Growth and Physiology of *Azotobacter chroococcum* in Continuous Culture

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SUMMARY

Azotobacter chroococcum (NCIB 8003) organisms, grown in continuous culture without fixed nitrogen, had chemical compositions at various dilution rates characteristic of nitrogen-limited populations. Fast-growing variants were selected for at high dilution rates; the efficiency of nitrogen fixation decreased with decreasing growth rate. In suitable media, carbon- and phosphate-limited populations were obtained and showed different compositions; they were very sensitive to inhibition by oxygen. Carbon-limited populations utilizing NH_4 under argon were not oxygen sensitive; they formed nitrogenase when they were N-limited. The chemical compositions of the various populations corresponded to theory for the nutritional state considered. Nitrogen fixation entrained a maintenance coefficient of 1·06 g. substrate/g. organism/hr compared with about 0·40 for ammonia assimilation. Assuming most of this maintenance was directed to respiratory protection of nitrogenase, an extrapolated maximum requirement of 4 moles ATP/mole N_2 fixed was observed. Attempts to repeat reports of (1) dependence of cytochrome pattern on nitrogen fixation and (2) increased efficiency of fixation with ultraviolet-irradiated N_2 were not successful with the strain of *A. chroococcum* used.

INTRODUCTION

Continuous culture is now an established technique for the study of microbial physiology. A body of work with heterotrophic aerobes such as *Klebsiella* (*Aerobacter*) *aerogenes* and *Torula utilis* in conditions of various nutrient limitations has established the profound effect of nutritional status on cell composition, enzymic activity and viability (Herbert, 1959; Malek & Fencl, 1966; Powell, 1967). The peculiar physiology of nitrogen-fixing bacteria makes them promising subjects for such studies, particularly the Azotobacteriaceae, which grow aerobically on simple media. Several papers making use of Azotobacter have been published; Malek (1952) and Macura & Kotkova (1953) investigated the morphological changes that occurred when *Azotobacter chroococcum* was cultivated in multi-stage continuous cultures; Marr & Marcus (1962) studied the kinetics of induction of mannitol dehydrogenase in carbon-limited and sulphate-limited cultures of *Azotobacter agilis*; Ierusalimsky, Zaitseva & Khmel (1962) and Khmel & Andreeva (1967) studied the behaviour of *Azotobacter vinelandii* in a variety of conditions and have determined the macromolecular composition of this organism at various growth rates; Zacharias (1963*a–c*) exposed nitrogen-fixing continuous cultures of *A. vinelandii* to u.v.-irradiated N_2 gas and observed an increase

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in the efficiency of nitrogen fixation (measured as mg. N fixed/g. glucose consumed); Aiba, Nagai, Nishizawa & Onodera (1967a, b) investigated the effect of carbon limitation on yield in nitrogen-fixing cultures of *A. vinelandii*, and, using Pirt's (1965) equation, obtained a value of 3·90 g. substrate/g. organism/hr for the maintenance coefficient.

These reports sometimes showed certain inconsistencies of detail and included no systematic study of the behaviour of a single strain in a variety of nutritional conditions. We report here a study of *Azotobacter chroococcum* in continuous cultures in which populations growing with and without fixed nitrogen were compared in three nutrient-limited states.

METHODS

Organism. Azotobacter chroococcum (NCIB 8003) was maintained on agar slopes of 'mannitol B' medium (Burk's medium as prescribed by Newton, Wilson & Burris (1953) with mannitol in place of sucrose) and stored at room temperature.

Growth conditions. Cultures were grown in 250 ml. chemostats of the design described by Baker (1968). Temperature was controlled automatically at 30°, and the pH value at $6·9 \pm 0·1$ in ammonia-grown cultures. No pH control was used with nitrogen-fixing cultures except with phosphate-limited populations, where the pH value was maintained at 6·9. A continuous culture vessel, capable of withstanding a pressure of 60 p.s.i., was constructed from Perspex (Imperial Chemical Industries Ltd.), the design and operation of which was similar to the system described by Baker (1968). Sterilization was effected by filling the vessel with 1 % β-propiolactone and leaving it overnight. It was then drained and flushed with 5 l. of sterile distilled water before admitting medium and inoculating.

