STUDIES ON THE ANTIVIRAL SUBSTANCE

MECHANISM OF ACTION OF BIHOROMYCIN, A NEW ANTIBIOTIC

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Effects of bihoromycin on the respiration, protein and nucleic acid syntheses of *Piricularia oryzae* was investigated, and a marked inhibition of nucleic acid synthesis was observed. Further experiment using *Bacillus subtilis* revealed that DNA synthesis was more profoundly affected than RNA synthesis by the antibiotic. When leaves of pinto-bean and tobacco were treated with bihoromycin, it was found that the former plant was highly sensitive to the antibiotic.

A number of substances effective against tobacco mosaic virus (TMV) multiplication in plant tissues have been reported, though none of them have been applied to the practical use as yet because of their weak inhibitory activity against TMV multiplication or high toxicity to host plants. Some of them inhibit specifically local lesion formation, and others specifically inhibit systemic symptom development and TMV multiplication. Actidione is classified as the former type[1], whereas blasticidin S and laurusin exert both types of action[2,3].

As reported in the previous paper[4], bihoromycin exhibits remarkable inhibition against the local lesion formation by TMV on the leaf of pinto-bean. It also inhibits the growth of *Piricularia oryzae* and *Bacillus subtilis* at the low concentration (below 1 μg/ml).

Results presented in this communication concern the mechanism of antimicrobial effects and host specificity of the antibiotic.

Materials and Methods

Bihoromycin was prepared as described in the previous report[4]. After purification by silica gel chromatography, it was recrystallized from hexane, These colorless needle-like crystals were used throughout the study. Since bihoromycin is practically insoluble in water, it was dissolved in ethanol and diluted with distilled water to various concentrations.

Amino acid mixture labeled with ^{14}C (acid hydrolysate of *Chlorella* proteins) was kindly supplied by the Institute of Applied Microbiology, the University of Tokyo. Other radioactive compounds, ^{32}P-H_3PO_4 (74.3 mC/ml), uracil-2-^{14}C (40.4 mC/mM), thymidine-2-^{14}C (54.5 mC/mM) and thymidine 5-triphosphate-2-^{14}C (45.5 mC/mM) were purchased from R.C.C., England. Unlabeled deoxyribonucleoside triphosphates were purchased from Sigma Chemical Company. Deoxyribonucleic acid (salmon sperm DNA) was obtained from Wako Pure Chemical Industries, Ltd.

P. oryzae, designated as strain P_2, was obtained from Division of Plant Pathlogy, National Institute of Agricultural Science, Tokyo. The fungus was grown in yeast-glucose medium[5] at 27°C on a reciprocal shaker. Mycelia at log phase stage of growth were collected by filtration, washed with 0.01 M tris buffer (pH 7.0) and homogenized with the same buffer for 30 seconds in a Waring blender. The homogenate was used for the incubation experiments.

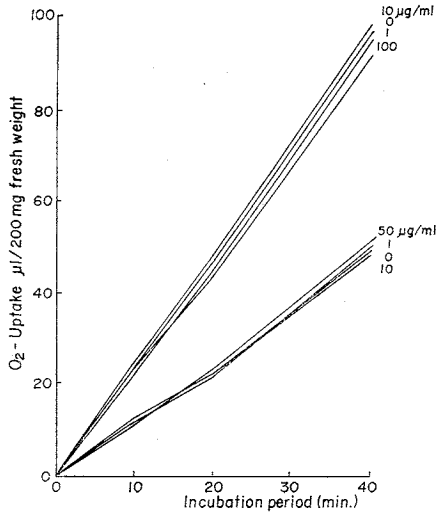
B. subtilis PCI 219 was grown in glutamate-glucose-mineral medium[6] at 30°C, and *Escherichia coli* strain B was grown in a medium containing 1.1% K_2HPO_4, 0.85% KH_2PO_4, 0.6% yeast extract and 1% glucose at 37°C. Cells in logarithmic phase were collected by centrifugation, washed with saline, and resuspended in 0.01 M tris buffer (pH 7.0).

Suspensions (5 ml) of fungal mycelia or bacterial cells were incubated in the presence of the antibiotic in L tubes. The radioactive precursors were added to the culture. The reaction was terminated by pouring the mycelia or cells into ice-cold ethanol or 10% cold perchloric acid. Preparation and fractionation of macromolecules from *P. oryzae* were carried out according to the method of OGUR and ROSEN[7], and from bacteria according to SCHMIDT, THANNHAUSER and SCHNEIDER.[8]

Cell-free extracts from *B. subtilis* or *E. coli* for DNA polymerization were prepared as follows: The washed cells were suspended in 50 mM tris buffer (pH 7.5) containing 10 mM 2-mercaptoethanol, 100 mM ammonium sulfate and bovine serum albumin (1 mg/ml), and sonicated for 15 minutes at 10 kc. The sonicates were centrifuged for 30 minutes at 16,000×g. This supernatant was used as the crude enzyme preparation, and DNA polymerase reaction, using salmon sperm DNA as a primer, was assayed by the method of OKAZAKI *et al.*[9]

Influence of the antibiotic on host plants of TMV, *i.e.* tobacco (*Nicotiana tabacum* L., bright yellow) and bean (*Phaseolus vulgaris* L., pinto bean), was examined by measuring the respiration of the slices of leaves of the host plants by WARBURG's manometric method.

