# EFFECTS OF L-METHIONINE-DL-SULPHOXIMINE ON THE ASSIMILATION OF NEWLY FIXED $NH_3$, ACETYLENE REDUCTION AND HETEROCYST PRODUCTION IN *ANABAENA CYLINDRICA*

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ABSTRACT: The addition of exogenous L-methionine-DL-sulphoximine (MSO) to $N_2$-fixing cultures of the blue-green alga *Anabaena cylindrica* results in over half of the newly fixed $NH_3$ being released into the medium. MSO also inhibits glutamine synthetase (GS) activity, has negligible effect on alanine dehydrogenase activity, and glutamate dehydrogenase activity under $N_2$-fixing conditions is negligible. In the presence of MSO, intracellular pools of glutamate and glutamine decrease, those of aspartate and alanine + glycine show little change, and the $NH_3$ pool increases. MSO alleviates the inhibitory effect of exogenous $NH_4^+$ on nitrogenase synthesis and heterocyst production. The results suggest that in $N_2$-fixing cultures of *A. cylindrica* the primary $NH_3$ assimilating pathway involves GS, and probably glutamate synthase (GOGAT), and that the repressor of nitrogenase synthesis and heterocyst production is not $NH_4^+$ but is GS, GOGAT, or a product of their reactions.

INTRODUCTION: Blue-green algae which possess peculiar differentiated cells called heterocysts fix $N_2$ and there is evidence that under aerobic conditions, those heterocysts are the loci of nitrogenase activity (1-4). In this paper we present evidence, based on the use of the analogue L-methionine-DL-sulphoximine (MSO) (5) that under $N_2$-fixing conditions the primary route of $NH_3$ assimilation involves glutamine synthetase (E.C.6.3.1.2) (GS) (6) and that inhibition of GS by MSO is accompanied by nitrogenase synthesis and heterocyst production even in the presence of exogenous $NH_4^+$.

## MATERIALS & METHODS

*Anabaena cylindrica* (CU1403/2a) was grown routinely in axenic continuous culture on $N_2$, or with 3 mM $NH_4^+$ (as $NH_4Cl$) (7). Aliquots of such material were taken as required and incubated with shaking (80 revs $min^{-1}$) in axenic batch culture in the light (3000 lux, continuous) at 26°C. The methods used to measure acetylene reduction, amino acid pools and enzymic activities (except GS activity (8)) are given elsewhere (9). Ammonia was assayed according to Solorzano (10). L-methionine-DL-sulphoximine, supplied by Sigma Ltd., London, was freshly prepared in distilled water as required and sterilised by filtration. Other chemicals were used at the highest purity available from the British Drug Houses, Poole.

## RESULTS

### *THE PRODUCTION OF EXTRACELLULAR $NH_3$ ON THE ADDITION OF MSO TO $N_2$-FIXING CULTURES OF* ANABAENA CYLINDRICA

The data in Table 1 show that when air-grown log phase cultures of *A. cylindrica* are incubated under air, or under $A/O_2/CO_2$ (77.96/22.00/0.04, v/v), for 24 h and MSO ( 1 μM) then added for 24 h, they continue to reduce acetylene. Under $N_2$, $NH_3$ equivalent to over half of the $N_2$ fixed (assuming a 3:1 ratio of $C_2H_2$ reduction:$N_2$ reduction, 11) is excreted into the medium in the presence of MSO, but in air-grown cultures without analogue, or under argon with or without analogue, there is no production of extracellular $NH_3$. These results indicate that the extracellular $NH_3$ is not due to cell autolysis, but is newly fixed $NH_3$ which is not incorporated into amino acids, because the key aminating pathway under $N_2$-fixing conditions is blocked by MSO.

