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Nitrogen Metabolism in Plant Cell Suspension Cultures

I. EFFECT OF AMINO ACIDS ON GROWTH

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ABSTRACT

Certain amino acids inhibit growth of tobacco (*Nicotiana tabacum* L. var. xanthi), tomato (*Lycopersicon esculentum*) carrot (*Daucus carota*), and soybean (*Glycerine max* L. co. Mandarin) cell cultures when nitrate or urea are the nitrogen sources but not when ammonia is the nitrogen source. These amino acids also inhibit development of nitrate reductase activity (NADH:nitrate oxidoreductase EC 1.6.6.1) in tobacco and tomato cultures. Threonine, the most inhibitory amino acid, also inhibits nitrate uptake in tobacco cells. Arginine, and some other amino acids, abolish the inhibition effects caused by other amino acids. We suggest that amino acids inhibit assimilation of intracellular ammonium into amino acids in cells grown on nitrate or urea.

Plant cell suspension cultures are usually grown in media containing nitrate or a mixture of nitrate and ammonia as nitrogen source (2, 5, 6, 9, 19). Addition of a mixture of amino acids or a protein hydrolysate enhances growth in many cases (2, 6, 9, 17–19), although the addition of single amino acids inhibits growth (7, 10, 17, 19). This inhibition of growth by single amino acids can be abolished by the addition of certain amino acids (7).

The addition of amino acids inhibits nitrate uptake and nitrate reductase activity in tobacco cell culture (7, 13, 14). Heimer and Filner (13) concluded that inhibition of growth in tobacco culture stems from a specific inhibition of nitrate assimilation.

The aim of this work was to examine whether inhibition of growth of plant cells in suspension culture by amino acids is a general phenomenon and whether it is caused by specific inhibition of nitrate assimilation.

MATERIALS AND METHODS

Culture. Tobacco culture (*Nicotiana tabacum* L. var. xanthi) was obtained from P. Filner; soybean culture (*Glycine max* L. cv. Mandarin) from O. L. Gamborg; tomato culture (*Lycopersicon esculentum*) from P. Preuss, and the carrot culture (*Daucus carota*) was initiated in our laboratory from carrot seeds which were germinated under aseptic conditions and transferred to growth medium.

Growth. Growth experiments were carried out in 250-ml Erlenmeyer flasks containing 50 ml of medium. Cultures were incubated at 28 C, in the dark, with shaking in a New Brunswick Gyrotory shaker at a speed of 150 rpm. The cultures were transferred every 14 days by inoculating 2.5 ml of grown culture into fresh medium with a wide mouth pipette. The inoculum for all experiments was grown in the same medium as in the experiment. Results of growth experiments were determined after 14 days.

The growth of cultures was determined on samples of 10 ml by measuring the settled cell volume in graduated centrifuge tubes, as described by Nickell *et al.* (17), unless otherwise stated. In the carrot culture there was formation of cell aggregates and therefore growth was estimated by filtration of cells through Whatman No. 1 filter paper. The dry weight was determined after drying of the cells at 60 C in a vacuum oven.

Media. Cultures were grown in T(NO_3) medium (Table I) unless otherwise stated. Growth hormones were added as follows: tobacco, 2,4-D, 0.5 mg/l; carrot, kinetin, 0.5 mg/l and 2,4-D, 0.05 mg/l; tomato, 2,4-D, 0.05 mg/l; and soybean, 2,4-D, 1 mg/l. In the last case 1 mM $(NH_4)_2SO_4$ was added as well (9). Soybean cultures were transferred to ammonia-deficient medium before conducting the growth experiments in order to eliminate any possible effect of ammonia carried over from the medium. Nitrogenless medium (T-N) was prepared by the replacement of KNO_3 with KCl (Table I). Growth medium containing ammonia as a sole nitrogen source (T(NH_4)) was prepared by the addition of 10 mM ammonium succinate solution to T-N medium (2, 11). Urea medium (T(urea)) was prepared by the addition of urea (10 mM) to the T-N medium. The pH of T (NH_4) and T(urea) was adjusted to 5.5, while that of T(NO_3) was adjusted to 4.5. These pH's were those giving optimal growth on the respective media. All media were sterilized by autoclaving at 121 C for 20 min. Urea was purified as described by Heimer and Filner (14) and was sterilized through a Millipore filter, 0.22 μm.

