***In situ* proton-NMR analyses of *Escherichia coli* HB101 fermentations in $^1\text{H}_2\text{O}$ and in D_2O**

Lothar Brecker,¹ Hansjörg Weber,¹ Herfried Griengl¹
and Douglas W. Ribbons^{1,2}

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 $^1\text{H}_2\text{O}$, 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_2O 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 (^1H). 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 $^1\text{H}_2\text{O}$ signal in the spectra which saturates the analogue digital converter of Fourier-transform (FT) spectrometers (Brown *et al.*, 1977). So proton-

Abbreviation: FT, Fourier-transform.

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, D₂O was used instead of ¹H₂O (Gaines *et al.*, 1996) or the water signal was suppressed with suitable pulse sequences (Guéron *et al.*, 1991; Hore, 1989; Ogg *et al.*, 1994; Smallcombe *et al.*, 1995).

We have recently monitored ¹H-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 ¹H₂O and the water signal was suppressed by a presaturation pulse sequence (Guéron *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 ¹H₂O and in D₂O media.

METHODS

Bacterial strain and cultivation. *E. coli* HB101 [F⁻ Pro⁻ Leu⁻ Thi⁻ (r_k⁻ m_k⁻) RecA, Sm^R] (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, ~10⁶ 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 Mg²⁺ trace elements stock solution was modified by the addition of concentrated HCl (8.0 ml l⁻¹), in place of citrate to prevent the divalent and trivalent salts precipitating. Stock solution [trace magnesium element solution (TMES)/HCl] consisted of: MgSO₄·7H₂O, 25.0 g l⁻¹; FeSO₄·7H₂O, 8.0 g l⁻¹; CuSO₄·5H₂O, 0.4 g l⁻¹; MnSO₄·H₂O, 0.4 g l⁻¹; CoSO₄·7H₂O, 0.4 g l⁻¹; ZnSO₄·2H₂O, 0.2 g l⁻¹; H₃BO₃, 0.04 g l⁻¹; Na₂MoO₄, 0.04 g l⁻¹. Ten millilitres of TMES was added to one litre of the potassium phosphate (50 mM), ammonium sulphate (10 mM), carbon (as specified) 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 OD₅₇₀ 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 D₂O vortex capillary was added to the cultures to avoid ¹H/D exchange reactions and kinetic isotope effects (Brecker *et al.*, 1999; Kalinowski *et al.*, 1984). For measurements with D₂O in the solvent, the deuterium signal was also used as an internal lock signal. The overwhelming water signal was suppressed with a presaturation method (Guéron *et al.*, 1991; Hore, 1989), in contrast to the experiments of Ogino

et al. (1978a, b, 1980). The following parameters were adjusted for a ¹H-frequency of 200 MHz: presaturation duration of 1.0 s, ¹H-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 ¹H₂O *in situ* without sampling of the cultures in the magnet, by one-dimensional FT ¹H-NMR. The example chosen was the complex mixed-acid anaerobic fermentation of glucose by *E. coli* (Wood, 1961). This has been investigated by ¹H-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 ¹H-NMR spectra, which gave satisfactory integral values and chemical shifts (see Fig. 2a, b).

Fermentations in ¹H₂O

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 ¹H-NMR spectra determined *in situ* because the spectra showed identifiable signals of all compounds in solution during the measurement time.

Fig. 1. 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 ($\pm 5\%$) 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 ($\pm 5\%$), 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 metab-

olism 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 H_2 and CO_2 . 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 the 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 D_2O). 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 pK_a 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 D_2O as solvent

Additional information is given about fermentations with different amounts of D_2O in the solvent, because it is also used as a reactant in metabolism. Deuterium in the solution is consequently built into the intermediates

Fig. 2. (a) ^1H -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 $\text{KH}_2\text{PO}_4/\text{NaOH}$ buffer, pH 7.1, in $^1\text{H}_2\text{O}$). The water signal (4.70 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.

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 ^1H -NMR spectra because the deuterium nucleus is not

directly detected by the ^1H -NMR measurements, but it influences the shape and number of the ^1H signals in a defined way. The relative amount of deuterium in a group of a molecule reduces the relative integral over the assigned characteristic ^1H -signal proportionally. This is related to standard signals of known concentration and pH in solutions with and without D_2O . More details about the structure are revealed by the shapes of the

Fig. 3. Kinetic profile of the *E. coli* HB101 fermentation of glucose and citrate from selected integrals of the data in Fig. 2(b). $\square$, Glucose; $\blacksquare$, citrate; $\circ$, acetate; $\blacklozenge$, formate; $\bullet$, ethanol; $\blacktriangle$, succinate; $\times$, lactate; $\triangle$, pyruvate.

signals. A deuterium nucleus has a nuclear spin of $I = 1$ and shows a coupling to all ^1H nuclei in geminal and vicinal positions. Each of these proton signals is then divided into three equidistant lines with the same integral. This signal is designated as a 'triplet' in this article. For a detailed explanation of D-couplings, see Sanders & Hunter (1987). Additionally, the shift of the proton signal of the deuterated compound changes slightly in comparison to that of the fully protonated compound.