Table I

The production of extracellular ammonia by $N_2$-fixing cultures of *Anabaena cylindrica* in the presence and absence of L-methionine-DL-sulphoximine

| Growth conditions | Sample | Analogue | n moles $NH_3$ fixed ($\mu$g chlorophyll *a*)$^{-1}$ 24 h$^{-1}$ | n moles $NH_3$ excreted ($\mu$g chlorophyll *a*)$^{-1}$ 24 h$^{-1}$ |
|---|---|---|---|---|
| $N_2$-fixing (incubated in air) | 1 | + | 106 | 62 |
| | 2 | + | 101 | 72 |
| | 3 | + | 104 | 69 |
| | 1 | - | 134 | 0.0 |
| | 2 | - | 144 | 0.0 |
| | 3 | - | 141 | 0.0 |
| Nitrogen starved (incubated in $A/O_2/CO_2$: 79.96/ 20.00/0.04, v/v) | 1 | + | 0.0 | 0.0 |
| | 2 | + | 0.0 | 0.0 |
| | 3 | + | 0.0 | 0.0 |
| | 1 | - | 0.0 | 0.0 |
| | 2 | - | 0.0 | 0.0 |
| | 3 | - | 0.0 | 0.0 |

The material was grown in air in continuous culture at 3000 lux and 26°C. Samples were transferred to 70 ml batch cultures and pretreated for 24 h with the appropriate gas phase. MSO (1 $\mu$M final concentration) was then added as shown and the material was assayed for $C_2H_2$ reduction, chlorophyll *a* and extracellular $NH_3$ before, and at the end of, the MSO treatment.

*EFFECTS OF MSO ON $NH_3$-ASSIMILATING ENZYMES AND ON AMINO ACID POOLS*

GS is the most active $NH_3$ assimilating enzyme present in $N_2$-fixing cultures of *A. cylindrica* and it has been postulated that the primary route of $NH_3$ assimilation in $N_2$-fixing cultures of this organism involves GS (6,9). Fig. 1*a* shows that on adding 1 μM MSO to $N_2$-fixing cultures of *A. cylindrica* extractable GS activity decreases to less than 10% of the initial level over a 12 h period while the production of extracellular $NH_3$ increases and the nitrogenase activity of the alga remains constant. The addition of MSO also leads to decreases in the intracellular pools of glutamine and glutamate (Fig. 1*b*). The intracellular pools of aspartate and alanine plus glycine show no such changes, while the intracellular pool of $NH_3$ increases (Fig. 1*c*).

*In vitro* studies on enzymes extracted from whole filaments of *A. cylindrica* show that GS activity is inhibited completely by 200 μM MSO, but this concentration of analogue had no effect on alanine dehydrogenase (E.C.1.4.1.1) (ADH) activity in the aminating direction. There is negligible inhibition of GS activity *in vitro* with 1 μM MSO indicating that the effect noted in Fig. 1*a* may be dependent on an accumulation of the analogue at its active site. MSO also inhibits glutamate synthase (E.C.2.6.1.53) (GOGAT) activity in *Klebsiella* (12), and in blue-green algae where GOGAT is also present (13) it probably exerts a similar effect. Glutamic dehydrogenase (E.C.1.4.1.3) (GDH) activity was negligible in the extracts with or without MSO. Thus, MSO inhibits GS activity in *A. cylindrica*, and probably GOGAT activity, while the other possible primary $NH_3$ assimilating enzymes

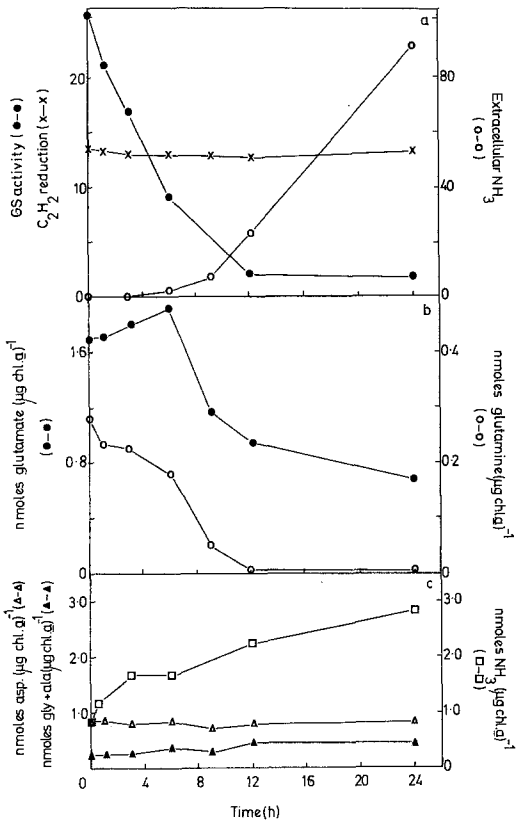


Fig. 1*a* The effect of MSO (1 µM) added at zero time on GS activity (●–●), nitrogenase activity (X–X) and extracellular $NH_3$ (O–O) of $N_2$-fixing *Anabaena cylindrica*. GS activity is expressed as n moles ADP formed (mg protein)$^{-1}$min$^{-1}$, nitrogenase activity as n moles $C_2H_4$ formed (µg chl *a*)$^{-1}h^{-1}$, and $NH_3$ as n moles (µg chl *a*)$^{-1}$.

