Glucose and Fructose Metabolism in *Zymomonas anaerobia*

By D. J. MCGILL* AND E. A. DAWES

Department of Biochemistry, University of Hull, Hull HU6 7RX, U.K.

(Received 29 July 1971)

Isotopic and enzymic evidence indicates that *Zymomonas anaerobia* ferments glucose via the Entner–Doudoroff pathway. The molar growth yields with glucose (5.89) and fructose (5.0) are lower than those for the related organism *Zymomonas mobilis* and the observed linear growth suggests that energetically uncoupled growth occurs. A survey of enzymes of carbohydrate metabolism revealed the presence of weak phosphofructokinase and fructose 1,6-diphosphate aldolase activities but phosphoketolase, transketolase and transaldolase were not detected. Fermentation balances for glucose and fructose are reported; acetaldehyde accumulated in both fermentations, to a greater extent with fructose which also yielded glycerol and dihydroxyacetone as minor products.

The genus *Zymomonas* includes but two species, *Zymomonas mobilis* and *Zymomonas anaerobia*. The former organism is responsible for the fermentation of the sap of *Agave americana* in the production of the Mexican alcoholic beverage pulque, and has been widely investigated since its isolation by Lindner (1928). Glucose fermentation balances with *Z. mobilis* (Kluyver & Hoppenbrouwers, 1931) suggested a yeast-type fermentation but subsequently, by a combination of isotopic and enzymic studies, the organism was shown to ferment glucose by the Entner & Doudoroff (1952) pathway (Gibbs & DeMoss, 1954; Raps & DeMoss, 1962; Stern, Wang & Gilmour, 1960; Dawes, Ribbons & Large, 1966). A growth yield study with *Z. mobilis* (Bauchop & Elsdon, 1960) was also consistent with this metabolic route, the ATP yield per mole of hexose being one half of the corresponding value for *Saccharomyces cerevisiae* which utilizes Embden–Meyerhof glycolysis.

In contrast *Z. anaerobia* is associated with cider spoilage ('cider sickness') and has not been intensively studied. The organism was originally isolated by Barker & Hillier (1912) and later re-isolated by Shimwell (1937) who named it *Achromobacter anaerobium*. Millis (1956) has described some of the biochemical characteristics of *Z. anaerobia* and presented evidence for an essentially quantitative conversion of glucose into ethanol and carbon dioxide. As part of a general biochemical investigation of this bacterium we have investigated its nutrition (Bexon & Dawes, 1970) and endogenous metabolism and survival (Dawes & Large, 1970); the present paper describes its carbohydrate

metabolism and molar growth yield coefficients for glucose and fructose. A preliminary report of some of this work has been presented to The Biochemical Society (McGill, Ribbons & Dawes, 1965).

EXPERIMENTAL

Materials

Organism. The strain of *Z. anaerobia* used was N.C.I.B. 8777 and the strain of *Z. mobilis* was N.C.I.B. 8938.

Media and growth of the organism. The standard medium used throughout this work contained (g/100 ml of water): Bacto yeast extract (Difco), 1; Bactopeptone (Difco), 1; and glucose, 1 (unless otherwise stated), pH 6.8. This was solidified for the preparation of stab-cultures, used for maintenance of the organism by the addition of 1.5 g of agar-agar (Hopkin & Williams Ltd., Chadwell Heath, Essex, U.K.). Over short periods (1 month) the organism was maintained at room temperature (20°C) in liquid media. For all experimental work it was grown at its optimum temperature of 30°C. Rapidly gassing cultures were used as inocula and semi-anaerobiosis was achieved by filling flasks to the neck with medium. Bulk cultures were grown in 1 litre flat-bottomed flasks with 2% (w/v) glucose as energy source and harvested by centrifuging at 2000g. Cells were washed twice with water before use.

Growth curves. Growth curves for *Z. anaerobia* and *Z. mobilis* were constructed by removal of 5 ml samples of culture from 1 litre flasks filled to the neck with medium and measuring the extinction at 620 nm against the appropriate uninoculated medium as a blank. Turbidity-dry weight calibration curves were prepared with cells grown in a similar manner, washed, and suspended in saline or water. There was no difference between curves constructed with saline and water suspensions under the conditions used.

Cell extracts. These were prepared by treating cell suspensions (8 ml portions, 50 mg dry wt./ml, cooled to

* Present address: Glenochil Research Station, Distillers Co. Ltd., Menstrie, Clackmannanshire, U.K.

4°C and surrounded by crushed ice) with an MSE ultrasonic disintegrator (MSE Ltd., Crawley, Sussex, U.K.) at 18–20 kHz and 1.0–1.5 A for 1 min, and recooling to 4°C before sonication for a further period of 1 min. The temperature was not allowed to rise above 15°C. The disrupted suspensions were pooled and held at 0°C before centrifuging at 6600g for 20 min at 0°C. The supernatants were re-centrifuged for a further 20 min at 6600g to yield a crude extract. Some supernatants were centrifuged at 105 000g for 2 h at 0°C on an MSE Superspeed 50 preparative centrifuge. All supernatants were stored at –20°C in 1–2 ml portions. The 105 000g particulate fractions were made to the same volume as the supernatant with water or 67 mM-phosphate buffer, pH 7.1, divided and stored in a similar manner.

Enzymes. Alcohol dehydrogenase, fructose diphosphate aldolase, glycerokinase, lactate dehydrogenase, phosphoenolpyruvate transphosphorylase and triose phosphate dehydrogenase were commercial preparations from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Alkaline phosphatase was obtained from British Drug Houses Ltd., Poole, Dorset, U.K. Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were products of Sigma Chemical Co., St Louis, Mo., U.S.A.

