Synthesis of Nitrogenase in the Cyanobacterium *Gloeotheca* (*Gloeocapsa*) sp. CCAP 1430/3

By PHILIP M. MULLINEAUX, ALAN E. CHAPLIN AND
JOHN R. GALLON*

Department of Biochemistry, University College of Swansea, Singleton Park,
Swansea SA2 8PP, U.K.

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$^{55}\text{FeCl}_3$ labelling and non-denaturing gel electrophoresis were used to study nitrogenase synthesis in *Gloeotheca* sp. CCAP 1430/3. Nitrogenase synthesis was inhibited by addition of NH_4^+ but was unaffected by elevated concentrations of O_2 . Upon transfer of cultures of *Gloeotheca* from light to darkness, there was initially a slight decrease in the rate of synthesis of nitrogenase but after 4–5 h there was an almost complete cessation of synthesis. This delayed effect of darkness on nitrogenase synthesis could not be related to any change in RNA synthesis, in protein synthesis or in the rate of breakdown of storage glucan.

In cultures of *Gloeotheca*, mRNA, including *nif* mRNA, was unstable, having a half-life of about 5 min. The synthesis of *nif* mRNA did not stop immediately upon transfer of cultures to the dark. If darkness exerts its effect on nitrogenase synthesis by inhibiting the synthesis of *nif* mRNA, it does so only after a lag of about 4 h.

INTRODUCTION

During the growth of illuminated cultures of *Gloeotheca* sp. 1430/3 in medium free of combined nitrogen, the rate of acetylene reduction (N_2 -fixation) fluctuated markedly (Mullineaux *et al.*, 1981*a*). Although the rate of acetylene reduction immediately fell by about 60% when illumination was removed (Gallon *et al.*, 1973) cultures transferred to the dark during a phase of increasing nitrogenase (EC 1.18.2.1) activity continued to exhibit this increase for up to 5 h (Mullineaux *et al.*, 1981*b*). Thus over this period, the rate of acetylene reduction in the dark, relative to that in the light, remained constant.

After about 5 h in the dark, the rate of acetylene reduction began to decrease until, after 12 h, nitrogenase activity had almost completely disappeared (Mullineaux *et al.*, 1981*b*). This decrease in nitrogenase activity after a period of darkness was not caused by exhaustion of carbon reserves, but apparently by a cessation of nitrogenase synthesis coupled with an irreversible inactivation of the enzyme, probably by O_2 . However, because nitrogenase synthesis was measured indirectly, other possible explanations have not yet been eliminated.

In this paper, a method is described for measuring the synthesis of nitrogenase in *Gloeotheca*, based upon incorporation of radioactivity from $^{55}\text{FeCl}_3$. The use of this method to study the effects of NH_4^+ , O_2 and darkness on nitrogenase synthesis is also reported.

METHODS

Growth of cultures. *Gloeotheca* sp. (*Gloeocapsa* sp. CCAP 1430/3; Culture Centre of Algae and Protozoa, Cambridge, U.K.) was grown in 15 l of sterile medium, either free of combined nitrogen (Tözüm & Gallon, 1979) or containing 2 mM- NH_4Cl (Mullineaux *et al.*, 1980).

Incorporation of ^{55}Fe into Fe-proteins of *Gloeotheca*. Cultures of *Gloeotheca* were transferred to medium, with or without NH_4^+ , containing 3.7 MBq l^{-1} of $^{55}\text{FeCl}_3$ [$7.02 \text{ GBq (mg Fe)}^{-1}$]. After 8 d growth under Osram white fluorescent lights, giving illumination at 2.5 klx ($17 \mu\text{E m}^{-2} \text{ s}^{-1}$), a broken-cell preparation was made under

Fig. 1. Effect of rifampicin on the incorporation of radioactivity from $[2\text{-}^{14}\text{C}]\text{uracil}$ into RNA. $[2\text{-}^{14}\text{C}]\text{uracil}$ was added, at zero time, to 15 ml of a 10-fold concentrated, illuminated culture of *Gloeotheca*. Samples (0.5 ml) were removed at the times indicated and the radioactivity in RNA measured as described by Doolittle (1972). Rifampicin (0.1 mg ml^{-1}) was added at the time indicated by the large arrow. The values of A and B indicate the maximum radioactivity incorporated into total and unstable RNA respectively. The points shown are the means of three separate determinations; the bars indicate the S.E. of these means.

