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NMR and Microorganisms

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Abstract

This article is an introduction to the use of NMR for the investigation of microbial physiology and metabolism. NMR parameters which determine the sensitivity and resolving power of the method are reviewed. A broad survey of current applications follows. Qualitative uses are described first; they include compound identification and localisation. Quantitative aspects, such as pH, concentration and flux measurements are then examined, as well as the corresponding experimental constraints. The review ends with suggestions of possible future developments in instrument capabilities aimed at improving sensitivity: higher fields, spectroscopic and imaging microprobes.

Introduction

The first report on the application of NMR to micro-organisms is that of Eakin *et al.* on the yeast, *Candida utilis* (1). During the past 27 years, many developments have taken place, which make *in vivo* NMR a very useful tool in the study of microbial physiology and metabolism. It is the purpose of this introductory review to provide the reader with an orientation to the field. It is comprised of four parts. We first list a few characteristics of the NMR technique which are deemed important for metabolic investigation. We then describe several applications of NMR, first as a qualitative tool then as a quantitative instrument. We have taken the liberty to mention advances which have taken place in related fields, such as biofluid analysis and NMR studies of mammalian cells or whole organs, whenever they were perceived as useful and readily adaptable to the case of micro-organisms. We end by mentioning various probable or desirable future developments.

Some Aspects of NMR of Importance in Microbiology

We assume that the reader has some knowledge of NMR. We will be mainly concerned with small molecules, observed either in homogenous solutions (supernatant or growth medium, cell extracts) or inside living cells.

Sensitivity

The sensitivity of the NMR experiment should probably be the foremost concern of prospective users. It depends on the following factors.

1) *Nature of the nucleus*: Each magnetic nucleus is characterised by a spin quantum number $I > 0$ and a gyromagnetic ratio γ. Several elements which play an important role in biochemistry have "low γ" nuclei (Mg, Ca) and present special problems. A further point is that nuclei with $I \geq 1$ (such as ^{14}N) have a quadrupole moment and display very broad lines when bound in a molecule. Exceptions include alkali, alkaline earth, and chlorine ions, provided they are not bound to a carrier macromolecule.

2) *Operating magnetic field*: The sensitivity depends roughly on the square of the static field. As the price of a spectrometer is also a steep function of the magnetic field, there is an obvious economic limitation here. It is compounded with the requirement of a wide sample access, because magnet prices also increase with sample bore.

3) *Active volume*: Provided the sample has a correct shape and is relatively homogenous, sensitivity is roughly proportional to the volume inside the radio-frequency coil. Thus, at constant concentration, a 20 mm tube should yield a signal about four times as intense as a 10 mm one.

4) *Concentration of resonant nuclei*: This is the main limiting factor but it depends on many variables, such as the effective concentration of the compound of interest and the relative abundance of the isotope being studied. Many elements have an abundant isotope devoid of magnetic moment while the magnetically active isotope is rare. Such is the case, to varying degrees, for C, O, Ca and S, and also for N because the main isotope is very difficult to use in metabolic studies, due to its broad signal. Enriched compounds are expensive and the range of available substances is limited. The case of hydrogen is almost the opposite; here, the intense solvent resonance tends to swamp the informative signals, and special pulse sequences must be used (2).

5) *Line-width*: The physics of NMR shows that the previous parameters determine the area of each signal. Therefore, the broader the signal, the smaller its amplitude and the more likely that it vanishes amidst noise and baseline artefacts. When working with supernatants or cell extracts, the contributions to the line-widths are those found for all solution work: solids suspended in the medium, paramagnetic impurities, viscous solution; they can all be partly or totally removed. In the case of living cells, the line-width is likely to be dominated by the sample's own heterogeneity. Some causes, such as gas bubbles, can be controlled.

6) *Accumulation time*: The signal to noise ratio increases as the square root of the number of scans accumulated. This result is exact insofar as the sample remains intact (cells remain viable, do not settle, gas bubbles are not formed). Further, the necessary number of scans puts a lower limit on the time resolution of each experiment. It may happen that a decent sensitivity cannot be reached because of limited instrument time and relaxation problems (see next paragraph).

