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The Role of N-Acetylcysteine in Protecting Synovial Fluid Biomolecules Against Radiolytically-mediated Oxidative Damage: A High Field Proton NMR Study

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High field proton (^{1}H) NMR spectroscopy has been employed to evaluate the abilities of the antioxidant thiol drug N-acetylcysteine and exogenous cysteine to protect metabolites present in intact inflammatory synovial fluid samples against oxidative damage arising from gamma-radiolysis (5.00 kGy) in the presence of atmospheric O_2. Although oxidation of urate to allantoin by radiolytically-generated $^{\bullet}$OH radical was readily circumventable by pre-treatment of synovial fluids with N-acetylcysteine (1.00 or 3.00×10^{-3} mol · dm^{-3}) or cysteine (1.00, 2.00 or 5.00×10^{-3} mol · dm^{-3}), both thiols offered only a limited protective capacity with respect to hyaluronate depolymerisation and the production of formate from carbohydrates in general. Radiolytic products generated from the added thiols (predominantly their corresponding disulphides) were simultaneously detectable in ^{1}H Hahn spin-echo spectra of gamma-irradiated synovial fluids, permitting a quantitative evaluation of the radioprotective capacity of these agents. It is concluded that the multicomponent analytical ability of high field ^{1}H NMR spectroscopy provides much useful molecular information regarding mechanisms associated with the radioprotectant actions of thiols in intact biofluids.

Keywords: N-acetylcysteine, antioxidant, $^{\bullet}$OH radical, radiolytic products, synovial fluid, ^{1}H NMR

INTRODUCTION

It has been known for over 40 years that certain thiols and disulphides exhibit a radioprotective ability if administered prior to exposure to sources of ionising radiation,[1–3] a phenomenon which presents the possibility that such compounds are able to confer protection of living organisms against the low levels to which they are being continuously exposed. Thiols such as cysteamine may act in this capacity by playing a role in neutralising the toxic effects of hydroxyl radical ($^{\bullet}$OH) (Eq. (1)) or, alternatively, by repairing damage to biomolecules oxidatively modified

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by $^{\bullet}OH$ radical (Eqs. (2) and (3)).

$$RSH + {}^{\bullet}OH \rightarrow RS^{\bullet} + H_2O \quad (1)$$

$$RH + {}^{\bullet}OH \rightarrow R^{\bullet} + H_2O \quad (2)$$

$$R^{\bullet} + RSH \rightarrow RH + RS^{\bullet} \quad (3)$$

Further radiolytically-induced chemical modifications to thiols include the subsequent dimerisation of thiyl radicals ($RS^{\bullet}$) to form the corresponding disulphide[4] (RSSR) (Eq. (4)) and the interaction of $RS^{\bullet}$ radicals with dioxygen to produce thiylperoxy radical species (Eq. (5)) which have been demonstrated in pulse radiolysis experiments.[5–7] In addition, sulphenyl radicals ($RSO^{\bullet}$) are also generated in the presence of O_2.[8] Radical ion dimers ($RSSR^{\bullet -}$) can arise from the reaction of thiyl radicals with further thiol[9] (Eq. (6)) or from the reduction of disulphides by radiolytically-generated aquated electrons ($e^-_{(aq.)}$) in systems depleted of O_2[10] (Eq. (7)).

$$2RS^{\bullet} \rightarrow RSSR \quad (4)$$

$$RS^{\bullet} + O_2 \rightarrow RSO_2^{\bullet} \quad (5)$$

$$RS^{\bullet} + RSH \rightarrow RSSR^{\bullet -} + H^+ \quad (6)$$

$$e^-_{(aq.)} + RSSR \rightarrow RSSR^{\bullet -} \quad (7)$$

However, Wefer and Sies[11] have presented evidence consistent with the oxidation of glutathione by superoxide anion ($O_2^{\bullet -}$) to its disulphide together with smaller quantities of sulphonate, a reaction which yields the aggressively-reactive singlet oxygen. Moreover, Aruoma *et al.*[12] have proposed that in the presence of O_2, sulphur-containing radical species arising from the interaction of physiologically-generated oxidants with penicillamine can inactivate α_1-antiprotease.

The antioxidant actions of N-acetylcysteine in a wide range of experimental systems are now well established. Examples of its ability to suppress oxidative damage in biological systems include inhibition of endotoxin-induced lung damage,[13] reduction of membrane damage by superoxide generating systems in porcine aortic endothelial cells,[14] protection of animals against paracetamol hepatotoxicity,[15] alleviation of diquat toxicity to hepatocytes,[16] and prevention of damage to human bronchial fibroblasts by tobacco smoke condensates.[17] Indeed, N-acetylcysteine has been employed as an effective therapeutic agent for the treatment of a range of human respiratory diseases.[18] Postulated mechanisms of action for this thiol drug include its ability to (1) increase intracellular concentrations of cysteine and hence glutathione and (2) scavenge oxidants such as $O_2^{\bullet -}$, hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl),[17] the latter of which is generated by the myeloperoxidase (MPO)–H_2O_2–Cl^- system.

Aruoma *et al.*[19] have recently investigated the antioxidant action of N-acetylcysteine with respect to its reactions with $^{\bullet}OH$ radical, $O_2^{\bullet -}$, H_2O_2 and HOCl, and have concluded that it reacts rapidly with $^{\bullet}OH$ radical (second-order rate constant, $k_2 = 1.36 \times 10^{10}\,mol^{-1} \cdot dm^3 \cdot s^{-1}$), is a powerful scavenger of HOCl, but reacts very slowly with H_2O_2 (at 25°C and pH 7.4). No evidence for the reaction of N-acetylcysteine with $O_2^{\bullet -}$ was obtained in these studies.

In view of the large amount of evidence available for the deleterious role of reactive oxygen-derived species in the pathogenesis of inflammatory joint diseases (reviewed in [20–22]), it is conceivable that N-acetylcysteine may have a therapeutic role to play here by modulating oxidative damage to endogenous biomolecules arising from the actions of such radicals *in vivo*. Indeed, the diminished antioxidant status of inflammatory synovial fluids renders biomolecules therein particularly susceptible to damage by biologically-generated oxidants.

We have previously reported the application of high field proton (^1H) Hahn spin-echo nuclear magnetic resonance (NMR) spectroscopy to the simultaneous detection of a variety of products derived from the reactions of radiolytically-generated reactive oxygen species (specifically $^{\bullet}OH$ radical) with components present in inflammatory knee-joint synovial fluid samples (e.g., low-molecular-mass oligosaccharides arising from hyaluronate fragmentation).[23] In this study

we have employed high field ^{1}H NMR spectroscopy to assess the ability of N-acetylcysteine to suppress radiolytically-mediated oxidative modifications to biomolecules present in inflammatory human knee-joint synovial fluid *in vitro*. The action of the naturally-occurring amino acid cysteine in regulating such oxidative damage has also been investigated in this manner for purposes of comparison.

MATERIALS AND METHODS

Reagents

N-acetyl-L-cysteine, L-cysteine and L-cystine were obtained from Sigma Chemical Co. (UK). All other reagents employed were of the highest possible grade and obtained from commercially available sources.

Synovial Fluid Samples

Knee-joint synovial fluid samples were drawn into clear sterile plastic tubes for therapeutic purposes from a total of 10 rheumatoid arthritis patients (American Rheumatism Association criteria) with inflamed knees and associated effusions. These samples were centrifuged immediately to remove cells and debris (2,500 r.p.m. for 20 min) and the supernatants either analysed within a few hours after collection, or stored at 4°C for a duration of 18 h. Storage in this manner was shown not to influence the results obtained in NMR experiments.

Synovial fluid supernatants (0.60 ml, $n=5$) were treated with μdm^3 aliquots of an aqueous stock solution of N-acetyl-L-cysteine to yield 1.00×10^{-3} and 3.00×10^{-3} mol · dm^{-3} concentrations of added thiol. Similarly, 0.60 ml portions of further synovial fluid supernatant samples ($n=5$) were treated each with μdm^3 aliquots of an aqueous *L*-cysteine stock solution to give final added thiol concentrations of 1.00, 2.00 and 5.00×10^{-3}mol · dm^{-3}. These samples were equilibrated at 4°C for a period of 18 h prior to gamma radiolysis and ^{1}H NMR analysis. Corresponding control samples containing no added N-acetyl-L-cysteine or L-cysteine were also stored in this manner.

Gamma-radiolysis of Synovial Fluids and Aqueous Thiol Solutions

Synovial fluid samples were subjected to gamma-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 level of 5.00 kGy (dose rate 288.6 Gy h^{-1}). Corresponding synovial fluids 'spiked' with increasing concentrations of N-acetyl-L-cysteine or L-cysteine, together with phosphate-buffered 2.00×10^{-2} mol · dm^{-3} aqueous solutions of N-acetyl-L-cysteine and L-cysteine (pH 7.00) were similarly irradiated.

