High Resolution NMR Spectroscopy and Some Examples of Its Use[1,2]

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Abstract

High resolution nuclear magnetic resonance (NMR) spectroscopy has gained wide acceptance in the study of fatty acids and other lipids. Some of the techniques are described and examples of identification and structure determination of fatty compounds are given. Nuclear magnetic resonance spectroscopy is of particular value in the examination of acids with various types of unsaturation, such as mono- and polyenoic, acetylenic, allenic and conjugated. Acids with a cyclopropene or cyclopropane ring have been detected and studied by NMR. Positional isomers of certain substituted acids can be identified by the NMR spectrum alone. Other lipids such as glyceryl ethers, phosphatidylcholines, sphingomyelin and plasmalogens have been investigated with the aid of NMR. Reference is made to studies of other compounds, including steroids, rotenones, gossypol, glycosides, sugars, amines, amino acids and polymers.

Introduction

NUCLEAR MAGNETIC RESONANCE spectroscopy (NMR) has become of great importance to organic chemists. It is estimated that at least one third of the current publications in this branch of chemistry include results from NMR spectra. Considerable use has been made of NMR in the study of fats and fatty acids (1) and it is valuable for practically all kinds of natural and synthetic products. Nuclear magnetic resonance has certain advantages over infrared spectroscopy but can best be used in conjunction with infrared and other forms of spectroscopy to provide the greatest amount of information. It is employed chiefly in the identification and determination of structure of organic compounds but there are many other applications in biochemistry and in medicinal, analytical and industrial chemistry.

Some good sources of information on NMR for lipid chemists are the following: Basic theory and practice (2); Practical discussion (3); Comprehensive text (4); Application to fatty acids and esters (1); Application to organic compounds and drugs (5); Application to sugars (6); Analytical uses (7); Recent developments (8); Index to NMR spectra in the literature (9); Collection of NMR spectra of organic compounds (10).

Materials and Methods

Equipment and Techniques

The capability of NMR has advanced rapidly in the last few years. Most laboratories have the NMR spectrometer operating at a frequency of 60 MHz but 100 MHz equipment is coming into general use. (The units hertz and megahertz replace the former units, cycles per second and megacycles per second.) Better resolution is obtained at 100 MHz and spectra can be produced with smaller samples. For advanced research, 220 MHz equipment is available.

Various techniques have been developed that extend the value of NMR spectra. Among these are the addition of deuterium oxide to suppress the signals of hydroxyl and amino protons, determination of spectra in different solvents to obtain information from solvent effects, application of decoupling (double resonance) to simplify complex signals and to identify related protons, repeated scanning and averaging by computer to obtain definitive spectra with very small samples (11), and prediction of chemical shifts and coupling constants on a theoretical basis. It is reported that resolution is improved considerably if dissolved oxygen is removed from the sample solution (12). The application of these techniques is described in some of the examples which follow.

Examination of Spectra

Tetramethylsilane (TMS) has become the standard reference material for hydrogen (proton) spectra. Unfortunately, as with infrared, two scales for the chemical shift have evolved. In the τ scale, TMS is assigned a value of 10 and the values increase from left to right on the chart along with increasing field strength. In the δ scale, TMS has a value of zero. For conversion, $\delta = 10 - \tau$.

Designation of various types of NMR spectra as AB, AX, ABX, etc. is customary and the lipid chemist can utilize this system to advantage. Interacting protons that are nearly alike in chemical shift are labelled A,B, etc. and M,N, or X,Y, is their chemical shift is considerably different from that of the A,B group. Two equivalent protons are designated as A_2 or X_2, etc. Thus the ethyl group $-CH_2CH_3$ is an A_2B_3 system and the vinyl group, as in $C_6H_5CH = CH_2$, is an ABX system. It is possible, in simple spectra, to recognize these and similar systems by inspection of the signal pattern. This provides a basis for the analysis of the spectrum and hence the structure of the compound (3).

