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ENERGY STATUS, GROWTH AND NITROGENASE ACTIVITY IN CONTINUOUS CULTURES OF *RHIZOBIUM* SP. STRAIN CB756 SUPPLIED WITH NH_4^+ AND VARIOUS RATES OF AERATION

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Continuous cultures of the cowpea-type *Rhizobium* sp., strain CB756, were grown in the presence of NH_4^+ at automatically controlled concentrations of dissolved O_2 and rates of aeration. Nitrogenase activity of steady-state cultures was only detected under microaeration conditions (dissolved O_2 typically <0.03 μM; aeration rate typically 0.6 μmol O_2/ml per h), when the cellular ATP pool size was 0.8–1.8 nmol/mg dry wt., (optimum 1.1) and the energy charge 0.6–0.7. At twice this aeration rate and dissolved O_2 concentration of about 0.15 μM, the yield of bacteria doubled, the ATP pool increased and energy charge increased to 0.8. With similar rates of O_2 supply but high concentration of dissolved O_2 (approx. 150 μM), cultures were NH_4^+-limited and the ATP pool and energy charge were slightly reduced. Amongst all of these O_2 supply conditions the total pool of adenosine phosphates was not significantly different (2.6 S.D. 0.7 nmol/mg dry wt.). In steady-state, O_2-limited cultures, concentrations of cyclic GMP were higher when nitrogenase was present. When rates of O_2 supply to steady-state cultures were changed, oscillations in bacterial energy status and growth rate were induced decreasing in amplitude until a new steady state was reached. This made it difficult to discern precisely the energy status in which nitrogenase activity was derepressed or repressed. However, generally, increases in nitrogenase activity followed decreases in ATP and energy charge and decreased nitrogenase activity accompanied increases in these energy parameters. These results are discussed in relation to the possible involvement of adenylation or deadenylation of glutamine synthetase and to the control of nitrogenase synthesis in the presence of NH_4^+. It is concluded that the small ATP pool size is responsible for failure of adenylylation of glutamine synthetase and is related to nitrogenase synthesis at microaeration rates.

Introduction

Little is known about the energy status [1] of cultures of the slow-growing types of legume root nodule bacteria such as the so-called cowpea *Rhizobium* sp. although molar yields of ATP (Y_{ATP}) and specific rates of ATP turnover have been estimated for the fast growing *R. trifolii* in chemostat studies [2]. Nitrogen fixation by various bacteria has a high ATP requirement [3–5] and nitrogenase activity in bacteroids from soybean nodules is correlated with ATP/ADP ratio [6]. It would be of interest to discern whether ATP concentration may also be involved in the control of synthesis of nitrogenase in those strains of *Rhizobium* spp. which are able to produce the enzyme in culture [7–9].

In *Klebsiella pneumoniae* it has been proposed that nitrogenase synthesis is promoted when glutamine synthetase is in the catalytically active, non-adenylylated form. Adenylylation of glutamine syn-

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Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

thetase is enhanced in the presence of glutamine or NH_4^+ and requires ATP. Synthesis of nitrogenase is repressed under these conditions (see review Ref. 10). In those strains of *Rhizobium* spp. which produce nitrogenase activity in culture, the presence of NH_4^+ does not always repress nitrogenase synthesis [12–16]. This was notable with O_2-limited cultures of the cowpea strain of *Rhizobium* sp. CB756 [12,13]. Here nitrogenase was positively correlated with the biosynthetic activity of glutamine synthetase as adenylylation of glutamine synthetase decreased with increasing O_2-limitation [13]. In the absence of NH_4^+ and with different N sources for continuous cultures, active nitrogenase was present at higher rates of O_2 supply and when dissolved O_2 concentrations were up to 1–2 μM [20,25]. These results suggested that restricted ATP supply in O_2-limited cultures might prevent adenylylation of glutamine synthetase in the presence of NH_4^+ and that this was related to failure to repress synthesis of nitrogenase. However, against this view Rao et al. [17] found substantial adenylylation of glutamine synthetase in batch cultures of *R. japonicum* with active nitrogenase.

