

## Sustained Photoproduction of Ammonia from Nitrate by *Anacystis nidulans*

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Conditions that lengthen the time during which L-methionine-DL-sulfoximine (MSX) promotes excretion of ammonia produced by photosynthetic nitrate reduction in *Anacystis nidulans* have been sought. If MSX was added every 24 h, maximal rates of ammonia production were maintained for 3 days. After this time, ammonia production ceased due to a specific deficiency of glutamine in the cells, which finally led to cell lysis. The effective ammonia production period could be further extended either by adding a low amount of glutamine at the end of the 3-day period or by allowing the cells to recover for 8 h in the absence of MSX after every 48-h period in the presence of inhibitor. In this way, a steady production of ammonia lasting for at least 10 days was achieved. The MSX-treated cyanobacterial cells thus represent a system relatively stable with time for the conversion of light energy into chemical energy through the photoreduction of nitrate to ammonia.

The photoreduction of nitrate to ammonia by photosynthetic organisms represents a remarkable process for the conversion of solar energy into chemical energy (4, 7, 8). Efficient photoproduction of ammonia from nitrate by cells of the cyanobacterium *Anacystis nidulans* has been achieved in our laboratory after specifically inhibiting glutamine synthetase, the first enzyme involved in the main ammonia assimilation pathway in this organism, with the glutamate analog L-methionine-DL-sulfoximine (MSX) (3, 11).

To be considered valuable for practical purposes, a system for the conversion of sunlight energy must operate at considerable rate and efficiency and must be relatively stable in time. The system constituted by MSX-treated *Anacystis* cells has been shown to produce ammonia at high rates (11) with relatively high efficiency (12), but until now the process has been only carried out during a limited period of time.

This communication reports the achievement of a steady photoproduction of ammonia from nitrate by MSX-treated *A. nidulans* cell suspensions, lasting for a period of at least 10 days.

### MATERIALS AND METHODS

**Growth of cells.** *A. nidulans* 1402-1 was grown photoautotrophically under continuous lighting (Sylvania daylight fluorescent tubes, giving an irradiance value of 25 W/m<sup>2</sup> at the surface of the culture vessels), at 40°C on a synthetic medium containing nitrate as the nitrogen source (5), in a stream of 2% CO<sub>2</sub> in air (vol/vol), which was bubbled through the culture at a flow rate of 1 liter/liter of cell suspension per h.

**Ammonia production experiments.** Cells from 2-day old cultures containing 20 to 25 µg of chlorophyll *a* (Chl) per ml were used. The cells were harvested by centrifugation, washed with culture medium, and finally suspended in the same medium to a density of 8 to 10 µg of Chl per ml. MSX was added to the cell suspension to a final concentration of 10 µM, and the cell suspensions were then incubated under standard culture conditions with the indicated modifications.

**Assays of enzyme activities.** Nitrate reductase and glutamine synthetase (transferase) activities were measured in toluenized cells as previously described (11). Nitrite reductase and NADH-alanine dehydrogenase were determined in cell-free extracts. Nitrite reductase activity was estimated according to Manzano et al. (10). NADH-alanine dehydrogenase was estimated as described by Rowell and Stewart (13).

**Extraction of cellular metabolites.** Perchloric acid was added to a sample of the cell suspension to give a concentration of 0.6 N. After 10 min at 0°C, the samples were centrifuged at 40,000 × *g* for 15 min. The supernatant was neutralized at 0°C with KOH. After removal of the KClO<sub>4</sub> precipitate by centrifugation (10,000 × *g* for 10 min), the supernatants were lyophilized. The lyophilized material was suspended to a small volume with water and used for the estimation of metabolites (3).

