only factor resulting in loss of β_H-crystallin.

When lens crystallins are denatured with guanidine-hydrochloride any buried thiol should be exposed for reaction with Ellman's reagent,

DTNB. At low levels of iodoacetamide, samples analyzed for sulfhydryl in the presence of guanidine contained approximately 6 μm-SH/mg protein of buried sulfhydryl (Figure 5), as indicated by the increase in total sulfhydryl at each of the alkylation ratios. After the exposed sulfhydryl had been alkylated (mg iodoacetamide/mg protein = 0.16), remaining buried thiol continued to be alkylated up to a ratio of 3 mg iodoacetamide/mg protein (Figure 6). Alkylation of previously buried sulfhydryl groups indicates that unfolding of the lens crystallins has occurred, exposing sulfhydryl groups.

Sulfhydryl data plotted on a log-linear scale (Figure 6) reveal a biphasic curve for the exposed sulfhydryl. The biphasic nature could mean that only very reactive sulfhydryl are alkylated up to a certain iodoacetamide/protein ratio, after which a less reactive class became available for alkylation, perhaps related to a conformational change in the protein. We interpret intersection of the two slopes to be the threshold point of alkylating this second class of sulfhydryl. Plotting the guanidine-exposed sulfhydryl (Figure 6) in the same manner gives a linear response, implying that at high levels of iodoacetamide, the lens protein has unfolded and reactivity of the buried thiol is directly dependent on the amount of iodoacetamide present.

To evaluate the relative reactivity of iodoacetamide with individual crystallin populations, alkylation with ^{14}C-iodoacetamide was performed. The elution profile of the alkylated protein with corresponding counts per minute (Figure 7) shows that alkylation follows the sulfhydryl population of each crystallin as reported in the literature (4), with

α-crystallin containing the least sulfhydryl, β-crystallin an intermediate amount, and γ-crystallin containing the most. Therefore, the alkylation of sulfhydryl is not specific for any one of the crystallins.

If loss of β_H-crystallin were due only to the alkylation of its sulfhydryl population, the other crystallins might be expected to exhibit similar changes, particularly β_L- and γ-crystallin which contain at least as high a sulfhydryl content as β_H-crystallin. The β_H-crystallin aggregate must contain unique properties that make it particularly susceptible to environmental changes. Other studies suggest the uniqueness of β_H-crystallin; for example, the presence of β_H-crystallin is sensitive to low tryptophan diets in rats (1), concentration of sucrose in homogenizing media (46), incubation of lenses in glucose-gree medium followed by exposure to cold (47), and galactose cataract (48). In another example, when the lens soluble protein fraction is dissociated with urea, β_H-crystallin is the only crystallin fraction that does not reaggregate unless very specific conditions are met (28). Similarly, only β_H-crystallin is not synthesized in a cell-free synthesizing system, and it is hypothesized to be formed by a post-synthetic modification of the β_L-crystallin (29).

Co-chromatography

Figures 8 and 9 show that alkylated β_L-crystallin co-chromatographs with non-alkylated β_L-crystallin. In contrast, β_H-crystallin shifts to an apparent lower molecular weight, co-chromatographing with β_L-crystallin. Alkylation of β_H-crystallin is not complete, i.e., some alkylated

β_H-crystallin is co-chromatographing with control β_H-crystallin, perhaps because of the long length of time (two days) before purified β_H-crystallin could be alkylated. This experiment confirms, however, that β_H-crystallin is not lost as insoluble material but co-chromatographs with β_L-crystallin.

Isoelectric Focusing

Since β_H- and β_L-crystallins have similar polypeptide composition (20), it is tempting to hypothesize that β_H-crystallin has been converted to β_L-crystallin by the loss of specific polypeptides with alkylation. Electrophoresis data do not support this theory, however. Isoelectric focusing of alkylated β_H- and β_L-crystallins reveals dissimilar patterns, as do controls (Figure 10), indicating that with alkylation β_H-crystallin is not converted to β_L-crystallin. An interesting difference in alkylated β_H-crystallin sample is the appearance of a band with a higher isoelectric point than any peptides in the control. Apparently, one of the polypeptides has been modified in some way so as to increase its positive charge. If previously buried basic amino acids were exposed during alkylation, net positive charge of the polypeptides could increase. Additionally, if iodoacetamide reacted with free sulfhydryl, which exists as the thiol anion (31), the total positive charge of the polypeptide would increase. Another possibility is that an aggregate of two or more polypeptide chains has been broken down into its component parts, one of which has a high isoelectric point.

