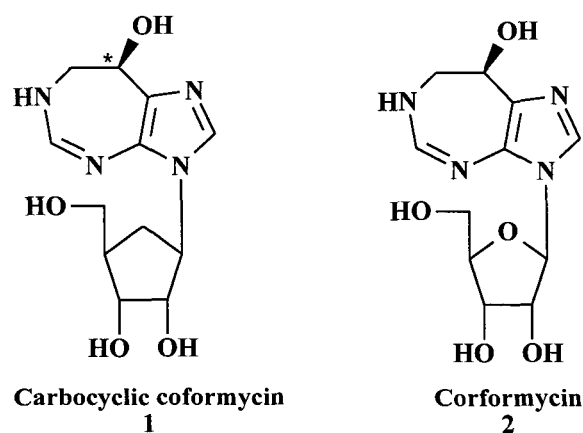# Adenosine-5'-Phosphate Deaminase

A Novel Herbicide Target

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Department of Biochemistry, AgrEvo UK Limited, Chesterford Park, Saffron Walden, Essex, CB10 1XL, United Kingdom (J.E.D., R.G.H., S.D.L.); and Hoechst Schering AgrEvo GmbH, D-65926 Frankfurt am Main, Germany (S.D.L.)

The isolation of carbocyclic coformycin as the herbicidally active component from a fermentation of *Saccharothrix* species was described previously (B.D. Bush, G.V. Fitchett, D.A. Gates, D. Langley [1993] Phytochemistry 32: 737–739). Here we report that the primary mode of action of carbocyclic coformycin has been identified as inhibition of the enzyme AMP deaminase (EC 3.5.4.6) following phosphorylation at the 5' hydroxyl on the carbocyclic ring in vivo. When pea (*Pisum sativum* L. var Onward) seedlings are treated with carbocyclic coformycin, there is a very rapid and dramatic increase in ATP levels, indicating a perturbation in purine metabolism. Investigation of the enzymes of purine metabolism showed a decrease in the extractable activity of AMP deaminase that correlates with a strong, noncovalent association of the phosphorylated natural product with the protein. The 5'-phosphate analog of the carbocyclic coformycin was synthesized and shown to be a potent, tight binding inhibitor of AMP deaminase isolated from pea seedlings. Through the use of a synthetic radiolabeled marker, rapid conversion of carbocyclic coformycin to the 5'-phosphate analog could be demonstrated in vivo. It is proposed that inhibition of AMP deaminase leads to the death of the plant through perturbation of the intracellular ATP pool.

Carbocyclic coformycin
1

Corformycin
2

Structures 1 and 2.

A novel carbocyclic analog of coformycin (Structure 1) isolated from a fermentation of *Saccharothrix* spp. was found to have excellent herbicide activity, controlling a wide range of species at <32 g ha^{-1} postemergence (Table I) (Bush et al., 1993). The symptoms resulting from treatment with this novel herbicide are cessation of growth, followed by paling and necrosis at the apical meristems. It is interesting that these effects are also characteristic of the inhibition of amino acid biosynthesis caused by glyphosate or compounds acting on acetolactate synthase (EC 4.1.3.18). An important clue, however, to the biochemical mode of action was the recognition that the related compound coformycin (Structure 2) is a very potent inhibitor of the enzyme adenosine deaminase (EC 3.5.4.4) (Nakamura et al., 1974; Cha et al., 1975). Adenosine deaminase catalyzes the hydrolytic deamination of adenosine to inosine. The 8-hydroxy-[1,3]-diazepin ring of coformycin is thought to mimic a transition state or high energy intermediate in this reaction (Frieden et al., 1980; Frick et al., 1986; Jones et al.,

1989). We have confirmed, however, that plants do not contain adenosine deaminase. The primary mode of action of carbocyclic coformycin is, in fact, inhibition of the related enzyme AMP deaminase following phosphorylation in vivo. The evidence that has led us to this conclusion is reported here.

MATERIALS AND METHODS

Adenosine deaminase (from calf intestinal mucosa), AMP deaminase (from rabbit muscle), and alkaline phosphatase (from bovine intestinal mucosa) were obtained from Sigma. [U-^{14}C]Adenosine (571 Ci mol^{-1}) and [8-^{14}C]AMP (953 Ci mol^{-1}) were obtained from Amersham. Coformycin was obtained from Calbiochem Novabiochem UK Ltd. (Nottingham, UK) Sephadex G-25M PD10 desalting columns (1.5 cm × 8 cm) were obtained from Pharmacia.

Plant Material and Growth Conditions

Peas (*Pisum sativum* L. var Onward) were grown in soil for 10 d at 25°C with a 16-h/8-h light/dark cycle. *Polygonum lapathifolium* plants were grown for 7 d in vermiculite at 25°C with a 16-h/8-h light/dark photoperiod.

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Abbreviation: I$_{50}$, concentration of test compound at which 50% of enzyme activity is inhibited.

Synthesis of Phosphorylated Carbocyclic Coformycin and Tritiated Carbocyclic Coformycin

Carbocyclic coformycin was isolated essentially as described by Bush et al. (1993). The 5'-monophosphate of carbocyclic coformycin was prepared from carbocyclic coformycin by a modification of the method described by Bennett et al. (1986) for the preparation of neoplanacin 5'-monophosphate. The phosphorylation reaction required 5 h, and the product was purified by column chromatography using C-18 reverse-phase silica 60 with water as eluent. The purity of the product was confirmed by cation-exchange chromatography as described below.

($\pm$)-Carbocyclic 8-tritiocoformycin was prepared using the following procedure: sodium borotritide (0.4 mg, 100 mCi) in 200 μL of dry methanol was instilled into a solution of 5 mg ($\pm$)-3-[(1β,2α,3α,4β)-2,3-dihydroxy-4-hydroxymethyl)cyclopentyl]-6,7-dihydroimidazo[4,5-d]-[1,3]diazepin-8(3H)-one in 500 μL of water at 0°C under argon. The reaction mixture was allowed to warm to ambient temperature for 1 h and then recooled to 0°C. A solution of 0.4 mg of sodium borohydride in 100 μL of dry methanol was added, and the temperature was allowed to rise to room temperature. After 2 h, solid carbon dioxide was added until the pH was between 7.0 and 8.0, and then the reaction mixture was concentrated in vacuo. The product was purified by reverse-phase chromatography on a C-18 silica column with water as eluent. This procedure yielded 1.8 mCi of racemic product of specific activity 400 Ci mol^{-1}.

Direct Spectrophotometric Assay for Mammalian Adenosine Deaminase

Nucleoside analogs were preincubated at the concentrations shown with 0.04 units of adenosine deaminase in 2.1 mL of phosphate buffer (0.1 M, pH 7.5) for 10 min at 25°C. The reaction was started by adding 100 μL of 0.8 mM adenosine to 700 μL of the preincubation mix. The decrease

Table I. *Efficacy of carbocyclic coformycin (% control) 26 d after postemergent application*

One-week-old plants were sprayed with carbocyclic coformycin in 40% (v/v) aqueous acetone containing 0.5% (v/v) Ethomeen T25 as a surfactant. The treated plants were kept in a greenhouse for 26 d prior to visual assessment of overall damage compared with control plants treated with the aqueous acetone and surfactant alone.

