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# IDENTIFICATION OF PRODUCTS FROM OXIDATION OF URIC ACID INDUCED BY HYDROXYL RADICALS

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The aim of the present study was to separate and characterise products formed by oxidation of uric acid by hydroxyl radicals with a view to probing for these products *in vivo* in clinical contexts. Aerated solutions of 200 $\mu$M uric acid, or its oxidation products, allantoin or parabanic acid, were exposed to gamma radiolysis, (52.0 Gy/min), as a source of HO· radicals, at pH 3.4 and 7.4. Aliquots were taken every 5 minutes for 20 minutes and oxidation products were separated by HPLC and analysed with a diode array detector. Identities of oxidation products were confirmed on the basis of similarity of retention times and absorbance spectra and peak purity parameters of known standards. Hydroperoxides were measured by tri-iodide formation in the 20 minute sample. Exposure of uric acid to such HO· fluxes produced a net loss of the parent compound with formation of a complex mixture of products with allantoin and parabanic acid being the predominant products at pH 3.4. The rate of uric acid degradation at physiological pH was slower and the distribution of oxidation products was different. A small but significant amount of uric acid hydroperoxide was detected at both pHs. A mechanism for uric acid oxidation under these conditions is presented.

INDEXING TERMS: Uric acid; allantoin, free radical; hydroxyl radical, high performance liquid chromatography.

## INTRODUCTION

Biochemical changes induced by free radicals have been implicated in a number of disease processes[1]. However, because of technical difficulties involved in radical detection *in vivo*, demonstration of temporal relationships between free radical production and the onset of pathophysiological damage remains elusive. Fresh strategies for measurement of free radical production are needed. Natural antioxidants provide the first line of defence against free radical damage, so oxidative changes to these compounds should reflect early increases in free radical fluxes before other biochemical or pathological damage is revealed[2].

Uric acid has been identified as one of the major antioxidants present in plasmas[3], and it has been postulated that quantitation of stable products resulting from uric acid oxidation may be a convenient way to determine an integrated measure of free radical flux[4]. There are no specific enzymes present in man which will oxidise uric acid, so the presence of uric acid-based oxidation products such as allantoin would imply that the parent molecule had been oxidised by processes

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outside metabolic control (ie free radicals or similar oxidising species). Halliwell and Gutteridge demonstrated increased allantoin: uric acid ratios in serum and synovial fluid of rheumatoid arthritis patients[4]. However, the analytical methods employed were nonspecific and not particularly sensitive. Studies employing electrochemical and biologically relevant oxidants suggest that there are a number of products formed during uric acid oxidation[5,6]. Thus, determination of allantoin alone may underestimate free radical reactions. The aim of this study was to identify the major products derived from uric acid and its major oxidation products, allantoin and parabanic acid when they were exposed to hydroxyl radicals, (HO·), produced from gamma irradiation of water.



## MATERIALS AND METHODS

### *Materials*

Uric acid, allantoin and parabanic acid were obtained from Sigma Chemical Co., (St Louis, Mo), whilst oxonic acid (potassium salt) and cyanuric acid were obtained from the Aldrich Chemical Co., (Milwaukee, Wis). Potassium iodide (AnalaR) came from BDH Chemicals (Australia) and methanol (HPLC grade) from Malinckrodt Pty. Ltd. (Aust.). Catalase was purchased from Boehringer Mannheim (Aust.). All other chemicals and solvents used were Analytical Reagent grade, purchased from Ajax Chemicals (Aust.). Oxaluric acid was prepared by alkaline hydrolysis of aqueous parabanic acid[7]. Water used in solution preparation was purified by passage through a Milli-Q water purification system (Millipore, Aust.). Glassware used in radiolysis was soaked for 12 hours in fresh concentrated AR grade nitric acid then rinsed 12 times in Milli-Q purified water then dried under dust-free conditions.

### *Methods*

i) *Radiolysis* Irradiations were performed on air saturated solutions in a 2,500 Ci $^{60}Co$ gamma source at 25 °C. Dose rates were measured by Fricke dosimetry[8] with the G-value for the formation of $Fe^{3+}$ taken as 15.5 ions/100 eV[9]. For conversion to SI units, ($\mu$mol. $J^{-1}$), G-value is multiplied by 0.1036. The dose rates varied from 50–57 Gy/min.

ii) *Chromatography* HPLC analysis was performed on a Varian LC Star System equipped with a diode array detector and Varian Star Workstation (Varian Chromatography Systems, Ca). Products were separated on C-18 reverse phase columns, (dimensions 250 × 4.6 mm), with particle size of 5 $\mu$m (either Ultropac TSK ODS-120T, from Pharmacia LKB Biotechnology, Sweden or Goldpak, Exsil 100, ODS, from Activon Scientific Products, Aust.). A volume of 50 $\mu$l of reaction mixture was applied to the column with a syringe loading injector, (Rheodyne, Ca). Mobile phases were as follows: (i) for pH 3.4 irradiations, (optimal pH for separation of products), 5mM $NH_4H_2PO_4/H_3PO_4$, pH 3.4; (ii) for pH 7.4 irradiations, 10mM $KH_2PO_4/H_3PO_4$, pH 3.4. The flow rate for both PH's was 1 ml/min and temperature was 25 °C. All chromatograms were run at 220 nm.

