Ketobutyrate decarboxylase activity in *Rhizoctonia solani*
by Robert Martin Quinn

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements of the degree of
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Abstract:

The non-oxidative decarboxylation of a variety of α -keto acids by a crude extract of *Rhizoctonia solani* was investigated. Manometric procedures, ^{14}C tracers, and paper and gas chromatography were used in the investigation. The best substrate for the enzyme preparation was found to be α -ketobutyrate. Other α -keto acids tested were pyruvate, α -ketovalerate, α -ketoiso-caproate, oxaloacetate, α -ketoglutarate, p-hydroxyphenylpyruvate, and phenylpyruvate. Thiamine pyrophosphate (TPP) and Mg^{++} appeared to be co-factors for the enzymatic decarboxylation of α -ketobutyrate. The K_m for α -ketobutyrate was 7.6×10^{-3} moles/ liter, and the energy of activation was 3980 calories. The optimum reaction rate was achieved at 32°C and at a pH of 6.2. Products of decarboxylation of the α -keto acids were CO_2 and the respective aldehyde. These products were recovered in stoichiometric amounts. The enzyme activity was found to be associated with the cell sap. Decarboxylase activity was inhibited in the presence of Hg^{++} , Ag^+ and norjavanicin.

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May 25, 1971

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IN RHIZOCTONIA SOLANI

by

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A thesis submitted to the Graduate Faculty in partial
fulfillment of the requirements of the degree

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ABSTRACT

The non-oxidative decarboxylation of a variety of α -keto acids by a crude extract of Rhizoctonia solani was investigated. Manometric procedures, ^{14}C tracers, and paper and gas chromatography were used in the investigation. The best substrate for the enzyme preparation was found to be α -ketobutyrate. Other α -keto acids tested were pyruvate, α -ketovalerate, α -ketoisocaproate, oxaloacetate, α -ketoglutarate, p-hydroxyphenylpyruvate, and phenylpyruvate. Thiamine pyrophosphate (TPP) and Mg^{++} appeared to be co-factors for the enzymatic decarboxylation of α -ketobutyrate. The K_m for α -ketobutyrate was 7.6×10^{-5} moles/liter, and the energy of activation was 3980 calories. The optimum reaction rate was achieved at 32°C and at a pH of 6.2. Products of decarboxylation of the α -keto acids were CO_2 and the respective aldehyde. These products were recovered in stoichiometric amounts. The enzyme activity was found to be associated with the cell sap. Decarboxylase activity was inhibited in the presence of Hg^{++} , Ag^+ and norjavanicin.

INTRODUCTION

Many enzymes are known to decarboxylate α -keto acids. Although oxidative decarboxylation of α -keto acids is more common in metabolic pathways of living organisms, several systems have been found which non-oxidatively decarboxylate α -keto acids. An extensive description and characterization of enzymes that non-oxidatively decarboxylate α -keto acids was presented by Barman (2).

The first report of an enzymatic decarboxylation of an α -keto acid was made by Neuberg and Karczag (12) in 1911. Pyruvic acid was non-oxidatively decarboxylated by a crude yeast preparation to form acetaldehyde and CO_2 .

Later work on decarboxylase activity in fungi was reported by Green et al. (5). Their work showed that crude yeast enzyme preparations decarboxylated several α -keto acids and pyruvate was the best substrate for the enzyme. They did not state, however, whether the decarboxylations were oxidative or non-oxidative. Compared to pyruvate, the rate of decarboxylation for α -ketoisovalerate was 88% as great; oxaloacetate, 32%; and α -ketoglutarate, 1%. The enzyme preparation did not decarboxylate phenylpyruvate, p-hydroxyphenylpyruvate, or acetoacetate, and α -ketobutyrate was not tested as a substrate. These investigators measured the production of CO_2 , but they did not indicate

whether the other products of decarboxylation were acids or aldehydes.

Long and Peters (10) compared decarboxylation rates on substrates with an enzyme preparation from pigeon brain. Pyruvate, α -ketobutyrate, and α -ketovalerate were used as substrates for the enzyme with α -ketobutyrate being the best substrate. The rates of decarboxylation of pyruvate and α -ketovalerate were 80% and 69% respectively of the rate of decarboxylation of α -ketobutyrate. They reported that the reactions studied were oxidative decarboxylations and the products were the respective organic acids and CO_2 .

