EVIDENCE FOR THE ASSIMILATION OF AMMONIA VIA THE GLUTAMINE PATHWAY IN NITRATE-GROWN *LEMNA MINOR* L.

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1. Introduction

Recent studies of nitrogen metabolism in higher plants suggest that ammonia assimilation may take place through the combined operation of glutamine synthetase and glutamate synthase, rather than the traditionally accepted route via glutamate dehydrogenase [1–3]. When plants of *Lemna mino* are grown under conditions where there is a restricted availability of ammonia high activities of glutamine synthetase and glutamate synthase are present, as the ammonia concentration is increased there is a decrease in the level of these two enzymes and an increase in the activity of glutamate dehydrogenase [4].

In order to investigate the route of ammonia assimilation the responses of nitrate-grown plants to two inhibitors, methionine sulphoximine and azaserine, have been determined. Methionine sulphoximine is a potent, irreversible inhibitor of glutamine synthetase [5] which although having some effect on bacterial glutamate synthase is without effect on glutamate dehydrogenase [6]. Azaserine is an analogue of glutamine and is an inhibitor of glutamine amide transfer reactions [6], including the glutamate synthase of *Lemna minor* [4]. The responses to these two inhibitors suggest that glutamine synthetase is the principal enzyme for ammonia assimilation and that the synthesis of glutamate occurs via glutamate synthase.

2. Materials and methods

Details for the growth of *Lemna minor* (Strain S1) have been described elsewhere [7]; 5 mM KNO_3 was used as the nitrogen source in the present experiments. The extraction and determination of enzyme activity were carried out as described previously [4]. Amino acids and ammonia were extracted and determined as described by Orebamjo and Stewart [8]; glutamate and glutamine were determined by the procedure of Ferguson and Sims [9]. Fractionation of extracts following the addition of ^{14}C-labelled $NaHCO_3$ was as described by Fletcher and Beevers [10]; radioactivity present in 2-oxoglutarate was determined after its enzymic conversion to glutamate, the glutamate being separated on Dowex-1-acetate. ^{14}C determinations were made by liquid scintillation counting.

3. Results and discussion

The additon of methionine sulphoximine brings about a rapid loss of glutamine synthetase activity: after 45 min no activity could be detected (fig.1). Accompanying this loss of the enzyme is a rapid accumulation of ammonia and a rapid decrease in glutamine content. The tissue concentration of glutamate shows an initial increase from 1.1 μmol/gfw to 1.5 μmol/gfw, but subsequently this decreases to 0.25 μmol/gfw. No changes in the levels of extractable glutamate synthase and glutamate dehydrogenase could be detected over the period of this experiment.

The decrease in glutamine content is consistent with the loss of glutamine synthetase and indicates a rapid turnover of glutamine in nitrate-grown plants. The rapid accumulation of ammonia suggests that methionine sulphoximine blocks the major route for the assimilation of ammonia which is derived from nitrate reduction. The initial rate of ammonia accumulation is 5–6 μmol/h/gfw, which is similar to the calculated rate of nitrate assimilation (5.7 μmol/h/gfw).

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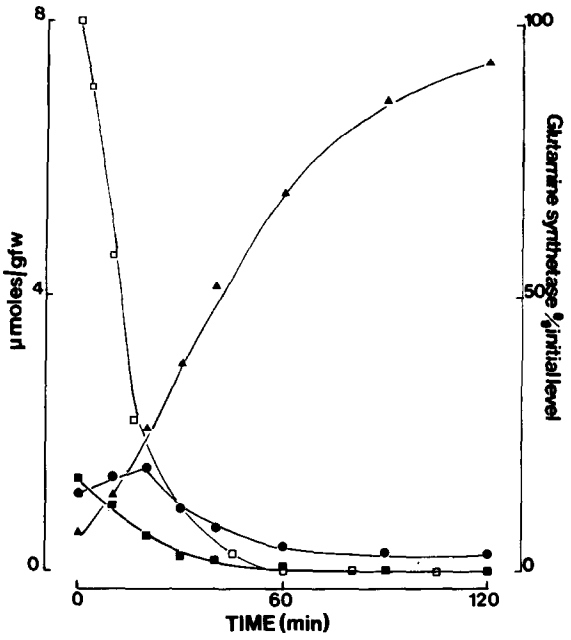

Fig.1. Response to methionine sulphoximine. 500 µM methionine sulphoximine added at zero time. (▲) Ammonia; (●) glutamate; (■) glutamine; (□) glutamine synthetase.