Species	Percent Control by Carbocyclic Coformycin	
	Application at 125 g ha^{-1}	Application at 32 g ha^{-1}
Sorghum halapense	100	35
Paspalum conjugatum	100	98
Cynodon dactylon	98	75
Imperata cylindrica	99	99
Cyperus rotundus	65	40
Convolvulus arvensis	90	50
Cirsium arvense	100	80
Rumex crispus	100	80
Taraxacum officinale	80	50
Sonchus arvensis	100	100

in A_{265} was measured spectrophotometrically over 2 min against a reference cuvette containing 800 μL of the preincubation solution.

Dissociation of Carbocyclic Coformycin from Mammalian Adenosine Deaminase

Adenosine deaminase (4 units) was incubated with 25 nM carbocyclic coformycin in 100 μL of phosphate buffer (0.1 M, pH 7.5) at 25°C for 10 min or 16 h. A 30-μL aliquot was then transferred to 3 mL of phosphate buffer (0.1 M, pH 7.5) containing 100 μM adenosine, and the reaction was followed as the decrease in A_{265} over 4 min against a reference cuvette containing all of the assay components except adenosine. An increase in the activity of the enzyme during the course of the assay is taken as evidence for the dissociation of the inhibitor. The activity of the inhibited enzyme was related to that of a control that was treated identically, but from which the carbocyclic coformycin had been omitted.

Direct Spectrophotometric Assay for Inhibition of Mammalian AMP Deaminase

Nucleoside analogs were preincubated with 0.04 units of AMP deaminase in 2.1 mL of 10 mM citrate buffer, pH 6.5 (2 M NaOH), 0.05% (w/v) BSA, and 33 mM KCl at 25°C for 10 min. The reaction was then started by adding 100 μL of 0.8 mM AMP to 0.7 mL of the preincubation buffer. The decrease in A_{265} was measured spectrophotometrically over 2 min against a reference cuvette containing 800 μL of the preincubation mixture.

Metabolism of [^{14}C]Adenosine and [^{14}C]AMP by Crude, Desalted Pea Seedling Extracts

Pea shoots were harvested close to soil level and homogenized in 2 volumes of ice-cold buffer containing Tris-HCl (0.1 M, pH 7.4, with 5 M NaOH), 0.1 mM DTT, and 0.4 M Suc using a Waring blender (1 min at top speed). The homogenate was passed through six layers of muslin and 2.5 mL was desalted on a Sephadex PD10 gel-filtration column (1.5 cm × 8 cm) that had been pre-equilibrated with the homogenization buffer. Protein was eluted using the same buffer. The assay contained, in a total volume of 250 μL, 50 mM Tris-HCl (pH 6.5), 50 mM KCl, 0.05% (w/v) BSA, and approximately 100 μg of protein. ATP (5 mM) was also included where indicated. The assay was started by the addition of either [^{14}C]adenosine (500 μM, 0.2 μCi) or [^{14}C]AMP (500 μM, 0.2 μCi). After incubation at 25°C for the time intervals indicated, the reaction was stopped by the addition of 250 μL of ice-cold 0.4 M HClO$_4$. Precipitated protein was removed by centrifugation (13,000g, 2 min), and 400 μL of the resulting supernatant was applied to a phenylsilane-bonded silica gel column (1 cm × 2 cm) that had been previously washed with 2 volumes of methanol and then 2 mL of 0.4 M HClO$_4$. Samples were eluted with 4 mL of 0.4 M HClO$_4$. The pH of the eluate was adjusted on ice to between 5.5 and 7.0 with 5 M KOH. The neutralized extract was centrifuged (13,000g, 2 min) and evaporated to dryness. The sample was then redissolved in 500 μL of 10

mM HCl and centrifuged (13,000g, 2 min) prior to HPLC analysis. The products of [^{14}C]adenosine metabolism were analyzed using reverse-phase chromatography, and the products of [^{14}C]AMP metabolism were analyzed by anion-exchange chromatography as described below.

Partial Purification of AMP Deaminase from Pea Shoots

AMP deaminase was partially purified from pea shoots essentially as described by Yoshino and Murakami (1980). Pea seedlings were harvested close to soil level and homogenized in 2 volumes of ice-cold phosphate buffer (pH 7.5) containing 0.1 mM DTT and 0.4 M Suc using a Waring blender (1 min, top speed). All of the following operations were carried out at 4°C. The homogenate was filtered through six layers of muslin and centrifuged at 100,000g for 20 min to remove cell debris. To the resulting supernatant was added (NH$_4$)$_2$SO$_4$ to 40% saturation while stirring constantly. The precipitate was collected by centrifugation (12,000g, 15 min) and dissolved in a minimum volume of phosphate buffer (pH 7.5), 0.1 mM DTT, then dialyzed overnight against 2 L of the same buffer. Insoluble material formed during the overnight dialysis were removed by centrifugation (10,000g, 10 min). The supernatant was applied to a phosphocellulose column (1.5 cm × 8 cm) that had been pre-equilibrated with 0.1 M phosphate buffer (pH 7.5). After loading, the column was washed with 14 mL of phosphate buffer (0.1 M, pH 7.5), and the enzyme eluted with 0.6 M phosphate buffer (pH 7.5). The partially purified enzyme was stored in aliquots at −70°C until required.

NH$_3$-Release Assay for Partially Purified Pea AMP Deaminase

A stopped spectrophotometric assay based on the method of McCullough (1967) was used in the purification of the plant enzyme and subsequent kinetic analysis. The assay contained (in a total volume of 150 μL) 60 mM citrate buffer (pH 7.1, with 5 M NaOH), 100 mM KCl, 0.1% (w/v) BSA, 1 μM diadenosine pentaphosphate, and approximately 0.01 units of AMP deaminase. The enzyme was transferred to the assay buffer by gel filtration on a Sephadex PD10 gel-filtration column (1.5 cm × 8 cm). The reaction was started by the addition of 0.6 mM AMP and 1.0 mM ATP and incubated at 25°C for 60 min. The reaction was stopped by the addition of 100 μL of reagent 1 (0.1 M phenol, 0.17 mM sodium nitroprusside) immediately followed by 100 μL of reagent 2 (0.125 M NaOH, 0.38 M Na$_2$HPO$_4$, and 5 mL of HOCl in a total volume of 500 mL). After a 60-min incubation at 55°C the A_{625} of the solution was determined.

