

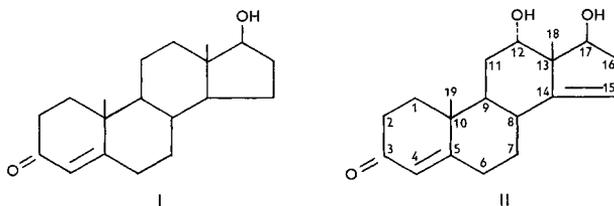
SOME RECENT STRUCTURAL AND ANALYTICAL APPLICATIONS OF HIGH RESOLUTION NUCLEAR MAGNETIC RESONANCE

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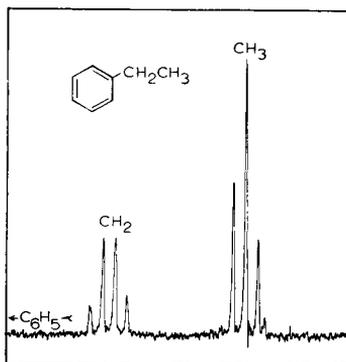
Recent instrumental improvements in high resolution NMR spectrometers, including audio-frequency modulation and phase-sensitive detection [4, 8, 9], thermal isolation of the magnet and control of its temperature, and careful attention to optimization of receiver noise figure and coil configuration, have permitted steady improvement in the sensitivity of these instruments. Fig. 1 shows the proton spectrum of a 1 per cent solution of ethyl benzene in a cylindrical glass cell of 4 mm internal diameter, taken at an operating frequency of 60 Mc in a magnetic field of approximately 14,100 oersteds. The weakest peak in the quartet represents approximately $\frac{1}{10}$ of the intensity arising from the two protons in the CH_2 groups, which corresponds to one proton per molecule in a 0.016 *M* solution. Since the volume of solution in the cell is typically 0.4 ml,¹ this means that a substance of molecular weight 300 can be studied effectively with as little as 2 mg of sample.

As an example of the type of structural information that can be obtained with small amounts of sample, let us consider an unknown steroid (II) resulting from a microbiological transformation of testosterone (I), and available in 2 mg quantity. Chemical evidence [5] suggested that hydroxylation at C_{12} or C_{18} had taken place. The NMR spectrum, shown in Fig. 2, proved to be quite informative when compared with the spectrum of the parent compound testosterone. Both compounds were studied in CDCl_3 solution to which a trace of tetramethylsilane had been added to serve as an internal reference compound. Chemical shifts are indicated in Fig. 2 over



¹ The volume can be reduced somewhat if cells with special configurations are constructed. Although the cell should be an ellipsoid of revolution to avoid disturbing the magnetic field homogeneity, a cylindrical cavity with length/diameter ratio 4:1 or greater appears to give satisfactory resolution with about half the standard volume of solution.

Fig. 1.—Proton spectrum of ethyl benzene, one percent by volume in CCl_4 .



the corresponding peaks and expressed in parts per million of the applied magnetic field according to the definition

$$\delta_i = \frac{(\nu_i - \nu_{\text{SiMe}_4})}{\nu_{\text{SiMe}_4}} \times 10^6,$$

where the expression in parentheses is just the frequency separation of the peaks as measured by the well known side-band method and ν_{SiMe_4} is 60 Mc.

The presence of two strong angular methyl peaks at $\delta = 1.13$ and 1.23 shows that C_{18} cannot be hydroxylated. Moreover, in addition to the triplet with $\delta = 3.72$ which is due to the proton on C_{18} and is found in both I and II, one other proton in II is found to be shifted to $\delta = 4.41$, indicating attachment of the hydroxyl to a ring. Observations of the 11α -proton in four 11β -hydroxylated steroids [10] average to $\delta = 4.43$,¹ but the hydroxyl group

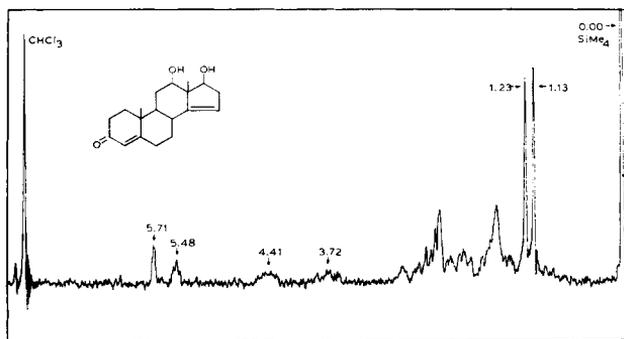


Fig. 2.—Spectrum of 2 mg of $\Delta^{14,14}$ -androstadiene- 17β , 12α -diol-3-one.

