IN VITRO METABOLISM OF CLAVICEPS PURPUREA (ERGOT)

by

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INTRODUCTION

The ergot fungus, Claviceps purpurea, a parasite of rye and barley, produces sympatholytic and sympathomimetic alkaloids of considerable pharmacological interest (88). The unique synthetic abilities of C. purpurea have induced the present undertaking of a comprehensive metabolic study of this organism.

Previous studies in this laboratory have indicated the presence of both the pentose cycle and the glycolysis-Krebs cycle pathways of glucose catabolism in this organism. On the basis of the rate of evolution of radioactive carbon dioxide from glucose labeled in various positions, it was concluded that about 90 per cent of the glucose was dissimilated by the glycolysis-Krebs cycle pathway (53, 54). The soluble succinic dehydrogenase of C. purpurea was found to possess interesting properties, including ease of solubilization, unusual stability, and rather high inhibition by o-phenanthroline, which was noncompetitive with succinate when PMS* was used as acceptor (46, 54). This thesis represents a continuation and extension of the metabolic studies previously reported. All the experiments were performed in vitro.

Amino acid decarboxylase. Amino acid decarboxylase activity, including glutamic decarboxylase, was first

*Refer to page 82 for meanings of abbreviations used in this thesis.

reported by Gale in microorganisms in 1940 (19). The pH optima of the decarboxylases were below neutrality. He resolved L-lysine, L-tyrosine, L-arginine, and L-ornithine decarboxylases into the apoenzymes and a soluble dialyzable extract, codecarboxylase (21). The decarboxylases were sensitive to inhibition by cyanide, hydrazine, hydroxylamine, and semicarbazide, suggesting that an α-keto group was involved (21). Gunsalus and co-workers observed that unless pyridine and nicotinic acid were added to the synthetic growth medium of Streptococcus faecalis in excess of simple growth requirements no tyrosine decarboxylase activity was produced (4), that the addition of pyridoxal to suspensions of Streptococcus faecalis activated the enzyme (82), and that ATP was necessary, in addition to pyridoxal, to activate the decarboxylase of dried cells (28). Baddiley and Gale (1) further showed that pyridoxal and ATP could replace codecarboxylase in the activation of soluble preparations of amino acid decarboxylases. Umbreit and Gunsalus showed that L-glutamic decarboxylase was activated by codecarboxylase or pyridoxal plus ATP (83). The cofactor was subsequently shown to be pyridoxal-5-phosphate (2, 29).

Amino acid decarboxylases are widespread in nature. The most ubiquitous is glutamic decarboxylase, which occurs in a variety of plants, animals, and microorganisms (16). Among the microorganisms which contain this enzyme are coliforms (19), clostridia, Proteus vulgarius and Proteus

morganii (22), Rhodotorula glutinis (53), and Fusarium vasinfectum (64). The pH optimum of the glutamic decarboxylases of microorganisms is about 4.5. The optimum is characteristically sharp; the enzyme possesses little activity below pH 3 or above pH 6.

Metzler, Ikawa, and Snell (59) proposed in 1954 a theory for the mechanism of action of pyridoxal phosphate-containing enzymes involved in amino acid metabolism. They proposed that a Schiff base is formed between the amino group of the amino acid and the formyl group of pyridoxal phosphate. The steps following Schiff base formation vary depending upon the particular type of enzyme. In the case of glutamic decarboxylase, carbon dioxide splits off from the Schiff base, the resulting amine (γ-aminobutyric acid) is removed by hydrolysis and pyridoxal phosphate is regenerated.

Respiratory system.

The respiratory system of yeast and mammalian mitochondria contains complex succinic and DPNH oxidase enzyme systems. These oxidase systems (succinic and DPNH oxidase, respectively) catalyze the oxidation of succinate and DPNH by molecular oxygen. The succinic oxidase chain of yeast and mammals contains a flavoprotein, succinic dehydrogenase, coenzyme Q and cytochrome b, the antimycin- and BAL-sensitive site or Slater factor, cytochrome c_1, cytochrome c,

and cytochrome oxidase (cytochromes *a* and a_3), in that order (12, 26, 39, 62, 78). Coenzyme Q and cytochrome *b* are both located between succinic dehydrogenase and the Slater factor. However, the exact relationships of coenzyme Q and cytochrome *b* to each other and to the rest of the respiratory chain have not been definitely established. Other substances or groups may be involved in the succinic oxidase chain, e.g., the disulfide bond of succinic dehydrogenase (41, 43). The DPNH oxidase chain begins with another flavoprotein, DPNH dehydrogenase and joins the succinic oxidase chain on the reducing side of the Slater factor. The flavoenzyme which is reduced by substrate, succinic dehydrogenase or DPNH dehydrogenase, is sometimes referred to as the primary dehydrogenase.

The spectrum of the reduced cytochromes of aerobic cells of many species (e.g., mammalian heart or yeast) observed in the microspectroscope contains bands of cytochromes *a* + a_3 at 603 mμ, cytochromes *c* + c_1 at 550(*c*) to 553(c_1), and cytochrome *b* at 563 mμ (40). Other cytochromes have been observed and are summarized in Dixon and Webb, p. 412-413 (16). In a survey of 45 species of fungi, particulate preparations of all species, with minor variations, contained the typical absorption bands of cytochromes *c*+c_1, *b*, and *a*+a_3 (7).

Ferricyanide and PMS serve as electron acceptors from both soluble and particulate succinic dehydrogenase of beef

heart and yeast. Methylene blue, DCIP, TTZ, cytochrome *c*, and oxygen, however, accept electrons only from particulate preparations (37, 75). Methylene blue and DCIP act between the Slater factor and substrate (78), exogenous cytochrome *c* acts between endogenous cytochrome *c* and cytochrome oxidase, and oxygen (39) and TTZ (62) accept electrons from cytochrome oxidase. Ferricyanide, in addition to accepting electrons from the primary dehydrogenase, also accepts electrons between the Slater factor and cytochrome oxidase (18). Incubation of heart muscle preparation in the presence of cyanide results in the loss of all of the methylene blue and DCIP activity (82), but in only one-half of the PMS activity (23, 41, 43). Incubation with cyanide had no effect on the methylene blue activity of brain or yeast mitochondria (23). Incubation with cyanide therefore inactivates the system between the sites at which PMS and methylene blue or DCIP accept electrons.

The relative maximum velocities of the artificial electron acceptors toward the succinic oxidase system of heart muscle preparation have been determined and compared to the rate of reaction with oxygen as electron acceptor (22). The rate with PMS is nearly equal to the rate with oxygen in fresh preparations suggesting that the concentration of primary succinic dehydrogenase limits the overall rate. The other artificial electron acceptors are less efficient in accepting electrons than PMS.

The succinic oxidase system has been reconstituted from soluble succinic dehydrogenase, cytochrome c, and heart muscle preparation, the succinic dehydrogenase activity of which had been inactivated by incubation at basic pH (42, 43). Hatefi has reconstituted the succinic oxidase system from preparations of succinic-coenzyme Q reductase, coenzyme Q-cytochrome c reductase, cytochrome oxidase, and cytochrome c (30). Tissieres (80) formed a complete succinic oxidase system by combining the base-inactivated oxidase of heart muscle preparation or of wild type Neurospora particles with an oxidase deficient Neurospora mutant.

Mitochondria.

Mammalian and plant mitochondria are double-membrane, usually elongated vesicles, 0.5-1.0 μ in length, which occur in the cytoplasm of cells. Lamellae which are continuous with the outer membrane extend into the interior of the mitochondria (68). Yeast mitochondria are similar in structure to the mammalian and plant mitochondria but are somewhat smaller, 0.4-0.6 μ long and 0.2-0.3 μ in diameter (84). Organelles corresponding to mitochondria are not observed in the cytoplasm of bacteria (16).

The cytochrome oxidase activity of the cell is localized exclusively in the mitochondria of plants, animals (16), and fungi (6, 13, 15). Although most of the Krebs

cycle enzymes are not limited to the mitochondrial fraction of animal and plant cells, mitochondria can oxidize all the intermediates of the Krebs cycle (16). The mitochondria of animals and plants also contain the enzymes involved in the β-oxidation of fatty acids, in oxidative phosphorylation, and in the oxidation of reduced DPN and TPN (16).

After disintegration of bacteria, particles ranging in size from less than 0.001 to 0.15 μ in diameter can be separated, which contain the bulk of the cytochrome oxidase activity and the bacterial cytochromes, succinic dehydrogenase and malic dehydrogenase activity, and nearly all of the cellular RNA (16, 60, 79, 81). Thus, biochemically these particles might be considered homologous with mitochondria, although in size and RNA content they resemble the smaller animal and plant microsomes more closely.

Yeast mitochondria oxidize most of the Krebs cycle intermediates and carry out oxidative phosphorylation (67, 85). They are thus morphologically and enzymatically homologous to the mitochondria of plants and animals. Particles that oxidize Krebs cycle derivatives have also been prepared from the hyphae of Allomyces macrogynus (5) and Blastocladiella Emersonii (10). Particles that contain the bulk of the cytochrome oxidase and succinic oxidase activity, and the cytochromes, have been reported in a variety of fungi (6, 7, 13, 15, 35).

If the mitochondrial membrane is disrupted by mechanical means (e.g., sonic disintegration, prolonged homogenization, or freezing and thawing) or osmotically by suspending the particles in water; more than half of the dry weight of liver mitochondria appears in solution, including the entire mitochondrial content of a number of enzymes, including L-glutamic dehydrogenase and fumarase, as well as nucleotide coenzymes (16, 32, 50). Aging the particles for several hours at 0°C. or treating them with 2,4-DNP also results in loss of bound DPN (33).

Increasing the shaking time in a high speed shaker with glass beads resulted in loss of α-ketoglutarate dehydrogenase, isocitrate dehydrogenase (67), and DPN (66) from yeast mitochondria. Further shaking resulted in loss of at least part of all Krebs cycle enzymes to the supernatant. During aging of yeast mitochondria, DPN is also lost (66).

Role of Iron in Succinic Dehydrogenase.

Both the soluble succinic dehydrogenase (36) and succinic-coenzyme Q reductase contain appreciable non-heme iron. Two forms of the soluble dehydrogenase, one with two iron atoms per molecule and one with four iron atoms per molecule have been reported (75). The iron in succinic dehydrogenase is thought to be mainly in the ferric state (56).

Ortho-phenanthroline is an effective chelator of metal ions. It has a high affinity for iron and forms a red ferro o-phenanthrolinate complex with the ferrous iron, which contains three moles of o-phenanthroline per mole of iron. Ortho-phenanthroline partially inhibits beef heart and yeast succinic dehydrogenase (73, 75). Ortho-phenanthroline at a concentration of 5.8×10^{-4} M inhibits the succinic dehydrogenase of C. purpurea about 60 per cent (46, 53). A red ternary, non-dialyzable complex is formed initially with succinic dehydrogenase of beef heart, which is not identical with ferro o-phenanthrolinate (36, 56, 72, 75). After prolonged incubation, the red ferro o-phenanthrolinate compound can be dialyzed, leaving denatured protein behind. The rate of formation of the iron complex with Triton-X is increased by bisulfite, p-chloromercurisulfinate, and urea (56).

