Putrescine metabolism in pea seedlings

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Abstract:

The effect of putrescine (Putr.) and N-carbamoylputrescine (N-CPutr.) fed to excised pea seedlings was studied. Contrary to great toxicity of higher than 0.5% Putr., N-CPutr. even at higher concentration was not toxic for the plants. The detoxication of plant cells by carbamoylation of Putr. was postulated. No differences were found in ornithine carbamoyltransferase (EC 2.1.3.3.) activity between plants fed with Putr. or N-CPutr. The most label from ^{14}C -putrescine was found in γ -aminobutyric acid. No labeled N-CPutr. was detected. The "oscillatory" mechanism of N-CPutr. synthesis in higher plants was postulated.

INTRODUCTION

The main path of putrescine synthesis in higher plants lead through decarboxylation of arginine to agmatine. Agmatine is metabolized to N-carbamoylputrescine and further to putrescine (Smith and Garaway 1964). It is known that many plants accumulate putrescine when the growing medium is deficient in potassium (Hacket, Sinclair and Richards 1965; Smith and Richards 1962; Smith 1963; Smith 1970). Potassium deficient barley plants show increased activity of N-carbamoylputrescine-amidohydrolase as compared with normal plants (Smith 1965).

In our previous work we have reported the carbamoylation of putrescine to N-carbamoylputrescine in the presence of ornithine carbamoyltransferase (EC 2.1.3.3.) (OCT) isolated from pea seedlings (Kleczkowski and Wielgat 1968) and bovine liver (Wielgat and Kleczkowski 1970).

In the present work some experiments concerning the pathway of putrescine metabolism in young pea seedlings are described.

MATERIAL AND METHODS

Reagents: putrescine-2 HCl and ethyleneglycol-monomethyl ether (Fluka, Switzerland). ^{14}C -putrescine-2 HCl with specific activity 21.8 $\mu\text{C}/\mu\text{mole}$ was purchased from the Radiochemical Centre, Amersham, England. Dowex 50 $\times$ 2, 200—400 mesh (BIO-RAD, USA). N-carbamoyl-putrescine-HCl was prepared from putrescine according to Smith and Garraway (1964). Carbamoyl phosphate, ammonium salt, was prepared by the Metzenberg, Marshall and Cohen method (1960). Other reagents were of Polish origin distributed by Biuro Obrotu Od-czynnikami Chemicznymi, Gliwice.

Plant material: 10—14-day-old pea seedlings, variety „Perła szlachetna” were used.

Enzyme activity determination: the assay method for putrescine carbamoylation activity was described previously (Kleczkowski and Wielgat 1968). N-carbamoylputrescine was determined colorimetrically according to Archibald (1944) as modified by Ratner (1955), and protein as described by (Lowry et al. (1951).

Plant feeding experiments: in experiments with potassium deficiency, the nutrient medium described by Smith (1963) was used. Pea seeds were germinated for 5 days on small trays on water moistened cotton wool. Young sprouts were separated from the cotyledones and placed on perforated porcelain discs, located on the liquid nutrient medium described by Smith (1963). Feeding experiments with excised 10—14-day-old pea seedlings fed with putrescine and N-carbamoylputrescine were performed as follows: the cut ends of pea seedlings excised 1 cm above cotyledones, were immersed after excision in a small reagent glass filled with 3 ml a suitable putrescine or N-carbamoylputrescine concentration. Control samples were fed the same concentration of sodium chloride or distilled water. The solutions of 2-HCl putrescine and N-carbamoylputrescine-HCl were neutral and did not require further neutralization. Time of feeding 24—72 hours.