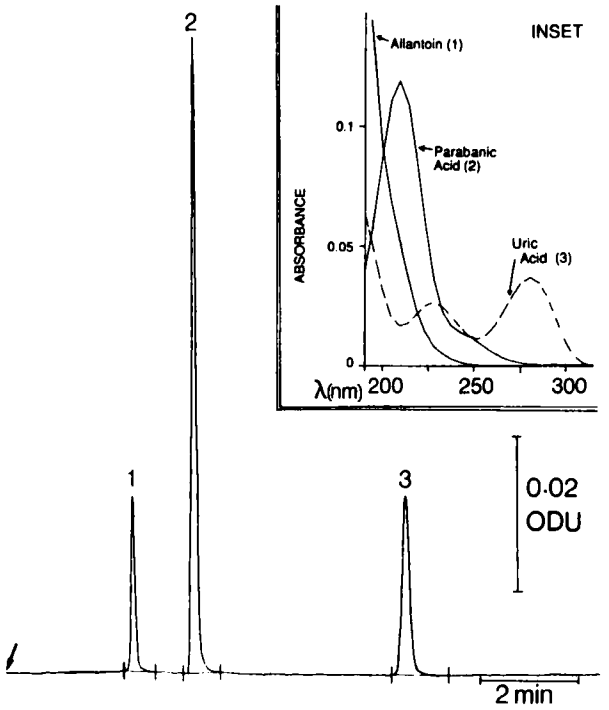


FIGURE 1 Chromatogram of standards run on the Activon column. Injection made at t = 0 min at the arrow. Mobile phase: 5mM $NH_4H_2$ $PO_4/H_3PO_4$, pH 3.4. Standards were dissolved in mobile phase. See Materials and Methods for other conditions. Peak 1: 72$\mu$M allantoin; Peak 2: 33$\mu$M parabanic acid; 18$\mu$M uric acid. INSET: UV spectra drawn by the diode array detector taken at the $t_{(R)}$ for each peak.

iii) *Hydroperoxide Analysis* Hydroperoxides were measured using an iodometric technique based on that of Hicks and Gebicki[10], before and after removal of radiolytically produced $H_2O_2$ by addition of catalase. A volume of 200$\mu$l of irradiated 200$\mu$M uric acid or controls (irradiated or unirradiated buffer) was added to 1.8 ml of a deoxygenated solution containing 6% (w/v) potassium iodide in 2:1 (v/v) methanol : acetic acid. Tri-iodide formation was measured as 360nm absorbance after the assay mixture was incubated for 20 minutes at room temperature. Hydroperoxide concentrations were calculated using $\varepsilon_{(360)}$ for $I_3^-$ of 28,000 litre.mole $^{-1}$. cm$^{-1}$ [10].

## RESULTS

### *Nonhydroperoxide products of uric acid oxidation*

i) *Chromatograph of Standards* Figure 1 shows a chromatogram of the parent uric acid (peak 3) and 2 major oxidation products, allantoin and parabanic acid (peaks 1 and 2 respectively). The Activon column, (Exsil ODS), gave reproducible retention times (2.63, 3.91 and 8.04 minutes for allantoin, parabanic acid and uric

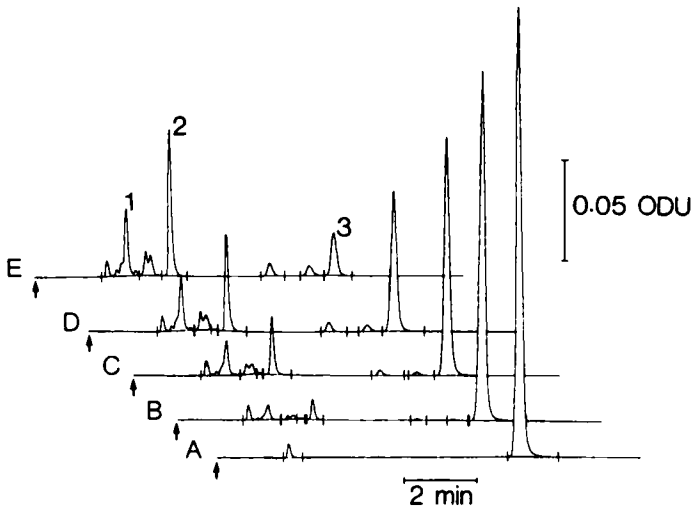


FIGURE 2a Chromatograms of 200μM uric acid irradiated in 5mM $NH_4H_2$, $PO_4/H_3PO_4$, pH 3.4. Dose rate, 52 Gy/min. Chromatograms A, B, C, D and E were samples taken at 0, 5, 10, 15 and 20 minutes radiolysis. Mobile phase, 5mM $NH_4H_2$ $PO_4/H_3PO_4$, pH 3.4. Column type, Activon. A volume of 50μl of reaction mixture was injected at t = 0 min at the arrows. See methods for further conditions. Peak 1, allantoin; peak 2, parabanic acid; peak 3, uric acid.

acid respectively). The Pharmacia column (TSK ODS) gave slightly longer retention times, typically 3.3, 5.0 and 10.8 min for allantoin, parabanic acid and uric acid (data not shown). The diode array detector allowed an absorbance spectrum of each peak to be drawn (Figure 1, INSET). Uric acid oxidation products were identified by computerised matching from standards stored in the library of the instrument on the basis of comparison of retention time, peak purity parameter and spectral similarity.

