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Human lens protein modification: the role of kynurenine and 3-hydroxykynurenine

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by

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Certification

I, Santiago Vazquez, declare that the work described in this thesis, submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy, in the Department of Chemistry, University of Wollongong, does not contain any material which has been accepted for the award of any other degree or diploma in this or any other University and to the best of my knowledge and belief contains no material by any other person, except where due reference has been acknowledged.

Santiago Vazquez

August, 2001
In loving memory of my father
A hundred times every day I remind myself that my inner and outer life depend on the labours of other men, living and dead, and that I must exert myself in order to give in the same measure as I have received.

Albert Einstein
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My supervisors, Professor Margaret Sheil and Associate Professor Roger Truscott for their personal and professional support;

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My wife Jenny who has provided me with unfathomable love, support and encouragement through the good times and the bad.
Publications

Sections of the work described in this thesis have been reported in the following publications:


Abstract

Kynurenine (Kyn) and 3-hydroxykynurenine (3-OHKyn) are present in the human lens where they play a protective role. With age, human lens proteins become increasingly yellow. A marked increase in protein colouration is also observed with the increasing severity of age related nuclear cataract. The molecular basis for the increase in protein colour with age is not well understood, however, it has been proposed, at least in part, to result from modification by UV filter compounds such as 3-OHKyn. Until recently, however, no direct evidence for these modifications has been found. To explore the role of these UV-filters in the lens, model studies have been used to develop and verify analytical methods (based on mass spectrometry in combination with HPLC). These methods enabled the direct analysis of amino acid modifications in human lens proteins.

Since UV filter compounds can often only be isolated in small quantities from the lens, this study used mass spectrometry as the basis for the identification and characterization of novel kynurenine-derived compounds. A combination of accurate mass measurement and tandem mass spectrometry was used to characterise the major fragment ions observed in the ESI mass spectrum of kynurenine. Three major fragmentation pathways were evident, resulting from the initial elimination either of ammonia, \( \text{H}_2\text{O} \) and \( \text{CO} \) or the imine form of glycine.

The major autoxidation products of 3-OHKyn as well as their relative rates of formation and the role that \( \text{H}_2\text{O}_2 \) played in mediating the autoxidation process was examined. Oxidation of 3-OHKyn generated a number of compounds, namely: xanthommatin (Xan), \( p \)-quinone, 4,6-dihydroxyquin-olinedihydroquinonecarboxylic acid (DHQCA) and a compound that has a structure consistent with that of hydroxyxanthommatin (OHXan). Xan was the major product formed initially, however, it was found to be unstable,
particularly in the presence of H$_2$O$_2$, and rapidly degraded to yield DHQCA. Hydrogen peroxide was formed rapidly upon oxidation of 3-OHKyn, and significantly influenced the rate of autoxidation.

Using an acid hydrolysis procedure in combination with HPLC and LC-MS we were able to detect covalent adducts of kynurenine bound to histidine and lysine residues. 3-OHKyn adducts of His and Lys residues, however, were not sufficiently stable under the acid hydrolysis conditions to enable them to be analysed by this method. Kyn modifications to Cys residues were found at very low levels due to their poor stability under physiological pH conditions.

Proteins isolated from human lenses were shown to contain significant levels of the coloured UV-filter, kynurenine, covalently bound to histidine and lysine residues at concentrations up to 3.6 and 0.2 nmol/mg of lens protein respectively. Analysis of human lens tissue over a wide range of ages indicated that the level of bound kynurenine increased with the age of the individual. The analysis of human cataract tissue, however, revealed that the level of Kyn modification to lens tissue decreased with the increasing severity of cataract. This indicated that Kyn once bound to lens protein may play a role in the progression of cataract.

Overall the work described in this thesis has provided strong evidence that post-translational modification of lens proteins by tryptophan metabolites appears responsible, at least in part, for the age-dependent colouration of the human lens and that covalently-bound Kyn plays a significant role in the progression of cataract.
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</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>δ</td>
<td>chemical shift</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1D</td>
<td>One dimensional</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2D</td>
<td>Two-dimensional</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-OHA</td>
<td>3-hydroxyanthranilic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-OHKG</td>
<td>3-hydroxykynurenine O-β-D-glucoside</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-OHKyn</td>
<td>3-hydroxykynurenine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGEs</td>
<td>advanced glycation end products</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AHBG</td>
<td>4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid O-glucoside</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ARN</td>
<td>age-related nuclear</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BHA</td>
<td>3-tert-butyl-4-hydroxyanisole</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CI</td>
<td>Chemical ionisation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CID</td>
<td>collisionally induced dissociation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLP</td>
<td>calf lens protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COSY</td>
<td>$^1$H-$^1$H correlation spectroscopy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cys-Kyn</td>
<td>cysteinylkynurenine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHQCA</td>
<td>4,6-dihydroxyquinolinequinonecarboxylic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMPO</td>
<td>5,5-dimethylpyrrole-N-oxide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DTNB</td>
<td>2,4-Dinitrothiocyananobenzene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EI</td>
<td>electron ionisation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPR</td>
<td>electron paramagnetic resonance</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionisation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fmoc</td>
<td>N-(9-flurenylmethoxycarbonyl)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSH-30HKG</td>
<td>glutathionyl-30H-kynurenine-glucoside</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSSG</td>
<td>oxidised glutathione</td>
<td></td>
<td></td>
</tr>
<tr>
<td>His-Kyn</td>
<td>histidylkynurenine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMBC</td>
<td>heteronuclear multiple bond correlation spectroscopy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HNE</td>
<td>4-hydroxy-2-nonenal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HO</td>
<td>hydroxyl radical</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IDO</td>
<td>indoleamine dioxygenase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kyn</td>
<td>kynurenine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LC/MS</td>
<td>liquid chromatography/mass spectrometry</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lys-Kyn</td>
<td>lysylkynurenine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>m/z</td>
<td>mass to charge ratio</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mr</td>
<td>molecular masses</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS/MS</td>
<td>tandem mass spectrometry</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance spectroscopy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OXHx</td>
<td>hydroxyxanthommatin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPD</td>
<td>a-phenylenediamine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCO</td>
<td>posterior capsular opacification</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ROESY</td>
<td>$^1$H-$^1$H rotating frame nuclear overhauser effect spectroscopy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT</td>
<td>retention time</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t-Boc</td>
<td>tert-butoxycarbony</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t-Boc-His</td>
<td>N-α-t-Boc-L-histidine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t-Boc-Lys</td>
<td>N-α-t-Boc-L-lysine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TDO</td>
<td>tryptophan dioxygenase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEAA</td>
<td>triethylammonium acetate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIC</td>
<td>total ion current</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xan</td>
<td>xanthommatin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1. Introduction

Age-related nuclear cataract, generally defined, is an opacification and associated
colouration of the central region of the human lens sufficient to impair vision. This form
of cataract is not static and if left untreated sight progressively deteriorates to the stage
where vision is severely impaired causing blindness.

Throughout the world, cataract accounts for nearly half of all blindness in man and is
most prevalent in developing countries. Cataract has been documented since very early
times and was recognized by the 28th World Health Assembly in 1975 as one of the
most important causes of avoidable blindness. It is more common in older individuals,
hence with ageing populations in many countries it is becoming an increasingly
important problem.¹

The condition is usually treated by surgery together with optical correction of the
resulting refractive deficit, however, surgery is expensive and may lead to ocular
damage. For example, the incidence of posterior capsular opacification (PCO) requiring
laser capsulotomy was ca. 19% following intraocular lens implantation.² In developed
countries, the availability of surgical services ensures that the majority of individuals
blind from cataract have sight restored, however, in developing countries surgical
services are limited, if available at all, resulting in many persons being needlessly
blind.¹³

To understand cataract a thorough knowledge of the normal lens is required and so in
section 1.1 a description of the normal human lens, its structure and composition,
metabolism and its vulnerability in relation to age-related changes is given. In the
following sections cataract, its effect on vision, the risk factors and current hypothesis
for cataract formation are discussed. Further sections deal with small molecules present in the lens and known reactions of small molecules with proteins.

Overall this thesis attempts to address the roles of kynurenine (Kyn) and 3-hydroxykynurenine (3-OHKyn) in age-related colouration of the human lens and the development of cataract. It is hoped that the work presented here may add to the understanding of the changes that occur during cataract formation and thus provide an insight into possible methods of preventing or delaying the disease process.

1.1. The Human Lens

1.1.1. Anatomy and Function

The lens is essential for normal human vision. A cross section of the gross anatomy of the eye is shown in Figure 1.1. The lens is located behind the iris and the pupil in the eye and is suspended in place by the zonular ligaments. These ligaments are fused to the capsule around the equatorial region and radiate outward to the ciliary muscle, where they are anchored.4,5 The normal human lens is a transparent, pale yellow, biconvex body contained within an elastic collagenous capsule. The capsule allows the passage of small molecules both into and out of the lens. The anterior surface is in contact with the aqueous humour on the corneal side and the posterior surface is in contact with the vitreous humour and faces the retina.

In combination with the cornea, the lens focuses light from an object in the field of view on to the retina. The simple curvature of the cornea accounts for about 80% of the focusing of light by the eye. Light from the cornea then passes through the pupil and reaches the lens.4 The lens achieves the remaining 20% or so of the focusing and determines the focusing range by changes in shape controlled by the action of the
sphincter-like ciliary muscle. To focus light from a distant object, the ciliary muscle relaxes, tightening the zonular fibres causing the lens to become flatter, minimizing the refractive power of the lens. To focus light from a near object, the ciliary muscle contracts, pulling the choroid forward releasing tension on the zonular fibres. This allows the lens to adopt a more spherical conformation resulting in greater refractive power.⁵

Figure 1.1: Gross anatomy of the human eye, adapted from van Heyningen.⁴

The eyeball reaches full size at puberty, but the lens continues to grow throughout life. The lens grows by the addition of layer upon layer of fibre cells around the original
nucleus, which is present at birth. Growth begins with the elongation of epithelial cells, which develop into fibre cells, in the equatorial region of the lens as shown in Figure 1.2. Nuclei and other intracellular organelles are lost as the fibre cells mature. This process occurs in the bow region of the lens, which, along with the epithelial layer, is the most metabolically active region of the lens. As fibre cells mature they elongate, eventually extending from the front of the lens curving round to the back of the lens where they meet in a region called the lens sutures. These sutures are visible to the naked eye in some lenses. The lack of DNA and RNA in the fibre cells means that there is little or no turnover of lens proteins as the cell ages. Therefore, cells contained within the nucleus are amongst the oldest in cells in the body and contain some of the oldest proteins, some of which predate birth.

Figure 1.2: A stylised cross section of the human lens showing the nucleus bounded by the bold line, adapted from J. Harding.
The equatorial diameter of the human lens increases throughout life, however, the rate of increase is substantially reduced after the second decade of life. At birth the diameter is approximately 5 mm and increases to 9-10 mm in a 20 year old. The thickness of the lens increases at a much slower rate than does the equatorial diameter. It only changes by about 0.75-1.5 mm throughout life.

The weight of the lens also increases throughout life. From 65 mg at birth, the mass of the lens increases to 125 mg by the end of the first year. The lens weight then increases at approximately 2.8 mg/year up to the age of 10 years. Thereafter, the mass of the lens increases at a slower rate (1.4 mg/year) to reach about 260 mg by the age of 90 years.

1.1.2. Composition

The physical appearance of the lens is unlike that of any other living tissue. It is devoid of nerves and blood vessels, yet it bears the normal complexity of living cells. Proteins comprise approximately 38% of the wet mass (the highest protein content of any tissue of the body). Also present in the lens are trace minerals common to other body tissues such as sodium and potassium. Potassium is more concentrated in the lens than in most tissues. Of direct concern for the present study, is the presence of a number of low molecular-weight compounds including 3-hydroxykynurenine O-β-D-glucoside (3-OHKG), 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid O-β-D-glucoside (AHBG), kynurenine (Kyn) and 3-hydroxykynurenine (3-OHKyn). The concentrations of these and other common small molecules in the human and bovine lens are given in Table 1.1.
Table 1.1: Major amino acids and low-molecular weight solutes in bovine and human lenses.\textsuperscript{11}

<table>
<thead>
<tr>
<th>Component\textsuperscript{a}</th>
<th>Bovine</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taurine</td>
<td>49.9</td>
<td>0.79</td>
</tr>
<tr>
<td>Serine</td>
<td>6.3</td>
<td>0.56</td>
</tr>
<tr>
<td>Proline</td>
<td>1.8</td>
<td>0.16</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>225.1</td>
<td>3.42</td>
</tr>
<tr>
<td>Glycine</td>
<td>93.3</td>
<td>0.79</td>
</tr>
<tr>
<td>Glutathione\textsuperscript{b}</td>
<td>-</td>
<td>1.5</td>
</tr>
<tr>
<td>Alanine</td>
<td>26.1</td>
<td>1.34</td>
</tr>
<tr>
<td>Ammonia</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>Urea</td>
<td>3.8</td>
<td>4.7</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>340.0</td>
<td>300.0</td>
</tr>
<tr>
<td>AHBG\textsuperscript{c}</td>
<td>-</td>
<td>20 - &lt;7</td>
</tr>
<tr>
<td>3-OHKyn\textsuperscript{c}</td>
<td>-</td>
<td>2 - &lt; 0.5</td>
</tr>
<tr>
<td>3-OHKG\textsuperscript{c}</td>
<td>-</td>
<td>200 - &lt; 8</td>
</tr>
<tr>
<td>Kyn\textsuperscript{c}</td>
<td>-</td>
<td>7 - &lt; 0.4</td>
</tr>
</tbody>
</table>

\textsuperscript{a} quantities are expressed as $\mu$g/g of protein (wet weight).

\textsuperscript{b} oxidised plus reduced (the major form) of glutathione.

\textsuperscript{c} quantities are expressed as $\mu$g/g of nuclear protein (dry weight)

for lenses ranging in age from 15-93 years.\textsuperscript{12}

Crystallins are the major proteins in the lenses of vertebrates and invertebrates. The crystallins of the human and bovine lens constitute more than 90% of the lens protein and comprise three main classes: $\alpha$, $\beta$ and $\gamma$, grouped by their aggregation behaviour and sequence homology. Their properties are summarized in Table 1.2.\textsuperscript{13} They are water-soluble proteins, however, certain crystallins may be partially associated with membranes and become insoluble with age.\textsuperscript{9} The function of crystallins in the lens is predominantly structural, that is, their abundance and short-range packing are required to maintain the refractive properties and stability of the lens.\textsuperscript{9,14} Although initially thought to be specific to the lens, it is now known that most crystallins also occur at low levels in tissues outside the lens.\textsuperscript{9}
Table 1.2: Properties of the different crystallins. 3,15,16

<table>
<thead>
<tr>
<th>Subunits</th>
<th>α</th>
<th>β</th>
<th>γ</th>
<th>γs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up to 9 minor subunits</td>
<td></td>
<td>Basic: 26-32 kDa Acidic: 23-25 kDa</td>
<td>20 kDa</td>
<td>24 kDa</td>
</tr>
<tr>
<td>Subunit molecular weight</td>
<td>20 kDa</td>
<td>ßH: 150-200 kDa ßL: c. 50 kDa</td>
<td>20 kDa</td>
<td>24 kDa</td>
</tr>
<tr>
<td>Native molecular weight</td>
<td>600-900 kDa</td>
<td>ßH: 0-8 ßL: 2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Number of subunits</td>
<td>30-45</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiol content</td>
<td>Low</td>
<td>High</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Subunit concentration in</td>
<td>αA 21.4 %</td>
<td>ßB1 8.8 %</td>
<td>γC 14.3 %</td>
<td>γs/ßA1 15.4 %</td>
</tr>
<tr>
<td>the young human lens</td>
<td>αB 6.3 %</td>
<td>ßB2 14.3 %</td>
<td>γD 2.5 %</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ßA3/ßB3 6.2 %</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ßA4 4.7 %</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1.1.3. Protection of the Lens from Oxidative Damage

Since the lens is permeable to numerous small molecules including oxygen, highly reactive oxygen species have the capacity to enter the lens or be formed by reaction with O₂ within the lens, and cause damage. The damage that may be caused includes oxidation of membrane lipids that may result in the formation of lipid peroxidation products such as malondialdehyde. Malondialdehyde can form cross-links between membrane lipids and proteins; polymerisation and cross-linking of proteins, which results in crystallin aggregation and inactivation of essential enzymes including those with an antioxidant role (e.g. catalase and glutathione reductase) 17,18.

These reactions have the potential to cause damage to the lens. The presence of a complex antioxidant system, in the lens, offers considerable protection, however, this system is not absolute and low level damage may accumulate throughout life. 16
1.1.3.a. Ascorbic Acid

Ascorbic acid is thought to play a major role in the antioxidant system in the lens since it is present in high concentrations in the outer layers of the lens, ascorbic acid rapidly reacts with superoxide anions, peroxide radicals and hydroxyl radicals to give dehydroascorbate. It also scavenges singlet oxygen, reduces thiyl radicals and is important in the prevention of lipid peroxidation. The ascorbic acid and glutathione systems are coupled in that dehydroascorbate reacts with the reduced form of glutathione to generate ascorbate and oxidised glutathione (GSSG).\(^{19-22}\)

1.1.3.b. Glutathione

Glutathione (GSH) is found in high concentrations in the lens (the levels are higher in the cortex than in the nucleus). GSH has many important roles in the lens including:\(^{11,17,19}\)

- maintenance of protein thiols in the reduced state, which helps to preserve lens transparency by preventing the formation of high molecular weight crystallin aggregates;
- protection from oxidative damage by detoxification of \(\text{H}_2\text{O}_2\);
- removal of xenobiotics; glutathione-\(S\)-transferase catalyses the conjugation of glutathione to hydrophobic compounds with an electrophilic centre and
- amino acid transport.

GSH has a half-life of 1-2 days and is constantly recycled by the \(\gamma\)-glutamyl cycle. GSH is synthesized from its constituent amino acids in a two step process, however, reduced GSH can also be taken into the lens from the aqueous humor.\(^{23}\) In the human lens the
production of GSH declines with age, furthermore, the levels in most forms of cataract are low.\textsuperscript{17}

\subsection*{1.1.4. UV-filters}

The young lens transmits approximately 90\% of visible light (400-700 nm) to the retina, however, transmission decreases with increasing age. The retina is protected from potentially damaging radiation in the ultraviolet (UV) region by absorption in the cornea and the lens. The cornea absorbs all wavelengths below 295 nm, while the lens absorbs strongly in the UVB (295-315 nm) and the UV/visible regions (315-400 nm).\textsuperscript{24,25} The major UV absorbers in the lens are free or bound aromatic amino acids (e.g. tryptophan), pigments (e.g. 3-hydroxykynurenine glucoside) and fluorophores.\textsuperscript{17}

Tryptophan absorbs more than 95\% of the radiation absorbed by all of the amino acids present in the human lens, however, this accounts for only 5\% of the total radiation absorbed by the lens. 3-OHKG has a greater relative absorbance than tryptophan and accounts for more than 80\% of all radiation absorbed by the lens. This is a consequence of the fact that greater than 90\% of the UV radiation that reaches the lens is UVA (315-400 nm), which falls within the optimal absorbance window of 3-OHKG (295-445 nm, absorption max 365 nm). Tryptophan, however, only absorbs light between 295-340 nm (absorption max 280 nm).\textsuperscript{16}

In the lens, short wave length radiation (less than 400 nm) can be quite damaging if it reaches the retina,\textsuperscript{26} and it is also the most vulnerable to dispersion and scattering. Hence, 3-OHKG, and other UV-absorbing compounds, act as light filters in the lens; protecting the retina, reducing chromatic aberration and promoting the formation of a sharper image on the retina.\textsuperscript{4} The major UV-filters in the lenses of humans and other higher primates, are derived from the metabolism of tryptophan.\textsuperscript{27} In decreasing order of
concentration, they are 3-hydroxykynurenine O-β-D-glucoside (3-OHKG), 4-(2-
amino-3-hydroxyphenyl)-4-oxobutanoic acid O-β-D-glucoside (AHBG), kynurenine
(Kyn) and 3-hydroxykynurenine (3-OHKyn).

Recent work in our laboratory has led to the discovery of glutathionyl-3-hydroxykynurenine (GSH-3-OHKynG) a novel fluorescent UV-filter present in older normal human lenses. The eyes of diurnal species other than primates may also contain UV-filters, however, these may be located in the retinal cones, cornea, macula lutea or the retinal capillaries.

1.1.5. Age-related Changes

The overall metabolic activity of the lens decreases with increasing age. This is attributed to decreasing enzyme activities in the cortex. A reduction in the activity and/or levels of many antioxidants also occurs with aging. The decrease in activity is greatest in the nucleus, thus proteins in this region of the lens are more susceptible to oxidative damage and therefore rely on the overlaying cortical fibres and epithelial layer to protect them.

1.1.5.a. Colouration of the Human Lens

The young human lens is very pale yellow to clear in colour, however, as an individual ages, an increase in lens colouration and fluorescence is observed as shown in Figure 1.3. During the earlier part of the sixth decade the colour intensifies, and this is primarily confined to the nucleus. This has an effect on colour perception. Since the change is gradual, it generally goes unnoticed by the individual. In artists, however, this change in colour perception affects the style and quality of their art in old age. Liebreich in 1872 used this fact to explain the change in use of colours by the elderly Turner and Mulready. The lens absorption makes blue objects seem dull and grey unless they are very bright blues; greens (made from mixing blue paint with yellow paint) appear
yellow. Liebreich suggested that an artist could compensate for the dullness of blue by using excessive amounts of colour or preferentially painting with the colours that still retain brilliance for them. In attempts to avoid these colour vision problems affecting his paintings, Monet carefully read the labels on the tubes of paint. He also maintained a regular order of colours on his palette.

Figure 1.3: Change in lens colouration with age. a) 6 months, b) 8 years, c) 12 years, d) 25 years, e) 47 years, f) 60 years.

This colouration of the lens is a part of aging and occurs in the normal, non-cataractous, lens. As an individual ages, the crystallins become increasingly coloured and in later adulthood these proteins become the major UV-absorbing species within the lens. The wavelength absorbed by the older lens extends to approximately 500 nm. It has been suggested that these yellow coloured proteins arise through the attachment of kynurenines or glycation products to the crystallins.
Carbohydrate-derived compounds such as glyceraldehyde react with lens proteins in vitro, causing yellowing and loss of protein thiols. The nonenzymatic glycation of lysine residues was first reported by Stevens et al. for lens proteins from diabetic rats and for lenses incubated in 30 mM glucose. In vitro experiments showed the continuous binding of sugars to the protein accompanied by opalescence, especially in the presence of oxygen. The authors suggested that the glycosylation caused a change in the conformation and thus an increased reactivity of thiol groups, protein-protein disulfide formation and aggregation.

Glycation and the subsequent Amadori rearrangement are also the first steps in the nonenzymic browning of food (Maillard reaction). Stevens et al. suggested that the later stages of the Maillard reaction produce advanced glycation end products (AGEs), which may be responsible for the yellow-to-brown pigmentation in human cataract. The structures of a number of possible AGEs are shown in Table 1.3. This reaction is initiated by the attachment of a sugar molecule (e.g. glucose) to an amino acid, normally lysine. In young lenses, 1.3% of the crystallin lysine residues are glycated while at the age of 50 years this increases to 2.7% and to approximately 4.2% in older lenses. In the later stages of the Maillard reaction, the bound sugar residues may condense with other lens proteins forming cross-links.
Table 1.3: Levels and structures of advanced glycation end products in cataractous and non- cataractous lens tissues (adapted from R. H. Nagaraj, unpublished data).

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>Tissue</th>
<th>Normal pmole/mg of protein</th>
<th>Cataractous pmole/mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentosidine</td>
<td><img src="image" alt="Pentosidine Structure" /></td>
<td>Skin aorta lens plasma brain</td>
<td>1-4</td>
<td>3-17</td>
</tr>
<tr>
<td>Vesperlysine</td>
<td><img src="image" alt="Vesperlysine Structure" /></td>
<td>Lens</td>
<td>20-40</td>
<td>36-60</td>
</tr>
<tr>
<td>Glyoxal-lysine dimer</td>
<td><img src="image" alt="Glyoxal-lysine Dimer Structure" /></td>
<td>Lens plasma</td>
<td>1-7</td>
<td>10-22</td>
</tr>
<tr>
<td>Methylglyoxal lysine-dimer</td>
<td><img src="image" alt="Methylglyoxal Lysine-Dimer Structure" /></td>
<td>Lens plasma</td>
<td>10-25</td>
<td>100-250</td>
</tr>
<tr>
<td>Argpyramidine</td>
<td><img src="image" alt="Argpyramidine Structure" /></td>
<td>Plasma aorta lens</td>
<td>10-50</td>
<td>50-350</td>
</tr>
<tr>
<td>Crossline</td>
<td><img src="image" alt="Crossline Structure" /></td>
<td>Immunological detection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrraline</td>
<td><img src="image" alt="Pyrraline Structure" /></td>
<td>Plasma kidney lens</td>
<td>2-15</td>
<td>4-22</td>
</tr>
<tr>
<td>Carboxymethyllysine</td>
<td><img src="image" alt="Carboxymethyllysine Structure" /></td>
<td>Skin aorta lens plasma</td>
<td>350-1780</td>
<td>2000-4500</td>
</tr>
<tr>
<td>Carboxyethyllysine</td>
<td><img src="image" alt="Carboxyethyllysine Structure" /></td>
<td>Lens</td>
<td>500-1250</td>
<td>Not detected</td>
</tr>
</tbody>
</table>
Currently there is little evidence that AGEs occur in vivo in body tissues, however some of the tissues that these AGEs have been found in are outlined in Table 1.3. The Amadori products are relatively stable and it is unlikely that they go on to form pigmented Maillard reaction products except in the most long-lived proteins, such as the lens crystallins and collagen.\textsuperscript{50}

In subsequent studies, brown pigments isolated from human cataracts after proteolytic digestion were shown to have UV absorbance spectra, fluorescence excitation spectra and chromatographic retention times similar to Maillard products synthesized from glucose and α-\textit{tert}-butyloxyacarbonyllysine.\textsuperscript{51} The fluorescence spectrum of protein from human cataract lenses is similar to that of bovine lens protein incubated with glucose or glucose 6-phosphate for 10 months at 37°C and pH 7.4.\textsuperscript{51} These authors suggested that at least part of the browning of the human lens could be Maillard browning brought about by reaction of long-lived proteins with glucose, glucose 6-phosphate, dihydroxyacetone, glyceraldehyde, their phosphorylated derivatives or with simple aldehydes.\textsuperscript{50}

Ascorbic acid acting via dehydroascorbic acid also forms covalent adducts with lens proteins causing browning, fluorescence, crosslinking and precipitation. Adduct formation and crosslinking occurred even under conditions where free radical formation was minimized, but both were inhibited by glutathione. These compounds were more effective than glucose and glucose 6-phosphate at causing protein precipitation and crosslinking, thus ascorbic acid may play a more significant role in the formation of these yellow pigments than glucose.\textsuperscript{52-54}
1.1.5.c. Post-translational Changes to Crystallins

Age-related changes in the lens crystallins occur as a result of both differential synthesis during development and postsynthetic modifications throughout life. Reported age-related changes to the crystallins include:\(^{17}\)

- loss of sulfhydryl and formation of disulfide bridges;
- phosphorylation of serine residues, particularly of \(\alpha\)A and \(\alpha\)B-crystallins;
- an increase in insoluble protein, that is \(\text{H}_2\text{O}\) insoluble (but soluble in s-methyl urea);
- accumulation of high molecular weight aggregates (predominantly composed of \(\alpha\)-crystallin but increasing in complexity with age);
- proteolysis of polypeptide chains;
- non-enzymic glycation;
- deamidation of glutamine and asparagine residues;\(^{15}\)
- racemization of aspartic acid residues;
- an increase in colour and non-tryptophan fluorescence and
- formation of covalent adducts with small molecules.

Although the significance of these changes to crystallins is not completely clear, it does seem reasonable to assume that the short-range spatial order of the crystallins is altered causing a decrease in transparency.
1.2. Cataract

1.2.1. What is Cataract?

The term cataract is derived from the Latin word *cataaracta* that is in turn derived from the Greek word *kataraktes*, which means waterfall (breakdown, down rushing). Ancient practitioners probably gave this name to the condition in the belief that the liquid content of the eye was cascading down. Defined strictly on the basis of the pathomorphological process involved, cataract is an opacification or loss of transparency in the lens of the eye.1 The vast majority of cataract is associated with aging and its incidence increases markedly with age.

