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Oxidative damage to hyaluronate and glucose in synovial fluid during exercise of the inflamed rheumatoid joint

Detection of abnormal low-molecular-mass metabolites by proton-n.m.r. spectroscopy

Martin GROOTVELD,*§ Edward B. HENDERSON,* Adrian FARRELL,* David R. BLAKE,* Harold G. PARKES† and Peter HAYCOCK‡

*The Inflammation Group, Bone and Joint Research Unit, The London Hospital Medical College, London E1 2AD, U.K., †Department of Chemistry, Birkbeck College, University of London, Gordon House, 29 Gordon Square, London WC1H 0PP, U.K., and ‡Department of Chemistry, Queen Mary College, University of London, Mile End Road, London E1 4NS, U.K.

Proton Hahn spin-echo n.m.r. spectroscopy was employed to detect abnormal metabolites present in rheumatoid synovial fluid that are derived from the deleterious generation of reactive oxygen radical species during exercise of the inflamed rheumatoid joint. A resonance attributable to a low-molecular-mass *N*-acetylglucosamine-containing oligosaccharide formed by the oxygen-radical-mediated depolymerization of synovial-fluid hyaluronate was clearly demonstrable when subjects with inflammatory joint disease were exercised. Moreover, formate, which may be derived from the attack of $OH^{\bullet}$ radical on synovial-fluid carbohydrates, was also readily detectable in these samples. γ-Radiolysis of rheumatoid synovial fluid samples and aqueous solutions of hyaluronate also gave rise to the production of the low-molecular-mass hyaluronate-derived oligosaccharide species and markedly elevated concentrations of (non-protein-bound) formate in the biological fluids. As expected, corresponding spectra of γ-irradiated blood serum samples obtained from normal volunteers did not contain the signal attributable to the low-molecular-mass oligosaccharide species, but the formate resonance (barely detectable in non-irradiated normal serum samples) became clearly visible. Additionally, a curious increase in the effective concentration of non-protein-bound low-molecular-mass metabolites such as acetate, citrate, lactate and glutamine was observed after γ-radiolysis of all biological fluids studied. The hyaluronate-derived low-molecular-mass oligosaccharide species and formate are suggested as novel markers of reactive oxygen radical activity in the inflamed rheumatoid joint during exercise-induced hypoxic/reperfusion injury.

INTRODUCTION

Hyaluronate (hyaluronic acid, hyaluronan) [1] is composed of a linear repeating disaccharide, β-D-glucuronyl-β-D-*N*-acetylglucosamine, of high molecular mass (upwards of 4000000 Da) that forms the central axis of the proteoglycan aggregates necessary for the functional integrity of articular cartilage and other extracellular matrices [2]. In its unaggregated form, hyaluronate is secreted continuously by the type II synoviocytes and comprises the major macromolecular species of the synovial fluid, being thereby responsible for the unique viscoelastic properties of what is otherwise effectively a simple plasma diffusate [3]. Hyaluronate is normally present in the serum only in very small amounts, of the order of 10–100 μg/l [4]. These concentrations have been found to be elevated 5–7-fold in the sera of patients suffering from rheumatoid arthritis [5].

In rheumatoid arthritis and other inflammatory arthritides, synovial hyaluronate is fragmented and depolymerized, with a corresponding reduction in synovial-fluid viscosity and an increase in the synovial concentration of diffusible hyaluronate saccharide species [6]. As normal and inflammatory synovial fluid contain no hyaluronidase activity [7], it has been inferred for some time that reactive oxygen metabolites are involved in the mechanism of hyaluronate depolymerization within the joint [8]. Broadly, the evidence derives from two approaches, namely (1) the demonstration of potential free-radical-generating systems within the joint, and (2) the demonstration of hyaluronate degradation by such systems *in vitro*, as evidenced by decreased viscometric parameters and apparent hyaluronate molecular mass as measured by gel-filtration techniques.

We have previously presented evidence for reactive-oxygen-radical-species-mediated damage to protein [9] and lipid components [10] found in the joints of arthritic patients, and have suggested hypoxic/reperfusion injury during exercise of the inflamed joint as a possible mechanism for perpetuating the generation of free radicals within the joint, leading to the chronicity of the inflammation [11]. Hypoxic/reperfusion injury involves the uncoupling of a number of intracellular redox enzyme systems, such as the conversion of xanthine dehydrogenase into its oxidase form, leading to the production of superoxide radicals. We have further demonstrated the generation of a reactive oxygen metabolite by synovium *in vitro* during hypoxia/reperfusion cycles by e.s.r. spectroscopy utilizing spin-trapping techniques [12]. Much of the toxicity to biological molecules associated with reactive oxygen radical species is attributable to the highly reactive hydroxyl ($OH^{\bullet}$) radical [13], the formation of which appears to be mediated by low-molecular-mass iron chelates such as iron–citrate or ternary iron–citrate–acetate complexes [14].

The recent development of high-field n.m.r. spectrometers with increased sensitivity, resolution and dynamic range has permitted the rapid study of complex mixtures of endogenous or exogenous species in biological fluids. Since the methodology has little or no requirement for pretreatment of samples, it is largely non-invasive. Useful dynamic information about the molecules present can be obtained from high-field high-resolution studies of human serum, plasma and urine [15–18]. The broad overlapping resonances arising from the large number of macromolecules present in biological fluids can be suppressed by spin-echo pulse sequences [19,20], leaving an n.m.r. spectrum that has

§ To whom correspondence should be addressed.

well-resolved signals arising from mobile portions of macromolecules and from many low-molecular-mass metabolites present at concentrations of approx. 0.10 mmol/l [15–18].

We present here direct evidence for reactive-oxygen-radical-species-mediated damage to hyaluronate in the synovial fluid of patients with inflammatory joint disorders. We have used high-resolution high-field proton Hahn spin-echo Fourier-transform n.m.r. spectroscopy to study oxidative damage to hyaluronate and glucose *in vitro* by reactive oxygen radical species generated by γ-radiolysis and to characterize the end products. We have used the same techniques to study oxidative damage to synovial-fluid hyaluronate *in vivo* by reactive oxygen radical species generated during hypoxic/reperfusion injury. We further present preliminary evidence for the detection of the end products of reactive-oxygen-radical-species-mediated hyaluronate degradation in the synovial fluid of patients with inflammatory joint disease and suggest that these joint-derived free-radical-damaged species may provide a sensitive index of disease severity, and possibly at-risk status in subclinical disease. The potential of this methodology to monitor such markers is discussed.

MATERIALS AND METHODS

Reagents

α-D-Glucose, D-sucrose, dextran (average molecular mass 79000 Da), *N*-acetyl-D-glucosamine and rooster comb hyaluronic acid were obtained from Sigma Chemical Co. Solutions of α-D-glucose, D-sucrose and dextran were prepared in double-distilled water.

