THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

INFLUENCE OF AMMONIUM ON GROWTH, PROTEIN SYNTHESIS AND NITROGEN ASSIMILATING ENZYMES IN PAUL'S SCARLET ROSE

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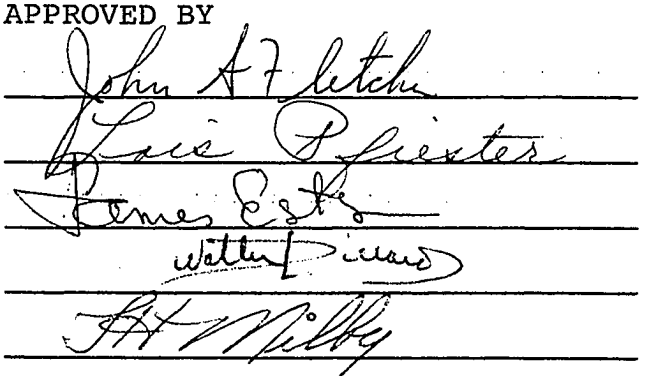
INFLUENCE OF AMMONIUM ON GROWTH, PROTEIN SYNTHESIS AND NITROGEN ASSIMILATING ENZYMES IN PAUL'S SCARLET ROSE

APPROVED BY



DISSERTATION COMMITTEE

ABSTRACT

Suspension cultures of Paul's Scarlet rose were grown on two defined media which differed only in their inorganic nitrogen content. Control medium possessed 24 mM NO_3^- and 0.91 mM NH_4^+ whereas, no NH_4^+ was present in the test medium. A comparison of fresh weight and protein content over a 14-day growth period showed that NH_4^+ caused a two-fold stimulation in both of these parameters of growth. It was shown that cells grown with NH_4^+, assimilated 60% of the absorbed inorganic nitrogen into amino acids as compared to only a 30% conversion by cells grown without NH_4^+.

A developmental study of several key enzymes in respiration and nitrogen assimilation indicated that NH_4^+ selectively enhanced the synthesis of nitrate reductase, glutamate dehydrogenase and glutamate synthetase. It is proposed that the increased activity of these enzymes was responsible for increased protein synthesis and growth.

ACKNOWLEDGEMENTS

I express my deep sense of gratitude and sincere thanks to Dr. John S. Fletcher for his inspiring guidance, constant advice and keen supervision during the course of this study and in the preparation of this manuscript. Appreciation is also expressed to the members of my advisory committee, Dr. Elroy L. Rice, Dr. James R. Estes, Dr. T.H. Milby, Dr. Lois Pfiester, and Dr. Walter L. Dillard for their advice and time.

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INFLUENCE OF AMMONIUM ON GROWTH, PROTEIN SYNTHESIS AND NITROGEN ASSIMILATING ENZYMES IN PAUL'S SCARLET ROSE

INTRODUCTION

In an earlier study it was observed that suspension cultures of Paul's Scarlet rose grown in a defined medium needed a supplemental amount of either NH_4^+ or glutamine for maximum growth to occur. Ammonium has also been shown to stimulate the growth of several other plant tissue cultures as well as some intact plants. The purpose of this investigation was to determine how NH_4^+ stimulates growth. Specific attention was given to the influence of ammonium on growth, protein synthesis and the development of nitrogen assimilating enzymes during the normal 14 day growth cycle of Paul's Scarlet rose.

The results of this study are presented in two papers prepared according to the instructions for contributors to Plant Physiology.

PAPER I

AMMONIUM INFLUENCE ON THE GROWTH AND NITRATE REDUCTASE ACTIVITY OF PAUL'S SCARLET ROSE SUSPENSION CULTURES

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 NO_3^- (24 mM) but differed in that NH_4^+ (0.91 mM) was present in control medium; whereas, no NH_4^+ was present in the test medium. A comparison of fresh weight increases over a 14 day growth period showed that NH_4^+ caused a two-fold stimulation in growth, and governed the pattern of development.

Ammonium also caused a two-fold increase in nitrate reductase activity but had little influence on the activity of representative enzymes from the Embden Meyerhof pathway or Kreb's cycle. Thus NH_4^+ 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 NO_3^- since the uptake of NO_3^- by intact cells was not influenced by the presence of NH_4^+ during the period of most rapid increase in nitrate reductase activity.

PAPER I

AMMONIUM INFLUENCE ON THE GROWTH AND NITRATE REDUCTASE ACTIVITY OF PAUL'S SCARLET ROSE SUSPENSION CULTURES

CHAPTER I

INTRODUCTION

In previous work with suspension cultures of Paul's Scarlet rose, cells were shown to grow with only NO_3^- serving as a nitrogen source, but maximum growth required a supplemental amount of either NH_4^+ or glutamine (21). Similar requirements were reported for suspension culture of soybean (1,23). Thus in these tissue culture systems a reduced form of nitrogen such as NH_4^+ is required for the maximum utilization of NO_3^- provided in the medium.

It is well accepted that NO_3^- induces the synthesis of nitrate reductase, the first enzyme in the pathway of NO_3^- reduction. However, the regulatory role played by NH_4^+, end-product of NO_3^- reduction, is less clearly understood. Ammonium has been shown to repress the induction of nitrate reductase in Neurospora, Chlorella, and Lemnaceae (9,14,17, 19). In most higher plants NH_4^+ either has no influence (2)

or it enhances the induction of nitrate reductase by NO_3^- (1,12,13,23).

Based on extensive studies with genetic hybrids of corn and wheat, Hageman and colleagues (7,8) proposed that the level of nitrate reductase in a plant governs the growth and yield of the plant. Despite the close correlation in hybrids between growth and nitrate reductase 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 nitrate reductase activity. Tissue cultures offer a simple system whereby the relationship between nitrate reductase activity and growth can be examined more directly.

The purpose of this study was to examine the influence of NH_4^+ on nitrate reductase activity in suspension cultures of Paul's Scarlet rose, and to determine the relationship between nitrate reductase activity and cell growth.

CHAPTER II

MATERIALS AND METHODS

Cell Suspension Cultures

This investigation was performed with non-photosynthetic 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 (21) 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) NO_3^-. During the transfer of cells, sufficient $(NH_4)_2SO_4$ was added to each control flask to give a 0.91 mM concentration of NH_4^+. A sterile syringe equipped with 0.22 mμ millipore filter was used for $(NH_4)_2SO_4$ additions. Similar additions of $(NH_4)_2SO_4$ were made on selected days during the growth cycle.

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

Nitrate and Ammonium Assays

Samples of 5 ml each were drawn aseptically from both control and without NH_4^+ grown cultures on each day. Following removal of cells by filtration the ammonium concentration of the medium was determined with an ammonium ion electrode (Model 95-10, Orion Research Inc., Cambridge, Mass., USA). The concentration of nitrate in the medium was determined with a nitrate ion electrode (Model 92-07, Orion Research Inc.).

Enzyme Assays

For the determination of nitrate reductase activity, one gram of cells was homogenized for 30 sec. with a Polytron 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 20,000 g for 15 minutes. The supernatant liquid was used immediately for enzyme assay. Nitrate reductase was measured according to the methods of Hageman and Flesher (11). The assay mixture contained 50 µmoles of potassium-phosphate buffer pH 7.5; 20 µmoles of KNO_3; 0.42 µmoles of NADH; 0.2 ml of enzyme extract, and deionized water to bring the volume up to 2.0 ml. The blank used was the complete assay mixture without NADH. The assay mixture was incubated at 30°C for 15 minutes. Following incubation, the reaction was stopped and the color was developed by the addition of 2 ml of a solution containing equal volumes of 1% w/v sulfanilamide in 3N HCl and 0.02% w/v N-(1-napthyl diamine

dihydrochloride) in deionized water. Precipitated protein was removed by centrifugation and the absorbance of the supernatants at 540 nm was measured. Nitrate reductase activity was expressed as μmol KNO_2^- formed per hour per gm fresh wt of tissue.

Extracts used for other enzyme assays were prepared by grinding (20 strokes) one gram of cells with a glass Ten Broeck 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 $MgCl_2$, 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 10,000 g yielding a crude mitochondrial pellet and clear supernatant. This supernatant liquid was used to assay for NAD-glyceraldehyde-3-P dehydrogenase and NADP dependent isocitrate dehydrogenase. The crude mitochondrial pellet was washed once with mitochondrial suspension (MS) medium (18) composed of 0.25 M sucrose, 2 mM EDTA, 5 mM cysteine-HCl, 50 mM tris-HCl buffer adjusted to pH 7.4. The suspension was centrifuged for 15 min at 10,000 g. 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 μmoles tris-

HCl buffer pH 7.5; 0.16 µmoles $MgSO_4$; 12 µmoles DL-isocitrate; 2 µmoles 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 3 ml, 120 µmoles of tris-HCl buffer pH 7.4; 0.16 µmoles $MnSO_4$; 12 µmoles DL-isocitrate; 2 µmoles NAD and 0.1 ml of mitochondrial suspension. The reactions for NAD and NADP dependent isocitrate dehydrogenases were initiated by the addition of enzyme preparations. The course of the reactions was followed by measuring the increase in absorbance at 340 nm and 25°C in 1 cm cuvettes using a Gilford spectrophotometer fitted with an external recorder.

Glyceraldehyde-3-P dehydrogenase activity was determined by a modification of the method of Cori et al., (4). A 2.8 ml reaction mixture contained 42 µmoles of sodium pyrophosphate buffer pH 8.5; 2 µmoles NAD; 10 µmoles cysteine-HCl; 51 µmoles sodium arsenate and 0.1 ml of enzyme preparation. The reaction was started by the addition of 0.2 ml of substrate (20 µmoles DL-glyceraldehyde-3-P). The course of the reaction was followed by measuring the increase in absorbance at 340 nm and 25°C.

CHAPTER III

RESULTS

Growth Kinetics

Control cells cultured in complete medium (Fig. 1) showed 3 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 NH_4^+ (Fig. 1), the pattern of growth was changed. The lag phase lasted five days. The growth rate increased on day five, and again on day 12. 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 NH_4^+ on growth was studied further by observing the changes in growth when NH_4^+ was added to cells previously cultured for 1 or 5 days in medium without NH_4^+ (Fig. 1). Upon addition of NH_4^+ on either day 1 or 5;

the growth rate and final fresh weight were increased in comparison to cells grown for 14 days without NH_4^+. However 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 the Development of Nitrate Reductase

There was no appreciable increase in nitrate reductase activity after the first day following the 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 μmoles KNO_2/hr/g fresh wt on day 5. In contrast to this, when cells were grown without NH_4^+ the maximum nitrate reductase activity of 2.2 μmoles KNO_2/hr/g fresh wt occurred on day 6 and it was only 1/2 of the activity present in control cells on day 5. The developmental patterns were the same when the data were expressed as μmoles KNO_2/hr/μg protein.

When NH_4^+ was added to cultures which had been previously grown for 1, 2 or 4 days without NH_4^+ there was a pronounced increase in nitrate reductase activity within 1 day. This was most apparent when NH_4^+ was added on day 2. In this case, nitrate reductase activity increased 8-fold during the first 24 hrs following NH_4^+ addition, as compared to a 3-fold increase in cells which did not receive the NH_4^+ addition. Furthermore the rate at which nitrate reductase activity increased during the first 24 hr period following NH_4^+ addi-

tion was equal to the most rapid rate at which the enzyme activity increased in the control cells. However when NH_4^+ 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. Thus the delayed addition of NH_4^+ caused a rapid increase in nitrate reductase activity but the period of rapid increase was of short duration. As a result of this the maximum enzyme activity of approximately 2.9 μmoles KNO_2/hr/g fresh wt was only 1/2 of the enzyme activity observed in the control cells on day 5.

Effect of Ammonium on the Development of Glyceraldehyde-3-P Dehydrogenase and Isocitrate Dehydrogenase

Cell extracts were assayed for glyceraldehyde-3-P dehydrogenase and isocitrate dehydrogenase. In control cells grown with NH_4^+ (Fig. 3) glyceraldehyde-3-P dehydrogenase activity increased rapidly and reached its maximum value on day 4. The activity of this enzyme also increased rapidly in cells grown without NH_4^+, but reached its maximum value one day later. The maximum activity attained in both cultures was approximately the same.

Both the particulate and the soluble isocitrate dehydrogenase were examined and their developmental patterns were observed to be about the same (Fig. 4). 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 NH_4^+ occurred

on day 5. The maximum activities of isocitrate dehydrogenase attained in cells grown in both media were approximately equal. It was interesting to note that 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 from most reports on this enzyme (3,25).

