Further Observations on Carbohydrate Metabolism and its Regulation in *Azotobacter beijerinckii*

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Certain enzymes of glucose catabolism in *Azotobacter beijerinckii* were studied and their activities in steady-state chemostat cultures were measured under various nutrient limitations. 2-Keto-3-deoxy-6-phosphogluconate aldolase was separated from 6-phosphogluconate dehydratase by affinity chromatography and the previously observed inhibition of the Entner-Doudoroff enzymes by tricarboxylic acids and ATP was attributed to chelation of Mn^{2+} and Mg^{2+} which activate the dehydratase. Glucose-6-phosphate dehydrogenase was unaffected by phosphoenolpyruvate while fructose-1,6-bisphosphate aldolase was activated by Co^{2+} , K^{+} or NH_4^{+} ions. Transketolase, transaldolase and triosephosphate isomerase were present but previous reports of 6-phosphogluconate dehydrogenase activity were shown to be artefacts. The findings confirm the major role of the Entner-Doudoroff pathway in glucose catabolism in *A. beijerinckii*. Pyruvate dehydrogenase, a key enzyme for carbon entry to the tricarboxylic acid cycle and to poly- β -hydroxybutyrate synthesis, was inhibited by acetyl-coenzyme A and NADH.

INTRODUCTION

Glucose is metabolized in *Azotobacter vinelandii* principally via the Entner-Doudoroff pathway as indicated by enzymic (Mortenson & Wilson, 1954; Mortenson *et al.*, 1955; Haaker & Veeger, 1976) and radiorespirometric studies (Still & Wang, 1964), and the same is true of *Azotobacter beijerinckii* (Senior & Dawes, 1971). Previously we have examined various aspects of the regulation of carbohydrate metabolism of *A. beijerinckii* in relation to poly- β -hydroxybutyrate biosynthesis (Senior & Dawes, 1971). Subsequently it became necessary to amplify some of this earlier work done with batch-grown organisms and the opportunity was taken to use the controlled conditions of the chemostat to make comparisons of oxygen- and carbon-limited bacteria for some of the enzymes. Attention was also directed to the role of the oxidative pentose phosphate pathway, entry to which is gained via 6-phosphogluconate dehydrogenase, since spurious assays for the latter enzyme can occur in the presence of an active Entner-Doudoroff system and an NADP-dependent glyceraldehyde-3-phosphate dehydrogenase, as noted for *Pseudomonas aeruginosa* by Blevins *et al.* (1975). As our previous investigations of the control of glucose metabolism and the tricarboxylic acid cycle in *A. beijerinckii* did not embrace pyruvate dehydrogenase, we have included it in the present survey which was designed to supplement the earlier work of Senior & Dawes (1971).

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METHODS

Organism. The organism was a capsuleless mutant of *Azotobacter beijerinckii* NCIB 9067, isolated by Senior *et al.* (1972), and now lodged as NCIB 11292.

Growth of the organism. Details of the chemostat, maintenance of the organism, growth media, growth of the organism for inocula, growth conditions, bacterial dry weight measurement, and control and determination of dissolved oxygen tension in the culture have been described previously (Senior *et al.*, 1972). The oxygen electrode (L. H. Engineering Co., Bells Hill, Stoke Poges, Bucks.) was sterilized *in situ* by autoclaving with the culture vessel. For oxygen-limited steady states (undetectable dissolved oxygen tension), the flow rates of oxygen and nitrogen were adjusted manually to the required values using the Flostat valves and rotameters. Nitrogen-limited steady states were established using argon as an inert carrier gas (flow rate 1 litre min⁻¹) to which the required amount of nitrogen was added by manual adjustment of the nitrogen Flostat valve; oxygen flow rate was adjusted automatically by the controller. For carbon-limited conditions, the nitrogen flow rate (1 litre min⁻¹) was adjusted manually and the oxygen flow rate was adjusted automatically by the controller.