Monoenoic Fatty Acids

Nuclear magnetic resonance spectra of a number of unsaturated acids are given in reference (1). Additional spectra were reported by Purcell et al. in 1966 (13). The complete series of C-18 monoenoic acids was examined by Gunstone and Ismail in 1967 (14). It was shown that identification by NMR spectra is possible for the four isomers with unsaturation closest to the carboxyl group and the four with unsaturation closest to the methyl group. The chemical shifts are given for all 16 isomeric acids.

Such spectra were used in several instances to determine the position of a double bond in acids isolated from natural sources, e.g., 3-hexadecenoic acid from *Helenium* seed oil (15), 4-dodecenoic acid from *Lindera* seed oil (16), and 5,11,14-eicosatrienoic acid in *Caltha* seed oil (17). The structure of synthetic acids was confirmed by their spectra, e.g., *trans*-2-*cis*-8,11,14-eicosatetraenoic acid (18).

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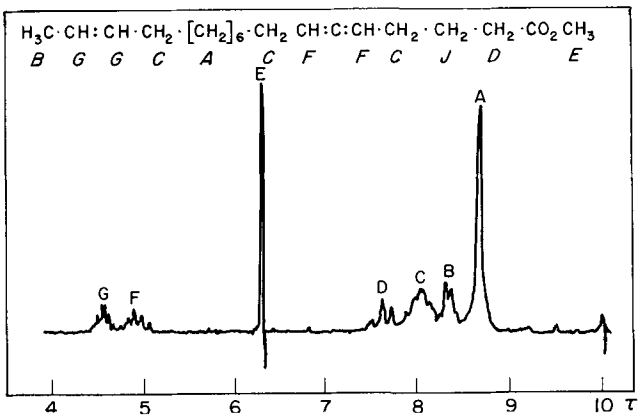


FIG. 1. Spectrum of methyl octadec-5,6,16-trienoate showing the allenic signal. Mikolajczak et al. (23), by permission.

Although the terminal methyl group gives a band near 9.1 τ in nearly all cases, it appears at 8.38 τ in 16-octadecenoic acid because it is deshielded by the adjacent olefine group (14). Thus one must not assume that absence of a band near 9.1 τ means that there is no terminal methyl (Fig. 1).

Dienoic Fatty Acids

Nuclear magnetic resonance spectra of a number of dienoic acids have been reported (19,20). The complete series of C-18 methylene-interrupted dienoic acids was examined by Christie and Holman (21). Of the 13 isomers, 5 that have the unsaturation near the middle of the chain (6,9 to 10,13) cannot be distinguished by NMR. The remaining 8 isomers can be identified individually. This is an ideal method for characterizing the acids quickly and nondestructively.

The allene group, CH = C = CH, provides a distinctive NMR band at 4.9–5.0 τ. It was observed in 5,6-octadecadienoic acid, isolated by Bagby et al. (22) from *Leonotis* seed oil, and in the unusual compound 5,6-*trans*-16-octadecatrienoic acid, discovered by Mikolajczak in *Lamium* seed oil (23) (Fig. 1).

Acetylenic Fatty Acids

Although there are no hydrogens at the triple bond (–C ≡ C–), the hydrogens on the adjacent carbons produce NMR peaks that help to locate the position of the triple bond in the chain. Spectra of some acetylenic fatty acids are described by Gunstone and Ismail (14) and by Purcell and Susi (19). Most of the C-18 mono-ynoic acids are included in these two reports. As with the enoic acids, identification by NMR is possible if the unsaturation is within four places from either end of the chain. Several C-12 acids are described in reference (14). It is pointed out that if the acid has 11 carbons or less, all of its mono-ynoic isomers can probably be identified.

