### CHAPTER-II

#### REVIEW OF LITERATURE

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2. REVIEW OF LITERATURE

A concise review of literature existing on the aspects considered relevant to the present investigation has been compiled. The literature was surveyed, as far as possible, up to the end of December, 1991.

Scope of the review. The review was planned to limit itself to the three main aspects marked out in the title of the present study, namely,

i) Ocular toxicology;

ii) Carbamylating agents as potential ocular toxicants, with special reference to MIC; and

iii) Changes in lens proteins induced by ocular toxicants, with special reference to lenticular cataracts.

2.1 Ocular toxicology (OT).

Earlier studies (1832 to around 1970). In view of the fact that in the initial stages of its growth, the field of OT remained as a part of the discipline of Ocular Biochemistry (OB), the earlier literature on OT is found widely dispersed in the source material(s) on OB. For this reason, it was considered cogent to refer first to some core/central information on OB itself to scan corroborative links. Studies on the biochemistry of the eye began in the early thirties of the last century with the publication of the analysis of the constituents of aqueous humour of the eye in 1832 by the renowned Swedish chemist Berzelius at Uppsala (9). This was followed by Mörner's investigations during 1893-94 on lens proteins (10). These contributions were followed later, in the present century, by publications
of monographs on the "Biochemistry of the Eye": Krause (11) (of Johns Hopkins Hospital) in 1934 was the first in this pursuit, followed in 1956, 1964 and 1970 by Pirie and van Heyningen of Nuffield Laboratory of Ophthalmology, University of Oxford, England (12), Lerman of University of Rochester, School of Medicine and Dentistry, New York (13) and Graymore of Institute of Ophthalmology, University of London, London, England (13a), respectively. In between, a number of reviews, symposia, and monographs on specialized areas of OB also appeared; amongst these, the prominent ones are: Kinoshita's review of 1964 entitled "Ophthalmic Biochemistry" (14), Graymore's monograph of 1965 on the "Biochemistry of Retina" (15), and Grignolo's Symposium of 1968 "International Symposium on the Biochemistry of the Eye" (16).

Although, the literature on OB cited above did include some notable aspects and literature on OT, yet the credit for compiling an exhaustive and encyclopedically comprehensive list of/literature on ocular toxicants that had become known over the past about one hundred years goes to Grant for his publication in 1962, its second edition in 1974, and later its third edition in 1986, of the "Toxicology of the Eye" (4,17,17a). Allen's monograph of 1964 "Industrial and Traumatic Pharmacology" (18), and that of Aronson and Elliot of 1972 "Ocular Inflammation" (19) are other sources of information on ocular toxicology. Prince's monograph of 1964 "Rabbit in Eye Research" (20) gives
additional details on some areas of OT, including the
investigative methodology on the subject.

It was felt that some details of the investigations
on OT that were pursued during the past over a century
(1850-1970) were warranted in order to afford an insight
into the type of studies that had attracted attention of
ocular toxicologists of those times; some details of ocular
lesions that were considered worth studying are presented
below in segmentary time-scale to highlight the genesis of
the scientific philosophy of that era.

1850-1900. The salient publications on OT of this era seem
to begin with Leber's monograph published in 1891 (21),
followed by his review of 1903 (22). These publications were
followed by a monograph on "Toxic Amblyopias" (impaired
vision) in 1896 by de Schweinitz (23); Carroll's
communication on the same topic ("Toxic Amblyopia") appeared
much later, only in 1956 (24).

1901-1936 (the pre-and the post-World War I period,
coincident with the peak period of the rise of coaltar
industry in Western Europe). This period saw explosive
developments in the field of industrial chemicals
particularly in Western Europe and U.S.A., though it was
notable in Germany. An enormous number of coaltar products,
viz. dyes, drugs and pharmaceuticals, plastics, war gases
and explosives, etc., became the specialities of this era.
The advent of the above commercial products brought mankind
(the industrial workers and the armed forces in particular)
face to face with occupational health hazards. Some of the salient publications on OT of this period are listed below for comparison: Uhthoff's monograph of 1901 on ocular damage from poisons (25, 25a); ocular damage from aniline dyes (26-29); ocular damage caused by drugs, pharmaceuticals and chemotherapeutics (30-33), ocular neuritis caused by intoxicants (34), and ocular damage and burns from chemicals in the chemicals industry (35-39).

1937-1974 (the pre- and the post- World War II period). Although, investigations on ocular damage (40-42) continued, yet a line of more refinements was also included, for example, on cornea (43-46) and retina (47-50). Investigations on industrial gases (51-52) and solvents (53) represent studies in the field of chemical industry. With repeated and incessant reports of cases of ocular damage observed in industrial workers, investigations in this direction also became quite frequent, as is apparent from communications and publications referred to in references (54-58); simultaneously, remedial measures against such ocular injuries were also undertaken (59). Concurrently, ocular effects of irritant drugs when applied directly to the conjunctiva (60), and the methods to study irritation and toxicity of substances applied topically to skin and mucous membranes (61) were also reported. The use of rabbit in eye research (20) and reference to the monument al compilatory work of Grant (17 and 17a) once again seem adequate to conclude this aspect.
More recent developments (1974-onward). In this period, a new era seems to have commenced in the field of OT. In recent years, with an overall change in the perceptions governing this sphere of scientific activity, the field of OT seems to have been perceived better as a part of the far wider (parent) field of environmental toxicology (ET), which undoubtedly is more comprehensive in scope, nature as well as in substance (cf. page 3). The principal reason for this change seems to be the fact that most of the present day environmental toxicants have been shown to possess ocular toxicity as well; this development is quite apparent from a deeper study and analysis of the latest (4th) edition of "Ocular Pathology" of 1991 (62).

The above fact seems true more in the realm of isocyanates, allied heterocumulenics and similar carbamylating agents.

2.2. Carbamylating agents as potential ocular toxicants.

2.2.1. Carbamylating agents (carbamylation of bioconstituents).

As mentioned earlier (page 4), several biological constituents are known to be readily carbamylated with potassium cyanate (K-O-C≡N), or with their more reactive versions, i.e. their tautomers (alkyl/aryl isocyanates: R/Ar-N=C=O); amongst the bioconstituents, the most prominent ones are proteins.

