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2/28/85

ABSTRACT

Title of Dissertation: Morphological and Cell Volume Changes
in the Rat Lens During the Formation
of Radiation Cataracts

Carmelann Bredehoft, Doctor of Philosophy, 1985

Dissertation Directed by: David C. Beebe

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Preliminary studies showed that x-irradiation caused an increase in the volume of postnatal rat lens epithelial cells. This increase in epithelial cell volume was followed by swelling of the underlying lens fibers, suggesting a correlation between impaired epithelial cell volume regulation and subsequent fiber cell swelling. To test this hypothesis, 4-week-old Sprague Dawley rats were injected with [^3H]-thymidine and, 24 hrs. later, their eyes irradiated with either 4 or 12 Gy (1 Gy = 100 rads). Lenses were examined with a slit lamp and cataracts graded on a scale of 1+ to 4+. Animals were killed 24 hrs., 3, 5, 15, and 30 wks. after exposure. Lenses were serially sectioned at 0.75 μm and average cell volumes of specific lens regions determined. The surface morphology of fiber cells from these lenses and their attachment at the posterior suture were examined by scanning electron microscopy. Lenses were also labelled with [^{35}S]-methionine and the incorporation of radioactivity into the crystallin, cytoskeletal and membrane protein fractions was examined by SDS-PAGE. Rats exposed to 4 or 12 Gy developed 0.5-1.5+ or 2.5-3.0+ cataracts, respectively, 10 to 16 wks.

after x-irradiation. Epithelial and equatorial cells of both groups did not significantly increase in volume during this period. Three wks. after irradiation with 12 Gy cortical fibers were disorganized and their volume was increased transiently. By 5 wks. cortical fibers returned toward control cell volumes, although their morphology remained altered. Cortical fiber volume in lenses irradiated with 4 Gy was not significantly different from control lenses throughout the period examined. Wet weight determinations showed that the localized swelling of the cortical fibers did not result in an increase in the wet weight of the entire lens. Autoradiography results indicated that affected fibers had been epithelial cells at the time of irradiation. Labelling of lens proteins continued to occur after x-irradiation. These results provide additional evidence that disturbances in fiber differentiation are involved with cataract formation, but do not support the hypothesis that a disturbance in epithelial cell volume regulation leads to fiber cell swelling. The possibility is discussed that previous results suggesting a defect in lens epithelial volume regulation in radiation cataracts may have been complicated by ocular inflammation.

MORPHOLOGICAL AND CELL VOLUME CHANGES
IN THE RAT LENS DURING THE FORMATION
OF RADIATION CATARACTS

by

Carmelann Bredehoft

Dissertation submitted to the Faculty of the Department of Anatomy
Graduate Program of the Uniformed Services University of the
Health Sciences in partial fulfillment of the
requirements for the degree of
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For Eric and My Family

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INTRODUCTION

The lens collects divergent light rays and converts them into a single image to be transmitted to the retina. The ability of the lens to focus light rays is dependent on its transparency since light passes throughout its cell membranes, cytoplasm, and extracellular spaces. Lens transparency depends on normal lens cell differentiation (Cogan, Donaldson, and Reese, 1952; Hanna and O'Brien, 1963; Worgul and Rothstein, 1977), osmotic balance (Kinsey and Reddy, 1965; Duncan, 1973; Philipson and Fagerholm, 1973; Kolata, 1980; Unakar and Tsui, 1980; Reddy et al., 1981) and protein synthesis (José, 1978; Reddy et al., 1980; Shinohara and Piatigorsky, 1980). In the transparent lens, light is minimally scattered because fiber cells and their proteins are densely packed in an orderly array. In addition, lens proteins are hydrated to a degree which minimizes the difference between their refractive indices and that of the surroundings (Bettelheim and Siew, 1982). If lens fibers become disorganized (Worgul, Merriam, Szechter, and Srinivasan, 1976) and/or their proteins become aggregated (Trokkel, 1962) substantial fluctuations will occur in their refractive and light scattering properties (Benedek, 1971). A lens opacity is proportional to the number, the molecular weight, and the difference in the refractive index of lenticular protein aggregates compared to the non-aggregated portion of the lens (Benedek, 1971). The development of radiation cataracts was characterized by a reduction in protein concentration and a decrease in lens volume (Philipson, 1969).

X-rays may cause cataracts by their effects on the mitotic activity (von Sallmann, Caravaggio, Munoz, and Drungis, 1957; Scullica,

Grimes, and McElvain, 1962), enzyme concentrations (Pirie, van Heyningen, and Flanders, 1955; Pirie, 1961; Matsuda, Giblin, and Reddy, 1980; Reddy et al., 1981) and various proteins of the lens cells (Trokkel, 1962; Giblin, Chakrapani, and Reddy, 1978; Bettelheim and Siew, 1982). A radiation cataract begins as an opacity at the posterior pole of the lens. This type of cataract is classified as a posterior, subcapsular, cortical opacity. Its location close to the visual axis heightens the severity of its effects on visual acuity (Trokkel, 1962). In the human, at high doses, progressive increase in the size of a radiation cataract gradually leads to complete opacification of the lens.

Cataract formation due to radiation may not be observed for months or years after the insult. This latent period varies inversely with dose (Schenken and Hagemann, 1975), and is affected by age (Cogan and Donaldson, 1951; von Sallmann, Tobias, Anger, Welch, Kimura, Munoz, and Drungis, 1955; Kandori, 1956; van Heyningen, 1975; Merriam and Szechter, 1975), species (von Sallmann et al., 1957; Leinfelder and Dickerson, 1960; Pirie, 1961), and the region of the lens exposed to radiation (Richard, Riley, and Leinfelder, 1956). Rats develop cataracts sooner than cats, dogs, and monkeys when exposed to equal amounts of radiation (von Sallmann et al., 1957). In addition, young rats (1 day to 6 wks.) develop cataracts earlier than adult rats (9-56 wks.) when exposed to doses of 3 to 9 Gy of x-ray (Merriam and Szechter, 1975). However, at this dose, once cataract formation begins, the opacity progresses faster and is more severe in adult lenses. At x-ray doses above 9 Gy, an opacity develops and progresses faster in young rat lenses, compared to adults (Merriam and Szechter, 1975).

Radiation-induced cataract can serve as a useful model for

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studying the process of cataractogenesis. The latent period allows careful examination of cytological changes prior to cataract formation. In addition, cellular changes seen in radiation cataracts, such as swollen lens fibers, "pseudoepithelial" cells, and an increase in the Na^+/K^+ ratio, have been observed in senile, steroid, galactose, and some types of hereditary cataracts (von Sallmann et al., 1955; Pirie, 1967; Palva, 1978; Eshaghian and Streeton, 1980; Pasino, 1982; Shinohara and Piatigorsky, 1982). These observations suggest that the study of cytological changes that occur in radiation cataracts could provide useful information about clinically- important human cataracts.

X-IRRADIATION AND ITS GENERAL CELLULAR EFFECTS

X-rays are a form of electromagnetic energy which originates outside the atomic nucleus. They are produced within an x-ray machine when a high-speed electron from a cathode ray tube hits an inner electron of a tungsten target and ejects this electron from its orbit (Fig. 1). The ejected electron is replaced by an electron from an outer shell causing x-rays to be emitted. The energy of the x-ray represents the difference in energies of the inner and outer orbital electron levels. The photons generated by the x-ray machine ionize and excite the atoms of the material through which they travel. The term rad is defined as the absorption of 100 ergs of energy per gram of matter and 1 Gy is equivalent to 100 rads. The following dose scale may be used to roughly quantify various amounts of absorbed radiation: 0-5 Gy is a small dose; 6-10 Gy, a moderate dose; 11-20 Gy, a large dose; and above 20 Gy, a massive dose (Casarett, 1968).

The energy from x-rays can ionize matter in different ways. It

can generate one ejected electron (2^0 electron), several ejected electrons, or a positron (e^+) and an electron (e^-) from the target atom. These charged particles are capable of ionizing other molecules directly or indirectly. In direct ionization, a molecule is ionized or excited by the charged particle itself. Indirectly, a molecule can receive the energy from the x-ray by transfer from another excited molecule. This indirect effect is common in aqueous solutions (e.g. cytoplasm) in which a water molecule may be ionized and then transfer its acquired energy to another molecule. Both direct and indirect effects can cause molecules to form ion pairs and free radicals which can affect the macromolecules of a cell. The interaction of proteins with ionizing particles results in degradation, inter- and intramolecular crosslinking and an alteration in tertiary and secondary protein structure. The interaction of nucleic acids and lipids with these charged particles cause single and double strand breaks of DNA molecules and the formation of lipid peroxides. These chemical changes are expressed at the cellular level as chromosomal aberrations, increased membrane permeability, and alterations in DNA structure and/or the mitotic cycle. Cells most likely to express these radiation-induced changes are those which divide frequently and differentiate after division. Those least likely to undergo changes after irradiation are highly differentiated, postmitotic cells.

X-IRRADIATION AND LENS MORPHOLOGY

Normal Lens Growth and Fiber Cell Differentiation

The lens is made up of a monolayer of epithelial cells, some of which are mitotically active, and an underlying mass of differentiated

lens fibers. The entire organ is surrounded by a thick basement membrane, the lens capsule, which acts as a barrier, keeping cells from entering or leaving the lens. The epithelium contains a central group of non-mitotic cells and a peripherally placed group of mitotically-active, germinative cells. Germinative epithelial cells synthesize DNA, divide and differentiate into lens fibers (Rafferty and Rafferty, 1981). As these cells differentiate they align in "meridional rows" and elongate at the equator to form the lens cortex (Fig. 2). The "mitotic pressure" generated by cell division in the germinative epithelial cells appears to be necessary for the migration of meridional row cells and their differentiation into lens fibers (Hayden and Rothstein, 1979). During differentiation the lens fiber cells increase in volume and elongate in anterior and posterior directions. This increase in lens cell volume along with the presence of microtubules and microfilaments are thought to play a role in fiber cell elongation (Byers and Porter, 1964; Piatigorsky, Webster, and Wollberg, 1972; Piatigorsky, 1975; Beebe, Johnson, Feagans, and Compart, 1982). The synthesis of lens crystallins and specialized plasma membranes accompanies cell elongation. During maturation, lens fibers lose most of their organelles and are internalized toward the center of the lens. In addition, mature fiber cells lose contact with the lens capsule and interdigitate anteriorly and posteriorly with fibers from the opposite side of the optical axis (Kuwabara, 1975). These mature fibers maintain their shape by a three-dimensional, cytoskeletal network and are connected to each other by extensive gap junctions (Maisel, Harding, Alcalá, Kuszak, and Bradley, 1981). The large number of these junctions suggests that lens fibers exist in a "syncytial" state with respect to the movement of water, ions,

and small metabolites (Rae, 1974).

The addition of newly formed lens cells and their elongation into fibers is a continuous process throughout the life of the organism. The uptake of radioactively labeled [^3H]-thymidine by the nuclei of epithelial cells, which undergo DNA synthesis and differentiation, has been used to monitor their normal migration into the lens fiber cortex (Hanna and O'Brien, 1963; Worgul et al., 1976; Bredehoft, 1982; Bredehoft and Beebe, 1982). Epithelial cells of young rats (up to 4 wks.-old) take 5 to 7 days to become lens fibers (Worgul et al., 1976; Bredehoft, 1982; Bredehoft and Beebe, 1982). With increasing age, the rate of fiber formation decreases. The labeling of nuclei in the cells in the germinative zone of a 40 wk.-old rat lens shows that these cells take more than 12 wks. to become lens fibers (Hanna and O'Brien, 1963).

Radiation-induced Epithelial Cell Damage

Radiation damage has been detected in the germinative epithelial cells prior to any manifestation of lens fiber cell damage (Poppe, 1942; Cogan and Donaldson, 1951; von Sallmann, 1952; von Sallmann et al., 1955; von Sallmann et al., 1957; Scullica et al., 1962; Palva and Palkama, 1978). Initially, there is a decrease in mitotic activity. In adult rats (10-12 wks.), within 1 hr. after x-irradiation (10 Gy), mitotic activity is depressed by 30% compared to unirradiated lenses (von Sallmann et al., 1957). Recovery from mitotic inhibition can occur as early as 24 hrs. after x-irradiation. It is characterized by an overshoot of the mitotic activity, clumping of chromosomes and formation of chromosomal bridges and fragments (Cogan and Donaldson, 1951; von Sallmann et al., 1955; Scullica et al., 1962). The compensatory increase

in mitosis persists for varying periods of time depending upon the animal species. In mice control levels of mitotic activity were reached after 4 wks., whereas, in rats and rabbits the counts of irradiated eyes approached that of control eyes after 8 wks. (von Sallmann et al., 1957; Scullica et al., 1962).

Mitotic inhibition is accompanied by a temporary depression in the number of cells undergoing DNA synthesis. Rats injected with [³H]-thymidine 1 hr. prior to 10 Gy irradiation showed a loss of labeled cells 3 days after exposure (Scullica, Grimes, and McElvain, 1963). Scullica et al. (1963) postulated that these cells died after release of the mitotic block that follows irradiation. Cell death was characterized by vacuolization and spaces devoid of cells in the germinative epithelium (Poppe, 1942; Scullica et al., 1962).

Direct radiation damage to the germinative epithelial cells alone can lead to cataractous changes (Alter and Leinfelder, 1953). Different regions of the rabbit lens were selectively shielded so that when only the germinative epithelial cells were x-irradiated, the same cataractous changes occurred as when the entire lens was exposed. In addition, x-ray cataracts did not form during hibernation of the ground squirrel, when mitosis ceases naturally (Leinfelder and Dickerson, 1960). These results suggested that cataracts form only when epithelial cells are permitted to migrate and differentiate into lens fibers. To test this hypothesis, cold depression and hormone stimulation of mitotic activity of epithelial cells were used after x-irradiation (Worgul and Rothstein, 1975). When a group of frogs were injected with triiodothyronine (T₃), which elevated the mitotic index, and exposed to 50 Gy they developed cataracts sooner than non-injected, irradiated controls. When a second group of frogs,

injected with T3, were kept at 10°C (which depresses mitotic activity) after x-irradiation, they did not develop cataracts. After returning some of these frogs to room temperature lens opacities were observed, but were less severe. These results indicated that a relationship exists between post-irradiation mitotic activity and cataract formation. Subsequently, it was found that cataracts did not develop in hypophysectomized frogs whose epithelial cells were confined to the resting phase of the mitotic cycle (Rothstein, Worgul, Medvedovsky, and Merriam, 1982).

Lens Fiber Damage

One sign of disturbance in fiber differentiation after x-irradiation is the misalignment of cells in the meridional rows at the lens equator. Worgul et al. (1976) proposed that distortion of cell alignment in the meridional rows leads to the formation of lens fibers which are incapable of the precise orientation necessary for lens transparency. In addition, meridional row cells have been found to undergo nuclear fragmentation and cytoplasmic disorganization within 12 hrs. after x-irradiation (Worgul and Merriam, 1980; Broglio and Worgul, 1982).

During cataract formation, the posterior subcapsular region becomes filled with undifferentiated "pseudoepithelial" cells and "differentiated" epithelial cells (Poppe, 1942; Friedenwald and Rytel, 1955; Kandori, 1956; Jose, 1978; Palva and Palkama, 1978; Eshaghian and Streeton, 1980; Merriam and Worgul, 1983; Rini, Worgul, and Merriam, 1983). Undifferentiated "pseudoepithelial" cells migrate toward the posterior lens pole and accumulate between the capsule and the cortical

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fiber mass. "Differentiated" epithelial cells are rounded "blister-like" cells which fill the posterior cortex. These cells are devoid of surface interdigitations and undergo blebbing at their capsular surface (Rini et al., 1983). In advanced stages of cataract formation these swollen "blister-like" cells rupture and dissolve, forming inter-fiber clefts filled with protein coagulum (Friedenwald and Rytel, 1955; Merriam and Worgul, 1983). Throughout the early stages of radiation cataract formation inner cortical and nuclear lens fibers remain unaffected (José, 1978; Merriam and Worgul, 1983; Rini et al., 1983).

X-IRRADIATION AND LENS BIOCHEMISTRY

Swelling and distortion of cortical fibers, especially in the posterior lens region, has been interpreted to be the result of alterations in lens permeability and metabolism (Pirie et al., 1955; von Sallmann and Locke, 1951; Lambert and Kinoshita, 1967; Duncan and Croghan, 1969). Glutathione decreases in concentration soon after x-irradiation (Pirie, van Heyningen, and Boag, 1953; Giblin, Chakrapani, and Reddy, 1979; Giblin and Reddy, 1981). Subsequently to this decrease is an irreversible increase in the passive permeability of lens membranes to cations and a reduction in active cation transport (Giblin, Chakrapani, and Reddy, 1976; Giblin and Reddy, 1982). These biochemical changes may lead to cataractous lenses which are abnormally hydrated and contain high molecular weight protein aggregates. Such radiation-induced alterations in lens structure and function are best understood in terms of normal cell volume and protein structure.

