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The application of nuclear magnetic resonance to pharmacological problems*

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Nuclear magnetic resonance (nmr) was first discovered in the investigation of fundamental properties of matter but rapidly became an important method in chemical research because of the wealth of detail it is capable of giving about molecular structure. The application to purely biological problems is more recent, but a number of the basic techniques for studying biological systems are now established and more extensive use of the method is desirable.

This survey is intended to accomplish two ends. Firstly to show that the basic principles of nmr spectroscopy are straightforward and easy to understand, and secondly to show the main ways in which this technique can be applied to problems of drug binding.

THE PRINCIPLES OF NUCLEAR MAGNETIC RESONANCE

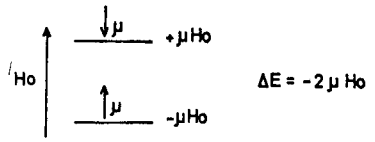
The resonance phenomenon

We will consider only the behaviour of the hydrogen nucleus in a magnetic field, since almost all the work to date has been concerned with the resonances of this atom. The hydrogen atom ^1H has a single proton nucleus with spin $I = \frac{1}{2}$ and a magnetic moment, μ. If the proton nucleus is placed in a powerful magnetic field, it aligns itself in the direction of the field like a tiny bar magnet. Because of the quantized nature of nuclear magnetic moments, only two orientations of the proton with respect to the field are permitted. It can be aligned either in the direction of the field, or in opposition to the field.

If it is given precisely the right amount of energy, a proton aligned with the field can be flipped into the antiparallel direction against the applied field. In so doing it must absorb energy, since this orientation is less stable than the original orientation with the field. It is this absorption of energy which is the basis of the observation of the magnetic resonance of the proton.

The energy associated with a proton aligned with the field H_0 is $-\mu H_0$, and against the field is $+\mu H_0$.

To induce transitions between these two orientations a quantum of energy of frequency exactly equal to ΔE, the energy difference between the two levels, is required The quantum of energy is given by $h\nu = \Delta E = -2\,\mu H_0$ where h is Planck's constant.



* Based on a demonstration presented at the 4th International Congress of Pharmacology, Basle, Switzerland, July 14–18, 1969.

Note that only the precise quantum ΔE will induce transitions since the two permitted orientations are exactly defined. The energy required is directly proportional to both the magnetic moment of the proton and the strength of the applied field.

At the magnetic field strengths currently available the resonance frequency falls conveniently in the radiofrequency range:

H_0 (K gauss)	ν (MHz)
14·1	60
23·5	100
51·7	220

For sensitivity reasons explained later it is necessary that ΔE shall be as large as possible. This implies that only nuclei such as protons with high μ values are suitable for biological systems. It also implies that the applied field H_0 should be as intense as possible.

The line widths of proton resonances for molecules in aqueous solutions commonly fall in the range 0·1 to 1·0 Hz in a total frequency of 10^8 Hz.

This implies that measurements are made to 1 part in 10^8–10^9 and the magnetic field must be homogeneous to the same order if full resolution of the spectra is to be obtained. The construction of magnets with this degree of homogeneity is difficult and accounts for a substantial part of the cost of the instruments.

The essential components of an nmr spectrometer are the magnet, sample probe, radiofrequency (rf) units and accessories for recording the spectra. The sample is contained in a cylindrical glass tube (usually 5 mm diameter, holding $\approx$0·5 ml). The radiofrequency power is supplied by coils adjacent to the sample and the induced power picked up by other coils. The strength of the magnetic field can be gradually increased until it is exactly right to give resonance at the radiofrequency employed.

The main limitation on the nmr of biological systems is the sensitivity of the method. This is limited by two factors.

The energy of the transitions $\Delta E = -2\mu H_0$ is very small, because nuclear magnetic moments are very small, even for the proton. Since ΔE is small, the populations of protons in the two orientations at equilibrium in a magnetic field are very nearly equal. The excess of nuclei in the lower energy level is only $\approx$1 in 10^5 in a field of 23,500 gauss ($\nu = 100$ MHz).

Since the radiofrequency field induces transitions between these two orientations with equal probability in both directions, there is only a net absorption of energy while an excess of nuclei is maintained in the lower energy level. As the power of the radiofrequency field is increased, it tends to equalize the populations of nuclei in the two energy levels. When this occurs the intensity of the observed signal decreases and eventually disappears. This phenomenon is termed saturation and limits the radiofrequency power which can be used and hence the sensitivity with which resonance can be detected.

The second factor determining the radiofrequency intensity at which saturation occurs and hence the sensitivity, is the rate at which nuclei return from the excited state to the lower energy level, restoring the necessary excess of nuclei in this level. This process is termed relaxation and for protons in liquids the half time is in the range 0·1 to 10 s. This slow relaxation together with the small value of ΔE account for the inherent insensitivity of nmr. In practice, to detect a proton signal in a single scan, a

concentration of 1–10 mM is required and to examine many biological materials, computer techniques are necessary to enhance sensitivity.

One advantage of the very small value of ΔE for nmr transitions is that no chemical changes are produced in the system by the absorption of energy at resonance. A second advantage is that nmr parameters which characterize the spectra are very sensitive to the chemical environment of protons in a molecule. It is this which accounts for the high information content of nmr spectroscopy and its particular attraction for biological systems.

The relation of nmr spectroscopy to the electromagnetic spectrum is shown to emphasize how small is the energy absorbed at resonance (Fig. 1).

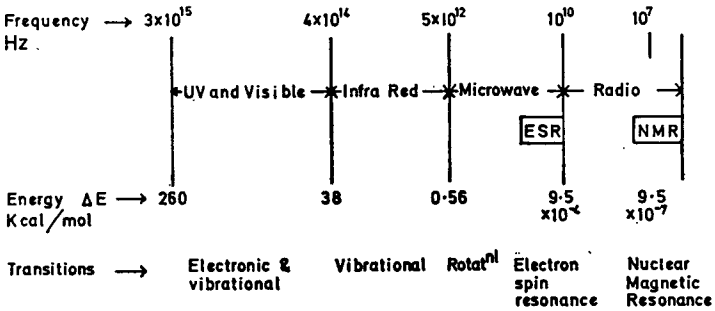


FIG. 1. The electromagnetic spectrum.

