Influence of Atmospheric Oxygen Concentration on Acetylene Reduction and Efficiency of Nitrogen Fixation in Intact *Klebsiella pneumoniae*

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SUMMARY

Oxygen-limited (N_2 -fixing) chemostat cultures of *Klebsiella pneumoniae* supplied with a N-free medium were established by introducing low atmospheric O_2 concentrations into the gas supply of anaerobic glucose-limited N_2 -fixing chemostat cultures; the molar growth yield for glucose and the efficiency of N_2 fixation (μg N fixed/mg glucose consumed) were increased (by up to 82 %) from the anaerobic values.

Acetylene-reducing activity was inhibited reversibly by O_2 in samples from O_2 -limited and anaerobic glucose-limited chemostat cultures. Oxygen uptake rates in samples from these chemostat cultures were similar, but C_2H_2 -reducing activity in samples from O_2 -limited chemostat cultures was more tolerant of low atmospheric O_2 concentrations, in part because of a higher population density. In the absence of glucose, O_2 was required at a low atmospheric concentration for C_2H_2 reduction in samples from either O_2 -limited or anaerobic glucose-limited chemostat cultures. The possibility is discussed that ATP generated from oxidative phosphorylation can be used for N_2 fixation in *K. pneumoniae*.

INTRODUCTION

Nitrogenase, the enzyme responsible for biological N_2 fixation from all sources examined so far, consists of two O_2 -sensitive proteins (see Eady & Postgate, 1974). However, nitrogenase in crude extracts prepared by decompression of an organism, is often less O_2 -sensitive than the component proteins, and shows some organism-to-organism differences in O_2 sensitivity. That from *Klebsiella pneumoniae* (Kelly, 1969) is more O_2 -tolerant than those from *Mycobacterium flavum* (Biggins & Postgate, 1971) and *Clostridium pasteurianum* (Kelly, 1969) but is less O_2 -tolerant than the air-stable preparation from *Azotobacter* (see Kelly, 1969, and Yates & Jones, 1974).

It has been suggested that the nitrogenase in intact *Azotobacter* is protected from O_2 by two mechanisms: respiratory protection, where a low dissolved O_2 tension is maintained by a controlled respiratory activity, and conformational protection, where the nitrogenase or its association with other proteins and membranes can undergo a controlled change so that the enzyme is both protected from O_2 and inactive towards reducible substrates (Dalton & Postgate, 1969; Yates, 1970; Drozd & Postgate, 1970; see also Yates & Jones, 1974). Other obligate aerobes such as *M. flavum* and *Derxia gummosa* are less O_2 -tolerant when fixing N_2 , probably because they do not possess such high respiratory rates as *Azotobacter* (see Yates & Jones, 1974).

Although O_2 probably represses nitrogenase synthesis in *K. pneumoniae* (St. John, Shah &

Brill, 1974), Klucas (1972) found that a species of *Klebsiella* synthesized nitrogenase when grown in a chemostat culture supplied with O_2 , provided that the medium contained a fixed N source (yeast extract) and the dissolved O_2 tension was at 10 mmHg or below. Klucas (1972) considered that nitrogenase activity did not depend on O_2 because C_2H_2 -reducing activity was irreversibly inhibited by low atmospheric O_2 concentrations. He suggested that O_2 tolerance occurred because of a high respiratory rate at low dissolved O_2 tensions (Harrison & Pirt, 1967; Harrison & Loveless, 1971), although he did not measure respiration rates.

Klebsiella pneumoniae M5a1 can grow and fix nitrogen on a solid medium in air (Hill, 1975). It then shows a colony dimorphism resembling that of *D. gummosa* in air, but unlike *D. gummosa* a low level of fixed N is required. Colony dimorphism in both organisms probably arises because only where respiratory activity is sufficient to lower the local O_2 tension, can N_2 fixation and subsequent growth to the large colony type take place. The present work shows that, like *D. gummosa* (Hill & Postgate, 1969), *K. pneumoniae* can grow in a liquid N-free medium supplied with O_2 under O_2 limitation. It also reports studies on the efficiency of N_2 fixation and the nature of O_2 inhibition of C_2H_2 -reducing activity in *K. pneumoniae* grown with and without O_2 .

