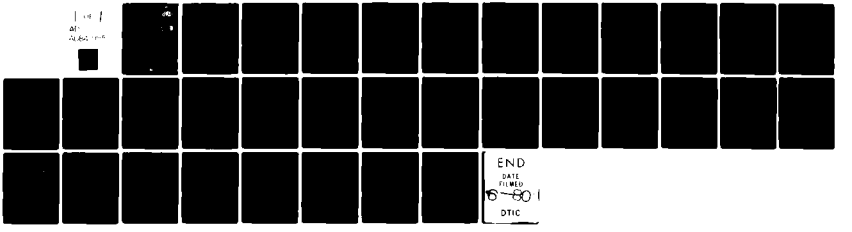
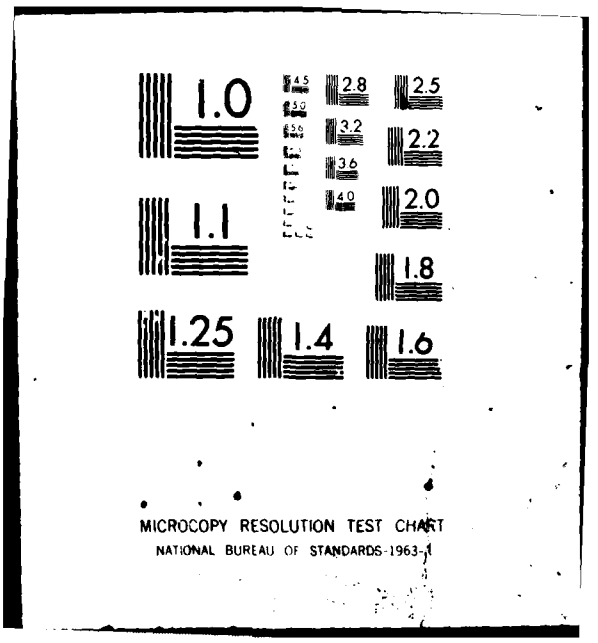
AD-A084 055 DUKE UNIV EYE CENTER DURHAM NC F/G 6/18
THE ORIGIN OF CATARACTS IN THE LENS FROM INFRARED LASER RADIATI--ETC(U)
JUL 77 M L WOLBARSHT, B S YAMANASHI, M A ORR DAMD17-74-C-4133
UNCLASSIFIED NL




1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0
4.5
5.0
5.6
MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS-1963-A

THE ORIGIN OF C[illegible] IN THE [illegible]
FROM INFRARED LASER RADIATION

Annual Progress Report

M. L. Wolbarsht
B. S. Yamanashi
M. A. Orr

Supported by
U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Washington, D.C. 20314

Contract DAMD 17-74-C-4133

Duke University Medical Center
Durham, North Carolina 27710

AD_____

THE ORIGIN OF CATARACTS IN THE LENS FROM INFRARED LASER RADIATION

Annual Progress Report

M. L. Wolbarsht
B. S. Yamanashi
M. A. Orr

Supported by
U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Washington, D.C. 20314

Contract DAMD 17-74-C-4133

Duke University Medical Center
Durham, North Carolina 27710

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
		9

4. TITLE (and Subtitle)
6 THE ORIGIN OF CATARACTS IN THE LENS FROM INFRARED LASER RADIATION

5. TYPE OF REPORT & PERIOD COVERED
Annual Progress Report, 9/1/75 - 8/31/76

6. PERFORMING ORG. REPORT NUMBER
1 Sep 75-31 Aug 76

7. AUTHOR(s)
10 M. L. Wolbarsht
B. S. Yamanashi
M. A. Orr

8. CONTRACT OR GRANT NUMBER(s)
15 DAMD 17-74-C-4133

9. PERFORMING ORGANIZATION NAME AND ADDRESS
Duke University Eye Center
Durham, North Carolina 27710

10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
62772A
16 3E62772A813.00.013 17

11. CONTROLLING OFFICE NAME AND ADDRESS
U.S. Army Medical Research and Development Command, Washington, DC 20314

12. REPORT DATE
7-20-77

13. NUMBER OF PAGES
27

14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)
11 20 Jul 77
12 34

15. SECURITY CLASS. (of this report)
Unclassified

15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)
Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)
cataracts, lens, infrared laser, gel electrophoresis, amino acid, chromatography, rabbit eyes

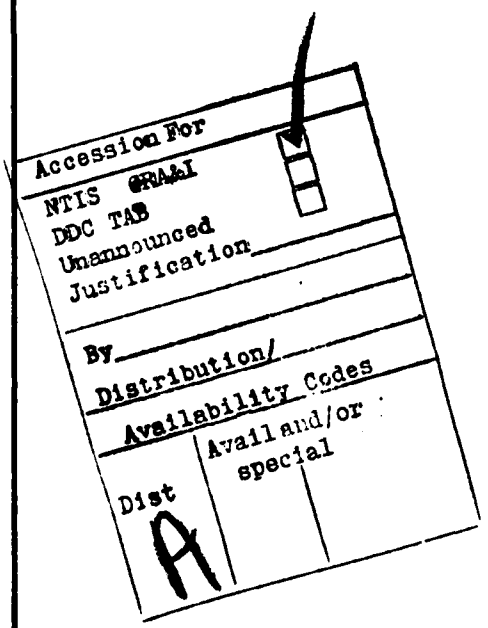
20. ABSTRACT (Continue on reverse side if necessary and identify by block number)
Changes in lens constituents, including proteins, following exposure to laser radiation have been used to detect the earliest changes possible in cataract formation. The results of current experiments are described. Calf, rabbit, rat, pig, and human lenses were incubated at various temperatures between 40-45°C. Purified lens crystallins were also incubated at temperatures from 40-45°C to determine the contribution of thermal elevation to denaturation possibly leading to cataractogenesis. Thin film

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

Unclassified

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

gel electrophoresis, column chromatography, electron probe x-ray microanalysis, and amino acid separation were the analytical methods used on the incubated lenses, as well as lenses from rabbit eyes exposed to a 1.06 micrometers CW neodymium laser *in vivo*.



THE ORIGIN OF CATARACTS IN THE LENS FROM INFRARED LASER RADIATION

M. L. Wolbarsht
B. S. Yamanashi
M. A. Orr

Annual Progress Report
Contract #DAMD 17-74-C-4133
1 September 1975 - 31 August 1976

Supported by
U.S. Army Medical Research and Development Command (SAMRDC)
Office of the Surgeon General
Department of the Army

LIST OF TABLES AND FIGURES

SUMMARY

Changes in lens constituents, including proteins, following exposure to laser radiation have been used to detect the earliest changes possible in cataract formation. The results of current experiments are described. Calf, rabbit, rat, pig, and human lenses were incubated at various temperatures between 40-45°C. Purified lens crystallins were also incubated at temperatures from 40-45°C to determine the contribution of thermal elevation to denaturation possibly leading to cataractogenesis. Thin film gel electrophoresis, column chromatography, electron probe x-ray microanalysis, and amino acid separation were the analytical methods used on the incubated lenses, as well as lenses from rabbit eyes exposed to a 1.06 μ CW neodymium laser *in vivo*.

FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal, Resources, National Academy of Sciences-National Research Council.

TABLE OF CONTENTS

INTRODUCTION

The purpose of this work was to investigate the mechanism of formation of lenticular cataracts following both acute and chronic exposure with infrared laser radiations. The experiments were designed to determine the specific initial interaction of laser radiation in the infrared region with the lens that leads to cataract formation.

