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PHYSIOLOGICAL ASPECTS OF N_2-FIXATION BY A *SPIRILLUM* FROM *DIGITARIA* ROOTS

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Summary—Studies of the physiology of the *Spirillum lipoferum* recognized as the major organism responsible for N_2-fixation in the roots of *Digitaria decumbens* cv *transvala* were performed in order to improve the methods of culture and help to explain the physiology of this N_2-fixing grass bacterial association.

Methods for isolation, purification and N_2-fixation assays are described. Acetylene concentrations used for N_2-ase activity measurements should be at least 12%, the Vmax of cultures in the log phase being at a pC_2H_2 of 0·12 atm and the apparent K_m 0·022 atm. Optimal temperatures for N_2-dependent growth are between 32 and 40°C, and little N_2-fixation is observed below 24°C. At 42°C the N_2-ase is inactivated. When cultures grown at 28 or 36°C are transferred to lower temperatures nitrogenase activity declines rapidly. One hour after transfer to 17°C activity is about half that before transfer and is maintained at this level for at least 8 h. After transfer to 10°C activity ceases after 1 h. Growth is very pH dependent, optimal growth on N_2 occurring only between pH 6·8 and 7·8. Nitrogen fixation below pH 5·5 and above 8·0 is less than one-quarter of the optimal. No N_2-fixation occurs in the absence of O_2 and maximal N_2-dependent growth is reached at 1·5% O_2 in the gas mixture bubbled through liquid cultures.

In contrast to previous reports, several sugars including glucose can be used by the *Spirillum* for N_2-fixation, but only when small amounts of starter nitrogen or organic acids are added to the medium. Efficiencies of N_2-fixation on malate and glucose are similar and about 60% of that of cells incorporating NH_4^+–N. Efficiency of NO_3^- incorporation is 74% of that of NH_4^+–N grown cultures. High observed efficiencies (52 mg N_2 fixed g^{-1} malate or glucose) are attributed to carbon limited growth at optimum or O_2 limited conditions, both facilitated by slow diffusion rates through the semi-solid agar medium used.

INTRODUCTION

Nitrogen fixation of economic importance in tropical grass–bacteria associations has now been well established (Balandreau and Villemin, 1973; Döbereiner and Day, 1975; Day *et al.*, 1975) but little is known yet about the mechanism and microorganisms involved. In the *Digitaria decumbens* cv *transvala* association and possibly a number of others, highly efficient N_2-fixing *Spirillum* forms have been identified and are thought to be responsible for N_2-fixation in the roots of field-grown grasses (Döbereiner and Day, 1975).

The morphological and physiological characteristics of the *Spirillum* given by Döbereiner and Day (1975) agree in every detail with the description of *Spirillum lipoferum* (Beijerinck, 1923), summarized in Bergey's Manual (Breed *et al.*, 1957), although revision by modern methods seems necessary. Beijerinck (1923) described this organism as a "Nitrogen fixing *Spirillum*" although he was not able to confirm this characteristic in pure cultures. Schröder (1932) with single-cell isolates grown in glucose malate medium concluded that even in media enriched with humic compounds there was no N_2-fixation and the organism was ignored as a N_2-fixer until Becking (1963) showed $^{15}N_2$ incorporation in the presence of yeast extract, with a *Spirillum* or *Vibrio* isolated from tropical soils and very probably identical with Beijerinck's *Spirillum lipoferum*.

The recent demonstration of extremely high efficiencies of N_2-fixation by the *Spirillum* isolated from *Digitaria* roots, when low concentrations of energy sources and semi-solid media were used (Döbereiner and Day, 1975), bring this organism into the forefront of interest among free-living N_2-fixing bacteria. In the present paper a number of physiological characteristics were studied to complement the earlier description.

MATERIALS AND METHODS

The *Spirillum* cultures were isolated from field-grown root pieces with highly active N_2-ase activity as determined by the C_2H_2 reduction method. The washed root pieces were placed into semi-solid N-free Na malate (0·5%) medium (2 ml in 7 ml flasks) with the following basic composition: KH_2PO_4, 0·4 g; K_2HPO_4, 0·1 g; $MgSO_4.7H_2O$, 0·2 g; NaCl, 0·1 g; $CaCl_2$, 20 mg; $FeCl_3$, 10 mg; $NaMoO_4.2H_2O$, 2 mg; 0·5% alcoholic solution of bromothymol blue, 5 ml; agar, 1·75 g; H_2O, 1 l. These *Spirillum* enrichment cultures form fine white pellicles 2–4 mm below the surface of the medium. The most active enrichment cultures as selected by C_2H_2 reductions were streaked

