^{31}P-NMR studies of *Mycoplasma gallisepticum* cells using a continuous perfusion technique

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^{31}P-NMR studies of *Mycoplasma gallisepticum* cells have been carried out using a continuous perfusion technique; these are the first such studies with this organism. Using this technique, glucose metabolism was monitored in the intact organisms, and cell extracts were prepared to identify the intermediates. Under glycolytic conditions, high levels of fructose-1,6-diphosphate were observed, indicating that this sugar may play a key role in the regulation of metabolism. The level of phospho*enol*pyruvate was low under normal glycolytic conditions, and did not increase during starvation. From the position of the internal inorganic phosphate peak, the intracellular pH was estimated. The cells were found to maintain an intracellular pH of ~7.1 over an investigated external pH range of 6.6–8.6.

^{31}P-NMR (Mycoplasma gallisepticum) *Metabolism* *Glycolysis*

1. INTRODUCTION

The ability to monitor metabolism in intact cells using non-invasive high field fourier-transform NMR spectroscopic techniques has significantly advanced our understanding of microbial energetics. NMR studies have, for example, been carried out on Gram-negative [1] and Gram-positive [2] bacteria, yeast cells [3], and amoebae [4]. There have, however, been no reports of the application of NMR to metabolism in mycoplasmas. Mycoplasmas are the smallest and simplest self-replicating prokaryotes, having the smallest genome of any free-living cell. They lack a cell wall and, as a result, are fragile and fastidious [5]. Most, if not all, mycoplasmas lack an oxidative phosphorylation system for ATP generation and use instead a system based on phosphorylation at the substrate level [6]. In non-fermentative mycoplasmas, ATP is formed by the arginine dihydrolase pathway [7]. Previous attempts to apply ^{31}P-NMR spectroscopy to the study of mycoplasma metabolism (Z. Gross and S. Rottem, unpublished), using dense cell suspensions, were unsuccessful. This was, most likely, because the packed, delicate cells could not survive the harsh conditions due to either hypoxia, the rapid consumption of nutrients, or the build-up of catabolic end-products. The recent development of continuous perfusion systems for NMR [8] for the study of microorganisms has opened the way for the effective application of ^{31}P-NMR to study mycoplasma metabolism under controlled conditions, which simulate as closely as possible the normal physiological state. The data presented in this study demonstrate the feasibility of performing

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NMR measurements to determine biochemical and physiological parameters on *Mycoplasma gallisepticum*.

2. MATERIALS AND METHODS

2.1. *Organisms and growth conditions*

Mycoplasma gallisepticum (strain A5969) was grown in Edward medium [9,10] containing 5% horse serum, 0.57% glucose, and 25 mM Hepes buffer adjusted to pH 7.8. The medium was inoculated from a frozen culture at an inoculum level of 1–2% and the cultures were incubated for 18–22 h at 37°C. Cells were harvested at the mid-exponential phase of growth (A_{640} = 0.22–0.25) by centrifugation at 12000 × *g* for 20 min. The cells were washed once and re-suspended in cold 0.25 M NaCl solution. Protein in the cell suspension was determined according to Lowry et al. [11]. Viability of the cells throughout the NMR analysis was measured using the colony forming units technique [12].

2.2. *Preparation of the cells for ^{31}P-NMR studies*

A cell suspension (containing 15–25 mg cell protein) was centrifuged to a pellet at 12000 × *g* for 3 min in an Eppendorf microfuge. The pellet was re-suspended in 0.25 M NaCl to a final volume of 0.6–0.8 ml and was pre-incubated at 37°C for 5 min. An equal volume of a 2.7% low-gelling-temperature agarose solution in Hepes-buffered saline (2.6 g/l Hepes buffer) containing KCl (0.2 g/l), $MgCl_2$ (0.15 g/l), and $CaCl_2$ (0.13 g/l) at 37°C was then added and the cell/gel mixture was gently, but thoroughly mixed. The cell/gel mixture, maintained at 37°C, was extruded under pressure, as described [13,14], into an NMR tube containing fresh growth medium, for which the horse serum was replaced with 0.5% bovine serum albumin. The gel casting process required ~5 min. The NMR tube was then attached to the perfusion system [8,13,14] and the flow of medium was adjusted to a rate of ~2.0 ml/min. The perfusion medium (100 ml) was recycled.