Medium B_6 contained (g./l. dist. H_2O): mannitol, 10; K_2HPO_4, 0·64; KH_2PO_4, 0·16; NaCl, 0·2; $MgSO_4.7H_2O$, 0·2; $CaCl_2$, 0·1; plus these trace elements (mg./l.): $FeSO_4.7H_2O$, 2·5; H_3BO_3, 2·9; $CoSO_4.7H_2O$, 1·2; $CuSO_4.5H_2O$, 0·1; $MnCl_2.4H_2O$, 0·09; $Na_2MoO_4.2H_2O$, 2·5; $ZnSO_4.7H_2O$, 1·2; nitrilotriacetic acid, 100 (Nitrilotriacetic acid is not a source of nitrogen for these bacteria). The pH value was $7·4 \pm 0·2$ and the medium was autoclaved in 20 l. batches at 121° for 45 min. NH_4^+-limited medium contained the ingredients for mannitol B_6 medium plus 0·15 g./l. NH_4Cl, which was sterilized separately in concentrated solution and added later. Mannitol was decreased to 1·5 g./l. for nitrogen-fixing C-limited cultures and to 2·0 g./l. for C-limited cultures grown with NH_4Cl (1·5 g./l.). K_2HPO_4 was lowered to 0·1 mg./ml. for P-limited cultures, KH_2PO_4 was omitted and KCl (0·55 mg./ml.) was added to give an equivalent value of K^+ ion. Non-nitrogen-fixing P-limited cultures were grown with 1·5 g./l. NH_4Cl. Continuous cultures were run for at least four doubling times after a change in nutritional condition or dilution rate to allow re-establishment of a steady state; constant extinction and pH value were taken as preliminary indications of a steady state.

Cultures were accepted as limited by NH_4, mannitol or phosphate when (1) the organism concentration was halved when the concentration of nutrient in the inflowing medium was halved, (2) doubling the concentration of all other medium components did not alter the organism concentration. The first criterion proved inapplicable to the special case of nitrogen-gas limitation as discussed later in this paper. Aeration was tested by using a Clark type of oxygen electrode (Protech Advisory Services Ltd, 21

High St, Rickmansworth, Herts.) and was adequate in all the systems reported on here.

Analytical procedures. Organism concentration (mg. dry wt/ml. culture), mannitol concentration, nitrogen content, cytochrome spectra and oxygen concentration were determined by previously described methods (Dalton & Postgate, 1968). Phosphate was determined by the method of Baginski, Foa & Zak (1967) with K_2HPO_4 as standard. Protein was determined with the Folin reagent based on Lowry, Rosebrough, Farr & Randall (1951) with bovine albumin serum (Sigma Chemical Co) as standard. Poly-β-hydroxybutyrate was determined by the method of Law & Slepecky (1961) as modified by Stockdale, Ribbons & Dawes (1968) with purified polymer from Azotobacter and sodium DL-β-hydroxybutyrate as standards. Ribonucleic acid was determined by the orcinol reaction (Morse & Carter, 1949) with yeast ribonucleic acid (Sigma Chemical Co.) as standard. Deoxyribonucleic acid was estimated by the method of Burton (1956) with calf thymus deoxyribonucleic acid (Sigma Chemical Co.) as standard. Polysaccharide was determined with the anthrone reagent based on a method devised by Mr P. J. Phipps (M.R.E., Porton, Wilts.) with glucose as standard.

Nitrogenase activity. Acetylene is reduced to ethylene by nitrogenase (Dilworth, 1966; Schöllhorn & Burris, 1967), with a specific activity about three times that observed for N_2 fixation by similar preparations. Acetylene reduction thus provides a rapid assay method for nitrogenase since the ethylene produced can be detected by vapour phase chromatography. Continuous culture samples (2 ml.) were transferred to 25 ml. conical flasks, gassed with different argon:oxygen mixtures (0·01 to 0·4 atm. O_2) for 2 min., and sealed with a Suba-Seal cap. Acetylene (1 ml.), freshly prepared from calcium carbide and water, was injected into the flask which was then transferred to a water bath at 30° with a shaking rate of 25 strokes/min. The flasks were set up as rapidly as possible because prolonged manipulation caused the activity of the population to decline. Two-ml. samples were taken from the flask by first injecting 2 ml. of the appropriate argon + oxygen mixture and then withdrawing a 2 ml. gas sample with an argon-flushed hypodermic syringe and needle. The sample was analysed for hydrocarbons in a Pye 104 gas-chromatography instrument fitted with a flame ionization detector, a 5 ft. Porapak R column of 4 mm. internal diameter at 45° with N_2 as carrier gas at a flow rate of 60 ml./min. The instrument was calibrated quantitatively for ethylene by injecting measured volumes of the gas accurately diluted with argon. The concentration of ethylene was taken as proportional to the peak height.

