

Rapid Communication

Adenylosuccinate Synthetase: Site of Action of Hydantocidin, a Microbial Phytotoxin

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The site of action of hydantocidin was probed using *Arabidopsis thaliana* plants growing on agar plates. Herbicidal effects were reversed when the agar medium was supplemented with AMP, but not IMP or GMP, suggesting that hydantocidin blocked the two-step conversion of IMP to AMP in the de novo purine biosynthesis pathway. Hydantocidin itself did not inhibit adenylosuccinate synthetase or adenylosuccinate lyase isolated from *Zea mays*. However, a phosphorylated derivative of hydantocidin, *N*-acetyl-5'-phosphohydantocidin, was a potent inhibitor of the synthetase but not of the lyase. These results identify the site of action of hydantocidin and establish adenylosuccinate synthetase as an herbicide target of commercial potential.

Hydantocidin is a phytotoxin isolated from cultures of *Streptomyces hydroscopicus* (Nakajima et al., 1991). This compound is a potent, nonselective herbicide when applied to emerged plants and is similar in efficacy to glyphosate. Significantly, it is minimally toxic to mice, with an oral 50%-lethal dose of >1000 mg/kg (Nakajima et al., 1991). Thus, hydantocidin would be a useful herbicide were it economically synthesized. It nevertheless constitutes an important lead in the discovery of novel mechanisms for weed control, a practice currently exploiting only about 15 known enzymatic targets (Duke, 1990). It seemed that defining the mode of action of hydantocidin should reveal an effective new target enzyme.

Adenylosuccinate synthetase catalyzes the first step in the two-step conversion of IMP to AMP in the de novo purine biosynthesis pathway (Stayton et al., 1983). The crystal structure of the bacterial enzyme has been elucidated (Poland et al., 1993), and the kinetic mechanism has been well described in ongoing investigations (most recently, Kang and Fromm, 1995). It is known to be the site of action of several antibiotics, including hadacidin and alanosine (Stayton et al., 1983). Inhibition of adenylosuccinate synthetase by alanosine was shown to have antiviral and antitumor activity (Murthy et al., 1966), suggesting the utility of inhibitors of this enzyme in therapeutic applications. Furthermore, the enzyme is a component of the quantitatively significant purine nucleotide cycle of mam-

malian muscle (Lowenstein, 1990) and pancreatic islets (Marynissen et al., 1992).

Surprisingly, there is only one literature report of adenylosuccinate synthetase from a plant source, that on the wheat germ enzyme (Hatch, 1966). The present study strongly implicates adenylosuccinate synthetase as the site of action of hydantocidin.

MATERIALS AND METHODS

Materials

Hydantocidin (for structures, see Fig. 1) was synthesized as described (Mio et al., 1991). NAH¹ and NAPH² were synthesized by variations of that procedure to be published elsewhere. Hadacidin was synthesized as described (Jahngen and Rossomando, 1982). All other reagents for biochemical analysis and yeast adenylosuccinate lyase were purchased from Sigma. Seeds of *Arabidopsis thaliana* Columbia were obtained from Lehle Seeds (Tucson, AZ).

Growth Experiments

Arabidopsis seeds were grown in Murashige and Skoog mineral medium, pH 6.0, supplemented with 10 g/L Suc and solidified with 8 g/L phytagar. Growth experiments were conducted in triplicate, in 24-well sterile plates, with 1 mL of medium per well. Probe compounds illustrated in Figure 1 were prepared as 2 to 5 mM stock solutions, filter sterilized, and added to the sterile medium before it was dispensed into the wells. Supplements to the medium such as those listed in Table I were prepared as 5 (adenine) or 20 (all others) mg/mL stock solutions, filter sterilized, and added directly to the wells prior to dispensing the medium. Seeds (20–50 mg) were sterilized in a 50-mL sterile polypropylene tube with 5-fold diluted commercial bleach

¹ Spectral data supporting the structure of NAH: ¹H NMR (DMSO-d₆) δ 2.44 (s, 3); ¹³C NMR (DMSO-d₆) δ 171.3, 168.9, 153.1, 97.2, 89.2, 70.8, 70.3, 61.7, and 26.1.

