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Characterisation of the Major Autoxidation Products of 3-Hydroxykynurenine Under Physiological Conditions

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3-Hydroxykynurenine (3-OHKyn) is a tryptophan metabolite that is readily autoxidised to products that may be involved in protein modification and cytotoxicity. The oxidation of 3-OHKyn has been studied here with a view to characterising the major products as well as determining their relative rates of formation and the role that H_2O_2 and hydroxyl radical ($HO^\bullet$) may play in modifying the autoxidation process. Oxidation of 3-OHKyn generated several compounds. Xanthommatin (Xan), formed by the oxidative dimerisation of 3-OHKyn, was the major product formed initially. It was, however, found to be unstable, particularly in the presence of H_2O_2, and degraded to other products including the *p*-quinone, 4,6-dihydroxyquinolinequinonecarboxylic acid (DHQCA). A compound that has a structure consistent with that of hydroxyxanthommatin (OHXan) was also formed in addition to at least two minor species that we were unable to identify. Hydrogen peroxide was formed rapidly upon oxidation of 3-OHKyn, and significantly influenced the relative abundance of the different autoxidation species. Increasing either pH (from pH 6 to 8) or temperature (from 25°C to 35°C) accelerated the rate of autoxidation but had little impact on the relative abundance of the autoxidation species. Using electron paramagnetic resonance (EPR) spectroscopy, a clear phenoxyl radical signal was observed during 3-OHKyn autoxidation and this was attributed to xanthommatin radical ($Xan^\bullet$). Hydroxyl radicals were also produced during 3-OHKyn autoxidation. The $HO^\bullet$ EPR signal disappeared and the $Xan^\bullet$ EPR signal increased when catalase was added to the autoxidation mixture. The $HO^\bullet$ did not appear to play a role in the formation of the autoxidation products as evidenced using $HO^\bullet$ traps/scavengers. We propose that the cytotoxicity of 3-OHKyn may be explained by both the generation of H_2O_2 and by the formation of reactive 3-OHKyn autoxidation products such as the $Xan^\bullet$ and DHQCA.

Keywords: 3-Hydroxykynurenine, tryptophan-oxidation, xanthommatin, quinone, hydrogen peroxide, hydroxyl radical, cataract

Abbreviations: DHQCA, 4,6-dihydroxyquinolinequinonecarboxylic acid; DMPO, 5,5-dimethylpyrroline-*N*-oxide; EPR, electron paramagnetic resonance spectroscopy; ESI-MS, electrospray ionisation mass spectrometry; $HO^\bullet$, hydroxyl radical; HPLC, high performance liquid chromatography; HRP/OPD, horseradish peroxidase/*o*-phenylenediamine; 3-OHKyn, 3-hydroxykynurenine; OHXan, hydroxyxanthommatin; $O_2^{\bullet-}$, superoxide; SOD, superoxide dismutase; UV, ultraviolet; Xan, Xanthommatin; $Xan^\bullet$, xanthommatin radical

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INTRODUCTION

3-Hydroxykynurenine (3-OHKyn) is a ubiquitous tryptophan metabolite that exhibits either pro- or anti-oxidant activity, depending on experimental conditions.[1–3] Levels of 3-OHKyn and its transamination product, quinolinic acid, are significantly elevated in the brain in pathological conditions such as dementia associated with human immunodeficiency virus (HIV) infection, hepatic encephalopathy, Parkinson's disease and Huntington's disease.[4–7] In neuronal cell cultures, 3-OHKyn has been found to be cytotoxic at concentrations as low as 1 μM.[3] 3-OHKyn is also present in primate lenses where it acts, together with its β-glucoside conjugate, as a UV-filter compound to filter light in the 295–400 nm region.[8,9] In human nuclear cataract, proteins in the lens become coloured, oxidised, cross-linked and insoluble.[10] We have proposed that aminophenols, in particular 3-OHKyn, may be implicated in these changes since incubation of 3-OHKyn with lens proteins, in the presence of oxygen, can reproduce the changes that occur in nuclear cataract.[11,12] The structures of the reactive intermediates of 3-OHKyn that are responsible for the protein modification are, however, not known.

For these reasons we examined the oxidation of 3-OHKyn to try to identify the reactive species involved and to gain an understanding of the possible involvement of H_2O_2 (produced as a by product) in the autoxidative reactions. Experimental conditions of pH (pH 7) and temperature (35°C) were chosen to mimic those of the lens. We show here that autoxidation of 3-OHKyn generates at least five compounds, some of which may be implicated in cellular protein modification.

MATERIALS AND METHODS

Materials

3-OHKyn, *o*-phenylenediamine (OPD), horseradish peroxidase (HRP), desferrioxamine (desferal), 5,5-dimethylpyrroline-*N*-oxide (DMPO) superoxide dismutase (SOD) and catalase were purchased from Sigma Chemical Co. (St Louis, MO, USA). Mannitol and H_2O_2 were obtained from Ajax (Australia). All gases used were compressed industrial grade (CIG, Chatswood, Australia). Water was purified using the Millipore Milli Q Plus 185 system. Phosphate buffers were prepared from AR grade NaH_2PO_4 and Na_2HPO_4, both obtained from Ajax (Australia).

High Performance Liquid Chromatography Analysis

The reversed phase HPLC system consisted of an LC 1150 HPLC pump (ICI instruments), a Rheodyne 7125 sample injector (equipped with a 20 μl sample loop) and a SD 2100 UV–Vis variable wavelength detector (ICI Instruments), set at 254 nm. Analytical separations were performed on a 250 mm × 4.6 mm Spherisorb S5ODS2 column (ICI Australia Operations Pty. Ltd., Dingley, Australia). Samples were analysed using an aqueous 4 mM ammonium acetate (buffer A)/acetonitrile 4 mM ammonium acetate (buffer B) gradient. The gradient was run at 0% buffer B for 10 min followed by an increase to 40% over 30 min and at a flow rate of 0.5 ml/min. When semi-preparative separations were required, a 25 mm × 10 mm Partisil-10 ODS-3 semi-preparative HPLC column (Whatman) was employed using the same eluents and gradient as described above with a flow rate of 3.0 ml/min. The solvents were degassed by sparging with helium for 10 min before use. Injection volumes were 20 μl for analytical runs and up to 4 ml for semi-preparative runs. The samples were adjusted to pH 7, where required, before HPLC analysis.

