Short Communication

Natural Abundance ^{13}C Nuclear Magnetic Resonance Spectra of Intact Fungal MyceliaToshiro MATSUNAGA, Akira OKUBO,
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High resolution ^{13}C nuclear magnetic resonance (NMR) spectroscopy has been applied to the studies of intact cells. This rapid and nondestructive technique provides information on the structure and dynamics of intracellular constituents *in vivo*. ^{13}C NMR studies have been reported for whole blood,¹⁾ yeasts,^{2,3)} *Neurospora crassa*,⁴⁾ *Escherichia coli*⁵⁾ and isolated rat liver cells.⁶⁾ These studies, however, were restricted to following the metabolic pathways of ^{13}C labeled compounds in the cells. Though the ^{13}C enrichment increases NMR sensitivity higher, only the enriched positions of the carbons in the metabolites can be detected.⁷⁾

In this communication we would like to show that it is possible to observe well resolved, natural abundance ^{13}C NMR spectra of a heavy metal tolerant fungus, *Penicillium ochro-chloron*⁸⁾ mycelia. From the NMR spectra at various stages of growth we characterized many cellular components and monitored the time course of the metabolism.

Penicillium ochro-chloron, strain ATCC 36741, was grown in 500 ml Erlenmeyer flasks containing 150 ml of the chemically defined medium⁸⁾ which contained 4% glucose as a carbon source at 30°C on a rotary shaker. Equal volumes of a single spore suspension were inoculated. The spores were obtained after 7 to 10 days incubation on Czapek's agar slants. After growth for 2, 3, 4, 6, 10 and 20 days, the mycelia were harvested by filtration on a Büchner funnel, washed three times with

150 ml of deionized water, packed into 10 mm diameter NMR tubes and measured immediately.

^{13}C NMR spectra were recorded at 25.1 MHz on a JEOL FX-100 spectrometer operating in the Fourier transform mode. ^{13}C NMR spectra were taken with 4500 scans of 90° pulses with 0.8 sec repetition time at 27°C with proton noise decoupling. The deuterium resonance of external D_2O was used as a frequency lock. Chemical shifts are given relative to external tetramethylsilane (TMS) at 0 ppm. After NMR measurement, the mycelia were lyophilized and weighed to follow the course of growth.

Figure 1 shows the natural abundance ^{13}C NMR spectra of *P. ochro-chloron* mycelia measured at different time after inoculation. Well resolved NMR spectra were successfully obtained in spite of the heterogeneity of the intact mycelia. The resonances were assigned on the basis of the chemical shifts and considerations of known metabolic pathways. Peaks 11, 12 and 13, which occur at 64.6, 70.6 and 72.2 ppm downfield from TMS respectively, are assigned to mannitol by comparison with the chemical shifts of the authentic sample. Peak 1 at 15.1 ppm is assigned to the terminal methyl carbon of fatty acids; peaks 2 to 7 at 23.8 to 34.8 ppm are assigned to the methylene carbons of fatty acids; peaks 24 and 25 at 129.0 and 130.7 ppm respectively are assigned to the olefinic carbons of unsaturated fatty acids; and peak 26 at 172.9 ppm is assigned to the carboxyl carbon of fatty acids.⁹⁾ The resonances in the region 62.4 to 83.8 ppm, including peaks 9, 10, 14, 15, 16, 17, 18 and 19, most likely represent the hydroxymethyl and hydroxymethylene carbons of polysaccharides and the resonances in the region 101.0 to 108.3 ppm, including peaks 22 and 23, represent the anomeric carbons of polysaccharides.¹⁰⁾ The line widths of the polysaccharides peaks are rather broader than those of mannitol and fatty acids peaks. Some of the polysaccharide resonances including the anomeric peak 23 at 108.3 ppm originate from the cell wall, because only the resonances were

FIG. 1. Natural Abundance ^{13}C NMR Spectra of *P. ochro-chloron* Mycelia. Each spectrum was taken at the time indicated on the right-hand side of the spectrum.

present in the ^{13}C NMR spectrum of the cell walls prepared with a MANTON GAULIN homogenizer. The weak peaks 8, 20 and 21 remain unidentified.

The ^{13}C NMR spectra changed with the age of the fungus. As shown in Fig. 2, the culture was in early logarithmic phase at 2 days, in late logarithmic phase at 4 days and entered stationary phase at 5 days. The mannitol peaks appeared at 2 days and their intensities reached a maximum at 4 days and then decreased. At 10 days, mannitol could no longer be detected. Mannitol is known to be a common polyol in fungi, but the metabolism and physiological role have not been elucidated well.⁽¹¹⁾ The fatty acids peaks were weak and broad at 2 days, became stronger abruptly at 4 days and were maintained till 20 days, showing the accumulation of fatty acids in the

stationary phase. In the ^{13}C spectrum of a lyophilized mycelium at 6 days which was re-suspended in chloroform, two new peaks were found at 63.2 and 70.0 ppm, together with the fatty acids peaks, which are attributed to glycerol carbons. This result indicates that the fatty acids which give resonances are constituents of triacyl glycerols. The intensities of the polysaccharides peaks also increased at 4 days.

The ability to exhibit narrow resonances depends strongly on the motional flexibility of the molecules. Whole mycelia consist of thousands of components, yet only mannitol, triacyl glycerols and polysaccharides which have a high degree of mobility and are relatively abundant in the mycelia yielded high resolution spectra. The sharp resonances of phospholipids associated with membranes or

FIG. 2. Growth Curve of *P. ochro-chloron*.

those of proteins were not observed.

The location and dynamic states of the mannitol, fatty acids and polysaccharides as well as further data to this assignment will be described elsewhere.

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