Carbon Dioxide Fixation in *Helminthosporium cynodontis*

By G. A. CLARKE* AND R. E. HARTMAN

Department of Biology, St Bonaventure University,
St Bonaventure, New York 14778, U.S.A.

(Received 2 April 1975, revised 2 September 1975)

SUMMARY

A mycelial suspension of *Helminthosporium cynodontis* (ATCC24938), grown on glucose-peptone-yeast extract broth and exposed to $\text{NaH}^{14}\text{CO}_3$ for 5 h, fixed significant quantities of ^{14}C into the following fractions (%): small molecular weight components, 7.4; lipid and lipoproteins, 3.9; nucleic acids, 59; the residual protein and cell wall fragments, 29.2. The labelled protein components were (%): aspartate, 39; glutamate, 18; cystine, 15; threonine, 9. Radioactive nucleic acid components were (%): adenine, 18; guanine, 18; cytidylate, 34; uridylate, 30. When the mycelium was grown in Czapek-Dox glucose medium and incubated in this medium plus $\text{NaH}^{14}\text{CO}_3$, the nucleic acid fraction contained 29.9% and the residual protein 49.5% of the cellular radioactivity. The removal of CO_2 from the atmosphere did not reduce growth. Pyruvate carboxylase (PC) and phosphoenolpyruvate carboxykinase (PEPCK) activities were demonstrated in extracts of *H. cynodontis*. Synthesis of PEPCK was stimulated under conditions promoting gluconeogenesis and was reduced under conditions promoting glycolysis, while PC synthesis was similar under both conditions.

INTRODUCTION

Among a variety of effects of CO_2 on the growth and morphology of filamentous fungi that have been reported are increased growth, enhanced germination of conidiospores, induction of yeast-phase cells in dimorphic fungi, and formation of thick-walled resistant sporangial cells (Hartman, Keen & Long, 1972). The few studies describing the metabolic basis for the utilization of CO_2 have generally suggested an anaplerotic function for CO_2 . Among the CO_2 fixation enzymes possibly related to anaplerotic biosynthesis that have been demonstrated in filamentous fungi are malic enzyme (Rick & Mirocha, 1968), pyruvate carboxylase and phosphoenolpyruvate carboxykinase (Hartman & Keen, 1973).

No studies describing the utilization of CO_2 by members of the genus *Helminthosporium* have been reported. The genus consists of a large number of soil saprophytes (Nelson, 1964) and plant pathogens (Dreschsler, 1923), and includes *H. cynodontis* which is a common pathogen of *Cynodon dactylon* or Bermuda grass (Dreschsler, 1923). We describe the utilization of $^{14}\text{CO}_2$ for biosynthesis and the demonstration of possible CO_2 fixation enzymes in *H. cynodontis*.

METHODS

Organism and cultivation. The organism used, *Helminthosporium cynodontis* strain RPP (ATCC24938), was described by White & Johnson (1971).

The media used in this investigation were GPY broth (glucose, 40 g; peptone, 10 g; yeast extract, 5 g; distilled water, 1 l) and a modified Czapek-Dox (CD) mineral salts

* Present address: Department of Biology, Roanoke College, Salem, Virginia 24153, U.S.A.

medium (NaNO_3 , 2.0 g; KH_2PO_4 , 1.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; KCl , 0.5 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g; ZnCl_2 , 0.00075 g; distilled water, 1 l), with glucose or succinate (2%, w/v) usually serving as the sole organic carbon source. The final pH of the CD medium was 5.5. Media were sterilized by autoclaving for 15 min at 121 °C. Phosphate was separately sterilized and added aseptically.

Cultures were maintained on GPY agar slants (GPY broth plus 1.5% (w/v) Bacto-agar). Experimental inocula were prepared by aseptically transferring the surface growth from two GPY agar slants to 75 ml GPY or CD-glucose broth in a 250 ml Erlenmeyer flask. This liquid culture was shaken on a New Brunswick rotary-type shaker at 275 rev./min for 36 h at 26 ± 2 °C. Generally 2 ml of this culture was transferred to 48 ml of fresh GPY or CD-glucose medium and incubated 1 to 2 days until maximum growth was observed, at which point the mycelia developed a light tan colour. One additional 2 ml transfer to 48 ml GPY broth generally resulted in a fine homogeneous mycelial suspension that was used as the source of inoculum for subsequent work.