RESULTS

Growth in nitrogen-fixing conditions

Azotobacter chroococcum was grown in medium B_6 without ammonia and steady states were established at various dilution rates. Figure 1 illustrates the influence of dilution rate on organism concentration: the dry-weight organism/ml. culture increased dramatically with decreasing growth rate. This pattern was obtained in three runs with this organism and the organism concentrations were reproducible within 10 % between runs 18 months apart. The pattern was obtained in two runs with a 100 ml. culture vessel and *Azotobacter vinelandii*; Dr D. Herbert and Dr D. W. Tempest also obtained such a pattern with nitrogen-fixing *A. vinelandii* of uncertain nutrient limitation (personal communication).

The curve also differed from theoretical (see Herbert, 1959) in that wash-out was not abrupt: steady states of low cell concentration were obtained at dilution rates between 0·3 and 0·4 hr⁻¹. This phenomenon was due to selection of fast-growing variants at the high flow rates because, when continuous culture was interrupted by removing 90 % of the culture and replacing it with fresh medium, the rate of growth of the 'batch' culture so obtained increased with increasing dilution rate. The doubling time of batch cultures obtained in this manner from a population growing at $D = $ 0·15 hr⁻¹ was 2·56 hr, identical with that of a batch culture from a slope on mannitol B_6 agar. From a dilution rate of 0·33 hr⁻¹ the doubling time in a batch culture was 1·97 hr.
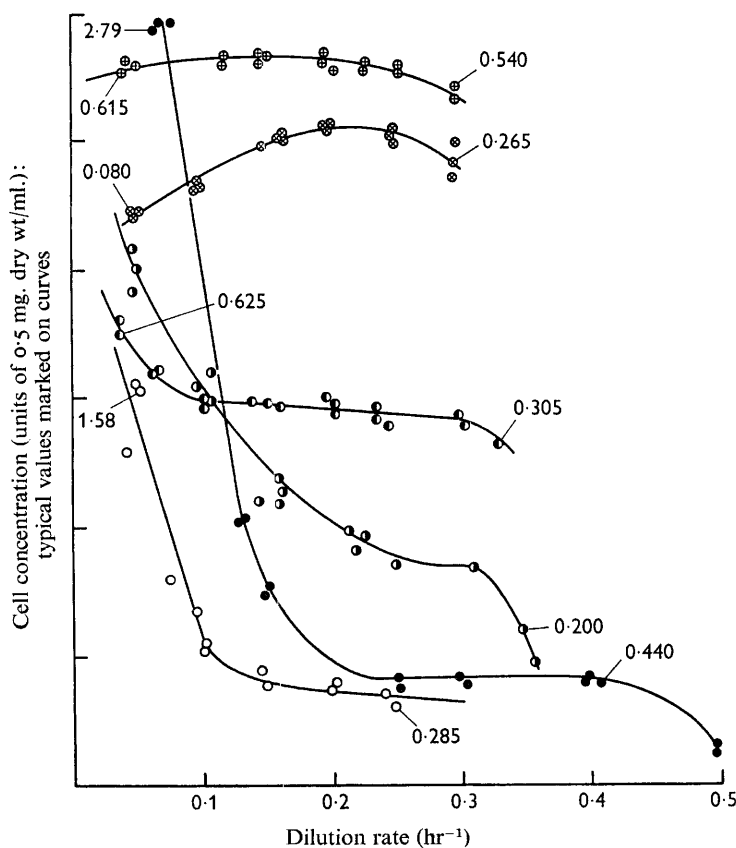

Fig. 1. Relations of cell concentration to dilution rate in continuous cultures of *Azotobacter chroococcum* of various nutritional types. Reasons for accepting the 'N₂-limited' conditions are given in the text. The ordinate is in units of 0·5 mg. dry wt organisms/ml., but the curves have been spaced out for clarity of presentation; cell concentrations at certain positions on the curves are therefore quoted. Only two series were run into the 'wash-out' stage. ◑, N₂-limited, N₂-fixing; ⊗, mannitol-limited, N₂-fixing; ○, phosphate-limited, N₂-fixing; ◐, NH₄ limited, NH₄-assimilating; ⊕, mannitol-limited, NH₄-assimilating; ●, phosphate-limited, NH₄-assimilating.

Table 1 illustrates the effect of dilution rate on macromolecular composition. Much of the increased cell mass at low growth rates was due to accumulation of polysaccharides and poly-β-hydroxybutyrate; RNA increased with increasing growth rate as it does with all other micro-organisms studied (Herbert, 1959). The efficiency of nitrogen

fixation in terms of carbon consumed increased with increasing growth rate (Table 2). Mannitol was normally detectable in samples from the culture vessel, usually in amounts exceeding 3 mg./ml.

