In situ proton-NMR analyses of Escherichia coli HB101 fermentations in $^{1}H_{2}O$ and in $D_{2}O$

Lothar Brecker,¹ Hansjörg Weber,¹ Herfried Griengl¹ and Douglas W. Ribbons¹,²

Author for correspondence: Douglas W. Ribbons. Tel: +43 316 873 8241. Fax: +43 316 873 8740. e-mail: sekretariat@orgc.tu-graz.ac.at

¹ Institute of Organic Chemistry, Technical University Graz, Stremayrgasse 16, A-8010 Graz, Austria
² Institute of Biotechnology, Technical University Graz, Petersgasse 12, A-8010 Graz, Austria

Experiments using one-dimensional Fourier-transform proton-NMR spectrometry for non-invasive analyses of microbial fermentations in situ, in vivo and in normal aqueous buffer are described. Analyses of the ‘mixed acid’ fermentation during growth of Escherichia coli on glucose and citrate were performed to identify and quantitatively estimate the concentrations of the two substrates provided and of the six products formed without sampling from the NMR tube. Identification of fermentation substrates and products was achieved by coincidence of selected diagnostic proton signals of individual compounds in the same solvent. The complete time course of growth of E. coli in the NMR tube correlated well with that of the same culture grown outside the magnet, with samples taken for proton-NMR analyses. The entire course of these in situ proton measurements during growth over 16–24 h was obtained automatically, usually unattended overnight. Thus, the utilization and formation of eight substances in the fermentation were monitored simultaneously, in normal $^{1}H_{2}O$, without sampling and individual analysis. Several metabolic changes could be readily detected during the fermentations. Additionally, the pH changes were estimated from the chemical shifts of the acetate signal as growth progressed. The effect of varying $D_{2}O$ concentrations in the solvent on growth rates and product yields was examined, and the increase in the complexity of signals given by these fermentations is described. This versatile and rapid method for the simultaneous, direct and automatic analysis of mixtures of many compounds has the potential to be extended to routine on-line analyses of industrial fermentations.

Keywords: proton-NMR, in situ analysis, deuterium-labelled products, Escherichia coli fermentations

INTRODUCTION

NMR spectroscopy is a helpful tool to analyse metabolic processes and solutes in living cells (Salhany et al., 1975) although it is quite an insensitive method (Braun et al., 1996). The NMR-active nuclei $^{31}P$, $^{13}C$ and $^{15}N$ have been used frequently (Gadian, 1983; Salhany et al., 1975; Weuster-Botz & de Graaf, 1996) to understand metabolic and physiological processes, including transport phenomena. These nuclei are present in many biological molecules and are excellent probes of metabolism and physiology. However, in the case of $^{13}C$ and $^{15}N$, the natural abundance and the sensitivities of the isotopes are very low and so the use of expensive or synthesized isotopically enriched substrates is usually necessary for sensitivity, selective spectral resolutions and short accumulation times.

Another NMR-active nucleus is the proton ($^{1}H$). It possesses two very useful properties: (i) it has a high natural abundance (99.98%) and the highest gyromagnetic ratio of all stable nuclei, leading to a high NMR sensitivity; (ii) the proton nucleus is present in almost all carbon-containing biological molecules, substrates and products. On the other hand, there are the disadvantages of a relatively small spectral range and an overwhelming $^{1}H_{2}O$ signal in the spectra which saturates the analogue digital converter of Fourier-transform (FT) spectrometers (Brown et al., 1977). So proton-
NMR measurements have been rarely used in the past to provide information about in vivo metabolic processes (Alam & Clark, 1989; Brown et al., 1977; Gaines et al., 1996; Morawski et al., 1997; Ogino et al., 1978a, b, 1980; L. Brecker, P. Urdl, W. Schmidt, H. Griengl & D. W. Ribbons, unpublished results). To avoid the large water signal in these cases, D$_2$O was used instead of H$_2$O (Gaines et al., 1996) or the water signal was suppressed with suitable pulse sequences (Guérion et al., 1991; Hore, 1989; Ogg et al., 1994, Smallcombe et al., 1995).

