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Amino Acid Metabolism of *Lemna minor* L.[1]

I. RESPONSES TO METHIONINE SULFOXIMINE

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ABSTRACT

When *Lemna minor* L. is supplied with the potent inhibitor of glutamine synthetase, methionine sulfoximine, rapid changes in free amino acid levels occur. Glutamine, glutamate, asparagine, aspartate, alanine, and serine levels decline concomitantly with ammonia accumulation. However, not all free amino acid pools deplete in response to this inhibitor. Several free amino acids including proline, valine, leucine, isoleucine, threonine, lysine, phenylalanine, tyrosine, histidine, and methionine exhibit severalfold accumulations within 24 hours of methionine sulfoximine treatment. To investigate whether these latter amino acid accumulations result from *de novo* synthesis via a methionine sulfoximine insensitive pathway of ammonia assimilation (*e.g.* glutamate dehydrogenase) or from protein turnover, fronds of *Lemna minor* were prelabeled with $[^{15}N]H_4^+$ prior to supplying the inhibitor. Analyses of the ^{15}N abundance of free amino acids suggest that protein turnover is the major source of these methionine sulfoximine induced amino acid accumulations. Thus, the pools of valine, leucine, isoleucine, proline, and threonine accumulated in response to the inhibitor in the presence of $[^{15}N]H_4^+$, are ^{14}N enriched and are not apparently derived from ^{15}N-labeled precursors. To account for the selective accumulation of amino acids, such as valine, leucine, isoleucine, proline, and threonine, it is necessary to envisage that these free amino acids are relatively poorly catabolized *in vivo*. The amino acids which deplete in response to methionine sulfoximine (*i.e.* glutamate, glutamine, alanine, aspartate, asparagine, and serine) are all presumably rapidly catabolized to ammonia, either in the photorespiratory pathway or by alternative routes.

It is now well established that GS[2] occupies a central position in plant N metabolism (12, 13). The GS-GOGAT cycle is thought to be responsible for the assimilation of most, if not all, of the ammonia derived from nitrate reduction and photorespiration (2–4, 10, 18, 19, 21, 23, 25). Studies with the potent inhibitor of GS, MSO, appear to rule out any major contribution of GDH to ammonia assimilation (1, 3, 8, 19, 21, 23). However, recent investigations with isolated plant mitochondria suggest that a small fraction of the ammonia generated from glycine decarboxylation can be directly reassimilated into glutamic acid via a mitochondrial GDH (26). The quantitative significance of this latter pathway *in vivo* still remains obscure.

In the course of investigations of the metabolic responses of *Lemna minor* to MSO, we have observed rapid accumulations of certain amino acids. This has prompted us to reevaluate whether or not an MSO insensitive pathway of ammonia assimilation (*e.g.* GDH) operates in the synthesis of certain amino acids when the GS-GOGAT pathway is blocked. We have reasoned that if amino acids such as Pro, Val, Leu, and Ile which accumulate in *L. minor* in response to MSO, are synthesized *de novo* via GDH, then these amino acids should be heavily labeled with ^{15}N when the plants are supplied with $[^{15}N]H_4^+$. In contrast, if these amino acids are derived from protein turnover (4–6), then these free amino acid pools should exhibit isotope dilution from ^{14}N-amino acid residues released from protein which was synthesized prior to the supply of $[^{15}N]H_4^+$. The results presented in this paper offer strong evidence in favor of protein turnover as the major source of the amino acids accumulated in response to MSO and fail to reveal any strong evidence in favor of the operation of a GDH pathway of ammonia assimilation. The ^{15}N-labeling data and amino acid pool size changes associated with MSO treatment provide insights as to the differential rates of turnover of specific amino acids and their accessibility to catabolism *in vivo*.

MATERIALS AND METHODS

Organism and Growth Conditions. *Lemna minor* L. was grown as described previously (16) on medium containing 5 mM KNO_3 as sole N source (22). Flasks (250 ml volume) containing 100 ml fresh medium were inoculated with 0.3 to 0.4 gfw of plants 48 h before administering MSO and/or $[^{15}N]H_4^+$. D,L-Methionine sulfoximine (MSO) was obtained from Sigma and $[^{15}N]H_4Cl$ (99% ^{15}N) from MSD Isotopes (St. Louis, MO). Solid $CaCO_3$ (100 mg/flask) was added to treatments receiving 5 mM $[^{15}N]H_4Cl$ in order to prevent medium acidification due to ammonia assimilation. In the experiment of Table I, 5 mM KNO_3 grown plants were supplied with 0.1 mM MSO alone.

