The Route of Ethanol Formation in *Zymomonas mobilis*

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(Received 10 August 1965)

1. Enzymic evidence supporting the operation of the Entner–Doudoroff pathway in the anaerobic conversion of glucose into ethanol and carbon dioxide by *Zymomonas mobilis* is presented. 2. Cell extracts catalysed the formation of equimolar amounts of pyruvate and glyceraldehyde 3-phosphate from 6-phosphogluconate. Evidence that 3-deoxy-2-oxo-6-phosphogluconate is an intermediate in this conversion was obtained. 3. Cell extracts of the organism contained the following enzymes: glucose 6-phosphate dehydrogenase (active with NAD and NADP), ethanol dehydrogenase (active with NAD), glyceraldehyde 3-phosphate dehydrogenase (active with NAD), hexokinase, gluconokinase, glucose dehydrogenase and pyruvate decarboxylase. Extracts also catalysed the overall conversion of glycerate 3-phosphate into pyruvate in the presence of ADP. 4. Gluconate dehydrogenase, fructose 1,6-diphosphate aldolase and NAD–NADP transhydrogenase were not detected. 5. It is suggested that NAD is the physiological electron carrier in the balanced oxidation–reduction involved in ethanol formation.

Considerable significance has been attached to *Zymomonas mobilis* (*Pseudomonas lindneri*) since it is cited as a model micro-organism for the anaerobic Entner & Doudoroff (1952) pathway of degradation of glucose (see, e.g., Gunsalus & Shuster, 1961; Wood, 1961). Evidence to support such claims has hitherto been based almost solely on isotopic data, and no enzymic details have been presented. It was the purpose of our investigation to confirm the assumptions of previous workers by demonstrating the presence of the relevant enzymes in this micro-organism, and particularly to examine the coenzyme specificities of the reactions involved in ethanol formation. Much of the work reported was carried out during 1960–61 and a preliminary account was communicated to The Biochemical Society at that time (Ribbons & Dawes, 1961).

Kluyver & Hoppenbrouwers (1931) showed that *Z. mobilis* ferments glucose to approximately 1.8 moles each of ethanol and carbon dioxide, and 0.2 mole of lactate/mole of hexose utilized. Gibbs & DeMoss (1951, 1954) and DeMoss (1953) confirmed the fermentation balance when washed cell suspensions were allowed to ferment glucose or fructose in the absence of a nitrogen source. Using specifically labelled [¹⁴C]glucose they showed that the carbon dioxide arose from C-1 and C-4 of the

glucose molecule. [2-¹⁴C]Glucose was fermented to yield ethanol labelled in the carbinol carbon atom. Earlier, DeMoss & Gibbs (1952) reported the presence of pyruvate decarboxylase ('carboxylase'), glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and fructose 1,6-diphosphate aldolase in cell extracts.

From the results of a radio-respirometric study Stern, Wang & Gilmour (1960) concluded that *Z. mobilis* ferments glucose by an Entner–Doudoroff pathway. A molar-growth-yield study by Bauchop & Elsdon (1960) is also consistent with this pathway, as these workers were able to demonstrate that the energy yield per mole of hexose was one-half of the corresponding value for *Saccharomyces cerevisiae*. We have confirmed this finding (Ribbons, Dawes & Rees, 1962) and details are presented in the next paper (Dawes, Ribbons & Rees, 1966). Since our work was completed a paper on the growth yield and growth characteristics of *Z. mobilis* has appeared (Belaich & Senez, 1965).

EXPERIMENTAL

Materials

Organism. The strain of *Z. mobilis* used was N.C.I.B. 8938. This strain is no longer motile, an observation confirmed by Dr J. L. Shimwell.

Media and growth of the organism. *Z. mobilis* was maintained initially as stab-cultures in nutrient agar containing glucose (2%, w/v). Slants of the same media were used

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subsequently when we found that *Z. mobilis* could tolerate and consume oxygen. Liquid cultures were grown at 30° in Erlenmeyer flasks containing (g./100 ml. of water): glucose, 2; Bacto yeast extract (Difco), 1; Bacto-Peptone (Difco), 1; pH 6.8. Bulk cultures (8 l.) were grown in 10 l. flat-bottomed flasks and were inoculated with 10 ml. of an actively gassing culture. Small volumes (up to 450 ml.) were harvested by centrifuging at 18000g for 5 min. Larger (8 l.) cultures were harvested at 55000g on a Sharples no. 1A laboratory Super-Centrifuge driven by compressed air. Cells were washed twice with either phosphate buffer (pH 7.0) or water, and then either used immediately or, alternatively, stored at -18° as a paste until required.

Cell extracts. These were prepared by submitting cell suspensions (approx. 5 g. wet wt./25 ml. of distilled water) to ultrasonic oscillation in either a Raytheon 9kcyc./sec. ultrasonic oscillator for 25–30 min., or an MSE ultrasonic disintegrator for 2 min.; cooling was achieved by running tap water (9–15°) or by crushed ice respectively. The resultant suspension was centrifuged at approx. 18000g for 30 min. Some extracts were further clarified in the Spinco (model L) preparative ultracentrifuge at 97000g or higher for 2 hr. at 0°.

