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Methanol oxidation and assimilation in *Hansenula polymorpha*

An analysis by ^{13}C n.m.r. *in vivo*

John G. JONES and Edward BELLION*
Department of Chemistry, University of Texas at Arlington, Arlington, TX 76019-0065, U.S.A.

The metabolism of methanol was monitored in whole cells of the methylotrophic yeast *Hansenula polymorpha* by using [^{13}C]methanol and n.m.r. *in vivo*. The main products observed under normal conditions were trehalose and glycerol, whereas cells that were starved before exposure to [^{13}C]methanol also accumulated glutamate, glutamine and alanine; formate was also more prominent in spectra from starved cells. Cells exposed to high methanol concentration together with high oxygenation oxidized methanol extensively, leading to formaldehyde accumulation; label was not found in any subsequent metabolic products, indicating possible cell inactivation. [^{13}C]Formate was incorporated into metabolic products in glucose-grown cells exposed to 150 mM-methanol for 3 h, but not in cells starved for 3 h, in which it was oxidized. At 21 °C such 3 h-starved cells showed a slower metabolism of [^{13}C]methanol compared with those at 37 °C, and also converted methanol into formate rather than into assimilation products. The labelling pattern in trehalose from starved cells at 37 °C was consistent with methanol assimilation via the pentose phosphate pathway. Lack of appearance of labelled formaldehyde and formate during metabolism under normal conditions suggests that the linear oxidation pathway is not a major contributor to methanol oxidation; their appearance in extreme conditions suggests instead a more likely role in detoxification.

INTRODUCTION

Hansenula polymorpha is a methylotrophic yeast that can use methanol as a sole source of carbon and energy [1]. Methanol is oxidized by an oxidase to generate formaldehyde, which is further oxidized or assimilated into carbohydrates (see Scheme 1). In common with other methanol-utilizing yeasts, there is compartmentation of the methanol oxidase and dihydroxyacetone synthase within peroxisomes, while the other enzymes associated with methanol metabolism are cytosolic. The peroxisomes are believed to have a role in containing the toxic and reactive products of methanol oxidation until they are further metabolized to less harmful compounds. Formaldehyde is assimilated enzymically via dihydroxyacetone synthase and also reacts non-enzymically with glutathione to produce *S*-hydroxymethylglutathione [2]. This is oxidized to *S*-formylglutathione, and the formate liberated upon subsequent hydrolysis may be oxidized to CO_2. NADH generated during this formaldehyde oxidation pathway has been postulated to provide energy for the cells, although an alternative hypothesis invoking the citric acid cycle as the energy-supplying pathway has been proposed [3]. H_2O_2 formed in the initial oxidation of methanol is removed by peroxisomal catalase [4].

A prominent feature of the methanol metabolism pathway in these organisms is the large excess capacity for methanol oxidation when compared with the activities of the subsequent enzymes involved in formaldehyde assimilation and oxidation [5]. Methanol enters the cells passively, and so its flow into the cell cannot be actively regulated; furthermore, the oxidation step is not inhibited by the products. This entails that the pathways for formaldehyde removal have to be efficiently regulated in order to prevent this lethal metabolite from accumulating and killing the cells. Also in this regard the open question arises concerning partitioning of formaldehyde between the assimilation pathway and the oxidation pathway, and the regulation of this important branch point, about which little is currently understood. In the present study we used ^{13}C n.m.r. to monitor the flow and fate of the carbon from [^{13}C]methanol into the metabolic pathways of this organism. ^{13}C n.m.r. has been widely used in micro-organisms to determine the carbon flow through key pathways such as glycolysis [6], gluconeogenesis [7] and amino acid synthesis [8,9]. The method allows simultaneous detection and quantification of labelled metabolites *in vivo* and allows analysis of partitioning of label between assimilation and oxidation. It is particularly suited to the study of methanol metabolism in yeast, because of the relatively low cost and high enrichment of commercially available [^{13}C]methanol.