3-Hydroxykynurenine Autoxidation

The standard autoxidation reaction mixture consisted of 3-OHKyn (2.2 mM) in sodium phosphate buffer (0.1 M) adjusted to the appropriate pH using 1 M solutions of NaOH or NaH_2PO_4.

The resultant mixture was then bubbled with oxygen (at approximately 15 ml/min) while immersed in a water bath at the specified temperature. Aliquots were taken at various time intervals for HPLC analysis. Where indicated, catalase (6300 units per addition) or SOD (1000 units per addition) was added to 5 ml of the standard autoxidation solution (pH 7 and 35°C) at hourly intervals. Mannitol (273 mg/5 ml) was also added to the autoxidation solution (pH 7 and 35°C) in some experiments as described below.

Preparation of Xanthommatin, Hydroxyxanthommatin and DHQCA

Hydroxyxanthommatin (OHXan) was obtained by semi-preparative HPLC purification from the 3-OHKyn autoxidation reaction mixture after 25 h of reaction at pH 7 and 25°C. For Xanthommatin (Xan) preparation, a 2.2 mM solution of 3-OHKyn in 0.1 M phosphate buffer (10 ml, pH 7) was bubbled with oxygen in the presence of catalase (6300 units per h) for 4 h. The Xan was then purified using semi-preparative HPLC as described above. DHQCA was synthesised from trimethoxybenzene as described previously.[13] Where indicated, the stability of the isolated 3-OHKyn autoxidation products was assessed by incubation in phosphate buffer as above. In specific cases, H_2O_2 was also added to give a final concentration of approximately 50 mM.

Assay of Hydrogen Peroxide

Hydrogen peroxide concentrations were measured using a HRP/OPD assay. Duplicate 10 µl aliquots of the autoxidation solution were placed in a micro-titer plate and frozen until all samples were collected. This was followed by the sequential addition to each well of 10 µl of distilled water, 50 µl of 0.2 M phosphate buffer (pH 5.0) containing HRP and 100 µl of 5 mg/ml OPD solution. This mixture was allowed to react for 5 min at room temperature and quenched by the addition of 30 µl of 1 M HCl. The absorbance was read at 490 nm and H_2O_2 concentrations calculated by reference to a standard curve.

Electrospray Ionisation-Mass Spectrometry Analysis

ESI-MS analysis was performed on a VG Quattro mass spectrometer (VG Biotech/Micromasss, Altrincham, UK). For electrospray analysis, fractions isolated by HPLC were either analysed directly or were lyophilised and then re-dissolved in 50% aqueous acetonitrile. The electrospray solvent was 49.5% (v/v) acetonitrile in H_2O which contained 1% (v/v) formic acid. This solvent was delivered at a flow rate of 10–15 µl/min. The scan rate was 100m/z per second and the scan range was from 100 to 900m/z. The MS source temperature was 75°C and a cone voltage of 30 V was routinely used.

Electron Paramagnetic Resonance Spectroscopy

3-OHKyn was made up to 500 µM in phosphate buffer (pH 7.4) that had been treated previously with washed Chelex-100 (Bio-Rad)[14] to minimise levels of contaminating transition metals. The mixtures were then incubated at 37°C while exposed to air (or bubbled with nitrogen) for the times specified below. Where indicated, DMPO was present at 0.1 M, catalase at 600 units/ml and H_2O_2 at 20 mM. EPR spectra were recorded at room temperature using a Bruker EMX X-band spectrometer with 100 kHz modulation and a cylindrical ER 4103TM cavity. Autoxidation mixtures were allowed to incubate for the times stated in the Results section and subsequently transferred to a flattened sample cell (WG-813-SQ, Wilmad, Buena, NJ, USA) with spectra recording initiated within 2 min. Hyperfine couplings were measured directly from the field scan. Typical EPR spectrometer settings were: gain 2×10^6, modulation amplitude 0.2 mT, time constant 0.16 s, scan time 84 s, resolution 1024 points, centre

field 348 mT, field scan 8 mT, power 25 mW, frequency 9.76 GHz, with 8 scans averaged.

RESULTS

Characterisation of the Major 3-Hydroxykynurenine Autoxidation Products

The formation of 3-OHKyn autoxidation products was monitored by analysing the reaction mixtures by HPLC at time intervals routinely ranging from 0.5 to 12.5 h. This time frame was chosen because earlier studies of protein modification by 3-OHKyn had shown that this occurs over a period of hours.[11,12] Autoxidation of 3-OHKyn resulted in the formation of at least five species. Figure 1 shows the HPLC profile of the reaction mixture after 2.5 h. The compounds with retention times of 5.3, 12 and 28 min were identified as DHQCA, 3-OHKyn and Xan, respectively (Figures 1 and 2), using ESI-MS, UV–Vis spectrophotometry and HPLC analysis with reference to authentic samples. The ESI (+ve) molecular ions and absorbance maxima for these species were: (i) DHQCA, m/z value = 236 with fragments at m/z values of 218 (H_2O loss) and 190 (formic acid loss), absorbance maxima at 350 and 460 nm; (ii) 3-OHKyn, m/z value = 225 with fragments at 208 (NH_3 loss), 179 (formic acid loss) and 152 (HCNCOOH loss), absorbance maximum at 365 nm; (iii) Xan, m/z value = 424 with minor fragments at m/z values of 407 (NH_3 loss), 361 (formic acid loss) and 351 (HCNCOOH loss), absorbance maximum at 436 nm. The compound eluting at 23 min was found to have an m/z value of 440 with fragments at m/z values of 423, 377 and 367 (consistent with the loss of NH_3, formic acid and HCNCOOH respectively), and an absorbance maximum at 485 nm; the latter is characteristic of a phenoxazone system.[15] Since the molecular mass of 439 for this compound is 16 above that of Xan, and the fragment ions observed in the ESI mass spectrum (all due to losses from the amino acid side chain) were analogous to those of Xan, we have denoted this species as OHXan. A possible structure is shown in Figure 2. The HPLC trace (Figure 1) also shows two groups of poorly resolved peaks, centred on retention times of 4.3 and 20 min respectively. Attempts to purify these compounds (designated as F 4.3 and F 20) were unsuccessful and their identities therefore are unknown.

