

## Methylamine Metabolism in *Hansenula polymorpha*: an In Vivo <sup>13</sup>C and <sup>31</sup>P Nuclear Magnetic Resonance Study

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Methylamine uptake, oxidation, and assimilation were studied in *Hansenula polymorpha*, a methylotrophic yeast. The constitutive ammonia transport system was shown to be effective at accumulating methylamine within cells cultured with methylamine or ammonia as a nitrogen source. [<sup>13</sup>C]methylamine oxidation rates were measured in vivo in methylamine-adapted cells by <sup>13</sup>C nuclear magnetic resonance and were found to be lower than its uptake rate into the cells. The <sup>13</sup>C label of methylamine was found exclusively in trehalose and glycerol, and [<sup>13</sup>C]formaldehyde was also extensively assimilated, indicating the presence of an assimilation pathway for the methylamine carbon. In vivo <sup>31</sup>P nuclear magnetic resonance analysis showed major differences in the endogenous polyphosphate levels and mean chain length during adaptation of the cells from ammonia to methylamine, indicating that methylamine accumulated in the vacuole in the same manner as basic amino acids and purines. [<sup>13</sup>C]glucose metabolism was drastically altered during adaptation of the cells from ammonia to methylamine as a nitrogen source. The total rate of glucose utilization and the rate of ethanol production fell. Direct trehalose synthesis from glucose increased, indicating a switch from carbon utilization for growth to that for storage. The rate of methylamine oxidation was sufficient to support a much higher flow of carbon into central biosynthetic pathways. These results suggest that this reduction in biosynthetic carbon flow, rather than nitrogen availability, was the main factor responsible for reducing the growth rate of the yeast when ammonia was replaced by methylamine as the nitrogen source.

A wide range of amines, including methylamine, can be readily utilized by yeasts as sources of metabolic nitrogen (23). These compounds are generally oxidized by flavin adenine dinucleotide-linked oxidases, which cleave the carbon-nitrogen bond and result in the formation of ammonia and an aldehyde residue (7). These enzymes are not constitutive but are rapidly induced when the organism is restricted to amines as the sole nitrogen source (11). Although some bacteria are able also to utilize the carbon from methylamine for growth and energy (17), yeast cannot so use the carbon residue, and therefore, a supplementary source of easily metabolized carbon such as glucose or acetate must be provided. The reason for the inability to exploit the carbon residue in this way remains a puzzle, although it has been suggested that perhaps the excess ammonia that would be generated causes repression of some metabolic enzymes (27). The induction of the oxidase enzymes has been the most studied aspect of yeast amine utilization, since these enzymes are imported and assembled within newly synthesized organelles, i.e., the peroxisomes (28). Relatively little is known about the mechanism of transport of amines into these cells or about the metabolic fates of the carbon and nitrogen constituents. It has been shown in the methylotrophic bacteria *Pseudomonas* strain MA that methylamine can be transported by a specific ammonia-inhibitable carrier in cells grown with methylamine as a nitrogen source only, but when the cells are grown on methylamine as their sole carbon, nitrogen, and energy source, a second carrier specific to methylamine and not inhibitable by ammonia is induced (1). In *Saccharomyces cerevisiae*, which is unable to utilize amines, methylamine is efficiently transported by the ammonia carrier (15). The process is dependent on a supply of readily fermentable carbon and is inhibited by

ammonia and basic amino acids but not by other organic amines (16). It is unclear whether the amine-utilizing yeasts induce a specific carrier in a similar manner to that of *Pseudomonas* strain MA or adapt in some other way to the substitution of ammonia in the medium by an amine.

In this study, methylamine transport into the cells was measured by the well-established method of monitoring the uptake of <sup>14</sup>C-labelled methylamine (15). To follow the fate of methylamine inside the cell, which is tedious with radioisotope methodology, we used in vivo <sup>13</sup>C nuclear magnetic resonance (<sup>13</sup>C-NMR) to observe <sup>13</sup>C-labelled methylamine. This method monitors the fate of the carbon and, simultaneously, reliably quantifies the rate of endogenous methylamine oxidation. <sup>13</sup>C-NMR has become a powerful and dependable tool for obtaining in vivo rates of metabolism for many metabolites in a wide range of organisms and conditions (3-5, 21, 26). We also used this technique to detect changes in glucose metabolism when methylamine was used as the nitrogen source. In addition, we used <sup>31</sup>P-NMR to observe cellular phosphate metabolism. It is accepted that phosphate and polyphosphate play a major role in nitrogen assimilation and metabolism within yeasts (6, 8, 22). The vacuole is considered to be the major storage site for basic nitrogenous metabolites as well as polyphosphates, and it is believed that the presence of negatively charged polyphosphate molecules is an effective thermodynamic sink for positively charged metabolites such as basic amino acids and purines (8, 22). This arrangement is considered to play a major role in the mechanism for sequestering ammonia and other basic nitrogenous compounds within yeast cells (6), and we wanted to determine whether this is the case for methylamine also.

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## MATERIALS AND METHODS

**Cell growth.** *Hansenula polymorpha* was grown in a mineral salts medium, pH 5.3, supplemented with vitamins, with 1% glucose as a carbon source and 25 mM ammonium chloride or methylamine hydrochloride as a nitrogen source. The cells were incubated overnight at 37°C in 2-liter shake flasks equipped with baffles, containing 0.5 liter of the growth medium. Cells were harvested in the mid-exponential phase of growth (culture optical density at 590 nm of 0.5 to 1.2 at 1-cm path length) by centrifugation at 4°C (2,500 × *g* [average], 10 min).

**<sup>14</sup>C-methylamine uptake.** Assays for methylamine uptake were based on a previous procedure used with *S. cerevisiae* (15). Cells were harvested, washed once with minimal medium containing 1% glucose and no nitrogen source, and resuspended in the same medium in a shake flask at 37°C. After a specified time, the cells were washed again with this medium and then resuspended to give a final optical density of approximately 1.0. The assays were performed with 3 ml of this suspension, and during the assay, the suspension was vigorously stirred and maintained at 37°C. [<sup>14</sup>C]methylamine (40 mCi/mmol; ICN Radiochemicals) was diluted with unlabeled reagent to give a specific activity of 2.5 × 10<sup>5</sup> to 2.7 × 10<sup>5</sup> cpm/μmol and a methylamine concentration of 40 mM. Samples, 0.5 ml, were removed at 2-min intervals for 8 min, and each was filtered through a 0.8-μm-pore-size Millipore membrane and washed with 5 ml of ice-cold water. The membrane was placed in a scintillation vial, covered with 5 ml of scintillation fluid (100 g of naphthalene, 0.5 g of 2,5-diphenyloxazole per liter of dioxane), and counted on a Beckman LS 230 scintillation counter. Uptake was linear for 10 min and then leveled off; uptake rates were determined from the linear portion of the curves.

