Journal of Industrial Microbiology, 6 (1990) 29–41
Elsevier

SIM 00257

The fermentation of xylose: studies by carbon-13 nuclear magnetic resonance spectroscopy

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(Received 21 June 1989; revised 19 September 1989; accepted 10 October 1989)

Key words: Xylose; Ethanol; Fermentation; NMR; *Pachysolen tannophilus*; *Pichia stipitis*; *Candida shehatae*

SUMMARY

The fermentation of D-xylose by *Pachysolen tannophilus*, *Candida shehatae*, and *Pichia stipitis* has been investigated by ^{13}C-nuclear magnetic resonance spectroscopy of both whole cells and extracts. The spectra of whole cells metabolizing D-xylose with natural isotopic abundance had significant resonance signals corresponding only to xylitol, ethanol and xylose. The spectra of whole cells in the presence of [1-^{13}C]xylose or [2-^{13}C]xylose had resonance signals corresponding to the C-1 or C-2, respectively, of xylose, the C-1 or C-2, respectively, of xylitol, and the C-2 or C-1, respectively, of ethanol. Xylitol was metabolized only in the presence of an electron acceptor (acetone) and the only identifiable product was ethanol. The fact that the amount of ethanol was insufficient to account for the xylitol metabolized indicates that an additional fate of xylitol carbon must exist, probably carbon dioxide. The rapid metabolism of xylulose to ethanol, xylitol and arabinitol indicates that xylulose is a true intermediate and that xylitol dehydrogenase catalyzes the reduction (or oxidation) with different stereochemical specificity from that which interconverts xylitol and D-xylulose. The amino acid L-alanine was identified by the resonance position of the C-3 carbon and by enzymatic analysis of incubation mixtures containing yeast and [1-^{13}C]xylose or [1-^{13}C]glucose. The position of the label from both substrates and the identification of isotope also in C-1 of alanine indicates flux through the transketolase/transaldolase pathway in the metabolism. The identification of a resonance signal corresponding to the C-1 of ethanol in spectra of yeast in the presence of [1-^{13}C]xylose and fluoroacetate (but not arsenite) indicates the existence of equilibration of some precursor of ethanol (e.g. pyruvate) with a symmetric intermediate (e.g. fumarate or succinate) under these conditions.

INTRODUCTION

The transformation of hardwood or agricultural residues to ethanol is an example of the conversion of renewable resources to liquid fuel and chemical feedstock. Relatively mild treatment of these renewable resources releases xylose, which can constitute up to 40% of the dry weight. Therefore, the efficiency and productivity of the fermentation of xylose to ethanol is important to the overall efficiency of the transformation.

Although the fermentation of xylose is carried out by some natural bacteria [9,19], recombinant bacteria [7,16], and several species of yeast [15,18,27], the yeast fermentations are currently the closest in development to commercial reality. However, the yeast fermentation is associated with limitations of productivity and efficiency. Therefore, in an attempt to understand these limitations more thoroughly, we have investigated the fermentation of xylose to ethanol in three species of yeast, *Pachysolen tannophilus*, *Candida shehatae*, and *Pichia stipitis*, by ^{13}C-nuclear magnetic resonance (NMR) spectrometry.

The putative fermentation pathway is conveniently divided into three parts [24,25]: the conversion of xylose to xylulose-5-phosphate, the conversion of xylulose-5-phosphate to fructose-6-phosphate, and the conversion of fructose-6-phosphate to ethanol. The reactions of the first part are established by the demonstration of the enzymes xylose reductase (aldose reductase) [26,29] and xylitol dehydrogenase [3,5] in these yeasts, and the inducibility of the enzymes by xylose [2,11]. Furthermore, xylose isomerase has not been demonstrated in any of these yeast strains. The second part of the pathway is presum-

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ably catalyzed by transketolase and transaldolase [8]. However, the demonstration of pentulose-5-phosphate phosphoketolase in some of these same species [6,20] raises the possibility of direct cleavage of pentose phosphate to an acetate derivative and glyceraldehyde-3-phosphate. The overall theoretical efficiency of the fermentation would be somewhat different with these two pathways. The third part of the fermentation pathway is the same as part of the pathway for alcoholic fermentation of glucose by *Saccharomyces cerevisiae.*

The efficiency of the fermentation is frequently limited by the production of the by-product, xylitol [24,25], due to either excessive production of reduced pyridine nucleotide or deficient production of oxidized pyridine nucleotide. Therefore, the addition of electron acceptors, 2-propanone (acetone) or acetaldehyde, [1,13] reduces the accumulation of xylitol. The effectiveness of oxygen [12,17,30], at low concentration, may be at least partly a direct effect of its role as an electron acceptor, but oxygen also seems to have a less direct role in the induction of enzymes [11] as well as in the production of biomass.

Nuclear magnetic resonance spectrometry has been an effective technique for the study of carbon metabolism in *E. coli* and *Saccharomyces cerevisiae* [4,22,28]. The Pasteur effect was demonstrated in derepressed *S. cerevisiae* metabolizing glucose. The ^{31}P-NMR spectra of extracts of cells metabolizing glucose demonstrated a decreased ratio of nucleoside triphosphate to nucleoside diphosphate level in anaerobic cells. The spectra of extracts of anaerobic cells demonstrated glucose-6-phosphate, fructose bisphosphate, α-glycerol phosphate, and 3-phosphoglyceric acid. The ^{13}C-NMR spectra demonstrated dihydroxyacetone phosphate, α-glycerol phosphate, fructose-1,6-bisphosphate, glutamate, aspartate, and acetate.

Ligthelm et al. [14] reported that the ^{13}C-NMR spectra of *Pichia stipitis* demonstrated that the anaerobic rate-determining step was the conversion of xylitol to xylulose, whereas under aerobic conditions it was the uptake of xylose. They also reported no preference for the anomers of xylose by the yeast and that the ethanol was labeled only in the C-2 position.

We report here the results of investigations by ^{13}C-NMR of the carbon metabolism by *Pachysolen tannophilus*, *Candida shehatae*, and *Pichia stipitis.*

MATERIALS AND METHODS

Materials. The strain of *P. tannophilus* (NRRL-4-2460) was from the Northern Regional Research Center. The strain of *Candida shehatae* (ATCC 22984) was from The American Type Culture Collection, and the strain of *Pichia stipitis* (CSIR 567) was from The South African Council for Scientific and Industrial Research. *Saccharomyces cerevisiae* (strain 20B12) was a gift from Dr. Gayle Knapp, Department of Chemistry and Biochemistry, Utah State University, Logan UT. The [1-^{13}C]xylose and the [2-^{13}C]xylose, both 99% enrichment, were from Omicron Biochemicals. All other chemicals, biochemicals and media components were from scientific suppliers.

Cell growth. The medium for cell growth contained: 10 g/l potassium phosphate (monobasic), 3 g/l yeast extract, 5 g/l peptone, 7.5 g/l ammonium chloride, 0.244 g/l anhydrous magnesium sulfate and either 45 g/l xylose or 30 g/l glucose (as the carbon source). Before sterilization the pH was adjusted to 5.5 with a small amount of sodium hydroxide, if necessary. The strains were preserved at 5°C as colonies on agar slants of the medium with glucose as the carbon source and were transferred every month.

All liquid cultures (500 ml) were incubated in 2-liter Erlenmeyer flasks at 30°C with rotary shaking at 130 rpm and an amplitude of 3 cm. The cell concentration was measured as the turbidity, A_{650} with a path length of 1 cm. The stock culture was inoculated into liquid medium with glucose as the carbon source and with malt extract (6 g/l). The fully grown (stationary phase, 24–48 h) culture was then inoculated into liquid medium with xylose and glucose (each at half the above concentration) as the carbon source.

