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Poly-β-hydroxybutyrate Biosynthesis and the Regulation of Glucose Metabolism in *Azotobacter beijerinckii*

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Azotobacter beijerinckii possesses the enzymes of both the Entner–Doudoroff and the oxidative pentose phosphate cycle pathways of glucose catabolism and both pathways are subject to feedback inhibition by products of glucose oxidation. The allosteric glucose 6-phosphate dehydrogenase utilizes both $NADP^+$ and NAD^+ as electron acceptors and is inhibited by ATP, ADP, NADH and NADPH. 6-Phosphogluconate dehydrogenase (NADP-specific) is unaffected by adenosine nucleotides but is strongly inhibited by NADH and NADPH. The formation of pyruvate and glyceraldehyde 3-phosphate from 6-phosphogluconate by the action of the Entner–Doudoroff enzymes is inhibited by ATP, citrate, isocitrate and *cis*-aconitate. Glyceraldehyde 3-phosphate dehydrogenase is unaffected by adenosine and nicotinamide nucleotides but the enzyme is non-specific with respect to NADP and NAD. Citrate synthase is strongly inhibited by NADH and the inhibition is reversed by the addition of AMP. Isocitrate dehydrogenase, a highly active NADP-specific enzyme, is inhibited by NADPH, NADH, ATP and by high concentrations of $NADP^+$. These findings are discussed in relation to the massive synthesis of poly-β-hydroxybutyrate that occurs under certain nutritional conditions. We propose that synthesis of this reserve material, to the extent of 70% of the dry weight of the organism, serves as an electron and carbon 'sink' when conditions prevail that would otherwise inhibit nitrogen fixation and growth.

Earlier fragmentary evidence (Mortenson & Wilson, 1954; Johnson & Johnson, 1961) and the subsequent radiorespirometric studies of Still & Wang (1964) suggested the operation of the Entner & Doudoroff (1952) pathway of glucose catabolism in *Azotobacter* as a major metabolic route. *Azotobacter vinelandii* was shown to possess hexokinase, phosphoglucomutase and phosphohexose isomerase, and also to produce intermediates of the pentose phosphate cycle, by Mortenson & Wilson (1955), who suggested that the $NAD(P)H/NAD(P)^+$ ratio influences the ratio of 6-phosphogluconate dissimilated by the two pathways. The present work was undertaken to demonstrate the presence of key enzymes of both the Entner–Doudoroff and pentose phosphate pathways in *Azotobacter beijerinckii* and to study their regulation, especially in relation to the formation of the storage compound poly-β-hydroxybutyrate, which occurs in this organism.

Our previous investigations of the route of biosynthesis of poly-β-hydroxybutyrate in *A. beijerinckii* revealed that the polymer is deposited to the extent of 70% of the dry weight of the organism in batch culture towards the end of exponential growth in nitrogen-free media containing 1% (w/v) glucose (Ritchie, Senior & Dawes, 1969). The initiation of polymer synthesis coincides with the attainment of zero oxygen concentration in shaken cultures, although under these cultural conditions the oxygen limitation could not be completely dissociated from the possibility of a simultaneous nitrogen limitation. The biosynthetic route, via coenzyme A esters (Ritchie & Dawes, 1969), involves the reduction of acetoacetyl-CoA to D(−)-β-hydroxybutyryl-CoA, a reaction catalysed by acetoacetyl-CoA reductase (Ritchie, Senior & Dawes, 1971) which utilizes both NADH and NADPH, although NADPH is oxidized at approximately fivefold the rate of NADH. The equilibrium of the reaction, at pH 7.3, favours the formation of D(−)-β-hydroxybutyryl-CoA, the substrate for the poly-β-hydroxybutyrate-granule-bound poly-β-hydroxybutyrate polymerase.

In an attempt to elucidate the regulatory mechanisms that control the biosynthesis of poly-β-hydroxybutyrate we have sought to correlate the regulation of the enzymes of glucose metabolism with the observed stimulation of polymer synthesis under conditions of oxygen limitation. A preliminary account of some of this work has been given (Senior & Dawes, 1970).

EXPERIMENTAL

Materials

Chemicals. 6-Phosphogluconate (sodium salt), glucose 6-phosphate, dithiothreitol, *N*-ethylmaleimide, *p*-chloromercuribenzoate, mersalyl, and ammonium sulphate, low in heavy metals (Pb <0.0001%), were obtained from BDH Chemicals Ltd. (Poole, Dorset, U.K.).

NAD^+, $NADP^+$, NADH, NADPH, fructose 1,6-diphosphate, bovine serum albumin fraction V, glyceraldehyde 3-phosphate diethyl acetal (barium salt), Dowex 50 resin (H^+ form), iodoacetamide, ATP, ADP, AMP, α-oxoglutarate, 5,5′-dithiobis-(2-nitrobenzoic acid), GSH and trisodium isocitrate were from Sigma (London) Chemical Co. (London S.W.6, U.K.). Yeast CoA (free acid) was from Boehringer Corp. (London) Ltd. (London W.5, U.K.). *cis*-Aconitate was supplied by Hopkin and Williams Ltd. (Chadwell Heath, Essex, U.K.), DEAE-cellulose DE-32 microgranular by Whatman Biochemicals Ltd. (Maidstone, Kent, U.K.). All other chemicals were of analytical grade.

Enzymes. Crystalline lactate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase were obtained from Sigma Chemical Co. and fructose diphosphate aldolase from Boehringer.

Organism. The organism used throughout this work was *Azotobacter beijerinckii* N.C.I.B. 9067.

Maintenance and growth of the organism. The basal medium (nitrogen-free) was prepared from two solutions of the following composition (g/l): solution A: glucose, 20.00; $MgSO_4,7H_2O$, 0.4; $CaCl_2$, 0.11; $FeSO_4,7H_2O$, 0.012; $Na_2MoO_4,2H_2O$, 0.01; solution B: K_2HPO_4, 2.0; NaCl, 0.4. The two solutions were autoclaved separately (15lb/in² for 20min) and combined in equal volumes after cooling. The pH of the complete medium was 7.7. For solid medium 2% (w/v) agar was added to the combined solutions.

Stock cultures of *A. beijerinckii* were maintained on slopes of the solid medium and subcultured every 4 weeks. For large-scale growth 10ml of liquid medium was inoculated from the slope and incubated at 30°C with continuous shaking. This subculture was then used to inoculate 2 litres of medium in a wide-necked 4 litre conical flask. The organisms were grown at 30°C on a gyrotary shaker (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.).

Harvesting of cells and preparation of crude cell extracts. The cells were harvested after 18h, corresponding to the end of exponential growth, by centrifuging at 5000*g* for 30min. The cells were washed three times with water and resuspended in approx. 5vol. of the buffer appropriate to the particular enzyme being studied. These buffers were as follows: for glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, 200mM-tris–HCl buffer, pH7.6, with subsequent cell disintegration by ultrasonic treatment [2min at 8A with a Soniprobe type 1130A (Dawe Instruments Ltd., London W.3, U.K.)]; 6-phosphogluconate hydrolyase and 3-deoxy-2-oxo-6-phosphogluconate aldolase, 100mM-potassium phosphate buffer, pH7.5, 200mM-imidazole–HCl buffer, pH7.5, or 250mM-tris–HCl buffer, pH7.5 (depending on the assay system used), followed by ultrasonic treatment; for glyceraldehyde 3-phosphate dehydrogenase, 200mM-potassium phosphate buffer, pH7.2, plus 10mM-2-mercaptoethanol, and disruption by passage through a French pressure cell (Milner, Lawrence & French, 1950) pre-cooled to 2°C; for citrate synthase, 200mM-sodium phosphate buffer, pH7.3, with subsequent cell disintegration by ultrasonic treatment; isocitrate dehydrogenase, 200mM-tris–HCl buffer, pH7.7, with subsequent ultrasonic treatment; NADH oxidase and NADPH–NAD^+ transhydrogenase, 200mM-potassium phosphate buffer, pH7.5, and disruption by French pressure cell. All disintegrated cell suspensions were centrifuged at 35000*g* for 20min and the supernatant was decanted.