**NMR procedures and parameters.** Cells for all NMR analyses were prepared by the above method. From a freshly harvested culture, the cell pellet was resuspended in a buffer consisting of minimal medium but with the phosphate concentration adjusted to 50 mM. For <sup>31</sup>P-NMR studies, the phosphate was replaced by 50 mM 2-(*N*-morpholino)ethanesulfonic acid-NaOH. The cell density was adjusted to 65 to 75 mg (dry weight) per ml on the basis of optical density measurements (14). This suspension, 2.5 to 3.0 ml, was used for routine NMR analysis. The cell suspension was prepared and stored on ice prior to the experiment and was used within 20 min of being prepared. During data acquisition, the cells were oxygenated by means of a 1-mm-inner-diameter polyethylene tube extending to the bottom of the sample tube with an oxygen flow rate of 15 to 20 ml/min. Oxygen electrode measurements showed that this arrangement maintained at least 50% oxygen-saturated buffer at these cell densities, thus ensuring that the cells were not oxygen limited. Lower oxygenation rates or higher cell densities resulted in a failure of the cells to utilize methylamine. The probe temperature was set to 25°C for the <sup>31</sup>P-NMR experiments and 37°C for the <sup>13</sup>C-NMR experiments.

All spectra were obtained with a Nicolet NIC-200 spectrometer operating at 80.99 MHz for <sup>31</sup>P-NMR and 50.33 MHz for <sup>13</sup>C-NMR and equipped with a 12-mm probe. Operating parameters for were as follows.

**<sup>31</sup>P-NMR.** <sup>31</sup>P-NMR data were obtained by using a pulse width of 8 μs (30°) and a delay of 250 ms, with a sweep width of 5,000 Hz. The points were stored in a 2-kilobyte data block and were zero filled to a 16-kilobyte block during spectral processing. The free induction decay was processed with 6 to 12 Hz of line broadening before being transformed.

D<sub>2</sub>O was added to a final concentration of 10% (vol/vol) to provide a lock signal just before insertion of the sample into the probe. The cell wall phosphomannan resonance was used as an internal standard for intensity measurements, while the external buffer P<sub>i</sub> was used as a chemical shift reference. The pH of the cell suspension was carefully measured just prior to the addition of any metabolites, and the chemical shift of the external P<sub>i</sub> was correlated with a chemical shift titration curve of the buffer. Typically, 1,500 to 2,000 scans (12 to 15 min) were taken with a cell density of 95 to 105 mg (dry weight) per ml of buffer.

**<sup>13</sup>C-NMR.** For <sup>13</sup>C-NMR, a pulse width of 10 μs, a delay of 340 ms, and a sweep width of 5,000 Hz were used for in vivo experiments and the data were saved and processed in the same way as for the <sup>31</sup>P-NMR experiments. A coaxially mounted 5-mm insert containing deuterobenzene provided both the lock signal and a reference for chemical shifts and intensities. Continuous broad-band decoupling was used for all spectra with a power output of 1.8 W; 1,800 accumulations (13 min) were obtained for each spectrum.

**Glutamate dehydrogenase assay.** Glutamate dehydrogenase (NADP-linked, EC 1.4.1.3) was assayed in cell extracts as described previously (27). Cell extracts were prepared by lysis of the cells following treatment with Zymolyase to digest the cell wall (28). The lysis buffer consisted of 50 mM Tris-HCl, pH 7.5, with phenylmethylsulfonyl fluoride freshly added to a final concentration of 1 mM, and glass beads were used in the lysis step for efficient cell breakage. The lysis suspension medium had a cell density of approximately 50% (vol/vol). After 5 min of vortexing with the beads at 2°C, the suspension was centrifuged at 15,000 × *g* (average) for 10 min, and the supernatant was retained. The volume of supernatant was adjusted with lysis buffer to give a protein concentration of 0.6 to 1.0 mg/ml. In some preparations that had low protein concentrations, the sample was concentrated by means of an Amicon ultrafiltration cell fitted with a YM-10 membrane (10,000-molecular-weight exclusion limit). Assay reagents were stored as stock solutions in 20 mM Tris-HCl, pH 7.6, and were replaced on a daily basis. NADPH was stored at -70°C in the same solution and was found to be usable over a period of 2 to 3 weeks. Assays were performed at 25°C on a Beckman DU-64 spectrophotometer equipped with the Kinetics Soft-Pac module and interfaced to an IBM PS/2 computer.

**Synthesis of [<sup>13</sup>C]formaldehyde.** Alcohol oxidase (Provesta Corp., Bartlesville, Okla.) (50 μl; specific activity, 525 U/μl) and a catalytic amount of catalase were added to 2.5 ml of minimal medium, pH 7.0, supplemented with 40 mM phosphate and equilibrated to 37°C in the NMR probe. [<sup>13</sup>C]methanol was added (12.5 μl, 120 mM), and the solution was oxygenated at 10 ml/min. The conversion of methanol to formaldehyde was monitored by <sup>13</sup>C-NMR using the same parameters as previously described, with 2-min. acquisition times (250 scans). The reaction was stopped, and the proteins were precipitated by the addition of trichloroacetic acid to a final concentration of 0.3%. The pH of the supernatant was adjusted to 5.5 with 1 M KOH, and the supernatant was stored at -70°C prior to the experiment.

## RESULTS

**[<sup>14</sup>C]methylamine uptake and glutamate dehydrogenase activities.** Kinetic parameters were measured for both ammonia-grown and methylamine-grown cells and were found to be similar. The *V*<sub>max</sub> and *K*<sub>m</sub> values are given in Table 1, which also shows a comparison of the effects of various

TABLE 1. Methylamine and glutamate dehydrogenase activities in glucose-ammonia-grown and glucose-methylamine-grown cells

Growth condition	Methylamine uptake results						Glutamate dehydrogenase activity <sup>a</sup> after incubation with:	
	$V_{max}^b$	$K_m$ ( $\mu$ M)	% of full activity after incubation with:				Ammonia	$\alpha$ -Ketoglutarate
			No inhibitor	Ammonia (0.5 mM)	Ethylamine (0.5 mM)	CCCP <sup>c</sup> (0.1 mM)		
Glucose-ammonia	13.7	226	100 (9.22 <sup>b</sup> )	4	79	2	250 $\pm$ 2 (3)	234 $\pm$ 8 (3)
Glucose-methylamine	11.0	320	100 (7.42 <sup>b</sup> )	9	79	3	150 $\pm$ 15 (2)	255 $\pm$ 14 (4)

<sup>a</sup> In nanomoles per minute per milligram of protein  $\pm$  standard error. Values in parentheses show the number of determinations made.

<sup>b</sup> In nanomoles per minute per milligram of cells (dry weight).

<sup>c</sup> CCCP, carbonylcyanide-*m*-chlorophenylhydrazone.

inhibitory reagents on methylamine transport for both cells. Ammonia was found to be a potent inhibitor of methylamine uptake in cells from both growth conditions, while ethylamine does not exhibit a significant inhibitory influence. These results suggest that methylamine is transported by a constitutive ammonia carrier in both cases. Addition of the protonophore carbonylcyanide-*m*-chlorophenylhydrazone (Sigma Chemical Co.) completely abolished transport under both conditions, indicating that the process was energy dependent and not passive.