Beinert and Lee, studying the electron spin resonance spectrum of submitochondrial particles and soluble succinic dehydrogenase, observed a signal at 1.94 gauss, which appeared upon the addition of succinate and which disappeared if the enzyme were denatured by heat, urea, or acid. The signal was inhibited by fumarate and malonate and was decreased by the addition of cyanide (3). The signal at 1.94 was confirmed by King, Howard and Mason (48) in the soluble succinic dehydrogenase. They also found that the signal decreased faster than PMS activity upon aging and

that the signal was present in the absence of substrate. Since the signal is in a region of the electron spin resonance spectrum characteristic of transition elements, Beinert and Lee (3) have proposed that this signal represents a new type of iron-containing electron carrier in mitochondria.

METHODS AND MATERIALS

Reagents

Semicarbazide, DEAE cellulose, L-malic acid, DCIP, and nicotinamide were obtained from Eastman Kodak Co., Rochester, N. Y.; TTZ and protamine sulfate from Krishell Laboratories, Portland, Ore.; DPNH, DPN, horse heart type III cytochrome c, α-ketoglutaric acid, ADP, PMS, and EDTA were obtained from Sigma Chemical Co., St. Louis, Mo.; glutamic acid was obtained from Mann Research Laboratories, Inc., New York, N. Y.; methylene blue from Coleman and Bell Co., Norwood, Ohio; ATP from Pabst Laboratories, Milwaukee, Wisc.; reagent KCN from Mallinckrodt Chemical Works, St. Louis, Mo.; glutathione and vitamin K from Nutritional Biochemicals Co., Cleveland, Ohio; vitamin E from Merck and Co., Inc.; o-phenanthroline from G. Frederick Smith Chemical Co., Columbus, Ohio; reagent succinic acid from Allied Chemical and Dye Corp., N. Y.; antimycin A from the Wisconsin Alumni Research Foundation, Madison, Wisc.; and fumaric acid from Smith-New York Co., Inc., Freeport, L. I. Potassium ferricyanide from J. T. Baker Chemical Co., Phillipsburg, N. J., was recrystallized three times from water. Buffer salts were reagent grade. Alumina C_γ was prepared by the method of Willstätter and Kraut (87).

In the studies of the respiratory particles and of the soluble succinic dehydrogenase, all reagents and buffers were dissolved in glass-redistilled water and all assay mixtures were diluted to volume in glass-redistilled water.

Culture of C. purpurea

Stock cultures of C. purpurea ATCC 9605 were maintained on potato-dextrose agar (54). Vegetative cultures were grown in 500 ml. Erlenmeyer flasks containing 100 ml. of a sucrose succinate medium (53, 54). When the hyphae were to be used in the study of L-glutamic decarboxylase, the medium was adjusted to pH 6.0 instead of pH 6.5. Each flask was inoculated with 2 ml. of a fully grown culture and incubated on a circular shaker for 48 hours at 25°C.

For the culture of large quantities of hyphae, the following procedure was followed. Ten liters of medium in a 20-liter carboy were inoculated with two 500 ml. flasks of the mycelial suspension described above. The culture was aerated with spargers attached through a cotton filter to an air-pressure line. After 48 hours the hyphae were harvested with a Sharples centrifuge. For the study of L-glutamic decarboxylase the cultures were grown for approximately 72 hours before harvesting.

Preparation of acetone powder

Nine volumes of acetone at -11°C. were added to one volume of hyphae. The mixture was blended for one minute, filtered with suction, rinsed with three volumes of acetone, refiltered, and finally dried under vacuum. Ten liters of medium yielded an average of 200 g. of hyphae (wet weight). This amount of hyphae gave 45 g. of acetone powder.

Sonic disintegration of hyphae

For sonic disintegration a Raytheon 200 watt, 10 kc. magnetostrictive oscillator was used at maximum power (1.2-1.3 amps).

Protein determinations

Protein concentration was determined by a modified biuret method (86). Crystalline serum albumin was used as a standard. Optical densities at 260 mμ and 280 mμ were determined with a Beckman DU spectrophotometer.

Non-fat total solids

The non-fat total solid content of suspensions of hyphae was determined by the following method. Water (9 ml.) was added to one ml. of hyphae. The sample was centrifuged and the supernatant fluid was discarded. Ethanol (95 per cent) was added and the mixture was centrifuged. The solid residue was heated at 75°C. for 6 hours and weighed.

Determination of γ-aminobutyric acid

γ-Aminobutyric acid was determined by the fluorometric method of Lowe et al. (52). Since the original procedure was designed as a micro-method, the quantities of materials prescribed were multiplied by 250 in this experiment. An Aminco-Bowman spectrophotofluorometer was used. Incident light was at 370 mμ and the emitted light was measured at 450 mμ.

Centrifugation

The centrifugal force that is given is the maximum force or the force exerted at the bottom of the centrifuge tube.

Ammonium sulfate fractionation

The amount of ammonium sulfate to add to attain a certain per cent saturation at 0°C. was determined with an equation given by King and Cheldelin (45).

Cytochromes

The cytochrome absorption bands of the respiratory particles were examined with a direct version, short dispersion microspectroscope according to Keilen and Hartree (40).

Measurement of L-glutamic decarboxylase activity

Enzymatic activity was determined by measuring the rate of carbon dioxide evolution using conventional Warburg

techniques at 36°C. The final concentrations in the vessels were 0.3 M phosphate buffer, pH 5.75, 0.04 M pyridoxal phosphate, and 0.02 M L-glutamic acid. The final volume after addition of enzyme and water was 2.5 ml. Activity was expressed as microliters of carbon dioxide evolved in 10 minutes at 36°C., and the Q_{CO_2} as microliters of carbon dioxide per 10 minutes per mg. of protein at this temperature. The specific activity of acetone powders was expressed as microliters per 10 minutes per mg. dry weight.

Determination of Michaelis constant and pH activity curve of L-glutamic decarboxylase

Pyridine-pyridine hydrochloride and glycine-glycine hydrochloride buffers (0.1 M) adjusted to constant chloride concentration with sodium chloride were used in the determination of Michaelis constants and the pH activity curve. The assay conditions were essentially the same as those used by Shukuya and Schwert (69). Michaelis constants and maximum velocities were determined by plotting the reciprocal of velocity vs. the reciprocal of substrate concentration as described by Lineweaver and Burk (51). The range of concentrations of L-glutamic acid used in the determination of the Michaelis constants was 0.02 M to 0.002 M. A Beckman zeromatic pH meter with a device for temperature compensations was used for pH determinations. The pH reported is the pH observed at the end of the assay.

Succinic dehydrogenase activity with artificial electron acceptors.

The activity of succinic dehydrogenase with PMS as acceptor was determined manometrically by the method of Singer (37). In the experiments in which only one concentration of acceptor was used, Warburg vessels contained 0.05 M succinate, 6.5×10^{-4} M PMS, 10^{-3} M KCN, sodium phosphate buffer (either 0.05 M, pH 7.7, or 0.033 M, pH 7.5, as indicated in the results section), enzyme, and water to 3.0 ml. Succinate and PMS were placed in the sidearm and tipped at zero time. Readings were made at 3 to 5 minute intervals and the maximum rate reported. In the methylene blue assay, 1.0×10^{-3} M methylene blue was substituted for PMS.

The system was the same for the spectrophotometric assay of succinic-acceptor reductase activity. All of the components were added to the cuvette except succinate, which was added at zero time. Wavelengths which were used with the various electron acceptors were: ferricyanide, 420 mμ; DCIP, 600 mμ; cytochrome c, 550 mμ. The millimolar extinction coefficients which were used at these wavelengths were 1.03 (79), 19.1 (26), and 19.7 (26), for ferricyanide, DCIP, and cytochrome c, respectively. The optical density change per unit time was divided by the millimolar extinction coefficient (units = $cm.^{-1}mM^{-1}$) and by the light path (1 cm.) to obtain the millimoles acceptor reduced per unit time per liter of solution; i.e., micromoles per unit time

per ml. of solution. This value was divided by the mg. of protein per ml. solution to give the specific activity in micromoles of acceptor reduced per unit time per mg. protein. To convert to the rate of oxidation of succinate the rate of reduction of acceptor was divided by two in the case of ferricyanide, since two moles of ferricyanide reduce one mole of succinate.

Units for respiratory enzymes.

When the enzymes were assayed manometrically, units are expressed as microliters per hour and specific activity is expressed in terms of the Q_{O_2} or microliters per hour per mg. of protein. Experiments in which total non-fat solids instead of protein were used to compute the Q_{O_2} are indicated in the results section. In spectrophotometric assays, the specific activity is given in micromoles of substrate oxidized per hour per mg. protein.

TTZ assay system.

The TTZ assay system contained 10^{-2} M TTZ, 0.06 M sodium phosphate, pH 7.5, 0.075 M succinate, enzyme, and water to a volume of 1.0 ml. After incubation at 35°C., 4 ml. of acetone were added, the mixture was centrifuged, and the optical density at 550 mμ was determined on the Beckman B spectrophotometer. The amount of TTZ reduced was determined by reference to a standard curve.

RESULTS

Amino Acid Decarboxylase Activity of Cell-Free Extracts

By Warburg manometry, the amino acid decarboxylase activity of cell-free extracts of C. purpurea was determined. Of the 21 amino acids tested, only L-glutamic acid was decarboxylated at an appreciable rate. It was therefore of interest to study this enzyme with the following objectives: (1) purification of the enzyme; (2) study of the properties of the enzyme and comparison with the enzyme from other sources; (3) interpretation of data in terms of proposed mechanisms of amino acid decarboxylation.

Properties of Crude Extracts of L-Glutamic Decarboxylase

Preparation of crude extract. For the following preliminary investigations, crude extracts were prepared either after sonic disintegration of the hyphae or from acetone powders. The first preparations were made by suspending the hyphae in 0.05 M phosphate buffer at pH 5.75, disintegrating in a sonic oscillator for 25 minutes, and separating the supernatant fluid after 10 minutes centrifugation at 10,000 x g. The protein concentration of the crude extract was 3 to 5 mg. per ml. and the Q_{CO_2} was 2 to 5.

Crude extracts from acetone powders were made by adding one g. of acetone powder to 30 ml. of 0.66 M

phosphate buffer at pH 8.0. After 30 minutes of stirring at 0°C., the suspension was centrifuged for 30 minutes at 10,000 x g, and the supernatant fraction was separated and retained. This extract had a protein concentration of 6 to 8 mg. per ml. and a Q_{CO_2} of 3 to 7.

Time course of CO_2 evolution. The rate of CO_2 evolution of crude extracts was linear with time for 90 minutes at 30°C. and for 30 minutes at 36°C.

Products of the reaction. The amount of CO_2 evolved was compared to the amount of γ-aminobutyric acid produced. In the Warburg bath under a nitrogen atmosphere, 9.8 micromoles of CO_2 were evolved in 3 hours. The amount of γ-aminobutyric acid produced in this period per Warburg vessel, determined fluorometrically with ninhydrin (52), was 10.7 micromoles. The γ-aminobutyric acid was identified chromatographically by comparison with an authentic sample of γ-aminobutyric acid. The mobile phase was butanol-acetic acid-water (4:1:5) and the spot was located with ninhydrin.