Uptake of NO_3^- and NH_4^+ from the Medium

The removal of NH_4^+ and NO_3^- from the medium was determined. During the first 3 days of growth, NH_4^+ was rapidly removed from the medium (Fig. 5), and by day 5 no NH_4^+ was detected in the medium. The uptake of NO_3^- from the medium of both cultures was almost identical during the first 6 days, the period during which nitrate reductase activity increased most rapidly. Slight differences in NO_3^- uptake were observed after the NH_4^+ was depleted from the medium. Thus NH_4^+ did not appear to influence the uptake of NO_3^-.

CHAPTER IV

DISCUSSION

In an earlier study (10) 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. In contrast to this, 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 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 (6,10,22). However, the present work showed that when cells were grown in MPR medium without NH_4^+ 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 NH_4^+ from the medium used by Nash and Davies. It follows that NH_4^+ governs both the rate and pattern of growth in suspension cultures of Paul's Scarlet rose.

Whenever cells were provided with NH_4^+ either at the beginning or during their growth cycle, the final fresh weight was increased. The presence of NH_4^+ also led to increased nitrate reductase activity. Cells transferred to medium with NH_4^+ developed 2 times as much nitrate reductase activity and grew twice as much as cells grown for 14 days in medium without NH_4^+. This tight correlation between the nitrate reductase activity and growth was consistent with the finding of Hageman that growth was proportional to the amount of nitrate reductase present in genetic hybrids of wheat and corn (7,8). However the present study also suggested that the time of maximum nitrate reductase activity was also important in governing the amount of cell growth. This was born out by observing that NH_4^+ added on different days during growth (day 1, 2, or 4) led to an earlier development of nitrate reductase but not a substantially greater amount of enzyme activity in comparison to the cells grown without NH_4^+ (Fig. 2). However, the earlier NH_4^+ 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 nitrate reductase development governed the growth of tissue culture cells.

We believe the stimulatory influence of NH_4^+ on nitrate reductase 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 mung bean seedlings (12). Addition of NH_4^+ on selected days during growth showed that the cation brought on an 8-fold stimulation of nitrate reductase 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 nitrate reductase activity had begun prevented any further increase in the enzyme (19). Thus the synthesis of nitrate reductase 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 nitrate reductase 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 representatives of the EMP pathway and the TCA 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.

Another possible explanation for the NH_4^+ stimulation of nitrate reductase activity was that NH_4^+ enhanced the uptake of NO_3^- from the medium, and this in turn caused an increased induction of nitrate reductase since it is well established that NO_3^- induces the synthesis of nitrate reductase (2). This possibility was discounted since the uptake of NO_3^- from the medium during the period of rapid nitrate reductase synthesis was almost identical in cultures grown with and without NH_4^+.

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PAPER I

LEGENDS FOR FIGURES

Figure 1. Influence of NH_4^+ on fresh weight increase. Control cells were grown in medium containing 0.91 mM NH_4^+. Other cells were grown in media with no NH_4^+, or NH_4^+ was added on day 1 or 5.

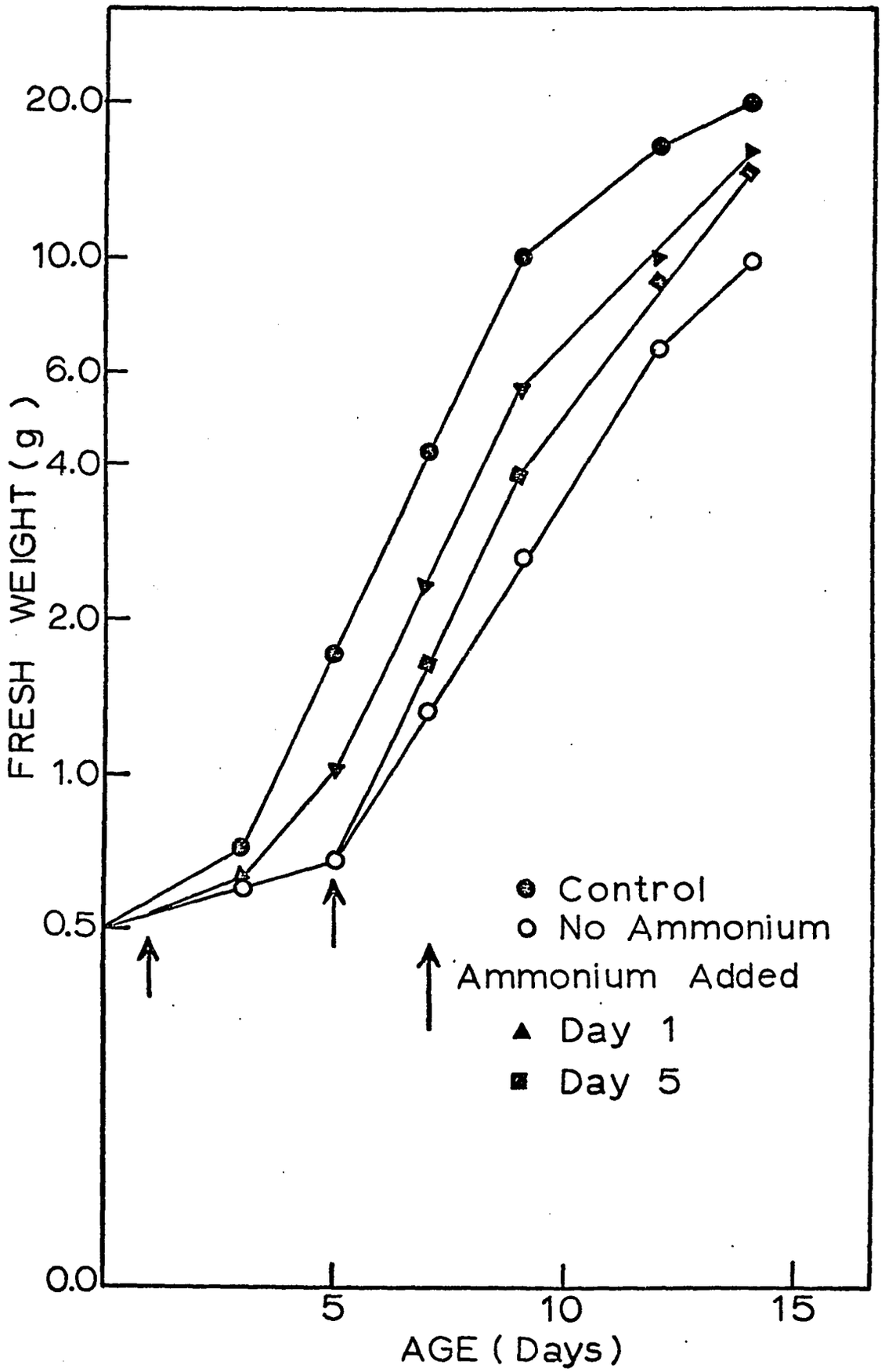
Figure 2. Influence of NH_4^+ on the development of nitrate reductase activity. Ammonium was provided as described in the legend of Fig. 1.

Figure 3. Influence of ammonium on the development of glyceraldehyde-3-P dehydrogenase activity. Ammonium was provided as described in the legend of Fig. 1.

Figure 4. Influence of ammonium on the development of isocitrate dehydrogenase activity. Ammonium was provided as described in the legend of Fig. 1.

Figure 5. Uptake of NO_3^- from the medium by control cells grown in medium with 0.91 mM NH_4^+ (■) versus uptake by cells grown in medium with no NH_4^+ (□). Uptake of NH_4^+ from the medium by control cells (▲). Changes in the nitrate reductase activity

of control cells (●) versus that of cells grown without NH_4^+ (○).


20.0
10.0
6.0
4.0
2.0
1.0
0.5
0.0
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5
10
15
AGE (Days)
Control
No Ammonium
Ammonium Added
Day 1
Day 5

Figure 1

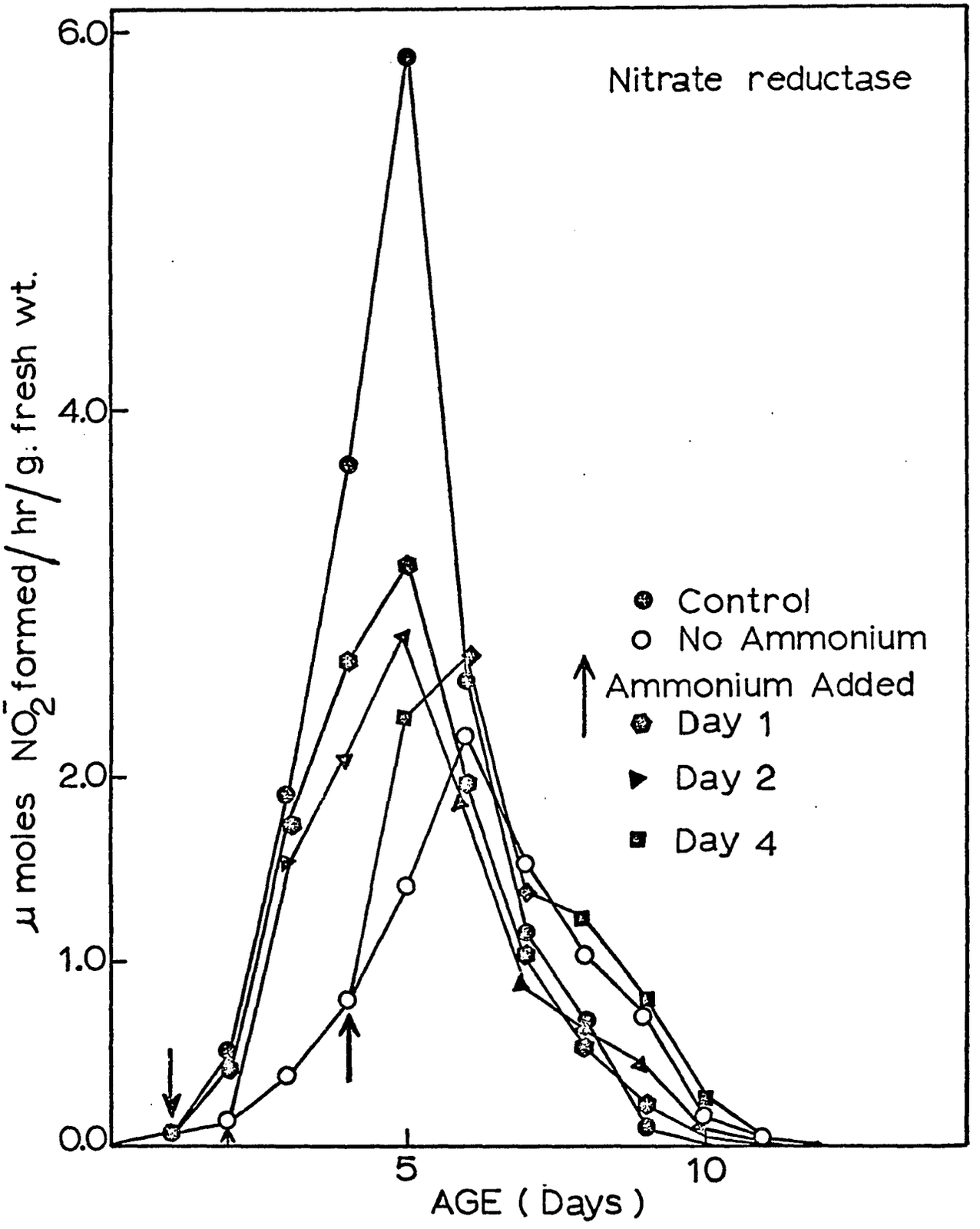
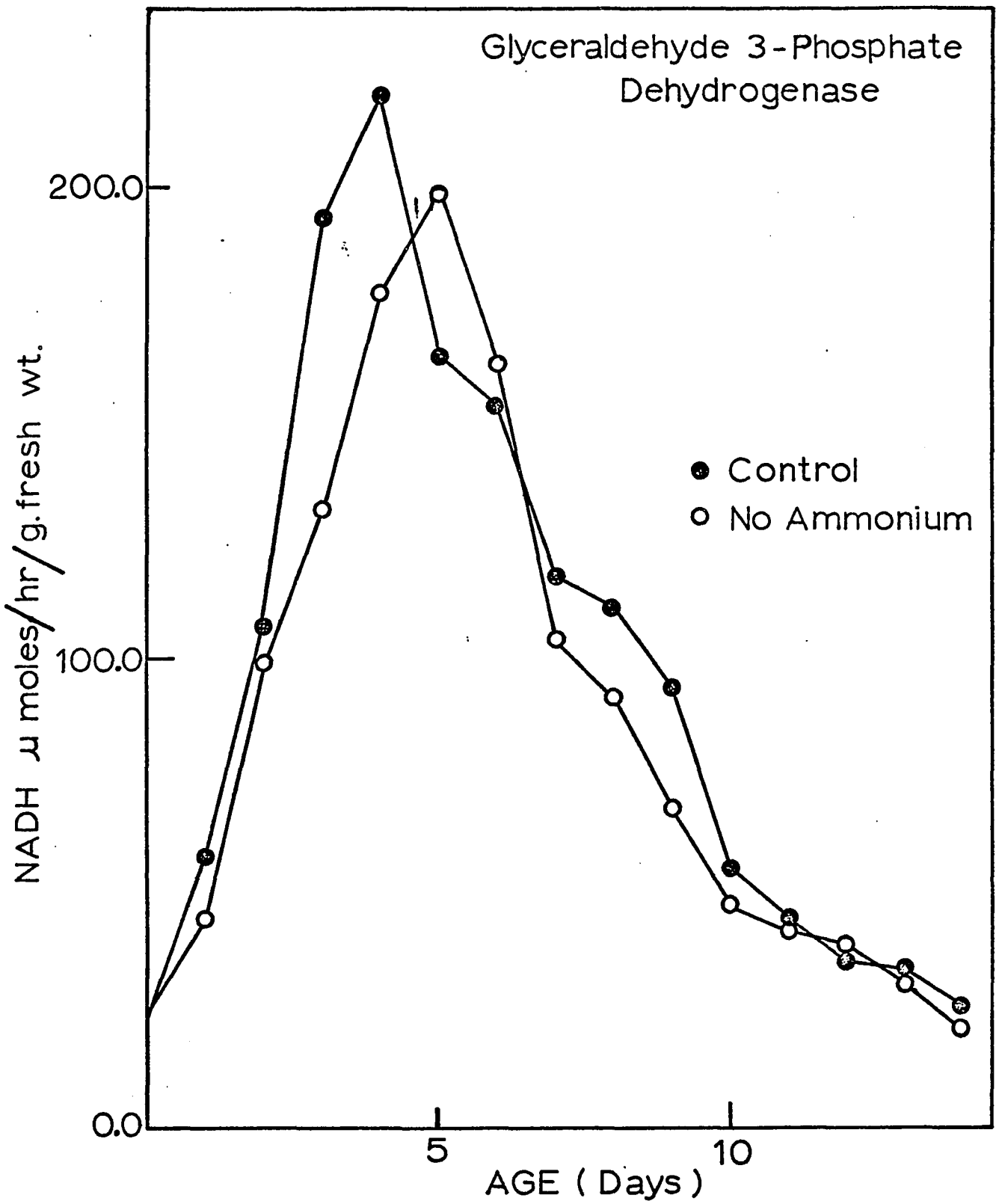