Figure 1 shows the uptake of methylamine following different periods of nitrogen starvation. In the cells grown on ammonia as a nitrogen source, optimal methylamine uptake occurred following about 60 min of nitrogen starvation and did not significantly change when this time was extended to 5 h. A similar result was reported from methylamine transport studies on *S. cerevisiae* grown with ammonia as a nitrogen source (15). With cells grown on methylamine as a sole nitrogen source, however, optimal uptake did not occur until the cells had been starved of nitrogen for 4 to 5 h.

During this experiment, the growth of the cells in the nitrogen-free medium was also monitored (Fig. 1). The glucose-ammonia-grown cells grew at a faster rate than those grown in glucose-methylamine medium. Also, the rate of growth in the glucose-ammonia-grown cells did not significantly change after 60 min, indicating that although the nitrogen uptake system was derepressed, there was no shortage of nitrogen for growth for at least 4 h after this period.

Glutamate dehydrogenase activities were found to be very similar in both sets of cells when  $\alpha$ -ketoglutarate was the baseline substrate and ammonia was used to start the reaction. When the order of substrate addition was reversed, the glucose-ammonia-grown cell extracts did not show a significant change in activity but the glucose-methylamine cell extracts showed a 40% decrease in specific activities (Table 1).

**<sup>13</sup>C-labelled methylamine metabolism.** Figure 2 shows a set of spectra obtained from methylamine-glucose-grown cells fed with <sup>13</sup>C-labelled methylamine. The label was found exclusively in metabolites of the assimilation pathway, with no label detected in either formate or bicarbonate. Trehalose was one of the main labelled products, accounting for all of the peaks in the cell extract spectrum except for a singlet at 63 ppm and another at 72 ppm. These were assigned as the C-1/C-3 and C-2 resonances of glycerol on the basis of a previous <sup>13</sup>C-NMR study of glycolysis in *S. cerevisiae* (4). The even distribution of the label in all three carbons of glycerol suggests that this product was not directly formed from newly synthesized dihydroxyacetone. During the assimilation of formaldehyde in methanol-adapted cells, the first product formed is dihydroxyacetone that is exclusively

labelled at C-1 and C-3 (24). A reductive pathway exists for the conversion of this metabolite to glycerol (5), resulting in the label being exclusively found in C-1 and C-3 of glycerol, with none found in C-2. The spectrum showed a slower evolution of the trehalose C-1/C-1' peak compared with that of the C-6/C-6' peak, indicating that a loss of hexose C-1 by the pentose phosphate pathway was occurring (20).

The rate of intracellular methylamine oxidation in the cells

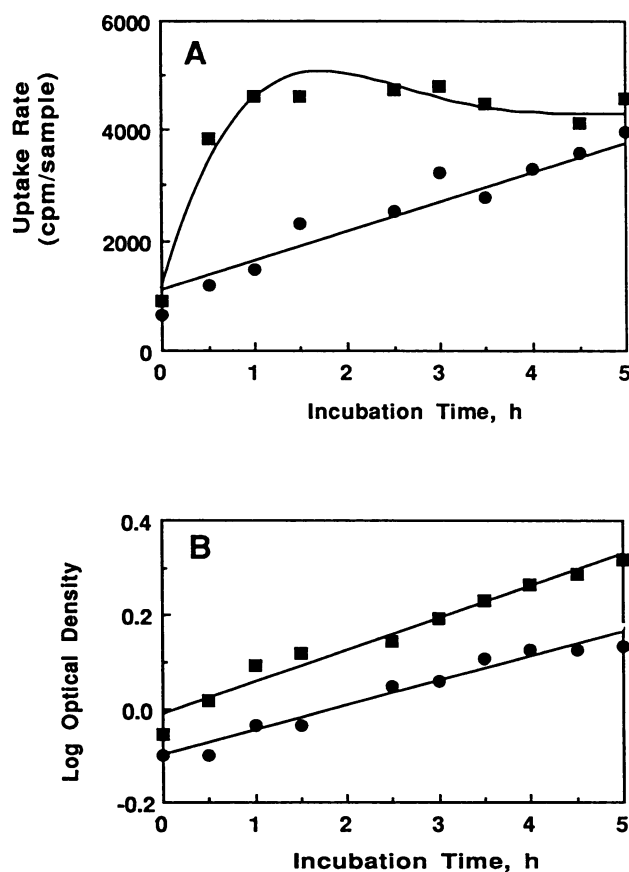


FIG. 1. (A) Comparison of methylamine uptake by glucose-ammonia-grown and glucose-methylamine-grown cultures of *H. polymorpha* preincubated in a nitrogen-free minimal medium containing 1% glucose for various periods. Methylamine uptake data have been normalized to a culture optical density of 1.0. Rates are expressed as counts per minute per sample incubated for 8 min in the assay medium. (B) Growth of the cultures in the preincubation medium during the experiment. Symbols: ●, glucose-methylamine-grown cells; ■, glucose-ammonia-grown cells.

