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The Role of Oxygen Limitation in the Formation of Poly-β-hydroxybutyrate during Batch and Continuous Culture of *Azotobacter beijerinckii*

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Azotobacter beijerinckii was grown in ammonia-free glucose–mineral salts media in batch culture and in chemostat cultures limited by the supply of glucose, oxygen or molecular nitrogen. In batch culture poly-β-hydroxybutyrate was formed towards the end of exponential growth and accumulated to about 74% of the cell dry weight. In chemostat cultures little poly-β-hydroxybutyrate accumulated in organisms that were nitrogen-limited, but when oxygen limited a much increased yield of cells per mol of glucose was observed, and the organisms contained up to 50% of their dry weight of poly-β-hydroxybutyrate. In carbon-limited cultures (D, the dilution rate, = 0.035–0.240h^{-1}), the growth yield ranged from 13.1 to 19.8g/mol of glucose and the poly-β-hydroxybutyrate content did not exceed 3.0% of the dry weight. In oxygen-limited cultures ($D = 0.049$–$0.252h^{-1}$) the growth yield ranged from 48.4 to 70.1g/mol of glucose and the poly-β-hydroxybutyrate content was between 19.6 and 44.6% of dry weight. In nitrogen-limited cultures ($D = 0.053$–$0.255h^{-1}$) the growth yield ranged from 7.45 to 19.9g/mol of glucose and the poly-β-hydroxybutyrate content was less than 1.5% of dry weight. The sudden imposition of oxygen limitation on a nitrogen-limited chemostat culture produced a rapid increase in poly-β-hydroxybutyrate content and cell yield. Determinations on chemostat cultures revealed that during oxygen-limited steady states ($D = 0.1h^{-1}$) the oxygen uptake decreased to 100μl h^{-1} per mg dry wt. compared with 675 for a glucose-limited culture ($D = 0.1h^{-1}$). Nitrogen-limited cultures had CO_2 production values *in situ* ranging from 660 to 1055μl h^{-1} per mg dry wt. at growth rates of 0.053–0.234h^{-1} and carbon-limited cultures exhibited a variation of CO_2 production between 185 and 1328μl h^{-1} per mg dry wt. at growth rates between 0.035 and 0.240h^{-1}. These findings are discussed in relation to poly-β-hydroxybutyrate formation, growth efficiency and growth yield during growth on glucose. We suggest that poly-β-hydroxybutyrate is produced in response to oxygen limitation and represents not only a store of carbon and energy but also an electron sink into which excess of reducing power can be channelled.

Under conditions of nutrient imbalance in bacterial cultures, usually when exhaustion of a single nutrient such as the nitrogen source limits growth in the presence of excess of carbon source, deposition occurs of relatively massive amounts of materials which may be considered as specialized reserves of carbon and/or energy. These materials are subsequently utilized by the cells, and Wilkinson (1959) has suggested certain criteria that should be met if a cellular component is to be considered as a specialized storage material.

Poly-β-hydroxybutyrate, a reserve peculiar to micro-organisms, has a widespread occurrence in both Gram-positive and Gram-negative bacteria. It is particularly characteristic of the nitrogen-fixing *Azotobacter*, our survey of which revealed that *Azotobacter beijerinckii* is remarkable in that it may accumulate up to 70% of its dry weight as polymer (Stockdale *et al.*, 1968). We have therefore used this organism for investigations of the mechanism and regulation of biosynthesis of poly-β-hydroxybutyrate when growth occurs on atmospheric nitrogen in a glucose–salts medium (Ritchie & Dawes, 1969; Ritchie *et al.*, 1971; Senior & Dawes, 1971). The present work with batch and continuous culture was designed to attempt to answer the specific question of what factors initiate the observed massive accumulation of poly-β-hydroxybutyrate. A preliminary account of part of this work has been given (Ritchie *et al.*, 1969).

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Experimental

Materials

Organism. The organism used was a spontaneous variant of *Azotobacter beijerinckii* N.C.I.B. 9067 that lacks the capsule of the wild-type and that is designated C^-. It was isolated from a chemostat after inoculation with the wild-type organism and operation for about 100h with glucose limitation; by this time the population was preponderantly capsuleless. The capsuleless organism in batch and continuous culture showed no tendency to revert to capsule formation. The wild-type and C^- organisms are indistinguishable in the standard bacteriological diagnostic tests (Jensen, 1954) for *Azotobacter beijerinckii* (D. A. McCarthy, unpublished work). The C^- organism had considerable advantages for the present work in that it grew more efficiently and could be centrifuged more readily. It was maintained on slopes of nitrogen-free medium (see below) and subcultured monthly.