Effect of CO_2 on growth. Mycelium (0.0212 mg dry wt) was added to 50 ml CD medium with 2% (w/v) organic carbon source (glucose, succinate, glycerol, or acetate) in 250 ml Erlenmeyer flasks and shaken for 62 h at 26 °C. The atmosphere in one set of flasks, containing the four different substrates, consisted of CO_2 -free air obtained by passing bottled gases (20% O_2 , 80% N_2) through Drierite, Ascarite, distilled water and mineral oil-soaked filters. The bottled gases thus treated entered the growth flasks at a rate of 5 ml/s. In control flasks (flasks containing CO_2), the atmosphere consisted of air passed through the system described minus the Ascarite filter.

CO_2 fixation. A 36 h GPY broth culture was harvested by centrifugation (1160 g), washed twice and resuspended in sterile 0.85% (w/v) NaCl. The washed mycelia were shaken for 5 h in 110 ml GPY broth containing 0.114 mCi $\text{NaH}^{14}\text{CO}_3$ (50 mCi/mmol). A 0.5 ml portion of the culture was filtered through an 8 μm Millipore filter, washed twice with distilled water, dried in a planchet and assayed for cellular ^{14}C . Since the second wash contained no radioactivity, it was assumed that all non-incorporated ^{14}C had been removed. To 2 ml of the culture filtrate was added 0.2 ml of 50% trichloroacetic acid (TCA). The acidified filtrate was flushed with CO_2 to remove residual $^{14}\text{CO}_2$, then 0.5 ml was dried in a planchet and assayed for ^{14}C in the excreted products.

The mycelium was fractionated by the method of Roberts *et al.* (1955). The nucleic acid fraction was hydrolysed in 6 M-HCl at 95 °C for 1 h and the hydrolysate was analysed by descending paper chromatography using the following solvent systems: isopropanol-HCl-water (170:41:39, by vol.; Wyatt, 1951); methanol-ethanol-HCl-water (50:25:6:19, by vol.; Kirby, 1955); methanol-HCl-water (70:20:10, by vol.; Kirby, 1955). Unlabelled nitrogen bases and nucleotides were run with the hydrolysate. The components were detected by their u.v. absorption. Protein was hydrolysed in 6 M-HCl in a sealed Pyrex tube at 121 °C for 6 h (Wang & Willis, 1965). The hydrolysate was evaporated to dryness and the residue resuspended in distilled water. The evaporation and resuspension processes were repeated three more times to remove residual HCl. The aqueous solution was assayed to determine the radioactivity in the residual protein fraction and was chromatographed (descending) using the following solvent systems: 2'-butanol-3% NH_4OH (120:40, by vol. Roland & Gross, 1954); *n*-butanol-water-acetic acid (5:5:1, by vol.; Block, 1950); ethanol-butyl alcohol-water-dicyclohexylamine (10:10:5:2, by vol.; Hardy, Holland & Nayler, 1955). Unlabelled amino acids were run with the hydrolysate. Amino acids were detected by the ninhydrin reaction. Amino acids containing less than 1% radioactivity were not included in Table 1.

Ribose and deoxyribose were separated by descending paper chromatography using the following solvent systems: *n*-butanol–acetic acid–water (4:1:5, by vol.; Block, 1952); collidine (Block, 1952); and isopropanol–acetic acid–water (3:1:1, by vol.; Clark, 1964). Ribose and deoxyribose were run with the cold TCA-soluble fraction and detected by *p*-anisidine hydrochloride spray in Block's system and aniline-acid-oxalate reagent in Clark's system.

