Analyses of Variously ^{15}N-Enriched Spermidines during Growth of *Candida boidinii* Cultured with ^{15}N-Enriched Ammonium Sulfate and α-^{15}N-Enriched L-Ornithine

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The analytical method for various ^{15}N-enriched spermidines was first applied for studies on polyamine metabolism of the yeast, *Candida boidinii* CBS 5777. Analyses were carried out during growth of the yeast in two defined media containing ^{15}N-enriched ammonium sulfate as a sole nitrogen source, and newly prepared α-^{15}N-enriched L-ornithine with ammonium sulfate as nitrogen sources. Observation in the former culture indicated that the ammonia pool was negligible, and the putrescine pool was larger than the decarboxylated *S*-adenosylmethionine pool in this organism. The latter culture suggested that α,δ-nitrogens of ornithine behaved equally throughout polyamine metabolism.

Keywords polyamine; nitrogen metabolism; yeast; ^{15}N-spermidine; GC-MS; ^{15}N-ammonium sulfate; pool size; α-^{15}N-L-ornithine

Stable isotopes are often useful when applied to studies otherwise impossible with radioactive isotopes. In the field of polyamine research, no attempt has been made to investigate polyamine metabolism from the aspect of nitrogen metabolism. In view of this fact, it was thought to be worth while analyzing ^{15}N-enriched polyamines derived from ^{15}N-enriched precursors in a biological system, though the metabolic pathway seems to have been almost elucidated using radioactive isotope labelled compounds.[1] As the biological system, culture of the yeast, *Candida boidinii* CBS 5777, was thought to be suitable, since the organisms can grow using various kinds of sole nitrogen sources, and the biochemistry of the yeast has been extensively studied by Haywood and Large,[2] reporting the presence of a polyamine interconversion system similar to that of mammalian cells.[3] The present paper deals with a manner of ^{15}N-enrichment of three nitrogens on spermidine molecule formed during the culture of the yeast in the presence of ^{15}N-enriched precursors, applying the method for gas chromatography–mass spectrometry (GC-MS) determination of various ^{15}N-enriched spermidines.[4]

Materials and Methods

Chemicals ^{15}N-Enriched ammonium sulfate (99% atom% ^{15}N, purity > 95%) was obtained from CEA, France. Potassium ^{15}N-enriched phthalimide was prepared in this laboratory from ^{15}N-enriched ammonium sulfate.[5] α-^{15}N-Enriched L-ornithine was prepared by optical resolution of α-^{15}N-enriched D,L-ornithine which was prepared according to the method of Roessler and Hesse[6] using potassium ^{15}N-enriched phthalimide, and was crystallized as monohydrochloric acid salt through a Dowex 1 × 8 (HCO_3^-) column. Optical resolution of the D,L-form was carried out similarly by the method of Greenstein,[7] which utilizes selective hydrolysis of the amide bond of α-monochloroacetylated L-amino acid with acylase I (Sigma). The hydrolysate containing α,δ-bis-monochloroacetylated ornithine (mostly D-form) and δ-monochloroacetylated ornithine (mostly L-form) was applied to a Dowex 1 × 8 (AcO^-) column, and was washed with water to collect the latter in a nonadsorbing fraction. The former was eluted with 2 M acetic acid. Each fraction, after hydrolysis with 6 N HCl, was applied to a Dowex 50 × 8 (H^+) column, and ornithine was stepwisely eluted with 0.5—2 N HCl. A crystalline monohydrochloric acid salt of α-^{15}N-enriched L-ornithine was obtained through a Dowex 1 × 8 (HCO_3^-) column. The optical purity was confirmed by a capillary GC[8] and high performance liquid chromatography (HPLC)[9] with a trace of contamination of α-^{15}N-enriched D-ornithine (less than 5%).

All other reagents and organic solvents were of analytical reagent grade and were commercially available.