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_2O in the media. In 98% D_2O media, the proton concentration is still very large (~ 3000 meq. l^{-1}) whereas carbon substrate proton concentrations of interest are ~ 0.5 – 200 meq. l^{-1} . These media were prepared by freeze-drying the original medium and redissolving the residue in $^1\text{H}_2\text{O}$ enriched with D_2O . For these fermentations, ^1H -NMR spectra were only taken initially and after 42 h incubation, while the progress of

Fig. 4. Cell density during an *E. coli* HB101 fermentation of glucose and citrate with 0 ($\bullet$), 50 ($\circ$) and 98 ($\blacklozenge$)% D_2O in the solvent (25% and 75% D_2O experiments not shown).

Table 1. Fermentation of glucose and citrate by *E. coli* HB101 in varying concentrations of D_2O

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

Substrate or product	$\text{D}_2\text{O}/^1\text{H}_2\text{O}$ (% v/v):				
	98	75	50	25	0
Glucose	9.3	7.3	6.1	0.0	0.0
Citrate	1.3	1.1	1.6	0.0	0.0
Succinate	0.7	0.5	0.5	0.5	6.0
Acetate	1.4	2.0	4.0	9.8	8.3
Lactate	9.6	12.6	11.5	8.0	9.8
Ethanol	0.4	0.6	1.4	13.2	8.3

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_2O 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

Fig. 5. Acetate signals in NMR spectra of culture supernatants from *E. coli* HB101 fermentations in 0 (a) and 75 (b) % D_2O after 42 h. Culture conditions were similar to those of Fig. 3, with D_2O -enriched ^1H_2O solvent (cf. Fig. 6).

Fig. 6. Theoretical composition of the acetate signal group in a solution with 75% D_2O . Sixteen per cent of the acetate molecules formed have a CH_3 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 CDH_2 group, showing a 'triplet' at 1.884 p.p.m. with a relative integral of 56×2 protons, and 28% possess a CDH_2 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 D_2O but were not related to the D_2O content (Table 1). On the other hand, calculations from the integrals showed that the amounts of deuterium in the products were related to the D_2O content in the solvent. There was an

isotopic discrimination of the species of succinate formed with D_2O 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 D_2O 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 CH₃-group, 56% with a CH₂D-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 CD₃ 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 × 16 (3 protons × 16%). The CH₂D group generates a 'triplet' at 1.884 p.p.m. with a coupling constant of ²J_{H-D} = 2.4 Hz and an integral of 2 × 56 (2 protons × 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 ²J_{H-D} coupling constant of 2.4 Hz and an integral of 1 × 28 (1 proton × 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 (Guéron *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 l⁻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, $^1\text{H}/\text{D}$ exchanges or inhibition of the fermentations as we show (Fig. 4 and Table 1). This can be circumvented by the use of a D_2O capillary added to the NMR tube (Brecker *et al.*, 1999; Kalinowski *et al.*, 1984) external to the reaction mixture. On the other hand, D_2O 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 D_2O 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 $^1\text{H}_2\text{O}$ and D_2O

Monitoring the time course of the *E. coli* HB101 fermentations by ^1H -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_3 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_3 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 D_2O 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 D_2O 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 $^1\text{H}/\text{D}$ in the metabolite as in the solvent. This is exactly the situation observed in all fermentations with different amounts of D_2O 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.

ACKNOWLEDGEMENTS

Support from Österreichische Nationalbank, project number 6404, and from the Austrian Science Foundation, projekt number P12763-CHE, is gratefully acknowledged. We also thank J. Plavec and M. Polak, National Institute of Chemistry, Ljubljana (Slovenia), for determining some spectra on a Varian Unity 600 MHz spectrometer. W. Steiner and G. Straganz (Technical University Graz) contributed substantially to this work.