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onto plates of the same basic composition but with Ca-malate and 5 ml of 10% (w/w) fresh yeast infusion/ l. added. After 1 week typical irregular, dry, white colonies, often with greenish centres, were selected, replicated into semi-solid Ca-malate medium with 15 ml yeast extract/l. and after 24 h again checked for C_2H_2 reduction. Purity of the most active of these isolates was checked again on agar plates before they were stored under oil in the semi-solid Ca-malate yeast medium. The most characteristic forms were observed in the N-free Na-malate medium, they are curved rods of varying sizes with one-half to one spiral turn and containing refractive lipid bodies. The cells are extremely active and the mode of motility is very characteristic. In Ca-malate medium the forms are less characteristic, having few lipid inclusions.

Acetylene reduction measurements were performed directly in the culture flasks fitted with rubber seals, 12% C_2H_4 was injected and C_2H_4 production was measured after 1 h by injecting 0·5 ml of the gas mixture into a Perkin–Elmer F-11 Gas Chromatograph with a Poropak N column at 100°C (Dart *et al.*, 1972). Care was taken not to disturb the semi-solid cultures with the pellicle because cracking of the surface or mixing stopped N_2-ase activity immediately.

Total N was determined by the semi-micro Kjeldahl procedure with HgO as the catalyst. Detailed methods of the individual experiments are given in the Results section.

RESULTS AND DISCUSSION

To improve the cultural methods and learn more about the physiology of the *Spirillum* several experiments were carried out, the results of which should lead to a better understanding of some aspects of the grass associations involving N_2-fixing spirilliform bacteria.

Apparent K_m *and Vmax for* C_2H_2

Uniformly inoculated semi-solid Ca-malate (0·25%) cultures (3 ml medium in 10 ml flasks) were incubated for 3 days at 36°C before assay. Four replicate vials were incubated with increasing concentrations of C_2H_2, the actual C_2H_2 concentration in the gas phase being determined by gas chromatography. Ethylene production was linear at all C_2H_2 concentrations for at least 2 h, the 1 h rates being presented in Fig. 1. Vmax was reached only at 12% C_2H_2, the apparent K_m for C_2H_2 being 0·022 atm. This is higher than the average reported for other free living N_2-fixing bacteria (0·005 atm, cited by Hardy *et al.*, 1973) but similar to the values obtained with *Beijerinckia fluminensis* (Döbereiner and Postgate, unpublished data). Fortunately these organisms do not represent the extreme C_2H_2 saturation difficulties of *Beijerinckia indica* cultures reported by Spiff and Odu (1973) and confirmed by the authors, Vmax being reached only between pC_2H_2 0·3–0·6 atm.

Optimal temperatures for N_2*-dependent growth and* N_2*-ase activity*

Spirillum cultures in semi-solid Ca-malate (0·25%) medium (3 ml in 10 ml flasks) were grown over a range of temperatures from 10–40°C. After 29 and 40 h N_2-ase activity was assayed at the growth temperature by C_2H_2 reduction as described in the Methods section. The results in Fig. 2A show temperature requirements typical of organisms from tropical environments, i.e. *Derxia* and *Azotobacter paspali* (Döbereiner, 1967). After 29 h little growth had occurred at 28°C while from 32 to 40°C N_2-ase activity was already almost at maximum for these cultures. After 40 h growth the cultures at 28°C had formed a pellicle and N_2-ase activity was 13 times

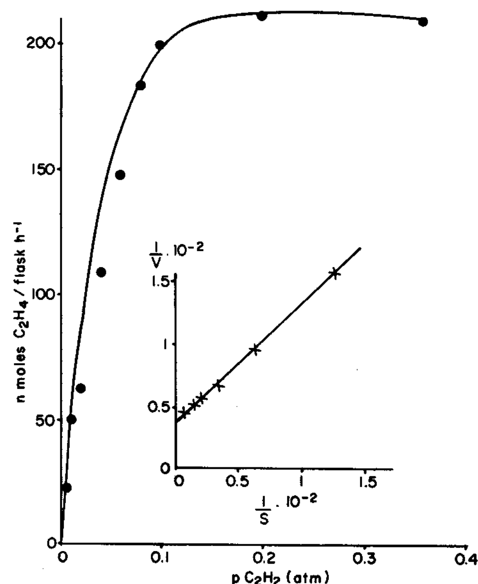

Fig. 1. The apparent K_m and V max of C_2H_2 reduction with *Spirillum lipoferum* grown in semi-solid Ca-malate cultures.