Media supplemented with amino acids were prepared by adding stock solutions of the amino acid to the media. The pH of the stock solutions was adjusted to 4.5 and the amino acid solutions were sterilized by filtration through a Millipore filter, 0.22 μm. All amino acids used (except glycine) were the L-form. Tryptone medium was prepared by adding Bacto-Tryptone (Difco) to T-N medium. The molarity of the tryptone was calculated in terms of nitrogen. Amino acids were used at 1 mM concentration.

Nitrate Reductase Assay. Cells were collected by filtration, rinsed with distilled H_2O, resuspended at a concentration of 1 g of cells (fresh weight) in 4 ml of ice cold 0.1 M potassium phosphate (pH 7.5) containing 1 mM EDTA and 1 mM dithioerythritol. Cells were disrupted with an MSE ultrasonic disintegrator operated for 1 min at 0 C. The homogenate was centrifuged at 10,000g for 20 min at 4 C and the supernatant liquid obtained was used as crude enzyme preparation. It was found that in order to measure nitrate reductase activity in tobacco cell extract, it was necessary to remove an unidentified inhibitor which behaved as if it were a small molecule. This was achieved by passing the cell extract through a Sephadex G-25 column and eluting with 0.1 M potassium phosphate (pH 7.5). The first fraction eluted contained the enzyme. The nitrate reductase activity was assayed using NADH as described by Filner (7). Enzyme activity is expressed

Table I. *Media Composition*

	Nitrate Medium T(NO_3)	Nitrogen-free medium T − N(H_4)
	760 mg/l	
I. Macronutrients		
$MgSO_4 \cdot 7H_2O$		
KNO_3	1800	
$NaH_2PO_4 \cdot H_2O$	300	300
$CaCl_2 \cdot 2H_2O$	200	200
KCl		900
Na_2SO_4		200
II. Micronutrients		
KI	0.5 mg/l	
Boric acid	0.2	
$MnSO_4 \cdot H_2O$	0.8	
$ZnSO_4 \cdot 7H_2O$	0.5	
$CuSO_4 \cdot H_2O$	0.02	
$Na_2MoO_4 \cdot 2H_2O$	0.01	
$CoCl_2 \cdot 6H_2O$	0.01	
III. Vitamins and growth factors		
Nicotinic acid	1 mg/l	
Pyridoxine HCl	0.5	
Thiamine HCl	0.5	
Fe citrate	5	
Myoinositol	100	
IV. Sucrose	20 g/l	

as nanomoles nitrate reduced/hr, and specific radioactivity is per g fresh weight.

Nitrate Uptake. Nitrogen-starved cells were inoculated into T-N medium containing 1 mM KNO_3. Aliquots were taken every hour for determination of the nitrate concentration in the cells.

Cells were collected by vacuum filtration on Whatman No. 1 filter paper, rinsed twice with T-N medium, and resuspended at a concentration of 1 g of cells (fresh weight) in 4 ml of distilled H_2O. Homogenate was prepared as described above. Nitrate was determined according to McNamara *et al.* (16).

RESULTS

Growth. Medium T(NO_3) is a modification of Tulecke medium (20), and is a good growth medium for all cultures tested with high growth rate and yield. The use of this medium permitted testing the cultures under similar conditions. We found a linear relationship between dry weight and settled cell volume in all cultures tested except for carrot which is not homogenous. The fresh weight-dry weight ratio for tobacco cells is 15.3. One gram per liter (dry weight) is equivalent to 17, 13, and 11% settled cell volume for tobacco, tomato, and soybean, respectively. Typical growth curves of tobacco and carrot cultures are shown in Figure 1.

