Tryptophan metabolism in the human lens

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IN THE

HUMAN LENS

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by

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DEPARTMENT OF CHEMISTRY

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DECLARATION

This is to certify that the work described in this thesis has not been submitted for a higher degree at any other university or institution,

Andrew M. Wood.
For my dearest Bernadette
This could never have been accomplished without your loving support.

For Aaron James
Having you made this a little more difficult but a lot more worthwhile.
Meanwhile the performance ended, and the amateur orchestra played the National Anthem. Conversation and billiards stopped, faces stiffened. It was the Anthem of the Army of Occupation. It reminded every member of the club that he or she was British and in exile. It produced a little sentiment and a useful accession of willpower. The meagre tune, the curt series of demands on Jehovah, fused into a prayer unknown in England, and though they perceived neither Royalty nor Deity they did perceive something, they were strengthened to resist another day. Then they poured out, offering one another drinks.

“Adela, have a drink; mother, a drink.”

They refused—they were weary of drinks—and Miss Quested, who always said exactly what was in her mind, announced anew that she was desirous of seeing the real India.

Ronny was in high spirits. The request struck him as comic, and he called out to another passer by: “Fielding! how’s one to see the real India?”

“Try seeing Indians,” the man answered, and vanished.

E.M. Forster
A Passage To India,
1924.
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ABSTRACT
The aim of this thesis was to discover more about the pathway in the human lens which leads to the formation of 3-hydroxykynurenine glucoside, the major UV-filter substance. This biosynthetic pathway has received scant attention since the discovery of the glucoside by van Heyningen in 1971.

In order to further examine the unique metabolism of tryptophan in human lenses, an intact lens organ culture system was employed. Radiolabelled tryptophan was added to the artificial aqueous humor of organ cultured-lenses and its incorporation was followed by analysis of the low molecular weight lens fraction by high pressure liquid chromatography and liquid scintillation counting.

Significant conversion of tryptophan into metabolites was observed in 24 hours. The major proportion of tryptophan radiolabel was found in 3-hydroxykynurenine glucoside, with smaller quantities detected in both kynurenine and 3-hydroxykynurenine. It was found, using tritiated 3-hydroxykynurenine, that synthesis of the 3-hydroxykynurenine glucoside is a diversion from normal tryptophan oxidative metabolism, in which 3-hydroxykynurenine is glucosylated by an enzyme system(s) localised primarily in the equatorial region of the lens.
The concentration of 3-hydroxykynurenine glucoside in the lens was, on average, approximately six times as great as the concentration of free tryptophan. Kynurenine and 3-hydroxykynurenine were present at much lower levels. No N\textsuperscript{\prime}-formylkynurenine, the immediate product of tryptophan oxidation, was detected in lens extracts.

The concentration of 3-hydroxykynurenine glucoside was found to decline from the levels in very young lenses to those aged about 40 years, after which the level was relatively constant. A similar trend was observed for the much lower concentrations of kynurenine and 3-hydroxykynurenine. No satisfactory explanation for this age-related change in lenticular tryptophan metabolites could be found.

Tryptophan, kynurenine, 3-hydroxykynurenine and 3-hydroxykynurenine glucoside were detected in the cortex and nucleus of the lens. The concentration of these compounds along the visual axis was found to be approximately twice as high as in the equatorial region. Conversion of tryptophan to 3-hydroxykynurenine glucoside, however, appeared to take place only in the organelle-containing cells of the lens. It was shown that epithelial explants from human lenses could also convert tryptophan to 3-hydroxykynurenine glucoside.
3-Hydroxykynurenine glucoside was found to efflux from cultured lenses at a linear rate. No further metabolism of 3-hydroxykynurenine glucoside was, however, detected, and it was therefore proposed that efflux represents the major, if not the only, pathway for removal of 3-hydroxykynurenine glucoside from lenses. 3-Hydroxykynurenine glucoside was also detected in human vitreous humor.

No evidence was found in the lens for the further metabolism of 3-hydroxykynurenine via the normal tryptophan oxidative pathway found in other mammalian tissues.
LIST OF ABBREVIATIONS

The following abbreviations of terms and tryptophan metabolites are used throughout this thesis. Standard terms e.g. L (litre) are not included.

TERMS

abs absorbance
AAH Artificial Aqueous Humor
dpm disintegrations per minute
HPLC High Pressure Liquid Chromatography
IDO indoleamine 2,3-dioxygenase
Km Michaelis-Menten enzyme constant
LSC Liquid Scintillation Counting
UV Ultraviolet
λ_max absorption maximum

Metabolites

Trp Tryptophan
3OHKG The O-β-D-Glucoside of 3-Hydroxykynurenine
3OHKyn 3-Hydroxykynurenine
Kyn Kynurenine
N-formylKyn N'-Formylkynurenine
3OHA 3-Hydroxyanthranilic Acid
5OHTrp 5-hydroxytryptophan
CHAPTER 1
CHAPTER 1
GENERAL INTRODUCTION

1.1 The O-β-D-Glucoside of 3-Hydroxykynurenine: A Little Known Metabolic Product of Tryptophan

Tryptophan (Trp), first identified as a result of apparently anomalous experimental results produced by early 20th century medical students (Hopkins and Cole, 1901a,b), was the first amino acid found to be nutritionally indispensable (Willcock and Hopkins, 1905), and subsequently given the label 'essential'.

The metabolism of ingested Trp as it is known in mammalian tissue is summarised in Fig. 1.1. As well as being a vital component of proteins, it is important in neurotransmission via the synthesis of serotonin (Falck, 1962; Johnson et al, 1977); the synthesis of enzyme cofactors (NAD, NADP) and, via complete oxidation to CO₂, the production of metabolic intermediates (e.g. acetyl-CoA). Decarboxylation of tryptophan gives tryptamine, a minor Trp metabolite (Hayaishi, 1976).

Not shown in Fig. 1.1 is a compound formed only in the eye lenses of humans and other primates, but not in the lens of any other species studied. This compound, a conjugate of 3-hydroxykynurenine and D-glucose, was first identified in the human eye lens by Ruth van Heyningen (1971a), and was subsequently shown by this same researcher to be a metabolic product of Trp (1973b). The O-β-D-glucoside of 3-
hydroxykynurenine (subsequently referred to as 3-hydroxykynurenine glucoside, or 3OHKG), is not as widely known as the Trp metabolite melatonin which is formed from serotonin solely in the pineal gland (Weissbach et al, 1960; Axelrod and Weissbach, 1961). That it has not been reported in any tissue other than the lens leads one to believe that it is a lens-specific molecule, and may therefore have a lens-specific function. Since the lens plays an important role in vision, it seemed worthwhile to find out more about the little known metabolic pathway that leads to the formation of 3OHKG, especially since, according to the early evidence of van Heyningen (1973,b), this compound appears to be the major Trp metabolite in the human lens.
Figure 1.1: Pathways of tryptophan metabolism.
Figure 1.2: The early steps in the oxidative pathway of tryptophan metabolism.
1.2 The Oxidative Pathway of Tryptophan Metabolism

1.2.1 Tryptophan Oxygenase or Indoleamine Oxygenase Catalyse the Cleavage of the Indole Ring to Yield N'-Formylkynurenine

The first step in the Trp oxidative metabolic pathway (Fig 1.2) is the opening of the indole ring. The formation of Kyn from Trp in liver was first observed by Kotake and Masayama (1936). They named the enzyme responsible for this process tryptophan pyrrolase. Knox and Mehler (1950) and Mehler and Knox (1950), subsequently showed that the formation of Kyn from Trp involved two enzymic steps, the initial one, involving the pyrrolase enzyme, yielding N'-formylkynurenine. The enzyme was shown to be haem dependent (Knox and Mehler, 1950; Tanaka and Knox, 1959; Hayaishi et al, 1957). Experiments involving the incorporation of $^{18}$O$_2$ into N-formylKyn demonstrated that molecular oxygen was involved in the reaction; the enzyme was therefore renamed tryptophan 2,3-dioxygenase (EC 1.13.11.11) (Hayaishi et al, 1957). In mammals, this enzyme is found only in the liver (Knox, 1955), and the rabbit liver enzyme was found to be specific for L-Trp (Tanaka and Knox, 1959). Kotake and Ito (1937), however, had previously identified D-Kyn in the urine of rabbits fed D-Trp, and demonstrated that this was not due to intestinal flora. Enzyme activity that converted D-Trp to D-Kyn was observed in rabbit small intestine homogenate (Higuchi et al, 1963; Higuchi and Hayaishi,
Having elucidated the oxygenase activity of the hepatic L-Trp specific enzyme, Osamu Hayaishi and his co-workers at The Department of Medicinal Chemistry, Kyoto University, were subsequently responsible for a great deal of research into the enzyme responsible for the initial cleavage of Trp in oxidative metabolism of extra-hepatic tissues.

Systems that generated superoxide (O$_2^-$) and H$_2$O$_2$, e.g. ascorbic acid, xanthine oxidase, glutathione reductase and their respective substrates, were found to activate the D-Trp enzyme (Yamamoto and Hayaishi, 1967). H$_2$O$_2$ itself, or H$_2$O$_2$ (but not O$_2^-$) producing systems such as those involving glucose oxidase or amino acid oxidase, however, would not support the enzyme activity (Yamamoto and Hayaishi, 1967). Superoxide was subsequently shown to be necessary for the activity (Hirata and Hayaishi, 1971; 1975) and incorporation of $^{18}$O$_2^-$ was reported in the reaction products (Hayaishi et al, 1977). The intestinal enzyme, but not the hepatic enzyme, was inhibited by superoxide dismutase (Hirata and Hayaishi, 1971).

Conversion of D-Trp to D-Kyn by tissues other than the intestine was also reported in rabbit lung, stomach, spleen and brain (Hayaishi and Hirata, 1973), and in rat brain (Gal et al, 1966; Tsuda et al, 1972). The enzyme activity was also found to be ubiquitously distributed throughout mouse (Yoshida et al, 1980),
rat (Cook et al, 1980) and human (Yamazaki et al, 1985) tissues.

In addition to the substrates L- and D-Trp, the enzyme was found to catalyse equivalent degradation of L- and D-5-hydroxytryptophan\(^1\), serotonin and tryptamine (Hirata and Hayaishi, 1972). The conversion of melatonin to N\(^{\gamma}\)-acetyl-N\(^{\beta}\)-formyl-5-methoxykynurenamine, both in vitro, and in rat brain slices in vivo, was also demonstrated (Hirata et al, 1974). All of these substrates possess both an indole ring and a substituted or unsubstituted amine group, and substrates that did not possess both these properties were not degraded. Having originally been tentatively named D-tryptophan pyrrolase, all of the discovered properties of the enzyme eventually led to it being known as indoleamine 2,3-dioxygenase (IDO) (EC 1.13.11.17). Like the hepatic enzyme tryptophan 2,3-dioxygenase, IDO was found to be haem dependent (Yamamoto and Hayaishi, 1967). Reviews of the research into this enzyme have been published by Hayaishi (1976) and Yoshida and Hayaishi (1987).

Although a range of indoleamines are substrates for IDO, the substrate with the highest activity as reported by Hayaishi (1976) was Trp. Both the D- and L- isomers had a reported \(V_{\max}\) of 140 mU mg\(^{-1}\) protein, a value fourteen times as high as that for 5OHTrp, which exhibited the next highest \(V_{\max}\). D-Trp, however, had a \(K_m\) (300 \(\mu\)M) fifteen times as high as that for L-Trp (20 \(\mu\)M). The

\(^1\) 5-Hydroxytryptophan is the intermediate formed when Trp is converted to serotonin (Fig. 1.2).
difference was even larger in a study of IDO in human tissues (Yamazaki et al, 1985). The $K_m$ for D-Trp (14.3mM) was 550 times that of the L-isomer (26μM). Although these findings indicate that the major role of IDO is the entry of L-Trp into oxidative metabolism in extra hepatic tissues, the *in vivo* metabolism of melatonin by this enzyme (Hirata et al, 1974), also indicates that IDO may be responsible for further metabolism of other Trp metabolites.

The induction of IDO was observed in the lungs of mice during viral infection and after intraperitoneal administration of bacterial lipopolysaccharide (Yoshida et al, 1979, 1981). The activities of other murine lung enzymes were not significantly altered during the infections. The immune inflammatory response can result in the stimulation of $O_2^-$ release from polymorphonuclear cells and macrophages (for review see Flohe et al, 1985). The induction of IDO during infection may therefore be a means of 'mopping up' the high levels of $O_2^-$. IDO induction was later shown to be due to interferons (Ozaki et al, 1988), a family of proteins that exert antiviral, cell-growth regulatory and immunomodulatory actions. Subsequently, it has been suggested that the depletion of available Trp may be important for control of rapidly dividing microbial pathogens and tumours (Carlin et al, 1989). Hydroxylated tryptophan metabolites e.g. 3-hydroxyanthranilic acid and 3-
hydroxykynurenine, have also been shown to be highly efficient scavengers of peroxyl radicals (Christen et al, 1990). Therefore the formation of these metabolites from Trp and the known requirement of IDO for $O_2^-$, a highly reactive species, suggests that the 100-fold induction of IDO reported in the lungs of mice following infection with viral pneumonia may represent an antioxidant defence system against inflammatory diseases which are known to be associated with a generalised oxidative environment (Flohe et al, 1985). It is also therefore conceivable that the metabolism of Trp in the human lens may aid in preventing the oxidation of crystallins.

1.2.2 A Non-specific Formamidase Hydrolyses N'-Formylkynurenine to Kynurenine

The hydrolysis of N'-formylkynurenine (N-formylKyn) to yield Kyn is catalysed by an enzyme that releases formate from a variety of arylformylamines. This formamidase (EC 3.5.1.9) does however show a preference for N-formylKyn (Greenberg, 1969). Since the activity of the enzyme is extremely high, and also because N-formylKyn readily undergoes non-enzymic hydrolysis (Dalgliesh, 1952) the apparent product of tryptophan oxygenase (or indoleamine oxygenase) is Kyn rather than N-formylKyn.
1.2.3 The Metabolism of Kynurenine

The next step in the pathway was found to be the hydroxylation of Kyn to 3OHKyn in a reaction catalysed by liver and kidney mitochondria (de Castro et al, 1956). The enzyme responsible for this reaction, kynurenine 3-hydroxylase (also called kynurenine 3-monooxygenase) (EC 1.14.12), was subsequently shown to exist in the outer mitochondrial membrane (Okamoto et al, 1967). It is specific for L-kynurenine and hydroxylation occurs only at the 3-position. Kynurenine 3-hydroxylase is also NADPH dependent.

Both Kyn and 3OHKyn are substrates for kynureninase (EC 3.1.3). This enzyme catalyses the hydrolytic cleavage of the side-chain, releasing alanine and either anthranilic (from Kyn) or 3-hydroxyanthranilic (from 3OHKyn) acid (Wiss and Fuchs, 1950). Braunstein et al (1949) found that the activity was greatly reduced in livers of pyridoxine deficient animals, but normal activity was restored in vitro by addition of pyridoxal phosphate. Subsequent investigations have shown conclusively that kynureninase is pyridoxal dependent (Wiss, 1949; Knox, 1953; Longenecker and Snell, 1955).

Although kynureninase can act on either Kyn or 3OHKyn, Wiss and Fuchs (1956) found that the enzyme from liver splits 3OHKyn about twice as rapidly as Kyn. Tanizawa and Soda (1979) reported a lower K_m for 3OHKyn ($1.3 \times 10^{-5}$) than for Kyn ($7.7 \times 10^{-4}$). Bender
and McCreanor (1985) showed that the initial rate of Kyn hydroxylation in rat liver is approximately three times as great as the initial rate of cleavage. These studies demonstrate that, although kynureninase acts directly on Kyn, under normal metabolic circumstances the major pathway for Kyn involves hydroxylation followed by kynureninase cleavage to 3-hydroxyanthranilic acid (and alanine).

Whilst tryptophan oxygenase is normally the rate-limiting enzyme in Trp oxidative metabolism, the basal activities of kynurenine hydroxylase and kynureninase are of the same order of magnitude as for this enzyme (Bender and McCreanor, 1985). As noted earlier, tryptophan oxygenase is a haem dependent enzyme, and its activity in rat liver can be increased by administration of haem (Badawy and Evans, 1975). Under circumstances of such increased tryptophan oxygenase activity, both kynurenine hydroxylase and kynureninase may be potential secondary rate-limiting enzymes of Trp oxidative metabolism.

Although the majority of Kyn is hydroxylated to 3OHKyn which is subsequently metabolised to 3OHA, kynurenine amino transferase (EC 2.6.1.7) can act on either Kyn or 3OHKyn to yield kynurenic or xanthurenic acid respectively. These are relatively unfavoured reactions, however, as the $K_m$ of the aminotransferase ($1.8 \times 10^{-3}$) is much higher than that of either kynurenine hydroxylase or kynureninase (Ueno et al, 1963). Therefore
transamination becomes significant when the other two enzymes are saturated, leading to an accumulation of Kyn and 3OHKyn (Bender, 1982). In addition, pyridoxal phosphate (vitamin B₆) deficiency leads to an increased excretion of xanthurenic and kynurenic acids due to a reduction in the activity of kynureninase (Lepkovsky and Nielsen, 1942; Knox, 1953).

1.2.4 3-Hydroxyanthranilic Acid is Converted to Acroleyyl Aminofumarate; a Branchpoint Of Trp Metabolism

The action of 3-hydroxyanthranilate oxygenase (EC 1.13.1.6) on 3OHA results in the formation of 2-acroleyl-3-aminofumarate. This metabolite may undergo non-enzymic cyclisation to quinolinic acid which continues down the pathway for production of nicotinamide nucleotides. Alternatively, picolinic carboxylase (EC 4.1.1.45) catalyses the formation of aminomuconic semialdehyde. This compound can undergo non-enzymic cyclisation to picolinic acid (Mehler and May, 1956; Mehler et al, 1958), or undergo further enzymic metabolism leading to complete oxidation, via Acetyl-CoA, to CO₂ and H₂O. This is the pathway followed by the majority of Trp that enters the oxidative pathway (Bender, 1982).

1.3 The Human Lens

The role of the lens is essentially to filter light and to control the further refraction of light that, having passed the cornea,
requires 'fine' focusing onto the retina. In mammals, this fine focusing is accomplished by contracting or relaxing the ciliary muscles (which are attached to the lens and to the ciliary body surrounding the eye by the suspensory ligaments), changing the lens curvature and hence its focal length (Curtis, 1979).

Unlike in any other organ, lens cells are never discarded. Instead of being replaced, older cells are surrounded by new cells. The cells derived from cuboidal epithelial cells differentiate and elongate into fibre cells, eventually losing all organelles, including nuclei. This lack of organelles, along with the orientation of the lens structural proteins—the crystallins—allows the passage of light through the lens with as little scattering as possible, thus facilitating the formation of the image on the retina. The lens is sandwiched between two bodies of transparent fluid; the aqueous humor on its anterior side and vitreous humor on its posterior side. The adult lens has no nerve or direct vascular supply; nutrients are transported from, and metabolic waste products removed into, the surrounding humors—mainly the aqueous humor. Aqueous humor is derived principally from plasma (by passive diffusion) and the ciliary epithelium (by passive diffusion and by active secretion) (Cole, 1984).
The lens can be divided into three distinct compartments: the nucleus, the cortex and the capsular region (Fig 1.3). The nucleus contains the very oldest cells, and is essentially the differentiated cells of the foetal lens. The cortex, which makes up the bulk of the lens body, consists of all the lenticular cells apart from the nucleus and the young cells of the anterior epithelium. The lens capsule, a collagenous layer which covers the outer surface of lens, has, beneath its entire anterior surface and a small part of its posterior surface, a single layer of fully functional epithelial cells. This layer is a metabolic 'powerhouse' of the lens, responsible for the active transport or diffusion of amino acids, sugars (e.g. glucose) and other small molecules into the lens from the aqueous humor and the
removal from the lens of metabolic waste products (e.g. lactate). Newly differentiated cortical cells retain their organelles and make up the 'lens bow', or 'bow region', the cells of which are also metabolically active. The remaining cortical cells which have lost their organelles, although unable to carry out, for example, mitochondrial dependent enzyme reactions, are still metabolically active; their major function appearing to be the synthesis of crystallins. The cells of the nucleus are not thought to synthesise protein, and are generally considered metabolically sluggish.

Although synthesis of crystallin is carried on throughout life, the turnover of these proteins is extremely slow. This means that, in the nucleus, just as the cells themselves are a remnant of the earliest stage of life, some of the proteins are also as old as the individual itself. The human lens is therefore an ideal subject for studies in aging (Dilley and Harding, 1975).

1.4 Unique Tryptophan Metabolism in the Primate Lens

The protein-free dialysate from a preparation of sixty lenses obtained from cataract operations, yielded fluorescent material that was separated from ninhydrin-positive compounds using column chromatography (van Heyningen, 1971a). This strongly yellow coloured material, lyophilised and applied to paper, separated into several fluorescent bands upon electrophoresis at pH 1.6. The major band, possessing a blue-white fluorescence, yielded equimolar
quantities of L-3-hydroxykynurenine and D-glucose when treated with β-glucosidase. 3OHKyn was identified by several methods, including comparing its UV spectrum with that of authentic compound; and treatment with kynureninase, which yielded alanine. Glucose was identified by enzymic treatment with hexokinase, glucose-6-phosphate dehydrogenase and glucose oxidase. Although the glucoside was found in the lenses of humans and other primates it was not identified in lenses of usual domestic or laboratory animals (van Heyningen, 1971a). The first measurement of the concentration of this compound in a cataract lens preparation gave an average value of 0.65 μmol g⁻¹ fresh lens. The compound was also found in normal human lenses.

Further studies by van Heyningen (1971b), showed that the fluorescent material isolated from lenses of humans, baboon (Papio spp.) and the grey squirrel (scurius carolensis leucotis) had an absorption peak at approximately 368 nm. Paper electrophoresis and chromatography of lens extracts from these three species indicated that the major fluorescent compound in the baboon lens was the same as that in humans—3-hydroxykynurenine glucoside. The squirrel pigment however ran differently from 3OHKG, and tests indicated that it was N-acetyl-3-hydroxy-L-kynurenine.

The major fluorescent compound in primate lenses, then, is a conjugate of glucose and 3OHKyn, the latter compound being a catabolite of the oxidative Trp pathway (Fig. 1.2). This glucoside
was confirmed as a metabolite of Trp by van Heyningen (1973b) using an intact human lens organ culture experiment. A lens was incubated for 20 hr in medium containing DL-[methylene-\textsuperscript{14}C]-tryptophan. The protein free-extract of this lens was subjected to paper electrophoresis and chromatography. An autoradiograph of the paper following this treatment is reproduced in Fig. 1.4.

The most intense area of radioactivity was found to be due to Trp, followed by 3OHKKG then Kyn. There were also three other lightly labelled areas but tests established that none of them was alanine\textsuperscript{1}, 3OHKyn, kynurenic acid or xanthurenic acid (see Fig. 1.2).

The identification of both Kyn and 3OHKKG in this experiment suggests that the formation of the glucoside is a diversion from the normal oxidative Trp pathway. From what is known about this pathway in other mammalian tissues (Section 1.2), indoleamine dioxygenase is most likely the enzyme that catalyses the initial cleavage of the Trp indole ring to form N'-formylkynurenine, as tryptophan oxygenase has only been found (in mammals) in the liver. N-formylKyn may be converted to Kyn via the action of formamidase (Section 1.2.2), and Kyn hydroxylated to 3OHKyn by kynurenine hydroxylase (Section 1.2.3). Although not detected in the result shown in Fig. 1.4, van Heyningen did report detection of trace amounts of 3OHKyn in another study (1973a).

\textsuperscript{1} She did mention that radiolabelled alanine was detected from radiolabelled Trp in one experiment.
Figure 1.4: Result of paper electrophoresis (pH 1.6) and chromatography (n-butanol:acetic acid: water 4:1:1) of the protein-free extract of a lens following incubation for 20 hr in a medium containing DL-[methylene-14C]-tryptophan. T, T' were due to Trp and an artificial radio-breakdown compound. F2 and F3 were 3OHKG and Kyn, respectively. The question marks represent areas of unidentified label. F1 was due to a fluorescent compound that was not radiolabelled. The lines represent the mobility of standard compounds. (reproduced from van Heyningen, 1973b).

Since kynurenine hydroxylase is a mitochondrial enzyme (Section 1.2.3), this series of reactions must be restricted in the lens to the anterior epithelial cell layer and the bow-region (Fig. 1.3), as the remaining bulk of the cells do not possess organelles, including mitochondria (Section 1.3).
It is following the formation of 3OHKyn that Trp metabolism in the human lens diverges from the normal pathway, as in the lens this compound is conjugated with glucose instead of following the normal pathway to 3-hydroxyanthranilic acid (Fig 1.2). Such a conjugation is not common in mammals. The only other known occurrence of a mammalian glucoside is a conjugate with bilirubin in dog bile (Heirwegh et al 1970; Fevery et al 1971). This glucoside is most likely formed as an alternative route of detoxification which usually involves glucuronide conjugation.

The enzyme that catalyses the formation of 3OHKG in the lens is not known, although studies with human liver microsomal fractions aimed at understanding bilirubin glucoside formation suggest that it is probably a uridine diphosphate (UDP) glucosyl transferase (Wong 1972).

Whatever the exact mechanism of 3OHKG formation, it appears to be an efficient one, as this compound is present in the lens in much higher quantities than its probable precursor 3OHKyn (van Heyningen, 1973a).

Despite the uniqueness of 3OHKG, only one other significant investigation has been carried out on this compound since the pioneering work of van Heyningen in the early 1970's. Bando et al (1981) isolated it from single lens extracts using column chromatography. It was found that its concentration in the lens decreased from infancy to 30-40 years of age, after which it
remained at a constant level. Only nine lenses were used in this study. The UV-spectrum of purified 3OHKG yielded $\lambda_{\text{max}}$ values of 263 nm and 365 nm; slightly shifted from the spectrum of 3OHKyn (267, 368 nm).

**1.5 The Possible Roles of 3-Hydroxykynurenine Glucoside**

All ultraviolet rays below 293 nm are completely absorbed by the cornea (Bachem, 1956) and cannot therefore have any direct effect upon the lens or retina. The presence in the human lens of a compound with a broad absorption centred around 365 nm (Bando et al, 1981) would indicate that a major role of this compound is to protect the retina from potentially damaging longer wavelength UV radiation that passes through the cornea.

The lenses of a variety of species contain UV-absorbing compounds. In primates these pigments are derived from Trp metabolism (van Heyningen, 1973b). In the grey squirrel the UV-absorbing pigment was found to be N-acetyl-3-hydroxykynurenine (van Heyningen, 1971b and confirmed by Zigman and Paxhia, 1988) which could also be derived from Trp. As well as terrestrial mammals, several species of shallow-dwelling fish also contain UV-absorbing pigments (Kennedy and Milkman, 1956; Bon et al, 1968; Zigman and Gilbert, 1978; Dunlap et al, 1989). Although some of these pigments have similar UV-spectra to kynurenines (Zigman, 1987), many are non-fluorescent and have been identified as
mycosporine-like amino acids (Dunlap et al, 1989; Thorpe et al, 1992b). Some fish species, however, do contain kynurenine-like pigments. In the gourami *Trichogaster trichopterus*, the pigment is 3OHKyn (Truscott et al, 1993), and several freshwater species were found to contain this Trp catabolite (Thorpe et al, 1992b).

Another possible role of these compounds is to enhance image quality by absorbing wavelengths that are most likely to cause chromatic aberration and light scatter (Walls and Judd, 1933a,b; Muntz, 1973, 1976; Douglas and McGuigan, 1989).

Since the most likely role of lens pigments is to absorb UV-radiation, it is interesting that kynurenine has been identified in the lens of the deep-sea fish *Stylephorus chordatus* (Thorpe et al, 1992a). The role of this fluorescent compound in a habitat where UV-rays cannot penetrate has yet to be elucidated, but maybe related to the filtering of bioluminescence (Thorpe et al, 1992a).

In the human lens 3OHKyn is apparently converted to 3OHKGG, a compound with very similar light absorbing qualities. Although its metabolic precursor can also perform this function, 3OHKyn is subject to both photochemical (Tomoda, et al, 1990) and non-photochemical oxidation (see Section 1.6). Glucosylation may also therefore take place in order to convert a relatively less stable UV-absorbing molecule into a more stable one, whilst maintaining maximum protection for the retina and lens from UV-radiation.
1.6 The Reactive Tryptophan Hypothesis of Human Nuclear Cataract Formation

Abnormal tryptophan metabolism is associated with a variety of medical conditions (for reviews see Bender, 1982 and Bender et al, 1987). Tryptophan and cataract have been linked for some time. Although an experimental cataract in rats can be induced by feeding a diet deficient in the essential amino acid (McAvoy et al, 1979), almost all the suggestive evidence for an involvement of Trp in cataract have come from experiments on humans.

There have been conflicting findings on the level of Trp in the serum of cataract patients compared with controls. Cotlier and Sharma (1980, 1981), Cotlier et al (1981) and Chadwick et al (1981), reported elevated levels of serum Trp in cases of cataract while Allegri and Angi (1981) and Libondi et al (1983) found no statistical difference in the serum Trp levels between cataract patients and control subjects. A more recent exhaustive study by Elderfield and Truscott (1993), into the levels of Trp and its metabolites in serum of cataract patients and age-matched controls favours the contention that there is in fact a difference in Trp levels between the two groups but that the levels of total Trp in cataract patients is lower than that of age-matched controls. Nevertheless, there is other evidence that a reactive Trp metabolite may be involved in nuclear cataract formation.