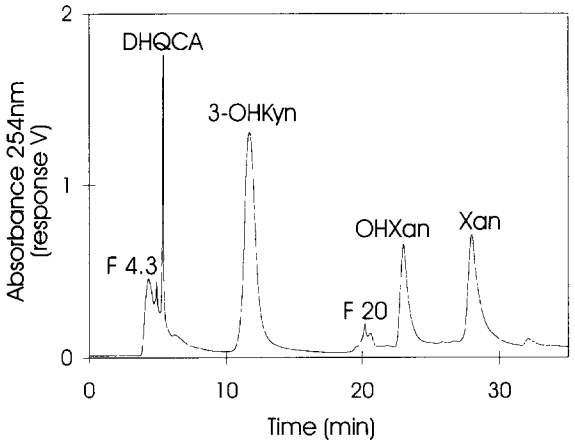


FIGURE 1 Reversed-phase HPLC trace of 3-OHKyn autoxidation products. 3-OHKyn (2.2 mM) was incubated in the presence of oxygen at pH 7 and 35°C for a period of 2.5 h. The reaction mixture was then analysed by HPLC as described in the Materials and Methods section. F 4.3, compound(s) of unknown identity which elute at 4.3 min; DHQCA, 4,6-dihydroxyquinolinequinonecarboxylic acid; 3-OHKyn, 3-hydroxykynurenine; F 20, compound(s) of unknown identity which elute at 20 min; OHXan; hydroxyxanthommatin; Xan, xanthommatin. A typical chromatogram is shown.

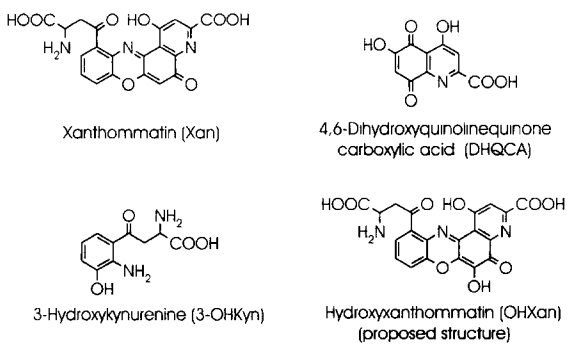


FIGURE 2 Structures of the major 3-OHKyn autoxidation products.

Autoxidation of 3-Hydroxykynurenine with Respect to Time

3-OHKyn autoxidation was monitored by performing HPLC analyses on aliquots of the reaction mixture taken at times ranging from 0.5 to 12.5 h. From the HPLC trace, a qualitative estimate of the amount of each species present was made based on the relative peak area at 254 nm. These values were plotted against time to give the product versus time profiles shown in Figure 3. The major autoxidation product of 3-OHKyn formed initially was Xan which reached a maximum concentration at *ca.* 2 h (Figure 3), after which its concentration fell to below the detection limit (*ca.* 10 h). In later experiments $Xan^{\bullet}$ was also detected (see EPR experiments below). Following the formation of Xan, increased production of OHXan (maximum concentration attained at *ca.* 7 h) was observed. This was accompanied by a relatively constant rate of formation of DHQCA and the compound(s) comprising F 4.3 (Figure 3). The compound(s) giving rise to the peak labelled F 20 accounted for less than 5% of the total 254 nm absorbance throughout the incubation.

Effects of pH and Temperature

As the pH of the autoxidation reaction was varied from 6 to 8, the rate of oxidation of 3-OHKyn increased by approximately 5-fold (over the initial 5 h of autoxidation) with a concomitant increase in the rate of formation of the subsequent autoxidation products (Figure 4). For the purposes of clarity, the data for 3-OHKyn and Xan only are shown in Figure 4. The effect of pH on the rate of autoxidation is probably due to the formation of the phenoxide ion in the initial autoxidation of 3-OHKyn, as this is predicted to facilitate electron loss.[16] When the reaction temperature was varied between 25°C and 35°C, the increase in temperature resulted in approximately a 3-fold increase in the rate of 3-OHKyn autoxidation (assessed during the first 5 h). This is also illustrated in Figure 4 (compare Figure 4, pH 7 condition, to Figure 3) as the experiments at the different pH values were conducted at 25°C in order to slow

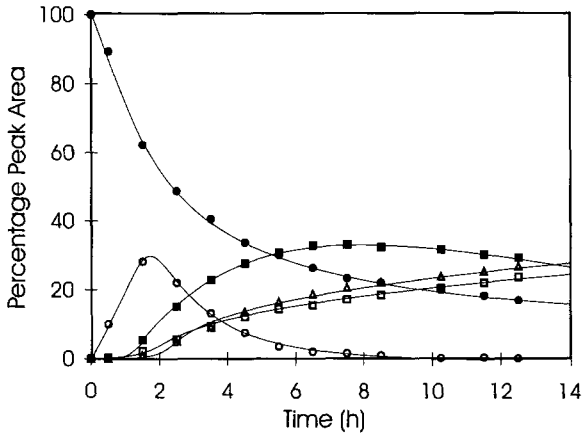


FIGURE 3 Autoxidation of 3-OHKyn at pH 7 and 35°C. 3-OHKyn (2.2 mM) was incubated at 35°C in 50 mM phosphate buffer (pH 7) and in the presence of oxygen. Aliquots taken at the times indicated were analysed by HPLC as described in the Materials and Methods section. (●, 3-OHKyn; ○, Xan; ■, OHXan; □, DHQCA; △, F 4.3). Abbreviations are described in the legend to Figure 1. Data are representative of 2 identical experiments.

