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Ammonia Exchange and Photorespiration in *Chlamydomonas*

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ABSTRACT

Two hours after the addition of L-methionine-DL-sulfoximine to the cell suspension, glutamine synthetase activity was inhibited by more than 90% in air-grown *Chlamydomonas reinhardii*. Cells continued to take up NH_3 from the medium provided that the concentration of dissolved CO_2 was high (equilibrated with 4% CO_2 in air). This NH_3 uptake, about 30% of the control, is discussed in terms of glutamate dehydrogenase activity. Without CO_2, or with a low CO_2 level, a NH_3 excretion was observed, the rate of which depended on the actual concentration of the dissolved CO_2. Experiments with $^{15}NH_3$ demonstrated that no NH_3 uptake was masked by this excretion and inversely that no excretion occurred during the uptake in the conditions where it took place. Furthermore, the NH_3 excretion observed in the absence of CO_2 increased when O_2 concentration rose to 15% and was inhibited when 10 millimolar isonicotinic acid hydrazide was supplied to the algal suspension. Thus, NH_3 excretion in the presence of L-methionine-DL-sulfoximine seems to be related to a photorespiratory process inasmuch as it presents the same properties with regard to the O_2 and the isonicotinic acid hydrazide effects. These results favor the hypothesis that NH_3 produced in the medium originates from the glycine to serine reaction. On the other hand, partial inhibition (50%) of photosynthesis by L-methionine-DL-sulfoximine was attributed to uncoupling between electron transfer and photophosphorylation due to NH_3 accumulation into the cell.

L-MSO[1] is known to be an irreversible inhibitor of GS (16) without effect on GDH (2, 13). Because of these properties, the use of this compound allowed a distinction between the two pathways capable of NH_3 assimilation (9). Thus, using L-MSO, Stewart and Rhodes on *Lemna minor* (20) and Cullimore and Sims on *Chlamydomonas* (5) concluded that NH_3 assimilation occurred exclusively via the GS-glutamate synthase pathway; GDH would be devoid of any anabolic role.

Furthermore, when a green plant is treated with L-MSO in the presence of air, NH_3 production takes place: this phenomenon has been observed both in higher plants like tobacco (18) or spinach (11) and in the green alga *Chlamydomonas* (4). This NH_3 production was shown to be light-dependent, abolished by DCMU, and absent when algae were bubbled with air supplemented with 3% CO_2 (4). From this, it was concluded that NH_3 originated from photorespiration. This conclusion agrees with the results of Keys *et al.* (8) who showed that reassimilation of NH_3 produced during the glycine to serine conversion was catalyzed by GS. However, Platt and Anthon (11) did not observe a decrease in the rate of NH_3 accumulation in spinach leaves under low O_2 concentration (2.2%), although in such conditions photorespiration is known to be largely decreased (3). In addition, they showed that L-MSO induced a rapid and complete inhibition of photosynthesis.

In this paper, we study the effect of CO_2 concentration on NH_3 exchange and photosynthesis in air-grown *Chlamydomonas* treated with L-MSO. Furthermore, we report on the effects of O_2 concentration and INH, an inhibitor of photorespiration, on NH_3 excretion which occurs in the presence of L-MSO. We discuss our results in relation to the NH_3-assimilating pathway and photorespiratory NH_3-recycling in algae.

MATERIALS AND METHODS

Plant Material. *Chlamydomonas reinhardii* (wild type 137 c) was kindly supplied by Dr. Pierre Bennoun. It was grown axenically and phototrophically under continuous fluorescent illumination with a quantum flux density of 40 to 50 $\mu E \cdot m^{-2} \cdot s^{-1}$ (400–700 nm). An air stream was bubbled through the continuously stirred culture at a rate of about 20 $L \cdot h^{-1}$. The culture medium contained 7.50 mM NH_4Cl, 0.45 mM $CaCl_2$, 0.04 mM $MgSO_4$, 4.12 mM K_2HPO_4, 2.67 mM KH_2PO_4, and 0.023 mM Fe·EDTA. One ml of trace element solution (7) was added to each L of medium. The pH was adjusted to 6.0.