METHODS

Organism and culture. *Klebsiella pneumoniae* M5a1, a gift from Professor P. W. Wilson, was maintained at 20 °C in air on 2% nutrient agar slopes and subcultured monthly. Chemostat cultures (200 ml fitted with PVC tubing; Baker, 1968) were inoculated with about 5 ml of an overnight culture grown on thioglycollate medium (Oxoid). The medium used for chemostat cultures contained (per litre): 12.06 g K_2HPO_4 , 3.4 g KH_2PO_4 , 26 mg $CaCl_2 \cdot 2H_2O$, 30 mg $MgSO_4$, 0.3 mg $MnSO_4$, 36 mg ferric citrate, 7.6 mg $Na_2MoO_4 \cdot 2H_2O$, 10 µg *p*-aminobenzoic acid, 5 µg biotin. The two vitamins are not in fact required by *K. pneumoniae*. Except where mentioned, growth was limited by glucose, present at from 15 to 30 mM. When required, sufficient $(NH_4)_2SO_4$, at 3 mM, was included to repress nitrogenase synthesis. Phosphate and $(NH_4)_2SO_4$ solutions were sterilized independently and subsequently added aseptically. Organisms were grown at 30 ± 0.5 °C under an atmosphere of either N_2 or $N_2 + O_2$ flowing at about 200 ml min⁻¹. Medium reservoirs were maintained under an atmosphere of N_2 . The buffering capacity of the medium was sufficient to maintain the pH at 6.7 ± 0.1 for the N_2 -fixing cultures and at 6.8 ± 0.1 for NH_4^+ -assimilating cultures. The criteria of glucose limitation were those of Hill, Drozd & Postgate (1972). The oxygen electrode, a Johnson, Borkowski & Engblom (1964) type (Western Biological, Sherborne, Dorset) fitted with a JB Laboratory Oxygen meter (Western Biological) and Griffin Nescro Recorder (Griffin & George, Wembley, Middlesex), was calibrated with distilled water at 30 °C in equilibrium with various $O_2 + N_2$ mixtures.

Assay for acetylene reduction. Nominally 25 ml conical flasks (35 ml) containing, when necessary, either 0.1 ml glucose or 0.1 ml glucose and 0.1 ml chloramphenicol (as specified) were flushed with Ar and capped with Suba-Seal closures (William Freeman Ltd, Barnsley, Yorkshire). Volumes of O_2 , to give the required atmospheric O_2 concentration, and of C_2H_2 (3.5 ml) were injected. Flasks were equilibrated at 30 °C for 5 min and the excess pressure released. Two ml of culture sample collected under N_2 were injected to start the assay, which was performed at 30 °C with a shaking rate of 84 strokes/min at an amplitude of 3.8 cm. Assays were stopped after the required incubation by injecting 0.1 ml of 40% (w/v) KOH and then gas samples (0.5 ml) were taken for analysis by gas chromatography.

Alternatively, for experiments to determine the percentage recovery of C₂H₂-reducing activity after exposure to O₂, gas samples (0.5 ml) for gas chromatography were removed at intervals during incubation and replaced by 0.5 ml Ar. Dilution of C₂H₄ was corrected for by measuring the concomitant dilution of C₂H₂. The amount of C₂H₄ produced was always <4% of the C₂H₂ present. During these experiments samples were flushed with Ar for 5 min by inserting 2 needles, one of which was connected to the Ar supply; after removing the needles, C₂H₂ (3.5 ml) was injected and the excess pressure released. Gas samples (0.5 ml) were injected into a Pye 104 gas chromatograph (Pye Unicam Ltd, Cambridge) fitted with a flame ionization detector and 45 cm column (1 mm internal diameter) filled with either Porapak R or Porapak N (mesh 100 to 120) and maintained at 37 °C with N₂ as carrier gas flowing at 7 ml min⁻¹. Ethylene peak heights were recorded and compared with standards.

