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*Short Communication*

# Oxidative damage to synovial fluid from the inflamed rheumatoid joint detected by $^1$H NMR spectroscopy

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## Introduction

The glycosaminoglycan hyaluronate (hyaluronic acid, hyaluronan) [1] is a linear repeating disaccharide, β-D-glucuronyl-β-D-*N*-acetylglucosamine, of very high molecular mass (>4 × $10^6$) which is a major component of the proteoglycan aggregates required for the functional integrity of extra-cellular matrices such as articular cartilage [2]. Hyaluronan is continually secreted in its unaggregated form by the type II synoviocytes and is largely responsible for the very high viscosity of knee-joint synovial fluid [3].

It has previously been demonstrated that in patients with rheumatoid arthritis or other inflammatory joint diseases, synovial fluid hyaluronate is depolymerized with a corresponding modification in the viscoelastic properties of synovial fluid and an increase in the synovial level of low-molecular-mass hyaluronate-derived saccharides [4]. This fragmentation is presumed to occur by the action of reactive oxygen radical species (RORS) [5] since (1) hyaluronidase activity is absent from both normal and inflammatory synovial fluid samples [6], and (2) there is currently a large quantity of experimental evidence implicating the involvement of these chemically-reactive oxygen metabolites in the pathogenesis of inflammatory joint diseases [7–9]. In addition to the presence of potential oxygen radical generating systems within the joint, this hypothesis is also supported by the demonstration of hyaluronate depolymerization by such systems *in vivo* as evidenced by decreases in apparent hyaluronate molecular mass and viscometric parameters.

We have previously suggested that hypoxic-reperfusion injury during perambulatory motion of the inflamed rheumatoid joint is a mechanism which perpetuates the deleterious production of oxygen-derived radical species within the joint, giving rise to the chronicity of the inflammation [9]. Much of the toxicity of RORS to biological systems is attributable to the highly reactive hydroxyl radical (·OH) [10], the formation of which appears to be mediated by low-molecular-mass iron chelates.

In this communication we present evidence for RORS-mediated damage to knee-joint synovial fluid in patients with inflammatory synovitis. Nuclear magnetic resonance (NMR) spectroscopy has been extensively used for investigating the metabolic profile of body fluids [11], particularly for monitoring the status and levels of low-molecular-mass endogenous components. We have employed high field proton Hahn spin-echo NMR spectroscopy to detect and characterize a series of low-molecular-mass species derived from the attack of radiolytically-generated RORS on hyaluronate. We have also utilized this technique to assess RORS-mediated oxidative damage to synovial fluid hyaluronate *in vivo* during hypoxic-reperfusion injury. In addition, the

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application of end-products derived from the reaction of ·OH radical with synovial fluid carbohydrate systems as markers of disease severity is discussed.

## Materials and Methods

*Reagents*

Rooster comb hyaluronic acid, α-D-glucose and *N*-acetyl-D-glucosamine were obtained from Sigma Chemical Co.

*NMR measurements*

$^1$H NMR measurements were conducted on a JEOL GSX-500 NMR spectrometer operating at 500 MHz for proton. All spectra were recorded at ambient probe temperature. Typically, 0.60 ml of serum, knee-joint synovial fluid or 10.0 mg ml$^{-1}$ solutions of hyaluronate in 10 mmol l$^{-1}$ phosphate buffer, pH 7.40, or aqueous solutions of α-D-glucose were placed in a 5-mm diameter NMR tube, and 0.10 ml of $D_2O$ 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 gated secondary irradiation at the water frequency. The Hahn spin-echo sequence $D[90°x\text{-}\tau\text{-}180°y\text{-}\tau\text{-collect}]$ was repeated 72–135 times with $\tau$ = 60 ms. Chemical shifts were referenced to external sodium 3-(trimethylsilyl)-1-propanesulphonate (TSP, $\delta$ = 0 ppm).

*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.

For the study of hypoxic-reperfusion injury, patients were subjected to exercise by isometric quadriceps contraction. This technique was performed as previously described [9].

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 rpm for 10 min to remove cells and debris. The supernatant was then stored at −70°C prior to treatment with gamma-irradiation and $^1$H NMR analysis.