Unless otherwise indicated cells for an NMR experiment were grown in liquid medium (5–8% inoculum) with xylose as the carbon source in an Erlenmeyer flask 80% full. After 18 h, the typical A_{650} was 15–20 and profuse budding was present. The cells were harvested by centrifugation (2500 × *g* for 20 min) and washed with deionized water. They were used immediately for experimental purposes. Ordinarily the strain was subcultured in this liquid medium (xylose) as many times as convenient.

NMR sample preparation. NMR spectra were determined on three types of samples: (1) whole cells in the presence of carbon source with normal isotopic abundance, (2) whole cells in the presence of carbon source with one carbon atom enriched with ^{13}C, and (3) extracts of cells that had been incubated with a carbon source enriched with ^{13}C.

For the first type of sample cells were suspended in sufficient water to give a calculated A_{650} of 250. The carbon source was added (300 mM final concentration) in

a sufficiently small volume that the cell concentration was essentially unchanged, and the resulting suspension was transferred to an NMR tube. Generally the NMR spectra with an unenriched carbon source required more scans than spectra with an enriched carbon source; but in the spectrum from the former type of sample, every carbon atom of the carbon source and carbon products could be detected by a resonance.

The second type of sample also consisted of whole cells, but the carbon source had one carbon atom enriched with ^{13}C. The spectrum from this type of sample is more sensitive than the first for the detection of carbon compounds derived from the labeled position, but does not detect the unenriched positions. The sensitivity of these experiments is much greater than that of the previous ones, but the fact that the sample is changing during data collection imposes sensitivity limits having to do with the signal-to-noise ratio of the NMR spectrometer.

The third type of sample consisted of extracts of cells that had been incubated with [^{13}C]xylose, or other isotopically enriched carbon source. For the preparation of extracts, washed cells were suspended in a solution of isotopically enriched carbon source to give a final solution containing carbon source (300 mM) and cells (A_{650} = 250). The solution was incubated at 30°C until the end of predetermined time periods, when a 10 ml sample was cooled quickly on ice and centrifuged (5000 × *g* for 10 min). The supernatant liquid was removed and the cells were suspended in 50 ml of perchloric acid (1 M). After the resulting suspension had set on ice for 10 min, it was centrifuged (approximately 2000 × *g* for 10 min). The supernatant liquid was removed and titrated to pH 7 with potassium carbonate (2.5 M). The precipitate was removed by centrifugation (approximately 2000 × *g* for 10 min) and the supernatant liquid was lyophilized. The resulting powder was suspended in water (1.0 ml), titrated to pH 5 to 7 with sodium hydroxide, centrifuged (approximately 2000 × *g* for 10 min), and placed into an NMR tube.

Since the extract sample is not changing during the collection of the NMR data, the number of scans that can be collected is not limited as in the case of the whole-cell experiments. Therefore, the sensitivity in the extract experiments is limited only by the amount of unenriched components in the extract.

NMR spectra. Samples were contained in standard sample tubes (10 mm o.d.), and chemical shift values were reported relative to dioxane (chemical shift 66.5 ppm) contained in a coaxial capillary tube. The NMR spectra were obtained on a Brucker, WH-400 spectrometer using a 75° observe pulse with a relaxation delay of 4 s between pulses. Low power proton broad band decoupling was applied during the relaxation delay, and switched to high power during data acquisition to remove C-H coupling.

Spectra of extracts were obtained at ambient probe temperature (ca. 25°C) and are the result of either 64 or 128 scans. The spectra of cell suspensions were obtained in either of two ways. (1) Samples of cells incubated at 30°C were taken at specific times. After they were cooled to 10°C, the spectra were obtained at the lower temperature. (2) Cells contained in an NMR sample tube were left in the spectrometer at 30°C and spectra were obtained at specific times. In both ways the spectra of unenriched samples is the result of 512 or 1024 scans, whereas the spectra of enriched samples is the result of 64 or 128 scans.

Standards. Spectra were determined for a number of compounds that are putative intermediates or products. These spectra (Table 1) served as standards in the analysis of data from the spectra of cells and extracts. The peak assignments (Table 2) were made by comparison of the standard spectra with previous peak assignments in the literature.

Data analysis. Each resonance in a spectrum was characterized by its chemical shift position (ppm) and its

TABLE 1

Chemical compounds whose NMR spectra were in the database for matching with unknown spectra

1-glycerol phosphate	glucose
2-fluoroacetate	glutamate
2-oxoglutarate	glyceraldehyde
2-propanol	glyceraldehyde-3-phosphate
2-propanone	glycerol
3-phosphoglycerate	isocitrate
acetaldehyde	lactate
acetaldehyde bisulfite	malate
acetate	phosphoenolpyruvate
alanine	pyruvate
arabinitol	succinate
aspartate	xylitol
citrate	xylose
dihydroxyacetone	xylulose
dihydroxyacetone phosphate	xylulose phosphate
ethanol	xylulose-5-phosphate
fructose-1,6-diphosphate	

TABLE 2

Peak assignments

Chemical shift	Compound	Position
PPM		
175	alanine	1
96.6	xylose	1b
92.2	xylose	1a
75.8	xylose	3b
74	xylose	2b
72.8	xylose	3a
71.9	xylitol	2,4
71.5	xylose	2a
70.8	xylitol	3
70.8	arabinitol	4
70.5	arabinitol	3
69.4	xylose	4a
69.2	xylose	4b
66.5	dioxane std	
65.1	xylose	5b
64.3	2-propanol	2
63.1	arabinitol	1
62.6	xylitol	1,5
60.8	xylose	5a
57.4	ethanol	1
30.2	2-propanone	1,3
23.2	acetate	2
23.6	2-propanol	1,3
16.7	ethanol	2
16.2	alanine	3

intensity, and these two pieces of data were stored in a computer file for each spectrum.

A computer program was written in which data could be entered into files for both standard spectra and unknown spectra. In order to compare intensities of the same peak between different spectra the intensity of each peak was normalized to an intensity of 10.0 for the external standard in that spectrum (peak intensity times the peak intensity of the external standard divided by 10.0).

The computer program could compare the spectra, unknown and standard, in two modes. In the first mode the resonances of a spectrum of a selected standard compound could be subtracted from those of the unknown spectrum, if all of the resonances matched within preset limits, usually 0.1 ppm. The subtracted resonances were in proportion to their intensities in the standard, with the resonance from the unknown spectrum that had the lowest intensity ratio to its match in the standard spectrum, completely subtracted. The fraction of the total carbon subtracted and the resulting difference spectrum were then reported. The difference spectrum could then be operated upon either again in this mode or in the alternative mode described below. The former mode was particularly useful for data from experiments involving substrates with natural isotopic abundance and for subtracting background resonances from spectra from experiments with isotopically enriched substrates.

In the second mode individual resonances in all or a selected list of standard spectra were matched to those of the unknown spectrum within preset limits, usually 0.1 ppm. The standard compounds and the position of the resonances in the spectra of those compounds that matched the unknown spectrum were reported. In addition the amount of carbon in the unknown spectrum that was accounted for by each standard compound that had one or more matching resonances was also reported. This latter mode of operation was particularly useful with unknown spectra from experiments with isotopically enriched substrates.

Chemical synthesis and analysis. [1-^{13}C]Xylitol was synthesized from [1-^{13}C]xylose (50 mg) by incubation of xylose at ambient temperature with sodium borohydride (0.13 g) in 2 ml of water. After 3 h, the reaction was stopped by the addition of glacial acetic acid until the foaming stopped. The sample was lyophilized. The lyophilized sample was then dissolved in methanol and dried on a rotary evaporator several times. Finally it was dissolved in water and lyophilized. The NMR spectrum revealed no resonance other than that of [1-^{13}C]xylitol.