Determination of protein. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). A calibration curve was prepared with crystallized bovine serum albumin. A sample of albumin was found on desiccation to contain 8.0% water, which was corrected for in the calibration.

Determination of pyruvate. The method of von Korff (1969) with lactate dehydrogenase was used. Samples were neutralized to pH7.0 with 0.1M-HCl before assay. A silica-glass cuvette (1cm light-path) contained: 0.3ml of 100mM-potassium phosphate buffer, pH7.4; 0.1ml of NADH (4mg/ml); 10μl of lactate dehydrogenase (100 units/mg, 5mg/ml); 0.1–0.3ml of sample; and deionized water to 3.0ml. The increase in E_{340} was monitored and used to calculate the original concentration of pyruvate.

Determination of total carbonyl groups. Total carbonyl groups (including pyruvate) were determined by semicarbazone formation. Reaction mixtures (0.5ml) were deproteinized by the addition of 0.5ml of 10% (w/v) trichloroacetic acid. After centrifugation, 0.5ml of supernatant was added to 1.0ml of semicarbazide reagent (10g of semicarbazide hydrochloride plus 15g of sodium acetate/litre), and 0.5ml of water. The mixture was incubated at 30°C for 15min after which time it was diluted to 5ml with water and the E_{250} of a sample, contained in a silica glass cuvette, was read (ϵ_{250}= 8350l·mol^{-1}·cm^{-1}; MacGee & Doudoroff, 1954).

Preparation and determination of acetyl-CoA. Acetyl-CoA was prepared from acetic anhydride by the method of Simon & Shemin (1953). Yields were 95–100% based on both hydroxamate assay (Lipmann & Tuttle, 1945) and thiol group titration with 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman, 1959).

Determination of CoA and thiol groups. An adaptation of the method of Ellman (1959) was used. The principle of the method is that 5,5′-dithiobis-(2-nitrobenzoic acid) reacts with the thiol reagent between pH7.0 and 8.0 to give a mixed disulphide with the release of the anion thionitrobenzoate, which is yellow ($\lambda_{max.}$ 412nm, ϵ_{412} of 13600l·mol^{-1}·cm^{-1}). The assay is only useful in the pH range 7.0–8.0, as 5,5′-dithiobis-(2-nitrobenzoic acid) decomposes outside this range. The reaction mixture contained 2.0ml of 67mM-sodium–potassium phosphate buffer, pH7.0; 0.2ml of 10mM-5,5′-dithiobis-(2-nitrobenzoic acid) in 67mM-sodium–potassium phosphate buffer, pH7.0; 25.0–200n-equiv. of thiol compound, and water to 3ml. The mixture was incubated at 30°C and its E_{412} read against a blank reaction mixture (minus thiol compound) until no further increase could be observed. ΔE_{412} was used to calculate the concentration of thiol groups.

Preparation of DL-*glyceraldehyde 3-phosphate.* The method was based on that in Sigma Technical Bulletin

no. 10. Dowex 50 resin (H^+ form; 1.5g wet wt.) was suspended in 6ml of deionized water in a boiling tube. DL-Glyceraldehyde 3-phosphate diethyl acetal (100mg of the barium salt) was added and mixed thoroughly. The reaction mixture was incubated at 100°C for 10min with periodic shaking, after which time the tube was plunged into an ice bath. The mixture was then centrifuged at 5000*g* for 5min and the supernatant decanted. The resin was resuspended in 2ml of water and recentrifuged. This washing procedure was repeated several times and the supernatants were combined and stored in 2ml portions at −20°C. The glyceraldehyde 3-phosphate content of samples stored under these conditions was found to decrease by approx. 2% per month. The percentage yield of enzymically active D-isomer was usually 40–43% (mol/mol).

Assay of prepared samples of DL-*glyceraldehyde 3-phosphate.* The assay was based on modifications of the methods of Fuller & Gibbs (1959) and of Pearce & Carr (1969) involving reduction of NAD by glyceraldehyde 3-phosphate dehydrogenase in the presence of arsenate.

Silica-glass cuvettes (3ml) contained: 200mM tris–HCl, pH8.5, 1.5ml; 0.17M-Na_2HAsO_4, pH8.5, 0.3ml; 0.20M-L-cysteine hydrochloride, pH7.0, 0.05ml; 0.1M-NaF, 0.60ml; 30mM-β-NAD, 0.05ml; and crystalline glyceraldehyde 3-phosphate dehydrogenase (750μg of protein/ml), 0.10ml. The reaction was initiated by the addition of 0.10ml of test solution and the reduction of NAD^+ measured at 340nm. The maximum E_{340}, attained after 3–5min at 25°C, was used to calculate the amount of glyceraldehyde 3-phosphate present. A control cuvette contained all components except the test solution, which was replaced by water.

Enzyme assays

All enzymes were assayed at their optimum pH. In the descriptions of assay systems, where a range of volumes of added enzymes or substrates is given, the highest value in each case represents the standard system used. Variations within the ranges indicated were made to study the effects of enzyme, substrate or co-factor concentration and volumes were always made up to the stated final reaction volumes by appropriate additions of water.

Glucose 6-phosphate dehydrogenase (EC 1.1.1.49). When assayed with 120mM-tris–HCl buffer, pH7.3, activity was 35% of that with 120mM-imidazole–HCl buffer, pH7.3. Silica-glass cuvettes (3ml) contained: 120mM-imidazole–HCl buffer, pH7.3, 1.5ml; 10mM-glucose 6-phosphate, 0.25ml; enzyme preparation, 0.1ml; 5mM-NAD^+ or -$NADP^+$, 0.5ml; and water to make a final reaction volume of 2.5ml. The reactants were pre-incubated at 30°C and the reaction was initiated with NAD^+ or $NADP^+$. The progress of the reaction was shown by the increase in E_{340}. With purified enzyme preparations micro-cuvettes (volume 0.5ml) were used containing: 200mM-imidazole–HCl buffer, pH7.3, 150μl; enzyme preparation, 100μl; 10mM-glucose 6-phosphate, 80μl; water to a final reaction volume of 0.4ml. The reaction was initiated with 5mM-NAD^+ or -$NADP^+$ (80μl). Where the effect of enzyme concentration was studied a range of 10–100μl of purified enzyme preparation was used and for studies of the effect of substrate concentration a range of 10–80μl of 5mM-NAD^+ or -$NADP^+$ was used with appropriate adjustment of water addition to maintain a reaction volume of 0.4ml.

6-Phosphogluconate dehydrogenase (EC 1.1.1.44). Silica-glass micro-cuvettes (volume 0.5ml) contained: 200mM-tris–HCl buffer, pH7.6, 150μl; enzyme preparation, 100μl; 20mM-6-phosphogluconate, 75μl; water to a total reaction volume of 0.4ml. The reaction was initiated by the addition of 75μl of 5mM-$NADP^+$ and followed at 30°C by the increase in E_{340}. Where the effect of enzyme concentration was studied a range of 20–100μl of purified enzyme preparation was used and for studies on the effects of substrate and cofactor concentration ranges of 5–75μl of 20mM-6-phosphogluconate and 20–75μl of 5mM-$NADP^+$ were used, the total reaction volume being maintained at 0.4ml by appropriate additions of water.

Glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.13). In initial experiments with crude extracts fructose 1,6-diphosphate in the presence of fructose 1,6-diphosphate aldolase (commercial preparation) was used as substrate. $NADP^+$ was reduced more rapidly than NAD^+ and was therefore used as a routine in assays. Silica-glass cuvettes (3ml) contained: 30mM-sodium pyrophosphate buffer, pH7.8, containing 4.0mM-cysteine, 1.9ml; 0.4M-Na_2HAsO_4, pH7.8, 0.1ml; 3mM-$NADP^+$, 0.10ml; 6mM-fructose 1,6-diphosphate, in 30mM-sodium pyrophosphate buffer, pH7.8, containing 4mM-cysteine, 0.30ml; aldolase solution (750μg of protein/ml), 30μl; crude extract, 0.1–0.4ml; and water to 3.0ml. The increase in E_{340} was measured at 30°C. The reaction was initiated with various components of the reaction mixture under identical preincubation conditions; it was found that initiation with $NADP^+$ gave the highest initial rates of reduction.

In assays with the partially purified enzyme, glyceraldehyde 3-phosphate was used as substrate. Silica-glass cuvettes (3ml) contained: 100mM-potassium phosphate buffer, pH7.2, plus 5mM-2-mercaptoethanol, 1.5ml; 0.4M-Na_2HAsO_4, pH7.2, 0.1ml; cell extract, 0.1–0.5ml; 3mM-NAD^+ or -$NADP^+$, 0.01–0.30ml; water to give a final reaction volume of 2.5ml. The reactants were pre-incubated for 3min at 30°C; the reaction was initiated with 0.2ml of 10mM-glyceraldehyde 3-phosphate and the increase in E_{340} measured. With undialysed crude extracts endogenous reduction of NAD^+ occurred, which was attributed to an NAD-specific D(−)-β-hydroxybutyrate dehydrogenase which acts on the large amounts of D-β-hydroxybutyrate produced by depolymerization of poly-β-hydroxybutyrate after cell disruption. Dialysis against water did not completely eliminate this endogenous reduction, but decreased it to a value that did not interfere with the glyceraldehyde 3-phosphate dehydrogenase assay.

6-Phosphogluconate hydro-lyase dehydratase (EC 4.2.1.12) *and 3-deoxy-2-oxo-6-phosphogluconate aldolase* (EC 4.1.2.14). These enzymes were assayed together because 3-deoxy-2-oxo-6-phosphogluconate was not available as a substrate. Two systems were employed. In the direct spectrophotometric assay silica glass cuvettes contained: 200mM-imidazole–HCl buffer plus 1mM-dithiothreitol, pH7.7, 2.0ml; lactate dehydrogenase, 0.05ml (100 units/mg; 1mg/ml); 10mM-NADH, 0.02ml; 50mM-6-phosphogluconate, 0.005–0.3ml (0.1ml for standard assay); enzyme preparation (50–750μg of protein; 125μg in standard assay); plus water to 3.0ml final

volume. The reaction, at 30°C, was initiated with 6-phosphogluconate, after prior determination of the rate of endogenous NADH oxidation. Decrease in E_{340} was monitored with a Unicam SP.1800 spectrophotometer. ϵ_{340} for NADH was taken as $6220 l \cdot mol^{-1} \cdot cm^{-1}$. For the determination of pyruvate and glyceraldehyde 3-phosphate yield from 6-phosphogluconate the reaction volume was doubled and 0.5ml samples were taken, deproteinized, neutralized and portions reacted with semicarbazide reagent as described above for the determination of total carbonyl groups, and with lactate dehydrogenase for pyruvate determination. The difference represented glyceraldehyde 3-phosphate.

Citrate synthase (EC 4.1.3.7). A continuous spectrophotometric method was employed by using an assay with 5,5′-dithiobis-(2-nitrobenzoic acid) adapted from that of Ellman (1959). Conversion of acetyl-CoA into CoA was monitored by the appearance of the E_{412} peak of the anion, thionitrobenzoate, in a silica-glass cuvette which contained: 200mM-sodium phosphate buffer, pH7.3, 2.0ml; 150mM-oxaloacetate (sodium salt, freshly prepared solution), 0.05ml; 10mM-acetyl-CoA, 0.02–0.20ml (0.02ml in standard assay); 10mM-5,5′-dithiobis-(2-nitrobenzoic acid), 0.2ml; enzyme preparation, 0.010–1.0mg of protein (0.02mg in standard assay), plus water to 3.0ml final reaction volume, temperature 30°C. The reactants, minus oxaloacetate, were preincubated so that the rate of endogenous acetyl-CoA hydrolysis could be measured. Identical reaction mixtures were subjected to direct assay and to delayed addition of 5,5′-dithiobis-(2-nitrobenzoic acid) at 1min and 2min periods after initiation of the reaction by addition of oxaloacetate. The E_{412} values obtained immediately after the addition of 5,5′-dithiobis-(2-nitrobenzoic acid) to the reactants were used to establish the initial rate of CoA liberation. This delayed addition technique was employed because of the interference by 5,5′-dithiobis-(2-nitrobenzoic acid) with inhibitor studies as noted in the Results section.

Isocitrate dehydrogenase (EC 1.1.1.42). A silica-glass cuvette contained: 200mM-tris–HCl buffer, pH7.7, 2.0ml; 10mM-$NADP^+$, 0.1ml; enzyme preparation, 59.0μg of protein; 150mM-isocitrate, 0.01ml; water to 3.0ml final volume. The reactants were incubated at 30°C for 2min before initiation of the reaction with isocitrate. The reaction course was monitored by ΔE_{340} measurement.

$NADPH$–NAD^+ transhydrogenase (EC 1.6.1.1) *and NADH oxidase* (EC 1.6.99.3). In all crude extracts NADH oxidase was present. It was assayed in a reaction mixture containing, in 3ml silica-glass cuvettes: 0.10M-potassium phosphate buffer, pH7.2, 1.5ml; crude extract, 0.1–0.5ml; 6mM-NAD(P)H, 0.1ml; and water to 2.5ml. The reaction was followed by decrease in E_{340} at 30°C. Extracts contained poly-β-hydroxybutyrate depolymerase and thus produced D(−)-β-hydroxybutyrate, which was readily oxidized to acetoacetate. The enzyme catalysing the oxidation of β-hydroxybutyrate was specific for NAD. Thus in undialysed extracts assay of NADH oxidation by NADH oxidase is obscured by the opposing reduction of NAD^+ in β-hydroxybutyrate oxidation. Consequently all crude extracts (approx. 50ml) used in this section of our work were dialysed against 5 litres of 100mM-potassium phosphate buffer, pH7.2, at 0°C for 24h to decrease the β-hydroxybutyrate to a concentration at which it did not interfere.

The NADH oxidase system was examined with both NADH and NADPH as electron donor. Crude cell extracts prepared for NADH oxidase assays also oxidized NADPH but at less than 5% of the rate with NADH. The addition of an equivalent concentration of NAD^+ stimulated NADPH oxidation 20-fold, suggesting the presence of a very active NADPH–NAD^+ transhydrogenase. This activity was assayed spectrophotometrically, the reaction cuvette containing: 0.10M-potassium phosphate buffer, pH7.1, 1.5ml; crude extract, 0.1–0.5ml; 6mM-NADPH, 0.1ml; 6mM-NAD^+, 0.1ml; water to 2.5ml. The decrease in E_{340} was measured at 25°C with various preincubation patterns. The order of addition of NADPH, NAD^+ and enzyme was varied to eliminate possible errors such as non-enzymic reactions. The action of $NADP^+$ as a carrier between NADPH and NADH oxidase was also investigated.

