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High Resolution ^{1}H NMR Investigations of the Reactivities of α-Keto Acid Anions with Hydrogen Peroxide

A. PERERA[a], H.G. PARKES[b], H. HERZ[a], P. HAYCOCK[c], D. R. BLAKE[a] and M. C. GROOTVELD[a,*]

[a]*The Inflammation Research Group, The London Hospital Medical College, 25-29 Ashfield Street, London E1 2AD, U.K.;* [b]*Department of Chemistry, Birkbeck College, University of London, 29 Gordon Street, London WC1H OPP, U.K.;* [c]*Department of Chemistry, Queen Mary and Westfield College, University of London, Mile End Road, London E1 4NS, U.K.*

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The chemical reactivity of various α-keto acid anions (β-hydroxypyruvate, β-phenylpyruvate, 2-ketobutyrate and 2-ketoglutarate) with hydrogen peroxide (H_2O_2) was investigated at physiological pH (7.4) and a temperature of 25°C. The initial concentration of the α-keto acid anions was kept constant at 1.00 mM whilst that of added H_2O_2 was varied from 0.25 to 1.00 mM, and the rate and extent of these reactions was evaluated using ^{1}H NMR spectroscopy. At all H_2O_2 concentrations utilised, the order of reactivity of the α-keto acid anions was β-hydroxypyruvate > β-phenylpyruvate > 2-ketobutyrate > 2-ketoglutarate. The results obtained are in agreement with a proposed mechanism for these reactions, involving nucleophilic attack of the *mono*-deprotonated peroxide species (HO_2^-) at the C-2 carbonyl group carbon centre. The antioxidant capacity of such α-keto acids is discussed in terms of their potential use as therapeutic agents in clinical conditions where H_2O_2 has been shown to play a critical role in the disease process, i.e., those involving 'oxidative stress'.

INTRODUCTION

The deleterious role of chemically-reactive oxygen radical species in the pathogenesis of many disease processes, e.g. inflammatory joint diseases,[1,2] is now a commonly documented phenomenon and the toxicity associated with increased superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) generation has been previously ascribed to the aggressively-reactive hydroxyl radical (·OH), the formation of which is promoted by 'catalytic' iron complexes.[3] The authors have previously presented evidence for oxygen radical-mediated oxidative damage to a variety of components (e.g. proteins, lipids, glycosaminoglycans) present in knee-joint synovial fluids obtained from patients with inflammatory joint diseases (reviewed in[4]).

However, H_2O_2 itself may contribute to the pathogenesis of inflammatory joint diseases. Notable examples of the toxicological effects of H_2O_2 include (1) oxidative inactivation of the enzyme glyceraldehyde-3-phosphate dehydrogenase (G-3-PDH) which may represent a critical phase of the mechanism of intrachondrocyte oxidant damage,[5] and (2) rapid consumption of the

*Corresponding author.

α-keto acid anions pyruvate, 2-ketoglutarate (2-oxoglutarate) and oxaloacetate via oxidative decarboxylation reactions, giving rise to a substantial decline in the cellular synthesis of ATP from both glucose and glutamine.[6] The relevance of the latter molecular mechanism to the pathogenesis of this disease process has been augmented by our own recent investigations which involved the application of high field proton (^{1}H) nuclear magnetic resonance (NMR) spectroscopy to simultaneously evaluate the antioxidant capacities of a range of polar, water-soluble low-molecular-mass endogenous metabolites present in ultrafiltrates of inflammatory synovial fluids.[22] These studies clearly demonstrated that consumption of added H_2O_2 was predominantly accounted for by its reaction with the α-keto acid anion pyruvate, generating acetate and CO_2 as products via an oxidative decarboxylation reaction.

We have previously applied high field ^{1}H NMR analysis to the detection of methionine sulphoxide in neutrophil-conditioned culture media subsequent to stimulation with phorbol esters,[8] experiments providing much useful information regarding the nature and levels of ROS generation by these cells.

The H_2O_2-scavenging ability of pyruvate (equation 1) present in certain types of cell culture medium is also of much interest in view of the cytoprotective properties of this component. Indeed, the incorporation of pyruvate in cell culture media is a practice which arises from early observations that this α-keto acid anion promotes the survival of cells at low plating densities.[9, 10] More recent investigations have also demonstrated the ability of pyruvate to substitute for serum[11] or conditioned medium[12] in supporting cell growth. Pyruvate release from astroglia cells has been implicated as an important aspect of the growth-promoting effect of conditioned cell culture media[13] and this agent can also block the toxic actions of exogenous, autoxidisable cysteine.[14] Since the oxygen tension of cell culture environments (usually 95% air) is much greater than that of most body fluids, cultured cells are rendered particularly susceptible to oxidative stresses and in view of the above evidence, the cytoprotective abilities of pyruvate in cell culture media are readily explicable by its powerful H_2O_2-scavenging properties. Indeed, O'Donnell-Tormey *et al.*[15] have recently reported that exogenous pyruvate has the ability to protect cultured cells against lysis induced by H_2O_2.

$$CH_3.CO.CO_2^- + H_2O_2 \longrightarrow CH_3.CO_2^- + CO_2 + H_2O \quad (1)$$

The above observations indicate a potential role for α-keto acid compounds as therapeutic agents for the treatment of clinical conditions which involve the excess, deleterious production of reactive oxygen species, e.g. inflammatory joint diseases. In this study we have employed high resolution ^{1}H NMR spectroscopy to determine (1) the relative rates of H_2O_2 consumption by the α-keto acid anions β-hydroxypyruvate, β-phenylpyruvate, 2-ketobutyrate and 2-ketoglutarate at physiological pH (7.40) and (2) the molecular nature of products generated. In this manner, valuable molecular information regarding the common mechanism of these reactions and structure-activity relationships was obtained. Such information may enable the design of further, more effective antioxidant α-keto acid compounds which may have potential therapeutic applications.