Alanine was assayed enzymatically according to Bergmeyer [31] by incubation of the sample with alanine dehydrogenase and NAD (1.0 mM) in potassium carbonate buffer (pH 10.5). The increase in the absorbance at 340 nm (A_{340}) was measured spectrophotometrically. It reflected the increase in NADH concentration (millimolar extinction coefficient 6.22), which is stoichiometric with the original concentration of alanine. The validity of the assay was confirmed with standard solutions of L-alanine.

RESULTS

NMR spectra

A representative spectrum of *P. tannophilus* in the presence of xylose of normal isotopic abundance (Fig. 1) shows absorbance by xylose, xylitol, and ethanol. That these three compounds account for greater than 99% of

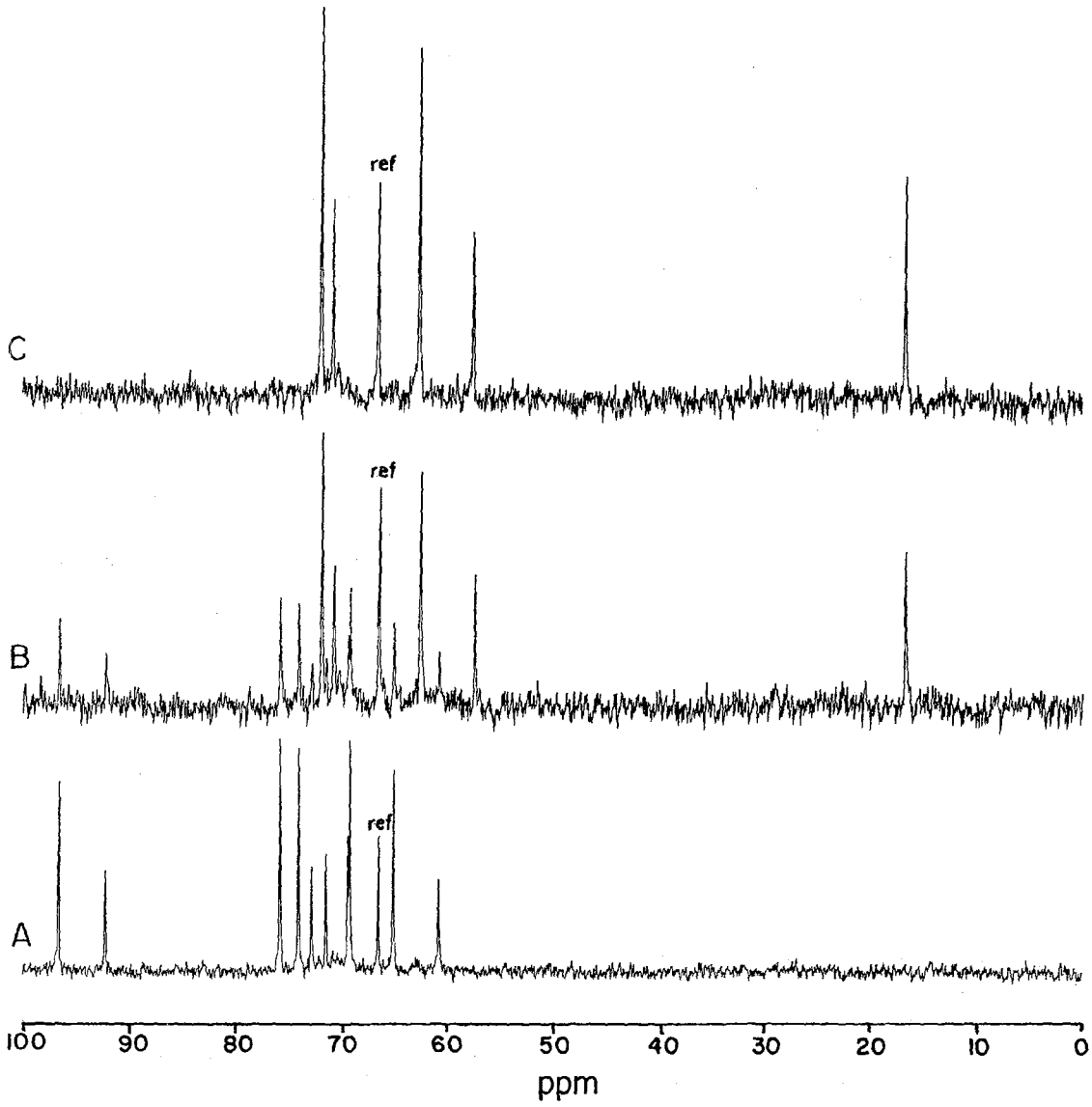

Fig. 1. NMR spectra of *P. tannophilus* in the presence of D-xylose. (A) Zero time, (B) 2 h, (C) 4 h. At least 99% of the total carbon was identified. The peak assignments are indicated in Table 2.

the total carbon in each spectrum indicates that there are no other significant detectable products or intermediates under these conditions. The only other metabolic carbon product that would not be detected under these conditions is carbon dioxide. Spectra for the other yeast strains, *Pichia stipitis* and *Candida shehatae*, were very similar (not shown). Although the estimation of the amounts of each compound in these spectra was attended by a standard error of measurement of about 15%, fermentation balance estimation could be performed with the data.

The time course of product appearance in the three strains (Fig. 2) indicated that total carbon seems to be lost during metabolism, especially by *P. tannophilus*. This loss would be consistent with the production of carbon dioxide in the production of ethanol. However, the fact that the loss was more severe with *P. tannophilus*, with slightly less

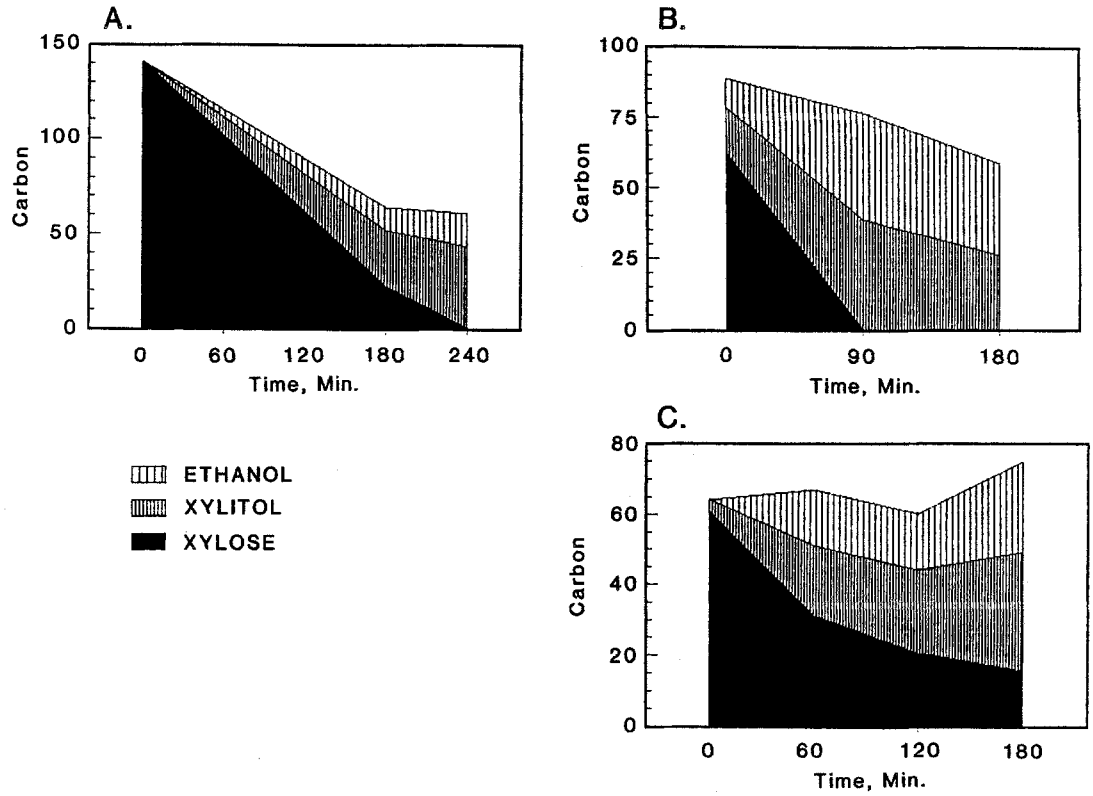

Fig. 2. Time course of xylose metabolism by (A) *P. tannophilus*, (B) *C. shehatae*, and (C) *P. stipitis*. The total carbon in each compound was calculated from the area under each resonance peak assigned as indicated in Table 2.

ethanol production, indicates that some additional process may also be responsible for the carbon loss. In all experiments, the more rapid metabolism of xylose by *C. shehatae* as compared to the other yeasts was evident.

