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PATHWAY OF AMMONIA ASSIMILATION IN ILLUMINATED *LEMNA MINOR*

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Key Word Index—*Lemna minor*; Lemnaceae; glutamate and glutamine biosynthesis; glutamate synthase; glutamine synthetase; $^{15}NH_3$ assimilation; computer models of $^{15}NH_3$ incorporation; pathway of NH_3 assimilation; sole operation of glutamate synthase pathway; role of asparagine.

Abstract—Plants of duckweed (*Lemna minor*) were grown under constant illumination and with a controlled supply of ammonium-N so as to maintain a constant low concentration. In two kinetic experiments (differing in illumination and N level) with ^{15}N-ammonia, plants were periodically harvested and their free amino acids analysed for ^{15}N abundance. Attempts were then made to fit the data by computer simulation models. Only models which had at least two or more intracellular compartments gave adequate fits. Two two-compartment models were tested fully. Both had in compartment 1 the glutamine synthetase–glutamate synthase cycle and in compartment 2 a second site of glutamine synthesis. In one model the glutamate for compartment 2 was derived by transport from compartment 1; in the second model it was synthesized from ammonia by glutamate dehydrogenase at a rate equivalent to 10% of the total N uptake. This second model was rejected after it was found that plants previously treated with methionine sulphoximine and aza-serine (inhibitors of the glutamate synthase cycle) were unable to incorporate ^{15}N. In spite of wide differences in labelling pattern between the two experiments the first model gave acceptable fits to both when different pool sizes were allowed for. Operation of the glutamate synthase cycle was confirmed by the correspondence between model and data for labelling of glutamine amide, glutamine amino and glutamic acid. Consideration of enzyme distributions suggested that compartment 1 (the glutamate synthase system) is the chloroplasts and compartment 2 the cytosol. Analysis of asparagine and neutral amino acids made it possible to construct balance sheets for N uptake in the two experiments. They suggest that all glutamine synthesized in the chloroplast is used for glutamate and asparagine synthesis and that the cytosol enzyme meets the need of the cell for glutamine *per se*. The high turnover rates for asparagine indicate that this compound is an important intermediate even under steady state conditions, and carries between 20 and 50% of the products of N assimilation.

INTRODUCTION

Until fairly recently the major route of ammonia assimilation had been considered to occur via the reductive amination of 2-oxoglutarate catalysed by glutamate dehydrogenase. However, the presence of high levels of a ferredoxin-dependent glutamate synthase in leaf chloroplasts and NAD(P)H- and ferredoxin-dependent enzymes in plant roots led to the assertion that most, if not all, of the nitrogen taken up by plants was assimilated through the glutamine synthetase–glutamate synthase cycle [1]. Although little doubt remains concerning the operation of this pathway in plants, no fully convincing argument has been put forward to exclude the parallel operation of the glutamate dehydrogenase pathway. Certainly the widespread distribution of glutamate dehydrogenase at high level attests to an important role for this enzyme.

In view of the ambiguity of the evidence from enzyme distribution, it is essential to apply other criteria to establish whether the glutamate synthase pathway functions exclusively in plants. An approach that was applied successfully in the food yeast *Candida utilis* was an analysis of the kinetics of ^{15}N incorporation into possible primary products of nitrogen assimilation [2, 3]. Unfortunately no comparable quantitative kinetic experiments have been carried out in higher plants with the precision necessary to yield definite conclusions. Thus, whilst some studies reveal that qualitatively the pattern of labelling of amino acids appears to support the glutamate synthase pathway in so far that ^{15}N abundance of the glutamine amide is greater than the α-amino of glutamate, this is not invariably so [1, 4–6].

None of the investigations reported so far has made use of one important feature of the assimilation of nitrogen via the glutamate synthase pathway namely that, provided the sizes of the glutamine and glutamate pools are known, it should be possible from measured rates of ammonia uptake to predict the nitrogen labelling in glutamic acid and in both the amide and amino groups of glutamine. This follows from the fact that in a cyclic system, the rate of synthesis of glutamine must only slightly exceed the

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rate of its utilization by glutamate synthase, while in the process of glutamine synthesis the glutamine amino-N is derived from glutamate at the same rate as the formation of glutamine amide from ammonia. This feature of the pathway has been tested here.

Duckweed was chosen as the experimental plant for the present investigations because of the high degree of uniformity of its tissues [7]. This justifies the use of a relatively simple analytical treatment in which differences between cell types are ignored and isotopic labelling is analysed in terms of its distribution between compartments within the cell. In addition the assimilation of nitrogen compounds by *Lemna* has been comprehensively studied (for recent review see [8]).

Plants of *Lemna minor* have been grown under constant illumination and with controlled nutrient input so as to achieve steady-state growth in the presence of constant low concentrations of ammonia. The results presented here derive from experiments in which $^{15}NH_4Cl$ is supplied to such plants.

