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The Regulation of Poly-β-hydroxybutyrate Metabolism in *Azotobacter beijerinckii*

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1. The enzymes β-ketothiolase, acetoacetyl-CoA reductase, acetoacetate–succinate CoA-transferase ('thiophorase') and D(−)-3-hydroxybutyrate dehydrogenase have been partially purified from crude extracts of glucose-grown nitrogen-fixing batch cultures of *Azotobacter beijerinckii*. The condensation of acetyl-CoA to acetoacetyl-CoA catalysed by β-ketothiolase is inhibited by CoASH, and the reverse reaction is inhibited by acetoacetyl-CoA. Acetoacetyl-CoA reductase has K_m for acetoacetyl-CoA of 1.8 μM and is inhibited by acetoacetyl-CoA above 10 μM. The enzyme utilizes either NADH or NADPH as electron donor. The second enzyme of poly-β-hydroxybutyrate degradation, D(−)-3-hydroxybutyrate dehydrogenase, is NAD^+-specific and is inhibited by NADH, pyruvate and α-oxoglutarate. CoA transferase is inhibited by acetoacetate, the product of hydroxybutyrate oxidation. In continuous cultures poly-β-hydroxybutyrate biosynthesis ceased on relaxation of oxygen-limitation and the rates *in situ* of oxygen consumption and carbon dioxide evolution of such cultures increased without a concomitant increase in glucose uptake. 2. On the basis of these and other findings a cyclic mechanism for the biosynthesis and degradation of poly-β-hydroxybutyrate is proposed, together with a regulatory scheme suggesting that poly-β-hydroxybutyrate metabolism is controlled by the redox state of the cell and the availability of CoASH, pyruvate and α-oxoglutarate. β-Ketothiolase plays a key role in the regulatory process. Similarities to the pathways of poly-β-hydroxybutyrate biosynthesis and degradation in *Hydrogenomonas* are discussed.

Poly-β-hydroxybutyrate is a specialized reserve of carbon and energy that accumulates in a variety of micro-organisms under conditions of nutrient imbalance. Very large amounts of the polymer are accumulated by *Azotobacter* species (Stockdale *et al.*, 1968) and by *Hydrogenomonas eutropha* (Schlegel *et al.*, 1961). Previous investigations of the pathway of poly-β-hydroxybutyrate biosynthesis in *Azotobacter beijerinckii* (Ritchie & Dawes, 1969; Ritchie *et al.*, 1971) and the demonstration that oxygen-limitation, rather than the more usually encountered nitrogen-limitation, initiates its accumulation in the cell, led to a study of the regulatory mechanisms that operate.

Senior & Dawes (1971) investigated the control of glucose metabolism in *A. beijerinckii* and found that both the Entner & Doudoroff (1952) and pentose phosphate-cycle pathways were subject to feedback inhibition by products of glucose oxidation that would accumulate under an oxygen deficiency. Glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, citrate synthase and isocitrate dehydrogenase were inhibited by either or both reduced nicotinamide nucleotides and ATP, and the combined action of 6-phosphogluconate dehydratase and 3-deoxy-2-oxo-6-phosphogluconate aldolase was inhibited by ATP, citrate, isocitrate or *cis*-aconitate. Chemostat studies by Senior *et al.* (1972) demonstrated that only oxygen-limitation of growth initiated accumulation of poly-β-hydroxybutyrate; nitrogen- and carbon-limitation were without effect. We have proposed that the synthesis of this reserve material serves as an electron and carbon 'sink' when conditions prevail that would otherwise restrict nitrogen fixation and growth (Senior & Dawes, 1971).

We have now investigated certain aspects of the degradation of poly-β-hydroxybutyrate and regulatory mechanisms which operate in the overall metabolism of the polymer in *A. beijerinckii* and have shown key roles for β-ketothiolase and D(−)-β-hydroxybutyrate dehydrogenase. Certain similarities of regulation are apparent with *Hydrogenomonas eutropha*, which has been studied by Oeding & Schlegel (1973).

Experimental

Cultures and cell extracts

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

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chemostat cultures a capsuleless mutant of *A. beijerinckii*, first isolated by Senior *et al.* (1972), was used.

Maintenance and growth of the organism for batch cultures. The basal medium (nitrogen-free) was prepared from two solutions of the following compositions. Solution A (g/l): glucose, 20.0; $MgSO_4,7H_2O$, 0.4; $CaCl_2$, 0.11; $FeSO_4,7H_2O$, 0.012; $Na_2MoO_4,2H_2O$, 0.01. Solution B (g/l): K_2HPO_4, 2.0; NaCl, 0.4. The two solutions were autoclaved separately [103 kN/m² (15 lb/in²) for 20 min] 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 10 ml 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.).

Growth of the organism in a chemostat. Details of the chemostat used, the medium for continuous culture and the growth conditions are given by Senior *et al.* (1972).

Harvesting of bacteria and preparation of crude cell extracts. The bacteria were harvested after 18 h, corresponding to the end of exponential growth, by centrifuging at 5000*g* for 30 min. The cells were washed three times with water and resuspended in approx. 5 vol. of the buffer appropriate to the particular enzyme being studied. These buffers were as follows: for β-ketothiolase, 100 mM-Tris–HCl, pH 7.3, plus 1.0 mM-dithiothreitol, with subsequent cell disintegration by ultrasonic treatment at 0°C [90 s at 8A with a Soniprobe type 1130A (Dawe Instruments Ltd., London W.3, U.K.)]; for D(−)-3-hydroxybutyrate dehydrogenase, 200 mM-potassium phosphate, pH 7.0, followed by ultrasonic treatment; for CoA transferase, 100 mM-Tris–HCl, pH 7.9, and disruption by passage through a French pressure cell (Milner *et al.*, 1950) pre-cooled to 2°C. All disintegrated cell suspensions were centrifuged at 35000*g* for 20 min at 0°C and the supernatants decanted.

Analyses

Determination of protein. Protein was determined by the method of Lowry *et al.* (1951). A calibration curve was prepared with crystallized bovine serum albumin. A sample of albumin was found on desiccation to contain 8.0% water and an appropriate correction was made.

Preparation and determination of acetyl-CoA. Acetyl-CoA was prepared from acetic anhydride and CoASH (Simon & Shemin, 1953).

Determination of CoA. An adaptation of the method of Ellman (1959) was used. The reaction mixture contained 2.0 ml of 67 mM-sodium–potassium phosphate buffer, pH 7.0, 0.2 ml of 10 mM-5,5′-dithiobis-(2-nitrobenzoic acid) in 67 mM-sodium–potassium phosphate buffer, pH 7.0, 25.0–200 nequiv. of thiol compound and water to 3.0 ml. The mixture was incubated at 30°C and its E_{412} read against a blank reaction mixture (minus CoA) until no further increase could be observed. $\lambda_{max.}$ of thionitrobenzoate was 412 nm and ϵ_{412} was assumed to be 13600 litre·mol^{-1}·cm^{-1}.

Determination of acetoacetate. Acetoacetate was determined by the spectrophotometric method of Walker (1954).

Determination of acetoacetyl-CoA. (*a*) Enzymic. Acetoacetyl-CoA was determined enzymically at 25°C with L(+)-3-hydroxybutyryl-CoA dehydrogenase. A cuvette (1 cm light-path) contained: 200 mM-potassium phosphate buffer, pH 7.5, 2.0 ml; 10 mM-dithiothreitol, 0.1 ml; 0.5 mM-NADPH, 10 μl; 50 μg of L(+)-3-hydroxyacyl-CoA dehydrogenase (approx. 4 units); 10–50 μl of acetoacetyl-CoA solution in a total volume of 2.5 ml. The reaction was initiated by addition of the enzyme and monitored spectrophotometrically at 340 nm until equilibrium was reached.

(*b*) Chemical. The u.v. spectrum of acetoacetyl-CoA in 0.1 M-Tris–HCl buffer, pH 8.8, shows a strong absorption peak at 260 nm, the contribution of the adenine moiety. In 0.1 M-Tris–HCl buffer, pH 8.8 plus 20 mM-$MgCl_2$, the spectrum of acetoacetyl-CoA is modified considerably and a new peak appears at 303 nm. This peak is produced by the absorption of the Mg^{2+}–enol form of acetoacetyl-CoA. The molar extinction coefficient at 303 nm was taken as 2.2×10^4 litre·mol^{-1}·cm^{-1} (Beinert, 1953).