2.3. *NMR measurements*

The NMR measurements were carried out using a JEOL GX-400 spectrometer equipped with a broad-band probe and a variable temperature unit. Acquisition parameters included: 20 kHz spectral window, 4096 data points, 18 μs ($\pi/2$) pulse, 0.1 s acquisition time, and 0.8 s pulse repetition time. The broad-band proton decoupler was gated off, except during the acquisition. Prior to Fourier transformation, the free-induction-decay signal was twice zero-filled and then exponentially multiplied so as to result in an additional 15 Hz line-broadening in the frequency-domain spectrum.

2.4. *Identification of intermediates during glucose metabolism*

Freshly prepared cells (80 mg cell protein) were re-suspended in a solution containing 10 ml of 0.15 M NaCl, 25 mM Hepes, 10 mM 2-mercaptoethanol, and 10 mM $MgCl_2$ adjusted to pH 7.5. The cells were starved by incubation at 37°C for 30 min with constant shaking. Glucose (50 mM) was then added and, after 2 min of incubation at 37°C, sugar metabolism was stopped by injecting the cells into 100 ml of boiling water. Insoluble materials were removed from the cell extract by centrifugation at 34000 × *g* for 60 min and the clear supernatant fluid was freeze-dried and reconstituted with 1.0 ml of 0.025 M Hepes buffer at pH 7.5. 1 ml of 100 mM Tris buffer, containing 10 mM EDTA at pH 7.5, was then added and the samples were subjected to NMR analyses.

3. RESULTS AND DISCUSSION

Fig.1 shows typical ^{31}P-NMR spectra obtained from starved and metabolizing *M. gallisepticum* cells. To achieve starvation, the cells were perfused with glucose-free medium. Glycolysis was then initiated by adding glucose (0.5%) to fresh medium. The spectrum of the metabolizing cells contained strong γ, α, and β ATP phosphate resonances at −5.2, −10.3, and −18.9 ppm, respectively (the external inorganic phosphate signal was set to 2.9 ppm [15]); additional resonances for the sugar phosphates of the Embden-Meyerhof pathway as well as intracellular inorganic phosphate were observed. Under starvation conditions, cellular concentrations of ATP decreased to below detectable levels within ~1 h of perfusion while the intracellular inorganic phosphate level increased. The intracellular inorganic phosphate level in glycolytic cells was only 10–15% of that observed in starved cells. The spectrum of glycolytic cells

