IN SITU ANALYSIS OF THE MICROBIAL FERMENTATION PROCESS BY NATURAL ABUNDANCE ^{13}C AND ^{31}P NMR SPECTROSCOPY. PRODUCTION OF ADENOSINE-5'-TRIPHOSPHATE FROM ADENOSINE

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1. Introduction

The application of ^{13}C NMR spectroscopy to in situ analysis of the complex microbial metabolites has been reported by Eakin et al. [1]. They studied the fate of [1-^{13}C] glucose on being fed to yeast cells, *Candida utilis*, by monitoring the ^{13}C NMR spectra of metabolites without separating them from the incubation broth. Use of 1-^{13}C-enriched substrate, as has been pointed out by Eakin et al. [1], would allow one to use a substrate concentration lower than several millimolar for its increased NMR sensitivity, although usual microbial fermentations, which are carried out in both laboratories and industry, use much higher substrate concentrations. The ^{13}C enrichment may still be essential to study the transient metabolites which might exist in the fermentation process only for a short period of time compared to the duration to obtain the ^{13}C NMR spectra of metabolites at natural ^{13}C abundance of 1.1%, or to search for the metabolites which might exist at very low concentration, either in intra- or extracellular medium. Despite the merit of higher NMR sensitivity described above use of the ^{13}C-enriched substrates in analyzing microbial reactions has obvious drawbacks. As only the enriched portion of the carbons in the metabolites can be detected, unexpected metabolites in the broth may hardly be characterized. The metabolites, by the same reason, which bear no carbons from the enriched part of the substrate may be overlooked.

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Recent advances in the ^{13}C NMR spectroscopy have improved the sensitivity quite a little [2,3], and therefore it seems now to be no reason to stick to the ^{13}C-enriched substrates, as far as one concerns to analyze the extracellular metabolites. In this communication we wish to present a clearcut example to prove a potential application of natural abundance ^{13}C and ^{31}P NMR spectroscopy to investigate various microbial fermentations.

2. Experimental

Frozen Baker's yeast cells (Oriental Yeast Co.) were suspended in cold acetone and were quickly collected by filtration. The acetone-treated dry cells were found to be permeable to fructose 1,6-bisphosphate (FBP). and were free of paramagnetic metal ions. The latter feature was essential to get sharp ^{31}P NMR spectra for the incubation broth. A typical run of the fermentation was following. To 175 mg adenosine (AR) in a 12 mm (O.D.) NMR sample tube was added 5 ml yeast cell suspension containing 500 mg of the dry yeast cells, 500 mg glucose, 150 mg KH_2PO_4, 300 mg K_2HPO_4, and 15 mg $MgSO_4 \cdot H_2O$. The resultant inhomogeneous mixture was incubated anaerobically in a NMR spectrometer (Varian XL-100-15) at a probe temperature of 34°C. ^{13}C and ^{31}P NMR spectra were obtained by Fourier transform method using a Varian 620-L computer with 16 K core memories, and an external fluorine lock unit served the field/frequency stabilization. Each of 4000 and 2000 free induction decays after

90° and 30° R_F pulses with repetition time of 0.5 sec were accumulated for the ^{13}C and ^{31}P spectra, respectively.

3. Results and discussions

The microbial phosphorylation of AR to adenosine 5′-triphosphate (ATP), which is coupled to the glycolytic degradation of glucose [4], has been investigated by ^{13}C and ^{31}P NMR spectroscopy. A typical trace of the natural abundance ^{13}C NMR spectra of a gross fermentation broth is given in fig.1, and the ^{31}P NMR spectra in fig.2. These spectra were measured during about 30 and 20 min, respectively, and hence they represent the 'time-averaged' spectra over the durations. As the whole reaction, however, took more than 30 h to complete, such duration for the NMR measurements therefore was not very significant in this case. Obviously use of a larger bore NMR sample tube or a higher frequency spectrometer reduce this type of uncertainty and would afford better time resolved profiles of the fermentation. The assignment of the NMR spectra was conveniently made by comparing the spectra to those of the standard samples, and/or by adding authentic samples directly to the broth. We have succeeded to identify glucose, trehalose, FBP, glycerol, ethyl