² Spectral data supporting the structure of NAPH: ¹H NMR (CD₃OD) δ 2.60 (s, 3); ¹³C NMR (CD₃OD) δ 173.3, 171.1, 154.8, 99.4, 88.9, 73.0, 72.1, 67.0, and 26.7.

Abbreviations: EPPS, *N*-(2-hydroxyethyl)piperazine-*N'*-3-propanesulfonic acid; NAH, *N*-acetylhydantocidin; NAPH, *N*-acetyl-5'-phosphohydantocidin.

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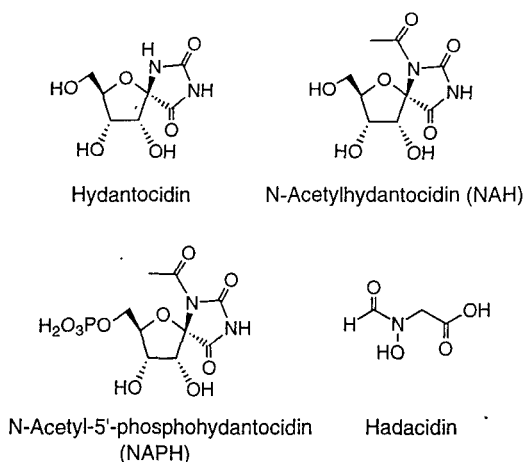


Figure 1. Structures of compounds used in this study.

followed by repeated washing in sterile water. About 12 seeds were added to each well using a 1000- μ L pipetter. The 24-well plates were sealed and kept at 27°C for 7 to 10 d prior to scoring the growth by means of a visual estimate of the size of the plants compared to those grown in the absence of added compounds.

Extraction and Isolation of Adenylosuccinate Synthetase and Lyase

Adenylosuccinate synthetase was partially purified from 4- or 5-d-old etiolated seedlings of *Zea mays*. All procedures were performed at 4°C. Seedlings were homogenized in a Waring blender with an equal volume (gram of tissue = milliliter of buffer) of 50 mM EPPS, pH 7.5, 5 mM MgCl₂, 1 mM EDTA, 5 mM sodium bisulfite, 5 mM DTT, 0.1 mM PMSF, and 100 mL/L glycerol. The extract was expressed through Miracloth (Calbiochem) sandwiched between layers of cheesecloth. To each liter of extract was added 4 g of protamine sulfate; the mixture was then stirred for 30 min and subjected to centrifugation at 45,000g for 60 min. The resulting supernatant was fractionated by ammonium sulfate precipitation. Retained were proteins precipitating at concentrations of ammonium sulfate greater than 45%, but less than 55%, of saturation. Similar methods were used to isolate adenylosuccinate synthetase from *Escherichia coli* cells and rat liver acetone powder (Sigma). The maize enzyme was further purified by chromatography on DEAE-cellulose (Whatman, DE 52). The column was initially equilibrated with 50 mM EPPS, pH 7.5, 5 mM MgCl₂, 0.1 mM EDTA, 100 mL/L ethylene glycol, and 1 mM DTT. Elution was with a linear, 14-bed-volume gradient of 0 to 0.4 M KCl dissolved in the same buffer. Yield of adenylosuccinate synthetase activity from DEAE-cellulose was 3 to 4 nmol min⁻¹ g⁻¹ fresh weight, and was 20-fold purified relative to the protamine sulfate supernatant. The HPLC conditions described below were used to assess the stability of IMP, GTP, and adenylosuccinate as indicators of the presence of interfering enzyme activities in this preparation. Activities of IMP-5'-nucleotidase, GTPase, and adeny-

losuccinate lyase were 3.1, 1.3, and 0.5%, respectively, of the activity of adenylosuccinate synthetase. These values were considered to be negligible.