MATERIALS AND METHODS

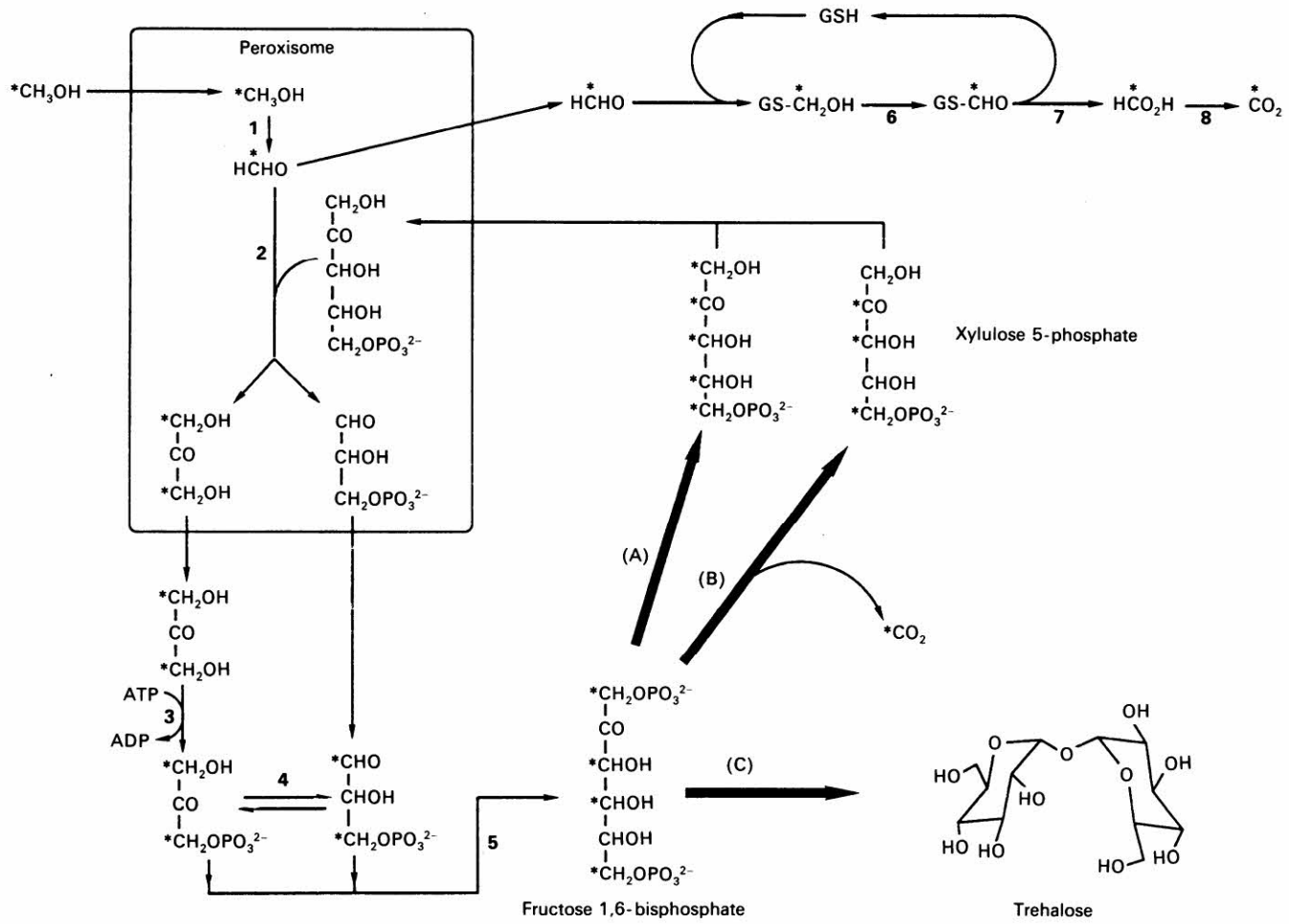
Cell growth

Hansenula polymorpha was grown in a mineral salts medium at pH 5.3 supplemented with vitamins, with 1% glucose (w/v) or 1% (v/v) methanol as a carbon source and 25 mM-NH_4Cl as a nitrogen source [1]. Cells were incubated for 14–18 h (glucose medium) or for 40–48 h (methanol medium) at 37 °C in 2-litre shake flasks equipped with baffles, containing 0.5 litre of the growth medium. They were harvested in mid- to late-exponential phase of growth (culture A_{590} 1.0–1.5, 1 cm path length) by centrifugation at 2500 $g_{av.}$ for 10 min at 4 °C. Dry weight values for the cells were obtained from a previous study [10].

N.m.r. procedures and parameters

Cell suspensions for all n.m.r. analyses were prepared by the same method. From a freshly harvested culture, the cell pellet was resuspended in a buffer consisting of minimal medium with the phosphate concentration adjusted to 50 mM. The cell density was adjusted to a value of 70–80 mg dry wt./ml. N.m.r. analyses were performed with 2.5–3 ml volumes of this suspension. The cell suspension was prepared and stored on ice before the experiment and was used within 20 min of being prepared. During data acquisition, the cells were oxygenated by means of a 1 mm-internal-diameter polyethylene tube extending to the bottom of the sample with an O_2 flow rate of 15–20 ml/min. Measurements with the oxygen electrode showed that this arrangement maintained at least 50%-O_2-saturated buffer at

* To whom correspondence and reprint requests should be addressed.



Scheme 1. Labelling of the major metabolic pathways of methanol metabolism in *Hansenula polymorpha*

Bold asterisks indicate major labelling sites, plain asterisks indicate random dilution of label into multiple sites. key to enzymes: **1**, alcohol oxidase; **2**, dihydroxyacetone synthase; **3**, dihydroxyacetone kinase; **4**, triose-phosphate isomerase; **5**, aldolase; **6**, formaldehyde dehydrogenase; **7**, *S*-formylglutathione hydrolase; **8**, formate dehydrogenase. Major metabolic routes depicted are: (A) re-arrangements via transaldolase and transketolase; B, pentose phosphate pathway; C, trehalose synthesis.

these cell densities. Lower oxygenation rates or higher cell densities resulted in failure of the cells to utilize methanol. Unless mentioned otherwise, the probe temperature was set to 37 °C for all the ^{13}C n.m.r. experiments.

