Ammonium Influence on the Growth and Nitrate Reductase Activity of Paul's Scarlet Rose Suspension Cultures

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ABSTRACT

Suspension cultures of Paul's Scarlet rose were grown in two defined media which differed only in their inorganic nitrogen content. Both possessed equal amounts of NO₃⁻ (24 mM), but differed in that NH₄⁺ (0.91 mM) was present in control medium; whereas, no NH₄⁺ was present in the test medium. A comparison of fresh weight increases over a 14-day growth period showed that NH₄⁺ caused a 2-fold stimulation in growth and governed the pattern of development.

Ammonium also caused a 2-fold increase in nitrate reductase activity but had little influence on the activity of representative enzymes from the Embden-Meyerhof pathway or citric acid cycle. Thus NH₄⁺ enhanced the nitrate reductase activity which was correlated with increased growth.

Ammonium had no influence on the in vitro activity of nitrate reductase which suggested that the stimulatory influence was due to an increased synthesis of the enzyme. The enhanced synthesis did not appear to be due to an increased availability of NO₃⁻ since the uptake of NO₃⁻ by intact cells was not influenced by the presence of NH₄⁺ during the period of most rapid increase in nitrate reductase activity.

The purpose of this study was to examine the influence of NH₄⁺ on NR activity in suspension cultures of Paul's Scarlet rose, and to determine the relationship between NR activity and cell growth.

MATERIALS AND METHODS

Cell Suspension Cultures. This investigation was performed with nonphotosynthetic suspension cultures of Paul's Scarlet rose grown in the dark. Cells were grown in 250-ml Erlenmeyer flasks containing 80 ml of MPR medium (22) with an initial pH of 5.5. Each new culture was started by inoculating a flask containing sterile medium with approximately 0.5 g of cells from a 14-day-old culture. The primary nitrogen source in all cultures was 24 mM NO₃⁻. During the transfer of cells, sufficient (NH₄)₂SO₄ was added to each control flask to give a 0.91 mM concentration of NH₄⁺. A sterile syringe equipped with a 0.22-nm Millipore filter was used for (NH₄)₂SO₄ additions. Similar additions of (NH₄)₂SO₄ were made on selected days during the growth cycle.

Fresh weight determinations were made at the times indicated by filtering the medium through Miracloth held in a Buchner funnel and weighing the cells immediately.

Nitrate and Ammonium Assays. Samples of 5 ml each were drawn aseptically from both control and without NH₄⁺-grown cultures on each day. Following removal of cells by filtration the NH₄⁺ concentration of the medium was determined with an NH₄⁺ ion electrode (model 95-10, Orion Research Inc., Cambridge, Mass.). The concentration of NO₃⁻ in the medium was determined with a NO₃⁻ ion electrode (model 92-07, Orion Research Inc.).

Enzyme Assays. For the determination of NR activity, 1 g of cells was homogenized for 30 sec with a Polytom homogenizer. The grinding medium consisted of 4 ml of cold 0.1 M tris-HCl buffer (pH 7.5) containing 0.01 M cysteine-HCl and 0.3 mM EDTA. The homogenate was pressed through Miracloth and centrifuged at 20,000g for 15 min. The supernatant liquid was used immediately for enzyme assay. Nitrate reductase was measured according to the methods of Hageman and Flesher (10).

The assay mixture contained 25 μmol of K-phosphate buffer, pH 7.5, 10 μmol of KNO₃, 0.42 μmol of NADH, 0.2 μl of enzyme extract, and deionized water to bring the volume up to 2 ml. The blank used was the complete assay mixture without NADH.

Extracts used for other enzyme assays were prepared by grinding (20 strokes) 1 g of cells with a glass TenBroek homogenizer. The grinding medium prepared according to Cooper and Beevers (3) consisted of 4 ml of 165 mM Tricine buffer (pH 7.5), 0.4 M sucrose, 10 mM KCl, 10 mM MgCl₂, 10 mM EDTA, and 10 mM β-mercaptoethanol. The homogenate was centrifuged for 10 min at 500 g to remove unbroken cells and cell fragments. The supernatant solution was then centrifuged for 15 min at 10,000g yielding a crude mitochondrial pellet and clear supernatant. This supernatant was used as assay for NAD-glyceraldehyde-3-P dehydrogenase.

In previous work with suspension cultures of Paul's Scarlet rose, cells were shown to grow with only NO₃⁻ serving as a nitrogen source, but maximum growth required a supplemental amount of either NH₄⁺ or glutamine (22). Similar requirements were reported for suspension cultures of soybean (1, 23). Thus, in these tissue culture systems a reduced form of nitrogen such as NH₄⁺ is required for the maximum utilization of NO₃⁻ provided in the medium.

It is well accepted that NO₃⁻ induces the synthesis of NR1, the first enzyme in the pathway of NO₃⁻ reduction. However, the regulatory role played by NH₄⁺, end-product of NO₃⁻ reduction, is less clearly understood. Ammonium has been shown to repress the induction of NR in Neurospora, Chlorella, and Lemnaeae (2). In most higher plants NH₄⁺ either has no influence or it enhances the induction of NR by NO₃⁻ (2, 11).

