SITE OF ACTION OF THE PHYTOTOXIN, HELMINTHOSPORAL

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Helminthosporal is a phytotoxin produced in culture filtrates of the fungus Cochliobolus sativus, Ito and Kurib., (conidial stage, Bipolaris sorokinianum, Sacc. in Sorokin, Shoemaker), which causes common root rot of cereals, particularly wheat and barley. The toxin is a sesquiterpenoid dialdehyde as established by de Mayo et al., (1962) and Corey et al., (1963, 1965). The structure is shown in Table 4. It is the purpose of this communication to report that helminthosporal is an inhibitor of mitochondrial electron transfer and oxidative phosphorylation. The site of action of the toxin on the electron transfer chain in plant mitochondria is between the level of flavoprotein dehydrogenase(s) and b-type cytochromes.

In early experiments, helminthosporal was found to inhibit barley germination and growth of the young seedlings. Roots of barley seedlings treated with helminthosporal show necrosis and stunting and effects very similar to those caused by the fungus when attacking barley (Spencer, 1965). An inhibitory effect on the germination of various plant seeds including rye, oat and susceptible or resistant lines of wheat has been noted. Spores of the fungus Monilinia fructicola are also very sensitive to the toxin (de Mayo et al., 1965). While investigating the mode of action of the toxin it was discovered that helminthosporal inhibits the respiration of roots and coleoptiles of barley and wheat of different strains. The toxin also inhibits

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the respiration of root or storage tissue from radish, squash, bean, lettuce, turnip, sweet and white potato (unpublished results).

In this report we examine the inhibitory effects of helminthosporal on plant and animal mitochondria. The oxidation of both succinate and NAD-linked substrates is inhibited strongly at concentrations of toxin lower than those inhibiting the respiration of intact or sliced plant tissue. Oxidative phosphorylation by mitochondria is also inhibited. The results are provided in Table 1.

Table 1

Inhibition by helminthosporal of substrate oxidation and oxidative phosphorylation in mitochondria

Mito. source	Substrate	(M) conc. Helminthosporal	Percent Inhibition O_2 uptake	P/O
Sweet potato	pyruvate	1×10^{-3}	95	100
" "	malate	"	82	83
" "	α-ketoglutarate	"	70	70
" "	succinate	"	70	95
Mouse liver	succinate	5×10^{-4}	30	53
" "	"	1×10^{-3}	55	72
" "	"	2×10^{-3}	82	100
Wheat coleoptile	"	1×10^{-3}	45	-

Table 2 shows the effect of helminthosporal on enzyme activities in mitochondria isolated from sweet potato, wheat coleoptile and mouse liver. The activities of NADH-oxidase, NADH-cytochrome c reductase, malate-cytochrome c reductase, succinate-cytochrome c reductase and succinoxidase were inhibited strongly. Since NADH-2,6-DCIP reductase, malate-2,6-DCIP reductase, NADH-menadione-cytochrome c reductase, succinic dehydrogenase and cytochrome oxidase activities were affected weakly or not at all by helminthosporal, the site affected by the toxin appears to be between flavoprotein dehydrogenase(s) and cytochrome c. Malate dehydrogenase is insensitive since no decrease in the

rate of 2,6-dichlorophenol indophenol reduction was seen in the presence of toxin. The antimycin A-insensitive succinoxidase activity of sweet potato mitochondria is partially sensitive to helminthosporal. Unpublished results show no evidence for titration of a mitochondrial site as in the case of rotenone (Ernster et al., 1963). NADH-oxidase and NADH-cytochrome c reductase activities in the microsomal fraction from wheat and sweet potato are not influenced by helminthosporal.

