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APPLICATIONS OF FOURIER-TRANSFORM, PROTON N.M.R SPECTROSCOPY TO STUDIES OF CARBOHYDRATE DERIVATIVES*

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ABSTRACT

The 100-MHz, proton n.m r. spectrum of 1,2,3,4-tetra-*O*-acetyl-β-D-ribopyranose and of methyl 2,3 4,5-di-*O*-isopropylidene-β-D-glucoseptanoside have been studied by the Fourier-transform method The results obtained illustrate the potential of this approach for increasing both the sensitivity and the resolution over those of a conventional n m r experiment, and for removing the unwanted degeneracy of some n m r transitions A study of the spectrum of a solution of the calcium chloride complex of D-allose illustrates the potential of Fourier-transform n m r for studying solution kinetics.

INTRODUCTION

Although the Fourier-transform method has been applied to proton nuclear magnetic resonance (n m r) spectroscopy since[2] 1965, very few such studies of carbohydrates have been reported to date[3] Our interests in this area have been directed principally towards the measurement of proton spin–lattice relaxation-times[1 4], but, during those studies, we have become increasingly aware of several additional advantages that the Fourier-transform, n m r method has over conventional, continuous-wave spectroscopy The present study is intended to document those advantages that relate to proton n m r studies of monosaccharides In particular, we shall demonstrate the immense improvements that can be routinely obtained in both sensitivity and resolution, we shall also provide a further example of the use of partially relaxed proton spectra as an aid to spectral assignments, and shall illustrate a study of a system that is changing with time.

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Although it is not appropriate to give a detailed discussion here, a brief comparison of the continuous-wave (c.w.) and Fourier-transform (F.t.) methods is relevant. In a typical c.w. measurement, a weak, observing radio-frequency field is scanned slowly through the appropriate frequency-range, exciting each transition in turn. For routine operation with a 100-MHz instrument, one scan takes of the order of 500 seconds, and an ~0.1-molar solution is needed to achieve an adequate signal-to-noise ratio, even under these conditions, a rather rigid compromise must be struck among various instrument settings. The signal-to-noise ratio can be improved by filtering out some of the noise, but this causes some loss of resolution, the best resolution is obtained by scanning very slowly, but then the resonances saturate readily (a property of their rather long relaxation-times), and the strength of the observing field must be lessened, again lowering the signal-to-noise ratio. Although it is possible to observe strong peaks, such as methyl resonances, down to concentrations of 10mM with one scan, establishing of the best combination of parameters, especially for line-width measurements, is tedious, and the results are poor. It is also worth noting that, unless further compromises can be accepted, if it is wished to time-average a number of scans in the c.w. mode, each scan requires the full 500 sec (or so) established for the best single scan.

In the F.t. mode[5], all of the resonances are excited at once, and the total time needed to acquire one scan is the pulse time (negligible) plus the acquisition time, the length of time that the free-induction decay (f.i.d.) is recorded. Like the c.w. experiment, the F.t. experiment necessitates a number of decisions that sometimes conflict. In theory, the f.i.d. is an exponential decay extending to infinity, in practice, the acquisition time is, typically, one to four seconds. In principle, then, time-averaging can be achieved by pulsing every second or two, and the immense improvement in signal-to-noise ratio that can be obtained by pulsing 1,000 times in as many seconds over scanning once in the c.w. mode for 1,000 sec has been documented[6]. However, the spins have a finite relaxation-time, and (just as saturation occurs in a c.w. scan at too high a power level) in the F.t. mode, sufficient relaxation-time must be allowed between pulses to achieve the maximum, undistorted signal-height for all of the transitions. Two approaches are possible here. (*1*) The pulse can be less than 90°, the spins are less removed from equilibrium, and the pulse interval can be shortened. However, the signal for each transient will be smaller as the maximum response is obtained from a 90° (or 270°) pulse. Alternatively, (*2*) a full 90°-pulse can be used, and a waiting period (in addition to the acquisition time) introduced between pulses. Each spectrum will then be at its full intensity, and fewer transients will be needed. In practice, it is often convenient to pulse rather rapidly, allowing some saturation of the slower-relaxing spins, while achieving maximum signal-to-noise ratio for the faster-relaxing ones. The relaxation times of protons of large organic molecules in solution are usually less than two or three seconds, so that, except for very dilute solutions, it is possible to gain a high signal-to-noise ratio in a reasonable time without saturating any signals (except, perhaps, those of the solvent).