Under these experimental conditions, the major primary radiolytic products of water are $^{\bullet}$OH ($G=2.7$), $e^-_{(aq.)}$ ($G=2.7$) and H$^{\bullet}$ ($G=0.5$) (Eq. (8)), where the G value denotes the 10^{-6} mol · dm^{-3} concentration of product generated per 10 Gy dosage. In the presence of atmospheric O_2, radiolytically-generated aquated electrons ($e^-_{(aq.)}$) are converted to $O_2^{\bullet-}$ (Eq. (9)).

$$H_2O \wwwarrow {}^{\bullet}OH, e^-_{(aq.)}, H^{\bullet}, H_2, H_2O_2, H_3O^+ \quad (8)$$

$$e^-_{(aq.)} + O_2 \rightarrow O_2^{\bullet-} \quad (9)$$

NMR Measurements

Proton NMR measurements on control and thiol-treated synovial fluid samples prior and subsequent to gamma-radiolysis were conducted on a JEOL JNM-GSX 500 (University of London Intercollegiate Research Services (ULIRS), Biomedical NMR Centre, Birkbeck College, London, UK) spectrometer operating in quadrature detection mode at 500 MHz for ^{1}H. All spectra were recorded at a probe temperature of 293 K.

Typically, 0.60 ml of sample was placed in a 5-mm diameter NMR tube and 0.07 ml of ^2H_2O was added to provide a field frequency lock.

The broad protein resonances were suppressed by the Hahn spin-echo sequence ($D[90°x–t–180°y–t$-collect]), which was repeated 128 times with $t = 60$ ms. The spectral width for these Hahn spin-echo spectra was 5,100 Hz. The intense water signal was suppressed by pre-saturation via gated decoupling during the delay between pulses. Chemical shifts were referenced to external sodium 4,4-dimethyl-4-silapentanoate-2,2,3,3-$^2H_4$ (TSP, $\delta = 0.00$ ppm). The methyl group resonances of alanine (1.487 ppm), lactate (1.330 ppm) or valine (1.050 ppm) served as secondary internal references in biofluid spectra.

One dimensional, single-pulse ^{1}H NMR spectra of control and gamma-irradiated L-cysteine solutions (containing 10.4% (v/v) added ^2H_2O) were also obtained on the JEOL JNM-GSX spectrometer using a pulse angle of 30–40° and a total delay between pulses of 3 s to allow full spin-lattice (T_1) relaxation of the protons in the samples investigated. Each spectrum corresponded to 49 free induction decays (FIDs) using 32,768 data points. The intense water signal was suppressed by pre-saturation via gated decoupling during the delay between pulses. Spectra were referenced to external TSP. ^2H_2O (0.07 ml) was added to 0.60 ml aliquots of control and gamma-irradiated N-acetyl-L-cysteine solutions and single-pulse ^{1}H NMR spectra of these samples were acquired using a Bruker WH 400 spectrometer (ULIRS Facility, Queen Mary and Westfield College, London, UK). Each spectrum corresponded to 65 FIDs using 6,557 data points, 30–40° pulses and a 3 s pulse repetition rate. The large water signal was suppressed as described above. Spectra were recorded at ambient probe temperature (293 K) and also referenced to external TSP.

RESULTS

Gamma-radiolysis of Aqueous Thiol Solutions

^{1}H NMR spectra (400 MHz) of control and gamma-irradiated (5.00 kGy) aqueous solutions of N-acetylcysteine demonstrated that N-acetylcystine was a major product derived from the attack of radiolytically-generated $^{\bullet}OH$ radical and, to a lesser extent, $O_2^{\bullet-}$ on the thiol. The ABX coupling system of this disulphide was readily detectable in spectra of gamma-irradiated samples (Figure 1). This product was not detectable in the solution of N-acetylcysteine allowed to equilibrate at 4°C in the presence of atmospheric O_2 for 18 h, indicating the high stability of this thiol under these conditions. A singlet resonance of low intensity located at 8.27 ppm was also present in spectra of gamma-irradiated N-acetylcysteine solutions, a signal which is presumably attributable to an amide (–NH–CO–CH_3) proton present in an alternative radiolytic product. Moreover, a small quantity of acetate (singlet at 1.93 ppm) was generated from the radiolytic degradation of the acetamido (–$NHCOCH_3$) functional group of the molecule, and a doublet centred at 1.41 ppm was present in spectra of gamma-irradiated samples, further indicating the diversity of radiolytic products arising from this thiol.

Gamma-radiolysis of aqueous cysteine solutions gave rise to cystine as the predominant radiolytic product. Its characteristic ABX coupling pattern was readily detectable in spectra of irradiated samples (Figure 2), and a reference spectrum of a solution of an authentic sample of L-cystine in ^2H_2O confirmed the identity of this product. In contrast to results obtained with N-acetylcysteine, incubation of aqueous solutions of cysteine in an O_2 atmosphere at 4°C for a period of 18 h was found to produce a small amount of cystine from the parent thiol, a product which presumably arises from its autoxidation via Eq. (10), followed by a combination of thiyl radicals generated to produce the corresponding disulphide (Eq. (4)).

$$RS^- + O_2 \rightarrow RS^{\bullet} + O_2^{\bullet-} \qquad (10)$$

After allowing for the small quantity of cystine already present in the control (unirradiated) cysteine solution, the intensity of the cystine-CH

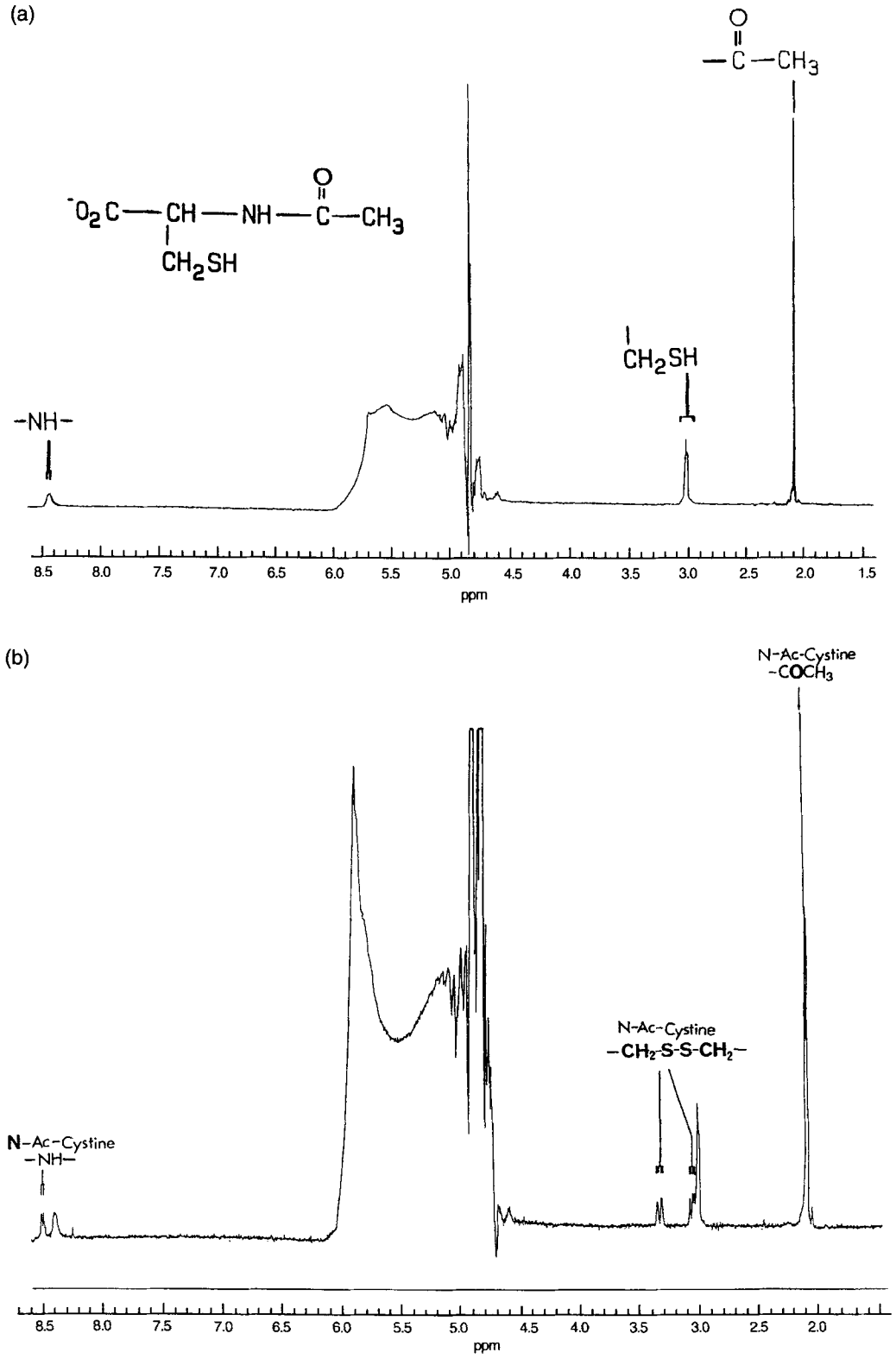

FIGURE 1 Single-pulse (400 MHz) ^{1}H NMR spectra of a 2.00×10^{-2} mol · dm^{-3} aqueous solution of N-acetyl-L-cysteine containing 2.00×10^{-2} mol · dm^{-3} phosphate buffer (pH 7.00) obtained (a) before and (b) after gamma-radiolysis at a dose level of 5.00 kGy. Abbreviations: A, acetate-CH_3.

