Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 5847–5851, October 1983
Biochemistry

^{13}C NMR studies of acetate metabolism during sporulation of *Saccharomyces cerevisiae*

(tricarboxylic acid cycle/glyoxylate cycle/glutamate/gluconeogenesis/fatty acid synthesis)

J. RICHARD DICKINSON*†, IAN W. DAWES*, ALAN S. F. BOYD‡, AND ROBERT L. BAXTER‡

*Department of Microbiology, University of Edinburgh, Edinburgh EH9 3JC, United Kingdom; and ‡Department of Chemistry, University of Edinburgh, Edinburgh EH9 3JJ, United Kingdom

Communicated by C. Auerbach, June 6, 1983

ABSTRACT **The sporulation of *Saccharomyces cerevisiae* in the presence of [2-^{13}C]acetate was studied by ^{13}C NMR spectroscopy. The fate of ^{13}C label was analyzed *in vivo* and in cell extracts. During the first 4 hr of sporulation the major metabolite produced from [2-^{13}C]acetate utilization was glutamate. From the labeling pattern observed it is concluded that both the tricarboxylic acid cycle and the glyoxylate cycle are operating. After about 4 hr trehalose is made. Comparison of the doublet/singlet ratios for C-1, 1^1 and C-6, 6^1 of trehalose shows a steady drop in the ratio of C-1, C-2-coupled species over trehalose labeled only at C-1 in the C-1, 2 segment of the molecule. The negative correlation of this ratio with that for the C-5, 6 segment indicates a cycling of glucose through the hexose monophosphate shunt. Subsequently fatty acid biosynthesis commences. Large amounts of saturated fatty acid were made. There were conspicuous differences observed in the metabolism of [2-^{13}C]acetate between sporulating and vegetatively growing cells.**

Diploid strains of *Saccharomyces cerevisiae* can undergo a developmental switch from vegetative growth to the alternative processes of meiosis and ascospore formation. This switch is dependent on the presence of two alleles of the mating-type locus *MAT* a and *MAT* α, it can only occur during a particular phase of the cell division cycle, and it is subject to regulation by the nutritional conditions operating (1). The molecular mechanisms responsible for this developmental switch are not known, and the basic changes in metabolism causing or accompanying sporulation are poorly understood and the subject of conflicting reports.

The present study was done to determine more precisely the metabolic changes that accompany sporulation. For this NMR spectroscopy is ideal because it enables an extensive and detailed analysis of the basic metabolic processes that occur. Moreover, yeast sporulation is very well suited to NMR analysis: ^{13}C-labeled acetate compounds are readily available, and sporulation occurs solely in acetate (2). From NMR studies it is possible to identify labeled intermediates, to quantitate some individual enzyme transformations, and to gain a broad insight into those pathways that are operating.

EXPERIMENTAL

S. cerevisiae strain S41, which has the genotype

$$\frac{MAT\text{a}\ HO\ arg\ 4\text{-}1\ cyh\ 1}{MAT\alpha\ HO\ arg\ 4\text{-}1\ cyh\ 1},$$

was grown at 30°C with shaking in a complex medium containing per liter: 10 g of yeast extract, 20 g of peptone, 10 g of potassium acetate (or [2-^{13}C]sodium acetate), 1 g of glucose, 0.2 g of adenine, and 0.2 g of uracil. Sporulation medium contained per liter: 10 g of sodium acetate, 0.2 g of adenine, 0.2 g of uracil in 100 mM potassium chloride, and 0.15 g of benzylpenicillin. Sporulation was induced by the method of Fast (2).

At intervals during sporulation, 1–1.25 × 10^9 cells were harvested by filtration and fixed in ice-cold 5% (wt/vol) perchloric acid. After 1 hr at 0°C the material was centrifuged for 5 min at 5,000 × *g*. Perchloric acid-soluble material was adjusted to pH 7.0 by adding ice-cold 5 M KOH. Potassium perchlorate was removed by centrifugation and the supernatant was freeze-dried. Perchloric acid-insoluble material was extracted twice with ethanol/diethyl ether, 3:1 (vol/vol), at 60°C (3) to isolate lipids. The lipid fraction was freeze-dried. Sporulation medium was retained when cells were harvested, freeze-dried, and used to estimate [2-^{13}C]acetate remaining in the medium.