MATERIALS AND METHODS

Reagents

Sodium dihydrogen phosphate and disodium hydrogen phosphate were obtained from BDH chemicals Ltd. (U.K.). Deuterium oxide, 2-ketoglutaric acid and 2-ketobutyric acid were purchased from Aldrich Chemical Co. (U.K.). Hydrogen peroxide (30% w/w), sodium β-

phenylpyruvate and lithium β-hydroxypyruvate were obtained from Sigma Chemical Co. (U.K.).

Sample preparation

Solutions of α-keto acid anions (1.00 mM) in ^2H_2O containing 10.00 mM phosphate buffer (pH 7.4) were prepared. The added hydrogen peroxide concentration ranged from 0.25 to 1.00 mM. The reaction mixtures containing α-keto acid anions and hydrogen peroxide were placed in 5-mm diameter NMR tubes (final volume 0.50–0.60 ml) and equilibrated at 25°C for increasing periods of time (0–8.00 hr.) prior to ^1H NMR analysis.

Proton NMR measurements

NMR measurements were conducted on either a Bruker AMX-600 spectrometer [University of London Intercollegiate Research Services (ULIRS), Department of Chemistry, Queen Mary and Westfield College, University of London] operating at 600 MHz in quadrature detection mode and a probe temperature of 300 K, or a JEOL JNM-GSX 500 spectrometer (ULIRS, Biomedical NMR centre, Department of Chemistry, Birkbeck College, London, U.K.) operating at 500 MHz in quadrature detection mode and a probe temperature of 293 K. For the Bruker AMX-600 spectrometer, each spectrum corresponds to 128 Free induction decays (FIDs), using 32,768 data points, 10 μs pulses and a 3.28s pulse repetition rate. For the JEOL JNM-GSX 500 spectrometer, typical pulsing conditions were: 128 FIDs using 32,768 data points, 45° pulse angle and a 5s pulse repetition rate, the latter to allow full spin-lattice (T_1) relaxation of the protons in the samples investigated. An exponential window function of 0.20 Hz was applied to each FID prior to Fourier transformation and the spectral width was 6,000 Hz. The water signal was suppressed by presaturation with gated decoupling during the delay between pulses.

Chemical shifts of resonances in spectra were referenced to either 50 or 200 μM solutions of sodium 3-trimethylsilyl-[2,2,3,3$^2H_4$]propionate (TSP, $\delta = 0.00$ ppm) in ^2H_2O sealed within a coaxial tube which was inserted into each NMR tube prior to the acquisition of spectra. The identities of components responsible for the resonances present in spectra were routinely assigned by (1) a consideration of characteristic chemical shift values, coupling patterns and coupling constants, and (2) comparisons with ^1H spectra of authentic standards.[23] The identity of reaction products was further confirmed by making standard additions of authentic, commercially-available compounds to reaction mixtures. Concentrations of reactants and products were determined by integration of their ^1H resonances and expressing their intensities relative to that of the 'internal-but-isolated' TSP standard.

RESULTS

Typical ^1H NMR spectra acquired on a reaction mixture containing 1.00 mM 2-ketobutyrate and an equivalent concentration of H_2O_2 with increasing equilibration time at a temperature of 25°C are exhibited in Figure 1. The spectra contain resonances of the α-keto anion reactant together with those of the product derived from its H_2O_2-mediated oxidative decarboxylation. The spectrum of 2-ketobutyrate shown in Figure 1(a) contains multiplet resonances centred at 1.07 (triplet) and 2.75 ppm (quartet). The product generated from the reaction of 2-ketobutyrate with H_2O_2 is propionate and the spectra shown in Figures 1(b) and (c) contain triplet (δ = 1.07 ppm) and quartet (δ = 2.19 ppm) resonances characteristic of this carboxylic acid anion. Similarly, Figure 2 shows ^1H NMR spectra of the α-keto anions β-hydroxypyruvate [-CH_2 group singlet at 4.70 ppm, (a)], β-phenylpyruvate [-CH_2 group singlet at 4.10 ppm and aromatic ring proton multiplets in the 7.20–7.40 ppm range, (c)] and 2-ketoglutarate [-CH_2 group triplets at 2.40 and 3.10 ppm, (e)], together with those of reaction mixtures containing each α-keto acid anion (1.00 mM) and H_2O_2 (1.00 mM) acquired after equilibration at 25°C for

