The Enzymes of Lipid Metabolism

Proceedings of the
Sixth International Conference
on the Biochemistry of Lipids
Marseille

Edited by:

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AVANT-PROPOS

L'ENZYMOLOGIE représente à l'heure actuelle l'une des branches les plus dynamiques de la Biochimie. Elle se subdivise en deux parties: (*a*) Ce que l'on peut appeler l'Enzymologie descriptive qui cherche à recenser d'une façon aussi complète que possible les nombreuses activités catalytiques dont disposent les êtres vivants et à écrire les réactions catalysées; (*b*) l'Enzymologie moléculaire qui a pour objet de purifier les enzymes, de déterminer leur structure et d'établir les relations existant entre la structure et l'activité. L'Enzymologie dans son ensemble touche donc à la physico-chimie classique pour la cinétique, à la Chimie organique et minérale pour les coenzymes, à la Chimie des protéines pour les purifications et les études des structures, à la biosynthèse des protéines pour l'élaboration des enzymes et enfin à la Chimie Théorique pour l'explication de l'effet catalytique.

Le métabolisme des lipides comporte des réactions d'hydrolyse, d'oxydation et de synthèse. Toutes ces réactions sont contrôlées par des enzymes spécifiques dont l'étude est en pleine expansion. Il était donc bon que la 6° Conférence Internationale sur la Biochimie des Lipides (International Conference on the Biochemistry of Lipids) organisée à Marseille en Juillet 1960 prit ce thème pour objet initial.

Le présent volume édité par Pergamon Press réunit la plupart des communications faites au cours de la réunion.

P. DESNUELLE

Septembre 1961

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NUCLEAR MAGNETIC RESONANCE AS A TOOL IN FAT RESEARCH

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As an introduction to the application of nuclear magnetic resonance (N.M.R.) spectroscopy in fat research, a short account is given of some fundamental features of this relatively new branch of spectroscopy.

N.M.R. is concerned with the transition between Zeeman levels, which atomic magnetic moments can undergo in a magnetic field. Some nuclei (among which ^1H is the most important and to which we shall confine ourselves) behave as tiny magnets, capable of taking up several orientations in a magnetic field. This is shown in Fig. 1. Each of these orientations cor-

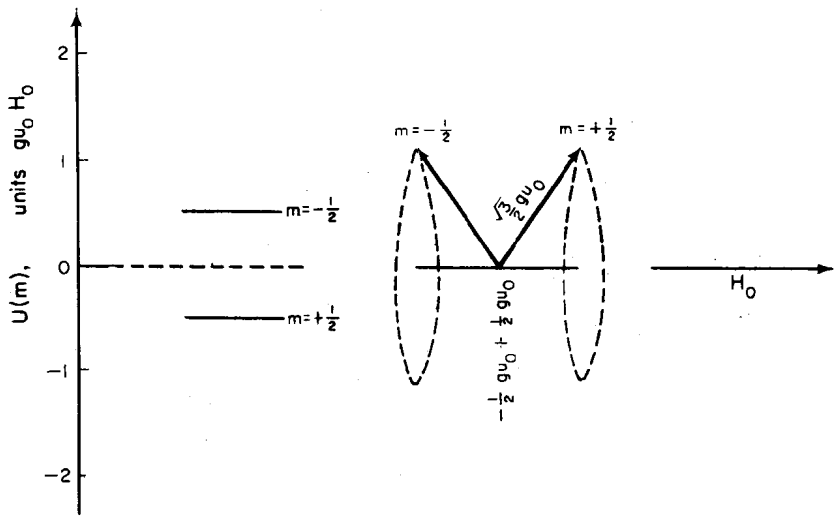


FIG. 1. Proton-spin orientations in a magnetic field.

responds, according to quantum mechanics, with a definite potential energy. When a system of such nuclei is subjected to radiation of a suitable frequency ν ($\nu = \Delta E/h$, in which ΔE is the difference in energy between two adjacent levels and h is Planck's constant), transitions between these energy levels are induced. Macroscopically, this leads to absorption of radiation at one particular frequency in a given magnetic field.

Experimentally, it has been found that absorption of energy occurs over

a range of frequencies, leading to a broad line in the case of solids or to a series of more or less sharp lines in the case of liquids. Apparently, not all nuclei are subjected to the same magnetic field. The atomic nucleus is thus employed to communicate a message about its (magnetic) surroundings and this information forms the subject of the nuclear magnetic resonance spectroscopy.