Effect of pyridoxal phosphate on stability. Exposing L-glutamic decarboxylase for a few minutes to temperatures of 55°C. or above resulted in considerable reduction of specific activity as well as in loss of total units. Addition of pyridoxal phosphate plus L-glutamic acid protected the enzyme from inactivation, but L-glutamic acid alone did not (Table 1). It was later found that the addition of

Table 1

EFFECT OF L-GLUTAMIC ACID AND PYRIDOXAL PHOSPHATE ON HEAT INACTIVATION OF L-GLUTAMIC DECARBOXYLASE

Incubation Time (min.)	Additions	µl/30 min.	Q_{CO_2}
0	Glutamate	63	4.0
	Glutamate + pyridoxal phosphate	61	4.0
10	Glutamate	20	2.3
	Glutamate + pyridoxal phosphate	41	7.0
20	Glutamate	13	2.3
	Glutamate + pyridoxal phosphate	18	3.9

Crude extracts of the enzyme were adjusted to pH 7.0 and then incubated at 55°C. The final concentrations of L-glutamic acid and pyridoxal phosphate were 0.15 M and 5×10^{-4} M, respectively. After incubation, the suspension was centrifuged 30 min. at 10,000 x g and the supernatant was used for assays and protein determination.

L-glutamate was not necessary for stabilization by pyridoxal phosphate. Concentrations of pyridoxal phosphate above 0.2 μM gave complete protection. The concentration that half stabilized the enzyme was about 0.1 μM (Table 2). Pyridoxal at a concentration of 10 μM did not stabilize glutamic decarboxylase.

Effect of pH on stability. The stability of the enzyme to heat in the pH range 3.75 to 5.5 was studied using crude extracts (Figure 1). The enzyme was almost totally inactivated at pH 3.75 at 0°C. for one hour. At pH 4.0 only about 20 per cent of the original activity remained. Above this pH the enzyme was stable at 0°C. If the enzyme were heated at 36°C. for 5 minutes, however, loss of activity was observed below pH 4.75. The enzyme was stable above pH 4.75 to 5.0 at 36°C.

Purification of L-Glutamic Decarboxylase

For kinetic studies, the enzyme was further purified. Fractionation with ethanol or acetone and adsorption on calcium phosphate or alumina C_γ gels were ineffective methods of purification. Small amounts of gel were used to remove some impurities however. In some of the first experiments the enzyme was precipitated at pH 5.2 and then extracted twice from the precipitate with 0.2 M phosphate buffer at pH 7.5. This procedure was omitted in later experiments because the yield was low. When

Table 2

THE STABILIZATION OF L-GLUTAMIC DECARBOXYLASE AT VARIOUS CONCENTRATIONS OF PYRIDOXAL PHOSPHATE

µM Pyridoxal phosphate	µl. per hr.
0 (no heat)	29
0	15
0.1	21
0.2	30
2	29
10	31
10 (Pyridoxal)	12

The enzyme (1.8 mg/ml) was heated in the presence of various concentrations of pyridoxal phosphate or pyridoxal for 7 min. at 50°C. The suspension was then assayed without centrifugation (0.9 mg. enzyme per Warburg flask).

Figure 1. Variation of stability with pH in a crude enzyme preparation. Per cent of original activity remaining after heating for five minutes at 36°C. is plotted as a function of pH. The activity before and after heating was assayed at 36°C. using 0.3 M phosphate buffer pH 5.75. Substrate concentration was 0.02 M and concentration of pyridoxal phosphate was 0.04 mM. The protein concentration was 5.6-7.0 mg/ml. After adjusting the pH to 5.75, 0.5 ml. of enzyme was added per Warburg flask for assay. The specific activity of the unincubated samples was 21-33.

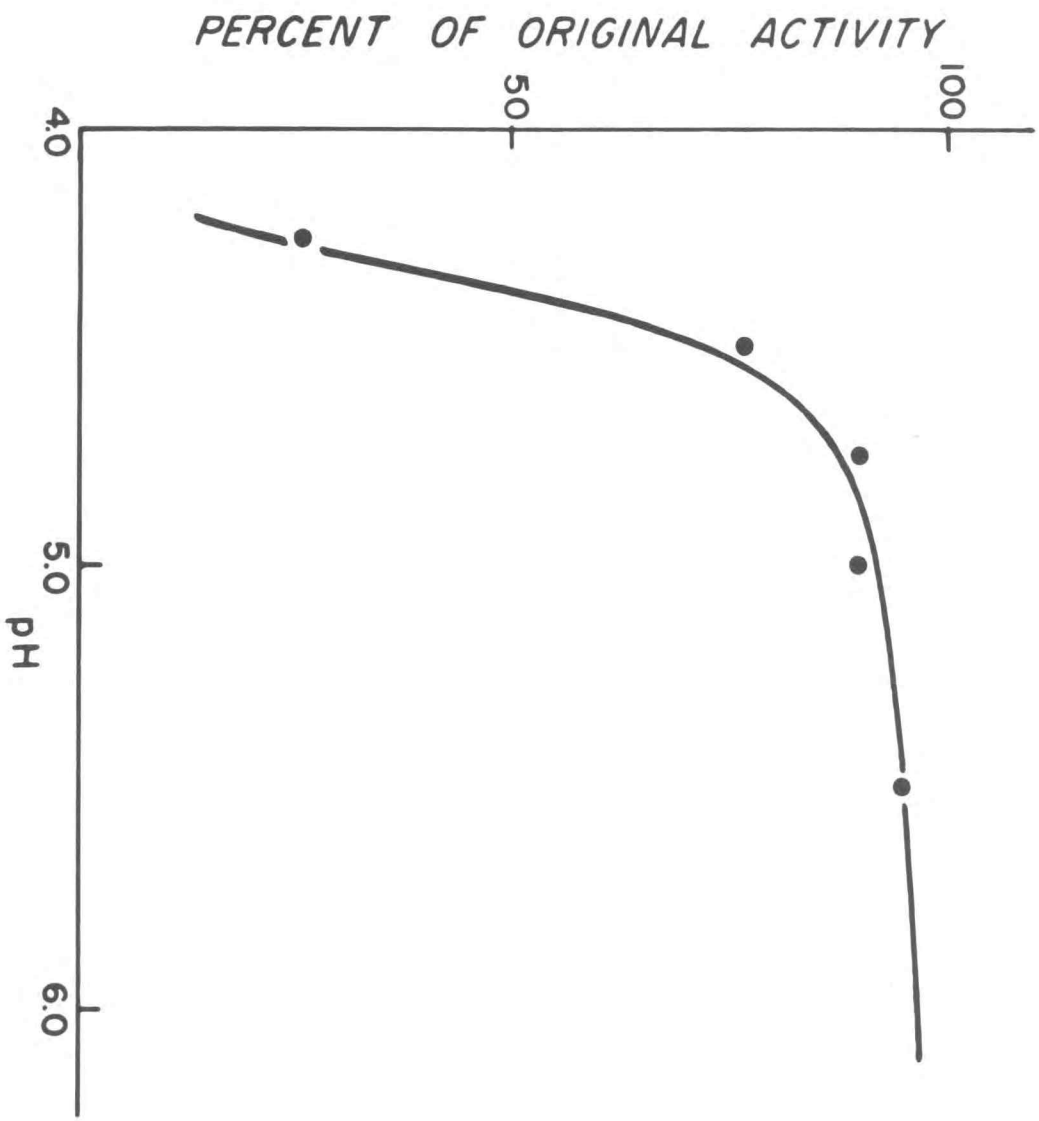

Figure 1

partially purified preparations of the enzyme were adsorbed on DEAE cellulose and the enzyme eluted stepwise with phosphate buffer, the enzyme was released sharply between 0.075 M and 0.1 M sodium phosphate, pH 7.0, with about 68 per cent yield. A yellow peak, presumably flavoprotein, came off at 0.05 M to 0.075 M; just preceding the colorless L-glutamic decarboxylase peak.

The purest fractions obtained with acid precipitation, ammonium sulfate fractionation, negative adsorption with alumina C_γ, and stepwise elution from DEAE cellulose, had a Q_{CO_2} of 630. This preparation was colorless and had no detectable absorption peak in the region 300 to 650 mμ at a concentration of 3.5 mg. per ml.

As a compromise between yield and purification, the following procedure was used for the preparation of L-glutamic decarboxylase for kinetic studies. All procedures were carried out at or near 0°C.

One gram of acetone powder was added with rapid stirring to 30 ml. of 0.06 M phosphate at pH 8.0. After 30 minutes of stirring the suspension was centrifuged for 30 minutes at 10,000 x g. Following the addition of 0.04 micromole of pyridoxal phosphate per ml. of supernatant liquid, the crude extract was heated at 50°C. for 10 minutes. After centrifugation for 20 minutes at 10,000 x g, enough 2 per cent protamine sulfate in 0.06 M phosphate, pH 8.0, was added to produce complete precipitation.

About 0.1 ml. of 2 per cent protamine sulfate was required per mg. of protein.

The resulting heavy precipitate was removed by centrifugation at 10,000 x g for 10 minutes. Solid ammonium sulfate was added to 0.70 saturation. After centrifugation at 10,000 x g for 10 minutes the precipitate was redissolved in the minimum volume of 0.002 M phosphate buffer, pH 7.0, and dialyzed for four hours against six liters of the same buffer. The preparation was dialyzed against an additional six liters overnight. The resulting material was centrifuged at 25,000 x g and the residue was discarded. The supernatant liquid was adjusted to 5.0 mg. of protein per ml. with 0.002 M phosphate at pH 7.0.

Alumina C_γ was added to the enzyme solution (0.34 mg. alumina C_γ per ml. of solution). After centrifugation, the supernatant fraction was brought to 0.40 saturation with solid ammonium sulfate and the mixture again centrifuged. The resulting supernatant liquid was diluted to 0.70 saturation with ammonium sulfate. After centrifugation, the precipitate was dissolved in the minimum volume of 0.02 M phosphate buffer, pH 7.0, and dialyzed for four hours against two two-liter portions of the same buffer. The resulting preparation was centrifuged at 25,000 x g and the precipitate was discarded.

The supernatant liquid was placed on a 1.5 x 30 cm. DEAE cellulose column which had been previously equilibrated

with 0.02 M phosphate buffer, pH 7.0. The enzyme was subjected to gradient elution between 100 ml. portions of 0.02 M and 0.35 M phosphate buffers, pH 7.0. The tubes containing the highest activity were combined and the solution was brought to 0.70 saturation with ammonium sulfate and centrifuged. The precipitate was dissolved in the minimum volume of 0.002 M phosphate buffer, pH 7.0, and dialyzed for two hours against two one-liter portions of the same buffer. The final enzyme preparation was stored at -11°C.

The specific activities and yields at the various stages in the purification procedure are shown in Table 3. The Q_{CO_2} of the final preparation was 111. This represents about a 77-fold purification of the starting material with a 22 per cent overall yield.

Properties of the Purified Enzyme

Stimulation of activity by pyridoxal phosphate. The activity of the enzyme, after fractionation between 37 and 48 per cent saturation with ammonium sulfate, was approximately 78 per cent greater in the presence of pyridoxal phosphate than in its absence. After the complete purification procedure, the activity was about 95 per cent greater in the presence of pyridoxal phosphate. Maximum stimulation of activity was obtained with 5 μM pyridoxal phosphate.

Table 3

PURIFICATION OF L-GLUTAMIC DECARBOXYLASE FROM *CLAVICEPS PURPUREA*

Treatment	Total Units	Q_{CO_2}*	Yield Per cent
Acetone Powder	87,000	1.45	100
Crude Extract	57,000	6.7	66
Heat	60,000	7.1	69
Protamine SO_4	54,000	13.7	62
0.70 $(NH_4)_2SO_4$	38,400	34.3	44
Alumina C_γ	33,600	35.5	39
0.40-0.70 $(NH_4)_2SO_4$	24,800	62.6	29
DEAE cellulose	19,200	111.0	22

*Q_{CO_2} is expressed in µl/10 min./mg. protein except that in acetone powder the Q_{CO_2} is in µl./10 min./mg. dry solid.

