Pyruvic Acid Metabolism and Ethanol Formation in *Erwinia amylovora*

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SUMMARY

Erwinia amylovora is the first member of the Enterobacteriaceae found to possess a thiamine pyrophosphate-dependent pyruvate decarboxylase yielding acetaldehyde and CO₂. In conjunction with an NAD-dependent ethanol dehydrogenase this represents the route of ethanol formation and explains the high ethanol yields previously reported. The organism also possesses an α -acetolactate decarboxylase and is thus able to produce acetoin by both the recognized microbial pathways. Fermentation balances for pyruvate with bacterial suspensions and extracts at pH 6.0 are recorded. NADH oxidase and lactate dehydrogenase are present but NADH-NADP transhydrogenase, coenzyme A-dependent acetaldehyde dehydrogenase, formate dehydrogenase or formate hydrogenlyase could not be detected. The findings are discussed in relation to the classification of *Erwinia* and the comparative biochemistry of the Enterobacteriaceae.

INTRODUCTION

Enzymic and isotopic studies have established the operation of the Embden-Meyerhof glycolytic and hexose monophosphate oxidative pathways of glucose metabolism in the plant pathogen *Erwinia amylovora* (Katznelson, 1958; Sutton & Starr, 1960); the Entner-Doudoroff pathway is absent (De Ley, 1962). Sutton & Starr (1959) reported a yield of 1.55 moles of ethanol per mole of glucose fermented, which suggested to us the possibility of a yeast-type pyruvate decarboxylase yielding CO₂ and acetaldehyde, which could then be reduced to ethanol by ethanol dehydrogenase. Pyruvate decarboxylase has, however, only a very limited distribution in bacteria and has not previously been found in the Enterobacteriaceae, in which family the genus *Erwinia* is placed. As the characteristic fermentation patterns of the Enterobacteriaceae reflect the pathways for the further metabolism of pyruvate, we have investigated the metabolism of this compound by *E. amylovora* in relation to the mechanism of ethanol formation and the comparative biochemistry of the Enterobacteriaceae.

METHODS

Organism. The organism used throughout this work was *Erwinia amylovora* EA 137S which was kindly supplied by Professor M. P. Starr (University of California, Davis, U.S.A.) who had taken the strain from a parent culture EA 137, originally isolated from fire-blight of apple by Ark (1937). Both EA 137 and EA 137S were plant pathogenic when tested by Ark during the studies of Sutton & Starr (1959) and were typical of *E. amylovora* in their biochemical reactions. For comparative studies of formate metabolism we have also used

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strains 100, 675 and 830 from the National Collection of Plant Pathogenic Bacteria, Harpenden, Herts.

Media and growth of the organism. *Erwinia amylovora* was maintained on Oxoid nutrient agar of composition (g./l. distilled water): Lab Lemco beef extract, 1.0; yeast extract, 2.0; peptone, 5.0; NaCl, 5.0; agar, 15.0; pH 7.4. The cultures were grown aerobically on nutrient agar slants at 30° for 36 to 48 h. and then stored at 4°. Subcultures were made at monthly intervals.

Liquid cultures were grown, as appropriate, in three different media. (a) Chemically defined medium contained (g./l. distilled water): NH_4Cl , 1.0; KH_2PO_4 , 4.0; K_2HPO_4 , 4.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; glucose, 4.0; sodium malate, 4.0; nicotinic acid, 2 mg. and 10 ml. of trace elements solution (see below). (b) Complex medium was medium A to which was added (g./l.): casamino acids (Difco), 2; yeast extract (Difco), 1. (c) Neopeptone medium contained (g./l.): NH_4Cl , 1.0; KH_2PO_4 , 4.0; K_2HPO_4 , 4.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; glucose, 4.0; neopeptone (Difco), 4.0; nicotinic acid, 2 mg. and 10 ml. of trace elements solution. The pH of media was adjusted to 6.8.