Batch-culture media. The basal medium (nitrogen-free) was prepared from two solutions of the following composition (g/l). Solution A: glucose 20.0 [for 1% (w/v) medium] or 10.0 [for 0.5% (w/v) medium]; $MgSO_4,7H_2O$, 0.4; $CaCl_2$, 0.11; $FeSO_4,7H_2O$, 0.012; $NaMoO_4,2H_2O$, 0.01; solution B: K_2HPO_4, 2.0; NaCl, 0.4. The two solutions were autoclaved separately [15lb/in² (103kN/m²) 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.

Continuous-culture media. The basal medium (nitrogen-free) had the following composition (g/l): glucose, 15.0 (1.5%, w/v); $MgSO_4,7H_2O$, 0.2; $CaCl_2$, 0.06; $FeSO_4,7H_2O$, 0.006; $Na_2MoO_4,2H_2O$, 0.01; nitrilotriacetic acid, 0.25; K_2HPO_4, 1.0; NaCl, 0.2. The pH of the complete medium was 6.8 at 30°C. The medium, in 80-litre batches, was sterilized by filtration through a Sartorius membrane filter (142mm) with a pore size of 0.2μm. A Sartorius cellulose pad prefilter (127mm) was employed (Sartorius, Göttingen, Germany). Sterile medium was collected in 20-litre batches in sterilized aspirators which were incubated at 30°C until required.

Inocula. All batch cultures and inocula for continuous culture were grown at 30°C on a gyrotary shaker (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.). For large-scale batch cultures 10ml of liquid medium was inoculated from a stock slope and incubated at 30°C with continuous shaking. This subculture was then used to inoculate 2 litres of batch-culture medium in a wide-necked 4-litre conical flask.

Chemostat cultures were checked microscopically each day for purity and also by inoculation, at 48h intervals, on to plates of solid 1% glucose–nitrogen-free medium.

Continuous-culture apparatus. A Porton-type chemostat (Herbert *et al.*, 1965) of 2-litre capacity was used. It possessed sufficient ports to accommodate probes for measurement and control of pH, temperature (heated by an external 250W i.r. lamp and cooled by an internal water coil), redox potential, oxygen concentration, medium inflow, gas inflow and effluent, culture effluent, and ports for inoculation, sampling of culture, antifoam addition and vessel drainage. The culture vessel, minus oxygen electrode but otherwise complete, was autoclaved at 20lb/in² (138kN/m²) for 1h. The vessel assembly was then mounted in its frame and the accessory equipment connected, with aseptic precautions. The oxygen electrode was sterilized by immersion for 1h in 10% (v/v) formaldehyde solution before insertion into the culture vessel. Medium was pumped into the vessel by a H.R. flow-inducer (Watson–Marlow Ltd., Marlow, Bucks., U.K.). To prevent back-growth from the culture vessel into the medium supply-lines a hot-water jacket (60°C) surrounded the medium line immediately before the inlet port.

Gas supply. All gases were obtained in cylinders from British Oxygen Co. Ltd. Gas flows were measured by rotameters (Series 1100, 300mm type; M. F. G. Rotameter Co. Ltd., Croydon, Surrey, U.K.) and regulated by Flo-stat regulators (G. A. Platon Ltd., Basingstoke, Hants., U.K.) at a working pressure of 10lb/in² (69kN/m²). The combined gases were passed through two Mackley filters (Microflow Ltd., Mackley Filter Division, Gateshead 8, Co. Durham, U.K.; NaCl penetration less than 0.001%), arranged in series, before flowing into the culture vessel. Effluent gases were passed through a cold-water condenser and a packed cottonwool filter before measurement of total effluent gas flow by a rotary gas-meter (Lange, Gelsenkirchen; Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K.). The effluent gas was dried by passage through a silica gel column (2.5cm × 30cm) and was then divided for analysis of oxygen by a paramagnetic analyser (Servomex Oxygen Analyser, Type OA 137; Servomex Controls Ltd., Crowborough, Sussex, U.K.) and carbon dioxide by an M.S.A. infrared analyser (Lira Model 300; Mine Safety Appliances Co. Ltd., Glasgow E.3, U.K.).

