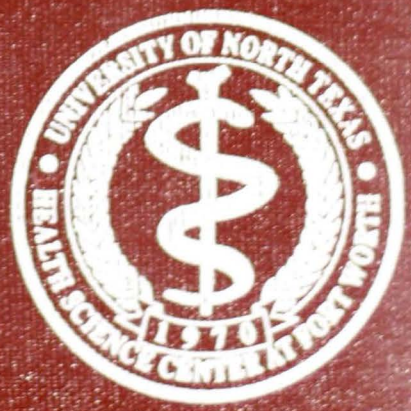
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HEALTH SCIENCE CENTER AT FORT WORTH
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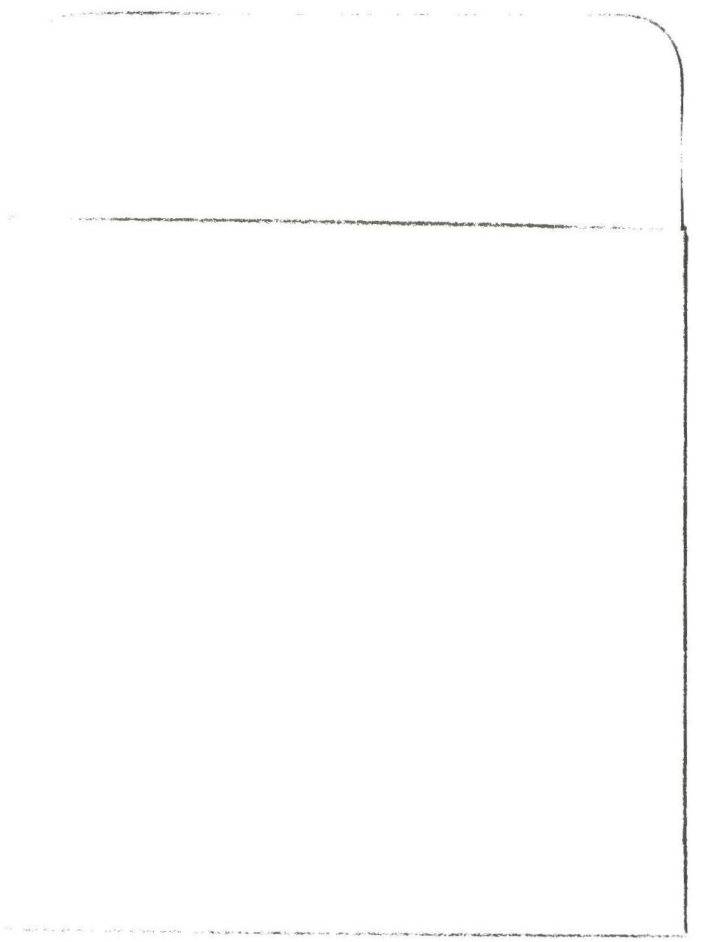

Jaime E. Dickerson, Jr., The Influence of Aging and Glycation on Protein-Thiol Mixed Disulfides in the Eye Lens. Doctor of Philosophy (Biomedical Sciences), June, 1994, 163 pp., 9 tables, 28 illustrations, bibliography, 116 titles.

The human lens is continually growing. As new cells are formed they differentiate into fiber cells which have no organelles, no protein synthesis or turnover.

Lens protein aging involves formation of very large aggregations and insoluble complexes. These are held together through disulfide linkages.

Reduced glutathione (GSH) is present in high concentrations. The oxidized form, (GSSG), (5% of the total) can form mixed disulfides with proteins. This can destabilize the protein conformation. Accumulation of mixed disulfides may increase the potential for further modification. The participation of a PSSG (protein/glutathione mixed disulfide) in the formation of a protein-protein disulfide becomes increasingly likely.

The purpose of this work is to document PSSG and protein-cysteine mixed disulfide (PSSC) accumulation in human lenses (through eight decades), and to identify a third mixed disulfide discovered in this research. The free thiol molecules GSH and cysteine were also quantitated for normal and cataractous lenses.

Glycation may alter conformation similar to mixed disulfides and potentiate mixed or protein-protein disulfide formation. This model was evaluated two ways. First, purified alpha crystallin was incubated with ascorbate and conformational changes were evaluated with CD spectroscopy. Second, rat lenses were cultured under high sugar conditions to determine if the resulting glycation influenced the level of mixed disulfides. Conversely, the effect of prior mixed disulfide formation on the extent of glycation in another purified crystallin, gamma, was evaluated.

The results indicate: GSH declines in the lens with age, cysteine exists in the lens albeit at relatively low levels, PSSG shows a triphasic pattern of accumulation, PSSC accumulates linearly with increasing age, the existence of a third mixed disulfide species, gamma glutamylcysteine mixed disulfide, detected in old or cataractous lenses, has been confirmed, glycation by ascorbic acid alters α- crystallin secondary structure, the influence of glycation is minimal on mixed disulfide formation, mixed disulfide formation affects glycation of gamma crystallin.

THE INFLUENCE OF AGING AND GLYCATION ON PROTEIN-THIOL MIXED DISULFIDES IN THE EYE LENS

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THE INFLUENCE OF AGING AND GLYCATION ON PROTEIN-THIOL MIXED DISULFIDES IN THE EYE LENS

DISSERTATION

Presented to the Graduate Council of the
Graduate School of Biomedical Sciences
University of North Texas
Health Science Center at Fort Worth
in Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Jaime E. Dickerson, Jr., B.A., B.S., M.S.
Fort Worth, Texas

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ARI	Aldose reductase inhibitor
BCA	Bicinchoninic acid
BSA	Bovine Serum Albumin
CD	Circular Dichroism
Cor	Cortex
CSO_3H	Cysteic acid
DHA	Dehydroascorbic acid
DTNB	Dithio-bis(nitrobenzoic acid)
DTPA	Diethylenetriaminepentaacetic acid
EDTA	Ethylenediaminetetraacetic acid
GSSC	Glutathione-cysteine mixed disulfide
GSH	Glutathione (reduced form)
GSO_3H	Glutathione sulfonic acid
GSSG	Glutathione (oxidized form) disulfide
HEPES	Hydroxyethylpiperazine ethanesulfonic acid
HEPPS	Hydroxyethylpiperazine propanesulfonic acid
HPLC	High performance liquid chromatography
HMW	High Molecular Weight

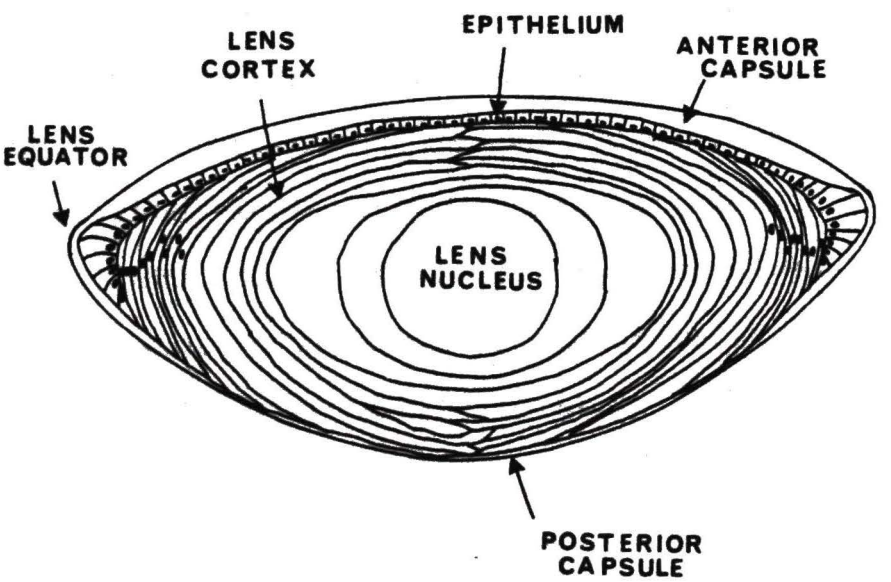
HRP	Horseradish peroxidase
MW	Molecular weight
Nuc	Nucleus
PBA	Phenylboronate agarose
PSSC	Protein-cysteine mixed disulfide
PSSG	Protein-glutathione mixed disulfide
PSSP	Protein-protein disulfide
SDS/PAGE	Sodium dodecyl sulfate/Polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid
WI	Water insoluble fraction
WS	Water soluble fraction

CHAPTER I

INTRODUCTION

There are two compelling reasons for studying ageing of the human eye lens. First, the lens is unique in that its biochemical history from embryonic development to old age may be read in the alterations its proteins contain. The lens is an avascular, continually growing tissue (Fig. 1) with new layers of fiber cells formed from the monolayer of epithelial cells throughout life and overlaying the older earlier formed cells. Its avascular nature means that the inner fiber cells are completely dependent on the outer layers for nutrition, removal of wastes and the metabolism of exogenous toxins. The transformation from epithelial cell to fiber cell involves many changes of both a morphological and biochemical nature. Perhaps most obvious is the loss of cellular organelles, including the nucleus, precluding further protein synthesis in the mature cells (c.f. Lieska 1992). Thus the protein molecules present in the center of the lens are as old as the individual and date to embryonic development of the lens. This represents a unique opportunity to study protein aging and the effects certain modifications have on protein functions. The second reason, and probably the single most important factor

Figure 1. Diagrammatic section of mammalian lens. (after Pirie and van Heyningen 1956)


EPITHELIUM
LENS CORTEX
ANTERIOR CAPSULE
LENS EQUATOR
LENS NUCLEUS
POSTERIOR CAPSULE

driving lens research, is cataract. Cataract is the opacification of all or part of the lens and may be caused by injury, disease, toxins, or congenital anomalies (Berman 1991). The most important factor is, however, the processes and changes associated with normal aging. Cataract is a leading cause of blindness throughout the world (van Heyningen 1975). In the United States, for example, more than 45% of persons between the ages of 75 and 85 years have decreased vision because of cataract (Young 1991). The prevalence is not uniform but varies substantially depending on geography, genetics, nutrition and occupational exposure to sunlight or other sources of ultraviolet radiation (Taylor et al. 1988, Young 1991, ch.7-8). Surgical treatment can restore vision but is expensive and in many countries is simply not available to all.

Human Cataracts

The term cataract encompasses a diverse collection of physicochemical and morphological features (Duncan 1981). Common to all cataracts are areas in the lens of refractive discontinuity or light scattering. The present discussion mainly addresses the senile or "age-related" cataract. From a very simplistic point of view there are two main types of senile cataract, cortical cataracts and nuclear cataracts. There are also many instances in which both types will be

present in the same lens. These are called mixed cataracts. Cortical cataracts are visible opacities in the outer layers of the lens and are often described as spokes, wedges, cuneiform or coronary shapes (van Heyningen 1975). These cataracts are characterized by disturbance in the regular order of the lens fibers which may be swollen or distorted with fluid filled gaps. This type of cataract often reflects a metabolic or physiological disturbance of some kind. The result is a discontinuity in the refractive index. If the cortical cataract is near the equator of the lens (i.e. generally beyond the edge of the pupillary opening) it will not interfere with vision until it has spread to more interior portions of the lens. If it is instead located at the posterior pole of the lens, it is called a posterior subcapsular cataract and even a small area of involvement in this part of the lens can greatly affect vision.

Nuclear cataracts are the result of protein modifications in the very center of the lens and involve the kinds of changes seen in normal aging albeit greatly exaggerated. Protein aggregates form which are large enough to scatter light. These changes are often accompanied by change of the normal pale yellow lens color through deeper shades of yellow, browns and sometimes nearly black. Obviously, light scattering elements and light absorbing

pigments located in the center of the lens along the optical axis will be very detrimental to vision.

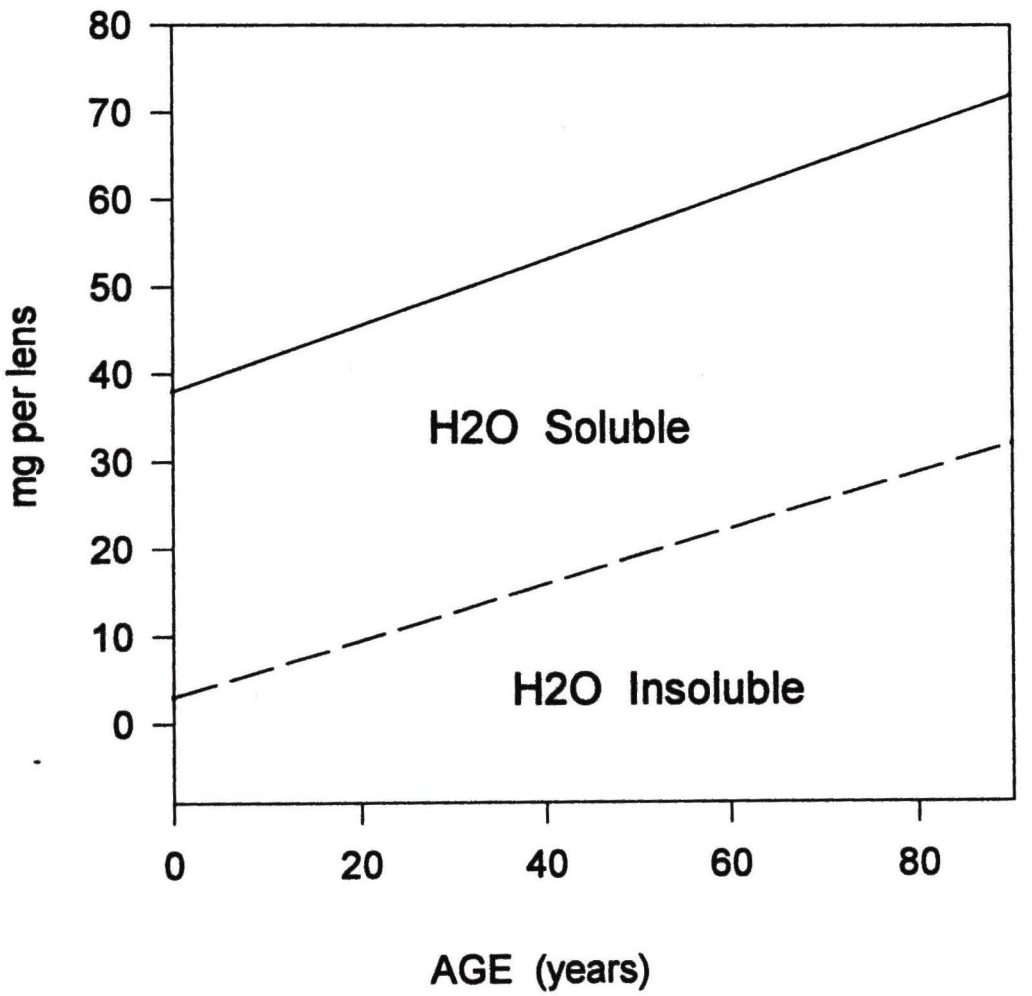
Aging and Human Lens Proteins

The human eye lens has a remarkably high protein content, approximately 35% on a wet weight basis (Spector 1984). Lens proteins accumulate a variety of modifications as they age. These include degradation of the carboxyl-terminus (Harding and Dilley 1976, Harding and Crabbe 1984), racemization of aspartic acids (Garner and Spector 1978), photooxidation of tryptophan (Pirie 1971, Zigler et al. 1976, Borkman 1977), phosphorylation (Spector, et al. 1985, Chiesa,Gawinowicz-Kolks and Spector 1987), O-linked N-acetyl glucosamine (Roquemore et al. 1992), glycation (Chiou et al. 1981) and formation of mixed disulfides with low molecular weight thiols (Lou, McKellar and Chyan 1986). It should be noted that not all of these post-translational changes are detrimental and in fact some (e.g. phosphorylation) may play important physiological roles in the lens. In fact, ordinarily the lens is able to perform its main functions, as a transparent refractive medium for light entering the eye, and accommodation to provide a sharp focus on the retinal surface in spite of the presence of many of these modifications.

The nature of the lens as a tissue in which turnover of proteins is virtually non-existent means that changes in these proteins are cumulative and thus several trends have been identified in the aging lens. Perhaps most profound is the progressive increase in the so-called "insoluble proteins"(Pirie 1968, Clark, Zigman and Lerman 1969, Spector, Roy and Stauffer 1975). The term "insoluble" does not imply that these proteins are insoluble in the lens but instead are insoluble under conditions *in vitro* in which they were formerly soluble. The amount of this insoluble protein is greater in the nucleus (inner,older) region of the lens then in the cortex (outer,younger) (Spector 1984) which suggests that accumulated modifications to the protein are involved in this insolubilization (Fig.2). In a similar fashion, a high molecular weight (HMW) protein fraction (so called because of its elution position in gel filtration) becomes increasingly prominent with age (Jedziniak et al. 1973, Spector, Li and Sigelman 1974, Jedziniak et al. 1975). Like the insoluble protein fraction, the HMW is much more pronounced in the nucleus reaching over 20% of the total protein by 80 yr while cortical levels are less than 5% (Jedziniak et al. 1975).

It is believed that the HMW proteins represent an intermediate stage in the proteins eventual insolubilization (Jedziniak et al. 1975, Roy and Spector 1976, Spector 1984) and the distinction between HMW and the insoluble protein

Figure 2. The change in dry weight of the normal human lens with aging. Protein is greater than 90% of the dry weight. (From Spector 1984).


80
70
60
50
40
30
20
10
0
mg per lens
H2O Soluble
H2O Insoluble
0
20
40
60
80
AGE (years)

fraction may be somewhat arbitrary, with insoluble proteins being operationally defined as those which precipitate at an average centrifugal force of 26,000 g (Roy and Spector 1976). It is interesting that cataractous (opaque) lenses do not necessarily have greater amounts of insoluble proteins or HMW fraction proteins than do age-matched transparent lenses. Although Jedziniak et al. (1973) reported an HMW (nucleus) value for a cataractous lens with nuclear sclerosis which was triple that of an old clear lens of similar age, further study showed that even this value was not unusual compared to other normal lenses in the sixth and seventh decades (Spector et al. 1974, Jedziniak et al. 1975).

The composition of the HMW aggregates seems to be primarily α- crystallin in the bovine lens (Spector et al. 1971) but in the human lens the situation is more complex. Amino acid analysis of the HMW from human normal and cataractous lenses reveal the composition to be different from α-, β- or γ- crystallins alone (Jedziniak et al. 1973). The aggregates appear to contain not only α- crystallin but also some β- as well as proteolytic products of approximately 11000 daltons (Roy and Spector 1976). There is also in cataractous lenses a 43000 dalton polypeptide which appears to be composed of portions of α- A and α- B crystallin chains joined by non-disulfide linkages (Spector and Roy 1978, Spector 1984).

It appears then that differences in the proteins and the forces holding them together are more germane to the issue of opacity than the actual quantities of HMW and insoluble fractions. Spector and Roy (1978) have shown that although the compositions of HMW aggregates (and the insoluble fraction) from normal and cataractous lenses are similar, the cataractous HMW aggregates are held together by disulfide linkages which are not present in the normal lenses. Further, the very large size of these covalently linked aggregates, especially within the insoluble protein fraction, may greatly exceed 10^6 daltons and is large enough to scatter light (Spector and Roy 1978).

From the above discussion it seems safe to conclude that as the lens ages changes occur in its proteins which eventually lead to the generation of very large aggregations containing altered or denatured proteins, truncated or partially degraded polypeptides and even some new polypeptide species which may have been formed from fragments of crystallins. In cataract these aggregations become covalently cross-linked through disulfide bridges and if of sufficient size are able to scatter light. Key to the understanding of lens ageing and cataractogenesis is understanding what sort of protein modifications precede the protein-protein disulfide formation.

Lens Protein Mixed Disulfides

The mammalian lens is endowed with a high concentration of glutathione which is mainly in the reduced form (GSH) (Table 1). Although humans and other primates have lesser amounts, the GSH is nevertheless at millimolar levels. These high levels of GSH have been the focus of a great deal of research over the past few decades. It is thought that the major biological function of GSH in the lens is to protect the lens from oxidative damage (Kinoshita 1964, Augusteyn 1979, Reddy 1979). Lens GSH has been found to be abnormally low in all types of cataracts (Tassman and Karr 1929, Pirie, van Heyningen and Boag 1953, Sippel 1966, Harding 1970, Truscott and Augusteyn 1977, Lou, et al. 1988 Lou, Dickerson and Garadi 1990) and declines with age (Harding 1970, Lou, et al. 1990). Kinoshita (1964) proposed that the glutathione was actually forming mixed disulfides with the lens proteins. It is the oxidized form of glutathione which is believed to participate in mixed disulfide formation according to the following reaction:

$$\text{Protein-SH} + \text{GSSG} \rightleftharpoons \text{Protein-SSG} + \text{GSH}$$

Attempts to quantify the amount of protein-glutathione mixed disulfide (PSSG or "bound" glutathione) employed a reductive approach in which the PSSG disulfides were

Table 1. GSH level in lenses from various mammals.

Animal	Age	Lens	μmole GSH/g	reference
cow	18 month	whole	4.47	1
cow	-	cortex	11.3-17.0	1
cow	-	nucleus	1.9-3.0	1
rabbit	6-15 month	whole	8.3-13.5	1
rabbit	-	cortex	8.7	1
rabbit	-	nucleus	3.4	1
horse	old	whole	3.1	1
squirrel	adult	whole	6.8	2
guinea pig	-	whole	4.1	1
guinea pig	-	cortex	14.0-19.6	3
guinea pig	-	nucleus	2.0-4.7	3
rat	2-11 month	whole	5.1-9.5	1
rat	2-11 month	cortex	9.4-15.4	1
rat	2-11 month	nucleus	1.8-4.0	1
rat	1.5 month	whole	4.5	2
monkey	1 year	whole	3.3	2

1. Pirie and van Heyningen 1956.
2. Lou and Dickerson 1992.
3. Padgaonkar, et al. 1994.

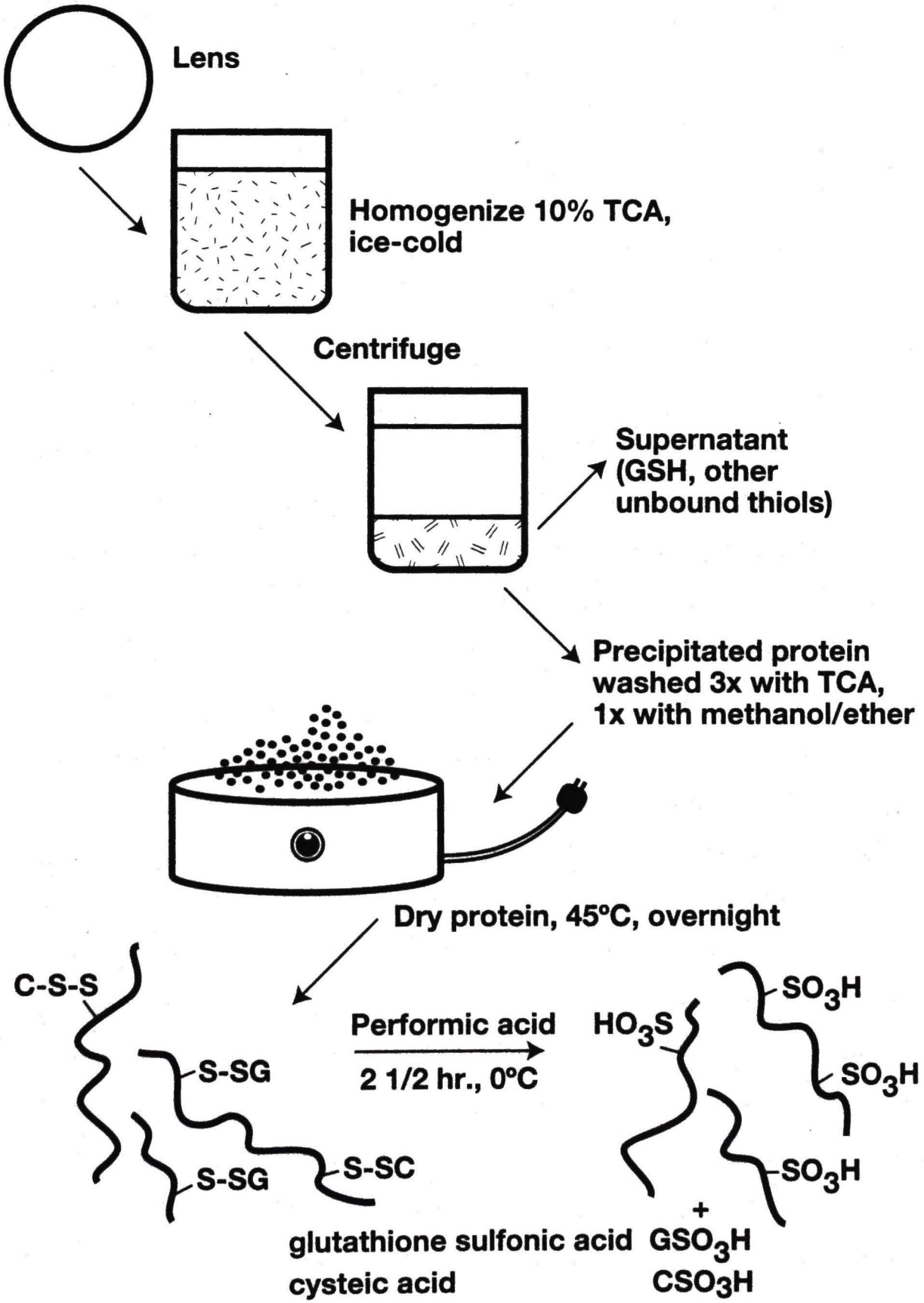
cleaved, with a powerful reducing agent such as sodium borohydride and the released thiols were quantitated colorimetrically with DTNB (Harding 1970, Reddy and Han 1976) or performic acid cleavage of the disulfide was followed by paper chromatography with ninhydrin detection of the resultant glutathione sulfonic acid (Harding 1970). Neither of these methods was satisfactory. The reductive method liberated thiols which were not separated (Harding 1970) and thus confirmation that the liberated thiol was GSH and not a mixture was not possible. The facility with which the reduced thiols undergo auto-oxidation complicated their chromatographic separation (Reddy and Han 1976), However from a qualitative standpoint, the identity of GSH as a major mixed disulfide component was established. Performic acid oxidation as employed by Harding (1970) did result in stable sulfonic acid derivatives of the liberated thiols but the paper chromatography detection method could not resolve glutathione sulfonic acid from other thiol derivatives which may have been present. Additionally, the procedure of incubating the lens homogenate overnight in TCA could certainly have generated oxidation of lens GSH and potentiated artifactual mixed disulfide formation. Therefore these values must be carefully considered.

A sensitive and accurate method for mixed disulfide analysis was developed by Lou et al. (1986,1987). This method employs performic acid oxidation to free the bound

thiols as stable sulfonic acids (Hirs 1956) and then utilizes anion-exchange amino acid analysis to separate and quantify the individual components (Fig. 3). This methodology has been the basis for several important findings. First, the chromatographic technique made possible the discovery that a second major protein-thiol mixed disulfide existed, namely cysteine (PSSC) and that at least for normal rat lenses was present at least seven-fold higher than PSSG (0.1 vs. 0.014 μmol gr^{-1}) (Lou et al. 1986). The high abundance of PSSC in the rat lens appears to be idiosyncratic; for human and monkey lenses the PSSG is the major mixed disulfide (Lou et al. 1986, Lou, Huang and Zigler 1989). The very high amount of PSSC relative to PSSG in the rat lens in spite of very high levels of free GSH and very low levels of free cysteine (Reddy and Kinsey 1966, Reddy, Klethi and Kinsey 1966, Reddy 1972) is a puzzle and is a question which is addressed in the present work.