^{13}C NMR spectra were obtained by using a Bruker WM 300 WB spectrometer (Bruker Spectrospin, Coventry, U.K.) operating at 75 MHz. For *in vivo* studies freshly harvested cells were resuspended in sporulation medium lacking acetate but containing 10% ^2H_2O and transferred to a 20-mm NMR tube (15 ml, 8.04 × 10^8 cells per ml). Immediately prior to the experiment 200 μl of 5 M [2-^{13}C]sodium acetate (90 atom %; Prochem, London) was added and the cell suspension was aerated at about 20 ml/min throughout accumulation (16 K time domain data points; acquisition time, 0.27 s; flip angle, 30°). High-power broad band decoupling was used during acquisition and low power decoupling during delays. Temperature was maintained at 28°C ± 2°C throughout. Spectra of aqueous extracts were recorded in 2% ^2H_2O solutions at pH 7.0 containing 2% dioxane as internal standard (16 K time domain data points; acquisition time, 0.54 s; flip angle, 36°; delay time, 2.5 s). Spectra of lipid extracts were run in C^2HCl_3 solution. Peak intensities were calibrated by comparison with those determined for known concentrations of the *bona fide* compounds relative to dioxane as internal standard at 67.4 ppm.

Amino acid pools were determined on perchloric acid extracts according to the method of Spackman (4).

RESULTS

***In Vivo* NMR Spectra.** Time elapsed ^{13}C NMR spectra of vegetatively grown cells resuspended in sporulation medium containing [2-^{13}C]acetate are shown in Fig. 1. Spectra taken over a period of 1 hr show the gradual diminution of the C-2 acetate resonance with the appearance of broad signals corresponding to the C-2, C-3, and C-4 of glutamate. Spectra of

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† Present address: Department of Microbiology, University College, Newport Road, Cardiff CF2 1TA, United Kingdom.

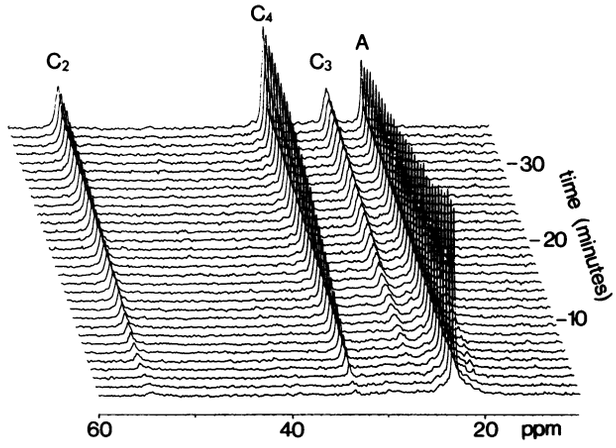

FIG. 1. Time course of [2-^{13}C]acetate metabolism by *S. cerevisiae* under aerobic conditions. Each spectrum is the result of 250 pulses. A, acetate C-2; C_2, glutamate C-2; C_3, glutamate C-3; C_4, glutamate C-4.

cells resuspended in 20% ^2H_2O showed that the acetate signal was derived from extracellular material; no glutamate was excreted into the medium. The appearance of ^{13}C label in the C-2, C-3, and C-4 of glutamate seen in Fig. 1 indicates that significant randomization of label occurs through tricarboxylic acid cycle intermediates. Signal broadness from enriched glutamate may be due to an increased rotational correlation time for the metabolite in the intracellular environment or to field inhomogeneity within the sample. Thus, it is not feasible to obtain quantitative data on the degree of randomization of label nor of the amount of metabolites produced from such spectra. However, quantification of the flux of ^{13}C into metabolites can be obtained from ^{13}C NMR spectra recorded in defined homogeneous solutions by calibrating the ^{13}C resonances of a known

