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Using Model Systems To Study Rat Lens Membrane Damage During Cortical Cataract Formation

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USING MODEL SYSTEMS TO STUDY RAT LENS MEMBRANE DAMAGE DURING CORTICAL CATARACT FORMATION.

by

Fusun Kilic

Department of Biochemistry

Submitted in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

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ABSTRACT

Cataract formation is a complex, multifactorial and multistep process: it is associated with cellular and biochemical changes in the lens. In the present work, steps in the pathway to cataract development were followed *in vitro* using two different reagents which induce cataract formation by two different mechanisms: elevated glucose (55.6 mM), as a model for *in vivo* diabetic cataractogenesis and an actin monomer-stabilizer, cytochalasin D (10^{-5} M, CD). Findings were utilized to classify and arrange the events in a common pathway and to focus on the mechanism of cataract production.

The development of cortical subcapsular opacity in cultured lenses was elucidated as a time-dependent phenomenon by measuring different parameters: i) distribution of the living and dead cells in the lens through a confocal microscope following their fluorescent staining, ii) appearance of the lens observed daily by a dissection microscope, and iii) changes in and/or leakage of intracellular components (γ-crystallin, LDH, and magnesium ion). The findings suggested possible mechanisms for the action of the cataract-causative agents, glucose and CD, which initiate membrane damage in the lens cells in both model systems and help locate these factors at common steps in their pathways to cataract.

Using this reproducible lens incubation system, various

potential anti-cataract agents e.g., antioxidants, sorbitol-lowering agent, and other agent were tested in an in vitro diabetic model systems. All these agents were relatively effective in preventing cataract formation in the in vitro diabetic model system. These findings support the hypothesis that the damage processes in the presence of glucose involve glycation and oxidative stress. The effect of an antioxidant, Vitamin C (VC), was also tested to explore the factors involved in the mechanism of CD-cataract formation. Prevention of damage by VC is consistent with the hypothesis that oxidative stress is associated with the disorganization of actin and cytoskeletal network during cataract formation.

The timing and localization of calcium uptake in the lens was studied in both cataractous model systems by radioactive and fluorescent labelling techniques. The lens calcium uptake increased parallel to the incubation time in cataract causative agents. The effect of CD was earlier and more severe than the glucose effect. The results of lens glucose-treatment, as well as inhibitors showed that elevated calcium could be one of the factors leading to cataract development. Finally, the role of calpain-proteolysis of spectrin/fodrin was studied during cataract formation process in both model systems. The results suggest that the proteolysis of spectrin/fodrin does not precede cataract formation but it is a consequence of pre-cataractous changes.

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THIS THESIS IS DEDICATED TO MY MOTHER, MY FATHER AND MY LOVELY HUSBAND, BILLOW WHO DID SO MUCH TO HELP ME TO FINISH THIS PROGRAMME.

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NOMENCLATURE

ATP	Adenosine 5'-triphosphate
β-Carot.	β-carotene
BSA	Bovine serum albumin
CaM	Calmodulin
CCRG	Cooperative cataract research group
CD	Cytochalasin D
CLSM	Confocal laser scanning microscopy
DHAA	Dehydroascorbic acid
DHLA	Dehydrolipoic acid
DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol bis(2-aminoethyl ether)-N,N,N',N'-tetra-acetic acid
FDA	Fluorescein diacetate
Glu.	Glucose
GSH	Glutathione
GSSG	Glutathione disulfide
HPLC	High performance liquid chromatography
IgG	Immunoglobulin G
LDH	Lactate dehydrogenase
PMSF	Phenylmethylsulfonyl fluoride
NAD(P)	Nicotinamide adenine dinucleotide(phosphate)
NBT	Nitrobluetetrazolium
PAGE	Polyacrylamide gel electrophoresis

PMSF	Phenylmethylsulphonyl fluoride
PMT	Photo multiplier tube
SDS	Sodium dodecyl sulphate
STZ	Streptozotocin
TCA	Trichloroacetic acid
TCLSM	Transmission confocal laser scanning microscope
Tris	Tris(hydroxymethyl) aminomethane
VC	Vitamin C
VE	Vitamin E

CHAPTER 1

INTRODUCTION : GENERAL ASPECTS OF LENS, PLASMA MEMBRANE AND CATARACT.

1.1 GENERAL AND CELLULAR STRUCTURE OF THE LENS

1.1.1 LENS ANATOMIC AND STRUCTURAL FEATURES

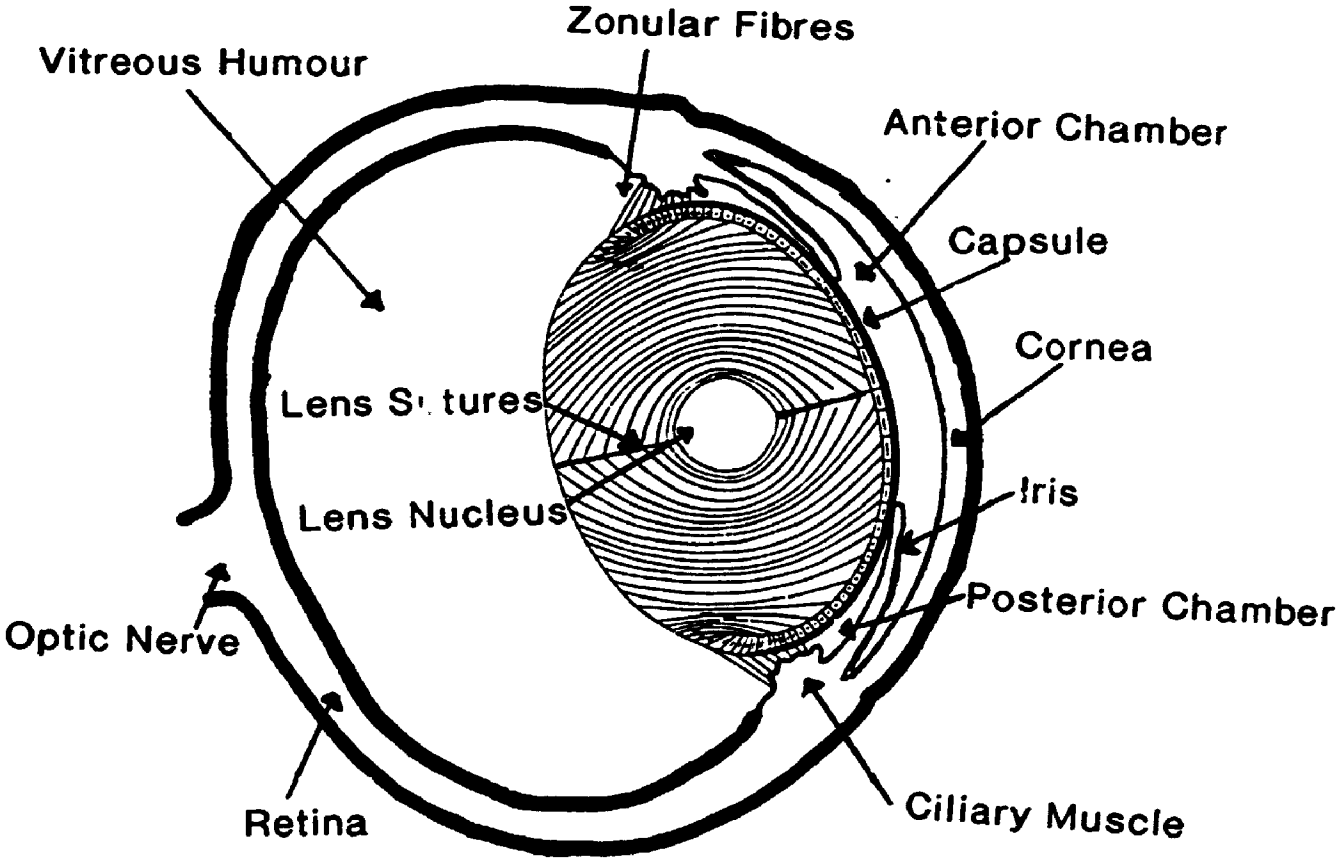
The lens is located behind the cornea between the iris and the vitreous humour and connected to the eyeball with the ciliary muscle (Berman, 1991). It lacks innervation and blood vessels. The lens is completely enclosed within a thin, transparent, elastic basement membrane, not fibrous but collagenous with a high carbohydrate content, called the capsule (Berman, 1991; Maisel, 1985; Duncan and Jacob, 1984; Rafferty, 1985; Hogan et al., 1975) **(Figure 1.1)**. Around the lens, the thickness of the capsule changes: the thinnest section is at the posterior pole and the thickest part of the capsule is at the anterior pole (Fisher and Pettet, 1972; Seland, 1974; Fels, 1970). The lens capsule is a selective barrier to diffusion into and out of the lens: low molecular weight components such as sugars, amino acids, and lactate appear to pass freely across the capsule. It is tightly attached to the epithelium (Harding et al., 1973).

1.1.2 LENS CELLS DIFFERENTIATION:

Only two types of cell exist in the lens; a single

FIGURE 1.1.
SCHEMATIC DIAGRAM OF MAMMALIAN EYE CROSS SECTION.

The lens is located behind the cornea between the iris and the vitreous humour and connected to the eye with the ciliary muscle. There are no blood vessels or nerves in the lens. The lens is enclosed by a basement membrane called the capsule. (Figure is modified from McAvoy, 1981).


Zonular Fibres
Vitreous Humour
Anterior Chamber
Capsule
Cornea
Lens S: tures
Lens Nucleus
Iris
Posterior Chamber
Optic Nerve
Retina
Ciliary Muscle

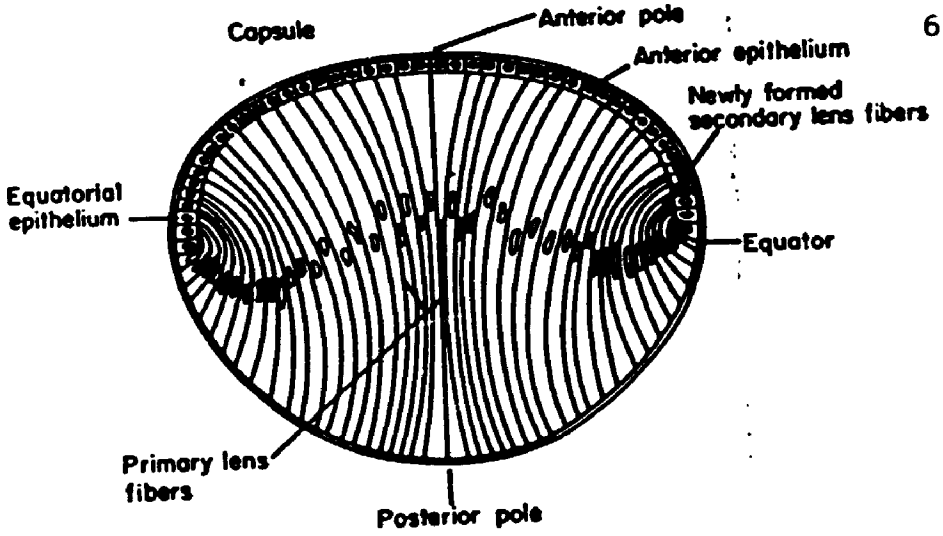
layer of epithelial cells on the hemispherical anterior surface, behind which lies the core of crystallin-rich fiber cells, elongated from anterior to posterior surface. The anterior and posterior chambers coincidentally divide the lens into two functionally distinct compartments: proliferation and elongation compartments (Harding et al., 1971; Reddan et al., 1975; Yamada, 1982; Piatigorsky, 1981; Rafferty, 1985; McAvoy, 1981).

The embryonic development of the fiber cells starts with the posterior cells of the lens vesicle. These cells cease division and differentiate to form the elongated primary lens fiber cells. The cavity of the vesicle is obliterated when the primary fiber cells contact the anterior cells of the lens vesicle. After the initial development of the lens and the formation of primary fiber cells, cells of the anterior epithelium continue to divide and then move to the equatorial zone where they differentiate into secondary lens fiber cells (McAvoy 1978, 1981) **(Figure 1.2, Panel A)**.

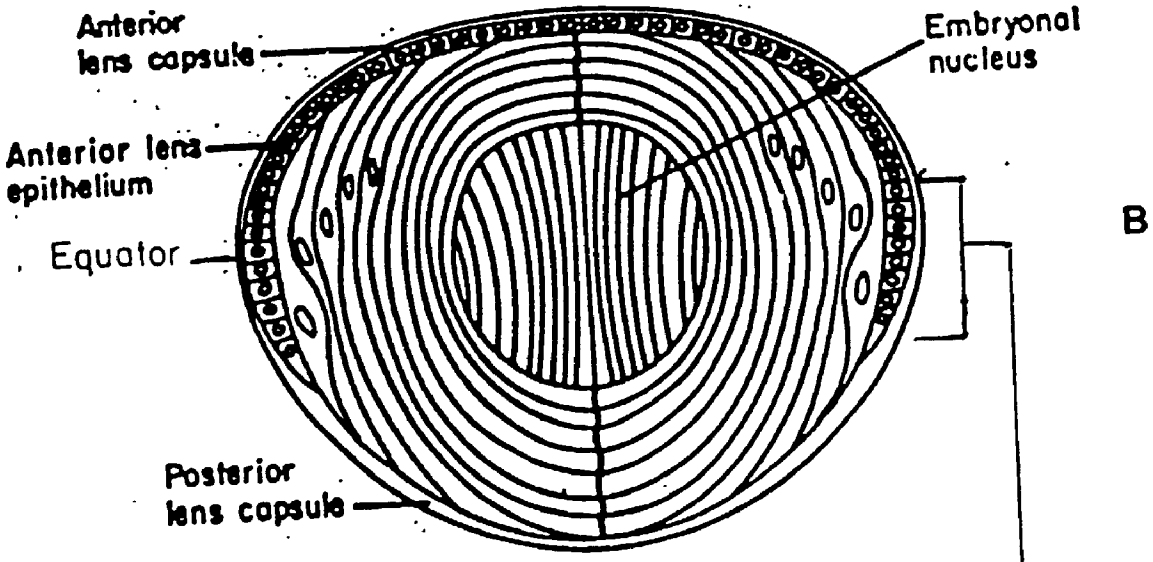
Throughout life no cells are lost from the lens. Instead, in the lens bow region, cells continually differentiate to form elongated fiber cells (secondary fiber cells) **(Figure 1.2, Panel B)**. Differentiated cells are added to the lens which increases in volume (Beebe et al, 1979), weight (Ehlers et al., 1968), and size (Hendrix and Zwaan, 1974).

FIGURE 1.2.
SCHEMATIC DRAWINGS OF THE LENS AND ITS DIFFERENTIATION.

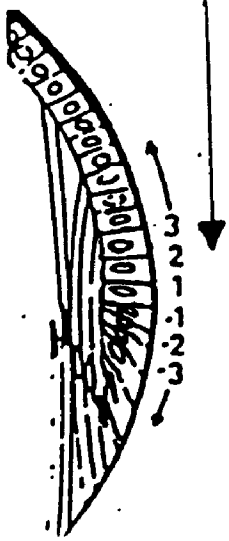
Newborn rat lens (Panel A) and mature rat lens (Panel B) showing the different layers of cells in the lens and the lens cortex and nucleus. Throughout life no cells lost from the lens. Instead, in the lens bow region, cells are involved in a cytodifferentiation process characterized by marked elongation. At the lens equator epithelial cells rotate 90° and elongate to give rise to the fiber cells (Panel C).


Capsule
Anterior pole
Anterior epithelium
Newly formed secondary lens fibers
Equatorial epithelium
Equator
Primary lens fibers
Posterior pole

A


Anterior lens capsule
Embryonal nucleus
Anterior lens epithelium
Equator
Posterior lens capsule

B


3
2
1
1
2
3

C

At the lens equator, the epithelial cells rotate 90° and elongate to give rise to the fiber cells (Coulombre and Coulombre 1963) (**Figure 1.2, Panel C**). The factors which initiate the fiber cell differentiation are normal constituents of the aqueous humour. Newly formed secondary fiber cells elongate as concentric layers on the primary fibers. The factors which initiate the elongation process are produced by the retina (Yamamoto, 1976). Secondary fibers are added throughout life by elongation of epithelial cells at the lens equator. The elongation process is characterized by the stimulation of crystallin synthesis, and the degradation of intracellular membranes (Spector, 1982; Kuwabara, 1975; Reeder and Bell, 1965; McDevitt et al., 1969). Differentiation of lens fibers from monolayer sheets of lens epithelial cells in vitro has been shown in rats (Hamada and Okada, 1977; Mousa and Trevithick, 1979), man (Hamada and Okada, 1978) and chicks (Okada et al., 1971, 1973).

As a result of epithelial cell proliferation followed by differentiation new fiber cells are continually added to the fiber mass. Previously formed fibers become progressively more internalized; the older fibers are found toward the central region (nucleus) and the younger ones toward the periphery (cortex). Newly formed, elongated secondary fibers which grow anteriorly and posteriorly towards the poles of the lens, meet the ends of the older

fiber cells and form the Y suture at the anterior and posterior poles of the lens. As the lens grows in size the sutures may become branched (Mann, 1964).

Kuwabara (1975) showed that fiber cells in the normal lenticular nucleus are relatively small and irregular in shape. The cytoplasm is uniform and finely granular in appearance. Mature lens fiber cells lack biosynthetic or energy-producing organelles and are therefore uniquely dependent on intercellular communication with the metabolically active anterior lens epithelial cells for the osmotic, ionic, and nutrient flux necessary to preserve lens functional integrity and to maintain lens homeostasis and prevent cataract formation (Berman, 1991). Ultrastructural studies have revealed an extensive network of gap junctional intercellular channels in the lens that link fiber to fiber, epithelial cell to epithelial cell, and epithelial cell to fiber (Musil, 1994). Many studies (Mathias and Rae, 1985; Miller and Goodenough, 1986; Goodenough et al., 1980) indicate that gap junctions serve to couple lens cells to each other. These channels would allow ions and small metabolites to pass to and from the deepest fibers of the lens.

In addition, evidence is accumulating that the formation of gap junctions is influenced by the adhesion molecule NCAM. The cell-cell adhesion molecules NCAM and N-cadherin are thought to be involved in epithelial-to-fiber

differentiation and in maintaining precise packing between fiber cells (Lo, 1988; Watanabe, 1989). Derangement of either process can result in congenital or late onset cataractogenesis (Zigler, 1990; Kaiser-Kupfer et al., 1983), and thus cell-cell adhesion molecules are likely to play an important role in the establishment and maintenance of lens transparency. The epithelial cells of chick and mammalian lenses synthesize both calcium-dependent (N-cadherin) and calcium independent (NCAM) cell-cell adhesion molecules whereas their levels decline in the cortical and nuclear fibers (Watanabe et al., 1989; Watanabe et al., 1992; Maisel and Atreya, 1990; Katar 1993). These molecules have been demonstrated to be involved in the maturation of lens gap junctions to a fiber-type morphology (Wat be et al., 1989). N-cadherin is localized in the lens and other organs within cytoskeleton-associated cell-cell specializations known as adherence junctions. Three closely related gap junction proteins (connexins), one in epithelial and two in fiber cells have been identified (Miller and Goodenough, 1986; Alcala and Maisel, 1985).

1.1.3 LENS PROTEINS AND TRANSPARENCY:

All of the detectable protein synthesis occurs in the epithelium and in newly formed, developing secondary fibers in the peripheral cortex. There are two major classes of proteins in the lens cells: the structural proteins called crystallins and cytoskeletal components (Harding and Dilley,

1976). The content of crystallins is more than 90% of all water-soluble lens proteins (Bloemandal, 1977). Crystallins are not evenly distributed throughout the lens; their total concentration increases from the cortex to nucleus (Bloemendal,1981).

The lens structural protein is composed of four major crystallins: α-, β- and γ-crystallin in vertebrates (Harding and Delay, 1976), and δ-crystallin in avian and some reptilian lenses (Bloemendal, 1982). The specific structure of crystallins maintains lens transparency. Age-related post-translational changes and aggregation of crystallins to form high molecular weight proteins disrupt the normal structure of the lens and decrease its transparency (Spector, 1982).

Approximately 35% of the weight of a lens consists of crystallins; water-soluble crystallin and insoluble α-crystallin. All γ-crystallins and one β-crystallin are monomeric proteins. Other crystallins have a tendency to form oligomers and are associated with high molecular masses. Studies on the crystallin polypeptides have indicated a high degree of conservation of these proteins making them a favourable system for the study of cellular differentiation during lens development (Bloemendal,1981). There is a sequence homology between β- and γ-crystallins. The relative amounts of β-/γ-crystallins vary with age. There is a gradual decrease of β-/γcrystallins and an

increase of α-crystallin with increasing age (Ringens et al., 1982; Ocken et al., 1977).

The cytoskeleton of the lens consists of three major classes of fibrous proteins: microtubules, intermediate filaments, and actin microfilaments (Maisel, 1985). Microtubules have the largest diameter of all cytoskeletal elements. The wall of microtubules is made of the single protein, tubulin. Found in epithelial and cortical fiber cells, microtubules play a role in both cell division and the determination of cell shape. They are mainly involved in cell elongation, but also maintain the general structure and polarity of the cell. In the lens, intermediate filaments of 7-11 nm diameter contain vimentin as the major constituent (Alcala and Maisel, 1985; Ramaekers and Bloemendal, 1981) and are found mostly in epithelial and cortical fiber cells.

The main components of microfilament structures are actin filaments, also called F-actin (filamentous actin), which are polymerized from globular actin monomers (G-actin). Polymerized and non-polymerized actin can interact with many proteins. Such "actin-binding proteins" regulate the degree of polymerization of actin and the stability, length and distribution of actin filaments (Karp, 1984). Actin microfilaments have been detected in both epithelial and fiber cells. Actin constitutes approximately 11% of the proteins of the newborn lens epithelial cells, while it

represents 1% of the proteins of the newborn lens fiber cells and the mature lens cortical fiber cells (Mousa and Trevithick, 1979). Microfilaments and/or microtubules may have a function in cell differentiation, possibly in the elongation process (Bloemendal, 1982).

The lens is a transparent, highly refractive structure. The focusing of images on the retina depends on the performance of the lens, which is dependent on its transparency. The refractive power and transparency of the lens require that the fibers maintain a precise cell-cell packing geometry and that the crystallins within them remain soluble (Lasser and Balazs, 1972). Also, the lens lacks chromophores and because of that it fully transmits incident light. In view of these factors, any damage or breakdown of the plasma membrane or the supramolecular organization in the lens has direct effects on transparency (Duncan and Jacob, 1984; Creighton et al., 1978).

1.2 LENS METABOLISM:

Metabolic processes that occur in other tissues also occur in the lens. Over the whole life-span, while maintaining transparency, the lens manifests continuous chemical change; this constitutes its metabolism. The magnitude of this metabolism and of the tasks involved may be appreciated from an overall consideration of lens metabolism.

The synthesis of major cell constituents requires

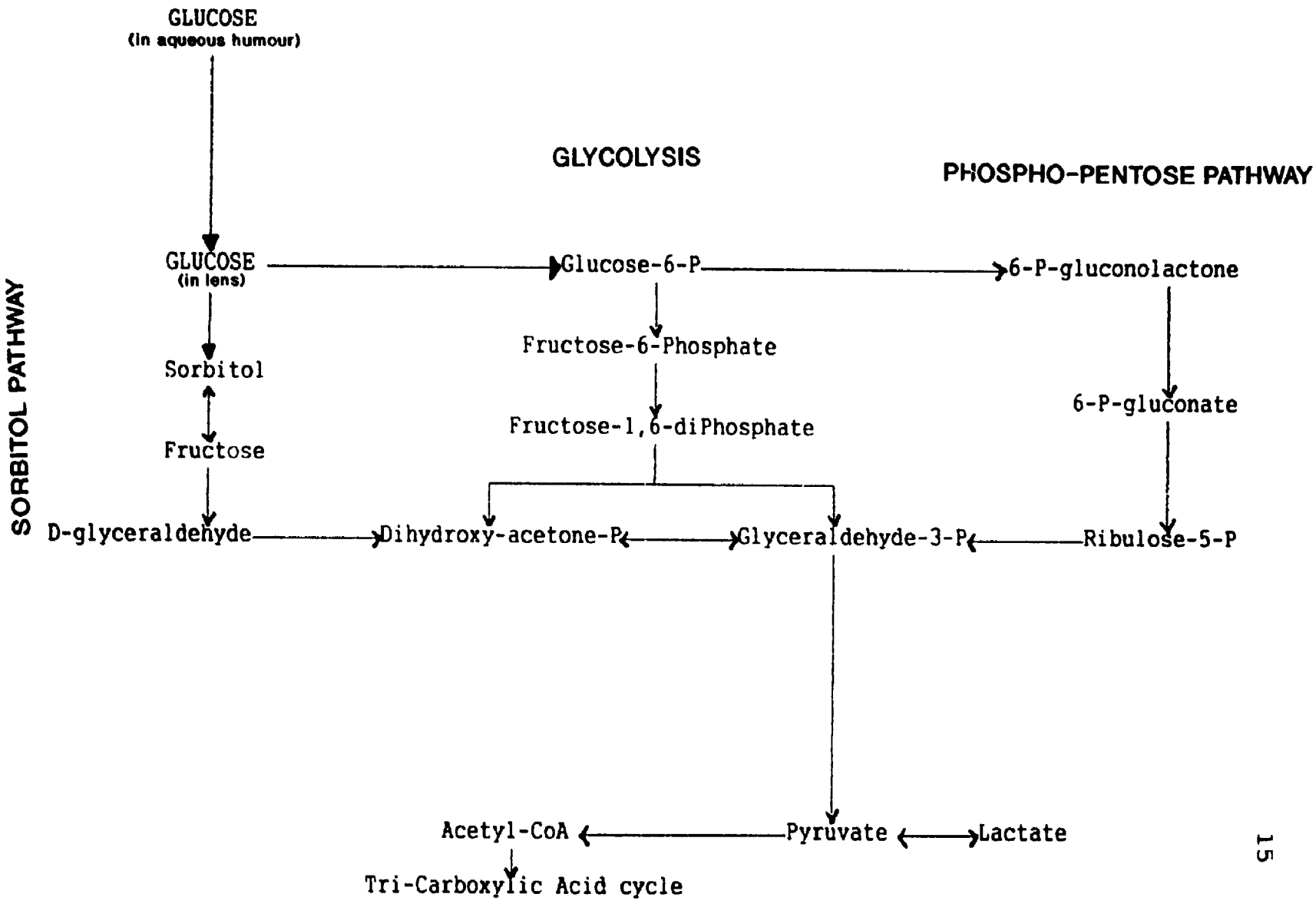
energy. In general, the energy derived by oxidation of foods is channelled into high-energy phosphate compounds, mainly ATP (Stryer, 1988). Like the cornea, the lens is devoid of a blood supply. For this reason the nutrients necessary for metabolic activities are derived by diffusion from the aqueous humour whose composition resembles a dialysate of blood plasma (Berman, 1991). Many compounds derived from the aqueous humour or fabricated by metabolic reactions have to be oxidized or reduced to be used by the lens cells. These compounds are used in coupled reactions to effect the synthesis of all types of essential small molecules and macromolecules: polysaccharides, proteins, lipids and nucleic acids. Of all the molecules involved in metabolic pathways, only lens carbohydrate, amino acid and calcium metabolism relate to the entire content of this thesis.

1.2.1 CARBOHYDRATE METABOLISM:

As many studies have already indicated, the lens obtains its major energy (70%) (Cheng and Chylack, 1985) in the form of ATP (Kinoshita, 1965) by glucose metabolism through one of the following pathways: the Embden-Meyerhof (glycolysis) pathway, the hexose monophosphate pathway, or the sorbitol pathway (Figure 1.3). A majority of the enzymes required for carbohydrate metabolism have been found in the epithelium or fibers in the cortex area since old fiber cells are devoid of mitochondria (Spector, 1982;

FIGURE 1.3.
GLUCOSE METABOLISM IN THE LENS.

Lens obtains its major energy source in the form of ATP by glucose metabolism through one of the following pathways: the Embden-Meyerhof (glycolysis) pathway, the hexose monophosphate shunt, or the sorbitol pathway. A majority of the enzymes required for carbohydrate metabolism have been found in the epithelium or fibers in the cortex area.


GLUCOSE
(in aqueous humour)
GLYCOLYSIS
PHOSPHO-PENTOSE PATHWAY
SORBITOL PATHWAY
GLUCOSE
(in lens)
Glucose-6-P
6-P-gluconolactone
Fructose-6-Phosphate
Sorbitol
6-P-gluconate
Fructose-1,6-diPhosphate
Fructose
D-glyceraldehyde
Dihydroxy-acetone-P
Glyceraldehyde-3-P
Ribulose-5-P
Acetyl-CoA
Pyruvate
Lactate
Tri-Carboxylic Acid cycle

Kuwabara, 1975).

1.2.1.1 GLYCOLYSIS:

Although the lens can metabolize the transported glucose via the other pathways, like the citric acid cycle, oxidative phosphorylation and α-glycerophosphate oxidation, thermodynamically these are not favoured reactions. Most of the glucose is metabolized and converted to lactate to be used by the young lens in the Embden-Meyerhof pathway (Harding and Crabbe, 1984; Cheng and Chylack, 1985). The glycolytic pathway is controlled by three enzymes: hexokinase, phosphofructokinase, and pyruvate kinase. The activity of hexokinase is controlled by the concentration of glucose-6-phosphate. The activity of phosphofructokinase is controlled by the ATP concentration, and the activity of pyruvate kinase is controlled by phosphofructokinase and ATP concentrations. The activities of these three enzymes remain constant throughout life in lens epithelial cells under healthy conditions. In diabetes the increasing glucose concentration in the aqueous humour will stimulate more glucose transport into the cell and along with the concentration of glucose, glucose-6-phosphate and the enzyme which convert[illegible]ruvate to lactate, lactate dehydrogenase, will increase (Harding and Crabbe, 1984; Cheng and Chylack, 1985; Harding, 1991).

1.2.1.2 THE PENTOSE PHOSPHATE PATHWAY:

In this pathway hexoses are converted to pentoses which are required for nucleic acid synthesis. The lens can metabolize 14% of glucose by the pentose phosphate pathway which is activated whenever there is a demand for NADPH (Kinoshita and Wachtl, 1958). The shunt activity of the epithelium is important in maintaining the cellular redox balance and protecting against oxidative damage (Harding and Crabbe, 1984; Cheng and Chylack, 1985; Harding, 1991). $NADP^+$ stimulates the two dehydrogenases: Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. The stimulation of an NADPH-generating system is used for the synthesis of fatty acid from acetyl-CoA, maintenance of reduced glutathione and the aldose reductase reaction. Glucose 6-phosphate dehydrogenase is regulated by the NADPH/$NADP^+$ ratio. NADPH and $NADP^+$ were found in the lens epithelium at much higher concentrations than in the cortex. When the glucose concentration is around 50-60 mM (10 times the normal value), it activates the pentose phosphate pathway which acts in conjunction with the sorbitol pathway. Stimulation of the pentose phosphate pathway is considered to represent the protective response of cells to natural oxidative insults, such as free radicals and peroxides.

1.2.1.3 THE SORBITOL PATHWAY:

There is a conjunction between the other pathways and

the sorbitol pathway: glucose is converted to glyceraldehyde -3-phosphate by either the Embden-Meyerhof or pentose phosphate pathways. Depending on the concentration of glucose, aldose reductase catalyses the conversion of glyceraldehyde 3-phosphate to glycerol 3-phosphate by reducing NADPH. Glyceraldehyde 3-phosphate is an intermediate in both glycolysis and pentose phosphate and has been found in the lens.

Two enzymes are involved in the sorbitol pathway: aldose reductase which uses NADPH, and polyol dehydrogenase which uses NAD^+. When the concentration of glucose that enters into the metabolic pathways is increased, this excess glucose is reduced to sorbitol by the catalytic effect of aldose reductase and NADPH. This sorbitol accumulates within the cell inducing an osmotic stress, because the other enzyme in this pathway, polyol dehydrogenase, is rate-limiting. It catalyses the conversion of sugar alcohol, sorbitol, to the keto-sugar, fructose (Harding and Crabbe, 1984; Cheng and Chylack, 1985; Harding, 1991). Aldose reductase activity can be inhibited by a wide variety of compounds which are usually heterocyclic, for instance, flavonoids (Reddy et al., 1992; Varma and Kinoshita, 1976; Kinoshita, et al., 1976).

1.2.2 AMINO ACID METABOLISM:

Amino acid metabolism is very important for the

synthesis of a variety of nitrogen-containing compounds. The ability to perform these syntheses depends upon the continuing availability of a pool of amino acids which are utilized as precursors. Extensive studies of all L-amino acids in the lens have shown that the L-amino acids are metabolized to provide energy and produce CO_2 and NH_3 (Cheng and Chylack, 1985; Harding and Crabbe, 1984). Like the other cells, lens cells do not contain a storage form of amino acids, nor do they synthesize a protein molecule if a normal constituent amino acid is lacking. Nutritionally essential amino acids can be supplied by diet. They are transported into the epithelial cells by an active transport system, diffuse toward the posterior pole and exit through the posterior capsule (Hoenders and Bloemendal, 1981).

The principal fate of amino acids is incorporation into proteins. Except for tryptophan and tyrosine, which are incorporated into lens proteins directly, amino acids are also metabolized after being taken up by the lens (Trayhurn and Van Heyningen, 1973). In addition to all these amino acids, the lens has sulphur-containing compounds which are a byproduct of cysteine catabolism in the lens. The ultimate metabolic fate of cysteine is the formation of inorganic sulphate and pyruvate. This involves initial removal of the amino group by aspartate transamination, and initial oxidation of organically bound sulphur. It is likely that in the lens cells the major pathway of cysteine catabolism

involves its oxidation to cysteine sulfinic acid. This intermediate product is also utilized for the synthesis of taurine (Reddy, 1967), a normal metabolite of cysteine. Taurine is found not only in the lens, but also in significant amounts in nerve tissue. Even though the role of taurine has not been established conclusively, it has been postulated to act as a scavenger of hypochlorite, an oxidizing species (Babior and Crowley, 1983). Some studies of the lens and retina show that taurine concentration must be kept at the physiological level for the normal function of the retina and the maintenance of lens clarity (Cheng and Chylack, 1985; Harding and Crabbe, 1984; Hoenders and Bloemendal, 1981).

1.2.3 CALCIUM METABOLISM:

The regulation of the free cytosolic [Ca^{2+}] in the lens cell is complex, since it has various important roles. To maintain the clarity of the lens, intracellular free and/or bound calcium must be at physiological levels. Either a decrease or an increase in the level of calcium appears to insult the lens plasma membrane and cause some complication in the lens (Delamere and Paterson, 1981; Harding, Chylack Jr, Susan, Lo and Bobrowski, 1983; Cheng and Chylack, 1985; Rafferty, Rafferty and Ito, 1994; Duncan, Williams and Riach, 1994).

Since extracellularly the level of Ca^{2+} is much higher

than intracellularly, there tends to be a movement of Ca^{2+} down its electrochemical gradient into the cell. There are at least two distinct pathways by which calcium efflux occurs in the lens cells. One is by a specific ATP-dependent Ca^{2+} pump which is linked to a Ca^{2+}, Mg^{2+}-activated ATPase (Cheng and Chylack, 1985) and an Na^{+}-Ca^{2+} exchange mechanism. Within the lens cells there are also reservoirs of Ca^{2+} maintained in the mitochondria and smooth endoplasmic reticulum by uptake processes. The lens Ca^{2+} level is also related to the glucose concentration in the cell, and glycolysis could facilitate calcium uptake. The bound calcium in the lens cells is distributed mostly along the fiber cell membrane (Delamere, et al., 1993; Hightower, et al., 1980).

1.3 CATARACT:

Transparency and lenticular elasticity necessary for accommodation, mediated by the lens capsule, are two major functional properties of the lens. Loss of transparency, which causes the lens to scatter significant amounts of light, is an early sign of cataractogenesis. Cataract, or opacity of the lens is one of the major causes of impaired vision and the leading cause of blindness worldwide. Cataract can occur as a result of disease, injury, exposure to toxic substances or the aging process (Zigler and Goosey 1981; Weale, 1981).

1.3.1 CLASSIFICATION OF CATARACTS:

In view of the fact that there has been no standardised cataract classification and grading, researchers who are involved in cataract study perform their studies with their own classification and grading methods. Three major types of cataract are now recognized clinically: cortical (anterior subcapsular), posterior subcapsular, and nuclear cataract. In addition, there is a great variety of types of metabolic damage that can cause more than 30 kinds of cataracts, such as (A) diabetic or sugar cataracts (i.e. galactose, xylose, arabinose, hypoglycaemic cataract), (B) cataract induced by drugs and other noxious substances (i.e. cyanate- and carbamylation-caused cataract, steroid-, alcohol-, iodoacetate-, dimethylsulphoxide-, methionine sulphoximine-, busulphan-induced cataract, and selenite cataract), (C) radiation cataracts (i.e. X-ray, β-, γ-, or neutron radiation cataract, atomic bomb cataract, UV, sunlight and visible radiation cataract, infrared, electric, microwave or ultrasound cataract), (D) hereditary, congenital cataracts (i.e. Nakano mouse, Philly mouse, Emory mouse cataract), (E) cold cataract, (F) traumatic cataract, and (G) osmotic cataract (Harding et al., 1985; Harding and Crabbe, 1984).

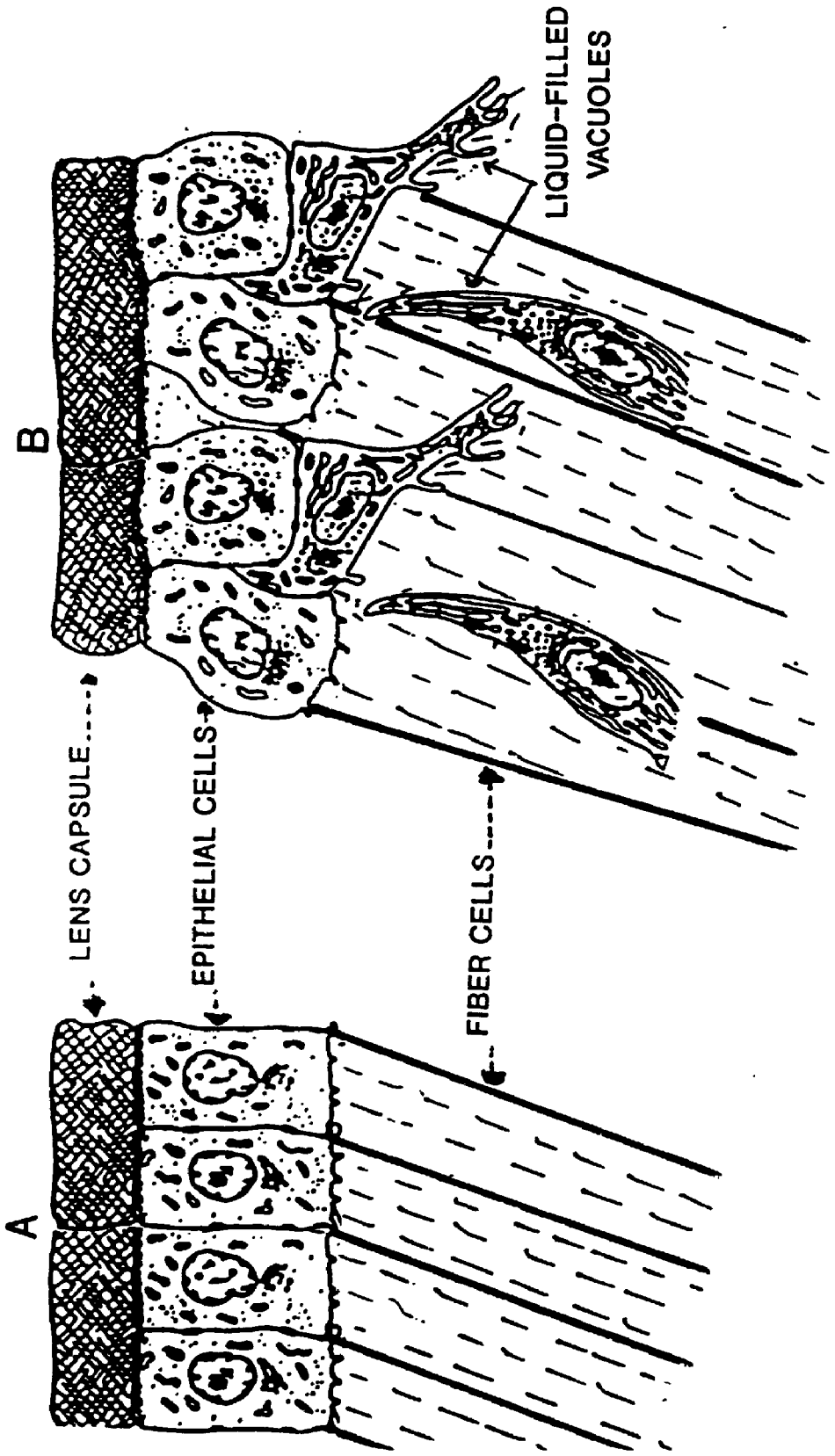
1.3.2 CHANGES IN LENS CELLULAR STRUCTURE ASSOCIATED WITH CATARACT:

The different classes of opacities are believed to be a result of basic changes in the lens. Nuclear cataract, which is classified as one of the three clinically recognized cataracts, has been attributed to the aggregation of the lens proteins (Jedziniak et al., 1973; Spector et al., 1973). Crystallin aggregates of high molecular weight form a density gradient which increases from the lens cortex toward the nucleus and decreases the amount of visible light transmission).

Cortical cataract, the most common form of senile cataract, starts with the proliferation of epithelium. In the extracellular matrix of proliferated epithelium, spindle-shaped cells appeared which contain some collagen, mucopolysaccharides, and calcium. Later, between epithelium and proliferated epithelium, a new basement membrane is produced (Font and Brownstein, 1974). This is followed by the disturbance of the regular order of fiber cells at the region just outside to the lens nuclear equator. Fiber cell breakage continues, reaching an extensive focal cellular degeneration which combines with realignment of anterior and posterior cell fragments. This is followed by the formation of globular bodies and large liquid-filled vacuoles (Creighton et al., 1978; Ross et al., 1982) **(Figure 1.4)**. Globular structures, resulting from localized fiber cell

FIGURE 1.4.
DIAGRAM OF GLOBULAR DEGENERATION.

Cellular degeneration is followed by the formation of the globular bodies and large liquid-filled vacuoles. Globular structures, resulting from localized fiber cell breakdown, are recognized as a characteristic of many types of lens opacities.


A
B
LENS CAPSULE
EPITHELIAL CELLS
FIBER CELLS
LIQUID-FILLED VACUOLES

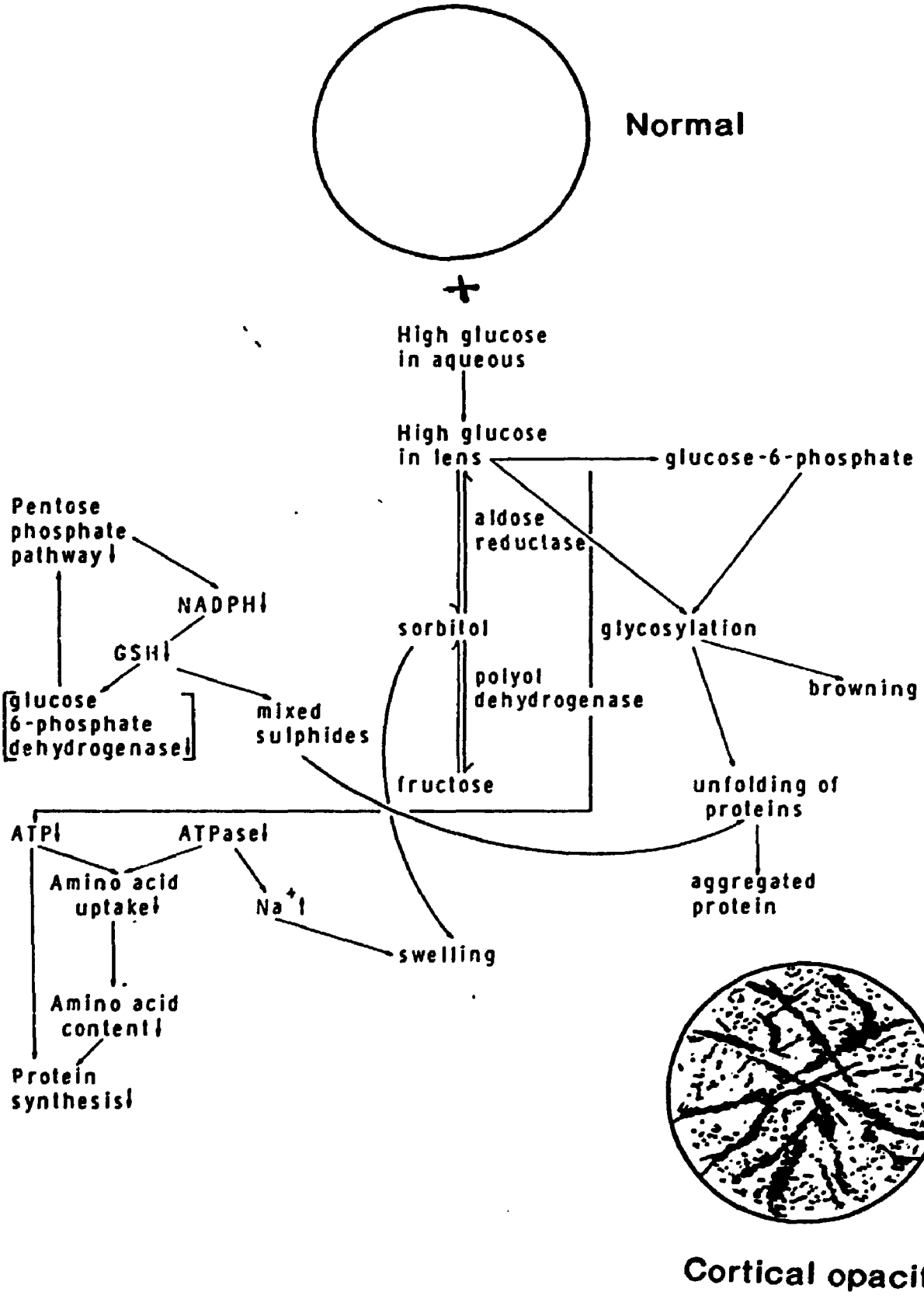
breakdown, are recognized as a characteristic of many types of lens opacities. A reduction in gap junction size and/or number is an early event in certain cataract models (Tanaka et al., 1980; Tanaka et al., 1990) and a feature of early age-related human cortical opacities (Vrensen et al., 1990), implicating derangement of gap junctions in cataractous lens pathology.

1.3.3 MOLECULAR CHANGES DURING CATARACTOGENESIS:

In the cataractous lenses there could be either a single mechanism or a combination of many mechanisms (Figure 1.5). The modification of lens proteins during cataract formation is mediated by enzymatic or nonenzymatic mechanisms. Amino acid analysis found that the amount of water-soluble proteins in the cataractous lenses was less than in healthy lenses perhaps the result of decreased protein synthesis during cataract formation (Berman, 1991). Although it has been possible to localize γ-crystallin (Shubert et al., 1970) and their mRNAs (Layden et al.,1988) in the rat lens, this has not been done during cataract formation, except using reporter genes (Goring et al., 1992). Nonenzymatic conformational changes were induced in bovine α-and γ-crystallins by incubation with glucose-6-phosphate (Beswick and Harding, 1987). In their model the formation of water-insoluble aggregates from water-soluble proteins, which could be the result of either their secondary and tertiary structural changes or a transamination reaction, caused the turbidity of the lens.

FIGURE 1.5.
THE EFFECT OF DIABETES ON LENS GLUCOSE METABOLISM.

During diabetes, high glucose in the aqueous humour is transported into the lens and excess glucose enters the glycolytic pathway. As a result of the metabolism of elevated glucose some changes occur which are tabulated in Table 1.1.