**Analytical methods.** Nitrate, nitrite, ammonia, γ-glutamyl hydroxamate, glutamate, and glutamine were estimated as described previously (11). Carbohydrates were estimated by the phenol-sulfuric method (6), total nitrogen by the Kjeldahl procedure, packed cell volume by centrifugation of the cell suspensions in calibrated hematocrit tubes, and cellular protein by a modification of the Lowry procedure (2) after pretreating the cells with 10% (wt/vol) trichloroacetic acid. Chl was determined spectrophotometrically in methanolic

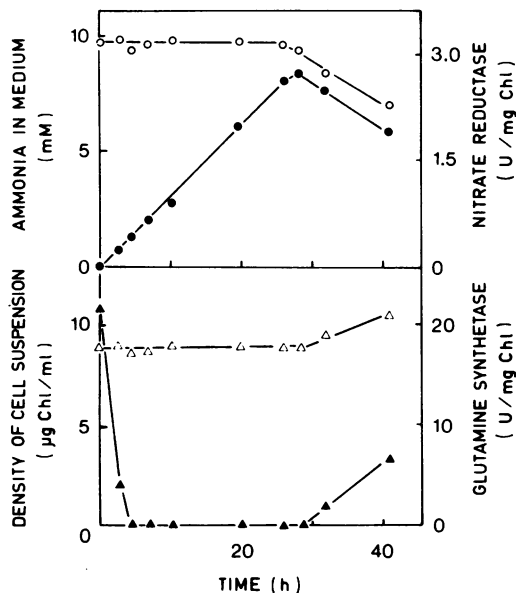


FIG. 1. Time course of the events leading to ammonia production and to its later cessation in MSX-treated *A. nidulans* cells. A cell suspension (150 ml) with a density of 9  $\mu\text{g}$  of Chl per ml and supplemented with 10  $\mu\text{M}$  MSX was incubated under standard culture conditions. Samples were withdrawn at the times indicated, and ammonia in the medium (●), density of the cell suspension ( $\Delta$ ), and cellular activity levels of nitrate reductase (○) and glutamine synthetase ( $\blacktriangle$ ) were determined.

extracts with the extinction coefficient given by MacKinney (9). The Chl and protein contents of MSX-treated *A. nidulans* cells were fairly constant, with values of 5.8 to 6.2  $\mu\text{g}$  of Chl and 110 to 130  $\mu\text{g}$  of protein per  $\mu\text{l}$  of packed cell volume (11). Measurements of the absorbance values at 628 and 680 nm of the cell suspensions for estimation of the phycocyanin/Chl ratio were performed by placing the sample-containing cuvette in the turbid-samples compartment of a Pye Unicam SP 1750 spectrophotometer.

## RESULTS AND DISCUSSION

The main events taking place after the addition of MSX to suspensions of *A. nidulans* cells in nitrate medium have previously been described (11) and can be summarized as follows. Glutamine synthetase becomes rapidly and completely inactivated, and ammonia appears in the medium concomitantly with this activity loss. Cell growth is prevented, and the rates of nitrate uptake and reduction increase about twofold. Ammonia accumulates in the medium at a constant rate of about 25  $\mu\text{mol}/\text{mg}$  of Chl per h.

The process of ammonia production continues for about 30 to 35 h without further additions, provided that nitrate is not limiting, and nitrate reductase activity remains at its original high

level during this period. As Fig. 1 shows, after this time, glutamine synthetase activity recovered, and the accumulation of ammonia in the medium ceased, followed by its utilization by the cells, which started to grow. Once glutamine synthetase became active, the antagonistic effects of ammonia (3, 5, 11) came into play; nitrate utilization was inhibited (data not shown), and the nitrate reductase level decreased rapidly (Fig. 1). Since, under standard culture conditions and in the absence of cells, MSX does not lose its activity for at least 48 h, the cessation of its effect on ammonia production can be related to the transformation of the inhibitor by the algal cells rather than to its spontaneous degradation.

It seems, therefore, that the temporary ceasing of ammonia photoproduction is due to the recovery of glutamine synthetase activity rather than to the induction of any other ammonia assimilation pathway. Thus, to lengthen the period of ammonia production, fresh MSX at its optimal concentration with respect to the density of the cell suspension (1 nmol/ $\mu\text{g}$  of Chl) (12) was added to the *Anacystis* cell suspension in which the effect of the original MSX had been lost. This addition resulted again in inactivation of the preexisting glutamine synthetase, allowing new maximal rates of ammonia production (data not shown). Following this approach, successive additions of 10  $\mu\text{M}$  MSX to the cell suspension every 24 h allowed maximal rates of ammonia production to be sustained for 3 days. During this period, cellular glutamine synthetase remained fully inactive, the nitrate reductase activity level being high, and about 90% of the nitrate taken up appearing in the medium as ammonia (Fig. 2). At this stage, the addition of MSX no longer resulted in maintenance of the process of ammonia production, and cell lysis of the suspension was observed shortly afterwards.