Isolation of Soluble Nitrogen Pools. Samples of 0.3 to 0.4 gfw of *Lemna* fronds were harvested at various times following addition of either 0.1 mM MSO, 5 mM $[^{15}N]H_4Cl$, or both, to the medium (see "Results" for details). Fronds were collected in a tea strainer, washed with 50 ml distilled H_2O, blotted dry with tissue paper, weighed, and immediately extracted in 10 ml methanol. The methanol extracts were phase separated by addition of 5 ml chloroform and 5 ml distilled H_2O, the upper aqueous phase was rotary evaporated to dryness, redissolved in 2 ml H_2O, and assayed for ammonia as described previously (16). The aqueous extracts were applied to 2 cm × 1 cm columns of Dowex 50-H^+ and amino acids eluted with 6 ml 6 M NH_4OH after washing with excess H_2O (10 ml). The amino acid fractions were rotary evaporated to dryness, redissolved in 2 ml H_2O, and applied to 2 cm × 1 cm columns of Dowex 1-acetate equilibrated

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[2] Abbreviations: GS, glutamine synthetase; GOGAT, glutamate synthase; GDH, glutamate dehydrogenase; gfw, gram fresh weight; MSO, methionine sulfoximine; *N*-HFBI, *N (O,S)*-heptafluorobutyryl isobutyl.

with water. Neutral and basic amino acids were eluted with 6 ml H_2O and the acidic amino acids (glutamate and aspartate) with 6 ml 2 M acetic acid. Both fractions were rotary evaporated to dryness and redissolved in 0.4 ml 60% methanol and the amino acids derivatized to their *N*-HFBI derivatives as described previously (16). In the experiment of Table I, the neutral plus basic and acidic amino acids were not separated. The derivatives were redissolved in 100 μl ethyl acetate:acetic anhydride (1:1 v/v) and 1 μl aliquots analyzed by GLC as described previously (16) except that the column used was a 30 m × 0.22 mm fused silica DB5 capillary column (J and W Scientific, Rancho Cordova, CA), the split ratio at the injector port was 20:1, injector temperature was 250°C, flame ionization detector temperature was 280°C, and the oven temperature program was 90°C for 4 min to 260°C at 6°C/min with helium as carrier gas at a linear flow velocity of 40 cm/s. An internal standard was not used for the present applications. Amino acids were quantitated by an external standard method (15).

GC-MS Analysis of Amino Acid Derivatives. The amino acid derivatives were diluted to 500 μl with ethyl acetate:acetic anhydride (1:1 v/v) and 2 μl aliquots analyzed by GC-MS essentially as described previously (16) but using the following modifications. The GC-MS system used was a model HP5996 (Hewlett-Packard, Palo Alto, CA) equipped with a HP9876A graphic printer and HP1000 computer system. All analyses were performed on a 30 m × 0.22 mm fused silica DB5 capillary column with helium carrier gas at 40 cm/s linear velocity, a source temperature of 200°C, injector temperature of 250°C, and an interface temperature of 280°C. The oven temperature program was 100°C to 270°C at 10°C/min. Analyses were performed in electron impact mode scanning over the mass range 220 to 370 atomic mass units throughout the chromatograms. The ^{15}N abundances of various amino acids were determined by plotting extracted ion current profiles and calculating current ratios as described previously (16). The ions monitored were Ala (240:241), Gly (226:227), Val (268:269), Thr (253:254), Ser (239:240), Leu, and Ile (282:283), Pro (266:267), Asp or Asn-amino N (284:285), Glu or Gln-amino N (280:281 or 298:299), and Lys (280:281).

RESULTS

Response of Nitrate Grown Plants of *L. minor* to MSO. When nitrate grown plants of *L. minor* were supplied with 0.1 mM MSO rapid changes in the soluble nitrogen pools were observed (Table I). The levels of Ala, Gln plus Glu, and Ser declined rapidly within the first 3 h, as free NH_4^+ accumulated (Table I). Asn plus Asp levels declined less rapidly but were depleted 10- to 20-fold after 10 h of MSO treatment (Table I). In contrast, several amino acids including Pro, Val, Leu, Lys, Ile, Thr, Phe, Tyr, His, Arg, and Met exhibited 4- to 10-fold accumulations within 28 h of incubation with MSO (Table I). The rates of accumulation of these amino acids ranged from 5 to 15 nmol/h·gfw (Table I).

There appear to be two possible explanations for these amino acid accumulations elicited by MSO. First, in the light of the finding that mitochondrial GDH can partly reassimilate ammonia derived from glycine decarboxylation (25), it seems plausible that the increased availability of NH_4^+ associated with MSO treatment saturates a mitochondrial GDH yielding glutamate which is then selectively channeled into amino acids such as Pro, Val, Leu, and Ile. Second, it is possible that protein turnover continues in the absence of protein synthesis in the MSO treated plants, generating free amino acids of which only Glu, Gln, Ala, Asn, Asp, and Ser are selectively catabolized to NH_4^+ in the photorespiratory pathway, or by alternative routes. This would leave a free amino acid pool which is enriched in the relatively poorly catabolized amino acids Pro, Val, Leu, Ile, Lys, Phe, Tyr (etc.) derived from protein hydrolysis. The latter explanation seems more likely in view of a previous report of an association between protein turnover and photorespiration (revealed by MSO) in *Chlamydomonas* (4).

