JOURNAL OF BACTERIOLOGY, Mar. 1996, p. 1756–1761 Vol. 178, No. 6
0021-9193/96/$04.00+0

Ethanol Transport in *Zymomonas mobilis* Measured by Using In Vivo Nuclear Magnetic Resonance Spin Transfer†

S. M. SCHOBERTH,[1]* B. E. CHAPMAN,[2] P. W. KUCHEL,[2] R. M. WITTIG,[1] J. GROTENDORST,[3] P. JANSEN,[3] AND A. A. DE GRAAF[1]

Institut für Biotechnologie-1[1] and Zentralinstitut für Angewandte Mathematik,[3] Forschungszentrum Jülich, D-52425 Jülich, Germany, and Department of Biochemistry, University of Sydney, Sydney, NSW 2006, Australia[2]

Received 12 September 1995/Accepted 4 January 1996

For the first time, unidirectional rate constants of ethanol diffusion through the lipid membrane of a microorganism, the bacterium *Zymomonas mobilis*, were determined, thus replacing indirect inferences with direct kinetic data. The rate constants k_1 (in to out) were 6.8 ± 0.4 s^{-1} at 29°C and 2.7 ± 0.3 s^{-1} at 20°C. They were determined by using ^1H selective nuclear magnetic resonance spin magnetization transfer. The measurements were done on 1-ml cell suspensions. No addition of radiotracers, withdrawing of aliquots, physical separation methods, or chemical manipulations were required. Until now, the rate constants of ethanol transport in microorganisms have been unknown because ethanol diffuses through the cytoplasmic membrane too quickly for radiolabel approaches. Net velocities of ethanol exchange were calculated from unidirectional rate constants and cytoplasmic volume, which was also determined with the same nuclear magnetic resonance experiments. The results (i) confirmed that ethanol would not be rate limiting during the conversion of glucose by *Z. mobilis* and (ii) indicated that ethanol can serve as an in vivo marker of cytoplasmic volume changes. This was verified by monitoring for the first time the changes of both cytoplasmic volume and extracytoplasmic and cytoplasmic concentrations of α and β anomers of D-glucose in cell suspensions of a microorganism. These findings may open up new possibilities for kinetic studies of ethanol and sugar transport in *Z. mobilis* and other organisms.

Ethanol is thought to diffuse freely across cell membranes of microorganisms, rapidly equilibrating intracellular and extracellular concentrations (6, 10, 14). However, this view is based on indirect evidence, on the grounds of measurements of cytoplasmic ethanol concentrations in *Saccharomyces cerevisiae* (6, 10, 14). The kinetics of ethanol transport could not be measured directly, since this process is too fast in microorganisms for conventional radiolabel approaches (10). To tackle this problem, i.e., being able to measure ethanol transmembrane exchange in *Zymomonas mobilis* in vivo, we adapted nuclear magnetic resonance (NMR) methods used to study transport phenomena in human erythrocytes (22).

We initiated this work with this anaerobic, gram-negative bacterium (38) since it is equivalent to *S. cerevisiae* in terms of the amounts of ethanol produced during fermentation of glucose and its alcohol tolerance for growth (14), and since we had previous NMR experience with *Z. mobilis* (4, 16, 29, 32, 36). To our knowledge, this is the first study to obtain unidirectional rate constants of ethanol transport in a microorganism (two brief preliminary reports on some aspects of these results have been given [31, 33]).

MATERIALS AND METHODS

Organism, growth, and preparation of cells for NMR spectroscopy. *Z. mobilis* ZM6 (ATCC 29191) was cultivated and harvested as described previously (32). Cell pellets were suspended at 6 g (wet weight) of cells per ml of potassium phosphate-$MgCl_2$ buffer (buffer 1 in reference 16) and stored in an ice bath. Preparation for NMR inversion transfer spectroscopy was as follows. NMR tubes (5-mm outside diameter [o.d.]) contained 800 μl of cell suspension, 26 μl of aqueous ethanol solution to give the final concentrations indicated in Results, ~30 μl of a 50 mM Dy-DTPA (Dysprosium-diethylenetriaminepentaacetic acid) solution (32), 126 μl of D_2O (99.9 atom% D; Sigma), and 6 μl of antifoam (Dehysan K996; Henkel, Düsseldorf, Germany). To obtain optimum separation of cytoplasmic and extracytoplasmic ethanol signals, the amount of Dy-DTPA solution was carefully adjusted to between 1 and 2 mM. The temperature was 29°C. For ^{13}C NMR inversion transfer control experiments, we used 10-mm-o.d. NMR tubes that contained 2.436 ml of cell suspension, [1-^{13}C]ethanol (99% enriched; Cambridge Isotope Laboratories) to give the final concentrations indicated in Results, 286 μl of D_2O (see above), 150 μl of 50 mM Dy-DTPA solution, and 20 μl of antifoam. The temperature was 20 or 29°C, as indicated in Results. To monitor glucose metabolism in vivo, cell pellets were suspended at 6 g (wet weight) of cells per ml of buffer which contained, per liter, 195.2 g of morpholineethanesulfonic acid (MES), 6.8 g of KH_2PO_4, 2.36 g of $MgCl_2 \cdot 6H_2O$, 7.44 g of EDTA, and 6 ml of 5 N KOH; to 2.8 ml of this suspension were added 10 μl of antifoam and ~15 μl of 0.5 M Dy-DTPA suspensions in 10-mm-o.d. NMR tubes. The reaction was started by addition of [1-^{13}C]glucose to cell suspensions. The temperature was 20°C.