Experimental Procedure. During exponential growth, cells were harvested by centrifugation and resuspended in a culture medium lacking NH_4Cl. NH_3 concentration was kept between 2 and 3 mg $NH_3 \cdot L^{-1}$ and Chl concentration between 3 and 6 μg Chl $\cdot ml^{-1}$. Cell suspension (200 ml) was transferred into a test flask and bubbled, depending on the experiment, either with air, CO_2-free air, 4% CO_2 in air, or gas mixtures containing different O_2 concentrations, at a rate of 10 to 20 $L \cdot h^{-1}$ when not specified otherwise. Illumination was provided by incandescent light with a quantum flux density of 1000 to 1100 $\mu E \cdot m^{-2} \cdot s^{-1}$ (400–700 nm). The temperature was 25°C during the assay. L-MSO was added in a final concentration of 0.5 mM at zero time. During the time experiment, we observed no significant change in Chl concentration and pH.

Ammonia Determination in the Medium. At timed intervals, 1.5-ml aliquots were taken. Cells were separated from the medium by filtration and NH_3 was determined colorimetrically as described by Weatherburn (23). The method was calibrated in the presence or in the absence of 0.5 mM L-MSO. When 10 mM INH was present, no coloration occurred. In this case, NH_3 was determined by isotopic dilution using ^{15}N. A known quantity of $^{15}NH_4Cl$ was added to the sample and final ^{15}N content was measured as described in the following section.

^{15}N Measurements. NH_3 was expelled by bringing the sample to pH 11 using 0.1 N NaOH, and was trapped on a glass fiber filter impregnated with 0.1 N H_2SO_4. After about 16 h, the filter was placed in one limb of a Rittenberg apparatus and 1 ml of 1 M LiOBr was introduced in the other limb. ^{15}N content of NH_3 was determined after its conversion into N_2 (15).

Photosynthesis Measurement. Algae (about 50 μg Chl) were filtered on a Metricel membrane filter GA 4 (0.8 $\mu m \times 42$ mm) to

[1] Abbreviations: L-MSO, L-methionine-DL-sulfoximine; GS, glutamine synthetase; GDH, glutamate dehydrogenase; INH, isonicotinic acid hydrazide.

form an artificial leaf. This filter was placed in a photosynthesis chamber containing 4 ml culture medium in order to avoid desiccation of the cells. The chamber was continuously flushed with air. The flow rate was monitored and regulated with a mass flowmeter (Air Liquide, RDS 101). CO_2 exchange was measured with an IR gas analyzer (Hartmann & Braun, URAS 2). The quantum flux density was 900 $\mu E\ m^{-2} \cdot s^{-1}$ (400–700 nm). Under these conditions, photosynthetic capacity was light-saturated and showed no degradation for 3 h. In some experiments, photosynthesis was directly measured in the test tube.

GS Activity Determination. After filtration, cells were permeabilized using liquid N_2 in a two-cycle freeze-thaw treatment. To prevent a penetration of external L-MSO into the permeabilized cells, cells were washed on the filter with 10 ml distilled H_2O prior to permeabilization. Synthetase activity was determined as described by Rhodes *et al.* (14).

Chl Content. After extraction with 90% methanol, Chl content was determined by the method described in Reference 17.