Enzyme purification

Crude cell extracts contained high activities of NADH oxidase, NADPH transhydrogenase and D(−)-β-hydroxybutyrate dehydrogenase. To study the regulatory action of NADH and NADPH on the enzymes examined in this work, each enzyme had to be purified to the extent of removing interfering systems such as NADH oxidase from the preparation. All ammonium sulphate fractionations were carried out at 0°C by adding the solid salt to the protein solution, in accordance with the data given by Dawson, Elliott, Elliott & Jones (1969). The percentage saturation values recorded are relative to full saturation at 0°C.

DEAE-cellulose (30g of DE-32 microgranular) was processed through the precycling washings with 1 litre each of 0.5M-HCl, 0.5M-NaOH and water as recommended by the manufacturers. The cellulose was then suspended in 1 litre of the appropriate buffer (200mM) and subjected to a vacuum to remove CO_2. The suspension was filtered at the pump and washed three times with 3 litre portions of boiled and cooled water. The cellulose was then resuspended in 1 litre of 5mM buffer at 0°C. A column (30.0cm × 2.5cm) was packed under N_2 pressure (15lb/in²). Then 1 litre of the appropriate eluting buffer, at 0°C, was slowly pumped through the column to complete equilibration. All columns were run at 4°C.

Glucose 6-phosphate dehydrogenase. A crude extract (10ml, 2.53mg of protein/ml) was dialysed overnight against 5 litres of water at 4°C. The extract was then loaded on the column of DEAE-cellulose and proteins were eluted from the column at 4°C by a continuous gradient of NaCl (0–800mM) in 800ml of 5mM-tris–HCl buffer, pH7.3. Fractions (8ml) were collected and assayed for both protein and glucose 6-phosphate dehydrogenase. The most active fraction (0.375mg of protein/ml; 8ml) was re-chromatographed on a similar DEAE-cellulose column with a continuous gradient of 0–300mM-NaCl in 300ml of 5mM-tris–HCl buffer, pH7.3, and identical elution conditions. The most active fraction (0.015mg of protein/ml, 8ml; specific activity 28.8μmol/min per mg of protein) was free of NADH oxidase and transhydrogenase activities, and represented an overall purification of 36.4-fold.

6-Phosphogluconate dehydrogenase. Because of the instability of this enzyme in all preparations other than crude extracts, fresh ammonium sulphate fractions were prepared for each separate experiment. A typical fractionation gave a tenfold increase in specific activity (final specific activity 0.875 μmol/min per mg of protein) and the most active fraction, 60–70% saturation precipitate, was free of NADH oxidase and transhydrogenase activities. Precipitates from crude extracts were separated by centrifuging (30000*g* for 5 min at 2°C), supernatants were decanted and the precipitated proteins re-dissolved in 200 mM-tris–HCl buffer, pH 7.6, and assayed for protein and 6-phosphogluconate dehydrogenase.

Glyceraldehyde 3-phosphate dehydrogenase: ammonium sulphate fractionation. Precipitates were sedimented at 30000*g* for 5 min at 2°C and re-dissolved in 200 mM-potassium phosphate buffer, pH 7.2. The two most active fractions, 40–50% and 50–60% saturation precipitates, with specific activities of 141.0 and 97.0 nmol/min per mg of protein, were then subjected to chromatography on DEAE-cellulose. The cellulose preparation was as for glucose 6-phosphate dehydrogenase chromatography except the buffer used for equilibration was 200 mM-potassium phosphate buffer, pH 7.2, and that used for elution was 5 mM-potassium phosphate buffer, pH 7.2. Proteins were eluted from the column by running a dual gradient with 300 ml of 0–200 mM-NaCl in 5 mM-potassium phosphate buffer, pH 7.20, followed by 300 ml of 200–600 mM-NaCl in the same buffer. Fractions (10 ml) were collected and chloride, protein and enzyme activities determined. Chloride was determined by titration with 0.1 M-$AgNO_3$ in the presence of 2,6-dichlorofluorescein indicator [0.1 g in 100 ml of 70% (v/v) ethanol]; the colour change at the end point is from white to pink. The most active fractions from the column, specific activities of 1.57 and 0.73 μmol/min per mg of protein representing a 31-fold purification, were made 5 mM with respect to 2-mercaptoethanol. After protein determination, bovine serum albumin (5 mg/ml final concentration) was added to stabilize the enzyme.

6-Phosphogluconate dehydratase and 3-deoxy-2-oxo-6-phosphogluconate aldolase. In crude extracts this system of two enzymes, assayed by both the semicarbazide and lactate dehydrogenase methods, was stable for up to 14 days, when stored at 0°C. However, activity was lost rapidly when attempts were made to purify either enzyme of the system. Large losses of activity were recorded when crude cell extracts were subjected to ammonium sulphate fractionation, DEAE-cellulose column chromatography, Sephadex G-100 and G-200 gel filtration and calcium phosphate-gel treatment. Various pH values and ionic conditions were employed. Fractions were recombined from ammonium sulphate treatment but activity was not enhanced. All experiments were carried out with crude extracts that had been dialysed against water.

Citrate synthase and isocitrate dehydrogenase. Citrate synthase was partially purified by two ammonium sulphate fractionations. The most active fraction, 50–60% saturation precipitate, from the first fractionation, contained most of the citrate synthase and isocitrate dehydrogenase activities. Therefore the fraction was split into two portions: one was re-fractionated with ammonium sulphate to purify further the citrate synthase (the most active fraction was the 40–55% saturation precipitate), and the other was used for all the isocitrate dehydrogenase assays. Precipitates from crude extracts were separated by centrifugation (30000*g* for 5 min at 2°C), the supernatants decanted and the precipitated proteins redissolved in 200 mM-sodium phosphate buffer, pH 7.3, and assayed for protein content, citrate synthase and isocitrate dehydrogenase activities. A typical fractionation increased the specific activity of citrate synthase from 0.17 to 2.34 μmol/min per mg of protein (14-fold purification), and that of isocitrate dehydrogenase from 0.36 to 5.86 μmol/min per mg of protein (16.2-fold purification). Both partially purified fractions were free of NADH oxidase, NADPH–NAD^+ transhydrogenase and D(−)-β-hydroxybutyrate dehydrogenase activity.