An acetylenic fatty acid recently found in a seed oil has a terminal acetylene group (24). The terminal proton gave the characteristic peak at 8.2 τ and confirmed the infrared band at 3,320 cm^{-1} for the same group. This unique acid had also a cyclopropene group as shown by the sharp singlet at 9.2 τ (Fig. 2). Autoxidation of soybean oil was found to produce an acetylenic compound as one of the products. It was identified as dec-1-yne and this was confirmed by comparing its NMR spectrum with that of a synthetic sample (25). The terminal acetylenic proton signal was a triplet at 8.2 τ and the alpha proton was at

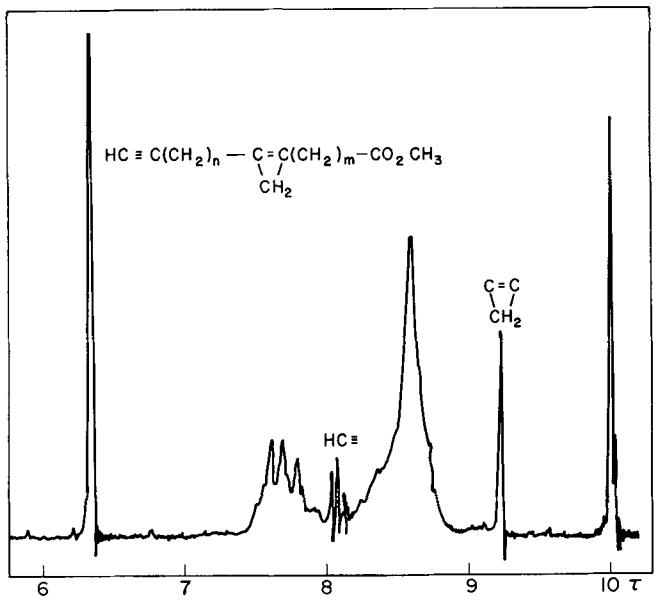
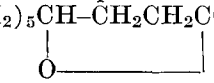


FIG. 2. Spectrum of a long-chain ester with terminal acetylene and cyclopropene groups (24).

7.9 τ. Another product of autoxidation was detected and identified by Fioriti et al. (26) using NMR and other techniques. This product was γ-decalactone $CH_3(CH_2)_5CH–CH_2CH_2CO$.



The CH signal was at 5.7 τ and the α- and β-CH_2 signals at 7.7 τ. Both are multiplets, although the CH bond is essentially a triplet.

Branched fatty acids and their NMR spectra are described by Sen Gupta and Peters (27) and examples are given of acids with three and four methyl groups. Branched acids have also been studied by other workers. Their spectra show marked differences in the CH_3 and CH regions.

Conjugated Fatty Acids

The protons of conjugated double bonds appear at low field because of deshielding by the multiple unsaturation. In a conjugated diene there is a complex multiplet centered at about 4.2 τ. This widely-split signal has been analyzed by Tallent et al. as it occurs in 13-hydroxy-*cis*-9,*trans*-11-octadecadienoic acid (coriolic acid) (28). The spectrum was taken at 100 MHz and double resonance was employed to aid in the analysis. It was possible to determine from the coupling constants which bond was *cis* and which was *trans*.

Cis-Trans Isomerism

Cis and *trans* configurations at a double bond give rise to different effects in the NMR spectra. In short chain acids the *cis* and *trans* forms are easily distinguished by the coupling constant for the olefinic protons, which is in the range 7–12 Hz for *cis* and 13–18 Hz for *trans* (1). It is not as simple in long chain acids where inspection of the spectra may give misleading results. The coupling of the double bond protons in oleic and elaidic acid has been elucidated by Schaumburg and Bernstein (29).

