The Apparent ATP Requirement for Nitrogen Fixation in Growing *Klebsiella pneumoniae*

By SUSAN HILL

*Agricultural Research Council, Unit of Nitrogen Fixation,
University of Sussex, Falmer, Brighton BN1 9QJ*

(Received 27 November 1975; revised 11 March 1976)

SUMMARY

The apparent ATP requirement for N_2 fixation in *Klebsiella pneumoniae* was high (the ATP/ N_2 molar ratio was 29 when estimated in anaerobic glucose-limited chemostat cultures) compared with that determined previously in *Azotobacter chroococcum* and in *Clostridium pasteurianum*. The high value was probably not due to unfavourable temperature, phosphate concentration or pH. The apparent ATP requirement for N_2 fixation was probably no lower in O_2 -limited chemostat cultures than in anaerobic glucose-limited chemostat cultures. When hydrogen was introduced into the atmosphere over the anaerobic glucose-limited chemostat culture, there was an increase in the apparent ATP requirement for N_2 fixation and in the activity of nitrogenase in intact organisms. A comparison of these increases suggests that some ATP is wasted by the ATP-dependent H_2 -evolving activity of nitrogenase.

INTRODUCTION

The reduction of N_2 by H_2 is exergonic (Bayliss, 1956). However, the reduction of N_2 to NH_4^+ by all nitrogenases so far examined requires ATP as well as a suitable reductant, such as sodium dithionite, reduced ferredoxin or reduced flavodoxin (Eady & Postgate, 1974). A substantial amount of ATP is hydrolysed during nitrogenase function *in vitro*. Values for the ATP requirement for the transfer of two electrons range between 4 and 5 (Zumft & Mortenson, 1975).

During growth, the maximum yield of micro-organisms, produced from 1 mol of the ATP generated from the carbon and energy source, may be lowered if a large proportion of the ATP is needed to synthesize a monomer (Stouthamer, 1973; Stouthamer & Bettenhausen, 1973), such as NH_4^+ from N_2 . Thus the decrease in molar growth yield (yield of organisms/mol carbon and energy source consumed) on replacing NH_4^+ with N_2 as nitrogen source, has been used to calculate the apparent ATP requirement for nitrogen fixation *in vivo* (the apparent ATP/ N_2 molar ratio).

In chemostat cultures, the energy requirement for maintenance can be estimated and the same nutritional limitation should minimize differences in the organisms' composition. A carbon and energy limitation probably ensures the most efficient use of the carbon and energy source for growth.

Daesch & Mortenson (1968) and Dalton & Postgate (1969) calculated an apparent ATP/ N_2 molar ratio from molar growth yield data obtained from carbon- and energy-limited chemostat cultures of *Clostridium pasteurianum* and *Azotobacter chroococcum* respectively. The ratio was 20 in the obligate anaerobe *C. pasteurianum* and as little as 4 to 5 in the obligate aerobe *A. chroococcum*. The marked difference in these ratios prompted the present work to estimate the apparent ATP/ N_2 molar ratio in the facultative anaerobe

Klebsiella pneumoniae. Because the ratio obtained (29) was so high, the influence of the culture conditions on it was investigated. During the course of this work, an apparent ATP/N₂ molar ratio of 30 in batch cultures of a *nif* constitutive mutant of *K. pneumoniae* was calculated by Shanmugam & Valentine (1975) from the excretion of NH₄⁺ by this mutant.

A preliminary report of some of the present work formed part of Hill, Drozd & Postgate (1972).

METHODS

Organism and culture. *Klebsiella pneumoniae* M5a1, a gift from Professor P. W. Wilson, was maintained and grown in anaerobic glucose-limited chemostat cultures or in O₂-limited chemostat cultures as described previously (Hill, 1976). Glucose limitation was tested using the criteria of Hill, Drozd & Postgate (1972), under the conditions specified in Table 1 but was not tested under all the conditions used, e.g. Figs 1 and 2 and Table 3. However, the residual glucose concentration was always less than 0.02 mM.

Cultures were grown under 1 atm (101 kPa) N₂ or N₂ plus other gases flowing at about 200 ml min⁻¹. Medium A was the nitrogen-free medium used for chemostat cultures described previously (Hill, 1976). Medium B contained, in addition, yeast extract (3 mg l⁻¹). When required, sufficient (NH₄)₂SO₄ or NH₄Cl was added to repress nitrogenase synthesis. Concentrated solutions of sodium pyruvate were sterilized by filtration, and when required, were added aseptically. Both ferric citrate, a constituent of medium A, and yeast extract were potential carbon and energy sources, and the latter was also a source of fixed nitrogen, but their concentrations were below the level which could support measurable growth; the fixed nitrogen provided by the yeast extract could not be detected by the method used for estimating N₂ fixation.

Modifications to the chemostat culture apparatus of Baker (1968) were as follows. To maintain the temperature below 25 °C, the culture vessel was wrapped with Calorex 15D cross-section tubing (CamLab Glass, Cambridge) through which water at the required temperature was circulated; the temperature of the culture was measured by a thermometer placed in a closed-end dip tube containing glycerol. For maintenance of steady states at various pH values, when the medium contained one tenth of the usual phosphate concentration, the pH of the culture was measured and controlled automatically, as described by Baker (1968), except that a demountable autoclavable combination electrode (Activion Glass, Kinglassie, Fife) was usually used and sodium hydroxide (1 M) was added by a T pump (F. A. Hughes, Epsom, Surrey). Addition of acid was not required.

Assay for acetylene reduction. The C₂H₂-reducing activity of a 2 ml sample of chemostat culture was measured under 0.1 atm C₂H₂ in Ar in the presence of 60 mM-glucose at 30 °C as described previously (Hill, 1976). To determine the rate of C₂H₂ reduction, generally four samples were incubated for four different time intervals (maximum 30 min), but occasionally one sample was incubated for 30 min, during which time gas samples were removed for analyses as described previously (Hill, 1976). Both methods showed a similar linear C₂H₂ reduction. The apparent K_m for C₂H₂ in Ar in samples from an anaerobic glucose-limited chemostat culture grown under 1.0 atm N₂ was 0.015 atm.

Analyses. Dry weight, protein content and the nitrogen content of organisms were estimated by the methods described previously in which the nitrogen content gave a reliable measure of the amount of N₂ fixed (Hill, 1976). The carbon and hydrogen contents of dried organisms were estimated at the same time as the nitrogen content using a model 185 CNH analyser (Hewlett Packard, F and M Scientific Division, Avondale, Pennsylvania,

U.S.A.) as described previously (Hill, 1976). The glucose concentration in the medium and the culture supernatant was measured by the enzymic method described previously (Hill, 1976); this did not detect glucose below 0.02 mM. The presence of NH₄⁺ in the supernatant from chemostat cultures supplied with (NH₄)₂SO₄ or NH₄Cl was determined by the method of Chaney & Marbach (1962). The pyruvic acid concentration in the medium or in the culture supernatant was estimated by the method of Freidemann (1957). Total volatile acids, removed from the culture supernatant by steam distillation, were titrated under N₂ against freshly standardized NaOH with phenol red indicator; double-glass-distilled water at pH 7.0 was used.

