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Influence of Protein Synthesis on NO_3^- Reduction, NH_4^+ Accumulation, and Amide Synthesis in Suspension Cultures of Paul's Scarlet Rose

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ABSTRACT

Changes in the concentrations of NH_4^+ and amides during the growth of suspension cultures of rose (*Rosa* cv. Paul's Scarlet) cells were examined. When cells were grown in medium possessing only NO_3^- as a nitrogen source, the concentrations of NH_4^+ and amides increased to 4.0×10^{-1} and 5.9 micromoles per gram fresh weight, respectively. The amounts of both constituents declined during the later stages of growth. When a trace amount of NH_4^+ was added to the NO_3^- base starting medium, the concentration of NH_4^+ in the cells was increased to 7.0×10^{-1} micromoles per gram fresh weight.

A comparison between the concentration of NH_4^+ in the cells (4.3×10^{-4} molar) with the K_m values for glutamate dehydrogenase (5×10^{-3} molar) and glutamine synthetase (1.7×10^{-5} molar) showed that the endogenous NH_4^+ would have to be concentrated 10-fold in compartments possessing glutamate dehydrogenase in order for the substrate to reach one-half the saturation level for that enzyme.

The influence of protein synthesis on the level of NH_4^+ and amides was examined by blocking protein synthesis with cycloheximide or puromycin and measuring changes in NH_4^+ and amide concentration over the subsequent 4 hours. The level of both NH_4^+ and amides showed substantial increases when protein synthesis was blocked. Ammonium accumulated to concentrations surpassing those reported by other authors to be toxic to plants. The reduction of NO_3^- did not appear to be influenced by the blockage of protein synthesis.

In the present study, tissue culture cells of increasing age were assayed for their NH_4^+ content to determine if the concentration of NH_4^+ fluctuated during cell growth and if it reached concentrations which were satisfactory for the functioning of both glutamate dehydrogenase and glutamine synthetase. These enzymes compete for NH_4^+ but differ greatly in their affinity for this common substrate (11, 16). Consideration was also given to the possibility that NH_4^+ may accumulate to toxic levels (11, 15, 25, 30) at certain stages of cell growth. A comparison between NH_4^+ and amide concentrations was made to evaluate the belief that the amides serve as a temporary depository of excess NH_4^+ (11).

The synthesis of certain amino acids is subject to end-product inhibition, as demonstrated by *in vitro* studies on isolated enzymes (4) and *in vivo* studies with tissue culture cells (5, 9, 10) and root tips (22). The question arises as to whether the early steps of nitrogen assimilation (nitrate reductase and nitrite reductase) are also subject to similar end-product control. Although there is evidence that several amino acids suppress the synthesis of nitrate reductase (3, 7) to our knowledge *in vitro* studies on nitrate reductase or nitrite reductase have not shown evidence of end-product regulation, and no *in vivo* studies have addressed this question. If the early steps of NO_3^- assimilation are not subject to control, but the later steps are, then abrupt cessation of protein synthesis would stop the flow of nitrogen into amino acids but would not curtail the reduction of NO_3^-. The result would be an accumulation of either NH_4^+ or, perhaps, storage compounds, such as amides. To investigate this question, we have subjected rose cells (*rosa* cv. Paul's Scarlet), actively engaged in protein synthesis, to cycloheximide or puromycin and compared NH_4^+ and amide changes to those occurring in control cells.

MATERIALS AND METHODS

Tissue Culture. Nonphotosynthetic suspension cultures of *Rosa* cv. Paul's Scarlet were used for this study. The cells were grown axenically in 250-ml Erlenmeyer flasks containing 80 ml of MPR medium (21). Cultures, which are hereafter referred to as 'NO_3^-' or as 'NO_3^- + NH_4^+,' contained 1,920 μmol NO_3^- or the same amount of NO_3^- + 72.8 μmol NH_4^+, respectively. Cultures were initiated in both types of medium by inoculation with 0.5 g of cells from 14-day-old NO_3^- + NH_4^+ cultures. Because of differences in the growth rates in the two media (17, 18) cells were grown for 14 days in the NO_3^- + NH_4^+ medium and for 21 days in the NO_3^- medium. At regular intervals during the growth cycles, 1- to 15-g samples of cells were harvested and homogenized in 80% ethanol with a Blackstone Ultrasonicator set at 80% of full power for 2 min. The ethanol-soluble compounds were separated from insoluble material by filtration. The ethanol-soluble samples were then assayed for their NH_4^+ and amide contents by steam distillation and titration (2).