All spectra were obtained with a Nicolet NIC-200 spectrometer operating at 50.33 MHz for ^{13}C, and equipped with a 12 mm probe. Operating parameters used were as follows. A pulse width of 10 μs with a pulse flip angle of 45°, a delay of 340 ms and a sweep width of 5000 Hz were used for experiments performed *in vivo* and the data were retained in 2 k of memory. A coaxially mounted 5 mm insert containing deuterobenzene provided both the lock signal and a reference for chemical shifts and intensities. The centre peak of the deuterobenzene triplet was assigned to 128 p.p.m. relative to tetramethylsilane (0 p.p.m.). Continuous broadband decoupling was used for all spectra with a power output of 1.8 W. A total of 1800 accumulations (13 min) were obtained for each spectrum.

Cell extracts were prepared by rapidly mixing the n.m.r. sample with 2.5 ml of 30 % (v/v) $HClO_4$ followed by immersion in liquid N_2. The mixture was freeze–thawed twice and the pH was adjusted to 7.0 by addition of 5 M-KOH. The cell debris and the $KClO_4$ precipitate produced were pelleted by centrifugation at 13000 $g_{av.}$ for 10 min. The supernatant was freeze-dried and resuspended in water containing 30 % (v/v) ^2H_2O. Spectra of the extracts were taken with a 2 s delay time and an 8 k or 16 k data block with the same decoupling parameters as for the experiments performed *in vivo*. Further processing of the data is described in the Figure legends.

RESULTS

[^{13}]Methanol metabolism by *Hansenula polymorpha*

Fig. 1 shows a time course of [^{13}C]methanol metabolism in a suspension of *Hansenula polymorpha* cells that were grown on methanol. The label was incorporated extensively into the trehalose carbon atoms with only minor amounts detected in formate, an oxidation pathway product. No free formaldehyde was detected, but a minor peak at approx. 66 p.p.m., which matched the known chemical shift of *S*-formylglutathione, was seen at the beginning of the experiment. This suggests that a significant fraction of carbon flow from formaldehyde went into the dihydroxyacetone synthase pathway rather than through the cytosolic oxidation pathway. The small degree of coupling between the (C3,C3′) and (C4,C4′) carbon atoms of trehalose seen in the cell-extract spectrum (Fig. 2*a*) indicates that the label was diluted into a large endogenous carbohydrate pool immediately after incorporation into dihydroxyacetone. Another feature of the trehalose labelling pattern was the lower rate of

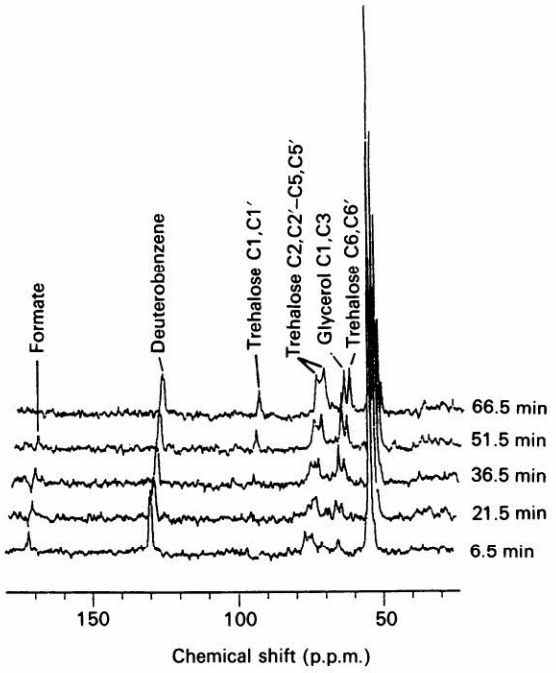


Fig. 1. ^{13}C-n.m.r. spectra of [^{13}C]methanol metabolism in a suspension of wild-type *Hansenula polymorpha*

[^{13}C]Methanol was added at zero time to a concentration of 0.3% (v/v) to a cell suspension maintained at 37 °C and oxygenated at a rate of 15 ml/min within a 12 mm-diameter n.m.r. tube. Spectra consist of 1800 scans (13 min). The free-induction decays were processed with 6 Hz of line-broadening and zero-filled to 16 k before Fourier transform. The chemical-shift scale corresponds to the uppermost spectrum.