Normal
High glucose in aqueous
High glucose in lens
glucose-6-phosphate
aldose reductase
Pentose phosphate pathway
NADPH
GSH
sorbitol
glycosylation
polyol dehydrogenase
browning
glucose 6-phosphate dehydrogenase
mixed sulphides
fructose
unfolding of proteins
ATP
ATPase
Amino acid uptake
Na+
aggregated protein
swelling
Amino acid content
Protein synthesis
Cortical opacity

This suggestion was also supported by Benedek (1971). All amino acids which are utilized for synthesis of animal protein require the L-configuration of amino acids. However, postsynthetically, transformation from the L-configuration to the D-form may occur. This is called racemization of amino acids. A decreased level of racemization was found in the water-insoluble fraction of cataractous lenses. Another very important development in the lens is oxidation of thiol groups during cataract formation (Spector et al.,1978; Zigman et al., 1982). These modifications initiate the formation of opacity in the lens.

In the opaque lens the amount of potassium was lower while the amounts of sodium and calcium were higher than in the normal lens (Table 1.1). Loss of glutathione and ATP along with excess amount of oxidative species were also found in cataractous lenses (Cheng and Chylack, 1985).

1.4 BIOCHEMISTRY OF LENS PLASMA MEMBRANE AND CYTOSKELETON:

Lens plasma membranes may be distinguished by either their density and solubility properties or their solubility in urea/water (Alcala and Maisel, 1985). Lens fiber plasma membranes are recognized as the major constituents of the water-insoluble fraction. Following studies on calf lenses (Alcala and Maisel, 1985; Harding and Crabbe, 1984; Bloemendal, 1982) two major protein components of fiber plasma membranes have been distinguished: MP26 and MP 34. MP26 represents about 95% of the lens membrane proteins and is the principal intrinsic protein of lenticular fiber

TABLE 1.1. SUMMARY OF CHANGES IN THE RAT LENS DURING DIABETI CATARACT FORMATION.

Increases	Decreases
Changes preceding the first opacities	
Sorbitol	Amino acid uptake
Fructose	Amino acid content
Glucose	Glutathione
Mitosis	Glucose-6-phosphate dehydrogenase
	6-Phosphogluconate dehydrogenase
	Pentose phosphate pathway
	Phosphofructokinase
	Aldolase
	ATP
	Lactic dehydrogenase
	Protein synthesis
	NADPH
	Fluorescence
Changes at the time of the first opacities	
Swelling and degeneration of cortical cells	
Later Changes	
Extracellular space	Sorbitol
	Fructose

The data shown in this table were obtained from the following references: van Heyningen 1959; Kinoshita et al., 1962; Spector et al., 1973; Creighton et al., 1978; Zigman et al., 1982; Cheng and Chylack, 1985; Alcala and Maisel, 1985; Beswick and Harding, 1987; Berman, 1991.

membranes in various species. MP34 is found in both epithelial and fiber cell plasma membranes. Anti-MP26 serum did not react with isolated MP34 or with epithelial membranes, which shows that MP26 synthesis occurs exclusively in lens fiber cells. Another significant difference between MP26 and MP34 is reflected by the fact that,in contrast to MP26, MP34 is not subject to gradual proteolytic degradation upon aging (Berman, 1991; Alc .la and Maisel, 1985; Harding and Crabbe, 1984; Bloemendal, 1982).

Underlying the plasma membrane of many eukaryotic erythroid and non-erythroid cell types is a cytoskeletal meshwork referred to as the membrane skeleton. The molecular organization and function of the lens cell membrane skeleton is not as clear as for the nonlenticular tissues, though the lens fiber cells are not exceptional and express a host of cytoskeletal components which are identical or related to those found in other tissue types. Initially, certain classes of lens polyribosomes were found to be associated with the fiber cells plasma membrane-cytoskeleton complex. Later it was shown that the polyribosomal population was attached to the plasma membrane-cytoskeleton complex via actin (Pollard, 1986; Bennett, 1989, 1990). A study of lens epithelial cells established that the organization of the cytoskeleton is different in the peripheral lens epithelium as compared to the central lens epithelium (Blanquet and Courtois, 1989).

Actin, tubulin, and vimentin-based filament systems are present in the lens fiber cell cytoplasm and are thought to participate in the elongation of lens fiber cells (Ellis et al., 1984). Also, these cytoskeletal proteins are thought to maintain the shape of the lens during visual accommodation, and in the genesis of cataract formation (Bloemendal, 1977; Bloemendal, 1981; Piatigorsky, 1981). The lens beaded filaments are thought to be comprised of filensin and the 49 kDa protein; these components are related to intermediate filament proteins and are unique to the cytoplasm of the lens fiber cell (Hess et al., 1993; Merdes et al., 1991). Actin and vimentin in the peripherial epithelium appear to be almost intact or slightly reduced after selective disruption of intermediate filaments and microfilaments, suggesting that these elements exist, most probably as an independent system in this region. In contrast, actin and vimentin from the central epithelium are largely removed after disruption of intermediate filaments and microfilaments. These observations suggest that microfilaments are involved in an inter-relationship with intermediate filaments in the central epithelium. Ramaekers et al., (1982) reported that vimentin is closely associated with the epithelial cell plasma membrane. The spatial relationships of actin-based and vimentin-based filaments of the peripherial epithelium and fiber cells are linked to profound changes in organization of major surface proteins

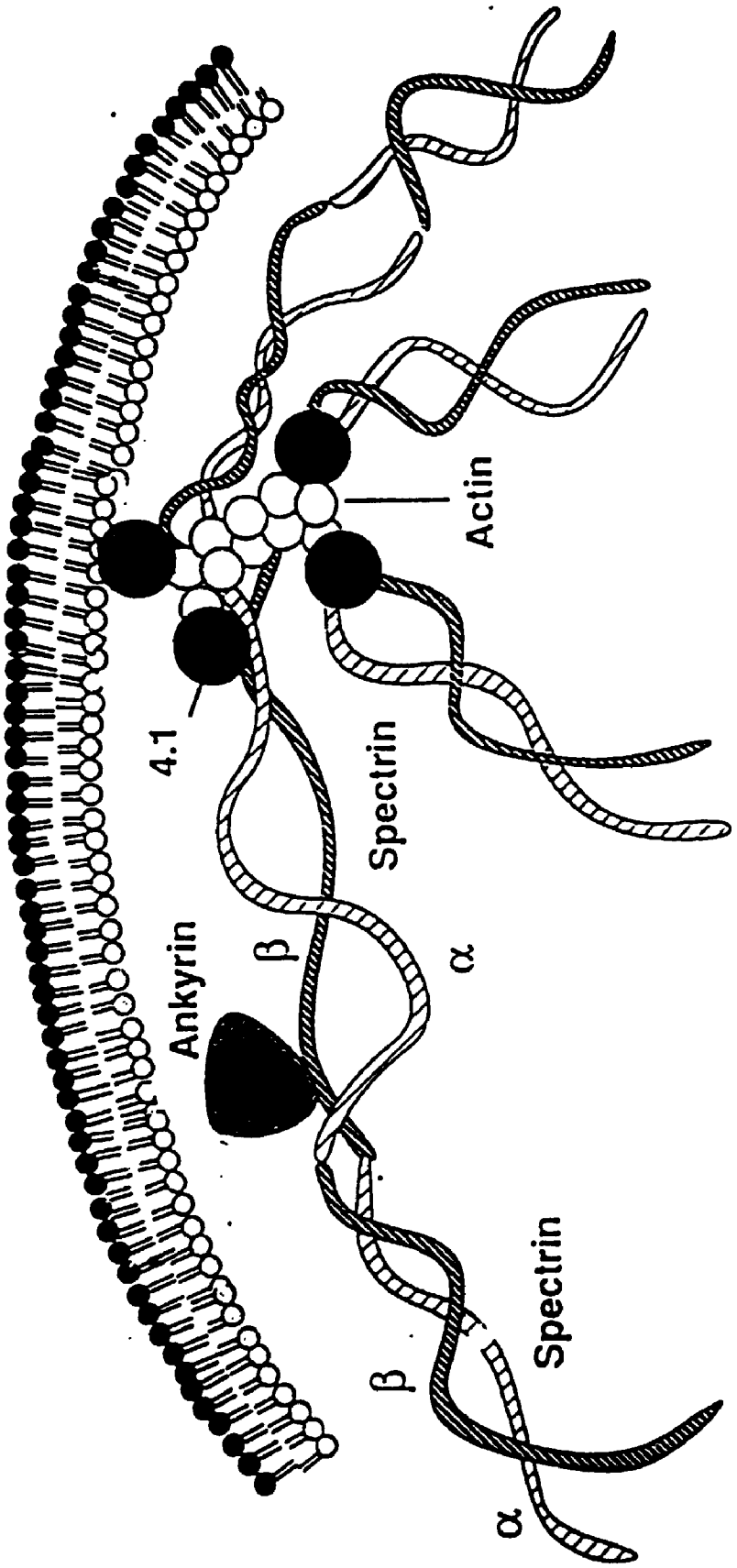
and glycoproteins facing the lens capsule (Ramaekers et al., 1982).

In the lens the following isoforms of erythrocyte membrane skeleton components are found : erythroid spectrin, nonerythroid spectrin (fodrin) (Yoon et al., 1989; Aster et al., 1984), protein 4.1, 4.9 (dematin) and adducin, as well as proteins which link the membrane skeleton to membrane: ankyrin and band 3, (Allen et al., 1987; Aster et al., 1984; Faquin et al., 1988; Granger and Lazarides, 1984; Green and Maisel, 1984; Kaiser, 1989; Lehto and Virtanen, 1983; Repasky et al., 1982) (Figure 1.6). The presence of the elements specified above suggest that the lens membrane skeleton is similar to that found in the erythrocyte and also may function to maintain membrane stability in the lens. Additionally, cytoskeletal components interact with specialized plasma membrane domains such as focal adhesions and adherence junctions via molecular bridges. These are also identified in the lens, including: vinculin, α-actinin, plectin, and plakaglobin (Bloemendal, 1981; Franke et al, 1987; Weitzer and Wiche, 1987; Lehto and Virtanen, 1985). In nonlenticular cells these proteins link integrins and cadherins to the cytoskeleton. In the lens, however, this relationship has not yet been studied.

The location of cytoskeletal proteins at the plasma membrane of the lens fiber and epithelial cells suggest that they may play a role in regulating the organization of the

FIGURE 1.6.
MEMBRANE-CYTOSKELETON ATTACHMENTS IN THE LENS.

Diagram shows the major interactions among spectrin, ankyrin, protein 4.1, and actin in the lens cell. These proteins maintain the shape of the lens during visual accommodation, and may be involved in the genesis of cataract formation. When the cytoskeleton is disrupted, there is much evidence to show that cell surface molecules which are stably associated with the cytoskeleton can be lost.


Actin
4.1
Spectrin
Ankyrin
β
α
β
Spectrin
α

membrane skeleton in this tissue. Under the cytoskeleton-disruption conditions there is much evidence to show that cell surface molecules which are stably associated with the cytoskeleton can be lost.

1.5 OBJECTIVES, RATIONALE AND SIGNIFICANCE OF THIS THESIS:

(a) Objectives:

The objectives of this work were: to utilize the technique of lens culture to focus on the mechanism of cataract and to classify and arrange the events in order during cataractogenesis. The pre-cataractous and cataractous indicators were distinguished by using different parameters in two in vitro model systems: 1) cytochalasin D-induced cataractogenesis, in which cytoskeletal network was disrupted and lens damage occurred rapidly within 24 hrs; 2) glucose-induced cataractogenesis, in which damage occured after 48 hrs and developed progressively up to 8-10 days as the lens slowly became opaque.

The development of cortical subcapsular opacity was elucidated as a time-dependent phenomenon. That enabled me to deduce the specificity of my hypothesis, to gain a clear understanding of cataract formation. What could initiate the membrane damage in the lens cells ? At which step in the process of the cataract formation does this occur?

In vitro studies always have advantages in exploring

the direct effects of the agents. Various known cataract preventive agents could be used to model protection of the lens and prevention of opacification. After experiments established the in vitro model systems and the indicator parameters, different cataract preventive agents were used to investigate their effects on cataract development.

(b) Rationale:

Recently, there has been increasing interest in the intracellular calcium level and its relation to cataract formation. This interest is based upon many significant experiments which suggest that insult to the membrane may be an early event in the cataract development process which could be caused by a high intracellular free calcium concentration. In exploring this hypothesis, the timing and localization in the lens of calcium uptake during the cataract formation is critical.

It is possible that a fluctuation in calcium concentration may initiate the calcium-dependent proteolysis and digestion of specific lens proteins which are calpain substrates. If the calpain is the cataract initiator, can a calpain inhibitor prevent cataract formation ?

(c) Significance :

The significance of this work is that it focuses on the mechanism of cataract formation in an in vitro system.

Moreover, it provides the biochemical evidence that the increase in cytosolic calcium is associated with the loss of transparency during the first 48 hours of cataract formation. This evidence stimulated my research to elucidate further the role of calcium in cataract formation. This work suggests that calcium-dependent proteolysis of the cytoskeletal protein spectrin/fodrin in the lens cells does not precede cataract formation but it is a consequence of pre-cataractous changes. To the best of my knowledge this is the first study to show that calcium-dependent proteolysis of spectrin/fodrin is not an immediate event in the mechanism of cataract formation.

CHAPTER 2

EXPERIMENTAL OPACIFICATION AND THE INDICATORS FOR PRE-CATARACTOUS AND CATARACTOUS LENSES

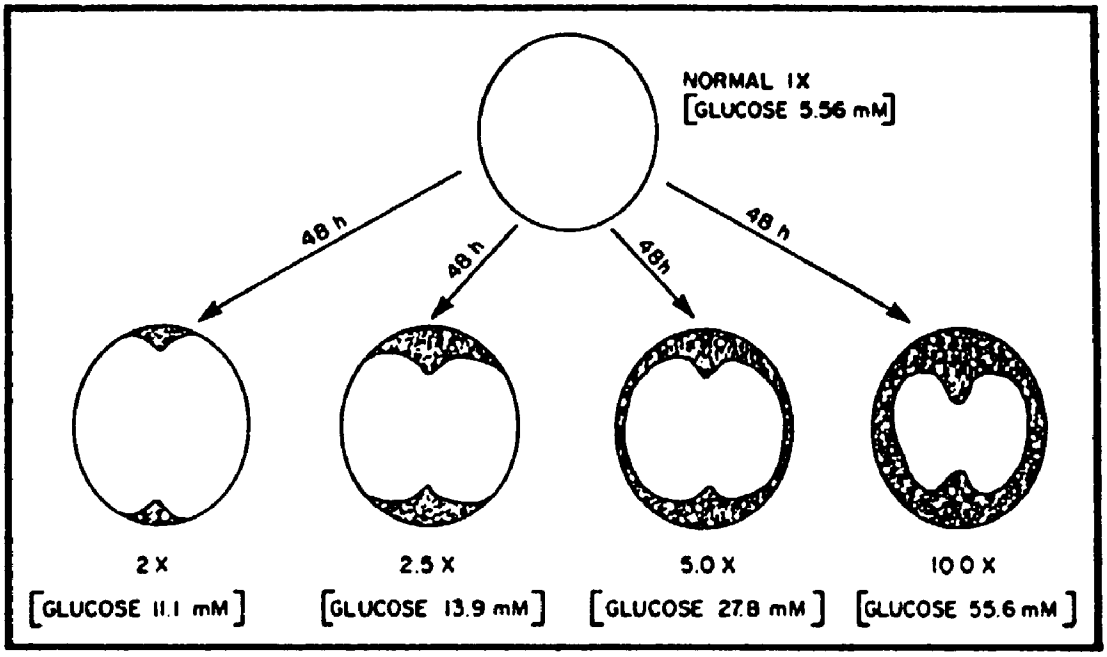
2.1 INTRODUCTION

In cataractogenesis, damage to lens membranes appears to be a major contributing factor (Ogise et al., 1993, Tumminia et al., 1994). As a result of membrane damage, the intracellular components of the lens - proteins (Tumminia et al., 1994), enzymes and ions- start to leak into the extracellular space during the process of cataract formation. Since cataract is the end product of a series of reactions, it is critical to be able to organize the events in sequence and elucidate the specific mechanisms used to achieve cataractogenesis. To clarify the order of changes occurring for pre- and cataractous lenses would be an important beginning.

The lens culture conditions first developed by Paterson and Fournier (1976) and then Creighton et al., (1980) maintain lens clarity in commercially supplied medium 199 with serum supplement for at least a two week incubation period. In an in vitro study of glucose on rat lenses Creighton et al., (1980) suggested that the depth of globular degeneration was roughly proportional to the glucose concentration in the incubation medium after 48 hr (Figure 2.1). Initially in my investigation, in vitro

FIGURE 2.1.
DIAGRAMMATIC REPRESENTATION OF GLOBULAR DEGENERATION.

Development of opacity in rat lenses after incubation for 48 hr in M199 with normal and elevated glucose concentration (modified from Creighton et al., 1980).


NORMAL 1X
[GLUCOSE 5.56 mM]
48 h
48 h
48h
48 h
2 X
[GLUCOSE 11.1 mM]
2.5 X
[GLUCOSE 13.9 mM]
5.0 X
[GLUCOSE 27.8 mM]
10.0 X
[GLUCOSE 55.6 mM]

culture conditions without serum were established to permit model studies, so that lactate dehydrogenase (LDH) and protein release could be followed with no interference from serum proteins. Once these conditions had been worked out, then two different reagents which represent two different mechanisms to achieve the same end, cataractogenesis, were used in the experiments reported here: elevated glucose (55.6 mM), as a model for *in vivo* diabetic cataractogenesis and an actin monomer-stabilizer, Cytochalasin D (10^{-5} M CD).

During the first 48 hr of culture, the lenses were observed to be clear and pre-cataractous, progressing to opaque (cataractous) after they have been incubated for 8 days. Lenses incubated under both conditions, either in glucose or CD, developed opacity at different times. I was able to observe the damage accompanying this opacity by various biochemical tests.

In vivo or *in vitro* development of sugar/diabetic cataract in mammals is accompanied by an accumulation of polyols in the lens (van Heyningen, 1959). The effects of this excess concentration of polyols could be seen at both cellular and molecular levels. Kinoshita *et al.*, (1962), suggested that the increased level of polyols causes an osmotic stress which is the primary cause of lens opacification. Many research teams have been working on the initial reactions following the polyol accumulation. The opacity of cortical cataract begins with the abnormal

differentiation of equatorial epithelium followed by the formation of globules and liquid-filled vacuoles in the cell cytoplasm. These globules cause the light scattering and reflection responsible for the opacity (Creighton et al., 1978; Harding, 1991; Unakar et al., 1978). Similar globular degeneration has been shown in lenses exposed to elevated glucose (Trevithick et al., 1981) or cytochalasin D (Mousa et al., 1979), an agent which disaggregates actin microfilaments (Pollard, 1976; Karp, 1991). Both methods will be described in the Materials and Methods sections (2.2) in detail.

There has been considerable recent interest concerning the role of intracellular free magnesium in the regulation of cell functions. Magnesium is a necessary co-factor for hundreds of enzymes and plays an essential role in protein synthesis. It stabilizes the structure of ribosomes and membranes. However, there has been no study of this cation either in healthy or in cataractous lenses until now. But in the lens epithelial cells a magnesium dependent ATPase was found (Bergner and Glasser, 1979). In other cells magnesium was shown to be one of the most abundant intracellular cations i.e.: in the rod outer segment, steady light is known to affect total magnesium content (Somlyo and Walz, 1985); magnesium fluxes in pancreatic islet cells are markedly modified by glucose (Henquin et al., 1983); and a number of chronic diseases, such as diabetes, essential

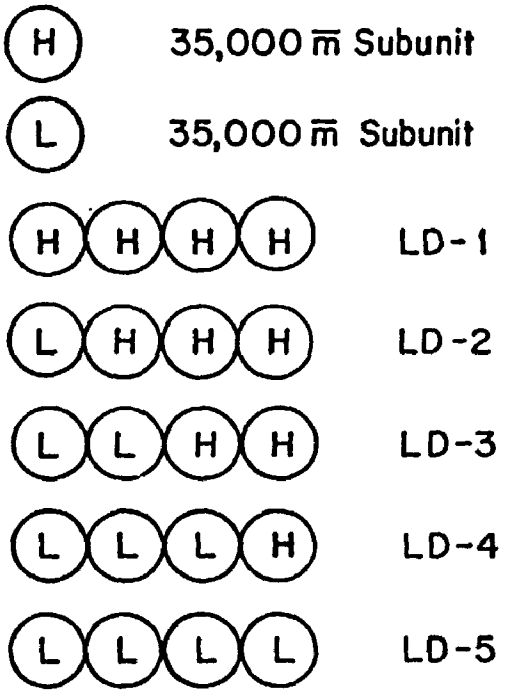
hypertension, renal vascular disease, circulatory shock and alcoholism, are associated with hypomagnesemia (Altura and Altura, 1981). However, evaluation of the role of magnesium as an acute or chronic regulator of lens cell function has been hampered by the lack of study of its role in the lens. One possible reason might be the lack of suitable methods for monitoring free cytosolic magnesium in the lens cells. The study reported here developed a fluorescent technique to visualize the location and distribution of intracellular free magnesium in lens cells during the precataractous stage in two model systems.

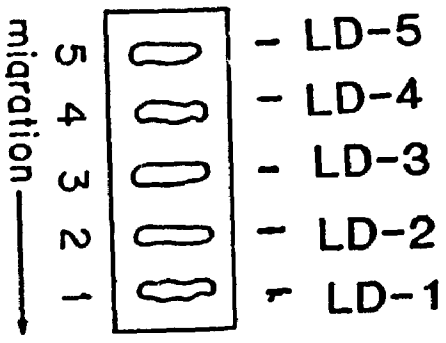
In a healthy mammalian cell, LDH is localized in the cytoplasm. When cells start to lose their integrity LDH release is often increased and this LDH appears in the extracellular medium (Bianca 1992; Kilic and Trevithick, 1995). In anaerobic glycolysis, the main source of energy for the lens, LDH (EC 1.1.1.27) is an important enzyme. The majority of pyruvate which is produced in glycolysis is converted into lactate by LDH using NADH (Stryer 1988; Berman, 1991). There are 5 LDH isoenzymes; the order of their electrophoretic migration in nondenaturing gels is arbitrarily assigned as LDH-5 to LDH-1 (Figure 2.2). LDH has been found in the lens of different species (Maisel, 1965; Gershbein et al., 1977). In the human lens LDH was found mainly in the cortex. Three isoenzymes, LDH-5, -4 and -3, have been reported in human lens. During the aging and

FIGURE 2.2.
THE SUBUNIT COMPOSITIONS OF LDH ISOENZYMES.

LDH has a molecular weight of 134 kDa and is composed of four peptide chains of two types: M and H. The subunit compositions of five isoenzymes, in order of decreasing mobility on a nondenaturing condition, is LD-5 to LD-1.

LACTATE DEHYDROGENASE ISOENZYMES





cataract formation LDH-3 was significantly low (Nagpal et al., 1991; Friedburg, 1973).

The effect of 55.6 mM glucose or 10^{-5} M CD on the healthy lens were studied with various parameters. These were: (i) the appearance of the lenses observed daily by a dissection microscope; (ii) the distribution of live cells; (iii) the leakage of the cytosolic enzyme, lactate dehydrogenase (LDH) into the incubation medium, (iv) the alteration of the intracellular free magnesium level. Also the leakage of γ-crystallin was supported by an in vivo study of diabetic rats. In vivo diabetes was induced by streptozotocin (STZ), which oxidatively damages the β-cells in the pancreatic islets of Langerhans. This impaired production of insulin causes diabetes in the rat. STZ diabetes has been found to be a good model system for studying diabetic cataract (Ross et al., 1982) and for showing large decreases in glutathione, ascorbate, taurine, cysteine and ATP in precataractous clear lenses (Mitton et al., 1993; 1994).

2.2 MATERIALS AND METHODS

REAGENTS: All chemicals were obtained from Sigma, Canlab (Toronto, Ontario), and/or Fisher, and were reagent grade or the highest purity available.

2.2.1 Animal Handling and Isolation of Eyes:

Animals (Wistar rat, female/male 200-220 g) were allowed to acclimatize to new surroundings for at least 1 week before use. Agway 3000 pellet feed and water were provided without limitation. Housing was in cage racks. All procedures of handling and experimentation met the guidelines of the Canadian Council on Animal Care and the Association for Research in Vision and Ophthalmology, and were designed, approved and monitored under the guidance of the Institutional Animal Care Committee System, and Veterinary Services at the University of Western Ontario.

The eyes were removed from CO_2-euthanized rats and placed in Minimum Essential Medium (Modified) with Earle's Balanced Salts with L-glutamine (M199) (serum free) for dissection. M199 was purchased from Flow Laboratories, Inc. (catalogue number: 10-201) and prepared as follows: 9.55 gm powder medium was dissolved in 800 ml distilled H_2O, and 2.2 gm $NaHCO_3$ added. The medium was adjusted to pH 7.4, sterilized by passing through a 0.20 µm filter and then 50 mg of gentamycin was added to a final volume of 1 litre

M199. Lenses with intact capsules were dissected from rat eyes following CO_2 euthanasia as described below.

2.2.2. Preparation of Rat Lenses, Problems and Their Solutions During *In Vitro* Study:

For *in vitro* study, eyes were cut peripherally in M199 using fine microscissors with one serrated edge (Fine Science Tools), and all layers around the lens capsule were removed at room temperature.

After dissection of all the lenses, each lens was weighed on a piece of parafilm then washed many times with M199. Then each was transferred into a 24-well tissue culture plate containing 1.5 ml M199 per well. The lenses were incubated for 24 hr in this medium at 35.5 °C in 5% CO_2: 95% air. After preincubation, any opaque lenses which had been damaged during dissection were discarded. To avoid contamination and keep the incubation conditions consistent for lens culture, the medium was changed after every 24 hr incubation period.

2.2.3 *In Vitro* Cataract Causative Agents: Glucose- and CD-Induced Cataractogenesis:

After a 24 hr preincubation in control medium (M199), lenses were examined to check whether there had been damage to the lens capsule during the dissection or whether any contamination had occurred. Then one set of lenses was

incubated with either M199 containing 55.6 mM glucose (10 times the normal concentration in the medium) or with M199 with a normal (5.56 mM) glucose concentration, which was used as a control.

One set of lenses was incubated with either 0.1% dimethylsulfoxide (DMSO), which was used as a control, or with 10^{-5} M CD (in 0.1% DMSO) in the presence of M199. The final incubation volume was 1.5 ml. The 24-well trays containing the lenses were incubated at 35.5 ^{0}C in 5% CO_2: 95% air. After every 24 hr, 0.05 ml aliquots were taken and assayed for LDH activity.

2.2.4 Lactate Dehydrogenase:

LDH activity was measured spectrophotometrically by the method of Moss et al., (1978), and Karlsson et al., (1974). Two ml of coenzyme solution (56 mM Tris buffer, 0.17 mM NADH and 5.6 mM EDTA, pH 7.4) were mixed with 0.05 ml of each sample from the lens medium, then incubated for 10 min at 37 ^{0}C. At the end of the incubation time the starter reagent (14 mM pyruvate 0.20 ml) was mixed and the absorbance at 340 nm was read immediately and followed for 3 min during which time the rate was linear. The LDH activity in international units at 37 ^{0}C is obtained as follows:

2.250 = total volume in cuvette, in ml,

0.05 = volume of sample in ml,

$$U/L\ (\mu mol/min/L) = \frac{\Delta A/min}{6.3 \times 10^{-3}} \times \frac{2.250}{0.05} = \frac{\Delta A}{min} \times 7143$$

6.3×10^{-3} = micromolar absorption coefficient of NADH at 340 nm.

$\frac{\Delta A}{min}$ = *average absorbance change (decrease) per* min

The specific activities were calculated from the linear portion (up to 3 min.) of the rate curve. The addition of M199 instead of sample served as a negative control. The total accumulated LDH leakage was calculated by adding the previous total to the daily units of LDH per gram of the initial wet weight of the lens, present in the culture medium. The medium was changed every day, and the LDH activity measured. Data represent the mean value of three experiments (n=3), each using a separate lens culture preparation. Also, standard errors (SE) are calculated as means ± SE (n=3) and percent SE values are tabulated.

2.2.4.1 Native Gel Electrophoresis and NBT Staining:

150 µl samples from incubation medium were run on 10% native PAGE to separate LDH isoenzymes. PAGE was performed using the buffer system of Dietz and Lubrano, (1968). The gels were stained for LDH by incubating them with 1 ml of 5.35 mM Na-lactate, 0.47 ml 8 mM NAD^+, 0.2 ml 8 mM phenazine methosulphate, 0.78 ml of 4 mM nitrobluetetrazolium, 5 ml of

Tris-HCl buffer 0.05 mM pH 7.5, and the volume of incubation assay was made up to 10 ml with water (Neilands, 1955). The gels were incubated at 37 °C.

2.2.5 Following the Time Course of Cataract Formation under a Dissection Microscope:

The formation of opacity on the incubated rat lenses was traced by examining them after every 24 hr period. The lenses were photographed through a binocular dissection microscope (Chylack, 1978) using a stereoscopic adapter.

2.2.6 Lens Proteins and γ-Crystallin:

2.2.6.1 <u>In Vitro</u> Study:

To estimate the amount of protein in the lens incubation medium the BioRad protein assay was followed. A standard curve was prepared with different amounts of IgG (0, 0.2, 0.4, 0.6, 0.8, 1. 1.2 µg) and the absorbance was read spectrophotometrically at 595 nm. The same procedure was followed for the incubation medium, and the amount of protein was calculated by using the standard curve.

2.2.6.1.1 SDS-Polyacrylamide Gel Electrophoresis:

150 µl samples from CD- and 55.6 mM glucose-supplemented M199 were run on 10% SDS-PAGE to observe the amount and variation of the proteins which leaked from the

lens into these incubation media. SDS-PAGE was performed using the buffer system of Laemmli (1970). Molecular weight markers included: B-galactosidase (116 kDa); phosphorylase b (92 kDa); bovine serum albumin (68 kDa); catalase (60 kDa); ovalbumin (43 kDa); lactate dehydrogenase (34 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa), and cytochrome c (12.3 kDa). The gel was stained with Coomassie Blue R-250.

2.2.6.1.2 Western Blotting:

25 µl samples from the lens incubation media (M199 containing 55.6 mM glucose) were run on 12.5% SDS-PAGE to observe the leakage of γ-crystallin. The gel was stained with Coomassie Blue. An identical gel was transferred to nitrocellulose and probed with polyclonal anti-γ-crystallin antibody as described by Towbin et al., (1979). The nitrocellulose was blocked by incubation at room temperature for 1 hr with 4% casein in Tris-buffered saline (20 mM Tris-HCl, pH 7.4, 0.15 M NaCl). Polyclonal antibody which was prepared by Linsclater et al., (1986), was purified on a protein A column, added directly to the blocking solution and incubated with the blot overnight at room temperature. As secondary antibody, peroxidase-conjugated goat anti-rat IgG (BioRad), diluted 1:5000, was used. The immunoreactive γ-crystallin was visualized using enhanced chemiluminescence (ECL, Amersham) according to the manufacturer's

instructions. ECL is a light-emitting non-radioactive method for detection of horseradish peroxidase-labelled antibody, using luminol as a reagent.

2.2.6.2 *In Vivo* Study:

2.2.6.2.1 *In Vivo* Diabetes Induction:

Diabetes was induced in Wistar rats (see section 2.2.1) by a single sterile intraperitoneal injection of streptozotocin (STZ) prepared in a citrate-phosphate buffer (2.3 g citrate monohydrate, 2.58 g sodium phosphate dibasic anhydrous in 200 ml of deionized water, pH: 4.5) and at a dose of 55 mg/kg. Dry STZ was dissolved in buffer, sterilized through a 0.20 µm size filter and animals were injected immediately using this solution by Mr. T. Dzialoszynski. After every 5 min period, the STZ solution was discarded and a fresh preparation made, as suggested by the manufacturer (Upjohn). Animals were monitored by two tests [urine glucose (TES-TAPE) and total glycated haemoglobin] to evaluate the degree of hyperglycaemia. The control animals received the same injections (volume per body weight) using the citrate-phosphate buffer without STZ.

2.2.6.2.2 Eye Examination:

The animals were examined using slit-lamp biomicroscopy by a veterinary pathologist with ocular specialization (Dr.

S.E. Sanford), after pupil dilation with Mydriacyl, at zero time and on a biweekly basis from week 4 until the end of the experiment. Lenses were graded as follows: 0, clear lens; 1, lenses showing visible posterior sutures; 2, lenses displaying isolated vacuoles; 3, coalesing peripheral vacuoles; and 4, peripheral coalescing vacuoles and radial streaks extending into central opacity.

A further analytical method based on the analysis of digitized images of the scattered light was also used by Mitton et al., (1989), using the Kevex image analysis program to evaluate the total area of scattered light greater than some minimum constant intensity level.

2.2.6.2.3 Urine Glucose and Glycated Haemoglobin Tests:

To test how predictive were the techniques we used each was tested (1) against the final glycohaemoglobin value at sacrifice (determined with Sigma Kit 4416), which is an indicator of the diabetic blood glucose levels integrated over time, or (2) to the blood glucose concentration, or (3) to each other.

During animal handling a drop of urine was collected on the test tape (TES-TAPE). Normal animals gave negative urine glucose results.

At the end of 12 weeks animals were anaesthetized using an intraperitoneal injection with 90 mg/kg ketamine hydrochloride and 6 mg/kg Rompun (Xylazine) by Mr. T.

Dzialoszynski. After 15 min, the animals were decapitated, the eyes removed into M199 (serum free) for dissection, and the whole blood was collected in Li/Heparin tubes (microvette, Sarstedt) and frozen at -70 °C until use.

Urine glucose and glycated haemoglobin tests were completed according to the kit instructions by our two technical assistants Ratan Bhardwaj and Beata Batarowicz.

2.2.6.2.4 Competition Assay Using Nitrocellulose Microtitre Plates:

γ-Crystallin leakage into the vitreous and aqueous humours was estimated by a sensitive technique which was developed based on a competition assay using nitrocellulose microtitre plates (Harlow and Lane, 1989).

γ-Crystallin was purified from a 1.5 x 100 cm Sepharose S-200 column (Linklater, 1985). Initially the column was washed with 0.05 M tris buffer, pH 7.6 containing 0.01 M sodium azide and 0.001 M β-mercaptoethanol and the fraction collector was set at 80 drops/tube. 20 lenses were weighed together (approximately 0.55 gram) and homogenized in 14.14 ml (column volume X 0.2) 0.05 M tris buffer. The tubes containing lenses or lens homogenates were always kept on ice. The lens homogenate was centrifuged at 26,000 g for 20

minutes at 4 °C. PMSF (0.001 v/v from a stock solution containing 0.1 mg PMSF/ml of ethanol) and leupeptin (2 mg/ml) were added to the lens extract before applying to the column. 140 fractions were collected and their absorbance at 280 nm was read.

γ-crystallin was labelled with ^{125}I as described (Fraker and Spect, 1978). A P-6 column in a 5 ml syringe was prepared, then washed with 10 ml buffer A (pH 6, 10 mM MES, 2 mM KI, 1 mM NaOH, 0.2 mM EDTA, 0.02% sodium azide and 1 mg/ml hemoglobin). The iodogen tubes were prepared by adding 8 µl iodogen (supplied by Pierce) which was dissolved in chloroform (1 mg/ml). After the chloroform was evaporated, the tubes were rinsed with borate buffer (pH 8.2, 6.25 mM borate, 145 mM NaCl, 0.1 mM EDTA), then placed in an ice bucket. 100 µl γ-crystallin (1 mg/ml, dialyzed against borate buffer) was mixed with 300 µCi ^{125}I and incubated on ice for 30 minute. The reaction was stopped by adding DTT (1 M, 2 µl) and buffer A (391 µl). The iodinated protein sample was applied to a P-6 column. 15 fractions (500 µl) were collected in Eppendorf tubes. From each fraction a 2 µl aliquot was mixed with NaOH (100 µl 0.1 M) prior to liquid scintillation counting. The fraction with the highest amount of radioactivity was used for competition

assays. Also, the percentage of labelling was calculated. γ-Crystallin (10 µl, ^{125}I labelled) was mixed with BSA (10 µl, 10 mg/ml) and TCA (500 µl, 10%) and after centrifugation, the supernatant was counted.

^{125}I-labelled γ-crystallin was mixed with authentic cold γ-crystallin or an unknown sample, appropriately diluted, prior to the addition to the microtiter plates (Harlow and Lane, 1988). The plates (Costar #8521) were prepared by incubation overnight with anti-γ-crystallin antibody (100 µl, 20 µg/ml) purified on a protein A column. Prior to the addition of the mixture the wells were washed twice with phosphate-buffered saline (PBS). To block nonspecific sites, the wells were wahed for 2 hr in 3% bovine serum albumin (BSA) in PBS at room temperature followed by two PBS washes. The mixtures of ^{125}I-γ-crystallin and cold γ-crystallin were incubated for 2 hr at room temperature in a final volume of 50 µl per well. After 4 PBS washes the remaining ^{125}I per well was read by scintillation counting.

2.2.7 Alteration in Lens Intracellular Free Magnesium Level During Cataract Formation:

Previously prepared magnesium-green (at a concentration

of 0.138 mM stock in 0.1 % DMSO) (Eberhard and Erne, 1991) was added to 1.5 ml culture medium to give a final concentration of 9.17 µM. After 0, 24 and 48 hr incubation, lenses were monitored for confocal images by Dr. D. Carter (Robarts Research Institute) on a prototype Transmission Confocal Laser Scanning Microscope (TCLSM) working in single-lens epifluorescence mode. The objective was an infinity corrected Mitutoyo Plan Apo x20/0.42NA dry lens which had a working distance of 20 mm. Both 488 nm and 514 nm lines from an Argon ion laser were used for illumination, and a 535 nm longpass filter was used in the detection arm. To obtain semi-quantitative results, x-y and x-z images were taken under standardized optical sectioning conditions (Dixon et al., 1991; Carter, 1993; Haugland, 1992) of the lens either at the equator region, or from the anterior through posterior side of the lens, which was optically sectioned through a depth of 2 mm with the photo multiplier tube (PMT) with the amplifier set at 200 µA/V. If the image intensity fell well outside this range, additional images were taken at 50 µA/V or 1 mA/V. Clear lenses were transparent enough to be scanned through their whole depth, although refractive index differences affect the spatial accuracy of these images.

2.2.9 Fluorescence Vital Staining to Determine Lens Cells Viability:

In this part of the study the effect of 55.6 mM glucose on incubated rat lenses was observed by vital staining of the rat lenses and compared with the healthy lens vital staining. Either fluorescein diacetate (FDA, 50 µg/ml, incubated for 5 min before scanning) (Mohr and Trounson, 1980) or the styryl dye RH414 (Molecular Probes T1111, 50 µg/ml, incubated for 3 min before scanning) (Betz, et al., 1992) was added into the incubation media at the end of 0, 24 and 48 hr. Lens cell membrane integrity was assessed by BioRad 600, confocal laser scanning microscopy (CLSM) combined with vital dye staining (Matsumoto, 1993).

2.3 RESULTS

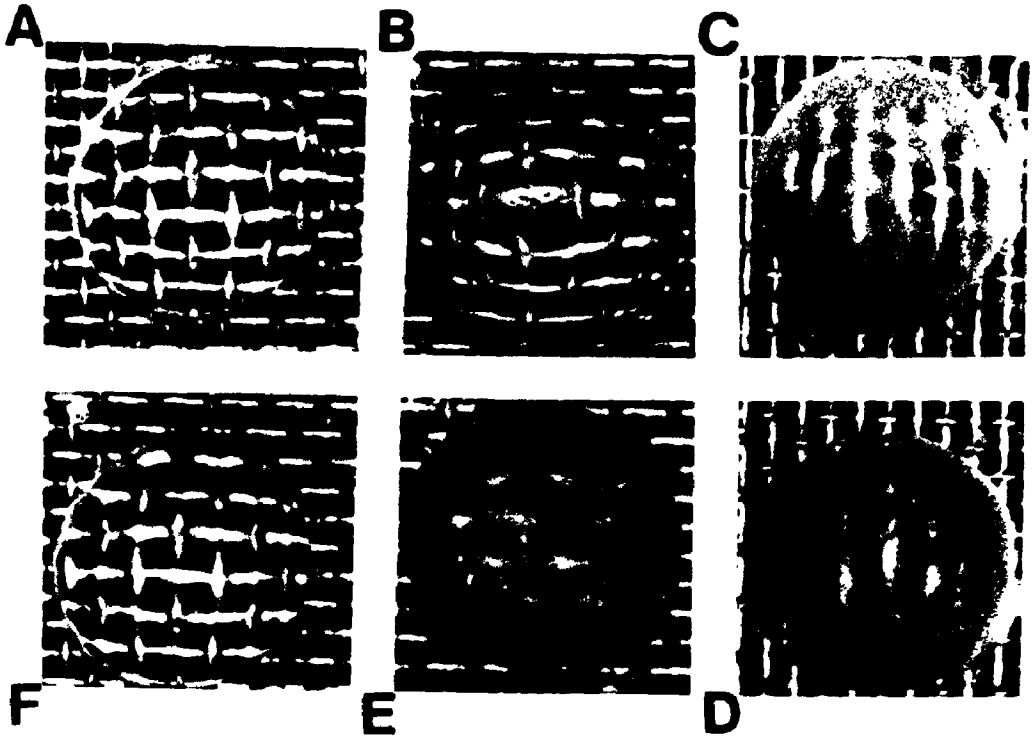
When Can We See the Development of Opacity Using the CCRG-Camera Dissection Microscope ?

One of the important parameters used to follow the effect of cataract-inducing agents is the observation of the appearance of the lenses by a dissection microscope during the incubation period. Following the dissection, lenses with intact capsules were incubated in M199 for 24 hr, to check whether any damage occurred to the capsule during dissection and cleaning. The lens appearance was examined through a dissection microscope, and only the healthy lenses were used in the rest of the procedure. At the end of 8 days, control lenses were clear in M199 without any supplement. Photomicrographs of lenses were obtained every second day using a Cooperative Cataract Research Group (CCRG) camera described by Chylack, (1978) (Figure 2.3, Panel A and F).

After 24 hr preincubation in M199, healthy lenses were removed to a medium with 55.6 mM glucose. During the first 24 hr in 55.6 mM glucose-containing medium, no opacity was observed. Between 24-48 hr, the lenses developed a slight opaque layer (cataract) (Figure 2.3, Panel B). After 4 days of incubation in the medium a visible opaque layer was observed (Panel C), and the depth of the lens opaque layer had clearly increased after 6 days (Panel D). After 8 days, the opaque area of the lenses was coloured red with the

FIGURE 2.3.
DEVELOPMENT OF OPACITY DURING LENS INCUBATION IN M199 CONTAINING 55.6 mM GLUCOSE.

Rat lenses were preincubated for 24 hr in M199 after eye dissection (Panel A). Panels B, C, D, E show the lenses after 2, 4, 6, 8 days incubation, respectively, in M199 containing 55.6 mM glucose. The control lens after 8 days in M199 is Panel F.


A
B
C
F
E
D

indicator dye from the medium, phenol red (Panel E).

The effect of CD on lens opacity was examined by incubating the rat lenses in M199 containing 10^{-5} M CD (in 0.1 % DMSO). After 24 hr pre-incubation in M199 (Figure 2.4), lenses were transferred to the medium containing 10^{-5} M CD and the progression of opacity was observed by a dissection microscope and photographed every second day. After 48 hr of incubation in CD-induced medium, the lens opacity was clearly recognizable (Figure 2.4, Panel B). The depth of the opaque layer grew visibly during the 4 days of CD-treatment (Panel C). After 6 days (Panel D), an outer opaque layer of the lens was coloured red with the indicator dye from the medium, phenol red, and the experiment was stopped. When the CD-treated lens was photographed, after 6 days, the photomicrography revealed a reduction in lens size. This may be due to the leakage of the cytosolic contents of the lens.

The Leakage of Lens Cytosolic Enzyme LDH into the Incubation Medium and its Correlation with the Cataract Development :

Cataract development in the presence of CD (Figure 2.4) or elevated glucose (Figure 2.5) was also followed by the measurement of LDH activity in the incubation medium. The leakage of LDH into the incubation medium was correlated with the development of lens opacity. A control group of, healthy lenses which were incubated in M199 appeared

FIGURE 2.4.

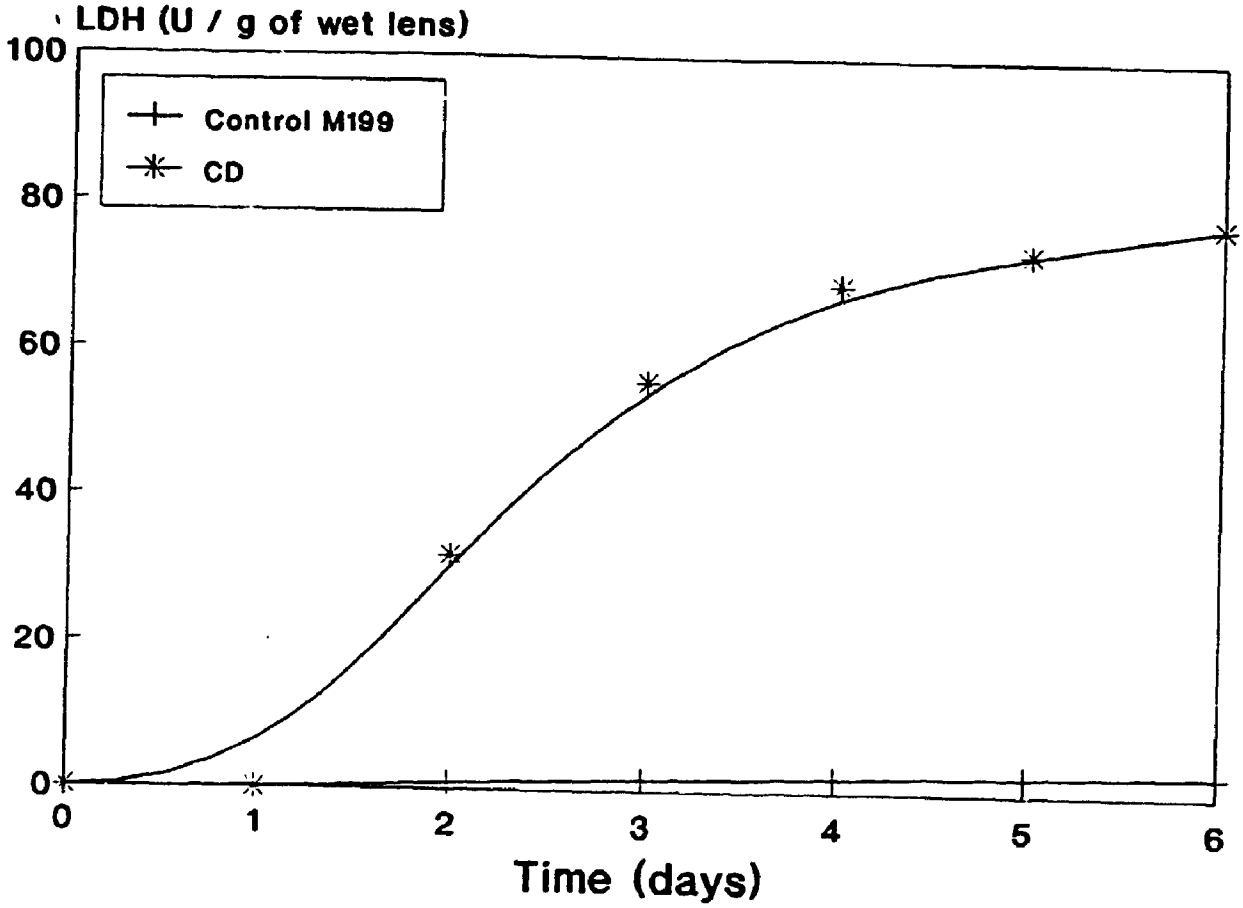
IN VITRO EFFECTS OF CD ON LDH RELEASE FROM INCUBATED LENSES.

Lenses were incubated in M199 or M199 containing 10^{-5} M CD in 0.1% DMSO. The LDH activity in the culture medium and the leakage per g lens was calculated as described in Materials and Methods (2.2.4). Data means ± SE (n=3) and percent SE are shown for each separate period of culture and culture condition. When the SE is lesser in magnitude than the dimensions of the point on the graph it is not shown.