7) *Relaxation delay*: Sensitivity depends on the spin lattice relaxation time T1, the pulse flip angle and the pulse repetition time. A relaxation delay is usually inserted before

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each excitation pulse. For quantitative work, a delay of at least $3T_1$ is required if the experimental signal is to be within 10% of the true signal. Shorter delays can be used, along with correction factors. A pair of spectra, recorded with respectively short and very long delays, are used to compute correction factors. This procedure relies on the assumption that relevant parameters do not evolve in time. For solution work, relaxation reagents can be added. They serve two useful purposes: decreasing T_1 and suppressing the Overhauser effect (next paragraph).

8) *Decoupling*: The spectra of nuclei other than hydrogen are usually recorded under complete proton decoupling. Since different nuclei may interact differently with surrounding protons, proton irradiation complicates the recording of quantitative spectra: one must resort to gated irradiation wherein the decoupling effect is retained, but the differential Overhauser enhancement is suppressed. Conditions for quantitative recording have been given (3,4). It is worth noting that the radio-frequency field used for decoupling is attenuated by ionic solutions, an effect which becomes important for large sample volumes and high frequencies. The higher the proton resonance frequency, the larger the spectral width required of the decoupler. One may either use higher decoupler power (at the risk of overheating the sample) or sophisticated decoupling schemes (5,6).

Inverse detection, where heteronuclear resonances are detected via proton signals, is making significant inroads in the field of *in vivo* NMR: it promises important gains in sensitivity and somewhat better resolution (7). The theoretical sensitivity advantage of inverse compared to straight forward detection of nucleus X is $(\gamma_H/\gamma_X)^3$. This large factor may not be fully realised because most reverse probes only accept 5 mm sample tubes and because of the deleterious effect of the intense and broad water signal. The method is also ineffective when applied to nuclei weakly coupled to protons, such as carboxylic carbons. Lastly, quantitation is difficult.

Implicitly, we have been considering the sensitivity required to detect a signal. It is usual to assume that a signal to noise ratio of at least 3 is necessary for detection. Better performance is required for quantification by use of line integrals. It has been shown (8) that the precision of any integral is numerically equal to the inverse of the signal to noise ratio; a signal which is ten times the noise will be integrated to a precision of 10%.

We have left aside the complicated problem of NMR visibility. It sometimes happens that a compound, abundant enough to be detected inside cells, is not seen (or seen with low sensitivity) on the experimental spectrum. A common explanation is that these small molecules are bound to large carrier macromolecules, with very slow motions. The corresponding signals are therefore broadened beyond recognition. A discussion is best left to specific cases.

Resolution

In the early applications of NMR to microbial physiology and metabolism, spectral resolution was rarely an issue. The ranges of ^{13}C and ^{15}N chemical shifts is such that few spectral overlaps were observed. Phosphorus spectra are somewhat crowded, for instance in the phosphomonoester region. This favourable state of affairs is changing because more complex systems are being investigated and also because proton NMR, which has a smaller frequency dispersion, is being used more often. In some cases higher operating fields will alleviate the problem, but the almost definitive solution is two- or multi-dimensional NMR , using multiple labelling and one- or multi-dimensional acquisition and processing. An example is provided by the recent investigation of glyphosate metabolism by Hutton *et al.* (9). Proton NMR investigations have also benefited from 2-D (COSY) techniques; see for instance the review by Navon (10) on proton-phosphorus 2D techniques.

Other Instrumental Requirements

Most experiments useful in metabolic NMR can be done using a typical high resolution spectrometer, provided it has multi-nuclear and reverse detection capabilities. Pulsed field gradients are becoming increasingly useful. They are used to shorten pulse sequences but also to weigh NMR signals according to the diffusion coefficients of the relevant molecules. A spin echo sequence is applied in the presence of a pair of pulsed gradients. The final signal intensity is a function of the diffusion coefficient of the compound under study. Thus, diffusion ordered two-dimensional spectroscopy is fast becoming a powerful analytical technique for complex mixtures (11). As already mentioned, a wide bore and ample spectrometer time are definite advantages.