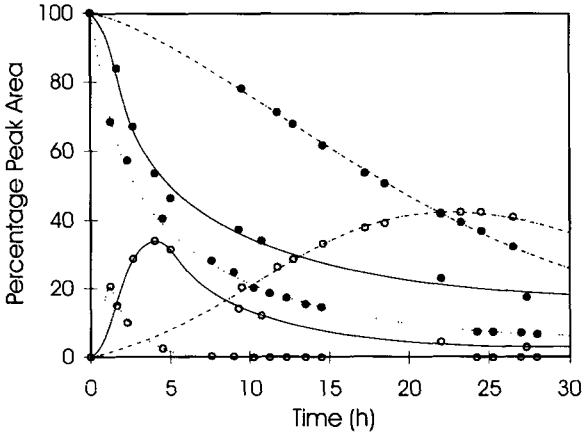


FIGURE 4 Autoxidation of 3-OHKyn at 25°C at pH 6 (dotted line), pH 7 (solid line) or pH 8 (broken line). 3-OHKyn (2.2 mM) was incubated at 25°C in 50 mM phosphate buffer at the indicated pH values and in the presence of oxygen. Aliquots taken at the times indicated were analysed by HPLC as described in the Materials and Methods section. (●, 3-OHKyn; ○, Xan). Abbreviations are described in the legend to Figure 1.

down the reaction rate and thereby more clearly assess differences due to pH effects. No new species were observed when either temperature or pH conditions were varied. When oxygen was excluded from the reaction mixture (at pH 7 and 35°C) by saturating the solutions with Ar, 3-OHKyn autoxidation was suppressed by > 99%. These results indicate that, like the autoxidation of other amino phenols (e.g. 3-hydroxyanthranilic acid[16]), the reaction conditions of pH and temperature have a marked effect on the rate of formation of autoxidation products and, as predicted, oxygen is essential. The origins and eventual fates of the major autoxidation intermediates were then examined by performing stability studies on HPLC-purified compounds.

Stability of Xanthommatin, Hydroxyxanthommatin and DHQCA

Since Xan was the major product observed during the initial stages of 3-OHKyn autoxidation, we examined its stability and the possibility that it may be an intermediate in the production of the other species. Xan in solution, even in the absence of oxygen, was inherently unstable, producing predominantly DHQCA with smaller amounts of 3-OHKyn (product ratio of approximately 32 : 1 respectively). The apparent rate of decomposition of Xan levelled off after approximately 24 h, indicating that this reaction may have reached equilibrium. A possible explanation for this is that DHQCA recombines with the available 3-OHKyn to produce Xan. In support of this, addition of a molar excess of 3-OHKyn to DHQCA at pH 7 in the absence of oxygen produced small amounts of Xan (approximately 4% yield with respect to the initial DHQCA concentration and based on 254 nm absorbance). When Xan was incubated in the presence of oxygen similar results were obtained, with the additional formation of the compound denoted F 4.3.

When purified OHXan was incubated at pH 7, either in the presence or absence of oxygen, its decomposition resulted in the production of F 4.3 and DHQCA, along with the peaks denoted at F 20 and trace amounts of 3-OHKyn (data not shown). The fact that the decomposition of both OHXan and Xan gave rise to DHQCA and F 4.3, confirmed the structural similarity of the two species noted above in the ESI-MS studies. DHQCA was relatively stable either in the presence or absence of oxygen. However, production of F 4.3 was observed after several hours of incubation in both instances. This suggests that F 4.3 may be one of the final 3-OHKyn autoxidation products.

Generation of Hydrogen Peroxide during 3-Hydroxykynurenine Autoxidation

It has been shown previously that autoxidation of 3-OHKyn generates H_2O_2 (e.g. [17]). H_2O_2 is generally thought to be produced via the dismutation of superoxide ions, although a minor pathway involving the dimerisation of $HO^\bullet$ may also occur.[18] In order to assess the rate of H_2O_2 production and the possibility that this product could itself play a role in formation of the autoxidation products, H_2O_2 generation was monitored.

Autoxidation of 3-OHKyn was accompanied by rapid formation of H_2O_2, producing almost a mole per mole equivalent of H_2O_2 throughout the time course experiments (Figure 5). The initial rate of H_2O_2 production (0–1 h) mirrored the consumption of 3-OHKyn and followed the formation of Xan (Figure 5 compared to Figure 3). Considering the temporal relationship between the formation of H_2O_2 and the autoxidation products derived from Xan, we suspected that the production of H_2O_2, and possibly $HO^\bullet$, may play a role in the formation of the Xan-derived autoxidation products. In order to address this possibility, we conducted the autoxidation experiments in the presence of catalase (to remove any H_2O_2 formed) or mannitol (a $HO^\bullet$ scavenger). The free radical trap, DMPO, was also used (see EPR experiments below).