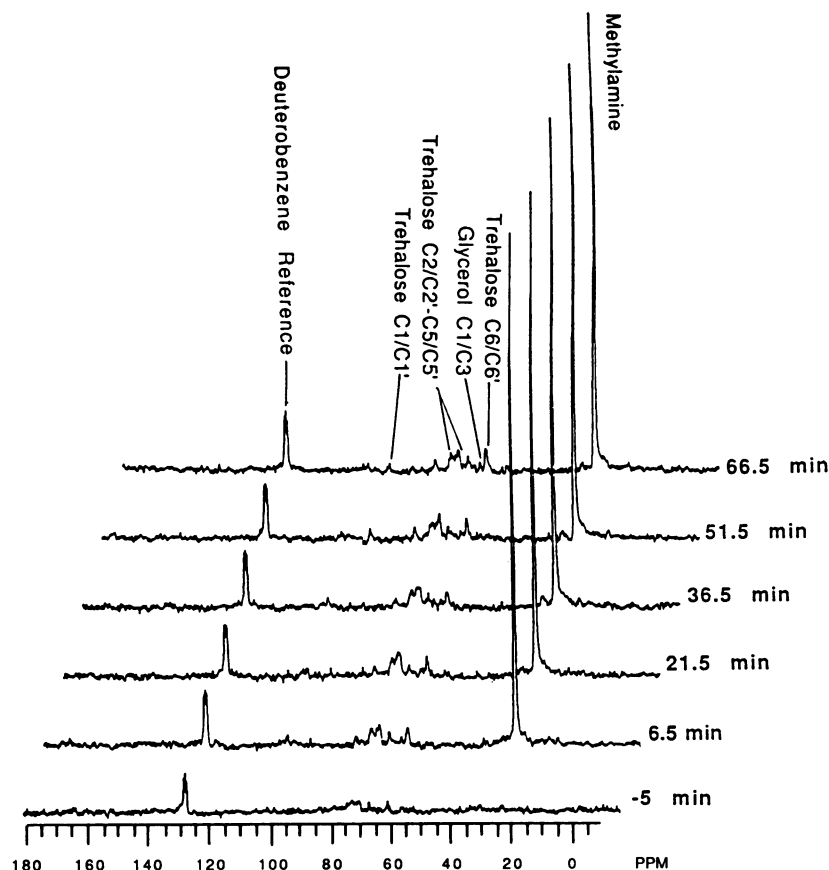


FIG. 2.  $^{13}\text{C}$ -NMR spectra (at 50.33 MHz) of  $^{13}\text{C}$  methylamine metabolism by glucose-methylamine-grown *H. polymorpha*.  $^{13}\text{C}$  methylamine was added at time zero to a concentration of 72 mM. The cells were oxygenated at a rate of 15 ml/min with a cell density of 65 mg (dry weight) per ml, and the probe temperature was maintained at 37°C. A total of 1,800 accumulations were taken per spectrum (13 min) with a 6-Hz line broadening applied to the free induction decay before Fourier transform. Times represent the midpoint of each set of accumulations. The chemical shift scale corresponds to the lowermost (-5 min) spectrum.

was also monitored by  $^{13}\text{C}$ -NMR. Although the internal and external pools of  $^{13}\text{C}$  methylamine in the cell suspensions were not distinguishable with  $^{13}\text{C}$ -NMR, the overall reduction of the methylamine intensity reflected its depletion by intracellular oxidation. Figure 3 shows the time course of the methylamine intensity over 60 min. After an initial burst of oxidation in the first 10 to 40 min of the experiment, which depleted the  $^{13}\text{C}$  methylamine by 7 to 10%, there was little additional metabolism of the label. The initial rate of oxidation was calculated to be about 9 nmol/min/mg (dry weight), which was close to the maximal uptake rate obtained in the  $^{14}\text{C}$  methylamine experiment. The labelling of assimilation products showed a rapid initial increase followed by a gradual reduction in peak intensities, reflecting the changes in the rate of label flow from methylamine during the experiment. As methylamine utilization slowed, labelled trehalose synthesis also decreased and its turnover was indicated by the slow disappearance of the signals.

The assimilatory route of carbon flow from methylamine-derived formaldehyde was supported by a series of experiments with  $^{13}\text{C}$ -labelled formaldehyde (Fig. 4). These experiments were conducted in the presence of glucose to repress possible synthesis of enzymes of the methanol (and therefore formaldehyde) assimilation pathway. In these spectra, following a rapid depletion of the added substrate, the bulk of the label was found in assimilation products, with only small

amounts detected in formate. Signals from natural-abundance  $^{13}\text{C}$  glucose were not observed, indicating that the assimilation products were produced as a result of  $^{13}\text{C}$  formaldehyde fixation. The formate resonance appeared as two peaks, one intracellular and the other from the more acidic external medium. This indicates that the cells actively secreted this metabolite as it was being produced. Formate is considered to be a toxic metabolite when it is allowed to accumulate in the cytosol, and many methylotrophic organisms actively secrete it to maintain low intracellular levels (18).

**$^{31}\text{P}$ -NMR results.** Figure 5 shows a comparison of the  $^{31}\text{P}$ -NMR spectra obtained from glucose-ammonia-grown cells, glucose-methylamine-grown cells, and glucose-ammonia-grown cells transferred to glucose-methylamine medium for 5 h prior to sampling. Major differences were found in the nature and distribution of phosphate metabolites in glucose-ammonia-grown and glucose-methylamine-grown cells. The glucose-ammonia-grown cells accumulated sugar phosphates after addition of glucose. From the ratio of the central to terminal polyphosphates, a mean chain length of 13 to 15 residues was calculated. After the addition of glucose, this ratio had decreased, indicating that hydrolysis of long-chain polyphosphates into shorter units had occurred (Table 2). This was also previously observed in *S. cerevisiae* cells harvested in the early to mid-exponential phase of growth

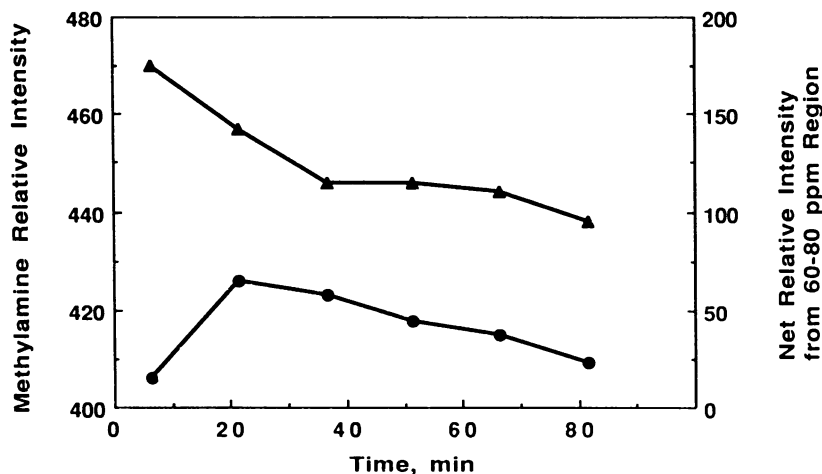


FIG. 3. Time course of the relative intensities of [ $^{13}\text{C}$ ]methylamine and  $^{13}\text{C}$ -labelled assimilation products from the spectra in Fig. 2. The integral values for the relative intensities of assimilation products were corrected for natural-abundance contributions by subtraction of the integral value obtained before the addition of [ $^{13}\text{C}$ ]methylamine. Symbols: ●, net relative intensity for 60- to 80-ppm region; ▲, methylamine relative intensity.

and placed under similar conditions (8). The vacuolar  $\text{P}_i$  peak could not be resolved from the external phosphate resonance, indicating that the interior of this organelle had a pH similar to that of the buffer (5.7 to 5.9). The presence of a vacuolar resonance which was similar in intensity to the cytosolic  $\text{P}_i$  signal was confirmed in cells resuspended in phosphate-free buffer (data not shown).

In cells grown on glucose-methylamine for the same length of time as cells grown on glucose-ammonia, (Fig. 5B), the

ratio of central to terminal polyphosphates had increased considerably, indicating that the polyphosphate in the glucose-methylamine-grown cells had a much longer mean chain length (Table 2). The total amount of polyphosphate was also higher compared with that in the glucose-ammonia-grown cells. After the addition of glucose, both the total amount of polyphosphate and its mean chain length increased, which is in direct contrast to data obtained for the glucose-ammonia-grown cells. Also, there was much less of