Determination of D(−)-3-*hydroxybutyrate.* D(−)-3-Hydroxybutyrate was determined by the enzymic method of Williamson *et al.* (1962) with NAD^+ and commercial D(−)-3-hydroxybutyrate dehydrogenase.

Preparation of acetoacetyl-CoA. CoASH (10–25 μmol) was dissolved in 2 ml of 0.2 M-potassium bicarbonate buffer, pH 8.0, at 0°C. A constant stream of nitrogen was passed through the solution. A ten-fold molar excess of diketen was added in portions (1 μl) from a 10 μl Hamilton syringe over a period of 1 h at 0°C. The reaction mixture was then adjusted to pH 3.0 with 1 M-HCl and the solution extracted three times with portions (2 ml) of diethyl ether. Preparations could be stored at −20°C without detectable degradation for a period of 2 weeks.

Other analyses. Glucose in chemostat culture supernatants, dry weights of chemostat cultures, poly-β-hydroxybutyrate content of cells, culture redox potential (E_h) and respiration rates *in situ* were determined as described by Senior *et al.* (1972).

Enzyme assays

β-Ketothiolase (*EC* 2.3.1.9). (*a*) Condensation reaction. A cuvette (1cm light-path) contained: 100mM-Tris–HCl buffer, pH7.3, plus 1mM-dithiothreitol, 1.5ml; partially purified enzyme preparation, 0.1ml (0.14mg of protein/ml, specific activity 14.9μmol/min per mg of protein); commercial L(+)-3-hydroxyacyl-CoA dehydrogenase, 1μl (10mg of protein/ml, 90 units/mg; 1 unit is 1μmol of product formed/min at 25°C); 11.58mM-NADH, 0.1ml; 14.34mM-acetyl-CoA, 0.05ml; water to a total volume of 3.0ml. The reactants, except for acetyl-CoA, were incubated at 30°C for 2min before addition of acetyl-CoA. The reaction was monitored by the change in E_{340}.

(*b*) Thiolysis of acetoacetyl-CoA. This assay depends upon the decrease of E_{303} of the Mg^{2+}–enol complex of acetoacetyl-CoA in Tris–HCl buffer, pH7.88, plus 20mM-$MgCl_2$, as it is converted into acetyl-CoA. Beinert (1953) quotes an ϵ_{303} at pH7.88 in 20mM-$MgCl_2$ of 1.268×10^4 litre·mol^{-1}·cm^{-1}. However, by a combination of enzymic and spectrophotometric assays, the ϵ_{303} for our chemically prepared acetoacetyl-CoA was found to be 1.726×10^4 litre·mol^{-1}·cm^{-1}.

A cuvette contained: 10mM-Tris–HCl buffer, pH7.88, plus 40mM-$MgCl_2$ and 1mM-dithiothreitol, 1.5ml; 3.44mM-CoASH in 100mM-Tris–HCl buffer, pH7.88, 0.05ml; 6–8mM-acetoacetyl-CoA (variation from batch to batch), 10μl; partially purified enzyme preparation, 10μl (0.14mg of protein/ml, specific activity 14.9μmol/min per mg of protein); water to a total reaction volume of 3.0ml. The reactants minus CoASH were incubated at 30°C for 2min before addition of CoASH. The reaction progress was monitored by the change in E_{303}.

Acetoacetate–succinate CoA-transferase (*EC* 2.8.3.5). An adaptation of the continuous spectrophotometric assay of Stern *et al.* (1956) was used. The enzyme catalyses the reaction:

$$\text{Acetoacetyl-CoA} + \text{succinate} \rightleftharpoons \text{acetoacetate} + \text{succinyl-CoA}$$

The assay allows the estimation of initial rates in either direction, but there are limitations. The assay depends upon the formation or degradation of the Mg^{2+}–enol complex of acetoacetyl-CoA in the range pH7.5–8.8. For each group of experiments the molar extinction coefficient for acetoacetyl-CoA was determined by a combination of enzymic and chemical methods, since the molar extinction coefficient is pH-dependent. ϵ_{303} max. occurs at pH8.8 in 100mM-Tris–HCl buffer plus 20mM-$MgCl_2$. ϵ_{303} decreases with decreasing pH but enzymic activity at pH7.9 was twofold that at pH8.8.

A cuvette (1cm light-path) contained: 100mM-Tris–HCl buffer, pH7.9, 2.5ml; 0.6M-$MgCl_2$, 0.1ml;

Table 1. *Partial purification of* D(−)-*3-hydroxybutyrate dehydrogenase by ultracentrifugation and ammonium sulphate fractionation*

Dialysed extract was centrifuged at 0°C for 4h at 300000g.

Sample	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Activity (units/ml)	Activity Total units	Specific activity (μmol/min per mg of protein)	Purification	NADH oxidase activity (%)
Cell extract	20.0	23.5	470.0	0.650	13.0	27.7	—	100
Dialysed centrifuged crude extract	15.0	12.0	180.0	0.506	7.6	42.2	1.53	4.5
$(NH_4)_2SO_4$ fractionation 0–40%-saturation ppt.	2.7	8.0	21.6	1.325	3.06	166.0	6.0	0.5
40–45%-saturation ppt.	2.5	7.8	19.4	0.725	1.81	93.5	3.38	0
Supernatant	15.0	9.16	137.5	0.140	2.10	15.3	0.55	0

150mM-disodium succinate, 0.1ml; enzyme preparation, 0.1ml of crude extract (5.13mg of protein/ml); 6.0–8.0mM-acetoacetyl-CoA (variations according to batch), 20μl; water to a total reaction volume of 3.0ml. The reactants minus succinate were incubated at 30°C for 2min to obtain the non-enzymic rate of acetoacetyl-CoA degradation, before addition of succinate and measurement of the subsequent decrease inE_{303}. The initial rate of reaction was unchanged by the initiation of the reaction with acetoacetyl-CoA rather than succinate.

Acetoacetyl-CoA reductase (*EC* 1.1.1.36). (*a*) Spectrophotometric assay. For the reduction reaction a cuvette (1cm light-path) contained: 100mM-potassium phosphate buffer, pH5.5, 1.5ml; 0.25mM-$MgCl_2,6H_2O$, 0.12ml; 12.5mM-dithiothreitol, 0.1ml; 6.0mM-NADPH, 0.1ml; 7–10mM-acetoacetyl-CoA (variations according to batch), 10μl; 0.1–0.4ml of cell extract (5mg of protein/ml); water to a total reaction volume of 2.5ml. A reference cuvette contained all reaction components except coenzyme and substrate. NADPH was destroyed at pH5.5 at a rate (ΔE_{340}/min) varying between 0.005 and 0.01, and assays had to be corrected for this destruction. Reactants, minus substrates, were preincubated at 30°C for 3min before addition of NADPH. The acid destruction of NADPH was recorded for 2min before addition of acetoacetyl-CoA and measurement of the initial rate of NADPH oxidation.

(*b*) Spectrofluorimetric assay. Because of the low K_m of acetoacetyl-CoA reductase for acetoacetyl-CoA, this assay involved the addition of very small quantities of acetoacetyl-CoA to the reaction mixture with consequent minute changes in E_{340}. The excitation wavelength was 340nm and the fluorescence recorded at 420nm with a Farrand recording spectrofluorimeter (Farrand Optical Co. Inc., Mount Vernon, N.Y., U.S.A.).

A cuvette contained: 100mM-potassium phosphate buffer, pH7.5, 1.0ml; 12.5mM-dithiothreitol, 0.1ml; 6.0mM-NADPH, 0.1ml; 10μl of acetoacetyl-CoA (56.2nmol); enzyme preparation (0.152μg of protein/ml), 20μl; water to a total reaction volume of 2.5ml. The reactants, minus acetoacetyl-CoA, were preincubated at 30°C for 3min before addition of substrate. Initial rates were recorded as the decrease in fluorescence at 420nm/min (arbitrary units were used for fluorescence).