It has been shown that the *threo* and *erythro* forms of vicinal dihydroxy long chain acids have a small difference in the chemical shift of the respective CH protons. This may provide a means of differentiating between *threo* and *erythro* forms. The

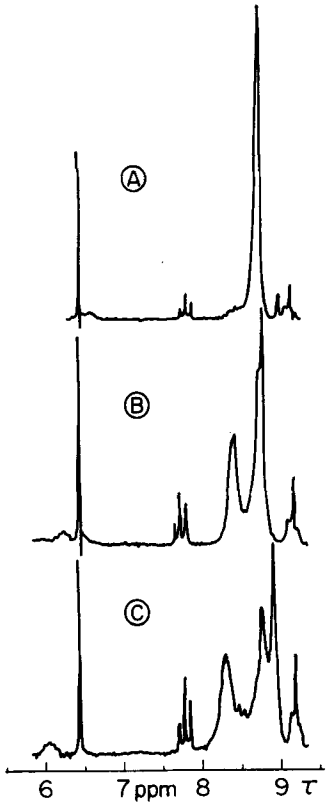


FIG. 3. Solvent effects. Methyl 12-hydroxystearate: A, in carbon tetrachloride, B, in pyridine, C, in quinoline. Tulloch (32), by permission.

O-isopropylidene derivatives of the same acids exhibit a larger difference in chemical shift for the CH protons (30).

In keto-enol tautomers, the CH_2 of the keto form, CH_2C, gives a peak at about 5.5 τ but the CH of

$$\begin{matrix} \| \\ O \end{matrix}$$

the enol form, $\begin{matrix} CH \\ | \\ OH \end{matrix}$, appears at 3–4.5 τ. Thus the areas of these peaks can be used to calculate the proportions of keto and enol forms in a compound at equilibrium. The enol form usually shows the OH peak as a broad line at low field. A study of the effect of solvents and of substituent groups on keto-enol equilibrium was made by Allen and Dwek by means of NMR (31).

Oxygenated Acids

Hydroxy fatty acids have been studied by Tulloch, who prepared all of the monohydroxystearic acids and examined their NMR spectra (32). He concluded that every one of the 17 isomers could be identified by determining the NMR spectrum in quinoline. Pyridine and CCl_4 were less satisfactory as solvents (Fig. 3). Dimethylsulfoxide has been used as a solvent for hydroxy acids.

Spectra of hydroxy compounds offer some difficulties in interpretation. It is helpful to add deuterium oxide to extinguish the signal of the hydroxyl protons. The technique of double resonance or spin decoupling is also very useful for these compounds. In the absence of D_2O, the protons of OH and COOH combine to give a single peak.

The effect of changing the solvent is important in determining structure. An example is an unusual

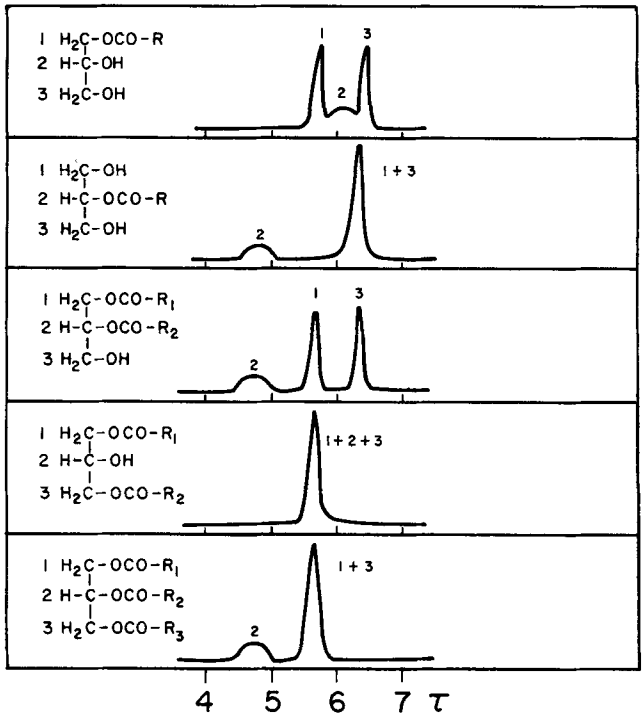


FIG. 4. Glyceryl proton peaks of mono-, di- and triglycerides. Serdarevich and Carroll (36), by permission.

acetal ester obtained from a seed oil (33). The ordinary spectrum in chloroform showed a signal partly hidden by the acetal and ester peaks and not readily identifiable. When recorded in benzene solution, this signal was shifted downfield and was seen as a sharp doublet. Its coupling constant provided evidence for the position of the protons in the molecule (33).

