Assimilative Pathway of Methanol in *Candida* sp. Incorporation of ^{14}C -Methanol, ^{14}C -Formaldehyde, ^{14}C -Formate and ^{14}C -Bicarbonate into Cell Constituents

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Received June 16, 1973

1) The incorporation of ^{14}C -methanol, ^{14}C -formaldehyde, ^{14}C -formate and ^{14}C -bicarbonate into a methanol-utilizing yeast, *Candida* N-16, was examined by paper-chromatography and radioautography.

2) At the earliest time period examined, the highest percentage of radioactivity fixed from ^{14}C -methanol or ^{14}C -formaldehyde into methanol-grown cells was found in fructose phosphate. The percentage distribution of radioactivity in fructose phosphate decreased as time elapsed. The radioactivity fixed from these compounds into glucose-grown cells was negligible compared with that fixed into methanol-grown cells.

3) The incorporation of ^{14}C -formate into methanol-grown cells was extremely low. The highest percentage of radioactivity fixed for short time incubation was found in serine. The incorporation pattern of glucose-grown cells was similar to that of methanol-grown cells.

4) At the earliest time period, over 70% of radioactivity fixed from ^{14}C -bicarbonate into methanol- or glucose-grown cells was found in aspartate.

5) These results suggest that in *Candida* N-16 methanol is specifically assimilated by a route with hexose phosphate as a primary stable intermediate.

A number of studies on the assimilation of methane, methanol and formate by bacteria have revealed the following pathways: the ribulose diphosphate cycle,^{1,2)} the glycine-serine pathway^{3~7)} and the allulose phosphate pathway.^{8~11)} In the case of methanol-utilizing yeast, several papers^{12~16)} have reported that methanol was oxidized via formaldehyde and formate to carbon dioxide. However, nothing was reported about the assimilative pathway of methanol until our previous observation that hexose phosphate appears to be an intermediate in the assimilation of methanol by *Candida* N-16.¹⁷⁾ To examine this observation in more detail, the incorporation of radioactivity from ^{14}C -methanol, ^{14}C -formaldehyde, ^{14}C -formate and ^{14}C -bicarbonate into cell constituents of methanol- and glucose-grown cells was investigated. The results presented in this paper support the previous conclusion that methanol is specifically assimilated to cell constituents through hexose phosphate in the organism. A part of this

work has already been published as a preliminary note.¹⁷⁾

MATERIALS AND METHODS

Growth of Candida N-16. The yeast was grown at 30°C in the following medium:¹⁵⁾ $(\text{NH}_4)_2\text{HPO}_4$, 3 g; KH_2PO_4 , 1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.002 g; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.002 g; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.002 g; yeast extract, 0.1 g; thiamine-HCl, 200 μg ; biotin, 4 μg and distilled water, 1 liter (pH 7.0). The medium was sterilized at 120°C for 15 min and then 10 g of methanol was added into 1 liter of the medium after cooling. When glucose was used as a carbon source, 10 g of glucose was added instead of methanol.

Incorporation of ^{14}C -methanol, ^{14}C -formaldehyde, ^{14}C -formate and ^{14}C -bicarbonate into cell constituents. Cells in a logarithmic growth phase were harvested by centrifugation, washed twice with 10 mM phosphate buffer, pH 7.3, and then suspended in the same buffer. The incorporation of ^{14}C -methanol, ^{14}C -formaldehyde, ^{14}C -formate or ^{14}C -bicarbonate was carried out in a flask containing 10 ml of a reaction mixture. The reaction mixture consisted of about 20 ~ 45 mg (dry wt.) of cells, ^{14}C -methanol (100 μCi , 20 μmoles), ^{14}C -formal-

dehyde (100 μ Ci, 6.25 μ moles), sodium 14 C-formate (160 μ Ci, 8 μ moles) or sodium 14 C-bicarbonate (200 μ Ci, 13 μ moles) and 10 μ moles of phosphate buffer, pH 7.3. Incubation was undertaken with stirring at 30°C. At suitable time intervals 1 ml of the mixture was removed and immediately transferred into 4 ml of hot ethanol. The incorporation of radioactivity into boiled-cells was examined for a control experiment.