RESULTS

Short term labelling of amino acids under steady state conditions

Experiment 1 was designed to determine the order of ^{15}N isotopic labelling of possible primary products of ammonia assimilation by duckweed. Indications that a steady state was achieved during the experiment are given by the stability of the pools of individual amino acids (Fig. 1a); the small fluctuations observed are within the error of the analytical methods employed. Fig. 1b shows that the pattern of labelling in the soluble amino acids is apparently in agreement with the operation of the glutamate synthase pathway since glutamine amide is labelled first and this is followed in turn by glutamate and the amino-N of glutamine. For this conclusion to be valid, however, it is necessary to assume that both glutamine and glutamate exist effectively in single pools within the cell [9]. If this is so then it should be possible to deduce from the rate of ^{15}N labelling in the amide group, the rate of nitrogen assimilation along this pathway. In fact this very simple model (Fig. 2) fails to fit adequately the experimental data and the rate of ammonia assimilation so deduced accounts for only *ca* 30% of that measured during the experiment. Similar arguments can also be applied to the glutamate data when this too is considered as a primary assimilant. Here again the rates of N assimilation deduced account for only a small fraction of that necessary to sustain steady-state growth. It is therefore necessary to conceive reaction models in which some of the amino acids are distributed between at least two compartments within the plant cell.

Of the various dual-compartment models considered only two gave a reasonable fit to the experimental data while accommodating the necessary rates of ammonia assimilation. Both models give a prominent position to the glutamate synthase cycle which is presumed to be located in compartment 1, while a second site of glutamine synthesis and associated glutamate and glutamine pools are in compartment 2. The two models differ only in the mode of origin of the second glutamate pool. In model A this pool is derived by the transport of glutamate from the cycle-generated glutamate pool of compartment 1, whilst in model B this pool is formed by primary assimilation via glutamate dehydrogenase.

Evaluations for both models are given in Fig. 3. Since both glutamine and glutamate are each assumed to exist in two cellular compartments, the calculated labelling is the resultant of the labelling in two distinct pools. In Fig. 3 the theoretical labelling in each pool is shown as well as the mean labelling which is fitted to the experimental observations. For each model the approach was to fit first the isotopic data for glutamine amide, adjusting the values for the two transfer coefficients and the relative sizes of the two metabolic pools so as to ensure that both the total pool size and the net rate of nitrogen assimilation were in agreement with the experimental observations. Once this had been

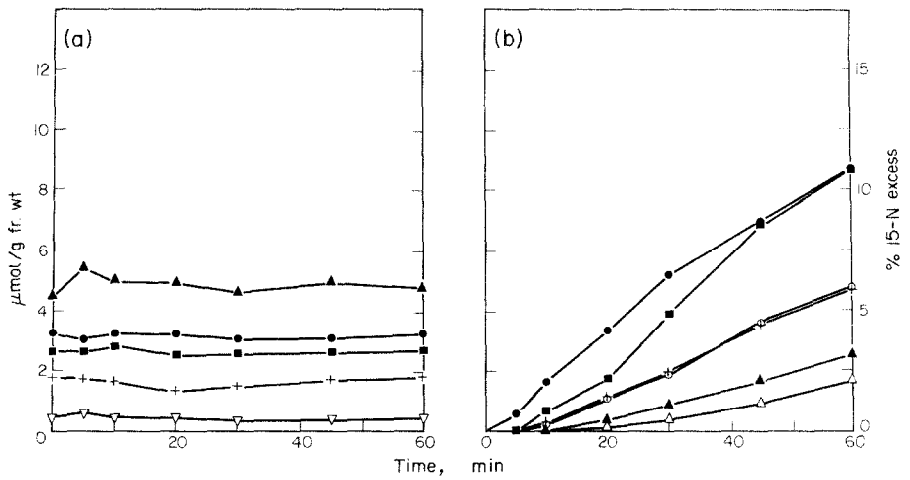

Fig. 1. Experiment 1: Amino acid pool levels and ^{15}N labelling (see text for details). (a) Level of soluble amino acids: glutamine, ●; glutamic acid, ■; neutral amino acids, +; asparagine, ▲; aspartic acid, ▽. (b) ^{15}N labelling: glutamine amide, ●; glutamine amino, ○; glutamic acid, ■; neutral amino acids, +; asparagine amide, ▲; asparagine amino, △.

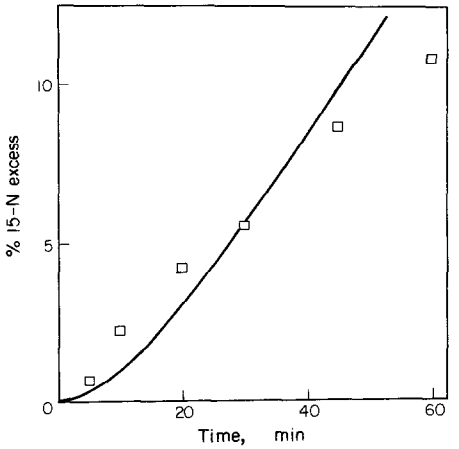

Fig. 2. Experiment 1: Labelling of glutamine amide calculated as a single pool. The computed curve is for a transfer coefficient of 24 nmol/min/g fr. wt.

