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Significance of yeast peroxisomes in the metabolism of choline and ethanolamine

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The yeasts *Candida utilis* and *Hansenula polymorpha* were able to grow in media containing choline or ethanolamine as the sole nitrogen source. During growth in the presence of these substrates, large peroxisomes developed in the cells, and extracts of choline-grown *C. utilis* cells contained increased levels of amine oxidase and catalase. Incubation of whole cells with choline in the presence of the amine oxidase inhibitor aminoacetonitrile led to excretion of dimethylamine and methylamine. Cytochemical experiments in which spheroplasts prepared from choline-grown cells were incubated with $CeCl_3$ and choline, trimethylamine, dimethylamine or methylamine revealed positively stained peroxisomes, whereas in the presence of 1 mM aminoacetonitrile staining was not observed. This indicated that choline was degraded via methylated amines and that peroxisomes played a role in its metabolism. A similar involvement of peroxisomes in choline degradation was observed in *H. polymorpha*. Cell-free extracts of ethanolamine-grown *C. utilis* and *H. polymorpha* also contained increased levels of amine oxidase and catalase. Ethanolamine was oxidized by cell-free extracts of both organisms after growth in the presence of ethanolamine or choline. Incubation of spheroplasts of ethanolamine- or choline-grown *C. utilis* with $CeCl_3$ and ethanolamine resulted in positively stained peroxisomes. In this organism peroxisomes were therefore also involved in ethanolamine degradation.

INTRODUCTION

The phosphatidyl derivatives of choline and ethanolamine are important components of yeast phospholipids and constitute approximately 35–50% and 30%,

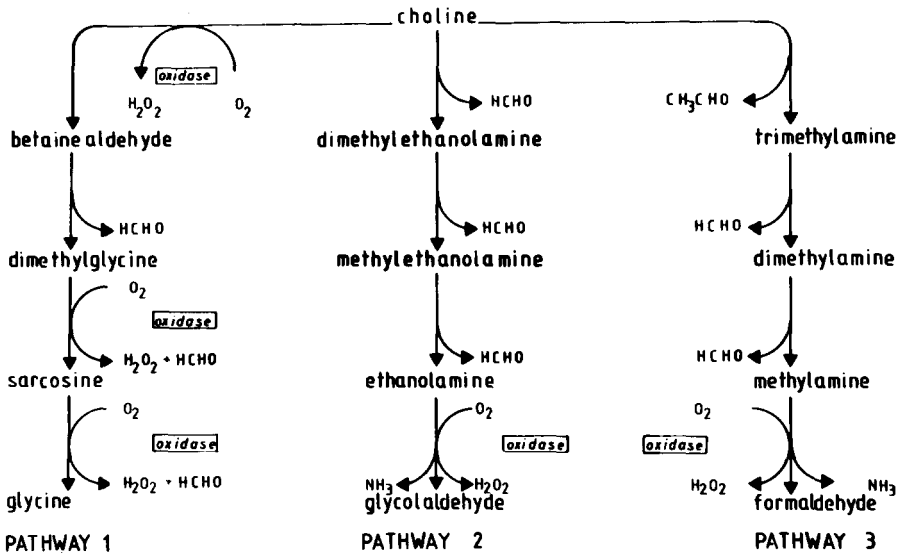


Fig. 1. Possible hydrogen peroxide-producing pathways of choline catabolism in yeasts.

respectively, of the total phospholipid fraction (Hunter and Rose, 1971). Surprisingly little is known about the metabolism of these compounds in yeasts. Kortstee (1970) found that several yeast species were unable to utilize choline as the sole carbon source. Van Dijken and Bos (1981) confirmed and extended this observation, but showed that many yeast species utilized choline as the sole source of nitrogen.

The metabolic pathway of choline degradation in yeasts has not yet been clarified. Some possible pathways are depicted in Fig. 1. Pathway 1 has been described for the bacterium *Arthrobacter* P1 (Levering et al., 1981) and in the fungus *Cylindrocarpon didymum* M-1 (Tani et al., 1977; Mori et al., 1980). Several hydrogen peroxide-producing enzymes are involved in this pathway, namely choline oxidase, dimethylglycine oxidase and sarcosine oxidase. Pathway 2 is hypothetical and to our knowledge so far its presence has not been described in any organism. This pathway is interesting since it possesses ethanolamine as an intermediate, the oxidation of which by amine oxidase was recently reported by Haywood and Large (1981) in *Candida boidinii*. The initial reaction of pathway 3, the cleavage of choline into trimethylamine and the two-carbon compound acetaldehyde has been reported to occur in a number of anaerobic bacteria (Hayward and Stadtman, 1959, 1960; Bradbeer, 1965). The product of this cleavage, trimethylamine, can be used by many yeasts as the sole nitrogen source (Van Dijken and Bos, 1981) and although its metabolism has not yet been completely elucidated, it is most likely that degradation occurs via dimethylamine and methylamine (Green and Large, 1983), as is also the case in bacteria

(Large, 1981). Methylamine is subsequently oxidized by a peroxisomal amine oxidase in the yeasts *Candida utilis* and *Hansenula polymorpha* (Zwart et al., 1980).