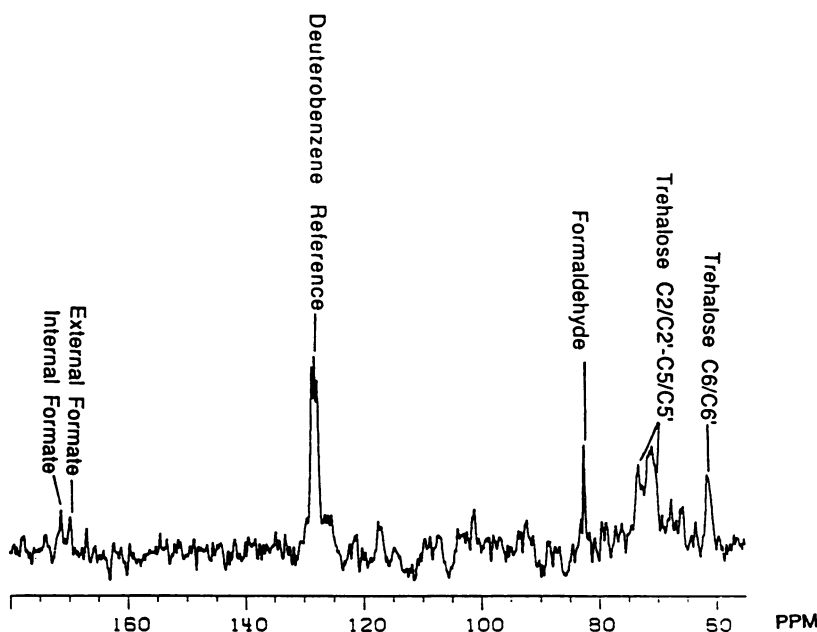


FIG. 4.  $^{13}\text{C}$ -NMR spectra (at 50.33 MHz) of [ $^{13}\text{C}$ ]formaldehyde metabolism in glucose-methylamine-grown *H. polymorpha*. Labelled formaldehyde was prepared (see Materials and Methods) in the NMR resuspension buffer, and the pH was adjusted to 5.5. The formaldehyde-paraformaldehyde concentration was calculated to be 80 mM in formaldehyde equivalents from the change in intensity of the methanol peak before and after the alcohol oxidase addition. The cells were resuspended in 2.4 ml of buffer to a density of approximately 78 mg (dry weight) per ml, glucose was added (110 mM final concentration), and data were collected immediately after the addition of 600  $\mu\text{l}$  of the formaldehyde solution. The spectrum consisted of 1,000 scans (8 min) and was processed in the same way as the [ $^{13}\text{C}$ ]methylamine spectra (Fig. 2). The methanol peak did not change in intensity during incubation with the cells.

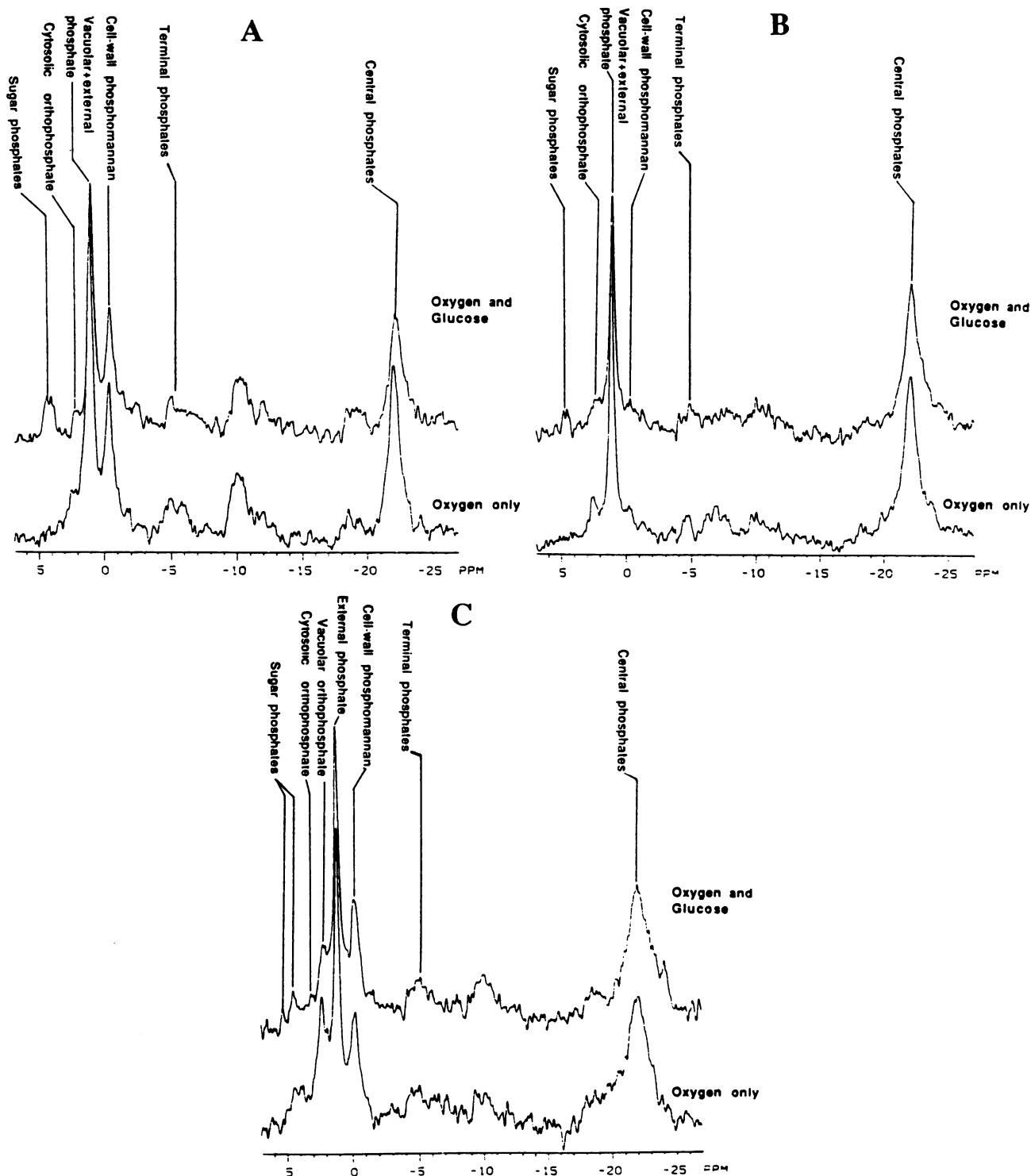


FIG. 5.  $^{31}\text{P}$ -NMR spectra (at 80.99 MHz) of glucose-ammonia-grown cells (A), glucose-methylamine-grown cells (B), and glucose-ammonia-grown cells incubated for 5 h in glucose-methylamine medium (C). Spectra consisted of 2,000 scans (10 min) and were taken immediately before and after the addition of glucose (110 mM). The cell densities were approximately 100 mg (dry weight) per ml of buffer, and no nitrogen source was present. The suspensions were oxygenated at a rate of 15 ml/min, and the probe temperature was maintained at 25°C. The free-induction decays were processed with a 12-Hz line broadening before Fourier transform.

TABLE 2. Polyphosphate relative intensities and central/terminal peak ratios in cells grown on glucose-ammonia and glucose-methylamine

Growth condition <sup>a</sup>	Polyphosphate intensity <sup>b</sup>		
	Central peak	Terminal peak	Central/terminal ratio
Glucose-ammonia (9)	110 ± 4	43.4 ± 3.3	2.64 ± 0.19
Glucose-methylamine (8)	177 ± 12	21.2 ± 1.3	8.60 ± 0.88

<sup>a</sup> Numbers in parentheses show the number of determinations made.