Little is definitively known about the mechanism of formation of cataracts following acute exposure to near infrared radiation from lasers. However, cataracts can be formed by chronic exposure to infrared from non-laser sources and it is likely that there are some similarities. This type of cataract is commonly called glass-blower's or steel-puddler's cataract. It is well known that any pathological disturbance of the eye can cause an increase in the susceptibility to any trauma producing cataract, and that some preexisting conditions may render certain individuals more susceptible than others to cataract following chronic exposure to infrared radiation. The variation in exposures leading to cataract from this cause makes analysis of the clinical data quite difficult. However, this type of problem can be overcome under laboratory conditions on animals. Even so, the actual mechanism, thermal or otherwise, has not been clearly formulated in the literature.

One widely accepted theory implicates the absorption of infrared radiation by the pigment of the iris as the major factor. As the temperature of the iris rises from this absorption, the surrounding regions are warmed, especially the anterior surface of the lens which is closely approximated to the iris (10). For exposures lasting a long time, perhaps years, this overheating of the lens could form a cataract. However, the calculated rise of temperature in the iris and anterior portions of the lens from the energy levels of these chronic exposures is very slight, certainly less than 1°C. However, the theory is that this increase in temperature of the anterior portion of the lens (which is the part in contact with the heated iris) will in time produce denaturation of a sufficient amount of lens protein in the anterior lens sutures to initiate cataract. The entire process has been compared with the formation of senile cataract in which there is a loss of ascorbic acid and a decrease in total lens protein and a decrease in the relative amount of soluble protein. This decrease in soluble protein is probably mostly in the α crystallin moiety.

In contrast with this theory involving heat conduction of the iris, a possibility also exists that the infrared radiation is absorbed directly by the lens. Absorption of near infrared by the lens is low, as shown in Fig. 1. Chronic IR exposures would not heat the lens more than that calculated by conduction from the iris, which is certainly less than 1°C. This suggests that if direct absorption by the lens is involved, it is more likely that some specific chemical process is stimulated or suppressed by the absorption of the infrared radiation, rather than some general protein denaturation or inactivation of enzyme systems due to a simple thermal process. It is also possible, although no direct evidence now exists, that absorption of infrared could cause local protein damage with a configurational change. If this protein were itself involved in the production of new proteins then abnormal (miscoded) proteins would be formed. As the turnover rate of proteins in the lens is extremely slow, this process would have a long half life. However even a few molecules of improperly coded protein could lead to a measurable loss of transparency.

The possibility of a non-thermal process in initiating IR cataract formation suggests a similarity between IR cataracts and ultraviolet-induced senile or brunescent cataracts. As the development of the brunescent cataract has been well documented, a review of these mechanisms together with an experimental technique used to elucidate them may be helpful in understanding the use of similar programs on infrared laser cataractogenesis. Brunescent (senile) cataract has long been characterized by a decrease in the total lens protein, mostly in the high molecular weight moiety (5), accompanied by a loss of ascorbic acid and a buildup of glutathione. The changes in lens protein have been examined in more detail and show that the crystallin fraction decreases directly as the insoluble albuminoid protein fraction increases (8). The conversion of the α crystallin to the insoluble albuminoid form does not seem to be the first step in the process. This conversion probably involves a conformational change in the portion of the protein that masks the sulfhydryl group (16) and that a complex may be formed between the soluble α crystallin and the trace amounts of glycoprotein that are present (7). However, similar changes occur in x-ray induced cataracts, UV cataracts, thermally activated glassblower's cataract, and nutritionally induced cataracts such as galactosemia. As this stage is common to all types, a better theory seems to be that different reactions, each specific to the particular type of trauma, act as a trigger for this common and later stage of degradation of the α crystallin.

One possible initial step in ultraviolet cataractogenesis is the photooxidation of tryptophan as indicated by the work of Zigman (34) and Kurzel *et al*. (13). Tryptophan is probably

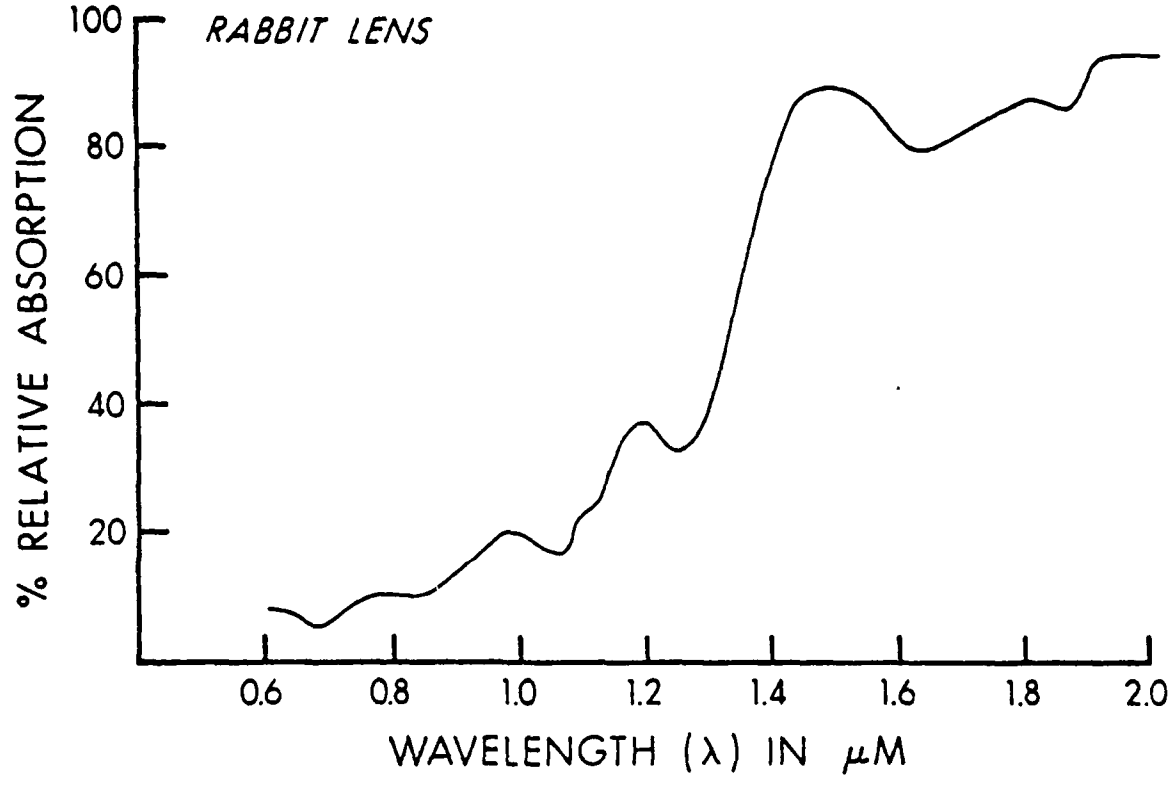


Figure 1. Absorption of rabbit lens. The absorption values are for normally incident light and normal transmitted light and are not corrected for scatter. Adapted from reference 1.

the most susceptible amino acid to UV degradation, as its excited states S_1 and T_1 have extremely low energy compared with all the other chromophoric amino acid residues in the lens protein. Also, the lowest excited state, T_1, of tryptophan, has a relatively long lifetime. Other aromatic amino acids, such as tyrosine, phenylalanine, and histine, which have excited states at higher energy levels than tryptophan, would probably transfer any absorbed energy to the lower energy excited states of tryptophan. This would effectively increase the lifetimes of the tryptophan excited states, maximizing the possibility that some excited state chemical reaction could take place. For this reason, the portion of the protein with tryptophan has the highest probability of acting as an immediate site for secondary chemical reactions such as oxidation.