Enzyme assays. Bacterial extracts were prepared by the methods of Senior & Dawes (1973) and, according to assay, were diluted to contain 50 to 750 µg protein ml⁻¹. All enzyme assays were performed at 30 °C under previously determined optimum conditions, except for transketolase, where shortage of substrate prevented establishment of the optimum. A Pye Unicam SP1800 recording spectrophotometer was used for all spectrophotometric assays.

6-Phosphogluconate dehydratase (EC 4.2.1.12) and *2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase* (EC 4.1.2.14). A combined assay for these enzymes measured pyruvate production from 6-phosphogluconate using lactate dehydrogenase and NADH. Silica glass cuvettes (1 cm light path) contained (ml): MnCl₂ (80 mM), 0.01; dithiothreitol (80 mM), 0.2; lactate dehydrogenase (1 mg ml⁻¹, 200 to 400 i.u. mg⁻¹), 0.01; NADH (10 mM), 0.2; bacterial extract, 0.05 to 0.2; 6-phosphogluconate (50 mM), 0.2; triethanolamine/HCl buffer (100 mM, pH 7.7) to 3.2 ml. The rate of any endogenous NADH oxidation was measured at 340 nm before initiating the reaction by adding 6-phosphogluconate.

6-Phosphogluconate dehydratase was measured as for the combined assay but sufficient partially purified KDPG aldolase from *A. beijerinckii* was added to ensure that the rate-limiting step was the conversion of 6-phosphogluconate to KDPG.

KDPG aldolase was assayed by measuring pyruvate formation from KDPG. Cuvettes contained (ml): NADH (10 mM), 0.2; lactate dehydrogenase (1 mg ml⁻¹, 200 to 400 i.u. mg⁻¹), 0.01; bacterial extract, 0.05 to 0.2; KDPG (30 mM), 0.3; triethanolamine/HCl buffer (100 mM, pH 7.7) to 3.2 ml, and the assay was conducted as for the combined enzymes.

6-Phosphogluconate dehydrogenase (EC 1.1.1.44). The reduction of NADP was measured at 340 nm with cuvettes containing (ml): MgCl₂ (50 mM), 0.1; NADP (5 mM), 0.2; 6-phosphogluconate (50 mM), 0.2; bacterial extract (50 to 750 µg protein ml⁻¹), 0.05 to 0.1; hydrazine (0.96 M), 0.2; triethanolamine/HCl buffer (100 mM, pH 7.7) to 3.2 ml.

A radiochemical assay was also used. The main compartment of Warburg flasks contained (ml): sodium [¹⁴C]gluconate (25 mM, 0.1 mCi mmol⁻¹), 0.5; Tris/HCl buffer (0.12 M, pH 7.5), 0.9; ATP (36 mM), 0.1; gluconate kinase (40 units ml⁻¹), 0.02; water, 0.08. The side-arm contained 0.5 ml bacterial extract and the centre well contained 0.05 ml Hyamine hydroxide and a roll of Whatman no. 541 filter paper. The system was incubated at 30 °C for 1 h to allow phosphorylation of the gluconate before the side-arm contents were tipped. After a predetermined time, the reaction was terminated by the addition of 2 M-H₂SO₄ (0.5 ml) and the incubation was continued for 1 h to allow absorption of the CO₂ by the Hyamine hydroxide. The contents of the centre wells were quantitatively transferred with methanol to volumetric flasks and the radioactivity was determined by scintillation counting of samples in Bray's (1960) fluid. Corrections were made for non-enzymic ¹⁴CO₂ release recorded with boiled extract controls.

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49). The reduction of NADP was measured at 340 nm with cuvettes containing (ml): NADP (5 mM), 0.3; bacterial extract, 0.1; glucose 6-phosphate (30 mM), 0.5; imidazole/HCl buffer (100 mM, pH 7.5) to 3.0 ml. The reaction was initiated by addition of the substrate.