Features of nmr spectra

A proton resonance signal is characterized by 3 parameters.

(1) The precise frequency ν at which resonance occurs for each set of protons in a molecule. There are practical difficulties in measuring the precise frequency and the resonance position is therefore measured with respect to a convenient standard (e.g. tetramethylsilane, TMS) which is arbitrarily assigned the value zero. The position of a resonance from this zero in dimensionless units (parts per million, ppm) is then termed the chemical shift, δ or alternatively τ which is $10-\delta$.

(2) The multiplicity of signals arising from a set of chemically equivalent protons in a molecule (e.g. methyl protons). This depends on the effect of the magnetic fields of adjacent protons, and is characterized by the coupling constant *J*.

(3) The lifetime of a nucleus in the excited state, and the width of the resonance absorption signals are characterized by the relaxation time T.

We now consider each of these parameters in turn.

The chemical shift

The resonances of protons in aqueous solution are spread over approximately 10 ppm of the total resonance frequency. This is a range of 1000 Hz for a resonance frequency of 100 MHz.

The resonance frequency is not exactly the same for all protons because in an applied field H_0, the effective field H_{eff} actually experienced by the protons is modified by their electronic environment, this is because electrons themselves have a magnetic moment which causes a local perturbation in the magnetic field experienced at the proton. The electron shielding is generally greater the higher the electron density around the proton, and we expect the shielding effect to decrease when the proton is in the vicinity of an electron withdrawing atom or group.

For example we find that for methyl groups, the shielding experienced decreases in the series

$$CH_3{\cdot}C > CH_3{\cdot}N > CH_3{\cdot}O—$$

with corresponding shifts in the frequency at which resonance takes place. A simple example is shown in Fig. 2. The spectrum of methyl acetate consists of two narrow lines of equal magnitude corresponding to the *acetyl* methyl and *ester* methyl. Note that the TMS reference signal falls at even higher field, where the methyl protons are very strongly shielded.

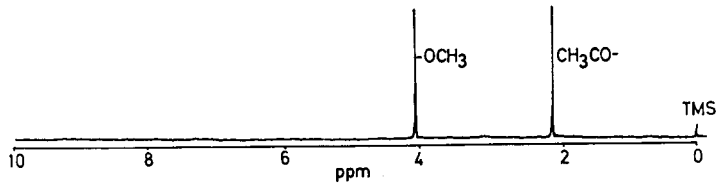


FIG. 2. The nmr spectrum of methyl acetate shows two equally sized resonances due to the CH_3O and CH_3CO groups. A small amount of tetramethylsilane (TMS) is included as a reference.

Approximate resonance positions are shown for protons in a range of simple chemical structures listed in Table 1.

The position of all these resonances can be understood in terms of the electronic shielding of the protons from the applied field H_0.

Especially of note are the resonances of aromatic ring protons (see benzene) which are very weakly shielded and consequently fall at low field strengths.

Table 1. *Chemical shifts of common groups*

		(ppm)
C*H*O	(acetaldehyde)	9·72
C_6H_6	(benzene)	7·26
$CHCl_3$		7·25
–C*H* =	(fumarate)	6·74
H_2O		5·0
CH_2OH	(ethanol)	3·59
CH_3OH	(methanol)	3·38
CH_3N	(trimethylamine)	2·12
CH_3CO	(acetone)	2·09
CH_3–COH	(t-butanol)	1·22
$-CH_2-$	(cyclohexane)	1·44
$-CH_3Si$		0

Spin coupling and spin coupling constants

In the spectrum of acetaldehyde (CH_3CHO) the methyl resonance consists of two signals of equal intensity, while the resonance for the single aldehyde proton is split into a quartet with intensities in the ratio of 1 : 3 : 3 : 1 (Fig. 3).

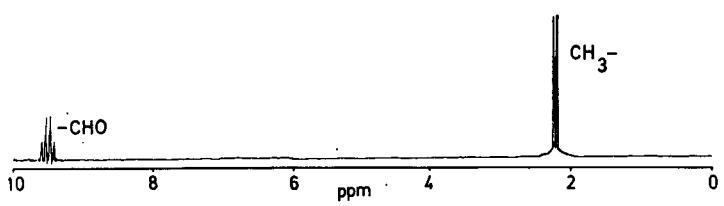
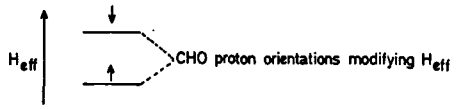


FIG. 3. The spectrum of acetaldehyde showing the splitting of the CH_3 signal into a doublet and CHO signal into a quartet by spin coupling.

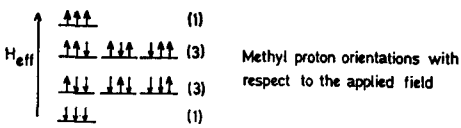
The separation between the resonances in both the doublet and the quartet is the same (Hz) and is termed the coupling constant, *J*.

The areas of the doublet and the quartet are in the ratio 3 : 1, which is the relative number of protons comprising each signal. Thus the signal area is directly proportional to the number of protons giving rise to the signal, and this frequently helps with the identification of resonances.

The multiplet structure of the acetaldehyde resonances can be readily understood once it is realized that the magnetic field experienced by a set of protons is affected by the magnetic field or nearby nuclei. For example the methyl (CH_3) protons of acetaldehyde experience the two distinct orientations allowed for by the magnetic moment of the adjacent –CHO proton.



These two orientations are equally probable so that half the methyl protons in the population experience H_{eff} perturbed by each orientation. This accounts for the observation of the methyl doublet. The -CHO proton experiences a field perturbed by a more complicated set of arrangements of the methyl protons. These are:



The expected ratio of intensities of the individual lines of the quartet is therefore 1 : 3 : 3 : 1, as observed in the spectrum.

