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Sustained Photoproduction of Ammonia from Dinitrogen and Water by the Nitrogen-Fixing Cyanobacterium *Anabaena* sp. Strain ATCC 33047

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Conditions have been developed that lengthen the time during which photosynthetic dinitrogen fixation by filaments of the cyanobacterium *Anabaena* sp. strain ATCC 33047 proceeds freely, whereas the subsequent conversion of ammonia into organic nitrogen remains blocked, with the resulting ammonia released to the outer medium. When L-methionine-DL-sulfoximine was added every 20 h, maximal rates of ammonia production (25 to 30 μmol/mg of chlorophyll per h) were maintained for about 50 h. After this time, ammonia production ceased due to a deficiency of glutamine and other nitrogenous compounds in the filaments, conditions which finally led to cell lysis. The effective ammonia production period could be further extended to about 7 days by adding a small amount of glutamine at the end of a 40-h production period or by allowing the cells to recover for 8 h in the absence of L-methionine-DL-sulfoximine after every 40-h period in the presence of the inhibitor. A more prolonged steady production of ammonia, lasting for longer than 2 weeks, was achieved by alternating treatments with the glutamine synthetase inhibitors L-methionine-DL-sulfoximine and phosphinothricin, provided that 8-h recovery periods in the absence of either compound were also alternated throughout. The biochemically manipulated cyanobacterial filaments thus represent a system that is relatively stable with time for the conversion of light energy into chemical energy, with the net generation of a valuable fuel and fertilizer through the photoreduction of dinitrogen to ammonia.

The conversion of solar energy into suitable redox energy through photosynthesis of the water-splitting type that is carried out by whole organisms is a process of great interest and significance. Light-driven synthesis of ammonia from dinitrogen and water by photosynthetic organisms represents an interesting system for the conversion of light energy into stored chemical energy, with the additional interest that the resulting compound is a valuable fuel and fertilizer (4, 7).

Filamentous cyanobacteria (blue-green algae) that are able to differentiate heterocysts are unique organisms in that they can fix N_2 under aerobic conditions, with light as the sole source of energy and water as the ultimate reductant, according to the following global equation:

$$N_2 + 3H_2O \xrightarrow{6e^-,\ \geq 12\ ATP} 2NH_3 + 3/2O_2$$

($\Delta E_0'$ [pH 7] = −1.10 V; $\Delta G_0'$ [pH 7] = +318 kJ/mol of ammonia)

where $\Delta E_0'$ is the standard redox potential change and $\Delta G_0'$ is the standard free energy change.

The achievement of effective production of ammonia from dinitrogen by N_2-fixing cyanobacteria requires (i) prevention of the incorporation of ammonia to carbon skeletons, which takes place mainly through the glutamine synthetase-glutamate synthase pathway (18), and (ii) overcoming the antagonistic effects of ammonia on dinitrogen fixation (4). Both requisites have been simultaneously fulfilled by interfering with the operation of the main ammonia assimilation pathway by using glutamate analogs, such as MSX (L-methionine-DL-sulfoximine) or PT (phosphinothricin [2-amino-4-(methylphosphinyl)-butanoic acid]) (6, 9, 12, 17).

To be considered practical, a system for the conversion of sunlight energy must operate at a considerable rate and efficiency and must be relatively stable with time. The system constituted by MSX- or PT-treated *Anabaena* sp. strain ATCC 33047 filaments has been shown to produce ammonia at high rates, in the range of 25 to 30 μmol/mg of chlorophyll per h (6, 9, 12), with relatively high efficiency, ca. 10% of its theoretical maximum, but until now the process has been only carried out for short periods of time. This communication reports the achievement of steady photoproduction of ammonia from dinitrogen by the cyanobacterium *Anabaena* sp. strain ATCC 33047, lasting for longer than 2 weeks.

MATERIALS AND METHODS

Growth of cells. *Anabaena* sp. strain ATCC 33047 was grown photoautotrophically, under continuous illumination (Sylvania day light fluorescent tubes, with an irradiance value of 25 W/m^2 at the surface of the culture vessels) at 40°C on a synthetic medium modified from BG11 medium as described previously (16), except that 25 mM $NaHCO_3$ was added. A stream of 2% (vol/vol) CO_2 in air was bubbled through the culture at a flow rate of 1.2 liters/liter of cell suspension per h.