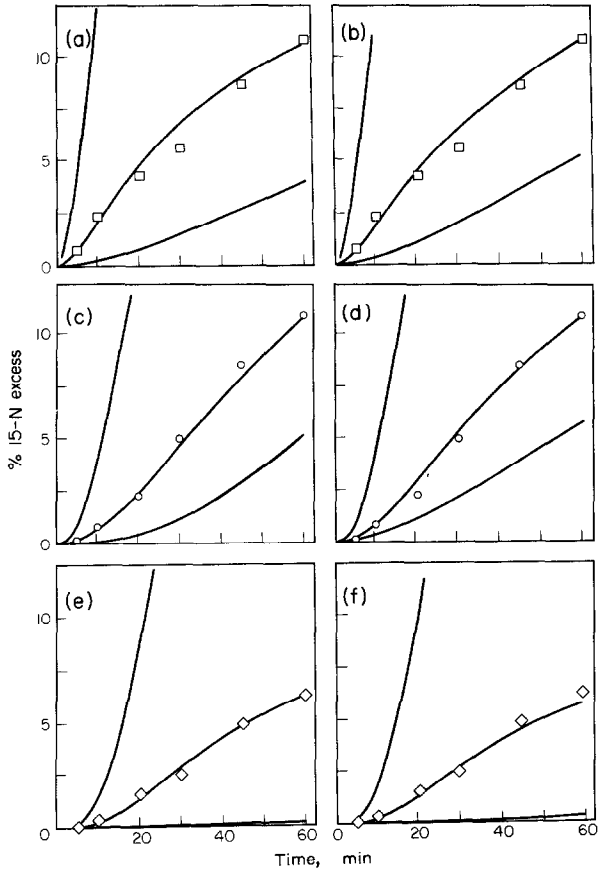

Fig. 3. Experiment 1: Computer simulation of labelling of glutamine and glutamic acid. In each graph the top line represents the labelling in compartment 1, the lowest line that in compartment 2, and the middle line the resultant mean. Two models are compared (see text): (a, c, e) model A; (b, d, f) model B. (a, b) Glutamine amide. (c, d) Glutamic acid. (e, f) Glutamine amino. The estimated pool sizes (μmol/g fr. wt) and transfer coefficients (nmol/min/g fr. wt) for model A are given in Fig. 8a; for model B they were, respectively: glutamine (compartment 1) 0.40, 70.0; (compartment 2) 2.70, 7.0; glutamic acid (compartment 1) 0.38, 60.0; (compartment 2) 2.47, 7.0.

achieved a similar procedure was adopted for the glutamic acid data. The critical test for the models rested in their ability to fit the data for the ^{15}N labelling of the amino group of glutamine. In both cases (Fig. 3e, 3f) an excellent fit was achieved which gives credence to the assertion that the glutamate synthase cycle is carrying most, if not all, of the nitrogen assimilation in this plant. It was not possible to distinguish between the two models in this regard but the fitted parameters for the primary assimilation of glutamate in model B suggest that no more than 10% of N assimilation can go via the glutamate dehydrogenase pathway.

The availability of ^{15}N isotopic data for the α-amino and amide nitrogen of asparagine enabled us to extend the analysis to this compound. With both models it was possible to fit satisfactorily the experimental observations by a simple reaction scheme. As an example the fit for model A is shown in Fig. 4(a and b). Here it was assumed that the asparagine was present in a single large pool and derived its amide nitrogen from the more highly labelled of the glutamine pools. Unfortunately isotope data were not available for aspartic acid, the putative precursor of the amino nitrogen, but it was evident from the close equivalence of the labelling in the amino and amide groups of asparagine that

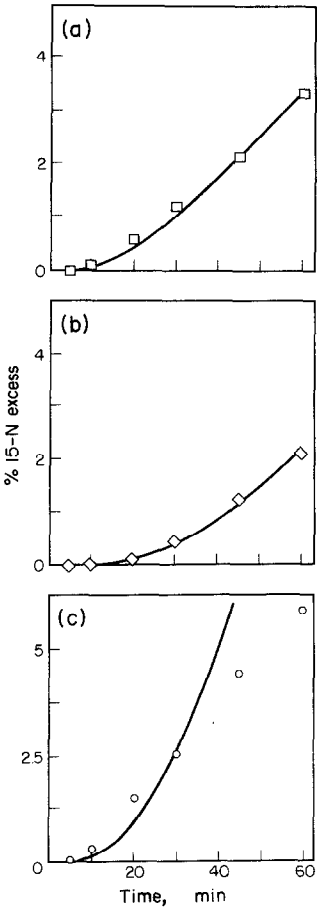

Fig. 4. Experiment 1: Computer simulation of labelling of asparagine and neutral amino acids (model A). (a) Asparagine amide. (b) Asparagine amino. (c) Neutral amino acids.

the amino precursor pool must be small. It was therefore presumed that only *ca* 10% of the total free aspartic acid was in the precursor pool and that this derived its nitrogen from the more highly labelled of the glutamate pools. With this pre-condition, a single transfer coefficient for asparagine accommodated satisfactorily both the amide and amino labelling in this compound.

Neutral amino acids clearly represent a very heterogeneous collection of compounds whose labelling and spatial distribution are likely to vary markedly. It is not surprising therefore that the curve shown in Fig. 4c fits the observations so badly. This was calculated on the assumption that each of the neutral amino acids is present in a single cellular pool, which derives its nitrogen from the more highly labelled glutamate pool. The approximate fit to the early points allows some estimate to be made of the total rate of glutamate utilization in this process.