RESULTS

Ammonia Exchange under Different CO_2 Concentrations in L-MSO-Treated Cells. In the presence of CO_2-free air, algae started to excrete NH_3 about 100 min after the addition of L-MSO (Fig. 1A). The excretion rate was 56 ± 3 nmol $NH_3 \cdot mg^{-1}$ Chl $\cdot min^{-1}$ (mean ± SD, six determinations). In air, NH_3 excretion started 150 min after the addition of the inhibitor. The excretion rate was very variable from one experiment to the other and was found to be 47 ± 30 nmol $NH_3 \cdot mg^{-1}$ Chl $\cdot min^{-1}$ (mean ± SD, 22 determinations). In the presence of 4% CO_2 in air, algae continued to take up NH_3 after the addition of L-MSO at a rate of 124 ± 17 nmol $NH_3 \cdot mg^{-1}$ Chl $\cdot min^{-1}$ (mean ± SD, six determinations). In the control experiments (Fig. 1B), the NH_3 uptake was about 45 nmol $NH_3 \cdot mg^{-1}$ Chl $\cdot min^{-1}$ under CO_2-free air and increased up to about 200 and 390 nmol $NH_3 \cdot mg^{-1}$ Chl $\cdot min^{-1}$ under air and 4% CO_2 in air, respectively. In every case, we observed a virtually complete inhibition of GS activity 120 min after the addition of the inhibitor (Table I). This L-MSO-insensitive NH_3 uptake occurring when algae were bubbled with air supplemented with CO_2 led us to assume that NH_3 excretion and NH_3 uptake could occur at the same time in L-MSO-treated cells.

In order to clear this point, we carried out an experiment using $^{15}NH_3$. Figure 2A shows that in air the $^{15}NH_3$ uptake was completely abolished about 150 min after the addition of L-MSO. NH_3 excretion consisted essentially of $^{14}NH_3$. From this, we concluded that there was no NH_3 uptake during the excretion. In 4% CO_2 in air, uptake occurred with the same constant rate for each isotopic species (Fig. 2B), thus proving that no excretion occurred during NH_3 uptake. Consequently, it appears that the CO_2 concentration affects NH_3 excretion in L-MSO-treated cells. The concentration of dissolved CO_2 is obtained through the equilibrium between the gas-liquid diffusion rate and photosynthetic CO_2 fixation. Thus, when the algal suspension was bubbled with air, the concentration of dissolved CO_2 was determined by the ratio between the air flow rate and the Chl content. To test the influence of the concentration