D(−)-3-*Hydroxybutyrate dehydrogenase* (*EC* 1.1.1.30). Crude extracts contained NADH oxidase; therefore assays were done to determine the inhibitory effect of KCN on NADH oxidase. Although activity remained in the presence of 2.0mM-KCN, it was diminished to 13.5% of the original value. This

Table 2. *Partial purification of CoA transferase by ammonium sulphate fractionation and DEAE-cellulose column chromatography*

Sample	Protein (mg/ml)	Total protein (mg)	Activity (units/ml)	Activity Total units	Specific activity (μmol/min per mg of protein)	Purification
Crude extract	5.13	385.0	0.704	52.8	0.14	—
45–52%-satd.-$(NH_4)_2SO_4$ ppt.	7.20	108.0	2.100	31.5	0.29	2.1
Most active fraction from DEAE-cellulose column	0.175		0.622	4.35	3.56	26.0

Table 3. *Partial purification of β-ketothiolase by ammonium sulphate treatment and DEAE-cellulose column chromatography*

Sample	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Activity (units/ml)	Activity Total units	Specific activity (μmol/min per mg of protein)	Purification
Cell-free extract	20.0	20.04	400.8	9.385	187.7	0.47	—
Supernatant from 40%-satd.-$(NH_4)_2SO_4$ fractionation	20.0	13.00	260.0	9.775	195.5	0.75	1.6
Most active fraction from DEAE-cellulose column	8.6	0.14	1.204	2.086	17.9	14.90	31.8

allowed an approximate estimation of crude extract activity to be made.

A cuvette (1cm light-path) contained: 200mM-potassium phosphate buffer, pH7.0, 2.0ml; cell-free extract (23.5mg/ml of protein), 50μl; 30mM-KCN, 0.2ml; 30mM-NAD, 20μl; 300mM-DL-3-hydroxybutyrate, 0.1ml (final concentration of D-isomer, 5mM); water to a total reaction volume of 3.0ml. The reactants, minus DL-3-hydroxybutyrate, were preincubated for 3min at 30°C, before addition of substrate. The initial rate of NAD^+ reduction (ΔE_{340}/min) remained linear for up to 5min at high substrate concentrations. With partially purified enzyme preparations NADH oxidase activity was less than 0.1% of the original so that KCN could be omitted from the reaction mixture and inhibition studies with NADH and NADPH carried out.

Enzyme purification

Ammonium sulphate fractionation. All ammonium sulphate fractionations were done at 0°C by adding the solid salt to the solution in accordance with the information given by Dawson *et al.* (1969). After the addition of salt a period of 30min was allowed for the completion of precipitation before centrifugation was commenced. The percentage saturation values recorded are relative to full saturation at 0°C.

Preparation of DEAE-cellulose. DEAE-cellulose (DE-32 microgranular; Whatman Biochemicals Ltd. Maidstone, Kent, U.K.) was processed as recommended by the manufacturers, and degassed under partial vacuum, before equilibration. All columns were run at 4°C and fractions collected by an Ultrorac automatic fraction collector (LKB, Stockholm, Sweden).

D(−)-3-*Hydroxybutyrate dehydrogenase.* When a crude extract was subjected to ammonium sulphate fractionation 86% of the total enzyme units were precipitated by 40% saturated salt. Since this fraction also contained most of the NADH oxidase activity, before ammonium sulphate fractionation a crude extract was centrifuged at 300000*g* for 4h at 0°C on a MSE Superspeed 65 ultracentrifuge; the supernatant was then dialysed against 5 litres of 5mM-potassium phosphate buffer, pH7.0, at 4°C for 18h. Precipitates from ammonium sulphate fractionation were centrifuged at 38000*g* for 10min at 0°C, and re-suspended in ice-cold 200mM-potassium phosphate buffer, pH7.0. The results of both purification procedures are shown in Table 1.

Acetoacetate–succinate CoA-transferase. A crude extract in 100mM-Tris–HCl buffer, pH7.9, was subjected to ammonium sulphate fractionation. Precipitated proteins were sedimented by centrifuging at 38000*g* for 20min at 0°C. Precipitates were resuspended in 100mM-Tris–HCl buffer, pH7.9. Table 2 shows the result of this fractionation. The most active fraction was dialysed against 5 litres of distilled water for 12h before DEAE-cellulose column chromatography. The column (20cm×2.5cm) was equilibrated with 5mM-Tris–HCl buffer, pH7.9, at 0°C before loading the dialysed protein solution on the column. Proteins were eluted with a continuous gradient of NaCl (0–160mM) in 800ml of 5mM-Tris–HCl buffer, pH7.9.

Fractions (5ml) were collected and assayed for protein and enzymic activity. The results of chromatography are also shown in Table 2. A 26.0-fold overall increase in specific activity was achieved

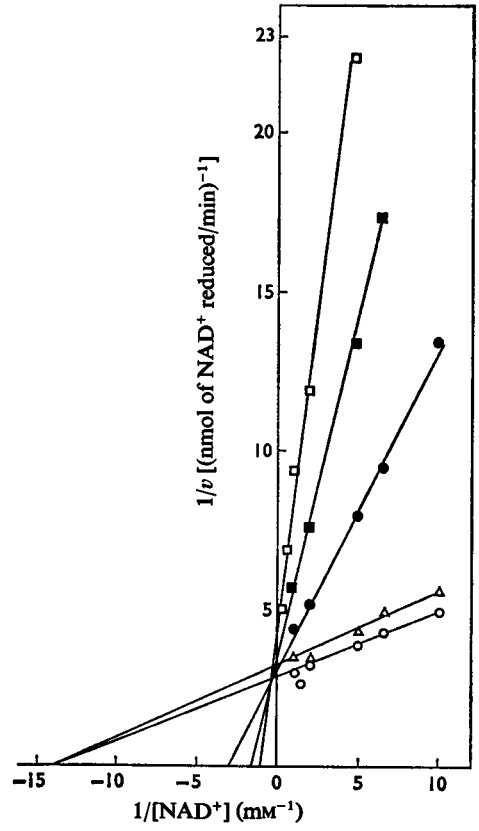


Fig. 1. *Lineweaver–Burk plot of the effects of NAD^+ and NAD(P)H concentrations on* D(−)-3-*hydroxybutyrate dehydrogenase*

Assays were conducted as described in the Experimental section with cuvettes that contained, in a total reaction volume of 3.0ml: D(−)-3-hydroxybutyrate, 5mM; 0.02ml of purified enzyme solution (0.16mg of protein); 30mM-NAD^+, 0.010–0.10ml (to give 0.10–1.0mM-NAD^+). ○, No additions; △, 0.122mM-NADPH; ●, 0.064mM-NADH; ■, 0.128mM-NADH; □, 0.255mM-NADH.

and the partially purified preparation was free of NADPH oxidase, NADPH–NAD^+ transhydrogenase, hydroxybutyrate dehydrogenase and acetoacetyl-CoA reductase activities.

β-Ketothiolase. A cell extract was made 40% saturated with ammonium sulphate and the precipitated proteins were removed by centrifuging at 38000*g* for 40min at 0°C. The supernatant was dialysed for 18h against two changes of 5 litre portions of 10mM-Tris–HCl buffer, pH7.3, plus 1mM-dithiothreitol. The protein solution was then loaded on a DEAE-cellulose column (30.0cm×2.5cm) and proteins were eluted at 4°C by running a linear gradient of KCl (0–300mM) in 2×300ml of 10mM-Tris–HCl buffer, pH7.3, plus 1mM-dithiothreitol. Eluted fractions were assayed for protein and enzymic activity. Details of the purification procedures are given in Table 3.

The most active fraction from DEAE-cellulose column chromatography was free of D(−)-3-hydroxybutyrate dehydrogenase, NADH and NADPH oxidases, NADPH–NAD^+ transhydrogenase, acetoacetyl-CoA reductase and acetyl-CoA hydrolase activities.

Acetoacetyl-CoA reductase. The enzyme was partially purified from crude extracts (specific activity 2.97μmol/min per mg of protein) by ammonium sulphate fractionation and DEAE-cellulose chromatography as described by Ritchie *et al.* (1971). The maximum specific activity attained was 67.2μmol/min per mg of protein, representing a 22.6-fold purification.