Nuclear magnetic resonance spectra of long chain epoxides were determined at 100 MHz by Aplin and Coles (34). It was stated in their preliminary report that *cis* and *trans* epoxides are readily distinguished by the chemical shift of the epoxide protons.

Glycerides and Glyceryl Ethers

Nuclear magnetic resonance spectra of glycerides have been studied in several laboratories (1,3,5,36). It was readily apparent that the isomeric mono- and diglycerides could be distinguished by NMR (36) (Fig. 4).

Good spectra of 1- and 2-monoglyceryl ethers were obtained by Serdarevich and Carroll (37). The peaks for the glyceryl protons are close together at 6.0 to 6.5 τ but it is possible to distinguish between the isomers. The OCH_2 group of the ether radical gives a peak very close to that of the CH_2 of the glyceryl radical. Wood and Snyder examined the glyceryl ethers from tumor tissues and obtained information on their composition by NMR (38). The compounds were identified as glyceryl ether diesters and contained no plasmalogens. Kates et al. (39) split the ether linkage in a natural glycerol diether by means of HI and obtained the alkyl iodide. It had the triplet at 6.8 τ representing $-CH_2I$ and a very strong doublet at 9.1–9.2 τ indicating several CH_3 groups. These data along with peak area measurements and comparison with a reference compound identified the alkyl chain as 3,7,11,15-tetramethylhexadecyl (dihydrophytyl).

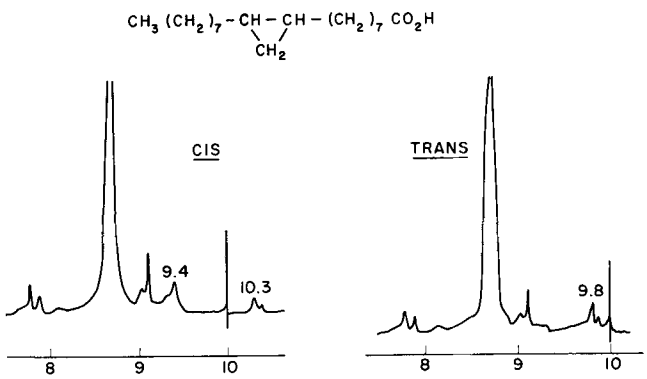


FIG. 5. Spectra of *cis*- and *trans*-9,10-methylene-octadecanoic acid. Blanchet et al. (42), by permission.

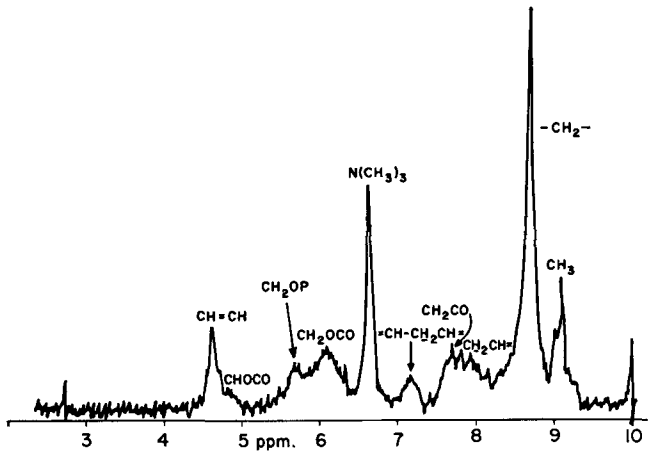
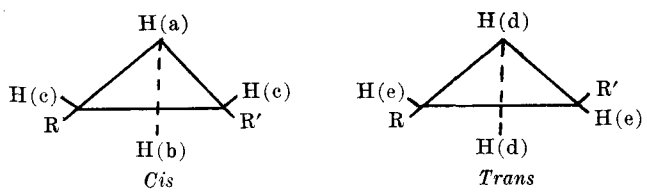


FIG. 6. Spectrum of phosphatidylcholine from egg yolk. Chapman and Morrison (46), by permission.