Chemicals. ADP (dilithium salt), ATP (disodium salt), fructose 6-phosphate (barium salt), glucose 6-phosphate (dipotassium salt), NADH (disodium salt), NADP (sodium salt), NADPH (tetrasodium salt) and 6-phosphogluconate (trisodium salt) were obtained from BDH Chemicals Ltd. Glyceraldehyde 3-phosphate diethyl acetal, NAD-free acid, phosphoenolpyruvate (sodium salt), and 3-phosphoglycerate (trisodium salt) were obtained from C. F. Boehringer und Soehne G.m.b.H. The monobarium salt of 3-deoxy-2-oxo-6-phosphogluconate was a generous gift from Dr W. A. Wood. Other reagents were either AnalaR grade or the best grade available.

Buffer solutions. Phosphate buffer was 67 mM-KH₂PO₄ adjusted to the required pH with 5 M-NaOH; glycylglycine buffer of the appropriate concentration was adjusted to the required pH with 5 M-NaOH. Pyrophosphate buffer was prepared from sodium pyrophosphate. Other buffers were prepared as described by Gomori (1955).

Radiochemicals. [U-¹⁴C]Glucose and [1-¹⁴C]glucose were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Chromatographic materials. Paper chromatography was performed with Whatman no. 1 paper. For column chromatography silicic acid (30–120 mesh; BDH Chemicals Ltd.) and Celite 545 (Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.) were used. The thin-layer adsorbent used was Keisegel Nach Stahl [Merck and Co. Ltd. obtained from Camlab (Glass) Ltd., Cambridge, U.K.].

Analytical methods

Chemical determinations. Glucose and fructose were measured by the method of Nelson (1944). Ethanol was determined by a colorimetric modification of the Cavett (1937–38) technique. The decrease in extinction at 360 nm of a standard dichromate solution (0.4258 g/l of water) was related in a linear manner to the ethanol concentration of the sample over the range 0–300 µg/ml. For all radiochemical work ethanol was measured as acetate;

neutralized medium was steam distilled in a Markham still and the distillate collected in potassium dichromate solution (10%, w/v, in 5 M-H₂SO₄). The oxidizing mixture was redistilled and the distillate titrated with M-NaOH with phenolphthalein as indicator. Acetaldehyde was determined by the method of Hullin & Noble (1953) for lactic acid with the omission of the preliminary boiling in conc. H₂SO₄ which serves to decarboxylate lactate to acetaldehyde. Lactic acid was determined by the Hullin & Noble (1953) method. CO₂ was measured in constant-volume Warburg respirometers at 30°C with a gas phase of N₂, and by a titrimetric method involving collection of the gas in carbonate-free M-NaOH in a bead tower, precipitation of the carbonate with M-BaCl₂ and back titration with 1 M-HCl (Neish, 1952). For large scale 'fermentation train' experiments the CO₂ was collected by passing the fermentation gases through tubes containing the sodium oxide reagent Carbasorb. Mannitol was determined by the periodate–chromotropic acid method of Snell & Snell (1962). Glycerol was determined by the method of Drawert & Kupfer (1963), the materials for which are sold in kit form by Boehringer. Glyceraldehyde-3-phosphate was determined by the alkali-labile phosphate procedure of Entner & Doudoroff (1952). Pyruvate was determined by the specific extraction procedure of Friedemann & Haugen (1943) and total keto acids by the semicarbazide method of MacGee & Doudoroff (1954). P_i was determined by the Fiske & SubbaRow (1925) procedure. Protein was determined by the biuret method of Stickland (1951). Polyhydroxy compounds were determined by the methods described by Neish (1952). Spectrophotometric measurements were made with Unicam SP.500 and SP.600 instruments with quartz cells of 1 cm light-path.

Chemical preparations. Hydroxypyruvic aldehyde was prepared by the method of Hesse, Ramisch & Renner (1956). The 2,4-dinitrophenylhydrazones of glyceraldehyde, dihydroxyacetone, hydroxypyruvic aldehyde and methyl glyoxal were prepared by the alcohol reflux method of Brady & Elsmie (1926).

Chromatography. Paper chromatography was carried out in the descending manner for analysis of fructose fermentation media with ethyl acetate–pyridine–water 12:5:4, by vol.), or propan-2-ol–water (4:1, v/v) as solvent. Spots or bands were located with the silver nitrate and benzidine–periodate dipping methods of Smith (1960). For detection of phosphate esters in Entner–Doudoroff assays with cell-free extracts of the organism, propan-1-ol–formic acid (90%, w/v)–water (6:3:1, by vol.) was used (De Ley, 1955).

Location reagents included benzidine–periodate (Smith, 1960); the spray for phosphate location of Burrows, Grylls & Harrison (1952); o-phenylenediamine (Dawson, Elliott, Elliott & Jones, 1969) for α-keto acids; and the thiobarbituric acid–periodate spray of Srinivasan & Sprinson (1959) for detection of 3-deoxy-2-oxo-6-phosphogluconate.

Column chromatography of 2,4-dinitrophenylhydrazones was carried out by the Wolfrom & Arsenault (1960) method and that of glycerol by the method of Neish (1952).

Thin-layer chromatography of 2,4-dinitrophenylhydrazones was by the method of Byrne (1965) with benzene–tetrahydrofuran (4:1, v/v). For derivatives acetylated on the plate benzene–tetrahydrofuran (24:1, v/v) was used.

Paper chromatography of hydrazine-free Entner-Doudoroff assay mixture. Samples (1 ml) removed from the assay mixture were evaporated on a boiling-water bath to 0.2 ml, and 20 μ l portions were chromatographed with reference compounds in the propanol-formic acid-water solvent of De Ley (1955). An *o*-phenylenediamine spray was used to detect α -keto acids (Dawson *et al.* 1969). To detect 3-deoxy-2-oxo-6-phosphogluconate the applied assay mixture and authentic 3-deoxy-2-oxo-6-phosphogluconate were treated with two drops of 2M-NaOH followed by 30 μ l of alkaline phosphatase solution (7.3 mg/100 ml of 0.1M-glycylglycine buffer, pH 7.0). The chromatogram was left at 18°C for 30 min before drying and chromatography as described.

Identification procedures. Fructose fermentation media were examined for non-volatile fermentation products as follows.