Isoelectric focusing of unfractionated lens preparations also shows the appearance of new bands with high isoelectric points (see arrows, Figure 9) that increase with high ratios of iodoacetamide. These bands do not correspond to any of the isolated crystallin bands and are probably due to changes in the γ-crystallin with alkylation. Alkylation of γ-crystallin is not possible because purified γ-crystallin precipitates at pH 8. One can examine γ-crystallin by separation from an alkylated whole lens homogenate, but this fractionation was not attempted here.

SDS Polyacrylamide Gel Electrophoresis

SDS gels (Figures 13) revealed only small changes in molecular weight of the alkylated samples. The alkylated samples in general have an increased number and clearer bands, perhaps by eliminating artifactual inter-peptide disulfide linkages. That changes with alkylation are seen indicates probable rearrangement of polypeptides in the aggregates, ultimately characterized as β_H- or β_L-crystallin. Data, however, do not permit explanation of the unique gel filtration behavior of alkylated β_H-crystallin.

Of course, even a minor change in characteristics of one of the crystallins could drastically affect the arrangement of fibers within the lens itself; the resulting disturbance of the uniformity of refractive index would cause opacity (3), so perhaps small changes in molecular weight and charge are significant.

Amino Acid Analysis

Amino acid composition of crystallin fractions was determined to discern whether any amino acids other than cysteine were being alkylated. As expected from the sulfhydryl data, amino acid analysis of acid-hydrolyzed samples (Tables II and III) indicated presence of carboxymethylcysteine. Alpha- and β_H-crystallins stored in the presence of iodoacetamide for approximately one week showed complete loss of histidine and appearance of peaks corresponding to 3-carboxymethyl-, and 1,3-carboxymethylhistidine; but this reaction was not important in the normal alkylation reaction, since samples were routinely separated from iodoacetamide by column chromatography immediately following alkylation. When these alkylated samples were analyzed, very small peaks corresponding to carboxymethylhistidine were observed, but lost histidine was impossible to estimate as compared to control samples. Possibly reaction with a specific histidine is necessary for the unfolding of β_H-crystallin but total loss of histidine was not necessary for the altered gel filtration properties of β_H-crystallin.

Samples were analyzed for cysteine by performic acid oxidation, but as cysteic acid and carboxymethylcysteine elute together, it was impoysible to observe loss of cysteic acid residues and the appearance of carboxymethylcysteine in alkylated samples.

Model for Loss of β_H-crystallin

Evidence presented to date has eliminated two of the hypotheses presented earlier for the apparent molecular weight shift of β_H-crystallin, namely that the protein has changed drastically in charge

or molecular weight. A third possibility, that β_H-cryrstallin has changed in shape, is more likely. Electron microscopy data (28) have indicated that β_H-cyrstallin aggregate is globular protein. Conformational changes and unfolding of lens crystallins with high concentrations of iodoacetamide have been indicated by the sulfhydryl data (Figures 5 and 6). Change in shape from a well-ordered, strictly arranged aggregate to a denatured, stretched-out molecule can certainly retard progress through the Sephadex or Sephacryl gel, causing elution at an apparent lower molecular weight. The process bringing about this change is obviously accelerated by iodoacetamide, although the mechanism is not clear.

Similar effects have been observed in polyacrylamide gels. Chrambach and Rodbard (49) have reported that random coils or extended rods will show a greater retardation in polyacrylamide gels than spherical molecules. Fawcett and Morris (50) have indicated that hydrophobic moieties which would likely be exposed with unfolding of protein tend to interact with polyacrylamide causing an increase in retardation.

Although sulfhydryl data (Figure 5) indicate changes in conformation of all the lens protein, only β_H-crystallin shows a marked change in its molecular sieving properties. Perhaps repeating sulfhydryl studies with purified β_H-crystallin would provide insights into its unique behavior.

Future Studies

In order to determine what characteristics of β_H-crystallin make it susceptible to chemical modification, in-depth studies of its

polypeptide subunits need to be performed on both control and alkylated preparations. Because the tryptophan-deficient cataract shows similar loss of β_H-crystallin as seen with alkylation, information obtained in these studies could be used to guide investigation of the tryptophan-deficient cataract in rat lens. Hopefully, some insights could be obtained about cataractogenesis in humans.

