Phytochemistry, 1967, Vol. 6, pp. 115 to 119. Pergamon Press Ltd. Printed in England

INHIBITION OF PLANT ADENYLOSUCCINATE SYNTHETASE BY HADACIDIN AND THE MODE OF ACTION OF HADACIDIN AND STRUCTURALLY RELATED COMPOUNDS ON PLANT GROWTH

M. D. HATCH

David North Plant Research Centre, C.S.R. Co. Ltd., Meiers Road, Indooroopilly, Queensland, Australia

(*Received* 16 *March* 1966)

Abstract—Plant adenylosuccinate synthetase was found to be competitively inhibited by hadacidin (N-formylhydroxyamino-acetic acid). Enzyme activity was reduced by 50 per cent when hadacidin was supplied at 1/600 the concentration of aspartic acid. The Ki was 1·1μM in the pH range from 6·4 to 7·75. Adenylosuccinate synthetase activity was unaffected by several other compounds which were structurally related to aspartic acid. Included amongst these compounds were a number of plant-growth retardants. Adenine, adenosine and AMP prevented the inhibition of wheat-seedling growth by hadacidin. It was concluded that the inhibition of plant growth by hadacidin is due to reduced *de novo* synthesis of AMP.

INTRODUCTION

HADACIDIN (N-formylhydroxyamino-acetic acid) was originally isolated from fungi[1, 2] and was found to inhibit the growth of bacteria,[3] tumour cells[1, 4] and plant tissues.[2, 5] The effect on tumour and bacterial growth was apparently due to inhibition of the *de novo* synthesis of AMP.[3, 4, 6] Furthermore hadacidin has been shown to inhibit *Escherichia coli* adenylosuccinate (AMPS) synthetase, an enzyme involved in AMP synthesis, by acting competitively with aspartic acid.[6] The recent identification of AMPS synthetase and AMPS lyase in wheat-germ extracts[7] provides evidence that the terminal steps for the *de novo* synthesis of AMP in plants are similar to those operating in animal tissues and micro-organisms.[8] The present paper reports that hadacidin is a potent inhibitor of plant AMPS synthetase and provides evidence that it inhibits plant growth by preventing AMP synthesis. The effect on AMPS synthetase of other plant-growth retardants which are structurally related to aspartic acid was also examined.

RESULTS

Assay and Properties of AMPS Synthetase

For the reaction catalysed by wheat-germ AMPS synthetase the Michaelis constant (K_m) of aspartate was previously found to be 0·43 mM at pH 8·0.[7] During the present studies a

[1] E. A. KACZKA, C. O. GITTERMAN, E. L. DULANEY and K. FOLKERS, *Biochemistry* **1**, 340 (1962).
[2] R. A. GRAY, G. W. GAUGER, E. L. DELANEY, E. A. KACZKA and H. B. WOODRUFF, *Plant Physiol.* **39**, 204 (1964).
[3] H. T. SHIGEURA and C. N. GORDON, *Cancer Res.* **22**, 1356 (1962).
[4] H. T. SHIGEURA and C. N. GORDON, *J. Biol. Chem.* **237**, 1932 (1962).
[5] G. E. ZAROOGIAN and R. W. CURTIS, *Plant Cell Physiol., Tokyo* **5**, 291 (1964).
[6] H. T. SHIGEURA and C. N. GORDON, *J. Biol. Chem.* **237**, 1937 (1962).
[7] M. D. HATCH, *Biochem. J.* **98**, 198 (1966).
[8] J. M. BUCHANAN, In *The Nucleic Acids* (Edited by E. CHARGAFF and J. N. DAVIDSON), Vol. 3, p. 304. Academic Press, New York (1960).

modified spectrophotometric procedure was employed, and at the pH used (7·6) the K_m was 0·65 mM. With this procedure the rate was linear for at least 20 min when GTP was continuously generated by phosphopyruvate kinase. When substrate levels of GTP were used the rate was the same initially but declined rapidly. A similar effect has been described for bacterial AMPS synthetase.[9] Further studies showed that GDP inhibits the wheat-germ enzyme. The initial rate was reduced by 50 per cent when 0·07 mM GDP was added with 0·19 mM GTP. This effect appeared to be competitive in nature and was not due to a reversal of the reaction.

During the present studies it was found that the activity lost during storage of the purified wheat-germ AMPS synthetase[7] was restored by thiol compounds. After storage for 2 days at −15°, 4 mM cysteine or 2-mercaptoethanol increased activity by more than 50 per cent. The enzyme was almost completely inhibited by 5 μM *p*-chloromercuribenzoate.

Inhibition of AMPS Synthetase by Hadacidin

The inhibition of AMPS synthetase by hadacidin was related to the concentration of aspartic acid in the reaction mixtures (Table 1). Activity was reduced by 50 per cent when the concentration of hadacidin was only 1/600 of the concentration of aspartic acid. The inhibitor constant (K_i)[10] at pH 7·7 was 1·1 μM. Inhibition by hadacidin was unaffected by pH over the range from 6·4 to 7·7 (Fig. 1) but declined at higher pH values.