Normal Lens Cell Volume

Normally, the ionic and osmotic balance of lens cells is maintained by an active Na^+ , K^+ -ATPase, located primarily in the epithelium (Bonting et al., 1963; Kinsey and Reddy, 1965; Duncan and Croghan, 1969; Duncan, 1973). This enzyme is involved in maintaining a concentration gradient of sodium and potassium ions between the lens tissue and the aqueous humor. The Na^+ , K^+ -ATPase pumps sodium ions from the lens into the surrounding medium in exchange for potassium. Energy required for the enzyme is supplied by ATP generated via anaerobic glycolysis. The Na^+ , K^+ -ATPase has been localized on the lateral surface of the epithelial cells and superficial cortical lens fibers (Kinoshita, 1963; Riley, 1970; Palva and Palkama, 1976). Like Na^+ , K^+ -ATPases in other tissues this enzyme can be inhibited by the cardiac glycoside, ouabain. The activity of this enzyme is dependent upon either the ionic concentrations of sodium and potassium or their electrochemical gradients. The maintenance of steady concentrations of sodium and potassium results in a constant cell volume which enables the lens to maintain a state of dynamic equilibrium.

Transport studies showed that ^{42}K penetrates the anterior surface of the lens more than three times as fast as it penetrates the posterior surface (Kinsey and Reddy, 1965). At the posterior surface sodium was found to penetrate more rapidly. In addition, removal of the capsule and epithelium abolished these differences between sodium and potassium permeability. A pump-leak system was proposed to account for the sodium and potassium fluxes. In this system K^+ ions are actively transported into the epithelium by Na^+ , K^+ -ATPase and diffuse to the outside of the

lens at the posterior capsule. Simultaneously, sodium ions are presumed to diffuse across the posterior part of the lens and are actively extruded by Na^+ , K^+ -ATPase.

In addition to transporting cations, the lens possesses a mechanism for the transport of glutathione (Reddy et al., 1980). This tripeptide, which contains L-glutamate, L-cysteine and glycine, acts as an antioxidant in its reduced form (GSH). It contains a reactive sulfhydryl group (-SH) which serves as a site for disulfide bond formation and prevents the oxidation of sulfhydryl containing proteins like Na^+ , K^+ -ATPase (Giblin et al., 1978).

Structural Lens Proteins

The synthesis of structural lens proteins is associated with proper ionic concentration and hydration (Kinoshita, 1965; Iwata and Kinoshita, 1971; Piatigorsky, Fukui, and Kinoshita, 1978; Kador, Fukui, Fukushi, Jernigan, and Kinoshita, 1980; Matsuda, Giblin, and Reddy, 1982). Approximately 35% of the lens wet weight is comprised of structural proteins. These proteins have been divided into water soluble and water-insoluble fractions (Mörner, 1894). The water-insoluble fraction has been further subdivided into a urea-soluble and urea-insoluble fraction. The lens varies in protein composition in its different regions and in animals of different ages (Harding and Dilley, 1976). Changes in lens protein composition results from alterations in the pattern of protein synthesis during cell differentiation and postsynthetic modifications occurring during aging (Harding and Dilley, 1976).

In mammals, the water soluble proteins consist of α , β and γ

crystallins (20, 19-30 and 20 kilodaltons, Kd, respectively). Crystallin classes are immunologically different from each other and each consists of a family of polypeptides which have related primary structures. Alpha and most β crystallins are multimeric proteins, while γ crystallin is monomeric.

Urea soluble proteins include cytoskeletal proteins, such as vimentin and actin (Hoenders and Bloemendal, 1981). Cytoskeletal proteins may play a role in lens fiber differentiation and in maintaining cell shape (Byers and Porter, 1964; Piatigorsky et al., 1972; Piatigorsky, 1975; Ramaekers, 1981). Analysis of this fraction by SDS polyacryamide gel electrophoresis has shown that it contains several components ranging from 20 to 200Kd (Fairbanks, Steck, and Wallach, 1971; Alcalá, Lieska, and Maisel, 1975).

The urea-insoluble fraction consists primarily of lens membrane components (Maisel, 1977; Broekhuysen and Kuchlmann, 1979; Horwitz, Robertson, Wong, Zigler, and Kinoshita, 1979; Roy, Spector, and Farnsworth, 1979). Membrane components include extrinsic and intrinsic proteins, carbohydrates and lipids. Specific membrane proteins have been isolated by using sucrose gradient centrifugation (Bloemendal et al., 1972), extensive urea-washings (Alcalá, Valentine, and Maisel, 1980), reaction with acid anhydrides (Roy et al., 1979), and sodium hydroxide (Russell, Robison, and Kinoshita, 1981). Extrinsic proteins like 43Kd polypeptides are more loosely attached to the membrane, can be removed without the use of detergents. They are free of lipids and are soluble in neutral aqueous buffers.

In contrast to extrinsic proteins, intrinsic proteins are embedded within the membrane structure. They are associated with

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membrane lipids and are extractable only with detergents or strong organic solvents. A predominant intrinsic lens membrane glycoprotein is a 26-27Kd polypeptide. This "main intrinsic polypeptide" (MIP) has been isolated from fiber membrane fractions of young human, calf, rat, and mouse lens (Alcalá et al., 1975; Horwitz et al., 1979; Roy et al., 1979; Tanaka, Russell, Smith, Uga, Kuwabara, and Kinoshita, 1980). Its presence appears to reflect the abundance of gap junctions in lens fiber cells of various species (Lasser and Galazo, 1972). Lens gap junctions have been found to possess morphological (Henderson, Eible, and Weber, 1979) and electro-physiological properties (Rae, 1979) similar to those of mouse liver gap junctions, which also contain a 26Kd protein. Following tryptic treatment, the 26Kd polypeptide converts into a 22Kd polypeptide (Henderson et al., 1979; Bowmann, de Leeuw, and Broekhuysse, 1980). Like the 26Kd polypeptide, the 22Kd polypeptide forms heat-induced high molecular weight aggregates which do not migrate in polyacrylamide gels and can be extracted from lens fiber membrane polypeptides by a chloroform-methanol mixture (Broekhuysse and Kuchlmann, 1979; Bowman et al., 1980).

Alterations in Cation Levels and Cell Volume After X-irradiation

One prominent effect of radiation exposure is a decrease in lens glutathione concentration which results in a series of changes that leads to an increase in membrane permeability (Pirie et al., 1953; Giblin et al., 1979; Matsuda et al., 1981). After x-irradiation of rabbit lenses, glutathione concentration decreases as early as 1 wk. after exposure (Matsuda et al., 1981). By 3 wks., GSH in irradiated whole lenses is reduced by 30% and continues to decrease to 25% of the control value at

7.5 wks. (Matsuda et al., 1981). This decrease in reduced glutathione has been shown to be associated with a reduction in the activity of the enzymes γ -glutamylcysteine synthetase and glutathione reductase (Pirie et al., 1953; Rathbun, Sethna, Skelnik, and Bistner, 1983; Rathbun, 1984). Gamma glutamylcysteine synthetase catalyzes the rate limiting step in glutathione synthesis. Glutathione reductase catalyzes the reaction which converts glutathione from its oxidized state (GSSG) back to its reduced form (GSH).

Accompanying this drop in glutathione is a decrease in Na^+ , K^+ -ATPase and cation pumping activity of whole x-irradiated rabbit lenses and isolated epithelium (Giblin et al., 1979; Matsuda et al., 1981, 1982). These changes in membrane permeability were not reversed by the regeneration of glutathione in the lens. This suggests that an irreversible conformational change in the sulfhydryl groups of Na^+ , K^+ -ATPase may be initiated by the loss of a critical amount of glutathione in the lens.

Despite the decrease in Na^+ , K^+ -ATPase activity 4 to 7.5 wks. after x-irradiation lenses are able to maintain near-normal total cation levels (Matsuda et al., 1982). Total Na^+ and K^+ ion concentration in x-irradiated lenses ranged from 26 to 31 μEq per lens and contralateral controls were 26 to 34 μEq per lens. It appears that as long as the exchange of Na^+ and K^+ was 1:1, normal lens hydration is maintained (Kinoshita, 1974; Matsuda et al., 1982). However, 1 wk. prior to complete lens opacity (8 wks. after x-irradiation) the 1:1 exchange of Na^+ for K^+ ion breaks down and there is an increase of Na^+ ions and water (Matsuda et al., 1982) into the lens. By 8 to 9 wks. mean total cation content of x-irradiated lenses increased to 48 μEq compared to 27 μEq in

the controls. In addition, mean wet weight of these lenses was 60 mg higher than the controls and water content increased to 86% of the total lens weight, compared to 67% in contralateral controls (Matsuda et al., 1982). These results show that although dramatic changes occur in permeability and active cation transport, irradiated rabbit lenses are able to maintain near normal hydration until complete lens opacification.

An alternate explanation for swelling of the lens at the time of lens opacification is that lens cell membranes may increase in their passive permeability to cations. This is suggested by the work of Palva and Palkama (1978) who found hydropic epithelial cells and vacuolated lens fibers but no loss of Na^+ , K^+ -ATPase in isolated epithelial preparations from control and x-irradiated rat lenses. Therefore, the possibility exists that both an increase in passive cation permeability and a decrease in cation transport may be causal agents in cataract formation.

Glutathione and High-Molecular-Weight (HMW) Protein Aggregates

Glutathione has also been implicated in the formation of HMW protein aggregates (Giblin, Chakrapani, and Reddy, 1978; Giblin and Reddy, 1978). These aggregates consist of disulfide linked proteins ranging in molecular weight between 10^6 and 10^9 daltons. Although reduced glutathione (GSH) concentrations in x-irradiated lenses were 25% of the controls at 1 wk. prior to cataract formation, loss of sulfhydryl groups was not observed until the time of complete lens opacification. This finding suggested that sulfhydryl groups may remain reduced at the expense of the lens reducing system (GSH) and only when a critically low

point is reached, oxidation of protein sulfhydryl group occurs. In addition, unfolding of lens proteins can occur at the end stages of cataract formation and cause protein sulfhydryl groups previously buried to become exposed and oxidized.

Possible Role of Membrane Proteins in Cataract Formation

Alterations in permeability and lens protein composition have led to the study of changes in membrane proteins during cataract formation. Since initial studies on erythrocyte membranes (Steck, Ramos, and Strapazon, 1976; Wilson, 1978) suggested a possible role of glycoproteins in membrane transport, Garadi et al. (1982) decided to examine glycoproteins in x-irradiated lenses. Carbohydrate moieties of lens glycoproteins were labeled by using galactose oxidase and tritiated borohydride, and these proteins were separated by SDS-PAGE and visualized by fluorography. These results showed that the glycoproteins identified were localized in the membrane fraction and that changes in the incorporation of tritium in these glycoproteins occurred in radiation-induced cataracts. An increase in tritium labeling of a 22Kd glycoprotein and a decrease of radioactivity in a 103Kd glycoprotein were found suggesting that changes do occur in the composition of lens membranes during cataract formation. However, the role of glycoproteins in regulating membrane processes remains to be established.

In addition to membrane glycoproteins, γ crystallin and the membrane proteins, 43Kd and 26Kd have been implicated in cataract formation. These proteins were found in isolated membrane fractions of human senile cataracts (Spector, Garner, and Roy, 1979; Garner, Roy, Rosenfeld, Garner, and Spector, 1981). The 43Kd extrinsic membrane

protein was present in disulfide-linked protein aggregates containing γ -crystallin. Also, a portion of 26Kd, an intrinsic membrane protein, was found in the non-membrane fraction. They proposed that physical damage to the lens, such as oxidative stress or osmotic shock, may cause membranes to break or become more porous. As a result, fiber membranes are compromised and vesicles, containing 26Kd and 43Kd, are formed. These membrane vesicles may act as nucleation sites for the formation of high molecular weight disulfide-linked aggregates.

Protein Synthesis and Ionic and Osmotic Balance Within the Lens

It has been shown that changes in ionic balance lead to a decrease in lens protein synthesis (Piatigorsky et al., 1978; Kador, Zigler, and Kinoshita, 1979; Piatigorsky, Kador, and Kinoshita, 1980; Shinohara and Piatigorsky, 1980; Shinohara and Piatigorsky, 1982). In the Philly mouse, a strain which develops hereditary cataracts between the third and eighth postnatal week (Kador et al., 1980), there was a net accumulation of Na^+ and loss of K^+ from the lens (Piatigorsky et al., 1980). Also, the incorporation of [^{35}S]-methionine into β and γ crystallins was markedly reduced. Negligible amounts of these crystallins were found in the medium of cultured Philly lenses. These results suggested that a correlation existed between alterations in protein metabolism and electrolyte concentrations. These data support the idea that selective degradation of crystallins and differential reduction in their synthesis is a primary cause for lowered amounts of soluble protein in cataracts. Similar changes in Na^+ and K^+ ions and a decrease in wet weight and crystallin synthesis was observed in rat lenses with galactose cataracts (Kador et al., 1979). By using in vitro

translation tests in a reticulocyte lysate Shinohara and Piatigorsky (1980, 1982) showed that in galactose-induced and in hereditary cataracts, the initial reduction in crystallin synthesis is caused by inefficient utilization of crystallin mRNAs. They suggested that this may be a general mechanism by which crystallin synthesis is initially reduced in cataracts.

A decrease in lens growth, characteristic of diabetic, galactosemic, and some types of hereditary cataracts, also occurs in radiation cataracts (Iwata and Kinoshita, 1971; Kinoshita, 1975; Kador et al., 1980). X-irradiated rabbit lenses have lower wet weights than unirradiated lenses, until the end stages of cataract formation (Matsuda et al., 1981, 1982). Slower lens growth may be due to a decrease in protein synthesis, an increase in proteolysis, and/or leakage of proteins from the hydrated lens. Garadi, Foltyn, Giblin, and Reddy (1984) used incorporation of [^{35}S]-methionine into x-irradiated rabbit lenses and found that changes in cation distribution affect not only crystallin synthesis, but also the membrane polypeptide synthesis of lens fiber cells. Crystallin synthesis was affected 4 wks. after x-irradiation. This coincided with alterations in Na^+/K^+ ratio. The decrease in crystallin synthesis became greater 5 to 7 wks. after x-irradiation, when the cation imbalance was larger. At 8 wks., when significant changes in hydration occurred, [^{35}S]-methionine incorporation was reduced to 20% of the control lenses in the crystallin, cytoskeletal, and membrane fraction of the lens fibers. Garadi et al. (1984) concluded that the Na^+/K^+ ratio and the availability of amino acids in the lens are important factors in the synthesis of lens proteins. They suggested that the decrease in dry weight observed in x-irradiated lenses may be caused by a decrease in

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protein synthesis and not protein leakage. Increased lens hydration, which is correlated with leakage, did not occur until the development of mature cataracts.

X-IRRADIATION AND INFLAMMATION

When studying hydration changes in radiation-induced cataracts in laboratory animals the following should be considered: species, age, total dose and inflammatory response of the animal's eye to x-rays. Inflammation alone can cause secondary, or complicated cataracts. Cytological effects of inflammation (endotoxin-induced) on the lens were similar to those caused by x-rays (Worgul and Merriam, 1979). These similarities were: depression in mitotic activity of germinative epithelial cells during the first 24 hrs., an increase in the mitotic activity of these cells within 72 hrs., and eventual disorganization of meridional rows.

Rabbits are prone to the early intraocular inflammatory effects of x-rays (Kinoshita and Lambert, 1967; Bito and Klein, 1981; Worgul and Merriam, 1981). When the eyes of these animals are exposed to doses of 2.5 to 15 Gy they develop various signs of inflammation. Within 3 to 6 hrs. after x-irradiation with 10 Gy, rabbit eyes developed engorged iridial blood vessels, decreased prostaglandin accumulation of the anterior uvea, increased protein levels in the aqueous humor and the presence of leukocytes in the normally cell-free anterior chamber (Worgul, Bito, and Merriam, 1977). Most of these changes did not begin to return to normal until 7 days after x-irradiation. Also, within 24 hrs. after 30 and 60 Gy x-irradiation, rabbit lenses had increased rubidium permeability that was greatest within the 1st wk. and declined during the

2nd and 3rd wk. after x-irradiation (Lambert and Kinoshita, 1967).

In addition, fiber cells located at the lens surface showed a small but significant increase in water content 28 days after x-irradiation with 60 Gy. Electronmicrographs of these superficial lens fibers showed that their fiber membranes had ruptured. These results suggested that the lens fibers may have ruptured secondary to increased hydration as a result of increased permeability.