CD + M199		Control (M199)		Day
LDH U/g lens± SE	%SE	LDH U/g lens± SE	%SE	
1.510 ± 0.020	1.33	0.150 ± 0.000	0.00	1
31.897 ± 0.901	2.83	0.304 ± 0.005	1.79	2
55.927 ± 0.858	1.53	0.670 ± 0.036	5.04	3
69.343 ± 0.886	1.28	1.049 ± 0.027	2.58	4
74.003 ± 0.701	0.95	1.428 ± 0.021	1.50	5
77.790 ± 0.745	0.96	1.908 ± 0.057	2.99	6

PHOTOMICROGRAPHS OF 10^{-5} M CD-TREATED LENSES.

(A): After preincubation in M199, day 0, each lens was transferred to M199 containing CD, and the effect of CD on lens transparency was observed by a dissection microscope. (B): 2 days; (C): 4 days; (D): 6 days.


LDH (U / g of wet lens)
100
80
60
40
20
0
Control M199
CD
0
1
2
3
4
5
6
Time (days)

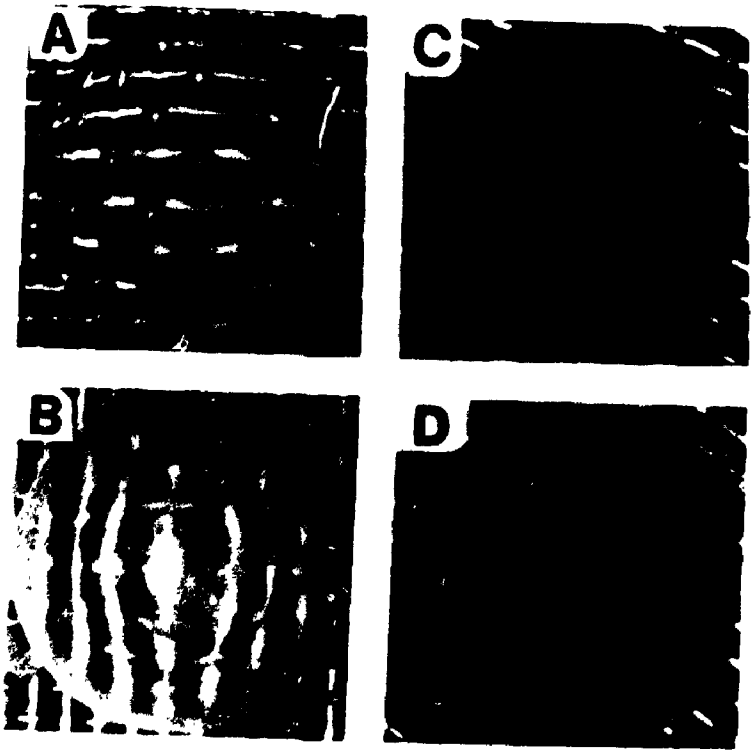
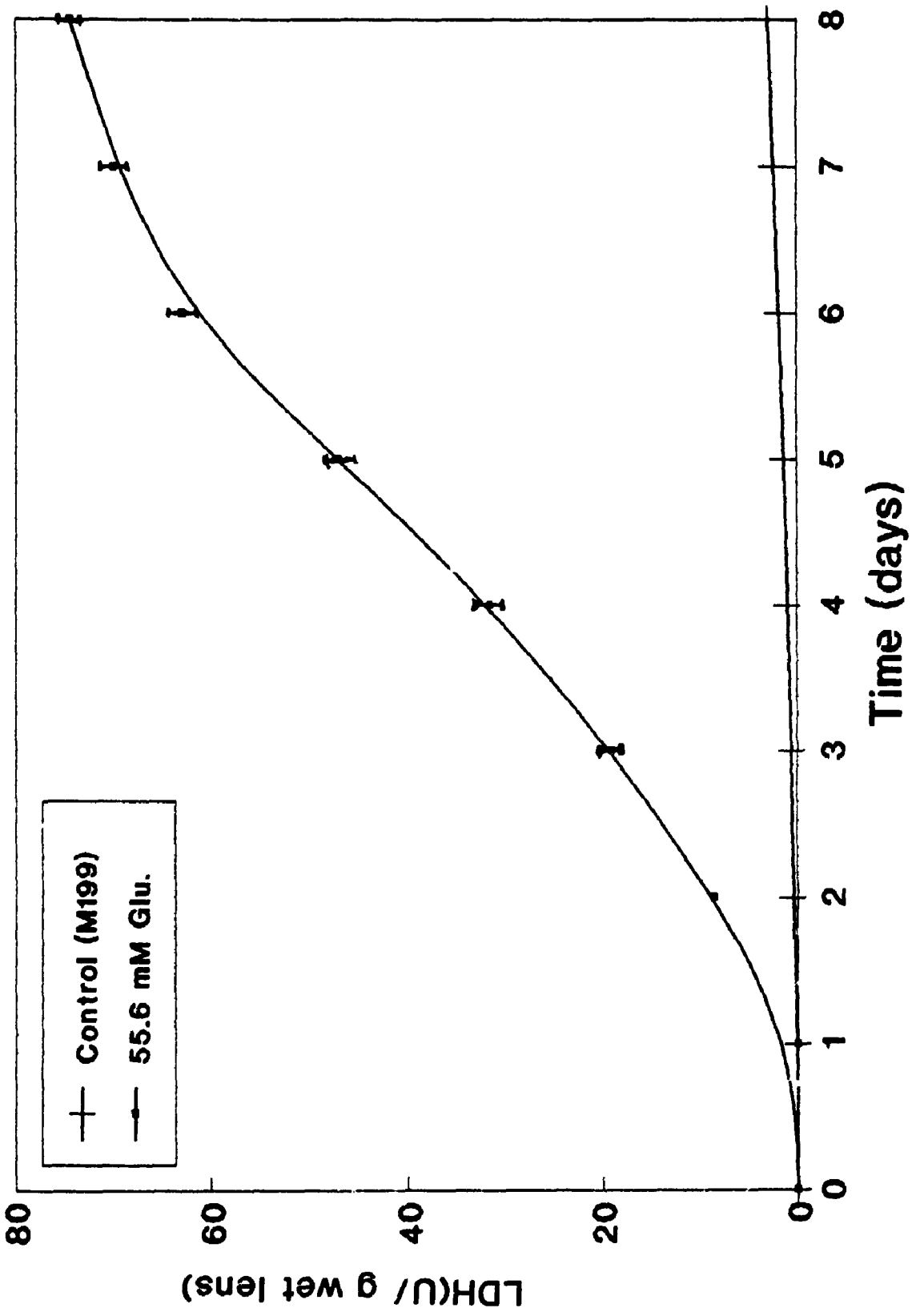

A
C
B
D

FIGURE 2.5.
IN VITRO EFFECTS OF GLUCOSE ON
RELEASE OF LDH BY INCUBATED LENSES.

After 24 hr preincubation in 1.5 ml M199, lenses were transferred to either fresh M199 or M199 containing 55.6 mM glucose. The LDH activity in the culture medium and the leakage per g lens was calculated as described in Materials and Methods (Section 2.2.4). Data (means ± SE (n=3) and percent SE) are shown for each separate period of culture and culture condition. When the SE is lesser in magnitude than the dimensions of the point on the graph it is not shown.

	Glucose-treatment		Control (M199)	
Day	LDH U/g lens ± SE	%SE	LDH U/g lens ± SE	%SE
1	0.750 ± 0.010	1.33	0.150 ± 0.000	0.00
2	8.497 ± 0.222	2.61	0.304 ± 0.005	1.79
3	18.973 ± 1.104	5.81	0.670 ± 0.036	5.04
4	31.403 ± 1.528	4.86	1.049 ± 0.027	2.58
5	46.967 ± 1.973	4.20	1.428 ± 0.021	1.50
6	62.913 ± 1.507	2.39	1.908 ± 0.057	2.99
7	69.930 ± 1.189	1.70	2.462 ± 0.080	3.28
8	74.397 ± 1.123	1.51	3.017 ± 0.106	3.52


LDH(U/ g wet lens)
80
60
40
20
0
0
1
2
3
4
5
6
7
8
Time (days)
Control (M199)
55.6 mM Glu.

optically clear and did not show any LDH leakage. The measured LDH results are summarized graphically in Figures 2.4 and 2.5.

During the first periods of 6, 12 and 24 hr LDH activity in the incubation medium containing 55.C mM glucose was negligible. At the end of 48 hr this activity had increased to 8.50 U/g of wet lens. During the first 4 days, leakage of LDH into the incubation medium from the glucose-treated lenses increased in a parallel manner to the depth of the lens opaque layer. At the end of 6 days the LDH leakage curve reached a plateau on the graph. After that point the rate of release of LDH activity into the glucose-induced medium was slowed down and became minimal, and the experiment was stopped at the end of 8 days (Figure 2.5).

The LDH level in the CD-induced culture medium was measured every day, along with the observation of their appearance by a dissection microscope. As summarized graphically in Figure 2.4, a linear increase in LDH release was confirmed to occur from day one to day 3, when analyzed by regression analysis, after which further release was markedly decreased, resulting in a plateau in the curve of LDH release versus time. The increased LDH leakage indicates rapid and extensive cellular damage to the lens by CD-mediated cataractogenesis. Between 24-48 hr of the incubation period, the leakage of LDH was 31.86 U/g of wet lens. There was no LDH activity in the samples taken from

the controls, either M199 or DMSO.

SDS-PAGE Separation of Incubation Media -

The effects of two additives - CD and 55.6 mM glucose - on rat lenses were confirmed by studying the leakage of other soluble lens proteins. The protein content of the incubation medium was measured by the BioRad standard protein assay then calculated daily using the standard curve which was prepared as described in Materials and Methods (Figure 2.6 A). The amount of soluble lens protein in the incubation medium was increased by exposure to CD and 55.6 mM glucose. The total accumulated leakage of lens proteins was calculated by adding the previous total to the daily amount present in the culture medium (Figure 2.6 B).

150 µl samples from CD-containing incubation medium (8 days) were run on 10% SDS-PAGE (Figure 2.7). CD caused an immediate leakage of proteins into the medium during the first 24 hr incubation (lane 3), after which there was almost no difference in the relative amount of different proteins, at different times.

The high glucose resulted in increasing leakage of lens cell intracellular protein into the incubation medium with increasing time (Figure 2.8, Panel A). 25 µl samples from the glucose-induced medium between days 1-8 of incubation were run on 12.5% SDS-PAGE respectively.

There were almost no differences in the patterns of

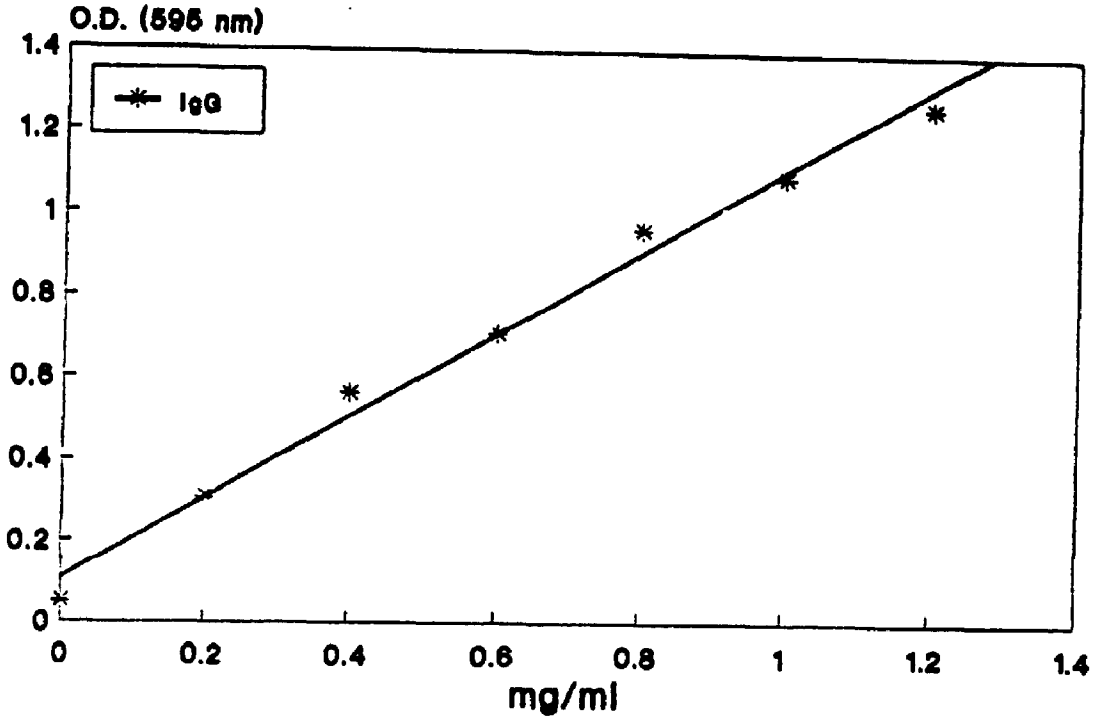
FIGURE 2.6.
BIORAD PROTEIN ASSAY FOR THE PROTEIN IN THE LENS INCUBATION MEDIA.

PANEL A. The absorbance of IgG detected by the BioRad assay was read spectrophotometrically at 595 nm. The standard curve was prepared to calculate the amount of proteins which leaked into the lens incubation medium.

PANEL B. The standard procedure was followed to measure the amount of protein from the daily incubation medium and the standard curve was used for the calculation. The amount of soluble lens protein in the incubation medium was increased by exposure to CD and 55.6 mM glucose. The total accumulated lens proteins leakage was calculated by adding the previous total to the daily amount present in the culture medium. Data (means ± SE (n=3) and percent SE) are shown for each separate period of culture and culture condition. When the SE is lesser in magnitude than the dimensions of the point on the graph it is not shown.

Day	55.6 mM Glucose		10^{-5} M CD	
	µg protein/mg lens ± SE	%SE	µg protein/mg lens ± SE	%SE
0	0.2 ± 0.018	9.00	0.2 ± 0.020	10.0
1	0.64 ± 0.030	4.69	1.4 ± 0.112	8.00
2	1.52 ± 0.087	5.72	4.9 ± 0.449	9.16
3	2.97 ± 0.170	5.72	11.1 ± 1.023	9.22
4	5.62 ± 0.225	4.00	19.3 ± 1.578	8.18
5	10.52 ± 0.653	6.21	30.3 ± 1.773	5.85
6	16.27 ± 1.250	7.68	42.3 ± 1.913	4.52
7	23.77 ± 1.548	6.51		
8	34.27 ± 1.778	5.19		

A


O.D. (595 nm)
IgG
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1
1.2
1.4
mg/ml

B

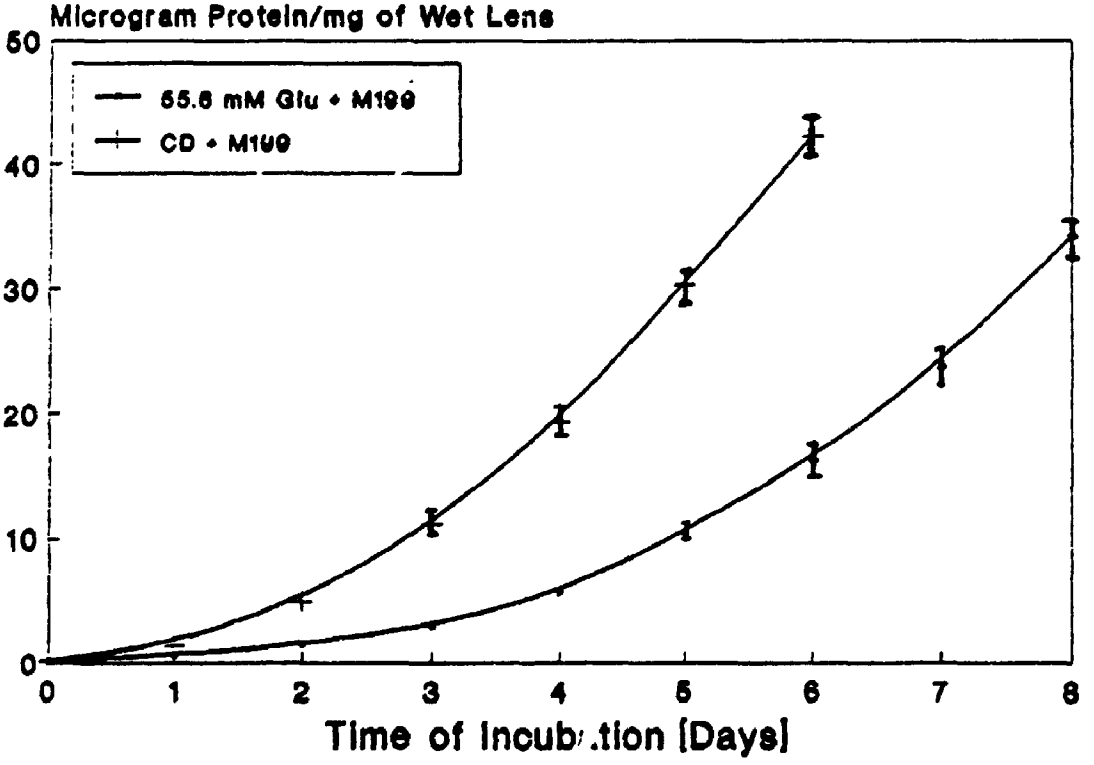

Microgram Protein/mg of Wet Lens
55.6 mM Glu + M199
CD + M199
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
Time of Incubation [Days]

FIGURE 2.7.
SDS-PAGE SEPARATION OF INCUBATION MEDIA PROTEINS.

150 µl samples from incubation medium were run on 10% SDS-PAGE to observe the amount and variation of the proteins which leaked into the incubation medium. The gel was stained with Coomassie Blue. The positions of molecular mass markers are as follows: B-galactosidase (116 kDa), phosphorylase b (94 kDa), bovine serum albumin (68 kDa), catalase (60 kDa), ovalbumin (43 kDa), lactate dehydrogenase (34 kDa), carbonic anhydrase (30 kDa). They are shown in lanes 1 and 9. Lane 2 is the sample from preincubation media, lanes: 3, 4, 5, 6, 7, 8 are the samples from the first through the end of sixth days of incubation in M199 with CD, respectively; i.e., lane 3-day 1, lane 4-day 2, lane 5-day 3, lane 6-day 4, lane 7-day 5, lane 8-day 6.

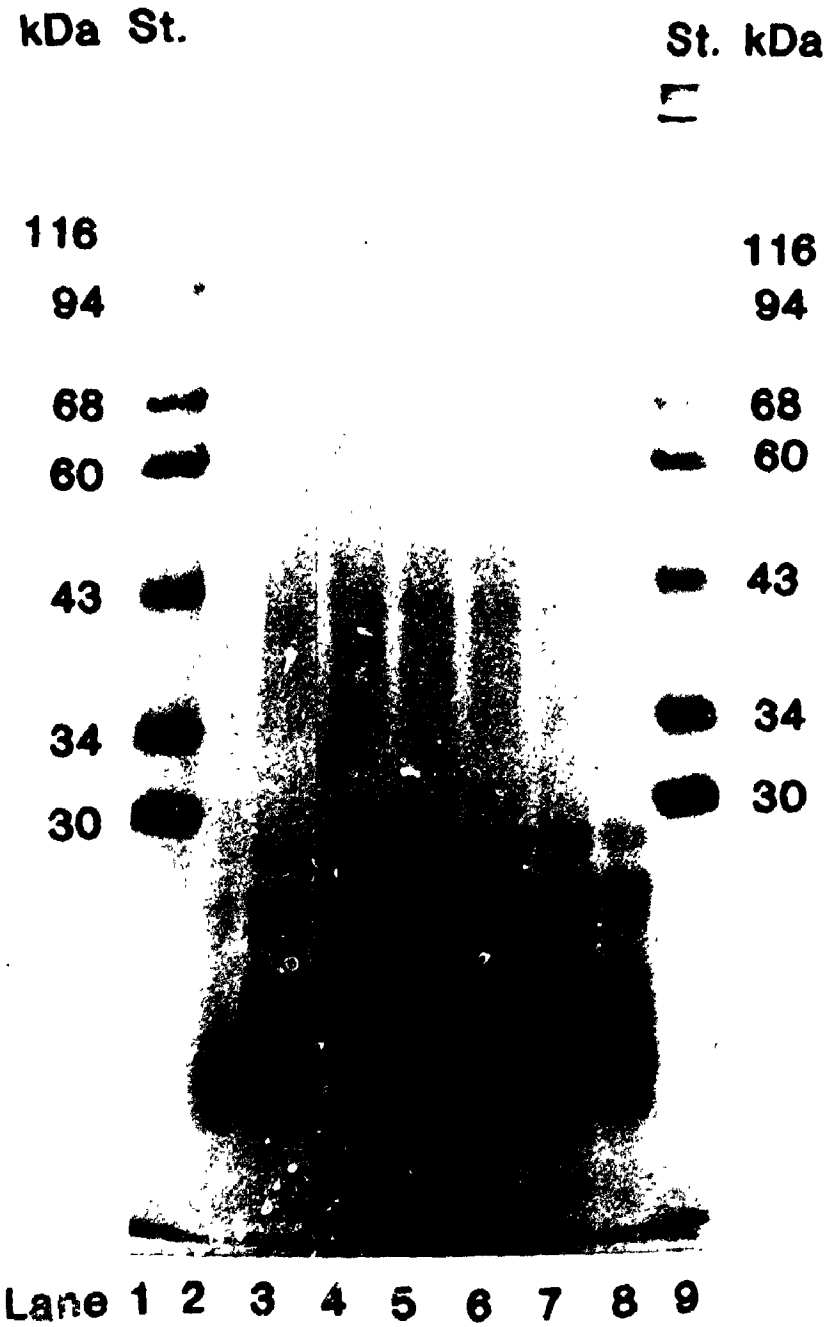
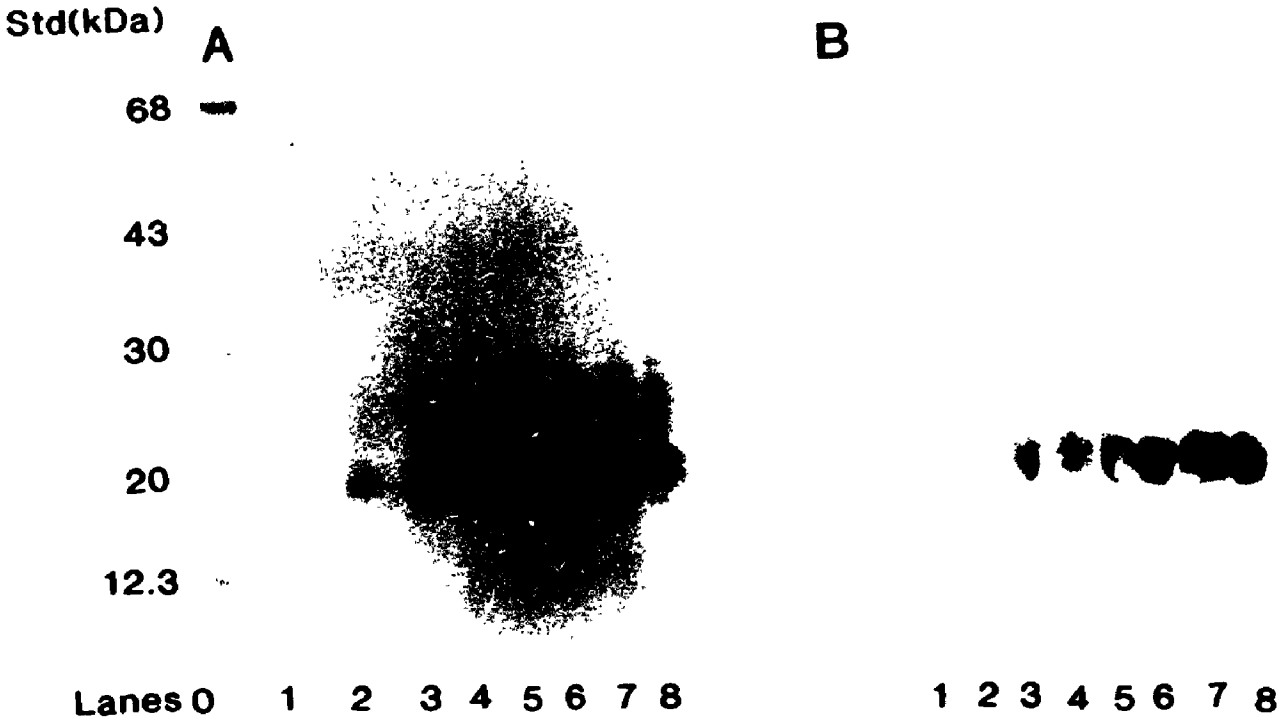

kDa St.
St. kDa
116
94
68
60
43
34
30
116
94
68
60
43
34
30
Lane 1 2 3 4 5 6 7 8 9

FIGURE 2.8.
THE IN VITRO EFFECT OF GLUCOSE ON THE LEAKAGE OF γ-CRYSTALLIN AND THE OTHER SOLUBLE LENS PROTEINS.

Proteins were immunostained with western blotting to reveal γ-crystallin: 25µl samples corresponding to days 1-8 (Panel A, lane 1-8), from incubation medium with 55.6 mM glucose were run on 12.5% SDS-PAGE to observe the amount and variation of the proteins which leaked into the incubation medium. The gel was stained with Coomassie Blue (A) and an identical gel transferred to nitrocellulose (B) which was probed with polyclonal antibody to γ-crystallin as described in Methods. The positions of molecular mass markers are as follows: bovine serum albumin (68 kDa), catalase (60 kDa), ovalbumin (43 kDa), lactate dehydrogenase (34 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa), cytochrome c (12.3 kDa). They are shown in Panel A, lane 0.


Std(kDa)
A
B
68
43
30
20
12.3
Lanes 0 1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8

proteins which leaked into the medium at different intervals. These are mostly crystallins, with molecular weights below 30 kDa.

When we compared the amount of proteins which leaked from the CD- with that from the glucose-induced lens, the protein leakage was slower in glucose-induced lenses.

Leakage of γ-Crystallin into the Incubation Medium During *In Vitro* Diabetic Cataract Formation :

25 µl samples from the lens incubation medium taken at day 8 were run on 12.5% SDS-PAGE (Figure 2.8, Panel A), transferred to nitrocellulose, and probed with polyclonal anti-γ-crystallin-antibody (Panel B). Western blotting revealed that the amount of protein identified as γ-crystallin increased when the lens became opaque. This was correlated with the release of LDH and other proteins into the culture medium, but the leakage of other lens proteins and LDH into incubation medium started earlier than the leakage of γ-crystallin.

Leakage of γ-Crystallin into Aqueous and Vitreous Humour in Diabetic Rat Lenses :

The leakage of γ-crystallin into the aqueous and the vitreous humour in the diabetic rat lens, was measured by using Competition Assays-Microtiter Plates, (Harlow and Lane, 1988).

Prior to the assay, γ-crystallin was isolated from rat lenses according to the procedure described in the Materials and Methods sections. γ-crystallin was purified on an S-200 column and the absorbance of the fractions was measured at 280 nm (**Figure 2.9, Panel A**). Then the purification was verified by SDS-PAGE and Western blotting results (Figure 2.9, Panel B and C).

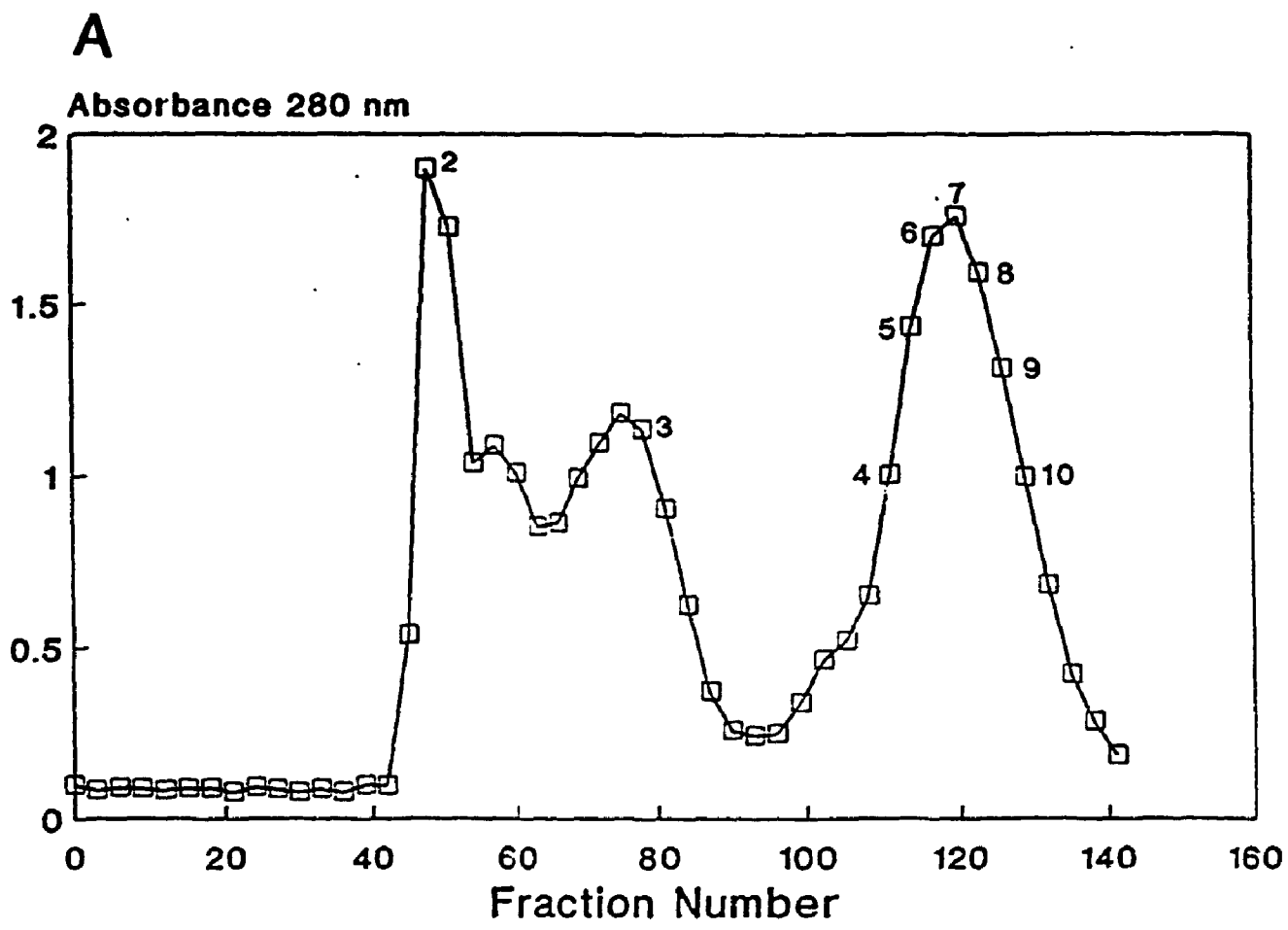
Prior to the assay, γ-crystallin was labelled with ^{125}I, and γ-crystallin antibody was purified on a Protein A column. Then the antibody was bound to each well in the microtiter plate. The remaining sites for protein binding on the nitrocellulose plate were saturated as required by incubating with blocking buffer. The antigen test solution (between 50-1000 ng) was added along with the standard labelled γ-crystallin solution to the wells. The labelled and unlabelled crystallin competed in binding to the antibody in the wells. At the end of incubation the wells were washed and the amount of labelled crystallin which was bound to the nitrocellulose was d termined by gamma counter to obtain a standard curve (**Figure 2.10**).

Diabetes was induced in 24 rats by intraperitoneal injection of streptozotocin as described in Materials and Methods (2.2). At the end of 12 weeks diabetic rats showed some physical changes on examination of their bodies, apart from the opacity in the eye lens. Cataract levels were graded, and diabetes was confirmed by measurement of

FIGURE 2.9.
LENS CRYSTALLINS AND PURIFICATION ON S-200 COLUMN.

PANEL A. S-200 column chromatography of rat lens extract, prepared as described in the text. α- and β-crystallin were eluted in the first 100 fractions. γ-crystallin was eluted fractions number 100-140 and the absorbance of those fractions was measured at 280 nm .

PANEL B and C. The purification of γ-crystallin was verified by SDS-PAGE (Panel B) and Western blotting results (Panel C). Aliquots (10 µl) of the fractions numbered on the graph (Panel A) were run on a 10% SDS-PAGE and stained with Coomassie Blue (Panel B) and an identical gel transferred to nitrocellulose which was probed with polyclonal antibody to γ-crystallin (Panel C) as described in Methods. The numbers of lanes in Panels B and C are identical to the numbers of the fractions identified by the numbers above the graph shown in Panel A. In Panel B, lane 1 shows molecular weight markers.


A
Absorbance 280 nm
2
1.5
1
0.5
0
0
20
40
60
80
100
120
140
160
Fraction Number
2
3
4
5
6
7
8
9
10

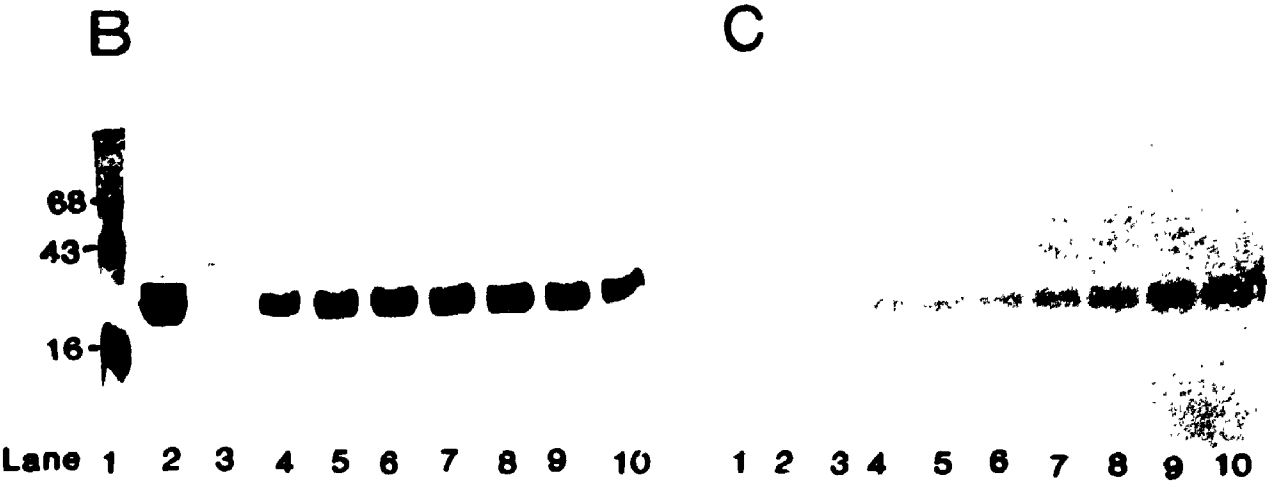

B
C
68
43
16
Lane 1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10

FIGURE 2.10.
DETECTING AND QUANTITATING ANTIGENS USING COMPETITION ASSAYS-MICROTITER PLATES.

γ-crystallin was labelled with ^{125}I, and γ-crystallin antibody was purified on a Protein A column. Then the antibody was bound to each well in the microtiter plate (Harlow and Lane, 1988). The labelled and unlabelled crystallin competed in binding to the antibody in the wells. The amount of labelled crystallin which was bound to the nitrocellulose was determined by gamma counter to obtain a standard curve.

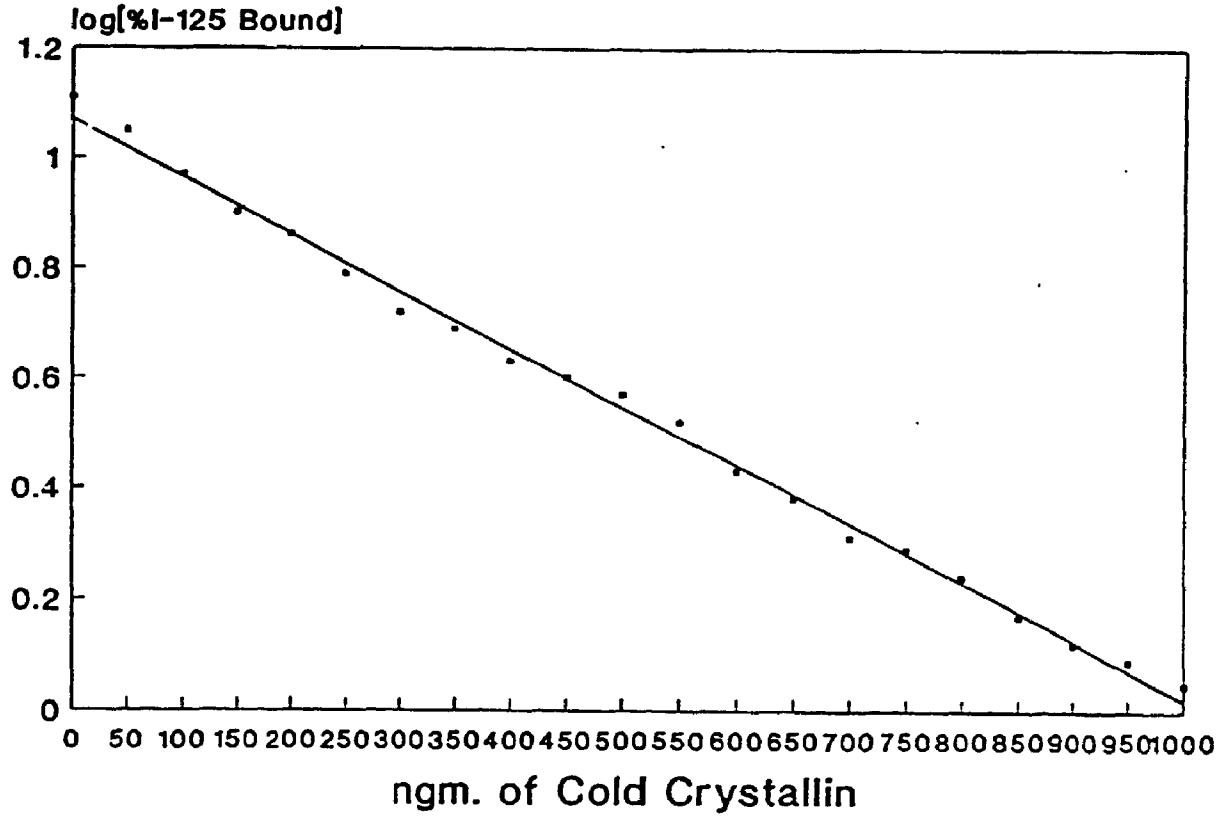


TABLE 2.1: LEAKAGE OF γ-CRYSTALLIN INTO AQUEOUS AND VITREOUS HUMOURS

	Leakage, % of normal	
HUMOURS	CONTROL	DIABETIC
Vitreous humour*	100 ± 23[a]	301 ± 55[a]
Aqueous humour**	100 ± 21.2[b]	331.8 ± 133.5[b]

[a]$p<0.0001$, [b]$p<0.01$.
* The amount of γ-crystallin = (29.7 ± 6.85) ng/µl.
** The amount of γ-crystallin = (15.94 ± 3.38) ng/µl.

glycohaemoglobin levels. Rats were killed, eyes removed and aqueous and vitreous humours collected. During the assay these samples were used as test solutions. The leakage of crystallin into the aqueous and vitreous humour during the diabetic cataract was determined by competition assay and calculated by using the standard curve. In diabetic animals the amount of γ-crystallin in the vitreous humour was increased from 100 to 301 ng/µl. In the aqueous humour, the normal value was changed from 100 to 331 ng (Figure 2.10 B).

Electrophoretic Separation of LDH Isoenzymes from Incubation Media and Lens Extract :

Figure 2.11 shows the electrophoretic separation of LDH isoenzymes from incubation media and lens extracts, using an enzymatic stain to illustrate their order of migration: LDH-5, 4, 3, 2, 1. Variations in intensity of staining may be an artifact of the staining method used, which is not as accurate as the spectrophotometric technique used to estimate total LDH. Alternatively (see Discussion in Section 2.4 page 101), possible proteolytic degradation of LDH may explain differences between samples. The standard sample used on the gel was LDH-3 (Figure 2.11, lane 1). Lanes 2, 3, 4 are 150 µl samples of the CD-induced culture medium after 2, 4, 6 days respectively. Lenses were homogenized at the end of 6 days incubation in CD or 8 days incubation in glucose-containing medium in 2 ml 0.05 M tris buffer. The

FIGURE 2.11.
ELECTROPHORETIC SEPARATION OF LDH ISOENZYMES FROM INCUBATION MEDIA AND LENS EXTRACT.

Samples from incubation medium were run on 10% native gel to separate LDH isoenzymes. The gel was stained with NBT as described in the text.

Lane 1, : is a commercially supplied sample of LDH-3 (0.5 µg).

Lanes 2, 3, 4: are 150 µl samples from the second, fourth and sixth day respectively of lens incubation medium with 10^{-5} M CD.

Lane 5 : is empty.

Lanes 6, 7, 8: are 10 µl samples from the lens extracts (either after 8 days incubation in glucose-treated or after 6 days CD-treated or from control, healthy lens respectively).

Lanes 9, 10 : are the samples from lens incubation medium which contained either (9) M199 or (10) M199 containing DMSO, at the end of eight days.

Lanes 11, 12, 13, 14 : are 150 µl samples from lens incubation medium M199 containing 55.6 mM glucose, after the second, eight, sixth and fourth day, respectively.


LDH
5
4
3
1 2 3 4 5 6 7 8 9 10 11 12 13 14

lens homogenate was centrifuged at 25,000 g for 20 min. 10 µl samples from the extract was run on native gel: lane 6, 7. A 10 µl sample from the extract of a control lens was run in Lane 8.

The isoenzyme pattern of LDH in the culture medium shows that lens LDH has three isoenzymes: LDH-5, 4, and 3 which leaked into the medium when the lens membranes are damaged. The intensity of the bands also reflects the amount of leakage, indicating that the amount leaked is in the following order: LDH 3> LDH 4> LDH 5.

When do the Lens Cells Start to Lose Integrity in the Cataract-Inducing Medium ?

T1111 is a water soluble dye which is fluorescent when embedded in a membrane. When healthy cells are exposed to the dye for 5 minutes, their shapes are revealed in outline as fluorescent, since T1111 has labelled the exposed outer surface of the plasma membrane lipid bilayer. The live cells which are stained with T1111 appear as a bright outline when their plasma membranes accumulate this fluorescent dye (Betz, et al., 1992) **(Figure 2.12, Panel A).** Optical sections were taken at 30 micron intervals in 48 hr incubated lenses (Panel B). In leaky or dead cells, the dye labels intracellular membranes, resulting in a highly fluorescent cytoplasm surrounding a characteristically unlabelled nucleus which is indicated by a arrowhead in the

FIGURE 2.12.
T1111 STAINING OF CONTROL LENS.

PANEL A. Lenses were incubated in M199 for 48 hr then the live cells were determined by fluorescent staining using T1111. The plasma membrane of the healthy cells which were exposed to fluorescent dye T1111, appeared as an outline under a confocal microscope. Dead cells were leaky and their cytoplasm was labelled, as shown in the figure with a white arrow. (Scale, 1 cm = 0.50 μm).

PANEL B. Optical sections are shown ordered left to right, in three successive rows. These were taken as a "z-series" of xy horizontal sections separated by a constant vertical 30 micron distance in the z-direction. Beginning at the underside of fiber cells of T1111-incubated control lenses, the sections proceeded upwards at 30 micron intervals until the top surface of the epithelium was reached in the bottom right corner of the panel. The central illumination artifact, which obscured the image, is seen at the lower right corner of the image in Panel B. (Scale, 1 cm = 0.50 μm x 3 = 1.50 μm).

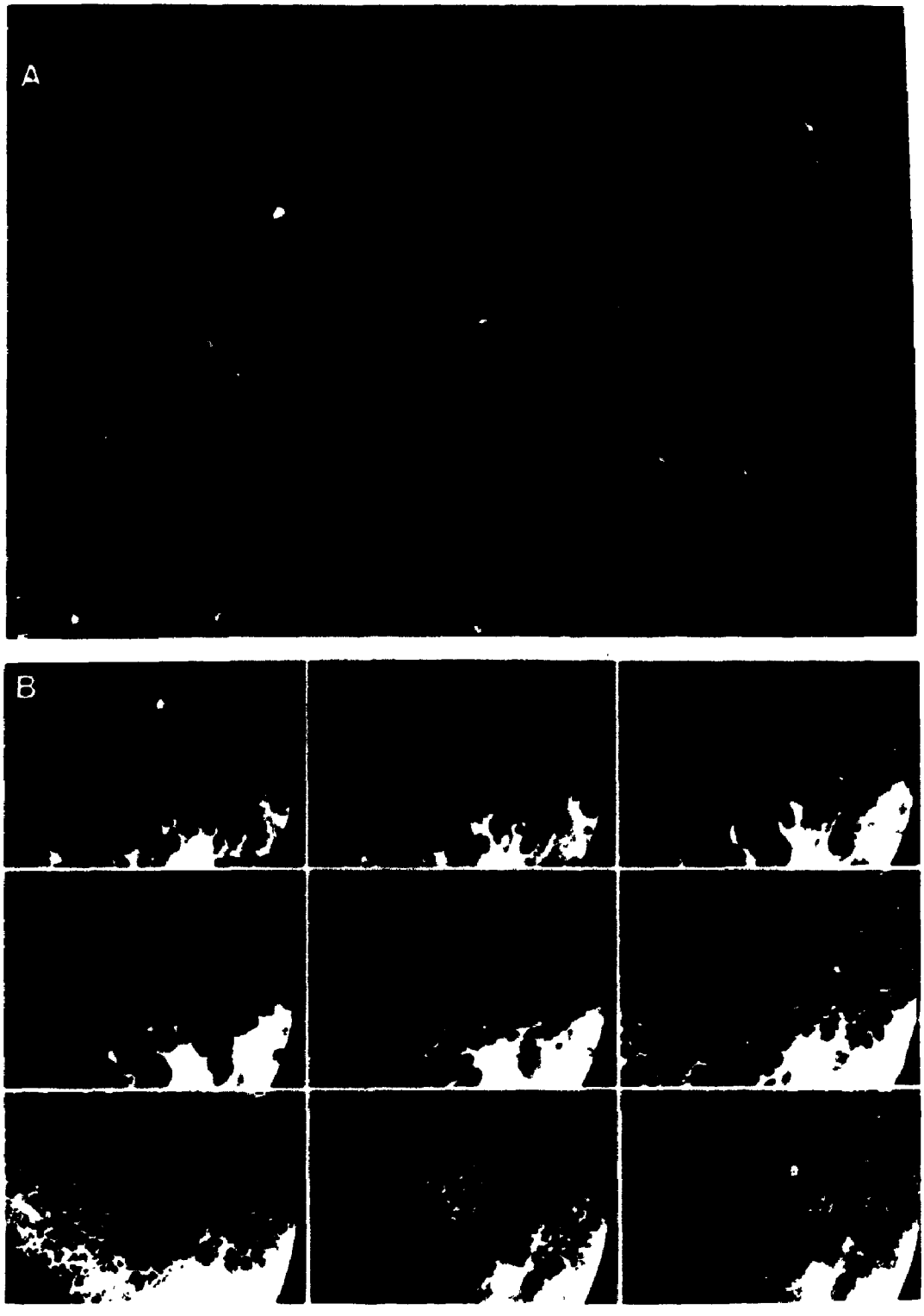
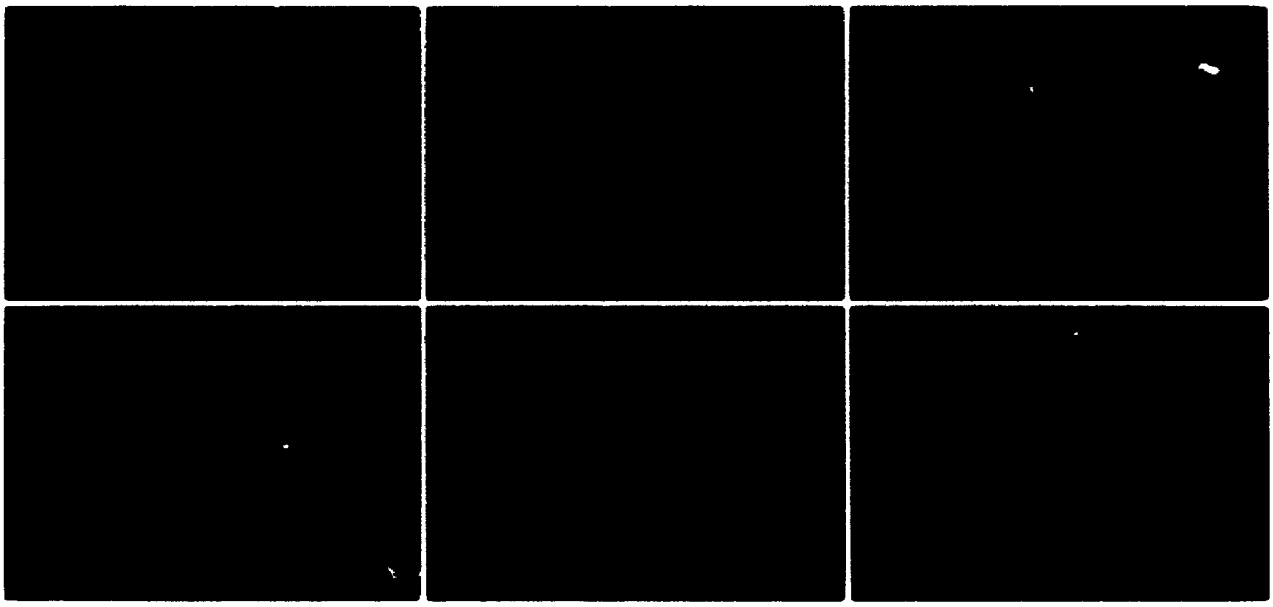

A
B

FIGURE 2.13.
FDA STAINING OF CONTROL LENS.