We have recently monitored $^1$H-NMR signal changes during enzymic reactions and anaerobic bacterial bio-transformations in situ (Brecker et al., 1999; Hickel et al., 1996; Pokorny et al., 1997, 1999). These measurements were made in H$_2$O and the water signal was suppressed by a presaturation pulse sequence (Guérion et al., 1991; Hore, 1989). Here, we describe the kinetics and stoichiometries of the mixed acid fermentation by an Escherichia coli K-12 strain, HB101, growing in the magnet, in normal H$_2$O and in D$_2$O media.

**METHODS**

**Bacterial strain and cultivation.** *E. coli* HB101 [F*<sup>−</sup>* Pro*<sup>−</sup>* Leu*<sup>−</sup>* Thi*<sup>−</sup>* RecA, Sm*<sup>+</sup>*] (ATCC 33694) was transferred from a slope into nutrient broth (≈ 5 ml) and used to inoculate mineral media (Morawski et al., 1997) supplemented with glucose (20 mM), citrate (5 mM, as chelator) and yeast extract (0.05%). After anaerobic incubation at 37 °C for 20 h, growth and gas formation had occurred and the culture was used (a loopful, ≈ 10$^6$ cells) to inoculate the same medium (0.7 ml) contained in a standard, non-sterile commercial NMR sample tube of 178 mm length and 5 mm outer diameter. It was incubated at 37 °C for 18 h in the magnet. Control experiments were done with cultures in static bottles and in NMR sample tubes outside the magnet. We used the same medium for two additional experiments, one without citrate and the other without glucose. For citrate-free media the Mg$^{2+}$ trace elements stock solution was modified by the addition of concentrated HCl (80 ml l$^{-1}$), in place of citrate to prevent the divergent and trivalent salts precipitating. Stock solution [trace magnesium element solution (TMES)/HCl] consisted of: MgSO$_4$, 7H$_2$O, 25.0 g l$^{-1}$; FeSO$_4$, 7H$_2$O, 8.0 g l$^{-1}$; CuSO$_4$, 5H$_2$O, 0.4 g l$^{-1}$; MnSO$_4$, H$_2$O, 0.4 g l$^{-1}$; CoSO$_4$, 7H$_2$O, 0.4 g l$^{-1}$; ZnSO$_4$, 2H$_2$O, 0.2 g l$^{-1}$; H$_2$BO$_3$, 0.04 g l$^{-1}$; Na$_2$MoO$_4$, 0.04 g l$^{-1}$. Ten millilitres of TMES was added to one litre of the potassium sulphate (50 mM), ammonium sulphate (10 mM), carbon (as specified) for formulation. The final proton concentration in the medium did not significantly alter the starting pH value from that obtained with media containing citrate. Cell mass was determined by OD$_{540}$ measurement and related to a standard curve.

**NMR measurements.** A 200 MHz narrow-bore magnet (Varian Gemini 200 system) and a 5 mm broad-band probe head were used. The NMR tube was rotated at 20 revolutions s$^{-1}$. A D$_2$O vortex capillary was added to the cultures to avoid $^1$H/$^2$H exchange reactions and kinetic isotope effects (Brecker et al., 1999; Kalinowski et al., 1984). For measurements with D$_2$O in the solvent, the deuterium signal was also used as an internal lock signal. The overwhelming water signal was suppressed with a presaturation method (Guérion et al., 1991; Hore, 1989), in contrast to the experiments of Ogino et al. (1978a, b, 1980). The following parameters were adjusted for a $^1$H-frequency of 200 MHz: presaturation duration of 10 s, $^1$H-pulse angle of 90°, an acquisition time of 2.0 s and a relaxation delay of 1.5 s. One hundred and twenty-eight scans were accumulated and one spectrum was provided in about 13 min. After a zero filling to 32k datapoints, the free induction decay was Fourier-transformed to spectra with a resolution of 0.11 Hz. The water signal (4-70 p.p.m.) was used as a reference. Between the spectral accumulations for the kinetic measurements, a delay time of 47 min was used. The control experiments external to the magnet were analysed only at the inoculation and end-point of the reactions.