Sources of materials

Chemicals. DL-3-Hydroxybutyrate, NADPH, NADH, NAD^+, $NADP^+$, bovine serum albumin fraction V, 2-oxoglutarate, ATP, 5,5′-dithiobis-(2-nitrobenzoic acid) and diketen were obtained from Sigma (London) Chemical Co. Ltd. (London S.W.6, U.K.). Dithiothreitol, ammonium sulphate ('low in heavy metals'), acetic anhydride, oxaloacetate, succinate and acetoacetate were from BDH Chemicals Ltd. (Poole, Dorset, U.K.). Yeast CoA (88% pure) was from Boehringer Corp. (London) Ltd. (London W.5, U.K.) and DEAE-cellulose DE-32 microgranular grade from Whatman Biochemicals Ltd. (Maidstone, Kent, U.K.). All other chemicals were of analytical grade.

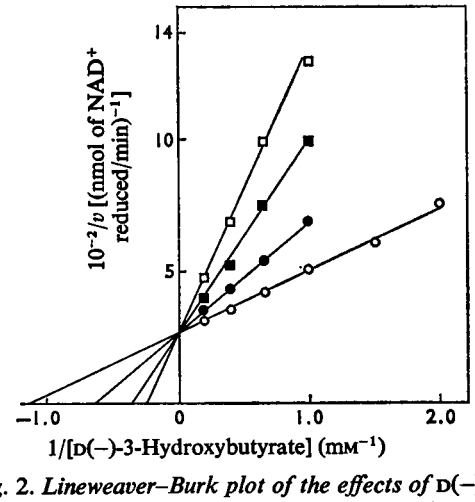


Fig. 2. *Lineweaver–Burk plot of the effects of* D(−)-3-*hydroxybutyrate and pyruvate concentrations on* D(−)-3-*hydroxybutyrate dehydrogenase*

Assays were conducted as described in the Experimental section with cuvettes that contained, in a total reaction volume of 3.0ml: 30mM-NAD^+, 0.1ml; 0.02ml of purified enzyme preparation (0.16mg of protein); 150mM-sodium D(−)-3-hydroxybutyrate, 0.01–0.10ml (to give 0.5–5.0mM-D(−)-3-hydroxybutyrate). Additions of sodium pyruvate were made to reaction mixtures (mM): 2.0 (□); 1.0 (■); 0.4 (●); none (○).

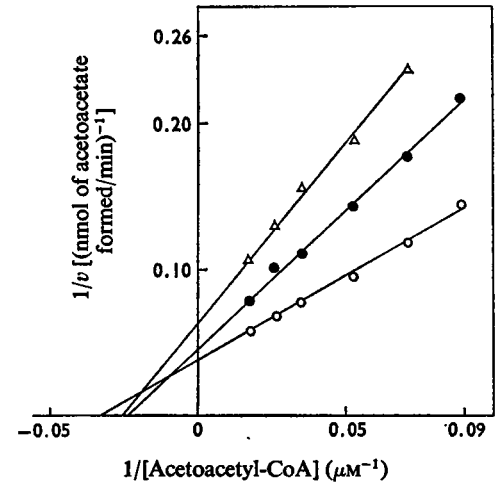


Fig. 3. *Lineweaver–Burk plot of the effects of acetoacetyl-CoA and acetoacetate concentrations on acetoacetate–succinate CoA-transferase ('thiophorase')*

Assays were conducted as described in the Experimental section with cuvettes that contained, in a total reaction volume of 2.5ml: 0.10ml of purified enzyme preparation (17.5μg of protein); 150mM-sodium succinate, 0.10ml; 600mM-$MgCl_2$, 0.05ml; 5.65mM-acetoacetyl-CoA, 6.0–30.0μl (to give 11.3–56.5 μM-acetoacetyl-CoA). Additions of sodium acetoacetate were made to reaction mixtures: 2.0mM (△); 1.0mM (●); none (○).

Enzymes. L(+)-3-Hydroxybutyryl-CoA dehydrogenase and D(−)-3-hydroxybutyrate dehydrogenase were from C. F. Boehringer und Soehne, G.m.b.H.

Results

D(−)-3-*Hydroxybutyrate dehydrogenase*

Activity was detected in all crude extracts of cells tested from different phases of growth in batch culture. The enzyme was very stable in both crude and partially purified extracts, being resistant to repeated freezing to −40°C and thawing. No loss of activity was detected over a period of 7 days at −40°C. Activity was unaffected by the presence of 1.0mM-dithiothreitol, 5.0mM-2-mercaptoethanol, 0.1mM-*p*-chloromercuribenzoate or 0.1mM-iodoacetamide. The pH optimum for the oxidation reaction was 8.4–8.5 when assayed in 200mM-Tris–HCl buffer.

The enzyme displayed Michaelis–Menten kinetics. The apparent K_m for NAD^+ was 70μM (Fig. 1). $NADP^+$ was not utilized as an electron acceptor in this reaction. Also shown in Fig. 1 is the competitive inhibition exerted by NADH (all experimental conditions are given in the legends to the figures). NADPH did not inhibit the reaction significantly. The apparent K_m for D(−)-3-hydroxybutyrate was 877μM (Fig. 2). Also shown in Fig. 2 is the competitive inhibition, with respect to D(−)-3-hydroxybutyrate, exerted by pyruvate (lactate dehydrogenase activity could not be detected in extracts of *A. beijerinckii*). L-Malate (0.5–5.0mM) and oxaloacetate (1.0–3.0mM) had no effect on activity. α-Oxoglutarate inhibited activity by competing with D(−)-3-hydroxybutyrate, giving kinetic patterns almost identical with those obtained for pyruvate inhibition.

Acetoacetate–succinate CoA-transferase

This enzyme had not previously been recorded in *A. beijerinckii*. All experiments were carried out with the 26-fold-purified enzyme preparation, which, when stored at 0°C, decreased in activity by 37% in 120h. Activity was unaffected by the presence of either 1.0mM-2-mercaptoethanol or 1.0mM-dithiothreitol. The enzyme displayed Michaelis–Menten kinetics and the apparent K_m for acetoacetyl-CoA was approx. 30μM (Fig. 3); the apparent K_m for succinate was 4.0mM. When the enzyme was assayed in the direction of acetoacetyl-CoA formation, maximum initial rates were 1% of those recorded for the reverse reaction. The enzyme was inhibited by acetoacetate (Fig. 3).

Activity was unaffected by NADH, NAD^+, NADPH and $NADP^+$ at 0.5mM, or by ATP, ADP

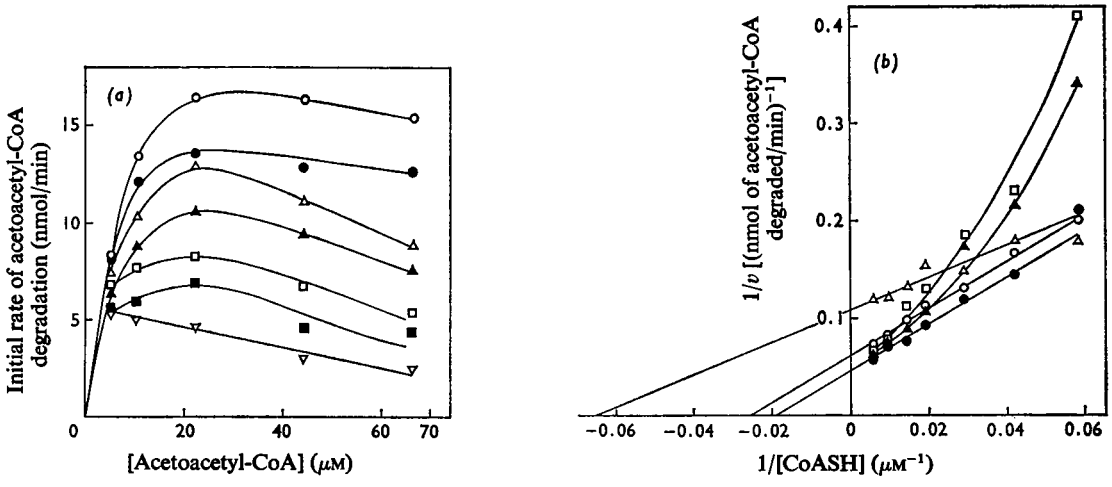


Fig. 4. *Effect of acetoacetyl-CoA and CoA concentrations on the thiolysis reaction of β-ketothiolase*

(*a*) Velocity versus acetoacetyl-CoA concentration in the presence of various concentrations of CoASH. Assays were conducted as described in the Experimental section with cuvettes that contained, in a total reaction volume of 3.0ml: 10μl of purified enzyme preparation (1.4μg of protein); 5.53–66.3μM-acetoacetyl-CoA. Concentrations of CoASH were (μM): 17.2 (▽); 24.08 (■); 34.4 (□); 51.6 (▲); 68.8 (△); 103.2 (●); 172 (○). (*b*) Lineweaver–Burk plot of data from (*a*) to show the effect of CoASH concentration in the presence of various concentrations of acetoacetyl-CoA. Cuvettes contained, in a total reaction volume of 3.0ml: 10μl of purified enzyme preparation (1.4μg of protein); 17.2–172μM-CoASH. Concentrations of acetoacetyl-CoA were (μM): 5.53 (△); 11.05 (○); 22.1 (●); 44.2 (▲); 66.3 (□).

and AMP (1.0–3.0mM) or by DL-3-hydroxybutyrate, D-lactate and pyruvate (3.0mM).