<sup>b</sup> Intensities (arbitrary units) obtained by integration of the 3- to 7-ppm and 21- to 25-ppm upfield regions of the spectra shown in Fig. 5 after addition of glucose in two separate experiments. In each experiment, the cells were sampled at least four times between 0 and 60 min after glucose addition and the intensity values were averaged. The standard errors of the average intensities for both sets of experiments are shown.

a change in sugar phosphate levels when glucose was added compared with glucose-ammonia-grown cells. Spectra of cells taken in a phosphate-free buffer also showed a much weaker vacuolar P<sub>i</sub> resonance than in the glucose-ammonia-grown cells (data not shown).

When glucose-ammonia-grown cells were harvested after 5 h of incubation in a glucose-methylamine medium and examined in nutrient-free buffer (Fig. 5C), the central polyphosphate resonance had increased at the expense of the terminal phosphate resonances, resulting from an increase of the mean polyphosphate chain length. When glucose was added to the sample, the total polyphosphate and its mean chain length increased slightly in the same manner as in the glucose-methylamine-grown cells. The sugar phosphate resonances were slightly weaker, but the intracellular P<sub>i</sub> resonance was strong and was resolvable into two peaks (Fig. 5C). The chemical shift of the upfield resonance indicated that it originated from an environment of pH 6.3 to 6.7, which indicates that the vacuolar pH had increased by about 0.5 pH units after exposure of the cells to methylamine, while the cytosolic pH was unchanged. These results indicate that the pH gradient across the vacuolar membrane was reduced in the presence of methylamine, suggesting that this basic species was accumulating within the vacuole. Similar effects were found in a <sup>31</sup>P-NMR study of *S. cerevisiae* cells that were exposed to a variety of different basic amines (8). Another prominent difference observed in the <sup>31</sup>P spectra between glucose-ammonia-grown and glucose-methylamine-grown cells was in the levels of cell wall phosphomannans. However, since cell wall synthesis is a complex and incompletely understood phenomenon, this aspect was not further pursued.

**Glucose metabolism.** [1-<sup>13</sup>C]glucose metabolism in glucose-ammonia-grown cells was compared with that in cells transferred to glucose-methylamine for 5 h (Fig. 6). Glucose-ammonia-grown cells metabolized the label in a similar manner to that of *S. cerevisiae*, with C-2-labelled ethanol being the major end product (4). The label was also detected in significant amounts in C-1/C-1' of trehalose, in contrast to *S. cerevisiae*, in which the label was detected in the glycogen C-1. The overall glucose utilization rate was too rapid to be determined from the data points under our experimental conditions but was estimated to be 64 nmol/min/mg (dry weight).

In cells transferred to glucose-methylamine, there was a major diversion of the label away from glycolysis towards trehalose synthesis, as seen by much greater label incorporation into trehalose and diminished ethanol production (Fig.

7). In methylamine-adapted cells, the glucose utilization rate was also drastically reduced to 35 nmol/min/mg (dry weight). The C-1/C-3 glycerol resonance and an unidentified resonance about 1 ppm downfield were also more prominent. The downfield resonance is characteristic of C-6 of either glucose-6-phosphate or glucose-1-phosphate. Both of these are precursors for trehalose synthesis, and their levels in the glucose-methylamine-grown cells should be elevated if trehalose synthesis from glucose is increased.

Despite the brief appearance of a sizable sugar phosphate resonance in the <sup>31</sup>P-NMR spectra of glucose-ammonia-grown cells when glucose was added to the medium, the <sup>13</sup>C-NMR spectra had very few contributions from glycolytic intermediates. This was probably the result of a combination of fast label flow through the pathway due to the higher temperature at which the <sup>13</sup>C-NMR experiment was run (37°C instead of 25°C) and a lower glucose concentration used in the <sup>13</sup>C-NMR experiment (42 versus 110 mM).

## DISCUSSION

Methylamine utilization by *H. polymorpha* was studied from the uptake of the compound from the external medium to its oxidation and storage within the cell. By using multinuclear NMR, we were able to follow its metabolism in vivo and at the same time monitor other basic processes such as glycolysis and phosphate metabolism in the cell. This enabled us to pinpoint significant metabolic differences in the overall cell metabolism when the nitrogen source was changed from ammonia to methylamine.

Our results indicate that methylamine uptake in both glucose-ammonia-grown and glucose-methylamine-grown cells was similar to that described in *S. cerevisiae* in terms of its inhibition profile and its kinetics (15, 16). We found no evidence of an inducible methylamine transport system in cells adapted to methylamine utilization. This is in contrast to bacterial methylamine adaptation, in which a transport system that is specific to methylamine is induced when the compound is either the sole nitrogen source or the sole carbon and nitrogen source (1). There was, however, a major difference in the time it took for the transport system to become derepressed when the cells were incubated in a nitrogen-free medium. Methylamine transport in the glucose-ammonia-grown cells became maximally derepressed after about 1 h of incubation, while in the glucose-methylamine-grown cells, this period had extended to 5 h. This suggests that the glucose-methylamine-grown cells had a greater store of endogenous nitrogen or a slower rate of metabolism or a combination of both. The slower growth rate in the glucose-methylamine medium supports the idea that the cell metabolism is limited in some manner when ammonia is replaced by methylamine as a nitrogen source. Currently, it is thought that the reduction in the growth rate of cells transferred from ammonia to methylamine may be due to a limitation of endogenous ammonia for amino acid synthesis (27). This was postulated to result from a slow methylamine oxidation rate within the cells.

The <sup>13</sup>C-NMR data showed that the initial rate of methylamine oxidation approached that of the uptake rate into the cell. However, this rate was only briefly sustained, even in cells that had been starved of nitrogen. The possibility that this step was being inhibited by formaldehyde or formate accumulation is not supported by our results. Neither of these products was ever observed during methylamine metabolism, and when the cells were challenged with formaldehyde, the rate of disappearance of this compound was far

