Regulation of the Key Enzymes of Methylated Amine Metabolism in *Candida boidinii*

By JEFFREY GREEN AND PETER J. LARGE*

Department of Biochemistry, University of Hull, Hull HU6 7RX, UK

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Nitrogen assimilation during growth of *Candida boidinii* on methylated amines as sole nitrogen source involves NADP-dependent glutamate dehydrogenase. Changes in enzyme activities during the adaptation of the yeast from growth on ammonium to growth on trimethylamine were examined. No ammonia, dimethylamine or monomethylamine could be detected in the medium during growth on trimethylamine. When two methylated amines were supplied together, they were used simultaneously, although monomethylamine was metabolized more quickly than the others. When cells were grown on a low concentration of ammonium plus higher concentrations of di- or trimethylamine, the ammonium was used first. NADP-dependent glutamate dehydrogenase was the first enzyme to be derepressed, followed by methylamine oxidase and formaldehyde dehydrogenase. Di- and trimethylamine mono-oxygenase activities only appeared when the ammonium concentration fell below 0.5 mM. At this point amine utilization could be detected and no diauxic lag was observed in the growth curve. During growth on limiting ammonium, there was an increase in the activity of methylamine oxidase (150-fold) and catalase (5-fold) in the absence of any amine, but no amine mono-oxygenase activity was detected. Addition of ammonium ions to cultures growing on dimethylamine produced an immediate repression of synthesis of methylamine oxidase, NADP-dependent glutamate dehydrogenase and the two amine mono-oxygenases. An inverse correlation was found between intracellular ammonium concentration and methylamine oxidase activity. Ammonium ions also inhibited the uptake of dimethylamine or trimethylamine by washed suspensions of dimethylamine-grown cells. It is concluded that the control of methylamine oxidase and catalase and (independently) of NADP-dependent glutamate dehydrogenase is by repression of enzyme synthesis by ammonium, while expression of amine mono-oxygenases seems to require the amine to be present in the medium. Formaldehyde and formate dehydrogenases seem also to be induced by their respective substrates.

INTRODUCTION

Many yeasts are able to grow on short chain primary, secondary or tertiary alkylamines as sole nitrogen source, while being unable to use them as carbon source (van Dijken & Bos, 1981). Until recently, very little was known about the nature of the enzymes involved in amine dissimilation. It has now been found that in a number of yeast species, including *Candida boidinii*, *C. utilis* and *Hansenula polymorpha*, secondary and tertiary amines are metabolized by mono-oxygenase systems (equations 1 and 2) (Green & Large, 1983a, b),

while primary amines are oxidized by amine oxidases (equation 3) (Zwart *et al.*, 1980; Haywood & Large, 1981).

Most yeasts contain at least two such amine oxidases (Haywood & Large, 1981; Green *et al.*, 1982, 1983), although only one is normally involved in growth on methylamine.

Zwart & Harder (1983), on the basis of chemostat studies, showed that both the methylotrophic yeast *H. polymorpha* and the non-methylotroph *C. utilis* behaved as if they were nitrogen-limited during growth on methylated or ethylated amines as nitrogen source. They suggested that the failure to utilize amines as carbon source was due to the strongly repressive effect of ammonia, which would under such conditions be formed in excess, and so would not allow expression of the key enzymes amine oxidase and catalase essential to amine dissimilation, so preventing growth. We have investigated growth of *C. boidinii* on alkylamines using a different experimental approach (batch culture instead of continuous culture), and we present here results which substantially support the observations and conclusions of Zwart & Harder (1983).

A preliminary report of some of these results was presented to the Fourth International Symposium on Microbial Growth on C₁-compounds in Minneapolis in 1983 (Large & Green, 1984).

METHODS

Organism and cultivation. *Candida boidinii* CBS 5777 was grown in batch culture at 30 °C and harvested as described by Haywood & Large (1981). Growth was followed by measuring the absorbance of the culture at 663 nm and converted to dry weight using a calibration curve.

Preparation of cell extracts. This has been described previously (Green & Large, 1983a).