Freeze-drying or continuous ether extraction was used for the concentration of non-volatile products in neutralized supernatants of complex fructose fermentation media. Ethanol extracts of freeze-dried media and water-soluble material in residues from ether extracts were used for paper chromatography as already described. Fructose media were subjected to continuous ether extraction and the extract taken to dryness. The residue was extracted with water and chromatographed on wet Celite columns as described by Neish (1952). The eluate was analysed for polyols by periodate oxidation (Neish, 1952).

For carbonyl-containing fermentation products, cell-free fermentation medium (30 ml) was continuously extracted with ether. The ether was dried over anhydrous Na₂SO₄ and flash-evaporated at room temperature. 2,4-Dinitrophenylhydrazine (200 mg), together with 20 ml of ethanol, was added to the dried extract and the suspension was refluxed for 16 h. Ethanol was removed on a rotary evaporator and 100 ml of warm nitrobenzene added to extract the residue. The nitrobenzene extract was diluted with 400 ml of benzene and subjected to column chromatography by the method of Wolfrom & Arsenaault (1960). Extrusion of the column, extraction of the main band with ethanol and rechromatography yielded an extract which was taken up in benzene. It was chromatographed by the thin-layer technique of Byrne (1965) which included treatment of the spots with either conc. HCl or an acetylation mixture before development.

Fermentation balances. Large-scale fermentation balance experiments were carried out by the 'train' technique of Neish (1952). Pure N₂ was bubbled at a constant rate of 20 ml/min through the fermentation flask containing about 5% (w/v) hexose (accurately determined in the initial medium) in the standard growth medium. CO₂ borne in the stream of N₂ was collected in tubes containing Carbasorb which were weighed before and after the experiment. Water vapour was collected in tubes containing anhydrous magnesium perchlorate. Hydrogen was determined by passing the N₂ stream over heated copper oxide at 450–550°C and collecting the water produced in weighed anhydrous magnesium perchlorate tubes. Volatile compounds were collected in a solid CO₂ trap placed next to the fermentation vessel. Clarification of the media before analysis was achieved by the zinc hydroxide precipitation procedure of Neish (1952) and the medium was finally neutralized with M-H₂SO₄.

Radiochemical fermentation balances. Fermentation

balances with [U-¹⁴C]glucose and [1-¹⁴C]glucose were carried out with a total of 10.1 ml of medium containing either 2% or 5% (w/v) (accurately determined) of appropriately labelled glucose, and 1% (w/v) each of yeast extract and Bactopeptone. The fermentation flasks were fitted at the top with a two-way stopcock and with a side port for adding media. The side port was closed with a rubber cap during fermentation. Actively gassing cultures of *Z. anaerobia* (0.1 ml) were used as inocula and fermentation was allowed to proceed for the period stated in the relevant Tables, after which time the fermentation was stopped by addition of 2 ml of 2M-HCl from a hypodermic syringe through the side port. CO₂ was collected in a bead tower as described by Neish (1952).

Small-scale fructose fermentation. By using the system employed for the radiochemical fermentation balance work, 5% (w/v) fructose (accurately determined), 1% yeast extract and 1% Bactopeptone in a volume of 10 ml were fermented for 48 h. The inoculum was 0.1 ml of an actively gassing *Z. anaerobia* culture. CO₂ was collected in a bead tower and glycerol was determined in the neutralized supernatant.

Radiochemical assays. All counting of radioactivity was done at infinite thinness. Solutions were plated on nickel-steel planchets with raised areas of 2.25 cm². Ethanol was assayed as acetate and CO₂ as the carbonate in NaOH. Other materials were plated directly from solution and dried under an i.r. lamp before counting to an error of 2% in an end-window Geiger-Müller tube (scaler type N5309, Ekco Electronics Ltd, Southend, Essex, U.K.). A Petri dish containing NaOH pellets was placed in the counting chamber to counteract the absorption of moisture by certain of the assayed materials.

Enzyme assays

All spectrophotometric determinations were carried out with a Unicam SP.800 instrument with the temperature controlled to 24°C and with quartz cells of 1 cm light-path. Unless otherwise stated all assays were started by the addition of cell extract. The assay methods for 6-phosphogluconate dehydrogenase and the enzymes of the Entner & Doudoroff (1952) pathway are given in the legends to the appropriate figures. NADH oxidase was measured spectrophotometrically by observing the decrease in extinction at 340 nm of NADH. The assay system contained: 67 mM-potassium phosphate, pH 7.0, 2.8 ml; 250 mM-MgCl₂, 0.1 ml; 10 mM-NADH, 0.05 ml; cell extract (either crude extract, 105 000g supernatant or particulate fraction), 0.1 ml. NADPH-NAD⁺ transhydrogenase was assayed as described by Dawes *et al.* (1966).

Glucose dehydrogenase and gluconate dehydrogenase were assayed colorimetrically with 2,6-dichlorophenol-indophenol as hydrogen acceptor. For glucose dehydrogenase the assay system contained: 0.1 M-veronal buffer, pH 6.6, 1.00 ml; 15 mM-KCN, 0.10 ml; 0.05% (w/v) dichlorophenol-indophenol, 0.25 ml; 75 mM-glucose, 0.55 ml; cell extract, 0.10 ml. The decrease in extinction at 600 nm was measured.

Hexokinase, gluconokinase, glucose 6-phosphate dehydrogenase, phosphohexose isomerase, triose phosphate dehydrogenase, fructose diphosphate aldolase and ethanol dehydrogenase were all assayed by measuring the rate of change in extinction at 340 nm due to oxidation or reduction of the appropriate nicotinamide nucleotide.

The hexokinase assay system contained: 0.12 M-tris-HCl buffer, pH 8.2, 1.1 ml; 250 mM-MgCl₂, 0.1 ml; 36 mM-ATP, pH 7.1, 0.8 ml; 10 mM-NADP, 0.3 ml; glucose 6-phosphate dehydrogenase (15 units/ml), 0.2 ml; 500 mM-glucose, 0.4 ml; cell extract, 0.1 ml. Hexokinase was also assayed with 0.4 ml of 500 mM-fructose instead of glucose.

