Nitrogen Metabolism of Lemna minor. I. Growth, Nitrogen Sources and Amino Acid Inhibition

K. W. Joy
Biology Department, Carleton University, Ottawa, Canada

Received January 23, 1969.

Abstract. Lemna minor grown in sterile culture on a minerals-sucrose medium can utilize as nitrogen source, in order of increasing growth rate: ammonia, nitrate, a mixture of glutamic and aspartic acids plus arginine, or a balanced mixture of amino acids (hydrolyzed casein). Maximum growth is found with nitrate plus hydrolyzed casein.

Many synthetic mixtures of amino acids are unable to support growth. Many single amino acids are inhibitory, and when added (at 2 mM or less) to cultures, growing in the presence of nitrate, cause a decrease in growth rate or even death of the plants (e.g. with alanine, valine, methionine or leucine). Some of these inhibitory effects are also found when the amino acid is added to cultures growing on ammonia or hydrolyzed casein. Arginine was the only amino acid of those tested which gave a marked stimulation of growth when added to cultures growing with inorganic nitrogen.

The rapid rate of growth, sterile nature of tissue, decreased biological variation of samples containing many plants and ability to utilize different culture media make this an attractive organism for studies on higher plant metabolism.

A considerable amount is known about the culture and growth of Lemna spp, but most studies have been concerned with growth and flowering behavior of the plant, rather than metabolism (see review by Hillman, 6).

For several reasons Lemna is well suited for use in metabolic and enzymological studies, yet relatively few workers have taken advantage of this. Rapid growth of uniform clonal material is possible, and the large number of plants taken in each sample tends to decrease the biological variation in an experiment. Another advantage is the possibility of sterile growth, excluding the chance that bacterial contamination is contributing to observed results. Sterile culture allows the plant to be grown on media containing sugars and amino acids, impossible under non-sterile conditions.

Several aspects of the mechanism and enzymology of nitrogen metabolism in Lemna are now being investigated. As a preliminary part of the metabolic study this paper describes the basic methods in use and the growth responses (and side effects) on various nitrogen sources. No attempt has been made to analyze or control growth as precisely as has been done elsewhere in growth studies (e.g. 1,9).

Materials and Methods

The sterile culture of Lemna minor was isolated by Dr. Ann Oaks, from local material, by repeated hypochlorite treatment (as described by Hillman, 6). The plants were grown in 1 liter Erlenmeyer flasks, each containing 200 ml medium. Hillman’s M medium (5) with the addition of 30 μM EDTA (Cleland and Briggs, 2) was used as basic medium. This medium, consisting of sucrose, minerals, and nitrates (20 mM), was modified where necessary by substituting for nitrates either ammonium chloride (7.5 mM) or acid hydrolyzed casein (Cas-AA) at 3 g/l. When nitrate was omitted CaCl₂ at 5 mM was added to the medium. Nitrate and hydrolyzed casein media were adjusted to pH 4.6, while pH of ammonia medium was stabilized by addition of about 300 mg solid CaCO₃ to each flask (see Results). Flasks containing medium were autoclaved for 20 min at 15 lb. pressure. L-isomers of amino acids were used.

Stock plants were maintained on medium solidified with agar, but in general inoculation of flasks was from a liquid culture growing on hydrolyzed casein. This practice was advantageous for 2 reasons, firstly, the plants tended to have quite short roots and were thus easily separated for transfer; secondly, any contamination showed rapidly in this medium. Usually 2 plants, with 5 or 6 visible fronds, were used to inoculate each flask. Inoculations were carried out in a sterile room.

1 This work was carried out in the Department of Botany, University of Toronto and was supported by a grant from National Research Council of Canada.
The plants were grown in growth cabinets with a 16 hr day of fluorescent plus tungsten light of about 1000 ft-c at the medium surface. The day temperature was 23°, while night temperature was 20°.

For most experiments plants were collected by pouring the medium through cheesecloth, then were rinsed and shaken free of loose water. The plants, spread in the cheesecloth, were pressed lightly for 20 sec between pads of paper towels to remove a constant proportion of water, and were then weighed.

Fresh weight was taken as the measure of growth, since in many experiments the whole sample was required for enzyme assays. Dry matter was usually 8 to 10% fresh weight. Higher dry weights (11-14%) were sometimes found when growth was poor, and were attributed to large accumulations of starch in such tissues. Unless otherwise noted, the fresh weights presented represent averages of at least 2 replicates. The experiments were repeated when the difference between replicates was greater than 10%. Methods of enzyme assay are described in a later paper (7).

Results

Nitrogen Source and Addition of Carbohydrate.