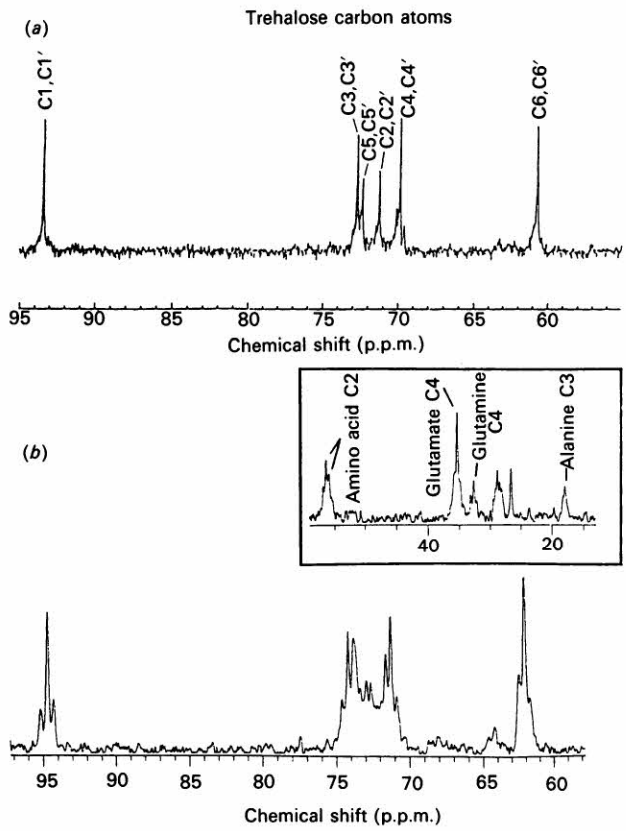


Fig. 2. (*a*) 75.5 MHz ^{13}C-n.m.r. spectrum of an $HClO_4$ extract obtained from the cell suspension shown in Fig. 1 after 73 min incubation, and (*b*) 50.33 MHz ^{13}C-n.m.r. spectrum of an $HClO_4$ extract of methanol-grown *Hansenula polymorpha* starved of methanol for 48 h before addition of label

(*a*) 75.5 MHz ^{13}C-n.m.r. spectrum of an $HClO_4$ extract obtained from the cell suspension shown in Fig. 1 after 73 min incubation. Spectrum consists of 12000 accumulations, which were saved in 16 k of memory and processed with 4 Hz line-broadening before Fourier transformation. (*b*) 50.33 MHz ^{13}C-n.m.r. spectrum of an $HClO_4$ extract of methanol-grown *Hansenula polymorpha* starved of methanol for 48 h before addition of label. Cell extracts were obtained in identical manner with that used for (*a*). Spectrum consists of 16000 scans collected in 16 k of memory and processed with 4 Hz line-broadening before Fourier transform.

label incorporation into the (C1,C1′) carbon atoms relative to the (C6,C6′) carbon atoms. Both of these sites are expected to be equivalently enriched since they are both derived from the symmetrically labelled C1 and C3 carbon atoms of dihydroxyacetone [11]. One explanation for this phenomenon could be that the (C1,C1′) carbon atoms produce a relatively weaker signal than the (C6,C6′) carbon atoms as a result of differences in nuclear Overhauser effects of relaxation times. However, spectra of authentic trehalose or trehalose in cell extracts show essentially similar peak intensities for these positions (see Fig. 2). Furthermore the (C1,C1′) peaks eventually approach the intensities of the (C6,C6′) peaks. The slower increase in (C1,C1′) spectral contributions relative to those of the (C6,C6′) is most probably a result of a loss of label at the C1 position of newly formed hexoses by pentose phosphate pathway oxidation (Scheme 1). The gradual increase of the trehalose (C1,C1′) peak is expected to occur as all the carbon atoms in the assimilation pool metabolites become enriched, resulting in a decrease in the contribution of unlabelled carbon atoms towards trehalose biosynthesis. No peaks due to natural-abundance ^{13}C were ever observed in experiments in which labelled methanol was omitted.

Metabolism by starved cells

[^{13}]Methanol metabolism was also monitored *in vivo* in cells that were starved (spectra not shown), such that the carbohydrate assimilation pool was depleted. Fig. 2(*b*) shows an $HClO_4$-extract spectrum obtained after 73 min incubation in [^{13}C]methanol, from cells that were starved of methanol for 48 h before the experiment. Analysis of the carbohydrate resonances in this cell-extract spectrum confirmed that the endogenous carbohydrate pool had been depleted before the addition of labelled methanol. The trehalose resonances consisted of multiplets that are the result of splitting by adjacent labelled carbon atoms. The (C1,C1′) and (C6,C6′) peaks have the simplest splitting pattern, since they are both resolvable from other resonances and can only have one neighbouring-carbon-coupling interaction. The centre line arises from a carbon atom that does not have a label adjacent to it, and the satellite doublets are the result of coupling by a labelled neighbouring carbon atom. The relative enrichments at the (C2,C2′) and (C5,C5′) positions were found to be 44%, compared with a minimal amount for freshly harvested cells. The overall intensities of the trehalose resonances were also greater in starved cells (Fig. 3*c*), possibly reflecting further de-repression of the trehalose synthesis pathway in response to starvation conditions [12–15].