Enzymes. Crystalline muscle aldolase was obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

Chemicals. NAD, NADP, NADH₂ and NADPH₂ were obtained from C. F. Boehringer und Soehne G.m.b.H., and from Sigma Chemical Co., St Louis, Mo., U.S.A. Triose phosphates were obtained as a solution of the esters (TEP solution) from C. F. Boehringer und Soehne G.m.b.H. 6-Phosphogluconate and glucose 6-phosphate were obtained as barium and sodium salts from Sigma Chemical Co. and from British Drug Houses Ltd., Poole, Dorset. The monobarium salt of 3-deoxy-2-oxo-6-phosphogluconate was a generous gift from Dr W. A. Wood. Other reagents were of analytical grade wherever possible.

Buffer solutions. Phosphate buffer was 0.067 M-KH₂PO₄ adjusted to the required pH with 5 N-NaOH; glycylglycine buffer of the appropriate concentration was adjusted to the required pH with 5 N-NaOH. Pyrophosphate buffer was 0.1 M-sodium pyrophosphate, pH 8.5. Other buffers (see Table 3) were prepared according to Gomori (1955).

Analytical methods

Chemical estimations. Glucose was determined by the method of Nelson (1944). Ethanol was estimated by dichromate oxidation of a neutral steam-distillate, followed by back-titration of the excess of 0.04 N-K₂Cr₂O₇ with KI and 0.05 N-Na₂S₂O₃ as described by Neish (1952). Lactate was estimated by the procedures of Hullin & Noble (1953) and Barker & Summerson (1941). Pyruvate was determined by the extraction procedure of Friedemann & Haugen (1943). Total keto acids were determined by the semicarbazide method of MacGee & Doudoroff (1954). Oxygen consumption and CO₂ evolution were measured in constant-volume Warburg respirometers at 30°. Evolution of CO₂ was always estimated with a gas phase of N₂ + CO₂ (95:5) (Umbreit, Burris & Stauffer, 1957). Spectrophotometric determinations were made with Hilger Uvispek, Unicam SP.500 and SP.600 instruments and quartz cells with 1 cm. light-path. Inorganic phosphate was determined by the method of Fiske & Subbarow (1925). Alkali-labile

phosphate was determined as described by Umbreit *et al.* (1957).

3-Deoxy-2-oxo-6-phosphogluconate was determined either by the semicarbazide method of MacGee & Doudoroff (1954) or by periodate oxidation followed by thiobarbituric acid treatment as described by Srinivasan & Sprinson (1959). The phosphorylated form of 3-deoxy-2-oxogluconate is not readily oxidized by periodate (MacGee & Doudoroff, 1954) and maximum colour formation required a 60 min. reaction period. Even then, as observed by Meloche & Wood (1964), the colour yield was much less than that obtained with the unphosphorylated compound. We found the molar extinction coefficient under these conditions to be 1500 at 548 mμ; that of the dephosphorylated compound is 44000 (Weissbach & Hurwitz, 1959). Hydrazine interferes with this reaction and was omitted from the assay mixture. Protein was estimated by the biuret method of Stickland (1951).

Chromatography. Descending paper chromatograms were developed in propan-1-ol-formic acid (90%, w/v)-water (6:3:1, by vol.) (De Ley, 1955). After drying at room temperature phosphate esters were located by dipping in the reagent of Burrows, Grylls & Harrison (1952) followed by drying at room temperature and irradiation with a u.v. lamp.

Enzyme assays. The assay methods for glucose 6-phosphate dehydrogenase, ethanol dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, NADH₂ oxidase, NAD-NADP transhydrogenase and the combined activities of 6-phosphogluconate dehydratase and 3-deoxy-2-oxo-6-phosphogluconate aldolase are given in the legends to the appropriate Figures.

Glucose 6-phosphate dehydrogenase in crude extracts was also assayed manometrically by O₂ consumption. Each flask contained: 20 μmoles of glucose 6-phosphate, 0.5 ml.; 0.3 M-glycylglycine buffer, pH 7.3, 1.0 ml.; 2 mM-NAD, 1.0 ml.; water, 0.3 ml.; cell extract, 0.2 ml. The centre well contained 0.2 ml. of 20% (w/v) KOH. Substrate was omitted from the control.

