Energetics of Biological Nitrogen Fixation: Determination of the Ratio of Formation of H_2 to NH_4^+ Catalysed by Nitrogenase of *Klebsiella pneumoniae* *in vivo*

By K. ANDERSEN AND K. T. SHANMUGAM

*Plant Growth Laboratory, Department of
Agronomy & Range Science, University of California,
Davis, California 95616, U.S.A.*

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Nitrogen fixation (Nif)-derepressed mutants of *Klebsiella pneumoniae* consumed, under optimum conditions, 7.5 to 8.5 mol glucose per mol N_2 fixed. The nitrogenase system of these mutants catalysed the production of about 1.3 mol H_2 per mol N_2 reduced. Almost one-third of the energy as ATP and reductant used by nitrogenase *in vivo* may be lost in H_2 production, since an ATP/2e ratio of approximately 4 was obtained. Nitrogenase-catalysed H_2 production was not substantially suppressed by increasing the partial pressure of N_2 from 0.2 atm (20 kPa) to 1 atm (101 kPa). In the absence of N_2 , H_2 production catalysed by nitrogenase increased about threefold. It is concluded that nitrogenase-catalysed H_2 production is of major importance in the overall efficiency of biological N_2 fixation *in vivo*.

INTRODUCTION

Reduction of N_2 or alternative substrates catalysed by nitrogenase requires ATP in addition to a suitable reductant. Determination of the *in vivo* energy requirement for N_2 fixation has been complicated by fixation being closely coupled to growth in free-living bacteria. Estimates of ATP/ N_2 molar ratios obtained by comparing molar growth yields of cultures supplied with N_2 or NH_4^+ as nitrogen source range from 4 to 5 for *Azotobacter chroococcum* (Dalton & Postgate, 1969), 20 for *Clostridium pasteurianum* (Daesch & Mortenson, 1968) to 29 for *Klebsiella pneumoniae* (Hill, 1976). Most *in vitro* studies with nitrogenase from several bacteria indicate a minimum requirement of four to five molecules of ATP per two electrons transferred (ATP/2e value). Higher or lower values have been reported, and the ratio has been reported to vary widely with pH, temperature and the ratio of the component proteins of nitrogenase (Ljones & Burris, 1972; see also Burns & Hardy, 1975; Orme-Johnson & Davis, 1977; Winter & Burris, 1976; Zumft & Mortenson, 1975).

All nitrogenase preparations isolated so far catalyse an ATP-dependent reduction of H^+ to H_2 , even in the presence of N_2 (Burns & Hardy, 1975; Zumft & Mortenson, 1975). However, it has been difficult to measure H_2 evolution mediated by nitrogenase *in vivo* because of the presence of hydrogenase systems which mediate uptake (aerobic organisms) or evolution (anaerobic organisms) of H_2 (Hyndman, Burris & Wilson, 1953; Dixon, 1972). Studies suggesting that nitrogenase-catalysed H_2 evolution occurs *in vivo* have been reported for *K. pneumoniae* (Hamilton, Burris & Wilson, 1964) and *Rhodopseudomonas capsulata* (Wall, Weaver & Gest, 1975). Inhibitor studies (Smith, Hill & Yates, 1976) support the notion that the lack of H_2 evolution from intact N_2 -fixing *Azotobacter chroo-*

coccum is due not to lack of nitrogenase-mediated H_2 evolution, but to a H_2 uptake system. H_2 evolution presumably catalysed by nitrogenase has also been observed in root nodules (Schubert & Evans, 1976) and in blue-green algae (Bothe *et al.*, 1977). Schubert & Evans (1976) reported that the loss of energy as ATP and reducing power, attributed to H_2 evolution, may be as high as 40 to 60 % of the total energy flow through nitrogenase in soybean and other legume root nodules. Some of this energy may be recouped by recycling H_2 through uptake hydrogenase (Schubert & Evans, 1976; Dixon, 1972). Hill (1976) estimated that 45 % of the total energy flow through nitrogenase probably resulted in H_2 evolution in *K. pneumoniae*. Recently, NH_4^+ assimilation has been blocked genetically (Shanmugam & Valentine, 1975) or chemically (Gordon & Brill, 1974; Stewart & Rowell, 1975; Weare & Shanmugam, 1976) thereby derepressing nitrogenase biosynthesis in free-living micro-organisms and leading to excretion of fixed N_2 as NH_4^+ . We have previously reported that mutant strains of *K. pneumoniae*, derepressed for nitrogen fixation and blocked in NH_4^+ assimilation, excrete large quantities of fixed N_2 as NH_4^+ (Shanmugam & Valentine, 1975; Shanmugam *et al.*, 1977a). Such strains provide a convenient tool for studying the physiology and energetics of N_2 fixation *in vivo*, where the energy consumption (as glucose) and the production of NH_4^+ can be determined directly using non-growing cells as described in this report. Construction of mutant strains blocked in the conventional hydrogen-producing system has also allowed investigation of nitrogenase-catalysed H_2 evolution *in vivo*.

METHODS

Bacterial strains. The isolation and characterization of *K. pneumoniae* mutant strains SK-24 to SK-60, and SK-512 have been described previously (Shanmugam, Chan & Morandi, 1975; Shanmugam, Morandi & Valentine, 1977b). Strains SK-48 and SK-49 were isolated as *Nif*⁻ derivatives of strain ASM-1 using the procedure described for the isolation of *Nif*-derepressed strains (Shanmugam *et al.*, 1975). These strains are blocked in NH_4^+ assimilation, require glutamate or glutamine for growth, and are derepressed for nitrogenase biosynthesis in the presence of NH_4^+ (except SK-48 and SK-49). Strains N-20 to N-27 were isolated as chlorate-resistant derivatives of strain SK-24; strain N-61 as a spontaneous chlorate-resistant derivative of SK-25, and strains N-6 and N-15 as chlorate-resistant derivatives of *K. pneumoniae* M5a1. The parent strains (strains M5a1 and SK-24) were mutated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as described by Shanmugam *et al.* (1975) and plated on L-broth or sucrose minimal medium with L-glutamate (1 mg ml⁻¹); both media contained sodium chlorate (2 mg ml⁻¹). Resistant clones were picked after incubating the plates anaerobically for 72 h at 25 °C. Strains blocked in the conventional hydrogen-producing system were selected as described below.

Cultivation of bacteria. The culture media used (L-broth, sucrose minimal medium or glucose minimal medium) were as described previously (Shanmugam & Valentine, 1975; Streicher, Gurney & Valentine, 1971) except that the Na/K phosphate concentration was increased to 0.1 M for more effective buffering.