The only cataracts in which this stage of photooxidation has been documented are acute forms, resulting from short exposures to the higher energies of ultraviolet. However, it has also been established by several groups (14, 6) that these UV-induced cataracts have many points in common with brunescent senile cataracts, which are a chronic and slowly developing form. This supports the hypothesis that brunescent cataracts probably result from chronic exposure to environmental near UV radiation. The usual source is sunlight, although artificial light sources with high level near UV could also act in this way.

The human lens is not normally subjected to radiation longer than 2000 nm or shorter than 293 nm, as the cornea absorbs very strongly in these regions. However, the lens absorbs most of the radiation from 300 to 400 nm and between 1100 and 1400 nm, as shown in Fig. 1. The relative effectiveness of various portions of the near infrared as a function of wavelength for cataractogenesis is not known at present. It seems likely that within the sensitive range the relative quantum efficiency for cataractogenesis has a relation to the photon energy with the higher energy photons the more effective. Most important, however, may be any strong absorption bands in tryptophan.

In brunescent cataracts, the photooxidation of tryptophan proceeds through several degradation steps: kynurenine to 3-hydroxykynurenine and its degradation via further hydrolyzed products of kynurenine. These latter degradation products can be detected by ultraviolet fluorescence techniques (13, 14). Glycoproteins have also been implicated with senile cataract formation (2) and particularly with the glycosidase activity in lenses. This enzyme activity may be specifically related

to the excited state of the glycoside of 3-hydroxykynurenine. Glycoproteins may also act as the source for "active" glucose molecules originally suggested by Spiro (24) and found by Spector (23) as the trigger for the onset of γ crystallin aggregate formation. However, the "active" glucose binds the low molecular weight crystallin components to each other to form the larger albuminoid moieties. Our current research program has sought to identify if a similar chain of degradation products can be connected with infrared cataractogenesis.

PREVIOUS RESULTS

The initial portion of this program distinguished between cataracts formed following heat transfer from the iris and those resulting from direct absorption of infrared radiation by the lens. In these studies both brown pigmented and white unpigmented rabbits were exposed to a CW neodymium laser. In some animals the iris was fully dilated, in others the iris was allowed to contract during the exposure. When the iris was dilated, the laser beam was confined to the lens, while in the undilated cases, a portion of the beam fell directly on the iris with the remainder directly on the lens. Cataracts were formed rapidly when the iris of the brown pigmented rabbits were exposed to a CW neodymium laser with 1 W contained within a 3 mm beam for two periods of 60 sec each, one day apart. Similar power levels of radiation delivered to the lens alone of brown pigmented rabbits, or to the iris or lens of white unpigmented rabbits produced minimal or nonobservable cataracts. Those cataracts formed in the brown pigmented rabbits when the iris was exposed initially appeared directly behind the iris and in generally confined to that region. This supports the model by Goldmann (10) for cataractogenesis by heat conduction from the site of absorption in the iris to the lens.

An analysis of the cataractous portion of the lens behind the iris showed no marked changes in the amino acid concentrations. The overall pattern of the amino acid distributions appeared similar to that in the corresponding portion of a normal lens. This indicates that the mechanism of cataract formation is probably denaturation of protein rather than oxidation or some other specific chemical reaction. A comparison experiment with 60 sec exposures at a 1 W power level in a 3 mm beam produced cataracts in the portion of the lens irradiated directly. An analysis of the cataractous portion of the lens showed no changes in the majority of amino acids. Cysteine, ornothine, and tryptophan were not found at significant levels. Methionine was reduced by a factor of 4 as compared to the normal lens, while the arginine and histidine levels increased by almost an order of magnitude. In the non-

cataractous portions of the lens, from the companion eye, there were also changes in histidine and arginine. Histidine increased by a factor of 4 in both the nucleus and cortex, while arginine increased by a factor of 2 in the cortex, but remained at approximately the same level in the nucleus. Although the power levels of those exposures were quite high, the changes in the lenses may be important features to examine following chronic exposure at lower levels. The amino acids changed detectably but it still seems likely that these were not significant enough to initiate cataractogenesis. As in all cases the laser beam was filtered to avoid contamination from visible or UV pump light. On the basis of these experiments we have inferred that the chief mechanism of cataract formation is most likely protein denaturation.

PRESENT PERIOD

During this period of the contract, exposures from a CW neodymium laser (1.06 μ) were used to produce cataracts with exposures at a single exposure of approximately 1 min at 1.5 W in a 3 mm beam. At these power levels cataracts were formed both in the part of the lens behind the iris and in the part of the lens which was directly exposed. This indicated that there is an intermediate power level which can produce cataracts both by heat conduction from the iris and by direct absorption in the lens itself. Isoelectric focused thin layer gel electrophoresis of all cataractous portions of the lens gave similar types of patterns with an almost complete disappearance of the soluble crystallins (α, β_H, β_L, and γ). This indicates a strong linkage between opacity formation and the complete precipitation of these crystallins. In the clear portions of the cataractous lenses the concentrations of the soluble proteins were almost unchanged except for the β_H crystallin fraction which had a markedly decreased motility. This decrease in motility may well be the initial step in the formation of the insoluble forms of all crystallins. This link is further borne out by similar changes in exposed lenses which did not develop cataracts. These lenses also showed a lowered motility of β_H fraction. Our working hypothesis at the present time is to use a lowering of the motility of β_H crystallin as the earliest indication of cataractogenesis.

Concurrent experiments on the possible direct effect of heat on the lens have been conducted by incubating extracted and homogenized lenses in physiological saline solution at temperatures from 37° to 45°C. The higher temperatures showed marked diminution of the α crystallin fraction. The loss of

the α crystallins, however, were not characteristic of lenses exposed in vivo to laser radiation. This implies either that the mechanism of laser induced cataractogenesis is not purely thermal; or that the intact lens, when heated, has some protective mechanism to prevent the degradation of the α crystallin by heat.

In pilot experiments an analysis of the γ crystallin fraction of lenses showed a marked change in motility following radiation. Incubation with ascorbic acid and glutathione accelerates these changes. This is opposite to the changes found in cataracts induced by near UV radiation which preferentially changes the γ crystallin fraction but does not affect α crystallin fractions. Thus at the present time we can almost certainly say that the IR cataract has an entirely different mechanism of formation than the UV-induced cataract.

The present data indicate that the disappearance of the soluble crystallins is probably by aggregation into insoluble albuminoid forms with a molecular weight greater than 1,000,000. In previous experiments high power level laser exposures initiated cataracts primarily behind the pigmented iris. In the present series of experiments lower power levels gave cataract development from direct absorption by the lens as well as by presumed heat transfer from the pigmented iris. We anticipate that even lower power levels and longer exposure durations will not show any cataract development from heat transfer from the iris. In all these cases cataractogenesis will result solely from direct absorption. Experiments now in progress are designed to find the difference between cataracts developed from long-term exposures, low-level exposures, and short-term high level exposures and between either of those and the cataracts formed in vitro whole lenses incubated at various temperatures between 40° to 45°C.