The trace elements solution contained (mg./l.): H_3BO_3 , 0.5; CaCO_3 , 10; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1; $\text{FeSO}_4(\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$, 10; KI, 0.1; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 1; MoO_3 , 1; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 5 (Starr, 1946). It was sterilized by Seitz filtration.

Glucose was autoclaved separately at 10 lb./in.² for 15 min. and, with the trace elements solution, added aseptically to the media.

The organism grew optimally at 28 to 30°; very little growth occurred above 34°. Growth was poor under anaerobic conditions in media B and C and absent in medium A.

Cultures (12.5 ml.) for use as inocula were grown at 28 to 30° in 50 ml. Erlenmeyer flasks in a Gallenkamp shaking bath (120 oscillations/min., stroke 3.8 cm.) or on a New Brunswick two-tier gyrotary shaker for 20 to 22 h. Larger cultures (1 to 2 l.) were grown in 4 l. wide-necked conical flasks on the gyrotary shaker at 30° for 12 h. In each experiment an inoculum (0.1 to 0.2%, v/v) of bacteria (0.8 to 1.0 mg. dry wt./ml.) from the appropriate medium was used. A lag phase of about 1 to 2 h. in aerobic and 4 to 5 h. in anaerobic culture was usually observed.

Preparation of washed bacterial suspensions. Bacteria were harvested at the onset of the stationary phase, washed twice with 0.076 M-K-phosphate buffer, pH 7.1, and suspended in an appropriate buffer solution. The organisms were either sedimented for 15 min. at 5000g or for 5 min. at 23,000g on an M.S.E. Major or Servall SS-1 centrifuge at room temperature (about 20°). Suspensions were used normally within an hour of preparation. Centrifuged bacterial pellets could be stored overnight at 4° without appreciable loss of activity.

Bacterial extracts. These were prepared by disrupting 10 ml. portions of bacterial suspensions (about 0.2 to 0.3 g. wet wt./ml.) with an M.S.E. ultrasonic disintegrator operated at 18 to 20 Kc./sec. with a current output of 1.3 to 1.5 A. 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°. The resulting preparations were centrifuged for 20 min. at 17,000g and 0° in the Superspeed unit of an M.S.E. Major centrifuge. The pale pinkish brown supernatant was transferred to pre-cooled Universal bottles and held at 4° or at -14° according to requirements. Extracts held at 4° were used within 1 to 2 h. of preparation and those stored at -14° were used within 72 h. without significant loss of enzymic activity. Sonication for 1 to 2.5 min. gave extracts with maximum specific activities for pyruvate decarboxylase. For acetaldehyde dehydrogenase assays extracts were also prepared by grinding with powdered glass (Strecker, Wood & Krampitz, 1950), by the Hughes's (1951) press and by the method of Lamanna & Mallette (1954).

Fermentation balances. The fermentations were carried out in replicate Warburg flasks each containing 1.8 ml. 0.1 M-K-phosphate buffer, pH 6.0; 0.5 ml. bacterial suspension (38.4 mg. dry wt./ml. of same buffer) or extract (34.1 mg. protein/ml. buffer); 0.5 ml. 0.1 M-pyruvate (side arm 1) and 0.2 ml. 5 N-H₂SO₄ (side arm 2). Chemical and biological controls were run in parallel with acid tipped at zero and final time. The atmosphere was N₂ and the temperature 30°. The reaction was started after 20 min. incubation by tipping pyruvate into the main compartment. Acid was tipped at the conclusion. Usually nine flasks were set up and the contents pooled for analysis. After taking the final reading flasks were cooled in ice before detachment from manometers. The contents were transferred quantitatively to pre-cooled centrifuge tubes, the bacteria sedimented and the supernatant clarified and neutralized with zinc hydroxide by the method of Neish (1952) before analysis. Residual pyruvate was determined.

Enzyme assays. The assay methods for pyruvate decarboxylase, NADH oxidase, alcohol dehydrogenase, lactate dehydrogenase and α -acetolactate decarboxylase are given in the legends to the appropriate Figures and Tables.