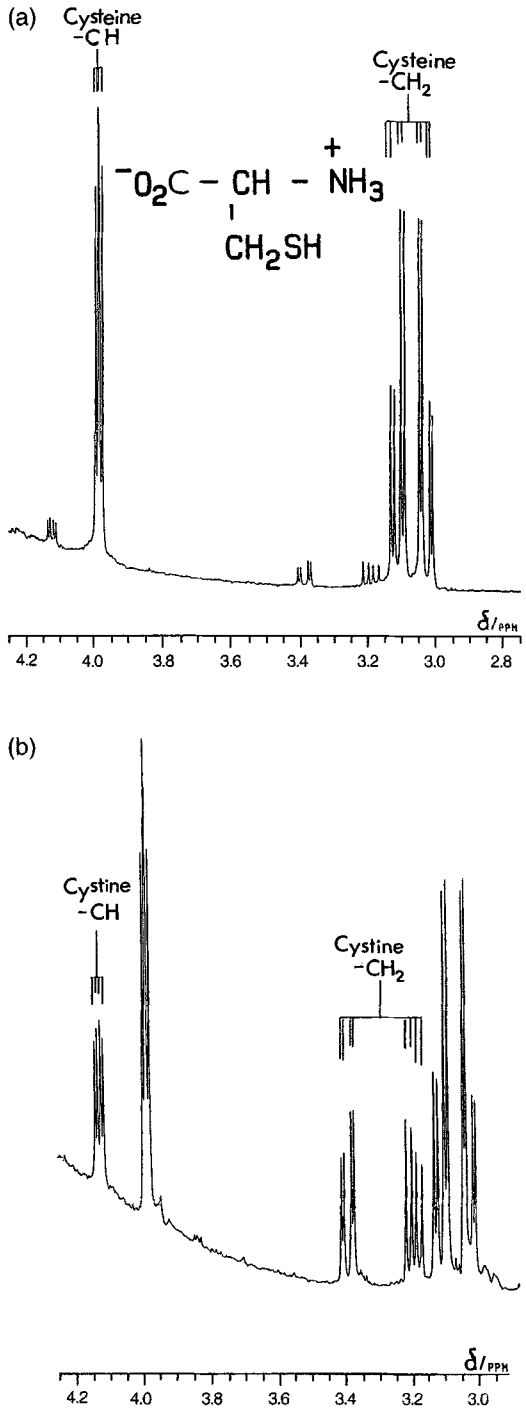

FIGURE 2 Single-pulse (500 MHz) ^{1}H NMR spectra of a 0.10 mol·dm^{-3} aqueous solution of L-cysteine containing 2.00×10^{-2} mol·dm^{-3} phosphate buffer (pH 7.00) obtained (a) prior and (b) subsequent to gamma-radiolysis (5.00 kGy).

proton resonance relative to that of the remaining cysteine in gamma-irradiated solutions was 0.28 ± 0.03 (mean ± standard error, $n = 4$) demonstrating a $22 \pm 2\%$ conversion of cysteine to its disulphide under these experimental conditions.

Hence, from these observations it is clear that the major products derived from gamma-radiolysis of aqueous solutions of both N-acetylcysteine and cysteine in this manner are the corresponding disulphides. The sulphonates of these thiols were not detected by ^{1}H NMR spectroscopy in our experiments.

Radioprotective Ability of N-acetylcysteine Towards Components Present in Inflammatory Synovial Fluids

As previously documented, modifications in the high field region of ^{1}H Hahn spin-echo NMR spectra mediated by radiolytically-generated $^{\bullet}$OH radical consisted of the production of an intense singlet resonance located at 2.032 ppm attributable to the N-acetyl-C$\underline{H}_3$ group protons of low-molecular-mass N-acetylglucosamine-containing oligosaccharide fragments derived from depolymerisation of the glycosaminoglycan hyaluronate, together with a marked rise in the concentration of non-protein-bound acetate, the latter predominantly arising from the reaction of $^{\bullet}$OH radical with the high levels of lactate present.[23] Smaller quantities of acetate are derived from the oxidative decarboxylation of pyruvate by radiolytically-generated H_2O_2 and/or further $^{\bullet}$OH radical. In addition, a singlet resonance located at 2.74 ppm of unknown identity was also generated subsequent to gamma-radiolysis.

The high field (aliphatic) region of typical ^{1}H Hahn spin-echo NMR spectra of control and gamma-irradiated (5.00 kGy) synovial fluid samples are shown in Figure 3. In Hahn spin-echo experiments, the amplitude of the observed signal (echo) at a time-point of $2\tau_2$ following the primary 90° pulse is critically dependent on (1) the intrinsic τ_2 value of the observed nuclear resonance,

(2) homonuclear spin–spin coupling (a phenomenon that can also give rise to phase modulation), and (3) a factor derived from diffusion of the molecule to a different applied magnetic field region during the re-focusing phase.[26] Moreover, at a τ_2 value of 60 ms ($\frac{1}{2}J$), doublets with J values of approximately 8.2 Hz, together with quartets, are inverted, whereas singlets and triplets remain positive (upright).[27] Subsequently, a time period of 120 ms elapses, sufficient to ensure that the net magnetisation associated with the majority of broad macromolecule resonances (i.e., those of short τ_2 values) decays to zero and hence do not contribute to the spectrum acquired.

Modifications in the low field (aromatic) region of these spectra arising from the potent oxidising actions of radiolytically-generated $^{\bullet}OH$ radical included the generation of (1) formate (singlet at 8.46 ppm) from carbohydrates in general (predominantly glucose), and (2) allantoin (doublet at 5.40 ppm) from urate (Figure 4), as previously reported.[23]

Equilibration of synovial fluid samples with 1.00 or 3.00×10^{-3} mol · dm^{-3} N-acetyl-L-cysteine prior to gamma-radiolysis exerted a selective, concentration-dependent influence on the quantities of NMR-detectable radiolytic products generated. At an added N-acetylcysteine concentration of 1.00×10^{-3} mol · dm^{-3}, the magnitudes of the allantoin-CH and formate-H signals were significantly diminished (Figure 4), whereas those of the acetate-CH_3 group and 2.74 ppm singlet resonances remained unaffected (Figure 3). On increasing the level of added N-acetylcysteine to 3.00×10^{-3} mol · dm^{-3}, the intensity of the allantoin-CH signal was not further reduced. However, the levels of NMR-detectable formate and acetate were elevated above those observed at an added N-acetylcysteine concentration of 1.00×10^{-3} mol · dm^{-3},