Resolved paper chromatograms were scanned with a Tracerlab 4 pi scanner to determine the radioactive peaks. Triplicate scans were obtained and the area under each peak on the chart recorder paper was cut, weighed and averaged to determine the relative radioactivity in each peak. The areas of radioactivity on each chromatogram were also detected by autoradiography with Kodak BB-54 X-ray film.

Radioactivity of the cells and cell fractions was measured in a Tracerlab 132M manual scaler with an FD-2 flow counter having an FD-2 Mono/mol window. Duplicate planchets were counted.

Preparation of cell extracts. Two 36 h cultures grown on CD mineral salts solution plus glucose or succinate were harvested by centrifugation at 2 °C and washed with 0.85% (w/v) NaCl. Subsequent procedures were carried out at about 4 °C. Mycelial pellet (2 g) was added to 30 ml of the following mixture: tris–CEK buffer [tris(hydroxymethyl)amino-methane], 0.1 M; dithiothreitol, 1 mM; EDTA, 0.1 mM; HCl to pH 7.6. Before cell disruption 30 mg of chromatographically-prepared trypsin inhibitor (TI) was added. To calculate the protein concentration of the extract the value of the TI was subtracted from the total protein. The mycelium was treated for 2 min in a Bronwil Biosonik III sonifier generating 63 W/cm² at the probe tip, then centrifuged for 10 min at 27000 *g*. The crude extract was dialysed against tris–CEK buffer, pH 7.6, for 4 h with hourly changes of buffer. The extract to buffer ratio for dialysis was 1:15 (v/v).

Cell-free extract ¹⁴CO₂ fixation. Cell extract (0.1 ml) was added to 1.6 ml portions of reaction mixtures that contained combinations of substrates and cofactors designed to demonstrate the presence of CO₂-fixing enzymes. The cell extract plus the complete reaction mixtures were incubated for 10 min at 30 °C. After addition of 0.18 ml of 50% TCA, precipitated protein was removed by centrifugation. The supernatant was flushed with CO₂ and neutralized with 0.7 ml of 6 M-NaOH, then 0.5 ml was dried in a planchet and assayed for radioactivity. Values of controls, in which the TCA was added to the extract before determining the non-enzymic retention of ¹⁴CO₂, were subtracted from the data before inclusion in the Tables.

Reproducibility of data. Duplicate reaction mixtures were used for each experiment. The data given are from duplicate experiments that agreed within 10%.

Protein determination. Protein was determined by method of Lowry *et al.* (1951). Bovine serum albumin (fraction V) was used as the standard.

Decarboxylation of the CO₂ fixation product. To test the susceptibility of the end-product of CO₂ fixation to decarboxylation, the method of Krebs & Eggleston (1945) was employed.

Whatman No. 1 filter paper (3 cm diam) was placed in the centre well of a Warburg vessel and moistened with 0.15 ml of 10% KOH. To the vessel, 2.5 ml of distilled water and 0.5 ml of the reaction mixture were added. Into one side arm was placed 0.5 ml potassium hydrogenphthalate buffer (7.6 g potassium hydrophthalate + 0.9 g NaOH in 50 ml H₂O), pH 5.5, and into the other side arm was placed 0.5 ml of aqueous KAlSO₄·12H₂O (saturated solution).

The vessel was stoppered immediately after the addition of 0.2 ml of 2 M-HCl and the contents of the side arms were combined. In the reaction, the decarboxylated ¹⁴CO₂ was

Table 1. *Distribution of ^{14}C in cell fractions and components of $H. cynodontis$ exposed to $\text{NaH}^{14}\text{CO}_3$*

Preparation*	Radioactivity	
	$10^3 \times \text{c.p.m./fraction}$	%
Total ^{14}C fixed	3223	100
Washed mycelium	2979	92
Supernatant	244	7.6
Mycelial fractions	2057	100
Low molecular weight	140	6.8
Ribose	—	(0)
Deoxyribose	—	(0)
Lipids	45	2.2
Nucleic acids	1313	63.8
Adenine		(18)
Guanine		(18)
Cytidylate		(34)
Uridylate		(30)
Residual protein and cell walls†	559	27.2
Aspartate		(39)
Glutamate		(18)
Cystine		(15)
Threonine		(9)

* Mycelia (192 mg wet wt) were exposed to 0.114 mCi of $\text{NaH}^{14}\text{CO}_3$ for 5 h. The second and third mycelial washes contained no radioactivity.

