4C Nuclear magnetic resonance spectroscopy of glycoprotein-derived carbohydrate chains

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1. Introduction

Nuclear magnetic resonance (NMR) spectroscopy is based on the observation that magnetic nuclei such as ^{1}H, ^{13}C, ^{31}P, and ^{15}N can absorb energy at characteristic radio frequencies when placed in a strong magnetic field. The resonance frequency of a particular nucleus, expressed as the chemical-shift (δ), is sensitive to its chemical environment, making NMR a valuable technique for structural studies. Furthermore, the resonance lines show fine structure, referred to as spin–spin (or scalar) coupling, originating from interactions with nearby nuclei sharing bonding electrons. Thus, a molecule typically has a characteristic NMR spectrum which can be used as a fingerprint. Information may also be gained on the dynamic properties of molecules by analysing NMR relaxation processes. Finally, NMR spectroscopy is useful for conformational studies because data can be obtained about distances between two nuclei that are close in space. The non-destructive character of NMR spectroscopy usually makes it the first technique to choose for a structural problem, and it is the only single method which can in principle give an *ab initio* structure for a novel carbohydrate. However, in practice it is often advantageous to combine NMR spectroscopy with other analytical methods.

This chapter focuses on the use of NMR spectroscopy to determine the structure of glycoprotein-derived carbohydrate chains. The main emphasis is placed on ^{1}H-NMR, because ^{1}H is the most commonly used nucleus for biologically interesting carbohydrates. Many of the concepts presented herein are valid not only for glycoprotein-derived carbohydrates but also for the vast array of other biologically important glycoconjugates.

2. Theory

NMR may look bewildering and frightful with the plethora of acronyms and sophisticated quantum mechanics often used to describe the behaviour of nuclear spins. However, in practice many of the experiments can be simplistically described in rather pictorial ways. For more comprehensive reviews of the theory of NMR, see, e.g. references 1–3.

2.1 One-dimensional (1-D) NMR

A simplistic version of the basic 1-D NMR experiment is depicted in *Figure 1* for two nuclei (A and B) with a spin of 1/2, e.g. protons. When the sample is placed in a static external magnetic field (B_0), all nuclei will be aligned in the applied field. Spins aligned in the same direction as the external field will possess a slightly lower energy than those oriented in the opposite direction, leading to a small net population difference between the two states. We can depict the resultant net magnetization of the sample with an *arrow* along the *z*-axis (*Figure 1A*). A simple 'pulse-and-collect' 1-D experiment starts with the application of a short, high-power, radio-frequency pulse perpendicular to the *z*-axis. This pulse gives rise to an additional transient magnetic field (B_1), which exerts a torque on the net magnetization of the sample, and forces it into the *x*–*y* plane to give transverse magnetization. Because the radio-frequency pulse is very short, typically 2–20 microseconds, there will be an inherent uncertainty in its frequency, and this uncertainty makes it possible to

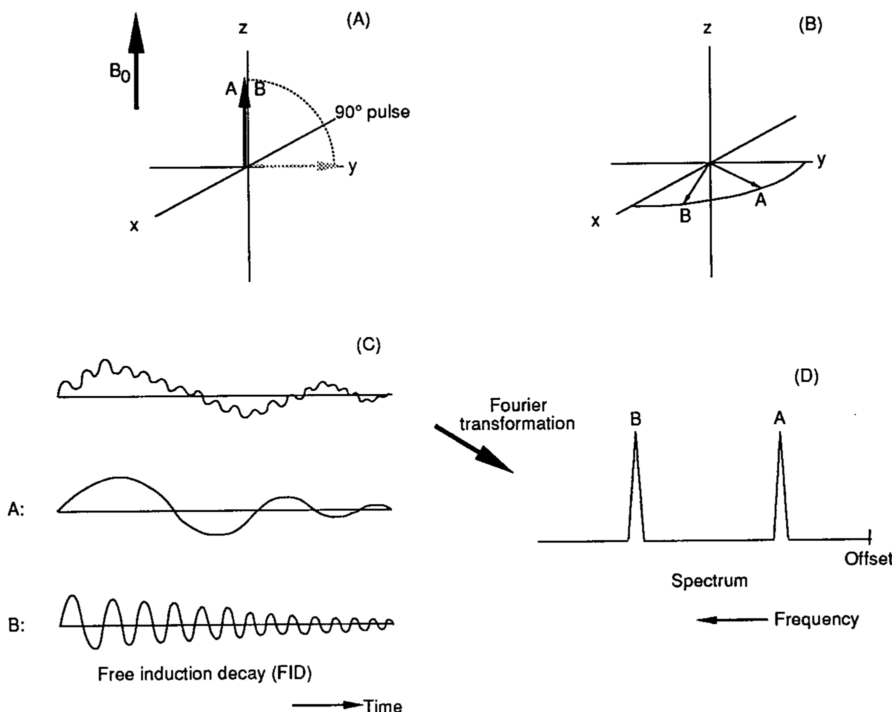

Figure 1. (A) Vector description of the net magnetization of a sample containing two nuclei with spin 1/2 when placed in a magnetic field. (B) The result after a 90 ° pulse. (C) The observed FID, consisting of the sum of the frequencies of A and B. (D) The resulting NMR spectrum obtained after Fourier transformation of the FID.

excite a broad range of ^1H frequencies with a single pulse. The net magnetization can now be envisaged as two vectors representing spins A and B that are moving (precessing) in the *x–y* plane (see *Figure 1B*, assuming no scalar spin–spin coupling). The precessing vectors A and B induce an oscillating radio-frequency signal in the *x–y* plane, and it is this signal which is measured in all NMR experiments. Owing to relaxation processes, this signal will decay and return to equilibrium, and is therefore called the free induction decay (FID). In our example the total FID is the sum of the frequencies from spins A and B in the sample (*Figure 1C*). The FID is a time-domain signal that can be converted by Fourier transformation into a frequency-domain spectrum, affording two peaks A and B (*Figure 1D*). The resonance positions of these peaks are called their chemical-shifts and are expressed as parts per million (p.p.m.) with respect to a reference signal. After the spins A and B have returned to equilibrium and have no components precessing in the *x–y* plane the next pulse can be given. By adding FIDs from several scans the signal-to-noise (S/N) ratio is improved, and by proper phase cycling of the pulses and the receiver certain artifacts can be removed.