Radiometric Assay for Partially Purified Pea AMP Deaminase

The assay contained (in a total volume of 150 μL) 60 mM citrate buffer (pH 7.1, with 5 M NaOH), 100 mM KCl, 0.1% (w/v) BSA, 1 mM diadenosine pentaphosphate, and approximately 0.01 unit of AMP deaminase. Enzyme was transferred to the assay buffer by gel filtration on a Sephadex PD10 gel-filtration column (1.5 cm × 8 cm). The reaction was started by the addition of [^{14}C]AMP (0.6 mM, 0.2 mCi) and ATP (1.0 mM) and incubated at 25°C for 60 min. The reaction was stopped by the addition of 160 μL of ice-cold 0.4 M HClO$_4$, and the mixture was kept on ice while the pH was adjusted to between 5.0 and 6.0 with 0.4 M KOH. The precipitate was removed by centrifugation (13,000g, 5 min), and the supernatant was analyzed by anion-exchange chromatography as described below.

Adenosine Kinase Assay

The assay contained (in a final volume of 100 μL) 50 mM Hepes (pH 7.35, with 5 M KOH), 1 mM MgCl$_2$, 2 mM DTT, 5 mM ATP, 1 μM diadenosine pentaphosphate, and 3 μg of protein from the phosphocellulose column in the above purification procedure for pea seedling AMP deaminase. The reaction was started by the addition of 0.5 mM adenosine or carbocyclic coformycin and incubated for 30 min at 30°C. The reaction was stopped by the addition of 100 μL of ice-cold 0.4 M HClO$_4$ and centrifuged (13,000g, 2 min). The pH of the supernatant was adjusted to between 5.0 and 6.0 using 0.4 M KOH and the samples re-centrifuged (13,000g, 2 min). The supernatant was analyzed by HPLC using anion-exchange chromatography as described below.

Measurement of Adenylates in *P. lapathifolium* Seedlings

Plants were sprayed with either 100 μM aqueous carbocyclic coformycin or 100 μM carbocyclic coformycin in 0.1% (v/v) Tween 20, 25% (v/v) methanol to runoff. Control plants were sprayed with water or 0.1% (v/v) Tween 20, 25% (v/v) methanol, respectively. The plants were incubated in the light at room temperature (approximately 20°C). At 1, 2, 4, and 6 h after treatment, samples containing 20 plants were harvested by excision at the level of the vermiculite and immediately frozen in liquid nitrogen. The frozen tissue was ground to a fine powder using a pre-cooled pestle and mortar, and then 50 μL of thymidine triphosphate (1 mM) was added as an internal reference standard to demonstrate that there were no significant losses of the nucleotides during the extraction procedure. To the mortar was then added 2 mL of ice-cold 0.4 M HClO$_4$, and the frozen acid was ground to a fine powder with the tissue. The extract was allowed to thaw and then it was transferred to a test tube and centrifuged (13,000g, 2 min). The supernatant was processed for HPLC analysis as described above. Anion-exchange chromatography was carried out as described below. In every case the recovery of the added thymidine triphosphate was at least 70% of that added.

Measurement of Adenylates in Pea Leaf Discs

Pea seedlings were grown as described above. Discs (1 cm in diameter) were cut from mature leaves using a cork borer and floated abaxial-surface downward on 100 μM aqueous carbocyclic coformycin or water (control). A vacuum was applied for 15 min and then released rapidly, and the discs were incubated in the light at room temperature (approximately 20°C). Samples of 5 discs were taken immediately after the vacuum had been released and then 1,

3, and 5 h later. The discs were rapidly frozen in liquid nitrogen and extracted and analyzed as described above.

Transpiration Feeding Experiments with Pea Seedlings

Pea shoots were excised near soil level and the stems were quickly washed with distilled water and placed upright in a 1-mL cuvette containing 2 mL of 100 μM carbocyclic coformycin or water (control). A section (approximately 2 mm) was cut from the base of the stem while it was immersed to prevent air locks from forming in the xylem. The seedlings were incubated in the light at room temperature (approximately 20°C) in a moving air stream. At intervals up to 5 h, individual seedlings were extracted and AMP deaminase, cytidine deaminase, and ATP levels were determined.

For the extraction of enzymes, seedlings were homogenized in 2 volumes of ice-cold citrate buffer (0.1 M, pH 7), 0.5 mM DTT using a pestle and mortar. The homogenate was passed through six layers of muslin and loaded onto a PD10 Sephadex gel-filtration column (1.5 cm × 8 cm) that had previously been equilibrated with 30 mM citrate (pH 7.1), 50 mM KCl, and 0.5% (w/v) BSA (assay buffer). The protein was eluted using the assay buffer, and protein and chlorophyll were measured in the eluate. The NH$_3$-release assay for AMP deaminase described above was modified for determination of enzyme activity in the treated pea seedlings. The assay contained (in a total volume of 500 μL) 60 mM citrate buffer (pH 7.1, with 5 M NaOH), 100 mM KCl, 0.1% (w/v) BSA, 1 μM diadenosine pentaphosphate, and 5 mM AMP. The reaction was started by the addition of 50 μL of desalted extract. At 15-min intervals from 0 to 60 min, the reaction was stopped by the addition of 420 μL of reagent 1 immediately followed by 275 μL of reagent 2 and processed as described above. The assay for cytidine deaminase was the same as the AMP deaminase assay described above, except that the AMP was replaced with 5 mM cytidine and the enzyme reaction was stopped at 10-min intervals between 0 and 30 min.

Perchloric acid extraction was carried out as described above, and ATP was determined using a biocounter (model M2500, Lumac, Landgraaf, The Netherlands). Samples were diluted 1:10,000 in water, and 100 μL was assayed according to the manufacturer's instructions.

Metabolism of [³H]Carbocyclic Coformycin in Vivo

Pea seedlings were supplied with 10 μM (400 Ci mol^{-1}) [³H]carbocyclic coformycin via the transpiration stream as described above. After 1, 2, 4, and 24 h of incubation, pairs of seedlings were rapidly frozen in liquid nitrogen, ground to a fine powder, and extracted in 2 mL of ice-cold 0.4 M HClO$_4$. The extracts were processed for HPLC analysis as described above except that the final sample was resuspended in 500 μL of water instead of 10 mM HCl. [³H]Carbocyclic coformycin, and the corresponding 5'-phosphate, were separated using cation-exchange chromatography as described below. Phosphatase treatment was carried out by the addition of 22 units of alkaline phosphatase to 250 μL

of the final sample. This was then incubated at 30°C for 30 min prior to HPLC analysis.

Association of [³H]Carbocyclic Coformycin with Protein

Pea seedlings were supplied with 10 μM (400 Ci mol^{-1}) [³H]carbocyclic coformycin via the transpiration stream as described above. After 2 and 24 h, pairs of seedlings were homogenized in buffer containing 0.1 M phosphate buffer (pH 7.5), 0.4 M Suc, and 0.1 mM DTT using a pestle and mortar. The homogenate was passed through six layers of muslin and loaded onto a Sephadex PD10 gel-filtration column (1.5 cm × 8 cm) that had previously been equilibrated with 30 mM citrate (pH 7.1), 50 mM KCl, and 0.05% (w/v) BSA (assay buffer). The column was eluted with 18 mL of assay buffer, and 500-μL fractions were collected. From each fraction, 50 μL was used to determine AMP deaminase activity using a single, 60-min incubation for each sample. Radioactivity was determined by liquid scintillation counting of a 200-μL aliquot from each fraction. A calibration curve for [³H]carbocyclic coformycin in the presence and absence of an equivalent amount of plant extract demonstrated that there was no detectable quenching by the plant material. Control experiments were carried out in which 500 μL of 10 μM (400 Ci mol^{-1}) [³H]carbocyclic coformycin was added to a pair of pea seedlings immediately before homogenization. The extract was then processed and analyzed exactly as described above.