Green et al. (6) studied a decarboxylase in fresh pig heart muscle. A solution of protein obtained from washed heart muscle catalyzed the non-oxidative decarboxylation of α -ketoglutarate forming CO_2 and succinic semialdehyde. The same preparation, however, carried out the oxidative decarboxylation of pyruvate and α -ketobutyrate. Besides CO_2 the products were acetoin (3-hydroxy-2-butanone) and propioin (4,4-hydroxy-3-hexanone) respectively.

Although these studies have shown that α -ketobutyrate is a substrate for oxidative decarboxylases from pigeon brain and heart muscle, there is no report of α -ketobutyrate being a

substrate for a non-oxidative decarboxylase found in the tissues of animals or fungi studied. Thus, one purpose of this report is to describe the presence and properties of an enzyme preparation from Rhizoctonia solani (Kühn) that non-oxidatively decarboxylates a number of α -keto acids with α -ketobutyrate serving as the best substrate for the enzyme (Fig. 1).

In R. solani and other fungi, it is often difficult, if not impossible, to demonstrate the presence of α -ketoglutaric acid oxidase (16). Dowler et al. (4) studied several decarboxylases in cell free extracts of ten representative fungi. In most of the fungi including R. solani the majority of enzymes in the citric acid cycle were found. However, α -ketoglutaric acid oxidase was not found in any of the extracts. Hence in R. solani, as well as in other fungi, there exists the possibility of a shunt within the Krebs cycle. This alternate pathway for decarboxylation of α -ketoglutarate could be a non-oxidative one. Thus, an additional purpose of this report is to discuss the possible importance of the non-oxidative α -keto acid decarboxylase activity in the metabolism of R. solani.

Figure 1. Scheme of non-oxidative decarboxylation of α -keto-
butyrate by an enzyme preparation from R. solani.

EXPERIMENTAL PROCEDURE

Culturing of R. solani: R. solani was grown in a medium containing 10 g glucose, 2.0 g asparagine, 1.0 g KH_2PO_4 , 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 mg $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.42 mg ZnCl_2 , 0.36 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ and traces of biotin and thiamin per liter of distilled water. The stationary cultures were grown at 25°C in one liter flasks containing 250 ml of medium.

Preparation of Cell Free Extract: One non-washed 10-day-old micelial mat, approximately 2.0 g dry weight, was ground in a Sorvall omnimixer for one minute at 4°C . This preparation was then centrifuged at 20,000 X g at 4°C for 10 minutes. The supernatant liquid was collected and centrifuged again as described. The final supernatant liquid was decanted and powdered $(\text{NH}_4)_2\text{SO}_4$ was slowly added to make a 75% saturated solution. This solution was centrifuged once under conditions as previously described. Assays showed that this precipitation did not greatly purify the enzyme. The pellet was resuspended in 0.5M phosphate buffer pH 6.2 unless otherwise specified. Preparations were kept at 4°C and were either used within 2-3 hours of preparation or frozen in this state for periods of time up to three months.

Chromatography: Two solvent systems were used in descending paper chromatography on Whatmen No. 1 filter paper;

(A) n-butanol-acetic acid-water (4:1:5) v/v/v and (B) n-pentanol-formic acid 5N (1:1) v/v. Organic acids and aldehydes were located on the chromatograms by using the alkaline silver nitrate reagent of Trevelyan (15), and an acid-base indicator containing methyl yellow and bromphenol blue (1).

Protein Determination: Protein determination in the enzyme preparations was done using the method of Lowry et al. (11).