Phosphoglucose isomerase (EC 5.3.1.9). The rate of formation of glucose 6-phosphate from fructose 6-phosphate was measured spectrophotometrically by coupling to glucose-6-phosphate dehydrogenase. Cuvettes contained (ml): MgCl₂ (100 mM), 0.2; NADP (10 mM), 0.2; fructose 6-phosphate (100 mM), 0.3; glucose-6-phosphate dehydrogenase (300 to 400 i.u. mg⁻¹, 1 mg ml⁻¹), 0.01; bacterial extract, 0.05; triethanolamine/HCl buffer (100 mM, pH 7.9, containing 0.1 M-hydrazine) to 3.16 ml. The substrate was checked for contamination with glucose 6-phosphate before initiating the reaction with the extract.

Fructose-1,6-bisphosphate aldolase (EC 4.1.2.13). The rate of formation of dihydroxyacetone phosphate

from fructose 1,6-bisphosphate directly, and indirectly via glyceraldehyde 3-phosphate and triosephosphate isomerase, was assayed spectrophotometrically by coupling to glycerol-1-phosphate dehydrogenase. Cuvettes contained (ml): NADH (10 mM), 0.2; CoSO₄ (30 mM), 0.1; glycerol-1-phosphate dehydrogenase (GDH)/triosephosphate isomerase (TPI) mixture (GDH, 100 to 200 i.u. mg⁻¹, 1 mg ml⁻¹; TPI, 1000 to 2000 i.u. mg⁻¹, 1 mg ml⁻¹), 0.01; bacterial extract, 0.1; fructose 1,6-bisphosphate (30 mM), 0.1; triethanolamine/HCl (100 mM, pH 7.7) to 3.0 ml. All reagents except the substrate were incubated for 3 min before initiating the reaction.

Phosphofructokinase (EC 2.7.1.11). The enzyme was assayed by converting the reaction product, fructose 1,6-bisphosphate, to glycerol 1-phosphate in the presence of aldolase, triosephosphate isomerase and glycerol-1-phosphate dehydrogenase and measuring the concomitant oxidation of NADH. Cuvettes contained (ml): fructose 6-phosphate (40 mM), 0.05; NADH (10 mM), 0.05; ATP (20 mM), 0.01; MgCl₂ (10 mM), 0.1; fructose-1,6-bisphosphate aldolase (9 units mg⁻¹, 10 mg ml⁻¹), 0.01; triosephosphate isomerase (2400 units mg⁻¹, 2 mg ml⁻¹), 0.01; glycerol-1-phosphate dehydrogenase (36 units mg⁻¹, 2 mg ml⁻¹), 0.01; bacterial extract (5 to 10 mg protein ml⁻¹), 0.01; triethanolamine/HCl buffer (100 mM, pH 7.6) to 3.0 ml. The reaction was initiated with ATP.

Triosephosphate isomerase (EC 5.3.1.1). The production of dihydroxyacetone phosphate from glyceraldehyde 3-phosphate was measured spectrophotometrically by coupling to glycerol-1-phosphate dehydrogenase. Cuvettes contained (ml): NADH (10 mM), 0.2; glycerol-1-phosphate dehydrogenase (200 i.u. mg⁻¹, 1 mg ml⁻¹), 0.01; bacterial extract, 0.01 to 0.04; glyceraldehyde 3-phosphate (5 mM), 0.7; triethanolamine/HCl buffer (100 mM, pH 7.7) to 3.0 ml. The reaction was initiated by the addition of substrate.

Transaldolase (EC 2.2.1.2). Activity was assayed spectrophotometrically by measuring the rate of glyceraldehyde 3-phosphate production from fructose 6-phosphate and erythrose 4-phosphate in a coupled reaction using glycerol-1-phosphate dehydrogenase and triosephosphate isomerase with NADH. Cuvettes contained (ml): EDTA (0.1 M, pH 7.7), 0.1; fructose 6-phosphate (100 mM), 0.05; NADH (10 mM), 0.07; GDH/TPI mixture (GDH, 100 to 200 i.u. mg⁻¹, 1 mg ml⁻¹; TPI, 1000 to 2000 i.u. mg⁻¹, 1 mg ml⁻¹), 0.01; bacterial extract, 0.04; erythrose 4-phosphate (10 mM) 0.2; triethanolamine/HCl buffer (200 mM, pH 7.7) to 1.3 ml. The endogenous rate of NADH oxidation was ascertained prior to initiating the reaction by addition of erythrose 4-phosphate.