Inhibition by hydroxylamine. The addition of 10^{-5} M hydroxylamine caused 80 per cent inhibition of activity. The addition of excess pyridoxal phosphate reversed this inhibition.

Effect of pH on activity. The effect of pH on activity is shown in Figure 2. The pH optimum was 4.8 to 5.2. The activity fell rapidly on either side and was practically zero below pH 3.0 and above pH 7.0. The omission of pyridoxal phosphate from the assay medium had no effect on the pH optimum.

Effect of pH on maximum velocity and Michaelis constant. Michaelis constants, determined by the method of Lineweaver and Burk, were 0.0169 M, 0.0174 M, and 0.0139 M, at pH 4.6, 5.25, and 5.65, respectively. Maximal velocities corresponding to the above Michaelis constants were 104, 104, and 90 µliters per 10 minutes at pH 4.6, 5.25, and 5.65, respectively, using pyridine buffers. K_m at pH 5.75 in 0.3 M phosphate buffer was 0.0140 M. A Lineweaver-Burk plot of the effect of substrate concentration on velocity at pH 5.25 is shown in Figure 3. The K_m at pH 4.6 was the same in the presence or absence of exogenous pyridoxal phosphate.

Oxygen uptake with L-glutamate, pyridoxal phosphate, and manganous ion. During an investigation of the effects of metal ions on L-glutamic decarboxylase activity, oxygen consumption was observed in the presence of L-glutamate,

Figure 2. Variation of activity with pH at 36°C. in purified enzyme preparation. Substrate concentration was 0.01 M. Concentration of pyridoxal phosphate was 0.04 mM. Glycine-glycine hydrochloride and pyridine-pyridine hydrochloride buffers (0.1M) were adjusted to 0.1M chloride ion concentration with NaCl. Each Warburg flask contained 0.36 mg. enzyme.

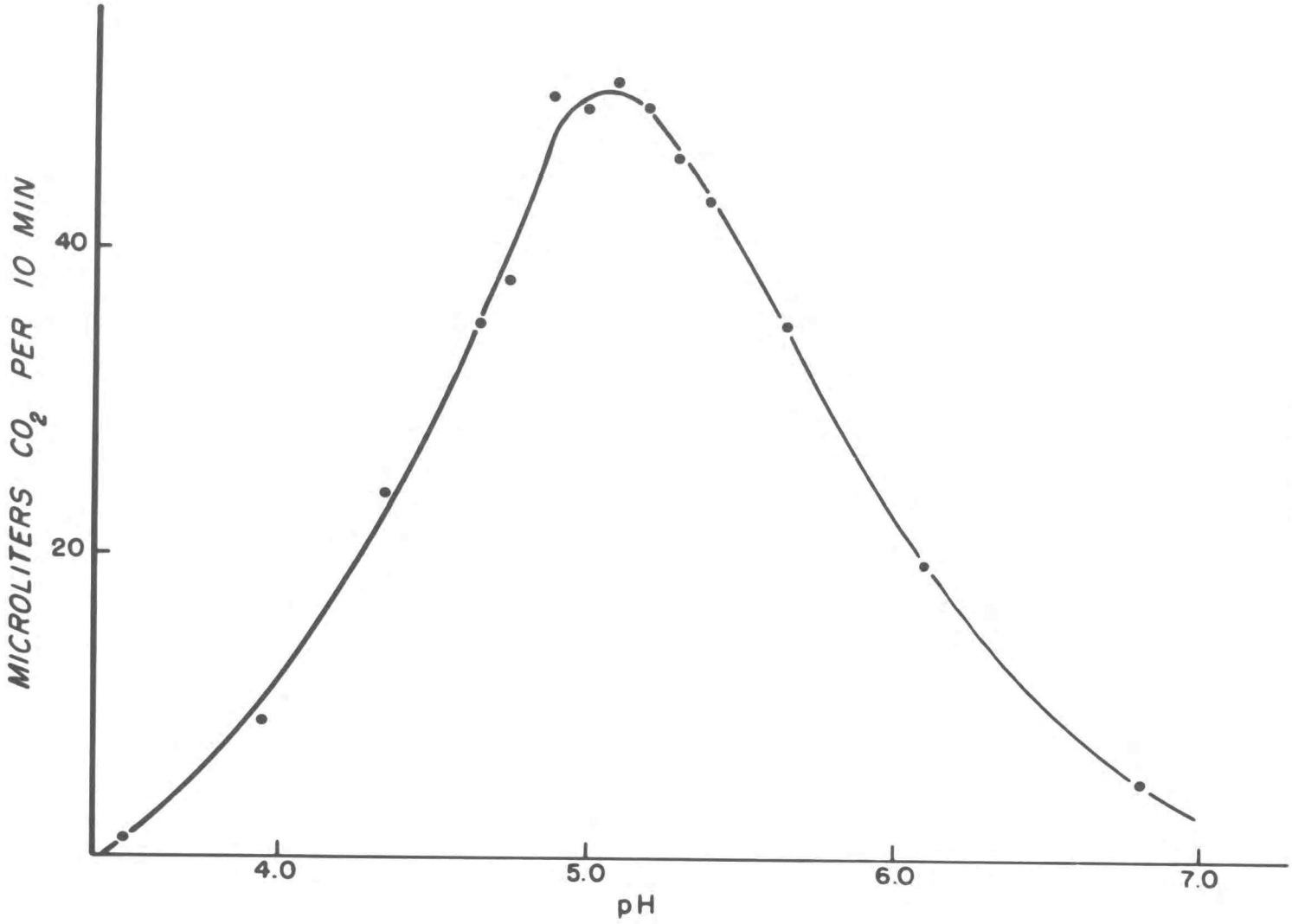

Figure 2

Figure 3. Lineweaver-Burk plot of reciprocal of activity in μliters per ten minutes vs. reciprocal of L-glutamic acid concentration in moles per liter. Concentration of pyridoxal phosphate was 0.04 mM. 0.1 M pyridine-pyridine hydrochloride buffer, pH 5.25, was adjusted to 0.1 M chloride ion concentration with NaCl. Each flask contained 0.34 mg. of protein.

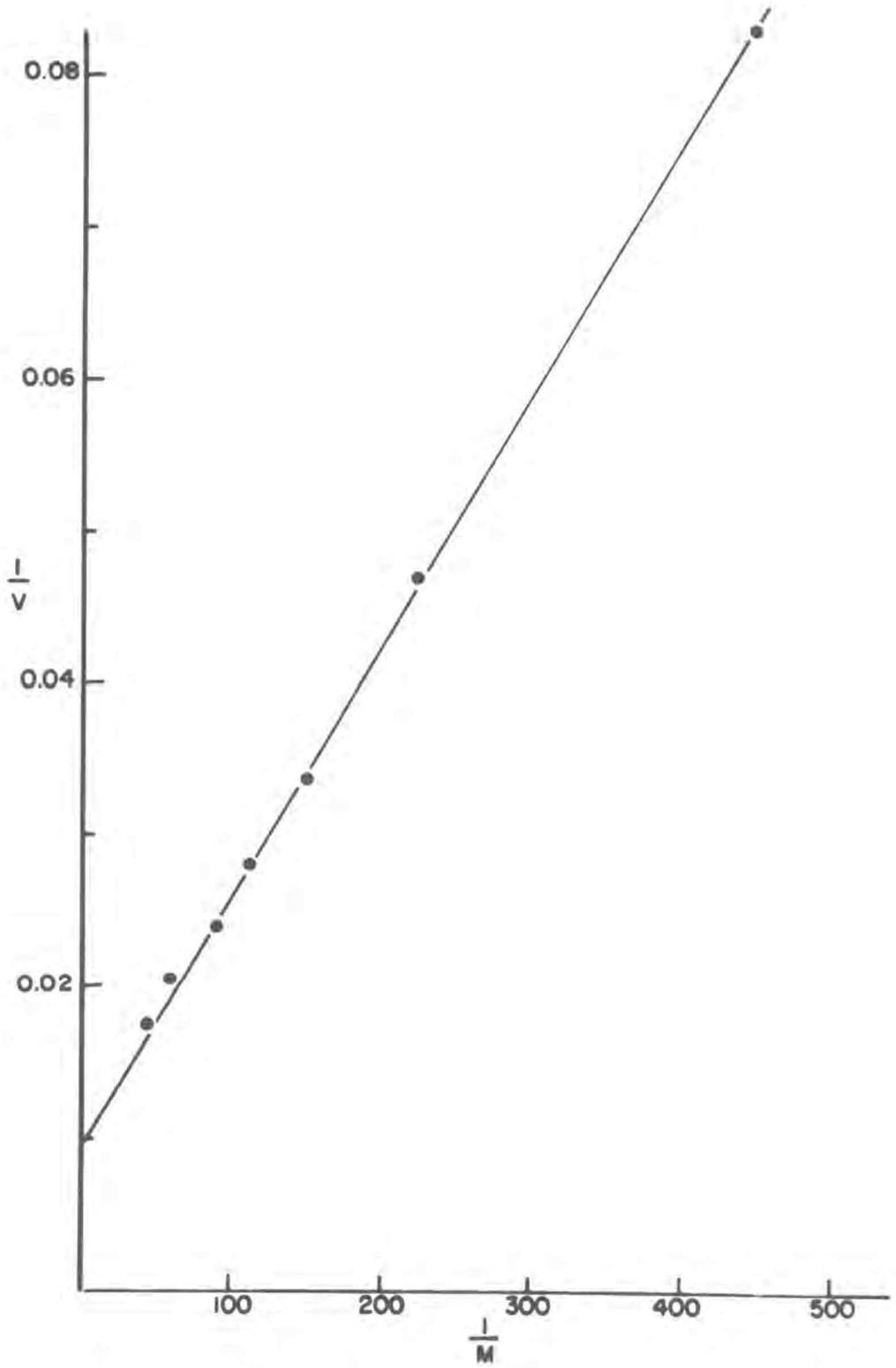

Figure 3

pyridoxal phosphate, and manganous chloride, and in the absence of enzyme. With a system containing 2×10^{-3} M L-glutamate, 2×10^{-3} pyridoxal phosphate, 0.2 M manganous chloride, and 0.16 M phosphate buffer, pH 5.6, in a final volume of 2.5 ml.; 60 microliters of oxygen were consumed per hour per Warburg vessel at 36°C. No oxygen consumption occurred in the absence of L-glutamate, pyridoxal phosphate, or manganous ion. The reaction was accompanied by bleaching of the yellow color of pyridoxal phosphate.

Respiratory Particles of Claviceps purpurea

Activity of particles prepared by various methods. Respiratory particles were prepared from hyphae of C. purpurea by suspending the hyphae in buffered sucrose, disintegrating the cells, and separating a mitochondrial fraction by centrifugation. Disintegration with the sonic oscillator, Potter-Elvehjem homogenizer, sand, or Waring blendor, yielded particles with some succinic oxidase activity. When the cells were blended with alumina, the particles possessed no succinic oxidase activity. Particles blended in the Potter-Elvehjem homogenizer were most active and those disintegrated by sonic irradiation were least active.

