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OCCURRENCE OF PYRUVATE DECARBOXYLASE IN *ERWINIA AMYLOVORA*

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Occurrence of a yeast-type pyruvate decarboxylase, that produces from pyruvate CO_2 and acetaldehyde in *Erwinia amylovora,* is demonstrated. Conditions for the maximum activity and co-factor requirements of the enzyme are determined. *E. amylovora* is placed in the family Enterobacteriaceae but the presence of pyruvate decarboxylase distinguishes it and alters its fermentation pattern from the other members of the family. The findings are discussed in relation to the comparative biochemistry of the family Enterobacteriaceae.

INTRODUCTION

Distribution of pyruvate decarboxylase in bacteria is very limited. So for only *Zymomonas mobilis* (De Moss, 1953; Dawes, Ribbons & Large, 1966), *Acetobacter suboxydans* (King & Cheldelin, 1954), *Zymosarcina ventriculi* (Bauchop & Dawes 1959), *Z. anaerobia* (McGill, Ribbons & Dawes, 1965), *Erwinia amylovora* (Haq, 1967; Haq & Dawes, 1971) and *E. herbicola* (Ntambi, 1978), are known to possess this enzyme. *E. amylovora* was the first member of the family Enterobacteriaceae reported to possess a pyruvate decarboxylase which yields acetaldehyde and CO_2 from pyruvate. Presence of this enzyme in *E. amylovora* alters the fermentation pattern of the organism from those of the other Enterobacteriaceae (Juni, 1952; Dawes & Foster, 1956; Haq, 1967) and it is, therefore, of a considerable interest from the stand point of comparative biochemistry of the family Enterobacteriaceae. Such findings may also lead to some agreement in the most contravorsal classification of the genus *Erwinia* as the organisms included in this group are heterogeneous in their biochemical and pathogenical characteristics (Waldee, 1945; Haq, 1967; Bergy's Manual of Determinative Bacteriology, 8th edition, 1974). In continuation of our previous work (Haq, 1967; Haq & Dawes, 1971; Haq, 1981) on the intermediate metabolism of *E. amylovora,* the present study provides further information on pyruvate decarboxylase present in the organism.

MATERIALS AND METHODS

Organism. The organism used in the present study was *Erwinia amylovora,* strain EA137S, kindly supplied by Professor M.P. Starr (University of California, Davis, U.S.A.).

Medium and Growth of the Organism. E. amylovora was maintained on Oxoid nutrient agar of composition (g/1. distilled water): Lab Lemco beef extract, 2.0; peptone 5.0; sodium chloride, 5.0; agar, 15.0 pH. 7.4. The cultures were grown aerobically on nutrient agar slants at 30°C. for 36 to 48 hr. and then stored at 4°C. Sub-cultures were made at monthly intervals. Colonies ranged from smooth to mucoid and when cells from a single colony of either type were plated, some of the daughter colonies were smooth and some mucoid. Since mucoid cells were difficult to suspend, the culture used in the present work was maintained in a predominately smooth state by periodic selection of smooth colonies.

Liquid cultures were made on medium of composition (g/1. distilled water): NH_4Cl, 1.0; KH_2PO_4, 4.0; K_2HPO_4, 4.0; $MgSO_4.7H_2O$, 0.2; glucose, 4.0; sodium malate, 4.0; casamino acids (Difco), 2; yeast extract (Difco), 1; micotinic acid, 2 mg. and 10 ml. of the following trace elements solution. The pH of the medium was adjusted to 6.8.

The trace elements solution contained (mg./1. distilled water) H_3BO_3, 0.5; $CaCO_3$, 10; $CuSO_4.5H_2O$, 1; $FeSO_4$ $(NH_4)_2SO_4.6H_2O$, 10; KI, 0.1; $MnSO_4.4H_2O$, 1; $ZnSO_4$ $7H_2O$, 5; (Starr, 1946). It was sterilized by Seitz filteration.

Glucose was autoclaved at 10 lb./in.^{2} for 15 min. and, with the trace elements solution, added aseptically to the medium autoclaved at 15 lb./in.^{2} for 15 min.