$^{15}NH_4Cl$ *assimilation in* Lemna *pretreated with methionine sulphoximine and aza-serine*

Experiment 2 was designed to enable us to distinguish between the two model systems proposed: by specifically inhibiting glutamine synthetase and glutamate synthase, enzymes which feature prominently in both schemes, it was hoped with ^{15}N to reveal whether additional routes of glutamate biosynthesis operate.

Conditions have already been established with *Lemna* under which the inhibitors methionine sulphoximine and aza-serine effectively block the glutamate synthase pathway [10]. Methionine sulphoximine, a potent inhibitor of glutamine synthetase [11] and aza-serine, an inhibitor of glutamine amide transfer reactions [12] are without effect on glutamate dehydrogenase [13].

Plants were grown under constant illumination on nitrate for 24 hr and 0.2 mM methionine sulphoximine and 0.2 mM aza-serine were then added. Enzyme assays revealed that the level of glutamine synthetase rapidly fell to zero whilst glutamate dehydrogenase activity was unaffected. After 2 hr exposure to these inhibitors, $^{15}NH_4Cl$ (94.34% ^{15}N abundance) was added to the medium to bring the final concentration to 2 mM. Plants were harvested at intervals and the ^{15}N abundance in the free glutamic acid was determined. No significant incorporation of ^{15}N into glutamic acid was observed over the 80 min of the experiment, by which time the glutamate labelling might have been expected to reach over 25% ^{15}N excess if 10% of the original rate of NH_3 assimilation had operated via the glutamate dehydrogenase pathway (Fig. 5). It seems unlikely therefore that, in spite of the fact that the levels of glutamate dehydrogenase are more than adequate to sustain the whole of the nitrogen assimilation (see Fig. 5), this enzyme has any role in the assimilation of nitrogen in illuminated plants of *Lemna*. Very similar experimental results have recently been reported for leaves of *Datura* [14] but in that case the discrepancy between these observations and the labelling pattern in the absence of methionine sulphoximine was not resolved. Some doubt must always remain concerning the interpretation of experiments using metabolic inhibitors however and so we attempted to confirm these observations in another experiment.

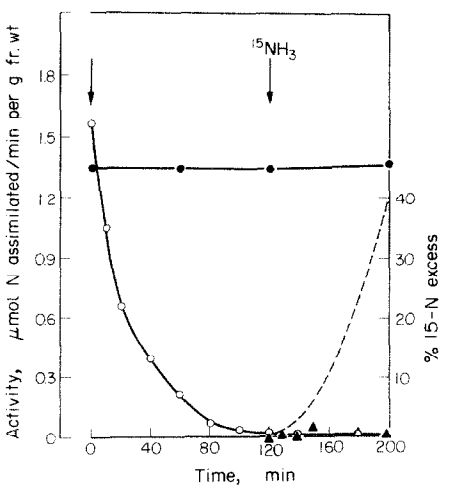

Fig. 5. Experiment 2: Incorporation of ^{15}N in glutamic acid and changes in the activities of enzymes following the addition of metabolic inhibitors to *Lemna*. NAD-glutamate dehydrogenase, ●; glutamine synthetase, ○; ^{15}N labelling in free glutamic acid, ▲; time of addition of 0.2 mM aza-serine and 0.2 mM methionine sulphoximine denoted by ↓; calculated ^{15}N labelling in glutamate assuming a transfer coefficient of 8.0 nmol/min/g fr. wt (equivalent to 10% of normal total uptake), - - -.

Amino acid labelling in Lemna *over a more extended period and under different conditions for growth*

An important test of the validity of any model is its adaptibility to meet modified physiological conditions which may produce alterations in the ratios of pools or changes in the contribution of pathways. In this third experiment plants were grown at a reduced light intensity (75 W/m^2) and provided with a 5-fold increase in ammonia availability. It was hoped that a reduction in light intensity would favour a reduction of the flux of nitrogen through the ferredoxin-dependent glutamate synthase pathway and that an increase in ammonia availability would elevate the activity of the glutamate dehydrogenase pathway. The results are summarized in Fig. 6 where data on the level of the individual soluble amino acids and their isotopic labelling are shown. In two respects the data for glutamine and asparagine are significantly different from those in the first experiment. Over the longer period of this experiment it is evident that there is a progressive fall in the size of both of the pools (Fig. 6a) and account has been taken of this in that a non steady-state model has been used in respect of these compounds in this experiment. Moreover, under the growth conditions applied here the level of both pools is about double that observed in the first experiment. One consequence of this is that the pattern of ^{15}N labelling is appreciably different (Fig. 6b) with glutamate now achieving more than twice the labelling of glutamine amide, which is even exceeded by the label in the combined neutral amino acids. In spite of these differences, it is still possible to fit satisfactorily with model A the labelling data for glutamine amide and glutamate merely by assuming that this major increase in glutamine level has occurred in the second less highly labelled pool and that it is in this pool that the departures from