Modern high resolution NMR puts stringent requirements on the stability of the magnetic field, of the spectrometer electronics and of the sample temperature. Because of the broader line-widths and simpler pulse sequences used, metabolic NMR is less demanding. For *in vivo* experiments, it is often possible to dispense with the field/frequency lock.

Solid state NMR and micro-imaging are two other important techniques. They both require wide-bore magnets, specific probes and special electronics.

Qualitative Information Obtained by NMR

We now turn to an overview of possible applications of NMR spectroscopy related to microbiology. We find it convenient to classify those, somewhat arbitrarily, as qualitative or quantitative.

Identification of Compounds

It is often the case that metabolic pathways produce unknown end products and that NMR is called upon to help in identifying these compounds. This will be particularly true for recently discovered organisms, or for some branches of secondary metabolism. As this type of work is mostly done on supernatants or cell extracts, the investigator can bring to bear all the tools of high resolution, solution NMR. Chemical shifts, signal multiplicities and coupling constant are all useful; two-dimensional correlation maps greatly facilitate the process. The wealth of data available and the fact that NMR, contrary to other characterisation methods, is not specific of any one class

of molecules are clearly advantageous. Some time ago, a novel molecule, 2,3-cyclo-pyro-diphosphoglycerate, was discovered on *in vivo* spectra and fully characterised; its role in gluconeogenesis was later established (12). Other metabolites, including osmolytes (13) and UDP amino sugars (14), have since been described. Kalic *et al.* (15) provide an instructive example (drawn from a study of a particular biofluid) of the power and limitations of this approach.

Identification of Metabolic Pathways or Branches in Pathways

Molecules involved in metabolism have a carbon backbone. It should therefore not be surprising to learn that the most powerful method to investigate a pathway consists in labelling carbon atoms in order to unravel their origin and fate. This can be done with high sensitivity with radioactive ^{14}C labelling, but it is very painstaking to obtain site-specific information inside a molecule. In contrast, ^{13}C NMR provides site information quite easily.

Analogous to the investigations reported in the previous paragraph, NMR spectroscopy can be used to identify new pathways or unknown branches. Given the present state of knowledge, discovering an entirely new pathway is an improbable event, but some steps are often clarified or confirmed by NMR. Again, it is mainly in the case of secondary metabolism, leading to complex molecules, that most applications will be found. We will only mention two extreme examples before referring readers to the references and the "Further Reading" section. Lelait and Grivet (16) used the labelling patterns of amino-acids (obtained after isolation and hydrolysis of proteins) to confirm the main metabolic pathways of acetogenic bacteria growing on ^{13}C labelled glucose or carbonate. Hajjaj *et al.* (17) grew the filamentous fungus *Monascus ruber* on $^{13}C_1$, $^{13}C_2$ and $^{13}C_1$-$^{13}C_2$ labelled acetate to delineate the complex biosynthesis of citrinin.

Hydrogen exchange with the solvent is readily characterised by NMR, provided the solvent atoms can be labelled in some way. Incubating a cell suspension in an $^1H_2O/^2H_2O$ mixture with a ^{13}C labelled substrate is a simple method; site-specific deuterium incorporation in the end products is revealed by the characteristic splitting of the carbon signals under deuterium coupling (18).

The previous paragraphs may have wrongly given the reader an impression that only labelling above the background abundance is useful. This is not the case, as the work of Pasternack *et al.* (19) testifies. These authors incubated *Saccharomyces cerevisiae* in the presence of [1-^{13}C]-glycine and formate in order to delineate one-carbon metabolism. NMR analysis of the metabolic products choline and adenine showed isotopic dilution, thus proving that formate can compete with glycine as a carbon donor.

NMR is also useful for investigations of ecosystems or complex bacterial populations. The presence of homoacetogens in the microflora of human feces was proven by Lajoie *et al.* (20) who detected doubly labelled acetate formed from H_2 and $^{13}CO_2$.