Having decided on the number of transients to acquire, the pulse strength, and

the interval between pulses, it is necessary to choose the acquisition time, which has a critical bearing on the resolution or sensitivity of the final, transformed spectrum As the free-induction decay proceeds, its intensity becomes lower, while the noise level remains constant Thus, the early part of the f i d contributes a great deal to the signal-to-noise ratio, while the information carried in the later stages is needed in order to achieve the best resolution A longer acquisition-time results in better and better resolution, until increasing noise nullifies the improvement However, with a given acquisition-time, it is possible[7] to weight, mathematically, the first or last stages of the f i d prior to Fourier transformation, in order to enhance either the sensitivity or the resolution of the final spectrum* Enhancement of sensitivity with loss of resolution may prove useful for very dilute samples, but we have found it more convenient to increase the number of transients When resolution enhancement is used, there is a large increase in the noise level, so that this procedure is usually applied after the acquisition of very many transients

RESULTS AND DISCUSSION

Probably the most widely publicized use of the Fourier-transform technique is for the routine improvement in the signal-to-noise ratio of n m r spectra A typical example of this is given in Fig 1, which shows a comparison between expending 1,000 sec on a single, continuous-wave scan of an n m r spectrum and 1,500 sec in acquiring 100 transients Without laboring the point, it is worthwhile noting that these spectra were made with an 0 1M solution contained in a normal, n m r tube Clearly, a very substantial, additional improvement could have been achieved by using the same weight of sample dissolved in a smaller volume of solution contained in a micro-cell

This approach has some peculiar limitations, one of which is illustrated by the spectrum shown in Fig 2B The broad hump across the baseline could arise from a number of different sources and, although it did not interfere with this particular experiment, it is worth noting that there are several methods that could have been used to minimize it These are to (*1*) concentrate the same amount of material in a micro-cell, or in one of the recently developed, 1-mm sample-tubes[9] that contain 5 μl of solution, and so lessen the number of transients, (*2*) use the computer to subtract the spectrum of a reference (probably a tube of the solvent), as in the operation of a double-beam spectrometer, or (*3*) use a probe having a lower proton "background" Finally, if the spin–spin relaxation-time of the background protons is much shorter than those of the sample, as is quite likely, delayed[10] F t spectroscopy might constitute a simple, instrumental solution, albeit with some loss of signal height

A more impressive, and useful, outcome of arithmetical manipulation of the f i d signal involves the resolution-enhancement routine[7], in which the later part of

*There is a variety of other methods[8] for mathematical manipulation, but these will not be discussed here

the f i d signal is weighted. The two spectra shown in Figs 1B and C clearly illustrate this point. The Fourier-transform spectrum shown in Fig 1B has a resolution comparable with that of a spectrum published previously[11]. The spectrum in Fig 1C, which shows a truly remarkable increase in resolution, was obtained by using the resolution-enhancement subroutine of a standard, Varian, F t program An acquisi-