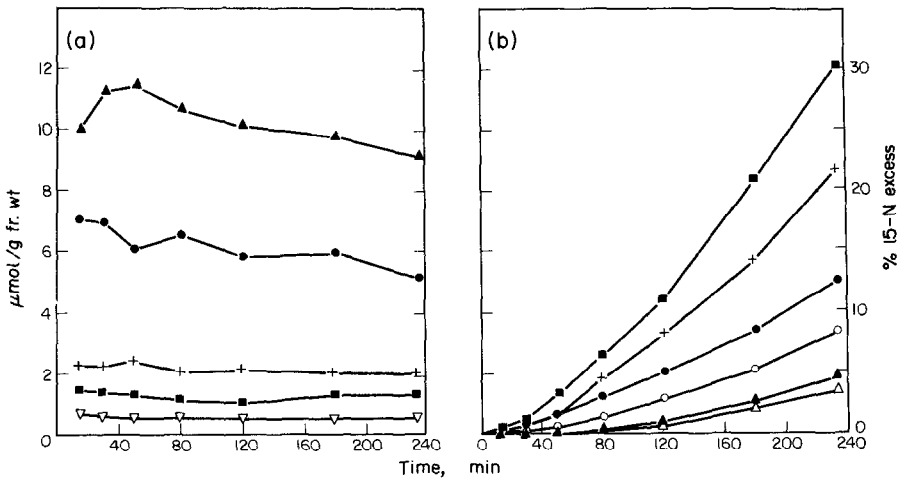

Fig. 6. Experiment 3: Amino acid pool levels and ^{15}N labelling. (a) Level of soluble amino acids: glutamine, ●; glutamic acid, ■; neutral amino acids, +; asparagine, ▲; aspartic acid, ▽. (b) ^{15}N labelling: glutamine amide, ●; glutamine amino, ○; glutamic acid, ■; neutral amino acids, +; asparagine amide, ▲; asparagine amino, △.

steady state have occurred. When these best-fit parameters are used to compute the labelling in the glutamine amino group, there is a good match with the experimental observations only for the first part of the experiment. By minor adjustments to these parameters, however, it is possible to obtain a reasonably satisfactory fit to all 3 curves over the duration of the experiment (Fig. 7). In this experiment again the labelling of the amino and amide groups of asparagine could be fitted to the same simple model. The expectation that the present experimental conditions might favour the participation of the glutamate dehydrogenase pathway was not borne out since model B, if anything, gave a slightly less good fit to the data.

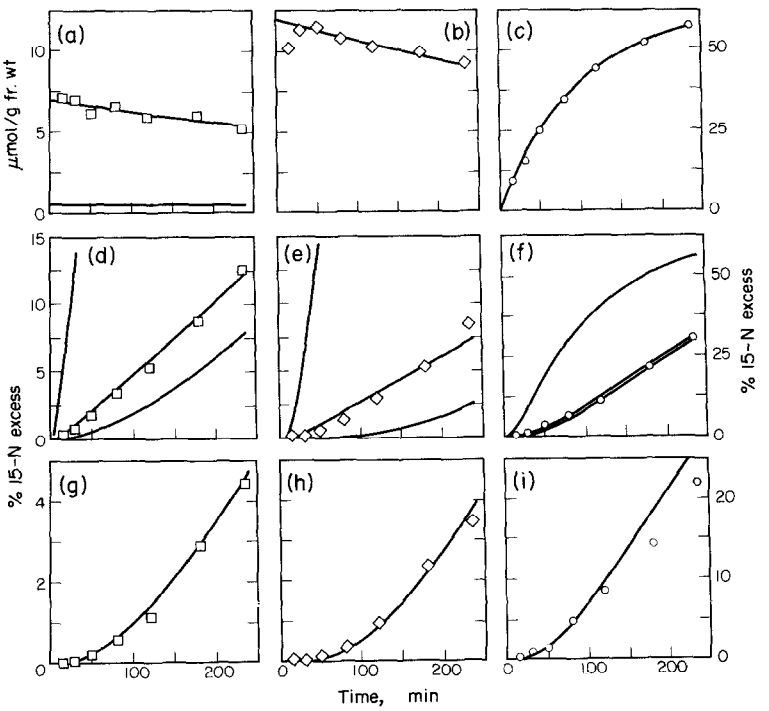

Fig. 7. Experiment 3: Computer simulation of pool levels and labelling in amino acids and amides (model A). (a) Glutamine level: the upper line is for total glutamine and the lower line for compartment 1 only; (b) asparagine level; (c) ammonia labelling in the culture medium; (d) glutamine amide labelling: compartment 1 (upper), compartment 2 (lower) and mean (centre); (e) glutamine amino labelling (scale as d); (f) glutamic acid labelling; (g) asparagine amide labelling; (h) asparagine amino labelling (scale as g); (i) neutral amino acid labelling.

DISCUSSION

While there is no longer any doubt that the glutamate synthase cycle is responsible for a major part of the N assimilation in plant leaves, there is still some uncertainty as to the extent to which, under different nutritional conditions, this pathway may be augmented by the action of glutamate dehydrogenase. Support for the operation of this second system has been derived from experiments in which glutamic acid has become more highly labelled than glutamine amide (see for example [6]). The present analysis reveals just how inadequate this interpretation is since it has been possible to fit satisfactorily both types of isotopic data using a relatively simple model based on the glutamate synthase cycle, the differences in labelling being achieved by minor adjustments to the sizes of cellular pools and rates of transfer between them. It is clear that far more complete experimental data are necessary (see for example [2, 3]) if an unequivocal answer to this question is to be realised from isotopic labelling experiments alone. It was for this reason that the inhibitor experiment was undertaken and the indications from this are clearly against even the relatively small rate of direct glutamate synthesis which has been suggested from the calculations based on model B.