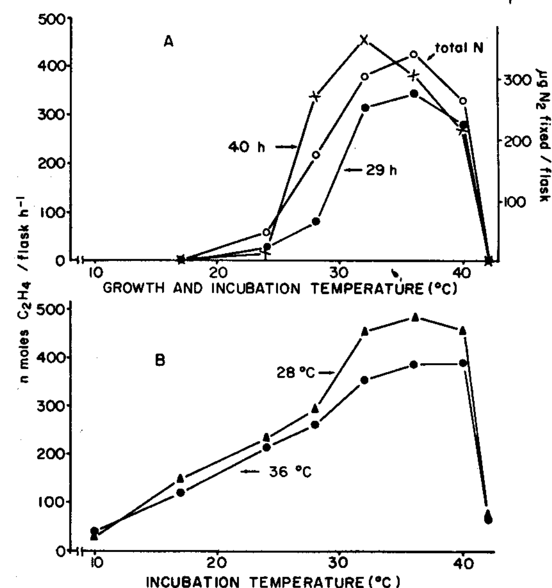

Fig. 2. A. The N_2-ase activity of *Spirillum lipoferum* cultures after 29 and 40 h growth at various temperatures. The N_2 fixed estimated by Kjeldahl analysis of the complete cultures after 2 days growth is shown. B. N_2-ase activity of *Spirillum lipoferum* cultures grown initially at 28 and 36°C, then transfered to a range of temperatures and assayed for N_2-ase activity 1 h after transfer.

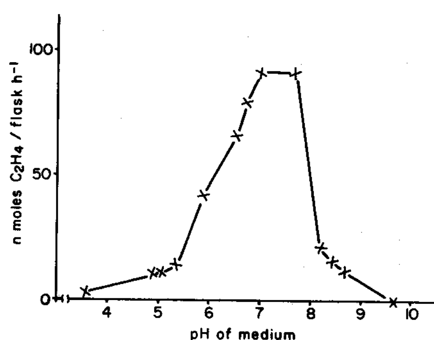

Fig. 3. N_2-ase activity of *Spirillum lipoferum* cultures grown over a range of pH conditions in 0·5% N-free galactose medium, after 45 h growth at 35°C.

higher than at 24°C. The N_2 fixed as determined by Kjeldahl analysis followed closely the C_2H_2 reduction curve, confirming optimal temperatures between 32 and 40°C. At 42°C the N_2-ase system was inactive.

In a second experiment *Spirillum* cultures were grown as above but at only two temperatures (28 and 36°C) for 27 h and then transferred to a 10–40°C temperature range for 1 h to equilibrate, after which rates of C_2H_2 reduction were determined. N_2-ase activity of cells grown at 28 or 36°C slowed progressively with cooler temperatures reaching almost zero after 1 h at 10°C (Fig. 2B). Transferring cells grown at 28°C to higher temperatures increased N_2-ase activity more than that of cells grown at 36°C.

The high temperature requirements of these organisms are of great ecological importance as in temperate regions soil temperatures seldom reach 28°C for any significant period. In the tropics, however, optimal temperatures for N_2-ase activity of this system occur during the main growing season almost daily, for most of the day. To observe how long reasonable activity could be maintained during the night, four additional cultures were assayed during 8 h after transferring them from 36 to 17°C. They maintained about half of their original activity at 36°C. Soil temperatures below 27°C were a major limiting factor to N_2-ase activity of *D. decumbens* grown in the field (Abrantes, Day and Döbereiner, unpublished data). Temperature requirements of *Spirillum* forms isolated from temperate regions (Schröder, 1932) should be interesting to investigate.

