

Original article

In vivo ^{13}C NMR studies of glucose catabolism by isolated rumen bacteria

J.P. Grivet ¹, J. Stevani ², G. Hannequart ² and M. Durand ²

¹ Centre de Biophysique Moléculaire, CNRS, 1A, avenue de la Recherche-Scientifique, 45071 Orléans Cedex 2 et Département de Physique, Université d'Orléans, France;

² Station de Recherches de Nutrition, INRA, 78350 Jouy-en-Josas, France

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Summary — Nuclear magnetic resonance (NMR) was used to study the metabolism of the rumen bacterial ecosystem. The fermentation of 1- ^{13}C -glucose was investigated. This system showed a transient build-up and decay of lactate labelled at the C_3 position. The degradation of lactate gave rise to volatile fatty acids. These results agree with *in vivo* data on diets rich in available carbohydrates.

^{13}C -NMR — glucose fermentation (rumen bacteria) — lactate — volatile fatty acids

Résumé — Etude en RMN du catabolisme du glucose 1- ^{13}C par des bactéries isolées du rumen. La RMN, technique non invasive, a été appliquée pour la première fois à l'étude du métabolisme de l'écosystème bactérien du rumen. Cette technique a permis de visualiser rapidement les cinétiques d'apparition et de disparition des divers métabolites de la dégradation du 1- ^{13}C glucose. Sa fermentation montre l'accumulation transitoire de lactate puis sa dégradation en acides gras volatils. Le marquage spécifique des molécules permet de mettre en évidence les principales voies métaboliques utilisées par les micro-organismes du rumen. Nos résultats sont en accord avec ceux des expériences menées *in vivo* avec des régimes concentrés.

^{13}C -RMN — fermentation de glucose (bactéries du rumen) — lactate — acides gras volatils

Introduction

The unique ability of ruminants to digest fibrous carbohydrates and to use non-protein nitrogen is due to the microbial activity which takes place in the reticulo-rumen. Three main groups of anaerobic microbes, bacteria, protozoa and fungi

interact and contribute to the huge fermentative activity of the rumen ecosystem. Diversity within the bacterial group is great. Total numbers of bacteria are $> 10^{10} \text{ ml}^{-1}$ and more than 20 different types attain numbers $> 10^7 \text{ ml}^{-1}$ (Hungate, 1966). Bacteria produce many different enzymes which allow the degra-

dation of polymers such as cellulose and hemicellulose into monosaccharides (hexoses and pentoses), which are then fermented. The relative amounts of the short-chain fatty acids and gas (CO_2 and CH_4) produced influence ruminant nutrition. Therefore, in order to manipulate rumen metabolism, it is important to know the primary fermentation products and the catabolic pathways used by the bacterial ecosystem under various conditions. However, the classical methods (Hungate, 1966) or ^{14}C -labelling techniques (Wallnöfer *et al.*, 1966) previously used are time-consuming and laborious; in this respect, *in vivo* nuclear magnetic resonance (NMR) is a promising technique which today is widely used for non-invasive studies of tissue and organ metabolisms (for recent reviews, see Avison *et al.*, 1986; Brindle and Campbell, 1987).

The main advantage of the NMR method is that it allows precise kinetic studies; all free metabolites present in the medium can be detected simultaneously, provided their concentrations and specific activity are high enough.

In this preliminary report, we describe the application of *in vivo* NMR methods to an investigation of glucose catabolism by bacteria isolated from the ovine rumen using 1- ^{13}C -glucose. As shown below, this system revealed the transient formation of lactate and the production of the main end-products, *i.e.* volatile fatty acids.

Materials and methods

Three Ile-de-France wether sheep, each equipped with simple rumen cannulas, were used to provide fresh rumen contents. The animals were given a mixed diet (barley straw 53, sugar beet pulp 26, tapioca 18 and urea 3). Samples of rumen digesta, taken 1 h after the morning meal, were combined and strained through 4 layers of surgical gauze. Bacteria

from the liquid phase were separated by differential centrifugation (Smith and McAllan, 1974). The bacterial pellet (40 g wet weight) harvested from 1 L of rumen fluid was washed twice with the NMR buffer, pH 6.8 (100 mM PIPES, 50 mM MES, 85 mM NaCl, 10 mM KH_2PO_4 , 10 mM K_2HPO_4 , 1 mM $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ purchased from Sigma) and suspended in a volume of buffer equal to the weight of the pellet. All centrifugations were performed under an N_2 atmosphere at 4°C. The density of the cell suspension was $\approx 10^{11}$ bacteria ml^{-1} . The bacterial suspension was conserved under N_2 at 4°C up to its utilization (usually 12 h).

