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Multicomponent Investigations of the Hydrogen Peroxide- and Hydroxyl Radical-Scavenging Antioxidant Capacities of Biofluids: The Roles of Endogenous Pyruvate and Lactate

Relevance to Inflammatory Joint Diseases

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High field proton (^1H) nuclear magnetic resonance (NMR) analysis of biofluids (healthy human blood sera and inflammatory knee-joint synovial fluids) has been employed to evaluate the hydrogen peroxide (H_2O_2)- and hydroxyl radical (°OH)- scavenging antioxidant capacities of a range of polar, low-molecular-mass endogenous metabolites therein. Data obtained indicate that consumption of H_2O_2 by pyruvate (generating acetate and CO_2 via an oxidative decarboxylation reaction) and °OH radical by lactate (generating pyruvate, and subsquently acetate and CO_2) may serve to protect alternative biofluid components (e.g., macromolecules) against reactive oxygen species-mediated oxidative damage in vivo. The mechanistic, physiological and potential therapeutic implications of these results are discussed with special reference to inflammatory joint diseases.

INTRODUCTION

There is currently a wealth of experimental evidence available suggesting a pathogenic role for chemically-reactive oxygen radical species in inflammatory joint diseases.[1–3] Much of the toxicity associated with elevated superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) generation has been ascribed to the potent oxidising actions of the hydroxyl radical (°OH), the production of which appears to be dependent on 'catalytic' iron complexes.[4] The molecular nature of such reactions is complex and similarly reactive oxo-iron species such as the ferryl ion (FeO^{2+}) are also involved. The iron ions for such complexes can be supplied in the acidotic environment of the inflamed rheumatoid joint by their reductant- and oxidant-mediated mobilisation from ferritin and haemoglobin respectively, the latter being released from erythrocytes which lyse during episodes of traumatic microbleeding.[51]

We have presented evidence previously for reactive oxygen radical-mediated oxidative dam-

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age to protein[5] and lipid[4,6] components present in the joints of arthritic patients, and have suggested hypoxic/reperfusion injury as a possible mechanism for perpetuating the excessive production of oxygen-derived free radical species within the joint, a phenomenon that may lead to the persistence of the inflammatory reaction.[8] The products which arise from oxygen radical attack on endogenous components can themselves stimulate further oxygen radical generation by inflammatory cells and again maintain the synovitis with progressive destruction of bone and cartilage.[9,10] We have also demonstrated the generation of a reactive oxygen metabolite by synovium *in vitro* during hypoxic/reperfusion cycles utilising electron spin resonance (ESR) spectroscopy coupled with spin-trapping techniques.[11] Moreover, we have recently employed high field proton Hahn spin-echo nuclear magnetic resonance (NMR) spectroscopy to detect abnormal low-molecular-mass species present in inflammatory synovial fluids which appear to be derived from oxygen radical-mediated oxidative damage to endogenous components, including the glycosaminoglycan hyaluronate.[12]

H_2O_2 may itself exert a deleterious role in the pathogenesis of inflammatory joint diseases, notable examples of its potential toxicological properties including (1) rapid consumption of the α-keto acid anions pyruvate, 2-oxoglutarate and oxaloacetate via oxidative decarboxylation reactions, giving rise to a major decline in the cellular synthesis of ATP from both glucose and glutamine,[13] and (2) inactivation of the enzyme glyceraldehyde-3-phosphate dehydrogenase (G-3-PDH) which has been postulated to be a key step in the mechanism of intrachondrocyte oxidative damage.[14] Additionally, diminishing intracellular ATP levels appear to represent a critical primary stage of cytolysis induced by H_2O_2,[15] an observation supported by Hyslop *et. al.*[16] who examined the mechanism of H_2O_2-mediated cell injury and found that the glycolytic and mitochondrial pathways of ADP phosphorylation were important intracellular targets.

The critical role of H_2O_2 in the pathogenesis of inflammatory joint diseases is further supported by our recent observation that it stimulates osteoclastic bone resorption at concentrations ≥ 10 nM.[17] Indeed, nanomolar concentrations of this reactive oxygen species enhance osteoclast motility at a constant pH value (7.4).

The powerful H_2O_2-scavenging capacity of pyruvate may be of much significance in view of the cytoprotective properties of this species. 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 when the level of oxidative stress is maximal.[18–21] More recent studies have demonstrated that pyruvate can substitute for serum[22–24] or conditioned medium[25] in supporting cell growth in culture. Culture chambers operating at pO_2 values of 95%(v/v) are, of course, examples of oxidatively-stressed systems. Release of endogenous pyruvate from astroglia cells has been implicated as an important aspect of the growth promoting role of conditioned culture medium,[26] and this metabolite can also suppress the toxic actions of exogenous, autoxidisable cysteine.[27]

The ready generation of H_2O_2 at several intracellular sites and its facile diffusion amongst cellular components requires an antioxidant which has the capacity to play a H_2O_2-scavenging role throughout the intracellular environment, and endogenous pyruvate is ably suited to this task since it arises in the cytosol as the final product of the glycolytic pathway, and is then transported to the mitochondrion for its oxidative decarboxylation[28] (producing acetyl coenzyme A which enters the tricarboxylic acid cycle). Indeed, Salahudeen *et. al.*[29] have recently suggested biochemical pathways that may be associated with its novel antioxidant role. Pyruvate is readily transported into and out of cells,[30] facilitating its role as both an intracellular and extracellular H_2O_2 scavenger.

The multicomponent analytical ability of currently-available high field NMR spectrometers permits the facile detection and quantification of

low-molecular-mass endogenous metabolites present in intact or ultrafiltered body fluids at sub-millimolar concentrations.[31–34] In this investigation we have employed high field, single-pulse proton (^{1}H) NMR spectroscopy to quantitatively assess the capacities of polar, water-soluble components present in normal serum and inflammatory synovial fluid ultrafiltrate samples to scavenge H_2O_2, and for the first time present evidence for the ability of pyruvate present in these biofluids to act as a powerful antioxidant in this context. Since the generation of °OH radical or a related powerful oxidant from added H_2O_2 was indicated in ca. 35% of the synovial fluid samples examined, we have also investigated the ability of pyruvate, and its product of anaerobic metabolism, lactate, to scavenge °OH radical (generated radiolytically) in chemical model systems.

MATERIALS AND METHODS

Reagents

Analytical grade hydrogen peroxide (30% (w/v) solution), thiourea (99+%), 5-aminosalicylic acid (99%), lithium acetoacetate (90–95%) human albumin (fraction V and fatty-acid free samples) and L(+) lactic acid (98%) were obtained from Sigma (U.K.). Deuterium oxide (99.9%), sodium acetate (99.99%), hydrogen peroxide (27.5% (w/v) solution), sodium 3-trimethylsilyl-(2,2,3,3-$^2H_4$)-propionate (99%), sodium pyruvate (98%), succinic acid (99%), sodium hydroxide (97%), sodium hydrogen phosphate (99%) and sodium dihydrogen phosphate (99%) were obtained from Aldrich. Trisodium citrate (99%) and sodium acetate (99%) were obtained from BDH. Deuterium oxide (99.8%) was also obtained from Flurochem Ltd. CF25 ultrafiltration membrane cones were obtained from Amicon. Acetone (99.8%) was obtained from Prolabo (Paris, France). A protein assay kit containing Coomassie brilliant blue G-250 dye and a series of bovine serum albumin calibration standards were obtained from Biorad. Dulbecco's phosphate-buffered saline was purchased from Oxoid.