Glyceraldehyde 3-phosphate dehydrogenase was also assayed manometrically. Each flask contained: 0.067 M-phosphate buffer, pH 7.1, 1.0 ml.; 0.1 M-glutathione, 0.2 ml.; 20 mM-ADP, 0.5 ml.; 2 mM-NAD, 0.2 ml.; triose phosphate ester solution (approx. 16 mM), 0.5 ml.; 0.08 M-MgCl₂, 0.1 ml.; cell extract, 0.5 ml. The centre well contained 0.2 ml. of 20% (w/v) KOH. Substrate was omitted from the control. Glucose dehydrogenase and gluconate dehydrogenase were both assayed by the reduction of 2,6-dichlorophenol-indophenol in tubes of the type described by Peel (1960). The assay system contained: 0.2 M-glycylglycine, pH 7.3, 2.0 ml.; 0.05% (w/v) 2,6-dichlorophenol-indophenol, 0.2 ml.; 20 mM-substrate, 1.0 ml.; water, 1.5 ml.; cell extract, 0.3 ml. The change in extinction at 600 mμ was measured over a period of 5 min.

Hexokinase was demonstrated spectrophotometrically by measuring the reduction of NAD by the glucose 6-phosphate produced by phosphorylation of glucose. The system contained: 0.08 M-NaHCO₃, 1.0 ml.; 0.4 M-MgCl₂, 0.1 ml.; 10 mM-glucose, 0.5 ml.; 50 mM-ATP, 0.3 ml.; 0.01 M-tris, pH 7.0, 0.5 ml.; cell extract, 0.1 ml.; 2 mM-NAD (or NADP), 0.1 ml. The NAD was added last and the change in E₃₄₀ measured. Gluconokinase was demonstrated spectrophotometrically in the following system: 0.1 M-glycylglycine, pH 7.0, 0.8 ml.; 50 mM-sodium arsenate, 1.0 ml.;

Table 1. *Fermentation of glucose during growth of Zymomonas mobilis*

Expt. no.	Glucose utilized (μ moles/ml.)	Ethanol formed (μ moles/ml.)	Molar ratio ethanol/glucose	Lactate formed (μ moles/ml.)	Molar ratio lactate/glucose
1	66	106	1.61	1.32	0.02
2	118.5	198.5	1.67	1.35	0.015
3	88	140.8	1.60	—	—

100 mM-glutathione, 0.1 ml.; 100 mM- NaHSO_3 , 0.2 ml.; 40 mM- MgCl_2 , 0.05 ml.; 10 mM-sodium gluconate, 0.3 ml.; 50 mM-ATP, 0.2 ml.; 2.0 mM-NAD, 0.1 ml.; 2.0 mM-NADP, 0.1 ml.; cell extract, 0.1 ml.

Pyruvate decarboxylase ('carboxylase') was assayed manometrically in the following system: 0.1 M-glycylglycine, pH 7.1, 1.5 ml.; cell extract, 0.5 ml.; 50 mM-sodium pyruvate, 1.0 ml.; the atmosphere was $\text{N}_2 + \text{CO}_2$ (95:5) and the temperature was 30°. The formation of acetaldehyde was detected by smell and established as the 2,4-dinitrophenylhydrazone. The CO_2 evolution in this system is not stoichiometric, an observation which we have also made with the pyruvate decarboxylases of *Zymosarcina ventriculi*, *Zymomonas anaerobia* and *Erwinia amylovora*.

RESULTS

Whole cell experiments

Products of glucose fermentation. Contrary to the reports of previous workers, we were unable to detect the formation of appreciable amounts of lactate by either growing or washed cells during glucose fermentation. The low lactate values have also been confirmed with our strain by Dr J. L. Peel (personal communication). Table 1 records the utilization of glucose and formation of ethanol. The average yield of ethanol (1.64 moles/mole of glucose fermented) is lower than that recorded by earlier workers but agrees well with Belaïch & Senez (1965), who used the same strain as us.

Oxidation of glucose by washed cell suspensions. When, early in this work, we observed that *Z. mobilis* could grow in the presence of oxygen, an examination was made of the oxidative capabilities of aerobically and anaerobically grown cells. Both types of cell oxidized glucose without lag and Q_{O_2} values of 60–80 (anaerobic) and 100–140 (aerobic) were recorded. When limited amounts of glucose were used, anaerobically grown cells took up 0.5–0.8 μ mole of oxygen/ μ mole of glucose added, and aerobic cells took up 0.7–1.5 μ moles of oxygen/ μ mole of glucose. These differences were consistent and reproducible for cells grown on media containing 2% (w/v) of glucose, and ratios of Q_{O_2} (aerobically grown)/ Q_{O_2} (anaerobically grown) were always between 1.3 and 2.5. Recently Belaïch & Senez (1965) reported much smaller differences between aerobically and anaerobically grown cells and we have therefore repeated our experiments with cells

Fig. 1. Pyruvate metabolism under conditions used for the Entner-Doudoroff enzymes assay. Reaction mixtures contained: 0.1 M-glycylglycine buffer, pH 7.4, 9.6 ml.; 0.1 M- Na_2AsO_3 , 0.4 ml.; cell extract, 0.5 ml. (8.2 mg. of protein); 50 mM-sodium pyruvate, 4.0 ml.; water to 20 ml. ●, No hydrazine; ○, 4.0 ml. of 0.56 M-hydrazine. Reactions were initiated by addition of pyruvate after incubation at 30° for 5–7 min. and samples (1 ml.) were withdrawn into 40% trichloroacetic acid (0.3 ml.). Pyruvate was estimated in the supernatant after removal of the protein by centrifugation.

grown on their medium. Under these conditions we confirmed their observations, suggesting that the oxidative capabilities of the cells are considerably affected by the medium on which they are grown.