The ethanol concentration in the chemostat culture sample was estimated immediately by the microdiffusion method of Neish (1957). The percentage recovery of ethanol during microdiffusion of a standard solution was $95 \pm 5\%$. The amount of ethanol lost by evaporation from the chemostat culture into the gas phase of the culture vessel was estimated by diverting the effluent gas stream from the culture for 3 h through a Drechsel bottle containing 100 ml 8.3 mM-potassium dichromate in 5 M-H₂SO₄. The remaining potassium dichromate was titrated (Neish, 1957), from which the amount of ethanol lost/ml chemostat culture was calculated (Fencl, 1966).

The rate of H₂ accumulation in the gas phase above the chemostat culture was measured by temporarily stopping the gas flow through the culture vessel. The gas inlet and outlet were shut off without stopping the inflow of medium. Gas samples (1 ml) were removed at approximately 5 min intervals through a Suba-Seal (William Freeman, Barnsley, Yorkshire) closure mounted in the lid of the culture vessel, and were analysed chromatographically in a Pye Unicam 104 gas chromatograph modified to take a Minigrade 625 gas density balance (Gow-Mac Field Instruments, Weybridge, Surrey) and fitted with a 150 cm column (4 mm internal diam.) filled with molecular sieve 5A, mesh 80 to 100. The column was maintained at 50 °C with either N₂ or Ar as carrier gas flowing at 50 ml min⁻¹ on the analytical side and 100 ml min⁻¹ on the reference side. Hydrogen peak heights were recorded and compared with standards. The amount of hydrogen produced during the dissimilation of 1 mol glucose was calculated from the rate of hydrogen accumulation in the gas phase (Fencl, 1966) using the average of the initial and final volumes of the chemostat and without correcting for the small change in dilution rate; the time taken for the estimate was small compared with the replacement time of the chemostat culture.

Other fermentation products in the culture supernatant were estimated as follows: acetate by the method of Rose (1955) using acetate kinase (Sigma); lactic acid by the method of Pryce (1969); 2,3-butanediol and acetoin plus diacetyl by the colorimetric methods of Neish (1952). Succinic acid, removed by continuous extraction with ether (Neish, 1952), was detected on the neutralized residue by the qualitative method of Snell & Snell (1953) and the amount of fluorescence was estimated visually. Since the formic acid concentration was very low, formic acid was removed by vacuum distillation and estimated by the method of Wood & Gest (1957) using chromotropic acid.

Definitions. Y_{glucose} is defined as g dry wt organisms/mol glucose consumed. Y_{ATP} is defined as g dry wt organisms/mol ATP generated from glucose dissimilation and is equal to $Y_{\text{glucose}}/2 \times (\text{part of glucose dissimilated}) + (\text{mol acetate/mol glucose consumed})$ (see Results, and Stouthamer & Bettenhausen, 1973) in which the dissimilated portion, P, of a consumed mol of glucose equals $[180 - (\% \text{ carbon content of organisms} \times Y_{\text{glucose}}/40)]/180$ (Stouthamer & Bettenhausen, 1973).

Table 1. *Influence of nitrogen source in medium B and dilution rate on growth parameters in anaerobic glucose-limited* chemostat cultures*

Cultures were maintained under 1.0 atm N₂, at 30±0.5 °C and at pH 6.8±0.2, effected by the buffering capacity of the medium.

Nitrogen source	Steady state	<i>D</i> (h ⁻¹)	Supplied glucose concn (mM)	†Y _{glucose} (g mol ⁻¹)	Dry wt content (%)			†Y _{ATP} (g mol ⁻¹)
					N	C	H	
N ₂	1	0.103	31.1	11.0	13.6	49.4	6.8	—
	2‡	0.103	31.1	11.1	12.8	47.5	6.9	4.2
	3‡	0.113	28.4	12.2	—	—	—	4.4
	4	0.120	30.3	11.2	13.3	47.5	6.6	4.1
	5	0.120	30.0	10.3	12.7	46.7	6.6	—
	6	0.125	28.1	11.4	12.5	—	—	4.2
	7	0.210	10.8	11.9	12.2	—	—	—
NH ₄ ⁺ §	8	0.102	15.8	27.2	—	—	—	11.6
	9	0.120	14.2	23.3	—	—	—	9.4
	10	0.138	13.2	25.6	—	—	—	—
	11	0.150	12.4	25.4	12.4	46.3	7.1	—
	12	0.210	14.1	28.6	—	—	—	11.8
	13	0.210	14.7	28.4	—	—	—	—
	14	0.210	12.8	27.5	13.0	47.2	7.1	—
	15	0.220	14.3	25.9	—	—	—	—

* The criteria for glucose limitation are described in Methods.

† Y_{glucose} and Y_{ATP} are defined in Methods.

‡ In steady states 2 and 3, medium B was replaced by medium A.

§ The NH₄⁺ concentration added to the medium was in excess of that required for growth: 3 mM-(NH₄)₂SO₄ for steady states 8, 9 and 10; 38 mM-NH₄Cl for 11, 14 and 15; 6 mM-NH₄Cl for 12; and 4.3 mM-(NH₄)₂SO₄ for 13.

RESULTS

Comparison of molar growth yields for glucose when ammonium ion is replaced by nitrogen as nitrogen source in anaerobic chemostat cultures

Steady states established in medium B will be referred to as N₂-fixing populations; those established in a similar medium containing excess (NH₄)₂SO₄ or NH₄Cl will be referred to as NH₄⁺-assimilating populations. At a dilution rate (*D*) near 0.1 h⁻¹, anaerobic glucose-limited chemostat cultures of both populations were established of approximately similar density (about 0.340 mg dry wt/ml). The molar growth yield for glucose (Y_{glucose}) was 56 % lower in N₂-fixing populations (11.2±1.0) than in NH₄⁺-assimilating population (25.4±2.1) (Table 1). In addition, in the N₂-fixing populations, the molar growth yield was not changed when the small amount of yeast extract was removed by replacing medium B by medium A (compare steady states 2 and 3 with 1, 4, 5 and 6 in Table 1).

Comparison of the energy budget for growth in ammonium-assimilating and in nitrogen-fixing populations

ATP yield from glucose dissimilation. When the nitrogen source was changed from NH₄⁺ to N₂, at *D* near 0.1 h⁻¹, there was no large change in the fermentation products (Table 2). Of the major products, acetate and H₂ concentrations were approximately similar in both populations, but ethanol was slightly lower in the NH₄⁺-assimilating population; CO₂ was not measured. Hence no gross changes occurred in the route of anaerobic breakdown of glucose. In *Klebsiella*, this occurs by the Embden–Meyerhof–Parnas pathway; it is

Table 2. Comparison of fermentation products in nitrogen-fixing and ammonium-assimilating populations