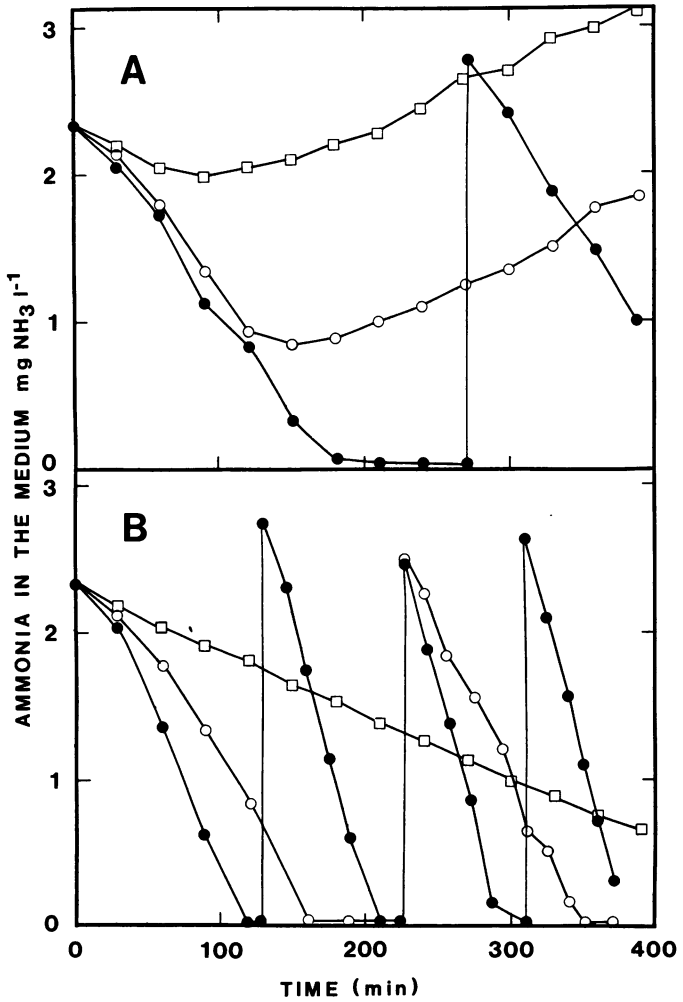

FIG. 1. A, NH_3 exchange in L-MSO-treated *Chlamydomonas* in different CO_2 concentrations in air, 5.8 μg Chl $\cdot ml^{-1}$. (□), CO_2-free air; (○), air; (●), 4% CO_2 in air. NH_4Cl was added at 270 min. B, Control without L-MSO. NH_4Cl was added at 125, 225, and 310 min.

Table I. *Effect of L-MSO on GS Activity under Different CO_2 Concentrations*

	GS Activity[a]		
	0 min	120 min	240 min
	nmol γ-glutamyl hydroxamate · mg^{-1} Chl · min^{-1}		
CO_2-free air	112	9	3
Air	87	3	2
Air + 4% CO_2	95	7	5

[a] Activity shown at times after L-MSO addition.

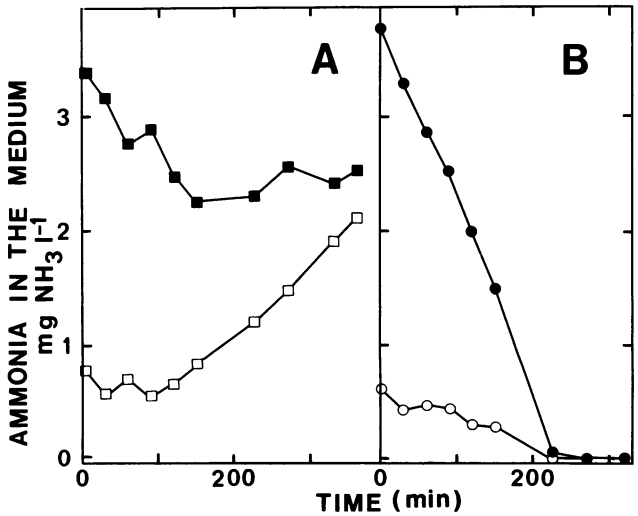

FIG. 2. Evolution of $^{14}NH_3$ and $^{15}NH_3$ species in L-MSO-treated *Chlamydomonas*. At zero time, L-MSO and $^{15}NH_4Cl$ were added to the algal suspension, 5.6 μg Chl $\cdot ml^{-1}$. A, air; (■), $^{15}NH_3$; (□), $^{14}NH_3$. B, 4% CO_2 in air; (●), $^{15}NH_3$; (○), $^{14}NH_3$.

of CO_2 on the NH_3 exchange in the presence of L-MSO when cells were bubbled with air, we performed the following experiment. Photosynthesis for a given Chl content was measured as a function of air flow rate (Fig. 3A). In this condition, photosynthesis was only dependent on dissolved CO_2 concentration. Therefore, we selected three flow rates corresponding to different concentrations of dissolved CO_2. After the addition of L-MSO, the NH_3 in the medium was measured. When cells were bubbled at a rate of 10 $L \cdot h^{-1}$, NH_3 excretion occurred at a rate of 40 nmol $NH_3 \cdot mg^{-1}$ $Chl \cdot min^{-1}$ (Fig. 3B). On the other hand, when the air flow rate was 45 $L \cdot h^{-1}$ or 80 $L \cdot h^{-1}$, we observed NH_3 uptake at a rate of 70 nmol $NH_3 \cdot mg^{-1}$ $Chl \cdot min^{-1}$ and 116 nmol $NH_3 \cdot mg^{-1}$ $Chl \cdot min^{-1}$, respectively. These results show that the NH_3 exchange in the presence of L-MSO is really dependent on the concentration of dissolved CO_2. When the concentration of dissolved CO_2 was low, NH_3 excretion took place; however, when the concentration of CO_2 dissolved was closer to the equilibrium with air, NH_3 uptake occurred.