The organism grew optimally at 28°C., no growth occurred above 34°C.

Cultures (12.5 ml.) for use as inocula were grown at

28° - 30°C. in 50 ml. Erlenmeyer flasks in a Gallenkamp shaking bath (120 oscillation/min., stroke 3.8 cm.) or on a New Brunswick two-tier Gyrotary shaker for 20 to 22 hr. Larger cultures were grown in 1 or 2 I. of medium in 4-I. wide-necked conical flasks on the Gyrotary shaker at 30°C. for 12 hr. In each experiment an inoculum (0.1 - 0.2%) of cells (0.8 - 1.0 mg. dry wt./ml.) from the medium was used. A lag phase of about 2 hr. in aerobic and 4 - 5 hr. in anaerobic culture was usually observed.

Preparation of Washed Cell – Suspensions. Bacteria were harvested at the onset of the stationary phase, washed twice with 0.1M. citrate phosphate buffer, pH 6.0, and suspended in the buffer solution. The cells were sedimented for 15 min. at 5000 g. or for 5 min. at 23000 g. on an M.S.E. Major or Sorvall 55–1 centrifuge at room temperature (about 20° - 22°C.). Cell suspensions were normally used within an hour of preparation. Centrifuge cell pellets could be stored overnight at 4°C. without appreciable loss of activity.

Cell-Extracts. These were prepared by disrupting 10 ml. portions of cell-suspension (about 0.2 - 0.3 g.wet wt./ml.) with an M.S.E. ultrasonic disintegrator operated at 18 - 20 Kc./sec. with a current output of 1.3 - 1.5 amp. Each sample was treated for three periods of 50 sec.; each separated by cooling intervals of 5 min. The sample vials were surrounded by crushed ice and the temperature of the contents never allowed to rise above 15°C. The resultant suspensions were centrifuged for 20 min. at 17000 g. at 0°C. in the Super Speed Unit of an M.S.E. Major centrifuge. The pale pinkish-brown supernatant was transferred to pre-cooled universal bottles and stored at 4°C. or at –14°C. according to requirements. Extracts stored at 4°C. were used within 1 - 2 hr. of preparation and those stored at –14°C. were used within 72 hr. without significant loss of enzymic activity.

Enzyme Assay. The assay methods for pyruvate decarboxylate are given in the legends to the appropriate Figures and Tables.

Pyruvate Apo-Decarboxylase. The preparation of pyruvate apo-decarboxylase was attempted by the methods of Holzer, Soling, Goedde and Holzer (1963) for yeast.

Buffers. All buffer were prepared according to Gomori (1955) unless otherwise stated; pH was determined with an E.I.L. 23A Direct Reading pH meter.

Chemical Determinations. Protein was determined by the biuret method of Stickland (1951), CO_2 evolution was measured by conventional Warburg manometry at 30°C under N_2 (Umbreit, Burris & Stauffer, 1964). Pyruvate and acetaldehyde were characterized as their 2,4-dinitrophenylhydrazones.

Chemicals. Chemicals of analytical reagent grade were used whenever possible. Pyruvic acid and acetaldehyde were always redistilled before use. 2,4-dinitrophenylhydrazones of pyruvic acid and acetaldehyde were prepared and recrystallized twice from ethanol. Sodium pyruvate was freshly prepared in each experiment from redistilled acid. Albumin (bovine fraction V) and pyrithiamine (neo) hydrobromide were obtained from Sigma Chemical Co; St. Louis, Missoure, U.S.A. Yeast extract and casamino acids were from Difco laboratories, Detroit, Muchigan, U.S.A. 2,4-Dinitrophenylhydrazine was obtained from Hopkin and Williams Ltd. Essex, U.K.

RESULTS

Demonstration of pyruvate decarboxylase in washed cell suspension.

The presence of a pyruvate decarboxylase in the organism was demonstrated by evolution of CO_2 and characterization of acetaldehyde formed in the anaerobic breakdown of pyruvate. The evolution of CO_2 was measured manometrically and acetaldehyde was characterized as its 2,4-dinitrophenylhydrazone.