Table I shows growth of *L. minor* with various nitrogen sources, in presence and absence of sucrose. Since sucrose doubled the growth rate, in all subsequent experiments sucrose was included in the medium. Light was required for optimum growth and even when sucrose was provided darkened or low light cultures grew at a much reduced rate (unpublished results). Several nitrogen sources were utilized, and growth was best with hydrolyzed casein. Nitrate was also a good source of nitrogen but ammonia, even in combination with nitrate, gave poor results. One initial problem with ammonia as nitrogen source was the acid drift of the medium. Within 2 weeks pH had fallen from 5.5 to about 3, when the whole culture would die. This acidification could be alleviated by changing the medium at weekly intervals, or more conveniently by adding a small amount of CaCO₃ to each flask. Even with this precaution, however, the growth rate was slow with ammonia and cultures did not look healthy after about 25 days.

Growth rates of cultures supplied with nitrate or Cas-AA are shown in Fig. 1. Under these conditions growth is not exponential, although part of the curve for nitrate grown cultures (21-36 days) appears to fit an exponential curve with a doubling time of 7 days. Growth is initially more rapid with Cas-AA, but the rate of increase is not maintained. For many metabolic experiments the period from 28 to 35 days is useful, giving a large amount of experimental material and a rapid rate of growth, a

![Fig. 1. Fresh weights of *L. minor* cultures grown for various times with hydrolysed casein (AA-C) or nitrate (NO₃⁻) as nitrogen source. The dashed line is an empirical curve for a doubling time of 7 days (but does not extrapolate to date of inoculation).](image)

Table I. Fresh Weight of *L. minor* Cultures Grown 17 Days With Various Nitrogen Sources, With and Without Sucrose

<table>
<thead>
<tr>
<th>Nitrogen source</th>
<th>Conc. (mg/l)</th>
<th>rate (mg/mg)</th>
<th>rate (mg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO₃⁻ (K⁺ + Ca²⁺)</td>
<td>20</td>
<td>1240</td>
<td>280</td>
</tr>
<tr>
<td>Cas-AA</td>
<td>approx 25</td>
<td>1850</td>
<td>875</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>7.5</td>
<td>860</td>
<td>400</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>7.5</td>
<td>665</td>
<td>...</td>
</tr>
</tbody>
</table>

1 Hydrolyzed casein.

Table II. Fresh Weight of *L. minor* Cultures Grown 29 Days With Various Amino Acid Mixtures as Nitrogen Source

<table>
<thead>
<tr>
<th>Fresh wt.</th>
<th>g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cas-AA</td>
<td>6.1</td>
</tr>
<tr>
<td>17 amino acids¹</td>
<td>Dead in 14 days</td>
</tr>
<tr>
<td>6 amino acids²</td>
<td>2.1</td>
</tr>
<tr>
<td>Arg + Gln + Asp³</td>
<td>6.9</td>
</tr>
<tr>
<td>Arg only³</td>
<td>1.7</td>
</tr>
</tbody>
</table>

¹ Asp, Glu, Pro, Gly, Ala, Val, Leu, Ile, Ser, Thr, all at 2 mm. Phe, Tyr, Trp, Cys, Met, His. Arg all at 1 mm.
² Asp, Glu, Pro, Gly, Ala, Val, all at 2 mm.
³ Each at 2 mm.
Amino Acid Mixtures. Hydrolyzed casein is a poorly defined medium, and an attempt was made to find a synthetic mixture of amino acids which would support growth. The results (table II) show that the most complex mixture tried, with 17 different amino acids, was inhibitory to growth, and caused death of the plants in 2 or 3 weeks. A number of different amino acid combinations were tried, and of those tried a simple mixture of arginine, aspartic and glutamic acids gave slightly better growth than Cas-AA, however at the concentration used (each amino acid at 2 mM) there would be little reserve of nitrogen for growth beyond 30 days.

Effect of Individual Amino Acids. In view of the inhibitory effects of some amino acid mixtures, plants were grown in media containing nitrate as the main nitrogen source, to which a range of single amino acids had been added. The results (table III) show that of the 13 amino acids tested 9 gave considerable inhibition of growth, and with alanine, valine, leucine, and methionine the effect was so strong that the plants died. Aspartic acid, ornithine, and citrulline had relatively small effects on growth, while arginine gave considerable stimulation of growth, more than doubling the yield given by media containing only nitrate nitrogen. Addition of Cas-AA to the nitrate medium gave further stimulation of growth.

Similar experiments were performed to determine the effect of varying concentrations of leucine and valine on growth of Lemna on nitrate (half strength, 10 mM) medium. Death of the plants was caused by 0.2 mM leucine and 0.1 mM valine, while at 0.05 mM both leucine and valine severely restricted growth but did not kill the plants. At 0.02 mM there was little effect of either amino acid on growth.