NMR spectroscopy. NMR spectra were recorded with a Bruker AMX-400 wide-bore spectrometer (32). In the ^1H experiments, the signal of H_2O was used for shimming, while in the ^{13}C experiments, D_2O enabled optimization of shims and field/frequency locking; broadband ^1H decoupling was used. To determine the kinetics of transmembrane ethanol exchange, spectra were obtained by using a δ-ordered inversion pulse sequence (27) followed by a variable delay of 0.01 to 25 s and then a (1 3 3 1) binomial water suppression 90° detection pulse (13) when the nucleus was ^1H or a 90° sampling pulse (27) when the nucleus was ^{13}C. For ^1H NMR, an 81° pulse flip angle avoided problems with zero crossing which affected the quantification of the concentrations from the spectra. For ^{13}C NMR, an inversion of 180° was used. As a control, estimates of T_1 obtained in the inversion transfer experiments were cross-checked with independent estimates of the T_1 values of the ethanol protons (the extracytoplasmic fluid was simulated by omitting cells from the NMR assay mixture, and the cytoplasmic space was assumed to be the same as that of a cell lysate supplemented with D_2O and ethanol).

The conversion of glucose to ethanol was monitored by using ^{13}C NMR experimental procedures previously described (32), replacing [1-^{13}C]xylose with [1-^{13}C]glucose. All ^{13}C spectra were processed by using Gaussian time windowing for resolution enhancement (32).

All ethanol ^1H spectra were quantified by using DOSIS (42). For ^{13}C signals, the built-in Bruker software MDCON was used.

Unidirectional rate constants. Unidirectional rate constants were determined from experimental NMR inversion transfer data as described for the study of human erythrocyte metabolism (22, 26). Complementary experiments (22) were performed except when indicated otherwise. The mathematical expressions of the analytical solutions of the Bloch-McConnell equations as given in reference

* Corresponding author.

† This paper is dedicated to Gerhard Gottschalk on the occasion of his 60th birthday.

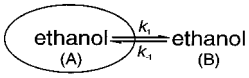

FIG. 1. Two-site transmembrane exchange scheme of ethanol in *Z. mobilis*. The shaded oval represents a cell of *Z. mobilis*. (A) and (B) denote cytoplasmic and extracytoplasmic ethanol, respectively. The rate constants, k_1 and k_{-1}, were measured under chemical equilibrium exchange conditions, i.e., net flux of ethanol = 0 and flux of molecules with inverted spin ≠ 0 (see Materials and Methods and Results).

26 were regressed onto the inversion transfer data that consisted of peak area versus delay time. The mathematical analysis was performed on an IBM mainframe ES/9000-620 using the SAS system version 6.08 (30). The fitting was done first with the procedure NLIN, using DUD (30) to find initial estimates, and then in the definitive run, including partial derivatives, with the method of Marquardt (30). The results yielded estimates of k_1 and k_{-1}. They were frequently cross-checked by using, in addition, two different analytical solutions of the Bloch-McConnell equations (9, 23).

Specific exchange rates. The specific transmembrane exchange rate at a given difference of the ethanol concentration between extracytoplasmic and cytoplasmic compartments, Δ_c, was computed according to equation 1,

$$\frac{v_A}{dw} = \frac{da}{dt}\frac{1}{dw} = k_1\Delta_c\frac{V_{in}}{dw} \quad (1)$$

where v_A is velocity (moles of A, i.e., cytoplasmic ethanol [Fig. 1], per second), a is the concentration of A, and V_{in}/dw is the specific cytoplasmic volume (dw = dry weight). The determination of V_{in} was a modification of the NMR method used by Hoffman and Gupta for erythrocytes (12): samples identical to those used for the experiments described in Table 1 were prepared, except that signals were acquired at equilibrium of exchange and Dy-DTPA was omitted, which resulted in a single ethanol signal. A coaxial capillary filled with glacial acetic acid at an appropriate dilution served as an external chemical shift and intensity standard. The fractions of the total cell volume occupied by solids (V_s [6]), by cytoplasm (V_{in}), and by extracytoplasmic space (V_{ex}) were computed as follows (ethanol-$^1H_{sus}$ denotes the ethanol-C-2 proton signal of the suspension, and ethanol-$^1H_{sup}$ denotes that of the suspension):

$$V_s = 1 - \frac{\text{ethanol-}^1H_{sus}}{\text{ethanol-}^1H_{sup}} \quad (2)$$

$$q = \frac{\text{ethanol-}^1H_{in}}{\text{ethanol-}^1H_{ex}} \quad (3)$$

$$V_{ex} = \frac{1 - V_s}{1 + q} \quad (4)$$

$$V_{in} = 1 - (V_{ex} + V_s) \quad (5)$$

From the known cell dry weight in the sample, the specific excluded space, V_s per milligram of dry weight [mg dw]), was obtained. This value was used to calculate V_s from the known cellular dry weight of a specific sample. As a control, V_s was also measured with ^2H NMR experiments, in which ethanol was replaced with D_2O and CD_3OD was used as the external standard (see Results).