Of special interest is the occurrence of one or more hydrogen peroxide-producing oxidases in each of these pathways. In yeasts as well as in many other eukaryotic organisms such oxidases are generally located in peroxisomes. Some examples of peroxisomal oxidases in yeasts are alcohol oxidase (Veenhuis et al., 1976), D-amino acid oxidase (Zwart et al., 1983), and amine oxidase (Zwart et al., 1980). Since a possible role of yeast peroxisomes in the metabolism of phospholipid components would add important new information to our knowledge of the function of these organelles, we have studied the metabolism of choline and ethanolamine in more detail in the yeasts *C. utilis* and *H. polymorpha*. The results of this study are presented in this paper.

Materials and Methods

Microorganisms and cultivation

Candida utilis NCYC 321 and *Hansenula polymorpha* de Morais et Maia CBS 4732 were grown in shake flask cultures or in continuous cultures at 30 °C and 37 °C, respectively, in the mineral medium of Van Dijken et al. (1976). Glycerol (0.1 % v/v), ethanol (0.25 % v/v), or glucose (0.25 % w/v) were used as a carbon source. Ammonium sulphate (0.25 % w/v), choline-HCl (0.2 % w/v), trimethylamine-HCl (0.25 % w/v), dimethylamine-HCl (0.25 % w/v), methylamine-HCl (0.25 % w/v), betaine-HCl (0.2 % w/v), dimethylglycine-HCl (0.1 % w/v), sarcosine-HCl (0.1 % w/v), glycine (0.2 % w/v), dimethylethanolamine (0.2 % v/v), methylethanolamine (0.2 % v/v), or ethanolamine (0.2 % v/v) were used as nitrogen sources. *Hyphomicrobium* X was grown in shake flasks in the mineral medium of Meiberg and Harder (1978), which contained methylamine-HCl (0.10 % w/v), dimethylamine-HCl (0.12 % w/v) or trimethylamine-HCl (0.15 % w/v) as the sole carbon and energy source. *Desulfovibrio desulfuricans* strain BH was grown anaerobically in a 20-l flask at 27 °C in the medium described by Widdel (1980) which contained choline-HCl (0.1 % w/v) as the sole carbon and energy source. Mineral media and ammonium sulphate were heat-sterilized for 15 min at 120 °C; carbon sources, organic nitrogen sources and vitamins were filter-sterilized.

Q_{O_2}-max determinations

The capacity of washed cell suspensions or spheroplasts to oxidize excess substrate (Q_{O_2}-max) was measured with a Clark type oxygen electrode (Biological Oxygen Monitor, Yellow Springs Instruments Co., Ohio, U.S.A.), in 50 mM sodium phosphate buffer pH 7.5 containing 30 mM substrate (for substrates

see Results section). The activity was expressed as nmol oxygen consumed · min^{-1} · mg (dr. wt)$^{-1}$.

Dry weight determinations

Cell dry weight was determined as described elsewhere (Zwart and Harder, 1983).

Enzyme assays and protein determination

Enzyme activities were measured in cell-free extracts. These were prepared by passing cells twice through a French pressure cell (American Instruments Company, Silver Springs, U.S.A.) at 0 °C and 137 MPa, followed by centrifugation at 30000 × *g* for 20 min to remove unbroken cells and debris. In some experiments cell-free extracts were made by ultrasonic treatment as described previously (Zwart et al., 1980), which were kept anaerobic by flushing nitrogen through the samples during the sonification procedure. Protein was measured according to Lowry et al. (1951). Amine oxidase, alcohol oxidase and catalase activity were determined as described previously (Zwart et al., 1980). Choline oxidase, dimethylglycine oxidase, and sarcosine oxidase were measured according to Levering et al. (1981). Choline dehydrogenase, dimethylglycine dehydrogenase and sarcosine dehydrogenase were assayed spectrophotometrically with NAD(P), or phenazine methosulphate/nitrotetrazolium blue (PMS/NTB) as electron acceptors (Oka et al., 1979). Cleavage of choline into trimethylamine and a C_2 component (probably acetaldehyde) was assayed anaerobically in the following reaction mixture (1 ml): 50 mM sodium phosphate buffer pH 7.5, 1 mM ADP, 10 mM pyruvate, 2 mM $FeSO_4$, and 0.2 ml anaerobically prepared cell-free extracts of yeasts, containing 15–20 mg protein · ml^{-1}. The reaction was started by the addition of choline (30 mM) and stopped after 30 min with 0.1 ml ice-cold perchloric acid (30% w/v). Precipitated protein was discarded by centrifugation and the supernatant adjusted to pH 7 with 1N NaOH. Any trimethylamine formed during this incubation was detected using cell-free extracts of *Hyphomicrobium* X, which contain trimethylamine dehydrogenase (Meiberg and Harder, 1978). A mono-oxygenase type reaction possibly involved in the oxidation of choline, choline phosphate, betaine, trimethylamine, dimethylamine, methylamine, dimethylethanolamine, methylethanolamine and ethanolamine was measured according to the procedure described for trimethylamine mono-oxygenase by Boulton et al. (1974) with the following modifications: cell-free extracts were prepared in the presence of 1% (v/v) Triton-X100 (Green and Large, 1983), NADPH, whereas KCN was omitted. Enzyme assays were performed at 28 °C (*C. utilis, Hyphomicrobium* X and *D. desulfuricans*) or at 37 °C (*H. polymorpha*). Amine oxidase and alcohol oxidase activity are expressed as nmol O_2 consumed · min^{-1} · mg protein^{-1}, catalase as ΔE_{240} · min^{-1} · mg protein^{-1}, amine mono-oxygenase as nmol NADH consumed · min^{-1} · mg pro-

tein^{-1} and choline cleavage as nmol trimethylamine produced · min^{-1} · mg protein^{-1}.