Intermediates

Xylitol. The substantial accumulation of xylitol in the fermentation of xylose raises the question of whether it is to be regarded as an intermediate, a product, or both. Although the metabolic evidence indicates that it is an intermediate [26,29], the question remains whether it is also a product. The NMR spectra of both *P. tannophilus* and *C. shehatae* in the presence of xylitol with both normal isotope abundance and with enrichment for ^{13}C showed that xylitol is essentially inert, although both intracellular and extracellular xylitol could be demonstrated in the spectra of the cell extracts and the cell-free medium respectively (not shown). In the presence of both xylitol and 2-propanone, the latter as an electron acceptor [1,13], there was a rapid and quantitative formation of 2-propanol, and a slow, incomplete formation of ethanol (Fig. 3). The amount of 2-propanol formed from 2-propanone was about the same as the amount of xylitol disappearing, but considerably greater than the amount of ethanol appearing. These results were confirmed in whole-cell experiments with [1-^{13}C]xylitol. The time courses (not shown) indicated that xylitol, in the presence of 2-propanone, was metabolized less rapidly and completely than xylose. Furthermore, no other carbon compounds could be identified in any of the experiments. The accumulated evidence indicated that xylitol is a product as well as an intermediate. In the presence of an electron acceptor it is metabolized to carbon dioxide and ethanol.

The time courses of spectra of both *C. shehatae* and *P. tannophilus* each in the presence of xylose and 2-propanone showed little or no formation of xylitol (not shown). Although the yield of ethanol was greater with added 2-propanone, the increase was insufficient to account for the disappearance of xylitol.

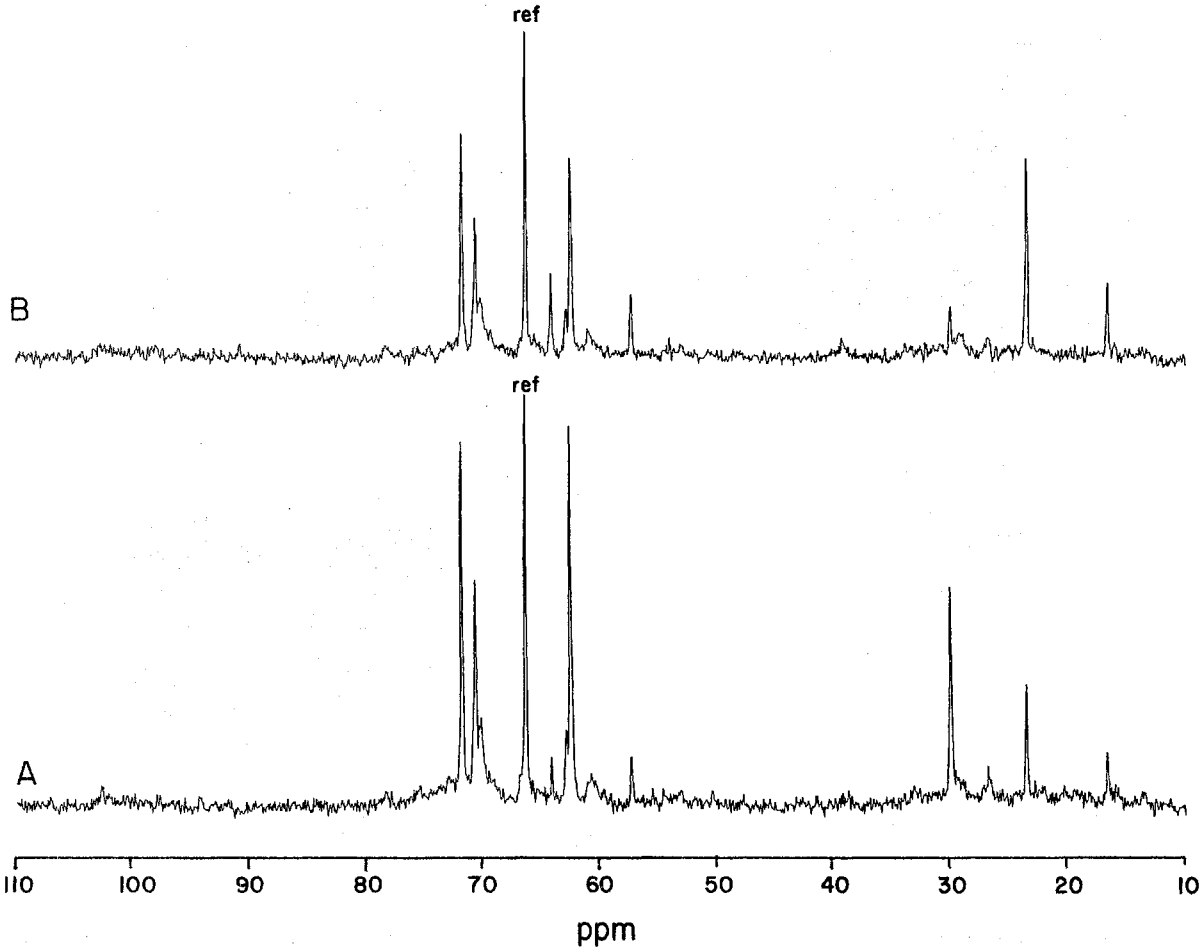

Fig. 3. NMR spectrum of *P. tannophilus* in the presence of xylitol and 2-propanone (acetone). (A) Zero time, (B) 2 h. The peak assignments in Table 2 were used to identify 98% of the total carbon.

D-xylulose. The fact that xylulose in water solution forms three species, two anomeric hemiketals and the ketone, causes each carbon to produce three absorbances in the NMR spectrum. Therefore, it is difficult to detect. The NMR spectrum (Fig. 4) of *P. tannophilus* in the presence of D-xylulose (natural abundance ^{13}C) showed xylitol, arabinitol, and ethanol. Although one of the arabinitol resonances overlaps one of the xylitol resonances, 4 of the 5 resonances of arabinitol were identifiable. Xylulose was not identified, even at zero time, and no other carbon resonances were seen. Ethanol and xylitol were present in about equal concentration, and the arabinitol was present in about half the concentration of xylitol. The time course indicated that the metabolism of D-xylulose is at least as rapid as that of D-xylose (time course not shown). Although xylulose certainly qualifies as a true intermediate, the production of arabinitol from xylulose but not from xylose is a source of some interest. Arabinitol (the D isomer) could arise from D-xylulose by reduction with the opposite prochirality at the C-2 position from that for the production of xylitol or from the oxidation of xylitol by xylitol dehydrogenase with either no or the opposite prochiral specificity and to produce L-xylulose, which could be reduced subsequently to arabinitol (Fig. 9). It would be interesting to learn whether there is a single enzyme responsible for the production of both arabinitol and xylitol. The fact that no arabinitol is identified in the metabolism of D-xylose can be explained