To determine the cellular situation leading to the cessation of ammonia production by *Anacystis*, the levels of various cell components, enzymatic activities related to nitrate metabolism, and amino acids involved in the glutamine synthetase-glutamate synthase pathway were studied in *Anacystis* cells which had been treated with MSX for 68 h and were compared with those in normal cells. The analysis of *Anacystis* cells subjected to MSX treatment for 68 h yielded data similar to those previously found in experiments of shorter duration (11). The cellular levels of protein, Chl, phycocyanin, and total nitrogen remained constant and equal to those of untreated cells, whereas the carbohydrate level increased linearly. The increase in the carbohydrate level, from 6.2  $\mu\text{g}/\mu\text{l}$  of cells at time zero to 39  $\mu\text{g}/\mu\text{l}$  of cells at 68 h, was interpreted as due to continued photosynthetic  $\text{CO}_2$  fixation while

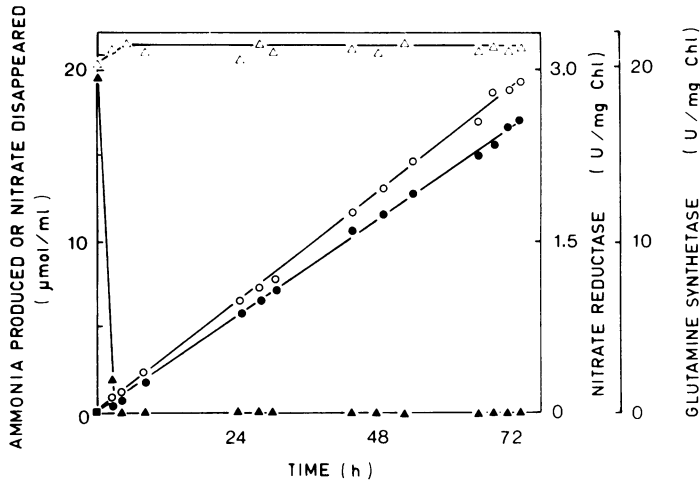


FIG. 2. Continuous ammonia photoproduction by MSX-treated *A. nidulans* cells for 3 days. Conditions were those described in the legend to Fig. 1, except that MSX (1.5 µmol) was added every 24 h. At 48 h, KNO<sub>3</sub> (1.5 mmol) was added. Samples were withdrawn at the times indicated, and ammonia (●) and nitrate (○) in the medium and cellular activity levels of nitrate reductase (△) and glutamine synthetase (▲) were determined.

MSX prevented the incorporation of ammonia into carbon skeletons and subsequent cell growth (11).

It is interesting that the phycocyanin levels typical of normal cells were maintained in MSX-treated *Anacystis* cells when the medium contained nitrate but not when it was free of nitro-

gen. As a consequence, MSX-treated *Anacystis* cells actively producing ammonia from nitrate did not suffer enhanced phycocyanin degradation, as is the case for cells subjected to nitrogen starvation (1). This contrasts with the situation observed for MSX-treated filamentous cyanobacteria under dinitrogen-fixing conditions, as