Excretion of trimethylamine, dimethylamine and methylamine by choline-grown yeasts

Since in the choline-based experiments gas-chromatographic methods for the quantitative determination of methylated amines (Yamada et al., 1976) gave unsatisfactory results, we decided to study excretion of the various possible intermediates of choline oxidation by yeasts by way of a biological assay. This method is based on the capacity of trimethylamine-grown cells of *Hyphomicrobium* X, to oxidize trimethylamine, dimethylamine and methylamine, whereas dimethylamine-grown cells only oxidize dimethylamine and methylamine, and methylamine-grown cells only oxidize methylamine (Meiberg and Harder, 1978). Excretion of trimethylamine, dimethylamine or methylamine by yeast cells grown in media containing choline as the sole nitrogen source can, therefore, be determined in a biological assay, using cells of *Hyphomicrobium* X as a probe. In order to obtain accumulation and excretion of intermediates of choline oxidation by yeasts, a washed cell suspension was incubated aerobically for 30 min in 0.1 M sodium phosphate buffer pH7.5, containing 20 mM choline-HCl and 1 mM aminoacetonitrile. This latter compound specifically inhibits amine oxidase and therefore blocks the further oxidation of methylamine. Samples were centrifuged in order to remove cells, and the supernatants tested for the presence of amines in the following way: 0.05 ml supernatant solution was mixed with 2.95 ml sodium pyrophosphate buffer (0.1 M pH 7.5) and to this mixture was added 0.02 ml of a concentrated (15–20 mg (dry wt) · ml^{-1}) suspension of cells of *Hyphomicrobium* X, grown either on methylamine, dimethylamine or trimethylamine. After addition of such cells, the oxygen consumption was measured. Endogenous oxygen consumption by *Hyphomicrobium* X in this assay system was negligible. Whole cells of *Hyphomicrobium* X were not capable of oxidizing choline, ethanolamine, methylethanolamine and dimethylethanolamine.

Preparation of spheroplasts

Spheroplasts were prepared with Zylomase 5000 (Kitamura et al., 1971) according to the method of Osumi et al. (1975).

Electron microscopy

Fixation. Whole yeast cells were fixed in 1.5% (w/v) $KMnO_4$ solution for 20 min according to Veenhuis et al. (1976). Spheroplasts were pre-fixed in 6% (w/v) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 30 min at 0 °C and, also after cytochemical experiments, post-fixed in a solution of 1% (w/v) OsO_4 and 2.5% (w/v) $K_2Cr_2O_7$ in 0.1 M cacodylate buffer, pH 7.2, for 45 min at room temperature. After dehydration in a graded alcohol series the cells were embedded in Epon 812. Ultrathin sections were cut with a diamond knife and

examined in a Philips EM201 or EM300.

Cytochemical staining. Catalase and oxidase activities were stained according to the methods described by Veenhuis et al. (1976). H_2O_2 production during choline oxidation was determined by a similar method modified as follows: spheroplasts were pre-fixed in 0.2% (w/v) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, in the presence of 1.5 M sorbitol as an osmotic stabilizer for 10 min at 0 °C. During the subsequent incubation the final reaction mixture also contained 1.5 M sorbitol. This modification was adopted since fixation in 6% (w/v) glutaraldehyde prior to incubation with substrate led to complete loss of the choline-oxidizing capacity of spheroplasts. After such incubations the spheroplasts were fixed in 6% glutaraldehyde and post-fixed in OsO_4/$K_2Cr_2O_7$ as described above.

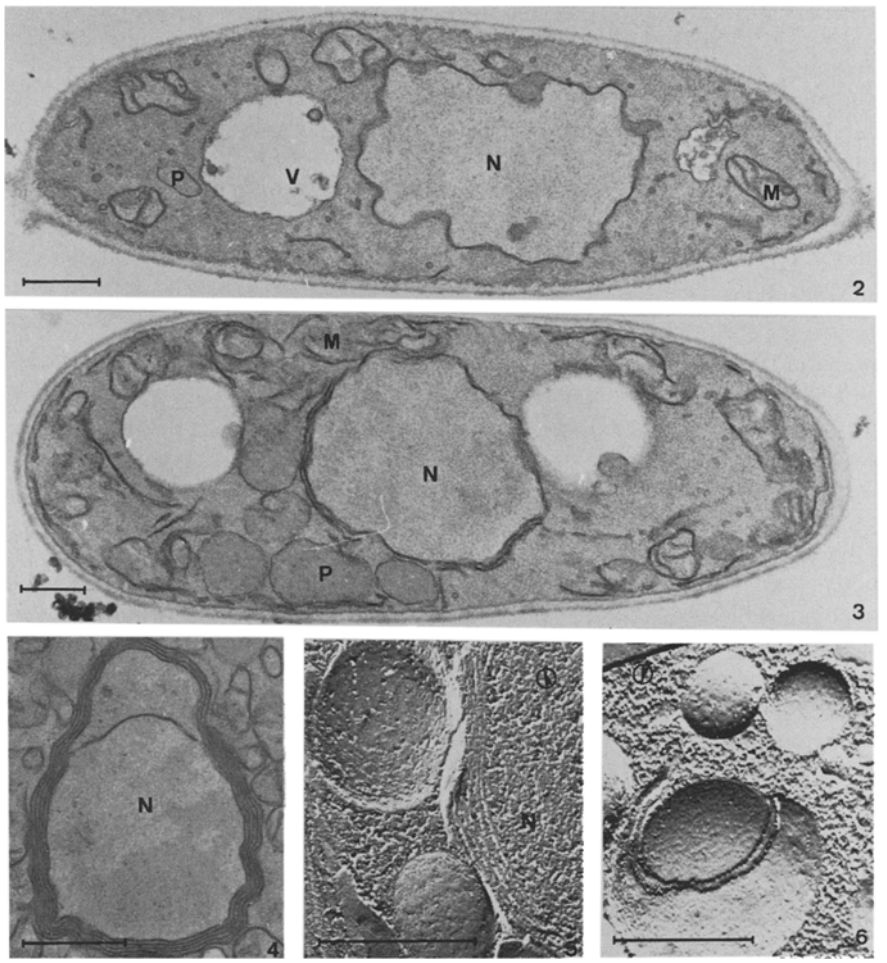
Freeze fracturing. Freeze etch replicas were prepared as described by Veenhuis et al. (1976).