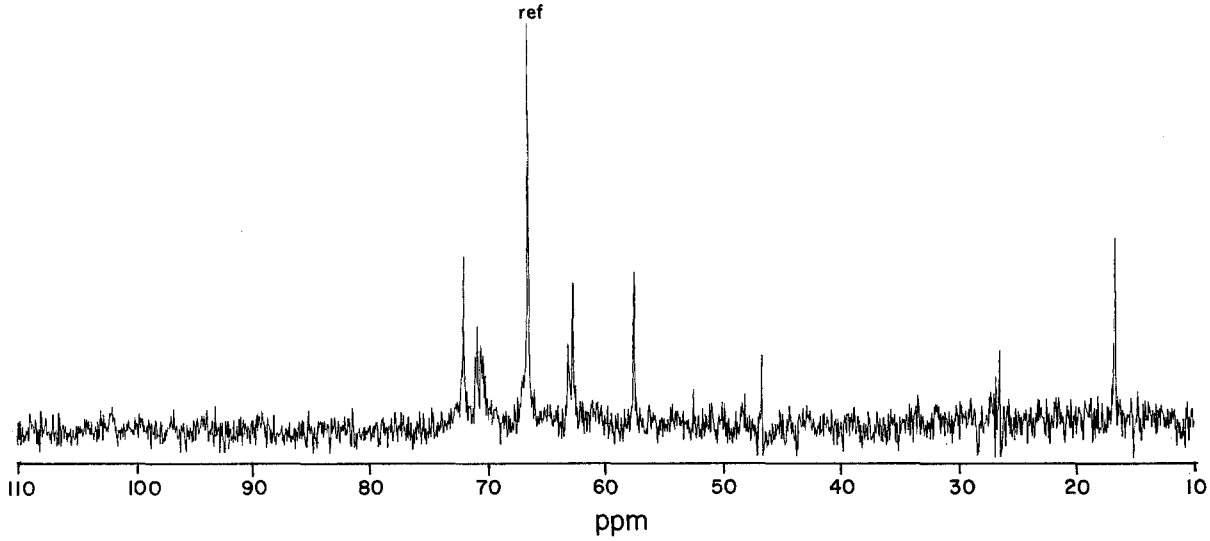

Fig. 4. NMR spectrum of *P. tannophilus* in the presence of D-xylulose at 90 min. The peak assignments indicated in Table 2 identified 84% of the total carbon.

by the hypothesis that the flux in the reduction of D-xylulose is less during the fermentation of xylose than during the fermentation of D-xylulose itself.

L-Alanine. The one unexpected compound that appeared in the spectra of cells and extracts of cells metabolizing D-xylose was the amino acid alanine (Fig. 5). The identification was confirmed by enzymatic analysis with alanine dehydrogenase. It was identified in experiments with both *P. tannophilus* and *C. shehatae*, but was seen only in experiments with xylose enriched with ^{13}C (C-1 position). The presence of a nitrogen source (ammonium sulfate) and the presence of metabolic inhibitors (following section), during the NMR experiments, resulted in enhanced amounts of alanine. It was also identified in spectra of *C. shehatae* and *S. cerevisiae* in the presence of [1-^{13}C]glucose. The fact that the alanine was labeled in the same position from both the xylose and the glucose confirms the flux through the transketolase/transaldolase pathway, which predicts the equivalence of the positions in these two compounds (Fig. 9). Although this pathway would also predict labeling of the C-1 of alanine in addition, the resonance of the carboxyl group depends upon the pH and is much more difficult to detect. However, at lower temperature (25°C) and in the presence of some inhibitors, the resonance of the carboxyl carbon (Fig. 6) was identified (175 ppm).

Other intermediates. Although most of the spectra contained no resonances for previously unidentified

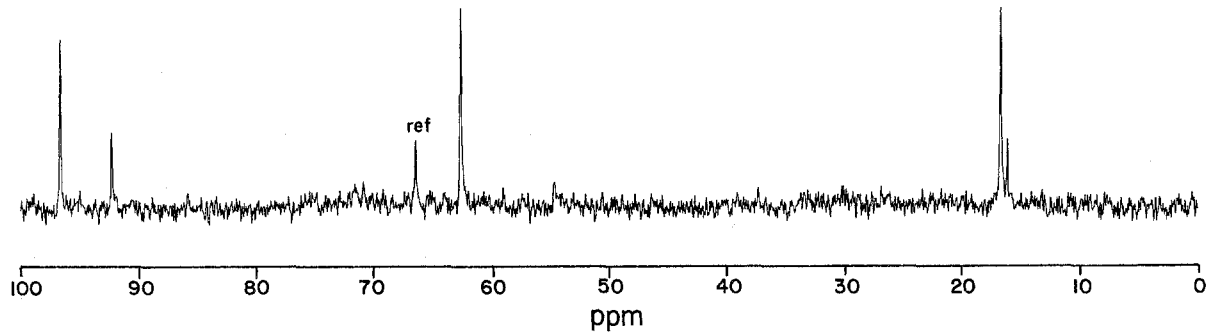

Fig. 5. NMR spectrum of *C. shehatae* in the presence of [1-^{13}C]xylose at 45 min. The peak assignments in Table 2 were used to identify 100% of the total carbon.

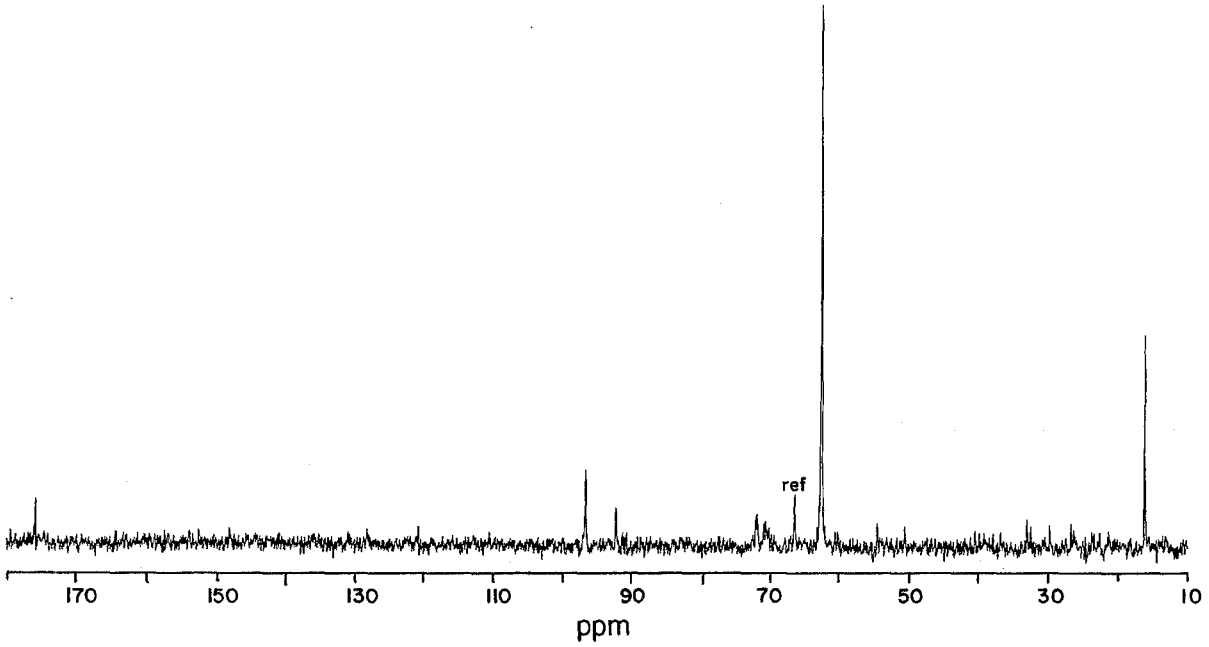

Fig. 6. NMR spectrum of *C. shehatae* incubated at 25°C in the presence of [1-^{13}C]xylose for 150 min. The peak assignments in Table 2 were used to identify 100% of the total carbon.