Several additional resonances between 15 and 60 p.p.m. (Fig. 2*b* inset) were detected in starved cells that were not seen in fed cells. These were identified as originating from the carbon atoms of glutamate, glutamine and alanine. These are key metabolites of nitrogen assimilation and are also produced during sporulation [7]. All these peaks showed extensive carbon–carbon coupling, suggesting that their carbon atoms originated from fully labelled

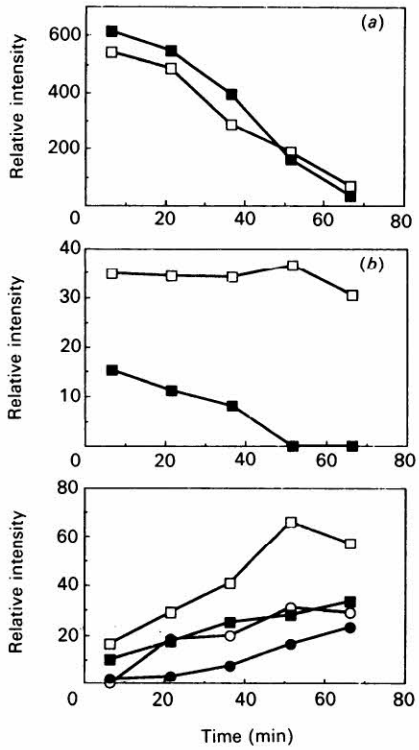


Fig. 3. Plot of relative-intensity changes with time of (*a*) labelled methanol, (*b*) labelled formate and (*c*) C1,C1′ (● and ○) and C6,C6′ (■ and □) carbon atoms of trehalose in freshly harvested (● and ■) and 48 h-starved cells (○ and □)

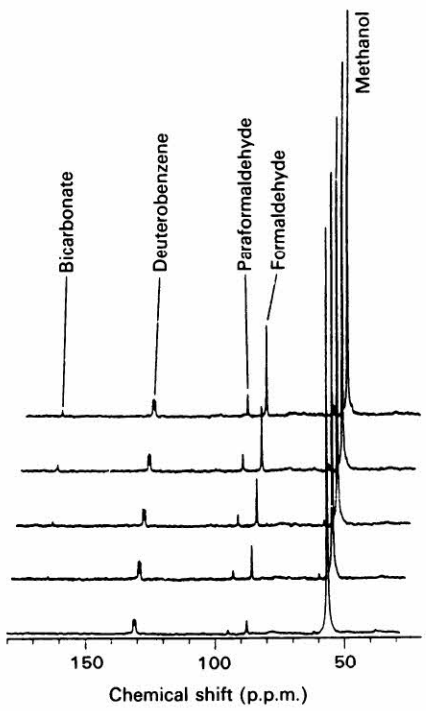


Fig. 4. 50.33 MHz ^{13}C-n.m.r. spectra of [^{13}C]methanol metabolism by cells exposed to high methanol concentrations and high oxygenation rates

Spectra were taken in the presence of 1.5% (v/v) [^{13}C]methanol added at zero time with 35 ml/min oxygenation rate. Cell densities were 60 mg dry wt./ml. All other parameters were as described for Fig. 1. Elapsed times for each spectrum were (from bottom) 6.5 min, 21.5 min, 36.5 min and 51.5 min. The chemical-shift scale corresponds to the uppermost spectrum.

citric acid cycle intermediates, which in turn could arise either from fully labelled glucose molecules or from singly labelled glucose that was subsequently scrambled in the citric acid cycle. The glutamate/glutamine ratio was calculated from their resolvable C4 peak area ratios and was found to be 2.9:1.

Methanol was oxidized in starved cells at a rate slightly lower than that found in freshly harvested cells, but the distribution of label into the metabolites was different (Figs. 3*a* and 3*b*). The formate resonance was more prominent in these spectra, indicating an increased flow of carbon through the oxidation pathway. This is expected to occur if the pool of assimilation cycle metabolites is reduced in size, leading to a decreased capacity for removal of formaldehyde by condensation with xylulose 5-phosphate (see Scheme 1).

Effect of excess methanol and high oxygenation

In order to determine the ability of the cells to cope with a high concentration of formaldehyde, we exposed cells to both higher concentrations of methanol and high oxygenation rates, and the results are depicted in Fig. 4. Formaldehyde rapidly accumulated and no labelling of assimilation products was observed. The oxidation rate of methanol, calculated from peak intensities, was also drastically decreased after an initial rapid burst. This is consistent with previous accounts in which *Hansenula polymorpha* cells that had maximal alcohol oxidase activities as a result of growth under methanol limitation were suddenly challenged with large amounts of methanol; that study reported rapid and irreversible cell inactivation and detection of formaldehyde in the medium [1].