Culture Conditions The yeast, *Candida boidinii* CBS 5777 (*C. boidinii*), was supplied from the Central Bureau voor Shimmel Culture, Delf., The Netherlands. *C. boidinii* maintained in YM medium, composed of 0.5 g of bacto-peptone (Difco), 0.3 g of yeast extract (Difco), 0.3 g of malt extract (Difco) and 1.0 g of glucose in 100 ml, was precultured at 30 °C in a defined medium (pH 6.0) containing 55 mM glucose, 7.5 mM ammonium sulfate, vitamins, and inorganic salts according to the method of Haywood and Large.[2] The organisms growing at log phase ($OD_{660} = 1.0$) were collected, and inoculated onto the defined medium containing 7.5 mM ^{15}N-enriched ammonium sulfate, or 100 μM α-^{15}N-enriched L-ornithine with 7.5 mM ammonium sulfate to show the initial $OD_{660} = 0.18$ or 0.1, respectively.

Polyamine Determination *C. boidinii* collected at various time intervals were washed three times with the defined medium lack of nitrogen source and containing 0.02% Brij 35, and suspended in 6 N HCl to be hydrolyzed at 120 °C for 48 h. Total polyamines were determined fluorometrically by the HPLC-OPA system.

Analysis of ^{15}N-Enriched Spermidines S1 to S8 stand for natural and 7 kinds of ^{15}N-enriched spermidines as shown in Table I. The original method[4] was modified by changing pentafluoropropionyl (PFP) derivatives to heptafluorobutylyl (HFB) ones, since HFB-spermidines were more stable than PFP-spermidines. All minor alterations of the method accompanied with this modification were successfully done. A column of 2% SE-30 was adopted for GC-MS (JEOL JMX-DX 300). The samples for GC-MS were prepared by carboxymethyl (CM)-cellulose column chromatography[10] of 6 N HCl hydrolysate of the cells.

TABLE I. Natural and ^{15}N-Enriched Spermidines

Spermidine	$NH_2(CH_2)_3NH(CH_2)_4NH_2$		
	N^1	N^4	N^8
S1	N	N	N
S2	^{15}N	N	N
S3	N	^{15}N	N
S4	N	N	^{15}N
S5	^{15}N	^{15}N	N
S6	N	^{15}N	^{15}N
S7	^{15}N	N	^{15}N
S8	^{15}N	^{15}N	^{15}N

Results and Discussion

Culture of *C. boidinii* with ^{15}N-Enriched Ammonium Sulfate as Sole Nitrogen Source *C. boidinii* growing at log phase in the preculture medium were collected and inoculated into the culture medium containing ^{15}N-enriched ammonium sulfate. The growth curve and polyamine contents per 10^8 cells were recorded during the incubation time for 12 h (Fig. 1). The organisms grew logarithmically during the first 5 h, keeping the contents of putrescine, spermidine and spermine about 4, 6 and 8 nmol per 10^8 cells, respectively. This strain had a noticeable feature in the high

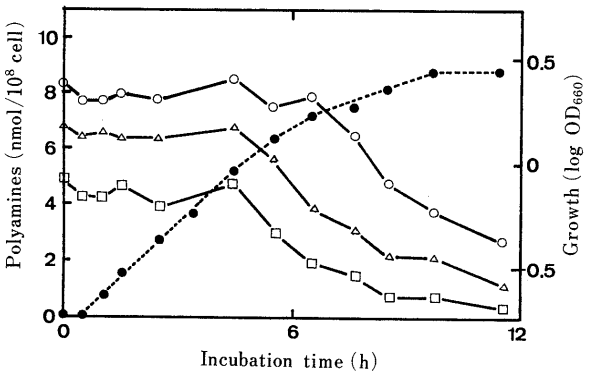

Fig. 1. Growth Curve and Polyamine Contents of *C. boidinii* Cultured with ^{15}N-Enriched Ammonium Sulfate

Details are described in Materials and Methods. ●---●, growth curve; □—□, putrescine; △—△, spermidine; ○—○, spermine.

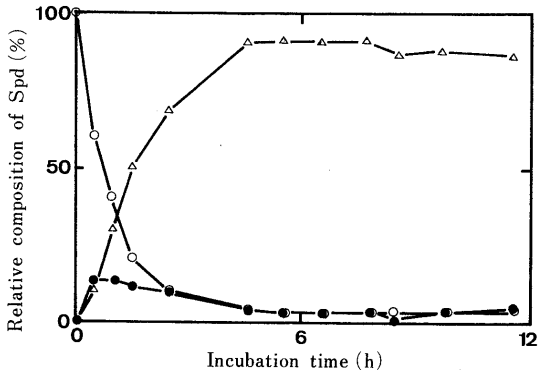

Fig. 2. Profile of Various Spermidines during Growth of *C. boidinii* Cultured with ^{15}N-Enriched Ammonium Sulfate

Spermidine samples in Fig. 1 were analyzed by GC–MS as described in Materials and Methods. ○—○, S1; ●—●, S2; △—△, S8.

contents of spermine compared with putrescine and spermidine. The rapid decrease after 5 h of incubation did not depend on their excretion to the culture medium. It might depend on their catabolism through the polyamine interconversion system or another metabolic pathway.