Relaxation processes

Relaxation processes limit the life time of a nucleus in the excited state. The absorbed energy is given up by two main processes.

(a) *Spin-lattice relaxation.* The absorbed energy of the excited nuclei is dissipated as random thermal motion throughout the assembly of nuclei in the sample (termed the lattice). This process acts directly to maintain the excess of nuclei in the lower energy level and is characterized by the relaxation time T_1.

This can be measured directly by applying a radiofrequency field of sufficient intensity to completely saturate the signal, and then following the recovery of the absorption signal with time, using a radiofrequency field of low intensity (Fig. 4). The exponential time course of the recovery is characterized by the time constant T_1.

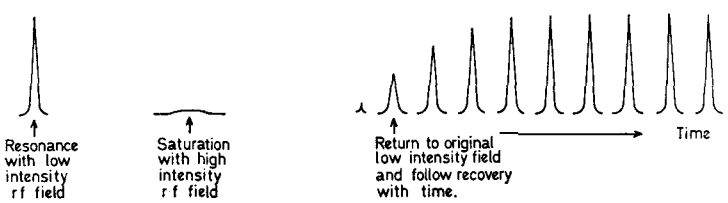


FIG. 4. The measurement of spin-lattice relaxation by saturation and recovery.

(b) *Spin-spin relaxation.* Two protons in the correct spatial relation to each other in the applied field are able to undergo a mutual exchange of orientations. This process does not affect the net number of nuclei in each orientation but does limit the life-time in the excited state. Spin-spin relaxation is characterized by the relaxation time T_2. The lifetime of the excited state directly determines the line width of resonance absorption signals so that long relaxation times give narrow lines in the spectrum and short relaxation times give broad lines. The exact relation is $T_2 = 1/\pi\Delta\nu$ where $\Delta\nu$ is the width of the line in Hz at half its maximum height, and T_2 is in seconds.

An important feature of the relaxation times is that they are connected with the motion of the molecules. Both relaxation processes occur more efficiently the longer the nuclei remain aligned with the applied field. Thermal motion tends to disorientate the nuclei, so that the faster the nuclei are tumbling in solution, the longer are the relaxation times. In all experiments described here the rate of molecular motion directly determines the relaxation times T_1 and T_2 and they are in fact equal in magnitude ($T_1 = T_2$). For this reason we will refer in future to a single relaxation time T which is directly proportional to the rate of molecular motion of the nucleus.

INTERACTION OF ANAESTHETICS WITH CYTO-MEMBRANES

We expect that when a small molecule (e.g. local anaesthetic) is partitioned into an ordered membrane structure, its molecular motion will be restricted whether it is inserted into the membrane lipid or bound to membrane protein. This steric interaction is readily detected from the increase in relaxation rate (1/T) of the small molecule which results in broadened resonances.

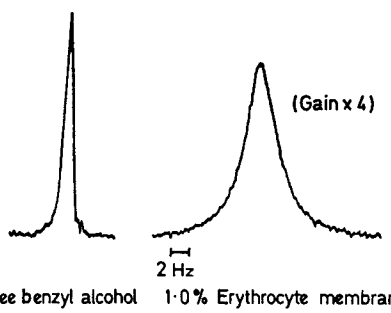


FIG. 5. The curve on the left shows the resonance due to the five aromatic protons of benzyl alcohol dissolved in water. The curve on the right shows the same concentration of benzyl alcohol in the presence of a 1% suspension of erythrocyte membranes. The line is broadened and reduced in amplitude (Metcalfe, Seeman & Burgen, 1968).

Fig. 5 shows the broadening of the aromatic proton line of benzyl alcohol in a 1·0% erythrocyte membrane suspension.

The benzyl alcohol molecules exchange sufficiently rapidly between the bound and free states to give a resonance whose line width is the weighted mean of the free and bound states. That is

$$\Delta\nu_{obs} = \frac{1}{\pi}\left[\alpha\left(\frac{1}{T_2}\right)_{membr} + (1-\alpha)\left(\frac{1}{T_2}\right)_{free}\right]$$

where α is the fraction of the alcohol molecules located in the membrane and $(1/T_2)_{membr}$ and $(1/T_2)_{free}$ are the values for the anaesthetic in the two separate locations.

The values obtained show that alcohol molecules in the membrane are slowed by at least three orders of magnitude in their rotation rates.

Experiments made with systems in rapid exchange are particularly easy because it is usually possible to work with high concentrations of the free drug compared with the concentration of the bound drug and so gain sensitivity.

This also means that the line width is under experimental control and that inconveniently broad signals need not be dealt with.