Adenylosuccinate lyase was extracted from 3-d-old etiolated seedlings of *Z. mays*. Seedlings were homogenized in a Waring blender with an equal volume of 20 mM potassium phosphate, pH 7.0, containing 1 mM DTT (KP buffer). The resultant slurry was filtered through two layers of cheesecloth, and the filtrate was subjected to 100,000g centrifugation for 60 min. The supernatant was used for adenylosuccinate lyase activity.

Enzyme Assays

Adenylosuccinate synthetase was assayed in the presence of 50 mM EPPS, pH 7.5, 10 mM potassium phosphate, 1 mM MgCl₂, 0.1 mM EDTA, and 100 mL/L ethylene glycol (assay buffer). Final concentrations of substrates were 3 mM aspartate, 0.2 mM IMP, and 0.2 mM GTP, but the sequence of addition varied as described in the figure legends (see "Results"). Anion-exchange HPLC was used to separate substrates from products and quantitate product formation. Following a 15-min reaction, 100 μ L of the reaction mixture were injected into a Hamilton PRP-X100 column equilibrated with 3% acetonitrile. Reactants were eluted with a convex gradient (curve = -2) to 6% 1 M potassium phosphate, pH 7, 12% 2 M KCl, and 3% acetonitrile delivered during a span of 30 min at a flow rate of 0.8 mL/min by a Perkin-Elmer 410 BIO pump. The column eluate was monitored at 270 nm using a Perkin-Elmer LC 480 diode array detector. The identities of the products of the adenylosuccinate synthetase reaction, GDP, and adenylosuccinate were determined by comparing their retention times and spectra with those of authentic standards. For the time-course studies, the identical reaction mixture was continuously monitored at 280 nm using a Beckman DU7 spectrophotometer.

Adenylosuccinate lyase was assayed in a reaction mixture of 1 mL of KP buffer containing 0.15 mM adenylosuccinate. The rate of the reaction was measured by monitoring the disappearance of the substrate at 280 nm.

RESULTS

Arabidopsis Growth Experiments

The mode of action of hydantocidin and other related compounds (Fig. 1) was initially probed by observing the growth of *Arabidopsis* seedlings in the presence of herbicidal compounds and putative antidotes. In the first phase, minimum lethal concentrations (those causing 95 to 100% inhibition of growth) of hydantocidin, NAH, NAPH, and hadacidin were determined to be 2, 12, 8, and 50 μ M, respectively. The ability of a range of classes of biosynthetic products to reverse growth inhibition was then tested. Despite the similarity between the symptoms caused by hydantocidin and glyphosate, aromatic amino acids in any combination did not reverse the growth inhibition of *Arabidopsis* caused by hydantocidin (not shown), consistent with the lack of structural similarity between hydantocidin

and any of the intermediates of the shikimate pathway. On the other hand, consistent with hydantocidin bearing a structural similarity to a nucleoside, inhibition of *Arabidopsis* growth caused by hydantocidin was readily reversed by the purine mononucleotides (not shown).

More detailed studies showed that growth inhibition was effectively reversed by AMP but not by GMP, IMP, or GTP (Fig. 2). The most effective reversion occurred with AMP, at a minimum optimal concentration of 0.5 mM. The pattern of protection by putative antidotes from the growth inhibition caused by NAH, NAPH, and hadacidin was similar to that observed with hydantocidin (Table I). Clearly, AMP, adenosine, and adenine were effective in reversing the inhibition caused by all of the compounds, whereas IMP and GMP were ineffective. Pyrimidines were also ineffective (not shown). These results suggest that hydantocidin exerts its herbicidal effect by inhibiting the conversion of IMP to AMP (Fig. 3). This hypothesis is supported by the knowledge that hadacidin is an inhibitor of adenylosuccinate synthetase (Markham and Reed, 1977). Because adenine and adenosine readily reversed the inhibition caused by these compounds, we eliminated the salvage pathway for synthesis of AMP (Fig. 3) from further consideration.