Compared to rabbits, post-weanling rats (greater than 4 wks.-old) have been found to be refractory to early ocular effects of x-rays when exposed to doses lower than 18 Gy (Worgul and Merriam, 1981). However, this may not be the case in early postnatal rats (1, 3 and 5 days). Preliminary studies showed that epithelial cells of early postnatal rat eyes irradiated with 15 Gy increased 26% in volume within 24 hrs. after x-ray exposure (Bredehoft, 1982; Bredehoft and Beebe, 1982). These cells continued to increase in volume over a 4 day period. By 5 days these cells were 54% greater in volume than the controls. Underlying lens fibers also increased in volume. At 3 days after x-irradiation they were 28% greater in volume, and by 5 days they were 77% greater in volume than the controls. These lenses developed diffuse cataracts within 5 days and mature cataracts by 1 month after x-irradiation. In addition, the developing retina of x-irradiated early postnatal rat eyes underwent massive cell death in the first 24 hrs. after x-ray exposure. By 5 days x-irradiated early postnatal rat eyes no longer contained any retinal cells. These results suggested that the early postnatal rat, like the rabbit may be highly sensitive to x-ray induced ocular inflammation due to its developing retina and hyaloid vascular system. Therefore, cataractous changes observed in x-irradiated rabbit and early postnatal

lenses raise the possibility that such changes may be due primarily to inflammation and only secondarily to x-rays.

SUMMARY

The following have been considered as causal agents in cataract formation:

1. Direct radiation damage to germinative epithelial cells results in the formation of aberrant lens cells. Some of these differentiate into "blister-like" fiber cells and occupy the lens cortex. Others migrate toward the posterior lens pole between the capsule and cortex to become "pseudoepithelial" cells. These aberrant cell types accumulate within the lens and contribute to cataract formation.
2. Lens glutathione concentration decreases after x-irradiation. After this decrease in glutathione there is a decrease in the activity of the Na^+ , K^+ -ATPase and alterations in the concentration of Na^+ and K^+ ions. Hydration remains normal until immediately prior to cataract formation when the 1:1 exchange between Na^+ and K^+ ions ceases. It has been suggested that when glutathione concentration reaches a critically low point the 1:1 Na^+/K^+ ratio is disrupted, an increase in the formation of HMW protein aggregates occurs, and the lens swells. Also, it is postulated that the decrease in reduced lens glutathione (GSH) concentration has been postulated to cause a conformational change in lens proteins and oxidation of their sulfhydryl groups.
3. As Na^+ concentration increases K^+ concentration decreases within the lens and the synthesis of lens proteins is inhibited. This may

- explain why the dry weight of x-irradiated lenses is lower than unirradiated lenses. This decrease in dry weight could also be due to an increase in protein degradation. Chemical studies of lens membranes have shown that the composition of intrinsic membrane glycoproteins becomes altered during radiation cataract formation.
4. Radiation-induced morphological and biochemical changes cause an increase in the light scattering properties of the lens due to membrane disorganization, vacuole formation and an increase in the difference between the refractive index of the lens proteins and their surroundings.

RATIONALE

Earlier developmental studies in our laboratory indicated that a marked increase in cell volume is associated with lens fiber differentiation (Beebe et al., 1982). Radiation studies suggested that direct damage to the germinative epithelial cells and disturbances in their differentiation into lens fibers are causal agents in cataractogenesis (Poppe, 1942; Cogan and Donaldson, 1951; von Sallmann et al., 1952; Alter and Leinfelder, 1953; Friedenwald and Rytel, 1955; von Sallmann et al., 1955; Kandori, 1956; von Sallmann et al., 1957; Worgul et al., 1976; Palva and Palkama, 1978; Rothstein et al., 1982). Therefore, we studied lens cell volume changes during fiber cell differentiation and cataract formation in x-irradiated early postnatal rat lenses (1, 3 and 5 days). We found that within 24 hrs. after a 15 Gy x-ray dose the volumes of epithelial cells increased significantly. These cells continued to increase in volume when compared to the controls. They differentiated into lens fibers over a 5 day period.

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Three days after x-irradiation the lens fibers underlying these swollen epithelial cells also began to increase in volume. By 5 days these lenses developed diffuse opacities. These preliminary results suggested a correlation between damage to epithelial cell volume regulation and subsequent fiber cell swelling.

To test this hypothesis and to determine whether earlier volume changes in early postnatal rat lenses (1, 3 and 5 days) were complicated by ocular inflammation, a series of experiments were carried out on 4 wk.-old Sprague-Dawley rats. Lenses of these animals were exposed to non-inflammatory doses of 4 and 12 Gy (Worgul and Merriam, 1981). Autoradiography and three-dimensional reconstruction were used to examine fiber cell movement during differentiation and changes in average cell volume of specific lens regions before and during cataract formation. To check for hydration changes of the whole lens after x-irradiation, wet weights of lenses were measured. In addition, protein labeling with [³⁵S]-methionine and separation of lens proteins with SDS-polyacrylamide gel electrophoresis were used to detect what proteins were present and being synthesized in the rat lens throughout radiation cataract formation. Attention was focused on the main intrinsic membrane polypeptide, MP 26. Also, scanning electron microscopy was used to examine the inner cortical and nuclear lens fibers and to determine whether the cortical fibers were making normal contact at the posterior suture region, where radiation cataracts develop.

MATERIALS AND METHODS

One hundred and ninety-two 4 wk.-old Sprague-Dawley rats weighing 80 to 90 gm (Taconic Farms and Charles River) were used. Control and experimental groups consisted of 96 animals each. The eyes of 48 experimental rats were x-irradiated with 4 Gy (1 Gy = 100 rads) and another group of 48 rats was exposed to 12 Gy. For autoradiography studies, 1 day prior to x-irradiation 24 of these experimental rats and 24 controls were injected i.p. with [^3H]-thymidine (New England Nuclear and ICN Pharmaceuticals, Sp. Act. 30 Ci/mmol) at a dose of 1.0 $\mu\text{Ci/gm}$ of body weight in 0.25 ml of sterile water.

Eyes of the remaining 72 experimental and 72 control animals were used for three-dimensional serial reconstruction, wet weight measurement of whole lenses and a pilot study involving protein separation and scanning electron microscopy.

ADMINISTRATION OF X-RAYS

A total dose of either 4 or 12 Gy was administered to each eye at a dose rate of 3.3 to 3.5 Gy/min. and 150 kilovoltage peak (kVp) through 0.25 mm Cu and 0.5 mm Al filters, from a Philips Industrial X-ray Unit, at a source-to-skin distance of 16-20 cm. A Victoreen 0.3 cc probe and Harshaw translucent dosimeters were used to estimate the amount of radiation received by the eye. These measurements indicated that the actual dose was approximately 10% higher than the nominal total dose due to x-ray scatter. Rats were anesthetized by i.p. injection of a sedative mixture containing Atropine sulfate (Lilly), Rompun (Maver-Lockhart) and Ketamine (Parke-Davis). Eyes of anesthetized rats were x-irradiated by

proptosing the eye through an 8 mm hole drilled in a 1 cm thick lead shield. The entire treatment platform, with the exception of the hole, was covered with lead bricks (Fig. 3(a), (b), (c) and see Appendix). During each x-irradiation the contralateral eye received less than 0.05 Gy. This was ignored when calculating the total dose. Animals whose eyes received 4 Gy were killed with an overdose of sodium pentobarbital at 24 hrs.; 5, 15 and 30 wks. and those whose eyes received 12 Gy were killed at 24 hrs.; 3, 5 and 15 wks. Time periods were chosen based on the findings of Merriam and Szechter (1975; Fig. 4). Control littermates were processed similarly to the irradiated rats with the exception of x-ray exposure.

SLIT-LAMP EXAMINATION

Prior to sacrifice both control and experimental animals were examined with a Carl Zeiss photographic bimicroscope (slit lamp). Cataractous changes were graded on a scale of 1+ to 4+ (Merriam et al., 1976; Fig. 5 and see Appendix).

THREE-DIMENSIONAL SERIAL RECONSTRUCTION

To ensure complete fixation of lenses used for three-dimensional reconstruction the posterior part of the globe and the cornea were removed. Lenses were processed according to the methods of Rae et al. (1983) and embedded in Spurr resin (1969; see Appendix). Because of technical difficulties in infiltrating and embedding the lens nucleus, only half of each lens was used. Due to the brittle nature of older lenses it was not possible to serially reconstruct lenses of the 30 wk.-old rats; however, representative single sections were obtained from

these animals.

Control and x-irradiated lenses were serially sectioned at 0.75 μm . Sagittal-sections of these lenses were taken through the central region. Twenty-four serial sections per lens were mounted, stained with 1% toluidine blue and pyronin, and photographed at a magnification of 20X with a Nikon photomicroscope. The epithelium of each lens section was photographed at a magnification of 500X and the equatorial and superficial cortical fibers at 300X. Tissue volume and average cell volumes of these regions were calculated according to Beebe et al. (1982; Fig. 6 and see Appendix). Areas of the lens regions were measured with a Hewlett Packard 9874-A digitizer and calculations were performed on a Hewlett Packard 9835-A computer. The following number of lenses were reconstructed: 16 controls, 9 experimental lenses x-irradiated with 4 Gy, and 15 experimental lenses x-irradiated with 12 Gy.

AUTORADIOGRAPHY

One day prior to x-irradiation 24 experimental (12 rats each for 4 and 12 Gy irradiations) and 24 control lenses were injected i.p. with [^3H]-thymidine (New England Nuclear and ICN Pharmaceuticals, Sp. Act. 30 Ci/mmol) at a dose of 1.0 $\mu\text{Ci/gm}$ of body weight. A total of 16 control and 24 experimental lenses were sagittally sectioned at 2 μm through the central region. Lens sections were mounted on glass slides and dipped in Kodak NTB-2 emulsion. After 8 wks., slides were developed in Kodak D-19, fixed in Kodak rapid fix and stained with 1% toluidine blue and pyronin (see Appendix). Nuclei were considered labeled if more than 4 silver grains covered them.

PROTEIN LABELING AND SEPARATION

An average of 8 to 12 rat lenses were used for each x-irradiation with 4 and 12 Gy. These lenses were incubated for 3 hrs. in 5 ml of Hams medium (with L-glutamine) containing 50 μ Ci/ml of [35 S]-methionine at 3, 5 and 15 wks. after 12 Gy irradiation and 5, 15 and 30 wks. after 4 Gy irradiation. Their lens fiber mass was dissected away from the capsule and epithelium and separated into crystallin, cytoskeletal, and membrane fractions according to Russell et al. (1981; Fig. 7 and see Appendix). These lens fractions were further separated by SDS-electrophoresis. Molecular weight standards used were: myosin (200Kd), β -galactosidase (130Kd), bovine serum albumin (68Kd), catalase (60Kd), ovalbumin (43Kd), chymotrypsinogen (25.7Kd). To be certain that the intrinsic fiber membrane protein MP 26, was isolated in the membrane fraction a membrane pellet was heated prior to being placed onto the gel. After heating MP 26 should aggregate and fail to migrate on the gel (Broekhuysse and Kuchlmann, 1979; Bowman et al., 1980). Autoradiograms were prepared on Kodak x-ray film from dried gels.

SCANNING ELECTRON MICROSCOPY

Eyes exposed to 4 Gy were removed at 5, 15 and 30 wks., and those eyes exposed to 12 Gy were removed at 3, 5 and 15 wks. Lenses were dissected from x-irradiated and control eyes in Puck's Saline G. An average of 4 lenses were used for each irradiation dose and time. They were prepared for scanning electron microscopy according to the methods of Kuzak and Rae (1982; see Appendix). Posterior poles of the lenses were split by applying pressure with a dissecting needle along the

natural fracture lines which occurred during critical point drying. Specimens were mounted on SEM studs and sputter coated with gold (Technics Hummer II). Coated lenses were examined on a JEOL JEM-35 scanning electron microscope at 20 KV.

STATISTICAL ANALYSIS

Standard statistical tests such as mean, standard deviation and variance were calculated for all data. Levels of significance were determined by the two-sample Student's t test. Data were considered significant when the probability of them occurring by chance was less than 5% ($p < .05$). Data from the control groups for eyes x-irradiated with 4 and 12 Gy and fixed 5 and 15 wks. after exposure were pooled. These lenses were taken from rats of the same age and were not statistically different from each other. Initially 2 controls and 2 experimental lenses were reconstructed for each dose and time. When differences were observed additional control and experimental lenses were reconstructed.

RESULTS

NORMAL LENS MORPHOLOGY

When germinative epithelial cells differentiate into lens fibers, they elongate, lose their cuboidal shape and appear as long thin crescents in lens sections. As a result, the nuclei of these lens fibers form a smooth arc, referred to as the lens bow (Fig. 8).

LENS MORPHOLOGY AFTER X-IRRADIATION

Lenses irradiated with 12 Gy showed several morphological differences from unirradiated controls. Twenty-four hrs. after x-irradiation the nuclei of a few epithelial cells in the transitional zone were fragmented (Fig. 9). By 3 wks. some of the lens bow nuclei were being displaced more posteriorly creating a disorganized appearance of the bow region (Fig. 10). In addition, cortical fibers lost their normal tapered, crescent morphology. The posterior portion of these fibers was lighter-staining than the anterior portion. At 5 and 15 wks. after x-irradiation with 12 Gy, disorganization increased in the cells of the lens bow and cortical fiber region. Lens bow nuclei were further displaced toward the posterior lens capsule and the disorganization of the fibers extended into the equatorial region (Fig. 11). At 15 wks. one lens showed multilayered nuclei beneath the lens capsule at the equator and superficial cortical region (Fig. 12).

Lenses irradiated with 4 Gy were not morphologically different from unirradiated lenses 24 hrs. and 5 wks. after x-ray exposure. Cell nuclei of irradiated lenses were not fragmented and lens bow structure

was normal. Cortical fibers of these lenses extended from the anterior epithelium to the posterior capsule in a thin, tapered, crescent.

Subtle morphological differences were observed at 15 wks. in lenses exposed to a 4 Gy dose. By 15 wks. 1 to 2 of the lens bow nuclei moved more posteriorly. These lenses maintained otherwise normal lens bow structure. Their equatorial and cortical fibers continued to show normal morphology (Fig. 13).

SLIT-LAMP EXAMINATION

Cataracts were not graded for every animal used at the designated times of 3, 5, 15 and 30 wks. due to technical difficulties with instrumentation. However, after x-irradiation with 12 Gy, 15 out of the 48 rats examined showed definite signs of 2.5 to 3.0+ cataracts between 10 and 16 wks. (Fig. 5; see Appendix). This type of cataract consisted of vacuoles and opaque dots densely populating the posterior region and sparsely occupying the anterior portion of these lenses. By 32 wks. the entire lens cortex was completely opaque for 8 out of the 15 rats whose eyes showed cataractous changes (Fig. 14). After x-irradiation with 4 Gy, the lenses of 8 out of the 22 rats examined, developed 0.5 to 1.5+ cataracts between 10 and 16 wks. after exposure. These lenses had a few opaque dots and vacuoles at the posterior suture. Of the 36 control rats examined, 3 developed cataractous changes throughout the experimental period. One control rat had bilateral nuclei cataracts and the other two control rats had 0.5+ cataracts (less than 5 vacuoles at the posterior lens pole underneath the lens capsule).

EFFECTS OF X-IRRADIATION ON REGIONAL LENS CELL VOLUME

Equatorial and Cortical Lens Fiber Volume

In control lenses, equatorial and cortical lens fibers increased significantly in volume over a 15 wk. period. Equatorial cells of 19 wk.-old rats were 43% greater in their volume than at 4 wks. of age (Table I; Col. C). Cortical lens fibers were 49% greater in volume at the end of this same 15 wk. period (Table II; Col. C). These changes in average cell volume were significant at $p < .025$. Contrary to controls, lenses irradiated at 4 wks. with 4 or 12 Gy did not show statistically significant increases in average equatorial or cortical fiber cell volume over the 15 wk. period (Tables I and II; Col. D).

However, a striking difference was observed when the average fiber cell volumes of irradiated and control lenses were compared at each interval. Lenses irradiated with 12 Gy showed a transient increase in their cortical fiber volumes at 3 wks. (Table II; Col. D; 12 Gy). These cortical fiber cells were 68% greater in volume than the controls (Graph 1). This increase was significant at $p < .005$. Interestingly, these cortical fibers returned toward control cell volumes by 5 and 15 wks. after x-irradiation (Graph 1) even though they remained morphologically altered (Fig. 11).

Epithelial Cell Volume

An increase in epithelial cell volume of control lenses over the period between 4 and 15 wks. was not observed (Table III; Col. C). The same situation existed for epithelial cells of lenses x-irradiated with

12 Gy (Table III; Col. D; 12 Gy). However, the epithelial cells of rats x-irradiated with 4 Gy at 4 wks. of age increased 32% in volume during the ensuing 15 wks. (Table III; Col. D; 4 Gy). This increase was significant at $p < .025$. During this time epithelial cells at 4 wks. showed a 32% reduction in volume by 9 wks. ($p < .01$; Table III; Col. D; 4 Gy). At 19 wks. these cells increased in volume 97% compared to their volume at 9 wks. ($p < .005$; Table III; Col. D; 4 Gy).

Effects of X-irradiation On Whole Lens Volume

To test whether whole lenses swell in response to x-irradiation, wet weights of irradiated and control lenses were measured. Over 12 wks. control lenses increased in wet weight by 58% (Table IV; Col. A, C). X-irradiated lenses also increased in wet weight, but at a slower rate than did the controls. Lenses x-irradiated with 12 Gy increased by only 28% over 12 wks. (Table IV; Col. A, D; 12 Gy), whereas, lenses exposed to 4 Gy increased 42% over 10 wks. (Table IV; Col. A, D; 4 Gy). The increase in wet weight and the differences in per cent increase were significant at $p < .005$.