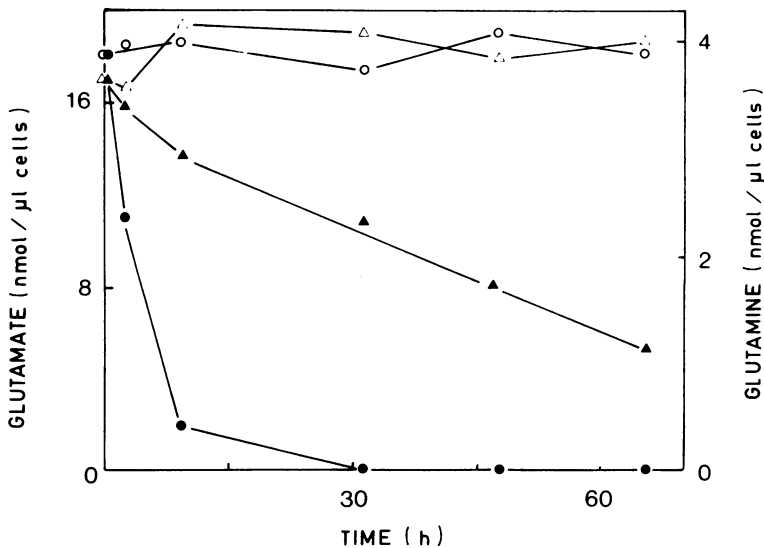


FIG. 3. Cellular levels of glutamine and glutamate in MSX-treated and untreated *A. nidulans* cells. Cells grown on nitrate medium were transferred to media containing KNO<sub>3</sub> (20 mM) (open symbols) or KNO<sub>3</sub> (20 mM) and MSX (10 µM) (closed symbols). Samples were withdrawn at the times indicated, and the cellular content in glutamine (circles) and glutamate (triangles) was estimated. Other conditions were those described in the legend to Fig. 1.

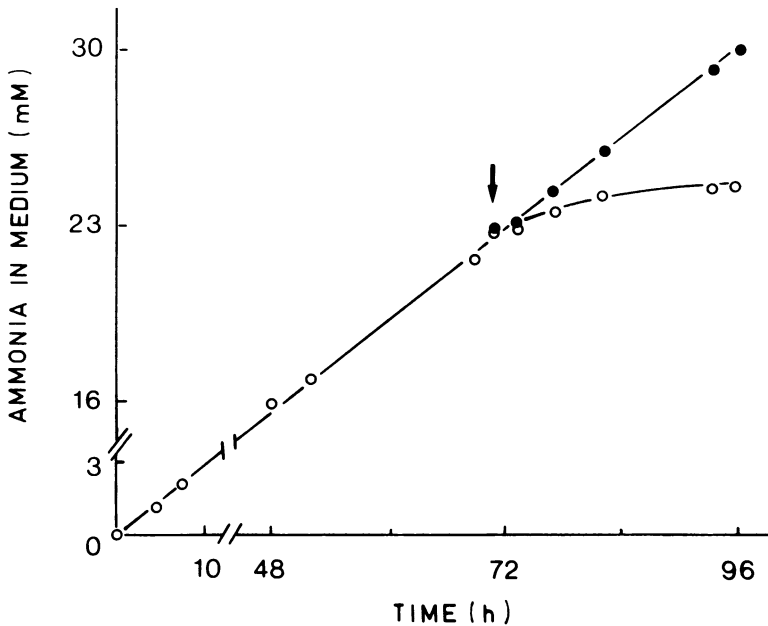


FIG. 4. Effect of glutamine addition on the prolongation of the ammonia production period by MSX-treated *A. nidulans* cells. Conditions were those described in the legend to Fig. 2, except that glutamine (0.5 mM) was added at 70 h (arrow) to one-half of the suspension (●); the other half was used as a control without any addition (○).

found for *Anabaena cylindrica* (14) and *Anabaena* sp. strain ATCC 33047 (unpublished data).

Although MSX-treated *Anacystis* cells lacked glutamine synthetase, they exhibited low but reproducible levels of NADH-alanine dehydrogenase (around 7 mU/mg of protein), levels of the same order of magnitude as those found in normal cells. This finding suggests that ammonia assimilation pathway(s) that may be secondary under normal conditions, can play an important role in the maintenance of the nitrogen balance in MSX-treated cells. The enzymes responsible for nitrate reduction, namely, nitrate reductase and nitrite reductase, in MSX-treated *Anacystis* cells were always present at about the same levels as in the untreated cells.

As a consequence of the inactivation by MSX of glutamine synthetase, the glutamine level in ammonia-producing cells decreased rapidly with time and became practically negligible 30 h after the addition of the inhibitor. This behavior was in contrast with that of untreated cells, which, under the same conditions, maintained their normal glutamine levels. The level of glutamate in MSX-treated *Anacystis* cells also decreased with time, but much slower; its value after 68 h was 35% that of normal cells (Fig. 3). This amino acid analysis indicates that *Anacystis* cells subjected to treatment with MSX become specifi-

cally deficient in glutamine and probably also in glutamine derivatives. Such a metabolic situation might eventually lead to the cessation of ammonia production.