The effect of addition of individual amino acids, at a concentration of 1 mM, on cultures of tobacco, carrot, tomato, and soybean is shown in Table II. Inhibition, when it occurred at all, was generally found in all of the cultures. Lysine strongly inhibited only the carrot culture, while glutamic acid inhibited strongly only the tobacco culture, and proline only the soybean. Isoleucine strongly inhibited the carrot and soybean cultures, aspartic acid and glycine only the tobacco and soybean cultures. Alanine, arginine, and glutamine either failed to inhibit, or inhibited only slightly, any of the cultures.

The very general effect of arginine in abolishing the inhibition caused by single amino acids is shown in Table III in the case of tobacco cells. Table IV shows that alanine was almost as effective with soybean and tomato, whereas isoleucine was an effective

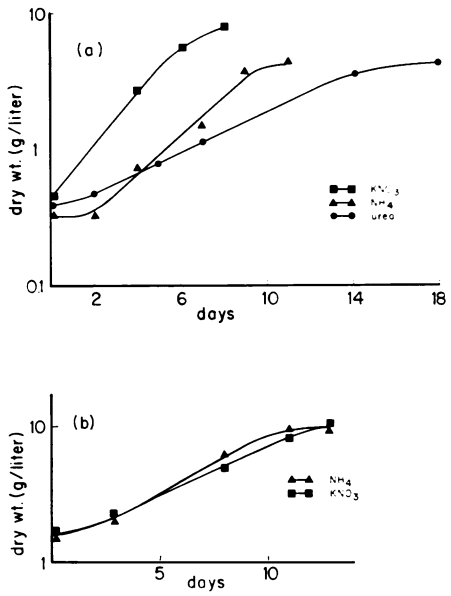

FIG. 1. Growth curves of tobacco and carrot cells on several media. a: Tobacco cells; b: carrot cells.

Table II. *Effect of Amino Acids on Growth of Plant Cell Cultures in Nitrate Medium*

Amino Acids Added	Settled Cell Volume			
	Tobacco	Tomato	Carrot	Soybean
	% of control			
None	100[1]	100[2]	100[3]	100[4]
Ala	73	97	110	125
Arg	100	87	80	125
Asp	11	95	77	35
Citrulline	11			
Cys	51	17	21	35
Glu	47	85	126	90
Gln		95	97	100
Gly	41	85	11	75
His	23	20	71	35
Ile	86	91	51	35
Leu	50	47	4	
Lys	77	87	10	125
Met	7	22	24	25
Orn	46			
Phe	7	37		25
Pro	83	91	97	25
Thr	14	72	61	35
Try	7	17	13	35
Tyr	54	35		
Val	12	45	72	30

[1] 75% settled cell volume.
[2] 90% settled cell volume.
[3] 4.8 g/l (dry wt).
[4] 35% settled cell volume.

Table III. *Antagonistic Effect of Arginine on Growth Inhibition by Other Amino Acids in Tobacco Cells*

Amino Acid Added	Settled Cell Volume
	% of control
Control (no amino acid added)	100[1]
Control (only arginine)	100
Asp	95
Cys	103
Glu	95
Gly	95
Ile	97
Leu	95
Lys	92
Met	95
Phe	94
Pro	103
Ser	108
Thr	94
Tyr	97
Val	95

[1] 70% settled cell volume.

Table IV. *Antagonistic Effect of Amino Acids on Growth Inhibition by Other Amino Acids*

Culture	Amino Acids Added	Settled Cell Volume
		% of control
Tobacco	Without amino acid	100[1]
	Thr	3
	Thr + Arg	94
	Thr + Ile	91
Tomato	Without amino acid	100[2]
	His	27
	His + Arg	82
	His + Ala	63
	His + Glu	59
	His + Ile	41
Soybean	Without amino acid	100[3]
	Thr	35
	Thr + Arg	101
	Thr + Ala	84
Carrot	Without amino acid	100[4]
	Thr	65
	Thr + Arg	94
	Thr + Ile	97

[1] 68% settled cell volume.
[2] 50% settled cell volume.
[3] 34% settled cell volume.
[4] 4.8 g/l (dry wt).

antagonist with tobacco and carrot cultures and, to a lesser extent, with tomato culture.

