Vol. 83, No. 2, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 28, 1978 Pages 688-696

EFFECTS OF 5-HYDROXYLYSINE ON ACETYLENE REDUCTION AND NH_4^+-ASSIMILATION IN THE CYANOBACTERIUM *ANABAENA CYLINDRICA*

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Received May 1,1978

ABSTRACT: 5-hydroxylysine, an analogue of glutamate and lysine, causes NH_4^+ production by N_2-fixing *A. cylindrica;* it also reversibly inhibits GS activity *in vitro* but has no effect on alanine dehydrogenase or GOGAT. On adding 5-hydroxylysine intracellular pools of glutamine, glutamate and aspartate decrease; those of alanine and serine increase. 5-hydroxylysine alleviates the inhibitory effect of NH_4^+ on heterocyst production and C_2H_2 reduction and in NH_4^+-grown cultures results in heterocyst synthesis and in C_2H_2 reduction. The data suggest that the GS-GOGAT pathway is the sole route of importance in primary NH_4^+ assimilation in *A. cylindrica,* that NH_4^+ alone does not inhibit nitrogenase and heterocyst production, and that GS and/or a product is involved in regulating the production of both.

INTRODUCTION: Cyanobacteria such as *A. cylindrica* fix N_2 under aerobic conditions in heterocysts (see 1,2) and the NH_4^+ produced enters amino acid metabolism via the GS-GOGAT pathway (3-6). We have shown that on adding the analogue MSO to N_2-fixing *A. cylindrica* GS activity is blocked and NH_4^+ which otherwise would have been assimilated is liberated extracellularly (6). We also observed that the effects of NH_4^+ on nitrogenase activity and heterocyst differentiation can be reversed by adding MSO. Such results have been independently confirmed (7-9). In studies on the effects of various analogues on NH_4^+ assimilation in cyanobacteria we found that the amino acid analogue HYL exhibits effects which are rather similar to those of MSO. Data obtained in such a study are presented here and their implications considered.

MATERIALS & METHODS: *Anabaena cylindrica* (CU1403/2a) was grown in axenic

Abbreviations: HYL, 5-hydroxylysine (2,6-diamino-5-hydroxyhexanoic acid); MSO, L-methionine-DL-sulphoximine; GS, glutamine synthetase (E.C.6.3.1.2); GOGAT, L-glutamine:2-oxoglutarate amino transferase (E.C.1.4.7.1).

0006-291X/78/0832-0688$01.00/0

continuous culture on N_2 (10), or with 3 mM NH_4^+ (as NH_4Cl), in which case 3 mM HEPES buffer, pH 7.4, was also added. Aliquots of such material were taken as required and incubated with shaking at 80 revs min^{-1} in axenic batch culture in the light (3,000 lux, continuous) at 26°C. The methods used to measure C_2H_2 reduction, chlorophyll concentrations, protein, free amino acid pools, and enzyme activities are those reported elsewhere (11). Ammonia was assayed according to Solorzano (12). HYL, L-glutamine (both Sigma Ltd., London) and NH_4Cl were freshly prepared as required and sterilised by filtration. Unless otherwise stated the chemicals used were the purest grades commercially available from the British Drug Houses, Poole.

RESULTS

THE PRODUCTION OF EXTRACELLULAR NH_4^+ ON THE ADDITION OF HYL TO N_2-FIXING ANABAENA CYLINDRICA

The data in Table 1 show that the addition of HYL at concentrations of 25 μM or higher to N_2-fixing *A. cylindrica* results in extracellular NH_4^+ production. The proportion of the total nitrogen fixed which is released increases from 37% at 25 μM HYL to 91% at 500 μM HYL. A concentration of 1000 μM HYL does not cause a greater release of NH_4^+, probably because of its general toxicity. There is no NH_4^+ production by cultures incubated with HYL under conditions of nitrogen-starvation. Table 1 shows further that highest rates of C_2H_2 reduction occur over 24 h at HYL concentrations of 75 μM and that higher concentrations cause some inhibition of activity. As Fig. 1 shows, with 75 μM HYL the rate of C_2H_2 reduction remains fairly constant over 24 h and the time course of NH_4^+ production is almost linear. No NH_4^+ is produced in the absence of HYL. Thus, extracellular NH_4^+ production is not due to cell autolysis but is probably due to the release of newly fixed NH_4^+ because the primary route of NH_4^+ assimilation is blocked by HYL.