Transketolase (EC 2.2.1.1). Activity was determined spectrophotometrically by measuring the rate of glyceraldehyde 3-phosphate production from ribose 5-phosphate and xylulose 5-phosphate in a coupled reaction with glycerol-1-phosphate dehydrogenase, triosephosphate isomerase and NADH. Cuvettes contained (ml): MgCl₂ (0.1 M), 0.1; NADH (10 mM), 0.07; GDH/TPI mixture (as above), 0.01; thiamin pyrophosphate (20 mM), 0.03; ribose 5-phosphate (50 mM), 0.1; xylulose 5-phosphate (10 mM), 0.1; bacterial extract, 0.1; triethanolamine/HCl buffer (200 mM, pH 7.7) to 1.3 ml. The reaction was initiated by addition of the extract and the endogenous rate of NADH oxidation was measured by omitting the substrates.

Pyruvate dehydrogenase (EC 1.2.4.1). The enzyme was assayed spectrophotometrically by the method of Jackson & Dawes (1976).

Preparation of KDPG. A mutant of *Escherichia coli* K12 (designated DF 1071-2B), lacking KDPG aldolase and isolated by Faik *et al.* (1971), was kindly provided by Professor Sir Hans Kornberg. An extract of this organism was used to convert 6-phosphogluconate to KDPG essentially as described by Faik *et al.* (1971) although the quantities were scaled up. The product was purified by the method of Meloche & Wood (1966).

Preparation of ω -aminoalkyl Sepharose. The method of Shaltiel & Er-Al (1973) was employed to prepare, from Sepharose 4B, derivatives of the type Sepharose-NH(CH₂)_nNH₂ where $n = 2, 3, 4, 5, 6$ or 8 (subsequently designated C₂, C₃ etc.).

Enzyme purification. The separation and purification of 6-phosphogluconate dehydratase and KDPG aldolase was attempted with various ω -aminoalkyl Sepharoses. Trials were conducted by applying crude extract (0.5 ml, about 4 mg protein) to small columns (0.5 × 4 cm) of the gels equilibrated at 4 °C with triethanolamine/HCl buffer (100 mM, pH 7.7), containing 1 mM-dithiothreitol. KDPG aldolase was detected in eluates from C₂, C₃ or C₄ columns but not in those from C₅, C₆ or C₈. A larger scale preparation was then undertaken with C₃ gel. Extract (2.5 ml, 20 mg protein) was applied to a column (1 × 5 cm) in a disposable 5 ml-syringe and the column was washed with 50 ml of the buffer. The molarity of the eluting buffer was increased to 150 mM (containing 1 mM-dithiothreitol) and 3 ml fractions were collected and monitored for KDPG aldolase. Activity was located in fractions 5 to 12 with the maximum in fractions 8 to 10; these latter fractions were pooled. This method resulted in a 10-fold purification of the aldolase.

Using a similar technique, the behaviour of 6-phosphogluconate dehydratase was studied. Eluates were supplemented with partially purified KDPG aldolase and monitored for activity, which was found in fractions from C₂ to C₄ columns but not from C₅ to C₈. A C₅ column was thus used, crude extract was applied and all unbound protein was eluted with 100 mM-triethanolamine/HCl (pH 7.7). The molarity

of the eluting buffer was increased stepwise to 150, 200, 300 and finally 600 mM but dehydratase activity was not detected in any fraction collected. A similar result was obtained when the eluting buffer contained 10 mM-dithiothreitol.