Except for the pairs shown in Table IV, no other amino acid was observed to reverse the inhibition caused by adding a single amino acid to one of the cultures; and an antagonist effective against one amino acid in a particular culture was effective for all inhibitory amino acids in that culture.

Effect of Amino Acids on Nitrate Reductase Activity. The characteristics of the nitrate reductase (NADH : nitrate oxidoreductase EC 1.6.6.1) in tomato and tobacco cultures are similar. The enzyme is NADH-specific with an optimal temperature of 25 C and an optimal pH of 7.8. Tobacco cell extract contains an inhibitor which can be removed by dialysis or gel filtration through a Sephadex G-25 column.

The enzyme is inducible by nitrate and the kinetics of the induction in tomato culture (Fig. 2) are similar to that of tobacco culture as described by Filner (7). Enzyme activity starts to develop within 1 hr after the addition of nitrate to the cells, and reaches its maximum after 12 hr. Enzyme level depends on culture age. It increased immediately upon transfer of an inoculum to fresh medium. It remained at a high level until the 7th day and declined quickly afterwards, even though the cells were still in the logarithmic phase (Fig. 3).

The effect of addition of amino acids on nitrate reductase activity in tomato and tobacco cultures was investigated in T(NO_3) medium to which 1 mM amino acids were added 6 days after inoculation (Table V). Amino acids inhibited nitrate reductase activity within 24 hr and the extent of inhibition was generally parallel to the inhibition of growth by amino acids in these cultures. Similar results were obtained when the amino acids were added at the time of inoculation (Behrend, Ph.D. dissertation, The Hebrew University, Jerusalem, Israel, 1976).

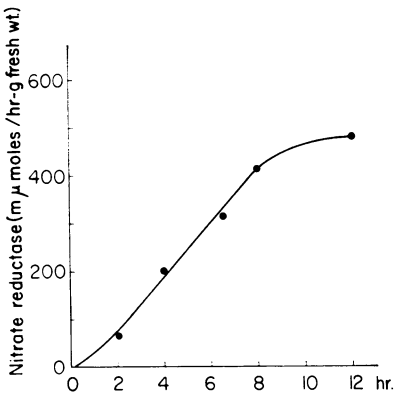

FIG. 2. Induction curve of nitrate reductase in tomato cell culture. T(NO_3)-grown cells were inoculated into fresh medium to give 50 g fresh weight/l. Aliquots of 50 ml were taken for nitrate reductase determination.

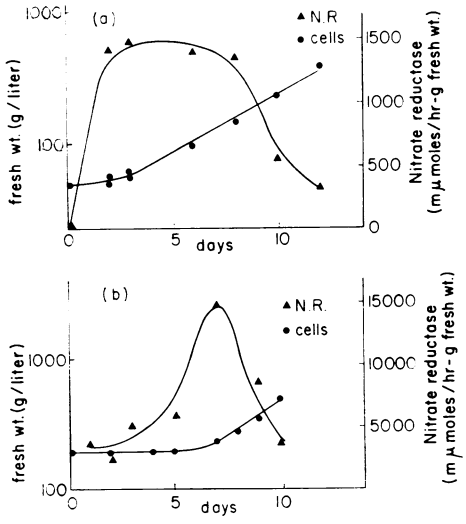

FIG. 3. Dependence of nitrate reductase level on culture age in tomato and tobacco cell culture. a: Tomato cells; b: tobacco cells.

Table V. *Effect of Amino Acids on Nitrate Reductase Activity in Cell Cultures*

Amino acids were added after 6 days of growth. Nitrate reductase was determined 24 hrs after the addition of amino acids.