Details of chemostat cultures are given in Table 1.												
Nitrogen source	D (h ⁻¹)	Equivalent† of total volatile acids (mol glucose dissimilated) ⁻¹	Fermentation products [mol (mol glucose dissimilated*) ⁻¹]									
			Acetate†	Ethanol†	Hydrogen†	Formate	Pyruvate	Lactate	Succinate	2,3-Butane-diol	Acetoin plus diacetyl	
N ₂	0.125	1.02	0.94	0.90‡	1.33	0.005	—	—	—	—	—	
	0.120	1.08	0.92	—	1.33	0.005	<0.01	—	≈0.14	0.012	0.001	
	0.103	—	0.97	—	—	—	—	—	—	—	—	
	0.103	—	0.84	—	—	—	—	0.04	—	—	—	
NH ₄ ⁺	0.120	0.96	0.93	—	1.36	0.01	—	—	—	—	—	
	0.138	1.03	—	0.80‡	—	—	—	—	—	—	—	
	0.190	—	—	0.73	—	—	—	—	—	—	—	
	0.102	—	0.85	—	—	—	—	0.02	—	—	—	
	0.210	—	0.97	—	—	0.016	<0.02	—	≈0.09	—	—	
	0.220	—	—	0.83	—	—	—	—	≈0.34	<0.017	<0.002	

* A greater proportion of the consumed glucose was dissimilated in the N₂-fixing populations than in the NH₄⁺-assimilating populations, therefore, for a comparison, the fermentation products are expressed as an amount produced during the dissimilation of 1 mol glucose. The dissimilated portion of a consumed mol glucose is defined in Methods.

† Estimates of total volatile acids, acetate and ethanol were made at least in duplicate on two separate occasions during a steady state. Estimates of H₂ production, measured as rate of H₂ evolution were made on two separate occasions during a steady state, except in the N₂-fixing population grown at D = 0.125 h⁻¹ where only one estimate was made. The difference between individual estimates and the corresponding mean values for total volatile acids, acetate, ethanol and H₂ was not greater than 8 %.

‡ The loss of ethanol from the culture by evaporation was measured, whereas other estimates of ethanol were corrected for the amount lost by evaporation.

assumed that pyruvate is subsequently cleaved by the thioclastic split to acetyl CoA as in *Escherichia coli*, in which case the yield of acetate indicates the amount of ATP produced from pyruvate cleavage (Hadjipetrou *et al.*, 1964). If ATP is generated during the anaerobic cleavage of pyruvate in *K. pneumoniae*, anaerobic growth ought to be supported by pyruvate. Anaerobic chemostat cultures were established, at D near 0.09 h^{-1} , of NH_4^+ -assimilating and of N_2 -fixing populations supplied with a modified form of medium B, containing sodium pyruvate (2 to 8 g l^{-1}) in place of glucose, and half the phosphate concentration. Although these chemostat cultures were not pyruvate-limited, growth occurred and was about 55 % lower per unit of pyruvate consumed in the N_2 -fixing than in the NH_4^+ -assimilating populations. In both populations the acetate production was approximately equal to the pyruvate consumption.

Thus the anaerobic dissimilation pathway of glucose through pyruvate to acetate in *K. pneumoniae* is similar with either NH_4^+ or N_2 , and yields approximately 2.9 mol ATP/mol glucose dissimilated: that is, 2 mol from the Embden–Meyerhof–Parnas pathway and 0.9 mol from pyruvate cleavage. The values of Y_{ATP} determined for the anaerobic glucose-limited chemostat cultures were 10.9 ± 1.5 for the NH_4^+ -assimilating and 4.2 ± 0.2 for the N_2 -fixing populations (Table 1).

Energy expenditure for growth. Besides N_2 fixation and concomitant NH_4^+ assimilation, there may be other metabolic differences between N_2 -fixing and NH_4^+ -assimilating populations. These differences could affect the energy expenditure for growth and thus contribute to the observed difference in the molar growth yields.

The carbon and nitrogen contents of washed organisms from both populations were similar: $47.8 \pm 1.6\%$ C and $12.9 \pm 0.7\%$ N for N_2 -fixing populations; $46.8 \pm 0.5\%$ C and $12.7 \pm 0.3\%$ N for NH_4^+ -assimilating populations. Thus non-nitrogenous storage products, such as polysaccharides, could not account for the high molar growth yield in the NH_4^+ -assimilating populations.

The low molar growth yield in the N_2 -fixing populations might be due to a large energy requirement for maintenance. If so, a twofold increase in the dilution rate might cause a marked increase in the molar growth yield. Increasing D from 0.1 h^{-1} to 0.2 h^{-1} altered the nutritional status of the N_2 -fixing population supplied with the usual glucose concentration (30 mM) because the residual glucose concentration increased to 0.5 mM. The new limitation was probably an intrinsic N_2 limitation: a limitation where some unknown physiological bottleneck associated with N_2 fixation controls growth and where a marked decrease in population density occurs when D is increased (Hill, Drozd & Postgate, 1972). A glucose-limited culture maintained at D of 0.21 h^{-1} , with the supplied glucose concentration lowered to 10.8 mM to compensate for a change in nutritional status, showed no significant change in molar growth yield. The molar growth yield in the NH_4^+ -assimilating glucose-limited populations was also not markedly increased when the dilution rate was increased from 0.1 h^{-1} to 0.2 h^{-1} (Table 1). Thus these data suggest that the energy requirements for maintenance in the NH_4^+ -assimilating and in the N_2 -fixing populations were similar.

Calculation of the apparent ATP/ N_2 molar ratio

Besides N_2 fixation, other anabolic processes occur in both N_2 -fixing and NH_4^+ -assimilating populations. It must be assumed that these anabolic processes have a similar energy requirement in both populations. The apparent ATP/ N_2 molar ratio can then be obtained from the Y_{ATP} values and the amounts of N_2 fixed.

$$\text{Apparent ATP}/\text{N}_2 \text{ molar ratio} = \frac{Y_{\text{ATP}}^{\text{NH}_4^+} - Y_{\text{ATP}}^{\text{N}_2}}{Y_{\text{ATP}}^{\text{NH}_4^+}} \times \frac{28}{F \times Y_{\text{ATP}}^{\text{N}_2}} \quad (1)$$

in which $Y_{ATP}^{NH_4^+}$ and $Y_{ATP}^{N_2}$ are the Y_{ATP} values for the NH₄⁺-assimilating and the N₂-fixing populations respectively, and F represents the proportion of nitrogen per g dry wt in the N₂-fixing population.

Using the average values for the molar growth yield estimates, the carbon and nitrogen contents of organisms, and the acetate production (Tables 1 and 2) for the steady states at *D* near 0.1 h⁻¹, the value for the apparent ATP/N₂ molar ratio is:

$$\frac{10.5 - 4.2}{10.5} \times \frac{28}{0.13 \times 4.2} = 31$$

If the route for NH₄⁺ assimilation in the N₂-fixing populations is by the glutamine synthetase, glutamate synthase pathway and that in the NH₄⁺-assimilating populations is by the glutamate dehydrogenase pathway (Brown, MacDonald-Brown & Meers, 1974), then only in the former populations will there be an ATP expenditure for glutamate production (2 mol ATP/mol N₂ fixed). Hence the apparent ATP/N₂ molar ratio in anaerobic glucose-limited chemostat cultures of *K. pneumoniae* will be about 29.