Enzyme assays. NAD- and NADP-dependent glutamate dehydrogenases (EC 1.4.1.2 and 1.4.1.4) and glutamate synthase (EC 1.4.1.14) were measured as described by Doherty (1970), glutamine synthetase (EC 6.3.1.2) by the method of Rowe *et al.* (1970), dihydroxyacetone kinase (triokinase) (EC 2.7.1.30) as described by van Dijken *et al.* (1978), and dihydroxyacetone synthase (EC 2.2.1.-) as described by O'Connor & Quayle (1980). Alcohol oxidase (EC 1.1.3.13) was assayed by replacing methylamine in the methylamine oxidase assay by 33 mM-methanol. Other enzyme assay methods have been described previously (Haywood & Large, 1981; Green & Large, 1983a). All enzyme activities are expressed as units or milliunits (respectively μmol or nmol substrate converted min^{-1}).

Chemical determinations. Protein was determined by the method of Bradford (1976), formate as described by Lang & Lang (1972), formaldehyde by the acetylacetone method of Nash (1953), ammonia by the indophenol method (Chaney & Marbach, 1962), glucose by the Boehringer GOD-Perid method (Werner *et al.*, 1970), methylamine by the enzymic method of Large *et al.* (1969), trimethylamine also enzymically (Large & McDougall, 1975). Dimethylamine was in some cases estimated enzymically by the procedure of Large & McDougall (1975) but using a dimethylamine dehydrogenase preparation from dimethylamine-grown *Hyphomicrobium* X (Meiberg & Harder, 1979). This method could only be used in the absence of trimethylamine. In other cases the colorimetric method of Dubin (1960) was used.

Determination of intracellular ammonium. Approximately 40 ml of culture was filtered through a 0.8 μm Sartorius membrane filter (47 mm diam.) and washed twice with 20 ml 0.1 M-acetate buffer pH 5.5. The filter was removed and immersed in 3 ml of freshly diluted 30% (v/v) HClO_4 for 10 min. The filter was removed and cell debris removed by centrifugation. The supernatant was brought to pH 7.0 with KOH and recentrifuged. Ammonium was measured in the supernatant.

Enzymic changes during adaptation from growth on ammonium to growth on trimethylamine. These experiments were carried out in vigorously aerated 20 l fermenters in a similar fashion to those described for adaptation to spermidine by Haywood & Large (1984).

RESULTS

Distribution of ammonium-assimilating enzymes, amine oxidases and other key enzyme activities in Candida boidinii grown on different nitrogen sources

The assimilation of ammonia in yeasts generally occurs by incorporation of ammonia into 2-oxoglutarate via NADP-dependent glutamate dehydrogenase (Brown, 1976) and ammonium limitation is generally reflected in a sharp increase in the activity of this enzyme. In some species, e.g. *Schizosaccharomyces pombe* (Brown, 1976), ammonium limitation results in an

increase in the glutamine synthetase–glutamate synthase system, while NADP-dependent glutamate dehydrogenase remains low. To investigate which of these systems was involved in ammonia assimilation in *C. boidinii*, cultures were grown on glucose with a number of different nitrogen sources. Cells were harvested in the late exponential phase of growth (A_{663} between 1.0 and 2.0). There was a fivefold increase in the specific activity of NADP-dependent glutamate dehydrogenase when the yeast was grown with methylated amines as sole nitrogen source, compared with cells grown on ammonium (Table 1), whilst the enzymes of the glutamine synthetase–glutamate synthase system could not be detected. The specific activity of NADP-dependent glutamate dehydrogenase was not affected by the nitrogen source used. In other experiments, low levels of glutamate synthase and glutamine synthetase were detected. It appears that growth on methylated amines is, effectively, nitrogen-limited and that the major pathway of ammonia assimilation in *C. boidinii* is via NADP-dependent glutamate dehydrogenase. The K_m^{app} for ammonia for this enzyme was determined to be 12.1 mM and the K_m^{app} for 2-oxoglutarate was 0.42 mM (the enzyme used was 10-fold purified).

