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SIMILARITIES IN ACTION BETWEEN STREPTOMYCIN AND HORDATINE, AN ANTIFUNGAL FACTOR IN BARLEY

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Abstract—Low concentrations of both streptomycin and hordatine inhibit protein synthesis in pea stem segments while affecting RNA synthesis and leucine uptake only slightly or not at all. This inhibition, as well as the antifungal activity of the compounds in a standard spore germination assay, is largely overcome in the presence of various divalent cations, e.g. Ca^{2+}, Mg^{2+}, Mn^{2+}. Studies with related compounds point to the importance, in both systems, of the two guanidine residues for high biological activity.

INTRODUCTION

PROTEIN synthesis in excised plant tissues is inhibited by low levels of streptomycin,[1] an action which probably underlies its inhibitory effects on plant growth.[2, 3] In the presence of certain divalent cations, e.g. Ca^{2+}, Mg^{2+}, Mn^{2+}, the phytotoxic effects of streptomycin, including inhibition of protein synthesis, are largely overcome.[4] This apparent reversal seems to be due to inhibition of streptomycin uptake by the cations, probably by competition for uptake sites.[4]

Hordatine A and related hordatines constitute a class of antifungal compounds which have been isolated from barley seedlings.[5] An early report by Ludwig *et al.*[6] demonstrated that the antifungal activity of hordatine-containing extracts was greatly reduced or abolished by various divalent cations, notably Ca^{2+}, Mg^{2+}, Mn^{2+}, while monovalent cations were ineffective. This parallelism with the action of streptomycin is of obvious interest, since both the hordatines and streptomycin are diguanidine compounds. The present report demonstrates further similarities in the biological effects of hordatine A, streptomycin, and other diguanidines.

RESULTS

Table 1 shows that incorporation of leucine into protein by pea epicotyl segments is inhibited by low concentrations of hordatine A (hereafter referred to as hordatine). At a concentration (50 μM) which inhibits protein synthesis by 50 per cent, hordatine is without effect on leucine uptake and inhibits RNA synthesis by only 10 per cent. These results closely parallel previous findings with streptomycin.[1]

It was of interest to determine whether simple diguanidines, or monoguanidine derivatives related to hordatine, were active as inhibitors of protein synthesis. The results in Table 2

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[5] A. STOESSL, *Can. J. Chem.* **45**, 1745 (1967).
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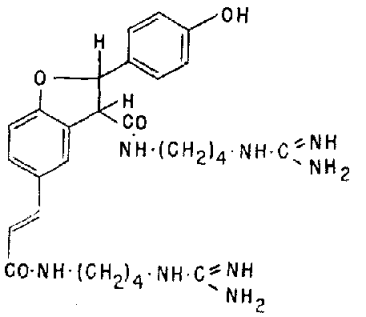
indicate that *n*-alkylene diguanidines are strongly inhibitory, while the corresponding *n*-alkylene diamines are without effect or slightly stimulatory. The monoguanidines are

TABLE 1. THE EFFECT OF HORDATINE ON PROTEIN AND RNA SYNTHESIS AND ON LEUCINE UPTAKE BY PEA SEGMENTS

Hordatine concn. (μM)	Specific activity (counts/min/mg)		^{14}C-Leucine uptake (counts/min/g fresh wt.)
	Protein	RNA	
0	1765	8393	22,810
20	1508 (85)	—	—
50	880 (50)	7555 (90)	22,220 (97)
100	570 (32)	—	—

Figures in parenthesis are per cent of control.



HORDATINE A

TABLE 2. THE EFFECTS OF VARIOUS COMPOUNDS ON INCORPORATION OF ^{14}C-LEUCINE INTO PROTEIN BY PEA SEGMENTS

Treatment	Specific activity (counts/min/mg protein)	% of control
Experiment 1		
Control	1525	—
Hexamethylene diguanidine	703	46
Decamethylene diguanidine	105	7
Hexamethylene diamine	1629	107
Decamethylene diamine	1584	104
Experiment 2		
Control	1885	—
Hordatine, 50 μM	1024	54
Agmatine	2111	112
p-Methoxycinnamoylagmatine	1830	97
p-Coumaroylagmatine	1809	96

All compounds except hordatine were used at 100 μM.

also essentially non-inhibitory at 100 μM; agmatine enhances leucine incorporation somewhat, while *p*-methoxycinnamoylagmatine and *p*-coumaroylagmatine have little or no effect.