Reactions catalysed by cell-free extracts

If an anaerobic Entner-Doudoroff pathway of glucose dissimilation occurs then 6-phosphogluconate should be metabolized to equivalent amounts of pyruvate and glyceraldehyde 3-phosphate in the presence of suitable trapping agents. Appropriate concentrations of hydrazine and arsenite are employed as a routine to prevent further metabolism of glyceraldehyde 3-phosphate and pyruvate

respectively in aerobic systems (Wood & Schwerdt, 1954). We have found that arsenite does not effectively prevent metabolism of pyruvate by extracts of *Z. mobilis* but that hydrazine alone will trap pyruvate (Fig. 1) as well as glyceraldehyde 3-phosphate. The formation of equivalent amounts of triose phosphate and pyruvate from 6-phosphogluconate in the presence of hydrazine is shown in Fig. 2. Approximately 70% conversion of 6-phosphogluconate into pyruvate and triose phosphate occurred; in other experiments between 60 and 70% conversion was consistently observed. Kovachevich & Wood (1955) also observed a similar conversion (64%) of 3-deoxy-2-oxo-6-phosphogluconate into pyruvate and triose phosphate with *Pseudomonas fluorescens*. In the absence of hydrazine pyruvate appears transiently and almost stoichiometric formation of triose phosphate is observed (Fig. 3); under these conditions the triose phosphate is not metabolized to pyruvate by crude extracts unless supplemented with NAD, ADP and inorganic phosphate. Some inorganic phosphate is usually liberated in the absence of hydrazine. These experiments are consistent with an Entner-Doudoroff pathway. The enzymic sequence common to all pathways of carbohydrate metabolism,

Fig. 2. Formation of pyruvate and glyceraldehyde 3-phosphate from 6-phosphogluconate in the presence of hydrazine. The reaction mixture contained: 0.1 M-glycylglycine buffer, pH 7.0, 9.6 ml.; 0.56 M-hydrazine, 4.0 ml.; water, 0.4 ml.; cell extract, 2.0 ml. (32.1 mg. of protein). It was incubated at 30° for 5 min. before the substrate, 6-phosphogluconate (4.0 ml., 10 mM), was added. Samples (2 ml.) were taken at intervals, added to 40% trichloroacetic acid (0.6 ml.), centrifuged and portions of the clarified solutions used for the analysis of pyruvate (●) and alkali-labile phosphate (Δ).

Fig. 3. Formation of pyruvate, glyceraldehyde 3-phosphate and inorganic phosphate from 6-phosphogluconate in the absence of hydrazine. The reaction mixture contained: 0.2 M-glycylglycine buffer, pH 7.3, 2.9 ml.; 10 mM-6-phosphogluconate, 1.2 ml.; water, 1.8 ml.; cell extract, 0.3 ml. (8.0 mg. of protein). ▲, Pyruvate; Δ, glyceraldehyde 3-phosphate; ■, inorganic phosphate. The horizontal broken line at 1.94 μmoles/ml. indicates the theoretical yield of pyruvate and glyceraldehyde 3-phosphate.

Fig. 4. Formation of pyruvate from glycerate 3-phosphate. The reaction mixture contained: 0.1 M-glycylglycine buffer, pH 7.1, 3.3 ml.; 20 mM-ADP, 1.0 ml.; 40 mM-MgCl₂, 0.1 ml.; 0.56 M-hydrazine, 2.0 ml.; cell extract, 1.6 ml. (12 mg. of protein); it was incubated at 30° for 5 min. for equilibration and then 10 mM-glycerate 3-phosphate (2.0 ml.) was added to initiate the reaction. Samples were treated as described in the legend to Fig. 2.

i.e. the conversion of glycerate 3-phosphate into pyruvate, was also demonstrated (Fig. 4).

NADH₂ oxidase. Attempts to determine by spectrophotometric methods the nicotinamide-

adenine dinucleotide specificities of the enzymes involved in the oxidation–reduction balance were initially hindered by the presence in crude cell extracts of a powerful NADH₂ oxidase, which could also be determined manometrically. This activity could be removed, however, by centrifuging at 105 000*g* for 2 hr. Table 2 shows the distribution of NADH₂ oxidase between the supernatant and particulate fractions. There was negligible NADPH₂-oxidase activity in our extracts.

Nicotinamide–adenine dinucleotide-linked dehydrogenases. The relevant enzymes for maintenance of a balanced oxidation–reduction in the *Z. mobilis* fermentation are glucose 6-phosphate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase and ethanol dehydrogenase.