A feature of the present analysis is the relative simplicity of the models used here and the ability of a two-compartment system to account reasonably well for the observed patterns of isotopic labelling. In view of this we have attempted to equate these theoretical compartments to actual locations within the plant. One possible interpretation is that compartment 1 corresponds to cells involved in photosynthetic N assimilation and compartment 2 to those growing cells actively involved in protein and nucleic acid synthesis. We have rejected this view because of the small size of the pools in compartment 1 whereas the mature photosynthetic cells constitute more than 70% of the *Lemna* plant [7]. This uniformity of tissue makes it possible that the compartments are intracellular and this view is supported by the intracellular distribution of the relevant biosynthetic enzymes. There is now some agreement that glutamine synthetase occurs both in the chloroplast and the cytosol [1, 8, 15, 16] so these two sites could correspond with our two compartments. Glutamate synthase, on the other hand, is probably located exclusively in the chloroplasts of photosynthetic tissue [1,15, 17] which suggests that these are equivalent to the first compartment of our model. The high rates of N assimilation that we attribute to this compartment accord well with the view that the chloroplasts are of major importance in the synthesis of N compounds of plants [17, 18]. While aspartate transaminase is present in chloroplasts [17–19] it seems that asparagine synthetase is located in the cytosol [20]. This poses a problem for our model since asparagine appears to be synthesized from the glutamine amide and aspartate pools of compartment 1. To account for this discrepancy one can only suggest that, although in the cytosol, asparagine synthetase may be associated in some way with the chloroplast boundary. Others have noted similar complications in regard to asparagine synthesis [21].

In many plants appreciable amounts of amino acids and amides appear localized within the cell vacuole [22–24] so one might expect that a 3-compartment system would be necessary to describe adequately the pattern of amino acid labelling in *Lemna*. It might indeed be possible to fit our experimental data more satisfactorily with a 3-compartmental system but in the absence of independent data as to the probable size of any vacuolar pools, we have deemed it unprofitable to undertake such a complex analysis, particularly since the differences between the calculated and observed values in our experiments might equally well be attributed to variations in cell type and activity within the plant tissues. In any case the *Lemna* used here was in active steady-state growth and on a restricted supply of N so that in these circumstances it is not too improbable that the vacuolar pools will be of negligible size and hence that the system will behave as if effectively in two compartments. There is indeed some experimental evidence in support of this; in Experiment 2 (see also [10]) the pools of glutamine and glutamic acid rapidly depleted following addition of the inhibitors, suggesting that these pools were being actively metabolized. This contrasts sharply with the situation in *Datura* leaves [14] where considerable storage pools remained intact in the presence of methionine sulphoximine.

More information about vacuolar pools is also required for a proper evaluation of another cycle of glutamine/glutamate transformation, the photorespiratory nitrogen cycle [25]. This scheme proposes that the throughput of N via the glutamate synthase pathway may, under conditions of active photorespiration, be far greater than the net rate of N assimilation and that the syntheses of glutamine and glutamate are located in the cytosol and chloroplast, respectively. We have examined the present ^{15}N data in relation to this model and conclude that the proposed system could operate in *Lemna* only if both chloroplastic and cytosol pools were relatively small, the major location of the cellular glutamate and glutamine being in the vacuole. Unless this assumption is made the dilution of isotope on entry into the glutamate pool would be such that it would be impossible to achieve the observed ratio of ^{15}N labelling in the amide and amino groups of glutamine.

An important feature of the photorespiratory nitrogen cycle is that it accords a specific role to the glutamine synthetase located in the cytosol, whereas only the chloroplast enzyme appears to be necessary for operation of the glutamate synthase cycle [15, 25]. Our own experiments, however, suggest an alternative role for the cytosol enzyme, namely that it is responsible for the major *output* of glutamine. As can be seen from the summarized results given in Fig. 8, the glutamine made in the chloroplast is almost exclusively used for the operation of the cycle and the provision of the amide group of asparagine. Thus, the chloroplast appears to be chiefly engaged in the synthesis of amino compounds and it falls to the cytosol enzyme to make the glutamine required for the synthesis of proteins and nucleic acids in the cytoplasm. The estimated output of *ca* 4 nmol/min/g fr. wt (Fig. 8a) is probably just adequate to supply both the glutaminyl residues of proteins and the glutamine and carbamyl phosphate used in nucleotide synthesis.