Extraction of 14 C-labeled compounds. The reaction mixture transferred to the hot ethanol described above was boiled for 20 min under a small reflux condenser and then centrifuged. The resultant precipitate was boiled with 5 ml of 20% ethanol and centrifuged. The combined supernatant was evaporated to dryness under reduced pressure at 45°C. The residue was dissolved in 100 μ l of 20% ethanol, 50 μ l of which was applied to filter paper (Toyo No. 50, 40 $\times$ 40 cm) for two-dimensional descending paper chromatography.

Chromatography and radioautography. The paper was developed at 30°C by using the following solvent system:¹⁸⁾ phenol-water (100:40, v/v) as the first solvent and *n*-butanol-propionic acid-water (142:71:100, v/v) as the second solvent. After chromatography the paper was exposed to X-ray film (Sakura X-ray film, non-screen type N) for a week to prepare radioautograms.

Dephosphorylation of phosphorylated compounds. Sugar phosphate located near a starting point of paper chromatography was eluted with 20% ethanol and then the eluate was hydrolyzed by acid phosphatase (wheat germ, Seikagaku Kogyo Co.) as follows. The eluate was evaporated to dryness. The resultant residue was dissolved in 50 μ l of water, and hydrolyzed by incubation with 100 μ g of acid phosphatase dissolved in 20 μ l of 20 mM acetate buffer, pH 4.8 for 20 hr at 30°C. Free sugars were separated by two-dimensional descending paper chromatography using the following solvent system: phenol-water (5:1, v/v) and *n*-butanol-pyridine-water (6:4:3, v/v).¹⁹⁾

Identification of labeled compounds. Radioactive compounds were eluted from paper chromatogram with 20% ethanol, and identified by co-chromatography with authentic compounds. A radioactive spot was detected by radioautography, and an authentic compound was detected by a spraying reagent. Sugars were identified by two-dimensional co-chromatography using the following solvent system: phenol-water and *n*-butanol-pyridine-water described above; phenol-90% formic acid-water (500:13:167, w/v/v) and *n*-butanol-propionic acid-water (47:22:31, v/v).¹⁰⁾ For the chromatography of amino acid, ethanol-water (7:3, v/v) was used as solvent. In order to discriminate serine from glycine, paper-electrophoresis was used.⁵⁾ It was carried out at 40 volts/cm in 0.75 N formate for 50 min.

For the chromatography of carboxylic acid, *n*-butanol-acetic acid-water (4:1:2, v/v) or ethyl acetate-acetic acid-water (3:1:1, v/v) was used.

Spraying reagents. The authentic compound was detected by spraying the following reagents: for ketose, 0.2% naphthoresorcinol in ethanol-phosphoric acid (10:1, v/v);²⁰⁾ for aldose, a reagent containing 2 g of diphenylamine, 2 ml of aniline and 10 ml of 80% phosphoric acid in 110 ml of acetone;²¹⁾ for amino acid, 0.02% ninhydrin in *n*-butanol; and for carboxylic acid, 0.1% bromocresol green (pH 5.0) in ethanol.

Measurement of radioactivity. Radioactive areas of paper chromatogram were cut out and placed in a vial containing 20 ml of a scintillation mixture, which contained 4% naphthalene, 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis-2-(5-phenyloxazolyl)-benzene in dioxane-cellosolve-toluene (3:3:10, v/v). Radioactivity was measured by a liquid scintillation spectrometer (Packard 'Tricab' Model 3320).

RESULTS

Incorporation of 14 C-methanol into cell constituents

The incorporation of radioactivity from 14 C-methanol into cell constituents of *Candida* N-16 grown on methanol was examined. Figure 1 and Fig. 2 show that the radioactivity of 14 C-methanol rapidly appeared in a number of metabolites during the incubation of the yeast. The variation with time of the percentage distribution of radioactivity incorporated from 14 C-methanol into non-volatile compounds is shown in Fig. 3. Over 70% of the total radioactivity fixed for the first 10 sec was found in phosphorylated compounds and 42% of the total radioactivity was in fructose phosphate. The variation curve of fructose phosphate showed a negative slope against time. The variation of malate also showed a negative slope, although the radioactivity of malate was relatively small at the earliest time period. However, all other compounds initially showed positive slopes. These results suggest that fructose phosphate may be a primary stable intermediate in the assimilation of methanol by *Candida* N-16. Besides, when glucose-grown cells were incubated with 14 C-methanol, the radioactivity fixed into non-volatile compounds was negligible.