pH effects on N_2*-dependent growth*

In these experiments the same basic medium was used but the phosphate buffer concentration was increased to 0·1 M and varying pH values were obtained by using different ratios of mono- and dibasic phosphates. Additional higher and lower values were obtained by adjusting with NaOH or H_2SO_4 respectively. The malate was replaced by galactose (0·5%) to avoid changes in pH by the utilization of malate. pH values were checked after sterilization and at the end of the experiment. The *Spirillum* does not produce acid with galactose or any other sugar. Five replicate cultures (3 ml semi-solid medium in 10 ml flasks) were incubated for 45 h at 35°C when N_2-ase activity was measured by C_2H_2 reduction. The results presented in Fig. 3 show that N_2-ase activity is very pH sensitive, optimal N_2-dependent growth occurring only between 6·8 and 7·8. Little growth and N_2-fixation occurred below pH 5·5 and above 9·0. Total N_2 incorporation as measured by Kjeldahl analysis showed very similar results. Considering that tropical soils in general, and also the soil from which these Spirilla were isolated seldom show a pH above 5·0, this highly specific pH requirement might at first seem surprising. Very similar pH requirements were observed with *A. paspali* cultures (pH 6·4–7·5) (Machado and Döbereiner, 1969). These observations support the recent findings (Döbereiner *et al.*, 1972; Day *et al.*, 1975; Döbereiner and Day, 1974) that good growth and active N_2-fixation are not occurring in the soil but only at the root surface or within root cells where these specific pH requirements can be met. N_2-fixing organisms occurring in soil remote from roots are probably primarily limited by carbon nutrition, pH limitations being secondary.

pO_2 *requirements for* N_2*-dependent growth*

Initial experiments in semi-solid medium showed little effect of the pO_2 in the gas phase above the culture either on efficiency or on N_2-dependent growth because the bacterial pellicle seems to adjust itself within the O_2 diffusion gradient in the position most favourable for optimal N_2-fixation and only at very low O_2 tensions in the gas phase was growth on the surface. To obtain more precise data about O_2 requirements and to enable studies in liquid culture, 120 ml flasks with 40 ml liquid Ca-malate (0·25%) medium were bubbled (50 ml/min) with gas mixtures of increasing O_2 concentrations in N_2 obtained by mixing cylinder N_2 with N_2–O_2–CO_2 mixture of 96:4:1 through flow meters. N_2-dependent growth was measured by Kjeldahl analysis after 2 days' growth at 35°C. The results of two such experiments with different strains (Fig. 4) show that optimal N_2-fixation is only possible between 1 and 2% O_2 in the gas mixture bubbled through the cultures which, if in equilibrium with the medium, should correspond to 30–60 μl O_2/100 ml, but probably is still lower due to the use of O_2 by the organisms. Dalton and Postgate (1969a) found that 22 μl O_2/100 ml (10 μM) was the highest pO_2 tolerated by carbon limited *Azotobacter* cultures. Exact measurements of this kind are only possible in continuous cultures, but the purpose of these experiments was to assess optimal cultural conditions for these organisms. It was,

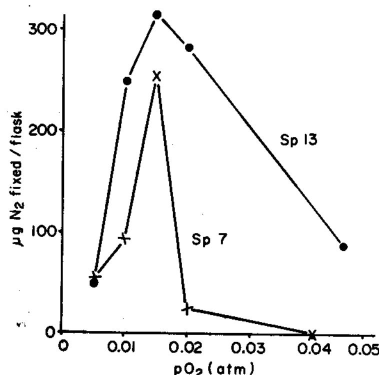

Fig. 4. N_2 fixed by two strains of *Spirillum lipoferum* grown in liquid cultures (N-free Ca-malate medium) bubbled at 50 ml min^{-1} with a range of O_2 concentrations in N_2.

Table 1. Effect of increasing concentration of starter N on N_2-dependent growth of *Spirillum lipoferum* in glucose medium, as evaluated by N_2-ase activity (*n* moles C_2H_4/flask . h^{-1})*

N starter (mM N in medium)	NO_3^- 1 day	NO_3^- 2 days	NH_4^+ 1 day	NH_4^+ 2 days	Yeast 1 day	Yeast 2 days
0	0	0	0	0	0	0
0.12	8	12	51	98	0	90
0.25	11	50	110	466	91	397
0.50	24	163	12	281	160	208
1.00	0	170	18	162	269	275

* 3 ml of semi-solid 0·25% glucose medium in 10 ml flasks inoculated with one drop of N-free medium. Values are means of 3 replicates. The whole experiment was repeated with a small inoculant (one loop) and showed similar but more irregular results and a longer lag phase before the onset of growth. In all experiments none of the cultures without starter showed nitrogen fixation or growth on glucose. The poor growth and N_2-fixation after 2 days at higher concentrations of NH_4^+ in the media was probably caused by acidification concomitant with growth.

however, concluded that even at the optimal O_2 concentration growth was slower and only half as efficient in terms of N_2 fixed/g malate used when compared with shallow layer batch cultures in undisturbed semi-solid medium, (32 mg N_2 g^{-1} malate as compared with 60 mg N_2 in semi-solid batch cultures with pO_2 0·04 atm. in the gas phase above the culture). This organism appears to have a very inefficient O_2 protection mechanism for its N_2-ase which explains its failure to grow on N-free solid medium incubated in air. Furthermore, the extreme O_2 sensitivity of N_2-ase activity on the roots of tropical forage grasses becomes more understandable if organisms of a similar physiological nature are predominantly involved.

