Nitrogen Fixation by Gloeocapsa

Abstract. *The continuous growth in a medium free of combined nitrogen and the experimental production of ethylene via acetylene reduction indicate that nitrogen fixation by blue-green algae is not solely confined to filamentous genera with heterocysts. Axenic cultures of* Gloeocapsa *sp., adapted to nitrate-free medium, form ethylene at rates comparable to those of species known to fix nitrogen.*

During routine testing of purification procedures (*1*) on blue-green algae, it became evident that clonal isolates of *Gloeocapsa* sp. which withstood conventional tests of purity (*2*) grew well through repeated transfers in a mineral medium, ASM-1 (*3*), minus its normal source of combined nitrogen. Subsequent investigation has produced valid evidence of atmospheric nitrogen-fixing ability in this member of the Chroococcales.

A report (*4*) that *Gloeocapsa minor* grew and fixed nitrogen in a nitrate-free medium was rejected (*5*), and it has been generally conceded (*6*) that no conclusive evidence of nitrogen fixation by coccoid blue-green algae exists. However in 1960, nitrogen fixation was strongly suspected in some coccoid blue-green algae isolated from soil crusts (*7*). Discovery (*8*) and development (*9–11*) of the acetylene reduction method as a means of indirectly measuring the ability to fix nitrogen has made it practical to test for this capacity in *Gloeocapsa.*

The original unialgal culture of *Gloeocapsa* sp. and the other test organisms in Table 1 were obtained from the Indiana University Culture Collection (*12*). We used 7-day-old, rapidly growing cultures. All cultures were grown in Delong culture flasks (300 ml) with 180 ml of ASM-1 medium minus nitrate except where noted. The flasks were shaken at 100 cycle/min under continuous, cool white, fluorescent lighting of 3240 lu/m^2 at 24°C.

Thickly concentrated cellular suspensions (5 ml) were added to 15 ml of sterile, nitrate-free media in serum bottles (40 ml) capped by rubber stoppers. The samples were gassed with acetylene at intervals and incubated horizontally for 30- and 60-minute periods as described. The ethylene produced by each of three duplicate samples per organism per time period was measured by gas chromatography. After each sample was measured, it was immediately diluted to 100 ml and blended for 30 seconds in a Waring Blendor. The optical density was measured at 650 nm. The sample was then filtered, dried at 50°C, and weighed. The gas mixture and the parameters of the gas chromatograph were identical to that described previously (*9*), except that a MicroTek GC-1600 gas chromatograph was used with an oven temperature of 60°C.

To protect against the possibility of a response being falsely attributed to *Gloeocapsa*, special control measures were used. We also tested uninoculated gassed samples and cultures of *Phormidium faveolarum* (an organism that does not fix nitrogen) which had been transferred to medium that did not contain combined nitrogen 48 hours before exposure to acetylene. Because earlier work (*13*) had shown that no appreciable nitrogen fixation occurred in blue-green algae after prolonged exposure to darkness, an 8-hour pretreatment period in darkness was arbitrarily adopted before incubation of dark-bottle and light *Gloeocapsa* controls. Also, as an additional measure *Gloeocapsa* that had been growing on complete ASM-1 medium was centrifuged, washed several times in water that had been distilled in glass, and resuspended in nitrate-free medium 18 hours before being gassed.

Table 1. Ethylene production. All results are expressed as the mean.

Organism	Optical density (650 nm)	Cells (mg)	Ethylene (nmole/min)	Ethylene (nmole mg cell^{-1} min^{-1})
Gloeocapsa sp.	0.072	19.3	2.16	0.112
Cylindrospermum sp.	.114	8.8	5.09	.578
Nostoc muscorum	.068	7.7	1.90	.246
Nostoc commune	.158	30.0	1.27	.042
Tolypothrix distorta	.058	10.0	1.78	.178
Phormidium faveolarum	.090	8.2	0.0	.0
Uninoculated controls			.0	.0
Gloeocapsa sp. controls:				
Dark bottles*	0.068	16.4	0.0	0.0
Predark treatment†	.082	20.5	.0	.0
Nitrate repressed‡	.104	24.7	.0	.0

* Serum bottles wrapped in aluminum foil. † Samples also receiving 8-hour pretreatment in darkness but light-incubated. ‡ Samples removed from complete ASM-1 only 18 hours before gassing.

The rates of ethylene production by those species known to fix nitrogen are in the same general range as those reported (*10*); these rates do not appear to be markedly greater than those of *Gloeocapsa,* especially if the extra weight contributed by its extensive gelatinous sheath material is considered. Thus, the ability of *Gloeocapsa* sp. to reduce acetylene would perhaps suggest that the heterocyst is not the site of nitrogen fixation (*14*), at least for this species of blue-green algae. This ability and the fact that *Gloeocapsa* has maintained its vigor and a seemingly normal growth rate over the past 9 months in a medium that is free of any trace of combined nitrogen should establish it as a nitrogen-fixing organism.