The experiments reported in this paper were designed to investigate the energy status of O_2-limited, NH_4^+-supplied continuous cultures in which nitrogenase activity was positively correlated with glutamine synthetase (biosynthetic) activity and where the glutamine synthetase adenylylation states increased with increasing supply of O_2 [12,13]. In addition, the same cultures were analyzed for content of cyclic AMP and cyclic GMP because of the recent report [18] that a high concentration of exogenous cyclic GMP prevented nitrogenase synthesis in a microaerophilic stationary phase culture of *R. japonicum*.

Materials and Methods

Bacteria. The cowpea strain CB756 (*Rhizobium* sp.) was used throughout as described previously [12,13].

Continuous cultures. The apparatus, equipped with O_2 electrode and feed-back stirrer controller has been described previously [13,19]. The growth temperature was 30°C. The medium contained glycerol (43 mM), sodium succinate (15 mM), ammonium sulphate (2.5 mM added; analyzed at 3.6 mM NH_4^+, apparently due to some precipitation during autoclaving), potassium phosphate (30 mM, pH 6.5), trace elements [20] and after autoclaving, $CaCl_2$ (0.7 mM) and $MgCl_2$ (0.14 mM). The dilution rate of the culture (600 ml for low and microaeration, 570 ml for high aeration) was usually near 0.05 h^{-1} but was 0.1 h^{-1} in some highly aerated cultures.

A range of aeration rates was used (0.3–1.6 μmol O_2/h per ml) in preliminary experiments, but in the main experiments there were three. Highly aerated cultures had steady-state dissolved O_2 concentrations of about 150–170 μM with O_2 solution rates [19] of about 4 μmol O_2/h per ml. In these cultures, O_2 usage was calculated from the O_2 solution rate for the prevailing stirring rate and the difference between the O_2 concentration in air-saturated medium and that measured in the culture. Cultures with low aeration rates had dissolved O_2 concentration levels of about 0.15 μM (the average for the controlling cycle) and solution rates of about 1 μmol O_2/h per ml. Microaeration provided a dissolved O_2 concentration close to or below the limit of measurement (0.03 μM) and O_2 solution rates of 0.3–0.7 μmol/h per ml. For low and microaeration rates, O_2 consumption by the culture was equal to the O_2 solution rate, since the residual O_2 was a negligible proportion of the O_2 supplied.

In all of these cultures, cell density was low and there was no perceptible difference in viscosity between the cultures of any condition used. Rates of O_2 solution therefore did not differ significantly from calibrated values. Furthermore, O_2 consumption rates calculated from O_2 solution rates agreed with values obtained for samples measured in a stirred O_2-electrode chamber [19].

Preliminary experiments established that cultures with dilution rates of 0.05–0.06 culture vol./h were O_2-limited up to aeration rates of 1.6 μmol O_2/h per ml (Fig. 1.). The cell dry wt. yields of such cultures increased linearly with increased O_2 supply and contained residual NH_4^+ (1.5–3 mM); washed bacteria contained 48–53% protein. In contrast, NH_4^+-limited cultures contained very low (<0.05 mM) or undetectable concentrations of NH_4^+ and the bacteria were 85–92% protein. With the exception of NH_4^+, all medium-supplied nutrients were in excess of requirements for culture densities at least up to 500 μg dry wt./ml.