Crude extracts were also assayed for methylamine oxidase, benzylamine oxidase, catalase (EC 1.11.1.6), trimethylamine and dimethylamine mono-oxygenases, as well as the enzymes involved in formaldehyde oxidation: formaldehyde dehydrogenase (EC 1.2.1.1), formate dehydrogenase (EC 1.2.1.2), and *S*-formylglutathione hydrolase (EC 3.1.2.12) (Table 1). NADP-dependent isocitrate dehydrogenase (EC 1.1.1.42) was also assayed. The results showed an increase in the activities of methylamine oxidase and catalase when alkylated amines were used as nitrogen source. It is noteworthy that methylamine oxidase was gratuitously induced at quite high activity in cells grown on benzylamine, which is not a substrate for the enzyme. The mono-oxygenases catalysing the degradation of tertiary and secondary amines were only produced by cells grown with secondary or tertiary amines, trimethylamine *N*-oxide or choline as sole nitrogen source.

Amines containing methyl groups induced high activities of the enzymes involved in formaldehyde oxidation, indicating that formaldehyde was completely oxidized to carbon dioxide. When ethylated amines were supplied as nitrogen source, the specific activities of formaldehyde and formate dehydrogenases were relatively low, reflecting the fact that these compounds are metabolized via acetaldehyde (Zwart & Harder, 1983). Methanol oxidase, a key enzyme in the metabolism of methanol, was not detected during growth on amines. From the observed enzyme patterns it can be concluded that amine oxidase has a fundamental role in the metabolism of all the alkylated amines investigated and that the aldehydes liberated from the alkyl groups are oxidized by way of dehydrogenases.

Changes in the specific activities of key enzymes during growth of C. boidinii with trimethylamine as sole nitrogen source

To investigate the roles of a number of key enzymes of methylated amine metabolism, the appearance of enzyme activities during the adaptation of *C. boidinii* from ammonium sulphate to trimethylamine as sole nitrogen source was followed. After growth on ammonium sulphate, *C. boidinii* was transferred to medium containing trimethylamine as nitrogen source and to medium containing ammonium sulphate. Samples were taken at intervals to measure growth and enzyme activity. In all these experiments the carbon source was glucose (55 mM).

Cells transferred to ammonium sulphate medium grew after a lag of 1.5 h and the levels of the enzymes investigated remained low and virtually unchanged during growth, except for a slight increase (twofold) in catalase activity during the stationary phase.

Cells transferred to trimethylamine medium grew after a slightly longer lag (2.5 h). During the period before growth began the synthesis of formaldehyde dehydrogenase, *S*-formylglutathione hydrolase, methylamine oxidase, catalase and a small (probably physiologically insignificant) amount of benzylamine oxidase was observed (Fig. 1*b*). The specific activities of formaldehyde dehydrogenase, catalase and methylamine oxidase followed each other closely; all reached peak activity just after the mid-exponential phase of growth, and *S*-formylglutathione hydrolase followed 3 h later. Formate dehydrogenase remained at barely detectable levels until 4 h into the stationary phase of growth; at this point a sharp increase in specific activity was noted.

Table 1. *Enzyme specific activities in crude cell extracts of C. bovidii grown on various nitrogen sources*

Enzyme	Nitrogen source†	Specific activity*									
		AS	MMA	MEA	Benz	DMA	DEA	TMA	TEA	TMAO	Chol
Trimethylamine mono-oxygenase		0	0	0	0	8	0	20	3	5	9
Dimethylamine mono-oxygenase		0	0	0	0	27	30	48	38	13	19
Methylamine oxidase		0	96	141	38	131	164	128	103	128	92
Benzylamine oxidase		0	8	6	95	0	—	6	—	2	0
Catalase		13	112	161	209	99	102	111	114	66	85
Formaldehyde dehydrogenase		64	160	73	49	199	79	361	67	260	130
S-Formylglutathione hydrolase		58	282	87	—	587	—	760	—	—	1480
Formate dehydrogenase		0	95	0	0	102	4	168	0	81	0
Glutamate dehydrogenase (NAD)		24	0	—	—	29	—	16	—	—	—
Glutamate dehydrogenase (NADP)		85	520	—	—	469	—	414	—	—	—
Isocitrate dehydrogenase (NADP)		82	118	84	97	84	98	83	115	110	90

* Values are in units (g protein)⁻¹ except for catalase, which is in units (mg protein)⁻¹. —, No data available.