Amino Acids	Nitrate Reductase	
	Tobacco	Tomato
	% of control	
Without amino acid	100[1]	100[2]
Ala	48	72
Arg	128	95
Asp	50	89
Cys	59	14
Glu	37	78
Gly	56	37
His	75	38
Ile	76	
Leu	78	60
Lys	60	94
Met	31	55
Phe	30	42
Pro	93	
Ser	56	90
Thr	22	78
Try	23	25
Tyr	32	18
Val	83	79

[1] 6100 nnoles/g fresh wt·hr.
[2] 1460 nnoles/g fresh wt·hr.

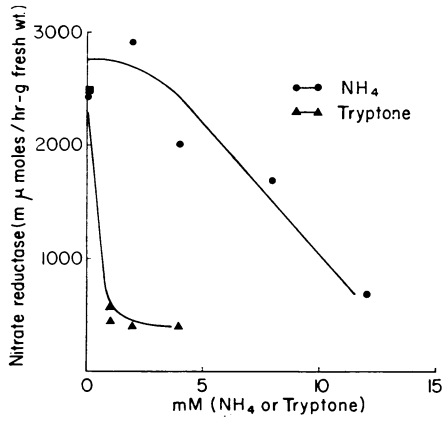

FIG. 4. Repression of nitrate reductase in tobacco cells by ammonium ion and tryptone. Tobacco cells were grown in T(NO_3) medium containing 4 mM KNO_3. Tryptone or ammonium succinate was added as indicated. Nitrate reductase was determined 72 hr after inoculation.

The activity of nitrate reductase obtained from tobacco culture after 6 days of growth was not affected by the addition to the *in vitro* enzyme reaction mixture of 1 mM concentrations of the following amino acids: arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine.

Figure 4 indicates that nitrate reductase in tobacco cells was repressed by increasing concentrations of ammonium as well as by low concentrations of tryptone. Tryptone, a mixture of amino acids, is clearly a more effective repressor than ammonium.

Effect of Amino Acids on Growth of Cultures Grown on Ammonium or Urea. Tomato, tobacco, and carrot cultures grow well on T(NH_4) medium as well as in T(urea) medium in terms of growth rate and final yield (Fig. 1).

Table VI shows the results of experiments to determine the effect of individual amino acids on the growth of cultures on ammonium medium (T(NH_4)). Except for cystine, which strongly inhibits tomato cell cultures, no inhibition was observed. On the contrary, in many cases some stimulation of growth was observed, particularly with the carrot culture.

When growing with urea as the nitrogen source (medium (T(urea)), inhibition of growth by individual amino acids was found, as is summarized in Table VII. With some exceptions, the inhibition was similar to that observed for cultures growing on nitrate as the nitrogen source (Table II).

The possibility that succinic acid itself functions as an antagonist to growth inhibition caused by amino acids was tested, and no beneficial effect of succinic acid could be observed (Table VIII).

Uptake of Nitrate. The rate of nitrate uptake by nitrogen-starved tobacco cells is exponential (Fig. 5). Uptake begins within 1 hr after transfer of the cells to medium containing nitrate and reaches a maximal level after 12 hr. The rate of uptake depends on nitrate concentrations with an apparent K*m* for the system of 0.7 μM (Fig. 6). The effect of several additions on nitrate uptake is shown in Table IX. The addition of cycloheximide or chloramphenicol to nitrogen-starved cells transferred to T(NO_3) medium inhibited nitrate uptake very severely. Threonine was also an effective inhibitor of nitrate uptake, and its effect could be reversed partly by isoleucine and completely by arginine.

Discussion. The phenomenon of inhibition of growth by single amino acids in plant cell cultures has been shown by Filner (7) in tobacco cell culture and by Gamborg (10) in soybean culture. In both cases inhibition of growth occurred in cells growing on nitrate as a nitrogen source. Heimer and Filner (7, 13, 14) showed that single amino acids inhibit not only growth of tobacco cell culture but also uptake of nitrate and activity of nitrate reductase in this culture. They concluded that single amino acids specifically inhibit nitrate assimilation and as a result growth is inhibited.

