

Nuclear Magnetic Resonance: a noninvasive technique in the study of life processes *in situ*

Ressonância Magnética Nuclear: uma técnica não invasiva para investigar processos biológicos *in situ*

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One of the most attractive aspects of the application of Nuclear Magnetic Resonance to study cellular physiology derives from the noninvasive and nondestructive characteristics of this method; in fact, not only is it possible to monitor substrate consumption and end-product formation, but it is also possible to follow the changes in the concentrations of intracellular metabolites in the same sample. Furthermore, various aspects of the cellular processes can be probed with the different nuclides that can be detected by NMR. For instance, ³¹P-NMR allows monitoring of the energetic status of the cell and the transmembrane proton gradient, and allows measuring of enzyme kinetics *in vivo*, whereas the fate of individual carbon (or nitrogen) atoms through different metabolic pathways can be followed by ¹³C-NMR (or ¹⁵N-NMR); cation gradients as well as cation transport over the cell membrane can be measured with ²³Na- or ³⁹K-NMR in the presence of shift reagents.

A major drawback of *in vivo* NMR techniques is low sensitivity which forces the use of dense cell suspensions in order to increase the total intracellular space that is accessible to detection. However, in many cases these sensitivity limitations are by far overcome by the unique opportunity provided by these measurements to monitor a number of biochemical parameters without disturbing the cellular organization. Examples of our work on the application of this technique to study bacterial physiology *in vivo* are presented.

Um dos aspectos mais atraentes na aplicação de técnicas de Ressonância Magnética Nuclear (RMN) para estudar fisiologia celular deriva das suas características não-destrutivas e não-invasivas; de facto, não só é possível seguir consumo de substratos e formação de produtos finais, mas também, na mesma amostra, detectar variações nas concentrações de metabolitos intracelulares sem interferência nos processos biológicos. A variedade de núclídeos acessíveis a detecção por esta técnica permite a investigação de facetas complementares dos processos celulares. Por exemplo, a RMN de ³¹P permite avaliar o estado energético celular, medir pH intracelular, determinar gradientes protónicos transmembranares ou medir velocidades de reacções *in vivo*, enquanto que a detecção de ¹³C (ou ¹⁵N) permite determinar o percurso de átomos individuais de carbono (ou azoto) ao longo de diferentes vias metabólicas. O transporte de catiões, tais como sódio ou potássio, pode ser estudado usando RMN de ²³Na ou ³⁹K em presença de agentes de desvio apropriados. A maior limitação desta técnica deriva da sua baixa sensibilidade intrínseca, que impõe o uso de suspensões celulares densas com o objectivo de aumentar o espaço intracelular sujeito a detecção. Contudo, em muitos casos, estas limitações são em grande medida ultrapassadas pela oportunidade única que esta técnica oferece de avaliar uma variedade de parâmetros bioquímicos sem perturbação da estrutura e organização celulares. Neste artigo serão usados como ilustração das potencialidades desta metodologia, exemplos dos estudos sobre fisiologia bacteriana efectuados por esta equipa de investigação.

1. Introduction

Nuclear magnetic resonance spectroscopy (NMR) is one of the most powerful analytical methods available to chemists today, allowing, for example, the qualitative and quantitative characterization of chemical mixtures, the measurement of reaction rates in the steady state, the determination of the isotopic distribution within molecules or the determination of the three-dimensional structure of proteins. The tremendous improvement achieved in the performance of spectrometers in the last two decades has made it possible for biochemists to benefit from the capabilities of this technique for carrying out measurements directly on living systems [1-5].

One of the most attractive aspects of the application of this method in the study of cellular processes derives from its noninvasive and nondestructive characteristics: not only is it possible to monitor substrate consumption and end-products formation, but also to follow changes in the levels of intracellular metabolites in the same sample. Moreover, with the high number of nuclides available to NMR, different aspects of cellular processes can be probed by taking a multinuclear approach. For instance, ^{31}P -NMR allows monitoring the intracellular pH [6,7], the energetic status of the cell and the dynamics of intracellular phosphate pools [8]; in favourable cases, enzyme kinetics can be studied *in vivo* [9,10], and unique information on compartmentation can be obtained. By using ^{13}C -NMR, the fate of individual carbon atoms are traced and carbon fluxes through several pathways can be determined [1,11]. Similar kind of information is in principle available by using ^{15}N -NMR, but more severe sensitivity limitations occur in this case. Cation gradients as well as cation transport over the cell membrane are measured with ^{23}Na - or ^{39}K -NMR [12,13]; calcium levels can be measured indirectly by the very sensitive ^{19}F NMR with suitable fluorinated probes [14]. Provided that the required probe heads are available, all this information can be obtained for a single sample by interleaved acquisition of the signals due to different nuclei.