Results

Ultrastructure of Candida utilis and Hansenula polymorpha during growth on choline or ethanolamine as the sole nitrogen source

In order to investigate a possible role of peroxisomes in the degradation of phospholipid components, we first studied the ultrastructure of *C. utilis* and *H. polymorpha* after growth on choline or ethanolamine as the sole nitrogen source. Figs 2 and 3 show micrographs of cells of *C. utilis,* grown in continuous cultures in the presence of 0.1% glucose as the carbon source and 0.25% ammonium sulphate (Fig. 2) or 0.2% choline (Fig. 3) as the nitrogen source, respectively. Compared with ammonium sulphate, growth on choline resulted in an increase in the number and size of peroxisomes which were scattered throughout the cells. Another striking feature of growth on choline was the synthesis of several membraneous layers, predominantly observed in close association with the nucleus (Fig. 4), but occasionally also surrounding other cell components such as mitochondria or vacuoles. These structures were not considered to be artifacts caused by specimen preparation for electron microscopy since they were also observed in untreated freeze-fractured cells (Fig. 5). In freeze-etch replicas the fracture faces of these membranes showed small particles (Fig. 6). Apparently, the presence of these membranes was related to the utilization of choline as a nitrogen source, since variation of the carbon source, i.e. glucose or ethanol instead of glycerol, did not produce any changes. The function of these membranes, however, remained obscure. As similarly described above for choline, growth of *C. utilis* on ethanolamine as the nitrogen source also led to the formation of several large peroxisomes in the cells.

A more complex situation was observed in *H. polymorpha.* Cells of this organism, grown in continuous cultures on glucose and ammonium sulphate already



Figs 2–6. Electron micrographs of cells of *Candida utilis* grown in continuous culture (D = 0.13 h^{-1}). M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole. The marker represents 0.5 μm. The arrow in Figs 5 and 6 indicates the direction of shadowing.

Fig. 2. Section of a glucose/ammonium sulphate-grown cell in which a single small peroxisome can be observed ($KMnO_4$).

Fig. 3. Section of a glucose/choline-grown cell showing several large peroxisomes ($KMnO_4$).

Figs 4 and 5. Details of glucose/choline-grown cells (Fig. 4, $KMnO_4$; Fig. 5, freeze-fractured) showing the nucleus surrounded by membraneous layers.

Fig. 6. Detail of the membraneous layers in glucose/choline-grown cells showing the substructure of their fracture faces after freeze-fracturing.

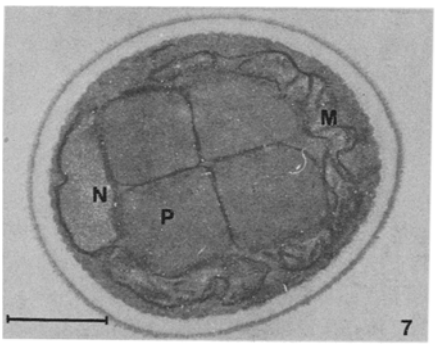
contained one or more large peroxisomes, mainly due to derepression of the synthesis of alcohol oxidase and catalase (Eggeling and Sahm, 1978; Egli et al.,

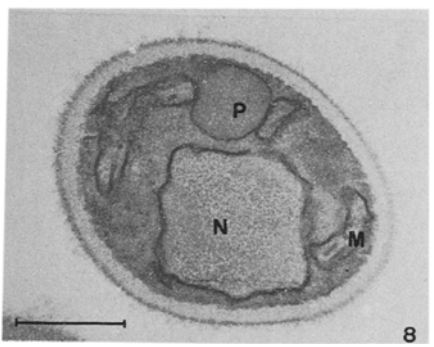
1980). During growth on choline instead of ammonium sulphate, the size and number of these organelles had significantly increased (Fig. 7). This was, however, not observed in ethanolamine-grown cells in which the peroxisomal size and number remained virtually unaltered as compared to growth on ammonium sulphate (Fig. 8). The membraneous layers observed in choline-grown *C. utilis*, were absent in *H. polymorpha*.