Formate metabolism

To estimate the flow of carbon through the formate oxidation step, which is thought to be the rate-limiting step for C_1 oxidation, [^{13}C]formate metabolism was monitored. Fig. 5 shows the time course of [^{13}C]formate metabolism in cell suspensions that were exposed to 150 mM-methanol for 3 h (*a*) or starved of carbon (*b*) after growth on glucose. Interestingly, in methanol-treated cells a significant portion of the label was incorporated into trehalose and other assimilation products, which, on the basis of their chemical shifts, were probably other carbohydrate-derived compounds. In the starved cells, however, the label was not incorporated into any detectable products, although the disappearance rate of formate for the starved cells was approx. 70% of that found for the methanol-treated cells, indicating its oxidation to CO_2. This supports the view that formate dehydrogenase activity is substantially de-repressed by starvation alone [16]. No resonances corresponding to *S*-formylglutathione were detected, suggesting that glutathione was not a significant participant in formate metabolism. Unfortunately, in methanol-grown or methanol-treated cells it was not possible to measure the rate of formate conversion into CO_2 by means of formate disappearance because formate was also unexpectedly assimilated.

Induction and temperature effects

A striking feature of methanol metabolism within methylotrophic yeasts is the extent and rapidity with which the enzymes and organelles that are unique to methanol metabolism are synthesized. Figs. 6(*a*) and 6(*b*) show the spectra of extracts obtained from wild-type cells that were grown with glucose as a carbon source, starved of carbon for 3 h and then exposed to methanol. At 37 °C, the optimum growth temperature, methanol was depleted to about 50% of the rate found for methanol-

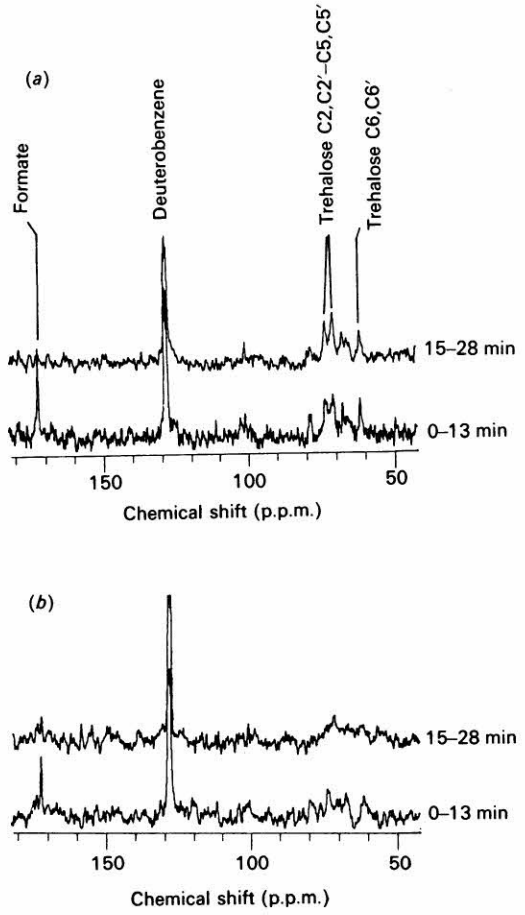


Fig. 5. [^{13}C]Formate metabolism in glucose-grown wild-type *Hansenula polymorpha*

Cells were (*a*) incubated in 150 mM-methanol at 37 °C for 3 h or (*b*) starved for 3 h at 37 °C before addition of 15 mM-[^{13}C]formate. Spectral parameters were as described for Fig. 1.

grown cells. A significant portion of the label was detected in assimilation products, with only transient labelling of formate. The total lack of the trehalose (C1,C1′) peak, 93 p.p.m., suggests that the pentose phosphate oxidation pathway was also active during the induction period. A similar experiment performed at 21 °C showed a slightly lower methanol utilization rate (approx. 80% of the 37 °C rate) and a different labelling pattern for the products. Here, most of the label appeared as formate, with little incorporation into assimilation products.

By use of this general methodology we were also able to evaluate the nature and extent of methanol utilization in a mutant of *Hansenula polymorpha* that was cold-sensitive for alcohol oxidase [17,18], and to show that its ability to metabolize methanol was restored following transformation with a plasmid containing the gene for alcohol oxidase (F. Guttierez, J. G. Jones & E. Bellion, unpublished work).

