The state of transparency of the eye lens determines an organism’s ability to visualize the world around itself. Thus the maintenance of a healthy and transparent eye lens is crucial for the effective functioning of an organism. In many organisms, a loss of lens transparency accompanies the process of ageing, and leads eventually to a condition termed cataract. This loss arises both from the accumulation of light-absorbing compounds within the lens, and from the increased scattering of incident light by the lens. Cataract occasionally arises precociously as well, from physical trauma, illnesses such as diabetes and diarrhoea, metabolic disorders, odd dietary and behavioural habits, and excessive exposure to sunlight. In order to understand the basic mechanisms underlying the loss of lens transparency, it is important to understand the basis of its generation and maintenance. This thesis presents a summary of attempts that were made to understand these two mutually complementary aspects of eye lens transparency; at the level of molecules, and using the tools and techniques of molecular biophysics and biochemistry.

The vertebrate eye lens consists, for the most part, of long enucleated fiber-shaped cells which are packed with very high concentrations of proteins called the crystallins. An ordered molecular arrangement of these proteins, in a glass-like state characterized by short-range order, is believed to be responsible for the low level of scattering of light by a normal lens. Covalent chemical modifications occurring in these protein molecules, especially through oxidative means, could result in changes in the light-absorbing properties and solubility of these proteins, as well as in their ability to remain packed in the spatially ordered arrangement that is necessary to ensure transparency. Thus, precipitation, colouration and aggregation of the crystallins within fiber cells could
drastically affect transparency in an adverse manner. It must also be noted that such an effect would tend to be permanent, since the lens, being enucleated, would be unable to repair or replace any of its damaged constituents. In the lens, oxidation could arise from the photosensitized or metabolic generation of reactive oxygen species and other free radicals, or from the direct photo-oxidation of susceptible amino acid residues. Since the lens is almost constantly bathed in light, photochemical mechanisms resulting in protein damage assume special importance.

Thus, investigations were initiated into the effects of oxidation through free radicals and direct photolysis on the covalent chemical structure, solubility, net charge, aggregative behaviour, tendency to crosslink, light absorption, three-dimensional structure, and stability of the crystallins and other select proteins. In a related vein, investigations were initiated into the formation, through inter-protein interactions, of the glass-like physiological state of proteins within the lens. This was done by using a variety of spectroscopic techniques to probe into the differences between the physical behaviour of the crystallins in dilute and concentrated solutions. The background, results and analyses of these investigations are presented in the thesis as follows.

Chapter 1 presents an overview of the architecture and molecular composition of the ocular lens. It describes the current understanding of the physical basis of lens transparency, and presents a brief review of available information on the oxidative changes that are associated with cataract formation. The last section of this chapter outlines the scope of this study and lists the questions that were asked.

Chapter 2 describes experiments performed to investigate the nature of the oxidative damage caused in crystallins and some "control" proteins upon reaction with the hydroxyl radical (·OH) which, in the lens, is generated by photosensitization or by the
Fenton reaction. The compound Bis(hydroperoxy)naphthaldiimide was used as a "photo-Fenton" reagent in order to photolytically generate pure ·OH, free of other oxyradicals. Some of the relevant findings are: (i) Trp residues are oxidized by ·OH to N-formylkynurenine and related compounds, but this in itself does not lead to the covalent aggregation of the protein; (ii) Tyr residues react with ·OH, but apparently do not produce dihydroxyphenylalanine or dityrosine. Nor do protein crosslinks occur as a result. Dityrosine seems to form only in proteins where the two reacting Tyr residues are appropriately oriented in space; (iii) the reaction with ·OH leads to covalent (non-disulfide) inter-molecular crosslinks in protein molecules; (iv) oxidation of His residues appears to be obligatory for such non-disulphide protein crosslinking. Histidine-free proteins do not form high molecular weight products upon reaction with ·OH. Protection of His residues by adduct formation in other proteins inhibits crosslinking; (iv) Lys residues seem to participate in the crosslinking reaction. Protection of the Lys residues by maleylation of the protein inhibits crosslinking; and (v) an oxidized crystallin is more acidic in nature than its parent protein, and has altered conformational features. The relevance of these findings to the cataract problem are discussed.

Chapter 3 presents an examination of the effects of irradiating γ-II crystallin with light of wavelengths corresponding to the absorption bands of its aromatic amino acids, in the presence of free radical scavengers. Since the scavengers remove the radicals generated by sensitization reactions from the photoproducts, no further oxidative damage of the protein occurs. Such direct photolysis leads to the destruction of the Trp and Tyr side chain moieties. The chromophores N-formylkynurenine and dityrosine are produced as a result, along with a number of other chromophores that have absorption and emission bands in the near-UV and visible regions. A number of these chromophores are found to exhibit fluorescent properties similar to those of compounds that accumulate in the lens.
with age. Limited photodestruction of aromatics leads to marked changes in the stability of the protein to denaturation by chemical methods; the protein unfolds at lower concentrations of guanidium hydrochloride and urea. Surprisingly however, the stability to denaturation by heat is increased upon photodamage. Changes are seen in the fluorescent properties of residual tryptophan moieties in the protein. Extended photodamage leads to major alterations in protein structure, and eventually to an unfolded state. The identification of dityrosine formed in the crystallins upon photolytic damage is discussed in some detail towards the end of the chapter. Also described are experiments which show that structural changes can occur to proteins in situ in spectrophotometer sample chambers, when they are exposed for long lengths of time to ultraviolet radiation (for instance, during spectral monitoring of long-term kinetic experiments).

The unusually high physiological concentrations of the crystallins in the lens must have a functional basis. Space filling considerations (outlined in chapter 4) suggest that conformational readjustments must characterize the generation of a glass-like order among crystallin molecules through inter-protein interactions, as protein concentration is raised to physiological levels. To investigate this possibility experimentally, novel methods of generating and (spectroscopically) examining concentrated solutions of these molecules were devised; the molecules were studied both in the form of individual solutions of purified crystallins and as natively constituted mixtures of the crystallins. Using UV-Vis absorption, fluorescence and CD spectroscopy as well as NMR spectroscopic methods, it is shown that the crystallins undergo conformational readjustments, transforming from a colligative mixture of independent globular entities to an open, extended, non-colligative array of interacting polypeptides, as protein concentration is raised from dilute (1 mg/ml) solutions to solutions of physiological concentration (~400 mg/ml). The use of fluorescence polarization anisotropy, ESR spectroscopic methods and red edge excitation
shift measurements shows that this transformation is accompanied by a dramatic reduction in the rotational mobility of the crystallins. Changes in the refractive indices (and, in some instances, the dispersive powers) of crystallin solutions with increasing protein concentration, are reported and interpreted. It is shown that (a) the refractive index of a crystallin solution rises linearly with protein concentration; (b) the rise in refractive index with concentration is characterized by different slopes for the three crystallins ($\gamma > \beta > \alpha$); and (c) the dispersive powers of solutions of $\alpha$-, $\beta$- and $\gamma$- crystallin of equivalent refractive index are not equivalent. These observations are interpreted in terms of the differential spatial localization of the crystallins in the lens, and the ability of the lens to correct for optical aberrations.