compounds, some of them contained resonances that corresponded to some of the carbon atoms of compounds, e.g. glutamate, pyruvate, 3-glycerol phosphate, in the set of standards. However, in every case these were eliminated from further consideration because of two or more of the following three criteria: (1) they were not repeated in subsequent experiments; (2) the resonance was a minor one of that compound in the absence of an expected major one for the same atom (or compound), e.g., occasionally a resonance corresponding to one of the minor resonances of one of the atoms of xylulose was present in the absence of a major resonance for the same carbon atom; (3) the position of the label could be accounted for only by a rearrangement of the label in addition to the accepted, direct pathway to the intermediate compound, in the absence of resonances expected from the direct pathway to the same compound. Attempts to promote the appearance of additional intermediates by variations in temperature, pH, and the presence of inhibitor (see below) were generally unsuccessful. In the most sensitive experiments, spectra of extracts, the resonances from unenriched carbon compounds appeared at about 1% of the intensity of the resonances of the enriched carbon atoms. These

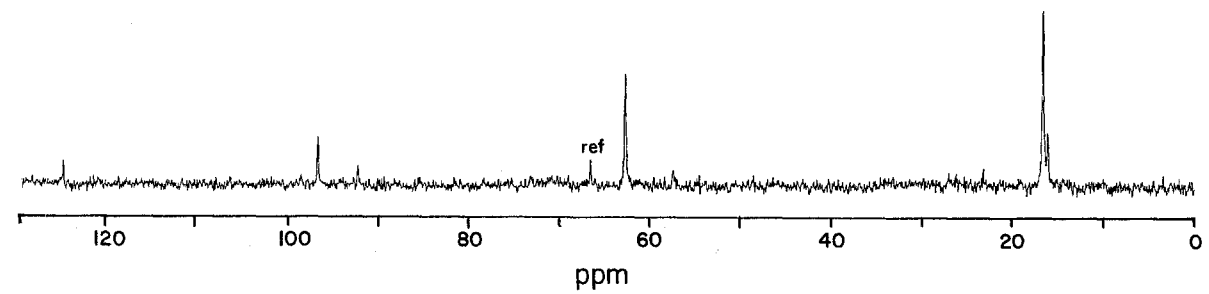

Fig. 7. NMR spectrum of *C. shehatae* in the presence of [1-^{13}C]xylose and fluoroacetate at 2 h. The peak assignments in Table 2 were used to identify 95% of the total carbon.

resonances from unenriched carbon atoms imposed a limit on the sensitivity of the spectra, and the 1% value was accepted as the limit of detection of intermediates (nonvolatile in these experiments).

Inhibitors

In attempts to increase the probability of the detection of intermediates, spectra were determined with the organisms metabolizing in the presence of various inhibitors.

Bisulfite. Bisulfite, meta-bisulfite, forms a complex with acetaldehyde, and its presence in anaerobic fermentations of glucose by *S. cerevisiae* leads to the formation of hydroxyl compounds in addition to ethanol, mostly glycerol, as a mechanism of electron disposal. The NMR spectra (not shown) of *C. shehatae* in the presence of xylose (normal abundance ^{13}C) and bisulfite (1.0 mM) showed resonances corresponding to xylitol, alanine, and the bisulfite complex of acetaldehyde, in order of decreasing quantity. The persistence of xylose and the fact that the quantities of xylitol and alanine are increased over that seen in control experiments indicated that the bisulfite imposes the same limitation in this system that it does in the *Saccharomyces* system fermenting glucose. In addition, the identification of the bisulfite complex of acetaldehyde in the spectrum confirms the existence of acetaldehyde as an intermediate. However, the fact that only 80% of the total carbon in the spectrum was identified indicates that there are some additional unexpected intermediates or products.

Fluoroacetate. Metabolically, fluoroacetate ordinarily forms the very toxic fluorocitrate, which inhibits the enzyme aconitase [10], and, therefore, inhibits the tricarboxylic acid cycle. The NMR spectra of *C. shehatae* incubated in the presence of [1-^{13}C]xylose and fluoroacetate

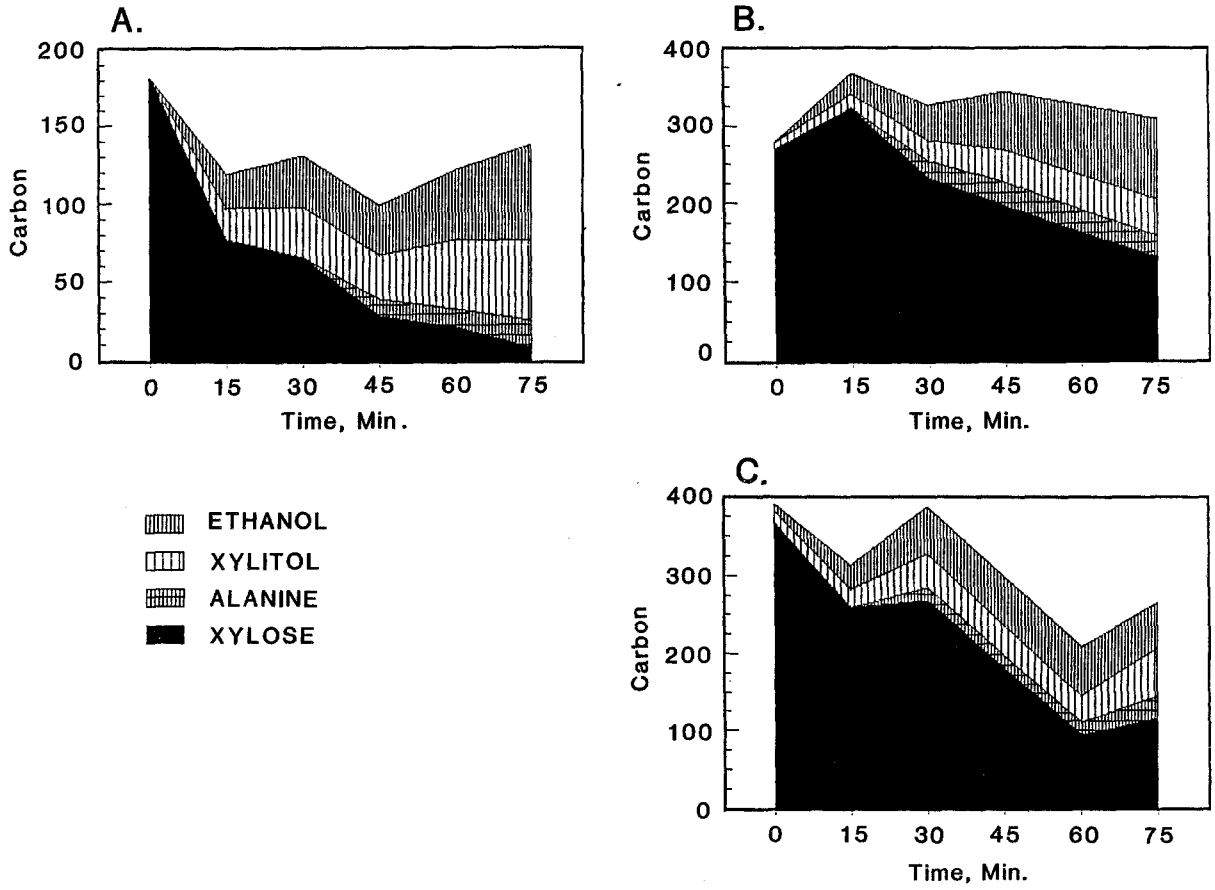

Fig. 8. Time course of the metabolism of [1-^{13}C]xylose by *C. shehatae* in the presence of (A) no inhibitor, (B) arsenite, and (C) fluoroacetate.