*Serum samples*

Serum samples were obtained from nine consenting, healthy male volunteers by allowing freshly drawn non-heparinized blood to clot. These samples were centrifuged and stored as described above.

*Gamma-radiolysis of biological fluids*

Synovial fluid, serum and aqueous solutions of hyaluronate were subjected to gamma-radiolysis in the presence of atmospheric oxygen using a $^{60}$Co source. The total dosage employed was varied from 0.048 to 5.0 × 10$^3$ Gy, at a dose rate of 4.76 Gy min$^{-1}$. Under these experimental conditions, the major primary radical species present are ·OH ($G$ = 2.7), $e^-_{(aq.)}$ ($G$ = 2.7) and H· ($G$ = 0.5), where the $G$-value represents the 10$^{-6}$ mol l$^{-1}$ concentration of product per 10 Gy dosage. Where appropriate, samples were saturated with $N_2O$ gas prior to irradiation in order to provide a relatively "clean" source of ·OH radicals.

## Results and Discussion

*NMR spectra of untreated and gamma-irradiated synovial fluid from rheumatoid patients and serum from normal controls*

Employment of the Hahn spin-echo technique to suppress broad resonances arising from relatively immobile macromolecules yields $^1$H NMR spectra containing well resolved signals attributable to mobile portions of macromolecules and many low-molecular-mass metabolites present in synovial fluid and blood serum. The high field (0.7–4.0 ppm) region of the 500 MHz $^1$H Hahn spin-echo NMR spectra of a typical rheumatoid synovial fluid obtained both prior to and following gamma-radiolysis (dose level 5.0 kGy) are shown in Figs 1(a) and 1(b), respectively. Subsequently to gamma-radiolysis, there are both qualitative and quantitative modifications in the spectra. The most striking qualitative difference between the two spectra consists of the presence of an intense singlet at 2.044 ppm in the spectrum of the irradiated sample. This signal, which is located slightly upfield to the broader resonances of *N*-acetyl-$CH_3$ protons of the mobile carbohydrate portions of *N*-acetylated glycoproteins, is probably attributable to the *N*-acetyl-$CH_3$ group protons of a molecularly mobile low-molecular-mass oligosaccharide derived from the radiolytic fragmentation of either synovial fluid hyaluronate or the polysaccharide moieties of *N*-acetylated glycoproteins. In addition, normalization of the