Glucose 6-phosphate dehydrogenase. This enzyme was non-specific with respect to the nicotinamide–adenine dinucleotide coenzyme; both NAD and NADP are reduced when the supernatants from the

ultracentrifuged cell-free extracts are incubated with glucose 6-phosphate. In phosphate buffer reduction of NADP is more rapid than that of NAD; substitution of tris–hydrochloric acid buffer results in the opposite behaviour. The relative activities of the two coenzymes were studied over a range of pH and with a variety of buffers (Table 3).

Ethanol dehydrogenase. Acetaldehyde causes rapid oxidation of NADH₂ when incubated in the presence of the supernatant fraction. NADPH₂ is not oxidized (Fig. 5*a*). Reversal of the reaction by the addition of ethanol was also demonstrated and a similar coenzyme specificity observed (Fig. 5*b*).

Triose phosphate dehydrogenase. This enzyme was shown to be NAD-specific (Fig. 6). Some impurity (e.g. glucose 6-phosphate) in the commercially available triose phosphates was responsible for the slight reduction of NADP observed. Fructose 1,6-diphosphate aldolase was not detected in these extracts since fructose 1,6-diphosphate did not cause reduction of NAD (by generation of glyceraldehyde 3-phosphate), although addition of crystalline muscle aldolase permitted immediate NAD reduction.

NAD–NADP transhydrogenase. Conclusions about coenzyme specificities could be invalidated by the presence of a transhydrogenase and therefore experiments were carried out to detect this enzyme in extracts (Fig. 7). The results of all such attempts were negative and we conclude that the enzyme is absent from *Z. mobilis*.

Other enzymes. The presence in extracts of hexokinase, gluconokinase, glucose dehydrogenase and pyruvate decarboxylase was established. Neither gluconate dehydrogenase nor fructose 1,6-diphosphate aldolase (Fig. 6) could be detected.

Formation of 3-deoxy-2-oxo-6-phosphogluconate as an intermediate in 6-phosphogluconate catabolism. In the presence of hydrazine, the formation of a

Table 2. *Distribution of NADH₂-oxidase activity in extracts of Zymomonas mobilis*

Particulate fraction is the sediment from centrifuging the crude extract at 105 000*g* for 2 hr. at 0°, taken up in 0.067 M-phosphate buffer, pH 7.0. Each cuvette contained: 0.067 M-phosphate buffer, pH 7.0, 2.5 ml.; enzyme solution (diluted fivefold), 0.1 ml.; 2 mM-NADH₂, 0.1 ml.

	Concn. of protein (mg./ml.)	Activity (μmoles of NADH ₂ oxidized/min./mg. of protein)	
		Specific	Total/ml.
Crude extract	22.20	0.10	2.22
Particulate fraction	12.05	0.17	2.07
Supernatant	7.27	0.03	0.22

Table 3. *Glucose 6-phosphate-dehydrogenase activity of extracts*

Each cuvette contained: buffer, 2.5 ml.; cell extract (105 000*g* supernatant), 0.1 ml. (0.71 mg. of protein); 2 mM-NAD or 2 mM-NADP, 0.1 ml.; 10 mM-glucose 6-phosphate, 0.1 ml., the last-named initiating the reactions.

Buffer	Concn. (M)	pH	Activity (Δ <i>E</i> ₃₄₀ /min./mg. of protein)	
			NAD	NADP
Pyrophosphate Tris–HCl	0.1	8.5	0.13	0.85
		8.4	1.09	1.36
		8.0	1.30	1.56
		7.0	1.35	1.05
		7.3	1.62	1.62
Glycylglycine	0.1	7.5	0.29	0.59
Phosphate	0.067	7.0	0.28	1.24
Phosphate–citrate	0.05	6.0	0.18	0.67
Citrate	0.05	6.0	0.02	0.18

Fig. 5. Coenzyme specificity and reversibility of ethanol dehydrogenase. (a) Each cuvette contained: 0.067M-phosphate buffer, pH7.0, 2.5 ml.; cell extract (105 000g supernatant diluted fivefold), 0.1 ml. (0.15 mg. of protein); 2mM-NADH₂ or 2mM-NADPH₂, 0.1 ml. ○, NADH₂; ●, NADPH₂, followed by NAD addition at the indicated time. Reactions were initiated by the addition of acetaldehyde at 3 min. Temperature, 23°. (b) Each cuvette contained: 0.1M-sodium pyrophosphate buffer, pH8.5, 0.5 ml.; water, 1.45 ml.; cell extract (105 000g supernatant), 0.25 ml. (1.9 mg. of protein); 3M-ethanol, 0.1 ml.; 2mM-NAD or 2mM-NADP, 0.1 ml. Ethanol was the last addition made after incubation for 5 min. at 22°. ○, NAD; ●, NADP.

keto acid from 6-phosphogluconate as determined by the semicarbazide method precedes the formation of pyruvate as determined by the Friedemann & Haugen (1943) technique (curves A and B respectively, Fig. 8). The difference between total keto acid and pyruvate (curve C) represents an intermediary keto acid precursor of pyruvate, presumably 3-deoxy-2-oxo-6-phosphogluconate (MacGee & Doudoroff, 1954). To demonstrate the formation of such a precursor directly, the thio-barbituric acid assay for β -formylpyruvic acid