N.m.r. measurements on biological fluids and hyaluronate

Proton-n.m.r. measurements were conducted on a JEOL JNM-GSX 500 (University of London Intercollegiate Research Service, Biomedical NMR Centre, Birkbeck College, London, U.K.) spectrometer operating at 500 MHz for ^{1}H. All spectra were recorded at a probe temperature of 20 °C. Typically, 0.60 ml of serum, knee-joint synovial fluid or 10.0 mg/ml solutions of hyaluronate in 10 mM-phosphate buffer, pH 7.40, were placed in a 5 mm-diameter n.m.r. tube, and 0.10 ml of ^2H_2O was added to provide a field-frequency lock. The intense water signal and the broad protein resonances were suppressed by a combination of the Hahn spin-echo sequence and the application of continuous secondary irradiation at the water frequency. The Hahn spin-echo sequence (90°–τ–180°–τ–collect) was repeated 72–135 times with $\tau = 60$ ms. Chemical shifts were referenced to external sodium 3-(trimethylsilyl)propane-1-sulphonate.

N.m.r. measurements on carbohydrates

For proton-n.m.r. measurements on control and γ-irradiated aqueous solutions of α-D-glucose, D-sucrose and dextran we used a Bruker WH400 (University of London Intercollegiate Research Service, Queen Mary College, London, U.K.) n.m.r. spectrometer equipped with a Bruker Aspect 3000 data system. A 0.60 ml portion of carbohydrate solution was placed in a 5 mm-diameter n.m.r. tube, and 0.10 ml of ^2H_2O was added to provide a field-frequency lock. The intense water signal was suppressed by continuous secondary irradiation at the water frequency. Single-pulse spectra were obtained with 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 employed. All spectra were recorded at ambient probe temperature (25.0 ± 1.0 °C) and referenced to sodium 3-(trimethylsilyl)propane-1-sulphonate ($\delta = 0$ p.p.m.).

Synovial fluid samples

Knee-joint synovial fluid was drawn into plastic sample tubes for therapeutic purposes from patients with moderately severe rheumatoid arthritis and associated knee effusions (age range 39–63 years).

For the study of hypoxic/reperfusion injury, patients were subjected to exercise by isometric quadriceps contraction. This technique was performed as previously described [11]. Briefly, the patients were rested with the knee immobilized in a splint for 1 h before aspiration. A local subcutaneous anaesthetic of 2% lignocaine (5 ml) was administered to the medial aspect of the knee joint, taking care not to inject any of the anaesthetic into the joint cavity. A 21-gauge needle was then inserted into the knee-joint cavity. A baseline sample (approx. 2 ml) of synovial fluid was then aspirated into a plastic syringe. The patient then performed isometric quadriceps contraction for a total period of 2 min (the needle was retained within the subcutaneous tissue, but withdrawn from the joint space during exercise). Synovial fluid samples (up to 2 ml) were aspirated immediately post-exercise, and thereafter at 2 min intervals for a total period of 8 min.

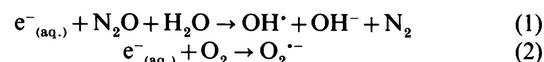
Immediately after aspiration, all synovial fluid samples were placed in plastic tubes and transported to the laboratory on ice, where they were centrifuged at 2500 rev./min for 10 min to remove cells and debris. The supernatant was then stored at −70 °C before treatment with γ-irradiation and proton-n.m.r. analysis.

Serum samples

Serum samples were obtained from seven consenting healthy male volunteers (age range 29–59 years) by allowing freshly drawn non-heparinized blood to clot. These samples were centrifuged and stored as described above.

γ-Irradiation treatment of biological fluids and carbohydrate solutions

Aqueous solutions of α-D-glucose, D-sucrose or dextran 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 5000 Gy (dose rate 4.76 Gy/min). Synovial fluid, serum and aqueous solutions of hyaluronate were similarly irradiated, but the total dosage employed was varied from 48 to 5000 Gy, at the same dose rate as above. Under these experimental conditions, the major primary radical species present are $OH^{\bullet}$ ($G = 2.7$), $e^-_{(aq.)}$ ($G = 2.7$) and $H^{\bullet}$ ($G = 0.5$) where the G value represents the μmol/l concentration of product per 10 Gy dosage. Where appropriate, these samples were also saturated with N_2O {to convert $e^-_{(aq.)}$ into $OH^{\bullet}$ radical [eqn. (1)]} or O_2 {to convert $e^-_{(aq.)}$ into $O_2^{\bullet -}$ [eqn. (2)]} before γ-radiolysis:



$$e^-_{(aq.)} + N_2O + H_2O \rightarrow OH^{\bullet} + OH^- + N_2 \quad (1)$$

$$e^-_{(aq.)} + O_2 \rightarrow O_2^{\bullet -} \quad (2)$$

The above dose rate was utilized to simulate the perpetual exercise-induced generation of biologically relevant concentrations of $OH^{\bullet}$ radical (1.285 μmol/l per min in the presence of atmospheric O_2 or in O_2-saturated fluids, and 2.570 μmol/l per min in N_2O-saturated fluids) in the inflamed rheumatoid joint.

RESULTS AND DISCUSSION

High-resolution proton-n.m.r. spectra of untreated and γ-irradiated synovial fluid from rheumatoid patients and serum from normal controls

Employment of the Hahn spin-echo technique suppresses broad resonances arising from relatively immobile macro-