¹ Since the measurements of reference [10] were reported in cps relative to benzene in an external annular cell at 40 Mc, it is necessary for comparison purposes to convert to the present scale by the relation

$$\delta_i(\text{ppm}) = (385 - 3/2 \nu_i (40 \text{ Mc})) / 60$$

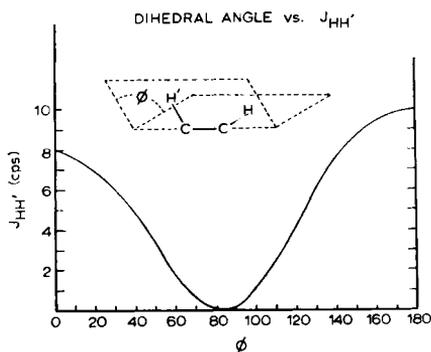


Fig. 3.—Dependence of spin-spin coupling constant for protons on adjacent carbon atoms as a function of dihedral angle (after Conroy).

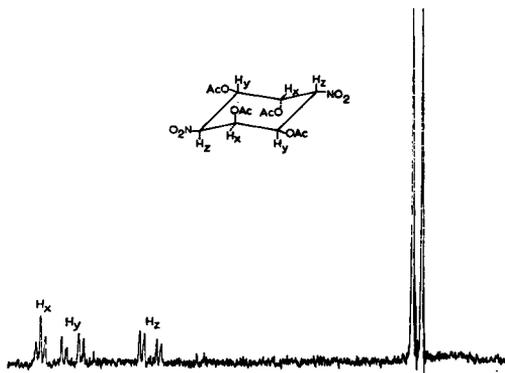
cannot be in the 11β -position because in that case it would shift the C_{19} peak from the value $\delta = 1.23$ observed in testosterone to approximately 1.50 (see [10]). However, the 12α -position for the hydroxyl would result in the 12β -proton bearing essentially the same equatorial relationship to the molecule as an 11α -proton. The angular methyl resonances would not be affected appreciably by a 12α -hydroxyl directed axially away on the reverse side of the molecule.

The closely spaced triplet at $\delta = 5.48$ shows that while the peak at $\delta = 5.71$ due to the olefinic proton on C_4 is unchanged, a new trisubstituted double bond has been introduced. The triplet structure indicates two neighboring protons on an adjacent carbon atom. Since the A-ring is apparently unchanged, according to the peak at 5.71, the new double bond can be located only from C_7 to C_8 , C_9 to C_{11} , or from C_{14} to C_{15} . The $\Delta^{9(11)}$ -group would, however, shift the C_{19} peak by more than 0.1 ppm beyond the $\delta = 1.23$ value observed for both I and II. The Δ^7 -group does not appreciably affect C_{18} , while a Δ^{14} -group has been found (11) to shift C_{18} by 0.27 ppm. Since the C_{18} resonance in II falls at 1.13 while in I it occurs at 0.82, the double bond has apparently been introduced at C_{14} and II must be $\Delta^{4,14}$ -andro-stadiene- 17β , 12α -diol-3-one.

Recent calculations by Karplus [6] and Conroy [2] have provided a firm basis for establishing molecular conformation from measurements of spin-spin coupling between protons on adjacent carbon atoms. An approximate reproduction of Conroy's results¹ is shown in Fig. 3. The dependence of the spin coupling, J_{HH} , on the dihedral angle between the two planes containing the carbon atoms and a proton is in agreement with numerous observations that in 6-membered ring systems the coupling between two adjacent axial protons is typically 8–11 cps while between an axial and equatorial, or two equatorial protons, the coupling diminishes to 2–3 cps.

¹ Permission to reproduce these results from the work of Dr. Harold Conroy, Yale University, New Haven, Conn., is gratefully acknowledged.

Fig. 4.—1,4-dinitroinositol tetra-acetate in CDCl_3 .