† Abbreviations: AS, ammonium sulphate; MMA, methylamine, MEA, ethylamine; Benz, benzylamine; DMA, dimethylamine; DEA, diethylamine; TMA, trimethylamine; TEA, triethylamine; TMAO, trimethylamine N-oxide; Chol, choline.

Fig. 1. Enzyme levels and the accumulation of metabolites during the growth of *C. boidinii* after transfer from medium containing 15 mM-ammonium sulphate as nitrogen source to medium containing 30 mM-trimethylamine hydrochloride. Cells grown on ammonium sulphate were harvested aseptically at 30 °C, washed with medium containing no nitrogen source and resuspended in complete medium with 30 mM-trimethylamine as sole nitrogen source. Samples were removed at intervals, the cells removed by centrifugation, and cells and supernatant medium stored at -20 °C. Later the pH and the content of formate and formaldehyde in the medium were measured and cell extracts prepared by French pressure cell and enzyme specific activities measured as described in Methods. (a) ●, Growth [log (cell dry wt ml⁻¹)]; ▲, pH of the medium; ○, formaldehyde concentration (μM); ▼, formate concentration (mM). (b) Enzyme specific activities during growth: ■, methylamine oxidase; ◇, benzylamine oxidase (× 20); ▼, catalase; ▽, formaldehyde dehydrogenase; △, S-formylglutathione hydrolase; □, formate dehydrogenase; ○, isocitrate dehydrogenase.

Dihydroxyacetone kinase and dihydroxyacetone synthase, key enzymes of formaldehyde assimilation in yeasts (van Dijken *et al.*, 1978; O'Connor & Quayle, 1980), were estimated during growth to determine whether the formaldehyde produced from the demethylation of trimethylamine was utilized as an auxiliary carbon source. Dihydroxyacetone synthase activity was not detected. Dihydroxyacetone kinase was present at repressed levels in both ammonium sulphate- and trimethylamine-grown cells. This confirms the view that the methyl groups of methylated amines are oxidized to carbon dioxide and not incorporated into cell material.

The formaldehyde dehydrogenase elevated during growth on trimethylamine was shown to behave in the same way as formaldehyde dehydrogenase from methanol-grown cells on polyacrylamide gel electrophoresis using a standard activity stain (R_m 0.58). It is almost certainly the same enzyme as that purified by Schütte *et al.* (1976).

Isocitrate dehydrogenase remained fairly constant in both sets of cells, with a specific activity of about $0.06 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$.

Formaldehyde and formate accumulated in the culture supernatant during growth on trimethylamine (Fig. 1*a*), methylamine or dimethylamine as nitrogen source. Neither was detected during growth on ammonium sulphate. The peak of formaldehyde accumulation (typically $40\text{--}70 \mu\text{M}$) always preceded the peak of formate. It might be expected that the concentration of formate accumulating would increase with increasing number of methyl groups in the amine provided as nitrogen source, and this was indeed the pattern observed. The highest concentrations of formate detected were respectively: with methylamine 4.4 mM , with dimethylamine 11.7 mM and with trimethylamine 21.1 mM . It was only in the stationary phase of growth, when formate dehydrogenase activity began to increase, that the external concentration of formate fell (Fig. 1*a, b*).

During growth on amines, ammonia did not accumulate in the medium, and no methylamine or dimethylamine was detected in the culture supernatants of trimethylamine-grown cells. Whereas at cessation of growth on ammonia the glucose in the medium was exhausted, in the amine cultures glucose and amine were both still present.

The data presented here confirm and extend the enzyme patterns observed by Zwart *et al.* (1980) and Zwart & Harder (1983) for the growth of the yeasts *Hansenula polymorpha* and *Candida utilis* on glucose with methylamine as sole nitrogen source.

Growth of C. boidinii on mixtures of two methylated amines

When *C. boidinii* was grown on a mixture of equimolar trimethylamine and methylamine (2.5 mM -each), both amines were utilized simultaneously, although the rate of consumption of methylamine was faster than that of trimethylamine. With corresponding mixtures of dimethylamine and methylamine, both substrates were used simultaneously, with methylamine once again being consumed at a slightly faster rate. Growing on a mixture of trimethylamine and dimethylamine (3 mM each), *C. boidinii* utilized both substrates simultaneously and at the same rate.