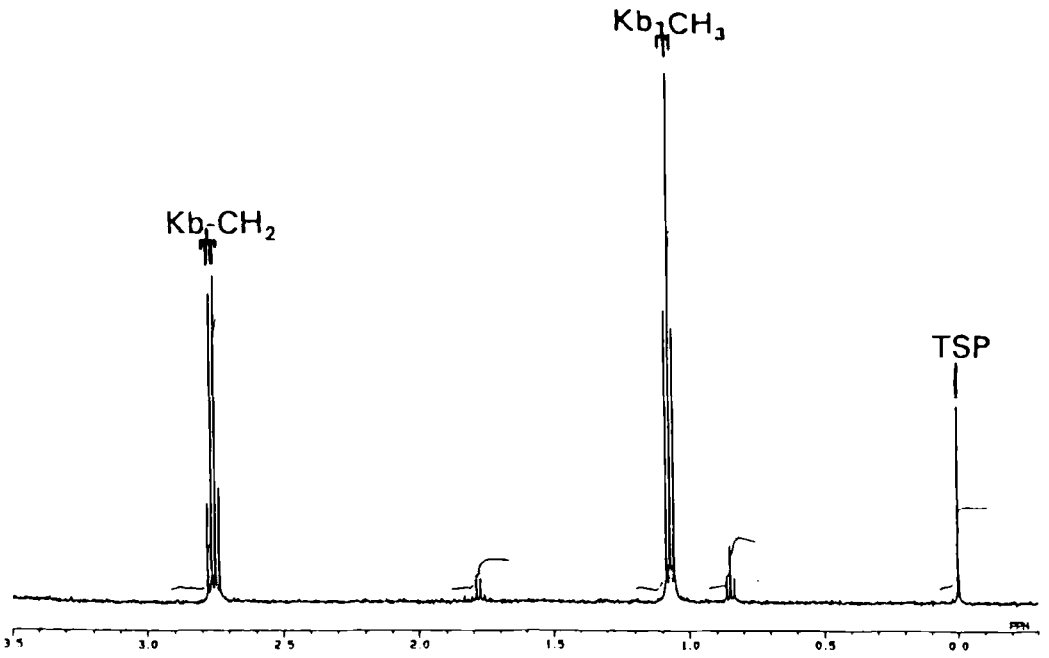

FIGURE 1 ^{1}H NMR spectra of (a) a solution of 2-ketobutyrate (1.00 mM) in $^{2}H_2O$ containing 10.00 mM phosphate buffer (pH 7.40), and a reaction mixture containing 1.00 mM 2-ketobutyrate and 1.00 mM H_2O_2 at (b) 15 min. and (c) 6.33 hr. after H_2O_2 addition. Abbreviations: Kb, 2-ketobutyrate-CH_2, -CH_3; Pr, propionate-CH_2, -CH_3.

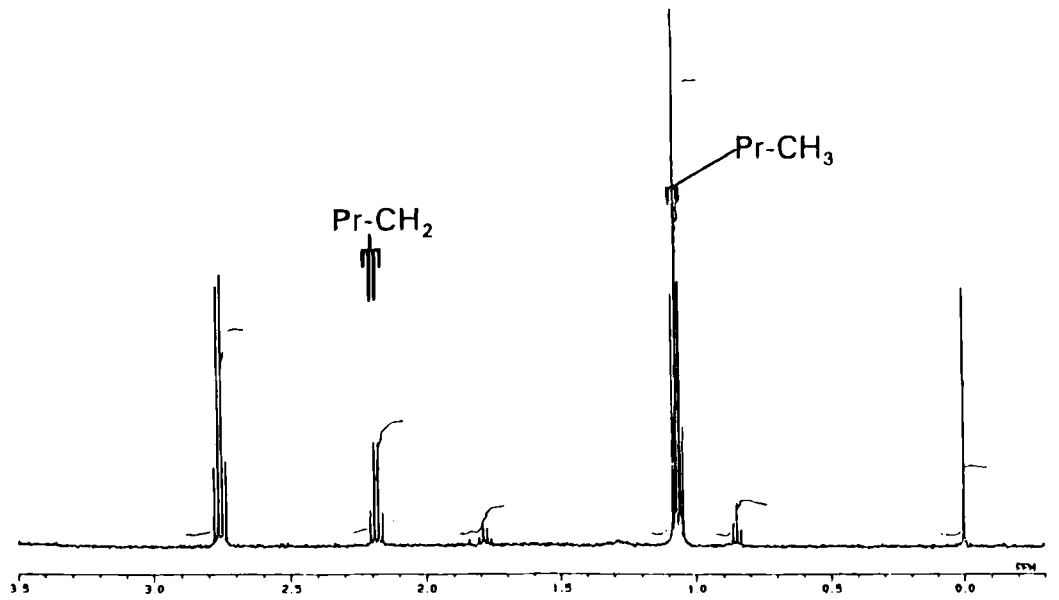

FIGURE 1b (*Continued*)

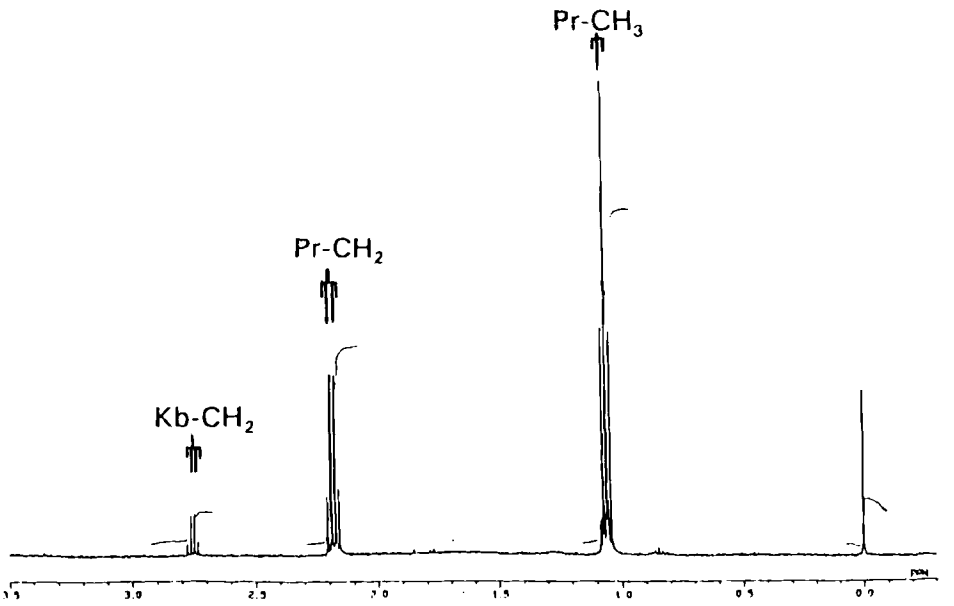

FIGURE 1c (*Continued*)