To denature protein, fractions containing AMP deaminase activity were pooled (fractions 6–11) to give a total volume of 3 mL. To this was added 100 μL of SDS (10% [w/v]). After heating to 100°C, the samples were centrifuged (13,000g, 5 min) and the supernatant fractionated by gel filtration as described above. Radioactivity in each of the fractions was determined by liquid scintillation counting of 200-μL aliquots.

HPLC Analysis of Radiolabel Associated with Protein

Pea seedlings were supplied with 10 μM (400 Ci mol^{-1}) [³H]carbocyclic coformycin for 48 h, the tissue was extracted and fractionated by gel filtration, and the fractions were analyzed as described above. Fractions containing AMP deaminase activity (fractions 5–12) were pooled and to the resulting 4.0 mL was added 1.0 mL of HClO$_4$ (0.4 M) to bring the pH of the solution to below 1.0. The sample was centrifuged (13,000g, 5 min) and the pH of the supernatant adjusted to 7.0 with KOH. The precipitate formed was removed by centrifugation (13,000g, 5 min), and the resulting supernatant was evaporated to dryness. The final residue was redissolved in 0.5 mL of water consistency and analyzed by HPLC using cation-exchange chromatography as described below.

Chromatography

Anion-exchange chromatography was carried out using a Partisil 10 SAX column (25 cm × 0.46 cm). The linear gradients were 7 mM KH$_2$PO$_4$, pH 4.5 (solvent A), and 0.25 mM KH$_2$PO$_4$, 0.5 mM KCl, pH 4.5 (solvent B), at a flow rate of 1.5 mL min^{-1}. Cation-exchange chromatography was

carried out using a Partisil SCX column (8.0 cm × 0.46 cm), 0.05 M $NH_4H_2PO_4$, pH 2.6, at a flow rate of 2 mL min^{-1}. Reverse-phase chromatography was carried out on a Spherisorb column (25 cm × 0.46 cm) using 95:5 10 mM KH_2PO_4:methanol (solvent 1) and 88:12 10 mM KH_2PO_4:methanol, pH 5.3 (solvent 2), with a step change at 6 min, at a flow rate of 1 mL min^{-1}. Radioactivity was determined using a heterogeneous cell radiodetector.

Chlorophyll and Protein Determination

Chlorophyll was extracted in 80% (v/v) acetone and determined by the method of Arnon (1949). Protein was determined according to the method of Bradford (1976), using a Coomassie protein assay reagent supplied by Pierce and BSA as standard.

RESULTS AND DISCUSSION

Inhibition of Adenosine Deaminase by Carbocyclic Coformycin

The carbocyclic analog of coformycin is a potent inhibitor of mammalian adenosine deaminase (Table II). Under the assay conditions used, coformycin itself is a more potent inhibitor. Inhibition by the carbocyclic analog of coformycin showed time dependence, and slow binding of the inhibitor to the enzyme was also evident in dissociation experiments. When the enzyme was incubated with 25 nM inhibitor for 10 min, then diluted 100-fold to reduce the concentration of unbound inhibitor effectively to zero, there was a gradual recovery of activity, indicating that bound inhibitor was being released (Fig. 1A). After a 16-h preincubation, there was no evidence for dissociation of the inhibitor for up to 24 h after dilution (Fig. 1B). The pattern of inhibition shown by carbocyclic coformycin, i.e. the formation of a rapidly reversible complex prior to the final, slowly reversible complex, is characteristic of tight binding, transition-state analogs (Schloss, 1989). The observed potent inhibition of adenosine deaminase suggested that this could be the target for carbocyclic coformycin in vivo. Evidence for the existence of this enzyme in plants was therefore assessed.
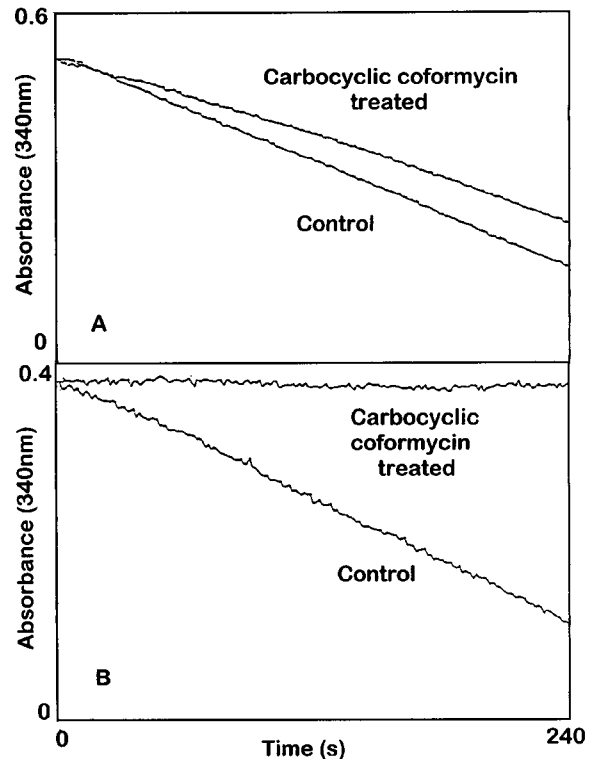

Figure 1. Release of carbocyclic coformycin from adenosine deaminase following dilution. Adenosine deaminase was preincubated with 25 nM carbocyclic coformycin for 10 min (A) and 16 h (B), then diluted 100-fold to reduce the concentration of unbound inhibitor effectively to zero. Enzyme activity was assayed immediately after dilution. The activity of the inhibited enzyme was related to an identically treated control from which the inhibitor had been omitted.

Evidence for the Absence of Adenosine Deaminase from Plants

Adenosine deaminase occupies a central position in purine metabolism (Fig. 2). Although there is good evidence for its existence in mammals, invertebrates, bacteria, and fungi (Schomburg and Salzmann, 1991), it could not be detected in barley (Butters et al., 1985; Brady and Hegarty,

Table II. *Inhibition of adenosine deaminase and AMP deaminase by carbocyclic coformycin and closely related structural analogs*

The compounds were preincubated with the enzyme for 10 min before the reaction was started by the addition of substrate. Substrate concentrations in each assay were as follows: adenosine deaminase: [adenosine] = 60 μM; AMP deaminase (rabbit muscle): [AMP] = 100 μM; AMP deaminase (pea): [AMP] = 640 μM. The assay for pea AMP deaminase also contained 5 mM ATP as an enzyme activator.