Several mathematical manipulations of the FID before Fourier transformation can also be used to improve the quality of the resulting spectrum. The S/N ratio is larger at the beginning of an FID than at the end. It is therefore possible to improve the S/N ratio of the final spectrum by multiplying the FID prior to Fourier transformation with a so-called window function, which gives more weight to the first part of the FID than to the last part. An increase in the S/N ratio always entails a decrease in the resolution of the spectrum. However, the resolution can be improved by multiplying the FID with a window function which gives more weight to the last part than to the first part. Such resolution enhancement is always detrimental for the S/N ratio. For 1-D ^1H-NMR spectra of oligosaccharides, a Gaussian multiplication is often applied to improve the resolution. Finally, zero-filling, i.e. adding zeros to the end of the FID before Fourier transformation, is used to improve the smoothness of lines and allowing peak maxima to be located more precisely.

2.2 Two-dimensional (2-D) NMR

The basic difference between 1-D and 2-D NMR spectroscopy is the addition of one or more radio-frequency pulses and a delay (t_1), which is incremented after every acquisition. Most 2-D NMR experiments can schematically be divided into four time periods: preparation, evolution (t_1), mixing, and acquisition (t_2) (*Figure 2*). The various 2-D pulse sequencies differ mainly in

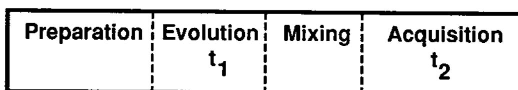

Figure 2. The different time periods in a 2-D NMR experiment.

the type of mixing period, which is the period during which spins are induced to interact with each other. The two main types of interactions which can be selected during the mixing period are:

- through space interactions, due to cross-relaxation (the nuclear Overhauser effect, NOE)
- through bond interactions, termed spin–spin (J-) coupling

During the preparation period the spins are allowed to return to thermal equilibrium, and during the acquisition period the transverse magnetization created by the last pulse is detected. The systematic incrementation of t_1 gives rise to a modulation in the amplitude of the peaks (A and B) in the spectra obtained after Fourier transformation in the t_2 direction. If the peaks are modulated only by their own frequency a second Fourier transformation in the t_1 direction gives rise to so-called diagonal peaks (see *Figure 3A*). However, if during the mixing period the spins have interacted with each other the second Fourier transformation will also reveal off-diagonal peaks, the so-called cross-peaks (see *Figure 3B*). Usually 2-D NMR spectra are presented as contour plots, analogous to the way mountains are represented on a map.

2.3 Three-dimensional (3-D) NMR

A 3-D NMR experiment can be visualized as a combination of two 2-D experiments, where the acquisition period of the first experiment and the

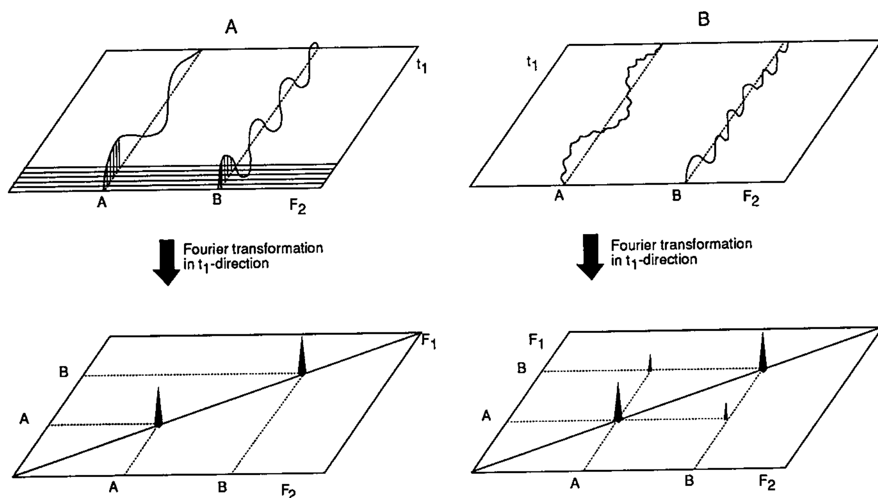

Figure 3. The origin of cross-peaks in a 2-D NMR experiment. (A) If the spins have not interacted with each other during the mixing period only diagonal peaks appear. (B) Modulation of the frequencies in the t_1-direction results in 2-D cross-peaks after a second Fourier transformation.