Acetaldehyde dehydrogenase. The method of Dawes & Foster (1956) was applied to crude, ultracentrifuged and ammonium sulphate-fractionated extracts with and without supplements of coenzyme A, MgSO₄ and glutathione. Cells were grown on media A, B and C and extracts were prepared in a variety of ways. Assays were run over a range of pH values in different buffers and at different temperatures (20 to 30°).

Pyruvate-formate lyase (thioclastic enzyme). The enzyme was assayed by the formate-pyruvate exchange reaction using [¹⁴C]-formate (Strecker, Wood & Krampitz, 1950). Each Warburg flask contained: 1 ml. 0.1 M-K-phosphate buffer, pH 6.8; 0.25 ml. 0.192 M-[¹⁴C]-formate (2×10^6 counts/min./ml.) in side arm 1; bacterial extract (15.7 mg. protein/ml.); 0.2 ml. 3 N-H₂SO₄ in side arm 2. Where appropriate, 0.2 ml. 0.2 M-pyruvate (in side arm 1 with formate) and 0.5 ml. of boiled aqueous extract of yeast (15 mg./ml.) or of *Erwinia amylovora* (from 0.2 g. cell wet wt./ml.) were added. The final volume in each flask was made to 3 ml. with distilled water and the reactions were run at 30° under N₂. Chemical controls (no enzyme) were set up with each reaction mixture. Acid was tipped after 15 min. to stop the reaction and the contents of the flasks were centrifuged at 17,500g for 15 min. Supernatants were treated with an equal volume of 2,4-dinitrophenylhydrazine (0.1 % (w/v) in 2 N-HCl) and incubated at 37° for 30 min. Pyruvate 2,4-dinitrophenylhydrazone was extracted into ethyl acetate and then into 10 % (w/v) Na₂CO₃. After acidification with 10 N-H₂SO₄ the hydrazone was extracted again into ethyl acetate, the organic layer separated and washed with water. After centrifuging to remove traces of water, the solution was concentrated and 0.05 to 0.1 ml. portions used for ascending paper chromatography in butan-1-ol saturated with 0.2 % ammonia. The two isomeric pyruvate 2,4-dinitrophenylhydrazones were cut out from the paper, eluted with 2 ml. distilled water, and portions (0.2 ml.) placed on nickel planchets (2.5 cm. diam., area 4.9 cm.²), dried and counted in a Nuclear Chicago gas flow counter for 100 min.

Formate dehydrogenase and formate hydrogenlyase were assayed by the methods of Bauchop & Dawes (1968). Methylene blue, benzyl viologen and cytochrome *c* were all tested as electron acceptors in the formate dehydrogenase assays.

Lactate dehydrogenase was assayed by the method of Wieland (1963).

Pyruvate apo-decarboxylase. The preparation of pyruvate apo-decarboxylase was attempted by the methods of Holzer, Söling, Goedde & Holzer (1963) for yeast, and of Morey & Juni (1968).

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

Chemical determinations. Pyruvate was determined by the toluene extraction method of Friedemann & Haugen (1943), acetaldehyde by the procedure of Johnson & Scholes (1954), ethanol by the enzymic method of Bonnichsen (1963) and lactate by the method of Hullin & Noble (1953). Acetoin, diacetyl and butane 2,3-diol were determined by the methods described by Neish (1952). Organic acids were extracted from fermentation liquors with ether, separated by partition chromatography on silicic acid columns according to Neish (1952) and titrated with 0.01N-NaOH with 0.5% (w/v) phenol red as indicator. CO₂-free air was bubbled through the solutions during titration. Protein was determined by the biuret method of Stickland (1951) and CO₂ evolution measured by conventional Warburg manometry at 30° under N₂ (Umbreit, Burris & Stauffer, 1964).