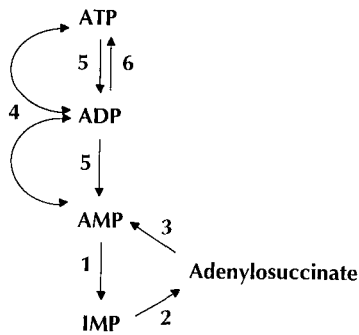|	I_{50}		
	Adenosine deaminase (calf intestinal mucosa)	AMP deaminase (rabbit muscle)	AMP deaminase (pea)
		μM	
Carbocyclic coformycin	0.025	1.8	120
Coformycin	0.011	1.2	nd
Carbocyclic coformycin-5′-phosphate	nd[a]	0.02	0.02

[a] nd, Not determined.

Figure 2. Pathways of purine metabolism in plants. 1, AMP deaminase (EC 3.5.4.6); 2, adenylosuccinate synthase (EC 6.3.4.4); 3, adenylosuccinate lyase (EC 4.3.2.2); 4, adenylate kinase (EC 2.7.4.3); 5, phosphatase (EC 3.1.3.1); and 6, ATP-regenerating pathways.

1966), cucumber (Staub et al., 1985), Jerusalem artichoke (Le Floc'h et al., 1982), lupin (Guranowski and Barankiewicz, 1979), or the seeds from 17 different plant families (Brady and Hegarty, 1966). Reports that it was present in yams (Osuyi and Ory, 1986) and spinach (Yoshino and Murakami, 1980) were based on measurements made in crude extracts and so must be considered inconclusive. It is therefore generally accepted that plants do not contain adenosine deaminase.

The above observations are supported by our own investigation of purine metabolism in plants. Table III shows the distribution of radiolabel 10 min after supplying [^{14}C]adenosine in the presence and absence of ATP to crude, desalted pea seedling homogenates. Adenosine alone was not metabolized, demonstrating that the plant extract does not contain an active adenosine deaminase. When ATP was included in the incubation, the [^{14}C]adenosine was rapidly converted to the phosphorylated adenosine nucleotides. Radiolabel also appeared in inosine monophosphate, presumably through the deamination of AMP via AMP deaminase. The small proportion of radiolabel that entered inosine must have resulted from the dephosphorylation of inosine monophosphate in the absence of adenosine deaminase. In addition to confirming that plants do not contain adenosine deaminase activity, the results of this experiment demonstrate the activity of the phosphorylating enzymes in the extract when they are provided with ATP.

In the absence of adenosine deaminase, the mechanistically related enzyme AMP deaminase offered an alterna-

tive potential target for carbocyclic coformycin in plants. The metabolism of [^{14}C]AMP by crude, desalted pea seedling extracts was investigated using the same approach as that described above for [^{14}C]adenosine. Again, the experiment was carried out in the presence and absence of ATP, which is a known allosteric activator of the plant enzyme (Le Floc'h and Lafleuriel, 1983). The results showed that in the absence of ATP, AMP is rapidly converted to adenosine through the action of phosphatases (Fig. 3A). The extract was also shown to contain AMP deaminase, which metabolized a smaller proportion of the label than the phosphatase activity. Some of the IMP produced is subsequently dephosphorylated to inosine. When ATP is included in the incubation, the distribution of radiolabel from [^{14}C]AMP is shifted from adenosine to ADP and ATP through the action of kinases (Fig. 3B). A greater proportion of the radiolabel is found in IMP and in inosine than in the absence of ATP, which is probably in part due to the stimulation of AMP deaminase by the ATP.

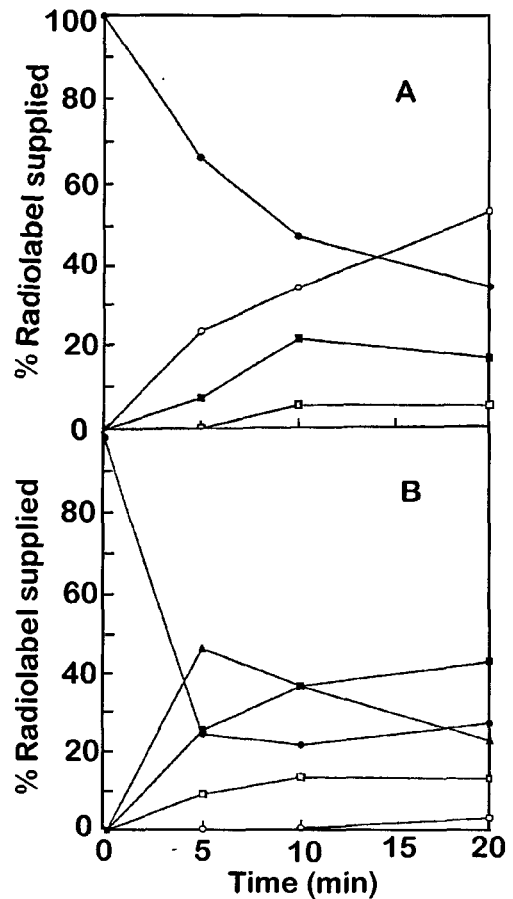A third related enzyme, adenine deaminase (EC 3.5.4.2), was ruled out as the target for carbocyclic coformycin

Figure 3. Distribution of radiolabel at intervals after supplying [^{14}C]AMP to a pea seedling homogenate in the absence of ATP (A), and with the addition of 5 mM ATP (B). [^{14}C]AMP was incubated with a crude, desalted homogenate prepared from 7-d-old pea seedlings. The reaction was stopped at the time intervals indicated by addition of ice-cold HClO$_4$ and radiolabel in AMP (●), adenosine (○), IMP (■), inosine (□), and ADP + ATP (▲) determined by HPLC analysis.

Table III. *Metabolism of [^{14}C]adenosine by crude, desalted pea seedling homogenates*

Pea shoots were harvested at 7 d and homogenized. [^{14}C]Adenosine (500 µM, 0.2 µCi) was incubated with the desalted homogenate. After 25 min the reaction was stopped by addition of ice-cold HClO$_4$, and the radiolabeled products were identified by HPLC analysis.

	Adenosine	Inosine	AMP	IMP	ADP	ATP
	% of radiolabel					
[^{14}C]Adenosine	100	0	0	0	0	0
[^{14}C]Adenosine + 5 mM ATP	40	2	20	15	15	8

because its distribution is restricted to certain specialized microorganisms (Schomburg and Salzmann, 1991). Our results therefore confirmed that plants contain AMP deaminase but not adenosine deaminase, and the possibility that the former enzyme was the target for carbocyclic coformycin was investigated further.