Determination and control of dissolved oxygen concentration in culture. A Mackereth (1964) oxygen electrode was used to determine the dissolved oxygen concentration in the culture. Before inoculation the culture medium was saturated with air and the maximum current output from the electrode recorded as 100% of air-saturation; oxygen-free nitrogen was passed through the medium to set the oxygen concentration at zero. The oxygen concentration of the culture could be controlled to any value between 0 and 100% air-saturation by means of a series 60 controller (Leeds and Northrup Ltd., Birmingham,

U.K.) by using a 3-action C.A.T. control unit. The control was based on the method first described by MacLennan & Pirt (1966). Several modifications were necessary because of the nitrogen-fixing capacity of our organism. The electro-pneumatic transducer, operated by compressed air (20lb/in^2; 138kN/m^2), fed its output signal to a control valve through which, in our system, pure oxygen was passed. If compressed air was used as the source of oxygen, attainment of a nitrogen-limited culture was impossible because of the irregularity of nitrogen gas flows.

Measurement of redox potential. Culture redox potential (E_h) was measured with a smooth platinum spade-electrode (Activion Ltd., Kinglassie, Fife, U.K.) by using a KCl salt bridge and a calomel reference electrode. The steam-sterilizable electrode was calibrated by the method of Jacob (1970).

Output current was measured by a high-resistance pH-meter (model 91B; Electronic Instruments Ltd., Richmond, Surrey, U.K.) to avoid polarity reversal at the electrode.

Determination of bacterial dry weight. A culture sample (20ml) was run into a pre-cooled (−20°C) universal 25-ml bottle. This immediately lowered the culture temperature to 6–8°C. A sample (5ml) was then dried to constant weight at 80°C in a tared vial. A further sample (6ml) was centrifuged at 35000*g* and 0°C for 15min and 5ml of the clear supernatant was dried to constant weight as described for the culture. By this method dry weights could be determined by difference with an accuracy ±5%. Bacterial densities were determined by measuring the E_{500}; suitable dilutions were made with water for cultures having $E_{500} > 0.50$.

Poly-β-hydroxybutyrate. Poly-β-hydroxybutyrate was assayed by the spectrophotometric method of Law & Slepecky (1961). A sample of culture (0.5–2.0ml) was added to 9.0ml of 10% alkaline hypochlorite solution (Williamson & Wilkinson, 1958) and incubated at 20°C for 24h, after which time the reaction mixture was centrifuged at 5500*g* for 45min to sediment the poly-β-hydroxybutyrate granules, and then the supernatant was decanted. The solid pellet was resuspended and washed successively with 10ml portions of water, acetone and ether. After drying (40°C for 2h) the white powder was dissolved in conc. H_2SO_4 (10ml) and heated for 10min at 100°C. After cooling the solution was read at 235nm against a conc. H_2SO_4 blank. A calibration curve was constructed with DL-β-hydroxybutyrate and purified polymer from *A. beijerinckii* (E_{235} of 1.0 was given by 6.3μg of poly-β-hydroxybutyrate/ml of reaction mixture). In our experience, when bacteria contain very small amounts of poly-β-hydroxybutyrate (0–1.5% of the dry weight) the content cannot be measured with an accuracy greater than ±1.0% of the bacterial dry weight, and we therefore record values in this range as <1.5%.

Metabolic quotients. Rates of CO_2 production *in situ* for cells grown in the chemostat were calculated from the expression:

$$\mu l \text{ of } CO_2 \text{ evolved/h per mg of dry biomass} = \frac{B T_f (60 \times 10^3 \times 273)}{x V T}$$

where B = % CO_2 content of effluent gas, T_f = total inflowing gas (ml/min), x = steady-state culture dry weight (mg/ml), V = total volume of culture (ml) and T is the culture temperature (°K).

Attempts to measure directly the rate of O_2 consumption *in situ* of chemostat cultures by using the relationship:

$$\mu l \text{ of } O_2 \text{ consumed/h per mg of dry biomass} = A - \frac{C T_f (60 \times 10^3 \times 273)}{x V T}$$

(where A = μl of O_2/min in inflowing gas and C is the % O_2 content of the effluent gas) were extremely unreliable and the errors very large because of the inherent difficulty of measuring a very small difference between two large experimentally determined values. Even with corrections applied to the equations for inflowing and effluent gas temperature and pressure, errors can vary between 12 and 460% (D. G. MacLennan, personal communication). We have therefore relied on the more easily measured rate of CO_2 production *in situ* and, since *A. beijerinckii* does not metabolize glucose under anaerobic conditions (see the Results and Discussion section) we have assumed