β-Ketothiolase

The maximum rate of reaction by the standard assay procedure in crude extracts was 0.468μmol of acetoacetyl-CoA cleaved/min per mg of protein. Activity in crude extracts diminished rapidly and shaking caused a more rapid decrease. Oxygen bubbled through a crude extract without added thiol destroyed activity completely in 1h. The addition of 1.0mM-dithiothreitol or 5.0mM-2-mercaptoethanol to both crude or partially purified extracts completely stabilized activity. The pH optimum for acetoacetyl-CoA formation (from acetyl-CoA) was 7.3, in either phosphate or Tris buffer. The restrictions of the direct spectrophotometric assay, with respect to pH, did not allow the pH optimum for the reverse reaction to be established.

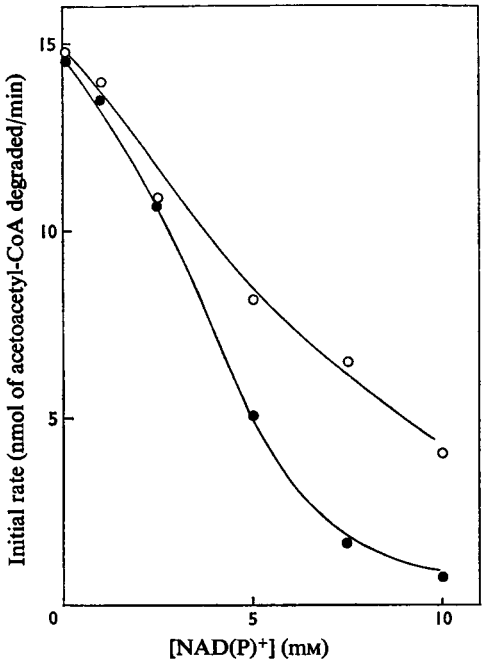


Fig. 5. *Plot of velocity versus NAD(P)+ concentration for β-ketothiolase assayed in the direction of thiolysis*

Assays were conducted as described in the Experimental section with cuvettes that contained, in a total reaction volume of 3.0ml: 10μl of purified enzyme preparation (1.4μg of protein); 172μM-CoASH; 22.1μM-acetoacetyl-CoA. Additions to the reaction mixture were such that concentrations of either nucleotide were 1.0–10.0mM. ○, NAD^+; ●, $NADP^+$.

The thiolysis reaction was inhibited by high concentrations of acetoacetyl-CoA but this inhibition was overcome by using high concentrations of CoASH. As the concentration of CoASH was decreased (Fig. 4*a*) the degree of inhibition exerted by low concentrations of acetoacetyl-CoA became more pronounced. Re-plotting the data in Fig. 4(*a*) as a Lineweaver–Burk double-reciprocal plot (Fig. 4*b*) shows that as the concentration of acetoacetyl-CoA is increased from 5.5 to 22.1μM the apparent K_m for CoASH is increased from ≃15μM at 5.53μM-acetoacetyl-CoA, through 40μM at 11.05μM-acetoacetyl-CoA, to reach a maximum of ≃53μM at 22.1μM-acetoacetyl-CoA. Above 22μM-acetoacetyl-CoA double-reciprocal plots of velocity versus CoA concentration became non-linear. The apparent $V_{max.}$ was also decreased by lowering the acetoacetyl-CoA concentration. Thiolysis was inhibited by both

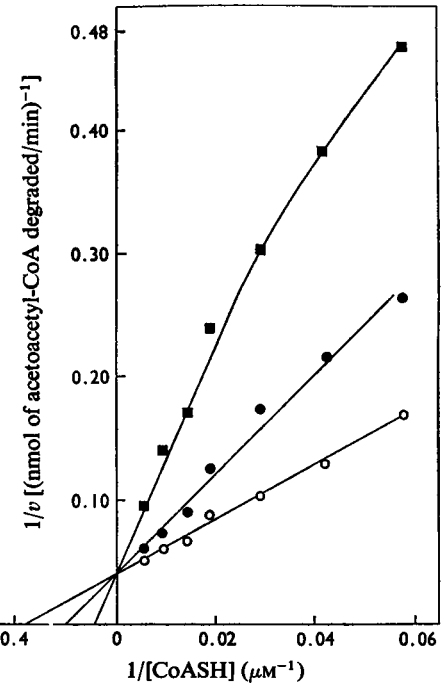


Fig. 6. *Lineweaver–Burk plot of the effects of CoASH and NAD+ concentrations on β-ketothiolase*

Assays in the direction of thiolysis were conducted as described in the Experimental section with cuvettes containing, in a total reaction volume of 3.0ml: 2μl of dialysed supernatant from 40% satd. ammonium sulphate fractionation (see Table 3; 13.0mg of protein/ml); 21.43μM-acetoacetyl-CoA; 17.2–172μM-CoASH. Additions of NAD^+ were made to the reaction mixtures, to final concentrations of: 2mM (●); 5mM (■). ○, No NAD^+.

NAD^+ and $NADP^+$ in the range 1–10mM (Fig. 5). NAD^+ inhibition was competitive with respect to CoASH when assayed with partially purified enzyme preparations (Fig. 6). NADH and NADPH had no effect on the activity of the enzyme.

The condensation reaction in the absence of free CoASH displayed Michaelis–Menten kinetics; the linear double-reciprocal plots of velocity versus acetyl-CoA concentration became non-linear (Fig. 7) and velocity versus substrate plots became sigmoidal in the presence of CoASH.

Acetoacetyl-CoA reductase

An extensive investigation of the properties of this enzyme has been carried out (Ritchie, 1968; Ritchie *et al.*, 1971; Senior, 1972). In a more recent series of experiments conducted with a Farrand recording spectrofluorimeter, an apparent K_m for acetoacetyl-CoA of approx. 1.9μM was obtained, compared with earlier spectrophotometric determinations, which were in the range 2.9–21.2μM (Ritchie, 1968; Ritchie *et al.*, 1971).

Measurements were complicated by the inhibitory effects of acetoacetyl-CoA at concentrations higher than 10μM (Fig. 8). No activators or inhibitors other than acetoacetyl-CoA were found. NAD^+ and $NADP^+$ (0.1mM), ATP (2mM), D(−)-3-hydroxybutyrate (2.0mM), acetoacetate (2.0mM) or acetyl-CoA (0.3mM) had no effect on activity.

Chemostat experiments

An investigation of the behaviour of *A. beijerinckii* in chemostat cultures showed that poly-β-hydroxybutyrate deposition occurs only during oxygen-limited growth (Senior *et al.*, 1972). We have now extended these observations to include the effects of the relaxation of oxygen-limitation with the subsequent transition to a carbon-limited culture.