FIG. 1. Radioautograms of Metabolites Produced from ^{14}C -Methanol by *Candida* N-16.

Cells (19 mg in dry wt.) were incubated with ^{14}C -methanol (100 μCi , 20 μmoles). At 10 sec and 60 sec incubation 1 ml of a reaction mixture was withdrawn, and then extracted with ethanol-water (See the text). The extracts were dissolved in 100 μl of 20% ethanol; 50 μl of this solution was used for paper chromatography.

Solvent system: phenol-water (100: 40, v/v), for the 1st; *n*-butanol-propionic acid-water (142: 71: 100, v/v), for the 2nd. A: 10 sec incubation. B: 60 sec incubation.

FIG. 2. Radioautogram of Hydrolyzates of Phosphorylated Compounds.

Phosphorylated compounds were eluted from paper chromatogram prepared from 15 sec incubation with ^{14}C -methanol. The eluates were hydrolyzed by acid phosphatase, and then subjected to paper chromatography.

Solvent system: phenol-water (5: 1, v/v), for the 1st; *n*-butanol-pyridine-water (6: 4: 3, v/v), for the 2nd.

Incorporation of ^{14}C -formaldehyde into cell constituents

The radioactivity incorporated from ^{14}C -formaldehyde into non-volatile compounds of methanol-grown cells was about 5% of that from ^{14}C -methanol under similar conditions. Owing to the chemical reactivity of formal-

FIG. 3. Time Course of the Percentage Distribution of Radioactivity Incorporated from ^{14}C -Methanol into Cell Constituents of Methanol-grown *Candida* N-16.

Cells (19 mg in dry wt.) were incubated with ^{14}C -methanol (100 μCi , 20 μmoles). The total volume was 10 ml. At the time indicated 1 ml of sample was removed and poured into 4 ml of hot ethanol. The analytical method is described in the text.

●—●, fructose phosphate; ○—○, glucose phosphate;
▲—▲, mannose phosphate; △—△, malate;
■—■, serine + glycine; □—□, aspartate;
▼—▼, alanine; ×—×, total radioactivity.

FIG. 4. Time Course of the Percentage Distribution of Radioactivity Incorporated from ^{14}C -Formaldehyde into Cell Constituents of Methanol-grown *Candida* N-16.

Cells (20 mg in dry wt.) were incubated in 1 ml of 10 mM phosphate buffer at pH 7.3 containing 2 μmoles of non-labeled formaldehyde for 2 min at 30°C , and then radioactive formaldehyde (100 μCi , 6.25 μmoles) was added to the suspension. The total volume was 10 ml. At the time indicated 1 ml of the reaction mixture was removed and poured into 4 ml of hot ethanol. The analytical method is described in the text.

●-●, fructose phosphate; ○-○, glucose phosphate
▲-▲, mannose phosphate; △-△, serine;
■-■, aspartate; □-□, malate;
▼-▼, glutamate; x-x, total radioactivity.

dehyde, ethanol-water soluble fractions obtained from boiled-cells showed a number of radioactive spots on chromatogram when incubated with ^{14}C -formaldehyde. Therefore, it was difficult to distinguish the enzymatic incorporation from the non-enzymatic incorporation. This technical difficulty could be reduced

FIG. 5. Time Course of the Percentage Distribution of Radioactivity Incorporated from ^{14}C -Formate into Cell Constituents of Methanol-grown *Candida* N-16.

Cells (32.3 mg in dry wt.) were incubated with sodium ^{14}C -formate (160 μCi , 8 μmoles). The total volume was 8 ml. At the time indicated 1 ml of the reaction mixture was removed and poured into 4 ml of hot ethanol. The analytical method is described in the text.

●-●, phosphorylated compounds;
○-○, aspartate; ▲-▲, serine;
△-△, malate; x-x, total radioactivity.

by the cells being incubated with a small amount of non-labeled formaldehyde and then radioactive ^{14}C -formaldehyde (100 μCi , 6.25 μmoles) having higher specific activity than ^{14}C -methanol (100 μCi , 20 μmoles) being added. The variation with time of the percentage distribution of radioactivity incorporated from ^{14}C -formaldehyde into non-volatile compounds is shown in Fig. 4. Over 50% of the total count fixed into non-volatile