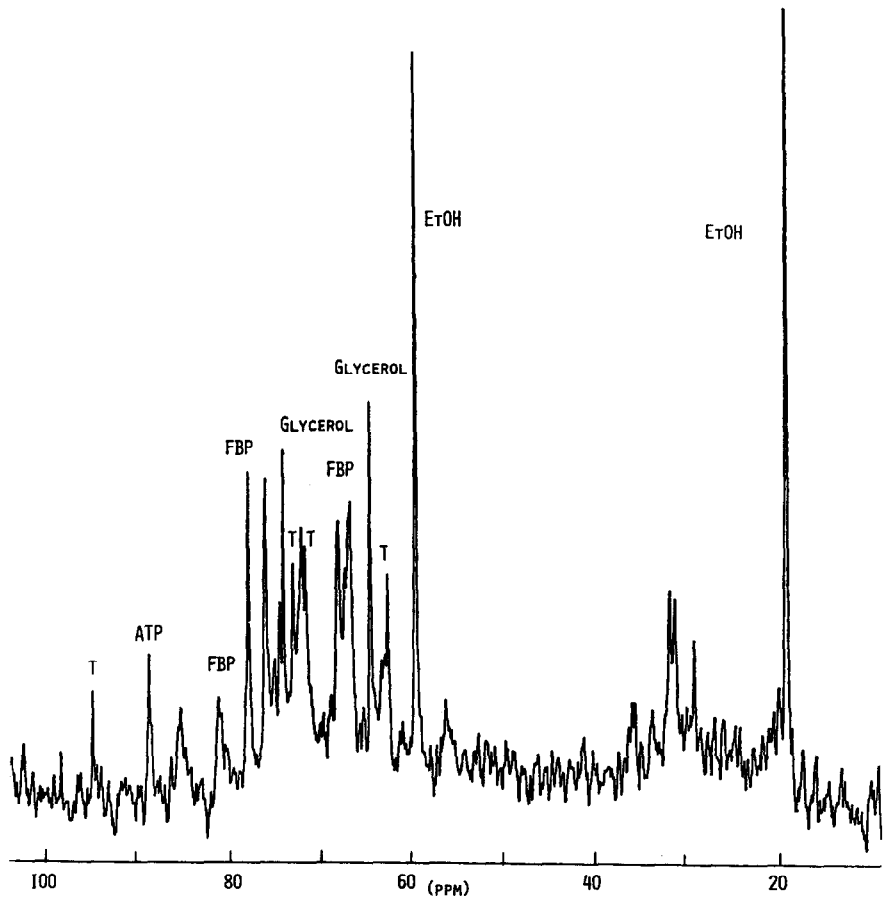

Fig.1. 25.2 MHz Proton decoupled ^{13}C NMR spectrum of a fermentation broth incubated at 34°C for about 10 h. The broth initially contained glucose and adenosine as substrates, and trehalose from the cultured Baker's yeast cells as assignable major carbon compounds. Chemical shift scale is given in ppm from the tetramethylsilane's carbon resonance in a separate sample tube.