Metabolic Expression in Genetically Modified Organisms

As the physiology and metabolism of naturally occurring micro-organisms become better known, genetically modified bacteria receive closer attention. The main question raised in connection with these organisms can be formulated as follows: are the implanted genes fully expressed or how are the desired functions operating? The work of Ansanay *et al.* (21) provides an illustration. These authors inserted the gene coding for the malolactic enzyme (from *Lactococcus lactis*) into the genomes of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Biochemical analyses, ^{13}C labelling (from [1-^{13}C]-glucose) and comparison of the two species showed that *S. cerevisiae* was indeed able to perform the malolactic fermentation, but with low yield due to a very inefficient malate transport system.

Distinction of Inside and Outside of Cell: Number of Cell Compartments

Several NMR parameters have been used to locate solutes either inside or outside of cells. The first method, historically, relies on the pH dependence of the chemical shift, specially that of phosphorylated compounds (*see* "*pH Measurements*"). The appearance of several "inside" inorganic phosphate signals is a proof of the existence of several cellular compartments, each with a distinctive pH. Another approach makes use of a non-toxic, non-permeating shift and/or relaxation reagent. It is also possible to recognise atoms bound to the outside of the cell membrane.

A more recent technique is based on diffusion weighted spectra (22). The "inside" molecules have a rather low effective diffusion coefficient, while the "outside" ones diffuse faster; moreover, they flow with the perifusion medium. It is possible to completely suppress extracellular contributions to the proton spectrum.

The existence of distinct metabolic pools and the assignment of some intermediates to a specific pool can be established by NMR examination of labelling patterns. The availability of mutants in which specific enzymes are missing is advantageous (19).

Channelling

Eukaryotes harbour multi-enzyme complexes (so-called metabolons). A current hypothesis is that a substrate can move from one active site to another without being released in the cytosol. This motion is assumed to be stereospecific and to preclude label scrambling. Such a mechanism has been investigated in yeast (23).

Quantitative Information

Let us now point to some quantitative measurements derived from NMR observations. The number of such applications is continuously growing, so that we can't even hope to compile a complete list.

Cell Volume

The determination of the volume of intracellular water is required for the calculation of any intracellular metabolite concentration. It is somewhat difficult to perform whatever the technique used, although the principle is simple. A non-permeating inert solute is added to the culture medium and a spectroscopic signal is recorded in the absence and in the presence of the cells. The difference is proportional to the excluded volume, occupied by the cells. On the other hand, the total water volume, both in the presence and absence of cells, is determined from the signal intensities of a freely diffusable compound. The difficulty is that one is looking at small variations of large signals. A careful determination of the intracellular volume for a suspension of *Enterococcus faecalis* by proton NMR is reported in (24).

Diffusion Coefficients

It was mentioned earlier (*see* "*Distinction of Inside and Outside Cell: Number of Cell Compartments*") that spin echo NMR, in the presence of pulsed field gradients, was sensitive to the diffusion of solute molecules. A quantitative determination of diffusion coefficients, both inside and outside the cell is possible. A recent application concerns the structure of granules (flocs) and biofilms. In anaerobic fermenters, bacteria often aggregate in granules, which sometimes show a defined concentric arrangement of bacterial species. It is of great interest to determine the flow of substrates and products in and out of these structures. The same is true for biofilms, whether natural or artificial. First results on water mobility on these structures have been presented (25).

Diffusion measurements can also be used for internal cell volume determination. The technique is more involved than the methods described in the previous section, but more reliable. Using a detailed mathematical model for the restricted diffusion of water with exchange between two compartments, Pfeuffer *et al.* (26,27) could determine volume changes in response to hypoosmotic and hyperosmotic conditions.