A striking example of the structural deductions which can be based upon this spin-coupling behavior is shown in Fig. 4. One of the possible isomers of 1,4-dinitroinositol tetra-acetate was investigated with the aim of determining its structure and conformation.¹ The strong, sharp peaks at the right-hand end of the spectrum indicate that the four acetate methyl groups are equivalent in pairs. The six ring-protons fall into three spin-spin multiplet patterns labelled H_x , H_y , and H_z and are thus also equivalent in pairs. A center or plane of symmetry is therefore suggested.

The groups H_y and H_z both display a large splitting characteristic of axial-axial spin-coupling and a smaller coupling which must be due to adjacent axial and equatorial protons. Therefore, H_y and H_z together represent four axial protons, and H_x represents two equatorial protons.

Assume for the moment that the nitro groups are not equatorial, as shown in Fig. 4, but axial. Then the protons on the same carbon atom would be the equatorial protons, H_x , and the remaining four protons would all be axial. This, however, requires the four acetate groups to be equatorial and *equivalent* which is contrary to observation.

Since the nitro groups have been shown to be equatorial, the protons on the same carbon atom are axial, either H_y or H_z . Each has one axial and one equatorial neighbor. The other axial protons also display a spin-coupling pattern indicative of one axial and one equatorial neighbor. These conditions and the equivalence in pairs of the three sets of protons can only be satisfied by the structure shown in Fig. 4 which possesses a center of symmetry.

While the analysis of chemical shifts and spin-spin coupling constants as exemplified by the preceding examples has been given much attention, quantitative measurements have often been neglected due to the more severe

¹ This work has been discussed in greater detail and submitted for publication elsewhere by S. W. Lichtenthaler and H. O. L. Fischer, Dept. of Biochemistry, University of California, Berkeley, Calif.

experimental difficulties. The problem deserves attention, however, since the strict theoretical dependence of the integrated intensities upon the number of nuclei being observed is a very great asset to NMR spectroscopy. It means that accurate quantitative analysis of mixtures can be carried out even if the spectra of the pure components have never been observed.

Due to its sharpness and suitability for integration, the absorption component of the NMR signals rather than the dispersion component is generally selected by adjustment of the r.f. phase-sensitive detector.

The peak height of the absorption signal is given by

$$V_{\max} = \frac{(\text{const}) M_o H_1 T_2}{1 + (\gamma H_1)^2 T_1 T_2}$$

(where M_o = net nuclear magnetic moment; H_1 = exciting radio frequency magnetic field; γ = magnetogyric ratio; T_1 = spin lattice relaxation time; and T_2 = spin-spin relaxation time), and since T_2 is inversely proportional to line width, peak height measurements are useful only if the lines are of identical width. On the other hand, the integral with respect to a magnetic field sweep is

$$\int v dH = \frac{(\text{const}) M_o H_1}{[1 + (\gamma H_1)^2 T_1 T_2]^{1/2}}$$

The net nuclear magnetic moment, M_o , depends both upon the number of nuclei/unit volume, N , and upon the excess population in the lower Zeeman level which, at room temperature, is inversely proportional to the absolute temperature, T . Furthermore, it is usually advantageous to integrate as a function of time; thus

$$\int v dt = \frac{(\text{const}) H_1 N}{T(dH/dt) [1 + (\gamma H_1)^2 T_1 T_2]^{1/2}}$$

The preceding equations are valid only for slow passage conditions which, in high resolution work, are rarely, if ever, met. They predict that, for power levels which give reasonable instrument performance and for typical values of T_1 and T_2 (a few seconds), the integrals may very well depend upon the relaxation times.

Measurement of the relaxation times is laborious and since the slow passage conditions are seldom met anyway, corrections are difficult to apply. It appears more profitable, therefore, to seek conditions which would lead to independence from the effects of relaxation. Examination of the term leading to "saturation", i.e. $(1 + (\gamma H_1)^2 T_1 T_2)^{-1/2}$, suggests that it is proportional to the decrease in the excess population of the lower Zeeman level over the time interval, T_1 , required for slow passage during which transitions