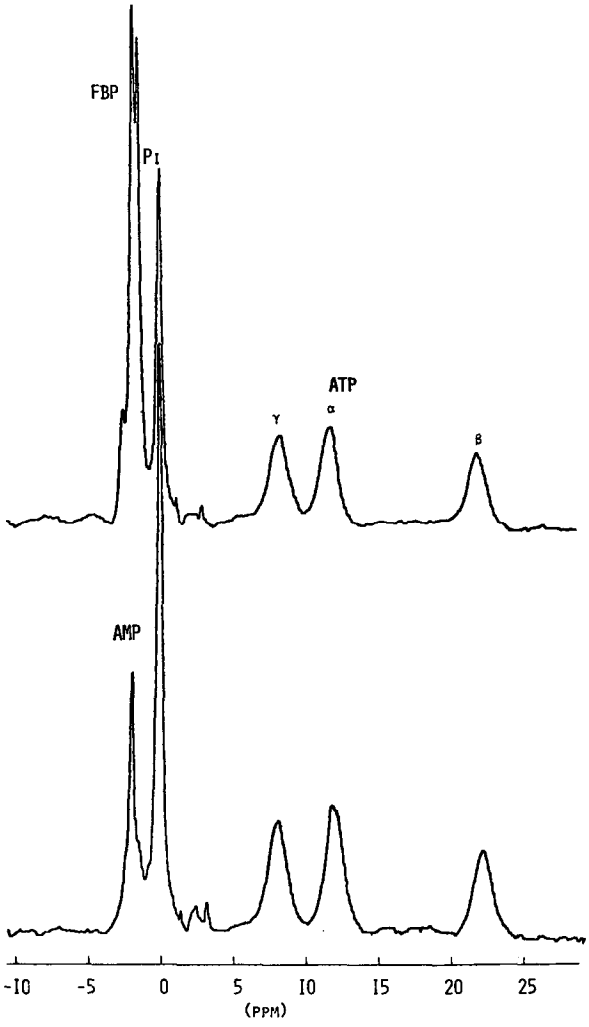

Fig.2. 40.5 MHz Proton decoupled ^{31}P NMR spectra of the broth. The top trace shows the spectrum for the broth incubated for 13 h and the bottom one for 28 h at 34°C. The initial broth only showed the P_i peak which was added as a substrate. The signal of FBP split into several lines due to various conformers [5]. The α- and γ-phosphorus peaks of ATP may be overlapped by the α- and β-phosphorus of ADP, respectively. The chemical shift refers to the P_i peak as to be 0 ppm.

alcohol, ATP, adenosine 5′-monophosphate (AMP) in the broth. In the course of analyzing the ^{13}C spectra we isolated and identified the 'glucose metabolite' which Eakin et al. [1] had speculated to be either intracellular glucose or glucose 6-phosphate in fact was extracellular trehalose. Trehalose is a well-known metabolite of glucose by yeasts [6,7], and was accumulated during cell culturing in our case (see fig.3). This may serve a good example of benefits to use a non-labeled substrate to identify the unexpected metabolite.

The time course of the reactions was tracked by periodically monitoring the ^{13}C as well as ^{31}P NMR spectra of the fermentation broth, and the results are shown in figs.3 and 4, respectively. In fig.3, the peak height of the characteristic resonances for each of the metabolites and in fig.4 the peak intensity estimated by multiplying the peak height with the width at the half-peak height were plotted to the incubation time. The profile of complex microbial metabolites in the broth are clearly seen in these figures. Glucose was extinct within initial 7 h and the concentration of FBP and inorganic phosphate (P_i) showed their respective maximum and minimum values at this period. Thereafter FBP, which is a key intermediate in the glycolytic cleavage of glucose, quickly decreased in its concentration and ATP came to be detected instead. The amount of ATP reached its maximum at around 18 h after the incubation had started, and then began to decrease, giving rise to AMP and P_i. Attempted detection of adenosine 5′-diphosphate (ADP) was hampered by serious line overlapping, but the accumulation was not very high as was evidenced by ^{31}P NMR spectroscopy (see fig.2). Glycerol and ethyl alcohol continually increased during the incubation period, while trehalose was gradually digested by Baker's yeast.

We have analyzed the concentration change of AR, ATP, and glucose in the broth also by conventional analytical methods: i.e. ATP and AR were analyzed by UV absorbance for the extracts of their respective spots on the paper chromatogram, and glucose was determined by the Schoorl's method [8]. The results were very consistent with what we got by the NMR method. Note that AR was not analyzable by in situ NMR measurement, because it was not soluble enough in the broth and the crystalline AR showed, of course, no narrow signal. In the case of UV analysis aliquots of the broth included solid AR were solubilized by adding hydrochloric acid before the analysis.