1.2.2. The Effect of Cataract on Vision

The effects that cataract have on vision are best demonstrated by the way failing eyesight affects artistic style. Many of the most famous artists working in France late in the 19th century suffered serious eye diseases. The best known example is that of Claude Monet (1840- 1926).55

As Monet’s impressionistic style was based on imprecision of detail, his visual difficulties did not become apparent until, when he was in his late 60s, a blurring of distant objects occurred in his paintings.56 Owing to his failing eyesight Monet found it necessary to alter his method of painting. Bright sunlight caused him difficulty so he simply changed the hours at which he painted.56 In addition to his problems with glare, his insensitivity to contrast and his diminished detail vision, Monet also complained about changes in his colour vision. He reported that reds appeared muddy, pinks dull, and that graduations or deep tones escaped him, he also said that he saw things in a yellow hue.38
Age-related nuclear cataracts produce many changes that alter colour perception. UV-light, entering the cataractous lens is converted into visible light by the fluorescent pigments and is randomly, scattered within the eye. Visible light is also scattered by protein aggregates. In addition to the defocusing of incident light, this scattering produces a veiling luminance that has the effect of desaturating colours. Furthermore, this desaturation also causes a change in the apparent hue (Abney effect) making objects appear more yellow.

Unlike other types of cataract, age-related nuclear cataracts absorb light, particularly short wavelength blue light. To a lesser extent this blue absorption also occurs with physiological aging as previously mentioned. The loss of blue and the apparent change in hue have the combined overall effect of making the world appear more yellow. This could explain Monet’s ‘muddy’ reds, ‘dull’ pinks and loss of deep tones.

What is of particular interest in Monet’s work is that he consciously painted a series of canvases of the same subject. His earlier (1899) and late (1923-25) paintings of the Japanese footbridge in the garden at Giverny demonstrate the remarkable change in colour and detail which occurred in Monet’s later paintings, see Figure 1.4. The paintings after 1915 became more abstract and the colours shifted from blue-greens to red-yellows. A French critic described his work in the following way: “The coloured symphony became more and more monochromatic for blues and yellows”. A person that has cataract may be experiencing the deterioration in sight shown in Figure 1.5.
Figure 1.5: Representation of the progressive deterioration of sight as part of the aging process: a) normal vision, b) shows the colour vision that may be experienced by an individual in their 6th decade, c) in addition to the change in colour vision a person with less severe cataract may also experience sensitivity to glare particularly in bright sunlight and during night time driving, d) the quality of vision that may be expected for a person with severe cataract. A veiling luminescence is apparent owing to the scattering of light by precipitated proteins. (Image adapted from Corel Photo-Paint 8)
1.2.3. Types of Cataract

Cataract can be classified by age of onset (e.g. congenital, juvenile or age-related cataract) or by the location of opacity within the lens (e.g. cortical or nuclear as shown in Figure 1.6).

Figure 1.6: A cut away diagram of a human lens displaying both cortical and age-related nuclear cataract. Also shown are diagrams of two whole lenses depicting a) age-related nuclear cataract and b) cortical cataract. Adapted from van Heyningen.4

Age-related cataracts may be further classified based on the colour of the lens using the Pirie classification.58 This may range from the light yellow colouration in the normal
aged lens to the pronounced brown colouration of a Type V or nigrescent lens as shown in Figure 1.7. In addition, cataract may be designated to be the result of, or secondary to, other ocular diseases, systematic disorders, and genetic or environmental influences. Although there are many forms of cataract, the type of cataract that gives rise to the majority of public health problems is age-related cataract which is the most common form.

**Figure 1.7:** Images of normal and age-related cataractous human lenses graded as types I-V using the Pierre classification method. a) a normal aged human lens, b) Type I cataractous lens, c) Type II cataractous lens, d) Type III cataractous lens, e) Type IV cataractous lens, f) Type V (brunescent) cataractous lens. Images provided by R. J. W. Truscott.
1.2.4. Severity of the Problem

The World Health Organization (WHO) estimated that in 1990, of the 38 million blind people in the world, cataract accounted for 41.8%, which is nearly 16 million people.\(^5^9\)

With the rapid aging of the population, the problem of blindness from cataract will become an even greater problem in future. For the period between 1980-2020 the projected increase in the elderly for the developed world is 186% while in developing countries the projected increase is 356%. On this basis, the WHO has estimated that there will be 54 million blind people aged 60 years or more by the year 2020.\(^5^9\) As a result, cataract surgery will continue to consume an increasing proportion of health care budgets in developed nations. In the U.S. current expenditure on cataract is estimated to be well over $3.4 billion annually.\(^6^0\) In developing countries, in which the majority of cataract blindness occurs, the number of new cataract cases outstrips the rate of surgical removal. In Africa alone, only about 10% of the 500 000 people that develop cataract blindness each year are likely to have their sight restored surgically. This has resulted, over time, in an accumulation of unattended persons blind from cataract, resulting in what is referred to as the “cataract backlog”.\(^1\)

1.2.5. Types of Treatment

With the present state of knowledge there is no proven means of preventing cataract or its progression to blindness. The condition is, however, amenable to surgical treatment, which, together with optical correction for the resulting refractive deficit, results in restoration of vision.\(^1\)

The modern sophistication of cataract surgery is the result of years of trial and error with surgical procedures and innovation. In former times when anaesthesia and fine
surgical instruments and techniques were not available, the cataractous lens was dislocated into the vitreous compartment to allow light transmission in the eye (couching of the lens). The benefit for the patient may have been short lived owing to frequent serious complications.\textsuperscript{16} Cataract surgery has now improved to the point where minimum discomfort to the patient, with small self-sealing incisions and folding intraocular lenses, and surgery performed under topical anaesthesia. Although 95\% of cataract surgeries improve vision, a small number of patients may encounter problems. Complications may include infection, bleeding and swelling, posterior capsular opacification or detachment of the retina.\textsuperscript{2,16} These are some of the more serious complications that may affect vision. Although surgical procedures have improved greatly in the twentieth century there has been very little progress in prevention or the inhibition of the progression of the disease mechanisms of cataract.

1.2.6. Risk Factors and Epidemiology

Epidemiological studies, of age-related cataract, have identified a number of risk factors for cataract as shown in Table 1.4. These risk factors may act synergistically and have a cumulative effect throughout life. The susceptibility of an individual to these insults may also be influenced by genetic differences.\textsuperscript{16}

The dominant risk factor in the general population is age. Thus, it is important to understand the changes that occur in the lens with age.\textsuperscript{61} Although not assigned a high relative risk (Table 1.4), the role of UV-light in the formation of cataract remains controversial despite some epidemiological evidence that supports an association between UV-B exposure and cortical cataract.
Table 1.4: Risk factors for age-related cataract classified according to relative risk (The smaller the number, the lower the risk).3,62,63

<table>
<thead>
<tr>
<th>Risk Factors and Relative Risk (1 → 10) for Age-related Cataract</th>
<th>1-2</th>
<th>2-3</th>
<th>4-5</th>
<th>5-6</th>
<th>&gt;10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Age</td>
</tr>
<tr>
<td>Black race</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lower caste</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lower education</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lower economic status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shorter height</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lower weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lower body mass index</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brown iris</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myopia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UV-B exposure</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Higher serum uric acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diuretics</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heat stroke</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zero consumption of milk and curds</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The causal link between UVB light and nuclear cataract is weak or nonexistent.64 An examination of six nuclear cataract studies by Dolin, in which individual exposure levels were assessed, found no association between solar UV-B and nuclear cataract.64 Furthermore, one of the first changes observed upon the exposure of lenses to sunlight in the laboratory was the destruction of tryptophan (tryptophan photolysis). This damage, however, was not found in brunescent cataractous lenses65,66 in fact, brown nuclear cataract could not be produced artificially in the laboratory using UV-light or sunlight exposure. Similarly, Barron et al.67 found that caged guinea pigs exposed to UV-radiation in the range 305-410 nm for a period of 9 months showed no appreciable
difference from the normal aging patterns. These results indicate that there is no
detectable tryptophan photolysis in the intact guinea pig lens by long wave UV-light.\textsuperscript{67}
Although changes in cortical proteins are observed upon \textit{in-vivo} exposure of squirrel
lenses to UV-light, it appears that nuclear proteins remain essentially unaffected.\textsuperscript{26}

Epidemiological studies have attempted to determine the role that sunlight plays in the
development of cataract in developing nations. A comprehensive review of these data
led Harding to conclude that sunlight was not responsible for a significant portion of the
cataracts beyond those observed in Western countries.\textsuperscript{68} Studies relating both equatorial
proximity and cataract and altitude and cataract showed no significant correlation.\textsuperscript{69} In
fact, a decrease in the prevalence of cataract amongst populations higher in the
mountains was recorded, even though the levels of UV-light are greater at higher
altitudes.\textsuperscript{70}

1.2.7. \textbf{Age-related (Senile) Cataract}

Nuclear cataract is the most common form of cataract in which the proteins become
brown\textsuperscript{58} cross-linked\textsuperscript{71} and insoluble\textsuperscript{65}. It is not clear why cataract is primarily a disease
of the elderly,\textsuperscript{72} however, there are several factors that are known to change with age.
For example, GSH is of pivotal importance in protecting the lens from oxidative
damage\textsuperscript{19} and in the vast majority of experimentally induced cataracts, a large decrease
in lenticular GSH is observed immediately prior to the onset of the cataract.\textsuperscript{3} It has been
postulated that as crystallins begin to become insoluble, as a result of aging, they
become isolated from the soluble anti-oxidants, GSH and ascorbate, and are thus more
easily damaged by oxygen species present in the lens.\textsuperscript{73}

A previous study has found that even the most advanced nuclear cataract lenses often
contained normal total levels of non-protein sulfhydryl (mostly reduced GSH), but the
levels in the nucleus were invariably very low or undetectable.\textsuperscript{74} This led to the recent proposal that within the lens an impediment to diffusion develops with age, resulting in a decrease in the availability of antioxidants. The lower level of GSH in the nucleus, may allow reactions to occur between lens crystallins, oxygen species and reactive low molecular weight metabolites. Hence, it has been proposed that in older lenses, this process could lead to nuclear cataract.\textsuperscript{75}

1.2.8. The UV-filter Hypothesis for Cataract Formation

3-OHKyn and 3-hydroxyanthranilic acid (3-OHA) are O-aminophenols. O-Aminophenols are readily oxidised at neutral pH and several of the oxidation products of 3-hydroxyanthranilic acid (3-OHA) have been characterized.\textsuperscript{76-78} It has been proposed that the reaction of oxidised aminophenols with lens proteins could lead to the changes known to accompany the formation of age-related nuclear cataract.\textsuperscript{78} Model studies have found that 3-OHA and 3-OHKyn react with proteins, under physiological conditions, to reproduce many of the changes that take place with the onset of cataract.\textsuperscript{42,79} In a related study, it has been shown that protein tanning was inhibited by the addition of GSH.\textsuperscript{42}

The finding that 3-OHKyn binds readily to proteins under oxidizing conditions has broader implications than the effects on ocular tissues. For example, in mice suffering from acute viral pneumonia, infection is accompanied by a one hundred fold increase in indoleamine dioxygenase\textsuperscript{80} (more below) and a thirteen fold increase in the level of 3-OHKyn.\textsuperscript{81}
1.3. Kynurenines

Although the kynurenine pathway of tryptophan metabolism has been known and used clinically in the assessment of patient status for many years, the study of kynurenines and their biological importance is still in its infancy. In 1981, it was discovered that quinolinic acid was a selective metabolite, interacting with a specific population of receptors in the mammalian brain. Since then, there has been a significant growth in the worldwide interest in quinolinic acid and other kynurenines.\(^8^2\)

1.3.1. The Kynurenine Pathway

The kynurenine pathway is the major route of tryptophan catabolism. In fact Ferstrom,\(^8^3\) showed that this pathway is responsible for 90% of tryptophan metabolism in mammalian peripheral tissues. The kynurenine pathway is summarized in Scheme 1.1. The first step of the pathway involves the oxidative cleavage of the heterocyclic ring of tryptophan. While many aromatic tryptophan metabolites arise via this pathway, none retains an intact indole nucleus.

Three distinct enzymes are able to catalyse the initial oxidative cleavage of the indole ring of tryptophan. The two haem enzymes, tryptophan dioxygenase (TDO, hepatic) and indoleamine dioxygenase (IDO, extrahepatic), are the major enzymes and act to produce N-formylkynurenine.\(^8^4\) A further enzyme that appears to be distinct from both of these has been identified in red blood cells, catalysing the oxidative cleavage of N,N-dimethyltryptamine to yield dimethylkynurenine.\(^8^5\)
Scheme 1.1: Tryptophan metabolism, the Kynurenine pathway.
IDO is widely distributed in mammalian tissues, having been identified in the stomach, lung and brain, however, it is not present in either the liver or kidney.\textsuperscript{86} IDO differs from TDO not only in its broader substrate specificity (L and D-tryptophan), but also in that it uses superoxide rather than O$_2$.\textsuperscript{87} The induction of TDO is accomplished by a number of physiological factors, however, IDO appears to respond mainly to pathological stress; it is induced by $\gamma$-interferon and bacterial lipopolysaccharide endotoxins.\textsuperscript{88,89}

1.3.2. The Metabolism of Kynurenine

Kynurenines are present in mammals, fungi, insects and many microorganisms. Within these organisms, kynurenine can be metabolised in a number of ways, as shown in Scheme 1.2. Kyn can undergo hydrolysis to anthranilic acid, catalysed by kynureninase; transamination to kynurenic acid, catalysed by aminotransferase or hydroxylation to 3-hydroxykynurenine, catalysed by kynurenine 3-monooxygenase (hydroxylase). Production of 3-OHKyn is the last major fate of kynurenine arising from the oxidation of physiological amounts of tryptophan, and leads into the pathways of either total oxidation or nicotinamide nucleotide synthesis. In addition, kynurenine and 3-hydroxykynurenine may be acetylated to a small extent, and in insects and some microorganisms, kynurenine and 3-hydroxykynurenine are substrates for the synthesis of ommochrome pigments and related compounds.\textsuperscript{82}
Scheme 1.2: The metabolism pathway for kynurenine.
1.3.3. Ommochromes

The ommochromes are a class of natural pigments which are found primarily among the arthropods. These pigments were assigned the name “ommochromes” because they are located in the ommatides of the insect eye. They are responsible for the colouration of the eyes as well as of certain other parts of the insect body and are acidic pigments, which have a red yellow or purple colour.

Scheme 1.3 shows the formation of ommochromes specifically from the oxidative condensation of 3-hydroxykynurenine to produce the phenoxazone ring system. The presence of a large number of ommochrome pigments in nature is possibly a consequence of the fact that the last steps of the synthesis can diverge in such a way that a variety of pigments are produced, such as ommatin and rhodommatin, for example. Scheme 1.3 shows xanthommatin, the yellow-brown pigment from insect eyes, is readily converted chemically into dihydroxanthommatin by the uptake of two hydrogen atoms as shown in Scheme 1.3. Dihydroxanthommatin readily oxidises in air into the more stable xanthommatin. Phenoxazones typically show a UV absorbance maximum at approximately 440 nm.
1.3.4. The Role of Kynurenines as Photosensitisers

It has been proposed that kynurenine metabolism generates two photosensitisers: kynurenic acid and N-formylkynurenine, that can generate oxygen radicals.\textsuperscript{92} 3-OHkyn and 3HKG, however, possess little photochemical activity.\textsuperscript{93}

Given the lack of evidence supporting a direct mechanism of UV-induced nuclear cataract, it has been suggested that if photooxidation is an important contributor to cataract formation, it may arise from a photosensitised reaction.\textsuperscript{94} For example, the loss of the glucoside of 3HKG has been observed following exposure to UV-A for long periods of time, with concomitant yellowing of the lens.\textsuperscript{95} Recently, Dillon \textit{et al.}\textsuperscript{44} also
concluded that 3HKG attaches to human lens proteins following exposure to UV-light, however, no data on the structure or the binding site of the bound moiety were provided.

1.4. Known Nonenzymatic Covalent Posttranslational Modification of Proteins by Small Molecules.

Non-enzymatic modification of protein can occur via various means such as deamidation, racemization or via covalent adduct formation. Such modifications may be either functional or potentially deleterious. Functional changes include the binding and release of retinal in the visual cycle, the formation in collagen and elastin of cross-links essential for their strength and acetylation by aspirin to relieve pain. Deleterious modifications may result from the introduction of foreign substances into the body or are the result of normally slow processes that become important only after middle age. Most modifications alter the charge on the protein and its hydrophobicity, thereby leading to a conformational change.

1.4.1. Protein Modifications by Lipid Peroxidation Products.

There is increasing evidence that aldehydes generated endogenously during lipid peroxidation (the oxidative deterioration of polyunsaturated fatty acids) are involved in most of the pathophysiological effects associated with oxidative stress in cells and tissues. Compared to free radicals, the generated aldehydes are stable and can diffuse within, or even escape from, the cell and attack targets far from the original site of production.

Among the aldehydes which originate from the peroxidation of membrane lipids, the α,β-unsaturated aldehyde, 4-hydroxy-2-nonenal (HNE) is believed to be largely responsible for the cytopathological effects observed during oxidative stress in vivo.
HNE causes protein damage by reacting with lysine amino groups, cysteine sulphydryl groups and histidine imidazole groups to form stable Michael addition-type reaction adducts as shown in Scheme 1.4.\textsuperscript{98-103}

\textbf{Scheme 1.4:} Formation of HNE-modified proteins. The RH group represents the histidyl imidazole, lysyl amino or the cysteinyl sulphydryl group of proteins.\textsuperscript{103}

\subsection*{1.4.2. Protein Modifications by Quinones}

Quinones are a ubiquitous class of compounds, which have been found in both natural products and endogenous biochemicals. They can be generated through metabolism of aromatic compounds with oxygen-containing substituents, including phenols, hydroquinones and catechols, by monooxygenases or peroxidase enzymes, metal ions and in some cases molecular oxygen.\textsuperscript{104} Some quinones are potent redox active compounds. They can undergo enzymatic and nonenzymatic redox cycling with their corresponding semiquinone radicals to generate superoxide anion radicals.\textsuperscript{104-106}

Superoxide radicals may dismutate spontaneously or following catalysis by superoxide dismutase, to produce hydrogen peroxide. In the presence of trace amounts of iron or other transition metals, hydrogen peroxide produces hydroxyl radicals. Hydroxyl radicals are powerful oxidizing agents that may damage essential macromolecules. For example, protein cysteine residues may be oxidised forming the disulfide bond which, in turn, can dramatically alter protein structure and function.\textsuperscript{107}
Quinones are also Michael acceptors and therefore can also damage organisms by covalently binding to cellular nucleophiles. For example, quinones react readily with sulphur nucleophiles such as GSH depleting cellular GSH levels. Cysteine residues on proteins can also add to quinones resulting in unwanted posttranslational modifications. As shown in Scheme 1.5, o- or p-diphenols (I) can undergo oxidation to the corresponding o- or p-quinone (II) followed by addition of GSH (III). The GSH conjugate can undergo a further series of sequential oxidations coupled with multiple additions of GSH to yield multiply substituted GSH conjugates (V).\textsuperscript{107}

In addition, some quinones can also react with nucleophilic amino groups on proteins or DNA.\textsuperscript{107} The addition of a sulphur atom to the quinone ring generates a quinone thiol ether, which is usually more redox active than the unsubstituted quinone. Experimental evidence supports a role for these GSH conjugates in the carcinogenic effects of polyphenolic antioxidants widely used in foods, for example, 3-\textit{tert}-butyl-4-hydroxyanisole (BHA).\textsuperscript{108,109}

\textbf{1.4.3. Other Xenobiotics}

A great variety of drugs and carcinogens have been found either to bind to proteins themselves or to produce reactive metabolites that may bind. Examples of xenobiotics include drugs such as glucocorticoids and acetaminophen or other chemical substances including epoxides, azo dyes, 2-acetylaminofluorene, carbon tetrachloride, chloroform and bromotrichloroethylene.\textsuperscript{50} Few studies, however, have been concerned with the exact nature of the change to the protein.

GSH protects the essential nucleophiles of tissue macromolecules against electrophilic attack by metabolites of drugs, but high drug doses deplete GSH and then the cellular
modification proceeds at a measurable rate. Protein thiol groups appear to be particularly vulnerable in this regard.\textsuperscript{50}

Scheme 1.5: The oxidative alkylation of quinones by glutathione.\textsuperscript{107}
1.5. Aims of the Project

The overall aim of the project was to determine the role and significance of the UV-filters kynurenine and 3-hydroxykynurenine in age-related colouration of lenticular protein and in the development of age-related nuclear cataract. Based on previous studies of lens protein modifications with age and in cataract, it was expected that any modifications would be present at very low levels in human lens and hence a significant proportion of the work described here involved the development and optimisation of suitable analytical methods for the analysis of biological samples. Mass spectrometry was chosen as the basis of these methods, owing to its high sensitivity and the capacity to analyse complex mixtures in combination with high pressure liquid chromatography (HPLC).

The first part of the study (described in chapter two), involved a comprehensive examination of the fragmentation of kynurenines observed by electrospray ionisation mass spectrometry and tandem mass spectrometry. This work was conducted, with a view to assisting in the characterization of any unknown reaction products of related structure found in the analyses of human lenses.

A second aspect of this work involved a detailed study of the oxidation of 3-OHKyn and the non-oxidative reaction of Kyn with protein, again as a basis for later work on human lenses (described in chapters three and four respectively). A method that enabled the search for novel biomarkers of UV-filter modification of human lens protein was developed and verified using model peptides and proteins, as described in chapter four. Finally, this method was applied to determine the quantities of modified amino acids present as a function of age of the lens and severity of cataract (chapter five).
2. A Study of Kynurenine Fragmentation using Electrospray Tandem Mass Spectrometry and Deuterium Exchange

2.1. Introduction

Kynurenine (Kyn) is found in the human lens along with related tryptophan metabolites. A number of unknown peaks are observed in the HPLC trace of lens extracts when the effluent is monitored at 360 nm. These peaks may correspond to novel derivatives of the known UV-filters. This chapter explores the MS characteristics of Kyn and related molecules. It is expected that a detailed knowledge of the fragmentation pathways will aid in the structural identification of novel UV-filter compounds isolated from human lenses.

A difficulty with the characterization of UV filter compounds and their derivatives is that often they can only be isolated from the lens or other tissue in very small quantities, thus precluding characterisation by nuclear magnetic resonance spectroscopy (NMR). Consequently, electrospray ionisation mass spectrometry (ESI-MS), which has inherently higher sensitivity than NMR, is invaluable for the characterization of new UV filter compounds. It must be recognised, however, that (ESI-MS), or in fact any MS method, cannot yield the same structural detail as NMR. The utility of mass spectrometry, however, has been demonstrated in the characterization of a novel fluorophore (glutathionyl-3OH-kynurenine-glucoside (GSH-3OHKG)) that our group has recently isolated from the human lens.32

There have been a number of reports in which mass spectrometry has been used for the analysis of Kyn and derivatives in biological samples.110-118 The only report of the fragmentation mechanisms of Kyn and its derivatives used electron ionisation (EI) and
therefore involved fragmentation of odd-electron ions with relative high internal energies\textsuperscript{119}. This chapter describes a detailed study of the fragmentation of Kyn observed in ESI-MS and tandem mass spectra (MS/MS) using a variety of techniques, including: precursor ion scans\textsuperscript{120,121} accurate mass measurements and deuterium exchange\textsuperscript{122-126} in combination with the more commonly-used product ion scans\textsuperscript{120,122}. This work has formed the basis for the study of Kyn and its derivatives and has been applied to the identification of novel Kyn derivatives isolated from the human lens outlined in Chapter 4. The multi-faceted approach employed here may also be a useful model for studies of other classes of amino acids and small molecules using ESI-MS and MS/MS.

2.2. Materials and Methods

2.2.1. Materials

Formic acid and acetonitrile (HPLC grade) were purchased from Ajax (Australia). Kynurenine was obtained from Sigma Chemical Co. (St. Louis, Mo, USA). D\textsubscript{2}O and DCl (purity 99.9\%) were obtained from Cambridge Isotope Labs (Andover, MA.). All gases used were compressed high purity obtained from BOC gases (Chatswood, Australia). Milli-Q\textsuperscript{®} water was used to prepare all solutions.

2.2.2. Electrospray Ionisation Mass Spectrometry

ESI mass spectra, precursor ion scans and product ion scans (for breakdown graphs) were recorded on a VG Quattro (VG Biotech, Altrincham, Cheshire, U.K.) which has a quadrupole hexapole quadrupole configuration in which each quadrupole has an m/z range of 4000. The instrument has been upgraded to meet Quattro II specifications. All spectra were acquired in positive ion mode at unit mass resolution using multichannel
analysis data acquisition. The instrument conditions used were: electrospray probe tip voltage 3.5 kV; 0.5 kV on chicane counter electrode; cone voltage 25-40 volts; nitrogen was used for both the bath and nebulizing gas with flow rates of 350 L/h and 10 L/h respectively; scan rate 100 m/z per second and typically 10-20 scans were summed to obtain representative spectra. The solvent (1% formic acid in 50% aqueous acetonitrile) was delivered using a Harvard Apparatus model 22 syringe pump (South Natick, MA, USA) operating at a flow rate of 10 μL/min. Kyn was dissolved in 50% aqueous acetonitrile (containing 1% formic acid) to give a concentration of 100 pmol/μL and 10 μL were injected for each analysis. The source temperature was set at 85°C. Both MS-1 and MS-2 were calibrated using a solution of sodium iodide (1mg/mL).

### 2.2.3. Tandem Mass Spectrometry

Tandem mass spectrometry (MS/MS) experiments on the VG Quattro were carried out in positive ion mode only, using the same ionisation and source conditions described above. For product ion scans of the non-deuterated species, the resolution of MS-1 was set to a minimum allowing the passage of 2-3 Da, while for precursor ion scans it was set to allow unit mass resolution. In both cases the collision gas was Ar gas at a pressure of 3.5x10⁻⁴ mbar and the collision energy was varied between 0 and 90 eV to achieve the desired level of fragmentation.

### 2.2.4. Accurate Mass Measurements

Accurate mass measurements and tandem mass spectrometry studies of deuterated Kyn were conducted on an Autospec oa-TOF magnetic sector mass spectrometer (VG Analytical Ltd. Wythenshawe, UK) equipped with an electrospray ion source. The mass spectrometer has a magnetic sector of EBE configuration as MS-1 and an orthogonal acceleration time-of-flight analyser as MS-2 i.e. EBE-TOF. The voltage on the
electrospray probe tip (ca. 8 kV) was optimised for individual samples to obtain the most stable and intense ion beam. Nitrogen was used as the nebulizing gas (concurrent to the solvent) and a counter current flow of warm nitrogen (80°C, 1 atm) was employed to assist with solvent evaporation.

Tandem mass spectrometry experiments on the EBE-TOF were carried out only in positive ion mode. Methane was used as the collision gas and the pressure in the collision cell was adjusted during the course of each run to maximize the diagnostic fragmentation obtained for each experiment. The voltage applied to the collision cell was 400 V. All tandem mass spectra were acquired over an m/z range of 50-250. Tandem mass spectra of product ions of interest were obtained by generating these ions via collisional activation in the source using raised cone and skimmer voltages. The ions of interest were then selected as the precursor ions in MS-1 and subsequently passed through the collision cell after which the MS/MS spectra were recorded by MS-2. The resolution in the MS/MS spectra was 500 (at 10% valley peak-to-peak) at m/z 122.

Accurate mass measurements were performed by setting the resolution in the magnetic sector analyser to 10 000 (at 10% valley peak-to-peak) and then scanning the accelerating voltage to span an m/z range of ± 20% of the ion of interest. Calibration of the instrument was achieved through the use of amino acid standards, which had m/z values within the m/z ±20% range for the particular product ion analysed.

2.2.5. Deuterium Exchange of Kynurenine

Deuterated Kyn, for labelling experiments, was produced by dissolving solid Kyn in D₂O with 0.01 M DCl, to facilitate both the exchange and ionisation process. This solution (concentration of 100 pmol/µL) was then infused into the mass spectrometer at a rate of 10 µL/min.
2.2.6. NMR Spectroscopy

One-dimensional (1D) $^1$H NMR spectra were recorded on a Varian Unity 400 spectrometer ($^1$H, 400 MHz; $^{13}$C, 100 MHz). All experiments were run in D$_2$O and referenced to residual HDO. Chemical shift values ($\delta$) are given in ppm.