Table 2
Inhibition of mitochondrial enzymes by helminthosporal

Activity measured	Percent inhibition		
	Sweet potato	Wheat coleoptile	Mouse liver
NADH-oxidase	57	82	-
NADH-2,6-DCIP reductase	-	5	-
NADH-cyt. c reductase	70	71	62
NADH-menadione-cyt. c reductase	5	-	-
Malate-2,6-DCIP reductase	0	0	0
Malate-cyt. c reductase	-	-	87
Succinoxidase	70	45	55
Succ.-cyt. c reductase	75	96	72
Succ. dehydrogenase	10	0	0
Cyt. c. oxidase	15	0	0

Final concs. of helminthosporal were: sweet potato (1×10^{-3}M); wheat (2×10^{-3}M); and mouse (1.3×10^{-3}M). Toxin was tested at 1×10^{-3}M on succinoxidase activity in wheat and mouse mitochondria.

In order to more clearly define the locus of action of helminthosporal between flavoprotein dehydrogenase(s) and cytochrome c, low temperature spectra were taken of sweet potato mitochondria oxidizing succinate with and without toxin. A low carotenoid variety of sweet potato, Pelican Processor, was used (Baker and Lieberman, 1962). Figure 1 shows the spectra. Upon adding toxin to mitochondria in air and then adding succinate to reduce the cytochromes, the absorption maxima at 549, 553, 557, 562 and 599 mμ are greatly diminished or do not appear, demonstrating that the toxin prevents the reduction of cytochromes b, c and a + a_3 (Baker and Lieberman, 1962). At lower concentrations of toxin the time required for anaerobiosis and full reduction of the cytochromes

decreases. Similar spectra are found with white turnip mitochondria which react much faster with helminthosporal.

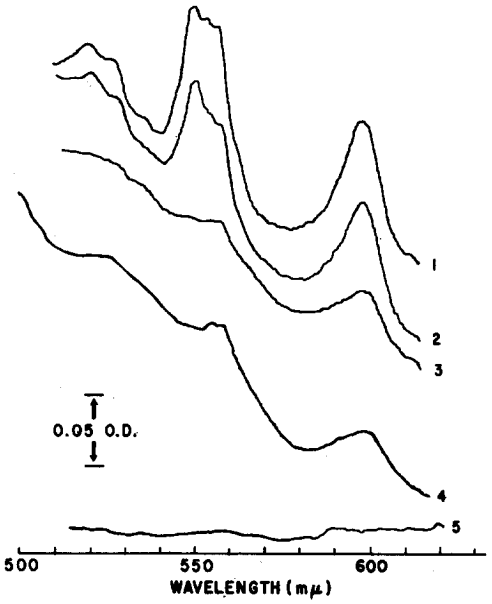


Figure 1. Low temperature (-196°C) spectra of sweet potato mitochondria. (1) Succinate-reduced cytochrome spectrum. Particles incubated with succinate 3-4 mins. before freezing. (2) Helminthosporal added to particles 5 min. before succinate added. (3) Helminthosporal added 10 min. before succinate. (4) Oxidized particles minus substrate. (5) Buffer baseline, no particles. Helminthosporal concentration was 2.5 x 10^{-3}M.

Helminthosporal affects oxidative phosphorylation in plant and mammalian mitochondria. For example, when succinate oxidation by mouse liver mitochondria is inhibited by 30% and 55%, the P/O ratio is inhibited by 53% and 72%, respectively. Compared with sweet potato mitochondria, oxidative phosphorylation by mouse liver mitochondria was more sensitive to helminthosporal. Helminthosporal also inhibits non-phosphorylating electron transfer thus distinguishing the toxin from energy transfer inhibitors such as oligomycin and atractylate. Unpublished data show that helminthosporal may inhibit all three sites of phosphorylation in the electron transfer chain. The partial reactions

in the phosphorylating sequence are inhibited by the toxin. Table 3 shows the effect of helminthosporal on dinitrophenol-induced ATPase activity and the ATP-P^{32} exchange reaction in mouse liver mitochondria.