Second, analysis of lenses in various cataract models for mixed disulfides has made clear the fact that both PSSC and PSSG generally increase following cataractogenic insult. These models include rat/H_2O_2 (Lou et al. 1986,1990), rat/photooxidative-rose bengal (Lou and Zigler 1986), rat/naphthalene dihydrodiol (Xu, Zigler and Lou 1992a) and rabbit/diquat (Yu et al. 1991) as well as insults in the lens culture system, UV radiation/squirrel (Zigman et al.

Figure 3. Oxidative method for release and quantitation of protein-bound thiols. Methodology of Lou et al. (1986,1987) involves oxidation of proteins in performic acid releasing bound thiols as the sulfonic acid derivatives. These may then be quantitated by anion-exchange amino acid analysis.


Lens
Homogenize 10% TCA, ice-cold
Centrifuge
Supernatant (GSH, other unbound thiols)
Precipitated protein washed 3x with TCA, 1x with methanol/ether
Dry protein, 45°C, overnight
C-S-S
S-SG
S-SG
S-SC
Performic acid
2 1/2 hr., 0°C
HO3S
SO3H
SO3H
SO3H
+
glutathione sulfonic acid GSO3H
cysteic acid CSO3H

1991), naphthalene/rat (Xu, Zigler and Lou 1992b) or hyperbaric oxygen/guinea pig (Giblin et al. 1992) cataracts *in vivo*.

Third and perhaps most important, the sequence of biochemical events from formation of mixed disulfides and disulfide-linked protein aggregates was established in a cataract model system. Lou et al.(1990) showed that PSSG increased in rat lenses cultured in 0.5 mM H_2O_2 in a time dependent fashion but began to decline by 48 hr in culture. Extending this work, Cui and Lou (1993) again demonstrated a time dependent increase in PSSG with a decline commencing between 48 and 72 hr of culture. This event coincided with nuclear opacification, appearance of PSSP aggregates in both water soluble and insoluble fractions and a drastic reduction in protein solubility. In addition after 48 hr of culture, PSSC was also elevated. Western blot analysis showed that the PSSP aggregates were composed of β- and γ-crystallins but not α- crystallin. This can be rationalized since β- and γ- are relatively rich in cysteine but not α-crystallin. It is somewhat at odds however with the composition of the HMW *in vivo* where α- is a significant component. These results are consistent with the notion that mixed disulfide formation is the primary event leading ultimately to protein-protein linkages. In this scheme GSSG formed through oxidative stress if not reduced to GSH will form PSSG. There are many more protein -SH groups than GSH

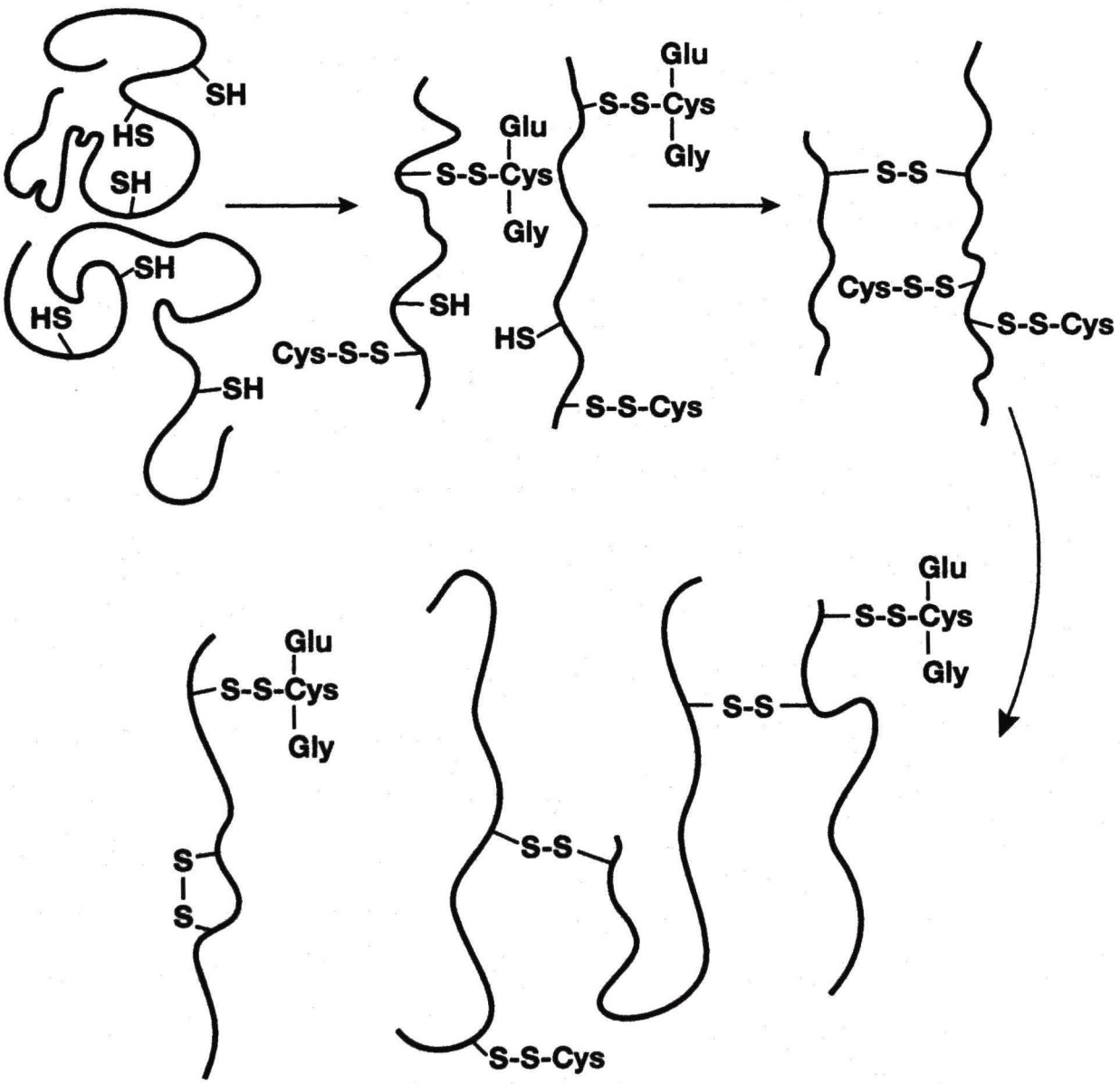
in the lens (Cui and Lou 1993). Thus GSSG once formed, can readily form PSSG and this modification can alter protein conformation (Liang and Pelletier 1988). This may expose other -SH groups (or other types of functional groups) for modification. The possibility that a protein -SH group will come into juxtaposition with a PSSG becomes greater as the level of PSSG increases. When this occurs, a protein-protein disulfide crosslink will form (Fig.4).

A major goal of the present work is to document the patterns of accumulation of these mixed disulfides in human lenses throughout the life span and to examine the levels seen in cataractous lenses. It will also be shown that there is a relation between PSSG and cataract formation in the human lens through the use of H_2O_2 as a cataractogenic agent in lens culture (as has been shown in rat lens).

Lenticular Thiol Levels

The formation of protein-thiol mixed disulfides presupposes that the thiol molecules, namely GSH and cysteine, are present and at sufficient levels such that mixed disulfides may form upon reaction of a protein -SH group and oxidized GSH or cystine (or alternatively a mixed disulfide of glutathione and cysteine, GSSC, could be the species involved).

Figure 4. Depiction of impact of thiolation of lens protein sulfhydryls causing unfolding of the polypeptide chain and subsequent aggregation via disulfide formation. (After Spector 1984)


SH
HS
SH
SH
HS
SH
Glu
S-S-Cys
Gly
Glu
S-S-Cys
Gly
SH
Cys-S-S
HS
S-S-Cys
S-S
Cys-S-S
S-S-Cys
Glu
S-S-Cys
Gly
S
S
Glu
S-S-Cys
Gly
S-S
S-S
S-S-Cys

The concentration of glutathione in the human lens is lower than for other species, ranging between 1.5 and 4.5 μmol gr^{-1} depending on age (Harding 1970). The amount of oxidized glutathione is generally much smaller (e.g. 5% of the total GSH)(Berman 1991). Reddy and Kinsey (1962) found that about 12% of the total GSH was oxidized for rabbit lens.

It is not difficult to imagine the source of the glutathione in the lens PSSG. There is a great deal of GSH in the lens, a certain portion of which is oxidized in the course of normal cellular events. This GSSG is, if not first reduced by glutathione reductase, free to react with protein -SH groups.

What is more perplexing is the origin of the PSSC, the cysteine mixed disulfide. The amount of free cysteine in the lens has always been considered to be quite low and as such has often been ignored. This is why previous efforts to quantitate lens protein mixed disulfides have never separated the individual components, instead considering all together as "protein-bound glutathione" (Harding 1970, Spector, Wang and Huang 1986) or "protein-bound sulfhydryls" (Truscott and Augusteyn 1977). The assumption that cysteine and other thiols were present at relatively minute levels compared to GSH has also influenced the reported free thiol levels as well. Probably the most common method for quantitation of free low molecular weight thiol molecules is

the spectrophotometric assay employing Ellman's reagent (DTNB)(Ellman 1959). Results are generally given as "GSH" rather than the more correct total free thiol.

The reason most workers consider cysteine levels in the lens to be insignificant is because of early reports that it was either not present in the lens or present at very low levels (Pirie and van Heyningen 1956, Reddy and Kinsey 1962, Reddy et al. 1966, Zigler et al. 1976,1977). Reddy's studies on rabbit lens used amino acid analysis to determine levels of the free amino acids in the lens. The earlier report was unable to detect any cysteine, while the latter reported 10 nmol gr^{-1}, a value over 1000-fold smaller than for free GSH. Clearly these low values are at odds with the observed presence of PSSC in lens at levels of similar magnitude to PSSG. Before invoking alternate sources for the bound cysteine it would be prudent to carefully consider the reliability of these early studies. In both, amino acid analysis was used with post-column ninhydrin detection. Cysteine quantitation has always presented a problem for this methodology. First, there is the problem of oxidation, but second and most serious is the inefficient color production. Ninhydrin reacts with α- amines to produce a blue color with an absorbance maximum at 570 nm. This reaction does not proceed readily for cysteine (Moore and Stein 1948) perhaps caused by interference from the sulfhydryl, as cystine and cysteic acid both have much

greater color yields (Moore and Stein 1948, Moore 1963). Therefore cysteine values obtained in this way will be underestimates of the actual amount present. The Zigler studies examined free amino acid levels in rat lenses as a function of age (Zigler et al. 1977) or in human lens with or without cataract (Zigler et al. 1976). Although a very different analytical method was employed for these studies (gas chromatography of N-trifluoroacetyl n-butyl esterified amino acids), only trace amounts of cysteine were detected. It may be that derivitization of cysteine is hindered for similar reasons as with ninhydrin.

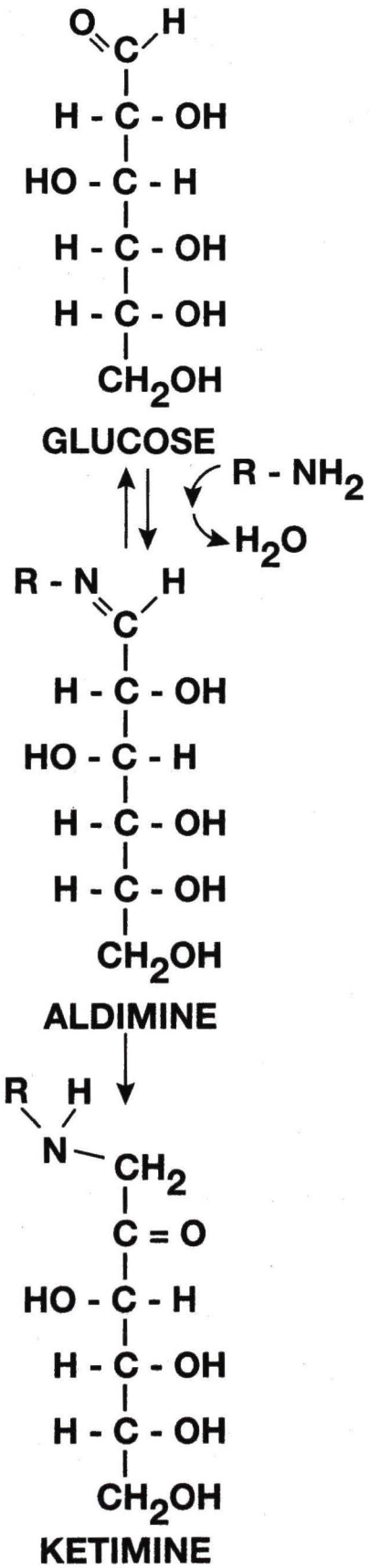
The actual amount of cysteine present in the different lens regions should be quantitated in order to better understand the formation of this mixed disulfide and to shed some light on why this mixed disulfide appears to be concentrated in the nucleus (Lou et al. 1990). Veltman and Lou (1993) measured free cysteine levels in rat lens cortex and nucleus and found that the cysteine levels were highest in the nucleus in agreement with the site of greatest PSSC accumulation. The present work examines the status of this thiol in human lens regions for both normal and cataractous lenses.

Glycation of Lens Proteins

Any alteration of the protein surface characteristics (e.g. charge, hydrophobicity or steric) could induce conformational changes allowing mixed disulfides and/or protein-protein disulfides to form. Glycation has been reported to effect these kinds of changes. Glycation is the non-enzymatic covalent addition of an aldose or ketose (or more generally any sugar-like molecule bearing a carbonyl group) to a protein's amino groups through the formation of a Schiff base and subsequent rearrangement to form a stable ketimine adduct (Fig 5). Typical agents used to study glycation include glucose, glucose-6-phosphate, fructose or ascorbate. It is important to distinguish glycation from glycosylation. In contrast to glycation, glycosylation is the enzymatic addition of sugars or oligosaccharides to a protein chain on Asn (N-linked) or Ser/Thr (O-linked). These processes are regulated and are normal features of some protein structures. They generally occur in discrete locations within the cell (e.g. endoplasmic reticulum /Golgi).

Glycation induced changes in a protein's conformation have been postulated to initiate cataractogenesis (Stevens et al. 1978). Glycation has been shown to alter the conformation and ligand binding characteristics of both human serum albumin and bovine α-crystallin. Conformational

Figure 5. Formation of glycated protein by the nonenzymatic addition of glucose to an amino group of the protein. A stable adduct is formed by an Amadori rearrangement of the aldimine to a ketimine.


O
H
C
H - C - OH
HO - C - H
H - C - OH
H - C - OH
CH2OH
GLUCOSE
R - NH2
H2O
R - N
H
C
H - C - OH
HO - C - H
H - C - OH
H - C - OH
CH2OH
ALDIMINE
R
H
N
CH2
C = O
HO - C - H
H - C - OH
H - C - OH
CH2OH
KETIMINE

changes were demonstrated for human serum albumin incubated with glucose (Shaklai, Garlick and Bunn 1984) and for lens crystallins incubated with either glucose (Liang and Chylack 1984) or glucose 6-phosphate (Beswick and Harding 1987). These studies have used fluorescence and circular dichroism (CD) spectroscopic techniques to evaluate the protein's conformational status.

Liang and Chylack (1984) incubated bovine calf α-crystallin with 55 mM glucose for one week. This treatment resulted in changes to the CD spectrum only in the near UV region (250-310 nm) suggesting changes in the protein tertiary structure. No change was observed in the far UV (<250 nm) suggesting little change in the secondary structure. Sulfhydryl micro-environment was also altered in the α-crystallin study (Liang and Chylack 1984). Beswick and Harding (1987) incubated bovine α-crystallin for 12 days with either 20, 50 or 100 mM glucose 6-phosphate. They also demonstrated alteration of the CD spectrum in the near UV. Interestingly, these changes were primarily increases in band intensity in contrast to those noted by Liang and Chylack (1984) which were slight decreases. The far UV region was, as in the former study, unaffected by glycation. Whether or not these changes were able to influence further protein changes was not addressed. Stevens et al.(1978) incubated bovine lens homogenates with either glucose 6-phosphate or glucose and reported an increase in protein-

protein disulfides. However, inspection of the published electrophoretic gel suggests a similar increase was also apparent in the control which was not glycated. Recently, others have reported similar findings (van Boekel and Hoenders 1991). Instructive as they are, these *in vitro* studies should not be extrapolated to the intact lens.

Whole lens studies (either *in vivo* or in culture) have been carried out primarily with lenses from diabetic rats (Monnier, Stevens and Cerami 1979, Perry, Swamy and Abraham 1987), galactosemic rats (Monnier et al. 1979) and human diabetic and senile cataractous lenses(Ansari, Awasthi and Srivastava 1980). The human lenses showed no correlation between the levels of PSSP or mixed disulfides and glycation (Ansari et al. 1980). The animal studies have been used as evidence that glycation promotes sulfhydryl oxidation.

The inherent complexity *in vivo* makes precise interpretation of data difficult. For instance, the high glucose level in the diabetic lens could promote not only glycation but also polyol accumulation, osmotic swelling (Kinoshita 1974), and perhaps even the generation of free radicals and hydrogen peroxide (Wolff, Jiang and Hunt 1991). Since all of these things may be occurring simultaneously, it is difficult to assign a causative role for any of these in sulfhydryl oxidation. It is therefore important to test alternative hypotheses in a system in which confounding processes may be controlled.

In this study the ability of glycation to produce conformational changes to lens proteins was evaluated *in vitro*. Purified bovine α- crystallin was incubated with ascorbic acid. Experiments similar to these have been carried out by others and have shown ascorbic acid (or the product of its oxidation, dehydroascorbate) to be a powerful glycating agent (Ortwerth and Olesen 1988, Prabhakaram and Ortwerth 1991,1992). Following incubation the conformational changes if any were evaluated with CD spectroscopy.

In the intact lens we have also employed *ex vivo* rat lens culture in order to evaluate the effect a high sugar environment and glycation have on mixed disulfide formation.

Purpose of This Study

The kinds of changes that take place in the human lens proteins during aging and ultimately cataract formation have been the focus of a concerted effort by many laboratories and literally hundreds of investigators over the past few decades. Indeed, much is now known about the kinds of changes that take place in the aging lens and in cataractogenesis. Less is known about the early changes which occur as a prologue to the aggregation, insolubilization and cross-linking of the lens proteins which are normal aspects of lens aging and occur to an

exaggerated degree in cataractogenesis. Cui and Lou (1993) have shown the importance of mixed disulfides in the formation of protein-protein disulfides in cultured rat lens exposed to oxidative stress. It seems reasonable to postulate that similar events occur in the human lens as it ages. The purpose of the present study is then to further establish the role protein-thiol mixed disulfides play in the process of aging as well as in cataract formation, both in organ culture model systems as well as in human senile cataract tissue. This will be attacked in several ways: 1) Document the pattern of mixed disulfide accumulation in human lenses (through nine decades) not only for PSSG but also for PSSC, protein-cysteine mixed disulfide. 2) Identify a hitherto unknown mixed disulfide species which was discovered in the course of this research. 3) Quantify the levels of the free thiols GSH and cysteine over the human lifetime and for the nucleus and inner and outer cortical regions of the lens. These are important because they represent the source for these modifications. The different lens regions are of different ages and biochemical environments, thus separate analysis of these regions may shed some light on differential mixed disulfide and HMW aggregate formation between these regions. 4) Mixed disulfides and free thiols were also quantitated in a number of cataractous lenses.

It is certainly possible that other types of protein modification such as glycation may alter protein conformation in a way analogous to that described for mixed disulfides and potentiate the formation of mixed or protein-protein disulfides as has been suggested by some investigators. This model was evaluated in two ways. First, purified alpha crystallin was incubated with ascorbate (a potent glycating agent) and conformational changes were evaluated with CD spectroscopy. Second, rat lenses cultured under high sugar conditions were used to determine if the high sugar milieu and resulting glycation influenced the level of mixed disulfides in these lenses. Conversely, the effect of prior mixed disulfide formation on the extent of glycation in another purified crystallin, gamma, was evaluated.

These studies have helped to delineate the role of protein thiolation in the normal aging process and in cataractogenesis. Appreciation for the role of mixed disulfides in lens aging may provide an avenue for therapeutic intervention in cataract.

CHAPTER II

MATERIALS AND METHODS

Materials

Reagents and Chemicals

Formic acid (88%) was purchased from the Mallinckrodt Chemical Co. (Paris, KY). L-cystinyl-bis-L-glycine was purchased from the Chemical Dynamics Corp. (South Plainfield, NJ). Hydrochloric acid (6M) and ninhydrin were products of Pierce Chemical Co. (Rockford, IL). Trione™ ninhydrin reagent was supplied by Pickering Laboratories Inc. (Mountain View, CA). TC-199 and penicillin-streptomycin (10,000 U each · mL^{-1}) were purchased from Gibco Laboratories, Inc. (Grand Island NY). Glutathione (oxidized and reduced forms), carboxypeptidases A and B, lithium chloride, calcium chloride (dihydrate), D-fructose, D-glucose, urea, D-xylose, xylitol, magnesium chloride, D-sorbitol, bovine serum albumin, cysteic acid, glutathione sulfonic acid, L-asparagine, zinc sulfate (0.3M sol.), and barium hydroxide (0.3M sol.) were all obtained from Sigma (St. Louis MO). Glycine, SDS-PAGE reagents, horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG and the

color development reagent were the products of Bio-Rad Laboratories (Richmond CA). Polyclonal rabbit antiserum for α- crystallin was the kind gift of Dr. Larry Takemoto of the University of Kansas. Acetic acid, sodium hydroxide (50%), sodium chloride, hydrogen peroxide (30%) trichloroacetic acid (TCA) and ammonium hydroxide (30%) were purchased from J.T. Baker Inc. (Phillipsburg NJ). D-[U-^{14}C]xylose was obtained from Amersham Corp. (Arlington Heights IL). AL01576 (spiro-(2,7-difluoro-9H-fluorene-9,4'-imidazolidine)-2',5'-dione) synthesized at Alcon Laboratories, Inc. (Fort Worth TX). Phenylboronate agarose (PBA-60) resin was purchased from Amicon Corp. (Danvers MA). The water used in these experiments was supplied by an Ahlfinger Water Co. (Dallas TX) water treatment system which supplied >17 Megaohm water. All other chemicals were of reagent grade.

Crystallins

Purified α- crystallin was obtained from bovine lenses as follows. Fetal calf eyes were obtained from a local slaughterhouse. The lenses were removed, decapsulated and placed in 4 mL/gram lens weight of 50 mM Tris, 0.2 M NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol, pH 7.4. The lenses were gently stirred with a magnetic bar at 4°C for 30 min. The nucleus was discarded and the resulting suspension was

disrupted in a ground glass #22 homogenizing tube. The homogenate was centrifuged (rcf=105,000g) and the supernatant was applied to a Sephadex G-200 column. Purified α- crystallin was obtained by elution with the same buffer (Zigler and Sidbury 1976) and by collecting and pooling fractions corresponding to the first peak (absorbance monitored at 280 nm). The identity of this material was verified to be α- crystallin by SDS/PAGE and Western blot analysis. Later experiments used bovine α-crystallin purchased from Sigma Chemical Co. (St. Louis, MO). The bovine γ- crystallin was obtained from Sigma.

Animals

The use of animals in this study conformed to the Association for Research in Vision and Ophthalmology (ARVO) Resolution on the Use of Animals in Reserch.

Male Sprague-Dawley rats were used exclusively for all lens culture experiments (Harlan Sprague-Dawley, Houston TX and Indianapolis IN). The average body weight of the rats was either 100 or 200 g depending on the experiment. Only 100 g rats were used for glycation measurements; both size classes were used in the mixed disulfide quantitation. The rats were sacrificed by asphyxiation with CO_2.

Human Lenses

Normal human lenses were obtained from the Florida Lion's Eye Bank, the San Diego Eye Bank and the Medical Eye Bank of Western Pennsylvania. The lenses were enucleated 15-24 hrs postmortem, stored at -80°C in an individual vial until use (1-4 weeks) except for those used in culture experiments which were used immediately. Cataractous lenses used in the mixed disulfide determinations were the kind gift of Dr. Venkat Reddy (Oakland University, Rochester, MI) and had been extracted from cataract patients in India. Cataractous lenses used for the free cysteine determinations were obtained from Dr. Seymour Zigman (University of Rochester, Rochester, NY). All had been shipped on dry ice and stored at -80°C prior to analysis.

Lenses split into cortex and nucleus portions for mixed disulfide analysis were dissected with a 4 mm (i.d.) cork borer. The core minus an approximately 1 mm slice from each end was considered nucleus (approx. 25-30% lens wet weight), the balance, (including capsule and epithelium), was considered to be cortex.

Methods I. Protein-Thiol Studies

Free Thiol and Protein-Thiol Mixed Disulfide Quantitation

Ice-cold TCA was added to lens homogenates for a final concentration of 10% (w/v), followed by centrifugation at 1600*g* for 15 min. The TCA supernatant was used for determination of free thiols (Lou et al. 1988). The protein precipitate was used for quantitation of protein-thiol mixed disulfides. Protein bound thiols were quantitated as the sulfonic acid derivatives released upon oxidation with performic acid (Lou et al. 1986,1988, Lou and Dickerson 1992, Dickerson and Lou 1993). The TCA pellet was washed three times with TCA and one additional time with methanol/ether (1:1, v/v). This pellet was then dried thoroughly on a 45°C hot block. After pulverizing with an agate mortar and pestle, the dried powder was reacted with performic acid in order to oxidize all thiol and or disulfide groups to the corresponding sulfonic acids. Freshly made ice-cold performic acid (4.5 mL 88% formic acid, 0.5 ml 30% hydrogen peroxide; allowed to react at room temperature for 1 hr; chilled on ice for 30 min prior to use) was added to the protein (≤ 24 mg/0.5 mL aliquot) and allowed to react (on ice) for a period of 2½ hr. The reaction was halted employing the method of Hirs (1956) in which the excess performic acid was removed through dilution

in 85 mL of ice-cold H_2O. This was then shell-frozen and lyophilized. Chromatography was carried out as follows. Samples were applied to the column (Amino-Pac® PA1, Dionex Corp.) in a 10 mM $Na_2B_4O_7$/120 mM NaOH/2% methanol mobile phase. Elution of the sulfonic acid derivatives was achieved through a sodium acetate gradient (0.15 to 0.5 M Na acetate, pH 13.2 over 4 min; 0.5 M maintained for 4 min). The flow rate was maintained at 1.0 mL/min. The column effluent was mixed with the ninhydrin reagent (0.76 mL/min) and heated through a 130°C reaction coil followed by absorbance measurement of the ninhydrin positive products at 570 nm.