A comparison line of experiments is also in progress to determine whether in vitro intact lenses have the same changes as the homogenized lenses when incubated at 45°C, or whether they resemble the in vivo lenses following laser exposure. In other experiments the effects of pulsed and CW neodymium lasers on in vivo lenses are compared. In future experiments the possibility of synergism between heat and laser exposures will also be investigated by exposing in vitro whole lenses to CW laser radiation at medium to low power levels while incubating at various temperatures between 40° to 45°C.

An attempt has been made to localize the position and amounts of certain trace elements in cataractous portions of the lens by electron probe x-ray microanalysis. To date

these tests have proved inconclusive; however, a change in technique has shown the possibility of more reliable data and further investigations along these lines are still in progress.

EXPERIMENTAL METHODS: DETAILS OF LASER EXPOSURE TECHNIQUES: RATIONALE OF ANALYTICAL TECHNIQUES FOR DETECTION OF CATARACTOUS CHANGES

Details of In Vivo Exposures

All laser exposures were made with a CW neodymium-YAG laser (Holobeam Model 250). It is a multi-mode CW laser with the majority of its output in a 1.065 μm beam, approximately 3 mm in diameter at the exit port. The beam was enlarged in collimated form to 24 mm by means of an 8X beam expander (Edmund Scientific). In Fig. 2 the laser output is shown as a function of the lamp input power to indicate the reproducibility of the output as a function of the input power. The power input was measured by a Scientech (Boulder, Colorado, Model 360) disc calorimeter which sampled the back beam of the laser. The ratio of the front to back beam of the laser output was measured periodically so that the front beam output could be accurately calculated by measuring the back beam power with an appropriate correction factor. The disc calorimeter was calibrated absolutely by passing a known current through a built-in heating resistor noting the calorimeter output. The measurements of laser power were relatively unaffected by the back reflection from the beam expander which had a constant 10% total air-glass reflection from all the surfaces.

Figure 3 shows the relative position of the eye of the exposed rabbit to the laser. In general, the right eye was exposed with a beam which impinged on the iris. The exposure to the left eye was positioned so that none of the radiation fell on the iris, but passed into the clear portion of the lens. A selected exposure level with a 3 mm beam was given to each eye twice with approximately a minute for each exposure with one day between exposures. Pigmented rabbits were used in most experiments, although some white unpigmented rabbits were used for control runs to indicate whether pigmentation of the rabbit played an important part. No significant differences between white and pigmented rabbits were seen in the lenses from the left eyes which were irradiated alone. However, the differences in the pigmentation of the iris produced lenticular reactions in the two types.

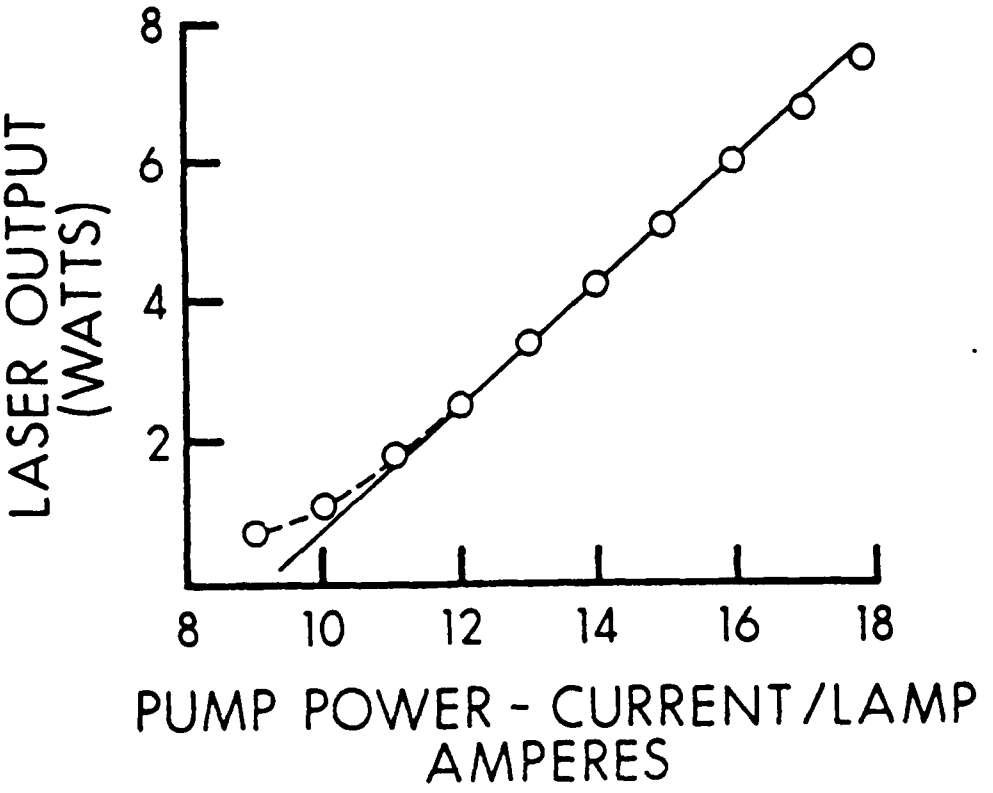


Figure 2. Control of laser power by changes in pump lamp current for Holobeam Model 250. The solid line shows a slope efficiency of 0.465%. Deviations below 2 W output made careful monitoring of the laser output necessary in this region. At higher power levels the departure from linearity was undetectable.

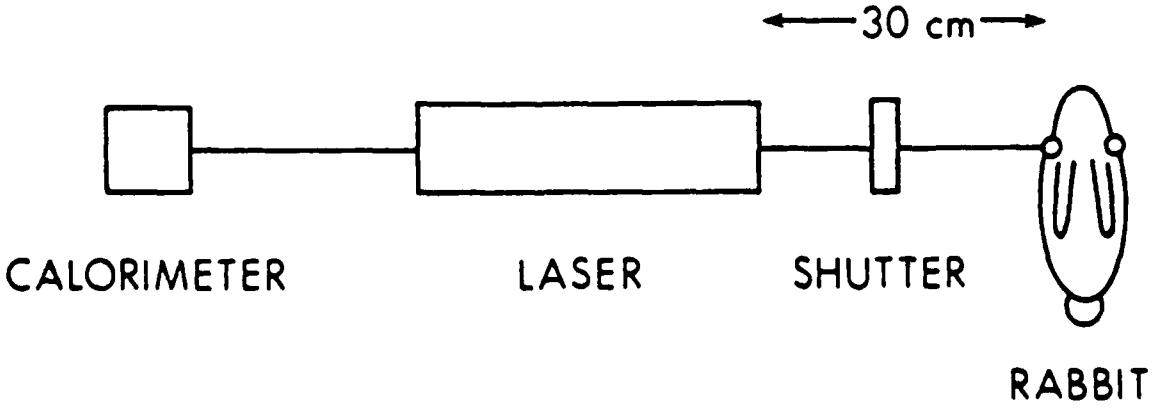


Figure 3. Diagram of laser exposure conditions for a rabbit eye. The animal holder was sufficiently rigid to maintain the animal in a fixed position during the exposure period.

