Carbon Dioxide Fixation by *Verticillium albo-atrum*

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SUMMARY

Verticillium albo-atrum required CO₂ for growth when glucose or glycerol was the sole carbon source but not when succinate or acetate was supplied. The yeast form of the fungus fixed ¹⁴C from NaH¹⁴CO₃ into extracellular metabolites (1 to 2 %), low-molecular-weight intracellular components (17 %), lipids (3 %), nucleic acids (34 %) and proteins (42 %). The only radioactive protein components were aspartate (33 %), glutamate (33 %), arginine+lysine (22 %), and leucine+isoleucine (6 %). Nucleic acid bases that contained appreciable ¹⁴C were uracil (27 %), cytosine (21 %), adenine (22 %), guanine (27 %) and thymine (4 %). Carbon dioxide fixation appeared to be important for the anaplerotic biosynthesis of four-carbon intermediates and for purine biosynthesis.

INTRODUCTION

Carbon dioxide stimulates growth and influences morphology in many fungi. Increased growth due to CO₂ occurs in *Curvularia*, *Cochliobolus* and *Chaetomium* (Macauley & Griffin, 1969), *Blastocladiella* (Cantino & Horenstein, 1956), *Fusarium* (Stover & Frieberg, 1958) and *Rhizopus* (Barinova, 1954) and enhanced germination of conidiospores in *Aspergillus niger* (Vakil, Rao & Bhattacharya, 1961). Formation of thick-walled resistant sporangial cells of *Blastocladiella emersonii* was induced by the addition of bicarbonate to the growth medium (Cantino, 1956). Addition of CO₂ to air promoted the growth of two species of *Trichophyton* as macrospores rather than as the normal mycelial forms (Chin & Knight, 1957). Carbon dioxide initiated swellings similar to appressoria on uredospore germ tubes of *Puccinia striiformis* (Macko & Fuchs, 1970) and induced production of the yeast phase in some dimorphic fungi such as *Mucor rouxii* (Bartnick-García, 1963), *Sporotrichum schencki* (Drouhet & Mariat, 1952), *Histoplasma farciminosum* (Bullen, 1949) and *Cladosporium wernickii* (Houston, Myer, Thomas & Wolf, 1969).

Verticillium albo-atrum causes a vascular wilt disease of cotton and is dimorphic (Keen, Wang, Long & Erwin, 1971). In stationary liquid culture the fungus produces mycelial growth while in shaken culture it grows predominantly as primary and secondary spores (Buckley, Wyllie & DeVay, 1969). Although the latter investigators referred to the spores as conidia, here they will be called the yeast form as proposed by Wang & Bartnicki-García (1970). The significance of the yeast form for pathogenicity is indicated by observations that infection of the upper parts of plants results from the upward movement of the spores (or yeast form) in the xylem vessels (Presley, Carns, Taylor & Schnathorst, 1966). We have examined the effects of CO₂ on the growth of the yeast form of *V. albo-atrum* and the utilization of NaH¹⁴CO₃ in biosynthesis.

METHODS

Organism and cultivation. One stock slope culture (inoculated with a single spore) of *Verticillium albo-atrum* (strain v3H, which causes defoliation of cotton plants; Erwin, Moje & Malca, 1965) was used. Except as noted in Table 1, the yeast form was grown in a glucose (25 g/l)–ammonium nitrate medium, pH 6.3, of Malca, Erwin, Moje & Jones (1966), which was modified by the addition of biotin (10 µg/l) and sterilized by autoclaving (121 °C, 15 min) the phosphate solution separately from the other components before combining aseptically. Eighty ml of culture was incubated in a 250 ml Erlenmyer flasks shaken at 80 to 90 reciprocal strokes per min at 25 °C.

Yeast-cell suspensions of *Verticillium albo-atrum* were prepared by filtering cultures through nylon net (1200 mesh per cm²) to remove any mycelial growth, and washing and resuspending the cells in sterile distilled water. Cells were sedimented by centrifuging for 5 min at 5000g.

Determination of ¹⁴CO₂ fixation. Aqueous NaH¹⁴CO₃ was obtained from International Chemical and Nuclear Corporation, Irvine, California (50 mCi/mM). It was diluted in 0.001 N-NaOH to prepare stock solutions.

To determine cellular ¹⁴C incorporation, yeast cells were incubated in NaH¹⁴CO₃, the suspension was centrifuged and the cells were washed twice and resuspended in 2.0 ml distilled water. The second wash contained no radioactivity, so it was concluded that contaminating extracellular ¹⁴C had been removed. Duplicate 0.5 ml samples of the suspension were assayed for radioactivity.

