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ALLANTOIN AND ALLANTOATE IN HIGHER PLANTS

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ABSTRACT

The amounts of amide nitrogen, ammonia, allantoin and allantoate were determined in *Phaseolus hysterinus* Dur. and *Glycine hispida* L. during a growth period of 28 days. *P. hys.* grown under different conditions showed remarkable differences in the amount of allantoate. Allantoinase was present in both plants and some properties of the enzyme were described. Allantoate was broken down in *P. hys.* in a manner yet unknown.

1. INTRODUCTION

About the metabolic pathway of allantoin in higher plants still little is known. The conversion of allantoin into allantoate, a reaction catalyzed by the enzyme allantoinase (allantoin amido-hydrolase EC 3.5.2.5), has been found already in 1929 by FOSSE and BRUNEL in *Soja hispida*. This enzyme has been shown to be widely distributed in higher plants (TRACEY, 1955).

Allantoicase (allantoate amidinohydrolase EC 3.5.3.4), has been reported only once in higher plants (*viz.* in *Soja hispida* by ÉCHEVIN and BRUNEL, 1937).

This enzyme was supposed to catalyze the breakdown of allantoate into 2 moles of urea and 1 mole of glyoxylate. This conversion has now been shown to be a two-step reaction (TRIJBELS and VOGELS, 1966a).

Other pathways of allantoate degradation in plants have not been reported. The aim of our investigation was to study the enzyme allantoinase and to find out the pathway along which allantoate is degraded in plants.

2. MATERIALS AND METHODS

Preparation of plant extracts: *Phaseolus hysterinus* Dur. (*P. hys.*) and *Glycine hispida* L. "Tübinger" (soybeans) were grown in the greenhouse under constant illumination for 16 hours a day (4800 lux/cm^2). In some cases plants were grown in another way described in the text.

To prepare extracts plants were stored for one night at —20°C, thawed, cut into small pieces and ground in a chilled mortar with quartz sand in 0.05 M Tris-HCl buffer, pH 7.4. The slurry was sonicated for 10 min in a MSE 500 W disintegrator and centrifuged at 10 000 g for 30 min. The cloudy supernatant was centrifuged at 100 000 g for one hour. The clear supernatant was used as enzyme

source. In some experiments the residue obtained by centrifugation at 10 000 g was used as enzyme source. All operations during the preparation of the extracts were performed at 4°C.

Allantoin, allantoate, ureidoglycine, ureidoglycolate and glyoxylate were determined according to TRIJBELS and VOGELS (1966b). Amide nitrogen was determined as free ammonia after hydrolysis of a sample in 2 N HCl for 3 hours. Free ammonia was determined with Nessler reagent. 7-^{14}C-allantoin was prepared from K^{14}CNO and 5-amino-hydantoin synthesized according to the method of BILTZ and GIESLER (1913).

Paperchromatography (descending development) was performed on Whatman No. 1 paper with phenol: water 4:1 (w/v) as solvent system. Allantoin, allantoate and urea were detected by spraying with p-dimethylaminobenzaldehyde in butanol: acetic acid 3:1. To detect radioactive compounds the dried chromatograms were passed through a Vanguard 880 automatic chromatogram scanner.

Allantoinase activity: the experiments were performed in mixtures containing per ml: 80 μmoles of TEA-HCl buffer, pH 7.4, 29 μmoles of allantoin, 0.3 μmoles of manganous sulphate and extract of soybeans or *P. hys.* containing 0.52 or 0.80 mg of protein, respectively. The mixtures were incubated at 30°C. The amount of allantoate formed was determined as described.

Unit of activity: one unit of allantoinase activity was defined as the amount, which will catalyze the transformation of 1 μmole of allantoin per minute under the conditions given. The specific activity was expressed in units per mg of protein.

Protein was determined according to LOWRY *et al.* (1951).