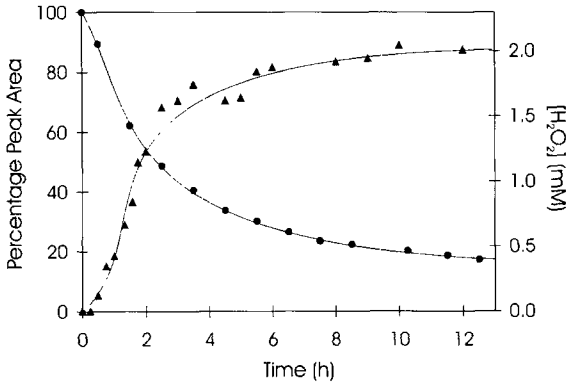


FIGURE 5 H_2O_2 concentration during the autoxidation of 3-OHKyn at pH 7 and 35°C. 3-OHKyn (2.2 mM) was incubated at 35°C in 50 mM phosphate buffer (pH 7) and in the presence of oxygen. Aliquots were taken at the times indicated and analysed for 3-OHKyn by HPLC and for H_2O_2 using the HRP/OPD assay as described in the Materials and Methods section. (●, 3-OHKyn; ▲, H_2O_2).

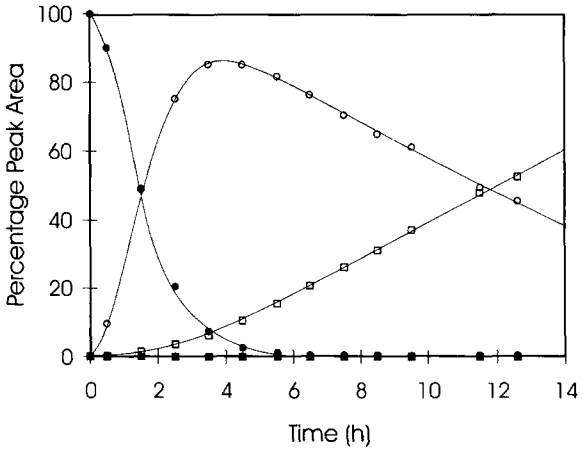


FIGURE 6 Autoxidation of 3-OHKyn at pH 7 and 35°C in the presence of catalase. 3-OHKyn (2.2 mM) was incubated at 35°C in 50 mM phosphate buffer (pH 7) and in the presence of oxygen. Catalase (6300 units) was added at hourly intervals. Aliquots taken at the times indicated were analysed by HPLC as described in the Materials and Methods section. (●, 3-OHKyn; ○, Xan; ■, OHXan; □, DHQCA). Abbreviations are described in the legend to Figure 1. The symbols for F 4.3 are obscured by the symbols for OHXan. Data are representative of two identical experiments.

Effect of Hydrogen Peroxide on 3-Hydroxykynurenine Autoxidation Products

Previous studies on the autoxidation of 3-hydroxyanthralinic acid, a structural analogue of 3-OHKyn, revealed that H_2O_2 plays an important role in the generation of specific autoxidation products.[19] In particular, cinnabarinic acid, a phenoxazone dimer of 3-hydroxyanthranilinic acid and structural analogue of Xan, was degraded by H_2O_2.[19] As noted above, we suspected that a similar reaction may also occur with Xan. This hypothesis was tested by performing the autoxidation of 3-OHKyn at pH 7 in the presence of catalase to remove H_2O_2 (produced during the course of autoxidation).

Figure 6 shows the relative peak areas of the major products of autoxidation in the presence of catalase. The addition of catalase to the reaction mixture led to a significant increase in the concentrations of Xan and DHQCA as well as suppression of OHXan and the compound denoted F 4.3 (Figure 5 compared with Figure 3). The initial rate (0–1 h) of 3-OHKyn autoxidation was not affected by the presence of catalase, however beyond this time, 3-OHKyn loss was accelerated and was not detected beyond 7 h (Figure 6). It appeared, therefore, that H_2O_2 had a marked effect on the relative concentrations of 3-OHKyn autoxidation products. The inferred role of H_2O_2 in the decomposition of Xan was confirmed by its addition to a solution of Xan which resulted in the formation of F 4.3 (data not shown). Similarly, addition of H_2O_2 to a solution of DHQCA resulted in the formation of F 4.3, again suggesting a role for H_2O_2 in the formation of F 4.3 (data not shown). The levels of F 20 detected were not significantly altered in the presence of catalase.

We also considered the possibility that the effect of catalase on the formation of the 3-OHKyn autoxidation products was not a direct result of H_2O_2 removal but rather a consequence of the inhibition of $HO^\bullet$ generation; the formation of which is known to occur in phosphate buffers (that contain trace amounts of transition metals) via the Fenton reaction.[20] However, in the presence of the $HO^\bullet$ scavenger, mannitol, no major effect on the course of the

autoxidation was observed. These results suggest a minor role for $HO^{\bullet}$ in the overall course of autoxidation under these conditions.

The initial oxyradical produced upon 3-OHKyn autoxidation is likely to be superoxide ($O_2^{\bullet -}$). We assessed a possible direct effect of $O_2^{\bullet -}$ on 3-OHKyn autoxidation rate by conducting the experiments in the presence of SOD that catalyses the dismutation of $O_2^{\bullet -}$ to H_2O_2. In the presence of SOD, 3-OHKyn autoxidation was not significantly affected (Figure 7). However, SOD did supress the levels of DHQCA detected by *ca.* 40% and increase the level of the compound denoted as F 4.3 by *ca.* 20% as assessed at the 5 h mark (data not shown). Superoxide therefore does not appear to play any part in the initial autoxidation of 3-OHKyn but may be involved in redox reactions of subsequent autoxidation products.