**RESULTS**

It was our purpose to analyse the metabolism of cells growing in H$_2$O in situ without sampling of the cultures in the magnet, by one-dimensional FT $^1$H-NMR. The example chosen was the complex mixed-acid anaerobic fermentation of glucose by *E. coli* (Wood, 1961). This has been investigated by $^1$H-NMR previously by correlation methods (Ogino et al., 1978a, b, 1980) using a continuous wave spectrometer and also by Alam & Clark (1989). We used a different strain of *E. coli*, HB101, which is a standard recipient strain in genetic transfers. An inoculated mineral medium (Morawski et al., 1997) containing glucose (20 mM), citrate (5 mM) and minor quantities of trace elements and yeast extract (0.05%) to satisfy auxotrophic requirements was incubated at 37 °C in the magnet. Cell growth was homogeneous throughout the whole NMR tube without stirring and the cell mass did not sediment or float. The capillary, the growing cells and the gases produced did not lead to significant deterioration of line shapes in the $^1$H-NMR spectra, which gave satisfactory integral values and chemical shifts (see Fig. 2a, b).

**Fermentations in H$_2$O**

During the fermentations we found several products, and two intermediates formed which accumulated in detectable concentrations. These are the same compounds that Ogino et al. (1978a, b, 1980) and Alam & Clark (1989) reported in their experiments. One of the intermediates that appears at an early stage of the metabolism is pyruvate. This is a key intermediate because four of the products are formed from it, whilst phosphoenolpyruvate is the precursor of succinate (Neidhardt et al., 1987; Wood, 1961). The second detectable intermediate is formate, which later partially decomposes to hydrogen and carbon dioxide. These two products are gaseous and leave the reaction mixture. The other products that accumulated in the culture are ethanol, acetate, lactate and succinate as expected (Neidhardt et al., 1987; Wood, 1961). A basic reaction scheme for glycolytic and citrate metabolism is shown in Fig. 1.

The progress of the *E. coli* HB101 fermentation was readily observed from the $^1$H-NMR spectra determined in situ because the spectra showed identifiable signals of all compounds in solution during the measurement time.
Major products and intermediates formed by E. coli HB101 during fermentation of glucose and citrate.

A reference spectrum of all compounds is shown in Fig. 2(a). The integrals over the signals indicate the relative concentrations (±5%) of the substrates, the intermediates and the products. So major organic compounds in the culture can be detected qualitatively and determined quantitatively from specific signal integrations (±5%), without taking samples from the fermentation and without chemical analyses. A stack plot of the spectra obtained from a fermentation shows the capability and thus the wide variety of other applications of this method (Fig. 2b). The time course of the fermentation showing substrates, intermediates and all accumulated products is shown in Fig. 3, and the capability, resolution and speed of this in situ analytical method is apparent.

Though we changed the conditions in the fermentation only slightly from those used by Ogino et al. (1978a, b, 1980) and Alam & Clark (1989), we demonstrated a different time course and several relationships not previously described between the reaction conditions and the progress of the fermentation. Formate, acetate, ethanol and succinate were formed at different rates and in different quantities during the time period. Also, pyruvate accumulated to a low concentration (~0.5 mM) and remained constant. A change in metabolism took place when citrate was totally consumed. The production of acetate, ethanol and succinate then became slower and a rapid production of lactate started. A parallel metabolic change occurred from the production of formate to its dissimilation to H2 and CO2. The concentration of pyruvate (~0.5 mM) remained relatively constant.

The stoichiometric non-equivalence of acetate and ethanol production and the utilization of citrate by E. coli HB101 suggested that growth on citrate as a single carbon source could occur. This would have been counter to expectations, as a nutritional differentiation amongst the Enterobacteriaceae is made on the basis that citrate alone does not support the growth of E. coli species (Koser, 1924; Dagley, 1954). To check the nutritional phenotype of strain HB101, citrate and glucose were supplied as single carbon sources to compare the growth characteristics. Glucose, but not citrate, as sole carbon source supported the growth of this organism. The amount of yeast extract (0.05%, w/v) in the media did not support significant growth. Control fermentations were not followed in detail by NMR measurements and they are not illustrated here.