Fig. 6. Coenzyme specificity of glyceraldehyde 3-phosphate dehydrogenase. Each cuvette contained: 0.1M-glycylglycine buffer, pH7.0, 1.0 ml.; 50mM-sodium arsenate, 1.0 ml.; 100mM-glutathione, 0.1 ml.; 100mM-NaHSO₃, 0.2 ml.; 40mM-MgCl₂, 0.05 ml.; triose phosphate esters, 0.2 ml. (approx. 16 mM), or 10 mM-fructose 1,6-diphosphate, 0.2 ml.; cell extract (105 000g supernatant), 0.1 ml. (0.71 mg. of protein); 2mM-NAD or 2mM-NADP, 0.1 ml., as shown. ○, Triose phosphate esters and NAD, followed by a second addition (indicated by arrow) of NAD after 4 min.; ●, triose phosphate esters and NADP, followed by a second addition (arrow) of NADP after 4.5 min. and NAD after 8.5 min. (arrow); △, fructose 1,6-diphosphate and NAD or NADP. Reactions were initiated by addition of the nucleotide. Temperature, 22°.

formed by periodate oxidation of 3-deoxy-2-oxo sugars (Weissbach & Hurwitz, 1959; Srinivasan & Sprinson, 1959) was applied.

The formation of 3-deoxy-2-oxo-6-phosphogluconate from 6-phosphogluconate as measured by this method and the subsequent appearance from it of an equivalent amount of triose phosphate (determined as alkali-labile phosphate) in the absence of hydrazine is shown in Fig. 9. Formation of the keto acid was very rapid, approaching the theoretical yield in the initial 2 min. of the reaction, and afterwards declining to a value representing about 25% of the theoretical yield. Pyruvate formation under these conditions was transient and very small. Samples of the incubation

Fig. 7. $NADH_2$ oxidase and transhydrogenase in (a) crude cell-free extracts and (b) high-speed supernatants (105 000g). (a) Each cuvette contained: 0.067M-phosphate buffer, pH 7.0, 2.5 ml.; 2 mM- $NADH_2$ or - $NADPH_2$, 0.1 ml.; crude cell extract (0.1 ml., 1.4 mg. of protein) was used to initiate the reactions at the arrows indicated by A. After 3.5 min. (arrow B) 2 mM- NAD (0.1 ml.) was added and after 5 min. (C) 2 mM- $NADH_2$ (0.1 ml.) was added to the $NADPH_2$ cuvette. $\circ$, $NADH_2$; $\bullet$, $NADPH_2$. Temperature, 24°. (b) Each cuvette contained: 0.067M-phosphate buffer, pH 7.0, 2.5 ml.; 2 mM- $NADH_2$ or - $NADPH_2$, 0.1 ml.; high-speed supernatant of the cell extract (0.1 ml., 0.5 mg. of protein) was used to initiate the reactions at the arrows marked D. After 4 min. (arrow E) 2 mM- NAD (0.1 ml.) was added to the $NADPH_2$ cuvette. $\circ$, $NADH_2$; $\bullet$, $NADPH_2$. Temperature, 24°.

Fig. 8. Formation of keto acids from 6-phosphogluconate in the presence of hydrazine. The incubation mixture contained, in a total of 10 ml.: 500 μmoles of glycylglycine, pH 7.0; 20 μmoles of 6-phosphogluconate; 2240 μmoles of hydrazine; cell extract (2.6 mg. of protein). Temperature, 30°. Samples (1 ml.) were removed at intervals into 1 ml. of 10% (w/v) perchloric acid and, after centrifuging, the supernatants were analysed as described in the Analytical methods section. No keto acids were detected in the control with 6-phosphogluconate omitted. $\circ$, Total keto acid (A); $\blacktriangle$, pyruvate (B); $\bullet$, difference (A - B) representing 3-deoxy-2-oxo-6-phosphogluconate (C).

mixture taken during the course of the reaction and deproteinized by hot ethanol were chromatographed on paper. Faint spots with the same R_F value as authentic 6-phosphogluconate (0.21–0.33) were found only in samples taken within the first 1.5 min. of the reaction. After 1.5 min. a spot with the same R_F value as authentic 3-deoxy-2-oxo-6-phosphogluconate (0.42–0.43) was observed but in later samples this disappeared.

DISCUSSION

Isotopic evidence (Gibbs & DeMoss, 1951, 1954; DeMoss, 1953) indicated that the Entner & Doudoroff (1952) route of glucose catabolism operates in *Z. mobilis*, but until 1961 (Ribbons & Dawes, 1961) there was no enzymic evidence to support this belief. *Z. mobilis* is one of the few bacteria that possess a yeast-type pyruvate decarboxylase, and can thus produce ethanol by the reduction of acetaldehyde. The only other ethanol-forming bacteria that possess this enzyme are *Zymomonas anaerobia* (D. J. McGill, D. W. Ribbons & E. A. Dawes, unpublished work), *Zymosarcina ventriculi* (Arbuthnott, Bauchop & Dawes, 1960) and *Erwinia amylovora* (A. Haq & E. A. Dawes, unpublished work) although, unlike the *Zymomonas*, the latter

two organisms also metabolize pyruvate by the thioclastic reaction. These various pathways of ethanol formation have been reviewed by Dawes (1963).