Aqueous solutions of sodium 5-aminosalicylate, sodium lactate and disodium succinate were prepared by neutralisation of the corresponding free acids with aqueous NaOH.

Serum and Synovial Fluid Samples

Serum samples were obtained from nine consenting healthy volunteers, age range 22–33 (3 female, 6 male) by allowing freshly drawn non-heparinized blood to clot. These samples were then centrifuged (3,000 r.p.m.) and the supernatant removed. Knee-joint synovial fluids (n = 11), were drawn into plastic sample tubes for therapeutic purposes from patients with moderately severe rheumatoid and osteoarthritis, and associated knee effusions (age range 35–76), and then immediately centrifuged as above to remove cells and debris.

Amicon ultrafiltration devices were washed twice with doubly-distilled water prior to use. Shortly after aspiration (ca. 3 hr.), the above biofluid samples were further centrifuged in centifree MPS-1 microselection tubes, 25 kDa molecular weight cut-off (Amicon) to remove macromolecules. These ultrafiltration devices were washed twice with doubly-distilled water prior to use.

Sample Preparation

10 µl of a 10 mM aqueous solution of H_2O_2 was added to 0.50 ml of ultrafiltered serum (SerUF) or ultrafiltered synovial fluid (SerUF) to yield a final concentration of 0.20 mM H_2O_2 and these samples were equilibrated for a period of 24 hr. at ambient temperature. Appropriate biofluid controls were treated in the same manner with doubly-distilled water replacing the hydrogen peroxide solution.

Four of the SFUF samples were equilibrated with 0.20 mM H_2O_2 in two stages with a 24 hr. interval between the first and second additions.

A further two of the SFUF samples were treated with the hydroxyl radical scavengers thiourea (5.00 mM) or 5-aminosalicylate (10.00 mM) prior to the addition of H_2O_2. Appropriate control samples containing no added H_2O_2 were also prepared.

Prior to ^1H NMR analysis, 0.20 ml of ^2H_2O was added to the samples to provide a field frequency lock. To 7 of the SFUF and all of the SerUF samples, the ^2H_2O added was buffered with 20 mM phosphate buffer (pH 7.40) containing a known concentration of the internal standard sodium 3-(trimethylsilyl)-(2,2,3,3-$^2H_4$)-propionate (TSP), usually 1.00 mM.

Equilibration of Aqueous Thiourea Solutions with Hydrogen Peroxide

1.00 M aqueous solutions of thiourea containing 20 mM phosphate buffer (pH 7.4) were treated with 0, 0.01, 0.02, 0.05, 0.10 and 1.00 M H_2O_2 and the mixtures were equilibrated at ambient temperature for 16 hr.. 0.50 ml aliquots of each of these samples were then added to 0.20 ml of ^2H_2O prior to ^{13}C NMR analysis.

γ-Irradiation Treatment of Aqueous Lactate and Pyruvate Solutions

10.00 mM aqueous solutions of lactate and pyruvate were subjected to γ-radiolysis in the presence of atmospheric O_2 using a ^{60}Co source (Department of Immunology, The London Hospital Medical College), at a total dose of 8.00 kGy (28 hr. at a dose-rate of 4.76 Gy/min.). Under these experimental conditions the major primary radiolytic products present are °OH (G = 2.7), $e^-_{(aq.)}$ (G = 2.7) and H° (G = 0.5), where the G value represents the concentration of product produced in μM per 10 Gy dosage. Virtually all of the $e^-_{(aq.)}$ generated reacts with available oxygen producing $O_2^{\circ-}$ (equation 1), which is relatively inert. 2.0 ml aliquots of the above solutions were also saturated with N_2O and γ-irradiated as described above. Under these conditions the concentration of °OH radical generated is effectively doubled (equation 2)

$$e^-_{(aq.)} + 0_2 \Rightarrow 0_2^{\circ-} \quad (1)$$

$$N_2O + H^+ + e^-_{(aq.)} \Rightarrow {}^\circ OH + N_2 \quad (2)$$

Samples of doubly-distilled water (untreated and N_2O-saturated) were similarly γ-irradiated.

Determination of Trace Levels of Protein in Biofluid and Human Albumin Ultrafiltrates

Protein concentrations in SFUF, SerUF and ultrafiltered human albumin solutions were determined by a microassay procedure (Biorad) in order to monitor the passage of trace levels of macromolecules through the ultrafiltration membranes employed in these investigations. A calibration curve prepared from standard solutions containing 5, 10, 15, 20 and 25 μg/ml bovine serum albumin was linear (correlation coefficient, r = 0.9995).

The concentration of protein present in inflammatory SFUF was 16.8 ± 1.7 μg/ml (mean ± standard deviation, n = 3) which is comparable to that found in ultrafiltered human albumin solutions (16.0 μg/ml). The protein level of healthy human SerUF samples, however, was 68.1 ± 9.6 μg/ml (n = 3).

Determination of Thiol in Biofluid and Human Albumin Ultrafiltrates

0.98 ml of ultrafiltered synovial fluid, serum or 50 mg/ml solutions of human albumin were treated with 0.02 ml of a 10.00 mM stock solution of 5,5′-dithiobis(2-nitrobenzoate) (DTNB) and the reaction mixture allowed to equilibrate at ambient temperature for a period of 30 min.. Following equilibration, the optical density of these solutions at a wavelength of 412 nm was measured against a blank solution containing 0.20 mM DTNB in phosphate-buffered saline (pH 7.40). 2-Mercaptosuccinate (5, 10, 20, 30, 50, 75 and 100 μM) was employed as a calibration standard for

these thiol determinations. A plot of absorbance at 412 nm versus 2-mercaptosuccinate concentration was linear (r = 0.9992) and yielded an an extinction coefficient (ε) of 1.41×10^4 M^{-1} cm^{-1} for the chromophoric 5-mercapto(2-nitrobenzoate) reaction product, similar to that previously reported.[7]

NMR Measurements

Proton NMR spectra were acquired using a JEOL JNM-GSX 500 NMR spectrometer (University of London Intercolleigate Research Services (ULIRS), Biomedical NMR centre, Birkbeck College, London, U.K.) operating at 500 MHz in quadrature detection mode and a probe temperature of 293 K. Each spectrum corresponds to 64 or 128 free induction decays (FIDs) using 32,768 data points, 3–7 μs pulses and a 5 s pulse repetition rate. The water signal was suppressed by presaturation with gated decoupling during the delay between pulses.