Nitrate reductase
6.0
4.0
2.0
1.0
0.0
μ moles NO_2^- formed/ hr/ g. fresh wt.
5
10
AGE (Days)
Control
No Ammonium
Ammonium Added
Day 1
Day 2
Day 4

Figure 2


Glyceraldehyde 3-Phosphate
Dehydrogenase
200.0
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0.0
NADH μ moles/hr/g. fresh wt.
Control
No Ammonium
5
10
AGE (Days)

Figure 3

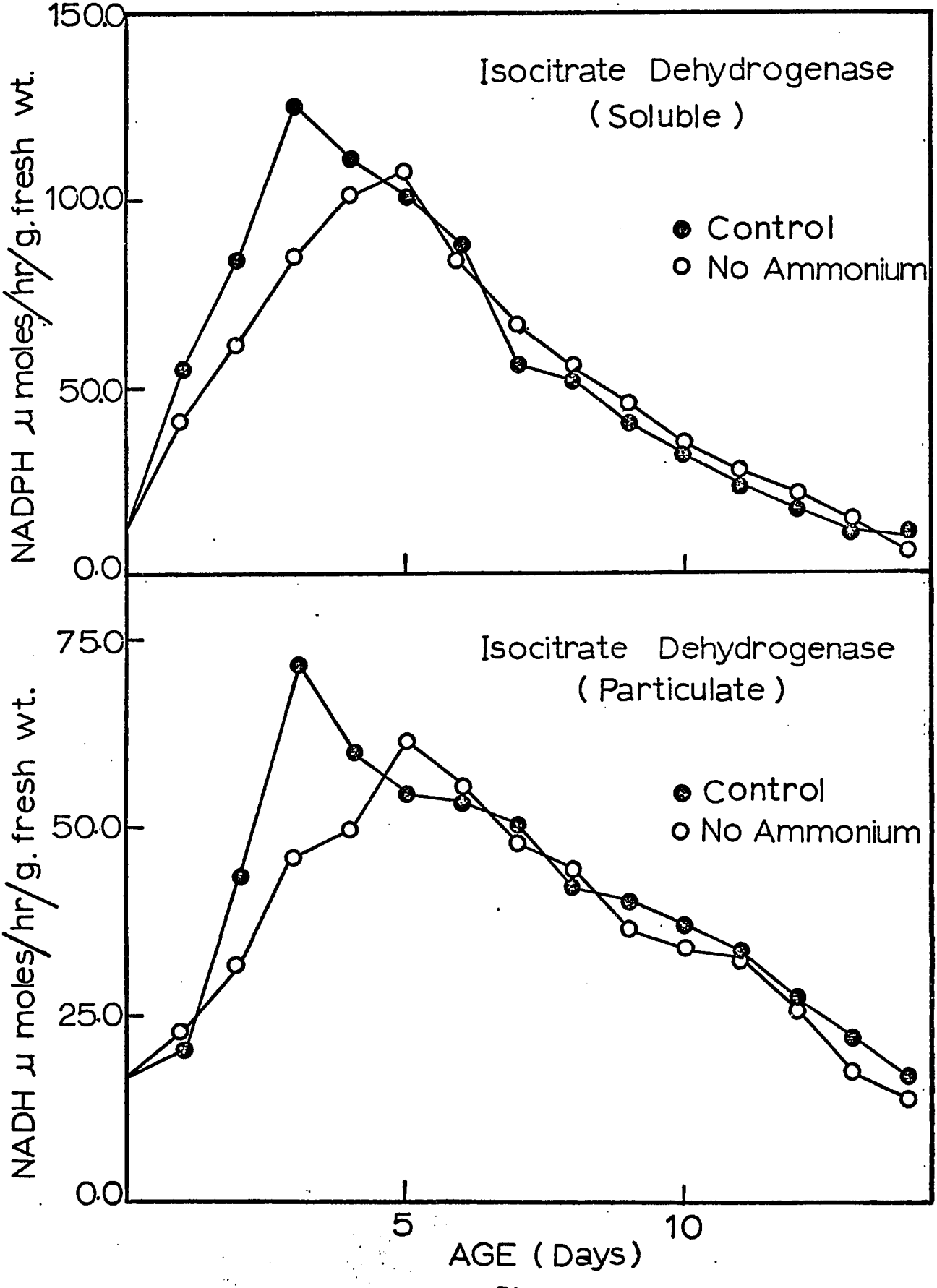
150.0
100.0
50.0
0.0
NADPH μ moles/hr/g. fresh wt.
Isocitrate Dehydrogenase
(Soluble)
Control
No Ammonium
75.0
50.0
25.0
0.0
NADH μ moles/hr/g. fresh wt.
Isocitrate Dehydrogenase
(Particulate)
Control
No Ammonium
5
10
AGE (Days)

Figure 4

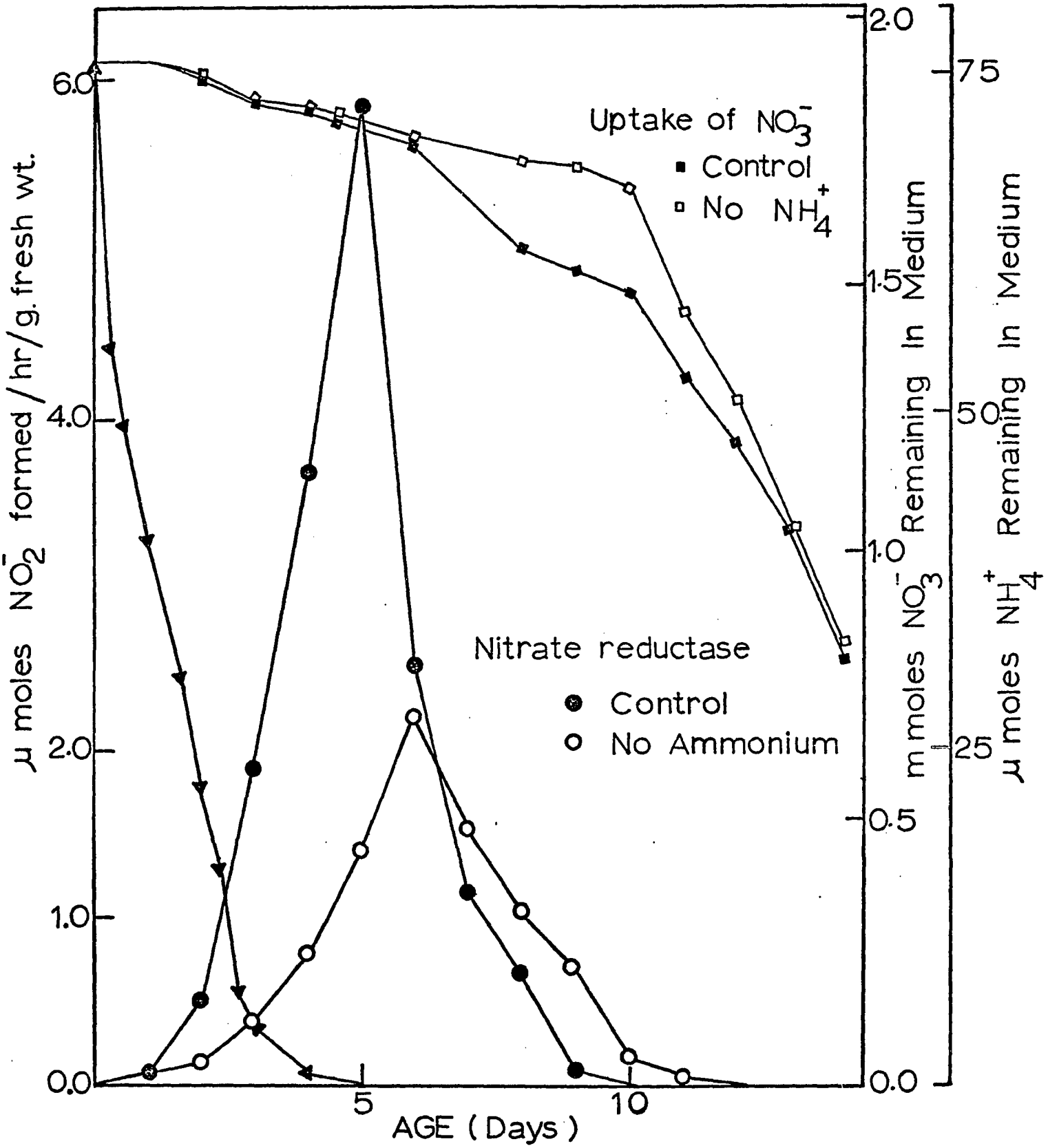
Uptake of NO_3^-
Control
No NH_4^+
Nitrate reductase
Control
No Ammonium
μ moles NO_2^- formed / hr / g. fresh wt.
m moles NO_3^- Remaining In Medium
μ moles NH_4^+ Remaining In Medium
AGE (Days)
6.0
4.0
2.0
1.0
0.0
2.0
1.5
1.0
0.5
0.0
75
50
25
5
10

Figure 5

PAPER II

INFLUENCE OF AMMONIUM ON PROTEIN SYNTHESIS AND NITROGEN ASSIMILATING ENZYMES IN PAUL'S SCARLET ROSE

ABSTRACT

The influence of NH_4^+ on protein synthesis was examined by growing suspension cultures of Paul's Scarlet rose on two defined media which differed only in their inorganic nitrogen content. Each medium contained 24 mM of NO_3^-, but only the control medium contained 0.91 mM NH_4^+. At the conclusion of a 14-day growth period cells grown with NH_4^+ possessed twice as much protein as those grown without NH_4^+. This two-fold difference held true for both the soluble and membrane-bound protein. The concentration of protein secreted into the medium changed with the age of the culture and the amount of secreted protein was significantly different when cells were grown in the presence of NH_4^+. Ammonium also caused a three-fold increase in the accumulation of soluble amino acids. There was no appreciable release of soluble amino acids into the medium over a 14 day growth period.

