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High resolution proton NMR investigations of rat blood plasma

Assignment of resonances for the molecularly mobile carbohydrate side-chains of 'acute-phase' glycoproteins

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An intense broad resonance at 2.14 ppm present in high field (400, 500 and 600 MHz) Hahn spin-echo ^{1}H-NMR spectra of rat blood plasma, but absent from those of human blood plasma is attributable to the presence of terminal *O*-acetylsialate sugars in the molecularly mobile carbohydrate side-chains of 'acute-phase' glycoproteins (predominantly α_1-acid glycoprotein). The presence of such alternative acetylsugars in the carbohydrate side-chains of rat plasma glycoproteins are of much physiological and experimental significance in view of the regular use of these animals in model systems of human inflammatory conditions.

^{1}H-NMR; Rat plasma; Acute-phase glycoprotein; *O*-Acetylsialate; *N*-Acetylated sugar; Inflammation

1. INTRODUCTION

High resolution proton (^{1}H) nuclear magnetic resonance (NMR) spectroscopy is a well established and powerful tool which enables the rapid simultaneous study of complex mixtures of endogenous or, where appropriate, exogenous components present in biological matrices [1–4]. The technique is largely non-invasive since it has little or no requirement for sample pretreatment. Indeed, much useful biochemical and clinical information can be obtained from ^{1}H-NMR investigations of body fluids such as blood plasma, urine, knee-joint synovial fluid and saliva. The broad overlapping resonances arising from the large number of macromolecules present are routinely suppressed by spin-echo Fourier-transform (SEFT) methods, resulting in profiles which contain many well-resolved resonances attributable to a wide variety of low-molecular-mass (non-protein-bound) metabolites and the mobile portions of macromolecules which are detectable at concentrations $> 5 \times 10^{-5}$ mol · dm^{-3}.

Our interest in the applications of high field biomedical NMR spectroscopy to the rapid diagnosis and assessment of a range of inflammatory diseases in humans, and the ability of animal model systems to mimic such clinical conditions, has led us to perform a comparative evaluation of the ^{1}H-NMR profiles of both healthy human and rat blood plasmas. Throughout the course of these investigations it became clear that whereas single-pulse and SEFT ^{1}H spectra of all human plasmas contained only two distinct glycoprotein resonances located at 2.04 and 2.08 ppm (attributable to the $-NHCOCH_3$ groups of *N*-acetylsugars present at the 5,5′- and 2,7- positions respectively in the branching carbohydrate side-chains [5–8]), corresponding spectra of all rat plasma samples examined contained an additional intense broad signal centred further downfield at 2.14 ppm.

In this communication we present evidence for the assignment of this 2.14 ppm rat plasma signal to glycoprotein acetylneuraminate sugars other than *N*-acetylneuraminate (sialate). The presence of these alternative acetylsugars in the branching carbohydrate side-chains of rat plasma 'acute-phase' glycoproteins is discussed in terms of their biochemical, physiological and pathological significance.

2. MATERIALS AND METHODS

2.1. *Proton NMR measurements*

Proton (^{1}H) NMR measurements on human and rat plasma samples were conducted on Bruker WH 400, Bruker AMX-600 (University of London Intercollegiate Research Services (ULIRS), Queen Mary and Westfield College facilities, University of London, UK) and JEOL JNM-GSX 500 (ULIRS, Biomedical NMR centre, Birkbeck College, University of London, UK) spectrometers operating in quadrature detection mode at operating frequencies of 400.13, 600.13 and 500.16 MHz respectively for ^{1}H. All spectra were recorded at a probe temper-

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ature of 298 K. Typically, 0.60 ml of biofluid 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 128–996 times with t = 68 ms for the Bruker spectrometers, and 60 ms for the JEOL JNM-GSX 500 facility. The intense water signal was suppressed by presaturation with gated decoupling during the delay between pulses. Proton Hahn spin-echo spectra of solutions of glycoproteins, bovine submaxillary mucin, *N*-acetylneuraminate and *N*-glycolylneuraminate in ^2H_2O were similarly acquired using 43–16,707 repetitions of the above pulse sequence.

Corresponding single-pulse (1D) spectra of rat plasma ultrafiltrates, and ^2H_2O solutions of glycoproteins and *N*-acetylsugars were also obtained. Typical pulsing conditions were: 116–5,363 free induction decays (FIDs) using 16,384 data points, 30–40° pulses and a 3 s pulse repetition rate, the latter to allow full spin-lattice (T_1) relaxation of the protons in the samples investigated.

Chemical shifts of resonances in spectra of biofluids, and glycoprotein, mucin and *N*-acetylsugar solutions were referenced to external sodium 3-trimethylsilyl[2,2,3,3-$^2H_4$]propionate (TSP; δ = 0.00 ppm). For plasma samples, the methyl group resonances of alanine (δ = 1.487 ppm), lactate (δ = 1.330 ppm) or valine (δ = 1.050 ppm) served as secondary internal references.

The relative intensities of each glycoprotein signal in 400 MHz ^1H Hahn spin-echo spectra of rat plasma and authentic rat and human α_1-acid glycoprotein and transferrin samples were determined by electronic integration.

2.2. *Sample collection and preparation*

Human and rat 'acute-phase' glycoproteins (α_1-acid glycoprotein and apotransferrin), bovine submaxillary mucin (containing ca. 12% (w/w) bound sialates), *N*-acetylneuraminic acid (from sheep submaxillary glands), and *N*-glycolylneuraminic acid (from porcine submaxillary glands) were purchased from Sigma, UK. Each glycoprotein was dissolved in deuterium oxide (^2H_2O) containing 2.86×10^{-2} mol · dm^{-3} phosphate buffer, pH 7.00 (meter reading), for ^1H-NMR analysis. The pH of stock solutions of *N*-acetylneuraminic and *N*-glycolylneuraminic acids was adjusted to 7.00 with 10 mol · dm^{-3} NaOD prior to dilution with phosphate-buffered ^2H_2O to yield 5.00×10^{-3} mol · dm^{-3} solutions. Bovine submaxillary mucin (20 mg) was added to 2.00 ml of ^2H_2O, the sample rotamixed, centrifuged to remove insoluble material and the supernatant (pH 5.5, meter reading) utilised for ^1H-NMR analysis and experiments with the enzyme neuraminidase described below.

Samples of whole blood were obtained from healthy male Wistar rats (n = 12) via cardiac puncture under anaesthesia (Sagatal) immediately prior to sacrifice by CO_2 asphyxiation. These samples were then centrifuged (2,000 × *g*) at 4°C and the plasma (heparinized) removed and stored at −20°C for a maximum duration of 72 h. Prior to ^1H-NMR analysis, rat plasma samples were thawed at ambient temperature. Relevant control experiments established that none of the criteria investigated were influenced by storage in the above manner. Where required, ultrafiltrates of rat plasma samples were obtained using Amicon centrifree micropartition devices (< 10 kDa molecular weight cut-off).