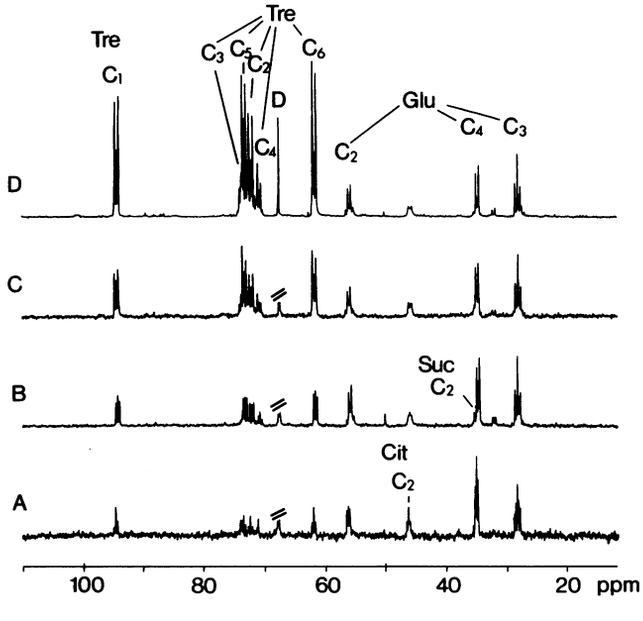

FIG. 2. Seventy-five-megahertz ^{13}C NMR spectra of cell-free perchloric acid lysates of *S. cerevisiae* after incubation with [2-^{13}C]acetate. Spectra A–D refer to lysates made after 1, 2, 4, and 12 hr of sporulation. Each spectrum is the result of 3,000 pulses. Spectrum A is shown with a 4× vertical scale and spectra B and C with a 2× scale with reference to dioxane (peak D in spectrum D) as internal standard. Tre C_1–C_6 refer to the carbon atoms of α, α^1-trehalose; Glu C_2–C_4 refer to the carbons of glutamate; and Suc C_2 and Cit C_2 refer to the C-2 carbons of succinate and citrate, respectively.

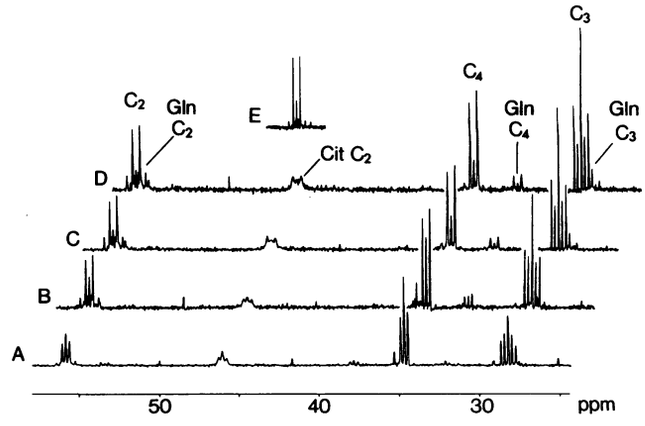

FIG. 3. Resolution-enhanced 75-MHz ^{13}C spectra of perchloric acid lysates; 16 K spectra were obtained as described in the legend to Fig. 2 and were subjected to Gaussian multiplication (GM = 0.5) with a line broadening factor of −2 Hz prior to zero filling to 32 K and Fourier transformation. Spectra A–D refer to lysates made after 1, 2, 4, and 8 hr of sporulation. Spectrum E (*Inset*) shows the multiplet corresponding to citrate C-2 obtained for the 8-hr sample after addition of 1 mM EDTA. C_2–C_4 refer to glutamate C-2, C-3, and C-4; Gln C_2–C_4 refer to glutamine C-2, C-3, and C-4; Cit C_2 refers to the C-2 of citrate.

metabolite by comparison with authentic compounds of known concentration under identical conditions.

Acetate Consumption During Sporulation. Acetate was continually taken up from the medium throughout sporulation; after 24 hr 70% of the acetate had been utilized. There was a concomitant increase of the ^{13}C-enriched bicarbonate signal at 167 ppm, paralleling acetate consumption and resulting in an increase of the pH of the medium (5).

Intermediary Metabolite Formation. ^{13}C NMR spectra of perchloric acid lysates from cells extracted after periods of 1, 2, 4, and 12 hr of sporulation are shown in Fig. 2. Significant is the appearance of multiplet signals corresponding to glutamate, glutamine, citrate, and trehalose. Concurrent with the changes in proportions of these metabolites are distinct changes in the ^{13}C multiplet structures, reflecting changes in the proportions of ^{13}C isotopomers with time. The changes in multiplicity of the glutamate C-2, C-3, and C-4 resonances are more clearly visible in the resolution-enhanced spectra shown in Fig. 3.