2.3. Results

2.3.1. Electrospray Ionisation Mass Spectrum of Kynurenine

The ESI mass spectrum of Kyn is shown in Figure 2.1. The spectrum shows the protonated molecular ion [M+H]$^+$ at m/z 209 and a series of ions that have been identified by accurate mass measurements and tandem mass spectrometry to be derived from Kyn. Table 2.1 summarizes the accurate mass measurements of the major ions in the ESI mass spectrum (Figure 2.1). In all but one case, the measured masses of the product ions are within ± 5 ppm of those expected from their proposed compositions. The exception was the ion at m/z 174 with a difference of 7.4 ppm. We remain confident of the composition proposed for this ion, however, since all other possible compositions had mass differences of greater than 50 ppm. Further, these assignments were supported by the results of tandem mass spectrometry experiments (see section 2.3.2).
Figure 2.1: The ESI mass spectrum of Kynurenine ($M_r = 208$).
Table 2.1: Accurate mass measurements of the major ions observed in the electrospray mass spectrum of protonated Kyn. The error is given as the ppm difference between the calculated and measured m/z.

<table>
<thead>
<tr>
<th>Observed m/z</th>
<th>Calculated m/z</th>
<th>Error (ppm)</th>
<th>Suggested Elemental Composition</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>209.0935</td>
<td>209.0926</td>
<td>-4.3</td>
<td>C_{10}H_{13}N_{2}O_{3}</td>
<td>[M+H]^+</td>
</tr>
<tr>
<td>192.0663</td>
<td>192.0661</td>
<td>-1.0</td>
<td>C_{10}H_{10}NO_{3}</td>
<td>-NH_{3}</td>
</tr>
<tr>
<td>174.0542</td>
<td>174.0555</td>
<td>7.4</td>
<td>C_{10}H_{8}NO_{2}</td>
<td>-(NH_{3}+H_{2}O)</td>
</tr>
<tr>
<td>163.0869</td>
<td>163.0871</td>
<td>1.2</td>
<td>C_{9}H_{11}N_{2}O</td>
<td>-(CO+H_{2}O)</td>
</tr>
<tr>
<td>146.0603</td>
<td>146.0606</td>
<td>2.1</td>
<td>C_{9}H_{9}NO</td>
<td>-(NH_{3}, H_{2}O+CO)</td>
</tr>
<tr>
<td>136.0767</td>
<td>136.0762</td>
<td>-3.7</td>
<td>C_{8}H_{10}NO</td>
<td>-(C_{2}H_{3}NO_{2})</td>
</tr>
<tr>
<td>120.0445</td>
<td>120.0449</td>
<td>3.3</td>
<td>C_{7}H_{8}NO</td>
<td>-(H_{2}O+CO, C_{2}H_{5}N)</td>
</tr>
<tr>
<td>118.0656</td>
<td>118.0657</td>
<td>0.8</td>
<td>C_{8}H_{8}N</td>
<td>-(C_{2}H_{3}NO_{2}, H_{2}O)</td>
</tr>
</tbody>
</table>

The relative intensity of all the product ions was found to increase with increasing cone voltage, indicating that they were formed predominantly via collisional activation in the intermediate pressure region of the source. Focussing effects in this region, which may cause differences in ion abundance on altering the cone voltage are not likely to be significant over this small mass range.\(^{127}\)

2.3.2. Tandem Mass Spectrometry of Kynurenine

The product ion spectrum of the protonated molecular ion of Kyn, m/z 209, yielded the same major fragment ions as those observed in the ESI mass spectrum of protonated kynurenine, albeit the relative intensity of each varied with collision energy. Figure 2.2 shows the relative intensity of the major product ions observed in MS/MS spectra obtained by varying the laboratory collision energy (E\(_{\text{lab}}\)).
Figure 2.2: Relative intensity of product ions versus laboratory collision energy ($E_{\text{lab}}$) in the product ion spectra of protonated kynurenine (m/z 209).
The most abundant product ion at low collision energies was the deaminated fragment at m/z 192. The relatively high abundance of this ion, even at the lowest collision energies, demonstrated that loss of NH$_3$ from the side chain of protonated Kyn was a particularly facile process. As the collision energy was increased, however, the intensity of this ion decreased whilst the intensity of the ions at m/z 74, 118, 120, 136 and 174 increased. The relative intensity of m/z 174, 136, 118 and 74 reached maxima at $E_{\text{lab}}$ values of approximately 20, 10, 40 and 55 eV respectively, while the intensity of the m/z 120 gradually increased with collision energy across the whole range examined. The relative intensity of the product ion at m/z 146 versus collision energy closely followed that evident for the ion at m/z 174 while that of m/z 163 decreased gradually over the collision energy range examined.

A series of precursor ions scans were also conducted on the major ions observed in the product ion spectrum. In these experiments, the second mass analyser (MS-2) was set to the m/z of the product ion of interest and MS-1 was scanned to identify all the precursors of that ion. Figure 2.3 shows the precursors ion scans for m/z 118, 120, 146, 192, 174, 163 and m/z 136.
Figure 2.3: Precursor ion scans for the product ions a) m/z 146 b) m/z 120 and c) m/z 118 of protonated kynurenine.
Figure 2.3: Precursor ion scans for the product ions d) m/z 136 e) m/z 163 f) m/z 174 and g) 192 of protonated kynurenine.
Precursor ion scans of m/z 118 (Figure 2.3(c)) and m/z 120 (Figure 2.3 (b)) showed that these ions were formed by the direct elimination of H₂O or C₂H₅N from ions at m/z 136 and 163, respectively. The precursor ion scan of m/z 146 revealed, however, that this product ion had three possible precursor ions. Elimination of H₂O and CO, from m/z 192, was the major decomposition pathway while the loss of CO and NH₃ from the ions at m/z 174 and 163, respectively, were minor decomposition routes as judged by the relative intensity of the precursor ions. Precursor ion scans, were also performed on the ions at m/z 136, 163, 174 and m/z 192 (Figure 2.3). These spectra showed that ions at m/z of 192, 163 and 136 result from the respective loss of the neutral species NH₃, H₂O and CO or the immonium form of glycine directly from the protonated molecular ion, while the ion at m/z 174 was produced by the loss of H₂O from m/z 192. The precursor ion scan shown in Figure 2.3 (e) suggests that formation of the ion at m/z 163 from the ion at m/z 192 may take place. This is the result of a minor alternate decomposition route involving the protonated molecular ion (C₁₀H₁₃N₂O₃). Initially H₂O is eliminated to give an ion at m/z 191 (C₁₀H₁₁N₂O₂) followed by elimination of CO to give the ion at m/z 163 (C₉H₁₁N₂O). The relationships between all of the product ions enabled identification of three major fragmentation pathways arising directly from the protonated molecular ion, as shown in Scheme 2.1.
Scheme 2.1: Fragmentation pathways of protonated Kyn. The bold arrows indicate major decomposition routes.
2.3.3. Tandem Mass Spectra of Deuterated Kynurenine

The accurate mass measurements and MS/MS data enabled the determination of the elemental composition and the origin of the product ions, however, definitive structures could not be assigned without further information. Hence, product and precursor ion scans were performed on Kyn that had been labelled using deuterium exchange to assist in the structural characterization of the major product ions. Kyn is amenable to deuterium labelling since it has six hydrogen atoms readily available for exchange in the region of the molecule where fragmentation occurs. It was also considered possible that exchange of a further hydrogen atom by deuterium occurs through enolization of the benzylic ketone. This possibility was explored using proton NMR analysis of Kyn. The \(^1\)H NMR spectrum of Kyn samples in D\(_2\)O and 0.1M DCl, is shown in Figure 2.4. We expected that the enolic proton would have been characterized by a broad peak at room temperature, because of slow exchange in the keto-enol conversion, absorbing at about the same range as the phenolic proton (\(\delta \approx 7.5 - 4.0\) ppm),\(^{128}\) however, no such peak was observed. Further, the integration ratio between the proton peaks for \(H_a\) and \(H_b:H_c\) (Figure 2.4 (a)) was 2:1, indicating that no enolization had occurred. If enolization had occurred an integration ratio of 1:1 would be expected. Bond et al. in the \(^13\)C NMR of Kyn over a range of pH also found no evidence for the formation of the enol that would cause a change in the \(^13\)C chemical shift.\(^{129}\)
Figure 2.4: $^1$H NMR spectrum of Kyn in 0.1M DCl in D$_2$O. a) $\delta$ 4-6 ppm region and b) $\delta$ 6-9 ppm region (aromatic protons).
The ESI tandem mass spectrum of $d_6$ deuterated Kyn, acquired on the EBE-TOF instrument is shown in Figure 2.5. The figure shows that multiple ions differing in mass by one unit resulted from particular neutral losses from deuterated Kyn. Since neutral losses resulted in the generation of differentially deuterated product various fragmentation mechanisms and rearrangements may be associated with each fragmentation event.

Product ion scans of specific fragment ions formed via collisional activation in the source enabled us to determine the number of exchangeable hydrogens in each ion, greatly assisting the process of assigning possible structures. These experiments were conducted on the EBE-TOF instrument as it enabled higher resolution precursor ion mass selection ensuring that no interference occurred from species in which Kyn was not deuterated to the same degree. Precursor ion scans, performed on the triple quadrupole instrument, were also used to confirm the precursor-product ion relationships for each of the deuterated species. These results are summarized in Scheme 2.2. The pathways shown in the scheme are analogous to those of undeuterated kynurenine (Scheme 2.1).
Figure 2.5: Product ion spectrum of deuterated Kyn ($d_6$) obtained using the EBE-TOF instrument.
Scheme 2.2: Fragmentation pathways of deuterated Kyn, determined from product spectra of deuterated Kyn (d6) fragments.
The same product ions were observed in the ESI mass spectra on both the EBE-TOF and triple quadrupole instruments, although some minor differences were apparent in the relative ion intensities. Figure 2.6 shows the product ion spectra of the ions at m/z 195 (d₃), m/z 167 (d₄) and m/z 140 (d₄). These ions equate to those observed at m/z 192, m/z 163 and m/z 136 in the ESI mass spectrum of unlabelled kynurenine. The difference in mass between the deuterated and non-deuterated form of a particular ion indicated its level of deuteration. It was evident from the spectra in Figure 2.6 that the initial decomposition of the deuterated molecular ion yielded predominantly one ion for each of the three major fragmentation pathways. Subsequent decomposition of these product ions revealed the presence of multiple structures for each product ion, as indicated by the differing levels of deuteration in the resulting ions. The suggested structures and fragmentation pathways for each of the more abundant ions in the spectra shown in Figure 2.6 are given in Schemes 2.3-2.7 presented in the discussion.
Figure 2.6: Product ion spectra of ions from deuterated kynurenine generated by in-source collisional activation a) m/z 195 \( (d_3) \) b) m/z 167 \( (d_4) \) c) m/z 140 \( (d_4) \) obtained on the EBE-TOF instrument.
2.3.4. ESI-MS of Related Kynurenine Compounds

Following the analysis of kynurenine by ESI-MS, a number of related compounds were also analysed to determine how the observed fragmentation compared with that of kynurenine. The compounds selected for analysis were representative of kynurenine-related compounds typically encountered in the analysis of small molecules within the lens. Understanding their ESI fragmentation behaviour aided in the identification of known and novel compounds through the work described in following chapters. Figure 2.7 shows the ESI mass spectra of the compounds 3-OHKyn, xanthurenic acid and kynurenic acid analysed by positive ion ESI-MS.

All of the major peaks observed in the ESI mass spectrum of 3-OHKyn, the hydroxylated form of Kyn (see structure in Figure 2.7 a)), correspond directly to those observed in that of Kyn, albeit, they are shifted by 16 Da to higher mass by the presence of the additional oxygen atom. The major ions observed were m/z: 225, 208, 190, 166, 162, 152, 136 and 110. The structures of kynurenic acid and xanthurenic acid only differ from each other by a hydroxyl group. In fact they are direct derivatives of Kyn and 3-OHKyn formed by deamination ring closure and subsequent oxidation. The ESI mass spectrum of kynurenic acid and xanthurenic acid (Figure 2.7 b) and c), respectively) are similar. They only display fundamental fragmentation involving the elimination of the carboxylic acid group, producing ions at m/z 144 and 160 respectively, or the elimination of water, producing ions at m/z 172 and 188 respectively, followed by the elimination of carbon monoxide. The sequence of elimination was confirmed by precursor ion scans in each case. Again, the ions formed for the respective compounds differ in mass by 16 Da.
Figure 2.7: The positive ESI-MS of a) 3-OHKyn, b) kynurenic acid and c) xanthurenic acid.
Picolinic (2-pyridinecarboxylic acid) and isonicotinic acid (4-pyridinecarboxylic acid), which only differ in the position of the carboxyl group, were analysed by positive ion ESI-MS and the spectra are shown in Figure 2.8. Although structurally quite similar, the ESI mass spectrum of picolinic acid and isonicotinic acid (Figure 2.8 a) and b)) demonstrate that the fragmentation behaviour is quite different. Isonicotinic acid only displays a molecular ion at m/z 124 while picolinic acid also displays the molecular ion (m/z 124) along with product ions at m/z 106 and 78 due to the sequential elimination of H₂O and CO. This dramatic difference in the fragmentation behaviour was attributed to the availability of exchangeable hydrogen during the fragmentation process.
Figure 2.8: Positive ESI-MS of a) picolinic acid (2-pyridinecarboxylic acid) and b) isonicotinic acid (4-pyridinecarboxylic acid) under the same ionisation conditions.
2.4. Discussion

2.4.1. Elimination of Ammonia from Protonated Kynurenine

Elimination of NH$_3$ from the protonated molecular ion to give the product ion at m/z 192, is the first step in the principal dissociation route of protonated Kyn. Dookeran et al. have studied the fragmentation of a large number of amino acids and found that the initial fragmentation steps of protonated $\alpha$-amino acids depend strongly on the identity of the R group in HNCH(R)CO$_2$H.$^{130}$ For aliphatic amino acids, sequential elimination of H$_2$O and CO is observed, while for aromatic amino acids the loss of NH$_3$ is observed.$^{130}$ Since kynurenine is an aromatic amino acid, our observations are in agreement with that study.$^{130}$ The ion resulting from elimination of NH$_3$ from Kyn was also observed as the base peak in the EI mass spectrum of Kyn.$^{119}$

Following the elimination of NH$_3$, a product ion is observed at m/z 146, which corresponds to the elimination of CO$_2$H$_2$. Chemical ionisation (CI) mass spectra of carboxylic acids also show loss of CO$_2$H$_2$ from protonated molecular ions as a two step process involving initial loss of H$_2$O to form a stable acylium ion, followed by elimination of CO.$^{131-134}$ In contrast, $\alpha$-amino acids cannot form stable acylium ions via the elimination of H$_2$O.$^{135}$ This has been rationalized by Tsang and Harrison,$^{136}$ who concluded that the possible acylium ion formed was higher in energy than its product ions, and may dissociate spontaneously losing CO.$^{136}$ It is interesting to note that we observed both direct elimination of CO$_2$H$_2$ from the deaminated product ion at m/z 192 and elimination via a pathway involving the formation of the intermediate acylium ion.
2.4.2. Elimination of C₂H₃O₂N from Protonated Kynurenine

The dissociation pathway involving the initial elimination of the immonium form of glycine (C₂H₃O₂N) to yield initially an ion at m/z 136, is proposed to occur via the formation of an ion/molecule complex as shown in Scheme 2.3. The breakdown graph for protonated Kyn (Figure 2.2) shows that the loss of NH₃ decreases rapidly in importance as the collision energy is raised.

The product ion peak at m/z 136 from loss of the imine initially increases in intensity but then decreases, as the fragment ion at m/z 74 becomes the most abundant ion. This observation is most readily explained by assuming the formation of an ion molecule complex a. At low internal energies, proton transfer occurs within the complex to form species b. With increasing internal energy, however, the complex may separate before proton transfer can occur and the immonium ion c is formed. Following the formation of the m/z 136 ion, H₂O is readily eliminated from b to generate a product ion at m/z 118.
Scheme 2.3: Proposed mechanism for elimination of the imine from protonated Kyn via the formation of an ion molecule complex.
2.4.3. Elimination of CO and H₂O from Protonated Kynurenine

The third major process occurring is the loss of CO₂H₂ directly from the molecular ion to yield the product ion at m/z 163. In this instance, the intermediate acylium ion (from loss of H₂O) was not observed, which is in contrast to the elimination of CO₂H₂ from the deaminated fragment (m/z 192). This suggests that the acylium ion formed directly from the protonated molecular ion is not stable and rapidly dissociates to give the ion at m/z 163. This is a characteristic fragmentation of amino acids as noted above in section 2.4.1. The m/z 163 ion further decomposes through one of two fragmentation pathways: either the loss of C₂H₅N to give the ion at m/z 120 or the loss of NH₃ generating the ion at m/z 146.

2.4.4. Elimination of Deuterated Ammonia

In one major fragmentation pathway, deuterated Kyn eliminates a molecule of ND₃ from the side chain resulting in the deaminated fragment ion at m/z 195. The product ion scan of this ion (Figure 2.6(a)) showed two pairs of ions for each dissociation step, thus suggesting the existence of parallel dissociation for the deaminated fragment (m/z 195). The existence of two dissociation pathways was interpreted in terms of the presence of two structural isomers of the deaminated fragment, the cyclized \( d \) and uncyclized \( e \) form as shown in Scheme 2.4. We suggest that the structures proposed in Scheme 2.4 direct the level of deuteration of the eliminated water by constraining the ability of the hydroxyl group to be protonated by nearby exchangeable hydrogen or deuterium. Protonation by nearby exchangeable hydrogen or deuterium is an important step in the elimination of water from carboxylic acids.

In \textit{ab initio} studies of protonated glycine, the loss of water can only occur when it is preceded by transfer of exchangeable hydrogen from nitrogen to the OH.\textsuperscript{137,138} The
influence of the location of the exchangeable hydrogen in the elimination of water was further explored using the related model compounds picolinic and isonicotinic acid (where the carboxylic group is the 2 or 4 position of the pyridine ring respectively). The ESI spectra are shown in Figure 2.8. The spectra show that the position of the carboxylic group relative to the source of exchangeable hydrogen plays a pivotal role in the elimination of water. When the source of exchangeable hydrogen is distant from the hydroxyl group, as in isonicotinic acid, no fragmentation occurs, while in picolinic acid, where the carboxylic acid is adjacent to the source of exchangeable hydrogen, facile loss of H$_2$O is apparent (Figure 2.8 a)). This suggests that the hydroxy group will draw on the nearest source of hydrogen or deuterium in the elimination process.

The prominent loss of HDO from e suggests the involvement of a source of exchangeable hydrogen in the structural rearrangements, which we suggest to be the two acidic hydrogens shown in e. Elimination of D$_2$O from d suggests the absence of hydrogen in the exchange process. We expect that the likely source of exchangeable deuterium or hydrogen would be the deuterium of the deuterated aromatic amine. Following the elimination of D$_2$O and HDO from f and g, carbon monoxide is subsequently eliminated to yield j and k, respectively.

The structures of the deaminated product ions f and g are similar to those expected for carboxylic acids in the gas phase. In fact, it might be expected that these ions would act as carboxylic acids during the elimination of water, producing a stable acylium intermediate. In the case of amino acids, however, the elimination of water produces an $\alpha$-amino acylium ion that undergoes facile exothermic loss of CO to form immonium ions. This rapid two-step decomposition may also be enhanced because the final product will be stabilized by the remaining positive charge. In the case of carboxylic
acids, however, the acylium intermediate is considerably more stable, which is possibly a consequence of the inability of the product ion to stabilise the charge beyond the production of the acylium ion.

The product ion spectra of the ions \( f \) and \( g \) indicated that the overall \((\text{[M+H}^+ - \text{H}_2\text{O} - \text{CO}]^+)\) decomposition process proceeded via parallel routes (Scheme 2.4). Elimination of the two neutral species occurs either without the detection of the acylium intermediate or with detection of the intermediates \( h \) and \( i \). This suggests the intermediates are sufficiently stable.

While the direct loss of formic acid cannot be discounted, given the rationale mentioned earlier, a possible reason for the absence of the intermediates in the parallel decomposition routes may be the facilitation of CO elimination by some means. For example by the ability of final product ions, \( j \) and \( k \), to better accommodate the charge.

2.4.5. Elimination of Deuterated Glycine as the Imine

The formation of an electrostatically-bound ion molecule complex prior to fragmentation (Scheme 2.3) has been proposed for the elimination of glycine as the imine. This could account for the production of complementary fragment ions from, in this case, the deuterated molecular ion.

At lower collision energies the fragment ion \( l \), as shown in Scheme 2.5, is expected to be the more prominent of the two possible ions formed. Further fragmentation of \( l \) leads to the elimination of \( \text{D}_2\text{O} \) (Figure 2.6 (c)) resulting in the formation of an indole type structure, \( n \), with two deuterium atoms. The formation of this stable cyclic structure was supported by the fact that additional product ions were observed in the tandem mass spectrum of \( m/z \) 120 (not shown).
Scheme 2.4: Major product ions and proposed structures observed in the product ion spectrum of [MH$^+$ - ND$_3$]$^+$ m/z 195.
Scheme 2.5: Major production observed in the production spectrum of [MH$^+$ - Imine]$^+$ m/z 140.
In association with the peak corresponding to \( n \), evident at m/z 120 (Figure 2.6 (c)), there was another peak one m/z higher. We proposed the fragment ion \( o \) has the same structure as \( n \) with the difference in m/z arising from the number of deuterium atoms associated with each fragment. The additional deuterium atom in structure \( o \) may be located on the carbon either \( \alpha \) or \( \beta \) to the nitrogen through H/D scrambling resulting from tautomerization of \( l \) to \( p \) and \( q \) (Scheme 2.5). This mechanism may also account for the peak at m/z 122 in Figure 2.6(c) that corresponds to the exchange of two deuterium atoms in the same manner.

At higher collision energies (>20 eV) the \( \alpha \)-glycyl cation \( m \) is expected to be the most prominent of the two ions. This species is essentially the immonium form of glycine and the analogous species has been previously observed in the CI mass spectrum of aspartic acid.\(^{136}\) Further, the tandem mass spectrum of \( m \) gave a product ion at m/z 29 (\( ^7\)DN=CH) produced by the elimination of D\(_2\)O and CO (not shown). This is in agreement with results reported by other groups.\(^{140-142}\) This ion is also apparent in the dissociation of glycine.\(^{143}\)

### 2.4.6. Elimination of Deuterated Water and Carbon Monoxide

For the pathway involving elimination of D\(_2\)O and CO, deuterated Kyn displays the dissociation chemistry expected for \( \alpha \)-amino acids.\(^{130}\) The carboxylic acid group dissociates through the elimination of D\(_2\)O and CO without the detection of the acylium intermediate. The apparently spontaneous decomposition of this acylium intermediate, the rationale for which has been presented above, gave rise to the immonium ion, structure \( r \) in Scheme 2.6. The product ion spectrum of \( r \) showed elimination of the enamine ND\(_2\)HC=CH\(_2\) or ND\(_2\)HC=CDH producing \( s \) or \( t \). The hydrogen/deuterium scrambling evident in the proposed structures for \( s \) and \( t \) suggests a contribution from
the aromatic nitrogen-bound deuterium during the elimination process. This would only be possible if two pathways existed for the elimination of the enamine, namely: path (a), direct elimination of the enamine from r or path (b), exchange of hydrogen and deuterium between the aromatic amine and the carbon α to the carbonyl group followed by elimination of the triply deuterated enamine by a similar mechanism to path (a). Further evidence for the structures s and t is provided via the presence of the product ions at m/z 93 and 94 corresponding to the elimination of carbon monoxide from the proposed structures respectively in the tandem mass spectra of t and s (not shown).

2.4.7. ESI-MS of Related Kynurenine Compounds

The ESI mass spectrum of 3-OHKyn (Figure 2.7) showed ions (formed by in-source collisional activation) that correlated with those observed in the ESI mass spectra of Kyn. Since all the major ions were shifted by 16 Da, we suggest that the phenolic group of 3-OHKyn does not play a role in directing the fragmentation routes of 3-OHKyn. Consequently, we expect that in the analysis of related compounds, any aromatic substituents present in Kyn would have, at best, only limited influence on the fragmentation patterns observed.
Scheme 2.6: Major product ions observed in the product ion spectrum of \([\text{MH}^+ - \text{D}_2\text{O} - \text{CO}]^+\) m/z 167.
The protonated forms of kynurenic acid and xanthurenic acid are very similar to the structures of the ions produced by deamination of Kyn and 3-OHKyn as a result of collisionally-induced dissociation. Both kynurenic acid and xanthurenic acid display the prominent loss of water followed by loss of carbon monoxide from the protonated molecular ion (Figure 2.7 b) and c)). The relationships between the ions produced from in-source collisional activation of kynurenic and xanthurenic acid were determined by precursor ion scans and are presented in Scheme 2.7. The fragmentation mechanism that accounts for the ions h and j in Scheme 2.4 suggests that elimination of H₂O and CO from kynurenic and xanthurenic acid proceeds via parallel decomposition routes to produce the ions v, w and y, z respectively (Scheme 2.7). By means of one route the acylium ion (arising through the loss of H₂O from the protonated molecular ion) is stable, producing the ions v and y. These ions may fragment further through the loss of CO to produce the ions w and z. In the parallel fragmentation pathway, the acylium ion is not stable (or at least is not detected) and the loss of H₂O and CO appears to occur in one step producing the ions w and z.

In both cases no further fragmentation is apparent following the formation of the ions w and z. Since the ions w and z are cyclized the ionisation process would need to be particularly energetic to result in fragmentation of these stable structures. In addition, the remaining hydroxyl groups on both structures are not likely to undergo further fragmentation since no source of exchangeable hydrogen remains following the elimination of H₂O and CO.
Scheme 2.7: Fragmentation pathways for kynurenic acid and xanthurenic acid determined from precursor ion scans.
3. Characterisation of the Major Autoxidation Products of 3-Hydroxykynurenine Formed Under Physiological Conditions

3.1. Introduction

The aminophenol 3-hydroxykynurenine (3-OHKyn) is found throughout the tissues of the human body. It is a precursor to NAD⁺ but its presence may also be deleterious to biological cellular processes particularly under oxidising conditions.⁸² The levels of 3-OHKyn and the corresponding transamination product, quinolinic acid, are significantly elevated in the brain in pathological conditions such as dementia associated with human immunodeficiency virus (HIV) infection, hepatic encephalopathy, Parkinson’s disease and Huntington’s disease.¹⁴⁴-¹⁴⁷ In neuronal cell cultures, 3-OHKyn has been found to be cytotoxic at concentrations as low as 1 μM, seemingly causing oxidative stress through the formation of hydrogen peroxide.¹⁴⁸

In human nuclear cataract, proteins in the lens become coloured, oxidised, cross-linked and insoluble.⁶⁵ It has been proposed that 3-OHKyn is implicated in these changes since 3-OHKyn is an ortho-aminophenol which can undergo both rapid and complex oxidation processes. Incubation of 3-OHKyn with lens proteins, in the presence of oxygen, can reproduce the changes that occur in nuclear cataract.⁴²,¹⁴⁹ The structures of the reactive intermediates of 3-OHKyn that are responsible for the protein modification, however, are not known. For these reasons the oxidation of 3-OHKyn, along with products purified from the autoxidation reaction have been examined here in an attempt to identify the reactive species possibly involved in protein modification. Secondly, the possible involvement of H₂O₂, produced as a by-product, in the autoxidative reactions has been explored. Experimental conditions of pH and temperature were chosen to mimic the conditions in the lens.
3.2. Materials and Methods

3.2.1. Materials

3-OHKyn, o-phenylenediamine (OPD), horseradish peroxidase (HRP), desferrioxamine (desferal), 5,5-dimethylpyrroline-N-oxide (DMPO) superoxide dismutase (SOD), ascorbic acid, metaphosphoric acid, 2,6-dichloroindophenol, glutathione and catalase were purchased from Sigma Chemical Co. (St Louis, MO, USA). Triethylammonium acetate buffer was obtained from Fluka Chemika-BioChemika (Buchs, Switzerland). Mannitol and H₂O₂ were obtained from Ajax (Australia). N-α-Fmoc-L-Lysine was obtained from Auspep Pty. Ltd. Parkville Australia. All gases used were compressed industrial grade (CIG, Chatswood, Australia). Venous blood was collected from the author in hepronised vacuum vials. Milli-Q® water was used in the preparation of all solutions. Phosphate buffers were prepared from AR grade NaH₂PO₄ and Na₂HPO₄, both obtained from Ajax (Australia). Chelex resin was obtained from Biorad (Australia).

3.2.2. Autoxidation of 3-Hydroxykynurenine

The standard autoxidation reaction mixture consisted of 3-OHKyn (2.2 mM) in sodium phosphate buffer (0.1 M) adjusted to the appropriate pH using 1 M solutions of NaOH or NaH₂PO₄. The resultant mixture was then bubbled with oxygen (at approximately 15 mL/min) while immersed in a water bath at the specified temperature. Aliquots were taken at various time intervals for HPLC analysis. Where indicated, catalase (6300 units per addition) or SOD (1000 units per addition) was added to 5 mL of the standard autoxidation solution (pH 7 and 35°C) at hourly intervals. Mannitol (273 mg/5 mL) was also added to the autoxidation solution (pH 7 and 35°C) in some experiments as
described below.