Activity of artificial electron acceptors toward succinic dehydrogenase of C. purpurea. For determination of the relative effectiveness of artificial electron

acceptors toward succinic dehydrogenase of C. purpurea, particles were prepared with sonic disintegration. The hyphae were rinsed with and suspended in a sucrose phosphate buffer containing 0.5 M sucrose, 10^{-3} M versene, and 0.1 M sodium phosphate, pH 7.5. The mixture was disrupted sonically for 90 to 120 seconds and centrifuged for 10 minutes at 1,000 x g in a Lourdes centrifuge. The supernatant liquid was centrifuged at 25,000 x g in the Spinco ultracentrifuge (no. 30 head) for 30 minutes. The pellet was resuspended in the sucrose phosphate buffer described above. The relative rates of succinate oxidation by the particles with various electron acceptors compared to PMS are shown in Table 4. Relative rates with soluble succinic dehydrogenase prepared by blending with alumina and ammonium sulfate fractionation (4) are also shown.

Preparation of respiratory particles. Hyphae of 48 hour cultures were washed successively with distilled water, glass-redistilled water, and buffered sucrose solution containing 0.5 M sucrose, 0.1 M sodium phosphate, pH 7.5, and 10^{-3} M versene. The hyphae were suspended in the latter solution and homogenized with 5 to 8 rapid strokes at maximum speed with a power-driven Potter-Elvehjem homogenizer. The resulting suspension was centrifuged at 500 x g for 10 minutes in a Lourdes centrifuge. The supernatant fraction was then centrifuged at 15,000 x g for 10 minutes and the supernatant portion was discarded.

Table 4

RELATIVE ACTIVITIES OF ARTIFICIAL ELECTRON ACCEPTORS TOWARD SUCCINIC DEHYDROGENASE

Electron Acceptor	Relative Activity	
	Soluble	Particles
PMS	(100)*	(100)*
Ferricyanide	3 *	32 *
Methylene blue	3	26 *
DCIP	0.5	18 *

Warburg vessels and cuvettes contained 0.05 M sodium phosphate buffer, pH 7.7, 10^{-3} M KCN, 0.05 M succinate, enzyme, and water to a final volume of 3.0 ml. In the ferricyanide assay 5 mg. of serum albumin was added. The PMS specific activity of the particles was 60 to 110 µl./hr./mg. in various assays. Protein concentration was 1.0-4.0 mg. per 3.0 ml. final volume. The PMS Q_{O_2} of the soluble enzyme was 100 to 350 and 2.4 to 3.2 mg. of protein was added per 3.0 ml. The temperature for the manometric assays of PMS and methylene blue activity was 36°C. The spectrophotometric assays for ferricyanide and DCIP activity were at 25°C. The ranges of concentrations of artificial electron acceptors used in the determination of the V_{max} were: PMS, 1.3-8.1 x 10^{-4} M; ferricyanide, 0.25-1.0 x 10^{-3} M; 0.3-1.3 x 10^{-3} M; 2.0-8.2 x 10^{-5} M. With the soluble enzyme a single concentration of methylene blue, 1.0 x 10^{-3} M, and of DCIP, 4.1 x 10^{-3} M, was used. A Beckman B Spectrophotometer was used for the spectrophotometric determinations. See methods for wavelengths and extinction coefficients.

* Per cent based on V_{max}.

The residue was resuspended in the buffered sucrose solution described above, dispersed with a Potter-Elvehjem homogenizer, and recentrifuged at 15,000 x g for 10 minutes. The supernatant fluid was discarded and the residue was resuspended in buffered sucrose. The succinic oxidase activity of these particles was 40 to 110 microliters of oxygen (4 to 10 micromoles of succinate) per hour per mg. protein at 36°C. The DPNH oxidase activity, measured spectrophotometrically at 340 mμ was 20 to 24 micromoles of DPNH oxidized per hour per mg. at 27°C.

Succinic oxidase activity of hyphae and respiratory particles of C. purpurea. Prior to homogenization, the washed hyphae had appreciable endogenous oxidase activity. The addition of succinate or succinate plus cytochrome c had little or no effect on the rate of oxygen consumption. The respiratory particles, on the other hand, had essentially no endogenous activity. The succinic oxidase activity was also stimulated 3- to 10-fold by the addition of cytochrome c. The amount of succinic oxidase activity recovered was about 3.5 per cent of the endogenous activity (Table 5).

Stability of respiratory particles. The succinic oxidase and DPNH oxidase activity were stable for 4 to 12 hours, after which time the activity decreased rapidly. In vacuo the succinic oxidase activity was stable for one to two days. The addition of vitamin K_1 or E also prolonged

Table 5

COMPARISON OF HYPHAE AND RESPIRATORY PARTICLES OF *CLAVICEPS PURPUREA*

	Mg./ml.	Total sample mg.	Q_{O_2}	Activity µl./hr.	Activity per cent
Hyphae	24	9600.	14	134,000	---
Particles	22	48.	99	4,750	3.5

Warburg vessels contained 0.05 M succinate, 0.033 M sodium phosphate, pH 7.5, 5.1×10^{-5} M cytochrome *c*, 0.5 ml. of suspended hyphae or 0.2 ml. of particles and water to 3.0 ml. Temperature was 36°C. For the hyphae, non-fat solids and the particles, protein, are given in mg./ml. The Q_{O_2} of the hyphae is µl./hr./mg. non-fat solids and of the particles, µl./hr./mg. protein.

the succinic oxidase activity of the particles. The succinic oxidase activity, as well as the succinic-DCIP reductase activity, was completely destroyed after the particles had been incubated at pH 9.4 to 9.5 at 36°C. for 15 minutes.

Efficacy of electron acceptors acting on the oxygen side of the Slater factor. The rate of oxidation of succinate with oxygen as electron acceptor was approximately the same as the rate with PMS as acceptor (Table 6). The activity with cytochrome c as acceptor was 50 per cent of the activity with PMS, and TTZ activity was 2 to 3 per cent of PMS activity.

Effect of incubation with cyanide on succinic-methylene blue reductase activity. Incubation of the particles with 0.05 M potassium cyanide for 13.5 hours at 20°C. had negligible effect on the succinic-methylene blue reductase activity of the particles.

Properties of the succinic-DPNH oxidase system. Some of the properties of the succinic oxidase and DPNH oxidase systems of C. purpurea are summarized in Tables 6 and 7. The DPNH oxidase system was assayed spectrophotometrically, by measuring the reduction of DPNH at 340 mμ, and manometrically. In the manometric determinations DPNH was generated from DPN in the presence of ethanol and alcohol dehydrogenase and the rate of oxygen consumption was measured. The results of the manometric assays closely paralleled the spectrophotometric

Table 6

SUCCINIC OXIDASE SYSTEM OF *CLAVICEPS PURPUREA*

Additions	Q_{O_2}	Micromoles succinate/ hr./mg.	Per cent
Succinate	36	--	--
Succinate + cytochrome *c*	100	--	--
Succinate + cytochrome *c* + KCN	0	--	--
Succinate + cytochrome *c* + Antimycin A	0		
Succinate + cytochrome *c*	99	8.9	97
Succinate + PMS + KCN (V_{max})	204	9.1	--
Succinate + cytochrome *c* + KCN (V_{max})	--	3.6	50
Succinate + PMS + KCN (V_{max})	161	7.2	--
Succinate + TTZ	--	0.13	4
Succinate + PMS + KCN	126	5.6	—

The temperature of all assays was 36°C. Succinic-cytochrome *c* reductase activity was determined using a Zeiss PMQ II spectrophotometer. Warburg vessels contained 2 to 3 mg. protein per 3.0 ml. In the succinic-cytochrome *c* reductase assay the protein concentration was 0.10 mg. per 3.0 ml. and in the succinic-TTZ reductase assay was 1.0 mg. per 1.0 ml. final volume.

All cuvettes and Warburg vessels contained 0.03 M sodium phosphate buffer, pH 7.5 and 0.05 M succinate. Other components were added as follows: 10^{-3} M KCN; 2 µg. antimycin A per mg. protein; 1.3 - 8.1 x 10^{-4} M PMS in the V_{max} determinations, and 6.5 x 10^{-4} M PMS when one level of the acceptor was used; 1.3-7.7 x 10^{-5} M cytochrome *c* in the determination of the V_{max} for succinic-cytochrome *c* reductase activity, and 5.1 x 10^{-5} M cytochrome *c* when one level was used. The conditions for the TTZ assay are described on page 17.

Table 7

DPNH OXIDASE SYSTEM OF CLAVICEPS PURPUREA

Additions	Wavelength mμ	Micromoles Substrate/ hr./mg.
DPNH	340	6.6
DPNH + amytal	340	2.1
DPNH + KCN	340	0.0
DPNH + cytochrome *c* + antimycin A	340	0.0
DPNH + cytochrome *c*	340	20.4
DPNH + cytochrome *c* + KCN	340	14.4
DPNH + cytochrome *c* + KCN	550	14.1
Reduced cytochrome *c* (V_{max})	550	40.5
Succinate + cytochrome *c* + KCN	550	1.9
DPNH + cytochrome *c*	340	20.0
DPNH + cytochrome *c* + succinate	340	15.1

All cuvettes contained 0.03 M sodium phosphate buffer, pH 7.5, and 0.14 mg. protein per 3.0 ml. Other components were added as follows: 10^{-3} M KCN 0.05 M succinate, 4 mM amytal, 5.1×10^{-5} M cytochrome *c*, and 1.5×10^{-4} M DPNH, and 36 micrograms antimycin A per mg. protein. The concentration of reduced cytochrome *c* was 1.0×10^{-5} M to 7.7×10^{-5} M. The temperature was 27°C. All assays were performed using the Model 11 Cary Recording Spectrophotometer.

observations. Both the DPNH and succinic oxidase activity was stimulated 3- to 10-fold by horse heart cytochrome c and was completely antimycin and cyanide sensitive. The DPNH oxidase activity was partially inhibited by amytal. DPNH oxidase activity measured at 340 mμ equalled cytochrome oxidase activity determined by using various concentrations of reduced cytochrome c and making a Lineweaver-Burk plot of the initial rates measured at 550 mμ to obtain a maximum velocity. A linear plot was obtained as expected from the data of Minneart (65). DPNH-cytochrome c reductase activity in the presence of KCN was only slightly less than the DPNH oxidase activity. The addition of succinate reduced the rate of DPNH oxidation measured at 340 mμ and slightly increased the rate of DPNH-cytochrome c reductase activity measured at 550 mμ. Succinate did not increase the rate of oxygen consumption when it was added to the alcohol-alcohol dehydrogenase DPN system. Neither ADP nor ATP appreciably altered either the DPNH or succinic oxidase activity.

Cytochromes. In the presence of succinate and in vacuo, distinct absorption bands at 553 and 560 mμ were visible in the microspectroscope. When cyanide was added, a very faint band could barely be seen at 605 mμ. When dithionite was added all of the bands intensified, although the cytochrome a band was still very faint.

Oxidation of Krebs cycle intermediates. The only Krebs cycle intermediates which were oxidized at an appreciable rate by the particles were succinate, malate, and fumarate and pyruvate combined (Table 8). There was slight fumarate-cytochrome c reductase activity. The rate of reduction of α-ketoglutarate, isocitrate, isocitrate plus TPN, oxalacetate, and pyruvate was zero. In the presence of added ADP, magnesium chloride, TPP, lipoic acid, and coenzyme A, however, there was some activity with α-ketoglutarate and pyruvate.

Sparking of the rate of reduction of cytochrome c was observed in the presence of fumarate or malate upon the addition of pyruvate. The sparking effect was relatively unstable and decreased considerably in 12 hours. Pyruvate sparked the oxidation of oxalacetate only in the presence of cofactors. The rate gradually decreased and was nearly zero in one minute. Likewise the addition of oxalacetate to a cuvette containing malate gradually decreased the rate of cytochrome c reduction to zero in about a minute.