Effect of O_2 Concentration on Ammonia Excretion in the Presence of L-MSO. To determine the influence of O_2 concentration on NH_3 excretion, we carried out two types of experiments. In the first one, we studied the effect of O_2 concentration in the absence of CO_2. Figure 4 shows that there was no NH_3 excretion under 0% O_2 in the gas phase. NH_3 excretion increased as a function of O_2 concentration up to 15% O_2. A maximum excretion rate of about 65 nmol $NH_3 \cdot mg^{-1}$ $Chl \cdot min^{-1}$ was obtained under O_2 concentration between 15 and 100% O_2.

In the second experiment, L-MSO-treated algae were bubbled with a gas mixture containing O_2 (1.5%), CO_2 (300 $\mu l \cdot l^{-1}$), balance N_2. NH_3 uptake decreased and stopped after about 350 min (Fig. 5). Moreover, the NH_3 excretion occurring in air stopped when the algae were bubbled with the same mixture containing low O_2 concentration. Gas flow rates and Chl content were controlled to avoid variations in the concentration of dissolved CO_2.

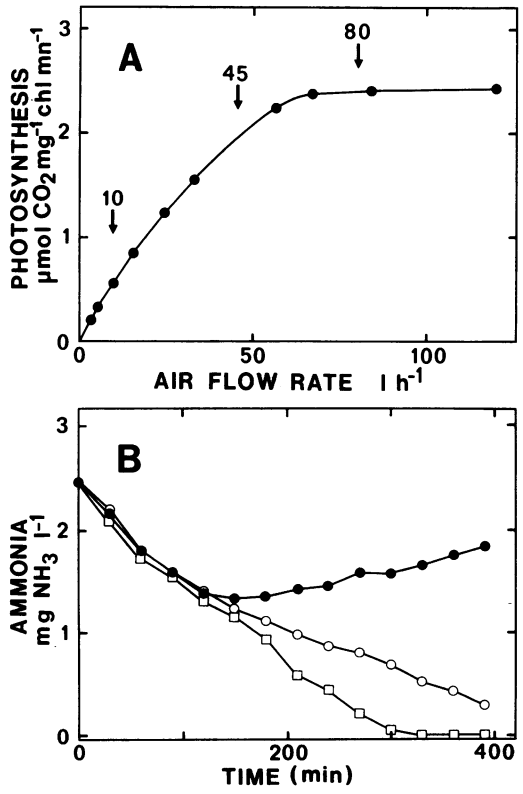

FIG. 3. A, Photosynthesis as a function of air flow rate. B, NH_3 exchange in L-MSO-treated *Chlamydomonas* bubbled with different air flow rates. After the addition of L-MSO, the algal suspension, 3.2 μg $Chl \cdot ml^{-1}$, was subdivided in three test flasks and bubbled with air at different flow rates. (□), 80 $L \cdot h^{-1}$; (○), 45 $L \cdot h^{-1}$; (●), 10 $L \cdot h^{-1}$.

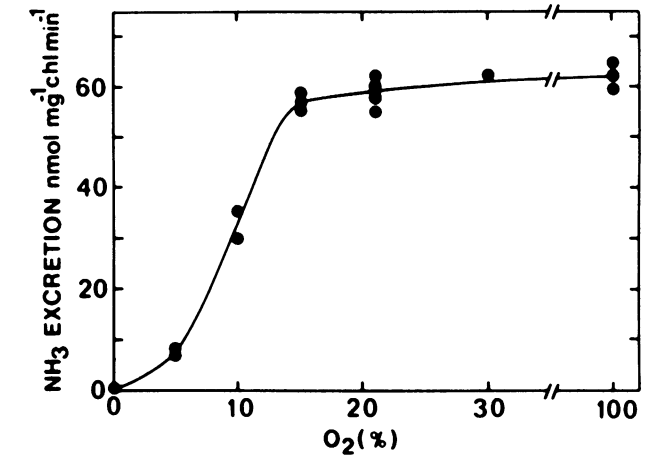

FIG. 4. Effect of O_2 concentration on NH_3 excretion in L-MSO-treated *Chlamydomonas*. Algae were bubbled with gas mixtures containing different O_2 concentrations, balance N_2. NH_3 excretion was determined between 3 and 6 h after the addition of L-MSO.