Blood was also collected from 8 consenting healthy male human volunteers and placed in sterile plastic vials containing lithium heparin. The blood was centrifuged (2,000 × *g*) at 4°C and the resulting plasma stored and thawed as described above prior to conducting NMR measurements.

Mild basic hydrolysis of the *O*-acetyl groups of rat plasma glycoprotein carbohydrate side-chain *O*-acetylsialate residues was conducted by adjusting 0.20 ml aliquots of 5 different rat plasma samples to pH 11.5 with 1.00 mol · dm^{-3} NaOH and allowing them to equilibrate at 37°C for a period of 18 h with corresponding untreated (control) samples. Each sample was then re-adjusted to pH 7.4 with 1.00 mol · dm^{-3} HCl and made up to a volume of 0.50 ml with ^2H_2O prior to ^1H-NMR analysis. SEFT ^1H-NMR spectra were also acquired on the control rat plasma samples.

Aliquots of six of the rat plasma samples obtained (0.40 ml) were each adjusted to pH 5.0 with 1.00 mol · dm^{-3} HCl and then treated with the enzyme neuraminidase (*Clostridium perfringens*; Sigma, UK, 5.8 or 8.7 units per ml) for 4.5 h at 37°C. Subsequently, samples were removed and their pH value retained at 5.0 to conserve *O*-acetylsilate sugars which do not appear to de-*O*-acetylate in the pH range of 3–6 [9]. All samples were then stored at −20°C for 65 h prior to conducting NMR experiments. SEFT ^1H-NMR spectra were acquired on neuraminidase-treated samples. Corresponding 0.40 ml aliquots of the above rat plasma samples untreated with neuraminidase but subjected to the same pH modification and equilibrated at 37°C for the same time periods served as controls which also underwent ^1H-NMR analysis. Further control experiments established that there were no differences between the SEFT ^1H-NMR spectra of these control samples and those adjusted to pH 5.0 as above and stored at 4°C for periods of up to 72 h. Where sufficient volumes of sample were available, control and neuraminidase-treated rat plasmas were ultrafiltered as described above prior to storage at −20°C and single-pulse ^1H-NMR spectra of the resulting ultrafiltrates obtained.

Solutions of bovine submaxillary mucin in ^2H_2O prepared as described above were also treated with *Clostridium perfringens* neuraminidase (7.4 units per ml). SEFT ^1H-NMR spectra of these solutions were acquired following their equilibration at ambient temperature for periods of 0, 2.95 and 6.67 h.

With the exception of an acetate signal located at 1.92 ppm, SEFT ^1H-NMR spectra of the above commercially available neuraminidase preparation did not contain any resonances in the chemical shift region of interest (1.70–2.30 ppm).

3. RESULTS

Fig. 1 shows the 0.80–2.80 ppm regions of typical 400 MHz SEFT ^1H-NMR spectra of healthy rat and human plasma samples. The broad protein envelope arising from the large number of macromolecules present in these samples is suppressed and only resonances attributable to molecularly mobile protons (i.e. those with long T_2 values) are present. Spectra of healthy human plasma samples contain intense broad resonances located at 2.04 ppm (signal I: $\Delta\nu_{1/2}$ = 7.8 Hz) and 2.08 ppm (signal II: $\Delta\nu_{1/2}$ = 11.6 Hz) which are attributable to the *N*-acetyl-CH_3 group protons of *N*-acetylsugars (*N*-acetylneuraminate and *N*-acetylglucosamine) 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), as previously reported by Bell et al. [8]. Corresponding SEFT ^1H-NMR spectra of rat plasma, however, contained a further intense broad singlet resonance located downfield at 2.14 ppm (signal III: $\Delta\nu_{1/2}$ = 8.3 Hz) in addition to the 2.04 and 2.08 ppm signals observed in human plasma. These distinctive broad resonances present in SEFT ^1H spectra of all rat plasma samples investigated (n = 12) are almost identical to those of an authentic, commercially-available sample of rat α_1-acid glycoprotein (Fig. 2a) which has a molecular mass of 44 kDa. The line-widths of the 2.04, 2.08 and 2.14 ppm signals in spectra of rat α_1-acid glycoprotein were 6.4, 3.8 and 6.6 Hz, respectively. As expected, the 2.14 ppm resonance was absent from corresponding spectra of human α_1-acid glycoprotein (Fig. 2b).