Cell suspensions for NMR were transferred under a stream of nitrogen to a sample tube sealed by a screw-cap and an elastomer septum. A hypodermic needle provided a vent for gaseous fermentation products. At time zero, labelled glucose was injected to a final concentration of 40 mM, which was about the quantity used in NMR studies on anaerobic bacteria (50 mM; Ezra *et al.*, 1983).

1- ^{13}C -glucose was obtained from Sigma and used as received; a 0.3 M stock solution was prepared containing $\approx 60\%$ of the β -anomer. The labelling was essentially total, as judged from the NMR spectrum.

Perchloric acid extracts were prepared for NMR spectroscopy using a method adapted from Herrero *et al.* (1985) and Nicolay *et al.* (1982) as follows. Four ml of cell suspension was sealed under nitrogen and brought to 39°C; the glucose solution was injected at time zero in order to reach a final concentration of 20 mM. At time T_e , the metabolism was quenched by adding ice-cold 70% perchloric acid until a final concentration of 6% (v/v) was obtained. The sample was vigorously shaken (Vortex) for 5 min, then frozen and thawed 3 times. The extract was centrifuged at 27 000 *g* for 10 min to remove cell debris. The supernatant was neutralized with KOH and again centrifuged at 3 000 *g* for 5 min to remove the precipitate of potassium perchlorate. To minimize the effect of paramagnetic impurities, EDTA was added to a final concentration of 20 mM. The pH was adjusted to ≈ 7 . The whole procedure was repeated for each value of T_e (0, 0.5, 1, 5, 15, 30 and 60 min) and the extracts were stored at -80°C until their spectra could be recorded.

NMR spectra were obtained at 75.4 MHz on a Bruker AM300 spectrometer under broadband proton decoupling (Waltz-16; see Shaka *et al.*, 1983) at 312 K in 10-mm tubes. The

following conditions were generally used for kinetic runs : 16 K data points for a spectral width of 18 500 Hz (240 ppm); 45° pulse angle, 1-sec relaxation delay; 300 scans : digital filtering equivalent to a 5 Hz broadening. Distorsionless enhanced polarisation transfer (DEPT) spectra (Pegg, Doddrell and Bendall, 1982) obtained on perchloric acid extracts, were recorded using generally similar conditions, except that a 1-Hz filter was used and between 500—1 000 scans. The sequence was optimized for $J_{CH} = 130$ Hz, and the conversion pulse was chosen so that CH and CH_3 signals would be positive and CH_2 lines negative; 10% (v/v) of 2H_2O was added to the extracts to provide a field-frequency lock. Cell suspensions were run without a lock. Spectra were referenced relative to either the β -glucose C-1 peak at 96.7 ppm or the resonance of dioxane at 67.4 ppm.

Resonances of perchloric acid extracts were assigned using literature values, spectra of authentic compounds recorded under similar

concentration and pH conditions and, in some cases, by adding the suspected compound to the solution and recording a new spectrum. These assignments were then used for the *in vivo* spectra.

Results

Figure 1 shows the aliphatic region of a DEPT spectrum of a perchloric extract prepared at $T_e = 5$ min. The following compounds and labelling positions have been assigned : C_3 -propionate, C_3 -lactate, C_2 -acetate, C_2 -propionate, dioxane, C_1 - α -glucose, C_1 - β -glucose. The chemical shifts of all the signals that could be assigned in the spectra of extracts are collected in Table I. At the present level of