RESULTS

Glucose 6-phosphate dehydrogenase. All the experiments were carried out with 36-fold purified enzyme. The enzyme, in either crude or purified preparations, could be stored at −30°C without loss of activity over a period of 3 days. The enzyme did not obey normal Michaelis–Menten kinetics. A double-reciprocal plot of initial rate of reaction

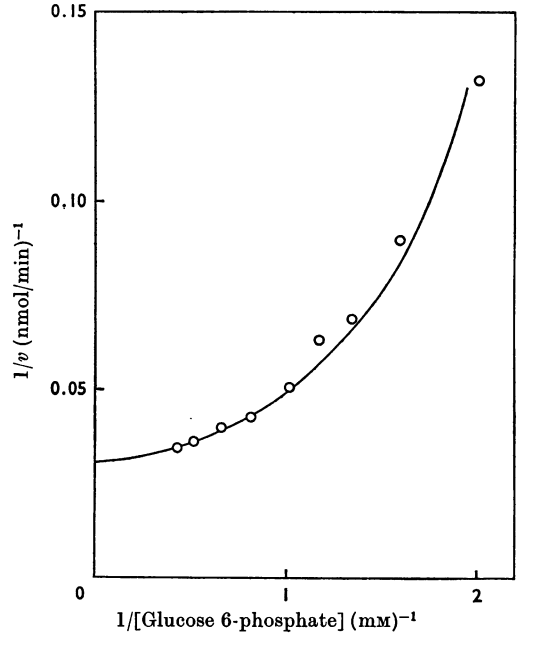

Fig. 1. Lineweaver–Burk plot of the effect of glucose 6-phosphate concentration on glucose 6-phosphate dehydrogenase activity, obtained with 36-fold purified enzyme. Assays were conducted as described in the Experimental section with micro-cuvettes which contained, in a total reaction volume of 0.4 ml: glucose 6-phosphate (0.5–2.5 mM); $NADP^+$, 1 mM; 0.75 μg of enzyme protein.

against substrate concentration with NADP at a saturating concentration (Fig. 1), indicates that glucose 6-phosphate interacts at site(s) other than the catalytic site(s). However, double-reciprocal plots of velocity versus NAD^+ or $NADP^+$ concentration were linear, giving K_m (NAD^+) 8.0×10^{-3} M and K_m ($NADP^+$) 1.92×10^{-4} M (Fig. 2) in the presence of saturating concentrations of glucose 6-phosphate. NADPH inhibits the enzyme non-competitively. In the presence of equimolar quantities (0.25 mM) of $NADP^+$ and NADPH, 47% inhibition of initial rate was observed and 62% inhibition when 0.25 mM-NADH was substituted for NADPH. The K_i for NADPH was 1.92×10^{-4} M and K_i for NADH, 1.98×10^{-4} M.

A typical velocity-versus-substrate-concentration profile in the presence of a saturating concentration of $NADP^+$ is shown in Fig. 3(*a*), and the sigmoidal nature of the curve is apparent. Fig. 3(*a*) also illustrates the effect of ATP and ADP on enzymic activity; an increase of ATP concentration gives an increased inhibition. ADP also inhibits the reaction but not to the same extent as ATP.

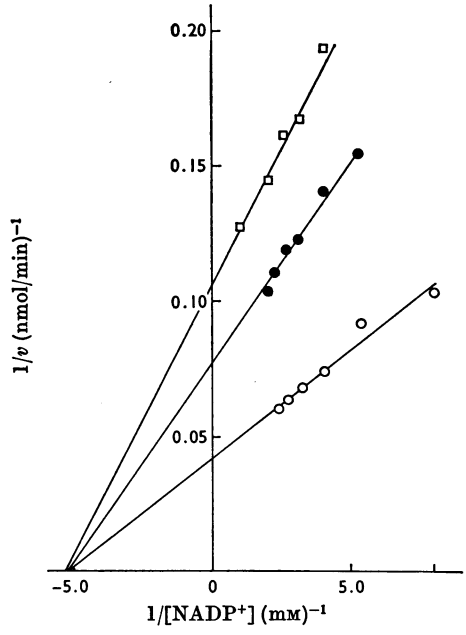

Fig. 2. Lineweaver–Burk plots of the effect of $NADP^+$ concentration on glucose 6-phosphate dehydrogenase activity showing inhibition by NADH and NADPH. Cuvettes contained: 1.5 ml of 200 mM-imidazole–HCl buffer, pH 7.3; 0.2 ml of 100 mM-glucose 6-phosphate; 25–200 μl of 5 mM-$NADP^+$ (to give 0.05–0.4 mM-$NADP^+$); 7.5 μg of enzyme protein; and 0.1 ml of 2.5 mM-NADPH or 2.5 mM-NADH as appropriate; total reaction volume 2.5 ml, temperature 30°C. ○, $NADP^+$ alone; ●, +0.25 mM-NADPH; □, +0.25 mM-NADH.

A Hill plot to determine the number of substrate–enzyme interacting sites is shown in Fig. 3(*b*). The average slope of the lines is +2.58, suggesting that there are at least three glucose 6-phosphate-binding sites on the enzyme. Fig. 3(*c*) shows a Hill plot for ATP inhibition. The average slope of the lines is −1.21, indicating at least two ATP-binding sites on the enzyme. The data used for the construction of Figs. 3(*b*) and 3(*c*) were derived from Fig. 3(*a*) and the value of $V_{max.}$ was taken as 39.4 μmol of $NADP^+$ reduced/min per mg of protein.

6-Phosphogluconate dehydrogenase. The enzyme in crude cell extracts in 200 mM-tris–HCl buffer, pH 7.0, retains full activity over 7 days when stored at −20°C. However, preparations partially purified by $(NH_4)_2SO_4$ fractionation are very unstable, 90% of the activity being lost within 12 h either at 0°C or −20°C. The enzyme was not stabilized by 5 mM-2-mercaptoethanol, 1 mM-$MgCl_2$ or 1 mM-dithiothreitol. At room temperature (20°C) the loss of activity was accelerated ($t_{\frac{1}{2}}$ approx. 30 min). However, by standardizing the assay procedure and adopting constant preincubation times, partial purification could be monitored and the kinetic constants determined.

The enzyme was specific for NADP (K_m 1.48×10^{-4} M) and the K_m for 6-phosphogluconate was 1.5×10^{-3} M and $V_{max.}$ (6-phosphogluconate) was 0.943 μmol/min per mg of protein. The determinations were carried out with a tenfold purified preparation of the enzyme. When assayed in the presence of equimolar concentrations of $NADP^+$ and NADPH (0.25 mM), the initial rate of reaction was 0.56 of that obtained with $NADP^+$ alone. If NADH (0.05 mM) was substituted for NADPH, then in the presence of 0.25 mM-$NADP^+$ the initial rate was 0.48 of that of the uninhibited reaction. On the basis of these and other results (Table 1) we conclude that NADH is a more effective inhibitor of 6-phosphogluconate dehydrogenase than is NADPH. The activity of the enzyme was unaffected by ATP, ADP or AMP (0.5–1.5 mM in each case).

Glyceraldehyde 3-phosphate dehydrogenase. All experiments were carried out with 31-fold purified enzyme. The enzyme was some sixfold more active with $NADP^+$ than with NAD^+, a ratio sustained throughout purification. The K_m for $NADP^+$ was 4.9×10^{-5} M, and K_m for NAD^+, 12.5×10^{-5} M; $V_{max.}$ for $NADP^+$ was 0.600 μmol/min per mg of protein and $V_{max.}$ for NAD^+, 0.105 μmol/min per mg of protein. Activity was inhibited by thiol-blocking reagents such as *p*-chloromercuribenzoate, mersalyl, iodoacetamide and *N*-ethylmaleimide (Table 2). Activity relative to the specific activity of the