Also, further differences were observed when comparing wet weights of x-irradiated and control lenses at each time period. The wet weight of lenses exposed to 12 Gy were reduced 21% ($p < .01$) at 5 wks. after x-irradiation and 14% ($p < .005$) at 15 wks. (Table IV; Col. B, D; 12 Gy). The wet weight of lenses exposed to 4 Gy were reduced 24% ($p < .01$) compared to the controls at 5 wks. (Table IV; Col. B, D; 4 Gy). This was the only time at which significant differences in wet weight were observed in animals x-irradiated with 4 Gy.

AUTORADIOGRAPHY RESULTS

X-irradiation did not appear to inhibit the movement of labeled epithelial cells into the lens fiber region. Both x-irradiated and control lenses had labeled nuclei in the germinative zone 48 hrs. after injection of [^3H]-thymidine (Fig. 15). At 3 wks. labeled nuclei reached the inner part of the lens bow (Fig. 16). By 15 wks. labeled nuclei were no longer detectable in either x-irradiated or control lenses (Fig. 17). One exception was a lens x-irradiated with 4 Gy. Labeled nuclei in this lens reached the deepest part of the lens bow at 15 wks. No label was detectable in any lens 30 wks. after x-ray exposure.

PROTEIN LABELING

Protein separation studies showed that up to 15 wks. after x-irradiation the labeling and accumulation of membrane, crystallin and cytoskeletal proteins was not markedly inhibited after exposure to 4 or 12 Gy. SDS polyacrylamide gels were used to determine the relative accumulation of these proteins in whole lenses at 3, 5, 15 and 30 wks. after x-ray exposure. A major intrinsic membrane protein, MP 26, was present within the membrane fraction of x-irradiated and control lenses for each period examined. This protein appeared in Coomassie-blue-stained gels as a prominent band which migrated near the molecular weight marker chymotrypsinogen (25.7Kd) (Fig. 18). To confirm the identification of this band as MP 26, a membrane fraction was heated prior to its application to the gel. Since MP 26 is a heat-sensitive protein (Broekhuysse and Kuchlmann, 1979; Bowman et al., 1980) partial aggregation of this band at the top of the gel substantiates its identification as MP

26 (Fig. 18). In addition, the lens crystallins (20Kd - 32Kd) and cytoskeletal proteins (43Kd - 56Kd) were identified on the basis of their migration relative to the molecular weight markers carbonic anhydrase (29Kd), ovalbumin (43Kd), and catalase (60Kd) (Fig. 19). These analyses did not reveal any obvious differences between control and x-irradiated lenses. Autoradiograms of the fiber mass of these lenses showed that incorporation of [³⁵S]-methionine into lens crystallins, cytoskeletal and membrane proteins continued after x-irradiation and during cataract formation (between 10 and 16 wks.) (Figs. 19, 20). By 30 wks. autoradiograms of the fiber mass of both control and experimental lenses showed that protein labeling in older lenses had slowed down.

SCANNING ELECTRON MICROSCOPY

A survey of the surface morphology of lenses irradiated with 4 and 12 Gy indicated that deep cortical and nuclear fibers were unaffected by x-irradiation when compared to the controls. These fibers interlocked normally with each other by ball and socket and flap and imprint interdigitations (Maisel et al., 1981; Kuszak et al., 1983; Fig. 21). In addition, they continued to make contact with the posterior lens capsule near the suture planes.

Unfortunately, surface morphology of cells in lens regions previously studied in the three-dimensional reconstructions was difficult to examine. Cells in the equatorial and superficial cortical region fractured through their cytoplasm instead of their membranes and cell to cell surface morphology was, therefore, not visible.

SUMMARY OF RESULTS (see Fig. 23)

1. Lenses irradiated with 12 Gy showed changes in their lens bow and cortical fiber morphology 3 wks. after x-irradiation. At that time, those lenses showed a 68% increase in their cortical lens fiber volume. Although the morphology of the lenses remained altered, cell volumes were not significantly different from control levels 5 and 15 wks. after x-irradiation. By 15 wks. these lenses developed 2.5 to 3.0+ cataracts which became mature (4+) 30 wks. after x-ray exposure.
2. The cells of lenses x-irradiated with 4 Gy did not change significantly in their morphology or cell volume. These lenses developed 0.5 to 1.5+ cataracts 30 wks. after x-irradiation.
3. X-irradiation with 4 and 12 Gy did not result in swelling of the lens, in fact, the increase in wet weight seen during normal growth was reduced in irradiated lenses in a dose-dependent manner.
4. X-irradiation did not inhibit the differentiation of epithelial cells into cortical lens fibers. Thus, those cells which changed in their morphology and volume and became cataractous were, in fact, germinative epithelial cells at the time of x-irradiation.
5. X-irradiation did not completely inhibit the labeling or accumulation of lens crystallins, cytoskeletal proteins or membrane (MP 26) proteins.
6. The surface morphology of inner cortical and nuclear fibers of irradiated lenses was unaffected by x-ray exposure.

DISCUSSION

CELL VOLUME MEASUREMENTS OF SPECIFIC LENS REGIONS

Proper hydration of the lens is essential for its transparency. Hydration changes of whole lenses, as measured by wet weight, have been observed in radiation, galactosemic and some types of hereditary cataracts (Pirie et al., 1953; Giblin and Reddy, 1978; Giblin et al., 1979; Matsuda et al., 1980, 1982; Kador et al., 1980; Piatigorsky et al., 1980). In the present study three-dimensional serial reconstruction was used to measure cell volume changes prior to and during radiation cataract formation. The average volume of cells in the epithelial, equatorial, and superficial cortical regions was measured with this technique, which is more sensitive than wet weight measurements of whole lenses.

Lenses X-irradiated With 12 Gy

A transient increase in cortical fiber volume was observed in lenses 3 wks. after x-irradiation with 12 Gy. This local swelling occurred at the time these cortical fibers lost their normal morphology without causing observable changes in light scattering. They lost their long, tapered, crescent appearance and became irregular in size and shape. In addition, their nuclei moved more posteriorly and the lens bow lost its smooth arc-like appearance. Although these cortical fibers remained irregular in size and shape, their cell volumes approached that of control values at 5 and 15 wks. after x-ray exposure. Between 10 and 16 wks., changes in light-scattering were observed in these lenses.

Epithelial and equatorial cell volume of these lenses were not significantly different from controls throughout the experimental period.

These results suggest that a transient, localized increase in cortical fiber volume, prior to cataract formation, may cause the morphological disruption seen in these cells. Alternatively, the loss of cellular organization observed in the cortical fibers may alter intercellular communication, membrane structure or some other critical aspect of the system used by these cells to maintain their volume. The possibility also exists that changes in fiber cell volume and morphology are unrelated. Since fiber cells continue to form after x-irradiation (Worgul and Rothstein, 1976; Bredehoft, 1982; Bredehoft and Beebe, 1982), those cortical fibers, which changed in cell volume and morphology at 3 wks. migrated toward the center of the lens by 15 wks. By this time the lens cortex contained newer lens fibers, with abnormal morphology but with normal cell volume. This situation makes it difficult to draw the simple conclusion that transient changes in fiber cell volume are responsible for alterations in fiber morphology or the reverse.

Lenses X-irradiated With 4 Gy

Lenses exposed to 4 Gy developed subtle cataractous changes between 10 and 16 wks. after x-irradiation. During this time, they were not significantly different from control lenses in their morphology or volume. These findings suggest that the transient swelling and altered fiber morphology observed in lenses x-irradiated with 12 Gy are dose-dependent phenomena. In addition, early cataractous changes seem to be able to occur without major alterations in morphology or cell volume.

This is a significant finding since it is thought that a cataract represents the end stage of injury to the dividing and differentiating lens cell population (Poppe, 1942; Cogan and Donaldson, 1951; von Sallmann et al., 1955, 1957; Scullica et al., 1962; Hanna and O'Brien, 1963; Worgul and Rothstein, 1977). Ideally, rat lenses x-irradiated with 4 Gy may represent the situation where a certain number of photon hits to the germinative epithelial cells were sufficient to cause observable light scattering changes. In this situation, the chance of looking at initial cataractous changes due to a primary effect is highly probable. This is supported by the finding that throughout their lifetime 4 wk.-old rats do not develop severe opacities (3+, 4+) when exposed to doses of 2 and 4 Gy (Merriam and Szechter, 1975).

When comparing the results obtained from lenses x-irradiated with 4 Gy to those of 12 Gy, the possibility exists that a larger number of photons may affect already existing lens fibers, as well as other parts of the epithelium besides the germinative zone. In these lenses, alterations in cell structure may represent the summation of a number of influencing factors in addition to the primary effect of x-rays on the epithelium. Therefore, lenses x-irradiated with 4 Gy represents a suitable model for looking at a primary causal agent in cataract formation. Additional studies of cataract formation should be pursued at this dose; however, a subcellular approach should be utilized since light scattering changes occurred without a detectable change in lens cell structure.

LENS CELLS CONTINUE TO MOVE AFTER X-IRRADIATION

The movement of cells undergoing DNA synthesis at the time of

x-irradiation was examined by autoradiography. Labeled nuclei of control and x-irradiated rat lenses were located in the germinative zone 48 hrs. after injection of [³H]-thymidine. Their labeled nuclei reached the inner bow region within 3 wks. By 15 wks. no labeled nuclei were detected in these lenses. Nuclei of these mature lens fibers became pyknotic and degenerated. These results indicate that x-irradiation does not inhibit fiber differentiation in 4 wk.-old rat eyes exposed to 4 and 12 Gy. From these results, it can be concluded that those cortical fiber cells, which transiently increased in volume and permanently changed in their morphology, were germinative epithelial cells at the time of x-irradiation. Similar findings were obtained for x-irradiated and unirradiated lenses of early postnatal (1, 3 and 5 days; Bredehoft, 1982; Bredehoft and Beebe, 1982) and 4 wk.-old rats (Worgul et al., 1976). Regardless of x-ray exposure in these animals it took a differentiating epithelial cell 5 and 7 days respectively, to reach the arc of the lens bow. These autoradiography results are significant because they connect the morphological changes in x-irradiated lens fibers with the primary site of x-ray damage, the germinative epithelial cells. Results from all 3 studies provide additional evidence that disturbances in fiber differentiation may be involved with cataract formation (Alter and Leinfelder, 1953; Cogan et al., 1953; Leinfelder and Dickerson, 1960; Worgul et al., 1975, 1976; Rothstein, 1980).

Contrary to the above findings, epithelial cells of 4 wk.-old rat lenses, irradiated with 24 Gy of gamma rays, did not enter the lens cortex until 8 wks. after exposure. At this time cataracts in these lenses were in advance stages of opacity (Hanna and O'Brien, 1963). In addition, labeled cells in the germinative zone of 40 wk.-old rat lenses

moved only a short distance after gamma irradiation before the [^3H]-thymidine label was lost. No labeled cells in these adult rats were found in the cortex at the time the clinical cataract was visible. These results indicated that irradiation slows the movement of germinative epithelial cells into the lens cortex and suggest that already existing lens fibers express radiation damage before the irradiated germinative epithelial cells ever reach the cortex.

It is difficult to explain the disparity between the rate of migration of labeled, irradiated germinative epithelial cells. One could think that the difference would be due to the strain of rat used. However, Worgul et al. (1976) repeated the work of Hanna and O'Brien (1963) using their 4 wk.-old Wistar strain rat and his own Columbia Sherman rat and found that these two strains displayed similar rates of fiber formation. Despite the differences that may exist as to whether x-rays slow down the rate of fiber formation, all 4 studies showed that x-irradiation does not block the process of fiber differentiation.

WHOLE LENSES DO NOT SWELL DURING EARLY AND MIDDLE STAGES OF CATARACT FORMATION

Three-dimensional serial reconstruction results showed that, overall, selected regions of x-irradiated lenses do not swell. These results suggested that x-irradiated lenses as a whole do not grossly swell during early and mid-cataract formation. Wet weight determinations confirmed that the entire lens does not swell after x-irradiation. In fact, lenses x-irradiated with 12 Gy showed a significant reduction in wet weight at 5 and 15 wks. after exposure. Lenses irradiated with 4 Gy had lower wet weights than controls at 5 wks. only. These results

indicate that the increase in wet weight seen during normal growth was retarded in x-irradiated lenses in a dose-dependent manner.

X-IRRADIATED LENSES CONTINUE TO INCORPORATE [^{35}S]-METHIONINE INTO PROTEINS

A decrease in lens growth rate (as measured by wet weight) appears to be a common feature in radiation (Pirie et al., 1953; Giblin et al., 1978, 1979; Matsuda et al., 1982), diabetic, galactosemic (Kinoshita, 1965) and certain kinds of hereditary cataracts (Kador et al., 1980; Iwata and Kinoshita, 1971). It has been suggested that this reduction in growth could be caused by a decrease in protein synthesis, an increase in proteolysis and/or a leakage of proteins from the hydrated lens (Kador et al., 1979; Shinohara et al., 1982; Garadi et al., 1984).

In the present study, stained gels and autoradiograms showed that rat lenses continued to accumulate protein and incorporate radioactive label after x-irradiation and during cataract formation. However, quantitative statements cannot be made about increases or decreases in the incorporation of radioactive label into proteins of x-irradiated lenses compared to the controls. The amino acid pool size, which affects the amount of radioactivity incorporated into protein was not determined in these experiments. Also, a decrease in the intensity of labeled protein bands was not consistently seen in autoradiograms of x-irradiated lenses compared to the controls. At times experimental lens proteins produced darker banding patterns than the controls and the reverse. This was confirmed by the fluctuating increase and decrease in the radioactive counts obtained for the protein fractions of experimental lenses. The variability in these data may be due to variation in the lenses of

different aged rats and/or the experimental technique used for protein separation. However, at 30 wks. after x-irradiation both control and experimental lenses lacked detectable bands on their autoradiograms. This was somewhat expected since protein synthesis in older lenses slows down.

Overall, these results suggest that x-irradiation does not significantly inhibit the accumulation or labeling of lens fiber proteins in cataractous rat lenses. In addition, from these data, the reduction in wet weight of x-irradiated lenses is not likely to be caused by the leakage of proteins from the hydrated lens, since whole x-irradiated lenses did not undergo gross swelling during cataract formation. A decrease in growth rate of these lenses may be caused by an increase in proteolysis or the leakage of proteins from damaged but not swollen lens fibers. Further studies are needed to test these possibilities.

In contrast to this present finding, Garadi et al. (1984) found that the incorporation of [^{35}S]-methionine into lens fiber cell proteins was reduced within 4 wks. in rabbit lenses x-irradiated with 20 Gy. By the end of 7 wks. post-irradiation, protein incorporation in irradiated rabbit lenses decreased to 20% of the control value. At this time, autoradiograms of x-irradiated lenses did not show any labeled bands while controls showed abundant incorporation. These animals developed complete opacities between 8 and 9 wks. after x-ray exposure. Although their data was quantitative and they did determine the specific activity (cpm/ μg protein) of [^{35}S]-methionine incorporated into lens proteins as a function of time after x-irradiation, they did not measure the specific activity (cpm/nmole of [^{35}S]-methionine) of the intracellular pool. Intracellular pool size is important, since it permits an accurate

measure of how much protein is synthesized within the normal lens cells. Without knowing actual amino acid pool size an increase in protein incorporation after x-irradiation could be the result of a decreased intracellular pool size and a decrease in protein synthesis may be due to a larger intracellular pool size. Before any quantitative statements are made about protein synthesis the intracellular amino acid pool size must be determined.

INFLAMMATION IS A COMPLICATING VARIABLE AFTER X-IRRADIATION

An additional explanation for the differences between these two sets of data is that rat and rabbit eyes respond differently to x-rays. Rabbits have been found to develop inflammatory changes, such as engorged iridial blood vessels, increased intraocular pressure and leukocytes in the anterior chamber soon after exposure to doses of x-ray as low as 2.5 Gy (Worgul et al., 1977). This appeared to be true for the rabbits used in Matsuda's studies (1980, 1981, 1982). After the eyes of the rabbits, used by Matsuda et al., were x-irradiated with 20 Gy, they developed inflammatory changes for the first 5 days after exposure (Reddy, personal communication). Unlike rabbits, rats have been found to be refractory to inflammation when exposed to doses of x-rays from 2 to 12 Gy (Worgul and Merriam, 1981). Rat eyes used in the present study did not show external signs of inflammation when exposed to doses of 4 or 12 Gy. It has also been shown that, by itself, inflammation may cause cataracts which morphologically resemble radiation cataracts (Worgul and Merriam, 1979). Based on these observations, it is possible that rabbits, unlike rats, may develop cataractous changes primarily due to inflammation and only secondarily to x-irradiation. This is supported by the fact that

4

after irradiation rabbit lenses developed mature cataracts many weeks earlier than rat lenses. Complications due to inflammation may be responsible for the differences observed in [^{35}S]-methionine incorporation into lens fiber proteins between x-irradiated rat and rabbit lenses.