Analyses

Glucose in culture media. Culture samples were centrifuged at 35000*g* and 0°C for 15min. A portion (1–100μl) of undiluted clear supernatant was added directly by Hamilton syringe to 5ml of blood-sugar reagent [glucose oxidase–peroxidase test combination; Boehringer Corp. (London), London W.5, U.K.] to avoid the errors that large dilutions would introduce. Deproteinization of supernatant samples was unnecessary. A calibration curve was prepared in the range 0–25μg of glucose/5ml of assay mixture. An E_{420} of 0.10 was given by 2.7μg of glucose per assay mixture.

that the rate of O_2 consumption equals the rate of CO_2 production when the organism oxidizes glucose in the chemostat. Potential (i.e. maximum possible) rates of O_2 consumption were determined manometrically. Samples (2ml) of culture from the chemostat were transferred to Warburg flasks which had 0.1ml of 20% (w/v) glucose in the side-arm and 0.1ml of 2M-KOH in the centre well. After equilibration at 30°C for 5min the endogenous rate of oxygen uptake was determined before the addition of glucose. After addition of glucose most samples exhibited a short lag (0.5–2min) before attainment of a linear rate of oxygen consumption.

Results and Discussion

Growth in batch culture

When the organism was grown in batch culture on 0.5% (w/v) glucose medium (Fig. 1*a*) the formation of poly-β-hydroxybutyrate occurred towards the end of exponential growth and the polymer accumulated to some 35% of the dry bacterial weight. The glucose in the culture was exhausted 22h after inoculation and poly-β-hydroxybutyrate biosynthesis ceased, as did further increase in biomass. The polymer was then degraded and by 24h the content had decreased to 17.5% (w/w) of biomass without significant concomitant decrease in dry weight, thus demonstrating the role of poly-β-hydroxybutyrate as a reserve material capable of furnishing carbon for cellular synthesis and nitrogen assimilation.

When excess of carbon was provided by increasing the glucose concentration in the medium to 2% (w/v), poly-β-hydroxybutyrate synthesis occurred towards the end of the exponential phase and continued after the growth showed transition to a slower rate, at about 16.5h (Fig. 1*b*); the polymer eventually attained a value of 74% of the dry bacterial weight at 31h.

Experiments with a Mackereth oxygen electrode inserted in batch cultures revealed that poly-β-hydroxybutyrate biosynthesis was initiated at about the time when the electrode registered zero current, indicating a dissolved oxygen concentration of zero, which coincided with the transition from exponential growth.

These findings led us to consider that oxygen limitation might be the critical factor that initiated the biosynthesis of the polymer in *A. beijerinckii*. This feature is of considerable interest, as nitrogen limitation has been shown to stimulate poly-β-hydroxybutyrate biosynthesis in *Bacillus megaterium* (Macrae & Wilkinson, 1958), the formation of other highly reduced compounds such as fatty acids and lipids in *Rhodotorula gracilis* (Kessell, 1968; Allen *et al.*, 1964) and the deposition of glycogen in *Escherichia coli* (Holme & Palmstierna, 1956). However, in a batch culture simultaneously fixing atmospheric nitrogen, an apparent oxygen limitation could well be masking a real nitrogen limitation and it was essential therefore to separate these two parameters. This was achieved by recourse to chemostat experiments.

Growth in chemostat continuous cultures

The chemostat, with oxygen and nitrogen controls, was used to separate the effects of these two types of nutrient limitation. Additionally, the effect of carbon limitation was studied. Tables 1 and 2 record the variation in polymer content with growth (dilution) rate under conditions of nitrogen- and oxygen-limitation respectively. Results for glucose-limited cultures are presented in Fig. 2. Clearly a massive