TABLE I. COMPARISON BETWEEN THE INCORPORATION OF ^{14}C -FORMALDEHYDE INTO GLUCOSE-GROWN CELLS AND THE INCORPORATION INTO METHANOL-GROWN CELLS

Candida N-16 was grown on the culture medium containing 1% (w/v) glucose or methanol at 30°C for 40 hr. Cells (ca. 2 mg in dry wt.) were incubated in 1 ml of 10 mM phosphate buffer at pH 7.3 containing 0.2 μmoles of non-labeled formaldehyde for 1 min at 30°C , and then radioactive formaldehyde (10 μCi , 0.64 μmoles) was added to the suspension. The total volume was 3 ml. After 2 min incubation, the reaction mixture was poured into hot ethanol. The analytical method is described in the text.

Cells	Radioactivity (counts/min)							Total
	Phospho- rylated compounds	Aspar- tate	Serine + Glycine	Gluta- mate	Malate	Citrate	Unidenti- fied com- pounds	
Methanol-grown	32532	5440	5211	2292	2501	1032	11308	60316
Glucose-grown	n.d. ^{a)}	2108	1148	1052	1092	284	972	6656

^{a)} not detectable.

FIG. 6. Time Course of the Percentage Distribution of Radioactivity Incorporated from ^{14}C -Formate into Cell Constituents of Glucose-grown *Candida* N-16.

Experimental conditions were the same those described in Fig. 5 except that glucose-grown cells were used.

●—●, phosphorylated compounds;
○—○, aspartate; ▲—▲, serine;
△—△, malate; ×—×, total radioactivity.

compounds for the first 20 sec was found in phosphorylated compounds. About 22% of the total radioactivity fixed was found in fructose phosphate, which showed the highest percentage of radioactivity at the earliest time period among compounds examined. The variation curve of fructose phosphate showed a negative slope against time. The percentage of radioactivity incorporated into aspartate and glutamate decreased with time.

However, the radioactivity fixed was relatively small in aspartate and glutamate. These results suggest that fructose phosphate seems to be a primary stable compound in formaldehyde-incorporation as well as in methanol-incorporation by methanol-grown cells.

Glucose-grown cells were also incubated with ^{14}C -formaldehyde. The incorporation of the radioactivity into glucose-grown cells was very small compared with that of methanol-grown cells. The incorporation pattern of glucose-grown cells was strikingly different from that of methanol-grown cells, as shown in Table I. In the case of glucose-grown cells, the incorporation into phosphorylated compounds including mainly hexose phosphate

was not observed at all. This result suggests that hexose phosphate may play a significant role in the pathway of formaldehyde-incorporation by methanol-grown cells.

Incorporation of ^{14}C -formate into cell constituents

Methanol-grown cells were incubated with sodium ^{14}C -formate, and the incorporation of radioactivity was examined. The incorporation of the radioactivity from ^{14}C -formate into non-volatile compounds of methanol-grown cells was very small compared with that from ^{14}C -methanol or ^{14}C -formaldehyde. Figure 5 shows the variation with time of the percentage distribution of radioactivity incorporated from ^{14}C -formate into non-volatile compounds. At the earliest time period the highest percentage of the total radioactivity fixed was found in serine (38%), and the percentage distribution decreased as time elapsed, suggesting that serine may be a primary compound in the formate-incorporation.

The incorporation of ^{14}C -formate into glucose-grown cells is shown in Fig. 6. The incorporation pattern of glucose-grown cells was similar to that of methanol-grown cells, and a primary compound from formate by glucose-grown cells was also serine.

Incorporation of ^{14}C -bicarbonate into cell constituents

Candida N-16 can not synthesize cell constituents from exogenous carbon dioxide in the absence of an energy source. Therefore, the incorporation of radioactivity into non-volatile compounds during the incubation of the yeast with sodium ^{14}C -bicarbonate was examined in the presence of methanol as an energy source. Figure 7 shows the distribution of radioactivity fixed from ^{14}C -bicarbonate into non-volatile compounds of methanol-grown cells for the first 120 sec. Most of the total radioactivity fixed for the first 15 sec was found in aspartate (72%) and malate (18%). No radioactivity was found in hexose phosphate at an earlier time. Only the percentage distribution of aspartate showed clear a

FIG. 7. Time Course of the Percentage Distribution of Radioactivity Incorporated from ^{14}C -Bicarbonate into Cell Constituents of Methanol-grown *Candida* N-16.