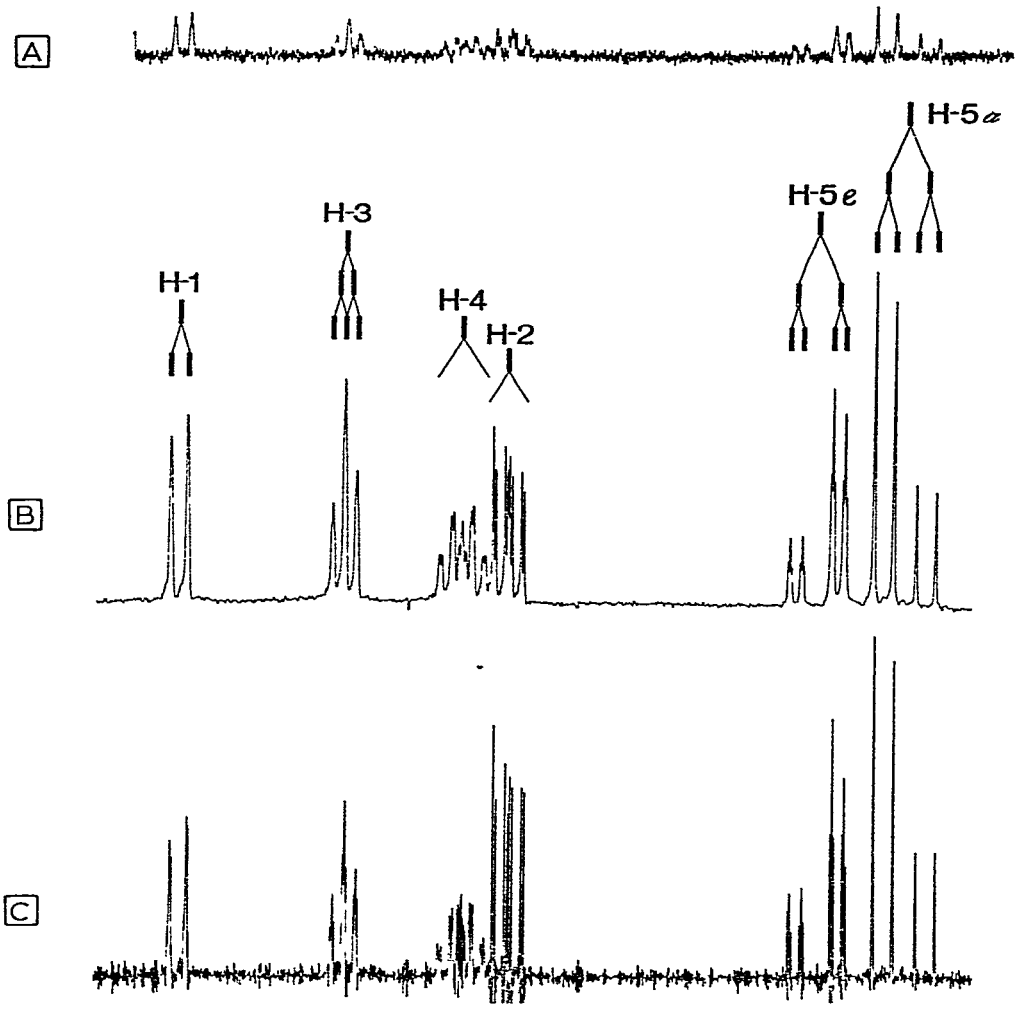

Fig 1 Partial, 100-MHz, proton n.m r. spectrum of 1,2,3,4-tetra-*O*-acetyl-*β*-D-ribopyranose in acetone-d_6 (0 1M) at 42° [A shows the result of a single, continuous-wave scan, with a sweep-width of 250 Hz and a total scan-time of 1,000 sec The spectrum in B shows the Fourier-transform summation of 100 transients, each with an acquisition time of 3 0 sec, and a delay time between successive transients of 12 sec, the total time used to obtain this spectrum was 1,500 sec. The spectrum given in C was derived from the same, free-induction decay signal as B, but a resolution-enhancement weighting-factor of 1 0 unit was applied immediately prior to the Fourier transform The marked increase in resolution and in the noise level should be noted]

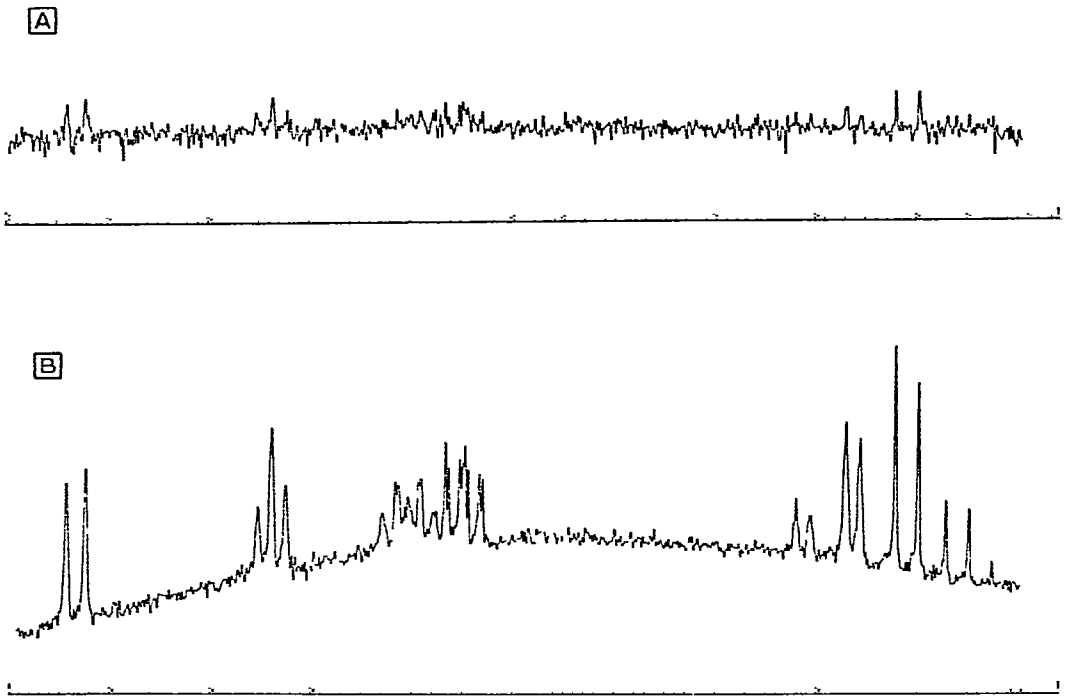

Fig 2 Partial, 100-MHz, proton n m r spectrum of 1,2,3,4-tetra-*O*-acetyl-*β*-D-ribopyranose in acetone-d_6 (6 mmolar) at 33° [A shows the result of a single transient, B shows that of the Fourier-transform summation of a total of 100 transients (500 sec total)]