THE EFFECT OF HYL ON GS AND ON THE INTRACELLULAR FREE AMINO ACID POOLS

The effect of HYL on GS activity was examined using both biosynthetic and transferase assays. Initially 75 μM HYL was added to filaments of *A. cylindrica* and GS activity in cell-free extracts determined 24 h later. There was no inhibition of the biosynthetic or transferase activities of the GS extracted from the HYL-treated filaments compared with untreated control

TABLE 1

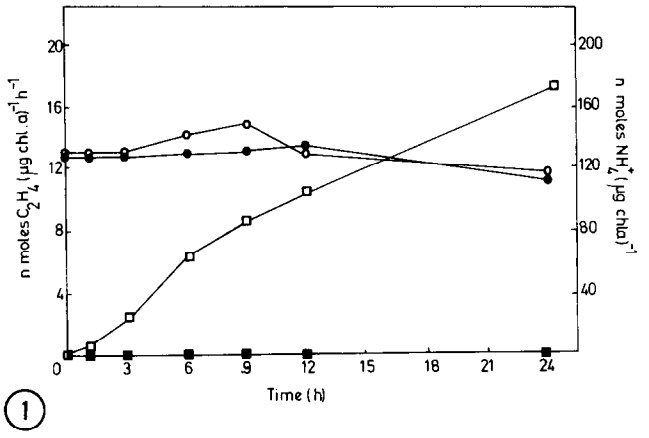
The effect of HYL on C_2H_2 reduction and NH_4^+ production by *A. cylindrica*

HYL (μM)	nmoles C_2H_4 μg chl a^{-1} h^{-1}*	nmoles NH_4^+ fixed μg chl a^{-1} 24 h^{-1}**	nmoles NH_4^+ released μg chl a^{-1} 24 h^{-1}***	% NH_4^+ released
0	9.7	164	0	0
25	9.8	166	61	37
50	10.4	171	128	75
75	11.1	180	138	77
100	9.5	158	128	81
500	2.8	116	106	91
1000	2.8	111	84	76

Samples were transferred from continuous cultures to 70 ml batch cultures shaken at 80 revs min^{-1}. HYL was added at 0 time and the material assayed for C_2H_2 reduction, chlorophyll *a* and extracellular NH_4^+ immediately and at intervals thereafter. Values are means of triplicates. No NH_4^+ was produced by control cultures incubated without HYL or with HYL under $Ar/O_2/CO_2$ (77.96/22.00/0.04, v/v). *Data are based on a 30 min incubation of material sampled after 24 h. **Data are based on a C_2H_2 reduction : N_2 reduction ratio of 3 : 1, and on samples assayed for C_2H_2 reduction over 30 min periods at 6 h intervals from 0 time. ***Extracellular NH_4^+ recorded after 24 h.

series which showed activities of 55 nmoles product formed mg $protein^{-1}$ min^{-1} and 305 nmoles product formed mg $protein^{-1}$ min^{-1}, respectively, in the biosynthetic and transferase assays. However, on adding HYL to extracted GS from N_2-grown cells there is an immediate inhibition of both biosynthetic and transferase activities with inhibition increasing with increase in HYL concentration up to 2 - 4 mM (Fig. 2). Furthermore, GS which is inactivated by 10 mM HYL can be completely reactivated by dialysing the extract against HYL-free buffer (Fig. 3). Thus, HYL can inhibit the activity of *A. cylindrica* GS but unlike MSO (6) its inhibitory effect is reversible. In this respect its effect on *A. cylindrica* GS is similar to that on mammalian GS (13,14).

Indirect evidence of GS inhibition *in vivo* comes from the finding (Fig. 4) that on adding HYL to N_2-fixing *A. cylindrica* the glutamine pool becomes