Resonances were referenced to internal sodium 3-trimethylsilyl-(2,2,3,3-$^2H_4$)-propionate (TSP, δ = 0.00 ppm). The methyl group resonances of lactate (δ = 1.330 ppm) or alanine (δ = 1.487 ppm) served as secondary internal references in spectra of biofluid ultrafiltrate samples. The identities of components responsible for the resonances present in SFUF and SerUF spectra were routinely assigned by a consideration of characteristic chemical shift values, coupling patterns and coupling constants. Where appropriate, standard additions of authentic standards were made to confirm assignments. Metabolite concentrations were determined by integration of their ^1H resonances and expressing their intensities relative to that of the internal TSP standard.

100 MHz ^1H-decoupled ^{13}C NMR spectra of aqueous thiourea solutions treated with increasing concentrations of H_2O_2 (0–1.00 M) were obtained on a Bruker WH 400 spectrometer using composite pulse proton decoupling. 600–1600 transients were collected into 32,768 data points and an exponential function corresponding to 3 Hz line—broadening applied prior to Fourier transformation.

Statistical Analysis of Metabolite Concentration Data

Tests for variance heterogeneity in the SFUF and SerUF concentrations of lactate, acetate, pyruvate and alanine were conducted using the F variance-ratio statistic.

In view of the large differences between the SFUF metabolite level variances and those of SerUF, data were $\log_e$-transformed in an attempt to homogenise sample group variability for each component measured prior to comparing mean values by a two-sample t-test. However, F variance-ratio tests on these $\log_e$-transformed data demonstrated that the SFUF lactate, acetate and pyruvate level variances remained significantly greater than those of SerUF, and hence the Aspin-Welch test for differences between the mean values of sample groups with unequal variances was employed.

A two-sample t-test was utilised to compare the mean pyruvate: acetate concentration ratio of SFUF with that of SerUF. For this comparison, the assumption of homogeneous intra-sample variances was satisfied (F-test).

RESULTS

High Field ^1H NMR Evaluation of the Interactions of Normal Serum Ultrafiltrate Components with Added H_2O_2

Figure 1 shows typical 500 MHz single-pulse ^1H NMR spectra of a SerUF sample obtained prior and subsequent to equilibration with H_2O_2 for 24 hr. at ambient temperature, and Table I(a) gives the levels of lactate, alanine, acetate, pyruvate and citrate determined by integration of the assigned resonances of these components and expressing their areas relative to that of the added TSP internal standard. The pyruvate-CH_3 group signal is present in a region of the

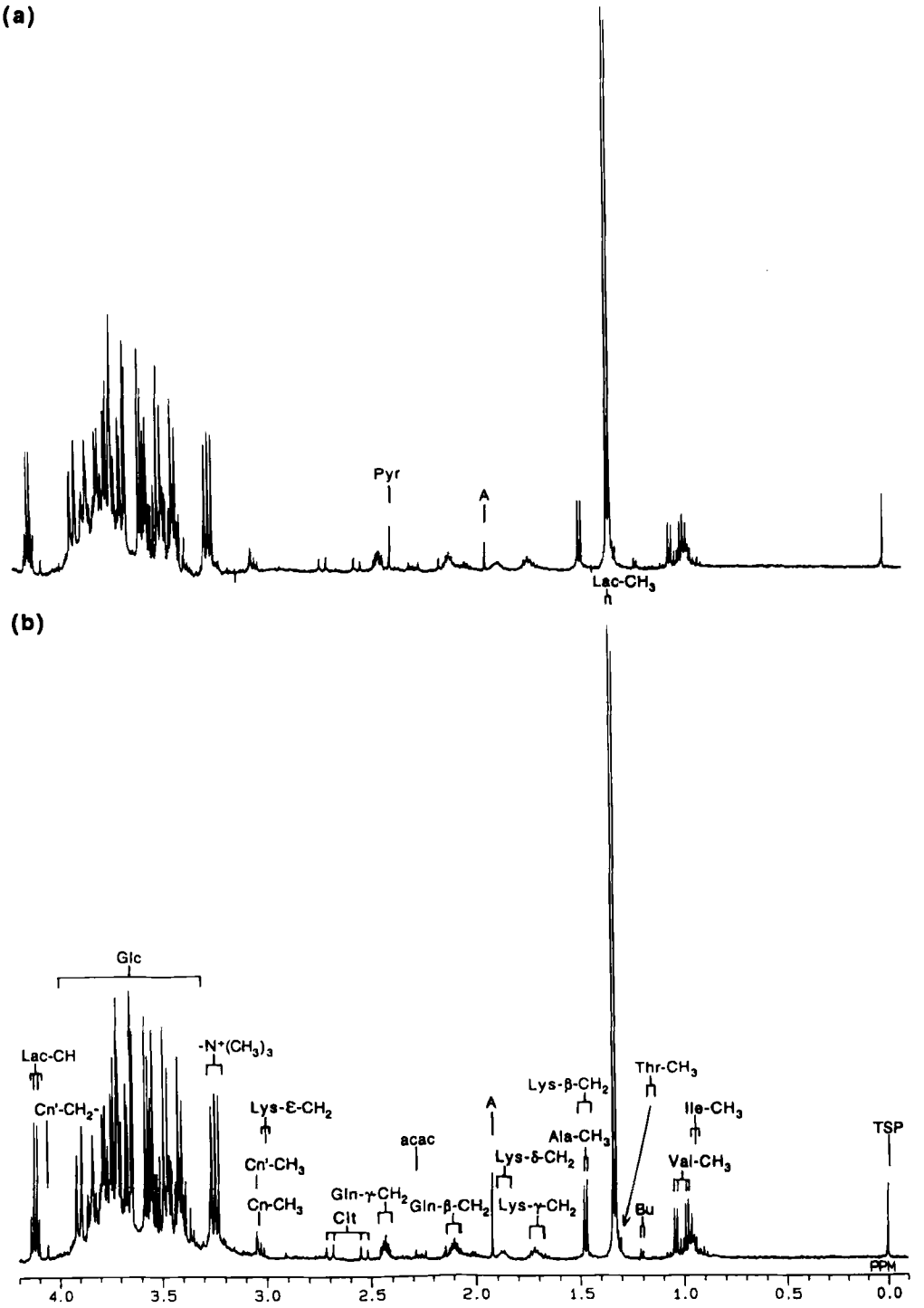

FIGURE 1 High field (aliphatic) region of 500 MHz single-pulse ^1H NMR spectra of a normal serum ultrafiltrate sample obtained (a), before, and (b), after equilibration with 0.20 mM H_2O_2 at ambient temperature for a period of 24 hr. Typical spectra are shown. Abbreviations: A, acetate; ac, acetone; acac, acetoacetate; Ala, alanine; Bu, 3-D-hydroxybutyrate; Cit, citrate; Cn', creatinine; Cn, creatine; Glc, glucose; Gln, glutamine; Ile, isoleucine; Lac, lactate; Lys, lysine; Suc, succinate; Thr, threonine; Val, valine.