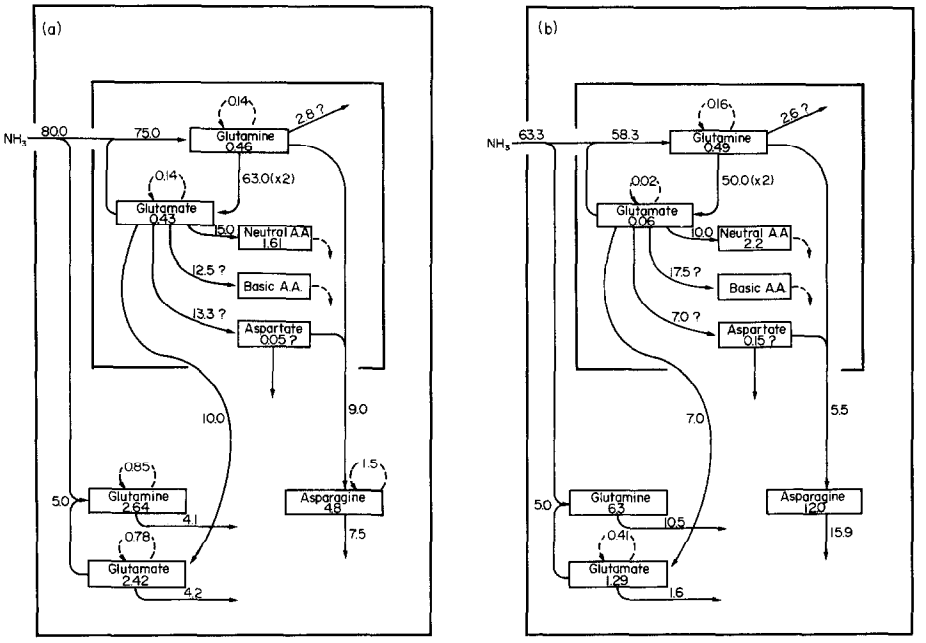

Fig. 8. A comparison of pool sizes and metabolic rates under differing cultural conditions (Experiments 1 and 3). The numbers in square boxes show the computed pool sizes (μmol/g fr. wt) and the annotations to the arrows the estimated transfer coefficients (nmol/min/g fr. wt). Quantities marked '?' are estimates based on balancing synthesis and utilization rather than on isotopic labelling. Arrows with broken lines indicate the rates required to maintain the pools in steady-state growth. (a) Experiment 1: (b) Experiment 3.

Comparison of the two kinetic experiments (Fig. 8) reveals many features of similarity. The major differences centre upon the fact that in Experiment 3 the glutamine and asparagine pools were no longer in steady state but instead were being depleted of nitrogen far faster than they were being replenished. One consequence (or cause?) of this was that the rate of ammonia assimilation was less in this experiment. The output of glutamate from the chloroplast was also less, presumably compensated for by the formation of glutamate consequential upon the utilization in transamidation reactions of the increased outflow of cytosol glutamine. There are some indications that there may have been similar effects on aspartate export as a result of asparagine utilization. These complexities could well explain the less good fit between the model and the data in this experiment, and they offer a fascinating glimpse of possible regulatory controls.

One other feature of the results that deserves special comment concerns the role of asparagine in the nitrogen metabolism of these plants. In both kinetic experiments the asparagine pool is large and rapidly becomes labelled. Even when it is assumed that this label is derived from the most highly labelled pools of glutamate and glutamine, remarkably rapid rates of asparagine synthesis and utilization are implied (Fig. 8a); partitioning of the asparagine between 2 pools only further increases this estimate of rate of synthesis and gives an appreciably worse fit to the data. It is also possible to rule out that the high labelling in asparagine amide results from reversible exchange of amide groups between asparagine and glutamine since if it were so the labelling of the asparagine amino group would not be simultaneously increased and the rate of synthesis calculated from the amino group would no longer accord with that derived from the amide. So we are thus faced with the fact that in Experiment 1 nearly 20% of the total nitrogen uptake passes through the asparagine pool. In Experiment 3 the position is even more exaggerated; because of the reduced assimilation and increased rate of asparagine utilization in this experiment, the output of the pool is equivalent to more than 50% of the ammonia uptake. All these observations confirm and extend the conclusions of Bauer *et al.* [5] as to the importance of asparagine in plant nutrition. Asparagine has long been recognized to act as a storage compound and as a medium for the intercellular transport of N; the present results suggest that it is also involved in intracellular transport and serves as a major intermediate in N metabolism.

EXPERIMENTAL

Growth. Plants of *Lemna minor* (strain S_1) were grown in a cylindrical glass vessel (24.5 cm dia) containing 2 l. of sterile basal medium [26] under continuous illumination (routine intensity 120 W/m^2 in the range 400–700 nm) at 25 ± 1°. The depth of medium in the culture vessel (4.5 cm) permitted satisfactory stirring by the action of two magnetic stirrers without causing damage to the plant roots. Under these conditions 10–15 g fr. wt of duckweed could be maintained in exponential growth for at least 2 days with a doubling time of 36 hr. Fresh culture medium was delivered to the apparatus using a calibrated peristaltic pump. The vol. of the medium was kept constant (2000 ± 50 ml) by a suck-out tube held at a

fixed level connected to waste via a pump. Either the flow rate or the concentration of N in the input medium could be adjusted to match the rate of NH_3 assimilation of the known wt of duckweed in the vessel.

In ^{15}N expts where plant material was sacrificed at intervals it was necessary to make progressive reductions of input to achieve a constant level of NH_3 in the medium.