Evolution of CO_2 from Pyruvate. Evolution of CO_2 from pyruvate by washed suspensions of cells grown aerobically on the liquid medium was carried out in Warburg flask under an atmosphere of nitrogen at 30°C. The flask containing KOH in the centre wells showed no change in gas pressure, whereas in other flasks without KOH, a gas was evolved indicating that the gas was CO_2 (Fig. 1). The evolution of CO_2 as the sole gaseous product in the anaerobic dissimilation of pyruvate by non-growing cells indicated the presence of a pyruvate decarboxylase in the organism.

Optimum pH for the Production of CO_2. The effect of pH on the production of CO_2 from pyruvate by intact cells under anaerobic conditions was measured manometrically. Citrate-phosphate buffers (0.1M) of pH values ranging from 5.6 to 7.6 were used. The reaction was carried out in Warburg flasks and the evolution of CO_2 was recorded at 10 min. intervals. The reaction was stopped after 20 min. by tipping the acid from the side arm when the gas was being evolved at a linear rate. The maximum rate of CO_2 evolution was recorded at pH 6.0 indicating that the optimum pH for anaerobic dissimilation of pyruvate by intact cells of *E. amylovora* is 6.0 (Fig. 2).

Variation of Pyruvate Decarboxylase Activity with Phase of Growth. Production of CO_2 and acetaldehyde during the metabolism of sodium pyruvate by washed cell-suspensions indicated the presence of pyruvate decarboxylase in the organism. It was observed that cells harvested at

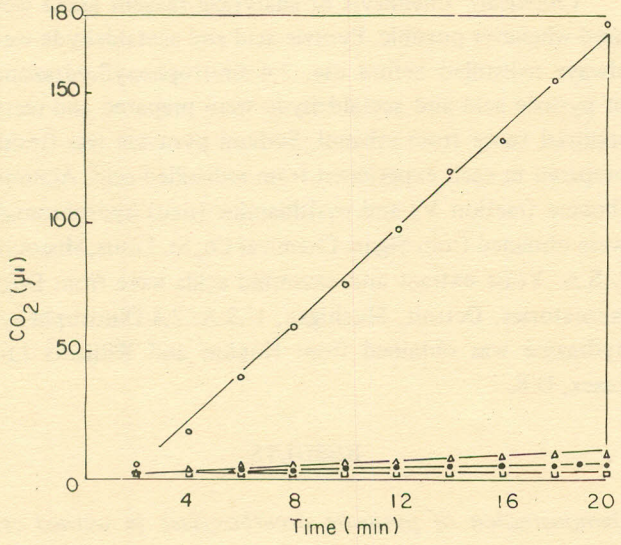

Fig. 1. Demonstration of pyruvate decarboxylase in washed cell-suspensions of E. amylovora. ○ Producting of CO_2; ● -Endogenous; △ -Chemical; □-KOH. The contents of each flash were: 0.5 ml. pyruvate (o.5M), or water in endogenous controls; 1.5 ml. citrate-phosphate buffer (0.1M, pH 6.0); 0.5 ml. cell suspension in buffer (7.4 mg dry weight/ml.), or buffer in chemical controls; 0.2 ml. water or KOH (20% w/v) in the centre cells and distilled water to make the volume to 3.0 ml. The reaction was started by tipping pyruvate into the main compartment followed for 20 minutes and readings taken at 2 minute intervals. Atmosphere nitrogen; temperature 30°.