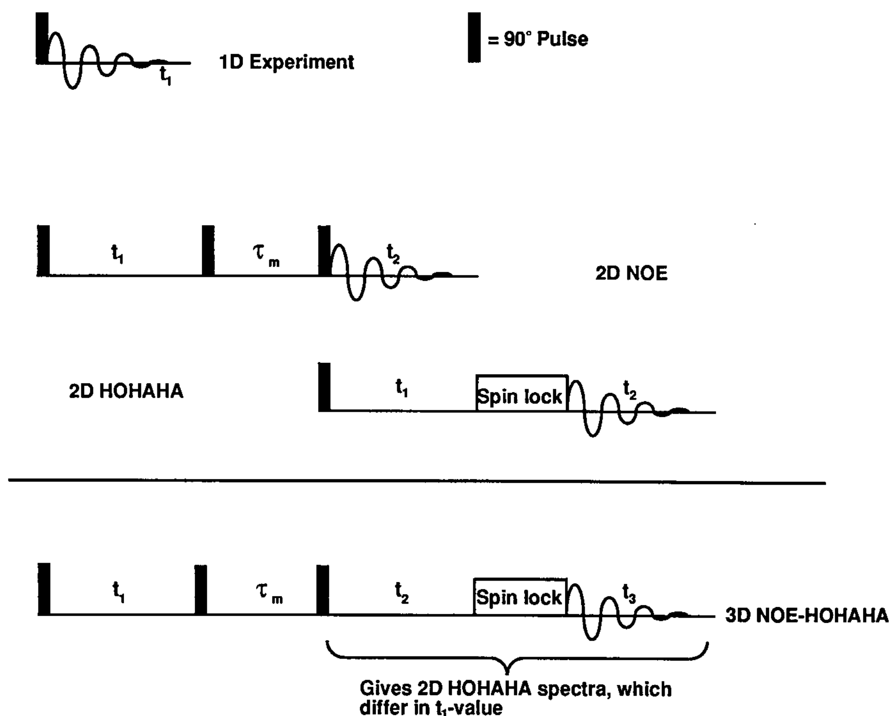

Figure 4. Schematic representation of 1-D, 2-D, and 3-D NMR experiments.

preparation period of the second one have been omitted. A 3-D experiment is thus recorded as a function of two variable delays (t_1 and t_2), both of which are independently incremented. As an example, consider the 3-D NOESY-HOHAHA experiment (*Figure 4*, see also Section 6). After Fourier transformation in both the t_3 and t_2 directions we obtain a series of 2-D HOHAHA spectra, which are modulated in the t_1 direction (*Figure 5A*). A third Fourier transformation in the t_1 direction yields the 3-D spectrum (*Figure 5B*), which consists of a body diagonal resembling a 1-D spectrum, diagonal NOESY and HOHAHA planes, as well as 3-D cross-peaks (see Section 7 for a real example).

3. Equipment

Currently there are four major manufacturers of high-field NMR spectrometers: Bruker, General Electric, JEOL, and Varian (mentioned in alphabetical order). Since the purchase of an expensive NMR spectrometer is a rare process, factors needed to be considered for this activity will not be

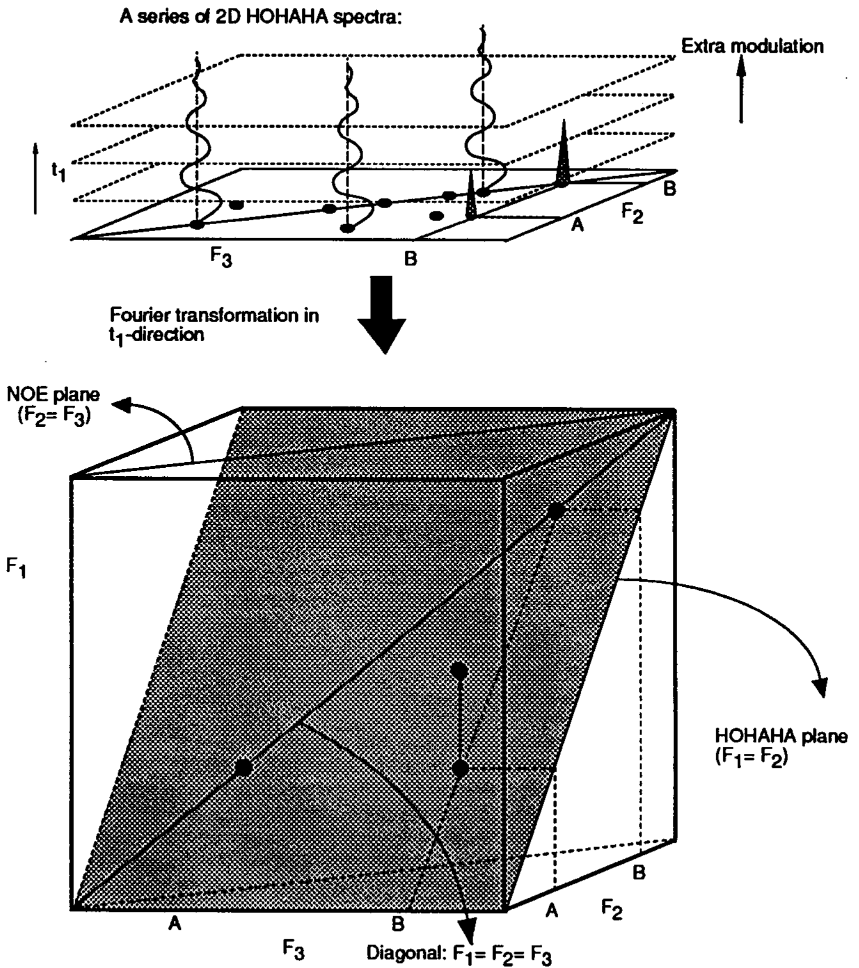

Figure 5. A 3-D NMR spectrum can be envisaged as a combination of several 2-D spectra which are modulated in the t_1 direction. The *upper part* shows the result after Fourier transformation in the t_3- and t_2-directions. The *lower part* shows the final cube containing the 3-D spectrum.

considered here [for some hints, see (3)]. All NMR spectrometers consist of:

- a magnet that can produce an intense and homogeneous field
- a radio-frequency transmitter
- transmitter and receiver coils, which are placed close to the sample in the magnet for perturbation of the nuclei in the sample and for the observation of their responses, respectively
- a radio-frequency receiver

- a computer which is required for manipulation of the radio-frequency pulses and for storing and handling the acquired data.

Among the most common external sources of problems for NMR spectroscopic work are:

- vibrations in the floor (which often can be overcome with a vibrational damper)
- temperature instabilities
- moving objects in the vicinity of the magnet, such as chairs and doors, that disturb the magnetic field.