Extraction of soluble amino acids. Samples of duckweed (equivalent to 1–1.5 g fr. wt) were washed with H_2O and then extracted with 50 ml MeOH at 0° for 24 hr. Filtrates were evapd to dryness at 35° and redissolved in 2 ml H_2O prior to chromatography.

Separation of amino acids and amides by ion exchange chromatography. Free NH_3 in the plant extract was separated from neutral and acidic amino acids by passage through a column (2×0.5 cm) of Zeo-Karb 225 (×12) (Na^+) resin equilibrated with 10 mM NaOAc. NH_3 was eluted using 8 ml 0.2 M NaPi buffer (pH 7.5). Neutral amino acids were separated from the acidic amino acids on a column (5×0.5 cm) of Dowex 1 (×8) (acetate) equilibrated with H_2O. Glutamate was eluted with 8 ml 0.2 M HOAc and aspartate with 4 ml 0.2 M NaOAc–HOAc buffer (pH 4.84). The neutral amino acid fractions were taken to dryness at 35° and redissolved in 1.5 ml H_2O. Glutamine was hydrolysed to glutamate and NH_3 by incubation with 2 units of glutaminase (Sigma: Grade III from *Escherichia coli*) at pH 5 and 30° for 2 hr. The amide N, as NH_3, was isolated from glutamate using Zeo-Karb 225 (×12) as described above. After the removal of the amide N of glutamine the asparagine was hydrolysed by incubation with 5 units of asparaginase (Sigma: Grade V from *Escherichia coli*) at pH 7 and 30° for 1 hr. Asparagine amide was recovered as NH_3 on Zeo-Karb 225. Glutamine and asparagine amino N were recovered as glutamate and aspartate by elution from Dowex 1 as described above.

The α-amino N was determined by the ascorbate–ninhydrin procedure [27]. NH_3 was determined by the PhOH hypochlorite method [28].

Determination of ^{15}N abundance. The colour complex from the ascorbate–ninhydrin reaction was decomposed by boiling the amino acid sample for 5 min with 0.25 ml 3.6 M H_2SO_4 in order to release N as NH_3. 2-Methoxyethanol was removed by acidic steam distillation (30 ml samples were collected). NH_3 was collected as 25 ml distillate in 0.01 M H_2SO_4 following the addition of 10 ml alkaline borate soln (0.15 M Na_3PO_4, 0.15 M sodium tetraborate, 0.6 M NaOH) to these samples.

Medium NH_3 and amide NH_3 were distilled directly using M NaOH. All NH_3 samples were evapd to dryness and taken up in 1.5 ml H_2O. 1 ml samples were converted to N_2 in evacuated Rittenberg tubes and the isotopic abundance measured [27].

Enzyme assays. Glutamate dehydrogenase was measured as in ref. [29] and glutamine synthetase [30].

Computer simulation of isotopic labelling. The computer simulation programme derives from the theoretical consideration of the kinetics of isotopic labelling given in earlier papers [2, 3]. If, in a quantity of cells Q, the compound B is derived from compound A with a transfer coefficient k_1, and is itself utilized in other syntheses with a combined transfer coefficient k_2, then

$$\frac{dB}{dt} = k_1 Q - k_2 Q.$$

Moreover, if the cells are growing with a specific growth rate a, then the steady state concentration of B is itself related to these same transfer coefficients, since

$$B/Q = (k_1 - k_2)/a.$$

From this it follows that

$$k_2 = k_1 - aB/Q$$

or

$$dB = Ba \cdot dt.$$

Under non steady-state conditions this relationship will not hold but instead

$$dB = Baf \cdot dt \quad (1)$$

where f is a correction factor; under these circumstances,

$$k_2 = k_1 - afB/Q$$

and the steady state may be regarded as a special case in which $f = 1.0$.

If A is labelled, its fractional abundance being $A^+ = A^*/A$ where A^* is the amount of labelled molecules in the total quantity A, then B will become labelled to $B^+ = B^*/B$ and

$$\frac{dB^*}{dt} = k_1 QA^+ - k_2 QB^+.$$

Substituting for k_2 we get

$$dB^* = k_1 QA^+ dt - (k_1 Q - afB)B^+ dt. \quad (2)$$

The quantity Q in this equation represents the cell mass which is itself growing, so any model must also include an incremental increase in Q,

$$dQ = Qa \cdot dt. \quad (3)$$

The simulation programme reiterated for each time increment dt a series of calculations in which operations analogous to those described by equations 1, 2 and 3 were performed for the various cellular pools. The labelling of the NH_3 in the culture soln was calculated by a similar process, the varying rate of input of fully labelled NH_3 to the culture vessel being simulated by the linear or quadratic equation giving best fit to the curve of pump rate against time; in Expt 1 the quantities of NH_3 in the medium were too small to allow direct determination of labelling, but for Expt 3 there was good agreement between observations and the values calculated in this way (see Fig. 7c). Output from the programme could be in tabular or graphical form. Tests showed that adequate accuracy was achieved when $dt = 0.1$ min and this value was used throughout. Curve fitting was performed by using the programme interactively with a Tektronix 4010 VDU. Best fit was judged by eye since it was unprofitable to prolong the computation to achieve a 'least squares' fit.

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