ii) *Radiolysis at pH 3.4* Initial uric acid oxidations were performed at pH 3.4 in a buffer identical to that of the mobile phase. Solutions of 200 μM uric acid prepared in 5mM $NH_4H_2PO_4/H_3PO_4$, pH 3.4, were exposed to gamma radiolysis (dose rate 52 Gy/min) for up to 20 minutes. The pH of the reaction mixture remained constant over this period. Chromatograms of the product distribution in the reaction mixture from a representative experiment are shown in Figure 2a. No allantoin or parabanic acid formation was observed in unirradiated controls over the course of the experiment or after extended periods of storage of uric acid stock solutions at low temperature. In the 5–20 minute treatments a mixture of 9 products as well as the initial uric acid were seen with allantoin and parabanic acid predominating (Figure 2a). Trace amounts of allantoin and parabanic acid could be detected after as little as 30 seconds irradiation, however, after an extended period of irradiation, (2 hours), parabanic acid was the only compound visible on the chromatogram (data not shown).

Standard curves of peak area at 220 nm versus product concentration were used to calculate the concentrations of uric acid, allantoin and parabanic acid, over the time course of the experiment. Kinetics of loss of uric acid and production of allantoin and parabanic acid are shown in Figure 2b. If a mass balance of these

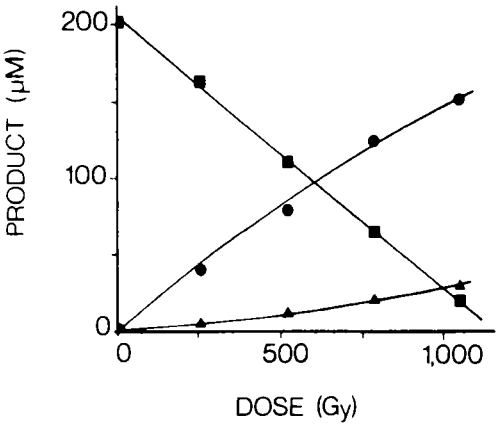


FIGURE 2b Kinetics of disappearance of uric acid and formation of allantoin and parabanic acid from experiment shown in Figure 2a. (■), uric acid; (●) allantoin; (▲), parabanic acid.

3 products is calculated at each time point concentrations of 202, 210, 200, 207 and 200 μM are obtained for 0–20 minute samples (Mean = 203.8 ± 4.5 (S.D.)). Thus, the major products of uric acid oxidation are allantoin and parabanic acid, with the other peaks which appear in Figure 2a only accounting for trace amounts of products. From this pattern of product formation, there are 2 likely routes for uric acid oxidation. (1) Oxidation of uric acid to allantoin follow by a further oxidative attack on allantoin with the production of parabanic acid or (2) parallel conversion of uric acid to allantoin (rapid) and parabanic acid (slow). In order to distinguish between routes (1) and (2), solutions of allantoin and parabanic acid were irradiated under the conditions employed for uric acid.

Figure 3a shows chromatograms of a solution of 240 μM allantoin in 5mM $NH_4H_2PO_4H_3PO_4$, pH 3.4 irradiated with Co gamma rays, (dose rate 52 Gy/min), over a 20 minute period. Parabanic acid, (peak 2), was the major product of allantoin oxidation. No change in peak area of allantoin and no additional peaks were observed in unirradiated controls, nor were there any post-irradiation changes in peak areas (data not shown). The kinetics of allantoin loss mirrored the formation of parabanic acid (Figure 3b) and plotting the decrease in allantoin concentration as a function of increase in parabanic acid concentration at each time point produced a linear relationship with a slope of $-1.05$ ($r^2 = 0.993$). The deviation from 1:1 stoichiometry for allantoin loss and parabanic acid formation may be explained by the formation of the product with a $t_{(R)}$ of around 9.7 minutes, (spectrally identical to the compound eluting immediately before uric acid in Figure 2a). The UV spectrum of this compound was very similar to the parabanic acid spectrum shown in Figure 1, (INSET), and may be indicative of parabanic acid radical dimerisation. The other minor products seen in the chromatogram E, Figure 2a, were not present after radiolysis of allantoin, further confirmation that they were derived from the parent uric acid.

Irradiation of 100 μM parabanic acid dissolved in 5mM $NH_4$ $H_2PO_4/H_3PO_4$, pH 3.4 (dose rate 52 Gy/min) resulted in no decrease in the concentration of this compound over a total dose identical to that used for radiolysis of uric acid or allantoin (data not shown).