VOLUME CHANGES IN EARLY POSTNATAL RAT LENSES MAY BE COMPLICATED BY INFLAMMATION

Another situation where inflammation may be a complicating variable is in the early postnatal rat lens (1, 3 and 5 days). In preliminary studies (Bredehoft, 1982), the epithelial cells of these lenses significantly increased in cell volume within 24 hrs. after x-irradiation with 15 Gy. These cells continued to increase in volume for 5 days. The lenses of these animals developed mature cataracts within 1 month after x-irradiation. This was not true for 4 wk.-old rats. These animals were found to be refractory to inflammatory changes below doses of 18 Gy (Worgul and Merriam, 1981). As shown in this study, epithelial cells of post-weanling rats did not significantly increase in volume during cataract formation. Mature cataracts developed in these animals at 32 wks. after x-irradiation with 12 Gy. These findings suggest that the volume changes observed in early postnatal rats were complicated by ocular inflammation. The initial increase in epithelial cell volume of the early postnatal rats lenses was possibly the result of inflammation following damage to the numerous mitotic cells in their radiosensitive retinas. X-ray damage to these cells leads to inflammation throughout the eye.

RADIATION CATARACTS MAY NOT BE OSMOTIC IN NATURE

Three-dimensional reconstruction data suggests that disturbances in fiber differentiation did not involve obligatory changes in epithelial or fiber cell volume which would lead to gross swelling of the entire lens as in osmotic-type cataracts (Friedenwald and Rytel, 1955; Piatigorsky et al., 1977; Kador et al., 1980). Previously, a dramatic change in lens hydration has been observed in sugar (Kinoshita, 1965; Kador et al., 1979) hereditary (Iwata and Kinoshita, 1971; Piatigorsky et al., 1978; Kador et al., 1980) and radiation cataracts (Friedenwald and Rytel, 1955; Lambert and Kinoshita, 1967; Garadi et al., 1984). Although the term osmotic has been used to refer to these cataracts, the rate at which gross accumulation of water occurs varies in these cataractous lenses. Sugar and hereditary cataracts develop due to an accumulation of osmotically active substances such as sugar and cations, respectively (Kinoshita, 1965; Piatigorsky et al., 1978; Kador et al., 1980). The hypertonicity created by these substances is immediately corrected by an influx of water causing vacuolization and complete lens opacification. In radiation cataracts increased lens hydration is not a primary causal agent. Initially alterations in enzyme activity and fiber membrane structure lead to light scattering changes long before the accumulation of water in the lens. Only at the time of mature radiation cataract formation has a change in cation permeability and lens hydration been noted (von Sallmann and Locke, 1951; Matsuda et al., 1982). The present findings on cell volume changes indicate that in radiation cataracts, unlike the other osmotic cataracts, the lens can develop advance cataractous changes (3+, see Appendix) without a change in its total

volume. This suggests that radiation cataracts are not any more "osmotic" than various other non-osmotic cataracts, including senile, cataracta complicata and steroid cataracts, whose end stages also result in an increase in lens water. Therefore, radiation cataracts should not be grouped with some hereditary and galactose cataracts, which are truly osmotic in nature.

DAMAGED LENS FIBERS RETURN TOWARD NORMAL CELL VOLUME

The findings presented here on radiation cataracts raise the question: What is responsible for the abnormal fiber morphology seen in lenses of animals x-irradiated with 12 Gy? One explanation is that after x-irradiation epithelial cells differentiated into lens fibers which transiently increased in their volume. This transient increase in cortical fiber volume may have caused these fibers to lose contact posteriorly, and round up into irregular sizes and shapes. To test this possibility the surface morphology of rat lens cells was examined at 3, 5, 15 and 30 wks. after x-irradiation. Irradiated lens fibers had the same type of complex interdigitations at their club-like terminal endings as control lenses. At times, the posterior ends of both x-irradiated and control fibers appeared to be globular in nature. This finding differed from that of Rini et al. (1984) who observed that only the ends of lens fibers irradiated with 12 Gy appeared as ellipsoidal or globular bodies. In addition, they found that subcapsular cortical fibers were edematous and devoid of surface morphology.

Although irradiated epithelial cells differentiated into morphologically-altered lens fibers which appeared to make contact at the posterior lens pole, this may not be true. These altered cortical fibers

may not make contact posteriorly, leaving a gap under the posterior capsule. This situation would force neighboring lens fibers to redistribute their volume and fill in the gaps left by the altered lens fibers (Fig. 22(A)). As a result, normal contact at the posterior lens pole appears to be maintained. This view helps to explain how morphologically-altered lens fibers can return toward normal cell volume.

Another possibility is that damaged epithelial cells differentiate into lens fibers which are able to extend anteriorly and posteriorly toward the suture regions; however, x-irradiation could force these fibers to redistribute their volume and develop "varicose" structures on their lateral surfaces. When these fibers are cut longitudinally these varicosities give the fibers the appearance of being irregular in size and shape (Fig. 22(B)).

Finally, radiation damaged epithelial cells may differentiate into abnormal lens fibers which do not extend all the way up to the overlying epithelium but maintain normal contact posteriorly. These damaged fibers will fill the posterior part of the lens cortex by crowding and squeezing neighboring fibers (Fig. 22(C)). When these fibers are sectioned they would give a disorganized appearance. In addition, as these damaged fibers fail to make contact anteriorly their nuclei would be displaced posteriorly. This would explain the posterior movement of the nuclei in the bow region seen after x-irradiation. Further morphological studies are needed to test these possibilities.

These additional morphological studies will require a clear outline of whole, individual lens fiber membranes. This could be obtained by incubating intact x-irradiated lenses in histochemical

reagents or fluorescent dyes like horseradish peroxidase or procion yellow. These reagents stain the extracellular spaces between fiber cells. Serial sections could be cut from these lenses and individual fiber cells could be reconstructed at critical times during cataract formation. A more selective technique would involve iontophoresing procion dyes into individual lens fibers through a glass capillary microelectrode according to the method of Rae (1974). This technique would also allow examination of adjacent lens fibers since these dyes readily cross electrotonic junctions between cells. By specifically localizing damage to individual lens cells the mechanism responsible for the cellular disorganization seen in several kinds of cataracts could be better studied.

SUMMARY

By selectively measuring cell volumes in specific lens regions with three-dimensional serial reconstruction the following has been shown:

1. The initial hypothesis suggesting a correlation between damage to epithelial cell volume regulatory mechanism and subsequent fiber cell swelling was not proven; the preliminary data which lead to the hypothesis were probably complicated by ocular inflammation.
2. After x-irradiation with 12 Gy cortical lens fibers showed a transient increase in their volume and a change in their morphology prior to any cataractous changes. During cataract formation these cortical fibers returned toward normal cell volume while their organization remained abnormal.

3. Although subtle changes in light scattering developed (0.5 to 1.5+ cataract) after x-irradiation with 4 Gy, cortical lens fiber morphology and volume were not significantly altered compared to the controls.
4. X-irradiation does not appear to inhibit fiber differentiation, nor markedly alter the accumulation and synthesis of lens proteins, nor cause gross swelling of the lens during early and middle stages of cataract formation.

Fig. 1. A Schematic illustration of how x-rays are generated from an x-ray machine. X-rays are produced when high-speed electrons from a cathode ray tube hit an inner electron of a tungsten target and knock this electron out of its orbit. The ejected electron is replaced by another electron from an outer shell of the tungsten target causing x-rays to be emitted. The energy of the x-ray represents the difference in energies of the inner and outer orbital electron levels. Modified from A. P. Casarett (1968).

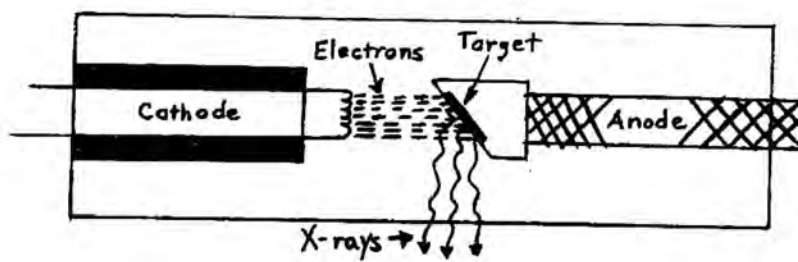


Fig. 1

Fig. 2. Schematic illustration of a section taken from a rat lens.

Laterally the lens fibers are cut longitudinally (arrow). At the top of this illustration the lens fibers are shown in cross-section (arrowhead). Different regions of the epithelium are labelled: central epithelial zone cells (CZ) are amitotic; germinative zone cells (GZ) are mitotically active cells, which become transitional zone cells (TZ). In the transitional zone, cells align into meridional rows (MR) prior to differentiating into lens fibers at the equator. These new fibers make up the lens cortex and their nuclei form the lens bow. As new fibers are added at the equator, older fibers within the cortex are internalized. The oldest lens fibers, form the region known as the nucleus, which is located in the center of the lens. Modified from Broglio and Worgul (1982).

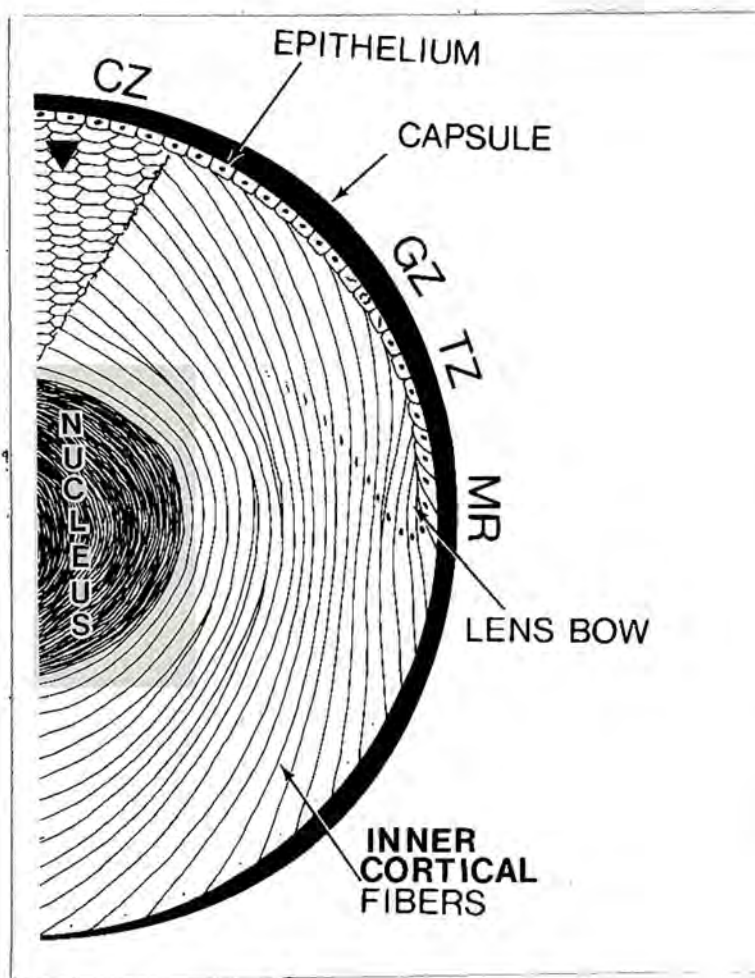


Fig. 2

Fig. 3. Device used to expose rats to x-rays. (a) Top view of acetate stand supporting a 1 cm thick lead shield drilled with an 8 mm hole. (b) Treatment platform, again shown from above, now covered with lead bricks except for the 8 mm hole (arrowhead). (c) Ortho-ray tube of x-ray unit (arrow) was placed directly over the hole at a distance of 20 cm from the proptosed eye of a rat, which had been placed onto a laboratory jack.

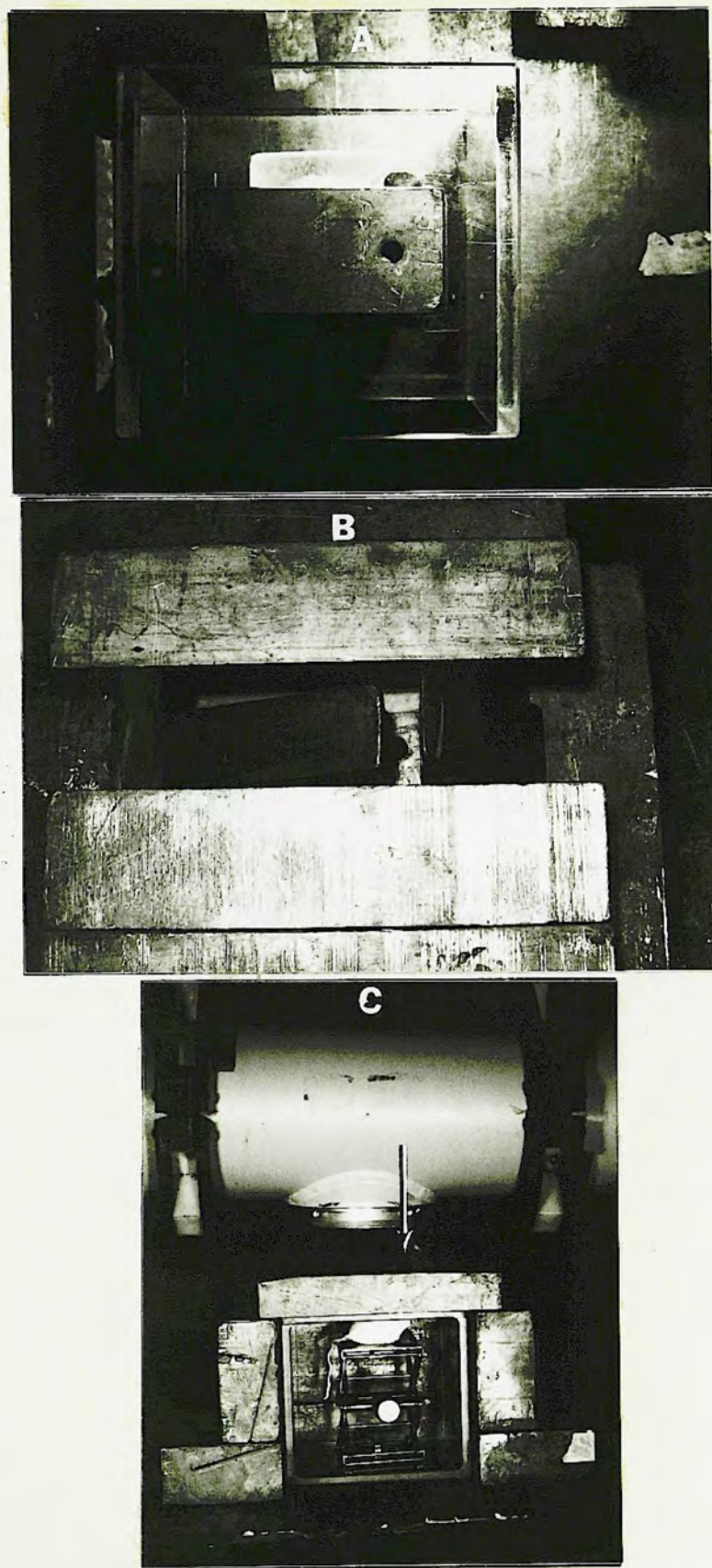


Fig. 3

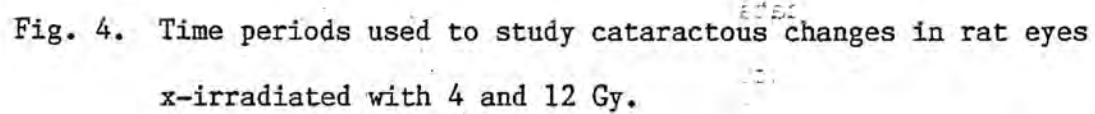


Fig. 4. Time periods used to study cataractous changes in rat eyes x-irradiated with 4 and 12 Gy.