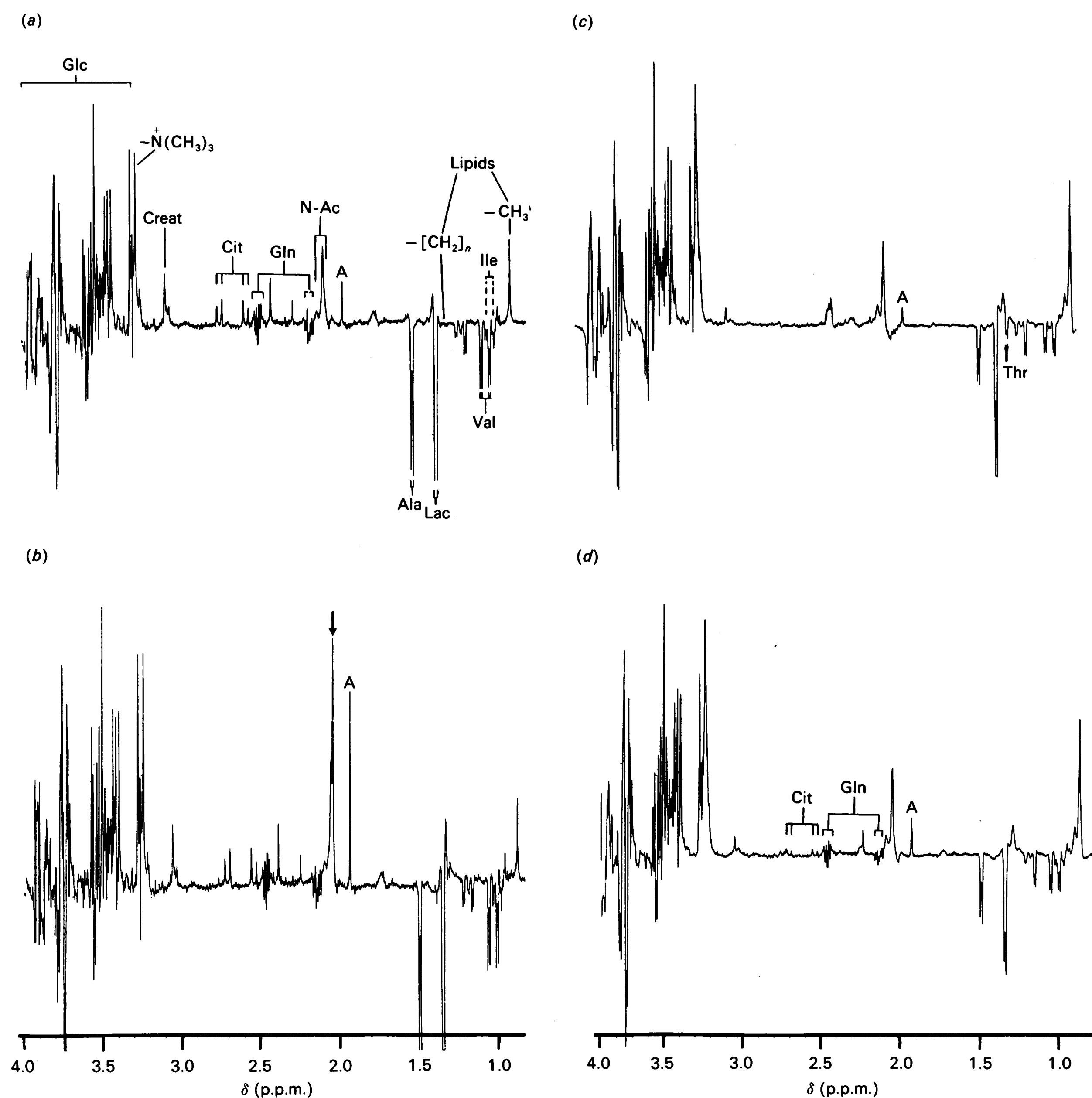


Fig. 1. Low-frequency (high-field) region of 500 MHz proton spin-echo n.m.r. spectra of (*a*) untreated synovial fluid obtained from a patient with rheumatoid arthritis, (*b*) as (*a*), but subjected to γ-irradiation treatment at a dose level of 5000 Gy, (*c*) untreated serum obtained from a normal volunteer, and (*d*) as (*c*), but exposed to γ-irradiation as in (*b*)

Typical spectra are shown. Abbreviations: A, acetate; Ala, alanine; Cit, citrate; Creat, creatinine; Glc, glucose; Gln, glutamine; Ile, Isoleucine: Lac, lactate; N-Ac, mobile portions of *N*-acetylated glycoproteins; Thr, threonine; Val, valine. The arrow in spectrum (*b*) denotes the intense sharp singlet at 2.044 p.p.m., attributable to a low-molecular-mass oligosaccharide species derived from the radiolytic degradation of synovial-fluid hyaluronate.

molecules, and signals attributable to highly mobile components are visible in proton-n.m.r. spectra of synovial fluid and blood serum. {In the n.m.r. sense, the word 'mobile' refers to molecules that undergo rapid molecular motion, e.g. tumbling. Slow molecular motion leads to short spin–spin relaxation times (T_2) and, consequently, broad n.m.r. resonances [21].} Figs. 1(*a*) and 1(*b*) show the low-frequency (high-field) region of the 500 MHz proton Hahn spin-echo n.m.r. spectra of a typical rheumatoid synovial fluid sample obtained before and after treatment with γ-irradiation in the presence of atmospheric O_2 at a dose level of 5000 Gy. There are both qualitative and quantitative differences between the two spectra, the most notable being the presence of an intense singlet at 2.044 p.p.m. in the spectrum of the irradiated sample. This resonance, which has a chemical-shift value characteristic of *N*-acetyl methyl protons and appears slightly upfield to the broader signals attributable to the *N*-acetyl methyl protons of relatively mobile portions of *N*-acetylated glycoproteins, is likely to be due to an *N*-acetyl methyl group located on a low-molecular-mass oligosaccharide species derived from the radiolytic degradation of either synovial-fluid hyaluronate or the polysaccharide moieties of *N*-acetylated glycoproteins. Additional qualitative changes induced by γ-irradiation treatment of the synovial fluid sample include the production of (i) an apparent multiplet in the 0.88–0.93 p.p.m. chemical-shift range and (ii) an

inverted doublet centred at 1.32 p.p.m. Moreover, when spectra are normalized to the valine and alanine methyl group resonances, there is clearly a large difference in the intensity of the acetate resonance between spectra, with smaller differences in the intensities of signals arising from citrate and lactate. Hence treatment with γ-irradiation induces a curious increase in the effective concentrations of these mobile (non-protein-bound) endogenous metabolites. The 500 MHz proton Hahn spin-echo n.m.r. spectra of untreated and γ-irradiated synovial fluid samples obtained from a total of eight rheumatoid patients demonstrated that the marked modifications in the spectra observed subsequent to irradiation treatment were always reproducible. Moreover, in several of these synovial fluid samples obtained from other rheumatoid patients, γ-irradiation treatment was found to produce a singlet resonance at 2.768 p.p.m. together with a decrease in the intensity of a singlet located at 2.384 p.p.m. (results not shown).

In order to determine whether the sharp singlet located at 2.044 p.p.m. was derived from oxidatively damaged hyaluronate or *N*-acetylated glycoproteins, corresponding spectra of control and γ-irradiated (5000 Gy) blood serum from normal volunteers were obtained (an example of which is given in Figs. 1*c* and 1*d*). These spectra clearly show that the sharp singlet at 2.044 p.p.m. is absent from the spectrum of the irradiated sample, indicating that this signal, primarily detected in all spectra of irradiated synovial fluid from rheumatoid patients, is solely derived from the radiolytically mediated depolymerization of hyaluronate. This observation was reproducible in n.m.r. spectra of serum samples obtained from six further normal volunteers. Further radiolytically induced qualitative differences in the two spectra are also apparent, including (i) loss of the well-defined threonine methyl group inverted doublet resonance at 1.275 p.p.m., (ii) the loss of a barely detectable inverted doublet at 1.091 p.p.m., (iii) the loss of an inverted doublet centred at 1.422 p.p.m., (iv) the production of an apparent singlet at 0.946 p.p.m., and (v) the appearance of a broad resonance at 1.726 p.p.m. Furthermore, some small changes in the complex pattern of glucose multiplets in the chemical-shift range 3.3–4.0 p.p.m. were also observed, indicating that radiolytically generated $OH^{\bullet}$ radical also attacks glucose to some extent.