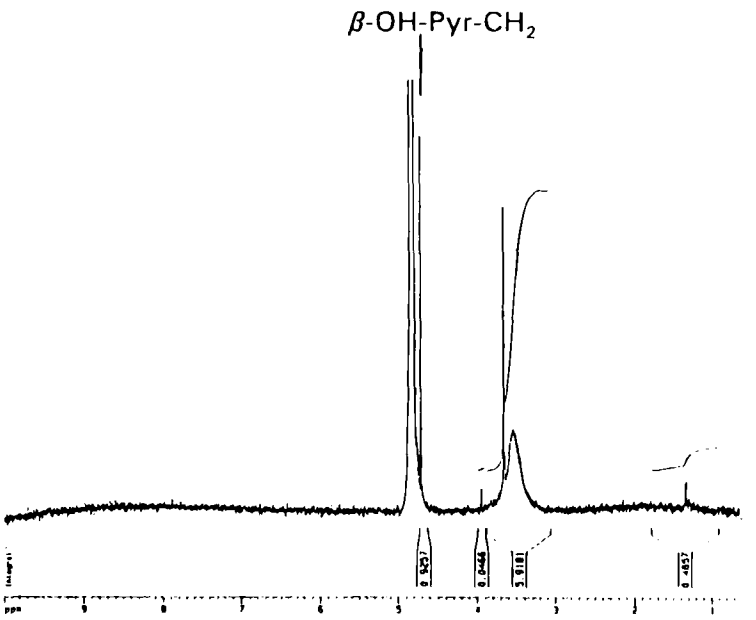

FIGURE 2a

FIGURE 2 ^1H NMR spectra of (a) a solution of β-hydroxypyruvate (1.00 mM) in ^2H_2O containing 10.00.mM phosphate buffer (pH 7.40) before H_2O_2 addition; (b) as (a), but 6.66 hr. after addition of 1.00 mM H_2O_2; (c) a solution of β-phenylpyruvate (1.00 mM) in ^2H_2O containing 10.00 mM phosphate buffer (pH 7.40) before H_2O_2 addition; (d) as (c), but 5.78 hr. after addition of 1.00 mM H_2O_2; (e) a solution of 2-ketoglutarate (1.00 mM) in ^2H_2O containing 10.00 mM phosphate buffer (pH 7.40) before H_2O_2 addition; (f) as (e) but 6.78 hr. after addition of 1.00 mM H_2O_2. Abbreviations: β-OH-Pyr, β-hydroxypyruvate-CH_2; β-OH-Ac, β-hydroxyacetate-CH_2; β-Ph-Py, β-Phenylpyruvate-CH_2; β-Ph-Py-Ar, β-phenylpyruvate aromatic protons; β-Ph-Ac, β-phenylacetate-CH_2; β-Ph-Ac-Ar, β-phenylacetate aromatic protons; Kg, 2-ketoglutarate-CH_2 groups; Suc, succinate-CH_2.

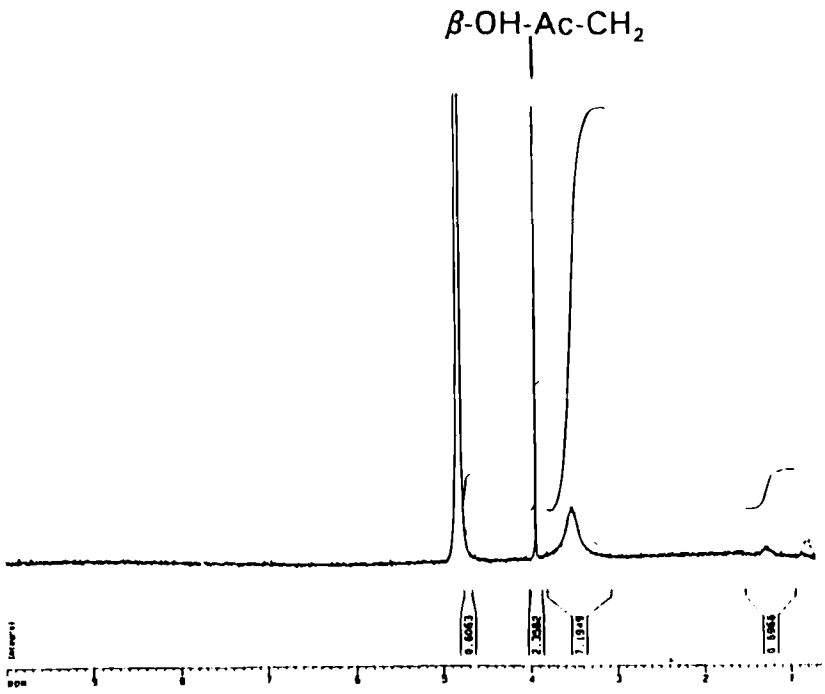

FIGURE 2b (*Continued*)

