Enzymes Catalyzing Anaplerotic Carbon Dioxide Fixation in Verticillium albo-atrum

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ABSTRACT

Pyruvate carboxylase and phosphoenolpyruvate carboxykinase activities were present in extracts from *Verticillium albo-atrum*. Nondialyzed extracts actively fixed $NaH^{14}CO_3$ when supplied either pyruvate and ATP or phosphoenolpyruvate (PEP) and ADP. Pyruvate plus NADH or NADPH did not produce CO_2 fixation; PEP alone or with phosphate also was ineffective. The carbon-14 was incorporated into aspartate and citrate by the crude extracts. Enzyme extracts dialyzed against pH 6.6 phosphate buffer utilized only pyruvate and ATP (pyruvate carboxylase activity); whereas, extracts dialyzed against pH 7.6 tris buffer required PEP and ADP for fixation of $^{14}CO_2$ (PEP carboxykinase). Oxalacetate was considered as the primary fixation product in both cases. Incubation of the crude extracts with oxalacetate for 30 min prior to dialysis enhanced pyruvate carboxylase activity. Both enzymes appeared to occur primarily in the cytosol although a significant level of PEP carboxykinase also was associated with the mitochondrial fraction.

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Additional key words: pyruvate carboxylase, phosphoenolpyruvate carboxykinase.

Verticillium albo-atrum is a dimorphic fungus (23). Infection of the upper parts of the host plant results from the upward movement of yeast-form cells in the xylem vessels (31). We (21) found that CO_2 enhanced the growth of yeast-form cells of the fungus on glucose or glycerol as the sole carbon source but not on succinate or acetate. The cells incorporated considerable radioactivity from $NaH^{14}CO_3$ into aspartate and glutamate of protein and into the four common nucleic acid bases. It was therefore hypothesized that anaplerotic CO_2 fixation was important in the growth of *V. albo-atrum* on hexoses.

Mirocha and co-workers have raised the possibility that dark fixation of CO_2 may be of nutritional importance in phytopathogenic fungi (28). Since concentrations of CO_2 are generally considered higher than ambient in the vessels of higher plants, anaplerotic CO_2 fixation is of possible nutritional significance in the pathogenic phase of vascular wilt fungi such as *V. albo-atrum*. We have, therefore, investigated the mechanisms for CO_2 fixation of this fungus. This report describes the identification and location of enzymes responsible for $^{14}CO_2$ fixation into four-carbon compounds.

MATERIALS AND METHODS.—*Preparation of cells.*—*Verticillium albo-atrum*, strain V3H, was grown in the glucose-ammonium nitrate medium of Malca et al. (27), modified by the addition of biotin at 1 μg/100 ml and prepared as previously described (21). Generally 50 ml of medium were used in 250-ml Erlenmyer flasks. Yeast-form cells were prepared by initially transferring spores from a slope culture to the synthetic medium containing glucose at 25 mg/ml and incubating for 4-5 days on a reciprocating shaker (80-90 strokes per min) at 25 C. A portion of the culture was added to fresh synthetic medium containing glucose at 5 mg/ml to yield an initial concentration of approximately 5×10^6 cells/ml. The culture was incubated for 48 hr, the yeast-form cells were separated by filtration through four layers of nylon netting (1,200 mesh/cm^2), washed once with deionized water, and pelleted by centrifugation at 34,000 *g* for 10 min at 4 C. Unless specified, all centrifugations were carried out under these conditions.

Preparation of cell extracts.—One gram of cells (wet weight) was combined with 7.5 g of acid-washed glass beads (Glasperlen, 0.45 - 0.50 mm diam, Van Waters & Rogers, Los Angeles) in 7.5 ml of buffer. Two buffers were generally used for cell breakage: 0.1 M potassium phosphate, pH 6.6, 1 mM dithiothreitol, 0.1 mM EDTA, 0.1 M KCl, (phosphate-breakage buffer); and 0.1 M tris, 1 mM dithiothreitol, 0.1 mM EDTA, 0.1 M KCl, and HCl to pH 7.6 (tris-breakage buffer). The cells were broken in a Bronwill MSK homogenizer for one min at approximately 4-10 C. When the tris-breakage buffer was used, Polyklar AT (GAF Corp., Calvert City, Kentucky) was added to the cell homogenate immediately after disruption at a concentration of 210 mg/g of cells.