To distinguish between these two possibilities, experiments were performed in which nitrate grown plants of *L. minor* were

Table I. *Time Course of Changes in Free Amino Acid and Ammonia Pools of Nitrate Grown L. minor Treated with 0.1 mM MSO*

Amino Acid	Time of Incubation with MSO (h)					
	0	2	3	10	19	28
	nmol/gfw					
Ala	144.3	24.2	9.5	11.5	9.7	13.1
β-Ala	4.5	8.8	7.9	9.8	13.3	12.3
γ-Aminobutyrate	24.7	8.6	7.6	5.4	3.6	4.4
Arg	31.1	42.5	38.9	49.7	48.5	84.8
Asn + Asp	1856.6	1445.9	1210.2	115.4	55.1	55.8
Gln + Glu	895.4	189.9	133.2	53.8	47.2	55.6
Gly	52.6	45.2	36.9	37.2	34.9	36.1
His	11.7	23.4	30.8	68.4	132.1	151.6
Homoserine	6.6	10.1	11.3	12.3	10.9	10.0
Ile	27.9	28.8	38.5	54.7	82.4	111.9
Leu	25.2	28.8	33.7	52.2	66.8	85.8
Lys	9.2	21.5	25.9	75.3	129.2	204.2
Met	4.7	9.12	16.4	45.2	65.8	72.2
Orn	5.6	5.7	5.9	7.2	8.4	10.4
Phe	28.9	36.6	54.6	105.4	255.4	309.4
Pipecolic acid	8.7	15.5	22.9	20.3	31.8	33.7
Pro	31.1	57.2	69.4	104.8	154.0	182.3
Ser	543.3	166.9	48.2	53.2	42.3	55.8
Thr	94.4	112.1	106.5	224.7	366.9	451.6
Tyr	17.8	21.6	32.2	70.9	115.7	232.5
Val	49.3	52.9	72.6	154.8	243.5	292.6
Total	3873.6	2355.3	2013.2	1332.2	1917.5	2466.1
NH_4^+	605.2	3350.1	5060.7	8543.2	9508.4	9687.3

supplied with $[^{15}N]H_4^+$ (5 mM, 99% $[^{15}N]$) and then treated with 0.1 mM MSO either immediately or after 12 h $[^{15}N]H_4^+$ assimilation. If amino acids such as Pro, Val, Leu, Ile, and Thr are derived from protein turnover then the free pools of these amino acids accumulated in response to MSO should be primarily ^{14}N enriched (*i.e.* derived from relatively unlabeled amino acid residues of protein). In contrast if these amino acids accumulate as a result of *de novo* synthesis from NH_4^+ via an MSO insensitive pathway of Glu synthesis (*e.g.* GDH), then these amino acids should remain heavily labeled with ^{15}N.

$[^{15}N]H_4^+$ Assimilation in the Presence and Absence of MSO. *a) Glu, Gln, Asn and Asp.* The results of Figure 1, A to D, show the changes in free pools of Glu (Fig. 1A), Gln (Fig. 1B), Asp (Fig. 1C), and Asn (Fig. 1D) in response to the addition of 5 mM $[^{15}N]H_4^+$ at zero time and MSO added either at zero time or after 12 h. The controls received only $[^{15}N]H_4^+$. The increased availability of NH_4^+ saturates a derepressed level of GS in nitrate grown plants (17, 18) and stimulates rapid accumulation of glutamine from 250 nmol/gfw at zero time to 3500 nmol/gfw by 12 h (Fig. 1B). The accumulation of Gln is severely inhibited by MSO supplied at zero time (Fig. 1B). The increased Gln and Asp (Fig. 1C) levels in response to NH_4^+ addition, as substrates of Asn synthetase, lead to substantial Asn accumulation which is again blocked by MSO (Fig. 1D). MSO elicits precipitous declines in the levels of Glu, Gln, Asp, and Asn when supplied either at zero time or after 12 h (Fig. 1), but the rates of depletion are somewhat slower than those observed in the absence of an exogenous supply of NH_4^+ (*cf.* Table I). It is possible that MSO uptake is partly inhibited by 5 mM NH_4^+.