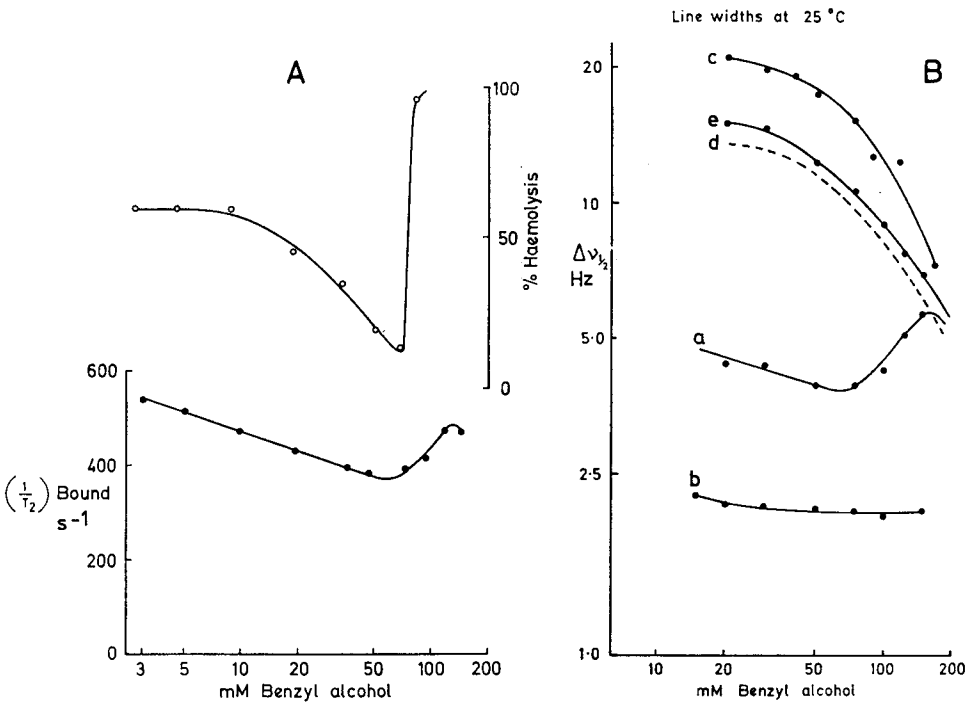


FIG. 6. A. Relaxation rate of benzyl alcohol aromatic protons bound to erythrocyte membranes as a function of alcohol concentration. The upper curve shows the degree of haemolysis of red cells in a hypotonic salt solution together with the alcohol.

B. Line width of the aromatic resonance of benzyl alcohol in the presence of 1% concentration of (a) erythrocyte membranes (b) lipid extracted from erythrocyte membranes (c) protein extracted from erythrocyte membranes (d) a 60 : 40 mixture of extracted protein and lipid and (e) erythrocyte membranes that had been pretreated with a high concentration (300 mM) of benzyl alcohol. The line width in the presence of the membrane at high alcohol concentrations corresponds to that of the appropriate mixture of the separate protein and lipid. The membrane has a smaller line broadening effect on the lower alcohol concentrations than expected from the contribution of the separated protein and lipid components.

If we now consider the relaxation rate of the anaesthetic in the membrane as a function of concentration, we find (Fig. 6A) that with increasing concentration of the alcohol the relaxation rate decreases to a minimum at 60 mM and then begins to increase again. These observations show that as the anaesthetic concentration is increased the anaesthetic molecules in the membrane initially find themselves in an increasingly fluid environment so that it is easier for the molecules to rotate. This trend is reversed at 60mM. It can also be seen that the nmr changes correlate with changes in the ease of hypotonic haemolysis of erythrocytes. The concentration of benzyl alcohol necessary to block peripheral nerve fibres is 35 mM.

Further analysis of this phenomenon has been possible by studying the behaviour of the alcohol in the presence of separated lipid and protein from the membrane.

It is found that at the highest concentration, the relaxation of the anaesthetic in the membrane corresponds to that in a mixture of protein and lipid of the correct proportions (Fig. 6B). However, in the lower concentrations, the relaxation rate in the membrane is much less than in the separated components and in these concentrations the organization of the membrane clearly reduces the degree of interaction of the anaesthetic with its components. The kind of results obtained with erythrocyte membranes can be extended to other membranes with generally similar results and also to other anaesthetics. In some such cases a number of resonances may be studied as has been done with xylocaine.

Xylocaine is a cationic local anaesthetic and competes for cationic binding sites in the membrane with inorganic cations. This phenomenon may be studied by nmr. For instance in the presence of Ca^{2+} a significant fraction of the xylocaine is displaced from the membrane (Fig. 7).

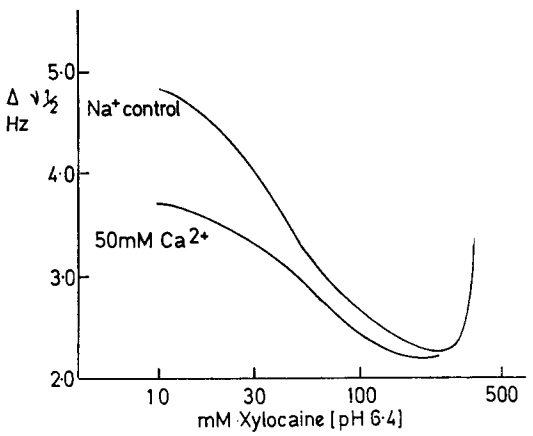


FIG. 7. Line width of the xylene methyl resonances of xylocaine in the presence of 1% erythrocyte membranes with 100 mM NaCl in the medium with this replaced by 50 mM $CaCl_2$. The lines are narrowed by the Ca ions particularly at low anaesthetic concentrations. The narrowing is due to reduced binding of anaesthetic consequent on competitition between Ca^{2+} ions and the xylocaine cation.

The number of molecules which can be examined directly by nmr is limited by the sensitivity requirement for concentrations > 10 mM. A wider range of molecules interacting with the membrane can be examined indirectly using a low concentration of benzyl alcohol as a reporter for changes induced in the membrane structure by other agents. An example of such an experiment where we can follow the relaxation changes of both reporter and perturbing agent simultaneously is shown (Fig. 8A). The upswing in the line width of the 15 mM benzyl alcohol resonance induced by neopentanol occurs simultaneously with the upswing for neopentanol itself. Thus the reporter benzyl alcohol and neopentanol are detecting the same changes in membrane structure.

Using this technique we have examined the line width changes induced in 15 mM benzyl alcohol by the n-alkyl alcohols. Up to hexanol (C_6) the form of the curve is similar to that for benzyl alcohol itself, and for each alcohol the upswing coincides with the lytic concentration range (Fig. 8B).