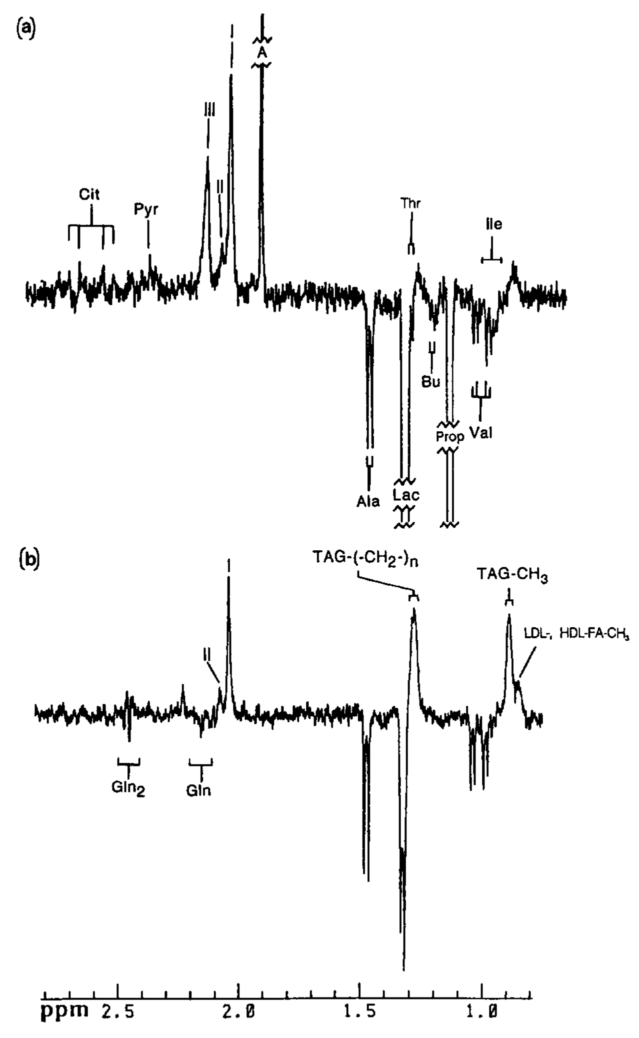

Fig. 1. 400 MHz ^{1}H Hahn spin-echo (t = 68 ms) spectra of the 0.80–2.80 ppm regions of (a) normal rat and (b) normal human blood plasmas. Typical spectra are shown. Resonances I, II (rat and human plasma) and III (rat plasma only) arise from the $-COCH_3$ group protons of acetylsugars present in the branching carbohydrate side-chains of 'acute-phase' glycoproteins (predominantly α_1-acid glycoprotein). The line-widths ($\Delta\nu_{1/2}$) of the rat plasma glycoprotein signals I, II and III are 7.2, 10.0 and 8.3 Hz, respectively. Abbreviations: A, acetate-CH_3; Ala, alanine-CH_3; Bu, 3-D-hydroxybutyrate-CH_3; Cit, citrate-CH_2; Gln_1 and Gln_2, β- and γ-CH_2 groups of glutamine respectively; Ile, isoleucine-CH_3; Lac, lactate-CH_3; LDL-, HDL-FA-CH_3, acyl chain terminal $-CH_3$ groups of low- and high-density-lipoprotein-associated fatty acids (predominantly cholesterol esters); Prop, propane-1,2-diol-CH_3; Pyr, pyruvate-CH_3; TAG-CH_3 and $-(-CH_2-)_n$, predominantly chylomicron- and very-low-density-lipoprotein (VLDL)-associated triacylglycerol acyl chain terminal-CH_3 and bulk $-CH_2-$ groups respectively; Thr, threonine-CH_3; Val, valine-CH_3. The propane-1,2-diol detectable in spectra of rat plasma is a component of the vehicle employed for administration of the Sagatal anaesthetic.

All three of these rat plasma glycoprotein acetylsugar signals were absent from single-pulse ^{1}H spectra of rat plasma ultrafiltrate (obtained by passage through an ultrafiltration membrane with a molecular weight cut-off of < 10 kDa), confirming the macromolecular nature of the components giving rise to them. Treatment of a typical rat plasma sample with rat α_1-acid glycoprotein (final concentration 2.0 mg/ml) resulted in a clear increase in the intensity of each of the 2.04, 2.08 and 2.14 ppm resonances (data not shown).

The 400 MHz SEFT ^{1}H-NMR spectra of rat and human apotransferrin are also compared in Fig. 2. Clearly, the spectrum of rat apotransferrin contains three carbohydrate side-chain signals centred at 2.04, 2.08 and 2.14 ppm ($\Delta\nu_{1/2}$ = 4.4, 3.0 and 4.0 Hz respectively), the latter being absent from the SEFT ^{1}H spectrum of the corresponding human sample. The relative intensities of the 2.04, 2.08 and 2.14 ppm rat apotransferrin signals were 1.00, 0.41 and 0.38, whereas for the SEFT ^{1}H spectrum of rat α_1-acid glycoprotein, these values were 1.00, 0.43 and 0.72, respectively, demonstrating that the branching carbohydrate side-chains of rat α_1-acid glycoprotein have a higher content of the species giving rise to the 2.14 ppm signal than those of rat apotransferrin. The intensity ratios of the 2.04 and 2.08 ppm signals were similar for both 'acute-phase' glycoproteins.

Since the 2.14 ppm rat plasma 'acute-phase' glycoprotein signal is conceivably ascribable to the *O*-acetyl-CH_3 group protons of glycosidically-bound *O*-acetylated *N*-acetyl- and *N*-glycolyl-neuraminates, the SEFT ^{1}H-NMR spectrum of this biofluid was compared with that of bovine submaxillary mucin (Fig. 3) which is known to contain a mixture of carbohydrate side-chain bound *N*-acetylneuraminate (Neu5Ac), *N*-glycolylneuraminate (Neu5Gc), *N*-acetyl-9-mono-*O*-acetylneuraminate ($Neu5,9Ac_2$), *N*-acetyl-7-mono-*O*-acetylneuraminate ($Neu5,7Ac_2$), *N*-acetyl-7,9-di-*O*-acetylneuraminate ($Neu5,7,9Ac_3$), *N*-acetyl-7,8-di-*O*-acetylneuraminate ($Neu5,7,8Ac_3$), and *N*-glycolyl-9-mono-*O*-acetylneuraminate (Neu9Ac5Gc), with relatively large amounts of the $Neu5,9Ac_2$ species. As expected, the 1.7–2.3 ppm regions of SEFT ^{1}H spectra of the bovine mucin sample bears a striking resemblance to that of rat plasma, and contains an intense broad singlet resonance located at 2.14 ppm. Moreover, a further similarity between these spectra is the presence of a weaker, broad singlet at 1.96 ppm ($\Delta\nu_{1/2}$ = 5.3 Hz in rat plasma). The broad *N*-acetyl-CH_3 group signal centred at 2.04 ppm in the mucin spectrum exhibits a clear heterogeneity, indicating several different, magnetically-distinct *N*-acetyl-CH_3 group environments for glycosidically-bound sialates.

These data suggest that the 2.14 ppm rat plasma glycoprotein carbohydrate side-chain signal is assignable to *O*-acetyl-CH_3 group protons present in a mixture of two or more of the above sialate *O*-acetyl esters. Indeed, ^{1}H-NMR analysis of rat plasmas at higher operating frequencies (500 and 600 MHz) demonstrated that their 2.14 ppm signal contains at least two overlapping components (e.g. Fig. 3a).

Previous reports have demonstrated that both 'free'