Chemicals and enzymes. Chemicals of analytical reagent grade were used whenever possible. Pyruvic acid, acetaldehyde and formic acid were always redistilled before use and *p*-hydroxydiphenyl was recrystallized from ethanol. 2,4-Dinitrophenylhydrazones of acetaldehyde and pyruvic acid were prepared and recrystallized twice from ethanol. Sodium pyruvate and sodium formate were freshly prepared in each experiment from the redistilled acids. Alcohol dehydrogenase, NAD, NADH, NADP, NADPH (sodium salts) were obtained from C. F. Boehringer und Soehne, Mannheim, Germany. Acetyl CoA, coenzyme A, pyrithiamine (neo) hydrobromide and albumin (bovine fraction V) were obtained from Sigma Chemical Co., St Louis, Missouri. α -Acetolactic acid acetate ethyl ester was from K & K Laboratories Inc., New York. Casamino acids, yeast extract and neopeptone were from Difco Laboratories, Detroit, Michigan; cocarboxylase and glutathione were from British Drug Houses, Poole, Dorset; 2,4-dinitrophenylhydrazine and powdered glass were from Hopkin & Williams Ltd., Essex, and silicic acid (100-mesh) from Mallinckrodt Chemical Works, St Louis, Missouri. Sodium [¹⁴C]-formate was obtained from the Radiochemical Centre, Amersham, Buckinghamshire.

α -Acetolactic acid was prepared by the hydrolysis of α -acetolactic acid acetate ethyl ester with NaOH, as described by Krampitz (1948). The mixture of ethanol and the sodium salts of α -acetolactic and acetic acids resulting from the hydrolysis was used as such in the experiments to assay α -acetolactate decarboxylase. The yield of CO₂ from α -acetolactate in the mixture was 84.3% of theoretical.

RESULTS

Fermentation balances for anaerobic pyruvate dissimilation. Table 1 records the fermentation balances for pyruvate with whole organisms and with extracts in phosphate buffer at pH 6.0. Lactate, acetate, acetaldehyde and CO₂ were the major products with intact organisms with acetoin, diacetyl and formate as minor products. In contrast, the corresponding fermentation by bacterial extracts resulted in a substantial increase in acetoin and CO₂, and a decrease in lactate and, to a lesser extent, acetate. Succinate was not determined in these experiments. With the exception of acetaldehyde, the products were those associated with fermentations of the Enterobacteriaceae, and the presence of the relevant enzymes was therefore investigated.

Enzyme assays

Detection and characterization of pyruvate decarboxylase. Washed suspensions of *Erwinia amylovora* evolved a gas, which was completely absorbed by KOH, from pyruvate in citrate-phosphate buffer at pH 6.0 under an atmosphere of N₂. As no other gas was released, this was assumed to be CO₂. The stoichiometry over the range of 2 to 10 μ moles of pyruvate was 0.64 mole CO₂/mole pyruvate, confirming the occurrence of competing reactions as indicated

Fig. 1. Pyruvate decarboxylase activity of whole organisms in relation to their growth cycle in complex medium. Growth, ○; pyruvate decarboxylase activity, $Q_{CO_2}^N$, ●.

Table 1. *Anaerobic dissimilation of pyruvate by bacterial suspensions and extracts of Erwinia amylovora*

Products	Bacterial suspension		Bacterial extract	
	Mole/mole pyruvate	Carbon recovery	Mole/mole pyruvate	Carbon recovery
Carbon dioxide	0.633	0.633	0.852	0.852
Acetaldehyde	0.207	0.414	0.172	0.344
Acetoin	0.0776	0.310	0.230	0.923
Butane 2,3-diol	—	—	0.036	0.144
Diacetyl	0.013	0.052	0.018	0.072
Acetic acid	0.224	0.448	0.132	0.264
Lactic acid	0.272	0.816	0.064	0.192
Formic acid	0.044	0.044	0.012	0.012
Ethanol	—	—	0.011	0.022
		2.717		2.825
Carbon recovery, (%)		90.3		94.2
Oxidation-reduction index		0.95		0.96