In Vitro Incubation Studies

The lenses from rats, rabbits, pigs, calves, and human eyes (obtained from the Eye Bank) were removed and placed in normal saline solution. Most lenses were homogenized before incubation while some were maintained as structural entities. The range of incubation temperatures finally selected in the majority of exposures was between 40° to 45°C. Higher temperatures produced cataracts so rapidly that they were not thought to be relevant to the present experimental design. Control runs used calf lenses to allow comparison of our techniques with former studies. For many years the calf lens has been the standard experimental material for gel electrophoresis and a large body of information is presently available on the analysis of the normal calf eye.

The lens homogenates, in physiological saline, were heated for up to 24 hr in a water bath at temperatures ranging from 37° to 45°C. No significant changes were seen below 40°C. The gel electrophoresis analyses were done at different times, some at half an hour, some at 12 hr, others were continued for the whole 24 hr. No significant precipitate occurred in any of the samples, but they did seem to become more opalescent or cloudy with the longer period of incubation. In order to determine which protein fractions of the lens were changed by incubation, calibrations were established with purified isolated lens crystallins that were available. These include the α, β_H, β_L, and γ crystallins. All were analyzed by the gel electrophoresis isoelectric focusing technique as established by Zigler (33). Both homogenized and whole lenses were tested as there are indications that the structural integrity of the lens prevents some changes from taking place which are observed in the homogenates. Measurements were also attempted on some lenses for ascorbic acid in a similar fashion to Weiter et al. (32) who incubated rabbit lenses during microwave exposures.

Samples of various purified lens protein were also incubated individually and in various combinations to determine (1) the effects of incubation at various temperatures on individual proteins and (2) any interaction among the purified proteins to either stabilized or synergized degradation. The purified lens proteins were also tested in buffered solutions in the following pH ranges: 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0. These samples were all heated at 44°C for 22 hr.

Gel Electrophoresis

The soluble lens proteins, which include the α, β, and γ crystallins, comprise over 90% of the dry weight of the lens. The function of the lens crystallins is to produce a matrix which is fully transparent to visible light and which remains so during lens accommodation. It seems likely that precisely ordered optical interactions among the various lens proteins would be required to produce such a transparent system. On the other hand, any disruption of this system would produce some opacification with frank cataract development if it is carried further.

Some of the changes which occur in the various crystallins can be conveniently studied after they are separated by isoelectric focused thin gel electrophoresis of the homogenized lens. This technique is now widely used to separate and characterize such protein mixtures. It is basically electrophoresis in a pH gradient which separates the various proteins according to their isoelectric points. As the thin layer polyacrylamide gel can be efficiently cooled even with high current densities, the experimental time can be markedly shortened. The exact procedure used was established by Zigler (33).

In this procedure the excised lenses were placed in cold distilled water, and immediately homogenized with a Willems Polytron (Brinkman Instruments). All insoluble material was removed from the lens homogenates by centrifugation at 27.00 g for 20 minutes at 4°C. The LKB 2117 Multiphor was used for isoelectric focused thin layer gel electrophoresis on polyacrylamide slab gels. Special, narrow range gels were prepared by the procedure recommended by LKB and were photopolymerized. For a broader spectrum, wide range gels (pH 3.5 - 10) were used, generally of the preformed types (LKB PagPlates). Lens samples (approximately 2 mg/ml concentration) from small applicator strips of Whatman 3 MM filter paper were applied to the surface of the gel midway between the anode and cathode. Initially the amperage was set at 50 mA. As the current dropped during focusing, the voltage was increased, but the maximum power was held below 30 W. Between one and one half to three hours the current stabilized, signaling equilibrium conditions. After an additional 20 minutes to allow the slower moving proteins to complete migration to their isoelectric points, the run was halted.

The gel was removed and stained directly for 20 minutes at 60°C with a standard staining mixture: 75 ml methanol, 155 ml water, 0.25 g Coomassie Brilliant Blue R, 7.5 g sulfosalicylic acid, and 25 g trichloroacetic acid. The plate was

then destained overnight at room temperature in a solution containing 1950 ml water, 750 ml ethanol and 240 ml glacial acetic acid. The initial standardization of the technique used frozen calf eyes from Pel-Freeze Biologicals, Inc. All test lenses were analyzed by the same procedure.

Amino Acid Analysis

The amino acid analysis followed conventional gas chromatographic techniques. Initially the lens samples were hydrolyzed by heating 6 normal HCl at 105°C in sealed evacuated tubes. Each sample was separated in two parts: one was heated for 24 hours, and the other for 48 hours in order to correct for the partial destruction of certain residues and to allow time for complete hydrolysis of hydrophobic amino acids. The values of valine and isoleucine were normally higher in the 48 hour hydrolysates; these values were therefore the more accurate. The test samples hydrolyzed for 72 hours showed no changes from the 48 hour values, thus long hydrolysis did not seem to be required in order to get accurate and reproducible values. As threonine and serine were progressively degraded by this procedure, their values were obtained by extrapolation back to zero hydrolysis time.

The amino acid levels were measured by gas chromatographic techniques following the conversion of the amino acids into their volatile derivatives. The hydrolyzed lenses were dyed in a dessicator and transferred in 0.1 normal HCl to Mini-aktor tubes (Regis Chemical Co.). This was evaporated and the hydrolyzed amino acids were converted into their n-trifluoroacetyl n-butyl esters by a variation of the method of Roach and Gerke (19). In this method 0.2 ml of 3 normal HCl n-butyl are added to dry sample tubes which are then closed, and heated to 100°C for 20 minutes. After cooling the tube is opened and the sample again dried. Finally 0.15 ml of 25% (volumetric) trifluoroaceticanhydride in methine fluoride is added to each tube which is sealed and heated to 150°C for 15 minutes. This procedure butylates the carboxyl groups of the amino acids while the amino groups had been trifluoroacetylated. The nonvolatile amino acids were thus converted to volatile derivatives which could be anodized with a gas chromatograph.

The gas chromatograph analyses used a Beckman CG 65 gas chromatograph equipped with a disc integrator and fitted with dual glass columns of 1/8" I.D. One column was 6 feet long and was packed with Tabsorb (Regis Chemical Co.). The oven temperature was programmed to rise from 70° to 225° at a rate of 10° per minute. This allowed resolution of 16 amino acids. The second column was 3 1/2 feet long packed with Tabsorb-HAC

(Regis Chemical Co.) and was used to quantitate the basic amino acids arginine and histidine. Relative molar response factors were determined for each amino acid by using the methods described above on a standard amino acid mixture with α amino acid isobutyric acid as internal standard. The molar response factors were determined concomitantly with each group of analyses in order to control possible variations associated with the conversion of the amino acids to their volatile derivatives or in the gas chromatograph itself. The amino acid compositions were determined as relative mole percent residues per 100 by dividing each amino acid integrator count by the appropriate response factor, summing the resulting values and finally deriving the total sum of each individual value. Some quicker procedures have been found more reliable during this present period, such as gel electrophoresis, and in other cases new techniques were tried, such as electron probe x-ray microanalysis. Therefore, only relatively few amino acid analyses were done in order to determine agreement with earlier values recorded during previous periods so as to allow a comparison of present experimental techniques with earlier work.

Electron Probe X-Ray Microanalysis

The concentrations of certain trace elements in the lens are known to increase or decrease with the formation of a cataract. For example, normal human lenses are high in zinc; this level is reduced by a factor of 4 in senile cataracts. However, senile cataracts are high in cobalt and iron, and especially high in copper, which was nearly 12 times the normal level. The reduction in zinc and elevation of copper in senile cataracts appear significant in view of the opposite trends for the levels of these trace elements in blood from these same patients (21). An increase in calcium accompanies degenerative changes in the lens such as normal aging, sclerosis, and cataract formation. In sclerotic lenses calcium may make up over 3% of the ash but in cataractous lenses having the same degree of sclerosis, the value rises to 5% (20).