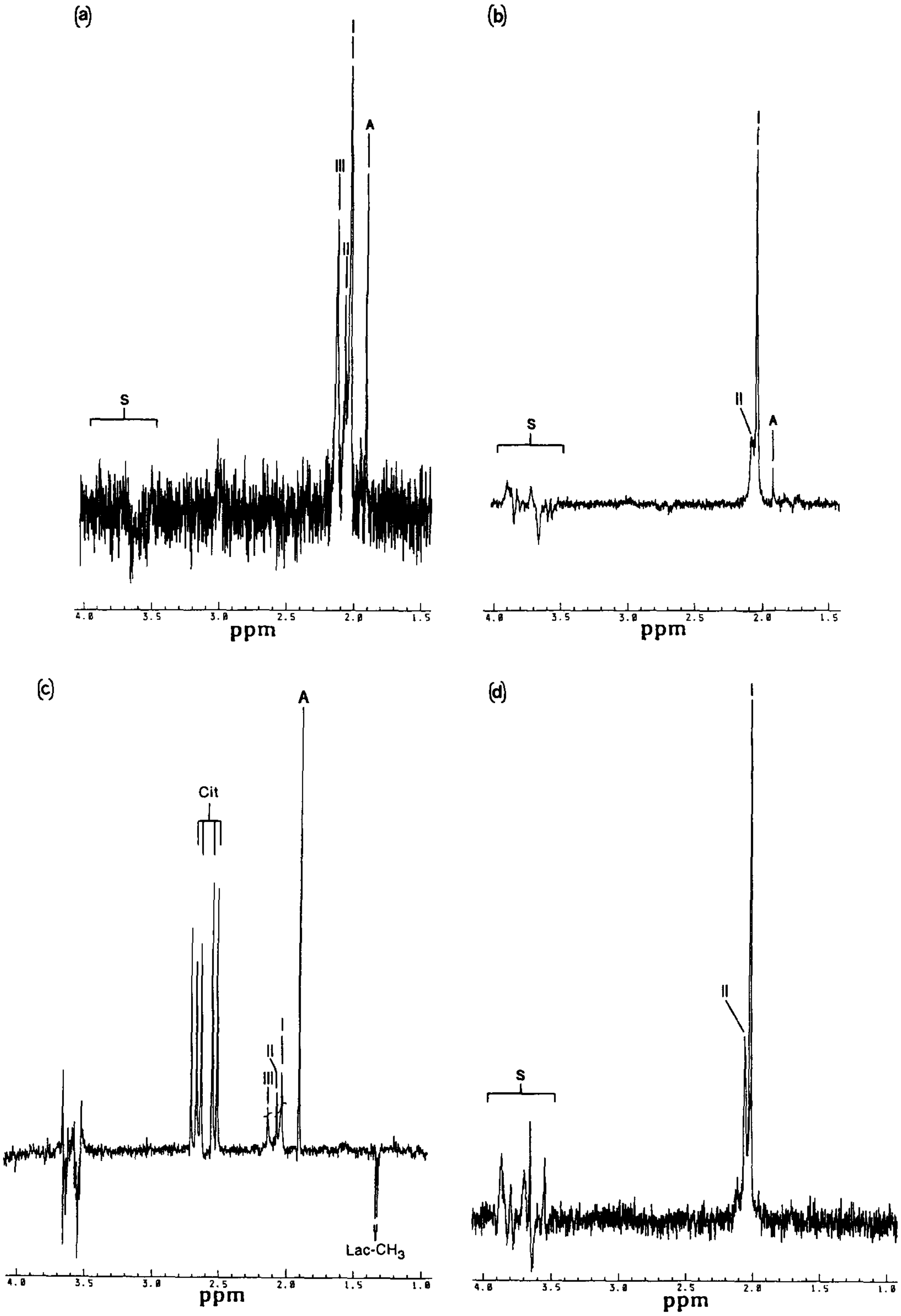

Fig. 2. Partial 400 MHz ^1H Hahn spin-echo NMR spectra of ^2H_2O solutions of (a) rat and (b) human α_1-acid glycoprotein. The concentrations of these proteins were 1.67 (rat) and 8.33 mg/ml (human). Corresponding spectra of ^2H_2O solutions of rat apotransferrin (1.67 mg/ml) and human apotransferrin (40 mg/ml) are shown in (c) and (d), respectively. Abbreviations: as in Fig. 1, with S representing side-chain sugar ring proton resonances. Acetate, citrate and lactate detectable in spectra of rat apotransferrin are impurities present in the commercially-available preparation employed for these studies.

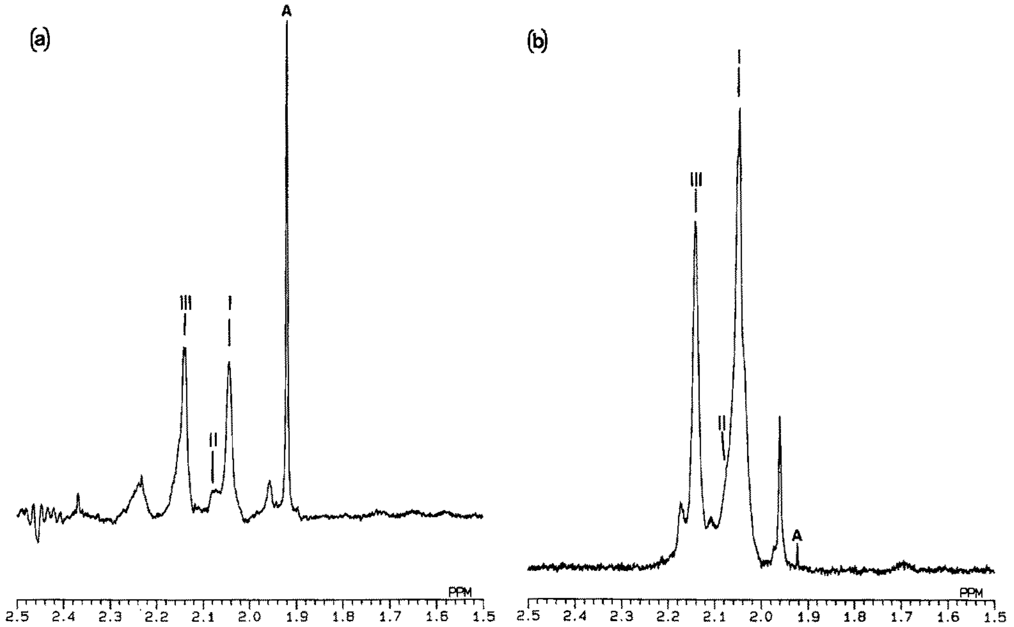

Fig. 3. 1.50–2.50 ppm regions of 500 MHz ^{1}H Hahn spin-echo (t = 60 ms) NMR spectra of (a) rat plasma and (b) bovine submaxillary mucin. Abbreviations: as in Fig. 1.

and glycosidically-bound sialates are de-*O*-acetylated in basic solution [9], and further evidence for the assignment of the broad 2.14 ppm rat plasma signal to *O*-acetylsialate species was provided by subjecting the intact biofluid to basic hydrolysis (pH 11.5) at 37°C for a period of 18 h, and monitoring modifications in the glycoprotein carbohydrate side-chain resonances. Fig. 4 shows typical partial 600 MHz SEFT ^{1}H-NMR spectra of a rat plasma sample acquired before and after hydrolysis in the above manner. These spectra clearly demonstrate the elimination of the 2.14 ppm glycoprotein signal on treatment with base, an observation accompanied by a clear increase in the intensity of the broader 2.08 ppm resonance which is partially attributable to the $-NHCOCH_3$ group protons of terminal Neu5Ac. As expected, a corresponding marked increase in the intensity of the acetate-CH_3 group signal (δ = 1.92 ppm) was observed following hydrolysis, confirming the liberation of this carboxylate anion from glycoprotein sugar *O*-acetyl groups. Modifications in the line-shape of the 2.04 ppm resonance were also notable following hydrolysis. These results were reproducible in all five samples investigated.

Although glycosidically-bound sialate *O*-acetyl-CH_3 groups are less susceptible to basic hydrolysis than the corresponding 'free' sugars [9], the complete disappearance of the 2.14 ppm signal observed indicates that the conditions employed here were adequate for their hydrolysis in intact rat plasma samples.