Relative ratios of various ^{15}N-enriched spermidines were then calculated at each incubation time, and the results were summarized in Fig. 2. Three kinds of spermidines were found during the incubation period. A main feature was the rapid decrease in the natural spermidine (S1) concomitant with a rapid increase in the triply ^{15}N-enriched one (S8). The $^{15}N^{1}$-enriched spermidine (S2) seemed to appear at the earliest stage of culture before the rise of S8, although the amount was low. These results allowed us to discuss the pool size of the precursors for spermidine biosynthesis. First, the ammonia pool should be negligible in this organism, since two nitrogens of putrescine moiety of spermidine observed were composed of either one of ^{14}N or ^{15}N. If there were a significant ammonia pool, mono ^{15}N-enriched putrescine should have been found especially at the early stage of culture, since putrescine was biosynthesized by the decarboxylation of ornithine supplied by *de novo* synthesis from ammonia. Direct analysis of putrescine by GC-MS also showed the absence of mono ^{15}N-enriched putrescine throughout the incubation period tested. Second, the presence of S2 and the absence of S6 suggested that the putrescine pool was larger than the

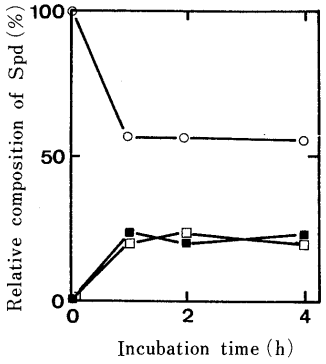

Fig. 3. Relative Composition of Various Spermidines in *C. boidinii* Cultured with α-^{15}N-Enriched L-Orinithine in the Presence of Ammonium Sulfate

Experimental conditions are described in the text. ○—○, S1; ■—■, S3; □—□, S4

decarboxylated S-adenosylmethionine pool. As arginine and ornithine should be included in the former pool and methionine in the latter, the results may be extended to the pool size of these amino acids in the cells: *e.g.* the arginine and/or ornithine pool may be larger than methionine.

Culture of *C. boidinii* with α-^{15}N-Enriched L-Ornithine in the Presence of Ammonium Sulfate It was of interest to know if α,δ-nitrogens of ornithine behave equally throughout polyamine metabolism. For this purpose, α-^{15}N-enriched L-ornithine was prepared, and applied for culture experiments to answer the following questions, that is, whether or not an exchange of N^{8}-primary nitrogen of spermidine occurrs *via* a transamination reaction, and whether or not polyamine biosynthetic enzymes are strictly ordered in cells like a multienzyme complex, as was imagined from the observation that new polyamines are derived from new precursors.[11] After preliminary experiments, 100 μM of α-^{15}N-enriched L-ornithine was added to the defined medium containing 7.5 mM ammonium sulfate. The amount of ornithine had no effect on the growth curve and polyamine contents, and the uptake was completed after 2 h when 53% of the cellular spermidine had been derived from the added ornithine (data using ^{14}C-ornithine not shown). This suggested that a preferential use of added ornithine for polyamine biosynthesis occurred under the conditions. The logarithmically growing organisms were collected at 1, 2 and 4 h after the addition of ornithine, and relative ratios of various spermidines were calculated. The results were summarized in Fig. 3. The ratio of S3 to S4 was kept at almost 1 to 1 during the incubation period of 4 h, and no significant difference was observed between the two spermidine species within the error of determination. Thus, it can be concluded that α and δ nitrogens of ornithine equally behaved throughout polyamine metabolism in *C. boidinii*, eliminating both the exchange reaction of N^{8} nitrogen of spermidine, and a strictly ordered arrangement of polyamine biosynthetic enzymes. The possibility of such an exchange reaction at the N^{1} position of spermidine may still remain.

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