The mechanism of cataract formation may be linked to these shifts of the lens inorganic constituents. The specific location of these elemental shifts within the lens can be monitored by electron probe analysis, often called energy dispersive x-ray analysis (EDX). This analysis is performed by discrimination of the x-rays generated in the specimen lens by the illuminating electron beam of the microscope. The techniques of x-ray microanalysis allows the measurement of both the wavelengths and intensities of the x-ray lines

generates a spectrum. Furthermore, it is possible to generate an x-ray map for a specified element and by correlating this with a scanning transmission electron microscope (STEM) map of the same region determine the precise location of the element. Changes in concentration and location of elements can thus reliably be detected.

In this technique whole lenses are excised and are placed directly into 2.5% gluteraldehyde buffered with 0.1 M cacodylate (room temperature, pH 7.4) for 1 hour. Following this primary fixation the specimens are placed in increasing concentrations of buffered gluteraldehyde in the following schedule:

gluteraldehyde %	minutes in solution
5	5
10	5
25	5
50	15

Undiluted Epon 812 was then introduced and after 1 hour replaced by the resin mixture without accelerator (left overnight at 4°C) Capsules were made on the next day from resin mixture with accelerator and were cured in a 60°C oven for 2 to 3 days. This direct gluteraldehyde-to-Epon technique has the advantage of being rapid and simple and minimizes the translocations of elements which occur with the conventional use of dehydration in graded alcohols prior to embedding.

The Epon embedded lenses were sectioned at a thickness of 5 μ, placed on gold grids, and lightly carbon coated. Both cataract and control specimens were examined at 20 KV on an ETEC scanning electron microscope with a KEVEX 5100 Si (Li) energy dispersive x-ray analyzer (Kevex Corporation, Burlingame, CA).

Galactose cataracts were used to standardize the electron probe x-ray microanalytical techniques. Rats (50 g) were fed a diet rich in galactose (1250 g galactose, 250 g casein, 250 g dry milk, 125 g butter, 37.5 g calcium carbonate, 25 g sodium chloride, 750 g graham flour) in order to induce cataract formation. At 10-14 days of galactose feeding a central cataract appeared and enlarged to fill most of the capsule.

EXPERIMENTAL RESULTS FROM PRESENT PERIOD; DETAILS AND DISCUSSION

Incubated Lenses

The initial experiments were conducted on normal calf lens homogenates as they are well characterized in previous experiments.

The heated samples showed changes as soon as a half an hour at 45° in the patterns shown by thin gel isoelectric focused electrophoresis. There was no significant precipitate, although all solutions became somewhat cloudy with longer incubation. There did not seem to be any additional change after 24 hours. The details of the thin layer gel electrophoresis are given below.

Isolated lens crystalline proteins were incubated to pinpoint possible changes in the homogenate or whole lenses. Control solutions of all proteins left at room temperature for 24 hours were unchanged. The α, β_H, β_L, and γ crystallins were analyzed separately by gel electrophoresis after incubation at temperatures ranging from 40° to 45°C for 22 hours and a detailed analysis is presented below.

The relative amounts of the various molecular weight fractions of the incubated calf lens homogenate was determined by column chromatography. Results to date show an increase in molecular weight of the α crystallin moiety. An additional peak also appeared which was composed of high molecular weight β crystallin. This aggregation seems to correlate well with any increase in incubation temperature.

Gel electrophoresis of the separated fractions made further characterization possible, although no changes in the individual patterns of the incubated lenses were seen.

Control lenses held at 4°C or at room temperature (20° to 25°C) for 24 hours were not changed detectably from freshly prepared samples measured the same day.

The isolated lens crystallins were unchanged as compared with fresh samples for periods of 24 hours at room temperature. However, some changes were found after incubation for 22 hours. The α crystallin showed changes quite comparable to those in the whole calf lens homogenate.

Column chromatography indicated that the α crystallin had shifted to a slightly higher molecular weight. The very high molecular weight soluble aggregate was much less than that seen in the whole lens homogenate indicating that traces of the β or γ crystallins may assist in the formation of this. Calcium (0.01 M $CaCl_2$) had no effect on α aggregation.

β_H crystallin tended to form a heavy flocculent precipitate in less than one hour at temperatures over 40°C. The isoelectric thin layer gel electrophoresis focusing results were not completely interpreted because of the overlap of

the many bands. For that reason samples of both the precipitate and the soluble material were disassociated in SDS solution. Electrophoresis of these samples shows marked differences in the insoluble fraction. It contained a high molecular weight band which was not present before heating. This component does not seem to result from the disulfate formation or at least not completely so. The sulfhydryl reagents did not disassociate any of the components into smaller units. Thus, non-disulfhyde covalent bonds may be involved in the aggregation. In addition, the inclusion of sulfhydryl reagents into samples of the original protein solution did not prevent or retard precipitation during incubation. The investigation of heated β_H crystallin by thin layer gel filtration of Sephadex G-200 indicates some breakdown of quartenary structure. Thus most of the soluble material after heat treatment for one and one half hours at 45°C is apparently reduced to the dimer state as compared with the 5-7 peptide chain native state.

β_L crystallin precipitated during the heat treatment but not as heavily as the β_H. The precipitation proceeded more slowly but did occur at lower temperatures than the β_H. In its native state β_L is a dimer and it does not contain the two relatively high molecular weight polypeptides, which probably account for the majority of the β_H precipitate. As with β_H the insoluble portion contains high molecular weight aggregates and is characterized by particular β_L polypeptides.

γ crystallin does not appear to be affected by the temperatures and incubation of durations that we used in the present trials. The minor γ component which seemed to be reduced in lens homogenates after prolonged heating is not present in original purified γ crystallin preparations.

Some solutions had more than one crystallin in order to determine whether some individual crystallins were stabilized by the presence of others. Adding β_L to β_H did not affect the onset of precipitation of β_H. α and γ crystallins in amounts equal to the β_H crystallin prevented the β_H precipitation for up to 24 hours, whereas under the same conditions the β_H alone would precipitate within one hour. This result is similar to those seen in the incubation of whole lens homogenates in which there is no β_H crystallin precipitation within the first 24 hours.

Buffering the lens protein solution seems to interact with heating, i.e. there is a pH dependency on the effect of heating.

The calf lens homogenate showed a decrease in the α crystallin fraction at pH 5.0 following incubation for 22 hours, but no detectable change at the higher pH of 7.0 and 8.0. The change at

pH 5.0 was similar to that seen in the calf lens homogenate in unbuffered saline. Thin layer isoelectric focused gel electrophoresis of all solutions showed no changes in the whole lens pattern. There was no observable precipitate in any of the samples.

The isolated α crystallin pattern appeared similar to that in the whole homogenate.

The γ crystallin solution remained completely clear during the entire incubation period and the electrophoresis pattern for pH 8.0 had the lowest isoelectric point missing completely, although other pH were unchanged from controls.

β_H solutions became cloudy rather quickly at the lower pHs and precipitated within about 4 hours. The same protein in unbuffered saline precipitates more slowly, although the precipitate is not as heavy. After 22 hours, all β_H samples contained some precipitate, although at pH 6.5 and 7.0 had the least.