Glucose-6-phosphate dehydrogenase was purified 36-fold by the use of C_6 -Sephacrose 4B gel which had been equilibrated with 20 mM-potassium phosphate buffer, pH 7.5. The bound enzyme was eluted with 100 mM-potassium phosphate.

Pyruvate dehydrogenase was partially purified from oxygen-limited organisms grown at a dilution rate of 0.1 h^{-1} . Crude bacterial extract (20 ml, $7.4\text{ mg protein ml}^{-1}$) was centrifuged at 100000 g for 1 h and 2 ml 1% (w/v) protamine sulphate was added to the supernatant. The resulting precipitate was sedimented at 12000 g for 10 min and to 18 ml of the supernatant was added $4.32\text{ g (NH}_4)_2\text{SO}_4$ to give 40% saturation. The precipitate was collected by centrifuging at 12000 g for 10 min, resuspended in 5 ml 0.05 M -potassium phosphate buffer (pH 7.0) containing 1 mM-mercaptoethanol, and applied to a column of 5 ml Sepharose 2B. The column was eluted with similar buffer and fractions between 105 and 180 ml displayed pyruvate dehydrogenase activity. A 44-fold purification was achieved.

Analyses. Protein, acetyl-CoA and glucose were determined by the methods previously described (Senior & Dawes, 1973; Senior *et al.*, 1972).

Chemicals and biochemicals. Chemicals were of analytical reagent grade where possible and were obtained from BDH or Hopkin & Williams. Biochemicals were obtained from Sigma or Boehringer. Diamines for the preparation of substituted Sepharose came from BDH, with the exception of the C_4 and C_8 amines which were obtained from Ralph N. Emanuel, Wembley (now the Aldrich Chemical Co., Gillingham, Dorset).

RESULTS

Entner-Doudoroff enzymes. The overall activity of the combined enzymes of this pathway (6-phosphogluconate dehydratase and KDPG aldolase) displayed an optimum pH of 7.9 and dependence on a thiol compound. The optimum concentration of dithiothreitol was 10 mM and, at equivalent thiol concentration, glutathione was equally effective. Crude extracts could be held at 0°C for 48 h without loss of activity if thiol compounds were added. Activity was stimulated by Mn^{2+} but above 0.25 mM inhibition occurred and at 5 mM the rate had decreased by about 25%; Mg^{2+} at 0.25 mM was only about 70% as effective as Mn^{2+} .

KDPG aldolase was separated from 6-phosphogluconate dehydratase by affinity chromatography on modified Sepharose 4B but all attempts to elute the active dehydratase failed. KDPG aldolase, purified 10-fold by this method, gave optimum activity in triethanolamine/HCl buffer at pH 8.4 and was unaffected by Mn^{2+} , Mg^{2+} or thiol compounds, indicating that 6-phosphogluconate dehydratase was the locus of the effects of these agents observed in the combined assay. The enzyme was stable in 150 mM -triethanolamine/HCl buffer, pH 7.7, at 4°C for 7 d without loss of activity. No inhibition was observed in the presence of ATP, citrate, isocitrate or *cis*-aconitate at concentrations of up to 5 mM .

Because attempts to separate and purify 6-phosphogluconate dehydratase were unsuccessful, inhibition studies were performed with crude extracts supplemented with partially purified KDPG aldolase to ensure that the rate-limiting step was the production of KDPG from 6-phosphogluconate. ATP (5 mM) caused 25% inhibition both in the absence and presence of 0.5 mM - Mn^{2+} . In contrast, in the presence of 5 mM - Mn^{2+} , ATP up to 5 mM relieved the inhibition produced by Mn^{2+} . This latter effect was attributed to ATP decreasing the effective concentration of Mn^{2+} , by chelation, towards the optimum of 0.25 mM . Citrate (5 mM) produced about 70% inhibition in the absence of added metal ions but had negligible effect in the presence of 0.55 mM - Mn^{2+} , and similar results were obtained with isocitrate and *cis*-aconitate. These findings suggest that the inhibition by tricarboxylic acids recorded by Senior & Dawes (1971) with unsupplemented extracts could be attributed to chelation of the activating metal ion by the inhibitor. The overall activity of the combined Entner-Doudoroff enzymes did not display any marked change in response to carbon or nitrogen limitation or to dilution rate (Table 1).