Previous investigations have shown that in NO_3^- grown plants

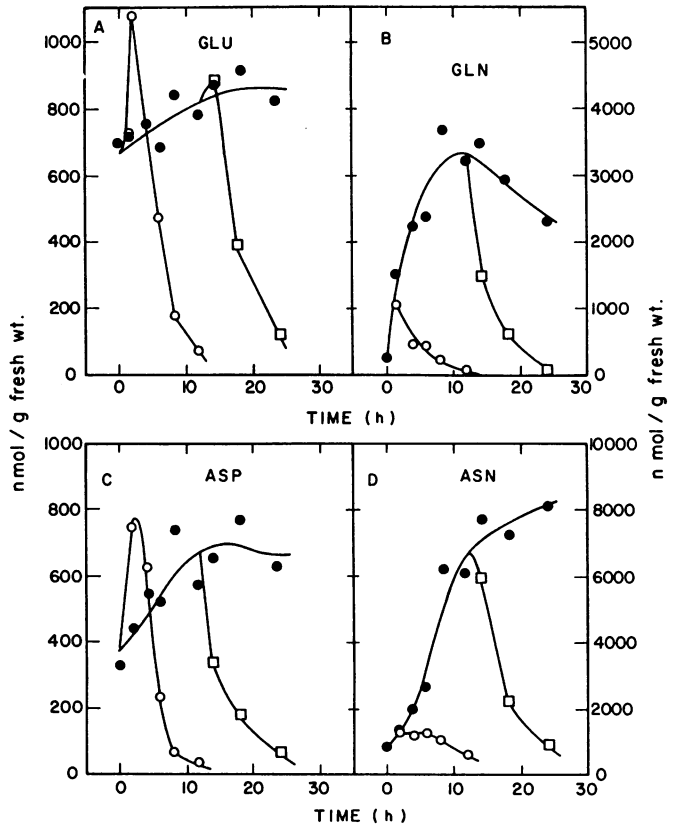

FIG. 1. Time courses of changes in the free amino acid pool sizes of Glu (A), Gln (B), Asp (C), and Asn (D) in *L. minor* supplied with 5 mM $[^{15}N]H_4Cl$ either alone at zero time (●), or together with 0.1 mM MSO at zero time (○), or supplied with 5 mM $[^{15}N]H_4Cl$ at zero time and then with 0.1 mM MSO at 12 h (□).

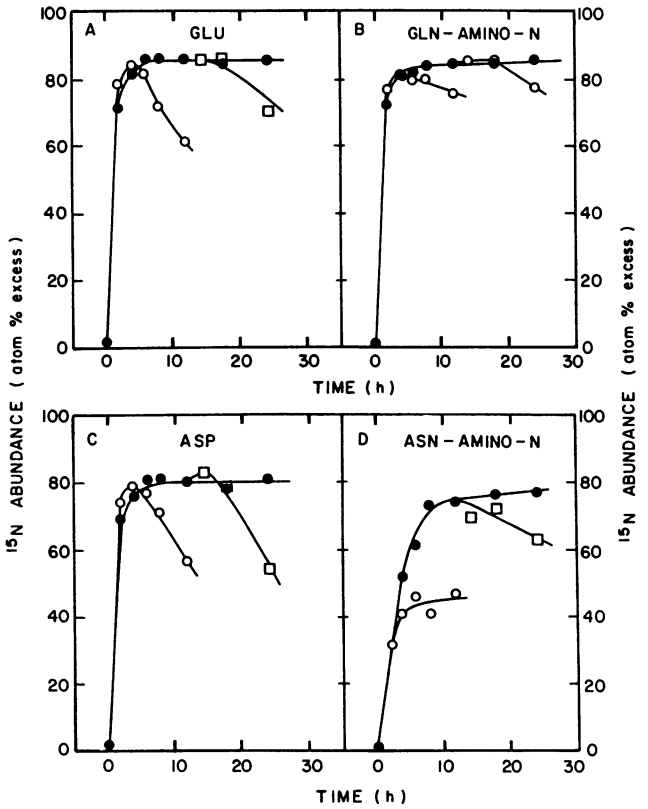

FIG. 2. Time courses of changes in the ^{15}N abundance of Glu (A), Gln (B), Asp (C), and Asn (D) in *L. minor* (see Fig. 1 legend for details of treatments).

of *L. minor*, MSO requires approximately 2 h to completely deactivate GS *in vivo* (23). Some $[^{15}N]H_4^+$ incorporation into amino acids via the GS-GOGAT cycle is therefore possible within the first 2 h, before GS is fully deactivated. Indeed the ^{15}N-labeling kinetics of Glu (Fig. 2A), Gln-amino N (Fig. 2B), Asp (Fig. 2C), and Asn-amino N (Fig. 2D) confirm that active assimilation of $[^{15}N]H_4^+$ via the GS-GOGAT cycle occurred within the first 2 to 4 h after simultaneous addition of $[^{15}N]H_4^+$ and MSO at zero time. No labeling of the amino-N moiety of Gln would have occurred if GS had been completely inhibited. Within the first 2 to 4 h of MSO treatment there appeared to be very little inhibition of ^{15}N incorporation into these amino acids, but beyond 4 h of MSO treatment the isotopic abundance of Glu (Fig. 2A), Gln-amino N (Fig. 2B), Asp (Fig. 2C), and Asn-amino N (Fig. 2D) began to decline as the pools of these amino acids depleted (Figs. 1A to 1D). In the case of Glu, Gln, Asp, and Asn, it is not possible to conclude that because a decrease in ^{15}N abundance is associated with MSO treatment, that this isotope dilution is due to protein turnover. An alternative explanation could be that for these amino acids there are stable, relatively metabolically inert, storage pools which do not become heavily labeled with ^{15}N and remain relatively uncatabolized in response to MSO. In other words, these labeling kinetics might equally be attributable to selective catabolism of heavily labeled metabolic pools. But such an explanation would not account for isotope dilution induced by MSO for an amino acid pool which accumulates rather than depletes in response to this inhibitor, as in the case of Pro, Val, Leu, and Ile.