The assay system for gluconokinase was: 0.12 M-tris-HCl buffer, pH 7.6, 1.1 ml; 250 mM-MgCl₂, 0.1 ml; 36 mM-ATP, pH 7.1, 0.8 ml; 10 mM-NADP, 0.3 ml; 6-phosphogluconate dehydrogenase (2 mg/ml), 0.1 ml; 50 mM-sodium gluconate, 0.5 ml; cell extract, 0.1 ml.

The glucose 6-phosphate dehydrogenase assay system contained: 0.1 M-tris-HCl buffer, pH 8.4, 2.5 ml; 250 mM-MgCl₂, 0.10 ml; 10 mM-NAD(P), 0.20 ml; 20 mM-glucose 6-phosphate, 0.50 ml; cell extract, 0.05 ml.

The phosphohexose isomerase assay system contained: 0.1 M-tris-HCl buffer, pH 7.0, 2.5 ml; 250 mM-MgCl₂, 0.1 ml; 10 mM-NAD, 0.1 ml; 20 mM-glucose 6-phosphate or fructose 6-phosphate, 0.2 ml; cell extract, 0.1 ml. The high activity of glucose 6-phosphate dehydrogenase in the extract eliminated the need for a separate addition of this enzyme and glucose 6-phosphate served as a control for the assay conditions.

The triose phosphate dehydrogenase assay system contained: 30 mM-sodium pyrophosphate-4 mM-cysteine buffer, pH 8.4, 2.25 ml; 0.4 M-sodium arsenate, 0.10 ml; fructose diphosphate aldolase (10 µg/ml), 0.10 ml; 10 mM-NAD or NADP, 0.20 ml; 24 mM-fructose 1,6-diphosphate, 0.25 ml; cell extract, 0.10 ml.

The fructose diphosphate aldolase assay system contained: 30 mM-sodium pyrophosphate-4 mM-cysteine buffer, pH 8.4, 2.35 ml; 10 mM-NAD, 0.20 ml; 0.4 M-sodium arsenate, 0.10 ml; water 0.10 ml; 24 mM-fructose 1,6-diphosphate, 0.25 ml; cell extract, 0.10 ml. The high activity of triose phosphate dehydrogenase in cell extracts eliminated the need for separate addition of this enzyme to the assay system.

The ethanol dehydrogenase assay system for reduction contained: 67 mM-potassium phosphate buffer, pH 7.0, 2.5 ml; 250 mM-MgCl₂, 0.1 ml; 10 mM-NADH or NADPH, 0.1 ml; 30 mM-acetaldehyde, 0.2 ml; cell extract, 0.05 ml.

The ethanol dehydrogenase assay system for oxidation contained: 73 mM-sodium pyrophosphate-22 mM-glycine-74 mM-semicarbazide hydrochloride buffer, pH 8.7, 2.4 ml; 10 mM-NAD or NADP, 0.2 ml; 30 mM-ethanol, 0.2 ml; cell extract, 0.2 ml.

Pyruvate decarboxylase was assayed manometrically (N₂ atmosphere with acid tipped at start and finish in separate sets of control and reaction flasks to correct for CO₂ retention and endogenous CO₂ production) at 30°C. Double side-arm flasks were used containing in the main compartment 200 mM-KH₂PO₄ buffer, pH 7.5, 1.6 ml; 250 mM-MgCl₂, 0.1 ml; water, 0.4 ml; and 105 000 g cell extract, (2.69 mg of protein/ml), 0.2 ml. The side arms contained respectively 40 mM-pyruvic acid, or water (control), 0.5 ml, and m-H₂SO₄, 0.3 ml. The acetaldehyde product was identified as its 2,4-dinitrophenylhydrazone by spectrum and melting point. The dissimilation of 3-phosphoglycerate to carbon dioxide and acetaldehyde was followed manometrically in an assay system similar to that for pyruvate decarboxylase, but with the addition of 0.2 ml of 200 mM-ADP and adjustment of the water in the main compartment to 0.2 ml.

6-Phosphofructokinase was assayed manometrically by

acid production and release of CO₂ from bicarbonate buffer (Raps & DeMoss, 1962) and by decrease in acid labile phosphorus. Extracts of *Escherichia coli*, an organism known to possess phosphofructokinase, were assayed simultaneously.

Transaldolase, transketolase and phosphoketolase were determined by the methods of de Vries, Gerbrandy & Stouthamer (1967). The assays were checked with extracts of organisms known to contain the enzymes, namely *Bifidobacterium bifidum*, kindly provided by Professor A. H. Stouthamer, and *Pseudomonas aeruginosa* 2F32 in use in this laboratory.

RESULTS

Whole-cell experiments

Growth curves. Growth curves for *Z. anaerobia* and *Z. mobilis* are compared in Fig. 1. It will be noted that both the growth rate and the total yield in 1% glucose medium are less with *Z. anaerobia*.

Molar growth yield coefficients. Molar growth yield coefficients for *Z. anaerobia* with glucose and fructose as energy sources gave *Y* (g dry wt./mol of substrate) values of 5.89 and 5.0 respectively (Figs. 2a and 2b). An experiment with [U-¹⁴C]-glucose as the energy source indicated that not more than 2% of the glucose utilized became cell carbon. These growth yields are considerably lower than the value of 10.5 (Bauchop & Elsdén, 1960) expected of an organism gaining 1 mol of

Fig. 1. Growth of *Z. anaerobia* and *Z. mobilis*. Organisms were grown in Bacto-yeast extract-Bactopeptone-1% (w/v) glucose medium. ○, *Z. mobilis*; ●, *Z. anaerobia*.

Fig. 2. Relationship between concentration of energy source and yield of *Z. anaerobia*. (a) Glucose; (b) fructose. The points are the average values of six individual determinations for each concentration.