A special growth flask was used in most experiments (Shanmugam *et al.*, 1977a). Bacteria were cultivated inside a dialysis bag with 25 ml medium suspended in 250 ml medium, allowing NH_4^+ and fermentation products to diffuse out, and glucose, the energy source, to diffuse in. Sampling ports closed with serum stoppers allowed aseptic removal or addition of samples from both inside and outside the dialysis bag. The medium, both inside and outside the dialysis bag, was constantly sparged with N_2 (> 99.996 % N_2 ; Liquid Carbonic, Chicago, Illinois, U.S.A.) or Ar (> 99.995 % Ar; Liquid Carbonic). Sparging rates were 10 to 30 ml min⁻¹. A growth-limiting quantity [4 to 8 µg (ml total culture volume)⁻¹] of freshly prepared, filter-sterilized, L-glutamate or L-glutamine was added (inside the dialysis bag) before inoculation. The inoculum culture (0.5 to 1 ml) was grown overnight in L-broth at 25 °C. Incubation was at 25 °C unless otherwise indicated. Samples were withdrawn at intervals from outside or inside the dialysis bag for determination of growth, NH_4^+ production, glucose consumption etc. Samples of the outgoing sparging gas (both inside and outside the dialysis bag) were collected and analysed for H_2 content. Growth was monitored by measuring the absorbance at 420 nm; a culture density of $E_{420} = 1$ corresponded to a cell protein concentration of 0.14 mg ml⁻¹. Samples of the culture were routinely examined for revertants to the parental phenotype (able to grow on NH_4^+ as nitrogen source) by plating on minimal medium containing NH_4^+ as the nitrogen source.

Analytical methods. Nitrogenase activity in whole cells was determined using the acetylene reduction assay as described by Shanmugam, Loo & Valentine (1974). Nitrogenase activity of the suspension inside

the dialysis culture flask was measured on samples removed under a stream of Ar with Ar-filled syringes, and transferred to Ar-filled assay flasks, taking care not to expose the samples to O₂. When H₂ evolution was measured, no acetylene was added to the assay flasks.

NH₄⁺ was determined after Conway diffusion using Nessler's reagent (Ballentine, 1957), or in untreated samples using an Orion model 95-10 ammonia electrode. The results from the two methods agreed well. Total ninhydrin-positive material excreted by the culture was determined as described by Spies (1957). Glucose was determined by the ferricyanide method (Umbreit, Burris & Stauffer, 1964) or enzymically using hexokinase (Calbiochem, Glucose Stat-Pack). Acetate was determined using the acetate kinase (Sigma)-hydroxylamine method (Holz & Bergmeyer, 1974).

H₂ was determined with a Varian model 920 gas chromatograph equipped with a 1.5 m × 6.4 mm column of 0.5 nm molecular sieve (30 to 60 mesh) and a thermal conductivity detector. N₂ was used as carrier gas (50 ml min⁻¹), and the column temperature was 40 °C.

Whole-cell protein was determined by the method of Drews (1965) using bovine serum albumin as a standard.

RESULTS

Excretion of NH₄⁺ from fixed N₂

Using a growth vessel in which the bacteria were grown inside a dialysis bag into which fresh medium could diffuse, and products such as NH₄⁺ could diffuse out (Shanmugam *et al.*, 1977a), the production of NH₄⁺ from N₂ was measured in a number of nitrogenase-derepressed mutant strains of *K. pneumoniae* blocked in the assimilation of NH₄⁺ into amino acids.

The specific rate of NH₄⁺ production was independent of the concentration of organisms inside the dialysis bag, and of the sparging rate, indicating that the transfer of N₂ to the liquid or diffusion of nutrients through the dialysis membrane were not limiting under the conditions used. The time-course for the production of NH₄⁺ by strain SK-25 is shown in Fig. 1. The culture stopped growing after about 1.5 days due to depletion of glutamine, an essential amino acid for the growth of this strain. However, NH₄⁺ production continued for almost 1 week after the stationary phase of growth was reached. The number of viable organisms was constant from the time growth stopped up to about 8 days, when a gradual decrease in viability occurred. The ratio of viable count to total cell count was determined by the slide culture technique according to Postgate (1969); up to 8 days the ratio was about 0.9. Organisms thus remained viable for long periods after they had stopped dividing, and even after they had lost most of their ability to fix N₂.

In a parallel experiment, chloramphenicol (0.2 mg ml⁻¹, sufficient to inhibit growth and nitrogenase biosynthesis completely) was added, both inside and outside the dialysis bag. Under these conditions, NH₄⁺ excreting activity was lost more rapidly (Fig. 1), decreasing by 50 % after 2 days. This indicates that protein synthesis is necessary to maintain maximum N₂-fixing activity.

The rates of NH₄⁺ production and the maximum amounts of NH₄⁺ produced by different Nif-derepressed strains of *K. pneumoniae* were determined (Table 1). All the Nif-derepressed mutant strains excreted large quantities of fixed N₂ as NH₄⁺. Mutations which lead to a decreased ability to assimilate NH₄⁺ into amino acids, for example, strains without glutamate synthase and glutamine synthetase, or glutamate dehydrogenase activities (Shanmugam *et al.*, 1975) result in NH₄⁺ excretion. NH₄⁺ was the main excretion product of fixed N₂ in these strains and accounted for more than 90 % of the ninhydrin-positive material excreted.

There was considerable variation in activity between the different strains. Rates of excretion as high as 3 μmol NH₄⁺ h⁻¹ (mg protein)⁻¹ were observed (strain SK-54). Some of the strains continued to excrete NH₄⁺ for almost 2 weeks (SK-29) and final levels as high as 450 μmol (mg protein)⁻¹ were accumulated (SK-25). Strain SK-24, which is only about 65 % derepressed for nitrogenase biosynthesis in the presence of NH₄⁺ (Shanmugam & Valentine, 1975), also has low NH₄⁺ excreting activity relative to the other strains, some

Fig. 1. Excretion of fixed N_2 as NH_4^+ during growth of the Nif-derepressed strain SK-25. Cultures were incubated anaerobically in dialysis culture flasks and analysed for growth (Δ), NH_4^+ production ($\circ$, $\bullet$) and glucose consumption ($\square$). In one experiment ($\bullet$), chloramphenicol (0.2 mg ml^{-1}) was added at the time indicated by the arrow.

Table 1. Excretion of fixed N_2 as NH_4^+ by Nif-derepressed strains of *K. pneumoniae*

Cultures were incubated in dialysis culture flasks and analyses were performed as described in Methods. The glucose/ NH_4^+ ratio is the minimum value determined from experiments like those described in Figs 1 and 5.