Measurement of oxygen uptake rate. Chemostat culture sample (2 ml) was introduced into an O₂ electrode respirometer (Rank Bros, Bottisham, Cambridgeshire) and when required glucose (0.1 ml) was added. The ambient O₂ tension in the sample was raised to approximately 135 mmHg by vigorous stirring and bubbling air through the sample. The respirometer lid was replaced and the O₂ uptake measured (potential O₂ uptake rate). When the O₂ tension had dropped to near zero, the sample was again exposed to an ambient O₂ tension of about 135 mmHg. This cycle was repeated, after which the subsequent O₂ uptake rate was measured.

The rate of O₂ uptake (Q_{O_2}) was calculated from:

$$Q_{O_2} [\text{nmol min}^{-1} (\text{mg bacterial protein})^{-1}] = \frac{r}{2p}$$

where p is the concentration of bacterial protein/ml and r the rate of depletion of O₂ concentration from the O₂-electrode cell at 30 °C (nmol min⁻¹), which had previously been calibrated with 2 ml of air-saturated distilled H₂O at 30 °C. No correction was made for the influence of glucose, medium components and fermentation products on the solubility of O₂.

Analyses. Dry weights were determined by washing organisms once with distilled H₂O and drying to constant weight in preweighed dry beakers at 80 °C. Dried material was cooled in the presence of a desiccant and then weighed; two empty beakers were included throughout the drying and weighing procedure as a control for the condensation of moisture in the air on the glass surfaces during weighing. A minimum of two estimates in duplicate were made on each steady state. The standard deviation of the method was 5%.

Measurements of extinction of culture, at 540 nm in an EEL Spectra against a water blank, were used only to monitor steady states.

Protein was estimated on washed organisms by the procedure of Lowry *et al.* (1951). A minimum of two estimates in duplicate was made on each steady state. The standard deviation of the method was 5%.

Total N of organisms was estimated on washed dried organisms, prepared as for dry weight, by a model 185 CHN analyser (Hewlett & Packard, F and M Scientific Division, Avondale, Pennsylvania, U.S.A.). Initially the standard deviation of estimates of percentage N in four samples of dried organisms from a single steady state was less than 1%, so subsequently, dried material from a single steady state was pooled and the N content estimated singly. Such estimates were more reproducible and not lower than measurements of the amount of N₂ fixed made by micro-Kjeldahl digestion of samples of culture and of the medium, with analysis of NH₃ (Chaney & Marback, 1962) diffused from the digests.

Glucose in cultures and media was estimated by a modification of the method using glucose oxidase and peroxidase described in Sigma's *Technical Bulletin No. 510*. The peroxidase-glucose oxidase enzyme was resuspended in 40 ml H_2O instead of the recommended 100 ml. Incubation was carried out in the dark and 0.5 ml of 0.5 M- H_2SO_4 was added to stabilize the light-sensitive coloured product (Møller & Ottolenghi, 1966) before E_{400} was recorded. This procedure did not detect concentrations of glucose lower than 0.02 mM. For estimates of the concentration of glucose in the medium, two separate dilutions differing by a factor of two were usually made, and each dilution was estimated in duplicate. The standard deviation of the method was 4%. 'Clinistic' reagent strips (Ames Co., Slough, Buckinghamshire) were used for the rapid detection of glucose in cultures; this qualitative method did not detect concentrations of glucose below about 0.3 mM.