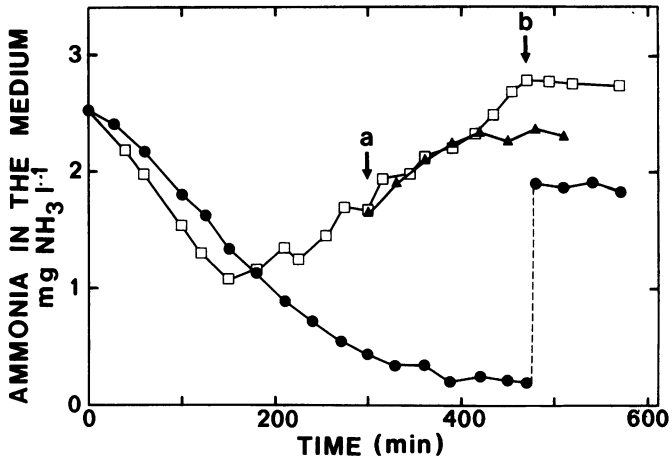

FIG. 5. Effect of INH and low O_2 concentration at CO_2 air level on NH_3 excretion in L-MSO-treated *Chlamydomonas*, 5.2 μg $Chl \cdot ml^{-1}$. (□), air; when indicated (a), the culture was subdivided in two parts and INH (10 mM) was added in one of them (▲); at time b, air was replaced in the other part by a gas mixture containing 1.5% O_2, 300 $\mu l \cdot L^{-1}$ CO_2, balance N_2. (●), 1.5% O_2, 300 $\mu l \cdot L^{-1}$ CO_2, balance N_2. NH_4Cl was added at 475 min.

Effect of INH on Ammonia Excretion in L-MSO-Treated Algae. INH is known to be an inhibitor of the photorespiratory pathway blocking the conversion of glycine to serine (10, 12). The addition of 10 mM INH stopped the NH_3 excretion observed in air in L-MSO-treated algae (Fig. 5). A complete inhibition of the excretion occurred about 10 min after the addition of INH.

Effects of L-MSO on Photosynthesis. In this experiment, we checked photosynthetic activity of L-MSO-treated algae bubbled with air and 4% CO_2 in air (Fig. 6). Measurements were performed with artificial leaves in the presence of air (see "Materials and Methods"). When algae were bubbled with air, the photosynthetic activity decreased and reached an equilibrium level corresponding to a 50% inhibition of the initial rate. Inhibition of photosynthesis and NH_3 excretion started approximately at the same time. On the other hand, when cells were bubbled with 4% CO_2 in air, NH_3 uptake occurred, and we observed no significant inhibition of photosynthesis.

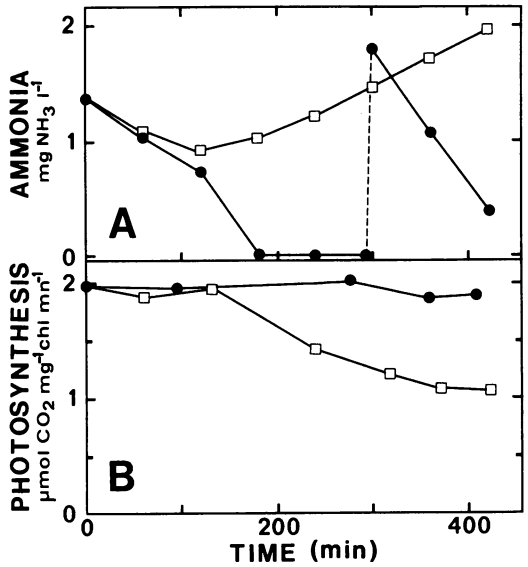

FIG. 6. A, NH_3 exchanges in L-MSO-treated *Chlamydomonas*, 5.6 µg Chl·ml^{-1}. (□), Air; (●), 4% CO_2 in air. NH_4Cl was added at 300 min. B, Photosynthesis measured with artificial leaves using algae treated as in A.