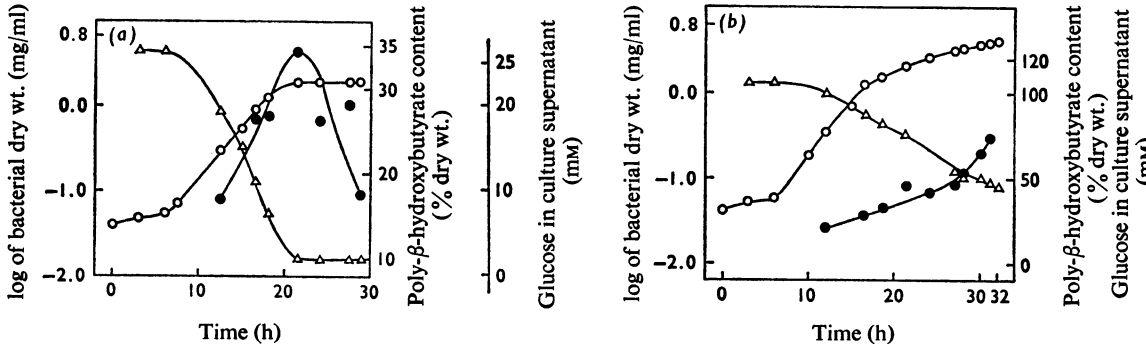

Fig. 1. *Growth and poly-β-hydroxybutyrate content of A. beijerinckii in nitrogen-free batch culture with limiting or excess of glucose*

For experimental details see the text. (*a*) Limiting glucose (0.5%, w/v); (*b*) excess of glucose (2%, w/v). o, Bacterial density; ●, poly-β-hydroxybutyrate content; △, glucose concentration in culture.

Table 1. *Variation of biomass yield and poly-β-hydroxybutyrate content with growth rate in nitrogen-limited chemostat cultures*

For experimental details see the text. Abbreviation: $Y_{av\,e^-}$, g of bacteria/available electron.

Growth rate D (h^{-1})	Yield of biomass (g/mol of glucose) (Y_1)	$Y_{av\,e^-}$	Poly-β-hydroxybutyrate content [% (w/w) of biomass]	Bacterial doubling time, t_d (h)
0.053	7.4	0.31	<1.5	13.07
0.079	10.8	0.45	<1.5	8.77
0.098	10.7	0.44	<1.5	7.11
0.130	12.5	0.52	<1.5	5.29
0.161	14.4	0.60	<1.5	4.31
0.186	16.3	0.68	<1.5	3.73
0.218	18.7	0.78	<1.5	3.18
0.234	19.7	0.82	<1.5	2.96
0.255	Culture washed out			2.72

Table 2. *Variation of biomass yield and poly-β-hydroxybutyrate content with growth rate in oxygen-limited chemostat cultures*

For details see Table 1 and the text.

Growth rate D (h^{-1})	Yield of biomass (g/mol of glucose) (Y_1)	$Y\,av\,e^-$	Yield of poly-β-hydroxybutyrate (g/mol of glucose) (Y_2)	$Y_1 - Y_2$	Poly-β-hydroxybutyrate content [% (w/w) of biomass]	Bacterial doubling time t_d (h)
0.049	50.9	2.12	22.6	28.3	45	14.14
0.075	49.7	2.07	21.4	28.3	43	9.24
0.102	48.4	2.02	21.6	26.8	45	6.79
0.121	55.7	2.32	22.1	33.6	40	5.71
0.146	70.1	2.92	17.6	52.5	25	4.75
0.158	64.0	2.66	19.3	44.7	30	4.40
0.164	49.0	2.04	15.6	33.4	32	4.22
0.192	52.9	2.20	10.6	42.3	20	3.61
0.227	63.3	2.64	16.1	47.2	25	3.05
0.252	55.3	2.31	10.7	44.6	20	2.75

deposition of the polymer occurs only under conditions of oxygen limitation.

Cultures grown with nitrogen- or carbon-limitation rarely contained more than 3% of their dry weight as polymer, whereas oxygen-limited cultures displayed values ranging from 20% at the highest specific growth rate ($D = 0.252\,h^{-1}$), to 45% at the lowest rate ($D = 0.049\,h^{-1}$). The very high contents of polymer recorded in batch cultures (65–74%) were never attained in continuous culture, which may reflect the fact that the former organisms had either ceased dividing or were growing at a rate very much lower than the minimum studied in the chemostat. Alternatively, it is possible that the slow-growing chemostat cultures might have contained a high proportion of dead bacteria.

Tables 1 and 2 and Fig. 2 also record the yields (Y_1) of bacteria (g/mol of glucose utilized) and of poly-β-hydroxybutyrate (Y_2; g/mol of glucose utilized). The difference ($Y_1 - Y_2$) represents a yield value relating cell mass, other than poly-β-hydroxybutyrate, to glucose consumed. In Tables 1 and 2 the value of $Y_{av\,e^-}$ represents the yield of cells as g/available electron (*av* e^-). Glucose was assumed to have an *av* e^- value of 24.