Ammonia production experiments. *Anabaena* sp. strain ATCC 33047 filaments from 2-day-old cultures, containing 20 to 25 μg of chlorophyll *a* (Chl) per ml, were used. The filaments were harvested, washed with culture medium, and finally resuspended in the same medium to reach a cell density of 8 to 10 μg of Chl per ml. The filament suspensions were incubated for 60 to 90 min in the light under standard

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culture conditions. Experiments were started by adding enough MSX to the cell suspension to achieve a ratio of 3.5 to 4 nmol of MSX per μg of Chl.

Assays of enzyme activities. Nitrogenase activity was measured in whole cells by the acetylene reduction technique (9). Glutamine synthetase (transferase) activity was determined in toluenized filaments (12). Alanine dehydrogenase was estimated in cell-free extracts as previously described (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, and the supernatant was neutralized at 0°C with KOH. After removal of $KClO_4$ by centrifugation (10,000 × *g* for 10 min), the supernatants were used for the estimation of metabolites.

Analytical methods. Ammonia was determined by the phenol-hypochlorite method as described previously (15). Carbohydrates were estimated by the phenol-sulfuric method (5). Chl was determined spectrophotometrically in methanolic extracts by using the extinction coefficient given by MacKinney (8). Ethylene was measured by using a PYE UNICAM 204 gas chromatograph equipped with a column filled with Porapak Q and a flame ionization detector. Glutamate and glutamine were determined according to Bergmeyer (3). γ-Glutamil hydroxamate was estimated after its reaction with $FeCl_3$ in acid medium (14). Cellular protein was determined by the Lowry procedure as modified by Bailey (2), by pretreatment of the filaments with 10% (wt/vol) trichloroacetic acid.

For dry-weight determination, 30-ml samples of the cell culture were filtered through preweighed dry filters (Whatman GF/C). The filters were washed with 2 volumes of distilled water and dried at 90°C until constant weight was reached. Relative phycocyanin levels were estimated spectrofluorimetrically by using an Aminco-Bowman spectrophotofluorimeter equipped with a HTV photomultiplier tube, type R44GS. For fluorescence measurements, filament suspensions (9 μg of Chl per ml) were illuminated with green light (590 nm, 5 W/m^2), which is mainly absorbed by phycocyanin, with the energy then transferred to chlorophyll. Chlorophyll fluorescence is proportional to the level of phycocyanin in the filament suspensions. Light intensity measurements were done with a YSI-Kettering model 65A radiometer.

Chemicals. MSX was purchased from Sigma Chemical Co., St. Louis, Mo. PT (6) was kindly supplied by P. J. Lea (Rothamsted, United Kingdom). Other chemicals were products of E. Merck AG, Darmstadt, Federal Republic of Germany.

RESULTS AND DISCUSSION

The addition of MSX to suspensions of *Anabaena* sp. strain ATCC 33047 filaments that were growing on air (78% N_2) as the nitrogen source caused a rapid inactivation of cellular glutamine synthetase, with cell growth consequently prevented. Nitrogenase activity increased gradually in response to MSX, reaching a stable level, which was around twofold higher than the initial level, about 4 h after its addition. Most of the ammonia (ca. 90%) resulting from N_2 fixation by the cells was released and accumulated in the outer medium (9; see also Fig. 1). The rate of ammonia production increased concomitantly with the increase in nitrogenase activity mentioned above, until it reached a maximum in correspondence with the establishment of a stable high nitrogenase level. The maximal rates of ammonia production under these conditions were 25 to 30 μmol/mg of Chl per h, and the process continued to be operative for about 20 h (Fig. 1). Figure 1 also shows that about 20 h after MSX addition glutamine synthetase activity started to recover and accumulation of ammonia in the medium ceased. These changes were followed by the utilization of the ammonia that was released by the filaments, with growth starting again. In parallel with the occurrence of ammonia uptake, the nitrogenase activity level decreased slightly.