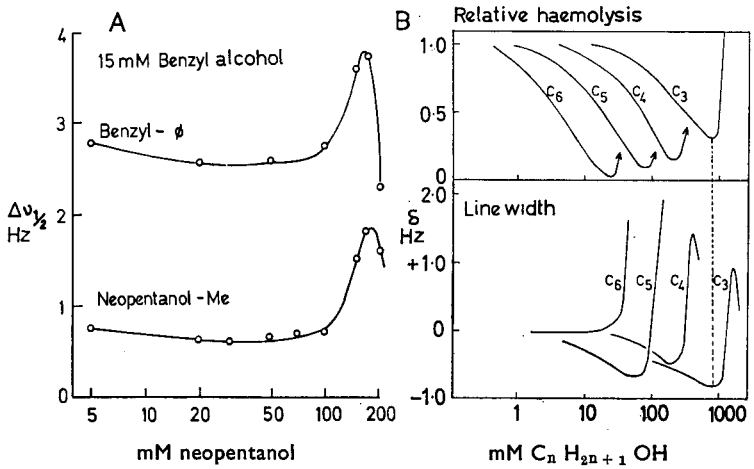


FIG. 8A. A 1% suspension of erythrocyte membranes in a medium containing 15% mM benzyl alcohol. Neopentanol was added as indicated in the abscissa and the aromatic resonances of benzyl alcohol and the methyl resonance of neopentanol measured. It can be seen that changing the concentration of neopentanol affects both its relaxation and that of the indicator benzyl alcohol.

B. A similar experiment except that the normal aliphatic alcohols from propyl to hexyl were added. Only the line widths of the benzyl aromatic resonance were measured (lower curves). The upper curves show the effect of the alcohols on haemolysis.

INTERACTIONS OF PROTEINS WITH SMALL MOLECULES

Aliphatic drugs such as acetylcholine can exist in multiple conformations (rotamers) in solution. Can we tell whether these exist for a sufficient time to be distinguishable species in drug-receptor interactions? We have mentioned previously that T, the nmr relaxation time in liquids depends on the motion of a proton with respect to its nearest neighbours. Thus for a methyl group the relaxation of each proton depends mainly on the motion of that proton with respect to the two others attached to the same carbon atom. Consider a methyl group $-CH_3$ attached to a group R. If rotation about the bond is free, relaxation can occur by intra-molecular rotation of the $-CH_3$ as in (a). If rotation about the bond is not permitted, relaxation can only occur as a result of tumbling of the whole molecule as in (b) (Fig. 9).

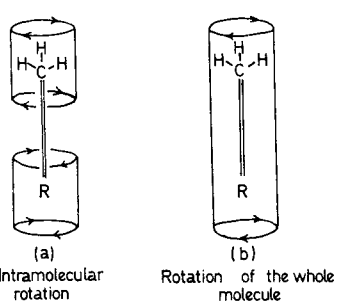


FIG. 9. Relaxation by intramolecular rotation.

In the first case (a) relaxation will be almost completely insensitive to the size of R, in the second (b) it will be directly dependent on the rotational tumbling rate of the molecule.

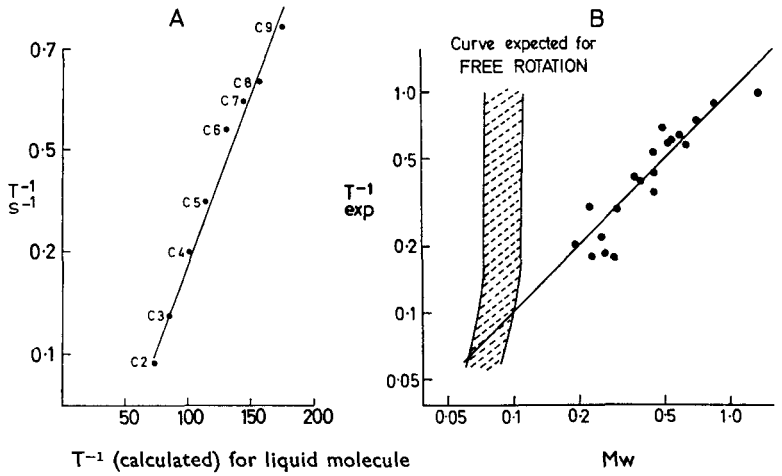


FIG. 10A. Relaxation of n-alkyltrimethylammonium methyl protons.

B. Comparison of experimental relaxation rates of methyl protons in alkylammoniums with values calculated for (i) free rotation around bonds (ii) relaxation only by molecular tumbling. (data from Nogrady & Burgen, 1969).

This is approximately proportional to the reciprocal of the molecular weight. In (Fig. 10A) the relaxations of the *N*-alkyl trimethyl ammoniums $C_nH_{2n+1}{}^+N(CH_3)_3$ are shown. It is clear that there is a strong dependence on molecular weight. It is possible to calculate the relaxation rates for the two extreme cases of Fig. 9 and compare them with the experimental results (Fig. 10B). It can be seen that the experimental values agree well with the calculation for a rigid molecule but do not agree at all with the supposition of free rotation around bonds. These results show that the drug rotates as a whole many times before rotation around the bonds occurs. The rotamers can therefore be considered as stable configurations during the time of collision with a receptor.

The drugs so far dealt with have C–C and C–N bonds. Bonds with oxygen –O– are usually much less restricted rotationally. This is confirmed by the relaxation measurements, thus the relaxations of $-O-CH_3$ or $-O-COCH_3$ groups are intermediate between rigid and free rotation. Two examples are shown in Fig. 11.

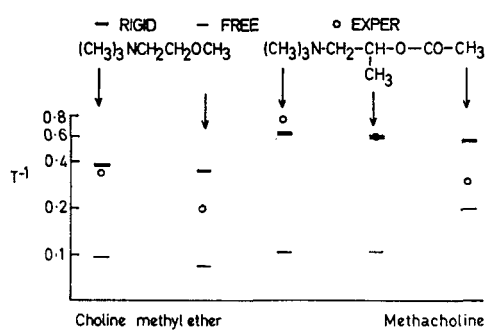


FIG. 11. Relaxation of methyl protons in choline methyl ether and methacholine (data from Nogrady & Burgen, 1969). The groups referred to are indicated by the tails of the arrows.

In choline methyl ether relaxation of the methyl groups on the nitrogen corresponds to that expected from a rigid molecule whereas the *O*-methyl is intermediate in relaxation between rigid and completely free rotation. It can be estimated that the methyl group can rotate once around the bond axis in every 2–3 rotations of the whole molecule. Similar results are seen with methacholine. The *N*-methyl and β-methyl groups behave as though rotation was not permitted whereas the acetyl methyl shows considerable freedom to rotate.