Homogenates were centrifuged for 15 min and the supernatant fractions were either assayed directly or dialyzed. Dialysis was generally carried out at 3 C with four hourly changes of buffer with a volume 15 times that of the extract. The dialysis bag was agitated during the dialysis period. Generally one of two buffers was used: 0.1 M potassium phosphate, pH 6.6 (phosphate-dialysis buffer); 2 mM tris, 0.1 mM EDTA, 1 mM dithiothreitol, and HCl to pH 7.6 (tris-dialysis buffer).

Fractionation of cells.—Twenty grams of cells (wet weight) were combined with 120 g of glass beads in 110 ml of sucrose-breakage buffer [0.1 M potassium phosphate, pH 6.6, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1 M KCl, 0.25 M sucrose, 0.15% (w/v) bovine serum albumin] and disrupted as above. The supernatant was decanted from the glass beads, centrifuged for 10 min at 2,500 *g*, and the pellet was

discarded. The supernatant was separated into a pellet fraction (presumed to be mitochondria) and a supernatant fraction by centrifugation for 20 min at 100,000 *g*. Two ml of the sucrose-breakage buffer containing 0.1 M oxalacetate and 0.55% Triton X-100 (Calbiochem, San Diego, California) were added to 10 ml of the supernatant. The mixture was incubated for 30 min at 23 C and centrifuged for 30 min at 100,000 *g* (Spinco Model L centrifuge). Equal portions of the supernatant were dialyzed against the phosphate-dialysis buffer, and against the tris-dialysis buffer. The dialyzed solutions were assayed for pyruvate carboxylase and phosphoenolpyruvate (PEP) carboxykinase, respectively, and analyzed for protein (cytosol, 100,000 *g* supernatant).

The pellet fraction from the 10,000 *g* centrifugation containing mitochondria was washed in the sucrose-breakage buffer and sedimented by centrifugation at 20,000 *g* for 20 min. The wet weight of the pellet was 0.64 g. The pellet was suspended in 5.5 ml of the phosphate-breakage buffer, combined with 6 g of glass beads, and treated for 1 min in the Bronwill MSK at 4 C. To 3 ml of the broken mitochondrial preparation was added 0.6 ml of the phosphate-breakage buffer containing 0.1 M oxalacetate, and 0.55% Triton X-100. The preparation was incubated for 25 min at 23 C. Portions of this preparation were dialyzed against the phosphate- and tris-dialysis buffers, and assayed for pyruvate carboxylase and PEP carboxykinase, respectively (mitochondrial homogenate). Another portion of the mitochondrial homogenate was centrifuged for 10 min at 34,000 *g*. Portions of this supernatant were dialyzed against the phosphate- and tris-dialysis buffers, assayed for pyruvate carboxylase and PEP carboxykinase respectively, and assayed for protein (mitochondrial supernatant).

Protein determination.—Generally protein was determined by the biuret method of Gornall et al. (17). In the cell fractionation study, protein was determined by the Lowry method (26). Bovine Serum Fraction V (albumin) was used as the standard.

Carbon dioxide fixation.—To assay for CO_2 fixation, reaction components were added to test tubes (13 × 100 mm) in a total volume of 1.5 ml. After equilibration at 28 C, the enzyme preparation and $NaH^{14}CO_3$ (50 mcuries/mmole) were added to the tubes which were immediately stoppered and incubated for 15 min. The reaction was stopped by the addition of 0.5 ml of 2 N HCl. Precipitated protein was removed by centrifugation. The supernatant was flushed with CO_2 for 15 min and a 0.5 ml aliquot was assayed for radioactivity. Controls in which the acid was added prior to the addition of the $NaH^{14}CO_3$ were run with each experiment. The radioactivity in this control was subtracted from that in the reaction mixtures. Preliminary studies demonstrated that CO_2 fixation was linear with time for at least 15 min. Except for the cell fractionation study, the radioactivity data reported in the tables was obtained from 0.5-ml aliquots of the acidified reaction mixtures.