Labeling Experiments: The purpose of the labeling experiments was to determine the nature and number of non-gaseous products produced by the decarboxylation of α -keto acids. The $(\text{NH}_4)_2\text{SO}_4$ precipitate containing 53 mg of protein was suspended in 3.0 ml 0.05M sodium acetate buffer pH 4.5. Labeled α -ketoglutarate containing 2.86×10^6 dpm in 0.1 ml distilled water was added and the reaction mixture was placed in a psychrotherm incubator shaker at 35°C and agitated at 100 rpm. Aliquots of 0.3 ml were taken out at 3, 20, 30, 60, 120, and 240 minute intervals and an equal volume of hot ethanol was added. A 20 λ portion of each aliquot was chromatographed in solvent B. Areas containing radioactivity was determined by a Packard Radiochromatogram scanner were shredded and counted by a liquid scintillation counter. Each counting vial contained 1.5 ml methanol and 13.5

ml toluene containing 4.0 g of 2,5-diphenyloxazole and 100 mg of p-bis-2(5 phenyloxazolyl) benzene per liter. The channels ratio method was used to correct for quenching. An experiment following the same procedure was also done in which the enzyme preparation had been boiled for 10 minutes. The area containing labeled succinic semialdehyde from one chromatogram was eluted with water, dried, redissolved in water and spotted with authentic succinic semialdehyde onto a chromatogram and developed two dimensionally in solvent systems A and B. An autoradiograph was made on NS-2T Kodak Medical X-ray film at 25° C for 4 days. The X-ray film was developed in D-19 (Kodak) Developer for 10 minutes and fixed in Kodak X-ray fixer for 10 minutes.

Enzyme Assay: A Gilson Differential Respirometer, model G 20, was used to assay for decarboxylase activity. All tests were run in Warburg flasks at 32° C and 100 oscillations per minute. Unless otherwise indicated, the total reaction mixture contained 30 μ moles of substrate, 1850 μ moles potassium phosphate buffer pH 6.2 containing the enzyme in 3.0 ml of distilled water. The substrate was held in the side arm in a 0.3 ml solution for an equilibration period of 20 minutes before each experiment was begun. KOH (0.2 ml of a 20% solution) was placed on a wick in the center well of a reference flask to absorb CO₂. The CO₂

evolution was measured by determining the difference in reading between the reference flask with KOH and the flask without KOH. As a control, a duplicate set of reaction flasks were run in which the enzyme preparation had been denatured by boiling for 2 minutes. All values were corrected to standard pressure at the termination of each experiment. Activity for the enzyme was reported as nmoles of CO_2 formed per minute from an average value during the first 0-60 minutes of the reaction. In order to compensate for the absorption of CO_2 by the buffer, all values of CO_2 should be multiplied by a factor of 1.5 to give absolute totals of CO_2 produced.

Stoichiometry: Gas chromatography on a F & M Biomedical Gas Chromatograph Model 400 was used to detect propanal produced in the decarboxylation of α -ketobutyrate. CO_2 evolution was simultaneously determined on the Gilson Respirometer. Aliquots of the reaction mixture were run isothermally in the gas chromatograph using a poropak-Q column 180 cm X 0.4 cm, a detector temperature of 250°C , a column oven temperature of 195°C and a carrier gas flow rate of 30-50 cc/minute. The enzyme reaction mixture contained 600 μmoles substrate, 1200 μmoles buffer and 24 mg protein. A control experiment was also done in which the preparation contained no substrate. After four hours, 2.0 ml of

a 0.31M solution of trichloroacetic acid was added to 2.0 ml of the reaction mixture and it was centrifuged at 20,000 X g for 5 minutes. Aliquots of 1 μ were subjected to gas chromatography. The quantity of propanal produced was determined by triangulation of peaks and a comparison made to peaks produced by known quantities of propanal.

Cellular Location of Enzyme: The culture used for mitochondrial enzyme isolation was grown on a shaker at 100 rpm in order to facilitate the formation of mycelial balls. These balls were strained on cheese cloth to remove the medium. The mycelium (35 g wet weight) was suspended in 35 ml of a solution containing 0.3M mannitol and 0.05% cysteine and hand ground in a 40 ml ground glass tissue grinder. The mixture was then centrifuged at 1000 X g at 4° C for 10 minutes. The pellet was discarded and the supernatant liquid was recentrifuged at 10,000 X g at 4° C for 5 minutes to pellet mitochondria. The supernatant liquid was treated the same as a regular enzyme preparation starting with the precipitation by $(\text{NH}_4)_2\text{SO}_4$. The mitochondrial pellet was resuspended in the mannitol-cysteine solution and centrifuged for 5 minutes at 10,000 X g at 4° C. The pellet from this centrifugation was resuspended in 10 ml 0.5M phosphate buffer pH 6.2. The suspension was placed in the MSK homogenizer

for 2 minutes and disrupted with 0.25-0.30 mm glass beads. This mixture was centrifuged for 10 minutes at 10,000 X g at 4° C.