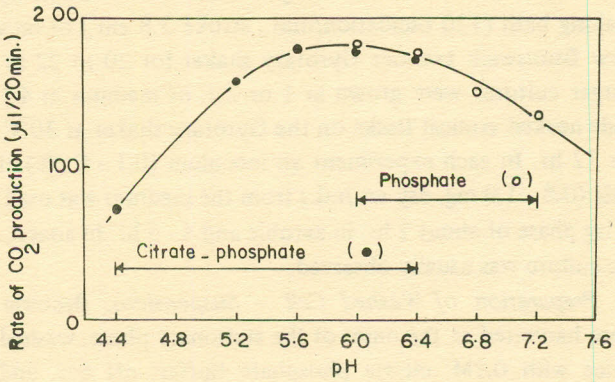

Fig. 2. Optimum pH for the production of CO_2. The contents of each Warburg flask were: 1.8 ml. citrate-phosphate buffer (o.1M pH ranged from 4.4 to 6.4) or phosphate buffer (0.1M pH 6.0 to 7.2); 0.5 ml. sodium pyruvate (0.5M), or water in endogenous controls in the side arm 1; 0.5 ml. cell-suspension (17.0 dry weight mg./ml. water); and 0.2 ml. sulphuric acid (3N) in side arm 2. The reaction was started by tipping the substrate into the main compartments under an atmosphere of nitrogen 30° and readings taken at 10 minute intervals for 20 minutes.

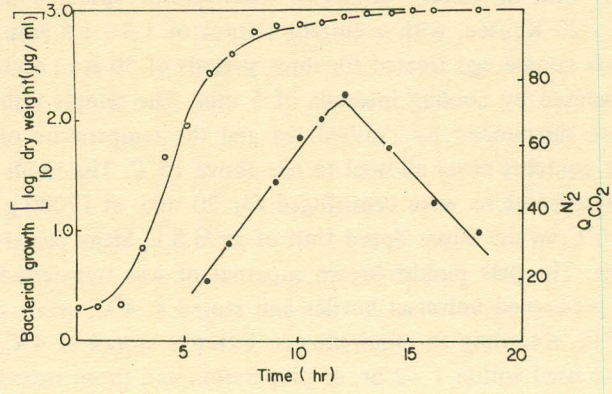

Fig. 3. ○- Growth curve; ● - Production of CO_2. Each Warburg flask contained: 1.5 ml. citrate-phosphate buffer (o.1M, pH 6.0); 0.5 ml. sodium pyruvate (0.5M), or water in endogenous controsl; 0.5 ml. Cell suspension in buffer (7.4 mg dry weight/ml.) or buffer in chemical controls, and water to make the volume to 3.0 ml. Atmosphere, nitrogen; temperature 30°. The reaction was followed for 20 minutes and readings taken at 2 minute intervals.

different times during the growth cycle displayed different enzymic activities. It was necessary, therefore, to determine the best time to harvest the cells in order to obtain maximum enzymic activity.

Cells grown aerobically were harvested from the liquid medium at different time intervals during the growth cycle (6 – 18 hr.), washed twice with citrate-phosphate buffer (0.1M, pH 6.0) and made into a suspension in the buffer. The reaction was started by tipping the substrate into the main compartments of Warburg flasks and evolution of CO_2 was recorded for 20 min. The cells harvested after 11 to 12 hr. growth, corresponding to the beginning of the stationary phase, displayed the greatest activity (Fig. 3). The optimum growth period for maximum enzymic activity under the standardized conditions used was thus 11 to 12 hr.

Stoicheiometry of the Reaction. Stoicheiometry of the reaction of decarboxylation of pyruvate by intact cells was observed in the Warburg apparatus. The evolution of CO_2 from 2 to 5 µmoles sodium pyruvate was measured manometrically at pH 6.0 and temperature 30°C. in citratephosphate buffer (pH 6) under nitrogen atmosphere. The reaction was started as described previously, it was run until no further gas evolution occurred and the acid tipped at the end after 5 hr. The relation between CO_2 evolution and pyruvate concentration is shown in Figure 4. It was found that only 64% of the theoretical yield of CO_2 was obtained, indicating the occurrence of competing reactions for the pyruvate.

Characterization of Acetaldehyde as its 2,4-dinitrophenylhydrazone. Acetaldehyde formed during the anaerobic dissimilation of pyruvate by intact cells was characterized as its 2,4-dinitrophenylhydrazone. Five ml. of sodium