To determine ¹⁴C fixation in the supernatant, 1.8 ml of supernatant fluids from the sedimented cells was acidified by the addition of 0.2 ml of 2 N-HCl and flushed with CO₂ for 15 min. Duplicate 0.5 ml samples were assayed for radioactivity.

Cells were fractionated by the method of Roberts *et al.* (1955). Protein was hydrolysed in a sealed glass tube in 6 N-HCl at 121 °C for 6 h (Wang & Willis, 1965). A sample of the hydrolysed protein that had been neutralized by repeated evaporation *in vacuo* was used to assay the radioactivity in the protein fraction. The nucleic acid fraction was hydrolysed in a stoppered tube with 11 N-perchloric acid at 100 °C for 1 h. After cooling, the hydrolysate was diluted with water, centrifuged and the resulting clear solution was used for paper chromatography.

Radioactivity was determined by adding samples to 10 ml of Bray's solution (Bray, 1960) and measuring the radioactivity in a liquid scintillation counter (Packard Tri-Carb). Generally total counts in excess of 10000 were collected; however, when this number was not obtained in 10 min, the 10 min count was used. Background corrections were made. Since the extent of quenching did not differ significantly among the samples (about 5 %), corrections were not applied.

RESULTS

Effect of CO₂ on growth. A CO₂-free atmosphere severely inhibited the growth of *Verticillium albo-atrum* in shaken cultures on glucose and glycerol, but had negligible effect upon growth on succinate or acetate (Table 1). Inhibition of growth on glucose and glycerol was also observed when commercial mixtures containing 80 % nitrogen and 20 % oxygen were bubbled through the cultures; these mixtures presumably lacked CO₂. Growth on glucose or glycerol in the CO₂-free cultures could be restored to the control values by bubbling pure CO₂ into the final water traps at 1 to 10 ml/min. Growth of the fungus was predominantly as the yeast form in all experiments. Bubbling air containing 10 % CO₂ through *Verticillium*

Table 1. *The effect of excluding CO₂ on the growth of Verticillium albo-atrum**

Carbon source	Growth†		Reduction in growth (%) in absence of CO ₂
	Air‡	Air minus CO ₂ ‡	
Glucose	4.8	0.28	94
Glycerol	5.2	0.14	97
Succinate	1.6	1.6	0
Acetate	2.1	2.2	0

* Inoculum was 10⁷ cells/ml; carbon sources were 1 % (w/v); flasks were shaken at 25 °C for 43 h except for the acetate set which was incubated for 67 h to achieve maximum growth.

† Numbers represent cells × 10⁸/ml as determined by haemocytometer.

‡ Aeration was at about 100 ml/min. Carbon dioxide removal was by passage through NaOH, Ascarite (A. H. Thomas Co., Philadelphia, Pennsylvania, U.S.A.), and water traps; control flasks were supplied by air passed through water.

Table 2. *Distribution of ¹⁴C in cellular fractions of Verticillium albo-atrum cells exposed to NaH¹⁴CO₃*

Preparation*	Total radioactivity	
	c.p.m.	%
Total ¹⁴ C fixed	2435900	100
Washed cells	2402300	98.6
Supernatant fluid	33600	1.4
Cellular fractions	2153500	100
Low molecular weight (cold-TCA-soluble)	374000	17.4
Lipids (EtOH-soluble, EtOH-ether-soluble)	64600	3.0
Nucleic acids (hot-TCA-soluble)	738900	34.3
Acid-EtOH, ether washes	28700	—
Protein (residual protein and cell walls)	913400	42.4

* A cell suspension (25 ml) containing 10⁸ cells/ml and NaH¹⁴CO₃ (5 µCi/ml) was incubated for 3 h; TCA = trichloroacetic acid; EtOH = ethyl alcohol; total ¹⁴C fixed was 3.8 % of that supplied.

cultures or supplementing cultures with KHCO₃ did not give growth greater than in controls aerated with atmospheric air.

Carbon dioxide fixation. Suspensions of yeast form *Verticillium albo-atrum* exposed to NaH¹⁴CO₃ for 3 h at 25 °C retained over 98 % of the ¹⁴CO₂ fixed inside the cells (Table 2). Of the ¹⁴CO₂ fixed, low-molecular-weight components contained 17.4 %, lipids 3.0 %, nucleic acids 34 %, and the protein fraction 42 %. Protein hydrolysates showed the greatest radioactivity in aspartate and glutamate (33 % each), while the arginine + lysine fraction had 22 %, the leucine + isoleucine fraction 6 % and glycine 3 %. No other amino acids contained significant radioactivity. Nucleic acid hydrolysates showed that the radioactivity was distributed in adenine (22 %), guanine (27 %), uracil (27 %), cytosine (21 %) and thymine (4 %) (Table 3).