4. Sample preparation

4.1 Choice of solvent

The final purification step in NMR sample preparation should be the removal from the sample of all protonated non-carbohydrate species, such as buffers and chelators. Anionic carbohydrates, (e.g. sialylated, sulfated, or phosphorylated) and large oligosaccharides are conveniently desalted on small (1 cm × 20 cm) gel filtration columns, (e.g. Bio-Gel P-2, Bio-Rad) eluted with water. The sample is recovered in the void volume and is ready for D_2O exchange after lyophilization. If the sample contains high amounts of acetate, as can be the case after high pH anion exchange chromatography, the use of 5 mM NH_4CO_3 as eluent instead of pure water facilitates the removal of the acetate. Small neutral oligosaccharides can be desalted on a mixed-bed of cation, (e.g. AG 50W, Bio-Rad) and anion, (e.g. AG 1, Bio-Rad) exchange resins.

For ^{1}H-NMR studies of carbohydrates the most commonly employed solvent is deuterium oxide (D_2O or ^2H_2O). This solvent closely resembles the natural aqueous environment and does not contain (significant amounts of) protons, which facilitates the observation of the ^{1}H atoms in the carbohydrate. Furthermore, simplification of spectra is achieved by exchanging labile protons like those of hydroxyl and amide groups with deuterium.

Commercially available D_2O is not always of satisfactory quality. It is advisable to record a test-spectrum of new batches of D_2O before any valuable compound is perhaps contaminated by impurities originating from the solvent. Since large differences have been observed in the pH (actually pD) of D_2O solutions from various manufacturers, a pH measurement of the D_2O may be useful. In particular when chemical-shift values are to be compared between closely related carbohydrates, a correct pH is of utmost importance, in particular for charged carbohydrates. For this purpose it is handy to use a small pH electrode which can fit into an NMR tube. However, in practice the easiest way to adjust the pH is often outside the narrow NMR tube. Addition of microlitre amounts of (commercially available) DCl or

NaOD is often enough for reaching correct pH values. Normally NMR spectra of oligosaccharides are recorded in neutral solutions. For the observation of amide NH atoms a low pH (pH 4–5) is advantageous, but care has to be taken not to hydrolyse labile glycosidic (or ketosidic) bonds, like those of fucose and neuraminic acid.

If the compound under investigation contains amide groups advantage can be taken of the excellent chemical-shift dispersion of the amide protons, by recording spectra in ^1H_2O as solvent (4). Typically a solution of 5–10 % (v/v) D_2O in ^1H_2O is used. The small amount of D_2O serves for the deuterium field-frequency lock signal. Alternatively, deuterated acetone may be used for the lock signal. The advantage of using acetone-d_6 is that it gives a sharper lock signal than D_2O and is less sensitive to small fluctuations in temperature. For observation of carbohydrate hydroxyl protons low temperatures are needed in order to slow down the exchange rate (5), and acetone or methanol has to be added to the sample to prevent freezing.

In some cases it may be useful to record NMR spectra in an aromatic solvent such as deuterated pyridine. This solvent may give rise to large changes in chemical-shifts of carbohydrate resonances when compared to D_2O, thereby helping in the identification of overlapping signals (6). Another way to improve the chemical-shift dispersion is to peracetylate the oligosaccharide (7), in which case a solvent such as dimethylsulfoxide (DMSO) needs to be used.

4.2 Exchange of labile protons with deuterons

There is usually no danger for denaturation in lyophilizing (freeze-drying), carbohydrates. Therefore, a convenient way to exchange labile protons with deuterons is to dissolve the dry oligosaccharide in a small amount of D_2O, let it stand at room temperature for about 30 minutes, and then lyophilize the sample. This is usually repeated at least once. For the deuterium exchange process it suffices to use less expensive D_2O, i.e. 99.8 atom-% D, and only use the most expensive '100%' D_2O for the actual NMR experiment. A detailed description of how samples can be exchanged in the NMR tube has been given (8), but utmost care must be taken because NMR tubes are very fragile.

Modern high-field NMR instruments are not particularly sensitive to minor amounts of solid particles in the sample. In many cases no filtering of the sample is therefore needed before the sample is transferred to the NMR tube, if care has been taken in the previous steps of sample handling. Glass wool and cotton wool, even after some 'cleaning' steps, are famous for introducing unwanted signals in ^1H-NMR spectra. Introduction of additional ^1H_2O is another drawback of many filtering procedures. A bench-top centrifuge can be sufficient to remove the largest solid particles, (e.g. from gel filtration or HPLC columns).

4.3 Paramagnetic impurities

Paramagnetic impurities are harmful to most types of NMR experiments. A convenient way to remove metal ions from the sample is to pass it in water through a small column of Chelex (Bio-Rad). If very small sample volumes are being handled, it may suffice to introduce a few grains of Chelex directly into the sample tube, allow it to stand with occasional gentle shaking, and finally remove the solids by centrifugation prior to transfer of the sample to the NMR tube. Another method is to add a small amount of the metal chelator EDTA to the sample. However, too much EDTA will obviously disturb the NMR spectrum.

Molecular oxygen also contains unpaired electrons which can cause dipolar relaxation of the sample protons and thereby reduce nuclear Overhauser effects (NOEs). Especially for small oligosaccharides, it can be worthwhile to remove dissolved oxygen from the solvent (see reference 9, pp. 215–17), but for large molecules this is generally not necessary.

4.4 Bacterial contamination

If D_2O is used as solvent there is usually no problem with bacterial or fungal contamination of the sample, since micro-organisms generally can not survive in too high concentrations of D_2O. More care has to be taken when experiments are performed with ^1H_2O as solvent. The use of a phosphate buffer, which may be convenient from an NMR point of view, and elevated temperatures around 37 °C, creates a good environment for micro-organisms. In these cases it is advisable to add a small amount (0.02%, w/v) of sodium azide. The addition of antibiotics or other compounds containing protons can introduce unwanted signals in the spectrum.