ATP per mol of hexose fermented. *Z. anaerobia* may have a high maintenance energy requirement or dissipate its energy in a manner that causes it to grow in a linear manner (Fig. 1). The lower yield with fructose confirms the chromatographic evidence that, in comparison with glucose fermentation, *Z. anaerobia* produces energetically wasteful products during fructose metabolism.

Products of glucose fermentation. The fermentation balances for glucose dissimilation by *Z. anaerobia* at the end of growth are recorded in Table 1. Clearly the stoichiometry for glucose fermentation resembles that of yeast, but with acetaldehyde a very minor product. The average ethanol yield of 1.78 mol/mol of glucose fermented agrees reasonably with the value of 1.87 recorded by Millis (1956).

Products of fructose fermentation. Table 2 records the utilization of fructose and production of ethanol, carbon dioxide and acetaldehyde at the end of growth. Two differences from the glucose fermentation are apparent, namely the increased production of acetaldehyde and the lower carbon

recovery, especially in terms of carbon dioxide and ethanol produced.

Glycerol and dihydroxyacetone as fructose fermentation products. After the observation of a low carbon recovery in the fermentation of fructose by *Z. anaerobia*, supernatants of fructose and glucose growth media exhausted of sugar were separately subjected to the periodate oxidation technique for mannitol determination (Snell & Snell, 1962). As a control, a yeast extract-peptone solution in proportions normally used for growth was also tested by the procedure. The extinctions recorded were for glucose medium, 0.508; fructose medium, 1.390; yeast extract-Bactopeptone, 0.392; and for a mannitol standard (20 μg/ml), 0.565.

The results suggested that polyol products were present in fructose media, and paper chromatography of an ethanol extract of neutral freeze-dried medium in the ethyl acetate-pyridine-water solvent yielded five spots with the silver nitrate detection agent, three of which corresponded in R_F value to glycerol, dihydroxyacetone and fructose markers. The remaining two unidentified spots were of much lower intensity. 2,4-Dinitrophenylhydrazine treatment of a duplicate chromatogram also indicated the presence of a spot corresponding in R_F value to dihydroxyacetone. The propan-2-ol-water solvent gave similar results.

A continuous ether extract of fructose medium was chromatographed on wet Celite and eluate fractions from the column were oxidized with periodate and the acid and formaldehyde produced were determined (Table 3). The formaldehyde produced/periodate consumed ratio and the acid produced/periodate consumed ratio indicate that glycerol is a fructose fermentation product of *Z. anaerobia*. An enzymic assay of fructose medium for glycerol after growth of the organism in small-scale culture revealed 0.054 mol of glycerol/mol of hexose fermented.

For analysis of the unknown carbonyl component of fructose media a continuous ether extraction of a washed cell-free medium was carried out. The extract was used for the preparation of 2,4-dinitrophenylhydrazones and the derivative was purified by the Wolfrom & Arsenault (1960) technique. T.l.c. of the purified compound with the corresponding hydrazones of dihydroxyacetone, glyceraldehyde, methylglyoxal and hydroxypyruvic aldehyde as standards by the methods of Byrne (1965) showed that dihydroxyacetone was a fructose fermentation product of *Z. anaerobia*.

Fermentation of [U- 14 C]glucose and [1- 14 C]glucose. Table 4 records the radiochemical balance for the fermentation of [U- 14 C]glucose. Comparison of the specific radioactivities of the CO_2 and ethanol with that of the substrate glucose reveals that no appreciable dilution of the labelled products by other

Table 1. *Fermentation of glucose during growth of Z. anaerobia*

Cells were grown in 150 ml of Bacto yeast extract–Bacto peptone medium, containing the indicated amounts of glucose, for 36 h. Ethanol oxidation number with respect to water = (–2). Carbon dioxide oxidation number with respect to water = (+2). Oxidation–reduction balance = mmol of ethanol produced $\times$ (–2) + mmol of carbon dioxide produced $\times$ (+2). Carbon balance = g-atom of carbon in products/g-atom of carbon fermented. n.d., not determined.

Expt. no.	Glucose fermented (mmol)	Products (mmol/100 mmol of glucose)			Oxidation–reduction balance	Carbon balance
		Ethanol	Carbon dioxide	Acetaldehyde		
1	42.70	176.9	180.0	0.003	+6.2	0.890
2	42.70	176.0	187.9	0.003	+23.8	0.887
3	40.75	168.5	198.0	0.004	+59.0	0.892
4	42.70	179.5	175.9	0.003	–7.2	0.890
5	42.16	185.0	189.5	n.d.	+9.0	0.933
6	42.16	184.2	190.5	—	+12.6	0.932
7	42.15	182.0	190.7	—	+17.4	0.925
8	42.20	175.5	190.4	—	+29.8	0.902
9	41.76	172.0	185.7	—	+27.4	0.883

Table 2. *Fermentation of fructose during growth of Z. anaerobia*

Cells were grown in 150 ml of Bacto yeast extract–Bacto peptone medium containing fructose of accurately determined concentration. Fermentation time for Expt. nos. 1, 3 and 4 was 48 h and for Expt. no. 2, 60 h.

Expt. no.	Fructose fermented (mmol)	Products (mmol/100 mmol of fructose)			Oxidation–reduction balance	Carbon balance
		Ethanol	Carbon dioxide	Acetaldehyde		
1	36.0	156.0	168.1	2.60	+24.0	0.809
2	99.0	152.6	151.7	1.33	–1.8	0.761
3	49.0	146.0	146.8	2.12	+1.6	0.739
4	49.0	148.2	151.7	1.87	+7.0	0.751

components of the medium occurred. Only 1.1% of the substrate glucose was incorporated into the cells. All the radioactivity present in C-1 of glucose appeared in CO₂; ethanol was devoid of activity (Table 5), a result incompatible with the operation of Embden–Meyerhof glycolysis.