A lens was incubated in M199 for 48 hr then the live cells were determined by fluorescent staining using FDA. The appearance of the equatorial region is seen in each of these 6 panels of this figure under the same light intensity and enlargement. FDA stains the cell cytoplasm of living cells: these cells appear fluorescent, but if they are dead FDA does not stain them and the appearance is dark on the screen. Several different sections through the equatorial region are shown to exhibit fluorescence after FDA staining, to indicate that this is not a local phenomenon. The PMT amplifier set at 200 µA/V. (Scale, 1 cm = 750 µm x 3 = 2.25 mm).



figures.

The same set of lenses was also stained with FDA (Figure 2.13) to confirm the T1111 result. FDA stains the cell cytoplasm of the living cells: these cells appear fluorescent; FDA does not stain dead cells, so they would appear dark on the screen (Mohr, et al., 1980). Lenses incubated in M199 containing FDA were uniformly labelled.

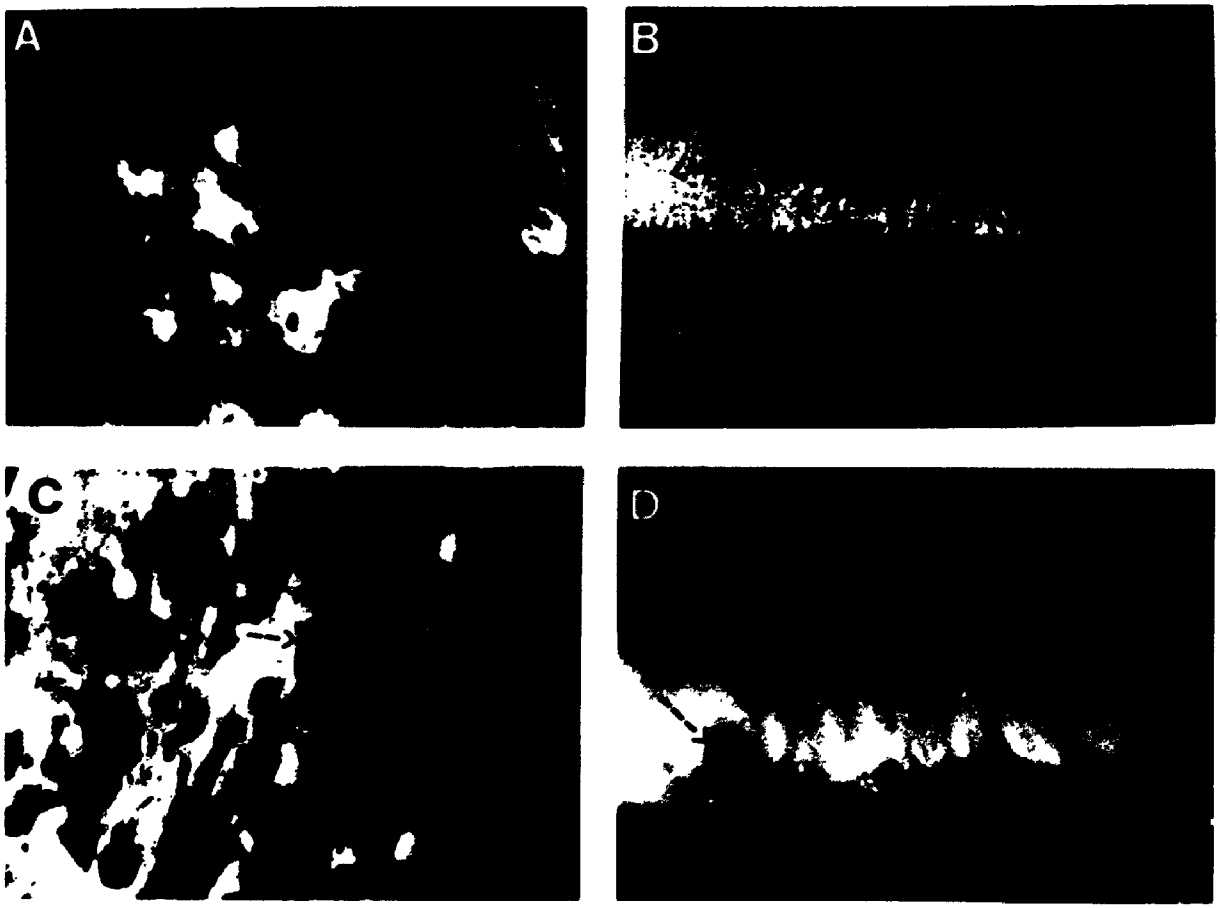
When 55.6 mM glucose was present in the medium, the staining pattern was quite similar to the control after 24 hr, but after 48 hr only a few epithelial cells showed cytoplasmic staining for T1111 (Figure 2.14, Panel A and B) and had no cytoplasmic labelling with FDA (Figure 2.14, Panel C and D). The effect of glucose could be seen on individual cells. Cell death in fiber cells is more difficult to determine, since mature fiber cells have very few remaining organelles. However, some fiber cells in glucose-incubated lenses were found to have a slightly brighter cytoplasm after this staining, suggesting that these are leaky while the remainder are intact. My results suggest that vital dye staining is a reliable and sensitive tool in evaluating lens preparations for in vitro study.

The Time Course of Changes in Intracellular Magnesium During CD- and Glucose-Treatment of Rat Lenses -

We (Dr. D.Carter and author) visualized the accumulation of magnesium in the pre-cataractous (either

FIGURE 2.14.
VITAL STAINING OF THE PRECATARACTOUS LENS CELLS WITH T1111 AND FDA.

After 48 hr incubation in glucose-induced medium, the vital staining of the lens equatorial cells was done by T1111 (Panel A and B) and FDA (Panel C and D). T1111 stains the plasma membrane of live cells and the cytoplasm of the dead cells while FDA labels the cytoplasm only of live cells. In the figure, the dead cells are shown with arrows. Panel B and D show the xz vertical optical section through the xy horizontal optical sections of areas shown in Panel A and C respectively (Scale, 1 cm = 0.50 µm x 2 = 1 µm).


A
B
C
D

glucose- or CD-induced) rat lenses with the magnesium-sensitive fluorescent dye magnesium-green-AM, by using the confocal microscopy techniques described above. To check my method after 24 hr preincubation in M199 lenses were transferred into M199 containing magnesium green-AM and intracellular magnesium in the lens equator region was localized (**Figure 2.15**). **Figure 2.16** shows the time course of changes in intracellular free magnesium in the same region (equator) evoked by the effect of either 55.6 mM glucose or CD in magnesium-green AM incubated rat lenses. Lenses were incubated in medium M199 containing Mg-green and 55.6 mM glucose or 10^{-3} M CD and examined after 0, 24 and 48 hr. Pictures from imaging on a prototype TCLSM were taken using the same enlargement and PMT amplifier set at 200 µA/V light intensity, to be able to compare the effect of additives on magnesium-uptake; they are illustrated in **Figure 2.16**. When the results from the lenses incubated in control media (Column A) and glucose- (Column B) or CD-induced media (Column C) are compared, the presence of cataract inducers enhanced the magnesium concentration at the lens equatorial cortex in a time-dependent manner.

FIGURE 2.15.
FLUORESCENT STAINING OF LENS INTRACELLULAR FREE MAGNESIUM WITH MAGNESIUM-GREEN.

Lenses incubated in M199 containing 9.17 µM magnesium-green were transferred to a TCLSM to take images of the equator region at two magnifications: low magnification (1 cm = 750 µm x 3 = 2.25 mm) and high magnification (1 cm = 1.5 µm x 3 = 4.5 µm) in order to see the intracellular free magnesium distribution.

Panel A: The appearance of the lens equator region after 24 hr incubation in M199 containing magnesium-green scanned with low light intensity (PMT was set at 200 µA/V) and low magnification.

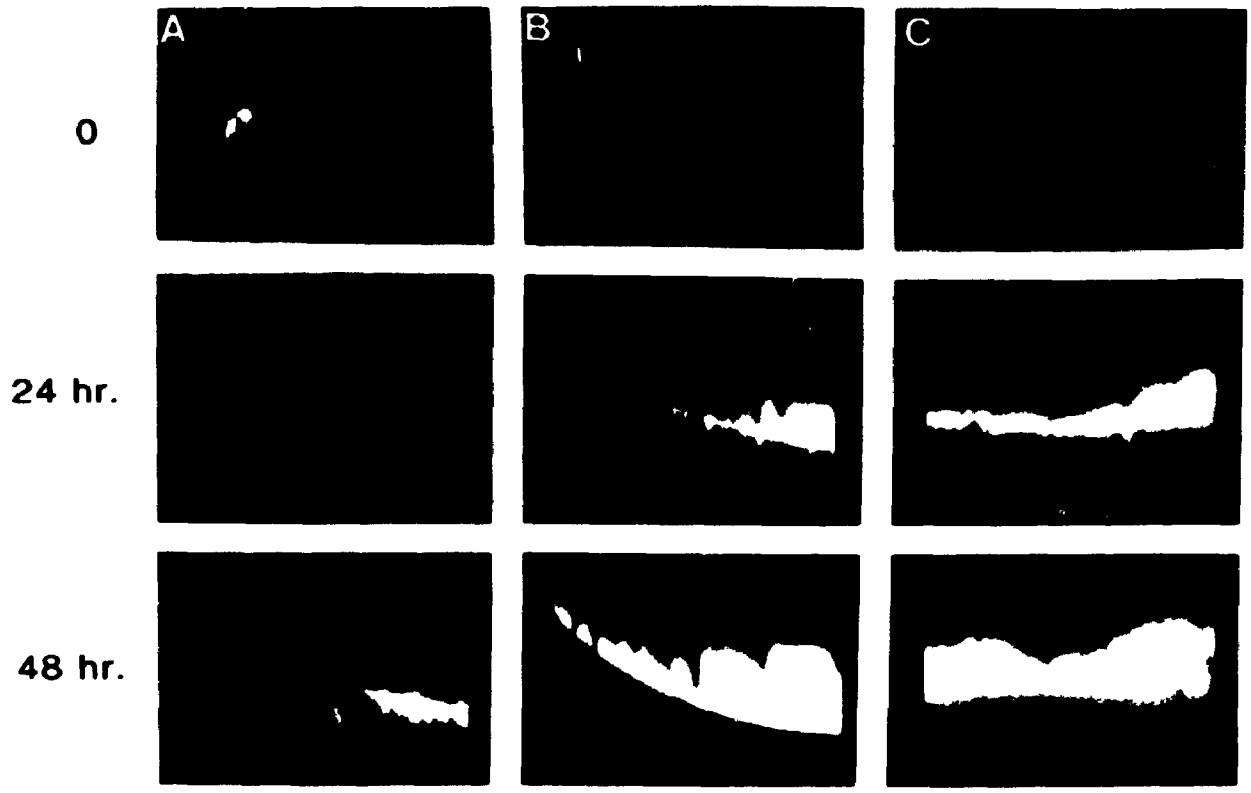
Panel B: The same area, lens equator region which is visualized in Panel A was imaged at a higher magnification and 50 µA/V light intensity. Upper level shows the xy images at a scale of 1 cm = 4.5 µm. The lower level of Panel B shows the xz image of the same area, viewed perpendicularly to the upper image.

Panel C: The area visualized in Panel A scanned one more time with high light intensity (50 µA/V) and high magnification. In the healthy, clear lens labelling with magnesium green was limited to the epithelial layer and to adherent tissue around the equator. The upper image shows an xy direction scanning and the lower a perpendicular xz direction scanning of the same area.

A B C

FIGURE 2.16.
THE EFFECT OF 55.6 mM GLUCOSE AND CD ON
LENS MAGNESIUM UPTAKE.

The effects on lens magnesium uptake of adding to M199 (Panel A, control) either 55.6 mM glucose (Panel B) or CD (Panel C) was tested by staining the intracellular free magnesium with the magnesium specific fluorescent dye magnesium-green, during 0, 24 hr, and 48 hr incubation. The PMT amplifier was set at 200 μA/V and the xz image was taken at a scale of 1 cm = 750 μm x 4 = 3.00 mm. The effect of CD on the magnesium uptake was earlier than it was with glucose. The volume of the fluorescently labelled area in CD-incubated lenses was much broader than the area in glucose-incubated lenses.


A
B
C
0
24 hr.
48 hr.

2.4 DISCUSSION

Cataract is the end product of a chain of biochemical reactions and cellular events. In cataractous lenses many compounds have been found either increased or decreased. For many years different researchers have speculated about the ingredients of cataractous lenses, even though the mechanism of cataract formation is still not well understood: Are membrane damage, cytoskeletal disruption and increased intracellular divalent ions the result or the effect of cataract formation? To know the order of these events during cataractogenesis will definitely be very helpful, both for its early diagnosis and for its pharmacological prevention.

In these experiments, several different indicators in pre-cataractous and cataractous lenses were compared in expectation that these findings will help to classify the immediate and late changes during cataractogenesis. This should eventually help to elucidate the mechanism. Experimentally two different cataract-inducers - glucose and CD - were used to treat the lenses <u>in vitro</u>. The effect of glucose on leakage of one of the lens proteins, γ-crystallin, was also studied in diabetic rats: this was expected to provide results complementary to those from the <u>in vitro</u> glucose model system.

Cataract development or the effect of the inducers, glucose and CD, were correlated and the three major

parameters were followed: i) distribution of the dead cells in the lens, ii) appearance of the lens observed daily by a dissection microscope, and iii) changes in intracellular components: leakage of lens soluble cytosolic proteins, γ-crystallin, LDH, and increased magnesium concentration.

My result suggests that glucose-induced cataractogenesis involves only low levels of epithelial cell mortality during the first 48 hr. The distribution of dead cells in the cataractous model was a good way to observe the results of cataract-causative agents and the amount of dead cells in the precataractous stage.

Although the appearance of the lens is a reliable way to follow the cataract formation, according to the observed order of the events, some parameters change earlier than the appearance observed in the dissection microscope, e.g., the leakage of the intracellular components of the lens. Using gel electrophoresis at the end of 48 hr of incubation, lens soluble proteins could be seen. However probing of the nitrocellulose transfer with anti-γ-crystallin-antibody for the same gel showed that either the amount of γ-crystallin was not enough to be determined by blotting or γ-crystallin leakage had not yet started. The effect of CD on lens opacity occured much earlier than that of glucose, suggesting that in CD cataract a disturbed cytoskeleton may precede oxidation, while in glucose the opposite is the case.

Similar results were observed for the lens intracellular magnesium content: the magnesium level as a function of time increased more in CD than in glucose medium. We (Dr. Carter and the author) conducted experiments to ensure that cataract-evoked changes in intracellular free magnesium were not caused by artifacts, i.e. (1) cataract induction by the dyes added, or (2) cataract induction by DMSO used to dissolve the dyes. Because of this concern the individual effects of magnesium-green and DMSO on the lens were examined with respect to: i) the amount of LDH in the medium and ii) the appearance of the lenses observed by a microscope. Both parameters showed that magnesium-green and DMSO have no damaging effect on the lens.

The leakage of γ-crystallin which was studied in vivo and in vitro or LDH could be a reliable parameter to correlate with the cataract formation. It is formally possible, however, that LDH and γ-crystallin might be degraded in lens bathing medium so that measurement of their concentration was an inaccurate reflection of leakage. We assumed, in these experiments, no differences in ratio of loss of LDH or γ-crystallin. We also measured LDH and γ-crystallin levels at identical time points between experiments, thereby minimizing potential differences. Future experiments will include measurements of LDH loss with time (using radiolabelled tracer LDH) in bathing medium

to determine whether variation in stability could lead to differences that do not reflect leakage ratio from lenses.

The leakage of γ-crystallin which was studied *in vivo* and *in vitro* could be a lens-specific parameter during cataract formation. Several factors led to our decision to determine the presence of cell damage by measuring LDH leakage : i) the leakage of LDH occurred much earlier than the leakage of γ-crystallin during cataract formation, ii) LDH measurement is a rapid technique and statistical analysis showed that it is reliable, and iii) risk is involved in using radioactive materials during competition assays. Using this reproducible lens incubation system, various other potential cataract preventive agents were tested during my study (chapter 3).

Since *in vitro* LDH leakage increases parallel to the release of other lens proteins and to the development of opacity in the lens, it appears that LDH leakage is a reasonable indicator of damage occurring during the formation of cataract. Another reason to rely on this technique is the pattern and order of lens LDH isoenzymes. My data shows three lens LDH isoenzymes : LDH 5, 4, 3 and the amounts released vary in the order LDH 3> LDH 4> LDH 5. The present results are consistent with the study of Nagpal *et al.*, (1991), which showed increases in 3 LDH isoenzymes leaking into aqueous and vitreous humours in human senile cataract.

Glucose is necessary for energy metabolism in all organisms, but it may have various toxic side effects depending on its concentration. Possible mechanisms of glucose damage include both glycation and oxidative stress. The biochemical modification of membrane proteins appears to be one of the serious effects of elevated glucose, resulting in damage to lens membrane structures (Wolff and Dean, 1987) during cataract formation (Spector and Garner, 1980; Augusteyn, 1981; Wells-Knecht et al., 1993). In diabetic, or sugar cataracts, globular degeneration and associated opacity occur first in the equatorial cortical subcapsular region of the lens (Creighton et al., 1980).

Cytochalasin D is a compound which binds to actin and blocks its spontaneous association into linear, helical polymers in the presence of ATP (Pollard, 1986; Brown and Spudich, 1979). The importance of actin polymerization and depolymerization in the lens cells is clear from the effect of CD in this and previous studies (Mousa et al., 1977, 1979). There are many cytoskeletal proteins which associate either with actin monomers or filaments (Karp 1984). The interactions of these proteins influence actin aggregation, stabilize the cytoskeletal mesh-work and affect the viscosity of the cytoplasm. Actin filaments also interact with many plasma membrane proteins, conferring shape and maintaining the integrity of the plasma membrane (Karp 1984). In cataract formation, many spontaneous reactions

occur: one of the lens actin monomer-stabilizing proteins might have a direct role in the actin-cytoskeleton interaction and disrupt their network during the cataract formation. As a consequence of this damage, the lens cell plasma membrane could lose its integrity and allow the discharge of intracellular components into globules resulting in globular degeneration.

At this point, with these findings, it was still difficult to outline the mechanism of cataract formation in both model systems. For this reason, different preventive agents were used to test the hypothesis that oxidative damage is associated with the disorganization of actin or glucose cataract. In view of this, I decided to extend my research to study the effect of different cataract preventive agents in these model systems (Chapter 3).

CHAPTER 3.

ANTIOXIDANTS AND OTHER CATARACT PREVENTIVE AGENTS IN EXPERIMENTAL CORTICAL CATARACTOGENESIS.

3.1 INTRODUCTION

The findings of modern science and technology allow the blindness of cataract to be prevented by three possible means: i) surgery, ii) eliminating the causes of cataract, and iii) anti-cataract drugs. Though costly, surgery is the only applicable clinical treatment: to remove cataract from the patients by replacing the lens with a plastic intraocular lens. Controlling the risk factors which cause cataract offers an early intervention to prevent cataract, just as controlling the blood glucose level in diabetic patients prevents cataract. The third method, involving anti-cataract drugs, has been used in only a few clinical trials, without success, although research with *in vivo* model systems has been successful in cataract prevention.

In vitro studies always have advantages in exploring the direct effects of the agents which cause cataract. This section of the thesis initially demonstrates the advantages of *in vitro* study using various known anti-cataract agents to model protection of the lens and prevention of opacification. Next, the effect of an antioxidant, vitamin C (VC), was tested to explore the factors involved in the mechanism of CD-cataract formation, *e.g.*, is oxidative

stress associated with the disorganization of actin and cytoskeleton during cataract formation ?

The leakage of LDH and the microscopic appearance of the lenses were chosen as the relevant parameters to correlate the effect of 55.6 mM glucose or 10^{-5} M CD with cataract development.

The agents used in the in vitro system could be classified into three groups: i) antioxidants, such as vitamins E and C and lipoic acid, ii) sorbitol-lowering agents, such as the aldose reductase inhibitor, Venoruton, and iii) other agents, such as taurine.

The effect of antioxidants in the in vivo glucose diabetic model system is well known. Using them in in vitro glucose- and CD-induced cataractous model systems, however, provided three distinct advantages : i) both models and systems were tested, and ii) the order of the pre- and cataractous parameters which were correlated with the opacity formation could be arranged in the cataract mechanism, and iii) the direct effects of the anti-cataract agents were also tested and compared with the in vivo study. Also, while the effect of sorbitol-lowering agents is known, venoruton, a mixture of hydroxyethylrutosides, is a drug not previously used against cataract. Using another agent, taurine, gave us the chance to test the effect of a different anti-cataract agent in an in vitro system.

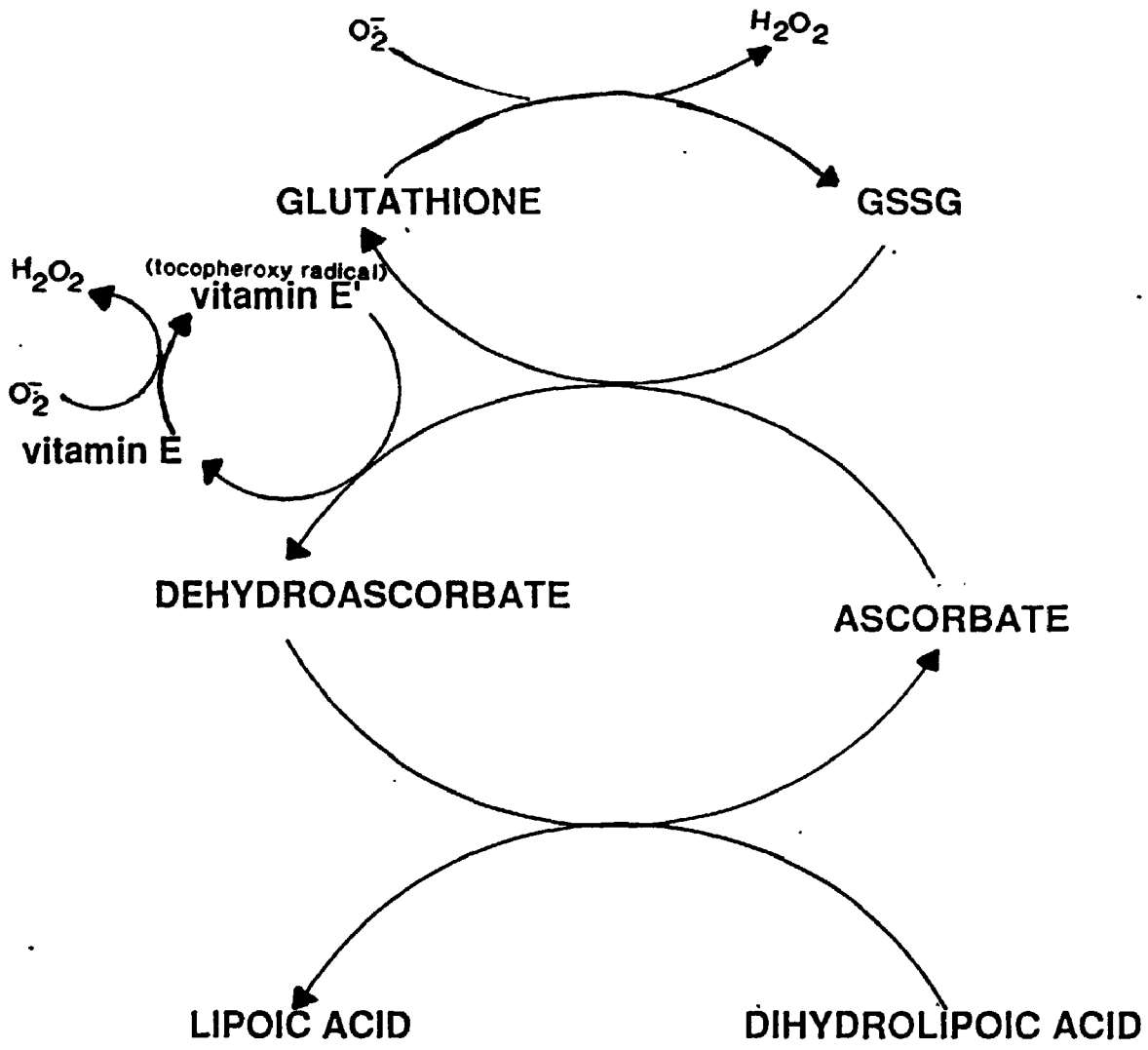
Oxidant stress may be a major contributor to the

etiology of cataract formation in the lens. Studies with ionizing radiation indicate that lens opacities similar to those caused by elevated glucose can form via free radical injury. ^{60}Co γ-irradiation causes globular degeneration *in vitro* (Ross *et al.*, 1983) and lens membrane damage *in vivo* (Ross *et al.*, 1990). ^{60}Co γ-irradiation is known to generate hydroxyl radical, superoxide anion, and singlet oxygen in air-saturated aqueous solution. Globular degeneration of the opaque subcapsular equatorial region of the lens is prevented by the addition of d-α-tocopherol to the medium in which lenses are incubated, subsequent to ^{60}Co γ-irradiation (Ross *et al.*, 1983). Wolff and Dean (1987) suggested that glycation of proteins is accompanied by generation of free radicals during the reaction of the sugar in the Amadori rearrangement.

Consistent with the hypothesis that this damage results from oxidant stress, it has been found that, *in vivo*, experimental diabetic cataractogenesis is prevented or reduced by a number of agents with antioxidant activity, such as α-tocopherol (Creighton and Trevithick, 1979; Stewart-DeHaan *et al.*, 1982), vitamin C (Linklater *et al.*, 1990), butylated hydroxytoluene (Linklater *et al.*, 1986), vitamin A (Linklater *et al.*, 1992; Clarke *et al.*, 1989) and a mixture of α-tocopherol, ascorbate and β-carotene (Kilic *et al.*, 1994). All these agents have antioxidant activity (Figure 3.1), and all are effective *in vivo* in prevention of

FIGURE 3.1
ANTIOXIDANT CYCLES.

Interrelations between antioxidants form one of the major defence mechanisms against tissue damage by free radicals and other reactive oxygen species [$O_2^{\cdot -}$, H_2O_2, $OH^{-\cdot}$, HOCl] (Figure is modified from Packer, 1992a).


O_2^-
H_2O_2
GLUTATHIONE
GSSG
H_2O_2
(tocopheroxy radical)
vitamin E'
O_2^-
vitamin E
DEHYDROASCORBATE
ASCORBATE
LIPOIC ACID
DIHYDROLIPOIC ACID

the globular degeneration (Ross et al., 1982; Yokoyama et al., 1994) caused by elevated glucose.

Antioxidants could act to prevent formation of lens opacities by several mechanisms (Packer et al., 1979). α-Lipoic acid is converted in vivo to the potent antioxidant dihyrolipoic acid (Handelman et al., 1994; Packer et al., 1995), and might relieve oxidant stress in the lens or be secreted into the incubation media (Figure 3.1). Antioxidants may serve as a source of reduced sulfhydryl groups (Handelman et al., 1994) that would aid in maintaining lens ascorbate and glutathione levels : these fall to low levels in several cataract models (Mårtensson and Meister, 1991; Mitton et al., 1993). Alternatively, antioxidants may stimulate formation of ATP during oxidative phosphorylation in the mitochondria. Lipoamide, a derivative of α-lipoic acid, functions as a prosthetic group (Barrera et al., 1982) in the mitochondria for the conversion of pyruvate to acetyl CoA, and α-ketoglutarate to succinyl CoA, and therefore α-lipoic acid has a direct catalytic role in maintaining the operation of the tricarboxylic acid cycle.

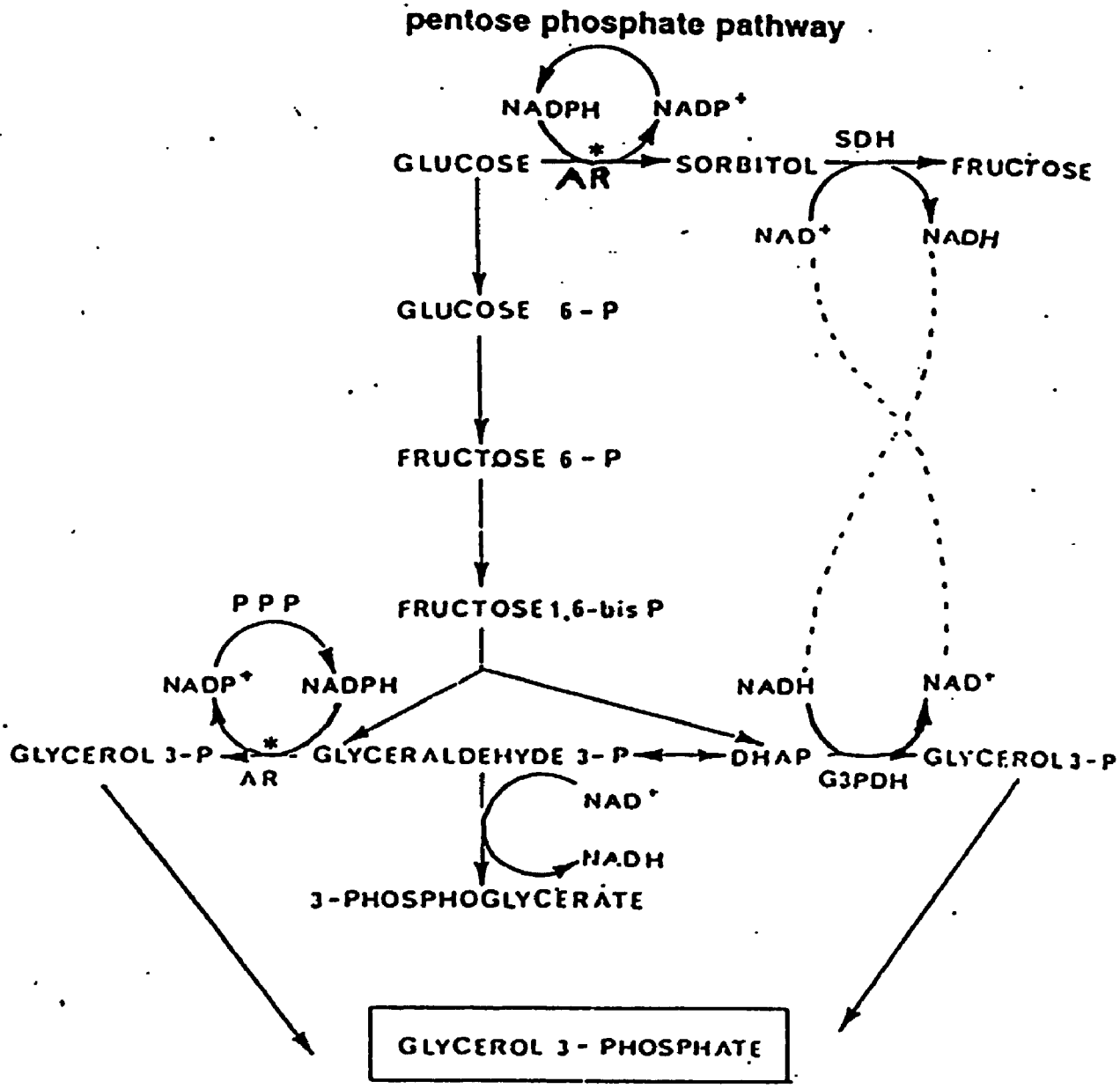
Glucose, after transport into the lens, is metabolized by either the glycolysis pathway or the overflow sorbitol pathway (Berman, 1991). In the sorbitol pathway two enzymes sequentially metabolize the transported glucose when its concentration is high: aldose reductase, which requires

NADPH, producing sorbitol and polyol dehydrogenase, which is rate limiting and requires NAD^+, oxidizing the sorbitol to fructose (Berman, 1991; Kinoshita et al., 1976) (Figure 3.2). As a result of its transport into the lens cells, glucose reacts with the lens cell proteins (Wolff and Dean, 1987; Cohen, 1986). Venoruton is a soluble bioflavonoid derivative which has two potential functions: as antioxidant and aldose reductase inhibitor.

It has been very well documented that diabetic cataract formation is preventable by aldose reductase inhibitors and antioxidants (Reddy et al., 1992; Chand et al., 1982). But a semisynthetic flavonoid, Venoruton, which is a mixture of mono- (about 5%), di- (about 34%), tri- (about 46%), and tetra- (approximately 5%) hydroxyethylrutosides (Wadworth et al., 1992), has not been used as a cataract preventive agent before. This derivative of rutin, which is obtained from buckwheat, has a similar antioxidant action to vitamin P (Balant et al., 1984). Its individual components have been shown to inhibit aldose reductase (Varma and Kinoshita, 1976), although the inhibition decreases with the degree of derivatization of the rutin. Venoruton was studied in diabetic patients and shown to improve microvascular perfusion and microcirculation and to reduce erythrocyte aggregation (Allen et al., 1990; Barrow et al., 1974). This agent also had a possible protective effect on the vascular endothelium (Belcaro et al., 1991).

FIGURE 3.2
THE PROPOSED EFFECT OF ALDOSE REDUCTASE INHIBITOR IN DIABETIC RAT LENS.

The pentose phosphate pathway is one of the ways to metabolize the glucose transported into lens cells. The enzyme aldose reductase (AR) converts glucose to sorbitol and the excess amount of sorbitol causes osmotic stress damage at the cellular and molecular levels. The effect of an AR inhibitor which could stop glucose conversion to sorbitol would also stop the loss of transparency.


pentose phosphate pathway
NADPH
NADP+
GLUCOSE
AR
SORBITOL
SDH
FRUCTOSE
NAD+
NADH
GLUCOSE 6-P
FRUCTOSE 6-P
FRUCTOSE 1,6-bis P
PPP
NADP+
NADPH
NADH
NAD+
GLYCEROL 3-P
AR
GLYCERALDEHYDE 3-P
DHAP
G3PDH
GLYCEROL 3-P
NAD+
NADH
3-PHOSPHOGLYCERATE
GLYCEROL 3-PHOSPHATE
increased 7-fold in
uncontrolled diabetes

In the streptozotocin diabetic rat during the early stages, even while the lenses remain clear under a dissection microscope, the sorbitol levels were found to be very high while antioxidants and lens amino acids and taurine were found to be lower than the physiological levels (Malone et al., 1990; Kasuya et al., 1992; Mitton et al., 1994). One effect of taurine could be scavenging hypochlorite which is an oxidizing species (Babior and Crowley, 1983). In white cells hypochlorite is produced by the enzyme myeloperoxidase, and it damaged the cell structure in a manner similar to other oxidizing agents. This might suggest that the role of taurine in the lens may be in part to prevent oxidative damage to the lens. Linklater et al., (1985) showed that addition of taurine to the diet of streptozotocin diabetic rats resulted in a decrease of γ-crystallin leakage into vitreous humour. Without taurine they found this leakage 4-5 fold higher than normal. In the experiments reported here, addition of taurine into the incubation medium was tested and the result was correlated with the amount of LDH in the culture media and the microscopic appearance of lenses.

3.2 MATERIALS AND METHODS

REAGENTS: All chemicals were obtained from Sigma (St. Louis, MO), Canlab (Toronto, Ontario), and/or Fisher (Toronto, Ontario) and were reagent grade or the highest purity available. R, S, and racemic lipoic acid were kindly provided by Asta Medica (Frankfurt, Germany). Venoruton was supplied by Zyma (Nyon, Switzerland).

3.2.1 *In Vitro* Vitamin C, Vitamin E and β-Carotene :

All of the antioxidants were prepared from stock prior to the assays described below: 1 M Ascorbic acid (Roche Canada Ltd.) (VC) in H_2O, and 10 mM d-α-tocopherol succinate (VE, 1162 I.U./g, Henkel Corp., Kankakee, IL) and 10 mM β-carotene (30% suspension in corn oil) were prepared in DMSO and adjusted to pH 7.4 then sterilized with a 0.20 μm filter and stored at -20 °C.

The antioxidants were added individually into the fresh M199, or M199 with glucose or with CD, to give a final concentration of 1mM/VC, 10 μM/VE and 10 μM/β-carotene.

The individual effect of antioxidants was examined by measuring the LDH leakage into the medium after each 24 hr period, as compared to an untreated concurrent control at the same time. LDH activity was measured spectrophotometrically by the method of Moss *et al.*, (1978) as described in Chapter 2.2.4.

VC did not interfere with the LDH assay. The level of VC in the incubation medium was analyzed by the method of Jagota and Dani (1982). 0.1 ml 10 X diluted Folin-Ciocalteu reagent was mixed with 1 ml M199 with or without 55.6 mM glucose and CD. After 10 min the absorbance (760 nm) was measured in a Bausch & Lomb spectrophotometer.

3.2.2 Combined Vitamin C, E, and β-Carotene :

3.2.4.1 In Vitro System

The antioxidants which were prepared and diluted as described above were all added together to give a final concentration of 1mM/VC, 10 μM/VE and 10 μM/β-carotene in M199, or M199 with 55.6 mM glucose.

3.2.4.2 In Vivo System - The Dietary Regimen :

The dietary regimen used the following components and concentrations: the basic diet was powdered rat food (Agway, Guelph, Ont.) No 3200. The following additives were mixed, as appropriate: i) dl-α-tocopherol (VE) (10 g/kg food, Roche Canada Ltd.) ii) β-carotene 30% suspension in corn oil, 4 g β-carotene/kg food in 13.2 g of suspension or, for c ntrols 9.2 g corn oil/kg food without any β-carotene (both from Roche Canada Ltd), and iii) ascorbic acid (VC) (2 g/kg food). To minimize any autooxidation of these additives, the food containing additives was freshly prepared by mixing at 2-3 day intervals and refrigerated in dark green plastic

bags purged with N_2 until use. Using the previously reported method for cataract grading by a veterinary pathologist (S.E.S), the animals were examined at zero time as usual and on a biweekly basis from week 4 until the end of the experiment.

3.2.3 α-Lipoic Acid Treatment :

After 24 hrs pre-incubation in M199 lenses were examined under a dissection microscope and the leakage of LDH in the incubation media was measured. Then lenses which appeared normal were used for the rest of the assay. α-lipoic acid (R-, S-, Racemic) (prepared previously in DMSO, neutralized to pH 7.4, sterilized with a 0.20 micron pore size filter and stored at-30°C) was added to fresh M199, or to M199 with glucose, to give a final lipoic acid concentration in the culture medium of 1 mM. Incubation was initiated by placing the lenses in the different media containing R-, S-, and racemic α-lipoic acid at 35.5°C.

3.2.4 Venoruton in the <u>In Vitro</u> System :

Just prior to starting the incubations, M199, or M199 + glucose was removed from the lens cultures, and venoruton (prepared previously at a concentration of 1 M in H_2O, sterilized with the 0.20 μm filter and stored in freezer) was added to fresh M199, or to M199 with glucose to give a final concentration of 1mM. The molecular weight of the

main constituent of Venoruton, was given us by the supplier as 742.7. The effect of venoruton was examined by measuring LDH leakage into the medium at 24 hr intervals, as compared to untreated concurrent controls.

3.2.5 Taurine Treatment :

5 M taurine was prepared in H_2O adjusted to pH 7.4 then sterilized with the 0.20 µm filter and stored at -20 °C. After 24 hr pre-incubation in M199, the effect of taurine was tested on the control (M199) and glucose-induced (M199 containing 55.6 mM glucose) in vitro diabetic rat lenses by adding it to their incubation medium to give a final taurine concentration of 5 mM.

3.2.6 Statistical Analysis :

The accumulated leakage of LDH was expressed as a function of duration of lens incubation (days 1-6). The linear portion of the curve of released LDH versus time was determined by inspection of the graph. The slopes of the linear portion of respective curves for controls, 55.6 mM glucose/CD and 55.6 mM glucose/CD plus treatment, were compared using the regression analysis program of the SPSSPC statistics package (Norusis, 1988).

Using the SPSSPC statistical analysis program, regression lines were fit to the equation:

y(accumulated LDH leakage) = constant + B_1(duration in days)

+ B_2(preventive agent treatment) + B_3(glucose treatment). The data used were the accumulated LDH units/g lens from appropriate days of the incubation period giving linear release as judged by inspection of the graphs. Each set of conditions for the different preparations added to the lens incubations was analysed statistically using values for the variables as follows:

glucose or CD: 0(control), 1(glucose or CD treatment)

preventive agent 0(control), 1(treatment)

duration: 1,2,3,4,5,6 days, as appropriate for linearity.

The regression analysis was performed using the SPSS statistical analysis program, using three data sets. The data included the data set for the following conditions: (1)control, (2) glucose or CD, (3) glucose or CD + preventive agents. The results are given as the value of the slope (B) ± SE for which each variable is responsible and the significance of each value.

Statistical analysis for <u>in vivo</u> experiment:

For determination of statistically significant differences between γ-crystallin levels of the control and diabetic rats subjected to different dietary regimens, the t-test (groups) program of the SPSSPC program was used to determine statistically significant differences between group means for Table 3.2 and 3.3.

Statistical analysis for DHLA groups:

To determine differences between DHLA levels in lenses incubated with R-, S-, or Racemic α-lipoic acid for 1, 2, or 3 days in control M199 or M199 with 55.6 mM glucose, the Instat statistics program for Macintosh (Graphpad Software, 1995) was used as follows by Dr. Garry Handelman of Dr. Lester Packer's laboratory at University of California, Berkeley, CA. The unpaired t-test of groups of all lenses incubated in (1) control M199, or (2) M199 with 55.6 mM glucose was used to compare these groups. To evaluate the effects of duration of incubation, the DHLA levels of all lenses incubated for 1, 2, or 3 days respectively were compared using the ANOVA analysis procedure of Instat. The day 2 data were significantly elevated by comparison to day 1 or 3. To evaluate the separate effects of R-, S-, or racemic α-lipoic acid, the DHLA levels of all lenses incubated in (1) R-, (2) S-, or (3) racemic α-lipoic acid were compared by the ANOVA analysis procedure of the Instat statistical analysis program (Graphpad Software, 1995). No significant differences were found.

3.3 RESULTS

Antioxidants as Anti-Cataract Agents:

The In Vitro Effect of VC on Glucose- and CD-Cataract Models:

To justify the subsequent in vivo study and to follow the steps in the pathway to cataract development in both cataract model systems, rat lenses were incubated in M199 containing 1 mM VC, along with either 55.6 mM glucose (Figure 3.3, Panel A and B) or 10^{-5} M CD (Figure 3.4, Panel A and B).

Lenses retained their normal clarity and appearance when cultured for 8 days in M199 with 1 mM VC and in 55.6 mM glucose with 1 mM VC. This result is consistent with the small amount of LDH found in the culture medium. In lenses exposed to elevated glucose there was a significant protection by VC. When analysed using the regression program of the SPSSPC statistical program (Norusis, 1988), the glucose-induced rate of LDH release was significantly ($p<0.00001$) decreased from 14.22 ± 2.85 U/g/day to 13.59 ± 2.62 U/g/day when VC was added. This is a reduction of 95.5% in LDH release compared to glucose-treated lenses.

When VC was added to the medium containing 10^{-5} M CD (Figure 3.4, Panel A and B), the leakage of LDH into the

FIGURE 3.3

IN VITRO PREVENTIVE EFFECT OF VC AGAINST GLUCOSE DAMAGE.

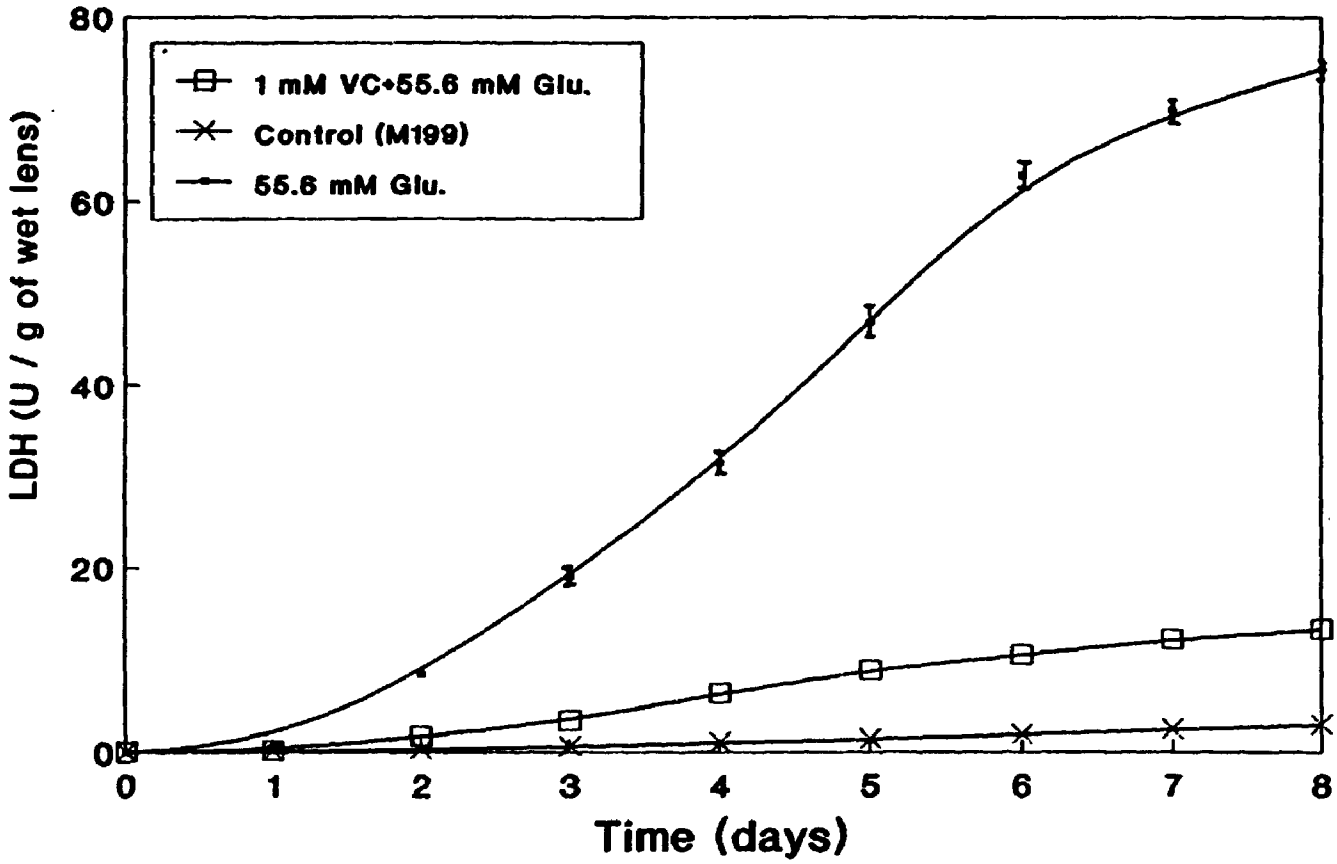
The effect of 55.6 mM glucose was tested in M199: 3 groups of lenses were incubated in M199 as follows:

(1) ⊟ 1 mM VC and 55.6 mM glucose;
(2) ✕ M199 (control);
(3) ● 55.6 mM glucose.