^{14}C -putrescine feeding experiments: the technique and age of plants were the same as described above for putrescine and N-carbamoylputrescine feeding. 2-HCl-putrescine (0.5%) supplemented with 20 $\mu\text{C}/2$ ml of ^{14}C -2-HCl-putrescine (spec. act. 21.8 $\mu\text{C}/\mu\text{mole}$) was used. Time of feeding was 60 min at room temperature, with 100 Watt lighting lamp and intensive air ventilation. After the feeding period, the plants were rinsed several times with distilled water to wash out the label, placed in Petri dishes and frozen in dry ice. The frozen plants were homogenized in a mortar with 5 ml of distilled water and centrifuged at 15 000 g for 15 minutes. The supernatant was collected and the sediment resuspended in water and again centrifuged as above. This manipulation was repeated three times. The pooled supernatant was deproteinized with trichloroacetic acid

(final concentration 5%). Protein sediment was centrifuged off, washed three times with 5% trichloroacetic acid and centrifuged as above. The acid was removed from the combined supernatants by shaking five times with equal volume of ethylether. After neutralization the traces of ether were removed by bubbling gaseous nitrogen through the solution.

The sample were evaporated to dryness in a vacuum evaporator at 30—40°, and the residue dissolved in 2—3 ml of water and fractionated on a Dowex 50 × 2 column according to the method described by Hammond and Herbst (1968). The fraction eluted with 0.6 N HCl was dried as above, once more rechromatographed on the same column and partitioned by descending paper chromatography on Whatman 1 paper, in *n*-butanol: acetic acid: water (4 : 1 : 5). Radioactive spots were eluted and rechromatographed twice in this solvent.

Labeled products were identified by two-dimensional paper chromatography. In the first dimension, the solvent was the same as above, and in the second one, phenol saturated with water was used. Identification was repeated by two dimensional thin-layer chromatography on Kiesel-gel G (Merck, Germany). The solvents used were as described above, or phenol was replaced by ethyleneglycol-monomethyl ether: propionic acid: water (70:15:15).

Developed chromatograms were sprayed with ninhydrin or Ehrlich reagent. Radioactivity was measured in a Geiger-Muehler counter.

RESULTS

To compare the effect of high concentration of putrescine as well as N-carbamoylputrescine on the survival of young pea plants, excised pea shoots were fed with these substances. The results of this experiment are shown in fig. 1. After 72 hours of feeding, plants fed the 1% solution of N-carbamoylputrescine behaved in the same manner as control samples fed on water or sodium chloride. Putrescine-fed plants after this period showed many necrotic brown spots and were completely withered. The behaviour of these plants during experiment is shown in table 1. Even the plants fed a 0.5% solution of putrescine showed after 72 hours significant symptoms of necrosis and were withered in 75%. In 1% putrescine after 48 hours the symptoms were the same as after 72 hours in 0.5% solution. The same relation was observed between 2% and 1% putrescine concentrations after 48 and 72 hours of feeding. In 1% solution of putrescine, the first symptoms of withering appeared already after 24 hours and in 2% putrescine after this time the plants were practically dead.

Shevyakova (1966) observed a similar effect when higher than 0.1% putrescine concentration was used during germination and growth of *Vicia faba* plants. Lower than 0.1% putrescine and cadaverine concen-

H_2O

NaCl

N-C Putr.

Putr.

Fig. 1. Behaviour of excised pea plants after 72 h feeding 1% solutions of H_2O , NaCl, N-CPutr. (N-carbamoylputrescine), Putr. (Putrescine-2HCl).

Table 1

Behaviour of excised pea plants after various periods of feeding with H_2O , NaCl, N-carbamoylputrescine-HCl and Putrescine-2HCl

Solution used	Appearance of plants after feeding for		
	24 h	48 h	72 h
H_2O	N	N	N
NaCl 0.5%	N	N	N
1.0%	N	N	slightly withered
2.0%	N	slightly withered	withered in 50%
N-carbamoylputrescine-HCl			
0.5%	N	N	N
1.0%	N	N	slightly withered
2.0%	N	slightly withered	withered in 50%
Putrescine-2HCl			
0.5%	N	withered in 30%	withered in 75% with yellowish-brown necrotic spots
1.0%	withered in 30%	withered in 60% with yellowish-brown necrotic spots	withered in 100% with big necrotic spots
2.0%	withered in 50% with small necrotic spots	withered in 95% with big necrotic spots	withered in 100% and almost dead