To study possible reversal of hordatine inhibition by divalent cations, cation concentrations were selected on the basis of earlier studies with streptomycin.[4] At the concentrations tested, the cations by themselves were known to have no more than marginal effects on leucine incorporation.[4] It can be seen from Fig. 1 that hordatine inhibition of protein synthesis is largely or completely overcome in the presence of Mg^{2+}, Mn^{2+}, Ca^{2+} or Zn^{2+}. These cations were previously found to be the most effective in reversing the action of streptomycin.[4] Co^{2+} and Fe^{2+}, which partially reverse streptomycin inhibition,[4] have little or no effect at the tested concentrations on the inhibitory action of hordatine.

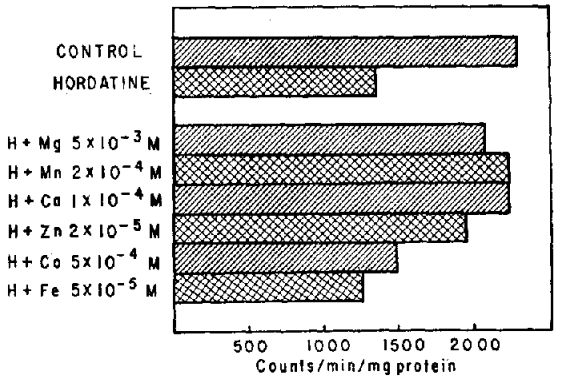


FIG. 1. THE EFFECT OF SIMULTANEOUS ADDITION OF DIVALENT CATIONS ON THE INHIBITION OF PROTEIN SYNTHESIS IN PEA SEGMENTS BY 50 μM HORDATINE.

Since hordatine and other diguanidine compounds showed affinities with streptomycin in their action on pea stem segments, it was of obvious interest to compare the antifungal activities of streptomycin and the other test compounds with that of hordatine. For this purpose a standard spore germination assay was employed, using as test organism *Monilinia fructicola*, which has been used previously[6] for screening activities of hordatine-containing extracts. The index of toxicity used in Table 3 is the concentration of the compound which

TABLE 3. INHIBITION OF *M. fructicola* SPORE GERMINATION

Compound	Concn. for 80–100% inhibition (μM)
Hordatine	20
Hexamethylene diguanidine	1600
Decamethylene diguanidine	25
Decamethylene diamine	800
Streptomycin	100
p-Methoxycinnamoylagmatine	400
p-Coumaroylagmatine	400
Agmatine	> 1600

caused at least 80 per cent inhibition of spore germination in different experiments. It is evident that with the exception of the C_6-diguanidine which is of low toxicity, there is a general correlation between antifungal activity and ability to inhibit protein synthesis in peas. Thus the most effective inhibitors of spore germination are hordatine, streptomycin

and the C_{10}-diguanidine while the C_{10}-diamine and the two agmatine derivatives are far less active. Agmatine itself is completely non-inhibitory at 1·6 mM.

It has been shown that the antifungal activity of crude hordatine extracts is suppressed in the presence of several divalent cations, and this observation has since been confirmed for purified hordatine.[7] From Table 4 it is evident that 1 mM Mg^{2+} offsets the inhibitory effects of streptomycin and C_{10}-diguanidine as well as that of hordatine. Similar results were obtained with Ca^{2+} and Mg^{2+} at 0·2 mM. Other cations such as Zn^{2+}, Co^{2+}, Fe^{2+} were not tested because these cations are themselves toxic at low concentrations.

TABLE 4. EFFECT OF 1 mM Mg^{2+} ON INHIBITION OF *M. fructicola* SPORE GERMINATION

Hordatine, M		$8{\cdot}10^{-5}$	$4{\cdot}10^{-5}$	$2{\cdot}10^{-5}$	$1{\cdot}10^{-5}$	$5{\cdot}10^{-6}$
% inhibition	−Mg	100	100	100	24	0
	+Mg	100	0	0	0	0
Decamethylene diguanidine, M		$1{\cdot}10^{-4}$	$5{\cdot}10^{-5}$	$2{\cdot}5.10^{-5}$	$1{\cdot}25.10^{-5}$	$6{\cdot}25.10^{-6}$
% inhibition	−Mg	100	100	100	81	34
	+Mg	83	48	18	0	0
Streptomycin, M		$8{\cdot}10^{-4}$	$4{\cdot}10^{-4}$	$2{\cdot}10^{-4}$	$1{\cdot}10^{-4}$	$5{\cdot}10^{-5}$
% inhibition	−Mg	100	100	100	91	66
	+Mg	82	65	53	20	0