by the fermentation balance. If higher initial concentrations of pyruvate were used, e.g. 13.3 mM instead of 3.3 mM, and the reaction halted before completion, a yield of 0.81 mole CO_2 /mole pyruvate was observed. The formation of acetaldehyde was suggested by its characteristic odour and established by the isolation of a 2,4-dinitrophenylhydrazone which gave a spectrum (E_{max} 356 nm.) and melting-point (148 to 151°) characteristic of acetaldehyde. The melting-point of acetaldehyde 2,4-dinitrophenylhydrazone is known to cover a range of 148 to 168.5° (Allen, 1930; Strain, 1935; Ross, 1953; Kramer & van Duin, 1954; Timmons, 1957), a fact usually attributed to the differing proportions of two isomers; an authentic specimen prepared from acetaldehyde under our conditions gave a melting point of 158 to 159°. The presence of pyruvate decarboxylase was therefore inferred.

Assays with bacteria harvested throughout the growth cycle indicated maximum decarboxylase activity at the onset of the stationary phase (Fig. 1) and the optimum pH was

6.0. Comparison of whole and disintegrated bacteria revealed a partial permeability barrier to pyruvate. For example, under comparable conditions reaction with 50 μ moles pyruvate was complete in 1 h. with disintegrated cells but took 3 h. with whole cells; the CO_2 yields were 85 and 63 % respectively.

Fig. 2. NADH oxidase. Cuvettes contained: 2.4 ml. 0.06 M-K-phosphate, pH 7.0; 0.2 ml. 1 mM-NADH or NADPH; 0.1 ml. bacterial extract (13 mg. protein/ml. buffer) and water to 3 ml. Assay 1, NADH; 2, NADPH. Reaction was initiated by addition of extract at A. 0.2 ml. 1 mM-NADH was added to assay 2 at B. Temperature 22°.

Table 2. *Effect of inhibitors on CO_2 -release from pyruvate*

Each Warburg flask contained: 1.5 ml. 0.1 M-citrate-phosphate buffer, pH 6.0; 0.5 ml. bacterial extract in the same buffer (5.1 mg. protein/ml.); 0.5 ml. 0.05 M-sodium-pyruvate (in the side arm)

Inhibitor	Concentration (mM)	$Q_{\text{CO}_2}^{\text{N}_2}$	Inhibition (%)
None		114	0.0
AgNO_3	0.1	92	19.2
	0.2	76	33.2
	2.0	0.0	100.0
CuSO_4	0.1	92	19.2
	0.2	81	28.9
	2.0	0.0	100.0
Pyrithiamine (neo)	0.1	121	0.0
	0.2	120	0.0
	2.0	125	0.0

The activity of many bacterial extracts was stimulated by the addition of 0.13 mM-thiamine pyrophosphate plus 6.4 mM- Mg^{2+} . Respective rates ($\mu\text{l. CO}_2/\text{mg. protein/h.}$) in two such experiments were (a) 200 and 232, and (b) 82 and 133. The enzyme proved extremely difficult to resolve, however, and methods satisfactory for other micro-organisms failed to produce complete resolution. While some variation occurred in different experiments, a fairly typical case gave rates ($\mu\text{l. CO}_2/\text{mg. protein/h.}$) of 88 for crude, 40 for 'resolved' and

73 for 'resolved' plus co-factors. AgNO_3 and CuSO_4 inhibited decarboxylase activity but pyrithiamine was without effect (Table 2).

NADH oxidase. Extracts of *Erwinia* contained an active NADH oxidase (Fig. 2) which had an optimum pH of 7.2 but retained more than 60 % of its activity over the pH range 6.4 to 8.0. The bulk of the enzyme could be precipitated by 20 to 40 % saturation with $(\text{NH}_4)_2\text{SO}_4$ but was completely sedimented by centrifuging at 105,000g for 90 min. It did not oxidize NADPH. As the enzyme interfered with spectrophotometric assays of NAD-linked enzymes it was removed routinely by ultracentrifuging at 105,000g for 90 min.