The emphasis in the present study is on ocular lens proteins as the concerned bioconstituent on the one hand, and on MIC as the concerned major carbamylating agent on the
other, though potassium cyanate has also been investigated. These two carbamylating agents form the central target in the present investigation.

1. Cyanate-urea system. The accumulation of cyanate in aqueous solution of urea is the net result of the reversible decomposition of cyanate, yielding NH$_4^+$ and CO$_3^{2-}$-ions; lower temperatures favour lower cyanate levels. Accumulation of cyanate ions [-O-CN$^-$] is most pronounced at a pH higher than 4 (63-65). The reversal of this phenomenon is the formation of urea by heating ammonium cyanate (thermal isomerization by prototropy or prototropic thermal isomerization; Wohler's synthesis of urea in 1928).

The effect of long contact of $\alpha$-crystallin with concentrated urea solution is similar to the effect of incubation of protein for a shorter time in KOCN-enriched urea solution; long contact eventually gives totally carbamylated $\alpha$-crystallin; enrichment of urea with cyanate enhances carbamylation of the protein (66).

Small amounts of cyanate in aqueous urea can carbamylate the $\alpha$- and the $\varepsilon$-NH$_2$ groups as well as the -SH groups of proteins, including those present in cysteine, GSH and urea-denatured $\alpha$-lactoglobulin; completely carbamylated proteins could be prepared readily with an excess of KOCN (67); the S-carbamyl group is stable below pH 5 but is removed quite readily at pH 8, thus making it possible for cyanate to be used as a reversible blocking group for -SH groups in proteins (68).
2. Isocyanate systems.

a) Exogenous carbamyations

Preparation techniques and scope. Smaller quantities of carbamylated biologicals have normally been prepared using lower alkyl or aryl isocyanates, such as methyl isocyanate, or phenyl isocyanate or their more active nitro- or dinitro- versions. Amongst these, MIC is known to be the most reactive agent.

Amongst the large biological substrates, proteins, polypeptides, enzymes, nucleic acids and polysaccharides have been employed; amongst the small molecules, oligopeptides, amino acids and amines, nucleotides and cyclic nucleotides, nucleosides and nucleic acid bases, and oligosaccharides and sugars, polyols, glycols, thiols, and hormones, etc. have been the recipients of the carbamylation process (68a).

The required isocyanates have usually been prepared from the Curtius thermal degradation of acyl azides in benzene or toluene; the required acyl azides have been prepared from hydrazine-activated sodium azide and redistilled acyl halide or anhydride, under phase-transfer-catalysis conditions (68b). Recently, the use of trimethylsilyl systems have been preferred.

For the rather large scale preparation of isocyanates needed for carbamylated biochemicals, and allied industrial products, direct phosgenation of the desired amine, ROH, RSH, ArOH or enol has been adopted (in the known
two-stage process); the substrate includes amines and amino acids; polyamino acids have been obtained through Leuch's anhydride.

The difficulty of handling gaseous, or even dissolved, phosgene has in recent years been circumvented in two ways: i) By using the solid substitute of phosgene, namely, triphosgene (TP; bis(trichloromethyl)carbonate, Cl₂C-O-CO-OCCl₃, m.p. 81-83°C), that is safer, and more convenient to handle, transport, and store; and ii) by totally avoiding the phosgene route, and, instead, using the newly developed reductive processes applied on the corresponding nitro/nitroso compounds to reach isocyanates (probably via the nitrenes). The solvents used for this purpose are the non-active hydrogen-containing solvents, like hydrocarbons, ethers (ether, dioxane, tetrahydrofurane, DMSO, DMF, and the like).

Scope. The present day developments detailed above could readily lead to a number of extremely dangerous but immensely promising newer isocyanates and thence to carbamylating agents and their congeners that are likely to throw new light on several aspects of OT and ocular toxicants. Detailed examination of the congeners, like Cl.NCO, OCN-NCO, diimino-carbenes C=N-N=C, dichloro-carbenes (including NGs and PIMs), and nitrenes, etc., is bound to change the existing concepts in the field of OT and of ocular toxicants; these studies would need more elaborate techniques to reach meaningful conclusions: labelling
techniques based on radiolabels, spin labels (the DOXYL-, the PROXYL-, and the TEMPO-labels), and spin traps, fluorescent labels, and visible chromogenic labels (dye labels) on the one hand, and advanced spectroscopic methodology (like mass-, NMR-, ESR-, CD-, etc.) on the other are needed. These findings could pave the way to newer clues to evolve and bring forth possibilities for the reversal of the damage caused to the ocular tissue by such ocular toxicants.

b) Endogenous carbamylations. In recent years, however, attention has also been drawn to the endogenous carbamylation of proteins by endogenously generated cyanate or isocyanate that arises in certain biochemical disorders, such as renal failure (ureamia), and severe diarrhoeas; this has been located in oriental countries like India and Pakistan. There are several reports (69-71) on the carbamylation of proteins even with urea itself, or, still better, with the urea-cyanate system (i.e. with cyanate dissolved in urea solution).

Isocyanatogens and micogens. Under the above category, of late another class of carbamylating agents has also emerged. These are isocyanatogens (micogens in the case of MIC), in the sense that these exogenous compounds release in-vivo isocyanates or MIC itself (72-76). Obviously, this category has quite promising significance from the experimental point of view. Two such series have been identified, and have been marked out for practical purposes.
Although these have been exemplified for the metabolic generation of MIC, other needed alkyl/aryl isocyanates are also implied. These categories are:

**Category I**

i) N-Nitroso-N-monomethylurea;

ii) p-Nitro-phenyl-N-monomethylurethane; and

iii) N-Monomethyl-formamide (NMF).