3-Hydroxykynurenine Autoxidation Assessed by EPR Spectroscopy

To investigate the possible formation of radical species during the initial stages of 3-OHKyn autoxidation, the reaction mixtures were analysed using EPR spectroscopy. Two major radical species were observed when the 3-OHKyn autoxidation reaction was allowed to proceed for 40 min at room temperature (Figure 8A). The hyperfine structures, line shapes and *g*-values (2.0057 and 2.0038) indicated are consistent with the presence of the DMPO–$HO^{\bullet}$ adduct and $Xan^{\bullet}$ respectively.[21–23] A novel HPLC-MS-EPR technique has been used to show that $Xan^{\bullet}$ is a major

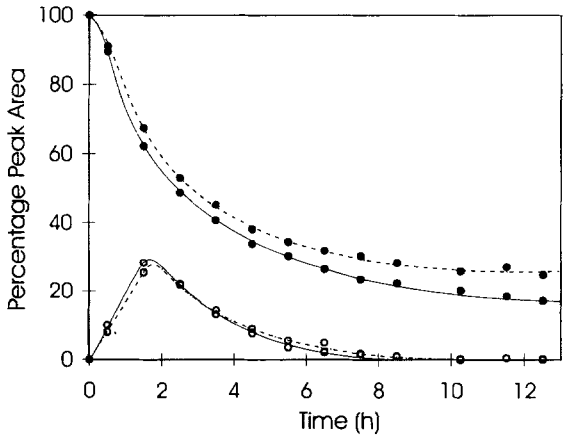


FIGURE 7 Autoxidation of 3-OHKyn at pH 7 and 35°C in the presence of SOD. 3-OHKyn (2.2 mM) was incubated at 35°C in 50 mM phosphate buffer (pH 7) and in the presence of oxygen (solid lines). SOD (1000 units per addition) was added at hourly intervals (broken lines). Aliquots taken at the times indicated were analysed by HPLC as described in the Materials and Methods section. (●, 3-OHKyn; ○, Xan). Abbreviations are described in the legend to Figure 1.

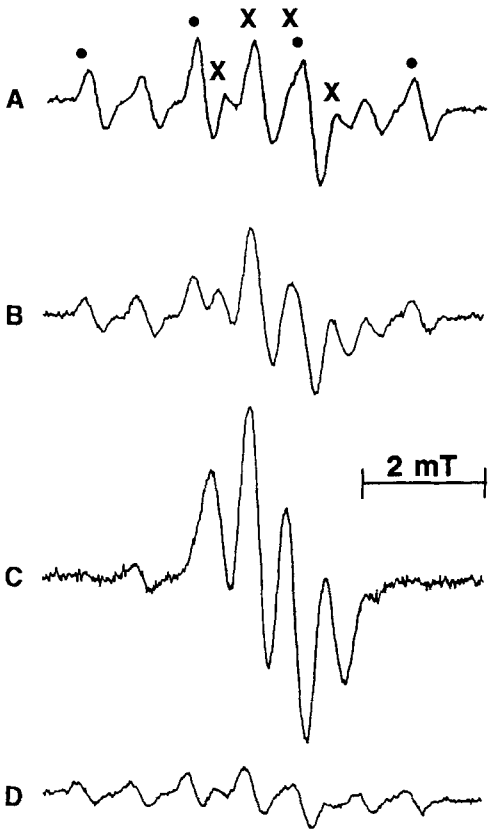


FIGURE 8 EPR spectrum of 3-OHKyn autoxidation products. 3-OHkyn (500 μM) was incubated in 50 mM phosphate buffer (pH 7.4) for 40 min at 22°C and in the presence of 0.1 M DMPO (A). Two prominent radical species were identified as DMPO–OH (●) and $Xan^{\bullet}$ (X). (*g*-values of 2.0057 and 2.0038 respectively). The unlabelled signals present in "A" were due to DMPO degradation-products. "B" to "D" are spectra collected after incubation of 500 μM 3-OHKyn for 60 min at 37°C with DMPO added approximately 2 min before scanning. Catalase (600 units/ml) or nitrogen was present throughout the incubations in "C" and "D" respectively.

3-OHKyn autoxidation product.[23] The g-values and hyperfine splittings for the phenoxyl radical detected in the present work appear to be identical to those previously determined for Xan$^\bullet$ (at $g = 2.004$), indicating they are the same species.[22,23] The two unlabelled signals present in Figure 8A are due to DMPO degradation-products and were therefore also detected in control incubations which contained no 3-OHKyn. We considered the possibility that the formation of Xan$^\bullet$ may be influenced by the H_2O_2 which is produced during the 3-OHKyn autoxidation reaction, or that HO$^\bullet$ could react with Xan to generate Xan$^\bullet$. To test these possibilities, the 3-OHKyn autoxidation was conducted in the presence of catalase or under nitrogen. For these experiments, DMPO was added 1 h after incubating the mixtures at 37°C and subsequently analysed (within 2 min) using EPR spectroscopy. In this case, two major radical signals were generated which were assigned as the DMPO–HO$^\bullet$ adduct and Xan$^\bullet$ (Figure 8B). This result was very similar to that shown in Figure 8A, the major difference being the relatively lower level of DMPO–HO$^\bullet$ detected. This may be a consequence of the long half life of the DMPO–HO$^\bullet$ adduct and the additional time provided for this adduct to form in the conditions used in Figure 8A compared to B. In the presence of catalase, no HO$^\bullet$ was produced while the levels of Xan$^\bullet$ were increased 2.4-fold (Figure 8C compared to 8B). This result indicates that neither H_2O_2 or HO$^\bullet$ are required for Xan$^\bullet$ production and also that H_2O_2 may contribute to the degradation of Xan$^\bullet$, perhaps to form other products (data shown previously indicates that H_2O_2 plays a role in Xan degradation). Under conditions where oxygen availability was limited by continuous bubbling with nitrogen, both the DMPO–HO$^\bullet$ and Xan$^\bullet$ signals were suppressed to 35% and 37% respectively (Figure 8D compared to 8B).

In order to assess whether H_2O_2 could directly contribute to Xan$^\bullet$ degradation, H_2O_2 was added to the autoxidation solution prior to analysis by EPR spectroscopy. The data shown in Figure 9A indicate the presence of the Xan$^\bullet$ formed after 3-OHKyn was incubated at 37°C for 100 min. When H_2O_2 was added to the reaction mixture 10 min prior to analysis by EPR spectroscopy, the Xan$^\bullet$ signal was abolished and a much less intense broad singlet was observed (Figure 9B). This implies that H_2O_2 plays a direct role in the degradation of Xan$^\bullet$.