A major drawback of NMR is its intrinsic low sensitivity, which limits *in vivo* observations to metabolites present at concentrations of the order of 0.5 mM or higher. This limitation imposes the use of dense cell suspensions in order to increase the total intracellular space available to detection and, therefore, problems in gas or nutrients supply may arise [5]. However, the low sensitivity turns out as an advantage when the method is applied to complex mixtures such as living cells and tissues since it results in relatively simple spectra as only a few number of resonances are apparent. In many cases, the sensitivity limitations are by far overcome by the unique opportunity provided by these measurements to monitor a number of biochemical parameters without disturbing the cellular organization.

To date, the *in vivo* NMR field has expanded tremendously. The availability of wide bore horizontal magnets of a size sufficient to accommodate a human body has provided an entirely new clinical dimension in the NMR applications. It is now possible to produce metabolic maps with concentration profiles of the major metabolites present, for example, in heart or human brain. Despite the clinical importance of these new developments, commonly referred to as MRS (magnetic resonance spectroscopy) and MRI (magnetic resonance imaging), here we will focus on the applications of *in vivo* NMR to study cell systems, either in suspensions, or when immobilized.

2. Phosphorus-31 NMR studies of whole cells

Energy metabolism

^{31}P has been the most popular nucleus in the studies of biological systems by *in vivo* NMR. This is due to the high relative sensitivity of this nucleus allowing direct detection of many important phosphorylated metabolites as well as noninvasive measurements of intracellular pH, in experiments performed with intact cells, tissues, organs or even humans [15].

The intracellular levels of phosphorylated compounds intimately involved in energy metabolism, such as ATP, ADP and AMP, sugar phosphates or inorganic phosphate, have been assayed by ^{31}P -NMR in many different types of living cells [2,5,15,16]; these noninvasive measurements are particularly useful since the rapid turnover of the observed metabolites and, eventually, their compartmentation in different cellular structures, make the interpretation of the results obtained by classical extraction procedures difficult.

Intracellular metabolites may become unobservable by NMR techniques as a consequence of binding to macromolecular structures, localization in environments with high viscosity, or association with paramagnetic ions; in any of these cases, the resonances may become too broad to be detected. Therefore, the question concerning the observability of di- and triphosphonucleosides in whole cells by NMR is of great importance, specially when considering the consequences for the calculation of phosphorylation potentials [5,17]. Several authors share the opinion that only the freely mobile molecules are available to participate in metabolism and, consequently, the fractions detected by NMR represent the true chemical activity of these metabolites. It is now well established that, in several types of mammalian cells, ADP and P_i are largely unobservable by *in vivo* NMR [5,17-21]. For this reason, considerably higher phosphorylation potentials have been measured in some systems by *in vivo* NMR than by destructive analytical methods [20,22,23].

^{31}P -NMR is the only noninvasive method available to measure intracellular pH: it is possible to estimate pH

in different intracellular compartments and to determine the magnitude of transmembrane pH gradients formed during metabolism in a noninvasive way [15,24]. These measurements are most often made by utilizing inorganic phosphate as an intrinsic probe, although the resonances due to other phosphorus compounds, such as glucose-6-phosphate, 2,3-bisphosphoglycerate and polyphosphate have also been used [15, 24-26]. The application of the method relies on the fact that in aqueous solution the proton exchange between the several ionization species is fast, leading to the observation of a single resonance at a frequency which depends on the relative concentrations of the ionization species. Thereby, the chemical shift of the resulting resonance can be used to determine pH, provided that a reliable calibration curve is available [24,27]. The precision of the method is usually on the order of 0.05 pH unit whereas accuracy is on the order of 0.1-0.2 pH unit. This high precision allows accurate determinations of pH changes although absolute pH values may be affected by the poorer accuracy.

A large volume of work have been done over the last two decades based on pH measurements by NMR: pH homeostasis was demonstrated in a large number of different cell types [28-35]; distinction between different cellular compartments was possible, and the method was applied, for example, to study the pH regulation in cytosolic and vacuolar compartments in yeast and plant cells [36-38].

Typical ^{31}P spectra of a bacterial cell suspension (Figure 1) are characterized by the presence of resonances due to nucleoside triphosphates and nucleoside diphosphates (mainly ATP and ADP), inorganic phosphate, oxidized and reduced forms of nicotinamide adenine dinucleotides (NAD(P)^+ and NAD(P)H) and a complex broad signal due to phosphomonoesters (PME), such as glucose 6-phosphate, glycerophosphate or AMP. The presence of two distinct resonances in the inorganic phosphate region (1 to 3 ppm), assigned to the intracellular and the extracellular inorganic phosphate, reveals the existence of a pH gradient across the cell membrane; from the chemical shifts of these signals, values of 7.1 and 6.5 were inferred for the intracellular and the extracellular pH, respectively. As shown in trace A (Figure 1), high levels of nucleoside triphosphates are detected in cells in the absence of added external substrate: a value of approximately $6 \mu\text{mol} \cdot \text{g}$ (cells dry weight) $^{-1}$ was determined by quantitative ^{31}P -NMR. In this organism the full pool of NTP is detected by NMR. In the absence of an external substrate the energy source for NTP synthesis in this particular bacterium (*Desulfovibrio gigas*) is polyglucose, an endogenous carbon reserve which can be detected in living cells by natural abundance ^{13}C -NMR. When the metabolism of polyglucose was inhibited by addition of fluoride, an inhibitor of the glycolytic pathway, the energy status of the cells decreased drastically as demonstrated by the disappearance of the NTP resonances (Figure 1B); a