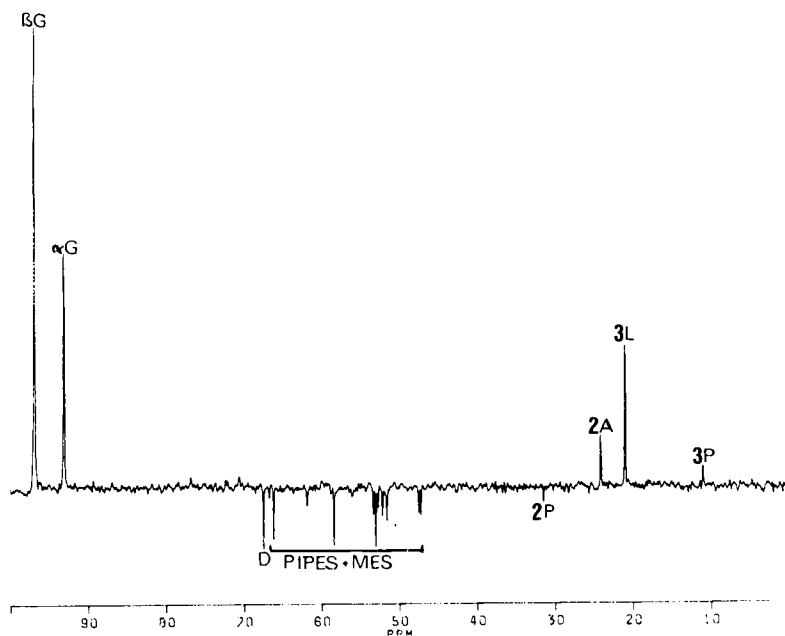


Fig. 1. Aliphatic region of the ^{13}C NMR spectrum of a $T_e = 5$ min perchloric acid extract. The DEPT sequence was used, so that CH_2 groups give negative lines, CH and CH_3 groups positive lines. 3P, [$3-^{13}C$] propionate; 2P, [$2-^{13}C$] propionate; 3L, [$3-^{13}C$] lactate; 2A, [$2-^{13}C$] acetate; α G [$1-^{13}C$ - α] glucose; β G, [$1-^{13}C$ - β] glucose; D, dioxane.

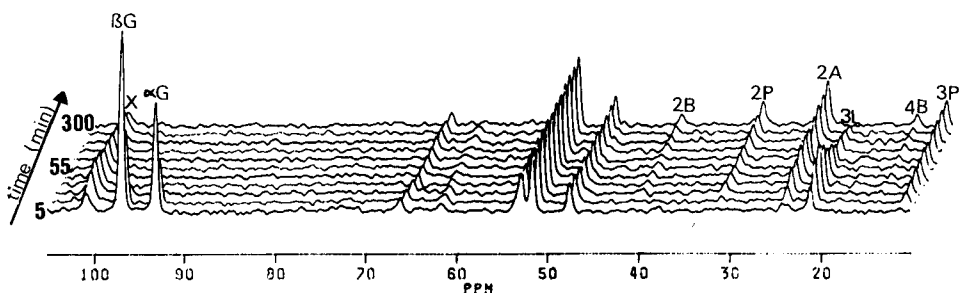


Fig. 2. Sequence of spectra (aliphatic region) acquired during a kinetic run. Each trace corresponds to a 10 min recording period. From bottom to top, the elapsed times at the middle of the period were, respectively, 5, 15, 25, 35, 45, 55, 65, 75, 85, 95 and 300 min. A gaussian filter (GB = 0.01; LB = -2) was applied before each Fourier transformation. 3P, [3-¹³C] propionate; 2P, [2-¹³C] propionate; 4B, [4-¹³C] butyrate; 2B, [2-¹³C] butyrate; 3L, (3-¹³C) lactate; 2A, [2-¹³C] acetate; α G, [1-¹³C- α] glucose; β G, [1-¹³C- β] glucose; X, polysaccharide.

sensitivity, no lines that could be ascribed to carbonyl or to carboxyl carbon atoms were detected.

An example of a series of kinetic spectra is presented in Figure 2. A plot of the relative amplitudes of several peaks of interest is given in Figure 3. All the compounds which were discovered during the survey using extracts are present. Further, a broad line appears at ca 100 ppm; this signal was assigned to a macromolecule since it was not present in spectra of extracts. This is very similar to the resonance position of C₁ in glycogen that is often accumulated in many bacteria (Rose, 1976).