The effect of methionine sulphoximine on the incorporation of ^{14}C-labelled $NaHCO_3$ is shown in fig.2. Following the addition of the inhibitor there is a decrease in ^{14}C-incorporation into glutamine. Initially, there is a very pronounced increase in the rate of incorporation into glutamate but subsequently the rate is markedly reduced. In contrast there is a progressive stimulation in the rate of incorporation into 2-oxo-glutarate, and this together with the accumulation of ammonia suggests that glutamate dehydrogenase can have only a minor role (if any) in the assimilation of ammonia by nitrate-grown plants. The initial increase in the labelling of glutamate could arise from the blocking of glutamine synthetase and the subsequent decrease in the rate of labelling from a reduced availability of glutamine for the glutamate synthase reaction.

Support for this view comes from the response of plants to azaserine. Following the addition of azaserine there is a rapid increase in glutamine content, a rapid decrease in glutamate and a small increase in ammonia content (fig.3). The increase in glutamine content is consistent with the inhibition of glutamine amide transfer reactions by azaserine and the pronounced decrease in glutamate suggests that a substantial part of the glutamate pool is derived from such reactions. Azaserine brings about marked changes in the incorporation of ^{14}C-bicarbonate into glutamate,

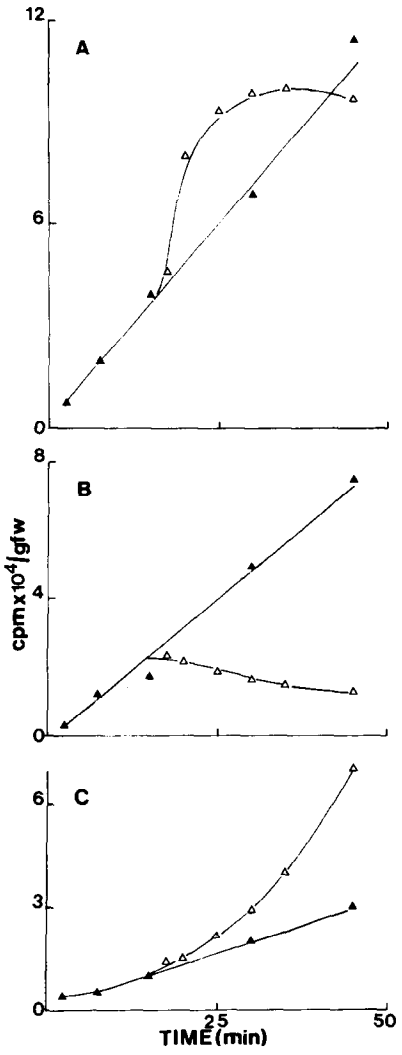

Fig.2. ^{14}C-labelled bicarbonate incorporation in the presence and absence of methionine sulphoximine. 0.4 µCi/ml $NaHCO_3$ added at zero time; 500 µM methionine sulphoximine added after 15 min. Closed symbols, control; open symbols, + MSO. (A) Glutamate (B) glutamine (C) 2-oxoglutarate.