Ogino et al. (1978a, b, 1980) reported that the pH-dependent chemical shifts of the proton signals in E. coli cultures allow a measure of the pH change. The pH value of the solution can therefore be estimated from the spectra. This behaviour is very pronounced for the methyl group of acetate. However, there is no absolute relationship between the shift and the pH value because the shift depends on several other conditions (e.g. temperature and the concentrations of salts and other compounds in the solution, including D2O). Nevertheless, if the starting and end points of the pH changes and the chemical shift are measured and calibrated, the pH value can be deduced from the chemical shift of the acetate signal in every spectrum obtained. For the monitored reaction, the pH value was calculated from the chemical shift of the acetate signal by comparison with standard solutions of known pH values (Ogino et al., 1978a, b, 1980). A pKw value of 4.5 was obtained from the 1H-NMR spectra. The results from fermentations showed that the pH value first rises from 6.8 to 7.3 after 3 h, and later decreases during growth to pH 5.1 (14 h). These changes are probably the consequence of the use of citrate at the beginning, and then of the production of several acids as products from glucose.

All these results were verified with parallel fermentations in static flasks of the same divided cultures. These were sampled and analysed by 1H-NMR, and a very similar progress of the fermentations was observed. The influence of the magnetic field or of the rotation of the NMR tube can therefore be excluded, or is minimal.

Effect of D2O as solvent

Additional information is given about fermentations with different amounts of D2O in the solvent, because it is also used as a reactant in metabolism. Deuterium in the solution is consequently built into the intermediates
and products of metabolism. So the kinetic effect of the deuterium can be detected as well as proton/deuterium exchange reactions. Furthermore, the position of the deuterium in these substances and the relative ratios of protons to deuterium give helpful and detailed information for investigations of physiology and metabolism. This information can be interpreted from the $^1$H-NMR spectra because the deuterium nucleus is not directly detected by the $^1$H-NMR measurements, but it influences the shape and number of the $^4$H signals in a defined way. The relative amount of deuterium in a group of a molecule reduces the relative integral over the assigned characteristic $^4$H-signal proportionally. This is related to standard signals of known concentration and pH in solutions with and without D$_2$O. More details about the structure are revealed by the shapes of the

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Fig. 2. (a) $^1$H-NMR of all starting substrates, intermediates and products detected during the E. coli HB101 fermentation of glucose and citrate in a standard solution (100 mM of each compound in 50 mM KH$_2$PO$_4$/NaOH buffer, pH 7.1, in $^4$H$_2$O). The water signal (4–7.0 p.p.m.) is suppressed, signals between ~3.7 and 5.7 p.p.m. are partly suppressed. (b) Stack plot of a kinetic measurement of the E. coli fermentation.
The cultures were repeated with 0, 25, 50, 75 and 98% HB101 fermentation described above (Figs 1, 2b and 3). We used this technique for an investigation of the compound.

...In 98% D$_2$O media, the proton signal of the deuterated compound changes slightly in comparison to that of the fully protonated compound. These media were prepared by freeze-drying the original medium and redissolving the residue in $^1$H$_2$O enriched with D$_2$O. For these fermentations, $^1$H-NMR spectra were only taken initially and after 42 h incubation, while the progress of growth was followed by the measurement of cell density (Fig. 4). The results showed that there is a kinetic hindrance due to the use of deuterium in metabolism. The cultures with the higher amounts of D$_2$O show a much slower progress of growth, product formation and use of the substrates than the reactions with less deuterium in the solution (Fig. 4 and Table 1).

We used this technique for an investigation of the E. coli HB101 fermentation described above (Figs 1, 2b and 3). The cultures were repeated with 0, 25, 50, 75 and 98% D$_2$O in the media. In 98% D$_2$O media, the proton concentration is still very large ($\sim$ 3000 meq. l$^{-1}$) whereas carbon substrate proton concentrations of interest are $\sim$ 0.5–200 meq. l$^{-1}$. These media were prepared by freeze-drying the original medium and redissolving the residue in $^1$H$_2$O enriched with D$_2$O. For these fermentations, $^1$H-NMR spectra were only taken initially and after 42 h incubation, while the progress of growth was followed by the measurement of cell density (Fig. 4). The results showed that there is a kinetic hindrance due to the use of deuterium in metabolism. The cultures with the higher amounts of D$_2$O show a much slower progress of growth, product formation and use of the substrates than the reactions with less deuterium in the solution (Fig. 4 and Table 1). In all five fermentations there was a switch in the metabolic progress after about 8 h that was not further investigated. The apparent stoichiometries of the products formed were influenced by the amount of deuterium supplied (Table 1). We did not attempt to assess the virtual reactions (Abeles et al., 1992; Sprinson & Rittenberg, 1951) of isotope exchange from these experiments, and the quantitative distribution of the two major pathways for glucose metabolism [glycolysis ($\sim$ 80%) and the pentose phosphate cycle ($\sim$ 20%)] (Neidhardt et al., 1987) was not calculated. Only the glycolytic metabolism of E. coli is considered in our