After every 24 hrs an aliquot was taken from each well and assayed for LDH activity, and the medium was changed. The LDH activity in the culture medium and the leakage per g lens were calculated as described in Materials and Methods. Data (means ± SE (n=3) and percent SE) are shown for each separate period of culture and culture condition. When the SE is lesser in magnitude than the dimensions of the point on the graph it is not shown.

	Glucose + M199		Control (M199)		VC + Glucose	
Day	U/g lens ± SE	%SE	U/g lens ± SE	%SE	U/g lens ± SE	%SE
1	0.750 ± 0.010	1.33	0.150 ± 0.000	0.00	0.250 ± 0.000	0.00
2	8.497 ± 0.222	2.61	0.304 ± 0.005	1798	1.697 ± 0.090	5.33
3	18.973 ± 1.104	5.81	0.670 ± 0.036	5.04	3.393 ± 0.178	5.26
4	31.403 ± 1.528	4.86	1.049 ± 0.027	2.58	7.322 ± 0.178	2.43
5	46.967 ± 1.973	4.20	1.428 ± 0.021	1.50	8.869 ± 0.238	2.69
6	62.913 ± 1.507	2.39	1.908 ± 0.057	2.99	10.565± 0.149	1.41
7	69.930 ± 1.189	1.70	2.462 ± 0.080	3.28	12.262± 0.160	1.30
8	74.397 ± 1.123	1.51	3.017 ± 0.106	3.52	14.390± 0.193	1.34

Panel A shows the lens prior to addition of glucose and VC into the incubation media. Panels B shows the lens after 8 days incubation in M199 containing 55.6 mM glucose along with 1 mM VC. Figure 2.3 Panel E showed the effect of 8 days incubation in M199 with 55.6 mM glucose.


1 mM VC+55.6 mM Glu.
Control (M199)
55.6 mM Glu.
LDH (U / g of wet lens)
80
60
40
20
0
0
1
2
3
4
5
6
7
8
Time (days)

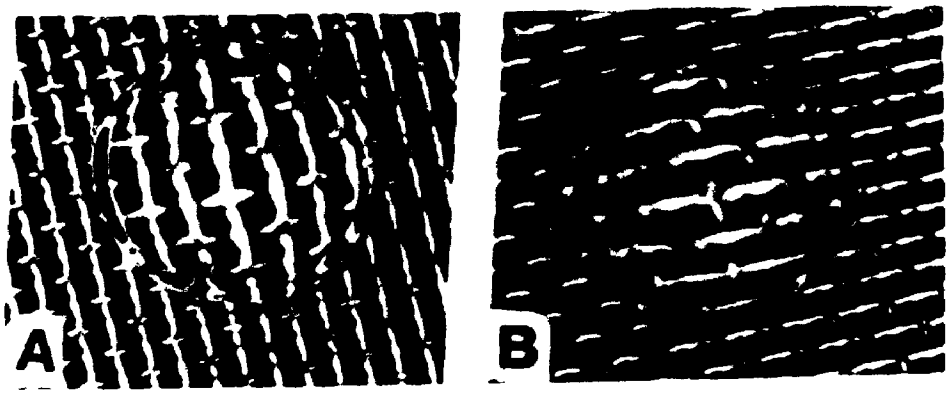

A
B

FIGURE 3.4
IN VITRO PREVENTIVE EFFECT OF VC AGAINST CD DAMAGE.

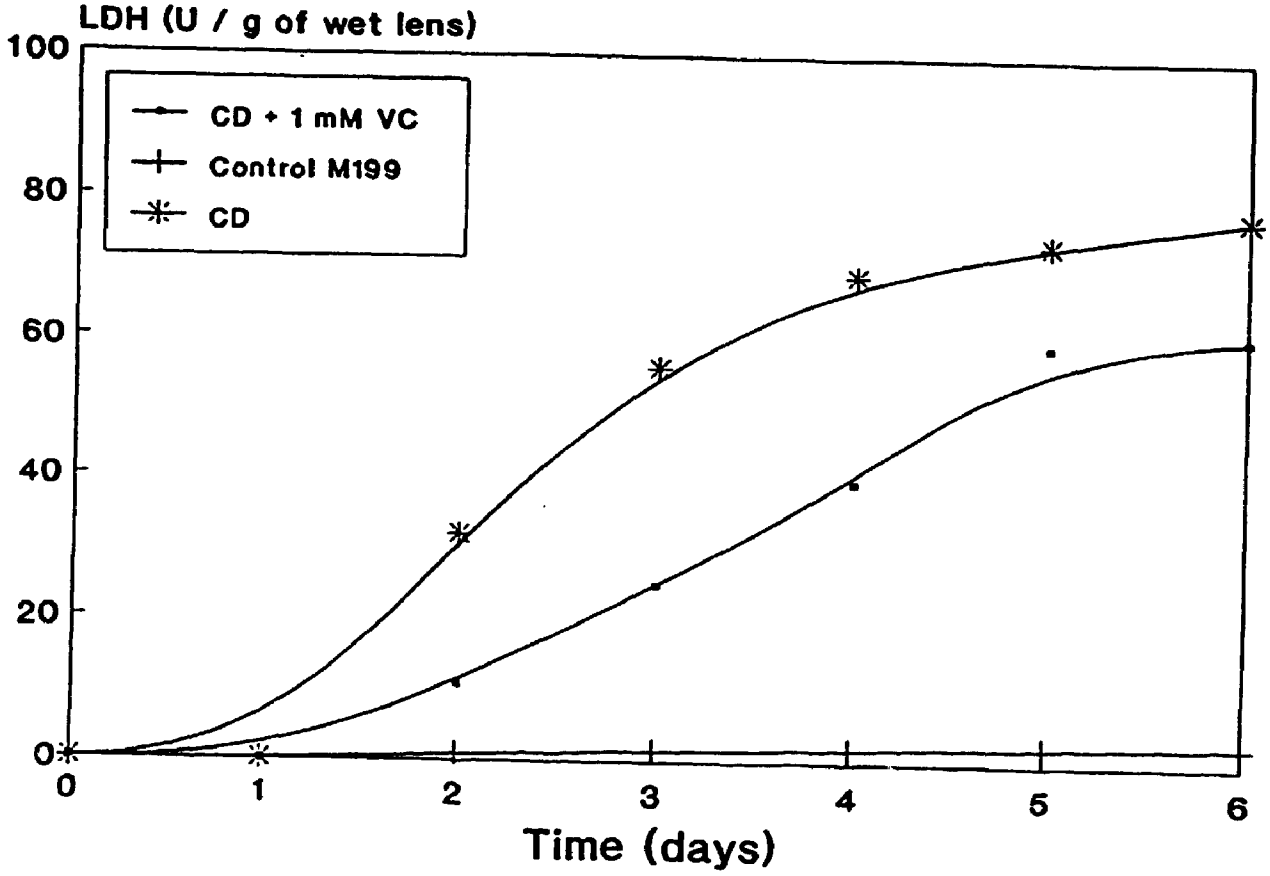
The effect of CD was tested in 0.1 % DMSO in M199. 3 groups of lenses were incubated in M199 as follows:

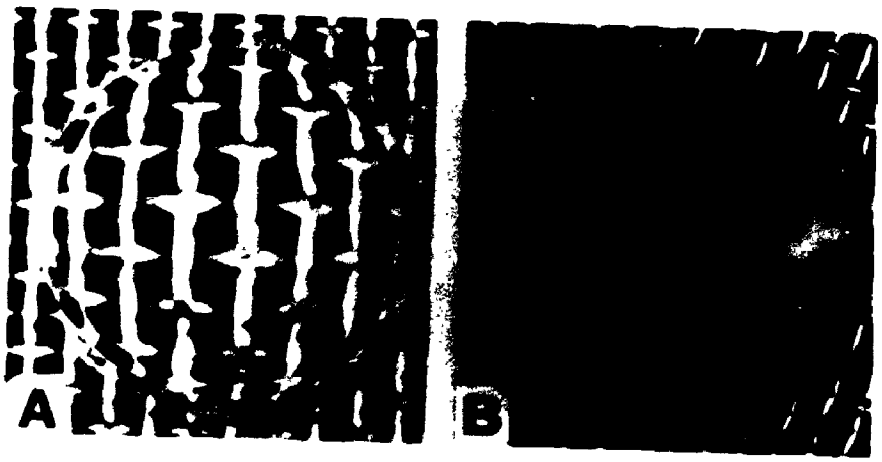
(1) —•— 1 mM VC and 10^{-5} M CD;
(2) —+— M199 (control);
(3) —✳— 10^{-5} M CD.

After every 24 hrs an aliquot was taken from each incubation and assayed for LDH activity, and the medium was changed. The LDH activity in the culture medium and the leakage per g lens was calculated as described in Materials and Methods. Data (means ± SE (n=3) and percent SE) are shown for each separate period of culture and culture condition. When the SE is lesser in magnitude than the dimensions of the point on the graph it is not shown.

	CD-treatment		Control (M199)		VC + CD-treat.	
Day	U/g lens ± SE	%SE	U/g lens ± SE	%SE	U/g lens ± SE	%SE
1	1.507 ± 0.020	1.33	0.150 ± 0.000	0.00	0.035 ± 0.000	0.00
2	31.897 ± 0.901	2.83	0.304 ± 0.005	1.79	10.350 ± 0.110	1.06
3	55.927 ± 0.858	1.53	0.670 ± 0.036	5.04	24.870 ± 0.063	0.26
4	69.343 ± 0.886	1.28	1.049 ± 0.027	2.58	39.407 ± 0.133	0.34
5	74.003 ± 0.701	0.95	1.428 ± 0.021	1.50	58.223 ± 0.214	0.37
6	77.790 ± 0.745	0.96	1.908 ± 0.057	2.99	60.500 ± 0.127	0.21

Panel A shows the lens prior to addition of CD and VC into the incubation media. Panel B shows the lens after 6 days incubation in M199 containing 10^{-5} M CD along with 1 mM VC. Figure 2.4 Panel D showed the effect of 6 days incubation in M199 with 10^{-5} M CD.


LDH (U / g of wet lens)
100
80
60
40
20
0
CD + 1 mM VC
Control M199
CD
0
1
2
3
4
5
6
Time (days)


A
B

incubation medium was significantly reduced as shown graphically in Figure 3.4. When analysed using the regression program of the SPSSPC statistical program (Norusis, 1988), the CD-treated lenses, the release rate for LDH was significantly ($p<0.0001$) decreased from 27.54 ± 3.33 U/g/day to 12.26 ± 2.80 U/g/day by addition of VC. This is a reduction of 44.5% in the release rate during days 1-3, for which LDH release was linear by inspection of the graphs.

A standard curve was prepared to calculate the level of VC in M199 containing glucose or CD (Figure 3.5). After 24 hrs the level of VC was 94% of the initial concentration in M199, and 75% in M199 containing either CD- or glucose indicating that the medium had a sufficient amount of antioxidant in both cases during the incubation interval (Table 3.1).

The Individual Effect of 10 μM VE and μM β-Carotene on <u>In Vitro</u> Diabetic Rat Lenses :

After 24 hr pre-incubation in M199 the lenses were examined under a dissection microscope and the levels of LDH were measured. Only the healthy lenses with undamaged intact capsules were transferred into either fresh M199 or M199 containing 55.6 mM glucose. Every day the amount of LDH in the incubation medium was measured and every second

FIGURE 3.5
STANDARD CURVE FOR ASCORBIC ACID ESTIMATION.

0, 1, 2, 3, 4, 4.5, and 5 µg VC in aqueous solution was mixed with dilute Folin-Ciocalteu reagent, then the absorbance was read at 760 nm. The level of VC in the incubation medium with/without additives during 24 hr incubation was estimated by using this standard curve.

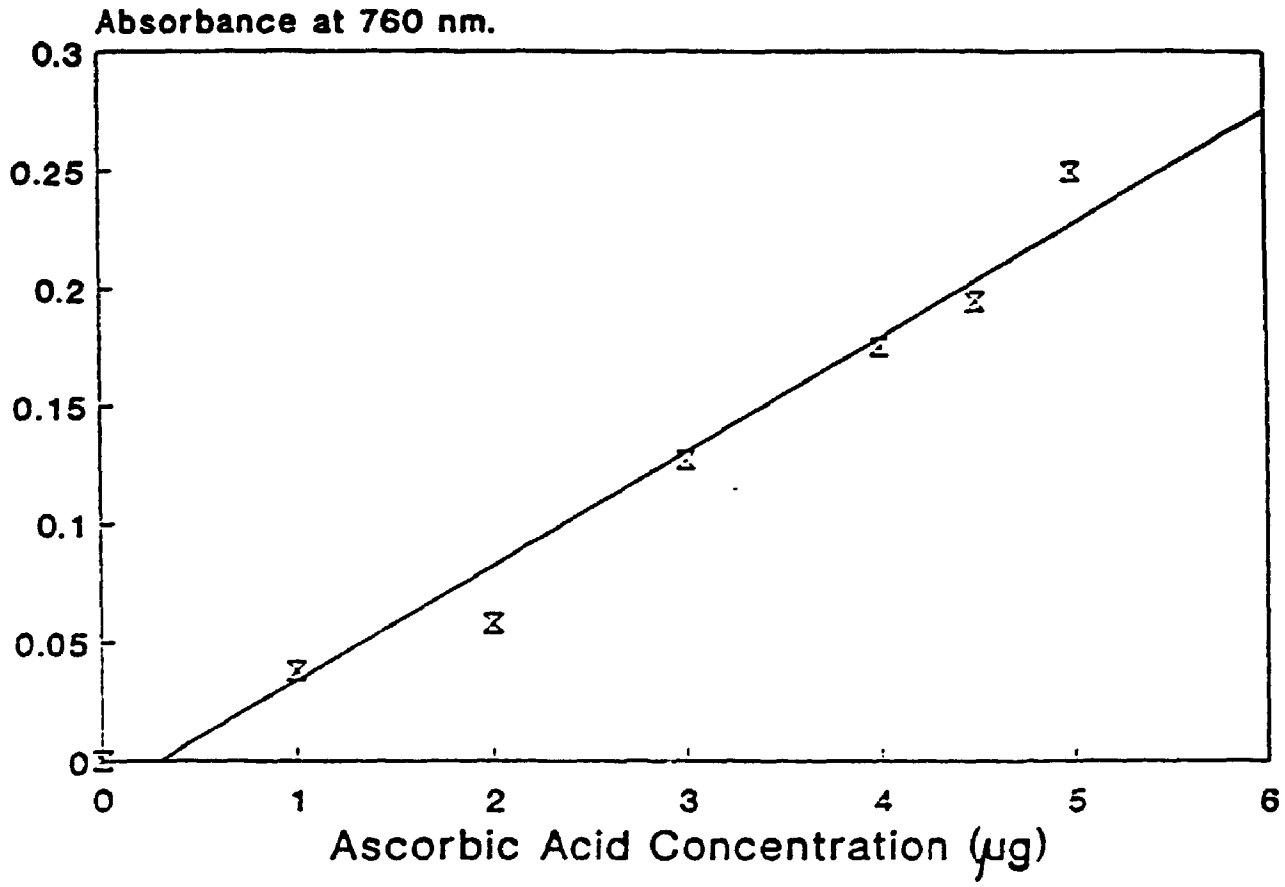

Absorbance at 760 nm.
0.3
0.25
0.2
0.15
0.1
0.05
0
0
1
2
3
4
5
6
Ascorbic Acid Concentration (µg)

TABLE 3.1 TIME COURSE OF VC CONCENTRATION IN M199, M199 + GLUCOSE AND M199 + CD.

VC (mM) at different time intervals in medium.

Medium	0 hr	4 hr	6 hr	8 hr	12 hr	24 hr
M199	0.930	0.925	0.915	0.905	0.879	0.871
M199+CD	0.860	0.840	0.823	0.719	0.709	0.699
M199+ Glucose	0.894	0.871	0.835	0.829	0.740	0.725

Identical aliquots of 1 M VC were added to each medium at 0 time and the amount of VC was determined after the times shown.

day the lenses were examined under a dissection microscope. At the end of 8 days incubation in the M199 containing 55.6 mM glucose under both conditions (either VE or β-carotene supplemented M199 containing 55.6 mM glucose) lenses appeared clear under the microscope and the amount of LDH in the incubation medium was similar to the control medium. The preventive effects of either VE (Figure 3.6, Panel A) or β-carotene (Figure 3.6, Panel B) on LDH release by in vitro diabetic rat lenses are promising.

In lenses exposed to elevated glucose there was a significant protection by VE . By regression analysis, the glucose-induced rate of LDH release is 27.32 ± 5.06 U/g/day ($p<0.00001$). The addition of VE reduces this rate by 25.08 ± 3.58 U/g/day, ($p<0.00001$). This indicates a strong protective effect of VE (91.7%) on glucose-treated lenses.

In lenses exposed to elevated glucose there was a large reduction in LDH leakage by lenses which were incubated with β-carotene. By regression analysis, the glucose-induced rate of LDH release is 27.32 ± 5.3 U/g/day ($p<0.00001$). The addition of β-carotene reduces this rate by 27.38 ± 3.75 U/g/day ($p<0.00001$). This indicates a strong reduction of damage (100%) by β-carotene.

FIGURE 3.6
ANTIOXIDANT: AS CATARACT PREVENTIVE AGENTS.

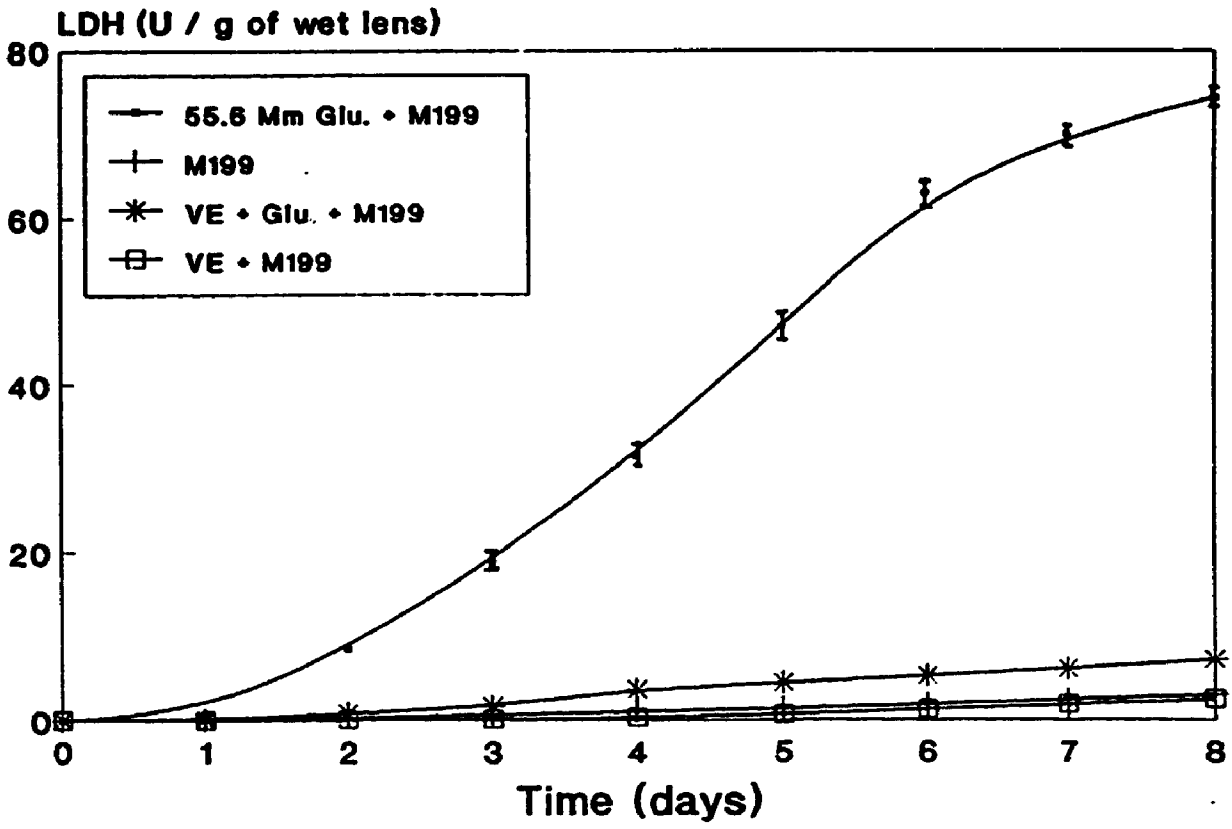
The preventive effects of VE (Panel A) and β-carotene (β-Carot.) (Panel B) were tested on the rat lenses incubated in M199 containing 55.6 mM glucose. The LDH activity of medium sample was measured and results are summarized graphically in both panels. At the end of 8 incubation days the total amount of LDH leakage was very low: the preventive effect on lens opacification could be also observed through a dissection microscope. This indicates a strong reduction of damage (100%) by either β-carotene or VE. Data (means ± SE (n=3) and percent SE) are shown for each separate period of culture and culture condition. When the SE is lesser in magnitude than the dimensions of the point on the graph it is not shown.

	Glucose-treatment		Control (M199)	
Day	LDH U/g lens ± SE	%SE	LDH U/g lens ± SE	%SE
1	0.750 ± 0.010	1.33	0.150 ± 0.000	0.00
2	8.497 ± 0.222	2.61	0.304 ± 0.005	1.79
3	18.973 ± 1.104	5.81	0.670 ± 0.036	5.04
4	31.403 ± 1.528	4.86	1.049 ± 0.027	2.58
5	46.967 ± 1.973	4.20	1.428 ± 0.021	1.50
6	62.913 ± 1.507	2.39	1.908 ± 0.057	2.99
7	69.930 ± 1.189	1.70	2.462 ± 0.080	3.28
8	74.397 ± 1.123	1.51	3.017 ± 0.106	3.52

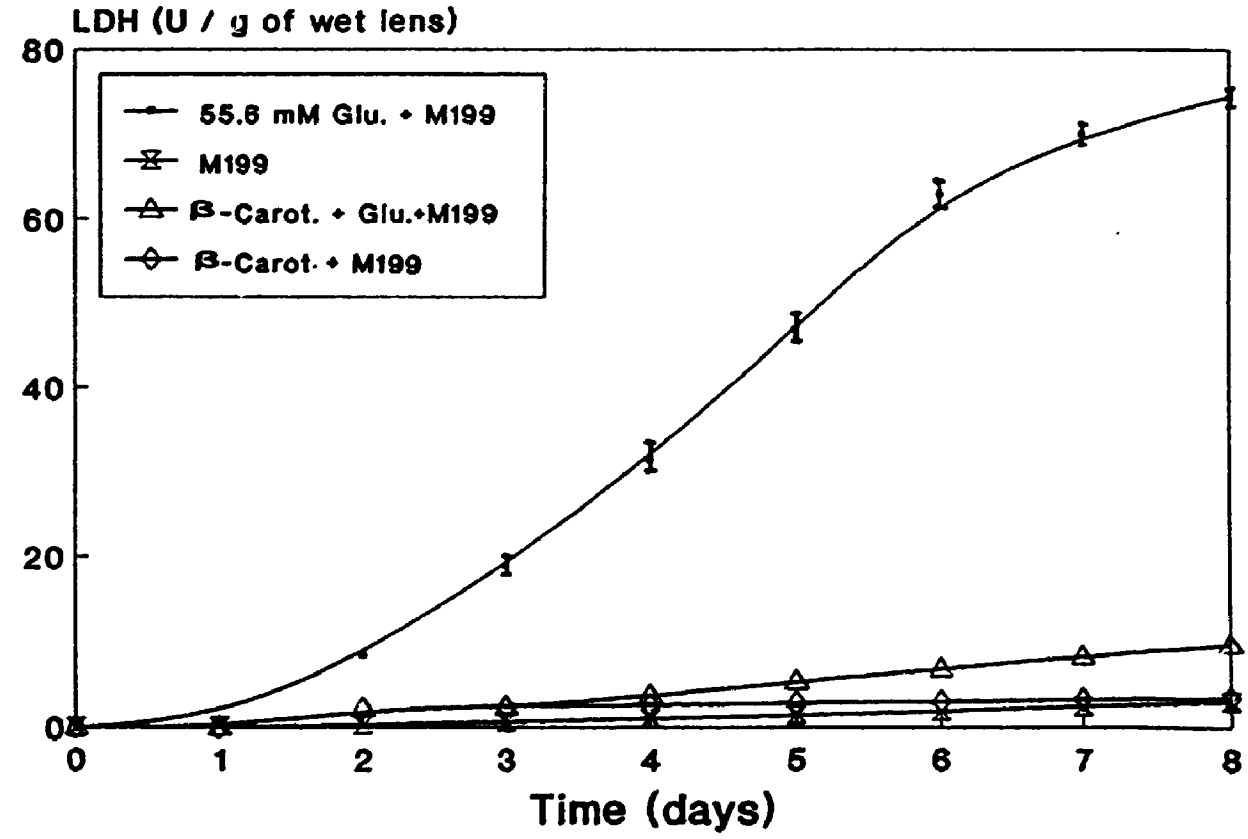
β-Carotene + Glu.		β-Carotene + M199	
U/g lens ± SE	%SE	U/g lens ± SE	%SE
0.010 ± 0.000	0.00	0.010 ± 0.000	0.00
2.181 ± 0.027	1.24	2.225 ± 0.032	1.42
2.377 ± 0.045	1.89	2.454 ± 0.045	1.83
3.683 ± 0.050	1.36	2.650 ± 0.043	1.62
5.312 ± 0.032	0.60	2.889 ± 0.055	1.90
6.870 ± 0.075	1.09	3.057 ± 0.043	1.41
8.294 ± 0.028	0.34	3.257 ± 0.069	2.12
9.656 ± 0.095	0.98	3.489 ± 0.072	2.06

VE + Glu.		VE + M199	
U/g lens ± SE	%SE	U/g lens ± SE	%SE
0.010 ± 0.000	0.00	0.010 ± 0.000	0.00
2.181 ± 0.025	1.15	0.079 ± 0.005	6.33
2.377 ± 0.029	1.22	0.123 ± 0.003	2.4
3.683 ± 0.036	0.98	0.350 ± 0.009	2.57
5.312 ± 0.047	0.88	0.727 ± 0.018	2.48
6.870 ± 0.063	0.92	1.293 ± 0.023	1.78
8.294 ± 0.057	0.69	1.883 ± 0.057	3.03
9.565 ± 0.074	0.77	2.473 ± 0.079	3.19

A


LDH (U / g of wet lens)
80
60
40
20
0
55.6 Mm Glu. + M199
M199
VE + Glu. + M199
VE + M199
0
1
2
3
4
5
6
7
8
Time (days)

B


LDH (U / g of wet lens)
80
60
40
20
0
55.6 mM Glu. + M199
M199
β-Carot. + Glu.+M199
β-Carot. + M199
0
1
2
3
4
5
6
7
8
Time (days)

Do Combined VC, VE and β-Carotene Prevent or Delay the Cataract ?

In Vitro :

VC, VE and β-carotene were added to the incubation media to give final concentrations 1 mM, 10 μM and 10 μM respectively. The usual procedure was followed for 8 days of incubation. The leakage of LDH into incubation medium increased very quickly. The effect of the combined vitamins on the control lenses which were incubated in M199 with elevated glucose was not as great as the individual effects when added singly. With combined treatment the LDH leakage was reduced, but by less than half of the simple treatment. Even the control lenses with mixed antioxidant but no glucose developed opacity during 8 days of incubation (Figure 3.7).

By regression analysis, the glucose-induced rate of LDH release is 27.32 ± 4.11 U/g/day ($p<0.00001$). The addition of VC, VE , and β-carotene combined reduced this rate by 15.27 ± 2.9 ($p<0.0053$). This indicates a 55.98% reduction of damage by this mixture.

FIGURE 3.7
COMBINATION OF VITAMINS: VC, VE, AND β-CAROTENE IN THE IN VITRO SYSTEM.

The individual preventive effect of vitamins in the in vitro glucose model system was observed. Also, the combination of vitamins in in vivo diabetic rat lenses were preventive. The total activity of LDH was measured from the daily incubation medium. The leakage was reduced significantly for the combination but not as much as for individual antioxidants. The leakage of LDH from 55.6 mM glucose incubated lenses were almost 75 U/g of lens, but with the vitamin combination the leakage of LDH was 40 U/g of lens.

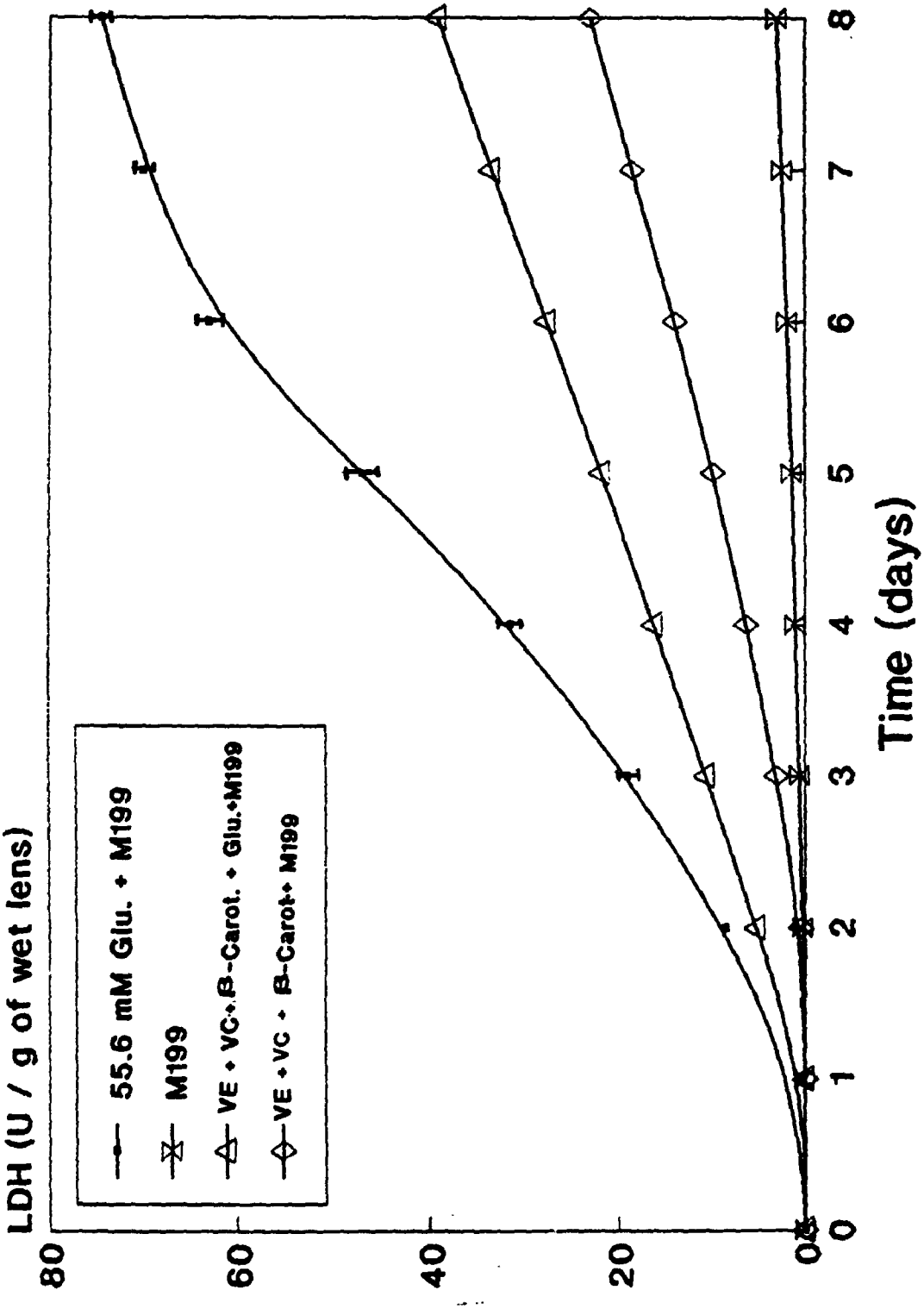
The treatments were as follows:

(1) 55.6 mM Glu+M199: M199 containing 55.6 mM glucose;
(2) M199: control medium 199;
(3) VE+VC+β-Carot+Glu+M199: M199 containing 10 μM vitamin E, 10 μM β-carotene, 1 mM vitamin C and 55.6 mM glucose.
(4) VE+VC+β-Carot.+M199: M199 containing 10 μM vitamin E, 10 μM β-carotene and 1 mM vitamin C.

Data (means ± SE (n=3) and percent SE) are shown for each separate period of culture and culture condition. When the SE is lesser in magnitude than the dimensions of the point on the graph it is not shown.

	Glucose-treatment		Control (M199)	
Day	LDH U/g lens ± SE	%SE	LDH U/g lens ± SE	%SE
1	0.750 ± 0.010	1.33	0.150 ± 0.000	0.00
2	8.497 ± 0.222	2.61	0.304 ± 0.005	1.79
3	18.973 ± 1.104	5.81	0.670 ± 0.036	5.04
4	31.403 ± 1.528	4.86	1.049 ± 0.027	2.58
5	46.967 ± 1.973	4.20	1.428 ± 0.021	1.50
6	62.913 ± 1.507	2.39	1.908 ± 0.057	2.99
7	69.930 ± 1.189	1.70	2.462 ± 0.080	3.28
8	74.397 ± 1.123	1.51	3.017 ± 0.106	3.52

β-Carotene+VC+VE+M199		β-Carotene+VE+VC+Glucose	
U/g lens ± SE	%SE	U/g lens ± SE	%SE
0.010 ± 0.000	0.00	0.010 ± 0.000	0.00
0.518 ± 0.006	1.16	5.232 ± 0.192	3.66
3.054 ± 0.046	1.51	10.606 ± 0.368	3.47
6.257 ± 0.106	1.69	16.148 ± 0.552	3.42
9.795 ± 0.171	1.75	21.739 ± 0.716	3.29
13.791 ± 0.344	2.49	27.497 ± 0.886	3.22
18.410 ± 0.368	2.00	33.388 ± 0.980	2.93
22.930 ± 0.401	1.75	39.217 ± 0.636	1.62


LDH (U / g of wet lens)
80
60
40
20
0
0
1
2
3
4
5
6
7
8
Time (days)
55.6 mM Glu. + M199
M199
VE + VC+ß-Carot. + Glu.+M199
VE + VC + ß-Carot+ M199

In Vivo :

The combination of dietary antioxidants including VC, dl-α-tocopherol and β-carotene was effective in preventing leakage of the lens protein γ-crystallin, into both aqueous and vitreous humours. When compared to the previous radioimmunoassay the binding assay used in these experiments was more sensitive, permitting the quantity of γ-crystallin leaking into the aqueous humour to be estimated more accurately. The differences in vitreous and aqueous leakage of γ-crystallin between diabetic control and diabetic dietary treatment group (29% decrease in vitreous γ-crystallin (**Table 3.2**), and 66% decrease in aqueous γ-crystallin, (**Table 3.3**) are highly significant and are correlated with each other.

TABLE 3.2 LEAKAGE OF γ-CRYSTALLIN INTO VITREOUS HUMOUR.

	Leakage as % of Normal*	
GROUP	NORMAL	DIABETIC
CONTROL	100 ± 23**	301 ± 55$^{**,+}$
DIET@	109.1 ± 28^{++}	214 ± 54$^{+,++}$
PERCENT CHANGE DIET/CONTROL	+9.1%	−28.9%

* Normal 29.7 ± 6.85 ng/μl

Probability of significant difference between groups.

** $p < 0.0001$
\+ $p < 0.007$
++ $p < 0.001$

The student t-test (groups) was performed to compare the results from each group of 12 lenses, and significances calculated according to the SPSSPC program (Norusis, 1980).

@The dietary regimen, described in Methods, consisted of Agway 3200 rat chow with the following additives: 10 g VE/kg food, 4 g β-carotene/kg food, 2 g VC/kg food.

TABLE 3.3 LEAKAGE OF γ-CRYSTALLIN INTO AQUEOUS HUMOUR.

	Leakage as % of Normal*	
GROUP	NORMAL	DIABETIC
CONTROL	100 ± 21.2**,+	331.8 ± 133.5**,+
DIET@	166.8 ± 59.0+	147.4 ± 41+
PERCENT CHANGE DIET/CONTROL	+66.8%	-65.6%

* Normal 15.94 ± 3.38 ng/μl γ-crystallin

Probability of significant differences between groups.

** $p < 0.01$
\+ $p < 0.003$

The student t-test (groups) was performed to compare the results from each group of 12 lenses, and significances calculated according to the SPSSPC program (Norusis, 1980).

@The dietary regimen, described in Methods, consisted of Agway 3200 rat chow with the following additives: 10 g VE/kg food, 4 g β-carotene/kg food, 2 g VC/kg food.

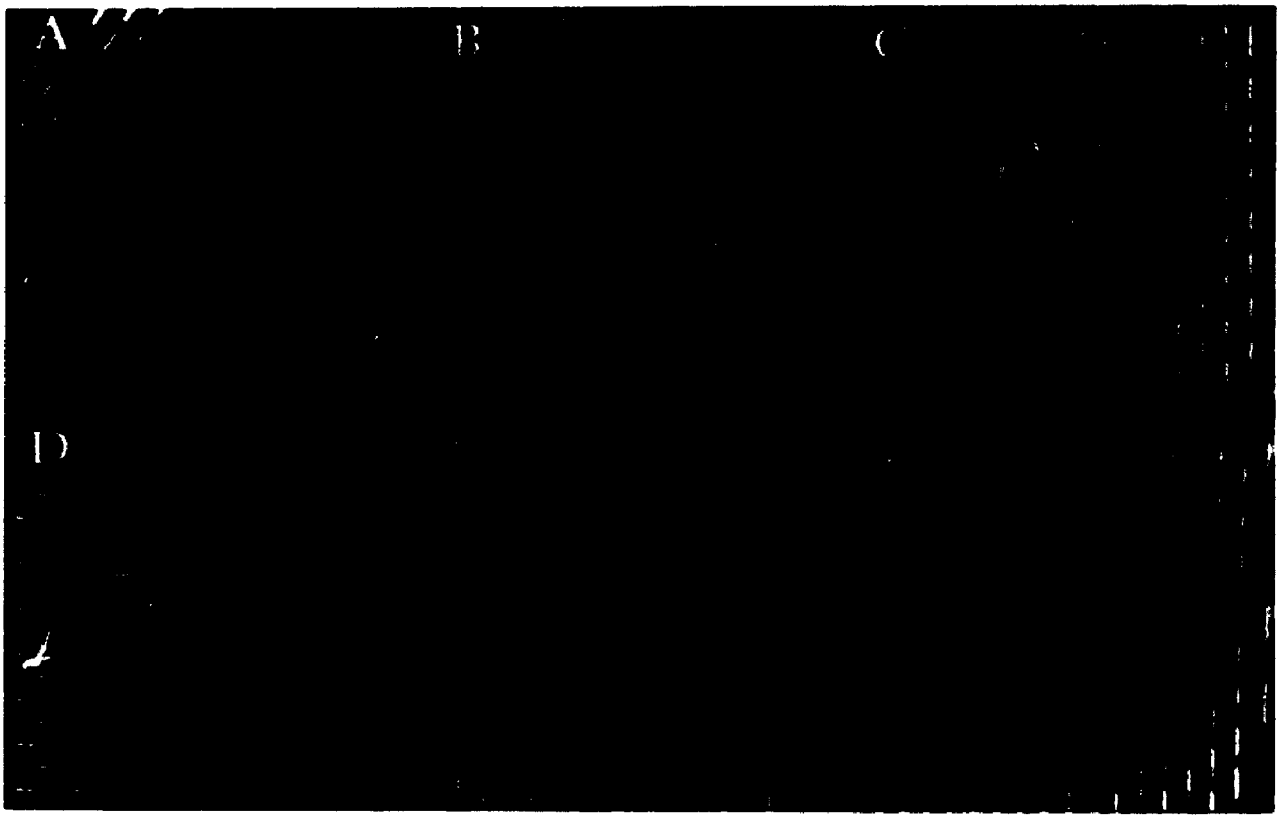
Lipoic Acid :

The upper panels in Figure 3.8 show the effects of lens incubation with M199 containing either 1 mM R-lipoic acid (Panel A), 1 mM racemic lipoic acid (Panel B), or 1 mM S-lipoic acid (Panel C). Lipoic acid addition to M199 had no effect on the healthy lens. However, in the presence of 55.6 mM glucose (lower panel, Figure 3.8), the addition of 1 mM R-lipoic acid (Panel D) was protective, while in the presence of racemic lipoic acid (Panel E) partial protection was observed, and for S-lipoic acid (Panel F) substantial lens opacity was observed. These findings are consistent with my previous studies of opacity induced by incubation at high glucose levels, and also demonstrate a protective effect of 1 mM exogenous R-lipoic acid in the incubation medium, and approximately half as much protection with 1 mM racemic lipoic acid in the medium. Racemic lipoic acid is half R- and half S-α-lipoic acid.

The leakage of LDH was also evaluated as a second marker for lens damage and its prevention by lipoic acid. Each individual experiment was replicated three times. After each 24-hr period, a sample was taken from the medium for LDH measurement and the lenses were transferred to fresh medium. The cumulative leakage of LDH into the medium was calculated by adding the daily amount of leakage per mg of

FIGURE 3.8
LIPOIC ACID: ANOTHER ANTIOXIDANT IN
IN VITRO GLUCOSE CATARACT MODEL SYSTEM.

Effects of different forms of lipoic acid on the development of opacities in normal rat lenses incubated for 8 days in M199 (top panel), or in 55.6 mM glucose (bottom panel). Addition of 1 mM R-lipoic acid (A), racemic lipoic acid (B), or S-lipoic acid (C) did not affect lens clarity at normal glucose levels. However, at high glucose levels, only 1 mM R-lipoic acid (D) prevented formation of the opacity. Prominent lens opacities developed during incubation with 55.6 mM glucose in the presence of 1 mM racemic lipoic acid (E), which was more pronounced with S-lipoic acid (F). Figure 2.3 Panel F shows the effect of 8 days incubation in M199 containing 55.6 mM glucose.


A
B
C
D

wet lens to the previous total. After 8 days, the experiment was terminated, and the lenses were removed to be photographed (Figure 3.9).

In experiments summarized graphically in Figure 3.9, the cumulative leakage of LDH from the lens began to display an increase after 48 hr incubation in elevated glucose. The curve for lens LDH leakage for the racemic lipoic acid mixture was located intermediate between the R-lipoic acid and S-lipoic acid curves (Figure 3.9). The slopes of the LDH release curve for the period 2-6 days were subjected to multiple regression analysis using as the dependent variable the total LDH released over the incubation period, to test whether the effect of added lipoic acid (R, S or racemic) was significant.

When three sets of conditions: (Table 3.4), i) lipoic acid control (M199 containing 1 mM Lipoic acid R, S or racemic), ii) glucose, 55.6 mM in M199, and iii) glucose, 55.6 mM in M199 containing 1 mM Lipoic acid (R, S or racemic), were subjected to multiple regression analysis, reduction of LDH leakage by R was approximately double that of racemic, and both R and racemic effects were statistically significant. There was a significant increase in LDH leakage for S-lipoic, the unnatural isomer.

Further analysis of the conversion to dehydrolipoate of

FIGURE 3.9
CUMULATIVE LDH IN INCUBATION MEDIUM OF RAT LENSES MAINTAINED UNDER DIFFERENT CONDITIONS.

Data (means ± SE (n=3) and percent SE) are shown for each separate period of culture and culture condition. When the SE is lesser in magnitude than the dimensions of the point on the graph it is not shown. Each curve represents the average for three experiments as follows:

(1) control (M199);
(2) 1 mM R-lipoic acid + M199;
(3) 1 mM S-lipoic acid + M199;
(4) 1 mM Rac.-lipoic acid + M199;
(5) 55.6 mM glucose;
(6) 55.6 mM glucose and 1 mM R-lipoic acid;
(7) 55.6 mM glucose and 1 mM S-lipoic acid;
(8) 55.6 mM glucose and 1 mM Rac-lipoic acid;

R-Lipoic Acid-M199		R-LipoicAcid+Glucose	
LDH U/gleas± SE	%SE	LDH U/gleas±SE	%SE
0.150±0.000	0.00	0.250±0.000	0.00
1.627±0.115	7.10	3.233±0.168	5.81
3.250±0.234	7.20	6.467±0.166	1.89
5.210±0.335	6.43	9.700±0.184	1.89
6.833±0.453	6.64	12.627±0.067	0.68
8.460±0.569	6.72	16.160±0.199	1.23
10.060±0.667	6.62	19.403±0.190	0.98
11.710±0.803	6.85	22.247±0.302	1.33

S-Lipoic Acid+M199		S-LipoicAcid+Glucose	
LDH U/g leas ± SE	%SE	LDH U/g leas ± SE	%SE
1.499±0.015	1.00	1.507±0.020	1.33
2.933±0.070	2.38	13.520±0.291	2.15
5.773±0.222	3.84	27.443±0.507	1.85
9.407±0.222	2.36	41.057±0.712	1.73
12.330±0.283	2.29	56.667±1.468	2.59
15.357±0.277	1.80	70.517±1.772	2.51
18.260±0.176	0.96	74.753±1.935	2.59
21.197±0.082	0.39	75.593±2.049	2.70

Rac-Lipoic Acid+M199		Rac-Lipo.Acid+Gluc.	
LDH U/g leas ± SE	%SE	LDH U/gleas ± SE	%SE
0.250±0.000	0.00	0.750±0.010	1.33
2.483±0.117	4.70	3.330±0.077	2.32
4.967±0.233	4.69	8.060±0.180	2.24
7.450±0.350	4.69	20.050±0.186	0.93
9.417±0.192	2.04	39.147±0.363	0.93
11.510±0.424	3.69	55.223±0.422	0.76
13.890±0.266	1.92	62.310±0.509	0.82
16.110±0.419	2.60	66.817±0.567	0.85

	Glucose-treatment		Control (M199)	
Day	LDH U/gleas ± SE	%SE	LDH U/gleas ± SE	%SE
1	0.750±0.010	1.33	0.150±0.000	0.00
2	8.497±0.222	2.61	0.304±0.005	1.79
3	18.973±1.104	5.81	0.670±0.036	5.04
4	31.403±1.528	4.86	1.049±0.027	2.58
5	46.967±1.973	4.20	1.428±0.021	1.50
6	62.913±1.507	2.39	1.908±0.057	2.99
7	69.930±1.189	1.70	2.462±0.060	3.25
8	74.397±1.123	1.51	3.017±0.106	3.52

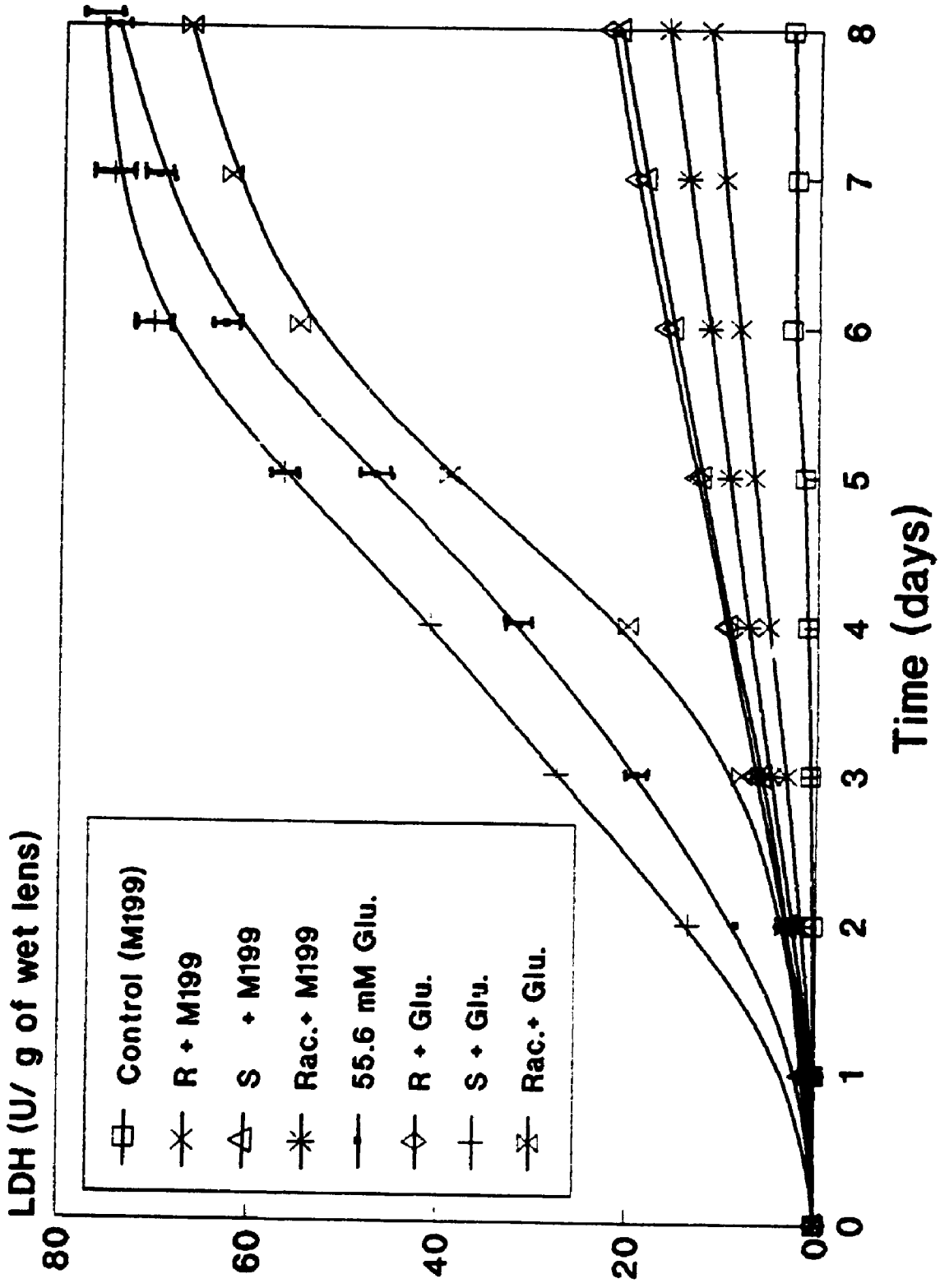

LDH (U/ g of wet lens)
80
60
40
20
0
0
1
2
3
4
5
6
7
8
Time (days)
Control (M199)
R + M199
S + M199
Rac.+ M199
55.6 mM Glu.
R + Glu.
S + Glu.
Rac.+ Glu.