Lens Protein Fractionation

Lens proteins were separated into water soluble and water insoluble fractions. Individual human lenses were homogenized in 4 mL 0.02 M sodium phosphate buffer, pH 7.4, 0.01 M EDTA, 0.1 M NaCl. The homogenate was centrifuged for 30 min at 17,000 g and 4°C. The supernatant was collected and added to an equal volume of 20% ice-cold TCA followed by centrifugation at 2,500 rpm for 15 min. The dry weight of the TCA pellets was used as equivalent to the quantity of WS protein. The pellet from the 17,000 g centrifugation was resuspended in 2 mL of the homogenizing buffer and re-centrifuged. This time, the supernatant was discarded with

the pellet saved as the water insoluble protein fraction (WI). Both the WS and WI portions were dried thoroughly, weighed and processed for mixed disulfide quantitation. The dry weight of the WI pellet was used as the amount of WI protein.

γ-Glutamylcysteine Production from Glutathione

Positive identification of γ-glutamylcysteine (γ-Glu-Cys) as a third important protein mixed disulfide component was made possible by producing γ-Glu-Cys through two distinct methods. In the first, glutathione was subjected to incomplete acid hydrolysis (Moore and Stein 1963) in order to generate a mixture containing all five possible fragments, namely, the three constituent amino acids glycine, cysteine and glutamic acid, as well as the two possible dipeptides, γ-glutamylcysteine and cysteinyl-glycine.

Reduced glutathione (GSH) (2 mg) was placed in a test tube with 1.0 mL 6 M HCl and vortexed. 0.33 mL of this mixture was placed in a vacuum hydrolysis tube (Pierce Chemical Co., Rockford, IL) and frozen on dry ice. The tube was then evacuated to 50 mtorr and placed on a hot block at 105°C for five hr and then cooled to room temperature. The hydrolysate was then frozen and lyophilized to dryness.

The GSH hydrolysate was reacted with performic acid exactly as described for the dried lens protein samples so that the sulfonic acid derivatives generated could be compared with authentic standards and the lens samples.

A second method of producing γ-glutamylcysteine involves carboxyl-terminal degradation of oxidized glutathione with carboxypeptidase (Strumeyer and Bloch 1962, Anderson and Meister 1985). Treatment of GSSG with this enzyme should cleave glycine and leave (bis)-γ-glutamylcysteine disulfide intact, as its peptide bond is a γ-carboxyl peptide bond rather than α-carboxyl and thus not a substrate for the enzyme. Oxidized glutathione (1 gr) was dissolved in 25 mL water and the pH was adjusted to 8.0 with NH_4OH. Carboxypeptidase A (40 mg, approx. 2,500 U) and carboxypeptidase B (10 mg, 1,050 U) were added to this solution (unit defined as hydrolysis of 1.0 mole hippuryl-L-phenylalanine min^{-1} at 25°C, pH 7.5) and the mixture was placed in a 37°C water bath for 8 hr. The enzyme was removed by ultrafiltration through centriprep-10 concentrators™ (Amicon, Danvers, MA), and an additional 10 mg (approx. 500 U) of carboxypeptidase A was added prior to an additional 14 hr incubation. The reaction mixture was again filtered to remove the enzyme and the resultant filtrate was frozen at -20°C prior to purification of the di-γ-glutamylcysteine. The di-γ-Glu-Cys was purified on a Dowex AG1 (8% cross-linking, formate form) anion exchange

column (Bio-Rad Laboratories, Richmond CA). After all glycine had been removed with water (525 mL), the dipeptide (along with remaining GSSG) was eluted with 4 M formic acid. This purification was carried out at 4°C. All formic acid fractions with a positive ninhydrin reaction were pooled and lyophilized. A portion of the dried residue was then subjected to performic acid oxidation exactly as outlined above in order to generate the sulfonic acid, γ-glutamyl cysteic acid.

Additional evidence that the product of this enzymatic digestion was γ-Glu-Cys was obtained by acid hydrolysis. If the product is γ-Glu-Cys, acid hydrolysis should yield equimolar amounts of glutamate and cysteine (or in this case cysteic acid since the performic acid oxidized form, γ-glutamylcysteic acid was used). Fractions corresponding to the presumed γ-Glu-Cysteic acid peak obtained from amino acid analysis were collected and subjected to acid hydrolysis as described above for GSH. Amino acid analysis was performed on the resulting products as follows. The reconstituted sample was applied to the column (Dionex Amino-Pac, PA-1) in a 0.23 M NaOH/0.007 M $Na_2B_4O_7$ mobile phase at a flow rate of 1.0 mL min^{-1}. This was continued for 10 min at which point a 4 min gradient changing from the above to 0.08 M NaOH/0.018 M $Na_2B_4O_7$/2% methanol ensued. The endpoint of this gradient was maintained for six min followed by a step change to 90% of this eluent and 10% 0.4

M Na acetate/0.001 M NaOH/2% methanol. A linear gradient was initiated at this point ending 10 min later with 100% of the acetate eluent. Glutamate and cysteic acid were identified by comparison with authentic standards which eluted at 14 min and 35 min respectively.

HPLC Assay for Free Cysteine and GSH

Frozen human lenses were dissected into three portions using stainless steel cork borers. A 4 mm (i.d.) borer was used to separate the outer cortex from the balance of the lens. Approximately 1 mm was shaved from both ends of the resultant core and added to the outer cortical ring. In a similar way, the core was divided into nucleus and inner cortex portions with a 2 mm (i.d.) borer.

The extraction and quantitation of the free thiols followed the method of Fahey and Newton (1987) with some modification. Lens pieces were homogenized in ice-cold 200 mM methane sulfonic acid (0.5 mL for nucleus, 1.0 mL for cortical pieces). An equal volume of 4 M Na^+ methane-sulfonate was added to each followed by centrifugation for 5 min at rcf = 14000g. The supernatants (0.5 mL) were mixed with 1.5 mL HEPPS buffer (200 mM HEPPS/200 mM methanesulfonate/5 mM DTPA) and monobromobimane to 3 mM. These were allowed to stand in the dark for 10 min. All

samples were re-centrifuged for 5 min and stored at -80°C pending analysis.

Chromatography of the derivatized samples employed a Dionex BioLC system equipped with a Beckman ultrasphere ODS-5 reverse phase column (4.6 x 250 mm). The mobile phase consisted of 0.25% glacial acetic acid (v/v)/57.4 mM sodium perchlorate (anhydrous) and an initial acetonitrile concentration of 6.5% (v/v), pH 3.9. Flow rate was set at 0.7 mL min^{-1}. The acetonitrile concentration was increased to 14.75% at 5 min and at 14 min a linear gradient commenced from 14.75% to 80% acetonitrile by 16 min. The system was returned to initial conditions at 20 min and the run terminated at 35 min. Dreivatized thiols were detected with a Dionex flourescence detector (ex.λ=265 nm, em.λ=455 nm).

A reagent blank was prepared in parallel with the lens samples. A few lens samples were pre-incubated with 5,5'-dithio-bis(2-nitrobenzoic acid)(DTNB), which would preempt bromobimane derivitization, in order to verify that detected peaks were indeed thiols.

Methods II. Lens Organ Culture

Rat or human lenses were cultured in modified TC-199 media as described by Zigler and Hess (1985).

Rat Lenses

Each culture experiment generally consisted of 24 lenses. Rats were sacrificed and the eyes were removed and enucleated through a posterior incision. Lenses were gently teased free of other ocular tissue and then placed one/well in a 24 well plate with 1.5 mL media/well and incubated in a 37°C, 5% CO_2 incubator. Media was exchanged for fresh every day for the duration of the experiment.

Human Lenses

Human lenses were cultured essentially as for the rat lenses. The main differences were that a six-well plate (9 mL/well) was used with one lens per well, and that the duration of the experiment was 36 hr with one change of media at 18 hr. One lens was maintained in normal media and the contralateral (to induce a cataract) received an aliquot of 0.3% H_2O_2 solution and 0.2 U of glucose oxidase in order to maintain the H_2O_2 level at 0.5-0.6 mM for the duration of the experiment (Lou et al. 1990). The concentration of H_2O_2

in the media was routinely checked with a YSI Model 27 analyzer (Yellow Springs Instruments, Yellow Springs, OH, USA) at the end of the study to verify correct H_2O_2 concentration. At 36 hr both lenses were removed from the media, washed in saline, blotted on filter paper and weighed. Gross morphological features were recorded.

Methods III. Glycation Experiments

Three different sugars or sugar-like compounds were selected as glycating agents for use in the following studies. In the early experiments ascorbate was used. In the presence of O_2 it is rapidly converted to DHA and in this form is a very potent glycating agent. It is also present in the lens in mM concentrations and thus had physiological relevance (Heath 1962). Ascorbate was used in the CD/conformational studies of bovine α- crystallin. Ascorbate was not used in the lens culture studies because it is already present at high levels in the lens and its instability in the presence of oxygen would make it more difficult to control media levels. Further, it is a redox active compound which could effect changes in lens GSH levels, thus potentiating mixed disulfide formation through non-glycation routes.

Xylose was chosen for the lens culture studies for a variety of reasons. First, xylose is an active glycating

agent; a good deal of xylose is present in the acyclic (reactive) form (Kaanane and Labuza 1989). Second, xylose is relatively inert physiologically. Although xylose can be metabolized in the lens by aldose reductase resulting in accumulation of xylitol and ultimately an osmotic-stress induced sugar cataract, in the presence of an ARI this reaction can be completely blocked. Xylose is not otherwise utilized by the cell therefore its effect on the cellular homeostasis should be limited to its glycation activity.

Fructose is frequently used as an osmolyte in normal control lens culture media (e.g. Kinoshita, Merola and Tung 1968, Zigler and Hess 1985) because it is not a substrate for aldose reductase (in fact it is the end product of the pathway). It became apparent during these experiments that fructose was as good a glycating sugar as xylose and thus other controls (xylitol, NaCl) were added.

Ascorbate Modification of Bovine α- Crystallin

The oxidized form of ascorbate, dehydroascorbate (DHA) is known to be a potent glycating agent and has been shown to glycate α- crystallin and form cross-linked products *in vitro* (e.g. Ortwerth and Olesen 1988). It is also well known that the aqueous humor (the fluid bathing the anterior portion of the lens) and the lens itself contain millimolar concentrations of ascorbate. Therefore experiments were

conducted to evaluate the potential for ascorbate (or DHA) induced structural changes to α- crystallin as detected by circular dichroism. Purified bovine α- crystallin was brought to 1 mg/mL in 0.1 M potassium phosphate, 1mM diethylenetriaminepentaacetic acid (DTPA), pH 7 which had been degassed by bubbling with argon for 30 min. This was dispensed into a sterile 24 well tissue culture plate through a 0.2 µm Gelman Acrodisc syringe filter. A radiolabeled 0.5 M solution of ascorbic acid was prepared from ^{14}C-ascorbic acid adjusted to a specific activity of 2.5 µCi/mmole. Zero, 10 or 20 µL of this solution was added to sample wells for 0, 5 or 10 mM final ascorbic acid concentrations. The plate was then placed in a 37°C incubator. After one or two weeks, aliquots were removed from the wells, and dialyzed against two 2 L changes of fresh phosphate buffer, and analyzed for ^{14}C-ascorbate incorporation. These were then used for CD spectroscopy. A second experiment utilized ^{14}C-ascorbic acid adjusted to a specific activity of 250 µCi/mmole, 100-fold greater than the first experiment, in an effort to increase the amount of radioactivity incorporated into the protein. As for the first trial, 0, 5 or 10 mM concentrations of ascorbate were used, however, airtight snap-top glass vials instead of a tissue culture plate were used to house the incubation mixtures and these were only incubated for one week.

The effectiveness of the dialysis in the first experiment in removing non-covalently bound ascorbate was evaluated with three replicates of each sample. One of each was again dialyzed as before. A second replicate was brought to 10% TCA by the addition of an appropriate volume of 100% (w/v) TCA. This was then allowed to stand on ice for 10 min followed by centrifugation in an Eppendorf microfuge to pellet the protein. This was repeated until the radioactivity in the wash was indistinguishable from background. A third replicate was dialyzed similar to replicate number one except that 200 mM cold ascorbate was added to the first dialysis buffer in order to "chase" any non-covalently bound ascorbate from the protein. The washed/dialyzed protein samples were all placed in scintillation cocktail for measurement of incorporated ascorbate.

Circular Dichroism Measurements

Circular dichroism (CD) spectra were obtained with an Aviv CD spectrometer (model 62 HDS). Samples were normalized to 0.13 mg protein mL^{-1} and placed in quartz cuvettes (0.2 cm pathlength). Spectra were recorded at 25°C, at 1 nm intervals with a 3 s dwell time.

Culture of Lenses in High Sugar Media

Ten separate lens culture experiments were conducted to assess the extent of lens protein glycation following one week of incubation in media containing either 30 mM xylose, 30 mM fructose and in two experiments, 30 mM xylitol or 15 mM NaCl. AL01576, an aldose reductase inhibitor(ARI), was added (10 μM) to the xylose media in all experiments to prohibit metabolism of the xylose to xylitol and thus prevent cataractogenic osmotic stress (Obazawa, Merola and Kinoshita 1974). Media was replaced daily until termination of the experiments. Harvested lenses were weighed and either frozen immediately on dry ice if processing could not be completed on the same day, or homogenized in 1.0 mL ice-cold 0.02 M EDTA. The homogenate was reserved for boronate affinity chromatography, and for quantitation of free GSH and lens protein mixed disulfides.

Xylose Penetration

In order for xylose glycation of lens proteins to occur during lens incubation, the xylose must penetrate the lens cells and attain effective intracellular concentrations. Xylose levels in rat lenses cultured in media with 30 mM D-xylose were measured after four and 12 hr. Four lenses were cultured in the xylose containing media as the experimental

group, another four lenses were cultured in fructose containing media (30 mM) as the control group. Three fresh lenses were also included as a third group for comparison with the cultured controls. Lenses (two in each group) were harvested at four and 12 hr. Lenses were removed with teflon covered forceps, blotted gently on filter paper and weighed. Free monosaccharides were extracted (Hayman et al. 1966) by homogenization in 0.5 mL ice-cold 0.3 M $ZnSO_4$. The homogenates remained on ice for 10 min followed by the addition of 0.5 mL 0.3 M $Ba(OH)_2$ and centrifugation for 15 min at 2,500 rpm. The supernatant (0.5 mL) was lyophilized pending xylose quantitation.

Xylose Measurements

The above dried samples were reconstituted in 1.0 mL 0.15 M NaOH and diluted 500-fold with the same solution. 50 µL of each was used for xylose measurement (Dickerson and Lou 1990) using a Dionex anion exchange chromatographic system with pulsed amperometric detection (PAD).

Boronate Affinity Chromatography

Phenylboronate agarose (PBA) chromatography was employed to determine the percentage of the lens proteins which were glycated. PBA resin binds reversibly to

molecules with cis-diol groups. PBA columns (2 mL bed volume) were prepared from Amicon PBA-60 (60-100 μmol boron mL^{-1}) gel and poly-prep plastic chromatography columns (Bio-Rad Laboratories, Richmond CA).

Prior to each assay, columns were washed with 6 mL 0.5 M NaOH followed by 6.0 mL 0.1 M acetic acid and then equilibrated with 12 mL starting buffer (100 mM Asn, 10 mM $MgCl_2$, pH 9.0). The above sample, 500-700 μL (1-2 mg protein) was then applied to the top of the column followed by 0.1 mL starting buffer. Columns were capped and allowed to equilibrate overnight in the cold room. Non-glycated protein was eluted from the column with 12-16 mL of reagent A (100 mM Gly, 10 mM $MgCl_2$, 7 M urea, pH 9), collected as four or six fractions. The column bound glycated protein was eluted with 6 mL of reagent B (100 mM sorbitol, 7 M urea) at room temperature and collected as one fraction.

The amount of protein present in these fractions was quantitated with the bicinchoninic acid assay (Pierce, Rockford IL) with bovine serum albumin as a standard.

Boronate Affinity Chromatography: Sample Preparation

Aliquots (0.4 mL) of the above homogenates were brought to 3 mL with 0.1 M potassium phosphate, pH 6.0 and reduced with $NaBH_4$ (10-20 μmole/mg protein) (Bookchin and Gallop 1968) in order to convert the Schiff base adducts and keto-

amine adducts to more stable hexitols. This mixture was allowed to react at room temperature for 10 min followed by an additional 50 min at 4°C. The samples were then transferred to dialysis bags (Spectra-Por 1, mol.wt. cut off=6-8,000, Spectrum Medical Industries, Inc., Los Angeles CA) and dialyzed against 4 L water (with one change) in the cold room. The dialyzed samples were then concentrated to about 100-200 µL in Centricon-10 tubes (Amicon Corp., Danvers MA) and diluted with 500 µL of sample buffer (7 M urea, 100 mM HEPES, 25 mM NaCl, 1 mM EDTA, pH 8.5). The samples were then applied to the affinity columns.

Bovine Serum Albumin/D-[U-^{14}C]Xylose Conjugates

Bovine serum albumin (BSA) glycated with radiolabeled xylose was prepared in order to test the PBA column for xylose/protein binding capability. The xylose modified BSA was prepared by incubating 8 mg BSA with 0.6 µmoles of D-[U-^{14}C]xylose (83 µCi/µmole) (1 wk, N_2 atm., 37°C) in 2 mL water. Following this incubation, the mixture was dialyzed exhaustively (until radioactivity could not be detected in the dialysis medium) and prepared for phenylboronate chromatography.

β-Elimination of O-Linked Sugars

The possibility that lens proteins were binding to the PBA columns due to sugar adducts in O-linkage to the proteins (covalently bound through enzymatic addition to Ser or Thr), was evaluated by comparing PBA column binding before and after treatment to cleave these linkages.

Cleavage of any protein-sugar O-linkages was accomplished through base-catalyzed β-elimination (Holt and Hart 1986). Aliquots of the above described $NaBH_4$ reduced lens homogenates were made 1M in sodium borohydride and 0.1M in NaOH. These were incubated for 18h at 37°. The samples were then dialyzed against 2x4L H_2O and 1x2L H_2O. These were then concentrated to about 100 µL, and prepared for boronate affinity chromatography as described above.

SDS-PAGE Profiles of PBA-60 Bound Lens Proteins

Homogenates from selected lenses, both fructose and xylose incubated, were analyzed by SDS-PAGE (Laemmli 1970) prior to fractionation on the PBA-60 affinity columns. Proteins which were eluted from the PBA-60 affinity columns in the "bound", glycated fractions for these same lenses were also analyzed. Samples were heated in boiling water for three minutes in 1% SDS + 1% β-mercaptoethanol prior to application on a 15% slab gel. Reducing conditions were

utilized because the intent was to determine if there were any obvious differences in the kinds of proteins present in the original homogenate and in the post-column glycated protein fraction; the presence of crosslinked species would make this more difficult. Approximately 10 µg of protein was loaded in each case. Electrophoresis commenced at 80 volts for 20 min followed by 130 volts until the dye front reached the bottom of the gel. Protein bands were stained with coomassie blue.

Synthesis of γ- Crystallin/Glutathione Mixed Disulfides

Commercially obtained γ- crystallin (Sigma) was reacted with glutathione as descibed by Slingsby and Miller (1985). A 1 mg mL^{-1} solution of γ- crystallin was prepared in H_2O. This was dialyzed against 4 L of water with three changes. The protein was finally equilibrated in 50 mM Tris/acetate pH 8.5 by dialysis against 2 L of this buffer. The concentration of the γ- crystallin sample was measured spectrophotometrically ($A^{1\%}_{1cm}$=21) and adjusted to 1 mg mL^{-1}, if necessary. The γ- crystallin was divided into three portions, one receiving 6.15 mg GSH (1 equivalent GSH; 1 mole GSH per mole γ- crystallin; MW = 20,000), one receiving 30.75 mg (5 eq. GSH) and one incubated without GSH. These samples were maintained at 20°C for 1 hr followed by a minimum of 18 hr in the -20°C freezer. It has been shown

that freezing and thawing enhances the reactivity of certain γ- crystallin cysteine residues towards low molecular weight thiols (Slingsby and Miller 1985). All samples were then thawed and dialyzed (to remove excess GSH and GSSG) in 0.1 M K^+ phosphate/10 mM DTPA pH 7 (4 L, two changes). Samples were then stored at -80°C.

Glycation of γ- Crystallin/GSH Mixed Disulfides

Samples were thawed and an aliquot (1 mL) of each was removed and used for mixed disulfide quantitation as described for lens proteins above. The balance of the samples (2.4 mL) were dispensed into small snap-cap vials, purged with N_2 and each given 50 μCi of D-[U-^{14}C] fructose. These were then placed in a 37°C incubator for 1 week. The proteins with incorporated fructose were separated from unbound fructose by precipitation from solution through the addition of 100% TCA to a final concentration of 10%. These were then placed on ice for 15 min followed by centrifugation for 15 min in an Eppendorf microfuge. The pellets were washed two additional times in TCA. Washed pellets were re-suspended in 0.5 mL 0.3 M K^- phosphate/0.15 M EDTA pH 7.6, and transferred to scintillation vials for measurement of incorporated radioactivity.

Methods IV. General Techniques

Protein Assays

The protein concentration of PBA column effluent fractions or of crystallin incubation mixtures was routinely measured using the bicinchoninic acid (BCA) assay, Pierce Chemical Co., Rockford, IL].

Western Blots

Proteins were transferred from SDS-PAGE gels onto 0.1 µm nitrocellulose (100 V/1 hr) for immunoblot analysis (Nakamura et al. 1988). The membranes were blocked with gelatin (3% in TBS), rocked for 1 hr at room temp., and then incubated with polyclonal antisera for α- crystallin (with 1% gelatin in TBS) overnight on the rocker table. The blots were rinsed with distilled water and washed twice with TBS for 10 min each. The secondary antibody, goat anti-rabbit IgG (HRP conjugated), was added in 1% gelatin/TBS and allowed to rock for 1 hr followed by washing as before. The membranes were then developed with HRP color developing reagent and the stained bands were compared to the original SDS-PAGE gel pattern.

Methods V. Statistical Analysis

Routine statistical methods are given in figure legends or table footnotes. More unusual tests are detailed below.

Human Lens Mixed Disulfides

Linear regression analysis was employed to evaluate the relationships between age and GSH, PSSG or PSSC. The inverse relationship of lens free GSH and protein-thiol mixed disulfides was evaluated using Kendall's coefficient of rank correlation (Sokal and Rohlf 1981).

γ-Glutamyl Cysteine

T-tests for the equality of means from samples with unequal variances (Sokal and Rohlf 1981) were employed to test the hypothesis that lenses from donors of age 60 or greater contain greater levels of γ-Glu-Cys mixed disulfide than those from younger donors, and to test the hypothesis that cataractous lenses have higher levels of γ-Glu-Cys mixed disulfide than do clear lenses of similar age. In some cases where only a single cataractous lens was available for a particular age group, a special t-test designed to compare a single variate with a group mean was employed (Sokal and Rohlf 1981).

Lens Culture/Glycation Experiments

Two-way analysis of variance (ANOVA) (weighted averages method for rx2 tables)(Steel and Torrie 1960) was used to analyze effects on mixed disulfide levels due to rat weight, culture conditions and the interaction between the two. Differences between pairs of means were evaluated using the approximate t-test method (ch.13,Sokal and Rohlf 1981). Two-way ANOVA for paired comparisons (Sokal and Rohlf 1981) was employed to test the data of Table 5. Single-factor ANOVA (Sokal and Rohlf 1981) was used to evaluate differences in lens GSH content, in mixed disulfide levels between the lens categories for the xylitol experiment, and in lens protein glycation levels. The Tukey-Kramer method for multiple comparisons (Sokal and Rohlf 1981) was used to evaluate differences between all pairs of means in the xylitol experiment, and among the treatment groups for the lens protein glycation study.

CHAPTER III

RESULTS

Effect of Age on Protein-Thiol Mixed Disulfide Level in Human Lenses

A total of 59 normal human lenses between 3 months and 88 years of age were processed and analyzed for free GSH, PSSG and PSSC levels. As shown in Fig. 6, human lens GSH decreased gradually with age showing a negative correlation ($r=-0.52$, $P<0.0005$). The value was highest in the first decade averaging between 3-4 μmol g^{-1} wet weight, decreasing to less than 2 μmol g^{-1} by the sixth decade.

The PSSC level was very low (0.01 μmol g^{-1} wet weight) during the first few years of age but gradually increased to 0.04 μmol g^{-1} by the third decade and continued to increase linearly until by 60 years the level had reached 0.1-0.12 μmol g^{-1} wet weight. This significant and positive correlation ($P<0.0005$) between PSSC and age is demonstrated in Fig.7.

The relationship between PSSG and age is more complicated. While PSSC increased monotonically with age, PSSG exhibited a triphasic pattern of accumulation (Fig. 8). The level of PSSG increased linearly between 3 months

Figure 6. Free GSH levels in normal human lenses as a function of age. Individual GSH level is expressed in μmol g^{-1} wet lens weight. Linear regression line shown, $r=-0.52$, $P<0.0005$.

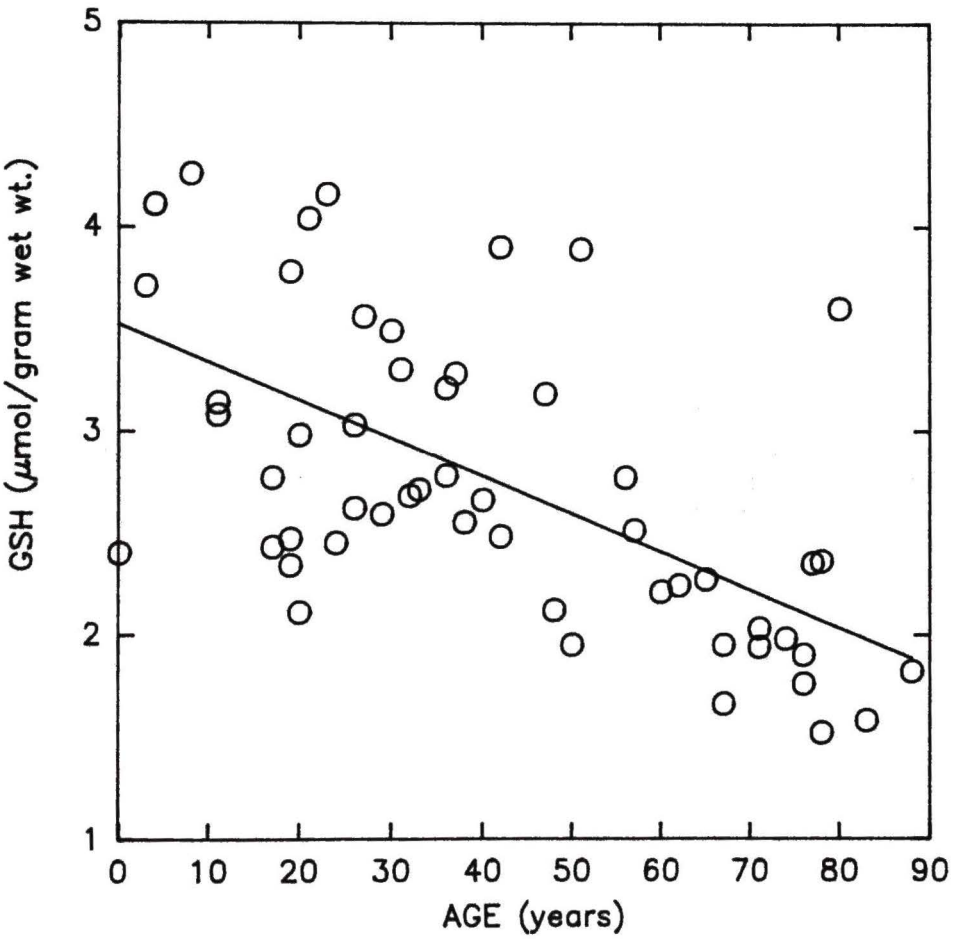

GSH (μmol/gram wet wt.)
AGE (years)
5
4
3
2
1
0
10
20
30
40
50
60
70
80
90

Figure 7. Protein-cysteine mixed disulfide (PSSC) in normal human lenses as a function of age. Individual lens PSSC level (measured as CSO_3H) is expressed in μmol g^{-1} wet weight. Linear regression line shown, $r=0.75$, $P<0.0005$.