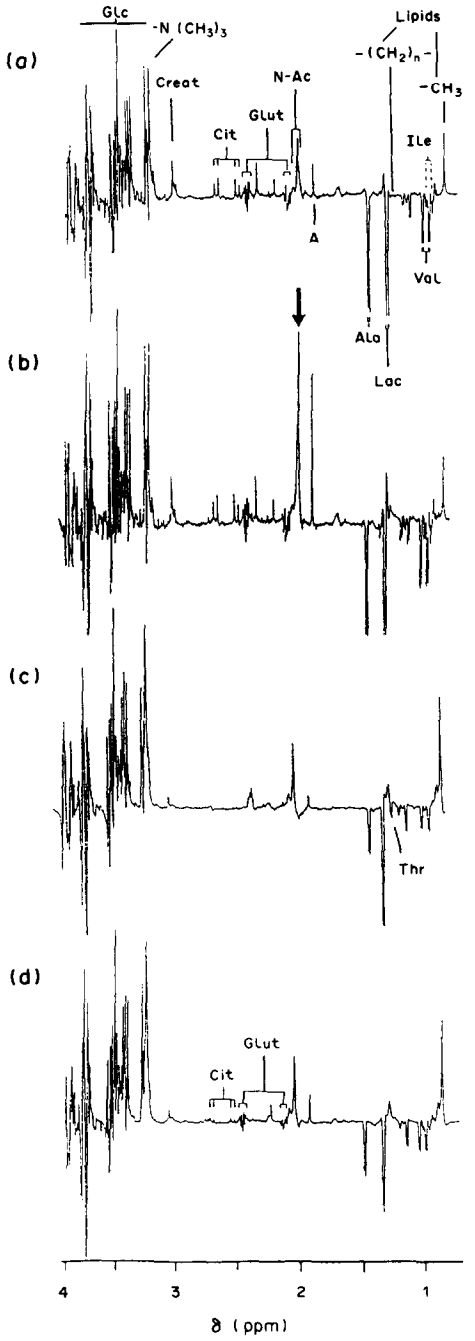


**Figure 1**
Low frequency region of 500 MHz $^1$H spin-echo NMR spectra of (a) untreated synovial fluid obtained from a patient with rheumatoid arthritis, (b) as (a), but subjected to gamma-irradiation treatment at a dose level of 5.0 kGy, (c) untreated serum obtained from a normal volunteer and (d) as (c), but exposed to gamma-irradiation as in (b). Typical spectra are shown. Abbreviations: A, acetate; Ala, alanine; Cit, citrate; Creat, creatinine; Glc, glucose; Glut, 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 ppm, attributable to a low-molecular-mass oligosaccharide species derived from the radiolytic degradation of synovial fluid hyaluronate.

intensity of resonances present in the spectra relative to those of the valine and alanine $-CH_3$ groups reveals radiolytically-induced increases in the intensities of the acetate $-CH_3$, citrate $-CH_2-$ and lactate $-CH_3$ group signals, the most notable being the rise in the effective concentration of molecularly mobile (non-protein-bound) acetate. Although it is possible that the increase in the level of NMR-detectable acetate is attributable to the attack of radiolytically-generated oxygen radicals (e.g. ·OH) or aquated electrons ($e^-_{(aq.)}$) on hyaluronate or the *N*-acetylated polysaccharide moiety of *N*-acetylated glycoproteins, it is likely that these modifications in the spectrum are a consequence of the radiolytically-mediated removal of these anionic metabolites from synovial fluid protein binding sites. $^1$H Hahn spin-echo NMR spectra (500 MHz) of untreated and gamma-irradiated (5.0 kGy) synovial fluid samples obtained from a total of eight rheumatoid patients demonstrated that the marked modifications in the spectra subsequent to irradiation treatment were always reproducible. Indeed, spectra of several of the samples investigated contained the sharp 2.044 ppm singlet resonance prior to gamma-radiolysis.

Figures 1(c) and 1(d) show corresponding $^1$H Hahn spin-echo NMR spectra of a typical sample of blood serum from a normal volunteer which were obtained before and after treatment with gamma-radiation at a dose level of 5.0 kGy. These spectra clearly demonstrate that the sharp oligosaccharide *N*-acetyl-$CH_3$ group signal at 2.044 ppm is absent from the spectrum of the irradiated sample, suggesting that this resonance, exclusively detected in the spectra of control or gamma-irradiated synovial fluid samples, is derived from the fragmentation of hyaluronate.

Further modifications in the spectrum of normal serum subsequent to treatment with gamma-irradiation include quantitative differences in the (normalized) relative intensities of the acetate, citrate and glutamate resonances. In the non-irradiated (control) serum, the characteristic AB coupling pattern of mobile citrate at 2.65 ppm is of very low intensity, and glutamine is not detectable whatsoever. Moreover, the non-protein-bound acetate concentration is relatively low. However, following treatment with gamma-irradiation at a dose level of 5.0 kGy, the relative intensities of both the acetate and citrate signals markedly in-

crease, and resonances attributable to mobile glutamine are now clearly visible. Hence, ·OH radical, $e^-_{(aq.)}$, $\cdot O_2^-$, or a combination of two or more of these species appear to have the ability to mediate the release of these endogenous metabolites from positively-charged protein binding sites. These radiolytically-mediated modifications in spectra were reproducible in a total of nine independent normal serum samples.

It should be noted that the production of NMR-detectable levels of the amino acid glutamine in biological fluids subjected to gamma-radiolysis may also be attributable to the oxygen radical-mediated degradation of synovial fluid and serum proteins. However, gamma-irradiation of an aqueous solution of human immunogammaglobulin G at a dose level of 5.0 kGy did not generate any NMR detectable glutamine (data not shown).