At this time, there seems little reason to believe that the ability to fix molecular nitrogen is extremely widespread in either *Gloeocapsa* or the very similar *Chroococcus* (*15*). Also, neither of two species of *Chroococcus* isolated pass fixation tests. Other than the fact that those species previously tested (*15*) definitely do not fix atmospheric nitrogen, perhaps the main reason that fixation has not been verified before is the apparent extremely slow rate of nitrogenase induction. Upon transfer from complete to nitrate-free medium, cultures fade, yellow, and appear completely deactivated after about 7 days. Perhaps depending upon the physiological state at transfer, this condition persists for periods of about 14 to 45 days, and then gradually, but slowly recedes. From the initial appearance of the slightest tint of green, recovery is usually complete within 2 weeks. In none of 14 other species that fix nitrogen has this response been observed; rather, they exhibit only a brief lag phase and then resume growth with no gross observable stress symptoms.

Because absolute evidence of bacterial contamination in blue-green algal cultures is established only by negative evidence and is theoretically impossible

to prove (*16*), experimental ethylene production in *Gloeocapsa* is doubly verified. This supportive evidence is offered by (i) the failure of samples to overcome nitrate repression within 18 hours since bacterial recovery from combined nitrogen repression should have been effected earlier (*17*); (ii) no acetylene reduction in dark bottles; and (iii) the 8-hour treatment in darkness that inhibited ethylene production in samples incubated in light for at least 60 minutes. Although this last-mentioned lack of response probably would not have been recorded in cultures unaccustomed to continuous lighting, it does indicate that acetylene reduction in the *Gloeocapsa* samples is truly light-dependent.

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18. Supported in part by FWPCA grants, WP-00805-04 and WP-00785-04.

5 May 1969; revised 23 June 1969

Maturation of Renal Organic Acid Transport: Substrate Stimulation by Penicillin

Abstract. *Renal* p-*aminohippurate transport in rabbits increased rapidly from birth to 4 weeks of age and then declined to that observed in adults. Penicillin administration to pregnant does or newborn rabbits stimulated the developing transport system, but did not increase the peak observed at 4 weeks. Therefore the continued presence of substrate (penicillin) during development significantly enhances the rate of maturation.*

The kidneys of newborn rabbits are histologically and physiologically immature (*1*). This is exemplified by the presence of a nephrogenic zone in the outer cortex and the development of a brush border in the proximal convoluted tubules during the first 4 to 5 weeks of life (*1*). Physiologically, it has been demonstrated that the renal transport mechanisms for organic ions in rabbits and several other species are not fully developed at birth (*2–4*). However, few attempts have been made to follow the development of specific kidney functions during the neonatal period. In contrast, investigation of the development of glycolytic and drug-metabolizing enzymes in the liver has been extensive (*5*). For example, it has been shown that the activity of hepatic drug-metabolizing enzymes is low at birth, with adult activity being reached at various times in the neonatal period (*5*). The mechanisms responsible for the increase in enzyme activity after birth have not been elucidated, although Dawkins (*5*) has suggested that one possible factor is substrate-induced stimulation of the enzymes. In this regard, it has been shown that a wide variety of drugs stimulate hepatic drug-metabolizing enzymes by a mechanism involving substrate stimulation (*6*). By analogy, it should be possible to stimulate a specific function of the kidney by challenging it with a substrate during the period of development. Thus, the objectives of this investigation were twofold: (i) to quantitate the maturation of renal organic acid transport in the newborn rabbit, and (ii) to stimulate the maturation of transport by treating either the pregnant doe or the newborn with a suitable substrate.

The technique developed by Cross and Taggart (*7*) was used to study in vitro the ability of renal tubules to actively transport the organic acid *p*-aminohippurate. Thin slices of kidney cortex were incubated in oxygenated media containing buffered salts and *p*-aminohippurate. At the end of the incubation period (90 minutes), the *p*-aminohippurate content of the slices and of the media was analyzed, and the transport that occurred was reported as the ratio of concentration in the slice to that in the medium (S/M) (micromoles of *p*-aminohippurate per gram of tissue divided by micromoles of *p*-aminohippurate per milliliter of medium). An S/M ratio greater than unity is indicative of active transport (*7*).

The pattern of development of organic acid transport was followed in young rabbits (ranging in age from 1 day to 8 weeks) and in adults. The S/M ratio of *p*-aminohippurate increases slowly from 1 day of age to about 2 weeks, and from this point to about 4 weeks of age the ratio increases rapidly, as does body weight. However, body weight continues to increase beyond 4 weeks of age, while the S/M ratio begins to decline until it reaches adult value. Rennick *et al.* (*2*) observed a similar peak in *p*-aminohippurate transport in 4-week-old puppies. At the present time we have no explanation for the significant increase in the S/M ratio at 4 weeks of age. In this regard, New *et al.* (*4*) indicated that the difference in *p*-aminohippurate accumulation in slices from newborn and adult rabbits is not due to differences in tissue water content. A rapid increase in activity during enzymatic maturation with a subsequent decline to adult activity is not a unique observation. Zorzoli (*8*) reported that the activity of various glycolytic enzymes was greater in kidney cortical tissue obtained from mice at 2 to 4 weeks of age than it was from adults.

Since a major excretory pathway of penicillin is active tubular secretion (*9*), it was thought that the prolonged presence of this drug in the body con-