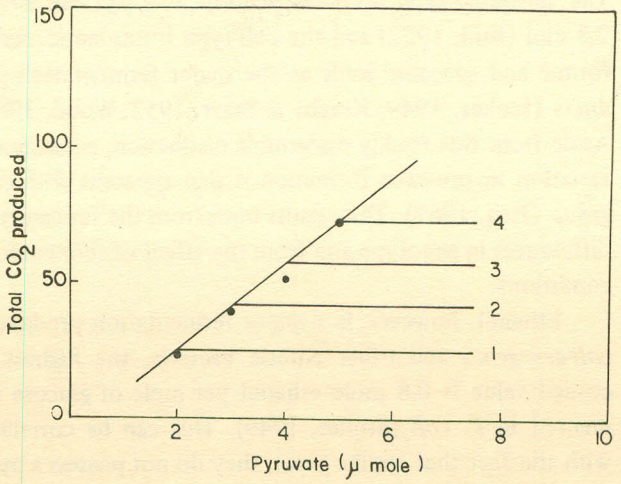

Fig. 4. Stoicheiometry of the reaction. Each Warburg flask contaned: 1.5 ml. citrate-phosphate buffer (0.1M, pH 6.0); 0.5 ml. sodium pyruvate (4 to 10 moles per ml.) or water in endogenous controsl; 0.5 ml. cell-suspension (16.6 mg. dry weight/ml. of buffer); 0.2 ml. H^2SO_4 (3N) and water to make the total volume to 3.0 ml. The reaction was carried out under atmosphere of nitrogen at 30°

pyruvate (0.5M) and 8.0 ml. of citrate-phosphate buffer (0.1M, pH 6.0) were pipetted into a Leurquin apparatus (Quickfit) test tube fitted with a side arm. 2.0 ml. of cell suspension (20 mg. dry wt./ml.) in the buffer was put in the side arm and oxygen-free nitrogen was continuously bubbled through the contents of the test tube. The tube was incubated at 30°C. in a water bath and was connected to another test tube of the Leurquin apparatus containing a solution of 2,4-dinitrophenylhydrazine (1.0 mg. per ml. of 2N HC1) at 10° - 15°C.

The reaction was started by tipping the cell-suspension into the tube and the acetaldehyde formed was swept by the continuous flow of nitrogen into the cold solution of 2,4-dinitrophenylhydrazine (2,4-DNP). The reaction was run for 2 hr. and then the solution of 2,4-DNP was held at room temperature (20° - 22°C.) for 3 hr. An orange-yellow precipitate of acetaldehyde 2,4-dinitrophenylhydrazone was formed. The precipitate was filtered on a small sintered glass funnel and washed with 2N HC1 until it was free from the hydrazine. Finally the precipitate was washed with distilled water to remove the hydrochloric acid and recrystallized from ethanol (Haq, 1967).

Spectrum Studies. The recrystallized acetaldehyde 2,4-dinitrophenylhydrazone obtained from the experiment was redissolved in ethanol (16.0 μg. per ml.), its spectrum recorded with a Unicam SP 800 Spectrophotometer and compared with the spectrum of authentic acetaldehyde 2,4-dinitrophenylhydrazone. They were identical and E_{max} in both cases was at 356 nm. (Fig. 5).

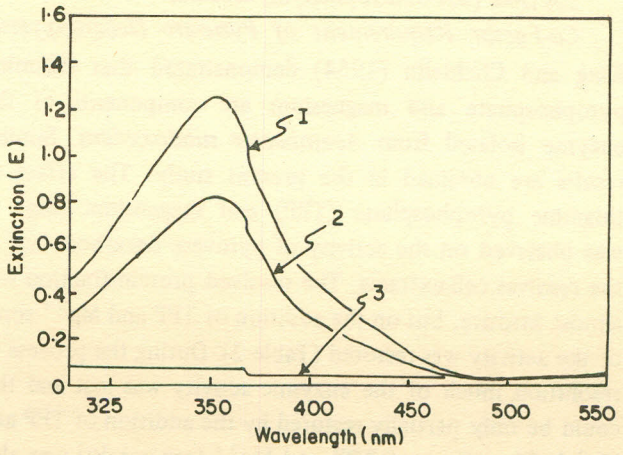

Fig. 5. Characterization of acetaldehyde as its 2,4-dinitrophenylhydrazone. 1. Acetaldehyde 2,4-dinitrophenylhydrazone (authentic); 2. Acetaldehyde 2,4-dinitrophenylhydrazone (isolated); 3. Solvent (ethanol).