Nif-derepressed strain	Phenotype*	NH_4^+ production			Rate of glucose consumption [$\mu\text{mol h}^{-1}(\text{mg protein})^{-1}$]	Glucose/ NH_4^+ (molar ratio)
		Maximum rate [$\mu\text{mol h}^{-1}(\text{mg protein})^{-1}$]	Time (days) with rate $> 50\%$ of maximum	Maximum production [$\mu\text{mol}(\text{mg protein})^{-1}$]		
SK-24	Glu^- , R	1.1	6	92	4.7	4.3
SK-25	Gln^- , S	2.5	6	365	9.5	3.8
SK-25	Gln^- , S	2.5	8†	450	—	—
SK-27	Gln^- , S	2.7	5	270	11.0	4.1
SK-28	Glu^- , R	2.2	5	280	8.6	3.9
SK-29	Glu^- , R	2.4	7‡	220	10.3	4.3
SK-37	Gln^- , R	2.8	5	280	10.6	3.8
SK-54	Glu^- , R	3.0	4	240	—	—
SK-55	Gln^- , S	2.0	4	230	8.1	4.0
SK-56	Gln^- , S	2.1	4	190	8.8	4.2
SK-57	Glu^- , R	2.0	8	320	8.5	4.3
SK-59	Glu^- , R	2.9	7‡	380	11.0	3.8
SK-60	Glu^- , R	2.0	—	—	8.2	4.1
SK-512	Gln^- , S	1.9	6	260	—	—
SK-48	Glu^- , Nif ⁻	0	—	—	2.1	—
SK-49	Glu^- , Nif ⁻	0	—	—	1.9	—

* R, Often reverted during the course of an experiment; $0.3 \text{ mg penicillin ml}^{-1}$ was added after incubation for 1 day to prevent growth of revertants (see text for further discussion). S, Genetically stable, no reversion was observed.

† Glutamine was fed very slowly [$0.5 \mu\text{g}(\text{ml total volume})^{-1} \text{ day}^{-1}$] into the culture throughout the experiment.

‡ This strain grew slowly during the entire incubation period (doubling time 8 days) and produced NH_4^+ for 13 days (11 days for SK-59).

of which are essentially fully derepressed (Shanmugam *et al.*, 1975). No clear difference was observed between glutamate- or glutamine-requiring strains in their ability to excrete fixed N_2 as NH_4^+ .

All except two of the strains examined (Table 1) stopped growing after about 1.5 days incubation. The exceptional strains, SK-29 and SK-59, continued to multiply very slowly during the entire course of the experiment (Table 1), and continued to produce NH_4^+ for 13 and 11 days, respectively. In another experiment, addition of small amounts of exogenous glutamine to a culture of strain SK-25 led to an increased period of NH_4^+ production (Table 1). These results, together with the chloramphenicol experiment described above, indicate that for NH_4^+ production to persist it may be essential to maintain a low rate of assimilation of nitrogen for synthesis of cellular proteins.

The Nif-derepressed strains were under a strong selective pressure for reversion to the parental phenotype which utilize NH_4^+ for growth. Many of the strains tended to be overrun during prolonged incubation by revertant types which consumed NH_4^+ (Table 1). To avoid this, penicillin (0.3 mg ml^{-1}) was used in some experiments to selectively destroy these rapidly multiplying types. Glutamine-requiring strains appeared to revert less frequently than glutamate-requiring strains. The reason for this is not known.

Effect of pH. Excretion of NH_4^+ by strain SK-25 was determined as a function of the pH of the incubation medium. In these experiments small numbers of organisms were used and the pH did not change significantly during the experiment. A broad pH optimum centred at pH 7 to 8 was observed (Fig. 2). At pH values below 7, both the rate and the duration of NH_4^+ production decreased. At pH 8.5, growth was very slow and stopped after 3 days; however, the rate and duration of NH_4^+ production were not significantly different from the values at pH 7.3.

Effect of temperature. An increase in the maximum rate of NH_4^+ production was observed with increasing temperature in the range 19 to 29 °C (Fig. 3). A pronounced decrease in the duration of NH_4^+ production occurred above 25 °C. At 34 °C, the maximum rate of fixation was maintained only for about 12 h after growth had stopped (Fig. 3). The maximum temperature at which *K. pneumoniae* M5a1 is able to grow on N_2 as nitrogen source is 35 to 37 °C (unpublished work; Hill, 1976). The pattern of NH_4^+ production was nearly the same regardless of whether the cultures were incubated at the higher temperature from the start of the experiment or grown at 25 °C before the temperature was shifted.

The apparent energy of activation for NH_4^+ production by nitrogenase was determined using strain N-20. Organisms were first incubated at 25 °C in the dialysis culture flask for 36 h, by which time growth had stopped and nitrogenase activity was at a maximum. Samples of the suspension were then incubated at different temperatures for short-term determination of the rate of NH_4^+ production. Control experiments, in which NH_4^+ production was measured using different numbers of organisms, established that N_2 diffusion into the liquid was not limiting. The energy of activation was calculated as 30 kJ mol^{-1} in the range 20 to 35 °C (Fig. 4). Below 20 °C, the energy of activation was higher. Burns (1969) and Hardy *et al.* (1968) reported an energy of activation of about 60 kJ mol^{-1} for N_2 reduction catalysed by *Azotobacter* nitrogenase *in vitro* in the same temperature range. This suggests that the rate-limiting steps in the overall process of reducing N_2 to NH_4^+ *in vivo* and *in vitro* were different.

Energy requirement for N_2 fixation

Nif-derepressed strains of *K. pneumoniae* excreted fixed N_2 as NH_4^+ whilst not growing, and provided a convenient experimental system for investigating the energy requirement for N_2 fixation *in vivo*. The energy requirement for N_2 fixation, in terms of mol glucose consumed per mol NH_4^+ produced, was determined for a number of the Nif-derepressed strains (for strain SK-25, see Fig. 1). These data were used to calculate the ratio of glucose

Fig. 2. Excretion of NH_4^+ from fixed N_2 by strain SK-25 grown in dialysis culture flasks in glucose minimal medium with 0.1 M-Na/K phosphate buffer at various pH values (○) or in glucose minimal medium with 0.13 M-Tricine [*N*-tris(hydroxymethyl)methylglycine]/NaOH buffer and 0.02 M-Na/K phosphate buffer at pH 8.5 (■). (a) NH_4^+ production during growth at pH 5.8 (○) and 8.5 (■); (b) maximum rates at various pH values. A small loss of NH_3 into the gas phase at pH 8.5 was corrected for by comparison with a standard run under identical conditions with $(\text{NH}_4)_2\text{SO}_4$ but no organisms.

Fig. 3. NH_4^+ production from N_2 by strain SK-25 grown in dialysis flasks: (a) at 29 °C (○) and 34 °C (■); (b) maximum rates at various temperatures.

consumed to NH_4^+ produced as a function of time (Fig. 5a). The energy requirement was high initially when the culture was still growing, and then reached a minimum of about 3.8 mol glucose per mol NH_4^+ when growth stopped. The rate of NH_4^+ production, as well as the NH_4^+ /glucose ratio, then gradually decreased with incubation time; after 7 days, 9 mol glucose were consumed per mol NH_4^+ produced. Table 1 summarizes the glucose requirement for N_2 fixation for various Nif-derepressed strains. The ratios are the minimum values determined during time-course experiments (as shown in Fig. 5a), and are based on the total rate of glucose consumption with no correction for basal levels of glucose catabolism. The minimum glucose requirement determined for strain SK-24 (Table 1) is in good agreement with our previously reported value of 4.8 mol glucose per mol NH_4^+ , obtained under slightly different experimental conditions (Shanmugam & Valentine, 1975).