The β_L solutions were similar to β_H in that precipitation was first observed in the pH 5.5 and 6.0 samples. After the total 22 hour period, all samples contained precipitates but with only slight amounts in pH 6.0, 6.5 and 7.0.

These results might indicate that some of the changes in the homogenized calf lens incubated in saline are in part due to the low pH. Consequently in future studies on lens homogenates and isolated lens proteins as well as whole lenses, solutions will be buffered to near neutral. Our trial runs along these lines indicate that the α crystallins show less changes although the γ crystallin shows effects not observed at the lower pH. β_H and β_L in neutral solution show much the same changes sas in unbuffered saline, except that the β_H precipitate appears more slowly, although the nature of it appears to be identical to that found in experiments in saline solution.

Laser Exposed Lenses

The results of typical exposures are shown in Fig. 4, which gives the analysis of various parts of the exposed and unexposed lenses by SDS gel electrophoresis. Where a visible opacity was present (Brown #1 and Brown #2), the opacified portion of the lens was studied separately. In these opaque portions, nearly all soluble crystallins (α, β_H, β_L, and γ) disappeared, implying that the opacity following the laser exposure resulted from the formation of high

Figure 4. Thin layer gel electrophoresis of rabbit lenses following laser exposure. #1, #2, and #3 are the controls. #1 is the whole lens; #2 is the nucleus; #3 is the cortex. #5, #6, and #7 are brown rabbits exposed in each eye for 7 seconds to 10 W. #4 is a portion from the dilated eye; #6 is a portion of the opacity formed; #5 and #6 are portions of the opacity from each eye; #7 is the rest of the lens from the undilated eye. #8, #9, #10, and #11 are from rabbits exposed to 2 W for 40 seconds. #8 and #9 are from the undilated eye; #10 and #11 are from the dilated eye. #11 is from the opaque portion of the dilated eye while #9 was slightly cloudy. #12 and #13 are from brown rabbit exposed to 2 W for 20 seconds. #12 is the whole lens from the nondilated eye; #13 is the whole lens from the dilated eye. See text for explanation of the patterns.

molecular weight albuminoids, a portion of which at least, was derived from these soluble crystallins. Clear portions of all exposed lenses that developed an opacity retained the soluble crystallins, but the β crystallin showed a significant change towards a lower motility, suggesting that agglutination of the β fractions is the initial step in the formation of the insoluble albuminoids. The portions of exposed lenses that did not develop an opacity showed a similar but smaller trend. From this it can be concluded that the IR effect on the lens is vibrational in nature with the β crystallin protein unfolding slightly to expose charged amino acid or sulfhydryl groups.

Another point to be examined is the indication that in some forms of cataract, the cortex of the lens decreases in size. Bo Phillipson (personal communication; 18) suggests that as the cortex gets thinner the nucleus gets larger, and that the decreased rate of fiber formation and protein synthesis in the enlarged nucleus contribute to the development of pathological degradation in the cortex following any environmental trauma.

The results of the amino acid analysis in this period were essentially similar to those found earlier. A record of a previous amino acid analysis is shown in Table I to indicate the normal levels and typical changes after exposure. Very few of the lenses were subjected to this type analysis during the current period. The majority were used to develop other test procedures. As compared with gel electrophoresis, the amino acid analysis could not detect as reliably the earlier stages of cataractogenesis. However, further amino acid characterization tests are planned to show that our manipulations during incubation at 37°C do not in essence change the picture seen earlier in the controls for laser exposure.

Electron probe x-ray microanalysis of biological structures has been attempted by several workers (3, 4, 9, 11, 12, 15, 17, 22, 25-31). These investigations were mainly concerned with analysis of minerals in hard and soft tissues, such as calcium in bone and the precipitates of excessive amounts of ions in pathological tissues. The method, as yet, has not been standardized in any part of the eye. We attempted to localize the elements sulfur, copper, and nickel. The experiments on control lenses with galactose cataracts in rats gave inconclusive results as backscattering from the gold grids interfered with the desired signal. Future studies will employ beryllium grids. Calcium analysis gave more reliable results. However, the changes in calcium levels are as yet too inconclusive to correlate with other changes in the lens or with cataractogenesis.

Table I

AMINO ACID ANALYSIS OF NORMAL AND LASER EXPOSED RABBIT LENSES

	Control		CW neodymium laser, 1.06 μ * 1.0 W exposure level, 3 mm beam*				
			right eye (beam impinged on iris)		left eye (beam did not touch iris)		
	brown cortex	brown nucleus	cortex	nucleus	cortex	nucleus	opacity
Alanine	5.1	4.8	4.9	4.9	4.3	4.4	4.8
Valine	5.9	5.5	6.0	6.1	6.0	6.1	5.6
Glycine	9.6	9.3	9.3	9.1	8.7	8.7	8.4
Isoleucine	3.9	4.0	4.1	4.6	4.4	4.7	3.7
Leucine	8.3	9.0	8.8	8.8	8.2	8.7	8.1
Proline	6.8	6.3	6.9	6.9	6.6	6.2	6.4
Threonine	3.4	2.8	3.5	3.5	3.4	3.1	3.2
Serine	9.6	9.8	9.9	10.4	9.8	10.1	9.0
Methionine	2.5	1.9	2.4	2.5	2.0	2.0	0.6
Phenylalanine	6.1	5.9	6.1	5.9	5.6	5.9	5.8
Aspartic acid	10.1	10.1	10.1	9.8	9.0	9.6	9.0
Glutamic acid	15.7	14.8	14.8	14.3	13.7	13.8	13.9
Tyrosine	4.7	6.1	5.3	5.0	5.0	6.0	4.7
Lysine	4.7	4.1	4.9	4.5	4.6	4.4	4.4
Histidine	trace	0.8	trace	0.81	2.7	0.7	5.3
Arginine	2.7	3.7	2.4	2.5	5.3	4.8	6.5
Cystine	0.8	1.2	0.9	0.4	0.6		

*Two exposures to each eye, one day apart: #1, 63 seconds; #2, 60 seconds

EXPERIMENTS IN PROGRESS AND POSSIBLE LINES OF FUTURE INVESTIGATION

Two major lines of investigation will be pursued in the coming period. One is the characterization of the initial changes in the *in vivo* lens following laser exposure. Both pulsed and CW lasers will be used to determine which may be characterized more easily. The second group of experiments will involve incubation and laser exposure of human lenses obtained from the Eye Bank.

The results from present laser exposures have not yet been completely analyzed. The experiments combining laser irradiation with incubation at higher than normal body temperature have not yet been completed sufficiently to allow analysis of possible synergism or equivalence. Additional lines that need further experiments are:

1. Incubation of lens to clearly delineate the contribution of pH to the degradation of the protein. More information is needed on the possible interaction of the proteins of various crystallins in preventing degradation of the crystallin during thermal or laser stress.

2. Measurements of changes in mobility of various protein fractions to indicate whether longer exposure levels and lower intensities are accompanied by similar changes in lens constituents at higher powers and shorter exposures take place or whether some new mechanism at present unknown begins to take place.