Based on extensive studies with genetic hybrids of corn and wheat, Hageman and colleagues (7, 8) proposes that the level of NR in a plant governs the growth and yield of the plant. Despite the close correlation in hybrids between growth and NR activity it is impossible, in intact plants, to rule out the contribution made to growth by other favorable characteristics such as increased photosynthesis, translocation, etc., which might accompany the genetic information for increased NR activity. Tissue cultures offer a simple system whereby the relationship between NR activity and growth can be examined more directly.

Abbreviations: NR: nitrate reductase; MS: mitochondrial suspension.
hydrogenase and NADP-dependent isocitrate dehydrogenase. The crude mitochondrial pellet was washed once with MS medium (18) composed of 0.25 mM sucrose, 2 mM EDTA, 5 mM cysteine-HCl, and 50 mM tris-HCl buffer adjusted to pH 7.4. The suspension was centrifuged for 15 min at 10,000g. The pellet obtained from the above centrifugation was suspended in 1 ml of MS medium and was used immediately for the enzyme assay.

The NADP-dependent isocitrate dehydrogenase activity was determined by the method of Kornberg (15). The reaction mixture contained, in a final volume of 3 ml, 250 µmol of tris-HCl buffer, pH 7.5, 0.16 µmol of MgSO₄, 12 µmol of DL-isocitrate, 2 µmol of NADP, and 0.2 ml of enzyme preparation.

The NAD-dependent isocitrate dehydrogenase was assayed according to the method of Coultate and Dennis (5). The reaction mixture contained, in a final volume of 4 ml, 120 µmol of tris-HCl buffer, pH 8.5, 2 µmol of NAD, 10 µmol of cysteine-HCl, 51 µmol of sodium arsenate, and 0.1 ml of enzyme preparation. The reaction was started by adding 20 µmol of DL-glyceraldehyde-3-P.

RESULTS

Growth Kinetics. Control cells cultured in complete medium (Fig. 1) showed three phases of growth (day 0 to 3, 3 to 9, and 9 to 14). These phases correspond to the lag, division, and expansion phases which are characteristic of some suspension cultures (24). At the conclusion of the 14-day growth period the cells weighed 19.7 g. This was a 40-fold increase in fresh weight.

When cells were grown in medium with no NH₄⁺ (Fig. 1), the pattern of growth was changed. The lag phase lasted until day 5, and this was followed by a second rate of growth which continued until day 12 whereupon a third rate of growth commenced. At no time did the growth rate of these cells equal that of the control cells. The fresh weight after 14 days of growth was 9.6 g. This was a 19-fold increase in fresh weight.

The influence of NH₄⁺ on growth was studied further by observing the changes in growth when NH₄⁺ was added to cells previously cultured for 1 or 5 days in medium without NH₄⁺ (Fig. 1). Upon addition of NH₄⁺ on either day 1 or 5, the growth rate and final fresh weight were increased in comparison to cells grown for 14 days without NH₄⁺. In both cases the fresh weight after 14 days of growth was still less than that of the control cells, and the separate phases of division and expansion were less distinct.

Effect of Ammonium on Development of Nitrate Reductase. There was no appreciable increase in NR activity after the first day following transfer of cells to fresh medium (Fig. 2). Subsequently, the activity in the control cells increased rapidly and attained a maximum value of 5.9 µmol of KNO₃/hr.g fresh weight on day 5. In contrast to this, when cells were grown without NH₄⁺, the maximum NR activity of 2.2 µmol of KNO₃/hr.g fresh weight occurred on day 6 and it was only half of the activity present in control cells on day 5. The developmental patterns were the same when the data were expressed on a per µg of protein basis.

When NH₄⁺ was added to cultures which had been previously grown for 1, 2, or 4 days without NH₄⁺ there was a pronounced increase in NR activity within 1 day. This was most apparent when NH₄⁺ was added to day 2. In this case, NR activity increased 8-fold during the first 24 hr following NH₄⁺ addition, as compared to a 3-fold increase in cells which did not receive the NH₄⁺ addition. Furthermore the rate at which NR activity increased during the first 24-hr period following NH₄⁺ addition was equal to the most rapid rate at which the enzyme activity increased in the control cells. However when NH₄⁺ was added on days 1, 2, or 4 the period of rapid increase in enzyme activity lasted for only 1 day; whereas, in control cells this rate continued for 3 days. The delayed addition of NH₄⁺ caused a rapid increase in NR activity but the period of rapid increase was of short duration. As a result of this the maximum enzyme activity of approximately 2.9 µmol of KNO₃/hr.g fresh weight was only half of the enzyme activity observed in the control cells on day 5. 