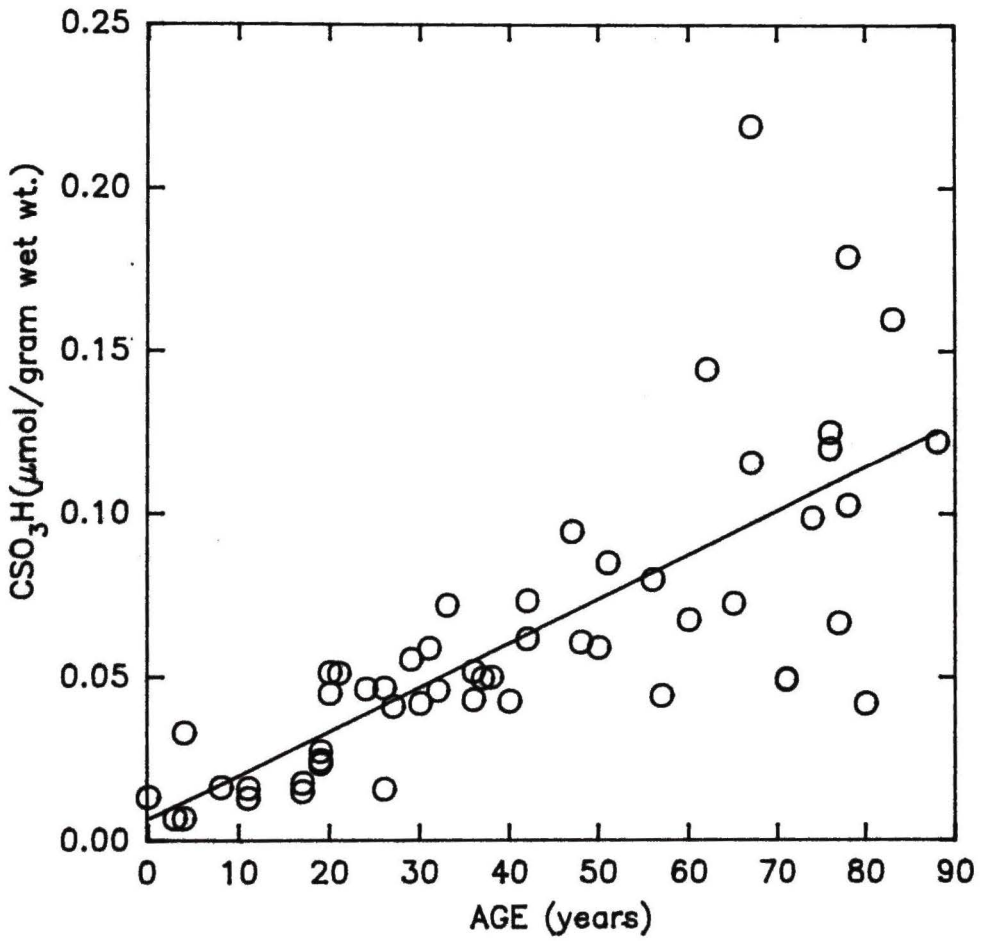
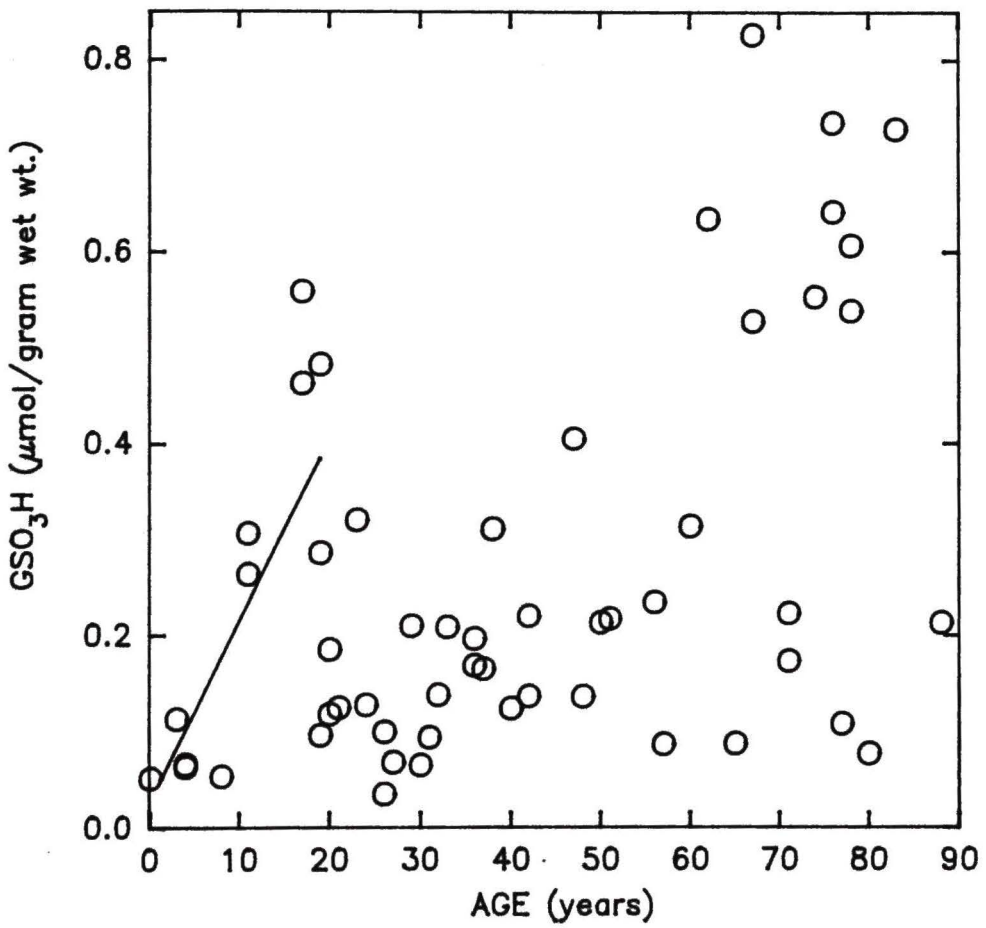

0.25
0.20
0.15
0.10
0.05
0.00
CSO_3H(μmol/gram wet wt.)
0
10
20
30
40
50
60
70
80
90
AGE (years)

Figure 8. Protein-GSH mixed disulfide (PSSG) in normal human lenses as a function of age. Individual lens PSSG level (measured as GSO_3H) is expressed in μmol g^{-1} wet weight. Linear regression line for data between zero and 19 years, $r=0.72$, $P<0.05$.


0.8
0.6
0.4
0.2
0.0
GSO_3H (μmol/gram wet wt.)
0
10
20
30
40
50
60
70
80
90
AGE (years)

and 19 years ($P<0.05$), with values from 0.05 μmol g^{-1} wet weight to 0.35 μmol g^{-1} wet weight. However, by 20 years, the level dropped back to 0.15-0.2 μmol g^{-1} wet weight and remained in this range for the next four decades. Linear regression analysis shows no significant trend ($P>0.05$) during this interval. After 60 years of age, PSSG began to increase again but with a high degree of variability; some lenses remained in the previous range (0.15-0.2 μmol g^{-1} wet weight), while others were much higher; 0.5-0.8 μmol g^{-1} wet weight.

In general it was observed that the level of GSH was inversely related to the level of PSSG in the same lens. In particular, the lenses with >0.5 μmol g^{-1} wet weight of PSSG generally had GSH values below 2 μmol g^{-1} wet weight. This reciprocal relationship is highly significant ($r=-0.34$. $P<0.01$, Kendall's coefficient of rank correlation).

Distribution of Protein-Thiol Mixed Disulfides in the Cortex and Nucleus Regions of Human Lens

The distribution of GSH, PSSG and PSSC in cortical and nuclear regions of the human lens was evaluated in 30 individual lenses from age 8 through 79 years. The lenses were grouped into eight decades with at least one and generally more lenses represented in each decade. The nucleus typically consisted of 25-30% of the lens wet weight

and the balance of the lens was considered cortex. As shown in Table 2 and Fig. 9, GSH levels in both nuclear and cortex regions gradually decreased with age, but the loss occurred earlier and more rapidly in the nucleus. Between the first and eighth decade, GSH declined 47% in the cortex and 66% in the nucleus. In contrast, both PSSG and PSSC increased gradually with age, but the increase was faster and more substantial in the nucleus. From the first to eighth decades, the PSSG level increased from 0.052 μmol g^{-1} wet weight to 0.388 μmol g^{-1} wet weight (7.5x) in the cortex and from 0.068 μmol g^{-1} wet weight to 0.955 μmol g^{-1} wet weight (14x) in the nucleus. Similarly, the PSSC level increased from 0.018 μmol g^{-1} wet weight to 0.086 μmol g^{-1} wet weight (5x) in the cortex and from 0.021 μmol g^{-1} wet weight to 0.154 μmol g^{-1} wet weight (7.3x) in the nucleus.

In general both PSSG and PSSC were evenly distributed in the cortex and nucleus in the young lenses but much higher levels of both PSSG and PSSC were found in the nuclear region of older lenses. The dramatic nature of these changes and the concomitant decrease in GSH can be seen best in the last column of Table 2, the ratio of total PSSG and PSSC to GSH. In the cortex of young lenses (first decade), PSSG+PSSC is only 1.5% the amount of the free GSH but this ratio gradually increases as the lens ages until by the eighth decade, it is 19%. In the nucleus, although the

Figure 9. Human lens cortical and nuclear GSH and protein-thiol mixed disulfides grouped by decade. A. GSH B. PSSG C. PSSC
Cortex, filled bars; Nucleus, hatched bars.

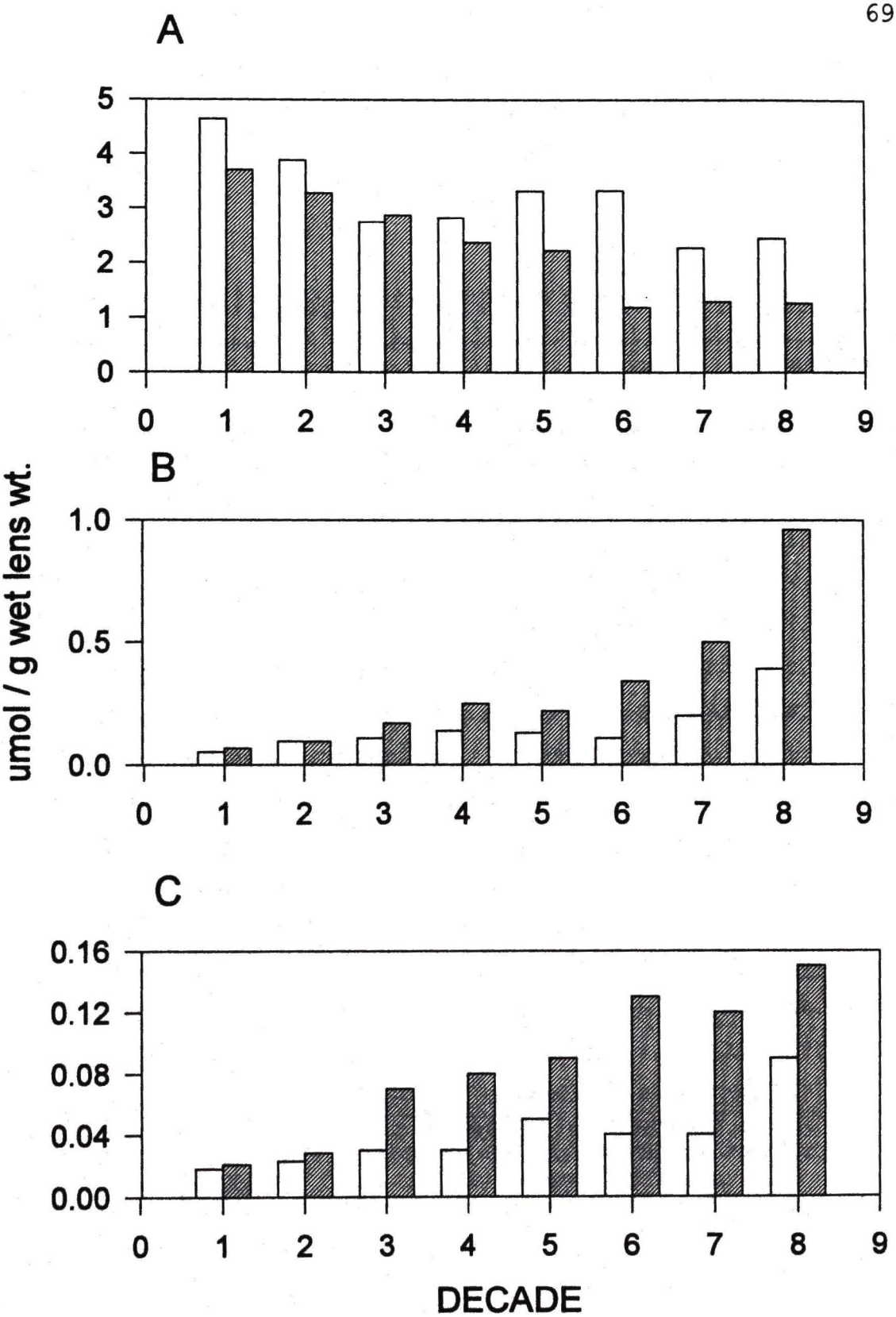

A
B
C
umol / g wet lens wt.
DECADE
0
1
2
3
4
5
6
7
8
9
0.0
0.5
1.0
0.00
0.04
0.08
0.12
0.16

Table 2. Distribution of protein-thiol mixed disulfides in human lens*.

Age (decade)	n		GSH	GSO_3H	CSO_3H	G+C	G+C/GSH (%)
1	1	Cor	4.64	0.052	0.018	0.070	1.5
		Nuc	3.70	0.068	0.021	0.089	2.4
2	1	Cor	3.89	0.097	0.023	0.120	3.1
		Nuc	3.28	0.096	0.028	0.124	3.7
3	6	Cor	2.75±0.94	0.11±0.04	0.03±0.01	0.14±0.04	5.1
		Nuc	2.88±0.28	0.17±0.06	0.07±0.01	0.24±0.06	8.3
4	6	Cor	2.83±0.51	0.14±0.03	0.03±0.01	0.17±0.03	6.0
		Nuc	2.38±0.24	0.25±0.10	0.08±0.02	0.33±0.12	13.9
5	4	Cor	3.32±0.84	0.13±0.05	0.05±0.01	0.18±0.06	5.4
		Nuc	2.23±0.57	0.22±0.06	0.09±0.02	0.31±0.07	14.0
6	6	Cor	3.33±1.92	0.11±0.04	0.04±0.01	0.15±0.05	4.5
		Nuc	1.20±0.30	0.34±0.16	0.13±0.06	0.47±0.21	39.0
7	2	Cor	2.28±0.31	0.20±0.02	0.04±0.01	0.24±0.03	10.5
		Nuc	1.30±0.42	0.50±0.22	0.12±0.06	0.62±0.28	48.0
8	4	Cor	2.45±0.29	0.39±0.01	0.09±0.02	0.47±0.03	19.2
		Nuc	1.27±0.38	0.96±0.07	0.15±0.02	1.11±0.08	87.4

CSO_3H, protein bound cysteine released as cysteic acid; GSO_3H, protein bound GSH released as glutathione sulfonic acid; G+C, GSO_3H+CSO_3H; Cor, cortex; Nuc, nucleus.
* Data is expressed as μmol g^{-1} wet lens weight, mean ± S.D.; n, number of samples.

first decade lens again showed a low PSSG+PSSC/GSH value of 2.4%, by the eighth decade the nuclear mixed disulfides were 87% of the amount of free GSH present.

Distribution of Protein-Thiol Mixed Disulfides in the Lens Proteins

In order to investigate whether PSSG or PSSC tend to occur in smaller molecular weight water soluble proteins (WS) or in the large aggregated water insoluble proteins (WI), the distribution of these two protein-thiol mixed disulfides in these two protein fractions was evaluated. Since the WI fraction increases during aging (Pirie 1968), two lenses from each age group (20s, 40s and 60s) were included for comparison. As shown in Table 3,the WS/WI ratio gradually decreased with aging. More PSSC and PSSG were found in the WI fraction than in the WS fraction for all ages. In these three age groups, the ratio PSSC/PSSG appeared to be constant both in the WS and WI protein fractions. The total mixed disulfide levels (per gram protein) in WS proteins increased moderately from the 20s to the 60s while the mixed disulfide content in the WI fraction was consistently high for all age groups.

Table 3. Distribution of protein-thiol mixed disulfides in proteins of normal human lenses*

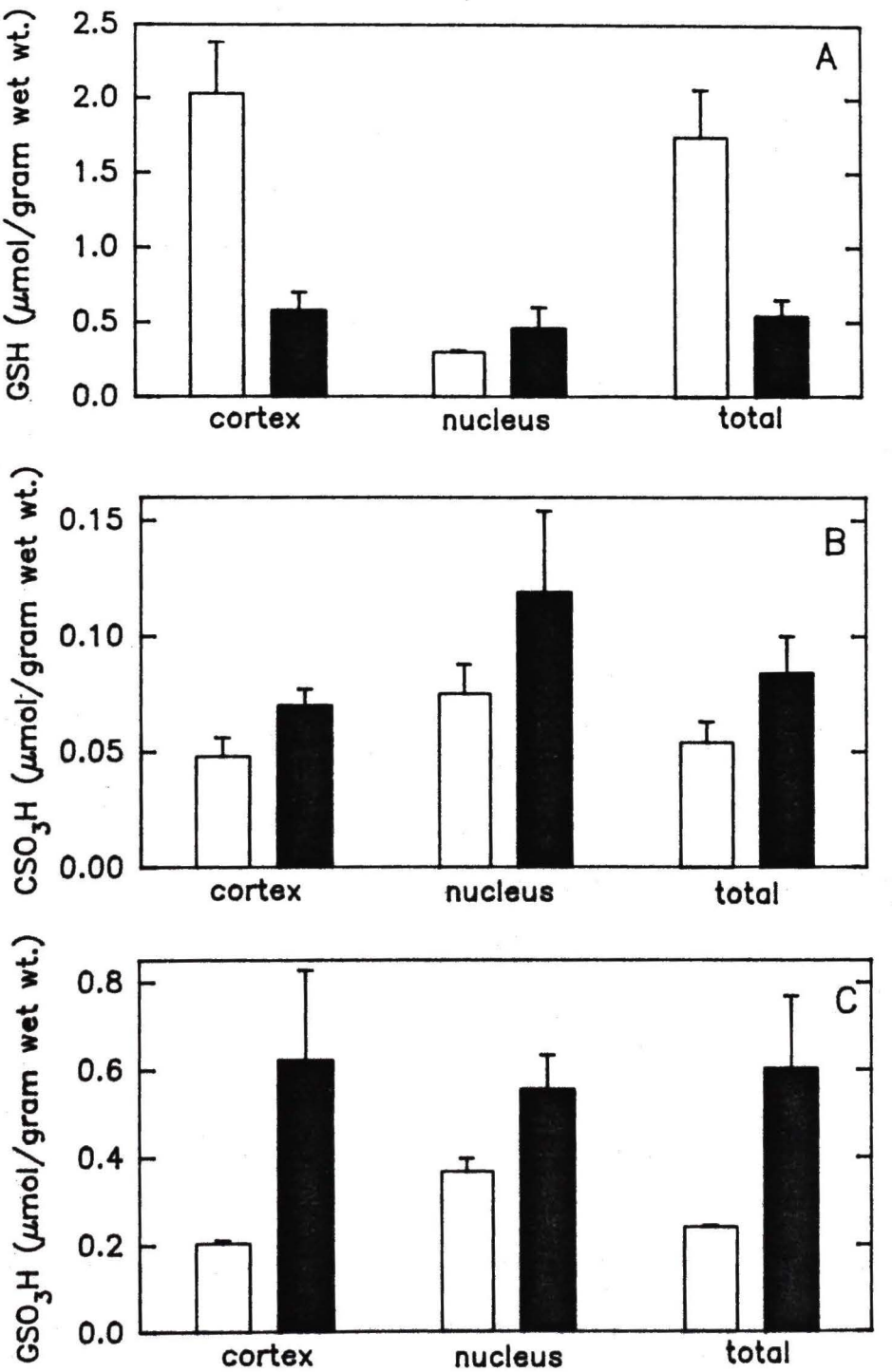
			WS			WI		
Sample	Age (years)	WS/WI	CSO_3H	GSO_3H	C/G	CSO_3H	GSO_3H	C/G
1	19	3.0	0.034	0.125	0.27	0.268	0.732	0.37
2	21	3.8	0.067	0.282	0.24	0.167	0.475	0.35
3	40	1.8	0.046	0.161	0.29	0.160	0.532	0.30
4	42	1.7	0.081	0.295	0.28	0.176	0.492	0.36
5	67	1.4	0.070	0.245	0.28	0.242	0.338	0.72
6	67	1.2	0.103	0.378	0.27	0.251	0.553	0.45

WS, water soluble proteins; WI, water insoluble proteins; C/G, CSO_3H/GSO_3H; CSO_3H, protein bound cysteine released as cysteic acid; GSO_3H, protein bound GSH released as glutathione sulfonic acid; WS/WI, dry weight of the water soluble fraction/dry weight of the water insoluble fraction.
* Data expressed as μmol g^{-1} dry protein weight.

Induction of Protein-Thiol Mixed Disulfide in Human Lens

Previous studies (Lou et al. 1986,1990) have shown that H_2O_2 stress induces PSSG formation in rat lenses in lens culture experiments and that this increase is related to the GSH loss and progressive opacification in the cortex. Therefore an attempt was made to examine whether human lens would be subject to similar changes under the same condition. Initial experiments indicated that human lens is more resistant to oxidative damage. The culture conditions for H_2O_2 (0.5 mM) to induce similar biochemical and morphological changes in human lenses as in the rat lens had to be modified by extending the H_2O_2 exposure to 36 hr. At this time point, the lens showed an overall haziness and spokes in the cortical region with considerable swelling when compared with the contralateral control lens. Biochemical changes in these lenses were quite pronounced. Figure 10 presents the averaged values of GSH, PSSG and PSSC in three pairs of 65-year-old normal lenses. In each case the level of these biomolecules in the oxidatively stressed lens was compared with its contralateral counterpart which was incubated in the absence of oxidant. H_2O_2 exposure caused the lens to lose more than 60% of the GSH (Fig. 10A); an over 50% increase in PSSG (Fig. 10C) and more than 30%

Figure 10. Effect of H_2O_2 (0.5 mM) exposure on the human lens GSH, PSSG, and PSSC in organ culture: A, GSH; B, CSO_3H; C, GSO_3H. The lens was dissected into cortex and nucleus for analysis. Nucleus represents 25-30% lens wet weight with the balance as cortex. Data are the results of three separate experiments; error bars = s. Open bars are control lens in TC-199 media. Black bars are the contralateral lens in media containing 0.5 mM H_2O_2 .


GSH (μmol/gram wet wt.)
2.5
2.0
1.5
1.0
0.5
0.0
A
cortex
nucleus
total
CSO3H (μmol/gram wet wt.)
0.15
0.10
0.05
0.00
B
cortex
nucleus
total
GSO3H (μmol/gram wet wt.)
0.8
0.6
0.4
0.2
0.0
C
cortex
nucleus
total

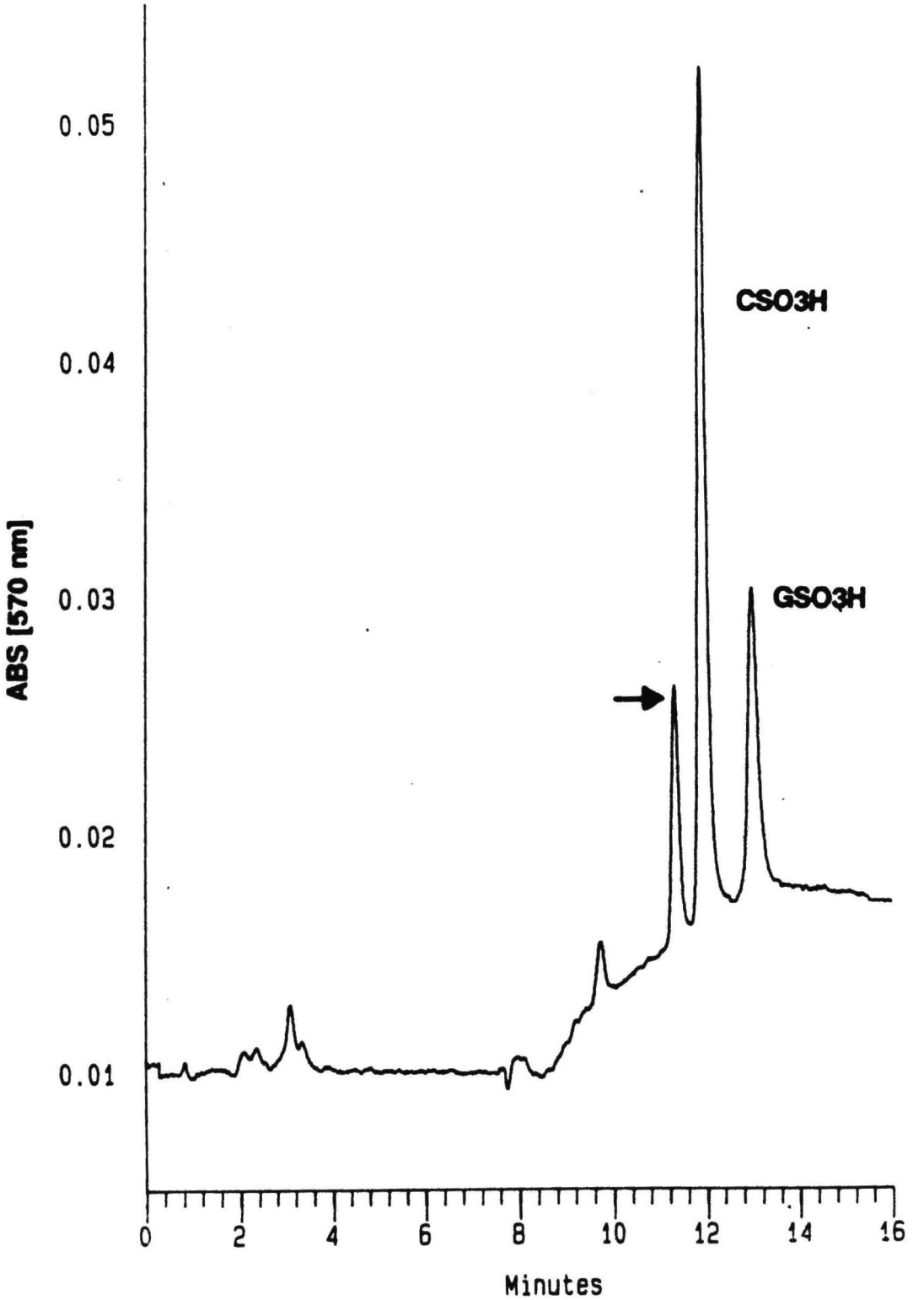
elevation in PSSC (Fig. 10B). Most of the GSH depletion occurred in the cortical region (a near 70% loss from control) with a more modest change in the nucleus. Cortical PSSG was elevated three-fold above the control but the increase in cortical PSSC was small. Nuclear PSSG and PSSC were both moderately elevated; the nuclear PSSC change was actually greater than for the cortex.