A developmental study of nitrogen assimilating enzymes showed that they reached their maximum activities on different days. The presence of ammonium enhanced the activities of nitrate reductase, glutamate dehydrogenase and glutamate synthetase. In contrast to this ammonium reduced the level

of glutamine synthetase during the first 4 days of growth. It is proposed that the increased activity of these enzymes was responsible for increased protein synthesis.

PAPER II

INFLUENCE OF AMMONIUM ON PROTEIN SYNTHESIS AND NITROGEN ASSIMILATING ENZYMES IN PAUL'S SCARLET ROSE

CHAPTER I

INTRODUCTION

In recent work from this laboratory (24) it was shown that when suspension cultures of rose cells were provided with NH_4^+ either at the beginning or during their growth cycle, the final fresh weight was increased. The presence of NH_4^+ also led to increased NR[1] activity. This appeared to be a selective stimulation of enzyme activity since NH_4^+ had no influence on the activity of glyceraldehyde-3-P dehydrogenase and isocitrate dehydrogenase. Thus, NH_4^+ appeared to stimulate the synthesis of NR which in turn led to increased growth.

In other systems it has been shown that NH_4^+ enhances the synthesis of GDH[2] (1,2,3,13,14,16,17,18,32,34); whereas, it reduces the activity of GS[3] (5,10,15,23,31,33,35). This

[1]NR: nitrate reductase
[2]GDH: glutamate dehydrogenase
[3]GS: glutamine synthetase

is of special interest since it is now recognized that these enzymes are members of alternate pathways leading from NH_3 to glutamate (7,9,19,20). Ammonia may be incorporated directly into glutamate through the action of GDH; or it may be incorporated into glutamine by GS and subsequently transferred to glutamate by GOGAT[4]. Since the synthesis of GDH and GS appear to be influenced in opposite manners by NH_4^+, it follows that the level of NH_4^+ in a cell may govern the flow of nitrogen through the alternate pathways.

In the present study the stimulatory influence of NH_4^+ on cell growth was studied further, by comparing the developmental pattern of key nitrogen assimilating enzymes (NR, GDH, GS and GOGAT) with the uptake of inorganic nitrogen and its assimilation into soluble and protein-bound amino acids.

[4]GOGAT: glutamate synthetase

CHAPTER II

MATERIALS AND METHODS

This investigation was performed with non-photosynthetic 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 with an initial pH of 5.5. The growth conditions and composition of the medium were the same as used previously (8,27).

Determination of Protein Contents

For the determination of cellular proteins, 1 g of cells was homogenized for 30 sec with a Polytron 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 centrifuged at 20,000 g for 15 min. The amount of protein present in supernatant (soluble) and in crude pellet (membrane-bound) was determined.

Proteins from 0.5 ml supernatant liquid was precipitated with 2.5 ml of cold 10% TCA and was used for protein assay. The crude pellet containing membrane bound proteins was homogenized for 30 sec in hot 80% ethanol and the homogenate was

centrifuged at 12,000 g for 10 min. The protein was then separated from the alcohol insoluble residue (pellet) according to the method of Osborn (30). Both the soluble and membrane-bound proteins were estimated by the method of Lowry, et al., (21), using bovine serum albumin as standard. Proteins present in the culture medium (secreted protein) was also estimated in a similar manner.

Determination of Soluble Amino Acids

One gram of cells was homogenized with 8 ml of 80% ethanol by using a VirTis grinder. The homogenate was filtered through glass fiber filter and the filtrate was evaporated to dryness on a waterbath at 37°C. The dried residue was dissolved in 10 ml of hot deionized water and a 0.5 ml sample was used to determine total alpha-amino nitrogen content with the ninhydrin-hydrindantin assay of Moore and Stein (25). The amount of soluble amino acids secreted into the medium was also determined in a similar manner.

Enzyme Assay

Nitrate reductase extracts were prepared in the same manner as described earlier for the determination of protein. Nitrate reductase was measured according to the methods of Hageman and Flesher (11). The reaction mixture contained 50 µmoles of potassium-phosphate buffer, pH 7.5, 20 µmoles of KNO_3, 0.42 µmoles of NADH, 0.2 ml of enzyme extract, and deionized water to bring the volume to 2 ml. The blank

consisted of the complete reaction mixture without NADH.

Extracts used for GDH, GS and GOGAT assays were prepared by grinding (20 strokes) 1 g cells with a glass Ten Broeck homogenizer. The grinding medium prepared according to Cooper and Beevers (6) consisted of 4 ml of 165 mM Tricine buffer (pH 7.5), 0.4 M sucrose, 10 mM KCl, 10 mM $MgCl_2$, 10 mM EDTA, and 10 mM β-mercaptoethanol. The homogenate was centrifuged for 7 min at 500 g to remove unbroken cells and cell fragments. The supernatant solution was then centrifuged for 15 min at 10,000 g yielding a crude mitochondrial pellet and supernatant. This supernatant was centrifuged at 100,000 g for 30 min, and the resulting clear supernatant was used for GOGAT and GS assays. No activity was detected when the pellets were assayed for these enzymes.

The crude mitochondrial pellet was washed once with MS[5] medium (22) composed of 0.25 M sucrose and 50 mM potassium-phosphate buffer adjusted to pH 7.5. The suspension was centrifuged for 15 min at 10,000 g. The pellet obtained from the above centrifugation was resuspended in 1 ml of MS medium and was used immediately for the GDH assay.

Glutamate dehydrogenase activity was determined by the method of Young (36). The reaction mixture contained, in a final volume of 3 ml, 75 μmoles potassium-phosphate buffer, pH 7.5, 10 μmoles α-ketoglutarate, 300 μmoles NH_4Cl, 0.3 μmoles

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[5]MS: mitochondrial suspension

NADH, and 0.2 ml of MS. The reaction was initiated by adding NADH, and the oxidation of pyridine nucleotide was followed at 340 nm and 25°C for 5 min. Controls were complete reaction mixture without NH_4Cl.

Glutamate synthetase was assayed according to the method of Fowler et al. (9). The reaction mixture contained in a final volume of 3 ml, 75 µmoles tris-HCl buffer, pH 7.5, 10 µmoles α-ketoglutarate, 15 µmoles L-glutamine, 0.3 µmoles NADH and 0.5 ml of enzyme preparation. The reaction was initiated by adding NADH, and the rate was measured at 340 nm and 25°C for 5 min. The blank used was the complete assay mixture without glutamine.

Glutamine synthetase activity was determined according to the method of Woolfolk et al. (35). The reaction mixture for the determination of transferase activity contained in a final volume of 2 ml, 90 µmoles L-glutamine, 60 µmoles sodium arsenate, 9 µmoles $MnCl_2$, 52 µmoles hydroxylamine (pH 7.0), 0.57 µmoles ADP and 60 µmoles imidazole buffer, pH 7.0. The reaction was initiated by the addition of 0.5 ml enzyme extract and was incubated at 37°C for 15 min. After incubation, the reaction was stopped and the color was developed by the addition of 0.5 ml of a solution containing equal volumes of 24% TCA, 6N HCl, and 10% $FeCl_3.6H_2O$ in 0.02N HCl. Precipitated protein was removed by centrifugation, and the absorbance of the supernatant at 540 nm was measured. The end concentration of hydroxymate was determined by reference

to a standard curve prepared by plotting the concentration of γ-glutamyl hydroxymate versus $O.D._{540}$. The enzyme activity was expressed as μmoles hydroxymate formed in 15 minutes.

The protein contents of the cell extracts for the above enzymes were also measured by the Folin method of Lowry, et al. (21).

CHAPTER III

RESULTS

Effect of Ammonium on Protein Synthesis

Analysis of protein extracted from control cultures grown with NH_4^+ showed that the synthesis of soluble protein was most rapid during the first 8 days; whereas, the synthesis of membrane-bound protein was more rapid thereafter (Fig. 1). At the conclusion of a 14-day growth period, the control culture contained 9 mg of membrane-bound protein and 13.7 mg of soluble protein. When the cells were grown in medium with no NH_4^+ (Fig. 1) there was a steady rate of protein synthesis of both the soluble and membrane-bound components. At the end of 14 days, cultures grown without NH_4^+ contained 5 mg of membrane-bound protein and 7 mg of soluble protein. Both values were considerably less than those in control cultures. Soluble protein was more plentiful than the membrane-bound proteins in both cultures.

Total cellular protein for both cultures was calculated by adding the values of membrane-bound and soluble proteins (Fig. 1). A comparison of protein content on day 14 between control and no NH_4^+ grown cells showed a two-fold difference.

No net protein synthesis occurred for control cells after 12 days of growth; whereas, cells grown without NH_4^+ showed sustained protein synthesis up to the end of growth cycle.

Protein synthesis was studied further by determining the amount of protein secreted into the medium (Fig. 3). Although an increase in intercellular protein was detected after 1 day of growth (Fig. 2), secretion of protein into the medium did not occur until day 4 and 5. For both cultures, the most rapid rate of protein secretion was between days 8 and 14. This was most pronounced in the control cells. After 14 days the control cells had secreted 17.5 mg of protein and the cells grown without NH_4^+ had secreted 7 mg.

Figure 4 shows the pattern of total protein synthesis (cellular plus secreted) per culture over a 14-day growth period. Rapid protein synthesis commenced two days following transfer of cells to fresh medium. At the conclusion of the 14 day growth period, the control cells had synthesized twice as much protein as the cells grown without NH_4^+.

Effect of Ammonium on Soluble Amino Acid Synthesis

The accumulation of soluble amino acids was different than that of protein synthesis. There was no appreciable accumulation of amino acids during the first six days following transfer of cells to fresh medium (Fig. 5). Subsequently, amino acids accumulated rapidly and attained a maximum value of 230 µmoles in control cells and 78 µmoles in cells grown

without NH_4^+. No appreciable amount of soluble amino acids was detected in the medium of either culture.

Relationship Between Uptake of Inorganic Nitrogen and Nitrogen Assimilation Into Amino Acids

Figure 6 shows the relationship between NO_3^- uptake and nitrogen assimilation into the amino acids of cells grown without NH_4^+. This graph is a plot of the µmoles of nitrogen contained in the various constituents which were examined. Uptake of NO_3^- continued at a reduced rate until day 10 whereupon the uptake shifted to a much faster rate. At the conclusion of a 14 day growth period, 1024 µmoles of nitrogen had been taken up by these cells. During this same time period only 308 µmoles of nitrogen were incorporated into soluble amino acids and protein. Thus, only 30% of the nitrate nitrogen removed from the medium was utilized for amino acid and protein synthesis.

The rate of NO_3^- uptake by the control cells during the first 6 days (Fig. 7) was very similar to that of the cells grown without NH_4^+ (Fig. 6). After day 6 the uptake of NO_3^- was more rapid. At the conclusion of 14 days, 1130 µ moles of nitrate nitrogen had been taken up by the cells. In addition to this 76 µmoles of NH_4^+ nitrogen was absorbed from the growth medium. A total of 1206 µmoles of inorganic nitrogen was taken up by control cells over a period of 14 days. At this time the amount of nitrogen present in soluble amino acids and protein was 722 µmoles. Approximately 60%

of the inorganic nitrogen which had been taken up was assimilated into soluble amino acids and protein.

Effect of Ammonium on the Development of Nitrogen Assimilating Enzymes

There was an appreciable increase in NR activity following transfer of cells to fresh medium (Fig. 8). The activity in the control cells increased rapidly and attained a maximum value of 28 n moles of KNO_2/min/mg protein on day 5. In contrast to this, when cells were grown without NH_4^+, the maximum NR activity of 16 n moles/min/mg protein occurred on day 6 and it was about one half of the activity present in control cells on day 5. Following day 5 or day 6 the enzyme activity declined rapidly and by day 10 minimum activity was reached.