3.2.3. Autoxidation of 3-OHKyn in the Presence of Glutathione or Ascorbic Acid.

Solutions of 3-OHKyn (2.5 mM) were oxidised in the presence of glutathione (GSH) (2.2 mM) or ascorbic acid (2.5 mM) at pH 7 (0.1 M phosphate buffer) at 35°C. The extent of oxidation of 3-OHKyn was followed by measuring the absorbance at 490 nm. The concentration of GSH was measured using the DTNB (2,4-Dinitrothiocyanatobenzene) assay\textsuperscript{74} and the concentration of ascorbic acid was determined using an assay adapted from that used for the analysis of vitamin C in food.\textsuperscript{150}

3.2.4. Autoxidation of 3-OHKyn in the Presence of Human Erythrocytes

Venous blood was sampled into evacuated hepronised tubes. The blood was subsequently centrifuged at 3000 rpm to separate the erythrocytes from the plasma. The supernatant plasma was removed and the remaining cells were resuspended with isotonic phosphate buffered saline to give the same concentration of erythrocytes as originally present in the blood. The cells were then centrifuged at 3000 rpm for five minutes, the supernatant was removed and the erythrocytes resuspended in phosphate buffered saline. The suspension was then stored on ice and used within three hours of purification.

To examine the autoxidation of 3-OHKyn in human erythrocytes 0.5mg/mL of 3-OHKyn was added to the solution. The reaction mixture was bubbled with oxygen and incubated at 35°C. A blank solution containing erythrocytes but without 3-OHKyn was also analysed. In each case the aliquots were analysed by removing a sample of the
suspended erythrocytes for centrifugation at 3000 rpm for 5 minutes. The supernatant was then analysed by analytical HPLC.

3.2.5. Autoxidation of 3-OHKyn in the Presence of Fmoc-L-Lysine

All reactions were carried out at 35°C and concentrations of 4.1 mg/mL of Fmoc-L-lysine (5 molar excess) and 0.5 mg/mL of 3-OHKyn in freshly prepared 35 mM phosphate buffer in 20% DMF/H₂O in a total volume of 10mL. The reaction mixture was sonicated for a short time before bubbling with oxygen that was humidified by bubbling through 20% DMF/H₂O, at a flow rate of 5 mL/min. The reaction mixture was incubated for a period of 80 hours. The 20% DMF/H₂O solvent system was chosen to solubilize the Fmoc-Lys as it was insoluble in water. After 80 hours, the mixture was lyophilised until the volume was reduced by approximately 1/3. The remaining solution was then analysed by analytical HPLC.

3.2.6. Blank Autoxidation of Fmoc-L-Lysine

8.2 mg of Fmoc-Lys was dissolved in 0.4 mL of DMF and to this was added 70 µL of 1 M phosphate buffer pH 7. The solution was made up to a total volume of 3.6 mL, incubated at 35°C and bubbled with oxygen for 5 days.

3.2.7. Blank Autoxidation of 3-OHKyn in DMF Solvent.

2.5 mg of 3-OHKyn was dissolved in 1 mL of DMF and to this was added 175 µL of 1 M phosphate buffer pH 7. The solution was made up to a total volume of 5 mL with H₂O, incubated at 35°C and bubbled with oxygen for 5 days.
3.2.8. High Performance Liquid Chromatography Analysis

The reverse phase HPLC system consisted of an LC 1150 HPLC pump (ICI instruments), a Rheodyne 7125 sample injector (equipped with a 20 µL sample loop) and a SD 2100 UV-vis variable wavelength detector (ICI Instruments), set at 254 nm for all analyses (unless otherwise specified). Analytical separations were performed on a 250 mm x 4.6 mm Spherisorb S50DS2 column (ICI Australia Operations Pty. Ltd., Dingley, Australia). Samples were analysed using an aqueous 4 mM ammonium acetate (buffer A) and acetonitrile 4 mM ammonium acetate (buffer B). The gradient used was 0% buffer B for 10 min followed by an increase to 40% buffer B over 30 min at a flow rate of 0.5 mL/min. When semi-preparative separations were required, a 25 mm x 10 mm Partisil-10 ODS-3 semi-preparative HPLC column (Whatman) was employed using the same eluents and gradient with a flow rate of 3.0 mL/min. The solvents were degassed by sparging with helium for 10 min before use. Injection volumes were 20 µL for analytical runs and up to 4 mL for semi-preparative runs. The samples were adjusted to pH 7, where required, before HPLC analysis.

3.2.9. Electrospray Ionisation Mass Spectrometry and Tandem Mass Spectrometry Analysis

Electrospray ionisation mass spectrometry (ESI-MS) analysis was performed on a VG Quattro mass spectrometer (VG Biotech/Micromass, Altrincham, UK). For electrospray analyses, fractions isolated by HPLC were either analysed directly or were lyophilised and then re-dissolved in 50% aqueous acetonitrile. The electrospray solvent was 49.5% (v/v) acetonitrile in H₂O which contained 1% (v/v) formic acid. This solvent was delivered at a flow rate of 10-15 µL/min by a Harvard Apparatus 22 syringe pump. The electrospray conditions used were as follows: nitrogen was used for both the bath and
nebulizing gas, flowing at 350 L/h and 10 L/h respectively. The capillary potential was 3.2 kV, HV lens 500 V and the cone voltages ranged from 20 V to 60 V. The source temperature was set at 85°C. The scan rate was 100 m/z per second and the scan range was from 100 to 700 m/z. Calibration of the mass spectrometer was achieved using NaI. Mass spectra were acquired in positive ion mode at unit mass resolution using multichannel analysis.

Tandem mass spectrometry (MS/MS) experiments were also carried out on the VG Quattro triple quadrupole mass spectrometer. The source conditions used were the same as those described above except that the resolution of the first quadrupole set was lowered slightly to allow greater transmission of the precursor ions. The collision cell was filled with argon gas at a pressure of 3.5x10^-4 mbar and the collision energy was varied between 25 and 45 eV to achieve the desired level of fragmentation.

3.2.10. Liquid Chromatography Mass Spectrometric Analysis

Liquid chromatography mass spectrometry (LC/MS) was carried out using an Applied Biosystems 140B solvent delivery system and 785A UV detector set at 360 nm. Adducts were separated using an Alltech Alltima 300 Å C18 column (250 mm x 2.1 mm, 5μm) at a flow rate of 200 μL/min, using a 0-80% (with 1% formic acid) gradient of over 40 min, a column oven temperature of 25°C and the source temperature maintained at 170°C. All mass spectra were acquired on the VG quattro mass spectrometer using the same conditions as described in section 3.2.9 except that the spectra were acquired in continuum mode.
3.2.11. Electron Paramagnetic Resonance Spectroscopy

3-OHKyn was made up to 500 μM in phosphate buffer (pH 7.4) that had been treated previously with washed Chelex-100 (Bio-Rad) to minimise levels of contaminating transition metals. The mixtures were then incubated at 37°C while exposed to air (or bubbled with nitrogen) for the times specified below. Where indicated, DMPO was present at 0.1 M, catalase at 600 units/mL and H₂O₂ at 20 mM. Electron paramagnetic resonance (EPR) spectra were recorded at room temperature using a Bruker EMX X-band spectrometer with 100 kHz modulation and a cylindrical ER 4103TM cavity. Autoxidation mixtures were allowed to incubate for different times as indicated in section 3.3 and subsequently transferred to a flattened sample cell (WG-813-SQ, Wilmad, Buena, NJ, USA). The spectra were recorded within 2 min. Hyperfine couplings were measured directly from the field scan. Typical EPR spectrometer settings were gain 2x10, modulation amplitude 0.2 mT, time constant 0.16s, scan time 84s, resolution 1024 points, centre field 348 mT, field scan 8 mT, power 25 mW, frequency 9.76GHz, 8 scans averaged for each spectrum.

3.2.12. Hydrogen Peroxide Assay

Hydrogen peroxide concentrations were measured using a HRP/OPD assay. Duplicate 10 μL aliquots of the autoxidation solution were placed in a micro-titer plate and frozen until all samples were collected. This was followed by the sequential addition to each well of 10 μL of distilled water, 50 μL of 0.2 M phosphate buffer (pH 5.0) containing HRP and 100 μL of 5 mg/mL OPD solution. This mixture was allowed to react for 5 min at room temperature and quenched by the addition of 30 μL of 1 M HCl. The absorbance was measured at 490 nm and H₂O₂ concentrations calculated by reference to a standard curve (from 0 to 3 mmol H₂O₂).
3.2.13. GSH Assay

The level of GSH in solution was determined using an 80 μL aliquot of the reaction mixture which was added to 3.68 mL of a solution consisting of 0.05 M Tris base and 0.02 M EDTA adjusted to pH 8.5 with HCl. 240 μL of 0.01 M DTNB in methanol was then added. The resulting solution was mixed and allowed to react for a minute before measuring the absorbance at 412 nm using a UV-Vis spectrophotometer.

3.2.14. Ascorbic Acid Assay

The level of ascorbic acid in solution was determined using a 100 μL aliquot to which was added 100 μL of metaphosphoric acid solution, 75 μL of 2,6-dichloroindophenol and 975 μL of H₂O. The resulting solution was mixed and allowed to react for a minute before measuring the absorbance at 520 nm using a UV-spectrophotometer. This absorbance was compared against a calibration curve to determine the concentration of ascorbic acid in solution. The metaphosphoric acid solution was made up from 0.4g of metaphosphoric acid, 1.6g of glacial acetic acid and 8 mL of methanol in 10 mL of H₂O. The 2,6-dichloroindophenol solution was prepared from 7.5 mg of 2,6-dichloroindophenol and 10 mL of AR grade acetone.

3.2.15. Preparation of Xanthommatin, Hydroxyxanthommatin and 4,6-Dihydroxyquinolinequinonecarboxylic Acid

Hydroxyxanthommatin (OHXan) was obtained by semi-preparative HPLC purification from the 3-OHKyn autoxidation reaction mixture after 25 h of reaction at pH 7 and 25°C. For the preparation of Xanthommatin (Xan), a 2.2 mM solution of 3-OHKyn in 0.1 M phosphate buffer (10 mL, pH 7) was bubbled with oxygen in the presence of catalase (6300 units per hour) for 4 h. The Xan was then purified using semi-preparative
HPLC as described above. 4,6-dihydroxyquinolinequione-carboxylic acid (DHQCA) was synthesised from trimethoxybenzene as described previously.\textsuperscript{151} Where indicated, the stability of the isolated 3-OHKyn autoxidation products was assessed by incubation in phosphate buffer as above. In specific cases, \( \text{H}_2\text{O}_2 \) was also added to give a final concentration of approximately 50 mM.

\subsection*{3.2.16. Examination of the Stability and Oxidation of Xanthommatin at pH 7}

Xan (2 mg) prepared as described earlier was dissolved in 5 mL of 0.1 M phosphate buffer pH 7, bubbled with oxygen continuously and incubated at 35°C. At the desired time intervals, 60\( \mu \)L aliquots were removed and analysed, in duplicate, by analytical HPLC using a 20\( \mu \)L loop.

\subsection*{3.2.17. Examination of the Stability and Oxidation of Hydroxyxanthommatin at pH 7}

OHXan was prepared as described earlier. 2 mg of OHXan was dissolved in 4 mL of 0.1 M phosphate buffer pH 7 bubbled with oxygen and incubated at 35°C. At the desired time intervals duplicate 60\( \mu \)L aliquots were removed and analysed by analytical HPLC using a 20\( \mu \)L loop.

\subsection*{3.2.18. Incubation of DHQCA with 3-OHKyn in the Absence of Oxygen}

The reaction mixture consisted of 3-OHKyn (1.0 mg) and DHQCA (0.5 mg) dissolved in 5 mL of sodium phosphate buffer (0.1 M) adjusted to the appropriate pH using 1 M solutions of NaOH or NaH\(_2\)PO\(_4\). The resultant mixture was then degassed using two freeze thaw cycles and sealed in five individual glass tubes, each containing 1 mL of the reaction mixture, under argon. The glass tubes were immersed in a water bath
maintained at 25°C and the reaction mixture was sampled by taking aliquots of an individual tube at various time intervals.

3.3. Results

3.3.1. Characterisation of the Major 3-Hydroxykynurenine Autoxidation Products

The products of autoxidation of 3-OHKyn were monitored by HPLC using aliquots taken from the reaction mixture at selected intervals. Figure 3.1 shows the HPLC profile after 2.5 h of 3-OHKyn (2 mM) autoxidation in 0.1 M phosphate buffer pH 7 and at 25°C. Four species were evident in the chromatogram. The major positive ESI ions and UV-visible absorbance maxima for each species are given in Table 3.1. The compounds with retention times of 5.3, 12 and 28 minutes were identified as DHQCA, 3-OHKyn and Xan, respectively, using ESI-MS, UV-vis spectrophotometry and HPLC analysis by comparison with synthetic standards. The UV-visible and ESI spectra for each of the synthetic standards are shown in Figures 3.2 to 3.4.

The compound eluting at 23 min has been tentatively assigned the structure shown in Figure 3.5 and denoted as hydroxyxanthommatin (OHXan). The rationale is as follows. The absorbance maximum of 486 nm is characteristic of a phenoxazone ring system. The molecular mass was 439 (observed m/z 440 for M+H+) which is 16 Da above that of Xan. Finally, the fragment ions observed in the ESI mass spectrum (all due to losses from the amino acid side chain) were analogous to those of Xan (Table 3.1).

The HPLC trace (Figure 3.1) also shows two groups of poorly resolved peaks, centred on retention times of 4.3 and 20 min respectively. Extensive efforts were made to purify these compounds (designated as F 4.3 and F 20) for the purpose of identification,
however, the inherent instability of F 20 and the short retention time for F 4.3 (co-eluting with the salt peak) precluded successful identification.

Table 3.1: Selected ions in the ESI mass spectra and absorbance maxima for the species DHQCA, 3-OHKyn, Xan and OHXan.

<table>
<thead>
<tr>
<th>Assignment</th>
<th>Retention Times (min)</th>
<th>Positive ESI ions (m/z)</th>
<th>UV-visible maxima (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHQCA</td>
<td>5.3</td>
<td>236 ([M + H⁺])</td>
<td>459</td>
</tr>
<tr>
<td></td>
<td></td>
<td>218 (H₂O loss)</td>
<td>349</td>
</tr>
<tr>
<td></td>
<td></td>
<td>190 (H₂O + CO loss)</td>
<td></td>
</tr>
<tr>
<td>3-OHKyn</td>
<td>12</td>
<td>225 ([M + H⁺])</td>
<td>371</td>
</tr>
<tr>
<td></td>
<td></td>
<td>208 (NH₃ loss)</td>
<td>265</td>
</tr>
<tr>
<td></td>
<td></td>
<td>179 (H₂O + CO loss)</td>
<td>229</td>
</tr>
<tr>
<td></td>
<td></td>
<td>152 (HCNCOOH loss)</td>
<td></td>
</tr>
<tr>
<td>Xan</td>
<td>28</td>
<td>424 ([M + H⁺])</td>
<td>436</td>
</tr>
<tr>
<td></td>
<td></td>
<td>407 (NH₃ loss)</td>
<td>235</td>
</tr>
<tr>
<td></td>
<td></td>
<td>361 (H₂O + CO loss)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>351 (HCNCOOH loss)</td>
<td></td>
</tr>
<tr>
<td>OHXan</td>
<td>23</td>
<td>440 ([M + H⁺])</td>
<td>655</td>
</tr>
<tr>
<td></td>
<td></td>
<td>423 (NH₃ loss)</td>
<td>486</td>
</tr>
<tr>
<td></td>
<td></td>
<td>377 (H₂O + CO loss)</td>
<td>359</td>
</tr>
<tr>
<td></td>
<td></td>
<td>367 (HCNCOOH loss)</td>
<td></td>
</tr>
</tbody>
</table>

3.3.2. Autoxidation of 3-Hydroxykynurenine as a Function of Time

From HPLC analysis of the autoxidation mixture at specific time intervals, qualitative measurements of species concentration were made based on the relative percentage peak areas determined at a wavelength of 254 nm (the optimum wavelength for the detection of compounds containing aromatic ring systems). These values were plotted against time to give the product versus time profiles shown in Figure 3.6 for the autoxidation of 3-OHKyn at pH 7 and 25°C.
Figure 3.1: A typical reversed-phase HPLC trace of 3-OHKyn autoxidation products. 3-OHKyn was incubated in the presence of oxygen at pH 7 and 35°C for a period of 2.5 h. The compounds shown are: F 4.3, DHQCA, 3-OHKyn, F 20, OHXan, and Xan.
Figure 3.2: Positive ESI mass spectrum of DHQCA (m/z 236) purified by HPLC from the autoxidation of 3-OHKyn. The UV-visible spectrum and structure are shown inset.
Figure 3.3: Positive ESI mass spectrum of xanthommatin (m/z 424) purified by HPLC from the autoxidation of 3-OHKyn. The UV-visible spectrum and structure are shown inset.
Figure 3.4: Positive ESI mass spectrum of 3-OHKyn (m/z 225). The UV-visible spectrum and structure are shown inset.
Figure 3.5: Positive ESI mass spectrum of hydroxyxanthommatin (m/z 440) purified by HPLC from the autoxidation of 3-OHKyn. The UV-visible spectrum and proposed structure are shown inset.
When the autoxidation of 3-OHKyn was carried out at pH 7 and 25°C, the major autoxidation product formed initially was Xan. It reached a maximum concentration at ca. 4 hours (Figure 3.6), after which the concentration fell below the limit of detection (ca. 35 h). In later experiments the xanthommatin radical Xan was also detected (see section 3.3.8). Following the formation of Xan, an increase in the production of OHXan was observed until a maximum concentration was reached at approximately 25 hours. This was accompanied by a relatively constant rate of formation of DHQCA and the compound(s) F 4.3 (Figure 3.6). The compound(s) giving rise to the peak labelled F 20 accounted for less than 5% of the total 254 nm absorbance throughout the course of incubation.

**Figure 3.6**: Relative percentage peak area versus time graph for the autoxidation of 3-OHKyn at pH 7 and 25°C in 50 mM phosphate buffer while bubbling with oxygen.
3.3.3. Effects of pH and Temperature

Following this initial examination of 3-OHKyn autoxidation, the effect of temperature and pH on the rate of autoxidation and distribution of products was examined. The experiments in which pH was varied were conducted at 25°C in order to slow the overall rate of reaction to a point where clear assessment of the differences owing to pH could be made. This is more important for the more basic pHs where reaction rate is increased. As the pH of the autoxidation reaction was varied, as shown in Figure 3.6 and Figure 3.7 a) and b), the rate of 3-OHKyn oxidation was approximately 5 to 10-fold greater at pH 8 compared to pH 6 (over the initial 5 h of autoxidation) with a concomitant increase in the rate of formation of the subsequent autoxidation products. The maximum concentrations of the autoxidation products were lower by as much as 25% at pH 8 compared with pH 6 indicating that the products are more stable at the lower pH.

The rate of 3-OHKyn autoxidation at 35°C was approximately 3 times greater cf. 25°C (Figure 3.8 showing the autoxidation of 3-OHKyn at 35°C versus Figure 3.6). This was assessed during the first 5 hours. No new species were observed over the pH and temperature ranges used here.
Figure 3.7: Effect of pH on the autoxidation of 3-OHKyn at 25°C. a) pH 6 and b) pH 8.
When oxygen was excluded from the reaction mixture (at pH 7 and 35°C) by saturating the solutions with argon, 3-OHKyn autoxidation was suppressed by >99%. These results obtained here are consistent with the studies of the autoxidation of other amino phenols (e.g. 3-hydroxyanthranilic acid) in that pH and temperature have a marked effect on the rate formation of autoxidation products and, as expected, oxygen is essential for the reaction to proceed.

3.3.4. Stability of Xanthommatin, Hydroxyxanthommatin and DHQCA

The origins and eventual fates of the major autoxidation species (DHQCA, Xan and OHXan) were followed by examining the stability of each species over an extended time (up to 50 hours). Since Xan was the major product observed during the initial
stages of 3-OHKyn autoxidation, the possibility was examined that it may be an intermediate in the production of the other species. Figure 3.9 shows the results when Xan was incubated at pH 7 and 35°C in the presence of oxygen. DHQCA was the predominant species formed together with lower levels of 3-OHKyn as shown in Figure 3.9. These data show that Xan in solution at pH 7 is inherently unstable.

![Graph showing the formation of DHQCA, 3-OHKyn, and Xan](image)

**Figure 3.9:** Products formed from the incubation of Xan at 35°C and pH 7 in presence of oxygen.

Although 3-OHKyn was produced in the reaction mixture and it was exposed to oxygen the formation of 3-OHKyn autoxidation products, apart from Xan, was not observed. It is possible that in the absence of the large amounts of active oxygen species normally
generated by the autoxidation of 3-OHKyn, the small amounts of 3-OHKyn produced by the decomposition of Xan slowly reoxidise regenerating Xan. Over time, this process would have the effect of slowly consuming 3-OHKyn and converting it to DHQCA via the decomposition of Xan. This is supported by the fact that the production of 3-OHKyn (Figure 3.9) reaches equilibrium after ca. 20 hours of incubation, and DHQCA formed continually over this same time period.

Figure 3.10 shows the abundance of products formed when purified OHXan was incubated, at pH 7 and 35°C in the presence of oxygen. OHXan decomposes to produce the unidentified species F 20, F 4.3, DHQCA and trace amounts of 3-OHKyn (after 40 hours). These same species were also observed when OHXan was incubated in the absence of oxygen (data not shown).

The fact that both OHXan and Xan decomposed to DHQCA and 3-OHKyn provides further evidence that there is structural similarity between the two species. This is consistent with the ESI-MS results whereby similar fragmentation was observed for both species.

When DHQCA was incubated at pH 7 and 35°C either in the presence or absence of oxygen, over the same time period no decomposition was detected. However, if H₂O₂, which is formed in the reaction solution during autoxidation, was added to a solution of DHQCA, F 4.3 was produced exclusively.

3.3.5. Generation of Hydrogen Peroxide During 3-Hydroxykynurenine Autoxidation

It has been shown previously that autoxidation of 3-OHKyn generates H₂O₂ (see, for example, Eastman et al.¹⁵¹). H₂O₂ is generally thought to be produced via the
dismutation of superoxide ions, although a minor pathway involving the dimerisation of HO* may also be involved.\textsuperscript{154}

In order to evaluate the rate of H$_2$O$_2$ production and its role in the formation of the autoxidation products, the concentration of H$_2$O$_2$ in solution was measured using a HRP/OPD assay over the course of 3-OHKyn autoxidation. These data, shown in Figure 3.11, indicated that the autoxidation of 3-OHKyn is accompanied by the rapid formation of H$_2$O$_2$, producing almost a mole per mole equivalent of H$_2$O$_2$ throughout the autoxidation at pH 7 and 35°C. The initial rate of H$_2$O$_2$ production (0-1 h) mirrored the consumption of 3-OHKyn and followed the formation of Xan (Figure 3.11 compared to Figure 3.8).

![Figure 3.10: Incubation of OHXan at 35°C and pH 7 in the presence of oxygen.](image-url)

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Considering the temporal relationship between the formation of H$_2$O$_2$ and the autoxidation products derived from Xan, it was suspected that the production of H$_2$O$_2$, and possibly HO', might play a role in the formation of the Xan-derived autoxidation products. To address this possibility, we conducted the autoxidation experiments in the presence of catalase (to remove any H$_2$O$_2$ formed) or mannitol (HO' scavenger). The free radical trap, DMPO, was also used (see section 3.3.7).

![Graph](image)

**Figure 3.11:** H$_2$O$_2$ concentration during the autoxidation of 3-OHKyn at pH 7 and 35°C, in the presence of oxygen (average of duplicate experiments).
3.3.6. Effect of Metal Impurities on 3-Hydroxykynurenine Autoxidation

Superoxide, hydrogen peroxide and the hydroxyl radical produced from the autoxidation of biomolecules have been implicated in a range of toxicities. Since a spin restriction exists on the direct reaction of oxygen with biomolecules, it has been hypothesised that their autoxidation is initiated by transition metals (e.g. Fe and Cu bound by the biomolecules).\textsuperscript{155} Hence, the possible influence of transition metals on 3-OHKyn autoxidation was investigated by addition of the metal chelators desferrioxamine or EDTA (in separate experiments) to the phosphate buffers prior to the addition of 3-OHKyn. The chelators should remove residual metal ions, however, this modification had no measurable effect on the autoxidation of 3-OHKyn. In addition, the buffers used in one autoxidation experiment were treated with Chelex resin in order to minimise metal contamination and once again the rate of autoxidation was unaffected. Although various methods were used in an effort to remove transition metal impurities, traces of these metals may still have been present in sufficient quantities to catalyse the autoxidation reaction. Since phosphate buffers are known to contain high levels of transition metal impurities, phosphate buffer was replaced with triethylammonium acetate (TEAA), which contains lower levels of transition metal impurities. The same temperature and pH conditions were used in the autoxidation. The data from HPLC analysis of 3-OHKyn autoxidation in TEAA buffer are shown in Figure 3.12. The replacement of the buffer resulted in the formation of all of the same species at similar levels to those found when using phosphate buffer with the exception of F 4.3.
The formation of OHXan and Xan were lower only reaching maximum values of ca. 13% and 21% respectively over the 15 hours of the experiment. This compares to values of ca. 33% (OHXan) and 28% (Xan) when phosphate buffer was used under the same temperature and pH conditions (Figure 3.8). The amount of DHQCA formed appeared to be the same in both experiments reaching a value of approximately 23%. The level of 3-OHKyn remaining after 15 hours of oxidation was higher when TEAA buffer was used in comparison to phosphate buffer. The rate of initial oxidation of 3-OHKyn was similar until the 3-4 hour mark where the rate levelled off with the percentage of 3-OHKyn formed, falling gradually to a value of ca. 59% after about 15 hours. In contrast when phosphate buffers were used, the level of 3-OHKyn fell to 16% after 15 hours of oxidation. From these data it appears that the formation of OHXan and
oxidation of 3-OHKyn were only significantly affected later in the oxidation by the use of TEAA buffer. These data suggest that transition metal impurities do not play a major role in the autoxidation of 3-OHKyn.

3.3.7. Effect of Hydrogen Peroxide, Hydroxyl Radicals and Superoxide on 3-Hydroxykynurenine Autoxidation Products

Previous studies on the autoxidation of 3-hydroxyanthranilic acid, a structural analogue of 3-OHKyn, revealed that H$_2$O$_2$ plays an important role in the generation of specific autoxidation products. In particular, cinnabarinic acid, a phenoxazone dimer of 3-hydroxyanthranilinic acid and structural analogue of Xan, was degraded by H$_2$O$_2$. As noted above, we suspected that a similar reaction may also occur with Xan. This hypothesis was tested by performing the autoxidation of 3-OHKyn at pH 7 in the presence of catalase to remove H$_2$O$_2$ (produced during the course of autoxidation). Figure 3.13 shows the relative peak areas of the major products of autoxidation in the presence of catalase.

The addition of catalase to the reaction mixture led to a significant increase in the concentrations of Xan and DHQCA whereas OHXan and the compound denoted F 4.3 were formed at much lower levels (Figure 3.13 compared with Figure 3.8). The initial rate (0-1 h) of 3-OHKyn autoxidation was not affected by the presence of catalase, however, after this time, 3-OHKyn loss was accelerated and it was not detected after 7 hours (Figure 3.13).

It appeared, therefore, that H$_2$O$_2$ has a marked effect on the relative concentrations of 3-OHKyn autoxidation products. The inferred role of H$_2$O$_2$ in the decomposition of Xan was confirmed by addition of H$_2$O$_2$ to a solution of Xan which resulted in the formation of F 4.3 (as determined by HPLC). Similarly, addition of H$_2$O$_2$ to a solution of DHQCA
resulted in the formation of F 4.3, again suggesting a role for H2O2 in the formation of F 4.3. The levels of F 20 detected were not significantly altered in the presence of catalase.

Figure 3.13: Autoxidation of 3-OHKyn at pH 7 and 35°C in the presence of catalase. 3-OHKyn. Catalase (6300 units) was added at hourly intervals.

It is possible that the effect of catalase on the formation of the 3-OHKyn autoxidation products was not only a direct result of the removal of H2O2 but a consequence of the inhibition of HO· generation; the formation of which is known to occur in phosphate buffers via the Fenton reaction.155 When the HO· scavenger, mannitol, was included only the production of OHXan was affected (ca. 3% lower compared to the standard
autoxidation at pH 7 and 35°C). These results imply a minor role for HO· in the overall oxidation but a possible agent in the formation of OHXan under these conditions.