It is useful to differentiate between a solid and a liquid (or solution) since essentially different information is obtained in the two cases. A nucleus in a solid lattice is subjected to the static and dynamic magnetic fields of its near neighbours; this leads to a spread in the fields, experienced by the various nuclei in the sample. The line shapes in this case are determined by intermolecular interactions and by motions of certain parts of the molecule. Hence, from the experimental curve, it is often possible to deduce useful information about the lattice. Applications to structural analysis of solid triglycerides will be given later.

In organic chemistry, however, N.M.R. is mostly applied to liquid samples. In this case, intermolecular influences are virtually absent, and, in consequence, the different magnetic fields within a molecule become apparent.

The hydrogen atoms in a molecule are subjected to a field of:

1. The (large) external field, causing the Zeeman levels to be split and enabling resonance to occur.
2. A field which is induced by the external field in the electron clouds in the molecule and which counteracts the external field. Since this induced field is different at different sites in the molecule, the hydrogen atoms in functional groups have their resonance frequencies shifted by small amounts. This effect, called the chemical shift, enables the various hydrogen-containing groups to be identified on the basis of empirical data.
3. A field, due to neighbouring nuclei, transmitted through the bonding

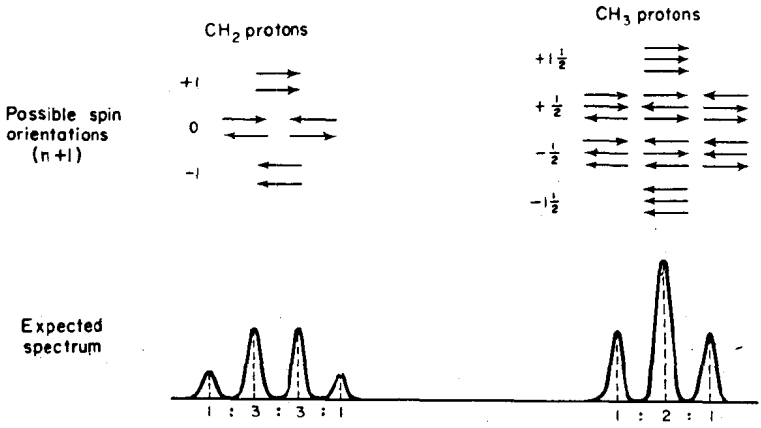


FIG. 2. Possible orientations of nuclear spins of ethyl group protons and expected spin–spin splitting pattern.

electrons. This effects an indirect spin–spin interaction, which gives information about the relative positions of various groupings.

Summarizing, it can be said that N.M.R. offers a group analysis; several physico-chemical effects become apparent in shifts of group frequencies. Moreover, the couplings between adjacent groups give rise to a hyperfine structure, from which additional structural details can be derived.

The combined effect of chemical shift and spin–spin coupling to be expected can be seen in Fig. 2 (spectrum of ethyl group).

The remainder of this paper is devoted to applications of N.M.R. spectroscopy for the investigations of lipids. The applications have been chosen mainly to elucidate the type of work that can be done with a high-resolution N.M.R. spectrometer.

IDENTIFICATION AND DETERMINATION OF UNKNOWN STRUCTURES

The N.M.R. spectrum of an unknown compound can be interpreted by a group analysis or merely given a finger-print to the identification. For this purpose it is essential to have a collection of spectra of pure compounds available. Relatively little has been published about the spectra of fatty acids and glycerides[1] but it is sometimes sufficient to have spectra of related compounds. The procedure for identification is fully analogous to that followed in infra-red spectroscopy for the same purpose: e.g. spectra of model substances are recorded and if necessary, modifications are effected in the unknown substance. Spectra of known fatty acids (see Figs. 3, 4) are useful

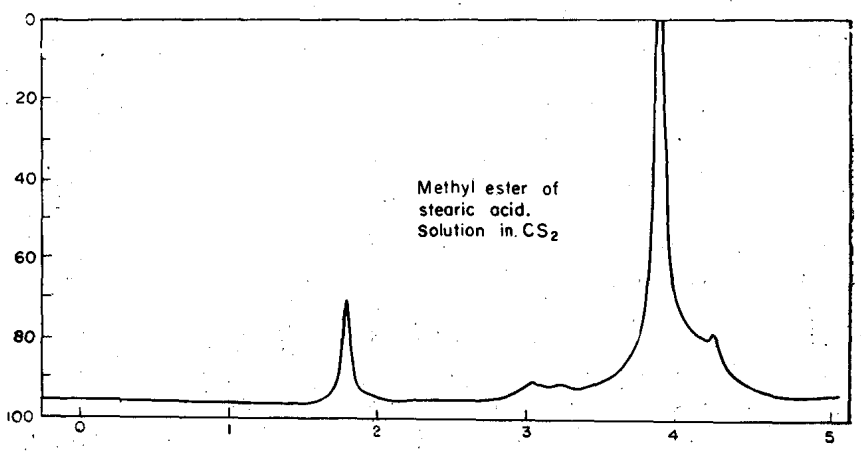
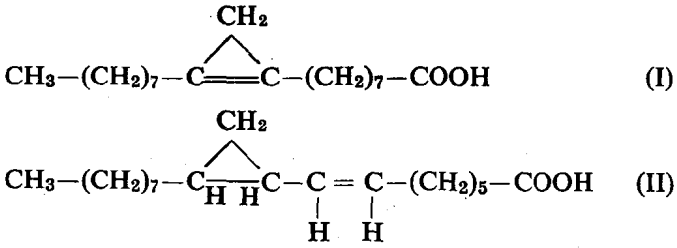