However, the most striking differences between the two spectra are notable in terms of quantitative differences in the (normalized) relative intensities of a number of resonances attributable to mobile low-molecular-mass endogenous metabolites. In the control serum, the characteristic AB coupling pattern due to mobile citrate is barely detectable, and the 'free' acetate concentration is very low. Furthermore, glutamine is not detectable whatsoever. However, following γ-irradiation treatment at a dose level of 5.0 kGy, the relative intensities of both the citrate and acetate resonances increase substantially, and resonances attributable to glutamine are now clearly visible. This indicates that $OH^{\bullet}$ radical, $e^{-}_{(aq.)}$, $O_2^{\bullet -}$, or a combination of two or more of these radical species have the ability to remove these endogenous metabolites from protein-binding sites. For example, if the carboxylate species acetate, citrate or lactate were bound to positively charged lysine residues in serum proteins before irradiation treatment, and one or more of these radiolytically generated oxygen radicals were able to attack a number of these protein lysine residues,

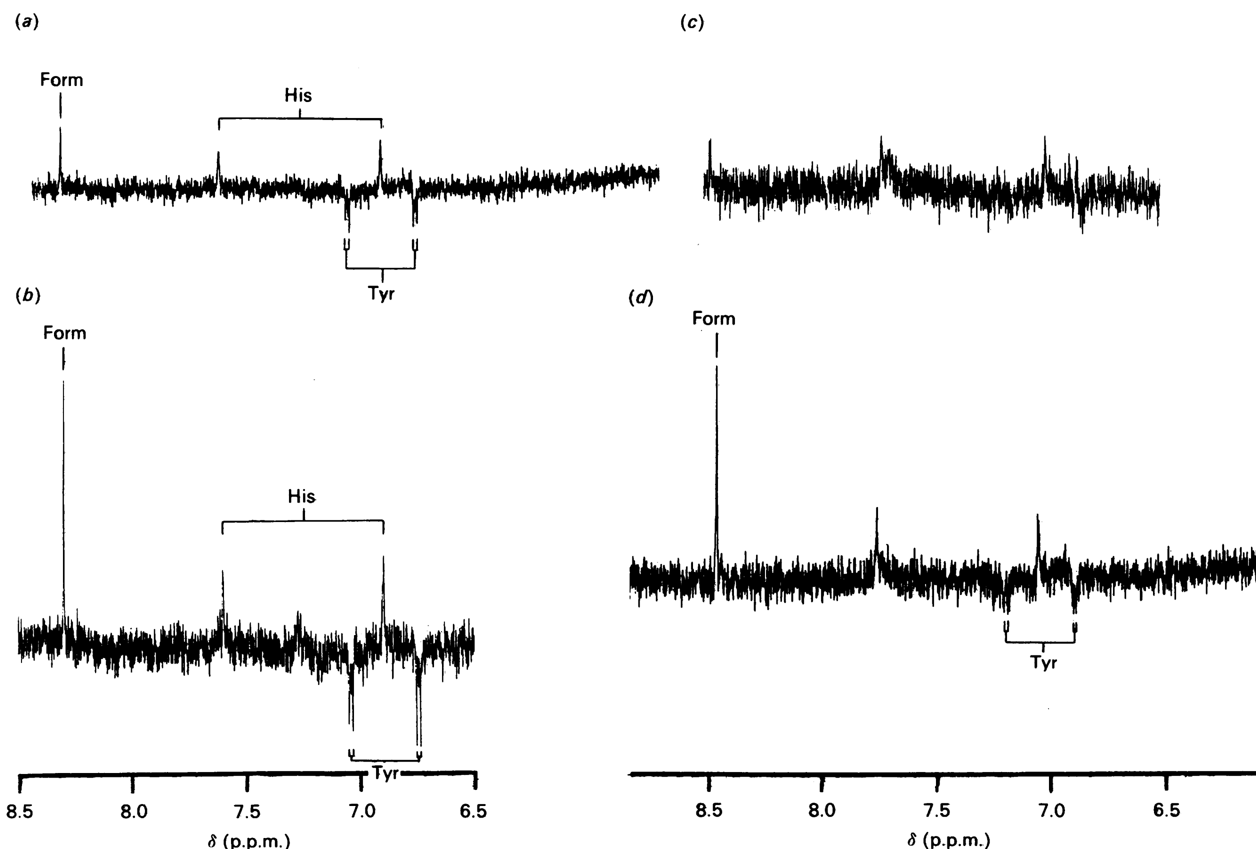


Fig. 2. Corresponding high-frequency (low-field) region of the 500 MHz proton spin-echo n.m.r. spectra of untreated and γ-irradiated (5000 Gy) biological fluids shown in Figs. 1(*a*)–(*d*)

Abbreviations: Form, formate; His, histidine; Tyr, tyrosine.

then the resultant chemical modifications of this particular amino acid are likely to affect its ability to bind anionic species, leading to their release from serum protein matrices. In accordance with this hypothesis, it is noteworthy that methyl-lactate does not bind to serum proteins, as assessed by proton Hahn spin-echo n.m.r. spectroscopy (J. D. Bell, personal communication). Alternatively, the radiolytically induced aggregation or denaturation of serum proteins may also give rise to the release of these metabolites from protein-binding sites.

Although citrate is known to react with OH$^•$ radical, it does so at a relatively low rate (second-order rate constant for citric acid at pH 1, $k_2 = 3.0 \times 10^7\ \mathrm{mol^{-1} \cdot l \cdot s^{-1}}$) [22]. Hence, in concentration terms, any small loss of non-protein-bound citrate (i.e. n.m.r.-detectable in terms of the Hahn spin-echo technique) appears to be more than compensated for by its release from serum proteins.

Prior saturation of rheumatoid synovial fluid samples with N_2O or O_2 did not give rise to any significant differences in the normalized intensity of the hyaluronate-derived oligosaccharide resonance at 2.044 p.p.m. after γ-radiolysis at a dose level of 5000 Gy (results not shown). This indicates that the OH$^•$-radical-induced fragmentation of synovial-fluid hyaluronate proceeds by a self-perpetuating chain reaction. Moreover, it appears that $e^-_{(aq.)}$ has little or no influence on hyaluronate depolymerization in samples irradiated in the presence of atmospheric O_2.