Since the increase in peroxisomal volume fraction during growth on choline or ethanolamine might indicate a possible function of the organelles in the degradation of these nitrogen sources, we decided to study their metabolism in the yeasts *C. utilis* and *H. polymorpha* in more detail.

Metabolism of choline in Candida utilis and Hansenula polymorpha

By definition peroxisomes are organelles which contain H_2O_2-producing oxidases and catalase (De Duve and Baudhuin, 1966). As shown in Fig. 1, several oxidases may be involved in choline degradation and attempts were made to detect activities of these enzymes in cell-free extracts of *C. utilis* and *H. polymorpha* after growth on choline as the nitrogen source. The results are shown in Table 1. Choline-grown cells of *C. utilis* contained amine oxidase, an enzyme not present in ammonium sulphate-grown cells, whereas catalase was present in enhanced levels. Activities of the other oxidases possibly involved in choline catabolism (Fig. 1) were not detected. In choline-grown cells of *H. polymorpha* amine oxidase was synthesized as well and in addition to an increased catalase activity an increase in the activity of alcohol oxidase was observed. As in *C. utilis* similarly also in *H. polymorpha* other oxidases with a possible function in choline metabolism were not detected. Therefore, the presence of amine oxi-





Figs 7–8. Electron micrographs of cells of *Hansenula polymorpha* grown in continuous culture ($D = 0.13\ h^{-1}$). M, mitochondrion; N, nucleus; P, peroxisome. The marker represents 0.5 μm.

Fig. 7. Section of a glucose/choline-grown cell containing several large cuboid-shaped peroxisomes ($KMnO_4$).

Fig. 8. Section of a glucose/ethanolamine-grown cell, showing a single peroxisomal profile ($KMnO_4$).

Table 1. H_2O_2-producing oxidase (nmol · min^{-1} · mg protein^{-1}) and catalase (ΔE_{240} · min^{-1} · mg protein^{-1}) activities in cell-free extracts of *Candida utilis* and *Hansenula polymorpha*, grown in continuous culture on glucose as the carbon source and choline, ethanolamine or ammonium sulphate as the nitrogen source ($D = 0.13\ h^{-1}$)

Enzyme	*C. utilis*			*H. polymorpha*		
	choline	ethanol-amine	$(NH_4)_2SO_4$	choline	ethanol-amine	$(NH_4)_2SO_4$
amine oxidase	82.5	23	0	22	17.8	0
alcohol oxidase	0	0	0	1310	206	177
catalase	25	22.3	5	47.6	12	22

dase in choline-grown cells of both yeast species indicated that degradation of choline may proceed via methylamine (pathway 3) or possibly ethanolamine (pathway 2), rather than via pathway 1 (Fig. 1). Apart from the absence of the typical enzymes in cell-free extracts, the view that pathway 1 was not operative during growth of cells on choline was supported by two additional observations. Firstly, *C. utilis* and *H. polymorpha* were unable to utilize dimethylglycine and sarcosine, two intermediates of pathway 1, as a nitrogen source. Secondly, washed cell suspensions of both organisms grown on choline were unable to oxidize dimethylglycine and sarcosine.

However, the above results did not give conclusive evidence whether in the yeasts studied choline was degraded via pathway 2 or 3 (Fig. 1). Growth experiments indicated that both *C. utilis* and *H. polymorpha* were able to utilize each and every intermediate of both pathways as a nitrogen source. Furthermore, washed cell suspensions of choline- but not ammonium sulphate-grown *C. utilis* rapidly oxidized choline and each intermediate of pathway 3, i.e. trimethylamine, dimethylamine and methylamine. Except for ethanolamine, oxidation of intermediates of pathway 2 by choline-grown cells was not observed i.e. addition of excess dimethylethanolamine or methylethanolamine to washed cell suspensions did not result in an increase in the already rather high endogenous rate of oxygen consumption.

The possible oxidation of the various intermediates of pathways 2 and 3 by way of a mono-oxygenase type reaction was studied using the assay of Boulton et al. (1974) for trimethylamine mono-oxygenase (see Materials and Methods). In cell-free extracts of choline-grown *C. utilis* NADH oxidation was observed in the presence of each of these intermediates (Table 2). Addition of choline, choline phosphate or betaine to the incubation mixtures did not lead to NADH oxidation (Table 2). In a control experiment with extracts prepared from cells grown in the presence of ammonium sulphate, oxidation of NADH consequent upon the presence of any of the intermediates of these pathways was not observed. Comparable results were obtained with choline-grown cells of *H. poly-*