Table 2. Nitrogenase activity (*n* moles C_2H_2/flask . h^{-1}) of *Spirillum lipoferum* grown on sugar media with different starters*

	No starter 1 day	No starter 2 days	Malate starter** 1 day	Malate starter** 2 days	Yeast extract starter (1%) 1 day
Glucose	0	0	38	324	88
Sucrose	0	0	113	660	164
Arabinose	0	0	0	289	0
Galactose	12	29	15	25	68
Lactose	n.d.	n.d.	0	294	44
Maltose	n.d.	n.d.	13	13	0
Rhamnose	n.d.	n.d.	0	0	54
Xylose	n.d.	n.d.	2	265	35
Mannose	n.d.	n.d.	14	0	119
Glycerol	1	0	62	119	133
Ethanol	0	0	3	63	14
Mannitol	0	0	0	0	0

* All substrates sterilized separately, giving a final concentration of 0·25% (3 ml semi-solid medium/10 ml flask, mean of 3 replicates). Activities of blanks without sugars or alcohols were subtracted.

** Three drops of a 24-h-old semi-solid malate (0·5%) culture were used as inoculant and starter. Drops of this inoculant remained intact in the semi-solid medium.

The use of sugars with and without starter nitrogen

Earlier observations (Döbereiner and Day, 1975) indicated that the *Spirillum* does not use sugars, not even glucose or sucrose. These observations were in contrast to the results of Schröder (1932) who observed good growth on glucose and fair growth on sucrose and the earlier work was therefore repeated. In numerous experiments with solid or semi-solid medium it was confirmed that no growth or N_2-fixation occurs on N-free glucose medium. When small amounts of NO_3^-, NH_4^+ or yeast extract were added to the medium growth started and when the NO_3^- or NH_4^+ was exhausted active N_2-fixation commenced (Table 1). Yeast extract in this respect was still more efficient and less inhibitory to N_2-fixation. In Table 2 N_2-dependent growth on a number of sugars with and without nitrogen or malate starters can be compared. Small amounts of malate had a slower but similar effect to nitrogen (Table 2). Glycerol and ethanol but not mannitol were used as substrates for growth when starters were used. With none of the sugars or alcohols tested was there any acid or gas production.

Efficiency of N_2*-fixation in semi-solid agar*

Spirillum has been shown to be very efficient when semi-solid agar cultures with low substrate concentrations incubated under pO_2 0·05 atm. were used (Day and Döbereiner, unpublished data). Under these conditions *Derxia* was nearly as efficient as *Spirillum* and even *Azotobacter* spp. gave considerably higher values than those usually reported in the literature. The role of low substrate concentrations in these highly efficient organisms needs study in continuous culture. To establish if under the conditions used in our experiments the cells were carbon limited the profile of N_2-ase activity throughout the growth of cultures at 0·05% and 0·25% malate was studied at two temperatures. Cultures were inoculated evenly (1 drop/3 ml medium) and incubated at 30 or 36°C. Three times a day three replicate vials were assayed for C_2H_2 reduction until no activity could be detected when it was assumed that all the substrate was exhausted. The vials were then combined in batches of 6 and analysed for total N by semi-micro Kjeldahl analysis. The results in Fig. 5 clearly show that after an initial colonization phase where the low substrate concentrations favoured growth, throughout much of the remaining time growth of the cultures at low substrate concentration was severely C-limited. Integrating total C_2H_2 reduction activity of the cells grown at 30°C and extrapolating the data to N_2 fixed using the theoretical 3:1 conversion factor gave values of 14·1 mg N g^{-1} substrate at 0·25% and 41·2 mg N g^{-1} substrate at 0·05% malate compared with 14·5 and 50·2 mg N g^{-1} malate determined by Kjeldahl analysis. There was faster growth but no difference in efficiency at 36°C. Because diffusion of malate through the agar medium is slower at 30°C than at 36°C the N_2-ase activity peak moved to the right (Fig. 5). In a third experiment at 33°C with 0·05% malate, C_2H_2 reduction estimates of efficiency was 53 mg N g^{-1} as