N — normal.

trations had a stimulating effect on germination and growth of several plants (Nezgovorova et al. 1966; Nezgovorova and Borisova 1967; Shevyakova 1966). In further work, the activity of ornithine carbamoyltransferase (OCT) toward putrescine as substrate, in plants fed with putrescine was determined. These results are presented in table 2. No influence of putrescine feeding on OCT activity was found up to a 0.25% concentration of putrescine in the feeding solution. A diminished activity of putrescine carbamoylation in the presence of OCT was

Table 2
Ornithine carbamoyltransferase (OCT) activity in excised pea plants,
fed putrescine for 48 h

Putrescine-2HCl concentration, %	OCT activity in μ moles of N-carbamoylputrescine/1 mg protein
0.10	0.260
0.25	0.250
0.50	0.206
1.00	0.180
2.00	0.060
Plant before excission	0.260
Excised plants immersed in H ₂ O for 48 h	0.255

Experimental conditions as described under methods.

observed at higher concentrations of putrescine (0.5—2%). It was probably caused by degeneration of the fed plants (see fig. 1 and table 1).

Shevyakova (1966) found that degradation of putrescine in higher plants begins by oxidation in the presence of diamine oxidase (EC 1.4.3.6.). She demonstrated that, when plants were fed a 0.1% concentration of putrescine, the activity of diamine oxidase rose about ten times as compared with control plants. At higher than 0.1% putrescine concentration no change in activity of this enzyme was observed and accumulation of putrescine occurred. Similarly Doisuke Yoshida (1969) found that degradation of ¹⁴C-putrescine in *Nicotiana tabacum* plants was inhibited by potassium or sulphur deficiency in the nutritional medium. It is possible, that at high concentrations of putrescine in plant cells, diamine oxidase is not able to metabolise this substrate and the accumulated putrescine causes degeneration and subsequent death of the plant.

The putrescine and ornithine carbamoylating activity in isolated pea germs, growing in potassium deficient medium was also determined (Table 3). Both activities were determined after 3 and 5 weeks of growth. There were no significant differences found in the activity of OCT between normal and potassium-deficient plants.

In the further experiments we tried to isolate the metabolic products

Table 3

Ornithine carbamoyltransferase (OCT) activity in normally growing and in potassium-deficient pea seedlings, with ornithine and putrescine as substrate

Growth medium	Age of plants weeks	
	3	5
	OCT activity in μ moles of product/1 mg protein	
	with ornithine as substrate	
normal medium	4.00	5.40
potassium-deficient medium	3.70	5.50
	with putrescine as substrate	
normal medium	0.17	0.35
potassium-deficient medium	0.17	0.35

Assay conditions as described under methods.

Table 4

Distribution of radioactivity in fractions isolated from pea seedlings after feeding with ^{14}C -putrescine

Isolated fraction	Counts/sec ^{14}C
1. Crude homogenate	7200
a) residue after centrifugation	140
b) supernatant	6950
2. Supernatant	
a) pellet after deproteinization	26
b) ether fraction	120
c) deproteinized and neutralized supernatant	5720
3. Dowex 50 $\times$ 2 column chromatography	
a) H_2O	320
b) 0.6 N HCl	4200
c) 6.0 N HCl	340
4. Rechromatography of 0.6 N HCl fraction on Dowex 50 $\times$ 2	
a) H_2O	4
b) 0.6 N HCl	3360
c) 6.0 N HCl	350
5. Paper chromatography and 2nd rechromatography of 0.6 N HCl fraction	
a) most radioactive spot identified by two-dimensional chromatography as γ -aminobutyric acid	1900

Experimental conditions as described under methods.

from pea seedlings fed ^{14}C -putrescine (Table 4). Above 26% of the total radioactivity present in the homogenate and above 56% of the total radioactivity after second rechromatography on Dowex 50×2 column was found in γ -aminobutyric acid. No ^{14}C -N-carbamoylputrescine was found there.