Influence of the environment on the apparent ATP/N₂ molar ratio

The apparent ATP/N₂ molar ratio of 29 in *K. pneumoniae* is higher than that in *C. pasteurianum* (Daesch & Mortenson, 1968) and in *A. chroococcum* (Dalton & Postgate, 1969). The ratio in *K. pneumoniae* might be influenced by both temperature and the chemical environment. Temperature and pH influenced the ATP requirement for electron transfer by nitrogenase preparations from *A. vinelandii* (minimum at 20 °C; Hadfield & Bulen, 1969) and from *C. pasteurianum* (minimum at pH 7.6; Jeng, Morris & Mortenson, 1970) respectively. Phosphate above 30 mM inhibited N₂ fixation by cell-free extracts from *K. pneumoniae* (Mahl & Wilson, 1968), from *A. vinelandii* (Bulen, Burns & LeCompte, 1965) and from *C. pasteurianum* (Mortenson, Valentine & Carnahan, 1963). Hydrogen, a product of anaerobic glucose dissimilation in *K. pneumoniae*, may have increased the ATP/N₂ molar ratio just as H₂ increased the ratio in a nitrogenase preparation from *A. vinelandii* by inhibiting N₂ reduction but not H⁺ reduction (Hadfield & Bulen, 1969). Oxygen, when supplied to an anaerobic glucose-limited chemostat culture of *K. pneumoniae*, increased the efficiency of N₂ fixation (μg nitrogen fixed/mg glucose consumed) (Hill, 1976). This increase could be due to a decrease in the apparent ATP/N₂ molar ratio.

Temperature. Steady-state molar growth yield values in N₂-fixing populations maintained at temperatures between 19 °C and 37 °C were slightly higher at 23 °C and 26 °C than at 30 °C; the molar growth yield in the NH₄⁺-assimilating populations maintained at 23 °C was similar to that at 30 °C (Fig. 1).

Phosphate concentration. Lowering the phosphate concentration to 8.4 mM, one tenth of the usual concentration, did not significantly influence the molar growth yield in either the N₂-fixing or the NH₄⁺-assimilating populations (compare values near pH 6.8 in Fig. 2 with those in Table 1).

pH. Using the medium with the low phosphate concentration, molar growth yields were estimated at various pH values (Fig. 2). The maximum molar growth yield in the N₂-fixing populations occurred at pH values between 6.6 and 7.5, indicating that the routine pH (6.8) was about optimum for growth. In the NH₄⁺-assimilating populations, the molar growth yield was not significantly different when the pH was changed from 6.8 to 7.3.

Atmospheric hydrogen concentration. Pengra & Wilson (1958) found that the assimilation of N₂ but not NH₄⁺ in batch cultures of *K. pneumoniae* was inhibited under 0.5 atm H₂. In the present work, replacing 1.0 atm N₂ by 0.5 atm Ar in N₂ did not alter the molar growth

Fig. 1

Fig. 2

Fig. 1. Influence of temperature on Y_{glucose} in N_2 -fixing populations and in NH_4^+ -assimilating populations. Chemostat cultures were supplied with medium B which contained, where indicated, NH_4^+ (as 3 mM- $(\text{NH}_4)_2\text{SO}_4$) in excess of that required for growth. The supplied glucose concentration was near either 30 mM (●) or 15 mM (○) and the residual glucose concentration was less than 0.02 mM. At 30 °C, both the average and the range of values are shown. Cultures were maintained under 1.0 atm N_2 , at D near 0.1 h⁻¹, and at pH 6.8 ± 0.2 , effected by the buffering capacity of the medium. The maximum variation at any one temperature was ± 0.5 °C.

Fig. 2. Influence of pH on Y_{glucose} in N_2 -fixing populations and in NH_4^+ -assimilating populations. Chemostat cultures were supplied with medium B, which contained 8.4 mM-phosphate (one tenth of the usual concentration) and, where indicated, NH_4^+ (as 3 mM- $(\text{NH}_4)_2\text{SO}_4$) in excess of that required for growth. The supplied glucose concentration was near either 30 mM (●) or 15 mM (○) and the residual glucose concentration was less than 0.02 mM. Cultures were maintained under 1.0 atm N_2 at D near 0.1 h⁻¹ and at 30 ± 0.5 °C. The maximum variation in pH at any pH value was ± 0.1 .

yield in either the N_2 -fixing or the NH_4^+ -assimilating populations. However, replacing this gas mixture by 0.5 atm H_2 in N_2 lowered the molar growth yield only in the N_2 -fixing populations (by 25 %, from 11.5 ± 0.8 to 8.7 ± 0.2 ; Tables 3 and 1). Increasing the atmospheric H_2 concentration from 0.5 atm to 0.65 atm H_2 in N_2 decreased the molar growth yield in the N_2 -fixing populations a further 24 % (from 8.7 ± 0.2 to 6.6), whereas replacing 0.5 atm Ar in N_2 with 0.8 atm Ar in N_2 had no significant influence (Table 3).

The amount of acetate produced/mol glucose dissimilated in the N_2 -fixing populations was lowered by replacing 1.0 atm N_2 by either 0.5 atm Ar, 0.5 atm H_2 or 0.65 atm H_2 in N_2 . Hydrogen did not affect the amount of acetate/mol glucose dissimilated in the NH_4^+ -assimilating populations (Tables 3 and 2). However, the Y_{ATP} values in the N_2 -fixing populations supplied with 0.5 or 0.65 atm H_2 in N_2 (Table 3) were significantly lower than those with either 0.5 atm Ar in N_2 (Table 3) or 1.0 atm N_2 (Table 1).

The nitrogen content of organisms in the N_2 -fixing populations was not affected by the various changes in the composition of the gas supply (Tables 3 and 1). Thus H_2 , besides causing a decrease in the molar growth yield in the N_2 -fixing populations, also inhibited N_2 fixation.

If the path of glucose dissimilation is not affected by H_2 , then the lowering of Y_{ATP} in the N_2 -fixing populations suggests that H_2 can cause a wastage of ATP in the N_2 -fixing

Table 3. Influence of atmospheric hydrogen concentration on growth parameters in nitrogen-fixing and in ammonium-assimilating populations

Chemostat cultures were maintained at D of 0.11 h^{-1} , at $30 \pm 0.5^\circ\text{C}$, and at $\text{pH } 6.8 \pm 0.1$, effected by the buffering capacity of the medium (medium B). The supplied glucose concentration was about 30 mM and 15 mM in the N₂-fixing and NH₄⁺-assimilating populations respectively, and the residual glucose concentration was less than 0.02 mM.

Nitrogen source	Supplied gas mixture, with N ₂ to 1.0 atm, (atm)		$\dagger Y_{\text{glucose}}$ (g mol ⁻¹)	Dry wt content (%)		Acetate produced/glucose dissimilated (mol mol ⁻¹)	$\dagger Y_{\text{ATP}}$ (g mol ⁻¹)
	H ₂	Ar		N	C		
N ₂	0.5	0	8.5	13.4	48.8	—	—
	0.5	0	8.8	—	—	0.73	3.4
	0	0.5	12.2	12.4	47.8	—	—
	0	0.5	10.7	—	—	0.70	4.3
	0.65	0	6.6	12.9	47.6	0.79	2.5
	0	0.8	10.4	13.5	49.6	—	—
*NH ₄ ⁺	0.5	0	25.5	—	—	0.99	10.3
	0.5	0	25.3	—	—	—	—
	0	0.5	25.0	—	—	—	—

* The NH₄⁺ concentration added to the medium (as 3 mM-(NH₄)₂SO₄) was in excess of that required for growth.