Insect eyes contain ommochromes; strongly pigmented
compounds that are derived from 3OHKyn (Butendant and Schafer, 1962; Linzen, 1967). In the cocoon of the silk moth, the active 'tanning' agent is 3OHA (Brunet, 1967; Brunet and Coles, 1974). Manthey et al (1992) subsequently demonstrated that one pathway for the tanning reaction involves coupling of 3OHA with tyrosine to form a benzocoumarin derivative. Other phenolic compounds have been implicated in the cross-linking of proteins in sclerotized insect cuticle (Anderson, 1971).

Carlin and Cotlier (1971) described low β-glucosidase activity in human lens homogenates. An increase in such a hydrolytic enzyme or, for some reason, the lack of glucosylation of 3OHKyn would result in increased levels of 3OHKyn in the lens, the oxidation products of which may be constituents of the dark insoluble part of nuclear cataracts. The sclerotising agent in the hardening of cockroach egg cases is 3,4-dihydrobenzoic acid, the reaction of which with protein is catalysed by a phenoloxidase. Prior to hardening the 3,4-dihydrobenzoic acid exists as the 4-O-β-glucoside, which is inert to the phenoloxidase. Sclerotisation can take place only on the release of a glandular β-glucosidase (Brunet, 1967). Although it has been identified in mammals only in the primate lens, 3-hydroxykynurenine glucoside has been identified previously in the silkworm Bombyx mori (Linzen and Ishiguro, 1966), and perhaps a similar reaction series occurs in that species.

The presence in the human lens of 3OHKyn, and the
knowledge that a related Trp metabolite, 3OHA, is utilised for protein cross-linking in the insect world (Brunet, 1967), led van Heyningen (1971a) to speculate that 3OHKyn may be involved in the protein cross-linking associated with the darkening of the lens nucleus in human cataract; in effect, that lens proteins become tanned or sclerotized as do moth cocoons or insect cuticle.

van Heyningen reported that no evidence was found, however, for the presence of 3OHKyn in chemical combination with brown material from nuclear cataracts; nor was any evidence found for N-acetyl-3OHKyn in chemical combination with squirrel lens proteins. In addition there were no ommochromes detected in the black nuclei of cataract lenses (van Heyningen, 1973b).

The product of 3OHKyn in normal Trp oxidative metabolism, 3-hydroxyanthranilic acid, was found to produce rapid brown colouration when incubated under oxidising conditions with a range of proteins including human crystallins (Truscott and Martin, 1989). The formation of the brown colouration coincided with a rapid decrease in sulphydral content. Further treatment of the proteins with H₂O₂ resulted in oxidation of methionine residues. A high percentage of both cysteine and methionine were found to be oxidised in the advanced nuclear cataractous lens (Truscott and Augusteyn, 1977) and H₂O₂ was found to be higher in the aqueous humor of some cataract patients compared with control patients (Spector & Garner, 1981; Spector, 1984). If the lens is capable of
normal Trp oxidative metabolism, then potentially 3OHA may form in the lens in a high enough concentration to cause protein aggregation\(^1\). This situation could theoretically occur if there is also a higher than normal oxidation potential in the lens. Such a state could be brought about by a decrease in the level of glutathione. The concentration of glutathione has been shown to decrease with aging in the human lens (Harding, 1970; Rathburn and Murray, 1991).

A recent study has shown that 3OHKyn is capable of modifying proteins under oxidising conditions (Stutchbury and Truscott, 1993).

Although there are several models for a reactive Trp metabolite being involved in cataract formation, at the present time there is no direct evidence of this in the lens. The best way to begin to answer whether or not Trp is involved in cataract formation is to find out more about the little studied metabolism of this amino acid in the human lens.

\(^1\) Ogino and Ichihara (1957) reported that treated urine from human cataract patients, but not that from controls, caused rapid development of cataract when injected into scorbutic guinea pigs. The cataractogenic agent was identified as 5-hydroxyanthranilic acid. This compound has been identified as a metabolite of anthranilic acid (Ueda et al, 1978).
CHAPTER 2
MATERIALS AND METHODS (GENERAL)

2.1 Materials

2.1.1 Human Lenses

Lenses were obtained post-mortem from eyes donated to The Sydney Eye Hospital Lions Eye Bank for possible corneal transplants. The lenses were dissected from the eyes by staff at the Sydney University Division of Ophthalmology located within The Sydney Eye Hospital and placed immediately into 5.0 mL sterile artificial aqueous humor (Section 2.2.2) in 30 mL plastic screwcapped vials (Bacto Laboratories Pty. Ltd., Liverpool, N.S.W., Australia) that had been sterilised by autoclaving (Section 2.2.1). The vials containing lenses were incubated at 35°C until transported to the laboratory by courier (a journey of 1 to 1.5 hr), usually within 24 hours. Permission to use this human tissue for experimental purposes was granted via application to the Human Experimentation Ethics Committees of The University of Wollongong and The Eastern Sydney Area Health Service.

2.1.2 Tissue Culture Medium

Auto-Pow version Minimum Essential Medium, Eagle (Modified); without glutamine or sodium bicarbonate, was purchased from ICN Biomedicals Australasia Pty. Ltd., Sydney, Australia. The medium was supplied in powder form in satchels.
requiring reconstitution to 1 L. The content of this medium is listed in Table 2.1. (Eagle, 1959; Yamane et al, 1968).

Table 2.1: Constituents of the Medium used for Whole Lens Organ Culture

<table>
<thead>
<tr>
<th>INGREDIENT</th>
<th>mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Arginine-HCl</td>
<td>126.4</td>
</tr>
<tr>
<td>L-Cystine,Na2</td>
<td>28.42</td>
</tr>
<tr>
<td>L-Histidine-HCl-H2O</td>
<td>41.90</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>52.50</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>52.50</td>
</tr>
<tr>
<td>L-Lysine-HCl</td>
<td>73.06</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>14.90</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>33.02</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>47.64</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>10.20</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>36.22</td>
</tr>
<tr>
<td>L-Valine</td>
<td>46.90</td>
</tr>
<tr>
<td>D-Ca Pantothenate</td>
<td>1.00</td>
</tr>
<tr>
<td>Choline Bitartrate</td>
<td>1.80</td>
</tr>
<tr>
<td>Folic Acid</td>
<td>1.00</td>
</tr>
<tr>
<td>i-Inositol</td>
<td>2.00</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>1.00</td>
</tr>
<tr>
<td>Pyridoxal-HCl</td>
<td>1.00</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.10</td>
</tr>
<tr>
<td>Thiamin-HCl</td>
<td>1.00</td>
</tr>
<tr>
<td>CaCl2-2H2O</td>
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</tr>
<tr>
<td>Dextrose</td>
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<tr>
<td>MgSO4</td>
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<tr>
<td>KCl</td>
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<tr>
<td>NaCl</td>
<td>6800.0</td>
</tr>
<tr>
<td>NaH2PO4-H2O</td>
<td>140.0</td>
</tr>
<tr>
<td>Succinic Acid</td>
<td>75.0</td>
</tr>
<tr>
<td>Na Succinate.6H2O</td>
<td>100.0</td>
</tr>
<tr>
<td>Phenol Red, Na</td>
<td>17.0</td>
</tr>
</tbody>
</table>

2.1.3 Chemicals

*Acetic acid (glacial), analytical reagent, was purchased from BDH Chemicals Pty. Ltd., Kilsyth, Vic., Australia.
Aqueous Counting Scintillant (ACS) was purchased from Amersham Australia, Pty. Ltd., Sydney.

HEPES (N-2-hydroxyethylpiperazine-N’-2-ethane sulfonic acid; cell culture tested), sodium acetate (trihydrate) and L-glutamine (cell culture tested) were purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A.

Milli-Q water was supplied through Millipore Pty. Ltd., Sydney, Australia.

Penicillin/streptomycin solution (50,000 U mL\(^{-1}\) penicillin/50 mg mL\(^{-1}\) streptomycin in 0.9% saline) was purchased from Boehringer Mannheim Australia Pty. Ltd., Sydney.

2.1.4 Radiochemicals

L-[\(^{14}\)C(U)]-Tryptophan; Specific Activity 611.8 mCi mmol\(^{-1}\); Concentration 20 \(\mu\)Ci mL\(^{-1}\), 0.033 \(\mu\)mol mL\(^{-1}\), was purchased from Du Pont (Australia) Ltd., Sydney.

L-[\(^{5}\)H]-Tryptophan; Specific Activity 32 Ci mmol\(^{-1}\); Concentration 1.0 mCi mL\(^{-1}\), was purchased from Amersham Australia Pty. Ltd., Sydney.

Glucose, D-[\(^{14}\)C(U)]-; Specific Activity 2.3 mCi mmol\(^{-1}\), was purchased from Du Pont (Australia) Ltd., Sydney.
2.2 Human Lens Organ Culture

2.2.1 Sterilisation

Materials and solutions required to be sterile for lens organ culture were autoclaved under standard conditions for the elimination of microorganisms including bacterial spores. The sterilisation protocol used was 121°C at 15 kPa pressure for 15 min.

Where fluids could not be autoclaved (e.g. L-glutamine) the appropriate volume was sterilised by filtering through a 0.22 μm membrane attached to a syringe. These syringe filters were purchased from ICN Biomedicals Australasia Pty. Ltd., Sydney.

2.2.2 Artificial Aqueous Humor

One satchel of tissue culture medium (Section 2.1.2) and 2.383g HEPES were dissolved, with stirring, in 800 mL Milli-Q water. The fluid was divided into 5x160 mL portions in 250 mL Pyrex bottles with screwcap lids (Corning Inc., NY, U.S.A.). The lids were left slightly loose and the fluid sterilised by autoclaving. After autoclaving, the lids were tightened, the bottles allowed to cool to room temperature and then stored at 4°C.

Completion of the artificial aqueous humor (AAH) was carried out in a Biological Safety Cabinet Class II (Email Westinghouse Pty. Ltd., Sydney, Australia) by addition of the following supplements to one bottle containing 160 mL of sterilised medium:-

1) 2.0 mL of 200mM L-glutamine solution in Milli-Q water
sterilised via a 0.22 \mu m filter;

2) 1.6 mL sterile (autoclaved) 1M NaOH (the medium changes colour from yellow to red due to the presence of phenol red indicator);

3) 0.4 mL penicillin/streptomycin sulphate solution (purchased sterile);

4) 36mL sterile (autoclaved) Milli-Q water.

The pH of 5 mL aliquots of AAH were checked and the pH of the sterile medium adjusted to 7.4 by dropwise addition of 1M NaOH or 1M HCl. The final AAH, containing 10mM HEPES, 2.0mM L-glutamine and 200 \mu g mL\(^{-1}\) streptomycin sulphate/200IU penicillin G, as per Marcantonio and Duncan (1983), was stored between uses at 4°C.

2.2.3 Lens Organ Culture

All manipulation of the lenses took place in the biological safety cabinet under sterile conditions. On arrival at the laboratory, lenses were transferred via a plastic spoon sterilised by swabbing in 70% alcohol to a piece of absorbent paper sterilised under UV light in the safety cabinet. The lens was blotted dry (and also any adhering vitreous humor was removed) by gentle rolling on the paper using the spoon, then placed in a 35mm diameter sterile plastic petri dish (Corning Inc., NY, U.S.A.) containing 4.5 mL AAH prewarmed to 35°C. The petri dishes with AAH were weighed
before and after addition of the lens in order to obtain the lens weight. This procedure was demonstrated by Dr. Julia Marcantonio of The University of East Anglia, Norwich, England, who advised on the setting up of the lens organ culture system.

Lenses were incubated at 35°C and the AAH changed every 24 hr by removing the old AAH with a sterile pasteur pipette and adding fresh AAH. Only lenses that appeared to have no damage to the capsule were used in lens organ culture and care was taken not to damage the capsule while manipulating the lens or changing the AAH.

2.3 Preparation of Lens Extracts

At the completion of an experiment involving lens organ culture, lenses were removed from the AAH, rinsed for approx. 30 sec in 0.9% NaCl or in fresh AAH, blotted dry on filter paper, transferred to 1.5 mL Eppendorf tubes and frozen at -20°C until analysed. When required for analysis, the lenses were allowed to thaw and were then homogenised in 0.5 mL 80% ethanol using a teflon homogeniser manufactured to fit the 1.5 mL tubes. The homogenate was left in an ice-bath (0°C) for 60 min, then centrifuged at 15,000 g for 15 min. The supernatant was collected and the pellet containing the lens protein re-extracted in a further 0.5 mL 80% ethanol. The supernatants from the two homogenates were combined and any remaining protein removed by
ultrafiltration through a polysulfone 10,000MW cut-off filter centrifuged at 15000 g (Activon Scientific Products Co. Pty. Ltd., Sydney, Australia). The ultrafiltrate was lyophilised and the protein pellet from the homogenates was stored at -20°C.

2.4 Analytical HPLC

2.4.1 Mobile Phase

The mobile phase (pH 4.5) was prepared according to the following formula (Elderfield et al, 1989):

1) 115 mL 200mM acetic acid (11.5 mL glacial acetic acid per L);

2) 85 mL 200mM sodium acetate (27.20 g CH₃COONa.3H₂O per L);

3) Combined and made up to 2 L with Milli-Q water.

The pH was adjusted to 4.5 using 1M HCl or 1M NaCl.

2.4.2 HPLC System

The HPLC system consisted of a K35D pump (ICI Instruments, Sydney, Australia), a Rheodyne 7125 sample injector fitted with a 100 μL sample loop and a Knauer Variable Wavelength Monitor. Chromatograms were recorded and peak areas integrated on a Shimadzu CR6A Chromatopac integrator. Analytical separations were performed on a 250 mm x 4.6 mm Spherisorb S5ODS2 column preceded by a 2 cm C130B guard column packed with C₁₈ packing.
material (Activon Scientific Products Co. Pty. Ltd., Sydney, Australia). The flow rate was 0.6 mL min\(^{-1}\).

2.5 Liquid Scintillation Counting

Two liquid scintillation counters were employed during the course of this project to measure the \(^3\)H- or \(^{14}\)C- label contained in AAH, lens tissue extracts or HPLC eluents. Aqueous Counting Scintillant (ACS), 10 mL per 1 mL sample was used in 28 mm scintillation vials (Bacto Laboratories Pty. Ltd., Liverpool, N.S.W., Australia). Samples were routinely counted for 5 min each.

2.5.1 Beckman LS 6000TA Counter

This machine possessed an Auto-DPM function that made running quench curves unnecessary when calibration standards were run prior to each batch of samples. This function was checked by counting samples of known dpm.

2.5.2 LKB 1219 counter

This machine required the calculation of quench curves for accurate determination of dpm values. An equal amount of \(^3\)H- or \(^{14}\)C- label was added to two series of 10 scintillation vials and 10 mL ACS added to each.

For \(^3\)H, 10 \(\mu\)L of L-[5-\(^3\)H]-tryptophan (Section 2.1.4) was
diluted to 1 mL in HPLC buffer and 10 μL added to each vial. This was equivalent to 222,000 dpm per vial.

For $^{14}$C, 10 μL of D-$[^{14}$C(U)]-glucose (Section 2.1.4) was diluted to 1 mL in HPLC buffer and 10 μL added to each vial. This was equivalent to 22,200 dpm per vial.

Varying amounts of CCl$_4$ as quenching agent (0, 5, 10, 15, 20, 30, 50, 75, 100 and 150 μL) were added to each vial and the quenched standards counted for quench curve calibration. The counting efficiencies ranged from 5-50% for the $^3$H curve and 75-90% for the $^{14}$C curve.
CHAPTER 3

THE DEVELOPMENT OF A LENS ORGAN CULTURE SYSTEM FOR THE INVESTIGATION OF TRYPTOPHAN METABOLISM IN HUMAN LENSES. IDENTIFICATION OF TRYPTOPHAN METABOLITES

3.1 Introduction

Most organs are linked by a nerve and blood supply and therefore it is difficult or impossible to maintain them in a viable state after removal from the body. Since the lens has neither blood nor nerves it is an ideal subject for 'whole organ' investigations. It is also one of the very few candidates for examining human metabolism in an excised organ.

The successful propagation of mammalian cells in culture created the opportunity for the study of metabolism at the cellular level. Both the cells and medium in which they are maintained and propagated can be analysed (Eagle, 1959), allowing the processes of metabolic breakdown within the cells as well as the output of metabolic by-products to be followed using a biological probe, such as a radiolabelled tracer. Cultures of normal and human cell lines, in particular, have been invaluable in the development of new treatments for human cancers. The advantage of using human cells in an in vitro system is that the metabolic processes studied are likely to be of greater relevance than those in an in vivo animal model, due to species variation.

Commercial tissue culture media containing all the essential
components for cell maintenance have been available for nearly four decades. One such medium (Yamane et al, 1968), has been one of those used successfully to maintain whole eye lenses, including those from human subjects. Previous studies of human lens metabolism under organ culture conditions have involved the use of radiotracer techniques to investigate active transport processes (Maraini and Pasino, 1983) and protein synthesis (Ringens et al, 1982). Hightower and Kinsey (1980), used electrophysical techniques to study bioelectric properties of post mortem lenses. They found that the normal membrane potentials of lenses stored at 4°C were recovered when the lenses were placed in culture at 37°C.

Whole lens organ culture is likely to prove important for investigating the mechanism of human cataract formation. Since this disorder, which is the major cause of blindness among the aged in all human populations, may be due to some impaired or abnormal metabolism in the lens, such a system provides an in vitro model for the study of those metabolic processes that may be implicated in the etiology of human cataract formation. These studies can take two forms:-

1) The incubation of intact cataractous lenses to investigate metabolic processes so that comparison can be made to normal lens metabolism. Lucas et al (1986) for example, were able to show that cataractous lenses retained the ability to respond to changes in
external calcium and osmolarity. Duncan and Jacob (1984), on the other hand, showed that the concentration of free Ca\textsuperscript{2+} in cataractous lenses was vastly higher than in clear lenses. Marcantonio and Duncan, (1987), compared protein synthesis in normal human and cataractous lenses.

2) The culture of clear lenses in order to investigate opacification due to the addition of suspected cataractogenic substances to the AAH. Hightower and Farnum (1985), for example, were able to induce opacities in human lenses upon loading the medium with calcium. This is one of at least thirty ways of inducing a lens opacity (for review see Harding, 1991).

Obviously it is important, if it is intended to extrapolate results back to the whole organism, that lenses remain viable under the conditions of culture. When using tissues one assumes that the biological material when removed from the deceased donor will continue to metabolise similarly in the artificial system as in the living person. If at least some of the normal metabolic pathways that are known to occur \textit{in vivo} can be demonstrated to be active \textit{in vitro}, this is supportive evidence for this assumption.

Glycolysis, one of the major cellular metabolic pathways, which generates ATP from glucose, provides the major source of energy for lenses (Kinoshita, 1965). Most of the glucose used by the lens is converted to lactate (Kuck, 1970), and exported from the lens as a metabolic waste product. The concentration of lactate in
human aqueous humor is approximately 6 to 9 times the level in plasma, the majority being derived from the ciliary body and lens (Riley, 1983). The concentration of glucose in the aqueous humor is about 75% of the plasma level (de Berardinis et al, 1965), due to its uptake by the lens and other ocular tissues. If lenses maintained in a tissue culture system, in which the incubating medium is an artificial aqueous humor (AAH), could be shown to be producing lactate in a reasonably constant manner, then it may be assumed that they remain metabolically viable in their artificial environment. Investigations of lactate output were therefore carried out.

The purpose of establishing the human lens organ culture system used in this study was the investigation of the metabolism of the essential amino acid tryptophan. Primate lenses are unique in that they appear to convert Trp into 3OHKG (van Heyningen, 1973b). Prior to embarking on this study experiments were undertaken to unambiguously identify the Trp metabolites present in the protein-free extract of a human lens.

3.2 Materials and Methods

3.2.1 Materials

*Tryptophan, kynurenine, 3-hydroxykynurenine and 3-hydroxyanthranilic acid were purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A.
*β-Glucosidase (EC 3.2.1.21)—7IU mg⁻¹ solid—was purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

*L-[5-³H]-Tryptophan; Specific Activity 32 Ci mmol⁻¹; Concentration 1.0 mCi mL⁻¹, was purchased from Amersham Australia Pty. Ltd., Sydney.

*The macaque monkey lens was obtained from Dr. Andrea Thorpe, of City University, London, U.K., during a visit to The University of Wollongong.

### 3.2.2 Methods

#### 3.2.2.1 General Methods

Refer to Chapter 2 for the following methods.

*Human Lens Organ Culture (2.1.1, 2.1.2 and 2.2)*

*Preparation of Lens Extracts (2.3)*

*Analytical HPLC (2.4)*

*Liquid Scintillation Counting (2.5)*

#### 3.2.2.2 Lactate Assays

The output of lactate into the AAH during lens incubations was measured spectrophotometrically using a diagnostic enzyme assay kit (Sigma Chemical Co., St. Louis, MO, U.S.A.) that employs lactate dehydrogenase (LDH) which, in the presence of NAD, converts lactate to pyruvate. The NAD is reduced to NADH and the concentration of lactate can be determined by measuring the
change in absorbance of NADH at 340 nm against a tissue culture medium blank. The extinction coefficient of NADH at 340 nm is 6.22 mM\(^{-1}\) cm\(^{-1}\). The total volume of reaction mixture in these assays was 3.0 mL, which included 100 µL AAH sample. The calculations were therefore as follows:

\[
\text{Conc. lactate (mM)} = \frac{\Delta A_{340}}{6.22} \times 30.
\]

3.2.2.3 UV Spectrophotometry

A Shimadzu UV-265 UV-visible Recording Wavelength Spectrophotometer was used for single wavelength absorbance in lactate assays and for UV-spectra of lens extracts and collected HPLC peaks.

3.2.2.4 Semi-preparative HPLC

This system consisted of a Waters 501 HPLC pump, a Rheodyne 7125 sample injector fitted with a 400 µL sample loop and a Knauer Variable Wavelength Monitor. Chromatograms were recorded on a Shimadzu CR6A Chromatopac integrator. Separations were performed on a 25 cm Whatman Partisil 10 ODS-3 column using a mobile phase of 0.1% acetic acid in 20% methanol (made up in Milli-Q water). The flow rate was set at 2.0 mL min\(^{-1}\).

3.2.2.5 Incubation of Lens in Radiolabelled Tryptophan

A human lens maintained under organ culture conditions was
incubated for 24 hr at 35°C in 4.5 mL AAH to which 100 µL L-[5-3H]-tryptophan was added (final concentration 50µM [20 µCi mL-1]). At the completion of the experiment, the lens was rinsed twice for 10 sec in unlabelled medium, blotted dry and frozen until analysed.

3.3 Results

3.3.1 Lactate is Produced by Healthy Lenses Under Organ Culture Conditions.

In order to test the accuracy of the assay, which is designed for samples of whole blood, a standard lactate solution in AAH was diluted, using AAH, and applied to the lactate assay. The concentrations obtained using the spectrophotometric calculation were within 5% of the standard values (Table 3.1).

Table 3.1: Determination of the accuracy of lactate assays in AAH using absorbance measurements of lactate standards.

<table>
<thead>
<tr>
<th>Standard Conc. (mM)</th>
<th>ΔA(340 nm)</th>
<th>Calculated Conc. (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.44</td>
<td>0.094</td>
<td>0.45</td>
</tr>
<tr>
<td>1.33</td>
<td>0.265</td>
<td>1.28</td>
</tr>
<tr>
<td>2.22</td>
<td>0.436</td>
<td>2.10</td>
</tr>
<tr>
<td>3.55</td>
<td>0.699</td>
<td>3.37</td>
</tr>
<tr>
<td>4.44</td>
<td>0.879</td>
<td>4.24</td>
</tr>
</tbody>
</table>

A 70 year old lens was placed into fresh AAH on arrival at the laboratory. In order to measure the effect of culture in the early stages, complete changes of AAH were carried out 1, 2 and 3 hr
after the commencement of the incubation. An earlier trial had shown that the quantity of lactate in 100 µL AAH after this short period in culture was too low for spectrophotometric measurement. 1.0 mL of these samples were therefore lyophilised and reconstituted in 100 µL Milli-Q water for lactate determination and the calculations were adjusted accordingly. 1.0 mL of lyophilised medium was used to prepare the blank for these readings. Another complete change of AAH was carried out 24 hr after commencement of the experiment and the AAH changed every subsequent 24 hr for 6 days. The incubation time and lens weight were used to express the lactate output of the lens as µmol hr⁻¹ g⁻¹ lens weight. The results of this experiment are shown in Table 3.2 and illustrated graphically in Fig. 3.1.

These results indicate that the lens took several hours to stabilise after being transported to the laboratory and placed into fresh AAH, as judged by the fluctuating lactate output during the first 3 hours.
Table 3.2: The measurement of lactate output into the AAH from a 70 year old human lens in culture.

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>ΔAbs</th>
<th>Conc. (mM)</th>
<th>Total (µmol)</th>
<th>Hours of Incubation</th>
<th>Output (µmol/hr/g)</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hr*</td>
<td>0.053</td>
<td>0.026</td>
<td>0.102</td>
<td>1</td>
<td>0.480</td>
<td>0.412</td>
</tr>
<tr>
<td>1 hr</td>
<td>0.038</td>
<td>0.018</td>
<td>0.073</td>
<td>1</td>
<td>0.344</td>
<td></td>
</tr>
<tr>
<td>2 hr*</td>
<td>0.236</td>
<td>0.114</td>
<td>0.455</td>
<td>1</td>
<td>2.138</td>
<td></td>
</tr>
<tr>
<td>2 hr</td>
<td>0.257</td>
<td>0.124</td>
<td>0.496</td>
<td>1</td>
<td>2.328</td>
<td>2.233</td>
</tr>
<tr>
<td>3 hr*</td>
<td>0.039</td>
<td>0.019</td>
<td>0.075</td>
<td>1</td>
<td>0.353</td>
<td>0.353</td>
</tr>
<tr>
<td>24 hr</td>
<td>0.336</td>
<td>1.621</td>
<td>5.834</td>
<td>21</td>
<td>1.304</td>
<td></td>
</tr>
<tr>
<td>24 hr</td>
<td>0.260</td>
<td>1.254</td>
<td>4.514</td>
<td>21</td>
<td>1.009</td>
<td>1.157</td>
</tr>
<tr>
<td>48 hr</td>
<td>0.225</td>
<td>1.085</td>
<td>3.907</td>
<td>24</td>
<td>0.764</td>
<td></td>
</tr>
<tr>
<td>48 hr</td>
<td>0.228</td>
<td>1.100</td>
<td>3.959</td>
<td>24</td>
<td>0.774</td>
<td>0.769</td>
</tr>
<tr>
<td>72 hr</td>
<td>0.190</td>
<td>0.916</td>
<td>3.299</td>
<td>24</td>
<td>0.645</td>
<td></td>
</tr>
<tr>
<td>72 hr</td>
<td>0.206</td>
<td>0.994</td>
<td>3.577</td>
<td>24</td>
<td>0.700</td>
<td>0.673</td>
</tr>
<tr>
<td>96 hr</td>
<td>0.269</td>
<td>1.297</td>
<td>4.671</td>
<td>24</td>
<td>0.914</td>
<td></td>
</tr>
<tr>
<td>96 hr</td>
<td>0.196</td>
<td>0.945</td>
<td>3.403</td>
<td>24</td>
<td>0.666</td>
<td>0.790</td>
</tr>
<tr>
<td>120 hr</td>
<td>0.133</td>
<td>0.641</td>
<td>2.309</td>
<td>24</td>
<td>0.452</td>
<td></td>
</tr>
<tr>
<td>120 hr</td>
<td>0.127</td>
<td>0.613</td>
<td>2.205</td>
<td>24</td>
<td>0.431</td>
<td>0.442</td>
</tr>
<tr>
<td>144 hr</td>
<td>0.205</td>
<td>0.989</td>
<td>3.559</td>
<td>24</td>
<td>0.696</td>
<td></td>
</tr>
<tr>
<td>144 hr</td>
<td>0.217</td>
<td>1.047</td>
<td>3.768</td>
<td>24</td>
<td>0.737</td>
<td>0.717</td>
</tr>
</tbody>
</table>

*These samples concentrated 10x before assay. This is accounted for in the calculations.

Figure 3.1: Lactate output of a 70 year old lens into the AAH during 6 days of organ culture. Complete changes of AAH were carried out at 1, 2, 3 and 24 hr then every subsequent 24 hr.
Fig. 3.2 shows the lactate output of a 69 year old lens in culture. Aliquots of AAH were removed at 6 and 12 hours during the first 24 hr of the incubation. A complete change of AAH was carried out at 24 hr and every subsequent 24 hr for a period of 11 days. This lens too displayed a very high lactate output in the first few hours after incubation in fresh AAH following transport to the laboratory. As for the lens in Fig. 3.1, the output had stabilised at a significantly lower level after 24 hr.

On arrival at the laboratory, this lens appeared to be in very good condition, as judged by its transparency when viewed through its anterior surface placed upwards against a millimetre square grid illuminated by a light source (lenses were placed in AAH in a covered petri-dish for this purpose). After 11 days in culture, the lens was still transparent although slightly swollen.
Another lens that arrived at the same time as that used to obtain the results in Fig. 3.2 was intact (the capsule did not appear to be damaged) but the cortex contained a severe opacity. No lactate was detected in samples of AAH from this lens when placed into culture even at the earliest time points. The appearance of the lens worsened daily and after 11 days the opacity had spread throughout the lens.