Effect of sonic disintegration on succinic oxidase activity of particles. The ease of solubilizing succinic dehydrogenase by sonic disintegration or by freezing and thawing followed by blending with alumina (46, 54) prompted a study of the effects of sonic disintegration of the respiratory particles on the distribution of succinic

Table 8

OXIDATION OF KREBS CYCLE INTERMEDIATES
BY PARTICLES OF *CLAVICEPS PURPUREA*

Substrate	Micromole cytochrome *c*/hr./mg. 0-6 hr. -cofactors	0-6 hr. +cofactors	12-13 hr. -cofactors
Malate	20	9	21
Malate + pyruvate	42	35	20
Malate + pyruvate + antimycin A		0	--
Fumarate	2	1	0
Fumarate + pyruvate	25	16	4
Fumarate + pyruvate + OAA*	--	2	--
OAA*	0	0	--
OAA + pyruvate	0	11	--
OAA + pyruvate (1 min.)	--	1	--
α-Ketoglutarate	0	3	--
Succinate	5.5	--	--
Succinate + OAA	0	--	--
Isocitrate	0	--	--
Isocitrate + TPN	0	--	--
Malate	12	--	--
Malate + OAA	8	--	--
Malate + OAA (1 min.)	0	--	--

The concentrations of substrates, cytochrome *c*, KCN, buffer, and DPN, the wavelength, and temperature, are the same as in the previous table. The concentration of antimycin A was 33 µg./mg. of protein, and of TPN was 1.4×10^{-4} M. The concentrations of cofactors were 0.04 M ADP, 7.2×10^{-4} M TPP, 8.7×10^{-4} M coenzyme A, 6.5×10^{-3} M lipoic acid, and 0.03 M $MgCl_2$. Cuvettes contained 0.12 mg. protein per 3.0 ml.

*OAA = oxalacetate

dehydrogenase between the soluble and particulate fractions. Since particles prepared from sonic extracts had relatively low oxidase activities compared to particles prepared by other methods, a study of the effects of sonic disintegration on activity with other acceptors was also undertaken. For comparison, particles prepared in the identical manner from beef heart were also studied.

About 7 g. of minced beef heart was homogenized in 30 ml. of the sucrose-phosphate solution (rf. page 35). Particles were then isolated by the exact method used to isolate particles from C. purpurea. The particles were diluted with sucrose-phosphate solution to about 3 mg. per ml. and 10 ml. were disintegrated with the sonic oscillator and then centrifuged at 143,000 x g in the Spinco (no. 40.2 head) for one hour. The residue was resuspended in sucrose-phosphate buffer. The entire procedure was repeated with a new preparation.

The succinic-PMS and succinic-cytochrome c reductase activities of the various preparations is shown in Table 9. Sonic treatment for 15 minutes resulted in a 56 per cent loss in PMS activity of particles derived from C. purpurea. The particles prepared from beef heart, to the contrary, were twice as active with PMS after sonic treatment. Most of the PMS activity of C. purpurea (73 per cent) became soluble after sonic treatment whereas only 27 per cent of the PMS activity from beef heart was

Table 9

EFFECT OF SONIC DISINTEGRATION ON THE RESPIRATORY PARTICLES OF *CLAVICEPS PURPUREA* AND BEEF HEART

Fraction	Mg./ml.	Q_{O_2}	PMS Activity	Cytochrome *c* µmoles/ hr./mg.	Cytochrome *c* Activity
C. Purpurea					
Particles	--	117	--	9	--
Sonic-treated particles	2.8	66	(100)	0	--
Residue	1.9	20	28	0	--
Supernatant fraction	0.8	184	73	0	--
Beef Heart					
Particles	--	172	--	16	--
Sonic-treated particles	2.9	364	(100)	10	(100)
Residue	1.7	423	70	13	79
Supernatant fraction	1.0	278	27	2	7

Warburg flasks contained 0.5 ml. of untreated and sonically disintegrated particles plus 0.5 ml. phosphate (0.1 M, pH 7.5)-sucrose (0.5 M). The residue and supernatant fractions (1.0 ml. each) were added without phosphate-sucrose. Succinate (0.05 M) 6.5×10^{-4} M PMS, 10^{-3} M KCN, and water were added to a final volume of 3.0 ml. The temperature was 36°C. The spectrophotometric assays were run at 27°C. at 550 mµ. The cuvettes contained 0.1 ml. enzyme, 10^{-3} M KCN, 0.05 M succinate, 0.033 M sodium phosphate, pH 7.5 and 5.1×10^{-5} M cytochrome *c* in a final volume of 3.0 ml.

solubilized. The PMS specific activity of the C. purpurea supernatant fraction was greater than whole sonic-treated particles; the specific activity of the supernatant fraction derived from beef heart particles was less.

The succinic-cytochrome c reductase activity of the particles of C. purpurea was destroyed completely by sonic treatment, but the succinic-cytochrome c reductase activity of beef heart was only decreased 40 per cent. The trend of the succinic oxidase activity of beef heart and C. purpurea particles was parallel to the effect on succinic-cytochrome c reductase activity.

To localize the break in the respiratory chain which resulted in loss of succinic-cytochrome c reductase activity the effect of time of sonic treatment on activity with PMS, DCIP, and cytochrome c, was determined (Table 10). After an initial drop in activity, the PMS activity remained fairly constant. The DCIP and cytochrome c reductase activity, however, continued to decrease to nearly zero in 10 minutes.

Inhibition of Soluble Succinic Dehydrogenase by o-Phenanthroline

Effect of preincubation. Preincubation had little effect on the inhibition of succinic-PMS reductase activity by o-phenanthroline. The per cent inhibition by 6.0×10^{-4} M o-phenanthroline was 51 per cent immediately after 5

Table 10

EFFECT OF TIME OF SONIC DISINTEGRATION ON SUCCINATE OXIDATION BY RESPIRATORY PARTICLES OF *CLAVICEPS PURPUREA*

Disintegration Time Minutes	Electron Acceptor		
	PMS[1]	DCIP[2]	Cytochrome c[2]
0	152	1.2	8.1
1	91	0.8	3.0
2	101	0.65	2.5
5	90	0.2	0.7
10	87	<0.02	<0.1
15	95	<0.02	<0.1

Protein concentration of particles was 2.9 mg./ml. Warburg vessels contained 0.05 M succinate, 0.05 M sodium phosphate, pH 7.7, 10^{-3} M KCN, 6.5×10^{-4} M PMS, 0.1 ml. enzyme, and water to 3.0 ml. The temperature was 36°C. In the DCIP assay the cuvettes contained 0.033 M sodium phosphate, pH 7.5, 10^{-3} M KCN, 0.2 ml. enzyme, and 6.2×10^{-4} M DCIP per 3.0 ml. final volume. The wavelength was 600 mμ. The assay system for the measurement of succinate-cytochrome c reductase was the same as in the previous table. The temperature for spectrophotometric measurements was 27°C.

[1]Activity is expressed in microliters oxygen/hr./mg. protein.

[2]Activity is expressed in micromoles succinate/hr./mg. protein.

minutes equilibration at 36°C. and was 59 per cent after 90 minutes preincubation at 36°C.

Type of inhibition. It was previously found that the inhibition of C. purpurea succinic dehydrogenase is not reversed by succinate, i.e., the inhibition is non-competitive with succinate (46, 54). To test whether the inhibition by o-phenanthroline was noncompetitive with PMS, reciprocal plots of activity vs. concentration of PMS at 1.0×10^{-4} M and 6.0×10^{-4} M o-phenanthroline (Figure 4) were made according to the method of Lineweaver and Burk (51). The concentration of succinate was 0.05 M throughout. The figure shows that the inhibition by o-phenanthroline is noncompetitive with PMS since the lines do not meet at the abscissa (infinite PMS concentration). The K_i was 6.0×10^{-4} M at 6.0×10^{-4} M o-phenanthroline and approximately 4.8×10^{-4} M at 1.0×10^{-4} M o-phenanthroline.

Inhibition with other chelators. The inhibition of C. purpurea succinic dehydrogenase by o-phenanthroline, α, α'-dipyridine, and 8-hydroxyquinoline, are compared in Table 11. At 10^{-3} M, only o-phenanthroline inhibited the enzyme. At 10^{-2} M all of the chelators were inhibitory. The contents of the Warburg vessels became very turbid at the higher level of the chelators at 36°C.

Reconstitution of succinic oxidase. Several attempts were made to reconstitute succinic oxidase from soluble

Figure 4. Lineweaver-Burk plot of reciprocal velocity vs. reciprocal of PMS concentration at two concentrations of o-phenanthroline. The assay system consisted of 0.05 M succinate, 10^{-3} M KCN, 0.05 M sodium phosphate, pH 7.7, 6.5×10^{-4} M PMS, o-phenanthroline as indicated, 0.2 ml. enzyme (12 mg. per ml.) and water to 3.0 ml. The temperature was 36°C. (A - no o-phenanthroline; B - 1.0×10^{-4} M o-phenanthroline; C - 6.0×10^{-4} M o-phenanthroline).

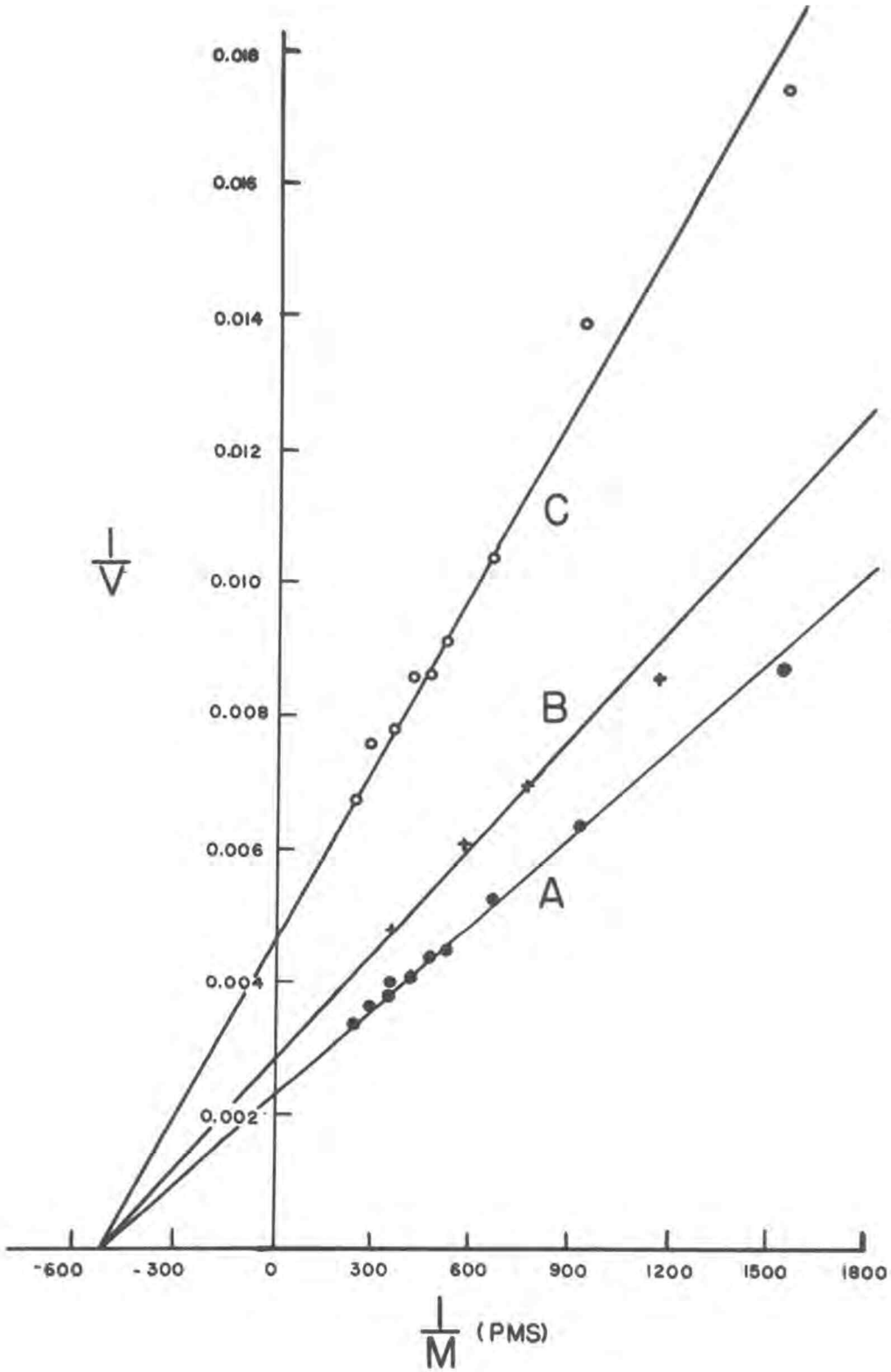
0.018
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.002
1/V
C
B
A
-600
-300
0
300
600
900
1200
1500
1800
1/M (PMS)