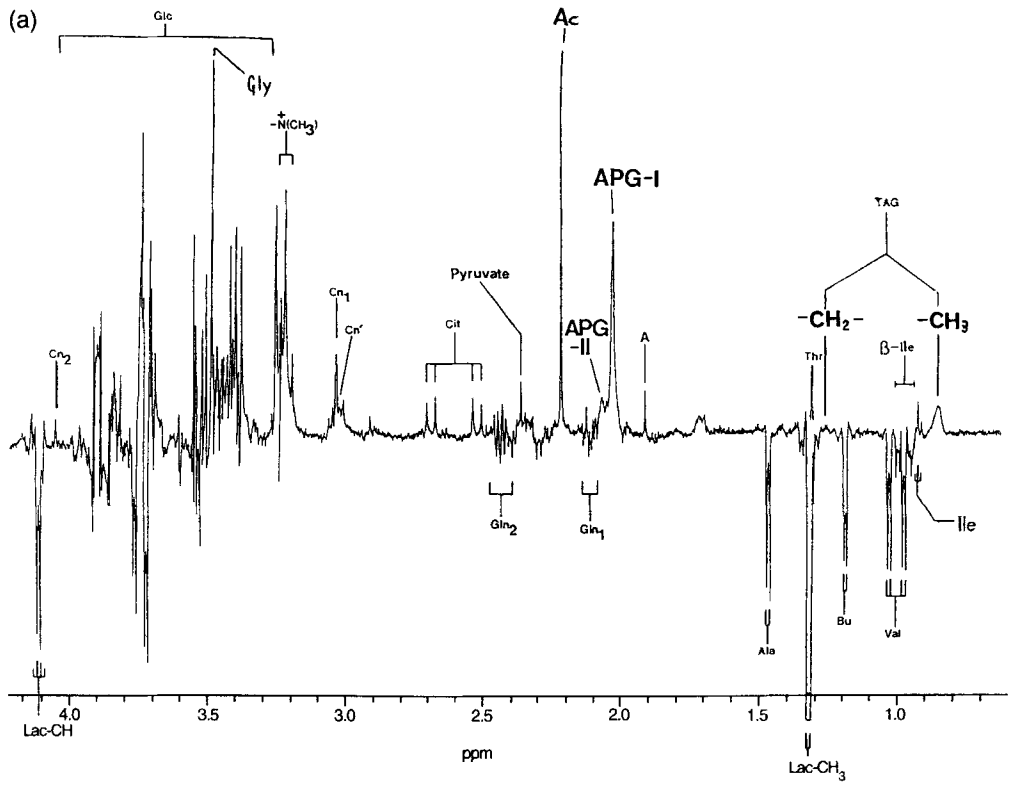

FIGURE 3(a)

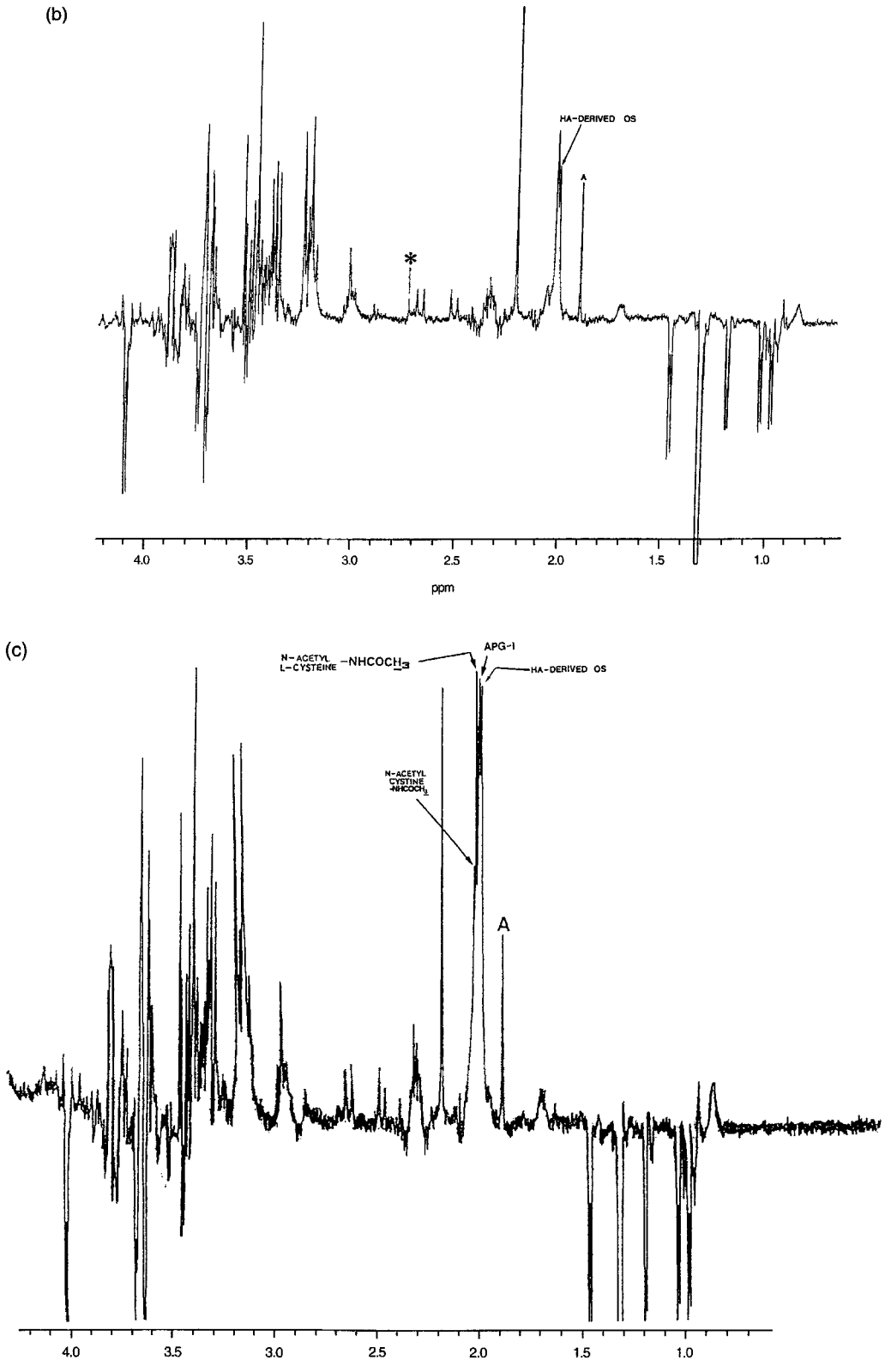

FIGURE 3(b) and (c)

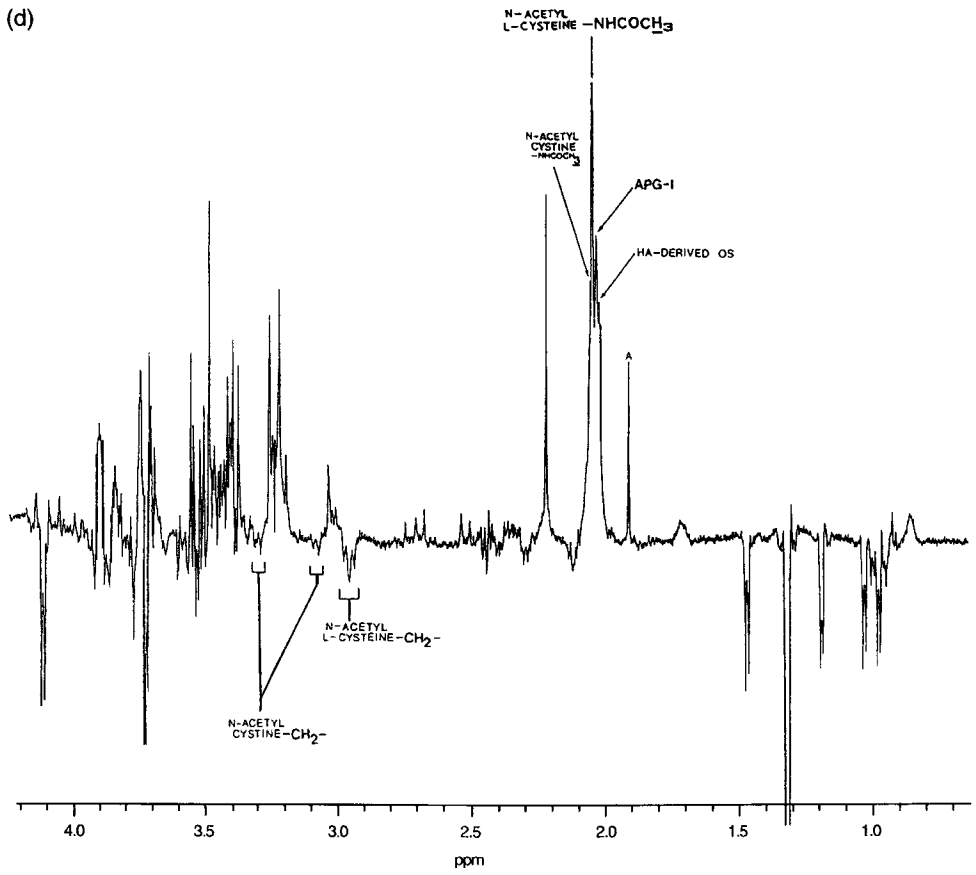

FIGURE 3(d)