Measurement of radioactivity.—Samples were added to 10 ml of Bray's scintillation fluid (7) and radioactivity was measured in a Packard Tri-Carb Model 3002 liquid scintillation counter. Except for the decarboxylation studies, the counting efficiency was similar among the samples so that quenching corrections were not applied. In the former, quenching was determined by using toluene-^{14}C as the internal standard.

Decarboxylation.—Decarboxylation was carried out according to the method of Krebs & Eggleston (24). This method is specific for removal of the beta-carboxyl of oxalacetate. A 0.5-ml aliquot of the reaction mixture was added to a Warburg vessel containing 1.0 ml of H_2O. One side-arm contained 0.5 ml of a saturated solution of $AlK(SO_4)_2 \cdot 12\,H_2O$ and 0.5 ml of a phthalate buffer (7.6 g potassium hydrogen phthalate + 0.9 g NaOH in 50 ml H_2O) was placed in the second side-arm. The components were then mixed and carbon dioxide was absorbed by 0.1 ml of NCS solubilizer impregnated on filter paper in the center well. The flasks were shaken for 3 hr at 25 C and radioactivity was measured in the filter paper and a 0.5-ml aliquot of the liquid phase.

2,4 Dinitrophenylhydrazone preparation.—The 2,4 dinitrophenylhydrazones were prepared essentially as described by Bacchi et al. (1). After termination of CO_2 fixation by the addition of 1.5 ml of 2 N HCl to 4.5 ml of reaction mixture, 12 μmoles of oxalacetate in 0.06 ml of 0.1 M tris-HCl buffer, pH 7.0, and 2 ml of 2,4 dinitrophenylhydrazine reagent [2,4 dinitrophenylhydrazine, 0.15% (w/v), in 2 N HCl] were added. After incubation for 1.5 hr at 23 C followed by 1 hr at 3 C, the precipitated hydrazone was removed by centrifugation at 34,000 *g* for 5 min. The supernatant was assayed to determine the quantity of radioactivity that did not precipitate as the hydrazone. An ethanol extract of the hydrazone precipitate was assayed for radioactivity and analyzed by thin-layer chromatography to determine the identity of the 2,4 dinitrophenylhydrazones. As controls, solutions containing 12 μmoles of oxalacetate, pyruvate, and α-ketoglutarate were treated in parallel with the reaction mixture and analyzed by thin-layer chromatography.

Chromatographic analyses.—The 2,4 dinitrophenylhydrazones of oxalacetate, pyruvate, and α-ketoglutarate were separated by thin-layer chromatography using Silica Gel GF 254 thin layer plates developed with benzene/methanol/glacial acetic acid (45:5:8) (v/v) as described by Bacchi et al. (1). Radioactivity on the TLC plates was detected using a Vangard Model 880 chromatogram scanner.

The organic acids were separated by descending paper chromatography (43) using Whatman No. 1 paper and two solvent systems: solvent system A, methyl isobutyl ketone:formic acid:water (100:28:17) (v/v/v); and solvent system B, diethyl ether:formic acid:water (50:20:5) (v/v/v). Radioactive compounds were identified by co-chromatography with authentic standards using the two solvent systems. One milliliter of the reaction

mixture was dried, resuspended in 0.2 ml of cold 0.13% (v/v) HCl in ethanol, incubated overnight at 4 C, and a 10-μliter aliquot was applied to each chromatogram. Aspartic acid was visualized by spraying with 0.25% (w/v) ninhydrin in acetone and heating at 105° for 5 to 10 min. Organic acids were visualized by spraying with a reagent consisting of 1 g glucose + 1 g aniline in 100 ml of methanol, drying the paper at room temperature and heating for 30 min at 105 C. Radioactivity on the chromatograms was detected prior to spraying, using the Vangard Model 880 gas flow chromatogram scanner. To determine relative concentrations of the radioactive products, duplicate strips were run and scanned. The areas under the recorder curves were cut out and weighed.