Ethanol dehydrogenase. The enzyme was assayed with ethanol and acetaldehyde as substrates (Fig. 3*a, b*). It showed a broader pH optimum with acetaldehyde than with ethanol (Fig. 3*c*), was specific for NAD and precipitated principally in the 40 to 60 % saturation $(\text{NH}_4)_2\text{SO}_4$ fraction.

Fig. 3. Reversibility, coenzyme specificity and pH optimum of ethanol dehydrogenase. (a) Ethanol as substrate. Cuvettes contained 2.4 ml. 60 mM-tris buffer, pH 8.5; 0.2 ml. 1 mM-NAD or NADP; 0.1 ml. 3 M-ethanol; 0.1 ml. bacterial extract (8.3 mg. protein/ml. buffer) and water to 3 ml. Curve 1, NAD; 2, NADP. Arrows indicate addition of ethanol. (b) Acetaldehyde as substrate. Cuvettes contained 2.4 ml. 0.6 M-K-phosphate buffer, pH 7.0; 0.2 ml. 1 mM-NADH or 0.1 ml. 1 mM-NADPH; 0.1 ml. 0.5 M-acetaldehyde; 0.1 ml. extract (8.3 mg. protein/ml. buffer) and water to 3 ml. Curve 1, NADH; 2, NADPH. Acetaldehyde was added at A to initiate reaction and 0.2 ml. 1 mM-NADH was added to 2 at B. (c) Effect of pH on ethanol dehydrogenase. Conditions as for (a) and (b) except bacterial extract (6.5 mg. protein/ml. water) and buffers: $\circ$, 0.06 M-citrate phosphate, $\square$, 0.06 M-K-phosphate; $\triangle$, 0.06 M-tris. Open symbols, acetaldehyde substrate; solid symbols, ethanol. The right-hand ordinate refers to ethanol substrate.

NAD-NADP transhydrogenase. The presence of this enzyme could not be detected.

Acetaldehyde dehydrogenase. Experiments with crude extracts, 105,000g supernatants and $(\text{NH}_4)_2\text{SO}_4$ fractions all failed to detect a coenzyme A-dependent acetaldehyde dehydrogenase of the *Escherichia coli* type (Pinchot & Racker, 1951; Dawes & Foster, 1956).

Lactate dehydrogenase. Efforts to demonstrate an NAD-dependent lactate dehydrogenase were frustrated by the presence of the very active pyruvate decarboxylase and ethanol dehydrogenase, and fractions freed of these enzymes did not display activity towards lactate. However, the presence of a rather weak lactate dehydrogenase, with optimum activity at pH 7.6–8.0, was demonstrated by coupling to ferricyanide and measuring ΔE_{405} (Fig. 4*a, b*).

Fig. 4. Lactate dehydrogenase assay. (*a*) Progress of reaction. Cuvettes contained: 1.4 ml. 0.1 M-K-phosphate, pH 8.0; 0.1 ml. 10 mM-potassium ferricyanide; 0.2 ml. 0.1 M-lithium lactate; 0.2 ml. bacterial extract (8.6 mg. protein/ml. buffer) (○) or water (control) (●) and water to 3 ml. After incubation for 15 min. at 22° the reaction was initiated by addition of the substrate. (*b*) Effect of pH. Conditions as for (*a*) except 0.1 M-K-phosphate (○) and 0.1 M-tris buffer (□) used and the reaction continued for 50 min.