Analysis of ^{13}C multiplets for a multiply labeled metabolite

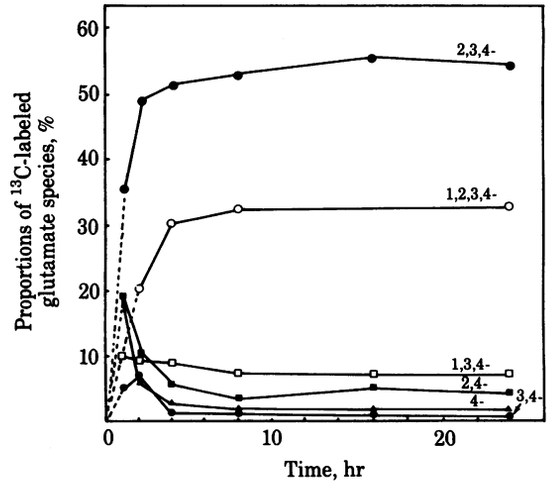

FIG. 4. Proportions of ^{13}C-labeled glutamate species in cell lysates as a function of time.

provides a means of quantifying the proportions of each labeled species in a complex mixture of isotopomers. Such information can in turn be used to estimate the relative contributions of the metabolic pathways involved in the formation of the compound (6–8). The correlation of ^{13}C–^{13}C multiplet/^{13}C–^{12}C center line intensity ratios with ^{13}C isotopomer populations is independent of the differences in T_1 and nuclear Overhauser effect for different carbons in a molecule. While a potential source of error arises from the fact that the T_1 values and nuclear Overhauser effect for ^{13}C adjacent to ^{12}C (central line) and ^{13}C adjacent to ^{13}C (multiplet) for specific carbon atoms may be different (9), these effects are significant only for nonprotonated carbons and are minimized when spectra are acquired under gated decoupling conditions allowing an adequate delay between pulses.

The kinetics of appearance of the various ^{13}C-labeled glutamate species is shown in Fig. 4. Analysis of the glutamate ^{13}C isotopomers is facilitated by consideration of the pathways involved. Condensation of [2-^{13}C]acetyl-CoA with oxaloacetate would give rise via the first turn of the tricarboxylic acid cycle to 2-oxoglutarate and hence glutamate labeled solely at C-4. Cycling of 2-oxo[^{14}-C]glutarate through the tricarboxylic acid cycle would give rise to [2,4-^{13}C]- and [3,4-^{13}C]glutamate and further cycling of the doubly enriched species would give rise progressively to 2,4-, 1,3-, and 2,3,4- and hence to 2,4-, 1,3,-4-, 2,3,4-, and 1,2,3,4-^{13}C-labeled glutamate (Fig. 5). No C-5-labeled glutamate species were observed, which precludes