Rotational stabilization of complexes

Just as anaesthetics are immobilized in a membrane, so drugs are restricted in their motion when interacting with a specific binding site. This is illustrated by the binding of drugs of the acetylcholine group to an antibody against the nicotinic agonist choline phenyl ether $C_6H_5{\cdot}O{\cdot}CH_2{\cdot}CH_2{\cdot}N\,(CH_3)_3$.

A simple example is tetramethylammonium (TMA). When it is mixed with antibody in the molar ratio of 1:10 (Ab:TMA) the relaxation rate is 6·8 s^{-1}. This value is the weighted mean of the relaxation rate in the free and bound states. From this we can calculate the relaxation rate in the bound state as 65 s^{-1} compared with the value of 0·09 s^{-1} obtained for free TMA. Thus the motion of the bound TMA is restricted by a factor of approximately 65/0·09 or $\approx$720.

This analysis can be applied to the more complex drug, methacholine, with three distinguishable resonances. Measurement of the relaxation rates of these three methyls showed that they are increased in the order: β–CH_3 131, $N(CH_3)_3$ 101, Acetyl CH_3 78 s^{-1}. Consider the following possibilities. (I) Only the $N(CH_3)_3$ group interacts with the antibody binding sites. (II) Only the β–CH_3 interacts. (III) Only the acetyl group interacts. (IV) The whole molecule interacts.

The calculated values of T_1 (s^{-1}) for the three methyls are shown in Table 2 for each possible type of interaction, together with the experimental values obtained.

Table 2. *Relaxation of the methyl groups of methacholine in combination with an antibody*

Interacting group	Estimated T^{-1} (s^{-1}) N-CH_3	β-CH_3	Acetyl CH_3
1. $N(CH_3)_3$ only	100	50–80	0·5
2. β-CH_3 only	70–90	100	0·5
3. Acetyl CH_3 only	1	1	100
4. Whole molecule	100	80	80
Experimental values	101	131	78

It is clear that the data are not consistent with any of the first three possibilities. The fourth could be correct or any combination of (I), (II), (III). In fact the order of relaxation rates would suggest that motion may be most restricted in the neighbourhood of the β-methyl group. However this must not be taken to mean that this is the group in the molecule contributing most strongly to binding.

Another example of the usefulness of relaxation measurements is shown in a study of the binding of sulphonamides to bovine serum albumin (BSA). This protein has a single binding site for sulphonamides.

With sulphacetamide the increase in relaxation rate of the aromatic protons is much greater than that for the acetyl methyl group (Fig. 12). This is best interpreted as showing that the primary site of immobilization is the aromatic ring.

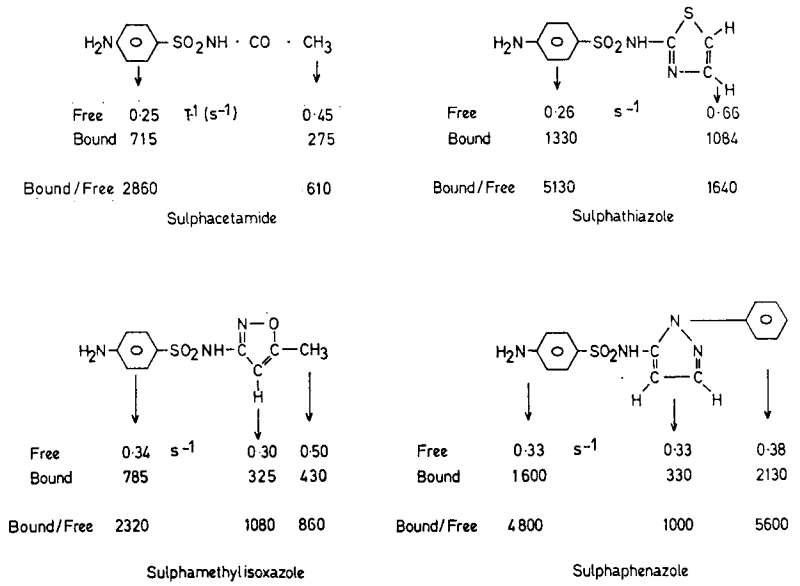


FIG. 12. Relaxation of protons in 4 sulphonamides free and bound to bovine serum albumin (Jardetzky & Wade-Jardetzky, 1965.)

Similar results were obtained with both sulphathiazole and sulphamethylisoxazole. On the other hand in sulphaphenazole which has two aromatic rings, both appear to be stabilized to the same extent. Since it was possible to demonstrate competitive displacement of one aromatic ring while the other remained bound, it follows that there are two distinct binding sites for the two aromatic rings of sulphaphenazole.

Chemical shifts in complexes

An alternative important method of studying binding is by looking for chemical shifts in the resonances of the bound molecule. This may occur as a result of changes in the magnetic environment of protons of the small molecule when complexed. The enzyme lysozyme hydrolyses certain glycosides containing hexosamines. Many hexosamines will act as inhibitors, for example *N*-acetylglucosamine (NAG). When NAG combines with the enzyme the acetyl methyl peak is displaced upfield and split into two. The two resonances correspond to the α- and β- anomers of NAG. Fig. 13A shows the nmr spectrum of β-NAG (acetamido methyl protons) free in solution and in the presence of lysozyme ($3{\cdot}0 \times 10^{-3}$M).

When the first spectrum was obtained some α-NAG had already appeared as a result of mutorotation caused by the enzyme. With time the β peak declined and the α peak grew until after approximately 20 min they were equal in size.

The difference in chemical shift for the two anomers shows that the acetyl group is in a different geometrical relation to the enzyme active centre in the two cases. In the free state the acetyl resonances of α- and β-NAG are indistinguishable. From studies of this kind the affinity constants of the two anomers can be determined individually in the presence of each other.

By adding another reporter substituent to the molecule as in NAG methyl glycoside, further information can be obtained. When the resonance shift was studied as a function of pH, two titratable groups with pK's of 4·7 and 6·1 could be demonstrated in the binding site in the neighbourhood of the acetyl group (Fig. 13B).