tion time of 3 0 sec was used, and the resolution-enhancement factor was varied in order to find the optimal value, with the original f i d stored on a tape Clearly visible in this spectrum is the presence of additional splittings in all resonances but those of H-2 and H-5*a*, which arise from long-range couplings For example, each of the two envelopes of the H-1 doublet is now seen to be a 1 3 3 1 type of quartet (*vide infra*), which indicates that H-1 is coupled with H-2, H-3, H-4, and H-5*e*! Most of the other ring-protons are also extensively coupled, even the proton shown as H-5*a*, which has only a geminal and a vicinal coupling clearly resolved, shows signs of further coupling A plot of resonances of H-5*e* and of H-5*a*, measured with the same sweep-width, but plotted with a five-fold expansion-factor, and shown in Fig 3, provides a clearer indication of the resolution obtained in this case the long-range couplings in the H-5*e* resonance are only ~0 5 Hz*

There is a limit to the increase in resolution possible, and this limit is related to the acquisition time. As mentioned previously, as either the acquisition time itself,

*It is worth noting here that the complete resolution of a splitting between two transitions with Lorentzian shape can only be achieved if the width of the half-height of each transition is equal to, or less than, one fifth of the splitting

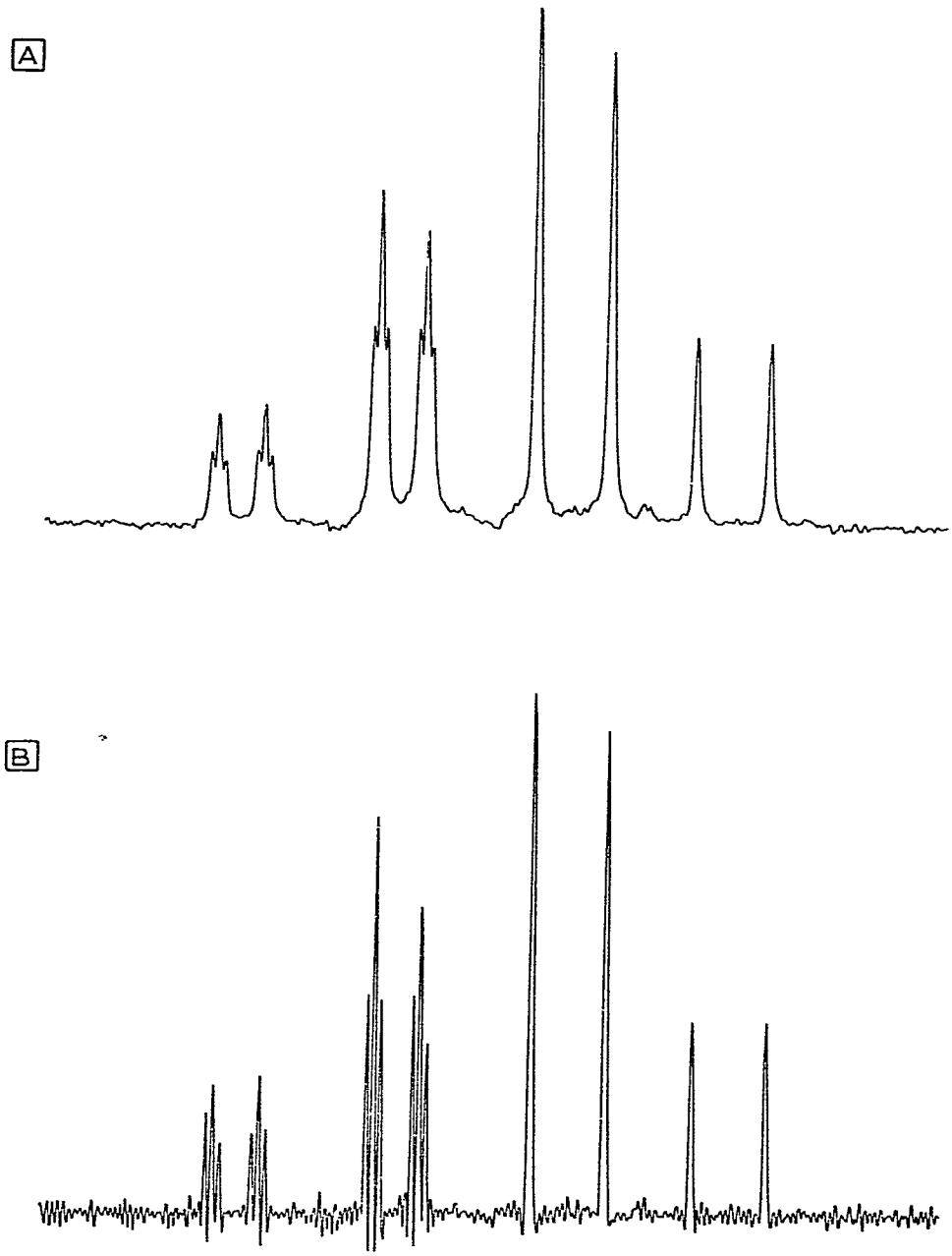

Fig 3. Both of these are expansions of the H-5*e* and H-5*a* resonances of 1,2,3,4-tetra-*O*-acetyl-*β*-D-ribopyranose given in Fig 1 [A corresponds to the trace in Fig 1B, and B to the trace in Fig 1C The very significant improvement in the resolution of a long-range coupling (~0 5 Hz) in the H-5*e* resonance is apparent. The fact that the half-height width of the H-5*e* transitions in B is less than that of the H-5*a* transitions implies the presence of a small, but unresolved, coupling to H-5*a*]