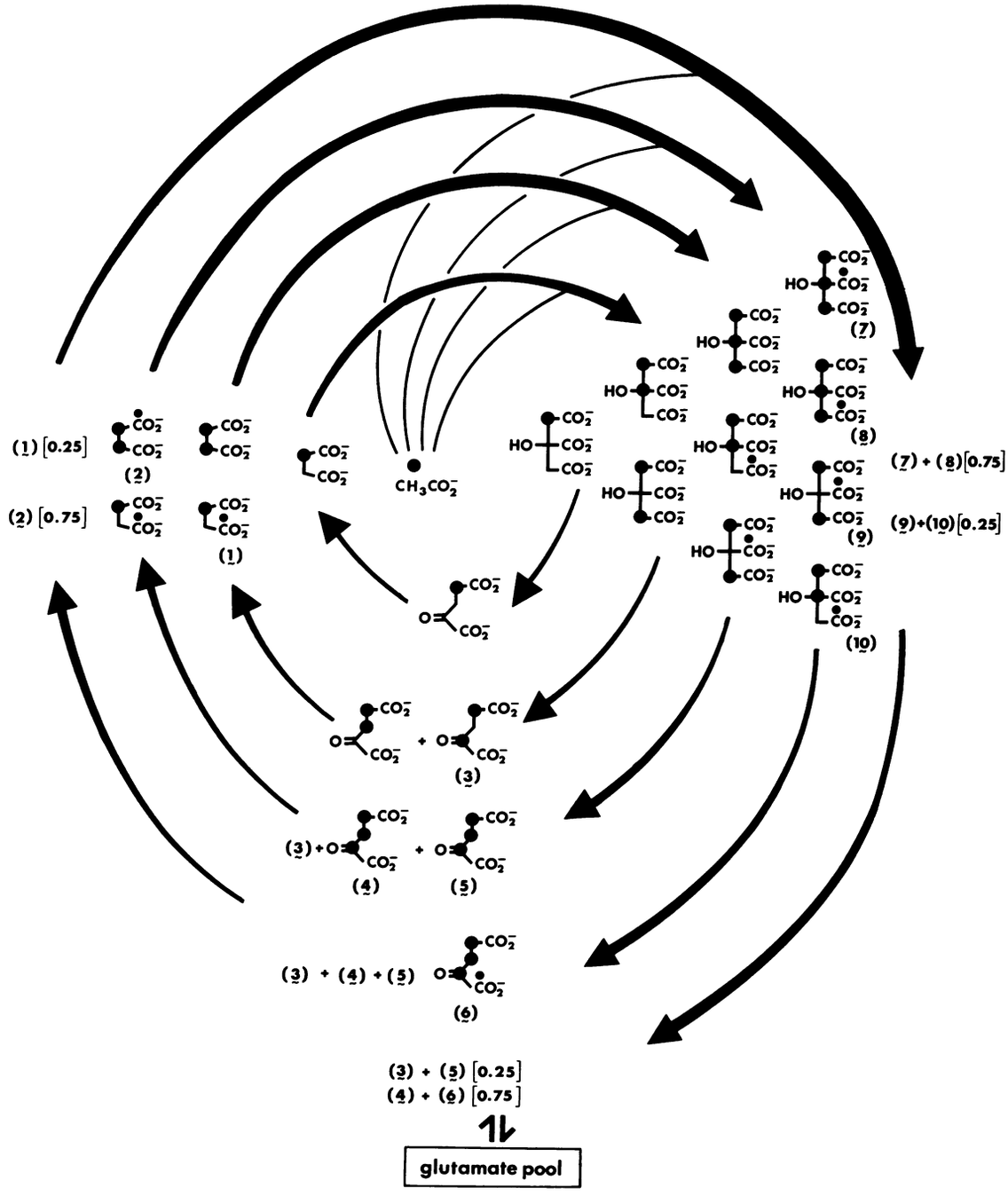

FIG. 5. ^{13}C isotopomer distribution for incorporation of [2-^{13}C]acetate on sequential turns of the tricarboxylic acid cycle. For clarity, no dilution via anaplerotic mechanisms is assumed. Similarly independent operation of the glyoxylate cycle sequentially gives rise to [2-^{13}C]citrate, [2,4-$^{13}C_2$]- and [2,3-$^{13}C_2$]citrate, and [2,3,4-$^{13}C_3$]citrate (8) exclusively.

"scrambling" of label from the C-2 to the C-1 of acetate, which could occur via the tricarboxylic acid cycle, pyruvate carboxylase, and pyruvate dehydrogenase (10). Net synthesis of glutamate solely from the tricarboxylic acid cycle would quickly deplete 2-oxoglutarate and other tricarboxylic acid cycle intermediates and generation of 2-oxoglutarate anaplerotically via the glyoxylate cycle alone would give rise to label located at the 4,3,4, and 2,3,4 positions on successive turns of the cycle. The accumulation of significant amounts of [1,2,3,4-^{13}C]glutamate indicates the synchronous operation of both cycles during the glutamate biosynthesis phase.

Amino acid pools were also determined during sporulation by direct analysis of perchloric acid-soluble material. For most of the amino acids the concentration fell or remained low up to 12 hr, with small increases from 12 to 24 hr. However, glutamate was present in far greater amounts and increased 3-fold in the first hr, reaching a maximum by 8 hr, and then declined. Incorporation of ^{13}C label from acetate into glutamate continues after net synthesis of glutamate has ceased, demonstrating a continuing equilibrium between intermediates. It would appear that a major function of the tricarboxylic acid and glyoxylate cycle enzymes during early sporulation is to provide glutamate. An increase of glutamate during sporulation has been reported (11), but this was believed to be derived from proteolysis (12).