$(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant liquid to make a 75% saturated solution. The solution was centrifuged at 20,000 X g for 10 minutes at 4° C and the pellet suspended in 3 ml 0.05M phosphate buffer pH 6.2.

Materials: R. solani which had been isolated from a cotton seedling in california was obtained from Dr. D. Mathre, Department of Botany and Microbiology, Montana State University. Sodium 2-ketoglutarate-5- ^{14}C with a specific activity of 90 $\mu\text{Ci}/\text{mM}$ was purchased from Amersham/Searle, Des Plaines, Illinois. P-hydroxyphenylpyruvate and phenylpyruvate were kindly supplied by Dr. E. E. Conn, Department of Biochemistry, University of California, Davis, California. All other α -keto acids were purchased from Sigma Chemical Co., St. Louis, Missouri. Succinic semialdehyde was prepared as described by Aronoff (1). All other chemicals used were reagent grade and the propanal was redistilled from a reagent grade solution. Norjavanicin, a toxin produced by Fusarium oxysporum, was obtained from Dr. H. Kern of the Institute for Special Botany, Technical University, Zurich, Switzerland.

RESULTS

Products of the Reaction: Two dimensional co-chromatography of authentic succinic semialdehyde and the product of the decarboxylation reaction followed by autoradiography showed that succinic semialdehyde was a product of enzyme activity using α -ketoglutarate-5- ^{14}C as the substrate. The spot on the autoradiogram was the same in position, size, and shape as the spot of authentic succinic semialdehyde on the chromatogram.

Figure 2 shows that the production of succinic semialdehyde was concomitant with the disappearance of α -ketoglutarate. A radiochromatogram scan indicated that there were only two areas of radioactivity on the chromatogram. As compared to the references, these areas contained α -ketoglutarate and succinic semialdehyde. There was no conversion of α -ketoglutarate to succinic semialdehyde in preparations containing boiled enzyme.

Stoichiometry: A comparison was made between the amount of CO_2 evolved and the amount of propanal produced from the decarboxylation of α -ketobutyrate. The results in Table I show that the amount of CO_2 produced in the reaction mixture was virtually identical to the amount of propanal produced. Some residual propanal existed in the enzyme preparation and this was accounted for in the calculations of experimental data.

Figure 2. The non-oxidative decarboxylation of α -ketoglutarate to form succinic semialdehyde by an enzyme preparation from R. solani. Experimental conditions are described in the Experimental Procedure. succinic semialdehyde $\Delta-\Delta$, α -ketoglutarate $O-O$.

TABLE I

Stoichiometric comparison of CO_2 evolution and propanal production from the non-oxidative decarboxylation of α -keto-butyrate (600 μmoles) by an enzyme preparation of R. solani. All other conditions are described in the Experimental Procedure.

Compound	μmoles
CO_2 evolved	23.01
Propanal produced	20.28
* α -ketobutyrate remaining	579.00

* Obtained by subtracting μmoles of product from the number of μmoles of α -ketobutyrate originally added to the reaction mixture.

Figure 3. Time course of CO₂ evolution of the non-oxidative decarboxylation of α-ketobutyrate by a decarboxylase from R. solani. The reaction mixture contained 13.5 mg of protein.

Reaction Properties: The time course of the enzymatic decarboxylation of α -ketobutyrate was essentially linear after the first 10 minutes (Fig. 3) while CO_2 production was virtually linear as a function of enzyme concentration (Fig. 4). The reaction was run at pH of 4.4, 4.8, and 5.6 in sodium acetate buffers and at pH of 5.8, 6.2, 6.6, 7.0, and 7.4 in potassium phosphate buffers. The enzyme had its greater activity with α -ketobutyrate at pH 6.2. It should be pointed out that the accuracy of CO_2 measurements by this manometric method suffers with increasing pH above 6.5 as a result of the retention of CO_2 by the buffer. This, however, was taken into account in buffers above 6.5 through 7.4 and the optimum conditions for the enzyme remained at 6.2. The heat stability of the enzyme was determined by exposing individual portions of the enzyme preparation to 35° , 45° , 55° , and 65° C for 5 minutes prior to starting the reaction. By interpolation of data recorded, the activity of the enzyme was reduced by 50% at 52° C. The enzyme remained active for at least 3 months when frozen at -20° C. Figure 5 shows that of the eight α -keto acids used as substrates for an enzyme preparation from R. solani, α -ketobutyrate was the best substrate for the enzyme.