DISCUSSION

On the basis of literature data, Smith and Garraway (1964) have proposed the following mechanism of putrescine synthesis in higher plants: (Schema 1).

Scheme 1. Pathway of putrescine synthesis in higher plants

As can be seen from the above scheme, N-carbamoylputrescine can be an intermediate product in the synthesis of putrescine from agmatine. In further work Smith (1965) established that barley plants degrade enzymatically N-carbamoylputrescine to putrescine. In our previous work

(Kleczkowski and Wielgat 1968) the enzymatic synthesis of N-carbamoylputrescine from putrescine and carbamoyl phosphate, in the presence of purified plant OCT was reported. This activity was about 100 times higher as compared with the reported by Smith (1965) activity of N-carbamoylputrescine amidohydrolase, which decomposes N-carbamoylputrescine to putrescine, ammonia and carbon dioxide in the presence of barley homogenate.

As all our earlier experiments were performed *in vitro* (Kleczkowski and Wielgat 1968; Wielgat and Kleczkowski 1970), we attempted to compare the effect of putrescine and N-carbamoylputrescine on the behaviour and survival of living plants, as well as to find in living cells fed with putrescine its supposed metabolite N-carbamoylputrescine. We hoped to find the answer, as to the physiological role of N-carbamoylputrescine in living plant cells.

Our results (Fig. 1, Table 1) proved clearly that pea plants fed a 2% solution of N-carbamoylputrescine for 72 hours behaved in the same way as those fed with sodium chloride (2%). On the other hand, when these plants were fed a 0.5% solution of putrescine, they withered after 48 hours and after 72 hours they were nearly dead.

This observation may suggest, that carbamoylation of putrescine in plants plays a role of detoxicant, when a large amount of putrescine is accumulated. If this suggestion would be true it was expected that putrescine-fed plants would accumulate non toxic N-carbamoylputrescine as well as toxic putrescine, but the results did not support our supposition (Table 4).

Sinclair (1967, 1969) found a correlation between arginine, agmatine and putrescine accumulation, as well as arginine decarboxylase and N-carbamoylputrescine amidohydrolase activity in barley plants, and the level of potassium and phosphorus in soil. Our attempts to find any differences in OCT activity between plants growing in normal and in potassium-deficient medium as well as in putrescine-fed plants (Table 2, 3) were without effect.

Contrary to our observations, Maretzki, Nickell and Thom (1968, 1969) studying the metabolism of arginine in sugarcane cell cultures found N-carbamoylputrescine as the major product of arginine catabolism. The discrepancy between our results and those of Maretzki et al. (1968, 1969) may be the result of different material as well as different substrates. Maretzki used arginine which can be decarboxylated to agmatine and agmatine can be metabolized to N-carbamoylputrescine. We attempted to show the reverse mechanism *in vivo*, from that proved *in vitro* involved in carbamoylation of putrescine to N-carbamoylputrescine (see scheme 1). In our study pea plants did not accumulate N-carbamoylputrescine but probably metabolized it further to γ -aminobutyric acid (see table 4).

It is known that citrulline and especially carbamoylaspartate are hardly to be detected in some cells, although both the above mentioned compounds are very important constituents of cell nitrogen metabolism.

We assume that like the considerable activity of carbamoylation of putrescine *in vitro*, this mechanism operates also *in vivo*, because OCT as well as putrescine are natural constituents of the plant cell. We were not able to find in detectable amounts any other ^{14}C -labeled product in plants fed ^{14}C -putrescine except γ -aminobutyric acid (Table 4). On this path of putrescine metabolism there is no place for N-carbamoylputrescine.