Fig. 3 records the effect of the sudden imposition of an oxygen limitation on a nitrogen-limited culture. A nitrogen-limited culture ($D = 0.233\,h^{-1}$) was grown with the dissolved oxygen concentration controlled at 10% of air saturation. On decreasing the oxygen supply rate to 0.25 of its original value, the oxygen concentration decreased rapidly and was accompanied by a decrease in E_h from +15 mV to −50 mV. At this point polymer accumulation commenced and the

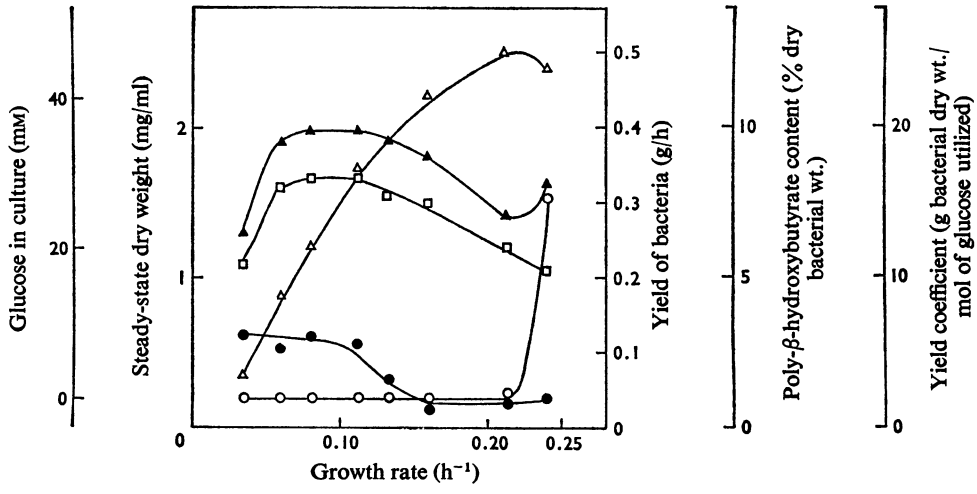

Fig. 2. *Variation of biomass yield and poly-β-hydroxybutyrate content with growth rate in glucose-limited chemostat cultures*

For experimental details see the text. ○, Glucose concentration in culture; ●, poly-β-hydroxybutyrate content; △, yield of bacteria (g/h); ▲, yield coefficient; □, steady-state bacterial dry weight.

E_h value, after an initial oscillation, rose to +30mV. Meanwhile the dissolved oxygen concentration in the culture remained at zero, as measured by the Mackereth electrode, and the glucose concentration in the culture increased from 26 to 70mM.

On imposition of the decreased oxygen supply on the nitrogen-limited culture, both E_{500} and culture dry weight increased rapidly; these increases could not be accounted for by the simple increase in polymer content during this period. Thus some increase in cell numbers probably occurred during this time, representing more efficient growth at low oxygen concentration, until the oxygen concentration reached a growth-limiting value after approx. 4h, and the culture began to wash out and re-adjust to the new environment.

The maximum value for polymer content, approx. 45% (w/w) of biomass, was not maintained in this oxygen-limited culture and fell to approx. 20% (w/w) after 32h, suggesting that synthesis of these high contents of polymer at fast growth rates is a transient phenomenon.

The growth-yield coefficient (g of dry biomass produced/mol of glucose utilized) ranged from 13.1 to 19.85 for glucose-limited cultures grown at rates between $0.035h^{-1}$ and $0.080h^{-1}$, and thence declined with increasing growth rate to give a final yield of 16.2 at $D = 0.240h^{-1}$. These values for yield are within the same range as those reported by Nagai *et al.* (1969, 1971) for *Azotobacter vinelandii* grown with carbon limitation, namely 5.4 and 21.4g/mol of glucose at $D = 0.1$ and $0.35h^{-1}$ respectively, and they attributed this variation in yield with growth rate to a decrease in oxygen concentration accompanying the increased dilution rate and causing an increase in yield. Similar results were obtained by Dalton & Postgate (1969*b*) for chemostat cultures of *Azotobacter chroöcoccum*. However, even with accurate control of dissolved oxygen concentration we have recorded large variations in growth yield over a wide range of growth rates. Growth yields from oxygen-limited cultures were substantially higher than those recorded for nitrogen- or glucose-limited cultures and varied between 49 and 70g/mol of glucose. These values may be compared with those obtained by Nagai *et al.* (1969, 1971) for oxygen-limited cultures of *A. vinelandii* (36.0–45.0g/mol of glucose). If values for oxygen-limited yields are corrected for poly-β-hydroxybutyrate content ($Y_1 - Y_2$, Table 2) it is clear that the increased yield under these conditions is not due entirely to polymer content and that the cells must therefore be growing more efficiently at lower oxygen concentrations.