RESULTS

The efficiency of nitrogen fixation in oxygen-limited chemostat cultures

An anaerobic glucose-limited chemostat culture of *K. pneumoniae* was established in the N-free medium at a dilution rate (D) of about 0.1 h^{-1} . By introducing O_2 into the gas supply gradually, in increments of about 0.004 atm after every two replacements of culture volume, steady states were obtained where the dissolved O_2 tension was apparently zero and the residual glucose concentration was less than 0.02 mM. These steady states were presumed to be O_2 -limited because (i) the dissolved O_2 tension was apparently zero and (ii) increases in supplied O_2 concentration, provided by increasing either the atmospheric P_{O_2} or the agitation rate, caused increases in the molar growth yield for glucose (Table 1). An increase in molar growth yield caused by providing O_2 suggests that some of the ATP required for growth was generated by oxidative phosphorylation.

In O_2 -limited chemostat cultures of a facultative anaerobe where ATP is generated by oxidative phosphorylation, the increase in molar growth yield for glucose should be influenced not only by the supplied atmospheric P_{O_2} and the agitation rate, but also by the dilution rate and the ratio of supplied concentrations of O_2 and glucose (Pirt, 1957). Although in the present work the dilution rate was maintained near 0.1 h^{-1} the differences in supplied glucose concentration (Table 1) may have influenced the magnitude of the molar growth yield.

The change from anaerobic glucose-limited growth to O_2 -limited growth did not influence the N content of the organisms. Therefore the efficiency of N_2 fixation, like the molar growth yield, was also increased. The largest increase was 82% (Table 1).

Requirement for oxygen for acetylene reduction

If O_2 -limited N_2 -fixing chemostat cultures of *K. pneumoniae* generate ATP by oxidative phosphorylation, N_2 fixation by these populations might require O_2 . This was found to be the case in samples not supplemented with glucose, where C_2H_2 -reducing activity required O_2 . The activity rose with increasing partial pressure to 0.02 atm; higher partial pressures of O_2 inhibited activity. Maximal activity was only 20% of that of an equivalent sample containing glucose (Fig. 1). The anaerobic C_2H_2 -reducing activity of samples from an O_2 -limited chemostat culture required the addition of glucose. When glucose was added, the only significant influence of atmospheric O_2 was to inhibit the activity above about 0.02 atm (Fig. 1).

Samples from an anaerobic glucose-limited N_2 -fixing chemostat culture behaved similarly. Under anaerobic conditions, C_2H_2 -reducing activity required glucose, and was

Table 1. *Increases of efficiency of N₂ fixation and molar growth yield for glucose (Y_{glucose}) in O₂-limited chemostat cultures*

<i>D</i> (h ⁻¹)	Supplied atmospheric <i>P</i> _{O₂} to culture	Agitation rate of culture (rev./min)	Supplied glucose concentration (mM)	Y _{glucose} (g mol ⁻¹)	Efficiency of N ₂ fixation (μg N ₂ fixed/mg glucose consumed)
0.103	0	558	31.1	11.1	7.9
0.104	0.005	558	31.1	12.4	9.0
0.106	0.035	558	35.3	15.6	11.0
0.110	0.019	595	23.9	18.2	13.2
0.110	0.025	595	25.1	20.4	14.5

Fig. 1. Influence of atmospheric P_{O_2} on C_2H_4 -reducing activity in samples from an O_2 -limited ($\square$, $\blacksquare$) and from anaerobic glucose limited ($\circ$, $\bullet$) chemostat cultures. Samples were either supplemented with 60 mM-glucose (closed symbols) or unsupplemented (open symbols). The standard deviations of estimates are indicated by bars to the right and left of the symbols, for samples from O_2 -limited and from anaerobic glucose-limited chemostat cultures respectively. The population density of the O_2 -limited chemostat culture was 0.434 mg dry weight/ml and that of the anaerobic glucose chemostat cultures was either 0.343 or 0.392 mg/ml; the O_2 -limited chemostat culture was supplied with atmospheric oxygen of P_{O_2} 0.019.

strongly inhibited by O_2 . However, in samples not supplemented with glucose, C_2H_2 -reducing activity required O_2 . The maximum activity under 0.02 to 0.025 atm O_2 was about 40 % of that in equivalent samples containing glucose (Fig. 1).