A pair of 15 year old lenses in apparently good condition were stabilised overnight in fresh AAH. At the commencement of lactate measurements the AAH was changed and aliquots removed at 8 and 16 hr. A complete change of AAH was carried out after 24 hr and the procedure was repeated for a further 24 hr. The results, illustrated in Fig. 3.3, indicate that there were only small changes in the lactate output over the incubation period. A colour change in the AAH from red to yellow was observed in 24 hr. Since the tissue culture medium contains phenol red, this would indicate a drop in pH probably due to a build-up of lactate, which may be the cause of the marginal drop in output at 24 hr from the maximum levels, usually attained by 16 hr. The trends were similar in both lenses for both days of the experiment. However, the lactate output had dropped slightly on the second day.
Figure 3.3: Lactate output of a 15 year old pair of lenses into AAH during 2 days of organ culture. Aliquots of AAH were removed at each time point and a complete change of AAH for each lens was carried out after the 24 hr sampling, as indicated by the arrow.

Three lenses that arrived at the laboratory at the same time appeared in good condition and were maintained in culture with daily changes of AAH for 7 days while preparation of material for an intended experiment was completed. After this period the lenses still looked healthy and lactate assays were carried out on the day 7 AAH. The lactate output over this final 24 hr period for these lenses, aged 67, 69 and 69 years, were 0.394, 0.220 and 0.248 μmol hr⁻¹ g⁻¹ lens respectively. These values are comparable to those in Fig’s 3.1 and 3.2. The lenses were subsequently used successfully in an experiment described in Chapter 6 (Section 6.3.6.1), demonstrating that even after a week in culture lenses can still be used experimentally.
3.3.2 The Major Long Wavelength UV Absorbing Compound in the Human Lens is the Glucoside of 3-Hydroxykynurenine

Having established an apparently viable human lens culture system, the identities of the tryptophan metabolites in human lenses reported in previous studies (van Heyningen, 1973a,b; Bando et al, 1981) needed to be confirmed.

A protein-free extract of 6 frozen human lenses was prepared according to the method in Section 2.3. The UV spectrum of this extract revealed a broad absorption peak with a maximum at 365 nm (Fig. 3.4).

Semi-preparative HPLC analysis of this extract using a detection wavelength of 365 nm resulted in the detection of one major peak and several minor peaks, as shown in Fig. 3.5.

The high absorbance of the major peak (5.97 min in Fig. 3.5) at 365 nm indicated that it was probably the glucoside of 3OHKyn (Bando et al, 1981). This peak was collected, lyophilised and reconstituted in 1.0 mL Milli-Q water. To one half of this sample, 1.0 mg of β-glucosidase was added. This sample and the untreated control were incubated at 35°C for 2 hr then reanalysed by semi-preparative HPLC. As illustrated in Fig. 3.6A, the control sample eluted at a similar retention time to the major peak in Fig 3.5. The retention time of the enzyme-treated sample, however, was significantly later than the control.
Figure 3.4: UV-spectrum of a protein-free human lens extract. The maximum was measured at 365 nm.

Figure 3.5: Semi-preparative HPLC chromatogram of a combined protein-free extract from 6 human lenses using a detection wavelength of 365 nm. Retention time in minutes printed above each peak.
Figure 3.6: (A) Control and (B) β-glucosidase treated halves of the major peak collected during the analysis seen in Fig. 3.5 and re-chromatographed by semi-preparative HPLC. The retention time of the treated sample has been shifted to that of authentic 3-hydroxykynurenine.

The retention time of authentic Trp metabolites on this system had been obtained previously. The retention time of the peak in Fig 3.6B corresponds to that of standard 3-hydroxykynurenine, confirming that the absorption detected in Fig. 3.4 is due mostly to the glucoside of 3OHKyn, as found by van Heyningen (1971a,b). It appears from Fig. 3.6A that some hydrolysis of this compound has occurred, as evidenced by the small peak corresponding to the retention time of 3OHKyn.

Further confirmation of the identity of the suspected 3OHKG peak was obtained by repeating the β-glucosidase experiment on the major peak from another semi-preparative HPLC analysis. This
time, UV-spectra of the control and enzyme-treated samples were measured (Fig. 3.7).

**Figure 3.7:** UV-spectra of a control (—) and β-glucosidase treated (—--) samples of the major peak detected by semi-preparative HPLC of protein-free human lens extracts. Maxima at 365 and 262 nm were measured for the control sample and at 368 and 267 nm measured for the treated sample.

The $\lambda_{\text{max}}$ values of the treated sample in Fig. 3.7 are the same as those for authentic 3OHKyn (Dawson et al, 1986); and the $\lambda_{\text{max}}$ values measured for the untreated semi-preparative HPLC peak correspond to those of the O-β-D-glucoside of 3-hydroxykynurenine as reported by Bando et al (1981).
van Heyningen (1971a,b) reported the presence of 3OHKG in baboon as well as human lenses but not in any non-primates she had studied. She speculated that this pigment is probably unique to the lenses of all primates. A protein-free extract from a macaque monkey lens was analysed by semi-preparative HPLC (Fig. 3.8A) and a peak was detected with a similar retention time to that of 3OHKG in human lenses. Fig. 3.8B shows the major peak from a human lens extract. In order to confirm the identity of the monkey pigment, it was collected and combined with a human lens extract. When this extract was analysed, only one peak was detected (Fig. 3.8C).
3.3.4 The Tryptophan Catabolites Kynurenine and 3-Hydroxykynurenine Are Present in the Human Lens.

In Section 3.3.2 it was established that the major absorbance at 365 nm in protein-free lens extracts is due to 3OHKG. In Fig. 3.5
retention times of minor peaks correspond to those of authentic 3OHKyn (7.26 min) and Kyn (9.33 min). When single lens extracts were analysed, these smaller peaks were not always detected. Later experiments using tritiated Trp suggested that the reason was that the semi-preparative column was retaining material. Since it would be necessary to analyse single lenses from culture experiments if any age-related differences were to be observed, and having confirmed the presence of 3OHKG, it was decided that, for further studies on Trp metabolism, another method of analysing the lenses was required.

Elderfield et al (1989) described a reversed-phase HPLC system that separates eight of the Trp metabolites. Using this procedure with a C8 column equivalent to that used by Elderfield et al (1989), it was found that 3OHKyn and 3OHKG (collected in 3.3.2) coeluted. A C18 column was found that adequately separated these two compounds. Although the use of this column made the analysis time much longer, a major objective of this project was to establish the level of 3OHKyn in human lenses; therefore a C18 HPLC column was used for all further analyses.

Trp metabolism in whole lenses can best be studied using radiolabelled precursor. In order to test that radiolabelled Trp is transported into the lens and can be incorporated using the in vitro culture system, a lens was stabilised overnight in AAH then incubated for 24 hr in AAH to which [5-3H]-L-tryptophan was
added. Half of the protein-free extract was analysed using the C_{18} analytical HPLC system and fractions of the eluent were collected every 2 min for LSC analysis. The HPLC chromatogram and resulting radioactive profile are compared in Fig. 3.9.

The peaks detected at 14.8, 17.7, 28.9 and 77.4 min (Fig. 3.9A) correspond to the retention times of standards of 30HKyn, 3OHKG, Kyn and Trp respectively. The detection wavelength was altered from 365 to 278 nm in order to detect the late-running Trp, which does not absorb at 365 nm. Radioactivity was detected at the retention times of these peaks as shown in Fig. 3.9B.

In order to confirm the identity of the compounds and to prove that the radioactivity was indeed associated with the assigned metabolites, the other half of the extract was chromatographed on HPLC and the three 365 nm absorbing peaks were collected separately. The largest peak was divided into two equal volumes and one part treated with β-glucosidase. Both samples were incubated for 2 hr at 35°C, lyophilised and re-run on HPLC at 365 nm. The peaks were collected and the associated radioactivity determined by LSC. The retention time of the enzyme-treated sample was shifted to that of 30HKyn, as were the majority of the counts (Table 3.3). The two small peaks were applied to separate lanes of a TLC plate. The plates were developed in n-propanol: 33% NH_{4}OH (7:3). After development the lanes were cut into consecutive segments of 5 mm each, starting at the origin, and
the segments counted by LSC. Standards of Kyn and 3OHKyn showed Rf values of 0.55 and 0.49 respectively. The Rf values for the major radioactive components corresponded to those of the standards in each case. These results indicate that the HPLC peaks in Fig. 3.10A detected at 365 nm are indeed 3OHKyn (14.8 min), 3OHKG (17.4 min) and Kyn (28.9 min).

Table 3.3: The retention times and associated radioactivity of β-glucosidase treated or control 3OHKG collected from a radiolabelled Trp lens incubation.

<table>
<thead>
<tr>
<th>Ret. Time</th>
<th>Control</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>dpm</td>
<td>33100</td>
<td>22400</td>
</tr>
</tbody>
</table>

Figure 3.9: (following page) HPLC chromatogram (A) and LSC profile (B) of one half of the protein-free extract from a human lens following incubation for 24 hr in AAH containing 50μM (20 μCi mL⁻¹) [5-³H]-L-tryptophan. Retention time in minutes is printed above each peak in (A).
(A) Retention times (in minutes):
- 17.472
- 14.8
- 28.945
- 64.99
- 77.427

(B) Radioactivity counts (dpm):
- 800,000

Retention Time (Minutes)
As discussed earlier, in order to be able to detect Trp by HPLC as well as LSC, which is important for quantification purposes (Chapter 4), the detection wavelength was altered from 365 nm, where Trp has no absorption; to 278 nm, where it has a $\lambda_{\text{max}}$. The Trp metabolites Kyn ($\lambda_{\text{max}}$ 257, 360; Dawson et al, 1986), 3OHKyn ($\lambda_{\text{max}}$ 267, 368; Dawson et al, 1986), and 3OHKG ($\lambda_{\text{max}}$ 263, 365; Bando et al, 1981) also have absorbance maxima that may allow their detection at 278 nm. However, as Fig. 3.10 indicates, the levels of Kyn and 3OHKyn are too low in the lens to be adequately detected at this wavelength. Although 3OHKG is detected, it is not resolved from other compounds present in the lens that absorb at 278 nm. The process of using an initial wavelength of 365 nm and altering it to 278 nm following the elution of Kyn was thus found the most satisfactory method for resolving and quantifying all the compounds of interest.
Figure 3.10: HPLC chromatogram of a protein-free human lens extract using a detection wavelength of 278 nm. The retention times of 3OHKyn, 3OHKG, Kyn and Trp respectively are marked with the 4 arrows from left to right.
3.4 Discussion

Human lens organ culture has been used successfully in several investigations into lens metabolism. The system used in this study, utilising a completely defined medium, was also used by Marcantonio and Duncan (1987) to study human lens protein synthesis following similar studies carried out on cultured bovine lenses (Marcantonio and Duncan, 1983). Despite these earlier successes, it was necessary to establish that the culture system was viable under the conditions used for this study.

The utilisation of glucose from aqueous humor to form lactate, which is subsequently exported into the aqueous humor as a metabolic waste product, is the major source of energy for the lens. The rate of formation of lactate was therefore determined in order to establish the metabolic viability of the lenses in culture. The average output over the first 24 hours for four lenses aged 15, 15, 69 and 70 was 0.95±0.32 μmol hr⁻¹ g⁻¹ lens. This figure is in close agreement with previous studies on human lenses. Wolfe and Chylack, (1986) found the average lactate production over 24 hr in twenty four lenses to be 0.95 μmol hr⁻¹ g⁻¹ lens. Their study also indicated that lactate production accounted for 97% of the glucose metabolised. Bassnett (1987) found an average lactate output of 1.20 μmol hr⁻¹ g⁻¹ for four human lenses.

Lenses that appeared healthy at the beginning of the culture period continued to produce lactate for many days with daily
changes of AAH. A 70 year old lens averaged $0.76 \pm 0.23 \text{ \mu mol hr}^{-1} \text{ g}^{-1}$ lens over a period of 6 days, and for a 69 year old lens, the output was $0.27 \pm 0.10$ over a period of 11 days. Although there was a marked difference in the values of these two similarly aged lenses, the output remained relatively constant over the duration of the culture.

Lenses typically took several hours to stabilise after being transported to the laboratory and placed in fresh AAH. This was associated with a fluctuating lactate output. Bassnett (1987) found that lactate output in rat lenses took 2-3 hours to stabilise in AAH after removal from the eye and therefore in his subsequent experiments, lenses were preincubated for 3 hours prior to commencement. Based on this data, all lenses used in experiments involving Trp metabolism were kept overnight in AAH at 35°C then transferred to fresh AAH at the beginning of the experiments. Since it appears from these lactate results that lenses continue to metabolise even after many days in culture, the added time used in stabilising the lenses probably results in them being more metabolically normal at the time of commencement of experiments. If experiments were started immediately upon arrival of the lenses at the laboratory after transferring to fresh AAH, the results obtained may have been anomalous due to the fluctuating metabolic output. This point is important because in some experiments where the output of 3OHKG into the AAH was 60
measured (Chapter 6), time samples were removed from the AAH between 2 and 24 hr.

Since after approximately 2 days in culture there is a drop in lactate output (Fig's 3.1 and 3.2), experiments were commenced as soon as possible. Since the culture vessel is a closed system, there will be, over time, a build up of lactate in the AAH, as opposed to the situation in vivo where lactate and other ions are removed via the aqueous humor. This build up of lactate will eventually lead to a drop in pH and a lowering of metabolic output. This can be seen even over 24 hr (Fig. 3.3). Therefore, lens incubation experiments were restricted to 24 hr in the same AAH.

As well as establishing that lenses maintain their major energy producing metabolic pathway in this culture system, a method of identifying lens Trp and its metabolites (i.e. Kyn, 3OHKyn and 3OHKG) has been utilised by slightly varying a previously developed HPLC procedure (Elderfield et al, 1989). The differences between this and the previous system are the use of a longer reversed-phase HPLC column in order to separate 3OHKyn from its glucoside, 3OHKG; and utilising the absorbance properties of the metabolites in order to detect them, rather than the amperometric and fluorescence detection used by Elderfield et al, (1989).

Further evidence that the lenses remained metabolically viable in culture was obtained from an initial experiment in which radiolabelled Trp was added to the AAH. Analysis of the lens after
24 hr revealed that influx of Trp from the AAH into the lens had occurred. Amino acid transport is an active process coupled to lactate production (Kinsey, 1965). In addition, radiolabel was also detected in the Trp metabolites Kyn, 3OHKyn and 3OHKG. This indicates that the enzymes of the pathway that produce 3OHKG from Trp (van Heyningen, 1973b) were active.

These culture conditions were exploited in the following chapters in order to investigate the little known lens specific metabolic pathway of tryptophan.
CHAPTER 4
CHAPTER 4
CHANGES IN TRYPTOPHAN METABOLITE LEVELS WITH AGE
IN THE HUMAN LENS

4.1 Introduction

Both physical and metabolic changes take place in the human lens during the aging process. Physical changes include an increase throughout life in weight and volume (Smith, 1883) and thickness (Brown, 1976); and age-related changes in fluorescence (Satoh, 1973; Kappelhof, van Best and Oosterhuis, 1986). Many age-related changes in metabolic processes have also been documented, one of the most notable being the decrease in the concentration of free glutathione in human lenses as a function of age (Harding, 1970; Rathburn and Murray, 1991). These alterations may adversely affect the visual acuity of the normal human lens. Also concurrent with these changes is crystallin aggregation. The mechanism of this aggregation, however, is not yet understood. This may be related to widespread post-translational modifications of lens proteins with aging (Harding and Dilley, 1976) which include deamidation (van Kleef, 1975; Dilley and Harding, 1975), phosphorylation (Sredy and Spector, 1984; Garland and Russell, 1985; Voorter et al, 1986; Chiesa et al, 1987) and proteolysis (David et al, 1989; Srivastava and Srivastava, 1989; Trayhurn and van Heyningen, 1976).

The young human lens is pale yellow in colour. With aging, however, the yellow colour deepens, this being especially noticeable
in the lens nucleus. This deepening colouration is not an opacity, and normal lenses remain transparent despite the yellowing. Whether this phenomenon, which is characteristic of age in all human lenses, is related to the cause of senile nuclear cataract is not known.

Studies carried out by Weale (1981, 1982, 1985, 1987 and 1988) clearly show that the absorbance of the human lens in the blue region of the electromagnetic spectrum (300-500 nm) increases markedly as a function of age. A systematic increase was observed in absorbance at 350, 400, 450 and 500 nm in intact lenses ranging in age from 0 to 85 years. This increase in absorbance causes a decrease in the amount of light of these wavelengths that is transmitted by the lens (Lerman and Borkman, 1976) and hence our sensitivity to blue light is markedly decreased as we age. Since many yellow pigments (e.g. xanthophyll and carotene) absorb in the blue region of the spectrum (Kennedy and Milkman, 1956), this loss of sensitivity to blue light along with an increase in the yellowing of the lens with age are probably more than a coincidental correlation. It is not known how or why these changes occur. The identity of the yellow 'age' pigment(s) is also unknown.

The human lens is known to contain quantifiable levels of 3-hydroxykynurenine glucoside (3OHKG), as determined by van Heyningen using paper electrophoresis and chromatography.
(1971a,b). This compound is formed from tryptophan via a lens-specific pathway (van Heyningen, 1973b). It has a $\lambda_{\text{max}}$ at 365 nm (Bando et al, 1981), and its effectiveness as a filter in this region of the spectrum was demonstrated by Wald (1952). In a darkened room illuminated only by a mercury vapour lamp (peak wavelength 365 nm), aphakic people\textsuperscript{1} were able to read an optometrists' chart from top to bottom while those with normal lenses could see nothing at all. The aphakic human eye was almost 1000 times as sensitive to light of wavelength 365 nm as the normal eye.

The concentration of 3OHKG has been shown previously, in a study of only nine human lenses aged from 3-83 years, to decrease from the younger lenses to those aged around 40 years, after which the level remained constant (Bando et al, 1981). Cooper and Robson (1969), also observed a marked decline with age in the concentration of a pigment extracted from human lenses which, although unidentified at the time, was certainly 3OHKG. As this compound is the major UV-absorbing species in the lens, preventing harmful UV rays from reaching the retina, this decrease may be especially significant if, as is widely assumed, UV-light plays a role in senile cataract formation.

The decrease in the levels of a compound with a $\lambda_{\text{max}}$ at 365 nm is surprising in the light of previously noted studies which demonstrate a clear age-related increase in the absorbance of the

\textsuperscript{1} Those who had undergone the removal of a cataractous lens
lens at this and other wavelengths in the same region of the spectrum. That the overall filtering capacity of the lens actually increases despite the loss of 3OHKG may be due to a generalised yellowing of lens proteins (Zigman, 1971; Lerman and Borkman, 1976; Spector, Roy and Stouffer, 1975). The deepening of the yellow colour of the lens begins in the nucleus which, due to the unique metabolic nature of the lens regarding lens turnover (See Chapter 1), possesses the oldest crystallins.

Other studies have led to the proposal that a reactive Trp metabolite may be involved in the process of human nuclear cataract formation, which is associated with the oxidation of lens crystallins (Truscott and Martin, 1989; Benavente and Truscott, 1991; Truscott et al 1991). The most likely candidate for such a reactive metabolite is 3-hydroxykynurenine (3OHKyn), the metabolic precursor of 3OHKG, as similar phenolic compounds have been implicated in the cross-linking of proteins in sclerotized insect cuticle (Anderson, 1971). It is also feasible that the colouration of lens proteins with age may result from an interaction with the crystallins and 3OHKyn over time (Stutchbury and Truscott, 1993).

For these reasons it seemed important to know more about the levels of Trp and its metabolic products in the human lens and how these levels change with age.

In this study on human lenses, fresh post-mortem lenses were maintained under tissue culture conditions for the
investigations on Trp metabolism which will be described in later chapters. Upon removal from culture, protein-free extracts of these lenses were analysed. The quantities of Trp, Kyn, 3OHKyn and 3OHKG were determined for each lens using HPLC and data combined with that obtained from similar analysis of lenses stored in liquid N₂.

4.2 Materials and Methods

4.2.1 Human Lenses

The lenses were obtained from the source as in Chapter 2 (Section 2.1.1), either in AAH (Section 2.2.2) or in cryogenic vials. The former had been obtained as soon as possible post-mortem and the latter had been stored for varying amounts of time in liquid N₂, where they had been placed immediately upon removal from the post-mortem eye. The lenses in AAH were weighed prior to the commencement of lens culture experiments. At the completion of experiments they were removed from AAH, rinsed in normal saline, blotted dry and frozen at -20°C until extracted. Lenses stored in liquid N₂ were weighed just prior to extraction.

4.2.2 Lens Extraction

(Refer to Section 2.3).

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4.2.3 HPLC

A 100 µL aliquot of reconstituted lens protein-free extract, representing typically 1/3 or 1/2 of the total, was analysed by HPLC and peak areas integrated according to Section 2.4. The solution used for reconstitution contained 0.01 mg mL⁻¹ 5-hydroxytryptophan (Sigma Chemical Co. St. Louis, MO, U.S.A.), which was used as an internal standard for quantification purposes (see Section 4.2.4). The monitoring wavelength was altered during the analysis from 365 nm (to detect Kyn, 30HKyn and 30HKG) to 278 nm (to detect 5-hydroxytryptophan [approx. 35 min] and Trp).

4.2.4 Standard Curves

For the Trp and 30HKyn standard curves, stock solutions were prepared in triplicate combining 2.0 mg Trp (MW 204.2), 2.2 mg 30HKyn (MW 224.2) and 1.0 mg 50HTrp in 10 mL Milli-Q water. These solutions, were diluted 1:10. Another stock solution containing only 0.1 mg mL⁻¹ 50HTrp was diluted 1:10 then used to further serially dilute the three stock solutions 1:2→1:5→1:10. This procedure resulted in three solutions containing Trp and 30HKyn in concentrations of 100µM, 50µM, 10µM and 1µM in 0.01 mg mL⁻¹ 50HTrp. Initial lens extract analyses indicated that for 30HKG (which was estimated according to the 30HKyn curve) and Trp, the range of the curves were not sufficiently high to accommodate some
experimental values. In order to ensure that the curve was linear at higher concentrations, a 500μM point was therefore added using duplicate solutions containing both 1.0 mg Trp and 1.1 mg 3OHKyn dissolved in 10 mL 0.01 mg mL⁻¹ 5OHTrp.

Duplicate solutions containing 5, 10, 20 and 50μM Kyn in 0.01 mg mL⁻¹ 5OHTrp were prepared for the Kyn standard curve. The range of this curve encompassed the quantities of Kyn detected.

100 μL of each solution was chromatographed on the analytical HPLC system starting with a detection wavelength of 365 nm and, after detection of the peaks of 3OHKyn or Kyn, the detection wavelength altered to 278 nm in order to detect peaks of 5OHTrp and Trp.

4.2.5 Quantification of Trp, Kyn, 3OHKyn and 3OHKG

Using the standard curves, the amount of Trp, Kyn and 3OHKyn in a protein-free lens extract could be determined by analysing 100 μL of reconstituted extract by HPLC. The peak area ratio relative to internal standard (5OHTrp) was compared to the appropriate standard curve in order to quantify the amount present in the injected extract. The amount of 3OHKG present was estimated using the 3OHKyn standard curve, as the former compound is unavailable commercially and attempts to synthesise it have so far been unsuccessful (Manthey et al unpublished results).
4.2.6 Recovery of Metabolites

Duplicate stock solutions containing 0.5 mg mL\(^{-1}\) each of Kyn and 3OHKyn were prepared in water. These solutions were then diluted 1:10 (50 µg mL\(^{-1}\)) and a further 1:50 (1 µg mL\(^{-1}\)) with 80% ethanol to provide the two concentrations for recovery experiments. A volume of 0.5 mL of these dilutions correspond to typical levels of 3OHKG and 3OHKyn respectively in lens protein-free extracts.

Four pieces of tissue of typical human lens weight (approx. 250 mg) were excised from a frozen bovine lens obtained from a local abattoir. Two of these samples were homogenised in 0.5 mL of the 80% ethanol solution containing 50 µg mL\(^{-1}\) of both Kyn and 3OHKyn and two in 0.5 mL 80% ethanol containing 1µg mL\(^{-1}\) of both Kyn and 3OHKyn. The extracts were then treated as normal (see Section 2.3); the second extraction was carried out using 80% ethanol containing no Kyn or 3OHKyn.

Since normally only 1/2 or 1/3 of a human lens protein-free extract was analysed, after lyophilising one of each pair of extracts was reconstituted in 200 µL internal standard and their duplicates were reconstituted in 300 µL internal standard. A 100 µL aliquot of each sample, representing 1/2 or 1/3 of the total extract, was analysed by HPLC in order to more closely mimic human lens analyses and the amounts of Kyn and 3OHKyn detected calculated from standard curves.
4.3 Results

4.3.1 Standard Curves

An internal standard was required that would run at a retention time far enough removed from the other compounds so as not to interfere with accurate peak area integration. The optimal wavelength chosen for the detection of Kyn, 3OHKyn and 3OHKG was 365 nm. The later eluting Trp does not absorb at this wavelength. It has a $\lambda_{\text{max}}$ at 278 nm and this wavelength was used for its detection. The Trp metabolite 5-hydroxytryptophan, (5OHTrp) formed in vivo via the 5-hydroxyindole pathway (Bender, 1982), had not been detected in any previously analysed lens extracts and the retention time of a commercial standard of this compound was sufficiently well resolved from that of Kyn to allow it to be used as an internal standard for this assay. It also possesses a $\lambda_{\text{max}}$ at 278 nm. The column used initially for these analyses, however, failed to separate Kyn from 5OHTrp sufficiently to allow precise quantification of Kyn. It was therefore decided to forego Kyn measurement during the accurate determinations of the other three compounds. Later, another column was found which separated Kyn from 5OHTrp satisfactorily and thus a Kyn standard curve could be calculated. Since only a fraction of a protein-free extract (usually 1/3 or 1/2) had been analysed, the remaining extracts were analysed on the new column in order to obtain the levels of Kyn.
Graphs of the average peak area ratios were plotted against the amount of compound injected in μmole and lines of best fit were generated by the graphics software using least squares analysis. The resulting standard curves are shown in Fig. 4.1. The standard deviation of the data, as shown by error bars, were also plotted. The straight line equations ($y=b+mx$) and correlation coefficients as calculated by the software are also given for each curve, as shown in Fig. 4.1.
Figure 4.1: Standard curves for determining quantities of Trp and its metabolites in lens protein-free extracts.
4.3.2 Quantification of Metabolites in Lens Extracts

Fig. 4.2 shows a typical HPLC chromatogram obtained for the quantification of 3OHKyn, 3OHKG, Kyn and Trp. The peak of the internal standard, 5OHTrp, is also shown.

![HPLC Chromatogram](image)

**Figure 4.2:** A typical HPLC chromatogram of lens protein free extract showing detection of 3OHKyn, 3OHKG and Kyn at 365 nm and 5-hydroxytryptophan (the internal standard used for quantification) and Trp at 278 nm.
The straight-line equations of the standard curves in Fig. 4.1 (y=b+mx) where:-

- \( y \) = peak area ratio of detected metabolite relative to 50HTrp;
- \( b \) = intercept of the curve on the y-axis;
- \( m \) = slope of the curve;
- \( x \) = amount of metabolite in \( \mu \)mole;

were used to calculate the quantities of compounds detected by HPLC. The amount of metabolite detected (x) can therefore be calculated from the peak area ratio (y) and slope of the curve (m) as \( x = (y + b)/m \).

### 4.3.3 Lens Extract Recovery Assays

The lens extraction process (Section 2.4) which employs 80% ethanol to obtain a protein-free sample for HPLC analysis involves several steps and some loss of metabolites may occur. In order to obtain a more accurate estimate of the quantity of these compounds present in the lens, assays were performed to determine the percentage loss of metabolites during the preparation of protein-free lens extracts.

The bovine lens, while containing the same percentage of protein as the human lens, does not metabolise Trp (Trayhurn and Van Heyningen, 1973). Therefore bovine lens tissue contains no Kyn or 3OHKyn and obtaining a protein-free extract involves removing the same quantity of protein on a per weight basis. The results of
these assays are shown in Table 4.1.

Table 4.1: The percentage recovery of known amounts of 3OHKyn and Kyn added to bovine lens tissue from which protein-free extracts were subsequently prepared.

