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MICROBIOLOGICAL PREPARATION OF D-[3,4-$^{13}C_2$]GLUCOSE

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SUMMARY

A simple and effective synthesis of D-[3,4-$^{13}C_2$]Glucose (60 atom % $^{13}C_2$) *via* ^{13}C enriched α,α' trehalose (**1**) produced from sodium [1-^{13}C]acetate by sporulating yeast is described.

Key words: Carbon-13, α,α' trehalose, *Saccharomyces cerevisiae*.

INTRODUCTION

The chemical synthesis of ^{13}C labelled sugars has received a great deal of attention in view of their value in investigations of metabolic pathways. However, while the intrinsic complexity of carbohydrate structures makes these challenging and interesting targets for development of chiral methods of total synthesis the time-consuming and labor intensive nature of the multistep syntheses involved in constructing specifically labelled monosaccharides, other than molecules labelled at chain terminii and C-2 positions[1], remains a strong disincentive to their construction and exploitation as probes for biological processes. Enzymatic syntheses present an attractive alternative to chemical syntheses but the flexibility of these are limited by the availability of useful and available enzymes, their substrate specificity and kinetics and the synthetic accessibility of appropriately labelled substrates[2]. The use of whole organisms to prepare labelled sugars is under normal conditions, even more limited, being typically restricted to the preparation of univerally or randomly isotopically enriched materials. In certain cases however production of specifically labelled sugars by fermentative methods is possible and here we describe a simple and effective preparation of D-glucose labelled at both the 3 and 4 positions with ^{13}C derived from [1-^{13}C]acetate by a method which exploits the specificity of acetate metabolism during ascospore development in specific strains of the yeast *Saccharomyces cerevisiae*. This material has proven of particular value as a probe for the examination of Entner-Doudoroff metabolism in bacteria[3].

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RESULTS AND DISCUSSION

Diploid strains of *S. cerevisiae* undergo a developmental switch from vegetative growth to the alternative process of meiosis and ascospore formation. This switch depends on the presence of two alleles of the mating-type locus MAT a and MAT α and is subject to regulation by the nutritional status of the organism[4]. Although the underlying molecular mechanisms are poorly understood it is now known that transition from a vegetative growth medium containing sugars to a sporulation medium in which acetate is the sole carbon source results in two sequential metabolic events[5]. The first phase, occuring immediately after transfer to an acetate rich sporulation medium, is characterised by the rapid catabolism of acetate with concurrent accumulation of L-glutamate. This initial process slows dramatically after the first two hours and is followed by a second, gluconeogenic, phase, which lasts for approximately six hours, during which time there is a steady synthesis of α,α' trehalose (**1**) which is accumulated as a reserve carbohydrate in the developing spores. The metabolism of acetate during this phase is shown schematically in the Figure. If the glyoxalate (a) and gluconeogenesis (b) pathways are considered in isolation we might expect ^{13}C from [2-^{13}C]acetate to be incorporated regiospecifically at the 1, 2, 5 and 6 positions of D-glucose-6-phosphate. The position is complicated however by randomisation of ^{13}C by the Kreb's cycle [pathway (c)] which generates a population of citrate isotopomers with a variety of ^{13}C enrichment patterns and the exchange of the C-1 and C-2 positions of D-glucose-6-phosphate *via* the pentose phosphate pathway (d). The net result is that ^{13}C from [2-^{13}C]acetate affords a complex mixture of isotopomeric trehalose species[5]. In contrast the predicted labelling pattern of D-glucose from [1-^{13}C]acetate is unaffected by Kreb's and pentose phosphate cycling of intermediates and should give rise to α,α' trehalose (**1**) labelled regiospecifically at the 3, 3' 4 and 4' positions. This effect can be usefully employed to effect a synthesis of D-glucose labelled with ^{13}C at the C-3 and C-4 positions.