FIGURE 3 High field (aliphatic) region of 500 MHz ^{1}H Hahn spin-echo NMR spectra of (a) a typical inflammatory synovial fluid sample; (b) as (a), but following gamma-radiolysis (5.00 kGy); (c), as (b), but treated with 1.00×10^{-3} mol·dm^{-3} N-acetylcysteine prior to gamma-radiolysis; (d), as (b), but treated with 3.00×10^{-3} mol·dm^{-3} N-acetylcysteine prior to gamma-radiolysis. Typical spectra are shown. Abbreviations: A, acetate-CH_3; Ac, acetone-CH_3; Ala, alanine-CH_3: APG-I and II; N-acetyl sugars present in the 5,5′- and 2,7-positions, respectively, of the molecularly-mobile carbohydrate side-chains of 'acute-phase' glycoproteins (predominantly α_1-acid glycoprotein); Bu, 3-D-hydroxybutyrate-CH_3; Cit, citrate-CH_2; Cn_1 and Cn_2, creatinine-N-CH_3 and -CH_2 groups respectively; Glc, glucose carbohydrate ring proton resonances; Gln_1, and Gln_2, β- and γ-CH_2 groups of glutamine respectively; Gly, glycine-CH_2; HA-derived OS, N-acetyl-CH_3 groups of N-acetylglucosamine residues present in oligosaccharide fragments arising from the radiolytic depolymerisation of hyaluronate; Ile and β-Ile, isoleucine terminal-CH_3 and β-CH_3 groups respectively; Lac-CH_3 and Lac-CH, lactate-CH_3 and -CH groups; -$N(CH_3)_3$, -$N(CH_3)_3$ groups of betaine, carnitine and choline; TAG-CH_3 and TAG-CH_2–, acyl chain terminal-CH_3 and bulk $(-CH_2-)_n$ groups respectively of chylomicron- and very low-density-lipoprotein (VLDL)-associated fatty acids (predominantly triacylglycerols); Thr, threonine-CH_3; Val, valine-CH_3. The asterisk in spectra (b), (c) and (d) denotes the radiolytically-generated 2.74 ppm singlet resonance.

the former to a value similar to that observed in gamma-irradiated samples that were not pre-equilibrated with the thiol.

In these experiments we were unable to accurately determine the intensity of (i.e., integrate) the singlet resonance attributable to the N-acetyl-CH_3 group protons of N-acetylglucosamine residues present in hyaluronate-derived oligosaccharide fragments since it is located very close to those of the 5,5′-position N-acetylsugars of the molecularly mobile 'acute-phase' glycoprotein carbohydrate side-chains (predominantly α_1-acid glycoprotein), N-acetylcysteine and N-acetylcystine, the latter arising from radiolytic

damage to the added thiol. However, the spectra obtained suggest that the amplitude of this oligosaccharide resonance, when normalised to that of the broader glycoprotein signal centred slightly downfield ($\delta = 2.04$ ppm), is reduced at an added N-acetylcysteine concentration of 3.00×10^{-3} mol · dm^{-3}, suggesting a limited suppression of radiolytically-mediated hyaluronate depolymerisation by this radioprotectant.

The radiolytic product N-acetylcystine was simultaneously detectable in ^{1}H Hahn spin-echo NMR spectra of gamma-irradiated synovial fluid samples pre-treated with N-acetylcysteine, demonstrating the radioprotectant/antioxidant activity of this thiol when present in intact biofluids. A substantial elevation in the concentration of N-acetylcystine generated (measured by the intensity of its –NHCOC$\underline{H}_3$ group proton resonance) was observed in gamma-irradiated synovial fluids on raising that of added N-acetylcysteine from 1.00×10^{-3} to 3.00×10^{-3} mol · dm^{-3}. Normalisation of the intensity of the N-acetylcystine-NHCOC$\underline{H}_3$ group signal to that of the alanine-CH_3 group revealed that

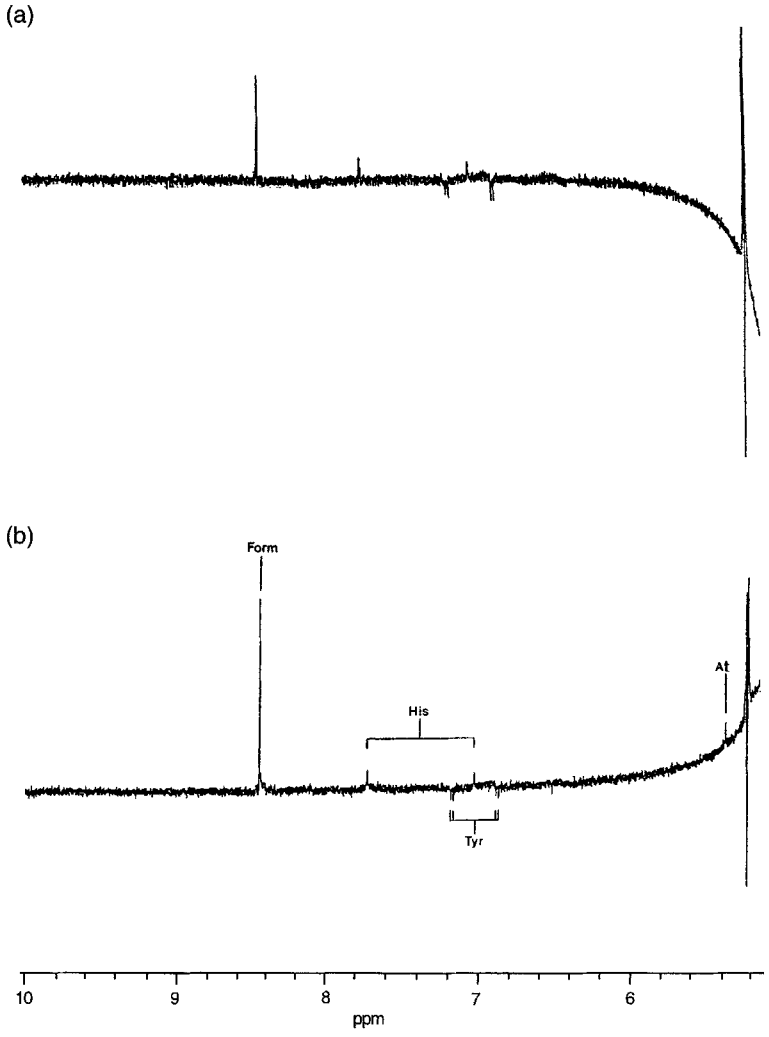

FIGURE 4(a) and (b)

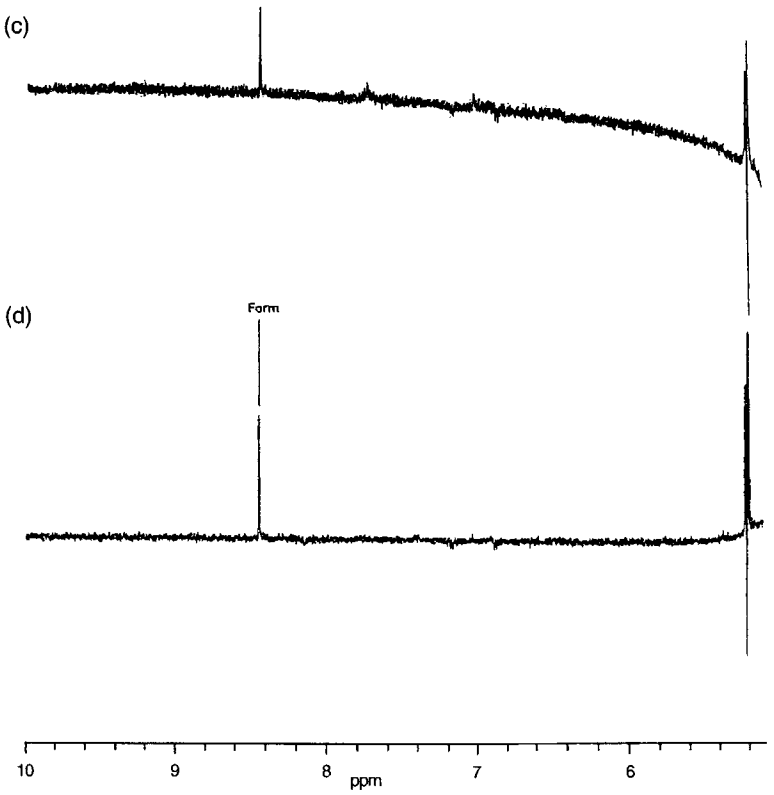

FIGURE 4(c) and (d)

FIGURE 4 Low field (aromatic) region of the 500 MHz ^{1}H Hahn spin-echo NMR spectra shown in Figure 3: (a) untreated (control) synovial; (b) as (a), but subsequent to gamma-radiolysis (5.00 kGy); (c) as (b), but treated with 1.00×10^{-3} N-acetylcysteine prior to gamma-radiolysis; (d) as (b), but treated with 3.00×10^{-3} mol · dm^{-3} N-acetylcysteine prior to gamma-radiolysis. Abbreviations: At, allantoin-CH; Form, formate-H; α-Glc, α-glucose anomeric ring proton; His, histidine imidazole ring protons; Tyr, tyrosine aromatic ring protons.

the level of this radiolytic product generated increased by ca. 60% on elevating the added N-acetylcysteine concentration in this manner. The approximate relative intensity of the N-acetylcystine to N-acetylcysteine-NHCOC$\underline{H}_3$ group resonances was 0.6 at both concentrations of added N-acetylcysteine.