Table 3. Detection of pyruvate thioclastic enzyme by pyruvate-formate exchange reaction

Reaction mixture	Isolated pyruvate 2,4-dinitrophenyl- hydrazone (mM)	Total radioactivity (counts/0.2 ml./ 100 min.)	Specific activity (counts/min./ μmole)
[¹⁴ C]-Formate	0	270	—
[¹⁴ C]-Formate + pyruvate	0.50	459	18
[¹⁴ C]-Formate + pyruvate + boiled yeast extract	0.49	1171	90
[¹⁴ C]-Formate + pyruvate + boiled <i>Erwinia amylovora</i> extract	0.52	1728	145

Pyruvate thioclastic enzyme. The production of formate in pyruvate fermentation suggested the presence of the thioclastic enzyme but no significant activity could be detected in crude extracts unless they were supplemented with boiled extracts of either baker's yeast or *Erwinia amylovora* (Table 3). Even then the incorporation of [¹⁴C]-formate into pyruvate was very low.

Formate dehydrogenase and formate hydrogenlyase. Neither intact organisms nor extracts produced H₂ and CO₂ from formate, nor did they reduce methylene blue or benzyl viologen in the presence of this substrate.

α-Acetolactate decarboxylase. Extracts decarboxylated α-acetolactate rapidly whereas CO₂ was evolved but slowly by intact bacteria. This indicated a permeability barrier to the substrate under the conditions used (Fig. 5).

Fig. 5. Decarboxylation of α -acetolactate by intact cells and extracts. Warburg flasks contained: 1.5 ml. 0.1 M-K-phosphate buffer, pH 6.0; 1.0 ml. 4.76 mM- α -acetolactate; 0.5 ml. bacterial suspension (20.7 mg. dry wt./ml.) or bacterial extract (9.8 mg. protein/ml.). Temperature, 30°. Atmosphere, N₂. The reaction was started by tipping the suspension or extract from the side arm to the main compartment. □, Intact bacteria; ○, bacterial extracts. Solid symbols are controls without substrate.

DISCUSSION

Considerable controversy has surrounded the classification of the genus *Erwinia*, which is placed in the Enterobacteriaceae family (*Bergey's Manual of Determinative Bacteriology*, 1957). It is generally agreed that *E. amylovora*, the causal organism of fire-blight, differs biochemically and pathogenically from the pectinolytic soft-rot *Erwinia* species (Martinec & Kočur, 1963; Edwards & Ewing, 1967) for which a new genus *Pectobacterium* was suggested by Waldee (1945), a differentiation supported by numerical taxonomy (Lockhart & Koenig, 1965). Martinec & Kočur (1963) have recommended that the genus *Erwinia* should include only two species and one variety, namely *E. amylovora*, *E. amylovora* var. *salicis* and *E. carotovora*. They have shown that the relationship between *Erwinia* and other genera of the Enterobacteriaceae is such that *E. amylovora* resembles *Shigella* and *E. carotovora* resembles *Klebsiella* and *Enterobacter*. Recent work on DNA base ratios indicates that *Erwinia* species have GC values within the span 50 to 56 % (Marmur, Falkow & Mandel, 1963; Fedorova, 1964; Starr & Mandel, 1969) which falls within the accepted range for the Enterobacteriaceae.

The Enterobacteriaceae are characterized by mixed acid fermentations of glucose with two principal variations in the formation of neutral compounds, typical of *Escherichia coli* and *Aerobacter aerogenes* respectively; the aerogenes type produces acetoin, diacetyl and butane 2,3-diol in addition to the ethanol produced in the coliform fermentation. It is a feature of these fermentations that the mechanism for alcohol production, via thioclastic fission of pyruvate to acetyl CoA, which is then reduced by a coenzyme A-dependent acetaldehyde dehydrogenase (Dawes & Foster, 1956), predicts a theoretical maximum yield of 1 mole ethanol/mole glucose fermented (Dawes, 1963). In practice, the highest yield recorded for *E. coli* is 0.8 mole/mole glucose (Stokes, 1949). Consequently the finding (Sutton & Starr, 1959) that *Erwinia amylovora* fermented glucose with the stoichiometry