Ammonia Exchange under Air in the Absence of L-MSO. In this experiment, we attempted to determine whether NH_3 excretion observed in the presence of L-MSO also occurred in untreated cells. $^{15}NH_4Cl$ was added to the algal suspension and the evolution of $^{14}NH_3$ and $^{15}NH_3$ was followed under the condition where the algal suspension was bubbled with air. Under such conditions, L-MSO induced $^{14}NH_3$ excretion. Each isotopic species disappeared with the same constant rate (data not shown), leading to the conclusion that no $^{14}NH_3$ excretion occurred, in agreement with earlier studies (4).

DISCUSSION

Our results show that, under certain conditions, the assimilation of NH_3 by *Chlamydomonas* can occur via another way than the GS-glutamate synthase pathway. When GS activity was virtually completely abolished by L-MSO (Table I), a CO_2-dependent NH_3 uptake was observed (Fig. 1). At present, it is generally accepted that NH_3 assimilation by higher plants occurs exclusively via the GS-glutamate synthase pathway (9). However, it was suggested that GDH could play an anabolic role in green algae, inasmuch as these algae contain a GDH with a low K_m for NH_3 (9). Our experiments using $^{15}NH_3$ (Fig. 2) show that under conditions where CO_2 limited photosynthesis, GS deactivation by L-MSO was followed by an abolition of NH_3 uptake. On the other hand, when photosynthesis was CO_2-saturated, a condition which was obtained when algae were bubbled with 4% CO_2 in air and which was approximated when algae were bubbled with a sufficiently strong air stream, GDH might have been responsible for this L-MSO-insensitive NH_3 uptake. If we assume that the NH_3 uptake observed in the presence of L-MSO is a measure of GDH contribution to NH_3 assimilation, GDH could cause about 30% of the total assimilation when photosynthesis is saturated with CO_2. The two pathways capable of NH_3 assimilation, the glutamate synthase cycle and the GDH pathway, have different energy requirements: the first way costs 1 ATP/NH_3 assimilated more than the second one. Thus, using the phosphorylating power to fix CO_2, algae will probably use the more economical pathway to fix NH_3. However, in order to obtain information on the possible anabolic role of GDH, it will be necessary to follow up ^{15}N incorporation into the first labeled amino acids under conditions where L-MSO does not completely inhibit NH_3 uptake.

Under some conditions, following GS deactivation, L-MSO induces an excretion of NH_3. Earlier studies showed that this excretion was light-dependent and absent in the presence of air supplemented with CO_2 (4). For these reasons, it was concluded that NH_3 originated from the photorespiratory pathway, which is known to produce NH_3 during the mitochondrial conversion of glycine to serine (8).

The NH_3 uptake observed in L-MSO-treated algae bubbled with 4% CO_2 in air might result from an excretion superimposed on an increased uptake. The use of $^{15}NH_3$ showed that no $^{14}NH_3$ excretion occurred under these conditions (Fig. 2B). However, it appears possible that $^{14}NH_3$ was produced and refixed inside the cell with no $^{14}NH_3$ excreted. This hypothesis seems to be substantiated by our experiment performed in the absence of L-MSO. Indeed, if we assume that the $^{14}NH_3$ excreted is produced during the glycine to serine conversion, NH_3 production is not induced by L-MSO. However, we did not observe any significant $^{14}NH_3$ release in the absence of L-MSO. This result can be explained by a rapid reassimilation of the $^{14}NH_3$ produced. Consequently, the transition between a NH_3 excretion and an uptake, observed when the concentration of dissolved CO_2 increased (Fig. 3), could result from the efficiency of such a NH_3-recycling. Therefore, the variability observed in the excretion rates when algae were bubbled with air was probably the consequence of this CO_2-sensitive NH_3-recycling, because Chl and air flow rates were not closely controlled during these experiments.