TABLE 3.4 REDUCTION BY LIPOIC ACID STREOISOMERS OF GLUCOSE-INDUCED LEAKAGE OF LDH*.

Form of α-Lipoic Acid Tested	(B) Leakage rate ± S E(B) Units LDH/g lens/day	Significance p<	% Difference from R
R	24.34 ± 3.23	p < 0.00001	0
Racemic	7.95 ± 3.38	p < 0.02	50.9
S	-7.89± 3.61	p < 0.03	100

* LDH leakage rate induced by glucose was 32 ± 3 units/g wet lens, per day.

Multiple regression analysis was performed as described in section 3.2.6.

TABLE 3.5 DIHYDROLIPOIC ACID CONCENTRATIONS IN RAT LENSES INCUBATED WITH LIPOIC ACID FOR 1, 2 AND 3 DAYS.

		DHLA Concentration ± SE nmol/lens (number of samples) after Incubation for:		
Form of Lipoic Acid Tested	Glucose in Medium	1 Day	2 Days	3 Days
R	5.5 mM	.125 ± .025 (2)	.273 ± .029 (3)	.115 ± .014 (3)
S	5.5 mM	.128 ± .063 (2)	.197 ± .029 (3)	.163 ± 0.047 (3)
Racemic	5.5 mM	.19 ± .0001 (2)	.19 ± .03 (2)	.126 ± 0.023 (3)
R	55.5 mM	.048 ± .09 (2)	.087 (1)	0.099 ± .011 (2)
S	55.5 mM	.197 ± 0.032 (3)	.121 (1)	.201 ± .031 (2)
Racemic	55.5 mM	.097 ± .013 (2)	.207 (1)	.161 ± .042 (2)

R, S and racemic α-lipoic acid was performed for every intact rat lens (Table 3.5). The HPLC analysis of both stereoisomers of lipoic acid was performed in Dr. L. Packer's laboratory by Dr. G.J. Handelman (University of California, Berkeley).

All three types of lipoic acid were converted by the rat lens to dihydrolipoate with similar efficiency (Figure 3.10, Panel A). Likewise, the rate of reduction was comparable for high and normal glucose (Panel B). For day 2, there was a 50% higher amount of dihydrolipoate found than for day 1 or day 3 (Panel C) ($p < 0.05$), but the biological meaning of this observation is unclear.

Sorbitol-Lowering Agent :

Venoruton, an Aldose Reductase Inhibitor :

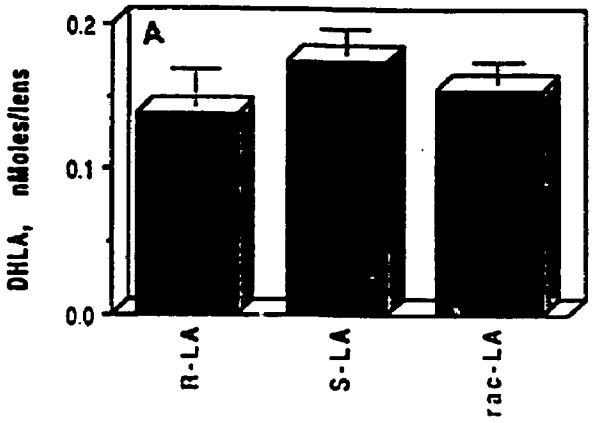
The effects of venoruton on healthy and glucose-treated cataractous lenses were observed by a dissection microscope and measurement of leaked LDH into the culture medium (Figure 3.11). Initially, the optimum concentration of venoruton was estimated by incubating the lenses in M199 with or without 55.6 mM glucose along with different concentrations of venoruton; 1 mM, 5 mM, 10 mM, for 8 days. Lenses incubated in M199 containing glucose and 5 mM and 10 mM venoruton developed opacity after 3 incubation days.

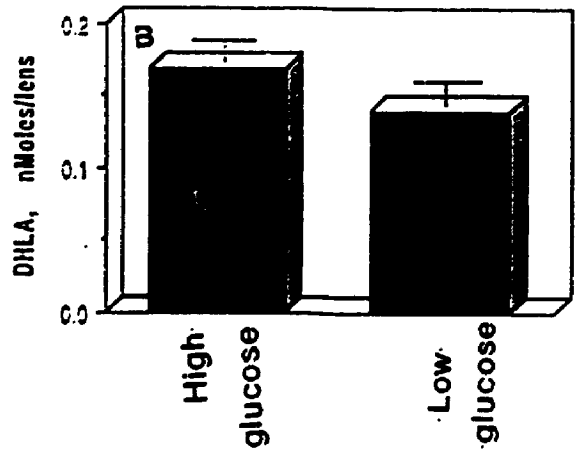
FIGURE 3.10
FROM LIPOIC ACID TO DIHYDROLIPOIC ACID (DHLA) :
A POWERFUL BIOLOGICAL REDUCTANT AND ANTIOXIDANT.

The amount of dihydrolipoate formed was measured in each intact rat lens. Reduction by intact rat lenses in culture of added lipoic acid (1 mM, in M199), to form dihydrolipoic acid in the lens.

A. Comparison of R, racemic, and S-lipoic acid
B. Effects of 5.56 mM and 55.6 mM glucose
C. Incubation for 24 h, 48 h, and 72 h. The DHLA concentration on day 2 was significantly different ($p<0.05$) from day 1 or 3 when DHLA concentrations for the different days were compared by ANOVA analysis using the INSTAT statistical analysis program.

The results for all samples were pooled accordingly to the appropriate comparison to be made:
For Panel A, results were all days were pooled to compare the DHLA levels after incubation with R, S, or racemic α-lipoic acid.
For Panel B, results for all R, S, and racemic samples were pooled to compare the DHLA levels after incubation in low glucose (5.56 mM) or high glucose (55.6 mM) in M199.
For Panel C, results for all R, S, and racemic samples at both high and low glucose levels were pooled to compare the DHLA levels after incubation for 1, 2, or 3 days in M199.


A
DHLA, nMoles/lens
0.2
0.1
0.0
R-LA
S-LA
rac-LA


B
DHLA, nMoles/lens
0.2
0.1
0.0
High glucose
Low glucose

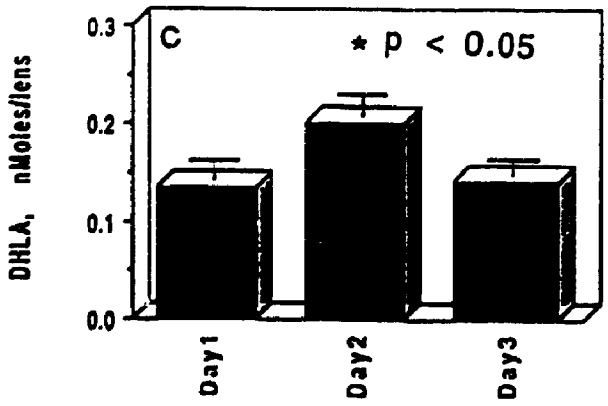

C
* p < 0.05
DHLA, nMoles/lens
0.3
0.2
0.1
0.0
Day1
Day2
Day3

FIGURE 3.11
VENORUTON: AN ALDOSE REDUCTASE INHIBITOR.

The effects of venoruton on healthy and glucose-induced cataractous lenses were examined by microscope and measurement of leaked LDH into the culture medium. After 24 hrs preincubation in 1.5 ml M199, lenses were transferred to fresh M199 containing: 55.6 Mm glucose; control, no additions; 55.6 mM glucose and 1 mM Venoruton; and control and 1 mM Venoruton.

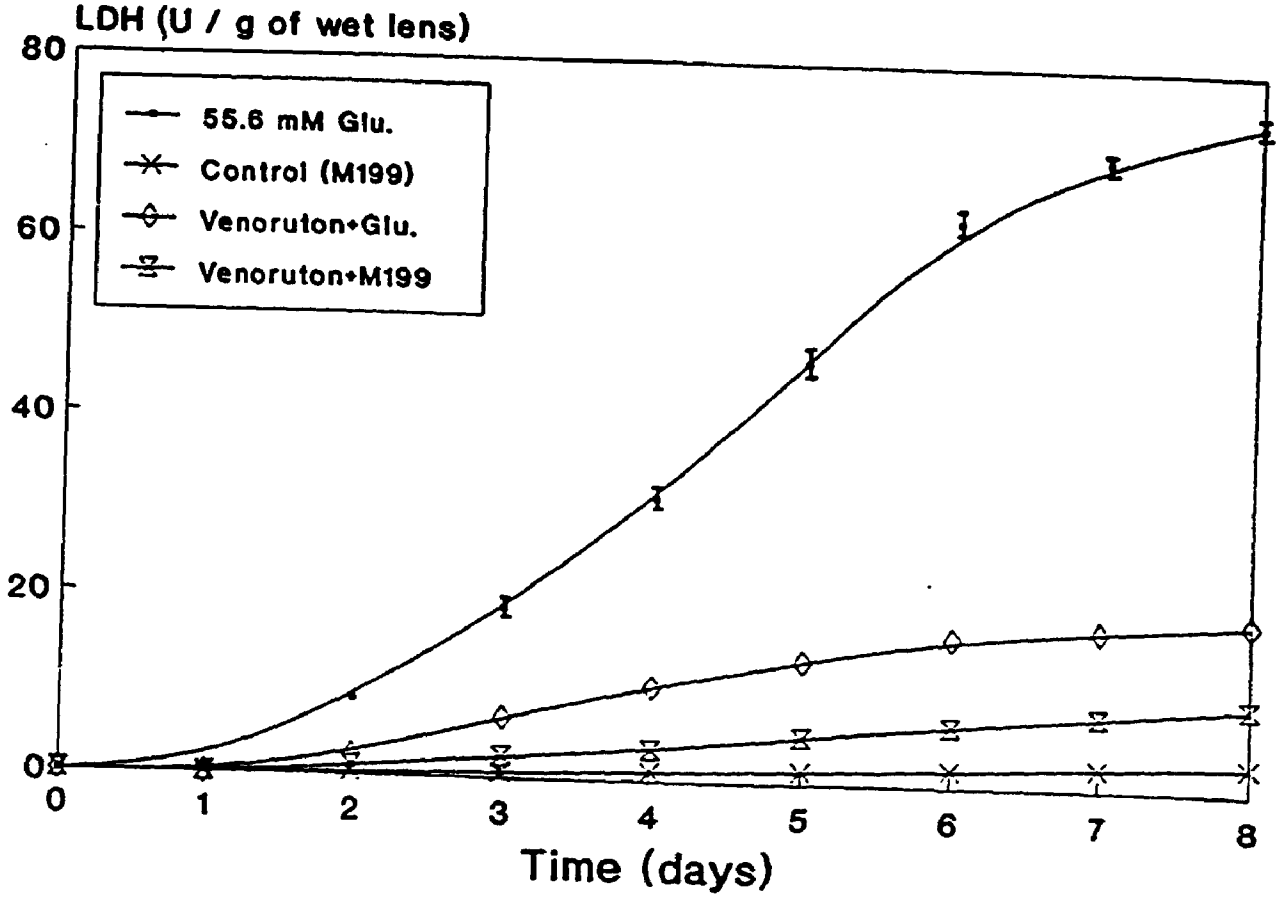
The LDH activity in the medium and the leakage per gram of lens was calculated as described in Materials and Methods. Data (means ± SE (n=3) and percent SE) are shown for each separate period of culture and culture condition. When the SE is lesser in magnitude than the dimensions of the point on the graph it is not shown.

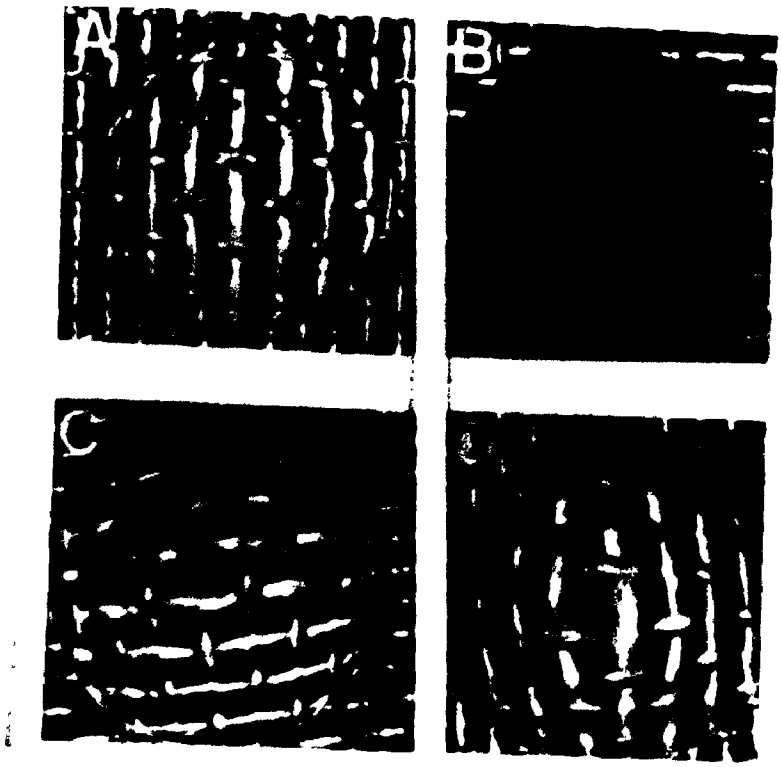
	Glucose-treatment		Control (M199)	
Day	LDH U/g lens ± SE	%SE	LDH U/g lens ± SE	%SE
1	0.750 ± 0.010	1.33	0.150 ± 0.000	0.00
2	8.497 ± 0.222	2.61	0.304 ± 0.005	1.79
3	18.973 ± 1.104	5.81	0.670 ± 0.036	5.04
4	31.403 ± 1.528	4.86	1.049 ± 0.027	2.58
5	46.967 ± 1.973	4.20	1.428 ± 0.021	1.50
6	62.913 ± 1.507	2.39	1.908 ± 0.057	2.99
7	69.930 ± 1.189	1.70	2.462 ± 0.080	3.28
8	74.397 ± 1.123	1.51	3.017 ± 0.106	3.52

Venoruton + M199		Venoruton + Glu.	
U/g lens ± SE	%SE	U/g lens ± SE	%SE
0.010 ± 0.000	0.00	0.010 ± 0.000	0.00
1.041 ± 0.019	1.86	2.234 ± 0.060	2.60
2.421 ± 0.045	1.86	6.703 ± 0.182	2.61
3.464 ± 0.064	1.86	10.471 ± 0.125	1.15
5.192 ± 0.096	1.86	13.657 ± 0.041	0.32
6.683 ± 0.063	0.93	16.483 ± 0.069	0.42
8.061 ± 0.078	0.96	17.662 ± 0.078	0.44
9.564 ± 0.097	1.02	18.833 ± 0.152	0.81

Panels show the lenses which were incubated 8 days in M199 containing 55.6 mM glucose with or without venoruton.

(A): control;
(B): 55.6 mM glucose;
(C): control and 1 mM venoruton;
(D): 55.6 mM glucose and 1 mM venoruton.


LDH (U / g of wet lens)
80
60
40
20
0
55.6 mM Glu.
Control (M199)
Venoruton+Glu.
Venoruton+M199
0
1
2
3
4
5
6
7
8
Time (days)


A
B
C

The optimum venoruton concentration was estimated as 1 mM, since lenses did not develop opacity at this concentration.

Our results show that lenses retained their normal appearance for 8 days in M199, either unsupplemented (Figure 3.11, Panel A) or with 1 mM venoruton (Panel C), when the medium was changed daily. Lenses were incubated in M199 with 55.6 mM glucose in the absence (Panel B) and the presence of 1 mM venoruton (Panel D). When 1 mM venoruton was added to the medium along with 55.6 mM glucose, lenses were strongly protected against the damage of glucose during 8 days of incubation.

In lenses exposed to elevated glucose there was a large decrease in the LDH leakage of the lenses which were incubated with venoruton. By regression analysis, the glucose-induced rate of LDH release was decreased from 27.26 ± 3.26 U/g/day ($p<0.00001$) to 18.75 ± 3.44 U/g/day ($p<0.0006$) when venoruton was added. This is a reduction of 68.75% in LDH release compared to that of glucose-treated lenses.

Anti-Cataract Agents : Taurine.

The effects of taurine on healthy and glucose-induced cataractous lenses were examined by using the same parameters: i) microscopic appearance of the lenses, and ii)

leakage of LDH into the culture medium and iii) the amino acid content (as a preliminary experiment). The amino acid analysis of lenses was only performed once. For this reason the graphics and the tables which present the results from amino acid analysis are not included in the thesis.

Initially the optimum concentration of taurine was estimated by incubating the lenses in M199 with or without 55.6 mM glucose along with the different concentrations of taurine; 1 mM, 5 mM, and 10 mM, for 8 days. Under all conditions lenses retained their normal appearance for 8 days, when the medium was changed daily.

When 5 mM taurine was added to M199 containing 55.6 mM glucose, the protection against the damage of glucose during 8 days of incubation was also determined in experiments summarized graphically in Figure 3.12. The cumulative leakage of LDH from the lens was reduced by the protective effect of taurine. By regression analysis, the glucose-induced rate of LDH release is 27.32 ± 5.0 U/g/day, ($p<0.00001$). Addition of taurine reduced this value by 25.27 ± 3.52 U/g/day, ($p<0.00001$). This is a reduction of 92.5% in the release rate during days 1-6, for which LDH release was linear by inspection of the graphs.

FIGURE 3.12
EFFECT OF TAURINE IN THE LENS INCUBATION MEDIUM ON LDH RELEASE OF LENSES IN ELEVATED GLUCOSE.

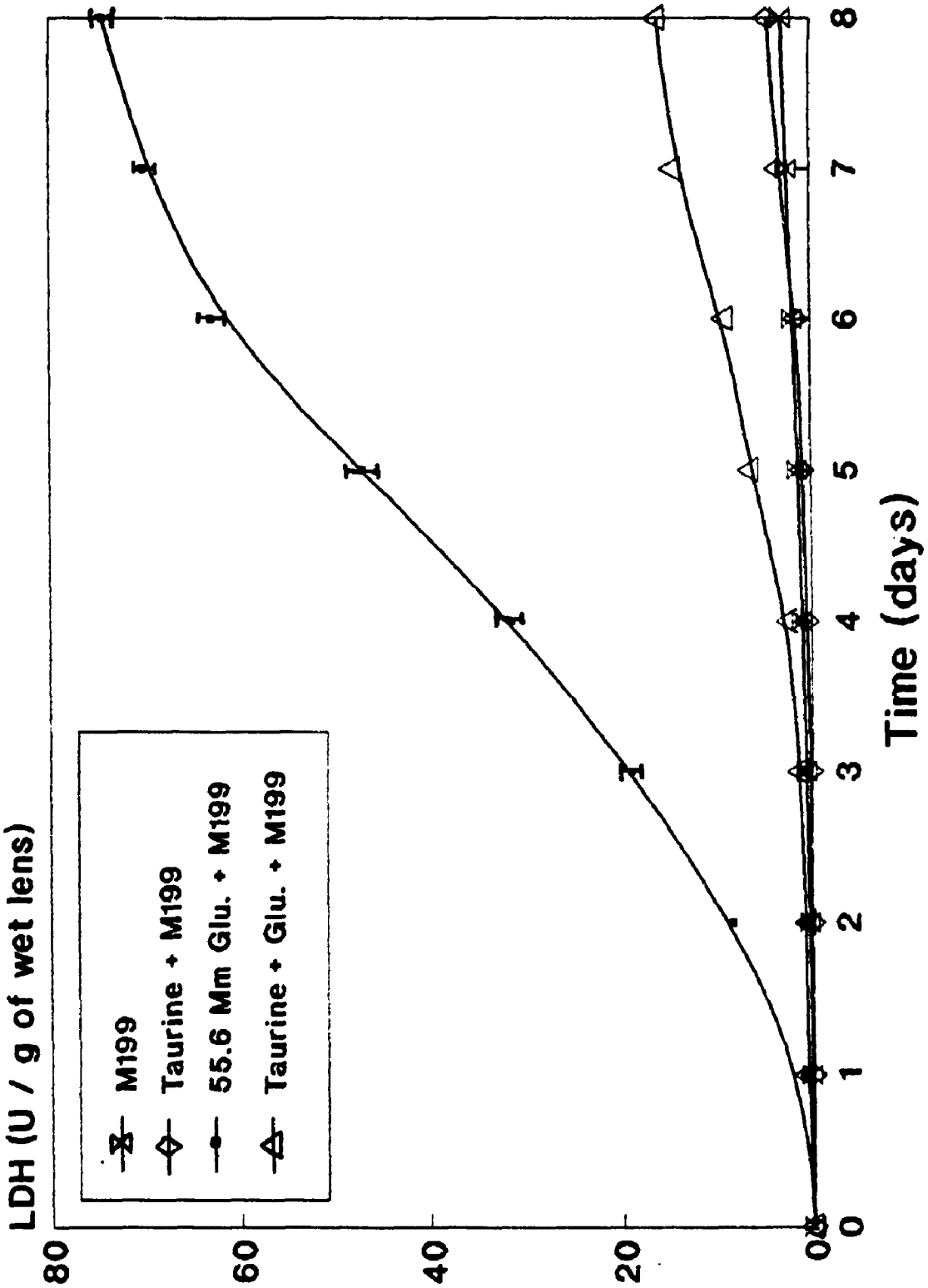
The effects of taurine on healthy and glucose-induced cataractous lenses were examined by measurement of leaked LDH into the culture medium. After 24 hrs preincubation in 1.5 ml M199, lenses were transferred to fresh M199 containing:

- control (M199);
- 5 mM taurine in M199;
- 55.6 mM glucose;
- 55.6 mM glucose and 5 mM taurine.

The LDH activity in the medium and the leakage per gram of lens was calculated as described in Materials and Methods. Data (means ± SE (n=3) and percent SE) are shown for each separate period of culture and culture condition. When the SE is lesser in magnitude than the dimensions of the point on the graph it is not shown.

	Glucose-treatment		Control (M199)	
Day	LDH U/g lens ± SE	%SE	LDH U/g lens ± SE	%SE
1	0.750 ± 0.010	1.33	0.150 ± 0.000	0.00
2	8.497 ± 0.222	2.61	0.304 ± 0.005	1.79
3	18.973 ± 1.104	5.81	0.670 ± 0.036	5.04
4	31.403 ± 1.528	4.86	1.049 ± 0.027	2.58
5	46.967 ± 1.973	4.20	1.428 ± 0.021	1.50
6	62.913 ± 1.507	2.39	1.908 ± 0.057	2.99
7	69.930 ± 1.189	1.70	2.462 ± 0.080	3.28
8	74.397 ± 1.123	1.51	3.017 ± 0.106	3.52

Taurine + M199		Taurine + Glucose	
U/g lens ± SE	%SE	U/g lens ± SE	%SE
0.000 ± 0.000	0.00	0.750 ± 0.010	1.33
0.016 ± 0.000	0.00	0.663 ± 0.051	7.69
0.216 ± 0.023	10.65	1.385 ± 0.096	6.93
0.553 ± 0.082	14.87	2.525 ± 0.156	6.18
0.938 ± 0.074	7.94	4.474 ± 0.313	7.00
1.466 ± 0.113	7.68	9.075 ± 0.460	5.07
3.318 ± 0.252	7.59	14.371 ± 0.306	2.13
5.170 ± 0.401	7.76	17.019 ± 0.636	3.74


LDH (U / g of wet lens)
80
60
40
20
0
0
1
2
3
4
5
6
7
8
Time (days)
M199
Taurine + M199
55.6 Mm Glu. + M199
Taurine + Glu. + M199

3.4 DISCUSSION

Generally the lens uses glucose as the major source of energy, but nevertheless, since it is an aldehyde it may still have various toxic side effects in the cell depending on its concentration, including nonenzymatic glycation and associated oxidative stress. During cataract formation accumulation of covalently attached sugar molecules to protein amino groups continuously damages lens membranes (Wolff and Dean, 1987). This damage could be the result of oxidative changes (Varma et al., 1977, 1984; Zigler and Goosey, 1984; Bhuyan and Bhuyan, 1984; Jernigan and Laranang, 1984; Ross et al., 1982, 1983; Garner et al., 1983) either directly (glycation-induced), or due to a decreased amount of tissue antioxidants such as glutathione (Mitton et al., 1993) or due to both. In the previous studies in the rat streptozotocin diabetes model of cataract, (Mitton and Trevithick, 1993; Mitton et al., 1993) glutathione falls significantly (66%) after only one week of diabetes, well before any morphological abnormalities such as cortical vacuoles can be seen. This decline continues as the morphological abnormalities appear, indicating that they may be causally related.

Antioxidant agents may stabilize the lens cells against loss of these critical components, and thus prevent damage leading to lens protein leakage, delaying cell death, and thus the onset of the subsequent opacification of the lens

(Trevithick et al., 1993; Mitton et al., 1993). Antioxidants may also prevent lipid peroxidation and resulting cell membrane damage (Zigler and Hess, 1985; Van Den Berg et al., 1989).

These data indicate that in vivo leakage of γ-crystallin into aqueous and vitreous humours is prevented by the dietary regimen of vitamins C and E and β-carotene, consistent with the hypothesis that antioxidant mixtures can reduce the lens membrane damage which is causing the leakage of intracellular components.

Mousa et al., (1979) previously showed that CD caused globular degeneration in the subcapsular cortex reversibly up to 2 hrs of in vitro lens incubation, after which time the globular degeneration became progressively less reversible. Even though CD is a reversible inhibitor, the extent of the globular degeneration and associated opacity increases progressively after the initial 2 hr period, suggesting that: i) further steps in the process of cataract formation, which could be oxidative damage, are initiated by CD; or, ii) CD damage over 2 hr could be overcome by the cell's natural defence system; or, iii) two hr CD-treatment does not disrupt the actin-cytoskeletal interaction enough to damage the membrane integrity. The data which were reported here are consistent with the hypothesis that oxidative stress is associated with the disorganization of actin and the cytoskeleton during globular degeneration,

but in the later stages the effects of CD and glucose cause the same end effect: cataractogenesis.

Studies of the effects of antioxidants indicate that different antioxidants can interact: i) glutathione can help to spare ascorbate in the buthionine sulfoxamine cataract model (Mårtensson and Meister, 1991), ii) in cultured hepatocytes tocopherol protects cellular glutathione concentrations (Glascott et al., 1992), and iii) ascorbate can regenerate tocopherol from the tocopheroxy radical (Niki et al., 1989; Packer et al., 1979; Yokoyama et al., 1994). Thus, a mixture of antioxidants may have particular benefits in prevention of cataract.

Even though in vivo studies with the individual dietary vitamins during the streptozotocin diabetic cataract showed protection against the lens damage, the in vitro combined vitamin protection was not as effective in reducing γ-crystallin leakage, perhaps because the ratio of concentration of the components was different.

The α-lipoic acid findings reported here indicate that lens opacities formed during incubation with high levels of glucose have an oxidative component which may contribute to the development of diabetic cataract. Dihydrolipoate formed in the lens from exogenous α-lipoic acid may act to protect oxidation-sensitive cellular components from damage, either by scavenging reactive oxygen species themselves, or by protecting reactive sulfhydryls in enzymes such as Na+/K+-

ATPase and glyceraldehyde-3-phosphate-dehydrogenase. The direct antioxidant effects of dihydrolipoate have been extensively documented by Packer and co-workers (Packer et al., 1995). The reduced form of α-lipoic acid, dihydrolipoic acid, has been shown to be a potent antioxidant, scavenging hydroxyl, superoxide, and peroxyl radicals, as well as singlet oxygen (Suzuki et al., 1991; Packer et al., 1995). In addition, it acts to regenerate antioxidants such as ascorbic acid from dehydroascorbate (Kagan, et al.,1992). This in turn may enhance the recycling of vitamin E (Packer, 1992). Antioxidants can exhibit recycling since they are interrelated by redox couples to each other as reported by Niki et al., (1982, Packer, 1992a,b), leading Packer to postulate the existence of a vitamin E cycle which maintains a pool of protective antioxidants. Thus lipoic acid may function as an antioxidant in several ways, both directly and indirectly.

The protective effect of lipoic acid in this model was highly stereospecific, with the R-enantiomer highly effective, the S-enantiomer significantly potentiating the damage, and the racemate providing partial protection. Our collaborative experiments with Dr. Packer's laboratory show that the whole lens reduces both lipoic acid enantiomers to dihydrolipoate with comparable efficiency. Hence, if the protective effects of lipoic acid supplementation are due to its reduction to dihyrolipoate (as seems likely), these

protective effects must be confined to relatively small, localized regions of the lens where the R-enantiomer is preferentially reduced.

The mitochondrial enzyme lipoamide dehydrogenase is almost completely specific to the R-enantiomer of lipoic acid (Pick et al., 1994). The findings reported here suggest that a region of the lens that is especially rich in mitochondria, such as the equatorial subcapsular cortex, may play a critical role in the etiology of high-glucose cataract. These cells might be especially sensitive to oxidant stress, and therefore benefit from additional dihydrolipoate formed in the mitochondria. Benefits to the cells might also occur via stimulation of ATP formation, since exogenous R-lipoic acid (but not S-lipoic acid) could stimulate oxidative pathways in the mitochondria.

The depletion of lens GSH during incubation with high glucose (Mitton et al., 1993) suggests a novel mechanism for the protective effects of exogenous R-lipoic acid. Dihydrolipoate can directly convert GSSG to GSH (Jocelyn, 1967). This would be of special importance if NADPH levels in lens tissues were to be depleted by glucose-driven stimulation of the aldose reductase pathway (De Mattia et al., 1994) thereby depriving the cell of the NADPH needed to maintain GSH in the reduced form. This possibility is highlighted by the fact that substantial lipoate-reduction in mitochondria-rich-tissues can occur by pathways

independent of NADPH formation.

Our results clearly show the preventive in vitro effect of venoruton in the glucose-induced cataract model system. This novel mixture of derivatives of the flavonoid rutin seems to protects the lens cell membrane against cataract damage. Venoruton may work either as an antioxidant or as an aldose reductase inhibitor or as both. When the results from the venoruton study are compared with the results of the VC study the leakage of LDH into the incubation medium was much less with VC than venoruton. Also, at the end of 8 incubation days, lenses protected with VC in the glucose-induced cataract model study appeared much clearer than with venoruton under the dissection microscope. Since venoruton has a chemical structure similar to many common antioxidants, we presumed that it might have an antioxidant role. If our hypothesis is true, then the preventive effect of venoruton on glucose-induced cataractous rat lenses may reflect a free-radical scavenging mechanism. Wolff and Dean, (1987) have suggested that the formation of cataracts of the lens by glucose stress might involve an increased production of reactive oxygen species. Increased levels of reactive oxygen species, whether due to an elevated rate of production, or to decreased antioxidant concentration (Wolff and Dean, 1987; Cohen 1986; Mitton et al., 1993), may contribute to damage and eventually death of cells.

Just as many studies defined the role of aldose

reductase in cataractogenesis (Berman, 1991; Kinoshita et al., 1976; Wolff and Dean, 1987), much evidence has already shown the effect of flavonoids in inhibiting aldose reductase (Varma et al., 1975). In this study, neither the activity of venoruton as an aldose reductase inhibitor nor its antioxidant activity was measured. Venoruton is a mixture of derivatives of the flavonoid rutin (Wadworth and Faulds, 1992). It has been shown by Varma et al. (1975), that the preventive role of rutin in diabetic cataractous lenses was 95% with 0.1 mM, which was less than the amount we used in this study. Since this study is consistent with the hypothesis that venoruton protected the lens cell membranes against cataractous damage, it is likely that a portion of this effect is due to its inhibition of aldose reductase.

Prior to observable opacity, the concentration of taurine in the diabetic rat lenses was found to be lower than the normal level. How taurine acts in the lens is still uncertain but in white blood cells, such as neutrophils and immature mononuclear phagocytes, the protective effect of taurine as an antioxidant was postulated to be due to its scavenging of hypochlorite ion (Babior and Crowley, 1983). Hypochlorite oxidizes: i) amines to chloramines and ammonia to monochloroamine:

$$H_2O_2 + Cl^- \text{----->} H_2O + OCl^-$$

$$OCl^- + NH_4^+ \longrightarrow NH_2Cl + H_2O$$

or ii) amino acids to monochloroamines

$$\begin{array}{l} NH_2 \\ | \\ RCH\text{-}CO_2H \end{array} + OCl^- \longrightarrow \begin{array}{l} HNCl \\ | \\ R.CH - CO_2H \end{array}$$

which decarboxylate to form aldehydes --> $CO_2 + NH_4Cl$ + RCHO. Such aldehydes were suggested (Crabbe and Wolff, 1984) as susceptible to oxidation to free radicals, and so taken in the context of H_2O_2 production initially causing this, may result in a "chain" of oxidative reactions and free radical formation. Also, aldehydes could have a toxic effect on the lens cells. Taurine is postulated to be a scavenger of excess hypochlorite by preventing the reactions with amino acids from proceeding past the formation of chloramines to yield aldehydes. The <u>in vitro</u> protective effect of taurine is consistent with Linklater's <u>in vivo</u> study (1985).

CHAPTER 4

CALCIUM and IT'S POSSIBLE ROLE IN ORGANIZATION OF MEMBRANE SKELETAL PROTEINS DURING CATARACT

4.1 INTRODUCTION

Calcium, one of the major divalent cations present in the lens cell cytoplasm (Delamere and Paterson, 1981), has a direct role during cataract formation by changing the organization of cytoskeletal proteins in cortical lens cells (Harding et al., 1983; Cheng and Chylack, 1985; Rafferty et al., 1994; Duncan et al., 1994). It is known that elevation of the calcium level to 1.8 mM, or Ca-deficient incubation medium induces an anterior and posterior opacity in rabbit lenses (Cheng and Chylack, 1985). Elevated calcium also causes lens fiber fragmentation resembling globular degeneration (Fagerholm, 1979; Srivistava, 1994).

The concentration and/or distribution of this divalent ion should be constant but in a physiologically correct range to avoid cataract formation. Despite the ubiquitous participation of Ca^{2+} in cellular functions, few studies have attempted to characterize Ca^{2+} uptake in rat lenses. The following questions still remain unanswered : After what duration of cataract formation does the lens calcium uptake increase ? What is the immediate action of elevated calcium in the lens at the cellular level?

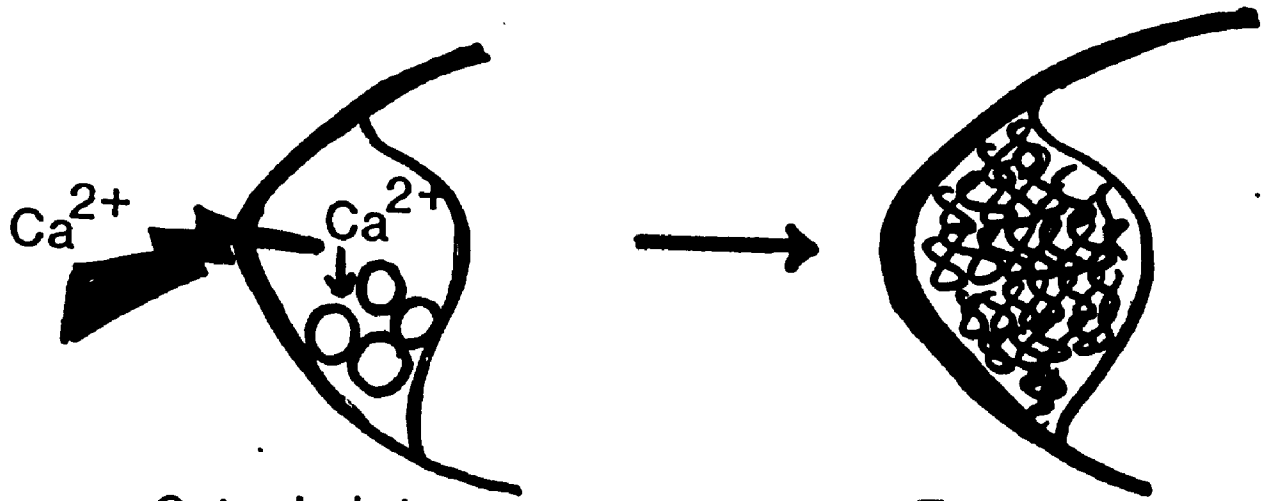
It may be postulated that during the precataractous

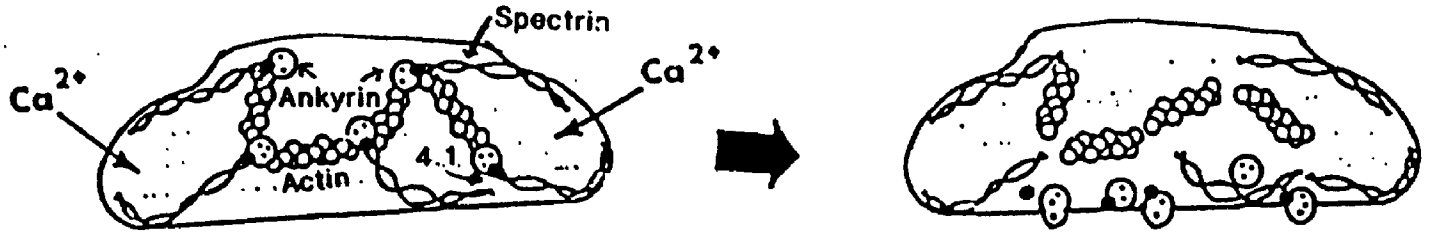
stage an increase of calcium concentration will activate calcium-dependent degradative processes, which mediate the loss of cellular viability (Rafferty et al., 1994; Duncan et al., 1994). Theses include activation of calcium-dependent neutral proteases with degradation of substrate proteins (David and Shearer, 1986; David et al., 1989) **(Figure 4.1)**, or disruption of the Ca-binding proteins (Bershadsky and Vasiliev, 1988; Roy et al., 1983; Ireland and Maisel, 1984), and activation of phospholipases and endonucleases (Bourguignon et al., 1988; Stryer, L., 1988). These activities, which are primarily responsible for cell killing, seem to be more specific for certain cell types, e.g., lens epithelial and fiber cells at the equatorial region (Creighton et al., 1980; Harding, J., 1991; Harding et al., 1982, 1983; Unakar et al., 1981(a), (b), 1978). In Figure 4.1 the possible immediate effect of calcium is proposed diagrammatically.

Intracellular free calcium has a central role in the activity of the calpain system. Calpain is a calcium-dependent neutral proteinase [EC.3.4.22.17] and can be inhibited by a protein, calpastatin. Slightly different calcium concentrations are required for proteolytic activity of calpain, for autolysis of calpain, or for calpain/calpastatin interaction. Lens calpain has been measured enzymatically (Yoshida et al., 1985; David and Shearer, 1986). Calpain was mostly found in the lens

FIGURE 4.1
THE IMMEDIATE EFFECT OF CALCIUM DURING CATARACT FORMATION.

In the cataractous lens the calcium concentration was found to be elevated. The highest concentration of calcium occurred at the lens equator. Initially, the proposed effect of calcium in the lens cells should be on the cytoskeletal network. A high calcium concentration will cause the dissociation of lens spectrin, ankyrin and protein 4.1. These changes result in the formation of blebs in the lens cells which causes the loss of transparency.


Ca^{2+}
Ca^{2+}
Cytoskeleton disruption
Equatorial globular degeneration


Spectrin
Ca^{2+}
Ankyrin
Ca^{2+}
4.1
Actin
A
B

epithelial cells and cortex cytosol. A smaller concentration was found in differentiating equatorial cells, associated with the plasma membrane (Anderson and Shearer, 1990; Yoshida et al., 1985). In the central lens nucleus calpain activity was still detectable but very low in old animals (David et al., 1989; Varnum et al., 1989).

What does calpain do in the lens? Does it initiate cataract formation? If it does, at what stage of cataract? Murachi (1989) has grouped the possible substrates of the calpains into three categories: i) cytoskeletal proteins, especially those involved in cytoskeletal/membrane interaction, ii) hormone receptors, and iii) enzymes, especially kinases and phosphatases. Calpain has been shown to be capable of degrading many of the diverse protein subunits of the eukaryotic cytoskeleton (Bershadsky and Vasiliev, 1988; Schliwa, 1986; Rousset, 1988). Functional elements of the cytoskeleton permeate the entire cell and are involved in cell attachment, cell movement, endocytotic processes, exocytosis, the intracellular transport of macromolecules and organelles, as well as the localization and stabilization of macromolecules and cell organelles (Zimmerman and Schlaepfer, 1984; Traub, 1985).

Lens calpain was tested for endogenous substrate specificity. α-Crystallin (Yoshida et al., 1984) and β-crystallin (David et al., 1993) but not γ-crystallin (David and Shearer, 1986) were shown to undergo limited cleavage by

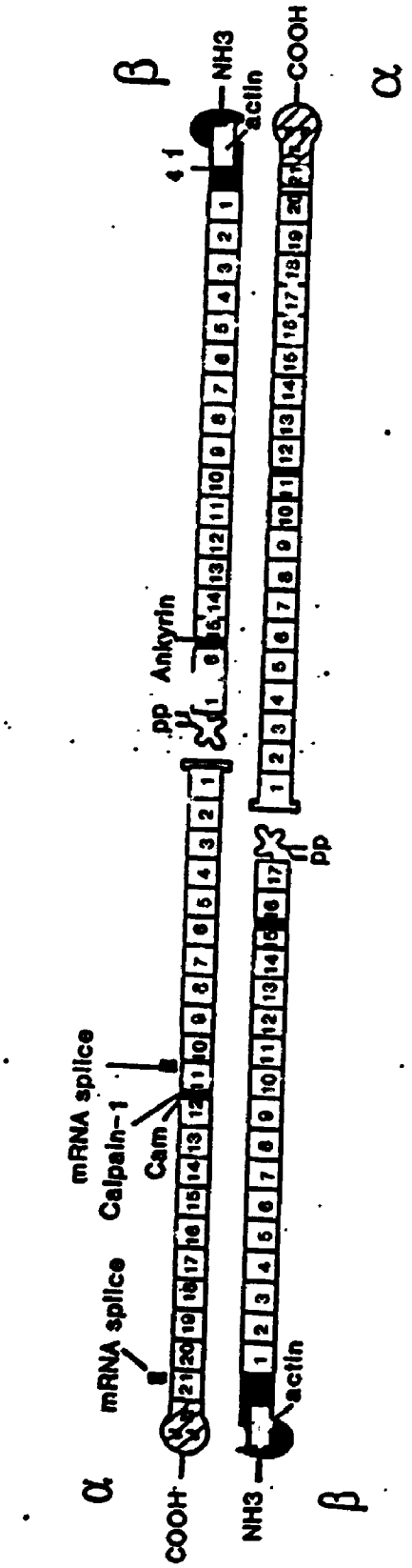
calpain. Lens cytoskeletal and membrane proteins also serve as calpain substrates. Unlike crystallin hydrolysis, the degradation of actin, vimentin and spectrin/fodrin was not limited (David and Shearer, 1986; Roy et al., 1983; Yoshida et al., 1984). Among the various substrate proteins for calpain, spectrin/fodrin has attracted a number of researchers (Truscott et al., 1990; Marcantonio and Duncan, 1991) who are interested in the transparency of the lens. This major cytoskeletal protein was the first to be described to undergo calpain-catalyzed proteolysis in different tissues. Sobue et al., (1988) reported that tumor promoters induce the reorganization of actin filaments and spectrin/fodrin; Saido, et al., (1993) showed the proteolysis and spatial resolution of fodrin in postischemic brain (Seubert et al., 1990; Nixon, 1986). But in the lens, whether proteolysis of spectrin/fodrin precedes cataract formation, or whether it is a consequence of pre-cataractous changes still remains controversial. For this reason it will be very important in completing the order of events in the pathway of cataract formation to investigate how the lens cytosolic proteins, which are the endogenous substrates of calcium-dependent neutral protease, are involved in cataract formation. Because of this the time course of proteolysis of spectrin/fodrin was studied in two cataract model systems.

Fodrin is a rod-shaped protein that lines the cortical

cytoplasm of lens cells. Fodrin shares a number of properties with spectrin, the principal component of the erythrocyte cytoskeleton: i) both proteins are comprised of two subunits (α and β) and likely exist in vivo as tetramers $(\alpha\beta)_2$ (Bennett, 1985; Glenney et al., 1982a), ii) both can bind actin and cross-link F-actin (Aster et al., 1986), iii) the higher molecular weight subunits of each bind calmodulin (each molecule of calmodulin contains four binding sites for calcium) and give similar peptide maps (Figure 4.2) (Glenney et al., 1982a; Carlin et al., 1983; Palfrey et al., 1982; Aster et al., 1984; Harris and Morrow, 1990), and iv) antisera raised against fodrin show some cross-reactivity with spectrin (Glenney et al., 1982b; Burridge et al., 1982). Additionally, fodrin (Levine and Willard, 1981; Goodman et al., 1981) and spectrin (Repasky et al., 1982) antibodies recognize proteins from a variety of tissues, suggesting that fodrin-like proteins are widely distributed among different cell types (Branton et al., 1981). It has been suggested that fodrin, in a manner similar to erythrocyte spectrin, may regulate cell shape and the position and lateral mobility of cell surface proteins (Burridge et al., 1982). However, the precise functions of fodrin and related proteins and the factors that regulate them in the lens during cataract formation are largely unknown. In the lens both α-, β-spectrin and α-fodrin were identified (Yoon et al., 1989; Aster et al., 1984) and

FIGURE 4.2
FEATURE OF SPECTRIN/FODRIN SEQUENCE.

The polypeptide chain is represented as a line running from the amino- to carboxyl-terminus. Some of the features of the sequence are marked.


α
β
COOH
NH3
mRNA splice
Calpain-1
Cam
mRNA splice
actin
pp
Ankyrin
pp
β
α
NH3
COOH
actin

calcium-dependent binding of calmodulin and lens spectrin/fodrin (Aster et al., 1986) was shown.

Calpain in lenses may be regulated by an endogenous inhibitor, calpastatin. The calpastatin to calpain ratio in rat lens was found to be low enough to detect calpain activity in crude lens homogenate (David and Shearer, 1984) but in human lens the amount of calpastatin exceeds calpain (David et al., 1989). Calpain may also be involved as a causative factor in diseases such as muscular dystrophy (Turner et al., 1988), heart and brain ischemia (Seubert et al., 1989) and Alzheimer's disease (Siman et al., 1990).