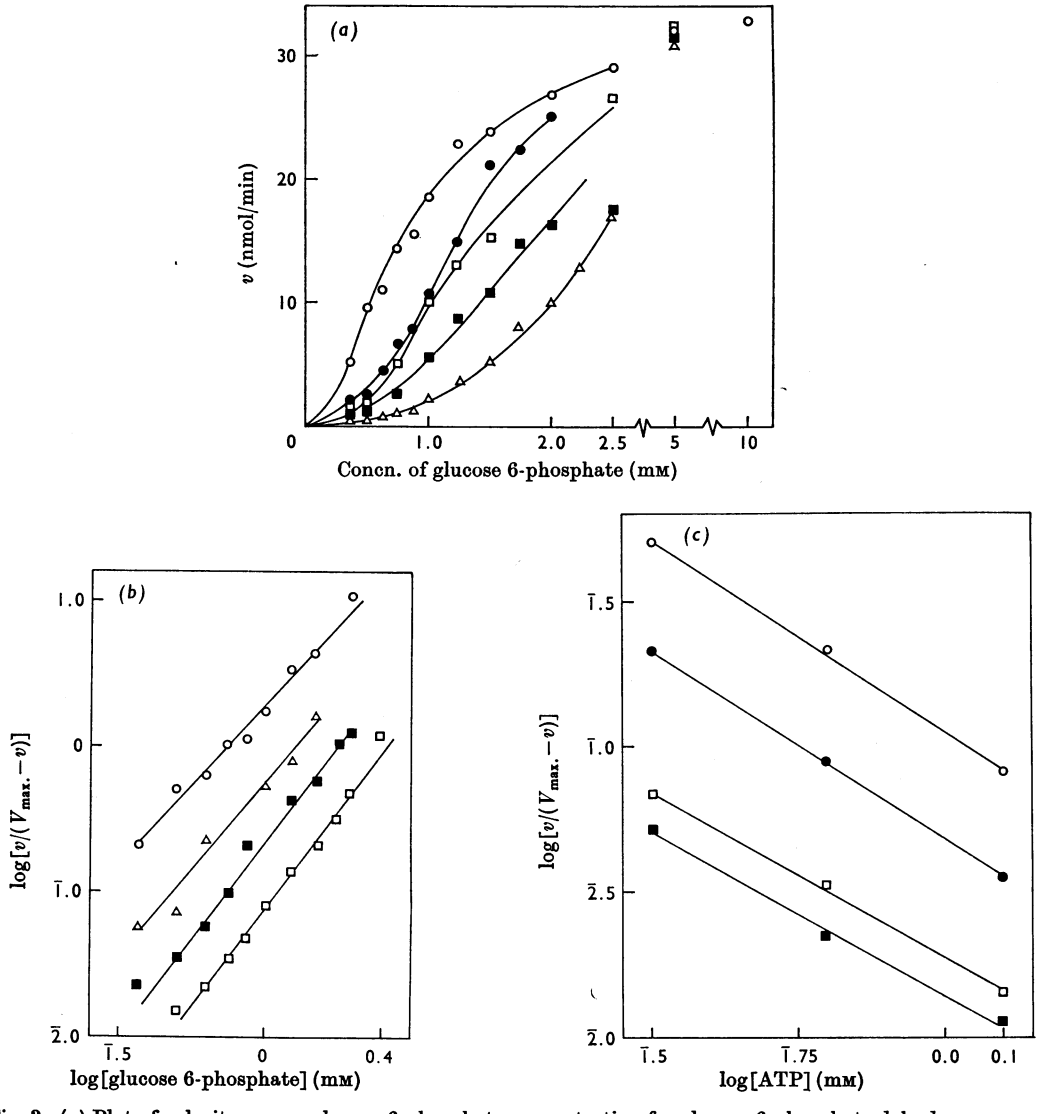

Fig. 3. (*a*) Plot of velocity versus glucose 6-phosphate concentration for glucose 6-phosphate dehydrogenase in the presence of a saturating concentration (1 mM) of $NADP^+$ showing the effects of ATP and ADP. Assays were carried out in micro-cuvettes as described in the Experimental section, with a total reaction volume of 0.4 ml. The range of glucose 6-phosphate concentrations used was 0.375–5.0 mM (and also 10 mM in the case of the uninhibited reaction), 0.75 μg of partially purified enzyme protein and ATP and ADP as indicated. ○, Control; □, 0.3125 mM-ATP; ■, 0.625 mM-ATP; △, 1.25 mM-ATP; ●, 1.25 mM-ADP. (*b*) Binding of glucose 6-phosphate. Results of (*a*) were re-plotted according to the Hill equation. Symbols are as for (*a*). (*c*) Binding of ATP. Results of (*a*) were re-plotted according to the Hill equation. Glucose 6-phosphate concentrations: ■, 0.375 mM; □, 0.50 mM; ●, 0.75 mM; ○, 1 mM.

purified enzyme was maintained, perhaps by an oxygen-scavenging mechanism, in uninhibited systems by thiol compounds such as 2-mercaptoethanol and dithiothreitol. Enzymic activity was not affected by the addition of either adenine nucleotides (AMP, ADP and ATP at 1 mM) or reduced nicotinamide nucleotides (0.25–0.5 mM).

6-Phosphogluconate dehydratase and 3-deoxy-2-oxo-6-phosphogluconate aldolase. The enzymes could be stabilized in crude dialysed extracts at

Table 1. *Modification of 6-phosphogluconate dehydrogenase activity by NADPH and NADH*

The assay systems contained: 200mM-tris–HCl buffer, pH7.6, 150μl; enzyme preparation, 50μl (46.3μg of protein); 20mM-6-phosphogluconate, 50μl; 2mM-$NADP^+$, 50μl; 2mM-NADPH or NADH, to give the final concentrations recorded, and water to 0.4ml. The reaction was at 30°C with final concentrations of 2.5mM-6-phosphogluconate and 0.25mM-$NADP^+$.

Inhibitor	Concentration (mM)	Initial rate (μmol/min per mg of protein)	Inhibition of initial rate (%)
None	—	0.847	0
NADPH	0.075	0.677	20
	0.125	0.542	36
	0.250	0.473	44
	0.500	0.000	100
NADH	0.050	0.407	52
	0.125	0.338	60
	0.250	0.000	100
	0.375	0.000	100
	0.500	0.000	100

Table 2. *Effects of thiol-blocking reagents on glyceraldehyde 3-phosphate dehydrogenase with $NADP^+$ as coenzyme*

The assay systems contained: 100mM-potassium phosphate buffer (without 2-mercaptoethanol), pH7.2, 1.5ml; 0.4M-Na_2HAsO_4, pH7.2, 0.1ml; enzyme preparation [5ml (5.3mg of protein) dialysed for 24h against 5 litres of 5mM-potassium phosphate buffer, pH7.2], 0.1ml; 10mM-glyceraldehyde 3-phosphate, 0.2ml; 3mM-$NADP^+$, 0.1ml; 2.5mM inhibitor to give the recorded final concentrations; water to give a final reaction volume of 2.5ml. Temperature 30°C.

Inhibitor	Concentration (mM)	Initial rate (μmol/min per mg of protein)	Inhibition (%)
None		0.414	0
Iodoacetamide	0.100	0.398	3.6
	0.200	0.378	8.6
	0.300	0.369	13.4
	0.400	0.378	8.6
	0.500	0.330	18.0
p-Chloromercuribenzoate	0.025	0.369	13.4
	0.050	0.245	40.8
	0.100	0.132	68.2
	0.150	0.000	100
N-Ethylmaleimide	0.100	0.398	3.6
	0.200	0.375	9.3
	0.300	0.375	9.3
	0.400	0.242	41.0
Mersalyl	0.025	0.399	3.5
	0.050	0.369	13.4
	0.100	0.283	31.5
	0.125	0.000	100
	0.150	0.000	100

0°C by the addition of 1mM-dithiothreitol or 3mM-2-mercaptoethanol. In the absence of dithiothreitol $t_{\frac{1}{2}}=3.5$ days. The specific activity of a cell extract stored at 0°C for 24h in the absence of dithiothreitol fell from 0.628 to 0.57μmol/min per mg of protein. The addition of 1–2.0mM-dithiothreitol after storage increased the specific activity to 1.165μmol/min per mg of protein. However, loss of activity in partially purified fractions could not be halted by addition of either 1mM-dithiothreitol, 2mM-GSH or 5mM-2-mercaptoethanol. The highest specific activity obtained, based on the

formation of semicarbazides of pyruvate and glyceraldehyde 3-phosphate, was 2.43 μmol of 6-phosphogluconate utilized/min per mg of protein.