conditions suitable for the extraction of nitrogenase (Hamadi & Gallon, 1981). All manipulations were performed under N_2 . After centrifugation for 15 min at 32000 g , 0.2 ml of the supernatant was subjected to electrophoresis on polyacrylamide slabs, using the non-denaturing conditions of Peterson & Wolk (1978). The buffer was 25 mM-Tris/NaOH (pH 9.0) containing 0.192 M-glycine. The gel was frozen and then cut into 1 mm slices using a Micklehurst gel slicer (Mickle Laboratory Engineering Co., Gomshall, Surrey). The slices were transferred to vials containing 0.2 ml distilled water. After standing overnight, 5 ml PCS emulsion scintillation fluid was added and the radioactivity in each slice was measured by scintillation spectrometry. The efficiency of counting of ^{55}Fe was 14%, and individual slices contained up to 500 c.p.m. above the background count of 60 c.p.m. Gels were stained for protein using 50 mg amido black in 100 ml 15% (v/v) acetic acid.

Measurement of nitrogenase synthesis. All operations were performed at 25°C . Exponentially growing cultures of *Gloeotheca* ($10^5\text{--}10^6\text{ cells ml}^{-1}$) were harvested 6–8 d after inoculation and were concentrated threefold by settling under gravity. Samples (50 ml) were then incubated, usually for 1–4 h, with 37 kBq ml^{-1} of $^{55}\text{FeCl}_3$. After centrifugation for 15 min at 20000 g , the pellet was resuspended in 1 ml Tris/glycine buffer (pH 9.0) previously bubbled with N_2 for 10 min and containing 1 mM-sodium dithionite. Cells were broken in a French press at 138 MPa, collected under N_2 and centrifuged at 32000 g for 15 min. A sample (0.2 ml) of the supernatant was subjected to electrophoresis, and its radioactive components were measured as described above.

The radioactivity in the peak corresponding to the Mo-Fe protein of nitrogenase was measured, and expressed, as $\text{Bq (mg protein)}^{-1}$, relative to the total amount of protein in the supernatant from centrifugation at 32000 g . The rate of synthesis of nitrogenase was calculated from the rate of increase of radioactivity in the Mo-Fe protein band. The efficiency of counting was 14% and samples contained at least 1200 c.p.m. above the background (zero time) incorporation of 1500 c.p.m. (see Fig. 3).

Measurement of the chemical stability of mRNA and its rate of synthesis. The rate of incorporation of $[2\text{-}^{14}\text{C}]\text{uracil}$ (7.4 kBq ml^{-1} ; specific activity 2.3 TBq mol^{-1}) into total RNA was measured using 0.5 ml samples removed from 15 ml cultures of *Gloeotheca* previously concentrated 10-fold and continuously bubbled with air (Doolittle, 1972). After a suitable incubation period, rifampicin (0.1 mg ml^{-1}) was added to these cultures and the disappearance of radioactivity from RNA was measured (Fig. 1). The efficiency of counting was 85% and samples contained up to 12000 c.p.m. above the background of 2000 c.p.m. From the rate of disappearance of radioactivity, the half-life of unstable RNA was calculated. If it is assumed that unstable RNA is predominantly mRNA (Doolittle, 1972; Smith & Carr, 1977), then the half-life of *Gloeotheca* mRNA is likely to be similar to that of unstable RNA. After all of the unstable RNA had disappeared from the cultures of *Gloeotheca*, the ratio of unstable RNA to total RNA (B/A in Fig. 1) was determined. This ratio was then used to calculate the rate of synthesis of unstable RNA according to the formula: Rate of synthesis of unstable RNA = Rate of synthesis of total RNA $\times$ [unstable RNA (B)]/[total RNA (A)]. The rate of synthesis of unstable RNA probably approximates to that of mRNA.

Functional stability of mRNA. This was calculated from the length of time which elapsed between addition of rifampicin (0.1 mg ml^{-1}) to cultures of *Gloeotheca* and the complete cessation of protein synthesis. Protein syn-

thesis was measured by incorporation of radioactivity from [^{14}C]protein hydrolysate into protein (Mullineaux *et al.*, 1980, 1981b). The efficiency of counting was 85% and samples contained 3000–7000 c.p.m. above the background of 250 c.p.m.