4.5 Choice of temperature

A stable temperature is a pre-requisite for obtaining good NMR spectra. Since the resonance frequency of water is temperature-dependent, this is especially crucial when using D_2O for the deuterium lock. In most work on glycoprotein-derived carbohydrates a temperature of 300 K (27 °C) has been used. Signals resonating under or close to the residual HDO-line in a ^1H-NMR spectrum can be visualized by either raising or lowering the temperature, because the chemical-shifts of the carbohydrate resonances are considerably less temperature-dependent than the chemical-shift of HDO. In NMR experiments where the signal intensities are dependent on the rotational correlation time (tumbling rate) of the molecule, lowering temperature can improve the results, as is the case for NOE experiments on oligosaccharides. In order to observe hydroxyl protons in aqueous solutions temperatures below 273 K (0 °C) are necessary, and can be reached by adding an organic solvent such as methanol or acetone to the sample solution. Large

polysaccharides exhibit broader resonance lines than small oligosaccharides, but by raising the temperature narrower lines can be obtained. For higher temperatures a ceramic spinner is needed, otherwise the spinner might be destroyed.

4.6 NMR tubes

High-field NMR spectrometers require high-quality NMR tubes, such as Wilmad 528 PP or 535 PP. The highest quality tubes are usually also the thinnest and most fragile ones! Much time spent on frustrating shimming can be saved by always using tubes of the same type. Carbohydrates do not generally stick to glass, but nevertheless a thorough cleaning of tubes is a prerequisite for good experiments. Drying of the tubes can be done by blowing filtered nitrogen through the tube. Unless very high sample concentrations are used, there will be some residual acetone visible in the NMR spectrum if one of the cleaning steps involves washing with acetone, and this can be used to calibrate ^{1}H-NMR spectra (2.225 p.p.m.). For higher amounts of material it is advisable to add a small amount of acetone to the solvent. The addition of undiluted acetone directly into the NMR tube should be avoided, because it usually leads to far too high concentrations. Chromic acid should be avoided for washing NMR tubes because it is paramagnetic.

5. The structural-reporter group concept

A ^{1}H-NMR spectrum recorded in D_2O of a disialylated diantennary *N*-linked oligosaccharide is shown in *Figure 6*. Characteristic features which are illustrated in this spectrum are the severe overlap of most of the ring protons in the region 3.5–3.9 p.p.m. and the occurrence of some well-resolved resonances outside this bulk region. Many of these resolved resonances have chemical-shift values which are strongly dependent on the structure of the carbohydrate chain; therefore, these signals have been named 'structural-reporter group' signals. By comparing the chemical-shift values and coupling constants of these structural-reporter groups of a vast number of closely related oligosaccharides it has been possible to establish empirical rules correlating chemical-shift values with carbohydrate structures.

D-Hexopyranosides in glycoproteins mostly occur in relatively fixed conformations ($^{4}C_1$). This allows the carbon-bound protons to be classified as either axial or equatorial; e.g. in *β*-glucose (*β*-Glc) all carbon-bound ring protons are axial. Anomeric protons (H-1) typically resonate at higher δ (p.p.m.) than other ring protons, because the C-1 atoms are bound to two oxygens, causing the H-1 atoms to be slightly more deshielded than other protons. In pyranosides equatorial protons often resonate at a higher δ than similar axial protons (compare, e.g. equatorial α-anomeric protons with axial *β*-anomeric ones). From the value of the three-bond coupling constants

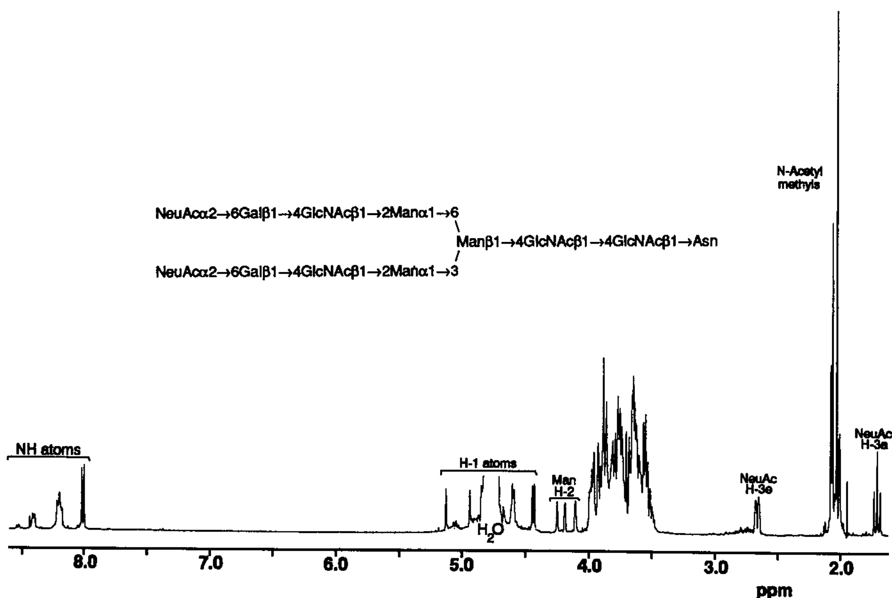

Figure 6. 1-D ^{1}H-NMR spectrum of an Asn-linked disialylated diantennary carbohydrate chain recorded in ^1H_2O containing 10% (v/v) D_2O and 20 mM potassium-phosphate, pH 5.1, at 300 K.

($^3J_{1,2}$) between the anomeric protons and the H-2 atoms it is possible to determine the anomeric configuration of the constituent monosaccharides in an oligosaccharide. For β-Gal and β-GlcNAc $^3J_{1,2}$ is typically 7–9 Hz, whereas for α-Fuc, α-Gal, and α-GlcNAc $^3J_{1,2}$ is 2–4 Hz. For mannose (Man) it is often not possible to determine the H-1/H-2 coupling constant because in both anomers it is around 1–2 Hz. However, by considering the chemical-shift values of the Man residues there is usually no problem in deciding whether a residue occurs in α or β configuration. Furthermore, it is possible to use the different intraresidual NOEs to distinguish between β-Man and α-Man residues. Sialic acid always occur in α-anomeric form when linked to another monosaccharide, but since sialic acids are ketosides they possess no anomeric protons.