† Amino acid components containing less than 1% radioactivity are omitted from the Table.

released and trapped on the KOH-saturated filter paper. After the reaction had proceeded, with shaking, for 2.5 h at 28 °C, the filter paper was removed, dried and assayed for radioactivity as previously described. Samples (0.15 ml) of the liquid portion of the reaction mixtures were added to identical pieces of filter paper, dried, and assayed.

RESULTS

Effect of CO_2 on growth. The effect of atmospheric CO_2 on the growth of *H. cynodontis* with glucose, sodium succinate, glycerol or sodium acetate as the organic carbon source in the CD medium was tested. With air as the atmosphere in the flask, growth on the various carbon sources was as follows (mg dry wt cells/flask): glucose, 241; sodium succinate, 89; glycerol, 45; sodium acetate, 49. When the CO_2 was removed, growth with glucose, sodium succinate and glycerol was not reduced; growth on acetate was occasionally reduced, sometimes by as much as 30% but no consistency was observed.

Cellular $^{14}\text{CO}_2$ fixation. A mycelial suspension grown in GPY broth fixed a significant quantity of radioactivity when incubated in GPY broth containing $\text{NaH}^{14}\text{CO}_3$ (Table 1). Among the cell fractions, the cold TCA-soluble (small molecular weight components) fraction contained 6.8% of the total radioactivity, the ethanol-soluble (lipid and lipoprotein) fraction contained 2.2%, the hot TCA-soluble (nucleic acid) fraction 63.8%, and the residual protein and cell wall fragments 27.2%. In the protein hydrolysate, aspartate (39%) was the most radioactive, while glutamate (18%), cystine (15%) and threonine (9%) were also labelled. In the nucleic acid hydrolysate, the pyrimidine nucleotides, cytidylate (34%) and uridylate (30%), had an approximately equal radioactivity which exceeded that of the

Table 2. *Effect of substrates and cofactors on ¹⁴CO₂ fixation by a cell-free extract*

Components added to the reaction mixture* (μ mol)	Radioactivity (c.p.m./0.5 ml)
None	0
PEP (10)	20
PEP (10), ADP (10)	497
PEP (10), KH ₂ PO ₄ (5), K ₂ HPO ₄ (5)	1
Propionate (10), ATP (2.5)	17
Pyruvate (10)	61
Pyruvate (10), ATP (10)	1093
Pyruvate (10), NADH (1)	5
Pyruvate (10), NADPH (1)	29

* The reaction mixture contained (μ mol): tris pH 7.0, 200; KCl, 100; MgCl₂, 3; dithiothreitol, 5; CoA, 0.6; biotin, 8×10^{-6} ; glutamate, 10; pyridoxal phosphate, 10^{-3} ; NaH¹⁴CO₃, 5 μ Ci; glutamate: oxalacetate transaminase, 10 units; protein, 202 μ g; total volume, 1.8 ml. Incubation was for 30 min at 30 °C.

purines, adenine (18%) and guanine (18%). No radioactivity was found in ribose and deoxyribose of the cold TCA-soluble fraction.

When mycelium was grown in CD-glucose broth and incubated in CD-glucose broth containing NaH¹⁴CO₃, radioactivity was distributed as follows (%): low-molecular-weight components, 11.2; lipids, 5.1; nucleic acids, 29.9; residual protein, 49.5.