Protons of the Cyclopropane Ring

Fatty acids containing a cyclopropane ring near the middle of the chain occur in seed oils and as products of bacterial metabolism. It was noted in 1959 with 40 MHz equipment that dihydrosterculic acid had NMR peaks centered at 9.4 and 10.3 τ (40). This was the *cis* isomer (*cis*-9,10-methylene-octadecanoic acid). The *trans* isomer, examined later, has a single complex band about 9.8 τ (41) (Fig. 5). The band at 10.3 τ is noteworthy because it represents one of the few situations in which a proton has a chemical shift higher than 10 τ.

Assignment of these bands to the four protons of the ring was not accomplished until 1967. Wood and Reiser (41), Blanchet et al. (42), and Minnikin (43) observed that the ratio of the bands at 9.4 and 10.3 τ for the *cis* isomer was 3:1. Thus the 9.4 band must contain the two equivalent protons, H(c), and also either H(a) or H(b).



In the *trans* isomer, the shielding is more nearly the same on all four protons and all appear in the complex band centered at 9.8 τ (9.4–10.1).

Examining the *cis* isomer, Minnikin argued that H(a) is heavily shielded by H(b) and the two H(c) protons and so appears at very high field, 10.3 τ; also that H(b) is deshielded by the two alkyl groups (R and R′) and so is shifted to lower field. Longone and Miller (44) rejected this interpretation and concluded that the peak at 10.3 τ is due to the *cis* proton H(b), because of the strong shielding effect of the long alkyl chains. Although this appears to be the correct assignment, it is possible that other factors are involved.

Nuclear magnetic resonance is of special value in studying cyclopropanoid esters because the main infrared band of the cyclopropane ring at 1,020 cm^{-1} may be partly obscured by the ester band at 1,015 cm^{-1}. (This ester band is not mentioned in the usual IR tables.) The data on cyclopropanoid acids enabled Conacher and Gunstone (45) to identify an unusual product from the rearrangment of an epoxy ester. Methyl vernolate [I] rearranged to give an ester with a keto group and a cyclopropane ring [II].

$$CH_3(CH_2)_4\overset{\overset{O}{\diagup\diagdown}}{CH}CHCH_2CH=CH(CH_2)_7CO_2Me \quad [I]$$

$$CH_3(CH_2)_4\overset{\overset{O}{\|}}{C}CH_2CH\cdot\overset{\diagup\diagdown}{CH}(CH_2)_7CO_2Me \quad [II]$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\quad O \qquad CH_2$$

The NMR spectrum showed that there were no double bonds in the product. Peaks in the region 9.5–10.3 τ were strong evidence for a cyclopropane ring. It was concluded that the product was a mixture of *cis* and *trans* isomers of the cyclopropanoid ester II.

Phospholipids

A number of phospholipids were studied in detail by Chapman and Morrison (46) and their spectra were recorded. Most of the phosphatidylcholines are sufficiently soluble in $CHCl_3$ to give useful spectra. The trimethylamino protons give a sharp line at 6.6 τ (Fig. 6). The same authors show the spectrum of sphingomyelin. The compound is insoluble in pure $CDCl_3$ but can be dissolved by adding 10% of deuteriomethanol. Reference has already been made to a phosphoglyceryl ether which was isolated by Kates et al. (39) from a bacterial phospholipid and identified by NMR.