<table>
<thead>
<tr>
<th>Amount Extracted (µg)</th>
<th>Amount Extracted (µmol)</th>
<th>Amount Detected (µmol)</th>
<th>Fraction Analysed</th>
<th>Total Amount (µmol)</th>
<th>% Rec.</th>
</tr>
</thead>
<tbody>
<tr>
<td>3OHKyn</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0.0022</td>
<td>0.0004</td>
<td>1/2</td>
<td>0.0008</td>
<td>36</td>
</tr>
<tr>
<td>0.5</td>
<td>0.0022</td>
<td>0.0003</td>
<td>1/3</td>
<td>0.0009</td>
<td>40</td>
</tr>
<tr>
<td>25</td>
<td>0.1115</td>
<td>0.0207</td>
<td>1/2</td>
<td>0.0415</td>
<td>37</td>
</tr>
<tr>
<td>25</td>
<td>0.1115</td>
<td>0.0114</td>
<td>1/3</td>
<td>0.0342</td>
<td>31</td>
</tr>
<tr>
<td>Kyn</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>36</td>
</tr>
<tr>
<td>0.5</td>
<td>0.0024</td>
<td>0.0006</td>
<td>1/2</td>
<td>0.0011</td>
<td>46</td>
</tr>
<tr>
<td>0.5</td>
<td>0.0024</td>
<td>0.0004</td>
<td>1/3</td>
<td>0.0012</td>
<td>49</td>
</tr>
<tr>
<td>25</td>
<td>0.1201</td>
<td>0.0248</td>
<td>1/2</td>
<td>0.0496</td>
<td>41</td>
</tr>
<tr>
<td>25</td>
<td>0.1201</td>
<td>0.0144</td>
<td>1/3</td>
<td>0.0432</td>
<td>36</td>
</tr>
</tbody>
</table>

These results indicate that less than half of the metabolites are recovered from the lens extract. Since the results are consistent for metabolite levels at both ends of the 3OHKyn standard curve, it can be assumed that the same level of recovery occurs at all levels in between. The quantities detected from lens extracts therefore were adjusted for this loss in order to obtain the actual quantities originally present in the lens. Since Trp is present in bovine lenses its percentage recovery could not be calculated. In the light of results for Kyn and 3OHKyn the quantities of Trp were determined assuming a recovery level of 40%.
4.3.3.1 Ultrafiltration Accounts For Most of the Loss of Metabolites

A 1.0 mL aliquot of a 1 μg mL\(^{-1}\) stock solution from Section 4.3.3 was ultrafiltered. The filtrate and 1.0 mL unfiltered solution were each lyophilised, reconstituted in 100 μL Milli-Q water and analysed by HPLC. According to the integrated peak areas, less than 50% of Kyn and 3OHKyn were recovered from the ultrafiltered sample; demonstrating that this procedure, which is carried out in order to remove residual protein from the lens extracts prior to HPLC, also accounted for the majority of loss of low molecular weight material.

4.3.4 The Concentrations of Tryptophan and its Metabolites in the Human Lens as a Function of Age

The final values for the concentrations of Trp, Kyn, 3OHKyn and 3OHKG, expressed as μmol g\(^{-1}\) lens wet weight, were graphed as a function of age (Fig. 4.3).

Lenses used in tissue culture experiments were plotted using a different symbol from those stored in liquid N\(_2\) in order to check for possible variation in results due to the differing post-mortem treatments. Fig. 4.3 shows no apparent difference between the two groups, especially among the older lenses, where there is an abundance of both types.
TRYPTOPHAN

(A)

KYNURENINE

(B)

μmol/g lens weight

AGE (Years)
Figure 4.3: (also on previous page) The concentration of Trp and its metabolites in the human lens as a function of age. (♦) Lenses used in tissue culture experiments. (□) Lenses stored in liquid N2.

The data suggests that the level of Trp in the lenses remains relatively stable from 15 years until well into old age. There were no lenses analysed between 5-15 years, but a pair of 5 year old lenses had the highest levels of Trp and another 5 year old lens had
a higher concentration than all but one of the older lenses.

In contrast to Trp, the levels of 3OHKG appear to begin to decline at about 15 years, and continue to do so until about age 40, after which the concentration remains relatively constant. The 32 year old lens with the exceptionally high concentration of Trp (Fig. 4.3A), did not have an unusually high level of either Kyn, 3OHKyn or 3OHKG for its age, suggesting that the activity of the enzyme that catalyses the cleavage of the Trp indole ring to form N'-formylkynurenine (N-formylKyn), the first intermediate in this pathway, is limiting. No N-formylKyn was detected in any lens, suggesting that the activity of the non-specific formamidase that converts this compound to Kyn is very high, as it is in the other organs (Bender, 1982). This compound is also hydrolysed non-enzymically.

The levels of Kyn and 3OHKyn followed approximately the same trend as that for 3OHKG from 15 years of age onwards. However, although the 3OHKG levels in three of the four 5 year old lenses approximated the levels in the 15-20 year old lenses, the same pattern was not apparent for Kyn and 3OHKyn, where the concentrations were generally lower in the younger lenses. This indicates that possibly the activities of kynurenine hydroxylase and the glucosylating enzyme are highest in very young lenses.

3OHKG is present in the lens in much higher quantities than the other three compounds. Its average concentration is 5.7 times
greater than that of Trp; and 27 and 68 times higher than the average concentrations of Kyn and 3OHKyn respectively.

4.3.5 The Weight of the Human Lens Increases Throughout Life

When the weights of the lenses used in this study were plotted as a function of age, it was found that there is a constant increase in lens weight throughout life (Fig. 4.4). Once again, there appears to be little difference between the cultured lenses and those stored in liquid N₂. The cultured lenses do appear, however, to have slightly higher weights when compared to frozen lenses of the same age. This may be due to partial dessication of frozen lenses. Since lenses were weighed at the beginning of the culture period, it is less likely to be due to swelling of cultured lenses.

![Figure 4.4: The weight of human lenses as a function of age. (■) Cryogenised lenses. (□) Cultured lenses.](image)

\[ y = 0.14763 + 1.5935 \times 10^{-3}x \]

\[ R^2 = 0.522 \]
4.3.6 The Actual Quantity of 3OHKG in Human Lenses Also Declines With Age

In order to check that the decrease in concentration of 3OHKG with age, expressed as \( \mu \text{mol g}^{-1} \text{lens} \) (Fig. 4.3D), is not simply a function of increasing lens weight (Fig. 4.4), the actual quantities of 3OHKG determined in the lenses were plotted as a function of age (Fig 4.5). Even when lens weight is not taken into account, it appears that the actual quantity of 3OHKG present in the lens generally decreases with age.

![Graph showing the actual quantity of 3OHKG in human lenses as a function of age.](image)

**Figure 4.5:** The actual quantity of 3OHKG in human lenses as a function of age.

4.4 Discussion

The object of this study was to determine the quantities of Trp and its metabolic products present in human lenses, and to see
if there is any age-related change in the levels of these compounds. In her pioneering work on this topic, Ruth van Heyningen (1971a,b; 1973a,b) identified two metabolites of Trp in the primate lens, both of which absorbed light of long UV wavelength. One of these, kynurenine, was a known intermediate of the metabolism of tryptophan in the mammalian liver and other organs. The other, the O-β-D-glucoside of 3-hydroxykynurenine, has not been identified in any other organ. Its aglucone, 3-hydroxykynurenine, is a metabolite of Trp, and has previously been identified in the human lens in trace amounts only (van Heyningen, 1973a). By analysing over 50 lenses with an age range of 0-91 years, the quantities of Trp, Kyn and 30HKG in human lenses have now been examined in greater detail than in any previous study, and 30HKyn has been quantified for the first time.

The results indicate that 30HKG is the major product of Trp metabolism in the human lens. The relative amounts of 30HKG and Trp suggest that the majority of Trp that is not incorporated into protein is efficiently converted to 30HKG. The very low quantities of Kyn and 30HKyn compared to 30HKG and the absence of N-formylKyn indicate that, if these compounds do not undergo any other metabolism except 30HKG formation, the activities of kynurenine formamidase [E.C. 3.5.1.9], kynurenine hydroxylase [E.C. 1.14.13.9] and the glucosyl transferase enzyme must be relatively high compared to IDO, the enzyme probably responsible for initial
Although there was little apparent alteration in the lenticular levels of Trp from 15 years of age onwards (Fig. 4.3A), there is a parallel age-related decrease in the concentrations of Kyn (Fig. 4.3B), 3OHKyn (Fig. 4.3C), and 3OHKG (Fig. 4.3D). These decreases were most marked up to the early twenties after which the levels declined more slowly, and were relatively constant after about 40 years of age.

That "...the growth of the lens does not cease with that of the rest of the body, but is continuous...throughout the whole period of life," was first observed by Smith (1883), who in a study of 156 post-mortem lenses aged 20-90 observed an increase with age in lens weight and volume. A more recent study by Harding et al (1977), confirm that there is a linear increase in weight in the human lens throughout life, and the lenses used in this study also conform to these findings (Fig. 4.4).

The age-related decrease in concentration of 3OHKG and its two metabolic precursors cannot be totally attributed to this weight increase, as the actual quantity per lens of 3OHKG also declines with age, as seen in Fig. 4.5.

The levels of Trp, Kyn and 3OHKG found in this study are in agreement with those found in previous studies. Using an ion exchange chromatographic method, Dickinson et al (1968), determined the concentrations of free amino acids in 12 human
senile cataractous (aged 54-84 years) and 2 clear eye bank lenses (37 years). The concentration of Trp in the cataractous lenses ranged from 0.06 (age 84) to 0.36 (age 83) mmol kg\(^{-1}\) lens water (average 0.22±0.11); and that of the eye bank lenses 0.15 and 0.16 mmol kg\(^{-1}\) lens water. These values were obtained assuming a water content of 65% of the total weight of each lens. Since mmol kg\(^{-1}\) lens water is equivalent to μmol g\(^{-1}\) lens water, these values were multiplied by 0.65 in order to convert them to units of μmol g\(^{-1}\) lens. The results converted in this manner are in the same range as the concentrations of Trp in Fig 4.3A.

Having isolated the low MW fraction from lens homogenates of 9 lenses ranging in age from 3-83 years using column chromatography, Bando et al (1981), used UV-spectrophotometry to estimate the content of 3OHKG in these lenses. The values obtained ranged from 0.235 μmol for the 3 year old lens down to 0.137 μmol for a 46 year old lens (although the older lenses had values only slightly higher than this lens). These values are in close agreement with those in Fig. 4.5. The concentration of 3OHKG was expressed as μmol g\(^{-1}\) lens protein to obtain a curve very similar in shape to that shown in Fig. 4.3D.

Having isolated Kyn and 3OHKG from lens protein-free extracts using paper electrophoresis, van Heyningen (1973a), was able to quantify these compounds by measuring their fluorescence.
The results were expressed as μmol g\(^{-1}\) lens weight, as has been done in this study. Trace amounts of 3OHKyn were also detected, although no quantities were reported. The 3OHKG values for 23 post-mortem lenses from 0-75 years old ranged from 0.7 to 4.0 μmol g\(^{-1}\) lens, with the higher values once again in the younger lenses. These results are similar to those in Fig. 4.3D; although the values tend to be slightly higher in the previous study. Kyn was detected consistently only in the younger lenses, which agrees with the age-related decrease for this compound shown in Fig. 4.3B. The Kyn values ranged from 0.01-0.1 μmol g\(^{-1}\) lens. A value of 5.85 μmol g\(^{-1}\) lens was obtained for 3OHKG in one exceptional 65 year old lens. The level of Kyn in this lens was also elevated. This result led van Heyningen to conclude that some common factor governs the occurrence of these Trp metabolites and the similar trends found in Fig. 4.3B-D adds weight to this argument.

All the lenses obtained for this study were obtained post mortem and not from cataract operations. It is interesting to note that van Heyningen's investigation (1973a) also included some lenses from cataract patients and the levels of 3OHKG in these lenses were generally much lower than in the post mortem lenses. Whether this is due to leakage of the cataractous lenses, a decrease in rate of synthesis or an increase in efflux is unknown. The levels of Trp found in cataractous lenses by Dickinson et al (1968), are no
different to the levels in normal human lenses found in this investigation.

That the levels of Trp remain constant while the levels of its metabolic products decline in a comparable manner, and since 30HKyn is the probable precursor of 30HKG, suggests that the decrease in concentration of 30HKG with age is not due to an impaired ability to glucosylate 30HKyn. The results instead suggest a decreased ability with age of the lens to metabolise the available Trp.

Potential control of the 30HKG biosynthetic pathway could take place at several levels. One possible site is at the level of the first enzyme which oxidatively cleaves Trp to N-formylKyn. This enzyme is assumed to be indoleamine dioxygenase (IDO) [E.C. 1.13.11.17] (Hayaishi, 1976) and would utilise superoxide as a cosubstrate (Hirata and Hayaishi, 1971;1975). Levels of lenticular superoxide may therefore affect the overall rate of 30HKG biosynthesis. Utilisation of superoxide in this manner as well as the potential antioxidant activity of 30HKyn (Christen et al, 1990) may add to the possible protective role of the biosynthetic pathway (Luthra and Balasubramanian, 1992). A caveat is that the oxidised 30HKyn formed may be highly reactive (Stutchbury and Truscott, 1993).

Bando et al (1982), showed that 30HKG can act as as a photosensitizer of rat lens soluble protein irradiated for 24 hr
under UV light ($\lambda_{\text{max}}$ 360 nm). Dillon and Atherton (1990) and Dillon et al (1990), however, found the excited singlet state of 3OHKG to decay rapidly and for it to be a relatively inefficient sensitizer for lens proteins, prompting them to suggest that 3OHKG acts as a filter for the retina from damaging radiation between 295-400 nm while also minimising photochemical insult to the lens.

While 3OHKG would appear to be an inefficient sensitizer of lens proteins, there are quantifiable, although low, levels of 3OHKyn present in lenses of all ages. The proposal that 3OHKyn may be implicated in human lens crystallin colouration was considered as a possibility by van Heyningen (1973b), but later discounted after failure to find xanthommatin, the dimeric oxidation product of 3OHKyn (Butenandt and Schafer, 1962). It has been proposed more recently that under oxidising conditions aminophenols produced by the human lenticular metabolism of tryptophan could play a role in the aetiology of senile nuclear cataract (Truscott and Martin, 1989; Truscott et al, 1991). In relation to this, both 3-hydroxyanthranilic acid (Truscott and Martin, 1989; Benavente and Truscott, 1991) and 3OHKyn (Stutchbury and Truscott, 1993) readily tan proteins. Although 3-hydroxyanthranilic acid has not been detected in the lenses studied, we have demonstrated in this investigation that significant amounts of free 3OHKyn are present in normal human lenses. Thus, if a lens is exposed to an oxidative stress, there is the potential for covalent modification of lens proteins by an
endogenous metabolite. 3OHKyn has also been shown to be cytotoxic for neural cells *in vitro*, an effect due at least in part to the generation of H₂O₂ (Eastman and Guilarte, 1990). Some cataract patients have been shown to have elevated aqueous humour levels of H₂O₂ (Spector and Garner, 1981).

In order for the scenario to be credible it must be demonstrated that free 3OHKyn is present in the lens (as shown in this chapter), and in particular in the lens nucleus; and that the synthesis of 3OHKyn takes place at a significant rate.
CHAPTER 5
THE TRANSPORT OF TRYPTOPHAN INTO HUMAN LENSES AND ITS INCORPORATION INTO 3-HYDROXYKYNURENINE GLUCOSIDE

5.1 Introduction
The previous chapter showed that, although the concentration of 3OHKG, the major lens UV filter compound, is much higher than the level of free Trp from which it is derived, suggesting an efficient metabolic process, there appears to be an age-dependent alteration in the human lenticular pathway responsible for converting Trp to 3OHKG. Results from a large number of human lenses covering the range of a normal human life-span revealed that the levels of this compound and its two immediate metabolic precursors decline dramatically from teenage years to about age 40; during which time the concentration of Trp remains relatively constant. In this chapter the metabolism of Trp in human lenses was investigated using an in vitro lens culture system with radiolabelled Trp to measure the incorporation of radiolabel into the lens over a period of 1-2 days.

van Heyningen (1973b) demonstrated incorporation in one cataractous lens after an incubation of 20 hr at 37°C in DL-[methylene-14C]-tryptophan. The autoradiogram of the protein-free extract of this lens (Fig. 1.4), following two dimensional electrophoresis and chromatography, revealed that most of the label was contained in Trp with lesser amounts (visually) in 3OHKG and
Kyn. No 3OHKyn was detected; nor was any evidence found for the formation of kynurenic acid or xanthurenic acid, possible metabolic products of Kyn and 3OHKyn respectively. There was also no evidence of $^{14}$C-alanine in this experiment. Alanine would have retained the label had Kyn or 3OHKyn been converted to anthranilic acid or 3-hydroxyanthranilic acid respectively.

The formation of 3OHKG in only 20 hr demonstrated by van Heyningen and the results of Chapter 4, which show that the metabolite does not accumulate in the lens but is maintained at a fixed level, indicate that significant formation is followed by removal of this compound.

In light of van Heyningen's result for one lens it was decided to further investigate the metabolism of Trp in intact normal human lenses using [U-$^{14}$C]-L-tryptophan and an incubation period of 24 hr. The use of universally labelled substrate would enable the detection of all possible catabolites. It was also hoped that the quantitative determination of label from LSC would enable an estimate of the rate of synthesis of 3OHKG. In addition, through analysis of the AAH as well as protein-free lens extracts following the 24 hr pulses it was hoped to determine the metabolic fate of 3OHKG.
5.2 Materials and Methods

5.2.1 Materials

*[^{14}C]-L-Tryptophan (this is the only form of radiolabelled Trp used in this chapter and is sometimes referred to simply as ^{14}C-Trp); [^{14}C]-D-Glucose—refer to Section 2.1.4.

*β-Glucosidase (EC 3.2.1.21)—7IU mg⁻¹ solid—was purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

*BCA Protein Assay Reagent was purchased from Pierce (Illinois, USA).

5.2.2 Methods

5.2.2.1 General Methods

Refer to previous sections for the following methods.

*Human lenses (2.1.1)

*Human Lens Organ Culture (2.2)

*Preparation of Lens Extracts (2.3)

*HPLC (2.4)

*Identification of Metabolites (3.3.2; 3.3.3).

*Quantification of Metabolites (4.2 and 4.3)

*Liquid Scintillation Counting (2.5)

5.2.2.2 ^{14}C-Trp Pulse Experiments

On arrival at the laboratory, lenses were removed from the
AAH in which they had been transported from the hospital and stabilised overnight in fresh AAH. The AAH containing 50μM Trp was changed again at the start of the experiment and [U-14C]-L-tryptophan was added to a final activity of 0.4μCi mL⁻¹. After 24 hours, lenses were removed from culture and rinsed twice for 10 seconds in unlabelled medium to remove any label adhering to the capsule. Lenses were frozen at -20°C until analysed. Samples of AAH from each culture (typically 500 μL) were lyophilised, reconstituted to 100 μL and analysed by HPLC.

5.2.2.3 Pulse-chase Experiments
Lens pairs were treated as in section 5.2.4 except that after 24 hours incubation in AAH containing [U-14C]-L-tryptophan one lens in each pair was transferred to fresh AAH containing 50μM Trp, but without [U-14C]-L-tryptophan, incubated for a further 24 hours, rinsed as before, blotted dry and frozen.

5.2.2.4 Analysis of Lens Protein
The quantity of protein in the lens precipitates obtained using the method described in Section 2.3, was measured using the BCA Protein Assay Reagent, which employs the bicinchoninic reaction (Smith et al, 1985, Wiechelman,1988). The standard (37°C; 30 min) protocol was used, according to the assay kit instructions. A standard
curve using the stock bovine serum albumin supplied with the assay kit was constructed. Stock 2.0 mg mL\(^{-1}\) BSA was diluted to a concentration range of 0.2-1.2 mg mL\(^{-1}\) using 3M urea, which according to the kit instructions does not interfere with the assay. The absorbance of the standard solutions were read in duplicate against a 3M urea blank at 562 nm. The average of the absorbance readings was plotted against the protein concentrations to give the standard curve depicted in Fig. 5.8. The lens protein pellets were dissolved in 3 mL 8M urea. A small portion of the digest was diluted 1:20 using 3M urea and the protein content assayed. The amount of total protein in mg was converted to μmol assuming a protein subunit molecular weight of 20,000. 1.0 mL of the digest was counted by LSC and the specific activity in dpm μmol\(^{-1}\) therefore calculated.

5.3 Results

5.3.1 Tryptophan Incorporation

Thirteen human lenses ranging in age from 48-91 years and two five year old lenses were pulsed for 24 hr in AAH containing [U-\(^{14}\)C]-L-tryptophan. All the lenses were transparent when viewed against a millimetre grid illuminated by transmitted light. All the lenses except the very young pair displayed the pale yellow colour characteristic of age except for one exceptional 76 year old pair. This
pair, despite being transparent throughout the whole lens, were a uniform brown colour from the nucleus to all but an outer rim of approximately 10% of the cortex. The boundary between the brunescence and the clear outer rim was perfectly circular. These were the only lenses of this type encountered during this project.

The protein-free extracts from these lenses were analysed by HPLC and a fraction collector used to collect 1 minute samples of the eluent. The amount of radiolabel detected in these samples was determined by LSC. In this way the amount of $^{14}$C-Trp transported into the lens could be detected as well as the radiolabel present in the Trp metabolites. The results of one such experiment are shown in Fig. 5.1.

The chromatogram at 365 nm (Fig. 5.1a) clearly identifies the major peak, 3OHKG, and the minor peaks of 3OHKyn and Kyn (as determined in sections 3.3.2 and 3.3.3). Significant amounts of radiolabel were detected at the retention times of 3OHKG and Kyn (Fig. 5.1b). This is not surprising considering the result in Section 3.3.4, where 5-$^3$H-tryptophan was used. The largest radioactive peak was due to unmetabolised Trp, which was not detected on the UV trace since it does not absorb at 365 nm.
Figure 5.1: The protein-free extract of a 75 year old human lens incubated for 24 hr in AAH containing 0.4 µCi mL\(^{-1}\) [U-14C]-L-tryptophan. (a) The HPLC chromatogram using a detection wavelength of 365 nm. The numbers above the peaks are retention times in minutes. The compounds were shown to be 3OHKyn (14.7), 3OHKG (17.1) and Kyn (29.7). (b) The radioactivity detected using LSC of eluted fractions from HPLC. Peaks of radioactivity were detected at the same retention times as the compounds detected in (a). The large late-running radioactive peak is due to [U-14C]-L-tryptophan. Tryptophan does not absorb at 365 nm and was therefore not detected by HPLC in (a).
Four million dpm were added to the AAH at the beginning of the pulse experiments and the quantity of label detected in the lenses after 24 hr show that this does not appear to have been limiting; being on average only 2.5% of the total added. Of the radiolabel that was transported into the lens, a significant amount was incorporated into Trp metabolites after 24 hr, as the radioactivity detected under the 3OHKG peak in Fig. 5.1b represents approximately 15% of that present in Trp.

To assess the degree of variability of 3OHKG biosynthesis in human lenses, the other lenses were analysed by HPLC, and the peaks of 3OHKyn, 3OHKG, Kyn and Trp were collected. The detection wavelength was altered from 365 to 278 nm, following detection of Kyn, so that Trp could be detected (see Section 4.2.3). The total amount of radiolabel in each compound was determined using LSC. The major incorporation, as judged by total dpm, was usually found into the metabolite 3OHKG; the amount of label detected ranging from 2,000-27,000 dpm. Kyn ranged from 400 to 8,200 dpm but was always significantly lower than 3OHKG except in one case (lens 229 Table 5.1). 3OHKyn ranged from no detectable radiation up to 4,400 dpm, but was again always much lower than 3OHKG and usually less than Kyn with one exception (lens 64 Table 5.1). In calculating the total dpm, the results of the lens extract recovery assays (Section 4.3.3) were taken into account, as a high proportion of the label would have been lost along with the low molecular
weight compounds during the preparation of the lens extracts. The raw data, presented as the dpm detected in Trp, Kyn, 3OHKyn and 3OHKyn glucoside, (Table 5.1), show varying rates of Trp transport into the lenses over the 24 hr incubation period. However, there is no correlation with age, the very young lenses being similar in this respect to many of the older lenses. The highest quantity of label present as Trp was in a 75 year old lens. There is nearly a ten-fold difference between this lens (approx. 162,000 dpm) and the lowest amount of Trp label detected in a 54 year old lens (approx. 17,000 dpm). Also, the highest amounts of label present as 3OHKG are in some of the lenses older than 70 years, and even one 81 year old lens displayed greater incorporation into 3OHKG than the 5 year old lenses, which were average in this respect compared to many of the older lenses.

The relative amounts of label as a percentage of the total dpm detected in all four compounds for the lenses for which all the information was available are given in Table 5.2.

Unmetabolised Trp accounts for more than 80% of the total except in three lenses where it is less than 75%. These are lenses 67 (67.1%), 69 (71.9%), and 128 (73.2%). These lenses also had an exceptionally high percentage of label present in 3OHKG; while the other lenses had less than 15% incorporation into this compound, these three had incorporation rates of 20% or greater.

Two of these lenses are the 76 year old brunescent pair.
mentioned previously and an 81 year old lens which was found to have an exceptionally low concentration of 3OHKG. At 0.1441 μmol 3OHKGg\(^{-1}\) lens weight, this level was very low even considering the age of the lens, and was easily the lowest level of 3OHKG among the pulsed lenses. Despite this, it incorporated label into 3OHKG faster than all but one other lens under the culture conditions. The brunescent pair by contrast had high levels of 3OHKG for their age.

The 75 year old lens with the highest total dpm (lens 50) was not exceptional for its percentage incorporation into 3OHKG. In appearance this was a normal lens and its analysis showed an unexceptional level of 3OHKG.

Only small percentages of the label were ever present in Kyn (max. 6.6%) or 3OHKyn (max. 4.2%). From these results it would appear that the major function of Trp metabolism in the lens is the formation of 3OHKG, although there is considerable variability in the rate with which this occurs in vitro.

If the age-related decrease in the level of 3OHKG in the lens demonstrated in Chapter 4 is due to higher rates of incorporation of Trp into 3OHKG in younger lenses, then it would have been expected that the 5 year old lenses in this study would have had the highest rate of incorporation of label into 3OHKG.
Table 5.1: The incorporation of radiolabel into metabolites of the 3-
hydroxykynurenine glucoside pathway in human lenses incubated for
24 hr in AAH containing 0.4 μCi mL\(^{-1}\) [U-\(^{14}\)C]-L-tryptophan.

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<th>LENS</th>
<th>AGE (Years)</th>
<th></th>
<th>Trp (dpm)</th>
<th>Kyn (dpm)</th>
<th>3OHKyn (dpm)</th>
<th>3OHKG (dpm)</th>
<th>TOTAL (dpm)</th>
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<td>48</td>
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<td>305</td>
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<td>544</td>
<td>7,115</td>
<td>74,833</td>
<td></td>
</tr>
</tbody>
</table>

ND=not determined; NA=not applicable

Table 5.2: The percentage incorporation of radiolabel into the 4
metabolites of the 3-hydroxykynurenine glucoside pathway in human
lenses incubated for 24 hr in AAH containing 0.4 μCi mL\(^{-1}\) [U-\(^{14}\)C]-L-
tryptophan.

<table>
<thead>
<tr>
<th>LENS</th>
<th>AGE (Years)</th>
<th>% Trp</th>
<th>% Kyn</th>
<th>% 3OHKyn</th>
<th>% 3OHKG</th>
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<td>91.6</td>
<td>2.5</td>
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<td>4.3</td>
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<tr>
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<td>81.7</td>
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<tr>
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<td>54</td>
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<td>2.7</td>
<td>0.9</td>
<td>10.3</td>
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<td>1.0</td>
<td>1.6</td>
<td>5.8</td>
</tr>
<tr>
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<td>2.2</td>
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</tr>
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<td>71.9</td>
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<td>20.0</td>
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<tr>
<td>72</td>
<td>76</td>
<td>95.6</td>
<td>0.9</td>
<td>0.0</td>
<td>3.5</td>
</tr>
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<td>64</td>
<td>78</td>
<td>90.0</td>
<td>1.4</td>
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<tr>
<td>128</td>
<td>81</td>
<td>73.2</td>
<td>2.1</td>
<td>1.1</td>
<td>23.6</td>
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<tr>
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<td>91</td>
<td>88.5</td>
<td>1.3</td>
<td>0.7</td>
<td>9.5</td>
</tr>
</tbody>
</table>

100
Although the study is hindered by a lack of lenses younger than 40, the 5 year old pair of lenses did have the highest concentrations of 3OHKG among the fifteen lenses used (Fig. 5.2A). The percentage incorporation of \(^{14}\text{C-Trp}\) into 3OHKG in the young lenses, however, was not exceptional (Table 5.2). In fact, since the older lenses had similar or higher quantities of radioactivity in 3OHKG compared with the young lenses (Table 5.1), their generally lower levels of 3OHKG (Fig 5.2A), meant that the specific activities of 3OHKG were often higher in the older lenses (Fig 5.2B). Although in Fig. 5.2 there is no apparent correlation between the concentration of 3OHKG and its specific activity after a 24 hr pulse in \(^{14}\text{C-Trp}\), it appears that lenses older than 60 years incorporated more label per unit of 3OHKG. If this is a real physiological phenomenon, then it may be that the decrease in 3OHKG with age demonstrated in Chapter 4 is due to a higher level of metabolism of this compound as the lens ages, which more than compensates for the apparent increased rate of synthesis.
Figure 5.2: The concentration (A) and specific activities (B) of 3OHKG in lenses after incubation for 24 hr in AAH containing [U-14C]-L-tryptophan.
5.3.2 Rate of Formation of 3OHKG

The specific activity of a compound is a measure of how much label is associated with a given quantity of that compound. Since 3OHKG is derived from Trp, the amount of radioactivity in 3OHKG (dpm) and the specific activity of Trp (dpm μmol⁻¹) in the lens at the end of the 24 hr incubation period, can be used to estimate the rate of formation of 3OHKG. Since Trp is an 11-carbon compound and one of these carbons is lost in the formation of 3OHKG, and since the ¹⁴C-Trp is universally labelled, the dpm determined for 3OHKG was multiplied by 11/10 (1.1) in order to make a more accurate correlation between the two compounds. The radiolabel detected in 3OHKG (dpm) was then divided by the specific activity of Trp (dpm μmol⁻¹) to obtain the quantity of newly synthesised 3OHKG (μmol).

The calculations for the 12 lenses for which all the necessary data had been obtained are displayed in Table 5.3. The final result was expressed in nmol. It must be borne in mind that this is a minimum value for 3OHKG synthesis, since the specific activity of Trp will increase over the 24 hr period. Trp was not quantified in 3 of the lenses (61, 51 and 50) in Table 5.1.

