Carbon Dioxide Fixation in Helminthosporium cynodontis

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SUMMARY

A mycelial suspension of Helminthosporium cynodontis (ATCC24938), grown on glucose-peptone-yeast extract broth and exposed to NaH\(^{14}\)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 NaH\(^{14}\)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 saprophyles (Nelson, 1964) and plant pathogens (Dreschsler, 1923), and includes \(H.\) cynodontis which is a common pathogen of Cynodon dactylon or Bermuda grass (Drechsler, 1923). We describe the utilization of \(^{14}\)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

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medium (NaNO₃, 2.0 g; KH₂PO₄, 1.0 g; MgSO₄·7H₂O, 0.5 g; KCl, 0.5 g; FeSO₄·7H₂O, 0.01 g; ZnCl₂, 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 CO₂ 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 CO₂-free air obtained by passing bottled gases (200, 80% O₂, 80% N₂) 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 CO₂), the atmosphere consisted of air passed through the system described minus the Ascarite filter.

CO₂ 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 NaH¹⁴CO₃ (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 ¹⁴C. Since the second wash contained no radioactivity, it was assumed that all non-incorporated ¹⁴C 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 CO₂ to remove residual ¹⁴CO₂, then 0.5 ml was dried in a planchet and assayed for ¹⁴C 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% NH₄OH (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)aminomethane], 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 27000g. 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 hydrogenphthalate +0.9 g NaOH in 50 ml H₂O), pH 5.5, and into the other side arm was placed 0.5 ml of aqueous KAISO₄·12H₂O (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 I. Distribution of $^{14}$C in cell fractions and components of H. cynodontis exposed to NaH$^{14}$CO$_3$

<table>
<thead>
<tr>
<th>Preparation*</th>
<th>Radioactivity</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total $^{14}$C fixed</td>
<td>3223</td>
<td>100</td>
</tr>
<tr>
<td>Washed mycelium</td>
<td>2979</td>
<td>92</td>
</tr>
<tr>
<td>Supernatant</td>
<td>244</td>
<td>7.6</td>
</tr>
<tr>
<td>Mycelial fractions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low molecular weight</td>
<td>2057</td>
<td>100</td>
</tr>
<tr>
<td>Ribose</td>
<td>140</td>
<td>6.8</td>
</tr>
<tr>
<td>Deoxyribose</td>
<td></td>
<td>(0)</td>
</tr>
<tr>
<td>Lipids</td>
<td>45</td>
<td>2.2</td>
</tr>
<tr>
<td>Nucleic acids</td>
<td>1313</td>
<td>63.8</td>
</tr>
<tr>
<td>Adenine</td>
<td>(18)</td>
<td></td>
</tr>
<tr>
<td>Guanine</td>
<td>(18)</td>
<td></td>
</tr>
<tr>
<td>Cytidylate</td>
<td>(34)</td>
<td></td>
</tr>
<tr>
<td>Uridylate</td>
<td>(39)</td>
<td></td>
</tr>
<tr>
<td>Residual protein and cell walls†</td>
<td>559</td>
<td>27.2</td>
</tr>
<tr>
<td>Aspartate</td>
<td>(39)</td>
<td></td>
</tr>
<tr>
<td>Glutamate</td>
<td>(18)</td>
<td></td>
</tr>
<tr>
<td>Cystine</td>
<td>(15)</td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td>(9)</td>
<td></td>
</tr>
</tbody>
</table>

* Mycelia (192 mg wet wt) were exposed to 0.14 mCi of NaH$^{14}$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 as much as 35; but no consistency was observed.

*Cellular $^{14}$CO$_2$ fixation.* A mycelial suspension grown in GPY broth fixed a significant quantity of radioactivity when incubated in GPY broth containing NaH$^{14}$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%), glutamic acid (18%), cystine (15%) and threonine (9%) were also labelled. In the nucleic acid hydrolysate, the pyrimidine nucleotides, cytidylate (34%), and uridylyl (30%), had an approximately equal radioactivity which exceeded that of the
Table 2. Effect of substrates and cofactors on $^{14}$CO$_2$ fixation by a cell-free extract