Behaviour of Intracellular Macromolecules

Since most enzymes operate inside the cell, one would be quite interested in establishing their properties *in situ*, as opposed to properties determined from cellular extracts, homogenous solutions or even crystals. As already mentioned, the possibility of observing well resolved NMR signals from intracellular macromolecules is remote. It has proved possible in some special cases. Some protons signals of haemoglobin are indeed observable in erythrocytes, because the molecule itself is quite concentrated, there is some segmental mobility and the resonances are shifted to an uncongested part of the spectrum by interaction with the paramagnetic iron atom. A approach which is rather powerful and more general is the biosynthetic labelling of the enzyme with a rare nucleus. This can be accomplished in good yield upon insertion of an inducible expression vector for the relevant gene in the microbe's genome. In the presence of the inductor, the enzyme is synthetised and, if a labelled amino-acid is present in the culture medium, it is incorporated. This technique has been very successfully applied to yeast and to mammalian cells (28). 5-fluorotryptophan was incorporated in various enzymes of the glycolytic pathway or of the Krebs cycle. Rotational correlation times and hence micro-viscosity coefficients could be derived from the observed line-widths.

pH Measurements

This staple of *in vivo* NMR uses either intrinsic probes (mainly phosphorylated compounds) or extrinsic reporter molecules. It is important to construct a calibration curve under conditions as similar as possible to those in the intracellular medium. Many synthetic indicators have been evaluated, including phosphonates (29) and fluorinated compounds (30). Fluorine derivatives have the advantages of a vanishing background and high pH sensitivity, but suffer from the drawback that they require a special spectrometer channel.

Concentrations

Non-invasive, real time, continuous determinations of concentrations represent the main uses of metabolic NMR. A few words of caution regarding experimental procedures have been offered in the section entitled "*Sensitivity*".

Relative concentrations are easily measured, but absolute concentrations are another matter; they can only be defined relative to some reference. The choice of a convenient reference merits some attention. The ideal compound gives a single sharp line well removed from any other signal of interest, does not interact with solutes or cells and is easily handled. For *in vivo* work, it is prudent to use an external reference, i.e. a sealed capillary containing the reference solution inside the main sample tube. An interesting substitute has been suggested (31). A dedicated electronic circuit (or even the decoupler) can be set to generate a signal similar to a free induction decay. This is picked up by the receiver coil, processed as a *bona fide* signal and produces a reference line, thus avoiding any contamination of the sample.

It must be pointed out that different concentrations are measured in biological NMR. Simplest to acquire and interpret are solute concentrations in the culture medium (or supernatant). One is mainly interested in substrates and end products, with perhaps a goal of establishing a carbon balance. Next come cell extracts, for which some biochemical questions must be considered: washing of cells and choice of extraction procedure. Disruption of cells (in a French press or by sonication), followed by protein (or nucleic acid) isolation and hydrolysis puts heavier emphasis on biochemical know-how but provides information on anabolic reactions and pathways. Finally, *in vivo* concentrations rely on free intracellular volume determinations.

To be significant, assays must refer to a known number of cells (or definite cell mass). It appears that the most common unit is the gram of dry cells.

Indirect Determination of Concentrations

In contrast to the direct concentration measurements referred to in the previous section, it is also possible to assay NMR invisible compounds through the effect they have on visible solutes. An early example was the determination of intracellular Mg^{++} through the variation of the chemical shift difference between the α and β phosphorus resonances of ATP (32). Many ^{19}F NMR indicators of Mg^{++} and Ca^{++} have been synthesised and evaluated (33). Oxygen concentrations can, within a limited range, be derived from spin-lattice (T_1) relaxation times.

Kinetics of Individual Reactions

In vivo reaction rates can be measured using the technique of magnetisation transfer. Many versions of this experiment are known (34): steady-state saturation, transient saturation, two-dimensional exchange spectroscopy (EXSY). The principle of the method can be briefly stated. Consider a nucleus that can exist in two chemical environments (say A and B) with distinct resonance frequencies (f_A and f_B) and can jump from one to the other (with rates k_A from A and k_B from B). An example is the ^{31}P nucleus within a phosphate group taking part in the reaction:

$$ADP + PCr \leftrightarrow ATP + Cr$$

The magnetisation of the γ phosphate of ATP evolves under two causes: spin-lattice relaxation and exchange with the PCr magnetisation. In the pulsed version we would look at the perturbation of (say) the ATP signal upon inversion of the PCr resonance (brought about by a weak selective pulse). In effect, we would be watching a competition between chemical exchange, which brings into the ATP site an inverted population of nuclei (negative signal) and spin-lattice relaxation which tends to restore a normal signal. The time scale of such an experiment is the relaxation time T_1 so that reactions much faster or much slower than the spin lattice relaxation cannot be investigated in this manner. The whole process is easily modelled by two first-order differential equations, the solutions of which can be fitted to the experimental results, allowing the determination of the rate constants for exchange. A recent example is given in (35) for the case of a genetically engineered yeast, while Roscher *et al.* (36) show how to apply the EXSY method.

Transmembrane Fluxes

The transport kinetics of ions, substrates or products across the cell membrane can be measured by NMR, provided inside and outside contributions can be recognised. Shift reagents can be used to create a difference in resonance frequencies; many such compounds are available for cation NMR, but interpretation of results can be difficult. The actual rate measurement techniques are quite diverse. If transport is slow, recording of successive spectra after injection of an external compound or resuspension of the cells in fresh medium may allow the monitoring of 'internal' and/or 'external' concentrations as functions of time. In the case of intermediate rate constants, the magnetisation transfer methods of the previous section are suitable. Here, the two exchanging sites are geometrically as well as spectroscopically different.

Because of its high sensitivity and ubiquitous occurrence, sodium has been the subject of many investigations. It has proved, however, rather difficult to quantify the NMR visibility of internal sodium, which has been variously reported to be in the range 30-60% (37,38). ^{23}Na is a spin 3/2 nucleus. When the ion moves slowly, or is bound to a partially oriented macromolecular structure (such as the cyto-skeleton), double and triple quantum transitions become observable and provide a possible technique to assay internal sodium. A complete theoretical treatment is available (39).

Potassium is difficult to observe and at least two substitutes have been proposed: rubidium and caesium. $^{133}Cs^{+}$ would seem to offer two advantages (40). Its quadrupole moment is 200 times lower than that of sodium, promising rather sharp lines, and its chemical shift is so highly sensitive to the environment that the internal and external ions have spontaneously resolved resonances.

The transport of organic solutes can be examined in much the same way, but it is usually slower than that of ions. The case of ethanol efflux from *Zymonas mobilis* is an exception. It is fast enough for magnetisation transfer methods to be applied (41).

Metabolic Flux

Metabolic fluxes, or ratio of fluxes at branch points, are the main quantitative data sought by workers interested in metabolism. They are the equivalent of the V_{max} and K_m of enzymology. They cannot be determined from NMR measurements alone when using a single label. This fact was well known of the practitioners of radioactive labelling who coined the term 'specific activity' (counts per minute per gram of product). It is sometimes masked in NMR; because of its low sensitivity, this analytical technique operates not with tracer amounts but with labelled compounds which form a notable proportion of the substrate. The difficulty can be traced to the fact that isotopic equilibrium (or flux) is different from substrate equilibrium (or flux). In order to stress the point, we submit to the reader a somewhat far-fetched analogy. Suppose that we wish to determine the flow rate of a mountain stream. This is rather laborious to do; we would have to measure the (cold) water speed at many points of the stream cross-section and sum all contributions. Instead, we may decide to dump a known number of fishes into the water and count how many of them pass under a bridge downstream. This method is the analogue of the simple NMR approach mentioned above.

The enrichment of a product P, that is the ratio [labelled P]/[total P], must be known before any conclusion on the corresponding flux can be drawn. The amount of P in a culture can be, in principle, determined with any analytical technique. For instance, Dominguez *et al.* (42) combined acidic and basic extraction procedures, HPLC and fluorescence assays with ^{13}C NMR analysis of labelling patterns to determine carbon fluxes in the central metabolism of *Corynebacterium glutamicum*.