Chemicals.—Sodium bicarbonate-^{14}C with a specific activity of 50 mcuries/mM was purchased from International Chemical and Nuclear Corp. (Irvine, California). Acetyl CoA, sodium salt; adenosine-5′-triphosphate, disodium (ATP); adenosine-5′-diphosphate, sodium (ADP); and aspartate:2-oxoglutarate aminotransferase were purchased from Sigma Chemical Co. (St. Louis, Missouri). 2-Phosphoenolpyruvic acid, trisodium (PEP); oxalacetic acid; tris(hydroxymethyl)-aminomethane, A-grade (tris); nicotinamide-adenine dinucleotide phosphate, reduced, tetrasodium (NADPH); nicotinamide-adenine dinucleotide, reduced, disodium (NADH); dithiothreitol; pyruvic acid, sodium; imidazole; (+) biotin; L-glutamic acid; and pyridoxal phosphate, A-grade, were obtained from Calbiochem (San Diego, California). Bovine serum albumin (Fraction V powder) was purchased from Nutritional Biochemicals (Cleveland, Ohio). NCS solubilizer was obtained from Amersham/Searle Corp. (Des Plaines, Illinois). Silica Gel GF 254 was purchased from Merck Chemicals, Darmstadt, Germany.

RESULTS.—*Carbon dioxide fixation by nondialyzed cell extracts.*—A nondialyzed cell extract prepared in the tris-breakage buffer exhibited high levels of $^{14}CO_2$ fixation when either pyruvate and ATP or PEP and ADP were present in the reaction mixture (Table 1). Phosphoenolpyruvate alone, or in the presence of phosphate, did not support fixation. Likewise, pyruvate, alone or in combination with NADH or NADPH, was ineffective. Pyruvate and NADH or NADPH also did not support fixation when $MgSO_4$ was substituted for $MnSO_4$ in the reaction mixture, when 30% CO_2 in N_2 was the atmosphere in the reaction vessel, or when the extract was prepared in phosphate-breakage buffer.

The only radioactive spots detected on paper chromatograms corresponded to aspartate (ninhydrin-reacting material) and citrate (glucose-aniline reacting material). They were detected in a ratio of 2:1, respectively, when reaction mixtures containing pyruvate and ATP or PEP and ADP were analyzed by paper chromatography using two solvent systems.

Carbon dioxide fixation by phosphate-dialyzed cell extracts.—A cell extract that had been prepared in the phosphate-breakage buffer and dialyzed against the phosphate-dialysis buffer required pyruvate and ATP for a high level of $^{14}CO_2$ fixation (Table 2). Phosphoenolpyruvate and ADP supported only 8% of the fixation obtained with pyruvate and ATP.

TABLE 1. Effect of substrates and cofactors on CO_2 fixation by a nondialyzed extract from *Verticillium albo-atrum*

Components added to the basic medium[a] (μmoles)	Radioactivity fixed (cpm/0.5 ml)
None	130
PEP (10)[b]	565
PEP (10), ADP (5)	35,058
PEP (10), KH_2PO_4 (10)	581
Pyruvate (10)	312
Pyruvate (10), ATP (5)	64,480
Pyruvate (10), NADH (1.5)	246
Pyruvate (10), NADPH (1.5)	465

[a] Basic medium (μmoles): tris, pH 7.2, 100; dithiothreitol 5; acetyl CoA, 0.5; $MnSO_4$, 2; KCl, 100; biotin, 4.1×10^{-5}; $KHCO_3$, 5; $NaH^{14}CO_3$, 5 μcurie; protein, 1 mg; total volume, 1.5 ml; incubation time: 20 min.

[b] Abbreviations used in the tables: PEP = phosphoenolpyruvate; ADP = adenosine diphosphate; ATP = adenosine triphosphate; NADH = reduced nicotinamide adenine dinucleotide; NADPH = reduced nicotinamide adenine dinucleotide phosphate.

The phenylhydrazone of the radioactive fixation product from pyruvate and ATP corresponded in chromatographic mobility to oxalacetate phenylhydrazone. However, only 2% of the total radioactivity of the reaction mixture precipitated as the phenylhydrazone. It was necessary, therefore, to establish the identity of the primary fixation product by another means.