The pattern of organism concentration against dilution rate and the presence of appreciable amounts of mannitol in the culture fluid indicated that the culture was not carbon-limited. This was confirmed by altering the mannitol concentration from 1 to 2 % at $D = 0.1$ and 0.2 hr^{-1}: no change in organism concentration occurred. Doubling the concentrations of other constituents of medium B_6 had no effect on cell concentration at these dilution rates, hence a component of the atmosphere must have

Table 1. *Selected data on macromolecular composition of*
Azotobacter chroococcum in continuous culture

Values as % dry weight organism; figures in parentheses indicate upper limits.

	Dilution rate (hr^{-1})	Protein	Poly-saccharide	PHB	RNA	DNA	Sum
N-limited, N$_2$-fixing	0·05	39·0	19·8	28·2	0·7	2·3	96·3
	0·10	40·0	10·3	27·1	6·9	2·7	87·0
	0·22	62·0	7·8	8·1	21·0	1·9	100·8
C-limited, N$_2$-fixing	0·05	54·6	(3·2)	(0·4)	7·1	—	—
	0·15	58·2	6·1	—*	16·8	2·4	—
	0·20	63·7	5·4	3·1	16·8	1·8	91·3
P-limited, N$_2$-fixing	0·05	37·8	10·0	31·1	7·8	1·2	87·9
	0·15	63·1	12·8	9·4	8·7	3·1	97·1
	0·20	56·2	8·4	—	13·6	2·5	—
N-limited, NH$_4$-assimilating	0·04	63·0	18·7	12·8	5·3	—	—
	0·15	69·5	8·1	8·5	12·8	—	—
	0·20	65·1	5·4	0·0	23·4	—	—
C-limited, NH$_4$-assimilating	0·05	57·5	6·8	3·9	5·4	—	—
	0·15	57·1	8·9	2·6	10·2	2·0	80·8
	0·27	63·4	5·1	—	13·8	1·8	—
P-limited, NH$_4$-assimilating	0·05	41·2	5·2	35·0	7·1	2·1	90·6
	0·16	58·0	12·0	1·0	12·1	3·4	86·5
	0·30	68·0	4·0	0·0	15·0	—	—

* — = not done

Table 2. *Efficiency of nitrogen fixation by A. chroococcum in continuous culture*

Analyses were performed on samples from steady state growth in air; the culture was N$_2$-limited at all growth rates (see text).

Dilution rate (hr^{-1})	μg. N fixed/mg. mannitol consumed
0·05	12·2–13·6
0·10–0·11	17·5–18·0
0·15–0·16	28·4–30·2
0·20–0·22	37·6–39·1
0·30	36·0

been limiting. Increasing the atmospheric pO_2 to 0.42 atm. at $D = 0.2$ hr^{-1} did not alter the organism concentration but the amount of mannitol consumed increased from 2.1 mg./ml. to 4.9 mg./ml.; an increased stirring rate at $pO_2 = 0.2$ atm. similarly increased the mannitol consumption without altering the organism concentration. Oxygen electrode measurements, with electrodes calibrated for molarity of dissolved oxygen in distilled water, showed an ambient oxygen concentration in the culture of

about 20 μM; when this was increased to above 45 μM, either by vigorous stirring or high pO_2, growth of the culture was inhibited as we reported earlier (Dalton & Postgate, 1968). Hence, by elimination, nitrogen was the growth-limiting nutrient.

For reasons given in the Discussion section it is intrinsically unlikely that such physical factors as stirring rates, pN_2 and solubility of N_2, limited access of nitrogen to the organism; this possibility was nevertheless tested by growing cultures at various pN_2 values using argon as diluent and keeping the pO_2 at 0·2 atm. At $D = 0·16\,hr^{-1}$ the organism concentration remained constant at 0·66 mg. dry wt/ml. between pN_2 values of 0·8 and 0·29, decreased to 0·61 at $pN_2 = 0·19$ and 0·41 at $pN_2 = 0·09$. An apparatus was designed to grow the bacteria in continuous culture under high pressures (it will not be described in detail here since the findings in this context were negative) and the steady state organism concentration was found to be constant (1·61 to 1·71 mg. dry wt/ml. at $D = 0·06\,hr^{-1}$) at total pressures of 1·5, 2·15 and 3·2 atm. of N_2 containing sufficient O_2 to keep the ambient concentration at 17 to 20 μM (at high pressures the pO_2 required to do this was less than 0·2; when a pO_2 of 0·2 was sustained at high pressures oxygen inhibition occurred). Hence the cell concentration was independent of pN_2 from 0·3 to 3 atm. despite nitrogen being physiologically the growth-limiting substrate; the question whether the population can be properly referred to as 'nitrogen-limited' is raised in the Discussion; for the purposes of this publication we shall call them 'N_2-limited'.