TABLE I H_2O_2-mediated modifications in the concentrations of NMR-detectable lactate, alanine, acetate, pyruvate and citrate in (a) inflammatory knee-joint synovial fluid and (b) normal blood serum ultrafiltrates. Samples were equilibrated with 0.20 mM H_2O_2 at ambient temperature for a period of 24 hr. Abbreviations: Lac, lactate; Ala, alanine; A, acetate; Pry, pyruvate; Glc, glucose; Form, formate; NM, no detectable modifications observed following H_2O_2 treatment. 'Trace' refers to levels which were too low to measure by electronic integration but still NMR-detectable at an operating frequency of 500 MHz. The SFUF-1 sample is an example of those in which the H_2O_2-induced elevation in acetate concentration observed was significantly greater than the corresponding decrease in that of pyruvate (75% greater).

(a) Normal serum ultrafiltrates

Sample	[Lac]/mM	[Ala]/mM	[A]/mM	[Pyr]/mM	[Cit]/mM
SerUF-1	1.36	0.23	0.06	0.04	0.02
SerUF-1 + H_2O_2	NM	NM	0.10	0.00	NM
SerUF-2	1.15	0.32	0.05	0.04	0.02
SerUF-2 + H_2O_2	NM	NM	0.09	0.00	NM
SerUF-3	2.04	0.41	0.05	0.07	Trace
SerUF-3 + H_2O_2	NM	NM	0.11	0.00	NM
SerUF-4	1.28	0.28	0.12	0.04	0.02
SerUF-4 + H_2O_2	NM	NM	0.16	0.00	NM
SerUF-5	2.04	0.36	0.10	0.07	0.03
SerUF-5 + H_2O_2	NM	NM	0.17	0.00	NM
SerUF-6	1.19	0.39	0.06	0.04	0.02
SerUF-6 + H_2O_2	NM	NM	0.10	0.00	NM
SerUF-7	1.89	0.24	0.05	0.09	Trace
SerUF-7 + H_2O_2	NM	NM	0.16	0.00	NM
SerUF-8	1.90	0.23	0.05	0.08	0.03
SerUF-8 + H_2O_2	NM	NM	0.13	0.00	NM
SerUF-9	1.58	0.29	0.04	0.08	0.02
SerUF-9 + H_2O_2	NM	NM	0.11	Trace	NM
SerUF-10	1.75	0.60	0.11	0.08	trace
SerUF-10 + H_2O_2	NM	NM	0.20	0.00	NM

(b) Inflammatory knee-joint synovial fluid ultrafiltrates

Sample	[Lac]/mM	[Ala]/mM	[A]/mM	[Pyr]/mM	[Cit]/mM
SFUF-1	2.61	0.29	0.08	0.06	trace
SFUF-1 + H_2O_2	NM	NM	0.15	0.02	NM
SFUF-2	5.59	0.41	0.32	0.18	trace
SFUF-2 + H_2O_2	NM	NM	0.48	0.00	NM
SFUF-3	2.73	0.40	0.09	0.16	0.18
SFUF-3 + H_2O_2	NM	NM	0.26	0.00	NM
SFUF-4	2.27	0.39	0.06	0.11	0.13
SFUF-4 + H_2O_2	NM	NM	0.17	0.00	NM
SFUF-5	1.71	0.31	0.07	0.08	0.11
SFUF-5 + H_2O_2	NM	NM	0.15	0.00	NM
SFUF-6	1.91	0.14	0.13	0.00	0.07
SFUF-6 + H_2O_2	NM	NM	NM	NM	NM
SFUF-7	4.25	0.22	0.07	0.09	0.11
SFUF-7 + H_2O_2	NM	NM	0.17	0.00	NM
SFUF-8	4.98	0.26	0.11	0.14	trace
SFUF-8 + H_2O_2	NM	NM	0.25	0.00	NM

spectrum which is occupied by many alternative singlet resonances with similar chemical shift values. Accurate determination of the pyruvate-CH_3 group signal chemical shift gave a value of 2.377 ± 0.002 ppm (mean ± standard deviation, n = 10).

The SerUF spectra clearly demonstrate that the only significant H_2O_2-mediated modifica-

tion arising is the elimination of the pyruvate-CH_3 group resonance with a corresponding increase in the intensity of that of the acetate-CH_3 group (δ = 1.92 ppm). Indeed, with the exception of small decreases in the concentration of 3-D-hydroxybutyrate observed in one SerUF investigated (which is presumably consumed by its H_2O_2-mediated oxidation to acetoacetate), integration of all other resonances present in spectra demonstrated no modifications in the concentrations of alternative NMR-detectable components subsequent to H_2O_2 treatment. The elevation in the intensity of the acetate-CH_3 group signal was quantitatively accounted for by the elimination of that of the pyruvate-CH_3 group in all samples examined (n = 9), as documented in Table I(a). These data are fully consistent with the oxidative decarboxylation of pyruvate to acetate and CO_2 by added H_2O_2 (equation 4), and suggest that endogenous pyruvate exerts a powerful H_2O_2-scavenging ability in human serum.

H_2O_2-Mediated Modifications in the ^{1}H NMR Profile of Inflammatory Synovial Fluid Ultrafiltrate Samples

High field ^{1}H NMR analysis of inflammatory SFUFs both prior and subsequent to treatment with 0.20 mM H_2O_2 also demonstrated the facile oxidative decarboxylation of pyruvate to acetate by this ROS (Figure 2). With the exception of small increases in the level of acetone in 2 of the samples investigated, no further H_2O_2-induced modifications in the concentrations of alternative NMR-detectable components were observed. Although one sample which contained no NMR-detectable pyruvate exhibited no observable modification in its spectrum, only 6 of the 11 SFUF samples investigated in this manner showed a quantitative transformation of pyruvate to acetate (examples of which are given in Table I(b)). For the remaining 4 SFUFs, the H_2O_2-mediated elevation in the concentration of acetate was greater than the observed decrease in that of pyruvate, indicating that further acetate arises from H_2O_2-mediated oxidative damage to an additional component. For these samples, the pyruvate was not completely degraded following H_2O_2 treatment. These results differ somewhat from those obtained with SerUF samples in which the H_2O_2-induced rise in acetate concentration was quantitatively accounted for by the consumption of pyruvate in all samples investigated (n = 9). The increase in acetate concentration unaccountable for by the reaction of H_2O_2 with SFUF pyruvate may arise from the prior generation of °OH radical via the interaction of low-molecular-mass electron donors such as ascorbate, the thiol cysteine or a "catalytic" iron(II) complex with H_2O_2, followed by the reaction of this extremely reactive radical species with lactate to generate pyruvate, the latter being oxidised to further acetate by H_2O_2 as previously described[35] (equations 3 and 4).

$$\underset{\text{Lactate}}{CH_3.CHOH.CO_2^-} \xrightarrow{{}^\circ OH} \underset{\text{Pyruvate}}{CH_3.CO.CO_2^-} \quad (3)$$

$$\underset{\text{Pyruvate}}{CH_3.CO.CO_2^-} \xrightarrow{H_2O_2 \text{ or } {}^\circ OH} \underset{\text{Acetate}}{CH_3.CO_2^-} + CO_2 \quad (4)$$

Thiol determination using the chromophoric DTNB reagent gave levels of 1.9 and 9.2 µM for two SFUF samples examined, values similar to that of a human serum albumin ultrafiltrate (3.4 µM). These data indicate that thiol detectable in SFUF is at least partially ascribable to the passage of small levels of albumin through the ultrafiltration membranes utilised in these investigations.