The product of $^{14}CO_2$ fixation was subjected to decarboxylation by the method of Krebs & Eggleston (24) which is specific for the beta-carboxyl of oxalacetate (Table 3). If oxalacetate were the primary fixation product in the basic reaction mixture containing pyruvate and ATP, then essentially all of

TABLE 2. Requirements for $^{14}CO_2$ fixation by a phosphate-dialyzed cell extract

Components added to the basic medium[a] (μmoles)	Radioactivity fixed (cpm/0.5 ml)
Pyruvate (10), ATP (5)	16,135
Pyruvate (10)	89
ATP (5)	75
Pyruvate (10), ADP (5)	1,724
PEP (10)	141
PEP (10), ADP (5)	1,286
ADP (5)	117
None	70

[a] Basic medium (μmoles): tris, pH 8.5, 100; $MgSO_4$, 3; KCl, 100; dithiothreitol, 5; $KHCO_3$, 5; biotin, 4.1×10^{-5}; $NaH^{14}CO_3$, 5 μcurie; protein, 0.86 mg; total volume, 1.5 ml; incubation time: 15 min.

TABLE 3. Decarboxylation of the radioactive product formed by the phosphate-dialyzed cell extract

Reaction mixture	Radioactivity[a] liquid paper (% of total)	
Basic[b]	3	97
Basic + transaminating[c] components	97	3

[a] Approximately 17,000 cpm were added to the vessels.
[b] Basic reaction medium (μmoles): tris, pH 8.5, 100; pyruvic acid, 10; ATP, 5; $MgSO_4$, 3; KCl, 100; dithiothreitol, 5; $KHCO_3$, 5; biotin, 4.1×10^{-5}; $NaH^{14}CO_3$, 5 μcurie; protein, 0.8 mg; total volume, 1.5 ml.
[c] Transaminating components (μmoles): pyridoxal phosphate, 0.04; glutamic acid, 10; aspartate aminotransferase, 12.8 units.

the radioactivity should be released from the reaction mixture and be absorbed by the NCS solubilizer on the paper in the center well of the reaction vessel. Such was the case. Furthermore, in the presence of L-aspartate: α-ketoglutarate aminotransferase and transaminating components, the radioactivity, as predicted, remained in solution and was chromatographically identified as aspartate. These data all support the argument that oxalacetate was the primary $^{14}CO_2$ fixation product and that pyruvate carboxylase was the enzyme responsible for fixation.

Since the preliminary data suggested that preincubation of the phosphate-prepared extract with oxalacetate prior to dialysis enhanced pyruvate carboxylase activity, an experiment was conducted to clarify this effect (Table 4). Immediately after centrifugation of the cell homogenate, 0.2 ml of phosphate breakage buffer with or without 20 μmoles of oxalacetate was added to 1 ml of the supernatant fraction. The supernatant was either dialyzed immediately (no oxalacetate added) or it was preincubated for 30 min at 2 C or 23 C in the presence or absence of oxalacetate. Incubation in the presence of oxalacetate prior to dialysis enhanced CO_2 fixation, but there was little difference whether the incubation was carried out at 2 C or 23 C. Substitution of pyruvate for oxalacetate during preincubation resulted in a decrease in CO_2 fixation of over 50% (Table 4).

TABLE 4. Effect of preincubation in the presence of oxalacetate on pyruvate carboxylase activity[a]

Incubation prior to dialysis	Radioactivity fixed (cpm/0.5 ml)
Experiment A	
No preincubation	3,940
30 min, 23 C + oxalacetate (20 μmoles/ml)	9,110
30 min, 23 C, no oxalacetate	3,030
30 min, 2 C + oxalacetate	7,040
30 min, 2 C, no oxalacetate	2,910
Experiment B	
30 min, 23 C + oxalacetate	12,820
30 min, 23 C + pyruvate (20 μmoles/ml)	5,950

[a] The reaction mixture had the composition of the first in Table 2.

Carbon dioxide fixation by tris-dialyzed cell extracts.—A cell-free extract prepared in the tris-breakage buffer and dialyzed for 30 hr against the tris-dialysis buffer required PEP and ADP for a high level of $^{14}CO_2$ fixation (Table 5). Pyruvate and ATP supported only 2.3% of the fixation obtained with PEP and ADP.