The initial oxyradical produced upon 3-OHKyn autoxidation is likely to be the superoxide anion (O$_2^-$). The effect of O$_2^-$ on the rate of 3-OHKyn autoxidation was assessed by conducting the standard autoxidation experiment at pH 7 and 35°C in the presence of the enzyme superoxide dismutase (SOD). SOD catalyses the dismutation of O$_2^-$ to H$_2$O$_2$. In the presence of SOD, 3-OHKyn autoxidation was not significantly affected (Figure 3.14), however, SOD did suppress the formation of DHQCA by ca. 40% and increased the concentration of F 4.3 by ca. 20% as determined at the 5 hour mark. This was possibly a secondary effect caused by the increased hydrogen peroxide concentration in solution (owing to dismutation superoxide) reacting with DHQCA to produce F 4.3. Superoxide therefore does not appear to play a part in the initial autoxidation of 3-OHKyn but may, via its conversion to H$_2$O$_2$, affect the product profile and redox reactions of subsequent autoxidation products.

3.3.8. 3-Hydroxykynurenine Autoxidation Assessed by EPR Spectroscopy

The radical species formed in solution during the initial stages of 3-OHKyn autoxidation, were examined using EPR spectroscopy as shown in Figure 3.15. Two major radical species were observed when the 3-OHKyn autoxidation reaction was allowed to proceed for 40 min at room temperature (Figure 3.15). The hyperfine structures, line shapes and g-values (2.0057 and 2.0038) are consistent with the presence of the DMPO-HO· adduct and Xan· respectively.$^{113,156,157}$
Figure 3.14: Autoxidation of 3-OHKyn at pH 7 and 35°C in a presence of SOD added at hourly intervals (1000 units per addition).

A novel HPLC-MS-EPR technique has been used to show that Xan' is the major 3-OHKyn autoxidation product in short time periods. The g-values and hyperfine splittings for the phenoxy radical detected in the present work appear to be identical to those determined for Xan' (at $g = 2.004$), indicating they are the same species. The two unlabelled signals present in Figure 3.15 a) arise from DMPO degradation-products and were therefore also detected in control incubations which contained no 3-OHKyn. The possibilities that either the formation of Xan' may be influenced by the H$_2$O$_2$ which is produced during the 3-OHKyn autoxidation reaction, or that HO' could react with Xan to generate Xan' were also considered. Hence, 3-OHKyn autoxidation was
examined by EPR in the presence of catalase and under nitrogen to eliminate H$_2$O$_2$. For these experiments, DMPO was added 1 hour after incubating the mixtures at 37°C and subsequently analysed (within 2 min) by EPR spectroscopy. In this case, two major radical signals were observed which were assigned as the DMPO-HO' adduct and Xan' (Figure 3.15 b)). This result was very similar to that shown in Figure 3.15 a), the major difference being the relatively lower level of DMPO-HO' detected. This may be a consequence of the long half life of the DMPO-HO' adduct and the additional time provided for this adduct to form under the conditions used in Figure 3.15 a) compared to b). In the presence of catalase, no HO' was produced while the levels of Xan' were higher by a factor of 2.4 (Figure 3.15 b) compared with c)). This result indicates that neither H$_2$O$_2$ or HO' are required for Xan' production and also that H$_2$O$_2$ may contribute to the degradation of Xan', perhaps by formation of other products (data shown previously indicates that H$_2$O$_2$ plays a role in Xan degradation). Under conditions where oxygen availability was limited by continuous bubbling with nitrogen, both the DMPO-HO' and Xan' signals were lower i.e. 35% and 37% respectively (Figure 3.15 d) compared to b)).

The data shown in Figure 3.16 indicates that Xan' was formed after 3-OHKyn was incubated at 37°C for 100 min. In order to assess whether H$_2$O$_2$ could directly contribute to Xan' degradation, H$_2$O$_2$ was added to the autoxidation reaction mixture prior to analysis by EPR spectroscopy. When H$_2$O$_2$ was added to the reaction mixture 10 min prior to analysis by EPR spectroscopy, the Xan' signal was absent and a broad singlet of lower intensity was observed (Figure 3.16). This implies that H$_2$O$_2$ plays a direct role in the degradation of Xan'.
Figure 3.15: EPR spectrum of 3-OHKyn autoxidation products. 3-OHKyn was incubated in phosphate buffer (pH 7.4) for 40 min at 22°C in the presence of 0.1 M DMPO (a)). Two prominent radical species were identified as DMP-OH (•) and Xan+ (x) (g-values of 2.0057 and 2.0038 respectively). The remaining spectra (b)-d)) were collected after incubation of 3-OHKyn for 60 min at 37°C with DMPO added approximately 2 minutes before scanning c) also contained catalase and d) was degassed with nitrogen.
3.3.9. Autoxidation of 3-OHKyn in the Presence of Cellular Antioxidants.

Whilst 3-OHKyn is present in the human lens, oxidation should be limited by lenticular antioxidants including GSH and ascorbic acid. To explore the effects of antioxidants on 3-OHKyn oxidation, the minimum levels of GSH and ascorbic acid required to inhibit the autoxidation of 3-OHKyn were determined by measuring long wavelength absorbance at 490 nm, since the 3-OHKyn oxidation products are coloured. This increase in absorption was associated with a concomitant decrease in the concentration of 3-OHKyn in solution as determined by HPLC. These data are shown in Figure 3.17. The oxidation of 3-OHKyn was effectively inhibited by the inclusion of GSH or ascorbic acid and only when their concentrations fell below the 1 milli-molar level did net oxidation of 3-OHKyn occur. Using the data shown in Figure 3.17, the minimum concentrations of GSH and ascorbic acid required to inhibit the oxidation of 3-OHKyn were calculated to be 0.3 mM and 0.1 mM respectively. When the concentrations of the
antioxidants fell below these values, the oxidation of 3-OHKyn proceeded at a rapid rate.

### 3.3.10. Autoxidation of 3-OHKyn in the Presence of Erythrocytes

The impact of 3-OHKyn autoxidation products within cells will depend largely on the physiological environment, in particular the presence of antioxidants and antioxidant enzymes. 3-OHKyn and other tryptophan metabolites have been found in the blood of patients suffering from hepatic encephalopathy and rheumatoid arthritis.\textsuperscript{145}

To determine the course of 3-OHKyn autoxidation in this extra-lenticular environment, autoxidation of 3-OHKyn was examined in the presence of isolated human erythrocytes in phosphate buffered saline (pH 7.4) at 35°C and bubbling with oxygen. The results of HPLC analyses of the reaction products are shown in Figure 3.18. The total oxidation of 3-OHKyn was complete within 5 hours. The two products resulting from the oxidation were determined to be xanthommatin and DHQCA on the basis of retention times and analysis by ESI-MS. HPLC analysis of the reaction mixture after 26 hours revealed that DHQCA was the major species remaining in the reaction mixture.
Figure 3.17: Autoxidation of 3-OHKyn in the presence of the cellular antioxidants a) ascorbic acid and b) glutathione. The oxidation of 3-OHKyn was monitored at 490 nm.
Figure 3.18: Autoxidation of 3-OH Kyn in the presence of human erythrocytes in phosphate buffered saline (pH 7.4) at 35°C.

Overall, the oxidation of 3-OHKyn in the presence of human erythrocytes closely resembled the oxidation of 3-OHKyn in the presence of catalase. A notable difference, however, was the delay of approximately 30 minutes in the onset of oxidation possibly due to the presence of cellular antioxidants.

3.3.11. Autoxidation of 3-OHKyn in the Presence of Fmoc-L-Lysine

Initial investigations of the modification of amino acids by autoxidation products of 3-OH Kyn focussed on N-(9-fluorenylmethoxycarbonyl) (Fmoc) lysine. This derivative was chosen since it had the advantage of increased retention time for the adducts
(compared with unmodified lysine) and the α amino group was protected from reaction. Autoxidation of 3-OHKyn was carried out in the presence of a 5 molar excess of Fmoc-Lys in phosphate buffered (pH 7) 20% DMF/H₂O. This buffer was determined by solubility experiments to be the optimum solvent for Fmoc-Lys. A period of 48 hours was chosen for the incubation based on prior data and it was found that the majority of 3-OHKyn had been consumed after this time.

After incubation the reaction mixture was a bright red-brown colour presumably because a large concentration of DHQCA was formed. This was confirmed by HPLC spiking experiments with the synthetic standard. Figure 3.19 shows the results of HPLC analysis of the reaction mixture. Eight adducts of Fmoc-Lys and 3-OHKyn autoxidation products with retention times between 20-40 minutes were observed. Each of the adduct peaks was isolated by HPLC and analysed by ESI-MS. The m/z for the protonated molecular ion ([M+H⁺]) of each identified species are given in Table 3.2.

Fractions F 32 (m/z 385) and F 35 (m/z 397) were determined, by blank autoxidation experiments on Fmoc-Lys, to be derived from the autoxidation of Fmoc-Lys (F 40, m/z 369) alone. MS/MS data (not shown) on each of these species suggests that F 32 arises from hydroxylation of Fmoc-Lys, while F 35 appeared to result from methylation of Fmoc-Lys. Since 3-OHKyn has an m/z value of 225 and Fmoc-Lys 369, only those species with m/z > 500 were investigated as possible adducts of 3-OHKyn and Fmoc-Lys.
Figure 3.19: HPLC separation of 3-OHKyn in the presence of a 10-fold molar excess of Fmoc-Lys after 48 hours of autoxidation.

Table 3.2: Positive ESI ions and retention times for the major peaks isolated from the autoxidation of 3-OHKyn in the presence of Fmoc-Lys.

<table>
<thead>
<tr>
<th>Fmoc adduct retention time (min)</th>
<th>Code</th>
<th>m/z of the protonated molecular ion/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>20.8</td>
<td>F 21</td>
<td>-</td>
</tr>
<tr>
<td>22.3</td>
<td>F 22</td>
<td>-</td>
</tr>
<tr>
<td>24.4</td>
<td>F 24</td>
<td>578/ 606</td>
</tr>
<tr>
<td>27.4</td>
<td>F 27</td>
<td>534/ 562</td>
</tr>
<tr>
<td>31.3</td>
<td>F 31</td>
<td>516</td>
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<td>32</td>
<td>F 32</td>
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<tr>
<td>38</td>
<td>F 38</td>
<td>542</td>
</tr>
<tr>
<td>40</td>
<td>F 40</td>
<td>369</td>
</tr>
</tbody>
</table>
These adducts were subjected to further structural analysis by MS/MS experiments. Only tentative structures (based around possible derivatives of 3-OHKyn) could be assigned to each of the species as a result of the MS/MS work. The majority of fragmentation appeared to involve the Fmoc-Lys moiety, rather than the 3-OHKyn moiety, thus prohibiting definite structural characterisation of the adduct. This fragmentation behaviour was evident in the tandem mass spectrum of Fmoc-Lys shown in Figure 3.20. Attempts were made to further elucidate the structures of these species by producing greater quantities for NMR analysis, however, their unstable nature coupled with their low yields prohibited this, consequently further structural analysis was abandoned.

To avoid changes in species after collection and concentration of peaks, LC/MS analysis (using formic acid buffered eluents) of the autoxidation mixture was undertaken to confirm the in vitro distribution of adducts. The LC separation of the autoxidation mixture, with detection at 254 nm is shown in Figure 3.21. The m/z for the protonated molecular ion ([M+H⁺]) of each identified species are given in Table 3.3. All of the species observed by manual collection of peaks from the HPLC separation followed by preconcentration and ESI analysis were also observed using LC/MS, however, a number of additional species were also observed. This may indicate either that those species not previously observed are less stable or that they elute closely with more concentrated species precluding ESI/MS analysis without further purification. The m/z values of the new species were 576, 680 and a number of species with m/z < 500. It appeared from the LC/MS that the species with m/z 576 represented an adduct formed through the addition of 3-OHKyn to Fmoc-Lys following 3-OHKyn deamination (with no apparent further oxidation). The LC/MS/MS and a possible structure is shown in Figure 3.22. This species demonstrated little fragmentation, however, a peak at m/z 369
attributable to the Fmoc-Lys group and m/z 425 showing the adduct bound to deaminated 3-OHKyn through the amine group of Fmoc-Lys. Reanalysis of the data obtained from the manual collection of HPLC peaks showed that this peak (m/z 576) was also present, however, it was a minor peak relative to the other adduct peaks and thus was not detected initially.
Figure 3.20: Positive ESI tandem mass spectrum of the Fmoc-Lys.
Figure 3.21: Direct LC/MS analysis of the autoxidation reaction mixture of 3-OHKyn in the presence of Fmoc-Lys.

Table 3.3: Positive ESI ions and retention times for the major peaks observed in the LC/MS of the autoxidation of 3-OHKyn in the presence of Fmoc-Lys.

<table>
<thead>
<tr>
<th>LC/MS retention time (min)</th>
<th>m/z of the protonated molecular ion</th>
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<tbody>
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<tr>
<td>36.66</td>
<td>562</td>
</tr>
<tr>
<td>36.97 (shoulder)</td>
<td>458</td>
</tr>
<tr>
<td>37.78</td>
<td>534</td>
</tr>
<tr>
<td>38.97</td>
<td>516</td>
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<td>385</td>
</tr>
<tr>
<td>41.00</td>
<td>454</td>
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</tr>
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</tr>
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</tr>
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</tr>
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<td>49.64</td>
<td>431</td>
</tr>
</tbody>
</table>
3.4. Discussion

The toxicity of 3-OHKyn has been largely attributed to its autoxidation with the ensuing generation of hydrogen peroxide and, in the presence of metals, the hydroxyl radical (HO•). The mechanism of 3-OHKyn autoxidation is not thought to involve direct reaction of 3-OHKyn with dioxygen since such a reaction is predicted to be kinetically unfavourable owing to spin restrictions. It is known, however, that in the presence of transition metals (e.g. Fe and Cu) the spin restriction can be circumvented thus permitting the apparent "autoxidation" of a variety of biomolecules.

The addition of chelators such as desferrioxamine and EDTA did not prevent 3-OHKyn autoxidation in the phosphate buffers used in the time-course studies presented here, however, under certain conditions chelated metals may still remain redox active, taking part in the autoxidation process. In addition, the buffers used for the EPR experiments were treated with Chelex resin in order to minimise metal contamination, yet under these conditions Xan' and HO• were still detected (Figure 3.15) These data may suggest that metals are not involved in 3-OHKyn autoxidation, however, it is possible that traces of metal impurities persist in the reaction mixtures and contribute to the observed autoxidation. Replacement of phosphate buffer with triethylammonium acetate buffer, which contains lower levels of transition metal impurities did, however, result in significant overall suppression of the formation of OHXan and oxidation of 3-OHKyn (Figure 3.12) although the initial rates of oxidation were unchanged.

The generation of H₂O₂ in the autoxidation of 3-OHKyn is most likely due to the initial reduction of oxygen by the phenoxide anion of 3-OHKyn to generate O₂•⁻ and its subsequent dismutation to yield (theoretically) 1 mole of H₂O₂ per mole of 3-OHKyn as shown in Scheme 3.1. The pKₐ of the phenoxy group of 3-OHKyn is reported to be
It therefore appears that the small fraction of 3-OHKyn that is in the phenoxide state at pH 7 is sufficient to allow autoxidation to proceed. It is also possible that other one-electron transfers contribute to oxygen reduction during the autoxidation of 3-OHKyn-derived products and that a proportion of the H₂O₂ (formed subsequently) reacts with at least some of the autoxidation intermediates (e.g. Xan). This suggests that the ca. 1:1 stoichiometry of 3-OHKyn loss and H₂O₂ production reflects the net effect of these reactions. Investigation of the physiologically relevant mechanisms of 3-OHKyn autoxidation clearly warrants further study.

Scheme 3.1: Mechanism of H₂O₂ generation via 3-OHKyn autoxidation.

Evidence that H₂O₂ is involved in the genesis of cataract has arisen from observations that both cysteine and methionine are highly oxidised in advanced cataractous lenses, while little or no oxidation is apparent in other amino acid residues. It has also been shown that these changes could be replicated by incubating lens proteins in H₂O₂. Furthermore, recent data, using sensitive HPLC assays to measure protein modification by OH⁻ (produced from H₂O₂ in the presence of redox-active metals via the Fenton
reaction) has provided further evidence that H$_2$O$_2$ is involved in nuclear cataract.$^{163,164}$ That study of cataractous lenses also showed OH$^-$ damage even at the earliest stages and became increasingly evident with the severity of cataract.$^{163}$ The possibility that UV exposure could lead to the production of similar hydroxylated amino acids was also investigated by Fu et al. At the wavelengths of UV-light that are transmitted by the cornea, no hydroxylated aliphatic amino acid residues were found.$^{163}$

Since epithelial cells play a significant role in protecting the lens against external sources of damaging agents, including hydrogen peroxide, it is most likely that the source of H$_2$O$_2$, relevant to nuclear cataract, lies within the lens. One possible internal source of H$_2$O$_2$ may be the small molecules that can readily oxidise in the presence of oxygen. The two molecules that need to be considered, in the primate lens, are 3-OHKyn and ascorbate. The production of H$_2$O$_2$ is dependent on both the availability of O$_2$ and the presence of antioxidants such as GSH.$^{61}$ In the cortex, the O$_2$ tension has been found to be 30 mm Hg. The oxygen tension in the nucleus has been postulated to be lower but the hardness of the tissue makes actual measurement quite difficult.$^{61}$ Recent data on chicken lenses, however, have shown that significant levels of O$_2$ are present in the nucleus.$^{165}$ Consequently the only limit to the oxidation of the 3-OHKyn and ascorbate is the availability of antioxidants. Their availability may be determined by their localised concentrations and their rates of regeneration from the oxidised forms.

Regeneration of oxidised GSH (GSSG) occurs primarily within the epithelium and outer cortex so GSSG has to diffuse to the lens cortex to undergo this process. Consequently, the mobility of antioxidants, rather than the rate of regeneration, may be the crucial factor in limiting oxidation in the lens interior. A common feature of age-related nuclear cataract is the low concentration of reduced glutathione in the centre of the lens. In fact, even in the most advanced nuclear cataract lenses, the cortex often contained normal
levels of GSH while levels in the nucleus were low or undetectable.\textsuperscript{74,75} This led to the recent investigation of the movement of labelled GSH in the lens.\textsuperscript{75} It was determined that in lenses from humans over the age of 40, a distinct zonal pattern of label distribution, was noted, whereby very little labelled GSH had penetrated to the centre of the lens. This limit to diffusion was also observed with small molecules such as D\textsubscript{2}O suggesting that diffusion of molecules such as ascorbate may also be impaired. The internal zone was found to have dimensions that coincided with those of the coloured region present in cataractous lenses. In another study, the concentration of GSH in the lens was found to decrease with age with the central region demonstrating a loss of GSH.\textsuperscript{166} In older, non-cataractous lenses the levels of GSH in the nucleus were found to fall below 1 mM.

From the work described here involving oxidation of 3-OHKyn at neutral pH (Figure 3.11) \textit{in vitro} it appears that H\textsubscript{2}O\textsubscript{2} is produced on an equimolar basis with the oxidation of 3-OHKyn. Oxidation studies on 3-OHKyn in the presence of the lenticular antioxidants GSH and ascorbate (Figure 3.17) showed that net oxidation of 3-OHKyn was observed only when the concentration of the antioxidant fell below 1 mM. This suggests that if the level of GSH and ascorbate in older human lens fall much below 1 mM, oxidation of 3-OHKyn would occur yielding H\textsubscript{2}O\textsubscript{2}, in turn giving rise to the characteristic protein oxidation typical of cataract.

An important objective of the present study was to investigate the course of 3-OHKyn autoxidation and gain an understanding of the structures of the products, some of which could potentially form adducts with proteins.\textsuperscript{42,149} It is unclear to what extent the cytotoxicity of 3-OHKyn can be solely ascribed to the formation of H\textsubscript{2}O\textsubscript{2} (and HO\textsuperscript{-}) compared with the deleterious reactions of 3-OHKyn oxidation products with cellular proteins (and possibly other macromolecules). Elucidation of reactive 3-OHKyn
autoxidation products is required in order to generate characteristic markers for modified proteins which may be useful for probing tissue samples, for example, in certain neurological disorders mentioned above, and in cataract.

The oxidation of o-aminophenols by air, cytochrome c, or ferricyanide has long been known to result in dimerisation of the initial substrate producing phenoxazones which absorb light in the visible regions. In the present study, when exposed to either oxygen or air, 3-OHKyn was found to react in the same manner to yield Xan (and its radical, Xan'). In previous studies, 3-OHKyn oxidation was induced by the addition of catalysts such as potassium ferricyanide and methaemoglobin/H₂O₂. Under these conditions, Xan was reported to be the sole oxidation product of 3-OHKyn. A recent article described in-line oxidation of 3-OHKyn coupled to HPLC, and detection with EPR spectroscopy or ESI-MS. This sophisticated experimental procedure allowed immediate identification of the species produced. These data also indicated that Xan was the principal oxidation product of 3-OHKyn. This is in agreement with the work outlined here which shows that, during the initial period of autoxidation, Xan and Xan' are the predominant products formed and this is accompanied by H₂O₂ production. From our investigations, however, Xan is only the first of at least five oxidation products. Further, prolonged autoxidation of 3-OHKyn leads to the eventual formation of just two species, DHQCA and F 4.3. DHQCA has not been reported previously as an autoxidation product of 3-OHKyn, although it has been described as a decomposition product of Xan. Butenandt et al. showed that incubation of Xan at pH 8 produced 3-OHKyn and DHQCA, though the quantities produced were not stated. The identification of DHQCA as an autoxidation product of 3-OHKyn is of interest since our laboratory has recently found that DHQCA reacts readily with lens proteins (Aquilina et al. unpublished results). This may be of particular relevance to lenticular
protein modification in senile nuclear cataract since the lens nucleus is metabolically inactive and proteins are therefore not turned over. In effect, the nuclear lens crystallins are as old as the individual and are thus more likely to show modification by 3-OHKyn autoxidation products.

Through the use of decomposition and oxidation studies of 3-OHKyn autoxidation intermediates, in this study, it was possible to elucidate possible product-precursor relationships (Scheme 3.2). From these data, it is clear that the autoxidation of 3-OHKyn generates Xan, Xan' and OHXan. We could find no direct evidence that Xan yields OHXan (e.g. by incubation with oxygen or H₂O₂). Addition of catalase, however, dramatically suppressed OHXan formation. A possible role for H₂O₂ or hydroxyl radicals in OHXan formation can therefore not be excluded, although the use of mannitol, a hydroxyl radical scavenger, did not significantly influence the course of the reaction. The use of TEAA buffer in place of phosphate buffer, which is known to contain lower levels of metal impurities, did significantly suppress the formation of OHXan. This provides indirect evidence that hydroxyl radicals generated from H₂O₂ in the presence of transition metals (Fenton reaction) may be important in the generation of OHXan.

The significance of the autoxidation of 3-OHKyn and the concomitant production of H₂O₂ under physiological conditions depends on the local environment, in particular the presence of antioxidant-enzymes such as SOD, GSH peroxidase and catalase. 3-OHKyn has been found in the blood and plasma of patients suffering from diseases such as hepatic encephalopathy. ³⁴ o-aminophenol, an analogue of 3-OHKyn, is known to cause toxic methemoglobinemia by oxidising intracellular haemoglobin in human erythrocytes. ¹⁷⁰ Tomada et al, incubated o-aminophenol in human erythrocytes and found 2-aminophenoxazine-3-one (the homologue of Xan), was formed by oxidative
dimerisation of the o-aminophenol.\textsuperscript{171} In order to approach extra-lenticular physiological conditions, the 3-OHKyn autoxidation reactions were conducted in the presence of isolated human erythrocytes under physiological conditions. The oxidation of 3-OHKyn in the presence of human erythrocytes has been carried out previously by Yamaguchi.\textsuperscript{172} Under similar conditions, Yamaguchi also observed the production of Xan, however, no reference was made to the production of DHQCA, as was observed here nor was the influence of cellular anti-oxidants considered.

Scheme 3.2: Major 3-OHKyn autoxidation products as determined from the experiments outlined here.
The similarity between the oxidation of 3-OHKyn in the presence of human erythrocytes and that of 3-OHKyn in the presence of catalase suggests that cellular antioxidant-enzymes, normally present within the erythrocytes, were released into the reaction mixture eliminating the effect of hydrogen peroxide. This was supported by the fact that obvious signs of erythrocyte lysis were evident after several hours of incubation. One notable difference between the autoxidation of 3-OHKyn in the presence of erythrocytes and that in the presence of catalase was the delay in the onset of 3-OHKyn oxidation (approximately 30 minutes) when erythrocytes were present. This delay can be attributed to the protection afforded by cellular antioxidants such as GSH or vitamin E leaching into the solution. This is consistent with the experiments in which the autoxidation of 3-OHKyn was examined in the presence of ascorbate or GSH, where it was observed that oxidation did not commence until the antioxidant levels fell well below 1 mM.

The reactions studied here in vitro might also be relevant to specific pathological situations where tryptophan metabolism via the kynurenine pathway is associated with oxyradical production. For example, human monocytes and macrophages, when primed with γ-interferon secrete small amounts of 3-OHKyn and have a high capacity to produce both \( \text{O}_2^* \) and \( \text{H}_2\text{O}_2 \). In addition, the low pH of inflammatory sites should suppress the autoxidation of 3-OHKyn. In the case of the lens nucleus (an avascular tissue), such inflammation is not possible, hence oxidation of 3-OHKyn may still occur.

As a model of the possible modifications that could occur to human lens proteins by the autoxidation of 3-OHKyn, it was autoxidised in the presence of Fmoc-derivatised lysine. Fmoc-Lys was chosen as the substrate because: derivatives were easily
detectable at 254 nm by analytical HPLC; the Fmoc group gave a increased retention
time allowing better purification; the ω-amino was blocked directing reaction to the
lysine side chain better simulating the situation in a protein and derivatives could be
easily deblocked under mild conditions to yield the adduct modified lysine adduct.

A large number of modifications to Fmoc-Lys were apparent from the autoxidation of
3-OHKyn in the presence of excess Fmoc-Lys at pH 7. Identification focussed on those
found to have an m/z value greater than 500 as it was thought that these would be the
most readily identifiable adducts. Through ESI/MS analysis of the individual peaks
following collection or direct LC/MS analysis of the reaction mixture it was found that
8 peaks fell into this category. All of the species formed were present in low quantities
and were also of limited stability thus precluding structural analysis by NMR. MS/MS
experiments were performed on each of these species, however, no structurally
diagnostic fragmentation of the adducts was evident so only tentative structures could
be suggested based only on adducts' masses.

One adduct to which a structure could be assigned with some degree of certainty was,
however, apparent in the LC/MS analysis of the reaction mixture. This adduct appeared
to be formed as a result of the binding of deaminated Kyn to Fmoc-Lys. It is interesting
that the mechanism of formation of this adduct does not require an oxidative
environment. All that is required is a sufficiently high pH to allow deamination of the
amino acid side chain of 3-OHKyn. The 3-OHKyn-Fmoc-Lys linkage of was confirmed
by LC/MS/MS (Figure 3.22). The binding of deaminated 3-OHKyn may be of relevance
to the colouration of human lens proteins with age as this modification of proteins could
occur in non-oxidative conditions and could also occur with other related kynurenines
containing the free amino acid side chain. It is interesting that despite such an oxidative
environment this species was observed. It must be noted, however, that the low concentration of this adduct in the mixture may be the result of oxidation. Greater quantities may result in an environment which is not as saturated with oxygen. The possibility that adducts may form between deaminated kynurenines and amino acids in less-oxidative environments, such as those present in the human lens, was investigated in the next section of work described in chapter 4.
4. Discovery of a Novel Protein Modification in Human Lens Protein.

4.1. Introduction

Lens proteins, once produced, show little or no turnover,\textsuperscript{176} thus, any post-translational modifications accumulate with time and may eventually contribute to age-related lenticular colouration and/or cataract.\textsuperscript{177} Recent work in our laboratory has led to the discovery of a novel fluorescent UV-filter, glutathionyl-3-hydroxykynurenine \(O-\beta-D\)-glucoside (GSH-3-OHKG), which was found to increase in concentration in the lens with age.\textsuperscript{32} The formation of GSH-3-OHKG was proposed to proceed initially via a Michael addition mechanism involving deamination of the 3-OHKG amino acid side chain to yield an \(\alpha,\beta\)-unsaturated ketone that is highly susceptible to nucleophilic attack and was found to react readily with the cysteine residue of glutathione (GSH).\textsuperscript{32} The proposed mechanism requires no oxygen or UV-light to initiate the reaction process, only suitable pH conditions which allow for the deamination of the UV-filter are required.