Growth of C. boidinii on mixtures of ammonium and a methylated amine

When a culture of *C. boidinii* grown with ammonium sulphate as sole nitrogen source was transferred to a medium containing both ammonium sulphate (5 mM) and dimethylamine (10 mM) as nitrogen sources, only the ammonium was utilized, and all the dimethylamine remained in the medium. Methylamine oxidase was not detected at any stage during growth. NADP-dependent glutamate dehydrogenase remained at a relatively low specific activity throughout [$0.3 \text{ units (mg protein)}^{-1}$].

A similar experiment with a lower concentration of ammonium sulphate (1.5 mM) and dimethylamine (14 mM) as nitrogen sources was performed (Fig. 2). The ammonium in the medium was utilized first. When the concentration of ammonium ions had fallen below 2 mM , derepression of NADP-glutamate dehydrogenase was evident. After 6 h growth, the ammonium concentration was below 1 mM and increased activities of methylamine oxidase and formaldehyde dehydrogenase were detected. At this point the dimethylamine provided began to be used and formate accumulation was detected. No diauxic lag was noted. Growth entered the stationary phase after 12 h and the specific activities of methylamine oxidase and formaldehyde dehydrogenase began to fall (Fig. 2). NADP-dependent glutamate dehydrogenase had begun to fall some 2 h earlier. Formate dehydrogenase activity began to increase after 17 h when the formate concentration in the medium had reached 3.8 mM .

The behaviour of dimethylamine and trimethylamine mono-oxygenases under these conditions was investigated in a further experiment (Fig. 3). The results confirmed those obtained in the previous experiments and showed that dimethylamine and trimethylamine mono-oxygenases were only expressed when the external ammonium concentration fell below 0.5 mM .

Similar experiments were performed in which only growth and substrate consumption were followed. On a mixture of ammonium sulphate (2 mM) and methylamine (3 mM), although no

Fig. 2. Growth of *C. boidinii* on a mixture of ammonium sulphate (1.5 mM) and dimethylamine (14 mM). Samples were removed at intervals from the culture growing at 30 °C, the cells removed by centrifugation, and cells and supernatant medium stored at -20 °C. Later the ammonium, dimethylamine and formate concentrations in the medium samples were measured, and cell extracts were prepared and enzyme specific activities measured as described in the legend to Fig. 1. ●, Growth [log (cell dry wt ml⁻¹)]; Δ, ammonium concentration in the medium; ○, dimethylamine concentration; ▲, NADP-dependent glutamate dehydrogenase; ■, methylamine oxidase; ▽, formaldehyde dehydrogenase; □, formate dehydrogenase.

Fig. 3. Growth of *C. boidinii* on a mixture of ammonium sulphate (1.25 mM) and dimethylamine (5 mM). Samples were removed at intervals from the culture and treated as described in the legend to Fig. 2. ●, Growth [log (cell dry wt ml⁻¹)]; Δ, ammonium concentration in the medium; ○, dimethylamine concentration. Enzyme specific activities during growth: ▲, NADP-dependent glutamate dehydrogenase; ■, methylamine oxidase; □, trimethylamine mono-oxygenase; ▽, dimethylamine mono-oxygenase.

diauxic lag was observed, the ammonium was used first and only when the ammonium concentration had fallen below 1 mM was consumption of methylamine detected.

Growth of C. boidinii on limiting ammonium sulphate

Candida boidinii was grown with ammonium sulphate (0.6 mM) as the sole source of nitrogen and the activity of a number of enzymes in cell-free extracts was followed during growth (Fig. 4). After 7.5 h the ammonium sulphate provided was exhausted and at this point methylamine oxidase activity was detected in cell extracts, even though there were no amines present in the culture medium. Catalase was also derepressed at this time. Trimethylamine and dimethyl-

Fig. 4. Growth of *C. boidinii* on limiting (0.6 mM) ammonium sulphate. Samples were removed at intervals from the culture and treated as described in the legend to Fig. 2. ●, Growth [log (cell dry wt ml⁻¹)]; Δ, ammonium concentration in the growth medium. Enzyme specific activities during growth: ▲, NADP-dependent glutamate dehydrogenase; ■, methylamine oxidase; ▼, catalase; ▽, formaldehyde dehydrogenase.

amine mono-oxygenases were not detected, nor was benzylamine oxidase. The specific activity of formaldehyde dehydrogenase remained at a repressed level. NADP-dependent glutamate dehydrogenase was derepressed throughout.