i) $\text{me-N-C=O}$

\[ \begin{array}{c}
\text{HN} \\
\text{O=N} \\
\text{NH}_2 \\
\text{H}_2\text{O} \end{array} \xrightarrow{} \begin{array}{c}
\text{me-N-C=O} \\
\text{NN} \\
\text{spontaneous} \end{array} \xrightarrow{-\text{N}_2} \begin{array}{c}
\text{me-N-C=O} \\
\text{labile intermediate} \\
\text{intermediate} \end{array} \]

ii) $\text{me-NH-C-O-NO}_2$

\[ \begin{array}{c}
\text{OH}^- \\
\text{H}_2\text{O} \end{array} \xrightarrow{} \begin{array}{c}
\text{[me-N-C-O-NO}_2] \\
\text{proton-abstracted} \end{array} \xrightarrow{} \begin{array}{c}
\text{me-N-C=O} \\
\text{labile intermediate} \end{array} \xrightarrow{} \begin{array}{c}
\text{[- HO-C(NO}_2]} \\
\text{proton-abstracted} \end{array} \]

iii) $\text{me-NH-C=O}$

\[ \begin{array}{c}
\text{NMF} \\
\text{cysteine} \\
\text{(-N-acyl)} \end{array} \xrightarrow{} \begin{array}{c}
\text{me-NH-CO-S-CH}_2-\text{C-COOH} \\
\text{NHAC} \end{array} \xrightarrow{} \begin{array}{c}
\text{me-N-C=O} + \text{N-Ac-cysteine} \\
\text{N-Ac-S(N-methylcarbamoyl)-cysteine} \\
\text{(urinary metabolite cysteine conjugate)} \end{array} \]

\[ \text{GSH} \xrightarrow{} \text{CH}_3\text{CH}_2-\text{O-CO-NH-CH-(CH}_2)_2\text{-CO-NH-CH-CO-NH-CH}_2-\text{COOme} \]

\[ \text{N-(ethoxycarbonyl)di-me ester} \]

\[ \text{derivative of the GSH conjugate from NMF} \]

\[ \text{(biliary metabolite)} \]
Category II

i) **S-linked series**: represented by S-(N-Me-carbamoyl) GSH/Cys (SMG) (76), and cysteine (SMC).

\[
\text{MIC+GSH} \rightarrow \text{G S-CO-NHme:} \\
+\text{Cys} \rightarrow \text{Cys-CO-NHme}
\]

ii) **O-linked series**: represented by the investigational antitumor caracemide (CAR) from DOW chemicals; synthesized from acetohydroxamic acid and MIC (73).

\[
\text{me-CO-N-O-CO-NHme} \\
\text{CO-NHme} \rightarrow \text{me-CO-NHOH + 2MIC}
\]

The above series provide a biological transport (carrier or vehicle) form of MIC; at the target site (i.e. the tissue bioconstituent), metabolic generation (in-vivo release) of MIC ensues.

In the case of nitroso-methylureas, the formation of a very labile diazo-intermediate (from the vicinially-positioned \(-N=0\) and the amidic \(-NH_2\)) leads to non-enzymatic (spontaneous) elimination of MIC.

The GSH conjugates act as carbamylating agents towards nucleophilic acceptors like cysteine. NMF works the same way via its biotransformation to SMG (or via some other unidentified similar system(s) as well). \(E_1CB\) (unimolecular elimination via the conjugate base) mechanism seems to be operative; proton abstraction from nitrogen results in the formation of a conjugate base which then eliminates the thiol-portion of the carbamoyl-thiol ester, and thus
releases NTC (or possibly other isocyanate moieties as well). The higher reactivity of SMC relative to that of SMG (or even of cysteine itself) shows the relative stabilities of the two conjugates in buffer systems: the proton abstraction from the carbamate nitrogen occurs by intramolecular transfer to the cysteine carboxylate ion, thereby facilitating E1cB-mediated elimination of MIC from the conjugate (72, 76).

These leads/clues could certainly be extended to explore several pending aspects of the desired/warranted investigations on cornea, retina and optic nerve, besides the left-overs from the present lenticular opacity for meaningful application programme(s) by selecting still better biologically-active "good leaving groups" in respect of conjugate bases referred to above.

2.2.2. Carbamylating agents-induced ocular lesions.

1. Cyanate-induced ocular lesions. The discovery by Cerami and Manning in 1971 (77) that sodium cyanate could inhibit the sickling phenomenon of sickle cell erythrocytes in-vitro revealed the potential of this compound in the chemotherapy of sickle cell anemia.

However, it also became known that cyanate was toxic to the ocular lens; animals (dogs) of early age developed more severe lenticular lesions; all dogs with cataracts evidenced other manifestations of toxicity, such as weight loss, and CNS and gastrointestinal involvement (78). In 28 months, the mean carbamylation achieved was 0.69
residue of cyanate per Hb-tetramer, a value comparable to that seen in studies on humans (79). Cornea was found more resistant than the lens; corneal opacities were associated with a higher mean level of carbamylation of Hb and a longer duration of dosage than lenticular lesions. The biochemical changes associated with corneal opacities were not investigated by these workers, and, thus, the detailed mechanism of ocular lesions remained to be determined. However, carbamylation of proteins is known to change the functional characteristics of several proteins (80); it seemed that a membrane structural protein(s) or enzyme(s) was altered leading to increased ionic flux and a compensatory increase in Na\(^+\), K\(^+\) pump. The metabolic studies performed on cataractous and normal lenses revealed increased Na\(^+\) and decreased K\(^+\) levels; GSH levels decreased by 34%; lactate leakage increased by 12%, and lactate level in cataractous lenses were increased by 30%; Rb-uptake increased by 26%; extracellular space which is related to altered transport of cations and is parallel to the degree of structural change increased by 61%.

Sodium cyanate-induced lens changes in the dogs paralleled the morphological changes seen in humans (81). Kinoshita has reported the in-vitro effects of cyanate on the lens (82).

Cyanate is known to affect the CNS; it causes axonal degeneration in neuropathy (83), and polyneuropathy in patients with sickle cell disease (84).
Cyanate-induced enzyme inhibition has also been reported (85-88).

2. Isocyanates-induced ocular lesions. Isocyanates have irritant effect upon the eyes, skin and the respiratory tract. The most severe symptoms of ocular and respiratory irritation are induced by vapors and fog from MIC (used as a reactive intermediate for pesticide manufacture) and toluene and allied diisocyanates (TDI is used in the manufacture of polyurethane foams).

Besides the above two most widely used isocyanates (MIC and TDI), experimental studies on the toxic and irritant effects have also been performed on the following isocyanates: ethyl-, octadecyl-, phenyl- and p-chloro-phenyl-isocyanates; p-phenylene-, toluene-, diphenylmethane- and 1,5-naphthalene-diisocyanates and triphenyl-methane triisocyanates (89). TDI and other polyisocyanates are released as vapors and fogs during the foam-forming reaction; these are severely irritating to eyes, skin and respiratory tract, and may induce sensitization (90-93).