3. EXPERIMENTAL

The variation of some nitrogenous compounds during growth of *P. hys.* and soybeans was determined (Figs. 1 and 2). It appeared that amides and free ammonia were most abundant in *P. hys.*, whereas in soybeans only the amides were on a comparable level. Allantoin was present only in small amounts in both plants during all growth stages tested. Allantoate was present in moderate amounts; soybeans contained more allantoate than *P. hys.* In *P. hys.* the ammonia seems to be in contraphase with the amides and allantoate. During the test period we could never demonstrate the presence of urea.

From Table 1 it can be seen that plants grown in daylight on tap water contained more allantoate than plants grown under the same conditions on 0.5 % $(NH_4)_2SO_4$ in tap water. These plants contained also more allantoate than plants cultivated in the greenhouse. The greatest allantoate accumulation occurred when plants were grown in the dark on tap water.

In both plants allantoinase was present: *P. hys.* and soybeans contained 6.6 and 5.8 U/g of seed, respectively (specific activity 0.032 and 0.074 U/mg of protein). The total amount of allantoinase in each plant was constant during the tested period of 28 days. The pH-optimum of allantoinase from both sources was found to be 7.4.

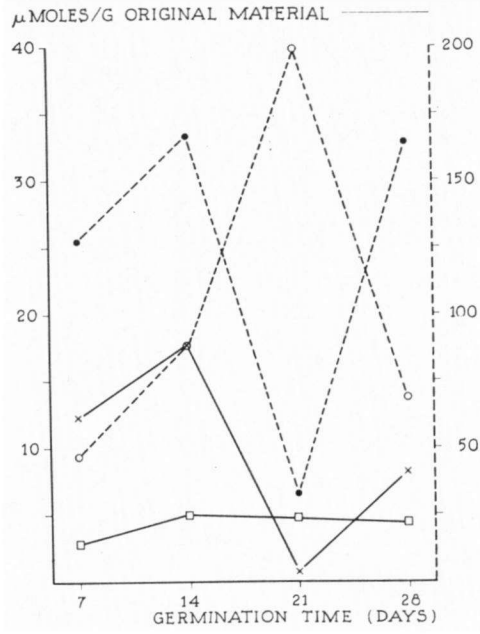

Fig. 1. Variation of some nitrogenous compounds during growth of *Phaseolus hysterinus:* allantoate (×————×), allantoin (□————□), amide nitrogen (●– – – –●) and NH_3 (o– – – –o).

The allantoinase reaction was completely inhibited by cysteine in a concentration of 8 mM. Phosphate ions too had an inhibiting effect on the enzymic activity. This is in accordance with the result found by LEE and ROUSH (1964). The K_m-values of the allantoinases from *P. hys.* and soybeans are 4.6×10^{-2}M and 1.4×10^{-2}M, respectively.

In contrast to the results of LEE and ROUSH (1964), who found an absolute specificity of the enzyme from soybeans for d-allantoin, we could not demonstrate such a specificity of the enzyme from both plants for d-allantoin, although optical rotation studies showed that in the case of soybean allantoinase d-allantoin was broken down about 10 times more rapidly than the l-form (Fig. 3). Manganous ions had only a slight activating effect on soybean allantoinase; the activity of allantoinase from *P. hys.* was not influenced by these ions.

On incubation of 25 mM sodium allantoate in 0.1 M phosphate buffer, pH 7.4, with the *P. hys.* residue at 30°C about 15 % of the substrate disappeared within one hour. Upon prolonged incubation up to 24 hours only 25–30 % of the substrate disappeared. However, we could not demonstrate the production of ureidoglycine, ureidoglycolate, glyoxylate or ammonia, which should be produced when allantoate was broken down according to one of the pathways known at this moment (VOGELS, 1966). After 24 hours of incubation only urea was found. Its presence has been demonstrated by paper-