The yield of bacterial cells from steady-state cul-

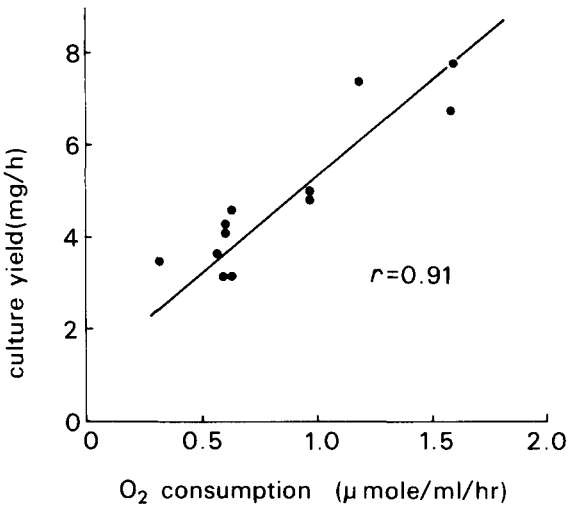

Fig. 1. Relationship between O_2 consumption and culture yield for O_2-limited, steady-state cultures of *Rhizobium* sp. strain CB 756.

tures was the product of effluent vol./h and culture density, estimated from a standard curve of dry wt. of water-washed cells and optical absorbance at 700 nm. In non-steady-state experiments in which changes in culture parameters were measured following altered O_2 supply, culture density changed rapidly due to increased or decreased growth and culture volume was also affected by frequent sampling in the period immediately following the change. In such experiments, calculations of culture growth were adjusted to take account of these factors.

Nitrogenase activity. Nitrogenase activity was measured at 30°C as C_2H_2 reduction, using culture samples collected in evacuated, capped vials and assayed with oxyleghaemoglobin as the source of O_2, as previously described [20]. Such assays provide optimal conditions and estimate the potential nitrogenase activity of the bacteria, but do not necessarily measure the activity of the culture itself, where conditions of O_2 supply in particular, may be less favourable.

Quantification of adenine nucleotides. Duplicate samples (3.0 ml) were collected through the sampling port hypodermic needle very rapidly (1–2 s) into evacuated blood sampling tubes containing 2.0 ml 1 N perchloric acid, which had been precooled to −20°C for 10 min in an ice/NaCl mixture (10 : 2, w/w). Immediately after the sample had been collected the temperature was 1–2°C and quenching was considered to be very rapid. After shaking and cooling for 3 min, the quenched samples were transferred to Corex centrifuge tubes and stood in ice for 25 min with occasional vigorous shaking. Cells were then removed by centrifugation for 10 min at $10\,000 \times g$ at 0°C. The extract was neutralized with calculated amounts of KOH and buffered with 1.0 ml of 0.25 M Hepe/magnesium acetate buffer, pH 7.5. The pH was further adjusted to between 6.8 and 7.0 and the extracts centrifuged at $10\,000 \times g$ for 10 min to remove $KClO_4$. An aliquot (0.9 ml) of the neutralized extract was incubated with 20 μg pyruvate kinase (Sigma) and 20 μmol phospho*enol* pyruvate to convert ADP to ATP. Another aliquot (0.9 ml) was incubated with the same mixture plus 20 μg adenylate kinase (Sigma) to convert AMP to ATP [21]. The original extract and the two incubated extracts were then used for assay of ATP by the luciferase-luciferin assay as described by Stanley and Williams [22]. Concentrations of ADP and AMP were estimated by difference.

The recovery of standards by the enzymatic conversion method was 96.4 ± 6.5% (mean and S.D.) for ATP, 94.8 ± 7.6% for ADP and 101.0 ± 17.7% for AMP. The recovery of standards by the whole extraction and conversion procedure in the presence of the bacteria was 78.9 ± 7.1% for ATP, 70.2 ± 3.4% for ADP and 80.0 ± 9.2% for AMP.

The cellular energy charge (EC) was calculated as

$$EC = \frac{[ATP] + \frac{1}{2}[ADP]}{[ATP] + [ADP] + [AMP]} \quad (1)$$

Assays of cyclic AMP and cyclic GMP. Duplicate samples (10–14 ml) of culture, containing 1.5–3.0 mg (dry wt.) (0.8–1.6 mg protein, Ref. 19), were collected and extracted as described by Lim et al. [18]. The extract was assayed by the specific radiochemical method [23], using kits prepared by New England Nuclear, Boston. The recovery of 50 000 cpm cyclic [^{3}H]AMP and cyclic [^{3}H]GMP with the extraction procedure in the presence of the bacteria was 69.5 and 71.4%, respectively (based on duplicate estimations which differed by less than 5% of the mean).