Cell-extract experiments

Enzyme assays. The specific activities of various enzymes of carbohydrate metabolism detected in extracts of *Z. anaerobia* are recorded in Table 6. The presence of a highly active glucose 6-phosphate dehydrogenase supports the operation of a hexose monophosphate oxidative pathway. The activity ascribed to a weakly active fructose diphosphate aldolase requires comment. Because of the highly active enzymes oxidizing hexose monophosphate and triose phosphate the activity observed could not have been the result of contamination by traces of these compounds. Similarly, the action of a phosphatase in cell extracts was discounted since the hexose monophosphates that would have been

formed during preincubation of substrate with the extract in the absence of NAD did not result, on subsequent addition of the nucleotide, in an initial increase in rate. Further, the activity was abolished by the addition of iodoacetate indicating that the enzyme link was through triose phosphate dehydrogenase. It seems likely, therefore, that a weak fructose diphosphate aldolase is possessed by *Z. anaerobia*, which correlated with the presence of phosphofructokinase.

NAD–NADPH transhydrogenase activity was absent and Fig. 3 demonstrates the presence of 6-phosphogluconate dehydrogenase in 105 000g supernatants. The enzyme is specific for NADP. The rate and extent of the reaction decreased with increasing 6-phosphogluconate concentration and the further addition of extract permitted the reaction to continue (Fig. 3). The maximum rate of reaction observed represented a low specific activity (0.02 μ mol/min per mg of protein).

The combined activities of the Entner–Doudoroff enzymes, i.e. 6-phosphogluconate dehydratase and 3-deoxy-2-oxo-6-phosphogluconate aldolase, were

Table 3. *Periodate oxidation of eluate from chromatography of extract of fructose fermentation medium*

Portions of eluate obtained by column chromatography of fructose fermentation medium of *Z. anaerobia*, together with standard solutions of glycerol and mannitol, were analysed for formaldehyde and acid produced on periodate oxidation as described in analytical methods. The theoretical yields are recorded in parentheses.

Sample	Ratio of μmol of formaldehyde produced to μmol of periodate consumed	Ratio of μmol of acid produced to μmol of periodate consumed
Unknown	$\frac{22.70}{21.50} = 1.06$	$\frac{11.75}{21.50} = 0.57$
Glycerol	$\frac{37.80}{36.75} = 1.01$ (1.00)	$\frac{18.75}{36.75} = 0.51$ (0.50)
Mannitol	$\frac{16.44}{41.75} = 0.39$ (0.40)	$\frac{34.50}{41.75} = 0.83$ (0.80)

Table 4. *Fermentation of [U- ^{14}C]glucose by *Z. anaerobia**

Cells were grown in 10.1 ml of Bacto yeast extract–Bacto peptone medium containing 2.78 mmol of [U- ^{14}C]glucose and analyses were performed after 30 h growth.

Material	Quantity (μmol)	$10^{-3} \times$ Radioactivity (c.p.s.)	$10^{-1} \times$ Sp. radioactivity		% of total radioactivity
			c.p.s./ μmol	c.p.s./ $\mu\text{g-atom}$ of C	
Glucose fermented	2.78	495	17.80	2.97	100.0
Carbon dioxide yield	5.20	152	2.92	2.92	30.7
Ethanol yield	5.05	282	5.58	2.79	57.0
Cells	11.38 mg	5.62	—	—	1.1
Supernatant (neutral)	50 ml*	15.12	—	—	3.1
Total recovery (%)	—	—	—	—	91.9

* Final volume after neutralization and dilution.

Table 5. *Fermentation of [1- ^{14}C]glucose by *Z. anaerobia**

Cells were grown in 10.1 ml of Bacto yeast extract–Bacto peptone containing [1- ^{14}C]glucose and analyses were performed after 48 h. The percentage recovery of isotope is given in parentheses.

Glucose fermented (mmol)	Ethanol formed (mmol)	CO_2 formed (mmol)	Cell yield (mg)	Radiochemical distribution (c.p.s.)				
				Glucose utilized	Ethanol	Carbon dioxide	Cells	Supernatant
1.19	2.35	2.15	2.37	111 200 (100)	back-ground	93 600 (84)	90.3 (0.8)	486 (4.3)

demonstrated (Fig. 4a). The initial appearance of a keto acid, followed by two compounds which appeared simultaneously in equimolar quantities and assayed respectively as pyruvate and an alkali-labile phosphate compound, is characteristic of this pathway which yields pyruvate and glyceraldehyde 3-phosphate. In the absence of hydrazine to trap pyruvate and the absence of added nicotinamide nucleotides and ADP, the glyceraldehyde 3-phosphate formed was not further metabolized (Fig. 4b). The pyruvate produced was rapidly decarboxylated and the concentration of alkali-labile material attained a constant value. Hydrazine affected the initial rate of the combined Entner–Doudoroff enzymes, e.g. specific activities were respectively 0.47 and 2.15 in its presence and absence. Proof of the identity of the keto acid

represented by the difference between total keto acids and pyruvate was obtained by paper chromatography of a hydrazine-free assay mixture; the R_F of one spot corresponded to 3-deoxy-2-oxo-6-phosphogluconate on spraying with thiobarbituric acid.

Under the various conditions of assay used transaldolase, transketolase and phosphoketolase activities could not be detected.

DISCUSSION

The isotopic and enzymic results show that the Entner & Doudoroff (1952) metabolic sequence serves as a major route from glucose to ethanol in *Z. anaerobia* and emphasize metabolic similarity to *Z. mobilis*. These two organisms are thus the

Table 6. *Survey of enzymes of carbohydrate metabolism*

The specific activities relate to the assay conditions described in the Experimental section.