or the weighting given the later stages of the f i d , is increased, there is an increase in the amount of noise introduced into the transformed spectrum The effect of altering the weighting factor is illustrated in the set of resonances shown in Fig 4A–E As the weighting factor is increased, there is a steady improvement in the resolution from spectrum 4A to 4D (accompanied, as always, by an increase in the noise level) The spectrum in Fig 4E shows the effect of applying too sharp a weighting function, only a small change causes the transition between an acceptable noise-level and a complete loss of signal

Previous studies from this laboratory have demonstrated that substantial differences exist between the spin–lattice relaxation-times of individual protons of carbohydrate derivatives[1 4] These differentials provide the basis of a rather powerful method for simplifying complex, proton n m r spectra Briefly, a 180°-pulse is applied to the nuclei, and this inverts their magnetization along the z-axis (see Fig 5) The usual process of spin–lattice relaxation then causes the magnetization to revert towards its equilibrium position, this is accompanied (see Fig 6) by a steady decrease

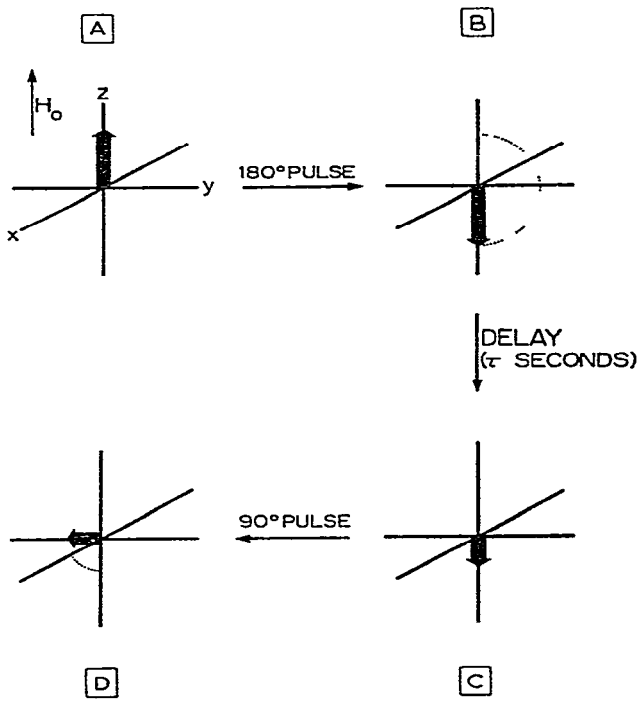

Fig 5 Diagrammatic representation of the rotating reference-frame model of a spin–lattice, relaxation-time measurement using a two-pulse sequence [In A, the magnetization of the nuclei is at thermal equilibrium with the lattice In B, this magnetization has been inverted through 180° by application of a 180°-pulse, the nuclei are no longer in thermal equilibrium, and the spin–lattice relaxation causes the magnetization to revert back along the z-axis towards its equilibrium position After a known delay time (pulse-delay), the residual magnetization C is assayed by the application of a 90°-pulse which tips the magnetization up into the x,y-plane D]