Inhibition of Mammalian AMP Deaminase

Coformycin and its carbocyclic analog were shown to be considerably less potent inhibitors of mammalian AMP deaminase than of adenosine deaminase (Table II). Since coformycin-5'-phosphate is reported to be a powerful inhibitor of AMP deaminase (Frieden et al., 1980), the 5'-phosphate analog of carbocyclic coformycin was chemically synthesized and, as anticipated, this proved to be a much better inhibitor than the unphosphorylated compound. After a 10-min preincubation with the enzyme in the absence of substrate, 50% inhibition of the mammalian AMP deaminase was achieved by 20 nM phosphorylated carbocyclic coformycin. It was not possible to use an extended preincubation period, nor to obtain meaningful kinetic data, because of the instability of the enzyme. Since the 5'-phosphate analog of carbocyclic coformycin proved to be a potent inhibitor of mammalian AMP deaminase, its ability to inhibit the plant enzyme was investigated.

Purification of Plant AMP Deaminase

AMP deaminase was partially purified from pea seedlings by an adaptation of the method of Yoshino and Murakami (1980). Using this procedure, a purification of about 20-fold with 30% yield was routinely achieved, and the specific activity of the final enzyme preparation was about 120 nmol mg^{-1} protein min^{-1}. There was no detectable AMP phosphatase (EC 3.1.3.1) or nucleotidase (EC 3.1.3.5) activity in the resulting preparation, which is important since these would deplete the substrate for AMP deaminase. Low levels of adenylate kinase (EC 2.7.4.3) activity were present in the partially purified extract and, since ATP was included in the assay as a potent allosteric activator of AMP deaminase, this could also interfere with the assay. Diadenosine pentaphosphate, a potent and highly specific inhibitor of adenylate kinase (Leinhard and Secemski, 1973), was therefore included in the assay in low concentrations (1 μM). At this concentration the diadenosine pentaphosphate has no effect on AMP deaminase activity itself. The K_m of the pea seedling AMP deaminase in the presence of 1 mM ATP was determined to be 400 μM. The K_m is reported as 250 μM for the spinach enzyme (Yoshino and Murakami, 1980) and 100 μM for the enzyme from Jerusalem artichoke (Le Floc'h and Lafleuriel, 1983), both values having been determined in the presence of 1 mM ATP.

Carbocyclic coformycin was found to be a considerably weaker inhibitor of plant AMP deaminase than of the corresponding mammalian enzyme (Table II). The affinity of these enzymes for carbocyclic coformycin mirrors their relative substrate affinities under the assay conditions used: mammalian AMP deaminase was determined to

have a K_m of 50 μM, whereas the pea enzyme was found to have a significantly lower affinity for AMP, with a K_m of 400 μM, as reported above. Inhibition of the pea enzyme by the 5'-phosphate, however, was as potent as that of the mammalian enzyme (I_{50} = 20 nM, [AMP] = 0.6 mM, [ATP] = 1 mM with 10-min preincubation), suggesting that AMP deaminase could be the target for the natural product following phosphorylation in vivo.

Effect of Carbocyclic Coformycin on Adenosine Nucleotides in Vivo

In theory, inhibition of AMP deaminase in vivo might be predicted to result in accumulation of AMP and depletion of the IMP pool. The effect of carbocyclic coformycin on the levels of purines in P. lapathifolium seedlings, which are particularly sensitive to the herbicide, was investigated. The levels of AMP and ADP showed no significant change over the time course of the experiment, remaining at about 20 and 35 nmol/mg protein, respectively, and the low amounts of AMP approaching the limits of detection of the HPLC analysis. There was, however, a significant increase in ATP in the treated plants, which was first apparent 2 h after spraying (Fig. 4). Similar results were obtained when the plants were treated with the inhibitor in a solution of 25% (v/v) methanol, 0.1% (v/v) Tween 20, which had been demonstrated to increase the uptake of the compound into the leaf (data not shown). The increase in ATP levels after 6 h is intriguing, since injury and death of plants following herbicide treatment is characteristically accompanied by a decrease in ATP levels (Raymond et al., 1987). As confirmation, we determined that treatment of P. lapathifolium seedlings with chlorsulfuron, a herbicide that acts through
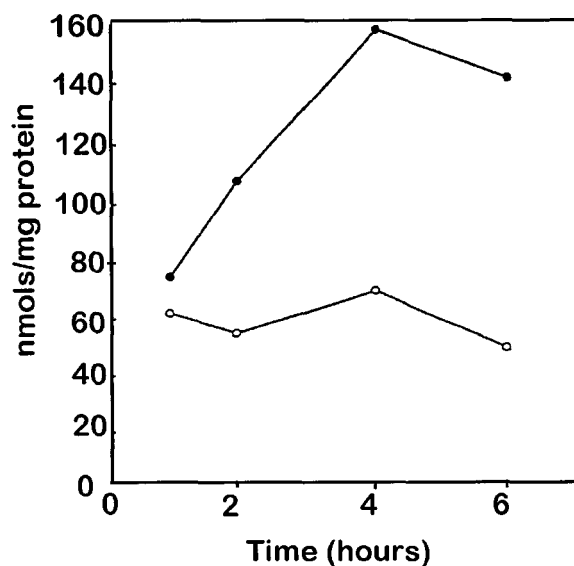

Figure 4. ATP levels in P. lapathifolium seedlings after treatment with carbocyclic coformycin. Seven-day-old plants were sprayed to run off with an aqueous solution of 100 μM carbocyclic coformycin (●) or water as a control (○). Plants were incubated in the light at 20°C. At the times indicated, seedlings were harvested and levels of adenine nucleotides determined.

the inhibition of acetolactate synthase (EC 4.1.3.18), re-sulted in a gradual decline in the ATP pool that was detectable within the first 2 h after spraying (data not shown).

We considered it possible that inhibition of AMP deaminase could result in a transient increase in AMP, which, because of the gradual uptake of carbocyclic coformycin following spraying, had remained undetected in the above experiments. Vacuum infiltration of pea leaf discs was therefore used to achieve a rapid and concerted entry of the herbicide into the cells. A significant increase in ATP was first seen after 5 h. No change, transient or otherwise, was detected in the levels of AMP; however, the earliest measurement was made 15 min after treatment (data not shown).

Measurement of the adenylate nucleotides provided no evidence for an accumulation of AMP in plants treated with the carbocyclic coformycin. AMP deaminase does not, however, mediate the flux in a simple linear pathway. Instead, it lies among a network of interconnected reactions, and there are alternative routes by which AMP could be metabolized if it were to accumulate (Fig. 2). Furthermore, the absolute levels of AMP in healthy plant tissues are low, and a large proportional change would be necessary to be detected in the above assays. The failure to demonstrate the accumulation of AMP does not, therefore, rule out AMP deaminase as the primary target of carbocyclic coformycin.

A consistent response of plant tissues to carbocyclic coformycin treatment was an increase in the level of ATP. It has been reported that deficiency of AMP deaminase in human erythrocytes is associated with an increase in the cellular ATP concentration (Dale and Norenberg, 1989; Paglia et al., 1989); thus, the effect of the herbicide on the ATP pool is consistent with a reduction in AMP deaminase activity.