Fig. 2 exhibits the high-frequency (low-field) region of typical proton Hahn spin-echo n.m.r. spectra of rheumatoid synovial fluid and serum from a normal volunteer both before and after γ-irradiation treatment at a dose level of 5000 Gy. The non-irradiated (control) rheumatoid synovial fluid sample exhibits a well-defined formate resonance at 8.388 p.p.m. and two inverted doublets centred at 6.893 p.p.m. attributable to p-tyrosine (AB

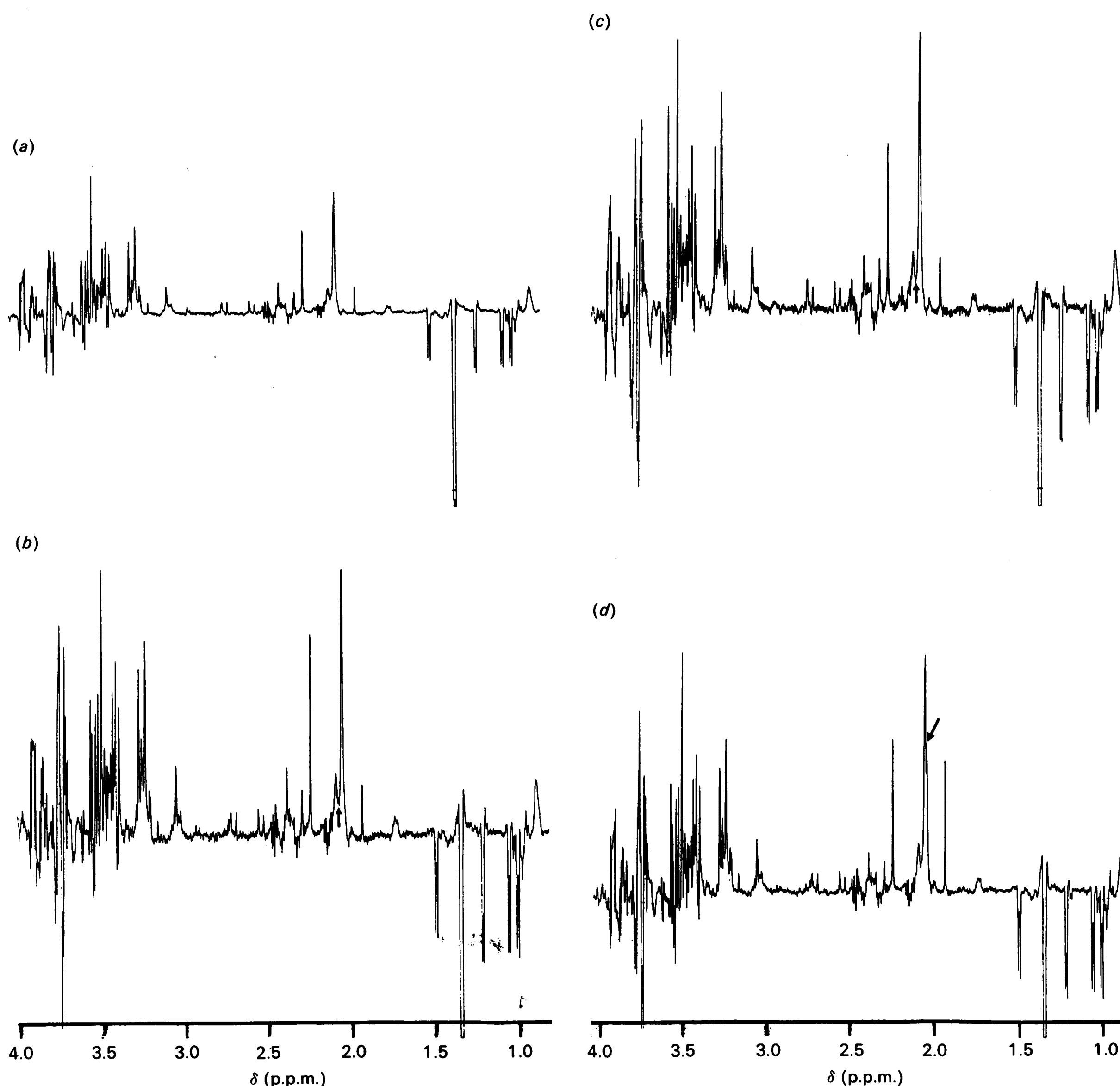


Fig. 3. Low-frequency region of 500 MHz proton spin-echo n.m.r. spectra of a sample of rheumatoid synovial fluid that was subjected to γ-irradiation treatment at doses of (*a*) 0, (*b*) 48, (*c*) 143 and (*d*) 5000 Gy

The arrows indicate resonances attributable to the *N*-acetyl methyl group in low-molecular-mass species derived from radiolytic damage to hyaluronate.

coupling system). After γ-irradiation treatment, the normalized intensities of the formate, and, to a somewhat lesser extent, *p*-tyrosine signals increase. Intriguingly, the formate and *p*-tyrosine signals are barely detectable in spectra of non-irradiated serum samples from normal human volunteers (Fig. 2*c*), but subsequent to γ-irradiation treatment their resonances are clearly visible, especially that of the formate proton (Fig. 2*d*). The presence of relatively high concentrations of formate (approx. 90–220 μmol/l, as estimated by the method of standard additions) may be attributable to its adverse production in the inflamed rheumatoid joint via reactive oxygen radical attack at monosaccharides such as glucose or polysaccharides such as hyaluronate. Formate is a well-known 'end product' of the radiolytic degradation of some common carbohydrates [23–25]. Its further production during radiolysis of these biological fluids may also be due to its release from protein-binding sites (as demonstrated for acetate, citrate and lactate in this study) as well as oxidative damage to carbohydrates. It should also be noted that γ-radiolysis of aqueous solutions containing CO_2 or HCO_3^- ion can also give rise to the production of formate [26]. However, generation of formate in this manner is retarded in oxygenated solutions, and hence is unlikely to be of any significance in the experiments conducted here. Indeed, γ-radiolysis of synovial fluid samples that were previously saturated with either N_2O or O_2 resulted in only small reductions in the intensity of the formate proton resonance relative to that obtained by irradiation of the same samples in the presence of atmospheric O_2.

The increase in the normalized intensity of the *p*-tyrosine-inverted doublets present in the low-field region of the spectrum after γ-irradiation treatment of rheumatoid synovial fluid or normal serum may be due to the hydroxylation of phenylalanine by radiolytically generated $OH^{\bullet}$ radical [27]. Aromatic compounds such as phenylalanine react extremely rapidly with $OH^{\bullet}$ radical, i.e. phenylalanine is a good $OH^{\bullet}$-radical scavenger [28, 29] (second-order rate constant $k_2 = 3.5 \times 10^9\ mol^{-1} \cdot l \cdot s^{-1}$) [22]. However, the low concentration of phenylalanine (approx. 64 μmol/l in human blood plasma) [30] severely restricts its ability to compete with alternative $OH^{\bullet}$ radical scavenger molecules such as glucose. Moreover, as noted for formate, it is also possible that the radiolytic degradation of serum or synovial-fluid proteins may give rise to the release of *p*-tyrosine normally bound to these macromolecules.