Reaction Requirements: The possibility of an oxygen requirement for the reaction was studied by running it in a nitrogen atmosphere. Oxygen was removed from the reaction mixture by bubbling nitrogen through the mixture prior to the beginning of the experiment. The enzyme did not require oxygen since the activity was unchanged from that of a reaction mixture run under standard conditions. Table II shows a definite increase in enzyme activity when the co-factors Mg^{++} and TPP were added to the reaction mixture (Experiment A). An attempt was made to remove these co-factors by dialysis and then the rate of reaction was observed after the addition of Mg^{++} and TPP (Experiment B). The results showed that much activity had been lost through dialysis which could not be completely restored. However, a noticeable increase in activity was observed when TPP was added.

Kinetics: The data from Figure 6 were plotted in the double reciprocal manner of Lineweaver and Burk (9). From Figure 7 the K_m for the enzyme was calculated to be $7.6 \times 10^{-3} M$. An Arrhenius plot using analysis by the method of least squares to obtain the best slope for the line showed that the activation energy for the enzymatic decarboxylation of α -ketobutyrate was 3980 calories (Fig. 8).

Figure 4. Relationship of protein concentration to the rate of CO_2 production from the non-oxidative enzymatic decarboxylation of α -ketobutyrate.

Figure 5. Rate of CO_2 production by the non-oxidative decarboxylation of various α -keto acids by a decarboxylase from R. solani. The reaction mixtures contained 7.8 mg of protein. The numbers above each bar represent the percentage of the rate of reaction relative to the rate of reaction with α -ketobutyrate.

TABLE II

Co-factor requirements for the non-oxidative decarboxylation of α -ketobutyrate. Experiment A was run under standard conditions except for the addition of 50 μ moles of TPP and 100 μ moles of Mg^{++} separately and combined to the reaction mixture which contained 13.5 mg of enzyme. The conditions for Experiment B were identical to Experiment A except the protein used in this test had been resuspended in a 0.01M tris buffer pH 8 containing 0.005M EDTA after the second centrifugation. The enzyme was dialyzed against 500 ml of 0.01M tris buffer pH 8 for 6 hours at 9° C. The buffer was changed every hour. The protein was then precipitated by the addition of $(NH_4)_2SO_4$ up to 75% saturation. This was then centrifuged and dissolved in distilled H_2O .

	CO ₂ Formed	
	Experiment A Non-dialyzed nmoles/min	Experiment B Dialyzed nmoles/min
Thiamine pyrophosphate + Mg^{++}	68.8	4.8
Thiamine pyrophosphate	67.7	10.5
Mg^{++}	49.9	5.1
Substrate only	42.0	3.3

Figure 6. Velocity of the non-oxidative decarboxylation reaction as a function of the molarity of α -ketobutyrate. The reaction mixtures contained 7.5 mg protein.

Figure 7. A Lineweaver-Burk plot of the data in Figure 6. The reciprocal of the reaction rate is plotted as a function of the reciprocal of substrate concentration. The reciprocal of the velocity is given in values X 1000.

Figure 8. Relationship between reaction velocity constant (K) and reaction temperature. Log of K is plotted as a function of the reciprocal of the absolute temperature at which the reaction was run. The reaction mixture contained 3.4 mg of protein.

Cellular Location of Enzyme: The enzyme preparation from mitochondria did not possess decarboxylase activity (Table III), however, enzyme activity was associated with the cell sap.