500 MHz SEFT ^{1}H-NMR spectra of a typical healthy rat plasma sample obtained prior and subsequent to treatment with the enzyme neuraminidase at pH 5.0 are shown in Fig. 5a and b, respectively. The latter spectrum contains a series of new resonances, e.g. intense sharp singlets located at 2.060, 2.068 and 2.126 ppm, and multiplets centred at 1.833 and 2.221 ppm. The 2.060, 1.833 and 2.221 ppm signals are assignable to 'free' Neu5Ac (Fig. 5c), consistent with previous reports [8], and those at 2.068 and 2.126 ppm to the *N*- and *O*-acetyl-CH_3 group protons, respectively, of Neu5,9Ac$_2$ (β-anomer) by a consideration of their chemical shift values and relative intensities (approximately 1:1), and reference to ^{1}H-NMR data available on a number of naturally-occurring, 'free' *O*-acetylated sialates [10]. The identity of the 2.060, 1.833 and 2.221 ppm resonances was confirmed by 'spiking' neuraminidase-treated rat plasmas with an authentic Neu5Ac standard. However, previously reported ^{1}H-NMR spectra of Neu5,9Ac$_2$ have shown that the H-3 axial (H-3$_{ax.}$) and equatorial (H-3$_{eq.}$) position proton resonances of this species have very similar chemical shift values to those of Neu5Ac [10], and hence the 1.833 and 2.221 ppm multiplets probably contain contributions from both sugars. Indeed, we were unable to resolve the H-3$_{ax.}$ and H-3$_{eq.}$ proton resonances of Neu5Ac and Neu5Gc in an equimolar mixture of the two sugars under similar experimental conditions at an operating frequency of 400 MHz. A consideration of chemical shift values suggests that the *O*-acetyl-CH_3 group of Neu5,9Ac$_2$ is the major contributor to the broad rat plasma 2.14 ppm signal when this sialate *O*-acetyl ester is glycosidally-bound. Indeed, this broad resonance was substantially reduced in intensity subsequent to neuraminidase treatment. As expected, the intensity of the 2,7-position *N*-acetylsugar signal (δ = 2.08 ppm) was also markedly diminished following treatment with this enzyme, as was that of the

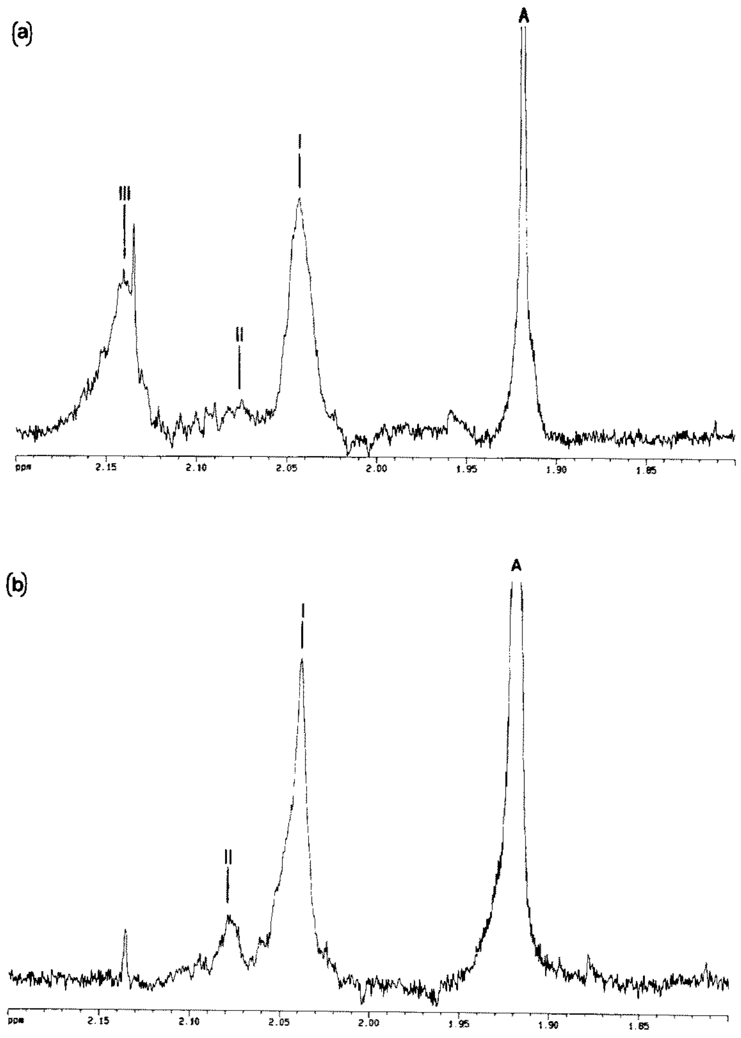

Fig. 4. Partial 600 MHz ^{1}H Hahn spin-echo (t = 68 ms) spectra of rat plasma (a) before and (b) after mild basic hydrolysis (pH 11.5) at 37°C for 18 h. Rat plasma samples were re-adjusted to pH 7.4 with 1.00 mol · dm^{-3} HCl prior to acquisition of spectra. Typical spectra are shown. Abbreviations: as in Fig. 1.

broad 1.95 ppm signal which is conceivably ascribable to a small quantity of Neu5,7Ac$_2$ present in the terminal position of rat plasma glycoprotein carbohydrate side-chains.

Further singlet resonances of lower intensity located at 1.982, 2.048, 2.099, 2.105, 2.130, 2.133 and 2.155 ppm generated by neuraminidase treatment of rat plasmas illustrate the diversity of terminal sialate sugars in the branching carbohydrate side-chains of glycoproteins present in this biofluid. Assignment of these additional signals is complicated by the experimental conditions employed here which deviate from those previously reported [10] (giving rise to small modifications in chemical shift values for some signals) and the occurrence of two anomeric forms for each sialate sugar. However, intensity considerations suggest that the 2.048 ppm resonance may be attributable to the $-NHCOCH_3$ group of the α-anomer of Neu5,9Ac$_2$. Furthermore, two of the additional signals may arise from the *N*- and *O*-acetyl-CH$_3$ groups of Neu5,7Ac$_2$. No resonances attributable to the $\text{-NH-CO-C}\underline{\text{H}}_2\text{OH}$ group protons of 'free' *N*-glycolylneuraminates (i.e. Neu5Gc and Neu9Ac,5Gc) were detectable in the 4.1–4.2 ppm chemical shift range of SEFT ^{1}H-NMR spectra of rat plasmas pre-equilibrated with neuraminidase, indicating that both Neu5Gc and Neu9Ac,5Gc are absent from the terminal position of the carbohydrate side-chains of glycoproteins present in this biofluid. The above observations were completely reproducible in a total of six different rat plasma samples investigated.