Further evidence for the radiolytically-mediated generation of N-acetylcystine from its thiol precursor was provided by the observation of its magnetically-inequivalent –C$\underline{H}_2$–S–S–C$\underline{H}_2$– proton resonances (part of its ABX coupling system) located at 3.08 and 3.29 ppm in these spectra at an added N-acetylcysteine concentration of 3.00×10^{-3} mol · dm^{-3}. These signals were only barely detectable at an added N-acetylcysteine level of 1.00×10^{-3} mol · dm^{-3}.

Each of the above observations were reproducible in a total of five different synovial fluid samples investigated.

Capacity of Exogenous Cysteine to Protect Synovial Fluid Biomolecules Against Radiolytically-mediated Oxidative Damage

High field ^{1}H NMR analysis demonstrated that equilibrium of knee-joint synovial fluids with increasing concentrations of L-cysteine (1.00, 2.00 or 5.00×10^{-3} mol · dm^{-3}) prior to gamma-irradiation treatment also gave rise to

concentration-dependent modifications in the nature and extent of radiolytic damage to biomolecules therein (Figures 5 and 6).

In the high field region of the resulting Hahn spin-echo spectra, these modifications comprised (1) a minor suppression of acetate production from lactate (and subsequently pyruvate) at the highest concentration of added cysteine employed (5.00×10^{-3} mol · dm^{-3}), and (2) a limited blockage of hyaluronate fragmentation throughout the whole added cysteine concentration range. However, as noted for N-acetylcysteine above, cysteine failed to exert an influence on the intensity of the 2.74 ppm singlet resonance.

Cysteine-induced modifications in the levels of radiolytic products detectable in the low field

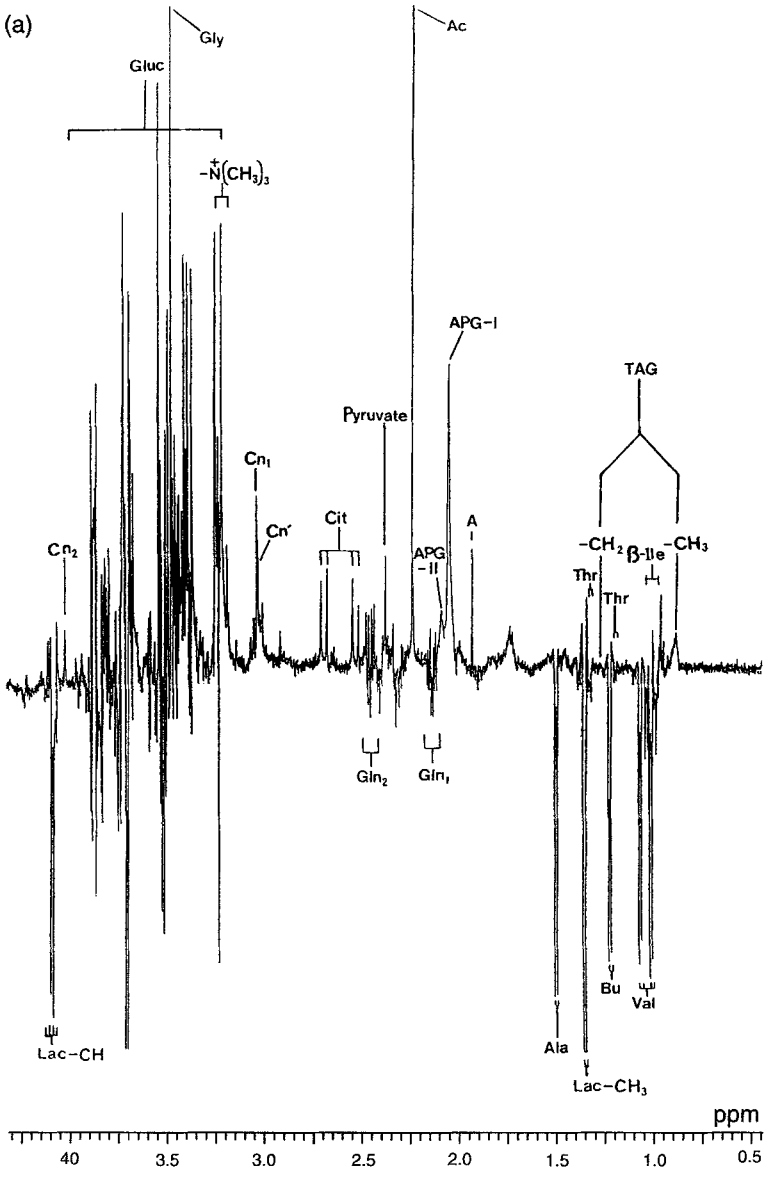

FIGURE 5(a)

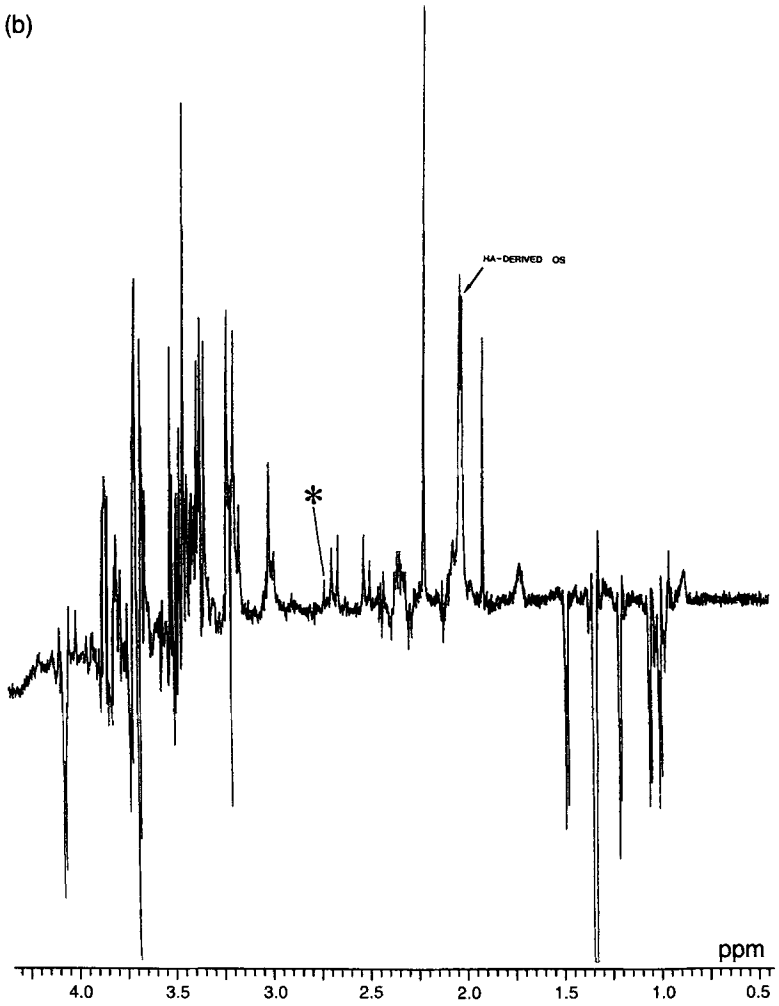

FIGURE 5(b)

region of Hahn spin-echo spectra included a substantial reduction in the concentration of allantoin arising from oxidative damage to urate. Indeed, this radiolytic product was completely undetectable at an added cysteine concentration of 5.00×10^{-3} mol · dm^{-3}. Although a limited inhibition of the level of formate generation was observed at an added concentration of 2.00×10^{-3} mol · dm^{-3}, a marked rise in the intensity of its 8.46 ppm resonance was noted at a cysteine concentration of 5.00×10^{-3} mol · dm^{-3}. This observation may reflect the ability of cysteine, and/or its major radiolytic product cystine, to displace this anionic metabolite from positively-charged protein binding-sites (e.g., lysine or arginine residues) at the highest level of added thiol. Previous ^{1}H NMR investigations have established that a pool of 'NMR-invisible', protein-bound anionic metabolites such as lactate can be mobilised from these macromolecular binding-sites by the addition of high levels of ammonium chloride (1.5 mol · dm^{-3}) to biofluids.[24]