The low field (high frequency) region of typical $^1$H Hahn spin-echo NMR spectra of rheumatoid synovial fluid and serum from a normal volunteer both prior to and following gamma-radiolysis (5.0 kGy) are exhibited in Fig. 2. The control (non-irradiated) spectrum of synovial fluid contains two inverted doublets centred at 6.893 ppm attributable to *p*-tyrosine, a well defined formate proton resonance located at 8.388 ppm, and two histidine aromatic ring proton resonances at 7.10 and 7.80 ppm. Subsequent to gamma-radiolysis, the normalized intensity of the formate proton resonance in spectra of rheumatoid synovial fluid or normal serum markedly increases. Formate is a well-known "end-product" of the radiolytic degradation of some common carbohydrates [12–14].

It should be noted that formate is also generated from gamma-radiolysis of aqueous $CO_2$ and/or $HCO_3^-$ solutions [15]. However, formate production via this mechanism is unlikely to contribute significantly to the radiolytically-dependent increase in its synovial fluid or serum concentrations observed here, since this process is markedly inhibited in oxygenated solutions.

*NMR studies of radiolytically-induced oxidative damage to hyaluronate and glucose*

Phosphate-buffered aqueous solutions of a commercial sample of hyaluronic acid were gamma-irradiated at dosage levels of 0.048, 0.143 and 5.0 kGy to provide confirmation that the sharp 2.044 ppm singlet observed in

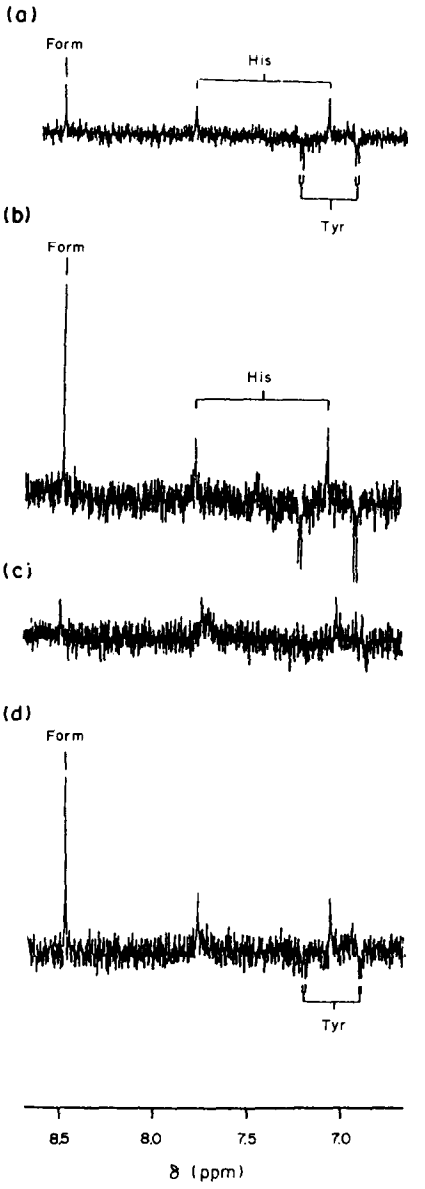


**Figure 2**
Corresponding high frequency region of the 500-MHz $^1$H spin-echo NMR spectra of untreated and gamma-irradiated (5.0 kGy) biological fluids shown in Fig. 1 (a–d). Abbreviations: Form, formate; His, histidine; Tyr, tyrosine.

gamma-irradiated (and some untreated) rheumatoid synovial fluid samples was derived from ·OH radical-mediated oxidative damage to hyaluronate.

The $^1$H Hahn spin-echo NMR spectrum of a non-irradiated sample contains weak lactate and acetate resonances located at 1.33 and 1.93 ppm, respectively [Fig. 3(a)]. Although acetate may arise from a limited amount of hydrolysis of the *N*-acetyl-glucosamine saccharide, both acetate and lactate are end-products from the metabolic degradation of hyaluronate [16].