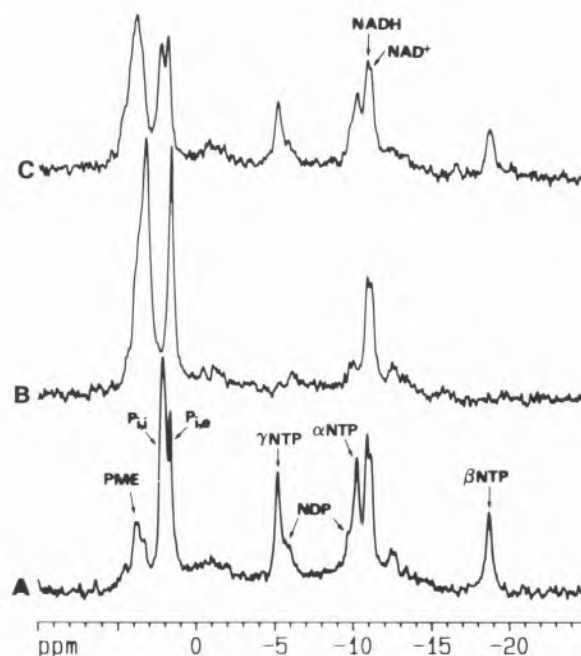


Figure 1 - *In vivo* ^{31}P -NMR spectra of a cell suspension of *Desulfovibrio gigas*. (A) initial spectrum under anaerobic conditions; (B) spectrum acquired after addition of 10 mM NaF to the cell suspension; (C) the argon atmosphere was switched to oxygen. Spectra were obtained at 202.5 MHz, at a probehead temperature of 33°C. PME, phosphomonoesters; $\text{P}_{i,i}$, intracellular inorganic phosphate; $\text{P}_{i,e}$, extracellular inorganic phosphate; NTP, nucleoside triphosphates.

concomitant increase in the intensity of the broad signal due to phosphomonoesters, indicating accumulation of phosphorylated metabolites of the glycolytic pathway, was also observed. Detailed analysis of perchloric acid extracts of cells treated with sodium fluoride showed that 3-phosphoglycerate and glycerol-3-phosphate were the two major intermediate metabolites that accumulated [39].

Most interesting is the observation that upon addition of an electron acceptor, such as oxygen, nitrite or thiosulfate, the synthesis of NTP would resume and consequently the energetic status of the cells would be nearly restored (Figure 1C). *D. gigas* is a strict anaerobe and, therefore, is unable to grow under aerobic conditions. However, as demonstrated by these experiments, it is capable of using oxygen for disposal of the reducing power derived from the utilization of internal carbon reserves, and thereby surviving under temporary aerobic conditions. In fact, not only is it able to utilize oxygen, but also the levels of cellular energization are enhanced by oxygen when aerobic and anaerobic conditions are compared. This can be appreciated in Figure 2 which illustrates an experiment where a cell suspension was subjected to repeated anaerobic/aerobic cycles: for a given cycle the levels of NTP are consis-

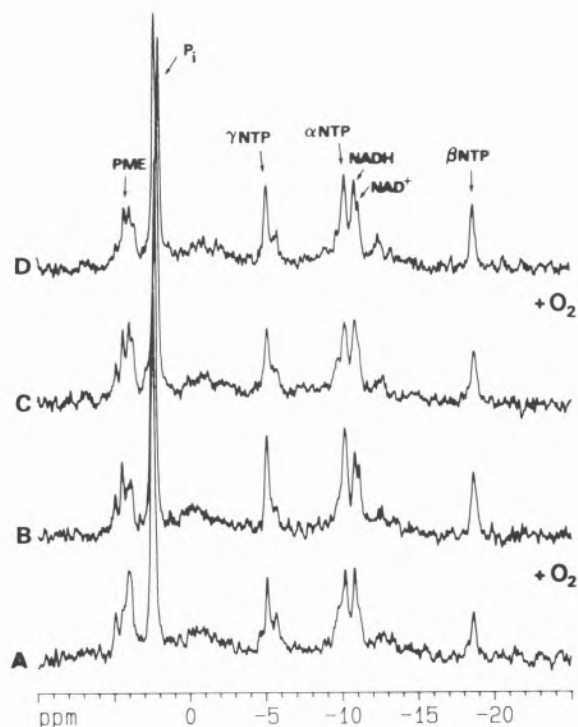


Figure 2 - Effects of alternating between anaerobic/aerobic conditions on the nucleoside triphosphates content of a cell suspension of *Desulfovibrio gigas*, as monitored by *in vivo* ^{31}P -NMR. Gases (argon or oxygen) were delivered directly to the cell suspension in the NMR tube. After acquisition of the first spectrum (A) under argon atmosphere, the gas phase was switched to oxygen and spectrum (B) was acquired; these anaerobic/aerobic cycles were repeated (spectra C-D). Spectra were obtained at 202.5 MHz, at a probehead temperature of 33°C. PME, phosphomonoesters; P_i , inorganic phosphate; NTP, nucleoside triphosphates.

tently higher when oxygen is delivered to the cells than when the cell suspension is made anaerobic by bubbling argon.