TABLE I

PROPERTIES OF CONTINUOUS CULTURES OF *RHIZOBIUM* SP. STRAIN CB756 DURING STEADY STATES WITH VARIOUS AERATION RATES

The medium contained 6 mM NH_4^+. The culture volume was 570–600 ml, depending on stirring rate. Y_{O_2} is the yield of cells (g) per mol O_2 consumed. AP is the total of ATP + ADP + AMP. Values in brackets are the numbers of steady states measured (*n*).

Culture state	Dilution rate (culture vol./h)	Dissolved O_2 (μM)	O_2 used (μmol/h per ml)	Culture density (μg/ml)	Culture yield (mg/h)	Y_{O_2}	Energy status of bacteria				Nitrogenase activity (nmol C_2H_4/h per mg)
							Total AP (nmol /mg)	ATP (nmol /mg)	ATP /ADP	Energy charge	
NH_4^+-limited, high aeration	0.055	176	0.80	292	9.2	20.2	2.95	1.87	2.7	0.75	0
	0.056	173	0.85	321	10.2	21.0	2.50	1.85	4.2	0.83	0
	0.108	1.45	1.32	320	19.7	26.2	3.53	2.52	4.5	0.79	0
O_2-limited, low aeration											
Mean	0.059	0.15	1.16	262	9.0	13.2	2.91	2.12	5.1	0.81	0
S.D. (n = 5)	0.006	0.03	0.09	13	1.8	3.6	0.90	0.70	2.0	0.02	
O_2-limited, microaeration											
Mean	0.054	<0.03	0.55	136	4.4	12.6	2.33	1.23	2.0	0.66	199
S.D. (n = 7)	0.004	–	0.16	18	0.9	2.9	0.52	0.25	0.7	0.05	44

Results and Discussion

Relationships between ATP pool size, energy charge and nitrogenase

Rhizobium sp. strain CB756 is a slow-growing type with a generation time of about 20 h in the O_2-limited conditions of these experiments (Table I). A steady state can be achieved usually within three generations of a shift in aeration. In preliminary experiments with steady-state, O_2-limited cultures, growing with dilution rates of about 0.05/h and O_2 supplied at 0.3–1.2 μmol/ml per h, nitrogenase was detected only when the cellular ATP pool size was between 0.8 and 1.8 nmol/mg dry wt., with a sharp optimum at 1.1 nmol/mg (Fig. 2). Between 1.0 and 2.0 nmol ATP/mg, nitrogenase activity was negatively correlated with ATP content (r = –0.95). The energy change of nitrogenase-active steady-state cultures was in the range 0.57–0.74. Between energy charge values of 0.65 and 0.8, energy charge was negatively correlated with nitrogenase activity (r = 0.95).

These relationships are not in conflict with previous results in which ATP/ADP ratios were positively correlated with nitrogenase activity in assays with bacteroids prepared from soybean root nodules [6]. In the experiments reported in the present paper, measurements of nitrogenase activity were made in standard shaken assays supplied with oxyleghaemoglobin. From other work, these conditions were known to produce ATP/ADP ratios which were opti-