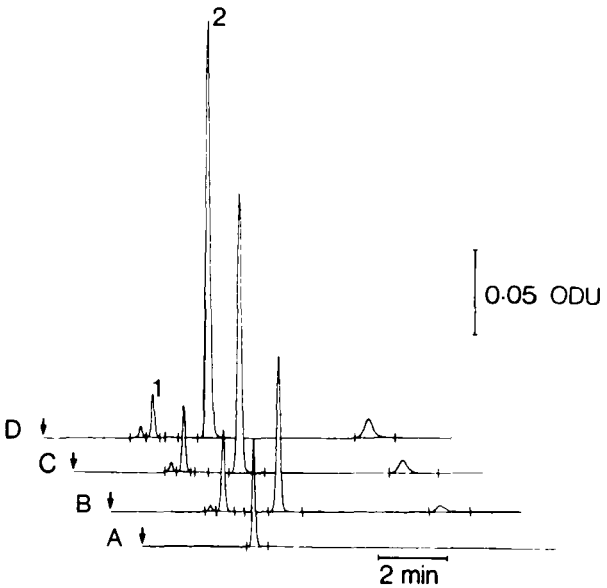


FIGURE 3a Chromatograms of 240μM allantoin irradiated in 5mM $NH_4H_2PO_4/H_3PO_4$, pH 3.4. Dose rate, 52 Gy/min. Chromatograms A, B, C, and D were samples taken at 0, 5, 10 and 20 minutes radiolysis. Mobile phase, 5mM $NH_4H_2PO_4/H_3PO_4$, pH 3.4. Column type. Pharmacia. A volume of 50μl of reaction mixture was injected at t = 0 min at the arrow. See Methods for further conditions. Peak 1, allantoin; peak 2, parabanic acid.

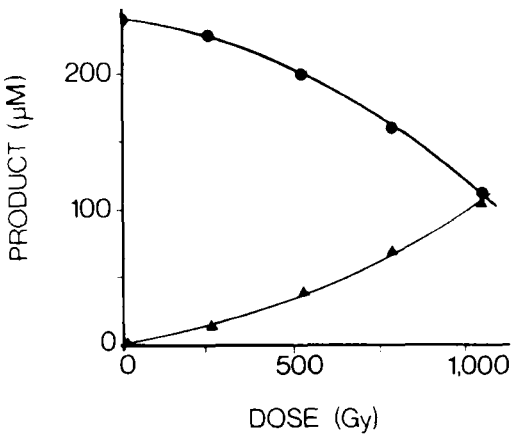


FIGURE 3b Kinetics of disappearance of allantoin and formation of parabanic acid from experiment shown in Figure 3a. (●), allantoin; (▲), parabanic acid.

iii) *Radiolysis at pH 7.4* Freshly prepared uric acid, (200 μM), in 1mM $K_2HPO_4/H_3PO_4$, pH 7.4, was exposed to gamma radiolysis (52 Gy/min) for up to 20 minutes. Figure 4a shows a typical chromatogram from the t = 20 minute irradiation. The pH remained constant over the period of radiolysis. No uric acid

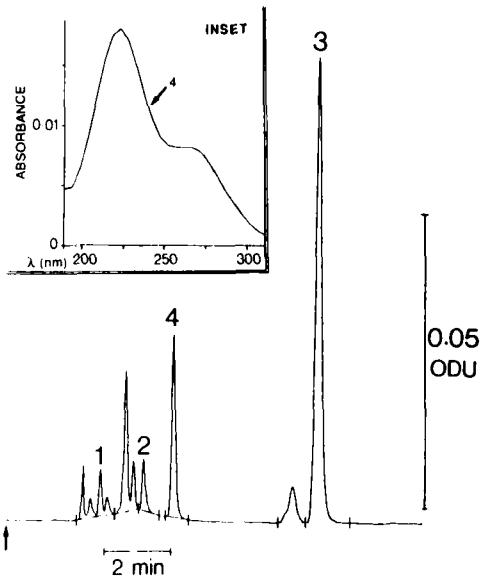


FIGURE 4a Chromatogram of 200μM uric acid irradiated in 1mM $K_2HPO_4/H_3PO_4$, pH 7.4. for 20 minutes. Dose rate, 52 Gy/min. Mobile phase, 10mM $KH_2PO_4/H_3PO_4$, pH 3.4. Column type, Activon. A volume of 50μl of reaction mixture was injected at t = 0 min at the arrows. See methods for further conditions. Peak 1, allantoin; peak 2, parabanic acid; peak 3, uric acid; peak 4, unidentified peak. INSET: UV spectra drawn by the diode array detector taken at the $t_{(R)}$ for peak 4 (4.5 min).

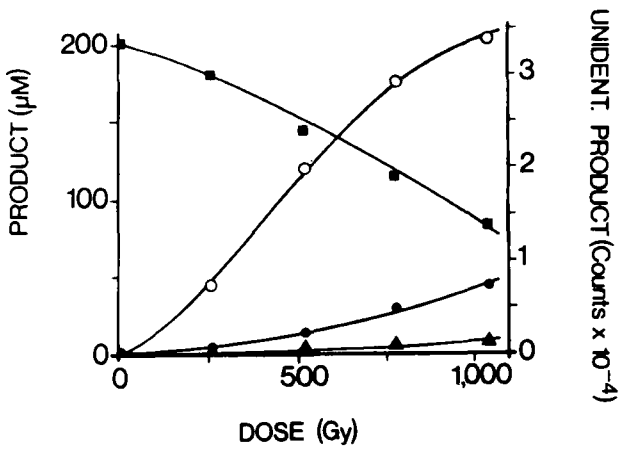


FIGURE 4b Kinetics of uric acid loss and oxidation product formation (experiment described in Figure 4a). (■), uric acid; (●), allantoin; (▲), parabanic acid; (○) peak area of unidentified product, $t_{(R)}$ = 4.5 min.