In this work it has been shown that inhibition of growth by single amino acids occurs also in carrot and tomato plant cells as

Table VI. *Effect of Amino Acids on Growth of Cell Cultures in Ammonium Medium*

Amino Acids Added	Settled Cell Volume		
	Tobacco	Tomato	Carrot
	% of control		
None	100[1]	100[2]	100[3]
Ala	105	107	134
Arg	115	110	150
Asp	115	103	
Cys	110	30	
Glu	115	116	148
Gln		116	144
Gly	90	91	
His	85	100	150
Ile	105	91	140
Leu	110	100	
Lys	100	100	112
Met			122
Pro	115	100	
Ser	85	84	140
Thr	71	70	137
Try	95		
Tyr	100		
Val	90	84	148

[1] 55% settled cell volume.
[2] 45% settled cell volume.
[3] 6.4 g/l (dry wt).

Table VII. *Effect of Amino Acids on Growth of Plant Cell Culture in Urea Medium*

Amino Acids	Settled Cell Volume		
	Tobacco	Tomato	Carrot
	% of control		
None	100[1]	100[2]	100[3]
Ala	102	100	84
Arg	100	103	84
Asp		100	
Asn	115	68	
Citrulline	15		
Cys	15	20	
Glu	54	89	140
Gln	70	92	140
Gly	20	31	7
His	12		
Ile	75	77	
Leu	28	57	
Lys	34	57	
Met	6		15
Orn	37		
Phe	41		4
Pro	100	100	100
Ser	14	80	9
Thr	7	17	3
Try	14		3
Val			15

[1] 50% settled cell volume.
[2] 45% settled cell volume.
[3] 6.6 g/l (dry wt).

Table VIII. *Effect of Succinic Acid on Growth of Tobacco Cells in T(NO_3) Medium plus Threonine*

Medium	Settled Cell Volume
T(NO_3)	95
T(NO_3) + 1 mM Thr	24
T(NO_3) + 1 mM Thr + 1 mM succinic acid	22
T(NO_3) + 1 mM Thr + 3 mM succinic acid	10
T(NO_3) + 1 mM Thr + 6 mM succinic acid	10
T(NO_3) + 1 mM Thr + 10 mM succinic acid	10

well as in tobacco and soybean (Table II). The inhibition is caused by most amino acids and in all the cultures tested can be abolished by arginine and isoleucine. We concluded that the effect of single amino acids on plant cell cultures is a general phenomenon.

The development of nitrate reductase activity is also inhibited by single amino acids in tobacco and tomato cultures and the extent of inhibition was generally parallel to the amino acid inhibition of growth (Table V).

Growth inhibition by amino acids could be explained by specific inhibition of one or more of the steps of nitrate assimilation leading to nitrogen starvation of cells. If this hypothesis were true, we would not expect growth inhibition of cells growing on nitrogen sources other than nitrate, such as ammonium and urea.

Plant cell cultures usually do not grow on ammonium as a sole nitrogen source (2, 19). However Gamborg (11) succeeded in growing soybean cells in an ammonium medium to which organic acids were added, and Butenko (2) described growth of tobacco and sunflower tumor tissue cultures on ammonium succinate as a sole source of nitrogen. Accordingly, medium was developed in which KNO_3 was replaced by ammonium succinate (Table I). Using this medium, it was found that amino acids do not inhibit growth of plant cells growing on ammonium (Table VI). We found that amino acids inhibit growth of plant cells growing on urea (Table VII). Heimer and Filner (14), on the contrary, found that amino acids did not inhibit growth of tobacco cells growing on urea. Heimer (Ph.D. dissertation, Michigan State University, 1970) found that threonine inhibited adaptation of tobacco cells from nitrate to urea, but that cells which had been adapted to urea were not inhibited by threonine or any other amino acid. We find that urea-adapted cells are inhibited by single amino acids. It should be noted that we used higher concentrations of amino acids (1 mM instead of 0.1 mM) in all our experiments since we did not observe inhibition by 0.1 mM concentration of amino acids. The reason for this difference is not clear; however, our growth medium had a different composition and contained a higher concentration of nitrogen. The fact that amino acids inhibit growth of tomato, tobacco, and carrot cultures growing on urea appears to rule out the hypothesis that amino acids specifically inhibit nitrate assimilation. The findings of Heimer