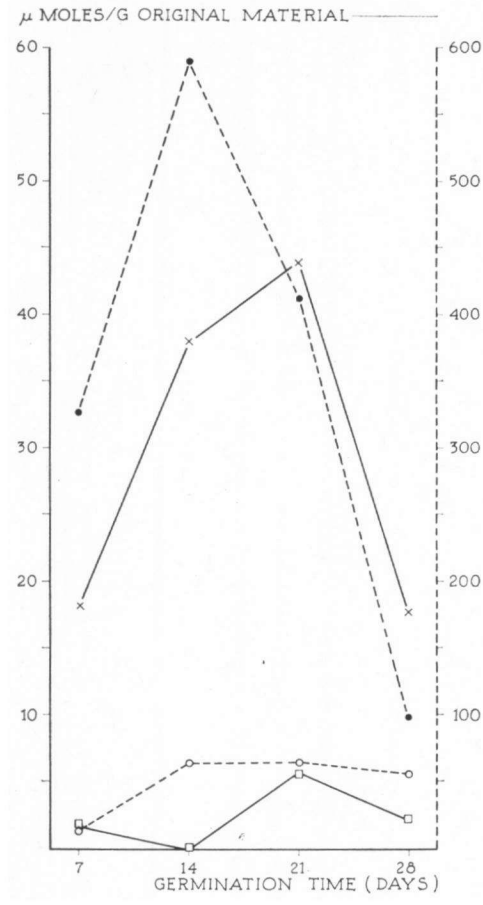

Fig. 2. Variation of some nitrogenous compounds during growth of *Glycine hispida:* allantoate (×————×), allantoin (□————□), amide nitrogen (●– – – –●) and NH_3 (○ – – – – ○).

TABLE 1

Amount of Allantoate present in *Phaseolus hysterinus* grown under different conditions

	Allantoate (μmoles/g original material)			
Germination time (days)	Daylight (tap water)	Daylight (0.5% $(NH_4)_2SO_4$)	Dark (tap water)	Greenhouse
3	10	11.5	16.1	—
6	15.7	13.5	22.5	—
9	29.7	19.5	—	13.9
15	52	31.2	—	15.2

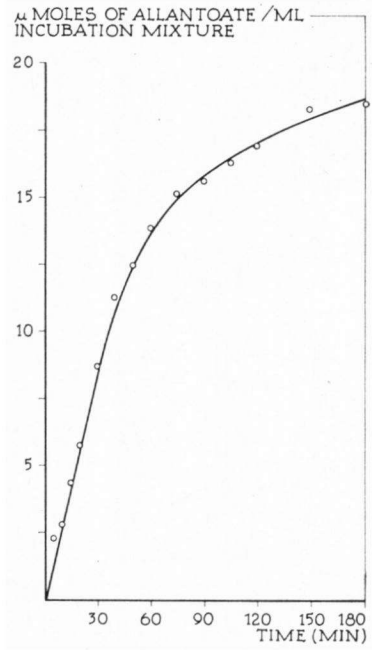

Fig. 3. Allantoate formation from allantoin by allantoinase from soybeans. Experiments were performed as described under Materials and Methods.

chromatography of an aliquot of the incubation mixture. On addition of urease to an aliquot of the incubation mixture ammonia was formed and after chromatography no urea could be detected.

Under similar conditions 7-^{14}C-allantoin was incubated: after 2, 4, 6 and 24 hours of incubation a 20 μl aliquot was taken and subjected to paperchromatography. After drying the paperstrips were scanned for radioactivity. In this case after 24 hours of incubation also only urea was found, outside allantoin and allantoate.

In soybeans we did not observe any disappearance of allantoate, under the same conditions. When the 100 000 g supernatant was used as enzyme source no disappearance of allantoate occurred in both plants.

4. Discussion

Plants grown in the dark show a much greater allantoate accumulation than plants grown in daylight or constant illumination. A similar effect was observed for *Soja hispida* (Échevin and Brunel, 1937) and *Trifolium sativum* (Fosse, de Graeve and Thomas, 1933). So, there may be some connection between allantoate degradation and photosynthesis. In agreement with this Reinbothe (1961) suggested that in chlorophyll-deficient leaves an allantoate degrading enzyme activity was absent. This hypothesis shall need further work to confirm this point of view.

Allantoinase is in higher plants a constitutive enzyme. The specific activity of allantoinase in our crude extracts of *P.hys.* and soybeans is much greater than that found for allantoinase from soybeans and mung beans (LEE and ROUSH, 1964; NAGAI and FUNAHASHI, 1961).

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