The 76 year old brunescent pair had by far the highest rates of formation of 3OHKG, at 25.6 and 13.2 nmol in the 24 hr culture period. In all the others less than 10 nmol were formed, the 81 year old with the lowest concentration of 3OHKG having the highest
quantity formed (8.3 nmol) among the lenses of normal appearance. The young pair of lenses had an unexceptional rate of 3OHKG formation.

**TABLE 5.3:** Estimates of the quantity of 3OHKG formed in 24 hr in human lenses incubated in AAH containing 0.4 μCi mL⁻¹ [U-14C]-L-tryptophan.

<table>
<thead>
<tr>
<th>Lens</th>
<th>Age (years)</th>
<th>dpm in 3OHKG</th>
<th>dpm in 3OHKG</th>
<th>Sp. Activity (dpm/μmol)</th>
<th>3OHKG formed in 24 hr (nmol)</th>
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<td>7,249</td>
<td>7,974</td>
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<td>4,670</td>
<td>8.27e+5</td>
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5.3.3 Pulse-chase Experiments

5.3.3.1 Turnover of 3OHKG

The major restriction on the use of whole lenses to study metabolic incorporation is that data from only one time point can be obtained. In this study, 24 hr was used as a convenient time period in which to measure Trp incorporation. It was found that metabolism of Trp was readily observable in single lenses in that time.
Assuming that a pair of lenses from the same individual are metabolically similar in vivo, then such lens pairs may be studied in vitro in order to provide incorporation data at two different time points. Comparison of the data may reveal evidence of metabolic breakdown and/or turnover. In order to do this, two pairs of lenses were incubated (one lens per culture dish) as before (Section 5.3.1) in AAH containing $^{14}$C-Trp. After 24 hr., both lenses were removed from the AAH containing radiolabel. One was rinsed to remove any label adhering to the lens capsule and frozen until analysed. The other lens was transferred to fresh AAH containing no $^{14}$C-Trp and incubated for a further 24 hr; in effect 'chasing' the radiolabel that was present in the lens after the 24 hr 'pulse'. This 'chased' lens was then rinsed as before and frozen until analysed. These 'chase' incubations were performed on the contralateral lenses of lens 50 (age=75) and lens 128 (age=81) in Table 5.1.

Fig. 5.1 shows the $^{14}$C-Trp incorporation from a 'pulsed only' lens from one of these experiments. The radiolabel profiles of this lens and its pulse-chased pair are compared in Fig. 5.3.

There were no new or larger radiolabelled peaks detected after the chase. No evidence was therefore found of either further metabolic products of 3OHKG or of any other pathway for Trp metabolism; e.g. if label had been detected at the retention time of 3-hydroxyanthranilic acid, this would have indicated that some Trp
in the lens is catabolised via the normal oxidative pathway present in other organs.

**FIGURE 5.3:** The radioactivity detected using LSC of eluted fractions from the HPLC of protein-free extracts from a pulsed-only lens (--) and a pulse-chased lens (---). The lenses were a pair from the same 75 year old individual. See Fig. 5.1 for identities of the radioactive peaks. The amount of radiolabelled tryptophan has reduced by approximately two-thirds during the chase and turnover of the tryptophan metabolites also appears to have occurred.

The quantity of radiolabel under the 3OHKG peak in the pulsed-chased lens was 50% less than in the pulsed lens. Assuming a similar incorporation at the end of 24 hr in both, this indicates that metabolism of 3OHKG has occurred during the subsequent 24 hr in culture. The radiolabel detected under Kyn was also much less in the 'chased' lens.

The specific activities of 3OHKG in this and the other pulse-
chase pair are illustrated in Fig. 5.4. Despite the differences in absolute specific activities of 30HKG between the two pairs, both showed a very similar decrease in specific activity following the 24 hr chase: 63% in A and 67% in B.

The accumulated data for these two pairs is summarised in table 5.4. The quantity of 30HKG present in the individual lenses of both pairs are similar but interestingly, the amount in both of the pulse-chased lenses was approximately 20% higher than that in the pulsed lenses. The quantity of Trp in the 81 year old pair was identical. Since the lenses of each pair were treated similarly until they arrived at the laboratory, and the 24 hr pulses were carried out concurrently, one may assume that the metabolic activity of the individual lenses in each pair in culture were similar. This is especially pertinent for the results shown in Fig. 5.4B, which are from lens 128—the 81 year old pair that displayed a high incorporation rate of label into 30HKG despite having a very low intrinsic level of the compound—and its pair, which had a similarly low concentration of 30HKG. That the concentration of 30HKG was unusually low in both lenses indicate that they were in a similar metabolic condition in vivo and therefore may have behaved similarly in vitro. The lower specific activity of 30HKG in the pulse-chased lens is most likely due therefore to significant removal of the compound during the 'chase', either by further metabolism or via some other means.
FIGURE 5.4: The specific activities of 3OHKG in protein-free lens extracts from 2 pulse-chased lens pairs. (■) Pulsed only lenses. (■■) Pulse-chased lenses. (A) A 75 year old lens pair (see Fig. 5.3). (B) An 81 year old pair. The decrease in specific activity was 63% in (A) and 67% in (B).
Table 5.4: Data obtained from protein-free extracts of lens pairs used in pulse-chase experiments (the values were obtained from analysis of 1/3 extracts and have not been adjusted to account for loss of metabolites during sample preparation).

<table>
<thead>
<tr>
<th>Lens</th>
<th>Age (Years)</th>
<th>dpm 3OHKG</th>
<th>Amount 3OHKG (µmol)</th>
<th>SA 3OHKG (dpm/µmol)</th>
<th>dpm Trp</th>
<th>Amount Trp (µmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>75</td>
<td>2,918</td>
<td>0.0206</td>
<td>141,946</td>
<td>21,658</td>
<td>ND</td>
</tr>
<tr>
<td>47</td>
<td></td>
<td>1,341</td>
<td>0.0250</td>
<td>53,696</td>
<td>5,852</td>
<td>ND</td>
</tr>
<tr>
<td>128</td>
<td>81</td>
<td>1,284</td>
<td>0.0047</td>
<td>273,579</td>
<td>7,144</td>
<td>0.0031</td>
</tr>
<tr>
<td>119</td>
<td></td>
<td>563</td>
<td>0.0060</td>
<td>94,084</td>
<td>2,367</td>
<td>0.0031</td>
</tr>
</tbody>
</table>

ND=not determined

5.3.3.2 Efflux of Tryptophan

The results depicted in Fig. 5.3 show that, assuming roughly equal levels of Trp in both lenses after the pulse, there has been an approximately 70% decrease in labelled Trp during the chase.

In order to see how much of the $^{14}$C-Trp loss during the chase was due simply to efflux of Trp from the lens, aliquots of the chase AAH from lens 119 were removed during the second 24 hr incubation period. Another single lens (lens 140—also aged 81) was pulse-chased as for lenses 50 and 119 specifically to gather data on this aspect. The aliquots of AAH were analysed by HPLC and peaks of Trp collected and counted by LSC. The total amount of dpm due to Trp in the AAH was calculated for each time point. The results for the two lenses are shown in Fig. 5.5. For lens 140 (Fig. 5.5A) it would appear that the dpm in the AAH is still increasing after 24 hr while

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for lens 119 (Fig. 5.5B) it appears to have slowed by this time. Despite the variability between the lenses, it is obvious that a significant amount of $^{14}$C-Trp present in the lens after a 24 hr pulse is lost by efflux during the subsequent chase. Since some of the Trp must inevitably be incorporated into protein, the reduction in the amount of labelled Trp in the lens during the chase can be attributed to lens efflux, protein biosynthesis and, as the results of Table 5.1 indicate, 3OHKG biosynthesis.

The quantity of labelled Trp in lens 128 (pulsed for 24 hr) was 53,600 dpm. Its pulsed-chased pair, lens 119, contained 17,800 dpm in Trp while 44,000 dpm Trp had effluxed into the AAH during the chase (total=61,800 dpm). This indicates that a high proportion (approx. 70%) of the Trp was lost from the lens due to a process of equilibration of the amino acid between the inside of the lens and the AAH. Since there was also a 50% loss of 3OHKG during the chase but no indication of any further metabolism the compound, it was decided to see if it too was released into the AAH.
Figure 5.5: The quantity of radiolabel detected in Trp in the AAH of two 81 year old lenses pulsed for 24 hr in AAH containing 0.4 μCi mL⁻¹ [U-14C]-L-tryptophan then 'chased' for a further 24 hr in AAH without label. (A) Lens 140; (B) Lens 119.
5.3.4 Efflux of 3OHKG into AAH

5.3.4.1 Identification of 3OHKG in AAH

Since the pulse-chase experiments indicated a significant loss of 3OHKG, it was thought that a route of turnover may simply be release into the AAH. Analysis of a sample of AAH from a 24 hr lens incubation by HPLC (365 nm detection) revealed a peak eluting at a retention time typical of 3OHKG. This peak was not detected in a sample of control medium. In order to determine whether the compound detected was in fact 3OHKG, 2x500 µL samples were treated as follows:-

1) Incubated for 4 hr at 35°C (Control)

2) Incubated for 4 hr at 35°C + 1 mg β-glucosidase.

At the end of the incubation period, the samples were lyophilised, reconstituted in 100 µL Milli-Q water and analysed by HPLC. In the enzyme-treated sample, the peak in the control sample was shifted from a retention time typical of 3OHKG to a retention time typical of 3OHKyn (Fig. 5.6). It seems therefore that 3OHKG is being released from the lenses into the AAH.
Figure 5.6: HPLC chromatograms at 365 nm of a peak collected from AAH following incubation of a lens for 24 hr. The peak was divided into two equal portions, one half treated with β-glucosidase and both portions re-run on HPLC. The chromatogram on the left is the control and that on the right the treated sample. Retention time in min printed above each peak.

5.3.4.2 Quantity of 3OHKGY Effluxed in 24 Hr

Samples of the AAH from the lenses used in 5.3.1 were analysed by HPLC. The quantity of 3OHKGY was estimated using the 3OHKyn standard curve (Section 4.3.1) and the total amount released into each AAH calculated. It was not necessary to take into account the factor for metabolite loss (Section 4.3.3), since no extraction or ultrafiltration was necessary. Samples were simply lyophilised then reconstituted in 100 µL Milli-Q water before analysis.

The quantity of 3OHKGY detected in each AAH is plotted with the estimate of the quantity formed in 24 hr from radioactive incorporation data (Table 5.3), in Fig. 5.7.
Figure 5.7: The quantity of 3OHKG detected in the AAH (■) and an estimate of the quantity of 3OHKG formed (□) in human lenses incubated for 24 hr in AAH containing 50 μM (0.4 μCi mL⁻¹) [U-14C]-L-tryptophan.

5.3.5 Incorporation of Trp Into Lens Protein

The results in Chapter 4 indicated a probable age-related change in the metabolism of Trp in human lenses, demonstrated by a decrease in the level of metabolites of the major Trp metabolic pathway as a function of age. Since the lens is continually synthesising protein, and since the level of free Trp appears to be relatively stable throughout life, this age-related change may be due to a greater percentage of available Trp being incorporated into
protein as the lens ages. In order to test this premise, the protein pellets precipitated in the preparation of the lens extracts following $^{14}$C-Trp pulses were analysed for the incorporation of Trp radiolabel.

5.3.5.1 Standard Curve

![Graph of BSA standard curve](image)

**Figure 5.8:** The BSA standard curve used for measuring the quantity of protein in precipitates obtained from lenses homogenised in 80% ethanol.

5.3.5.2 Determination of Protein in Lens Precipitates

The protein pellet from each lens was dissolved in 3.0 mL 8M urea. It was found that diluting a small aliquot 1:20 with 3M urea produced absorbance readings within the range of the standard curve. Duplicate samples were measured and the average absorbance readings used to determine the protein concentration.
This value was multiplied by \(60^1\) in order to determine the total quantity of protein in the original 3 mL solution and divided by 20,000 to convert this value to \(\mu\)mole protein.

The total dpm in the protein pellet was determined by counting 1.0 mL of the 8M urea protein solution by LSC. In order to test the accuracy of the LSC readings in the presence of urea and protein, the protein pellet from a lens that had not been in contact with radioactivity was dissolved in 3.0 mL 8M urea. An amount of \(^{14}\)C-glucose containing 22,000 dpm was added to two scintillation vials. One was left as a control and to the other 1.0 mL of the protein solution was added. The LSC readings were 22,300 for the control and 20,900 for the protein; a difference of 6%. This was considered acceptable and no adjustments to raw dpm values were made. The results for the 24 hr \(^{14}\)C-Trp pulsed lenses are summarised in Table 5.5 and the protein incorporation graphed in Fig. 5.9.

With the exception of one lens (no. 51), there was generally a higher quantity of dpm detected in protein in the older lenses compared to the two five year old lenses. The young lenses also had a generally lower protein content (with the exception of no. 65), as would be expected, and the specific activities showed no trend with age, the very young and very old lenses being similar in this respect. The lenses in the sixties age-group had the highest values.

\(^1\) A 1:20 dilution of a 3.0 mL solution
Table 5.5: The incorporation of radiolabel into protein in human lenses incubated for 24 hr in AAH containing 0.4 μCi mL⁻¹ [U-14C]-L-tryptophan

<table>
<thead>
<tr>
<th>LENS (Years)</th>
<th>AGE (Years)</th>
<th>TOTAL PROTEIN (mg)</th>
<th>TOTAL PROTEIN (μmol)</th>
<th>TOTAL dpm in PROTEIN</th>
<th>SPECIFIC ACTIVITY (dpm/μmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>59</td>
<td>5</td>
<td>34.46</td>
<td>1.723</td>
<td>8,417</td>
<td>4,885</td>
</tr>
<tr>
<td>73</td>
<td>5</td>
<td>26.80</td>
<td>1.340</td>
<td>8,690</td>
<td>6,468</td>
</tr>
<tr>
<td>61</td>
<td>48</td>
<td>41.78</td>
<td>2.089</td>
<td>10,078</td>
<td>4,824</td>
</tr>
<tr>
<td>66</td>
<td>53</td>
<td>48.80</td>
<td>2.440</td>
<td>17,993</td>
<td>7,374</td>
</tr>
<tr>
<td>51</td>
<td>54</td>
<td>47.24</td>
<td>2.362</td>
<td>5,673</td>
<td>2,402</td>
</tr>
<tr>
<td>224</td>
<td>63</td>
<td>52.54</td>
<td>2.627</td>
<td>31,624</td>
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</tr>
<tr>
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<td>45.79</td>
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</tr>
<tr>
<td>65</td>
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<td>26.37</td>
<td>1.318</td>
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<td>10,169</td>
</tr>
<tr>
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<td>76</td>
<td>71.03</td>
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<td>23,260</td>
<td>6,550</td>
</tr>
<tr>
<td>69</td>
<td>76</td>
<td>46.68</td>
<td>2.334</td>
<td>18,365</td>
<td>7,869</td>
</tr>
<tr>
<td>72</td>
<td>76</td>
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<td>9,447</td>
</tr>
<tr>
<td>64</td>
<td>78</td>
<td>53.86</td>
<td>2.693</td>
<td>19,374</td>
<td>4,966</td>
</tr>
<tr>
<td>128</td>
<td>81</td>
<td>48.04</td>
<td>2.402</td>
<td>16,060</td>
<td>6,686</td>
</tr>
<tr>
<td>120</td>
<td>91</td>
<td>50.57</td>
<td>2.528</td>
<td>17,781</td>
<td>7,032</td>
</tr>
</tbody>
</table>

The specific activities of protein after the 24 hr pulses also do not indicate any trend with age. Surprisingly, the raw counts (total dpm) into protein provide some evidence that more Trp is incorporated into protein in older lenses (Fig. 5.9). If this is so, then, since the concentration of free Trp remains relatively constant throughout life (Fig. 4.3A), the age-related decrease in metabolites of the 3OHKG pathway (Fig. 4.3B-D) may possibly be explained by relatively more free Trp being utilised for protein synthesis in older lenses. However, incorporation experiments (Section 5.3.2) did not provide evidence for an age-related decrease in the conversion of Trp to 3OHKG.
Figure 5.9: The incorporation of radiolabel into protein in human lenses incubated for 24 hr in AAH containing 0.4 μCi mL⁻¹ [U-14C]-L-tryptophan.

5.3.6 The Percentage Incorporation of Trp Radiolabel Into the Metabolites of the 3OHKG Pathway and Protein

Having analysed the incorporation of Trp into its metabolites and protein, and having also established a major route for the removal of 3OHKG, the major Trp metabolite, there was still inadequate data to explain the observed decrease in levels of Kyn, 3OHKyn and 3OHKG that had been found to occur with increasing age.
The radioactivity present in a lens after a 24 hr pulse with [U-14C]-L-tryptophan can be divided into three categories:-

i) free $^{14}$C-Trp;

ii) $^{14}$C-Trp incorporated into metabolites of the 30HKG pathway;

iii) $^{14}$C-Trp incorporated into lens protein.

It was decided to directly compare all of these sources of radiolabel for each lens. This was done by summing the dpm and then calculating the percentage for each category (Table 5.6). The total dpm detected in each lens showed no correlation with age (Fig 5.10).

![Figure 5.10: The total quantity of radiolabel detected as a function of age (including free Trp, Kyn, 3OHKyn, 3OHKG and protein) in human lenses incubated for 24 hr in AAH containing 0.4 μCi mL$^{-1}$ [U-14C]-L-tryptophan.](image)
The data comparing incorporation into 3OHKG and protein are tabulated in Table 5.6. While the five year old lenses have a 1:1 ratio of dpm detected in the 3OHKG pathway to dpm detected in protein, in the older lenses this ratio is generally much less than 1. Three exceptions to this observation are the 76 year old brunescent pair and the 81 year old single lens with the unusually low concentration of 3OHKG discussed previously. While displaying a percent incorporation into protein comparable to lenses of a similar age, these lenses had the highest percentage incorporation into 3OHKG, being approximately double the values found in other lenses. The other nine lenses above age 40 by contrast had from 2-5 times more radiolabel incorporated into protein than into 3OHKG. [The protein in lens 50 (Table 5.1) was not determined]. Although this raises the possibility that there may be an decrease in the relative proportion of Trp being used for 3OHKG synthesis in older lenses, the lack of young lenses made it difficult to draw firm conclusions. It must be concluded that there is insufficient data at present to explain the age-related decline in Trp metabolites found in Chapter 4. It is hoped that in the future resources and a greater number of normal human lenses may become available to further investigate this particular aspect of lens Trp metabolism.
Table 5.6: The comparative radiolabel present in free Trp, the metabolites of the 3-hydroxykynurenine pathway and precipitated protein in human lenses incubated for 24 hr in AAH containing 0.4 μCi mL⁻¹ [U-14C]-L-tryptophan.

<table>
<thead>
<tr>
<th>Lens Age</th>
<th>Free Trp</th>
<th>Glucoside pathway</th>
<th>Protein</th>
<th>TOTAL</th>
<th>% TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>59</td>
<td>63,327</td>
<td>9,505</td>
<td>8,417</td>
<td>81,250</td>
<td>77.9</td>
</tr>
<tr>
<td>73</td>
<td>95,593</td>
<td>8,767</td>
<td>8,690</td>
<td>113,050</td>
<td>84.6</td>
</tr>
<tr>
<td>61</td>
<td>ND</td>
<td>3,708</td>
<td>10,078</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>66</td>
<td>25,858</td>
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<td>17,394</td>
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<td>31,624</td>
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<td>26,788</td>
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</tr>
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<td>13,405</td>
<td>98,506</td>
<td>79.1</td>
</tr>
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<td>31,893</td>
<td>23,260</td>
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<td>80,499</td>
<td>31,433</td>
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<td>130,297</td>
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</tr>
<tr>
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</tr>
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<td>89,260</td>
<td>60.0</td>
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<td>66,202</td>
<td>8,632</td>
<td>17,781</td>
<td>92,614</td>
<td>71.5</td>
</tr>
</tbody>
</table>

NA=not applicable; ND=not determined

5.3.7 Post Death Time Delay to Commencement of Experiments

Biographical data on the organ donors revealed that the eyes were enucleated 4 to 22 hr after death. The eyes were then dissected by staff of The Sydney Hospital and the lenses transferred to supplied vials containing 5 mL AAH (Section 2.1.1) and stored in an incubator at 37°C until transported by road to the facility where the experiments were carried out, a journey of 90 km. Lenses were then stabilised overnight in fresh AAH before commencement of the 24 hr pulse experiments.
Although the total number of lenses is not large, no correlation could be found between experimentally observed Trp transport and metabolism in the lens and either (i) the time of enucleation, (ii) placing the lenses into AAH at the eye hospital or (iii) commencement of the experiment; following death of the donor. The data for the latter as a function of total transport of $^{14}$C-radiolabel into the lens and its incorporation into 3OHKG are given in Fig 5.11.

Figure 5.11: The total radioactivity (■) and percentage radiolabel incorporated into 3OHKG (□) in human lenses incubated for 24 hr in AAH containing 0.4 μCi mL$^{-1}$ [U-14C]-L-tryptophan plotted against the time interval between death and the beginning of 24 hr pulse.
5.4 Discussion

The most abundant lens metabolite of Trp in the cultured human lens is 3-hydroxykynurenine glucoside, and this was also the metabolite most highly labelled, as judged by incorporation of radiolabel from Trp in a 24 hr period. The objective of this chapter was to obtain data on the synthesis and degradation of this unique metabolite and possibly to elucidate the cause of the alteration in Trp metabolite levels that occur as a function of age in vivo (Chapter 4).

In an attempt to obtain data on these aspects, lenses of different ages were incubated for 24 hr in AAH containing 50μM Trp to which 14C-radiolabelled Trp was added. The concentration of Trp in human aqueous humor has been determined in previous studies (Barber, 1968; Dickinson, Durham and Hamilton, 1968) to be 30μM.

Radiolabelled Trp was transported into the lens from the AAH and substantial incorporation of the label into Trp metabolites was detected in 24 hr. The major incorporation was into 3OHKG, and label was also detected in Kyn and 3OHKyn, as one would expect if 3OHKyn were an intermediate in a pathway involving glucosylation as a final step (Knox and Mehler, 1950). Post-mortem treatment of lenses appeared to have no bearing on these results (Section 5.3.7). Van Heyningen (1973b), was able to demonstrate incorporation of
[methylene-$^{14}$C]-Trp into 3OHKG and Kyn, but not into 3OHKyn. Had there been any other metabolites of the normal liver Trp oxidative pathway, e.g. 3-hydroxyanthranilic acid, then these could not have been detected by radiolabel, as the only labelled carbon would be located in alanine following the action of kynureninase. Even using U-$^{14}$C-Trp, however, no evidence of 3-hydroxyanthranilic acid could be found in this present study. If there is any other metabolism of 3OHKyn except glucosylation, then it occurs either too slowly to be detected in 24 hr or so quickly that 3-hydroxyanthranilic acid is further oxidised before any build up of the compound occurs. The latter is conceivable since 3-hydroxyanthranilic acid is rapidly oxidised in vivo (Bender, 1982).

The present study was hampered by the lack of lenses, especially lenses younger than 40 years of age. It was demonstrated in Chapter 4 that the concentration of Trp metabolites declines from a young age until approximately age 40. Only two 5 year old lenses became available during this study, and all the other lenses were over 40 years old.

With three notable exceptions, the percentage incorporation of Trp into 3OHKG (Table 5.2) was no different in the two young lenses than in the older lenses. This was unexpected, as results in Chapter 4 led to the expectation that young lenses may have a greater rate of conversion of Trp to 3OHKG. When expressed on an incorporation per
μmole basis it appeared that lenses older than 60 in fact incorporated label into 30HKG more rapidly than younger lenses (Fig. 5.2), leading to speculation that perhaps the decline in levels with age is not because older lenses lose the ability to convert Trp to 30HKG but because the processes involved in metabolism/efflux of this compound increase with age. However, there was no correlation of 30HKG loss into the AAH (the only route of turnover found), with age (5.3.4.2). Nor was any age-related difference observed when incorporation of Trp into protein was tabulated on a per μmole basis (5.3.5.2).

Pulse-chase experiments revealed that, as well as Trp being converted rapidly to 30HKG, the turnover of 30HKG is also rapid. Although no evidence for further catabolism of this compound was observed, 30HKG was detected in the AAH (5.3.4.1), indicating efflux from the lens as a means of removal. Evidence that this is the actual physiological route of turnover may possibly be obtained by analysing the eye humors, where the compound must be initially present upon leaving the lens. The efflux from the lens may be an active transport process, as 30HKG, like Trp, is charged at physiological pH, and cell membranes are impermeable to the diffusion of charged molecules (Stryer, 1981).

If passive diffusion or efflux of 30HKG into the AAH was the only route for removal of this compound, it would be expected that rates of loss and formation would be similar after 24 hr in culture.
The results (Fig. 5.7) do not indicate this and in fact efflux rates are often higher. The rate of efflux in the absence of evidence of further metabolism of 3OHKG (see Chapter 7) is probably the more realistic figure. The rate of synthesis on the other hand is likely to be a rough estimate, since the quantity formed (Table 5.3) was based on the radiolabel associated with 3OHKG inside the lens. The results in Section 5.3.3.1, however, showed that significant efflux of 3OHKG probably occurs in this time. Therefore, if some of the 3OHKG is released into AAH within 24 hr of synthesis, then the dpm value for 3OHKG inside the lens will not take into account this loss of radiolabelled 3OHKG. In addition, the specific activity of Trp used to calculate the rate of formation of 3OHKG is a maximum value at the end of the 24 hr culture period. Therefore the estimate of 3OHKG synthesis is probably significantly lower than the actual value. All that can be noted is that both the efflux and formation of 3OHKG occur in low nmolar amounts in 24 hr in vitro.

A higher rate of efflux could in theory provide an explanation for the decrease in concentration of 3OHKG with age demonstrated in Chapter 4. If older lenses were more permeable to small molecules, then 3OHKG levels would decrease due to greater loss of the compound. Assuming that release of 3OHKG into the surrounding humor is the only or major route of turnover of this compound in vivo, this is not borne out by the efflux results in Fig. 5.7, in which no age-related correlation of either synthesis or efflux can be
All the lenses examined were able to incorporate Trp radiolabel into 3OHKG at a significant rate. The most surprising result was in an 81 year old lens that had a comparatively high rate of incorporation into 3OHKG yet possessed the lowest actual concentration of this compound. This resulted in a very high specific activity (Fig 5.2). This lens and its pair, used in a pulse-chase experiment (5.3.3.1), both had unusually low concentrations of Trp as well as 3OHKG. They did not display a greater than normal efflux of 3OHKG in 24 hr, however; nor was there anything unusual about the lactate output (lens 128=0.74 μmol lactate hr\(^{-1}\) g\(^{-1}\); compare with typical results in Chapter 3). The reasons behind the low concentration but high rate of incorporation of 3OHKG in these lenses is unclear.

The other two lenses with unusually high rates of 3OHKG formation, by contrast, had high, although not exceptional, concentrations of 3OHKG but normal levels of Trp. This pair of lenses (76 years of age) were of unusual appearance in that, although transparent, the bulk of the lens was brown in colour and was surrounded by a ring of clear colourless cortex. In relation to the potential for protein colouration by 3OHKyn (Stutchbury and Truscott, 1993), it is particularly noteworthy that this pair of lenses with marked brunescence also displayed the highest flux through
the 3OHKG pathway. Thus in these two lenses there would be expected to be a high level of production of 3OHKyn, a metabolite which has been shown to readily bind to crystallins, and to form brown coloured proteins. Further study of such unusual lenses may provide an insight into whether Trp metabolism plays a role in the age-related colouration of human lenses.

The percentage of $^{14}$C-Trp incorporated into protein compared to that converted to 3OHKG was much lower in the two 5 year old lenses than in most of the lenses older than 40 (5.3.6; Table 5.6). This was the only evidence found that may possibly explain the higher concentration of 3OHKG in younger lenses (Fig. 4.3D). This aspect is worthy of further investigation on more lenses, especially if young lenses become available.

It would seem that the Trp which is available for conversion to 3OHKG remains relatively constant over time, since free Trp levels are constant and the amount of Trp being incorporated into protein also seems relatively unaffected by lens age. The latter observation is in accord with that of Marcantonio and Duncan (1987) who demonstrated active protein synthesis in human lenses above age 60; and Satoh (1972) who demonstrated a linear increase with age in total lens protein. Since the quantity of label found in protein was quite constant across the age range studied, but incorporation into 3OHKG varied, it would appear likely that 3OHKG biosynthesis is not
regulated indirectly by the demands placed on free Trp for protein incorporation, but is instead controlled independently.

Potential control of the 3OHKG biosynthetic pathway could take place at several levels. One possible site is at the level of the first enzyme which oxidatively cleaves Trp to N'-formylkynurenine. This enzyme is assumed to be indoleamine dioxygenase (IDO) (EC. 1.13.11.17) and would utilise superoxide as a cosubstrate (Hayaishi, 1976). Levels of lenticular superoxide may therefore affect the overall rate of 3OHKG biosynthesis. Utilisation of superoxide in this manner as well as the potential antioxidant activity of 3OHKyn (Christen et al, 1990) may add to the possible protective role of the biosynthetic pathway (Luthra and Balasubramanian, 1992). A caveat is that the oxidised 3OHKyn formed, may be highly reactive.
CHAPTER 6
CHAPTER 6
THE DISTRIBUTION AND TURNOVER OF TRYPTOPHAN METABOLITES IN HUMAN LENSES

6.1 Introduction

The metabolic fate of 3OHKG, from evidence gained using in vitro studies (Chapter 5), appears to be its release from the lens into the surrounding humor. The compound was detected in the AAH following lens incubations (Section 5.3.4) in similar quantities to its apparent rate of formation over the same time period (Section 5.3.2). In addition, no other products of Trp metabolism other than Kyn, 3OHKyn and 3OHKG were detected to indicate any further metabolism of 3OHKG.