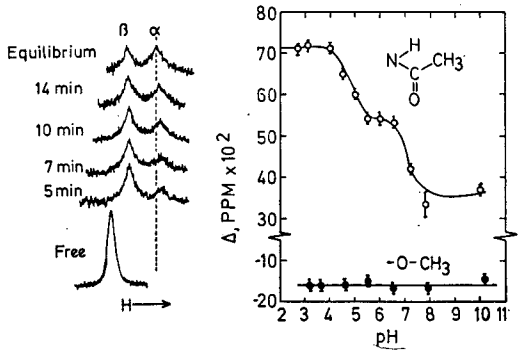


FIG. 13A. Resonance of the acetyl methyl of NAG in the free state and in the presence of lysozyme. The resonance is shifted upfield and split into two corresponding to the β- and α-anomers of NAG which are clearly in different magnetic environments.

B. In NAG-methyl glycoside the acetylmethyl is shifted upfield by lysozyme, the ether methyl downfield. The former shows a two step change with pH, the *O*-methyl is unaffected (Dahlquist & Raftery, 1968).

These are sufficiently far away from the *O*-methyl not to affect it. The lack of effect on the *O*-methyl strongly suggests that a proton-dependent conformation change in the enzyme is unlikely to account for the changes.

SPECTRA OF PROTEINS

Amino-acids give straightforward nmr spectra, the main features of which are shown in Fig. 14. The α-protons all give lines in the same region (4·2–4·8 ppm). The other aliphatic protons give lines upfield in the range 1·0–3·8 ppm. The aromatic amino-acids give lines due to their ring protons at 7–8 ppm with the exception of the C_2 proton of histidine which is at 8·7 ppm. In small peptides there are small shifts from the values in the free amino-acids due to peptide bond formation and the spectra of fully unfolded proteins are similar and indeed are close to the sum of the resonances of the constituent amino-acids. They are complicated mainly by the large number of nearly coincident lines.

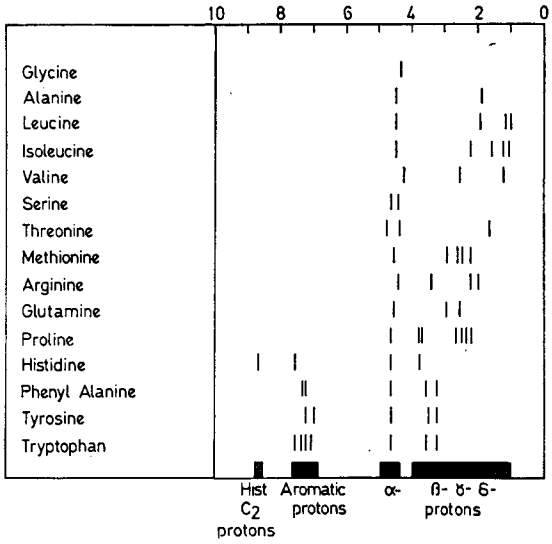


FIG. 14. Position of resonances in amino-acid spectra.

In native proteins however two important changes occur. (a) The individual lines become broadened because of the relative rigidity and restriction on motional freedom in the native protein structure. (b) Lines are shifted by electronic interactions with neighbouring residues, so that the resonances for a particular amino-acid become spread over a range. Those changes result in a drastic smearing of the details of the spectra, which appear as envelopes with few sharp lines evident. This is illustrated in the spectrum of ribonuclease. At increasing temperatures, the spectra become progressively sharper, and in the denatured state at 80°, considerable detail from the constituent amino-acids is apparent (Fig. 15).

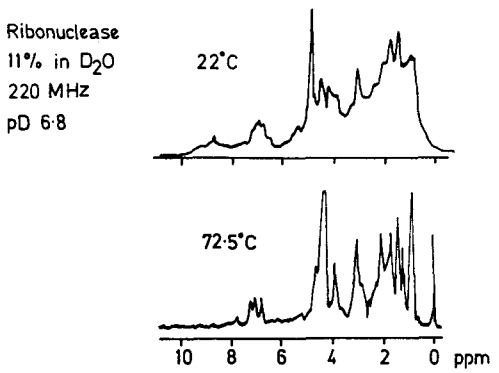


FIG. 15. Spectrum of ribonuclease in the native state at 22° C partially unfolded at 72·5° C.

Despite these formidable difficulties it is proving possible to obtain useful information about the role of individual amino-acids in the formation of protein complexes. For instance, in ribonuclease dissolved in a buffer at pH 5·37 four small resonances can be observed down field from the aromatic region (Fig. 16A).

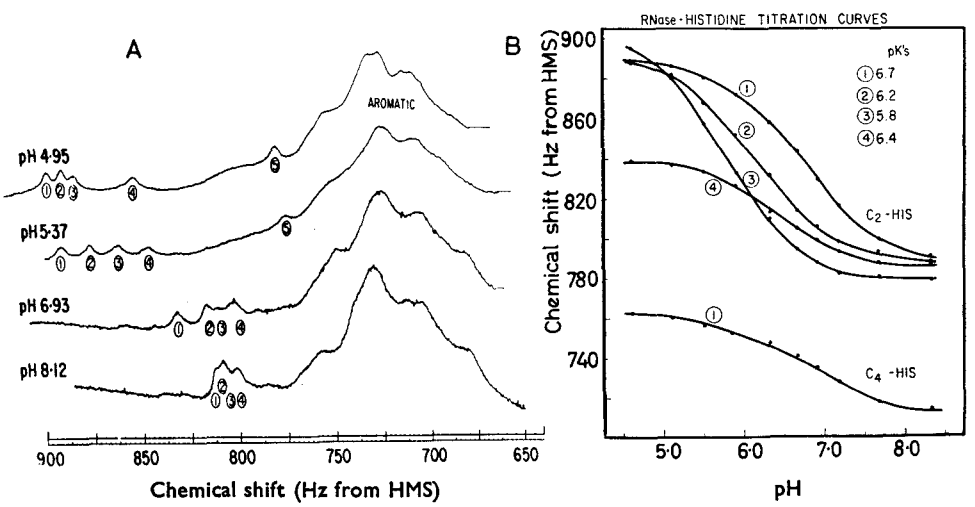


FIG. 16A. Spectrum of the aromatic region of the spectrum of ribonuclease. The peaks 1, 2, 3, 4 are the C_2 protons of the four histidines in the enzyme.