$\dagger Y_{\text{glucose}}$ and Y_{ATP} are defined in Methods.

organisms. The apparent ATP/N₂ molar ratio in populations supplied with 0.5 atm H₂ in N₂, calculated by equation 1 from the growth parameters for the N₂-fixing and comparable NH₄⁺-assimilating populations, was 40: this is 38 % higher than that found with either 0.5 atm Ar in N₂ or 1.0 atm N₂. Furthermore, when the gas supply to the N₂-fixing population was changed from either 1.0 atm N₂ or 0.8 atm Ar in N₂ to 0.65 atm H₂ in N₂, the molar growth yield decreased by 36 %: this decrease, if associated specifically with the N₂-fixing process, was equivalent to a 120 % increase in the apparent ATP/N₂ molar ratio.

Atmospheric oxygen concentration. If ATP is generated by oxidative phosphorylation in O₂-limited chemostat cultures, the apparent ATP/N₂ molar ratio cannot be determined by equation 1 because the amount of ATP produced from glucose dissimilation cannot be estimated directly. However, any lowering in the apparent ATP/N₂ molar ratio should correspond to an increase in Y_{ATP} in the N₂-fixing populations. Y_{ATP} in a N₂-fixing O₂-limited chemostat culture may be determined if it is assumed that the energy requirement for growth, excluding that required for N₂ fixation, is not influenced by the change from anaerobic glucose limitation to O₂ limitation. Then Y_{ATP} in the NH₄⁺-assimilating populations should not be influenced by this change in nutritional status and can be used to calculate the total ATP production from glucose dissimilation during O₂-limited growth.

$$\text{Mol ATP/mol glucose dissimilated} = {}^0_2Y_{\text{glucose}}^{\text{NH}_4^+} / (\text{P}^{\text{NH}_4^+} \times Y_{\text{ATP}}^{\text{NH}_4^+}), \quad (2)$$

in which ${}^0_2Y_{\text{glucose}}^{\text{NH}_4^+}$ and $\text{P}^{\text{NH}_4^+}$ are the molar growth yield and the dissimilated portion of a consumed mol glucose, respectively, in the NH₄⁺-assimilating O₂-limited population. A similar production of ATP from glucose dissimilation during O₂-limited growth in both NH₄⁺-assimilating and N₂-fixing populations, should allow the determination of the Y_{ATP} in the latter population (${}^0_2Y_{\text{ATP}}^{\text{N}_2}$):

$${}^0_2Y_{\text{ATP}}^{\text{N}_2} = {}^0_2Y_{\text{glucose}}^{\text{N}_2} / \text{P}^{\text{N}_2} \times (\text{mol ATP/mol glucose consumed}), \quad (3)$$

in which ${}^0_2Y_{\text{glucose}}^{\text{N}_2}$ and P^{N_2} are the molar growth yield and the dissimilated portion of a consumed mol glucose, respectively, in the N₂-fixing O₂-limited populations.

Table 4. *Influence of nitrogen source on growth parameters in oxygen-limited chemostat cultures*

Cultures were maintained at D of 0.11 h^{-1} , at $30 \pm 0.5^\circ \text{C}$ and at pH 6.7 ± 0.1 , effected by the buffering capacity of the medium (medium A). Oxygen was supplied at the pressures indicated, together with nitrogen, to give a total pressure of 1 atm. The dissolved oxygen tension was apparently zero and the residual glucose concentration was less than 0.02 mM .

Nitrogen source	Supplied P_{O_2} (atm)	Supplied glucose concn (mM)	$\dagger Y_{\text{glucose}}$ (g mol $^{-1}$)	Increase in Y_{glucose} from anaerobic value	Dry wt content (%)		Acetate produced/glucose dissimilated (mol mol $^{-1}$)	$\dagger \text{ATP}$ yield/glucose dissimilated (mol mol $^{-1}$)	$\S Y_{\text{ATP}}$ (g mol $^{-1}$)
					N	C			
N_2	0.019	23.9	18.2	1.6	13.1	48.0	0.94	—	4.3
$\ast \text{NH}_4^+$	0.019	23.9	38.9	1.5	13.3	48.2	0.95	4.82	—
N_2	0.025	25.1	20.4	1.8	12.8	48.0	1.03	—	3.6
$\ast \text{NH}_4^+$	0.025	25.1	48.8	1.9	13.0	48.2	1.01	6.63	—

$\ast$ The NH_4^+ concentration added to the medium (as $12 \text{ mM} \cdot (\text{NH}_4)_2\text{SO}_4$) was in excess of that required for growth.

$\dagger Y_{\text{glucose}}$ is defined in Methods.

$\ddagger$ These values were calculated by equation 2, see text.

$\S$ These values were calculated by equation 3, see text.

Table 5. *Influence of the composition of the supplied gas mixture for growth on the specific acetylene-reducing activity in samples, and on the ratio of the potential rate of nitrogen fixation to the observed rate of nitrogen fixation*

Details of the chemostat culture are given in Tables 1, 3 and 4; cultures were maintained at D of 0.11 h^{-1} . Acetylene reduction in samples was measured under $0.1 \text{ atm C}_3\text{H}_2$ in Ar, see Methods.

Supplied gas mixture, with N_2 to 1.0 atm,			Protein content (% dry wt)	Specific C_2H_2 -reducing activity* [nmol (mg protein) $^{-1} \text{ min}^{-1}$]	Rate of N_2 fixation in chemostat culture [nmol (mg protein) $^{-1} \text{ min}^{-1}$]	Potential/observed rate of N_2 fixation
H_2	Ar	O_2				
0	0	0	76	64 ± 4 (2)	11.9	1.8
0.5	0	0	74	106 ± 4 (2)	11.4	3.1
0	0.5	0	78	75 ± 4 (2)	10.9	2.3
0.65	0	0	77	170 ± 13 (2)	11.0	5.2
0	0.8	0	75	83 ± 2 (2)	11.2	2.5
0	0	0.019	76	63 ± 15 (4)	11.4	1.9
0	0	0.025	72	73 ± 12 (2)	11.6	2.1

$\ast$ The number in parenthesis indicates the number of estimates, some of which, for the O_2 -limited chemostat cultures, were a single 10 min assay.

In an O_2 -limited chemostat culture of a facultative anaerobe, the proportion of ATP generated from oxidative phosphorylation during the dissimilation of glucose will depend, at least, on the ratio of supplied glucose concentration to the oxygen solution rate and on the dilution rate, which will affect the amount of available O_2 (Pirt, 1957). If these parameters are kept constant, the yields of ATP from glucose dissimilation in NH_4^+ -assimilating and in N_2 -fixing populations might be similar.

A N_2 -fixing O_2 -limited chemostat culture was established at D of $0.11\ h^{-1}$, by the method described previously (Hill, 1976), and the molar growth yield estimated. The culture was then repressed for N_2 fixation by adding excess $(NH_4)_2SO_4$ to the medium reservoir, and the molar growth yield was estimated during the subsequent steady state (Table 4). In both the N_2 -fixing and NH_4^+ -assimilating populations, the molar growth yields were larger than in comparable anaerobic cultures, and increased with the increase in atmospheric O_2 concentration.