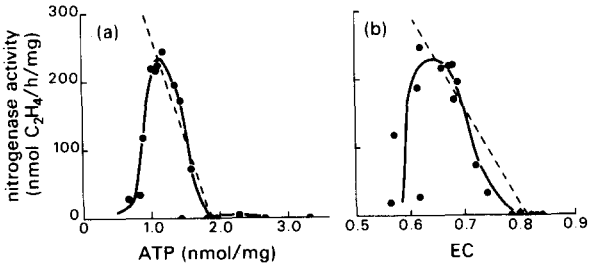

Fig. 2. Nitrogenase activity in samples from continuous cultures of *Rhizobium* sp. strain CB756 in relation to (a) ATP concentration and (b) energy charge in the bacteria. All data are from cultures with steady rates of nitrogenase activity. Between 1 and 2 nmol ATP/mg dry wt. of cells, nitrogenase activity of samples was negatively correlated (r = –0.95) with the ATP pool size and energy charge of the culture. EC, energy charge.

mum for nitrogenase activity [6,25–27]. However the energy parameters were measured using samples taken from the chemostat and refer to the conditions in which nitrogenase was produced; they do not refer to the energy status during the assays of nitrogenase activity. The standard nitrogenase assay probably reflects the total amount of enzyme present but a quantitative measurement of the actual amount of nitrogenase protein (as in Ref. 18) would have been preferable. However, this was not possible with the low-density cultures used, without taking unacceptably large samples.

Comparison of steady-state values with three different aeration rates

Data from 15 steady states are given in Table I. In these experiments, O_2 supply was adjusted to give microaeration conditions (about 0.5 μmol O_2/h per ml of culture), with active nitrogenase in the cultures, or low aeration conditions (about 1.0 μmol O_2/h per ml) with no detectable nitrogenase activity. Both of these conditions produced O_2-limited cultures since yield increased linearly with O_2 supply up to 1.6 μmol O_2/h per ml, Fig. 1), excess NH_4^+ was present and the bacteria contained 51–53% protein. A third condition, with high concentrations of dissolved O_2 (145–176 μM) was added for comparison. With two rates of dilution and analysis of effluent culture for cell yield and protein content and residual NH_4^+ [13], results showed that these highly aerated cultures were NH_4^+-limited.

Highly aerated, NH_4^+-limited cultures responded to increased supply of the limiting nutrient with increased O_2 consumption (cf. Ref. 16), increased total adenosine phosphates and ATP, but energy charge did not alter significantly (Table I).

In cultures in which the dissolved O_2 concentration was reduced by three orders of magnitude and O_2 became the limiting nutrient (low aeration, Table I), Y_{O_2} (g of cell dry wt. produced per mol of O_2 consumed) was reduced significantly but there were only small changes in the energy status of the bacteria.

Upon further reduction of the dissolved O_2 concentration and stirring rate (microaeration) so that O_2 consumption was halved compared with low aeration, culture yield and all measured energy parameters were reduced (for example energy charge declined from 0.81 to 0.66, and ATP from 2.1 to 1.2 nmol/mg) but there was no significant change in Y_{O_2} (Table I). In these conditions there were quite high levels of nitrogenase activity in the bacteria. It is noteworthy that, whilst energy charge values for highly aerated cultures and cultures with low aeration were normal for actively growing bacteria, the cellular ATP pools of these slow-growing cultures were at the low end of the observed range for other bacteria (2–10 μmol/g; Ref. 1). Under microaeration, the ATP pool size declined still further and this would be consistent with failure of adenylylation of glutamine synthetase in the presence of an excess of NH_4^+ due to shortage of ATP, as observed in previous work [12, 13]. The failure of repression of nitrogenase synthesis under these conditions may mean that adenylated glutamine synthetase is involved in repression as suggested for *Klebsiella pneumoniae* (reivewed in Ref. 10). Alternatively the diminished ATP pool and energy charge may be common to both regulation of glutamine synthetase activity and nitrogenase synthesis.

It has already been found that, under the conditions which favour nitrogenase synthesis, nitrogen fixation in the chemostat provides only a relatively small proportion of the nitrogen assimilated by the bacteria [25]. In the O_2-limited conditions in which nitrogenase is induced, the energy status of the culture is sufficient to sustain only low cell densities, leaving little capacity for the utilization of ATP in nitrogenase activity. The rates of nitrogen fixation which have been observed in chemostat cultures [25] account for less than 1% of the energy used in growth, if it is assumed that 4 mol of ATP are hydrolyzed per electron pair transferred to nitrogen [24] and that $Y_{ATP} = 10$ (Ref. 2). In contrast, in root nodules where bacterial growth is suppressed, bacteroids are able to devote a much greater proportion of their available energy to nitrogenase activity.