b) Pro, Val, Leu, and Ile. After a 4 h lag, MSO elicited accumulations of Pro (Fig. 3A), Val (Fig. 3B), Leu (Fig. 3C), and Ile (Fig. 3D) at rates similar to those observed in the absence of an exogenous ammonium supply (*cf.* Table I). The corresponding ^{15}N-labeling kinetics for these amino acids are shown in Figure

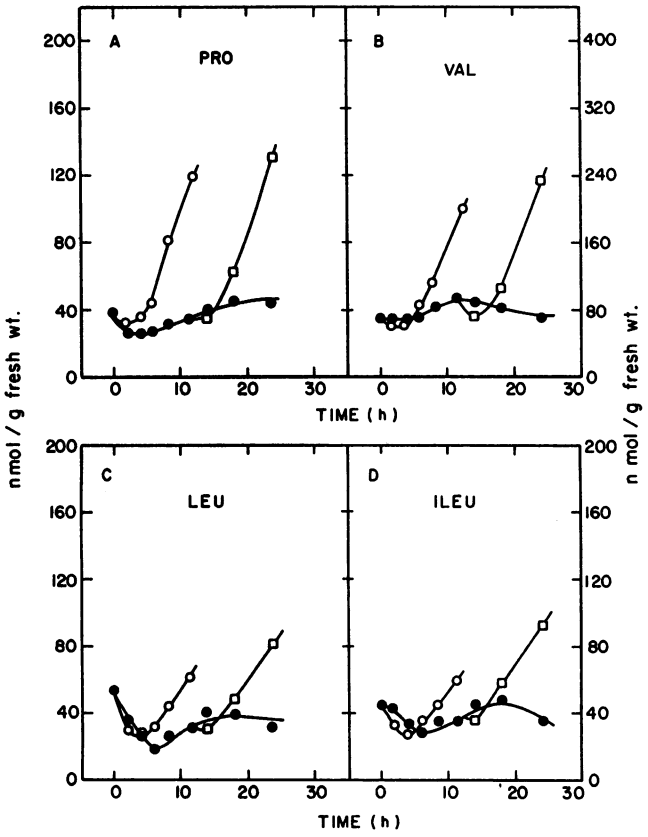

FIG. 3. Time courses of changes in the free amino acid pools of Pro (A), Val (B), Leu (C), and Ile (D) in *L. minor* (see Fig. 1 legend for details of treatments).

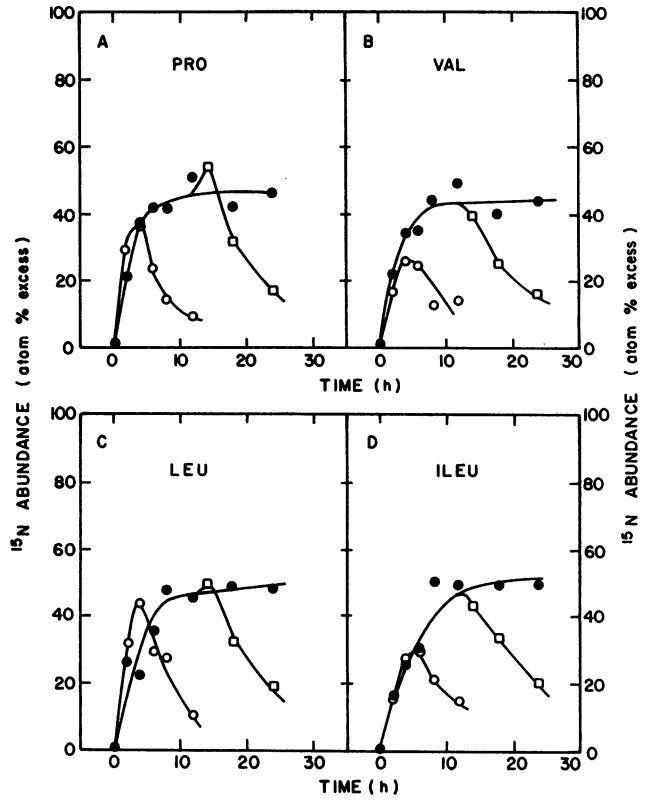

FIG. 4. Time courses of changes in the ^{15}N abundance of Pro (A), Val (B), Leu (C), and Ile (D) in *L minor* (see Fig. 1 legend for details of treatments).