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**Table 1. Fermentation of glucose and citrate by E. coli HB101 in varying concentrations of D$_2$O**

Values are concentrations of substrates and products in mM (meq. $^1$H$^{-1}$) calculated from selected signals, and were measured after 42 h growth at 37°C.

<table>
<thead>
<tr>
<th>Substrate or product</th>
<th>D$_2$O/$^1$H$_2$O (%)</th>
<th>98</th>
<th>75</th>
<th>50</th>
<th>25</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>9·3</td>
<td>7·3</td>
<td>6·1</td>
<td>0·0</td>
<td>0·0</td>
<td>0·0</td>
</tr>
<tr>
<td>Citrate</td>
<td>1·3</td>
<td>1·1</td>
<td>1·6</td>
<td>0·0</td>
<td>0·0</td>
<td>0·0</td>
</tr>
<tr>
<td>Succinate</td>
<td>0·7</td>
<td>0·5</td>
<td>0·5</td>
<td>0·5</td>
<td>6·0</td>
<td>6·0</td>
</tr>
<tr>
<td>Acetate</td>
<td>1·4</td>
<td>2·0</td>
<td>4·0</td>
<td>9·8</td>
<td>8·3</td>
<td>8·3</td>
</tr>
<tr>
<td>Lactate</td>
<td>9·6</td>
<td>12·6</td>
<td>11·5</td>
<td>8·0</td>
<td>9·8</td>
<td>9·8</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0·4</td>
<td>0·6</td>
<td>1·4</td>
<td>13·2</td>
<td>8·3</td>
<td>8·3</td>
</tr>
</tbody>
</table>

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**Fig. 3.** Kinetic profile of the E. coli HB101 fermentation of glucose and citrate from selected integrals of the data in Fig. 2b. □, Glucose; ■, citrate; ○, acetate; ●, formate; ●, ethanol; ▲, succinate; ×, lactate; Δ, pyruvate.

**Fig. 4.** Cell density during an E. coli HB101 fermentation of glucose and citrate with 0 (●), 50 (○) and 98 (●)% D$_2$O in the solvent (25% and 75% D$_2$O experiments not shown).

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**Fig. 5.** Acetate signals in NMR spectra of culture supernatants from *E. coli* HB101 fermentations in 0 (a) and 75 (b)% D₂O after 42 h. Culture conditions were similar to those of Fig. 3, with D₂O-enriched ¹H₂O solvent (cf. Fig. 6).

**Fig. 6.** Theoretical composition of the acetate signal group in a solution with 75% D₂O. Sixteen per cent of the acetate molecules formed have a CH₃ group. This group induces a singlet in the ¹H-NMR spectrum at 1.896 p.p.m. with a relative integral of 16×3 protons. Fifty-six per cent of the acetate molecules have a CDH₂ group, showing a ‘triplet’ at 1.884 p.p.m. with a relative integral of 56×2 protons, and 28% possess a CDH₂ group inducing a ‘quintet’ at 1.872 p.p.m. with a relative integral of 28×1 proton. Summation of these three signals forms the signal group shown on the right. A detailed explanation is given in the text.

discussion. The relative ratios of the products were different from the fermentations with and without D₂O but were not related to the D₂O content (Table 1). On the other hand, calculations from the integrals showed that the amounts of deuterium in the products were related to the D₂O content in the solvent. There was an isotopic discrimination of the species of succinate formed with D₂O in all cases and also, to a lesser extent, exchange into acetate. The most interesting fact is that lactate was produced as the major product in D₂O although there was still citrate in the solution. So the time course of the formation of fermentation products
was also influenced by the amount of deuterium in the water.