TABLE 5. Requirements for CO_2 fixation by a tris-dialyzed cell extract

Components added to the basic medium[a] (μmoles)	Radioactivity fixed (cpm/0.5 ml)
PEP (10), ADP (5)	31,767
PEP (10)	1,477
PEP (10), KH_2PO_4 (10)	489
ADP (5)	120
Pyruvate (10)	35
Pyruvate (10), ADP (5)	195
Pyruvate (10), ATP (5)	724
None	80

[a] Basic medium (μmoles): tris, pH 7.2, 100; $MnSO_4$, 2; acetyl CoA, 0.5; KCl, 100; dithiothreitol, 5; $KHCO_3$, 5; biotin, 4.1×10^{-5}; $NaH^{14}CO_3$, 5 μcurie; protein, 0.8 mg; total volume, 1.5 ml; incubation time: 15 min.

Only 5% decarboxylation was observed with reaction mixtures containing PEP and ADP (Table 5), indicating that the radioactivity was not in oxalacetate. Instead, paper chromatography showed that citric acid was the major radioactive substance. Although the extract had been dialyzed, oxalacetate and acetyl CoA, the substrates for citrate synthase would be present if oxalacetate were the primary product of CO_2 fixation, since acetyl CoA was one of the components of the reaction mixture. Tokunaga et al. (36) found high activities of citrate synthase in *V. albo-atrum*. In order to corroborate the suspicion that oxalacetate was in fact the primary $^{14}CO_2$ fixation product, it was necessary to find conditions that would supress citrate synthase activity without inhibiting the CO_2-fixation enzyme. A cell-free extract prepared in tris-breakage buffer, incubated for 75 min at 28 C in the presence of oxalacetate, and dialyzed for 18 hr against the tris-dialysis buffer, was found to fix a high level of $^{14}CO_2$ in the reaction mixture containing PEP and ADP. However, no citrate accumulation occurred and the radioactive product was quantitatively decarboxylated. It was therefore concluded that oxalacetate was the initial CO_2 fixation product and that PEP carboxykinase was the enzyme involved.

TABLE 6. Pyruvate carboxylase and phosphoenolpyruvate carboxykinase in cell fractions

Fraction	Radioactivity 10^3 cpm/ml fraction	Specific activity[a] 10^3 cpm/mg protein
Pyruvate carboxylase[b]		
Cytosol (100,000 *g* supernatant) Basic reaction mixture	41.5	32
Mitochondrial homogenate (disrupted 10,000 *g* particles)	1.7	
Mitochondrial supernatant (35,000 *g*) Basic reaction mixture	0.9	1.5
PEP carboxykinase[c]		
Cytosol (100,000 *g* supernatant) Basic reaction mixture	60.5	34
Mitochondrial homogenate (disrupted 10,000 *g* particles)	5.9	
Mitochondrial supernatant (35,000 *g*) Basic reaction mixture	3.2	5.3

[a] In the calculation of specific activity, the quantity of protein contributed by the bovine serum albumin in the breakage buffer was substrated from the total protein assayed in the cytosol fraction.

[b] Pyruvate carboxylase was prepared by dialyzing the fraction against the phosphate-dialysis buffer. The following reaction mixture was used (μmoles): tris, pH 7.9, 100; $MgSO_4$, 3; KCl, 100; biotin, 4.1×10^{-5}; $KHCO_3$, 5; pyruvate, 10; ATP, 5; $NaH^{14}CO_3$, 5 μcurie; 0.1 ml fraction; total volume, 1.5 ml; incubation time, 10 min. The radioactivity of the reaction mixture minus pyruvate was subtracted from all values.

[c] PEP carboxykinase was prepared by dialyzing the fraction against the tris-dialysis buffer. The following reaction mixture was used (μmoles): imidazole, pH 5.9, 100; $MnSO_4$, 2; KCl, 100; $KHCO_3$, 5; dithiothreitol, 5; ADP, 5; PEP, 10; $NaH^{14}CO_3$, 5 μcurie; 0.1 ml fraction; total volume, 1.5 ml; incubation time, 10 min. The radioactivity of the reaction mixture minus PEP was subtracted from all values.