In Vivo Inhibition of AMP Deaminase

Since experiments on AMP deaminase in vitro had shown that phosphorylated carbocyclic coformycin was a potent inhibitor, we decided to investigate the effect of carbocyclic coformycin on extractable AMP deaminase activity in plant tissues. When pea seedlings were supplied with the herbicide via the transpiration stream, it was observed that the extractable AMP deaminase activity began to decrease within 15 min and declined to 20% of the control value after 1 h (Fig. 5). Cytidine deaminase, which was used as an enzyme control for nonspecific effects on the seedlings or on the assay due to the herbicide treatment, showed no consistent change in activity over this period. The protein and chlorophyll contents of the seedlings were also unaffected (data not shown). As in previous experiments, an increase in ATP was also observed but first became apparent after 4 h of treatment and is therefore secondary to the inhibition of AMP deaminase. The plants showed no visible symptoms up to 24 h after treatment.

The decrease in extractable AMP deaminase activity was also observed when pea seedlings were treated with concentrations of carbocyclic coformycin as low as 1 μM over
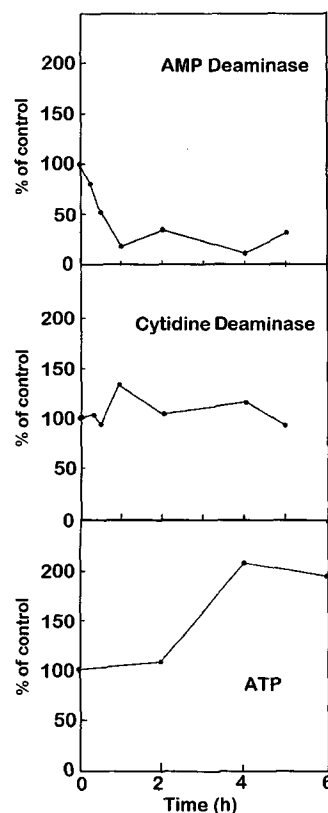

Figure 5. Extractable activity of AMP deaminase and cytidine deaminase and levels of ATP in pea seedlings following transpiration feeding with 100 μM aqueous carbocyclic coformycin. Seven-day-old pea seedlings were excised and supplied with 100 μM aqueous carbocyclic coformycin or water alone (control) via the transpiration stream. At the times indicated, seedlings were homogenized and assayed for AMP deaminase and cytidine deaminase activity or extracted in $HClO_4$ for ATP determination. Data represent activity in the treated samples as a percentage of the control. Activities in control (t = 0): AMP deaminase = 19 μmol g^{-1} fresh weight min^{-1}; cytidine deaminase = 70 μmol g^{-1} fresh weight min^{-1}; and ATP = 333 nmol g^{-1} fresh weight min^{-1}.

a 24-h period. During this time the seedlings took up a total of about 1 mL of the solution. It is very unlikely, therefore, that the intracellular carbocyclic coformycin will have achieved a concentration that could account for the inhibition of AMP deaminase, since only 40% inhibition was observed with 100 μM of the compound in vitro. Furthermore, the inhibitor must remain bound to the enzyme throughout the extraction, gel filtration, and assay procedure, and so must be very tightly associated with it. Phosphorylated carbocyclic coformycin is a considerably more potent inhibitor, with an I_{50} of only 20 nM (Table II). These results can best be explained if the carbocyclic coformycin is phosphorylated in vivo, and the aim of the following experiments was to provide evidence for this.

Metabolism of [³H]Carbocyclic Coformycin

Pea shoots were supplied with carbocyclic 8-tritiocoformycin (10 μM, 400 Ci mol^{-1}) via the transpiration

stream for up to 4 h. The increase in ATP levels characteristic of carbocyclic coformycin treatment was again observed. After 4 h, two major radiolabeled compounds were detected, one being carbocyclic coformycin itself, and the other, which accumulated gradually, having the same retention time as the 5'-phosphate analog (Fig. 6). Treatment of the extract with phosphatase converted this second major peak back to a compound with the same retention time as carbocyclic coformycin, implying a phosphorylated metabolite. There is good evidence, therefore, that carbocyclic coformycin is rapidly phosphorylated in vivo.

Association of [³H]Carbocyclic Coformycin 5'-Phosphate with Protein

Gel-filtration analysis of the protein fraction of pea seedlings that had been supplied with radiolabeled herbicide by transpiration feeding for 2 h revealed that a small amount of radiolabel was associated with the protein (Fig.
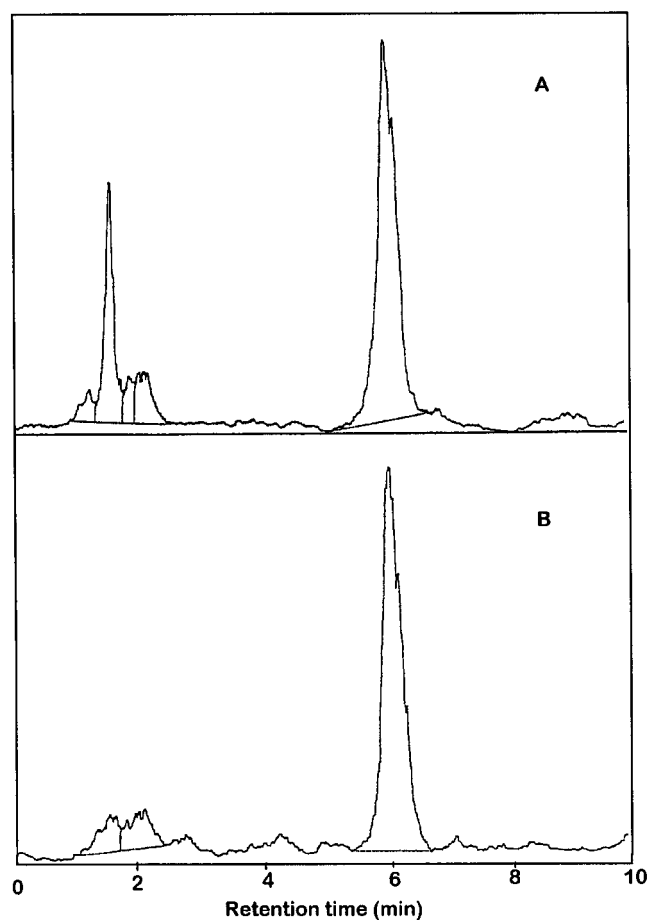

Figure 7. Distribution of radiolabel and protein in gel-filtration fractions of homogenates prepared from pea seedlings after 2 and 24 h of transpiration feeding with [³H]carbocyclic coformycin after 2 h of transpiration feeding (A) and after 24 h of transpiration feeding (B). Seven-day-old pea seedlings were excised and supplied with 10 mM[³H]carbocyclic coformycin for 2 or 24 h. The seedlings were then homogenized, and the resulting homogenate was applied to a Sephadex PD10 gel-filtration column. Radioactivity (●) and AMP deaminase (○) activity were determined in 0.5-mL fractions eluted from the column.