At this point it is worth stressing that the non-destructive characteristics of the NMR measurements allowed to monitor, for a single sample, changes in the levels of intracellular phosphorylated metabolites in response to different external conditions.

Detection of unusual phosphorylated metabolites

NMR provides a straightforward way for detecting the unexpected; this is a very important advantage in the studies involving living systems since unknown metabolites can be readily detected without need for separative techniques or special sample treatment. Several unusual metabolites were first detected by *in vivo* NMR. This was the case for cyclic 2,3-diphosphoglycerate, the unique compound detected in sev-

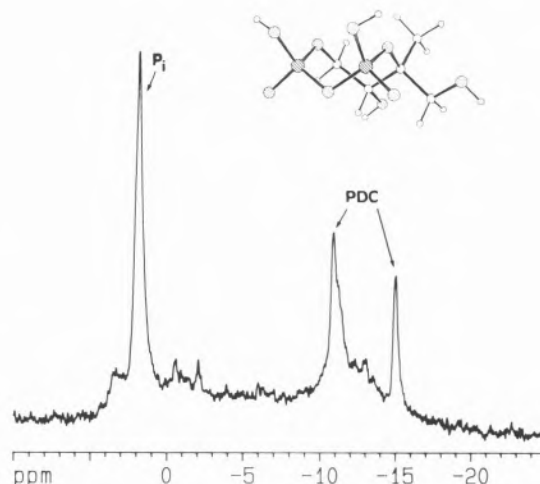


Figure 3 - *In vivo* ^{31}P -NMR spectrum of a cell suspension of *Desulfovibrio desulfuricans* ATCC 27774, showing the presence of high intracellular amounts of a novel phosphoric anhydride diester compound (3-methyl-1,2,3,4-tetrahydroxybutane-1,3-cyclic bisphosphate). The spectrum was obtained at 202.5 MHz, at 33°C. The inset shows the minimum-energy conformation found for the RS isomer of the compound. P_i , intracellular inorganic phosphate; P_i , extracellular inorganic phosphate; PDC, phosphoric anhydride diester compound.

eral strains of methanogenic archaea [40,41], and which was found to play an important role in the carbohydrate metabolism of these organisms. The presence of high amounts of a novel phosphorus-containing compound in *Desulfovibrio desulfuricans* was also revealed by *in vivo* ^{31}P -NMR [42]. The spectrum obtained from a cell suspension of this strain (Figure 3) is dominated by two strong resonances in the phosphoric anhydride diester region; the chemical shift of one of the phosphorus resonances (-14.8 ppm) is rather unusual in ^{31}P -NMR spectra of living systems, and immediately suggested the uncommon nature of this metabolite. Following extraction and partial purification, its structure and molecular conformation were fully elucidated using a combination of multinuclear NMR techniques [43], and the novel metabolite was identified as 3-methyl-1,2,3,4-tetrahydroxybutane-1,3-cyclic bisphosphate (inset in Figure 3). The intracellular concentration was found to be independent of the stage of growth, ruling out the possibility of being a phosphorus reserve material; so far the physiological role of this metabolite has not been elucidated.

3. Carbon-13 NMR in living systems

The major disadvantage of carbon-13 for NMR applications derives from the low receptivity of this nucleus which is due to a low natural abundance (1.1%) combined with a low absolute sensitivity. As a

consequence of this, the large majority of the metabolic studies utilizing this nuclide use isotopically enriched substrates. The major drawback of these experiments is related with the high cost of specifically labelled compounds; however, this disadvantage is by far overcome by the valuable information unravelled by these labelling studies which allow tracing a specific carbon atom through different metabolic pathways, identifying metabolic pathways and measuring carbon fluxes. The ^{13}C labelling/NMR detection approach allows the identification of intermediate metabolites and end-products without the need to use elaborate analytical and separation procedures and also provides details on the distribution of the label within the same molecule [11,44]. Current studies are directed towards obtaining information about *in vivo* metabolic rates and control of fluxes of central pathways such as glycolysis, gluconeogenesis, and the tricarboxylic acid cycle (TCA). Although most reactions of central pathways of metabolism are well established, there is a severe lack of information on *in vivo* regulation within the different organs and species. *In vivo* ^{13}C -NMR is an indispensable tool in these studies [11]. Examples of the utilization of this method are the determination of metabolic fluxes through the branch point of the TCA cycle and the glyoxylate shunt in *Escherichia coli* and yeast [45,46], the metabolism of pyridine nucleotides in *E. coli* and *Saccharomyces cerevisiae* [47], sugar transport processes in *Zymomonas mobilis* [48] or methanol metabolism in *Hansenula polymorpha* [49].