The long-chain aldehydes obtained on hydrolysis of plasmalogens from bacteria were examined by Goldfine (47), who made good use of NMR to aid in the identification. It was proved in this way that some of the aldehydes (isolated as dimethylacetals) had a *cis*-cyclopropane group. They were converted to the corresponding acids (chain length C-17 and C-19) and identified by comparison with synthetic samples of the cyclopropane acids. In this work, 10 mg of the aldehyde acetal gave an excellent NMR spectrum, with 60 MHz equipment. The peaks for the *cis* cyclopropane ring were 9.4 and 10.37 τ (Fig. 7).

Slotboom et al. (48) synthesized a plasmalogen and checked the structure of the unsaturated ether group by comparison of the NMR spectrum with that of the glyceryl ether obtained from a natural plasmalogen. The spectra showed that the two compounds had the same structure but the natural compound was *cis* and the synthetic one was *trans*. The acetylated derivative, 1-alkenyloxy-2,3-diacetyl glycerol, was prepared for this purpose. The *cis*-olefinic proton peak was a doublet at 4.1 τ with J 6.5 Hz.

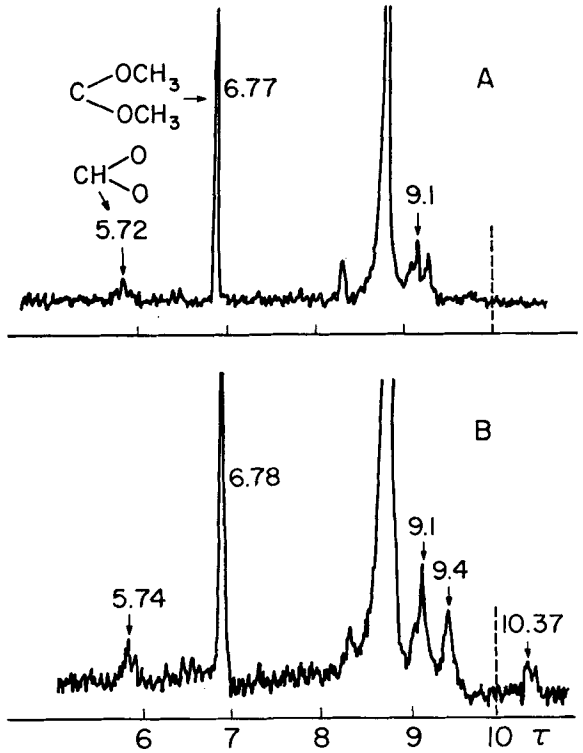


FIG. 7. Spectra of A, octadecanal dimethylacetal; B, aldehyde dimethylacetal from a bacterial plasmalogen, containing a cyclopropane group. Goldfine (47), by permission (redrawn).

The signal of the *trans*-form was at 3.7 τ, J 12.5 Hz, characteristic of a *trans* vinyl ether bond.

Steroids

Steroids have been studied extensively and their spectra are the subject of a book by Bhacca and Williams (49). Recent work by Scallen and Krueger constitutes an excellent example of the joint use of infrared and NMR (50). Samples as small as 5 mg are used. Detection of the important double bond at C-24 in steroids is feasible. In a saturated cholestanol the methyl groups at C-26 and C-27 give peaks at 9.08 and 9.18 τ but when there is a double bond at C-24, these signals are shifted downfield to 8.33 and 8.40 τ.

The spectra of 265 different steroids were recorded by Zurcher and the chemical shifts of all of these are given (51).

Amines, Amino Acids and Proteins

Nuclear magnetic resonance has found wide application in determining the structure of amines and amino acids. Spectra of amines of pharmaceutical interest have been reviewed recently by Eisdorfer et al. (52). Alkyl groups attached to nitrogen are very often identified by NMR. Solvent effects are useful in this connection, particularly solvents of different degrees of acidity. The N-methyl group of secondary amines is readily distinguished from the N-methyl groups of tertiary amines (53). Acetone or dimethyl sulfoxide can be used as solvent.