Test application of a drop of m- and p-TDI on rabbit eyes caused immediate pain, lachrimation, swelling of the lids, and conjunctival reaction; corneal epithelium was also damaged, and this could be reduced by prompt flushing with water (89,90).

3. MIC and micogens-induced ocular lesions. MIC is a powerful and unbearable lachrimator; in fact all such isocyanates have strong irritant action on eyes and also on
respiratory tract and skin. Micogens like nitrosomethylurea (and even nitrosoamines) also cause similar ocular damage (75).

The reports available on the ocular lesions caused by MIC and micogens are reviewed below; in view of the anticipated spurt in the number of publications following the Bhopal MIC gas leak disaster, the account that follows has logically been arranged in two chronological periods, namely, the pre- and the post-Bhopal disaster periods.

**The pre-Bhopal disaster period (i.e. upto December 2, 1984):**

The ocular lesions induced by MIC (and allied isocyanates, and related compounds) had been reported by Lewin (1913)(31), Watrous (1947)(56), Mann (1948)(40), Schreuk (1955)(89) and Zappe (1957)(90). These communications were supplemented and integrated in the monographs by Grant (1962, 1974 and later in 1986; 4,17,17a), Allen (1964;18), and Aronson and Elliot (1972; 19).

These publications were followed by elaborate investigations undertaken by Harding and his collaborators at the Nuffield Laboratory of Ophthalmology (University of Oxford, England) during the years 1979 onward; the reports gathered by them upto the end of November 1984 had brought forth experimental evidence that MIC did induce serious ocular lesions arising from its powerful capability of carbamylating bovine lens proteins that, in turn, induced conformational changes in their structure, and, further,
that such developments did lead to lenticular cataracts (69,70,94-96). These results were indeed exceptionally significant, because previous reports had not emphasized such a powerful ocular damage so explicitly.

The post-Bhopal disaster period (from December 3, 1984 onward).

Just after the MIC-disaster, several reports (97-103) started appearing in the international media like Nature, Lancet, JAMA, Br J Ind Med, J Occup Med, and the like, on the scientific and medical aspects of the MIC-induced damage caused to the population of Bhopal. During the initial stages of the disaster, two important developments took place that do deserve attention; these were:

i) immediately after the gas-leak, there started the "cyanide controversy" (104,105); it was contradicted by Nemery et al. of Medical Research Council Unit of England in November 1985 (106), and by Jameson et al. of National Toxicology Program (NIEHS) in 1986 (107). Later, it was set to rest by elaborate investigations of Vijayaraghavan et al. in 1987 (108), and of Varma et al. in 1988 (109);

ii) the second development concerned the ocular damage caused by the "toxic gases" (including MIC) released from the Union Carbide plant in Bhopal. Investigations on this aspect were sponsored by the Royal Commonwealth Society for the Blind (England) under the leadership of Andersson and his team from the Department of Occupational Health,
London School of Hygiene and Tropical Medicine, London, Moorfield Eye Hospital, London and Chattisgarh Eye Hospital, Raipur, Madhya Pradesh, India. This team visited the site of the disaster to assess possible long-term visual disability among the survivors; the preliminary assessment and survey took place on December 11-17, 1984, and the findings became available on December 22-29, 1984 (110); the results of the follow-up (completed with the help of additional team from the Department of Ophthalmology, Gandhi Medical College, Bhopal) were made available on March 30, 1985 (111).

The preliminary findings of the first limited enquiry in the first fortnight of the disaster (110) concerned with the severe acute effects and revealed that the toxic gas was intensely irritating to the eyes, and nose and throat and leads to corneal ulceration and to pulmonary oedema; the main trouble was found to be streaming eyes, photophobia, profuse lid oedema, and superficial corneal ulceration; slit-lamp examination showed discrete superficial lesion in all cases; no case of blindness and no evidence of irreversible eye damage was observed, though there were possibilities for long term problems. The follow-up (111) stressed the need for investigating the long-term effects of MIC and of substances involved in the episode, including its metabolites.

Harding and Rixon (1985)(112) published their findings on the lens opacities induced in rat lenses by MIC.
in in-vitro investigations; other findings on the MIC-induced ocular damage came from Dwivedi et al. (1985)(113), Gassert et al. (1986)(114), Salmon et al. (1985)(115), Andersson et al. (1986)(116), Gupta et al. (1987)(117), Khurrum (1987)(118), Raizada (1987)(119), and Andersson et al. (1988)(120) and (1990)(121). The clinical studies on MIC reported by Misra et al. (1987)(122), and Misra et al. (1988)(123) also detailed the ocular lesions caused by MIC. During 1987-1990, several reports (124-127) became available on the MIC-caused health hazards.

2.3. Change in lens proteins induced by ocular toxicants.

2.3.1. Structural organization of the eye. The lens is a clear avascular tissue suspended by zonular fibers between the aqueous humor and the anterior face of the vitreous body and its function is to allow free passage of light, and focus it on the retina. Hence, it was considered appropriate to include a brief and concise account of anatomical structure and physiological function of the different parts of the eye; this summary has been abstracted from Ocular Pathology 1991 (62).

The eye is composed of three primary layers. The outer coat, the tunica fibrosa, is composed primarily of collagen-elastic tissue and provides a protective outer wall. The cornea forms the anterior one sixth of this layer, the sclera the posterior five sixths. The sclera is white and opaque as a result of random, irregular layering of its collagen fibers. In contrast, the corneal collagen
lamellae are arranged in a parallel fashion and therefore have a geometrically regular appearance. This renders the cornea transparent to incoming light.

The uvea or tunica vasculosa is the middle layer of the eyes and lies immediately inside the sclera. This richly vascular and pigmented tissue consists of three parts: the iris, the ciliary body, and the choroid; these together with aqueous and vitreous humor constitute the dynamics of the transport systems in the eye (128). The most important function of the uvea is the vascular supply to the eye, particularly to the outer portion of the sensory retina via the choriocapillaris.