No major difference in efficiency was observed between the different strains. The glucose

Fig. 4. Arrhenius plot for NH_4^+ production from N_2 in strain N-20. Suspension (2 ml) grown in the dialysis culture flask for 36 h was transferred anaerobically to 25 ml flasks filled with an atmosphere of N_2 . The flasks were incubated at the temperatures indicated. At 30 min intervals, H_2 evolution was measured, samples were removed, centrifuged, and NH_4^+ and glucose concentrations were determined as described in Methods. The rates given are the linear rates observed during the first 1.5 h of incubation.

Fig. 5. Glucose requirement for production of NH_4^+ from N_2 in strain SK-25. (a) During growth at pH 7.3 and 25 °C (calculated from data in Fig. 1); (b) minimum glucose requirement as a function of growth pH using glucose minimal medium with 0.1 M-Na/K phosphate buffer (○) or glucose minimal medium with 0.13 M-Tricine/NaOH buffer + 0.2 M-Na/K phosphate buffer (■); (c) minimum glucose requirement at pH 7.3 as a function of growth temperature.

requirement varied from 3.8 (strain SK-25) to 4.3 (SK-24) mol glucose per mol NH_4^+ , although there was a threefold variation in rates of N_2 fixation between different strains (Table 1). The Nif⁻ strains SK-48 and SK-49 consumed glucose at a much lower rate under the same conditions (Table 1).

Effect of pH. The influence of pH in the incubation medium on the minimum glucose requirement for production of NH_4^+ from N_2 was investigated for strain SK-25 (Fig. 5b). The glucose requirement was lowest at pH 8.5 and increased sharply below about pH 6.5. As shown in Fig. 2, the duration and rate of NH_4^+ production also dropped significantly at low pH.

Effect of temperature. The influence of temperature on the minimum glucose requirement for N_2 fixation was determined in a similar manner (Fig. 5c). The glucose requirement increased with increasing temperatures from 19 to 34 °C.

To estimate the energy requirement for N_2 fixation in terms of ATP consumption the

fermentation products must be measured, since *K. pneumoniae* can obtain ATP from the clastic cleavage of pyruvate to acetate, in addition to that obtained through the Embden–Meyerhof–Parnas pathway to pyruvate (Hadjipetrou *et al.*, 1964). From the levels of acetate produced by several Nif-derepressed strains (Table 4), the apparent ATP requirement for N_2 fixation was calculated by assuming an ATP yield of 2 + (mol acetate/mol glucose) per mol glucose fermented.

*Isolation and characterization of mutant strains blocked in
the conventional hydrogen-producing system*

Klebsiella pneumoniae can evolve H_2 at a high rate via the conventional hydrogen-producing system coupled to carbohydrate fermentation. The rate of H_2 evolution by strains SK-24 and SK-25 was still high when nitrogenase biosynthesis was repressed by aspartate plus glutamine as described by Shanmugam & Morandi (1976) (see Table 2). Mutant strains blocked in the conventional hydrogen-producing system were isolated as described in Methods. Chlorate-resistant clones (unable to assimilate nitrate to ammonia) were assayed for nitrogenase activity and H_2 evolution. Those clones which produced H_2 only in the presence of nitrogenase activity were selected for subsequent experiments. Both H_2 production and nitrogenase activities in strains N-15 to N-61 were more than 99 % repressed by aspartate plus glutamine (Table 2). The Nif⁻ strain N-23 did not produce H_2 . When strains N-15 to N-61 were allowed to derepress nitrogenase, the rates of production of H_2 and NH_4^+ increased in parallel.

Cyanide inhibited H_2 evolution and nitrogenase activity in strains SK-24 and N-22 (Fig. 6). With strain N-22 these two activities were inhibited in parallel. With strain SK-24, where H_2 evolution is mainly mediated by the conventional hydrogen-producing system, this activity was the more strongly inhibited. For example, 1 mM-NaCN produced a 40 % inhibition of H_2 evolution and only a 15 % inhibition of nitrogenase activity. These observations suggest that strains N-15 to N-61 are blocked in the conventional hydrogen-producing system (not repressed by amino acids), and that H_2 production is mediated by nitrogenase and is repressed by amino acids. Strain N-6 is apparently only partially blocked (Table 2). Cultures of strains N-20 to N-27 were also assayed for H_2 uptake. Organisms growing in a medium containing aspartate plus glutamine, conditions which completely repress nitrogenase, were incubated with 1 % H_2 in Ar. No uptake of H_2 was detected (Table 2). Resting suspensions also did not take up H_2 when incubated with benzyl viologen or methylene blue (2 mM each) as electron acceptors.

Nitrogenase activity, measured as H_2 uptake with 2 mM-benzyl viologen as acceptor (Pichinoty, 1969), was detected in extracts of strain N-20. The block is therefore apparently not in hydrogenase itself, but rather in the electron transport system coupling to hydrogenase.

Nitrogenase-catalysed H_2 evolution

Since strains N-20 to N-61 produced NH_4^+ and H_2 only via the nitrogenase system, they can be used to determine the relative rates of formation of these two products *in vivo*. NH_4^+ production and H_2 production were determined for these strains (see Fig. 7 and Table 3). With 100 % N_2 as the sparging gas, the rates of NH_4^+ production and H_2 evolution by strain N-20 were 1.90 and 1.20 $\mu\text{mol h}^{-1}$ (mg protein)⁻¹, respectively. Under these conditions, nitrogenase catalysed the formation of 0.63 mol H_2 per mol NH_4^+ produced. The range of H_2/NH_4^+ ratios determined for strains N-22 to N-61 was 0.6 to 0.7 (Table 3), with an average molar ratio of 0.65. When the sparging gas was changed to Ar (at 56 h, Fig. 7), NH_4^+ production stopped and the H_2 production increased from 1.2 to 3.3 $\mu\text{mol h}^{-1}$ (mg protein)⁻¹. A decrease in H_2 evolution and a resumption of NH_4^+ production occurred when the sparging gas was changed back to N_2 at 79 h.

Thus when N_2 is not available as a substrate, nitrogenase will catalyse a rapid energy-

Table 2. *Repression of H₂ evolution in chlorate-resistant strains of K. pneumoniae by repressors of nitrogenase biosynthesis*

Flasks (5 ml) with 1 ml glucose minimal medium containing L-glutamate (0.1 mg ml⁻¹) or L-aspartate plus L-glutamine (each 1 mg ml⁻¹) were inoculated and incubated overnight at 25 °C in a gas phase of Ar. H₂ production (in Ar atmosphere) and acetylene reduction (in N₂ atmosphere) were measured after 1 h incubation as described in Methods. H₂ uptake was measured in an atmosphere of 1 % H₂ in Ar. All results are expressed as $\mu\text{mol h}^{-1} (\text{mg protein})^{-1}$.