breakdown was observed in unirradiated control samples over the same time interval. There were qualitative and quantitative differences in product distribution when uric acid was irradiated at physiological pH as distinct from pH 3.4 (compare Figure 2a, chromatogram E). The major qualitative differences were the appearance

of 2 peaks, one at $t_{(R)} = 3.4$ minutes, (the second peak after allantoin in Figure 4a), and the other at 4.5 minutes (peak 4, Figure 4a). The extent of uric acid destruction and allantoin and parabanic acid formation was also lower at pH 7.4 (Figure 4b). These lower levels of allantoin may also be in part responsible for the lower steady state concentration of parabanic acid. At physiological pH, parabanic acid was also converted to the open chain, oxaluric acid, which elutes as a doublet immediately before allantoin with retention times of 2.2 and 2.4 minutes respectively (Figure 4a).

Freshly prepared solutions of allantoin and parabanic acid were irradiated at pH 7.4 under conditions identical to uric acid oxidation. Figure 5a shows a chromatogram of products formed after irradiation of 200$\mu$M allantoin for a period of 20 minutes. Again, no allantoin breakdown was observed in an unirradiated control preparation of allantoin after the same time period. The major products were parabanic acid and the product with a $t_{(R)}$ of around 4.5 minutes (spectrally identical to peak 4 in Figure 4a), along with a significant amount of oxaluric acid. The other peaks seen in Figure 4a (uric oxidation) were not observed during allantoin radiolysis (Figure 5a), thus they must have been minor products of uric acid oxidation. Kinetics of allantoin loss and parabanic acid formation are shown in Figure 5b. The extent of oxidation of allantoin was less at high pH than at the lower pH (52% of initial concentration remaining after 20 minutes irradiation at pH 3.4 compared to 70% remaining at pH 7.4). Also, the apparent stoichiometry of allantoin loss: parabanic acid formation dropped from about 1:1 at pH 3.4 to 3:1 at 7.4. This latter decrease may be accounted for by the formation of the compound with $t_{(R)} = 4.5$ minutes (Figure 5b).

When 100$\mu$M parabanic acid (in 1mM $K_2HPO_4$/ $H_3PO_4$, pH 7.4) was irradiated for 20 minutes, (dose rate, 52 Gy/min), the rate of conversion of the parent compound to oxaluric acid was not significantly different from that in an unirradiated control (data not shown). Control studies demonstrated that this was a hydrolytic process. Conversion of parabanic acid to oxaluric acid proceeded in a linear fashion over a 5 hour incubation at room temperature. The rate of conversion was unaltered whether the incubation was performed under argon or air, only 10% of the initial 100$\mu$M parabanic acid remaining after each treatment. The rate of oxaluric acid formation from parabanic acid decreased as a function of pH over the range 7.4-3.4 with no conversion observed over a similar 5 hour period at pH 3.4 (data not shown). The oxaluric acid formed during uric acid and allantoin irradiation at pH 7.4, (Figures 4a and 5a respectively), probably arose by the same radiation-independent process.

iii) *Characterisation of Unknown Peaks* The peaks with retention times of 3.4 minutes and 4.5 minutes, (Figures 4a and 5a), were further characterised. Both compounds were found to be highly reactive intermediates. Solutions of 200$\mu$M uric acid and allantoin, each irradiated for 20 minutes were used as the source of the 3.4 minute peak and 4.5 minute peak respectively. Each peak was individually collected and stored at $-17\,^{\circ}C$ with a view to analysis by mass spectrometry. When the collected peak with a $t_{(R)}$ of 3.4 minutes was rechromatographed immediately, a mixture of allantoin, parabanic acid, oxaluric acid and the original $t_{(R)} = 3.4$ minute peak was observed. When the 3.4 minute peak was collected and rechromatographed for a second time, a similar distribution of compounds was observed (data not shown). This apparent rapid decomposition of the peak prevented accumulation of an amount sufficient for mass spectrometry.

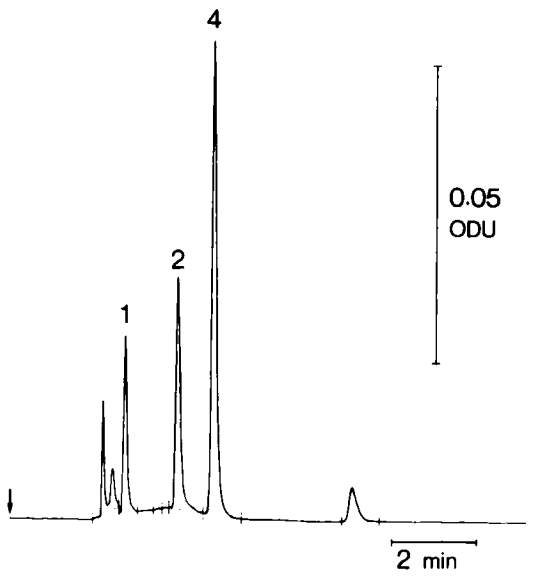


FIGURE 5a Chromatogram of 200μM allantoin irradiated in 1mM $K_2HPO_4/H_3PO_4$, pH 7.4 for 20 minutes. Dose rate, 52 Gy/min. Mobile phase, 10mM $KH_2PO_4/H_3PO_4$, pH 3.4. Column type, Activon. A volume of 50μl of reaction mixture was injected at t = 0 min at the arrows. See methods for further conditions. Peak 1, allantoin; peak 2, parabanic acid; peak 4, unidentified peak at $t_{(R)}$ = 4.5 minutes.