This chapter focuses on the covalent modification of lens crystallins in the absence of oxygen by UV-filters, in particular, kynurenine (Kyn) and 3-hydroxykynurenine (3-OHKyn). The formation of GSH-3-OHKG\textsuperscript{32} and our work in chapter 3 demonstrating the direct modification of Fmoc-Lys by 3-OHKyn led us to investigate the role of Kyn and 3-OHKyn in the modification of human lens proteins. Since Kyn and 3-OHKyn have the same amino acid side chain as 3-OHKG, they should also be able to undergo deamination of the side chain and subsequently react with nucleophilic amino acids. In a model system, reaction of Kyn with calf lens protein (CLP) under non-oxidative conditions generated coloured, fluorescent protein.\textsuperscript{178} Analysis of tryptic peptides from Kyn-modified calf lens protein (CLP) revealed that all modifications
were located at His, Cys or Lys residues. As such, our investigation of the reaction of Kyn and 3-OHKyn with amino acids focused on these three amino acids.

The applicability of enzymic digestion of naturally-occurring protein followed by HPLC analysis in order to observe UV-filter modifications to human lens protein is limited by two factors. First, UV-filter modifications to crystallins may be distributed over many possible reactive sites in proteins, effectively diluting the concentration of each modified peptide; this is further complicated by the co-elution of background peptides derived from many parent proteins interfering with purification and subsequent ESI-MS analysis. Second, modified proteins are often not amenable to enzymic digestion owing to the crosslinked and insoluble nature of aged proteins. Consequently, acid hydrolysis may be more suited to the analysis of modified lens protein. Acid hydrolysis reduces protein to the amino acids, thus limiting the number of modified amino acids to three and enables digestion of proteins that are not otherwise amenable to enzymic digestion. It was proposed that acid hydrolysis would be followed by HPLC analysis for intact Kyn or 3-OHKyn modifications, however, stability of the modified amino acids is important for the success of this procedure. Hence, the applicability of the acid hydrolysis procedure for the analysis of in vivo modification of model proteins by Kyn or 3-OHKyn was investigated.

4.2. Materials and Methods

4.2.1. Materials

All organic solvents and acids were HPLC grade (Ajax, Auburn, NSW, Australia). Milli-Q® water (purified to 18.2 MΩ/cm²) was used in the preparation of all solutions. Human lenses were obtained from local sources (Figtree Private Hospital, NSW) or
from the National Disease Research Interchange, U.S.A. Fresh calf lenses (< 2 year old) were obtained from Parish Meats, Yallah, NSW, Australia. The amino acids (N-α-t-Boc-L-histidine, N-α-t-Boc-L-lysine and cysteine) and Kyn sulphate salt were obtained from Sigma (St. Louis, MO, U.S.A.). HCl (6 M) sequencing grade was purchased from Pierce (Rockford, Illinois, U.S.A.).

4.2.2. Synthesis and Purification of Kyn and 3-OHKyn-modified Amino Acids

3-OHKyn or the sulphate salt of Kyn (50 mg) were dissolved in 50 mM Na₂CO₃/NaHCO₃ buffer, pH 9.5 (30 mL). The amino acids (N-α-t-Boc-L-histidine, N-α-t-Boc-L-lysine or cysteine) were added to the buffer in a 10-fold molar excess. The pH was readjusted to 9.5 with 0.1 M NaOH if required, and then the resulting solution was bubbled with argon, sealed, wrapped in foil and incubated at 37°C for 48 hours. After adjusting the pH to between 4-5 with glacial acetic acid, the resulting mixture was separated by semi-preparative or analytical HPLC using the methods detailed below. The amino acid adducts were deprotected by incubation of the adducts at 37°C in 6 M HCl overnight. Following lyophilisation, the adducts were purified using semi-preparative or analytical HPLC.

4.2.3. Acid Hydrolysis of Kyn-modified Amino Acids and Lens Protein.

Kyn-modified lens protein (approx. 10 mg) or Kyn-modified amino acids (N-α-t-Boc-L-histidine, N-α-t-Boc-L-lysine or L-cysteine) (2 mg) were hydrolysed with 6 M HCl (1 mL) for 24 hours at 110°C in an evacuated hydrolysis tube. After hydrolysis, the sample was lyophilised overnight and then dissolved in 100 μL of H₂O. The pH of the solution was adjusted to between 5 and 6 with approximately 100 μL of 1 M Na₂HPO₄. The resultant solution was made up to 500 μL with 0.1 M NaH₂PO₄. The solution was
then centrifuged at 14 000 rpm for 2 minutes and the supernatant was analysed by reversed phase HPLC.

4.2.4. Incubation of an Octapeptide with Kyn

The octapeptide Val-His-Leu-Thr-Pro-Val-Glu-Lys (2.3 mg) was dissolved in 50 mM Na$_2$CO$_3$/NaHCO$_3$ buffer, pH 9.5 (10 mL). The sulphate salt of Kyn (1.3 mg) was added and the pH of the resulting solution was readjusted to 9.5 with 0.1 M NaOH as required. The tube was wrapped in foil, bubbled with argon, sealed and incubated at 37°C for 2 days. After incubation, the sample pH was adjusted to 6 with glacial acetic acid and analysed by semi-preparative HPLC with detection at 254 nm. All the peaks detected by HPLC were subsequently collected and analysed individually by ESI-MS. Following analysis of the Kyn-modified peptide peaks, they were pooled and the resulting sample was subjected to the normal acid hydrolysis procedure. Following acid hydrolysis, the sample was lyophilised and neutralised as described in the acid hydrolysis procedure and subsequently analysed by LC/ESI-MS.

4.2.5. Incubation of Calf Lens Protein with Kyn or 3-OHKyn

Calf lens protein (CLP) (50 mg) was dissolved in 10 mL of 50 mM Na$_2$CO$_3$/NaHCO$_3$ buffer (pH 9.5). 3-OHKyn or the sulphate salt of Kyn (10 mg) was added and the pH of the resulting solution was readjusted to 9.5 with 0.1 M NaOH if required. The tube was wrapped in foil, bubbled with argon, sealed and incubated at 37°C for 4 days. Purification of the protein (tanned and untanned) from the reactants was achieved with the use of a Sephadex G25 PD-10 column (Amersham, Pharmacia, Biotech) that had been previously conditioned with Milli Q® water. The collected protein fraction was lyophilised and further extracted with ethanol to ensure that all unreacted Kyn was removed.
The rate of reaction of Kyn with CLP was monitored by measuring the change in UV-absorbance of protein with respect to time. Aliquots of the reaction mixture were taken at 0, 22, 66 and 116 hours. The protein mixture was separated from the reaction mixture using a Sephadex G25 PD-10 column using the procedure detailed above. The purified protein was dissolved in 5.00 mL of H$_2$O (volumetric flask) and the absorbance at 360 nm was measured for each aliquot.

### 4.2.6. HPLC

Reversed-phase HPLC (RP-HPLC) was performed on a Beckman System Gold® HPLC system equipped with a 127S solvent module and a model 166 UV-Vis detector. For analytical scale separations, a Varian (Microsorb-MV™ C18, 100 Å, 5μm, 4.6 mm x 250 mm) column was used with the following mobile phase conditions: solvent A (aqueous 4 mM ammonium acetate, pH 6.5) for 5 min followed by a linear gradient of 0-50% solvent B (80% acetonitrile/H$_2$O, 4 mM ammonium acetate) over 20 min, followed by a linear gradient of 50-100% solvent B over 15 min and re-equilibration in the aqueous phase for 15 min. The flow rate throughout the separation was 1 mL/min. Semi-preparative separations were performed using the same conditions as those for the analytical separations except that a semi-preparative column (Hypersil® BDS C18, 5 μm, 10 mm x 250 mm) was used with a flow rate of 3 mL/min.

### 4.2.7. Mass Spectrometry

Electrospray ionisation mass spectra were acquired on a VG Quattro triple quadrupole mass spectrometer (VG Biotech Ltd., now Micromass, Altrincham, U.K.). Samples were dissolved in 50% aqueous acetonitrile, containing 1% formic acid, and introduced into the mass spectrometer by a Harvard Apparatus 22 syringe pump (South Natick MA) at a rate of 10 μL/min. Nitrogen was used as both the bath and nebulizing gas,
flowing at 350 L/h and 10 L/h respectively. The capillary voltage was 3.2 kV and the cone voltage ranged from 20 V to 60 V. The source temperature was set to 85°C. Calibration of the mass spectrometer was achieved using NaI. Spectra were acquired in positive ion mode at unit mass resolution using multichannel analysis. Typically 10-20 scans (rate 100 m/z per second) were summed to obtain representative spectra.

For tandem mass spectrometry, the conditions used were the same as those described above except that the resolution of the first quadrupole was set to a minimum to increase transmission of the selected precursor ion. The collision gas used was argon at a pressure of 3.5x10⁻⁴ mbar and the laboratory collision energy was varied between 25 and 45 eV to achieve the desired level of fragmentation.

LC/ESI-MS was carried out using an Applied Biosystems 140B solvent delivery system and 785A UV detector set at 360 nm with the VG Quattro mass spectrometer. Kynurenine adducts were separated using an Alltech Alltima C18 column (250 mm x 2.1 mm, 5μm, 300 Å) at a flow rate of 200 μL/min, using a 0-80% acetonitrile/H₂O (each containing 1% formic acid) gradient over 40 min, a column oven temperature of 25°C, and the source temperature maintained at 170°C. All spectra were acquired in continuum mode with representative spectra obtained by summing 10-50 scans.

4.2.8. NMR Spectroscopy

N-α-t-Boc-L-histidine, N-α-t-Boc-L-lysine) ¹H NMR spectra were recorded on a Varian Unity 400 spectrometer (¹H, 400 MHz; ¹³C, 100 MHz). Two-dimensional (2D) spectra were acquired on a Bruker Avance 400 spectrometer (¹H, 400 MHz; ¹³C, 100 MHz). For each compound, the 2D experiments performed were: ¹H-¹H correlation spectroscopy (COSY); ¹H-¹H rotating frame nuclear overhauser effect spectroscopy
(ROESY) and heteronuclear multiple bond correlation spectroscopy (HMBC). All experiments were run in D$_2$O and referenced to residual HDO. Chemical shift values ($\delta$) are given in ppm.

### 4.2.9. Fluorescence and UV-Visible Spectroscopy

Fluorescence spectra were obtained on a Hitachi F-4500 fluorescence spectrometer (Tokyo, Japan) in three dimensional scan mode. Slit widths were routinely 5 nm for excitation and 5 nm for emission and the scan speed was 12 000 nm/min. UV-visible absorbance spectra were obtained using a Shimadzu UV-265 spectrophotometer (Kyoto, Japan). The slit width used was 1.0 nm and the sampling interval was 0.5 nm.

### 4.2.10. Isolation of Human Lens Proteins

Individual lenses were placed over dry ice on a glass sheet until frozen. The nucleus was cored (using a 6 mm cork borer) and separated from the cortex. The nucleus was then homogenized in absolute ethanol. After cooling for an hour at -20°C the homogenate was centrifuged for 20 min at 14 000 rpm. The supernatant liquid was removed and the pellet was re-extracted in 80% ethanol and again centrifuged. The supernatant was discarded and the pellet was dried under vacuum.

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### 4.3. Results

#### 4.3.1. Kynurenine Binding to Amino Acids

In previous work by Garner et al., Kyn has been found to covalently modify His, Lys and Cys residues in tryptic peptides of calf lens protein. Initial investigation of Kyn and 3-OHKyn-modifications to proteins, therefore, involved synthesis of authentic standards of Kyn and 3-OHKyn-modifications of these amino acids. Kyn and 3-OHKyn
adducts of N-α-t-Boc-L-histidine, N-α-t-Boc-L-lysine and L-cysteine were prepared at pH 9.5. Basic conditions were used as they promote the formation of the reactive α,β-unsaturated ketone of Kyn and 3-OHKyn, which is the initial step in the reaction with nucleophiles, ie. the amino acids His, Lys and Cys. t-Boc protected amino acids were used in the case of histidine and lysine to prevent the modification of the α-amino group. Cys was left unprotected, as preliminary investigations showed that Kyn preferentially reacted at the sulfhydryl group rather than the α-amino group.

Following the reaction of Kyn or 3-OHKyn with each of the amino acids, the reaction mixtures were analysed by HPLC (after adjusting the pH of the reaction mixtures to ca. 5.5 using acetic acid). The respective Kyn-modified amino acid adducts were found to be present in each of the reaction mixtures and definitive identification was made by ESI-MS of the isolated peaks and by direct LC/ESI-MS. Representative HPLC traces showing the reaction mixtures of Cys, N-α-t-Boc-L-histidine and N-α-t-Boc-L-lysine with Kyn are shown in Figure 4.1.
Figure 4.1: HPLC traces of the reaction mixture of: a) Kyn and L-cysteine, b) Kyn and N-α-t-Boc-L-histidine, c) Kyn and N-α-t-Boc-L-lysine. Kyn and each of the amino acids were incubated at pH 9.5 and 37°C for 48 hours (UV detection at 360 nm).
The major components in each of the reaction mixtures were identified by ESI-MS as the unreacted amino acid (t-Boc form in the case of Lys and His), kynurenine (retention time = 14.5 min) and the Kyn-t-Boc-amino acid adduct (retention time of ca. 19 min for t-Boc-Lys-Kyn and t-Boc-His-Kyn, and 14.3 min for Cys-Kyn) along with kynurenine yellow, the intra-molecular cyclization product of deaminated Kyn (retention time of 15.7 min).

4.3.2. Characterisation of the Kynurenine-modified Amino Acids

Following synthesis and purification (semi-preparative HPLC) of Kyn-modified t-Boc-His, t-Boc-Lys and Cys, characterisation was carried out using a combination of UV-Visible spectroscopy, 3D fluorescence spectroscopy, tandem mass spectrometry and NMR spectroscopy.

Initial analysis of the Kyn-modified amino acids by ESI-MS showed that the molecular masses (Mr) of the Kyn-modified amino acids were 446 Da, 437 Da, and 312 Da respectively for t-Boc-His-Kyn, t-Boc-Lys-Kyn and Cys-Kyn (the presence of the t-Boc group adds 101 Da to the mass of the amino acid). Further structural analysis by tandem mass spectrometry, was also carried out as discussed in section 4.3.3.

The UV-visible spectra of all three Kyn-modified amino acids, shown in Figure 4.1 were very similar to each other and to that of free Kyn. Two absorption maxima were apparent below 300 nm and a broad maximum at ca. 360 nm. These broad maxima tailed into the visible region of the spectrum giving rise to the characteristic pale yellow appearance of all the adducts.

The three-dimensional fluorescence spectra of the Kyn-modified amino acids are shown in Figure 4.2. The 3D-fluorescence spectra were quite similar for all of the adducts.
His-Kyn demonstrated an excitation maximum at 410 nm and emission maxima at 490 and 525 nm while t-Boc-Lys-Kyn had an excitation maximum of 410 nm and emission maxima at 490 and 527 nm. The excitation and emission maxima of the Cys-Kyn adduct differed slightly from those of the t-Boc protected amino acids. Cys-Kyn demonstrated an excitation maximum at 392 nm and emission maxima at 490 and 527 nm. By contrast, Kyn has an absorption maximum at 360 nm but exhibits little fluorescence (spectrum not shown).

Figure 4.2: UV-Vis (slit width 1.0 nm, sampling interval 0.5 nm) and (inset) three-dimensional fluorescence spectra of a) Cys-Kyn, b) His-Kyn and c) t-Boc-Lys-Kyn (slit widths, Ex 5 nm/Em 5 nm).
Figure 4.2: UV-Vis (slit width 1.0 nm, sampling interval 0.5 nm) and (inset) three-dimensional fluorescence spectra of a) Cys-Kyn, b) His-Kyn and c) t-Boc-Lys-Kyn (slit widths, Ex 5 nm/Em 5 nm).
NMR analysis was used to determine the structures of all the Kyn-amino acid adducts. 1D and 2D-NMR spectra were acquired for each of the three Kyn adducts to confirm the site of covalent attachment. One-dimensional $^1$H chemical shifts for each of these adducts are shown in Table 4.1. The $^1$H-NMR chemical shifts revealed four aromatic protons for each compound (H-2, H-3, H-4 and H-5; referring to the notation used in the structures given in Table 4.1), with chemical shifts found to be consistent with an unmodified Kyn aromatic ring (Table 4.1). Analysis of the aliphatic region of bound Kyn indicated the presence of three protons in the Kyn side chain. From their chemical shifts, two protons were located at C-8 while the other was at C-9. This indicated that the amino acid modification of Kyn was at C-9 and not C-8. In Table 4.1 the $^1$H chemical shifts displayed for Kyn and the respective amino acid in each of the Kyn-modified amino acids are similar to the chemical shift of the individual amino acids and Kyn. Significant differences in chemical shift were only observed for protons in close proximity to the sites of covalent attachment. For example, H-13 of His has a chemical shift of 3.24 ppm in the free state but when bound in His-Kyn, the proton signal shifts downfield to 7.3 ppm.

The site of amino acid modification to Kyn (through C-9) was confirmed by performing rotating frame nuclear Overhauser effect spectroscopy (ROESY) on each of the adducts. ROESY NMR experiments are particularly useful for determining short inter-proton distances through space. The diagnostic ROESY correlations (cross peaks) are given in Figures 4.3-4.5. In each case, cross peaks were observed between H-9 of Kyn and the pertinent protons of the amino acids Lys, His and Cys (also H-11 and H-13 in the case of His). In heteronuclear multiple bond correlation (HMBC) experiments, which allow through bond correlation of protons with carbon atoms, carbon-proton cross-peaks were
observed, again confirming the structures of the Kyn-modified amino acids (data not shown).

The structures determined via NMR spectroscopy were consistent with those predicted for a mechanism involving nucleophilic attack by the sulfhydryl or amine groups of the amino acids on the α,β-unsaturated side chain of deaminated Kyn and covalent attachment at C-9 of Kyn.
Table 4.1: $^1$H and assignments for Cys-Kyn. The atom numbering adopted is shown in Figures 4.3, 4.4 and 4.5.

<table>
<thead>
<tr>
<th>Atom Label</th>
<th>Kyn</th>
<th>Lysine</th>
<th>Histidin</th>
<th>Cysteine</th>
<th>t-Boc-Lys-Kyn</th>
<th>t-Boc-His-Kyn</th>
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<tr>
<td>H3</td>
<td>6.56</td>
<td>-</td>
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<td>-</td>
<td>6.7</td>
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</tr>
<tr>
<td>H4</td>
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<td>-</td>
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<td>-</td>
<td>6.8</td>
<td>6.8</td>
<td>7.30</td>
</tr>
<tr>
<td>H8</td>
<td>3.58</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.6</td>
<td>3.8</td>
<td>3.70</td>
</tr>
<tr>
<td>H9</td>
<td>4.29</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4.0</td>
<td>5.3</td>
<td>3.90</td>
</tr>
<tr>
<td>H11</td>
<td>-</td>
<td>2.99</td>
<td>8.58</td>
<td>2.93</td>
<td>3.1</td>
<td>8.65</td>
<td>3.30</td>
</tr>
<tr>
<td>H12</td>
<td>-</td>
<td>1.68</td>
<td>7.29</td>
<td>4.55</td>
<td>1.6-1.8</td>
<td>-</td>
<td>4.18</td>
</tr>
<tr>
<td>H13</td>
<td>-</td>
<td>1.44</td>
<td>3.24</td>
<td>-</td>
<td>1.7</td>
<td>7.3</td>
<td>-</td>
</tr>
<tr>
<td>H14</td>
<td>-</td>
<td>1.8</td>
<td>4.73</td>
<td>-</td>
<td>1.65</td>
<td>3.2</td>
<td>-</td>
</tr>
<tr>
<td>H15</td>
<td>-</td>
<td>4.32</td>
<td>-</td>
<td>-</td>
<td>3.8</td>
<td>4.1</td>
<td>-</td>
</tr>
<tr>
<td>H18,19,20</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.3</td>
<td>1.1</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 4.3: Structure of N-α-t-Boc-L-histidylnurenine showing selected proton-proton correlations determined from its ROESY NMR spectrum.
Figure 4.4: Structure of N-α-t-Boc-L-lysylkynurenine showing selected proton-proton correlations determined from its ROESY NMR spectrum.
Figure 4.5: Structure of Cys-Kyn showing selected proton-proton correlations determined from its ROESY NMR spectrum.
4.3.3. Tandem Mass Spectrometry of the Modified Amino Acids

The structures of Cys-Kyn, t-Boc-Lys-Kyn and t-Boc-His-Kyn were investigated further by tandem mass spectrometry (MS/MS). MS/MS, in conjunction with chromatographic techniques, was an important tool for the identification of Kyn-modified amino acids since Kyn-modifications in the biological systems examined here were present at low concentrations making identification by other techniques (such as NMR) difficult. Figures 4.6, 4.7 and 4.8 show the MS/MS spectra of the t-Boc-Lys-Kyn, Lys-Kyn, t-Boc-His-Kyn, His-Kyn and Cys-Kyn adducts respectively. Tables 4.2, 4.3 and 4.4 present suggested structures of the major product ions observed in these spectra.

The product ions for each of the Kyn-modified amino acids are consistent with the site of protonation being at the α-amino group, and thereby directing initial fragmentation towards the amino acid moiety. In the case of t-Boc-His-Kyn and t-Boc-Lys-Kyn, this resulted in the loss of the t-Boc group generating the product ions at m/z 347 and 338 which are essentially the protonated molecular ions of His-Kyn and Lys-Kyn respectively. For each of the adducts the presence of the respective amino acid was confirmed by the observation of a product ion for the protonated amino acid (m/z 147, 156 and 122 for Lys, His and Cys respectively).

The MS/MS spectra of the protonated molecular ions of His-Kyn, Lys-Kyn and Cys-Kyn showed a number of product ions characteristic of the Kyn moiety. In particular, a diagnostic ion at m/z 192, representing the deaminated form of Kyn, was observed in the MS/MS of all the Kyn-modified amino acids. Other product ions characteristic of Kyn were observed at m/z 174, 146, 136 and 120 (see section 2.3.1 for details of the fragmentation of Kyn). Through MS/MS studies the location of the covalent modification of Kyn by the amino acids Lys and Cys was confirmed. MS/MS of
protonated Lys-Kyn and Cys-Kyn each resulted in product ions containing a portion of the respective amino acid along with a portion of Kyn, indicated by the ions at m/z 203 and 202 respectively (Table 4.2 and 4.4). The proposed structures of these ions are consistent with the \textsuperscript{1}H and ROESY NMR data that suggested that C-9 of Kyn is the site of covalent attachment by the amino acid.

The MS/MS spectra of the Kyn-modified amino acids provide a unique product ion fingerprint for the identification of these adducts in biological samples. Initial identification can be made by observation of the molecular ion and an associated product ion (both of which are of relatively high relative abundance). The most appropriate ions for each of the amino acids are: m/z 338 and 203 for Lys-Kyn; m/z 347 and 301 for His-Kyn; and m/z 313 and 202 for Cys-Kyn, these ion pairs being the protonated molecular ion and a unique prominent product ion. Definitive identification can be made by comparison of the product ion distribution of the biologically-derived adduct with that of the synthetic standard.
Figure 4.6: ESI tandem mass spectra of the protonated molecular ion of a) N-ο-Benzyl-Lys-Kyn b) Lys-Kyn.
Table 4.2: Major product ions and their proposed structures observed in the ESI tandem mass spectrum of protonated *t*-Boc-Lys-Kyn and Lys-Kyn.

<table>
<thead>
<tr>
<th>Observed Ions (m/z)</th>
<th>Lys-Kyn</th>
<th><em>t</em>-Boc-Lys-Kyn</th>
<th>Proposed Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>338</td>
<td>438</td>
<td><img src="image" alt="Proposed Structure" /></td>
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<td>-</td>
<td>203</td>
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<td>136</td>
<td>128</td>
<td>128</td>
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</table>
Figure 4.7: ESI tandem mass spectra of the protonated molecular ion of a) N-α-t-Boc-His-Kyn b) His-Kyn.
Table 4.3: Major product ions and their proposed structures observed in the ESI tandem mass spectrum of protonated \( t \)-Boc-His-Kyn and His-Kyn.

<table>
<thead>
<tr>
<th>Observed Ions (m/z)</th>
<th>His-Kyn</th>
<th>( t )-Boc-His-Kyn</th>
<th>Proposed Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td><img src="image" alt="Proposed Structure" /></td>
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<tr>
<td>-</td>
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</tr>
<tr>
<td>347</td>
<td>347</td>
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<tr>
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</tr>
<tr>
<td>109</td>
<td>109</td>
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<td></td>
</tr>
</tbody>
</table>
Figure 4.8: ESI tandem mass spectra of the protonated molecular ion of Cys-Kyn.
Table 4.4: Major product ions and their proposed structures observed in the ESI tandem mass spectrum of protonated Cys-Kyn.

<table>
<thead>
<tr>
<th>Observed Ions (m/z)</th>
<th>Proposed Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>313</td>
<td><img src="image1" alt="Structure 313" /></td>
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<tr>
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</tr>
<tr>
<td>174</td>
<td><img src="image6" alt="Structure 174" /></td>
</tr>
<tr>
<td>146</td>
<td><img src="image7" alt="Structure 146" /></td>
</tr>
<tr>
<td>122</td>
<td><img src="image8" alt="Structure 122" /></td>
</tr>
</tbody>
</table>
Given the structural similarity between Kyn-modified and 3-OHKyn-modified amino acids, and the fact that comprehensive characterization of the Kyn-modified amino acids had been carried out, characterization of the 3-OHKyn-modified amino acid analogues was limited to MS/MS studies. The MS/MS spectra of each 3-OHKyn modified amino acids are shown in Figure 4.9. In each spectrum, the major product ions relate well with those observed for the respective Kyn-modified amino acids, albeit the product ions containing the 3-OHKyn moiety were shifted up by m/z 16 (owing to the presence of the additional oxygen atom). The product ion at m/z 208 is common to the MS/MS spectra of all the 3-OHKyn modified amino acids and is the hydroxylated equivalent of the product ion at m/z 192 for Kyn-modified amino acids. The similarity in structure between the Kyn and 3-OHKyn modified amino acids is also evident in the distribution of product ions in the respective MS/MS spectra. As with the Kyn-modified amino acids, 3-OHKyn-modified amino acids yielded product ions including m/z 110, 136, 152, 162, 190 and 208, which were representative of free 3-OHKyn (see section 2.3.4). In addition, two structurally diagnostic ions were observed at m/z 202 and 203 for Cys-3-OHKyn and Lys-3-OHKyn, respectively. These ions were also observed in the MS/MS spectra of Cys-Kyn and Lys-Kyn and demonstrate the attachment of the amino acid through C-9 of the amino acid side chain.
Figure 4.9: ESI tandem mass spectra of the protonated molecular ion of: a) Lys-3-OHKyn, b) His-3-OHKyn and c) Cys-3-OHKyn.
4.3.4. Stability of the Kyn and 3-OHKyn-modified Amino Acids Towards Acid Hydrolysis

The first step in the development of a HPLC method for the detection of Kyn and 3-OHKyn in proteins involved an investigation of the stability of the Kyn and 3-OHKyn-modified amino acid adducts towards acid hydrolysis over a 24 hour period at 110°C in 6 M HCl. Aliquots of the acid hydrolysis mixtures of each of the adducts were removed after 24 hours and the quantity of adduct remaining was measured by HPLC. Table 4.5 shows the percentage recovery for each of the Kyn and 3-OHKyn-modified amino acids. These data indicate that the Kyn-modified amino acids were more stable than the equivalent adducts of 3-OHKyn. Lys-3-OHKyn and His-3-OHKyn were not detected at all after the 24 hour hydrolysis period, presumably owing to the formation of degradation products during the early stages of hydrolysis. Consequently, acid hydrolysis was not considered suitable for detection of 3-OHKyn-modified amino acids. The Kyn-modified amino acids were recovered with ≥ 96% efficiency and thus were considered to be possible targets in human protein hydrolysates.

Table 4.5: Recovery of the Kyn-modified amino acids after acid hydrolysis in 6M HCl and 110°C for 24 hours.