Vegetatively grown cells of *S. cerevisiae*, strain S41, were harvested by centrifugation, washed and resuspended in sporulation medium in which the sole carbon source was sodium [1-^{13}C]acetate. After eight hours the cells were harvested, lysed with perchloric acid and the neutralised, delipidated perchloric acid extract subjected to ion exchange chromatography. Trehalose was isolated by crystallisation from the neutral fraction and characterised by ^{1}H and ^{13}C nmr spectroscopy and by mass spectrometry. In the proton spectrum, integration of the intense H-3,3' and H-4,4' ^{13}C satellite signals (centred at 3.76 and 3.36 ppm respectively) indicated an enrichment level of *ca* 70 atom % ^{13}C at both positions. The {^{1}H} ^{13}C nmr showed two doublet resonances ($^{1}J_{3,4}$ 40.4Hz) offset by 0.03 ppm from singlet signals at 69.14 and 72.11 ppm corresponding to singly enriched C-4, 4' and C-3, 3' species. Integration of the doublet and singlet signals showed a ratio of doubly enriched $^{13}C_2$

to singly enriched $^{13}C_1$ monomer units of 6:1, indicating that each glucose unit of the trehalose produced was 60% [3,4-$^{13}C_2$], 5% [3-^{13}C], 5% [4-^{13}C] and 30% [3,4-$^{12}C_2$]. This was confirmed by mass spectroscopy (FAB, +ve ion, glycerol) which showed a major ion at $m/_z$ 443 ($^{13}C_4{}^{12}C_8H_{22}O_{11}$+H)$^+$ and ions at 442, 441, 440 and 439 ($^{12}C_{12}H_{22}O_{11}$+H)$^+$ in a ratio of 58:8:3:28. Acid hydrolysis of the isolated trehalose afforded enriched D-glucose, which was chromatographically identical to authentic unenriched material. Crystallisation of a sample from water afforded the α-D-anomer. Mass spectroscopy of the crystalline enriched material was consistent with the analysis of the isolated trehalose approximating to 60% D-[3,4-$^{13}C_2$]glucose, 10% D-[3-^{13}C] and [4-^{13}C]glucose and 30% unenriched material. The isolated yield of enriched D-glucose was 85mg/gm of sodium [1-^{13}C]acetate fed to the culture.

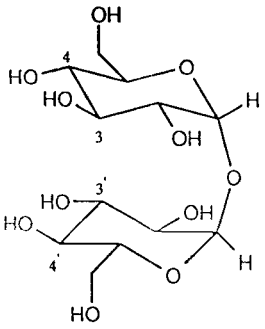
EXPERIMENTAL

Mass spectra were recorded using a Kratos MS50 TC instrument and nmr spectra using a Bruker WM360 spectrometer. *S. cerevisiae* strain S41 was originally obtained from H. O. Halvorson, Brandeis University, Waltham, Mass.

Growth of the organism: *S. cerevisiae* strain S41 was grown with shaking (200rpm) on a New Brunswick orbital shaker at 30° in 250cm^3 Erlenmyer flasks each containing 100cm^3 medium. Vegetative growth was sustained in a complex medium containing 10g yeast extract, 20g of peptone, 10g potassium acetate, 1g of glucose, 0.2g of adenine and 0.2g of uracil per litre. After 12 h growth the cells were removed by centrifugation (2000 X g, 20min) , washed with water and resuspended in a similar volume of sporulation medium containing 1g sodium [1-^{13}C]acetate (98 atom % 13C), 20mg of adenine, 20mg of uracil, 0.75g potassium chloride and 15mg of benzylpenicillin per 100cm^3.