Inhibition Analyses: The effect of possible activators or inhibitors on the enzymatic decarboxylation of α -ketobutyrate is shown in Table IV. As indicated, the rate of decarboxylation was not influenced by the presence of valine, isoleucine or succinate. Results did not vary when valine, isoleucine or succinate were present in concentrations of .01, .005, .001, .0005, and .0001 molar. Hg^{++} and Ag^+ severely limited activity of the enzyme while Zn^+ increased activity of the enzyme. Cu^{++} did not affect the rate of decarboxylation. Norjavanicin (7) which is known to effect TPP dependent enzymes reduced the activity of the enzyme by 50% (Table II).

TABLE III

Nannamoles of CO_2 produced by the non-oxidative decarboxylation of α -ketobutyrate by an enzyme preparation (1.5 mg) from the mitochondria of R. solani and an enzyme preparation (3.8 mg) from a mitochondrial free preparation of R. solani.

Treatment	Specific Activity of Protein nMoles CO_2 /min/mg protein
mitochondrial enzymes	0
mitochondrial free enzymes	6.1

TABLE IV

Effects of potential activators and inhibitors on the production of CO_2 from the non-oxidative decarboxylation of α -keto-butyrate by an enzyme preparation from R. solani. Additions of 50 μmoles of valine, isoleucine and succinate, 5 μmoles $\text{Hg}(\text{NO}_3)_2$, AgNO_3 , ZnSO_4 , CuSO_4 , and 0.45 μmoles norjavanicin were added separately to the complete 3 ml reaction mixture. 25.4 mg of protein was used in each reaction mixture.

Treatment				nMoles of CO_2 produced/min
Complete reaction mixture only				90.9
"	"	"	+ valine	89.2
"	"	"	+ isoleucine	86.2
"	"	"	+ succinate	86.0
"	"	"	+ Hg^{++}	20.7
"	"	"	+ Ag^+	15.8
"	"	"	+ Zn^{++}	156.4
"	"	"	+ Cu^{++}	89.8
"	"	"	+ norjavanicin	48.3

DISCUSSION

The enzyme preparation of R. solani exhibited activity with a wide variety of α -keto acids (Fig. 5). Although this activity was more general, it was very similar to the partially purified yeast decarboxylase activity reported by Green et al. (5). It is interesting to note that the rate of decarboxylation of pyruvate, α -ketoisovalerate, and oxaloacetate were in the same proportions for both the enzymes from yeast and R. solani. With α -ketoglutarate the activity of the enzyme from R. solani was substantially greater than the activity of the enzyme from the yeast. α -Ketobutyrate was not tested as a substrate for the yeast enzyme by Green et al. (5), and it is possible that it could have had its best activity with α -ketobutyrate also. Since the enzyme preparation used in these experiments was not purified, more than one decarboxylase may have been present. Therefore, the data in Figure 5 may not necessarily represent the activity of one decarboxylase. For instance, the dicarboxylic acids may have a specific decarboxylase and the monocarboxylic acids may have another.

The experiments with α -ketoglutarate-5-¹⁴C as a substrate for the enzyme showed that succinic semialdehyde ¹⁴C was the only nongaseous product formed in the decarboxylation reaction. Stoichiometry accounted for equal amounts of CO₂ and propanal

produced by the decarboxylation of α -ketobutyrate (Table I).

It was assumed that the amount of α -ketobutyrate remaining after decarboxylation could be determined by simply subtracting the number of moles of CO_2 or propanal produced from the number of moles of α -ketobutyrate in the initial reaction mixture.

Krampitz (8) indicated that most decarboxylases contain thiamine pyrophosphate (TPP) and magnesium loosely bound as co-factors. The information in Table II suggests these co-factor requirements for the enzyme preparation from R. solani. Only a 50% increase in activity resulting from the addition of these co-factors indicates that the co-factors may have been already bound to a large percentage of the enzyme. A threefold increase in activity was noted when TPP was added to an enzyme preparation that had been dialyzed against an alkaline buffer in order to remove the co-factors. There is no apparent explanation for the inability of the preparation to be activated by the addition of both Mg^{++} and TPP. The experimental results may have been altered by the great deal of enzyme activity which was lost during the dialysis process (Table II). Norjavanicin reduced

the activity of the enzyme 50% at a concentration of 1150 μ molar (Table IV). At this same concentration, Kern et al. (6) reported an 80% reduction in activity of pyruvate decarboxylase. This toxin is known to effect only TPP dependent enzymes by reacting with TPP.