<table>
<thead>
<tr>
<th>Adduct</th>
<th>Percentage Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys-Kyn</td>
<td>96 ± 2%</td>
</tr>
<tr>
<td>Lys-Kyn</td>
<td>96 ± 2%</td>
</tr>
<tr>
<td>His-Kyn</td>
<td>99 ± 1%</td>
</tr>
<tr>
<td>Cys-3-OHKyn</td>
<td>84 ± 2%</td>
</tr>
<tr>
<td>Lys-3-OHKyn</td>
<td>0%</td>
</tr>
<tr>
<td>His-3-OHKyn</td>
<td>0%</td>
</tr>
</tbody>
</table>
4.3.5. Modification of a Model Octapeptide by Kyn

The feasibility of the acid hydrolysis technique for the detection of adducts in human lens proteins was explored further by Kyn modification of an octapeptide (VHLTPVQK) containing both His and Lys residues. The octapeptide was modified by Kyn using the procedure described in section 4.2.4. The semi-preparative HPLC trace of the resulting reaction mixture is shown in Figure 4.10. The HPLC profile shows two large peaks along with a series of smaller, later eluting peaks. The two large peaks with retention times of 15.6 and 16.6 minutes were determined, by ESI-MS, to be unreacted Kyn and kynurenine yellow respectively. Following individual collection of the smaller peaks and subsequent ESI-MS analysis, two fractions with retention times of 18.8 and 20.3 minutes containing Kyn-modified peptides were found (Figure 4.10). The presence of the Lys and His residues in the peptide resulted in two peptide fractions that contained Kyn-peptide adducts. The ESI-MS of the pooled Kyn-modified peptide fractions (18.8 and 20.3 min) collected from HPLC (shown inset in Figure 4.10) revealed a singly charged species at m/z 1114 and a doubly charged species at m/z 557.5. A molecular mass of 1113 Da for the modified octapeptide was that expected for the modification by a single deaminated Kyn moiety.
Figure 4.10: The HPLC separation of the reaction mixture of Kyn and the octapeptide VHILTPVQK. The two Kyn-modified peptide containing fractions are denoted by • and *. The ESI mass spectrum of the combined Kyn-modified peptide fractions is shown inset.
MS/MS fragmentation of the peptide bonds will produce b series ions if the peptide positive charge remains at the N-terminus, or y series ions if the charge is retained at the peptide C-terminus. The b and y ion fragmentations produce a ladder of peptide ions whose mass difference give sequence information according to which amino acid has been lost. The location of the Kyn modification was determined by the difference between the m/z of the b-series product ions generated MS/MS spectra of the unmodified peptide fraction, shown in Figure 4.11, with those of the Kyn-modified peptide shown in Figure 4.12. Differences in the spectra were observed in the b-series ions b₂, b₃ and b₄. These ions (b₂= m/z 428.5, b₃= m/z 541.7 and b₄= m/z 642.6) in the MS/MS spectrum of the Kyn-modified peptide were higher than the equivalent ions in the MS/MS spectrum of the unmodified peptide by 191 m/z. This difference in the m/z of the b-series ions may be explained by the covalent attachment of the Kyn moiety to the His residue in the peptide. A clear MS/MS spectrum of the later eluting Kyn-modified fraction was not obtained, however, three y-series ions (y₂= m/z 467, y₃= m/z 567 and y₆= m/z 877;) were observed as a result of in-source collisionally induced dissociation during the ESI-MS of this fraction suggesting that the Kyn modification occurred at the Lys residue of the peptide.

ESI-MS analyses of the remaining HPLC fractions, showed that these peaks did not result from the direct modification of the peptide by Kyn. These were attributed to unreacted peptide (RT = 19.1 min) along with impurities in the peptide sample. Three species identified were the formylated peptide (RT= 19.9 min) and what appeared to be the octapeptide with a Glu residue in place of a Gln residue (RT= 21.7 min). These species were not observed in the reaction mixture at time zero thus were assumed to form as a result of the incubation.
Figure 4.11: ESI tandem mass spectrum of the [M+H]$^+$ ion of octapeptide VHLTPVQK. The origin of the major product ions is shown inset.
Figure 4.12: ESI tandem mass spectrum of the [M+H]^+ ion of Kyn-modified octapeptide VHLTPVQK. The origin of the major product ions is shown inset.
The two Kyn-modified peptide fractions were pooled and subjected to the acid hydrolysis procedure as outlined in section 4.2.3. Following this, the hydrolysate was lyophilised overnight then dissolved in Milli Q H₂O and analysed by LC/ESI-MS (1% formic acid was used as the HPLC buffer) with UV-Vis detection at 360 nm. Figure 4.13 represents the chromatographic profiles for the LC/ESI-MS separation monitoring the: a) absorbance at 360 nm for coloured species; b) total ion current (TIC) of eluted species detected by the mass spectrometer and c) and d) ion current for the ions at m/z 338 and 347 representing the protonated molecular ions of Lys-Kyn and His-Kyn, respectively. In the 360 nm trace (Figure 4.13), two coloured peaks were apparent with retention times of 15.3 and 15.8 minutes. These two species were also apparent in Figure 4.13 b). Note the TIC peaks were offset by 0.1 minute owing to the time that elapsed while the sample travelled from the LC UV-detector to the mass spectrometer. These peaks each corresponded with the peaks in the ion chromatograms for m/z 338 and 347 (Figure 4.13 c) and d)), indicating that the coloured peaks arose from the Lys-Kyn and His-Kyn adducts, respectively. Further confirmation of the identity of each adduct was gained from LC-MS/MS. The tandem mass spectrum of each adduct (not shown) was compared to that of the synthetically-prepared standards (Figure 4.6 and 4.7). In both cases the same ions and similar relative abundances were apparent, confirming the identity of these species as Kyn-modified Lys and His. A number of other peaks, with retention times of 4.7, 5.8, 6.6 and 12.4 minutes, were also apparent in the total ion chromatogram (TIC). The ESI mass spectra of these peaks (not shown) were consistent with the amino acids proline, histidine, valine and leucine, respectively, generated by the acid hydrolysis of the octapeptide.
Figure 4.13: LC-MS of Kyn-modified peptide (VHLTPVQK) hydrolyzate. a) UV response at 360 nm, b) total ion current, c) ion chromatogram for Lys-Kyn (m/z 338), d) ion chromatogram for His-Kyn (m/z 347).
4.3.6. Modification of Calf Lens Protein by Kynurenine

The same conditions used for the modification of amino acids and peptides by Kyn were also used for the modification of calf lens protein (CLP). The rate of modification of crystallin by Kyn, at pH 9.5, was determined by measuring the increase in absorption at 360 nm from increasing crystallin colouration owing to incubation with Kyn.

The Kyn-modified CLP was separated from free small molecules present in the reaction mixture, including Kyn and kynurenine yellow (all of which contribute to colouration), using a PD-10 size exclusion column and subsequent solvent extraction of the lyophilised protein with ethanol. The increase in UV-light absorption of the purified CLP was found to be quite rapid. The reaction reached equilibrium within 20 hours, after this time the absorbance was relatively constant showing no apparent further increase in colouration (data not shown).

The 3D-fluorescence spectrum of Kyn-modified CLP, shown in Figure 4.14 was taken after 160 hours of incubation of CLP with Kyn. Kyn-modified CLP has an excitation maximum at 380 nm and emission maxima at 450 (major) and 480 (minor) nm. The 3D-fluorescence spectrum of Kyn-modified CLP displays one major peak as opposed to the Kyn-modified amino acids which display two major peaks of similar intensity (Figure 4.14) and the excitation/emission maxima are at shorter wavelengths for the Kyn modified CLP (380/450, 480 cf. 410/490, 525). The UV-Vis spectrum, however, is very similar to that of the Kyn-modified amino acids, displaying an absorption maximum at 360 nm.
CLP was also reacted with 3-OHKyn in a similar manner to that used for the preparation of Kyn-modified CLP. The rate of reaction of 3-OHKyn with protein, however, could not be followed by UV absorption because the protein-bound 3-OHKyn mixture was found to undergo oxidation as evidenced by a deep brown colouration of the 3-OHKyn-modified protein, which interfered with detection at 360 nm.

**4.3.7. Acid Hydrolysis of Kyn and 3-OHKyn-modified Calf Lens Protein**

Kyn and 3-OHKyn-modified CLP samples were subjected to acid hydrolysis after size exclusion purification. The resulting hydrolysate was analysed by LC/MS and these results are shown in Figure 4.15. In the case of Kyn-modified CLP, the major coloured peaks were identified by correlation between the UV absorbance at 360 nm (Figure 4.15
(a)) and the ion currents for the protonated molecular ions of c) Cys-Kyn at \( m/z \) 313, RT= 18.4 and 18.7 min (isomers), d) Lys-Kyn at \( m/z \) 338, RT= 15.8 min and e) His-Kyn at \( m/z \) 347, RT= 16.4 min. Further, the LC/MS/MS spectra of each of these protonated molecular ions, shown in Figure 4.16, were the same as those for the synthetic standards (Figures 4.6-4.8) except that the signal-to-noise ratio was lower owing to there being less sample. As a control experiment, Kyn was also added to unmodified CLP immediately prior to hydrolysis. No ions corresponding to Kyn adducts were observed in these experiments, thereby eliminating the possibility of artefactual formation of these adducts during the acid hydrolysis procedure by a reaction of any remaining free Kyn.

In the case of 3-OHKyn-modified CLP, only the Cys-3-OHKyn adduct was observed. This is in agreement with the acid hydrolysis experiments on the individual 3-OHKyn-modified amino acids which showed that the His and Lys-3-OHKyn adducts are not stable under the conditions used for acid hydrolysis.
Figure 4.15: LC-MS analysis (formic acid buffer) of acid hydrolysed Kyn-modified CLP. a) UV absorbance at 360 nm. b) total ion current. ESI ion currents corresponding to the protonated molecular ions c) m/z 313, Cys-Kyn, d) m/z 338, Lys-Kyn, e) m/z 347, His-Kyn, respectively.
Figure 4.16: LC-MS/MS of the coloured peaks from the hydrolysate of Kyn-modified CLP. From the tandem mass spectra these peaks were identified as a) Cys-Kyn, b) Lys-Kyn and c) His-Kyn.
4.3.8. Acid Hydrolysis of Human Lens Proteins

Once the applicability of the acid hydrolysis method for Kyn-modified CLP was established, human lens proteins were then examined. Figure 4.17 shows the HPLC trace of aged lens protein following acid hydrolysis in which the presence of two major peaks corresponding to His-Kyn and Lys-Kyn were evident. (It should be noted that this HPLC system employed ammonium acetate as the buffer as this system was better suited to the analysis of human protein hydrolysates. This is different from the LC/MS chromatogram shown in Figure 4.15, which utilises formic acid buffer. With the use of formic acid the Kyn-modified amino acids had longer retention times). The identity of these peaks was confirmed by spiking the sample with the synthetic standards. In addition, following a second HPLC purification step to minimize interference by co-eluting hydrolysis products, the individual peaks were collected and analysed by ESI-MS and MS/MS as shown in Figure 4.18. It must also be noted that protein hydrolysate contained very high levels of background amino acids produced as a direct result of the hydrolysis procedure. These amino acids had the effect of increasing the retention times of species relative to the synthetic standards analysed using the same buffer conditions in the absence of excess background amino acids. It was assumed the excess amino acids present in the hydrolysate acted as ion pair reagents giving rise to the longer retention times. In contrast to the model CLP system, where higher levels of the adducts were present, the Kyn-adducts were not detected by direct LC/ESI-MS analysis of the hydrolyzate. This may be a consequence of the high concentration of amino acids present in the hydrolysate suppressing the ionisation of the relatively low levels of the Kyn-modified amino acids.
Figure 4.17: HPLC trace of acid-hydrolysed human lens protein (nuclear). A 200 µl aliquot was analysed by analytical HPLC using ammonium acetate buffer with detection at 360 nm.
Figure 4.18: ESI tandem mass spectra of [M+H]$^+$ ions of a) His-Kyn and b) Lys-Kyn purified from human lens protein following acid hydrolysis.
Also evident in the chromatograms of human lens protein samples were peaks (not shown in Figure 4.17) that eluted between 10 and 15 min. These were also observed at similar levels in the acid hydrolysates of unmodified CLP and probably arose from acid degradation products of tryptophan.\textsuperscript{181}

A very small peak, observed at a retention time of 12.4 min, was identified by spiking experiments as Cys-Kyn, however, the identity of the peak could not be confirmed by ESI-MS, again owing to the high amino acid background. The possibility that the Cys adduct may be selectively oxidised and thus lost was also considered. Hence, acid hydrolysis of the human lens protein was also performed in the presence of an antioxidant system (phenol/mercaptoacetic acid) as described by Fu et al.\textsuperscript{163} that enables good recovery of readily-oxidised amino acids such as DOPA. This added precaution, however, did not result in an increase in the yield of Cys-Kyn, so it is suggested that the observed level of Cys-Kyn reflected the levels present in the lens. One other possible explanation for the low concentration of Cys-Kyn found in lens protein is that it is inherently unstable at the near neutral pH of the human lens. This instability was confirmed by experiments on Cys-Kyn at pH 7.2 where approximately 50\% decomposed at 37°C over 20 hours (this is discussed in greater detail in section 5.3.4).

All human lenses analysed to date have contained measurable levels of His-Kyn and Lys-Kyn. Although 3-OHKyn-Cys could survive the acid hydrolysis procedure it was not observed in human lenses. A notable difference between the human lens protein and the Kyn-modified CLP was that the levels of Kyn-modified amino acids were significantly lower in the human lens protein. This may be a consequence of the elevated pH and high Kyn concentration conditions used in the model experiment generating higher levels of the reactive deaminated Kyn intermediate. In addition to the
effect of pH, it is likely that other processes such as reduction and conjugation with GSH compete for the $\alpha,\beta$-unsaturated ketone derivative of Kyn when it is formed within the lens.\textsuperscript{61}

### 4.4. Discussion

Kyn has been shown to be present in the primate lens as a UV-filter, but it is not limited to the lens. The function of Kyn in the lens has long been thought to provide localized protection to the retina and decrease chromatic aberration. Since Kyn is not readily oxidised, in contrast to 3-OHKyn, it has generally been believed to be benign and unable to bind with protein, thus solely fulfilling its function as a UV-filter compound.

In recent work we have identified a novel UV-filter compound, the 3-OHKG adduct of GSH. This novel compound results from the non-oxidative covalent modification of deaminated 3-OHKG by the peptide GSH.\textsuperscript{32} The levels of 3-OHKG attached to human lens protein, as a function of age, were determined in further work.\textsuperscript{182} Human lens protein was base hydrolysed releasing AHAG which was used as an indirect measure of the level of 3-OHKG bound to the protein. Model studies were also carried out on modification of CLP by Kyn to identify the primary sites of reaction.\textsuperscript{178} Analysis of tryptic peptides of Kyn-modified crystallins revealed that all of the coloured peptides contained either His, Cys or an internal Lys residue.\textsuperscript{178}

Although Kyn and 3-OHKyn are present in the human lens at lower levels than 3-OHKG, the preceding evidence indicates that Kyn is at least capable of directly contributing to the age related colouration of human lens protein. To determine if these UV-filters are involved in this process, biochemical markers are required. Kyn and 3-OHKyn were reacted with the nucleophilic amino acid His, Lys and Cys to produce
covalent adducts, which would potentially serve as biomarkers. Arginine was not used as a biomarker as it was not identified as a site of covalent modification by Kyn in Kyn-modified CLP.

The synthetic Kyn-modified amino acids were characterised by UV-Vis and fluorescence spectroscopy, tandem mass spectrometry and NMR spectroscopy. Not surprisingly, all the adducts displayed similar UV-Vis and fluorescence characteristics. Initially mass spectrometry was used to characterise the structure of the novel adducts. Fragment ions identifying each of the components present were evident i.e. the respective amino acids and the Kyn. In the MS/MS spectra of Lys-Kyn and Cys-Kyn, two prominent ions identified at m/z 203 and 202 respectively confirming the proposed structures of the Kyn-modified amino acids. These ions comprised of a portion of the respective amino acid covalently bound to a portion of Kyn (see Tables 4.2 and 4.4). The ions at m/z 203 and 202 were also observed in the MS/MS spectra of Lys-3-OHKyn and Cys-3-OHKyn, respectively, confirming that the structures of the 3-OHKyn-modified amino acids only differed from those of Kyn by an aromatic hydroxyl group. Further confirmation of the Kyn-modified amino acid structures was gained using $^1$H NMR correlation experiments (ROESY). For each Kyn-modified amino acid the NMR experiments clearly showed the cross peaks between the amino acid and the Kyn side chain, confirming linkage through the side chain. The information gained from the NMR characterization of the Kyn adducts in combination with MS/MS studies on the Kyn amino acid adducts was used in assigning structures for the 3-OHKyn analogues.

By far the simplest way to utilise these adducts as biomarkers was to use them as individual indicators of amino acid modification in proteins. One possible alternative would be the analysis of modified tryptic peptides, however, this is much more difficult.
since these adducts would be dispersed over many possible sites of modification. To utilise the Kyn-modified amino acids as biomarkers it was necessary to establish if the protein could be hydrolysed to the individual amino acid level while leaving the modified amino acids intact. This required acid stability data for the various adducts. Subjecting the various amino acids to acid hydrolysis conditions revealed some disparity in the applicability of the acid hydrolysis technique to the various adducts. All of the Kyn amino acid adducts displayed acceptable levels of stability towards acid hydrolysis conditions (≥ 96%). While 3-OHKyn adducts were not suited to the acid hydrolysis procedure as only Cys-3-OHKyn showed stability. Since Cys-Kyn is only detected at very low concentrations in human lenses this was also expected to be the case for the 3-OHKyn analogue. This was in fact the case in human lenses, Cys-3-OHKyn (or the other 3-OHKyn-modified amino acids) was not observed in the HPLC of human lenses following acid hydrolysis. Given the inability to observe any of the 3-OHKyn modifications only the identification and quantification of Kyn-modified amino acids, in the aged lens, was undertaken (see Chapter 5).

The applicability of the acid hydrolysis method for the analysis of Kyn modifications to human lens proteins was verified using a simple peptide (VHLTPVQK) and CLP, which were both modified by Kyn at elevated pH (9.5). The modified protein or peptide was then subjected to acid hydrolysis. The octapeptide contained two possible sites of reaction with Kyn, the His and the Lys residues. The LC-MS of the hydrolysate of the Kyn-modified peptide demonstrated the presence of both His-Kyn and Lys-Kyn adducts. This confirmed that both residues could be modified and that the acid hydrolysis procedure was useful for the identification of these modifications. In order to mimic the situation in human lenses more closely, crystallins purified from calf lenses were modified with Kyn and subjected to the acid hydrolysis the and subsequent
analysis procedure. LC-MS of the hydrolyzate showed the presence of Lys-Kyn, His-Kyn and Cys-Kyn. Their identities were confirmed by performing MS/MS analysis as each of the adducts eluted from the column. In a control experiment the presence of free Kyn with unmodified CLP did not result in the formation of the Kyn-modified amino acids during the acid hydrolysis procedure, hence the adducts observed in the hydrolysis of Kyn-modified CLP were entirely derived from the modification process and any Kyn modification, were amenable to analysis with this method.

Using the acid hydrolysis procedure developed as part of this work we have, for the first time, shown that Kyn is covalently conjugated to human lens proteins. The acid hydrolysis of human lens protein revealed that His-Kyn and Lys-Kyn were the major adducts with lower levels of Cys-Kyn present. The full characterisation of the levels of the amino acids in the lens with respect to age are given in the following chapter (Chapter 5). 3-OHKyn was also expected to bind to lens proteins as it bound to amino acids in the same manner as Kyn. An experiment was performed to modify CLP with 3-OHKyn followed by acid hydrolysis and RP-HPLC analysis, however, only the Cys-3-OHKyn adduct was observed in the chromatogram of the hydrolyzate. This was consistent with the recovery following acid hydrolysis of the individual 3-OHKyn amino acids which revealed that only the Cys-3-OHKyn adduct was present at detectable levels. In the acid hydrolysis of human lens tissue none of the 3-OHKyn-modified residues were detected. We know that the acid hydrolysis is not well suited to the analysis of His or Lys-3-OHKyn adducts, however, from the initial characterisation experiment the Cys-3-OHKyn adduct was expected to be observed along with the Kyn-amino acid adducts. A possible reason for the failure to detect Cys-3-OHKyn in human lenses was that the protein bound Cys-3-OHKyn adduct may be readily oxidised within the lens forming a cross link that we are yet to characterise.
The lower than expected level of Cys-Kyn implies that a variety of factors may play a role in determining the final concentration of the adduct. In addition to their pKa values, the extent to which residues are modified is likely to be influenced by the tertiary structure of the protein and accessibility of residues for reaction, as well as the adduct stability at physiological pH. The low level of Cys-Kyn observed may also reflect the lower abundance of Cys residues in crystallins compared to histidine and lysine as shown in Table 4.6. At present, the best explanation for the observed HPLC pattern would appear to be the relative stabilities of the Kyn adducts at physiological pH. These issues are examined and discussed in greater detail in Chapter 5.

Table 4.6: The content of the nucleophilic amino acids Cys, Lys and His in the major crystallin classes.

<table>
<thead>
<tr>
<th>Crystallin</th>
<th>% Cys</th>
<th>% Lys</th>
<th>% His</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>0.3</td>
<td>4.3</td>
<td>4.1</td>
</tr>
<tr>
<td>β</td>
<td>1.7</td>
<td>3.7</td>
<td>3.4</td>
</tr>
<tr>
<td>γ</td>
<td>3.3</td>
<td>2.7</td>
<td>3.0</td>
</tr>
</tbody>
</table>

The majority of covalent modifications to lens proteins reported to date have been found at very low levels, except for carboxymethyllysine (approximately 300 pmol/mg of protein). The levels of the Kyn adducts that we have described here are comparable to those of carboxymethyllysine. These findings are also consistent with those from previous model experiments where the reaction between Kyn and nucleophilic amino acids was investigated.

The modification reaction proceeds via initial deamination of the Kyn amino acid side chain followed by a Michael addition of the nucleophilic amino acid residue to the
resultant α,β-unsaturated ketone. The reaction mechanism is summarized in Scheme 4.1. As noted previously, the rate of Kyn adduct formation is dependent on two factors. First, deamination of the Kyn amino acid side chain, which has been found to be most efficient at pHs 9-10, with a much reduced, but still apparent, rate at physiological pH.\textsuperscript{179} Secondly, the nucleophilicity of the amino acids, which is related to their pKa values; and attributed to the nucleophilicity of the deprotonated forms.\textsuperscript{184,185} NMR spectroscopy revealed that in His-Kyn, one of the imidazole nitrogen atoms reacted to a greater extent with the deaminated Kyn. This finding is in agreement with the reaction of 4-hydroxy-2-nonenal with His.\textsuperscript{186} Steric hindrance appears to direct reaction to the τ nitrogen, the nitrogen furthest from the histidine side chain, for relatively bulky molecules such as Kyn.
Scheme 4.1: Suggested mechanism for the formation of the Kyn amino acid adducts in proteins. Formation of the His-Kyn adduct is shown as model for the other Kyn-modified amino acids.
The consequences of Kyn binding to lens protein are not yet understood. Kyn has the potential to render the protein photo-reactive and we have confirmed this in preliminary experiments. Although not detected during this work, 3-OHKyn is also expected to be bound to lens proteins. The consequences of 3-OHKyn binding to human lenses may be more deleterious than when Kyn is bound. When 3-OHKyn binds to protein through the amino acid side chain, as described here, the reactive o-aminophenol is still able to undergo oxidation reactions that, in turn, may lead to localized formation of superoxide and hydrogen peroxide as described in Chapter 3. Oxidation of bound 3-OHKyn could also produce protein cross-links and may also result in protein colouration, precipitation and cataract formation. This is the subject of ongoing work in our laboratory.

Nucleophiles, such as GSH, present in the lens in relatively high concentrations, may compete with the amino acid residues for the unsaturated ketone derivative of Kyn and therefore protect crystallins from modification. After middle age, the concentration of GSH in the nuclear region of the lens decreases to < 1 mM and this diminished protection could therefore contribute to the non-oxidative attachment of UV-filters in this part of the lens. It has been proposed that the development of an internal lens barrier, to the diffusion of small molecules such as GSH, after age 40-50 may be the primary event responsible for these changes, which leads to lens colouration and ultimately to age-related nuclear cataract formation.

Although protein-bound Kyn has been shown in this study to be present in the lens, tryptophan metabolites can also be increased in a variety of other disease states of other tissue. For example Kyn and 3-OHKyn, together with quinolinic acid and kynurenic acid, are found in the central nervous system (CNS) and their concentrations increase significantly in response to CNS inflammation, systemic immune response and HIV
infection. The role of Kyn and 3-OHKyn in these conditions is not yet known, but their binding to proteins may play a part in the genesis of these diseases. These modifications may also occur in other disease states where Kyn and Kyn metabolites are increased via the up regulation of indoleamine 2,3-dioxygenase (IDO).
5. The Role of Kynurenine Modified Protein in Age-related Cataract and the Colouration of the Human Lens.

5.1. Introduction

With aging, lens crystallins become increasingly coloured and fluorescent, increasing the filtering capacity of the lens. There have been suggestions as to the reason for this increase in protein modification, ranging from glycation to UV-filter compound mediated modification. Several advanced glycation end products (AGEs) have been identified including Pentosidine and carboxymethyl lysine, however, the exact glycation agent responsible for forming these AGEs in vivo remains unknown. An analysis of the levels of adducts in the proteins indicated that the increase in protein colouration may be attributed largely to covalent modification by UV-filter compounds present in the lens. Specifically, kynurenine (Kyn) and 3-hydroxykynurenine O-β-D-glucoside (3-OHKG) have been shown to bind to nucleophilic amino acids following deamination of the aliphatic side chain, a process that occurs readily at neutral pH.

In chapter 4, the Kyn-mediated modification of nucleophilic amino acid residues from aged human lens protein was described, verifying that kynurenine is able to modify human crystallins in vivo in the same manner as 3-OHKG. Using the techniques described in the previous chapter we aimed to obtain more quantitative data on the changes in protein-bound Kyn as a function both of age and of the severity of cataract in the human lens. It was thought that these data might provide an insight into the role, if any, of Kyn in the development of lenticular colour with age and the progression of age-related cataract. In addition, model studies were carried out on the Kyn-modified amino acids and Kyn-modified calf lens protein (CLP) with a view to characterising...
their behaviour under physiological pH conditions and providing an explanation for the 
low level of Cys-Kyn observed in the lens analysed in chapter 4.

5.2. Materials and Methods

The methods used in the analysis of human lens proteins are the same as those used in 
the previous chapter with the exception of those detailed here.

5.2.1. Stability Studies of the Kyn-modified Amino Acids at pH 7.2

Each of the synthetic Kyn-modified amino acids (His-Kyn, 4.6 mg; Lys-Kyn, 4.8 mg; 
Cys-Kyn, 3.6 mg) was dissolved in 3 mL of 0.1 M phosphate buffer (pH 7.2). The 
solution was degassed with argon and sealed in a glass vial and wrapped with 
aluminium foil to eliminate light. The glass vial was immersed in a water bath and 
incubated at 37 °C. Aliquots (20 μL) of the reaction mixture were removed at time 
intervals and analysed by HPLC. Following sampling, the solution was again sealed 
under argon and wrapped in aluminium foil.

5.2.2. Incubation of Kyn-modified Cysteine at pH 7.2 in the Presence of Excess 
N-α-t-Boc-His and N-α-t-Boc-Lys

Synthetic Cys-Kyn (1.0 mg, 3.2 μmol) together with 26.3 mg of N-α-t-Boc-histidine 
and 23.4 mg of N-α-t-Boc-lysine (ca. 20 molar excess) were dissolved in 0.2 M 
phosphate buffer pH 7.2 (2 mL). The final pH was adjusted to 7.2 using 0.1 M NaOH if 
required. CHCl₃ (20 μL) was added to the solution to inhibit bacterial growth. The vial 
containing the reaction mixture was bubbled with argon (to eliminate oxygen), sealed, 
wrapped in foil and incubated at 37°C. Aliquots (100 μL) were removed from the 
reaction mixture at 0, 6.75, 23.75, 30.2 and 49.5 hours. After each sample was taken, the
vial was again bubbled with argon, sealed and wrapped in foil. Duplicate 20 μL samples of each aliquot were analysed directly by analytical HPLC.

5.2.3. Incubation of Kyn-modified Calf Lens Protein at pH 7.2 in the Presence of Excess N-α-t-Boc-Histidine

Kyn-modified calf lens protein (28.5 mg), purified using a PD-10 column, was added to 30.0 mg of N-α-t-Boc-histidine in 0.2M phosphate buffer pH 7.2 (5 mL). If required, the pH was readjusted to 7.2 using 0.1 M NaOH and 20 μL of CHCl₃ was added to the solution to inhibit bacterial growth. The vial was bubbled with argon, sealed, wrapped in foil and incubated at 37 °C. Aliquots (1.0 mL) were taken from the reaction mixture at 0, 22, and 46 hours. After sampling, the reaction mixture was again bubbled with argon, sealed and wrapped in foil. Each aliquot was prepared for analysis by separation of the protein fraction from small molecules using a Sephadex G25 PD-10 column using the procedure described in section 4.2.5. The separated protein and small molecule fractions were pooled and lyophilised. The small molecule fraction was redissolved in a total of 200 μL of HPLC solvent A and analysed by HPLC. The protein fraction was hydrolysed according to the hydrolysis procedure described above (section 4.2.3) and subsequently analysed by HPLC.