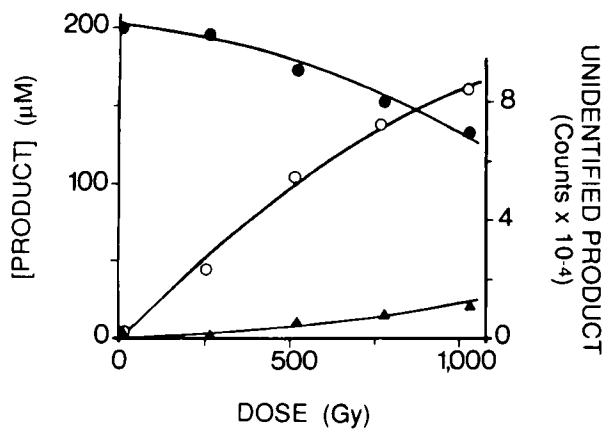


FIGURE 5b Kinetics of allantoin loss and product formation (experiment described in Figure 5a). (●) allantoin; (▲) parabanic acid; (○) peak area of unidentified product, $t_{(R)}$ = 4.5 min.

When the 4.5 minute peak was collected into a vessel at − 17°C, then rechromatographed immediately, a quantitative conversion of this peak to parabanic acid was observed. Thus neither of these compounds was stable enough to be collected for subsequent analysis by mass spectrometry, and HPLC/MS was not available to us.

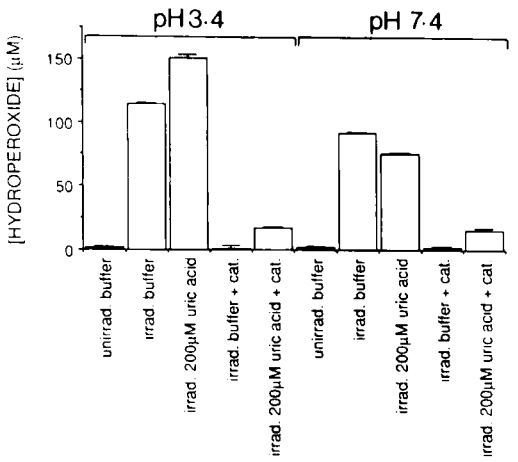


FIGURE 6 Hydroperoxide levels in irradiated uric acid. Dose rate, 52 Gy/minute; Irradiation time, 20 minutes. Uric acid solutions prepared in 5mM $NH_4H_2PO_4/H_3PO_4$, pH 3.4 or 1mM $K_2HPO_4/H_3PO_4$, pH 7.4. Catalase, (379 units/ml), where necessary was added 10 minutes before hydroperoxide assay.

## HYDROPEROXIDE PRODUCTS ASSOCIATED WITH URIC ACID OXIDATION

Oxygen centred radicals formed during oxidation of uric acid have been postulated to be involved in inactivation of enzymes such as alcohol dehydrogenase[11] and $\alpha_1$-antiproteinase[12]. The presence of uric acid hydroperoxides was implied, but neither study measured their formation directly. Figure 6 shows the hydroperoxide levels in unirradiated controls, irradiated controls and irradiated 200$\mu$M uric acid solutions in the presence and absence of catalase. There was a small but significant amount of hydroperoxide detected in the irradiated uric acid treatments after hydrogen peroxide produced during radiolysis was removed by addition of catalase. The extent of hydroperoxide production at pH 3.4 was not significantly different from that at pH 7.4 (17.5 ± 0.4 $\mu$M at pH 3.4 and 15.3 ± 1.3 $\mu$M at pH 7.4). Interestingly, the amount of tri-iodide reactive hydroperoxides, in the uric acid treatment irradiated at low pH without subsequent catalase treatment was significantly greater than that of the sum of the irradiated buffer alone and the catalase treated irradiated uric acid. This trend was reversed at physiological pH, where the presence of uric acid decreases the total amount of tri-iodide reactive material produced.

## DISCUSSION

The present study has examined uric acid oxidation at the pH optimum for chromatographic separation of the products, (3.4), and at physiological pH, (7.4). A reproducible flux of HO· radicals was generated by gamma radiolysis of water. Whilst this process produces a range of oxidising and reducing species[13], it is

chemically clean and thus offers advantages over other biochemical or chemical sources of free radicals employed in other studies of uric acid oxidation. A comparison of the reactivities of the radicals, produced under the present experimental conditions suggests that the hydroxyl radical, (HO·), was the species most likely to initiate uric acid oxidation. The rate constant for the uric acid/HO· reaction is diffusion controlled ($k = 7.2 \times 10^9$ litre.mole$^{-1}$.second$^{-1}$ at pH 6-7)[14]. Reaction between superoxide and uric acid is probably not significant. When uric acid was exposed to superoxide radicals from the xanthine/xanthine oxidase system, no oxidation products were observed until the addition of $Fe^{3+}$ to stimulate HO· formation[6]. Willson *et al* observed no loss of uric acid chromophore, (292 nm absorbance), in irradiated aqueous, aerated solutions containing exccess formate (where hydroxyl radicals were quantitatively converted to superoxide)[15].