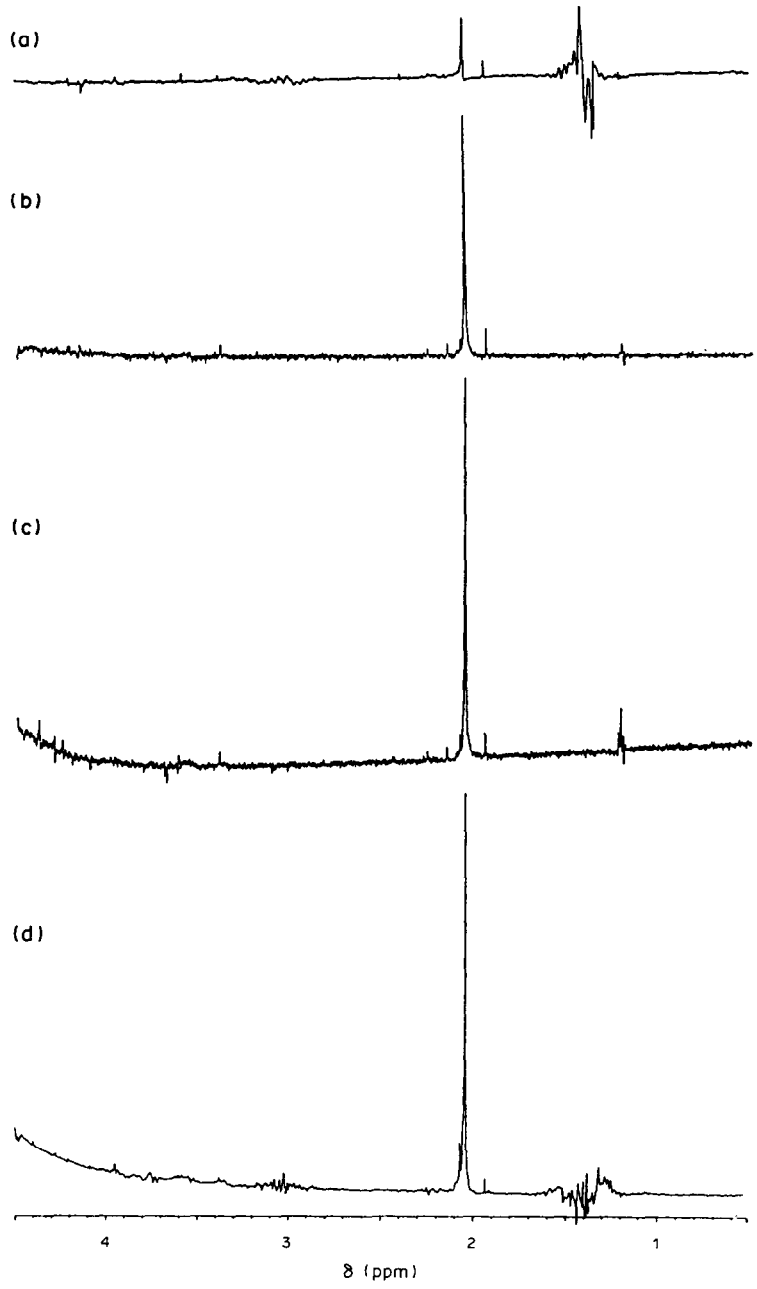


**Figure 3**
Low frequency region of 500-MHz $^1H$ spin-echo NMR spectra of a commercial sample of hyaluronate exposed gamma-irradiation at dose levels of (a) 0, (b) 48, (c) 143 and (d) $5.0 \times 10^3$ Gy.

The spectrum also has a series of very weak singlets and multiplets in 3.3–4.0 ppm carbohydrate proton chemical shift range. Moreover, the sharp 2.044 ppm singlet is also clearly visible, demonstrating that commercial samples of hyaluronate contain small quantities of low-molecular-mass saccharide species.

Corresponding spectra of 10 g $l^{-1}$ solutions of hyaluronate obtained subsequent to gamma-radiolysis at dose levels of 0.048, 0.143 and 5.00 kGy are exhibited in Figs 3(b), 3(c) and 3(d), respectively. At a dose of 48 Gy, the 2.044 ppm singlet markedly increases in intensity, and two additional sharp singlets located at 2.070 and 2.140 ppm are also present in the spectrum. The 2.070 ppm signal further increases in intensity at a dose level 143 Gy, as indeed does the 2.044 ppm singlet.