4, A to D. All four amino acids exhibited similar patterns of pronounced isotope dilution induced by MSO (Fig. 4). These data provide clear evidence that the accumulations of Pro, Val, Leu, and Ile induced by MSO are not the result of *de novo* synthesis from $[^{15}N]H_4^+$. Rather these free amino acids must accumulate from ^{14}N-labeled precursors; most probably ^{14}N-amino acid residues of protein.

c) Ala, Gly, Ser, and Thr. Ala and Ser (Fig. 5, A and C, respectively) exhibited patterns of pool size increases in response to NH_4^+, and pool size decreases in response to MSO which were similar to those of Gln, Glu, Asn, and Asp (Fig. 1). MSO treatment, either at zero time or after 12 h, led to isotope dilution of Ala and Ser (Fig. 6, A and C, respectively). The corresponding data obtained for Gly (Figs. 5B and 6B) is somewhat difficult to interpret since in the photorespiratory N cycle, Glu, Ala, Asn, and Ser are all potential precursors of Gly (14, 24), and Ser is the product of glycine (10). One would expect Gly to remain at least as heavily labeled as its precursor and product, Ser, at all times. Gly was, however, somewhat less heavily labeled than serine in the MSO treatments applied at zero time (*cf.* Fig. 6, B and C). It is possible that a very small metabolic pool of Gly, serving as intermediate in the photorespiratory pathway and carrying N flux from the catabolism of Glu, Gln, Ala, Ser, Asp, and/or Asn to NH_4^+, is tightly compartmentalized in the mitochondria away from larger cytosolic, vacuolar, and/or chloroplastic Gly pools which label less heavily and which are subject to isotope dilution from sources such as protein turnover or glutathione catabolism (11). Thus the labeling kinetics of the bulk Gly pool may not necessarily reflect the true labeling kinetics of the mitochondrial Gly pool. For these reasons, the labeling kinetics of Gly are not necessarily inconsistent with Gly being a principal intermediate in the catabolism of Glu, Gln, Ala, Ser, Asp, and/or Asn to NH_4^+ in MSO treated plants.

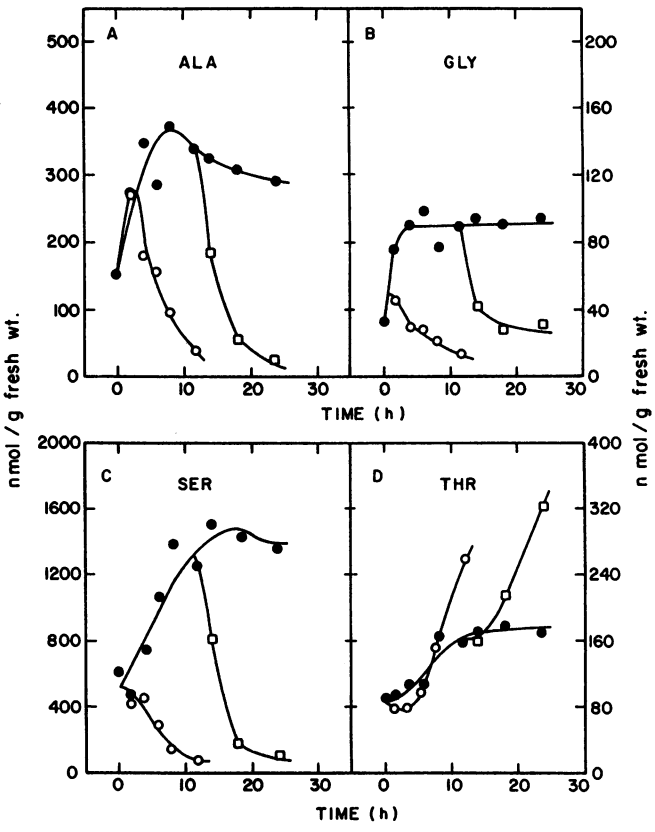

FIG. 5. Time courses of changes in the free amino acid pools of Ala (A), Gly (B), Ser (C), and Thr (D) in *L. minor* (see Fig. 1 legend for details of treatments).