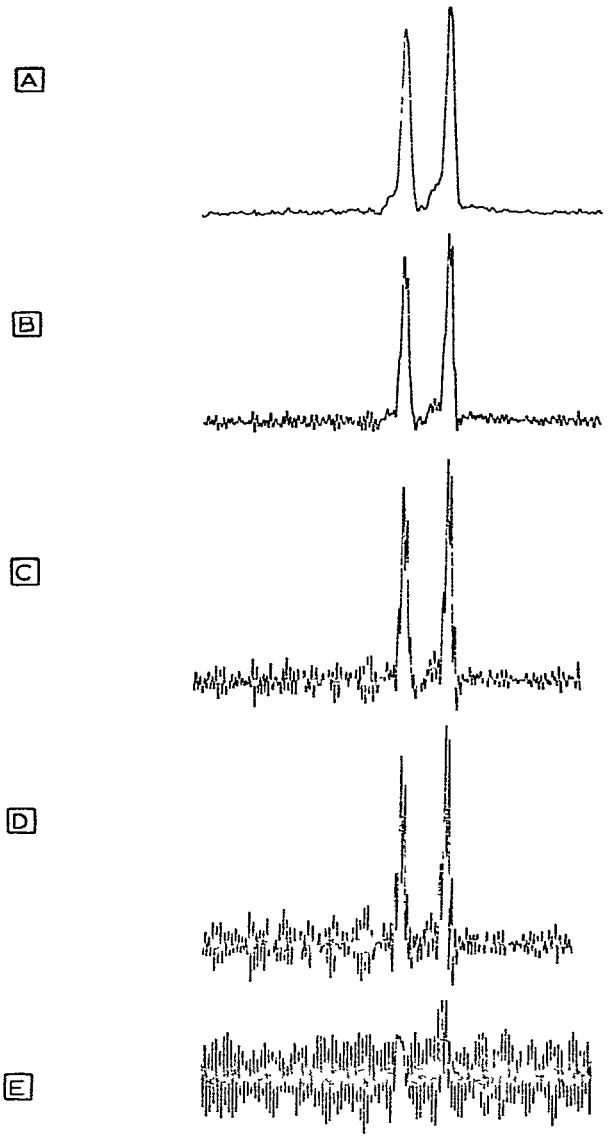

Fig 4 The H-1 resonance of 1,2,3,4-tetra-*O*-acetyl-*β*-D-ribopyranose in acetone-d_6 solution (0 1M) at 42°. [All of the spectra were based on the free-induction decay signal resulting from 100 transients with an acquisition time of 3 0 sec and a pulse-delay time of 12 sec (total time, 1 500 sec) A is the result of direct, Fourier transformation, with no resolution enhancement B was obtained by applying a resolution-enhancement factor of 1 5, C, a factor of 1 0; D, a factor of 0 8, and E, a factor of 0 7 The progressive increase both in resolution and noise level should be noted In D, the H-1 resonance is clearly visible as a doubletted quartet with long-range couplings of ~0 5 Hz The spectrum shown in E illustrates the effect of applying too sharp a weighting function]

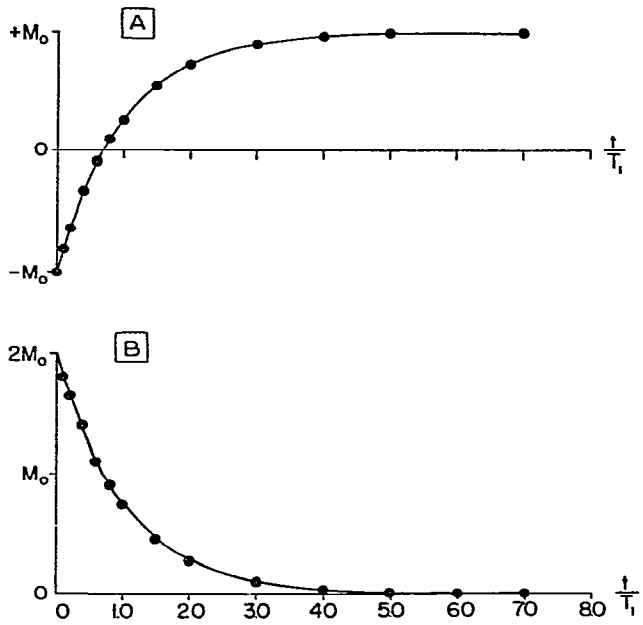

Fig 6 Diagrammatic representation of the recovery of magnetization along the z-axis (M_z) following its inversion by a 180°-pulse, from its initial value ($-M_0$) to $+M_0$ [The exponential, recovery curve shown in A depicts the return of magnetization that would be found in a typical "two-pulse" experiment The curve in B would be obtained from a "three-pulse" sequence and plots ($M_0 - M_t$), which decreases from an initial value of $+2M_0$ to zero at infinite time in this class of experiment, transitions with the shorter relaxation-time (higher relaxation-rate) decrease most rapidly towards zero intensity]