The nitrogen and carbon contents of organisms in the O_2 -limited chemostat cultures (Table 4) were similar to those in anaerobic glucose-limited chemostat cultures (Table 1), so the increase in molar growth yield apparently caused by O_2 was not due to large amounts of non-nitrogenous storage products. The acetate production in the O_2 -limited chemostat cultures suggested that the ATP produced from substrate phosphorylation was not affected by the changes in either nitrogen source (Table 4) or nutritional status (Tables 4 and 1). Thus the increases in molar growth yield in the NH_4^+ -assimilating populations were probably due to additional ATP production by partial aerobic metabolism.

Y_{ATP} in the N_2 -fixing O_2 -limited chemostat culture supplied with $0.019\ atm\ O_2$ (Table 4) was similar to that found in N_2 -fixing anaerobic glucose-limited chemostat cultures (Table 1); the value in the N_2 -fixing O_2 -limited chemostat cultures supplied with $0.025\ atm\ O_2$ was lower (Table 4). Hence these Y_{ATP} values suggest that, with O_2 , the apparent ATP/ N_2 molar ratio is about the same as or higher than in anaerobic glucose-limited chemostat cultures.

Level of nitrogenase in nitrogen-fixing populations

Hydrogen inhibition of N_2 fixation in anaerobic cultures might have led to an increase in the level of nitrogenase. The C_2H_2 -reducing activity of chemostat culture samples under $0.1\ atm\ C_2H_2$ in Ar with added glucose, assumed to be a measure of the amount of active nitrogenase within the organisms, increased progressively in N_2 -fixing populations under N_2 with 0.5 or $0.65\ atm\ H_2$. This increase was attributed to the H_2 since there was only a marginal increase when N_2 was diluted with 0.5 or $0.8\ atm\ Ar$ (Table 5). Comparable C_2H_2 -reducing activity in samples from the O_2 -limited cultures was similar to or slightly higher than that in samples from chemostat cultures supplied with $1.0\ atm\ N_2$ (Table 5).

The theoretical conversion factor of 3 was used to calculate the potential rate of N_2 fixation from the C_2H_2 -reduction rates. Even in the N_2 -fixing populations supplied with $1.0\ atm\ N_2$, the ratio (potential/observed rate) was greater than 1 (Table 5), indicating that the nitrogenase in these organisms was apparently not operating at a maximum rate for N_2 reduction during growth under anaerobic glucose limitation. In the N_2 -fixing populations supplied with 0.5 and $0.65\ atm\ H_2$, the ratio was 72 % and 188 % higher, respectively, than with $1.0\ atm\ N_2$; the ratio was only slightly higher if H_2 was replaced by either 0.5 or $0.8\ atm\ Ar$. In the N_2 -fixing populations supplied with $0.019\ atm\ O_2$, the ratio was similar to that with $1.0\ atm\ N_2$; with $0.025\ atm\ O_2$ it was slightly elevated (Table 5).

DISCUSSION

The apparent ATP/N₂ molar ratio in glucose-limited chemostats of *K. pneumoniae* M5a1 obtained (29) is similar to that found in batch cultures of a *nif* constitutive mutant of *K. pneumoniae* SK24 by Shanmugam & Valentine (1975), who calculated the ratio from the NH₄⁺ excretion and concomitant glucose consumption by this mutant. On the other hand, the ratio was lower (20) in sucrose-limited chemostats of *C. pasteurianum* (Daesch & Mortenson, 1968) and much lower (4 to 5) in mannitol-limited chemostats of *A. chroococcum* (Dalton & Postgate, 1969).

However, such comparisons may be misleading for the following reasons. In each case, calculation of the ratio required the determination of the amount of ATP generated from the carbon and energy source. Both in the mutant SK24 (Shanmugam & Valentine, 1975) and in *A. chroococcum* (Dalton & Postgate, 1969), the amount of ATP generated from the carbon and energy source was assumed, not estimated. Although Dalton & Postgate (1969) corrected the molar growth yield data of *A. chroococcum* for utilization of mannitol for maintenance, including respiratory protection, they assumed that Y_{ATP} in the NH₄⁺-assimilating population was 10.5 and that the amount of ATP generated from mannitol was similar in the NH₄⁺-assimilating and N₂-fixing populations. More recent work concerning respiration in *Azotobacter* (Yates & Jones, 1974) suggests that these assumptions are probably invalid. The ratio may be influenced by the nutritional status of the organisms. Daesch & Mortenson (1968) stated that their N₂-fixing cultures were 'limited in sucrose to varying degrees', which suggests that the limiting nutrient was not sucrose.

The high ATP/N₂ molar ratio in glucose-limited cultures of *K. pneumoniae* M5a1, grown anaerobically under 1.0 atm N₂, was probably not due to an unfavourable aspect of the environment. Though the efficiency of nitrogen fixation was increased by providing O₂ (Hill, 1976), the apparent ATP/N₂ molar ratio was probably not decreased, because Y_{ATP} in the N₂-fixing O₂-limited cultures was similar to or lower than that in the N₂-fixing anaerobic glucose-limited cultures. The validity of the estimate of Y_{ATP} in the N₂-fixing O₂-limited cultures depends on the assumption that the yield of ATP from glucose dissimilation during O₂-limited growth was similar in both the NH₄⁺-assimilating and in the N₂-fixing populations. The growth conditions were designed to minimize any differences. However, Harrison & Loveless (1971) obtained 'uncoupled growth', indicated by a lowering of growth yield without a concomitant increase in fermentation products, in glucose-limited chemostat cultures of *Klebsiella aerogenes* NCIB8017 by lowering the O₂ supply so that it was just limiting (NH₄⁺ was the nitrogen source). Harrison & Pirt (1967) observed a somewhat similar behaviour in NH₄⁺-limited chemostat cultures of this organism. *Klebsiella aerogenes* NCIB8017 can fix N₂ (Dixon, 1972), and probably did so when NH₄⁺-limited at zero or low O₂ tensions in Harrison & Pirt's experiments. The 'uncoupled growth' in *K. aerogenes* was associated with fluctuations in the dissolved O₂ tension. In the O₂-limited cultures of *K. pneumoniae* supplied with either NH₄⁺ or N₂ as nitrogen source, the dissolved O₂ tension apparently remained near zero. Therefore 'uncoupled growth' probably did not occur in these cultures.

The apparent ATP/N₂ molar ratio in growing *K. pneumoniae* is higher than the ratio of between 12 and 15 calculated from the reported values of the ATP requirement for electron transfer by nitrogenase preparations from various organisms (Zumft & Mortenson, 1975). However, such a comparison may also be misleading because the amount of ATP hydrolysed per electron pair transferred can be influenced by the ratio of the two nitrogenase components and the assay conditions (Zumft & Mortenson, 1975); in addition, proton reduction

is probably never completely suppressed by N₂ *in vitro* (Hadfield & Bulen, 1969). On the other hand, Shanmugam & Valentine (1975) suggested that in *K. pneumoniae* there may be a high ATP requirement for the production of a suitable reductant for nitrogenase function. This seems unlikely because reduced flavodoxin is a reductant in *K. pneumoniae* (Yoch, 1974) and the breakdown of pyruvate could supply electrons for its rereduction.