DISCUSSION

The CO₂ requirement for growth on glucose and glycerol but not succinate or acetate suggests an anaplerotic role for CO₂ in the biosynthesis of four-carbon compounds when the fungus is grown on C₃ compounds or hexoses. The lack of a CO₂ requirement on acetate is consistent with enzymatic evidence for the glyoxylate cycle in the fungus (Tokunaga *et al.*

Table 3. *Radioactivity of protein and nucleic acid extracts*

Component	Radioactivity	
	c.p.m.	%
Protein hydrolysate*		
Glutamate	1500	33
Aspartate	1500	33
Arginine + lysine	1000	22
Leucine + isoleucine	300	6
Glycine	150	3
Nucleic acid hydrolysate†		
Guanine	570	27
Adenine	460	22
Uracil	580	27
Cytosine	450	21
Thymine	80	4

* Amino acids were separated by descending paper chromatography with butanol:acetic acid:water (4:1:5) in one direction and high-voltage electrophoresis in 0.02 M-sodium borate, pH 9.2, in the second direction by means of techniques devised by Drs T. G. James and E. Noltmann, Department of Biochemistry, University of California, Riverside (manuscript in preparation). Unlabelled amino acid standards were run with the hydrolysates. Ninhydrin-reacting areas were cut from the chromatograms and the paper added to scintillation vials for counting. Amino acids with less than 2 % of the radioactivity were not included in the table.

† Nitrogen bases were separated by descending paper chromatography with either 95 % EtOH:ammonium acetate (7:3) or isopropanol:12 N-HCl:water (170:41:39). Standards were run with the hydrolysate, u.v. light-absorbing (254 nm) areas were cut from the chromatogram and added to scintillation vials for counting.

1969). Succinate reduces the CO₂ requirement for growth of the Phycomycete, *Aqualin-derella fermentans* (Emerson & Held, 1969).

The low level of growth in the absence of CO₂ with glucose or glycerol as carbon sources presumably results from a deficiency of the glyoxylate pathway enzymes. This deficiency would result in a shortage of four-carbon intermediates in the absence of CO₂. Repression of glyoxylate enzymes during growth on glucose has been demonstrated in several organisms. When *Escherichia coli* was grown in a medium containing a limiting amount of glucose, the glyoxylate cycle was repressed but when the glucose became exhausted, isocitrate lyase was derepressed and the bacteria grew at the expense of the acetate which had accumulated in the medium during growth on glucose (Holms & Bennett, 1971). *Rhizopus nigricans* exhibited enhanced synthesis of malate synthase (Wegener, Schell & Romano, 1967) and isocitratase (Wegener & Romano, 1964) when grown on acetate and decreased synthesis of these enzymes when grown on glucose.

The preferential uptake of ¹⁴C from carbonate into those amino acids directly synthesized from TCA cycle intermediates (aspartate, glutamate, arginine, and isoleucine; Table 3) also supports the anaplerotic function of CO₂ fixation in the synthesis of four-carbon compounds by *Verticillium albo-atrum*. Similar incorporations of ¹⁴CO₂ into amino acids by several fungi have been reported. *Penicillium chrysogenum* incorporated high levels of ¹⁴CO₂ into aspartate, glutamate, and arginine (Gitterman & Knight, 1952), *Neocosmospora vasinfecta* fixed carbon into aspartate and glutamate and a lesser quantity into tyrosine (Budd, 1969), and uredospores of *Uromyces phaseoli* used CO₂ in the synthesis of aspartate, glutamate, and alanine (Staples & Weinstein, 1959).

The radioactivity associated with uracil, cytosine, and thymine (Table 3) can also be attributed to anaplerotic CO₂ fixation since it has been generally established that aspartate

is a precursor of pyrimidines (Mahler & Cordes, 1971). The radioactivity of adenine and guanine, on the other hand, probably resulted from the direct carboxylation that occurs in the synthesis of purines (Mahler & Cordes, 1971). *Neocosmospora vasinfecta* incorporated labelled CO₂ into guanine, adenine, uridylate, cytidylate and thymidylate (Budd, 1969). Germinating conidiospores of *Aspergillus niger* fixed high levels of ¹⁴C into uridine, cytosine, and adenine with lower levels into guanine and thymine (Yanagita, 1963), and uredospores of *Uromyces phaseoli* incorporated more ¹⁴C into purine than into pyrimidines (Stallknecht & Mirocha, 1971).

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