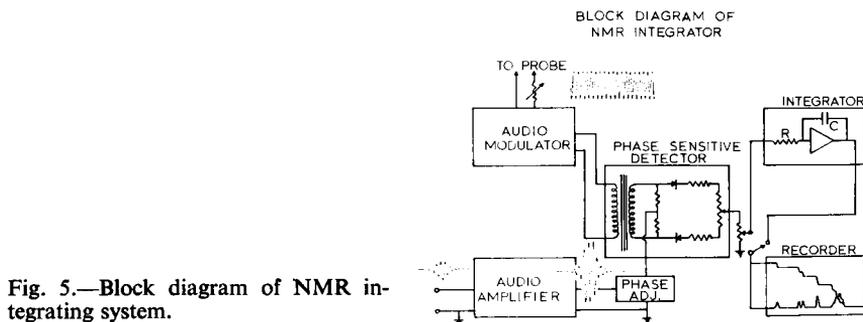


Fig. 5.—Block diagram of NMR integrating system.

occur with probability $(\gamma H_1)^2 T_2$. A simple modification of the equation which should make it more valid for rapid passage through resonance is to replace the time T_1 by the actual time of passage, $\Delta H/dH/dt$. Since the line width, ΔH , is $1/\gamma T_2$ we have then

$$\int v dt = \frac{(\text{const}) H_1 N}{T(dH/dt)} [1 - 1/2 \gamma H_1^2 / (dH/dt) + \dots]$$

for $\gamma H_1^2 / (dH/dt) \leq 1$.

This equation predicts that even at high r.f. power levels the integrals can be made to be *independent* of relaxation times for sufficiently fast sweep rates.

It would appear that a signal-to-noise problem might arise since, in order to keep $\gamma H_1^2 / dH/dt \leq 1$ an n -fold increase in sweep rate can only be accompanied by a \sqrt{n} -fold increase in H_1 , resulting in an integral smaller by \sqrt{n} . However, the faster sweep permits obtaining n -times as many observations per unit time, and statistical averaging of these n observations restores a factor \sqrt{n} in the reliability of the result. It is often convenient to use a digital voltmeter when a large number of observations are desired, rather than measuring from a strip chart recording.

Increasing the sweep rate to about 5 cps/sec for integrating, instead of the usual 1–2 cps/sec rate for recording the absorption spectrum, reduces the errors due to random drifts in the magnetic field correspondingly. This has permitted integration accuracy better than 1 per cent without any difficulty from this source of instability. Another source of error must be eliminated, however, before such accuracy can be achieved with weak signals. This is due to variation in the D.C. output of the r.f. detector due to instabilities in probe balance, transmitter power level changes, and stray coupling in leads. Fortunately, this problem is greatly reduced by the use of audio-frequency modulation of the magnetic field at a frequency of one

Relative NMR intensities in pure compounds.

Compound	No. of protons	$\frac{\text{Integral of group}}{\text{Total integral}} \times$		No. of protons
Ethyl benzene	10	C_6H_5 4.99	CH_2 2.00	CH_3 3.01
1,2-dichloropropane	6	$\text{CH}_2 + \text{CH}$ (unresolved) 2.99		CH_3 3.01
1,1,2-trichloroethane	3	CHCl_2 0.998	CH_2Cl 2.002	—
Acetophenone	8	C_6H_5 4.98	—	CH_3 3.02
Diethyl fumarate	12	$(\text{CH}=\text{C})_2$ 2.01	$(\text{CH}_2)_2$ 3.98	$(\text{CH}_3)_2$ 6.02
Ethyl acetate	8	CH_2 2.04	CH_3s (unresolved) 5.96	

Fig. 6.—Integrated intensities of distinguishable groups of protons expressed as a fraction of the total intensity.

or two thousand cps. Several authors [1, 3, 9] have discussed the effect of such modulation on the NMR signals. A set of audiofrequency side bands are obtained along with an audio-frequency signal at the usual field strength. By suitable adjustment of modulation amplitude and audiofrequency phase, the side bands can be essentially completely rejected by a phase-sensitive detector while the center-band signal, now free from low frequency drifts after traversing an RC coupling circuit, is converted back to D.C. and recorded or integrated. A block diagram is shown in Fig. 5.

The strong signals from pure compounds provide a convenient test for the linearity of the entire system of r.f. amplifiers, detector, audio-amplifier, phase detector and integrator. Fig. 6 shows the relative intensities of signals from various groups of protons in pure organic liquids. In nearly every case the ratios of the measured integrals agree with those calculated from the molecular formulae to better than 0.5 per cent.