Labelling experiments and ^{13}C -NMR analysis have also proved very useful in biosynthetic studies. Substrates isotopically enriched on specific positions are provided to the cells and the ^{13}C enriched sites of biosynthetic molecules are identified. This approach has been used, for instance, in the elucidation of biosynthesis of aromatic compounds in yeast [50], antibiotic production by *Streptomyces parvulus* [51], alginate biosynthesis in *Pseudomonas aeruginosa* [52], isoprenoid synthesis in several bacteria [53], or nonactin biosynthesis from acetate, propionate and succinate by *Streptomyces* [54].

Although natural abundance ^{13}C -NMR is in general not useful in metabolic studies, it is the technique of choice for detection and identification of major metabolites, such as freely mobile carbon reserves or compatible solutes (small organic solutes that accumulate intracellularly in response to changes in salinity and/or temperature). In this latter area several novel organic solutes have been recently detected and characterized: *di-myo*-inositolphosphate in the hyperthermophile *Pyrococcus woesei* [55], β -mannosylglycerate in *Rhodothermus marinus* [56] and *Pyrococcus furiosus* [57].

Two examples of our own work were selected in order to illustrate the potentialities of *in vivo* ^{13}C -NMR to elucidate carbon metabolism: in the first one, lactic acid bacteria were the object of study, and information was withdrawn from the labelling pattern analysis of meta-

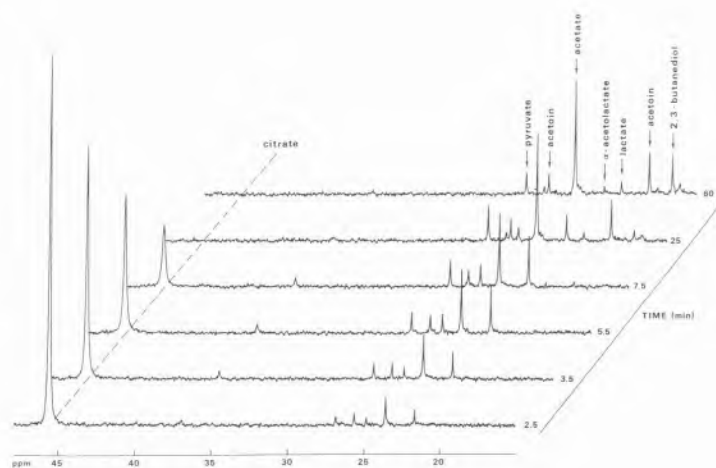


Figure 4 - ^{13}C -NMR spectra showing the time-course for the consumption of $[2,4\text{-}^{13}\text{C}]$ citrate (20 mM) by a cell suspension of *Lactococcus lactis* at pH 5.5. The spectra were obtained at 125.8 MHz, at a probehead temperature of 30°C . Each spectrum represents 30 s of accumulation.

bolites excreted to the external medium; the second example deals with polyphosphate accumulating bacteria where the flux of label is determined from analysis of intracellular carbon reserves *in vivo*.

Studies involving analysis of labelling patterns of extracellular compounds

Citrate is an important precursor of flavour compounds (acetate and diacetyl) in lactic acid bacteria, organisms playing an important role in the production of fermented foods, such as milk derivatives, olives or wine.

The metabolic pathway of citrate degradation by lactic acid bacteria was until recently subject of controversy, due to chemical instability of some of the intermediates in the catabolism, such as α -acetolactate. Two alternative pathways were proposed for the biosynthesis of flavour compounds: one involving the condensation of hydroxyethyl-thiamin pyrophosphate with acetyl-CoA and the other involving the condensation of two molecules of pyruvate to originate α -acetolactate. In the experiment shown in Figure 4, citrate labelled on carbons 2 and 4 was supplied to a cell suspension of *Lactococcus lactis*, and spectra were acquired consecutively until substrate exhaustion. Citrate was consumed at a rate of $180\ \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g (cells dry weight)}^{-1}$ and the label was found on the methyl groups of the end-products acetate, lactate, acetoin and 2,3-butanediol. Resonances due to labelled pyruvate and α -acetolactate were also transiently observed, indicating the existence of carbon flux through pyruvate, and demonstrating that α -acetolactate is in fact the intermediate metabolite in the production of acetoin and 2,3-butanediol [58].

Carbon 13 NMR was also successfully used to characterize the pathways of glucose [59] and citrate

[60] catabolism in *Oenococcus oeni*, a lactic acid bacterium involved in wine production. From the analysis of label present in the end-products (acetate, lactate and erythritol) derived from glucose selectively labelled on different carbon atoms, it was possible to elucidate the pathway for synthesis of erythritol by these wine bacteria [59].