DISCUSSION

It has been shown that in addition to having antifungal activity towards the test organism *Monilinia fructicola*, both hordatine and streptomycin inhibit protein synthesis in pea segments. Studies with related compounds point to the importance, in both biological systems, of the two guanidine residues for high activity. Furthermore, in both systems the inhibitory effects of streptomycin and of hordatine are overcome by the same divalent cations. In the case of streptomycin inhibition of protein synthesis, it was found previously[4] that the apparent cation reversal is due to inhibition of streptomycin uptake. It seems likely that reversal of hordatine inhibition has a similar basis, though no direct evidence is available on this point.

Alkyl guanidines are known to inhibit energy transfer reactions in animal mitochondria[8] and it was recently reported that synthalin (decamethylene diguanidine) is an inhibitor of energy transfer in spinach chloroplasts.[9] However, the concentration of synthalin required for 50 per cent inhibition (0·2 mM) is about forty times greater than that which causes 50 per cent inhibition of protein synthesis in peas. Further, in the mitochondrial work the structure-activity relations appeared to differ considerably from those of the present study, e.g. mono-guanidines were highly active, while streptomycin was virtually inactive.[8] For these reasons, and because hordatine and streptomycin inhibit protein synthesis at concentrations which have only small or negligible effects on RNA synthesis and on leucine uptake, it is most improbable that these diguanidine compounds are acting here as inhibitors of energy transfer reactions.

The correlation between the ability of the different compounds to inhibit protein synthesis in pea segments and their ability to inhibit fungal spore germination is by no means exact.

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However, considering the divergence of these two biological systems and that factors such as relative penetration rates may well vary considerably, the correlation is sufficiently remarkable as to suggest that a study of the action of hordatine on fungal protein synthesis might be profitable. In this connection it may be of interest that at 50 μM (cf. Table 1), exogenous hordatine inhibits leucine incorporation into protein of 4-day barley coleoptiles by only 15 per cent (unpublished results).

EXPERIMENTAL

Chemicals

Streptomycin was used as the sulfate; hexamethylene diamine and decamethylene diamine were the dihydrochlorides. Alkylene diguanidines, as the dihydrochlorides, were obtained from Professor G. E. Blackman, Dept. of Agriculture, Oxford University. Agmatine dihydrochloride, *p*-methoxycinnamoyl-agmatine hydrochloride, *p*-coumaroylagmatine acetate and hordatine A diacetate were supplied by Dr. A. Stoessl of this Institute.

Protein and RNA Synthesis

Segments from the third internode of 7-day etiolated peas were incubated in the appropriate autoclaved medium (buffered in 5 mM maleate, pH 5·5, and containing 15 mg/l penicillin G) for 1 hr before addition of L-leucine-1-^{14}C (31·0 mc/mM; 0·25 μc) or adenine-8-^{14}C (2·42 mc/mM; 0·5 μc) for a further 2 hr. Incorporation into protein or RNA was determined by a modified Schmidt–Thannhauser procedure as described previously.[1] That the radioactivity in the 1 N NaOH hydrolysate of the washed pellet from ^{14}C-leucine-labelled tissue represented incorporation into polypeptide material was checked as follows. Aliquots of a 1 N NaOH supernatant were adjusted to 6 N NaOH for complete hydrolysis at 110° for 16 hr in evacuated sealed tubes. After neutralization (HCl) the hydrolysate was desalted on Dowex 50-X8 and the amino acids eluted with ammonia.[10] Of the radioactivity applied, 93–98 per cent was recovered in the ammoniacal eluate. Recoveries of standard L-leucine-1-^{14}C were in the same range. Aliquots of the eluate were concentrated and subjected to chromatography in two directions on "Avicel" thin layers, using the solvent systems of Wyatt *et al.*[11] Autoradiography revealed that the radioactivity was associated exclusively with ninhydrin-positive material occupying the same region as standard L-leucine. *Antifungal activity* was determined in serial dilutions by a standard spore drop assay,[12] using *Monilinia fructicola*.

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