500 MHz single-pulse ^{1}H-NMR spectra of ultrafiltrates derived from control and neuraminidase-treated rat plasmas (Fig. 5d and e) confirmed that all of the new, sharp resonances generated via the actions of this enzyme could be assigned to low-molecular-mass, 'free' sialate sugars. The intensities of each of the two Neu5,9Ac$_2$ acetyl group substituent signals expressed relative to that of *N*-acetyl-CH$_3$ group of Neu5Ac in

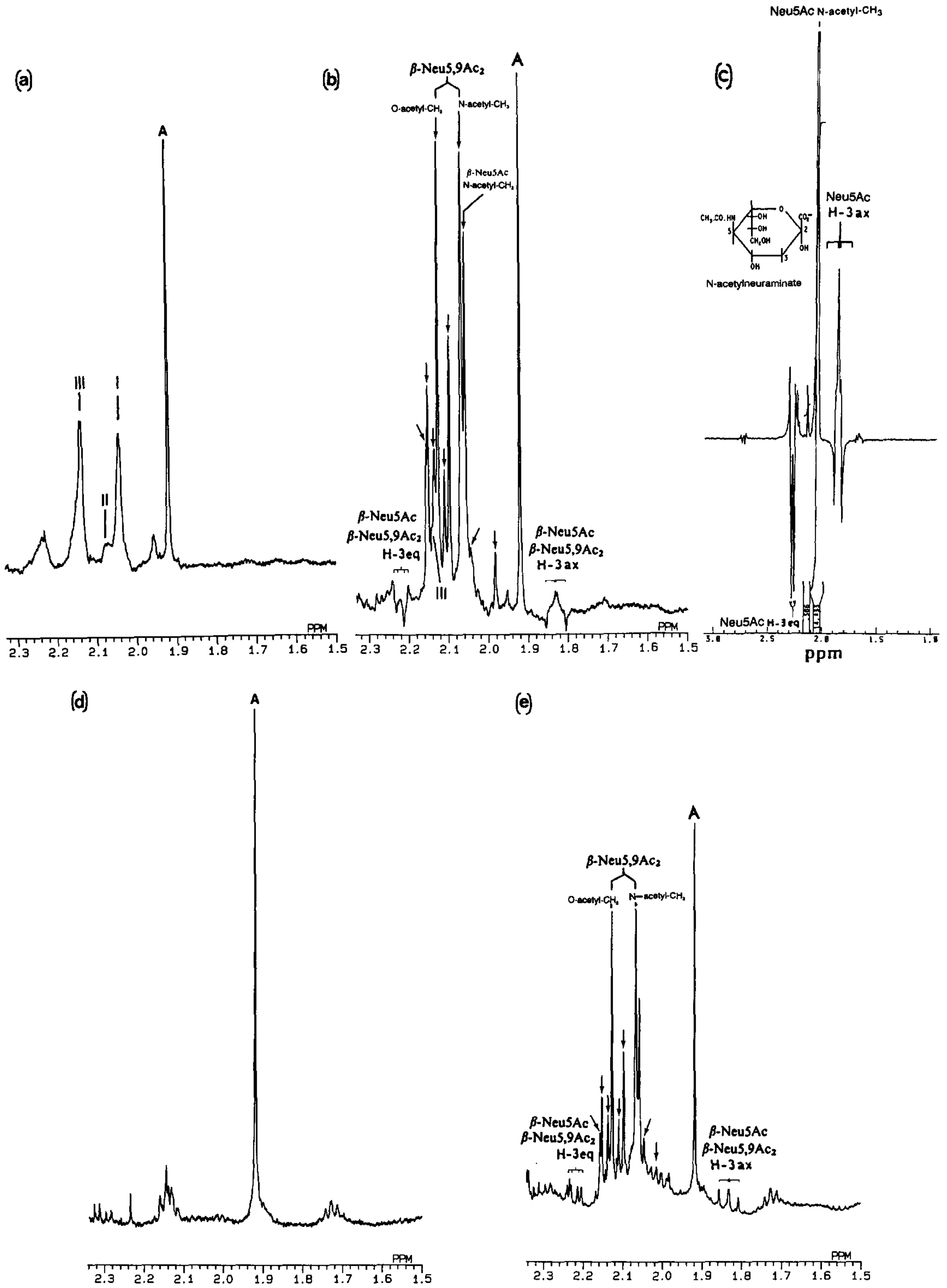

Fig. 5. 1.50–2.30 ppm regions of 500 MHz ^{1}H Hahn spin-echo NMR spectra of rat blood plasma (a) before and (b) after treatment with the enzyme neuraminidase at pH 5.0 and 37°C (section 2). (c) Partial 400 MHz SEFT ^{1}H NMR spectrum of a 5.00×10^{-3} mol · dm^{-3} solution of *N*-acetylneuraminate in ^{2}H$_2$O; (d) and (e) 1.50–2.30 ppm regions of 500 MHz single-pulse ^{1}H-NMR spectra of ultrafiltrates obtained from control and neuraminidase-treated rat plasma samples respectively. Typical spectra are shown. The 1.50–2.30 ppm regions of 500 MHz SEFT ^{1}H-NMR spectra of a solution of bovine submaxillary mucin in ^{2}H$_2$O (pH 5.5) before and at 2.95 and 6.67 h after incubation with neuraminidase at ambient temperature (section 2) are shown in (f), (g) and (h) respectively. Abbreviations: as in Fig. 1, with Neu5Ac-*N*-acetyl-CH$_3$, H-3$_{ax.}$ and H-3$_{eq.}$ representing *N*-acetylneuraminate *N*-acetyl-CH$_3$, H-3 axial and H-3 equatorial proton resonances, respectively, and Neu5,9Ac$_2$-*N*-acetyl-CH$_3$, *O*-acetyl-CH$_3$, H-3$_{ax.}$ and H-3$_{eq.}$ representing *N*-acetyl-9-mono-*O*-acetylneuraminate *N*-acetyl-CH$_3$, *O*-acetyl-CH$_3$, H-3 axial and H-3 equatorial proton resonances, respectively. Further new resonances for 'free' sialate sugars appearing subsequent to enzyme treatment are indicated by the arrows in (b), (e), (g) and (h).

