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EFFECTS OF ANTIMETABOLITES ON PRODUCTION OF THE PHYTOALEXIN PISATIN

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Abstract—The effect of *p*-fluorophenylalanine, puromycin dihydrochloride, cycloheximide and patulin on the synthesis of pisatin was investigated. Their actions differed. In some cases pisatin synthesis was stimulated and in others it was inhibited. These incompatible effects make any definite conclusions as to whether protein synthesis is required for pisatin synthesis impossible.

INTRODUCTION

PISATIN synthesis in pea pods is stimulated by germinating fungal spores,[1] fungal culture filtrates,[2] respiratory inhibitors,[3] heavy metal ions,[3] amino acids,[3] some acids of the Krebs cycle[4] and a number of antibiotics and other specific inhibitors of protein synthesis.[5,6] In an attempt to determine whether new enzyme synthesis is required for the formation of pisatin, a number of antimetabolites, which are believed to inhibit protein synthesis, were tested for their ability to affect the production of pisatin by pea-leaf discs, both in the presence of an inducing culture filtrate and alone. A culture filtrate was used in preference to a fungal spore suspension in order to avoid interaction between the antimetabolites and the germinating spores, which might influence production of inducing substances, and in preference to a heavy metal salt as being closer to natural conditions of infection.

RESULTS AND DISCUSSION

A culture filtrate of *Penicillium expansum* CMI 37128 induced the formation of large amounts of pisatin.[4] The amounts of pisatin produced in the presence of this culture filtrate, and the effects of the various antimetabolites used are shown in Table 1.

p-Fluorophenylalanine is believed to be incorporated into protein in place of phenylalanine and thus to result in formation of non-functional proteins.[7] It inhibited pisatin formation in the presence of culture filtrate at concentrations above 10^{-3} M and did not itself induce pisatin formation. The inhibition could be explained either as the result of an inhibition of enzyme synthesis required for pisatin formation or, in view of the report that phenylalanine is readily incorporated into pisatin,[8] as a direct competitive inhibition of

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pisatin synthesis. It might be possible to decide between these alternative explanations by the use of other amino acid analogues.

TABLE 1. PISATIN CONCENTRATION IN DIFFUSATES OBTAINED FROM PEA-LEAF DISCS

Treatment	Pisatin concentration (μg/ml)
p-*Fluorophenylalanine* (26 hr)	
Culture filtrate control	27·2
4×10^{-4} M in culture filtrate	37·6
4×10^{-3} M in culture filtrate	17·5
4×10^{-2} M in culture filtrate	1·0
Distilled water control	1·0
4×10^{-4} M in distilled water	1·0
4×10^{-3} M in distilled water	1·0
4×10^{-2} M in distilled water	1·0
Puromycin dihydrochloride (28 hr)	
Culture filtrate control	52·6
10^{-4} M in culture filtrate	65·3
10^{-3} M in culture filtrate	70·5
Distilled water control	1·0
10^{-4} M in distilled water	16·6
10^{-3} M in distilled water	21·5
Cycloheximide (48 hr)	
Culture filtrate control	96·4
3×10^{-6} M in culture filtrate	69·2
3×10^{-5} M in culture filtrate	14·5
3×10^{-4} M in culture filtrate	1·8
Distilled water control	12·7
3×10^{-6} M in distilled water	29·8
3×10^{-5} M in distilled water	31·5
3×10^{-4} M in distilled water	2·2
Patulin (42 hr)	
Culture filtrate control	115·6
6×10^{-6} M in culture filtrate	108·6
6×10^{-5} M in culture filtrate	113·8
6×10^{-4} M in culture filtrate	74·5
6×10^{-3} M in culture filtrate	2·6
Distilled water control	8·7
6×10^{-6} M in distilled water	8·7
6×10^{-5} M in distilled water	37·7
6×10^{-4} M in distilled water	47·7
6×10^{-3} M in distilled water	12·3

Even at 10^{-3} M, puromycin enhanced the inducing activity of culture filtrates and itself acted as an inducer. Cycloheximide also induced pisatin formation at low concentration, but was inhibitory and non-inducing at 3×10^{-4} M. Cycloheximide, although itself capable of inducing pisatin synthesis at low concentrations, consistently reduced the inducing activity of culture filtrate. Patulin, which is of particular interest as being a normal metabolite

of *P. expansum*, was itself an inducer of pisatin synthesis at low concentrations and its slightly depressing effect on pisatin synthesis in the presence of a *P. expansum* culture filtrate suggest that it was already present in optimal or super-optimal concentration. Clearly, though patulin induces pisatin synthesis, it cannot alone account for the activity of *P. expansum* culture filtrates.

Thus, while it is clear that some inhibitors of protein synthesis will induce pisatin synthesis, any conclusions as to whether new enzyme synthesis is required for its formation will probably not be satisfactorily explained until we know more of the nature of the inducing agents in fungal culture filtrates and of the manner in which they act.

EXPERIMENTAL

Materials

Penicillium expansum CMI 37128 was obtained from the Commonwealth Mycological Institute, Kew, England. *p*-Fluorophenylalanine, cycloheximide and puromycin hydrochloride were commercial samples. Patulin was kindly supplied by Professor P. W. Brian.

Procedure

Culture filtrate of *P. expansum* was prepared after growth in a medium consisting of 1·0 g ammonium tartrate, 1·0 g KH_2OPO_4, 0·5 g $MgSO_4.7H_2O$, 12·5 g glucose and 1 l. distilled water for 4 days at 22°. Induction of pisatin synthesis was studied by placing drops of solution, sterilized by membrane filtration, on pea (*Pisum sativum* cv. Greenfeast) leaf discs 8 mm in dia., kept in Petri dishes on moist filter paper. After incubation in darkness at 24° for 24–48 hr the diffusates were collected and the concentration of pisatin in the diffusate was measured. The diffusate was centrifuged at 4000 rev/min and 5 ml was extracted by partitioning four times with 5 ml of redistilled petroleum ether, b.p. 40–60°. The extract was evaporated to dryness at 40° under vacuum, the residue redissolved in ethanol and the pisatin present assayed spectrophotometrically as described by Cruickshank.[9] The u.v. absorption of the inhibitors did not interfere with the spectrophotometric assay. Similar results to those shown in Table 1 were obtained on two occasions.

All the experiments were repeated twice, giving similar results. Pea leaves were selected from the same node of plants of similar age; only leaves which were visually similar were used. The discs were cut, bulked, chosen at random and dispensed into the various treatments at random. In excess of 150 discs were used for each treatment. The extraction procedure was shown to yield highly reproducible results.

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