The inner layer of the eye, the tunica nervosa, composed of sensory retina, pigment epithelium, and the optic nerve develops embryologically as an anteriorly protruding portion of the brain. This tunic is derived from the two-layered neuroectodermal optic cup. In the posterior aspect of the eye the original inner layer of the optic cup forms the sensory retina and the outer layer develops the retinal pigment epithelium. The space between these layers, the original cavity of the optic vesicle, forms the subretinal space in the adult eye. In the adult, it is normally merely a potential space and reappears only in cases of pathologic retinal detachment. The anterior aspect of the tunica nervosa develops into the two-layered epithelia of the iris and the ciliary body.
2.3.2. **The lens components.** The lens consists of three components: from the outer surface inward they are the lens capsule, the lens epithelium, and the lens substance. The lens capsule is a secretory product of the lens epithelium. It functions as a metabolic barrier and may be largely responsible for the elasticity and shaping of the lens during accommodation. The lens epithelium is confined to the anterior surface and the equatorial lens bow. It contains only a single layer of epithelial cells which, in course of terminal differentiation, lose their nuclei as well as other cytoplasmic elements such as mitochondria and microsomes. Most of the fiber cells of the tissue are devoid of cytoplasmic components. Thus, the lens is a clear avascular anaerobic tissue with limited metabolic activities, very essential for its physiological function—via glycolysis for ATP production and hexose monophosphate pathway for the supply of reduced nucleotides to maintain the redox state through the glutathione cycle coupled with ascorbic acid.

At the stage of primary embryonic nucleus, the lens fibers extend from the anterior to the posterior pole and the lens is spheric. As growth proceeds, pole-to-pole growth is not achieved and thus the lens becomes a flattened biconvex sphere. Growth of the lens by deposition of new fibers occurs throughout the lifespan. Growth is slower after the second decade, and the lens does not increase much in size thereafter because of a relative loss of hydration and shrinkage of the lens nucleus. Thus, the morphological
structure of the tissue is arranged so that the dedifferentiated fiber cell is not eliminated but is pushed in towards the centre of the tissue. The oldest fiber cells are in the centre of the lens and the youngest cells are at periphery. Fiber cells produced at all stages of life from fetus to old age are present, which is the major factor in the genesis of the lenticular opacity.

The lens substance is a product of the continuous growth of the epithelium and consists of the cortex and nucleus, the transition between the two being gradual.

Most of the metabolic activity of the tissue is confined to the epithelial cell layer and a narrow peripheral band of cortical fiber cells. The cells are rich in enzymes and have extensive protein metabolism. The enzymes whose activity in the lens epithelium is higher than those in the cortex and nucleus include the acid hydrolases (acid phosphatase, N-acetyl-β-D-glucosaminidase, α-D-mannosidase, α-L-fucosidase and β-D-glucuronidase) (129), lactate dehydrogenase (130), aldehyde reductase II (131) and aldose reductase (131-133). However, the level of leucine peptidase activity which is present in all the layers except the epithelium, gradually decreases towards the nucleus (134).

The lens contains a remarkably high concentration of protein, ranging from 35% to 40% of the total wet weight of the human lens, with higher values reported for other species. Most of this protein is the structural protein
represented by the water soluble protein(s), the crystallins, whose only function is to contribute to the transparency by maintaining an essential refractive index gradient (135); the rest is contributed by the water insoluble proteins, the albuminoids. For instance, the concentration of the protein fractions (11) in the bovine lens are as follows: Alpha crystallin (31.74%), Beta crystallin (53.39%), Gamma crystallin (1.46%) and Albuminoid (12.50%). There are also very small amounts of nucleoproteins, mucoproteins and lipoproteins in the lens.

Even at extremely high concentrations of crystallins, a properly functioning lens needs to maintain the short-range ordering of the crystallins; otherwise lens opacification (i.e. cataract) occurs (136,137) as a result of increased light scattering. Recent observations showed that under normal physiological conditions a low molecular weight crystallin particularly γ-II which contains a high degree of exposed thiols may be maintaining the transparency of the lens through a reductive effect by inhibiting the intrinsic tendency of α-crystallin to aggregate, an association reaction implicated in the loss of lens transparency (138). Not only is the overall crystallin concentration in the nuclear lens fiber cells higher than in the cortical cells, but the relative proportions of the α-, β- and γ-crystallins also vary with age and location within the lens, as a result of differential synthesis during development (139,140). Perhaps, most interesting is the
fact that γ-crystallins appear to be enriched in the lens nucleus, the region in which senile cataract most commonly manifests itself (134,139-140).

2.3.3. Molecular organization of lens crystallins and changes with age.

Different aspects of the physiology and the biochemistry of the lens have been reviewed by various workers (13a,134,141) dealing with function and macromolecular composition (141), chemical constituents (13), isolation and characterization of the lens proteins and the changes in structural characteristics with development, aging and cataract (134).

With aging the protein in the human lens undergoes a number of molecular and macromolecular changes, such as yellowing, the formation of new nontryptophan fluorescent compounds covalently attached to the protein, marked increases and decreases in the molecular weight of the polypeptides and a drastic increase in the aggregative properties of the protein as summarized by Castineiras (142). These modifications occur slowly and eventually lead to the formation of opacification of the lens and a resultant decrease in visual acuity. These changes are aggravated, resulting in a spontaneous eye opacification especially in 85% of octogenarians having some degree of lenticular opacification, beyond middle age (143). In many types of acquired cataracts (62) such as traumatic (chemicals including those from environmental toxicants and
radiant energy), metabolic disorders, drug-induced, and opacities caused by associated intraocular disease such as uveitis etc, the structural changes associated with senescent cataract would be aggravated and accelerated to appear at an earlier age.

The most prominent biochemical observations associated with aging or in the various types of cataracts are increase in the proportion of high molecular weight proteins (soluble to insoluble), and a change in the redox system more so, in favour of the oxidized state of the lens or its membrane (134, 144, 145). It is becoming increasingly clear that conformational perturbations, disulfide bonding and aggregation or cross-link formation, common phenomena in the aged and cataractous lens, are closely related to the precise molecular features of lens crystallins (146-150). Thus, the modern trends are related to understand the molecular events underlying this process.