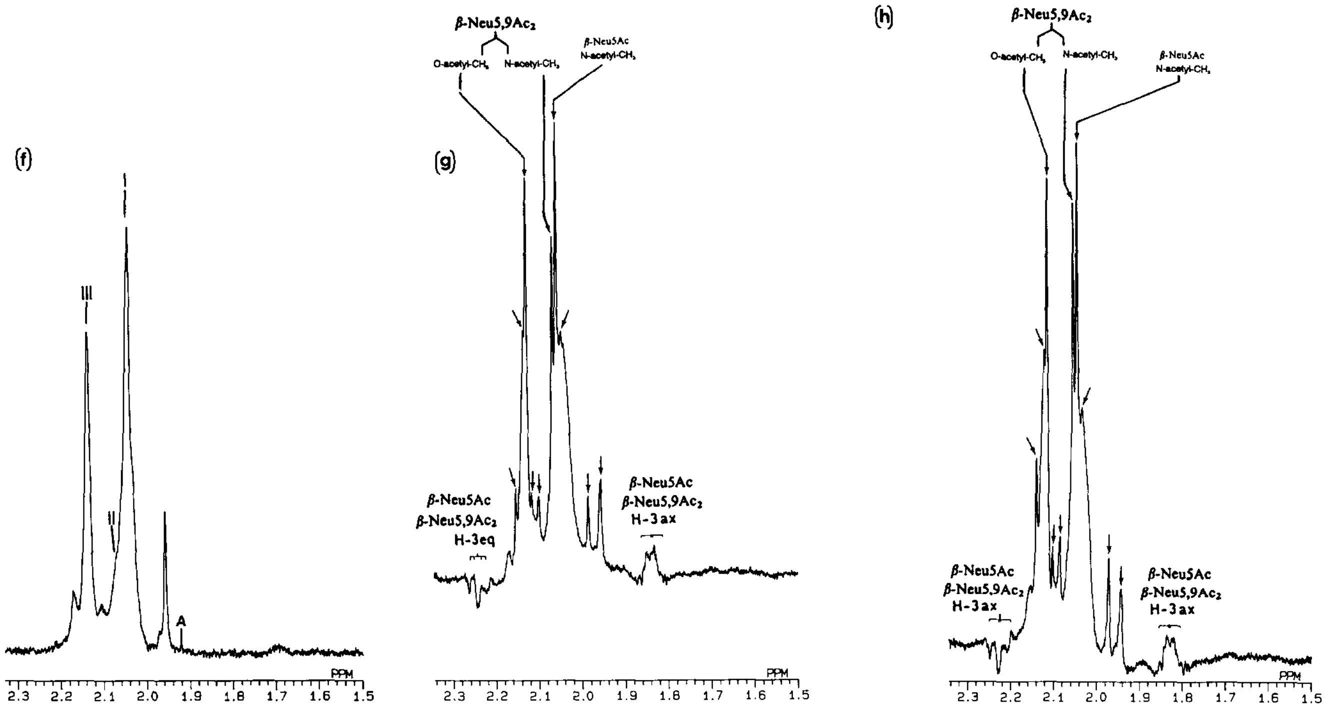

Fig. 5 (*continued*)

ultrafiltrates of neuraminidase-treated rat plasma samples was ca. 1.5:1.0. Additional weak resonances at 2.110 (singlet) and 2.019 ppm (apparent triplet) were also present in spectra of ultrafiltrates obtained from neuraminidase-treated rat plasmas. Treatment of these *O*-acetylsilate-containing ultrafiltrates with NaOH (final pH 11.5) for 3.00 h at ambient temperature gave rise to the complete removal of all singlet resonances assignable to sialate *O*-acetyl-CH_3 groups with a corresponding marked elevation in the intensities of those attributable to Neu5Ac and acetate (data not shown), observations consistent with the lability of these NMR-sensitive reporter groups towards base.

Typical SEFT ^{1}H-NMR spectra of a solution of bovine submaxillary mucin in ^2H_2O obtained before and at two different time intervals after treatment with neuraminidase at ambient temperature are shown in Fig. 5f–h. Clearly, the cleavage of terminal sialates from the branching carbohydrate side-chains of this glycoprotein by neuraminidase gives rise to a range of new, sharp signals in a time-dependent manner, i.e. singlets at 1.955, 1.984, 2.045, 2.060, 2.069, 2.104, 2.125, 2.130 and 2.156 ppm, and multiplets at 1.840 and 2.232 ppm. The 2.060 ppm singlet resonance is attributable to the $-NHCOCH_3$ group protons of Neu5Ac, and those at 2.069 and 2.125 ppm to the *N*- and *O*-acetyl-CH_3 groups, respectively, of Neu5,9Ac_2. Although the multiplets centred at 1.840 and 2.232 ppm are partially ascribable to the H-$3_{ax.}$ and H-$3_{eq.}$ protons, respectively, of Neu5Ac, these signals are also likely to contain major contributions from Neu5,9Ac_2, Neu5Gc and Neu9Ac,5Gc in view of the availability of relatively high levels of these sialates in bovine submaxillary mucin [10]. Sharp singlets located at 4.14 and 4.16 ppm were also detectable in SEFT ^{1}H-NMR spectra of neuraminidase-treated mucin samples (data not shown), consistent with the enzymic liberation of *N*-glycolylneuraminates such as Neu5Gc and Neu9Ac,5Gc from the terminal position of this glycoprotein's carbohydrate side-chains. Certain of the additional singlet resonances in the spectra shown in Fig. 5g and h are conceivably attributable to the *N*- and *O*-acetyl-CH_3 groups of the β-anomer of Neu5,7Ac_2 (1.984 and 2.156 ppm) and the *N*-acetyl-CH_3 group of the α-anomer of Neu5,9Ac_2 (2.045 ppm), and the remaining signals may arise from the *N*- and *O*-acetyl-CH_3 group protons of Neu5,7,9Ac_3, Neu5,8,9Ac_3 and Neu5Gc,9Ac in view of the prevalence of these sialates in bovine submaxillary mucin. Interestingly, a sharp doublet resonance of very weak intensity at 1.34 ppm was also generated in the spectrum on neuraminidase treatment (data not shown). This signal may be attributable to the *O*-lactyl-CH_3 group of sialate *O*-lactyl esters, indicating the release of terminal *N*-acetyl- or *N*-glycolyl-9-*O*-lactylneuraminates.

With the exception of the 'free' *N*-glycolyl- and *O*-lactyl-substituted neuraminates generated, the above results are similar to those obtained on neuraminidase treatment of rat plasmas and demonstrate the time-dependence of the enzymic release of different sialate species from the terminal position of mucin's carbohydrate side-chains.

4. DISCUSSION

The broad 2.14 ppm 'acute-phase' glycoprotein resonance present in SEFT ^{1}H-NMR spectra of rat plasma, but absent from those of all human plasma samples examined demonstrates some degree of biological specificity in terms of the chemical nature of the branching carbohydrate side-chain acetylsugars responsible for it. Indeed, in 1970 Schoop [11] reported that whereas human 'acute-phase' glycoproteins contain only Neu5Ac, serum glycoproteins of other mammals possess *O*-acetylated neuraminate derivatives (*N*-glycolyl, *O*-acetyl, *N*-,*O*-diacetyl and triacetyl-neuraminates) in addition to Neu5Ac. Previous high resolution ^{1}H NMR studies of blood plasma obtained from a variety of vertebrates (excluding rats) have provided evidence for some 'between species' heterogeneity in the chemical shift values and intensities of glycoprotein sugar acetyl-CH_3 group resonances [12], and hence the additional 2.14 ppm signal detectable in SEFT ^{1}HNMR spectra of rat plasma, ascribable to *O*-acetyl-CH_3 groups present in sialate *O*-acetyl esters, is not unexpected. The data presented in this study clearly demonstrate that α_1-acid glycoprotein, and, to a lesser extent, transferrin, contribute to each of the three glycoprotein signals observed in SEFT ^{1}H spectra of rat plasma, but haptoglobin and α_1-antitrypsin may also augment the lineshape and intensity of these resonances. In corresponding spectra of human plasma, the 2.04 and 2.08 ppm glycoprotein *N*-acetylsugar resonances are predominantly attributable to α_1-acid glycoprotein (25.0% (w/w) Neu5Ac and *N*-acetylglucosamine [13]) with haptoglobin, transferrin and α_1-antitrypsin (containing 10.0, 3.6 and 3.8% (w/w) *N*-acetylsugars respectively [13]) contributing only to a relatively minor extent [8].