TIME PERIODS FOR CATARACT FORMATION

- 24 hrs. - prior to cataract formation, soon after x-irradiation
- 3 wks. - prior to cataract formation for 12 Gy
- 5 wks. - prior to cataract formation for 4 Gy;
early cataract formation for 12 Gy
- 15 wks. - cataract formation for 12 Gy;
early cataract formation for 4 Gy
- 30 wks. - cataract formation for 4 Gy

* Based on the findings of G. R. Merriam and A. Szechter

Fig. 4

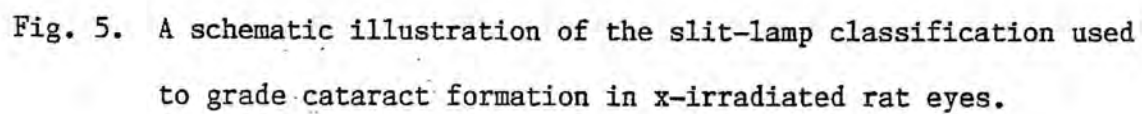
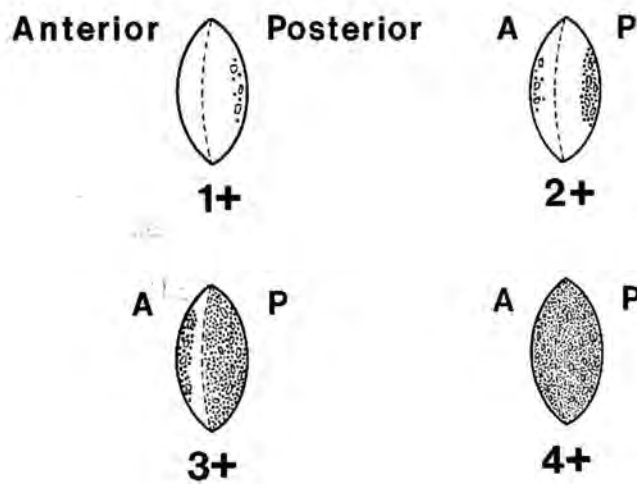


Fig. 5. A schematic illustration of the slit-lamp classification used to grade cataract formation in x-irradiated rat eyes.

CATARACT CLASSIFICATIONS *



*based on the findings of G.R. Merriam Jr. & A. Szechter

Fig. 5

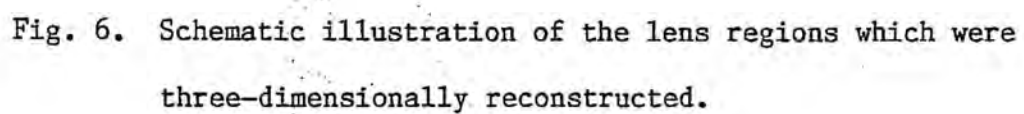
The figure is a schematic illustration of lens regions, which were three-dimensionally reconstructed. It is located in the center of the page, above the caption. The illustration itself is very faint and appears to be a collection of small, scattered points or a very light, irregular shape, possibly representing a lens or a cluster of points in a 3D reconstruction. It is not clearly defined and blends into the background.

Fig. 6. Schematic illustration of the lens regions which were three-dimensionally reconstructed.

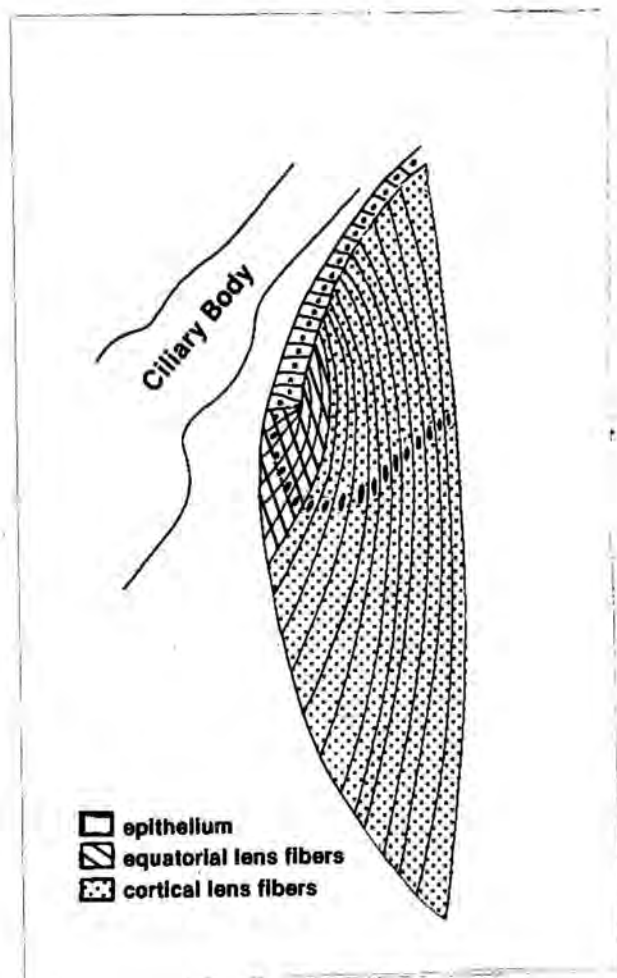
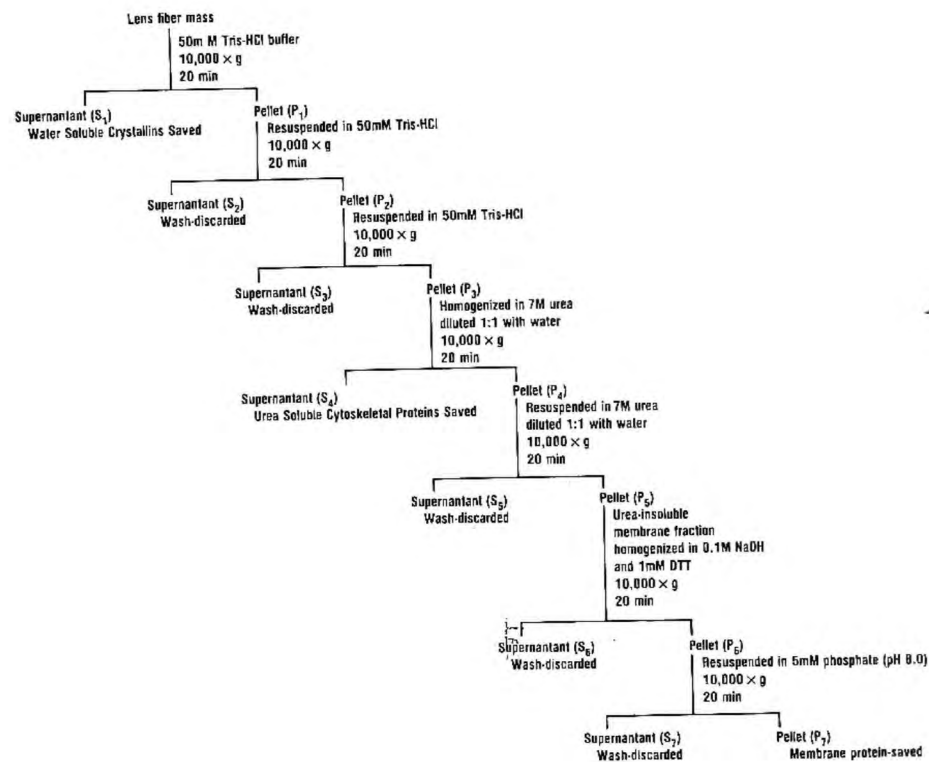


Fig. 6

Fig. 7. Flow chart of the separation technique used to isolate the crystallin, cytoskeletal, and membrane fractions of rat lenses.

Fig. 7



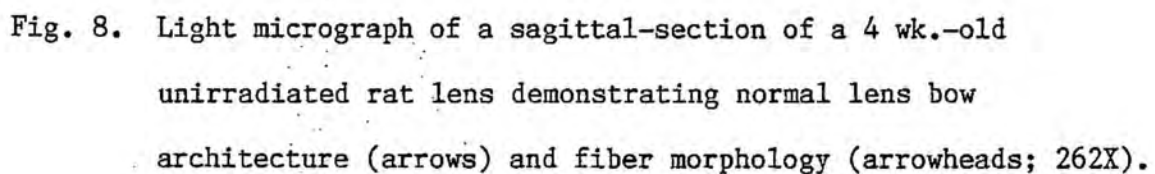


Fig. 8. Light micrograph of a sagittal-section of a 4 wk.-old unirradiated rat lens demonstrating normal lens bow architecture (arrows) and fiber morphology (arrowheads; 262X).

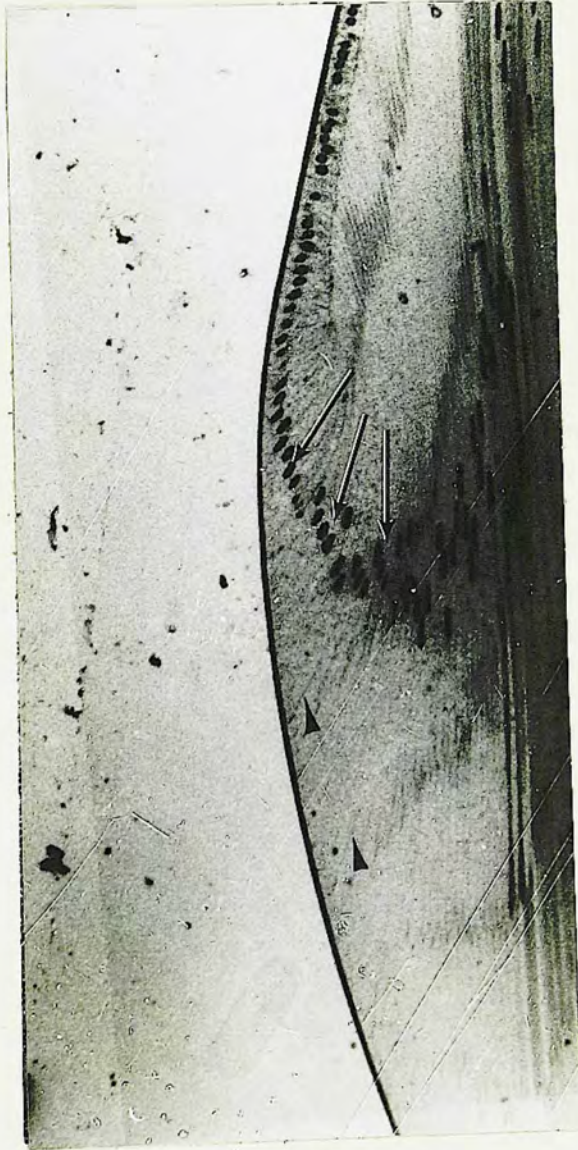


Fig. 8

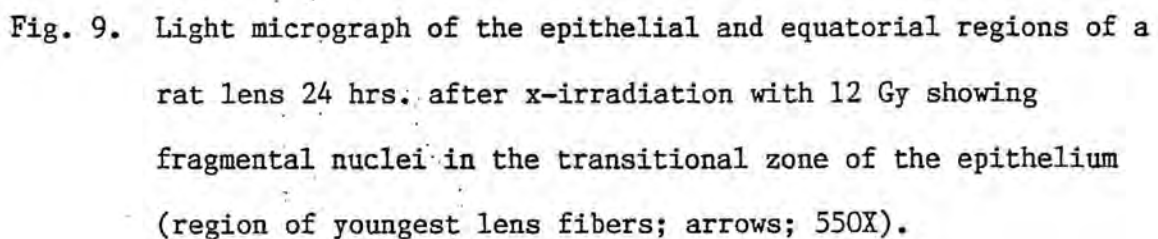


Fig. 9. Light micrograph of the epithelial and equatorial regions of a rat lens 24 hrs. after x-irradiation with 12 Gy showing fragmental nuclei in the transitional zone of the epithelium (region of youngest lens fibers; arrows; 550X).

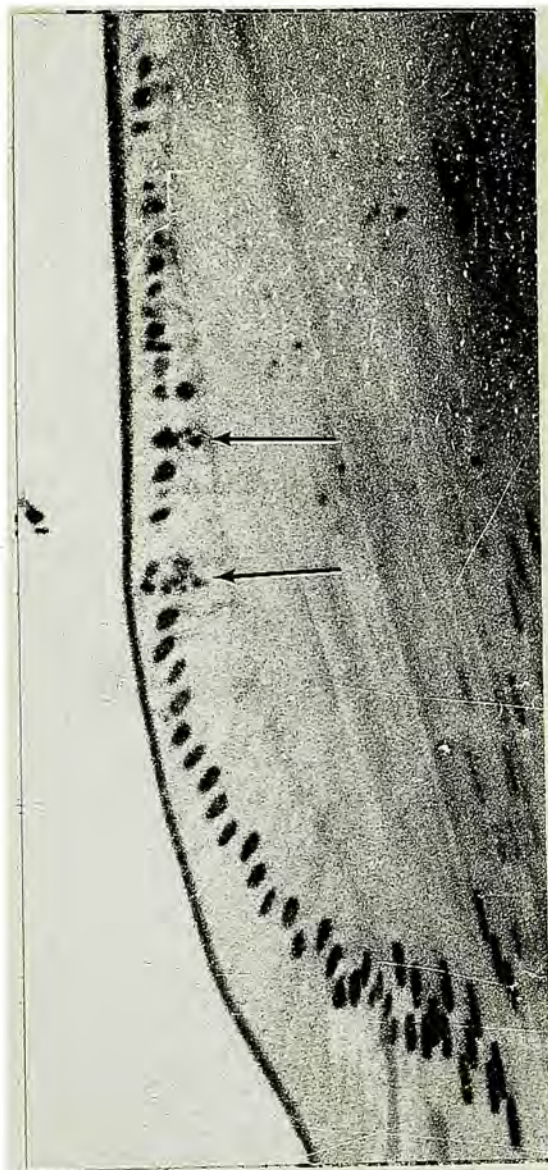


Fig. 9

Fig. 10. Light micrograph of a sagittal-section of a rat lens 3 wks. after x-irradiation with 12 Gy showing displacement of the lens bow nuclei toward the posterior lens pole (arrows) and the loss of the smooth, tapered, crescent appearance of the superficial cortical lens fibers (arrowheads; 262X).



Fig. 10

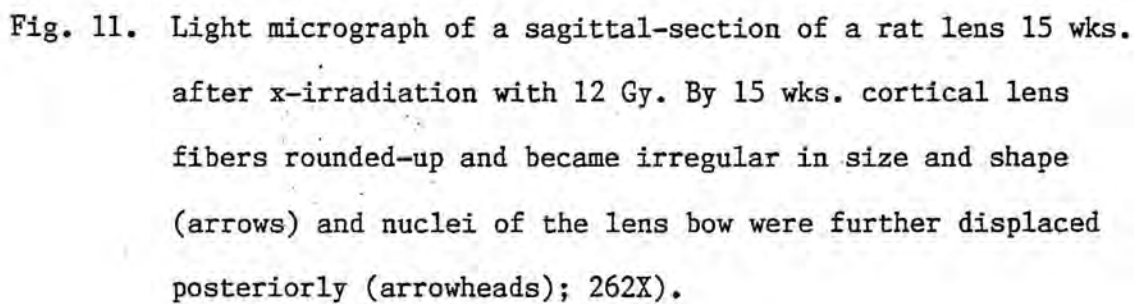


Fig. 11. Light micrograph of a sagittal-section of a rat lens 15 wks. after x-irradiation with 12 Gy. By 15 wks. cortical lens fibers rounded-up and became irregular in size and shape (arrows) and nuclei of the lens bow were further displaced posteriorly (arrowheads); 262X).



Fig. 11

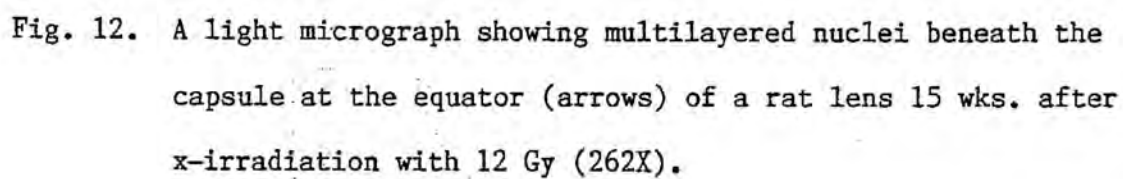


Fig. 12. A light micrograph showing multilayered nuclei beneath the capsule at the equator (arrows) of a rat lens 15 wks. after x-irradiation with 12 Gy (262X).



Fig. 12

Fig. 13. A light micrograph of a sagittal-section of a rat lens 15 wks. after x-irradiation with 4 Gy. One to two lens bow nuclei (arrows) moved more posteriorly while equatorial and cortical fibers maintained normal morphology (132X).



Fig. 13

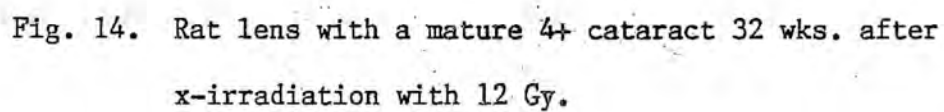


Fig. 14. Rat lens with a mature 4+ cataract 32 wks. after
x-irradiation with 12 Gy.