Since under standard culture conditions and in the absence of filaments MSX keeps active for at least 48 h, the cessation of the effect of the inhibitor on ammonia production could be related to its transformation by the cyanobacterial cells rather than to its spontaneous degradation. The temporary interruption of ammonia production is apparently due to the recovery of glutamine synthetase activity rather than to the induction of any other ammonia assimilation pathway. Accordingly, to lengthen the period of ammonia production, fresh MSX at its optimal ratio with respect to the amount of cells (3.5 to 4 nmol/μg of Chl) was added to suspensions of *Anabaena* sp. strain ATCC 33047 in which the MSX effect had been lost. This addition resulted again in inactivation of preexisting glutamine synthetase and allowed anew maximal rates of ammonia production (data not

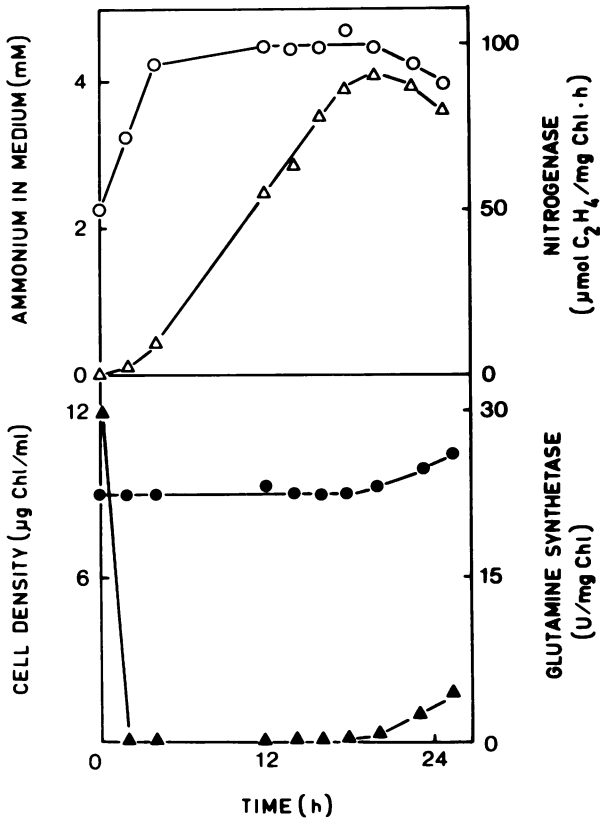

FIG. 1. Time course of the events leading to ammonia production and its later cessation in MSX-treated *Anabaena* sp. strain ATCC 33047 filaments. A filament suspension (150 ml) with a density of 9 μg of Chl per ml that was supplemented with 35 μM MSX was incubated under standard culture conditions. Samples were withdrawn at the times indicated, and ammonia in the medium (△), density of the filament suspension (●), and cellular activities of nitrogenase (○) and glutamine synthetase (▲) were determined.

shown). Following this approach, successive additions of MSX to the filament suspension every 20 h allowed maximal rates of ammonia production to be maintained for about 48 h (Fig. 2). During this period, glutamine synthetase remained fully inactive, and the level of nitrogenase activity was at least twofold higher than that in untreated filaments. Nevertheless, after 48 h in the presence of MSX, the ammonia production rate decreased progressively, in spite of the fact that glutamine synthetase remained inactive. This cessation in ammonia production seems to be a consequence of a loss of nitrogenase activity (data not shown). Cell lysis of the suspension was observed shortly afterwards.

To determine the cellular situation leading to the cessation of ammonia production by *Anabaena* sp. strain ATCC 33047, the levels of various cell components, enzymatic activities related to nitrogen metabolism, and amino acids involved in the glutamine synthetase-glutamate synthase pathway were measured in filaments which had been treated with MSX for 36 h and were compared with those in untreated filaments. Table 1 shows the results obtained. The values for the chlorophyll and protein content were only slightly lower in MSX-treated filaments than in the untreated ones. However, remarkable differences were found regarding the levels of carbohydrates and phycocyanin. The carbohydrate and phycocyanin levels in MSX-treated filaments were threefold higher and fivefold lower, respectively, than those in untreated filaments. The reduction in the phycocyanin level in MSX-treated cells was also evident from the change in the absorbance at 628 nm/absorbance at 680 nm ratio of the cell suspensions, which decreased from 0.91 to 0.72 as a consequence of the prolonged treatment with MSX. The differences in carbohydrate and phycocyanin content were interpreted as being due to continued photosynthetic CO_2 fixation in the presence of MSX, which otherwise prevented the incorporation of ammonia derived from N_2 fixation into carbon skeletons. Actually, the C/N ratio increased in MSX-treated *Anabaena* sp. strain ATCC 33047 filaments from 4.2 at zero time to 6.8 at 36 h. The MSX-treated cells thus appear to be nitrogen starved (1). Such an induction of nitrogen starvation by MSX has been also reported for N_2-fixing *Anabaena cylindrica* (19), but it is in contrast with the situation noticed in MSX-treated *Anacystis* cells producing ammonia from nitrate, which did not exhibit enhanced phycocyanin degradation (10, 11).