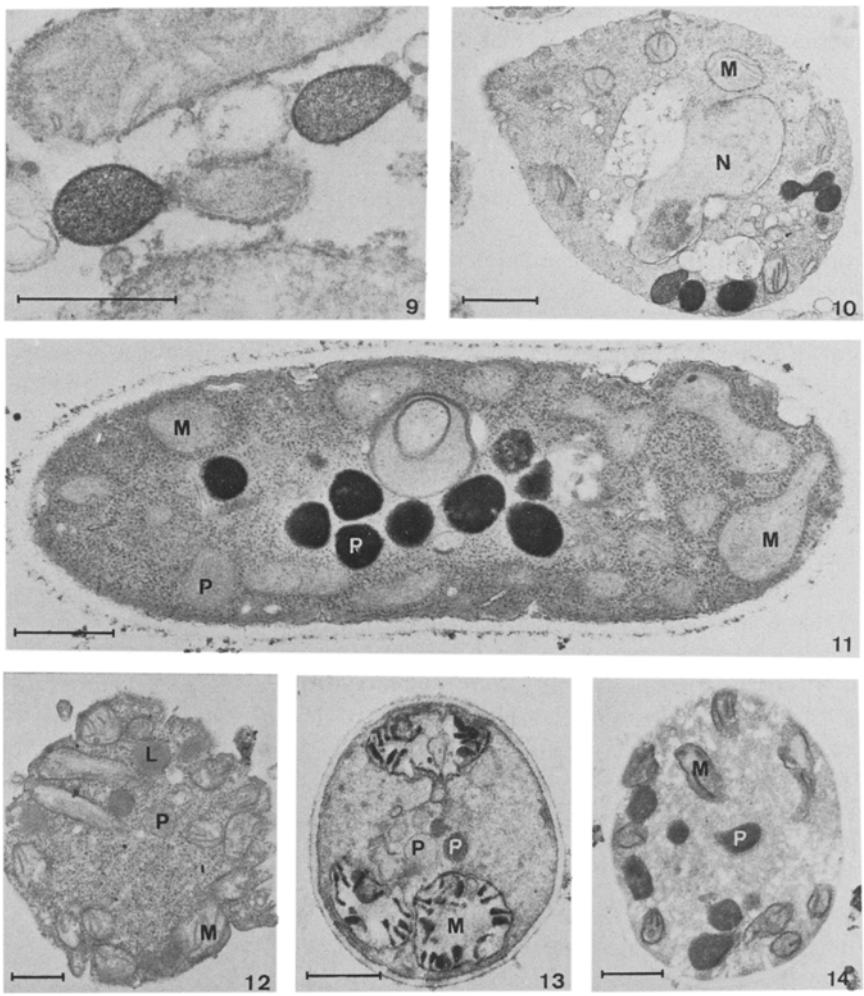
morpha (Table 2). These results may indicate the presence of one or more mono-oxygenase activities involved in the oxidation of intermediates of pathway 2 and 3. However, the observed substrate-dependent oxidation of NADH is not necessarily due to the activity of such mono-oxygenases. It cannot be ruled out that other enzymes are involved in the production of formaldehyde from the various intermediates derived from choline. Reduction of formaldehyde thus formed by an NADH-linked formaldehyde reducing enzyme as recently discovered in some methylotrophic yeasts (Hou et al., 1982) would also lead to NADH oxidation in the assay systems used. Similarly reduction of glycolaldehyde could lead to NADH consumption. A method of discriminating between the two possibilities is to exploit the known sensitivity of trimethylamine mono-oxygenase activity to carbon monoxide (Green and Large, 1983). When carbon monoxide was used in the above assay system, only trimethylamine-dependent NADH oxidation was significantly inhibited in cell-free extracts of both choline-grown *C. utilis* and *H. polymorpha*. This suggests that trimethylamine mono-oxygenase is present in such cells and that this enzyme may play a role in choline metabolism.

In order to obtain more conclusive information whether enzymes of pathway 2 or 3 were involved in choline degradation, we measured excretion products of choline-grown cells in a reaction mixture containing 20 mM choline and 1 mM aminoacetonitrile, a compound which specifically inhibits amine oxidase (Haywood and Large, 1981). Upon incubation of washed cell suspensions of choline-grown *C. utilis* in this reaction mixture we were able to identify dimethylamine and methylamine as intermediates of choline degradation using a biological assay with cells of *Hyphomicrobium* X (see Materials and Methods). Both compounds were excreted in low concentrations (0.35 mM of each), whereas

Table 2. Substrate-dependent oxidation of NADH (nmol NADH consumed $\cdot min^{-1} \cdot$ mg protein^{-1}) in cell-free extracts of *Candida utilis* and *Hansenula polymorpha*, grown in batch cultures (glucose + choline) and harvested in the exponential growth phase at an optical density (OD_{663}) of 1.5

Substrate	Oxidation of NADH in extract of	
	C. utilis	*H. polymorpha*
choline	0	0
choline phosphate	0	0
betaine	0	0
trimethylamine	40	37
dimethylamine	98	99
methylamine	55	–
dimethylethanolamine	107	136
methylethanolamine	116	99
ethanolamine	45	37

–, not determined.



Figs 9–14. Electron micrographs of cells of *Candida utilis* grown in batch cultures in the presence of glucose and choline. L, lipid; M, mitochondrion; N, nucleus; P, peroxisome. The marker represents 0.5 μm.

Fig. 9. Micrograph of isolated peroxisomes released from the cells by osmotic shock, positively stained after incubation with $CeCl_3$ and methylamine (glutaraldehyde-OsO_4/$K_2Cr_2O_7$).

Fig. 10. Section of a spheroplast with positively stained peroxisomes after incubation with $CeCl_3$ and ethanolamine (glutaraldehyde-OsO_4/$K_2Cr_2O_7$).

Figs 11–12. Sections of spheroplasts, prefixed in 0.2% glutaraldehyde + 1.5 M sorbitol, showing positively stained peroxisomes after incubation with $CeCl_3$ and choline in the presence of 1.5 M sorbitol as an osmotic stabilizer (Fig. 11). Staining is absent in control experiments in which choline is omitted from the incubation mixture (Fig. 12).