γ-Irradiation of a separate synovial fluid sample at three different dosage levels (48, 143 and 5000 Gy) initially gave rise to a weakly intense relatively sharp singlet at 2.076 p.p.m. at the two lower doses studied (Fig. 3). Although this signal was not present in the spectrum obtained from the sample exposed to the higher dosage level of 5000 Gy, the very intense sharp singlet

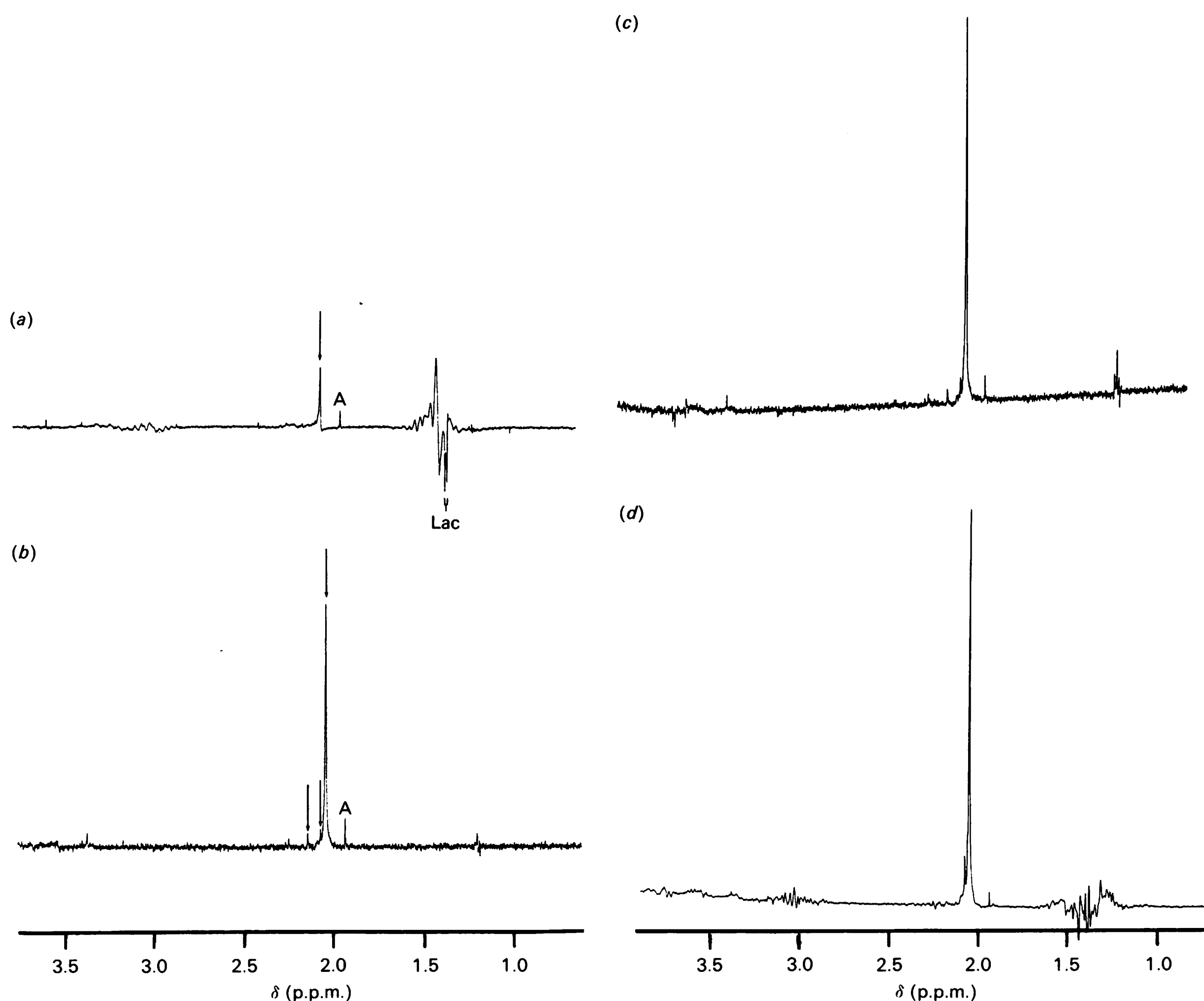


Fig. 4. Low-frequency region of 500 MHz proton spin-echo n.m.r. spectra of a commercial sample of hyaluronate exposed to γ-irradiation at dose levels of (*a*) 0, (*b*) 48, (*c*) 143 and (*d*) 5000 Gy

located at 2.044 p.p.m. attributable to a proposed low-molecular-mass *N*-acetylglucosamine-containing oligosaccharide was present, as indeed it was in all synovial fluid samples that were irradiated at this dosage. This indicates that both primary and secondary *N*-acetylglucosamine-containing radiolytic products are derived from radiolytically depolymerized hyaluronate.

These observations were reproduced by exposing a further rheumatoid synovial fluid sample to the same various dosages of γ-irradiation. However, the spectrum obtained after exposure of this alternative sample to a dosage of 5000 Gy exhibited two further relatively weak sharp singlets at 2.109 and 2.122 p.p.m. in addition to the very intense sharp singlet at 2.044 p.p.m. (results not shown), suggesting an increased heterogeneity of *N*-acetyl methyl group environments in depolymerized hyaluronate.

Proton Hahn spin-echo n.m.r. studies of radiolytically induced oxidative damage to hyaluronate

In order to confirm that the sharp singlet at 2.044 p.p.m. observed in irradiated (and some non-irradiated) rheumatoid synovial fluid was derived from oxidatively damaged hyaluronate, phosphate-buffered aqueous solutions of commercially available hyaluronate were subjected to γ-radiolysis at dose levels of 0, 48, 143 and 5000 Gy.

The 500 MHz proton Hahn spin-echo n.m.r. spectrum of non-irradiated hyaluronate has weakly intense lactate and acetate resonances at 1.33 and 1.93 p.p.m. respectively (Fig. 4*a*). The acetate is possibly a product derived from a small amount of hydrolysis of the *N*-acetylglucosamine moiety. However, it is important to note that both acetate and lactate are terminal products derived from the metabolic breakdown of hyaluronate *in vivo* [31]. In addition, a number of very weakly intense singlets and multiplets in the 3.3–4.0 p.p.m. carbohydrate proton chemical-shift range are present. Interestingly, the sharp singlet at 2.044 p.p.m. is also present in the spectrum, indicating that a small quantity of the proposed low-molecular-mass oligosaccharide product resulting from the oxidative degradation of hyaluronate is present in commercial samples of this polysaccharide.

Figs. 4(*b*), 4(*c*) and 4(*d*) show the corresponding spectra of 10 mg/ml solutions of hyaluronate after exposure to a γ-irradiation source at dose levels of 48, 143 and 5000 Gy respectively. At the lowest dosage employed (48 Gy), the sharp 2.044 p.p.m. singlet increases markedly in intensity, and two further sharp singlets located at 2.070 and 2.140 p.p.m. are also present. At a dose level of 143 Gy, the 2.044 p.p.m. signal further increases in intensity, as does the 2.070 p.p.m. singlet. Additionally, the carbohydrate resonances in the 3.3–4.0 p.p.m. range, probably attributable to low-molecular-mass oligo-, di- or mono-saccharides, increase in intensity. The spectrum obtained from the sample exposed to a dose level of 5000 Gy shows that the 2.044 p.p.m. signal does not further increase in intensity, indicating that an autocatalytic (self-perpetuating) depolymerization process is involved. However, the 2.070 p.p.m. signal does increase in intensity at this high dose level, and a complex low-molecular-mass carbohydrate resonance pattern is now clearly visible.