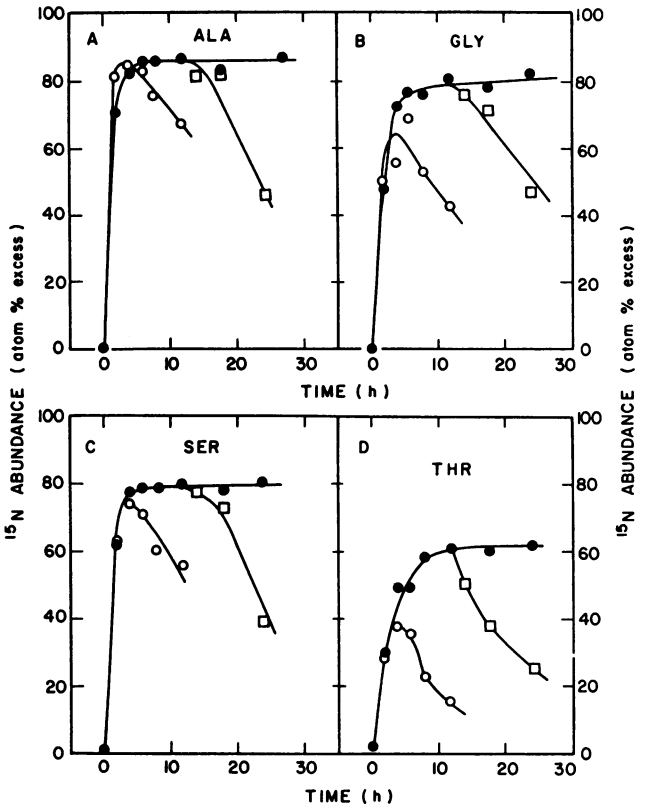

FIG. 6. Time courses of changes in ^{15}N abundance of Ala (A), Gly (B), Ser (C), and Thr (D) in *L. minor* (see Fig. 1 legend for details of treatments).

The question arises; could Asp or Asn catabolism contribute to the accumulation of amino acids of the Asp family (*e.g.* Thr and Lys) in response to MSO? The results of Figures 5D and 6D indicate that the Thr that is accumulated in response to MSO is far less heavily ^{15}N-labeled than either Asp (Fig. 2C) or Asn-amino N (Fig. 2D), especially in the treatments with MSO applied after 12 h $[^{15}N]H_4^+$ assimilation. Protein turnover represents the simplest explanation for this accumulation of $[^{14}N]$ Thr. Similar results to those of Thr have been observed for Lys (results not shown). It seems plausible that amino acids such as Phe, Tyr, His, and Met, which also accumulate in response to MSO (Table I), all have a common origin in protein catabolism. However, in these experiments we did not measure the ^{15}N abundance of Phe,Tyr, His, or Met.

DISCUSSION

The present results confirm the established importance of GS in plant N metabolism (1, 2, 4, 8, 10, 12, 13, 15, 23, 25). Inactivation of this enzyme by MSO triggers rapid metabolic changes which cascade into all facets of amino acid metabolism in complex ways. The labeling data appear to rule out any major contribution of GDH to NH_4^+ assimilation in support of previous findings with *L. minor* (19, 23). Protein turnover appears to be the major source of the amino acids that accumulate in response to MSO. It is necessary to envisage, however, that these amino acids (*e.g.* Pro, Val, Leu, Ile, and Thr) are not readily catabolized *in vivo*. It is possible that these amino acids are specifically sequestered in the vacuole and are thus rendered inaccessible to catabolism. The present results resemble a previous report of an association between protein turnover and photorespiration in *Chlamydomonas* which was revealed by MSO (4), but suggest that in *L. minor* only certain amino acids released from protein (*i.e.* Glu, Gln, Ala, Ser, Asp, and Asn) are selectively catabolized to NH_4^+ either in the photorespiratory N pathway or by alternative routes. Thus, only the Glu, Gln, Ser, Ala, Asn, and Asp pools are rapidly depleted in the MSO treated plants, leaving a free amino acid pool enriched in those poorly catabolized amino acids derived from protein hydrolysis. Further work is required to directly prove that Glu, Gln, Ala, Ser, Asn, and Asp are all processed via glycine to NH_4^+. Asn may be metabolized to Asp and NH_4^+ by asparaginase or may be transaminated to oxosuccinamate and then deaminated to NH_4^+ independently of Gly metabolism (7, 20), or Asn may participate directly in photorespiration as an N-donor for Gly (24).

Davies (5) and Davies and Humphrey (6) have directly measured protein turnover rates in nitrate grown plants of *L. minor* and have estimated a protein half-life of approximately 80 h. If *L. minor* has a protein content of about 5 mg/gfw and if we make the simplifying assumption that this protein is comprised of 20 amino acids each representing 5% of the protein hydrolysate, then a crude estimate of the rate of release of each of the 20 amino acids from protein corresponds to about 10 to 15 nmol/h·gfw. This is within the range of the observed rates of accumulation of individual amino acids such as Pro, Val, Leu, Ile, and Thr in the MSO treated plants (*i.e.* 5–15 nmol/h·gfw). Johansson and Larsson (9) have suggested that MSO induces shortage in amino-N for maintenance of functional integrity of the photosynthetic apparatus in *Lemna gibba*. It is possible that these amino-N shortages may activate protein catabolism. Nitrogen deficiencies have been shown to stimulate protein turnover in *L. minor* (6).