TABLE 1. Effect of MSX on the levels of different cell components in *Anabaena* sp. strain ATCC 33047[a]

Time (h) and treatment	Cell component			
	Chl[b]	Phycocyanin[c]	Protein[b]	Carbohydrates[b]
0	31	100	700	79
36 (without MSX)	30	100	650	94
36 (with MSX)	26	20	550	284

[a] A suspension of *Anabaena* sp. strain ATCC 33047 filaments containing 7.9 μg of Chl (250 μg [dry weight]) per ml was divided into two halves, to one of which MSX (35 μM) was added at zero time and 20 h later. After 36 h of incubation under standard growth conditions, the cell density values reached were 37.0 μg of Chl (1,214 μg [dry weight]) per ml and 7.8 μg of Chl (304 μg [dry weight]) per ml for the MSX-free and MSX-containing suspensions, respectively.
[b] Values are in micrograms per milligram (dry weight).
[c] Values are percentages of the control at zero time.

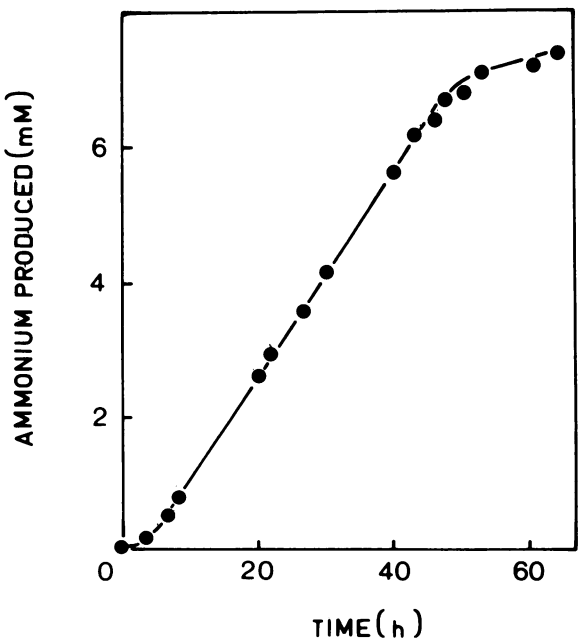

FIG. 2. Continuous ammonia photoproduction by MSX-treated *Anabaena* sp. strain ATCC 33047 filaments. Conditions were the same as those described in the legend to Fig. 1, except that MSX (35 μM) was readded every 20 h. The ammonia in the medium was determined at the times indicated.

MSX-treated *Anabaena* sp. strain ATCC 33047 filaments lacked glutamine synthetase activity and exhibited low, although reproducible, levels of NADH-alanine dehydrogenase (ca. 14 mU/mg of protein), about twofold higher than those found in untreated filaments. This activity might be responsible for the assimilation of low amounts of ammonia, since the nitrogen deficiency was more severe in MSX-treated *Anabaena* sp. strain ATCC 33047 kept under argon atmosphere than in that maintained under air. In fact, after 36 h of treatment, the former had lost most of its phycocyanin, whereas the latter still retained ca. 20% of the initial level.

As a consequence of the inactivation of MSX of glutamine synthetase, the glutamine level in ammonia-producing filaments decreased rapidly with time and became practically negligible 9 h after the addition of the inhibitor. This behavior contrasted with that of untreated filaments, which, under the same conditions, maintained their initial glutamine level. The level of glutamate in MSX-treated *Anabaena* sp. strain ATCC 33047 filaments also decreased with time but did so much more slowly than that of glutamine, its value after 36 h being 75% of that in untreated filaments (Fig. 3). This amino acid analysis indicates that the *Anabaena* sp. strain ATCC 33047 filaments that were subjected to treatment with MSX, in addition to suffering from general nitrogen starvation, became specifically 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 deficiencies and thus prolong the ammonia production period, two different attempts were made. One of them was the addition to the cell suspension of glutamine (0.2 mM) simultaneously with the normal readdition of MSX 40 h after the experiment had been initiated. The data in Fig. 4 show that this glutamine treatment resulted in sustained ammonia production at a constant rate for an extra period of at least 32 h, a phenomenon that did not occur in a control that received the normal