Glucose-6-phosphate dehydrogenase. This enzyme is regulated allosterically by ATP, ADP, NADH and NADPH (Senior & Dawes, 1971) and in these respects bears a marked resem-

Table 1. *Specific activities of various enzymes of glucose metabolism in steady-state chemostat cultures of A. beijerinckii*

Enzyme	Specific activity [nmol min ⁻¹ (mg protein) ⁻¹]	Limiting nutrient	Dissolved oxygen tension* (% air satn)	Dilution rate (h ⁻¹)
Glucose-6-phosphate dehydrogenase	2100	Carbon	5.0	0.10
	1100	Oxygen	ND	0.10
Phosphoglucose isomerase	550	Carbon	5.0	0.10
Entner–Doudoroff enzymes	1710	Carbon	5.0	0.10
	1690	Carbon	5.0	0.20
	1870	Carbon	7.5	0.10
	1610	Carbon	10.0	0.12
	1750	Carbon	10.0	0.21
	1870	Nitrogen	5.0	0.14
	1810	Nitrogen	10.0	0.14
Fructose-1,6-bisphosphate aldolase	95	Carbon	10.0	0.12
	73	Oxygen	ND†	0.11
	76	Oxygen	ND	0.11
Triosephosphate isomerase	5000	Carbon	10.0	0.12
	2700	Oxygen	ND†	0.11
	3100	Oxygen	ND	0.11
Transketolase	56‡	Carbon	10.0	0.12
Transaldolase	100	Carbon	10.0	0.12

ND, Not detected.

* For carbon-limited cultures the nitrogen flow rate was set at 1 litre min⁻¹ and the oxygen flow rate was adjusted automatically to produce the required dissolved oxygen tension. For oxygen-limited cultures the oxygen content of the inflowing gas was 1 % (v/v) with a total gas flow of 1 litre min⁻¹ (except where indicated).

† Oxygen content of inflowing gas 2.8 % (v/v); total flow rate 1 litre min⁻¹.

‡ At pH 7.7, see text.

blance to the corresponding enzyme from *Alcaligenes eutrophus* H16 (Tunail & Schlegel, 1972) which, however, is additionally subject to allosteric inhibition by phosphoenolpyruvate. In contrast, we found the *A. beijerinckii* enzyme was unaffected by 0.5 mM-phosphoenolpyruvate although some inhibition occurred at 2 mM.

6-Phosphogluconate dehydrogenase. The assay for this enzyme gave a steadily increasing reaction rate if 6-phosphogluconate was added last to the assay system and a faster, linear rate if NADP were the final addition. This suggested that the substrate for the observed reaction was being generated from 6-phosphogluconate as would be the case if the Entner–Doudoroff enzymes converted 6-phosphogluconate to pyruvate and glyceraldehyde 3-phosphate and the latter compound served as substrate for the glyceraldehyde-3-phosphate dehydrogenase which is NADP-dependent in *A. beijerinckii* (Senior & Dawes, 1971). To test this possibility the assay was performed in the presence of increasing concentrations of hydrazine, which sequesters glyceraldehyde 3-phosphate. In the presence of 82 mM-hydrazine, which completely inhibits glyceraldehyde-3-phosphate dehydrogenase, NADP reduction was abolished. In comparable experiments with extracts of *Escherichia coli* or with commercial preparations of 6-phosphogluconate dehydrogenase, hydrazine did not inhibit NADP reduction, and addition of the *A. beijerinckii* extract to the *E. coli* extract did not affect the activity of the latter (Fig. 1).