Lactic acid bacteria involved in the production of fermented foods occur in complex media such as milk, meat and wine. Therefore, for the detailed characterization of the fermentation processes it is necessary to study the interactions between the catabolism of different substrates. The interpretation of results from experiments where multiple substrates are supplied is often difficult since the origin of carbon in the end-products cannot be exactly determined. Whenever different substrates are converted to a common intermediate metabo-

lite, the information supplied by analytical methods is insufficient to fully characterize the co-metabolic processes. This is the case for citrate and glucose metabolism in the homofermentative lactic acid bacterium *Lactococcus lactis*, where pyruvate is the central intermediate in the catabolic pathways of both sugar and citrate. By using glucose and citrate labelled on suitable positions, it is possible to determine precisely the origin of each carbon atom in the end-products. According to the metabolic pathways proposed for citrate and sugar catabolism in *L. lactis* (Figure 5), the utilization of [2,4-¹³C]citrate will produce [2-¹³C]acetate, [3-¹³C]lactate, and acetoin or 2,3-butanediol labelled on C-1 and C-4, whereas [1-¹³C]acetate, [2-¹³C]lactate and acetoin or 2,3-butanediol labelled on C-2 and C-3 are derived from the metabolism of [2-¹³C]glucose. The experiment was carried out and the extent of isotopic enrichment in the end-products evaluated from ¹H-NMR spectra of supernatant solutions obtained after centrifugation of the cell suspensions. The presence of an additional substrate caused a stimulation on the rate of citrate utilization and the pattern of end-products was changed; acetate, acetoin and 2,3-butanediol represented more than 80% of the end-products of citrate alone, however, when glucose was also added, 80% of the citrate was converted to lactate. Significant amounts of unlabelled acetate and lactate were found in these spectra, due to the utilization of endogenous reserve compounds even in the presence of exogenously added substrates.

The information derived from the above mentioned experiments can be obtained with certainty from other analytical methods only if all the end-products are determined, the carbon recoveries are 100% for each substrate, the metabolic pathways are well-known, and the contribution of endogenous reserve compounds is negligible [58].

Metabolic studies involving detection of intracellular components

In vivo ¹³C-NMR detection of intracellular pools of metabolites in bacterial suspensions metabolizing isotopically enriched substrates is often precluded by the low concentrations of metabolites and the small proportion of the sample volume occupied by intracellular space. A much more favourable situation is found with organs, such as liver, heart or brain, where many intermediates of glycolysis and the TCA cycle have been monitored *in vivo* [61-64]. Despite these unfavourable conditions, some examples of direct detection of glycolytic intermediates have been reported [65]. Here we will report on our studies on carbon metabolism by a mixed culture of polyphosphate accumulating bacteria obtained from a wastewater treatment plant at Beirolas. Conventional wastewater plants for biological phosphorus removal work on the basis of anaerobic/aerobic cycles. During the anaerobic stage

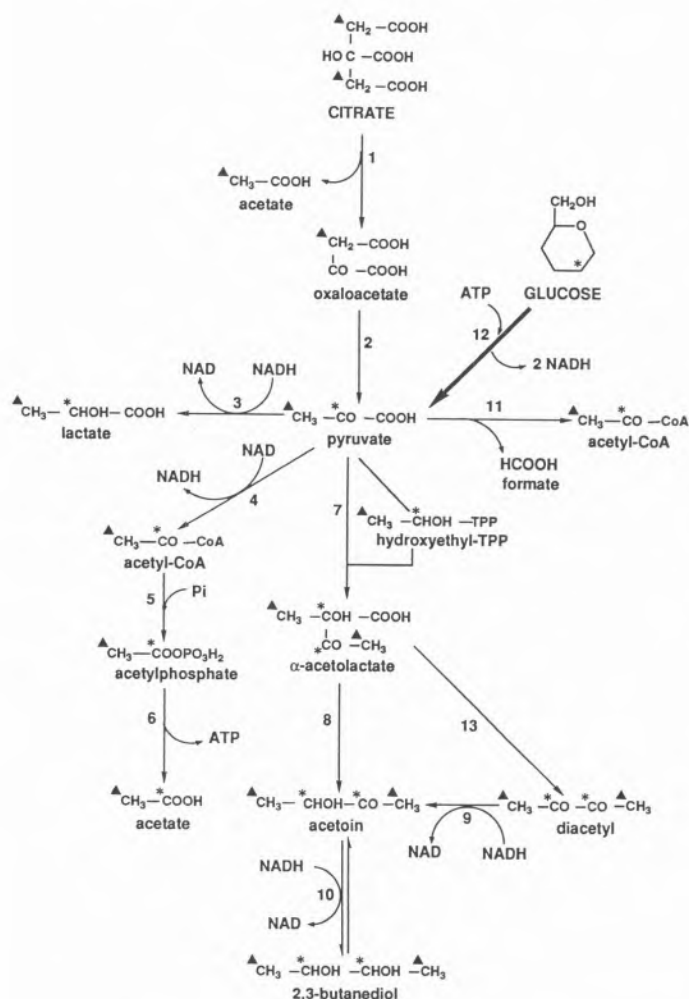


Figure 5 - Pathways of citrate and glucose metabolism in *Lactococcus lactis* showing the labelling pattern on the intermediates and end-products derived from the utilization of [2,4-¹³C]citrate (▲) and [2-¹³C]glucose (*).