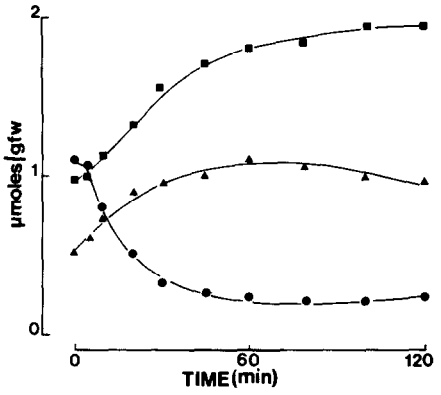

Fig.3. Response to azaserine. 250 μg/ml azaserine added at zero time. (▲) Ammonia; (●) glutamate; (■) glutamine.

glutamine and 2-oxoglutarate (fig.4). There is a rapid decrease in the labelling of glutamate and an increased labelling of glutamine and 2-oxoglutarate. This is particularly marked in the case of 2-oxoglutarate where there is a 12-fold increase in the rate of ^{14}C-incorporation. Although an increase in ^{14}C-incorporation into glutamine and a decrease into glutamate is to be expected from an inhibition of glutamine amide transfer reactions, it is difficult to see why this should result in such a marked increase in the labelling of 2-oxoglutarate. This increase in labelling of 2-oxoglutarate and the decrease in label accumulating in glutamate cannot be explained by the synthesis of glutamate via glutamate dehydrogenase. Rather these results suggest glutamate is synthesized in a reaction requiring glutamine amide transfer and 2-oxoglutarate, namely the reaction catalysed by glutamate synthase.

The responses of nitrate grown plants to methionine sulphoximine and azaserine are consistent with glutamine synthetase being the principal enzyme of ammonia assimilation and with the synthesis of glutamate by glutamate synthase. Problems inevitably arise in interpreting results obtained from the use of inhibitors, in particular it is difficult to establish their specificity in vivo. However experiments with the yeast *Candida utilis* indicate that the response of nitrate-grown cells to methionine sulphoximine is quite different from that of *Lemna minor.* Although there is an 85% loss of glutamine synthetase over 60 min, a decrease in glutamine from 78 to 10 μmol/

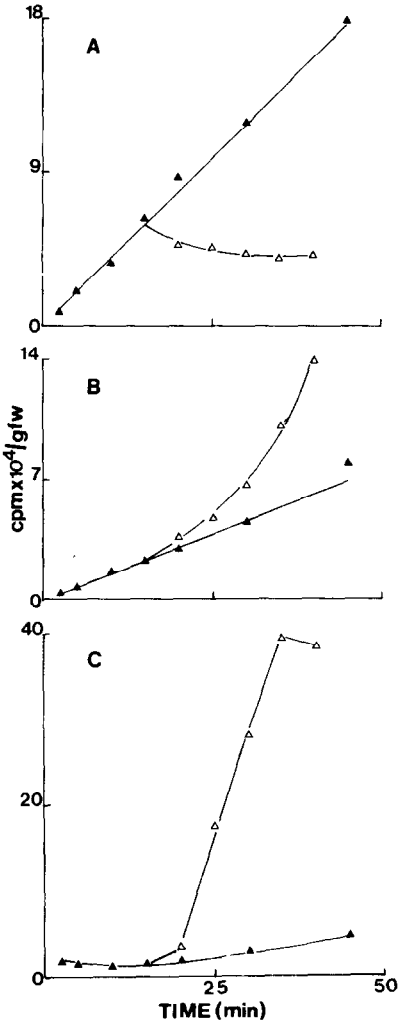

Fig.4. ^{14}C-bicarbonate incorporation in the presence and absence of azaserine. Details as for fig.2.

gdw, there is no increase in ammonia content and there is an increase in glutamate from 90 to 140 μmol/gdw. Detailed studies of ammonia assimilation in this yeast have been carried out by Sims and his co-workers, and results from both ^{15}N-incorporation studies and enzymological investigations show that the primary and major product of ammonia assimilation is glutamate, the synthesis of which is catalysed by an NADP-linked glutamate dehydrogenase [11,12]. The response of *Lemna* to methionine sulphoximine resembles that of nitrogen-fixing cells of *Anabaena cylindrica* [13].

^{15}N-incorporation studies suggest that *Anabaena* assimilates ammonia via the glutamine pathway [14]. The similarity in response of *Lemna* and *Anabaena* to methionine sulphoximine supports the view that in nitrate-grown plants of *Lemna* ammonia assimilation occurs via the glutamine pathway.

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