Changes in energy status and growth of cultures during derepression of nitrogenase

When the O_2 supply to a steady-state culture was decreased from the low to the micro level, growth was arrested for about 1 h. Nitrogenase activity was detected 1.5 h later and increased slowly to a maximum after about 30 h (see also Ref. 13). A typical experiment is illustrated in Fig. 3. The results suggest

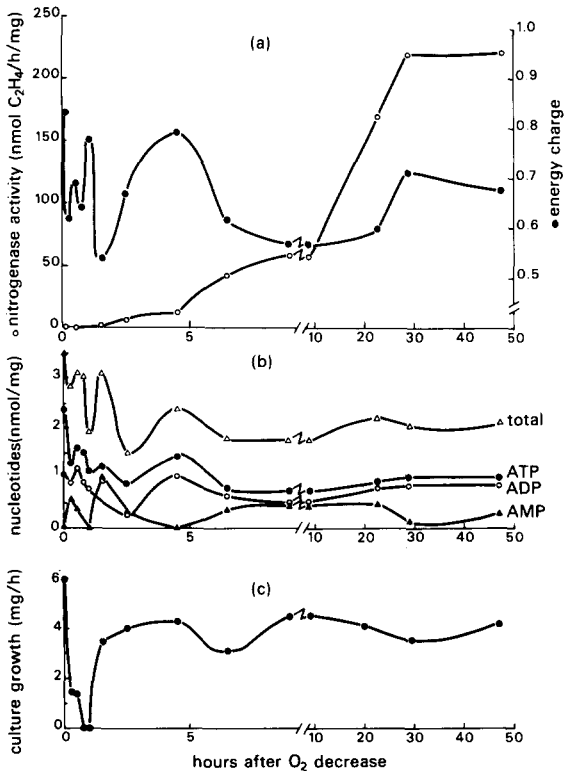

Fig. 3. The time course of changes in growth rate and energy status of continuous culture of *Rhizobium* sp. strain CB756 during the derepression of nitrogenase activity following the reduction in O_2 supply from 1.3 to 0.6 μmol O_2/ml per h. (a) Nitrogenase activity (○) and energy charge (●); (b) total adenosine phosphates (△), ATP (●), ADP (○) and AMP (▲); (c) culture growth (●).

that upon derepression, the *nif* genes may be transcribed in relation to growth. Therefore, nitrogenase activity only reaches a maximum value after one complete growth cycle, the generation time in these cultures being about 18–20 h. Other factors contributing to the slow increase in nitrogenase activity could be the low energy status of the culture and possible degradation of nitrogenase resulting from exposure of the culture surface to O_2.

The change in O_2 supply initiated oscillations in growth rate and energy status which persisted with decreasing amplitude and freqency for at least one generation time (Fig. 3), finally stabilizing near the mean values for the microaeration steady states reported in Table I. These oscillations prevented establishment of a clear relationship between any of the energy parameters and nitrogenase synthesis. However, nitrogenase was initially detected following a decline in ATP pool size and in energy charge. Also, the rapid increase of nitrogenase activity between 4.5 and 9 h corresponded with a decline in ATP and energy charge following rapid oscillations, during which nitrogenase activity increased only slowly (Fig. 3a and b). Similar checks and accelerations in nitrogenase increase were reported previously [13], and they corresponded with temporary increases in glutamine synthetase adenylylation, about 5 h after O_2 supply was reduced. In other similar experiments, there were temporal, quantitative differences from those shown in Fig. 3 but the same qualitative features were present.