inorganic phosphate is released by the biomass and carbon substrates (mainly short chain fatty acids) are taken up and stored as carbon reserves such as polyhydroxyalkanoates. In the aerobic stage carbon reserves are utilized while inorganic phosphate is taken up and intracellularly accumulated as polyphosphate [66]. Enrichment of activated sludge in polyphosphate accumulating microorganisms is only possible due to this metabolic coupling of polyphosphate hydrolysis with synthesis of carbon reserves under anaerobic conditions. In order to elucidate carbon/phosphorus metabolism in this biological system, acetate labelled with ^{13}C on the methyl group was provided to a cell suspension in the beginning of the first anaerobic stage, and the fate of label through anaerobic/aerobic cycles monitored *in vivo* by ^{13}C -NMR. It was possible to follow the flux of label from acetate to hydroxybutyrate /hydroxyvalerate co-polymer in the first anaerobic stage (Figure 6), then to monitor the conversion of these units into glycogen in a subsequent aerobic stage (Figure 7), and afterwards, by subjecting the same sludge to a second anaerobic stage, to observe the flux of labelled carbon from glycogen to the hydroxyvalerate and hydroxybutyrate units (not shown). The uptake/release of inorganic phosphate and the extracellular pH were monitored by ^{31}P -NMR in the same experiments (not shown here). The data provide an unequivocal demonstration of the involvement of glycogen in the biological phosphorus removal process. On the basis of these ^{13}C labelling data, a biochemical model for the synthesis of polyhydroxyalkanoates from acetate and glycogen was elaborated in which the tricarboxylic acid cycle is proposed as an additional source of reduction equivalents. According to this study, with 1 C-mol acetate 1.48 C-mol P(HB/HV) are synthesized, and 0.70 C-mol glycogen are degraded anaerobically, while 0.16 P-mol phosphate is released. In the aerobic stage 1 C-mol of P(HB/HV) is converted to 0.44 C-mol glycogen [67].

4. Proton NMR of living systems

Proton NMR spectra from small metabolites in living animals, perfused organs or cell suspensions should in theory be easily obtained, since the sensitivity of proton detection is much higher than that of ^{13}C or ^{31}P . Thus ^1H NMR methods should allow the detection of lower metabolite concentrations with improved time resolution when compared with ^{13}C detection, without the need of using labelled precursors. Despite these advantages, *in vivo* proton NMR has not been extensively used due to a narrow chemical shift window of ^1H and to interference from the large water signal in biological samples. At present, this can be overcome with special techniques that reduce the water resonance or selectively excite a given spectral region [15,44,68], and the number of useful *in vivo* NMR studies has increased considerably [69-71].

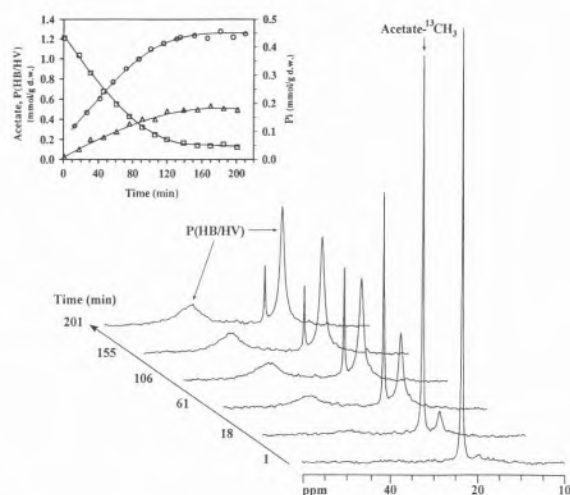


Figure 6 - Time course of phosphate release, acetate consumption and P(HB/HV) formation by activated sludge under anaerobic conditions as monitored by *in vivo* ^{13}C -NMR. The corresponding data are shown in the inset. The cell suspension was supplied with $[2-^{13}\text{C}]$ acetate at time zero. Spectra were acquired sequentially at the times indicated, alternating between phosphorus and carbon detection. Each spectrum represents 8 min of acquisition. The HB content was determined from the intensities of the resonances due to the methyl group of hydroxybutyrate at 19.7 ppm, after correction for signal saturation; Symbols: inorganic phosphate (\circ); acetate (\square); P(HB/HV) (\triangle). Resonance assignments: $\text{CH}_3(\text{B}_4)$ in P(HB/HV), 19.7 ppm; $\text{CH}_2(\text{B}_2, \text{V}_2)$ in P(HB/HV), 40 ppm; methyl group in $[2-^{13}\text{C}]$ acetate, 23.9 ppm.