Previous investigations of the depolymerization of hyaluronate have indicated that it proceeds by a mechanism involving direct oxidative cleavage of glycosidic linkages between *N*-acetylglucosamine and glucuronate [32,34]. Such cleavage is likely to give rise to low-molecular-mass *N*-acetylglucosamine-containing oligosaccharide species with high mobility in aqueous solution, as demonstrated here. Moreover, our results are in accordance with the findings obtained by McNeil *et al.* [33], who have suggested that the depolymerization of hyaluronate by OH$^{\bullet}$ radical may be ordered to some extent with rapid progression from high- to low-molecular-mass species, i.e. proceeding by a chain reaction. However, Balazs *et al.* [34] have suggested that, in addition to the direct oxidative cleavage of glycosidic linkages, elimination of the C-5 hydrogen atom gives rise to uronate radicals, which are stabilized by interaction with the carboxylate

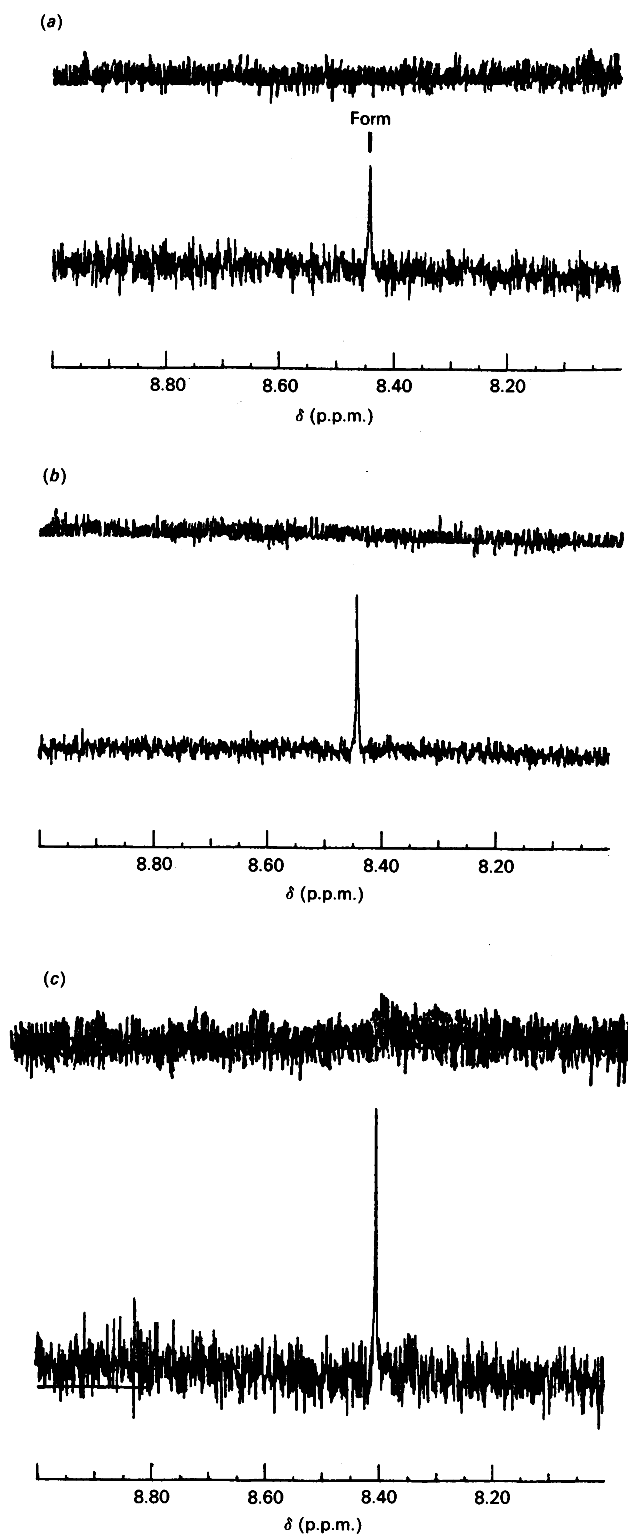


Fig. 5. High-frequency (low-field) region of 400 MHz proton-n.m.r. spectra of aqueous solutions of (*a*) α-D-glucose (0.10 mol/l), (*b*) D-sucrose (0.05 mol/l) and (*c*) dextran (20 mg/ml) obtained before and after γ-radiolysis at a dose level of 5000 Gy

group. Such uronate radicals may have the ability to interact further and form cross-linked hyaluronate-degradation products.

It is also important to note that hyaluronate is also susceptible to attack from radiolytically generated aquated electrons ($e^{-}_{(aq.)}$), the reaction being quite rapid (second-order rate constant, $k_2 = 1.4 \times 10^8\ mol^{-1} \cdot l \cdot s^{-1}$) [34].

γ-Irradiation treatment of aqueous solutions of model carbohydrates

γ-Irradiation of aqueous solutions of α-D-glucose, D-sucrose and dextran was conducted to monitor the production of formate as a terminal end product of their radiolytic degradation. Fig. 5 exhibits the low-field region of high-resolution (400 MHz) proton-n.m.r. spectra of aqueous solutions of (*a*) α-D-glucose (0.10 mol/l), (*b*) D-sucrose (0.05 mol/l) and (*c*) dextran (20 mg/ml), both before and subsequent to γ-irradiation treatment at a dose level of 5000 Gy. After γ-radiolysis, the formate proton singlet located at 8.388 p.p.m. is clearly detectable for all carbohydrates studied. At the given concentrations of carbohydrate irradiated, the intensity of the formate resonance produced decreases in the order dextran > sucrose > glucose.

Interestingly, proton-n.m.r. spectra of non-irradiated aqueous solutions of *N*-acetyl-D-glucosamine that were obtained on samples either immediately after purchase or subsequent to storage at room temperature for considerable periods of time both contained the characteristic formate proton resonance. Moreover, proton Hahn spin-echo spectra of commercial samples of hyaluronate also contained the formate signal (results not shown).

Hence it is clear that formate is indeed a terminal end product resulting from radiolytically mediated oxygen-radical attack on these model systems. These observations are in accordance with Phillips & Moody [35], who conducted some early pioneering studies on the γ-irradiation treatment of carbohydrates.

Application of proton Hahn spin-echo n.m.r. spectroscopy to the study of oxidative damage to hyaluronate in knee-joint synovial fluid during hypoxic/reperfusion injury

In general, examination of the proton Hahn spin-echo n.m.r. spectra of routine (unirradiated) rheumatoid synovial fluid samples revealed that the sharp hyaluronate-derived oligosaccharide resonance at 2.044 p.p.m. was present in only a small proportion of these samples. However, it is important to note that this observation is likely to be a direct reflection of the exercise status of the patient's inflamed knee joint(s) at the time at which the samples were aspirated. Exercise-induced hypoxic/reperfusion injury, a consequence of a number of pathophysiological and biochemical phenomena present within the inflamed joint, is a process that is mediated by the deleterious generation of reactive oxygen radical species such as $OH^{\bullet}$ radical. This process can give rise to cell and tissue damage after the reintroduction of O_2 to the synovium during exercise.