Enzyme Assays

To determine which of the two enzymes that convert IMP to AMP were inhibited by hydantocidin and its deriv-

atives, the enzymes were extracted and assayed for inhibition. The compounds illustrated in Figure 1 were tested as potential inhibitors of adenylosuccinate lyase from yeast as well as maize after preincubation with the enzyme from 0 to 60 min. At 1 mM, well in excess of the concentration required for 100% inhibition of *Arabidopsis* growth, none of these compounds inhibited adenylosuccinate lyase from either source (data not shown).

As expected, maize adenylosuccinate synthetase was strongly inhibited by 10 μM hadacidin (Fig. 4). Neither hydantocidin (Fig. 4) nor NAH (not shown) inhibited the synthetase at a concentration of 0.5 mM. This was surprising because, as noted above, hydantocidin did not inhibit adenylosuccinate lyase. Based on the hypothesis that hydantocidin could exert its effect by mimicking a nucleotide, the phosphorylated derivative NAPH (Fig. 1) was synthesized. Consistent with the results presented in Table I, NAPH almost completely inhibited adenylosuccinate production at a concentration of 2 μM (Fig. 4). As with the maize enzyme, adenylosuccinate synthetase isolated from *E. coli* and rat liver was inhibited by NAPH and hadacidin but not by NAH or hydantocidin (data not shown).

The degree of inhibition of maize adenylosuccinate synthetase by NAPH was observed to increase with time, the duration of which was a function of the concentration of NAPH (Fig. 5). In contrast, the degree of inhibition by hadacidin was not measurably time dependent (Fig. 5). To determine whether the time dependence was due to mod-