Permeability and diffusion coefficients for ethanol. The transmembrane permeability coefficient, P (centimeters per second), was calculated according to equation 6,

$$P = k_1\frac{V_{in}}{A} \quad (6)$$

where A denotes the cytoplasmic boundary surface area (see references 19, 25, and 34 and Results).

The translational diffusion coefficient, D_{mem} (square centimeters per second), was calculated according to equation 7,

$$D_{mem} = \frac{Pd_{mem}}{K} \quad (7)$$

where d_{mem} denotes the thickness of the lipid bilayer of the cytoplasmic membrane and K is the partition coefficient for ethanol in the lipid bilayer-water system (see reference 34 and Results).

Cell surface area. An approximate estimate of the surface area, A, of an average cell was obtained as follows. Swings and de Ley (38) give the cell size of *Z. mobilis* as 2 to 6 μm by 1 to 1.5 μm. Our own microscopic observations indicated that a prolate spheroid with diameters of about 2 by 1 μm is a mathematically realistic model of the average cell. The volume (V) and the surface area (A) of a prolate spheroid are given by equations 8 and 9, where l (length) and d (diameter) denotes the main and small axes (for equation 8, see textbooks of mathematics; for equation 9, see reference 7):

$$V = \frac{\pi}{6}ld^2 \quad (8)$$

$$A = \frac{\pi}{2}d\left(d + \frac{l^2}{\sqrt{l^2 - d^2}}\arcsin\frac{\sqrt{l^2 - d^2}}{l}\right) \quad (9)$$

In order to fit the figure of 1.173 μm^3 for an average cell volume, l and d were calculated by using equation 8 and found to be 2.077 and 1.039 μm ($l = 2 \times d$), using the equation solver in Excel (Microsoft). The cytoplasmic boundary surface area, A, was calculated by using equation 9, assuming (i) that the cytoplasm had also the shape of a prolate spheroid; (ii) that the cytoplasm had a volume of 80% of the total cell volume (35), and (iii) that the distance from the cytoplasmic membrane to the cell wall was the same throughout the cell. Using again the equation solver in Excel, we calculated a cytoplasmic surface area of 5.2 μm^2 and a volume of 0.938 μm^3 per cell.

Cell dry weight, cell counts, and cell volume. Dry weight was determined as described previously (8), but with 20 mM ammonium acetate as the washing agent. Cell counts and mean cell volume were determined with an ELZONE 280 particle counter (Particle Data Europe, Consdorf, Luxembourg, Belgium). When cell suspensions were analyzed immediately after dilution (1:100, vol/vol) into aqueous 3% (wt/vol) NaCl, mean cell volume was not affected.

RESULTS

Unidirectional rate constants for transmembrane ethanol exchange in *Z. mobilis*. To obtain maximum signals, ^1H instead of ^{13}C NMR (32) was used for most of the ethanol exchange experiments in this study. Figure 2 shows a typical ^1H spectrum of ethanol. The binomial water suppression also suppressed the ethanol-C-1 protons, however (Fig. 2); therefore, only the proton signals of ethanol-C-2 were used for quantification. A comparison of data acquired with a pulse sequence whereby the binomial pulse train was replaced with a simple 90° pulse ensured that the ethanol-C-2 proton signals were not affected by the water suppression procedure. The rate constants were computed from the data of spin inversion transfer experiments

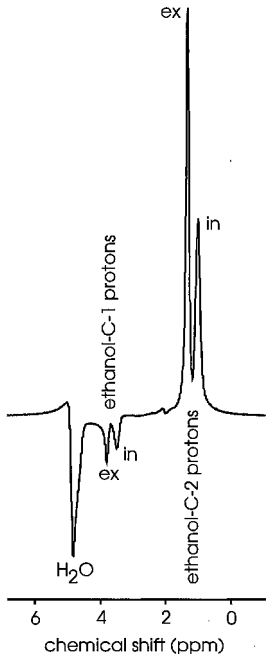

FIG. 2. ^1H NMR spectrum of ethanol in a suspension of *Z. mobilis*. Cell suspensions were supplemented with 1,200 mM (final concentration) ethanol. Dy-DTPA was used to cause separate extracytoplasmic (ex) and cytoplasmic (in) resonances. The spectra were acquired under relaxed conditions, mixing time of 12 s (see Materials and Methods and Fig. 2). The areas of the resonance lines of the proton signals ethanol-C-2 were proportional to the concentration of extracytoplasmic and cytoplasmic ethanol. The inversion of the proton signals of water and ethanol-C-1 was due to the binomial water suppression pulse sequence (phase roll).