Attempts were also made to assay 6-phosphogluconate dehydrogenase isotopically by measuring ¹⁴CO₂ production from 6-phospho[1-¹⁴C]gluconate in the presence of arsenite to prevent pyruvate oxidation. Controls incorporated [1-¹⁴C]pyruvate to prevent the possibility of error due to ¹⁴CO₂ release from this keto acid, which is produced by Entner–

Fig. 1. Effect of hydrazine on the assay for 6-phosphogluconate dehydrogenase in *A. beijerinckii*, *E. coli* and yeast. Assays for 6-phosphogluconate dehydrogenase were carried out in the presence and absence of 82 mM-hydrazine with extracts from *A. beijerinckii* and *E. coli*, and with commercial 6-phosphogluconate dehydrogenase from yeast (Sigma; P0507): *A. beijerinckii* extract, hydrazine absent (1), present (2); *E. coli* extract, hydrazine absent (3), present (4); combined *A. beijerinckii* and *E. coli* extracts, hydrazine present (5); yeast 6-phosphogluconate dehydrogenase, hydrazine absent (6), present (7).

Doudoroff activity. Convincing evidence for the oxidative decarboxylation of 6-phosphogluconate could not be obtained and it was concluded that 6-phosphogluconate dehydrogenase does not play a significant role in the metabolism of *A. beijerinckii*.

Transketolase and transaldolase. In the absence of 6-phosphogluconate dehydrogenase, production of pentoses from fructose 6-phosphate and glyceraldehyde 3-phosphate by the non-oxidative reactions of the pentose cycle must be inferred and the enzymes catalysing these reactions were sought and found (Table 1). Crude extracts from carbon-limited organisms gave an optimum pH of 7.7 for transaldolase but because of a shortage of substrate it was not possible to ascertain the optimum pH for transketolase which was therefore assayed at the same pH.

Phosphoglucose isomerase. This enzyme was present (Table 1) and displayed optimum activity between pH 7.7 and 8.2 in triethanolamine/HCl buffer.

Fructose-1,6-bisphosphate aldolase. Low activities were observed unless extracts were supplemented with either Co^{2+} , K^+ or NH_4^+ ions (Table 2). Maximum activity was observed in the presence of Co^{2+} which was subsequently used in all assays. Mercaptoethanol or cysteine at 1 mM final concentration abolished activity but 1 mM-glutathione was without effect. The specific activities of the aldolase from carbon- and oxygen-limited *A. beijerinckii* are given in Table 1.

Phosphofructokinase. All efforts to detect this enzyme in *A. beijerinckii* extracts failed under conditions which gave satisfactory assays with *E. coli* extracts and samples of the commercial rabbit muscle enzyme.

Triosephosphate isomerase. Activity was significantly higher in extracts prepared from carbon- than from oxygen-limited organisms (Table 1).

Pyruvate dehydrogenase. The partially purified enzyme, which was free from β -keto-thiolase, acetoacetyl-CoA reductase and NADH oxidase, was not inhibited by phosphoenolpyruvate (10 mM), AMP (1 mM), ATP (1 mM), acetoacetate (1 mM) or 3-hydroxybutyrate (10 mM). Acetyl-CoA showed competitive inhibition with respect to CoASH, and NADH inhibited in a non-compulsory ordered manner. The apparent K_m for pyruvate was 0.5 mM, for NAD 0.15 mM and for CoASH 42.5 μM . This enzyme thus displays properties similar

Table 2. *Effect of cations on fructose-1,6-bisphosphate aldolase activity*

Cation	Concn (mM)	Specific activity [nmol NADH utilized min ⁻¹ (mg protein) ⁻¹]
None	—	7
Co ²⁺	0.1	97
	1.0	94
	2.0	92
K ⁺	50.0	82
NH ₄ ⁺	50.0	68
K ⁺ + Co ²⁺	50.0 + 1.0	95

to pyruvate dehydrogenases from other sources in being inhibited by a short-chain acyl-CoA ester (Erfe & Sauer, 1969) and by NADH (Hansen & Henning, 1966).