We showed above natural abundance ^{13}C NMR spectroscopy has excellent features to the in situ

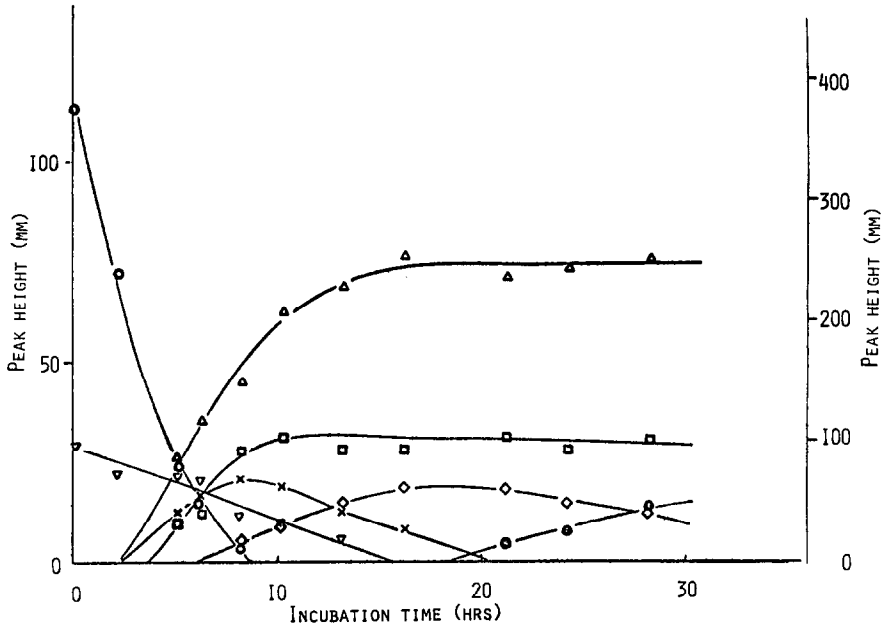

Fig.3. The time course for the phosphorylation of adenosine to ATP using Baker's yeast cells, analyzed by in situ ^{13}C nuclear magnetic resonance spectroscopy. Vertical scale at the right is for the glucose peak height which was very intense at the beginning, and the intensity for the other metabolites' spectra should refer to the left scale. The incubation time of each point represents the middle of the duration for NMR measurements which were about 30 min each. Symbols denote as following: (○) glucose, (△) trehalose, (△) ethyl alcohol, (□) glycerol, (X) FBP, (◇) ATP, (●) AMP.

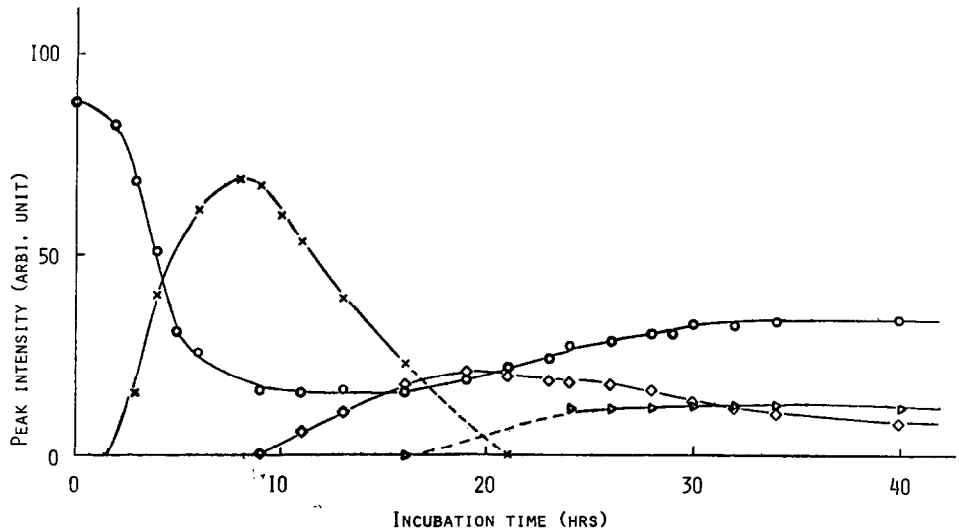

Fig.4. The time course obtained by in situ ^{31}P NMR spectra for the phosphorylation of adenosine to ATP in a Baker's yeast cell suspension incubated at 34°C. The incubation time of each point is again the middle of the time between starting and finishing the NMR measurements (about 20 min). Symbols denote as following: (○) inorganic phosphate (P_i), (X) FBP, (◇) ATP, (▷) AMP.

analysis of the cultured broths for various microbial fermentations. The usefulness of ^{31}P NMR, as was noted before [9,10], is complemental to the ^{13}C NMR method, but obviously is limited to the metabolites which have phosphorus.

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