Tolerance to oxygen of acetylene reduction

The tolerance of C_2H_2 -reducing activity to low atmospheric O_2 concentrations was slightly higher in glucose-supplemented samples from the O_2 -limited chemostat cultures than from the anaerobic glucose-limited chemostat cultures (Fig. 1). Tolerance of C_2H_2 -reducing activity to 0.03 atm O_2 in glucose-supplemented samples from various O_2 -limited

Table 2. *Tolerance of C₂H₂-reducing activity to 0.03 atm O₂ in samples from various O₂-limited and anaerobic glucose-limited chemostat cultures. For comparison, the population density and the increase in molar growth yield (Y_{glucose}) are also shown*

Supplied atmospheric P _{O₂} to culture	Population density (mg dry weight/ml)	Increase in Y _{glucose} from anaerobic value	Tolerance of C ₂ H ₂ -reducing activity to 0.03 atm O ₂ *
0	0.392	0	41 (1)
0	0.343	0	49 (1)
0	0.346	0	46 ± 13 (4)
0.005	0.387	Marginal	41, 43 (2)
0.035	0.549	1.4	59 (1)
0.019	0.434	1.6	69 ± 4 (3)
0.025	0.515	1.8	88 (1)

* The tolerance of activity, defined as the percentage of C₂H₂-reducing activity found under 0.03 atm O₂ compared with the activity found anaerobically, was estimated as C₂H₄ produced after 10 min incubation in samples supplemented with glucose. The number in parenthesis indicates the numbers of estimates; where the number of estimates exceeds 2, the standard deviation is given.

and anaerobic glucose-limited chemostat cultures are compared in Table 2. The tolerance was greater in samples from O₂-limited than from anaerobic glucose-limited chemostat cultures, and can be correlated with the increase in molar growth yield from the anaerobic value rather than with the increase in population density (Table 2).

Reversal of oxygen inhibition of acetylene-reducing activity

Acetylene-reducing activity was completely inhibited by 0.2 atm O₂ over samples removed from an anaerobic glucose-limited chemostat culture and supplemented with glucose and chloramphenicol. Activity was partially restored when the O₂ had been removed by flushing the samples with Ar for 5 min (Fig. 2*a*). Maximum activity in samples exposed to O₂ was preceded by a lag period and depended on the time of exposure to O₂; the activity was 52 % lower in samples exposed for 25 min than in samples exposed for 12 min.

Complete inhibition of C₂H₂-reducing activity under 0.2 atm O₂ also occurred in samples from O₂-limited chemostat cultures, and this also was partially reversible. The percentage recovery of C₂H₂-reducing activity (see legend to Fig. 2) after 12 min exposure to 0.2 atm O₂ was 43 % in samples from the anaerobic glucose-limited chemostat culture, and 46 and 68 % with O₂-limited populations grown with 0.005 and 0.035 atm O₂ respectively (Table 3). The lag period following exposure to O₂ before C₂H₂-reducing activity became linear was 43 % shorter in samples from the O₂-limited chemostat cultures supplied with 0.035 atm O₂ (Table 3).

In the control samples which contained chloramphenicol and glucose, the rate of C₂H₂-reduction in the first 12 min was lower than the rate observed after the sample had been flushed with Ar for 5 min (Fig. 2*a*). Samples from the O₂-limited chemostat cultures supplied with 0.035 atm O₂ showed no such increase (Fig. 2*b*; Table 3).

During the prolonged incubation following flushing with Ar, anaerobic C₂H₂-reducing activity in samples from both O₂-limited chemostat cultures declined slightly (Fig. 2*b*). Whether this decrease was due to specifically unstable nitrogenase, exhaustion of substrates for C₂H₂ reduction or appearance of an inhibitor was not established, but chloramphenicol was not involved since the same effect was observed in its absence.