ATP expenditure for hydrogen evolution by nitrogenase in growing K. pneumoniae

The ATP requirement for N₂ fixation will be influenced by the ratio of H₂ to NH₄⁺ production. Having excluded other possibilities, therefore, the high apparent ATP/N₂ molar ratio in growing *K. pneumoniae* may be due to a large proportion of the ATP consumed by nitrogenase resulting in H₂ evolution.

The level of nitrogenase, estimated by C₂H₂ reduction, in glucose-limited chemostat cultures of *K. pneumoniae* under 1.0 atm N₂ was 1.8 times greater than was required to account for the rate of N₂ reduction during growth. If all this enzyme was fully active during growth, 45 % of the total ATP it utilized (13 mol ATP) would have been diverted to H₂ evolution. This 45 % may be an underestimate because C₂H₂ may not have completely suppressed the H₂-evolving activity of nitrogenase. Although C₂H₂ differs from N₂ by suppressing the amount of H₂ evolved by nitrogenases *in vitro* almost completely (Hardy, Burns & Parshall, 1971), some H₂ evolution accompanied C₂H₂ reduction by the nitrogenase from *K. pneumoniae* (Eady *et al.*, 1972). Thus the ATP requirement for N₂ reduction in growing *K. pneumoniae* may be 16 or less.

On the other hand, the C₂H₂-reducing activity in samples may have led to an overestimate of the nitrogenase activity during growth; the change from glucose limitation in the chemostat culture to nitrogen limitation in the test samples (under 0.1 atm C₂H₂ in Ar) may have released a control of activity. For example, an inhibition by ADP may have been released, although during growth the adenylate energy charge is probably maintained at a high level (Chapman, Fall & Atkinson, 1971). In addition, C₂H₂ inhibits the conventional hydrogenase in *K. pneumoniae* (Smith, Hill & Yates, 1976) and this may have influenced the nitrogenase activity in the samples.

It has been assumed that H₂ is evolved by nitrogenase in growing *K. pneumoniae*. Attempts to demonstrate this reaction in samples from glucose-limited anaerobic chemostat cultures have not been convincing (unpublished results) because this organism produces H₂ via the conventional hydrogenase in amounts much greater than that proposed for the H₂-evolving activity of nitrogenase. However, in an anaerogenic strain of *K. pneumoniae*, evidence suggested that H₂ evolution occurred by nitrogenase activity in intact organisms (Hamilton, Burris & Wilson, 1964).

The experiments with H₂ in the atmosphere over the culture support the view that this strain wasted some ATP via the H₂-evolving activity of nitrogenase *in vivo*. Hydrogen inhibits N₂ fixation in growing organisms (Burris, 1971) and in nitrogenase preparations, but not the ATP-dependent H₂-evolving activity of nitrogenase *in vitro* (Hadfield & Bulen, 1969; Hwang, Chen & Burris, 1973). As expected, H₂ partly inhibited N₂ fixation in the anaerobic glucose-limited chemostat culture of *K. pneumoniae*, but it caused an increase in the apparent ATP/N₂ molar ratio of 38 % with 0.5 atm H₂ and, probably, 120 % with 0.65 atm H₂. Furthermore, the specific acetylene-reducing activity of samples from these populations in the absence of H₂ was increased by 66 % with 0.5 atm H₂ and by 166 % with 0.65 atm H₂. Figure 3 compares the increase in nitrogenase activity and the apparent ATP/N₂ molar ratio with changes in atmospheric H₂ concentration. The increase in the apparent

Fig. 3. Influence of atmospheric H_2 concentration on the increase in nitrogenase activity and apparent ATP/ N_2 molar ratio in growing *K. pneumoniae*. The nitrogenase activity (●) is shown as the ratio of the potential rate of N_2 fixation to the observed rate of N_2 fixation (see Table 5). The apparent ATP/ N_2 molar ratio in *K. pneumoniae* (○) is shown together with the ATP/ N_2 molar ratio in an unfractionated preparation of nitrogenase from *Azotobacter vinelandii* (□), under various atmospheric H_2 concentrations (calculated from the data of Hadfield & Bulen, 1969).

ATP/ N_2 molar ratio in *K. pneumoniae* caused by H_2 was similar to that found by Hadfield & Bulen (1969) with a nitrogenase preparation from *A. vinelandii*. Thus, the greater expenditure of ATP for N_2 fixation in chemostat cultures of *K. pneumoniae* caused by H_2 could be accounted for by the increase in the proportion of ATP utilized for H_2 evolution.

Since H_2 was produced by the conventional hydrogenase during growth under 1.0 atm N_2 , a high dissolved H_2 concentration in the environment of the nitrogenase could cause inhibition of N_2 fixation by H_2 and hence wastage of ATP. This seems unlikely, because the apparent ATP/ N_2 molar ratio was not decreased in cultures where the endogenous H_2 concentration was probably low, i.e. under O_2 limitation, where the ratio was similar to or higher than that under anaerobic glucose limitation. Indeed, the organisms from cultures under O_2 limitation, like those under anaerobic glucose limitation, contained about twice as much nitrogenase as they needed, so they probably wasted ATP too.

Growth under glucose or O_2 limitation probably led to a limited supply of ATP. A low concentration of ATP, but a high adenylate energy charge, can increase the proportion of H^+ to N_2 reduced by nitrogenase (Silverstein & Bulen, 1970). If this situation occurs in chemostat cultures, the loss of ATP through increased H^+ reduction might leave so little for N_2 reduction that the population becomes effectively nitrogen-limited and responds by synthesizing extra nitrogenase. Furthermore, this nitrogen limitation would be exaggerated when H_2 , an inhibitor of N_2 reduction but not of H^+ reduction, was supplied to the culture.

I thank Professor J. R. Postgate, Drs R. R. Eady, M. G. Yates, B. E. Smith and R. N. F. Thorneley for helpful discussion and reviewing the manuscript, and Mr E. Kavanagh for technical assistance.

REFERENCES

- BAKER, K. (1968). Low cost continuous culture apparatus. *Laboratory Practice* **17**, 817-824.
 BAYLISS, N. S. (1956). The thermodynamics of biological nitrogen fixation. *Australian Journal of Biological Sciences* **9**, 364-370.
 BROWN, C. M., MACDONALD-BROWN, D. S. & MEERS, J. L. (1974). Physiological aspects of microbial inorganic nitrogen metabolism. *Advances in Microbial Physiology* **11**, 1-52.