When the structural-reporter group method is applied, care has to be taken that the same temperature, solvent, pH, and calibration is used as for the model compounds. The spectrometer frequency does not affect the chemical-shift values nor the coupling constants, but the appearance of the spectrum changes with changing magnetic field because the relative magnitudes of the coupling constants and the chemical-shifts will differ. In some cases it may even be helpful to record a spectrum at lower field because the different appearance of the spectrum can occasionally reduce overlap. The chemical-shifts of some resonances are considerably more sensitive to change in pH

than others, e.g. in neuraminic acids the chemical-shift values of the H-3 protons are pH-sensitive due the protonation of the nearby carboxyl group.

The severe resonance overlap in ^{1}H-NMR spectra of carbohydrates often gives rise to multiplet patterns showing virtual couplings. These occur when one proton is coupled to another proton, which in turn is strongly coupled to a third proton, i.e. it possesses the same chemical-shift as its coupling partner. In case of virtual coupling the observed multiplet pattern of a signal can not directly by interpreted in terms of actual coupling constants, but computer simulation of the spectrum can aid in its interpretation. A well-known example is the anomeric proton signal of the reducing GlcNAc in *N,N'*-diacetylchitobiose (GlcNAcβ1→4GlcNAc), the common core unit in *N*-linked carbohydrate chains.

The structural-reporter group method has to be used with utmost care and should preferably be extended with further NMR experiments when completely novel carbohydrate structures are investigated. Whenever feasible the structural-reporter group method should be combined with data obtained by other means. Helpful and crucial information can be the molecular mass of the compound (obtained for example by fast atom bombardment mass spectrometry, FAB-MS), because in some cases non-carbohydrate substituents might be present, which do not affect the chemical-shifts of distal structural-reporter groups. The elution properties of an oligosaccharide in different (liquid) chromatographic systems may provide information that can aid in the interpretation of the NMR spectrum.

An inherent drawback of NMR spectroscopy, as compared to many other spectroscopic techniques, is its rather low sensitivity. However, modern high-field NMR spectrometers operating at a ^{1}H frequency of 500 or 600 MHz can usually yield a reasonable one-dimensional (1-D) ^{1}H-NMR spectrum from about 10–20 nanomol of sample. Consequently, for a tetrasaccharide with a molecular mass of 700 Da as little as ten micrograms of the oligosaccharide is sufficient to obtain a spectrum.

5.1 *N*-linked carbohydrates

For a comprehensive review on the use of ^{1}H-NMR structural-reporter groups for the determination of the structure of *N*-linked oligosaccharides see (10). The most employed structural-reporter group signals occurring in *N*- and *O*-linked carbohydrates are collected in *Table 1*. Briefly, if an *N*-linked oligosaccharide belongs to the known classes of compounds a 1-D ^{1}H-NMR spectrum in D_2O can be used as a fingerprint. From this spectrum alone it is often possible to determine the complete primary structure of the oligosaccharide, encompassing the branching pattern, the type of fucosylation, the type of linkage for neuraminic acid, and so on. Additional information can be gained if a spectrum is also recorded in ^1H_2O as solvent, thereby taking advantage of the favourable chemical-shift dispersion of the amide protons.

Table 1. ^{1}H-NMR structural-reporter group signals for carbohydrate chains of glycoproteins

Anomeric protons
Amide protons
Man H-2 atoms
GalNAc-ol H-2, H-3, H-4, and H-5 atoms
Sialic acid H-3 atoms
Fuc H-5 and H-6 atoms
Gal H-3 and H-4 atoms
Protons shifted out of the bulk region due to glycosylation shifts
Protons shifted out of the bulk region due to the presence of non-carbohydrate substituents like acyl, sulfate, and phosphate groups
Protons belonging to non-carbohydrate substituents like *O*-methyl, *N,O*-acetyl, and *N*-glycolyl groups

5.2 *O*-linked carbohydrates

The ^{1}H-NMR structural-reporter groups of *O*-linked oligosaccharides have recently been reviewed (11). Since the terminal sequences of *N*- and *O*-linked oligosaccharides are very alike, the same type of structural-reporter groups can be used for these structural elements in both classes of oligosaccharides. The core structures around the Ser/Thr-bound GalNAc residue can usually readily be identified from characteristic patterns of GalNAc-ol H-2 and H-5 signals, in combination with resonances from the GalNAc-bound monosaccharide residues (11, 12).

5.3 Automation: a database computer program

Recently, a database computer program that uses ^{1}H-NMR chemical-shift values has been developed for computer-assisted identification of complex carbohydrates (13). This program operates on a database that contains tables of ^{1}H-NMR chemical-shift values and corresponding carbohydrate structures, and literature references taken from the Complex Carbohydrate Structure Database [CCSD, (14)]. From an input list of chemical-shift values, the program generates an output list of partially or completely matching carbohydrate structures. Even though this approach does not provide a fully automatic identification of carbohydrate chains it requires much less labour than any classical way. For novel compounds it can be used to recognize structural elements that occur in known carbohydrates.

5.4 Identification of non-carbohydrate substituents

5.4.1 Phosphorus-containing substituents

Attachment of a phosphate group to a monosaccharide residue typically gives rise to a shift increment of $\Delta\delta$ = 0.25–0.55 p.p.m. of the proton bound to

the substituted carbon atom. ^{31}P is an NMR active nucleus (spin 1/2) and consequently heteronuclear couplings are often visible on the shifted signal. Frequently, it is far from trivial to identify the location of a phosphate group in an oligosaccharide. To this end a $^{1}H[^{31}P]$ relayed spin-echo difference (RESED) experiment has been devised (15). By this method it is possible to selectively observe subspectra of phosphorylated residues in complex carbohydrates. Glycoprotein-derived carbohydrate chains may also contain 2-aminoethylphosphate or 2-aminoethylphosphonate substituents.