Melting Point. The melting point of isolated and recrystallized acetaldehyde 2,4-dinitrophenylhydrazone was determined with an "Electro-thermal apparatus" and was compared with those of authentic acetaldehyde 2,4-dinitrophenylhydrazone and 2,4-dinitrophenylhydrazine, which are given in the Table 1. The mixed melting point of the isolated acetaldehyde 2,4-dinitrophenylhydrazone was close to that of authentic one which proved the formation of acetaldehyde in the anaerobic dissimilation of pyruvate. Hence the presence of a pyruvate decarboxylase was inferred.

In various publications (Allen, 1930; Strain, 1935; Ross, 1953; Kramer & Van Duin, 1954; Timmons, 1957) it was found that the melting point of acetaldehyde 2,4-dinitrophenylhydrazone recorded varies from 148 to 168.5°C. This variation could be due to the mixture of 2 isomers of the compound in varying amounts or it might be due to the methods of preparation, and the impurities present in the hydrazone.

Table 1. Melting points

Compound	Melting point °C.
Ac.DNP (Authentic)	158 – 159
Ac.DNP (Isolated)	148 – 151
Pyr.DNP	213 – 214
2,4 DNP	194 – 198

Ac.DNP (Acetaldehyde 2,4-dinitrophenylhydrazone)
Pyr.DNP (Pyruvic acid 2,4-dinitrophenylhydrazone)

2,4 DNP (2,4-dinitrophenylhydrazine).

Co-Factor Requirement of Pyruvate Decarboxylase. King and Cheldelin (1954) demonstrated that thiamine pyrophosphate and magnesium are components of the enzyme isolated from *Acetobacter subdoxydans.* Similar results are obtained in the present study. The effect of thiamine pyrophosphate (TPP) and magnesium (Mg^{2+}) was observed on the activity of pyruvate decarboxylase in the resolves cell-extracts. The resolved protein-fraction was almost inactive, but on the addition of TPP and Mg^{2+} some of the activity was restored (Table 2). During the process of resolution much of the enzymic activity was lost and this could be only partially restored by the addition of TPP and Mg^{2+}. The effect of TPP and Mg^{2+} (separately) was also observed; both of these co-factors partially reactivated the enzyme.

Table 2. Co-factor requirements of pyruvate decarboxylase

Components	Specific activity moles/mg. protein/hr.
Holoenzyme	16.60
Protein fraction*	2.63
Protein fraction* + TPP + Mg^{2+}	3.78
Protein fraction* + TPP	3.26
Protein fraction* + Mg^{2+}	3.58

*Resolved protein fraction.

The enzyme was assayed spectrophotometrically by the method of Holzer, Soling, Goedde & Holzer (1963). Each cuvette contained: 1.5 ml. maleate buffer (0.2M, pH 6.6); 0.2 ml. pyruvate (0.1M); 0.2 ml. TPP (2μg./ml. water); 0.1 ml. $MgSO_4$ (0.5M); 0.2 ml. $NADH_2$ (1mM); 0.1 ml. cell extracts (Holoenzyme, 4.5 mg. or protein fraction, 12.3 mg. protein/ml. of buffer) and water to make the volume to 3.0 ml. The reaction was run for 5 min. at 22°C.

DISCUSSION

Erwinia amylovora, a plant pathogenic bacterium, is placed in the tribe Erwiniae of the family Enterobacteriaceae (Bergy's Manual of Determinative Bacteriology, 8th ed. 1974). Organisms of this group are characterized by their ability to ferment glucose or pyruvate into a great variety of end products (Haq, 1967) and on the basis of their fermentation patterns and the products formation, they can be divided into two groups; one group resembling to *Acetobacter aerogenes* and the other to *Escherichia coli.* The aerogenes-type produces acetoin, diacetyl and butane 2,3 diol (Juni, 1952) and the coli-type forms lactic, acetic, formic and succenic acids as the major fermentation products (Stokes, 1949; Kraght & Starr, 1952; Wood, 1961). Aside from this readily discernible distinction, considerable variation in products formation is also apparent with each group (Haq, 1967). This results both from the fundamental differences in genotype and from the effect of fermentation conditions.