Figure 6. HPLC traces showing distribution of radiolabel following transpiration feeding of pea seedlings with [³H]carbocyclic coformycin. Seven-day-old pea seedlings were excised and supplied with 10 μM [³H]carbocyclic coformycin for 4 h before extraction in perchloric acid. The distribution of radiolabel in the final sample before and after treatment with alkaline phosphatase was determined by cation-exchange chromatography before phosphatase treatment (A) and after phosphatase treatment (B).
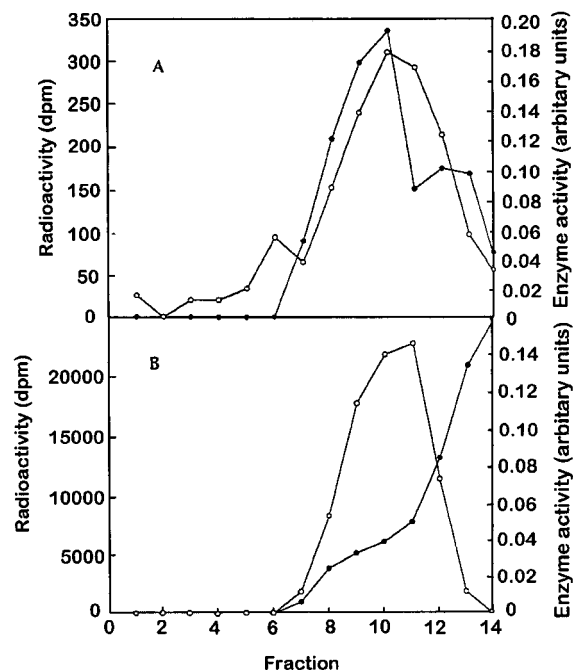
7A). There was, however, no decrease in the total amount of extractable activity of AMP deaminase. The lack of inhibition of AMP deaminase almost certainly results from insufficient uptake of the herbicide. There was no association of radiolabel with the protein if the inhibitor was added to the seedlings immediately prior to extraction. Following a 24-h feeding period (Fig. 7B), 240 times more radiolabel was associated with the protein fraction and AMP deaminase was inhibited by 60%. In the experiments using the unlabeled compound described above, 80% inhibition of the enzyme was observed after only 1 h of feeding; however, the concentration of inhibitor supplied was considerably higher.

Treatment of the protein with SDS to denature it resulted in the release of the radiolabel, demonstrating that the radiolabel is not covalently incorporated into the protein but is reversibly bound. The identity of the radiolabeled compound extracted from the protein fraction was determined by HPLC analysis. A single peak was detected that co-eluted with the 5'-phosphate analog of carbocyclic coformycin and was converted back to a compound with the retention time of carbocyclic coformycin upon phosphatase treatment (data not shown). We believe that these results provide good evidence that the decrease in extractable AMP deaminase is the result of inhibition by phosphorylated carbocyclic coformycin in vivo.

Route of Phosphorylation of Carbocyclic Coformycin in Vivo

Adenosine kinase would appear to be the most likely enzyme to mediate the phosphorylation of carbocyclic coformycin in vivo; however, no phosphorylation of either coformycin or its carbocyclic analog by the partially purified enzyme from pea seedlings was detected using ATP as the phosphate donor (data not shown).

Other Possible Modes of Action of Carbocyclic Coformycin

Since carbocyclic coformycin is a nucleoside analog that can undergo phosphorylation in vivo, the possibility of incorporation into nucleic acids arises. There was, however, <1% incorporation of the radiolabeled compound into nucleic acids in pea seedlings up to 24 h after it was supplied (data not shown). Carbocyclic coformycin does not, therefore, act as a substrate for nucleic acid synthesis. Potential mutagenic effects were investigated using an Ames test in *Salmonella typhimurium*. At a concentration of 880 mg plate^{-1}, the results were negative, indicating that at this dose level carbocyclic coformycin has no ability to induce frame-shift gene mutations or base pair substitutions. Taken together, these results indicate that the primary mode of action of carbocyclic coformycin does not involve interference with nucleic acid synthesis.

Carbocyclic coformycin shows structural similarities to the cytokinins and so it was tested in a bioassay for cytokinin-like activity, which involves the assessment of betacyanin induction after application of the test compounds to etiolated *Amaranthus* seedlings. Although at a concentration of 2 parts per million it retarded root and hypocotyl growth in the *Amaranthus* seedlings, there were no other visible symptoms. Carbocyclic coformycin does not, therefore, appear to act as a cytokinin analog.

Summary of Evidence and Possible Cause of Death

It has been shown that there is a very rapid and dramatic decrease in the extractable activity of AMP deaminase after plants are treated with carbocyclic coformycin. This correlates with a strong, noncovalent association of phosphorylated carbocyclic coformycin with the enzyme. The 5'-phosphate analog has been shown to be a potent, tight binding inhibitor of plant AMP deaminase in vitro, and evidence has been provided that there is rapid phosphorylation of carbocyclic coformycin in vivo, although the actual route of phosphorylation has not been identified. Taken together, these observations provide overwhelming circumstantial evidence that AMP deaminase is the primary target for carbocyclic coformycin.

How, then, does a decrease in AMP deaminase activity lead to the death of the plant? As already discussed, AMP deaminase, in the absence of adenosine deaminase, is involved in the degradation of purines in plants. If AMP deaminase serves a purely catabolic function, it is perhaps surprising that inhibition has such drastic consequences for the plant. The rapid and dramatic changes in the overall nucleotide pool size are probably related to the vital role

proposed for AMP deaminase in the short-term stabilization of the intracellular adenylate energy charge (Chapman and Atkinson, 1973). The adenylate energy charge represents the relative saturation of the adenylate pool in phospho- and hydro-bonds and is expressed as the ratio (Pradet and Raymond, 1983):

$$\frac{ATP + \frac{1}{2}ADP}{ATP + ADP + AMP}$$

It is widely accepted that in animals and microorganisms the adenylate energy charge in actively metabolizing cells is maintained between 0.85 and 0.94 (Saglio et al., 1980; Raymond et al., 1987), although lower values have been reported for plant cells. If the level of ATP falls because the rates at which it is consumed and regenerated are in imbalance, there will be an increase in the concentration of AMP through the action of the freely reversible adenylate kinase (EC 2.7.4.3):

$$ATP + AMP \rightleftharpoons 2ADP$$

Excess AMP will be rapidly deaminated through the action of AMP deaminase, and the adenylate energy charge will be maintained at a high value.

Although there may have been perturbations in the AMP and ADP pools that were below the limits of detection of our assay, the most significant changes were observed in ATP levels. AMP accumulating as a result of inhibition of AMP deaminase would be rapidly converted to ADP through the action of adenylate kinase. In normal, healthy tissues, the rate of ATP regeneration is limited by supply of the substrates ADP and Pi. The observed buildup of ATP, therefore, results from the increased availability of ADP.

Inhibition of AMP deaminase will, through its effects on adenylate metabolism, have consequences for almost every aspect of metabolism, which explains why this enzyme should represent such a potent herbicide target. This novel mode of action offers the potential for the discovery of new, potent herbicides through biochemical design or by screening for inhibitors of the enzyme.

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