Table 3

Inhibition by helminthosporal of the DNP-induced ATPase and the ATP-P^{32} exchange reaction in mouse liver mitochondria

	"Partial" Reaction[1]			
(M) conc. helminthosporal	ATPase (μmoles P_i released)		ATP-P^{32} exchange rate (μmoles P_i/min.)	
0	3.19; 4.02	(0)	0.285; 0.380	(0)
2×10^{-5}	2.66; -	(15)	- -	-
2.7×10^{-4}	- -	-	0.288	(22)
2×10^{-4}	2.06; 3.19	(27)	- -	-
5.4×10^{-4}	- -	-	0.057; 0.095	(74)
1×10^{-3}	- 0.43	(89)	0.013; 0.010	(95)
2×10^{-3}	0.17; 0.00	(98)	0.004 -	(97)

[1]Results show values for two separate experiments. Average percent inhibition given in parentheses. Mitochondria were prepared by Schneider's method and washed twice for the ATP-P^{32} experiment.

Phosphate release from ATP is inhibited by 98% at 2×10^{-3}M helminthosporal and by 27% at 2×10^{-4}M. The ATP-P^{32} exchange reaction is almost blocked at 1×10^{-3}M helminthosporal and is inhibited by 74% at 5.4×10^{-4}M. The phytotoxin does not act strictly as an uncoupler like DNP or as an energy transfer inhibitor, viz, oligomycin. It has a dual effect on electron transfer and oxidative phosphorylation as do some other inhibitors of these processes. Further experiments to more fully establish the site(s) of inhibition of oxidative phosphorylation by helminthosporal with relation to DNP and oligomycin action are in progress.

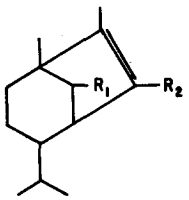
Helminthosporol and its air oxidation product, helminthosporic acid, isolated by Tamura et al. (1963), are growth-promoting substances which stimulate rice and cucumber seedlings, the production of α-amylase in barley endosperm (Okuda et al., 1967) and the release of reducing sugars from rice seed endosperm (Mori et al., 1965). We found that helminthosporal inhibits gibberellic acid (GA_3)-induced α-

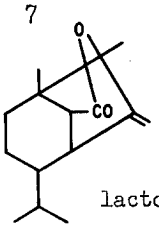
amylase formation by barley endosperms and the aldehyde mono-acid inhibits barley germination (unpublished data). Therefore, it was of interest to test analogs of helminthosporal on succinate-malate oxidation and oxidative phosphorylation by mitochondria isolated from sweet potato or mouse liver. The results for sweet potato mitochondria are given in Table 4.

Table 4

Effect of helminthosporal and analogs on P/O ratio and the oxidation of succinate and malate by sweet potato mitochondria

Analog		Percent inhibition			
		Succinate		Malate	
R_1	R_2	O_2 uptake	P/O	O_2 uptake	P/O
CHO	CHO	74	100	98	100
CH_2OH	CHO	68	93	58	58
COOH	CHO	13	1	37	19
CH_2OH	COOH	21	16	41	32
COOH	COOH	42	14	42	25
lactone		42	7	44	18





lactone

Helminthosporal: R_1=CHO; R_2=CHO. Compounds tested at 1 x 10^{-3}M.

Modification of the substituents at R_1 and R_2 contributes to the degree of inhibition. Among the analogs tested, helminthosporal showed the highest inhibitory effect on O_2 uptake and oxidative phosphorylation. Weaker effects on these processes were noted with the more oxidized analogs. In light of their plant growth-promoting activities even at concentrations as high as 1.4 x 10^{-3}M (Okuda et al., 1967; and Sakurai and Tamura, 1965), the inhibition we have observed with helminthosporol (R_1=CH_2OH: R_2=CHO) and helminthosporic acid (R_1=CH_2OH: R_2=COOH) is very curious. Also, the aldehyde mono-acid (R_1=COOH: R_2=CHO) was found to inhibit germination of barley seed to the same extent as helminthosporal but it had only a minor effect on plant and mammalian mitochondria.

The results reported here suggest that the phytotoxic activity of helminthosporal may be due to inhibition of mitochondrial electron flow and oxidative phosphorylation. Helminthosporal had no direct effect on a number of other cellular reactions which were tested.

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