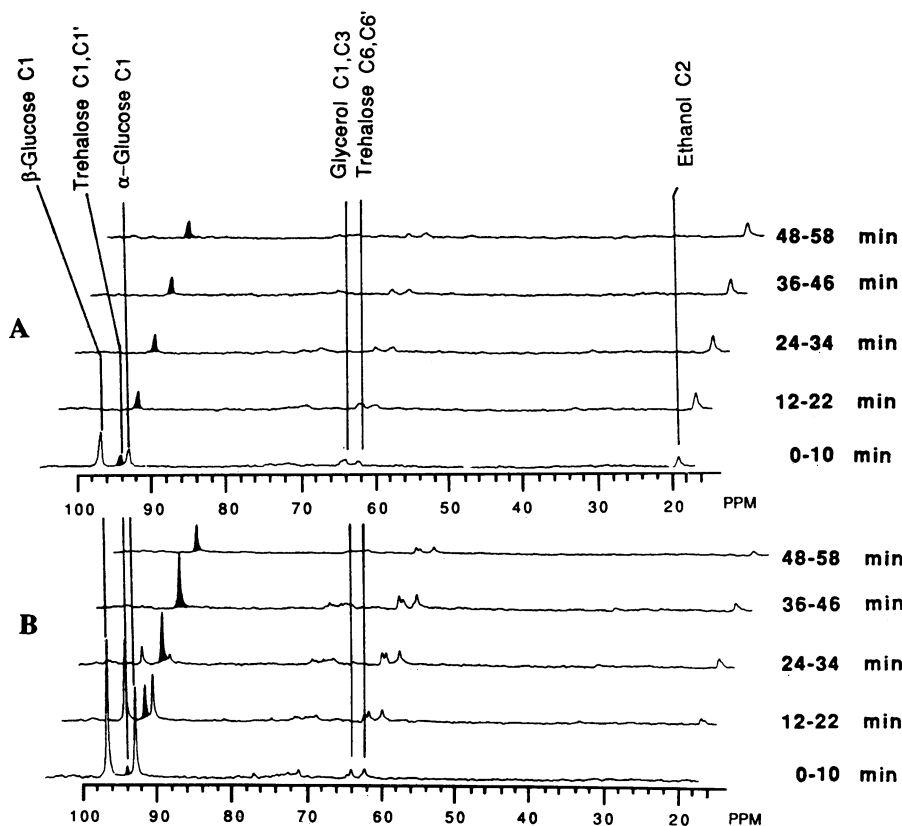


FIG. 6.  $^{13}\text{C}$ -NMR spectra (at 50.33 MHz) of  $[1-^{13}\text{C}]$ glucose metabolism by glucose-ammonia-grown cells (A) and by glucose-ammonia-grown cells transferred to glucose-methylamine medium for 5 h (B).  $[1-^{13}\text{C}]$ glucose was added at time zero to a concentration of 72 mM. The cells were oxygenated at a rate of 15 ml/min with a cell density of 65 mg (dry weight) per ml, and the probe temperature was maintained at 37°C. A total of 1,400 accumulations were taken per spectrum (10 min), with a 6-Hz line broadening applied to the free induction decay before Fourier transform. Times represent the midpoint of each set of accumulations. The chemical shift scale corresponds to lowermost spectrum (0 to 10 min).

in excess of the methylamine oxidation rate, in agreement with the known high levels of formaldehyde dehydrogenase under these conditions (27). This suggested that a much higher rate of methylamine oxidation could be sustained before the cells experienced any degree of formaldehyde toxicity. This apparent excess capacity for formaldehyde removal is consistent with the ability of these cells to utilize dimethylamines and trimethylamines, where the molar ratio of formaldehyde to ammonia during the oxidation of these compounds is two and three times that for methylamine (23).

The destination of the methylamine carbon was found to be trehalose, indicating that a significant fraction of the formaldehyde generated by methylamine oxidation had been assimilated into carbohydrates rather than oxidized to formate and bicarbonate. During growth of *H. polymorpha* on methanol, there is high activity of dihydroxyacetone synthase (DHAS), a transketolase that donates a ketose fragment from xylulose-5-phosphate to formaldehyde, resulting in the assimilation of the single carbon of formaldehyde into triose (24). A Western immunoblot analysis of extracts from glucose-methylamine-grown cells by using antiserum obtained against purified DHAS from methanol-grown cells gave a negative result (data not shown), indicating that a different transketolase was responsible for this reaction in glucose-methylamine-grown cells. This enzyme was most likely the normal or classical transketolase acting nonspecifically,

as suggested by Waites and Quayle (25). This enzyme has been recently confirmed to be able to perform this function in a DHAS-negative mutant of *H. polymorpha* growing on xylose-methanol mixtures in which substantial methanol carbon was assimilated (2). Thus, a possible reason for the failure of this organism to grow on methylamine as a sole carbon source could be due to its inability to induce DHAS in sufficient quantities under these conditions. Despite high activities of formaldehyde dehydrogenase in these cells (27), we observed no carbon flow through the oxidative pathway except when the cells were challenged with formaldehyde. The possibility that the carbon in this pathway was being rapidly converted to  $\text{CO}_2$  (and hence lost from the sample) was probed by monitoring the rate of  $[^{13}\text{C}]$ formate oxidation. This reaction was found to be torpid in comparison with the very rapid rate of formaldehyde disappearance (data not shown), again in agreement with the known comparatively low (approximately 30-fold lower than formaldehyde dehydrogenase) activity of formate dehydrogenase (27). This indicates that the role of the oxidative pathway is to scavenge excess formaldehyde that is not assimilated into carbohydrates. Our results indicate that the combination of these pathways is very efficient at removing formaldehyde as soon as it is produced. Also, the products of assimilation appeared to be predominantly directed towards trehalose synthesis and the pentose phosphate pathway. This latter



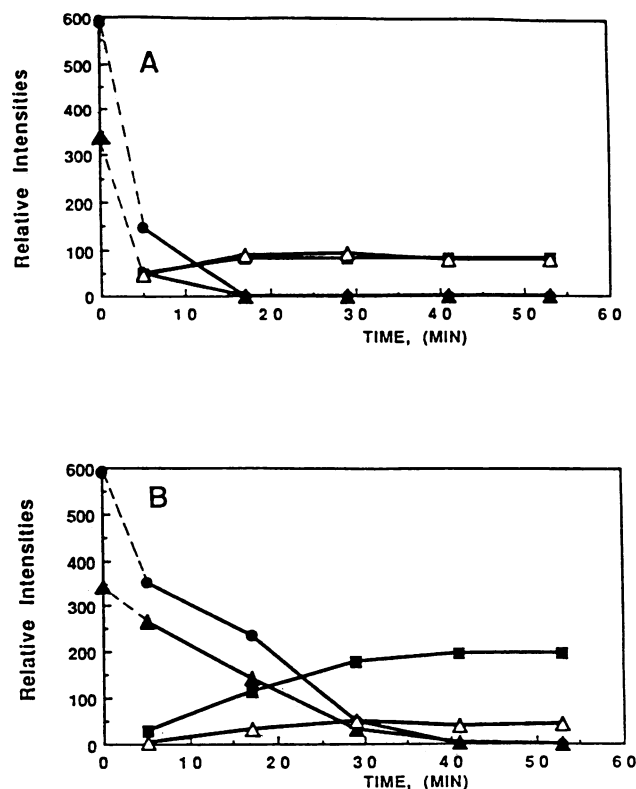


FIG. 7. Comparison of the relative intensities of various <sup>13</sup>C-labelled metabolites following the addition of [1-<sup>13</sup>C]glucose to glucose-ammonia-grown cells (A) and glucose-ammonia-grown cells transferred to glucose-methylamine medium for 5 h (B). Symbols: ●, β-glucose C-1; ▲, α-glucose C-1; ■, trehalose C-1/C-1'; △, ethanol C-2.

pathway may be important in supplying pentose phosphates needed for the regeneration of xylulose-5-phosphate, the ketose donor for formaldehyde assimilation.