Figure 4

Table 11

INHIBITION OF SOLUBLE SUCCINIC DEHYDROGENASE OF *CLAVICEPS PURPUREA* BY CHELATORS

Inhibitor	Concentration Moles/liter	Per cent Inhibition
o-Phenanthroline	10^{-3}	62
	10^{-2}	100
8-Hydroxyquinoline	10^{-3}	7
	10^{-2}	98
α, α'-Dipyridine	10^{-3}	0
	10^{-2}	73

The assay system consisted of 0.05 M succinate, 10^{-3} M KCN, 0.05 M sodium phosphate, pH 7.7, 6.5×10^{-4} M PMS, inhibitors as indicated, 0.4 ml. enzyme containing 6.8 mg. of protein (Q_{O_2} equals 67), and water to 3.0 ml. The temperature was 36°C.

succinic dehydrogenase prepared by blending of the hyphae with alumina and ammonium sulfate fractionation (46, 54), cytochrome c and oxidase. The oxidase preparation was made by incubating either C. purpurea respiratory particles or Keilin-Hartree heart muscle preparation for 15 minutes at pH 9.4 to 9.5. No reconstitution of succinic oxidase activity was observed. Even with the addition of succinate before blending with alumina and dialysis under nitrogen, the soluble enzyme was still inactive as far as reconstitution was concerned.

Stimulation of Oxygen Consumption by Ascorbate and Cytochrome c in the Presence of High Levels of Cyanide

A few preliminary manometric measurements of cytochrome oxidase activity were made using ascorbate and cytochrome c. In the course of these determinations a nonenzymatic stimulation of oxygen consumption occurred when high levels of cyanide were added (Table 12).

Table 12

EFFECT OF CYANIDE CONCENTRATION ON THE NONENZYMATIC OXIDATION OF A SYSTEM CONTAINING CYTOCHROME c AND ASCORBATE

KCN Concentration moles/liter	Microliters per hour
--	32
10^{-4}	35
3×10^{-4}	31
10^{-3}	35
3×10^{-3}	53
10^{-2}	62

Warburg vessels contained 5.1×10^{-5} M cytochrome c, 2.8×10^{-2} M ascorbate, 0.07 M sodium phosphate, pH 7.5, and water to 3.0 ml. Temperature was 36°C.

DISCUSSION

L-Glutamic Decarboxylase of C. purpurea

General Properties. The properties of L-glutamic decarboxylase from C. purpurea were found to be similar to those of the enzyme from other microbial sources. In common with the decarboxylase from E. coli, purified preparations of the enzyme were stable, and had higher activity when exogenous pyridoxal phosphate was added (69). The activity in the C. purpurea enzyme system was 95 per cent greater when pyridoxal phosphate was added compared to only 25 per cent in the E. coli system, indicating that the C. purpurea decarboxylase had been depleted of the cofactor to a greater extent.

Effect of pH on activity. The pH-activity curve of L-glutamic decarboxylase from C. purpurea corresponds closely to the curves obtained by Najjar and Fischer (63) of the enzyme in E. coli, although Najjar and Fischer used acetate buffers, in contrast to pyridine and glycine buffers for the present study.

The decrease in enzyme activity observed as the pH is increased above the optimum or decreased below it may be due to one of three causes: (1) irreversible loss in activity due to instability of the enzyme in this pH range, (2) decrease in affinity of enzyme for substrate caused by reversible conversion of free enzyme or substrate into

an inactive form, or (3) decrease in V_{max} as a result of the reversible formation of an inactive enzyme-substrate complex.

Effect of pH on stability. The activity of the enzyme fell rapidly as the pH was lowered below the optimum. There was practically no activity at pH 3.5. The enzyme was also progressively more labile as the pH decreased. Activity was almost completely destroyed after exposure of the enzyme to a pH of 3.75. Epps reported loss of activity by L-tyrosine decarboxylase at pH 4.5 (17). The optimum pH for the enzyme was 5.5. The data are consistent with the conclusion that the decrease in activity below the optimum is due to inactivation of the enzyme.

In contrast to the instability of the enzyme in acid solution, the enzyme was stable above 4.8, and the decrease in activity above the optimum must therefore be the result of some factor other than the denaturation of the enzyme.

Michaelis constants. The Michaelis constants of the enzyme for the substrate from C. purpurea are quite similar in magnitude to those reported by Najjar and Fischer (63) in E. coli, despite the difference in buffers used. The K_m values of the enzyme in C. purpurea were practically constant over the pH range of 4.6-5.75. This is consistent with the observation of King and Lucas (44) that the acidity had little effect on the stability constant for the

formation of the Schiff base from glutamic acid and pyridoxal phosphate.

The K_m values of L-glutamic decarboxylase of C. purpurea and those of E. coli reported by Najjar and Fischer are shown in Table 13. From the low K_m at pH 5.0 they concluded that the free enzyme is in an inactive form at pH 4.0, an active form at pH 5.0, and an inactive form at pH 5.6. The reduction in K_m near pH 5.0 was not observed in the enzyme system of C. purpurea. This would suggest that neither the free enzyme nor the substrate change to an inactive form above or below the pH optimum. Further data will be needed to see whether the differences in data and interpretation can be resolved. It is not likely that the discrepancy represents a basic difference in the mechanism of action of the enzyme from C. purpurea and E. coli.

Effect of pH on velocity. The rate of decarboxylation at a concentration of 0.01 M glutamic acid decreased with increasing pH above 4.8-5.2, whereas the K_m for glutamic acid was essentially constant over the same pH range. A decrease of affinity of enzyme for substrate would be expected to result in an increase in the K_m. The maximum velocity, which is not affected by the affinity, was also less at pH 5.65 than at 5.25. Therefore, the decrease in activity is not due to a change in affinity of enzyme for substrate. Neither is it due to instability of the enzyme

Table 13

K_m OF L-GLUTAMIC DECARBOXYLASE AT VARIOUS pH VALUES

C. purpurea		*E. coli* (63)	
pH	K_m(M)	pH	K_m(M)
4.6	0.0169	4.0	0.0166
5.25	0.0174	5.0	0.0045
5.65	0.0139	5.6	0.0143
5.75	0.0140		

at higher pH, since the enzyme can be heated at 50°C. at pH 7.5 without loss of activity, but is essentially inactive at pH 7.5. The most likely reason for the reduction in activity over the pH range 5.2 to 7.0 is that the enzyme-substrate complex is changing to an inactive form through loss of a proton. If maximum velocities at several pH values within this range were available, it would be possible to calculate the pH for this transition. Lacking these data, the pH was estimated from the half-maximum velocity in the pH activity curve and from the limited number of maximum velocities which were determined by use of the Henderson-Hasselbalch equation. The pH values obtained by the two methods were 5.8 and 6.5, respectively, suggesting that the pH for dissociation of the enzyme-substrate complex occurs in this pH range.

The pH for the dissociation of the pyridinium ion in a Schiff base of pyridoxal with valine is 5.9 (58), and of pyridoxal phosphate with valine is 6.3 (14). It is therefore possible that the decrease in activity above the pH optimum results from conversion of the pyridinium form of the enzyme-substrate complex to the uncharged pyridine ring.

Stabilization by pyridoxal phosphate. The stabilization of D-amino acid oxidase by FAD studied in detail by Burton (9) is analogous to the stabilization of L-glutamic acid by pyridoxal phosphate. By comparing the

stabilization constants (Stabilization constant = concentration of the substance at which one-half the enzyme activity is lost that would have been lost without the substance.) and K_m's of FAD and alanine for D-amino acid oxidase, Burton was able to show that the stabilization is related to the affinity of cofactor or substrate for the enzyme (or apoenzyme). Boyer et al. (8) had previously shown that the stabilization constant of caprylate for albumin was equal to the affinity of caprylate for albumin.

The K_m of C. purpurea glutamic decarboxylase for pyridoxal phosphate was not determined. However, the K_m of 3×10^{-7} M of pyridoxal for tyrosine decarboxylase of inactive Streptococcus faecalis whole organisms (27) is fairly close to the concentration of pyridoxal phosphate for half-stabilization of 1×10^{-7} M observed for C. purpurea, supporting a relationship between affinity and stabilization. L-glutamate did not appear to protect the enzyme. This may be because its primary binding site is on the aldehyde group of pyridoxal phosphate rather than on the apoenzyme.

The phosphate group of pyridoxal phosphate has no effect on the nonenzymatic reactions with amino acids (58). However, analogous enzymatic reactions occur only with pyridoxal phosphate. Pyridoxal is inactive. This evidence has pointed to the conclusion that the phosphate group

functions as a point of attachment of the cofactor to the apoenzyme.

The relationship between binding and stabilization gives a means of determining the role of the phosphate group, since binding of enzyme to substrate (or apoenzyme to cofactor) is necessary for stabilization to occur. Pyridoxal did not stabilize the apoenzyme of C. purpurea and hence did not bind to the apoenzyme. Since pyridoxal phosphate did stabilize the apoenzyme, the phosphate group is therefore necessary for the binding of pyridoxal phosphate to L-glutamic decarboxylase apoenzyme.

Nonenzymatic oxidation with pyridoxal phosphate, manganous ion, and L-glutamic acid. The rapid, nonenzymatic consumption of oxygen with pyridoxal phosphate, manganous ion, and L-glutamic acid is probably analogous to the reactions reported by Ikawa and Snell (34) and by Mazelis (57).

Ikawa and Snell first reported the oxidative deamination of amino acids by pyridoxal and metal salts. After 30 minutes at 100°C. the products from the reaction of glutamic acid with pyridoxal in the presence of copper ion were ammonia and α-ketoglutarate. Pyridoxal was destroyed in the course of the oxygen consumption, as determined by manometric experiments. The high temperature employed by Ikawa and Snell is not necessary, since the rate of oxygen consumption under appropriate conditions

was found to be rapid at 36°C. Repeating the determinations made by Ikawa and Snell with the present system at 36°C. and making simultaneous manometric measurements might help to further characterize this reaction.