Fig. 2. Reversible inhibition of C_2H_2 -reducing activity by $0.2 \text{ atm } O_2$ in samples from (a) an anaerobic glucose-limited chemostat culture and (b) an O_2 -limited chemostat culture supplied with $0.035 \text{ atm } O_2$. Samples, supplemented with 60 mM -glucose and 0.31 mM -chloramphenicol were exposed for 12 min initially to either $0.2 \text{ atm } O_2 + 0.1 \text{ atm } C_2H_2$ in Ar (○) or $0.1 \text{ atm } C_2H_2$ in Ar (●). Both samples were then flushed with Ar for 5 min, when C_2H_2 to 0.1 atm was injected. Gas samples were removed at intervals for analyses. The percentage recovery of C_2H_2 -reducing activity in the O_2 -treated sample (see Table 3) was estimated by comparing the rate of subsequent linear anaerobic C_2H_2 reduction in the O_2 -treated sample with the final rate of C_2H_2 reduction in the control sample.

Potential oxygen uptake rates

A higher potential for O_2 removal might account for the greater tolerance of C_2H_2 -reducing activity towards O_2 in samples from O_2 -limited chemostat cultures compared with those from anaerobic glucose-limited ones. Therefore the potential O_2 -uptake rates in samples from O_2 -limited N_2 -fixing chemostat cultures, an anaerobic glucose-limited N_2 -fixing chemostat culture and an anaerobic glucose-limited NH_4^+ -assimilating chemostat

Table 3. *Comparison of recovery of anaerobic C₂H₂-reducing activity after exposure to 0.2 atm O₂ for 12 min in glucose- and chloramphenicol-supplemented samples from O₂-limited and anaerobic glucose-limited chemostat cultures*

Each estimate was made in duplicate, therefore the range of estimates is indicated.

Supplied atmospheric P _{O₂} to culture	Population density (mg dry weight/ml)	Recovery of C ₂ H ₂ -reducing activity		Increase in C ₂ H ₂ -reducing activity in control sample† (%)
		Recovery of activity after O ₂ treatment* (%)	Lag after O ₂ treatment before linear C ₂ H ₂ reduction (min)	
0	0.346	43 ± 3	64 ± 8	25 ± 6
0.005	0.387	46 ± 3	58 ± 7	17 ± 4
0.035	0.549	68 ± 6	35 ± 2	0

* The estimate of percentage recovery of C₂H₂-reducing activity after O₂ treatment is defined in the legend to Fig. 2.

† See text.

Table 4. *Comparison of potential O₂-uptake rates (Q_{O₂}) and apparent sensitivity of O₂ uptake to O₂ in samples from O₂-limited and from anaerobic glucose-limited chemostat cultures*

N source for culture	D (h ⁻¹)	Supplied atmospheric P _{O₂} to culture	Population density (mg dry weight/ml)	Potential Q _{O₂} * [nmol (mg bacterial protein) ⁻¹ min ⁻¹]		Sensitivity of O ₂ uptake*†
				Without added glucose	With added glucose (60 mM)	
N ₂	0.103	0	0.346	188 ± 11 (3)	299 ± 40 (4)	64, 57 (2)
	0.104	0.005	0.387	198 ± 22 (3)	288 ± 12 (4)	24 ± 5 (3)
	0.106	0.035	0.549	256 264 (2)	305 320 (2)	11 (1)
NH ₄ ⁺	0.102	0	0.429	90 ± 22 (3)	231 ± 28 (3)	10 ± 6 (3)

* The numbers in parenthesis indicate the number of estimates; where the number of estimates exceeds two the standard deviation is given.

† The apparent sensitivity of O₂ uptake to O₂ is defined as the percentage loss of O₂ uptake rate in a sample containing glucose after three consecutive exposures to an ambient O₂ tension of approximately 135 mmHg (see Methods).

culture were compared. In all samples the potential O₂-uptake rate was increased by adding glucose. When glucose was added the potential O₂-uptake rates were not significantly different in samples from these chemostat cultures (Table 4). After a second and third exposure to the same dissolved oxygen tension in the respirometer, the subsequent O₂-uptake rate in samples was lower. This apparent sensitivity of the O₂-uptake mechanism was more marked in samples from the anaerobic glucose-limited N₂-fixing chemostat culture than in samples from the O₂-limited N₂-fixing chemostat cultures, and was probably in part associated with the N₂-fixing process because samples from the anaerobic glucose-limited NH₄⁺-assimilating chemostat cultures did not show the same sensitivity (Table 4).