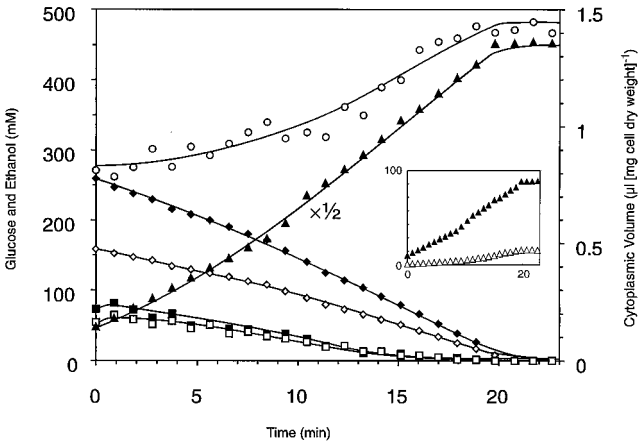

FIG. 3. Conversion of glucose to ethanol by *Z. mobilis* monitored with in vivo NMR. A cell suspension of *Z. mobilis* that contained MES buffer (see Materials and Methods) was supplemented with D-[1-^{13}C]glucose 2 min prior to time zero at 20°C. Spectra were accumulated every 54 s. After quantification, the ethanol signal intensities (inset) were tentatively used to estimate the extracytoplasmic and cytoplasmic volumes as indicated in the text. This enabled the calculation of the specific cytoplasmic volume and hence the compartmental concentrations of ethanol and glucose (main diagram). The inset shows original normalized peak areas of ethanol signals. In the inset, the time scale (*x* axis) is the same as in the main diagram; normalized peak areas for ethanol signals are given on the *y* axis. Symbols: ◆, extracytoplasmic β-glucose; ◇, extracytoplasmic α-glucose; ■, cytoplasmic β-glucose; □, cytoplasmic α-glucose; ▲, intra- and extracytoplasmic ethanol in the main diagram (concentration times 0.5) and extracytoplasmic ethanol in the inset; △, cytoplasmic ethanol in the inset; ○, specific cytoplasmic volume. For details, see the text.

as described in Materials and Methods. Table 1 lists the kinetic constants obtained from 10 such experiments.

Specific efflux rates of ethanol. Efflux rates were computed as described in Materials and Methods. V_s was determined with four experiments with cell suspensions containing ethanol and two assays with the supernatant (see Materials and Methods). This yielded 0.103 ml of excluded volume per ml of assay mixture ($V_{s\ \mathrm{ethanol}}$) or 0.804 μl (mg dw)$^{-1}$ (^{2}H NMR control experiments [see Materials and Methods] yielded $V_{s\ \mathrm{D_2O}}$ of 0.706 μl [mg dw]$^{-1}$). Exchange rates were calculated by using equation 1; values for k_1 were taken from Table 1, and V_{in} was calculated as described in Materials and Methods. The specific efflux rates for arbitrarily different compartmental ethanol concentrations of 1 and 100 mM are given in Table 2.

Transmembrane permeability and diffusion coefficients for ethanol. The coefficients were calculated as described in Materials and Methods. Values for k_1 and V_{in} were as specified above. Values for P_{ethanol} at 20 and 29°C thus obtained are listed in Table 2. The diffusion coefficient, D_{mem} (square centimeters per second), was calculated from *P*, assuming that the value of d_{mem} for *Z. mobilis* (see Materials and Methods) would lie somewhere between 4 nm (34) and 8 nm (24). Since the partition coefficient, *K*, of ethanol between lipid bilayer and water was not known, that between hexadecane and water, 5.7×10^{-3}, was taken (34). This yielded values for D_{mem} of 0.4×10^{-8} to 0.8×10^{-8} cm^2 s^{-1} (20°C) and 0.85×10^{-8} to 1.7×10^{-8} cm^2 s^{-1} (29°C).

Ethanol as an in vivo marker of extracytoplasmic and cytoplasmic volume changes. The value of the specific cytoplasmic volume of *Z. mobilis* depends on the ratio of extracytoplasmic and cytoplasmic glucose (37). We tried to determine whether such volume changes, brought about during conversion of glucose to ethanol, could be observed directly in real time in cell suspensions. Two experiments were done, on the basis of our previous results obtained with xylose (32) and other data (33). They were started by the addition of D-[1-^{13}C]glucose to the suspension of *Z. mobilis* (see Materials and Methods), and ^{13}C NMR spectra were acquired every 54 s. From the ethanol signal intensities, extracytoplasmic and cytoplasmic volumes (ethanol spaces) and specific cytoplasmic volumes were calculated as described above. From these figures, compartmental concentrations of ethanol and glucose and the specific cytoplasmic volume of *Z. mobilis* were obtained for each of the 25 datum points (Fig. 3).

DISCUSSION

It is known that diffusion of small molecules, such as ethanol, through the lipid membrane is very rapid (34), and hence accurate estimates of exchange rates are difficult to obtain with radiotracers. NMR spin transfer spectroscopy, however, operates in short time domains: observing the individual signals of extracytoplasmic and cytoplasmic ethanol nuclei with a time resolution of 0.1 ms (i.e., after a mixing time of 0.1 ms) was easily accomplished in the experiments presented here. And since the total reactant concentrations were not altered at any time during the ethanol exchange experiments, accumulation of spectra could be repeated ad libidum for each time point, thus enabling us to improve the precision at will.

Ethanol transmembrane exchange rates. For the first time, direct kinetic data (unidirectional rate constants) for transmembrane ethanol exchange in a bacterium can be given (Table 1). From the data presented, it appears that in *Z. mobilis*, the rate constants for the exchange are independent of ethanol concentration and are temperature dependent. This result was to be expected (see references 14 and 37 for literature on ethanol tolerance of this organism), but it served as a further confirmation of the fidelity of the NMR method. Using our data, we can calculate specific efflux rates for any given concentration difference, Δ_c, of ethanol, which was not possible before. The value at $\Delta_c = 1$ mM at 29°C (Table 2) compares favorably with data from a recent study: *Z. mobilis* converting glucose to ethanol in cascaded fluidized bed reactors at 30°C reaches a maximum ethanol production rate of 0.83 μmol min^{-1} (mg dw)$^{-1}$ (41). The kinetic data (Table 2) may also be used to decide whether ethanol efflux can be rate limiting in the catabolism of glucose (i.e., whether cytoplasmic ethanol can accumulate). First, for example, Struch et al. report a maximum glucose uptake in fermenting *Z. mobilis* of 0.75 μmol min^{-1} (mg dw)$^{-1}$ at 30°C (37). Assuming stoichiometric conversion of glucose to ethanol, this would yield an efflux rate for ethanol of 1.5 μmol min^{-1} (mg dw)$^{-1}$. This rate corresponds to a concentration difference, $\mathrm{ethanol_{in}} - \mathrm{ethanol_{out}}$, of