5.3. Results

5.3.1. The Amino Acids Modified by Kyn in Calf Lens Protein.

Analyses of tryptic peptides from crystallins exposed to Kyn at high pH, have revealed that Kyn modification occurs primarily at nucleophilic amino acid residues including His, Lys and Cys.¹⁷⁸
As shown in section 4.3.6, Kyn modification of bovine crystallins was induced by incubating purified lens proteins with Kyn at pH 9.5 for 48 hours at 37°C. Following Kyn-modification, the protein was isolated from the reaction mixture and acid hydrolysed (6M HCl, 110°C, 24 hours) to yield the individual Kyn-modified amino acids. Following lyophilisation and neutralization, the resulting hydrolysate was analysed by LC-MS (Figure 4.14). Under the basic conditions used, Lys residues were found modified to the greatest extent, with Lys-Kyn accounting for 43% of the peak area at 360 nm. His-Kyn and Cys-Kyn accounted for 36% and 21% of the total modification by Kyn respectively.

5.3.2. The Levels of Kyn-modified Amino Acids in Non-Cataractous Human Lenses

Crystallins isolated from 20 individual human lenses ranging in age from 16 to 80 years were analysed, in duplicate, using the acid hydrolysis procedure (section 4.2.3) to determine the degree of protein modification to the protein by Kyn. The concentrations of the adducts present are quoted in terms of the nmol of adduct present per mg of dry lens protein and were quantified by HPLC using a calibration curve for each adduct. These units were chosen because other modifications to human lens proteins were quoted in these terms. All human lenses analysed contained measurable levels of His-Kyn and Lys-Kyn. Quantification of Cys-Kyn in human lens protein was complicated by the low levels present, particularly in older lenses, and by the presence of a coeluting peak. For this reason, the levels of Cys-Kyn are not presented in the following graphs. When Cys-Kyn was clearly observed the concentration was found to have a similar, but lower, value to that of Lys-Kyn. Possible causes for the low level of Cys-Kyn in human lenses are discussed in sections 5.3.4 through 5.3.6.
5.3.2.a. Kyn-modified Amino Acid Levels in the Nuclear Region

Figure 5.1 shows the concentrations of His-Kyn and Lys-Kyn in the nucleus of normal (non-cataractous) human lenses. The concentrations of these Kyn-modified amino acids were found generally to increase as a function of age. The data shows a considerable degree of scatter in the Kyn-adduct levels, particularly in older individuals, suggesting that environmental or genetic factors may influence the degree of Kyn adduction in the human lens.

The concentration of the His-Kyn adduct found in human lens protein from the nucleus varied from a concentration of 0.01 nmol/mg of protein in a 16 year old individual to a maximum value of 3.6 nmol/mg of protein in an 72 year old individual. The concentration of the Lys-Kyn found in human lens protein was found to increase from a value of 0.008 nmol/mg of protein in a 16-year-old lens to a maximum concentration of 0.20 nmol/mg of protein in the lens of a 72-year-old individual. Although the levels of both adducts were similar in the lenses of younger individuals (< 30 years) the concentration of His-Kyn was approximately 20 fold greater than that of Lys-Kyn in the oldest lenses. Over the age range analysed, the increase in His-Kyn concentration (ca. 360 fold) is also far greater than Lys-Kyn (ca. 25 fold).

5.3.2.b. Kyn-modified Amino Acid Levels in the Cortical Region

Figure 5.2 shows the concentration of His-Kyn and Lys-Kyn in the cortices of normal human lenses. Up to the age of 70, the concentrations of both adducts remained relatively constant, however, the lenses of individuals older than ca. 70 demonstrated substantially elevated levels of both Kyn-modified amino acids. Once again, older lenses showed a considerable degree of scatter in Kyn-adduct concentrations.
Figure 5.1: Nuclear levels of Kyn-modified amino acids as a function of age of the lens. a) Lys-Kyn, b) His-Kyn.

The level of the His-Kyn adduct in the cortex was constant at a level of ca. 0.004 nmol/mg of protein in the lenses of individuals younger than 30. The His-Kyn content increased from 0.037 nmol/mg of dry protein in the sixth decade to a maximum value of 2.5 nmol/mg of dry protein in a 72 year old lens.
Figure 5.2: Cortical levels of Kyn-modified Lys and His as a function of lens age. a) His-Kyn, b) Lys-Kyn.
The level of Lys-Kyn in cortical protein displayed a similar trend to that of His-Kyn. The Lys-Kyn adduct value remained constant at ca. 0.005 nmol/mg of dry protein in the lenses of individuals < 30 years. This increased only slightly to 0.017 nmol/mg of dry protein in lenses between 50 and 60 years, but increased to a maximum value of 0.07 nmol/mg of dry protein from a 72 year old lens. His-Kyn levels were also low and relatively constant up until the seventh decade. Although the levels of both adducts were similar in the lenses of younger individual (< 30 years) the concentration of His-Kyn was more than ca. 30 times greater than that of Lys-Kyn in the oldest lenses.

Since age-related lenticular colouration is known to be more prominent in the nucleus than in the cortex, the concentration of Kyn-modified His and Lys residues in the nucleus was compared to those in the cortex for individual lenses as a function of age. The ratio of nuclear-to-cortical content of both Kyn-modified amino acids are shown in Figure 5.3. Although there is some degree of scatter in the ratios, it was found that if the nuclear level of the Kyn-modified amino acids was high the cortical level was also high for a given lens. The His-Kyn level in the nucleus is approximately 5 times that found in the cortex. This value remains quite stable across the range of ages analysed. The ratio of Lys-Kyn, however, does not remain constant, increasing from ca. 2 in the late teens to ca. 7 in the seventh decade.
Figure 5.3: Ratio of nuclear-to-cortical concentrations of the Kyn-modified amino acids with respect to age. a) Lys-Kyn, b) His-Kyn.
5.3.3. The Levels of Kyn-modified Amino Acids in the Nuclear Region of Cataractous Human Lenses

To elucidate whether modification of nuclear protein by Kyn may have a role in the development of age related nuclear cataract, protein from the nuclear region of lenses of various cataract types (graded I-IV according to the Pirie system\textsuperscript{58}) as well as aged normal lenses (70-80 years) were analysed using the acid hydrolysis method. Four lenses were analysed for each lens type and analyses were performed in duplicate. Only the nuclear protein from the lenses was analysed owing to limited availability of whole lens tissue. The quantities of His-Kyn and Lys-Kyn in each of the nuclear protein samples, plotted according to lens type are shown in Figure 5.4. As expected, a reasonable degree of variation in the data is observed, however, both His-Kyn and Lys-Kyn displayed similar trends across the range of lens types analysed. The His-Kyn level was found to fall by approximately four-fold from an initial level of 2.14 nmol/mg of protein in type I cataract to 0.53 nmol/mg of protein in type IV cataract. Type I lenses are removed from patients as a result of cortical cataract and would be expected to have values similar to those of aged normal lenses. The level Lys-Kyn fell from an initial value of 0.11 nmol/mg of protein in type I cataract to 0.03 nmol/mg of protein in type IV cataract, also a four-fold loss. Here, as in the nuclei of normal lenses, the level of His-Kyn was approximately 20 times greater than that of Lys-Kyn.
Figure 5.4: Nuclear levels of Kyn-modified Lys and His in aged normal lenses and in cataractous lenses. The error bars show standard deviation for \( n = 4 \) for each lens type. a) His-Kyn, b) Lys-Kyn.
5.3.4. Stability of Kyn-modified Amino Acids Under Physiological Conditions.

Since Cys is the preferred site of reaction for Kyn in proteins at physiological pH,\(^{195}\) it was considered that the observed differences in distribution of the Kyn moiety may reflect either the peculiar conditions present within the intact lens (e.g. protein-protein interactions), or the stabilities of the different Kyn-modified amino acids. The stability of synthetic Kyn-modified His, Lys and Cys under physiological conditions was determined by HPLC over a period of 100 hours as shown in Figure 5.5.

All Kyn-modified amino acids displayed some degree of instability under these conditions. His-Kyn was the most stable adduct, with 87% of the original concentration remaining after 90 hours of incubation while 65% of Lys-Kyn remained after this time. Cys-Kyn was the most labile of the three modified amino acids; its concentration fell to < 50% within 24 hours and 27% after 93 hours of incubation.

Decomposition of the Kyn-modified amino acids initially yields the deaminated form of Kyn. In the absence of nucleophiles, this α,β-unsaturated ketone appears relatively stable at pH 7, although it can undergo slow intramolecular cyclization to yield kynurenine yellow or react again with free amino acid to regenerate the Kyn adduct. The HPLC gradient used here did not resolve Kyn yellow (low level concentration) from the α,β-unsaturated ketone. The ability of deaminated Kyn to react again with free amino acid after decomposition, may explain the appearance of the stability curves which appear to approach equilibrium (Figure 5.5).
Figure 5.5: The stability profiles, at pH 7.2 and 37°C, for the individual Kyn-modified amino acids. a) His-Kyn (□), b) Lys-Kyn (Δ) and c) Cys-Kyn (○) and production of the α,β-unsaturated ketone ( )
5.3.5. Incubation of Cys-Kyn with Excess t-Boc-Histidine and t-Boc-Lysine Under Physiological Conditions

The α,β-unsaturated ketone corresponding to deaminated Kyn, formed from the decomposition of Cys-Kyn, could conceivably react with another amino acid. Such a pathway was investigated by incubating Cys-Kyn with a 20 fold molar excess of α-t-Boc-His and α-t-Boc-Lys at pH 7.2, resulting in the formation of t-Boc-His-Kyn and t-Boc-Lys-Kyn as shown in Figure 5.6. α-t-Boc derivatives were used to protect the α-amino group from reaction, thus mimicking the reactive sites of these residues in proteins.

![Figure 5.6](image)

**Figure 5.6:** Incubation of the Cys-Kyn adduct with a 20 fold molar excess of t-Boc-His and t-Boc-Lys at pH 7.2 and 37°C. t-Boc-His-Kyn (∇), t-Boc-Lys-Kyn (∆), Cys-Kyn (○) and the α,β-unsaturated ketone (▪).
Incubation of Cys-Kyn in the presence of the α-t-Boc amino acids resulted in the decomposition of Cys-Kyn (63% lost after 50 hours) and the formation of Kyn-modified His and Lys, as well as formation of the α,β-unsaturated ketone. Over the course of the incubation, the rate of decomposition of Kyn-Cys slowed. The levels of α-t-Boc-His-Kyn and α-t-Boc-Lys-Kyn increased during the incubation, with the concentration of α-t-Boc-His-Kyn increasing at approximately 7 times the rate of Boc-Lys-Kyn. After 50 hours of incubation t-Boc-His-Kyn was the most abundant species (1.2 μmole) compared with 0.19 μmol of Lys-Kyn formed after this time. The α,β-unsaturated ketone reached a maximum level at 22 hours (1.2 μmole).

5.3.6. Incubation of Kyn-modified Bovine Crystallin with t-Boc-Histidine Under Physiological Conditions

Although the transfer of the Kyn moiety to His and Lys was observed readily in the model system employing Cys-Kyn, it was important to see if this phenomenon could also be observed with intact proteins. The decomposition of protein-bound Kyn was therefore examined as a guide to the situation in human lenses.

Kyn-modified CLP was incubated in phosphate buffer (pH 7.2) at 37°C with a large molar excess of t-Boc-His. Figure 5.7 shows the changing distribution in Kyn-modified amino acids in CLP as a result of incubation at pH 7.2. The protein was sampled at various time intervals, purified by size exclusion, acid hydrolysed and analysed by HPLC. The small molecule fraction was analysed directly by HPLC.
Figure 5.7: The changing distribution in Kyn-modified amino acids in Kyn-modified CLP following incubation in phosphate buffer at pH 7.2 and 37°C. His-Kyn (□), Lys-Kyn (△) and Cys-Kyn (○).

Under these conditions, the redistribution of the Kyn moiety within the protein appeared to be essentially complete within 24 hours. The level of His-Kyn within the protein increased rapidly from 1.3 μmole to 2.2 μmole per μmol of protein over the initial 24 hours of incubation after which it remained constant. The increase in modified His was accompanied by a fall in the level of modifications to both Cys and Lys residues from 0.73 to 0.33 μmole/μmole of protein and 1.5 to 0.85 μmole/μmole of protein respectively after 96 hours of incubation. The level of Cys-Kyn remained relatively constant after the initial 24 hours of incubation while that of Lys-Kyn continued to decrease over the entire incubation. The formation of an α,β-unsaturated ketone and t-Boc-His-Kyn was detected in the small molecule fraction extracted from size exclusion chromatography. The formation of Kyn yellow was only detected by analytical HPLC after 24 hours of incubation.
5.4. Discussion

The purpose of the work described in this chapter was to determine the role, if any, of Kyn in age-related colouration of the human lens and the development of age-related nuclear (ARN) cataract. To achieve this, the level of Kyn modification to the nuclear and cortical protein of normal lenses and cataractous lenses was quantified using the acid hydrolysis procedure developed in chapter 4.

Analyses of nuclear protein from normal human-lenses, ranging in age from 16 to 80 years, have revealed that in vivo modification of the nucleophilic amino acids by Kyn was found to increase in concentration as a function of age. A considerable degree of scatter was apparent in these data. On the basis of other analyses of human lenses, however, this is not unexpected.\textsuperscript{12} Although there was a high degree of variability, lenses which demonstrated high levels of His-Kyn, for example, also had corresponding high levels of Lys-Kyn indicating that the observed scatter in these data was not artefactual. This also suggests that both Lys and His adducts are derived from the same phenomenon (\textit{i.e.} deamination of Kyn).

The concentrations of His-Kyn and Lys-Kyn in the nuclear region of human lenses were found to be low and constant up until about middle age (ca. 40 years). The analysis of the cortical protein from these lenses revealed that the levels of Kyn modification of His and Lys residues remained relatively constant from ca. 20 years through to the sixth decade (Figure 5.2). After 70 years, the level of His-Kyn and Lys-Kyn in cortical protein appears to increase significantly. One possible explanation for the variations in the level of Kyn modifications to crystallins observed as a function of age or lenticular region may be that the variation is related to the decrease in GSH concentration found with age and lenticular region. GSH is a nucleophile present in the lens in relatively
high concentrations, and it competes with the amino acid residues of proteins for reaction with the unsaturated ketone derivative of Kyn and therefore protects crystallins from modification.\textsuperscript{166,196,197} After middle age, the concentration of GSH in the nuclear region of the lens decreases to $< 1$ mM \textsuperscript{12,166} and this diminished protection could therefore exacerbate the non-oxidative attachment of UV-filters in this part of the lens. It has been proposed that the development of an internal lens barrier (to the diffusion of small molecules between the cortex and the nucleus) after age 40-50 may be the primary event responsible for these changes, which lead to lens colouration.\textsuperscript{61} In agreement with this, the levels of Kyn-adducts in lens nuclear protein were 2-7 fold higher than those in the cortex of a given lens. In the cortex, where the concentration of GSH is higher than in the nucleus after the second decade of life,\textsuperscript{166} the level of Kyn modification to cortical protein remains relatively low until approximately the sixth decade. This coincides with a drop in the cortical level of GSH in normal human lenses.\textsuperscript{166}

The majority of covalent modifications of human lens proteins, which may contribute to its colouration, reported to date have been found at very low levels, except for carboxymethyllysine (approximately 0.3 nmol/mg of protein)\textsuperscript{183} and 2-amino-3-hydroxyacetophenone O-$\beta$-D-glucoside (AHAG) (ca. 0.2 $\mu$g/mg of lens protein). The levels of the Kyn adducts that have been found here are comparable to those of carboxymethyllysine and AHAG suggesting that covalent binding of Kyn to lens proteins appears to be one mechanism for age-related colouration of the human lens. The full consequences of Kyn binding to lens protein are not yet understood. Kyn itself may undergo further reaction, for example oxidation, or Kyn addition potentially may render the protein photo-reactive.\textsuperscript{198} Although colouration, crosslinking and insolubilisation increase with the progression of cataract the molecular basis for these phenomena are not yet known.
If a measured parameter is really linked to the development of cataract, one would expect to see a measurable change in the lens centre in type II lenses, and for this to be more pronounced in type III and still more in type IV lenses. This is true, for example, in protein sulfhydryl groups, protein crosslinking, insolubilisation and methionine sulfoxide and hydroxylation. Such a pattern was observed in proteins His-Kyn and Lys-Kyn concentrations measured in the nuclei of types I-IV age-related nuclear cataract lenses. There, was a pronounced decrease in concentrations of both with the progression of cataract. Concentrations of the Kyn adducts in aged normal and type I cataract samples represent Kyn-modifications to protein over an entire lifetime. We do not yet know if bound Kyn derivatives participate in further reactions although it has been shown here that His-Kyn, Lys-Kyn and Cys-Kyn vary in their stabilities. The subsequent substantial fall in Kyn adduct levels with the development of nuclear cataract suggests that changes occur in the lens between cataract types I and II that make these adducts unstable. Type I cataracts have no nuclear involvement and are surgically removed because of cortical opacification. One of the most notable changes with the onset of nuclear cataract (Type II) is that the nuclear levels of GSH fall from ca. 42 nmol/lens in Type I cataract to ca. 16 nmol/lens in Type II cataract and remain at this low level through to Type IV cataract. A fall in lenticular GSH would be expected to cause an increase in Kyn binding to crystallins, however, the levels of Kyn adducts were found to be lower, rather than higher, in the nuclei of type II lenses. Since the cataractous lens becomes progressively more coloured, further reaction (e.g. oxidation) of protein-bound Kyn (and other UV-filters), is suggested by these data. Such reactions may result in the colours that characterises age-related nuclear cataract. Oxidation of Kyn-adducts may also play a role in other reactions that lead to crystallin crosslinking and insolubilisation. This finding is supported by recent work of Aquilina et al. which
showed that Kyn may significantly alter the properties of crystallins leading to their intense colouration (brown) crosslinking and insolublisation (A. Aquilina, unpublished results).

In all lenses analysed the amount of His-Kyn was consistently higher than that of Lys-Kyn particularly in older lenses. Cys-Kyn typically represented a low percentage of the total amount of Kyn-modified amino acids and in some cases was not observed at all. This result was unexpected since a model study under physiological conditions had shown that the primary site of modification of the lens protein, α-crystallin was the Cys residue. Presumably, if only the rate of reaction of deaminated Kyn with nucleophilic residues is considered, modification by Kyn should occur primarily at Cys residues over those of His and Lys residues. When averaged across all crystallins the most abundant residue is Lys (3.6%) closely followed by His (3.5%) while Cys is also present at significant levels (1.8%). The differing abundances of the various residues, therefore, does not explain why the concentration of His-Kyn is significantly greater than that of Lys-Kyn or Cys-Kyn in aged human lenses. Factors such as pKa values, nucleophilicity and protein conformation are likely to influence reactivity of individual residues towards Kyn.

The stability of the Kyn-modified amino acids may also influence the in vivo distribution. In agreement with this hypothesis, incubation of the Kyn-modified amino acids under physiological conditions revealed significant differences in their stabilities. His-Kyn was the most stable, while Cys-Kyn was the least stable, its concentration falling to ca. 50% of its original value in this time period. Decomposition of all Kyn-modified amino acids resulted in the production of an α,β-unsaturated ketone (2) along with the respective amino acid. The deamination of Kyn, the reaction of the
α,β-unsaturated ketone with nucleophilic amino acids and the decomposition of the adducts is summarised in Scheme 5.1. Once produced by deamination of Kyn, or the decomposition of a Kyn-amino acid adduct, the α,β-unsaturated ketone can react via one of two possible routes: intramolecular cyclisation forming Kyn yellow or reaction with another nucleophilic amino acid. The former is slow at pH 7 and the latter reaction can effectively regenerate the Kyn-modified amino acid.

Scheme 5.1: The formation of the Kyn modified amino acids His-Kyn, Lys-Kyn and Cys-Kyn along with Kynurenine yellow, from Kyn following deamination.

The formation of the reactive α,β-unsaturated ketone from Kyn-modified amino acids and its subsequent reaction with amino acids was modelled using the most labile of the Kyn-modified amino acids, Cys-Kyn. Under physiological pH conditions, the
incubation of Cys-Kyn in the presence of excess $t$-Boc-His and $t$-Boc-Lys resulted in a redistribution of ca. 46% of the Kyn moiety from Cys to $t$-Boc-His (40%) and $t$-Boc-Lys (6%) in 50 hours. This model system demonstrated the mobility of the Kyn moiety at pH 7.

Since the Kyn moiety was, over time, able to migrate from the least stable to the most stable Kyn-modified amino acids, it is possible that the same mechanism of Kyn transfer may exist in long lived proteins such as those in the human lens. Hence, a model system was employed with a view to characterising the behaviour of the Kyn-modified amino acids in a protein. Kyn-modified calf lens protein was incubated at pH 7 and 37°C in the presence of free $t$-Boc-His (Figure 5.7). Within the first 24 hours, the distribution of the Kyn moiety across the nucleophilic residues had changed significantly. After 96 hours 82% of the Kyn moiety released by decomposition of both Cys-Kyn and Lys-Kyn had resulted in the net formation of His-Kyn. The remaining 18% had reacted to form $t$-Boc-His-Kyn along with some 2.

In Kyn-modified CLP, the redistribution of the Kyn moiety, once eliminated from its initial site of reaction, occurs on two levels. First, the re-reaction with the protein from which it was released appears to be the most favoured since formation of His-Kyn in the protein accounted for 82% of the released Kyn. Second, if the deaminated Kyn diffuses away from the original protein it becomes available to react elsewhere, this accounted for < 18% of the Kyn released by Kyn-modified CLP. Even though the concentration of free His ($t$-Boc-His) in solution was ca. 10 times greater than that of protein His, the observed reaction of the $\alpha,\beta$-unsaturated ketone towards protein His was favoured. In the lens this dynamic process may eventually result in the formation of His-Kyn residues in preference to the other Kyn-modified amino acids.
Although not investigated here, the consequences of 3-OHKyn binding to human lens protein are likely to be even more deleterious. When 3-OHKyn binds to protein through the amino acid side chain, as described here for Kyn, the reactive $o$-aminophenol functionality is still able to undergo oxidation reactions that, in turn, may lead to localized formation of superoxide and hydrogen peroxide (see section 3.4). Oxidation of bound 3-OHKyn may also produce protein cross-links$^{149}$ and may also result in protein colouration, precipitation and cataract formation.
6. Conclusions

The aim of this project was to gain a greater insight into the roles of kynurenine and 3-hydroxykynurenine in age-related lens colouration and age-related nuclear cataract. A multifaceted approach was undertaken here with the eventual goal of the direct analysis of human lens protein for the presence of specific modifications. The initial aspect of this work involved characterization of the fragment ions observed in electrospray and tandem mass spectra of protonated kynurenine. This was complemented by a detailed investigation of autoxidation reactions of 3-OHKyn, which led to the investigation of the modification of amino acids by deaminated Kyn. Finally, a procedure involving acid hydrolysis with analysis by liquid chromatography mass spectrometry (LC-MS) was used for the determination of the levels of Kyn-modified amino acids in human lens proteins.

The study of kynurenine fragmentation demonstrated that collision induced dissociation (CID) reactions of protonated kynurenine offer a potential means for structural characterization using tandem mass spectrometry. Protonated kynurenine displayed a diverse range of fragmentation routes and mechanisms, however, three major fragmentation pathways were evident, elimination of ammonia, H₂O and CO or the imine form of glycine. Deuterium exchange experiments, coupled with product ion scans, provided the greatest source of structural information and revealed the variety of structural rearrangements that the fragment ions underwent during CID, which are not normally observed owing to the isobaric nature of the ions formed. These routes cover many aspects of conventional dissociation chemistry and this understanding of the fragmentation behaviour of protonated Kyn will aid in the structural characterization of other Kyn derivatives which may be isolated in small quantities from the lens.
The autoxidation of 3-OH Ky n was found to be complex, resulting in the formation of three major and several minor products. Two of the major species were identified as Xan and DHQCA and the third tentatively identified to be the hydroxylated form of Xan, OHXan. Xan (and Xan') were the major initial products, while DHQCA and OHXan were significant products during later stages of the autoxidation. Autoxidation of 3-OHKyn also generated mole-for-mole concentrations of $\text{H}_2\text{O}_2$ and indicated that $\text{H}_2\text{O}_2$, rather than $\text{O}_2^-$ or $\text{OH}^-$, plays an important role in the autoxidation process.

Oxidation reactions in the presence of glutathione and ascorbic acid, both lenticular antioxidants, resulted in the delayed onset of 3-OHKyn autoxidation. This effect was only apparent when the antioxidant concentrations were greater than ca. 1 mM. The sequestering of transition metals through the addition of EDTA to the reaction mixture (to chelate transition metal impurities) had no significant effect on the autoxidation process. The use of relatively metal free buffer solutions, however, did affect the rate of 3-OHKyn oxidation, indicating that transition metals may have some role in the autoxidation.

The search for possible 3-OHKyn mediated modifications of lens protein was modelled by the autoxidation of 3-OHKyn in the presence of Fmoc-Lys. Of the numerous species produced, one of particular interest resulted from the deamination of 3-OHKyn, and the deaminated from covalently binding covalently binding to Fmoc-Lys. Kyn is also readily undergoes deamination and this allowed the synthesis of the covalent adducts of Kyn and 3-OHKyn with the nucleophilic amino acids His, Lys and Cys. Following structural characterization, an investigation of the suitability of these adducts for use as biomarkers in the analysis of human lens protein was undertaken. Kyn-modified amino acids but not their 3-OHKyn analogues were found to be suited to the acid hydrolysis
procedure used. Hence only the determination of Kyn adduct levels in human lens proteins was pursued.

Proteins isolated from human lenses were found to contain significant levels of the UV-filter kynurenine, covalently bound to histidine and lysine residues. Identification was confirmed by synthesis of the kynurenine-amino acid adducts and comparison of the chromatographic retention times and mass spectra of these authentic standards with those of corresponding adducts isolated from human lenses following acid hydrolysis. Using calf lens proteins as a model, covalent binding of kynurenine to lens proteins has been shown to proceed via side chain deamination in an analogous manner to that observed for the related UV-filter, 3-hydroxykynurenine O-\(\beta\)-D-glucoside. Levels of His-Kyn and Lys-Kyn were low in the nuclei of human lenses younger than 30, but thereafter increased in concentration with the age of the individual, reaching levels as high as 3.6 nmol/mg and 0.20 nmol/mg of lens protein for His-Kyn and Lys-Kyn respectively. The levels of these adducts were in all cases 2-7 times greater in the nuclei compared with the cortex of human lenses. Post-translational modification of lens proteins by tryptophan metabolites, therefore, appears to be at least in part responsible for the age-dependent increase in colouration and fluorescence of the human lens.

In the nuclei of cataractous lenses, the levels of His-Kyn and Lys-Kyn were found to fall with the increasing severity of cataract, suggesting that Kyn may also have a role in the progression of cataract.

The levels of Cys-Kyn in human lenses were always very low or below the detection limit. Although the Kyn moiety binds covalently to lens protein, it was shown in model studies that it is able to migrate from the most labile modified amino acids to the residue that forms the most stable Kyn adduct. This was consistent with the observation that the
level of Kyn-modified Cys in human lenses was always much lower than that of His-Kyn and Lys-Kyn. It appears that the decomposition of the initially formed Kyn-amino acid adducts allows regeneration of the deaminated form of Kyn, which is then able to react with other nucleophilic amino acid residues. Hence, over time there is a redistribution of the Kyn adducts favouring the formation of the more stable His adduct.

The reaction of Kyn with protein was extensively investigated and it has also been shown that 3-OHKyn is able to modify nucleophilic amino acids residues in the absence of O₂. Since post-translational modification by Kyn and 3-OHKG has been documented, 3-OHKyn-mediated modification of lens protein is also likely to occur, however, it was not possible to quantify 3-OHKyn-mediated changes in the human lens, because of the instability of these adducts during acid hydrolysis. The consequences of covalent attachment of 3-OHKyn will probably be more deleterious than those of Kyn, since once bound, 3-OHKyn is still able to undergo oxidation, possibly resulting in the formation of protein-protein crosslinks and the localised production of hydrogen peroxide. Such a crosslink has been observed in model studies performed currently in this laboratory.

Therefore as a result of the work described here, Kyn has been shown to be involved in normal human lens colouration and also in the progression of age-related nuclear cataract. The full implications of Kyn binding to lens proteins are not yet fully understood. While the exact mechanisms and processes involved are still unclear, the work in this thesis has provided a sound basis for further attempts to define the role of Kyn and 3-OHKyn in changes to the human lens with age and in the onset of cataract.
References