Strain	Parent strain	Glutamate*		Aspartate + glutamine		
		Nitrogenase activity†	H ₂ production	Nitrogenase activity†	H ₂ production	H ₂ uptake
SK-24	ASM-I	1.70	11.3	0	7.5	—
SK-25	ASM-I	2.70	12.7	0	9.9	—
N-6	M5aI	1.54	9.6	0	0.6	—
N-15	M5aI	0.94	7.5	0	0.04	—
N-20	SK-24	0.89	3.9	0	0.02	< 0.03
N-20	SK-24	1.21	0.03‡	—	—	—
N-22	SK-24	1.38	5.2	0	0.02	< 0.03
N-23	SK-24	0	0	0	0	—
N-25	SK-24	0.77	1.9	0	0	< 0.03
N-27	SK-24	1.95	2.7	0	0	< 0.03
N-61	SK-25	2.61	3.1	0	0	—

* Glutamine was added for strains SK-25 and N-61.

† Acetylene reduction.

‡ In the presence of 0.2 atm (20 kPa) acetylene.

Fig. 6. Inhibition of nitrogenase activity (Δ) and H₂ evolution ($\circ$) in strains SK-24 (a) and N-22 (b) by cyanide. Cultures were grown overnight in glucose minimal medium containing 0.1 mg L-glutamate ml⁻¹. NaCN was then added and nitrogenase activity (acetylene reduction) and H₂ evolution were determined (see Methods).

dependent H₂ evolution *in vivo*. The total rate of electron flow through nitrogenase (to H⁺ plus N₂), calculated from the data in Table 3, shows a decrease of 20 to 25 % under Ar compared with N₂ for all the strains tested. A similar decrease in the rate of glucose consumption was observed under Ar (Table 3). Under these conditions, the rate of glucose consumption was linear with time, since growth had stopped after about 30 h (compare Fig. 7 and Fig. 1). Strains SK-24 and SK-25 also consumed 20 to 25 % less glucose under Ar than under N₂.

H₂ production under N₂ was not due to non-saturation with N₂. When a culture which had reached a constant level of nitrogenase activity was sparged with different concentrations of N₂ in Ar, there was little increase in the rate of H₂ production until the sparging gas

Fig. 7. Nitrogenase-catalysed H_2 evolution and NH_4^+ production in a strain blocked in the conventional hydrogen-producing system. Strain N-20 was incubated in the dialysis culture flask as described in Methods and sparged with N_2 or Ar as indicated. At intervals H_2 evolution (■) and NH_4^+ in the supernatant (○) were measured.

Table 3. *Determination of the nitrogenase-catalysed formation of H_2 and NH_4^+ in chlorate-resistant strains of *K. pneumoniae**

Experiments were performed as described for Fig. 7. Results for production of NH_4^+ and H_2 and for glucose consumption are expressed as $\mu\text{mol h}^{-1} (\text{mg protein})^{-1}$.

Strain	Gas phase	NH_4^+ produced	H_2 produced	$\frac{H_2^*}{NH_4^+}$	Glucose consumed	$\frac{\text{Glucose}^*}{NH_4^+}$	$\frac{\text{Glucose}^*}{H_2}$
N-20	N_2	1.90	1.20	0.63	7.41	3.90	
	Ar	0	3.30		5.94		1.8
N-22	N_2	1.51	1.06	0.70	6.07	4.02	
	Ar	0	2.78		5.19		1.9
N-25	N_2	1.32	0.88	0.67	5.16	3.91	
	Ar	0	2.25		4.33		1.9
N-27	N_2	1.41	0.84	0.60	5.44	3.86	
	Ar	0	2.54		4.50		1.8
N-61	N_2	1.55	1.01	0.65	—	—	

* Molar ratio.

contained less than 20 % N_2 . A Lineweaver-Burk plot of the data gave an apparent K_m for N_2 of 0.06 atm (6 kPa). This is somewhat lower than the apparent K_m for N_2 reported for nitrogenase from *K. pneumoniae* *in vitro* [0.21 atm (21 kPa); Parejko & Wilson, 1971]. Acetylene completely suppressed nitrogenase-catalysed H_2 evolution (Table 2).

The H_2/NH_4^+ molar ratio as a function of pH and temperature was determined in short-term experiments with a non-growing culture of strain N-20 (Fig. 8). The pH dependence of the rate of NH_4^+ production was similar to that observed in long-term experiments with SK-25 (Fig. 2). There was little change in the rate of NH_4^+ production and in the H_2/NH_4^+ molar ratio in the range pH 6.5 to 8.5. At pH values below 6, a pronounced drop in the rate of NH_4^+ production and a simultaneous rise in the H_2/NH_4^+ ratio was observed. At pH 4.8, the ratio increased to 1.9 and the rate of NH_4^+ production decreased to 25 % of the maximum. The pH dependence of glucose consumption was also similar to that in strain SK-25 (Fig. 5b). At pH 4.8, 8.0 mol glucose were consumed per mol NH_4^+ produced (Table 4).

The H_2/NH_4^+ molar ratio remained constant at temperatures below about 20 °C but increased with temperatures above 20 °C (see Fig. 8b). The rate of NH_4^+ excretion increased with temperature to 40.4 °C, the highest temperature tested (Fig. 4). The glucose consumption in these experiments agreed well with the temperature dependence observed for

Fig. 8. (a) Nitrogenase-catalysed production of H_2 and NH_4^+ in strain N-20 as a function of pH using glucose minimal medium with 0.1 M-Na/K phosphate buffer (○) or glucose minimal medium with 0.13 M-Tricine/NaOH buffer and 0.02 M-Na/K phosphate buffer (■). The culture was allowed to induce a maximum level of nitrogenase in the dialysis culture flask and then 2 ml samples were transferred anaerobically into flasks (25 ml) filled with N_2 and containing 2 ml buffer at the desired pH. A small loss of NH_3 to the gas phase at pH 8.9 was corrected for by comparison with a standard run under identical conditions with $(NH_4)_2SO_4$ but no organisms. H_2 evolution and NH_4^+ production were determined as described for Fig. 4. (b) Nitrogenase-catalysed production of H_2 and NH_4^+ in strain N-20 as a function of temperature at pH 7.3. The experiment was performed as described for Fig. 4.

Table 4. *Energetics of N_2 fixation by Nif-derepressed strains of K. pneumoniae*

For details, see Methods.