Samples that were equilibrated with two sequential additions of 0.20 mM H_2O_2 with a 24 hr. interval between them (n = 4) displayed no further modifications subsequent to the first H_2O_2 treatment. As expected, pre-incubation of two of the SFUF samples which gave a virtually stoichiometric conversion of pyruvate to acetate following H_2O_2 treatment with the °OH radical scavenger 5-

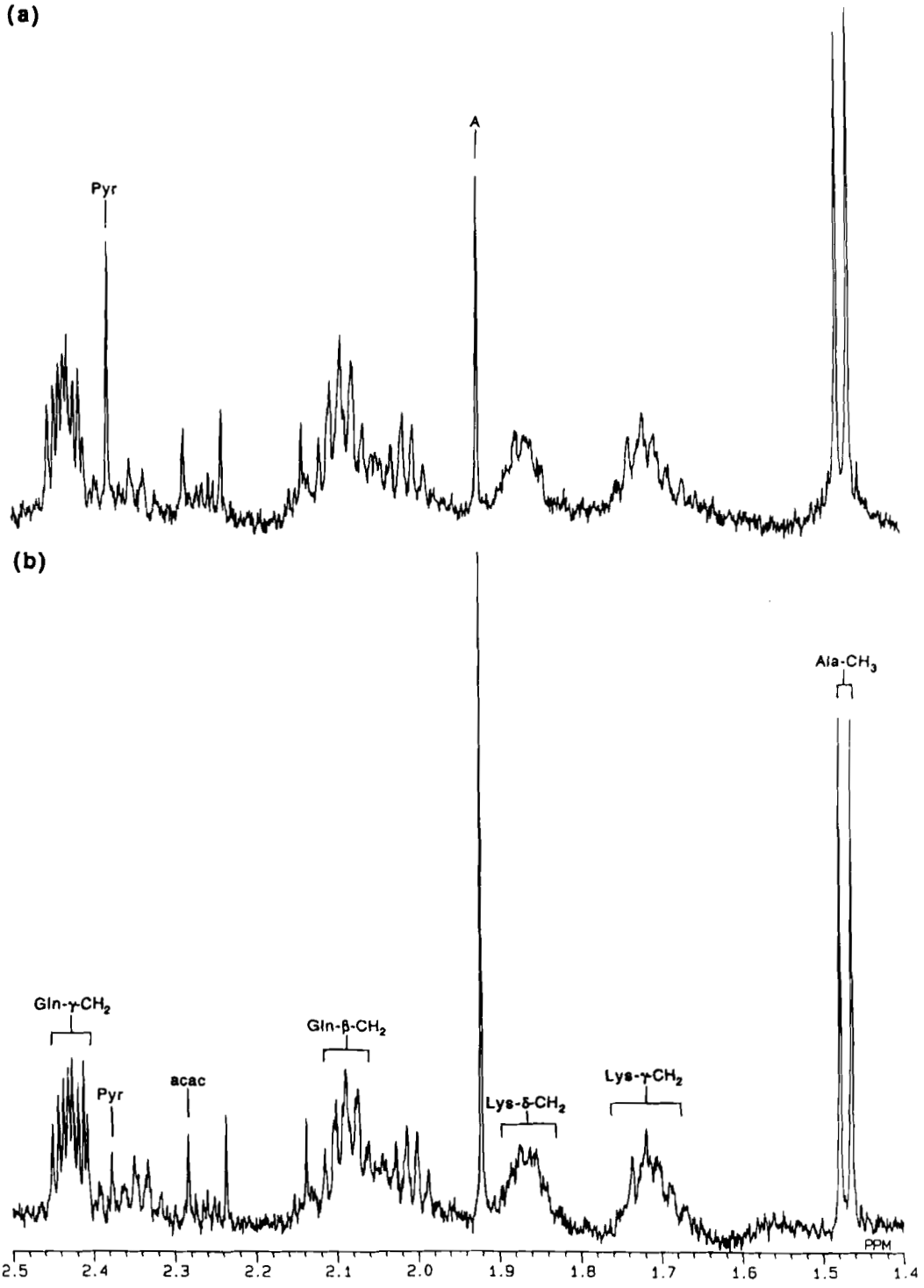

FIGURE 2 1.40–2.50 ppm regions of 500 MHz single-pulse ^1H NMR spectra of an inflammatory synovial fluid ultrafiltrate sample obtained (a), before, and (b), after equilibration with 0.20 mM H_2O_2 at ambient temperature for a period of 24 hr.. Typical spectra are shown. The spectra illustrated are those of a sample in which the H_2O_2-induced rise in acetate concentration observed was greater than the reduction in that of pyruvate. Abbreviations: as Figure 1.

aminosalicylate (10.00 mM) failed to suppress the oxidative decarboxylation of pyruvate in any detectable manner. However, their prior treatment with thiourea (5.00 mM), also a potent °OH radical scavenger, prevented ca. 50% of the pyruvate from being oxidised to acetate in each case (data not shown). ^{13}C NMR spectroscopy, however, demonstrated that prolonged equilibration (16 hr.) of a 1.00 M solution of thiourea with increasing concentrations of H_2O_2 (0, 0.01, 0.02, 0.05, 0.10, and 1.00 M) at ambient temperature diminished the intensity of this scavenger's characteristic methyl group signal (δ = 185.0 ppm) and generated a new resonance located at 180.5 ppm which was only detectable at the highest level of added H_2O_2 (data not shown). The intensity of the 180.5 ppm signal expressed relative to that of thiourea was approximately 1:1 in this sample. It is conceivable that this new resonance is attributable to formamidine disulphide, a known thiourea oxidation product.

Statistical analysis of the $\log_e$-transformed control sample metabolite concentration data of Table I by the Aspin-Welch statistic for sample groups with heterogeneous variances demonstrated that the mean SFUF lactate level (3.010 mM) was significantly greater than that of SerUF (1.603 mM), $p < 0.005$. No significant differences between the inflammatory synovial fluid and normal serum ultrafiltrate mean levels of alanine, acetate and pyruvate were found.

The metabolite levels given in Table I presumably reflect only the non-macromolecule-bound fraction of these biomolecules and hence are somewhat lower than those previously reported (e.g., lactate levels in intact inflammatory synovial fluid[36]). Indeed, recent ^1H NMR investigations have established that a pool of 'NMR-invisible', protein-bound metabolites such as lactate can be displaced from these macromolecular binding sites by the addition of high levels of ammonium chloride ($\geq$ 0.5 M) to biofluids.[37] The higher concentrations of lactate present in inflammatory SFUF reflect the hypoxic status of the inflamed rheumatoid joint and also serve to offer increased protection of alternative, critical biomolecules present in this matrix against damage induced by the potent oxidising actions of °OH radical.