Rates for CO_2 production *in situ* are presented in Table 3 for carbon-, nitrogen- and oxygen-limited chemostat cultures together with their variation with growth rate. Also recorded is the calculated CO_2 evolution per doubling time, which may be equated with the oxygen consumption per doubling time, and is thus a measure of growth efficiency with respect to oxygen. The assumption that the rate of CO_2 production equals the rate of O_2 consumption was

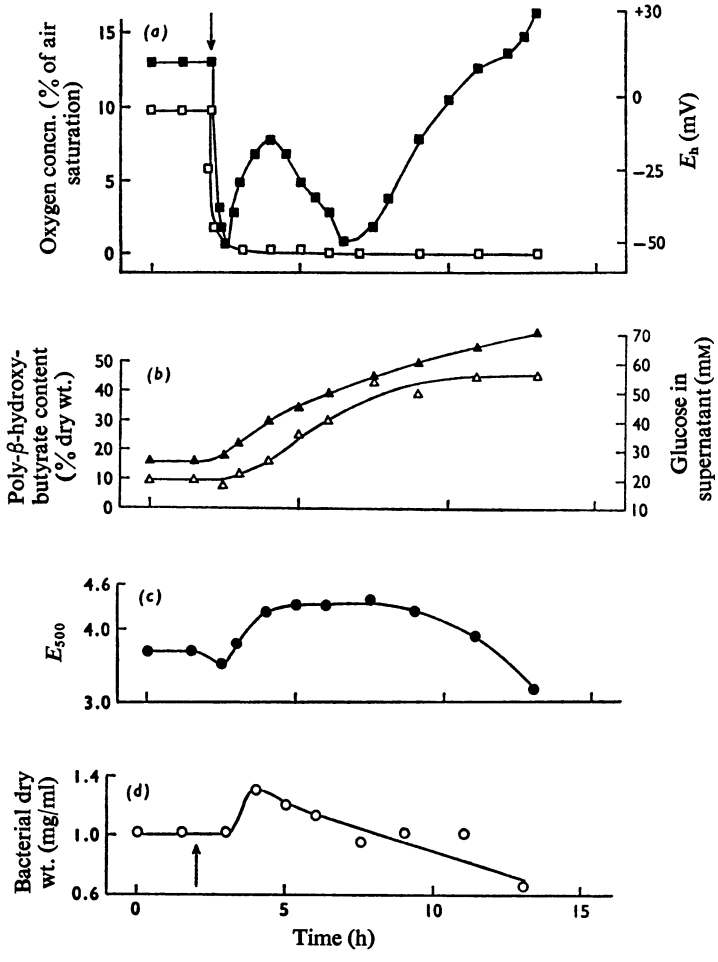

Fig. 3. *Effect of imposition of oxygen-limitation on a nitrogen-limited chemostat culture of A. beijerinckii*

The nitrogen-limited culture growth rate was $0.233\,h^{-1}$ and at the point indicated by the arrow the oxygen supply rate was decreased from 100ml/min to 25ml/min, imposing an oxygen limitation; 40min later the oxygen supply rate was decreased further to 15ml/min. (*a*), □, Culture dissolved-oxygen concentration; ■, redox potential *in situ*; (*b*), △, poly-β-hydroxybutyrate content; ▲, culture glucose concentration; (*c*), ●, culture turbidity (E_{500}); (*d*), ○, bacterial dry weight.

justified for *A. beijerinckii* by subjecting an oxygen-limited chemostat culture ($D = 0.095\,h^{-1}$) to complete oxygen starvation. When nitrogen replaced the oxygen–nitrogen mixture and the inflowing medium was stopped, CO_2 evolution fell to zero and the glucose concentration in the culture remained constant.