The effects of O_2 concentration and INH on the NH_3 excretion occurring in L-MSO-treated algae indicate that NH_3 excretion and photorespiration are closely linked. Under low O_2 concentration, at the CO_2 air level, conditions where photorespiration is largely decreased (3), no NH_3 excretion occurred (Fig. 5). Platt and Anthon (11) did not observe any significant effect of a decrease in O_2 concentration on NH_3 accumulation rates when spinach leaves were supplied with L-MSO. NH_3 production in the presence of L-MSO could result from different mechanisms in algae and in higher plants. It is generally accepted that dark respiration in leaves saturates at about 2% O_2, while photorespiration is not yet saturated at 100% O_2 (3). Our results show that NH_3 excretion in the absence of CO_2 was half-saturated when the O_2 concentration in the gas phase was about 10% O_2. This suggests that a photorespiratory process, rather than a respiratory process, is involved in the metabolic pathway leading to the excretion of NH_3. Furthermore, we observed that INH, an inhibitor of the glycine to serine conversion both in algae (12) and in higher plants (10), stopped NH_3 excretion (Fig. 5). Consequently, it seems probable that the reaction 2 glycine → serine + CO_2 + NH_3 belonging to the photorespiratory pathway would be responsible for NH_3 excretion in L-MSO-treated *Chlamydomonas*.

It seems likely that the major process for CO_2 loss during photorespiration in higher plants occurs during this reaction (19, 22). If it is the same in algae, the activity of photorespiration could be determined in measuring NH_3 excretion in the presence of L-MSO. Thus, photorespiration would not occur in air-grown *Chlamydomonas* when the concentration of dissolved CO_2 is close to the equilibrium with air. This is suggested by the fact that the uptakes observed when L-MSO-treated algae were bubbled either with air at a sufficient flow rate or with 4% CO_2 in air were not significantly different; if NH_3 really originates from photorespiration, no NH_3 should be produced under 4% CO_2 in air as under saturating air flow rates. Photorespiration would only occur at low concentrations of dissolved CO_2. This is in agreement with recent studies showing that an O_2-sensitive CO_2 release occurred at subsaturating dissolved inorganic carbon levels (1). The formation of glycolate is generally assumed to be the consequence of the ribulose-1,5-bisphosphate oxygenase activity (22). In higher plants, the *in vitro* K_m (O_2) of this activity has been reported to be 33% (6), and the K_m (O_2) for glycolate formation by intact

chloroplasts to be 23% (21). However, we observed in *Chlamydomonas* that saturation of NH_3 excretion occurred when the O_2 concentration was higher than 15% O_2 and half-saturation for 10% O_2 (Fig. 4). This deviation can be due either to the fact that the K_m (O_2) values for algae and higher plants are quite different or, if not, to the fact that one step of the photorespiratory pathway limits the utilization of glycolate and therefore causes its excretion by algae.

Measurements of NH_3 excretion rates were obtained when the photosynthetic capacity was partially inhibited (Fig. 6). Photorespiration and photosynthesis are very closely related, so we attempted to find out how L-MSO inhibited photosynthesis. It was suggested (11) that the inhibition of photosynthesis by L-MSO could result from either a direct action on CO_2 uptake or an uncoupling of phosphorylation following NH_3 accumulation. Our results clearly show that the effect of L-MSO on photosynthesis is indirect because it occurred only when algae excreted NH_3 (Fig. 6). Thus, the uncoupling effect is probably responsible for the inhibition observed.

In conclusion, L-MSO appears to be a useful tool to study the NH_3 production associated with photorespiration *in vivo*. However, in our experimental conditions, photosynthesis was partially inhibited, due to the uncoupling effect induced by NH_3 accumulation into the cell. By following directly in short time experiments the NH_3 production into the cell, the uncoupling effect should be overcome. We are presently performing such experiments.

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