Mitochondrial and post-mitochondrial supernatant fractions of cell homogenates were examined for glutamate dehydrogenase (GDH) activity. Activity was observed only in the mitochondria. The developmental pattern of GDH was similar in both cultures with the maximum activities occurring on day 2 (Fig. 9). The maximum value for control cells was 150 n moles NAD/min/mg protein as compared to 82 n moles NAD/min/mg protein for cells grown without NH_4^+. In both cultures, the GDH activity decreased rapidly after day 2 and by day 5 it was only half the activity present on day 2.

The assay for glutamate synthetase (GOGAT) was performed with the supernatant liquid obtained following a

100,000 g centrifugation. The developmental pattern of GOGAT (Fig. 10) was similar to that of GDH (Fig. 9). Following transfer of cells to fresh medium the GOGAT activity increased more rapidly in the control culture than in the culture grown without NH_4^+ and after 24 hr there was a two-fold difference. The maximum enzyme activities attained in cells on day 2 were 16.8 n moles NAD/min/mg protein and 11.5 n moles NAD/min/mg protein for control and no NH_4^+ grown cultures respectively. The activities decreased rapidly between days 2 and 5, and then remained relatively constant thereafter.

The effect of NH_4^+ on the development of glutamine synthetase (GS) showed a different pattern (Fig. 11) than that of NR, GDH and GOGAT (Figs. 8,9, and 10). There was no appreciable increase in GS activity during the first 4 days of growth in the control cultures. Subsequently, the enzyme activity in control cells increased rapidly and attained a maximum value of 812 n moles hydroxymate/min/mg protein on day 8. In contrast to this when cells were grown without NH_4^+, there was a substantial increase in enzyme activity after the first day. The maximum activity of 870 n moles hydroxymate/min/mg protein occurred on day 6. This value was approximately equal to that of control cells. Thus the presence of NH_4^+ in the medium delayed the synthesis of GS and the maximum activity, although approximately equal to that of the control, occurred two days later as compared to the maximum activity for cells grown without ammonium.

CHAPTER IV

DISCUSSION

Cells grown for 14 days in MPR medium containing 0.91 mM NH_4^+ possessed two times as much protein as cells grown without NH_4^+. This difference held true for both soluble and membrane bound proteins. During the later stages of growth a considerable amount of protein was secreted by the cells into the medium. In control cultures as much as 40% of the total protein synthesized was recovered in the medium. This amounted to 17.5 mg of protein which is lower than that reported by Moore (26) and greater than that reported by Olson (28).

When cells were grown in medium containing NH_4^+, the NH_4^+ was absorbed rapidly and after 4 days of growth it had been depleted from the medium. Since the amount of nitrogen taken up in the form of NH_4^+ exceeded the amount of nitrogen incorporated into protein during the first four days, it was possible that NH_4^+ served as the primary source of nitrogen during early growth. However, after day 5 the nitrogen entering protein must have come from NO_3^- since from that point on the μmoles of nitrogen in protein exceeded the amount of NH_4^+ nitrogen provided in the starting medium.

The presence of NH_4^+ in the medium had little influence on the uptake of nitrate, but had a pronounced influence on the assimilation of nitrogen from nitrate into amino acids. When cells were grown in medium containing NH_4^+, approximately 60% of the absorbed NO_3^- was utilized for amino acid synthesis whereas, only 30% of the absorbed NO_3^- was incorporated into the amino acids of cells grown without NH_4^+.

The activities of three enzymes NR, GDH, and GOGAT, were increased when cells were grown in medium with NH_4^+. The greatest influence was on NR where the activity was increased approximately 2-fold. This result agrees closely with the reported stimulation of NH_4^+ on NR activity in soybean tissue cultures (4), and mung bean seedlings (12). Ammonium increased the activities of GDH and GOGAT 1.8 and 1.5-fold respectively. This result closely resembles the reported stimulation by NH_4^+ of GDH activity in cucumber leaves (22), and of GOGAT activity in carrot tissue cultures (7).

Glutamine synthetase (GS) activity was repressed when cells were grown in medium with NH_4^+. The results of this study corroborated the findings of Rhodes et al., (31) where the specific activity of GS in Lemna minor was markedly reduced when either NH_4^+ or glutamine was present in the growth medium. In rose cells the reduced synthesis of GS during the initial 4 days of growth coincided with the period when NH_4^+ was in the medium. Soon after this supply was depleted, the synthesis of GS proceeded at a much faster rate.

It has been demonstrated that NH_4^+ in the medium suppressed the activity of GS and stimulated the activity of GDH during the first five days of growth, a period when protein synthesis was rapid. However, at all times the specific activity of GS was greater than that of GDH. In view of the higher affinity of GS for NH_3 (29), it follows that the inhibition of GS and the enhancement of GDH may not be great enough to influence the flow of nitrogen through the alternate pathways of nitrogen incorporation, GDH versus GS and GOGAT. A more restrictive feature in rose cells may be the low level of GOGAT whose maximum activity was only 1/10 that of GDH. This result was the opposite of what had been observed in sycamore cell suspensions (9) and carrot tissue cultures (7). This difference is probably due to our consideration of mirochondrial GDH; whereas, the other authors only reported soluble GDH activity. The level of GDH in the mitochondria may be critical in nitrogen assimilation since this compartment is the only recognized site of α-ketoglutarate synthesis which serves as a substrate for both GDH and GOGAT.

We propose that the enhanced synthesis of protein and increased growth brought on by the presence of NH_4^+ was due to its stimulation of certain key enzymes which include NR, GDH, and GOGAT. Additional studies must be completed to establish how NH_4^+ has caused these increased activities.

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PAPER II

LEGENDS FOR FIGURES

Figure 1. Influence of NH_4^+ on the synthesis of membrane-bound and soluble proteins. Control cells were grown in medium containing 0.91 mM NH_4^+ (●). Other cells were grown in media with no NH_4^+ (○).

Figure 2. Influence of NH_4^+ on the synthesis of total cellular protein.

Figure 3. Amount of protein secreted into the medium for control and no NH_4^+ grown cells.

Figure 4. Ammonium influence on the pattern of total protein (cellular plus secreted) synthesis

Figure 5. Effect of ammonium on the accumulation of soluble amino acids.

Figure 6. Relationship between NO_3^- uptake and nitrogen assimilation into protein and amino acids of cells grown without NH_4^+.

Figure 7. Relationship between nitrogen uptake (NO_3^- and NH_4^+) and its assimilation into protein and amino acids of cells grown in medium containing 0.91 mM NH_4^+.

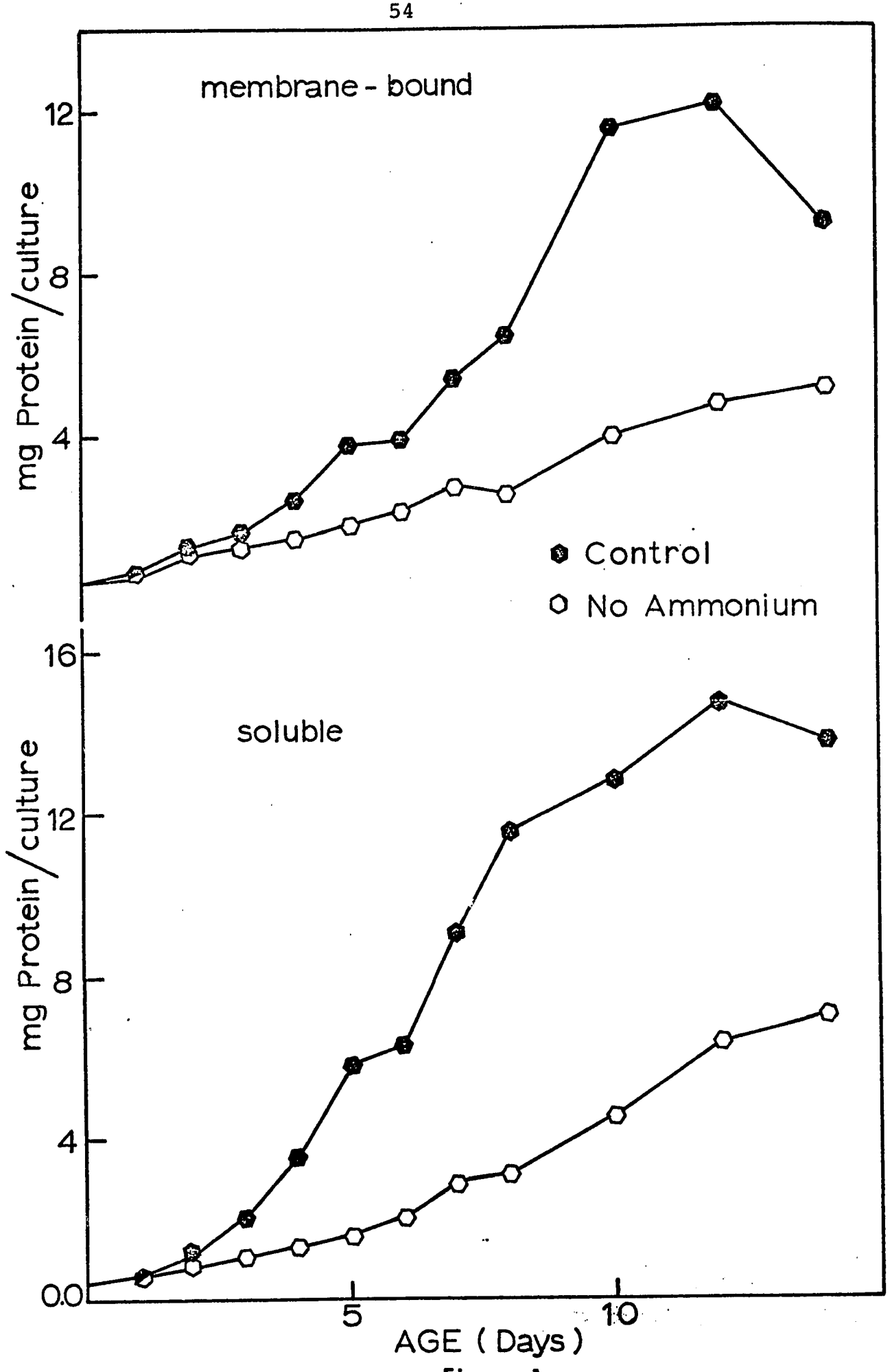
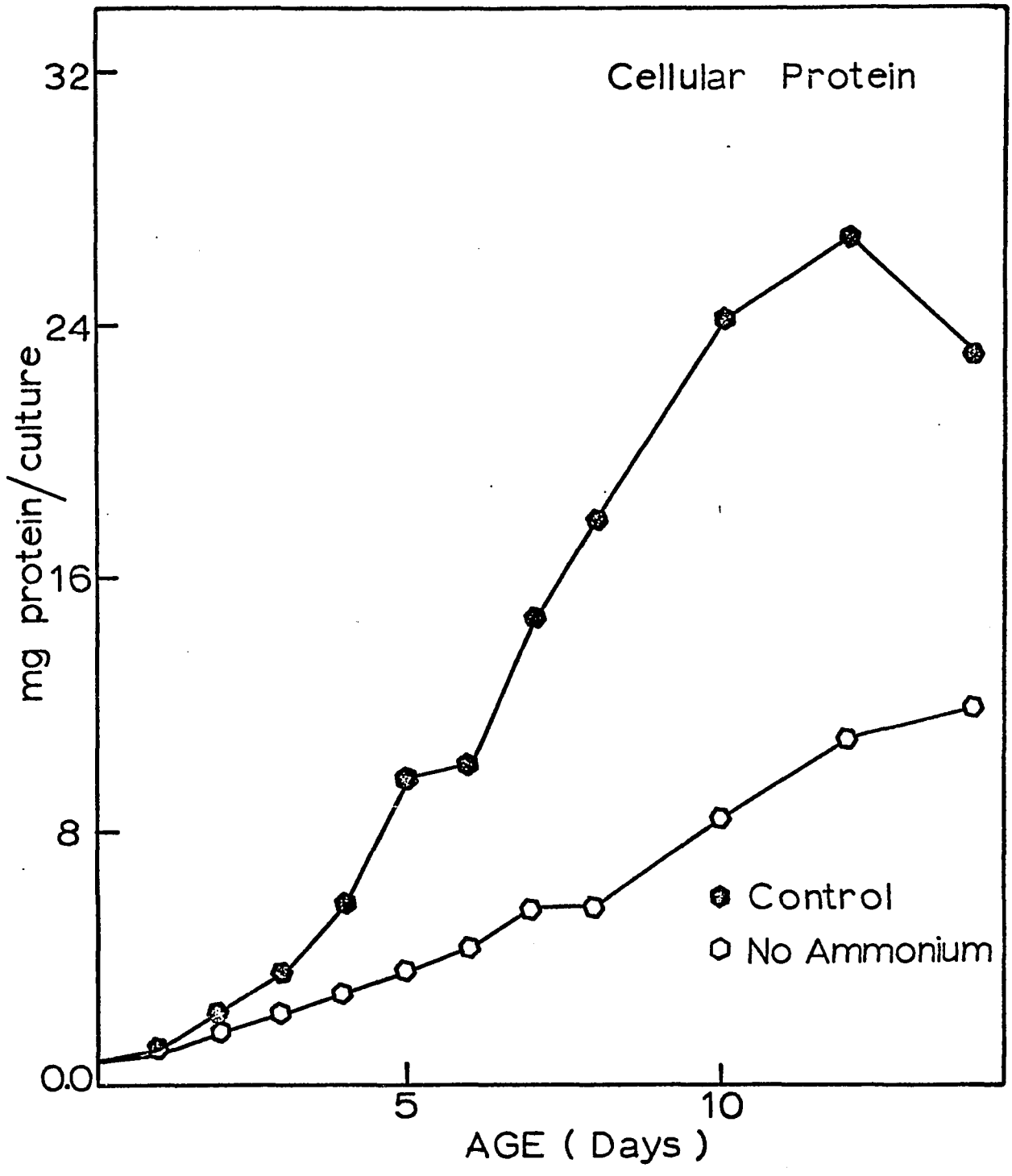

membrane - bound
mg Protein/culture
12
8
4
Control
No Ammonium
soluble
mg Protein/culture
16
12
8
4
0.0
5
10
AGE (Days)