However, at a dose level of 5.00 kGy the

intensity of the 2.044 ppm signal does not increase further, indicating that the ·OH radical-mediated hyaluronate depolymerization is an autocatylitic (self-perpetuating) process. It should also be noted that hyaluronate is susceptible to depolymerization by radiolytically-generated aquated electrons ($e^-_{(aq.)}$), the reaction proceeding quite rapidly (second-order rate constant, $k_2 = 1.4 \times 10^8$ $mol^{-1}$ l $s^{-1}$) [17].

The high frequency region of the spectra of gamma-irradiated hyaluronate or α-D-glucose solutions contained the formate porton singlet signal at 8.388 ppm. Gamma-radiolysis of aqueous hyaluronate solutions which were previously saturated with $N_2O$ to remove $e^-_{(aq.)}$ gave rise to only a small decrease in the intensity of this resonance, indicating that formate is indeed a terminal product derived from ·OH radical attack on these model systems.

*Assessment of oxidative damage to hyaluronate in knee-joint synovial fluid during hypoxic-reperfusion injury*

In order to investigate the influence of RORS generation during exercise-induced hypoxic-reperfusion injury on the production of the molecularity mobile hyaluronate-derived oligosaccharide species by $^1H$ NMR spectroscopy, a group of patients with inflamed knees ($n = 4$) were subjected to exercise by isomeric quadricep contraction (for a 2-min period) and synovial fluid samples were collected at 2-min intervals. The low frequency (1.0–4.0 ppm) region of typical spectra of synovial fluid samples obtained from a rheumatoid patient that were aspirated at (a) 2 min pre-exercise, (b) immediately following exercise and (c) 2 min post-exercise are shown in Fig. 4. Subsequent to exercise, the 2.044 ppm singlet resonance (initially present in the spectrum of the pre-exercise sample) increases in intensity relative to that of the broader signal attributable to the *N*-acetylated glycoprotein methyl group, implicating the reactive-oxygen radical mediated fragmentation of hyaluronate. The relative intensity of the 2.044 ppm resonance increases further at the 4-min post-exercise time point, but subsequently decreases to its previous pre-exercise "steady-state" level at the 6 and 8-min post-exercise time-points (data not shown). This indicates that the elevated levels of the hyaluronate-derived oligosaccharide species are further degraded by RORS, or alternatively, are rapidly cleared from the synovium. These observations were reproducible in corresponding samples obtained from two of the three remaining rheumatoid patients that were subjected to exercise.

Spectra of synovial fluid samples from a series of further patients ($n = 4$) did not contain the sharp hyaluronate-derived oligosaccharide—$NHCOCH_3$ group resonance at 2.044 ppm, indicating that it was unresolved from the relatively broad *N*-acetylated glycoprotein —$CH_3$ group signals located slightly downfield. As expected, spectra of ultrafiltrates of these samples contained this oligosaccharide signal (data not shown). It should also be noted that the intensity of this resonance in spectra of synovial fluid samples is likely to be determined by the exercise status of the patients inflamed knees at the time at which the samples were collected.

Breakage of the critical glycosidic linkage in synovial fluid hyaluronate by ·OH radicals indirectly generated by the occurrence of one or more transient ischaemial reperfusion events during exercise of the inflamed rheumatoid joint rapidly gives rise to the formation of low-molecular-mass *N*-acetyl-glucosamine-containing oligosaccharide species, as demonstrated here.

In addition, ·OH radical-mediated oxidative damage to low-molecular-mass carbohydrates present in synovial fluid (predominantly glucose) can give rise to a wide range of further products. These include acids produced via attack at the extremities of the molecule (e.g. oxidation of $C_1$ in glucose yields gluconic acid), and aldehydes via ring cleavage. Radiolytic degradation of hexoses in aqueous solution (largely attributable to the indirect action of ·OH radical) gives rise to lower saccharides, aldonic and uronic acids, and three, two- and one-carbon aldehyde fragments [18]. Moreover, both formate and carbon dioxide are produced in the final stages of oxidative damage to simple mono- and disaccharides. However, it is important to note that formate is itself a powerful ·OH radical scavenger [19]. Hence, the relatively high levels of formate observed in rheumatoid synovial fluid may reflect its perpetual regeneration via the further interaction of RORS with endogenous carbohydrate systems located in the inflamed rheumatoid joint.