Most of the literature concerning the mechanism of cataract formation is concerned with the function of calcium and calcium-dependent proteolysis in the lens during cataract formation. To date, the timing of calcium uptake by the precataractous diabetic lens has not been well defined but some insights have emerged from studies of the increased calcium concentration in cataractous lenses. The rationale of this chapter is initially to show the increase of calcium concentration with cataract formation. In this section, the intracellular free calcium (Ca^{2+}) uptake and movement in pre-cataractous rat lenses was investigated by fluorescent and radioactive labelling techniques. The results should enable us to deduce the specificity of this intracellular divalent cation, to gain a clearer understanding of its mechanism of action and to order the

events occuring during cataract formation. Also, this in turn led to my study of calpain in the *in vitro* diabetic cataract and its immediate substrate. Finally the effect of a calpain inhibitor peptide (Sigma C-9181, CIP) was studied in rat lenses during *in vitro* diabetic cataract formation.

4.2 MATERIALS AND METHODS

REAGENTS: All chemicals were obtained from Sigma, Canlab (Toronto, Ontario), and/or Fisher and were reagent grade or the highest purity available. Ca-green-AM (as the potassium salt) was purchased from Molecular Probes Inc., Eugene, Oregon, and ^{45}Ca was from ICN, Radiochemicals, Irvine, CA. Anti-fodrin-antibody was supplied from ICN (Cat.No: 69-327-1). The calpain inhibitor peptide was obtained from Sigma (C-9181, amino acid sequence: Asp-Pro-Met-Ser-Ser-Thr-Tyr-Ile-Glu-Glu-Leu-Gly-Lys-Arg-Glu-Val-Thr-Ile-Ile-Pro-Lys-Tyr-Arg-Glu-Leu-Leu-Ala).

4.2.1 ^{45}Ca Uptake and Movement Into the Incubated Rat Lenses:

Movement of ^{45}Ca was estimated in the normal and/or glucose-treated lenses during the first 48 hrs as described below. Lenses were incubated in incubation medium M199 or M199 with 55.6 mM glucose added; each contained 1 µCi ^{45}Ca, and incubations were for 0, 24 and 48 hrs. At the end of the incubation time lenses were rinsed rapidly with nonradioactive M199 to remove excess external ^{45}Ca, then frozen in liquid nitrogen and cut into two halves. Each half was freeze-dried and the movement of radioactive calcium into the incubated lenses was made visible by exposing the flat cut surface on a X-ray film by autoradiography. For ^{45}Ca uptake measurements, lenses were

incubated as described above in the radioactive solution, and at the end of each incubation period lenses were rinsed rapidly with nonradioactive solution to remove excess superficial ^{45}Ca, then homogenized with a rotating knife homogenizer (Ultra-Turrax, TeKmar, Cincinnati, Ohio) in 2 ml of 10% (w/v) trichloroacetic acid (TCA). After removal of the proteins by centrifugation, radioactivity in the 0.2 ml aliquots of the supernatant was counted, each in 10 ml of Ready Solv (HP/b, Beckman, Palo Alto, CA) with a Beckman LS 9800 liquid scintillation spectrometer.

4.2.2 Preparation of Lenses for Confocal Microscopy -

Previously prepared calcium-green (at a concentration of 0.138 mM stock) (Eberhard and Erne, 1991) was added into 1.5 ml culture medium to give a final concentration of 9.17 µM. After 0, 24 and 48 hrs incubation, lenses were monitored for confocal images described in chapter 2.2.8.

4.2.3 The Effect of VC and EGTA on Lens Calcium Uptake -

Just prior to starting the incubations, ascorbic acid was added as described in chapter 3.2, or 3.6 M EGTA was added to culture medium containing 55.6 mM Glucose and 9.17 µM Ca-green, to give a final concentration of 1 mM or 3.6 mM respectively. Lenses were scanned by confocal microscopy at 0, 24 and 48 hrs of incubation. The effects of VC and EGTA on lens LDH leakage was determined and compared to untreated

concurrent controls.

The level of VC in the incubation medium was also analyzed by the method of Jagota and Dani (1982) as described in Chapter 3.

4.2.4 Spectrin/Fodrin in the Cataractous Lenses :

Healthy and 55.6 mM glucose-incubated rat lenses were homogenized in 2 ml Laemmli 3X sample buffer (at boiling temperature 90 °C) which had 0.1 mM leupeptin added, at the end of each incubation day (0-8 days). After centrifugation at 37,000xg (Aster et al., 1984), 20 µl extract from each sequentially obtained daily sample was subjected to SDS-PAGE for 45 min at 200 mA. The gel concentration was 10%. Then the western blotting procedure described in chapter 2.2.6.1.2. was followed. 1:1500 diluted monoclonal-anti-α-fodrin antibody (Ylikoski et al., 1990) was used to probe the proteins which were transferred to nitrocellulose.

4.2.5 Effect of Calpastatin on In Vitro Diabetic Rat Lenses:

A synthetic oligopeptide corresponding to an exon of the human calpastatin gene (Maki et al., 1989) was supplied by Sigma. In this study it is referred to as calpain inhibitor peptide (CIP). From the previously prepared stock CIP (7.97×10^{-4} M) 19 µl was added into either control M199 or glucose-containing M199. Rat lenses with intact capsules

were incubated in this culture medium for 8 days. Every day one lens from M199 and one lens from glucose-M199 was homogenized. At the end of 8 days, samples from extracts of glucose-M199 and M199 incubated lenses were analysed by SDS-PAGE and Western blotting.

4.3 RESULTS

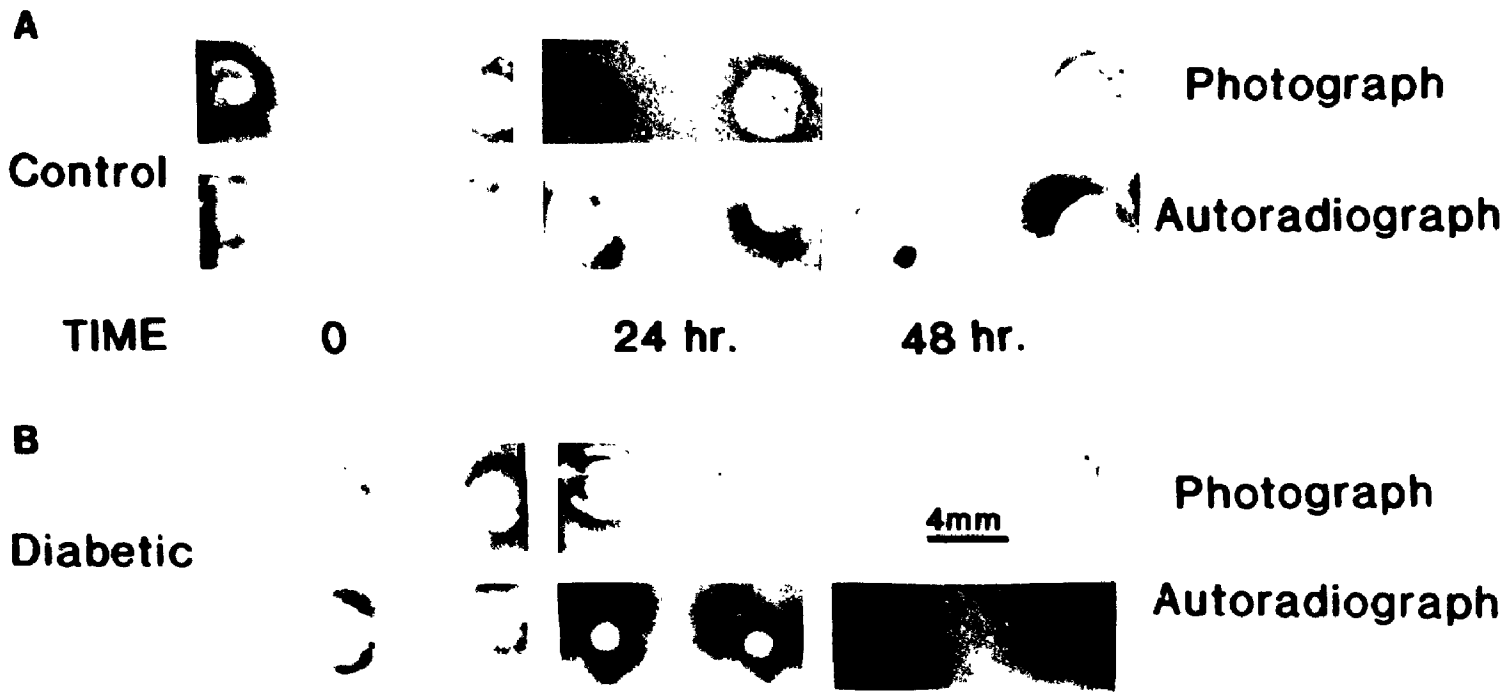
Calcium Uptake and Movement :

The combination of a fluorescence digital imaging system with radioactive calcium, ^{45}Ca, permits the rapid movement of this divalent cation into healthy and glucose-treated rat lenses to be followed.

In this section the calcium uptake was studied with a radioactive labelling technique. The effect of glucose on calcium movement was compared with the control lenses. Lenses were incubated in M199 containing ^{45}Ca following the procedure described in the Materials and Methods section (4.2.2). The visualization of radioactive calcium movement into the incubated lenses is shown in **Figure 4.3.** The first row of each of panels A and B are the pictures of lyophilized lenses under the dissection microscope. In the second row of panels are the autoradiography pictures of the same lenses. In glucose-treated lenses the accumulation of radioactivity increased with time around the equatorial area. The difference in the radioactively labelled area of glucose and control lenses could be seen at 0 time. When calcium ^{45}Ca was used to follow the movement of free calcium into control lens, for 0, 24 and 48 hrs of incubation, it appeared only in the intact capsule. In glucose-treated lenses the radioactive labelling after 48 hrs was more intensive than after 24 hrs or in control healthy lenses **(Figure 4.3, Panel A and B)** and strongly localized at the

FIGURE 4.3
RADIOACTIVE CALCIUM UPTAKE
BY CATARACTOUS AND HEALTHY LENS.

Lenses were incubated in 1 μCi ^{45}Ca with M199 either (control, Panel A) or glucose-induced medium (diabetic, Panel B) for 0, 24 and 48 hrs. At the end of the incubation time lenses were frozen and cut into two hemispherical pieces. Each piece was freeze-dried and the movement of radioactive calcium into the incubated lenses with time was visualized by exposing the flat cut surface on an X-Ray film by autoradiography.


A
Control
Photograph
Autoradiograph
TIME
0
24 hr.
48 hr.
B
Diabetic
4mm
Photograph
Autoradiograph

equatorial outer cortex of the lens (Figure 4.4).

As a continuation of this study, the uptake of ^{45}Ca by incubated healthy and glucose-treated lenses was determined by counting with a liquid scintillation spectrometer (Figure 4.5). The uptake of calcium also increased with increasing time. The radioactive labelling of control lenses did not increase with time as much as the glucose treated lenses. To confirm the radioactive labelling result, a calcium specific fluorescent dye, calcium-green, was also used.

Initially, to test the method a control lens was incubated in M199 containing 9.17 μM Calcium-green. At the end of 0, 24 and 48 hrs it was imaged on a TCLS Microscope. The calcium stained area appeared as a crescent with the maximum intensity (50 μA/V) at the lens equator (Figure 4.6 Panel A). The small fluorescently stained surface area did not increase with incubation time. The initial pattern of intracellular free calcium-staining did not show any increase or difference during further incubation under the usual control conditions (Figure 4.7, Panel A). By contrast, in elevated glucose (Panel B) the uptake and distribution of calcium changed in a time-dependent manner. These changes were most pronounced in cells around the equatorial region of the lens. This result was consistent with the results from the radioactive calcium (^{45}Ca)-incubated lens.

FIGURE 4.4
LOCALIZATION OF CALCIUM UPTAKE BY RADIOACTIVE LABELLING.

Calcium uptake of a lens which was incubated for 48 hrs in M199 containing glucose and 1 µCi ^{45}Ca: a magnified view of Figure 4.4. Panel B, 48 hr to show better the isotope localization. The photograph of the lyophilized lens is shown in Panel A, autoradiograph in Panel B and the localization on the schematic lens diagram Panel C.


A
1mm B

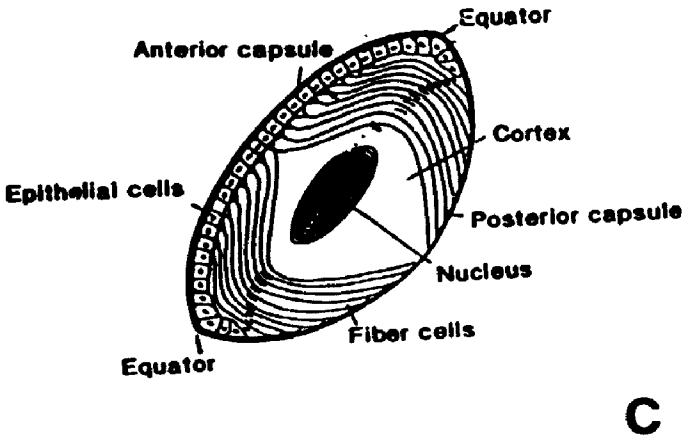

Equator
Anterior capsule
Cortex
Epithelial cells
Posterior capsule
Nucleus
Fiber cells
Equator
C

FIGURE 4.5
THE AMOUNT OF CALCIUM UPTAKE BY
HEALTHY AND CATARACTOUS LENSES.

Lenses were incubated with 1 µCi ^{45}Ca either in M199 (control) or medium containing 55.6 mM glucose (diabetic). At the end of the 0, 24 and 48 hrs they were homogenized, proteins were TCA-precipitated, and the amount of soluble radioactivity in the supernatant was counted with a liquid scintillation spectrometer. Data (means ± SE (n=3) and percent SE) are shown for each separate period of culture and culture condition. Error bars are not shown when the SE is less than 1 mm on the graph.

Time	Contro. (M199)		Glucose + M199	
	DPM/mg lens ± SE	%SE	DPM/mg lens ± SE	%SE
0 hr	148.509 ± 2.436	1.64	241.751 ± 10.014	4.14
24 hr	289.063 ± 3.524	1.22	658.991 ± 8.123	1.23
48 hr	373.468 ± 14.475	3.88	1000.326 ± 15.16	1.52

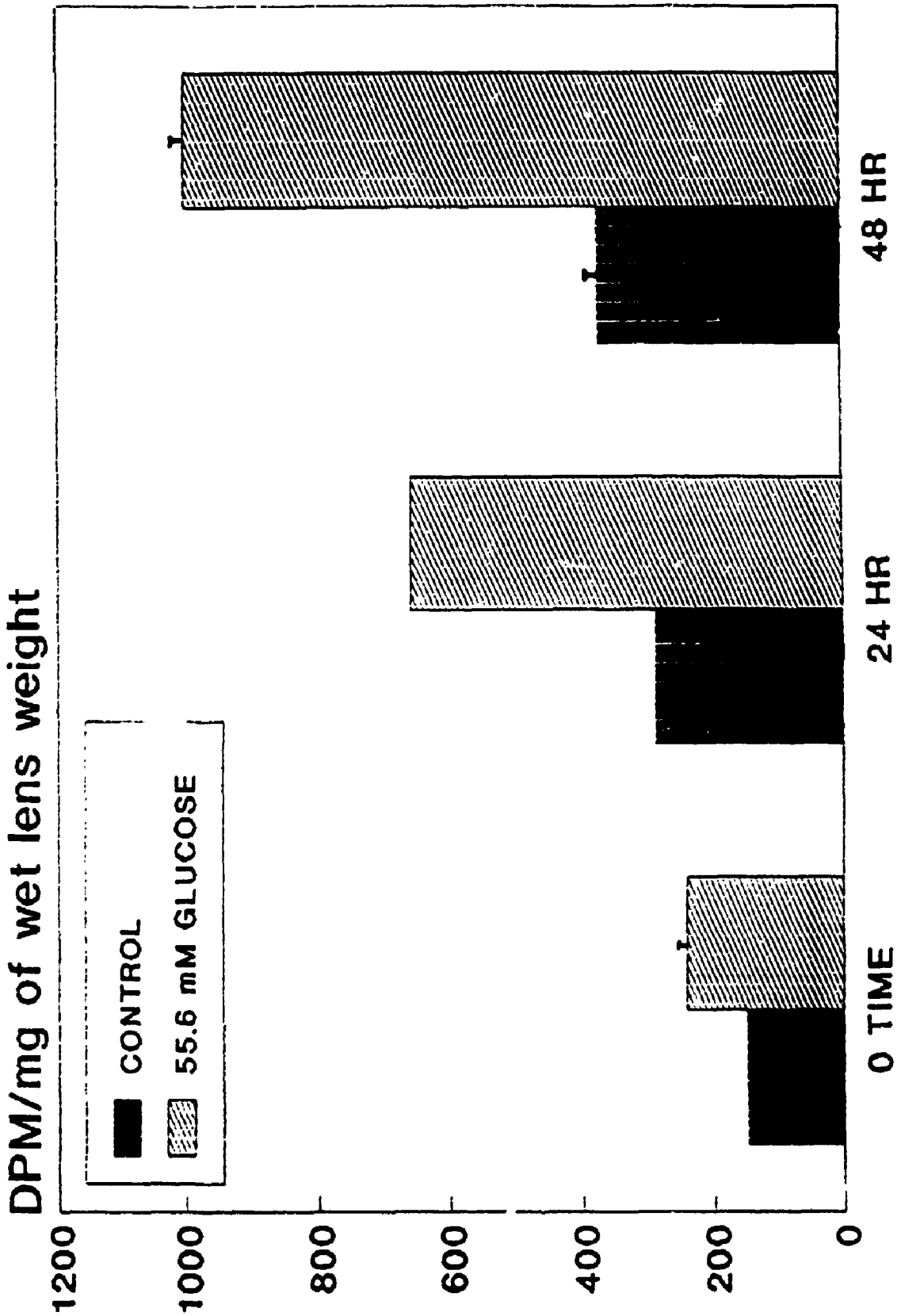
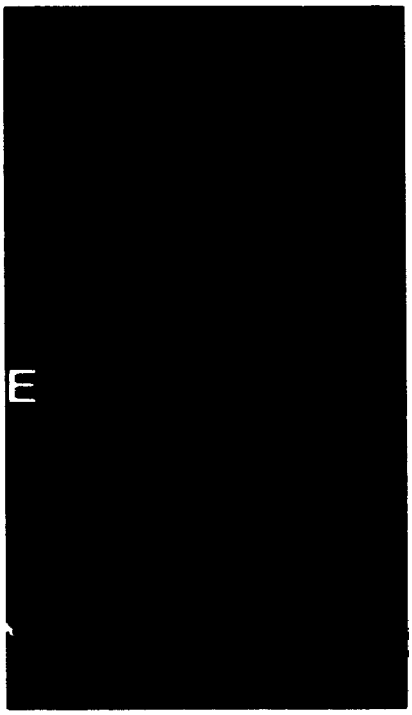

DPM/mg of wet lens weight
1200
1000
800
600
400
200
0
CONTROL
55.6 mM GLUCOSE
0 TIME
24 HR
48 HR

FIGURE 4.6
CALCIUM GREEN: CALCIUM SPECIFIC FLUORESCENT DYE.

Lenses incubated in M199 containing 9.17 µM calcium-green were transferred to a TCLSM to take images of the equator region at two magnifications: low magnification (1 cm = 750 µm x 3 = 2.25 mm) and high magnification (1 cm = 1.5 µm x 3 = 4.5 µm) in order to see the intracellular free calcium distribution.

Panel A: The appearance of the lens equatorial region (E) after 24 hr incubation in M199 containing calcium-green scanned with low light intensity (PMT was set at 200 µA/V) and low magnification. The upper level shows the xy and the lower level shows the xz images of the same region. In panel A (lower) the xz image is labelled to show the location of the equator (E). This was not appropriate for the upper part since the xy cross-section is taken through the equator.

Panel B : The same area in Panel A was imaged at a higher magnification and 50 µA/V light intensity. Upper level shows the xy images and the lower level of Panel B shows the xz image of the same area with high light intensity (50 µA/V).

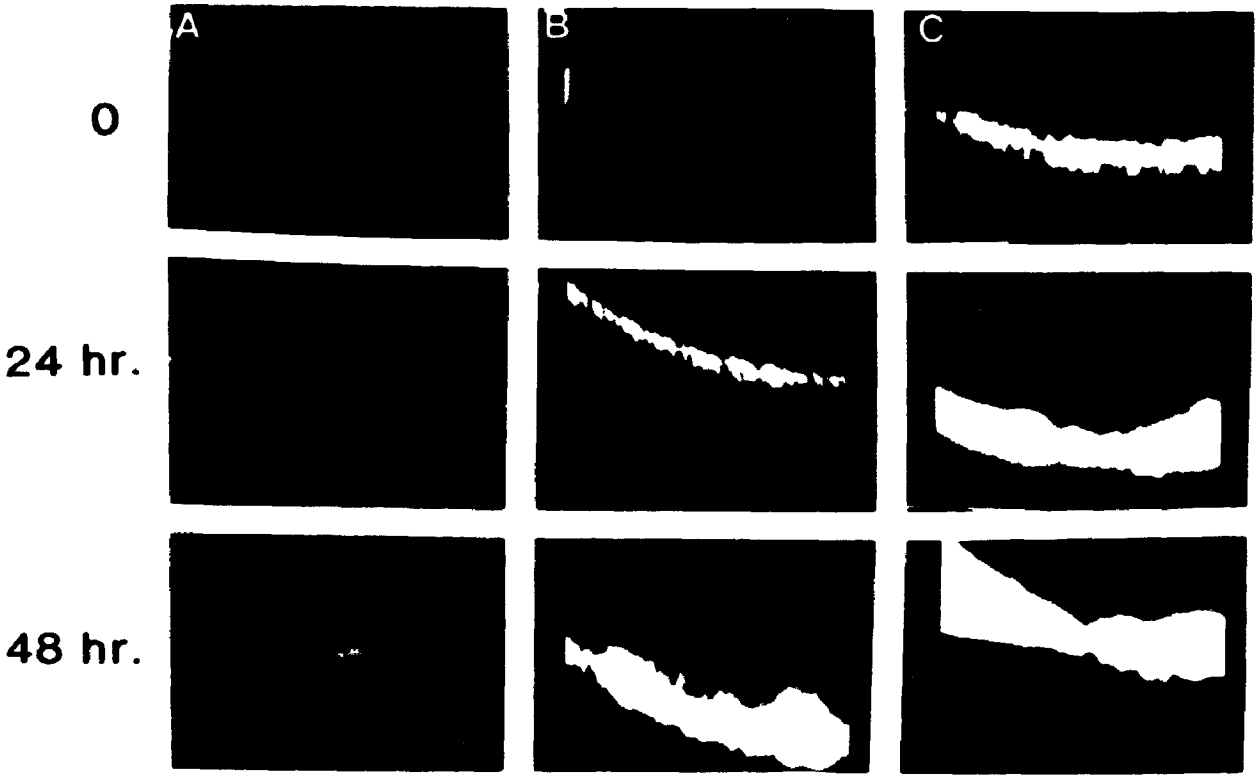


A

B

FIGURE 4.7
THE EFFECT OF CD AND GLUCOSE
ON CALCIUM UPTAKE. CONFOCAL STUDY.

The effect on lens calcium uptake of adding to M199 (Panel A, control) either 55.6 mM glucose (Panel B) or CD (Panel C) was tested by staining the intracellular free calcium with the calcium specific fluorescent calcium-green dye during 0, 24 hrs, and 48 hrs incubation. The PMT amplifier was set at 200 μA/V and the xz image was taken at a scale of 1 cm = 750 μm x 4 = 3.00 mm. The calcium uptake was affected earlier by CD than in the case of glucose. The fluorescently labelled area in the CD-incubated lens was much broader than that for the glucose-incubated lens.


A
B
C
0
24 hr.
48 hr.

The Effect of CD on Calcium Uptake :

By using the techniques described above, the time course of the effect of CD on lens calcium uptake was observed (Figure 4.7, Panel C).

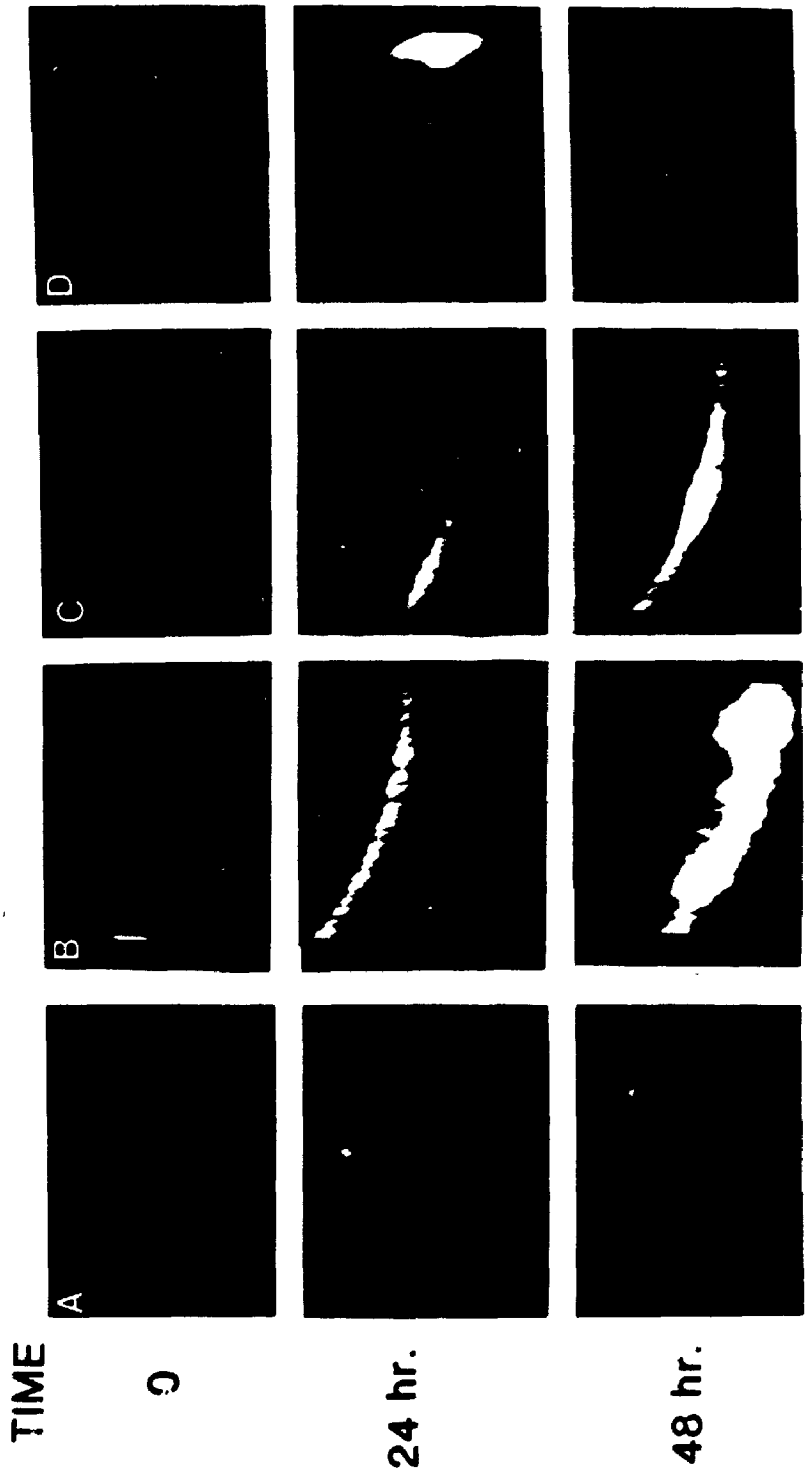
Lenses were incubated in medium M199 containing Ca-green and 10^{-5} M CD and examined after 0, 24 and 48 hrs. Pictures from imaging on a prototype TCLSM are illustrated in Figure 4.7, Panel C. When the results from the lenses incubated in control media, high glucose, and CD containing media are compared, the presence of glucose and CD can be seen to have enhanced the calcium uptake in a time-dependent manner. But the fluorescently labelled area was much larger in CD-treated lenses at a much earlier time. This indicates that the severe effect of CD on the lens cells also occurred earlier than that of glucose.

The Effect of VC and EGTA on Lens Calcium Uptake :

The preventive effect of VC on protein leakage by the glucose-treated lens was previously studied (Kilic and Trevithick, 1995) An antioxidant, VC protected the lens cell plasma membranes during cataract formation. When the membrane is damaged, it would be expected that the calcium uptake would be easier and/or quicker in the glucose-treated lenses (Figure 4.8 Panel B). To understand whether the preventive effect of VC is earlier than the calcium movement, we incubated lenses with M199 containing VC,

FIGURE 4.8
EGTA, VC AND CALCIUM UPTAKE.

Lenses were incubated in 9.17 µM calcium-green in M199 (Panel A) and M199 + 55.6 mM glucose (Panel B). After 0, 24, and 48 hrs lenses were transferred to a TCLSM to take images of the equator region. The effect on lens calcium uptake of adding to M199 a calcium specific divalent ion chelator (3.6 mM EGTA) (Panel C) or an antioxidant (1 mM VC) (Panel D) was tested, by staining as in Panel A and B. The PMT amplifier was set at 200 µA/V and the xz image was taken at a scale of 1 cm = 750 µm x 4 = 3.00 mm.


TIME
0
24 hr.
48 hr.
A
B
C
D

glucose and calcium-green for 0, 24 and 48 hrs (Panel D). The calcium movement into the VC protected lenses was prevented.

EGTA, which is a calcium specific chelator, was used to explore the relation between intracellular and medium calcium levels. Lenses were incubated in M199 with 55.6 mM glucose and 3.6 mM EGTA (Panel C). The fluorescent labelling results showed that calcium entrance into the incubated lenses was decreased.

The preventive effects of EGTA and VC on glucose-treated lenses during 48 hrs incubation were observed under the dissection microscope (Figure 4.9). The amount of LDH in the culture medium was also measured to confirm the effect of both agents during 48 hrs incubation.

Spectrin/Fodrin Degradation in Glucose- and CD-Treated Lens:

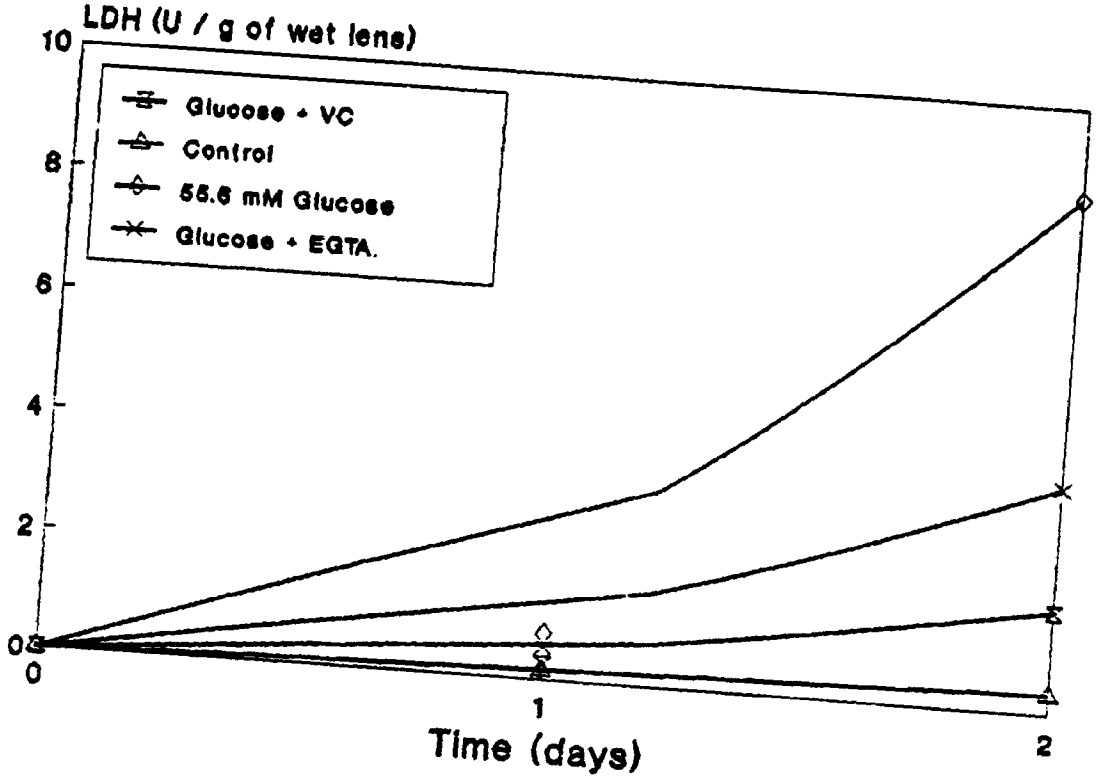
After the timing and localization of immediate calcium movement into the pre-cataractous rat lenses, one of the anticipated effects of calcium was that it would activate a neutral proteinase, calpain. For <u>in vitro</u> diabetic rat lenses, while developing opacity during the first 48 hrs, the measured LDH and the calcium uptake both increased. Extracts from lenses incubated in M199 for 8 days (prepared as described in section 4.2.4) were run on SDS-PAGE and transferred to nitrocellulose paper and both subunits of spectrin/fodrin visualized by immunostaining

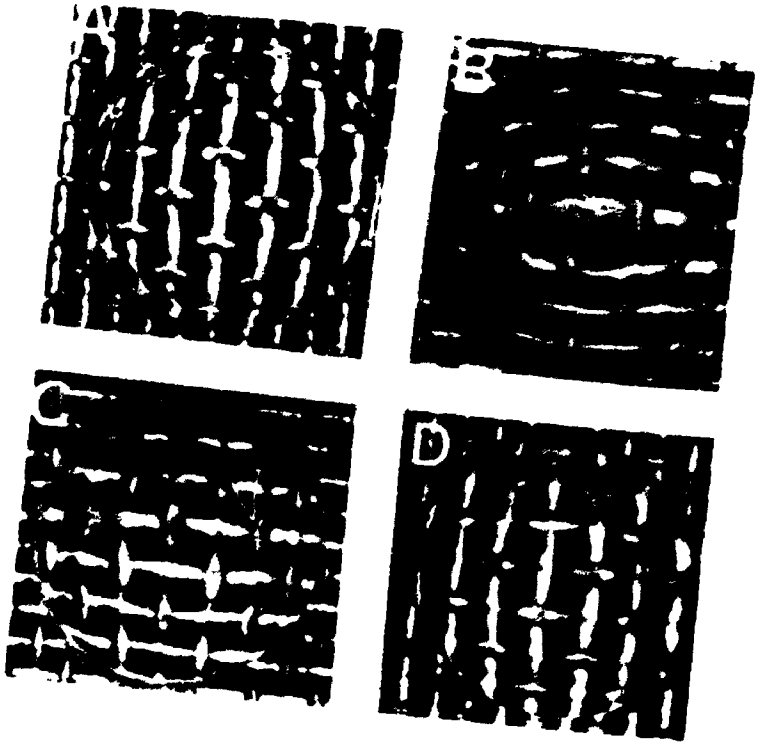
FIGURE 4.9
THE EFFECT OF EGTA AND VC ON LENS TRANSPARENCY.

After 24 hr preincubation in 1.5 ml M199, lenses were transferred to fresh M199 containing the following additives: (A) ⊸ 55.6 mM glucose and 1 mM VC;
(B) ⊸ control, no additions;
(C) ⊸ 55.6 mM glucose;
(D) ⊸ 55.6 mM glucose and 3.6 mM EGTA.
Data (means ± SE (n=3) and percent SE) are shown for each separate period of culture and culture condition. When the SE is lesser in magnitude than the dimensions of the point on the graph it is not shown.

hr	Glu.+ VC		M199		Glucose		Glu.+EGTA	
	U/g lens ± SE	%SE	U/g lens ± SE	%SE	U/g lens ± SE	%SE	U/g lens ± SE	%SE
0	0.00±0.00	0.00	0.00±0.00	0.00	0.00±0.00	0.00	0.00±0.00	0.00
24	0.25±0.00	0.00	0.15±0.00	0.00	0.75±0.01	1.33	0.53±0.01	1.89
48	1.70±0.09	5.33	0.34 ± 0.005	1.47	8.50±0.22	2.61	3.74±0.03	0.80

At the end of 48 hrs incubation lenses were photographed by using a CCRG camera (Chylack, 1978). The lens in Panel A was incubated in M199 and for Panel B, in M199 containing 55.6 mM glucose. Panels C, D show the lenses which were incubated, respectively, in 1 mM VC and 3.6 mM EGTA containing glucose-induced M199.


LDH (U / g of wet lens)
10
8
6
4
2
0
0
1
2
Time (days)
Glucose + VC
Control
55.6 mM Glucose
Glucose + EGTA.


A
B
C
D

(Figure 4.10). Several lower molecular weight reactions were also recognized but these may be the spectrin/fodrin cleavage products of other enzymes, because they appeared even after leupeptin was added into homogenization buffer. It is also possible that the anti-fodrin-antibody cross-reacted with other smaller molecular weight cytosolic lens proteins. To test the specificity of the antibody and the possibility of calpain digestion of spectrin/fodrin during homogenization, a different (nonlenticular) cell type, L6(Myoblast) cells containing spectrin/fodrin was also prepared under similar conditions, without homogenization. They were prepared in Laemmli buffer and run in the first 2 lanes beside the molecular weight standard. L6 cells (whether boiled in buffer or not) also produced a similar spectrin/fodrin pattern on nitrocellulose by immunoblotting (Figure 4.11). This indicates that the low molecular weight (140-150 kDa) protein band recognized by the antibody was not an artifact of calpain proteolysis during homogenization, since L6 cells which were extracted without homogenization, also showed a similar pattern.

The time course of glucose cataract effects on spectrin/fodrin proteolysis was followed. In glucose-treated lenses after 8 days (Figure 4.11), anti-fodrin-antibody detected no intact spectrin/fodrin, suggesting that it had been digested.

By disrupting actin filaments and the other lens

FIGURE 4.10
LENS SPECTRIN/FODRIN.

A control lens which is incubated in M199 for 8 days was homogenized in Laemmli 3X sample buffer (containing 0.1 mM leupeptin) at 90°C and centrifuged at 26,000 g for 20 min. A 25 µl sample from the lens extract was subjected to 10% SDS-PAGE and electrotransferred to nitrocellulose and immunostained with fodrin antibodies (Panel A). An identical gel was stained with Coomassie Brilliant blue (Panel B, lane 2). Both subunits of spectrin/fodrin were immunostained. Molecular weights of standard proteins are indicated in Lane 1, Panel B.

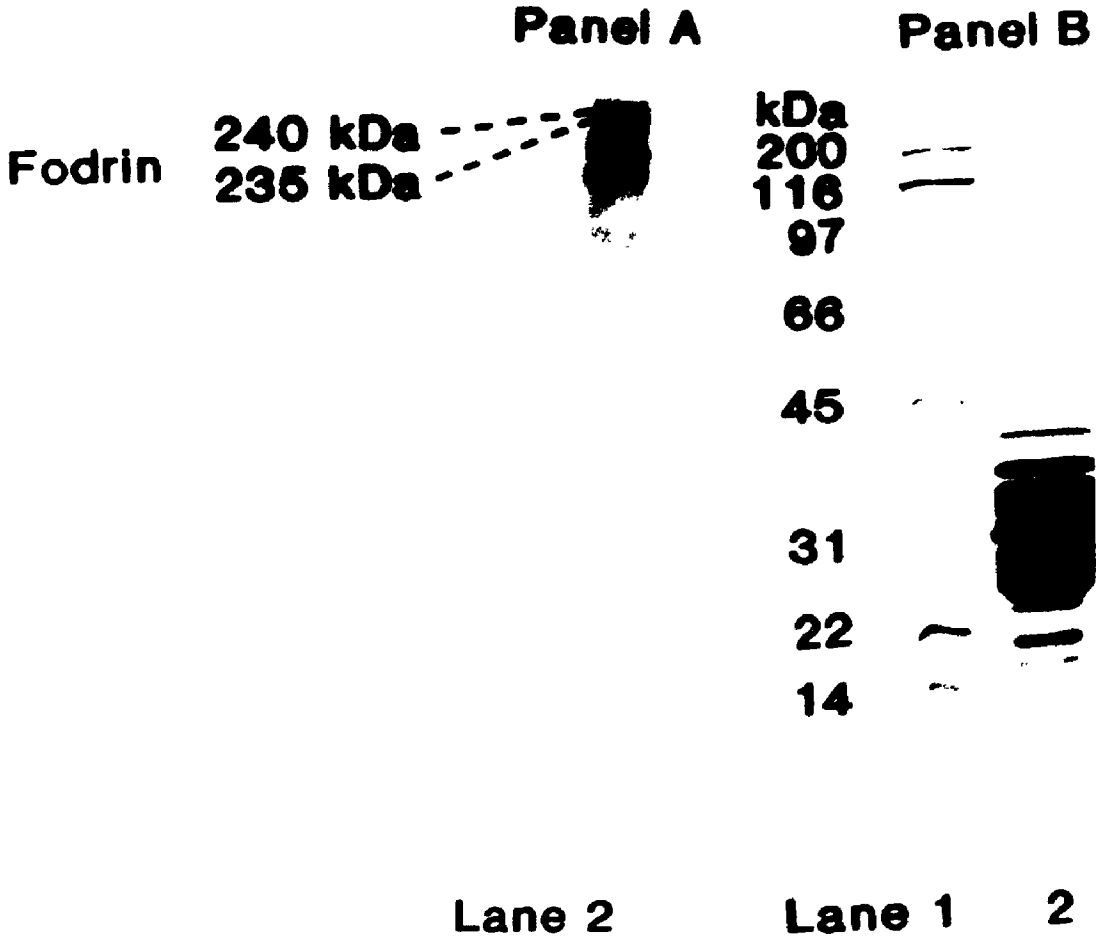

Panel A
Panel B
Fodrin
240 kDa
235 kDa
kDa
200
116
97
66
45
31
22
14
Lane 2
Lane 1
2

FIGURE 4.11
EFFECT OF GLUCOSE ON SPECTRIN/FODRIN PROTEOLYSIS.
A TIME COURSE STUDY.

Lenses were homogenized in Laemmli 3X sample buffer (at elevated temperature 90 °C) which has 0.1 mM leupeptin, and centrifuged at 26,000 g for 20 min. 25 µl of supernatant from selected of sequentially obtained daily samples (from day 0 in M199, or day 4 and 8 in M199 containing 55.6 mM glucose) were loaded on 10% SDS-PAGE (Lane 3, 5 and 4 respectively). On the SDS-PAGE the Coomassie Blue-stained detectable proteins were low molecular weight lens proteins around 20-30 kDa, which are mostly lens crystallins. When the identical gel was transferred to a nitrocellulose and probed with fodrin antibody, the 240 and 235 kDa subunits of spectrin/fodrin appeared as one broad band. To test the specificity of antibody and our method another type of cells, L6 myoblast cells were prepared in sample buffer as follows.
The order of the lanes for Western blotting and SDS-PAGE as follows:
Lane (0) Molecular weight standards.
Lane (1) L6 cells in samle buffer (agitated 5 min at 90°C).
Lane (2) L6 cells in sample buffer (agitated 5 min at room temperature).
Lane (3) Extract from the control lens (at day 0 in M199) was prepared as described above.
Lane (4) Extract from the lens incubated 4 days in 55.6 mM glucose and prepared as described above (Lane 3).
Lane (5) Extract from the lens incubated 8 days in 55.6 mM glucose and prepared as described above (Lane 3).
A similar extract of a control lens incubated 8 days in M199 is shown in Figure 4.10, for comparison.

FODRIN IN THE LENS

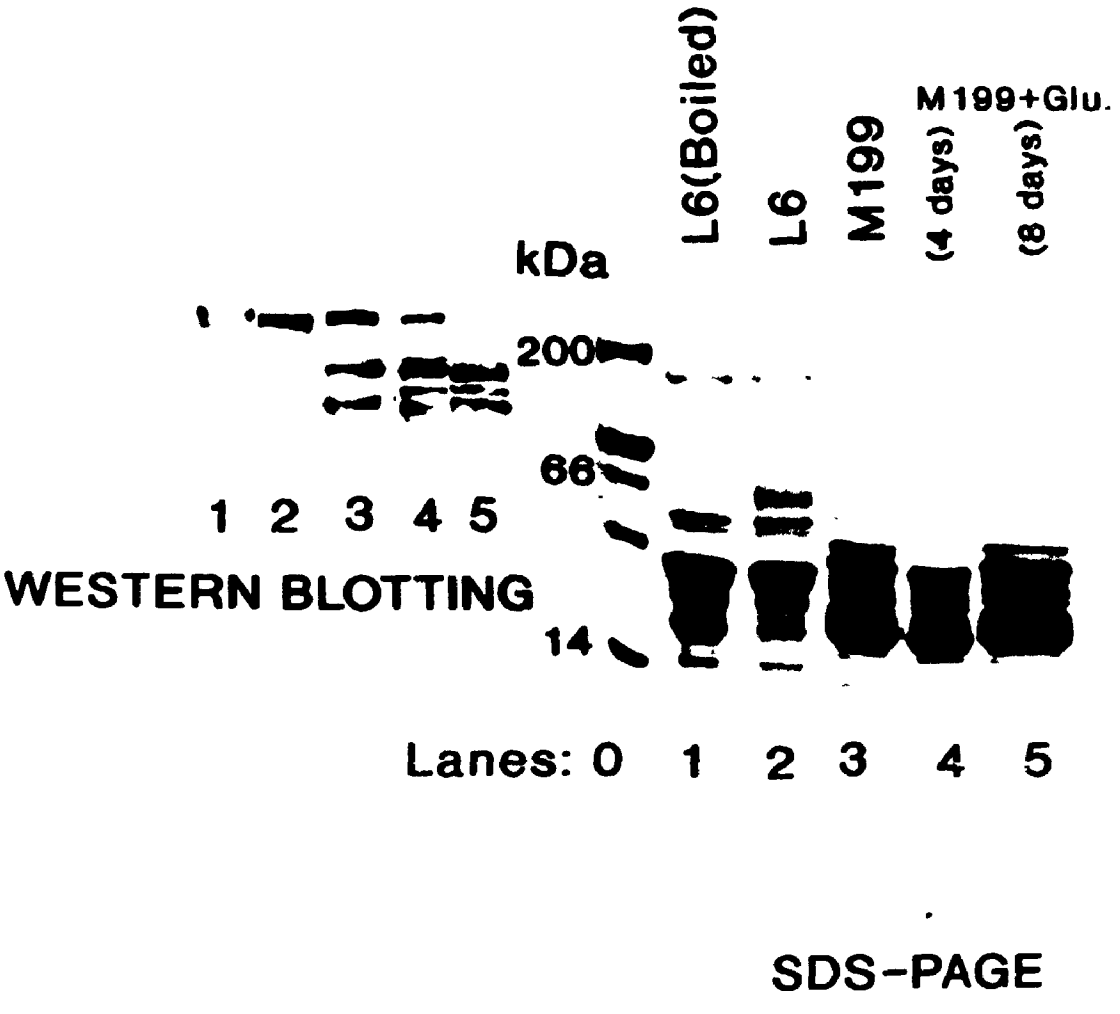
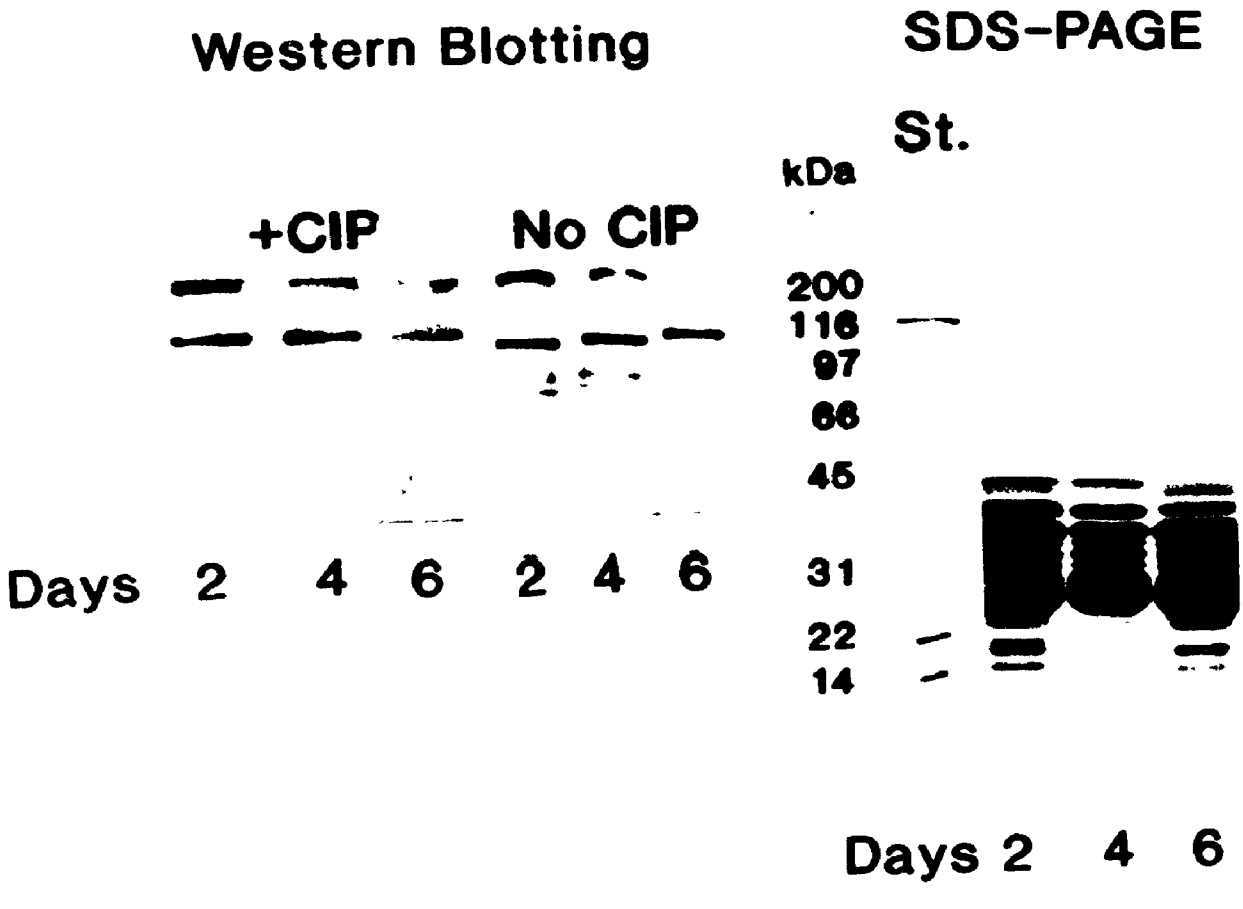


FIGURE 4.12
EFFECT OF CD ON SPECTRIN/FODRIN PROTEOLYSIS.
A TIME COURSE STUDY.