Before relaxation of oxygen-limitation the culture was controlled at 30°C and pH6.6 with the dissolved oxygen concentration at less than 0.5% of air saturation. The redox potential (E_h) was +50mV, the steady-state dry weight 1.95mg/ml, glucose in the culture supernatant 37.6mM and the nitrogen gas was supplied in excess (flow rate through the 2 litres of culture was 1 litre/min). The specific growth rate was 0.101 h^{-1} and the cellular poly-β-hydroxybutyrate content was 48.0% of the dry biomass. At zero time (Figs. 9 and 10) the oxygen concentration was raised

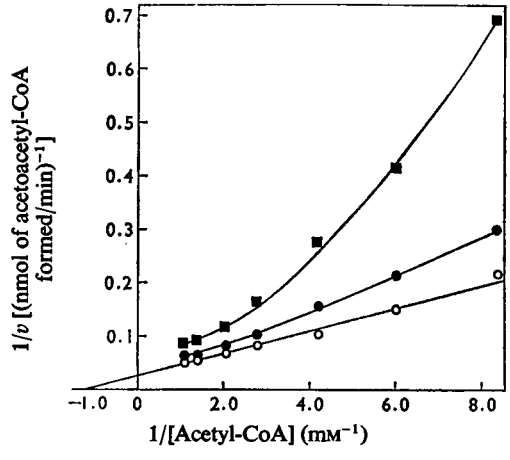


Fig. 7. *Lineweaver–Burk plot of the effect of CoASH concentration on the condensation of acetyl-CoA catalysed by β-ketothiolase*

Assays were conducted as described in the Experimental section with cuvettes containing, in a total reaction volume of 3.0ml: 0.1ml of purified enzyme preparation (14μg of protein); commercial L(+)-3-hydroxyacyl-CoA dehydrogenase, 1μl (10mg of protein/ml, 90 units/mg); 11.58mM-NADH, 0.1ml; 0.12–0.91mM-acetyl-CoA. Additions of CoASH to reaction mixtures were: 34.4μM (●); 103.2μM (■); none (○).

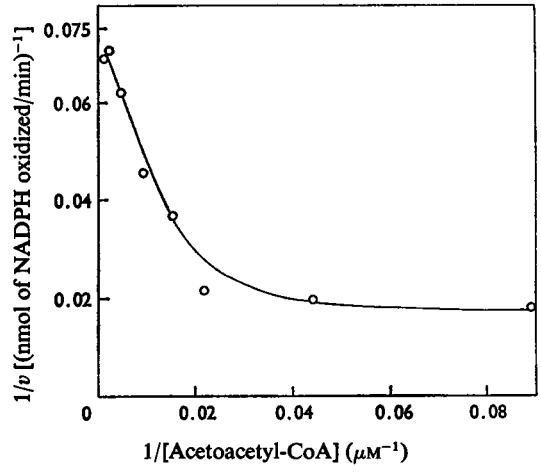


Fig. 8. *Lineweaver–Burk plot of the effect of increasing concentrations of acetoacetyl-CoA on acetoacetyl-CoA reductase*

Assays were conducted as described in the Experimental section with cuvettes containing, in a total reaction volume of 2.5ml: 0.02ml of purified enzyme preparation (0.158mg of protein/ml); 6.0mM-NADPH, 0.03ml; 0.005–0.40ml of 5.62mM-acetoacetyl-CoA (to give 11.25–900μM-acetoacetyl-CoA).

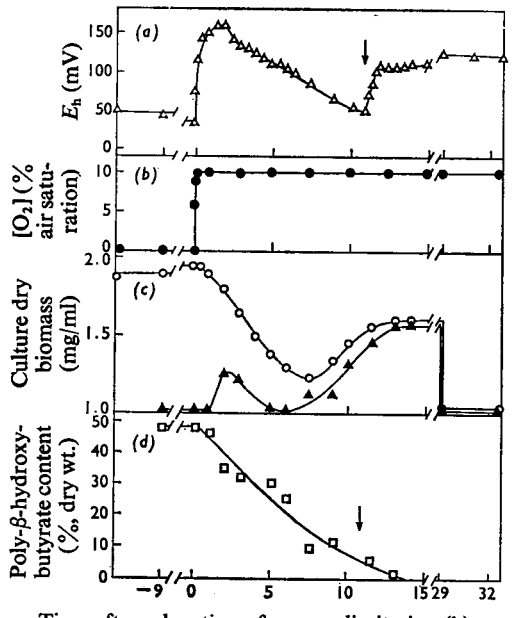


Fig. 9. *Effect of relaxation of oxygen-limitation on an oxygen-limited chemostat culture of A. beijerinckii*

The oxygen-limited culture specific growth rate was $0.101\,h^{-1}$ and at zero time the oxygen supply rate was increased automatically by the concentration controller to maintain a dissolved oxygen concentration of 10% of air saturation. The arrows indicate the time when the culture glucose became exhausted. (*a*) △, Redox potential *in situ*. (*b*) ●, Culture dissolved oxygen concentration. (*c*) ○, Culture dry weight; ▲, culture dry weight minus poly-β-hydroxybutyrate content. (*d*) □, Bacterial poly-β-hydroxybutyrate content.

by the controller to 10% of air saturation. Attainment of 10% saturation took 2 min and this concentration was maintained for the remainder of the experiment. E_h became more positive and the culture dry weight and poly-β-hydroxybutyrate content decreased (Fig. 9). The culture glucose concentration, after an initial decrease during the first 30 min after relaxation of oxygen-limitation (Fig. 10), remained constant for a further 1.5 h. For the next 7 h E_h became more negative, and the culture dry weight decreased along with the culture glucose concentration (Figs. 9 and 10).

At 11.5 h the culture became glucose-limited and the poly-β-hydroxybutyrate content was then 6.0% (w/w) of the dry weight. At the point of glucose-limitation (arrowed in Fig. 9) E_h became more positive (increasing from +50 mV to +120 mV) and remained between +110 mV and +120 mV for the remainder of the experiment. During the 4 h before glucose-limitation the culture went through a period of batch growth and, by the time of glucose-limitation, had 'overshot' the value of steady-state dry weight dictated by the continuing supply of glucose. Thus during the period 15–29 h, the culture dry weight fell from 1.6 to 1.03 mg/ml, the latter value representing the dry weight of a new, glucose-limited steady state (with inflowing medium glucose concentration at 55 mM). After 13.0 h the cellular poly-β-hydroxybutyrate content was <1.5% (w/w) of the dry weight and remained at this low value for the remainder of the experiment.

By subtracting the poly-β-hydroxybutyrate content from the culture dry weight (Fig. 9) it can be seen that the initial and final dry weights, disregarding polymer content, were equal.

The rates of oxygen uptake and carbon dioxide evolution (μl of oxygen or carbon dioxide utilized or evolved/h per mg dry wt. of culture) *in situ* given in Fig. 10 were calculated from the dry weights shown in Fig. 9. Both rates agree fairly closely. Before the relaxation of oxygen-limitation the rates of oxygen consumption and carbon dioxide evolution were 75.5 and 60.2 respectively. When the oxygen concentration was increased (zero time, Figs. 9 and 10) respiratory activity increased immediately, and continued to increase until glucose became limiting (Fig. 10). The rate of oxygen consumption reached 622, representing an 8.3-fold increase, and carbon dioxide evolution was 568, a 9.5-fold increase.

At the point of glucose exhaustion the rates of oxygen uptake and carbon dioxide evolution decreased, probably caused by the 'overshoot' phenomenon already mentioned. However, by 29 h the rates had re-adjusted to 567 for carbon dioxide evolution and 592 for oxygen consumption.

Discussion

Any mechanism for the regulation of poly-β-hydroxybutyrate metabolism in *A. beijerinckii* must account for the observation that biosynthesis of the polymer is inhibited and its degradation proceeds freely under conditions of either molecular nitrogen- or carbon-limitation of growth, and that only when cultures are oxygen-limited does poly-β-hydroxybutyrate accumulate (Senior *et al.*, 1972). In discussing these regulatory phenomena it is convenient to consider separately the physiological and enzymological aspects.