(1 mM) had a previously unidentified resonance corresponding to the methyl group of acetate (Fig. 7), whose accumulation would be expected in the absence of the forward operation of the tricarboxylic acid cycle (Fig. 9). In addition, the labeling and relative quantities of the previously identified compounds are somewhat different. The C-1 position of ethanol (57.5 ppm) contains isotope in addition to the C-2 position. The isotope in the C-2 position is predicted by a number of pathways, but that in C-1 indicates equilibration with a symmetric compound

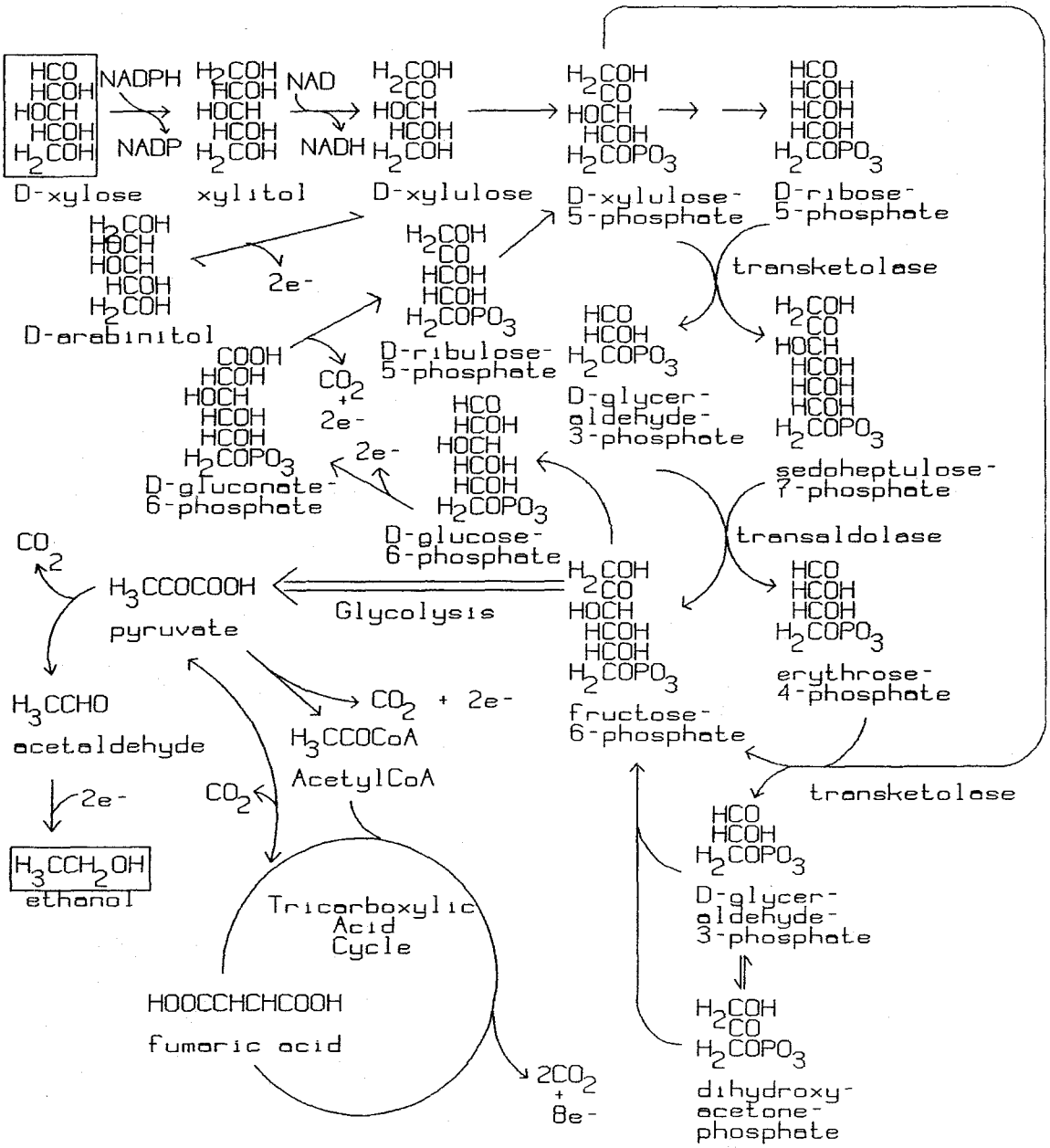

Fig. 9. Principal metabolic pathways involved in the fermentation of xylose. The starting material, xylose, and the desired product, ethanol, are each enclosed in a box. Where the identities of electron acceptors are not known explicitly, they are indicated as "e-." the amount of electrons and carbon dioxide produced in the tricarboxylic-acid cycle is that amount from one mol of acetyl-CoA.

such as fumarate or succinate, via the anaplerotic fixation of carbon dioxide by pyruvate (Fig. 9).

In the presence of fluoroacetate (Fig. 8C) there was less xylitol relative to ethanol than in its absence.

Arsenite. The principal enzymatic effect of arsenite is to form a complex with reduced lipoic acid and, therefore, inhibit its reoxidation [21,23]. Metabolically, in yeast it will inhibit the production of acetyl coenzyme A from pyruvate but not the decarboxylation of pyruvate nor the production of acetaldehyde. Although the NMR spectrum of *C. shehatae* in the presence of [1-^{13}C]xylose and sodium arsenite (1 mM) showed only resonances corresponding to the expected carbon atoms of xylose, xylitol, ethanol and alanine; the relative quantities of ethanol and alanine were increased in proportion to xylitol (Fig. 8). Attempts to confirm this observation with *P. tannophilus* were unsuccessful.

DISCUSSION

Xylose fermentation. The efficiency of the fermentation of xylose to ethanol is ordinarily limited by the formation of xylitol [24,25]. Although the formation of xylitol can be attenuated or eliminated by the careful control of the partial pressure of dissolved oxygen or the addition of an electron sink, e.g. 2-propanone, [1,13] the commensurate increase in the efficiency of ethanol production is not realized.

Since the electron balance in the conversion of xylose to ethanol is zero, there must be an adventitious source of electrons for xylitol production. Therefore, there must be a previously unidentified oxidation of xylose, or one of its products, stoichiometric with the xylitol produced. Since the NMR experiments reported here failed to show an oxidation product, it must be carbon dioxide. Two possible pathways are apparent: (1) the oxidation of acetate in the tricarboxylic acid cycle and (2) an oxidative cycle in which pentose phosphate is converted to hexose phosphate by a combination of transketolase and transaldolase, and the hexose phosphate is converted back to pentose phosphate by oxidative decarboxylation. That the tricarboxylic acid cycle is a significant source of electrons in *C. shehatae* is indicated by the results with arsenite and fluoroacetate (Fig. 8). However, the inability to extend this observation to other organisms brings this hypothesis into question. The question of whether the hexose phosphate cycle is also a significant source of electrons can be answered, if either some crucial intermediates (hexose or triose) could be identified with the NMR spectra or specific inhibitors for the participating reactions could be identified.

NMR. The use of ^{13}C-NMR is certainly a convenient and elegant method for the detection of metabolic products and intermediates. However, it is associated with definite limits in sensitivity. The sensitivity associated with the detection of ^{13}C-intermediates is limited by the natural abundance of the isotope (ca. 1%) in the most prevalent carbon compounds in the incubation. The fact that a labeled intermediate present in 1% of the concentration of the most prevalent unenriched compound will give the same signal magnitude for the resonance imposes a limit of about 1% on the necessary concentration of intermediates.