Carbon dioxide fixation by cell fractions.—On the basis of carbon dioxide fixation by cytoplasmic and mitochondrial fractions, pyruvate carboxylase appeared to be located exclusively in the cytosol (Table 6). Phosphoenolpyruvate carboxykinase activity was also found in the cytosol, but significant activity was associated with the 10,000 *g* fractions, presumably containing mitochondria. Acetyl CoA did not enhance the activity of any of the fractions.

DISCUSSION.—*Verticillium albo-atrum* cells contain pyruvate carboxylase [EC 6.4.1.1] and PEP carboxykinase [EC 4.1.1.32] based on the specific $^{14}CO_2$-fixation requirements of ATP and pyruvate in phosphate-prepared extracts and of PEP and ADP in tris-extracts, respectively. We were unable to demonstrate the presence of additional oxalacetate- or malate-synthesizing enzymes (25) in this fungus (Table 1). The primary fixation product of dialyzed extracts supplied either pyruvate and ATP or PEP and ADP is clearly oxalacetate. The accumulation of label in aspartate and citrate by nondialyzed extracts indicates the presence of transaminase and citrate synthase activity. These same reactions would explain the high in vivo labeling of the aspartate and glutamate moieties of protein by whole cells of *V. albo-atrum* exposed to $NaH^{14}CO_3$ (21).

Although pyruvate carboxylase has been reported from several animal tissues (1, 22, 38, 41) and microorganisms (3, 8, 11, 15, 16, 20, 30, 33), its occurrence in filamentous fungi had previously been recognized only for *Aspergillus niger* (6) and *Penicillium camemberti* (35).

The observed stimulation of pyruvate carboxylase by oxalacetate is apparently due to stabilization of the enzyme, since the effect is still observed after oxalacetate is removed from the extract by dialysis. Utter & Scrutton (40) cited the unpublished observation of J. J. Irias and M. F. Utter that addition of oxalacetate protects mammalian pyruvate carboxylase against inactivation at 2 C; whereas addition of pyruvate accelerates the rate of inactivation. They attributed these effects to changes in conformation of the enzyme.

Phosphoenolpyruvate carboxykinase occurs in many higher (3, 12, 29, 39) and lower (1, 9, 34, 42) animals. The enzyme has been reported in *Saccharomyces cerevisiae* (10), *Pasteurella pestis* (5), and *Nocardia corallina* (4). The only filamentous fungi in which PEP carboxykinase has been previously found are *Aspergillus niger* (43) and *Neurospora crassa* (13).

Pyruvate carboxylase seems to be located only in the cytosol of *V. albo-atrum*. Conversely, the enzyme has been reported to occur only in the mitochondria of chicken liver (22), superovulated rat ovary (14) and lactating rabbit mammary gland (18). It has been found in both the cytoplasm and mitochondria of rat liver and adipose tissue (32), lactating rat mammary gland (18), cow liver (2), and the fat body of the adult male desert locust (29).

Although PEP carboxykinase occurs in both the cytosol and the mitochondria of *V. albo-atrum*, most of the activity is associated with the former. A similar relationship was found in rat liver, but not in guinea

pig liver (29) and *Tetrahymena pyriformis* (34), where most of the activity is associated with the mitochondria. Phosphoenolpyruvate carboxykinase was associated only with the mitochondria of rabbit liver, mouse liver, and hamster liver (29).

The experiment to ascertain the location of pyruvate carboxylase and phosphoenolpyruvate carboxykinase inside of the cell did not exclude the possibility of disruption of the mitochondria and subsequent leakage of the enzymes into the cytosol during fractionation. However, since the bulk of both enzymes was found in the cytosol, mitochondrial damage as an explanation for the data is valid only if it is assumed that the bulk of the mitochondria were damaged. This possibility seems unlikely since the mitochondrial preparation method was similar to the efficient method used for *Neurospora crassa* (19).

The presence of classical anaplerotic CO_2-fixing enzymes in *V. albo-atrum* is probably of importance in the net synthesis of Tricarboxylic acid cycle intermediates used for additional biosyntheses and also for gluconeogenesis when the organism is grown on C_3 or C_4 compounds (21). Any nutritional importance of these enzymes during pathogenesis (23) is unclear. However, the high CO_2 environment presumed to occur in the vascular system of the host plant and the existence of autotrpophic energy-generating systems in this fungus (37) are consistent with such a possibility.

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