Effect of Ammonium on Development of Isocitrate Dehydro-
genase and Glyceraldehyde-3-P Dehydrogenase. Both the particulate and the soluble isocitrate dehydrogenase were examined and their developmental patterns were observed to be about the same (Fig. 3). For example in control cells the maximum activity for both the particulate and the soluble enzyme appeared on day 3; whereas, the maximum level for both enzymes in cells grown without NH₄⁺ occurred on day 5. The maximum activities of isocitrate dehydrogenase attained in cells grown in both media were approximately equal. The soluble, NADP-requiring isocitrate dehydrogenase was more plentiful in the young cells; whereas, the particulate bound, NAD-requiring enzyme was the predominant form of the enzyme in the older cells, an observation which differs with previous reports on this enzyme (3, 25).

In control cells grown with NH₄⁺ glyceraldehyde-3-P dehydrogenase activity increased rapidly and reached its maximum value on day 4 (data not shown). The activity of this enzyme also increased rapidly in cells grown without NH₄⁺, but reached its maximum value 1 day later. The maximum activity attained in both cultures was approximately the same.

**Uptake of NO₃⁻ and NH₄⁺ from Medium.** The removal of NH₄⁺ and NO₃⁻ from the medium was determined. During the first 3 days of growth, NH₄⁺ was rapidly removed from the medium (Fig. 4), and by day 5 no NH₄⁺ was detected in the medium. The uptake of NO₃⁻ from the medium of both cultures was almost identical during the first 6 days, the period during which NR activity increased most rapidly. Slight differences in NO₃⁻ uptake were observed after the NH₄⁺ was depleted from the medium. Thus NH₄⁺ did not appear to influence the uptake of NO₃⁻.

**DISCUSSION**

In an earlier study (9) it was shown through DNA determinations and microscopic examination of rose cells that the lag and division phases were periods of growth when the rate of cell division exceeded the rate of expansion; whereas, during the expansion phase the reverse was true, the rate of expansion exceeded the rate of division. Nash and Davies (20) did not observe these phases of growth in their work with suspension cultures of Paul's Scarlet rose. In the present study cells grown for 14 days in 80 ml of MPR medium had a 40-fold increase in fresh weight and a final yield of 19.7 g. Furthermore the growth kinetics showed distinct phases of growth comparable to those reported in previous work from this laboratory (6, 9, 21). The present work showed that when cells were grown in MPR medium without NH₄⁺ only a 19-fold increase in fresh weight occurred, the fresh weight yield was reduced to 9.6 g, and the division and expansion phases were less distinct. These results were strikingly similar to those of Nash and Davies where a 16-fold increase in fresh weight and a final yield of 12 g was reported after 14 days growth in 90 ml of medium (20). We believe the difference in growth of Paul's Scarlet rose as reported by Nash and Davies versus that of our laboratory was due primarily to the omission of NH₄⁺ from the medium used by Nash and Davies. It follows that NH₄⁺ governs both the rate and pattern of growth in suspension cultures of Paul's Scarlet rose.

Whenever cells were provided with NH₄⁺ either at the beginning or during their growth cycle, the final fresh weight was increased. The presence of NH₄⁺ also led to increased NR activity. Cells transferred to medium with NH₄⁺ developed twice as much NR activity and grew twice as much as cells grown for 14 days in medium without NH₄⁺. This tight correlation between the NR activity and growth was consistent with the finding of Hageman that growth was proportional to the amount of NR present in genetic hybrids of wheat and corn (7, 8). The present study also suggested that the time of maximum NR activity was also important in governing the amount of cell growth. This was borne out by observing that NH₄⁺ added on different days during growth (day 1, 2, or 4) led to an earlier development of NR but not a substantially greater amount of enzyme activity in comparison to the cells grown without NH₄⁺ (Fig. 2). However, the earlier NH₄⁺ was added in the growth cycle the greater the fresh weight yield was on day 14 (Fig. 1). Thus both the amount and
time of NR development governed the growth of tissue culture cells.

We believe the stimulatory influence of NH$_4^+$ on NR activity was due to enhanced enzyme synthesis since NH$_4^+$ had no in vitro influence on the enzyme, an observation which is consistent with the work of others (16, 19). The magnitude of the NH$_4^+$ stimulation when it was included in the starting medium was approximately the same as reported for soybean tissue cultures (1) and mug bean seedlings (12). Addition of NH$_4^+$ on selected days during growth showed that the cation brought on an 8-fold stimulation of NR activity in 24 hr. This result was the opposite of what has been observed in Chlorella cultures where NH$_4^+$ added after the development of NR activity had begun prevented any further increase in the enzyme (19). Thus the synthesis of NR in rose cells was stimulated by NH$_4^+$, and the mechanism responsible for this appears to be characteristic of higher plants (1, 12, 13), but not algae and fungi (14, 17, 19).

The enhanced activity of NR by NH$_4^+$ may have been a general influence of NH$_4^+$ on enzyme synthesis. To examine this possibility, glyceraldehyde-3-P dehydrogenase and isocitrate dehydrogenase were studied as representative of the Embden-Meyerhof-Parnas pathway and the tricarboxylic acid cycle, respectively. Since NH$_4^+$ did not substantially alter the level of these enzymes it was concluded that NH$_4^+$ had a selective influence on the synthesis of certain enzymes which included nitrate reductase.

LITERATURE CITED