The energy of activation for the non-oxidative decarboxylation of α -ketobutyrate was 3980 calories (Fig. 7). This is in the range of the energy of activation for a large number of enzyme catalyzed reactions reported by Sizer (12).

Decarboxylase activity was found in the cell sap surrounding the mitochondria (Table III). That mitochondria of R. solani possess enzyme activities commonly associated with the Krebs cycle was demonstrated by Tolmsoff (14). Recently, this finding was confirmed by Mathre (unpublished) using mitochondrial preparations from R. solani. Thus, although decarboxylase activity might be a part of an alternate pathway of the Krebs cycle, it did not appear to be in direct association with other Krebs cycle enzymes that are associated with the mitochondrion. There is the possibility, however, that the enzyme could be easily extracted during separation of the mitochondria. The possibility of an alternate pathway within the Krebs cycle would involve the non-oxidative decarboxylation of extra mitochondrial α -ketoglutarate

to yield succinic semialdehyde. This aldehyde could then be oxidized to succinic acid by succinic semialdehyde dehydrogenase. The succinic acid could then be incorporated into the remainder of the Krebs cycle within the mitochondria. Although succinic semialdehyde dehydrogenase was not assayed for in R. solani it has been previously described in basidiomycetous fungi (3). This shunt would effectively bypass the step of oxidative decarboxylation of α -ketoglutarate and account for the inability of Dowler et al. (4) and other investigators (16) to demonstrate the presence of α -ketoglutarate dehydrogenase among the Krebs cycle enzymes they described.

Two amino acids for which α -ketobutyrate serves as a precursor were added to the enzyme reaction mixture to determine if activation or inhibition of this reaction may occur. No effect was observed (Table IV). If α -ketobutyrate decarboxylase is an important enzyme in the metabolism of valine or isoleucine, no apparent allosteric regulation occurs at the levels of these amino acids at the concentrations that were tested. Nevertheless, this enzyme could regulate the levels of α -ketobutyrate that would be eventually used for amino acid synthesis in the cell. Succinate, another possible product of the metabolism involving α -ketobutyrate, also showed no effect on enzyme

activity at the concentrations tested. There also exists the possibility that valine, isoleucine or succinate may have an allosteric effect at concentration not tested, although this is unlikely considering the range of concentrations tested.

Green et al. (5) found that by adding Ag^+ , Hg^{++} , and Cu^{++} to their partially purified yeast decarboxylase 100% inhibition of the enzyme was achieved. They also reported that Zn^{++} activated rather than inhibited the enzyme preparation. Data from the non-oxidative decarboxylase from R. solani showed similar but not identical results. Hg^{++} and Ag^+ greatly reduced the activity of the enzyme preparation. Cu^{++} , however, had almost no effect on activity. The addition of Zn^{++} increased the activity of the enzyme by 2/3 (Table IV). Green et al. (5) suggest that Zn^{++} may be acting as a co-factor in place of Mg^{++} in the yeast carboxylase. This might also explain the increase in activity seen in the enzyme preparation from R. solani when Zn^{++} was added. Although additions of Mg^{++} by itself did not activate the enzyme to a great extent in undialyzed preparations, additions of Mg^{++} to the dialyzed preparation doubled the activity of the enzyme (Table II).

SUMMARY

The non-oxidative decarboxylation of α -keto acids by a crude extract of Rhizoctonia solani was investigated using a manometric procedure. The possible importance of this enzyme in the metabolism of the fungus was discussed. The best substrate for the enzyme preparation was α -ketobutyrate. Thiamine pyrophosphate and Mg^{++} were possible co-factors for the enzymatic decarboxylation of α -ketobutyrate. The K_m for α -ketobutyrate was 7.6×10^{-3} moles/liter, and the energy of activation was 3980 calories. Optimum catalysis for the reaction was achieved at $32^{\circ} C$, pH 6.2. Products of the action of the enzyme preparation on α -keto acids were CO_2 and the respective aldehyde. Decarboxylase activity was inhibited in the presence of Hg^{++} , Ag^{+} , and norjavanicin.

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