REFERENCES

- Abeles, R. H., Frey, P. A. & Jencks, W. A. (1992). *Biochemistry*. Boston & London: Jones & Bartlett.
- Alam, K. Y. & Clark, D. P. (1989). Anaerobic fermentation balance of *Escherichia coli* as observed by in vivo nuclear magnetic resonance spectroscopy. *J Bacteriol* **171**, 6213–6217.
- Braun, S., Kalinowski, H.-O. & Berger, S. (1996). *100 and More Basic NMR Experiments*. Weinheim: VCH.
- Brecker, L., Pogorevc, M., Griengl, H., Steiner, W., Kappe, T. & Ribbons, D. W. (1999). Synthesis of 2,4-diketoacids and their aqueous solution structures. *New J Chem* **23**, 437–446.
- Brown, F. F., Campbell, I. D., Kuchel, P. W. & Rabenstein, D. C. (1977). Human erythrocyte metabolism studies by ^1H spin echo NMR. *FEBS Lett* **82**, 12–16.
- Dagley, S. (1954). Dissimilation of citric acid by *Escherichia coli* and *Aerobacter aerogenes*. *J Gen Microbiol* **11**, 218–227.
- Gadian, D. G. (1983). *Nuclear Magnetic Resonance and its Application to Living Systems*. Oxford: Clarendon Press.
- Gaines, G. L., Smith, L. & Neidle, E. L. (1996). Novel nuclear magnetic resonance spectroscopy methods demonstrate preferential carbon source utilisation by *Acinetobacter calcoaceticus*. *J Bacteriol* **178**, 6833–6841.
- Guéron, M., Plateau, P. & Decorps, M. (1991). Solvent signal suppression in NMR. *Prog NMR Spectrosc* **23**, 135–209.
- Hickel, A., Gradnig, G., Griengl, H., Schall, M. & Sterk, H. (1996). Determinations of the time course and enzymic reaction by ^1H -NMR spectrometry: hydroxynitrile lyase catalysed transhydrocyanation. *Spectrochim Acta Part A Mol Spectrosc* **52**, 93–96.
- Hore, J. P. (1989). Solvent suppression. *Methods Enzymol* **176**, 64–77.
- Kalinowski, H.-O., Berger, S. & Braun, S. (1984). ^{13}C -NMR-Spektroskopie, p. 72. Stuttgart & New York: Georg Thieme Verlag.
- Koser, S. A. (1924). Correlations of citrate utilization by members of the colon-aerogenes group with other differential characteristics with habitat. *J Bacteriol* **9**, 59–77.
- Lütgens, M. & Gottschalk, G. (1980). Why a co-substrate is required for anaerobic growth of *Escherichia coli* on citrate. *J Gen Microbiol* **119**, 63–70.
- Morawski, B., Eaton, R. W., Rossiter, J. T., Guoping, S., Griengl, H. & Ribbons, D. W. (1997). 2-Naphthoate catabolic pathway in *Burkholderia* strain JT 1500. *J Bacteriol* **179**, 115–121.

- Neidhardt, F. C., Ingraham, J. L., Brooks Low, K., Magasanik, B., Schaechter, M. & Umberger, H. E. (editors) (1987). *Escherichia coli* and *Salmonella typhimurium: Cellular and Molecular Biology*. Washington, DC: American Society for Microbiology.
- Ogg, R. J., Kingsley, P. B. & Taylor, J. S. (1994). WET, a T_1 - and B_1 -insensitive water-suppression method for in vivo localized ^1H NMR spectroscopy. *J Magn Reson B* **104**, 1–10.
- Ogino, T., Arata, Y., Fujiwara, S., Shoun, H. & Beppu, T. (1978a). Use of proton correlation NMR spectroscopy in the study of living cells. Anaerobic metabolism of *Escherichia coli*. *J Magn Reson* **31**, 523–526.
- Ogino, T., Arata, Y., Fujiwara, S., Shoun, H. & Beppu, T. (1978b). Proton correlation nuclear magnetic resonance study of anaerobic metabolism of *Escherichia coli*. *Biochem* **17**, 4742–4745.
- Ogino, T., Arata, Y. & Fujiwara, S. (1980). Proton correlation nuclear magnetic resonance study of metabolic regulations and pyruvate transport in anaerobic *Escherichia coli* cells. *Biochem* **19**, 3684–3691.
- Pokorny, D., Steiner, W. & Ribbons, D. W. (1997). β -Ketolases – forgotten hydrolytic enzymes? *Trends Biotechnol* **15**, 291–296.
- Pokorny, D., Brecker, L., Pogorevc, M., Steiner, W., Kappe, T. & Ribbons, D. W. (1999). Proton-nuclear magnetic resonance analyses of the substrate specificity of a β -ketolase from *Pseudomonas putida*, acetopyruvate hydrolase. *J Bacteriol* **181**, 5051–5059.
- Salhany, J. M., Yamane, T., Shulman, R. G. & Ogawa, S. (1975). High resolution ^{31}P nuclear magnetic resonance studies of intact yeast cells. *Proc Natl Acad Sci USA* **72**, 4966–4970.
- Sanders, K. M. & Hunter, B. K. (1987). *Modern NMR Spectroscopy*. Oxford/New York/Toronto: Oxford University Press.
- Smallcombe, S. H., Patt, S. L. & Keifer, P. A. (1995). WET solvent suppression and its applications to LC NMR and high-resolution spectroscopy. *J Magn Reson A* **117**, 295–303.
- Sprinson, D. B. & Rittenberg, D. (1951). Nature of the activation process in enzymatic reactions. *Nature* **167**, 484.
- Weuster-Botz, D. & de Graaf, A. A. (1996). Reaction engineering methods to study intracellular metabolite concentrations. *Adv Biochem Eng Biotechnol* **54**, 75–108.
- Wood, W. A. (1961). Fermentation of carbohydrates and related compounds. In *The Bacteria*, vol. II, pp. 59–149. Edited by I. C. Gunsalus & R. Y. Stanier. New York/San Francisco/London: Academic Press.

Received 2 July 1999; revised 16 August 1999; accepted 19 August 1999.