Physiological aspects of the biosynthesis and degradation of poly-β-hydroxybutyrate

Senior *et al.* (1972) speculated that oxygen-limited growth would produce cells that had critical rate-

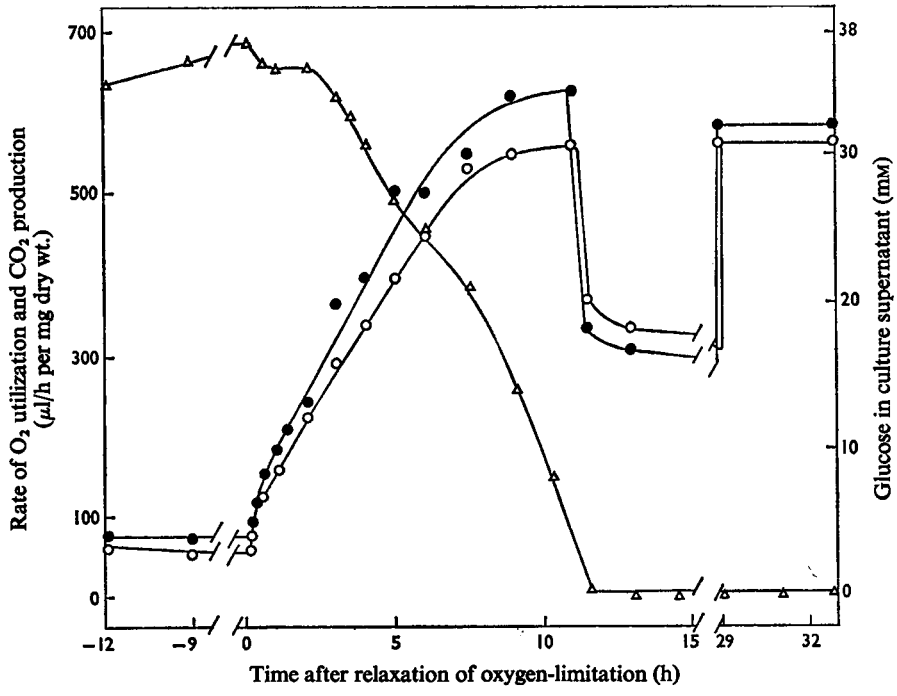


Fig. 10. *Effect of relaxation of oxygen-limitation on an oxygen-limited chemostat culture of A. beijerinckii*

Experimental details are as given in the text and in the legend to Fig. 9. △, Culture glucose concentration; ●, rate of oxygen utilization *in situ*; ○, rate of evolution of carbon dioxide *in situ*.

limiting steps in their metabolism of glucose and acetyl-CoA. On the assumption that oxygen-limited growth conditions would yield cells that had a high NAD(P)H/NAD(P)$^+$ ratio, we suggested on the basis of previous findings (Senior & Dawes, 1971) that the inhibition, by reduced nicotinamide nucleotides or by adenine nucleotides or by both, of glucose 6-phosphate dehydrogenase [inhibited by NAD(P)H, ATP and ADP], 6-phosphogluconate dehydrogenase [inhibited by NAD(P)H], the combined activity of the Entner & Doudoroff (1952) pathway enzymes (inhibited by ATP, citrate, isocitrate and *cis*-aconitate), citrate synthase (inhibited by NADH) and isocitrate dehydrogenase [inhibited by NAD(P)H and ATP] would effectively decrease the rate of acetyl-CoA metabolism via the tricarboxylic acid cycle. Accumulated acetyl-CoA would then be condensed to acetoacetyl-CoA, reduced to D(−)-β-hydroxybutyryl-CoA and polymerized to poly-β-hydroxybutyrate, with the concomitant reoxidation of NAD(P)H. Conversely, on the relaxation of oxygen limitation, we suggest that these effects are reversed and polymer synthesis ceases when acetyl-CoA is again oxidized via the tricarboxylic acid cycle.

Previous experiments with chemostat cultures subjected to rapid changes of environment (Senior *et al.*, 1972) revealed that poly-β-hydroxybutyrate synthesis was stimulated by oxygen-limited growth, whereas the present results show that relaxation of oxygen-limitation, in the presence of excess of exogenous glucose, led to a loss of the polymer from the culture. Because unrestricted growth occurred under these conditions, and the culture was not in a steady state, it is not possible to say with certainty whether only polymer synthesis ceased or whether cessation of synthesis was accompanied by polymer degradation. Our results are inconclusive on this point. However, the unrestricted growth that followed the relaxation of oxygen-limitation was accompanied by a rapid and instantaneous increase in the rates of oxygen utilization and carbon dioxide evolution without a significant decrease in the concentration of exogenous glucose (Fig. 10: during the period 0–3h). These findings suggest that during oxygen-limited growth certain catabolites of glucose metabolism are accumulated. Similar observations have been made with *Hydrogenomonas eutropha* under polymer-synthesizing conditions (oxygen- or nitrogen-limited growth; Schuster & Schlegel, 1967), when the

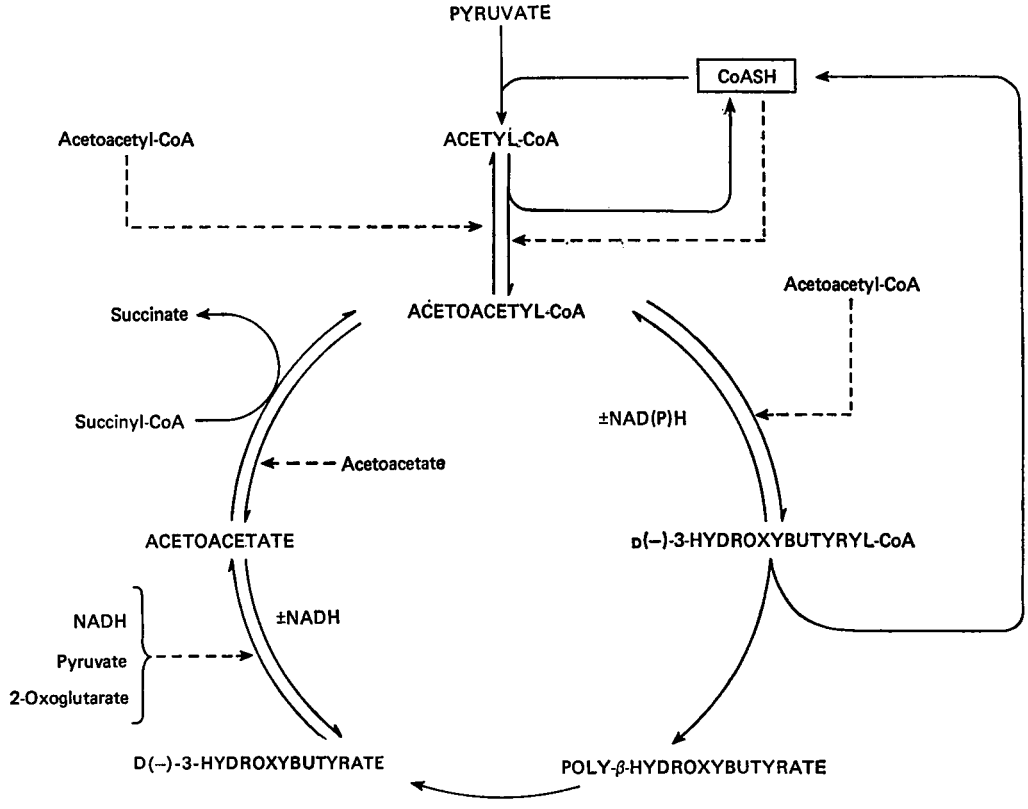
intracellular pyruvate pool can be as high as 11.0mM (H. G. Schlegel, personal communication).

Regulation of enzyme activities

Poly-β-hydroxybutyrate biosynthesis proceeds via CoA esters and free CoASH is released at two points, namely during the condensation of two acetyl-CoA molecules to yield acetoacetyl-CoA and during the final reaction of biosynthesis, β-hydroxybutyrate polymerization (Fig. 11).

Our discovery of acetoacetate–succinate CoA-transferase (thiophorase) in *A. beijerinckii* not only completed the pathway of poly-β-hydroxybutyrate degradation but revealed a cyclic process (Scheme 1). CoA transferase, first demonstrated by Stadtman (1953) in *Clostridium kluyveri*, was also shown to be highly active in pig heart (Stern *et al.*, 1956). The pig heart enzyme catalysed the transfer of CoA to succinate from acetoacetyl-CoA with a $K_{eq.}$ of 8.82×10^3 M at pH8.1 in favour of succinyl-CoA formation. The K_m values of the pig heart enzyme for succinate and acetoacetyl-CoA, 1mM and 720μM respectively, may be compared with those for the *A. beijerinckii* enzyme, namely 4mM for succinate and 28.5μM for acetoacetyl-CoA. The pig heart enzyme is product-inhibited when assayed in either direction (Stern *et al.*, 1956; Hersh & Jencks, 1967). With the *A. beijerinckii* enzyme, acetoacetate inhibits the succinate-stimulated degradation of acetoacetyl-CoA by competing with the latter substrate. Although the equilibrium constant for this reaction does not lie in favour of poly-β-hydroxybutyrate degradation, under conditions of low NAD(P)H, high CoASH and thus low acetoacetyl-CoA concentrations, together with a high acetoacetate concentration, the reaction would proceed towards acetoacetyl-CoA formation.