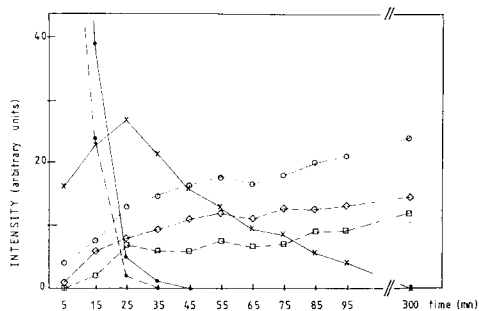


Fig. 3. Plot of signal intensities corresponding to the spectra of Fig. 2. Concentrations are rough estimates obtained as explained in the text. Key to symbol: ● : [1-¹³C- α] glucose; ○ : [1-¹³C- β] glucose; x : [3-¹³C] lactate; □ : [2-¹³C] propionate; ◇ : [3-¹³C] propionate; ⊙ : [2-¹³C] acetate.

Table I. Chemical shifts and assignments of variable peaks in Fig. 2.

δ (ppm)	Assignment	I_{300} (mM)
11.03	C ₃ -propionate	3.0
14.13	C ₄ -butyrate	1.0
21.10	C ₃ -lactate	0.6
24.20	C ₂ -acetate	4.8
31.55	C ₂ -propionate	2.4
40.45	C ₂ -butyrate	1.0
47.0	?	0.6
93.3	C ₃ - α -glucose	0.0
97.0	C ₃ - β -glucose	0.0

Intensities (I_{300} , uncorrected for saturation effects) have been measured on the last spectrum of Fig. 2 (recorded at time 300 min). Chemical shifts (δ) are relative to TMS at $d = 0$ ppm.

Spin lattice relaxation times were measured at the end of a kinetic run. Values for protonated carbons were all in the range 0.5–2 sec. If it was assumed, as a first approach, that line widths, nuclear Overhauser effect (NOE) enhancements and saturation factors were roughly equivalent for CH, CH₂ and CH₃ groups, the vertical axis of Figure 3 could be approximately labelled in concentration units, using the initial glucose concentration as a calibration. The concentrations estimated from a spectrum recorded at a very long time under quantitative conditions are reported in Table I. Furthermore, we did not observe spin-spin couplings, which means that most molecules carried isolated ¹³C atoms.

Discussion

The total estimated ¹³C concentration at the end of the kinetic run was ≈ 10 mM compared to an initial glucose concentration of 40 mM. Much of the label became NMR invisible. This effect could be due to loss of such labelled gases as CO₂ (≈ 10% according to Wallnöfer *et al.*, 1966) and CH₄. It is most likely that incorporation into non-mobile macromolecules by the micro-organisms occurred; a similar phenomenon has been observed by Demeyer and Henderickx (1967) with ¹⁴C-labelled glucose. We observed a transient increase of a signal at ≈ 100 ppm; this resonance has not been assigned yet. However it may reasonably be assumed that it corresponds to the synthesis of an α-glucan used as a storage form of glucose, as described by Smith and McAllan (1974) for rumen bacteria.

This system exhibited a transient build-up and decay of lactate labelled at the methyl (C₃) position. Figure 3 leads us to propose that the lactate was derived from

the glucose and was fermented as soon as the glucose was depleted. It has been shown that most lactate-utilizing bacteria do not consume lactate when carbohydrates are present (Russell and Baldwin, 1978).

The degradation of lactate gave rise to acetate, propionate and butyrate in almost quantitative yield. Pyruvate, the key intermediate of glucose metabolism (Demeyer and Henderickx, 1967), was not detected because its stationary concentration was under the NMR detection limit. Two molecules of pyruvate were formed from one molecule of glucose by the Embden—Meyerof pathway (EMP) pathway, so only half of the acetate and propionate produced was labelled. Since propionate was singly labelled, we can estimate that an almost equal number of labelled molecules of acetate and propionate were formed. However, in similar conditions of incubation (unpublished results), the molar ratio of total acetate over total propionate (estimated by gas chromatography) was close to 2.0 in the supernatant of the incubation. This apparent discrepancy could be explained if the concentration of propionate was higher and that of acetate lower inside the cells than in the liquid environment.

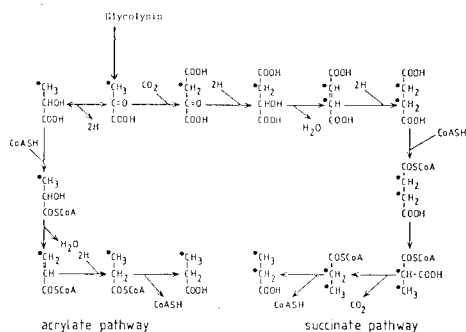


Fig. 4. Two different pathways of the fermentation of pyruvate to propionate in the rumen (from Counotte *et al.*, 1981).