The length of time which elapsed between addition of rifampicin (0.1 mg ml^{-1}) to cultures of *Gloeotheca* and the interruption of incorporation of ^{55}Fe into Mo–Fe protein of nitrogenase was used to estimate the functional stability of *nif* mRNA (the mRNA coding for the proteins of nitrogen fixation). However, because of the short (10 min) incubation period involved, the method described above was modified, as follows. Immediately after incubation with $^{55}\text{FeCl}_3$, cells were broken in the French press. The broken-cell suspension was centrifuged at $32\,000\text{ g}$ for 15 min and the supernatant was concentrated 50-fold by ultrafiltration, under N_2 , in an Amicon ultrafiltration unit (Amicon, Woking, Surrey, U.K.) fitted with an XM-100 membrane that excluded proteins of molecular weights greater than 150000. Samples (0.2 ml) of this concentrated extract were then subjected to electrophoresis as described above. The efficiency of counting was 14% and samples contained 5000 to 8500 c.p.m. above the background of 2000 c.p.m.

Measurement of the rates of synthesis of rRNA and tRNA. Cultures of *Gloeotheca* were concentrated 10-fold as described above and 30 ml samples were incubated for a suitable period with 37 kBq ml^{-1} of [^{32}P]orthophosphate. Each sample was then centrifuged at $32\,000\text{ g}$ for 15 min at 4°C and the pellet was resuspended in $10\text{ ml } 0.1\text{ M}$ Tris/HCl buffer (pH 7.5) containing 4 mM-MgCl_2 and 1 g sodium lauryl sulphate l^{-1} . Cells were broken in a French press at 138 MPa and 1 ml diethylpyrocarbonate (Sigma) was then added to each extract in order to inactivate nucleases (Solymosy *et al.*, 1968). Following incubation for 5 min at 37°C , RNA was extracted (Solymosy *et al.*, 1968) and rRNA was separated from tRNA by selective precipitation (Kirby *et al.*, 1967). That these samples contained no DNA was demonstrated by electrophoresis (Dingman & Peacock, 1968).

After separation, each RNA fraction was dissolved in $3\text{ ml } 0.5\text{ M-NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer (pH 7.4) containing 0.4 M-NaCl , and its RNA content was measured spectrophotometrically (Solymosy *et al.*, 1968). The radioactivity in 0.2 ml of each RNA fraction was then measured by scintillation spectrometry in 10 ml PCS scintillation fluid. The efficiency of counting was 95% and individual samples contained 300–1400 c.p.m. above the background (80 c.p.m.).

Other measurements. Nitrogenase activity was measured in cultures of *Gloeotheca* using the acetylene reduction technique (Tözüm *et al.*, 1977). Cell density was measured as previously described (Tözüm *et al.*, 1979). Protein was determined by the method of Bailey (1962) using bovine serum albumin as standard.

Chemicals. All radiochemicals were purchased from Amersham. Unless stated otherwise, all other chemicals were obtained from BDH.

RESULTS

Radioactivity was incorporated into N_2 -fixing cultures of *Gloeotheca* during 8 d incubation in medium containing $^{55}\text{FeCl}_3$. When cell-free extracts of these cultures were subjected to PAGE at pH 9, a peak of radioactivity was consistently found 10–20 mm from the origin (Fig. 2a). This peak was tentatively identified as the Mo–Fe protein of nitrogenase, for the following reasons: (1) because it contained radioactivity from $^{55}\text{FeCl}_3$, it must have been an Fe-containing polymer; (2) it gave a stained band upon reaction with amido black and, furthermore, when eluted from the gel had a UV spectrum similar to that of the Mo–Fe protein of other nitrogenases (Lowe *et al.*, 1980) having an absorption maximum at 275–280 nm and a broad shoulder between 325 and about 410 nm; (3) it was missing from extracts of cultures of *Gloeotheca* which had been grown in medium containing NH_4Cl and which did not fix N_2 (Fig. 2b); (4) it behaved, during electrophoresis, in a manner almost identical to the behaviour of the Mo–Fe protein of nitrogenase from *Anabaena variabilis* (Peterson & Wolk, 1978); (5) it was retained by a membrane ultrafilter which excluded proteins of molecular weight greater than 150000. The Mo–Fe protein of *Anabaena cylindrica* has a molecular weight of 220000 (Hallenbeck *et al.*, 1979), much greater than that of, for example, the Fe-protein of nitrogenase (60000). A second peak of radioactivity, at about 75 mm from the origin, was tentatively identified as the Fe-protein of *Gloeotheca* nitrogenase (see Peterson & Wolk, 1978). However, this peak was not always found, perhaps because of its extreme sensitivity to O_2 . For example, 5 min exposure of *Gloeotheca* extracts to air had little effect upon the radioactivity incorporated into the peak 10–20 mm from the origin, but completely abolished the peak 75 mm from the origin.