DISCUSSION

^{13}C n.m.r. was used to monitor the flow of carbon from methanol into the oxidative and assimilatory pathways in the methylotrophic yeast *Hansenula polymorpha*. Trehalose was the main assimilatory product detected in all our studies, and the labelling pattern correlates well with the analysis by Waites *et al.* of [^{14}C]methanol label distribution in triose and hexose assimilation products [11]. The synthesis of this disaccharide during methanol metabolism in yeast has not been studied, but its formation during change from vegetative growth to sporulation in *Saccharomyces cerevisiae* has been studied both with ^{13}C n.m.r. and with other methods [7,13]. Trehalose synthesis in yeast and fungi is generally associated with periods of decreased growth rate, starvation and differentiation processes [14,15]. Our results indicate that growth on minimal medium with methanol as a sole carbon source represents a suboptimal condition for *Hansenula polymorpha*, and this has important implications in terms of growth rates on this substrate and the yield of cell material per unit of methanol. Methylotrophic yeasts in general show much lower growth rates and lower yields of cell material per g of methanol oxidized when compared with methanol-utilizing bacteria [19]. The lower growth yield has been attributed to the differences in methanol-oxidation enzymes between yeast and bacteria [20]. Bacterial methanol oxidation is coupled to the generation of reducing equivalents, but in yeast no energy is directly obtained from this step since alcohol oxidase ultimately transfers the reducing equivalents to O_2 with concomitant formation of H_2O_2. The lower growth rate of yeast on methanol when compared with growth on other carbon sources such as glucose is not clearly understood. Methanol oxidation to formaldehyde does not appear to be the rate-limiting step for methanol metabolism under standard culture conditions [5]. Our study suggests that the diversion of assimilated carbon from the major biosynthetic pathways to the synthesis of trehalose and glycerol could contribute to the lower growth rate.

Most studies of methanol utilization by yeast have used chemostat systems rather than batch cultures. The chemostat allows precise control of the cell density and substrate concentration in the medium, unlike a batch culture, where both are constantly changing. However, we were able to correlate some important aspects of methanol metabolism in our batch cultures to data obtained from chemostat studies. From the methanol oxidation rate, assuming a 37% conversion into cell material [1], the rate of carbon assimilation was estimated to be about 2% of the cell mass per h. This leads to a doubling time of approx. 34 h, which corresponds to a chemostat dilution rate of 0.03 h^{-1}, which was used in a study of methanol metabolism by this organism. Under these conditions, high concentrations and activities of alcohol oxidase were measured, and when a pulse of 1% (v/v) methanol was added the cells became inactivated as a result of formaldehyde poisoning due to excessive oxidation of methanol. This did not occur at higher dilution rates, where the cells had lower concentrations and activities of methanol oxidase [1]. Our observations of [^{13}C]formaldehyde production during addition of 1.5% (v/v) methanol to the suspension agrees well with the chemostat results, and probably reflects the increased synthesis of alcohol oxidase during the late-exponential phase of growth as methanol in the culture medium becomes scarce.

[^{13}C]Methanol metabolism was observed in yeast cells that were grown on a medium containing glucose as a carbon source and transferred to one with methanol as the sole source of carbon. Following transfer into methanol medium, there is a rapid synthesis and assembly of peroxisomes and their key enzymes, alcohol oxidase, catalase and dihydroxyacetone synthase [21–23]. Our results show that the cells were able to oxidize methanol at 50% of the rate of methanol-grown cells after only 60–80 min of incubation in methanol-containing medium, which is consistent with the rapid and extensive synthesis of the peroxisomal enzymes [24,25]. During the early stage of cell adaptation to methanol we detected *S*-formylglutathione, the product of formaldehyde dehydrogenase. The appearance of this metabolite may have been due to a lag in the induction of formate dehydrogenase activity or a result of formaldehyde leakage into the cytosol from partially assembled peroxisomes that were unable to partition the metabolite from the cytosol. *S*-