¹⁴CO₂ fixation by cell-free extracts. Table 2 demonstrates the effects of substrates and cofactors on CO₂ fixation by non-dialysed cell-free extracts obtained from CD-glucose-grown cells. The reaction mixtures had a neutral pH to increase the chances of observing multiple enzyme activities. At a neutral pH, Mn is more effective than Mg in promoting carboxylation by pyruvate carboxylase (PC; EC. 6.4.1.1) (Hartman & Keen, 1974a) and by phosphoenolpyruvate carboxykinase (PEPCK; EC. 4.1.1.32) (Hartman & Keen, 1974b). Maximum fixation was achieved with pyruvate and ATP, suggesting the presence of PC. Approximately 50% of the maximum activity was obtained in the presence of phosphoenolpyruvate (PEP) and ADP, suggesting the presence of PEPCK. The significance of fixation with PEP and ADP was unclear, because it could have resulted from the pyruvate kinase-mediated transfer of phosphate from PEP to ADP to form ATP and pyruvate which could then be carboxylated by PC. Since pyruvate plus NADH or NADPH supported no fixation, the possibility of the malic enzyme, EC. 1.1.1.38, was eliminated. PEP plus phosphate or propionate plus ATP resulted in no activity, eliminating PEP-carboxytransphosphorylase, EC. 4.1.1.38, and propionyl carboxylase, EC. 6.4.1.3, as possible carboxylating enzymes. PEP on its own supported no fixation, eliminating PEP carboxylase, EC. 4.1.1.31.

PEPCK synthesis may be enhanced by growth of the mycelium on citric acid cycle intermediates (Flavell & Fincham, 1968; de Torriontegui, Palacian & Losada, 1966; Ruiz-Amil *et al.* 1965; Hofer, Becker & Betz, 1970; Hartman & Keen, 1974b). Therefore the cofactor requirements for CO₂ fixation by a dialysed extract prepared from *H. cynodontis* grown on succinate were investigated. In reaction mixtures containing the basic components for the PEPCK system (Table 4) but with the combination of substrates and cofactors listed in Table 1, the cell extract produced significant CO₂ fixation only in the presence of PEP and ADP. Apparently the fungus contains PEPCK.

Since it has been reported that biotin and CoA are frequently necessary for PC activity but not for PEPCK activity, the effect of these compounds on CO₂ fixation by a dialysed

Table 3. $^{14}\text{CO}_2$ fixation by dialysed extracts from glucose-grown and succinate-grown cells

Reaction mixture* buffer, pH; divalent cation (μmol)	Growth medium: carbon source	Reaction mixture, substrates (μmol)	Specific activity (c.p.m./ μg protein)
Tris (200), pH 8.0; MgCl_2 (3)	Glucose	Pyruvate (15), ATP (7.5)	3.00
	Succinate	Pyruvate (15), ATP (7.5)	3.23
MES† (100), pH 6.0; MgCl_2 (3)	Glucose	PEP (10), ADP (5)	0.40
	Succinate	PEP (10), ADP (5)	1.67

* Reaction mixture components used in all assays: dithiothreitol, 5 μmol ; KCl, 100 μmol ; glutamate, 10 μmol ; pyridoxal phosphate, 10^{-3} μmol ; KHCO_3 , 3 μmol ; $\text{NaH}^{14}\text{CO}_3$, 5 μCi ; glutamate:oxalacetate transaminase, 10 units; protein, 220 μg (glucose-grown), 120 μg (succinate-grown); total volume, 1.8 ml. In addition to the above components, the pyruvate-ATP reaction mixture also contained biotin, 8×10^{-6} μmol , and CoA, 0.6 μmol . Incubation was for 15 min at 30 °C.

† MES, 2-(*N*-morpholino) ethane sulphonic acid.

Table 4. Decarboxylation of the radioactive product formed by the dialysed cell extract

Reaction mixture	Radioactivity		
	Added to vessel (c.p.m.)	Liquid (%)	Paper (%)
Pyruvate carboxylase system (PC)*			
Basic reaction mixture	1082	90.1	9.9
Reaction mixture minus transaminating components†	1015	17.8	82.2
Phosphoenolpyruvate carboxykinase system (PEPCK)‡			
Basic reaction mixture	879	89	9
Reaction mixture minus transaminating components†	894	5	91

* PC reaction mixture: tris pH 8.0; 200 μmol ; pyruvate, 10 μmol ; ATP, 2.5 μmol ; MgCl_2 , 3 μmol ; KCl, 100 μmol ; biotin, 8×10^{-6} μmol ; CoA, 0.6 μmol ; dithiothreitol, 5 μmol ; KHCO_3 , 3 μmol ; $\text{NaH}^{14}\text{CO}_3$, 5 μCi ; transaminating components†; protein, 320 μg ; total volume, 1.8 ml.