The lenses incubated either in M199 containing CD along with Calpain Inhibitor Peptide (+ CIP) or in M199 containing CD (No CIP). At the end of each incubation day (2-4-6 days), they were homogenized in 2 ml Laemmli 3X sample buffer (at elevated temperature 90 °C) which has 0.1 mM leupeptin. 25 µl lens extract from selected sequentially obtained daily samples (from day 2, 4 and 6) were applied to 10% SDS-PAGE. Intact spectrin/fodrin could barely be recognized at the end of 4 incubation days, but by 6 days α-fodrin antibody could not detect any intact spectrin/fodrin. A control lens incubated in M199 without CD is shown in Figure 4.10.

When CIP was used along with CD, spectrin/fodrin digestion was partially prevented.


Western Blotting
SDS-PAGE
St.
kDa
+CIP
No CIP
200
116
97
66
45
31
22
14
Days 2 4 6 2 4 6
Days 2 4 6

cytoskeletal network by CD-treatment the proteolysis of spectrin/fodrin was speeded up and there was no intact spectrin/fodrin in the CD-treated lens extract at the end of 6 incubation days (Figure 4.12).

Can Lens Transparency be Protected by Calpain Inhibitor Peptide (CIP) ?

The effect of CIP was tested on both cataract model systems: CD- and glucose-treated rat lenses. Lenses were incubated in either M199 control or glucose-M199 medium containing CIP during 8 days. Every day one lens from each sample was homogenized and daily LDH leakage was also measured. At the end of the experiment a 25 µl sample from each day's lens extract was run on SDS-PAGE and the protein was probed with anti-α-fodrin antibody.

At the end of 7 days incubation in M199 containing CIP and 55.6 mM glucose, intact spectrin/fodrin could be detected on the nitrocellulose immunoblot (Figure 4.13). The LDH leakage was reduced (Figure 4.14) and the lens maintained its transparency after 8 days incubation in glucose-M199 medium with CIP added (Figure 4.15). The protective effects of CIP were seen on lens transparency and also in preserving spectrin/fodrin intact.

The mechanism of CD cataract was obviously different

FIGURE 4.13
CIP IN THE GLUCOSE CATARACT MODEL SYSTEM.

The effect of CIP was tested on glucose-treated lenses. 8 lenses were incubated either in M199 containing glucose along with Calpain Inhibitor Peptide (+ CIP) or in M199 containing glucose (No CIP). At the end of every 24 hrs, each lens was homogenized in 2 ml Laemmli 3X sample buffer (at elevated temperature 90 °C) which contains 0.1 mM leupeptin. 25 µl supernatant from selected sequentially obtained daily samples (from day 4, 5, 6 and 7) were applied to 10% SDS-PAGE. Then the proteins were transferred to nitrocellulose by electroblotting and detected by anti-fodrin-antibody. At the end of 7 incubation days intact spectrin/fodrin could still be recognized by immunoblotting.

In Vitro Effect of Calpain Inhibitor on Fodrin

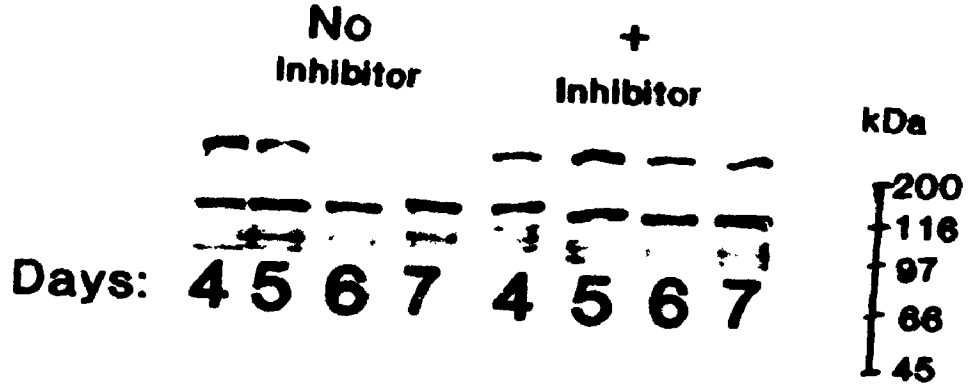


FIGURE 4.14
EFFECT OF CIP ON LENS TRANSPARENCY IN
THE GLUCOSE MODEL SYSTEM.

After 24 hr preincubation in 1.5 ml M199, lenses were transferred to fresh M199 containing the following additives: (1)✳ 55.6 mM glucose and CIP;
(2)+ control, CIP + M199;
(3)—•— 55.6 mM glucose .
The LDH activity in the culture medium and the leakage per g lens was calculated as described in Materials and Methods. The control case shows that the amount of CIP which was used during the experiment does not cause any damage, since a negligible amount of LDH accumulation was found in the media at the end of 8 days. Data (means ± SE (n=3) and percent SE) are shown for each separate period of culture and culture condition. When the SE is lesser in magnitude than the dimensions of the point on the graph it is not shown.

	Glucose + M199		CIP + M199		CIP + Glucose	
Day	U/g lens ± SE	%SE	U/g lens ± SE	%SE	U/g lens ± SE	%SE
1	0.750 ± 0.010	1.33	0.050 ± 0.000	0.00	0.150 ± 0.000	0.00
2	8.497 ± 0.222	2.61	0.234 ± 0.005	2.13	1.747 ± 0.160	9.18
3	18.973 ± 1.104	5.81	0.571 ± 0.036	6.30	3.366 ± 0.418	12.4
4	31.403 ± 1.528	4.86	1.119 ± 0.039	3.49	7.233 ± 0.119	1.66
5	46.967 ± 1.973	4.20	1.344 ± 0.051	3.79	9.000 ± 0.645	7.16
6	62.913 ± 1.507	2.39	2.105 ± 0.077	3.66	10.610± 0.703	6.63
7	69.930 ± 1.189	1.70	3.517 ± 0.080	2.27	12.311± 0.035	0.28
8	74.397 ± 1.123	1.51	3.647 ± 0.106	2.91	14.050± 0.057	0.39

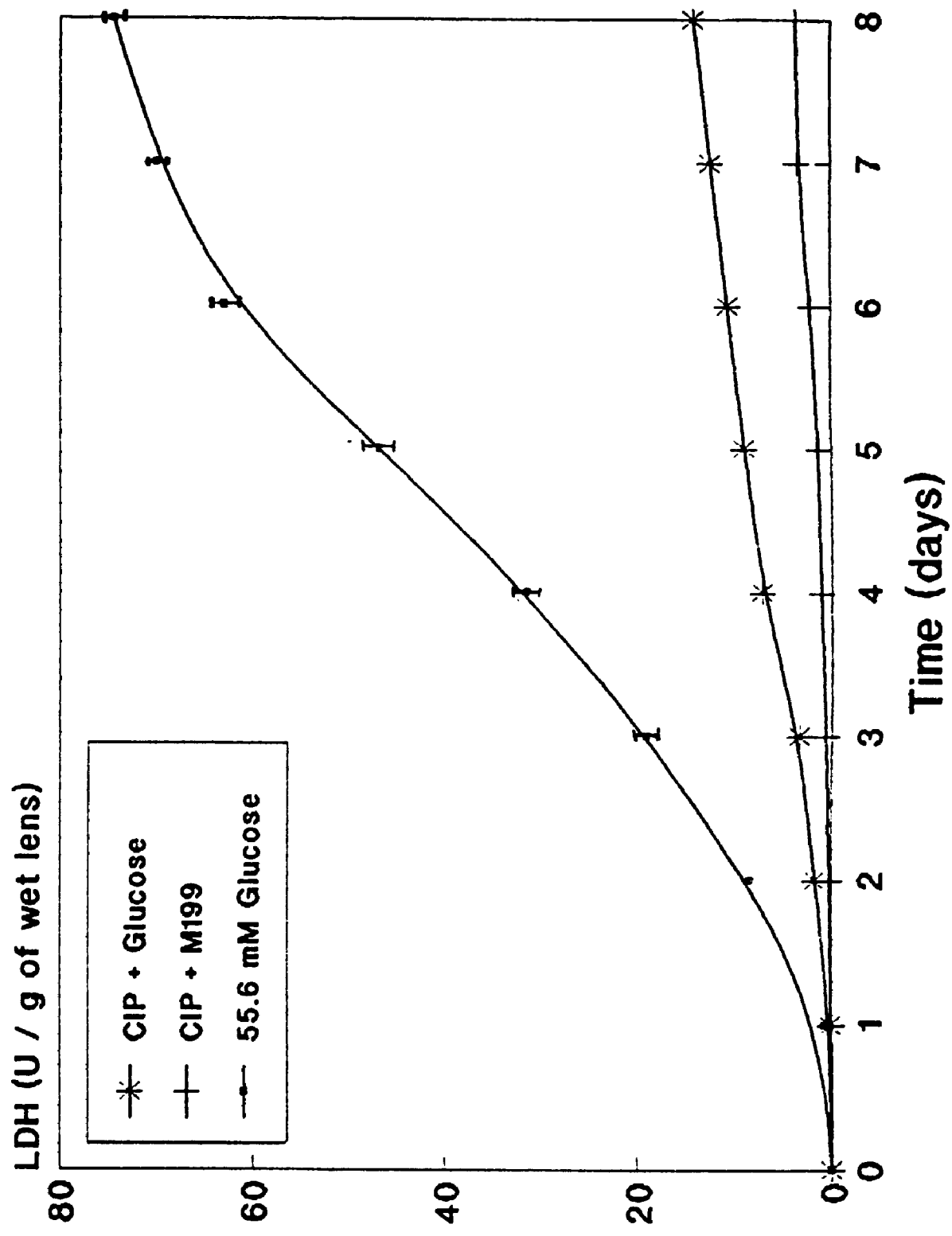
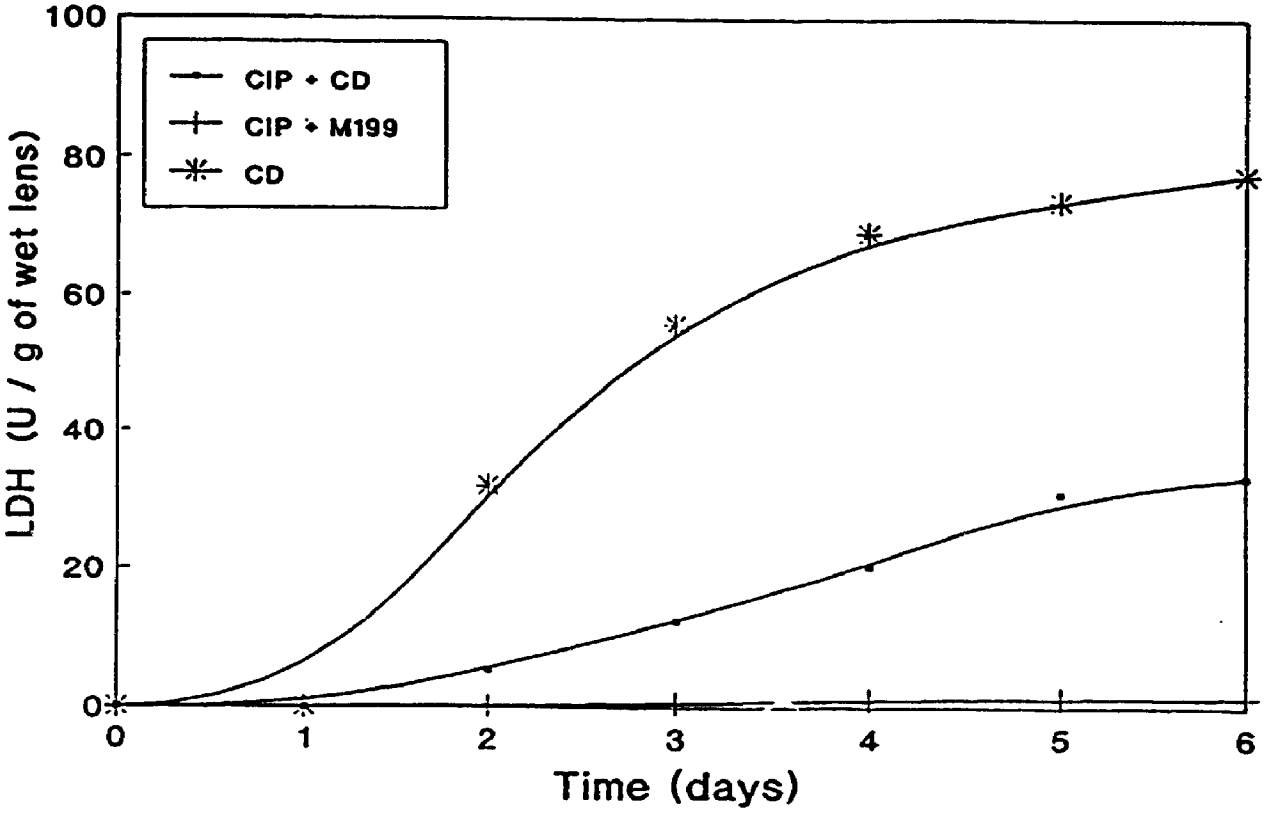

LDH (U / g of wet lens)
80
60
40
20
0
0
1
2
3
4
5
6
7
8
Time (days)
CIP + Glucose
CIP + M199
55.6 mM Glucose

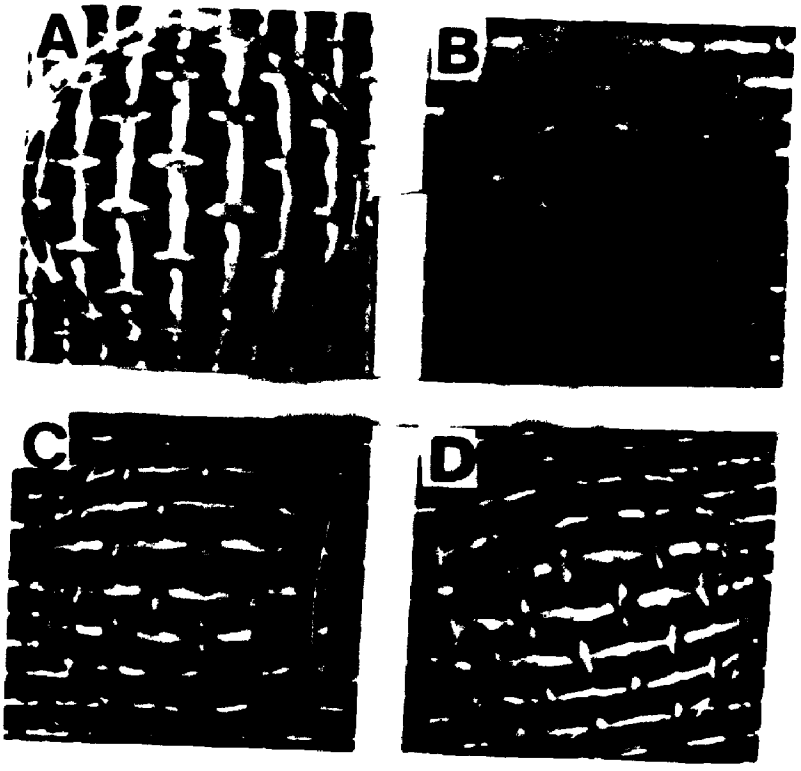
FIGURE 4.15
EFFECT OF CIP ON LENS TRANSPARENCY IN THE CD MODEL SYSTEM.

Lenses were incubated in M199 containing the following additives: (1)—•— CD and CIP;
(2)—+— control, CIP + M199;
(3)—✳— CD.
The LDH activity in the culture medium and the leakage per g lens was calculated as described in Materials and Methods. Data (means ± SE (n=3) and percent SE) are shown for each separate period of culture and culture condition. When the SE is lesser in magnitude than the dimensions of the point on the graph it is not shown. CIP slowly reduced the leakage of LDH from the CD-treated lenses.

	CD		CIP + M199		CIP + CD	
Day	U/g lens ± SE	%SE	U/g lens ± SE	%SE	U/g lens ± SE	%SE
1	1.507 ± 0.020	1.33	0.050 ± 0.000	0.00	0.075 ± 0.000	0.00
2	31.897 ± 0.901	2.83	0.234 ± 0.005	2.13	5.421 ± 0.057	1.04
3	55.927 ± 0.858	1.53	0.571 ± 0.036	6.30	12.410 ± 0.211	1.70
4	69.343 ± 0.886	1.28	1.119 ± 0.039	3.49	20.193 ± 0.098	0.49
5	74.003 ± 0.701	0.95	1.344 ± 0.051	3.79	30.790 ± 0.182	0.59
6	77.790 ± 0.745	0.96	2.105 ± 0.077	3.66	32.821 ± 0.005	0.02

The protective effects of CIP were also followed by using a CCRG camera, Chylack, (1978). The lens in Panel A was incubated in M199 and Panel B, in M199 containing CD. Panels C, and D show the lenses which were incubated, respectively, in CIP + M199 and CIP containing glucose-induced M199.


CIP + CD
CIP + M199
CD
LDH (U / g of wet lens)
100
80
60
40
20
0
0
1
2
3
4
5
6
Time (days)


A
B
C
D

than glucose cataract. The effect of CD on the proteolysis of spectrin/fodrin was more rapid than CIP; this result can be seen in Figure 4.12. CIP prevented proteolysis to some extent but was not as effective as in the glucose model. At the end of 6 days of incubation in M199 containing CD along with CIP, spectrin/fodrin proteolysis was partially protected. The result of this experiment was also confirmed with the leakage of lens LDH (Figure 4.15) into incubation media and with the appearance of the lenses (Figure 4.15 Panel A and B) after 6 days.

4.4 DISCUSSION

This section of my study has three objectives: i) to study the timing and localization of the first calcium entrance into diabetic rat lenses, ii) to discover whether spectrin/fodrin proteolysis precedes cataract formation or is a consequence of pre-cataractous changes, and iii) to study the effect of calpain inhibitor during cataract formation. Since many simultaneous reactions occur in cataract formation, it is critical to be able to organize the events in order and to elucidate the mechanism. To clarify the timing of lens calcium uptake would be an important beginning.

It has already been shown that the initial opacity of cortical cataract is caused by globular degeneration of the equatorial lens fiber cells and associated abnormal proliferation of equatorial epithelial cells (Creighton et al., 1980; Harding, 1991; Harding et al., 1982; Unakar et al., 1981a; Unakar et al., 1978; Unakar et al., 1981b). This is followed by the disturbance of the regular order of fiber cells at the region just external to the lens equator. Similar globular degeneration has been shown in lenses exposed to elevated glucose (Creighton et al., 1982) or cytochalasin D (Mousa et al., 1979), an agent which disaggregates actin microfilaments. This suggested that elevated calcium concentrations which would also affect microfilaments might be involved in the globular

degeneration induced by glucose.

The findings reported here indicate that the equatorial cortex was the first target area for calcium uptake into the lens during glucose treatment which resulted in elevated calcium-green fluorescence, and ^{45}Ca uptake in the first 24 hrs incubation. In the equatorial cortex the calcium uptake increased parallel to the eventual formation of globular degeneration (Creighton et al., 1982). In this part of the study, with these findings we expected to discover whether the membrane-cytoskeleton interactions and/or calpain type protease activation initiate or stimulate the opacification process.

Prevention of Ca^{2+} uptake, opacity, and LDH leakage by VC is consistent with a causative role for calcium in the process of cataractous globular degeneration leading to the local opacity which is seen first at the lens equator in incubated lenses. The role played by O_2-derived free radicals in the injury to cytoplasmic and organelle membranes during diabetes has been suggested by the work of Wolff and Crabbe (1985) but is still not well characterized. Antioxidant agents, which may stabilize the lens cells against loss of these critical components, may i) prevent lens damage leading eventually to protein leakage by the lens cells, ii) delay lens cell death, and thus iii) delay the onset of the subsequent opacification of the lens, possibilities which were all confirmed by the study reported

here. The ability of an antioxidant to prevent calcium uptake or damage to the lens might be an indication of the following hypothesis: as a consequence of pre-cataractous damage the intracellular level of free calcium increases, enhancing the cataract formation process. Can we stop this increase ?

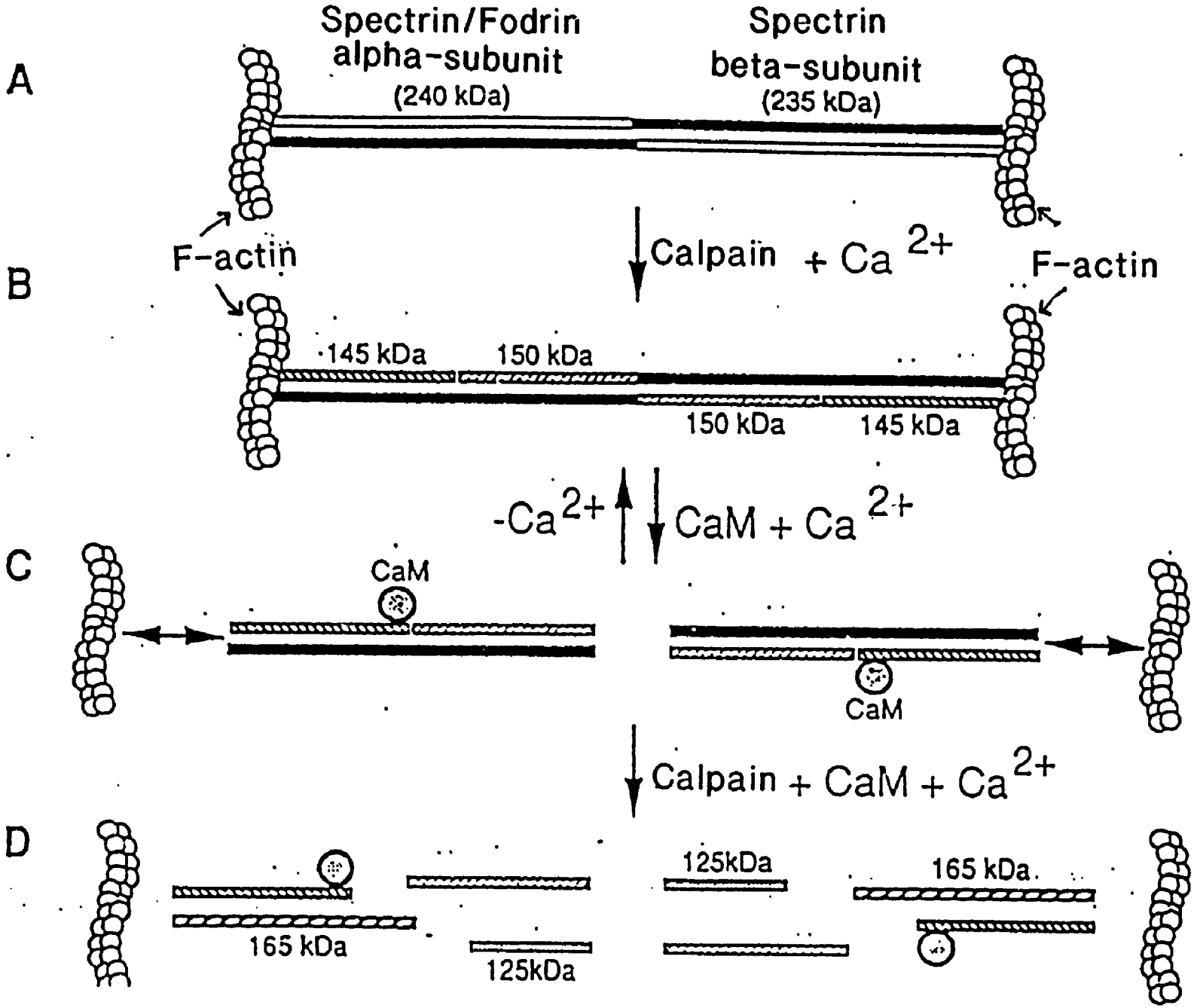
In almost all tissues, high levels of calcium often cause severe damage by decreasing the interaction of cytoskeleton proteins with the cell plasma membrane (Niggli and Burger, 1987) or initiate calcium-dependent proteolysis (Saido et al., 1994). Thus, one possible consequence is that membrane-cytoskeleton interactions and/or calpain type protease activation initiate or stimulate the opacification process. Another possible explanation of calcium-dependent plasma membrane damage might be: the effect of elevated level of calcium on calcium-binding proteins which have roles in cell membrane integrity. Calcium also has a role on promotion of actin polymerization, trafficking of actin cross-linking proteins, cell-signalling, signal transduction etc.. Our results confirm those of Duncan et al., (1993) and Rafferty et al., (1994) in showing that maintenance of calcium concentration at a physiological level is very important for transparency and cell integrity of the lens. During the increase or fluctuation of cytosolic [Ca^{2+}] all these functions are disrupted and membrane integrity is threatened. But, what is the mechanism by which calcium acts

in the lens during cataract formation ?

Spectrin/fodrin, structurally and functionally, has been a well- studied, major cytoskeletal protein which was the first to be described to undergo calcium-dependent proteolysis in different tissues (Saido et al., 1994). Proteolysis of spectrin/fodrin was detected in the lens specifically, and shown to occur there during cataract formation (David et al., 1993). Spectrin/fodrin is among the most susceptible endogenous substrates of the calcium-activated neutral proteinase, calpain. Our studies indicate that calcium-activated neutral proteinase is present in lens cells, although calpain activity toward spectrin/fodrin was low under our experimental conditions. The preceding discussion indicates that in the lens the intracellular free calcium concentration and proteolysis of spectrin/fodrin by a calcium activated proteinase, calpain, is regulated by interaction between spectrin/fodrin and calmodulin (Aster et al., 1986). These interactions, schematically represented in Figure 4.16, illustrate the following: i) Intact spectrin/fodrin is attached from one end to F-actin and from the other end to its other subunit. When the cellular free calcium concentration increases, it immediately activates the calcium-dependent neutral proteinase, calpain. ii) Calpain initially digests spectrin/fodrin α-subunits into two peptides; 145 and 150 kDa. iii) Cleavage of α-spectrin/fodrin subunits exposes a calmodulin-binding site

FIGURE 4.16
DIAGRAM OF POSTULATED CALCIUM-DEPENDENT SPECTRIN/FODRIN -CALPAIN INTERACTION IN THE LENS CELLS.

Initially calpain is activated by the increase intracellular free calcium concentration (Step A). The proteolysis of spectrin/fodrin (Step B) will expose the calmodulin (CaM) binding (Step C) and CaM will bind free calcium. When the free calcium concentration decreases, the cells will attempt to increase the calcium uptake. If the cell free calcium level exceeds the endogenous [CaM] then any excess amount of free cellular calcium will activate calpain leading to the digestion of spectrin/fodrin which eventually will dissociate the (spectrin/fodrin)-(F-actin) complex and α and β subunits (Step D).


A
Spectrin/Fodrin
alpha-subunit
(240 kDa)
Spectrin
beta-subunit
(235 kDa)
F-actin
Calpain + Ca 2+
F-actin
B
145 kDa
150 kDa
150 kDa
145 kDa
-Ca2+
CaM + Ca 2+
C
CaM
CaM
Calpain + CaM + Ca2+
D
165 kDa
125kDa
125kDa
165 kDa

on spectrin/fodrin which possibly is buried in the intact protein tertiary structure. iv) When spectrin/fodrin binds calmodulin, the intracellular free calcium level decreases, because every calmodulin binds 4 calcium molecules. v) To compensate for the intracellular free calcium level, the cell will increase its calcium uptake. vi) When the endogenous calmodulin is saturated with calcium then the excess amount of calcium will activate calpain. At that time dissociation of spectrin/fodrin from F-actin and from its subunits occurs. Alternatively, it is possible that these steps may not be in this order and/or time sequence. Another possibility is that of a concerted mechanism: everything could happen at the same time, or yet another possibility is that the proteolytic activity varies with the location of lens cells.

On the basis of the relative rates of spectrin/fodrin degradation, in the CD and the glucose models, the activity of calcium-activated neutral proteinase, calpain, should be greater on its other substrates like vimentin, actin, and α-crystallin. On the other hand, calpain ir ɔitor peptide, while protecting lens transparency in the glucose model against calpain degeneration, could not stop it in CD-treated lenses. This might suggest that once the cytoskeletal network is disrupted, calpain activity toward its substrate becomes higher. Perhaps CD causes a dissociation of the F-actin-Spectrin/fodrin complex which

enhances the proteolysis and calpain activity in the cells.

In future investigations, examining the proteolytic responses of different calpain substrates in the lens, such as α-, β-crystallin, actin or vimentin, may be a particularly fruitful application of the cell biology approaches used in this study. In addition to providing clues to the physiological functions of calpain, localization of proteolyzed spectrin/fodrin in the lens cells may yield useful information about the differential vulnerability of lens cells to cataractous damage.

CHAPTER 5

REVIEW OF RESULTS CONCLUSIONS and FUTURE STUDY.

A disease, injury, exposure to toxic substances, or the aging process can cause opacity in the lens, cataract, which is the leading cause of blindness (Harding and Crabbe (1984). Cellular and biochemical changes in human cataract have been extensively studied for many years, and research on experimentally induced cataracts and *in vitro* model systems leave no doubt that cataractogenesis is a complex, multifactoral and multistep process, one that is associated with aging and which may be initiated by oxidative changes. To date, the detailed mechanism and order of events is not well understood.

In the present work we have developed *in vitro* cortical cataract model systems using rat lenses for studying the mechanism of cataract formation. Initially, optimum medium and incubation conditions for the *in vitro* system were determined: rat lenses with intact capsules kept their clarity for 8 incubation days in M199 without serum. Once these conditions had been worked out, then two different reagents which represent two different mechanisms to achieve the same end, cataract, were used in the experiments reported here: elevated glucose (55.6 mM) and cytochalasin D (CD, 10^{-5} M). During the first 48 hours of culturing with cataract-inducers, the lenses were pre-cataractous,

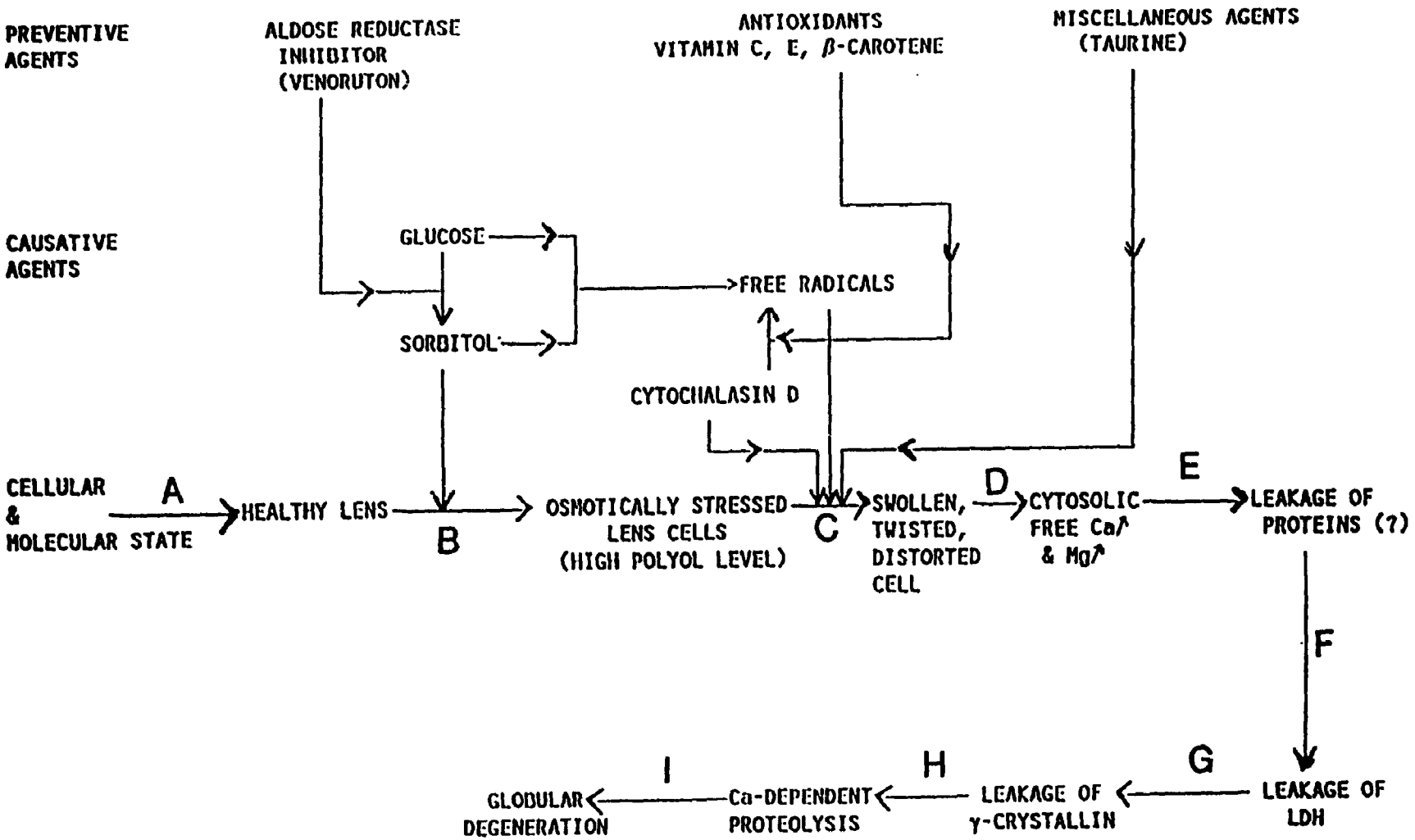
progressing to the cataractous state after they have been incubated for 8 days. To provide complementary results to those from the in vitro glucose model system, the effect of glucose on the lens was studied in diabetic rats. Physical and biochemical changes occurring in incubated lenses appeared to resemble those occurring in vivo. These observations indicate that our in vitro system is trustworthy and reliable for the study of cataract development in organ culture.

Glucose and CD both acted similarly to produce cataract. Occurrence of changes suggested that there should be a common pathway leading to cataract. We combined all our findings to propose a pathway of events in the pathogenesis of cataract (Figure 5.1). The differences between the two agents made it possible to emphasise different parts of this common pathway, leading us to distinguish the differences between the two cataract-inducers, and then to place them correctly in the common pathways.

Cataract development or the effect of inducers, glucose and CD, were correlated and followed using three major parameters: i) distribution of the dead cells in the lens, ii) daily appearance of the lens under a dissection microscope, and iii) changes in and leakage of lens soluble cytosolic proteins, γ-crystallin and LDH, and increased magnesium concentration.

FIGURE 5.1
THE SUMMARY OF OUR FINDINGS.

The possible multistep scheme of cortical cataractogenesis, based on involvement of elevated glucose and cytochalasin D and different preventive agents in the overall process of cataract formation.


PREVENTIVE AGENTS
ALDOSE REDUCTASE INHIBITOR (VENORUTON)
ANTIOXIDANTS VITAMIN C, E, β-CAROTENE
MISCELLANEOUS AGENTS (TAURINE)
CAUSATIVE AGENTS
GLUCOSE
SORBITOL
FREE RADICALS
CYTOCHALASIN D
CELLULAR & MOLECULAR STATE
A
HEALTHY LENS
B
OSMOTICALLY STRESSED LENS CELLS (HIGH POLYOL LEVEL)
C
SWOLLEN, TWISTED, DISTORTED CELL
D
CYTOSOLIC FREE Ca↑ & Mg↑
E
LEAKAGE OF PROTEINS (?)
F
LEAKAGE OF LDH
G
LEAKAGE OF γ-CRYSTALLIN
H
Ca-DEPENDENT PROTEOLYSIS
I
GLOBULAR DEGENERATION

Distribution of the dead cells in the lens during the precataractous changes was useful in localizing those lens cells which are first affected by the cataract-inducers. During the confocal study we mostly focused on the cells located in the equatorial region. The results indicated that CD- induced cataract formation occurs earlier than glucose cataract, and that high levels of epithelial cell mortality occurred during the first 24 hr of incubation in CD-incubated lens as visualized by FDA and T1111 localization. Our results suggest that glucose-induced cataractogenesis involves only low levels of epithelial cell mortality during the first 48 hours. Also, the fluorescent staining of the dead cells indicated that all of the lens cells do not lose their integrity at the same time during cataract formation. A growing number of dead cells in the lens should be related to an increasing stage of cataract. Perhaps the amount of the leakage of some intracellular ingredient depends on the number of dead cells during the different stages of the cataract.

Even though some parameters changed earlier than the appearance of the lenses, under a dissection microscope, using this method to follow the development of cataract could be a reliable technique along with biochemical analyses under some conditions: for example, to follow the effect of an anti-cataract drug, or to follow an effect of cataract-inducing agents (Chylack, 1978).

Our *in vitro* and *in vivo* studies gave us similar results for the leakage of γ-crystallin as an indication of cataract formation. But the western blotting of the soluble lens proteins which leaked into the incubation medium during the first 48 hr of incubation, indicated that γ-crystallin was one of the proteins in the medium but it was not the first lens protein released. This result was obtained using both inducers (glucose and CD) although at different times. The effect of CD on lens opacity occurred much earlier than glucose, suggesting that in CD, induced damage is more severe and rapid. The CD-disturbed cytoskeleton may precede oxidation, while in glucose the opposite order occurs. The combination of oxidative damage with CD-disruption of the cytoskeletal network, may result in more dead cells at the very early stage of cataract formation than occurs in the glucose-induced cataract model. Subsequently, more protein leakage was found for CD. Similar results were seen in the lens intracellular magnesium content: the magnesium uptake was found to be greater, and occurred earlier in the CD- than the glucose-treated lens.

The next parameter to correlate with the *in vitro* cataract formation was the leakage of LDH. Its leakage occurred much earlier than the leakage of γ-crystallin or other detectable soluble lens proteins during cataract formation. LDH measurement is a rapid technique without a risk of radioactivity, and statistical analysis showed that

it is reliable. We used this information as the basis for further culturing and for the establishment of both in vitro systems. Using this reproducible lens incubation system, we tested various potential anti-cataract reagents in our two cataractous model systems. The uptake of magnesium also indicated the timing of cataract formation and the first targets of both cataract-inducers. We were not able to quantify the actual concentration of magnesium or calcium because the confocal microscope we used did not have the ability to simultaneously detect the fluorescent emission at high and low wavelengths. If this had been possible we could have quantified the actual concentration of magnesium or calcium.

The study of anti-cataract agents in vitro has two advantages: i) their direct effects on cataractous lenses can be tested, and ii) it is possible to identify the step in the pathway of cataractogenesis at which the treatment of cataract inducer is effective. Initially, to test the hypothesis that: "the disrupted cytoskeleton network precedes oxidation", we obtained the results from the study of the antioxidant, VC on the CD-induced cataract model system. When treatment with VC partially reduced the effect of CD on lens opacity, it strengthened the hypothesis about the possible mechanism of CD on the lens cells. The in vitro antioxidant study in the glucose-induced cataract model gave us the chance to test the direct effect of this

drug on healthy and cataractous lenses. Consistent with the hypothesis that this damage results from oxidant stress, it has been found that, *in vitro*, experimental diabetic cataractogenesis is prevented or reduced by a number of agents with antioxidant activity, such as VC, VE, β-carotene, taurine, and R-, and racemic-α-lipoic acid. All these agents have antioxidant activity, and all were effective *in vitro* in prevention of the globular degeneration damage caused by elevated glucose. Until we observed the direct effects of these antioxidants we could only suspect that, according to suggestions in the literature, damaging mechanisms of glucose might possibly be due to glycation and oxidative stress. But our *in vitro* studies and consistent findings gave strong support for our hypothesis. As a part of this study a novel drug, venoruton, an aldose reductase inhibitor, was studied which led to some promising results regarding its possible clinical application in the future.

Finally the role of the elevated level of intracellular free calcium was studied during the cataract formation. The role of elevated calcium has been studied in various cells during different diseases. In the cataractous lens the calcium level was found to be higher than normal (Cheng and Chylack, 1985; Rafferty *et al.*, 1994; Duncan *et al.*, 1994). We asked: when did calcium uptake start, and where is its target cell in the lens? Some studies have localized

calcium in isolated lens cells and in the lens cells (Creighton et al., 1980; Harding, J., 1991; Harding et al., 1982, 1983; Unakar et al., 1981(a), (b), 1978) but our results both from radioactive and fluorescent labelling in vitro in the rat lens serves to extend the work of others. Also, our study on the time course of lens calcium uptake showed clearly that in the glucose-treated lens between 24-48 hr incubation, calcium uptake was increased and was enhanced by glucose and time. These findings related calcium to cataractogenesis. When we investigated the literature on other diseases and tissues, the most common effect of calcium was the breakdown of the cytoskeletal protein network (Zimmerman and Schlaepfer, 1984; Traub, 1985). We were aware that the neutral proteinase calpain, and its inhibitor, calpastatin, have been found in the lens (Yoshida et al., 1985; David and Shearer, 1986). The substrates for calpain were reported to be α-crystallin, ß-crystallin, actin, vimentin and spectrin (David and Shearer, 1986; Roy et al., 1983; Yoshida et al., 1984; David et al., 1993). But still I did not know which of these substrates are digested by the initial calpain activity resulting from the elevated intracellular free calcium concentration. An equally pressing question is whether the calpain activity has an immediate role in cataract formation ? In an other words, which comes first ? Does stimulated calpain activity result in or result from cataract formation ?

It has been suggested that the membrane-cytoskeletal components and their interactions in red blood cells may functions in a similar manner in lens cells (Gandolfi et al., 1988). Their interactions in lens and red blood cells may also be similar. Defects in the protein components of the membrane skeleton cause red cells to take on abnormal shapes, create unstable membranes and lead to damage in such cells with reduced cellular deformability in the circulation, e.g., hereditary hemolytic anemias (Goodman, Shiffer, 1983; Sheetz, 1983). In the light of this information, the elevated calcium and parallel increase of the opacity led us to study the function of calcium-activated protease during cataract formation. The time course of the proteolysis of lens spectrin/fodrin was studied in both cataract model systems, to explore whether proteolysis of spectrin/fodrin precedes cataract formation, or whether it is a consequence of pre-cataractous changes. The time course of an experiment with glucose-treated lenses showed that intact spectrin/fodrin was digested totally at the end of 6 incubation days. The same experiment with CD-treated lenses showed this digestion occurred much earlier than in the glucose-treated lenses. From our previous findings we know that cataract formation started after 48 hr in glucose-treated lenses and after approximately 24 hr of incubation in CD-treated lenses. Our conclusion, then, is that proteolysis of spectrin/fodrin might not have an

immediate effect on cataract formation, but it might enhance the development of cataract. Following the initiation of the cataract process, proteolysis of spectrin/fodrin may occur as we proposed and described in Chapter 4.4.

To summarize what we know about calcium-calpain-spectrin/fodrin-cataract : i) intracellular free calcium started to increase in the very early stage of cataract formation, ii) calpain was found in lens cells, iii) lens cytoskeletal proteins and crystallins were shown to be calpain substrates, and iv) proteolysis of spectrin/fodrin did not occur parallel to the cataract formation.

There are several possible interpretations of this summary: either, i) calpain activity toward spectrin/fodrin is low under our experimental conditions, or ii) the principal fate of spectrin/fodrin in the in vivo system is its proteolysis but whether it will cause any damage to the plasma membrane or not totally depends on the rate of its in situ synthesis, or, iii) the nature of calpain-catalyzed proteolysis of spectrin/fodrin is not digestive; it may proceed in a limited manner resulting in alteration, rather than destruction, of the structure and function of spectrin/fodrin in the lens cells, or, iv) the activity of calpain is greater on its other substrates such as vimentin, actin, and α-crystallin, or v) proteolysis of spectrin/fodrin by calpain is regulated by the spectrin/fodrin-calmodulin interaction, since the α-subunit of

spectrin/ fodrin has affinity for the calcium-dependent binding of calmodulin as described in Chapter 4.4. It is the last interpretation, in our opinion, which is the most plausible of the five.

To summarize our findings about calcium-calpain-spectrin/fodrin-cataract: calcium-activated calpain proteolysis is involved in the cataract development, and proteolysis of spectrin/fodrin is a consequence of pre-cataractous changes. By using calpain inhibitor peptide in both systems the proteolytic cleavage of spectrin/fodrin and the development of opacity were prevented.

Our findings are consistent with the hypothesis that in glucose-treated lenses membrane damage initially results from oxidant stress (Figure 5.1). The study with antioxidants was consistent with this idea. The increased calcium-uptake then could activate calpain which would digest its substrate. Although a course of cataractogenesis for CD-treated lenses is different, it acted similarly to produce cataract: CD- first disrupted the cytoskeletal network then damaged the cell plasma membrane. Membrane failure led to elevated intracellular free calcium which activated calcium-dependent proteolysis.

FURTHER AVENUES OF RESEARCH BASED ON THIS WORK

The quantification of the calcium-uptake of incubated lenses or equatorial epithelial cells in glucose or CD

models over time will help to identify the type of calpain in the system. Two types of calpain have been identified in the lens (Anderson et al., 1994) : to initiate the in vivo activity of μ-calpain (calpain I) requires micromolar, and m-calpain (calpain II) requires millimolar concentrations of calcium. Future experiments using the same conditions, and analyzing the degradation of α-, and β-crystallin, vimentin and actin, will definitely shed light on the calpain-substrate hypothesis and on the cataract pathway.

We have the fodrin antibody (a generous gift from Dr. Saido from University of Tokyo, Japan) which is specific to the proteolytic 150 kDa form of spectrin/fodrin α-subunit. The immunohistochemical study of calpain-catalyzed spectrin/fodrin proteolysis in cataractous lens will identify the cells which have the proteolyzed spectrin/fodrin. This in turn will strengthen the role of this major cytoskeletal protein in the lens and the effect of its digestion during cataract formation.

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