Comparison of the relative amounts of free and bound GSH revealed that the ratio shifted from 4:1 (GSH:PSSG) to just 2:1 after the oxidative stress.

Identification of γ-Glutamylcysteine/Protein Mixed Disulfide in Human Lens

Analysis of cataractous human lenses from China (Lou,M.F., Zigler,J.S., Huang,Q.-L., Xu,G.T. and Hu,T.-S., unpublished data) for mixed disulfide content revealed that in many instances, a third peak eluting at 11.5 minutes was present in addition to those corresponding to GSO_3H at 13.0 min and cysteic acid at 12.0 min (Fig 11). The possibility that this peak represents a third mixed disulfide species seems strong since these peaks are the product of performic acid oxidation of the lens proteins; the appearance of the peak following performic acid treatment is evidence of the disulfide nature of the attachment.

Figure 11. Chromatogram showing mixed disulfide profile from cataractous human lens. CSO_3H, cysteic acid; GSO_3H, glutathione sulfonic acid. Arrow identifies position of the γ-glutamyl cysteine sulfonic acid peak. Full analytical details given in Materials and Methods.


0.05
0.04
0.03
0.02
0.01
ABS [570 nm]
CSO3H
GSO3H
0
2
4
6
8
10
12
14
16
Minutes

Given that the new peak is a small thiol compound in disulfide linkage with the proteins, two possibilities emerge. The compound is either an intermediate in glutathione metabolism or it is a relatively unrelated thiol compound of either endogenous or exogenous origin which appears under conditions of abnormal lens metabolism. Because of the normal high concentration of GSH in the lens and the presence of key enzymes in its metabolism (Reddy et al. 1966), the former hypothesis is the more parsimonious.

Four major compounds in the GSH synthetic and degradative pathways bear the thiol functional group. These are γ-glutamylcysteinylglycine (GSH), γ-glutamylcysteine (γ-Glu-Cys), cysteinylglycine (CG) and cysteine (Cys). Of these four, GSH and Cys are already accounted for as the two primary mixed disulfide peaks. Thus only γ-Glu-Cys and CG remain as candidates for the identity of the unknown peak. Authentic CG sulfonic acid prepared from commercially available (bis)-CG disulfide, coelutes with cysteic acid and not with the unknown. γ-Glu-Cys, its disulfide and also its sulfonic acid derivative are not available commercially.

Incomplete acid hydrolysis of GSH was attempted in order to generate both dipeptides as well as the three component amino acids. Based on the chromatography of authentic standards, two large peaks corresponding to glutamic acid/glycine and cysteic acid/CG sulfonic acid, with lesser peaks for GSO_3H and γ-Glu-Cys were expected.

This is what was found (Table 4). Note that the γ-Glu-Cys peak was identified deductively by elimination through positive identification of all others. Also note that the retention time for this peak, 11.5 minutes, is in agreement with the retention time for the unknown mixed disulfide from the cataractous lenses.

A more specific method of generating γ-Glu-Cys was through carboxypeptidase digestion of oxidized glutathione (GSSG). In this scheme only the α peptide bond between cysteine and glycine is cleaved resulting in (bis)-γ-Glu-Cys disulfide and glycine. This technique followed by separation and removal of glycine and performic acid oxidation resulted in a single peak in addition to the GSO_3H (Fig. 12), the γ-Glu-Cys sulfonic acid peak. Acid hydrolysis of this isolated γ-Glu-Cys peak followed by amino acid analysis revealed equimolar amounts of Glx and cysteic acid confirming the identity of this product. As can be seen from panels A and B, the γ-Glu-Cys exactly coelutes with the unknown peak in the cataractous lens sample.

Further investigation of this new thiol compound in normal and cataractous human lenses revealed that lens age also influences its accumulation. The level of γ-Glu-Cys is <5 nmol/lens in normal (i.e. transparent) human lenses below the age of 60 (Fig. 13). In older normal lenses (>60 y) the level of γ-Glu-Cys mixed disulfide is significantly higher ($p<0.05$), increasing over seven-fold between the fifth and

Table 4. Acid hydrolysis products of glutathione.

Peak	Retention Time (min)	Identity
1	9.6	Glu/Gly
2	11.5	γ-GluCys
3	12.0	Cysteine/CysGly
4	13.0	Glutathione

GSH was hydrolyzed under vacuum in 6 M HCl for 5 hr at 105°C. The dried hydrolysate was oxidized with performic acid and analyzed by anion-exchange amino acid analysis.

Figure 12. A. γ-Glu-Cys sulfonic acid prepared by oxidation of the carboxypeptidase digest of GSH. B. Cataractous human lens. Note identity of retention time between cataractous peak and γ-Glu-CysSO_3H. 1.: γ-Glu-CysSO_3H, 2: CSO_3H. 3: GSO_3H. Chromatographic conditions as for Fig. 8.

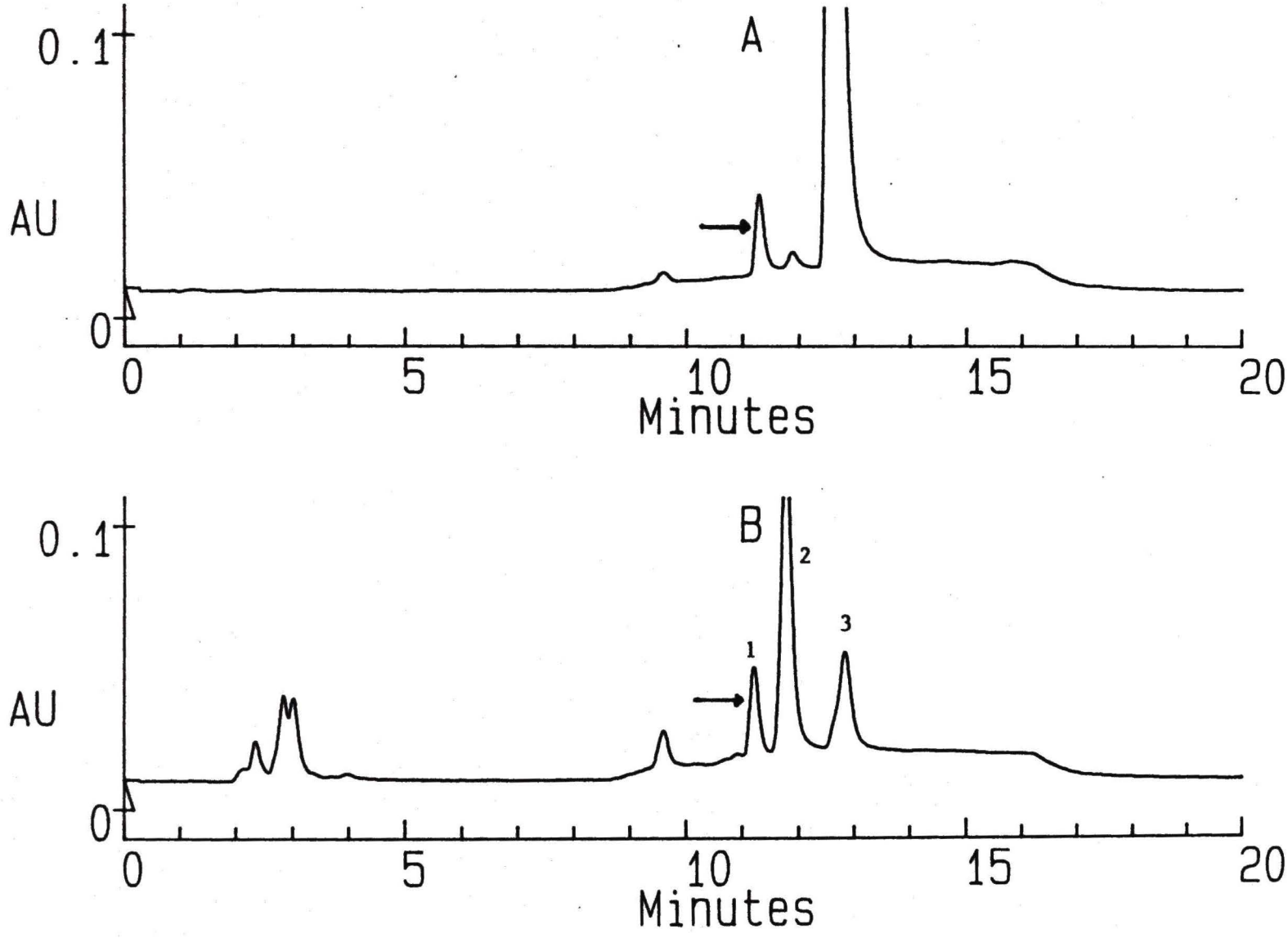
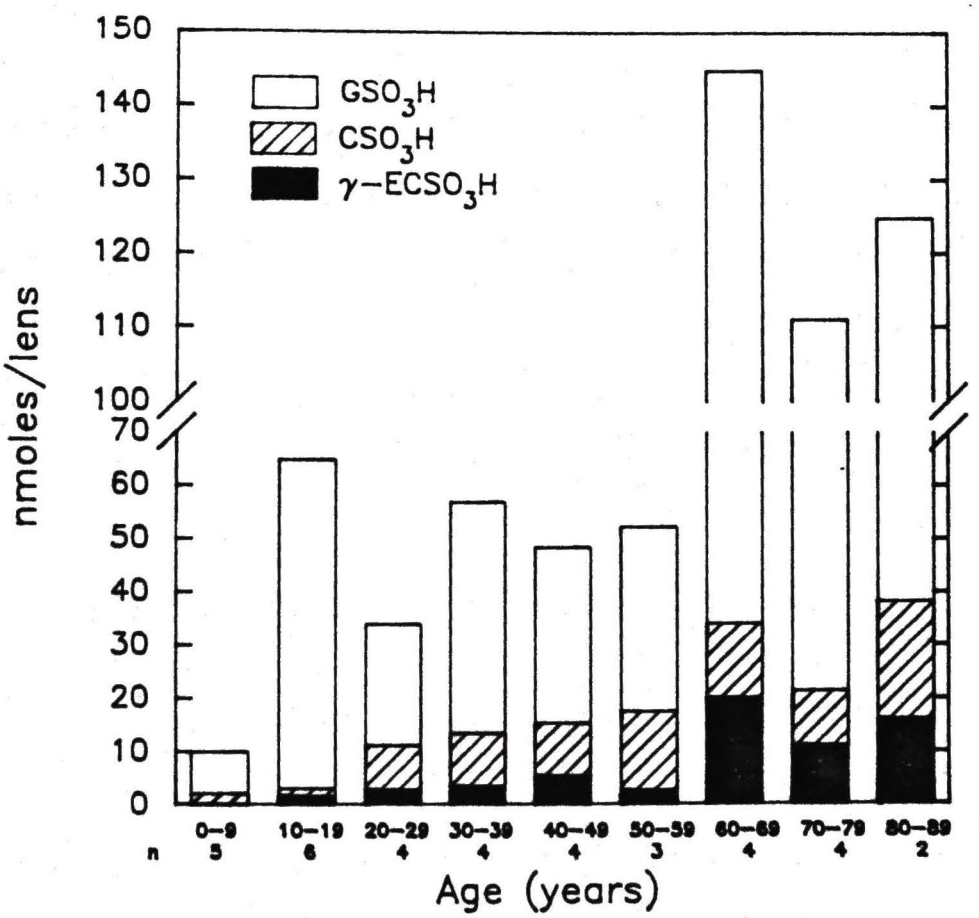

0.1
AU
0
A
0
5
10
15
20
Minutes
0.1
AU
0
B
1
2
3
0
5
10
15
20
Minutes

Figure 13. The level of the three major protein bound thiols in the human lens as a function of age. Linear regression analysis shows: γ-Glu-Cys mixed disulfide, no significant linear relationship; t-test demonstrates that the mean γ-Glu-Cys mixed disulfide level for lenses ≥60 yr is significantly greater than for those less than 60 ($p < 0.05$). PSSG; significant linear relationship between 0 and 20 yr ($p < 0.05$) and no significant trend between 20 and 60 yr. PSSC; significant linear increase with age ($p < 0.0005$). Regression lines are for PSSG (0-20 yr) and PSSC (0-88 yr).


150
140
130
120
110
100
70
60
50
40
30
20
10
0
nmoles/lens
GSO3H
CSO3H
γ–ECSO3H
n
0–9
5
10–19
6
20–29
4
30–39
4
40–49
4
50–59
3
60–69
4
70–79
4
80–89
2
Age (years)

sixth decades. Thirteen cataractous lenses with nuclear opacities ranging in severity from immature to hypermature were grouped according to age and contrasted with clear lenses of similar age (Fig. 14). As can be seen from the figure, the cataractous lenses have appreciably greater levels of γ-Glu-Cys mixed disulfide at all ages and may be distinguished statistically from age-matched clear lenses in all cases except for the 50-59 age group. Although the mean for this group is 20-fold higher than for the clear lenses, the variability is also great (range = 7.0-158.4) and a test for outliers was unable to reject any of the variates.

Free Thiol Levels in Normal Human Lenses

Glutathione

The monobromobimane/HPLC assay for thiols is, in contrast to Ellman's non-specific spectrophotometric method (Ellman 1959), able to distinguish GSH from other thiol species. It was assuring, therefore, to find that the human lens GSH data from this study (Fig. 15) corroborates the earlier data (Fig. 6; Harding 1970). This not only supports the validity of the earlier data but also underscores the preponderance of GSH relative to other lens thiols. As can be seen in Fig. 15, all three lens regions show a significant, linear decline in GSH with age. The outer

Figure 14. The mean level of γ-Glu-Cys mixed disulfide in clear and cataractous human lens for the five decade intervals between 30 and 79 yr.
ns, not significantly different ($p>0.05$); * and ***, significantly different from clear lenses of the same age interval at $p<0.05$ and $p<0,001$ respectively. Error bars are ± one SE. Statistical details are given in Materials and Methods. Clear lenses (i.e. with no obvious opacity) were obtained post-mortem from U.S. eye banks and cataractous lenses were obtained from cataract surgeries performed in India.

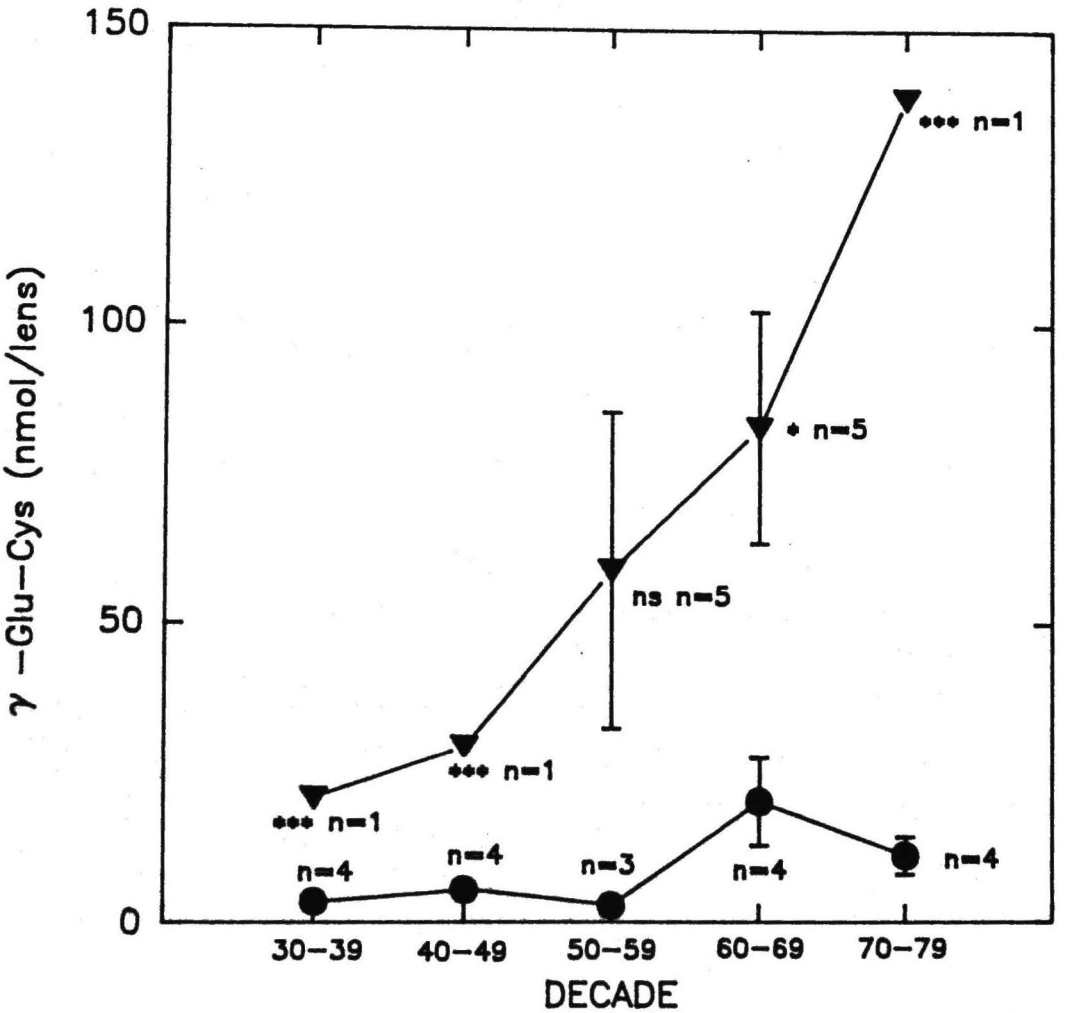
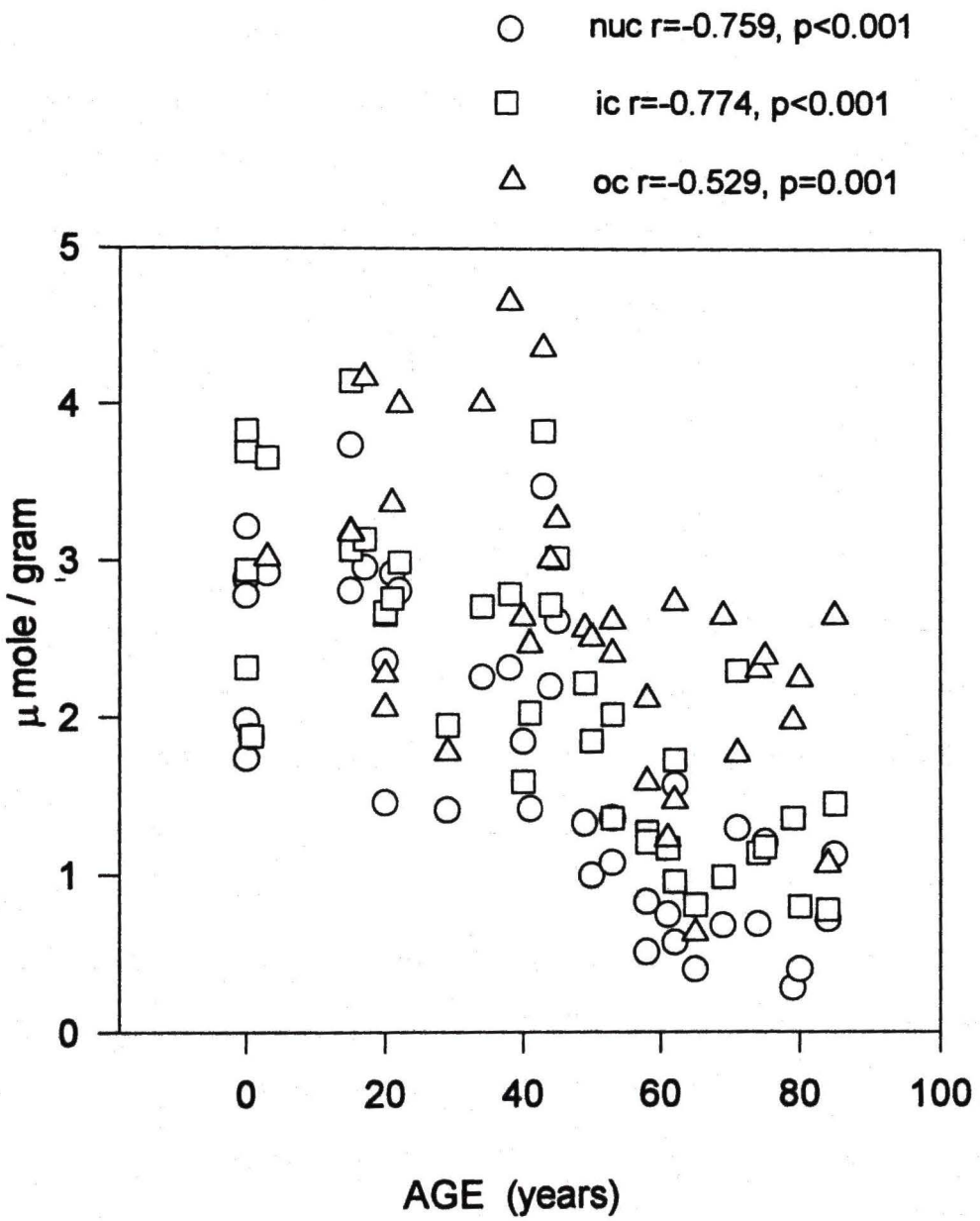

150
100
50
0
γ -Glu-Cys (nmol/lens)
*** n=1
* n=5
ns n=5
*** n=1
*** n=1
n=4
n=4
n=3
n=4
n=4
30-39
40-49
50-59
60-69
70-79
DECADE

Figure 15. Free GSH content of normal human lens outer cortex, inner cortex and nucleus as a function of age. Lenses were dissected as described in Materials and Methods resulting in outer cortex, approx. 55% of lens wet weight; inner cortex, approx. 35%; nucleus approx. 10%. Quantitation accomplished via monobromobimane derivitization of thiols, followed by reverse-phase HPLC and fluorescent detection (see Methods). Open circle: nucleus. Closed circle: inner cortex. Triangle: outer cortex. All regression lines are significant at $p \leq 0.001$.

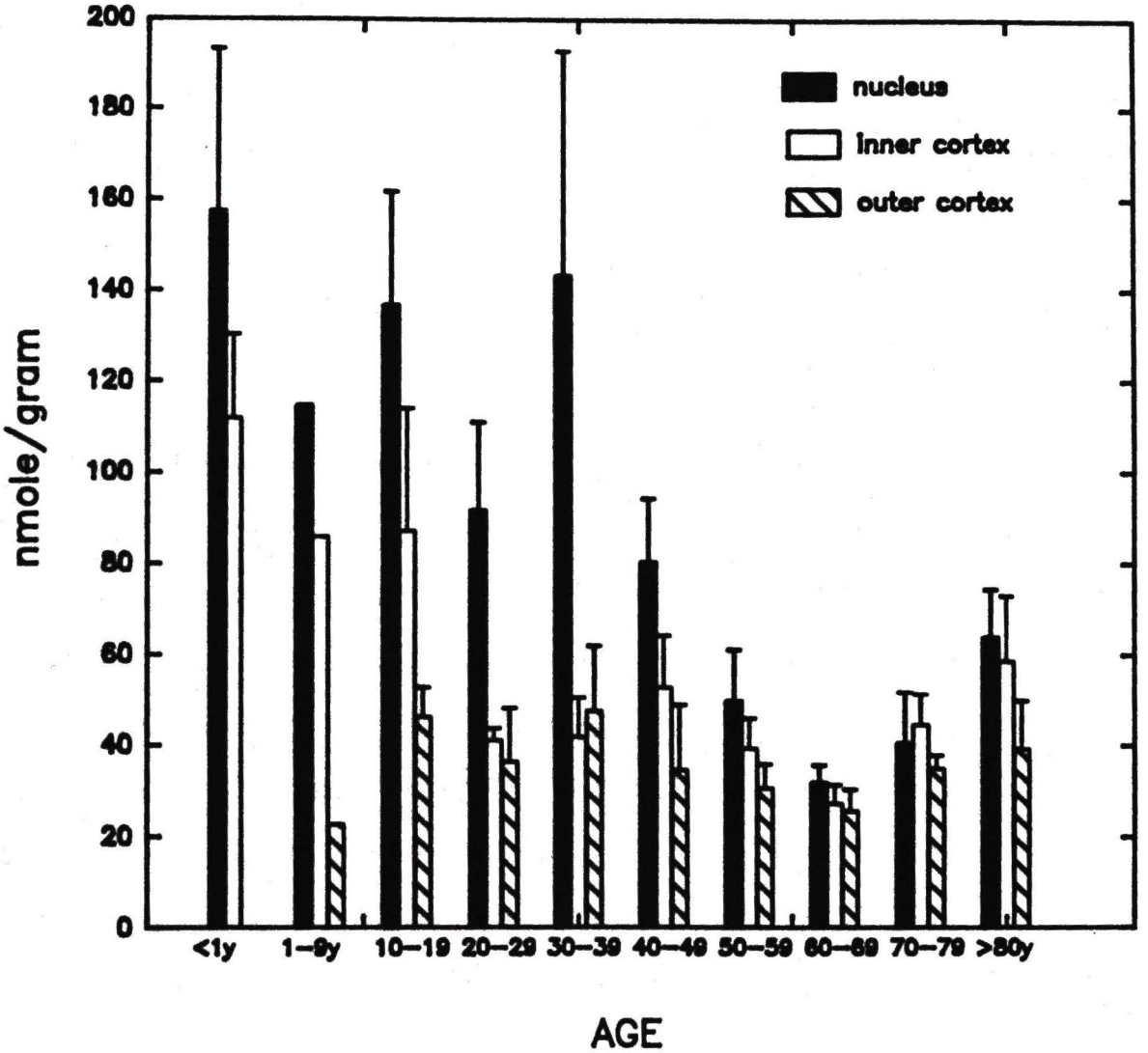

nuc r=-0.759, p<0.001
ic r=-0.774, p<0.001
oc r=-0.529, p=0.001
µ mole / gram
0
1
2
3
4
5
0
20
40
60
80
100
AGE (years)

cortex is richest in GSH for all ages and the nucleus poorest. Average values for outer cortex ranged from 3.6 μmol g^{-1} at infancy to 1.5 μmol g^{-1} at 100 years. Inner cortex and nucleus had similar, albeit somewhat lower values for very young ages (nucleus, 2.8 μmol g^{-1}, inner cortex 3.4 μmol g^{-1}) but the slopes of the regression lines for these two regions were steeper than for outer cortex (-0.759, -0.774 vs -0.529) indicating a faster overall rate of loss of GSH from these regions.