Repression of various enzyme activities by addition of ammonium ions

The effect of excess ammonium sulphate on methylamine oxidase, trimethylamine and dimethylamine mono-oxygenases and NADP-dependent glutamate dehydrogenase was investigated by adding ammonium sulphate (15 mM) to exponential-phase cultures of *C. boidinii* containing dimethylamine as nitrogen source and glucose (150 mM) as carbon source. The results obtained (Fig. 5) indicated that the addition of ammonium completely blocked the synthesis of all four enzymes. After the addition of ammonium sulphate, the rate of dimethylamine consumption was reduced and formate ceased to accumulate in the medium, suggesting that the rate of dimethylamine metabolism had been severely reduced. (Formate dehydrogenase levels were basal throughout.) In control experiments in which ammonium sulphate was not added, the enzyme activities investigated continued to rise during the period of growth considered.

Effect of intracellular ammonium concentration on methylamine oxidase expression

Candida boidinii was grown on glucose with a number of different nitrogen sources. The cultures were harvested in the mid-exponential phase of growth ($A_{663} \sim 1.0$). Intracellular ammonium and the levels of ammonium in the culture supernatants were determined, as well as the activity of methylamine oxidase in cell extracts. The results are summarized in Table 2, and indicate that methylamine oxidase is only expressed when the intracellular concentration of ammonium is low. In crude cell extracts ammonium concentrations up to 30 mM did not inhibit methylamine oxidase activity.

From these results it can be concluded that methylamine oxidase synthesis in *C. boidinii* is regulated by a repression/derepression mechanism in which the intracellular concentration of ammonium plays an essential role.

Studies on dimethylamine uptake by non-growing cells of C. boidinii

Dimethylamine-grown *C. boidinii* was suspended in 70 mM-potassium phosphate, pH 7.0, to a density of 1.76 mg cell dry weight ml⁻¹. The suspension was equilibrated at 30 °C for 15 min with 5 mM-dimethylamine with shaking. Samples were then taken at intervals and the cells were removed immediately by rapid centrifugation. After the rate of dimethylamine uptake had been established, ammonium sulphate (5 mM) was added. The results (Fig. 6) showed immediate inhibition of dimethylamine uptake. Similar results were obtained for trimethylamine uptake.

Fig. 5. The effect of ammonium addition on enzyme activities during growth of *C. boidinii* on dimethylamine as initial nitrogen source. A culture of *C. boidinii* was grown on dimethylamine as sole nitrogen source and samples of supernatant and cells removed at intervals. At the point shown by the arrow, ammonium sulphate was added to a final concentration of 15 mM. ●, Growth (only the first and last points on the growth curve are shown); Δ, ammonium concentration in the medium; ○, dimethylamine concentration; ▼, formate concentration. Enzyme specific activities during growth: ▲, NADP-dependent glutamate dehydrogenase; ■, methylamine oxidase; □, trimethylamine mono-oxygenase ($\times 10$); ▽, dimethylamine mono-oxygenase ($\times 10$).

Fig. 6. Inhibition of dimethylamine uptake by ammonium ions in washed cell suspensions of *C. boidinii*. The arrow denotes time of ammonium addition. ●, Dimethylamine concentration; ○, ammonium concentration in supernatant after removal of cells by rapid centrifuging.

Table 2. Effect of intracellular ammonium concentrations on methylamine oxidase expression

Nitrogen source	Ammonium concentration		Specific activity of methylamine oxidase [units (mg protein) ⁻¹]
	Medium (mM)	Cellular [nmol (mg cell dry wt) ⁻¹]	
Ammonium sulphate	10.8	54.10	0
Glutamate	0.035	29.74	0
Methylamine	0	0	0.062
Trimethylamine	0	2.56	0.157
Potassium nitrate	0	19.33	0

The presence of methionine sulfoximine (1 mM), a specific inhibitor of glutamine synthetase, did not prevent this inhibition, although the rate of ammonium uptake was reduced.