1*b*,*c* The effect of MSO (1 µM) added at zero time on intracellular amino acid and $NH_3$ pools of $N_2$-fixing *Anabaena cylindrica*. ●-●, glutamate; O–O, glutamine; ▲–▲, aspartate; ▲–▲, glycine + alanine; □–□, $NH_3$.

are either unaffected (ADH) by MSO, or show negligible activity in these cultures (GDH).

*EFFECTS OF MSO ON NITROGENASE ACTIVITY AND HETEROCYST PRODUCTION*

Fig. 2 shows that when $NH_4^+$ (3.0 mM) is added to heterocystous $N_2$-fixing cultures of *A. cylindrica*, nitrogenase activity is almost completely inhibited within 12 h in the absence of MSO, due probably to an inhibition of nitrogenase synthesis, coupled with protein turnover, as others have reported (14). However, when MSO is added at the same time as the $NH_4^+$, there is no marked or prolonged inhibition of nitrogenase activity. The initial slight drop in activity seen in the presence of MSO probably reflects the time required for effective concentrations of MSO to accumulate within the organism. Thus, MSO relieves the inhibitory effect of $NH_4^+$ on nitrogenase, and the results suggest that $NH_4^+$ *per se* is not the repressor of nitrogenase synthesis.

Figs. 3*a*,*b* show the effects of adding MSO to cultures which have lost their nitrogenase activity on the addition

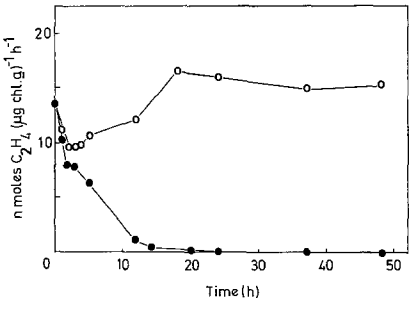


Fig. 2. The effect of $NH_4^+$ (3.0 mM) added at zero time on nitrogenase activity in *A. cylindrica* in the presence (O–O) and absence (●–●) of MSO (1 µM).

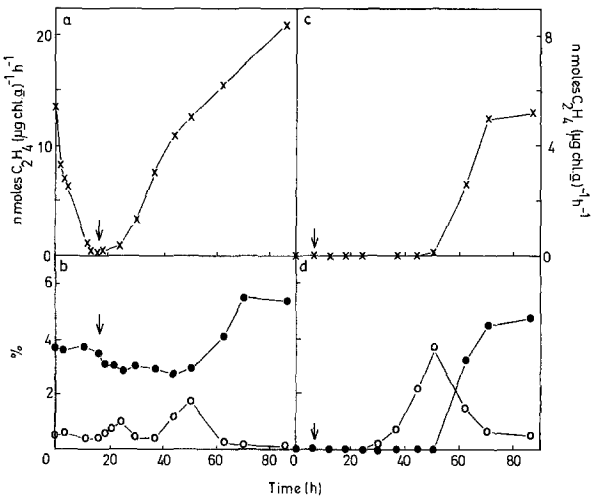


Fig. 3*a,b* The effect of adding $NH_4^+$ (3.0 mM) at zero time to a $N_2$-fixing culture of *A. cylindrica* and the subsequent effect of adding MSO (1 μM) at the time arrowed. **X–X**, nitrogenase activity; **●–●**, mature heterocysts as % of total cells; **O–O**, proheterocysts as % of total cells.

3*c,d* The effect of adding MSO (1 μM) at the time arrowed to a culture of *A. cylindrica* previously grown on $NH_4^+$ (3.0 mM) until there were no heterocysts or nitrogenase activity. **X–X**, nitrogenase activity; **●–●**, mature heterocysts as % of total cells; **O–O**, proheterocysts as % of total cells.

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of $NH_4^+$ (Fig. 3*a*) but which still possess heterocysts (Fig. 3*b*). It is seen that within 10 h of adding MSO, nitrogenase activity restarts and increases to a level which is higher than that of the original material. This recovery of nitrogenase activity precedes the formation of new proheterocysts and mature heterocysts.