K_m *of continuously multiplying cells.* Extrapolation of the figures relating pN_2 to organism concentration gave a K_m for nitrogen fixation by actively multiplying bacteria of 0·07 atm., higher than most recorded values for Azotobacter (0·02 to 0·05 atm.: Wilson & Roberts, 1954; Parker & Scutt, 1960), but in close agreement with Strandberg & Wilson's (1967) value of 0·066 atm. obtained with $^{15}N_2$.

Test of irradiated N_2. Zacharias (1963 a–c) observed that u.v.-irradiated nitrogen was fixed more efficiently (in terms of carbon consumed) than non-irradiated gas by continuous cultures of *Azotobacter vinelandii*. To test for a comparable effect with *A. chroococcum* a quartz tube, 30 × 2·5 cm., was attached to the gas-inlet port and N_2 was irradiated while passing along the tube at about 200 ml./min. by a lamp emitting 30 watts at 253·7 nm set 3 mm. distant from the tube before mixing to pN_2 of 0·8 with oxygen. No significant change in efficiency of nitrogen fixation or organism concentration occurred in 2- or 3-day steady states at $D = 0·21\,hr^{-1}$, whether the lamp was on or off.

Growth with ammonium as nitrogen source

Since nitrogen fixation was limiting growth in the cultural condition just described, comparable continuous cultures were established under an argon + oxygen mixture of pO_2 0·2 atm. with NH_4Cl as growth-limiting substrate; automatic pH control was used to compensate for acidity generated as NH_4^+ was assimilated; with 1·4 mM-NH_4Cl the cell concentration was 0·21 mg./ml. at $D = 0·2\,hr^{-1}$ and was doubled at 2·8 mM-NH_4Cl; the former concentration was adopted to obtain the plot of organism concentration against growth rate in Fig. 1; the form of the curve is very similar to that shown by nitrogen-fixing populations. Table 1 lists representative analyses illustrating comparable trends in polysaccharide, poly-β-hydroxybutyrate and RNA contents. The ambient oxygen concentration was about 15 μM; the population did not show oxygen sensitivity of the kind reported by Dalton & Postgate (1969) for nitrogen-fixing cultures, though increasing the oxygen concentration to 52 μM caused a 25 % lowering of the organism concentration at $D = 0·105\,hr^{-1}$.

Nitrogenase content of NH₄-limited cells. Ammonium is known to prevent synthesis of nitrogenase but, since the ammonium concentration in an ammonia-limited continuous culture is vanishingly small, ammonium repression might not operate in such conditions. Figure 2 shows that whole cells assayed by the acetylene reduction technique showed 'nitrogenase' activity and illustrates that activity depended on pO_2; sufficient O_2 to allow growth and metabolism was necessary, but too much was inhibitory. Substitution of nitrogen for the argon in the gas phase of the continuous culture enhanced the cell concentration considerably, but decreased the specific 'nitrogenase' activity; the population decreased and its activity increased on returning to argon again (Fig. 3). The values used to plot Fig. 3 refer to optimal pO_2 values in the test system; these differed according to the density of the population being tested.
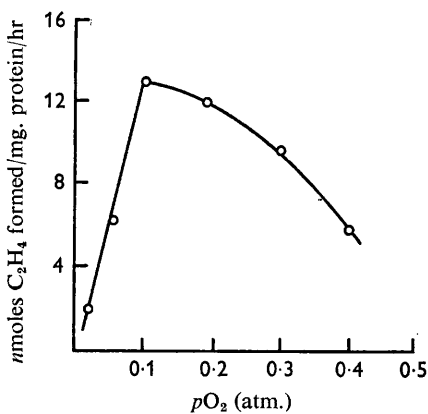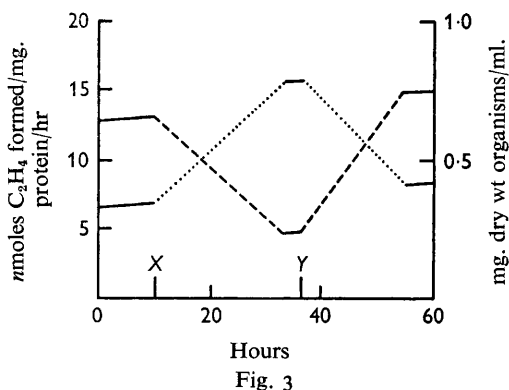

Fig. 2. Acetylene reduction by *Azotobacter chroococcum* grown in NH₄-limited continuous culture under argon + oxygen: effect of pO_2. Samples from continuous culture were gassed with argon + oxygen mixtures and sealed in conical flasks, C_2H_2 was injected to start reaction. Rates of ethylene production were obtained by gas chromatography of samples at timed intervals. For details see text.