The effect of the presence of various amines on the utilization of dimethylamine by *C. boidinii* was investigated. Tetramethylammonium chloride, triethanolamine and methylamine all reduced the rate of dimethylamine uptake. Dimethylamine uptake had a sharp pH optimum at pH 6.0, suggesting that dimethylamine is transported as the dimethylammonium ion. At pH values of 5 and 7, uptake was only 50% of the maximum, and at pH values of 4 and 8, it was only 16%. The low level of uptake at pH 4.0 is significant, since late in the growth cycle of the cells the pH of the growth medium falls below this value (Fig. 1).

DISCUSSION

Growth of *C. boidinii* in the presence of a methylated amine as nitrogen source resulted in an increase in the activities of the formaldehyde-oxidizing enzymes: formaldehyde dehydrogenase, *S*-formylglutathione hydrolase and formate dehydrogenase (as also observed by Zwart *et al.*, 1980). The presence of these enzymes ensures the removal of the potentially toxic formaldehyde resulting from the demethylation of the nitrogen source; their effectiveness is shown from the low levels of formaldehyde found in the growth medium (Fig. 1). When growth was supported by ethylated amines the levels of the formaldehyde-oxidizing enzymes remained low (Table 1). Similar results using cells of *C. utilis* grown in continuous culture have been observed by Zwart & Harder (1983). Being a methylotroph, *C. boidinii* is capable of utilizing formaldehyde as a carbon source, but under the growth conditions studied, the enzymes of formaldehyde assimilation (the dihydroxyacetone pathway, van Dijken *et al.*, 1978) were present at repressed levels. Thus, most of the formaldehyde produced must be oxidized to carbon dioxide and water in a two-stage process, in which the formaldehyde (via *S*-formylglutathione) is immediately converted to formate and excreted into the culture medium where it accumulates until late in the growth cycle when formate dehydrogenase becomes active. This is in contrast to yeasts grown on methylated amines in continuous culture, where formate does not accumulate and high levels of formate dehydrogenase can be detected (Zwart & Harder, 1983). The formaldehyde dehydrogenase present in glucose/amine-grown cells did not differ in electrophoretic mobility from the enzyme present in methanol/ammonium-grown cells (purified by Schütte *et al.*, 1976), suggesting that it is probably not a different protein.

The oxidation to CO₂ and water of the aldehydes produced by dealkylation of the amines used as nitrogen source results in the production of NADH (equations 4–6).

This NADH could be used to supply the initial mono-oxygenase reactions and to yield energy via oxidative phosphorylation. Since NADH has been shown to be a significantly better electron donor for dimethylamine mono-oxygenase of *C. utilis* than NADPH (Green & Large, 1983*a*), and since NADH–NADP⁺ transhydrogenase has been shown to be absent from *C. utilis* (Bruinenberg *et al.*, 1983), it seems possible that NADH formed in aldehyde oxidation is used to supply the mono-oxygenases. Enzymes which generate NADPH, such as glucose-6-phosphate dehydrogenase (J. Green, unpublished) and isocitrate dehydrogenase (Table 1, Fig. 1*b*) were not elevated during growth on di- or trimethylamine, compared with growth on ammonium. In contrast, during growth on nitrate (which has an obligate requirement for NADPH, for nitrate and nitrite reductases), Bruinenberg *et al.* (1983) have demonstrated clearly that glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are significantly elevated in activity, irrespective of the carbon source of the cells.

There is evidence to suggest that growth with alkylated amines as sole nitrogen source is associated with a physiological state of nitrogen limitation (see Zwart & Harder, 1983). Our results would bear this out, since accumulation of ammonium was never detected in culture

supernatants and only very low levels of intracellular ammonium were detected in cells grown on amines, compared with cells grown on other nitrogen sources (Table 2). Growth with methylated amines as sole nitrogen source also resulted in a significant increase in the activity of NADP-dependent glutamate dehydrogenase, a condition associated with nitrogen limitation (Dalton, 1979). The levels of the glutamine synthetase-glutamate synthase system were low or undetectable, thus ammonium is probably assimilated by NADP-dependent glutamate dehydrogenase in *C. boidinii*, as in *C. utilis* and *H. polymorpha* (Zwart & Harder, 1983). The reason for our failure to detect glutamine synthetase may merely be that the assay conditions were not optimal.