The high molecular weight aggregates in the insoluble proteins are derived from the soluble proteins as a mixture of all the three groups of crystallins while in the water-soluble proteins these are of four types: (1) high molecular weight aggregates of \( \alpha \)-crystallin which increase with age in clear lenses, and calcium and/or glucose may promote its formation. The many thiol groups of \( \beta \)-and \( \gamma \)-crystallins could bind a cloud of \( \alpha \)-crystallin molecule by disulfide bonds such that \( \alpha \)-crystallin would form 90% of the aggregate; (2) high molecular weight may be derived from a
mixture of lens crystallins; (3) disulfide cross-linked proteins of high molecular weight, and (4) non-disulfide covalently cross-linked proteins of high molecular weight; the last two are isolated in a dissociating media. The latter aggregates appear to be a further stage after the progression from the conformationally altered protein of nuclear cataract to disulfide formation (134).

In addition to covalent disulfide crosslinks, there are other possibilities of adduct formation between the lens proteins and other endogenous compounds, such as carbohydrates, (glucose, glucose-6-phosphate (151-159), galactose (160,161); ascorbic acid (and its oxidation products) (162-164); oxidation products of amino acids (tryptophan, tyrosine, cysteine, methionine and histidine) (142,165-172) and their degradation products (173,174). These alter the UV and fluorescence spectral characteristics, usually a red shift in the excitation and emission maxima from those of the normal proteins.

These large protein aggregates would scatter light and contribute to the visual impairment in cataract. It has thus become evident, that the transparency of lens proteins, their main optical feature, is closely related to the molecular spatial arrangement (secondary and tertiary structure) of the crystallins which in turn is determined by their primary structure (175). The secondary structures of the crystallins are very similar; all have mostly $\beta$-pleated sheet structure (147,176). Considerable differences,
however, have been noted in the tertiary structures of these proteins (147,148). Recent studies have shown that among the three different classes of crystallins (\(\alpha\), \(\beta\) and \(\gamma\)), the tryptophan (147), and the cysteine (148) residues of \(\alpha\)-crystallin are the most exposed.

\(\alpha\)-crystallin, the major structural crystallin in mammalian lens and in the high molecular weight proteins, is a heterogenous population of large multisubunit aggregates, with an average molecular weight of about 800,000 under most of the isolation conditions. In-vitro studies with purified \(\alpha\)-crystallin showed that the size and polydispersity of \(\alpha\)-crystallin are influenced by a variety of factors such as temperature, ionic strength, age of the tissue, as well as the type of the buffer used (177, 178). Hence different quaternary structures have been proposed for the aggregates on the basis of studies conducted with such different preparations (179,180). It is composed of two closely related gene products (\(\alpha A_2\) and \(\alpha B_2\); \(M_\gamma \approx 20,000\)) which are subjected to a wide variety of covalent post-translational modifications which are responsible for the disruption of the organized ordered structure of lens fibers. There is a direct gene controlled expression of \(A_2\) and \(B_2\) chains and the formation of a homogeneous macromolecule, \(M_\gamma\) of approximately 700,000 (181). This is followed by post-translation reactions leading to the formation of \(A_1\) and \(B_1\) chains and changes in the aggregate structure reflected in physical heterogeneity and higher molecular weight and
finally limited proteolytic degradation and racemization primarily detected in the protein isolated from the older sections of the tissue (182). Also, a slow nonenzymatic deamidation reaction (183-185) and a cAMP mediated phosphorylation of the serine hydroxyl groups of the \( \alpha \)-crystallin \( A_2 \) and \( B_2 \) chains specifically of the cortex in the formation of \( A_1 \) and \( B_1 \) chains and the \( \beta \)-crystallin \( B_2 \) (\( \beta B_p \)), in their N- and C-terminal domains (186,187) are being implicated. It is suggested that this metabolically controlled phosphorylation (possible control by extracellular factors) may be associated with the terminal differentiation of the lens epithelial cells and the intracellular organization of the lens fiber cells.

The \( \beta \)-crystallins are a heterogeneous population of oligomers, ranging from dimers and trimers (\( \beta_d; M_r \approx 50,000-60,000 \)) to hexamers (\( \beta_h; M_r \approx 200,000 \)) with the different forms participating in a reversible concentration-dependent self-association equilibrium (188,189). There are a number of different \( \beta \)-crystallin polypeptides which range in \( M_r \) from about 22,000 to 32,000, but all are closely related and show a high degree (>40%) of sequence homology to themselves and the \( \gamma \)-crystallin (190,191).

The third class of crystallins is the monomeric (or low \( M_r \)) \( \beta_b \) (191) and \( \gamma \)-crystallins. These are a highly homologous (\( \approx 75-95\% \)) family of six or seven gene products, each with a \( M_r \) of about 20,000. The major crystallins of the bovine lens are \( \gamma \)-II, \( \gamma \)-III A, \( \gamma \)-IIIB, and \( \gamma \)-IV A with
more than 75% sequence homology (192,193) which confer a remarkably high stability (150,194) that is of utmost biological significance as the lens transparency depends upon the unperturbed native state of the protein. However, this conformational stability could be reduced by change of redox state as demonstrated by glutathione reaction monitored by thermodynamics of thermal and athermal denaturation of γ-crystallins (195). A distinctive feature of the γ-crystallins is the presence of unusually large amounts of free sulfhydryl groups (five to seven per polypeptide chain) which could be easily susceptible to oxidation and formation of non-covalent adducts, with non-tryptophan fluorescence. This has been justified to a great extent by demonstrating that bovine γ-II, the major gene product of the γ-crystallin manifest significant redox activity (196,197). Inspite of a high degree of sequence homology and similarity in secondary structure, the microenvironments of tryptophan, tyrosine, and cysteine residues of γ-crystallins in solution vary greatly (198,199) and differ from their α and β counterparts in cryoprecipitation (140) and photoinduced (149,199,200) changes, and the various γ-fractions differ in their denaturation behaviour (150) and in susceptibility to cryoprecipitation and ease and extent of photoaggregation (199,200). Exhaustive reviews have appeared on the evolutionary aspect and expression of crystallins and their genes, in addition to the biochemistry and biophysics of the
lens. The latest concerned reviews are those of Wistow and Piatigorsky (135), and Schoenmakers et al. and Summers et al. (192, 193).