Fig. 3. 'Nitrogenase' activity of ammonium-limited *Azotobacter chroococcum* in continuous culture. An ammonium-limited continuous culture was growing under A + 20 % (v/v) O_2 in a steady state at D. The atmosphere was changed to air at *X* and back to argon + oxygen at *Y*. Ethylene production rates were then at the optimal pO_2 value for the population being tested. (See Fig. 2). . . ., bacterial concentration; – – –, acetylene-reducing activity.

Carbon-limited populations

Nitrogen-fixing, carbon-limited cultures. The establishment of steady states in mannitol-limited continuous cultures was difficult because of the oxygen-sensitivity reported by Dalton & Postgate (1968); inhibition of growth by over-aeration occurred readily, and was associated with formation of a black pigment. Steady states were successfully established in which the concentration of mannitol and no other component of the environment determined the cell concentration when the stirring rate was adjusted so that the dissolved oxygen did not exceed 23 μM; for all experiments reported here the stirring rates were adjusted manually to maintain the oxygen concentration at between 5 and 10 μM at a pO_2 of 0·2 atm.

Figure 1 includes a plot of bacterial concentration against dilution rate. Unlike the comparable curve for N₂-limited populations, a sharp wash-out was observed and the

yield declined dramatically at low dilution rates. Table 1 records some data on the organism composition: polysaccharide or poly-β-hydroxybutyrate were low and did not accumulate at low dilution rates; more RNA appeared the faster the cells grew. These findings are consistent with the normal behaviour of carbon-limited populations in a chemostat.

Ammonium-grown, carbon-limited cultures. No difficulty from oxygen inhibition was experienced in establishing mannitol-limited cultures under argon + 20 % (v/v) oxygen; 3 g. $NH_4Cl/l.$ provided excess NH_4^+ in the presence of 2 g. mannitol/l.; pH control was necessary. Stirring was adjusted to produce an oxygen concentration of about 40 μM. The curve relating cell concentration to dilution rate (Fig. 1) is typical of that of a normal carbon-limited aerobic organism with little significant deviation from linearity. (The wash-out range above $D = 0.3$ hr^{-1} was not studied.) It differed markedly from the pattern obtained when the population was fixing nitrogen. On the other hand, the data on cell composition (Table 1) show similar trends.

Phosphate-limited populations

Nitrogen-fixing, phosphate-limited cultures. Mannitol B_6 medium with K_2HPO_4 lowered but with K^+ restored to its normal molarity as described in Methods gave phosphate-limited populations according to our usual criteria. The populations showed oxygen sensitivity comparable to carbon-limited, nitrogen-fixing cultures (and again showed pigment formation when over-aerated); and the pH value tended to fluctuate because of the lower buffering power. To obtain the data plotted in Fig. 1 the dissolved oxygen was held at about 10 μM under $pO_2 = 0.2$ atm. by manual control of the stirring rate, and automatic pH control to 6.9 was used. The pattern of organism concentration against dilution rate resembled that of N_2-limited population but Table 1 shows, as one might expect on theoretical grounds, that the increased dry weight at low dilution rates was due largely to storage of poly-β-hydroxybutyrate, not polysaccharide, which requires phosphate for its deposition. RNA showed the usual pattern.

Ammonium-grown, phosphate-limited cultures. A comparable chemostat was set up with 3 g. $NH_4Cl/l.$ in the medium and an atmosphere of A + 20 % (v/v) O_2. It was phosphate-limited according to the usual criteria and showed no abnormal sensitivity to aeration; oxygen was held about 25 μM and pH at 6.8. Fig. 1 shows the curve relating organism concentration to dilution rate; it was essentially similar to that for nitrogen-fixing population but the yield (wt organism/wt P) was higher and wash-out did not occur until $D = 0.5$ hr^{-1}. Table 1 illustrates that poly-β-hydroxybutyrate was again the main storage product responsible for deviation from linearity at low dilution rates.

Morphological observations

Morphological changes consistent with those reported by other workers using *Azotobacter vinelandii* were observed in all these experiments and will not be reported in detail. Generally speaking, azotobacters tended to be coccoid at dilution rates from 0.15 to about 0.3 hr^{-1}, large and bacillary from 0.3 hr^{-1} to wash-out. Below 0.15 hr^{-1} long, filamentous forms appeared and were common at 0.03 hr^{-1}. N_2-, NH_4^+- and particularly PO_4-limited populations showed pronounced clear areas under phase contrast at low dilution rates. These stained with Sudan Black and were presumably poly-β-hydroxybutyrate.