The physiological condition of nitrogen limitation during growth with amines as nitrogen source is probably due to feedback repression of methylamine oxidase synthesis by ammonium. In the presence of high levels of intracellular ammonium, methylamine oxidase is repressed (e.g. nitrate-grown cells, Table 2), whereas under ammonium limitation the repression is released. Amines are not required as inducers of methylamine oxidase. Catalase appears to be regulated in the same way and the synthesis of these two peroxisomal enzymes may be closely linked. Benzylamine oxidase or trimethylamine and dimethylamine mono-oxygenases do not behave in this way; they appear to be induced by their respective amine substrates. This might explain why benzylamine oxidase is never induced in *C. boidinii* without methylamine oxidase also being formed (Haywood & Large, 1981). A repression/derepression control mechanism for an enzyme involved in nitrogen catabolism is uncommon in micro-organisms. Regulation by nitrogen catabolite repression has been reported for asparaginase II in *Saccharomyces cerevisiae* (Roon *et al.*, 1982), in which ammonium or a metabolite of ammonium acts as the repressor molecule. Repression of amine oxidase synthesis has also been observed in *H. polymorpha* when transferred from methanol/methylamine medium to methanol/ammonium medium (Veenhuis *et al.*, 1981). The regulatory effect of the ammonium ion on methylamine oxidase synthesis could explain the inability of methylotrophic yeasts, such as *C. boidinii*, to grow on amines as sole carbon source (van Dijken & Bos, 1981). Utilizing such compounds as a carbon source would result in the accumulation of ammonium ions and thus in the repression of methylamine oxidase and hence cessation of growth. Similar conclusions to those presented here have been made by Zwart & Harder (1983) in a chemostat study of amine metabolism in *C. utilis* and *H. polymorpha* (see also the discussion in Large & Green, 1984).

Apart from the influence of ammonium on methylamine oxidase synthesis, it also inhibited the uptake of dimethylamine by washed cell suspensions of *C. boidinii* (Fig. 6) and it is known to prevent methylamine uptake in *C. utilis* (Zwart, 1983; Zwart & Harder, 1983).

The enzymes of formaldehyde metabolism are regulated quite separately, only being synthesized in the presence of formaldehyde. Formate dehydrogenase was only synthesized when the level of formate excreted into the culture medium had reached high levels (> 5 mM). This may reflect the fact that this enzyme has a high K_m for formate, 13 mM (Schütte *et al.*, 1976), thus a large amount of enzyme would need to be synthesized for the efficient metabolism of low levels of formate. Hence in the interests of economy formate dehydrogenase may only be produced when the concentration of formate is sufficient for the enzyme to operate efficiently.

The observation that amine-grown cultures contained both glucose and amine when growth ceased is puzzling. It may be that a vitamin or mineral is limiting growth, though evidence for this could not be obtained (data not shown). It was found that eventually (over 2 d) the amine-grown cultures attained the same level of growth as ammonium sulphate cultures, and it is possible that the fall in pH of the medium could be responsible for the cessation of growth, since dimethylamine is taken up very poorly by *C. boidinii* at pH values below 4.0.

A further factor that needs to be taken into account in interpreting these results is the dissolved oxygen tension, which decreases rapidly to very low levels in this type of batch culture. Since *C. boidinii* is a facultative fermentative yeast, ethanol is produced in these cultures. Although the K_m values for oxygen of the mono-oxygenases and oxidase involved in methylated amine metabolism are very low (Haywood & Large, 1981; J. Green & P. J. Large, unpublished results), it seems likely that the activities of the enzymes might be affected. Moreover, indirect effects may also play a role, since ethanol is known to be strong repressor of the enzymes metabolizing C_1 -compounds in yeasts.

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