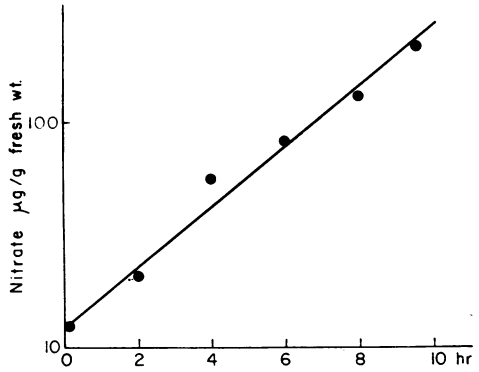

FIG. 5. Nitrate uptake by tobacco cells.

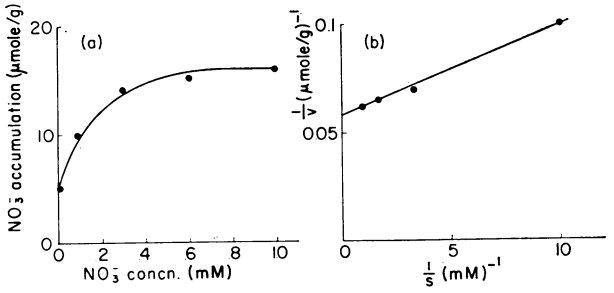

FIG. 6. Dependence of the rate of nitrate accumulation on the concentration of nitrate in the medium for tobacco cells. The nitrate concentration was measured after 10 hr.

Table IX. *Effect of Cycloheximide, Chloramphenicol, and Threonine on Nitrate Uptake*

Additions to Medium	NO_3^- Accumulation after 24 hr
	µmoles/g fresh wt
None	12.0
Cycloheximide (1 µg/ml)	0.67
Chloramphenicol (20 µg/ml)	1.60
Thr	2.40
Thr + Ile	6
Thr + Arg	13

that threonine inhibits adaptation of tobacco cells from nitrate to urea also suggest that the effect of threonine is more complicated than repression of the nitrate assimilation system.

In order to understand the inhibition process, the effect of amino acids on nitrate uptake was examined. Nitrate uptake was exponential in cells which had been starved for nitrogen and transferred to nitrate-containing medium (Fig. 5). The development of the nitrate uptake system in these cells was inhibited by protein synthesis inhibitors and also by threonine (Table IX). The kinetics of the uptake in nitrogen-starved cells and its inhibition by protein synthesis inhibitors indicate that the nitrate uptake system is also inducible, as was found for nitrate reductase (1, 7) and nitrite reductase (1, 3).

The inhibitory effects of amino acids on plant cells could be explained by at least two possibilities. (*a*) Amino acids inhibit growth by one mechanism and nitrate assimilation by another. (*b*) Growth and nitrate assimilation are both inhibited as a secondary result of the effect of amino acids on a third system.

The first possibility can hardly be accepted since there is an obvious parallelism between the effect of amino acids on growth (either on nitrate or urea) and on nitrate assimilation. Therefore, it seems likely that single amino acids inhibit another system, and as a consequence nitrate uptake and nitrate reductase are inhibited. Since cells growing on nitrate or urea are inhibited by single amino acids and since both nitrate and urea are converted to ammonium, we suggest that the inhibition point must be in one or more steps of ammonium assimilation, thus leading to nitrogen starvation and inhibition of nitrate uptake and nitrate reductase.

It is generally considered that glutamic dehydrogenase is the enzyme that is chiefly responsible for incorporation of ammonium into the α-amino group of amino acids (12), but glutamate synthase (4, 15), alanine dehydrogenase (8), and several other pathways for ammonium assimilation have also been demonstrated. It is possible that the pathway of intracellular ammonium assimilation in cells growing on nitrate or urea is different from that of cells growing on ammonium.

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