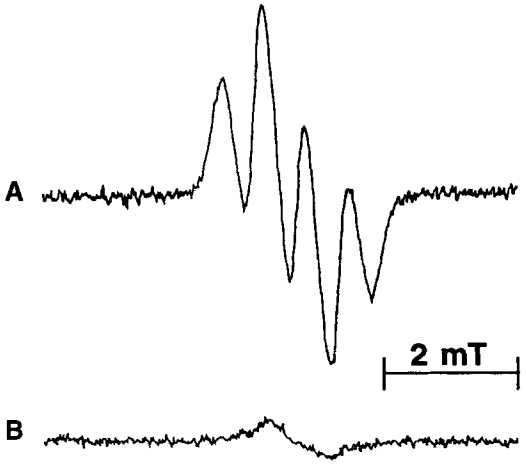


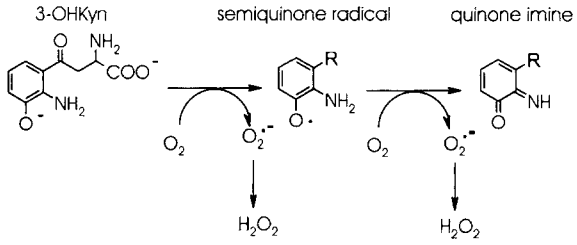
FIGURE 9 EPR spectrum of Xan$^\bullet$ in the presence or absence of H_2O_2. 3-OHkyn (500 μM) was incubated in 50 mM phosphate buffer (pH 7.4) for 100 min at 37°C (A). A parallel incubation was made up to 20 mM H_2O_2 10 min before EPR analysis (B).

DISCUSSION

The toxicity of 3-OHKyn has been largely attributed to its autoxidation with the ensuing generation of H_2O_2 and HO$^\bullet$.[3,17,24] Although not addressed in the present report, the mechanism of 3-OHKyn autoxidation is probably not a result of its direct reaction with dioxygen as this is predicted to be kinetically unfavourable due to spin restrictions.[20] It is known, however, that in the presence of transition metals (e.g. Fe and Cu) this restriction can be circumvented thus permitting the apparent "autoxidation" of a variety of biomolecules.[20] Paradoxically, the addition of chelators such as desferrioxamine and EDTA did

not abolish 3-OHKyn autoxidation in the phosphate buffers used in our time-course studies (data not shown). Replacement of phosphate buffer with triethylammonium acetate buffer resulted in the formation of all of the expected species with only a slight lowering in their rates of formation (data not shown). In addition, the buffers used for the EPR experiments were treated with Chelex resin in order to minimise metal contamination,[14] yet Xan$^{\bullet}$ and HO$^{\bullet}$ were still detected (Figure 8). These data infer that metals are not involved in 3-OHKyn autoxidation, although, it is possible that traces of metal persist in the reaction mixtures and contribute to the autoxidation observed. It may be that traces of metal are introduced with the 3-OHKyn itself (possibly as a chelate) or that traces are present in the reaction cells.

The generation of H_2O_2 in our experimental system is most likely due to the initial reduction of oxygen by the phenoxide anion of 3-OHKyn to generate $O_2^{\bullet-}$ and its subsequent dismutation to yield (theoretically) 1 mol of H_2O_2 per mole of 3-OHKyn (Scheme 1). The pK_a of this phenoxyl is reported to be 9.6.[25] It therefore appears that the small fraction of 3-OHKyn that is in the phenoxide state at pH 7 is sufficient to allow autoxidation to progress. It is also possible that other



SCHEME 1 Mechanism of H_2O_2 generation via 3-OHKyn autoxidation. It is proposed that 3-OHKyn is oxidised via two one-electron losses to yield superoxide ($O_2^{\bullet-}$) which may form H_2O_2 after dismutation. The semiquinone and/or quinone immine then undergo condensation reactions to form Xan (not shown). At higher pH, the increased formation of a phenoxide at C3 of the kynurenine ring could facilitate oxygen reduction. Note also that trace amounts of transition metals (e.g. Fe or Cu) may contribute to 3-OHKyn autoxidation (see text for further details). R, the amino acid side chain of 3-OHKyn.

one-electron transfers contribute to oxygen reduction during the autoxidation of 3-OHKyn-derived products and that a proportion of the H_2O_2 (formed subsequently) reacts with at least some of the autoxidation intermediates (e.g. Xan). This suggests that the *ca.* 1 : 1 stoichiometery of 3-OHKyn loss and H_2O_2 production reflects the net effect of these reactions. Investigation of the physiologically relevant mechanisms of 3-OHKyn autoxidation clearly warrant further study.

The primary objective of the present study was to investigate the course of 3-OHKyn autoxidation and to gain an understanding of the structures of the autoxidation products, some of which could potentially form adducts with proteins.[11,12] It is unclear to what extent the cytotoxicity of 3-OHKyn can be ascribed to the formation of H_2O_2 (and HO$^{\bullet}$) compared with the deleterious reactions of 3-OHKyn oxidation products with cellular proteins (and possibly other macromolecules). Elucidation of reactive 3-OHKyn autoxidation products is required in order to generate characteristic markers for modified proteins which will be useful for probing tissue samples, e.g. in the neurological disorders mentioned above and in cataract.