Combining results derived from several techniques and several samples should be considered with some caution. Several investigators have proposed methods using only NMR. One approach (43) uses proton NMR and ^{13}C labelling; on the proton spectrum, both unlabelled and labelled product can be observed, provided the C-H coupling constant is large enough, as is the case for CH_n groups. Wendisch *et al.* (44) have developed a frequency selective spin echo difference technique which remains useful for non-protonated carbons. Any magnetically active nucleus coupled to carbon could be used; Lutz *et al.* (45) have taken advantage of ^{31}P-^{13}C couplings to determine the specific enrichment of phosphorylated metabolites.

The most powerful method is probably the analysis of amino acid multiplet patterns arising when micro-organisms are fed with uniformly labelled glucose, as first described by Gagnaire and Taravel (46) and perfected by Szyperski *et al*. One is sometimes interested in the global turnover or renewal rate of a whole family of molecules, for instance amino acids. Roberts *et al*. (47) used ^{15}N NMR to measure the free amino-acid turnover rate in methanogens.

Up to now, we have been mainly concerned with pure species; however, some quantitative work has been done on mixed cultures and complete ecosystems. Wolin *et al.* (48) examined the colonisation of the digestive tracts of infants by Bifidobacteria. These micro-organisms use a unique pathway of hexose catabolism to produce acetate and lactate. One third of the acetate molecules are derived entirely from the C_3 carbon. Fermentation of [3-^{13}C]-glucose thus yields doubly labelled acetate, with a characteristic spectrum. It was shown that 70% of the acetate produced by a bacterial suspension was due to Bifidobacteria.

Are Isotopic Substitutions Innocuous?

After reading the previous sections, the reader has no doubt noticed that isotopic labelling is one of the main tools of metabolic NMR. He or she may well wonder whether substituting a nucleus by a lighter or heavier isotope has any effect on the biochemical reactions being studied. A short answer is no, except possibly for the hydrogen isotopes. Isotope effects on chemical and biochemical reactions have been extensively studied during the last fifty years. Chemical equilibria are slightly displaced upon isotopic substitution, but the effect is too small to have observable consequences on living systems.

Of greater import are the modifications of rate constants. Hydrogen exchanges can be slowed down by a factor of ten when protium is replaced by deuterium. This is probably the reason why eukaryotes and higher organisms cannot live on heavy water. Kinetic isotope effects are much smaller for heavier nuclei. Organic reactions rates can be reduced by a few percent when ^{12}C is replaced by ^{13}C. The resulting small concentration changes can be monitored by mass spectrometry and have been used as probes of reaction mechanisms (49).

Metabolic pathways operating in micro-organisms comprise many enzymatic reactions; a given metabolite is often synthesised by several routes. The end result is that apparent isotope effects become small (a few per mil, not detectable by NMR) and difficult to interpret, except when they can be related to a single rate limiting step. The $^1H/^2H$ couple is again an exception, with variations in relative abundance of several percent. We refer the reader to the literature (50) for the many interesting applications of site-specific natural isotope fractionation.

Probable and/or Desirable Developments

We will end by listing some technical developments that should in the near future make NMR more productive or easier to use for the microbiologist. This list is not speculative, as the technology already exists for these improvements.

High Field, Wide Bore Magnets

Higher fields will mean more sensitivity, allowing observation of low γ nuclei and/or the use of lower enrichment for rare nuclei. As structural biochemists reach for ever increasing fields, one may hope that many 11.7 T (500 MHz for protons) spectrometers will be converted to metabolic work. A similar trend is seen in biofluid analysis, although here the driving force is the necessity of improving the resolution of proton spectra.

Microprobes

Manufacturers as well as several research groups (51) are developing probes for very small volumes (1-100 microlitres). They could be used in conjunction with very small scale cultures.

High Pressure Probes and NMR of Gases

It is now possible to do high resolution NMR under pressures of 500 MPa (5000 atm)(52). It is conceivable that gas consumption and production (H_2, CH_4) by methanogens or acetogens and interspecific hydrogen transfer could be studied *in vivo* with these specialised probes.