Table 3 records the results of an experiment to determine the products of 6-phosphogluconate degradation and variation of initial rate of reaction with enzyme concentration. A sample, taken 2.0h after initiation with 6-phosphogluconate, was analysed for total carbonyl groups by the semicarbazide reagent, and for pyruvate by the method of von Korff (1969). Samples, to determine the initial rate of carbonyl-group formation, were also taken at 0, 1, 2, 3 and 5min. This extract did not catalyse any further metabolism of either glyceraldehyde 3-phosphate or pyruvate. When the direct spectrophotometric method was employed the yield of NAD^+, calculated from ΔE_{340}, and thus the yield of pyruvate, was 94%, 1.0 μmol of 6-phosphogluconate giving 0.47 μmol of pyruvate.

Enzyme activity was unaffected by ADP (0.33–1mM), AMP (0.33–1mM), NADPH (0.053–0.42mM), NADH (0.06–0.2mM), $NADP^+$ (0.5mM), NAD^+ (0.5mM) or acetoacetate (3.33mM). However ATP, citrate and, to a lesser extent, isocitrate and *cis*-aconitate all inhibited activity at physiological concentrations (Table 4).

A control reaction was carried out to determine whether citrate could be converted into isocitrate in crude extracts, in the presence of NADH to ascertain whether the effect observed with citrate

Table 3. *Formation of pyruvate from 6-phosphogluconate by crude dialysed extracts*

Each reaction system contained: 200mM-imidazole–HCl buffer plus 1mM-dithiothreitol, pH7.7, 0.8ml; 50mM-6-phosphogluconate, 0.1ml; bacterial extract, 10–60 μl; water to a final volume of 1.5ml. They were incubated at 30°C and samples (0.2ml) taken at 1 and 2min (for determination of the initial rate) and a sample (0.1ml) at 2h for total yield. Each was deproteinized and assayed for pyruvate and total carbonyl groups as described in the Experimental section. Each assay system contained 5 μmol of 6-phosphogluconate. For 'pyruvate yield as % of substrate' 1mol of pyruvate/mol of 6-phosphogluconate = 100%.

Protein (mg)	Initial rate of carbonyl-group formation (μmol/min)	Total carbonyl compounds (μmol)	Total pyruvate (μmol)	Pyruvate yield as % of products	Pyruvate yield as % of substrate
0.156	0.37	7.10	3.72	52.4	74.4
0.312	0.76	7.50	3.73	49.7	74.6
0.468	1.11	7.76	3.83	49.4	76.6
0.624	1.63	7.16	3.46	48.3	69.2
0.770	1.84	8.26	4.15	50.3	83.0
0.926	2.25	8.50	4.24	50.0	84.8

Table 4. *Effect of ATP, citrate, isocitrate and* cis*-aconitate on the activity of the Entner–Doudoroff pathway enzymes*

Each assay contained 200mM-imidazole–HCl buffer, plus 1mM-dithiothreitol, pH7.7, 2.0ml; lactate dehydrogenase (100 units/mg; 1mg/ml), 0.050ml; 10mM-NADH, 0.030ml; 50mM-6-phosphogluconate, 0.030ml; cell extract (500 μg of protein), 0.020ml; plus water to a final volume of 3.0ml.

Concn. of ATP (mM)	Initial rate* (nmol/min)	Concn. of citrate (mM)	Initial rate* (nmol/min)	Concn. of isocitrate (mM)	Initial rate* (nmol/min)	Concn. of *cis*-aconitate (mM)	Initial rate* (nmol/min)
0	36.20	0	28.60	0	29.5	0	33.3
0.25	26.50	0.050	24.70	0.25	28.9	0.25	32.6
0.50	25.30	0.125	22.20	0.50	19.3	0.50	28.9
0.75	15.65	0.25	16.80	0.75	19.3	0.75	24.1
1.0	12.05	0.50	18.20	1.0	19.3	1.0	22.9
2.0	8.43	0.75	7.90	2.0	9.6	2.0	14.5
3.0	7.82	1.0	7.90	3.0	6.0	3.0	15.7
4.0	6.03	2.0	4.30	4.0	4.8	4.0	12.1
5.0	4.82	3.0	2.4	5.0	4.8	5.0	9.6
		4.0	0.0				
		5.0	0.0				

* Pyruvate formation.

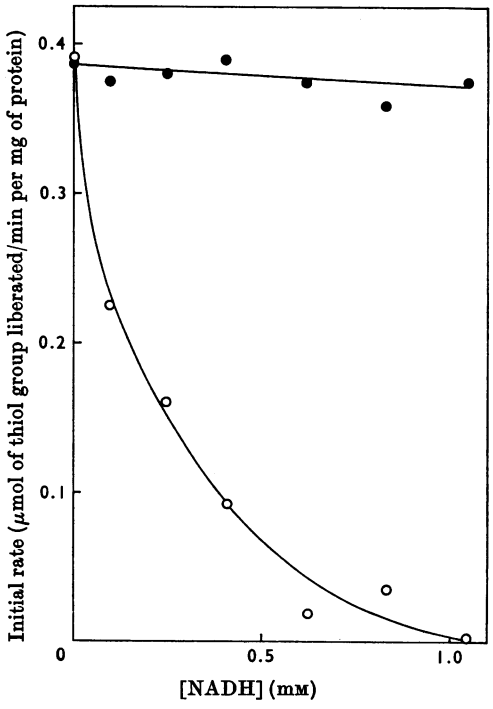

Fig. 4. Inhibition of citrate synthase by NADH and reactivation by AMP. The assays were carried out by the delayed addition technique described in the Experimental section. Cuvettes contained in addition 0–0.2ml of 15.75mM-NADH, to give final concentrations of 0–1.05mM-NADH and, as appropriate, 20μl of 150mM-AMP. ○, NADH inhibition; ●, reactivation by 1mM-AMP.

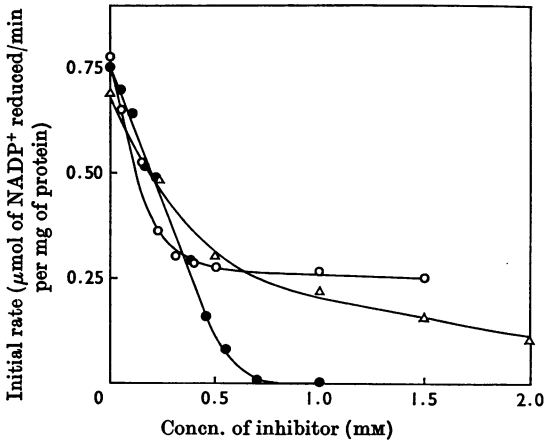

Fig. 5. Inhibition of isocitrate dehydrogenase by ATP, NADH and NADPH. The assays were as described in the Experimental section with the addition of either ATP, NADH or NADPH to give the final concentrations recorded. △, ATP; ●, NADH; ○, NADPH.

might be due to isocitrate. The isocitrate dehydrogenase of this organism was found to be NADP-specific, and addition of citrate to a complete assay mixture did not produce any increase or decrease in E_{340} of either $NADP^+$ or NADH respectively.