Strain	Gas phase	Temperature (°C)	pH	Glucose consumed [$\mu\text{mol h}^{-1}$ (mg protein) $^{-1}$]	Glucose* consumed NH_4^+ produced	Acetate* produced glucose consumed	Apparent ATP/ N_2 value†
SK-24	N_2	25	7.3 ¹	4.7	4.3	0.94	25
SK-25	N_2	25	7.3 ¹	9.5	3.8	0.76	21
SK-25	Ar	25	7.3 ¹	7.7	—	0.86	—
SK-28	N_2	25	7.3 ¹	8.6	3.9	0.84	22
SK-37	N_2	25	7.3 ¹	10.6	3.8	0.75	21
N-20	N_2	25	7.3 ²	7.4	3.9	0.89	23
N-20	Ar	25	7.3 ²	5.9	1.8‡	0.90	5§
N-20	N_2	25	8.9 ³	6.0	4.0	1.30	26
N-20	N_2	25	4.8 ³	3.7	8.0	0.56	41
N-20	N_2	40	7.3 ⁴	13.4	6.3	0.38	30
SK-48	N_2	25	7.3 ¹	1.9	—	1.34	—
SK-48	N_2	25	4.8 ³	1.8	—	0.60	—
SK-48	N_2	40	7.3 ⁴	5.9	—	0.41	—

¹⁻⁴ Samples taken from the experiments described in Table 1, Figs 7, 8(a) and (b) respectively.

* Molar ratio.

† Calculated as described in the text.

‡ Mol glucose consumed per mol H_2 produced.

§ Apparent ATP/ H_2 value.

SK-25 in long-term experiments (Fig. 5c). The glucose requirement for N_2 fixation, the levels of acetate formed from fermentation, and the calculated apparent ATP/ N_2 values under different conditions are presented in Table 4.

DISCUSSION

Energy (ATP) requirement for N₂ fixation

Nitrogen fixation in *K. pneumoniae* *in vivo* was most efficient below 25 °C, and at a pH of 7 to 8. The minimum energy requirement for N₂ fixation was about 4 mol glucose consumed per mol NH₄⁺ produced for the Nif-derepressed strains and varied little from strain to strain, although the rate of fixation varied threefold. The Nif⁻ strains SK-48 and SK-49 consumed glucose at 25 % of the rate of N₂-fixing strains (Table 1). This indicates that the major part of the energy derived from fermentation of glucose was used for N₂ fixation in these Nif-derepressed strains under the present experimental conditions.

The observed increase in glucose/NH₄⁺ molar ratio with time (Fig. 5*a*) could not be explained by a change in the fermentation pattern because the acetate/glucose molar ratio did not change significantly during the incubation, nor by an increase in the amount of energy lost through nitrogenase-catalysed H₂ evolution. The H₂/NH₄⁺ molar ratio was not significantly increased after 6 days incubation of strain N-20 in the dialysis culture flask, although the rate of NH₄⁺ excretion had decreased to 45 % of its maximum value.

The increase in glucose/NH₄⁺ ratio with time (Fig. 5*a*) may be caused in part by a decreased rate of fixation increasing the relative contribution of 'maintenance' energy requirement in the overall rate of glucose consumption.

The energy requirement for N₂-fixation in terms of ATP may be estimated when the fermentation pathways for glucose are known. In *K. aerogenes*, acetate production is a measure of the ATP obtained through the clastic cleavage of pyruvate (1 mol ATP per mol acetate) (Hadjipetrou *et al.*, 1964; Stouthamer & Bettenhausen, 1973). Hill (1976) reported that *K. pneumoniae* could grow anaerobically on pyruvate producing 1 mol acetate per mol pyruvate consumed and we obtained similar results (Table 4). An apparent ATP requirement of 21 to 25 ATP/N₂ was calculated for N₂ fixation in some of the Nif-derepressed strains at pH 7.3 and 25 °C. If the rate of glucose consumption in the Nif⁻ strains SK-48 and SK-49 (Table 1) measures 'maintenance' energy requirement for the organism, then the apparent ATP/N₂ value for the nitrogenase reaction is about 14 to 16. This is considerably lower than the value of 29 ATP/N₂ estimated by Hill (1976) for *K. pneumoniae* growing on N₂, but is closer to the value estimated for *C. pasteurianum* (Daesch & Mortenson, 1968).

If the nitrogenase-catalysed H₂ production in the presence of N₂ (H₂/NH₄⁺ molar ratio of 0.65) is also considered, an apparent ATP/2e ratio of about 4 is deduced (calculated value 3.7 for strain N-20). This agrees with minimal values of 4 to 5 ATP/2e reported in most recent *in vitro* studies (Burns & Hardy, 1975; Zumft & Mortenson, 1975; Watt *et al.*, 1975). The real energy cost in terms of ATP equivalents to a cell fixing N₂ under conditions where respiration is possible, may be considerably higher than about 14 to 16 ATP/N₂. A H₂/NH₄⁺ ratio of 0.65 corresponds to 4.3 pairs of low potential electrons per N₂ reduced, electrons which potentially could have given 13 ATP if used in oxidative phosphorylation (assuming a P/O ratio of 3). The total energy cost may therefore be close to 30 ATP equivalents per N₂, or 35 to 40 ATP equivalents including the 'maintenance' energy requirement of the organism.

Nitrogenase *in vitro* is most efficient at slightly alkaline pH and relatively low temperatures. ATP hydrolysis is uncoupled from substrate reduction for clostridial nitrogenase below pH 6 (Jeng, Morris & Mortenson, 1970) and for nitrogenase from *A. vinelandii* at temperatures above 20 °C (Watt *et al.*, 1975). The glucose requirement for N₂ fixation increased at pH values below pH 7 (Fig. 5*b*) and temperatures above 20 °C (Fig. 5*c*). The apparent ATP/N₂ requirement (Table 4) also increased: to 41 at pH 4.8 and 25 °C, and to 30 at 40 °C and pH 7.3. This large increase at pH 4.8 may be due mainly to the low rate of fixation (25 % of the rate at pH 7.3), causing the relative contribution of the cells 'maintenance' energy requirement to increase. If the glucose consumption observed for the

Nif⁻ strain SK-48 at pH 4.8 measures the 'maintenance' energy requirement, then the apparent ATP/N₂ value for the nitrogenase reaction is 20. A similar correction gives an apparent ATP/N₂ value of 27 for the nitrogenase reaction at 40 °C. If the observed nitrogenase-catalysed H₂ evolution (Fig. 8*a, b*) is also considered then the apparent ATP/2e values are 3.0 at pH 4.8 (25 °C) and 4.0 at 40 °C (pH 7.3). These values are, though, only approximations.