A two-sample t-test established that there was no significant difference between the mean pyruvate:acetate concentration ratios of SFUF (1.050) and SerUF (1.107). Tests for variance heterogeneity between the untransformed normal serum and inflammatory synovial fluid ultrafiltrate metabolite levels yielded F ratios (where F = SFUF variance/SerUF variance) of 14.58 ($p << 0.005$), 13.35 ($p << 0.005$) and 9.61 ($p << 0.005$) for lactate, acetate and pyruvate respectively, demonstrating a wide 'between patients' scatter in the SFUF levels relative to those of SerUF. These observations are attributable to the wide variability of clinical expression associated with inflammatory joint diseases.

Treatment of Pyruvate and Lactate with H_2O_2 or Radiolytically-Generated °OH radical in Aqueous Solution

High field ^1H NMR analysis of chemical model systems containing pyruvate or lactate was also conducted in order to further investigate and evaluate the abilities of these endogenous metabolites to scavenge H_2O_2 and °OH radical, the latter generated by γ-radiolysis.

Figure 3 exhibits partial 500 MHz single-pulse ^1H NMR spectra of aqueous sodium pyruvate solutions (10.00 mM) equilibrated with 0, 0.10, 0.25, 0.50 and 1.00 mM H_2O_2. The intensity of the pyruvate-CH_3 group signal at 2.377 ppm decreases with increasing H_2O_2 concentration, a spectral modification which is accompanied by the production of an acetate-CH_3 group resonance (δ = 1.92 ppm), the latter increasing proportionately with increasing levels of added H_2O_2. Indeed, integration of these singlet resonances demonstrated that the H_2O_2-induced oxidative decarboxylation of pyruvate observed was quantitatively accountable for by the concentration of acetate generated (Equation 4). The singlet located at 1.50 ppm in these spectra is attributable to an impurity present in the commer-

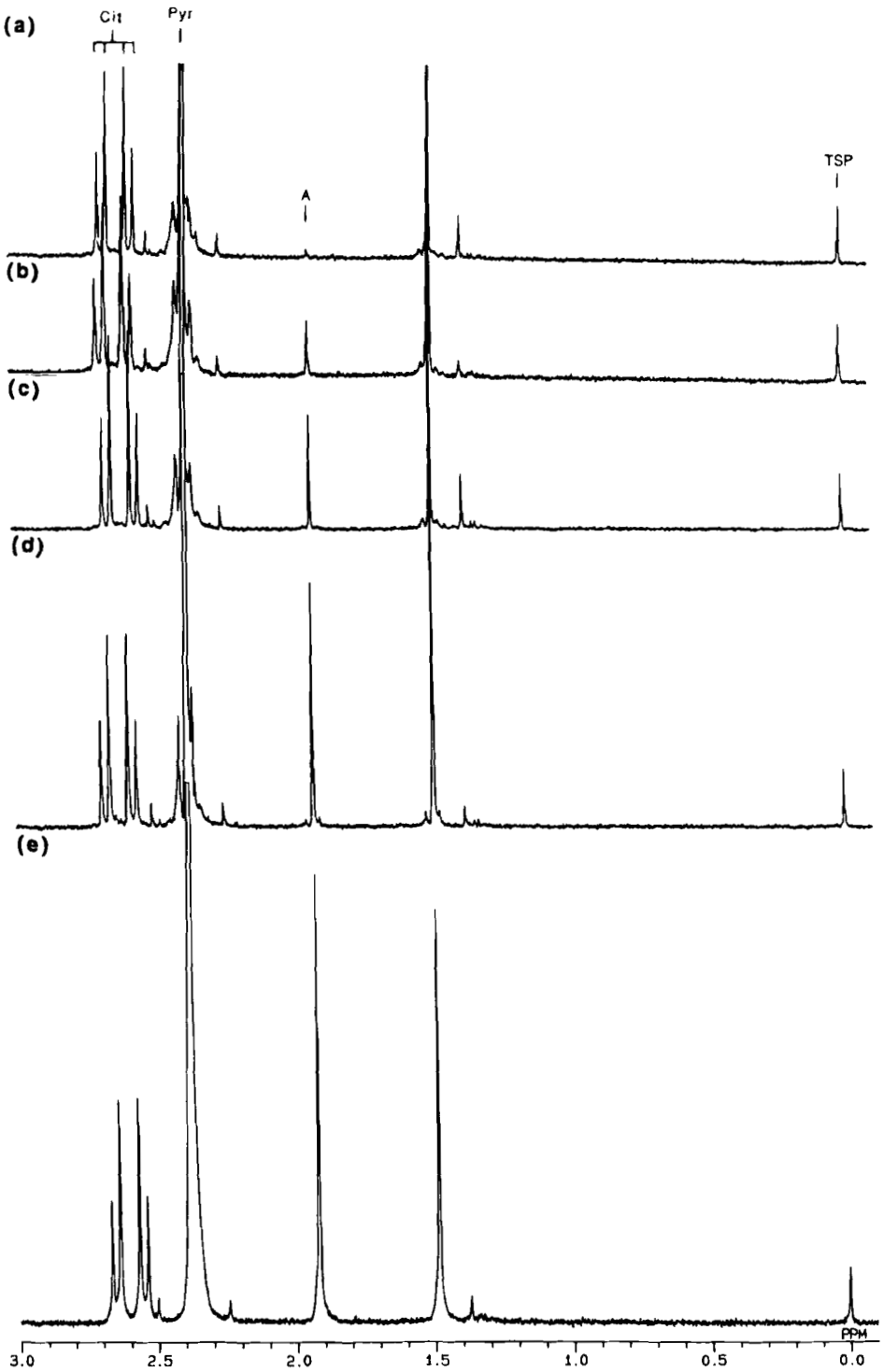

FIGURE 3 Partial 500 MHz ^{1}H NMR spectra of 10.00 mM aqueous solutions of sodium pyruvate (also containing 1.00 mM trisodium citrate) equilibrated with (a) 0, (b) 0.10, (c) 0.25, (d) 0.50 and (e) 1.00 mM H_2O_2 for 24 hr. at ambient temperature. Abbreviations: as Figure 1. The singlet resonance located at 1.50 ppm in these spectra is ascribable to an impurity present in the commercially-available pyruvate sample employed for these investigations.

cially-available pyruvate sample employed for these investigations.

For a reaction mixture containing 10.00 mM pyruvate and 5.00 mM H_2O_2 [in 20 mM phosphate buffer (pH 7.40)] incubated at a temperature of 37.0°C, the conversion of pyruvate to acetate and CO_2 had proceeded almost to completion at a time-point of 6.33 min. after mixing (i.e., consumption of 46% of the initial pyruvate concentration).