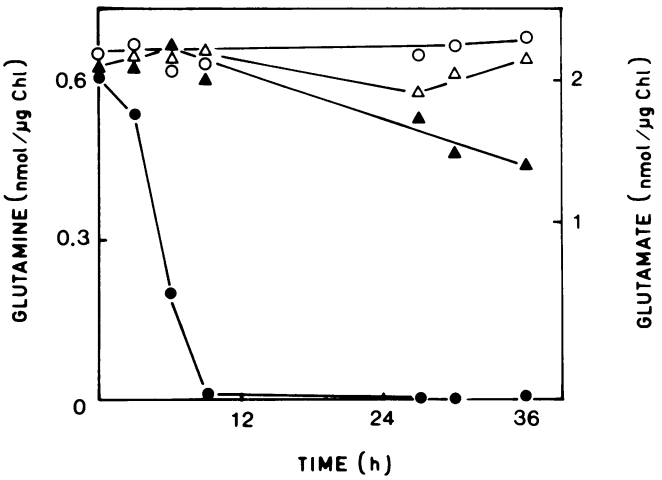

FIG. 3. Cellular levels of glutamine and glutamate in MSX-treated and untreated *Anabaena* sp. strain ATCC 33047 filaments. An air-grown suspension of filaments containing 9 μg of Chl per ml was transferred either to a medium free of combined nitrogen source (open symbols) or to the same medium supplemented with 35 μM MSX (closed symbols). Samples were withdrawn at the times indicated, and the cellular content of glutamine (circles) and glutamate (triangles) was estimated. Other conditions were the same as those described in the legend to Fig. 2.

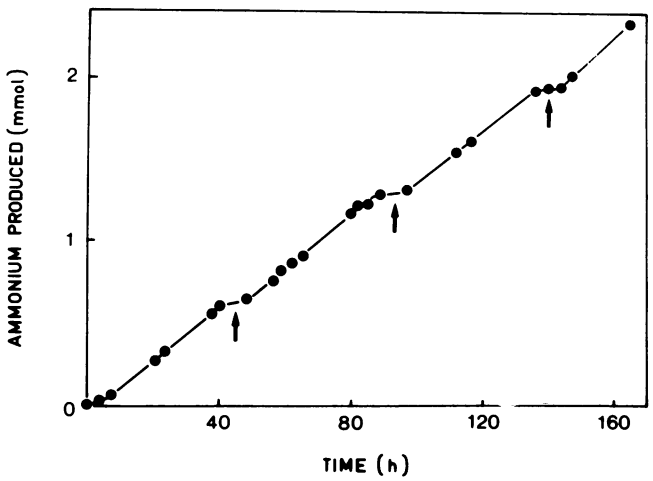

FIG. 5. Effect of the recurrent addition and removal of MSX on the photoproduction of ammonia by *Anabaena* sp. strain ATCC 33047 filaments. A filament suspension (150 ml) with a density of 9.8 μg of Chl per ml was maintained for 40 h in the standard culture medium supplemented with 35 μM MSX. After this time cells were harvested and washed with fresh medium lacking MSX. After 8 h in the absence of the inhibitor, and once the cell load had been corrected to 10 ± 0.2 μg of Chl per ml, MSX (35 μM final concentration) was readded. Successive periods of ammonia production (ca. 40 h) and cell recovery (ca. 8 h) (arrows) were alternated as indicated.

addition of MSX (35 μM) but did not receive glutamine. The glutamine treatment did not result in cell growth (measured as an increase in Chl) or in any increase in glutamine synthetase activity but allowed the maintenance of the high nitrogenase activity level. Glutamate (0.2 mM) could not replace glutamine in sustaining ammonia production, a result which suggests that cessation of the process is related to glutamine deficiency.