Human Lens Cysteine

The amount of free cysteine in human lens also declines with age (Fig. 16). There are some important differences between GSH and cysteine, however. Between the infancy and 49 years of age, the cysteine level is highest in the nucleus. These nuclear values are generally between 130 and 160 nmole g^{-1} wet weight until approximately 40 years of age after which a precipitous drop occurs (i.e. to 80 nmole g^{-1} at 40-49 years and to approximately 35 nmole g^{-1} by 60-69 years). The nuclear cysteine appears to increase in the older lenses ≥ 80 years to about 60 nmole g^{-1}. Inner cortical values generally follow the nuclear trend except that they are lower in cysteine. These values range from a high of 110 nmole g^{-1} in infants to 40-60 nmole g^{-1} for all ages ≥ 20. The lowest values for all ages were recorded in

Figure 16. Human lens free cysteine as a function of age. Data from the same samples used in Fig. 15. n = number of lenses in each age group. Error bars = s.


200
180
160
140
120
100
80
60
40
20
0
nmole/gram
nucleus
inner cortex
outer cortex
<1y
1–9y
10–19
20–29
30–39
40–49
50–59
60–69
70–79
>80y
AGE

the outer cortex where free cysteine was always approximately 40 nmole g^{-1}. It must be pointed out that this gradient of increasing cys concentration from outer cortex to nucleus is opposite the GSH concentration gradient.

Free Cysteine in Cataractous Lenses

Cataractous lenses from two individuals were available for free cysteine measurements. The data are shown in Fig. 17. As can be clearly seen, the amount of free cysteine in the outer cortex is comparable to that of age matched controls with clear lenses. However the inner lens regions have dramatically elevated levels of this thiol. Inner cortex from the cataractous lenses is at 110-160 nmol g^{-1}, at least three-fold higher than normal. The nucleus with over 400 nmol g^{-1} cysteine has over 10x as much free cysteine as is found in the age matched control group.

Glycation Induced Lens Protein Conformational Changes

Ascorbate Modification of Bovine α- Crystallin

The modification of α- crystallin structure through covalent addition of ascorbate was attempted in vitro. Figure 18 shows the amount of ^{14}C-ascorbate bound per mg

Figure 17. Human cataract lens free cysteine. Two human cataractous lenses analyzed for free cysteine. Data are contrasted with 70-79 year old age class from Fig.16.

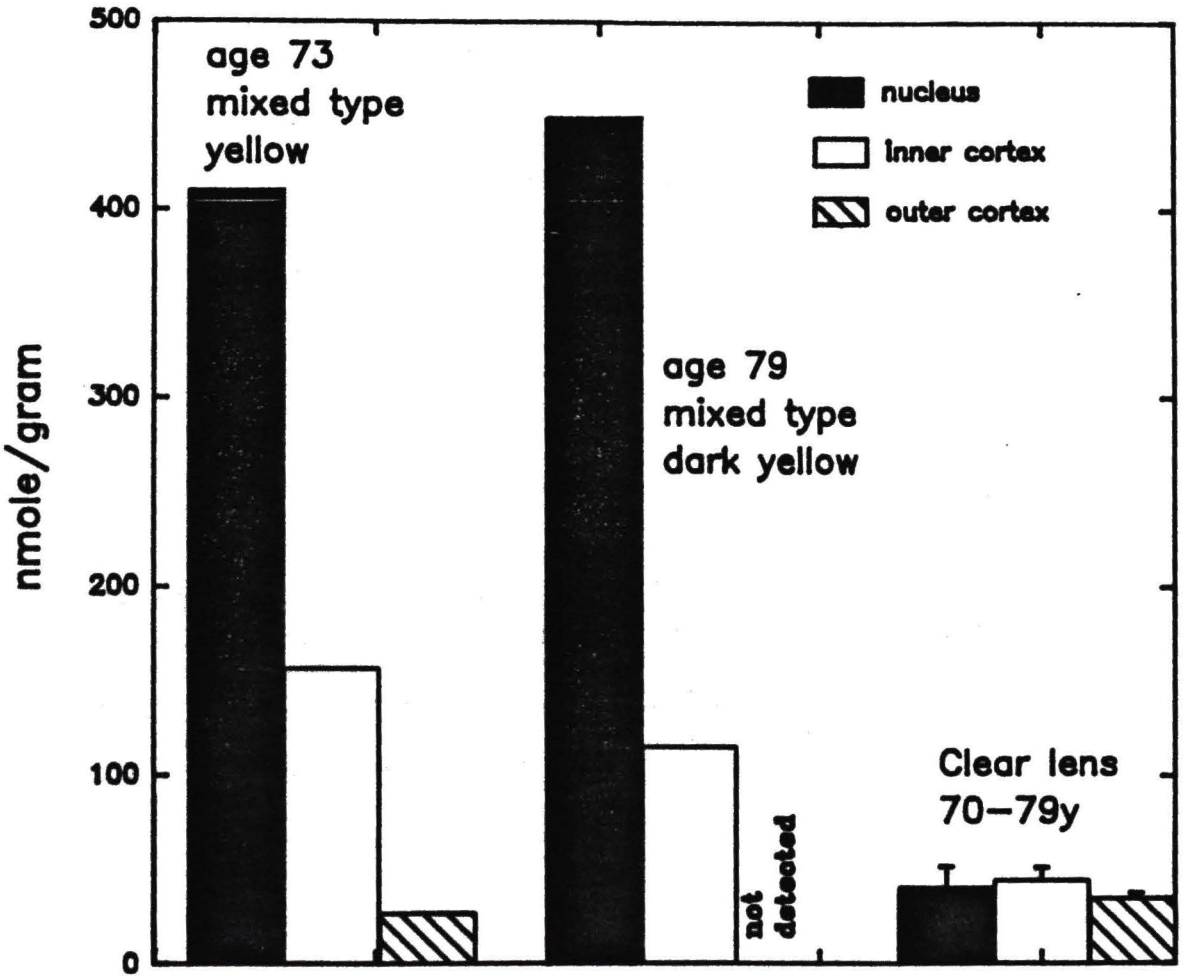
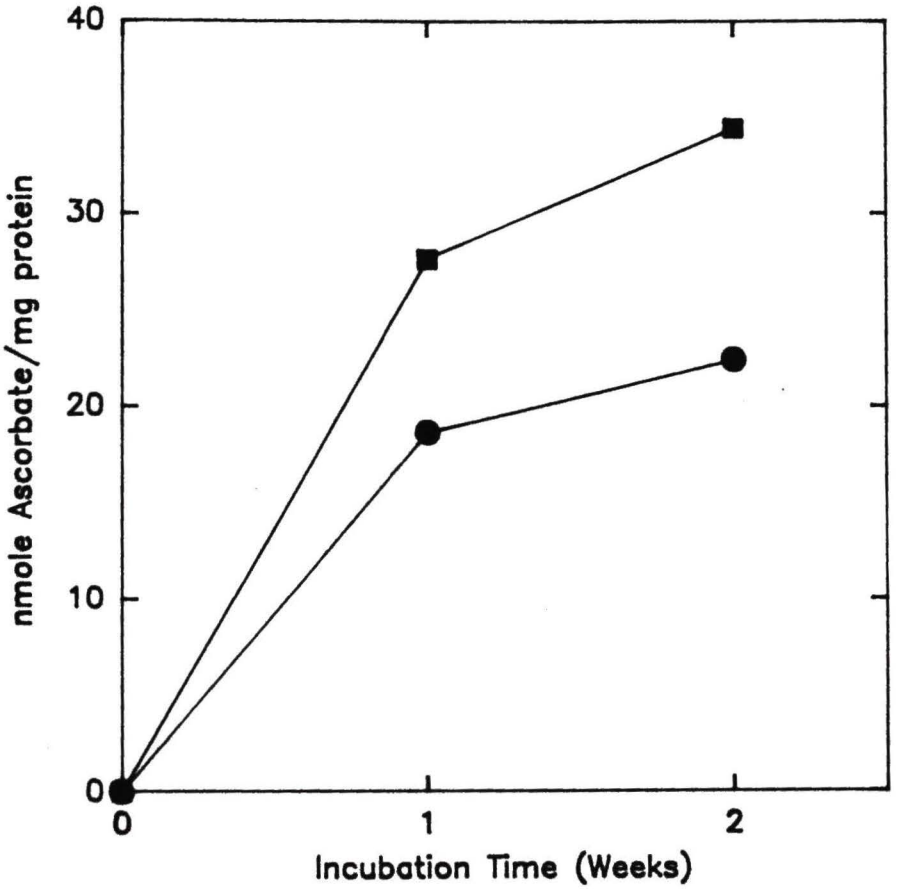

500
400
300
200
100
0
nmole/gram
age 73
mixed type
yellow
nucleus
inner cortex
outer cortex
age 79
mixed type
dark yellow
not detected
Clear lens
70–79y

Figure 18. ^{14}C-Ascorbate incorporation by bovine α-crystallin incubated with either 5 mM (circles) or 10 mM (squares) ascorbic acid for one or two weeks. Specific activity of the ascorbic acid was 2.5 μCi/mmole. The protein concentration was 1 mg/mL; 370 μL aliquots of total 1.0 mL reaction mix were used for scintillation count.


40
30
20
10
0
0
1
2
nmole Ascorbate/mg protein
Incubation Time (Weeks)

protein. Note that the amount of ^{14}C-ascorbate bound is both concentration and time dependent. The 5 mM ascorbate incubation resulted in binding of 19 nmoles of ascorbate per mg protein by one wk. This is the equivalent of 15 ascorbate molecules per α- crystallin molecule (40 subunits); the 10 mM ascorbate incubation resulted in 50% greater ascorbate binding (i.e. stoichiometry approximately one ascorbate for every two subunits). It is important to consider that free diffusion of air into the reaction mixtures was allowed as incubations were carried out in a tissue culture plate. When a follow-up experiment was conducted in which reaction mixtures were sealed in air-tight vials, much less incorporation of ascorbate occurred (Table 5).

The possibility that ascorbate modification of the protein had caused conformational changes was examined by CD spectroscopy. The far UV region of the CD spectra for the one week incubated α- crystallin are shown in Fig. 19. The band at 215 nm was diminished in the 5 mM sample and to an even greater extent for the 10 mM ascorbate-treated sample. Samples incubated with either 5 or 10 mM ascorbate under similar conditions except that diffusion of air was restricted showed no difference in their CD spectra (Fig. 20).

The presence of non-disulfide cross-links formed as a result of ascorbate modification of α- crystallin was examined with SDS/PAGE following reduction of the protein

Table 5. The influence of air on the glycation of α-crystallin by ascorbate.

Ascorbate Concentration	Aerobic[1]	Anaerobic[2]
	Ascorbate Incorporated (nmol mg protein^{-1})	
5 mM	18.6	2.30 ± 0.67
10 mM	27.6	4.53 ± 0.66

[1]Aerobic, one experiment. [2]Anaerobic, three experiments, mean ± s. Protein concentration for all experiments was 1 mg mL^{-1}. Incubation period was for one week.

Figure 19. Circular dichroism spectra for bovine α-crystallin incubated in the presence of air with either 0(....), 5(——) or 10(---) mM ascorbic acid for one week. Aviv CD spectrometer, 0.2 cm path length, all protein concentrations normalized to 0.13 mg/mL (Pierce BCA assay).

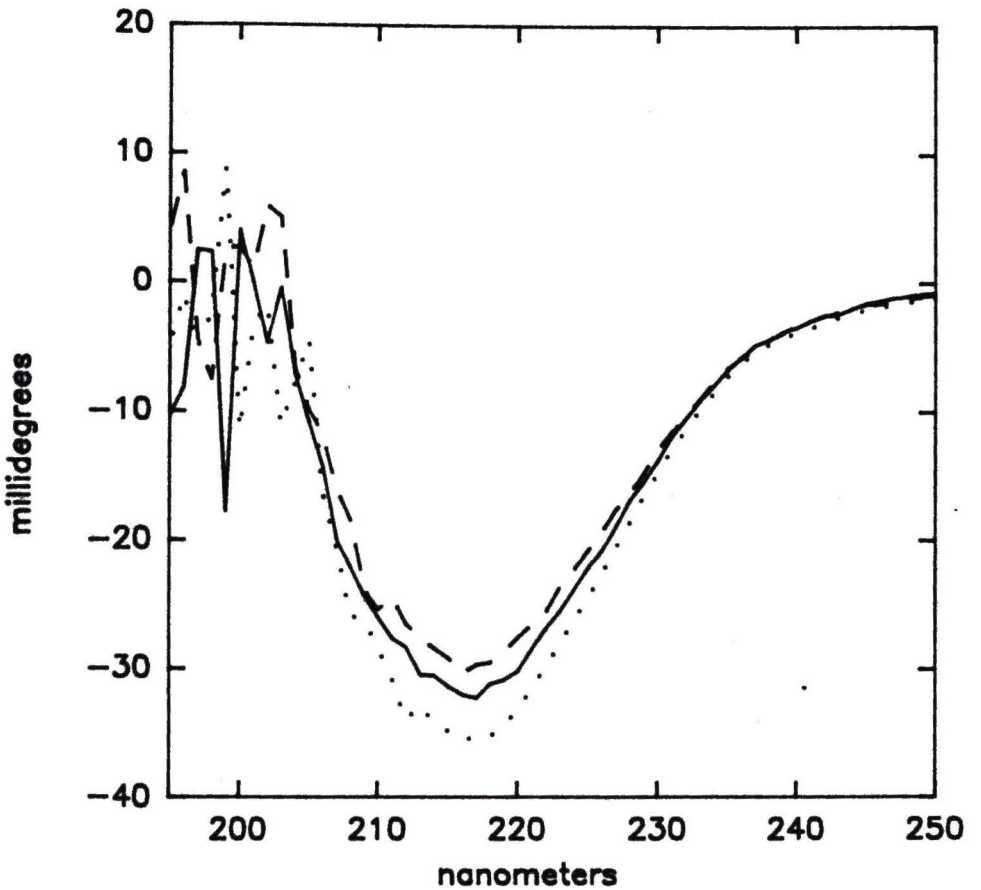
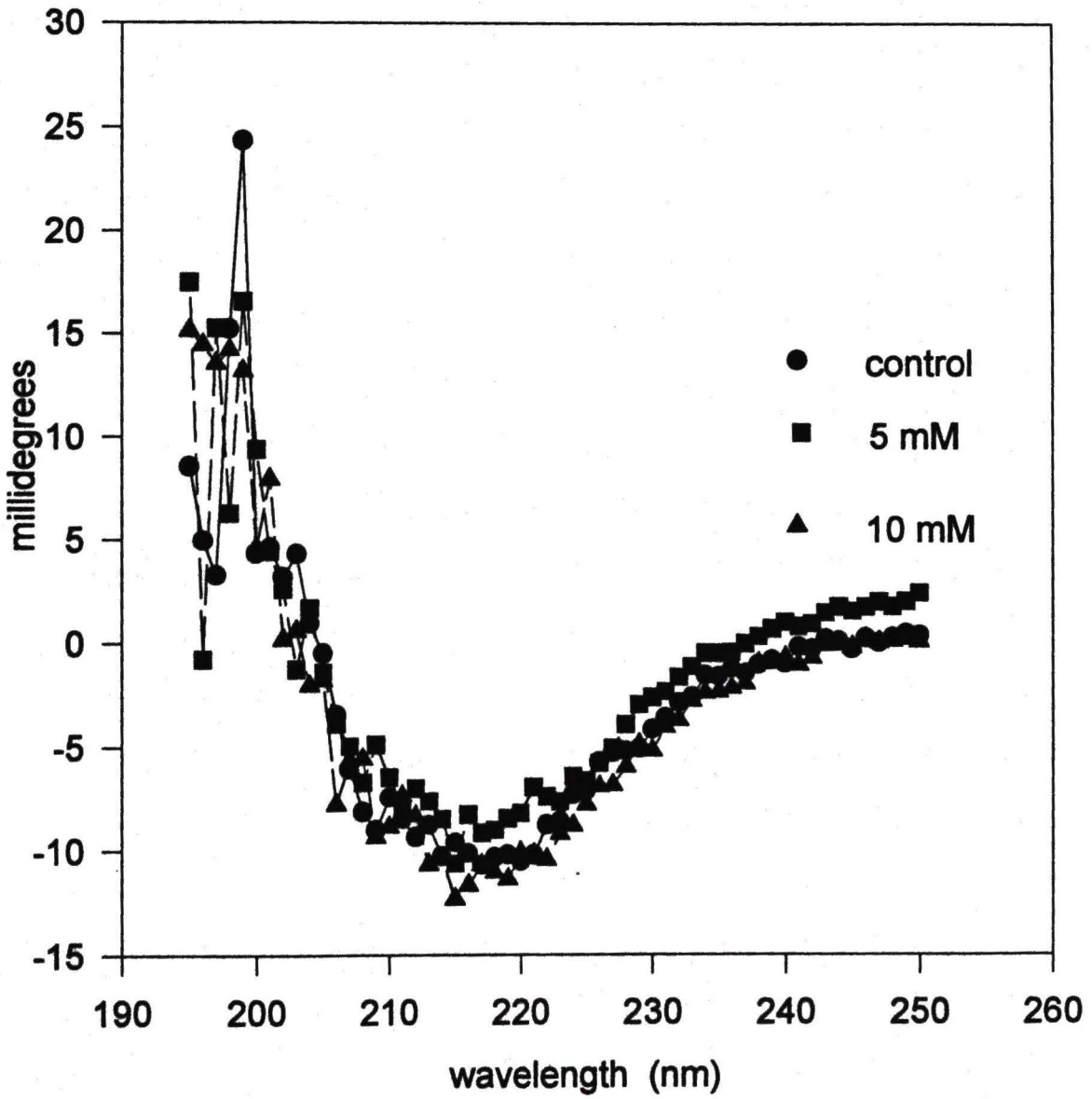

20
10
0
−10
−20
−30
−40
millidegrees
200
210
220
230
240
250
nanometers

Figure 20. Circular dichroism spectra for bovine α-crystallin incubated in air-tight vials with 0(....), 5(——) or 10(---) mM ascorbic acid for one week. Aviv CD spectrometer, 0.2 cm path length, all protein concentrations normalized to 0.13 mg/mL (Pierce BCA assay).


30
25
20
15
10
5
0
-5
-10
-15
millidegrees
190
200
210
220
230
240
250
260
wavelength (nm)
control
5 mM
10 mM

with 2-mercaptoethanol. SDS/PAGE of the 2-mercaptoethanol treated samples from this experiment is shown in Fig. 21. Note the large amount of non-reducible cross-linked material at the top of the gel for samples incubated with ascorbate. This material was present in all of the ascorbate incubated samples, albeit to different degrees depending on the ascorbate concentration and the duration of the experiment. Accompanying the appearance of cross-linked material was a diminution of the main α-crystallin bands at 20 kD. These changes did not occur in either the one or two-week incubated controls. This result suggests that high molecular weight aggregates were formed as a result of ascorbate conjugation with α- crystallin.

Although SDS/PAGE analysis suggests that glycation and cross linking by ascorbate was occurring (Fig 21), it is possible that some of the radioactivity measured, (Fig 18, Table 5), was the result of non-covalently bound ascorbate (Ortwerth, personal communication). This type of binding could potentially have influenced the CD results. To assess this possibility, and evaluate the effectiveness of dialysis in removing unbound ascorbate, a study was carried out in which the ascorbate was not only removed by dialysis, but in parallel treatments by repeated TCA washes and by dialysis against "cold" "chase" ascorbate. The results are shown in Table 6. Ascorbate incorporation was uniformly lower than in the previous study. This is perhaps due to the

Figure 21. SDS/PAGE 15% mini-gel of bovine α-crystallin incubated (in presence of air) for one or two weeks with or without ascorbic acid (AA). Lane 1: 10mM AA, 2 wk. Lane 2: 5mM AA, 2wk. Lane 3: Control (no AA), 2 wk. Lane 4: 10mM AA, 1 wk. Lane 5: 5mM AA, 1wk. Lane 6: Control (no AA), 1 wk. All samples had been reduced with 2-mercaptoethanol prior to electrophoresis. 80 volts for 20 min then increased to 130 volts until dye front had reached bottom of gel(1.5-1.75 hr).

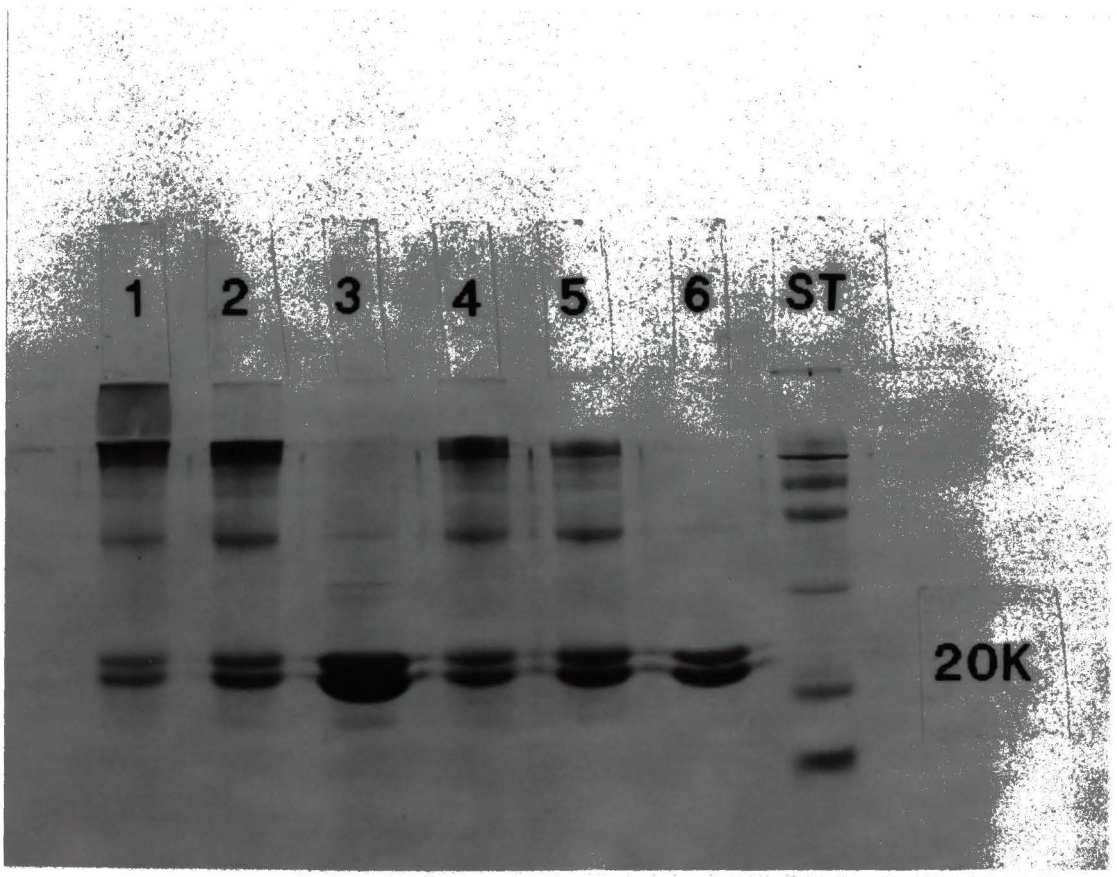

1
2
3
4
5
6
ST
20K

Table 6. ^{14}C-ascorbate incorporation in α- crystallin samples: Unbound label removed by various methods.

Ascorbate Concentration (mM)	Dialysis	Dialysis with "Chase"	TCA Wash
5	2.96	2.30	1.63
10	4.53	5.19	3.87

Data are expressed as nmol ascorbate mg protein^{-1}.
Experimental details are given in Materials and Methods.

incubation conditions. The first experiment was contained in a tissue culture plate which allows free exchange of gases with the well contents. The second trial's samples were all contained in air-tight snap-cap vials. Since oxygen must be available to convert ascorbic acid to dehydroascorbic acid (DHA), the reactive species, decreased availability of oxygen in the second trial could account for the diminished binding of label. Although the TCA washing gave the lowest values for ascorbate incorporation, the dialysis samples had values of similar magnitude. The 5 mM dialysis sample bound 2.96 nmol mg^{-1}, the 5 mM dialysis "chase" sample bound 2.30 nmol mg^{-1} and the TCA washed sample bound 1.63 nmol mg^{-1}. Similarly, for the 10 mM treatments, the dialysis sample bound 4.53 nmol mg^{-1}, the dialysis "chase" bound 5.19 nmol mg^{-1} and the TCA washed sample bound 3.87 nmol mg^{-1}. The results of this control experiment allow it to be concluded that dialysis alone is sufficient to remove the non-covalently bound ascorbate from the α- crystallin preparation. Therefore the results of the first ascorbate incubation (Table 5, Figs. 18,19) are valid.

Culture of Rat Lenses in High Sugar Media

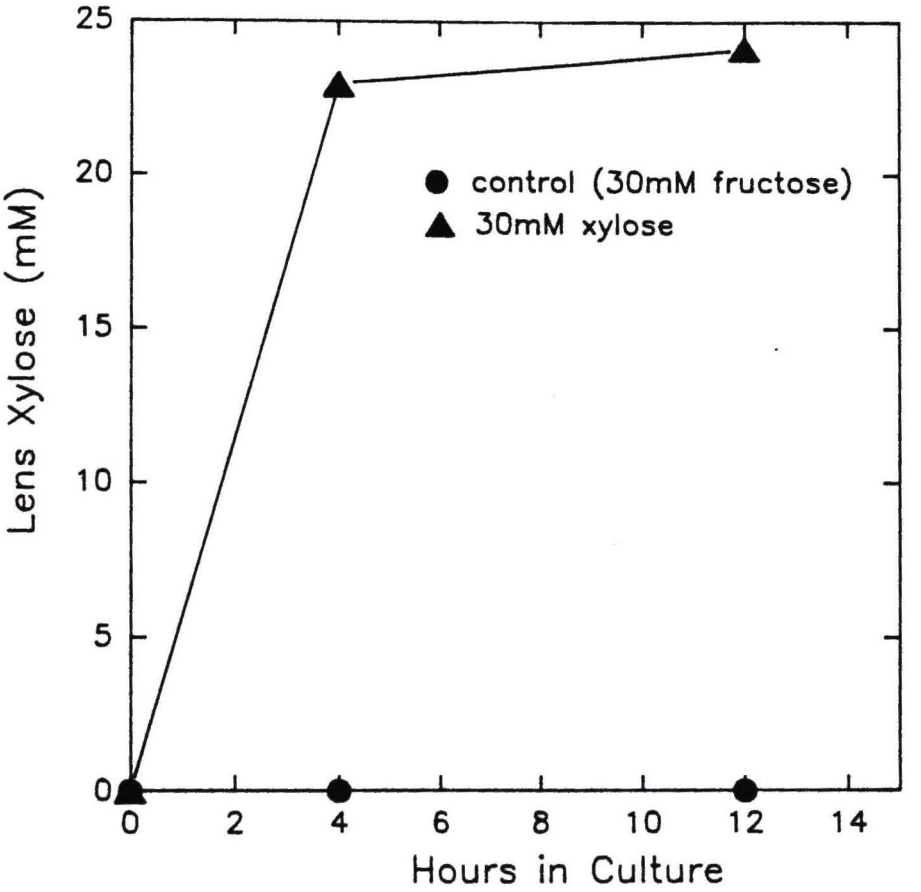
The *in vitro* experiments described above clearly demonstrate that protein glycation has the potential for conformational destabilization. However, this system is a

highly simplified model for events occurring *in vivo*. The more realistic lens organ culture model was employed to evaluate effects of glycation of lens proteins *in situ*. This system utilizing high sugar media has been well established (e.g. Kinoshita et al. 1968). Xylose was chosen over other sugars because of the favorable equilibrium between cyclic and acyclic forms [the acyclic form is the active glycating agent (Kaanane and Labuza 1989)], and because in the presence of an aldose reductase inhibitor (ARI), it is relatively inert metabolically. Thus, when used with an ARI, its action can be limited to protein glycation.