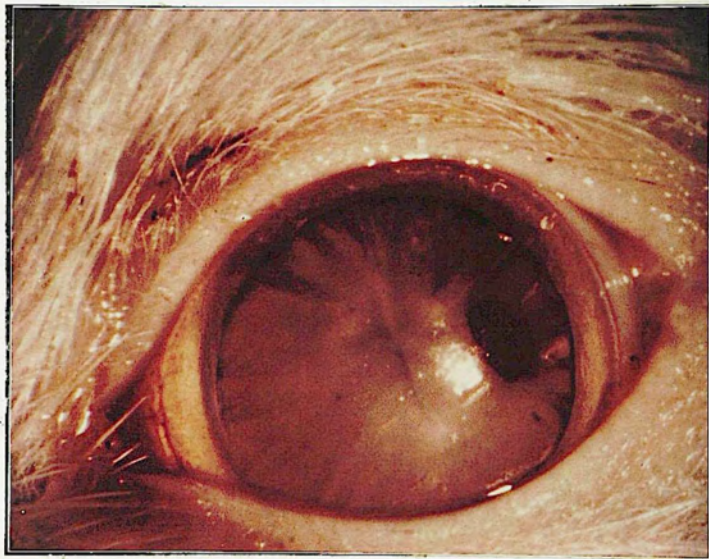


Fig. 14

Table I

3
Average Equatorial Cell Volume (μm $\pm$ SD)

A. Age	B. Post-irradiation Time	C. Control Lenses	D. Irradiated Lenses	
			4 Gy	12 Gy
4 wks.	24 hrs.	1,576 $\pm$ 422	1,708 $\pm$ 179	1,759 $\pm$ 208
7 wks.	3 wks.	1,888 $\pm$ 827	—	2,650 $\pm$ 483
9 wks.	5 wks.	1,524 $\pm$ 891	1,129 $\pm$ 1,562	1,778 $\pm$ 618
19 wks.	15 wks.	*2,258 $\pm$ 74	2,833 $\pm$ 925	1,886 $\pm$ 566

* This 43% increase in control equatorial cell volume of 19 wk.-old rats is significant at $p < .025$ compared to the control values for 4 wk.-old rats.

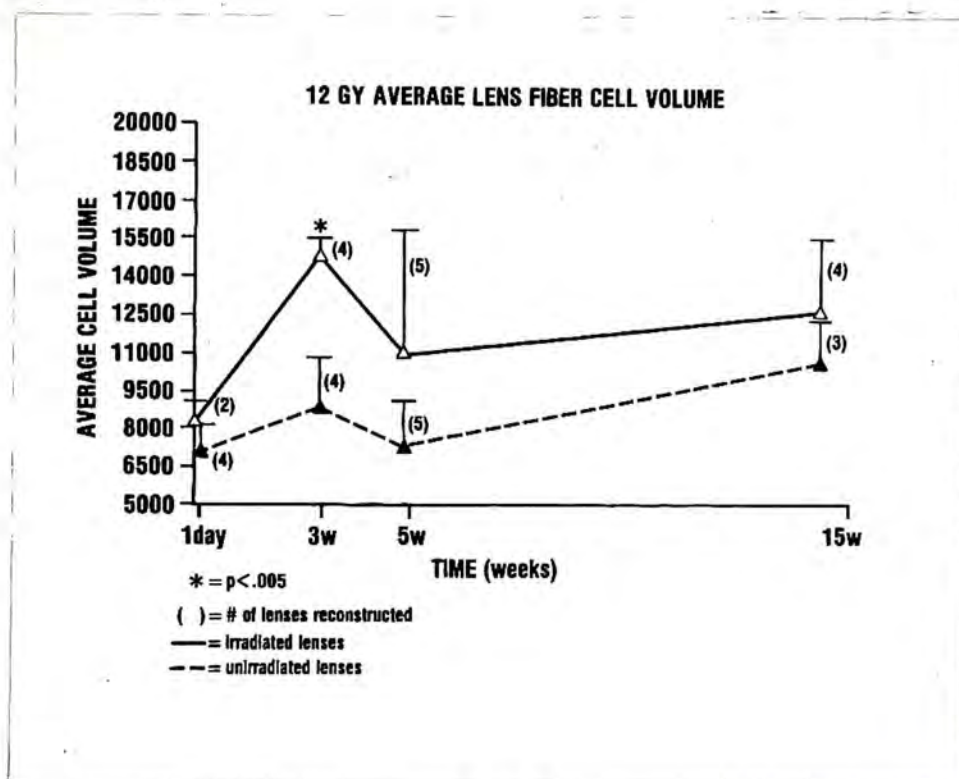
Table II

Average Cortical Lens Fiber Volume ($\mu\text{m} \pm \text{SD}$)

A. Age	B. Post-irradiation Time	C. Control Lenses	D. Irradiated Lenses	
			4 Gy	12 Gy
4 wks.	24 hrs.	7,155 $\pm$ 993	8,710 $\pm$ 2,453	8,317 $\pm$ 737
7 wks.	3 wks.	8,851 $\pm$ 2,016	-----	*14,870 $\pm$ 663
9 wks.	5 wks.	7,317 $\pm$ 1,765	6,592 $\pm$ 1,301	10,996 $\pm$ 4,910
19 wks.	15 wks.	*10,635 $\pm$ 1,830	14,131 $\pm$ 5,728	12,695 $\pm$ 2,863

* This 49% increase in control cortical fiber cell volume of 19 wk.-old rats is significant at $p < .025$ compared to the control values for 4 wk.-old rats.

* This 68% increase in cortical fiber cell volume at 3 wks. after x-irradiation with 12 Gy is significant at $p < .005$ compared to unirradiated controls.



Graph 1

Table III

3
Average Epithelial Cell Volume (μm $\pm$ SD)

A. Age	B. Post-irradiation Time	C. Control Lenses	D. Irradiated Lenses	
			4 Gy	12 Gy
4 wks.	24 hrs.	495 $\pm$ 129	491 $\pm$ 18	516 $\pm$ 46
7 wks.	3 wks.	540 $\pm$ 69	-----	555 $\pm$ 161
9 wks.	5 wks.	460 $\pm$ 207	*331 $\pm$ 25	506 $\pm$ 113
19 wks.	15 wks.	561 $\pm$ 84	*652 $\pm$ 76	583 $\pm$ 157

* This 97% increase in epithelial cell volume of 9 wk.-old rat lenses irradiated with 4 Gy is significant at $p < .005$ compared to the values obtained for 19 wk.-old rat lenses.

* This 32% increase in epithelial cell volume of 19 wk.-old rat lenses irradiated with 4 Gy is significant at $p < .025$ compared to the values obtained for 4 wk.-old rat lenses.

Table IV

Average Wet Weight (mg $\pm$ SD)

A. Age	B. Controls	C. Post-irradiation Time	D. Irradiated Lenses	
			4 Gy	12 Gy
7 wks.	30 $\pm$ 2	3 wks.	—	31 $\pm$ 2
9 wks.	39 $\pm$ 6	5 wks.	32 $\pm$ 2	32 $\pm$ 2
19 wks.	46 $\pm$ 3	15 wks.	45 $\pm$ 3	40 $\pm$ 2
34 wks.	54 $\pm$ 5	30 wks.	53 $\pm$ 6	—

Fig. 15. A dark-field micrograph of labeled nuclei (arrows) found across from the ciliary body (CB) in the germinative epithelium of an x-irradiated rat lens 48 hrs. after injection of [^3H]-thymidine (1200X). Similar results were observed in control lenses. The high background, due to experimental technique, did not obscure labeled nuclei. When sections were focused through these nuclei more than 4 silver grains were located directly on top of them.

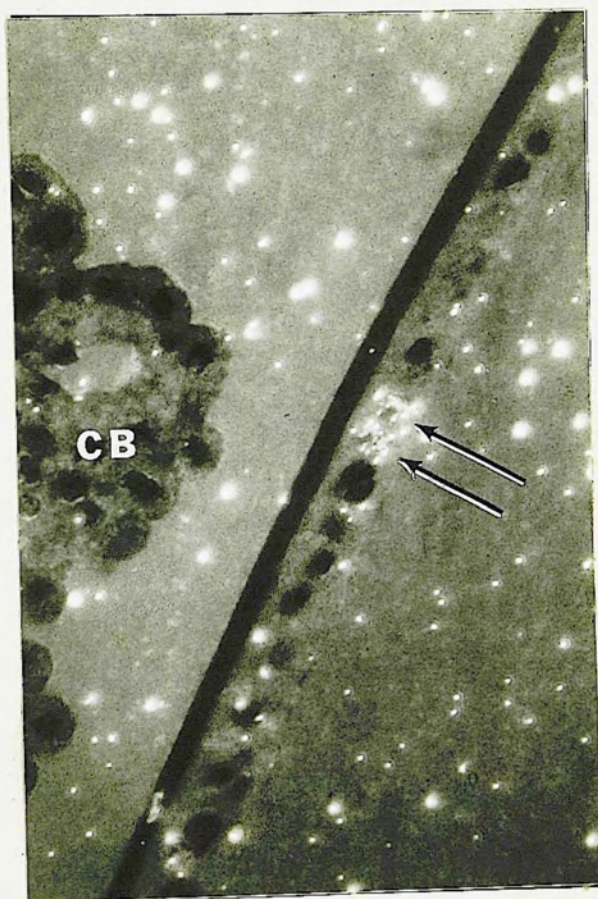


Fig. 15

Fig. 16. A dark-field micrograph of labeled nuclei (arrows) in the inner part of the bow region of a control rat lens 3 wks. after injection of [^3H]-thymidine (440X). Similar results were observed in irradiated lenses.

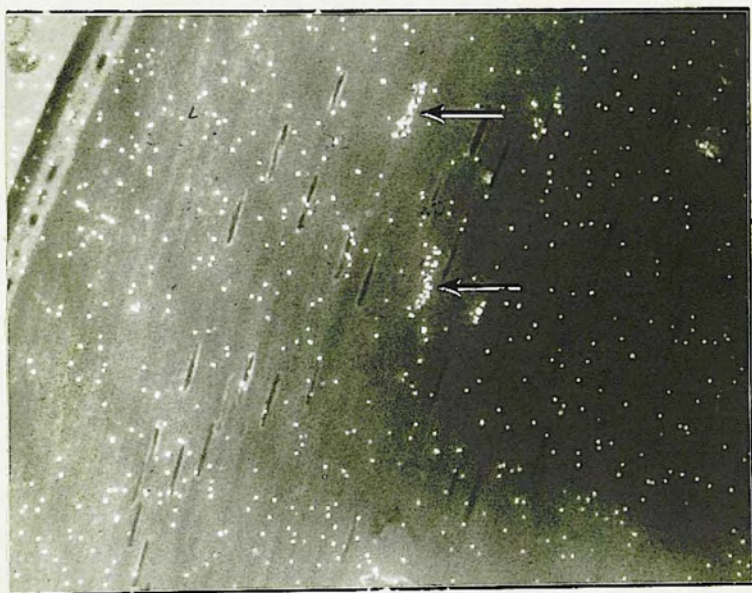


Fig. 16

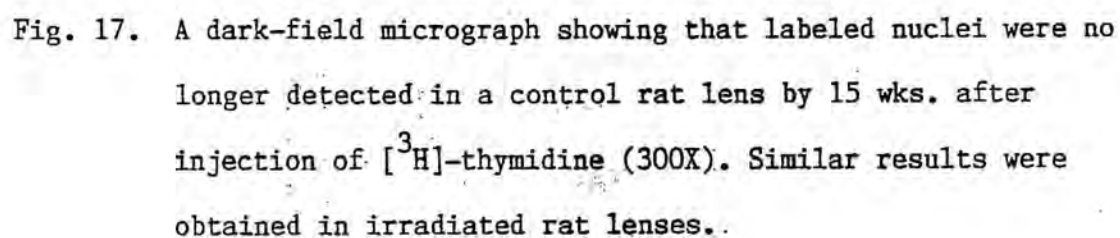


Fig. 17. A dark-field micrograph showing that labeled nuclei were no longer detected in a control rat lens by 15 wks. after injection of [^3H]-thymidine (300X). Similar results were obtained in irradiated rat lenses..



Fig. 17

Fig. 18. SDS-PAGE, Coomassie-blue-stained gel of x-irradiated rat lens proteins 30 wks. after exposure to 4 Gy showing the migration of an unheated sample of MP 26 (lane D) relative to molecular weight marker chymotrypsinogen (25.7Kd; lane H). When heated at 100⁰C for 10 min. prior to its application to the gel, both MP 26 and membrane associated proteins aggregated at the top of the gel (arrow; lane E). The crystallin (lane B) and cytoskeletal (lane G) fraction did not aggregate at top of gel when heated at 100⁰C for 10 min. These proteins migrated in the same manner as those in the unheated crystallin (lane A) and cytoskeletal (lane F) sample. Molecular weight standards used were: myosin (200Kd), β -galactosidase (130Kd), bovine serum albumin (68Kd), catalase (60Kd), ovalbumin (43Kd) and chymotrypsinogen (25.7Kd) (lanes C and H).

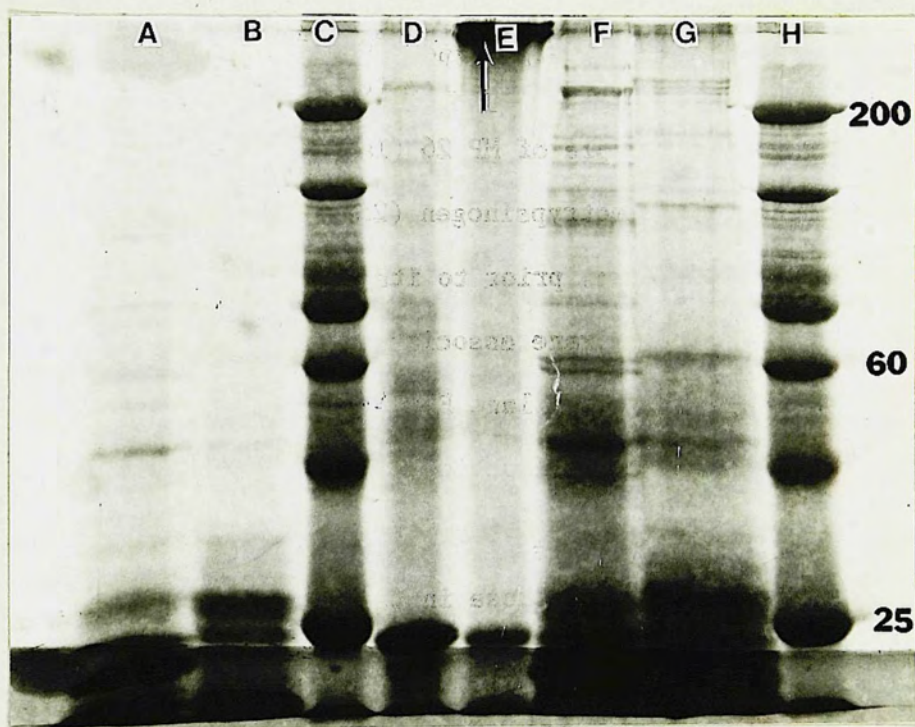


Fig. 18

Fig. 19. SDS-PAGE, Coomassie-blue-stained gel of control and x-irradiated rat lens proteins 15 wks. after exposure to 4 Gy showing the accumulation of their crystallin, cytoskeletal, and membrane protein fractions. Similar results were obtained for control and x-irradiated rat lenses 15 wks. after exposure to 12 Gy.

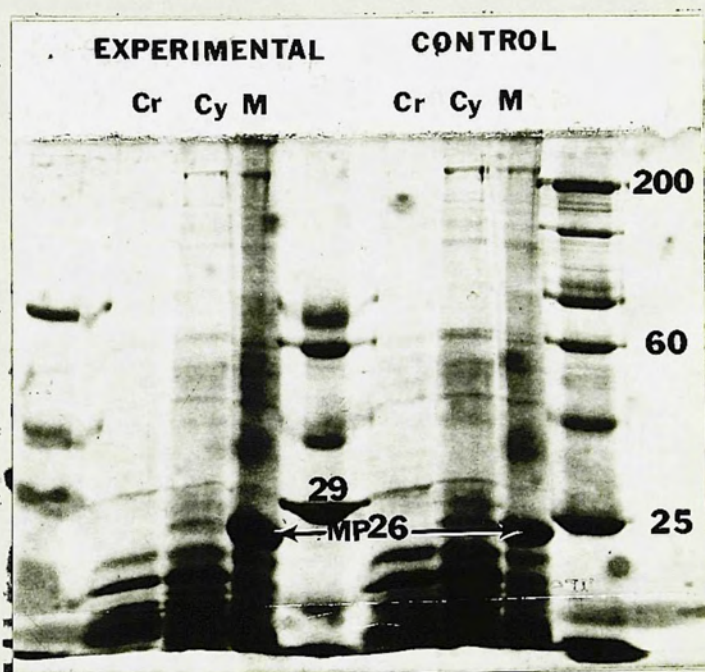


Fig. 19

Fig. 20. Autoradiogram of control and x-irradiated rat lenses 15 wks. after exposure to 4 Gy demonstrating the incorporation of label into crystallin, cytoskeletal, and membrane protein fractions. Although it may appear as though there is a decrease in the incorporation of radiation label in the crystallin (cr) and cytoskeletal (cy) fraction of x-irradiated lenses, this was not a common trend for these lenses throughout the experimental period when compared to the controls. Variability between control and irradiated lenses maybe due to variation in the lenses of different aged rats and/or the experimental technique used for protein separation. Similar labeling was observed in control and x-irradiated rat lenses 15 wks. after exposure to 12 Gy.