The deuterium exchange reactions to give deuterated metabolites during the fermentations showed that protium was not preferred to deuterium. The change in the signal structure and in the chemical shifts in isotopic products obtained gives additional information about metabolism. An example is the comparison of the spectra of the acetate signals from fermentations with 0 and 75% D₂O (Fig. 5). In the case of 0% D₂O, the methyl group of acetate gave the usual singlet (~ 2.00 p.p.m.). In the 75% D₂O solution three acetate species with different amounts of deuterium should be produced. These are about 16% with a CH₂-group, 56% with a CH₃D-group and 28% with a CHD₂-group, as calculated from the glycolytic pathway (Neidhardt et al., 1987) for the case of no ¹H/D discrimination. A CH₃D group is not formed because one proton in the product acetate does not exchange with protium/deuterium from the solvent during metabolism (Neidhardt et al., 1987). The three isotopic species of acetate generated showed different signals with slightly altered chemical shifts that partly overlapped. The signals in the deuterium-enriched solution showed a shift compared to the normal acetate signal in ¹H₂O. The whole signal group consisted of three individual signals. The first one is a singlet formed from the CH₃ group at 1.896 p.p.m. that has an integral of 3 x 16 (3 protons x 16%). The CH₃D group generates a ‘triplet’ at 1.884 p.p.m. with a coupling constant of ²JH-D = 2.4 Hz and an integral of 2 x 56 (2 protons x 56%) over the whole signal. A ‘quintet’ is formed from the CHD₂ group. For a detailed explanation of the D-couplings causing a ‘quintet’, see Sanders & Hunter (1987). The chemical shift of this ‘quintet’ is 1.872 p.p.m., with a ³JH-D coupling constant of 2-4 Hz and an integral of 1 x 28 (1 proton x 28%). The overlap of the signals leads to a signal group of five lines in the relative ratio of about 30:15:18:3:1 (Figs 5 and 6). This distribution of deuterium into acetate indicates that the use of deuterium is not discriminated against relative to the use of hydrogen except in the kinetics of the fermentation. The results also fitted exactly the use of water in metabolism that was predicted (Neidhardt et al., 1987).

### DISCUSSION

The main purpose of this report is to demonstrate the advantages of using FT ¹H-NMR experiments for non-invasive analyses of anaerobic fermentations. A detailed discussion of the biochemical data obtained is premature and further investigations are required.

### The ¹H-NMR measurement technique

A non-invasive analytical technique to obtain information about metabolism is desirable. ¹H-NMR spectroscopy is, in spite of its low sensitivity, such a method. Spectra can be taken directly in situ at any stage of the fermentation without sampling the culture. This can be done simultaneously for several compounds, automatically and without attention overnight (Figs 2b and 3). The increase in cell mass only slightly influences the quality of the spectra, so that measurements in vivo and during growth are possible. Additionally, the large number of protons in ¹H₂O is not a significant problem, although they are also detected by our method, because the signal can be suppressed by special measurement techniques (Gueron et al., 1991; Hore, 1989; Ogg et al., 1994; Smallcombe et al., 1995). The suppression of the ¹H₂O signal also partially suppresses signals from solutes in the 3.7–5.7 p.p.m. range, but the extent of this can be easily assessed by reference to standard solutions (Fig. 2a).

These three advantages (in situ, in vivo and in aqua) as well as simultaneous analysis of several substrates and products and of pH changes distinguish the method from other analytical techniques. If there are no samples taken from a culture there is no possibility of influencing the fermentation process or perturbing the experimental conditions. This makes the method superior to all invasive techniques and analyses. Additionally, the data are collected directly from the growing cultures. There is no loss of time, and expensive labour is reduced. Further, the structures and the concentrations of the detected substances are not influenced by losses due to self-decomposition during concentration, chemical or physical treatments, or extraction, separation, chromatography or derivatization. The separation of each compound is automatically achieved via the characteristic signals in the spectra obtained.

The ¹H-NMR method described has more advantages than disadvantages compared with the use of other NMR-active nuclei. One reason is its much higher sensitivity because of a natural abundance of 99-98% and the largest gyromagnetic ratio of all nuclei (Braun et al., 1996). It is not necessary to use isotopically labelled compounds except for specific purposes. A second reason is the presence of protons in all organic molecules so that most accumulated metabolites in concentrations of ≥ 1–2 meq. ¹H·¹ can be detected. Other advantages are the distinguishing shifts of each signal and the ¹H-couplings that lead to characteristic signal forms for identification of isotopically formed species (Figs 5 and 6).