A more stringent test is the analysis of small samples (25 mg) for total hydrogen. In this case the unknown samples are compared with a standard, using tubes with identical cross sectional areas. Fig. 7 shows the results of comparing three complex natural products against naphthalene as a standard. The naphthalene was run in triplicate to establish the precision of the measurement. The percentage of hydrogen by weight agrees in nearly all cases with the calculated value to within the standard deviation of the measurement, which is about 0.5 per cent of the percentage present. This accuracy, and

Total hydrogen analyses by NMR for complex organic molecules.

Sample	Wt. (mg)	% H by wt.	% H (NMR) ^a
C ₁₀ H ₈ (A)	25.1	6.29	6.31 ± 0.03 ^b
C ₁₀ H ₈ (B)	25.3	6.29	6.28 ± 0.01
C ₁₀ H ₈ (C)	25.3	6.29	6.28 ± 0.02
C ₂₇ H ₄₄ O ₃	25.1	10.65	10.58 ± 0.05
C ₁₅ H ₁₈ O ₆	25.2	6.26	6.27 ± 0.04
C ₁₆ H ₂₀ O ₆	25.1	6.80	6.81 ± 0.03

^a Based on average integral of naphthalene solutions.

^b Standard deviation.

Fig. 7.—Total integrals compared with weight percent hydrogen for several compounds in identical sample cells.

the non-destructive nature of the analysis, may make the NMR method more attractive than the usual combustion method when a limited amount of sample is available.

As an example of the analytical possibilities of the technique, a mixture of steroids with isomeric double bonds was studied. Fig. 8 shows the separate signals obtained from the olefinic proton on the 5-membered ring and the terminal methylene protons. Electronic integration of this portion of the spectrum established the relative amounts of the isomers to the nearest percent, reproducible in separate sample preparations.

Finally, the use of intensity measurements to arrive at and confirm an assignment of spectral lines is illustrated in Fig. 9. Each carbon atom of the cyclohexane ring has one proton. A large number of configurational and conformational isomers are possible. However, the presence of a typical small-large-large-small pattern characteristic of two protons with a small chemical shift and a comparable spin-coupling constant [7] is confirmed by

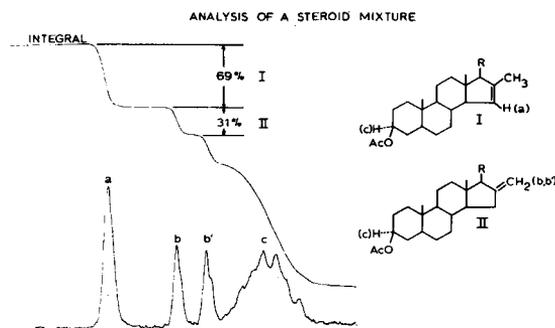


Fig. 8.—Analysis of a mixture of isomers.

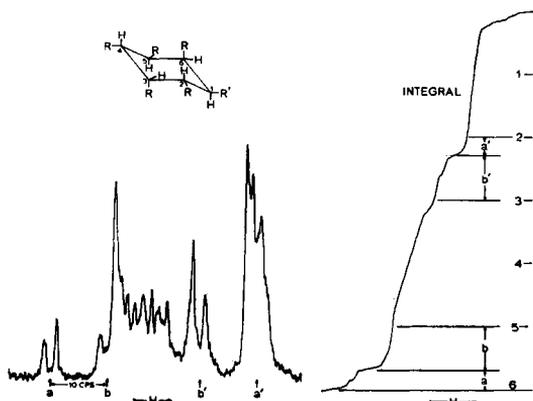


Fig. 9.—Confirmation of spectral assignments using quantitative information.

the integral curve. Fig. 9 shows that the right hand peak in the spectrum is larger than the value corresponding to $\frac{2}{3}$ of the total intensity by an amount just equal to the area of the doublet, a , on the left. Therefore, it conceals a similar doublet, a' . The large spin-coupling, 10 cps, requires two adjacent axial protons, while the doubling of each line of the pattern with a much smaller spacing shows that each axial proton has an equatorial neighbor. This sequence of protons occurs only for the particular isomer and conformation shown in Fig. 9; consequently, all other structures which were under consideration could be eliminated.

Accurate area measurements of NMR signals have proven so useful in the applications described in this paper and in a host of other problems that this capability can be regarded as almost essential. The analytical usefulness of the instrument is so greatly extended that there can be little doubt that widespread application to problems in research and routine analysis will result.

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