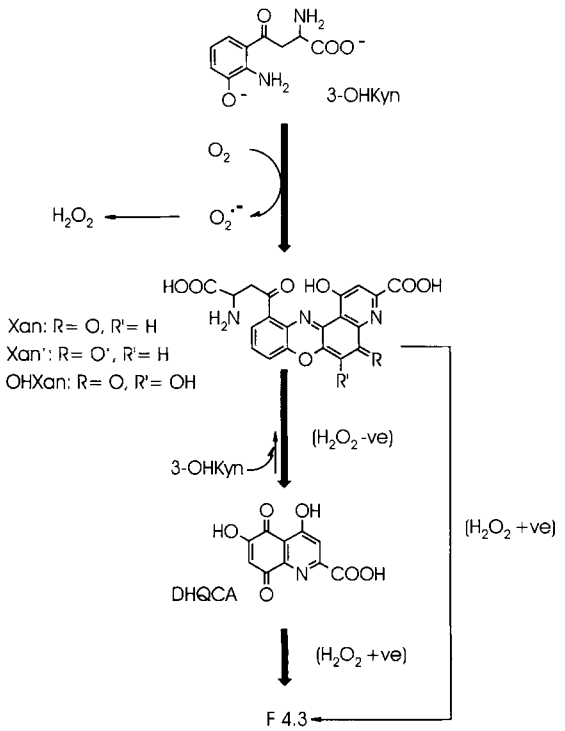
The oxidation of *o*-aminophenols by air, cytochrome c, or ferricyanide is accompanied by dimerisation with the formation of phenoxazones[26] which absorb light in the visible regions.[27] In the present study, when exposed to either oxygen or air, 3-OHKyn was found to react in an analogous manner to yield Xan (and its radical, Xan$^{\bullet}$) and OHXan. In previous studies, 3-OHKyn oxidation was induced by the addition of catalysts such as potassium ferricyanide and methaemoglobin/H_2O_2.[28,29] Under these conditions, Xan was reported to be the sole oxidation product of 3-OHKyn. A recent article described in-line oxidation of 3-OHKyn coupled with HPLC, and detection with EPR spectroscopy or ESI-MS.[23] The sophisticated experimental procedure utilised in that work allowed immediate identification of the species produced. The data

indicated that Xan was the principal oxidation product of 3-OHKyn.[23] This is in agreement with our work which shows that, during the initial period of autoxidation, Xan (and Xan$^{\bullet}$) is the predominant product formed and this is accompanied by H_2O_2 production. From our investigations, however, Xan is only the first of at least five oxidation products.

Prolonged autoxidation of 3-OHKyn leads to the formation of two major species, DHQCA and F 4.3. DHQCA has not previously been reported as an autoxidation product of 3-OHKyn, although it has been described as a decomposition product of Xan.[30] Butenandt showed that incubation of Xan at pH 8 produced 3-OHKyn and DHQCA, though the quantities produced were not stated.[30] The identification of DHQCA as an autoxidation product of 3-OHKyn is of interest since our laboratory has recently found that DHQCA is able to react with proteins (unpublished results). This may be of particular relevance to lenticular protein modification in senile nuclear cataract since the lens nucleus is metabolically inactive and proteins are therefore not turned over. In effect, the nuclear lens crystallins are as old as the individual and are thus more likely to be modified by 3-OHKyn autoxidation products. Due to the difficulty of isolating sufficiently pure quantities of the compound denoted F 4.3, its structure remains to be determined.

Through the use of stability, decomposition and oxidation studies of purified compounds, we were able to elucidate possible product–precursor relationships (Scheme 2). From this data, it is clear that the autoxidation of 3-OHKyn generates Xan, Xan$^{\bullet}$ and probably OHXan. We could find no evidence that Xan yields OHXan directly (e.g. by incubation with oxygen or H_2O_2). Catalase addition did, however, dramatically suppress OHXan formation. A possible role for H_2O_2 in OHXan formation can therefore not be ruled out.

The physiological significance of the role of H_2O_2 in the generation of 3-OHKyn autoxidation products will depend also on the local environment, in particular the presence of enzymes such



SCHEME 2 Major 3-OHKyn autoxidation products. It is proposed that 3-OHKyn is initially autoxidised to generate Xan and the closely related Xan$^{\bullet}$ and OHXan. After further degradation/autoxidation DHQCA and the compound attributed to the peak(s) denoted as F 4.3 are generated. H_2O_2, which is produced as a byproduct (see Scheme 1), can either stimulate (+ve) or inhibit (−ve) the pathway as indicated parenthetically.

as GSH peroxidase and catalase. In order to approach extralenticular physiological conditions, we also conducted the autoxidation reactions in the presence of isolated erythrocytes, at a physiological concentration, in phosphate buffered saline (pH 7.4). The HPLC profile of this reaction mixture was almost identical to that of 3-OHKyn autoxidation in the presence of catalase (data not shown). Since there was also some evidence of erythrocyte lysis after several hours incubation, it is likely that antioxidant enzymes (catalase, SOD and GSH peroxidase) normally present within the erythrocytes[31] were released into the reaction mixture.

The reactions we have studied *in vitro* might also be relevant to specific pathological situations

where Trp metabolism via the kynurenine pathway is associated with oxyradical production. For example, human monocytes and macrophages which are primed with interferon-γ secrete small amounts of 3-OHKyn and have a high capacity to produce both $O_2^{\bullet -}$ and H_2O_2.[32] In addition, the low pH of inflammatory sites[33] would be predicted to suppress the autoxidation of 3-OHKyn and the products we have detected in the present report. However, in the case of the nucleus of the lens (an avascular tissue), such inflammation is not possible.

In conclusion, the autoxidation of 3-OHKyn is complex, resulting in the formation of three major and several minor products. Two of the major species have been identified as Xan and DHQCA and the third appears to be OHXan. Xan (and Xan$^{\bullet}$) are the major products formed initially, while DHQCA and OHXan appear to be quantitatively important 3-OHKyn autoxidation products at later stages. These data show that high concentrations of H_2O_2 are generated throughout the reaction and indicate that H_2O_2, but not $O_2^{\bullet -}$ or HO$^{\bullet}$, plays an important role in determining the autoxidation pathway. We hypothesise that DHQCA and possibly Xan$^{\bullet}$ may play a role in the modification of proteins by 3-OHKyn and this could contribute to its cytotoxicity.

Acknowledgements

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