FIG. 3. N.M.R. spectrum of methyl ester of stearic acid, dissolved in CS_2.

for determining the structures of unusual fatty acids. An example of this is the structure of sterculic acid, a component fatty acid from the oil of the

seeds of *Sterculia foetida*, for which a chemical structure determination yielded the following possible structures:



(I)

(II)

From the N.M.R. spectrum, the first structure could be unambiguously confirmed. If structure II were the correct one, the N.M.R. spectrum should exhibit an absorption at the position characteristic of double bond protons (position O in Fig. 4). The absence of such a peak indicates that structure I must be preferred.

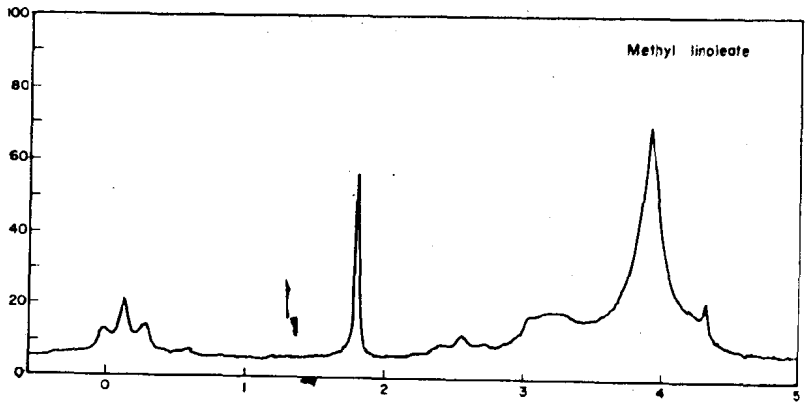


FIG. 4. N.M.R. spectrum of methyl linoleate.

It is not always possible, definitely to establish the structure of an unknown compound, but it is usually easy to ascertain whether or not certain functional groups (epoxy-, keto-, hydroxyl-groups, etc.) are present. It is often a matter of convenience whether N.M.R. or I.R. is used for this purpose.

For the identification of fatty acids, a graph has been recorded (Fig. 5) showing the position of the CH_3 or CH_2 resonance, relative to an external H_2O reference. Part of this shift is due to bulk susceptibility differences and not to a true chemical shift. If the unknown acid (or methylester) is a pure compound, this may be a rapid method of measuring the chain length of normal saturated fatty acids.

INTERACTIONS IN THE LIQUID PHASE

To explain the intramolecular effects (chemical shift, spin–spin coupling) it has been assumed that no intermolecular interaction occurs in liquids.