Instead of increasing the effective concentration of a gas, one could think of increasing the apparent magnetic moment (or the γ factor) of the nuclei. This seemingly ludicrous goal can be reached in two cases: ^3He and ^{129}Xe, using optical pumping techniques (53). Although of doubtful biochemical significance in themselves, these gases could serve to explore the geometric structure of microbial aggregates (flocs, granules), much as is now being developed for lung and vascular imaging.

Spectroscopic Imaging

Magnetic resonance imaging, or MRI, is a well known non-invasive investigative technique, useful in material science and in medicine. It is usually implemented with large, horizontal bore magnets. A micro-scale version of MRI uses a vertical bore magnet. The ultimate resolution is estimated as 10 micrometers, although current images show details at about 50 micrometers. This is sufficient to investigate heterogeneous samples, such as granules, biofilms or bioreactors as mentionned earlier (*see* "*Diffusion Coefficients*"). Using special pulse sequences, it is possible

to perform spectroscopic imaging, wherein a three-dimensional concentration map of several abundant metabolites is constructed for an organ such as the brain. The spatial resolution is of the order of half a centimetre. The challenge consists in combining a high spatial resolution and chemical sift information.

Data Banks and Data Processing

To this author's knowledge, no NMR data bank useful for metabolism exits at the present time. The many compilations of chemical shifts that do exist mainly concern organic chemistry: spectra were recorded in organic solvents, for neutral molecules. We believe that it would be useful to collect chemical shifts, coupling constants, pK's of molecules frequently encountered in metabolic work, along with an accurate description of conditions: pH, ionic strength, concentration, temperature.

The use of NMR spectroscopy for research in physiology or metabolism involves the recording and analysing of a great number of spectra and there is a need for the automation of the whole process. Software for the automatic treatment and analysis of spectra is available but, in the author's opinion, no single program can yet handle the variety of spectra and practical problems met when performing *in vivo* NMR.

Conclusion

We hope that this overview of possible NMR applications in microbiology will encourage readers to consider the use of NMR whenever a problem arises in their own research.

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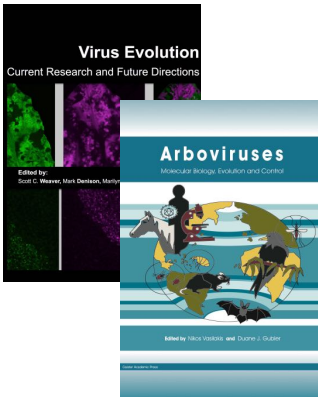

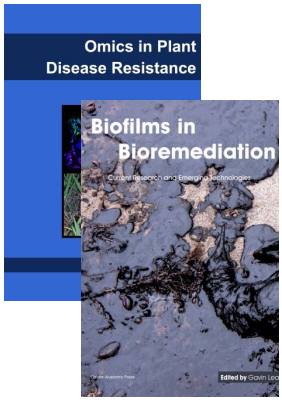

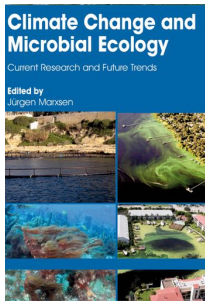

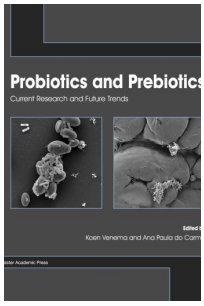

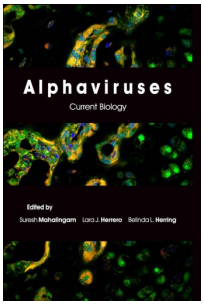

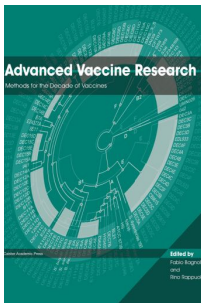

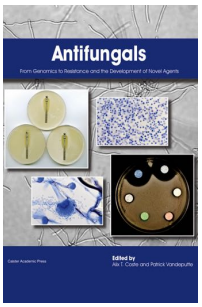

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