Mutants blocked in the conventional hydrogen-producing system

The *Klebsiella* strains used in the present study for quantitative determination of nitrogenase-mediated H₂ evolution were isolated as chlorate-resistant clones. The mutant strains N-20 to N-27 did not take up H₂ nor evolve H₂ unless nitrogenase was present (Table 2). The patterns of inhibition of H₂ evolution and nitrogenase activity by cyanide were identical in these strains. It is concluded that these strains in addition to being blocked in nitrate reduction (K. T. Shanmugam, unpublished work), were also blocked in the conventional hydrogen-producing system, thus suggesting that a reduction step, shared by both pathways, may be altered. Similar pleiotropic effects of mutations leading to chlorate resistance have been produced in *K. aerogenes* (Stouthamer *et al.*, 1967) and *Escherichia coli* (Azoulay & Marty, 1970) giving strains defective in the formate-hydrogenlyase system even though hydrogenase was still present.

The chlorate-resistant strains N-20 to N-27 were apparently not impaired in their energy metabolism since their glucose requirement for N₂ fixation was the same as for the other derepressed strains described before (Tables 1 and 3). Stouthamer (1967) observed that chlorate-resistant strains of *K. aerogenes* which did not produce gas also gave the same growth yield on glucose as the wild type.

Nitrogenase-catalysed H₂ evolution

Several different strains had H₂/NH₄⁺ molar ratios of 0.6 to 0.7 at pH 7.3, 25 °C and 100 % N₂ (Table 3) which agrees with values reported for nitrogenase *in vitro*. Even in the presence of 100 % N₂, the H₂/NH₄⁺ molar ratio for purified nitrogenase from *A. vinelandii* drops to only 0.5 to 0.8 (Hadfield & Bulen, 1969; Hwang, Chen & Burris, 1973; Burns & Hardy, 1972). Hill's (1976) study indicated that the H₂/NH₄⁺ molar ratio was probably significantly higher than 0.65 in glucose-limited chemostat cultures of *K. pneumoniae*. This difference may be due to ATP limitation since Silverstein & Bulen (1970) reported that low ATP concentrations caused an increase in the H₂/NH₄⁺ ratio for isolated nitrogenase from *A. vinelandii*.

When N₂ was replaced by Ar, the production of H₂ markedly increased in cultures of *K. pneumoniae* (Fig. 7). Since the rate of glucose consumption stayed at a high level when N₂ became limiting (Table 3), additional energy must have been lost through nitrogenase-catalysed H₂ production. The energy requirement, expressed as ATP/2e value, is apparently the same for nitrogenase-catalysed H₂ production as for NH₄⁺ production (Table 4). These observations agree with *in vitro* studies of nitrogenase where ATP/2e values are the same for reduction of N₂, H⁺ and other substrates (see, for example, Hadfield & Bulen, 1969) or slightly lower for H⁺ than for N₂ (Hwang & Burris, 1973).

In conclusion, energy lost through nitrogenase-catalysed H₂ production comprising at least 30 %, and, under unfavourable conditions of temperature and pH, even more, is an important factor in the energy requirement for biological N₂ fixation.

Energy sparing effect

In a number of *in vitro* studies the electron turnover rate for nitrogenase was the same when N₂ plus H⁺ were substrates (N₂ atmosphere) or H⁺ alone (Ar atmosphere) (see

Burns & Hardy, 1975). The rate of both electron turnover for nitrogenase and glucose consumption by intact organisms under Ar atmosphere was 75 to 80 % of the corresponding rate under N₂ (see Table 3). Acetate production is consistent with the fermentation pattern being unchanged (Table 4).

Therefore, in the absence of N₂, a 20 to 25 % energy sparing can occur *in vivo* but not apparently *in vitro*. Organisms may have evolved mechanisms that allow a partial shut-down of the electron flow through nitrogenase when N₂ is limiting or when other nitrogen sources become available. Ludden & Burris (1976) recently reported the presence of an activating factor needed for nitrogenase activity in *Rhodospirillum rubrum*.

Energy supply may be the rate-limiting step of symbiotic N₂ fixation (Hardy & Havelka, 1975). To use bacteria such as root nodule bacteria (*Rhizobium*) to the best advantage, genetic and physiological factors which lead to greater efficiency of N₂ fixation have to be identified so that the most efficient conditions or organisms can be selected. We have demonstrated that nitrogenase-catalysed H₂ production can be changed by physical means, and it may be possible to construct mutants whose nitrogenase works more efficiently. Recently, H₂ uptake hydrogenase, which permits organisms with this enzyme system to recoup some of the ATP expended on nitrogenase-catalysed H₂ evolution, have been described (Dixon, 1972; Smith *et al.*, 1976). Optimizing this system is perhaps more likely to improve the energy efficiency of symbiotic N₂ fixation in important crop plants.

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REFERENCES

- AZOULAY, E. & MARTY, B. (1970). Étude du système multi-enzymatique hydrogène lyase chez *Escherichia coli* K12 et ses mutants chlorate-resistants. *European Journal of Biochemistry* **13**, 168-173.
- BALLENTINE, R. (1957). Determination of total nitrogen and ammonia. *Methods in Enzymology* **3**, 984-995.
- BOTHE, H., TENNIGKEIT, J., EISBRENNER, G. & YATES, M. G. (1977). The hydrogenase-nitrogenase relationship in the blue-green alga *Anabaena cylindrica*. *Planta* **133**, 237-242.
- BURNS, R. C. (1969). The nitrogenase system from *Azotobacter*. Activation energy and divalent cation requirement. *Biochimica et biophysica acta* **171**, 253-259.
- BURNS, R. C. & HARDY, R. W. F. (1972). Purification of nitrogenase and crystallization of its Mo-Fe protein. *Methods in Enzymology* **24**, 480-497.
- BURNS, R. C. & HARDY, R. W. F. (1975). *Nitrogen Fixation in Bacteria and Higher Plants*. New York: Springer Verlag.
- 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. O. D. (1972). Hydrogenase in legume root nodule bacteroids: occurrence and properties. *Archiv für Mikrobiologie* **85**, 193-201.
- DREWS, G. (1965). Untersuchungen zur Regulation der Bacteriochlorophyll-Synthese bei *Rhodospirillum rubrum*. *Archiv für Mikrobiologie* **51**, 186-198.
- GORDON, J. K. & BRILL, W. J. (1974). Derepression of nitrogenase synthesis in the presence of excess NH₄⁺. *Biochemical and Biophysical Research Communications* **59**, 967-971.
- HADFIELD, K. L. & BULEN, W. A. (1969). Adenosine triphosphate requirement of nitrogenase from *Azotobacter vinelandii*. *Biochemistry* **8**, 5103-5108.
- HADJIPETROU, 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**, 139-150.
- 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. & HAVELKA, U. D. (1975). Nitrogen fixation research: a key to world food? *Science* **188**, 633-643.
- HARDY, R. W. F., HOLSTEN, R. D., JACKSON, E. K. & BURNS, R. C. (1968). The acetylene-ethylene