Changes in energy status and growth of cultures during repression of nitrogenase

Following the increase of O_2 supply to microaerated steady-state cultures, nitrogenase activity decayed exponentially with a half-time of about 0.5 h (Fig. 4a, Table IIA) in some experiments, or more slowly when the dissolved O_2 concentration was lower (Table II, B and C). These results indicate that degradation of nitrogenase by O_2 may be substantial in these cultures.

Again the change in O_2 supply initiated oscillations in growth and energy metabolism, but these were much shorter-lived than with decreased O_2 supply (cf. Fig. 3b and c with 4b and c). Energy charge was close to a new equilibrium value about 1.5 h after the change (Table II) and growth rate and ATP concentration were close to their new equilibrium values within 5–6 h. The apparently anomalously high values of ATP/ADP in experiments A and C (Table II) are due to very low values for ADP during periods of rapid ATP formation. They illustrate the superiority of using energy charge values rather ATP/ADP ratios to describe energy status of the culture. With lower rates of increased O_2 supply, the ATP increases were lower, and decay of nitrogenase slower (Table IIC).

Content of cyclic AMP and cyclic GMP in steady states

From four steady state cultures with lower aeration and zero nitrogenase activity, values of 5.2 ± 1.1 (mean ± S.D.) pmol cyclic AMP/mg dry wt. and 0.12 ± 0.05 pmol cyclic GMP/mg were obtained.

TABLE II

EFFECTS OF INCREASED O_2 SUPPLY ON CONTINUOUS CULTURES OF *RHIZOBIUM* SP. STRAIN CB756 WHICH WERE INITIALLY IN MICRO-AERATED, STEADY STATES WITH ACTIVE NITROGENASE

The early course of experiment A is seen in Fig. 3. Other details as in Table I. At zero time, the stirring rate and head-space O_2 concentration was increased to raise the concentration of dissolved O_2 to about 0.1 μM, using the feed-back stirrer controller. O_2 consumption rates were calculated from the time trace of the O_2 electrode current, the observed maximum and minimum stirrer speeds, and the O_2 solution rate calibration for those stirrer speeds. The concentration of dissolved O_2 is the average for the controlling cycle. Because of frequent sampling, the culture volume and dilution rate changed and this was taken into account when calculating culture yield, O_2 usage, etc.

Expt. No.	Time from change (h)	Dissolved O_2 (μM)	O_2 used (μmol/h per ml)	Culture density (μg/ml)	Culture yield (mg/h)	Y_{O_2}	Energy status of bacteria				Nitrogenase activity (nmol C_2H_4/mg per h)
							Total AP (nmol/mg)	ATP (nmol/mg)	ATP/ADP	Energy charge	
A	0	0.02	0.63	136	4.6	12.2	2.7	1.4	1.7	0.69	170
	0.5	0.14	1.37	146	15.9	21.1	2.6	2.5	24.1	0.98	89
	1.5	0.14	1.48	160	12.4	16.5	2.4	2.1	8.5	0.89	38
	2.5	0.14	1.37	162	6.4	9.5	2.8	2.2	3.4	0.89	21
	4.5	0.16	1.45	182	10.8	14.9	2.4	2.0	21.6	0.84	5
	6.5	0.12	1.57	201	11.7	14.2	3.0	2.2	3.5	0.82	2
B	0	0.02	0.60	125	4.2	11.7	2.1	1.0	1.1	0.67	221
	0.5	0.08	1.42	129	9.9	12.5	2.2	1.4	3.1	0.74	182
	1.5	0.11	1.22	135	7.6	12.0	2.5	2.1	5.4	0.92	109
	2.5	0.03	1.42	138	6.2	8.7	1.5	1.3	4.7	0.91	107
	4.5	0.11	1.10	148	7.1	12.9	2.0	1.4	2.5	0.86	66
	6.5	0.11	1.30	161	8.6	12.7	2.2	1.7	4.2	0.83	22
C	0	0.02	0.57	121	3.7	10.8	2.6	1.2	1.3	0.62	246
	0.5	0.02	1.20	121	3.7	5.5	2.4	2.0	9.7	0.87	249
	1.5	0.11	1.18	125	5.8	9.6	2.7	2.1	3.3	0.88	218
	2.5	0.11	0.95	127	4.8	10.2	2.1	1.7	4.4	0.91	140
	4.5	0.07	1.00	132	5.2	10.6	2.2	2.1	24.9	0.97	103
	6.5	0.11	1.16	142	6.7	11.4	1.9	1.6	7.1	0.92	66