Inhibitors. Ten-day-old cultures grown in NO_3^- medium were used for the inhibitor studies, because of their rapid rate of protein synthesis (17) and their low NH_4^+ content (Table I). Preliminary studies were performed, as previously described (8), with either acetate-U-^{14}C or leucine-U-^{14}C to determine the effectiveness of various concentrations of cycloheximide and puromycin to inhibit protein synthesis of 10-day-old cells grown in NO_3^- medium. Repeated experiments showed that 3.6×10^{-6} M cycloheximide and 10^{-4} M puromycin inhibited protein synthesis by approximately 85% and 40%, respectively.

The effect of these inhibitors on the accumulation of NH_4^+ and amides by rose cells was determined by incubating a 25-g sample of 10-day-old cells in media containing either 3.6×10^{-6} M cycloheximide or 10^{-4} M puromycin. The incubations were carried out in sintered glass funnels containing 50 ml of medium which had been filtered from 10-day-old cultures. Air forced up through the funnel provided aeration and mixing. Following 0, 2, and 4 h of incubation, cell aliquots were harvested, and the ethanol-soluble compounds were extracted as described in the previous section. The ethanol-soluble fractions were then subjected to steam distil-

Table I. *Endogenous Concentrations of NH_4^+ and Amides during Growth of Cells in NO_3^- and in NO_3^- + NH_4^+ Medium*

Age	NO_3^- Medium				NO_3^- + NH_4^+ Medium			
	NH_4^+		Amides		NH_4^+		Amides	
days	*μmol/g fresh wt*	*mM*	*μmol/g fresh wt*	*mM*	*μmol/g fresh wt*	*mM*	*μmol/g fresh wt*	*mM*
1	0.1 (0.01)[a]	0.10[b]	1.0 (0.1)	1.1	0.5 (0.1)	0.50	1.8 (0.6)	1.9
2	0.4 (0.05)	0.43	2.8 (0.5)	3.0	0.7 (0.1)	0.77	4.8 (2.2)	5.1
5	0.1 (0.01)	0.10	5.9 (1.0)	6.3	0.3 (0.1)	0.31	3.0 (1.0)	3.2
7	—[c]	—	1.2 (0.1)	1.2	0.18 (0.01)	0.19	1.6 (0.3)	1.7
11	—	—	1.2 (0.3)	1.3	0.18 (0.01)	0.19	1.0 (0.1)	1.1
16	—	—	0.9 (0.1)	1.0	0.12 (0.02)	0.11	1.2 (0.4)	1.2
21	—	—	1.2 (0.4)	1.3	—	—	0.7 (0.1)	7.2

[a] Numbers in parentheses; SE of the mean, with three replications.
[b] The mM values were estimated based on total water content of the cell.
[c] Dashes, below the detection level of the assay used.

lation to determine the amounts of NH_4^+ and amides present, or to column chromatography and ninhydrin staining to determine the quantities of glutamate, aspartate, glutamine, and asparagine present.

Steam Distillation of NH_4^+ and Amides. Ethanol-soluble fractions recovered from cell extracts were transferred to Kjeldahl flasks and evaporated to dryness with an air jet, as described by Barker and Volk (2). The steam distillation procedure of these authors was also used to determine the amounts of NH_4^+ and amides present in individual samples. Immediately following the distillation of each fraction into boric acid solution, samples were titrated back to the original pH of the boric acid with a standard potassium biiodate solution. Experiments in which known amounts of $(NH_4)_2SO_4$, glutamine, or asparagine were added separately to cell extracts confirmed the accuracy of the methods used.

Chromatographic Separation of Amino Acids and Amides. Ethanol-soluble fractions recovered from cell extracts were evaporated to dryness in a vacuum and rinsed with chloroform to remove lipids. The residue was dissolved in three aliquots of H_2O totaling 20 ml and passed through a Dowex-50 (H^+) column. Adhering amino acids were eluted with 50 ml of 1 N NH_4^+OH, which was subsequently air-dried and redissolved in 20 ml H_2O. This solution containing the amino acids was then passed through a Dowex-1 (acetate) column to separate glutamate and aspartate from the other amino acids. Glutamate and aspartate were eluted sequentially with 400 ml of a linear gradient from 0 to 2 N acetic acid.

The eluant recovered from the Dowex-1 (acetate) column was adjusted to 1 N with HCl and autoclaved for 2 h to hydrolyze amide bonds. The hydrolyzed samples were then dried, redissolved in H_2O, and passed through Dowex-1 (acetate) columns. These columns were then eluted with acetic acid, as described above. Fractions collected from the Dowex-1 columns were air-dried at 35°C and taken up in 2 ml H_2O. Those fractions containing glutamate or aspartate were identified by titrating each sample with 8.8×10^{-4} M NaOH, using phenolpthalein as an endpoint indicator. Samples containing amino acids were then pooled and quantified with a ninhydrin-hydrindantin assay (20).