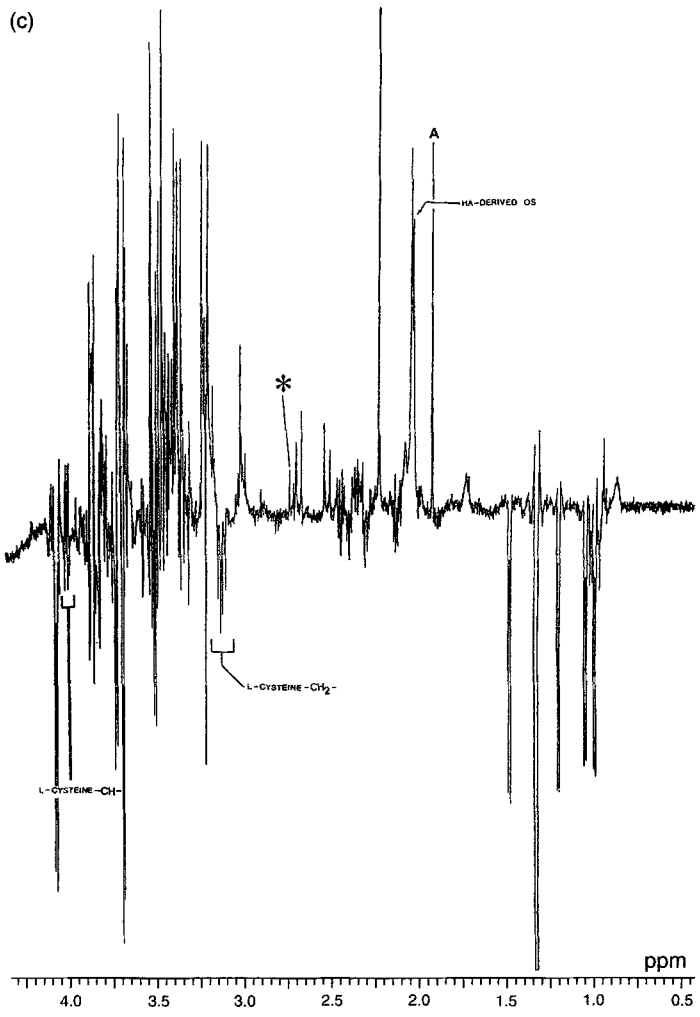

FIGURE 5(c)

Although cysteine itself was readily detectable in ^{1}H Hahn spin-echo spectra of synovial fluid at the lowest concentration added (1.00×10^{-3} mol · dm^{-3}), overlap with the resonances of endogenous metabolites such as glucose prevented detection of the cystine-CH and -CH_2 group signals.

The above modifications in ^{1}H Hahn spin-echo NMR spectra of gamma-irradiated synovial fluids arising from their pre-treatment with 1.00, 2.00 or 5.00×10^{-3} mol · dm^{-3} cysteine were observed in all five samples investigated.

DISCUSSION

The multicomponent analytical ability of high field ^{1}H NMR spectroscopy offers major advantages over alternative analytical techniques in that it permits the rapid, simultaneous study of the status and levels of a wide range of metabolites present in biological samples and generally requires no knowledge of sample composition prior to analysis. As detailed in this investigation, the technique is readily applicable to the facile detection and quantification of a variety of

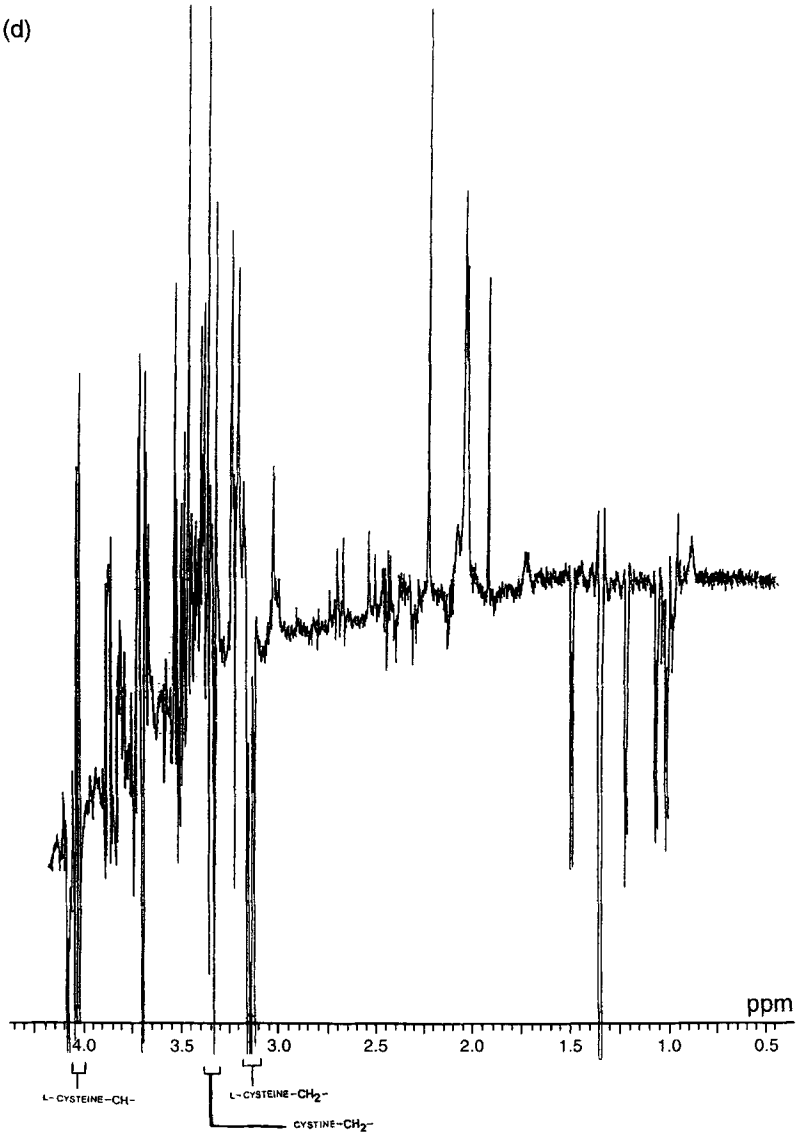

FIGURE 5(d)

FIGURE 5 High field (aliphatic) region of 500 MHz ^{1}H Hahn spin-echo NMR spectra of (a) inflammatory synovial fluid; (b) as (a), but following gamma-radiolysis (5.00 kGy); (c) as (b), but treated with 1.00×10^{-3} mol · dm^{-3} cysteine prior to gamma-radiolysis; (d) as (b), but treated with 5.00×10^{-3} mol · dm^{-3} cysteine prior to gamma-radiolysis. Typical spectra are shown. Abbreviations: as in Figure 3.

products derived from radiolytically-mediated oxidative damage to biomolecules present in intact human body fluids, and allows both qualitative and quantitative assessments of the ability of radioprotectant and/or antioxidant agents to suppress such damage. Indeed, a rapid characterisation of the molecular nature of products arising from the interaction of radiolytically-generated oxygen radical species with exogenous antioxidants while present in whole biological fluids is also achievable, e.g., detection of N-acetylcystine proton resonances in ^{1}H Hahn

spin-echo spectra of knee-joint synovial fluids containing added N-acetylcysteine.

The substantial reduction in the intensity of the allantoin-CH group doublet resonance observed on pre-equilibration of samples with N-acetylcysteine demonstrates that this thiol drug offers protection of synovial fluid urate against oxidative damage induced by the actions of radiolytically-generated $^{\bullet}OH$ radical. However, only limited protection of hyaluronate against $^{\bullet}OH$ radical-mediated fragmentation was observed, consistent with the suggested autocatalytic nature of this reaction.[25] Although N-acetylcysteine suppresses the generation of formate (arising from radiolytic damage to synovial fluid carbohydrates) at an added concentration of $1.00 \times 10^{-3}\,mol \cdot dm^{-3}$, the level of this product in gamma-irradiated synovial fluids returns to a value close to its control value on raising the concentration of this added thiol to