In an early pioneering study Holleman[38] demonstrated the ability of pyruvate and alternative α-keto acids to reduce H_2O_2 to H_2O, a reaction involving decarboxylation of the α-keto acid at the one-carbon position. Moreover, more recent physicochemical investigations have demonstrated the thermodynamic favourability and rapid rates of these reactions, and provided detailed information regarding their common mechanism.[39,40]

Untreated aqueous solutions of pyruvate equilibrated in the presence of atmospheric O_2 at ambient temperature for periods of 72 hr. or more were also found to generate acetate, consistent with the slow autoxidation of this α-keto acid anion. However, the concentration of acetate generated via autoxidation over a 24 hr. period was reproducibly very low relative to that observed following H_2O_2 treatment.

Equilibration of lactate (10.00 mM) with H_2O_2 alone (0.10–1.00 mM) generated no ^{1}H NMR-detectable products (data not shown).

As expected, gamma-radiolysis of aqueous pyruvate solutions (10.00 mM) produced high levels of acetate (ca. 1.25 mM), demonstrating that °OH radical as well as H_2O_2 oxidises pyruvate to acetate and CO_2. Low concentrations of formate were also generated. Similar gamma-irradiation treatment of 10.00 mM aqueous solutions of lactate, however, yielded low levels of pyruvate (ca. 90 μM) in addition to approximately 1.0 mM acetate, conforming to the reaction scheme given in equations 3 and 4. Moreover, higher levels of formate were generated on gamma-radiolysis of lactate than those arising from equivalent concentrations of pyruvate. Samples that were previously saturated with N_2O to yield a relatively 'clean' source of °OH radical did not give rise to any significant modifications in the concentration of each NMR-detectable radiolytic product. The 0.00 to 2.50 ppm regions of single-pulse ^{1}H NMR spectra of γ-irradiated pyruvate and lactate solutions are shown in Figure 4, and concentrations of the radiolytic products generated are given in Table II.

DISCUSSION

The multicomponent analytical ability of high field proton NMR spectroscopy offers major advantages over alternative techniques in that it permits the rapid, simultaneous study of the status and levels of a wide range of metabolites present in human body fluids, and generally requires no knowledge of sample composition prior to analysis. These advantages facilitate the rapid characterisation of the molecular nature of components that would not normally be expected to be present in the samples investigated, including those arising from the attack of reactive oxygen species (ROS) on endogenous components.

High field ^{1}H NMR analysis of healthy human serum ultrafiltrates before and after treatment with 0.20 mM H_2O_2 demonstrated that the major oxidant-mediated modification in these samples involved a complete conversion of the α-keto acid anion pyruvate to acetate and CO_2 in all samples examined. For inflammatory synovial fluid ultrafiltrate samples, however, the elimination of pyruvate by added H_2O_2 was quantitatively accounted for by corresponding increases in the concentration of acetate in only 6 out of a total of 11 samples subjected to ^{1}H NMR analysis in this manner. Indeed, for 4 of the samples investigated, the H_2O_2-induced elevation in acetate concentration was greater than the decrease observed in that of pyruvate, an observation which suggests that such further acetate arises from the reduction of H_2O_2 by a 1-electron reductant to form °OH

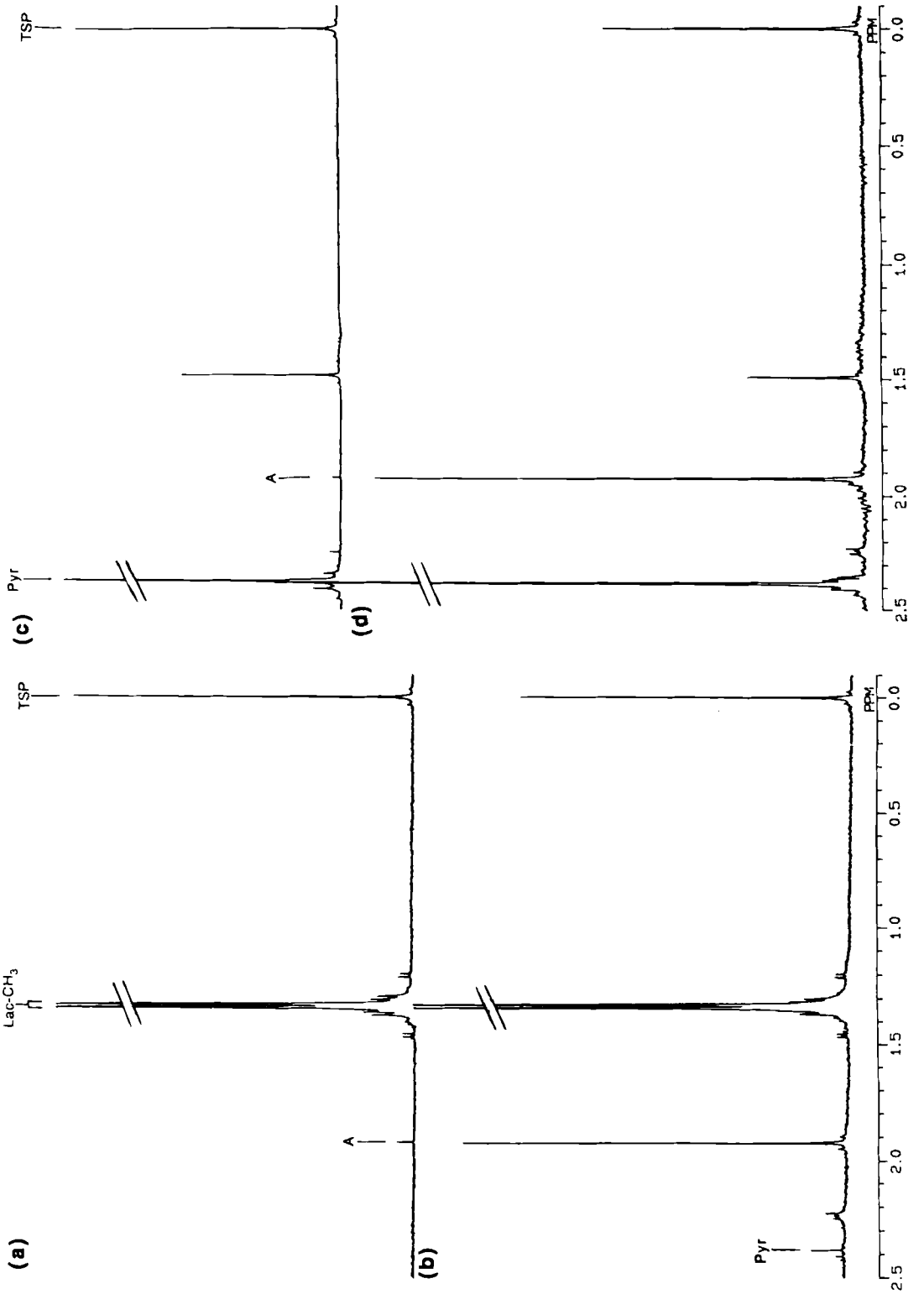

FIGURE 4 Partial 500 MHz ^{1}H NMR spectra of aqueous solutions of pyruvate and lactate obtained before and after gamma-radiolysis (8.00 kGy). Samples were saturated with N_2O prior to gamma-irradiation treatment. (a), 10.00 mM lactate (control); (b), as (a), but following gamma-radiolysis; (c), 10.00 mM pyruvate (control); (d), as (c), but following gamma-radiolysis. Abbreviations: as Figure 1.