DISCUSSION

The increase in efficiency of nitrogen fixation. The increases in molar growth yield and efficiency of N₂ fixation when O₂ was supplied to the chemostat culture suggests that a proportion of the ATP required for N₂ fixation and growth was generated by oxidative

phosphorylation. On the other hand, these increases may reflect a lowering of the amount of ATP required for N₂ fixation. However, estimates of the apparent ATP requirement for N₂ fixation in O₂-limited chemostat cultures of *K. pneumoniae* (Hill, unpublished) were not lower than those in anaerobic glucose-limited chemostat cultures (Hill, Drozd & Postgate, 1972). Additional circumstantial evidence in the present work suggests that ATP produced from oxidative phosphorylation could be used for N₂ fixation in the O₂-limited chemostat culture, since C₂H₂-reducing activity in samples from this chemostat culture required O₂ when glucose was not provided. This phenomenon was also observed in samples from an anaerobic glucose-limited chemostat culture, which implies that the enzymic apparatus for oxidative phosphorylation is present during anaerobic growth. Other facultative anaerobes growing anaerobically are known to contain some of the components of oxidative phosphorylation (see Wimpenny, 1969). *Klebsiella aerogenes* [NCIB8017; this strain has been shown to fix N₂ (Dixon, 1972)] grown anaerobically in glucose-limited chemostat culture supplied with NH₄⁺ contained cytochromes *a*₁, *a*₂ and *b*₁ (Harrison, 1972). Thus, contrary to the conclusions of Klucas (1972), it seems likely that ATP produced from oxidative phosphorylation can be used for N₂ fixation in *K. pneumoniae*.

Protection of nitrogenase from oxygen. Klucas (1972) found that the inhibition of C₂H₂-reducing activity under 0.1 atm O₂ in a species of *Klebsiella* was irreversible, although the length of exposure to O₂ and the time of incubation under anaerobic conditions following O₂ treatment were not specified.

In samples from anaerobic glucose-limited and O₂-limited chemostat cultures of *K. pneumoniae* a proportion of the C₂H₂-reducing activity, completely inhibited under 0.2 atm O₂, returned when O₂ was removed. This return of activity was not due to protein synthesis because chloramphenicol was present and is known to inhibit protein synthesis in this strain of *K. pneumoniae* (Tubb & Postgate, 1973). However, in the control samples, removal of C₂H₂ seemed to activate primarily inactive nitrogenase and comparable activation may have happened in the O₂-treated samples, although the magnitude of this effect in the control samples indicates that it could only account for a part of the recovery. When O₂ treatment of samples from the anaerobic chemostat culture was lengthened from 12 to 25 min, the recovery of C₂H₂-reducing activity was less. This indicates that the irreversible damage caused by O₂ increased with time. Consequently, recovery of activity was probably due to the remaining undamaged nitrogenase, and was not caused by activation of inactive nitrogenase. Thus a proportion of the C₂H₂-reducing activity, completely inhibited by O₂, was reversible.

It follows that nitrogenase in *K. pneumoniae* may be protected from O₂ by some process analogous to the conformational change proposed to explain the 'switch on-off' phenomenon in *Azotobacter* (see Yates & Jones, 1974). Hence this phenomenon, whereby O₂ damage to nitrogenase is prevented and C₂H₂ (and by implication N₂) reduction does not occur, is not a specific characteristic of obligate aerobic N₂ fixers.