The key reactions of the Entner–Doudoroff system are dehydration of 6-phosphogluconate, by the enzyme 6-phosphogluconate dehydratase, to 3-deoxy-2-oxo-6-phosphogluconate, which is split by a specific aldolase to yield 1 mole each of pyruvate and glyceraldehyde 3-phosphate/mole; the triose phosphate is then metabolized to pyruvate by reactions common to glycolysis. Since anaerobic operation of the Entner–Doudoroff system involves a balanced oxidation–reduction between glucose 6-phosphate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase and ethanol dehydrogenase, we were interested in the coenzyme specificities of these enzymes. Of additional interest is the reason why the Embden–Meyerhof pathway of glucose catabolism apparently does not operate, despite the presence of fructose 1,6-diphosphate aldolase (DeMoss & Gibbs, 1952), although we did not detect this aldolase in our extracts. The latter problem was solved when Raps & DeMoss (1962)

Fig. 9. Formation of 3-deoxy-2-oxo-6-phosphogluconate, pyruvate and alkali-labile phosphate from 6-phosphogluconate in the absence of hydrazine. The incubation mixture contained, in a total volume of 11 ml.: 50 μ moles of glycylglycine, pH 7.0; 20 μ moles of 6-phosphogluconate; cell extract (5.3 mg. of protein). Temperature, 30°. Samples (1 ml.) were removed at intervals into 1 ml. of 10% (w/v) perchloric acid and, after centrifuging, the supernatants were analysed as described in the Analytical methods section (3-deoxy-2-oxo-6-phosphogluconate by the thiobarbituric acid method). No products were detected in the control with 6-phosphogluconate omitted. O, 3-Deoxy-2-oxo-6-phosphogluconate; ●, pyruvate; Δ , alkali-labile phosphate.

showed that *Z. mobilis* lacks phosphofructokinase, one of the enzymes unique to glycolysis.

Our experiments show that the enzymes necessary for the production, from 6-phosphogluconate, of 3-deoxy-2-oxo-6-phosphogluconate and its subsequent fission to pyruvate and glyceraldehyde 3-phosphate are present in extracts of the organism, thus substantiating the operation of the Entner-Doudoroff pathway. Further, the 3-deoxy-2-oxo-6-phosphogluconate and glyceraldehyde 3-phosphate finally present at equilibrium account for all of the 6-phosphogluconate metabolized.

Of the three oxidoreductase enzymes involved in the fermentation balance, the reactions catalysed by glucose 6-phosphate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase presumably reduce the nicotinamide nucleotide and the reduction of acetaldehyde, mediated by ethanol dehydrogenase, re-oxidizes the coenzyme. Since glucose 6-phosphate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase in other organisms are known to have varying coenzyme specificities, the question of coenzyme specificity in *Z. mobilis* was

of some importance. Glucose 6-phosphate-dehydrogenase activity with both NAD and NADP has been observed in *Leuconostoc mesenteroides* (DeMoss, Gunsalus & Bard, 1953), *Pseudomonas fluorescens* (Wood & Schwerdt, 1954) and *Xanthomonas phaseoli* (Hochster & Katznelson, 1958). The last workers were also unable to detect transhydrogenase activity in *X. phaseoli*, although Wood & Schwerdt (1954) found the enzyme in *P. fluorescens* and suggested that the observed activity of glucose 6-phosphate dehydrogenase with NAD in that organism was due to the coupled action of transhydrogenase and an NADP-specific glucose 6-phosphate dehydrogenase. The other oxidative enzyme, glyceraldehyde 3-phosphate dehydrogenase, has been shown to be completely specific for NAD in *P. fluorescens* (Frampton & Wood, 1961) and in *X. phaseoli* (Hochster & Katznelson, 1958).

Our work has confirmed the observation of DeMoss & Gibbs (1952) that glucose 6-phosphate dehydrogenase in *Z. mobilis* is active with both NAD and NADP, and has shown in addition that the glyceraldehyde 3-phosphate and ethanol dehydrogenases are specific for NAD. As in *X. phaseoli* (and unlike *P. fluorescens*), transhydrogenase is absent from *Z. mobilis* and it thus appears likely that, although the glucose 6-phosphate dehydrogenase in the organism is active with both NAD and NADP, only the former is the physiological electron carrier in the balanced oxidation-reduction of anaerobic glucose fermentation. Similar conclusions about the role of NAD in the phosphoketolase-mediated fermentation of glucose by *Leuconostoc mesenteroides* have been reached by DeMoss (1961). Under growth conditions it is possible that the non-specific glucose 6-phosphate dehydrogenase of *Z. mobilis* furnishes NADPH₂ for general biosynthesis.