Figure 1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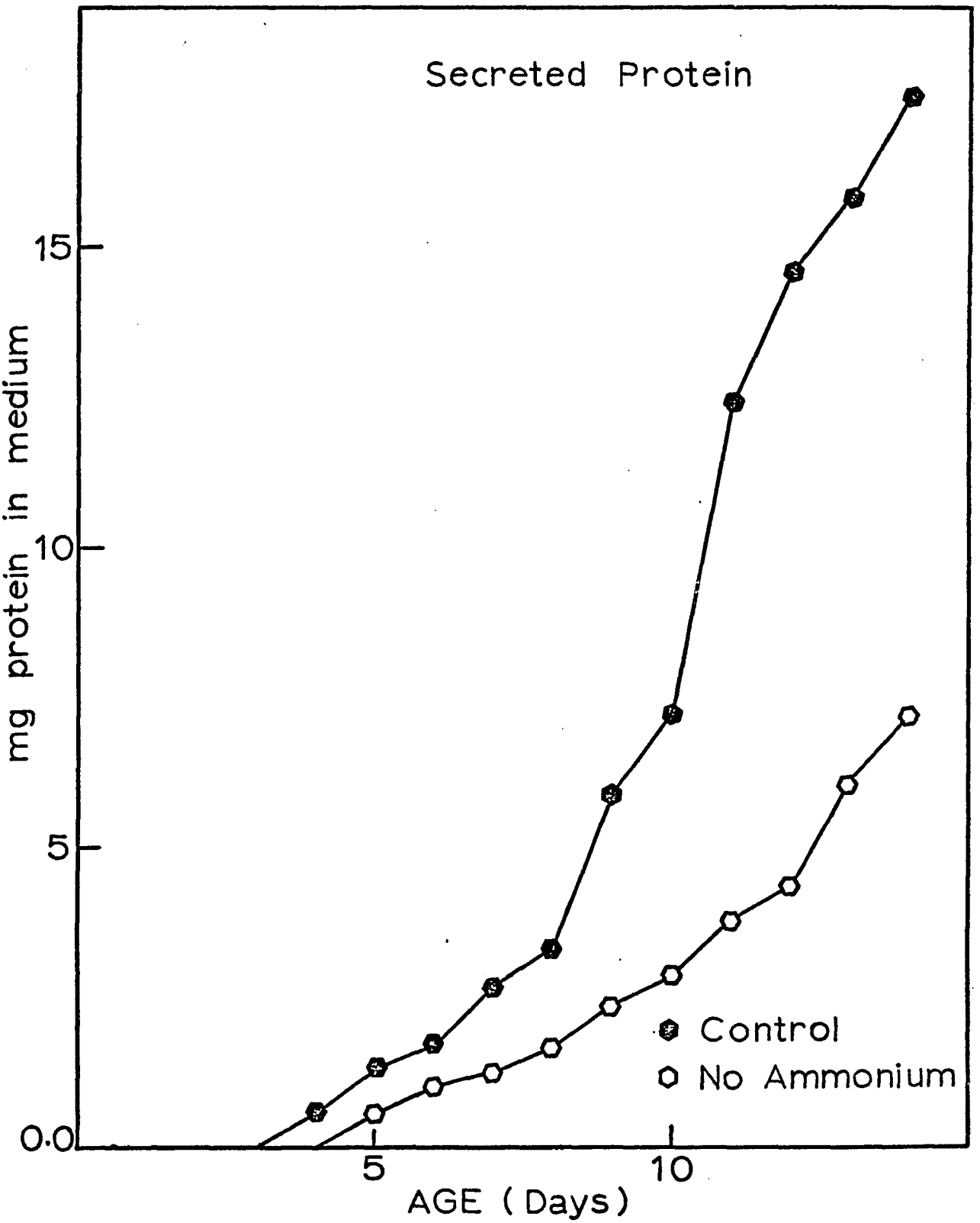
24
16
8
0.0
mg protein/culture
5
10
AGE (Days)
Control
No Ammonium

Figure 2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mg protein in medium
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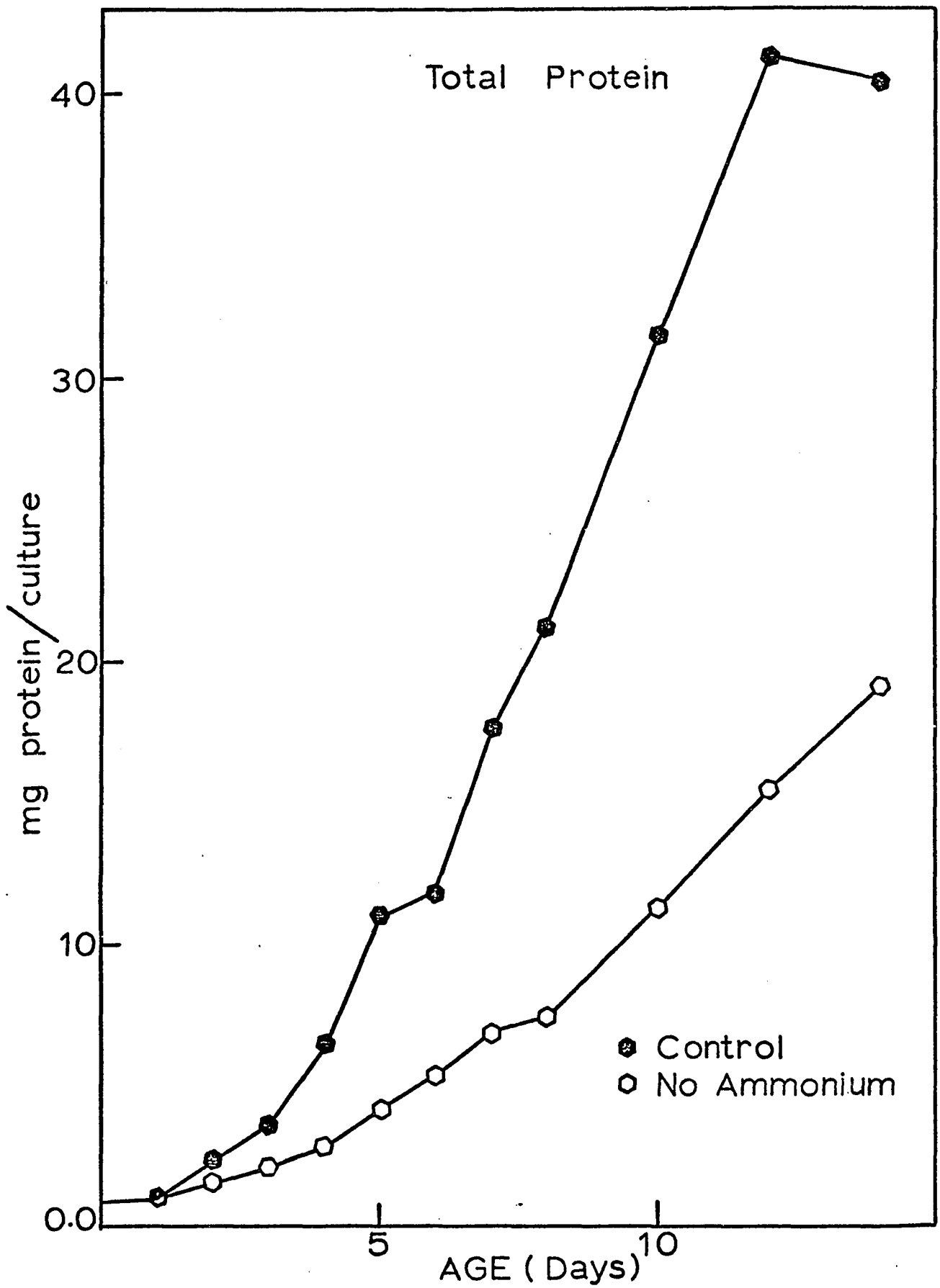
10
5
0.0
5
10
AGE (Days)
Control
No Ammonium

Figure 3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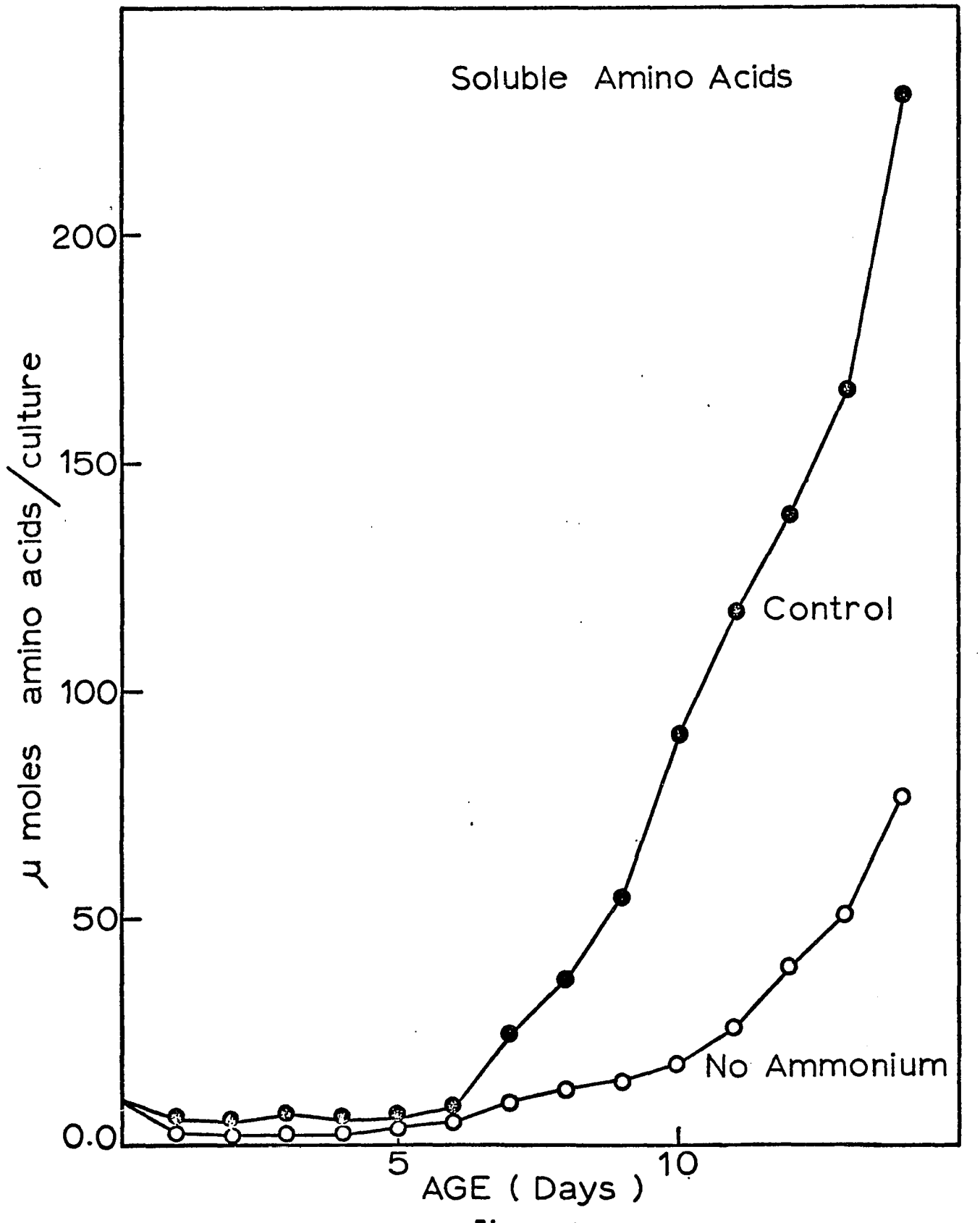
10
AGE (Days)
Control
No Ammonium