Computer simulation studies are in progress to interpret the data shown in Figures 1 to 6 in terms of flux and compartmentation (*cf.* 15, 19). Preliminary calculations suggest that the rate of Pro synthesis from Glu is approximately 40 nmol/h·gfw in the absence of MSO. When we assume that Pro is released from protein at 10 nmol/h·gfw and that 38% of the free Pro pool is in a metabolically inactive storage compartment, this rate of synthesis of Pro accounts for the observed isotopic labeling kinetics of Pro in the absence of MSO. If it is further envisaged that all 50 nmol/h·gfw of Pro are utilized in protein synthesis, and that both *de novo* Pro synthesis and Pro utilization in protein synthesis cease after a lag of 4 h in response to MSO, but that protein turnover remains active in the presence of MSO, then these assumptions account for the observed increase in pool size of Pro at a rate of 10 nmol/h·gfw (Fig. 3A) and the observed decrease in isotopic abundance of proline (Fig. 4A) in response to MSO added at zero time.

Similarly, the labeling kinetics of Thr can be accommodated by assuming that in the absence of MSO, Asp is synthesized from Glu at a rate of 1000 nmol/h·gfw, and that Asp donates nitrogen to Thr at a rate of 80 nmol/h·gfw, with a further 20 nmol/h·gfw of Thr derived from protein turnover. Of this total of 100 nmol/h·gfw of Thr, 60 nmol/h·gfw is envisaged to be utilized for protein synthesis and 40 nmol/h·gfw is envisaged to be used for Ile synthesis. It is necessary to assume that approximately 10% of the free Thr pool is in a metabolically inactive compartment. When *de novo* Thr synthesis and Thr utilization in protein synthesis and Ile synthesis are each envisaged to be severely impaired after a lag of 4 h in response to MSO added at zero time, then these assumptions account for both the accumulation of Thr at a rate of 20 nmol/h·gfw from protein turnover (Fig. 5D) and the observed isotopic dilution of Thr (Fig. 6D).

The data for Asn-amino N (Figs. 1D and 2D) suggest that Asn-amino N is initially synthesized at a rate of 600 nmol/h·gfw from Asp with a further 20 nmol/h·gfw from protein turnover. About 6% of the free Asn pool appears to be localized in a metabolically inactive storage compartment. Of the total of 620 nmol/h·gfw Asn-amino N produced, we envisage that 60 nmol/h·gfw are utilized in protein synthesis, and that initially only 110 nmol/h·gfw of Asn is catabolized (either by the asparaginase, oxosuccinamate, and/or photorespiratory pathways) (7, 20, 24).

This leaves 450 nmol/h·gfw which is simply accumulated in the free pool. This model accommodates the relatively slow depletion of Asn in response to MSO added at zero time, and accommodates the rapid accumulation of Asn in its absence. MSO is envisaged to block Asn utilization in protein synthesis and Asn synthesis after a lag of 4 h when added at zero time, but leaving Asn catabolism rate and the rate of release of Asn from protein unimpaired.

To account (a) for the reduced rate of accumulation of Asn after 10 h ammonia assimilation, and (b) the more rapid depletion of Asn caused by MSO when added after 12 h ammonia assimilation (Fig. 1D), it is necessary to envisage that the rate of Asn catabolism markedly increases after about 10 h of ammonia assimilation, from 110 nmol/h·gfw to about 600 nmol/h·gfw. The rate of *de novo* synthesis of Asn may in turn increase during this time from 600 nmol/h·gfw to about 800 nmol/h·gfw. In the above models the Glu pool serving as a precursor to Pro and Asp (which in turn donates N to Thr and Asn-amino N) is assumed to achieve a maximum of 86% ^{15}N abundance. These models are simplistic in the sense that they do not account for cytosolic and chloroplastic sites of amino acid biosynthesis (19), but nevertheless seem to accomodate the observed labeling data assuming modest protein turnover rates (*i.e.* rates of release of amino acids from protein of approximately 10 to 20 nmol/h·gfw). Comparable studies with other specific inhibitors of amino acid biosynthesis would be extremely useful in verifying these fluxes deduced so far. The present calculations suggest that asparagine may become a major intermediary metabolite in response to NH_4^+ nutrition in *Lemna*. Flux via the amino N group of Asn may be as high as 800 nmol/h·gfw after 12 h of NH_4^+ assimilation, implying that the total flux via both the amide and amino N groups may be as high as 1600 nmol/h·gfw; perhaps 10 to 20% of the total N assimilation of the *Lemna* plant. It appears that the availibility of Asp and Gln may be important regulatory factors in Asn synthesis and accumulation; rapid depletion of both Asp and Gln in response to MSO appears to severely curtail Asn synthesis and accumulation.

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