- assay for N_2 fixation: laboratory and field evaluation. *Plant Physiology* **43**, 1185–1207.
- HILL, S. (1976). The apparent ATP requirement for nitrogen fixation in growing *Klebsiella pneumoniae*. *Journal of General Microbiology* **95**, 297–312.
- HOLZ, G. & BERGMAYER, H. U. (1974). Acetate determination with acetate kinase and hydroxylamine. *Methods of Enzymatic Analysis* **3**, 1528–1532.
- HWANG, J. C. & BURRIS, R. H. (1973). Nitrogenase catalysed reactions. *Biochimica et biophysica acta* **283**, 339–350.
- HWANG, J. C., CHEN, C. H. & BURRIS, R. H. (1973). Inhibition of nitrogenase-catalysed reductions. *Biochimica et biophysica acta* **292**, 256–270.
- HYNDMAN, L. A., BURRIS, R. H. & WILSON, P. W. (1953). Properties of hydrogenase from *Azotobacter vinelandii*. *Journal of Bacteriology* **65**, 522–531.
- JENG, D. Y., MORRIS, J. A. & MORTENSON, L. E. (1970). The effect of reductant on inorganic phosphate release from adenosine 5'-triphosphate by purified nitrogenase of *Clostridium pasteurianum*. *Journal of Biological Chemistry* **245**, 2809–2813.
- LJONES, T. & BURRIS, R. H. (1972). ATP hydrolysis and electron transfer in the nitrogenase reaction with different combinations of the iron protein and the molybdenum-iron protein. *Biochimica et biophysica acta* **275**, 93–101.
- LUDDEN, P. W. & BURRIS, R. H. (1976). Activating factor for the iron protein of nitrogenase from *Rhodospirillum rubrum*. *Science* **194**, 424–426.
- ORME-JOHNSON, W. H. & DAVIS, L. C. (1977). Current topics and problems in the enzymology of nitrogenase. In *Iron-Sulfur Proteins*, vol. 3, pp. 15–60. Edited by W. Lovenberg. New York: Academic Press.
- PARJEKO, R. A. & WILSON, P. W. (1971). Kinetic studies on *Klebsiella pneumoniae* nitrogenase. *Proceedings of the National Academy of Sciences of the United States of America* **68**, 2016–2018.
- PICHINOTY, F. (1969). Recherche des activités formiate-oxydase, hydrogene-lyase, hydrogenase et formiate-deshydrogenase chez quelques *Enterobacteriaceae*. *Annales de l'Institut Pasteur* **117**, 3–15.
- POSTGATE, J. R. (1969). Viable counts and viability. *Methods in Microbiology* **1**, 611–628.
- SCHUBERT, K. R. & EVANS, H. J. (1976). Hydrogen evolution: a major factor affecting the efficiency of nitrogen fixation in nodulated symbionts. *Proceedings of the National Academy of Sciences of the United States of America* **73**, 1207–1211.
- SHANMUGAM, K. T. & MORANDI, C. (1976). Amino acids as repressors of nitrogenase biosynthesis in *Klebsiella pneumoniae*. *Biochimica et biophysica acta* **437**, 322–332.
- 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.
- SHANMUGAM, K. T., LOO, A. S. & VALENTINE, R. C. (1974). Deletion mutants of nitrogen fixation in *Klebsiella pneumoniae*: mapping of a cluster of *nif* genes essential for nitrogenase activity. *Biochimica et biophysica acta* **338**, 545–553.
- SHANMUGAM, K. T., CHAN, I. & MORANDI, C. (1975). Regulation of nitrogen fixation. Nitrogenase-derepressed mutants of *Klebsiella pneumoniae*. *Biochimica et biophysica acta* **408**, 101–111.
- SHANMUGAM, K. T., MORANDI, C., ANDERSEN, K. & VALENTINE, R. C. (1977a). Genetic engineering with nitrogen fixation. In *Proceedings of the III International Conference on Enzyme Engineering*, (in the Press). Edited by K. Pye.
- SHANMUGAM, K. T., MORANDI, C. & VALENTINE, R. C. (1977b). Nitrogenase derepressed mutants of *Klebsiella pneumoniae*. In *Iron-Sulfur Proteins*, vol. 3, pp. 1–14. Edited by W. Lovenberg. New York: Academic Press.
- SILVERSTEIN, R. & BULEN, W. A. (1970). Kinetic studies of the nitrogenase-catalysed hydrogen evolution and nitrogen reduction reaction. *Biochemistry* **9**, 3809–3815.
- SMITH, L. A., HILL, S. & YATES, M. G. (1976). Inhibition by acetylene of conventional hydrogenase in nitrogen-fixing bacteria. *Nature, London* **262**, 209–210.
- SPIES, J. R. (1957). Colorimetric procedures for amino acids. *Methods in Enzymology* **3**, 467–477.
- STEWART, W. D. P. & ROWELL, P. (1975). Effect of L-methionine-DL-sulphoximine on the assimilation of newly fixed NH_3 , acetylene reduction and heterocyst production in *Anabaena cylindrica*. *Biochemical and Biophysical Research Communications* **65**, 846–856.
- STOUTHAMER, A. H. (1967). Nitrate reduction in *Aerobacter aerogenes*. II. Characterization of mutants blocked in the reductions of nitrate and chlorate. *Archiv für Mikrobiologie* **56**, 76–80.
- 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.
- STOUTHAMER, A. H., BETTENHAUSEN, C., VAN HARTINGSVELD, J., VAN'T RIET, J. & PLANTA, R. J. (1967). Nitrate reductions in *Aerobacter aerogenes*. III. Nitrate reduction, chlorate resistance and formate metabolism in mutant strains. *Archiv für Mikrobiologie* **58**, 228–247.
- STREICHER, S., GURNEY, E. & VALENTINE, R. C. (1971). Transduction of the nitrogen-fixing genes in *Klebsiella pneumoniae*. *Proceedings of the National Academy of Sciences of the United States of America* **68**, 1174–1177.
- UMBREIT, W. W., BURRIS, R. H. & STAUFFER, J. F. (1964). *Manometric and Biochemical Techniques*. Minneapolis: Burgess Publishing Company.
- WALL, J. D., WEAVER, P. F. & GEST, H. (1975). Genetic transfer of nitrogenase-hydrogenase activity in *Rhodopseudomonas capsulata*. *Nature, London* **258**, 630–631.
- WATT, G. D., BULEN, W. A., BURNS, A. & HADFIELD, K. L. (1975). Stoichiometry, ATP/2e values, and energy requirements for reactions catalysed by nitrogenase from *Azotobacter vinelandii*. *Biochemistry* **14**, 4266–4272.

- WEARE, N. M. & SHANMUGAM, K. T. (1976). Photo-production of ammonium ion from N_2 in *Rhodospirillum rubrum*. *Archives of Microbiology* **110**, 207–213.
- WINTER, H. C. & BURRIS, R. H. (1976). Nitrogenase. *Annual Review of Biochemistry* **45**, 409–426.
- ZUMFT, W. G. & MORTENSON, L. E. (1975). The nitrogen-fixing complex of bacteria. *Biochimica et biophysica acta* **416**, 1–52.