TABLE 1. Unidirectional rate constants estimated for transmembrane exchange of ethanol in *Z. mobilis*[a]

Experimental conditions	Influx, k_{-1} (s^{-1})	Efflux, k_1 (s^{-1})
29°C, 1,300 mM ethanol	4.45 ± 0.06	7.26 ± 0.31
29°C, 1,200 mM ethanol	3.17 ± 0.33	6.98 ± 0.35
29°C, 781 mM ethanol (C)	3.29 ± 0.46	6.65 ± 0.96
29°C, 96 mM ethanol	3.22 ± 0.36	6.36 ± 0.89
20°C, 835 mM ethanol (C)	1.61 ± 0.12	2.52 ± 0.19
20°C, 696 mM ethanol (C)	1.22 ± 0.17	2.97 ± 0.41

[a] Results of NMR inversion transfer experiments performed on cell suspensions (116 to 128 mg dw ml^{-1}) supplemented with ordinary reagent-grade ethanol or with ^{13}C-labeled ethanol as a control (C). The first four rows show the results of two experiments each (complementary experiments; see Materials and Methods). Means and standard errors are given.

TABLE 2. Selected characteristics of *Z. mobilis* found in this study[a]

Temp (°C)	Parameter				
	Specific ethanol efflux at $\Delta_c = 1$ mM (μmol min^{-1} [mg dw]$^{-1}$)	Specific ethanol efflux at $\Delta_c = 100$ mM (μmol min^{-1} [mg dw]$^{-1}$)	Total cytoplasmic vol in sample (V_{in}) (ml ml^{-1})	Specific cytoplasmic vol (V_{in}/dw) (μl [mg dw]$^{-1}$)	Permeability coefficient for ethanol (cm s^{-1})
20	0.42 ± 0.15	41.5 ± 15.3	0.307 ± 0.06	2.5 ± 0.3	0.5×10^{-4}
29	0.99 ± 0.03	99.4 ± 2.7	0.306 ± 0.3	2.4 ± 0.3	1.2×10^{-4}

[a] Mean cell dry weights were 122 ± 8 (20°C) and 126 ± 3 (29°C) mg ml^{-1}.

1.5 mM (calculated by using equation 1 and the values given in Table 2, assuming that the specific ethanol efflux rates at 29 and 30°C were almost the same). Even for a moderate (in terms of *Z. mobilis*) ethanol concentration of ~200 mM, this would be less than 1% (mol mol^{-1}) concentration difference between cytoplasm and extracytoplasmic space. The same holds true when a similar comparison is done with V_{max} values of glucose uptake published for suspensions of *Z. mobilis*, 0.2 to 0.33 (5) and 0.816 (2), and of V_{max} values of glucose uptake obtained with the glucose facilitator of *Z. mobilis* expressed in *Escherichia coli*, 0.41 μmol of glucose min^{-1} (mg dw)$^{-1}$ (40).

Second, the maximum rate of extracytoplasmic glucose consumption in the experiment depicted in Fig. 3 was an average of 0.22 μmol (mg dw)$^{-1}$ min^{-1} (between 15 and 19 min) at 20°C. This corresponds to double the rate of ethanol production, namely, 0.44 μmol (mg dw)$^{-1}$ min^{-1}. The concentration of ethanol during this period changed from about 700 to 800 mM. For this specific efflux rate of ethanol at 20°C, it can be calculated from Table 2 that even at a concentration difference of ethanol of as low as 0.2 to 0.3% (vol/vol), ethanol efflux would still have doubled the rate of glucose influx.

Thus, we conclude that (i) ethanol transport is not rate limiting and (ii) cytoplasmic ethanol accumulation is not likely to occur in the catabolism of glucose of *Z. mobilis*, confirming previous indirect evidence (6, 10, 15) with direct experimental data.

Permeability and diffusion coefficients for ethanol transmembrane diffusion. The value of the permeability coefficient, *P*, at 29°C for *Z. mobilis* (Table 2) compares well with the findings of Guijarro and Lagunas (10) for the permeability coefficient of ethanol in yeast cells at 30°C: 3×10^{-4} cm s^{-1}. Other permeability coefficients reported for ethanol in single cells, such as 6×10^{-4} cm s^{-1} (rabbit erythrocyte, 25°C [15]) and 3×10^{-4} cm s^{-1} (ox erythrocyte, 25°C [15]), are also of the same order of magnitude as those for *Z. mobilis* at approximately the same temperature (Table 2). Considering some uncertainties about the effective membrane surface area in *Z. mobilis* (due to membrane-bound proteins [24]), and in the absence of data on other bacterial systems, they seem to confirm the fidelity of the NMR method used in this study.