When uric acid was exposed to gamma radiolysis at pH 3.4 and 7.4, allantoin and parabanic acid were the major products, (Figures 2b and 4b), with allantoin levels always being higher than parabanic acid levels over the time course of the experiment. These results suggest two possibilities. 1) a sequential formation of allantoin, then parabanic acid; or 2) a parallel formation of both compounds from uric acid with the rate of allantoin formation being faster than the rate of parabanic acid formation. Separate irradiation of allantoin, (Figures 3b and 5b), and parabanic acid indicate that the former process is that most likely to be operating.

Figure 7 presents a hypothetical overall mechanism for uric acid oxidation at pH 3.4 and 7.4 which is consistent with the data presented here. Hydroxyl radicals can react with organic molecules by hydrogen abstraction or addition. These processes would form uric acid radicals (i) and (ii) respectively (Figure 7). Our results favour the hydroxylation process with subsequent ring opening ie the right hand arm of Figure 7. Addition of HO· to the 4,5 double bond of the carbon skeleton has previously been considered the primary reaction in purines[16]. Allantoin was the first stable product formed, as suggested by Figures 2b and 4b, probably via a symmetrical intermediate postulated by previous workers[17].

The present results demonstrated that allantoin was also susceptible to hydroxyl radical attack. At pH 3.4, (Figure 3a), this reaction produced parabanic acid virtually exclusively, by expulsion of the urea moiety from the parent allantoin. No urea peak could be observed on any of the chromatograms as it had virtually no absorbance at 220 nm. The product distribution was more complex at pH 7.4 (Figure 5a). Reactive transients with retention times of 3.4 minutes and 4.5 minutes were observed. Further experiments showed that the yield of the 4.5 minute compound decreased with pH (data not shown). The UV spectrum of this compound, (Figure 4a, INSET), was different from the other common potential oxidation products of uric acid (oxonic acid, cyanuric acid or oxaluric acid) and co-chromatographed with none of these under our experimental conditions (data not shown). We also produced low yields of this product when allantoin oxidation was induced by permanganate (data not shown). A previous study by Brandenberger and Brandenberger[18], where uric acid was oxidised by permanganate, identified another 2,4-dihydroxy −1,3,5 - triazine compound, allantoxaidine, (a decarboxylated form of oxonic acid). The UV spectrum of allantoxaidine has some superficial similarity with the $t_{(R)} = 4.5$ minute product[18]. Unambiguous identification of these transients required HPLC/MS to which this laboratory does not have access. However given the aparently high reactivity of these compounds, it is unlikely that they will be stable enough to be detected *ex vivo*.