The various chemical characteristics of the three crystallins (134) have been outlined in Table given below:

<table>
<thead>
<tr>
<th>Property</th>
<th>Class of crystalin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrophoretic mobility (towards the anode, at pH 8-9)</td>
<td>α- High</td>
</tr>
<tr>
<td>Range of isoelectric points (pH)</td>
<td>4.8-5.0</td>
</tr>
<tr>
<td>Molecular weight (Daltons)</td>
<td>Over 5x10^5</td>
</tr>
<tr>
<td>Molecular form</td>
<td>Aggregate</td>
</tr>
<tr>
<td>Thiol content</td>
<td>Low</td>
</tr>
<tr>
<td>N-terminal amino acid</td>
<td>Masked (N-acetylated methionine)</td>
</tr>
</tbody>
</table>

Not only the individual structural characteristics of the crystallins but also the interactions of these proteins among themselves would be another important factor in maintaining the transparency of the lens. These proteins have an extensive network of interacting charges on their surface which may contribute to their thermodynamic stability, and partially define the degree of water retention in the lens. Induced perturbation of the normal crystallin tertiary structure has been observed in the
presence of sugar molecules (154-158,201), in photosensitizing reactions (165,199,200,202-206) and in the aging process (134,151-153, 162,207-210). Some crystallins appear to be more susceptible to such chemical or photochemical insults than others (149), suggesting that some tertiary structures may, in fact, be more vulnerable to damage in-vivo. γ-crystallin was found to be more readily glycated than α- and β-crystallin in streptozotocin induced diabetic rats (211). Also rate and site of glycation of rat α-, β- and γ- crystallins differed (212).

2.3.4. Mechanism of post-translational modification. Increasing evidence has been accumulating in support of the hypothesis of Stevens et al. (213) that the free amino groups are of importance in maintaining the state of crystallins and modification of these by post-synthetic reactions, such as carbamylatation (69,95,157,214), non-enzymatic glycation (213,215) and formation of isopeptide bond (216) leads to the conformational changes in the crystallins which result in the formation of high molecular weight protein aggregates. In addition, certain other types of non-enzymatic modifications such as racemization (217,218), deamidation (183-185), deamination (185), disulfide bond formation (134,146-150) and dityrosine formation (142,219) of lens crystallins were also implicated as causative factors in the development of lenticular opacity, whereas reactions involving acetylation (220-224) would be conferring protection against crystallin
aggregation which is mediated through post-translational modification such as carbamylation, non-enzymatic glycation, and isopeptide bond formation, etc.

**Acetylation.** Evidence for this has been derived from the multiple action of aspirin, i.e. acetyl salicylate (O-acetoxy-benzoic acid) as a therapeutic agent in various pathological conditions such as arthritis (225) and has an anti-inflammatory and anti-platelet effect (226). Acetylation of free amino groups of many proteins such as hemoglobin (227), albumin (228), immunoglobulins (229), erythrocyte membrane peptides (230) and lately various crystallins preferably at the ε-amino group of the lysine (220) has been demonstrated. It has also been shown that both carbamylation and glycosylation would be inhibited by aspirin as a result of acetylation of ε-NH₂ group (220-224). These in-vivo and in-vitro studies suggested the potential therapeutic use of aspirin against non-enzymatic post-translational modification (glycation and carbamylation) (220,224,231).

Other post-translational modifications such as phosphorylation (186,187), and deamidation (183-185), etc., have already been dealt with above. Various hypotheses have been put forward for the post-translational modification of the protein with respect to carbamylation and glycation in aging and cataract of different etiology (clinically or experimentally produced). The common feature in these two processes are the formation of stable carbamylated or
glycated proteins by combining with free amino group (N-terminal or \(\varepsilon\)-amino group) in the proteins.

Carbamylation: Carbamylation of lenticular proteins leading to opacity has already been dealt with in detail in the previous section, i.e. carbamylating agents which includes the carbamylation by denaturing agent (urea or urea in the presence of cyanate) \((66,69-71,78,82,95)\), methyl isocyanate \((112,121)\), and the special feature of non-enzymatic transfer reaction mediated by carbamylated glutathione \((76,232)\) or cysteine \((232)\) to form a stable N-carbamylated product with free amino groups (N-terminal or \(\varepsilon\)-amino groups).

Glycosylation. Two major hypotheses put forward for the age-related glycation and the etiology of lenticular opacity are the disarrangement of the normal metabolism involving the osmotic hypothesis \((132)\) and the oxidative damage \((151-153)\). Both of these mechanism lead to non-enzymatic glycation resulting in the disorganization of the lenticular proteins, and hence the loss of transparency of the lens.

Osmotic hypothesis. As already stated, the lens being relatively inactive metabolically and the metabolic activity being of the anaerobic type, most of the lenticular energy metabolism is utilized in the maintenance of transport processes. Under normal conditions, glucose metabolism is preferentially through glycolysis for energy purposes, and through hexose monophosphate pathway for the supply of reducing units (NADPH) to maintain the redox state mediated by GSSG/GSH in the aqueous phase and the lipid soluble
vitamin E at the membrane level. Glucose is preferentially phosphorylated by hexokinase and a part of it could be diverted to the polyol pathway via the formation of sorbitol by utilizing the necessary nucleotide cofactor (NADPH) generated from the hexose monophosphate pathway. The limiting factors for the polyol pathway are the availability of the substrate (glucose) and the cofactor, reduced nucleotide (NADPH). Thus, excess glucose or increase in the NADPH/NADP⁺ ratio would be promoting the polyol pathway. This pathway is constituted by the enzymes aldose reductase and polyol dehydrogenase. In the animal lens (233), a higher specific activity of aldose reductase relative to hexokinase and polyol dehydrogenase, sufficient regeneration of NADPH (the cofactor for aldose reductase), and the levels of NADPH adequate to inhibit polyol dehydrogenase, would promote the accumulation of sorbitol from glucose. This accumulation of sorbitol is the basis of the osmotic hypothesis. The sorbitol so formed is not rapidly converted to fructose and being relatively impermeable through most of the biological membranes accumulate intracellularly, creating a hyperosmotic effect resulting in increased hydration and membrane dysfunctions. This leads to total electrolyte imbalance (a decrease in the K/Na ratio), a decrease in biochemical constituents (reduced GSH levels and ATP and a leakage of amino acids) and eventually a loss
of total osmotic integrity depicted by Kinoshita (233a) as given below (Fig. A).

Calcium is also known to play a role in cataract formation. In lenses with near normal sodium content, the high calcium concentrations were associated with highly localized opacities, while nuclear cataracts had a low calcium content (234).