TABLE 1. INHIBITION OF AMPS SYNTHETASE BY HADACIDIN

Ratio of concn. of aspartate to hadacidin	% Inhibition	
	With 0·66 mM aspartate	With 3·3 mM aspartate
45	90 (15 μM)*	91 (75 μM)
330	61 (2 μM)	62 (10 μM)
670	46 (1 μM)	49 (5 μM)
1500	25 (0·44 μM)	28 (2 μM)
3500	16 (0·19 μM)	16 (0·9 μM)

* The concentration of hadacidin is given in brackets.

Effect of Other Compounds on AMPS Synthetase

Several compounds which are structurally related to aspartic acid were tested for inhibitory effects on AMPS synthetase. Like hadacidin, some of these are known to inhibit plant growth. The compounds examined were succinic 1,1-dimethylhydrazide (B-995), maleic 1,1-dimethylhydrazide (C-011), maleic hydrazide, azaserine, zeatin, maleic monoamide, succinic monoamide, D-aspartate, asparagine, L-glutamate and β-alanine. None of these compounds inhibited AMPS synthetase by more than 10 per cent when provided at the same concentration as L-aspartate.

Effect of Hadacidin and Adenine Derivatives on Wheat-seedling Growth

Inhibitory effects of hadacidin on whole cells have been prevented by adenine.[3, 11] During the present studies hadacidin was found to inhibit root and shoot growth of pea, lentil and

[9] I. LIEBERMAN, *J. Biol. Chem.* **223**, 327 (1956).
[10] M. DIXON and E. C. WEBB, In *Enzymes*, p. 329. Longmans, London (1962).
[11] J. L. MEGO, *Biochem. Biophys. Acta* **70**, 221 (1964).

wheat seedlings. The effect of hadacidin on wheat-seedling growth with or without various nucleotide bases or their derivatives was examined (Table 2). Hadacidin was most effective when supplied from the commencement of the germination treatment. This may have been due in part to an effect on the onset of germination rather than on subsequent growth. However, considerable effects were observed when the inhibitor was supplied after 2 days' germination in water when shoot and root growth had already commenced. Complete or almost complete reversal of the inhibitory effect of hadacidin was observed with adenine, adenosine and AMP. Under the same conditions guanosine and cytidine were ineffective. Some nucleotide bases or their derivatives caused a significant reduction in growth in the absence of hadacidin.

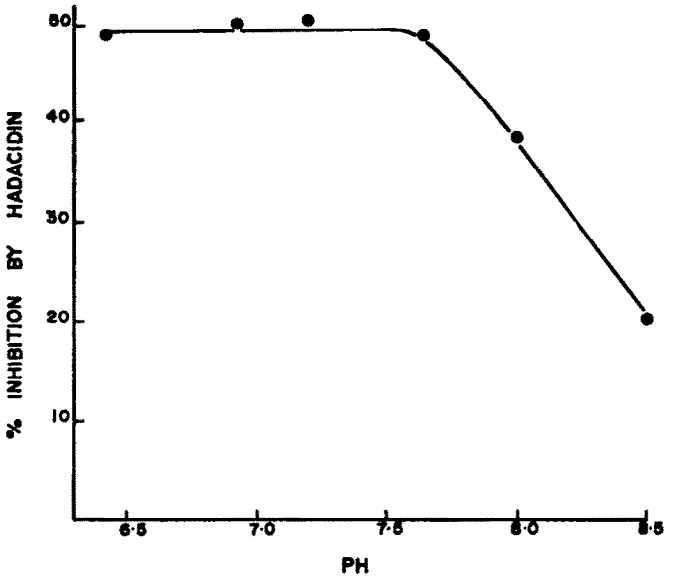

FIG. 1. THE EFFECT OF pH ON THE INHIBITION OF AMPS SYNTHETASE BY HADACIDIN.
The concentrations of aspartic acid (3 mM) and hadacidin (5·5 μM) were chosen so that the inhibition at pH 7·7 was approximately 50 per cent.

DISCUSSION

Compared with its effect on the *E. coli* enzyme,[6] hadacidin was an even more effective inhibitor of wheat-germ AMPS synthetase. The K_i for the bacterial enzyme is four times that of the plant enzyme and the ratio of the concentration of hadacidin to aspartic acid required to give 50 per cent inhibition is sixteen times greater for the bacterial enzyme. These differences were not due to the different reaction pH employed in the two studies.

Shigeura[12] studied the effect on AMPS synthetase of a series of compounds which were structurally similar to hadacidin. This work provided some information about the structural features of hadacidin which were essential for activity. However, the structural property of hadacidin which allows it to compete so effectively with aspartic acid in the reaction catalysed by AMPS synthetase remains obscure. During the present studies several compounds which appeared to be as or more closely related to L-aspartic acid than hadacidin were found to be

[12] H. T. SHIGEURA, *J. Biol. Chem.* 238, 3999 (1963).