Figure 4


Soluble Amino Acids
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150
100
50
0.0
μ moles amino acids / culture
Control
No Ammonium
5
10
AGE (Days)

Figure 5

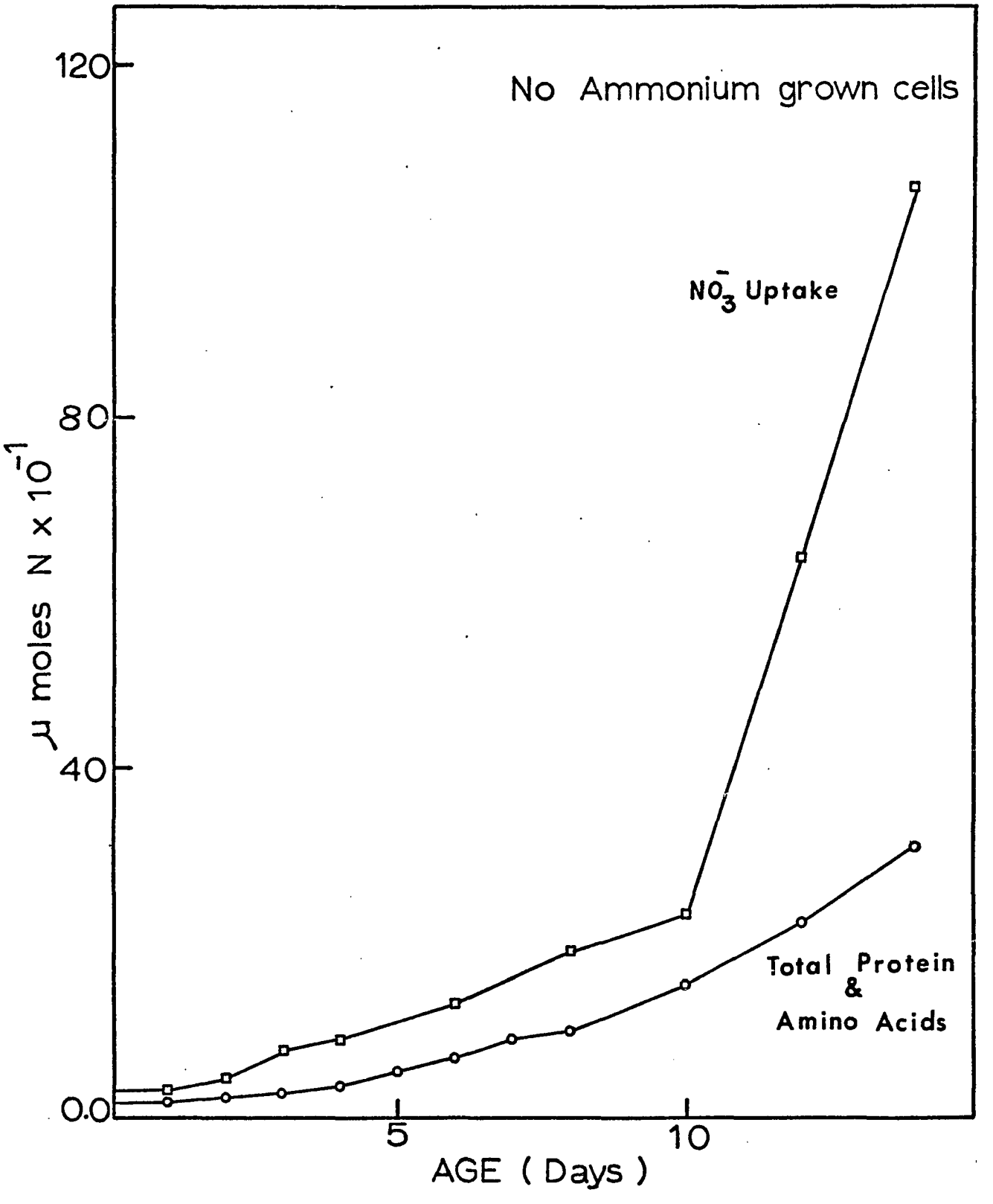
No Ammonium grown cells
NO_3^- Uptake
Total Protein
&
Amino Acids
120
80
40
0.0
μ moles N x 10^{-1}
5
10
AGE (Days)

Figure 6

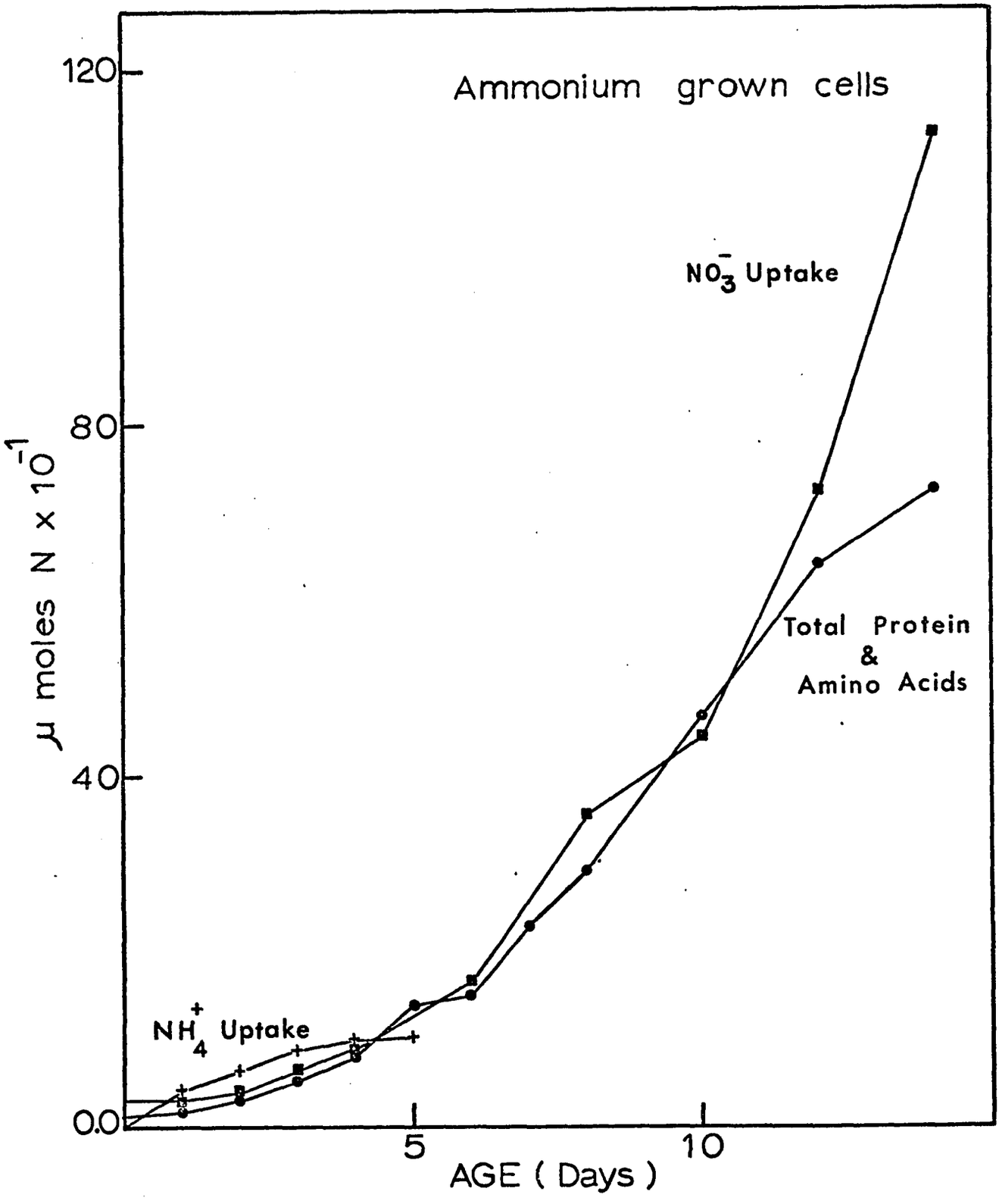
Ammonium grown cells
120
80
40
0.0
μ moles N x 10⁻¹
NO₃⁻ Uptake
Total Protein
&
Amino Acids
NH₄⁺ Uptake
5
10
AGE (Days)

Figure 7

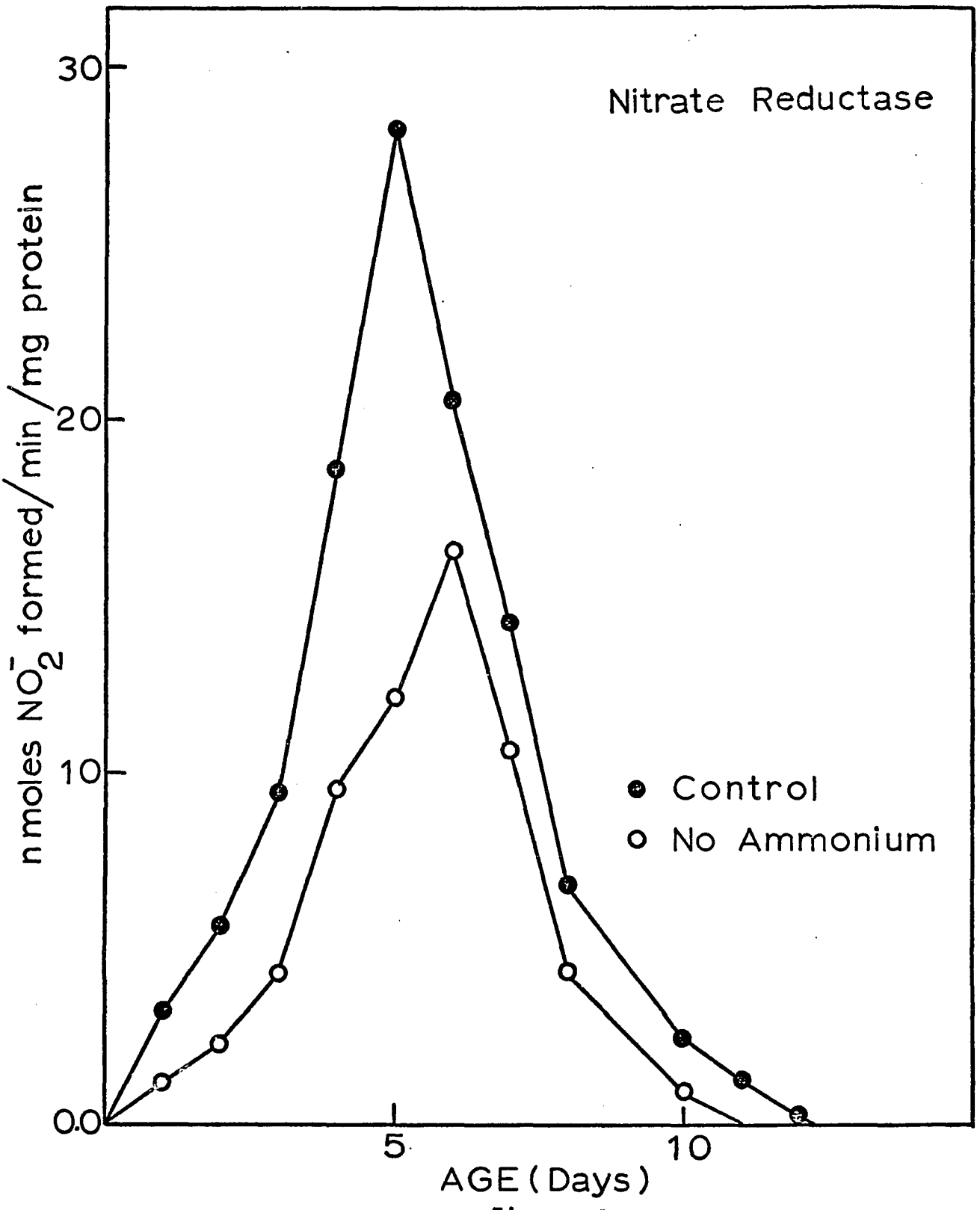
Nitrate Reductase
30
20
10
0.0
nmoles NO_2^- formed/min/mg protein
Control
No Ammonium
5
10
AGE (Days)