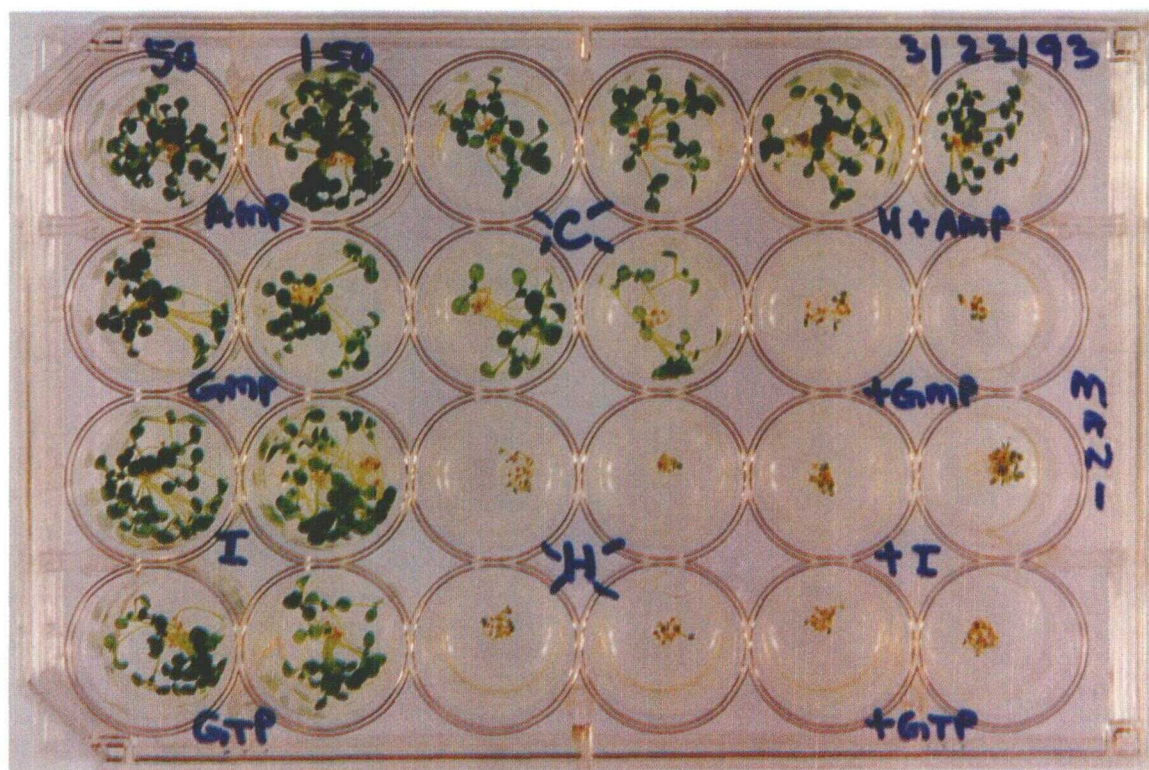


Figure 2. Inhibition of *Arabidopsis* growth by hydantocidin and its reversal by AMP. I, IMP; H, hydantocidin; C, control (no supplements). Antidotes were present at 1 (columns 1 and 5) or 3 (columns 2 and 6) mg/mL. Hydantocidin was present at 2 μM . The left two columns probe the effect of antidotes alone; the center two, the effect of hydantocidin alone; and the right two, hydantocidin and antidotes given in combination.

Table 1. Effect of various antidotes on the inhibition of growth of *Arabidopsis* caused by hydantocidin, NAH, NAPH, or hadacidin

Hydantocidin, NAH, NAPH, and hadacidin were present at 2, 12, 8, and 50 μM , respectively. None of the antidotes had any effect on the growth of *Arabidopsis* without the inhibitors. Growth was evaluated by means of a visual estimate of the size of the plants compared to those grown in the absence of added compounds.

| Compound ^a | Antidote ^b | Percent Growth |
|--|-----------------------|----------------|
| None | None | 100 |
| Hydantocidin, NAH, NAPH, or hadacidin | None | 0–5 |
| | IMP | 0–5 |
| | AMP | 80–100 |
| | GMP | 0–5 |
| | Adenine | 60 |
| | Adenosine | 80 |

^a For structures, see Figure 1. ^b Antidotes were present at 2 mg/mL, except adenine, which was at 0.5 mg/mL.

ification of NAPH by the enzyme preparation, the enzyme was combined with NAPH and incubated in the absence of the substrates (GTP, IMP, and aspartate) for 20 min, then the reactions were started by addition of substrates. The ensuing progress curves were not linear at the inhibited reaction rates but, rather, were identical to those shown in Figure 5. To determine whether the time required for the onset of maximal inhibition was due to the formation of a

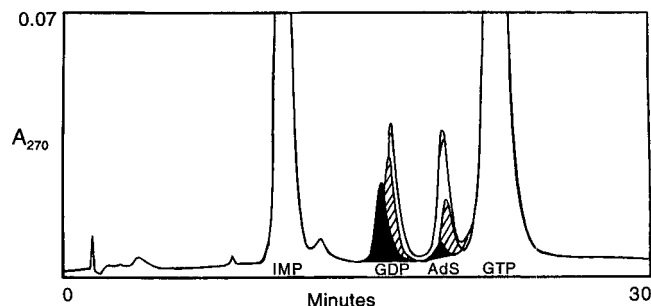


Figure 4. Inhibition of maize adenylosuccinate synthetase by NAPH and hadacidin. Anion-exchange HPLC showing the amount of adenylosuccinate (AdS) formed in 15 min in the presence of no inhibitor (open), 500 μM hydantocidin (open), 10 μM hadacidin (cross-hatched), or 2 μM NAPH (filled). Enzyme preparation (0.11 mg) was diluted with assay buffer (see "Materials and Methods") to a volume of 170 μL . To this was added 20 μL of test compound or water and 8 μL of a mixture of aspartate and GTP. After a 15-min preincubation, reactions were started with the addition of 2 μL of IMP and allowed to proceed for 15 min before 100 μL were injected into the column. The appearance of a greater effect of the inhibitors on adenylosuccinate production than on GDP production is due entirely to the presence of GDP, a contaminant in the stock solution of GTP, at the start of the enzymatic reaction.