Estimates for the membrane diffusion coefficient for ethanol in *Z. mobilis*, D_{mem}, were ~5×10^{-8} cm^2 s^{-1} at 20°C and ~1×10^{-8} cm^2 s^{-1} at 29°C (obtained with uncertainties about the exact value of the partition coefficient for ethanol between the lipid bilayer and water, the thickness of the lipid fraction, and the effective area of the cytoplasmic membrane). As expected, the latter value is lower than that for diffusion of ethanol through the bulk biomass of *Z. mobilis*, i.e., $D_{eff,p} = 1.28 \times 10^{-6}$ cm^2 s^{-1} (41).

Specific cytoplasmic volume. In our investigation, the total cytoplasmic space, $V_{in\ ethanol}$, was 0.31 ml ml^{-1}. In a recent NMR study with *E. coli*, Axe and Bailey (1) also used dense cell suspensions. The volume compositions given by these authors were (in milliliters per milliliter; the values for *Z. mobilis* in the present paper are in parentheses): 62% (59%) extracytoplasmic space, 20% (31%) cytoplasmic space, and 18% (10%) excluded space. The values for the specific cytoplasmic ethanol space of *Z. mobilis* found in this study (Table 2) lie within the range found with chemical tracer techniques (difference between ^3H_2O and [^{14}C]taurine space): 2.2 to 2.4 (28), 3 (37), and 3.3 (5) μl (mg dw)$^{-1}$. It is speculative to cite the definitive mean cytoplasmic volume or the concentration of proteins that might affect it, but we present here two possible methods of determination.

(i) Assuming that the cytoplasm of *Z. mobilis* occupies 80% of the average cell volume (see Materials and Methods) and using the average specific cell number of 3.03×10^9 (mg dw)$^{-1}$, an average total cytoplasmic volume of 0.354 ml (ml of sample volume)$^{-1}$ for the experiments depicted in Table 1 was calculated. The difference from the average $V_{in\ ethanol}$ (Table 2) was therefore 13% (vol/vol), which could be attributed to compounds such as large proteins or cell wall constituents that would exclude ethanol. If we attribute this difference solely to large cytoplasmic proteins, the true cytoplasmic volume of *Z. mobilis* would have been 2.8 μl (mg dw)$^{-1}$, and hence the true cytoplasmic concentration of ethanol would have been 87% of the extracellular value.

(ii) An approximate estimate for protein concentrations in vivo in *Z. mobilis* can be obtained as follows. If the chemical shift difference between extracytoplasmic and cytoplasmic ^{31}P NMR signals of dimethyl methylphosphonate in *Z. mobilis* cell suspensions, namely, 29.2 Hz (experiments not shown), is a measure of protein concentration in the same manner as in erythrocytes (3, 20), it would correspond to 100 mg of protein per ml of cytoplasmic space. Calibrating protein concentration versus cell size as has been done with erythrocytes (18) might enable the monitoring of the true cytoplasmic volume in *Z. mobilis* as a function of time with NMR.

The previous protocols used to measure cytoplasmic volume in a suspension of bacteria, both biochemical methods (e.g., reference 28 and references cited therein) and NMR techniques (1, 11), involve adding at least two different markers to the sample and performing various steps of physical separation and mixing. The method presented here is straightforward; it does not rely on removal of the cells from the NMR spectrometer and is noninvasive, and hence it can be used to monitor real-time volume changes as a function of time.

Ethanol as an in vivo marker of cytoplasmic and extracytoplasmic volume. Ethanol appears to be suitable as an in vivo marker of cytoplasmic volume in *Z. mobilis* because of its high specific efflux rate at minute concentration differences between cytoplasmatic and extracytoplasmatic space (see above). Thus, for the first time for a bacterium, it was possible in this investigation to monitor the change of cytoplasmic space and of extracytoplasmic and cytoplasmic concentrations of the α and β anomers of D-glucose during its conversion to ethanol (Fig. 3).

Maximum change of cytoplasmic volume occurred between 13 and 14 min (Fig. 3). The results compare favorably with

findings published by Struch et al. (37); these authors studied the relation between the specific cytoplasmic volume and the concentration difference between extracytoplasmic and cytoplasmic glucose; their experiments involved running several separate fermentations and performing time-exacting radiolabel experiments, but in contrast, the results obtained here were with 3-ml cell samples, and the same information was obtained in about 20 min.

The specific cytoplasmic volume, $V_{\text{in ethanol}}$, found after glucose consumption (about 1.5 μl [mg dw]$^{-1}$; Fig. 3) is lower than the average figure of 2.5 μl (mg dw)$^{-1}$ which was obtained in the kinetic studies of ethanol exchange (see above). This may be due to the osmotic pressure exerted by MES buffer (130 mM) that was added in the experiment depicted in Fig. 3; MES is not taken up into the *Z. mobilis* cytoplasm under our experimental conditions (32).

In a recent NMR study, Axe and Bailey (1) used [^{13}C]ethanol and [^{13}C]glucose as markers to determine the cytoplasmic volume in cell suspensions of *E. coli*. Their method cannot be applied in vivo since cell lysates were necessary.

Hockings and Rogers (11) determined the cytoplasmic volume of *Enterococcus faecalis* with ^{1}H NMR. Their method, too, cannot be applied in vivo. However, these authors found that the NMR methods gave a better estimation of the cytoplasmic water volume than the conventional radiolabel methods.