Enzyme	Bacterial extract	Co-factor	Specific activity ($\mu\text{mol/min per mg}$ of protein)
Glucose dehydrogenase	Crude		0.002
Hexokinase		ATP	
Glucose	Crude		0.140
Fructose	Crude		0.126
Gluconokinase	Crude	ATP	0.01
Phosphohexose isomerase	Crude		0.42
Phosphofructokinase	Crude	ATP	0.074*
Fructose 1,6-diphosphate aldolase	Crude		0.03
Glucose 6-phosphate dehydrogenase	Crude	NAD	3.84
	Crude	NADP	3.72
6-Phosphogluconate dehydrogenase	105 000g supernatant	NADP	0.02
	Crude	NAD	no activity
6-Phosphogluconate dehydratase + 3-deoxy-2-oxo-6-phosphogluconate aldolase	Crude		2.15*
Triose phosphate dehydrogenase	Crude	NAD	0.89
		NADP	0.01
Pyruvate decarboxylase	Crude		2.15
Ethanol dehydrogenase	Crude	NAD	2.20
		NADP	no activity
	105 000g supernatant	NADH	0.43
		NADPH	no activity
NADH oxidase	Crude		0.050
	105 000g supernatant		0.020
	105 000g particles		0.080
NADPH oxidase	Crude		0.003
NADPH-NAD transhydrogenase	Crude		no activity
Transketolase	Crude		no activity
Phosphoketolase	Crude		no activity
Transaldolase	Crude		no activity

* Assayed at 30°C instead of 24°C.

only bacteria currently known to utilize the Entner-Doudoroff pathway in association with a pyruvate decarboxylase.

Of the established routes of ethanol formation (Dawes, 1963) only phosphoketolase cleavage of pentose phosphate (Heath, Hurwitz & Horecker, 1956) could also explain the labelling pattern observed and this enzyme was not detected. Further, the 6-phosphogluconate dehydrogenase of *Z. anaerobia* is only weakly active, specific for NADP and, in the shown absence of NAD-NADPH transhydrogenase, could not couple with the NAD-specific ethanol dehydrogenase. Its function is most probably to produce pentose for nucleic acid biosynthesis and, in conjunction with glucose 6-phosphate dehydrogenase, to furnish NADPH for fatty acid biosynthesis. These observations correlate with the inability of the organism to grow with pentoses as the energy source (Millis, 1956). As

glucose 6-phosphate dehydrogenase is active with both NAD and NADP, anaerobic growth presumably implies that NAD is the physiological electron carrier for fermentation.

Raps & DeMoss (1962) reported the presence of fructose diphosphate aldolase in *Z. mobilis* extracts but were unable to detect a phosphofructokinase. We have found both enzymes, weakly active, in *Z. anaerobia* but our isotopic results indicate they are not involved in the main fermentation route and no metabolic role can as yet be assigned to them. Wolin (1964) and de Vries *et al.* (1967) have shown that fructose diphosphate is an obligatory co-factor for lactate dehydrogenase in some organisms; however, lactate dehydrogenase has not been detected in *Z. anaerobia*.

An NAD-independent glucose dehydrogenase and a gluconokinase of very low activity were found in extracts of *Z. anaerobia*; their role in overall

Fig. 3. 6-Phosphogluconate dehydrogenase assay. Reaction cuvette A initially contained: tris-HCl buffer, pH 7.6, 120 μ mol; 6-phosphogluconate, 0.1 μ mol; NADP, 2 μ mol; $MgCl_2$, 37.5 μ mol; NaF, 15 μ mol; cell extract (0.62 mg of protein) in a total volume of 3.0 ml. Reaction cuvettes B and C contained the same as A except that 2.5 and 5.0 μ mol of 6-phosphogluconate, respectively, were added and the total volume made up to 3.0 ml. At arrow 2 additional cell extract (0.62 mg protein) was added to B and C. The reaction was started by addition of NADP at arrow 1.

glucose metabolism cannot therefore be a significant one.

The low growth yields of *Z. anaerobia* and *Z. mobilis* remain a matter for conjecture. Senez (1962) suggested that energy metabolism may not always be coupled in an economic manner to the synthesis of cellular constituents, and thus result in 'energetically uncoupled growth'. The proposed agent of energy wastage was an adenosine triphosphatase acting in a controlled fashion on the ATP pool. Belaïch & Senez (1965) found that the rate of glucose uptake by *Z. mobilis* was similar for cells growing in both simple and complex media but they could not equate the rate of metabolism of the hexose with the energy produced. The 'maintenance' concept (Mallette, 1963; Marr, Nilson & Clark, 1963) was studied by Pirt (1965) who found by continuous culture techniques that the growth rate of an organism at low growth rates was in some cases proportional to the yield of cells. He suggested that the energy required to maintain an organism at low growth rates was an important proportion of the total energy being produced. The low growth yield coefficients of both *Z. anaerobia* and *Z. mobilis* may, then, be attributable to linear growth, perhaps

Fig. 4. Assay of Entner-Doudoroff enzymes. (a) Assay in the presence of hydrazine; the incubation mixture contained in a total of 30 ml: 1.2 mmol of glycylglycine, pH 7.0; 150 μ mol of 6-phosphogluconate; 6.72 mmol of hydrazine, pH 7.0; and cell extract (10.8 mg of protein). The temperature was 30°C. The reaction was started by addition of the cell extract that had been preincubated at 30°C for 10 min. In the control water replaced 6-phosphogluconate. Samples (2 ml) were removed at intervals into 2 ml of 2M-HClO₄ and, after centrifugation, the supernatants were analysed as described in the Experimental section. No reaction products were detected in the control tube. ●, Pyruvate; ○, alkali-labile phosphate (representing glyceraldehyde 3-phosphate); □, total keto acids minus pyruvate (representing 3-deoxy-2-oxo-6-phosphogluconate). (b) Assay in the absence of hydrazine; as the reaction was more rapid than in its presence the quantity of substrate was doubled. The incubation mixture contained in a total of 29 ml: 1.0 mmol of glycylglycine buffer, pH 7.0; 300 μ mol of 6-phosphogluconate; and cell extract (10.8 mg of protein). Temperature and reaction conditions were as for (a). Samples (2 ml) were removed at intervals into test-tubes and heated on a boiling-water bath for 2.5 min to stop the reaction. Analyses were performed as for assay (a). The symbols are also as for assay (a).

caused by the leakage of important metabolic intermediates in to the growth medium (W. W. Forrest, personal communication).