in the intensity of magnetization to zero intensity, followed by a recovery back to its equilibrium value If the spectrum is sampled with a 90°-pulse applied at precisely the time when the magnetization has decayed to zero intensity, no resonance signal will be detected. As individual protons have different T_1 values, they will each have a different "null time" and, hence, each can be made to "disappear" in turn[12]

This process is illustrated in Fig 7, with reference to methyl 2,3 4,5-di-*O*-isopropylidene-*β*-D-glucoseptanoside The normal, ^{1}H n m r spectrum is shown in Fig 7A, together with a diagrammatic, first-order assignment The spectra in Figs 7B, 7C, and 7D show the effect of increasing the delay time between the 180°-pulse and the 90°-pulse In Fig 7B, essentially all of the magnetization is still inverted However, it may be seen that the more rapidly relaxing nuclei have already started to revert to their normal, positive-going intensity, and the spectrum in Fig 7C now shows both H-6 resonances in their normal absorption mode. In the spectrum shown in Fig 7D, these two resonances have been joined by H-5, which is the next most rapidly relaxing proton, that same spectrum now shows the other protons with their magnetization essentially at the null point

From these spectra, it is obvious that selection of the correct delay-time between

the 180°-pulse and the 90°-pulse enables a rather clear distinction to be made between individual resonances that have different T_1 values Although not illustrated here, the removal of unwanted solvent-resonances by this technique is invaluable[1,12], especially where quite dilute solutions are being studied

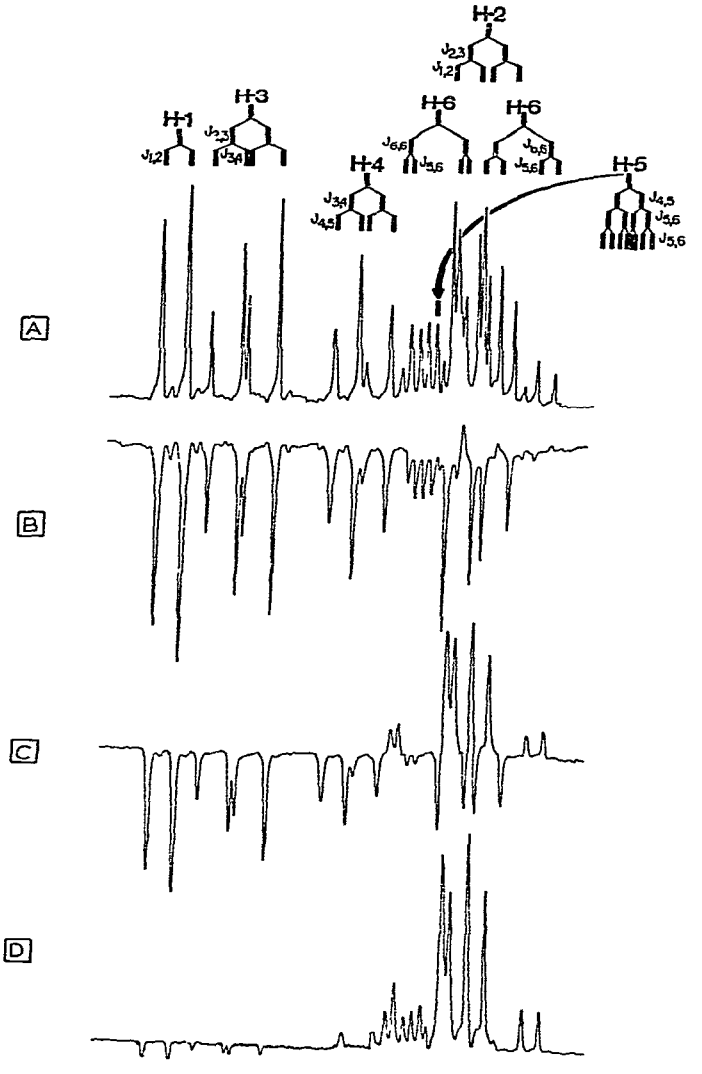

Fig 7 Partial, 100-MHz, ^{1}H n m r spectra of methyl 2,3 4,5-di-*O*-isopropylidene-*β*-D-glucoseptanoside in benzene-d_6 solution (0 2M) using an acquisition time of 4 0 sec [A and B were each obtained by using 9 transients, and C and D, by using 16 transients The spectrum shown in A is the normal spectrum That in B was obtained by inverting the magnetization with a 180°-pulse and then sampling the residual magnetization after 1 0 sec by using a 90°-pulse The spectra in C and D were obtained in the same way, but using pulse-delay times of 1 6 and 2 5 sec, respectively]