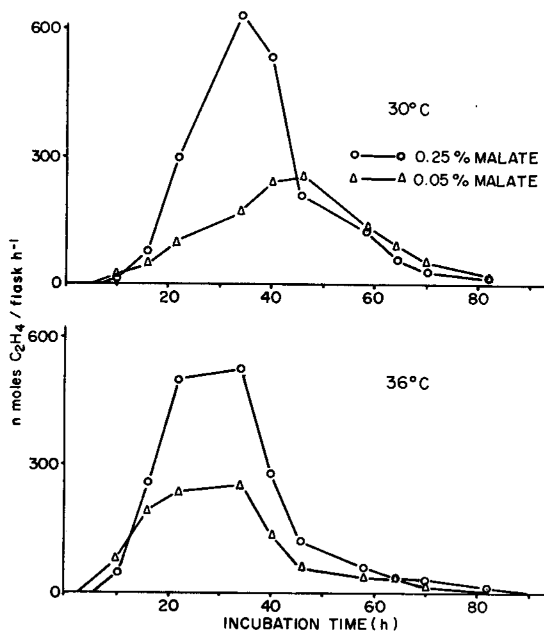

Fig. 5. N_2-ase activity profiles throughout the growth cycle of *Spirillum lipoferum* cultures grown in semi-solid malate medium at two substrate concentrations (0·05 and 0·25%) and two incubation temperatures (30° and 36°C).

compared with 59 mg obtained by Kjeldahl analysis. The $C_2H_2:N_2$ conversion factor for *Spirillum* cultures grown in semi-solid medium appears to be very close to the theoretical 3:1.

The extremely high efficiencies observed in these and in previous experiments probably can be explained by the simultaneous carbon and O_2 limiting conditions prevailing in the semi-solid agar cultures where availability of both is limited by diffusion to the growth pellicle. This pellicle seems to be localized at the position where the dissolved O_2 concentrations are just enough for respiration without inhibiting N_2-fixation. The pellicle usually starts to form far below the surface of the medium (down to 5 mm) and rises to the surface as O_2 demand in the culture increases, so that throughout much of the growth cycle the organisms grow under optimum, i.e. just O_2 limited conditions. Dalton and Postgate (1969a, 1969b) attributed the problems of maintaining carbon-limited *Azotobacter* cultures in chemostats to the difficulty of meeting exactly the O_2 demand, as such cultures show extreme sensitivity to O_2. It is possible that similar high efficiencies occur in the grass root environment where carbon and O_2 availability are probably limited, and are therefore much higher than those usually extrapolated from *Azotobacter* cultures grown in air and with 1 or 2% sugar.

Table 3. Efficiency of substrate utilization by *Spirillum lipoferum* grown on N_2 or combined nitrogen

Nitrogen source (14 mM N)	mg N incorporated/g substrate consumed Malate 0.05%(w/v)	Glucose 0.05%(w/v)***
N_2	52.5*	50.1
$(NH_4)_2SO_4$	86.4	79.7
$Ca(NO_3)_2$	63.5	65.1
NH_4 malate 0.10%	65.0	n.d.
NH_4 malate 0.05%**	87.0	n.d.

* Means of 5 replicate flasks.
** 7 mM N.
*** Inoculated with inoculant including yeast extract.

Efficiency of substrate utilization with combined nitrogen

The efficiencies of N_2-fixation under the conditions described above were compared with that of the incorporation of mineral-N. Semi-solid Ca-malate or glucose (0·05%) cultures (20 ml in 120 ml flasks) were grown at 36°C with or without 14 mM N in the form of $(NH_4)_2SO_4$, $Ca(NO_3)_2$ or ammonium malate (5 replicates/treatment). After 8 days, the semi-solid cultures were diluted, heated to 80°C and centrifuged at 10,000 *g* for 10 min. The cells were analysed by semi-micro Kjeldahl procedures, results being presented in Table 3. As expected, NH_4^+ incorporation was most efficient followed by NO_3^- utilization with 73% and N_2-fixation with 61% of the efficiency of the NH_4^+ grown cells. The efficiency of ammonium malate at higher concentrations (0·1% malate) was similar to that of NO_3^-. Efficiencies with malate and glucose were similar. The results are in the same range as the NH_4^+ incorporation cited by Dalton and Postgate (1969b) for *Aerobacter aerogenes* (75 mg N_2 g^{-1} glycerol).

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