Cytochrome contents of Azotobacter

Table 3 illustrates the relative peak heights of standard suspensions of *Azotobacter chroococcum* obtained in various nutrient conditions. Peaks of cytochromes a_1, a_2, b_1, c_4 and c_5 were seen but insufficient cytochrome o (Jones & Redfearn, 1967) was produced to register. No evidence for an association of cytochrome content with nitrogen fixation (Lisenkova & Khmel, 1967) was obtained, though modest variations with nutritional status occurred, notably a high content of cytochromes a in the N-limited populations. The carbon-limited population was examined under high purity commercial A, H_2, N_2 and O_2 in an attempt to detect oxidation of the cytochrome peaks by N_2 (Wilson, 1958). None was found, though O_2 produced the theoretical oxidation of all components.

Table 3. *Cytochrome content of Azotobacter chroococcum from various nutrient limitations*

Peak heights relative to $c_4 + c_5$ taken as 100.

Cytochrome	N_2	NH_4	C (N_2-grown)	C (NH_4^+-grown)	PO_4 (N_2-grown)	PO_4(NH_4^+-grown)
					Nutrient limitation	
a_2^α	31·5	31·4	6·5	16·7	11·5	7·5
a_1^α	22·6	18·2	6·5	6·7	1·8	1·5
b_1^α	93·6	90·3	78·8	76·3	80·4	74·6
b_1^β	63·5	67·4	59·0	61·2	64·8	63·4
$(b+c)^\gamma$	275·0	225·0	128·0	572·0	435·0	276·0
O.D. $(c_4 + c_5)^\alpha$	0·266	0·258	0·279	0·214	0·190	0·206

DISCUSSION

Nitrogen-limitation in Azotobacter

Nutrient limitations in continuous cultures are usually operational characters: the type of nutrient limitation (carbon/energy, nitrogen source, sulphur source etc.) is imposed by the experimenter and determined by (1) dependence of cell concentration on the concentration of growth-limiting substrate, and (2) independence of cell concentration from moderate changes in the concentration of any other substrate. When the growth-limiting nutrient is relatively insoluble (e.g. with oxygen) the first criterion applies to the concentration of dissolved substrate; such limitations can be detected empirically if, when the flow of liquid medium is discontinued, the cell concentration increases. *Azotobacter chroococcum* growing in mannitol B_6 medium showed the characteristics of an organism limited by N_2: independence of other components, increasing cell density with decreasing dilution rate, continuation of growth when medium flow stopped. But if the first criterion is applied strictly, difficulties arise. At $D = 0·2$ hr^{-1}, for example, the oxygen solution rate was about 70 mmole O_2/l./hr, which, allowing the $p N_2$ and solubility of N_2, is equivalent to a nitrogen solution rate of 140 mmole N_2/l./hr. From the nitrogen content of the culture the nitrogen fixation rate was about 0·67 mmole N_2/l./hr, so obviously physical access of N_2 to the bacteria was not limiting and this fact is reflected in the K_m value of 0·07 atm. obtained here. It follows that some intrinsic property of the organisms leads to N_2-limitation at all

growth rates tested in the routine medium. This situation is probably also true of *A. vinelandii* and can be extended to the majority of batch cultures of either species. These, in conventional media, will normally be N_2-limited until they reach a sufficient density to become oxygen-limited. Daesch & Mortenson (1968) described continuous cultures of *Clostridium pasteurianum* in which growth continued when the flow of medium ceased which, though they did not interpret their results in this way, is evidence that they were N_2-limited in the sense used here.

We have no information on the physiological 'bottleneck' which leads to N_2-limitation in these organisms, but it is clearly of survival value since carbon- and phosphate-limitations lead, when nitrogen is being fixed, to excessive sensitivity to oxygen. Dalton & Postgate (1968) presented evidence that the oxygen sensitivity of nitrogen-fixing Azotobacter, which is almost absent from ammonia-grown populations but which is most marked in carbon- and phosphate-limitation, is an expression of respiratory protection of nitrogenase. Nitrogenase was regarded as damaged or inactivated by oxygen, but the organism's respiration normally scavenges oxygen and keeps it away from the enzyme. Thus the sensitivity of nitrogen-fixing Azotobacter depends upon the population's capacity to respire oxygen away; this will be high with nitrogen-limited populations because of the excess of carbon substrate available; it will be low with carbon-limited populations and may be so with phosphate-limited populations. Thus the carbon-limited populations which we obtained were probably in a state of fine balance between nitrogen limitation and oxygen inhibition; the physiological meaning of the term 'carbon-limited' becomes doubtful when limitation by one metabolic patterning (C) may lead to intoxication of a second pathway (N) by a substrate for the third (O).