Most of the kinetic data in our study were obtained with unlabeled ethanol; the experiments with [1-^{13}C]ethanol served only to confirm the reliability of the ^{1}H NMR methods. And even [1-^{13}C]glucose might be replaced with unlabeled sugar, as our results with xylose transport in *Z. mobilis* indicate (32).

Conclusions. The enormous potential of in vivo NMR spectroscopy holds not only for applications in the study of enzyme reactions but also for almost any membrane transport process (17, 21, 22), whether fast (time scale of about 0.01 to 10 s) or slow (time scale of about 1 min or more). Examples that used both time scales are given in this report. While the techniques presented here were exemplified with kinetic studies of ethanol transmembrane diffusion and glucose transport in *Z. mobilis*, the general methods should be applicable to other fast transmembrane exchange reactions in this and other microorganisms.

ACKNOWLEDGMENTS

This work was supported by the Bundesministerium für Forschung und Technologie of Germany (R.M.W. and A.A.D.G.) and the National Health and Medical Research Council of Australia (B.E.C. and P.W.K.).

In Jülich, we thank K. Striegel for excellent technical assistance and S. Rittershaus and O. Heller for providing data on cell size and number. We thank U. Wiszkowski, E. Heisler, and A. Michels for assistance in the inversion transfer experiments at 20°C and E. D. Rodríguez Messmer for assistance in computation. We thank S. Bringer-Meyer, R. Krämer, and H. Sahm for critically reading the manuscript. We are indebted to B. Radüchel (Schering AG, Berlin, Germany) for providing samples of Dy-DTPA.

In Sydney, we thank B. Bulliman for advice on computational problems, N. Scott for the dimethyl methylphosphonate mix, and the Department of Microbiology for providing their facilities to grow cells of *Z. mobilis* for the dimethyl methylphosphonate experiment.

REFERENCES

1. **Axe, D. D., and J. E. Bailey.** 1995. Transport of lactate and acetate through the energized cytoplasmic membrane of *Escherichia coli*. Biotechnol. Bioeng. Lett. **47:**8–19.
2. **Belaich, J. P., J. C. Senez, and M. Murgier.** 1968. Microcalorimetric study of glucose permeation in microbial cells. J. Bacteriol. **95:**1750–1757.
3. **Berthon, H. A., and P. W. Kuchel.** NMR studies of erythrocyte metabolism. Adv. Mol. Cell. Biol., in press.
4. **de Graaf, A. A., R. M. Wittig, U. Probst, J. Strohhäcker, S. M. Schoberth, and H. Sahm.** 1992. Continuous-flow NMR bioreactor for *in vivo* studies of microbial cell suspensions with low biomass concentrations. J. Magn. Reson. **98:**654–659.
5. **DiMarco, A. A., and A. H. Romano.** 1985. D-Glucose transport system of *Zymomonas mobilis*. Appl. Environ. Microbiol. **49:**151–157.
6. **Dombek, K. M., and L. O. Ingram.** 1986. Determination of the intracellular concentration of ethanol in *Saccharomyces cerevisiae* during fermentation. Appl. Environ. Microbiol. **51:**197–200.
7. **Fichtenholz, G. M.** 1966. Differential- und Integralrechnung, vol. II, p. 226–227. VEB Deutscher Verlag der Wissenschaften, Berlin.
8. **Gerhardt, P.** 1981. Diluents and biomass measurement, p. 504–507. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
9. **Grotendorst, J., P. Jansen, and S. M. Schoberth.** 1994. On solving the McConnell equations in biochemistry. MapleTech **1:**56–62.
10. **Guijarro, J. M., and R. Lagunas.** 1984. *Saccharomyces cerevisiae* does not accumulate ethanol against a concentration gradient. J. Bacteriol. **160:**874–878.
11. **Hockings, P. D., and P. J. Rogers.** 1994. ^{1}H NMR determination of intracellular volume in cell suspensions. Arch. Biochem. Biophys. **311:**383–388.
12. **Hoffman, D., and R. K. Gupta.** 1986. NMR measurement of intracellular water volume. J. Magn. Reson. **70:**481–485.
13. **Hore, P. J.** 1983. Solvent suppression in Fourier transform nuclear magnetic resonance. J. Magn. Reson. **55:**283–300.
14. **Ingram, L. O.** 1986. Microbial tolerance to alcohols: role of the cell membrane. Trends Biotechnol. **2:**40–44.
15. **Jones, R. P.** 1988. Intracellular ethanol—accumulation and exit from yeast and other cells. FEMS Microbiol. Rev. **54:**239–258.
16. **Kalnenieks, U., A. A. de Graaf, S. Bringer-Meyer, and H. Sahm.** 1993. Oxidative phosphorylation in *Z. mobilis*. Arch. Microbiol. **160:**74–79.
17. **Kirk, K.** 1990. NMR methods for measuring membrane transport rates. NMR Biomed. **3:**1–16.
18. **Kirk, K., and P. W. Kuchel.** 1985. Red cell volume changes monitored using a new NMR procedure. J. Magn. Reson. **62:**568–572.
19. **Kirk, K., and P. W. Kuchel.** 1986. Equilibrium exchange of dimethyl methylphosphonate across the human red cell membrane measured using NMR spin transfer. J. Magn. Reson. **68:**311–318.
20. **Kirk, K., and P. W. Kuchel.** 1988. Physical basis of the effect of hemoglobin on the ^{31}P NMR chemical shifts of various phosphoryl compounds. Biochemistry **27:**8803–8810.
21. **Kuchel, P. W.** 1990. Spin-exchange NMR spectroscopy in studies of the kinetics of enzymes and membrane transport. NMR Biomed. **3:**102–119.
22. **Kuchel, P. W., K. Kirk, and G. F. King.** 1995. The use of NMR in the study of membrane transport and diffusion, p. 247–327. *In* H. J. Hilderson and G. B. Ralston (ed.), Physico-chemical methods in the study of biomembranes. Plenum Press, London.
23. **Led, J. J., and H. Gesmar.** 1982. The applicability of the magnetization-transfer NMR technique to determine chemical exchange rates in extreme cases. The importance of complementary experiments. J. Magn. Reson. **49:**444–463.
24. **Neidhardt, F. C., J. L. Ingraham, and M. Schaechter.** 1990. Physiology of the bacterial cell. Sinauer Associates, Sunderland, Mass.
25. **Raftos, J. E., B. T. Bulliman, and P. W. Kuchel.** 1990. Evaluation of an electrochemical model of erythrocyte pH buffering using ^{31}P nuclear magnetic resonance data. J. Gen. Physiol. **95:**1183–1204.
26. **Robinson, G., B. E. Chapman, and P. W. Kuchel.** 1984. ^{31}P NMR spin-transfer in the phosphoglyceromutase reaction. Eur. J. Biochem. **143:**643–649.
27. **Robinson, G., P. W. Kuchel, B. E. Chapman, D. M. Doddrell, and M. G. Irving.** 1985. A simple procedure for selective inversion of NMR resonances for spin transfer enzyme kinetic measurements. J. Magn. Reson. **63:**314–319.
28. **Ruhrmann, J., and R. Krämer.** 1992. Mechanism of glutamate uptake in *Zymomonas mobilis*. J. Bacteriol. **174:**7579–7584.
29. **Sahm, H., S. Horbach, J. Strohhäcker, R. Welle, and A. A. de Graaf.** 1994. Novel pathway of glycerol-3-phosphate formation. FEMS Microbiol. Lett. **120:**37–44.
30. **SAS Institute Inc.** 1989. SAS/STAT user's guide, version 6, 4th ed., vol. 2. SAS Institute Inc., Cary, N.C.
31. **Schoberth, S. M., B. E. Chapman, P. W. Kuchel, R. Wittig, J. Grotendorst, P. Jansen, and A. A. de Graaf.** 1994. *In-vivo* NMR spectroscopy to study transmembrane diffusion of ethanol in the bacterium *Zymomonas mobilis*, p. 43. *In* L. P. Ingman, J. Jokisaari, and J. Lounila (ed.), 12th European Experimental NMR Conference (EENC 94).
32. **Schoberth, S. M., and A. A. de Graaf.** 1993. Use of *in vivo* ^{13}C nuclear magnetic resonance spectroscopy to study sugar uptake in *Zymomonas mobilis*. Anal. Biochem. **210:**123–128.
33. **Schoberth, S. M., A. A. de Graaf, B. E. Chapman, P. W. Kuchel, and H. Sahm.** 1992. Use of *in vivo* NMR spectroscopy to study transport kinetics of metabolites, including sugars, in bacterial cell suspensions, L-19, p. 10. *In*