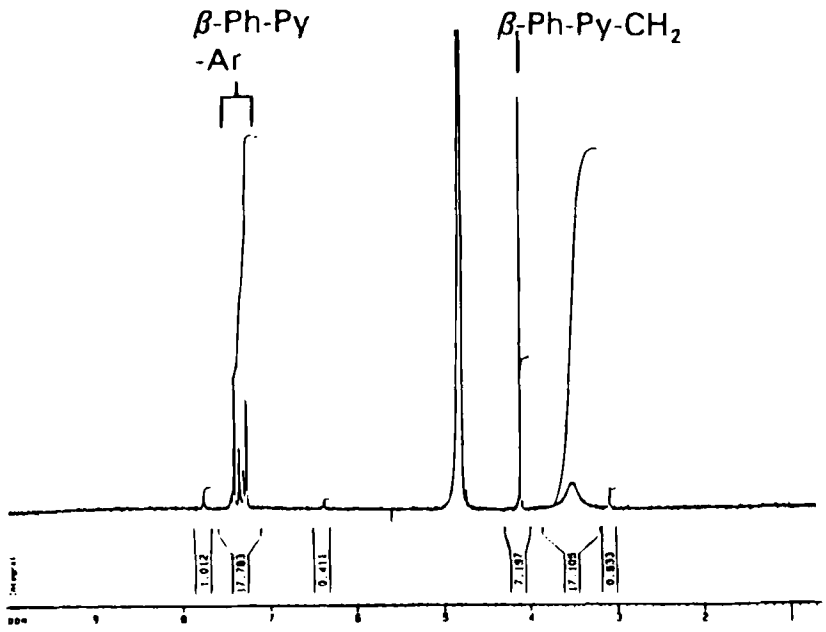

FIGURE 2c (*Continued*)

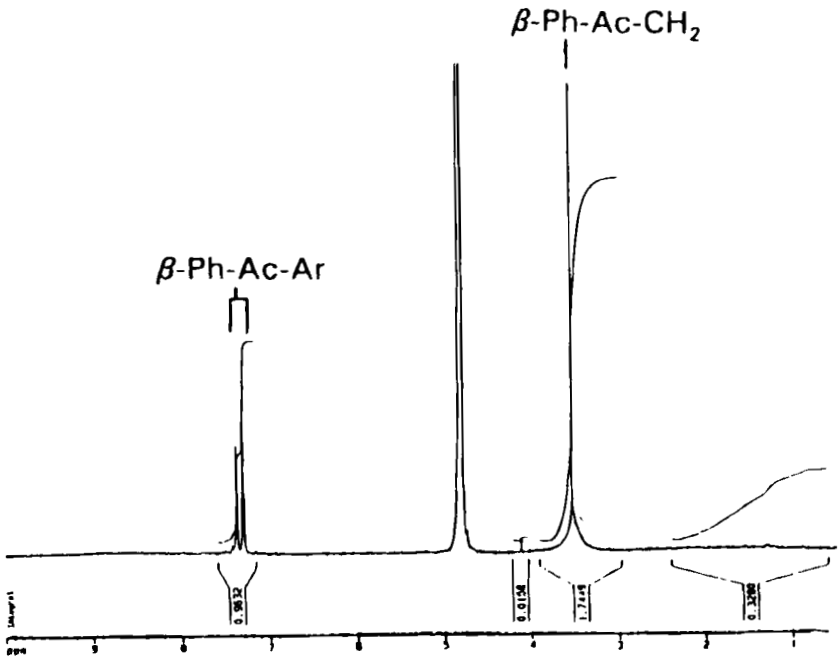

FIGURE 2d (*Continued*)

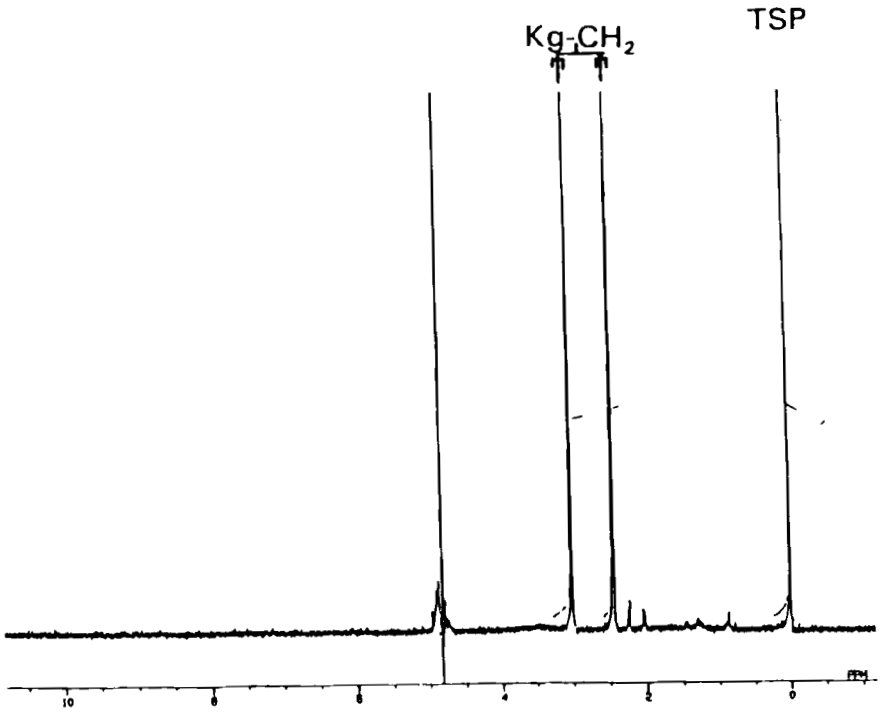

FIGURE 2e (*Continued*)