The experiments described above show the

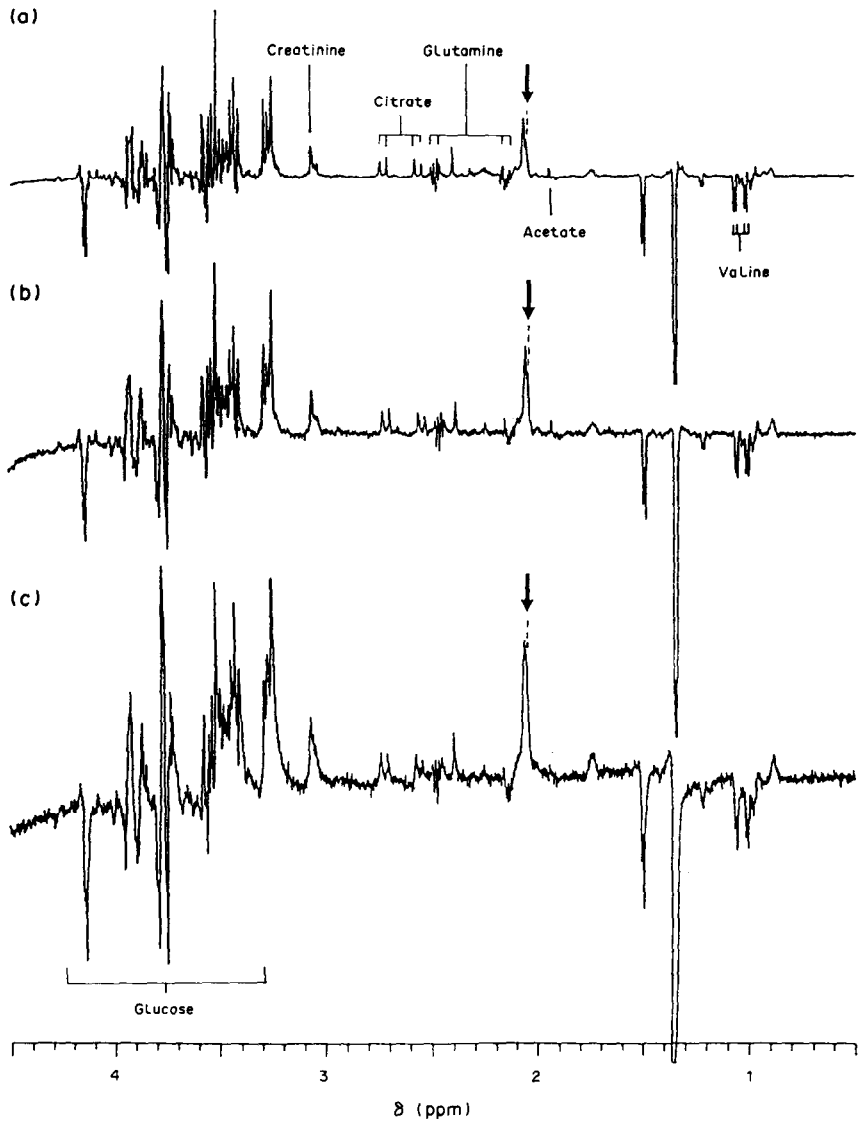


**Figure 4**
Low frequency region of 500-MHz $^1H$ spin-echo NMR spectra of samples of synovial fluid obtained at increasing time-points from a rheumatoid patient subjected to exercise by isometric quadriceps contraction for a period of 2 min. (a) 2 min pre-exercise, (b) immediately following exercise 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.

application of proton Hahn spin-echo NMR spectroscopy for the study of synovial fluid from rheumatoid patients, particularly during hypoxic-reperfusion injury.

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