The presence of gluconokinase and the absence of gluconate dehydrogenase suggests that gluconate is metabolized via 6-phosphogluconate and the Entner-Doudoroff enzymes. Although our results do not permit us to conclude whether oxidation of glucose precedes phosphorylation, this seems unlikely since direct oxidation of glucose is not known to be coupled with nicotinamide nucleotide reduction, which is mandatory for ethanol formation.

The technical assistance of Mrs Irene Thomson, Mr J. Hastings and Miss Elaine Gibson is gratefully acknowledged.

REFERENCES

- Arbuthnott, J. P., Bauchop, T. & Dawes, E. A. (1960). *Biochem. J.* **76**, 12P.
- Barker, S. B. & Summerson, W. H. (1941). *J. biol. Chem.* **138**, 535.
- Bauchop, T. & Elsdon, S. R. (1960). *J. gen. Microbiol.* **23**, 457.

- Belaich, J. P. & Senez, J. C. (1965). *J. Bact.* **89**, 1195.
- Burrows, S., Grylls, F. S. M. & Harrison, G. S. (1952). *Nature, Lond.*, **170**, 800.
- Dawes, E. A. (1963). *J. gen. Microbiol.* **32**, 151.
- Dawes, E. A., Ribbons, D. W. & Rees, D. A. (1966). *Biochem. J.* **98**, 804.
- De Ley, J. (1955). *Naturwissenschaften*, **42**, 96.
- DeMoss, R. D. (1953). *J. cell. comp. Physiol.* **41**, Suppl. 1, 207.
- DeMoss, R. D. (1961). *Bact. Proc.* p. 166.
- DeMoss, R. D. & Gibbs, M. (1952). *Bact. Proc.* p. 145.
- DeMoss, R. D., Gunsalus, I. C. & Bard, R. C. (1953). *J. Bact.* **66**, 10.
- Entner, N. & Doudoroff, M. (1952). *J. biol. Chem.* **196**, 853.
- Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
- Frampton, E. W. & Wood, W. A. (1961). *J. biol. Chem.* **236**, 2571.
- Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
- Gibbs, M. & DeMoss, R. D. (1951). *Arch. Biochem. Biophys.* **34**, 478.
- Gibbs, M. & DeMoss, R. D. (1954). *J. biol. Chem.* **207**, 689.
- Gomori, S. (1955). In *Methods in Enzymology*, vol. 1, p. 138. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Gunsalus, I. C. & Shuster, C. W. (1961). In *The Bacteria*, vol. 2, p. 1. Ed. by Gunsalus, I. C. & Stanier, R. Y. New York: Academic Press Inc.
- Hochster, R. M. & Katznelson, H. (1958). *Canad. J. Biochem. Physiol.* **36**, 669.
- Hullin, R. P. & Noble, R. L. (1953). *Biochem. J.* **55**, 289.
- Kluyver, A. J. & Hoppenbrouwers, W. H. (1931). *Arch. Mikrobiol.* **2**, 245.
- Kovachevich, R. & Wood, W. A. (1955). *J. biol. Chem.* **213**, 757.
- MacGee, J. & Doudoroff, M. (1954). *J. biol. Chem.* **210**, 617.
- Meloche, H. P. & Wood, W. A. (1964). *J. biol. Chem.* **239**, 3511.
- Neish, A. C. (1952). *Rep. nat. Res. Coun. Canad. no. 46-8-3: Analytical Methods for Bacterial Fermentations* (2nd revision).
- Nelson, N. (1944). *J. biol. Chem.* **153**, 375.
- Peel, J. L. (1960). *Biochem. J.* **74**, 525.
- Raps, S. & DeMoss, R. D. (1962). *J. Bact.* **84**, 115.
- Ribbons, D. W. & Dawes, E. A. (1961). *Biochem. J.* **81**, 3P.
- Ribbons, D. W., Dawes, E. A. & Rees, D. A. (1962). *Biochem. J.* **82**, 45P.
- Srinivasan, P. R. & Sprinson, D. B. (1959). *J. biol. Chem.* **234**, 716.
- Stern, I. J., Wang, C. H. & Gilmour, C. M. (1960). *J. Bact.* **79**, 601.
- Stickland, L. H. (1951). *J. gen. Microbiol.* **5**, 698.
- Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1957). *Manometric Methods*, 3rd ed., pp. 22, 278. Minneapolis: Burgess Publishing Co.
- Weissbach, A. & Hurwitz, J. (1959). *J. biol. Chem.* **234**, 705.
- Wood, W. A. (1961). In *The Bacteria*, vol. 2, p. 99. Ed. by Gunsalus, I. C. & Stanier, R. Y. New York: Academic Press Inc.
- Wood, W. A. & Schwerdt, R. F. (1954). *J. biol. Chem.* **206**, 625.