An increase in C₂H₂-reducing activity in the absence of protein synthesis *de novo* was observed in an anaerobic N₂-fixing SO₄²⁻-limited chemostat culture of *K. pneumoniae* by Tubb & Postgate (1973), who suggested that activation of inactive nitrogenase might account for the increase in activity. Since I did not observe any increase in C₂H₂-reducing activity in samples from anaerobic glucose-limited chemostat cultures with chloramphenicol when C₂H₂ was present, it is likely that C₂H₂ inhibits the increase in nitrogenase activity; the inhibition was temporarily lifted when samples were flushed with Ar for 5 min.

Samples from O₂-limited chemostat cultures, when compared with those from anaerobic glucose-limited chemostat cultures, showed (i) a greater tolerance of C₂H₂-reducing activity

to O_2 (Fig. 1; Table 2) and (ii) a greater percentage and faster recovery of C_2H_2 -reducing activity following complete inhibition of activity by O_2 (Table 3). Although the initial potential O_2 -uptake rates were similar in samples from O_2 -limited and from anaerobic glucose-limited chemostat cultures, samples from the former chemostat cultures showed a greater capacity for continued O_2 uptake. Oxygen-limited chemostat cultures also had a greater population density and a higher molar growth yield than the anaerobic glucose-limited chemostat cultures. The higher population density, together with a greater capacity for continued O_2 uptake, would increase the rate of O_2 removal and thus prevent O_2 from reaching the O_2 -sensitive sites of the C_2H_2 -reducing process. On the other hand, the degree of recovery of C_2H_2 -reducing activity after complete inhibition by O_2 may reflect the supply of a limiting substrate rather than the level of nitrogenase present.

Oxygen uptake, supported by the endogenous source of reducing power in samples from anaerobic glucose-limited and from O_2 -limited chemostat cultures, could account for the ability of these unsupplemented samples to reduce C_2H_2 in low atmospheric O_2 concentrations. Under these conditions the endogenous source of reducing power would probably supply electrons for O_2 uptake, concomitant ATP production and for C_2H_2 reduction.

Thus nitrogenase in *Klebsiella* is probably protected from O_2 by both respiratory protection and conformational protection as in *Azobacter*. The potential O_2 -uptake rates in samples from anaerobic glucose-limited and from O_2 -limited chemostat cultures of *K. pneumoniae* were similar to those found by Harrison & Loveless (1971) in samples from anaerobic glucose-limited and from O_2 -limited chemostat cultures of *Klebsiella aerogenes* (NCIB8017) supplied with NH_4^+ . The potential O_2 -uptake rates of *K. aerogenes* (NCIB8017) in samples from glucose-limited (Harrison & Loveless, 1971) and from NH_4^+ -limited (Harrison & Pirt, 1967) chemostat cultures increased when the O_2 supply to the culture was lowered. In the latter culture at zero or low O_2 tensions the organisms were probably fixing N_2 . On the other hand, respiratory activity in *Azotobacter* increases in response to an increase in dissolved O_2 tension (Yates & Jones, 1974). Thus this difference in response of respiratory activity to dissolved O_2 tension between *Azotobacter* and *Klebsiella* may account in part for the occurrence of N_2 fixation in *Klebsiella* only during anaerobic growth or under or near O_2 limitation. Although the ATP produced from oxidative phosphorylation in O_2 -limited N_2 -fixing chemostat cultures of *K. pneumoniae* can probably be used for N_2 fixation and growth, reducing power for nitrogenase activity may be available only from anaerobic processes. Pyruvate or formate can support C_2H_2 reduction in crude extracts of *K. pneumoniae* (Yoch, 1974). The formate dehydrogenase associated with evolution of H_2 from formate in the coli-aerogenes group is O_2 sensitive (Peck & Gest, 1957; Ruiz-Herrera & Alvarez, 1972). Therefore the supply of reducing power for nitrogenase activity in intact *K. pneumoniae* may operate only at zero or very low O_2 tensions and, like nitrogenase (St. John *et al.*, 1974), may be repressed by O_2 .

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