The second approach to extend the ammonia production period was to allow the filaments to recover from their deficiencies by themselves. This 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 ammonia production for at least 7 days. The long period in the presence of MSX (around 40 h) resulted in net ammonia production, whereas the shorter period in its absence (around 8 h) did not. During the recovery periods, glutamine synthetase

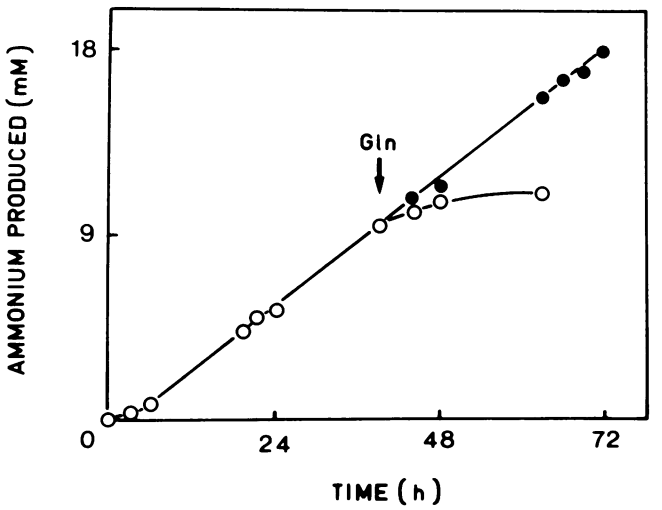

FIG. 4. Effect of glutamine addition on the prolongation of the ammonia production period by MSX-treated *Anabaena* sp. strain ATCC 33047 filaments. Conditions were the same as those described in the legend to Fig. 2, except that glutamine (0.2 mM) was added after 40 h (arrow) to one-half of the suspension (●), with the other half used as a control without glutamine addition (○).

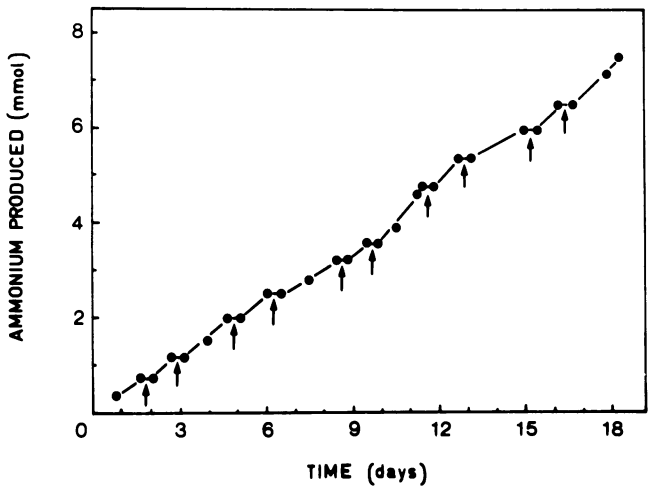

FIG. 6. Effect of the alternance of MSX and PT on the photoproduction of ammonia by *Anabaena* sp. strain ATCC 33047 filaments. Conditions were the same as those described in the legend to Fig. 5, except that the successive periods of ammonia production proceeded alternately in the presence of MSX (ca. 40 h) and of PT (ca. 20 h), with recovery periods (ca. 8 h) in the absence of any inhibitor between them (arrows).

activity increased from 0 mU to 140 to 280 mU/mg of protein (7 to 14% of the level in normal cells), allowing glutamine synthesis, increase of the phycocyanin level (from 20 to 25% to 50 to 80% of the initial one), and slight growth (from 9.8 to about 10.5 μg of Chl per ml in 8 h). Surprisingly, after 7 days of ammonia production, resistance to MSX appeared. This phenomenon was probably due to an adaptation of the filaments to MSX and made it necessary to increase the MSX concentration up to 100 to 150 μM to again achieve complete inactivation of cellular glutamine synthetase.

The effect on the duration of the ammonia production process of alternating treatments with two different glutamine synthetase inhibitors, namely, MSX and PT, has also been tested. PT is another glutamate analog which effectively inhibits glutamine synthetase and allows ammonia production at high rates by *Anabaena* sp. strain ATCC 33047 (6). The treatment including the use of both MSX and PT consisted of a first period in the presence of MSX (ca. 40 h), followed after a short interval of recovery (ca. 8 h) by a second period (around 20 h) in the presence of PT. Figure 6 shows that this sequential treatment with MSX and PT permitted continuous ammonia production for longer than 2 weeks, provided that recovery periods in the absence of either inhibitor were also alternated throughout.

The process of biological ammonia photoproduction from N_2 by adequately treated living cyanobacterial cells represents the net gain of a valuable compound from abundant and inexpensive substrates, namely, air and water, at the expense of only sunlight as the source of energy. Since the system operates, once it has been stabilized as described herein, at a considerable rate (see reference 4 for a comparison of ammonium production rates by different biological systems) and with a reasonable energy conversion efficiency (around 2%), it could prove eventually valuable for practical purposes.

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