A series of patients with inflamed knees ($n = 4$) were subjected to exercise by isometric quadriceps contraction (for a period of 2 min) in order to assess the influence of reactive oxygen radical generation during exercise-induced hypoxic/reperfusion injury on the production of this molecularly mobile hyaluronate-derived oligosaccharide species by proton-n.m.r. spectroscopy. Fig. 6 exhibits the high-field (0.8–3.0 p.p.m.) region of typical spectra obtained from samples of synovial fluid that were aspirated at (*a*) 2 min pre-exercise, (*b*) immediately after exercise and (*c*) 2 min post-exercise. After exercise, the 2.044 p.p.m. singlet (present initially in the spectrum of the pre-exercise sample) clearly increases in intensity relative to the broader *N*-acetylated glycoprotein methyl group resonance located slightly downfield, indicative of the reactive-oxygen-radical-dependent depolymerization of hyaluronate. At the 4 min post-exercise time point the relative intensity of the 2.044 p.p.m. resonance increases further, but at the 6 min and 8 min post-exercise time points the intensity of this resonance decreases somewhat to reach its previous pre-exercise 'steady-state' level (results not shown), suggesting that the higher concentrations of hyaluronate-derived oligosaccharide species produced during hypoxic/reperfusion injury are rapidly

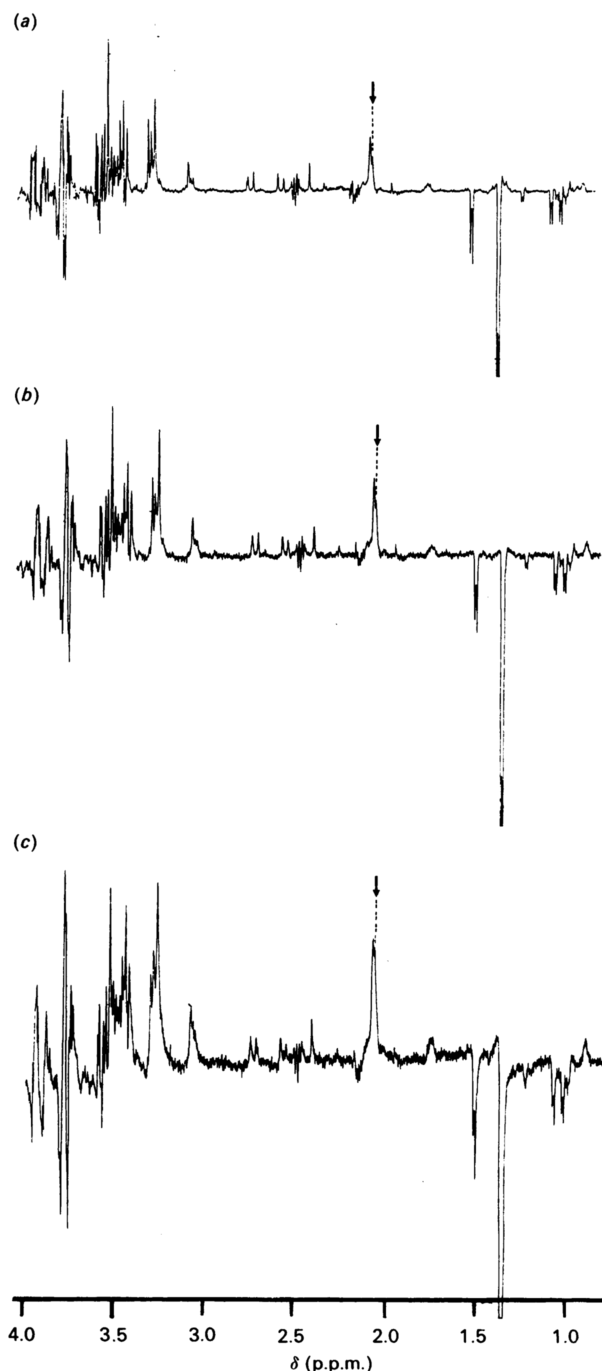


Fig. 6. Low-frequency region of 500 MHz proton spin-echo n.m.r. spectra of samples of synovial fluid obtained at increasing time points from a rheumatoid patient subjected to exercise by isometric quadriceps contraction for a period of 2 min

The spectra were obtained (*a*) 2 min pre-exercise, (*b*) immediately after exercise and (*c*) 2 min post-exercise. The arrows denote the signal attributable to the *N*-acetyl methyl group protons of a low-molecular-mass oligosaccharide species derived from oxygen-radical-mediated oxidative damage to hyaluronate.

cleared from the synovium, or, alternatively, are further degraded by reactive oxygen radical species. These observations were reproducible in two of the three remaining rheumatoid patients that were subjected to exercise.

The glycosidic linkages in hyaluronate are critical to the retention of its polymeric structure in synovial fluid. The scission of these linkages by OH$^{\bullet}$ radical indirectly generated by the occurrence of one or more transient ischaemic events during exercise of the inflamed rheumatoid joint rapidly leads to the production of low-molecular-mass oligosaccharide species, as demonstrated here.

Moreover, a wide-ranging series of further products derived from the oxidative degradation of low-molecular-mass carbohydrates present in synovial fluid, such as that derived from hyaluronate depolymerization as above or the simple monosaccharide glucose, is also expected during hypoxic/reperfusion injury. For these lower sugars, OH$^{\bullet}$ radical attack at the extremities of the molecule yields acids (e.g. gluconic acid is derived from oxidation of C-1 in glucose) and ring cleavage produces aldehydic species. Indeed, the products arising from the radiolytic degradation of hexoses in aqueous solution (largely attributable to the indirect action of OH$^{\bullet}$ radical) include lower saccharides, uronic acids and aldonic acids, as well as C_3, C_2 and C_1 aldehydic fragments [36]. In addition, both formate and CO_2 are produced in the final stages of oxidative damage to simple mono- and di-saccharides. Hence the relatively high concentrations of formate observed in rheumatoid synovial fluid samples may be a reflection of the interaction of OH$^{\bullet}$ radical with endogenous carbohydrate systems in the inflamed rheumatoid joint. However, it is important to note that formate is itself a powerful OH$^{\bullet}$-radical scavenger ($k_2 = 3.2 \times 10^4\ \mathrm{mol^{-1} \cdot l \cdot s^{-1}}$) [37,38], although its effective synovial fluid concentration largely precludes its competition with alternative scavengers (e.g. glucose).

In theory, the potentially deleterious production of these species during hypoxic/reperfusion injury could be inhibitable by the employment of novel anti-inflammatory drugs that have the ability to scavenge reactive oxygen radical species such as $O_2^{\bullet -}$ or OH$^{\bullet}$, and experiments to test this hypothesis are required. The experiments performed here demonstrate that proton Hahn spin-echo n.m.r. spectroscopy is readily applicable to the study of oxidative damage to biomolecules occurring in the inflamed rheumatoid joint during hypoxic reperfusion injury.

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