Figure 8

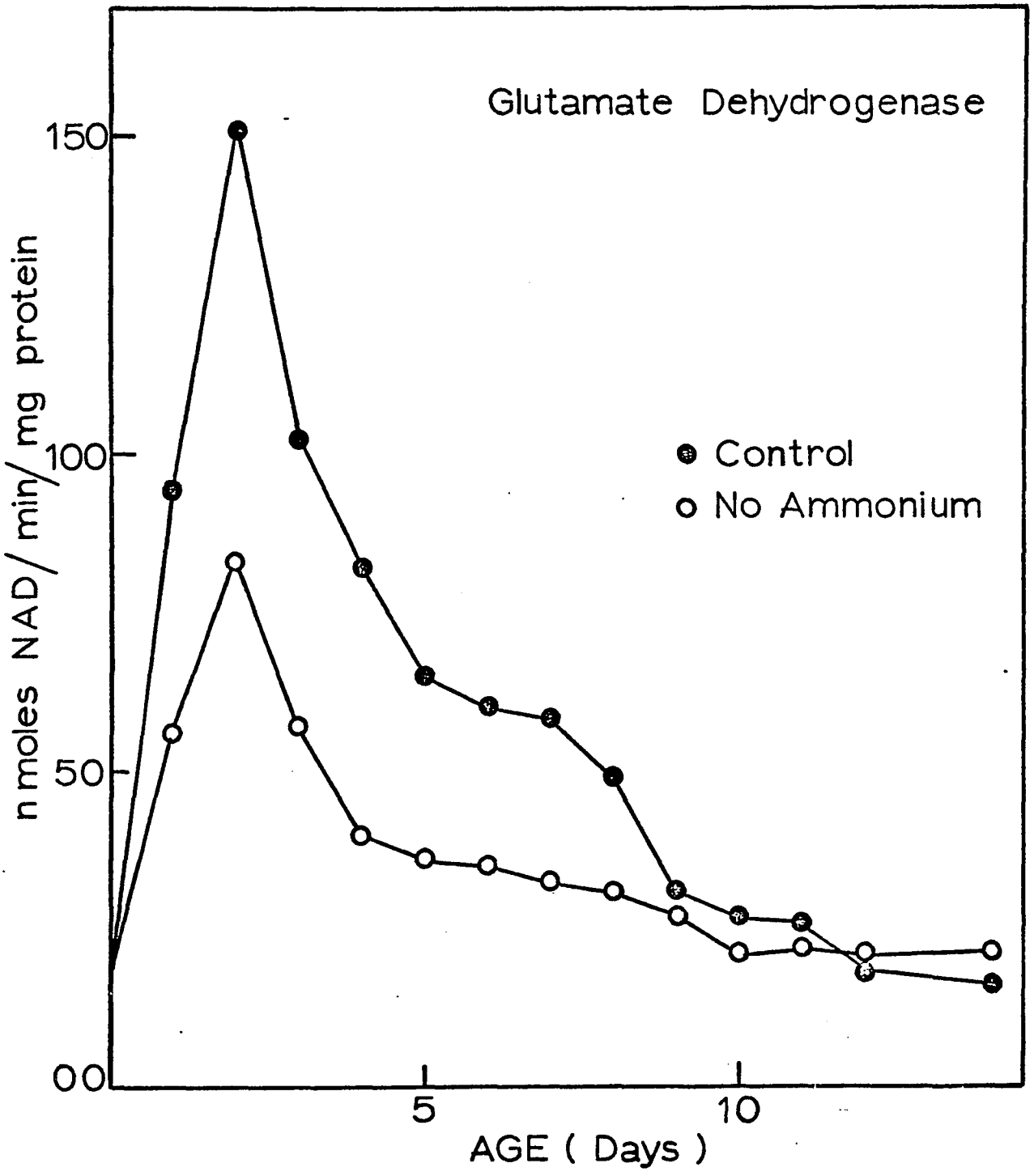
Glutamate Dehydrogenase
150
100
50
00
n moles NAD/ min/ mg protein
Control
No Ammonium
5
10
AGE (Days)

Figure 9

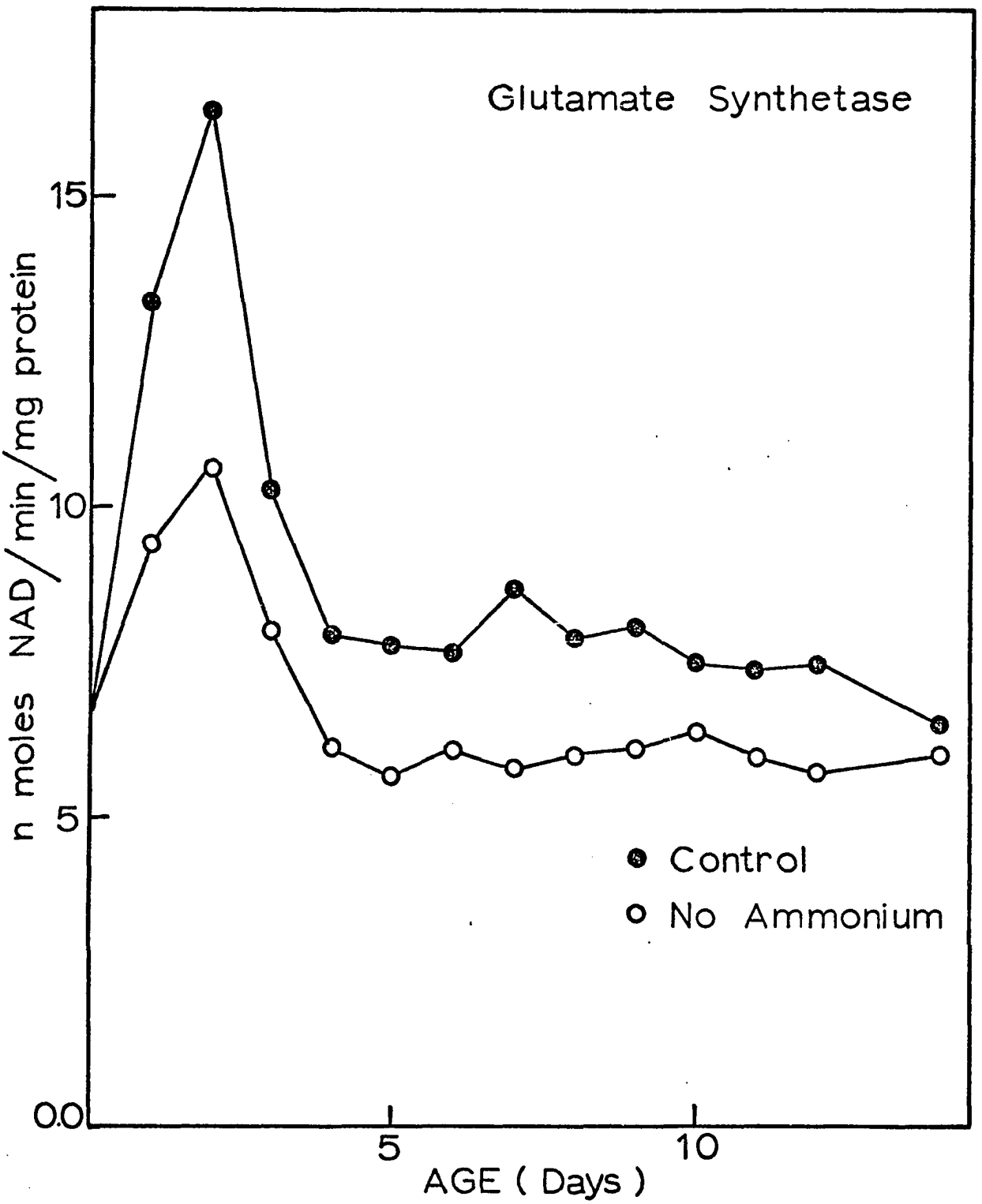
Glutamate Synthetase
n moles NAD/min/mg protein
15
10
5
0.0
5
10
AGE (Days)
Control
No Ammonium

Figure 10

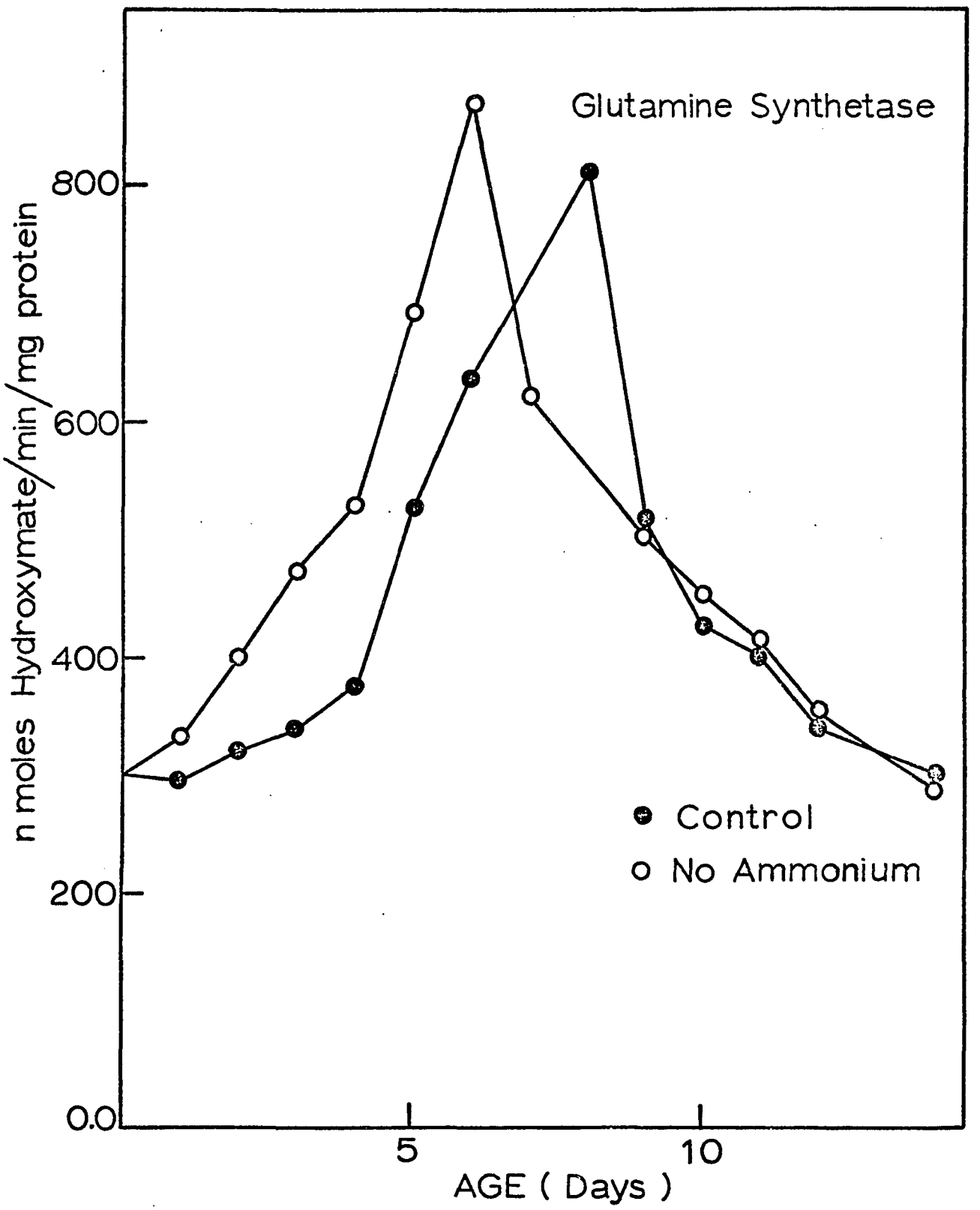
Glutamine Synthetase
n moles Hydroxymate/min/mg protein
800
600
400
200
0.0
5
10
AGE (Days)
Control
No Ammonium

Figure 11