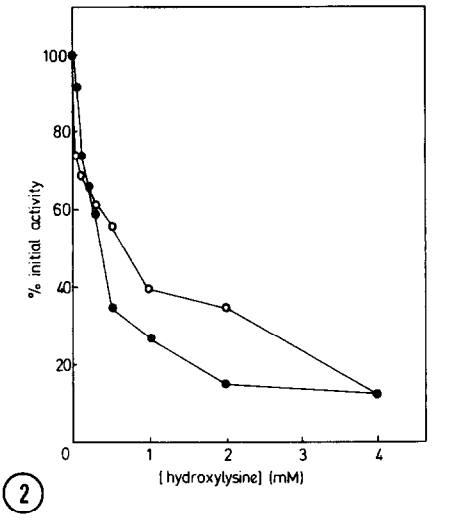
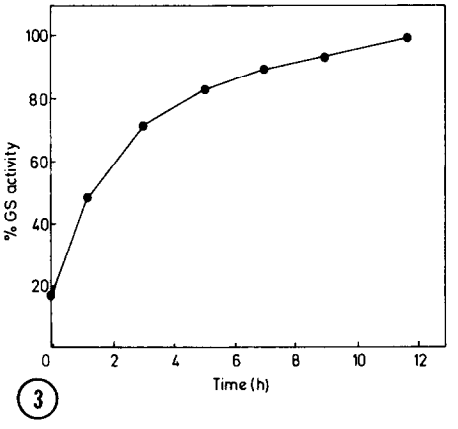


Fig. 1. Effect of 75 μM HYL added at 0 time on C_2H_2 reduction and NH_4^+ production by N_2-fixing *A. cylindrica*. ●-●, C_2H_2 reduction in absence of HYL; O-O, C_2H_2 reduction in presence of HYL; ■-■, NH_4^+ production in absence of HYL; □-□, NH_4^+ production in presence of HYL. In this and other Figs. values are the means of triplicate determinations unless otherwise stated.

Fig. 2. Effect of HYL on the *in vitro* biosynthetic (O-O) and transferase (●-●) activities of GS from N_2-fixing *A. cylindrica*. Activities of the untreated enzyme extracts were: biosynthetic activity, 55 nmoles product formed mg protein^{-1} min^{-1}; transferase activity, 305 nmoles product formed mg protein^{-1} min^{-1}.



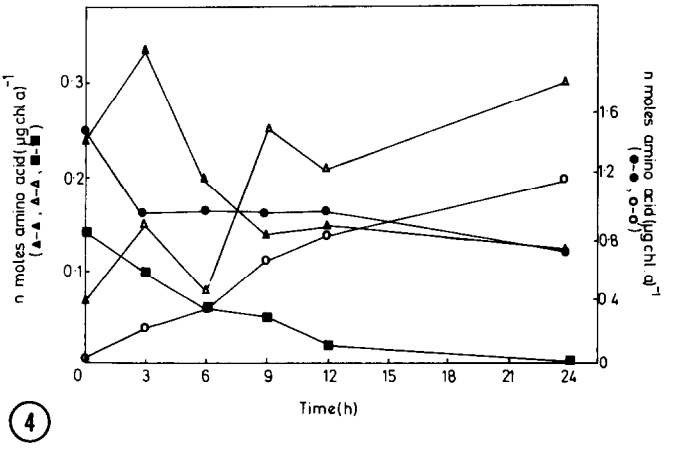


Fig. 3. Effect of dialysis, begun at 0 time, on the biosynthetic activity of GS previously inactivated *in vitro* by 83% on adding 10 mM HYL. The extract used was a 35,000 g x 30 min supernatant fraction containing 7 mg protein ml^{-1}. After inactivation the extract was dialysed against Tris-HCL buffer (50 mM, pH 7.5) and tested for activity at intervals thereafter. The 100% activity of the untreated control series remained steady at 50-55 nmoles product formed mg protein^{-1} min^{-1}. There was no reactivation of the enzyme in an undialysed control series. Values are means of duplicates.

Fig. 4. Effect of 75 μM HYL added at 0 time on the intracellular free amino acid pools of N_2-fixing *A. cylindrica*. ●-●, glutamate; O-O, serine; ▲-▲, aspartate; ▲-▲, alanine; ■-■, glutamine.