SUMMARY

In the tryptophan-deficient cataract, loss of the $_H$-crystallin has been reported (1). This same phenomenon has been described if lenses are alkylated with high levels of iodoacetamide. Study of the alkylation reaction has yielded the following conclusions.

(1) Gel-chromatography of alkylated lens homogenates revealed that the β_H-crystallin was eliminated in both young and old rats only at very high concentrations of iodoacetamide (approximately a 50 mm excess of iodoacetamide to total sulfhydryl, equivalent to a mg iodoacetamide/mg protein ratio of 1.)

(2) Protein analysis of soluble and insoluble preparations indicated that β_H-crystallin was not converted to insoluble material with alkylation (Table I).

(3) Free-sulfhydryl determinations of soluble lens protein at increasing levels of iodoacetamide (Figure 5) demonstrated that the exposed sulfhydryl was essentially alkylated at a 0.16 mg iodoacetamide/mg protein ratio. Buried sulfhydryl remained at a constant level of approximately 6 μm-SH/mg protein until a 1 mg iodoacetamide/mg protein ratio; then this sulfhydryl also became alkylated, implying that unfolding of the lens protein had occurred, exposing previously buried sulfhydryl.

Log-linear plots of free-sulfhydryl at increasing levels of iodoacetamide resulted in a linear response to total sulfhydryl (buried and exposed) but a biphasic response of the exposed sulfhydryl.

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(4) Alkylation with ^{14}C-iodoacetamide demonstrated that the alkylation reaction was not preferential for any one of the crystallins; radioactivity corresponded to the relative sulfhydryl content of each crystallin.

(5) Co-chromatography of soluble lens protein with radioactive β_H- and β_L-crystallin, respectively (Figures 8 and 9), demonstrated that alkylated and non-alkylated β_L-crystallin co-chromatographed but alkylated β_H-crystallin shifted to an apparent lower molecular weight and co-chromatographed with β_L-crystallin.

(6) Isoelectric focusing of isolated crystallin fractions (Figure 12) revealed no changes in isoelectric point with alkylation of α- and β_L-crystallin; alkylated β_H-crystallin had at least one additional band with a higher isoelectric point than any of the non-alkylated β_H-polypeptides.

Focusing of unfractionated lens soluble protein at different levels of iodoacetamide resulted in the appearance of new bands in the alkylated sample (Figure 11). These bands, with isoelectric points above pH 9, did not correspond to any bands seen in the focused patterns of the isolated crystallins (Figure 12). Since alkylated γ-crystallin was the only crystallin not focused (see results), the new bands probably corresponded to the alkylated γ-crystallin.

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ALKYLATION OF RAT LENS CRYSTALLINS

WITH IODOACETAMIDE

by

Linda Rose Haynes

(ABSTRACT)

Alkylation of lens proteins with iodoacetamide during homogenization of tissue (50 millimolar excess) immediately followed by gel-permeation chromatography yielded a crystallin population devoid of β_H-crystallin. This result occurred in lens homogenates from both young (100 g) and older (400 g) male rats. Beta$_H$-crystallin was not converted to insoluble protein with alkylation. Each crystallin fraction reacted with radioactive iodoacetamide in proportion to sulfhydryl content; at a ratio of 1 mg iodoacetamide/ mg protein total free-sulfhydryl of the crystallins had reacted after 1 hr at pH 8, 25°C. Alkylated α-, β_L-, and γ-crystallin fractions demonstrated no altered chromatographic behavior on Sephacryl S-200; only alkylated β_H-crystallin was altered so that it co-chromatographed with control or alkylated β_L-crystallin.

SDS polyacrylamide gels of isolated crystallin fractions revealed small changes in molecular weight; bands were more distinct and increased in number with alkylation. Isoelectric focusing revealed no changes in isoelectric point with alkylation of β_L- and α-crystallin;

alkylated β_H-crystallin had at least one new band with an isoelectric point higher than any polypeptide in the control. Two new bands of high isoelectric point appear with isoelectric focusing of alkylated lens preparations; intensity increased with the degree of alkylation. These bands probably correspond to alkylated γ-crystallin. From the above data, it was concluded that altered gel-permeation characteristics of alkylated β_H-polypeptides were not due to large changes in molecular weight or charge but to an altered conformation of β_H-crystallin.