Xylose Penetration

In order for lens protein glycation to occur in organ culture, the glycating agent must first enter the cytosol of the lens cells. Although it is known that xylose can enter the lens (Obazawa et al. 1974), I wanted to know more precisely the concentration inside the lens and how rapidly it was attained. Xylose levels in lenses cultured with 30 mM xylose were rapidly elevated (Fig.22). The level had reached a plateau of 23 mM by four hours. Weights of the 30 mM xylose and 30 mM fructose cultured lenses were virtually identical at four hours. Fructose cultured (30 mM) lenses and fresh control lenses had no detectable xylose.

Figure 22. Xylose levels in rat lenses cultured with or without 30 mM xylose. Measurements made by anion exchange chromatography with pulsed amperometric detection (see Methods).

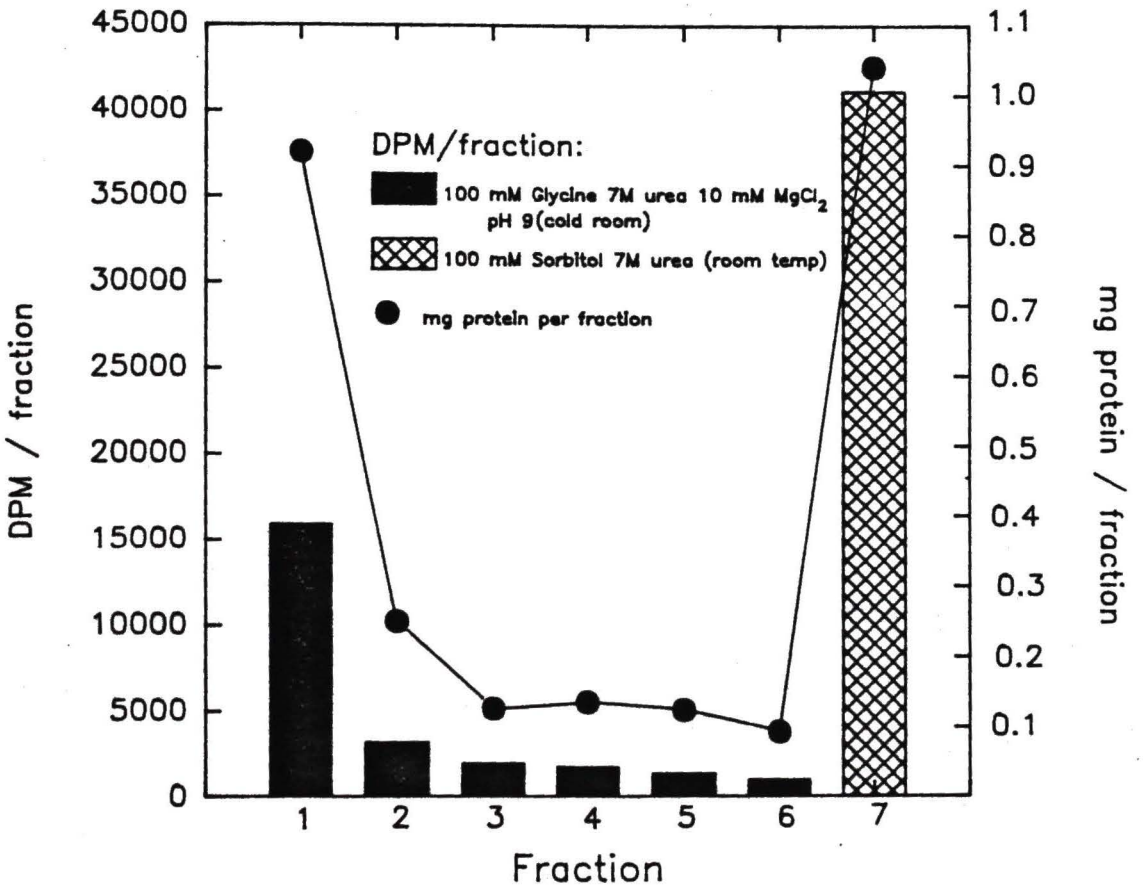

25
20
15
10
5
0
Lens Xylose (mM)
control (30mM fructose)
30mM xylose
0
2
4
6
8
10
12
14
Hours in Culture

Separation and Quantitation of Glycated Proteins

The ability of phenylboronate agarose (PBA) to bind protein-xylose conjugates was tested with bovine serum albumin/xylose conjugates prepared for this reason. If this model xylated protein could be retained on the PBA columns, it should be possible to use them successfully with homogenates of rat lenses which had been cultured in a xylose medium. The results are demonstrated in Fig.23. Bovine serum albumin incubated with D-[U-^{14}C]xylose was exhaustively dialyzed and applied to a 2 mL PBA-60 column as outlined above in METHODS. Unbound (and presumably unglycated) protein was eluted from the column in the first glycine wash along with some radioactivity. The succeeding glycine fractions contained background levels of protein and radioactivity. The sorbitol wash eluted a high specific radioactivity fraction containing the protein-xylose conjugates. Thus the selectivity of the method was assured and this technique was employed to quantitate xylose glycated lens proteins.

Rat lenses were cultured for one week in either 30 mM xylose + ARI or 30 mM fructose. An ARI was included in the xylose culture media to prevent the metabolism of xylose to xylitol and the associated osmotic stress and cataract formation (Kinoshita 1974). Because fructose is not a substrate for aldose reductase an ARI is not necessary in

Figure 23. Binding of glycated (xylated) BSA (preparation described in Methods) to PBA-60 chromatography column. Bars represent the total radioactivity present in each fraction, filled circles are the total protein present as measured by the BCA protein assay.


45000
40000
35000
30000
25000
20000
15000
10000
5000
0
DPM / fraction
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
mg protein / fraction
DPM/fraction:
100 mM Glycine 7M urea 10 mM MgCl2 pH 9(cold room)
100 mM Sorbitol 7M urea (room temp)
mg protein per fraction
1
2
3
4
5
6
7
Fraction

the fructose containing media. The incubation period was set at one week to allow ample time for the glycation reactions to occur. Glycation *in vivo* is considered to be a slow process (Berman 1991). Lenses cultured in either 30 mM xylose + ARI or 30 mM fructose were found to have 23.6% and 23.2% of the protein glycated, respectively as determined by PBA-60 chromatography (Fig.24). These two groups did not differ significantly from each other ($P>0.05$) but were both different from uncultured controls ($P<0.05$) in which 18.1% of the protein bound to the PBA gel.

O-Linked Sugar Adducts

Many proteins are glycosylated by specific enzyme systems on Asn amide nitrogen (N-linked) or on Ser/Thr hydroxyls (O-linked). It has recently been reported that α-crystallin is a glycoprotein with single O-linked N-acetyl glucosamine units at specific serines in its polypeptide chain (Roquemore et al.) The possible overestimation of protein glycation levels because of glycosylation on Ser or Thr residues was evaluated. Measurements of PBA-60 affinity binding for five samples before and after a procedure designed to remove O-linked saccharides (see METHODS) were conducted. Table 7 summarizes the data on both fructose and xylose incubated lenses. The deglycosylation of the lens proteins did not decrease the amount of protein binding to

Figure 24. Glycated protein (as percent of total protein) in rat lenses cultured in media containing 30 mM xylose (+10 μM AL01576) or fructose, or not cultured. Data are mean ± s.

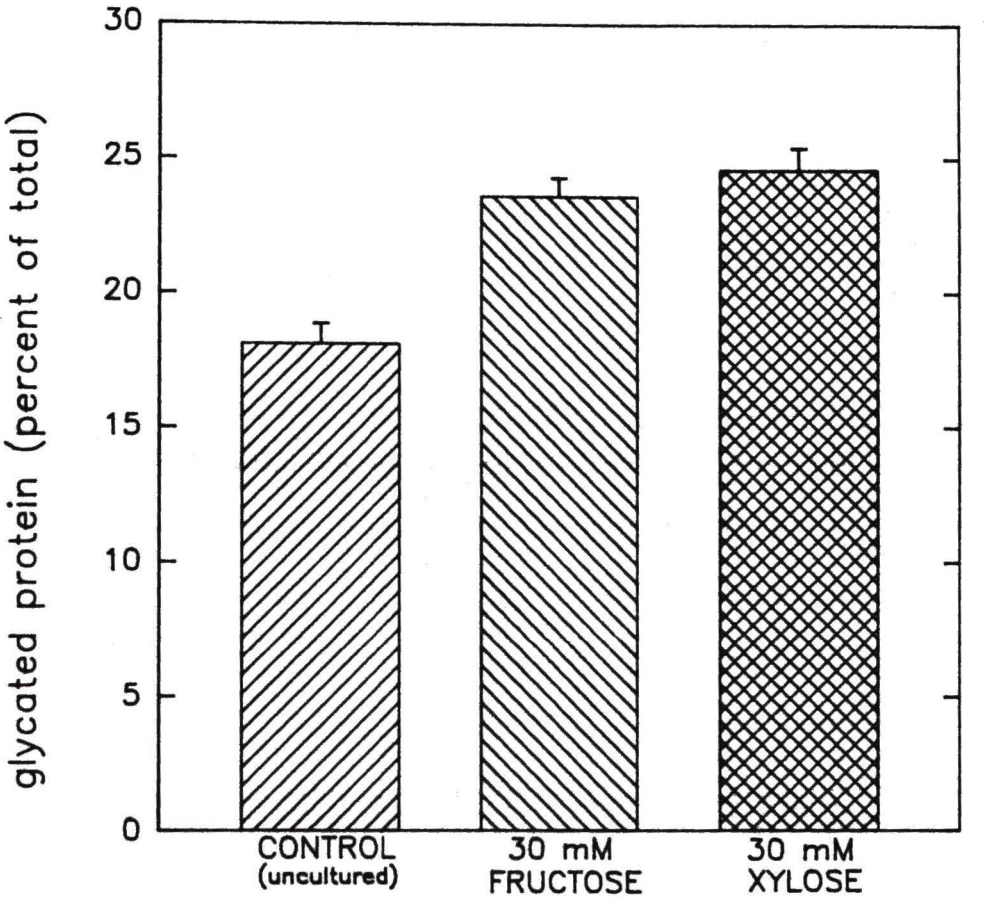

glycated protein (percent of total)
30
25
20
15
10
5
0
CONTROL
(uncultured)
30 mM
FRUCTOSE
30 mM
XYLOSE

Table 7. Percent Glycated Protein Before and After Treatment to Remove O-Linked Sugars.

SAMPLE	Before	After
1	27.1	28.9
2	26.2	29.0
3	31.9	27.6
4	23.8	28.2
5	26.5	28.5

Samples were incubated for 18h in 1M $NaBH_4$/0.1M NaOH. Two-way ANOVA for paired comparisons found no significant difference between the two groups ($P \rangle 0.05$). For further details, see Methods.

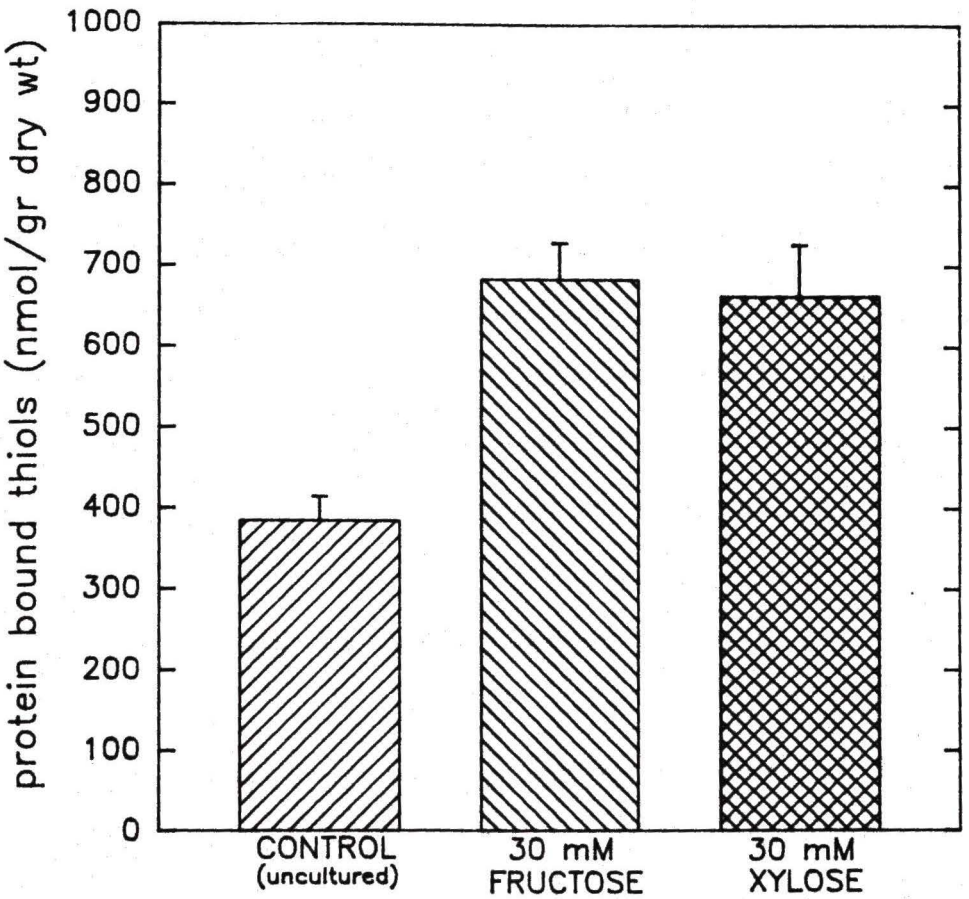
the PBA-60 columns. ANOVA for paired comparisons showed no significant difference ($P>0.05$) in percent of protein bound before or after this treatment. It must be concluded then that none of the PBA binding is due to O-linked glycosyl units and that the increase in glycation values seen following the incubation period is indeed the result of glycation processes occurring during the experimental period.

Lens Protein-Thiol Mixed Disulfides

Lens mixed disulfide data were collected from experiments which used lenses from rats of either 100 g or 200 g mean body weight. Rat body weight was included in the statistical analysis as a second treatment category. Two-way ANOVA did not identify any significant effect due to rat body weight and no significant interaction between body weight and culture conditions for mixed disulfide levels.

The protein-thiol mixed disulfide levels (sum of PSSC and PSSG) in cultured rat lenses were significantly higher in lenses cultured in either 30 mM xylose (689.1 nmol/gr dry wt) or 30 mM fructose (646.3 nmol/gr dry wt) than for uncultured controls (432.0)($P<0.001$)(Fig. 25), however, the two different sugar cultured groups were not statistically distinguishable from each other($P>0.05$).

Figure 25. Total protein-thiol mixed disulfides as measured by performic acid oxidation and anion exchange amino acid analysis (see Methods) from rat lenses cultured with either 30 mM xylose (+10 μM AL01576), 30 mM fructose or not cultured. Data are mean ± s.


protein bound thiols (nmol/gr dry wt)
1000
900
800
700
600
500
400
300
200
100
0
CONTROL
(uncultured)
30 mM
FRUCTOSE
30 mM
XYLOSE

GSH Levels

GSH levels were uniformly low after one week of culture under any of the culture conditions when compared to fresh lenses (Fig. 26). Cultured lenses had GSH values ranging between 1-2 µmol GSH per gram wet lens weight. In a typical experiment, mean GSH levels for xylose+ARI cultured lenses (1.5 µmol/g), fructose cultured lenses (1.6 µmol/g) and xylitol cultured lenses (1.74 µmol/g) were indistinguishable statistically ($P>0.05$). In contrast, fresh lenses had a mean GSH value about 4 fold greater at 6.5 µmol/g.

SDS-PAGE of PBA-60 Bound Protein

SDS-PAGE analysis of lens proteins present in both fructose incubated and xylose+ARI incubated lens samples before PBA-60 fractionation and in the sorbitol-eluted fraction was carried out in order to determine if any of the lens proteins were preferentially glycated. No obvious differences were found (Fig. 27). Both xylose and fructose incubated lenses showed more intense bands at ≈70 kD in the sorbitol fractions and fainter bands in the 20 kD region, suggesting that some oligimerization of the 20 kD proteins (mainly α and γ- crystallins) was taking place. Since 2-mercaptoethanol was present in the sample buffer,

Figure 26. Lens GSH in lenses cultured for one week with either 30 mM xylose+10 μM AL01576 (n=6), 30 mM fructose (n=6), 30 mM xylitol (n=6) or not cultured (n=4). Data are mean ± s.

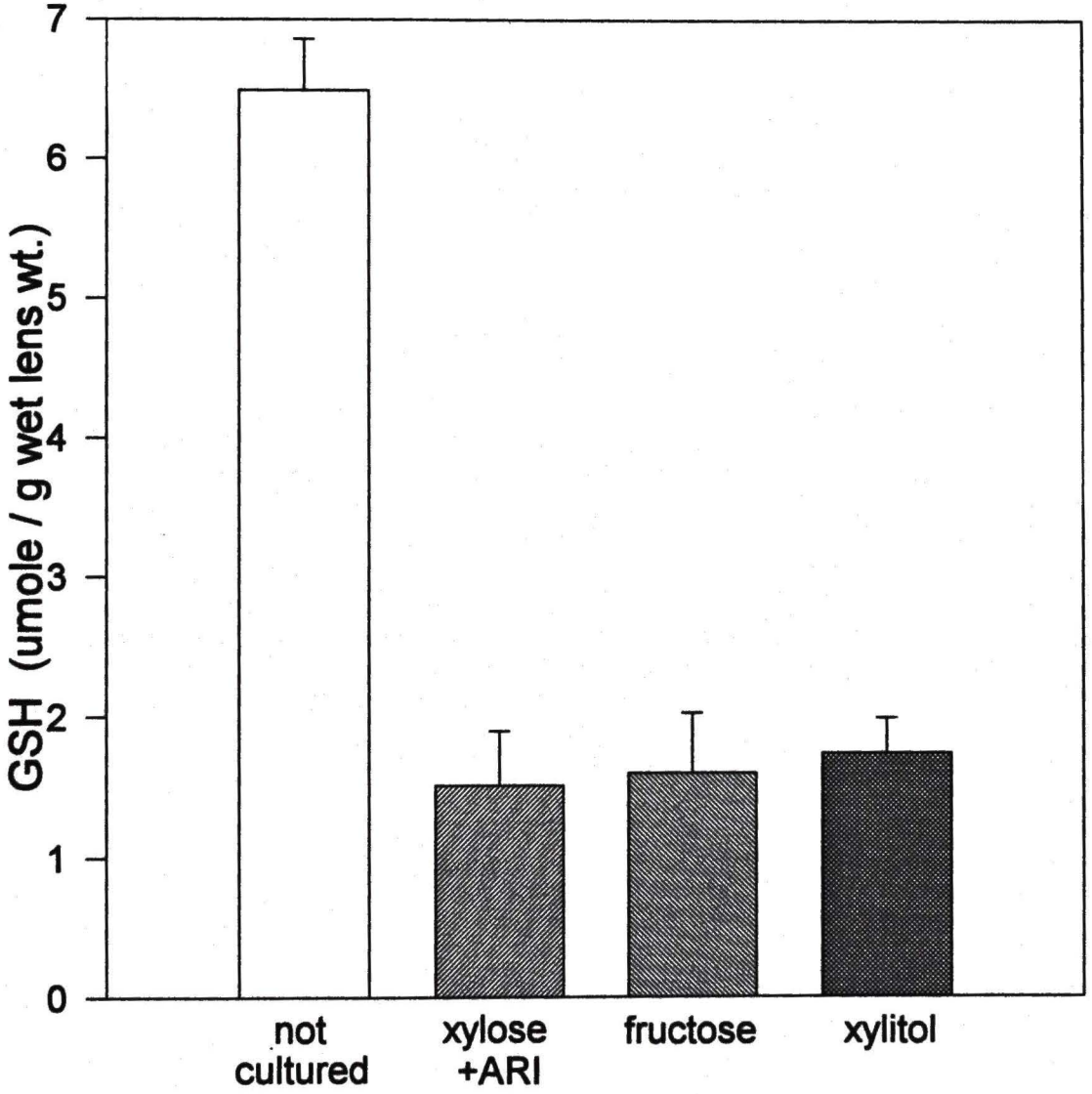
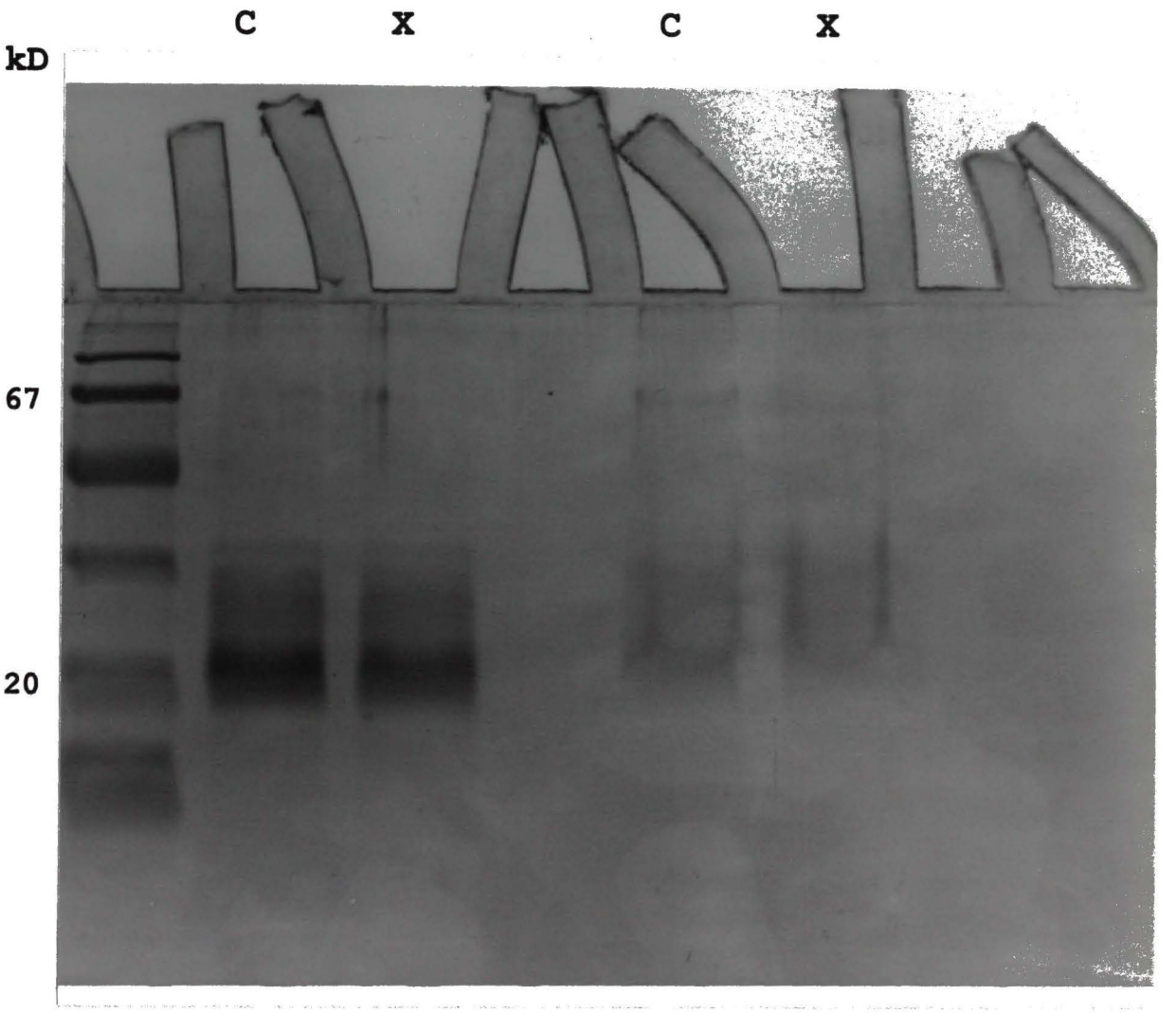

GSH (umole / g wet lens wt.)
0
1
2
3
4
5
6
7
not cultured
xylose +ARI
fructose
xylitol

Figure 27. SDS-PAGE gel of lens proteins from rat lenses cultured with 30 mM xylose (+ 10 µM AL01576)(=X) or uncultured (=C). All samples, 10 µg. Lane 1, MW markers; lanes 2-3, uncultured control (C,lane 2) or xylose cultured (X,lane 3) prior to PBA-60 fractionation; lanes 4-5, glycated protein fraction from PBA-60 columns for uncultured lens (C,lane4) or xylose cultured lens (X,lane 5). All samples were reduced with 2-mercaptoethanol in the sample buffer to eliminate disulfide cross-links. Electrophoretic conditions are described in Methods.


C
X
C
X
kD
67
20
Lane 1
2
3
4
5

these linkages are of a non-disulfide nature. Interestingly, no higher molecular weight material at the stacking/separating gel boundary was visible.