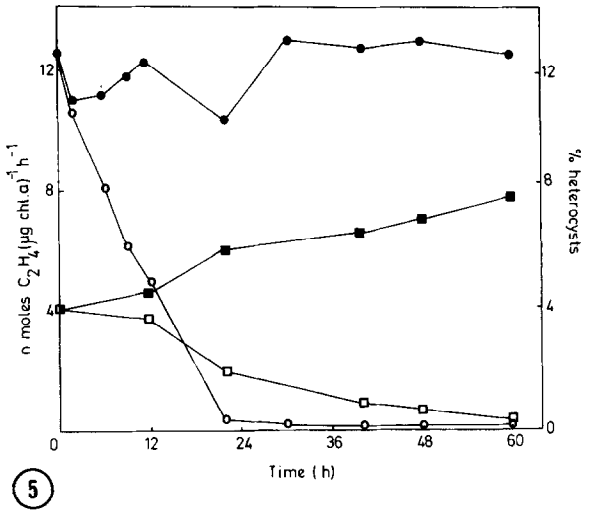
undetectable over 24 h and the glutamate pool decreases to 60% of its original level. There is also a decrease in aspartate which may arise from glutamine or glutamate by transaminase-mediated reactions (15) and an increase in alanine, probably due to alanine dehydrogenase activity (16), and in serine. Rather similar findings are observed when GS is inhibited irreversibly by MSO (6). Of the other potentially important enzymes involved in primary NH_4^+-assimilation glutamate dehydrogenase activity is negligible in *A. cylindrica* and alanine dehydrogenase activity is unaffected by HYL concentrations up to 1000 μM. The latter concentration does not affect GOGAT activity either.

EFFECTS OF HYL ON C_2H_2 REDUCTION AND ON HETEROCYST FORMATION

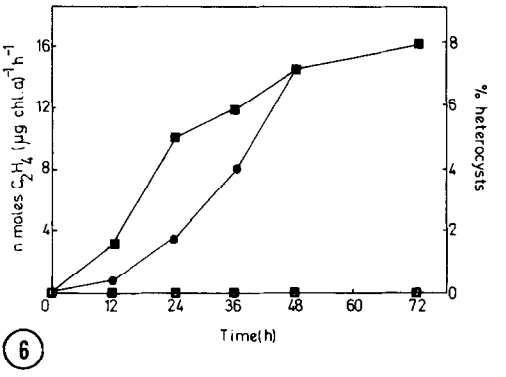
In *A. cylindrica* 3.0 mM NH_4Cl results in an inhibition of both C_2H_2 reduction and heterocyst production. However, such inhibitions by NH_4^+ can be prevented by adding HYL and HYL actually stimulates heterocyst production in cultures supplied with NH_4^+ (Fig. 5). Fig. 6 shows further that on adding HYL to NH_4^+-grown non-N_2-fixing heterocyst-free cultures, heterocysts are synthesised and C_2H_2-reducing activity develops. There is no heterocyst production or C_2H_2 reduction without HYL. Thus, NH_4^+ alone does not repress nitrogenase synthesis and heterocyst differentiation in *A. cylindrica*.

THE EFFECT OF HYL ON A. CYLINDRICA *IN THE PRESENCE AND ABSENCE OF ADDED GLUTAMINE*

Fig. 7 shows the effect of adding HYL and glutamine to N_2-fixing *A. cylindrica*. It is seen (Fig. 7*a*) that glutamine alone has no effect on growth whereas HYL inhibits growth and that this inhibition can be reduced by 40% by simultaneously adding glutamine. Glutamine also partially prevents NH_4^+ production in the presence of HYL (Fig. 7*b*). Fig. 7*c* shows that the addition of HYL stimulates heterocyst production whereas glutamine alone depresses it. Glutamine alone also inhibits nitrogenase activity but when added with HYL stimulates it relative to the control (Fig. 7*d*), probably because low amounts of glutamine or its products are required for protein biosynthesis, although in high concentration these compounds may inhibit nitrogenase activity.



(5)



(6)

Fig. 5. Effect of 75 µM HYL on the inhibition of C_2H_2 reduction and heterocyst formation which occurs on transferring N_2-grown cultures to NH_4^+ (3.0 mM)-containing medium at 0 time. C_2H_2 reduction in presence (●-●) and absence (O-O) of HYL; heterocyst frequency in presence (■-■) and absence (□-□) of HYL.

Fig. 6. Effect of adding, at 0 time, 75 µM HYL to a NH_4^+-grown culture of *A. cylindrica*. Heterocyst frequency in presence (■-■) and absence (□-□) of HYL; C_2H_2 reduction in presence (●-●) and absence (O-O) of HYL.

Studies using 0.5 mM ^{14}C-glutamine showed that 75 µM HYL did not affect its uptake.

DISCUSSION: The above data allow several conclusions to be drawn on the inter-relations of NH_4^+ assimilation, C_2H_2 reduction, heterocyst production, GS activity, and glutamine or products of its metabolism, in *A. cylindrica*.