Putrescine in *Vicia faba* is oxidatively deaminated to γ -aminobutyric aldehyde (Shevyakova 1966) and further oxidized to γ -aminobutyric acid. According to the scheme proposed by Smith and Garraway (1964), N-carbamoylputrescine may be an intermediary product in the synthesis of putrescine from agmatine. If we consider the above mentioned data, then the only role which putrescine carbamoylation can play in plant cells is the detoxication of the cell medium from large amounts of putrescine which is highly toxic for plants (see fig. 1 and table 1). In this assumption, the mechanism of putrescine carbamoylation should be "oscillatory". When a high concentration of putrescine is accumulated in the cell, the mechanism of carbamoylation starts. Successively after removal of the excess of putrescine through its conversion to nontoxic N-carbamoylputrescine, the reverse mechanism comes into play, N-carbamoylputrescine is again converted to putrescine, which is normally metabolized to γ -aminobutyric acid.

In some plants such as *Nicotiana tabaccum* ^{14}C -N-carbamoylputrescine is a good precursor of the nicotine-ring synthesis (Doisuke Yoshida 1966).

We found in our study that plant OCT is an enzyme which, beside the carbamoylation of ornithine and putrescine, catalyses additionally but in a smaller degree the carbamoylation of agmatine, spermidine, spermine (Kleczkowski and Wielgat 1968) and lysine (Kleczkowski 1965; Jasiorowska and Kleczkowski 1970).

CONCLUSIONS

1. The effect of putrescine and N-carbamoylputrescine fed to young pea seedlings was studied. It was found that 0.5% putrescine after 24 hours of feeding caused significant withering and appearance of yellowish-brown necrotic spots. Higher than 0.5% putrescine concentrations caused damage of the plants and death. N-carbamoylputrescine even at higher concentrations was not toxic for the plants. A detoxication mechanism of plant cells by carbamoylation of toxic putrescine to non toxic N-carbamoylputrescine is postulated.

2. There were no differences found in ornithine carbamoyltransferase (ornithine and putrescine as substrates) activity between normal and potassium-deficient plants as well as between water and 0.5‰ putrescine-fed plants.

3. ^{14}C -putrescine fed to excised pea seedlings was metabolized to γ -aminobutyric acid. No ^{14}C -N-carbamoylputrescine was detected. The "oscillatory" mechanism of N-carbamoylputrescine synthesis in higher plants is suggested. It could be based on the conversion of the excess of toxic putrescine to non toxic N-carbamoylputrescine, which can be again converted to putrescine and metabolized further to γ -aminobutyric acid.

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Metabolism putrescyny w siewkach grochu

Streszczenie

1. Podczas dokarmiania kiełków grochu roztworami putrescyny i N-karbamoiloputrescyny stwierdzono, że rośliny dokarmiane 0,5‰ roztworem putrescyny przez 24 godziny wykazują wyraźne objawy wędnięcia i nekrozy. Wyższe niż 0,5‰ stężenie putrescyny powoduje zamieranie roślin. N-karbamoiloputrescyna nawet w wyższych stężeniach nie wykazuje efektu toksycznego na dokarmiane rośliny. Postuluje się karbamoilację putrescyny jako mechanizm detoksykacji w okresach zwiększonego nagromadzenia tego związku.

2. Nie stwierdzono istotnych różnic w aktywności karbamoilotransferazy ornitynowej (ornityna i putrescyna jako substraty) w preparatach z roślin rosnących na normalnym i ubogim w potas podłożu, jak również między roślinami dokarmianymi putrescyną i kontrolnymi.

3. Po dokarmianiu roślin ^{14}C -putrescyną, większość radioaktywności stwierdzono w kwasie γ -aminomasłowym. Nie wykryto radioaktywnej N-karbamoiloputrescyny. Sugeruje się „wahadłowy” mechanizm karbamoilacji putrescyny w komórce roślinnej w przypadku jej większego nagromadzenia się.

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