Xylitol or NaCl Cultured Lenses

Fructose has long been used in lens organ culture as an osmolyte "filler" for normal control media (e.g. Kinoshita et al. 1968, Obazawa et al. 1974, Zigler and Hess 1985). After the first lens culture experiment was completed in this study it became clear that during a one week incubation, fructose was not an inert "filler" molecule but was instead as active a glycating agent as xylose. Clearly, proper control treatments were needed. The possibility that protein-thiol mixed disulfides form in rat lenses in culture regardless of the presence or absence of a glycating sugar was investigated by substituting 30 mM xylitol or 15 mM NaCl (= 30 mOsM) for the xylose or fructose in the culture media. Lenses cultured for one week in media containing 30 mM xylitol instead of a sugar were analyzed for GSH and mixed disulfides in parallel with lenses cultured in 30 mM xylose+ARI, 30 mM fructose and age matched uncultured controls. Xylitol was chosen because it has no aldehyde or keto functional group and thus can not participate in Maillard reactions. In addition xylitol is not readily converted to xylulose by lens polyol dehydrogenase (Obazawa

et al. 1974). NaCl was used for similar reasons. The results are shown in Table 8. All cultured lenses contained significantly higher levels of mixed disulfides ($P<0.05$) than the uncultured controls. The cultured lens groups, including xylitol and NaCl cultured lenses had statistically indistinguishable levels of mixed disulfides ($P>0.05$) except that the xylose + ARI cultured group was significantly lower than the NaCl and xylitol cultured lenses. It seems then that the presence or absence of a glycating sugar makes little difference regarding mixed disulfide status. What is more important is the actual length of time the lenses are exposed to the culture environment.

Influence of Protein Thiolation on Subsequent Glycation of Bovine γ -Crystallin

Bovine γ- crystallin was incubated with either 0, 1, or 5 equivalents of GSH to assess the effects differential thiolation may have on glycation of the same protein. Table 9 shows that the 1 equivalent treatment resulted in a 30-fold increase in the amount of bound GSH and the five equivalent treatment produced a 48-fold increase in PSSG. PSSC was also increased 10 and 15-fold respectively, because a small amount of cysteine is present as a contaminant in the commercially obtained GSH. The PSSC values were small

Table 8. Total Protein-Thiol Mixed Disulfides in Cultured Rat Lenses.

	Uncultured	Xylose+ ARI	Fructose	Xylitol	NaCl
Mean	456	564	696	712	779.7
s	90	87	47	119	73.9
n	4	6	6	7	3

Data are nmoles/gr dry wt.
Rat lenses were cultured for one week in 30 mM xylose+ARI, fructose, xylitol or 15 mM NaCl. Xylose media included AL01576 (10 μM), an aldose reductase inhibitor. Mixed disulfides were quantitated as described in methods. Means connected by underline are not significantly different (Tukey-Kramer multiple comparisons; $P > 0.05$).

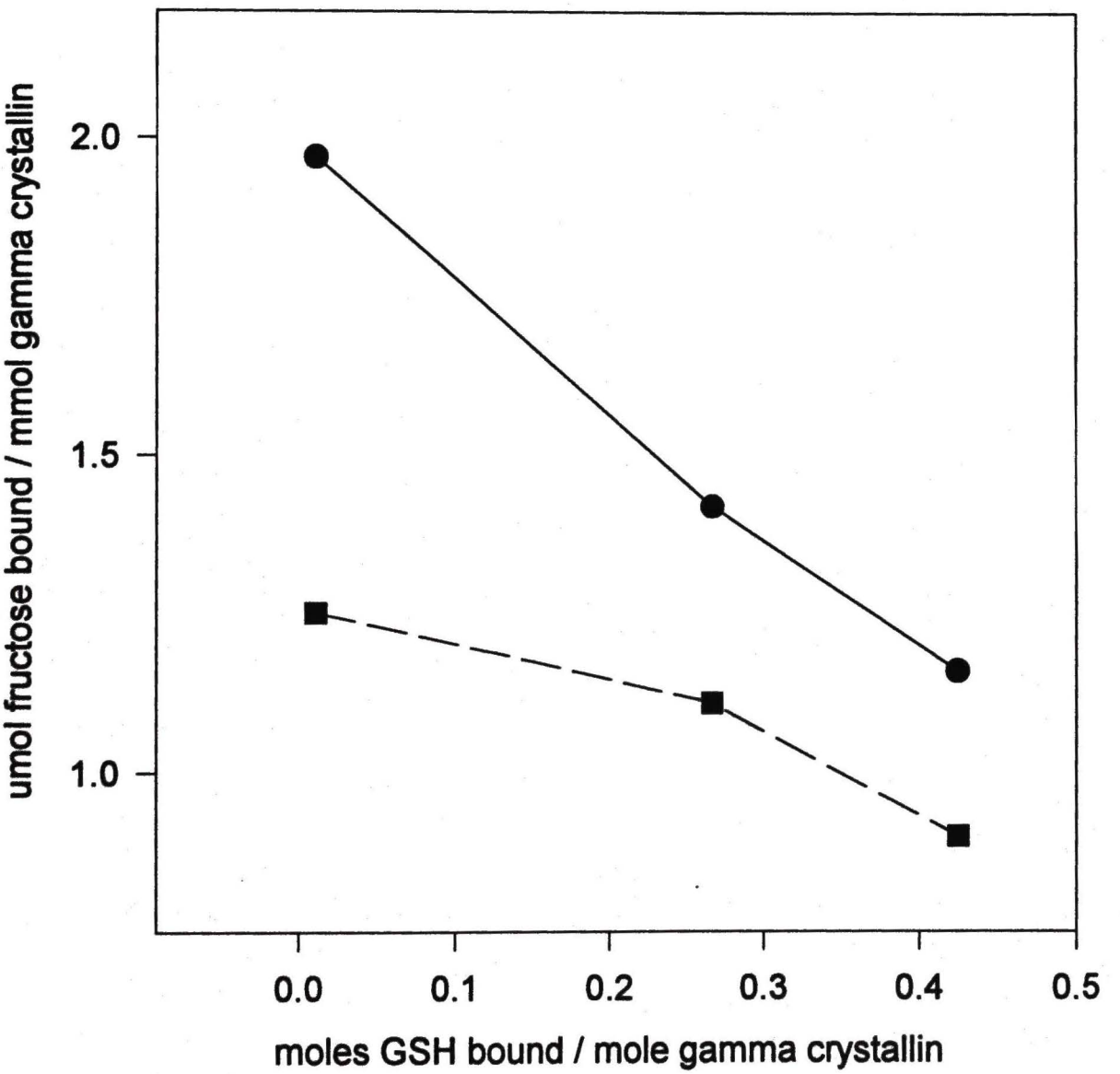
Table 9. Formation of γ- crystallin/GSH mixed disulfides in vitro.

GSH	0	1 equivalent	5 equivalent
Mean	0.4	11.8	18.9
n	2	2	2

Data are expressed as μmol GSO3H released g protein^{-1} following performic acid oxidation as described in Materials and Methods. γ-crystallin (1 mg mL^{-1}) was reacted with either 0, 1 or 5 equivalents of GSH (1 equivalent GSH defined as 1 mole GSH per mole of γ- crystallin, see Methods).

relative to the PSSG in both GSH incubated samples, at 13% of the PSSG amount for the one equivalent treatment and 12% for the five equivalent treatment. These samples were then incubated with ^{14}C-fructose to induce glycation. After one week, all three samples were glycated however as can be seen from Fig 28, an inverse relation between amount of mixed disulfide modification and glycation by fructose was observed. The γ- crystallin which was not thiolated incorporated about 1.97 μmoles fructose mg^{-1} of γ-crystallin. The one equivalent sample had 1.42 μmoles mg^{-1} of γ- crystallin and the five equivalent sample bound 1.16 μmoles fructose. This experiment was repeated with essentially the same outcome (Fig. 28).

Figure 28. Glycation of γ- crystallin by ^{14}C-fructose following differential thiolation of the protein. Protein was incubated with GSH as describe in Table 9 and Methods, followed by one week incubation with ^{14}C-fructose. Each line represents a separate experiment.


umol fructose bound / mmol gamma crystallin
2.0
1.5
1.0
0.0
0.1
0.2
0.3
0.4
0.5
moles GSH bound / mole gamma crystallin

CHAPTER IV

DISCUSSION

Age and Mixed Disulfide/Thiol Status in Human Lens

The proportion of the glutathione pool involved in protein-thiol mixed disulfides in normal lenses is very low; at about 1-9% of free GSH. In spite of this, the human lens contains an impressive amount of these modified proteins. The effects of aging and oxidative stress on the degree of protein S-thiolation in human lens has never been explored before. A total of 95 donor lenses were analyzed to answer these questions. These lenses were selected and processed so that the same sample was evaluated for both GSH and protein-thiol mixed disulfides.

The finding that GSH depletion occurs in lens with age (Harding 1970, Lou et al. 1990) was confirmed. The steady and progressive depletion of GSH between the first decade and the sixth decade amounted to a 40% decrease. Lou et al. (1990) demonstrated in the rat lens that aging affected not only GSH depletion (50% decrease) but also resulted in an increase in protein-thiol mixed disulfides. Similar observations were found in human lens in this study.

PSSC was accumulated slowly and linearly with age. This seems to be a simple reflection of the amount of time a given polypeptide chain is exposed to potential random modification of the available free cysteines.

The change in PSSG with age appeared to be triphasic (Fig. 8). In contrast to Harding (1970), the PSSG level in clear lenses is not nearly as high as reported or as uniformly consistent across age groups.

The earlier results of Harding (1970) must be viewed as ambiguous since the method they employed was not specific for protein bound GSH measuring instead total thiols bound to the protein. The method did not require free GSH removal from the lens homogenate; the overnight incubation of the lens homogenate in TCA will generate PSSG artificially.

In the present study, PSSG increases from birth to about twenty years of age at which point it decreases to a level which is maintained for the next four decades, followed by a marked increase in the number of lenses with high levels of PSSG during the sixth decade. The former change may be a unique physiological phenomenon in young, growing lenses since it may require an active- protective mechanism to prevent oxidation of the protein thiols so that the nascent structural proteins may retain the optimal conformation to be packed properly. Protein S-thiolation has long been proposed as a protective mechanism in other cell types (Brigelius 1985). The correct folding of a

protein has also been shown to be profoundly influenced by disulfides formed during this process (Oas and Kim 1988). The young lens is known to exhibit a relatively high metabolic activity. There may be enzymes to regulate protein S-thiolation and S-dethiolation by non-protein thiols (Hatakeyama, Tanimoto and Mizoguchi 1984), just as enzymes are involved in phosphorylation (Chiesa et al. 1987) or addition/removal of O-linked sugar moieties (Roquemore et al. 1992).

A potential target for enzymatic thiolation is γ-crystallin. The γ- crystallin content is known to be maximal in pre-natal bovine lens, (about 22% of the total lens proteins), and declines to about 9% at birth (Pierscionek and Augusteyn 1988). Since this thiol-rich protein is also high in the young human lens (Thomson and Augusteyn 1985), S-thiolation of γ- crystallin by the abundant GSH may be partially responsible for the initial sharp rise in the PSSG level observed in the first two decades. If thioltransferase (glutaredoxin) is involved it is not surprising that only PSSG levels seem to be under enzymatic regulation. Gravina and Mieyal (1993) show that for a variety of synthetic protein-thiol mixed disulfide constructs including papain, hemoglobin and BSA, in disulfide linkage with GSH, cysteine or cysteamine, only the PSSG mixed disulfides served as substrates for GSH-dependent dethiolation by red blood cell thioltransferase. Thus a

PSSC linkage is permanent with regard to the action of thioltransferase.

The second increase in PSSG may be a reflection of the health of the individual lens as well as an effect of aging since the older lenses showed wide variation in PSSG levels; some maintained a low level while others were as much as two or three-fold higher. These latter cases with elevated levels of PSSG could be due to protein modification induced by oxidative stress. There is certainly a need for further experimentation to clarify the factors responsible.

Interestingly, the aging effect on lens thiol and protein S-thiolation parallels the presbyopic changes and other biophysical alterations of the lens associated with age (Koretz and Handelman 1985, Koretz, Handelman and Brown 1984). It may be possible that there is some relation between the underlying cause of altered accommodation in the lens and lens protein modifications as are documented here.

Regional Distribution of Mixed Disulfides

Human lens PSSG and PSSC are distributed quite evenly in the nuclear and cortical regions of the young lens but as the lens ages, the nucleus appeared to accumulate both mixed disulfides more rapidly, particularly PSSC. This preferential association of PSSC with the WI fraction or nucleus is very intriguing. A similar situation has been

observed in the rat lens by Lou et al. (1990) except that in rat lenses the spatial distribution of PSSG and PSSC is even more striking. PSSC is found in the WI fraction and in the nucleus, while PSSG is associated more with the cortical region and with the WS proteins. These findings could be interpreted to suggest that PSSC may play a very important role in protein aggregation. Alternatively, the presence of elevated PSSC in the nucleus in general and the WI proteins in particular, may be a trivial consequence of the fact that PSSC is not a substrate for thioltransferase or other dethiolating enzyme activities. As a result it accumulates particularly in the older regions of the lens.

The difference in size between GSH and cysteine may also play a role in the distribution of their protein mixed disulfides. The smaller size of cysteine may allow it access to protein sulfhydryls which are sterically buried to GSH. PSSC formed in this manner would also tend to be somewhat buried and thus less likely to participate in the disulfide exchange reactions leading to protein-protein cross-links than would PSSG formed on more exposed regions of the protein. PSSP would then be formed at the expense of PSSG and not PSSC. PSSC would thus be carried along with the protein in its eventual aggregation and insolubilization. While this scenario is speculative, it is consistent with many well known facts. 1) γ(II)- crystallin is known to have seven cysteine residues of which only two to three are

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accessible to GSH (Slingsby and Miller 1985). 2) Normal lens PSSC is formed in appreciable amounts in spite of a 30-100 fold greater concentration of GSH suggesting a different pool of target protein sulfhydryls. 3) PSSC is apparently not involved in PSSP formation in experimental cataract models such as the rat lens culture/H_2O_2 system (Cui and Lou 1993). 4) In experimental cataracts, once the oxidative insult is removed and the lenses are allowed to "recover", PSSG levels often decline while levels of PSSC are much slower to reverse or are permanent (Cui and Lou 1993, Padgaonkar et al. 1994).

Lens Clarity and Protein-Thiol Mixed Disulfides

Opacification has been found previously to accompany the accumulation of protein-thiol mixed disulfides in humans (Harding 1970, Truscott and Augusteyn 1977, Anderson and Spector 1978, Lou et al. 1989) and in animal models (Lou and Zigler 1986, Xu et al. 1992a,b, Cui and Lou 1993, Padgaonkar et al. 1994). The ex vivo culture of human lenses with H_2O_2 demonstrated directly that human lens opacification and mixed disulfide accumulation are congruent phenomena. The cataract which was induced in culture bore many similarities to human senile cataract. Importantly, a cataract could be induced in old human lenses (65 years), albeit with more resistance to oxidation than non-primate animals. These

lenses showed not only cortical haziness with considerable swelling but also demonstrated a loss of GSH and a corresponding increase in PSSG and PSSC, a phenomenon that is quite similar to H_2O_2 induced cataract in cultured rat lenses (Lou et al. 1990).

γ-Glutamylcysteine/Protein Mixed Disulfide

The patterns of accumulation of the three mixed disulfide species in humans are quite different from each other (Fig. 13). The pattern of γ-Glu-Cys protein mixed disulfide accumulation (Figs. 13,14) is noteworthy in that it appears to an appreciable extent only in old or infirm lenses. The amount of this mixed disulfide is even more dramatically elevated in cataractous lenses. The significance of γ-Glu-Cys protein mixed disulfides is not clear. It is apparent that the presence of this mixed disulfide is indicative of severe perturbation to the lens, which may have altered its glutathione metabolism.

The synthesis of glutathione involves the sequential action of two synthetase enzymes. The first step is the formation of the γ-glutamylcysteine dipeptide from glutamate and cysteine. This is catalyzed by γ-glutamylcysteine synthetase and is the flux-generating step (Rathbun 1984). This enzyme is feedback inhibited by GSH at levels well below normal values for the lens (K_i=0.4mM) (Rathbun 1984).

The second enzyme in the pathway, glutathione synthetase, is thought to be in close association with γ-glutamylcysteine synthetase, perhaps as part of a multi-enzyme complex (Rathbun, Sethna and Van Buskirk 1977) and its activity has been reported to be 77-fold greater than γ-Glu-Cys synthetase activity in adult human lens (Sethna, Holleschau and Rathbun 1982/83). These facts indicate that opportunities for the formation of protein-γ-Glu-Cys mixed disulfides are very limited in the normal lens and that utilization of γ-Glu-Cys in the synthesis of GSH is tightly coupled with its production. The appearance of γ-Glu-Cys mixed disulfide is thus unlikely unless the synthesis and utilization of γ-Glu-Cys has been uncoupled through damage to one or both of these enzymes. Declining levels of free GSH are normally associated with old or cataractous lenses (Harding 1970, Rathbun and Murray 1991), and have been attributed to a drastic reduction in cysteine transport (Rathbun and Murray 1991). A drop in GSH synthetase activity resulting from damage or modification of the enzyme is certain to accelerate the further depletion of GSH. Severe depletion of GSH could remove the feedback inhibition of the γ-Glu-Cys synthetase. Under these circumstances a surplus of γ-Glu-Cys may result which is then available for forming mixed disulfides. Although the above scenario is speculative, the data are generally supportive of such a scheme.

Lenticular Cysteine Levels and Mixed Disulfides

Even though plausible hypotheses can be proposed as to why there are different modes of accumulation for PSSG and PSSC (as well as PSSγGluCys), they amount to no more than "houses of cards" if it can not be demonstrated that a pool of cysteine is available for mixed disulfide formation. Without free cysteine one must invoke alternative mechanisms to account for the observed presence of PSSC such as degradation of PSSG in situ or worse yet, that it is an artifactual observation. The results outlined in Fig. 16 demonstrate clearly two things. First, free cysteine is in fact present in the lens in concentrations much higher than had previously been reported (Pirie and van Heyningen 1956, Reddy and Kinsey 1962, Reddy et al. 1966, Zigler et al. 1976,1977), This is certainly due to the newer methodology with its greater sensitivity and accuracy. Second, that the region of highest concentration is in the nucleus, coincident with the region of highest PSSC.

Although the correlation between PSSC and free cysteine is a satisfying result it brings forward a much more difficult question: How is cysteine maintained at a higher concentration in the nucleus than in the cortex? A piece of the answer may be that cysteine is the limiting substrate in GSH biosynthesis as all other substrates are present at saturating levels (Rathbun 1989). Given this, if the GSH

biosynthetic enzymes are present and functional, cysteine levels will be maintained at a low level as utilization for GSH production would increase commensurate with any cysteine concentration rise. If however the enzymes are not present, are damaged or if one of the other substrates becomes limiting (e.g. ATP), the cysteine level could rise without a compensatory increase in utilization. This may be the case in the older regions of the lens. Other systems normally utilizing cysteine (e.g. protein synthesis) are also operating at an extremely low level or not at all in the nucleus. Sethna et al. (1982-83) showed a 16-fold decline in the activity of γ-glutamylcysteine synthetase (first and rate limiting step in GSH biosynthesis) over an 83-year time frame in human lens. It has also been reported that the level of this enzymatic activity is even lower in human cataracts (Rathbun 1989). This could provide a partial explanation for the very high cysteine levels in human cataract (Fig. 17). It seems less likely that the lens has a system exclusive to the nucleus for actively concentrating cysteine. If this were true, a cysteine transporter of some sort should be present on the membranes of the inner fiber cells and at a higher level (or activity) than on the outer fiber and epithelial cells. No system of this type and distribution has yet been described in the lens.

Protein Glycation: A Role in Mixed Disulfide Formation?

The data shown in Figs. 18-21 clearly show that modification of a proteins amino groups through glycation (in this case with ascorbic acid), may effect structural changes in the substrate protein. If this is a general phenomenon it follows that these modifications may influence the rate or occurrence of other types of protein modification.

Protein glycation is a relatively slow process (Berman 1991, ch.5, p.244) which usually involves experiments lasting several weeks if not months (Stevens et al. 1978, Monnier et al. 1979, Perry et al. 1987, van Boekel and Hoenders 1991). Fructose and xylose are however relatively good glycating agents when compared to glucose, due in part to the favorable equilibrium between cyclic and acyclic (reactive) forms (Kaanane and Labuza 1989). In addition, xylose has been shown to enter bovine lens and is not phosphorylated once inside (Pirie and van Heyningen 1956). It is convert to lactate (Pirie and van Heyningen 1956) and in the presence of an ARI is not metabolised by aldose reductase. In this study both sugars increased the percent glycated protein level from 18.1% to about 23% of the lens protein (Fig. 24). It has been postulated by some (Stevens et al. 1978, Monnier et al. 1979, Perry et al. 1987, van Boekel and Hoenders 1991) that glycation of lens proteins

facilitates the oxidation of sulfhydryls and involvement in disulfide cross-links. Disulfides form from thiols exclusively by oxidation with other disulfides (Jocelyn 1972, ch.4, p.94), Since mixed disulfide formation has been shown to be prerequisite to PSSP formation (Cui and Lou 1993), the question is then, is the glycation observed in this study sufficient to alter the environment and thus modify the protein sulfhydryl groups?

Protein-thiol mixed disulfide analysis of lenses from this and other experiments (Fig.25, Table 8) shows a dramatic elevation (from approx. 400 to approx. 700 nmol/g) in the amount of lens protein thiolation for lenses cultured one wk in either 30 mM fructose, 30 mM xylose + ARI, 30 mM xylitol or NaCl (Table 8). Since xylitol is unable to participate in Maillard reactions and there was no potential glycating agent present (beyond physiological levels) in the NaCl treatment, it must be concluded that the glycation by xylose or fructose has no effect on protein-thiol mixed disulfide formation.

The relatively high background level of protein glycation seen in the uncultured controls is somewhat perplexing. Perry et al. (1987) report much lower levels (<2%) in normal rats, although others have recently shown normal levels of approximately 20%, similar to the present study (Prabhakaram and Ortwerth 1992, Ortwerth, personal communication). The possibility that this high background

level was due to glycosylation (the enzymatic addition of sugars to a protein on Asn, Ser or Thr) instead of glycation (Schiff base formed between sugar carbonyl and protein amino group) was investigated because of recent reports that α-crystallin may exist with O-linked *N*-acetyl glucosamine (Roquemore et al. 1992). Samples which were treated under conditions designed to promote the elimination of O-linked sugars exhibited the same degree of binding to boronate before and after this treatment (Table 7). In addition, if the α- crystallin was artifactually elevating the measured level of glycated protein because of glycosyl adducts, the SDS-PAGE of the glycated protein fraction should have been enriched with this protein; this was not the case (Fig. 27).

If all of the boronate binding is due to Maillard products, it could be argued that the initial level of glycation (≈18%) had already exerted the conformational changes necessary to promote protein-thiol mixed disulfide formation and that further glycation would be of no consequence to the protein structure. This may in fact be true but it would be disingenuous. Supporters of the glycation hypothesis tout the conformational destabilizing effects of glycation as a result of either the high sugar levels in diabetes mellitus or the accumulated glycation products with age. Baseline levels, no matter how high, in young animals do not provide support for their position. Rather it is the increase in glycation during the course of

the disease, or aging (or culture) which should have accelerated this effect further. If very high sugar levels in the lens environment can be shown to enhance glycation and yet show no significant effect on mixed disulfide status, their hypothesis is falsified.

In the mammalian eye lens, formation of protein-thiol mixed disulfides between the structural proteins and small molecular weight thiols has been shown to be involved in the normal aging process and in cataractogenesis (Lou et al. 1989,1990; Lou and Dickerson 1992, Dickerson and Lou 1993, Cui and Lou 1993). Such protein modification has been attributed to oxidative stress to the lens (Lou and Dickerson 1992), compromised cellular defenses (Reddan et al. 1988) and to decreased GSH biosynthetic capacity (Dickerson and Lou 1993). It has recently been demonstrated that excessive protein-thiol mixed disulfide formation may be a crucial step in the formation of protein-protein disulfides ultimately leading to lens opacification(Cui and Lou 1993).

In contrast to the pace of glycation, mixed disulfide formation can occur quickly. Studies in our laboratory have shown appreciable increases in lens mixed disulfides following only a few hours culture in media containing only a modest amount of H_2O_2 (0.5mM) (Lou et al. 1990, Lou and Dickerson 1992, Cui and Lou 1993).

Perhaps the most compelling argument for the independence of mixed disulfide formation from protein glycation is in the free GSH data (Fig. 26). Note that GSH is depleted to only about 20% of its original level following one week in culture, irrespective of media type including xylitol. Given this redox environment, it is not necessary to invoke glycation as an explanation for the mixed disulfides formed in these long term culture experiments.

The facilitation of changes in a protein's primary structure by an initial different modification is a well known phenomenon. Consider for example degradation of proteins following ubiquitination (Ciechanover 1987), or the "molecular wear and tear" of enzymes (Sun, Yuksel and Gracy 1992). The fact that glycation occurs in lens and to significant degrees over an individual's lifetime is not at issue. Neither is the fact that glycation, like many types of protein modification, can alter conformation. This has been well documented (e.g. Liang and Chylack 1984, Beswick and Harding 1987). What is under scrutiny is the assumption that these changes precede or are prerequisite for oxidative changes to protein thiol groups. The evidence of this study and others (Ansari et al. 1980) does not support this contention.

The possibility that a relation does exist between glycation and mixed disulfide formation can not be ruled

out. It is the author's speculation that glycation may not be a major influence on mixed disulfide formation.

Protein Thiolation: Influence on Mixed Disulfides

Lens protein thiolation with GSH has been shown to effect conformational changes. Liang and Pelletier (1988) demonstrated conformational destabilization and unfolding for both α- and γ(II)- crystallins following attachment of GSH. α- crystallin but not γ(II) was made more susceptible to proteolysis by this modification. Whether or not these changes were sufficient to influence the glycation potential of the modified protein has not been assessed until now.

The data of Fig. 28 clearly show that glycation of γ(II)- crystallin by fructose is moderately inhibited by increasing levels of GSH-thiolation. This result may be explained in a number of ways but the two most obvious explanations are that 1) the added carboxyl groups from the GSH participate in salt links (electrostatic interactions) with the proteins amino groups blocking the formation of Schiff bases between the protein and the fructose;2) thiolation of exposed cysteine residues brings bulky GSH moiety into close proximity with amino groups or reduces their accessibility in some other way (e.g. conformational shift of the protein), preventing glycation.

Regardless of the mechanism, the end result is that modification by GSH altered the further modification potential of this protein in a very tangible way. If as was proposed earlier, the sharp increase in PSSG in human lens during the first two decades is serving a protective function for γ- crystallin, it may be that thiolation of this protein protects not only sulfhydryls but amino groups as well.

Future Directions

It is a truism in science that for every question answered several new questions emerge. This work is no exception. The results reported here suggest continued research in several areas.

1) Is thioltransferase involved in a regulated thiolation of lens proteins during the first twenty years of life? What is the function of this phenomenon? What proteins are thiolated? How is this activity regulated?

2) The GSH synthetic enzymes should be studied in relation to the sudden appearance of γ-glutamylcysteine and the levels of free cysteine in lenses of various ages as well as different lens regions.

3) The possibility that other types of protein modification (racemization, destruction of tryptophan, etc.) may influence mixed disulfide formation and protein

aggregation should be explored.

4) A more thorough study of the effect of γ- crystallin thiolation and the effect on glycation could be carried out to better understand this phenomenon. Because the tertiary structure of γ- crystallin is known (Blundell et al. 1981) it might be possible to produce mutants with substitutions for crucial cysteine or lysine residues.

Further study and eventual understanding of the protein modifications occurring on the path to cataract may facilitate the development of biochemical interventions.

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