Abstracts of the XV International Conference on Magnetic Resonance Biological Systems.
34. **Stein, W. D.** 1986. Transport and diffusion across cell membranes. Academic Press, New York.
35. **Stock, J. B., B. Rauch, and S. Roseman.** 1977. Periplasmic space in *Salmonella typhimurium* and *Escherichia coli*. J. Biol. Chem. **21:**7850–7861.
36. **Strohhäcker, J., A. A. de Graaf, S. M. Schoberth, R. M. Wittig, and H. Sahm.** 1993. ^{31}P nuclear magnetic resonance studies of ethanol inhibition in *Z. mobilis*. Arch Microbiol. **159:**484–490.
37. **Struch, T., B. Neuss, S. Bringer-Meyer, and H. Sahm.** 1991. Osmotic adjustment of *Z. mobilis* to concentrated glucose solutions. Appl. Microbiol. Biotechnol. **44:**518–523.
38. **Swings, J., and J. de Ley.** 1977. The biology of *Zymomonas*. Microbiol. Rev. **41:**1–46.
39. **Weast, R. C., and M. J. Artle (ed.).** CRC handbook of chemistry and physics, 60th ed. CRC Press, Boca Raton, Fla.
40. **Weisser, P., R. Krämer, H. Sahm, and G. A. Sprenger.** 1995. Functional expression of the glucose transporter of *Zymomonas mobilis* leads to restoration of glucose and fructose uptake in *Escherichia coli* mutants and provides evidence for its facilitator action. J. Bacteriol. **177:**3351–3354.
41. **Weuster-Botz, D.** 1993. Continuous ethanol production by *Zymomonas mobilis* in a fluidized bed reactor. Part I. Kinetic studies of immobilization in macropore glass beads. Appl. Microbiol. Biotechnol. **39:**679–684.
42. **Wittig, R., M. Möllney, W. Wiechert, and A. A. de Graaf.** Interactive evaluation of NMR spectra from *in vivo* isotope labelling experiments. Comput. Appl. Biotechnol., in press.