Mazelis measured the rate of reduction of iodine spectrophotometrically at room temperature in the presence of pyridoxal phosphate, amino acid, and manganous ion. Because his system was modified by the addition of iodine and by use of light to activate the reaction, it is difficult to compare the manometric measurement of oxygen consumption with his data.

Respiratory System

General properties. The respiratory particles of C. purpurea have many properties in common with plant and mammalian mitochondria; i.e., the appearance of the cytochromes in the microspectroscope, the cyanide sensitivity of cytochrome oxidase, the antimycin sensitivity of succinic- and DPNH-cytochrome c reductase, the antimycin insensitivity of succinic-DCIP reductase activity, and the amytal sensitivity of DPNH oxidase activity. Succinic oxidase and DPNH oxidase are not separate chains since the two activities are not additive when both substrates are added simultaneously. This too is a common characteristic of the particulate respiratory systems of plants and mammals, e.g., the Keilin-Hartree heart

muscle preparation. The ability of mammalian cytochrome c to serve as electron donor and acceptor in the C. purpurea oxidase system also suggests similarities between the two systems, although it is possible that mammalian cytochrome c is acting merely as an artificial electron acceptor and donor. However, bacterial cytochrome systems which are markedly different from the common systems of plants and mammals cannot use mammalian cytochrome c as electron acceptor and so the ability of the C. purpurea system to use mammalian cytochrome c may have some functional significance.

There was no evidence for microsomal antimycin-insensitive DPNH-cytochrome c reductase or various cytochromes common to bacteria.

Cytochrome oxidase activity. There appears to be a correlation between the relative intensities of the cytochrome bands and the ratio of DPNH oxidase to cytochrome oxidase activity in heart muscle preparation and in C. purpurea respiratory particles. The a band of C. purpurea particles was very faint compared to the b and c bands, whereas the intensity of the a band of heart muscle preparation was the same as the b and c bands (38). The DPNH oxidase activity was just equal to cytochrome oxidase activity in C. purpurea particles, whereas the cytochrome oxidase activity of Keilin-Hartree heart muscle preparation was 4 to 5 times the DPNH oxidase

activity (77). The cytochrome a band in the spectrum of respiration particles was due to cytochrome oxidase. Evidently, relative to other components of the respiratory system, the C. purpurea respiratory particles contain less cytochrome oxidase than heart muscle preparation does.

Limiting reaction in succinic oxidase. The succinic oxidase and succinic-PMS reductase activities were nearly equal. These two activities are also roughly equal in Keilin-Hartree heart muscle preparation (22). It would appear, as proposed by Singer for heart muscle preparations, that the limiting reaction in succinic oxidase of C. purpurea is the reaction with succinic dehydrogenase. The limiting nature of the succinic dehydrogenase of C. purpurea particles is further emphasized by the low succinic-cytochrome c reductase activity compared to the amount of DPNH-cytochrome c reductase activity. The succinic-cytochrome c reductase activity of C. purpurea respiratory particles was only about 13 per cent of the DPNH-cytochrome c reductase activity. In the Keilin-Hartree heart muscle preparation, the succinic- and DPNH-cytochrome c reductase activities are about equal. The low succinic dehydrogenase activity may be due to the solubilization of succinic dehydrogenase during disintegration of the hyphae of C. purpurea. MacDonald et al. (46, 54) have shown that the succinic dehydrogenase of C. purpurea is easily solubilized. Experiments, which

will be discussed later, confirmed this observation.

Comparison of respiratory particles with mitochondria. The fact that Krebs cycle enzymes are associated with the particles indicates that they are analogous to the mitochondria in other organisms. Like yeast and brain mitochondria (23), the particles exhibited little or no inactivation by cyanide (Tsou effect). The Tsou effect is observed in the simpler Keilin-Hartree heart muscle preparation.

The respiratory particles of C. purpurea possessed very little endogenous oxidase activity and required absolutely the addition of exogenous DPN for the oxidation of DPN-linked substrates. Also, some of the Krebs cycle activities appeared to be absent, or nearly so; i.e., fumarase, α-ketoglutarate dehydrogenase, and isocitrate dehydrogenase. These properties are characteristic of aged or otherwise damaged mitochondria of other organisms, e.g., beef heart and yeast mitochondria, and suggest that the respiratory particles of C. purpurea have lost enzymes and coenzymes through the mitochondrial membrane during their preparation.

Permeability of respiratory particles to PMS. The increase in succinate-PMS reductase activity after sonic disintegration of beef heart mitochondria appears to be due to the breaking of the mitochondrial membrane to permit access of PMS to the dehydrogenase. Singer previously has

reported that the addition of calcium, which causes swelling of mammalian mitochondria resulted in an increase in succinic-PMS reductase activity (76). He concluded that calcium increased the permeability of the mitochondria to PMS much as sonic disintegration has done in the present experiment.

With the C. purpurea particles the PMS activity decreased somewhat. This indicates that in contrast to the beef heart mitochondria, there was no permeability barrier to PMS by C. purpurea respiratory particles. The ability of PMS to enter the respiratory particles of C. purpurea is probably related to the exit of DPN and some of the Krebs cycle enzymes and suggests that the particle, as isolated, is unusually permeable to a variety of compounds.

Effect of sonic disintegration on succinic oxidase system. The succinic-cytochrome c reductase activity of C. purpurea was gone after 15 minutes of sonic disintegration. The succinic-DCIP reductase was also inactive and the succinic-PMS reductase activity was mainly in the soluble fraction. The loss of succinic-cytochrome c reductase activity of beef heart particles from sonic disintegration was only about 37 per cent, and the degree of solubilization of succinic dehydrogenase from beef heart particles was also much less.

The loss in succinic-cytochrome c reductase activity of C. purpurea can be attributed to solubilization of

succinic dehydrogenase. The residual succinic-PMS reductase activity in the particles could be due to nonspecific binding of soluble succinic dehydrogenase to the particles, or to the fact that the soluble succinic dehydrogenase has been inactivated for DCIP and cytochrome c but not for PMS as acceptor.

The small amount of soluble succinic-cytochrome c reductase activity after sonic disintegration of beef heart particles is of some interest since it is antimycin sensitive. The small residual succinic-cytochrome c reductase in the soluble fraction from C. purpurea particles was antimycin insensitive.

It was mentioned earlier that the properties of the succinic and DPNH oxidase systems of C. purpurea were common among particulate oxidase systems in plants and mammals. On the other hand, the structural lability of the particles as reflected in the ease of solubilization of succinic dehydrogenase and the permeability of fresh particles to various substances is quite different from mammalian (beef heart) mitochondria.

The question may be asked why respiratory particles of beef heart and C. purpurea have such different permeability characteristics, and such differences in binding power for succinic dehydrogenase. The lipid "cement" of the mitochondria is definitely implicated and a comparative quantitative and qualitative study of the lipids of

mammalian and C. purpurea respiratory particles may reveal some striking differences.

Reconstitution of succinic oxidase of C. purpurea. Succinic oxidase activity could not be regained by combining C. purpurea or beef heart oxidase and soluble succinic dehydrogenase of C. purpurea. King (47) has observed that the ability of beef heart succinic dehydrogenase to reform the succinic oxidase system is even more labile than the succinic-PMS reductase activity. However, the recombination of components was done within 3-4 hours of the beginning of the experiment and in some experiments the soluble enzyme was kept under nitrogen during most of the experiment. Furthermore, the soluble succinic dehydrogenase of C. purpurea is exceptionally stable (46, 54) compared to beef heart succinic dehydrogenase as far as activity with PMS as acceptor is concerned. While this does not give direct information about the stability of the soluble succinic dehydrogenase of C. purpurea for reconstitution, it would be somewhat surprising to find exceptional stability with PMS to be accompanied by exceptional lability for reconstitution. The failure to reconstitute does not appear to be the result of lability of the soluble dehydrogenase.

It may be that there is a relationship between the ease of solubilizing the enzyme and difficulty in recombining with the particle. Also the specific activity of

the soluble succinic dehydrogenase of C. purpurea was much less than that of preparations from beef heart used by Keilin and King (42, 43). Consequently, contaminating proteins in the soluble preparation may have prevented the reconstitution.

Increase in oxygen uptake by ascorbate and cytochrome c in the presence of high concentrations of cyanide

The stimulation of oxygen uptake by high concentrations of cyanide in the presence of ascorbate and cytochrome c may be due to nucleophilic attack on dehydroascorbate, which would shift the equilibrium between ascorbate and dehydroascorbate in the direction of dehydroascorbate.

Soluble Succinic Dehydrogenase of C. purpurea

Inhibition by o-phenanthroline. o-Phenanthroline was found to be a noncompetitive inhibitor of the soluble succinic dehydrogenase of C. purpurea with respect to PMS. MacDonald et al. (46, 54) had previously shown that the inhibition by o-phenanthroline with respect to succinate was also noncompetitive. The noncompetitive nature of the inhibition of C. purpurea by o-phenanthroline with respect to PMS was somewhat unexpected, since Singer had reported that this inhibition was competitive for yeast (73) succinic dehydrogenase.

The inhibition also appeared to be free of some of the complications encountered by Singer and co-workers.

The inhibition was not altered by preincubation with the inhibitor and could vary up to 100 per cent depending on the concentration of o-phenanthroline. This is in contrast to the soluble succinic dehydrogenases of beef heart and yeast reported by Singer. With these enzymes o-phenanthroline produced progressively greater inhibition with time when the enzyme was incubated with substrate and only partially inhibited succinic dehydrogenase activity (72, 75).

The K_i values were 6.0×10^{-4} M at an o-phenanthroline concentration of 6.0×10^{-4} M and approximately 4.8×10^{-4} M at 1.0×10^{-4} M. The K_i was calculated on the assumption that one mole of enzyme combines with one mole of inhibitor. The kinetic data are consistent only with the combination of one mole each of enzyme and inhibitor even though in nonenzymatic chelation with iron three moles of o-phenanthroline combine with one mole of inorganic iron.

Since o-phenanthroline is an effective chelator of ferrous iron, the o-phenanthroline probably combines with the iron moiety of succinic dehydrogenase. Since the inhibition is noncompetitive when measured with PMS, the site at which PMS acts must not be iron but some other part of the succinic dehydrogenase molecule. Hypotheses which involve iron as an electron carrier in succinic-PMS reductase activity must be rejected since this model leads

to either competitive or mixed type of inhibition. The reaction of o-phenanthroline with enzyme-bound iron may cause a change in the tertiary structure of the enzyme so that the PMS cannot react.

Iron may still act as an electron carrier beyond the site at which PMS accepts electrons, since the present experiment has considered only the reactions with PMS. The increase in the ESR signal attributed to a transition element in the presence of succinate (59) suggests that iron may be involved in the transfer of electrons. It would be interesting to test the effect of o-phenanthroline on this ESR signal.

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APPENDIX I.

ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
DCIP	Dichlorophenolindophenol
DNP	Dinitrophenol
DEAE	Diethylaminoethyl
DPN	Diphosphopyridine nucleotide
DPNH	Reduced diphosphopyridine nucleotide
EDTA	Ethylenediamine tetraacetate
OAA	Oxalacetate
PMS	Phenazine methosulfate
RNA	Ribonucleic acid
TPN	Triphosphopyridine nucleotide
TPP	Thiamine pyrophosphate
TTZ	Triphenyltetrazolium chloride