Amino acids have been studied extensively and many NMR spectra have been recorded (54). The acids can be dissolved in alkaline D_2O or in a strong acid, e.g., trifluoroacetic acid. In the spectrum of alanine, determined in D_2O, the typical bands for CH_3CH are seen, also a peak for HDO at 5.3 τ. The protons of NH_2 and COOH are replaced by deuterium (54). In addition to the signals from the groups attached to nitrogen, other parts of the molecule give useful peaks in the NMR spectra. Thus, an unusual amino acid studied by Fowden (55) contained a cyclopropane ring and terminal methylene group. It was identified by NMR.

Proteins and related materials present a difficult problem because of the large molecule size and low solubility. Progress up to 1962 in this field has been reviewed (56). Nuclear magnetic resonance spectra of ribonuclease, lysozyme and hemoglobin have been obtained. Work is in progress at the present time with 220 MHz equipment and computer averaging. Ribonucleic acid, mol wt 28,000, has given an NMR spectrum under these conditions (57).

Sugars

Mabry et al. examined sugars and glycosides by NMR after converting them to the trimethylsilyl ethers (58). In this way the compounds become soluble in CCl_4 and the troublesome OH peaks are eliminated. The whole procedure requires less than an hour and is especially useful for flavanoids. The trimethylsilyl protons give a signal very close to 10 τ, which does not interfere with the main part of the spectrum. Methoxy, acetyl or isopropylidene derivatives of sugars may also be used. Applications of NMR in carbohydrate chemistry have been reviewed by Hall (6).

Spectra of Other Classes of Compounds

Nuclear magnetic resonance gives information on the structure and configuration of natural insecticides such as rotenones. Crombie and Lawn (59) studied 20 of these compounds and recorded the τ values. The NMR spectrum of gossypol has been determined recently (60).

Spectra of polymeric materials can be obtained. These substances give relatively simple spectra which show the structure of the polymer. The sample must be a mobile liquid or solution. If necessary, it can be examined at elevated temperatures, e.g., up to 180 C.

Ethylene oxide monomer and polymer (in CCl_4) both have a one-line spectrum since all of the protons are equivalent in each of the two forms. The signal in the polymer is at lower field than in the monomer. Spectra of methyl methacrylate polymers, consisting of different stereochemical forms, are radically different (61). The isotactic polymer has a regular chain of dextro, levo or racemic forms but the syndiotactic polymer has alternating dldl or other mixed chains, resulting in distinctive spectra.

Quantitative Applications

A method for determining molecular weight by NMR was worked out by Barcza (62). A known weight of a reference compound is added to the unknown sample. The area of a peak in the reference spectrum is compared with the area of a recognizable peak in the unknown spectrum and the molecular weight is calculated from these two areas. The method appears to be most suitable for substances of m.w. of 200 or less. Certain reference compounds are recommended, which can be removed from the sample after the determination.

A review of analytical applications of NMR, including 700 references, was compiled by Lustig and Moniz (7). Examples of quantitative analysis by NMR have appeared in pharmaceutical chemistry. Spectra of aspirin, phenacetin and caffeine were ob-

tained by Hollis (63). From these a method was devised for the rapid analysis of mixtures of the three drugs, as used in headache tablets. The analysis requires only 20 min. It is merely necessary to powder the sample, shake with $CDCl_3$, warm to dissolve the three components, and determine the spectrum. A solution of caffeine (50 mg/ml) serves as a standard for calibration. Average deviation was 1% to 3% for the active ingredients.

Many drugs can be determined quantitatively by dissolving or extracting them with a suitable solvent and obtaining the NMR spectra with an internal standard (64). Meprobamate has been determined in this way, using acetone as solvent and malonic acid as standard. Other closely related drugs are easily distinguished by their spectra. The procedure is said to be rapid, accurate and specific.

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