Pulse-chase analysis has been employed on single lenses in the following study to further investigate the efflux of 3OHKG into the AAH, and to assess the distribution of 3H-Trp and its metabolites in various regions of the lens following exposure to the radiolabel. In addition, the quantities of Kyn, 3OHKyn and 3OHKG were determined in these regions. In her early work on the compound, van Heyningen (1973a) determined that 3OHKG is present in both cortex and nucleus and that the levels were similar in each. This aspect has been examined further.

Having identified and quantified 3OHKyn in the whole lens (Chapter 4), it was important to determine the distribution of this compound throughout the lens, and especially, due to the reactive
metabolite hypothesis of cataract formation (Truscott et al., 1991), to establish its presence or absence in the nucleus, as changes in the proteins that accompany age-related colouration occur predominantly in the proteins of the nucleus (Dilley and Pirie, 1974; Truscott and Augusteyn, 1977).

In addition, radiolabelled 3OHKyn was employed to gather further evidence on the above aspects and also to confirm the presence of a glucosyl transferase enzyme in the lens.

6.2 Materials and Methods

6.2.1 Materials

*L-[5-3H]-Tryptophan:—refer to Section 2.1.4 (this compound is referred to throughout the text as 3H-Trp).

*Bovine Serum Albumin (BSA) and Hyamine (methylbenzethonium hydroxide) [1.0M in ethanol] were purchased from the Sigma Chemical Co. St. Louis, MO, U.S.A.

*Human Vitreous Humor was obtained from the same source as human lenses (Section 2.1.1). At the time of removal of lenses from donor eyes, the vitreous humor was taken up in a 1 mL syringe and frozen at -70°C until analysed.

*Phosphate buffer pH 6 was prepared according to Dawson et al (1986).

*C18 Sep Pak Cartridges were purchased from Waters, MA, U.S.A.

*Acetonitrile (HPLC grade) was purchased from BDH Laboratory
*10,000 MW centrifugal ultrafilters were purchased from Activon Scientific Products, Sydney, Australia.

6.2.2 Analysis of Lens Segments

Using a 3 mm cork borer, a core was taken through the centre of the frozen lens from the anterior to the posterior sides. This core, subsequently referred to as the visual axis, was divided, as carefully as possible, into anterior cortex (CA), nucleus (N) and posterior cortex (Cp) using a scalpel, as shown in Fig. 6.1. The doughnut-shaped remainder of the lens following removal of the visual axis is referred to as the equatorial region (Eq).

![Diagram of lens segments](attachment:image.png)

**Figure 6.1**: Diagrammatical cross-section of a lens, viewed perpendicularly to the lens equator, showing the division of a central core removed with a 3 mm cork borer into anterior cortex (CA), nucleus (N), posterior cortex (Cp) and the remaining equatorial region (Eq).

The pieces of tissue were placed into preweighed Eppendorf
tubes and the tubes reweighed. Protein-free extracts were then prepared according to Section 2.3, (except that visual axis segments were extracted using only 100μL 80% ethanol), and analysed by HPLC according to Section 2.4.

6.2.3 The Rate of Efflux of 3-Hydroxykynurenine Glucoside

Aliquots (500 μL) of AAH removed at various time intervals during lens incubations were lyophilised, reconstituted in 100 μL Milli-Q water and chromatographed on HPLC with detection at 365 nm in order to quantify 3OHKG.

6.2.4 Analysis of Human Vitreous Humor

Human vitreous samples (approx. 1 mL) were transferred to 1.5 mL Eppendorf tubes and ground gently with a teflon homogeniser, then ultrafiltered through a 10,000MW cutoff centrifugal membrane filter. The filtrate was lyophilised, reconstituted in 100 μL Milli-Q water and chromatographed by HPLC.

6.2.5 Pulse-chase Experiments

Human lenses maintained under organ culture conditions (see Section 2.2) were incubated at the start of the experiments in 5 mL AAH to which 100 μL L-[5-3H]-tryptophan had been added (final concentration 50μM [20μCi mL⁻¹]). The lenses were first of all
incubated for 24 hr at 35°C, then transferred to 30 mL sterile plastic screwcap vials containing 6 mL fresh AAH. The vials were swirled gently for 5 min, then 2x500 µL aliquots of AAH were removed and frozen at -20°C. (These were the 'zero' time point samples). The vial was then incubated at 35°C and 2x500 µL aliquots of AAH removed 2, 5, 8 and 24 hr after the start of the chase. One of the aliquots removed at each time point was used to determine the release of 3OHKG, and the other, the release of protein into AAH. Tall screwcapped vials were used in preference to the shallow petri dishes normally used for lens culture experiments so that the lenses remained wholly immersed even when the final aliquots of AAH were removed. At the completion of the experiment, the lenses were rinsed twice for 10 sec in unlabelled medium, and frozen at minus 20°C until analysed.

6.2.6 Leakage of Lens Protein Into the AAH

A 500 µL aliquot of AAH collected at each time point during the lens chase was assayed for lens protein. Since only minute amounts of lens protein, if any, would be expected in the AAH, protein was measured by taking advantage of the fact that a proportion will be radiolabelled following incorporation of ³H-Trp. BSA (1.0 mg) was added to each sample in order to obtain a visible protein pellet. This was achieved by adding 250 µL of 4 mg mL⁻¹ BSA to each 500 µL sample of AAH. The protein was then
precipitated by adding 750 μL 20% TCA and leaving overnight at 4°C. The protein was pelleted by centrifugation at 15,000 g and washed 3 times in 1 mL 2% TCA then once with ether. This method has been used successfully by Marcantonio and Duncan (1986) to measure labelled protein released from bovine lenses in culture. The pellets were transferred to scintillation vials, dissolved in 200 μL hyamine (Kabayashi and Maudsley, 1974) and counted by LSC.

6.2.7 ³H-3-Hydroxykynurenine Experiments

6.2.7.1 Preparation of Tritiated 3-Hydroxykynurenine

Tritiated 3-hydroxykynurenine was prepared in the laboratory of Assoc. Prof. Mervyn Long, (The University of New South Wales, Kensington, N.S.W., Aust.), using the following method.

Approx. 3 mg of 3-hydroxykynurenine was transferred to a constricted tube and 65 μL of 18M H₂SO₄ and 100 μL HTO (5 Ci mL⁻¹) were added. The mixture was degassed on a vacuum line, sealed and placed into an oven at 110°C for 24 hr. When removed from the oven, the solution was neutralised with 5M NaOH, transferred to another constricted tube, evaporated under vacuum and sealed.

6.2.7.2 Purification of Tritiated 3-Hydroxykynurenine

The solid material obtained above was dissolved in 1 mL Milli-Q Water. Indicator paper showed the solution to be very acidic. It
was divided into 2 x 0.5 mL aliquots. To one aliquot was added 0.5 mL phosphate buffer (Dawson et al, 1986), making the pH $\approx 6$.

A C$_{18}$ 'Sep-Pak' was solvated with 60% acetonitrile followed by several washes with Milli-Q Water. The pH 6 solution was taken up in a syringe and applied to the 'Sep-Pak'. A yellow coloured fraction was eluted upon application of 60% acetonitrile, and 2 x 0.5 mL aliquots of this fraction were lyophilised overnight.

The lyophilised samples were each reconstituted in 100 μL Milli-Q Water and applied separately to the analytical HPLC system with the detection wavelength set at 365 nm. Large peaks eluting at the retention time of standard 30HKyn were detected. These peaks were collected into 1.5 mL Eppendorf tubes.

The radioactivity in 100 μL (from a total of 1.5 mL) of the collected material was measured using LSC. This sample was found to contain $2 \times 10^6$ dpm; the total radioactivity was therefore $3 \times 10^7$ dpm in 1.5 mL. Using the 30HKyn standard curve (Section 4.3.1) the amount of 30HKyn present in the HPLC peak was found to be approximately 300 nmol. The specific activity of this material was therefore calculated to be:

$$\frac{3 \times 10^7 \text{dpm}}{300 \text{ nmol}}$$

or $1 \times 10^8 \text{ dpm μmol}^{-1}$

or $45\mu\text{Ci μmol}^{-1}$. 

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6.2.7.3 Lens Culture Experiments

Human lenses maintained under organ culture conditions were placed in fresh AAH to which was added 100 μL of the purified \(^3\)H-3OHKyn (final conc. 4.5μM [4.44x10^3 dpm mL\(^{-1}\)], which had previously been sterilised through a 0.22 μm syringe filter. After 24 hr, the lenses were removed from the AAH, rinsed twice for 10 sec in 0.9% saline and frozen at -20°C until analysed.

6.3 Results

6.3.1 Tryptophan Metabolites Are Distributed Throughout The Lens

Most of the lenses used for experiments in this chapter were divided into the four regions as described in Section 6.2.2 and protein free extracts of each region were analysed by HPLC. The aim of this procedure was to obtain information on the distribution and relative concentrations of Trp metabolites throughout the lens.

6.3.1.1 The Concentration of 3OHKG is Highest Along the Visual Axis

Initial analyses of the visual axis segments revealed that 3OHKG is distributed relatively evenly throughout the anterior cortex, nucleus and posterior cortex (Table 6.1), although lens 784 showed significant variation from this pattern.
Table 6.1: The concentration of 30HKG in various regions of human lenses (μmol g⁻¹ lens).

<table>
<thead>
<tr>
<th>LENS</th>
<th>AGE</th>
<th>CA</th>
<th>N</th>
<th>CP</th>
<th>Eq</th>
</tr>
</thead>
<tbody>
<tr>
<td>150</td>
<td>U</td>
<td>0.5229</td>
<td>0.7190</td>
<td>0.7466</td>
<td>ND</td>
</tr>
<tr>
<td>139</td>
<td>U</td>
<td>0.5367</td>
<td>0.6132</td>
<td>0.7249</td>
<td>ND</td>
</tr>
<tr>
<td>134</td>
<td>U</td>
<td>0.9160</td>
<td>0.7667</td>
<td>0.6954</td>
<td>ND</td>
</tr>
<tr>
<td>140</td>
<td>81</td>
<td>0.4221</td>
<td>0.4486</td>
<td>0.4367</td>
<td>ND</td>
</tr>
<tr>
<td>142</td>
<td>75</td>
<td>0.8567</td>
<td>0.9871</td>
<td>0.8229</td>
<td>0.4610</td>
</tr>
<tr>
<td>190</td>
<td>76</td>
<td>1.1848</td>
<td>0.8703</td>
<td>1.0011</td>
<td>0.3720</td>
</tr>
<tr>
<td>784</td>
<td>77</td>
<td>0.2489</td>
<td>0.3720</td>
<td>0.1978</td>
<td>0.2614</td>
</tr>
</tbody>
</table>

U=unknown; ND=not determined

Lenses 150, 139, 134 and 140 were analysed specifically for the purpose of comparing the concentration of 30HKG in the lens cortex and nucleus. Lenses 142, 190 and 784 were also used in pulse-chase experiments and the equatorial region of these lenses was analysed as well as the visual axis in order to study the short-term incorporation of radiolabelled Trp throughout the lens (Section 6.3.5). It was found, however, that the level of 30HKG in the equatorial region of all 3 lenses (Table 6.1) was lower than that of the nucleus, ranging from 43-70% of the nuclear value. In lenses 142 and 190 the concentration in the equatorial region was much lower than throughout the visual axis.

6.3.1.2 Kynurenine and 3-Hydroxykynurenine Are Present Throughout the Lens

Kyn and 3OHKyn were not detectable along the visual axis of the lenses in Table 6.1 at the integrator settings used for 30HKG. This may have been due to the small amount of tissue analysed.
from the visual axis (5-20 mg) compared to the equatorial region (approx. 200 mg), and not to absence of the metabolites in these sections of the lens. In order to better determine whether Kyn and 3OHKyn are present in all of the lens regions, as well as to gather more information on the distribution of 3OHKG throughout the lens, four more lenses were analysed and the sensitivity of the integrator was increased for the visual axis extracts. These lenses were 2 pairs from individual donors aged 37 and 88. The HPLC chromatograms from one of each pair (Fig. 6.2), show that Kyn and 3OHKyn, as well as 3OHKG, are present throughout the lens. The results, summarised in Table 6.2, show that, as in Table 6.1, the concentration of 3OHKG is lower in the equatorial region than along the visual axis. Also, the concentrations of both Kyn and 3OHKyn were noticeably lower in the equatorial region than in the visual axis.

The averages (μmol g⁻¹ lens) of the CA (0.8576±0.5989; n=11), N (0.8940±0.4668; n=11) and Cp (0.8555±0.4680; n=11) values for 3OHKG in Tables 6.1 and 6.2 show that the concentration of this metabolite, although variable among lenses, is relatively constant along the visual axis of individual lenses, and that this level is approximately double that of 3OHKG in the equatorial region (0.4744±0.2698; n=7). Along with the data for Kyn and 3OHKyn (Table 6.2), it appears that, although all three previously identified lens Trp metabolites are distributed throughout the lens, they are not distributed evenly, their levels being lower in the equatorial
segment of the lens containing the bow-region (see Section 1.3).

Table 6.2: The concentration of Trp metabolites in various regions of human lenses

<table>
<thead>
<tr>
<th>LENS/SECTION</th>
<th>Concentration (nmol/g lens tissue)</th>
<th>Kyn</th>
<th>3OHKyn</th>
<th>3OHKG</th>
</tr>
</thead>
<tbody>
<tr>
<td>258 (Age=88)</td>
<td>CA 8.1</td>
<td>4.6</td>
<td>389.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N 11.7</td>
<td>4.1</td>
<td>648.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CP ND</td>
<td>10.4</td>
<td>684.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eq. 2.3</td>
<td>1.9</td>
<td>178.1</td>
<td></td>
</tr>
<tr>
<td>274 (Age=88)</td>
<td>CA 8.6</td>
<td>6.1</td>
<td>754.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N 15.5</td>
<td>3.5</td>
<td>949.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CP 7.4</td>
<td>3.2</td>
<td>759.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eq. 4.7</td>
<td>2.3</td>
<td>344.7</td>
<td></td>
</tr>
<tr>
<td>257 (Age=37)</td>
<td>CA 71.1</td>
<td>23.7</td>
<td>2288.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N 61.1</td>
<td>25.1</td>
<td>1967.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CP 48.8</td>
<td>21.5</td>
<td>1933.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eq. 20.3</td>
<td>8.8</td>
<td>1030.3</td>
<td></td>
</tr>
<tr>
<td>260 (Age=37)</td>
<td>CA 33.6</td>
<td>7.1</td>
<td>1511.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N 40.7</td>
<td>15.3</td>
<td>1492.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CP 23.1</td>
<td>10.1</td>
<td>1408.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eq. 13.7</td>
<td>5.5</td>
<td>673.6</td>
<td></td>
</tr>
</tbody>
</table>

ND=not determined

6.3.1.3 The Level of Tryptophan is Also Higher Along the Visual Axis

Although figures were obtained for only three lenses, it was found that the levels of Trp were also lowest in the equatorial region (Table 6.3).

Table 6.3: The concentration of free Trp in various regions of human lenses (μmol g⁻¹ lens wet weight).

<table>
<thead>
<tr>
<th>LENS</th>
<th>AGE</th>
<th>CA</th>
<th>N</th>
<th>CP</th>
<th>Eq</th>
</tr>
</thead>
<tbody>
<tr>
<td>142</td>
<td>75</td>
<td>0.1525</td>
<td>0.3050</td>
<td>0.1125</td>
<td>0.1075</td>
</tr>
<tr>
<td>190</td>
<td>76</td>
<td>0.2140</td>
<td>0.1805</td>
<td>0.2050</td>
<td>0.1025</td>
</tr>
<tr>
<td>784</td>
<td>77</td>
<td>ND</td>
<td>0.1875</td>
<td>ND</td>
<td>0.1210</td>
</tr>
</tbody>
</table>

ND=not determined
Figure 6.2 (above and on following page): Chromatograms at 365 nm of visual axis protein-free extracts from a 37 year old (lens 257) and 81 year old (lens 274). Peaks of 3OHKyn, 3OHKG and Kyn were detected and are labelled for the CA.
6.3.2. The Release of 3-Hydroxykynurenine Glucoside Into the AAH.

In Chapter 5 it was demonstrated that 3OHKG was present in nmolar quantities in the AAH following a 24 hr lens incubation. However the AAH was examined at only one time point. In order to obtain more information on the release of 3OHKG from the lens, aliquots of AAH were removed at various times during 24 hr incubations of several lenses. Analysis of the AAH by HPLC at 365 nm revealed that the quantity of 3OHKG in the AAH increased with time (Fig. 6.3).

6.3.2.1 3-Hydroxykynurenine Glucoside Effluxes From the Lens at a Linear Rate.

Taking into account the volume of AAH remaining at each
sampling time, the volume of AAH analysed and the amount of 30HKG detected, the total amount of 30HKG effluxed at each time point was calculated. Graphing these results produced a curve that was linear, and so a rate of efflux could readily be determined. Some examples are shown in Fig. 6.4.

6.3.2.2 Half-life of 30HKG in the Lens

Based on the linear rate of efflux and the quantity of 30HKG in the lens, a half-life of 30HKG could be calculated for each lens on which efflux analysis had been carried out (Table 6.4). According to these results, the total quantity of 30HKG in the lens is turned over in a time period ranging from less than 2 days (lens 38) to approximately 9 days (lens 51). This assumes that efflux is the only mechanism in the lens for removal/metabolism of 30HKG.

Table 6.4: Half-life of 30HKG in the lens based on the quantity in the lens and the rate of efflux observed in culture.

<table>
<thead>
<tr>
<th>Lens</th>
<th>Age (years)</th>
<th>30HKG (µmol)</th>
<th>30HKG efflux (µmol/hr)</th>
<th>Half-life (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>51</td>
<td>54</td>
<td>0.222</td>
<td>1.01e-3</td>
<td>110</td>
</tr>
<tr>
<td>56</td>
<td>54</td>
<td>0.136</td>
<td>1.08e-3</td>
<td>63</td>
</tr>
<tr>
<td>37</td>
<td>64</td>
<td>0.058</td>
<td>1.40e-3</td>
<td>21</td>
</tr>
<tr>
<td>38</td>
<td>64</td>
<td>0.044</td>
<td>1.16e-3</td>
<td>19</td>
</tr>
<tr>
<td>142</td>
<td>75</td>
<td>0.068</td>
<td>8.38e-4</td>
<td>41</td>
</tr>
<tr>
<td>190</td>
<td>76</td>
<td>0.086</td>
<td>1.74e-3</td>
<td>25</td>
</tr>
<tr>
<td>140</td>
<td>81</td>
<td>0.110</td>
<td>1.08e-3</td>
<td>51</td>
</tr>
</tbody>
</table>

The half-life was calculated by dividing the total amount of 30HKG present in each lens by the rate of efflux (determined from the linear curves). This value was then halved.
Figure 6.3: Chromatograms of AAH at 365 nm removed during a 24 hr lens incubation showing an increasing quantity of 3OHKG. The 3OHKG peak is labelled with its retention time in min for each chromatogram.

Figure 6.4: The efflux of 3OHKG from human lenses in culture into the AAH over a 24 hr time period. Lenses 38 (□), age=64, $R^2=0.986$; 51 (○), age=54, $R^2=0.992$; 56 (■), age=54, $R^2=0.952$; and 190 (●), age=76, $R^2=0.999$.
6.3.3 Labelled Protein Accumulates in the Chase AAH

At the same time that samples of chase AAH were removed for 3OHKG analysis, a further aliquot of AAH was used to assay for leakage of lens protein. In these experiments, any radiolabel detected in TCA precipitates of the AAH must be due to leakage of protein that had previously incorporated the tritiated Trp transported into the lens from the AAH. Counting of the TCA precipitates by LSC revealed that some protein leakage did occur. Calculating the total amount of label present in the AAH at each sampling point and graphing (Fig. 6.5) revealed that protein was accumulating in the AAH during the incubations.

Figure 6.5: Radiolabel associated with protein in the chase AAH of lens 142 (○) and lens 190 (□).
Although all lens cells synthesise protein, the cells of the bow region are the major site of protein synthesis. The detection of radiolabel in the TCA precipitates is likely to be due to some disruption of the lens capsule since death, resulting in leakage of small amounts of newly synthesised lens protein into the AAH. This result raises the possibility that the appearance of 3OHKG in AAH may also be due to leakage caused by capsular damage, and not via a normal active transport or diffusion process. If the release of 3OHKG from the lens is a real phenomenon \textit{in vivo}, then it would be expected that it would be present in the surrounding ocular humor. Samples of human vitreous humor were obtained to test this.

6.3.4 Tryptophan, Kynurenine and 3-Hydroxykynurenine Glucoside Are Present in Human Vitreous Humor.

Having found evidence \textit{in vitro} that a major route of turnover of 3OHKG is via lens efflux, it seemed that, if this were a real physiological phenomenon, that the compound may be detected in the transparent humor that surrounds the lens \textit{in vivo}.

A sample of human vitreous humor was analysed by HPLC using a detection wavelength of 365 nm and a small peak running at the retention time of 3OHKG was detected. Similar sized peaks were also detected near the retention times of 3OHKyn and Kyn.

In order to determine whether or not these peaks were due to Trp metabolites, and to see if Trp itself is also found in the vitreous,
the following series of samples were prepared and analysed by 
HPLC:-

1) Standards of 3OHKyn, Kyn and Trp;
2) Sample of vitreous humor;
3) Sample of vitreous humor 'spiked' with 3OHKyn, Kyn and 
   Trp;
4) Sample of vitreous humor 'spiked' with 3OHKG collected 
   from a previous lens extract analysis;
5) Suspected 3OHKG peak, collected from the HPLC of a 
   vitreous sample, and incubated for 2 hr at 35°C with β-glucosidase.

The detection wavelength was altered from 365 to 278 nm 
after detection of Kyn in order to detect Trp. The chromatograms 
from these HPLC analyses are reproduced in Fig. 6.6 (A-E).

Figure 6.6: [following 2 pages] Chromatograms relating to 
analyses of human vitreous humor. Peaks are labelled with 
retention times in min.
(A) Standards of 3OHKyn (14.0), Kyn (29.8) and Trp (75.8). The 
detection wavelength was altered from 365 nm to 278 nm after 
elution of Kyn in order to detect Trp.
(B) A sample of human vitreous humor chromatographed under the 
same conditions as for (A).
(C) A sample of human vitreous humor ‘spiked’ with the same 
standards as in (A).
(D) A sample of human vitreous humor ‘spiked’ with some 3OHKG 
collected from a lens protein free extract.
(E) Reanalysis of the suspected 3OHKG peak from a human vitreous 
humor sample following treatment with β-glucosidase.
Fig. 6.6(B) shows one peak at a retention time slightly later than that of 3OHKyn standard, another at a retention time typical of 3OHKG and two other peaks at almost the exact retention times of Kyn and Trp as seen in Fig. 6.6(A). In Fig. 6.6(C), these two peaks are seen to coelute with standards of Kyn and Trp but the 3OHKyn
standard has a slight ‘shoulder’, indicating that the 14.5 min peak in (B) may not be 3OHKyn. Fig. 6.6(D) shows that 3OHKG elutes at almost the exact time as the peak seen in (B) and (C); and in Fig. 6.6(E) the suspected 3OHKG from a vitreous sample when treated with β-glucosidase elutes at the retention time of 3OHKyn standard.

This set of results indicates strongly that 3OHKG, as well as Trp and Kyn, are found in human vitreous humor. The results are less clear for 3OHKyn. It may not be present in vitreous, as the small peak preceding 3OHKG did not appear to coelute with the 3OHKyn standard and its retention time was also always slightly later. Further studies are necessary to determine whether or not the compound is 3OHKyn.

Four samples of vitreous humor were weighed, prepared for HPLC and analysed. The quantity of 3OHKG was estimated using the 3OHKyn standard curve (Section 4.3.1) and the concentrations calculated. The results are shown in Table 6.5.

<table>
<thead>
<tr>
<th>Vitreous Sample</th>
<th>Age (Years)</th>
<th>weight (g)</th>
<th>Amount 3OHKG (nmol)</th>
<th>Conc. 3OHKG (μmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>60</td>
<td>1.077</td>
<td>0.8356</td>
<td>7.8e-4</td>
</tr>
<tr>
<td>2</td>
<td>56</td>
<td>0.9989</td>
<td>0.5333</td>
<td>5.3e-4</td>
</tr>
<tr>
<td>3</td>
<td>72</td>
<td>0.7869</td>
<td>0.4347</td>
<td>5.5e-4</td>
</tr>
<tr>
<td>4</td>
<td>57</td>
<td>0.9597</td>
<td>0.8353</td>
<td>8.7e-4</td>
</tr>
</tbody>
</table>

Table 6.5: The level of 3OHKG detected in 4 samples of human vitreous humor.
6.3.5 Pulse-chase Experiments

6.3.5.1 Transport and Incorporation of Tryptophan Throughout the Lens

In order to gain information on the dynamics of 3OHKG synthesis and movement within the lens, three pulse chase experiments were performed as described in 6.2.5. Protein-free extracts of CA, N, Cp and the equatorial region were analysed by HPLC and peaks of 3OHKG and Trp quantified using the standard curves in Section 4.3.1. It had already been found in Section 6.3.1 that the concentration of these compounds was generally higher along the visual axis than in the equatorial region. The peaks were also collected and counted by LSC in order to determine the distribution of 3OHKG and Trp after 48 hr. Table 6.6 summarises the data from these experiments.

It is apparent that there is considerable variability in the distribution of radiolabelled Trp throughout the lenses following the 24 hr chase and 24 hr pulse. In each case for which the data was available the specific activity of Trp and 3OHKG is lowest in the nucleus.
Table 6.6: The label (dpm) detected in and specific activity of 30HKG and Trp in Sections of 3 lenses after a 24 hr pulse in AAH containing 20μCi mL\(^{-1}\) L-[5-\(^3\)H]-tryptophan followed by a 24 hr chase in unlabelled AAH.

<table>
<thead>
<tr>
<th>Lens/Region</th>
<th>Age</th>
<th>30HKG (dpm)</th>
<th>30HKG (dpm/μmol)</th>
<th>TRYPOTPHAN (dpm)</th>
<th>TRPOTPHAN (dpm/μmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>142</td>
<td>75</td>
<td>3,700</td>
<td>451,600</td>
<td>11,200</td>
<td>7,800,000</td>
</tr>
<tr>
<td>CA</td>
<td></td>
<td>800</td>
<td>119,000</td>
<td>4,200</td>
<td>2,000,000</td>
</tr>
<tr>
<td>N</td>
<td></td>
<td>1,200</td>
<td>161,200</td>
<td>15,200</td>
<td>14,000,000</td>
</tr>
<tr>
<td>CP</td>
<td></td>
<td>48,200</td>
<td>529,000</td>
<td>639,400</td>
<td>30,000,000</td>
</tr>
<tr>
<td>Eq</td>
<td></td>
<td>67,500</td>
<td>2,800,000</td>
<td>523,800</td>
<td>21,000,000</td>
</tr>
<tr>
<td>190</td>
<td>76</td>
<td>0</td>
<td>0</td>
<td>7,800</td>
<td>2,400,000</td>
</tr>
<tr>
<td>CA</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N</td>
<td></td>
<td>0</td>
<td>0</td>
<td>3,800</td>
<td>2,300,000</td>
</tr>
<tr>
<td>CP</td>
<td></td>
<td>0</td>
<td>0</td>
<td>3,800</td>
<td>2,300,000</td>
</tr>
<tr>
<td>Eq</td>
<td></td>
<td>67,500</td>
<td>2,800,000</td>
<td>523,800</td>
<td>21,000,000</td>
</tr>
<tr>
<td>784</td>
<td>77</td>
<td>300</td>
<td>140,800</td>
<td>900</td>
<td>a</td>
</tr>
<tr>
<td>CA</td>
<td></td>
<td>300</td>
<td>41,500</td>
<td>600</td>
<td>b</td>
</tr>
<tr>
<td>N</td>
<td></td>
<td>500</td>
<td>379,800</td>
<td>187,800</td>
<td>b</td>
</tr>
<tr>
<td>CP</td>
<td></td>
<td>33,200</td>
<td>541,700</td>
<td>814,300</td>
<td>28,700,000</td>
</tr>
</tbody>
</table>

a: Trp peak not integrated; b: Trp peak not detected

Although the visual axis generally has a higher level of 30HKG than the outer regions, recently synthesised 30HKG does not appear to diffuse rapidly into the centre of the lens. In lens 142, which has a similar concentration of 30HKG throughout the visual axis (Table 6.1), and in which the weights of these analysed lens segments were also similar, the diffusion of 30HKG was very slow into the nucleus, both in terms of dpm detected and specific activity. The diffusion of Trp was similarly slowest into the nucleus. In lens 190, no labelled 30HKG had penetrated any part of the visual axis despite the high value in the equatorial region, and, although labelled Trp was present in the CA and CP, none had reached the
nucleus. In lens 784, smaller quantities of label than in lens 142 were present in the visual axis, but once again the nucleus contained the lowest values.

Although only three lenses were analysed, there appears to be no common feature in the diffusion into either $C_A$ or $C_P$. In lens 142 the specific activity of 30HKG in the $C_A$ is much higher than in the $C_P$, and of a similar level to the equatorial region, but in lens 784 the situation is reversed. The specific activity of Trp is highest in the $C_P$ of lens 142, but the values are almost equal in lens 190.