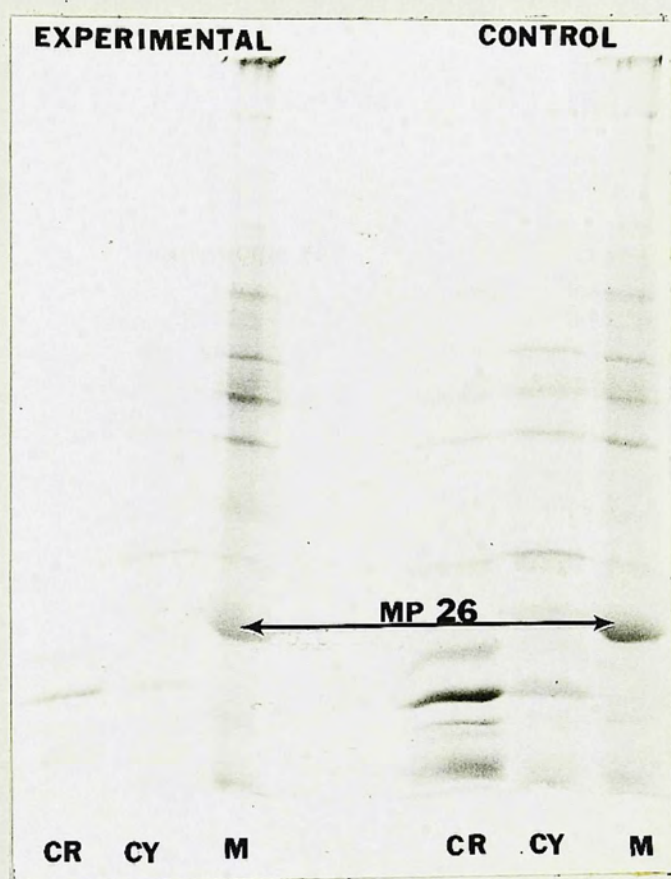


Fig. 20

Fig. 21. Scanning electron micrograph of inner cortical fibers of a control rat lens. The outlined area represents the hexagonal shape of a lens fiber when fractured in cross section. The inset, at lower right-hand corner, illustrates the following types of fiber interdigitations shown here in this scanning electron micrograph: ball (short arrow) and socket (arrowhead) and flap (open arrowhead) and imprint (asterisks). A lens fiber is made up of 4 short sides and 2 long sides. A ball-like structure setting ontop of a short stalk is located at the angle formed by the short sides. This ball-like structure fits into a complementary shaped socket which is formed at the angle of the short and long side of an adjacent fiber. The flap and imprint interdigitation also exists at the angles formed by the short and long sides of each lens fiber. This interdigitation consists of a tongue-like extension which is formed at the angle of the short and long side of the lens fiber. This tongue-like extension fits into a complementary imprint which is formed on the surfaces of the short sides of the adjacent lens fiber. Balls and flaps and complementary sockets and imprints are repeated in an alternating pattern along the length of the fibers. This same type of interdigitation pattern was observed in x-irradiated lenses (6440X).

Modified from J. Kuszak, J. Alcala and H. Maisel (1980).

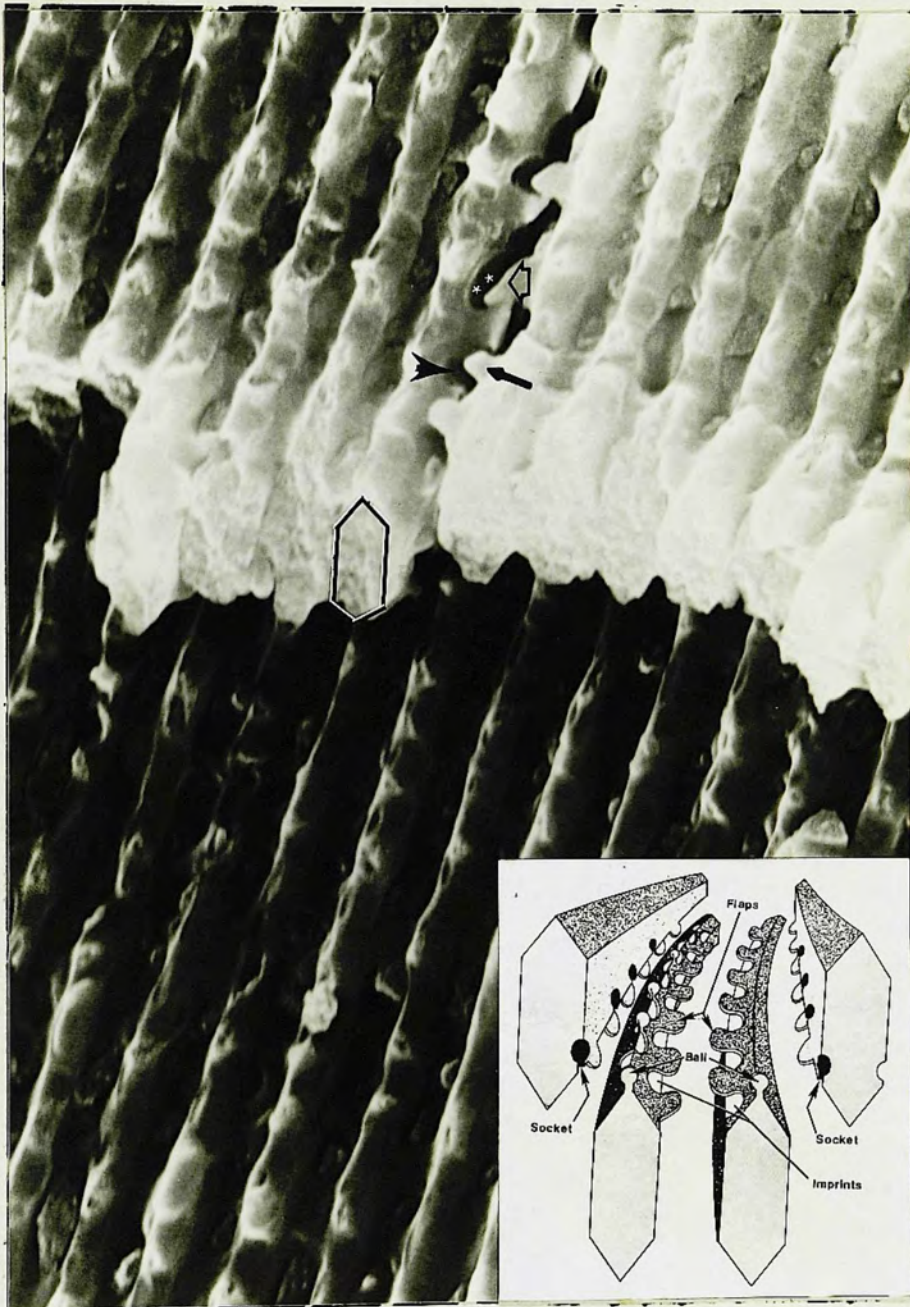


Fig. 21

Fig. 22. Schematic illustration showing 3 different ways in which morphologically altered lens fibers returned toward normal cell volume. (A) After x-irradiation, damaged epithelial cells may differentiate into cortical fibers which do not make contact posteriorly leaving a gap under the posterior capsule, forcing neighboring fibers to redistribute their volume in order to fill in the gaps left by the damaged fibers. (B) X-irradiation could force altered cortical fibers to redistribute their volume and develop "varicose" structures on their lateral sides. (C) Newly differentiated lens fibers may not extend all the way up to the overlying epithelium while maintaining normal contact posteriorly. These damaged fibers and their nuclei will fill the posterior part of the lens cortex by crowding and squeezing neighboring fibers.

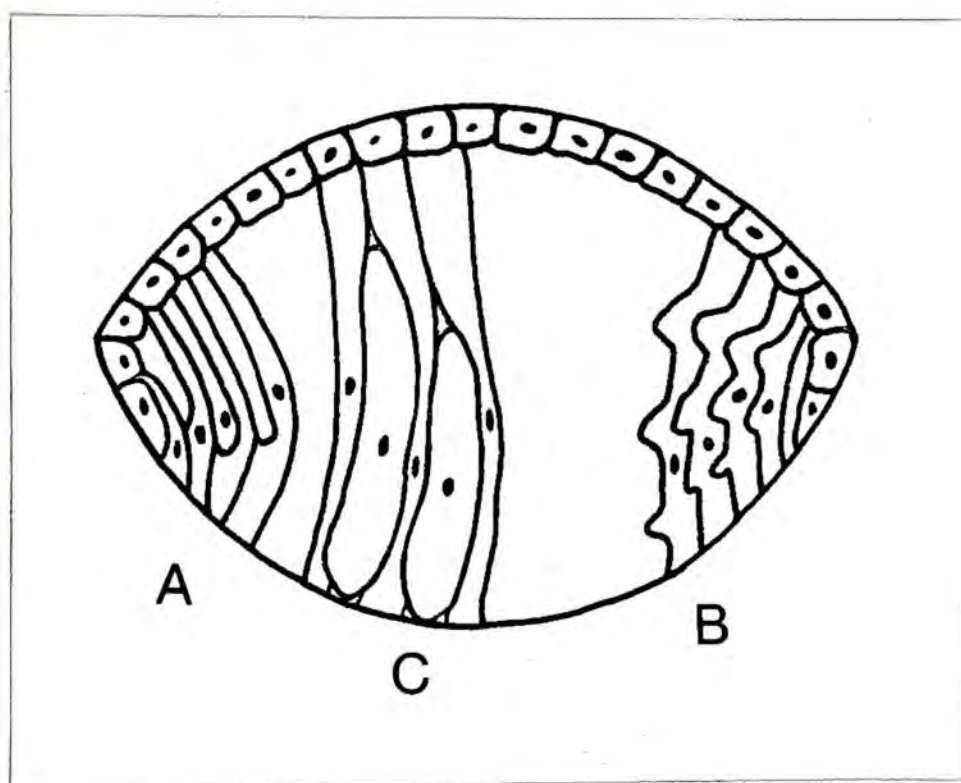


Fig. 22



Fig. 23. A schematic illustration showing methodology and results obtained from 4 wk.-old rat lenses x-irradiated with 4 and 12 Gy. The right side of this illustration represents the methodology and results of lenses x-irradiated with 12 Gy, whereas, the left side represents that of 4 Gy lenses. The center part of this diagram represents the time frame of cataract formation.

APPENDIX

X-IRRADIATION SET-UP

1. Anesthetized rats were placed on their side on a laboratory jack.
2. The jack was elevated until the whole eye pushed into an 8 mm hole drilled in a 1 cm thick lead shield.
3. The lead shield was supported by an acetate stand.
4. The entire treatment platform except for the hole in the lead shield was covered by lead bricks.

SLIT-LAMP CLASSIFICATION (Merriam et al., 1976)

- 1+ - vacuoles and opaque dots in the posterior cortex and subcapsular region (Fig. 5).
- 2+ - a dense opacity in the posterior cortex and the appearance of vacuoles and opaque dots in the anterior cortex (Fig. 5).
- 3+ - completely opaque posterior cortex and a dense opacity in the anterior cortex (Fig. 5).
- 4+ - opacity of the entire lens (Fig. 5).

TISSUE PREPARATION FOR SERIAL RECONSTRUCTION

1. Lenses were fixed in 10% neutral formalin in 0.08 M phosphate buffer (pH 7.4) for 72 hrs. at 25°C.
2. Rinsed in 0.08 M phosphate buffer.
3. Postfixed in 2% osmium tetroxide for 1 1/2 hrs. at 4°C and washed in 0.08 M phosphate buffer for 24 hrs. at 25°C.
4. Dehydrated in a graded series of ethanol (30, 50, 70, 95 and 100%)

- over a 2 hr. period and stored overnight in 100% ethanol.
5. Further dehydrated in a 3:1, 1:1 and 1:3 mixture of ethanol:propylene oxide for 15 min. each and 100% propylene oxide for 1 hr.
 6. Infiltrated in a 3:1, 1:1 and 1:3 mixture of propylene oxide:Spurr resin for 1 hr., 24 and 8 hrs., respectively, and left in 100% Spurr overnight.
 7. Embedded in Spurr and polymerized under vacuum at 70°C for 24 hrs.

THREE-DIMENSIONAL SERIAL RECONSTRUCTION (Beebe et al., 1982)

1. Sections of half-lenses for each time period were cut with a glass knife on a Dupont-Sorvall JB-4 microtome.
2. Microtome section-thickness calibration was confirmed by setting it at 0.75 μm and measuring the distance advanced in 100 revolutions using a Scheer-Tumico dial-reading micrometer and dividing by 100.
3. Cells in each region were followed through subsequent serial sections by tracing their nuclei from the photographic prints onto transparent acetate sheets and overlaying the acetate sheets.
4. Each cell in the reconstructed area was scored only once by counting none of the nuclei in the first section, only those nuclei that appeared for the first time in succeeding sections, and all of the nuclei present in the final section of the series.
5. Area determinations were obtained by outlining the three different areas of interest from a template and measuring each area using a Hewlett Packard 9874-A digitizer.
6. Tissue volumes were calculated by multiplying the measured areas by the section thickness (0.75 μm) and the number of serial sections

(22-24 serial sections per lens).

7. Average cell volumes were calculated by counting the nuclei in the reconstructed regions and dividing this number into the total volume of the region.
8. Calculations were performed using a Hewlett Packard 9835-A computer.

AUTORADIOGRAPHY (Cowan, 1972)

1. Lens sections were dipped in Kodak NTB-2 nuclear track emulsion at 40°C, air dried for 48 hrs. and stored in light-tight boxes with Drierite desiccant at 4°C.
2. Slides exposed for 8 wks. were developed in Kodak D-19 at 18°C for 4 min. and fixed in Kodak rapid fix at 18 to 20°C. for 6 min.
3. Slides were washed and stained with 1% toluidine blue and pyronin.

PROTEIN SEPARATION

Isolation of Crystallins (Water-Soluble Fraction)

1. Lens fiber mass was suspended in 7 times the lens weight of 50 mM Tris-HCl buffer (containing 5 mM Ethylenediamine Tetraacetic Acid (EDTA), 10 mM DL-Dithiothreitol (DTT) and 0.2% sodium azide; pH 7.4) and homogenized with a glass tissue grinder.
2. Homogenate was centrifuged at 10,000 xg in an SS-34 rotor in the Sorvall RC-5 Superspeed centrifuge for 20 min. at 4°C.
3. The supernant containing the water soluble lens crystallins was frozen and stored in liquid nitrogen for further analysis.

Isolation of Cytoskeletal Proteins (Urea-Soluble Fraction)

1. Pellet was rehomogenized with Tris-HCl buffer and centrifuged at 10,000 xg for 20 min. three times.
2. Washed pellet was homogenized in 7 M urea in 50 mM Tris-HCl buffer (pH 7.4), diluted 1:1 with water and centrifuged at 10,000 xg for 20 min.
3. Supernant containing cytoskeletal proteins was stored in liquid nitrogen for further analysis.

Isolation of Lens Membrane Proteins (Urea-Soluble Fraction)

1. Urea insoluble pellet was rehomogenized in 7 M urea, diluted 1:1 with water and spun at 10,000 xg for 20 min.
2. Pellet was homogenized in 0.1 M NaOH and 1 mM DTT and kept in an ice bath for 15 min.
3. Membrane fraction was centrifuged at 10,000 xg for 20 min. and resuspended in 5 mM phosphate buffer (pH 8.0) for 10 min.
4. Purified membrane pellet was obtained by centrifugation at 10,000 xg for 20 min. and stored in liquid nitrogen for further analysis.

SDS-PAGE

1. Electrophoresis was performed on a 1.5 mm thick gel slab with a 4% stacking and a 13% separating gel.
2. Membrane pellets were solubilized in 100 μ l of 1X sample buffer (containing 0.1% phenol red, 10% glycerol, 1% 2-mercaptoethanol and 8 M urea).
3. To measure amount of radioactivity present in each sample 5.0 μ l of

membrane sample, 10 μ l of crystallin and cytoskeletal fractions were counted in a Beckman LS 9000 Scintillation counter.

Equivalent amounts of radioactivity for each fraction were loaded onto the gel. Crystallin and cytoskeletal fractions were mixed with equal volumes of 2X sample buffer.

4. Electrophoresis was performed at 140 volts for 4 hrs. at 25°C.
5. Gels were stained in 0.1% Coomassie-blue in 50% methanol, 10% acetic acid overnight and destained electrophoretically in 5% acetic acid for 20 min.
6. Gels were dried for 2 hrs. under vacuum on a BioRad Model 224 Gel Slab-Dryer.

SCANNING ELECTRON MICROSCOPY

1. Lenses were fixed in 2.5% glutaraldehyde in 0.07 M sodium cacodylate buffer (pH 7.4) for 72 hrs.
2. They were washed for 24 hrs. in 0.07 M phosphate buffer (pH 7.4) and postfixed for 24 hrs. in a 1% solution of osmium tetroxide.
3. They were dehydrated in a graded series of ethanol (30, 50, 70, 95, and 100%) and left overnight in a 100% ethanol.
4. Lenses were critical point dried in a Ladd Critical Point Dryer.

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