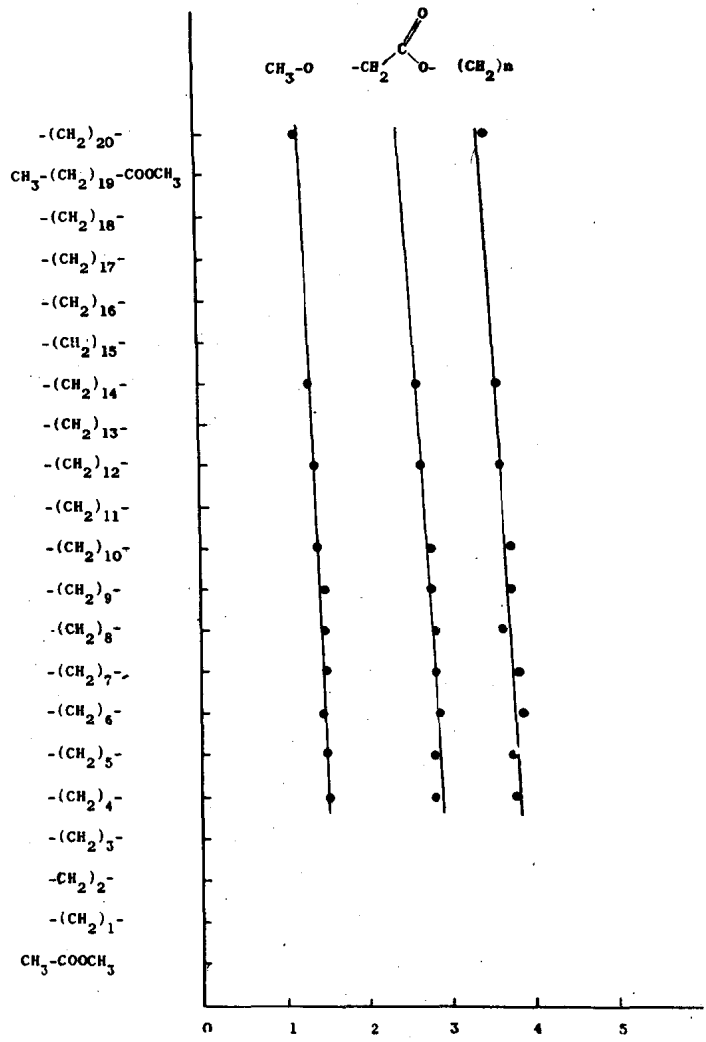


FIG. 5. Relation between the chain length of fatty acids and the shift of some groups in the molecule.

But, in most cases, this is too naïve a view. Often, quite strong interactions between molecules may occur: association, polymerization, hydrogen-bonding, proton-exchange. In the case of carboxylic acids, several examples of these phenomena are known. The chemical shifts of hydrogen atoms involved in hydrogen bonds are mostly to low field, due to the strong electronegativity of oxygen.

As an example of association in the liquid phase leading to a change in the chemical shift value, a plot of the resonance absorption peak of the carboxyl group in palmitic acid, dissolved in carbon disulphide, is given in Fig. 6. It appears that dimerization occurs down to very low concentrations (0·6 mol %).

This picture is still more complicated for the lower fatty acids and for solutions in more polar solvents, where hydrogen bonds can occur between acid molecules and between acid and solvent. N.M.R. offers, together with infra-red measurements, a rapid means of estimating the presence and strength of hydrogen bonds.

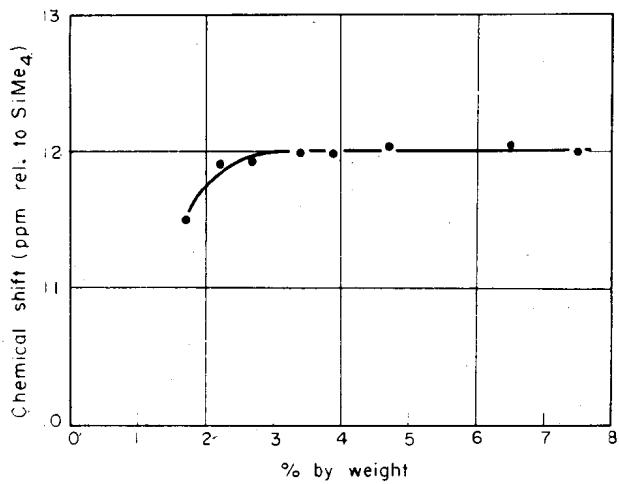


FIG. 6. Chemical shift of the carboxyl group of palmitic acid as a function of the concentration.

QUANTITATIVE APPLICATIONS

Two different types of measurements can be mentioned, which make use of the fact that in N.M.R. spectroscopy the area of an absorption peak is proportional to the number of nuclei contributing to it.

The first application is the measurement of the amount of fat present in the liquid form in mixtures of solid and liquid fats. As liquids give rise to very sharp absorption lines, in contrast to solids, it is possible to distinguish between these two forms and to measure the amount of liquid present. For this purpose it is essential to be able to measure the area of a peak quickly and accurately.

The second type of measurement is used to determine quantitatively molecular structures, an example of which is the determination of the degree of branching of fatty acids.