TABLE 2. INHIBITION OF WHEAT SHOOT AND ROOT GROWTH BY HADACIDIN AND REVERSAL OF INHIBITION BY ADENINE, ADENOSINE AND AMP

Experiment	Treatment*			Growth after 6 days				
					Shoots		Roots	
	0–12 hr	12–48 hr	Remaining 4-day period	Germination (%)	Wt. (g)	% Control	Wt. (g)	% Control
1	Water	Water	Water	66	1·34	—	1·03	—
	Adenine	Adenine	Adenine	64	1·22	—	0·97	—
	AMP	AMP	AMP	67	0·99	—	0·46	—
	Had.	Had.	Had.	33	0·08	6	0·0	0
	Had.+adenine	Had.+adenine	Had.+adenine	62	0·85	70	0·58	61
	Had.+AMP	Had.+AMP	Had.+AMP	58	0·61	63	0·38	81
2	Water	Water	Water	66	0·84	—	0·82	—
	Water	Adenine	Adenine	52	0·59	—	0·52	—
	Water	Adenosine	Adenosine	55	0·64	—	0·53	—
	Water	Cytidine	Cytidine	49	0·75	—	0·63	—
	Water	Had.	Had.	50	0·11	13	0·18	22
	Water	Had.+adenine	Had.+adenine	54	0·43	73	0·49	94
	Water	Had.+adenosine	Had.+adenosine	66	0·41	65	0·52	97
	Water	Had.+cytidine	Had.+cytidine	53	0·06	8	0·10	16
3	Water	Water	Water	65	0·94	—	0·73	—
	Water	Water	Adenosine	62	0·88	—	0·61	—
	Water	Water	Guanosine	49	0·79	—	0·43	—
	Water	Water	Cytidine	63	0·98	—	0·72	—
	Water	Water	Had.	60	0·38	40	0·27	37
	Water	Water	Had.+adenosine	68	0·91	103	0·76	125
	Water	Water	Had.+guanosine	55	0·32	40	0·22	50
	Water	Water	Had.+cytidine	60	0·40	41	0·19	27

* Wheat seeds (2·5 g, approx. 60 seeds) were soaked in the solutions shown for 24 hr to allow imbibition of water and were then grown on filter paper saturated with the treatment solutions. The growth temperature was 22° and solutions were changed every 12 hr. The concentration of the nucleotide bases or their derivatives was 1 mM and that of hadacidin (Had.) was 0·05 mM in Expt. 1 and 3 and 0·1 mM in Expt. 2. Individual experiments were not replicated but for six replicate batches of 60 seeds grown in water the standard deviation was 0·07 for a mean shoot weight of 1·01 and 0·05 for a mean root weight of 0·83.

ineffective as inhibitors. Included amongst these were the plant-growth retardants B-995, C-011 and maleic hydrazide. The mode of action of these compounds apparently differs from that of hadacidin. Succinic monoamide is a possible metabolic breakdown product of B-995, and maleic monoamide of C-011 and maleic hydrazide. Neither of these monoamides affected AMPS synthetase activity.

Studies with whole cells have provided evidence that hadacidin affects growth,[3,4] chloroplast development[11] and ATP levels[13] by preventing the conversion of IMP to AMP or adenine. The present studies showed that the inhibitory effect of hadacidin on root and shoot growth of wheat seedlings was prevented by adding adenine, adenosine or AMP but not guanosine or cytidine. These studies, together with the studies on the purified wheat AMPS synthetase, provide strong evidence that the action of hadacidin on plants is largely or solely due to the inhibition of the *de vovo* synthesis of AMP.

EXPERIMENTAL

Materials. Adenine, adenosine, cytidine, guanosine and GDP were obtained from Sigma Chemical Co. (St. Louis, Mo.) and the monoamides of succinic acid and maleic acid from K & K Laboratories Inc., New York. The sources of other biochemicals were as previously stated.[7] The following chemicals were gifts, hadacidin (Merck Sharp & Dohme), B-995 and C-011 (United States Rubber Co.), azaserine (Park-Davis and Co.) and zeatin (Dr. D. S. Letham).

Preparation and assay of AMPS synthetase. The enzyme was prepared as previously described[7] but a modified assay procedure was used. Reaction rates were determined in quartz cuvettes, with a 1 cm light path, by measuring the change in extinction at 277·5 mμ over a period of 15 min. Reaction mixtures contained enzyme, tris-HCl buffer, pH 7·7 (10 μmoles), $MgCl_2$ (4 μmoles), phosphopyruvate (0·35 μmole), pyruvate kinase (1·4 units), ATP (0·05 μmole), GTP (0·07 μmole), IMP (0·25 μmole) and L-aspartate (2 μmoles) in a total volume of 0·7 ml. Measurements were made at 30°. To start the reaction enzyme was added to the cuvettes which contained all the other components including inhibitors.

Studies on seedling growth. The procedure for growing and treating seedlings is described in the legend of Table 2. After 6 days shoots and roots were collected, the roots were blotted dry with filter paper then the fresh weight of each was determined. The per cent or germination was calculated from the number of seeds which showed visible shoot or root growth.

[13] K. H. SHULL and S. VILLA-TREVINO, *Biochem. Biophys. Res. Commun.* 16, 101 (1964).