The discovery of a CoA transferase in *A. beijerinckii* poses the question of how the cell regulates poly-β-hydroxybutyrate metabolism to prevent the occur-



Scheme 1. *Regulation of poly-β-hydroxybutyrate metabolism in A. beijerinckii*

Inhibition is indicated by broken lines.

rence of an energetically wasteful process whereby acetoacetyl-CoA formed by degradation is re-cycled to poly-β-hydroxybutyrate.

There is unfortunately no detailed information available concerning the regulation of the poly-β-hydroxybutyrate depolymerizing enzyme(s) associated with the membrane of the polymer granules. It is a complex system that has been studied in *Rhodospirillum rubrum* (Merrick *et al.*, 1962), *Bacillus megaterium* (Gavard *et al.*, 1966; Griebel & Merrick, 1971) and *Micrococcus halodenitrificans* (Sierra & Gibbons, 1963) but which has not yet been resolved in *A. beijerinckii*. Therefore there is some doubt, but it seems possible that the regulation of polymer degradation could be achieved by the inhibition of D(−)-3-hydroxybutyrate dehydrogenase by NADH (competing with NAD^+), pyruvate and 2-oxoglutarate (competing with 3-hydroxybutyrate). This NAD^+-specific enzyme is not affected by NADPH and is similar in pH optimum, substrate specificity and Michaelis constant to the enzymes isolated by Schindler & Schlegel (1963) from *Hydrogenomonas eutropha* H 16, and by Shuster & Doudoroff (1962) from *R. rubrum*. Inhibition of activity by pyruvate, D-lactate and DL-2-hydroxybutyrate was recorded by Delafield *et al.* (1965) with a purified enzyme from *Pseudomonas lemoignei*, an organism capable of degrading exogenous poly-β-hydroxybutyrate.

The regulation of the *A. beijerinckii* enzyme by pyruvate and 2-oxoglutarate is probably exerted when glucose catabolism and the tricarboxylic acid cycle are operating and the need to oxidize accumulated polymer, in its role as a reserve of carbon and energy, is minimal. A similar pattern of regulation of the 3-hydroxybutyrate dehydrogenase exists in *Hydrogenomonas eutropha*; pyruvate and NADH inhibit the enzyme but the other active oxo acid is oxaloacetate (V. Oeding & H. G. Schlegel, personal communication) and not 2-oxoglutarate as in *A. beijerinckii*. Oxaloacetate is not effective with the *A. beijerinckii* enzyme, which may reflect the importance of 2-oxoglutarate as a key metabolite in the incorporation of reduced nitrogen into amino acids by this nitrogen-fixing organism. The major pathway of NH_4^+ incorporation into amino acids in *Azotobacter vinelandii* is via a reductive transamidation, whereby one molecule each of glutamine and 2-oxoglutarate are converted into two glutamate molecules with concomitant oxidation of NAD(P)H (Nagatani *et al.*, 1971).

A finer control of the *A. beijerinckii* enzyme is exerted by the competitive inhibition by NADH, rendering the enzyme very sensitive to changes in the NADH/NAD^+ ratio brought about, for example, by the imposition or relaxation of oxygen-limitation of growth. The polymer would not be degraded on relaxation of oxygen-limitation until both the NAD^+ concentration had increased and the pyruvate concentration decreased, the latter condition being achieved on exhaustion of exogenous glucose.

During conditions of unrestricted growth in the presence of excess of oxygen (i.e. no polymer accumulation), the steady-state concentration of CoASH would be expected to be high, mediated by the action of citrate synthase, citrate formation serving as a sink for acetyl groups and simultaneously releasing free CoASH. Restriction of citrate synthase activity as a result of NADH accumulation during oxygen-limitation (Senior & Dawes, 1971), would lead to a decrease in the high steady-state concentration of CoASH, with a concomitant increase in the concentration of acetyl-CoA.

A substantial increase in acetyl-CoA concentration would be required to saturate the β-ketothiolase (K_m for acetyl-CoA = 0.9mM) and thereby initiate the synthesis of polymer. It is apparent that the steady-state concentrations of CoASH and acetyl-CoA may well be the critical factors governing the regulation of poly-β-hydroxybutyrate biosynthesis and degradation at the level of β-ketothiolase. This enzyme consequently occupies a key position in polymer metabolism, governing both biosynthesis and degradation. We have no evidence to suggest that more than one β-ketothiolase is present and we therefore assume that a single enzyme catalyses both the condensation of acetyl-CoA and the thiolysis of acetoacetyl-CoA.

Under conditions of unrestricted growth high concentrations of CoASH and low concentrations of acetyl-CoA would ensure that not only was the rate of acetoacetyl-CoA synthesis low, through the suboptimum concentration of acetyl-CoA, but that synthesis was inhibited by CoASH. The relief by CoASH of acetoacetyl-CoA inhibition of acetoacetyl-CoA thiolysis would, in turn, ensure that only when CoASH was present at high concentrations would degradation proceed.

Oeding & Schlegel (1973) have purified and characterized the β-ketothiolase from *Hydrogenomonas eutropha* and we have benefited from discussions with them; it seems clear that the *H. eutropha* and *A. beijerinckii* β-ketothiolases are very similar. The K_m values for acetyl-CoA are high and condensation is inhibited by CoASH. Acetoacetyl-CoA inhibits the thiolysis reaction and inhibition is relieved by CoASH. These kinetics suggest the enzyme is product-inhibited in both directions, although an allosteric type interaction cannot be completely eliminated. Although NAD(P)$^+$ inhibits the *A. beijerinckii* and *H. eutropha* enzymes at millimolar concentrations, Oeding & Schlegel (1973) and ourselves agree that this inhibition is not likely to be of physiological significance.

Our previous, rather curious and apparently inexplicable, finding that acetoacetyl-CoA inhibited acetoacetyl-CoA reductase (Ritchie, 1968; Ritchie *et al.*, 1971; Senior, 1972) is now probably explained

on the basis that only during conditions when NAD(P)H concentrations are high (e.g. during oxygen-limited growth) would reduction of acetoacetyl-CoA, and consequently synthesis of polymer, proceed. This effect would counter the tendency for cycling leading to energy wastage.

An overall scheme of regulation to explain our findings *in vivo* can now be proposed. Conditions for poly-β-hydroxybutyrate biosynthesis, namely high NAD(P)H, low CoASH and high acetyl-CoA concentrations, are produced during oxygen-limitation of growth, which thus stimulates biosynthesis of the polymer. Simultaneously these conditions inhibit degradation and prevent unrestricted cycling of metabolism. Conditions that favour a high intracellular concentration of $NAD(P)^+$, e.g. resulting from relaxation of an oxygen-limitation of growth, would restrict biosynthesis but would not stimulate degradation until the steady-state concentration of acetyl-CoA decreased and that of CoASH increased, as a result of the supply of glucose catabolites, such as pyruvate, becoming restricted. These latter effects would then enable the thiolysis of acetoacetyl-CoA catalysed by β-ketothiolase to proceed.

The scheme proposed for the regulation of poly-β-hydroxybutyrate metabolism accords with the recognized features for the deposition and utilization of a reserve material. Thus it ensures that accumulation occurs only when the supply of exogenous carbon is in excess of the requirements for growth and maintenance, and that degradation takes place only when the supply of exogenous carbon is exhausted. Further, degradation is accompanied by the formation of NADH, which can be re-oxidized with the concomitant production of energy. To test experimentally the validity of the proposed regulatory processes it will now be necessary to measure the intracellular concentrations of CoASH, acetyl-CoA, pyruvate, 2-oxoglutarate, NADP(H) and $NAD(P)^+$ under various growth conditions. This information should then enable the regulation of poly-β-hydroxybutyrate metabolism in *A. beijerinckii* to be characterized completely.

We are indebted to Miss Mechthild Gottwald for the experiments on oxo acid inhibition of D(-)-3-hydroxybutyrate dehydrogenase, to the Science Research Council for the award of a studentship to P. J. S. and to the Agricultural Research Council for a Grant-in-aid. We are also grateful to Prof. H. G. Schlegel and Mr. V. Oeding for discussions and for an exchange of manuscripts before publication.

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