In branched fatty acids, the ratio of protons in terminal CH_3-groups to those in (CH_2+CH)-groups is

$$\frac{3(m+1)}{2n-3m-2},$$

where n is the chain length (excluding the carboxyl group) and m is the degree of branching. This ratio can easily be determined from the spectrum. As an

example, the identification of a 4× branched C_{20} acid, isolated from butter fat, was performed with the aid of its N.M.R. spectrum (Fig, 7). In the same

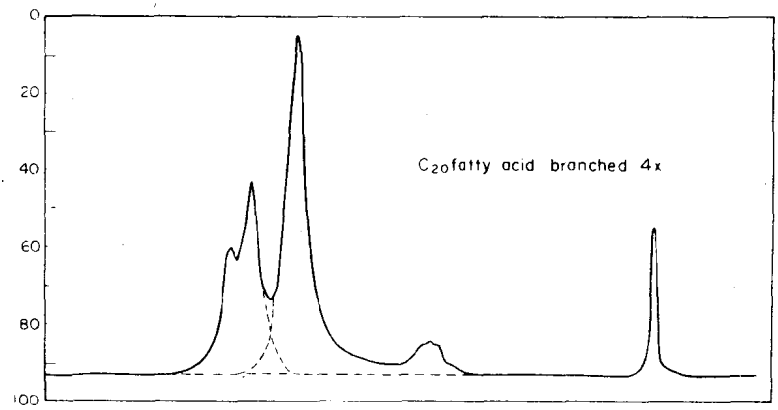


FIG. 7. N.M.R. spectrum of a C_{20}-fatty acid, branched four times.

way it is possible to determine the average degree of branching in mixtures of fatty acids.

Methods are now becoming available of measuring peak areas to an accuracy of about 2 per cent.

POLYMORPHISM IN SOLID TRIGLYCERIDES

It is well known that solid triglycerides occur in several polymorphic forms, which can be distinguished by X-ray diffraction methods. Chapman, Richards and Yorke[2] recorded the N.M.R. spectrum of a number of triglycerides in various polymorphic forms and compared the results with those obtained by X-ray and infra-red measurements. These measurements were, of course, made in the solid phase and consequently the broad lines expected from solid compounds were found. The width of the line indicated the freedom of motion assigned to the structural elements and from these data conclusions regarding the crystallographic form can be made. In general, the α-form shows a rather narrow line, consistent with the greater motional freedom in the hexagonal form

The N.M.R. method has not in this case, contributed new knowledge, but it confirms from an entirely different angle, the X-ray and infra-red measurements.

In addition to the foregoing examples of N.M.R. spectroscopy, I should like to mention a related technique, which seems promising for use in biochemical problems, namely electron spin resonance[3]. Just as the nucleus possesses a magnetic dipole moment, the electron has one, which is very much greater. In diamagnetic compounds, two electrons are paired and no resulting moment can be observed. Sometimes, however, unpaired electrons

are present, for instance with free radicals, and then a phenomenon, similar to the one described for nuclei, can occur: electron spin resonance.

This form of spectroscopy can give information about reactions involving free radicals; it can measure the concentration of (stable) free radicals and it may give information about structure if radicals are produced by irradiation.

ACKNOWLEDGEMENT

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PRESERVING CONCENTRATION OF THE LIPASE OF THE PANCREATIC JUICE

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DURING the last two years we experimented with extracts of hog pancreas trying to purify the lipase. It was our intention to avoid a high loss of activity and to investigate why the lipase was very often denaturated. We could confirm almost all of the results published during the last years by P. Desnuelle and his co-workers.

One year ago we analysed the contents of a human pancreatic cyste to see whether it was a real pancreatic juice. We found a very strong lipase activity and it was surprising that this lipase was more stable than our preparations. We decided to drain the pancreatic ductus of a hog to prove whether the lipase of the juice had other properties than the extracted one and to investigate whether there was an efficient method of stabilizing the lipase.

The experiments had been carried out with the pancreatic juice of a hog. At first we used Neurath's method and implanted two polyethylene tubes in the ductus: one directed towards the gland and the other through the ductus into the intestine. But we never used the possibility of draining the liquor into the intestine, since we have found that the juice could be replaced completely by feeding the hog with fresh pancreatic glands. The volume of the juice as well as its lipase activity was increased by feeding lipids and fresh pancreas. By this way we could gain up to 1200 ml of juice daily. As often as possible the material was collected and frozen at −20°C.

There is no doubt that the best method for the determination of lipase consists in the titration of the fatty acids liberated by the lipase, especially according to the method described by P. Desnuelle[1]. However, for a rapid determination, requiring only very small amounts of enzyme, we used the clearing of a fat emulsion by the lipase, as described by B. Borgström[2]. Using a microphotometer[3] we required 0·5 ml of the emulsion and 0·03 –0·06 ml of the lipase solution. Unfortunately this method is very sensitive towards ions especially towards calcium and magnesium and it fails if the solution contains soap or other surface active substances.

Starting from the experience of R. Willstätter, that glycerol stabilizes the lipase, we analysed the stabilizing activity of different substances. Among all of them, butanol or methylpropanol have the best qualities. Their stabilizing power is at least equal to that of glycerol but they do not increase