The potential rate of oxygen consumption for oxygen-limited cultures was lower than for carbon-limited cultures, indicating a possible repression or inhibition of certain enzyme(s) concerned with oxidative activity. Although the precise locus of this effect has not been ascertained we have shown that citrate synthase and isocitrate dehydrogenase are subject to regulation by NADH, as are several other enzymes of glucose catabolism (Senior & Dawes, 1971). As the oxygen-limited bacterium continues to manufacture and utilize large quantities of acetyl-CoA for the production of poly-β-hydroxybutyrate, it is possible that the oxidative rate-limiting reaction(s) occurs within the tricarboxylic acid cycle. Our results with a nitrogen-limited culture subjected to a sudden imposition of oxygen limitation support the hypothesis

Table 3. *CO_2 production in situ of glucose-, nitrogen- and oxygen-limited chemostat cultures of A. beijerinckii*

For experimental details see the text. For both glucose- and nitrogen-limited cultures the dissolved oxygen concentration was controlled to 10% of air saturation.

Limiting nutrient	Growth rate D (h^{-1})	CO_2 production ($\mu l\ h^{-1}$/mg dry weight)	CO_2 evolved per doubling time (μl/mg dry weight/t_d)
Glucose	0.035	185	3618
	0.060	297	3438
	0.080	393	3402
	0.100	675*	—
	0.113	516	3150
	0.133	716	3726
	0.160	874	3790
	0.214	1179	3816
	0.240	1328	3807
Nitrogen	0.053	660	8640
	0.079	707	6228
	0.098	1032	7335
	0.131	1052	5580
	0.161	1094	4725
	0.186	1065	3960
	0.218	999	3168
	0.234	1055	3123
Oxygen	0.049	49	648
	0.076	56	509
	0.102	120, 100*	815
	0.121	116	675
	0.146	90	428
	0.157	91	400
	0.164	200	842
	0.192	218	781
	0.252	152	428

* Rate of CO_2 production equated with rate of O_2 consumption determined manometrically.

of a rate-limiting tricarboxylic acid cycle where acetyl-CoA, no longer being oxidized at a rapid rate, is channelled into poly-β-hydroxybutyrate biosynthesis. Under conditions of oxygen limitation, the rate of re-oxidation of NADH and NADPH, via NADH oxidase and NADPH–NAD^+ transhydrogenase, would be expected to decrease. We suggest that, under these conditions, the re-oxidation of NAD(P)H is principally achieved via poly-β-hydroxybutyrate biosynthesis rather than by electron transport to oxygen as the ultimate electron acceptor (Ritchie *et al.*, 1969; Senior & Dawes, 1971). It would seem therefore that the most suitable environment for the growth of this strict aerobe is, perhaps surprisingly, one in which the oxygen concentration is growth-limiting. Under such conditions the growth yields are greater, the oxygen requirement is lower, and the cell accumulates a storage polymer which confers advantages for maintenance of viability during periods of starvation. Since the natural habitat of *A. beijerinckii* is soil, the oxygen concentration of which in both gaseous and liquid phases is variable (Greenwood, 1968), the growth-inhibitory effects of oxygen concentrations in excess of about 20% of air saturation on the *Azotobacter* (Bulen *et al.*, 1963; Dalton & Postgate, 1969*a*) may be countered by the organism increasing its oxidative activity, thus lowering the environmental partial pressure of O_2 to a more acceptable value. This process was called 'respiratory protection' by Dalton & Postgate (1969*a*) who proposed that the mechanism operated in growing *Azotobacter* to protect the nitrogenase, whereas in non-growing bacteria the appropriate components of the system assumed a conformation that prevented access of oxygen to the sensitive sites. Drozd & Postgate (1970) confirmed the scavenging role of augmented respiration with *Azotobacter chroöcoccum*. Hill & Postgate (1969) showed that *Derxia gummosa*

preferred a low partial pressure of oxygen for growth when fixing molecular nitrogen; Hill (1971) suggested that the inhibition by oxygen of nitrogen fixation in this organism can be overcome when small amounts of fixed nitrogen are present in agar medium, because the respiratory activity accompanying the resulting initial phase of growth lowers the concentration of the local oxygen supply sufficiently to release the nitrogenase system from inhibition.

In the absence of both exogenous substrate and intracellular reserve materials this process of respiratory protection would be impossible and viability would be threatened. Cells possessing a large quantity of readily oxidized poly-β-hydroxybutyrate would have a distinct advantage in their ability to increase their oxidative activity in the absence of exogenous substrate.

All the *Azotobacteriaceae* examined by Stockdale *et al.* (1968) accumulated poly-β-hydroxybutyrate and it may be speculated that the polymer serves a similar role in all these organisms, functioning as both a storage material and as a means of regulating the oxygen environment of their natural habitat for maintenance of viability.

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