Ethanol, however, is a minor fermentation product of *coli-aerogenes* and other enteric bacteria, the highest recorded value is 0.8 mole ethanol per mole of glucose fermented in *E. coli* (Stokes, 1949). This can be correlated with the fact that, unlike yeast, they do not possess a pyruvate decarboxylase. Infact, it has been shown that *E. coli* cleaves pyruvate by th : thioclastic reaction and ethanol is formed by reduction of the C_2-moiety (acetyl coenzyme A) thereby produced (Dawes & Foster, 1956). The theoretical maximum yields of ethanol in this mechanism is 1 mole per mole of glucose fermented (Dawes, 1963). Instead, Sutton & Starr (1959, 1960) reported that *E. amylovora* produced yields of ethanol as high as 1.55 mole per mole of glucose fermented. Such high quantities of ethanol formation cannot be accounted for by the coli-form mechanism. A yield of ethanol greater than unity per mole of glucose could only be due to the existence of pyruvate decarboxylase in the organism (Dawes, 1963). Presence of this enzyme in *E. amylovora* has been reported previously (Haq 1967; Haq & Dawes, 1971) and the present study provides information on its cofactor requirements and conditions for maximum activity.

Presence of a pyruvate decarboxylase was established by the production of CO_2 and acetaldehyde from pyruvate (Figs. 1, 5 & Table 1). Assays with bacteria harvested throughout the growth cycle indicated maximum decarboxylase activity at the onset of stationary phase (Fig. 3) and optimum pH was 6.0 (Fig. 2). The stoichiometry over the range of 2 to 5 moles of pyruvate was 0.64 mole CO_2 per mole of pyruvate (Fig. 4), confirming the occurrence of competing reactions as reported by Haq (1967).

Salts of heavy metals such as $CuSO_4$, $AgNO_3$ and $Hg(NO_3)_2$ which are inhibitory for pyruvate decarboxylase in *A. suboxydans* (King & Cheldelin, 1954) and *Z. ventriculi* (Bauchop & Dawes, 1959) also inhibited pyruvate decarboxylase in *E. amylovora* (Haq, 1967; Haq & Dawes, 1971). Green *et al* (1941) showed that thiamine pyrophosphate (TPP) and magnesium (Mg^{2+}) were essential components of yeast pyruvate decarboxylase. King & Cheldelin (1954) also reported that, like yeast, TPP and Mg^{2+} were cofactors of pyruvate decarboxylase isolated from *A. sub-*

oxydans. Similar results were obtained with cell extracts of *E. amylovora* in the present study. Almost complete resolution of the enzyme was obtained and on addition of TPP and Mg^{2+} the activity was partially resorted (Table 2). This indicated that TPP and Mg^{2+} are also cofactors of the *E. amylovora* pyruvate decarboxylase and confirmed the occurrence of a yeast-type pyruvate decarboxylase in the organism.

E. amylovora is thus one of the few bacteria that possess a yeast-type pyruvate decarboxylase and is the first organism in the family Entereobacteriaceae demonstrated to have this enzyme. Wilson (1974) reported that *E. uredovora, E. milletiae, E. salicis, E. ananas* and *E. atroseptica* produced CO_2, yields of 0.58 to .82 mole per mole of pyruvate and assumed the presence of pyruvate decarboxylase in the organisms. But she did not demonstrate the presence of acetaldehyde; another product of the decarboxylation of pyruvate. However, Ntambi (1978) demonstrated and confirmed the presence of a pyruvate decarboxylase in *E. herbicola.* Presence of pyruvate decarboxylase in Erwinias, as stated before, is of a considerable interest from the stand point of comparative biochemistry of the family Enterobacteriaceae and explains the atypical fermentation balance of *E. amylovora* with high yields of ethanol from glucose and acetaldehyde from pyruvate. (Sutton & Starr, 1958, 1959; Haq & Dawes, 1971). It would be interesting to know the distribution of pyruvate decarboxylase in other members of the genus *Erwinia.* Such studies may as well remove the controversy surrounded the classification of the genus *Erwinia.*

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