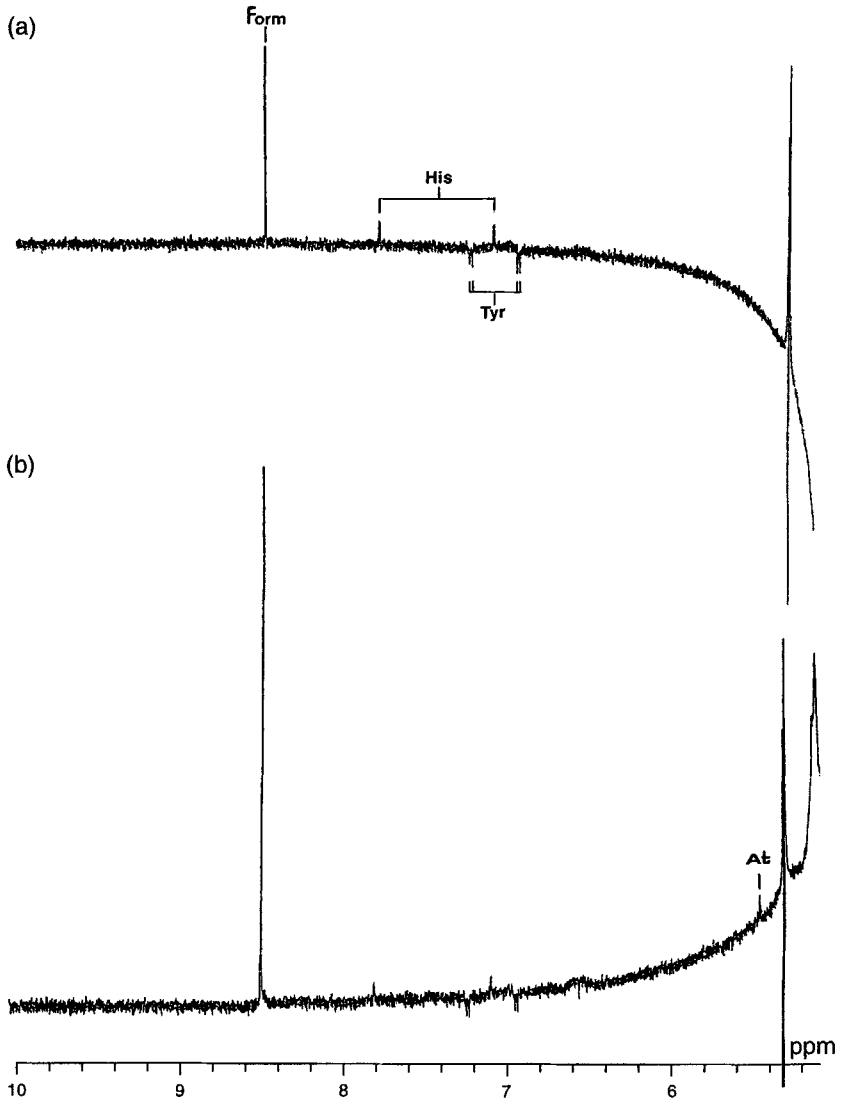

FIGURE 6(a) and (b)

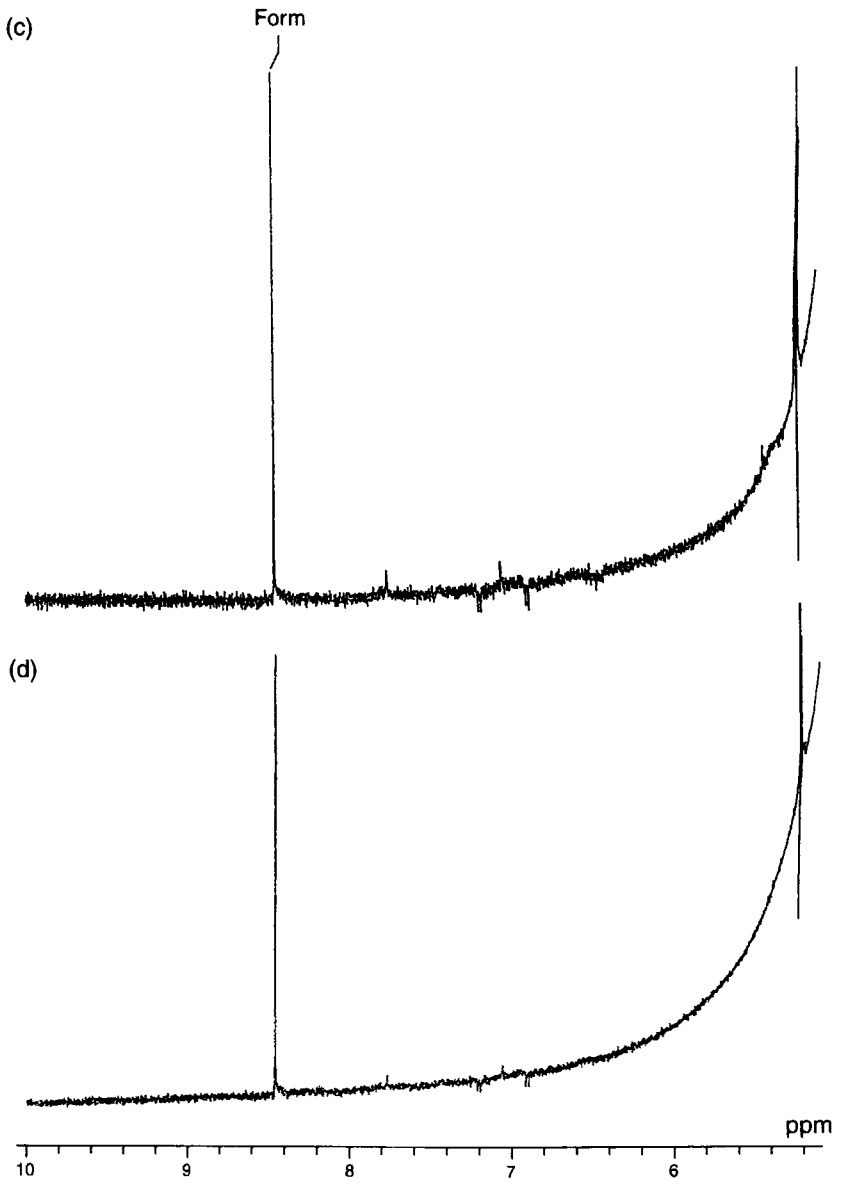

FIGURE 6(c) and (d)

FIGURE 6 Low field (aromatic) region of the 500 MHz ^{1}H Hahn spin-echo NMR spectra shown in Figure 5: (a) untreated (control) synovial fluid; (b) as (a), but subsequent to gamma-radiolysis (5.00 kGy); (c) as (b), but treated with 1.00×10^{-3} mol · dm^{-3} cysteine prior to gamma-radiolysis; (d) as (b), but treated with 5.00×10^{-3} mol · dm^{-3} cysteine prior to gamma-radiolysis. Abbreviations: as in Figure 4.

3.00×10^{-3} mol · dm^{-3}. This observation is probably attributable to the displacement of this anionic component from positively-charged protein binding-sites by the high level of added N-acetylcysteine. However, sulphur-containing radicals arising from the attack of radiolytically-generated $^{\bullet}$OH radical on N-acetylcysteine may also play a role in influencing levels of molecularly-mobile, NMR-detectable formate. The thiol cysteine exerted a similar concentration-dependent

influence on the level of formate detectable in ^{1}H Hahn spin-echo spectra of gamma-irradiated synovial fluids.

The radiolytically-mediated elevation in synovial fluid acetate concentration observed at an added N-acetylcysteine concentration of $3.00 \times 10^{-3}\,mol \cdot dm^{-3}$ is explicable by its generation from the attack of $^{\bullet}OH$ radical on N-acetylcysteine itself and/or its mobilisation from positively-charged protein binding sites as discussed for formate above. This increase in acetate concentration at high levels of the added thiol was not observed when cysteine was employed as an $^{\bullet}OH$ radical scavenging radioprotectant, consistent with its direct radiolytically-mediated production from the N-acetyl-CH_3 group of N-acetylcysteine while present in intact synovial fluids.

The role of N-acetylcysteine-derived oxysulphur radicals in contributing towards radiolytic damage in this system is unclear at present, but Aruoma *et al.*[12] detected only a very small increase in the ability of this thiol to protect α_1-antiprotease against damage induced by radiolytically-generated $^{\bullet}OH$ radical on changing the irradiation atmosphere from pure N_2O to 80% (v/v) N_2O/20% (v/v) O_2.

The radioprotective capacity of exogenous cysteine was similarly characterised by a substantial inhibition of radiolytically-mediated allantoin generation from urate (a complete suppression occurring at an added concentration of $5.00 \times 10^{-3}\,mol \cdot dm^{-3}$), a limited reduction in the level of low-molecular-mass oligosaccharide species arising from the radiolytic fragmentation of hyaluronate, and a decrease in the concentration of formate generated at an added cysteine concentration of $2.00 \times 10^{-3}\,mol \cdot dm^{-3}$. A marginal reduction in the intensity of the acetate-CH_3 group signal at an added cysteine concentration of $5.00 \times 10^{-3}\,mol \cdot dm^{-3}$ was also noted.

In conclusion, the experiments performed here provide much useful information regarding the particular biofluid components for which exogenous thiols such as N-acetylcysteine offer protection against radiolytic damage. Such information is also of much physiological and clinical significance in view of the adverse toxicological properties associated with radiolytic products derived from various biomolecules,[23] e.g., formate arising from radiolytic damage to carbohydrates. The technique employed here is equally applicable to investigations of the precise molecular mechanisms underlying the radioprotectant and/or antioxidant actions of alternative therapeutic agents such as non-steroidal anti-inflammatory drugs (NSAIDS).

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