To overcome the MSX-promoted glutamine deficiency and thus prolong the ammonia production period, two different attempts were made. One of them consisted of the addition to the cell suspension of glutamine at low concentrations (0.2 to 0.5 mM) simultaneously with the normal readdition of MSX at the end of the 3-day period. The data in Fig. 4 show that this glutamine treatment resulted in sustained ammonia production at constant rate for at least 24 h, a phenomenon that did not occur in a control without glutamine addition, in which the rate of ammonia production slowed with time until it became negligible. Interestingly enough, the glutamine treatment did not result in cell growth (measured as Chl) or in any increase in glutamine synthetase activity, but the levels of nitrate and nitrite reductases doubled. No increment in the ammonia production rate was observed, however, suggesting that the normal nitrate and nitrite reductase levels are high enough to maintain the process at its maximal rate. Glutamate could not replace glutamine in sustaining ammonia production, a result which confirms the assumption that the cessation of the process is specifically related to glutamine deficiency.

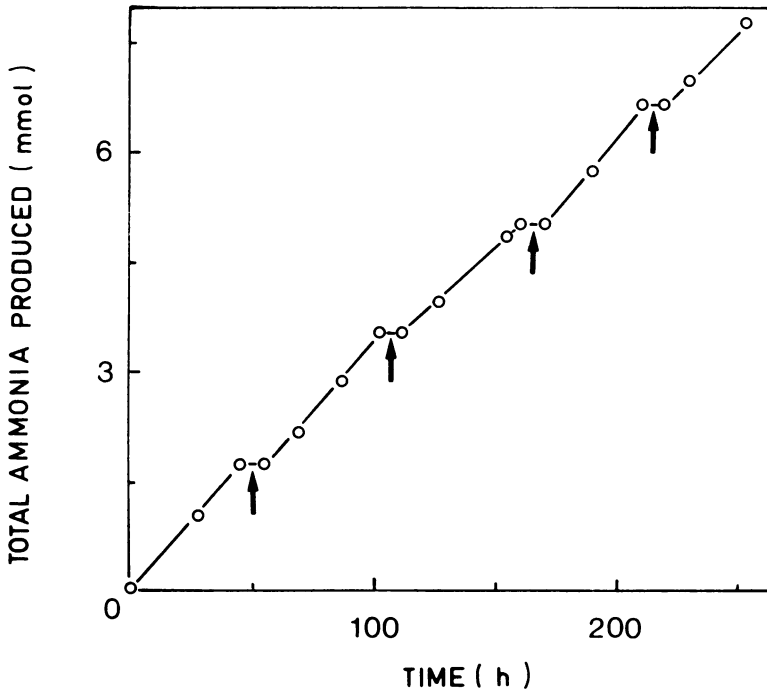


FIG. 5. Effect of the recurrent addition and removal of MSX on the photoproduction of ammonia by *A. nidulans* cells. A cell suspension with a density of  $9 \mu\text{g}$  of Chl per ml was maintained for 48 h in nitrate culture medium supplemented with  $10 \mu\text{M}$  MSX. After this time, cells were harvested and washed three times with fresh nitrate medium lacking MSX. After 8 h in the absence of the inhibitor, and once the cell loading had been corrected to  $9.0 \pm 0.2 \mu\text{g}$  of Chl per ml, MSX ( $10 \mu\text{M}$  final concentration) was readded. Successive periods of ammonia production (around 48 h) and cell recovery (around 8 h) (arrows) were alternated as indicated. The last readdition of MSX was to reach a final concentration of  $100 \mu\text{M}$ .

The second approach to extend the ammonia production period consisted of allowing the cells to recover from glutamine deficiency by themselves. This goal was achieved by alternating exposure to MSX with periods in which the glutamine synthetase inactivator was removed. Figure 5 shows that this sequential treatment allowed continuous production of ammonia at high rates for at least 10 days. The long period in the presence of MSX (around 48 h) resulted in net ammonia production, whereas the shorter period in its absence (around 8 h) did not. During the recovery periods, glutamine synthetase activity increased from 0 to 150 to 300 mU/mg of protein (10 to 20% of the level in normal cells), which allowed glutamine synthesis and a slight growth (from 9.0 to about  $10.6 \mu\text{g}$  of Chl per ml in 8 h). Surprisingly, after day 8 of ammonia production, resistance to MSX appeared. This phenomenon was probably due to an adaptation of the cells to MSX, making it necessary to increase the MSX concentration up to  $100 \mu\text{M}$  to again achieve complete inactivation of cellular glutamine synthetase.

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