RESULTS

Fluctuations in NH_4^+ and Amide Levels during Culture Growth. When cells were transferred to medium without NH_4^+, the NH_4^+ concentration increased during the first 2 days, reaching a peak level of 0.40 μmol/g fresh weight before it declined to an undetectable level on day 7 (Table I). The accumulation of amides was slower during early growth, and it reached the highest concentration (5.9 μmol/g fresh weight) on day 5, whereupon the amides declined to a steady concentration which prevailed during the remainder of the growth period. Thus during the first 2 days of growth, NO_3^- was reduced more rapidly than NH_4^+ was assimilated into organic form. A partial explanation for this may be the high activities of nitrate and nitrite reductase that prevailed during the early growth of this culture as compared to later growth (14).

The addition of NH_4^+ to the medium increased the intracellular amounts of NH_4^+ such that, at its highest level (0.73 μmol/g fresh weight on day 3), it was 75% higher than the highest level observed in cells grown without NH_4^+ (Table I). In contrast to this, the highest level of amide accumulation was 25% less in the NH_4^+-treated cells. Thus, the concentration of NH_4^+ fluctuated 2-fold without causing an increase in amide accumulation.

To determine if the fluctuations in NH_4^+ concentration during culture growth were sufficient to have a bearing on either NH_4^+ toxicity or enzymic utilization of NH_4^+, it was necessary to express NH_4^+ concentrations as mM values. Inasmuch as the ratio of fresh to dry weight changes during growth (18), the H_2O content of cells at each age was determined by subtracting the dry weight (18) from the fresh weight at each age. These H_2O values were used to calculate the mM concentration of NH_4^+ at each age by assuming that the NH_4^+ was evenly distributed throughout the cell, which is highly unlikely.

Effects of Inhibitors on Protein Synthesis. Provision of either cycloheximide or puromycin increased the endogenous level of NH_4^+ and amides (Table II). There was a correlation between the extent to which protein synthesis was inhibited and the amount of NH_4^+ and amides which accumulated. An 85% inhibition of protein synthesis for 4 h by cycloheximide caused NH_4^+ and amide levels to increase to values which were 2,300% and 650% of the control values, respectively. A 40% inhibition by puromycin resulted in NH_4^+ and amide values which were 360% and 230% of the controls, respectively. The levels of NH_4^+ and amides were higher in the control cells than they were when observed in the developmental study (Table I). We have no explanation for this difference.

The makeup of the accumulated amides was studied further by repeating the inhibitor experiments and analyzing the soluble extracts for glutamine and asparagine by column chromatography in conjunction with the ninhydrin-hydrindantin assay. Both asparagine and glutamine accumulated (Table III) with the latter, accounting for 68% of the total. The increases in the amides were accompanied by decreases in glutamic and aspartic acids, though not in equimolar amounts.

DISCUSSION

The concentration of NH_4^+ in cells changed during growth of the cultures. Ammonium was most plentiful in young cultures, a

Table II. *Effect of Cycloheximide or Puromycin on the Accumulation of NH_4^+ and Amides in 10-Day-Old Cells*

	NH_4^+			Amides		
	0 h	2 h	4 h	0 h	2 h	4 h
			μmol/g fresh wt			
Cycloheximide						
Control	0.1 (0.02)[a]	0.1 (0.03)	0.1 (0.01)	2.5 (0.3)	4.4 (0.2)	2.6 (0.7)
Treatment		0.9 (0.05)	2.3 (0.35)		21.8 (7.0)	16.9 (6.7)
% Control		610	2,300		500	650
Puromycin						
Control	0.1 (0.01)	0.1 (0.001)	0.1 (0.01)	1.8 (0.5)	1.4 (0.02)	1.4 (0.1)
Treatment		0.4 (0.01)	0.4 (0.01)		3.7 (0.1)	3.3 (0.4)
% Control		300	360		260	230

[a] Numbers in parentheses, SE of the mean, with three replications.

Table III. *Effect of Cycloheximide on the Accumulation of Glutamate, Aspartate, Glutamine, and Asparagine*

Time	Control				Cycloheximide			
	Glu	Asp	Gln	Asn	Glu	Asp	Gln	Asn
h				*μmol/g fresh wt*				
0	5.7 (0.1)[a]	3.1 (0.1)	1.2 (0.04)	0.61 (0.02)				
2	5.6 (0.1)	2.8 (0.1)	2.0 (0.2)	0.84 (0.05)	3.3 (0.1)	2.7 (0.3)	11.4 (1.1)	5.2 (0.2)
4	5.8 (0.4)	3.0 (0.1)	1.8 (0.2)	0.76 (0.05)	3.0 (0.2)	1.6 (0.1)	9.2 (0.8)	4.4 (0.2)