† Transaminating components: pyridoxal phosphate, 10^{-3} μmol ; glutamate, 10 μmol ; glutamate:oxalacetate transaminase, 10 units.

‡ PEPCK reaction mixture: MES pH 6.0, 100 μmol ; PEP, 10 μmol ; ADP, 2.5 μmol ; MgCl_2 , 3 μmol ; KCl, 100 μmol ; dithiothreitol, 5 μmol ; KHCO_3 , 3 μmol ; $\text{NaH}^{14}\text{CO}_3$, 5 μCi ; transaminating components†; protein, 150 μg ; total volume, 1.8 ml. Incubation was for 15 min at 30 °C.

extract from succinate-grown cells was determined. Since dialysed extract required biotin and CoA for maximum fixation supported by pyruvate plus ATP, but not for CO_2 fixation from PEP plus ADP, the presence of both PC and PEPCK in this organism is again suggested.

Next, the relationship between growth on different carbon sources and the specific activity of both PC and PEPCK was studied (Table 3). Dialysed extracts obtained from both glucose-grown and succinate-grown cells and tested in the pyruvate plus ATP reaction mixture yielded almost equal specific PC activity. The extract obtained from succinate-grown cells and reacted in the PEP plus ADP reaction mixture contained four times the specific PEPCK activity of the extract from glucose-grown cells.

Since oxalacetate (OAA) is the product of both PC and PEPCK, the radioactivity in the product of reaction mixtures where these enzymes are active should be removed by decarboxylation with the method of Krebs & Eggleston (1945). This method specifically decarboxylates the β -carboxyl of OAA. It can be seen (Table 4) that in both reaction mixtures

containing no transaminase, the radioactivity was in a product which was specifically decarboxylated, namely OAA. In the basic reaction mixtures containing transaminating components that specifically convert OAA to aspartate, the radioactivity was not susceptible to decarboxylation.

DISCUSSION

When *H. cynodontis* was grown on four different carbon sources in the presence and absence of exogenously added CO₂, no requirement of CO₂ for growth was demonstrated. This finding may be due to high respiratory CO₂ production as reported for *Neocosmospora* by Budd (1969). With much endogenous CO₂ production, the removal of CO₂ may not have been complete.

The high level of radioactivity associated with aspartate in *H. cynodontis* supports the anaplerotic function of CO₂ fixation in this organism. Uredospores of *Puccinia recondita*, *Uromyces phaseoli* (Staples & Weinstein, 1959) and mycelium of *Mucor rouxii* (Bartnicki-Garcia, 1963) also fixed ¹⁴C predominately into aspartate as an early product while yeast phase cells of *Verticillium albo-atrum* (Hartman *et al.* 1972) distributed the ¹⁴C equally between aspartate and glutamate with lesser quantities in arginine-lysine.

Equal radioactivity was found in cytidylate and uridylate as well as in adenine and guanine, with the pyrimidine nucleotides being more strongly labelled. The importance of anaplerotic CO₂ fixation is suggested by the radioactivity in the pyrimidines since aspartic acid is a common precursor. Radioactivity in the purines, however, probably resulted from the direct carboxylation which is generally considered to occur in the biosynthesis of purines (Mahler & Cordes, 1971). Similar labelling patterns to those of *H. cynodontis* have been demonstrated in *Aspergillus oryzae* (Tanaka, Ono & Yanagita, 1966) while approximately equal labelling of purines and pyrimidines was demonstrated in middle-exponential-phase mycelia of *Neocosmospora vasinfecta* (Budd, 1969) and in yeast-phase cells of *V. albo-atrum* (Hartman *et al.*, 1972). This was not the case in germinating uredospores of *U. phaseoli* in which more ¹⁴C was fixed into purine nucleotides than in pyrimidine nucleotides at all times sampled (Stallknecht & Mirocha, 1971).