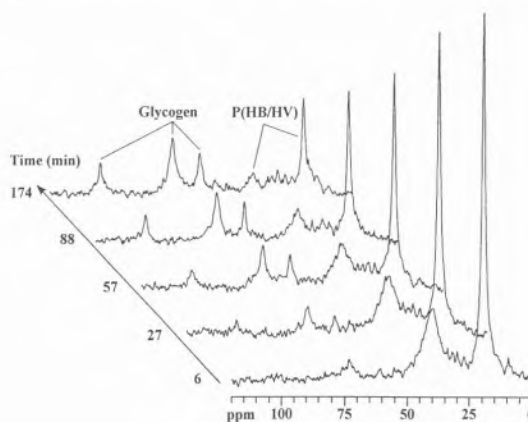


Figure 7 - Time course for the aerobic degradation of P(HB/HV) by activated sludge under aerobic conditions, as monitored *in vivo* by ^{13}C -NMR. Following the experiment shown in Figure 6, oxygen was provided at time zero and spectra were acquired sequentially at the times indicated, alternating between phosphorus and carbon detection. The decrease of resonances due to P(HB/HV) at 19.7 and 40 ppm and the build-up of glycogen (C_1 at 100.2 ppm, C_2+C_5 at 71.7 ppm and C_6 at 61.1 ppm) are clearly observed. The values represented for glycogen refer to the amounts directly detected by NMR.

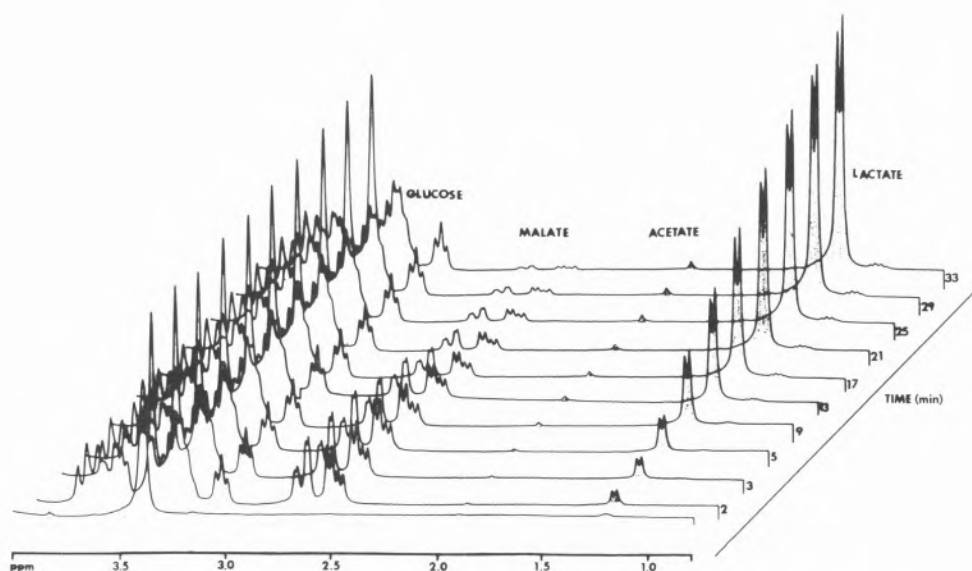


Figure 8 - Time-course for the consumption of L-malate plus glucose (45 mM each) at pH 3.5 by a cell suspension of *Oenococcus oeni* as monitored by ^1H -NMR (300.1 MHz).

^1H -NMR is also an ideal analytical technique to monitor, on line, kinetics of product formation and substrate consumption. An example is shown in Figure 8 where this technique was used to measure the activity of the malolactic enzyme in a cell suspension of *Oenococcus oeni*. Malolactic fermentation consists of the decarboxylation of L-malate to L-lactate and CO_2 , and its rate is commonly measured by manometric techniques. However, the evaluation of malolactic activity as a function of released CO_2 is only possible when carbon dioxide is not produced by other metabolic reactions. In this experiment, (Figure 8) L-malate was supplied to a cell suspension of *O. oeni* and spectra were acquired consecutively until malate exhaustion. The rate of malolactic fermentation was easily determined by integration of the relevant resonances. Furthermore, when mixed substrates are used the extent of their utilization can also be evaluated from the production of acetic acid which is readily detected by proton NMR (Miranda, M., Veiga-da-Cunha, M., Loureiro-Dias, M. C. and Santos, H., unpublished results).

5. Concluding remarks

Although metabolic studies by NMR have begun only in the 1970s, the field has expanded rapidly and is presently making major contributions to the understanding of metabolic biochemistry in relation to cell physiology. Despite the enormous progress, NMR is unlikely to replace existing methods for metabolic analysis, mainly due to intrinsic insensitivity; however, *in vivo* NMR remains as an indispensable complement because of its valuable noninvasive characteristics.

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