5.4.2 Sulfates

A sulfate group bound to a monosaccharide residue generally gives rise to a shift increment of $\Delta\delta$ = 0.5–0.7 p.p.m. of the proton attached to the substituted carbon atom. Unlike ^{31}P, the most abundant sulfur isotope (^{32}S) is not NMR active. Therefore, the location of a sulfate group can not be established by NMR as elegantly as for phosphorus-containing substituents. However, by assigning the shifted resonance to a particular proton and connecting it to an independently assigned structural-reporter group, (e.g. H-1) the attachment position(s) of sulfates can be determined.

5.4.3 Other substituents

If enzymic methods are used to release carbohydrate chains from a glycoprotein, it is possible to isolate oligosaccharides bearing acetyl and/or glycolyl substituents. Both types of substituents can readily be recognized by ^{1}H-NMR spectroscopy, in particular when they occur on neuraminic acid (16).

6. Two-dimensional NMR of oligosaccharides

During the last decade a vast number (hundreds) of 2-D NMR experiments have been proposed. In the following, some of the most useful experiments for structural studies on glycoprotein-derived oligosaccharides will be discussed.

6.1 Homonuclear Hartmann–Hahn (HOHAHA) spectroscopy (17, 18)

The HOHAHA experiment is one of the most important 2-D NMR techniques for carbohydrates since it enables correlations to be obtained between all protons in a scalar coupling network which, in the case of oligosaccharides, means a single monosaccharide residue. The idea behind the experiment is to eliminate the chemical-shift difference between scalarly coupled protons, i.e. to create a situation where the protons are strongly coupled. The assignment of HOHAHA spectra can often be ambiguous because cross-peaks originate both from direct and indirect connectivities. In

such cases it may be helpful to record several HOHAHA spectra with different mixing times, to compare the spectra with a COSY spectrum, or to compare it with a NOESY or ROESY spectrum where typical intraresidue cross-peaks can aid the assignments. For oligosaccharides having rather long relaxation times it is possible to use HOHAHA mixing times of considerable length, typically 100–120 milliseconds. Optimal Hartmann–Hahn transfer is obtained when the carrier frequency is positioned between the resonance positions of the protons of interest.

For Gal and GalNAc, where the coupling constants between the H-4 and H-5 protons are small (< 1.5 Hz), it can be difficult to obtain complete subspectra by HOHAHA. Usually the magnetization transferred from the well-resolved H-1 atom stops at the H-4. A triple-quantum filtered (TQF) COSY experiment can reveal the H-5, H-6, and H-6′ connectivities, but unless at least one of these signals has been independently assigned, the TQF-COSY experiment is of limited use for assignment purposes. For Fuc (6-deoxy-L-galactose) the H-5 signal can usually easily be assigned from a cross-peak to the well-resolved methyl (H-6) atoms. In neuraminic acids the small three-bond coupling constant $^3J_{6,7}$ (<1.5 Hz) prevents magnetization transfer from the well-resolved H-3 protons further than H-4, H-5, and H-6.

One of the most common artifacts in HOHAHA spectra are the occurrence of cross-peaks due to NOE effects in the rotating frame, i.e. ROESY-type cross-peaks. These cross-peaks are easily recognized because of their different signs, as compared to the diagonal peaks and the HOHAHA cross-peaks. In cases where there is a problem with the ROESY-type of cross-peaks, a clean-TOCSY sequence may reduce the problem (19). A general problem with HOHAHA experiments, especially when using long spin-lock times, is slight heating of the sample. This is seen as a change of the lock signal level and it may result in increased t_1 noise. It is a good custom to allow a warming-up period for a HOHAHA experiment by starting the experiment for five to ten minutes, adjusting the most critical shims at the end of this period, and then restarting the experiment. This should also be considered when several 2-D experiments are run sequentially, by adding an equilibration time in between the experiments.

In general, HOHAHA spectra are more sensitive than COSY spectra. The in-phase transfer of magnetization prevents the cancellation problem of anti-phase cross-peaks as can occur in COSY-type spectra. For oligosaccharides studied in D_2O the spectral width is small (below 5 p.p.m.) and good results have been obtained with the MLEV-17 HOHAHA mixing scheme.

6.2 Correlated spectroscopy (COSY) (20)

The COSY experiment was the first 2-D NMR experiment to be proposed. It is still one of the most commonly used pulse sequences, but in many cases it can be preferable to record a HOHAHA spectrum with a short mixing time

instead of a COSY experiment, especially if sensitivity poses a problem. Unlike HOHAHA experiments there is no net magnetization transfer in COSY-type spectra. COSY spectra provide information on directly coupled protons. Numerous variations on the COSY-theme have been proposed (20). From the fine structure of the cross-peaks in COSY-type experiments information can be extracted about coupling constants, which in turn can be used to estimate dihedral angles in carbohydrates.

6.2.1 Double-quantum filtered (DQF) COSY (18)

When the cross-peaks in a COSY spectrum are phased to be absorptive, the diagonal is dispersive; this often obscures cross-peaks located close to the diagonal. The advantage of a DQF-COSY over an unfiltered COSY is that single quantum cross-peaks around the diagonal (cross-peaks between lines in a multiplet) are eliminated. The DQF-COSY experiment has been widely applied in structural studies on oligosaccharides.

6.2.2 Triple-quantum filtered (TQF) COSY (18)

Ideally, TQF-COSY spectra contain signals from only such protons which are mutually coupled to two other protons. This means for hexopyranosides (Gal, GlcNAc, GalNAc, and Man) the H-5, H-6, and H-6′ atoms, for xylose the H-4, H-5a, and H-5e signals, and for neuraminic acid the H-3a, H-3e, and H-4 trio, as well as the group of H-8, H-9a, and H-9e resonances. However, in practice TQF-COSY spectra are not always easy to interpret due to the absence of cross-peaks in case of overlapping signals or due to the occurrence of artifactual cross-peaks which have passed the triple quantum filter.

6.2.3 Relayed COSY (or RELAY) (18)

A RELAY experiment consists of the COSY sequence supplemented by one or more additional coherence transfer steps. In comparison with HOHAHA methods, the RELAY experiment is rather insensitive but has the advantage that it is possible to determine beforehand the number of relayed connectivities that should appear.