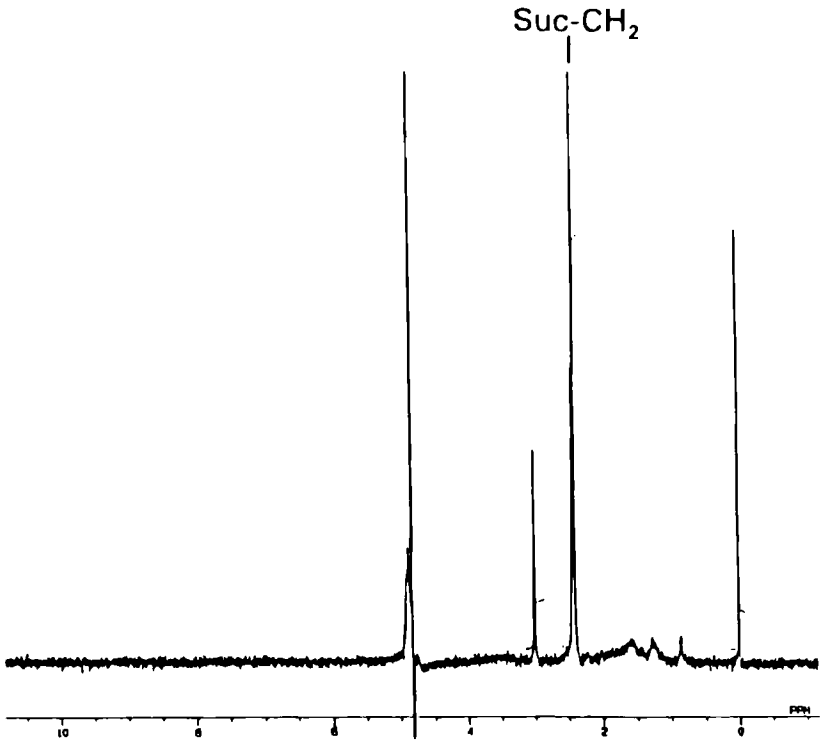

FIGURE 2f (*Continued*)

periods of 6.66 hr. (β-hydroxypyruvate), 5.78 hr. (2-phenylpyruvate) and 6.78 hr. (2-ketoglutarate). In the case of β-hydroxypyruvate, the product formed is β-hydroxyacetate which has a characteristic -CH_2 group singlet resonance located at 3.95 ppm [Figure 2(b)]. Reaction of β-phenylpyruvate with H_2O_2 produces β-phenylacetate which has a -CH_2 group singlet resonance located at 3.55 ppm [Fig. 2(d)] and the spectrum shown in Figure 2(f) contains a -CH_2 group singlet resonance at 2.40 ppm ascribable to the reaction product succinate which arises from the oxidative decarboxylation of 2-ketoglutarate by H_2O_2.

Figure 3 shows plots of the percentage of α-keto acid anion remaining against time for 1.00 mM concentrations of α-keto acid anion treated with (a) 0, (b) 0.25, (c) 0.50, (d) 0.75 and (e) 1.00 mM H_2O_2. It is evident that in the absence of added H_2O_2, β-phenylpyruvate, 2-ketobutyrate and 2-ketoglutarate are stable for periods of up to 7.5 hr. However, approximately 8% of the β-hydroxypyruvate was found to oxidise to its corresponding decarboxylation product within 5.7 hr., a reaction presumably effected by atmospheric O_2. In the presence of 0.25 mM H_2O_2 (Fig. 3(b)), the conversion of 2-ketoglutarate (6%), 2-ketobutyrate (14%), β-phenylpyruvate (19%) and β-hydroxypyruvate (32%) to succinate, propionate, β-phenylacetate and β-hydroxyacetate respectively occurred within 30 min. The percentage of each α-keto acid anion remaining decreased further with increasing time, i.e. after equilibration periods of up to 7 hr. At an added concentration of 0.5 mM, H_2O_2 reacted with 52% of the β-hydroxypyruvate present within 20 min. (Fig. 3(c)). However, the quantity of 2-ketoglutarate, 2-ketobutyrate and β-phenylpyruvate consumed was only 12%, 22% and 26% respectively within this time interval. After time periods of 5.8–6.5 hr., the percentage decomposition of the α-

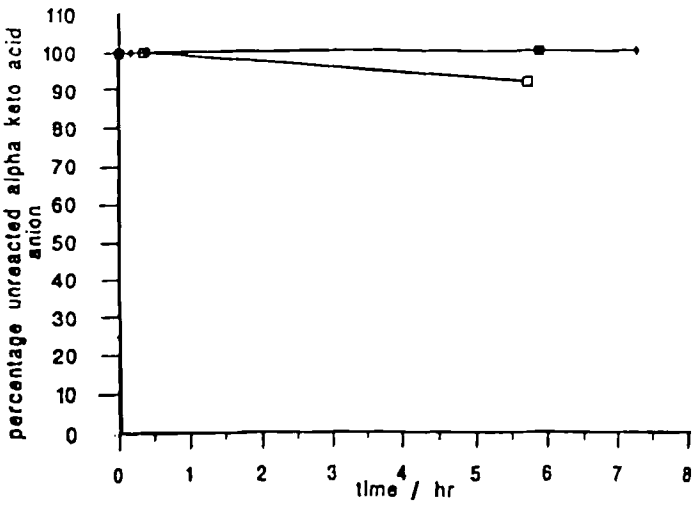

FIGURE 3 Plots of the levels of α-keto acid anion remaining (expressed as a percentage of their initial concentrations) against time for 1.00 mM α-keto acid anions treated with (a) 0; (b) 0.25; (c) 0.50; (d) 0.75 and (e) 1.00 mM H_2O_2. Abbreviations: □ hydroxypyruvate, ■ phenylpyruvate, ◇ 2-ketobutyrate, ◆ 2-ketoglutarate.