TABLE II Concentrations of NMR-detectable products arising from gamma-radiolysis of aqueous solutions of lactate and pyruvate (10.00 mM) both with and without N_2O pre-saturation. The values given have been corrected for trace levels of acetate and formate detectable in samples of gamma-irradiated doubly-distilled water.

Sample	Pre-saturation with N_2O	[A]/mM	[Pyr]/mM	[Form]/mM
10.00 mM Lactate	–	1.08	0.09	0.20
10.00 mM Lactate	+	1.04	0.09	0.22
10.00 mM Pyruvate	–	1.26	8.64	0.40
10.00 mM Pyruvate	+	1.23	8.31	0.40

radical which in turn participates in the two-stage reaction sequence depicted in Equations 3 and 4.

Experiments on chemical model systems involving the treatment of aqueous solutions of pyruvate and lactate with H_2O_2 or °OH radical-generating systems demonstrated the powerful °OH radical scavenging ability of lactate (second-order rate constant, $k_2 = 4.8 \times 10^9$ mol.$^{-1}$dm^{3}s^{-1}[41]), further confirming the importance of the above 2-step reaction sequence. These model systems were found to be particularly appropriate in terms of their ability to successfully mimic ROS-mediated oxidative damage to these components present in intact inflammatory SFUF samples.

Suitable candidates for the electron-donor required to effect the conversion of H_2O_2 to °OH radical in synovial fluid ultrafiltrates include ascorbate (giving rise to the ascorbyl radical), low-molecular-mass thiols such as cysteine and glutathione (generating corresponding thiyl radicals), and trace levels of 'catalytic' iron(II)-complexes. However, the concentrations of (reduced) ascorbate in rheumatoid synovial fluid are extensively depleted,[42] an observation consistent with the marked susceptibility of this essential nutrient to oxidative damage mediated by excessive ROS production, and indicative of its diminished ability to promote the deleterious generation of °OH radical from H_2O_2 in this environment. Levels of the 'free' amino acid cysteine in inflammatory synovial fluid are not known to us, but are approximately 20 μM in healthy human blood plasma.[43] However, this thiol is not readily NMR-detectable in complex biofluid sample mixtures in view of its higher-order coupling pattern (ABX). Estimates of reduced glutathione (GSH) concentrations in normal human plasma have shown it to be present in only trace quantities (ca. 1–2 μM).[44]

The above observations, however, appear to be consistent with previous reports by Rowley *et al.*[4] and Gutteridge[45] that approximately 40% of inflammatory synovial fluids contain trace levels of 'catalytic', bleomycin-detectable iron complexes with the ability to promote °OH radical generation from phagocytically-generated H_2O_2. Indeed, recent observations have suggested that at least some of this non-transferrin-bound iron is present as iron-citrate complexes.[46]

Any °OH radical generated in SFUF samples from added H_2O_2 may also attack the amino acid alanine to form pyruvate by the reaction sequence depicted in equations 6–8.[47] Consumption of pyruvate produced via this mechanism by H_2O_2 (Equation 4) may also account for the observation that added H_2O_2 induces an increase in acetate concentration that is greater than the decrease in that of pyruvate observed in certain of the SFUFs examined. However, alanine levels were unaffected by added H_2O_2 in all biofluid ultrafiltrate samples investigated.

$$H_3N^+CH(CH_3)CO_2^- + {}^\circ OH \Rightarrow H_3N^+{}^\circ C(CH_3)CO_2^- + H_2O \quad (6)$$

$$2H_3N^+{}^\circ C(CH_3)CO_2^- \Rightarrow H_2N^+= {}^\circ C(CH_3)CO_2^- + H_3N^+CH(CH_3)CO_2^- \quad (7)$$

$$H_2N^+={}^\circ C(CH_3)CO_2^- + H_2O \Rightarrow CH_3COCO_2^- + NH_4^+ \quad (8)$$

Unfortunately, the procurement of normal synovial fluid samples was perturbed by the very small volumes (≤0.3 ml) obtainable from normal knee-joints, and hence we have not been able to compare and contrast H_2O_2-induced modifications in the ^1H NMR profiles of normal and inflammatory synovial fluid ultrafiltrates at this stage.

The H_2O_2-scavenging properties of pyruvate and further α-keto acids are well known, and O'Donnell-Tormey *et al.*[48] have elegantly demonstrated the specificity of H_2O_2 consumption by pyruvate, 2-oxoglutarate and oxaloacetate using gas chromatography coupled with mass spectrometric detection. This unique H_2O_2-scavenging ability suggests an important role for α-keto acids as antioxidants in biological systems. Indeed, earlier investigations demonstrating the cytoprotective effects of pyruvate or pyruvate-containing cell culture media[18–21] lend further support to this hypothesis. Intriguingly, Salahudeen *et al.*[29] have recently presented experimental data regarding the ability of pyruvate to protect renal tissue against H_2O_2-mediated injury and suggested a potential therapeutic role for it in diseases in which an elevated production of H_2O_2 is implicated.

The data presented here also provide much evidence to suggest that ROS-mediated oxidative damage to macromolecules (e.g. proteins) and low-molecular-mass components in inflammatory synovial fluid will be largely circumventable by (1) prior consumption of H_2O_2 by available pyruvate, and (2) scavenging of °OH radical (arising from the single electron reduction of any residual H_2O_2) by lactate which is present at very high levels in this biofluid and reacts extremely rapidly with this radical species. However, it should be noted that possible exceptions to this proposal include those involving a localised, 'site-specific' generation of °OH radical possibly arising via the reaction of 'catalytic' redox-active transition metal ions complexed by either high- or low-molecular-mass endogenous chelating ligands, e.g. Cu(II) ion-induced oxidative damage to human serum albumin is localised to the three N-terminal amino acids (H_2N-*Asp.Ala.His*-)[49] which cooperatively provide a powerful binding site for this metal ion.[50] Moreover, reactions of an autocatalytic, self-perpetuating nature (i.e. those which require only very low levels of °OH radical to initiate relatively high levels of damage) such as lipid peroxidation are also possible exceptions.

The above observations indicate the possible therapeutic applications of pyruvate, 2-oxoglutarate, oxaloacetate and synthetic α-keto acids in inflammatory joint diseases. Indeed, perturbations of H_2O_2-mediated oxidative damage to biomolecules in synovial fluid and ultimately, adjacent tissue, may prove to be an effective therapeutic avenue in these conditions, together with those in which H_2O_2-dependent 'oxidative stress' has also been incriminated.

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