The method is better for the detection of products than intermediates. The method is particularly convenient for carbon-balance studies in fermentation and cell culture, because a single spectrum yields sufficient information to quantitate the significant substrates and products with the exception of carbon dioxide. In addition, most substrates and products are present in the medium at a convenient concentration (several grams per liter). Although the precision of quantitation was a limitation in the present study, this could be improved by modifications in the protocol for spectrometer operation.

ACKNOWLEDGEMENTS

This project was partially supported by funds from The Tennessee Valley Authority, Biomass Research Program. We acknowledge the use and assistance of the NMR Facility of the Comprehensive Cancer Center, which is supported by USPHS Grant CA 13148.

REFERENCES

1 Alexander, N. 1986. Acetone stimulation of ethanol production from D-xylose by *Pachysolen tannophilus*. Appl. Microbiol. Biotechnol. 25: 203–207.
2 Bolen, P.L. and R.W. Detroy. 1985. Induction of NADPH-linked D-xylose reductase and NAD-linked xylitol dehydrogenase activities in *Pachysolen tannophilus* by D-xylose, L-arabinose, or D-galactose. Biotechnol. Bioeng. 27: 302–307.
3 Chiang, C. and S.G. Knight. 1960. Metabolism of D-xylose by molds. Nature (Lond.) 188: 79–81.
4 den Hollander, J.A., K. Ugurbil, T.R. Brown, M. Bednar, C. Redfield and R.G. Schulman. 1986. Studies of anaerobic and aerobic glycolysis in *Saccharomyces cerevisiae*. Biochemistry 25: 203–211.
5 Ditzelmueller, G., C.P. Kubicek, W. Woeher and M. Roehr.

1984. Xylitol dehydrogenase from *Pachysolen tannophilus*. FEMS Microbiol. Lett. 25: 195–198.

6 Evans, C.T. and C. Ratledge. 1984. Induction of xylulose-5-phosphate phosphoketolase in a variety of yeasts grown on D-xylose: a key to efficient xylose metabolism. Arch. Microbiol. 139: 48–52.

7 Ingram, L.O., T. Conway, D.P. Clark, G.W. Sewell and J.F. Preston. 1987. Genetic engineering of ethanol production in *Escherichia coli*. Appl. Environ. Microbiol. 53: 2420–2425.

8 Lachke, A.H. and T.W. Jeffries. 1986. Levels of enzymes of the pentose phosphate pathway in *Pachysolen tannophilus* Y-2460 and selected mutants. Enz. Microbiol. Technol. 8: 353–359.

9 Lacis, L.S. and H.G. Lawfore. 1988. Ethanol production from xylose by *Thermanaerobacter ethanolicus* in batch and continuous culture. Arch. Microbiol. 150: 48–55.

10 Lalspeich, W., A. Peters and T. Wilson. 1952. The inhibition of aconitase by 'inhibitor fractions' isolated from tissues poisoned with fluoroacetate. Biochem. J. 51: 20–25.

11 Ligthelm, M.E., B.A. Prior and J.C. du Preez. 1988. The induction of D-xylose-catabolizing enzymes in *Pachysolen tannophilus* and the relationship to anaerobic D-xylose fermentation. Biotechnol. Lett. 10: 207–212.

12 Ligthelm, M.E., B.A. Prior and J.C. du Preez. 1988. The oxygen requirements for yeasts for the fermentation of D-xylose and D-glucose to ethanol. Appl. Microbiol. Biotechnol. 28: 63–68.

13 Ligthelm, M., B.A. Prior and J.C. du Preez. 1989. Effect of hydrogen acceptors on D-xylose fermentation by anaerobic culture of immobilized *Pachysolen tannophilus* cells. Biotechnol. Bioeng. 32: 839–844.

14 Ligthelm, M.E., B.A. Prior, J.C. du Preez and V. Brandt. 1988. An investigation of D-(1-^{13}C)xylose metabolism in *Pichia stipitis* under aerobic and anaerobic conditions. Appl. Microbiol. Biotechnol. 28: 293–296.

15 Maleszka, R. and H. Schneider. 1982. Fermentation of D-xylose, xyliltol, and D-xylulose by yeasts. Can. J. Microbiol. 28: 360–363.

16 Neale, A.D., R.K. Scopes and J.M. Kelly. 1988. Alcohol production from glucose and xylose using *Escherichia coli*. Appl. Microbiol. Biotechnol. 29: 162–167.

17 Neirinck, L.G., R. Maleszka and H. Schneider. 1984. The requirement of oxygen for incorporation of carbon from D-xylose and D-glucose by *Pachysolen tannophilus*. Arch. Biochem. Biophys. 228: 13–21.

18 Nigam, J.N., R.S. Ireland, A. Margaritis and M.A. Lachance. 1985. Isolation and screening of yeasts that ferment D-xylose directly to ethanol. Appl. Environ. Microbiol. 50: 1486–1489.

19 Patel, G.B., C.R. Mackenzie and B.J. Agnew. 1986. Fermentation of xylose and hemicellulose hydrolysates by an ethanol-adapted culture of *Bacteroides polypragmatus*. Arch. Microbiol. 146: 68–73.

20 Ratledge, C. and J.E. Holdsworth. 1985. Properties of a pentulose-5-phosphate phosphoketolase from yeasts grown on xylose. Appl. Microbiol. Biotechnol. 22: 217–221.

21 Sanadi, D.R., M. Langley and F. White. 1958. Oxidative decarboxylation of alpha-keto acids. Biochim. Biophys. Acta 29: 218–219.

22 Schulman, R.G., T.R. Brown, K. Ugerbil, S. Ogawa, S.M. Cohen and J.A. den Hollander. 1979. Cellular applications of ^{31}P and ^{13}C nuclear magnetic resonance. Science 205: 160–166.

23 Searls, R.L. and D.R. Sanadi. 1960. Alpha-ketoglutaric dehydrogenase, isolation and some properties of a flavoprotein component. Biochem. Biophys. Res. Commun. 2: 189–190.

24 Skoog, K. and B. Hahn-Haegerdal. 1988. Xylose fermentation. Enzyme Microb. Technol. 10: 66–80.

25 Slininger, P.J., P.L. Bolen and C.P. Kurtzman. 1987. *Pachysolen tannophilus*: properties and process considerations for ethanol production from D-xylose. Enzyme Microb. Technol. 9: 5–15.

26 Smiley, K.L. and P.L. Bolen. 1982. Demonstration of D-xylose reductase and xylitol dehydrogenase in *Pachysolen tannophilus*. Biotechnol. Lett. 4: 607–610.

27 Toivola, A., D. Yarrow, E. Van den Bosch, J.P. Van Dijken and W.A. Scheffers. 1984. Alcoholic fermentation of D-xylose by yeasts. Appl. Environ. Microbiol. 47: 1221–1223.

28 Ugerbil, K., H. Rottenberg, P. Glynn and R.G. Schulman. 1982. Studies of bioenergetics in wild-type and adenosintriphosphatase(1-) *Escherichia coli* cells. Biochemistry 21: 1068–1075.

29 Verduyn, C., R. Van Kleef, J. Frank, H. Schrender and J.P. Van Dijken. 1985. Properties of the NAD(P)H-dependent xylose reductase from the xylose-fermenting yeast *Pichia stipitis*. Biochem. J. 226: 669–677.

30 Verduyn, C., J. Frank Jzu, J.P. van Dijken and W.A. Scheffers. 1985. Multiple forms of xylose reductase in *Pachysolen tannophilus*. FEMS Microbiol. Lett. 30: 313–317.

31 Williamson, D.H. 1985. Determination with alanine dehydrogenase. In: Methods of Enzymatic Analysis. (Bergmeyer, H.U., Ed.), Vol. 8, pp. 341–344, VCH, Weinheim.