Trehalose Synthesis. Trehalose production began by 4 hr but the major increase occurred between 4 and 8 hr, an approximately 5-fold increase occurring over this period. The labeling pattern of the trehalose produced reveals significant equilibration between the dicarboxylic acid pools of the tricarboxylic acid and glyoxylate cycles. Phospho*enol*pyruvate derived solely from the glyoxylate cycle without tricarboxylic acid intervention should be labeled exclusively at C-3 and C-2 on the first turn of the cycle and after successive cycles will be labeled at C-3 and C-2 (10). Phospho*enol*[2,3-$^{13}C_2$]pyruvate would in turn give rise via gluconeogenesis to [1,2,5,6-$^{13}C_4$]glucose monophosphate together with [5,6-$^{13}C_2$]glucose monophosphate derived from condensation of [2,3-$^{13}C_2$]glyceraldehyde 3-phosphate with endogenous dihydroxy[^{12}C]acetone phosphate and [1,2-$^{13}C_2$]glucose monophosphate from dihydroxy[1,2-$^{13}C_2$]-acetone phosphate and unenriched glyceraldehyde 3-phosphate. On the other hand, phospho*enol*pyruvate derived from an oxaloacetate pool fully randomized by tricarboxylic acid cycling would contain a mixture of 1,2,3- and 2,3-^{13}C-labeled species. Examination of the C-5 multiplet of trehalose (Fig. 2) shows a steady increase of both quartet and doublet intensities with time, reflecting a significant degree of equilibration of the C_4 dicarboxylic acid pools. The proportional increase of both 5,6- and 4,5,6-^{13}C-labeled glucose moieties in trehalose contrasts with a relatively small proportion of C-6-labeled species produced from phospho*enol*[3-^{13}C]pyruvate at the start of gluconeogenesis (Fig. 6).

Comparison of the doublet/singlet ratios for C-1, 1′ and C-6, 6′ of trehalose (Fig. 7) shows a steady drop in the ratio of C-1, C-2-coupled species over trehalose labeled only at C-1 in the C-1,2 segment of the molecule. The negative correlation of this ratio with that for the C-5,6 segment is indicative of a constant cycling of synthesized glucose through the hexose monophosphate shunt. It is interesting to note that net glutamate production ceases when trehalose begins to be labeled and therefore there is a switch in the fate of acetate from glutamate production to gluconeogenesis. This identifies an important metabolic "landmark" in sporulation. It is noteworthy that a considerable proportion (up to 30%) of the labeled trehalose is generated via the operation of the pentose phosphate pathway. This will give rise to the generation of NADPH, which can be used in the reductive biosynthesis of fatty acids.

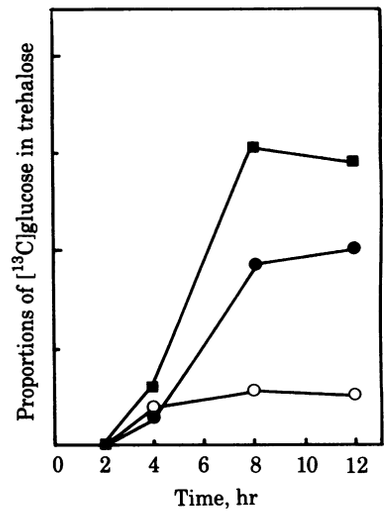

FIG. 6. Proportions of ^{13}C-labeled glucose 4,5,6-^{13}C isotopomers in trehalose produced as a function of time. Relative proportions are based on analysis of trehalose C-6, C-5, and C-4 multiplets as described in the text. ■, 5,6-^{13}C; ●, 4,5,6-^{13}C; and ○, 6-^{13}C.

Lipid Synthesis. Commencing at about 4 hr saturated fatty acids labeled in the expected even positions began to appear, as indicated by the presence of equal enrichment at the C-16 and C-18 positions; no label was detected in odd-numbered C atoms. Very little unsaturated fatty acid synthesis was detected during sporulation. Because most of the fatty acids of vegetative cells are unsaturated (13) it is clear that there are differences in the regulation of fatty acid synthesis between sporulating and vegetative cells. A previous report (13) had suggested that the proportion of unsaturated fatty acids increases during sporulation.