It is not uncommon to find that micro-organisms have a different fermentation product pattern with fructose than with glucose, e.g. the products of glucose fermentation by *Leuconostoc dextranicum* are predominantly lactate, ethanol, carbon dioxide, glycerol and acetate whereas with fructose, mannitol essentially replaces glycerol as a polyol product (Nelson & Werkman, 1940). With *Z. anaerobia* both glycerol and dihydroxyacetone account for a carbon balance ratio of at least 0.027 unit (value taken from glycerol determination) and are minor products of fructose fermentation. Further, acetaldehyde, a very minor product of glucose fermentation, is of much greater significance in fructose fermentation.

The balance equation for glucose fermentation during growth of *Z. anaerobia* is:

and for *Z. mobilis* (Kluyver & Hoppenbrouwers, 1931):

For fructose fermentation the corresponding equation for *Z. anaerobia* is:

The fermentation of fructose by *Z. mobilis* presumably resembles that for glucose since, unlike *Z. anaerobia*, the growth yields recorded (Dawes, Ribbons & Rees, 1966) for this organism are similar for both sugars. *Z. anaerobia* differs from *Z. mobilis*, however, in its inability to grow on sucrose (Millis, 1956).

We are indebted to the Science Research Council for the award of a research studentship to D. J. McG., to Miss Joyce Bexon for the assays of phosphofructokinase and Mr M. P. Stephenson for the assays for phosphoketolase, transketolase and transaldolase.

REFERENCES

- Barker, B. T. P. & Hillier, V. F. (1912). *J. agric. Sci.* **5**, 67.
 Bauchop, T. & Elsdon, S. R. (1960). *J. gen. Microbiol.* **23**, 457.
 Belaich, J.-P. & Senez, J. C. (1965). *J. Bact.* **89**, 1195.
 Bexon, J. & Dawes, E. A. (1970). *J. gen. Microbiol.* **60**, 421.
 Brady, O. L. & Elsmie, G. V. (1926). *Analyst, Lond.*, **51**, 77.
 Burrows, S., Grylls, F. S. M. & Harrison, G. S. (1952). *Nature, Lond.*, **170**, 800.
 Byrne, G. A. (1965). *J. Chromat.* **20**, 528.
 Cavett, J. W. (1937-38). *J. Lab. clin. Med.* **23**, 543.

- Dawes, E. A. (1963). *J. gen. Microbiol.* **32**, 151.
 Dawes, E. A. & Large, P. J. (1970). *J. gen. Microbiol.* **60**, 31.
 Dawes, E. A., Ribbons, D. W. & Large, P. J. (1966). *Biochem. J.* **98**, 795.
 Dawes, E. A., Ribbons, D. W. & Rees, D. A. (1966). *Biochem. J.* **98**, 804.
 Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M. (Eds.) (1969). *Data for Biochemical Research*, 2nd ed., p. 516. Oxford University Press.
 De Ley, J. (1955). *Naturwissenschaften* **42**, 96.
 de Vries, W., Gerbrandy, S. J. & Stouthamer, A. H. (1967). *Biochim. biophys. Acta*, **136**, 415.
 Drawert, F. & Kupfer, G. (1963). *Z. Lebensmittelunters. u.-Forsch.* **123**, 3.
 Entner, N. & Doudoroff, M. (1952). *J. biol. Chem.* **196**, 853.
 Fiske, C. H. & SubbaRow, Y. (1925). *J. biol. Chem.* **66**, 375.
 Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
 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.
 Heath, E. C., Hurwitz, J. & Horecker, B. L. (1956). *J. Am. chem. Soc.* **78**, 5449.
 Hesse, G., Ramisch, F. & Renner, K. (1956). *Chem. Ber.* **89**, 2137.
 Hullin, R. P. & Noble, R. L. (1953). *Biochem. J.* **55**, 289.
 Kluyver, A. J. & Hoppenbrouwers, W. H. (1931). *Arch. Mikrobiol.* **2**, 245.
 Lindner, P. (1928). *Atlas mikrosk. Grundl. Gärungsk.* **3** Aufl. 2.
 MacGee, J. & Doudoroff, M. (1954). *J. biol. Chem.* **210**, 617.
 Mallette, M. F. (1963). *Ann. N.Y. Acad. Sci.* **102**, 521.
 Marr, A. G., Nilson, E. H. & Clark, D. J. (1963). *Ann. N.Y. Acad. Sci.* **102**, 536.
 McGill, D. J., Ribbons, D. W. & Dawes, E. A. (1965). *Biochem. J.* **97**, 44 P.
 Millis, N. F. (1956). *J. gen. Microbiol.* **15**, 521.
 Neish, A. C. (1952). *Analytical Methods for Bacterial Fermentations*, 2nd revision. Rep. nat. Res. Council. Canada no. 46-8-3.
 Nelson, M. E. & Werkman, C. H. (1940). *Iowa State Coll. J. Sci.* **14**, 395.
 Nelson, N. (1944). *J. biol. Chem.* **153**, 375.
 Pirt, S. J. (1965). *Proc. R. Soc. B*, **163**, 224.
 Raps, S. & DeMoss, R. D. (1962). *J. Bact.* **84**, 115.
 Senez, J. C. (1962). *Bact. Rev.* **26**, 95.
 Shimwell, J. L. (1937). *J. Inst. Brew.* **43**, 507.
 Smith, I. (1960). In *Chromatographic and Electrophoretic Techniques*, vol. 1, p. 250. Ed. by Smith, I. London: William Heinemann (Medical Books) Ltd.
 Snell, F. D. & Snell, C. T. (1962). *Colorimetric Methods of Analysis*, vol. 3, p. 65. Toronto: D. Van Nostrand Co. Inc.
 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.
 Wolfom, M. L. & Arsenault, G. P. (1960). *Analyt. Chem.* **32**, 693.
 Wolin, M. J. (1964). *Science, N.Y.*, **146**, 775.