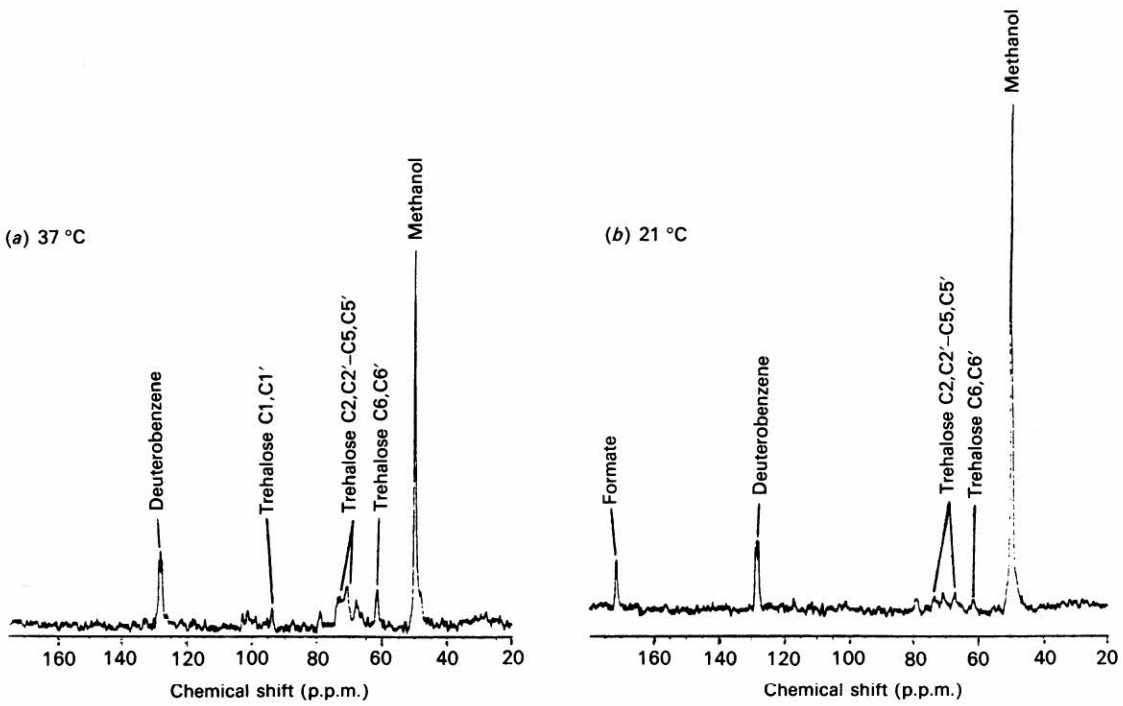


Fig. 6. 50.33 MHz cell-extract ^{13}C-n.m.r. spectra of [^{13}C]methanol metabolism by a suspension of glucose-grown *Hansenula polymorpha* after incubation in [^{13}C]methanol for (*a*) 40 min at 37 °C and (*b*) 40 min at 21 °C

Cells were preincubated for 3 h in minimal medium containing no carbon source at 37 °C before addition of methanol (0.3 %, v/v). Samples (1 ml) were taken for $HClO_4$-extract analysis at the assigned times. Spectra consisted of 19000 scans stored in 16 k of memory and processed with 8 Hz line-broadening.

Formylglutathione and *S*-hydroxymethylglutathione are the predominant metabolites in the cytosolic oxidation pathway, presumably because these compounds are much less toxic than free formaldehyde and formate. The absence of significant resonances from the glutathione adducts also suggests that the cytosolic pathway is not the major route of oxidation in these organisms under batch-culture conditions. This contrasts with studies of chemostat cultures, which indicate, on the basis of measurements of enzyme activities, a strong flow of carbon through this pathway. These contrasting results can be reconciled by the fact that methanol oxidase activity in chemostat cultures, especially those grown at low dilution rates, is generally much higher than in batch cultures in the exponential phase of growth [1]. High methanol oxidase activity in the cells produces a greater potential for formaldehyde overproduction and leakage into the cytosol. These conditions may cause the elevation of cytosolic oxidation pathway activity as an additional means of protection from formaldehyde poisoning. However, recently Sibirny *et al.* [26] have shown, by using mutants of *Hansenula polymorpha* lacking either formaldehyde dehydrogenase or formate dehydrogenase, that these enzymes are not indispensable for growth on methanol, since such mutants are able to sustain cell yields with methanol similar to those of the wild-type, but are more sensitive to higher exogenous methanol concentrations. On the basis of this and other evidence they propose that the linear formaldehyde oxidation pathway is not the major energy-supplying pathway, and instead suggest that the citric acid cycle fulfils that role. Although our current results cannot allow us to comment on the latter hypothesis, the observed lack of significant flux through the linear pathway in our studies points to a more probable role for the direct formaldehyde oxidation pathway in formaldehyde detoxification rather than energy production. If this is the case then the question of the regulation of the partition of formaldehyde between assimilation and oxidation assumes less importance.

Finally, our study showed that a pathway operates for the incorporation of label from formate into assimilation products, even in the presence of a substantial flow of carbon originating from unlabelled methanol. This appears to be a significant route, since the products were detected in measurable amounts under the experimental conditions. These findings suggests that, even in the presence of ample concentrations of methanol, carbon flow from formaldehyde to formate is not substantial, enabling the reverse reaction to occur by introducing a pulse of formate into the system. Normally carbon assimilation occurs from formaldehyde in the reaction with xylulose 5-phosphate catalysed by dihydroxyacetone synthase or transketolase. Assimilation of formate would therefore require its prior reduction, although the exact mechanism by which this reduction occurs is unclear. Its unlikely to have occurred via formaldehyde dehydrogenase, since this enzyme is unable to reduce free formate [27] and the synthesis *in situ* of *S*-formylglutathione, which could be reduced, is unlikely. Furthermore neither of the glutathione derivatives of formaldehyde and formate, which are the true substrates for formaldehyde dehydrogenase, was detected in this experiment. It is possible that the reduction was catalysed by an endogenous aldehyde dehydrogenase; however, this did not occur in *Escherichia coli*, in which formaldehyde and methanol were not produced when the organism was similarly challenged with formate [28]. Another reasonable possibility would be formation of N^{10}-formyltetrahydrofolate, followed by its cyclization and reduction to N^5N^{10}-methylenetetrahydrofolate, which could then release formaldehyde. Alternatively, there could be a specific enzyme for direct formate reduction present in *Hansenula polymorpha*; however, this question was not further pursued in the present study.

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