Fig. 13. Section of an unfixed cell, showing positively stained peroxisomes after incubation with DAB and choline, indicating in vivo H_2O_2 production. Note that not all peroxisomes show reaction products. Staining of the mitochondria is due to cytochrome c peroxidase ($KMnO_4$).

Fig. 14. Section of a spheroplast prefixed with 0.2% glutaraldehyde + 1.5 M sorbitol after incubation with DAB and H_2O_2 in the presence of 1.5 M sorbitol, showing positively stained peroxisomes and mitochondria (glutaraldehyde-OsO_4/$K_2Cr_2O_7$).

excretion of trimethylamine was not detected. Similar results were obtained with choline-grown *H. polymorpha*. In control experiments performed in the absence of aminoacetonitrile and in cells grown in the presence of ammonium sulphate, excretion of the above components was not observed.

The results of the above experiments suggest that in both yeasts studied choline degradation probably proceeds via trimethylamine, dimethylamine and methylamine (pathway 3, Fig. 1), and not via dimethylethanolamine, methylethanolamine and ethanolamine (pathway 2, Fig. 1). Unfortunately, the first reaction of pathway 3, namely the production of trimethylamine from choline could not be demonstrated in cell-free extracts. This is a well-known reaction in several anaerobic bacteria (Hayward and Stadtman, 1959, 1960; Bradbeer, 1965) and occurs for example in *D. desulfuricans* during growth on choline. In experiments in which extracts of choline-grown *D. desulfuricans* strain BH were used in order to test the assay system, a specific activity of 90 nmol $\cdot$ min^{-1} $\cdot$ mg protein^{-1} of the choline cleavage enzyme was measured. However, in cell-free extracts prepared from choline-grown *C. utilis* or *H. polymorpha*, activities of this enzyme were not detectable.

Metabolism of ethanolamine in Candida utilis and Hansenula polymorpha

When *C. utilis* was grown in media containing ethanolamine as the sole source of nitrogen, amine oxidase activity was detected in cell-free extracts (Table 1). This enzyme is probably involved in the oxidation of both methylamine and ethanolamine since a similar enzyme purified from *C. boidinii* showed activity with both compounds (Haywood and Large, 1981). Comparable results were obtained with *H. polymorpha* indicating that the process of ammonia liberation from ethanolamine in both yeast species resulted from direct oxidation of the substrate by amine oxidase.

Cytochemical staining experiments

Both the ultrastructure of the cells and the presence of amine oxidase and catalase after growth in the presence of choline and ethanolamine suggested that peroxisomes may play a role in the degradation of these compounds in the two yeast species under study. The subcellular localization of amine oxidase and catalase was further investigated by cytochemical staining techniques. Amine oxidase activity was demonstrated using a direct method for the detection of H_2O_2-producing oxidases, namely by incubation of cells or spheroplasts with $CeCl_3$ and the oxidase substrate (Veenhuis et al., 1976). After incubation of spheroplasts of choline-grown *C. utilis*, prefixed in 6% glutaraldehyde in a reaction mixture containing 50 mM aminotriazole, 5 mM $CeCl_3$ and 20 mM methylamine staining was confined to the peroxisomal matrix (Fig 9). When ethanolamine was used as substrate the peroxisomes were also positively stained (Fig. 10). Identical incubations in the presence of choline did not result in any staining

of microbodies or cytoplasm. Biochemical experiments revealed that this absence of staining could be explained by a complete loss of the ability of the spheroplasts to oxidize choline that was caused by the pre-fixation in 6% glutaraldehyde. Such a pre-fixation in cerium-based incubations is essential since cerous ions only very slowly penetrate into living, intact cells. Formaldehyde could also not be used as a pre-fixative, since this compound completely inhibits amine oxidase activity (Zwart et al., 1980). Therefore we investigated the influence of a very mild glutaraldehyde pre-fixation on the choline-oxidizing capacity of spheroplasts. The results obtained indicated that after a short pre-fixation in 0.2% glutaraldehyde in the presence of 1.5 M sorbitol, inactivation of the capacity to oxidize choline was largely prevented. This mild fixation in 0.2% glutaraldehyde was sufficient to facilitate the penetration of cerous ions into the spheroplasts, but was inadequate in preventing them from swelling or rupturing during the subsequent incubations in hypotonic incubation mixtures. Therefore the presence of the osmotic stabilizer during the final incubations was essential, since the choline-oxidizing activity was rapidly lost in its absence. Incubations of spheroplasts pre-fixed as described above with 5 mM $CeCl_3$ and 20 mM choline resulted in electron-dense deposits in the majority of the peroxisomes present (Fig. 11). Some of the organelles, however, remained unstained after this procedure for reasons unknown. It was noted that peroxisomal staining only occurred in spheroplasts which had retained a significant portion of the cell wall (compare Fig. 11) and possessed an electron density of the cytoplasm similar to that of whole cells. It was invariably absent in organelles in spheroplasts which appeared swollen or partly disrupted. This may be due to the fact that activities of enzymes involved in the initial conversion of choline are in some unknown way dependent on the structural integrity of the cells, since they were also rapidly inactivated during the preparation of cell-free extracts and osmotic disruption of spheroplasts (see above). Similarly as with choline, the peroxisomes were also stained when trimethylamine, dimethylamine or methylamine were used as substrates. During incubations with choline in the presence of carbon monoxide, staining was strongly – although not fully – inhibited, suggesting a choline degradation pathway via trimethylamine mono-oxygenase, which is carbon monoxide-sensitive (Green and Large, 1983). Incubations with the above substrates in the presence of 1 mM aminoacetonitrile did not result in staining of the peroxisomes, suggesting that amine oxidase was responsible for the production of H_2O_2 during choline oxidation. In addition, in control experiments without substrate staining was also absent (Fig. 12).