<table>
<thead>
<tr>
<th>Components added to the reaction mixture* (μmol)</th>
<th>Radioactivity (c.p.m./0.5 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>PEP (10)</td>
<td>20</td>
</tr>
<tr>
<td>PEP (10), ADP (10)</td>
<td>497</td>
</tr>
<tr>
<td>PEP (10), KH$_2$PO$_4$ (5), K$_2$HPO$_4$ (5)</td>
<td>1</td>
</tr>
<tr>
<td>Propionate (10), ATP (2.5)</td>
<td>17</td>
</tr>
<tr>
<td>Pyruvate (10)</td>
<td>61</td>
</tr>
<tr>
<td>Pyruvate (10), ATP (10)</td>
<td>1093</td>
</tr>
<tr>
<td>Pyruvate (10), NADH (1)</td>
<td>5</td>
</tr>
<tr>
<td>Pyruvate (10), NADPH (1)</td>
<td>29</td>
</tr>
</tbody>
</table>

* The reaction mixture contained (μmol): tris pH 7.0, 200; KCl, 100; MgCl$_2$, 3; dithiothreitol, 5; CoA, 0.6; biotin, 8×10$^{-6}$; glutamate, 10; pyridoxal phosphate, 10$^{-5}$; NaH$_4$CO$_3$, 5 μCi; glutamate: oxalacetate transaminase, 10 units; protein, 202 μg; total volume, 1.8 ml. Incubation was for 30 min at 30 °C.

When mycelium was grown in CD-glucose broth and incubated in CD-glucose broth containing NaH$_4$CO$_3$, radioactivity was distributed as follows (%): low-molecular-weight components, 11.2; lipids, 5.1; nucleic acids, 29.9; residual protein, 49.5.

$^{14}$CO$_2$ fixation by cell-free extracts. Table 2 demonstrates the effects of substrates and cofactors on CO$_2$ 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 Torrontegui, Palacian & Losada, 1966; Ruiz-Amil et al. 1965; Hofer, Becker & Betz, 1970; Hartman & Keen, 1974b). Therefore the cofactor requirements for CO$_2$ 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 CO$_2$ 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 CO$_2$ fixation by a dialysed
Table 3. $^{14}$CO$_2$ fixation by dialysed extracts from glucose-grown and succinate-grown cells

<table>
<thead>
<tr>
<th>Reaction mixture*</th>
<th>Growth medium:</th>
<th>Reaction mixture, substrates (μmol)</th>
<th>Specific activity (c.p.m./μg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>buffer, pH; divalent cation (μmol)</td>
<td>carbon source</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tris (200), pH 8.0; MgCl$_2$ (3)</td>
<td>Glucose</td>
<td>Pyruvate (15), ATP (7.5)</td>
<td>3.00</td>
</tr>
<tr>
<td>Succinate</td>
<td>Pyruvate (15), ATP (7.5)</td>
<td>3.23</td>
<td></td>
</tr>
<tr>
<td>MES† (100), pH 6.0; McCl$_2$ (3)</td>
<td>Glucose</td>
<td>PEP (10), ADP (5)</td>
<td>0.40</td>
</tr>
<tr>
<td>Succinate</td>
<td>PEP (10), ADP (5)</td>
<td>1.67</td>
<td></td>
</tr>
</tbody>
</table>

* Reaction mixture components used in all assays: dithiothreitol, 5 μmol; KCl, 100 μmol; glutamate, 10 μmol; pyridoxal phosphate, 10$^{-5}$ μmol; KHCO$_3$, 3 μmol; NaH$^{14}$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

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

* 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; NaH$^{14}$CO$_3$, 5 μCi; transaminating components†; protein, 320 μg; total volume, 1.8 ml.

† Transaminating components: pyridoxal phosphate, 10$^{-5}$ 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; McCl$_2$, 3 μmol; KCl, 100 μmol; dithiothreitol, 5 μmol; KHCO$_3$, 3 μmol; NaH$^{14}$CO$_3$, 5 μCi; transaminating components‡; protein, 150 μg; total volume, 1.8 ml. Incubation was for 15 min at 30 °C.

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 ^14^C predominately into aspartate as an early product while yeast
phase cells of *Verticillium albo-atrum* (Hartman et al. 1972) distributed the ^14^C 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 uredo-
spores of *U. phaseoli* in which more ^14^C 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 demon-
strated with pyruvate plus ATP (the substrates for PC), and with PEP plus ADP (the sub-
strates 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, 1974a, 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 demon-
strated are *A. niger* (Woronick & Johnson, 1969), *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 NaH$^{14}$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


CO₂ fixation in H. cynodontis