covalent bond between NAPH and adenylosuccinate synthetase, an enzyme-inhibitor complex was formed by incubating a mixture of enzyme, 0 or 5 μM NAPH, and substrates for 20 min, then passing the mixture through Sephadex G-25 equilibrated with assay buffer. When NAPH was present in or omitted from the mixture, 97 or 86% of the activity applied to the gel-filtration columns was recovered, respectively. A control mixture that was not passed through Sephadex G-25 was totally inactive. Thus, inhibition of adenylosuccinate synthetase by NAPH is reversible.

Linear, inhibited reaction rates were obtained when enzyme was incubated with NAPH, GTP, and aspartate prior to initiating the reaction with IMP (see Fig. 4 legend for details). Using this procedure with varying concentrations of NAPH, a 50%-inhibitory value of 140 ± 15 nM was obtained.

DISCUSSION

The reversal of the growth-inhibitory effect of hydantocidin in *Arabidopsis* plants by AMP but not by IMP or GMP suggested that the target enzyme was adenylosuccinate synthetase or adenylosuccinate lyase. This conclusion was strongly supported by the observation that the pattern of rescue was identical to that of the known adenylosuccinate synthetase inhibitor hadacidin. Furthermore, IMP was shown to be capable of entering *Arabidopsis* plants and reversing growth inhibition caused by formycin B, a toxic analog of adenine (data not shown).

The absence of inhibition of either adenylosuccinate synthetase or adenylosuccinate lyase by hydantocidin was perplexing, but models suggested a structural analogy between hydantocidin and the *anti* conformer of inosine.

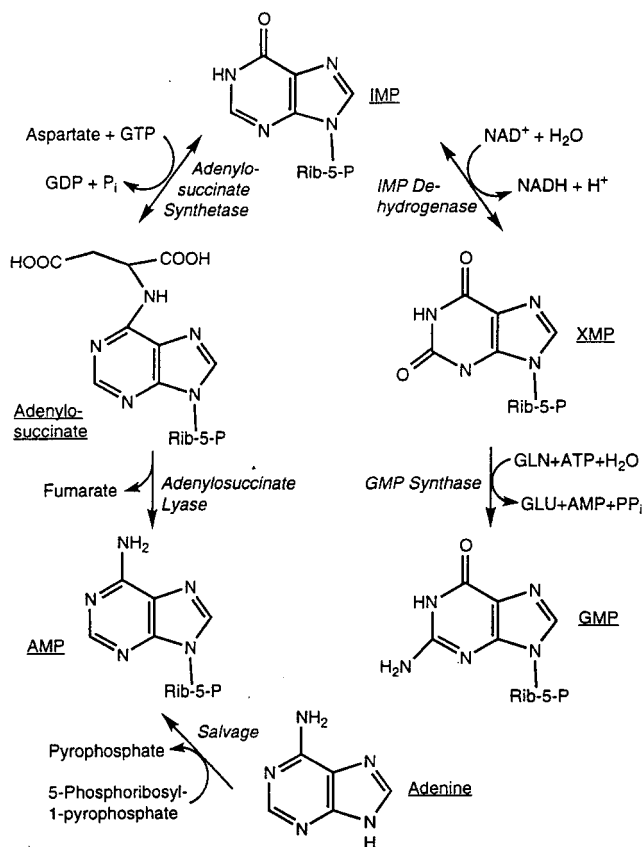


Figure 3. Conversion of IMP to AMP and GMP, and salvage of adenine.