The Metabolic Fate of Acetate in Vegetatively Growing Cells. To compare sporulation and vegetative cell metabolism, the pattern of [2-^{13}C]acetate labeling of cellular constituents was determined in cells grown in presporulation medium containing [2-^{13}C]acetate as the major energy source. Only 1.1% of cellular glutamate was ^{13}C-labeled; the remainder (observed due to natural abundance) derives from the growth medium. Vegetative cells also contained slightly less than half the amount of citrate compared with sporulating cells. The trehalose con-

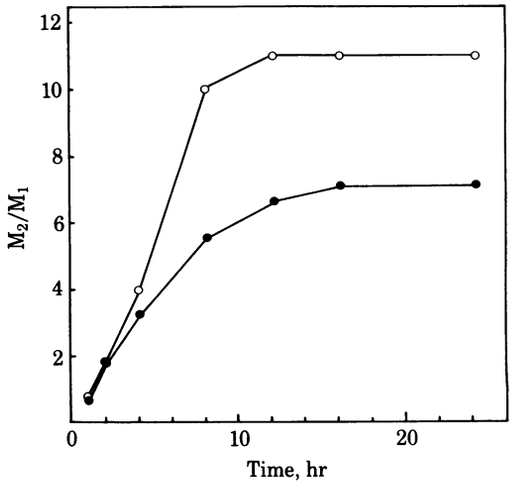

FIG. 7. Doublet/singlet (M_2/M_1) ratios for C-6, 6^1 (○) and C-1, 1^1 (●) resonances of α, α^1-trehalose during the course of sporulation.

tent of vegetative cells was only 15% of that found in the latter stages of sporulation, and virtually no label from [2-^{13}C]acetate was incorporated into trehalose under vegetative conditions. This is another major difference between cells growing vegetatively on acetate media and sporulating cells: there is no significant gluconeogenesis from acetate in vegetative cells to make the storage carbohydrate trehalose. The levels of ^{13}C incorporation into fatty acids by vegetative cells were also extremely low. It appears that vegetative cells growing on acetate in a rich medium use acetate mainly for respiration, whereas sporulating cells use acetate for production of intermediates for biosynthesis.

DISCUSSION

This work has provided a comprehensive insight into several aspects of the metabolism of sporulating cells. Several shifts in metabolism occur: the first is a very rapid change in the operation of the tricarboxylic acid and glyoxylate cycles, such that the intracellular glutamate concentration increases. Subsequently, beginning just before 4 hr into sporulation, when premeiotic DNA synthesis occurs (14), acetate begins to be converted to trehalose via gluconeogenesis. At about 4 hr saturated fatty acid synthesis begins and continues throughout sporulation, without any significant formation of unsaturated fatty acids from acetate. These results confirm previous studies on the level of glutamate (11) and the synthesis of trehalose and fatty acids (1) and extend them to show the contribution of acetate to their formation and their timing relative to each other. They also highlight the role of the glyoxylate cycle in cells sporulating on acetate, as indicated by the studies of Miyake *et al.* (15) and the demonstration that a mutation affecting isocitrate lyase in *S. cerevisiae* leads to asporogeny (16). The contribution of the hexose monophosphate pathway to trehalose biosynthesis was unexpected.

A major discovery has been that the tricarboxylic acid cycle early in sporulation furnishes a high intracellular pool of glutamate, and it appears that apart from trehalose synthesis one of the main uses of acetate is the production of glutamate. Acetate is not used for this in vegetative cells. From the pattern of labeling of glutamate it is clear that a high proportion of the 2-oxoglutarate produced in the first part of the tricarboxylic acid cycle is converted directly to glutamate. With the anaplerotic role of the glyoxylate cycle indicated it is apparent that the 2-oxoglutarate dehydrogenase and succinyl thiokinase activities of the tricarboxylic acid cycle may be reduced during sporulation. Either or both of these activities may be regulated during sporulation to effect the metabolic fluxes observed.

The importance of high intracellular glutamate levels to the sporulating yeast cell is not clear. After transfer of cells to sporulation medium the concentration of all amino acids decreases, except for proline and glutamate (11, 17). Because glutamate can act as a nitrogen source for *S. cerevisiae* and is the first intermediate in the assimilation of NH_4^+, it is clear that the switching of cells from vegetative growth to sporulation is not a direct consequence of the lack of available nitrogen.

These ^{13}C NMR studies can now be extended to investigate other aspects of sporulation control—for example, glucose repression. Metabolism in a variety of mutants that are affected in sporulation, including those altered in the initiation (18, 19), or the later stages, of sporulation (1), can now be studied. Moreover, other cellular processes such as nutritional control over cell proliferation are amenable to this type of analysis.

We are grateful to Dr. R. Ambler for access to the amino acid analyzer and to Margaret Daniel for technical assistance. This research was supported by grants from the Science and Engineering Research Council.

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