Thus, in contrast to the earlier reports that increase or decrease in the level of calcium induces cataracts, the recent studies by Duncan and Jacob (234) have conclusively demonstrated that an increase in intracellular calcium does change the organization of proteins within the membrane gap junctions which in turn affect the ease with which substances can pass from one lens cell to the next, and induce cellular uncoupling. Further, calcium diffusion within the cell is extremely slow, presumably because of the
interaction between calcium and charged molecules within the lens, and this in turn help to localize the opacity.

**Non-enzymatic oxidation:** The oxidation of NADPH can also be facilitated by monosaccharide autoxidation (132). It has been shown (235) that monosaccharides D-glucose, D-\(\alpha\) , L- and DL-glyceraldehyde and other hydroxyaldehydes \([RCH(OH)CO(R')]\), are able to autoxidize under physiological conditions, forming dicarbonyl compounds and hydrogen peroxide via reactive intermediates of dioxygen and carbon-centered free radicals, as summarized in Fig. B.

FIG. B(Crabbe). A mechanism for the autoxidation of monosaccharides, showing the formation of superoxide, hydrogen peroxide and hydroxyl radicals. \(R, R' = \text{alkyl or H}\). The ene-diol and the ene-diol oxyradical may be metal-ion coordinated (235).
The same autoxidizable monosaccharides which initiate oxidative damage (132) are the substrates for aldose reductase. It has been observed that in vitro NADPH oxidation does not reflect monosaccharide reduction. The NADPH is oxidized by the conjugate acid of superoxide, produced by monosaccharide autoxidation as shown in Fig. C. (132).

\[ \text{AUTOXIDATION} \]

\[ \text{NADPH} \rightarrow \text{A.R. inhib} \rightarrow \text{O}_2 \rightarrow \text{NADP}^+ \]

\[ \text{H}_2\text{O}_2 \rightarrow \text{NADP}^+ \rightarrow \text{O}_2 \]

\[ [\text{ENZ-NADPH OXID}^3] \rightarrow \text{NADPH OXID}^3] \]

**Fig. C (Grabher) Diagrammatic representation of superoxide-mediated NADPH oxidation by autoxidizing monosaccharides causing an apparent aldose reductase activity in vitro (S.P. Wolff, DPhil thesis, Oxford). Binding of nucleotide to the 'enzyme' causes a 1000-fold increase in rate (132).**
This reaction which proceeds via the NADP⁺ radical, is accelerated 1000-fold if the nucleotide is present as enzyme-nucleotide complex. Since NADPH is also bound to bovine lens aldose reductase; this binding increases the ability of NADPH to be oxidized by the autoxidizing monosaccharides.

The two routes by which the tissue damage could be caused by autoxidizing monosaccharides have been summarized in Fig. D. (132).

**FIG. D (Grabbe).** Two routes by which autoxidizing monosaccharides can cause tissue damage. (132).
Glutathione could be important in protection against damage by dicarbonyls and free radicals. The effect of lipid peroxidation could be crucial for changes at the cell membrane, while both dicarbonyls and free radicals could cause protein modification.

It has been observed that a variety of compounds inhibit the autocatalytic, superoxide-mediated oxidation of NADPH in-vitro, delay or prevent cataract in experimental diabetic animals, and inhibit sorbitol production in cultured tissues and experimental diabetic animals. These so-called aldose reductase inhibitors are antioxidants. Generally, antioxidants may function as reducing compounds, free radical chain interruptors, quenchers or inhibitors of singlet oxygen formation, and as inactivators of prooxidant metals through chelation or reduction to less reactive, lower oxidation states. It, therefore seems probable that aldose reductase inhibitors act by preventing the oxidative damage initiated by autoxidizing monosaccharides.

Oxidative stress of any form could promote the non-enzymatic modification of the protein as in the case of glycosylation of proteins (151-153) in general. In this process, glucose reacts with protein amino groups via nucleophilic addition to form a chemically reversible Schiff base adduct, which subsequently rearranges to the more stable but still chemically reversible Amadori product. Amadori products then slowly undergo a series of further reactions with amino groups on other proteins to form
glucose-derived intermolecular crosslinks, such as the recently characterized advanced glycosylation product 2-(2-furoyl)-4(5)-(2-furanyl)-IH-imidazole. Compounds like aminoguanidine (236) which intercept the complex rearrangement to the formation of the adduct would prevent the crosslinking or aggregation formation. The sequence of reactions is presented in the Fig. E. (236).

Fig. E Sequence of nonenzymatic glycosylation product formation that leads to cross-linking of proteins through irreversible advanced glycosylation products such as 2-(2-furoyl)-4(5)-(2-furanyl)-IH-imidazole. Prevention of glucose-derived protein cross-linking by reaction of aminoguanidine with early glycosylation products is shown at lower right. (236).

In vivo, nonenzymatic glycosylation is a normal process and occurs particularly in long-lived organelles and with compounds which have a very low turnover, such as the erythrocyte membrane, hemoglobin, collagen and several other proteins including the lens crystallins (134,237,238).

In the case of lens crystallins, these post-translational reactions may result in the accumulation of modified polypeptides in the nucleus where little or no protein turnover can be detected in the older lenses (134,142).
Not only glucose or its epimers (galactose), ascorbic acid (162,163) and its oxidation products dehydroascorbic acid and diketogulonic acid (164) are also capable of forming covalent adducts with proteins by similar mechanism; in fact the reaction is rapid and effective with the oxidation products leading to extensive protein-protein crosslinking. However, high dietary intake of ascorbic acid does not affect the formation of oxidation products as monitored by the fluorescence spectrum of murine lenses (162); rather it has a protective effect on maintaining the transparency of the lens. The fact that ascorbic acid requires oxidation to produce active glycating species is consistent with the observed inhibition of ascorbic acid glycation by reducing agents such as GSH (239). Therefore, the high levels of glutathione present in the normal lens act by maintaining ascorbate in the reduced state and preventing the formation of the reactive species such as dehydroascorbic acid and 2,3 diketogulonic acid.

The changes in the structural organization of the individual soluble protein fraction could be demonstrated by physicochemical methods such as UV and near-UV CD, fluorescence (intrinsic and extrinsic), phosphorescence (240), spectroscopic methods like NMR etc. (241,242), electrophoresis (PAGE and SDS-PAGE), Raman spectra (243-246) and hydrodyanamic methods (95,138,177,190,247-251).