Following addition of $^{55}\text{FeCl}_3$ to a culture of *Gloeotheca*, incorporation of radioactivity into the presumed Mo–Fe protein of nitrogenase was linear during the first 4 h (Fig. 3) but thereafter declined. There was always significant radioactivity in this protein band at zero time

Fig. 2. Incorporation of ^{55}Fe into the Fe-containing proteins of (a) N_2 -fixing and (b) NH_4^+ -grown *Gloeotheca* cultures. *Gloeotheca* was grown for 8 d in medium containing $^{55}\text{FeCl}_3$. The cells were then broken, and centrifuged at 32000 g, after which 0.2 ml supernatant (containing approximately 0.2 mg protein) was subjected to PAGE at pH 9.0. The radioactivity in 1 mm slices of the gel was then measured. The results are from a single experiment but are typical of eight independent experiments in which great care was taken to exclude O_2 during electrophoresis.

Fig. 3. Incorporation of radioactivity from $^{55}\text{FeCl}_3$ into the Mo-Fe protein of nitrogenase. Illuminated cultures of *Gloeotheca*, concentrated threefold, (50 ml) were incubated with $^{55}\text{FeCl}_3$, after which the cells were broken and centrifuged at 32000 g. Samples of the supernatant (0.2 ml, containing 0.15–0.2 mg protein) were subjected to electrophoresis and the radioactivity in the peak corresponding to the Mo-Fe protein of nitrogenase was measured. The results shown are the means of three experiments using cultures without any additions (Δ) or preincubated for 30 min with chloramphenicol (0.1 mg ml^{-1}) ($\blacktriangle$). The bars indicate the S.E. of these means.

(equivalent to 1500–2000 c.p.m. under the conditions of detection), probably the result of non-specific adsorption of ^{55}Fe on to protein. This was not apparent from the earlier experiments in which much less $^{55}\text{FeCl}_3$ had been administered. Chloramphenicol (0.1 mg ml^{-1}) had no effect on this adsorption but almost completely abolished the subsequent increase of radioactivity in this band (Fig. 3), suggesting that this increase is the result of protein synthesis and not of exchange of ^{55}Fe with pre-existing Fe in nitrogenase. Addition of 2 mM- NH_4Cl , 30 min prior to $^{55}\text{FeCl}_3$, also completely inhibited incorporation of radioactivity into this, but no other, protein. This supports the suggestion that the peak of radioactivity shown in Fig. 2(a) represents a component of nitrogenase, because NH_4^+ represses the synthesis of nitrogenase in almost all N_2 -fixing organisms (Eady, 1981).

Table 1. *Synthesis of nitrogenase, tRNA and rRNA in cultures of Gloeocapsa incubated in the light, and after transfer to the dark*

Nitrogenase synthesis was measured as the rate of increase of radioactivity from $^{55}\text{FeCl}_3$ into the Mo-Fe protein of nitrogenase and is expressed relative to the total amount of protein present in the extract of *Gloeocapsa*. RNA synthesis was measured as the rate of incorporation of radioactivity from $[^{32}\text{P}]\text{orthophosphate}$ into each RNA fraction and is expressed relative to the RNA content of that fraction. The rates of synthesis are from a single experiment, but the figures in parentheses, expressing these rates as a percentage of the rates of synthesis in parallel cultures, incubated in the light, are the means of at least three experiments ($\pm$ S.E.).

Illumination (klx)	Incubation period in dark (h)	Nitrogenase synthesis [Bq min ⁻¹ (mg protein) ⁻¹]	Synthesis of tRNA [Bq min ⁻¹ (mg RNA) ⁻¹]	Synthesis of rRNA [Bq min ⁻¹ (mg RNA) ⁻¹]
2.5	0	9.33 (100)	6.67 (100)	15.00 (100)
0	0.25-4	6.17 (66 $\pm$ 5)	2.67 (40 $\pm$ 2)	4.17 (28 $\pm$ 2)
0	4-7	0.25 (3 $\pm$ 2)	2.67 (40 $\pm$ 2)	4.17 (28 $\pm$ 3)
0	8-10	0.12 (1 $\pm$ 1)	2.67 (40 $\pm$ 3)	4.17 (28 $\pm$ 2)

Table 2. *Synthesis of total RNA and unstable RNA in cultures of Gloeotheca incubated in the light, and after transfer to the dark*

The rate of incorporation of radioactivity from $[2\text{-}^{14}\text{C}]\text{uracil}$ was used as a measure of the synthesis of total RNA. The ratio of unstable RNA to total RNA was calculated from the effect of rifampicin (0.1 mg ml^{-1}) on the incorporation of radioactivity into RNA (see Fig. 1). This ratio was then used to calculate the rate of synthesis of unstable RNA from the rate of synthesis of total RNA.