Isolation of trehalose: After 8 h growth in sporulation medium the cells were removed by filtration, washed with water and lysed with ice-cold 5% (wt/vol) perchloric acid. After 1 h at 0° the material was centrifuged (5000 X g, 5min) and the soluble fraction adjusted to pH 7 with ice cold 5M KOH. The precipitated potassium perchlorate was removed by centrifugation (5000 X g, 10min) and the supernatent was freeze dried. The freeze dried material was washed with ether-methanol (1:1, 10cm^3) to remove lipids, dissolved in water (10cm^3) and passed through a column of AG50X8 resin (200mesh, 10cm^3, H^+ form) eluted with 20cm^3 water. The eluate was then applied to a column of AG1X4 resin (200mesh, 10cm^3, OH^- form) and eluted with 20cm^3 water. The total aqueous eluate was concentrated under vaccuum (1mm Hg, 35°) on a rotary evaporator and the residue was crystallised from aqueous alcohol affording trehalose as its dihydrate ; m 95-96°, (95mg). Spectroscopic data are given in the text.

Hydrolysis of trehalose: Enriched trehalose (70mg) was dissolved in aqueous H_2SO_4 (2M, $2cm^3$) and heated in a sealed tube at 100° for 6 h. The solution was neutralised with sat. aq. $BaCO_3$, filtered and the filtrate evaporated to dryness under nitrogen. The residue was dissolved in water ($2cm^3$), applied to a column of AG1X4 resin (200mesh, $5cm^3$, OH^- form) and eluted with water ($10cm^3$). The total eluate was applied to a column of AG50X8 resin (200mesh, $5cm^3$, H^+ form) and eluted with water ($20cm^3$). Evaporation of the eluate under vaccuum afforded D-glucose (65mg, 88%), which co-chromatographed with authentic material on paper using n-butanol: acetic acid: water (4:1:5) as eluate. Crystallisation of a sample of the product from water afforded enriched α-D-glucose (m 141-2 °)



(1)

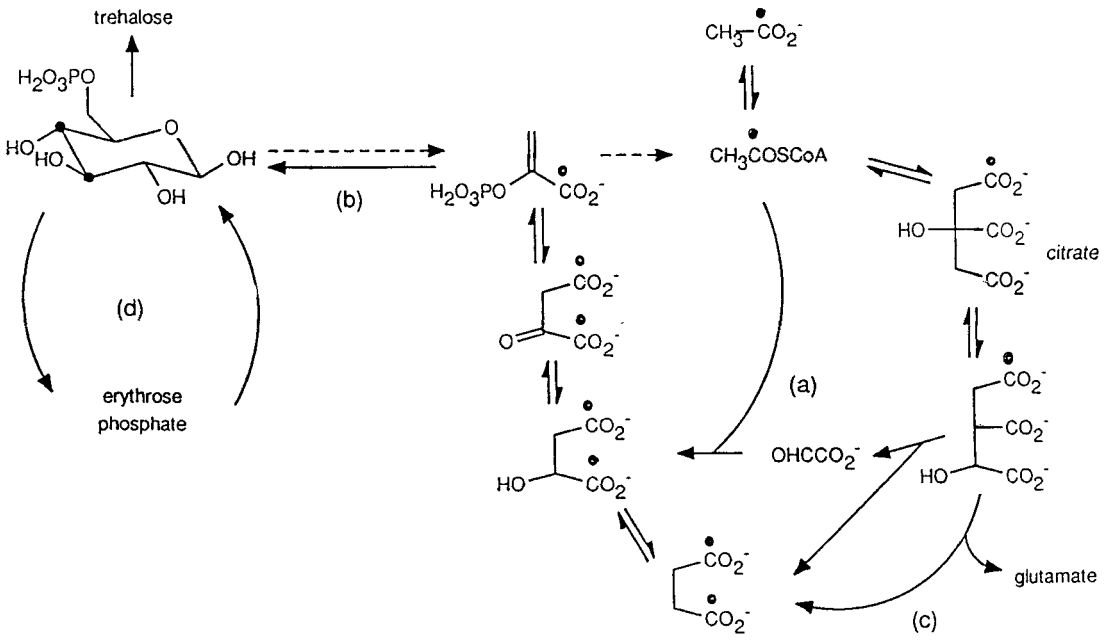


Figure: Schematic outline of the pathways of acetate metabolism in sporulating *S. cerevisiae*. The fate of the C-1 of acetate is shown.

ACKNOWLEDGEMENTS

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