The maximal activity of glutamate dehydrogenase was similar in cells from both growth conditions. This suggests that the endogenous ratio of ammonia to α-ketoglutarate in glucose-methylamine-grown cells was not different to that in glucose-ammonia-grown cells; i.e., there was not any appreciable limitation or starvation in terms of nitrogen as a result of methylamine utilization. This finding is in agreement with results from a chemostat study of yeast methylamine metabolism in which it was found that specific activities of glutamate dehydrogenase did not vary much when the nitrogen source was changed from ammonia to methylamine unless the carbon source was limiting, in which case a drastic increase (18-fold) in the specific activity of this enzyme occurred (27). Preincubation of the extract from glucose-methylamine-grown cells with ammonia resulted in a reproducible decrease in the measured glutamate dehydrogenase activity; this effect did not occur with extract from glucose-ammonia-grown cells. This difference in behavior towards ammonia suggests that perhaps a different form of the enzyme exists in glucose-methylamine-grown cells, a form that is inhibitable by ammonia. In support of this notion is the observation that addition of ammonia (35 mM) to a continuous culture of *Candida utilis* growing on methylamine as the sole nitrogen source resulted in a rapid decrease in glutamate dehydrogenase specific activity which was

slowly reversed as the ammonia was consumed (27). Further work will be needed to verify this concept.

Changing the nitrogen source from ammonia to methylamine had a profound effect on glucose metabolism in these cells. Our results indicate that a major portion of the glucose was directly converted to trehalose without any participation in the glycolytic pathway when methylamine was the nitrogen source. The amount of carbon that entered glycolysis and the citric acid cycle (and hence was available for nitrogen assimilation) was severely reduced and appeared to be one of the main rate-limiting steps in the growth of this organism under these conditions. The induction of trehalose synthesis from glucose in yeast cells and fungi is triggered by exposure of the organisms to a wide variety of suboptimal growth conditions (10, 12, 13, 19). This process has been found to be an important metabolic landmark in the onset of sporulation, signalling the point at which carbon was diverted from the assimilatory and energy-producing pathways to that of storage (20). Trehalose synthesis in cells grown in a minimal medium featuring glucose as a carbon source has also been found to be induced by omitting the nitrogen source (ammonia) (12). When ammonia was restored to the medium, this synthesis ceased and the trehalose was instead hydrolyzed. There are no data published regarding the effects on trehalose metabolism of replacing ammonia with other nitrogen sources such as amines. However, it is well known that when glucose is replaced by a suboptimal carbon source such as galactose or maltose, trehalose synthesis is stimulated (12, 13, 20). Our results suggest that the same process occurs when ammonia is replaced by a suboptimal nitrogen source such as methylamine; a more detailed investigation of the regulation of trehalose synthesis in this organism is currently under way in this laboratory.

The methylamine uptake rate was found to exceed the oxidation rate under our experimental conditions, indicating that this metabolite accumulates within the cells. The <sup>31</sup>P-NMR data suggest that vacuolar polyphosphate synthesis is stimulated by changing the nitrogen source from ammonia to methylamine. In a study of arginine uptake in *S. cerevisiae* (8), the total polyphosphate levels and the mean chain length increased sharply when the cells were supplied with both P<sub>i</sub> and arginine. Also, the high-molecular-weight polyphosphate was found to sequester arginine very effectively in a model system free of any enzymes, with release of the amino acid occurring only in the presence of vacuolar polyphosphatases. This binding property was common to basic amino acids, with no such activity found in experiments with neutral or acidic species. The polyphosphate is thought to act as a cation exchanger, thus selectively sequestering basic amino acids that are positively charged under physiological conditions. While no data have been published for the interaction of alkylamines with polyphosphate, it is reasonable to propose that they bind in the same manner, provided that the amine group is sufficiently basic to be predominantly protonated under physiological conditions. Methylamine has a sufficiently high pK<sub>a</sub> value (10.6) to meet this criterion and on this basis should bind effectively to polyphosphate. It is interesting to speculate whether vacuolar sequestration of methylamine (and other basic alkylamines) is a mechanism for storage or a detoxification route that prevents them from accumulating in the cytosol. Methylamine does dissipate pH gradients in the same manner as ammonia, and therefore, its levels have to be tightly regulated. In *Pseudomonas* strain MA, excess methylamine is temporarily converted to γ-glutamylmethylamide and the basic nature of the methylamine nitrogen is neutralized (9). Polyphosphate may play a similar

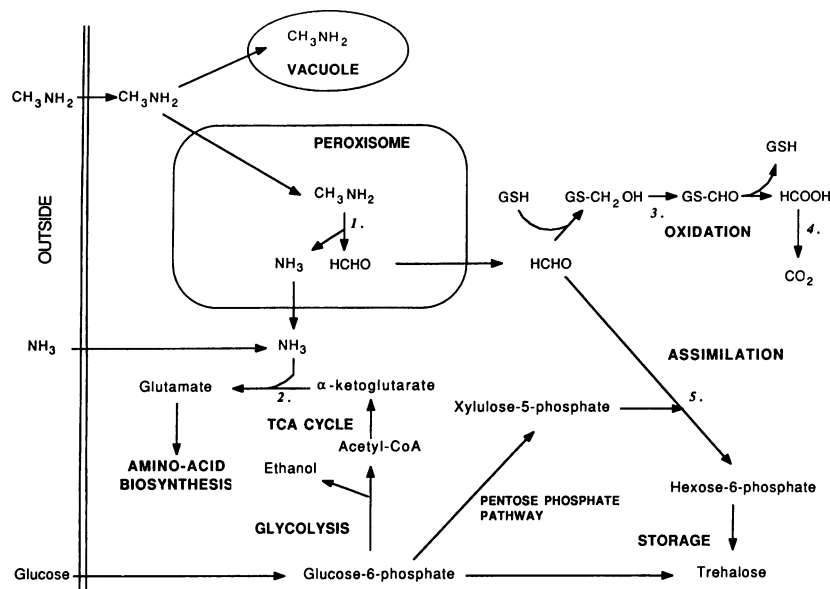


FIG. 8. Pathways of carbon and nitrogen metabolism during growth of *H. polymorpha* on glucose-methylamine. Enzymes: 1, amine oxidase; 2, glutamate dehydrogenase; 3, formaldehyde dehydrogenase; 4, formate dehydrogenase; 5, classical transketolase and glycolytic enzymes.

role in yeast cells, enabling the organism to accumulate large amounts of this metabolite without suffering any toxic effects. However, this is energetically costly, since the synthesis of polyphosphate bonds and the maintenance of a large pH gradient across the vacuolar membrane requires a substantial supply of ATP. This may be an important factor in restricting the rate of carbon assimilation in these cells by diverting ATP away from biosynthetic pathways. A summary of the metabolism of both carbon and nitrogen during growth of *H. polymorpha* on glucose with methylamine as the nitrogen source is depicted schematically in Fig. 8.

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