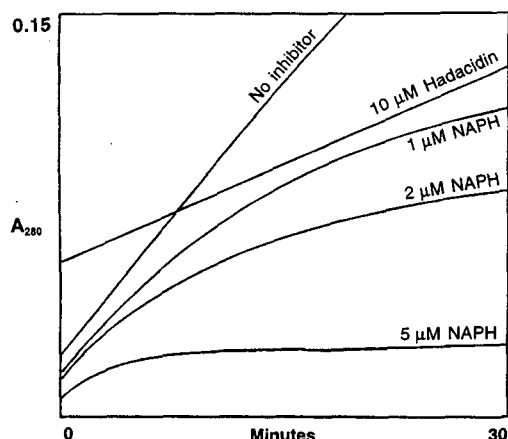


Figure 5. Time course of the adenylosuccinate synthetase reaction: inhibition by hadacidin and NAPH. Enzyme preparation (0.11 mg) was combined with inhibitors in a volume of 192 μL , and reactions were started promptly with the addition of 8 μL of a mixture of GTP, IMP, and aspartate. The rate of formation of adenylosuccinate in the control reaction was 0.6 $\mu\text{M}/\text{min}$.

Hence, we hypothesized that hydantocidin may need to be phosphorylated at the 5' position to be an inhibitor of one of these enzymes. Indeed, once synthesized and tested, NAPH proved to be a potent inhibitor of adenylosuccinate synthetase, exhibiting the time-dependent inhibition displayed by many tight-binding inhibitors (Schloss, 1988). The inactivity *in vitro* of NAH indicates that of the two modifications of hydantocidin present in NAPH, phosphorylation rather than acetylation accounts for its inhibitory activity. Because hydantocidin was more potent in the *Arabidopsis* bioassay than NAH, it is possible that 5'-phosphohydantocidin would be a better inhibitor than NAPH.

The interpretation that these results elucidate the mode of action of hydantocidin rests on the assumption that plants are able to phosphorylate hydantocidin, which has not been investigated. The situation is similar to that of the antibiotics 5-ethynyl-1- β -D-ribofuranosylimidazole-4-carboxamide (Balzarini et al., 1993) and ribavirin (Streeter et al., 1973; Willis et al., 1978), which are antiviral compounds that must be phosphorylated *in vivo* to become inhibitors of IMP dehydrogenase. Prior to the discovery of the mechanism of their phosphorylation *in vivo*, those compounds were shown to be inactive on the target enzyme unless phosphorylated. The low level of toxicity of hydantocidin to life forms other than plants may arise from an inability to phosphorylate hydantocidin or an ability to rapidly dephosphorylate 5'-phosphohydantocidin. Selective toxicity is apparently not due to differential inhibition of adenylosuccinate synthetase by NAPH, because the enzyme isolated from *E. coli* or rat liver was inhibited by NAPH (not shown). Inhibition of wheat germ adenylosuccinate synthetase by hadacidin and growth inhibition of wheat seedlings and its reversal by adenine derivatives have been reported (Hatch, 1967).

While this manuscript was in review, a report appeared showing a similar pattern of reversion of growth inhibition

due to hydantocidin in *Arabidopsis* growth tests (Heim et al., 1995). In addition, treatment of *Arabidopsis* plants with hydantocidin resulted in reduced incorporation of [^{14}C]Gly into adenine but not guanine nucleotides and depleted pools of ATP. As in our study, hydantocidin did not inhibit adenylosuccinate synthetase or lyase, but an inhibitor of the synthetase was extracted from plants treated with hydantocidin. Our finding that a phosphorylated derivative of hydantocidin is a potent inhibitor of the synthetase is complemented by an experiment in the earlier report in which an undefined inhibitor of the synthetase was formed in a time-dependent manner in a mixture of hydantocidin, ATP, MgCl_2 , and an extract of *Setaria faberi*.

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