The rates of synthesis are from a single experiment, but the figures in parentheses, expressing these rates as a percentage of the rates of synthesis in parallel cultures, incubated in the light, are the means of four experiments ($\pm$ S.E.), as are the ratios unstable RNA/total RNA.

Illumination (klx)	Incubation period in dark (h)	Synthesis of total RNA [Bq min ⁻¹ (10 ⁶ cells) ⁻¹]	Unstable RNA/ Total RNA	Synthesis of unstable RNA [Bq min ⁻¹ (10 ⁶ cells) ⁻¹]
2.5	0	1.00 (100)	0.50 $\pm$ 0.10	0.50 (100)
0	0.25-4	0.33 (33 $\pm$ 8)	0.19 $\pm$ 0.05	0.06 (12 $\pm$ 6)
0	4-7	0.31 (31 $\pm$ 6)	0.18 $\pm$ 0.04	0.06 (12 $\pm$ 5)
0	7-10	0.32 (32 $\pm$ 5)	0.19 $\pm$ 0.07	0.06 (12 $\pm$ 6)

Because of the adsorption of ^{55}Fe on to protein, nitrogenase synthesis was always estimated by measuring the rate of incorporation of ^{55}Fe into the Mo-Fe protein of nitrogenase, not simply the amount of incorporation.

After 6 h incubation of cultures with $^{55}\text{FeCl}_3$, incorporation of radioactivity into the Mo-Fe protein ceased. This may have been the result of a high turnover of nitrogenase, causing rapid equilibration between unincorporated $^{55}\text{FeCl}_3$ and ^{55}Fe -labelled protein.

When illuminated cultures of *Gloeotheca* were incubated for up to 4 h under an atmosphere of pure O_2 , it was not possible to detect any decrease in the rate of synthesis of the Mo-Fe protein of nitrogenase. However, nitrogenase activity could not be detected in *Gloeotheca* cultures incubated in the light for 60 min under O_2 (A. F. Hamadi, unpublished observation), suggesting that nitrogenase synthesis continues under pure O_2 but that the enzyme is rapidly inactivated.

Between 15 min and 4 h after transfer of *Gloeotheca* cultures from light to darkness, the rate of synthesis of the Mo-Fe protein of nitrogenase continued at 66 % of its rate in the light. However, between 4 h and 7 h after transfer to darkness, the rate of synthesis markedly decreased and normally reached zero after 8 h in the dark (Table 1). This supports the earlier conclusions about nitrogenase synthesis in *Gloeotheca*, which were based on studies of nitrogenase activity during a light-dark transition (Mullineaux *et al.*, 1981 b).

Following reillumination of *Gloeotheca* cultures, previously incubated for 12 h in the dark, there was no incorporation of ^{55}Fe into the Mo-Fe protein of nitrogenase for 10 h. However, between 10 h and 12 h after reillumination, ^{55}Fe incorporation was observed at a rate of about 85% of that occurring prior to the original transfer to darkness. This recommencement of incorporation of ^{55}Fe coincided approximately with the reappearance of nitrogenase activity (Mullineaux *et al.*, 1981 b).

Fig. 4. Effect of rifampicin on the incorporation of radioactivity from [U-¹⁴C]protein hydrolysate into protein. [U-¹⁴C]Protein hydrolysate was added at zero time to 50 ml cultures of *Gloeotheca*, previously concentrated 10-fold. After incubation, with illumination at 2.5 klx, 2 ml samples were removed into methanol and the radioactivity in protein was measured as described by Mullineaux *et al.* (1980). Rifampicin (0.1 mg ml⁻¹) was added to the incubation at the time indicated by the arrow. The points are the means of five separate experiments; the bars show the s.e. of these means.

Within 15 min of transfer of *Gloeotheca* cultures to darkness, synthesis of tRNA, rRNA and unstable RNA decreased by 60, 70 and 90%, respectively (Tables 1 and 2). However, the rate of RNA synthesis remained constant thereafter and did not reflect the decrease in nitrogenase synthesis that occurred about 4 h later.