3-¹³C-Lactate degradation gave rise to propionate. Two possible routes are shown in Figure 4. The succinate pathway resulted in a mixture of equal quantities of 2-¹³C and 3-¹³C-propionate due to total randomization of the label in the succinate step. The acrylate pathway gave only 3-¹³C-propionate, as the carbon skeleton of lactate remained intact (Prins *et al.*, 1975). Therefore the excess of 3-¹³C-propionate could be attributed to the acrylate pathway. From our data it can be estimated that only about 10% of the propionate was formed *via* the acrylate pathway. A complete quantitation of the present spectra is underway in our laboratories.

This first approach shows that these *in vitro* results are in agreement with *in vivo* data on lactate turnover and its contribution to volatile fatty acid production with a diet rich in available carbohydrates (Mackie *et al.*, 1984). The NMR method is applicable to a complex anaerobic ecosystem and will be used for studying the effects of mineral deficiencies in rumen bacteria metabolism.

References

- Avison M.J., Hetherington H.P. & Shulman R.G. (1986) Application of NMR to studies of tissue metabolism. *Annu. Rev. Biophys. Biochem. Phys. Chem.* 15, 377-402
- Brindle K.M. & Campbell I.D. (1987) NMR studies of kinetics in cells and tissues. *Quart. Rev. Biophys.* 19, 159-182
- Counotte G.H.M., Prins R.A., Janssen R.H.A.M. & Debie M.J.A. (1981) Role of *Megasphaera elsdenii* in the fermentation of DL-(2-¹³C) lactate in the rumen of dairy cattle. *Appl. Environ. Microbiol.* 42, 649-655
- Demeyer D.I. & Henderickx H.K. (1967) Methane production from glucose *in vitro* by mixed rumen bacteria. *Biochem. J.* 105, 271-277
- Ezra F.S., Lucas D.S., Mustacich R.V. & Russell A. (1983) ³¹P and ¹³C NMR studies of anaerobic glucose metabolism and lactate transport in *Staphylococcus aureus* cells. *Biochemistry* 22, 3841-3849
- Herrero A.A., Gomez R.F. & Roberts M.F. (1985) ³¹P NMR studies of *Clostridium thermocellum*. *J. Biol. Chem.* 260, 7442-7454
- Hungate R.E. (1966) *The Rumen and its Microbes*. Academic Press, New York
- Mackie R.I., Gilchrist F.M.C. & Heath S. (1984) An *in vivo* study of ruminal microorganisms influencing lactate turnover and its contribution to volatile fatty acid production. *J. Agric. Sci.* 103, 37-51
- Nicolay K., Hellingwerf K.J., Kaptein R. & Konings W.N. (1982) ¹³C NMR studies of acetate metabolism in intact cells of *Rhodospseudomonas sphaeroides*. *Biochim. Biophys. Acta* 720, 250-258
- Pegg D.T., Doddrell D.M. & Bendall M.R. (1982) Proton polarization transfer enhancement of a heteronuclear spin multiplet with preservation of phase coherency and relative component intensities. *J. Chem. Phys.* 77, 2745-2748
- Prins R.A., Lankhorst A., Van Der Meer P. & Van Nevel C.J. (1975) Some characteristics of *Anaerovibrio lipolytica*, a rumen lipolytic organism. *Antonie Leeuwenhoek J. Microbiol.* 41, 1-11
- Rose A.H. (1976) *Chemical Microbiology, An Introduction to Microbial Physiology*. Butterworths, London, Boston, p. 81
- Russell J.B. & Baldwin R.L. (1978) Substrate preferences in rumen bacteria, evidence of catabolite regulatory mechanisms. *Appl. Environ. Microbiol.* 36, 319-329
- Shaka A.J., Keeler J.H., Frankiel T. & Freeman R. (1983) An improved sequence for broadband decoupling: Waltz 16. *J. Magn. Reson.* 52, 335-338
- Smith R.H. & McAllan A.B. (1974) Some factors influencing the chemical composition of mixed rumen bacteria. *Br. J. Nutr.* 31, 27-34
- Wallnöfer P., Baldwin R.L. & Stagno E. (1966) Conversion of ¹⁴C labelled substrates to volatile fatty acids by rumen microbiota. *Appl. Microbiol.* 14, 1004-1010