B. From the chemical shifts in experiments of the type shown in A, titration curves for the four histidines can be constructed (Meadows, Jardetzky & others, 1968).

These are likely to be due to the C_2 protons of histidine residues and this identification can be established by examining the shift as a function of pH. The groups show marked changes in chemical shift in the range pH 5–8 and imidazoles are the only basic groups ionizing in this pH range. The identification has been confirmed by alkylation of some of the histidines with iodacetate. There are in fact only four histidines in ribonuclease at positions 12, 48, 105 and 119 in the peptide chain. By various biochemical manipulations these have been identified unambiguously with peaks 2, 4, 1, and 3 respectively. We then have the remarkable result that the ionization of the four histidines can be individually determined (Fig. 16B). On addition of the inhibitor 3′-cytidylic acid to the enzyme some very clear cut changes are seen (Fig. 17A). The C_2 resonance of histidine 119 is moved down field by 60 Hz so that it appears below 105 which is itself unaffected by the inhibitor. Histamine 12 undergoes a smaller concurrent movement down field. Histamine 48 is hardly affected.

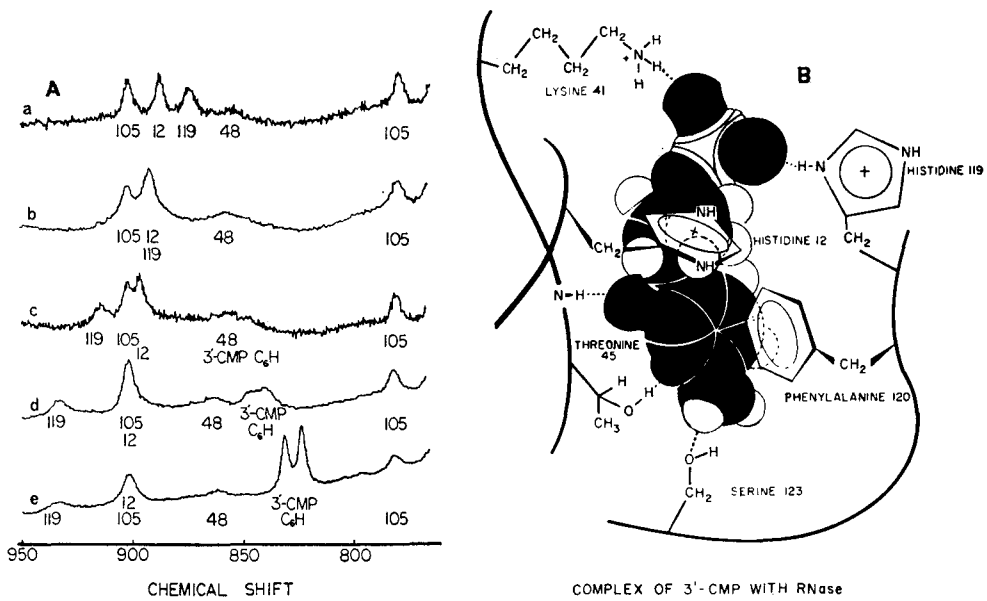


FIG. 17A. Histidine resonance region of ribonuclease in the presence of 3′-cytidylic acid. (a) Ribonuclease alone (6·5 mM). (b) Plus 2 mM 3′-CMP. (c) Plus 5 mM 3′-CMP. (d) 10 mM 3′-CMP. (e) 30 mM 3′-CMP (Meadows & Jardetzky, 1968).

B. Structure of the complex between ribonuclease and 3′-CMP (Meadows, Roberts & Jardetzky, 1969).

At the same time the doublet due to the proton in the 6 position of the nucleotide is broadened and shifted down field. From comparison with the crystal structure the structure of the complex appears to be as in Fig. 17B. The down field shift of the 119 resonance is due to a hydrogen bond interaction with the phosphate of the nucleotide, whereas the down field shift of histidine 12 is due to interaction with the ribose. The down field shift of the cytidine H_6 is due to the ring current from phenylalanine 120 which is lying in contact with it. The lack of effect on the other histidines is because they are not contributing to the formation of the complex. By contrast, in the complex formed by 5′-cytidylic acid with the enzyme, histidine 119 is not involved but the phosphate reacts instead with the 6 amino-group of lysine 41. This offers a neat

example of what nmr can offer to structure activity studies in the way of distinguishing complexes in which the mode of interaction is fundamentally different.

The application of these techniques to other amino-acid residues is clearly more difficult both because the resonances are not separated and because the convenience of the pH dependence of the histidine resonance is not available. In certain cases the spectrum can be simplified by making a protein in which most of the normal amino-acids have been replaced by deuterated amino-acids; these will not give signals at the proton frequency. Staphylococcal nuclease has been prepared with all but tyrosine, tryptophane and histidine deuterated with a remarkable simplification of the spectrum and used to study interactions of this enzyme with substrates and inhibitors.

SUMMARY AND CONCLUSIONS

Nuclear magnetic resonance has unique properties to offer in the study of molecular interactions, especially in view of its great sensitivity to changes of molecular geometry. While the method is inherently insensitive it is frequently possible to take advantage of rapid exchange to enhance the sensitivity and to improve the resolution, but in any case the development of computer techniques such as signal averaging and Fourier transform spectroscopy can improve the sensitivity enough to make it relatively easy to study the smaller macromolecules and even to consider working with less sensitive nuclei. Large macromolecular structures such as nucleic acid and cell membranes cannot be studied by high resolution nmr because of the very rapid relaxation rates associated with these large structures, but indirect study by rapidly exchanging probes can be carried out.

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