Assignment of the broad rat plasma 2.14 ppm resonance to *O*-acetylsialate sugars (predominantly Neu5,9Ac$_2$) present in the terminal position of 'acute-phase' glycoprotein carbohydrate side-chains is based on (i) comparisons of the 1.7–2.3 ppm regions of SEFT ^{1}H spectra of this biofluid with that of bovine submaxillary mucin, a rich source of *O*-acetylsialates (Fig. 3); (ii) the susceptibility of sialate *O*-acetyl groups to mild basic hydrolysis (Fig. 4); and (iii) the identification of selected *O*-acetylsialate species in spectra of neuraminidase-treated rat plasmas and ultrafiltrates derived therefrom (Fig. 5).

Examination of single-pulse ^{1}H spectra of ultrafiltrates obtained from neuraminidase-treated rat plasmas afforded an estimate of the relative quantities of Neu5Ac and Neu5,9Ac$_2$ liberated from glycoproteins present in this biofluid. Approximately 50% more Neu5,9Ac$_2$ than Neu5Ac was present in these samples, a value that should be considered as a lower limit in view of the increased resistance of glycosidically-bound *O*-acetylsialates to neuraminidase cleavage [9].

It should also be noted that some of the Neu5,9Ac$_2$ detectable in spectra of neuraminidase-treated rat plasmas may result from the migration of an *O*-acetyl group from the 7-position in Neu5,7Ac$_2$ to the primary hydroxyl group at the 9-position where it has enhanced stability [9,10]. However, Kamerling et al. [10] have reported that incubation of Neu5,7Ac$_2$ at pH 5.0 and 37°C does not give rise to significant *O*-acetyl group migration within 24 h.

Interestingly, the fraction of the broad 2.14 ppm signal remaining in SEFT ^{1}H spectra of rat plasma subsequent neuraminidase treatment may represent glycosidically-bound sialate species containing a 4-*O*-acetyl functional group, since 4-*O*-acetylation of sialate blocks the cleavage of these terminal sugars from sialocarbohydrate chains by commercially available preparations of this enzyme [20,21].

Specific functions associated with the presence of glycosidically-bound *O*-acetylsialates in biological systems include a diminished rate of cleavage from sialocarbohydrate chains by neuraminidase [37], an involvement in environmental adaption [38] and the binding of influenza C viruses [39], and an ability to influence alternate pathway complement activation [40] and bacterial antigenicity [41]. Hence, such differences in the physiochemical properties of the glycoprotein carbohydrate side-chains between rat and human blood plasmas are likely to have important biochemical, physiological and pathological ramifications.

Although previous suggestions for the functional status of glycoprotein carbohydrate side-chains include their non-specific actions as lubricants and stabilisers which confer negative charge [14], such moieties now have a well established role in molecular recognition processes [15,16]. Indeed, recent evidence suggests that the carbohydrate side-chains of glycoproteins function as 'address labels' which direct compartmentalisation either intra- or extracellularly [14,17], and have regulatory roles which involve their interactions with hormone receptors and lectin-like species located on cell membranes. Also of particular relevance to this area of investigation are the observations that: (i) a property of the carbohydrate moiety of α_1-acid glycoprotein exerts a regulatory function towards various immune responses [18]; and (ii) modifications in glycoprotein terminal sugar residues during synthesis or subsequent to partial degradation give rise to their intercompartmental movement such as that involved in the endocytosis of material following macrophage membrane binding [17,19].

These data are also of much interest in the context of analytical biochemistry in view of reports that many conventional methods for the analysis of glycoconjugates give rise to the destruction of sialate *O*-acetyl substituents [9], and *O*-acetylation of sialate interferes with the commonly employed periodate-thiobarbituric acid assay system for determining trace levels of free *N*-acetyl- and *N*-glycolylneuraminates [21,22]. The facile, non-destructive analysis of glycoprotein carbohydrate side-chains in biofluids by high field ^{1}H-NMR spectroscopy both prior and subsequent to neuraminidase treatment serves to offer much useful information regarding the precise molecular nature and levels of sialate sugars present.

The concentrations of human plasma 'acute-phase' glycoproteins are of much clinical interest since they are known to be markedly elevated in a range of abnormal clinical conditions, e.g. inflammatory joint diseases, cancer, certain liver diseases, and also during surgical trauma [23–27]. Indeed, inflammation arising from any form of tissue injury induces an increase in the levels of these 'acute-phase' proteins which are predominantly synthesized in liver parenchymal cells [28] in response to cytokines such as interleukins-1 and -6. Since cytokines released from cells at the inflammation site differentially induce this response [29], the nature and chronicity of inflammation may be influenced by the molecular structure, functional status and plasma levels of the glycoproteins generated. Hence, the precise chemical nature of the branching carbohydrate side-chains of these proteins is likely to play an important role in the progression of inflammatory diseases such as synovitis, and Sharif et al. [30] have recently suggested that extracellular glycoproteins and glycosaminoglycans bearing terminal *N*-acetylglucosamine residues may act as inflammatory stimuli in rheumatoid arthritis.

The presence of terminal *O*-acetylsialate sugars in the molecularly mobile carbohydrate side-chains of rat 'acute-phase' glycoproteins is of much significance in view of the regular use of these animals in models of human inflammatory conditions. Previous investigations involving the measurement of 'acute-phase' reactants in animals are generally limited to models of chronic inflammation [31]. However, in these experimental models, modifications in the serum levels of 'acute phase' proteins correlate well with disease activity, its progression and effective therapeutic intervention [32].

In rats, both α_1-acid glycoprotein and α_2-macroglobulin are sensitive 'acute-phase' reactants that have been employed to quantitate the 'acute-phase' response [33]. Since the molecularly-mobile carbohydrate moiety of α_1-acid glycoprotein is predominantly responsible for the three NMR-detectable glycoprotein signals present in SEFT ^{1}H spectra of rat plasma, further high field ^{1}H-NMR studies are likely to be of much use in determining the biofluid concentrations of this glycoprotein in experimental models of inflammation employing these animals. Moreover, high field ^{1}H-NMR analysis of rat plasma samples both prior and subsequent to neuraminidase treatment as described here permits an evaluation of any time-dependent modifications in the relative levels of particular glycoprotein sialate sugars (e.g. Neu5Ac and Neu5,9Ac$_2$) during progression of the disease process, and may yield much useful diagnostic information.

The results obtained here are also of much interest in view of the powerful abilities of one or more heat-resistant macromolecular components of rat serum (A.R. Moore, personal communication) and air pouch inflammatory exudate [34] to protect cartilage against degradation (monitored by glycosaminoglycan release). Indeed, the capacity of rat air pouch inflammatory exudate to exert such activity has been suggested to arise from the biochemical actions of 'acute-phase' proteins [34]. Intriguingly, in early investigations pertinent to this research area Highberger et al. [35] and Franzblau et al. [36] found that α_1-acid glycoprotein exerts an influence on the spacing of collagen fibres formed from soluble collagen. Moreover, the desialyzed forms of this protein do not exhibit this properly [36] indicating that its carbohydrate side-chain terminal sialate sugars are involved in the production of striated collagen. Hence, a comparative evaluation of the relative abilities of rat and human plasmas, and α_1-acid glycoproteins, to inhibit cartilage deterioration is warranted.

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