Since, in contrast to cerous ions, living yeast cells are readily penetrated by diaminobenzidine (DAB), a possible in vivo H_2O_2-production during choline oxidation was also investigated by DAB-based incubations (Veenhuis et al., 1976). Aerated incubations of unfixed intact cells with DAB and 20 mM choline, also resulted in positively stained peroxisomes (Fig. 13). However, in the same way as described above for the cerium-based incubations in this case also not

all the organelles present in one cell were stained when completely unfixed cells were incubated with DAB and choline (Fig. 13). The presence of catalase activity in these organelles was demonstrated after incubation of spheroplasts with DAB and H_2O_2 (Fig. 14). Staining of the mitochondria after the DAB-based incubations was independent of the presence of substrate and is most probably due to staining of cytochrome c peroxidase (Todd and Vigil, 1972).

Discussion

Growth of the yeasts *C. utilis* and *H. polymorpha* in media containing choline or ethanolamine as the sole nitrogen source was accompanied by the presence of several relatively large peroxisomes in the cells compared to cells grown in the presence of ammonium sulphate. It is well substantiated that the presence of an increased peroxisomal volume fraction in yeasts may indicate a possible function in the degradation of a certain compound (Osumi et al., 1975; Kawamoto et al., 1977; Zwart et al., 1980, 1983; Veenhuis et al., 1983).

Experimental evidence for the involvement of a peroxisomal H_2O_2-producing enzyme in the metabolism of choline in *C. utilis* was obtained from cytochemical staining experiments (Figs 11 and 13). The only detectable peroxisomal oxidase in choline-grown cells of this organism was amine oxidase and since staining was absent after incubations with choline in the presence of 1 mM aminoacetonitrile, we concluded that amine oxidase was responsible for the observed H_2O_2 production from choline. After incubations with $CeCl_3$ and methylamine, amine oxidase activity was demonstrated cytochemically in all the peroxisomes present in the cells (Fig. 9). Therefore, the reasons for the absence of staining in part of the peroxisomal population after incubations in the presence of choline (Figs 11 and 13) are unknown. Most probably amine oxidase is also involved in choline metabolism in *H. polymorpha*. Alcohol oxidase, the second H_2O_2-producing peroxisomal enzyme whose activity had increased drastically, did not react with choline or one of its metabolic products. A very likely explanation for the synthesis of enhanced levels of this enzyme under these conditions is derepression caused by the methyl groups present in the choline molecule (Eggeling and Sahm, 1978).

Excretion of dimethylamine and methylamine, observed after incubation of whole cells of both strains with choline and 1 mM aminoacetonitrile, indicated that methylamine rather than ethanolamine was the internal substrate for amine oxidase. Methylamine is probably produced via a pathway involving trimethylamine and dimethylamine (pathway 3, Fig. 1) in which an NADH-dependent trimethylamine mono-oxygenase is involved. A similar enzyme was present in *C. utilis*, grown on trimethylamine as the sole nitrogen source (Green and Large, 1983). When the presence of this enzyme was tested in extracts of choline-grown *C. utilis* cells, it was found that a number of the possible intermediates of choline

metabolism gave a positive reaction in the assay system. However, only in the case of trimethylamine oxidation could the observed activity be attributed to a mono-oxygenase type reaction, because its activity was sensitive to inhibition by CO. In the present study we did not investigate a possible accumulation of the intermediates of pathway 2 as depicted in Fig. 1 and for that reason the possible occurrence of this route cannot be fully excluded. Unfortunately the primary reaction of the suggested pathway of choline degradation, i.e. the cleavage of choline into trimethylamine and a C_2 component, was not detectable in cell-free extracts of *C. utilis* or *H. polymorpha* by the biological assay system used. This assay was, however, adequate for measuring the same reaction in cell-free extracts of *D. desulfuricans*, grown anaerobically on choline.

Our results demonstrate an indirect but essential role of peroxisomal amine oxidase in choline metabolism in *C. utilis* and *H. polymorpha* when these organisms utilized choline as the sole nitrogen source. In contrast, amine oxidase plays a direct role in the degradation of another phospholipid component, ethanolamine. Therefore, peroxisomes in these yeasts have an important function in the degradation of these compounds in order to produce nitrogen necessary for the synthesis of cell material. Choline or ethanolamine cannot be utilized as carbon source because of the relatively high nitrogen to carbon ratio of the compounds as compared to cell material. Growth on these compounds as carbon source would inevitably result in ammonia accumulation and excretion and we have previously shown that the synthesis of amine oxidase is severely repressed by ammonia (Zwart and Harder, 1983). Thus the physiological role of peroxisomes in choline and ethanolamine degradation is dependent on the nitrogen status of the cells and explains why these compounds are not used as sole carbon sources by these yeasts.

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