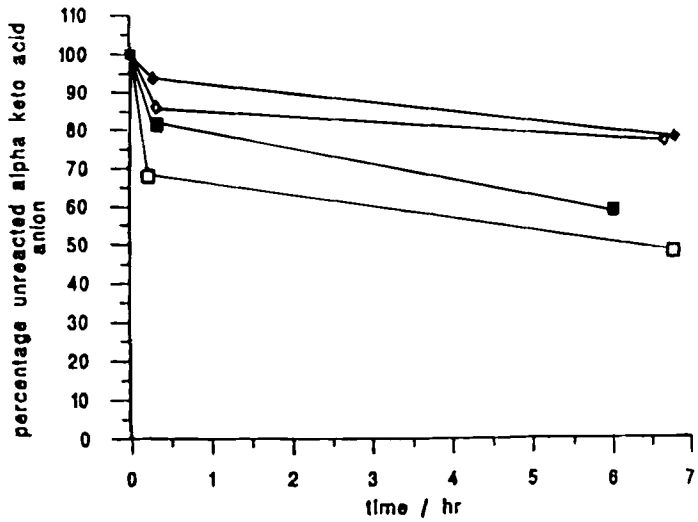

FIGURE 3b (*Continued*)

keto acid anions were 39%, 46%, 67% and 85% for 2-ketoglutarate, 2-ketobutyrate, β-phenylpyruvate and β-hydoxypyruvate respectively.

Similar trends in reactivity were noted at added H_2O_2 concentrations of 0.75 and 1.00 mM (Fig. 3(d) and (e) respectively). Indeed, at the highest concentration of H_2O_2 investigated (1.00 mM) the reaction between β-hydroxypyruvate and H_2O_2 was very rapid, almost 82% of the compound disappearing within the first 20 min. The corresponding reactivity of the other α-keto acid anions were β-phenylpyruvate (52%), 2-ketobutyrate (28%) and 2-ketoglutarate (21%). After time periods of 5.8 to 6.8 hr., the reactions of β-hydroxypyruvate and β-phenylpyruvate with 1.00 mM H_2O_2 were almost complete and the levels of 2-ketoglutarate and 2-

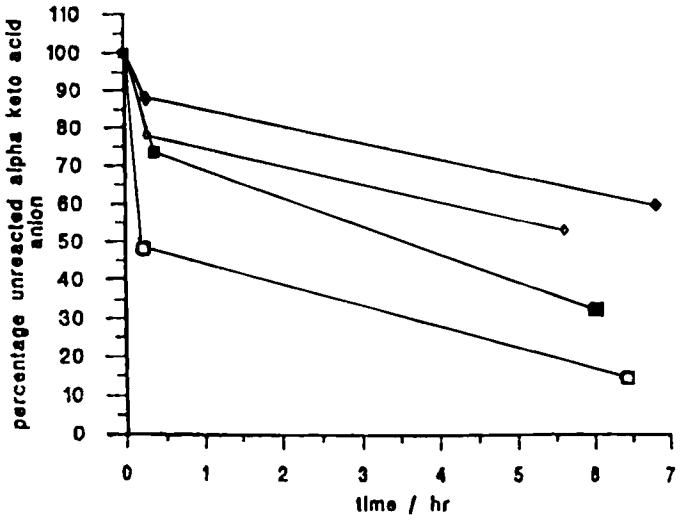

FIGURE 3c (*Continued*)

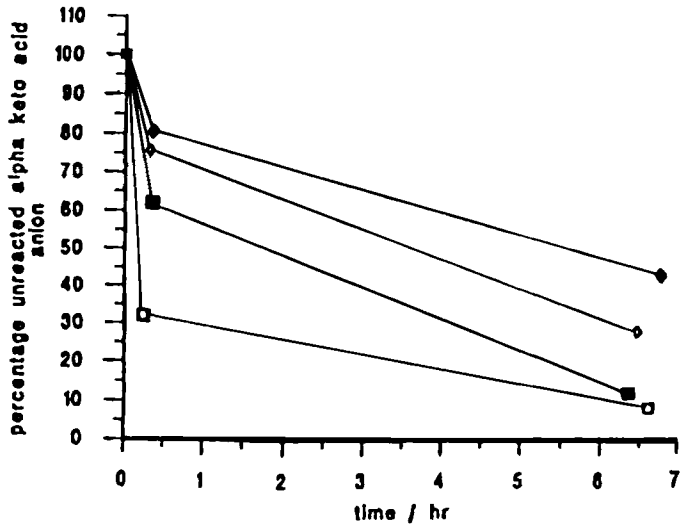

FIGURE 3d (*Continued*)

ketobutyrate remaining were 17 and 26% of their initial concentrations respectively. The greater than stoichiometric conversion of β-hydroxypyruvate to β-hydroxyacetate observed at added H_2O_2 concentrations of 0.25, 0.50 and 0.75 mM are presumably ascribable to oxidation of this α-keto acid anion by atmospheric O_2, as indicated in Figure 4(a). At all four concentrations of added H_2O_2, the order of rates for the reactions between the α-keto acid anions and H_2O_2 was β-hydroxypyruvate > β-phenylpyruvate > 2-ketobutyrate > 2-ketoglutarate.