Maintenance coefficients in Azotobacter

Deviations from linearity in curves relating yields to dilution rates of carbon-limited continuous cultures are attributed to 'maintenance' factors (Herbert, 1959). From Fig. 1 it is clear that the 'maintenance coefficient' (Powell, 1967) of carbon-limited, nitrogen-fixing populations of *Azotobacter chroococcum* is dramatically different from that of comparable ammonia-utilizing organisms. Plots of $1/Y$ against $1/D$ (Pirt, 1965) from the data in Fig. 1 gave a maintenance coefficient of 1·06 g. mannitol/g. dry. wt organism/hr when fixing nitrogen compared with about 0·04 for ammonia-grown cells and with values of 0.055 for *Escherichia coli* (Schulze & Lipe, 1964) and 0·094 for *Aerobacter cloacae* (Pirt, 1965). Aiba *et al.* (1967a) reported a value of 3·90 for *A. vinelandii*. These very large maintenance coefficients are presumably mainly expressions of 'respiratory protection': carbon substrate diverted to prevent access of oxygen to the nitrogenase system.

ATP consumed in nitrogen fixation

At a given growth rate, the yield of carbon-limited continuous cultures fixing N_2 differed from the yield of one using NH_4^+ by an amount representing two factors: the carbon substrate expended in respiratory protection and that required to generate ATP specially for nitrogen fixation. This difference decreased with increasing growth rate, as would be expected if the need for respiratory protection declined. By plotting the reciprocal of the difference in yield against the reciprocal of the dilution rate and extrapolating to a hypothetical 'infinite' growth rate, a yield difference corresponding

to minimum respiratory protection can be obtained which, using the widely accepted value of 10·5 for Y_{ATP} (Bauchop & Elsden, 1960), may be translated into ATP available for nitrogen fixation. Extrapolation of this kind gave a value of between 4 and 5 moles ATP available/mole N_2 fixed. Cell-free preparations consume about 15 moles ATP/mole N_2 (Silver, 1967); it follows that growing organisms are much more efficient in their ATP economy. This calculation errs on the side of over-estimation, because Y_{ATP} is not likely to be much less than 10·5 and may well be greater with an aerobe; moveover, extrapolation used assumes that the whole extrapolated yield difference represents ATP for nitrogen fixation and some may not. The value of 4 to 5 mole ATP/mole N_2 with Azotobacter contrasts with about 30 mole ATP/mole N_2 reported by Daesch & Mortenson (1968) for *Clostridium pasteurianum*.

Efficiency of nitrogen fixation. The efficiency of nitrogen fixation is sometimes expressed as mg. N fixed/g. carbon source consumed, and Jensen (1953), on the basis of batch culture analyses indicating efficiencies of 10 to 15 mg. N/g., decided that the contribution of free living Azotobacter to the soil economy was negligible since carbon sources capable of supporting significant nitrogen fixation in agricultural terms were not available. However, in chemostat culture, particularly at low pO_2 values, efficiencies of 30 to 40 mg. N fixed/g. carbon were obtained, which may be compared with the efficiency of incorporation of ammonia by a normal heterotroph (*Aerobacter aerogenes* incorporated about 75 mg. N/g. glycerol, calculated from data of Postgate & Hunter, 1962). Soil is not always well aerated and its micro-environments generally resemble more closely nutrient-limited chemostats than laboratory batch cultures; it may be that the contributions of Azotobacter to soil fertility have been under-estimated.

Characteristics of Azotobacter in continuous culture. The dependence of chemical composition on nutritional status which is general among micro-organisms (Herbert, 1961) followed the usual lines once the condition of N_2-limitation in 'ordinary' culture was recognized. N-limitation, via either N_2 or NH_4^+, leads to increased synthesis of non-nitrogenous polymers at low growth rates and the steady state cell concentration increased with decreasing growth rate. RNA increased with growth rate in all conditions of nutrient limitation. DNA/cell remained constant within experimental error; protein varied consistently with the proportion of other polymers.

Selection of mutants of high growth rate during 'wash-out' was originally considered an improbable event (Herbert, Elsworth & Telling, 1956) but there is now evidence that continuous culture at low growth rate favours the selection of variants of low μmax; the fast-growing variants obtained here may thus have originated by a reverse process.

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