6.3 Nuclear Overhauser effect spectroscopy (NOESY) (9, 18)

NOESY methods have been widely applied in structural work on oligosaccharides because cross-peaks are obtained between protons close in space, and this information can be used in the form of structural constraints when calculating three-dimensional structures of oligosaccharides (21). However, care has to be taken when interpreting NOEs of oligosaccharides in terms of definite conformations. As previously pointed out (22) the observed NOEs are ensemble averages originating from all different conformations present of the oligosaccharide in solution. The distance constraints obtained from NOESY spectra always need to be evaluated by force-field calculations of

energetically favourable conformers before three-dimensional structures of oligosaccharides can be obtained.

NOEs are often used to determine linkage positions between monosaccharides, but the results should be examined critically since the strongest NOE between adjacent monosaccharide residues need not always be the proton at the linkage carbon.

For small oligosaccharides the NOE effects may be very weak, due to an unfavourable molecular rotational correlation time (τ_c). In such cases NOESY measurements at lower temperatures, thorough degasing of the solvent (removal of oxygen), or ROESY experiments may solve the problem.

6.4 Rotating-frame nuclear Overhauser effect spectroscopy (ROESY) (23)

The ROESY experiment was originally termed CAMELSPIN. The principal advantage of the ROESY experiment, in comparison with NOESY, is that spin-locked NOEs are always positive and increase with increasing τ_e. The use of ROESY cross-peak intensities in a quantitative sense should be done with utmost care. Not only are the intensities dependent on the distance from the offset frequency but several other factors have to be taken care of, such as Hartmann–Hann effects (24).

6.5 Heteronuclear multiple-quantum coherence spectroscopy (HMQC) (25, 26)

The HMQC experiment belongs to a family of heteronuclear inverse (proton detected) experiments which have gained enormous popularity in the last few years. With an HMQC experiment it is possible to correlate carbon resonances with their directly bonded protons. Because the chemical-shifts of ^{13}C nuclei are usually well dispersed and therefore more readily assigned, the HMQC experiment is of great value for the assignment of ^{1}H resonances.

6.6 Heteronuclear multiple-bond spectroscopy (HMBC) (25)

This HMBC experiment generates cross-peaks between ^{13}C and ^{1}H atoms separated by several chemical bonds. The most beautiful way of sequencing oligosaccharides is to use long-range ^{1}H–^{13}C couplings over the glycosidic linkages, allowing the unambiguous determination of linkage positions. However, due to the insensitivity of this technique it is seldom possible to apply it in practice for dilute solutions.

7. Three-dimensional (3-D) NMR

Currently, there are only a few reports on the application of 3-D NMR to structural carbohydrate problems. Nevertheless, the potential use of 3-D NMR on carbohydrates are enormous and due to the small chemical-shift

dispersion in carbohydrate NMR spectra these molecules are ideally suited for 3-D NMR. In principle, any two 2-D methods can be combined into a 3-D experiment.

The non-selective homonuclear 3-D NOE-HOHAHA experiment was the first 3-D NMR method which was performed on an oligosaccharide (27). In *Figure 7* an F_3 cross-section (F_1–F_2 plane) is shown from a 3-D NOE-HOHAHA spectrum of a disialylated diantennary oligosaccharide linked to Asn. The cross-section is taken at the F_3-position of Man-4 H-2 at 4.19 p.p.m. The most notable 3-D cross-peak is indicated with an *arrow* and proves the existence of a NOE between Man-4 H-5 and Man-3 H-2. This cross-peak (F_1, F_2, F_3) occurs at F_1 = Man-3 H-2, F_2 = Man-4 H-5, and F_3 = Man-4 H-2, thereby originating from an NOE transfer between Man-3 H-2 and Man-4 H-5, followed by a HOHAHA transfer between Man-4 H-5 and Man-4 H-2.

The heteronuclear 3-D $^1H/^{13}C$ HMQC-NOESY experiment has been performed at natural ^{13}C abundance on the same disialylated diantennary compound (28). This experiment is extremely powerful since it enables '2-D NOESY' slices to be generated taken at selected ^{13}C chemical-shift values. In particular the observation of NOEs between carbohydrate protons resonating within the 3.5–3.9 p.p.m. bulk region may be mentioned.

The main limitation of heteronuclear (^{13}C and ^{15}N) NMR techniques are their low sensitivity because of the low natural abundance of these isotopes. Biosynthetic incorporation of ^{13}C and ^{15}N into glycoproteins expressed in cell cultures will certainly trigger many detailed NMR studies on intact glycoproteins in solution.

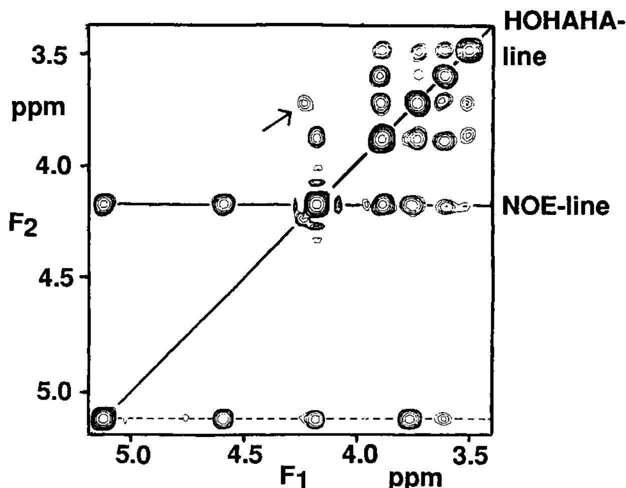

Figure 7. F_3 cross-section (F_1–F_2 plane) from a 3-D NOE-HOHAHA spectrum of a disialylated diantennary oligosaccharide linked to Asn. The cross-section is taken at the F_3-position of Man-4 H-2 at 4.19 p.p.m. See text for further details.

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