The enzymes were not affected by either succinate, fumarate, malate or oxaloacetate (all at 5mM). In an attempt to study the 6-phosphogluconate dehydratase, a crude extract was treated with borohydride by using a method identical with that described by Grazi, Meloche, Martinez, Wood & Horecker (1963). Treatment with $NaBH_4$, in either the presence or the absence of pyruvate, led to a complete loss of enzymic activity, neither extract catalysing the formation of a semicarbazide-positive product from 6-phosphogluconate.

Citrate synthase. All experiments were carried out with 14-fold partially purified enzyme. When the direct spectrophotometric method was used, i.e. with 5,5′-dithiobis-(2-nitrobenzoic acid) present before and after initiation of reaction with oxaloacetate, the K_m for oxaloacetate was 6.25×10^{-3}M and for acetyl-CoA, 1.82×10^{-4}M. No effectors were found and none of the following compounds inhibited or stimulated activity: NADH (0.21–1mM), NAD^+ (0.33–0.66mM), NADPH (0.25–1mM), $NADP^+$ (0.33–0.66mM), ATP (5–10mM), ADP (1–3.33mM), AMP (1–3.33mM), α-oxoglutarate (6.66mM), glutamate (5mM), aspartate (5mM) and acetoacetyl-CoA (0.04–0.2mM). However, when 5,5′-dithiobis-(2-nitrobenzoic acid) was added after the reaction had been initiated with oxaloacetate, the K_m for oxaloacetate was decreased to 1.25×10^{-3}M whereas the K_m for acetyl-CoA (1.35×10^{-4}M) did not change significantly, and the enzyme became sensitive to NADH and to AMP reactivation of the NADH inhibition (Fig. 4). No effect was detected with NADPH, $NADP^+$, NAD^+, ADP, α-oxoglutarate, glutamate, aspartate or acetoacetyl-CoA at the concentrations recorded above.

Isocitrate dehydrogenase. All experiments were carried out on 16.2-fold purified enzyme. The enzyme was found to be NADP-specific and activity was inhibited by ATP, NADH and NADPH (Fig. 5). ADP (3mM), AMP (3mM), NAD^+ (0.1mM), α-oxoglutarate (4mM) and glutamate (10mM) did not affect enzymic activity. K_m for $NADP^+$ was 1.0×10^{-4}M, and for isocitrate, 1.75×10^{-4}M.

DISCUSSION

Our studies of certain enzymes involved in glucose metabolism and the further oxidation of acetyl-CoA, which catalyse formation of NADPH and NADH, in relation to their role in the formation and function of poly-β-hydroxybutyrate in the cell, have revealed that almost all are inhibited by

either or both of these reduced nucleotides at physiological concentrations. A similar regulatory system has been found in *Hydrogenomonas*, another poly - β - hydroxybutyrate - producing organism (Schindler & Schlegel, 1969). The similarities of the glucose 6-phosphate dehydrogenases of these organisms are striking, both in substrate specificity and ATP, NADPH and NADH inhibition; they display almost identical allosteric kinetics and non-competitive inhibition by NADPH and NADH. The 6-phosphogluconate dehydrogenase of *A. beijerinckii*, although strongly inhibited by both NADPH and NADH, is unaffected by ATP, ADP or AMP.

Johnson & Johnson (1961) observed that the glyceraldehyde 3-phosphate dehydrogenase of *Azotobacter agilis* reduced $NADP^+$ more rapidly than NAD^+, but they did not isolate the enzyme or study its kinetics. We have isolated and characterized a similar enzyme from *A. beijerinckii* which reacts with $NADP^+$ at some sixfold the rate with NAD^+ but is unaffected by reduced nicotinamide nucleotides and adenosine nucleotides. The enzymes of the Entner & Doudoroff (1952) pathway have been partially characterized and found to be inhibited by ATP and the three tricarboxylic acids of the tricarboxylic acid cycle. Although we have not yet been able to separate the activities of 6-phosphogluconate dehydratase and 3-deoxy-2-oxo-6-phosphogluconate aldolase, the formation of 3-deoxy - 2 - oxo - 6 - phosphogluconate has been demonstrated by the assay procedure of Kersters & De Ley (1968).

Weitzmann & Jones (1968) found that NADH inhibited and AMP reactivated the citrate synthase of *A. vinelandii* when assayed directly with 5,5′-dithiobis-(2-nitrobenzoic acid). Our findings with *A. beijerinckii* are slightly different in that, although this direct assay does not affect catalytic activity significantly, the regulation of activity by AMP and NADH is abolished in the presence of this reagent, implying that the regulatory site(s) of the *A. beijerinckii* enzyme may possess an essential thiol group. Isocitrate dehydrogenase, the enzyme which Barrera & Jurtshuk (1970) suggest may provide the major portion of the reducing equivalents necessary for nitrogen fixation in *A. vinelandii*, is specific for $NADP^+$ in *A. beijerinckii* and its activity is inhibited by ATP, NADH and NADPH, findings in accordance with those of Barrera & Jurtshuk (1970). Chung (1970) showed that *A. vinelandii* possesses an NAD-linked NADPH transhydrogenase facilitating the conversion of NADPH into NADH, which is then oxidized by a particulate NADH oxidase. A similar system exists in *A. beijerinckii* enabling NADPH to be rapidly oxidized by the NADH oxidase in the presence of catalytic quantities of NAD^+. We suggest that this offers the cell a means of adjusting its contents of NAD(P)H and $NAD(P)^+$ according to metabolic requirements.

In assessing the relevance of these findings to poly-β-hydroxybutyrate biosynthesis several other factors must be considered, namely nitrogen fixation, oxygen limitation and the availability of glucose. The formation of poly-β-hydroxybutyrate requires large quantities of acetyl-CoA and reducing equivalents, either as NADH or NADPH. Our results with batch cultures indicate that oxygen limitation of growth may be the 'trigger' that initiates poly-β-hydroxybutyrate biosynthesis, although nitrogen limitation could not be ruled out under these cultural conditions. With an oxygen limitation one might expect tricarboxylic acid-cycle activity to diminish and glucose oxidation rates to decrease through the inhibitory effects of accumulating NADPH, NADH and citrate. The problem posed is how the organism can continue to fix atmospheric nitrogen and manufacture vast quantities of poly-β-hydroxybutyrate in this situation. We suggest that not only is the biosynthesis of poly-β-hydroxybutyrate a mechanism by which a readily available reserve of carbon, reducing power and energy is accumulated, but also that the actual synthesis serves a regulatory purpose. Thus the extensive utilization of NADPH and NADH which its biosynthesis entails releases the inhibition of glucose oxidation and citrate metabolism by a quasi-fermentation phenomenon. This release would allow nitrogen reduction and fixation of NH_4^+ into carbon skeletons to continue at an increased rate and hence permit the growth rate to increase under conditions that would otherwise impose a severe limitation on it.

The problem of the relative significance of nitrogen limitation and oxygen limitation for poly-β-hydroxybutyrate biosynthesis has been studied in a chemostat. During growth ($D = 0.233 h^{-1}$) in a medium containing 1.5% (w/v) glucose, but limited by atmospheric nitrogen, with oxygen partial pressure controlled at 10% of air saturation, *A. beijerinckii* contains less than 5% (w/v) of poly-β-hydroxybutyrate (P. J. Senior & E. A. Dawes, unpublished work). These findings seem to eliminate nitrogen-limitation as the initiating factor for poly-β-hydroxybutyrate synthesis and support the role of oxygen limitation as the controlling parameter. Under conditions of either oxygen- or nitrogen-limitation cells completely devoid of poly-β-hydroxybutyrate have yet to be observed and it appears there is a continuous basal rate of polymer synthesis independent of these growth conditions.

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