3. Electron probe microanalysis of calcium shifts, to accompany the tests for the role of ascorbic acid and ascorbates.

4. Examination for possible formation of free radicals by electron spin resonance techniques.

ACKNOWLEDGEMENTS

In order to accomplish this work we were assisted by many of our colleagues, both with their time and experimental expertise and by the loan of equipment. Dr. Ian Matheson of the Department of Biochemistry, University of Georgia, was very helpful in loaning laser and laboratory facilities and assisting with the exposure of the rabbit eyes. Dr. Sam Zigler supervised our procedure and performed the majority of the work of the gel electrophoresis and the amino acid analysis, assisted with all

incubation procedures, and loaned us much needed equipment. Dr. Peter Ingram and his staff at the Research Triangle Institute were helpful when we contracted for his services in the electron probe x-ray microanalysis.

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences/National Research Council.

REFERENCES

1. Campbell, C. J. Selected ocular effects from an experimental neodymium laser. Trans. Amer. Ophthal. Soc. 66: 636-672, 1968.

2. Carlin, R. and Cotler, E. Glycosidases of the crystalline lens. Invest. Ophthal. 10:887-897, 1971.

3. Coleman, J. R. and Terepka, A. R. Electron probe analysis of the calcium distribution in cells of the embryonic chick chorioallantoic membrane. I. A critical evaluation of techniques. J. Histochem. Cytochem. 20:401-413, 1972a.

4. Coleman, J. R. and Terepka, A. R. Electron probe analysis of the calcium distribution in cells of the embryonic chick chorioallantoic membrane. II. Demonstration of intracellular location during active transcellular transport. J. Histochem. Cytochem. 20:414-424, 1972b.

5. Davson, H. "The Eye, Vol. I, Vegetative Physiology and Biochemistry." Academic Press, New York, 1962.

6. Dilley, K. J. and Pirie, A. Changes to the proteins of the human lens nucleus in cataract. Exp. Eye Res. 19: 59-72, 1974.

7. Dische, Z. The glycoproteins and glycolipoproteins of the bovine lens and their relation to albuminoid. Invest. Ophthal. 4:759-778, 1965.

8. Dische, Z., Borenfreud, E., and Zelmenis, G. Changes in lens proteins of rats during aging. Arch. Ophthal. 55: 471-483, 1956.

9. Galle, P. Cytochimie sur coupre ultrafined par spectrographie des rayons. X. Electron Microscopy II, 79-80, Proc. 6th Int. Congr. Electr. Micros. (Ueda, R., ed.), Maruzen, Tokyo, 1966.

10. Goldmann, H. Genesis of heat cataract. Arch. Ophthal. 9:314, 1933.

11. Ingram, P., Morosoff, N., Pope, L., Allen, F. and Tisher, C. Some comparisons of the techniques of sputter (coating) and evaporative coating for scanning electron microscopy. Scanning Electron Microscopy (Part I), Proc. 9th Annual SEM Symposium, IIT Research Institute, Chicago, Ill., 1976.

12. Kritz, W., Hohling, H. J., Schnerman, J., von Rosensteil, A. P., and Hall, T. A. Methodik der Elektronenstrahl-mikroanalyse und ihre Anwendung in der Histologie. Aeta histochemica (Suppl. 12), 329-332, 1972.

13. Kurzel, R. B., Wolbarsht, M. L., and Yamanashi, B. S. Spectral studies on normal and cataractous intact human lenses. Exp. Eye Res. 17:65-71, 1973a.

14. Kurzel, R. B., Staton, G., Borkman, R. F., Wolbarsht, M. L. and Yamanashi, B. S. Tryptophan excited states and cataract in the human lens. Nature 241:132, 1972b.

15. Lehrer, G. M. and Berkeley, C. Standards for electron probe microanalysis of biologic specimens. J. Histochem. Cytochem. 20:710-715, 1972.

16. Merola, L. O. and Kinoshita, J. H. The reactivity of the sulfhydryl groups in normal bovine lens. Amer. J. Ophthal. 44(5, Pt. II):326-331, 1957.

17. Mizuhira, V., Nakamura, H., Yotsumoto, H., and Namae, T. Elemental analysis to the biological sections. II. Comparative examinations by the electron probe x-ray microanalyzer (XMA) and the transmitted scanning image system (TSEI) using JSM-13. J. Electr. Micros. 21:242-243, 1972.

18. Nordmann, J. and Eisenkopf, M. The thickness of the human lens cortex in the different types of senile cataract. Invest. Ophthal. 15:425-427, 1976.

19. Roach, D. and Gehrke, C. W. The gas-liquid chromatography of amino acids. J. Chromatog. 43:303-310, 1969.

20. Salit, P. W. Calcium content and weight of human cataractous lenses. Arch. Ophthal. 9:571-578, 1933.

21. Shlopak, T. V. Nauk. Zap., Ivano-Frankivsk. Derzh. Med. Inst. No. 5, 72, 1962.

22. Spear, G. S., Slusser, R. J., Tousimis, A. J., Gaylor, C. G., and Schulman, D. Cystinosis. An ultrastructural and electron probe study of the kidney with unusual findings. Arch. Path. 91:206-221, 1971.

23. Spector, A., Freund, T., Li, L-K, and Augustyn, R. C. Age dependent changes in the structure of crystallins. Invest. Ophthal. 10:667, 1971.

24. Spiro, R. and Spiro, M. J. Enzymatic synthesis of the hydroxylysine-linked disaccharide of basement membranes and collagens. Fed. Proc. 27:345, 1968.

25. Tousimis, A. J. Electron probe x-ray microanalysis of medical and biological specimens. In "Symposium on X-ray and Electron Probe Analysis," Special Technical Publication 349, pp. 193-206, American Society for Testing and Materials, Philadelphia, 1963a.

26. Tousimis, A. J. Electron probe microanalysis of biological specimens. In "Third International Symposium on X-ray Optics and X-ray Microanalysis," pp. 539-557 (H. H. Pattee, Jr., V. E. Cosslett, and A. Angstrom, eds.), Academic Press, New York, 1963b.

27. Tousimis, A. J. Combined scanning electron microscopy and electron probe analysis of biological tissues. 27th Ann. Proc. EMSA, pp. 34-35, 1969a.

28. Tousimis, A. J. A combined scanning electron microscopy and electron probe microanalysis of biological soft tissues. Proc. Second Ann. Scanning Electron Microscope Symposium, pp. 217-230, IIT Res. Inst., Chicago, 1969b.

29. Tousimis, A. J. Electron probe microanalysis of biological structures. In "X-ray and Electron Probe Analysis in Biomedical Research," pp. 87-103 (K. M. Earle and A. J. Tousimis, eds.), Plenum Press, New York, 1969c.

30. Tousimis, A. J. Electrolyte localization in biological tissue by electron probe analysis. Proc. 7th Int. Cong. Electron Microscopy. Grenoble Microscopie electronique 1: 487, 1970.

31. Tousimis, A. J. and Adler, I. Electron probe x-ray micro-analyzer study of copper within Descemet's membrane in Wilson's disease. J. Histochem. Cytochem. 11:40-47, 1963.

32. Weiter, J. J., Finch, E. D., Schultz, W., and Frattali, V. Ascorbic acid changes in cultured rabbit lenses after microwave irradiation. Ann. N.Y. Acad. Sci. 247:175-188, 1975.

33. Zigler, J. S., Jr. Comparative studies on a heterogeneous family of vertebrate lens proteins--the beta crystallins. Ph.D. Thesis, Department of Zoology, Duke University, 1975.

34. Zigman, S. Eye lens color formation and function. Science 171:807, 1971.