indicated a deviation from the Enterobacteriaceae pattern. We have now shown the presence in the organism of a yeast-type pyruvate decarboxylase, which converts pyruvate to acetaldehyde and CO_2 . This enzyme has not been recorded in any other member of the Enterobacteriaceae family. Indeed it is of very limited occurrence in bacteria and previously only *Acetobacter suboxydans* (King & Cheldelin, 1954), *Zymomonas mobilis* (De Moss, 1953), *Z. anaerobia* (McGill, Ribbons & Dawes, 1965) and *Sarcina ventriculi* (Bauchop & Dawes, 1959) were known to possess the enzyme. The pyruvate decarboxylase of *Erwinia amylovora* seems to differ from the other recorded examples in having a very tightly-bound thiamine pyrophosphate prosthetic group. The presence of an NAD-dependent ethanol dehydrogenase permits the reduction of acetaldehyde to ethanol and the two enzymes can thus account for the high yield of ethanol observed in the fermentation of glucose. When pyruvate replaces glucose as the fermentation substrate there is available the equivalent of 4H less reducing power, thus accounting for the low yield of ethanol and the accumulation of free acetaldehyde observed in this work.

A coliform-type of thioclastic reaction converting pyruvate to acetyl CoA and formate was inferred from the incidence of formate in the fermentation products, although the yield was low. Evidence for the presence of the enzyme has been obtained but [^{14}C]-formate incorporation was weak. While negative results can never be conclusive, our inability to detect a coenzyme A-dependent acetaldehyde dehydrogenase, coupled with the low activity of the thioclastic reaction, suggests that the coliform route of ethanol formation is not significant in *Erwinia amylovora*. The fact that the yield of acetate was considerably in excess of that of formate indicates the existence of other reactions leading to acetate, e.g. coupled oxidation-reduction of pyruvate to lactate, acetate and CO_2 (Fig. 6).

The presence of pyruvate decarboxylase is of interest in relation to acetoin formation. Two mechanisms are known in micro-organisms (Juni, 1952), one characteristic of organisms possessing pyruvate decarboxylase and the other, in those that do not have the enzyme, involving α -acetylactate as an intermediate, together with α -acetylactate decarboxylase. *Erwinia amylovora* thus appears to be the first bacterium recorded with both pyruvate decarboxylase and α -acetylactate decarboxylase enzymes. While we have not been concerned with evaluating their relative significance in acetoin formation, it may be noted by comparing the results in Table 1 that a large increase in acetoin yield resulted in a much smaller decrease in acetaldehyde yield, indicating that diversion of acetaldehyde cannot account for much of the additional acetoin formed. Since a survey of *E. amylovora* by Martinec & Kočur (1964) revealed that 12 of the 49 strains studied did not produce acetoin, it would be instructive to

Fig. 6. Fermentation of pyruvate of *Erwinia amylovora*.

know whether these organisms lacked both pyruvate decarboxylase and α -acetolactate decarboxylase.

Martinec & Kočur (1964) reported that sodium formate was utilized by all 49 strains of *Erwinia amylovora* examined in their taxonomic study of this species. Utilization was judged on the basis of the increase in pH in a modified Koser's medium (containing formate) with phenol red as indicator (Dr M. Kočur, personal communication). All our attempts to detect the metabolism of formate by intact organisms and extracts of *E. amylovora* have failed and we attribute the positive diagnostic bacteriological test to an artifact of the method.

While the effect of pH was incidental to our investigation it is well known that the products of coli-aerogenes fermentations are influenced considerably by this parameter. At low pH the yield of lactate increases at the expense of acetate, formate, H_2 and CO_2 , as does acetoin and related compounds.

Thus *Erwinia amylovora* differs from all other Enterobacteriaceae in possessing a pyruvate decarboxylase, which explains the atypical fermentation balance recorded by Sutton & Starr (1959) with high ethanol yield. It would be interesting to know the distribution of pyruvate decarboxylase in other members of the genus *Erwinia*, particularly in relation to the pectinolytic organisms.

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