Several observations lead to the conclusion that PC and PEPCK are present in *H. cynodontis*. The CO₂ fixation was anaplerotic. Maximum fixation by extracts was demonstrated with pyruvate plus ATP (the substrates for PC), and with PEP plus ADP (the substrates for PEPCK). Biotin and CoA deletion decreased fixation with the former substrates but did not affect fixation with the latter. The radioactivity of the extract fixation product was removed by a method specific for OAA. Finally, PEPCK synthesis was stimulated under conditions that promote gluconeogenesis (growth on succinate) and was reduced under conditions promoting glycolysis (growth on glucose), while PC synthesis was similar under both conditions. Similar findings have been reported for these enzymes from other sources such as *V. albo-atrum* (Hartman & Keen, 1974*a, b*).

Pyruvate carboxylase has been reported to occur in the filamentous fungi, *Aspergillus niger* (Bloom & Johnson, 1962), *Penicillium camemberti* (Stan & Schormüller, 1968) and the yeast-phase cells of *V. albo-atrum* (Hartman & Keen, 1973).

In addition to *H. cynodontis*, other filamentous fungi in which PEPCK has been demonstrated are *A. niger* (Woronick & Johnson, 1960), *Neurospora crassa* (Flavell & Fincham, 1968), and yeast-phase cells of *V. albo-atrum* (Hartman & Keen, 1973).

The presence of the established anaplerotic enzyme, pyruvate carboxylase, and the high

level incorporation of ^{14}C from $\text{NaH}^{14}\text{CO}_3$ into aspartate, glutamate, and the pyrimidines strongly indicate the importance of CO_2 in the synthesis of citric acid cycle intermediates and related biosynthetic precursors in *H. cynodontis*.