[a] Numbers in parentheses, SE of the mean, with two replications.

feature which was enhanced if NH_4^+ was provided in the medium. The changing concentrations of NH_4^+ in the cells were compared with the K_m values of glutamine synthetase and glutamate dehydrogenase. The concentration of NH_4^+ never reached the 5×10^{-3} M K_m for glutamate dehydrogenase which Givan (11) has reported as a low estimate based on numerous studies with the enzyme. In contrast to this, when an average K_m (1.7×10^{-5} M) was calculated for glutamine synthetase (27), the concentration of NH_4^+ in the cells was more than adequate for the enzyme. Thus, it appeared that the low concentration of NH_4^+ in rose cells would permit only glutamine synthetase to function effectively, unless isolated compartments in the cell provided glutamate dehydrogenase with a higher concentration of NH_4^+. Presumably NH_4^+ would have to accumulate in the mitochondria, since this is the location of the enzyme in rose cells (19).

When protein synthesis was inhibited by either cycloheximide or puromycin, NH_4^+ and amides accumulated. After a 4-h incubation in cycloheximide, a net increase of approximately 16.5 μmol/g fresh weight of NH_4^+ plus amides accumulated in the treated cells, as compared to that in controls. Inasmuch as the only source of nitrogen provided to these cells was NO_3^-, it follows that these soluble forms of reduced nitrogen arose from either NO_3^- reduction or protein degradation. Estimated rates of protein turnover, which range from 0.1 to 7% per h (9, 12, 13, 26, 29), can only account for a fraction of the reduced nitrogen which accumulated. Therefore, it can be concluded that, in rose cells, reduced protein synthesis and the resulting accumulation of unused amino acids do not inhibit the reduction of NO_3^- in the same manner as that in which amino acid synthesis is inhibited (9, 10).

Another aspect of the present study was to consider the potential toxicity of NH_4^+ (11) and the reported secondary effects of cycloheximide (13, 15, 23). Although NH_4^+ has long been considered toxic to plants (11), only a few studies have examined its mode of action. Krogman *et al.* (15) found that 50% of the photophosphorylation of isolated spinach chloroplasts was inhibited by NH_4^+ concentrations ranging from 6×10^{-4} to 4×10^{-3} M. Puritch and Barker (25) found that endogenous concentrations of NH_4^+ ranging from 1.6×10^{-2} to 2.4×10^{-2} M inhibited photosynthesis and caused ultrastructural changes in tomato chloroplasts. Vines and Wedding (30), who studied the effects of NH_4^+ on respiration, found that 3×10^{-3} M NH_4^+ stopped the oxidation of substrates provided to beet root mitochondria. These workers also established that NH_3 was the toxic substance.

In the present investigation, the highest concentration of NH_4^+ found in cells grown in NO_3^- medium was 4.3×10^{-4} M, and, in $NO_3^- + NH_4^+$ medium, it was 7.7×10^{-4} M. These concentrations of NH_4^+ were below the toxic levels reported in the studies cited above. In contrast to this, when protein synthesis was inhibited by either cycloheximide or puromycin, NH_4^+ accumulated to concentrations surpassing those reported to be toxic, despite the increased synthesis of amides. This raises the possibility that inhibitors of protein synthesis may have secondary effects on metabolism which are due to increased concentrations of NH_4^+. In keeping with this suggestion, it can be speculated that the secondary effects of cycloheximide should not be uniform for all plants, since the response of plants to NH_4^+ varies with the species (21). This speculation is consistent with the mixed reports concerning cycloheximide influence on such processes as respiration and ion uptake in higher plants (1, 6, 9).

The possible direct effect of cycloheximide on amide metabolism has been debated by several authors (13, 23, 28, 31). Oaks and Johnson (23) and Jones (13) found that corn root tips respond to cycloheximide with an increase in glutamine and a decrease in asparagine. This was interpreted to indicate that cycloheximide inhibited a glutamine-dependent asparagine synthetase by acting as a glutamine analog. In later work, Stulen and Oaks (28) showed that cycloheximide inhibited asparagine synthetase in root tips by inhibiting the development of asparagine synthetase activity, presumably by inhibiting protein synthesis. Furthermore, low concentrations of cycloheximide had no effect on the *in vitro* asparagine synthetase activity. Wheatley and Oaks (31) found that modifications in the structure of cycloheximide could not separate its inhibition of asparagine synthesis from its inhibition of protein synthesis in corn root tips. The present study supports the position that cycloheximide does not directly affect the *in vivo* activity of asparagine synthetase by acting as a glutamine analog, because a)

asparagine accumulated in cycloheximide treated cells, and b) puromycin had the same effect on rose cells as did cycloheximide, even though it has a very different chemical structure.

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