- BULEN, W. A., BURNS, R. C. & Lecompte, J. R. (1965). Nitrogen fixation: hydrosulfite as electron donor with cell-free preparations of *Azotobacter vinelandii* and *Rhodospirillum rubrum*. *Proceedings of the National Academy of Sciences of the United States of America* **53**, 532–539.
- BURRIS, R. H. (1971). Fixation by free-living micro-organisms: enzymology. In *The Chemistry and Biochemistry of Nitrogen Fixation*, pp. 105–160. Edited by J. R. Postgate. London and New York: Plenum.
- CHANEY, A. L. & MARBACH, E. P. (1962). Modified reagents for determination of urea and ammonia. *Clinical Chemistry* **8**, 130–132.
- CHAPMAN, A. G., FALL, L. & ATKINSON, D. E. (1971). Adenylate energy charge of *Escherichia coli* during growth and starvation. *Journal of Bacteriology* **108**, 1072–1086.
- DAESCH, G. & MORTENSON, L. E. (1968). Sucrose catabolism in *Clostridium pasteurianum* and its relation to N₂ fixation. *Journal of Bacteriology* **96**, 346–351.
- DALTON, H. & POSTGATE, J. R. (1969). Growth and physiology of *Azotobacter chroococcum* in continuous culture. *Journal of General Microbiology* **56**, 307–319.
- DIXON, R. A. (1972). *Genetics of nitrogen fixation in coliform bacteria*. D. Phil. thesis, University of Sussex.
- EADY, R. R. & POSTGATE, J. R. (1974). Nitrogenase. *Nature, London* **249**, 805–810.
- EADY, R. R., SMITH, B. E., COOK, K. A. & POSTGATE, J. R. (1972). Nitrogenase of *Klebsiella pneumoniae* purification and properties of the component proteins. *Biochemical Journal* **128**, 655–675.
- FENCL, Z. (1966). A theoretical analysis of continuous culture. In *Theoretical and Methodological Basis of Continuous Culture of Microorganisms*, pp. 67–153. Edited by I. Malek and Z. Fencl. New York and London: Academic.
- FREIDEMANN, T. E. (1957). Determination of α -keto acids. In *Methods in Enzymology*, vol. 3, pp. 414–418. Edited by S. P. Colowick and N. O. Kaplan. New York and London: Academic.
- HADFIELD, K. L. & BULEN, W. A. (1969). Adenosine triphosphate requirement of nitrogenase from *Azotobacter vinelandii*. *Biochemistry, New York* **8**, 5103–5108.
- HADIPIETROU, L. P., GERRITS, J. P., TEULINGS, F. A. G. & STOUTHAMER, A. H. (1964). Relation between energy production and growth of *Aerobacter aerogenes*. *Journal of General Microbiology* **36**, 139–149.
- HAMILTON, I. R., BURRIS, R. H. & WILSON, P. W. (1964). Hydrogenase and nitrogenase in a nitrogen-fixing bacterium. *Proceedings of the National Academy of Sciences of the United States of America* **52**, 637–641.
- HARDY, R. W. F., BURNS, R. C. & PARSHALL, G. W. (1971). The biochemistry of N₂ fixation. *Advances in Chemistry Series* **100**, 219–247.
- HARRISON, D. E. F. & LOVELESS, J. E. (1971). The effect of growth conditions on respiratory activity and growth efficiency in facultative anaerobes grown in chemostat culture. *Journal of General Microbiology* **68**, 35–43.
- HARRISON, D. E. F. & PIRT, S. J. (1967). The influence of dissolved oxygen concentration on the respiration and glucose metabolism of *Klebsiella aerogenes* during growth. *Journal of General Microbiology* **46**, 193–211.
- HILL, S. (1976). Influence of atmospheric oxygen concentration on acetylene reduction and efficiency of nitrogen fixation in intact *Klebsiella pneumoniae*. *Journal of General Microbiology* **93**, 335–345.
- HILL, S., DROZD, J. W. & POSTGATE, J. R. (1972). Environmental effects on the growth of nitrogen-fixing bacteria. *Journal of Applied Chemistry and Biotechnology* **22**, 541–558.
- HWANG, J. C., CHEN, C. H. & BURRIS, R. H. (1973). Inhibition of nitrogenase-catalysed reductions. *Biochimica et biophysica acta* **292**, 256–270.
- JENG, D. Y., MORRIS, J. A. & MORTENSON, L. E. (1970). The effect of reductant in inorganic release from adenosine 5'-triphosphate by purified nitrogenase from *Clostridium pasteurianum*. *Journal of Biological Chemistry* **245**, 2809–2813.
- MAHL, M. C. & WILSON, P. W. (1968). Nitrogen fixation in cell-free extracts of *Klebsiella pneumoniae*. *Canadian Journal of Microbiology* **14**, 33–38.
- MORTENSON, L. E., VALENTINE, R. C. & CARNAHAN, J. E. (1963). Ferredoxin in the phosphoclastic reaction of pyruvic acid and its relation to nitrogen fixation in *Clostridium pasteurianum*. *Journal of Biological Chemistry* **238**, 794–800.
- NEISH, A. C. (1952). *Analytical Methods for Bacterial Fermentation*, Report no. 46-8-3, 2nd rev. Saskatoon: National Research Council of Canada.
- NEISH, A. C. (1957). Chemical procedures for separation and determination of alcohols. In *Methods in Enzymology*, vol. 3, pp. 255–263. Edited by S. P. Colowick and N. O. Kaplan. New York and London: Academic.
- PENGRA, R. M. & WILSON, P. W. (1958). Physiology of nitrogen fixation by *Aerobacter aerogenes*. *Journal of Bacteriology* **75**, 21–25.
- PIRT, S. J. (1957). The oxygen requirement of growing cultures of an *Aerobacter* species determined by means of the continuous culture technique. *Journal of General Microbiology* **16**, 59–75.
- PRYCE, J. D. (1969). A modification of the Barker–Summerson method for the determination of lactic acid. *The Analyst* **94**, 1151–1152.
- ROSE, I. A. (1955). Acetate kinase of bacteria. In *Methods in Enzymology*, vol. 1, pp. 591–595. Edited by S. P. Colowick and N. O. Kaplan. New York and London: Academic.

- SHANMUGAM, K. T. & VALENTINE, R. C. (1975). Microbial production of ammonium ion from nitrogen. *Proceedings of the National Academy of Sciences of the United States of America* **72**, 136-139.
- SMITH, L. A., HILL, S. & YATES, M. G. (1976). Inhibition by acetylene of conventional hydrogenase in nitrogen-fixing bacteria. *Nature, London* (in the Press).
- SNELL, F. D. & SNELL, C. T. (1953). *Colorimetric Methods of Analysis Including some Turbidometric and Nephelometric Methods*, vol. 3, pp. 381-382. Princeton, New Jersey: Van Nostrand.
- STOUTHAMER, A. H. (1973). A theoretical study on the amount of ATP required for synthesis of microbial cell material. *Antonie van Leeuwenhoek* **39**, 545-565.
- STOUTHAMER, A. H. & BETTENHAUSEN, C. (1973). Utilization of energy for growth and maintenance in continuous and batch cultures of microorganisms. *Biochimica et biophysica acta* **301**, 53-70.
- WOOD, H. G. & GEST, H. (1957). Determination of formate. In *Methods in Enzymology*, vol. 3, pp. 285-292. Edited by S. P. Colowick and N. O. Kaplan. New York and London: Academic.
- YATES, M. G. & JONES, C. W. (1974). Respiration and nitrogen fixation in *Azotobacter*. *Advances in Microbial Physiology* **11**, 97-135.
- YOCH, D. C. (1974). Electron transport carriers involved in nitrogen fixation by the coliform *Klebsiella pneumoniae*. *Journal of General Microbiology* **83**, 153-164.
- ZUMFT, W. G. & MORTENSON, L. E. (1975). The nitrogen-fixing complex of bacteria. *Biochimica et biophysica acta* **416**, 1-52.