REFERENCES

- BARTNICKI-GARCIA, S. (1963). Symposium on biochemical basis of morphogenesis in fungi. III. Mold-yeast dimorphism of *Mucor*. *Bacteriological Reviews* **27**, 293-304.
- BLOCK, R. J. (1950). Estimation of amino acids and amines on paper chromatograms. *Analytical Chemistry* **22**, 1327-1332.
- BLOCK, R. J. (1952). *Paper Chromatography, a Laboratory Manual*. New York: Academic Press.
- BLOOM, S. J. & JOHNSON, M. J. (1962). The pyruvate carboxylase of *Aspergillus niger*. *Journal of Biological Chemistry* **237**, 2718-2720.
- BUDD, K. (1969). The assimilation of bicarbonate by *Neocosmospora vasinfecta*. *Canadian Journal of Microbiology* **15**, 389-398.
- CLARK, J. M. (1964). *Experimental Biochemistry*, p. 17. San Francisco: W. H. Freeman.
- DRECHSLER, C. (1923). Some graminicolous species of *Helminthosporium*. *Journal of Agricultural Research* **24**, 641-740.
- FLAVELL, R. B. & FINCHAM, J. R. S. (1968). Acetate-nonutilizing mutants of *Neurospora crassa*. II. Biochemical deficiencies and the roles of certain enzymes. *Journal of Bacteriology* **95**, 1063-1068.
- HARDY, T. L., HOLLAND, D. O. & NAYLER, J. H. C. (1955). One phase solvent mixtures for the separation of amino acids. *Analytical Chemistry* **27**, 971-974.
- HARTMAN, R. E. & KEEN, N. T. (1973). Enzymes capable of anaplerotic carbon dioxide fixation in *Verticillium albo-atrum*. *Phytopathology* **63**, 947-953.
- HARTMAN, R. E. & KEEN, N. T. (1974a). The pyruvate carboxylase of *Verticillium albo-atrum*. *Journal of General Microbiology* **81**, 21-26.
- HARTMAN, R. E. & KEEN, N. T. (1974b). The phosphoenolpyruvate carboxykinase of *Verticillium albo-atrum*. *Journal of General Microbiology* **81**, 21-26.
- HARTMAN, R. E., KEEN, N. T. & LONG, M. (1972). Carbon dioxide fixation by *Verticillium albo-atrum*. *Journal of General Microbiology* **73**, 29-34.
- HOFER, M., BECKER, J. & BETZ, A. (1970). Metabolism of the obligatory aerobic yeast *Rhodotorula gracilis*. II. Pyruvate kinase and phosphoenolpyruvate carboxykinase activities at various metabolic states. *Archiv für Mikrobiologie* **71**, 111-115.
- KIRBY, K. S. (1955). Some new solvent systems for the paper chromatography of nucleic acid degradation products. *Biochimica et biophysica acta* **18**, 575-576.
- KREBS, H. A. & EGGLESTON, L. V. (1945). Metabolism of acetoacetate in animal tissues. *Biochemical Journal* **39**, 409-419.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265-275.
- MAHLER, H. R. & CORDES, E. H. (1971). *Biological Chemistry*, 2nd edn., pp. 820-834. New York: Harper & Row.
- NELSON, R. R. (1964). The perfect stage of *Helminthosporium cynodontis*. *Mycologia* **56**, 64-69.
- RICK, P. D. & MIROCHA, C. T. (1968). Fixation of CO_2 in the dark by the malic enzyme of bean and oat stem rust uredospores. *Plant Physiology* **43**, 201-207.
- ROBERTS, R. B., ABELSON, P. H., COWIE, D. B., BOLTON, E. T. & BRITTEN, R. J. (1955). *Studies of Biosynthesis in Escherichia coli*, pp. 13-14. Publication No. 607. Washington, D.C.: Carnegie Institution.
- ROLAND, J. F., JR. & GROSS, A. M. (1954). Quantitative determination of amino acids using monodimensional paper chromatography. *Analytical Chemistry* **26**, 502-505.
- RUIZ-AMIL, M., DE TORRONTGUI, G., PALACIAN, E., CATALINE, L. & LOSADA, M. (1965). Properties and function of yeast pyruvate carboxylase. *Journal of Biological Chemistry* **240**, 3485-3492.
- STALLKNECHT, G. F. & MIROCHA, C. J. (1971). Fixation and incorporation of CO_2 into ribonucleic acid by germinating uredospores of *Uromyces phaseoli*. *Phytopathology* **61**, 400-405.
- STAN, H. J. & SCHORMÜLLER, J. (1968). Regulation of pyruvate carboxylase of *Penicillium camemberti* by the adenylate system. *Biochemical and Biophysical Research Communications* **32**, 289-294.
- STAPLES, R. C. & WEINSTEIN, L. H. (1959). Dark carbon dioxide fixation by uredospores of rust fungi. *Contributions, Boyce Thompson Institute for Plant Research* **20**, 71-82.
- TANAKA, K., ONO, T. & YANAGITA, T. (1966). Further observations on the carbon dioxide incorporation into RNA in the early phase of conidia germination in *Aspergillus oryzae* with special reference to soluble RNA synthesis. *Journal of General and Applied Microbiology* **12**, 329-336.
- DE TORRONTGUI, G., PALACIAN, E. & LOSADA, M. (1966). Phosphoenolpyruvate carboxykinase in gluconeogenesis and its repression by hexoses in yeasts. *Biochemical and Biophysical Research Communications* **22**, 227-231.

- WANG, C. H. & WILLIS, D. L. (1965). *Radiotracer Methodology in Biological Science*. Englewood Cliffs, New Jersey: Prentice-Hall.
- WHITE, J. P. & JOHNSON, G. T. (1971). Zinc effects on growth and cynodontin production of *Helminthosporium cynodontis*. *Mycologia* **63**, 548–561.
- WORONICK, C. L. & JOHNSON, M. J. (1960). Carbon dioxide fixation by cell-free extracts of *Aspergillus niger*. *Journal of Biological Chemistry* **235**, 9–15.
- WYATT, G. R. (1951). The purine and pyrimidine composition of deoxypentose nucleic acids. *Biochemical Journal* **48**, 584–590.