There are also some disadvantages of the ¹H-NMR method. The spectral range for the protons is low (~ 12 p.p.m.) so that signals may overlap. This can lead to difficulties in the interpretation of some more complex spectra, for example carbohydrates and other signals close to the suppressed ¹H₂O signal. Other problems are a relatively low sensitivity in comparison to other analytical methods and an inherent error of about ± 5% in the integrations of signals. Many of these reasons are of less consequence when magnets with higher fields are used, for example 600 MHz.

Further possible problems are the presence of a deuterated compound (D₂O) in the sample that is used routinely for a lock. If it is in the solvent, it can lead to...
several influences on the fermentation. There can be kinetic isotope effects, $^1$H/$^2$H exchanges or inhibition of the fermentations as we show (Fig. 4 and Table 1). This can be circumvented by the use of a $^2$H$_2$O capillary added to the NMR tube (Brecker et al., 1999; Kalinowski et al., 1984) external to the reaction mixture. On the other hand, $^2$H$_2$O in the solvent can be a helpful probe to analyse kinetic isotope effects and isotope distributions. Additional information can be obtained about the preferential use of the isotope when $^2$H$_2$O is part of the solvent used in metabolism. The incorporation of deuterium into the intermediates and products gives further insights into metabolism.

### E. coli HB101 fermentation in $^1$H$_2$O and $^2$H$_2$O

Monitoring the time course of the E. coli HB101 fermentations by $^1$H-NMR revealed a large number of details concerning metabolic events as the cultures progressed (e.g. Fig. 2b and Fig. 3). First, citrate (5 mM) supplied as a metal ion chelator in the media was used preferentially to glucose during growth. Citrate alone does not support growth (Dagley, 1954; Lütgens & Gottschalk, 1980), and this characteristic is routinely used to differentiate E. coli strains from other members of the Enterobacteriaceae (Koser, 1924). Lütgens & Gottschalk (1980) convincingly showed that citrate (20 mM) was metabolized by E. coli B when a co-substrate, such as glucose, l-lactate or peptone, was also provided. From chemical and enzymic assays they concluded that the co-substrates were required to provide reducing equivalents to reduce oxaloacetate formed by citrate lyase (Fig. 1).

In the absence of a co-substrate, oxaloacetate is formed from citrate (Dagley, 1954; Lütgens & Gottschalk, 1980). Enzymes for metabolism of oxaloacetate to C$_4$ compounds (pyruvate or phosphoenolpyruvate), for example oxaloacetate decarboxylase and malic enzyme, are absent or of very low activity, respectively (Lütgens & Gottschalk, 1980). Oxaloacetate is thus an electron sink for the formation of succinate, whilst inability to form C$_4$ intermediates from citrate can account for the lack of growth on citrate alone. This conclusion is supported by the high acetate to succinate ratio in glucose plus citrate fermentations (Figs 2b and 3), compared with the almost equimolar formation of acetate and ethanol with glucose alone (Wood, 1961).

The use of deuterium in the solvent for the fermentation provides evidence that water is used as co-substrate. Growth rates progressively decreased with increased $^2$H$_2$O in the media (Fig. 4). The number and positions of the deuterium atoms bonded to carbon atoms in the metabolites show which hydrogen atoms come from the substrate (glucose or citrate) and which from the $^2$H$_2$O in the solvent. This is discussed for acetate. Acetate is formed from the C-1 and C-2 and from the C-5 and C-6 carbon atoms of glucose in equal amounts by glycolysis (Neidhardt et al., 1987; Wood, 1961). In acetate, one or two hydrogen atoms come from the substrate and are retained at the same carbon atom. The other hydrogen atoms are derived from the water and should have the same ratio of $^1$H/$^2$H in the metabolite as in the solvent. This is exactly the situation observed in all fermentations with different amounts of $^2$H$_2$O which we describe here (Figs 5 and 6). The E. coli HB101 strain therefore does not prefer one hydrogen isotope to the other, except kinetically.

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