DISCUSSION

Reaction of α-keto acid anions with H_2O_2 gives rise to the formation of carboxylate anions of chain length one carbon less than the parent α-

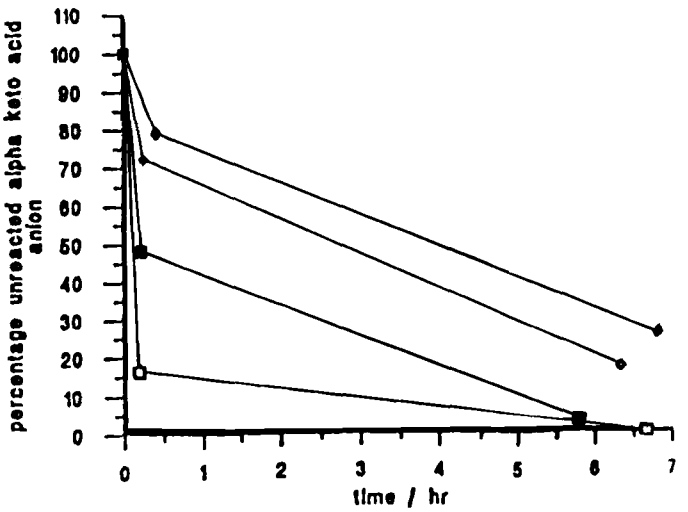

FIGURE 3e (*Continued*)

keto acid anion, (equations 1–5).[16] The formation of the corresponding carboxylic acid anions was directly observed by ^{1}H NMR analysis of the reaction mixtures.

$$\underset{\beta\text{-hydroxy-pyruvate}}{OH\text{-}CH_2\text{-}CO\text{-}CO_2^- + H_2O_2} \longrightarrow \underset{\beta\text{-hydroxy-acetate}}{OH\text{-}CH_2\text{-}COO^- + CO_2 + H_2O} \quad (2)$$

$$\underset{\beta\text{-phenyl-pyruvate}}{C_6H_5\text{-}CH_2\text{-}CO\text{-}CO_2^- + H_2O_2} \longrightarrow \underset{\beta\text{-phenyl acetate}}{C_6H_5\text{-}CH_2\text{-}CO_2^- + CO_2 + H_2O} \quad (3)$$

$$\underset{\text{2-ketobutyrate}}{CH_3\text{-}CH_2\text{-}CO\text{-}CO_2^- + H_2O_2} \longrightarrow \underset{\text{propionate}}{CH_3\text{-}CH_2\text{-}CO_2^- + CO_2 + H_2O_2} \quad (4)$$

$$\underset{\text{2-ketoglutarate}}{{}^-O_2C\text{-}(CH_2)_2\text{-}CO\text{-}CO_2^- + H_2O_2} \longrightarrow \underset{\text{succinate}}{{}^-O_2C\text{-}(CH_2)_2\text{-}CO_2^- + CO_2 + H_2O} \quad (5)$$

In all four reactions above a common mechanism involving nucleophilic attack on the C-2 carbonyl group carbon atom followed by decarboxylation has been previously proposed [16] (Equation 6).

$$R-\underset{\displaystyle O}{\overset{\displaystyle \|}{C}}-CO_2^- + H_2O_2 \longrightarrow R-\underset{\displaystyle OH}{\overset{\displaystyle OOH}{\overset{|}{\underset{|}{C}}}}-CO_2^- + R-CO_2^- + CO_2 + H_2O \quad (6)$$

The formation of the above tetrahedral intermediate is considered to occur via a pre-equilibrium, and the subsequent decarboxylation step is an irreversible process.[17, 18] Moreover, recent evidence obtained indicates that the attacking species present at pH values below 3.8 is H_2O_2, but at higher pH values the HO_2^- anion has been implicated as the attacking nucleophile [16]. Therefore, at a pH of 7.4 as utilised in these investigations, the nucleophile involved is presumably HO_2^- which attacks the C-2 carbonyl group carbon centre of the α-keto acid anion. The presence of strongly electron-withdrawing substituents such as -OH or $-C_6H_5$ at the C-3 carbon centre facilitates attack of the HO_2^- nucleophile at the C-2 carbonyl group carbon, and hence enhances the reaction rate. The order of reac-

tivity of the four α-keto acid anions with H_2O_2 observed in the present study is consistent with the proposed mechanism of reaction where the hydroxyl and phenyl substituents at the C-3 carbon give rise to a faster reaction rate between the α-keto acid anion and H_2O_2. The observed order of reactivity was the same throughout each concentration of H_2O_2 utilised.

Consumption of H_2O_2 (the precursor to the *highly toxic* ·OH radical) *in vivo* by such compounds may prove an effective therapeutic avenue for the treatment of inflammatory synovitis together with alternative diseases in which H_2O_2-mediated injury has been implicated. Pyruvate and 2-ketoglutarate are endogenous α-keto acid anions and millimolar concentrations of pyruvate in blood plasma achieved via its systemic administration are well tolerated in humans without any deleterious effects.[19] Moreover, the facile transport of this α-keto acid anion into cells[20] and mitochondria[21] ensures that increases in its systemic levels are accompanied by elevations in those of the extracellular space and, more specifically, the mitochondrial compartment. Hence, the intra-articular administration of pyruvate or alternative α-keto acids may provide a safe as well as an efficient therapy for inflammatory joint diseases. The results presented here demonstrate the enhanced reactivity of exogenous α-keto acid anions such as β-hydroxypyruvate and β-phenylpyruvate with H_2O_2 when compared to endogenous 2-ketoglutarate, and the phenyl substituent at the 3-position of β-phenylpyruvate may also serve to scavenge any ·OH radical present. In view of these observations, the therapeutic potential of compounds such as β-hydroxypyruvate and β-phenylpyruvate in inflammatory joint diseases is noteworthy and the structure-activity relationships observed here may permit the design of more efficient H_2O_2 scavengers.

Acknowledgements

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