DISCUSSION

Results with chemostat-grown organisms confirm our previous findings with batch cultures that glucose is metabolized in *A. beijerinckii* principally via the Entner–Doudoroff pathway and also demonstrate that the oxidative pentose phosphate pathway is not of significance in this organism. The absence of phosphofructokinase precludes the operation of glycolysis. Pentoses required for nucleic acid synthesis can be synthesized from fructose 6-phosphate and glyceraldehyde 3-phosphate by the non-oxidative reactions catalysed by transketolase and transaldolase, both enzymes being present. As phosphoglucose isomerase, fructose-1,6-bisphosphate aldolase and triosephosphate isomerase are all present, fructose 6-phosphate and glyceraldehyde 3-phosphate can be produced from glucose 6-phosphate, and it seems that the main function of fructose-1,6-bisphosphate aldolase would be in pentose formation, its activity being very much lower than that of the combined Entner–Doudoroff enzymes.

Previous reports of the presence of 6-phosphogluconate dehydrogenase in *Azotobacter vinelandii* (Mortenson & Wilson, 1954; Haaker & Veeger, 1976) and *A. beijerinckii* (Senior & Dawes, 1971) are probably explained as artefacts caused by the operation of the Entner–Doudoroff enzymes on 6-phosphogluconate to produce glyceraldehyde 3-phosphate, which is then oxidized by the NADP-dependent glyceraldehyde-3-phosphate dehydrogenase; a similar situation applied with *P. aeruginosa*, since Blevins *et al.* (1975) have shown that mutants devoid of 6-phosphogluconate dehydratase are unable to metabolize glucose. However, the situation in *A. vinelandii* was initially somewhat confused. Thus Mortenson & Wilson (1954) reported 6-phosphogluconate oxidation by extracts and claimed that the dehydrogenase differed from others in not requiring NAD(P) as its hydrogen acceptor; subsequently they stated that all attempts to demonstrate the presence of the 6-phosphogluconase had failed (Mortenson *et al.*, 1955). No explanation for these discrepancies was offered.

Separation of 6-phosphogluconate dehydratase from KDPG aldolase permitted identification of the dehydratase as the locus of action of the inhibitory effects of ATP and tricarboxylic acids reported by Senior & Dawes (1971). However, these effects depended on the concentration of divalent metal ions present and the extent to which the tricarboxylic acids and ATP sequestered the available activating ions; they would not, therefore, seem to be of physiological significance and the major regulatory site for hexose phosphate metabolism is glucose-6-phosphate dehydrogenase, allosterically modulated by ATP, ADP, NAD and NADP but virtually unaffected by phosphoenolpyruvate.

While a systematic comparison of the effects of different nutrient limitations was not our prime objective, such observations as were made indicated that the levels of the in-

vestigated enzymes of carbohydrate metabolism were higher in carbon-limited than in oxygen-limited organisms.

Poly- β -hydroxybutyrate synthesis in *A. beijerinckii* is initiated by diversion of acetyl-CoA, destined for oxidation via the tricarboxylic acid cycle, to acetoacetyl-CoA formation as a result of oxygen limitation, effected by the intracellular concentrations of free CoASH and NADH (Senior & Dawes, 1973). In addition to this fine control, the levels of activity of NADH oxidase, isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase all decreased while those of β -ketothiolase and acetoacetyl-CoA reductase increased, when an oxygen limitation was imposed; in contrast, citrate synthase and pyruvate dehydrogenase were relatively unaffected (Jackson & Dawes, 1976). Pyruvate dehydrogenase is a multi-component system and the complex in *A. vinelandii* has been the subject of detailed biochemical study (Bresters *et al.*, 1975; De Abreu *et al.*, 1977).

The demonstration of the fine control of pyruvate dehydrogenase in *A. beijerinckii* by NADH indicates that when an oxygen limitation is imposed the rate of acetyl-CoA formation from pyruvate will decrease until poly- β -hydroxybutyrate synthesis is initiated and the NADH/NAD ratio restored to near its original value; at a dilution rate of 0.1 h^{-1} this takes almost 2 h (Jackson & Dawes, 1976).

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