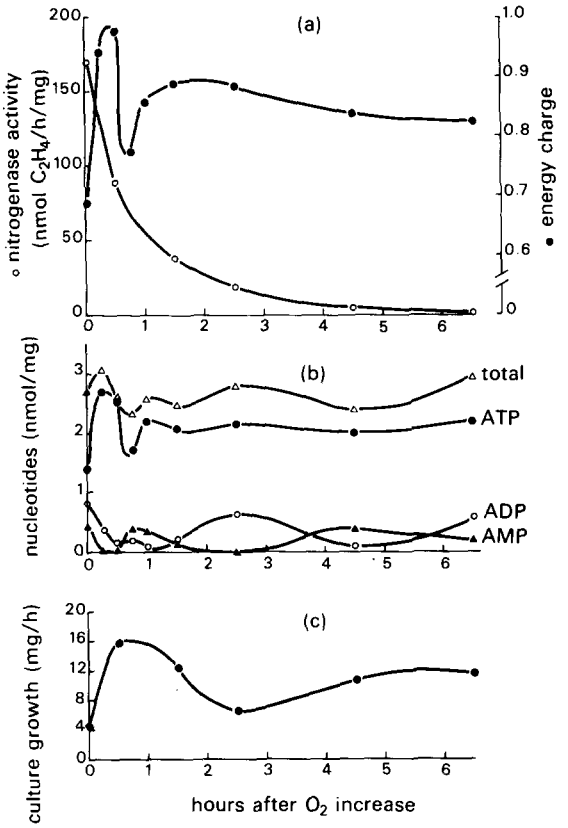

Fig. 4. The time of changes in growth rate and energy status of continuous cultures of *Rhizobium* sp. strain CB756 during repression of nitrogenase activity following the increase in O_2 supply from 0.6 to 1.4 μmol O_2/ml per h. Symbols as in Fig. 3.

From cultures with microaeration and active nitrogenase, values were 4.6 ± 0.4 and 0.41 ± 0.07, respectively for cyclic AMP and cyclic GMP. In terms of cell protein, these values should be doubled since the average protein content for this strain in these cultures is 53% dry wt. [19]. Lim et al. [18] reported that cyclic GMP increased from 0.25 to 2.6 pmol/mg protein when stationary phase cultures of *R. japonicum* with active nitrogenase were subjected to high aeration. They also found that expression of nitrogenase activity was inhibited by exogenous cyclic GMP at 0.1 mM, which is about three orders of magnitude higher than the endogenous concentration. Our results from O_2-limited continuous cultures do not support a role of cyclic GMP in repressing steady-state levels of nitrogenase activity in CB756.

Addendum

During revision of the manuscript, the work of Upchurch and Mortenson [28] came to our attention. These authors examined *Klebsiella pneumoniae*, *Clostridium pasteurianum* and *Azotobacter vinelandii* for changes in energy parameters during repression and derepression of nitrogenase in the presence or absence of NH_4^+. They found that energy charge of 0.8 or more was associated with repression and values in the range 0.53–0.75 were obtained with derepressed cultures. Although the bacteria and the form of our experiments were different, energy charge being varied by aeration rates, the values for energy charge of cultures producing nitrogenase were identical. This agreement seems to be strong evidence for a general role for energy status of cells in the regulation of nitrogenase synthesis.

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