Figs. 3*c,d* present data on the effects of adding MSO to cultures which had been grown routinely on $NH_4^+$ to deplete the filaments of nitrogenase and of heterocysts. On the addition of MSO, nitrogenase activity restarts and new heterocysts are formed, but in contrast to the results shown in Figs. 3*a,b*, recovery of nitrogenase activity takes 40 h from the time of addition of MSO, and the return of activity parallels the production of new mature heterocysts. Figs. 3*c,d* also show that nitrogenase activity remains undetectable when proheterocysts only are present. In sum, the addition of MSO relieves the inhibitory effects of $NH_4^+$ on nitrogenase activity and heterocyst production and while nitrogenase activity returns within 10 h when existing heterocysts are present, there is no recovery in heterocyst-free cultures until after 40 h when new mature heterocysts develop.

DISCUSSION: The data presented here show that on the addition of MSO to $N_2$-fixing cultures of *A. cylindrica*, newly fixed $NH_3$ is excreted, as also happens in *Azotobacter* (15). This indicates that the major route of assimilation of newly fixed $NH_3$ is blocked by MSO. The enzymic data and the amino acid pool data suggest that the pathway which is blocked involves GS and probably GOGAT. These results thus fit the view of Stewart *et al.* (9) based on $^{15}N$ kinetic data, that of Lea and Miflin (13) based on enzymic data, and that of Lawrie *et al.* (16) based on $^{14}C$-labelling kinetics, that the GS-GOGAT pathway is probably the primary route of $NH_3$ assimilation in $N_2$-fixing cultures of blue-green algae. The fact that not all the $NH_3$ fixed was excreted over 24 h (Table 1) appears to be due to the time required for the MSO to exert its effect. This is borne out by the data in Fig. 1*a* on the time

course of inhibition of GS by 1 μM MSO and by the time course of $NH_3$ excretion. When $NH_3$ excretion begins virtually all the $NH_3$ fixed is excreted.

The finding that nitrogenase synthesis and heterocyst production still occur in the presence of $NH_4^+$ when MSO is available has a bearing on the ways in which these are regulated at the molecular level. First, the results enable us to distinguish between the two proposals put forward by Fogg (17) to explain the regulation of heterocyst development in *A. cylindrica*. He postulated that heterocysts were formed from vegetative cells when the concentration within the vegetative cell *"of a specific nitrogenous inhibiting substance, probably ammonia, or some simple derivative of ammonia, falls below a critical level"*. Our data suggest that $NH_4^+$ is not the regulator and that Fogg's latter possibility is more likely. Second, the results showing nitrogenase synthesis in the presence of $NH_4^+$ suggest that as in *Klebsiella* (18,19,20) $NH_4^+$ *per se* does not regulate nitrogenase synthesis directly either. Third, the results suggest that the regulator of both nitrogenase synthesis and heterocyst formation is probably GS, GOGAT or a product of their reactions. It has been postulated that in *Klebsiella* GS exerts a positive control over nitrogenase, possibly with catalytically active GS acting to switch on the *nif* operon (18,20). Other possible regulators of the *nif* operon may be glutamine, glutamate or GOGAT (15). Our results suggest that if any of these enzymes or compounds regulate the *nif* operon in *A. cylindrica*, they appear to do so in a negative manner, with nitrogenase activity being highest when their activities (GS and GOGAT) or levels (glutamine and glutamate) are low.

Fourth, the results show that nitrogenase activity and heterocyst production are affected similarly by the addition of MSO. This could be due to the regulator acting independently, but in a similar manner, on both heterocyst (*het*) and $N_2$-fixing (*nif*) genes, or on a common regulatory gene. It is unlikely that MSO acts only on the *het* gene which, when expressed and heterocysts are produced, provides the reducing environment essential for active nitrogenase because when MSO is added to heterocyst-containing nitrogenase-less cultures (Fig. 3*a*,*b*), nitrogenase activity becomes detectable before new mature heterocysts develop. Nevertheless, the presence of mature heterocysts is an essential pre-requisite for nitrogenase activity in these aerobic cultures, with no activity occurring either in non-heterocystous filaments or in those with proheterocysts only (Fig. 3*c*,*d*). Further studies in this area are in progress.

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