The chemical stability of unstable RNA was identical in cultures of *Gloeotheca* incubated either in the light or in the dark. The half-life of unstable RNA was 5 ± 0.5 min (s.e., 8 determinations), which is similar to that reported in other cyanobacteria (Doolittle, 1972; Smith & Carr, 1977; Meeks *et al.*, 1977). Although unstable RNA may include precursors of rRNA as well as mRNA, the stability of mRNA is likely to be similar to these values. Indeed, in illuminated cultures of *Gloeotheca*, the functional stability of mRNA (Fig. 4) was similar to the chemical stability of unstable RNA, having a half-life of 6 ± 1 min (s.e., 5 determinations). Because of the low rate of protein synthesis (Mullineaux *et al.*, 1981*b*), the functional stability of mRNA could not be measured in the dark. Neither the chemical stability of unstable RNA nor the functional stability of mRNA was greatly affected by the presence of NH₄Cl in the growth medium (half-life = 6 ± 1.5 min; s.e., 4 determinations). This contrasted with the mRNA of *Klebsiella pneumoniae*, which was more stable under N₂-fixing conditions than in the presence of NH₄⁺ (Eady *et al.*, 1981).

Measurement of the functional stability of *nif* mRNA was more difficult. Although complete inhibition of the incorporation of ⁵⁵Fe into the Mo-Fe protein occurred within 20 min of adding rifampicin to illuminated cultures of *Gloeotheca*, it was not possible to calculate a half-life for *nif* mRNA from these data because nitrogenase synthesis could not be measured over less than 10 min. However, it appears that the stability of *nif* mRNA is similar to that of total mRNA.

DISCUSSION

Synthesis of nitrogenase in *Gloeotheca* differs from that of the enzyme in other N₂-fixing organisms, for example *K. pneumoniae* (Eady *et al.*, 1978) and *Azotobacter chroococcum* (Robson, 1979), in that it is not repressed by O₂. In *Gloeotheca*, the ability to synthesize nitrogenase in the presence of O₂ in order to replace enzyme inactivated by O₂ affords one explanation of the ability of this organism to fix N₂ aerobically (Gallon, 1981). In heterotrophic bacteria such as *K. pneumoniae* and *A. chroococcum*, synthesis of nitrogenase under conditions where the enzyme is inactive would be wasteful, hence the repression of nitrogenase synthesis by inhibitory concentrations of O₂ is an advantage to these organisms. However, in a photoautotroph like *Gloeotheca*, the metabolic energy needed to support nitrogenase synthesis may be more readily available, and there would therefore be less of a disadvantage in maintaining the synthesis of nitrogenase

even in the presence of O₂. Indeed, in allowing aerobic N₂-fixation, O₂-insensitive synthesis of nitrogenase may be an advantage to *Gloeotheca*. If, among cyanobacteria, the inability of O₂ to inhibit nitrogenase synthesis is confined to *Gloeotheca*, it may explain why, unlike most other non-heterocystous cyanobacteria, *Gloeotheca* can fix N₂ aerobically.

Within 4 h following transfer of *Gloeotheca* cultures from light to darkness, synthesis of tRNA, rRNA and unstable RNA declined rapidly (Tables 1 and 2). At the same time, there was a sharp decrease in the rate of protein synthesis (Mullineaux *et al.*, 1981*b*). Thereafter, these rates remained constant. In contrast, synthesis of nitrogenase decreased slightly during the first 4 h of darkness but virtually ceased within the following 3 h (Table 1). Although the initial fall in the rate of nitrogenase synthesis upon transfer to darkness may be related to the simultaneous decrease in RNA and protein synthesis, the subsequent cessation of nitrogenase synthesis after about 5 h in the dark could not be related to any non-specific effect on RNA or protein synthesis, or even to depletion of glucan reserves (Mullineaux *et al.*, 1968*b*). It may therefore be specific to nitrogenase.

In *Gloeotheca*, mRNA, including *nif* mRNA, is very unstable. Consequently, synthesis of *nif* mRNA must continue during the first 4 h of darkness in order to permit the continued synthesis of nitrogenase. Thus the inhibition of nitrogenase synthesis that occurs 4–5 h after the removal of illumination cannot be a result of inhibition of *nif* mRNA synthesis occurring immediately on transfer to darkness. Any inhibition of the synthesis of *nif* mRNA must occur shortly before nitrogenase synthesis ceases. However, whether the effect of darkness on nitrogenase synthesis is exerted at the level of transcription of *nif* mRNA remains to be seen. Furthermore, the mechanism by which darkness inhibits nitrogenase synthesis is at present unknown.

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