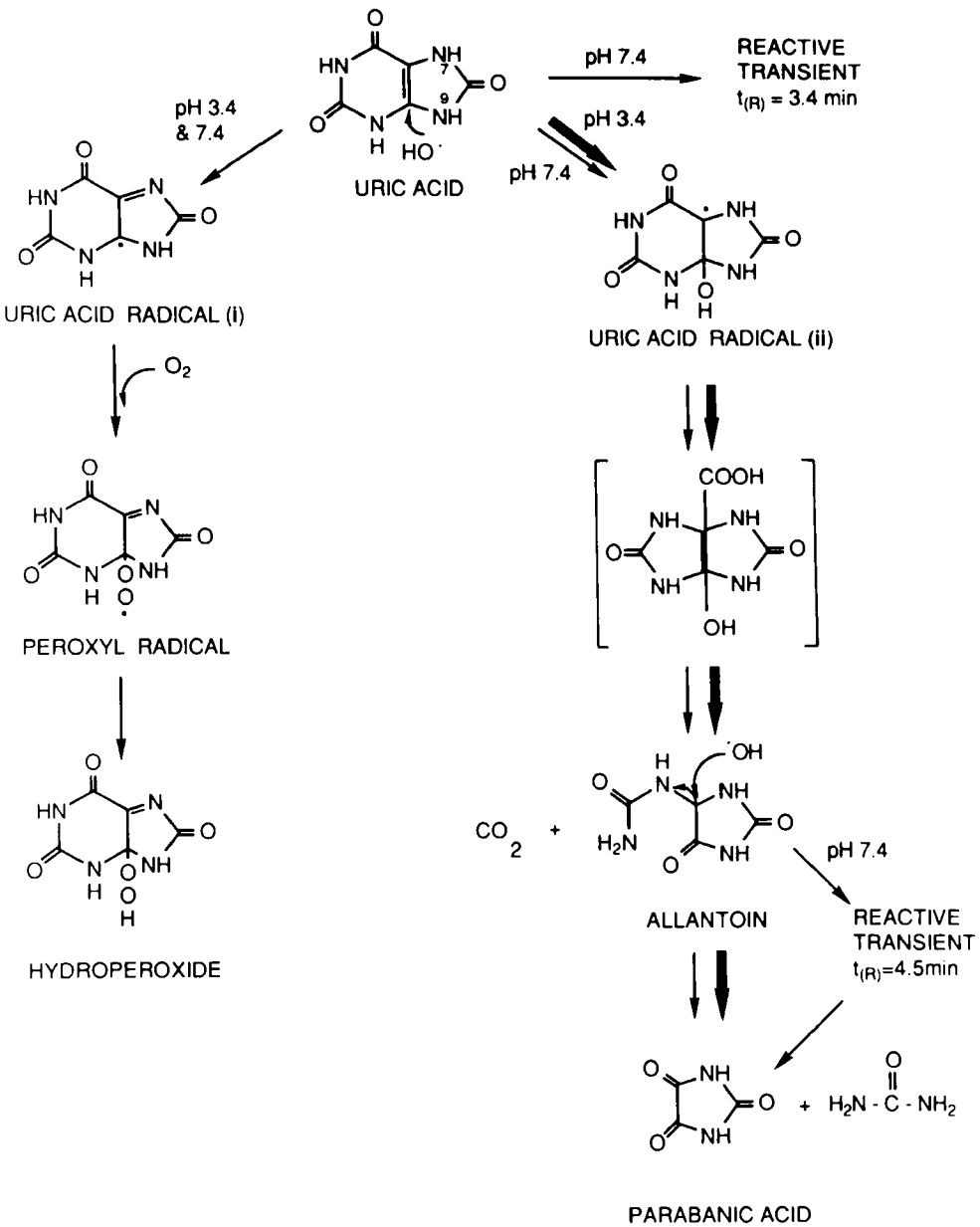


FIGURE 7 Proposed mechanism of uric acid oxidation by hydroxyl radicals at pH 3.4 and 7.4.

Although the flux of HO· was independent of pH from pH 3.4 to 7.4[13], the rate of uric acid oxidation decreased with increasing pH (Figures 2b and 4b). The G(− uric acid), ie the radiation chemical yields for uric acid destruction, were 0.176 μmol. $J^{-1}$ at pH 3.4 and 0.112 μmol. $J^{-1}$ at pH 3.4. and 0.112 μmol. $J^{-1}$ at

pH 7.4. Yields of a similar order have been observed before for HO·-induced destruction of uric acid and other purines[19]. Three explanations were presented to account for this phenomenon. i) Purines were less reactive at neutral pH, than the more acid pH. ii) The purine molecule may also be attacked at a site at neutral pH which does not result in loss of chromophore. This may be a possibility, as nitrogen-centred radicals arising from the $N_7$ and $N_9$ positions on the 5 membered ring of uric acid oxidised by permanganate or a peroxidase system have been observed by fast-flow ESR[20]. iii) A mechanism involving a back-reaction to regenerate the initial purine molecule was also suggested[19]. This back reaction may involve a charge transfer phenomenon, which would be favoured at pH 7.4, where uric acid possesses a single negative charge, over pH 3.4, where the molecule is fully protonated. A similar charge transfer mechanism has been postulated to account for some of the *in vivo* antioxidant properties of uric acid[21].

Hydroperoxide formation from uric acid (left arm of Figure 7) has been notoriously difficult to measure. Oxygen consumption during purine radiolysis has been previously found to be low[21,22] and it was originally presumed that purine hydroperoxides were not formed during radiolysis of aerated, aqueous solutions[23]. The data in Figure 6 show reproducible yields of hydroperoxide - containing products were detected after catalase treatment, (G(Hydroperoxide) = 0.041 $\mu$mol. $J^{-1}$ at pH 3.4 and 0.053 $\mu$mol $J^{-1}$ at pH 7.4). These values are the same order of magnitude as a previous study which reported the radiation chemical yield of urate hydroperoxide to be 0.031 $\mu$mol. $J^{-1}$ (measured by oxygen consumption)[21]. It was assumed that the hydroperoxides were derived solely from uric acid (as outlined in Figure 7), however formation of hydroperoxides in other products cannot be completely ruled out as post-column iodometric assay of each compound was beyond the scope of this study.

An unexpected observation was the increased amount of tri-iodide reactive material in uric acid irradiated at low pH without subsequent catalase addition. This extra hydrogen peroxide formation may arise from expulsion of a perhydroxyl radical from dimers of the uric acid peroxyl radicals[22]. This type of reaction may not be favoured at pH 7.4, well above the pKi for the perhydroxyl/superoxide couple[13]. Although uric acid has been considered an efficient *in vivo* antioxidant[2,3], the reaction between uric acid and oxygen centred radicals may nevertheless produce transients which may be responsible for the inactivation of enzymes such as alcohol dehydrogenase and $\alpha$1-antiproteinase[11,12]. This extra hydrogen peroxide, may be the species responsible for this phenomenon.

In summary, a range of products have been shown to be formed by HO·-induced oxidation of uric acid, the major products being allantoin and parabanic acid. Measurable amounts of allantoin can be found *in vivo*, especially at loci containing large amounts of uric acid, such as the rheumatoid joint[4]. Increased allantoin : uric acid ratios have also recently been reported in the vitreous humour of patients with cataract[24]. The extent of further oxidation of allantoin *in vivo* will depend on the rate constant for the allantoin/HO· reaction, (not known but probably approaching diffusion controlled levels), and the relative concentrations of other HO· scavenging species. Also, the physiological implications of the presence of these products have not been investigated. Products arising from uric acid oxidation (including allantoin) have been postulated as having a role in the development of Reye's Syndrome[25], however no analyses were performed to identify unambiguously the responsible compound(s). Use of the analytical techniques described here may allow elucidation of these potentially toxic species associated with uric acid oxidation.

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