Cells (44.7 mg in dry wt.) were incubated in 1 ml of a diluted culture medium (a dilution rate, 1/4) containing 80 μmoles of non-labeled methanol for 4 min at 30°C , and then radioactive sodium ^{14}C -bicarbonate (200 μCi , 13 μmoles) was added to the suspension. The total volume was 10 ml. At the time indicated 1 ml of the reaction mixture was removed and poured into 4 ml of hot ethanol. The analytical method is described in the text.

●—●, phosphorylated compounds;
○—○, aspartate; ▲—▲, fumarate;
△—△, malate; ×—×, total radioactivity.

FIG. 8. Time Course of the Percentage Distribution of Radioactivity Incorporated from ^{14}C -Bicarbonate into Cells Constituents of Glucose-grown *Candida* N-16.

Experimental conditions were the same as those described in Fig. 7 except that glucose-grown cells were preincubated with 80 μmoles of non-labeled glucose instead of methanol in the case of methanol-grown cells.

●—●, phosphorylated compounds;
○—○, aspartate; ▲—▲, fumarate;
△—△, malate; ×—×, total radioactivity.

negative slope against time, suggesting that aspartate may be a primary stable compound in bicarbonate-incorporation. A similar experiment was carried out with glucose-grown cells. The variation with time of the percentage distribution pattern of the radioactivity fixed was very similar to that observed in the methanol-grown cells as shown in Fig. 8. A primary stable compound formed from bicarbonate by glucose-grown cells was also aspartate.

DISCUSSION

For the assimilation of methane, methanol and formate by 'one carbon'-utilizing bacteria, three pathways have been reported: the glycine-serine pathway,^{3~7)} in which serine is formed by condensation of 5,10-methylenetetrahydrofolate with glycine; the allulose pathway,^{8~11)} in which hexose phosphate is formed by condensation of formaldehyde with ribose-5-phosphate; and the ribulose diphosphate cycle,^{1,2)} in which carbon dioxide is incorporated into 3-phosphoglycerate in the presence of ribulose-1,5-diphosphate. In the case of methanol-utilizing yeast, the previous paper¹⁷⁾ reported that at the earliest time of incubation of *Candida* N-16 with ^{14}C -methanol, the radioactivity was mainly incorporated into fructose phosphate. The present paper described that the highest percentage of the radioactivity fixed from ^{14}C -formaldehyde was found in fructose phosphate at the earliest time period and that the percentage distribution decreased as time elapsed, whereas ^{14}C -formate was fixed mainly into serine, and ^{14}C -bicarbonate into aspartate. Furthermore, there appeared to be a quantitative difference in the amount of [^{14}C]-fixation from these compounds; high fixation was observed in methanol- and formaldehyde-incorporation, and low fixation in formate- and bicarbonate-incorporation. Besides, the incorporation of the radioactivity from formate into serine for short time incubation and the incorporation from bicarbonate into aspartate were observed not only in methanol-grown cells but also in glucose-grown cells, while the for-

mation of hexose-phosphate from methanol or formaldehyde was observed only in methanol-grown cells. This suggests that the incorporation of methanol or formaldehyde into hexose phosphate may play a significant role in the assimilation of methanol by *Candida* N-16.

The previous work¹⁵⁾ has indicated that methanol is oxidized via formaldehyde and formate to carbon dioxide by *Candida* N-16, and enzymes capable of catalyzing these reactions have been found in cell free extracts. These results suggest the assimilative pathway of methanol in *Candida* N-16 is as follows: Methanol→Formaldehyde→Hexose phosphate→Cell constituents.

Foster and Davis²²⁾ have proposed the classification of methane- and methanol-utilizing bacteria into two types: an obligate methylotroph which is capable of growing only on methane and methanol, and a facultative methylotroph which is capable of growing on multicarbon compounds and methanol. It has been reported that in obligate methylotrophs methanol can be metabolized either by the serine pathway or the allulose pathway, while in facultative methylotrophs methanol can be metabolized only by the serine pathway.^{22~25)} According to this classification, *Candida* N-16 corresponds to an organism belonging to the facultative group, because the yeast can be grown on multicarbon compounds as well as methanol. Nevertheless, *Candida* N-16 can assimilate methanol through a way analogous to the allulose pathway. This appears to be most interesting in view of comparative biochemistry.

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