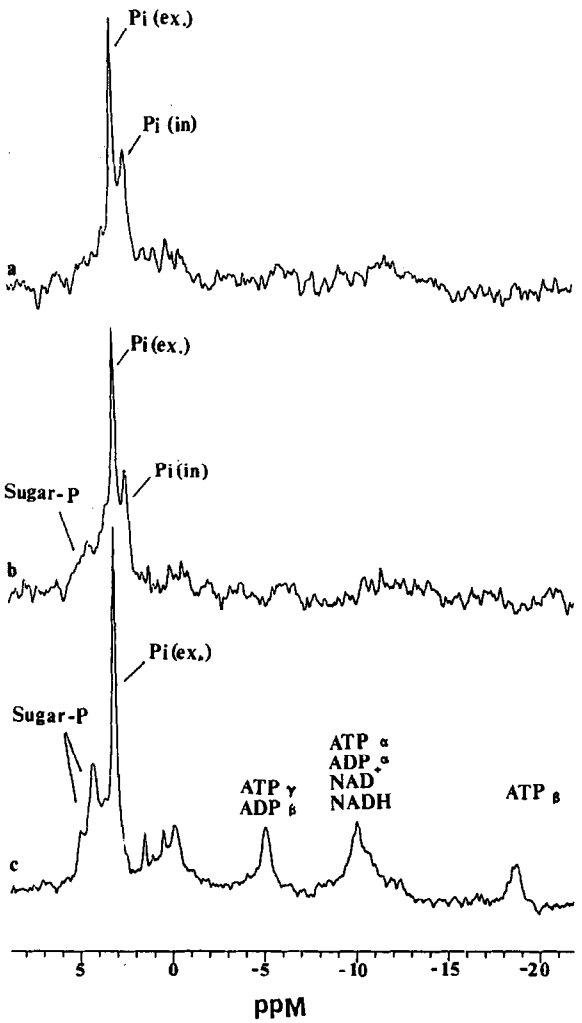

Fig.1. ^{31}P-NMR spectra of starved and glycolyzing *M. gallisepticum* cells. The various traces resulted from averaging 1000 transients: a 0.8 s pulse repetition time was used. The cells were prepared as described in section 2: (a) cells perfused with a glucose-free medium; (b) glucose added to the medium and acquisition of the spectrum begun; (c) spectral acquisition begun 20 min after the addition of glucose. The external pH was ~7.8. P_i(ex.), extracellular inorganic phosphate; P_i(in), intracellular inorganic phosphate.

was practically the same using freshly prepared gel/cell threads or gel/cell threads that were perfused for 10–12 h with glucose containing medium, suggesting that the cells were metabolically active within the threads. To determine viability, cells were removed from the gel/cell threads by vortex-mixing the threads into buffer, followed by pipetting onto plates. It was found that 60 ± 15% of the viability, relative to time 0, was maintained after 12 h in the perfusion system, indicating the feasibility of carrying out long-term experiments with metabolically active cells. To identify the sugar phosphate metabolites, cell extracts were analyzed (fig.2). The predominant metabolites that were identified were: fructose-1,6-diphosphate (δ 4.01 and 4.21), glucose-6-phosphate (δ 4.92), phospho*enol*pyruvate (δ 0.18), and an unidentified component (labelled 'x' at δ 1.23). Other Embden-Meyerhof metabolites (such as, glyceraldehyde-3-phosphate, dihydroxyacetone phosphate, 3-phosphoglycerate, 2,3-diphosphoglycerate, or 2-phosphoglycerate) or acetylphosphate were not detected. The high levels of fructose diphosphate suggest that this component plays a major role in mycoplasma metabolism. Fructose diphosphate was previously shown to play a role in metabolic regulation [16]. In several bacteria examined, the level of phospho*enol*pyruvate is increased upon starvation, apparently as a result of inhibiting the pyruvate kinase by the increased inorganic phosphate levels [17]. In *M. gallisepticum*, however, the phospho*enol*pyruvate level in starved cells was lower than in glycolytic cells, even though the inorganic phosphate levels were very high. This indicates that in mycoplasma pyruvate

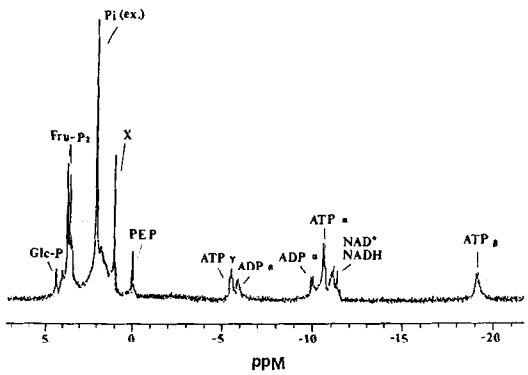

Fig.2. ^{31}P-NMR spectra of inorganic phosphate and sugar phosphate intermediates in the cell extract of glycolyzing cells. Glc-P, glucose 6-phosphate; Fru-P_2, fructose 1,6-diphosphate; P_i(ex.), extracellular inorganic phosphate; x, unidentified intermediate; PEP, phospho*enol*pyruvate.

kinase activity is not affected by intracellular inorganic phosphate levels.

The intracellular pH, determined from the position of the intracellular inorganic phosphate resonance [15], was approx. 7.1, and was, to a large extent, maintained in the presence of a wide variation in external pH. Thus, over a range of external pH from ~6.6 to 8.6, the internal pH was maintained to within 0.3 pH units, becoming slightly more alkaline under high external pH conditions and slightly more acidic under low external pH conditions. These results are in good agreement with those measured using the benzoic acid partition technique [18].

The NMR perfusion technique allows us to avoid the problems associated with the use of dense cell suspensions and permits us to continue to address a number of questions in mycoplasmal cell physiology. Such studies, preferably in combination with ^{14}C-fluorography, will enable us to follow the time course of fluctuations in the concentration of intermediates in cells maintained under varying conditions.

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