Filner (3) implicated amino acids in control of growth of cultured tobacco cells through action on nitrate reductase. For this reason the effect of some amino acids were tried with main nitrogen sources other than nitrate. The results are also given in table III. Although not exactly the same as with nitrate, the results show that alanine, leucine, valine, and methionine again gave markedly inhibitory effects with either ammonia or Cas-AA.

In the experiments described above the amino acid was present in the flask at time of inoculation. Amino acids were also added to well established cultures (table IV). During 3 days incubation, cultures with fresh nitrate or nitrate plus arginine continued to grow, but growth was arrested when leucine or valine were added. The higher level of nitrate in the fresh medium induced the formation of additional nitrate reductase and to some extent, nitrite reductase. Arginine had no inhibitory effect on this induction. Leucine and valine severely checked the production of these 2 enzymes, although it is clear that some induction did occur and the enzyme levels in the tissue were quite high.

Table IV. Effect of Addition of Some Amino Acids on Growth and Enzyme Levels in Established Cultures of L. minor

Cultures were grown 36 days on nitrate media, then transferred for 3 days to fresh NO₃ medium with or without amino acids added at 2 mM (arginine at 1 mM).

<table>
<thead>
<tr>
<th>Nitrate reductase</th>
<th>Nitrite reductase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh g</td>
<td>mU/g</td>
</tr>
<tr>
<td>Original (Day 36)</td>
<td>9.3</td>
</tr>
<tr>
<td>3 days</td>
<td>+ NO₃⁻ (Day 39)</td>
</tr>
<tr>
<td></td>
<td>+ NO₃⁻ + Arg</td>
</tr>
<tr>
<td></td>
<td>+ NO₃⁻ + Leu</td>
</tr>
<tr>
<td></td>
<td>+ NO₃⁻ + Val</td>
</tr>
</tbody>
</table>

Discussion

In culture, hydrolyzed casein, nitrate, or ammonia will support the growth of Lemna. Ammonia however, is a relatively poor nitrogen source, even when pH drift is controlled or when nitrate is also supplied, suggesting that some toxicity must be involved.

A "balanced" mixture of amino acids, such as complex hydrolyzed casein, or a simple mixture of arginine, aspartate, and glutamate support growth. Only 1 amino acid (arginine) of those tried was stimulatory to growth of Lemna when supplied together with inorganic nitrogen, but it is found that some mixtures and many single amino acids depress growth. This obtains even when another...
easily utilized nitrogen source is present, showing that there is a positive inhibitory action, rather than an effect due to nitrogen deficiency caused by inability to use the single amino acid. Leucine, valine, and methionine have an inhibitory effect even when present in conjunction with hydrolyzed casein, which already contains these particular amino acids, their addition causing approximately a 5-fold increase in concentration. There is no indication of how such inhibition may be caused. Nakashima (8) investigated flowering and frond number of *L. gibba*, and found that some amino acids, including alanine, valine, and leucine, inhibited flowering and also depressed growth, as found here.

Filner (3) found that many amino acids repressed nitrate reductase formation in cultured tobacco cells, and concluded that the accompanying decrease in growth was a direct consequence of nitrogen starvation caused by repression of this enzyme. In *Lemna*, leucine and valine are found to affect growth and nitrate reductase (table IV) but the effect on growth is also found when the plants are utilizing ammonia or Cas-AA as their source of nitrogen. when nitrate reductase is absent in any case (7), or when considerable amounts of the enzyme is present (table IV). It therefore appears that in *Lemna* (and possibly also in cultured tobacco cells) the effect on nitrate reductase may be secondary, synthesis of the enzyme being retarded by a slowing of some other area of metabolism, rather than the effect on nitrate reductase being the primary cause of cessation of growth. Inhibition of growth and repression of nitrate reductase may of course be 2 distinct and separate effects of the amino acids. There is as yet no indication of which areas of metabolism might be affected. A single amino acid in gross excess could interfere in the delicate mechanisms of amino acid synthesis, intracellular transport or protein synthesis but it is less easy to understand how a lesser excess in a balanced mixture can also cause such severe effects. Many other workers have noticed inhibitory effects of single amino acids (4, 10).

The beneficial effect of arginine is exceptional, and the fact that arginine (and to some extent citrulline) increase growth on nitrate medium suggests that arginine is closely associated with the rate limiting step in normal nitrate growth. Filner (3) and others (4, 10) have also noted growth promotion with arginine.

**Acknowledgments**

I thank Dr. Ann Oaks for supplying the initial sterile culture, Mrs. P. Merrilees for capable technical assistance, and Miss S. Alim who helped with some of the initial experiments.

**Literature Cited**