For the F t method, the final advantage discussed here follows from the fact that the 90°-pulse used to assay the magnetization of the sample excites all of the nuclear resonances simultaneously As the entire spectrum is sampled rather rapidly (commonly less than 2 sec of acquisition time), it is possible to study systems whose composition is changing as a function of time

The example illustrated in Fig 8 is the mutarotation of a freshly dissolved sample of the calcium chloride complex of α-D-allopyranose The fall in intensity

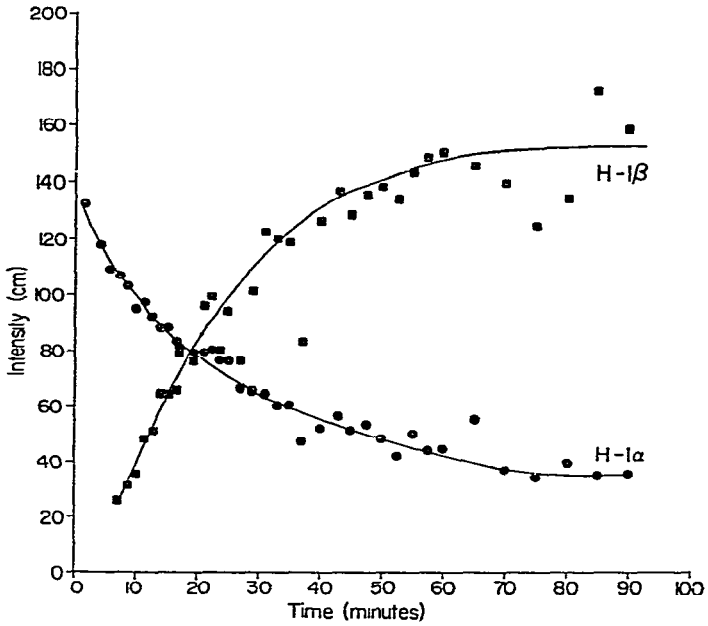

Fig 8 Mutarotation of a freshly mixed solution of 0 15 molar α-D-allopyranose–calcium chloride in 99 96% D_2O at 33° [The intensities were obtained by adding the peak heights of the two transitions of each anomeric proton]

of the H-1α transitions, and the corresponding rise in that of the H-1β transitions, are plotted in Fig 8 In this experiment, the two-pulse sequence previously described was used to partially nullify the HOD peak This was necessary because, although the anomeric region of the spectrum of D-allose at 33° is well separated from the HOD peak, the spinning side-bands of the latter completely obscure the resonances of β-D-allopyranose, and distort those of the α anomer Partial nulling of the residual-water peak allowed both transitions to be monitored cleanly

CONCLUSIONS

Access to a Fourier-transform, n m r spectrometer makes possible, on a routine basis, a number of useful n m r experiments Spectra can be obtained rapidly

from rather small quantities of material, with the added advantage that systems changing as a function of time can also be studied. The possibilities of data manipulation can be exploited in two, quite distinct, ways. On the one hand, the magnetization of the nuclei being studied can be so manipulated that resonances can be selectively eliminated from the observed spectrum A particular set of conditions under which to obtain the basic, free-induction decay signal having been selected, it is then possible to process the data prior to Fourier transformation so that the resolution of the frequency-domain spectrum is significantly enhanced The resolution obtained in this way is so much higher than any previously attainable that it will be necessary to study anew the entire area of long-range couplings Finally, it should be noted that the chemical manipulation of spin–lattice relaxation-times by paramagnetic species[13] provides a further level of control, and this should further extend the application of T_1 values in n m r. studies

EXPERIMENTAL

The n m r measurements were made as described previously[1]. The sample of 1,2,3,4-tetra-*O*-acetyl-β-D-ribopyranose was prepared by the standard method[14]. Methyl 2,3 4,5-di-*O*-isopropylidene-β-D-glucoseptanoside[15] and hydrated α-D-allopyranose–calcium chloride complex[16] were provided by one of us (J D S).

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