6.3.5.2 3-Hydroxykynurenine Glucoside is Labelled in the Chase AAH

It has been shown previously that 30HKG effluxes from organ cultured human lenses in a linear manner (Section 6.3.2.1). To determine if the efflux represented 'old' or newly synthesised 30HKG, samples of the chase AAH were removed during the chase phase of two pulse-chase experiments for analysis and counting of 30HKG. As shown in Fig. 6.7A the amount of 30HKG in the AAH increased with time in both lenses and the rate of efflux was linear. The specific activity of 30HKG was highest in the two early time points (Fig. 6.7B). This suggests that much of the efflux can be accounted for by newly synthesised 30HKG, especially since the specific activity of 30HKG in the AAH is much higher than in the lens itself (compare the specific activity values in Fig. 6.7B with
those in Table 6.7).

Figure 6.7: (A) The efflux of 3OHKG into the AAH of two pulse-chased lenses. (■) Lens 190; (□) Lens 142. (B) The specific activity of 3OHKG at sampled time-points during the chase in the same two lenses. (■) Lens 142; (■) Lens 190.

6.3.6 Transport and Incorporation of 3OHKyn Into Human Lenses *In Vitro*

Results in this and the previous chapter, following on from the pioneering work of van Heyningen (1973b), indicate that 3OHKG is a
product of Trp metabolism and the most likely pathway is via the intermediates N-formylKyn, Kyn and 3OHKyn. It seemed pertinent, as part of a study into this metabolic pathway, to prove conclusively that 3OHKG is indeed formed via glucosylation of 3OHKyn, and not via an unknown mechanism.

The easiest way to test this is by using the radiolabelled precursor $^3$H-3OHKyn in a lens incubation experiment. If 3OHKyn could be shown to be the immediate precursor of 3OHKG, then an enzyme must be present in the lens to carry out the glucosylation, and no other mechanism would be necessary.

In addition to this, if, in a lens culture experiment a metabolite of Trp, apart from Trp itself, could be shown to enter the lens from the AAH and be metabolised to 3OHKG, the possibility would be raised that a similar occurrence may take place in vivo.

6.3.6.1 3-Hydroxykynurenine is the Precursor of 3-Hydroxykynurenine Glucoside

Three lenses were used in an experiment to test the transport and incorporation of $^3$H-3OHKyn from the AAH. The lenses were a pair aged 69 and a single lens aged 67. According to van Heyningen (1973b), Kyn is present in human aqueous humor in much lower levels (10-100 fold) than in the lens and “...the concentration of kynurenine increases in a human lens incubated in a medium containing kynurenine,” (van Heyningen, 1973a). It seemed feasible
therefore that 3OHKyn in the AAH, could be transported into the lens and act as a precursor of 3OHKG.

The protein-free extract from each lens following incubation for 24 hr in AAH containing 4.5μM (a total of 2x10^6 dpm) purified ^3H-3OHKyn was analysed by HPLC. Peaks of 3OHKyn and 3OHKG were detected using λ=365 nm and were collected separately. The amount of radioactivity in the collected peaks was determined using LSC. The results are shown in Table 6.7 and Fig. 6.8. The specific activities of 3OHKyn and 3OHKG were also calculated and tabulated (Table 6.8).

Table 6.7: The amount of label detected in 3OHKyn and 3OHKG after incubation of lenses for 24 hr in AAH pulsed with 2x10^6 dpm ^3H-3OHKyn.

<table>
<thead>
<tr>
<th>LENS</th>
<th>3OHKyn (dpm)</th>
<th>3OHKG (dpm)</th>
<th>% (3OHKG/TOTAL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>19,000</td>
<td>11,500</td>
<td>38%</td>
</tr>
<tr>
<td>2</td>
<td>29,600</td>
<td>9,300</td>
<td>24%</td>
</tr>
<tr>
<td>3</td>
<td>8,500</td>
<td>8,800</td>
<td>51%</td>
</tr>
</tbody>
</table>

Table 6.8: The specific activities of 3OHKyn and 3OHKG after incubation of lenses for 24 hr in AAH pulsed with ^3H-3OHKyn.

<table>
<thead>
<tr>
<th>Lens</th>
<th>3OHKyn (dpm/µmol)</th>
<th>3OHKG (dpm/µmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.2e+7</td>
<td>228,000</td>
</tr>
<tr>
<td>2</td>
<td>5.0e+7</td>
<td>204,400</td>
</tr>
<tr>
<td>3</td>
<td>7.1e+6</td>
<td>118,800</td>
</tr>
</tbody>
</table>

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These results show that 3OHKyn was able to enter the lens from the AAH, as judged by the detection of tritium label under the 3OHKyn HPLC peak. Label was also detected under the 3OHKG peak, and in all three lenses it was a high proportion of the total label detected in these two compounds, ranging from 24-51%. The formation of 3OHKG is almost certainly therefore a lens-specific divergence from the oxidative Trp catabolism that occurs in other tissues, since radiolabel from 3OHKyn, a ‘normal’ Trp catabolite, was rapidly incorporated into 3OHKG, a lens-specific Trp metabolite.

In the three lenses used for this study the amount of 3OHKyn entering the lenses varied by a factor of almost 3 but the label incorporated into 3OHKG was relatively constant. This may indicate a constant but low activity of the glucosyl transferase enzyme.
responsible for the formation of 3OHKG, with substrate in excess of capacity accumulating in the lens. Nothing is known about the properties of this enzyme at the present time.

That 3OHKyn can enter the lens in vitro from the AAH suggests that it could probably do so in vivo if it were present in the aqueous humor. This may have implications in the etiology of senile nuclear cataract in humans, since 3OHKyn is a Trp metabolite which becomes reactive on oxidation and can tan proteins in a manner similar to that which takes place with the onset of cataract (Stutchbury and Truscott, 1993).

6.3.6.2 Glucosylation of 3-Hydroxykynurenine Takes Place in the Equatorial Region

Four more lenses were incubated in 3H-3OHKyn under the same conditions as in 6.3.5.1. Incubation was followed however, by dissection into CA, N, Cp and equatorial regions in order to assess the incorporation of label throughout the lens during the incubation period. Protein free extracts of each lens segment were chromatographed on HPLC using a detection wavelength of 365 nm and peaks of 3OHKyn and 3OHKG collected and counted by LSC (Table 6.9). These lenses are the same four discussed in Section 6.3.1.2.
Table 6.9: Radiolabel present in peaks of 3OHKyn and 3OHKGT from HPLC and LSC analysis of protein free extracts from lens segments following 24 hr lens incubation in 3H-3OHKyn.

<table>
<thead>
<tr>
<th>Lens</th>
<th>Age</th>
<th>Ant. Cortex</th>
<th>Nucleus</th>
<th>Post. Cortex</th>
<th>Equatorial</th>
</tr>
</thead>
<tbody>
<tr>
<td>258</td>
<td>88</td>
<td>761</td>
<td>176</td>
<td>139</td>
<td>7442</td>
</tr>
<tr>
<td>3OHKyn</td>
<td></td>
<td>309</td>
<td>92</td>
<td>0</td>
<td>2588</td>
</tr>
<tr>
<td>3OHKGT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>274</td>
<td>88</td>
<td>302</td>
<td>1324</td>
<td>152</td>
<td>4187</td>
</tr>
<tr>
<td>3OHKyn</td>
<td></td>
<td>92</td>
<td>178</td>
<td>0</td>
<td>2341</td>
</tr>
<tr>
<td>3OHKGT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>257</td>
<td>37</td>
<td>234</td>
<td>53</td>
<td>413</td>
<td>2371</td>
</tr>
<tr>
<td>3OHKyn</td>
<td></td>
<td>117</td>
<td>0</td>
<td>81</td>
<td>2513</td>
</tr>
<tr>
<td>3OHKGT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>260</td>
<td>37</td>
<td>643</td>
<td>106</td>
<td>953</td>
<td>3358</td>
</tr>
<tr>
<td>3OHKyn</td>
<td></td>
<td>275</td>
<td>53</td>
<td>215</td>
<td>2635</td>
</tr>
</tbody>
</table>

Label was found in all regions of the lens but, (as in 6.3.5.1) with the exception of lens 274, label was slow to reach the nucleus compared to the CA and Cp regions. Of the label that was able to penetrate into the nucleus of lens 274, however, very little was found to be due to 3OHKGT, the vast majority (88%) being 3OHKyn which had apparently diffused into the nucleus and avoided glucosylation. In the equatorial regions of the four lenses, by contrast, from 26 to 51% of the label had been incorporated into 3OHKGT. A high proportion of the label in three of the CA segments (lenses 258, 257 and 260) was also glucosylated. The equatorial region possesses most of the epithelial cells as well as the bow-region (Section 1.3), which contains recently differentiated fibre cells that still possess organelles. These results would suggest that
the enzyme that carries out the glucosylation is located only in the
the metabolically active young cells of the lens epithelial cell layer
and bow-region, and that any 3OHKyn which has not been
glucosylated in the outer regions of the lens is able to diffuse into
the nucleus.

6.3.6.3 3OHKG Effluxes From the Lens Close to the Site of Synthesis

AAH samples (500 µL) from the four lenses in 6.3.6.2 were
lyophilised and analysed by HPLC. Detection of 3OHKG was expected
considering the results obtained in 5.3.4.2 and 6.3.2. The specific
activities of 3OHKG in the AAH (average $6.5 \times 10^6 \pm 5.7 \times 10^5$ dpm
µmol$^{-1}$) were much higher than in the equatorial region of the lens
which ranged from 65,500 to 173,700 dpm µmol$^{-1}$. It is probable
(Section 6.3.6.2) that glucosylation takes place in the outer region of
the lens, in the anterior epithelial cell layer and the bow-region. It
is also probable, in light of the high specific activity of 3OHKG
detected in the AAH, that the efflux of 3OHKG takes place from the
cells in which it was formed i.e. 3OHKG effluxes from the lens close
to the site of synthesis. The bow-region cells and some epithelial
cells close to the lens equator are adjacent to the vitreous humor.
Human vitreous humor has been shown to contain 3OHKG (6.3.4).

The quantity of 3OHKyn detected in the AAH of these
cultures was also calculated and counting of the peaks by LSC
revealed an average specific activity of 3OHKyn of 8.69x10^7±0.423x10^7 dpm μmol⁻¹. The specific activity of the purified 3H-3OHKyn (Section 6.2.7.2) was calculated to be 1x10^8 dpm μmol⁻¹. The quantity of label used was therefore not a limiting factor in these experiments.

6.4 Discussion

In previous chapters the presence of Trp and its oxidation products Kyn, 3OHKyn and 3OHKG was established in human lenses, and the synthesis and efflux of these metabolites was found to be readily detectable in vitro over a time period of 24 hr. In this chapter it has been established that these same metabolites are present in all regions of the lens, although they are not distributed evenly throughout the lens body. The higher concentrations tend to occur in and around the lens nucleus.

The concentration of 3OHKG was found to be highest along the visual axis, and, despite variance in actual levels between lenses, within individual lenses the concentration was generally similar throughout this lens core, and, on a per gram weight basis, approximately twice as high as that found in the equatorial region. Whether or not there is a gradient of concentration from the lens capsule to the nucleus cannot be ascertained from the method of analysis used—for that information several concentric lens layers would have to be analysed—but the lower concentration in the
equatorial region compared to the visual axis may be the due to such a gradient. This would mean that the concentration near the outer edge of the lens would be low. This could be explained by the lens capsule being very permeable to 3OHKG. 3OHKG was found to increase in quantity in the AAH at a linear rate over a 24 hr period and this may indicate either active transport or simple diffusion. That this phenomenon is probably a real physiological event and not due to lens damage was evidenced by the detection of 3OHKG in samples of human vitreous humor.

The greater concentration of 3OHKG along the visual axis is in accord with the function of the compound as a UV filter. A similar distribution pattern in the lens was observed for a 488 nm-excitation fluorophor using a laser Raman microprobe (Barron, Yu and Kuck, 1988). Interestingly the 488 nm fluorophor increased in intensity in lenses older than 10 years; approximating a time at which the lens levels of 3OHKG decline steeply (Chapter 4). This may support the contention that the 488 nm fluorophor is derived from 3OHKG (Barron et al., 1988) or, as seems more likely on the basis of our data, from an intermediate in the biosynthetic pathway such as 3OHKyn, since the latter compound shows a similar age-related decrease and readily autoxidises to produce dimers which absorb light in the 488 nm region, and 3OHKyn-tanned proteins also develop a long wavelength fluorescence (Stutchbury and Truscott, unpublished data.)
van Heyningen's analysis (1973a), of the cortex and nucleus revealed similar concentrations, on a per g lens basis, of 3OHKG in both regions. Analysis of normal post-mortem lenses aged 40 to 75 years revealed concentrations in nuclei of 1.45±0.72 µmol g⁻¹ lens (n=8) and in cortex of 1.26±0.38 µmol g⁻¹ lens (n=8). These measurements are within the range of the values found in anterior cortex, nucleus and posterior cortex in the present study (Section 6.3.1). It was only when the remaining equatorial region was analysed that a differing level was discovered (Table 6.1). It cannot be ascertained from van Heyningen's paper how the lenses were dissected. It is stated, however, that post-mortem lenses were frozen until examined. If the lower levels of Trp metabolites in the equatorial region (Section 6.3.1) are due to a higher water content in this area of the lens, then the lenses in the previous study may have become dessicated during storage, resulting in similar values of 3OHKG concentration in cortex and nucleus; i.e. expressed as µmol g⁻¹ lens protein, the levels may be fairly constant. It is also possible that the lenses examined in the present study absorbed water during culture, resulting in elevated levels of metabolites (on a per mass lens basis) in the equatorial region. These lenses were also frozen following completion of culture experiments. The total weight of CA, N, Cp and Eq. portions following dissection of the frozen lenses ranged from 50 to 100% of the lens weight before
commencement of the experiments. Variable proportions of dessication had taken place while the lenses were frozen, yet analysis still revealed higher concentrations of Trp metabolites along the visual axis compared to the equatorial region. It is conceivable that van Heyningen (1973a) also analysed cortex from along the visual axis, resulting in her finding only slightly lower levels of 3OHKG in cortex compared with the nucleus.

Pulse-chase experiments using $^3$H-Trp and 24 hr pulse experiments using $^3$H-3OHKyn demonstrated that label did not always reach the visual axis segments within the time period of the experiments, and was especially slow in reaching the nucleus. The $^3$H-3OHKyn experiments also indicated that, once beyond the metabolically active cells of the epithelium and lens bow, no apparent further glucosylation of 3OHKG occurs, as the vast majority of radiolabel detected in nucleus and Cp was due to 3OHKyn, suggesting that it had escaped glucosylation. Both sets of radiolabelled experiments also demonstrated that the specific activity of 3OHKG in the AAH was much higher (1-2 orders of magnitude) than in the lens itself, and the highest values were in the early time points, indicating that some of the efflux can be attributed to recently synthesised metabolite.

The appearance of recently synthesised 3OHKG so rapidly in the AAH, the high specific activity of 3OHKG in the AAH compared
to the lens, the paucity of label detected in the nucleus, the higher concentrations of metabolites in the nucleus and the presence of 3OHKG in human vitreous humor all contribute to a possible model of the synthesis, distribution and efflux of Trp metabolites in the human lens. In this model, Trp is transported into the lens from the aqueous humor and is converted to 3OHKG in the anterior epithelial cell layer and possibly the bow region. This would be consistent with the known requirement for a mitochondrial enzyme in the hydroxylation of Kyn (Okamoto et al., 1967). Some 3OHKG is effluxed into the surrounding ocular humor soon after synthesis, while some, along with any unmetabolised Trp and small quantities of unmetabolised Kyn and 3OHKyn, diffuses further into the lens. Once beyond the organelle-containing cells no further metabolism takes place, so that free Trp, Kyn, 3OHKyn and 3OHKG are able to diffuse to all regions of the lens. Diffusion is slow in the inner regions of the lens, however, as evidenced by the results in Table 6.6. This model indicates a metabolically dynamic outer lens and metabolically sluggish inner cortex and nucleus, and is consistent with other studies of lens metabolism. The results also point to a considerable variability between lenses in the rate of diffusion into the visual axis core from the active cortex.

The constant formation, flux and efflux results in a half-life of 3OHKG of less than 2 to approximately 9 days in the lenses studied (Table 6.5). That a significant quantity of recently synthesised
3OHKG is effluxed from the lens is further evidence that the rates of formation of this compound estimated in Section 5.3.2 are minimal values. A more accurate estimate of the rate of formation may be the rate of efflux, since if a lens is to maintain a certain level of 3OHKG then efflux must equal rate of formation. The efflux of 3OHKG is 1.19±0.30 (n=7) nmol hr⁻¹ according to the rates in Table 6.4.

In Section 4.4 it was speculated that there may be potential for covalent modification of lens crystallins by 3OHKyn, especially if the lens were exposed to an oxidative stress. The modification may lead to the yellowing of the lens that is most obvious in the lens nucleus and possibly even to senile nuclear cataract. The highest concentrations of 3OHKyn are found along the visual axis including the nucleus. It is theoretically possible that unglucosylated 3OHKyn may undergo oxidation in the lens and its oxidation products become covalently incorporated into protein. This effect would be exacerbated by an increase in oxidative stress on the lens, possibly by H₂O₂, the level of which has been shown to be elevated in the aqueous humor of some cataract patients (Spector and Garner, 1981). A hypothetical decrease in the activity of indoleamine oxygenase, which converts Trp to N-formylKyn, could possibly result in a higher level of the superoxide anion (O₂⁻), which may also cause an increase in oxidative stress on the lens.

It is possible for 3OHKyn to enter the lens from AAH and
undergo glucosylation (Section 6.3.6). The activity of the glucosylating enzyme appears to be low. A concentration of 3OHKyn in AAH of only 4.5μM was sufficient to apparently saturate the enzyme, as shown in Fig. 6.7, where free ³H-3OHKyn inside the lens equalled or exceeded the amount of ³H-3OHKG. Therefore it is conceivable that an increase in 3OHKyn blood levels in vivo may lead to its increased concentration in the aqueous humor and subsequently increased levels in the lens.

It is interesting to note that there is no firm evidence that 3OHKyn is present in the vitreous humor (Section 6.3.4). This needs to be investigated further, possibly using a fluorometric analysis of the uncertain vitreous compound (Shibita and Onodera, 1991).

The possible reactivity of 3OHKyn with lens crystallin may be a reason for its conversion to the more inert 3OHKG. Both compounds possess very similar UV-spectra (Fig. 3.7); therefore if a major role of the glucoside is as a protection against UV radiation, its formation may take place to maintain the UV absorbing fluorophor at a high level while preventing protein modification by 3OHKyn which may possibly occur if the lens is placed under increased oxidative stress, which could be brought about, for example, by a reduction in the levels of reduced glutathione. An age-related decline in the levels of reduced glutathione has been observed (Harding, 1970; Rathbun and Murray, 1991).
CHAPTER 7
OTHER EXPERIMENTS PERTAINING TO HUMAN LENS
TRYPTOPHAN METABOLISM

7.1 Introduction

It has been established that 3OHKG undergoes efflux under organ culture conditions in the same time period that synthesis of the compound was detected (Chapters 5 and 6). Although synthesis and efflux could not be precisely correlated, both processes are of the same order of magnitude, and it is possible that efflux into the AAH may be the sole fate of 3OHKG following its formation in the lens. The presence of this compound in human vitreous humor (Section 5.3.4) is strong evidence that efflux also occurs in vivo.

Of particular interest is whether or not 3OHKG can be further metabolised by the lens, and also whether lens Trp metabolism results in the formation of any other compounds apart from Kyn, 3OHKyn or 3OHKG. For example, does 3OHKyn undergo conversion to 3OHA and eventual complete oxidation via the normal Trp oxidative metabolism that occurs in other tissues?

In order to help answer these questions, the possibility of using cultured lens epithelial explants was investigated to see if synthesis of 3OHKG could be observed in this system. Earlier results from whole lenses had suggested that 3OHKG was being biosynthesised in the anterior zone (Chapter 6). Since Trp has been shown to be taken up by the whole lens in vitro and converted to 168
3OHKG which then diffuses throughout, or is released from the lens, then a viable preparation of lens epithelium may be able to metabolise Trp from the bathing medium and release the metabolic products back into the medium. Another advantage of using such explants is that they can be kept for several days without frequent changes of medium (e.g. see Lin et al, 1991). Thus the medium can be sampled for accumulated metabolites during time-course studies.

The major feature used for the identification of 3OHKG in the early parts of this study (Chapter 3) was its hydrolysis to a compound with the same retention time as 3OHKyn on HPLC, following treatment with commercially available β-glucosidase (isolated from almonds). Carlin and Cotlier (1971), had previously reported very low activities of a β-glucosidase (as well as other glycosidases) in human lens homogenates using fluorescence-based assays. It was decided to investigate the β-glucosidase activity in human lenses using ortho- and para-nitrophenyl glycosides which are known chromogenic substrates of β-glucosidase, yielding yellow coloured compounds with $\lambda_{\text{max}}$ at 400 or 420 nm when hydrolysed. The existence of such enzyme activity would provide one possible route for the degradation of 3OHKG; namely its hydrolysis to 3OHKyn. In addition, radiolabelled 3OHKG collected from previous HPLC analyses was added to an epithelial explant and a lens homogenate in attempts to detect any hydrolysis of the compound.
radiochemically.

In other experiments, the presence of $^{14}$C-alanine was tested for in an epithelial preparation involving incubation with $^{14}$C-Trp, and N-formylKyn was synthesised in order to check for its existence in lens extracts.

7.2 Materials and Methods

7.2.1 Culture of Epithelial Explants

In an initial trial, an individual whole capsule with adhering epithelium from a human lens was explanted into a 35 mm sterile plastic petri dish containing 1.5 mL medium 199 with Earl's salts as described previously by McAvoy and Fernon (1984). The concentration of Trp in the medium was 50 µM and $\{U-^{14}$C$\}$-L-tryptophan was added to the medium to a final concentration of 0.4 µCi mL$^{-1}$. (This procedure was carried out in the laboratory and under the supervision of Dr. John McAvoy of Sydney University). Further epithelial studies were carried out using EMEM (Sections 2.1.2; 2.2.2) modified from that used in whole lens cultures by buffering with 20mM NaHCO$_3$ instead of NaOH and incubating in a 5% CO$_2$ incubator at 35°C.

7.2.2 Lens Homogenate Experiment

Tritiated 3OHKG was obtained by chromatographing the
remaining protein-free extract from a previously pulsed lens on HPLC. The 3OHKG peak was collected, lyophilised and reconstituted in 1.2 mL Milli-Q water.

A 20 year old lens in culture was homogenised in 0.6 mL phosphate buffered saline with glucose (pH 7.4). This buffer contained 40.5 mL Na\textsubscript{2}HPO\textsubscript{4}.2H\textsubscript{2}O (36.61 g L\textsuperscript{-1}; 0.2M) and 9.5 mL NaH\textsubscript{2}PO\textsubscript{4}.H\textsubscript{2}O (27.6g L\textsuperscript{-1}; 0.2M) diluted to 100 mL with Milli-Q water with NaCl and D-glucose added to concentrations of 0.9% and 5mM respectively. The homogenate was divided into 3 Eppendorf tubes and 100 \( \mu \)L \(^3\)H-3OHKG, containing an estimated 20,000 dpm, was added to each sample. One sample was immediately frozen at -20°C (time=0). The other tubes were incubated at 35°C (1 drop of chloroform was added to maintain sterility). One sample was removed after 6 hr and the remaining sample removed 24 hr after the start of the incubation. The homogenates were stored frozen at -20°C until analysed. Before analysis, the samples were ultrafiltered through a 10,000MW cut-off polysulfone filter (Activon Scientific Products Co. Pty. Ltd., Sydney, Australia), centrifuged at 15000 g. The ultrafiltrate was lyophilised, reconstituted in 100 \( \mu \)L Milli-Q water and analysed by HPLC. Peaks of 3OHKyn and 3OHKG were collected and counted by LSC.
7.2.3 Pulse-pulse Experiment

A pair of lenses from an individual donor were incubated at the start of the experiment in separate culture vessels containing 4.5 mL fresh AAH to which 100 μL L-[5-3H]-tryptophan was added (final concentration 50μM [20 μCi mL⁻¹]). After 24 hr, one of the lenses was removed from culture, rinsed for 30 sec in 'cold' AAH to remove adhering label, then blotted dry and frozen at -20°C. The other lens was transferred to fresh AAH containing L-[5-3H]-tryptophan and pulsed for a further 24 hr, then rinsed, blotted dry and frozen until analysed.

Protein free extracts of each lens were prepared as previously (Section 2.3) and one half of each extract was analysed by HPLC (Section 2.4). Fractions of HPLC eluent were collected every 2 min into scintillation vials and the quantity of radiolabel present in each 2 min fraction was determined by LSC (Section 2.5).

7.2.4 Glycosidase Assays

7.2.4.1 Substrates

o-Nitrophenyl galactoside (oNP-gal), o-nitrophenyl glucoside (oNP-glu), p-nitrophenyl galactoside (pNP-gal), p-nitrophenyl glucoside (pNP glu) and β-glucosidase were purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A.
7.2.4.2 Test of the Assays

Duplicate assays containing 100 µL 1 mg mL\(^{-1}\) (7U mL\(^{-1}\)) β-glucosidase in citrate buffer pH 5.0 (Dawson et al, 1986) and 100 µL aqueous 1.0mM oNP-gal, oNP-glu, pNP-gal or pNP-glu were prepared in glass test tubes and incubated in a water bath at 37°C for 1 hr. The reactions were stopped by addition of 1.0 mL glycine/NaOH buffer pH 10.2 (Dawson et al, 1986). Blanks were prepared by mixing 100 µL glycoside with 100 µL citrate buffer and treating them as for the assay tubes. Absorbances for each assay were read against the appropriate blanks at 400 nm for pNP-gal and pNP-glu and at 420 nm for oNP-gal and oNP-glu.

7.2.4.3 Glycosidase Assays on Lens Homogenates

Lenses were removed from culture (frozen lenses were not assayed for β-glucosidase activity) and immediately homogenised in 500 µL Milli-Q water. Assay tubes were set up in duplicate containing 100 µL citrate buffer pH 5.0, 50 µL 1mM oNP-gal, oNP-glu, pNP-gal or pNP-glu, and 50 µL lens homogenate. The tubes were incubated in a 37°C water bath for 1 hr and the reaction stopped by addition of 1.0 mL glycine/NaOH buffer pH 10.2. Blanks were prepared by incubating 100 µL buffer and 50 µL lens homogenate under the same conditions and adding 50 µL 1mM oNP-gal, oNP-glu, pNP-gal or pNP-glu following addition of the glycine/NaOH buffer.
Assay and blank tubes were centrifuged for 20 min at 3000 g and the supernatants read against the appropriate blank at 400 nm for pNP-gal and pNP-glu and at 420 nm for oNP-gal and oNP-glu assays.

7.2.5 Amino Acid Analysis

The amino acid analysis system consisted of an LC 1150 HPLC pump (ICI Instruments, Sydney, Australia), an AS 2000 automatic injector with variable volume injection and a Knauer Fixed Wavelength UV-visible detector in series with a programmable LC 1250 Fluorescence detector. Chromatograms were recorded and data processed using the DP 800 Chromatography Management System (ICI). Analytical separations were performed on a 150 mm x 4.6 mm 3µm Spherisorb S3ODS2 column (ICI). A gradient system consisting of 20mM phosphate buffer/15% methanol pH 7.0 (buffer A) and 90% acetonitrile (buffer B); 18-99% B was used at a flow rate of 1.0 mL min⁻¹.

7.2.6 Synthesis of N'-Formylkynurenine

The procedure was based on that used by Dalgleish (1952). A mixture of 0.1 mL acetic anhydride and 0.2 mL 98% formic acid was cooled in an ice-bath and added to 0.208g (1 mmol) DL-kynurenine (Sigma) dissolved in 0.45 mL 98% formic acid. The mixture was left to stand at room temperature for 2 hr then transferred with shaking
to 3 mL ether in a round-bottomed flask and left to stand in an ice-bath for 3 hr. The product was filtered through a Buchner funnel, allowed to dry as much as possible under vacuum filtration, then sealed in a stoppered glass tube. A small fraction was dissolved in analytical HPLC buffer and analysed by HPLC at 320 nm. A peak of Kyn and a much larger peak with a retention time of 56 min were detected. A semi-prep HPLC column (Section 3.2.2.4) and a mobile phase of 7% methanol was used to purify larger amounts (5 mg) of material since a lyophilised peak from the analytical HPLC system would contain acetate salt from the buffer. The identity of the purified peak was confirmed using UV-spectrophotometry and mass spectrometry. Purified N-formylKyn was stored sealed from light and under dessication to prevent hydrolysis to Kyn.

7.2.7 Mass Spectrometry

Analysis was performed on VG Quattro Triple Quadrupole mass spectrometer (Fisons Instruments, Altrincham, Chesire, England) using an electrospray ionisation source and an SFC 500 Microflow Pump. The flow rate was 5μL min⁻¹ and the source temperature was 65°C.
7.3 Results and Discussion

7.3.1 Experiments With Lens Epithelial Explants

7.3.1.1 An Epithelial Explant Metabolised Trp to 3OHKG

In a preliminary experiment, the epithelial explant from a 69 year old lens was cultured in medium containing \([U^{\text{14C}}]\)-L-tryptophan. Samples of medium were removed for analysis after 3 and 48 hr.