First, the data which show NH_4^+ production on the addition of HYL to N_2-fixing cultures and its inhibition of GS but not of GOGAT or alanine dehydrogenase support the view that GS is involved in primary NH_4^+ assimilation and that even in the presence of high levels of NH_4^+ possible alternative routes of NH_4^+ assimilation such as glutamate dehydrogenase and alanine dehydrogenase are unimportant in primary NH_4^+ assimilation. This is also borne out by the lack of growth in the presence of HYL even when excess NH_4^+ is available and by the fact that some algal growth occurs in the presence of HYL when exogenous glutamine is added (Fig. 7). These conclusions support

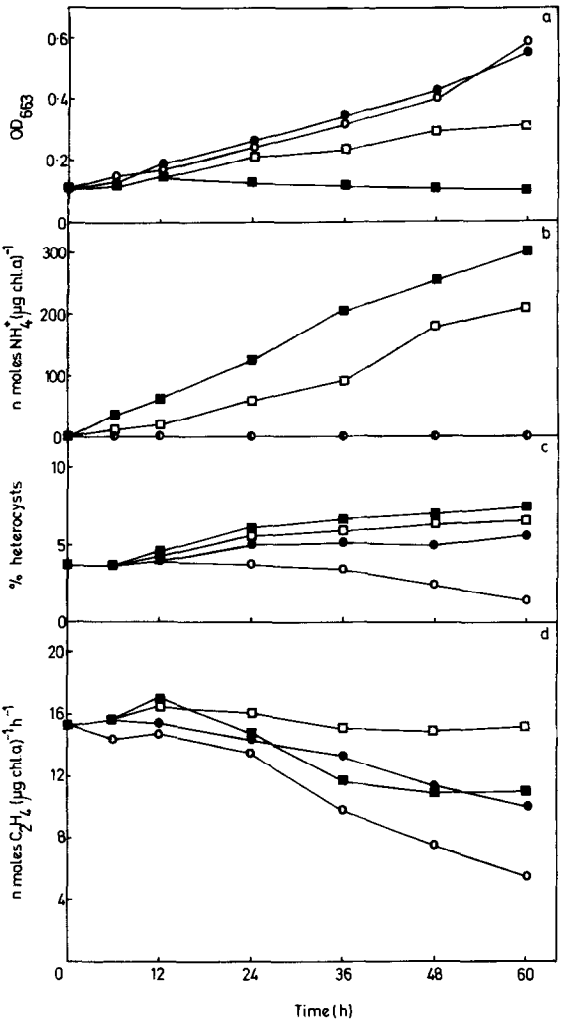


Fig. 7. Effect of 0.5 mM glutamine and 75 µM HYL added at 0 time on (a) growth, (b) NH_4^+ production, (c) heterocyst frequency and (d) C_2H_2 reduction by *A. cylindrica*. ●-●, untreated cultures; O-O, + glutamine; ■-■, + HYL; □-□, + glutamine + HYL.

the view of Lea and Miflin (4) for cyanobacteria in general and the views of others for *A. cylindrica*, based on other evidence (3,5,6,8,9). They show further that compounds which inhibit GS reversibly may be as important in regulating NH_4^+ production as are compounds such as MSO which inhibit the enzyme irreversibly (6).

Second, the findings that heterocysts are synthesised and nitrogenase

activity develops in the presence of NH_4^+ when GS is inhibited suggests that NH_4^+ is either not involved in the regulation of heterocyst synthesis and nitrogenase or, as seems more likely in view of recent findings for *Klebsiella* (17), it is not the sole regulator and that GS may also be implicated directly or indirectly in regulating both. The similar response of both to NH_4^+ and to GS inhibition may indicate that *het* and *nif* gene transcription and/or expression may both have a common regulatory mechanism which is affected by GS or a product. It may be noted in this respect that genetic studies on *Nostoc* spp. show that revertants of *het*$^-$*nif*$^-$ mutants commonly develop both heterocysts and nitrogenase activity simultaneously (7).

Finally, the fact that added glutamine inhibits heterocyst production and nitrogenase activity suggests that glutamine or a product may be involved, directly or indirectly in the inhibition of both (see also 18). This may also explain, in part at least, why the addition of HYL, which inhibits glutamine production, prevents inhibition of both nitrogenase activity and heterocyst development by glutamine, and why glutamine which allows growth in the presence of HYL results in decreased NH_4^+ production. The precise mechanism by which this regulation takes place requires study.

Acknowledgements: We thank S.R.C., N.E.R.C., the Royal Society and the Association of Commonwealth Universities for financial support and Gail Alexander for technical assistance.

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