In the 48 hr medium, large HPLC peaks were detected at the positions of 3OHKG and Kyn (no peaks were detected in the 3 hr chromatogram). Significant amounts of radiolabel were detected at these positions in the 3 and 48 hr samples, and the quantity of label had increased in the time period between removal of the aliquots. A significant amount of radioactivity was also observed at 6 min It is possible that this may be another, as yet unknown, product of Trp catabolism.
Figure 7.1: Incubation of the epithelial cell layer of a 69 year old lens at 37°C in medium containing 0.4 μCi mL⁻¹ [U-14C]-L-tryptophan. (A) The HPLC chromatogram of a sample of medium after 48 hr incubation. At 365 nm, peaks of 3OHKG (17.5 min) and Kyn (27.6 min) were detected whilst at 278 nm Trp (65.9 min) was detected. Trp was present in the original medium and the other metabolites have been transported into the medium from the lens cells. (B) The radioactivity detected using LSC of eluted fractions from the HPLC of samples of medium after 3 hr (---) and 48 hr (—) incubation. The levels of radioactivity detected at the retention times of 3OHKG and Kyn show that synthesis of these compounds from Trp has occurred during the incubation period. The difference in retention times for Kyn and Trp than in previous results reflects the use of a different HPLC column. For one column, variation in HPLC retention as judged by chromatography of standards was less than 10%.
7.3.1.2 Epithelial Explant Fails to Metabolise 30HKG

Radioactive 30HKG was obtained in the following way: the protein free extract fractions remaining from the 24 hr $[^{14}\text{C(U)}]$-L-tryptophan incubations (Chapter 5) were run on HPLC and the peaks of 30HKG were collected. These ‘hot’ peaks were lyophilised and pooled in 150 µL Milli-Q water.

An epithelial explant was prepared from an 81 year old lens. It was incubated overnight, and then 100 µL of the medium was analysed by HPLC. A peak of 30HKG was detected and the culture was therefore considered to be metabolically active.

Fresh medium (3 mL) containing the $^{14}\text{C}$-30HKG was added to the explant. A zero time sample of 500 µL was immediately removed and the culture reincubated. Further 500 µL aliquots were removed at 4, 8, 16.5 and 48 hr. The medium samples were lyophilised, reconstituted in 100 µL Milli-Q water and analysed by HPLC at 365 nm. The peaks of 30HKG were collected. Although no 30HKyn was detected, eluent was collected from 14-16.5 min (30HKG was running at 17+min), in case the 30HKG was hydrolysed by β-glucosidase. The collected fractions were counted by LSC. The quantity of radioactivity associated with the 30HKG peaks did not decrease (Fig. 7.2). There was therefore no evidence of degradation of 30HKG in 48 hr. Radiolabel detected at the position of 30HKyn was only slightly higher than background levels, and may have been in
fact due to overlap with the 3OHKG peak.

![Graph showing radiolabel detection in 3OHKG in samples of medium from an 81 year old lens epithelium explant](image)

Figure 7.2: The radiolabel detected in 3OHKG in samples of medium from an 81 year old lens epithelium explant to which $^{14}$C-3OHKG had been added.

7.3.1.3 Build Up Of Kyn in Epithelial Explants

A pair of epithelial explants were incubated without change of medium for three days, and aliquots were removed at 24 and 48 hr during the incubations. HPLC analysis and calculation of the total quantity of Trp metabolites (Fig. 7.3) present in the media at each sampling point showed that the amount of 3OHKG did not increase in an apparently linear manner, as it did in AAH in whole lens incubations (Section 6.3.2.1). The relative quantity of Kyn, however, although only 4% of that of 3OHKG after 24 hr, had increased to 20%.
at 48 hr and by 72 hr the level of Kyn in the medium was 63% of that of 3OHKG (Fig. 7.3). The level of 3OHKyn in the medium, while not detected after 24 hr, was 14% of the 3OHKG level after 48 hr and had increased only slightly (16%), at 72 hr.

Figure 7.3: The quantities of Kyn, 3OHKyn, and 3OHKG released by an epithelial explant over a period of three days in the same medium.

The quantities of Kyn and 3OHKyn in whole lenses quantified in Chapter 4 were, on average, 4% and 1.5% respectively of the amount of 3OHKG. In these epithelial preparations, however, levels of Kyn and, to a lesser extent, 3OHKyn have become a much higher proportion of the total Trp metabolites. In addition, while the levels of 3OHKyn and 3OHKG appear to have plateaued by 48 hr, Kyn has
increased in an apparently linear manner to 72 hr. It would appear that the activities of kynurenine hydroxylase and the glucosyl transferase enzyme may have declined markedly in the later stages of the incubation. In the case of kynurenine hydroxylase, an NADPH dependent enzyme, a possible cause may be a fall in the level of NADPH after an extended period in the same culture medium. That the level of 3OHKyn has remained static while apparently no glucosylation has been taking place provides evidence for a lack of kynureninase activity in lens cells, which would result in formation of 3-hydroxyanthranilic acid. Kynureninase, although not requiring NADPH, is pyridoxyl dependent. The culture medium contains equal quantities of both pyridoxyl and nicotinamide. Nothing is known about the requirements of the glucosylating enzyme.

The accumulation of precursors in the medium of epithelial explants whilst the level of 3OHKG remains constant may support the suggestion that no mechanism exists in the epithelial cells for the metabolism of Trp via the oxidative metabolism found in other tissues; i.e. the higher than normal levels of Kyn and 3OHKyn are not removed by hydrolysis to anthranilic or 3-hydroxyanthranilic acid. The apparent fall in kynurenine hydroxylase activity may mean that the enzyme has been inactivated or that lens Trp metabolism is unable to provide nicotinamide cofactors. This invites speculation that, if for some reason glucosylation became inhibited in vivo, or if the capacity of the glucosyl transferase were exceeded, Kyn and
3OHKyn could build up in higher than normal quantities. The implications of this scenario are interesting in light of the reactivity of 3OHKyn with lens proteins (Stutchbury and Truscott, 1993).

Although the major part of this study has involved whole lens experiments, these initial investigations with human lens epithelial explants have shown that this procedure is worthy of further development, and may well prove useful in further studies on lens Trp metabolism. This may be especially so in investigations on the enzymes involved in this pathway.

7.3.2 3OHKG is Not Degraded in Lens Homogenates

Whether 3OHKG would be taken up as well as released by an epithelial explant is uncertain. In addition, if any 'factors' responsible for breakdown of the glucoside are intracellular and are not released by the epithelial cells into the medium, then the result shown in Section 7.3.1.2, in which an epithelial preparation did not breakdown 3OHKG, is inconclusive.

In order to further investigate the possible breakdown of 3OHKG by the lens, a lens homogenate experiment employing $^3$H-3OHKG was carried out according to Section 7.2.2 (Fig 7.4).
Figure 7.4: The radiolabel detected in 3OHKG in samples of a 20 year old lens homogenate to which $^3$H-3OHKG had been added.

The radioactivity detected under the 3OHKG peak in the sample after 24 hr incubation is 8% lower than at the earlier time points. This was not associated with an increase in label coeluting with 3OHKyn, however, and so may not be attributable to glucosidase activity on the 3OHKG. The size of the 3OHKG HPLC peak from the 24 hr sample was also 8% smaller than the other two samples, indicating that the lower dpm value obtained in the 24 hr sample is most likely due to a lower recovery during the processing of the homogenates prior to HPLC.

The recovery of dpm from each homogenate sample in this experiment (approx. 8,000 against a calculated 20,000) can be explained by the ultrafiltration prior to analysis, which, as demonstrated in Section 4.3.3.1, results in approximately 40%
recovery of metabolites.

This result provides additional evidence that no further metabolism of 3OHKG takes place in the lens.

7.3.3 Glycosidase Assays

Treatment of 1mM aqueous solutions of four glycosides (Section 7.2.4.1) with commercial β-glucosidase under the conditions described in Section 7.2.4.2 resulted in varying degrees of hydrolysis (Table 7.1).

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th>Abs (420)*</th>
<th>Nitrophenyl formed/hr (μmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>oNP-gal</td>
<td>0.539</td>
<td>0.031 (31%)</td>
</tr>
<tr>
<td>oNP-glu</td>
<td>0.477</td>
<td>0.027 (27%)</td>
</tr>
<tr>
<td>pNP-gal</td>
<td>Abs (400)**</td>
<td>1.366 1.275 0.087 (87%)</td>
</tr>
<tr>
<td>pNP-glu</td>
<td>1.512</td>
<td>1.534 0.100 (100%)</td>
</tr>
</tbody>
</table>

*ε=21,300; **ε=18,300

Incubation of the glycosides tested in Table 7.1 with homogenates from a 72 year old and two pairs of lenses 79 and 83 years old, however, resulted in no detectable hydrolysis using UV-spectrophotometry. Since a notable feature of mammalian glycosidases is the coexistence of highly specific inhibitors (Conchie et al, 1959), the possible inhibitory activity of lens homogenates was checked by assaying β-glucosidase activity in homogenates of 62 and
64 year old lenses using pNP-glu. No inhibition of glucoside activity was detected.

The lack of glycosidase activity by UV-spectrophotometry is not surprising in the light of the study by Carlin and Cotlier (1971). They measured lens β-glucosidase activity to be 0.045 μmol g⁻¹ lens hr⁻¹; a value they acknowledge is too low for determination by spectrophotometric methods. They used instead fluorometric procedures based on the degradation of 4-methylumbelliferyl glycosides. The peak activity of β-glucosidase was around pH 5, although there was only a minor drop in activity at physiological pH.

Although the β-glucosidase activity in human lens as measured by Carlin and Cotlier (1971) is very low compared to other tissues (Conchie et al, 1959), it is still significant when compared to the lens concentration of 3OHKG. According to the range of concentrations of this compound found in Fig. 4.3D, a β-glucosidase activity of 0.045 μmol g⁻¹ lens hr⁻¹ would result in total turnover of 3OHKG in 2 to 50 hr. This assumes that firstly the results of Carlin and Cotlier using the fluorescence assay for glucoside activity are valid and secondly that 3OHKG is a substrate of the lens glycosidases. As described earlier, experiments using a lens homogenate and lens epithelial preparation, however, failed to detect any hydrolysis of 3OHKG. There is little possibility that lens β-glucosidase is a lyzosomal enzyme that was not released by lens homogenisation, as lyzosomes
have never been identified in the lens and glycosidases were found not to be activated by treatment with Triton-X-100 (Carlin and Cotlier 1971).

From results presented in this thesis, it may be that the values obtained by Carlin and Cotlier (1971), are in fact artifactual results produced by the fluorometric analysis. Another possibility is that the glycosidases in the lens do not hydrolyse 3OHKG or that other lens components inhibit the lenticular glycosidase. Either way, it would appear from the results presented here, that an increase in lens concentration of the possible cataractogenic agent 3OHKyn (Stutchbury and Truscott, 1993), is not likely to occur due to hydrolysis of 3OHKG, as first suggested as a possibility by van Heyningen (1971a).

7.3.4 Assay For $^{14}$C-Alanine

LSC analyses of lens protein free extracts following incubation of lenses in radiolabelled Trp and separation on HPLC often resulted in a small radioactive peak with a retention time of 6 min. This radioactive peak was much more prominent in the $^{14}$C-Trp epithelium experiment (Fig. 7.1B). A sample of L-[U-$^{14}$C]-alanine (150 mCi mmol$^{-1}$; purchased from Amersham, Sydney, Australia) containing approx. 400,000 dpm was chromatographed on HPLC. LSC analysis showed that the major radioactivity eluted from the column.
at a retention time of 6 min.

No trace of 3-hydroxyanthranilic acid (3OHA), the metabolic product of 3OHKyn in the ‘normal’ oxidative metabolism of Trp, had been detected by either HPLC or LSC in any lens incubation experiments. However, the conversion of 3OHKyn to 3OHA also results in the release of alanine, which, in the $^{14}$C-Trp experiments, would also carry the radiolabel if formed. If the 6 min peak were alanine, this could be a result of some Trp in the lens being metabolised via the ‘normal’ pathway; or it could also be a result of kynureninase activity on Kyn, releasing alanine and anthranilic acid. For this reason the 6 min peak seen in Fig 7.1B was tested by amino acid analysis (AAA) to see if the detected radiolabel was due to alanine.

A 1 µL sample of L-[U-$^{14}$C]-alanine (approx. 22,000 dpm) in ‘cold’ carrier was run through the amino acid analyser and 30 sec fractions were collected around the known retention time of standard alanine on this system. The fractions were counted by LSC. Another sample of the 48 hr medium from the $^{14}$C-Trp epithelial experiment (Section 7.3.1.) was run on HPLC and the eluent was collected between 4 and 7 min after starting the analysis. One-third of this material was counted on LSC and it contained approx. 700 dpm. The remaining material was lyophilised and run on the amino acid analyser (AAA). Fractions at the same retention time were
collected as for the $^{14}$C-alanine standard.

The radioactivity of the $^{14}$C-alanine standard was recovered (Table 7.2). No radioactivity was recovered, however, from the approx. 1,500 dpm analysed from the 6 min HPLC peak. No evidence was therefore found from this assay for Trp metabolism involving the production of alanine. The existence of the 6 min radioactive peak, however, means that the existence of other, as yet unknown, products of Trp catabolism besides Kyn, 3OHKyn and 3OHKG cannot be ruled out.

Table 7.2: The radioactivity detected in $^{14}$C-alanine standard and in the 6 min radioactive HPLC peak when analysed using amino acid analysis.

<table>
<thead>
<tr>
<th>AAA RET. TIME (min)</th>
<th>14C-Ala Standard dpm</th>
<th>6 min HPLC Peak dpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>14-14.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>14.5-15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15-15.5</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>15.5-16</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>16-16.5</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>16.5-17</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>17-17.5</td>
<td>121</td>
<td>0</td>
</tr>
<tr>
<td>17.5-18</td>
<td>1717</td>
<td>0</td>
</tr>
<tr>
<td>18-18.5</td>
<td>4960</td>
<td>0</td>
</tr>
<tr>
<td>18.5-19</td>
<td>9820</td>
<td>0</td>
</tr>
</tbody>
</table>

7.3.5 N'-Formylkynurenine is Not Detected In Human Lenses

Due to the high activity of the formamidase that acts on N'-
formylkynurenine (N-formylKyn) (Greenberg, 1969; Bender, 1982), the first product of Trp oxidative metabolism appears to be Kyn. Since Trp metabolism in the lens has been shown to be different to that in other organs, it was decided to test for the presence of this compound in human lenses. N-formylKyn is an unstable compound and can hydrolyse to Kyn non-enzymically. It is not available commercially. Therefore, in order to determine its retention time on HPLC, its synthesis was required. This was carried out (Section 7.2.6) and the identity of the purified compound was confirmed by UV-spectrophotometry (Fig. 7.5) and mass spectrometry (Fig. 7.6).

No N-formylKyn was detected in lens extracts even at a detection wavelength of 320 nm (the compound has a $\lambda_{\text{max}}$ at 321 nm) suggesting that in human lenses, as in other tissues, formamidase may be quite active; or, less likely, that non-enzymatic breakdown within the lens is comparable to the rate of synthesis.

Another possibility is that, if N-formylKyn is present in the lens, it is hydrolysed by the extraction process. To calculate recovery data 100 $\mu$L of a 0.06 mg mL$^{-1}$ solution of purified N-formylKyn was chromatographed on HPLC with detection at 320 nm (to give a 100% recovery figure), then 0.5 mL of 0.06 mg mL$^{-1}$ N-formylKyn in 80% ethanol was used to prepare a protein-free extract from 250 mg bovine lens tissue. (The bovine lens does not metabolise Trp [Trayhurn and van Heyningen, 1973] and will therefore not contain
N-formylKyn). Based on integrated HPLC peak areas it was determined that approx. 50% of the compound had hydrolysed to Kyn when taken through the extraction procedure. This percentage loss would not prevent the detection of the compound in human lens extracts if it were present in any significant quantity.

![UV-spectrum of synthesised N'-formylkynurenine.](image)

**Figure 7.5:** The UV-spectrum of synthesised N'-formylkynurenine. $\lambda_{\text{max}}$ were detected at 230, 260 and 321 nm (compare with Dalgliesh, 1952).
Figure 7.6: Mass spectrum of synthesised N'-formylkynurenine (MW 236).
7.3.6 Pulse-pulse Experiment

Epithelial and homogenate experiments with radioactive 3OHKG present, failed to detect any possible further metabolism of this metabolite. In intact lenses loss of radioactive 3OHKG can be explained experimentally by the rapid appearance of the compound in AAH. In a variation on this procedure, a pair of lenses were pulsed separately for 24 hr in L-[5-3H]-tryptophan. One of the pair was then removed from culture whilst the other was given a further 24 hr pulse in order to compare the radio-profiles of the lenses after 24 and 48 hr.

LSC analysis of the protein free extracts of these lenses following HPLC (Fig. 7.7), demonstrated that the amount of radioactive Trp in the double-pulsed lens was approximately twice as high as in the single-pulsed lens.

Since the lenses were a pair from the same individual, it may be expected that the extent of Trp incorporation was similar in both after the first 24 hr pulse. That Trp apparently continues to be transported into the lens from the AAH during the second day in culture is further evidence that the lens culture system, employed for the bulk of experiments used in this study, works well, as there was apparently no decrease in the metabolic rate of conversion of Trp to 3OHKG during the second 24 hr incubation. The quantity of label in Kyn is similar in both lenses but the quantity associated
with 3OHKG is twice as high in the double-pulsed lens. This suggests that Kyn is efficiently hydroxylated then glucosylated, and that the apparent rate of synthesis of 3OHKG is apparently linear over a 48 hr period. The lack of detection of radiolabel after 48 hr in compounds apart from those of the 3OHKG pathway indicates that this is the major route of free Trp metabolism in the human lens. If there are other as yet undiscovered pathways, they play only a quantitatively minor role in the overall metabolism of lenticular tryptophan.

Figure 7.7: The radioactivity associated with HPLC eluent of the protein free extracts of an 82 year old lens pair given one (---) or 2 (-) 24 hr pulses in AAH containing 20 µCi mL⁻¹ L-[5-³H]-tryptophan. The radioactive peaks at 18, 30 and 75 min are due to 3OHKG, Kyn and Trp respectively.
APPENDIX A

HUMAN FOETAL LENSES

During the course of this study a pair of lenses from a 20 week old foetus were obtained and incubated for 24 hr in AAH (Section 2.2.2) containing [U-14C]-L-tryptophan at the same concentration as that used for adult lenses (Chapter 5). Upon analysis of protein-free extracts by HPLC, a small peak was detected at the retention time of 3OHKG. Using the integrated peak areas and the 3OHKyn standard curve (4.3.1), values of 0.0061 and 0.0122 μmol g⁻¹ lens were estimated for these lenses. These values would appear at the very lower region of Fig. 4.4D but show that, if the peak detected at 17 min in Fig. 1A is 3OHKG, formation of this metabolite is a process that is initiated before birth. The Trp peak was detected at 78 min.

Fractions of HPLC eluent were collected every 2 min and analysed by LSC. As seen in Fig. 1B, although radiolabelled Trp had been transported into the lens in 24 hr, there was no evidence of incorporation into Kyn, 3OHKyn or 3OHKG; in contrast to results obtained in Chapter 5 (see Table 5.1). If the 3OHKG pathway does occur at this stage of human development, the metabolic rate is much slower than in post-natal lenses.
Figure 1: The HPLC chromatogram (A) and LSC profile (B) of the protein-free extract of a 20 week old human foetal lens incubated for 24 hr in AAH containing 50μM (0.4 μCi mL⁻¹) [U-14C]-L-tryptophan. Retention times are printed above detected peaks in (A).
APPENDIX B

GOURAMI LENSES CONVERT TRYPTOPHAN INTO 3-HYDROXYKYNURENINE

Introduction

The lens UV-filter compound present in the three-spot gourami Trichogaster trichopterus has been identified as 3-hydroxykynurenine (Truscott et al, 1993); a substance also found in the human lens (this thesis and Wood and Truscott, 1993). In primates, 3-hydroxykynurenine is the immediate precursor for the major UV-filter substance 3-hydroxykynurenine glucoside (van Heyningen, 1971a,b; 1973a,b).

It has been proposed that aminophenols such as 3-hydroxykynurenine may be involved in the aetiology of human cataract (Truscott et al, 1991). However it has been difficult to test this hypothesis since no non-primate animal model for this pathway has been described. In humans, 3-hydroxykynurenine and the glucoside are biosynthesised in the lens from tryptophan (Wood and Truscott, 1993; van Heyningen, 1973b).

It was therefore of considerable interest to determine if the 3-hydroxykynurenine present in the gourami lens was derived from lenticular metabolism of tryptophan. This can be readily determined by using intact lenses incubated in artificial aqueous humor (AAH) containing radiolabelled tryptophan.

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Materials and Methods

Fish were purchased from local aquaria and killed by decapitation. Lenses were dissected from the eyes and transferred to sterile petri dishes (Crown Scientific, Sydney Aust.) containing artificial aqueous humor as described by Duncan et al (1981, 1990) and incubated at 25°C (the normal environmental temperature of these species). Radiolabelled tryptophan, either L-[\(^{14}\)C(U)]- (Du Pont, Sydney Aust.) or L-[5-\(^{3}\)H]- (Amersham, Sydney Aust.) as indicated in the text was added to the AAH where necessary. At the completion of incubation experiments, lenses were homogenised twice in 0.5 mL 80% ethanol, centrifuged at 15,000 g for 20 min and the pooled soluble fraction from the two extractions ultrafiltered using a 10,000 MW cut-off centrifugal ultrafilter (Activon Scientific, Sydney Aust.). The filtrate was lyophilised, reconstituted in Milli-Q water (Millipore, Sydney Aust.) and chromatographed by HPLC. Liquid scintillation counting of collected HPLC peaks was also performed.

Results and Discussion

Prior to commencing this study a range of 'gouramis' available from local aquaria were surveyed. Fig. 1 shows the HPLC traces of lens extracts from two such fish; the three-spot gourami (A) and pink kissing gourami (B). It is readily apparent that the profiles differ markedly. The three-spot gourami extract is dominated by 3-
hydroxykynurenine (Truscott et al., 1993) and some kynurenine is also present. In contrast the pink kissing gourami HPLC profile contains little or no 3-hydroxykynurenine or kynurenine. The compounds absorbing at 365 nm in this case may represent mycosporine-like amino acids (Thorpe et al., 1992b) and this finding is consistent with the phylogenetic classification of the two anabantoids within the order Perciformes. The kissing gourami Helostoma temmincki is the only species of the genus within the family Helostomatidae whereas the three-spot gourami is a member of the family Belontiidae (Goldstein, 1971).

The three-spot gourami was found to contain approximately 1.5 nmol of 3-hydroxykynurenine and 0.059 nmol tryptophan per mg lens and the kissing gourami 0.056 nmol tryptophan per mg lens.

For studies on tryptophan metabolism therefore, lenses from the three-spot gourami were used. Initially, gourami lenses were placed into AAH containing $^{14}$C-labelled Trp (0.58μM, 0.4 μCi mL$^{-1}$) for 18 hr and the protein-free extract separated by HPLC. Under these conditions 3-hydroxykynurenine elutes at 15 min, kynurenine at 31 min and tryptophan at 81 min. This preliminary experiment revealed that tryptophan was taken up by the lenses and some conversion into kynurenine and 3-hydroxykynurenine was also apparent in this time (Fig. 2).
Figure 1
Figure 1 (previous page): HPLC traces of extracts from gourami lenses: (A) three-spot gourami *Trichogaster trichopterus* and (B) kissing gourami *Helostoma temmincki*. At approximately 33 min the wavelength of detection was changed from 365 nm to 278 nm to allow quantification of tryptophan. 3-hydroxykynurenine (3OHKyn) elutes at 15 min, kynurenine (Kyn) at 31 min and Trp at 81 min. The peak at 40 min is the internal standard 5-hydroxytryptophan.

![HPLC trace](image)

**Figure 2:** HPLC trace of a three-spot gourami lens extract following 18 hr incubation in AAH containing $^{14}$C-Trp. Conditions as for Fig. 1.

In order to follow the kinetics of the transformation, time-course experiments were conducted using readily available $^3$H-Trp at two levels: 0.156μM and 50μM tryptophan. The results are depicted in Fig. 3 and Fig. 4.

At the low tryptophan concentration, which was used to
provide a brief 'pulse' of the amino acid, Trp appears to be taken up by the lenses and then declines steeply, presumably due to incorporation of label into protein. The free tryptophan is converted rapidly to kynurenine. After an initial delay, label then appears in 3-hydroxykynurenine. Such a labelling pattern would be expected if kynurenine were an intermediate in the conversion of Trp into 3-hydroxykynurenine.

A similar time course pattern was obtained when lenses were incubated in AAH containing 50μM tryptophan (Fig. 4), a level approximating that which would be expected in normal human aqueous humor (Dickinson et al, 1968).

In this study no attempt was made to optimise the composition of the AAH used for the fish lens incubations and this factor may have contributed to the standard errors shown in Fig. 4. For this reason the time course data should be regarded as indicative only. Nevertheless it seems clear that the gourami lens, like the human, is able to metabolise tryptophan to 3-hydroxykynurenine and therefore may usefully serve as a readily accessible model for studies on the lenticular formation of UV-filter substances derived from tryptophan.

Figure 3 (following page): Time course of incorporation of label from $^3$H-Trp into lens (a) tryptophan, (b) kynurenine and (c) 3-hydroxykynurenine. Trp was added to AAH at a final concentration of 0.156μM. Lenses were incubated (3 lenses per petri-dish containing 2 mL AAH) at 25°C for the times indicated, rinsed with AAH lacking Trp, then extracted together and chromatographed by HPLC. At the retention times corresponding to the metabolites, fractions were collected and dpm determined by LSC.
Figure 3
Figure 4
Figure 4: (preceding page) Time course of incorporation of label from $^3$H-Trp into lens (a) Trp, (b) Kyn and (c) 3OHKyn. Trp was added at a final concentration of 50μM and incubated as described for fig 3. Lenses were extracted individually, (mean ± S.D.; n=3).

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APPENDIX C

POSSIBLE IMPLICATION OF THIS STUDY: THE POTENTIAL ROLE OF 3-HYDROXYKYNURENINE AS A CATARACTOGENIC AGENT

It has been proposed that primate lens proteins may be susceptible to a particular post-translational modification as a result of the presence within the lens of the pathway for 3-hydroxykynurenine glucoside (30HKG) biosynthesis (Truscott et al, 1991; Wood and Truscott, 1993). Specifically, the molecule of interest in this regard is 3-hydroxykynurenine, which has been shown to react readily with proteins in the presence of oxygen. The coloured proteins which result have been modified primarily through covalent interaction with amino groups (Stutchbury and Truscott, 1993).

Such processes may be implicated in the normal increase in colouration of human lenses which is responsible for the well documented lessening of the ability to see blue light in older people. This 'blue-deprivation' seems to be associated with an increased crystallin pigmentation (Spector, Roy and Stauffer, 1975).

It is conceivable that 30HKyn could produce more substantial modification of crystallins in the lens when reduced glutathione levels fall markedly. This has been documented in the lens nucleus in the earliest stage (Type II) of senile nuclear cataract (Truscott and
In order for the scenario to be credible it must be demonstrated that free 3OHKyn is present in the lens, including the lens nucleus; and that the synthesis of 3OHKyn takes place at a significant rate. In this thesis measurable levels of 3OHKyn have been reported in the nuclei of human lenses. It has also been demonstrated that 3OHKG synthesis in organ cultured human lenses occurs at a surprisingly rapid rate, such that readily quantifiable labelling of 3OHKG and its metabolic precursor, 3OHKyn, can be observed following a 24 hr incubation in AAH containing radioactive Trp. Lenticular 3OHKyn can also be derived potentially from sources outside the lens since labelled 3OHKyn entered lenses in culture and was transformed, at least partially, into 3OHKG. Pulse experiments with radiolabelled 3OHKyn revealed that free 3OHKyn was found in the nucleus after 24 hr. This may represent metabolite in excess of glucosylation capacity.

Attempts were made to discover if 3OHKG could be broken down by lens extracts, since this could serve as an additional pathway for removal of 3OHKG and may also function as another source of lenticular 3OHKyn. Lens homogenates were used to measure spectrophotometrically the hydrolysis of known substrates of β-glucosidase. No such hydrolysis could be observed, however. Nor was any hydrolysis of $^{14}$C- or $^3$H-3OHKG collected from incorporation experiments observed in either a lens epithelial culture in 48 hr or a
lens homogenate in 24 hr. No evidence therefore could be obtained for 3OHKyn formation in lenses as a result of 3OHKG hydrolysis. It would appear that 3OHKG may be a stable molecule within the lens and that efflux may be the chief or only means for removal.

If more were known about the control of lens levels of 3OHKG it may be possible to artificially regulate the concentration using drugs. This may be useful if UV light can be demonstrated unambiguously to be involved in senile cataract formation since the glucoside, whilst known to absorb otherwise damaging UV radiation, is a poor photosensitiser (Dillon and Atherton, 1990; Dillon, Wang and Atherton, 1990). The influence on lenticular 3OHKyn levels may need to be taken into consideration.

Whilst the proposal that 3OHKyn may be implicated in human lens crystallin colouration is attractive, and indeed was considered as a possibility by van Heyningen (1973), definite proof must await the isolation of characteristic adducts from aged or senile nuclear cataract lenses, if indeed they do exist.
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Publications and Submissions Arising From This Thesis

Papers


Conference Presentations


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