Fig. 1. Effect of bihoromycin on the O_2-uptake of *Piricularia oryzae*



Results

1. Effect of Bihoromycin on the Mycelia of *P. oryzae*

As described in the previous paper[4], antimicrobial spectra of bihoromycin indicated singnificant inhibitory effect on the growth of *P. oryzae* (M.I.C. 0.5 µg/ml).

Fig. 1 shows the effect of bihoromycin on O_2 uptake by mycelia of *P. oryzae*. No effect was observed even up to the concentration of 50~100 µg/ml of the antibiotic on the endogenous respiration as well as exogenous one. In

Table 1. Effect of bihoromycin on protein and nucleic acid synthesis

Concentrations of antibiotic (mcg/ml)	^{14}C-Amino acids incorporated into				^{32}P-Incorporated into nucleic acids	
	soluble protein		insoluble protein			
	40 min.	80 min.	40 min.	80 min.	15 min.	30 min.
none	4,068* (100)***	8,616 (100)	1,374 (100)	2,512 (100)	1,172** (100)	2,271 (100)
0.5	3,262 (80.2)	6,707 (77.8)	1,294 (94.2)	2,214 (88.1)	489 (41.7)	1,126 (49.6)
5.0	2,987 (73.4)	6,209 (72.1)	934 (68.0)	1,830 (72.9)	388 (3.31)	562 (24.7)
50.0	2,865 (70.4)	6,129 (71.0)	808 (58.8)	1,822 (72.5)	239 (20.4)	397 (17.5)

Mycelial suspension of *P. oryzae* was fractionated 5 ml portions into L tubes. After incubation with antibiotic for 30 minutes, 0.5 µC of ^{14}C-amino acids or 5 µC of ^{32}P was added. Further incubation was carried out at 27°C in Monoshin incubator. Protein and nucleic acid fractions were prepared by the method of OGUR and ROSEN. Protein content was determined by the method of LOWRY *et al.*, and nucleic acid content was measured by optical density at 260 mµ. The radioactivity was counted with gas flow counter or GEIGER-MÜLLER counter.

* specific activity (cpm/mg protein). ** specific activity (cpm/OD at 260 mµ). *** percent incorporation.

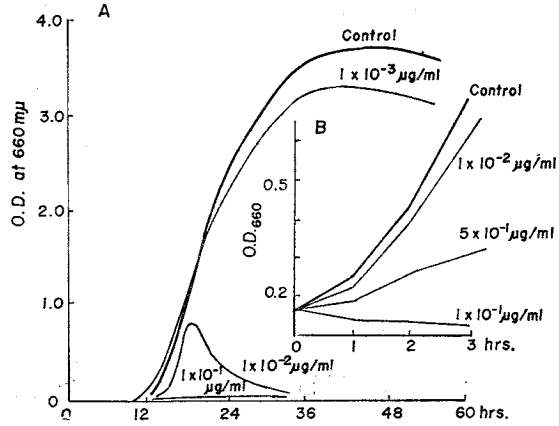
the latter case 0.1 M glucose was added as a substrate after starvation of the mycelia for 20 hours at 27°C.

Effect of bihoromycin on macromolecular syntheses was investigated. As shown in Table 1, the incorporation of ^{32}P-H_3PO_4 into nucleic acid fraction was remarkably inhibited by bihoromycin, while ^{14}C-amino acid mixture incorporation into soluble or insoluble protein fractions was not so affected. These results suggest that the primary site of action of bihoromycin may be inhibition of RNA or DNA synthesis.

Fig. 2. The effect of bihoromycin on the growth of *Bacillus subtilis* PCI 219

The spores of *B. subtilis* were inoculated into glucose-glutamate-mineral medium, and cultivated on reciprocal shaker at 30°C. (A)

Growing cells at 21 hours cultivation were transferred to fresh medium. (B)



2. Effect of Bihoromycin on Nucleic Acid Synthesis of Bacteria

Bihoromycin inhibited the growth of *B. subtilis* PCI-219 at low concentration, but it did not exhibit inhibitory effect on the growth of *E. coli* B even at 50 μg/ml. The growth of *B. subtilis* was inhibited by bihoromycin at 0.01 μg/ml when the antibiotic was present before initiating cell division, while the cell growth at logarithmic phase was inhibited and cell lysis was induced at 0.1 μg/ml (Fig. 2).

As shown in Fig. 3A, the incorporation of uracil-2-^{14}C into RNA fraction was completely inhibited at minimum inhibitory concentration (0.1 μg/ml), but only slight inhibition was observed at 0.01 μg/ml. DNA synthesis, however, was affected more profoundly by bihoromycin (Fig. 3B). Incorporation of thymidine-2-^{14}C into DNA fraction was inhibited even at 0.01 μg/ml.

The difference was more remarkable in the case of shorter preincubation of the drug with cells (Table 2). When cells were treated with bihoromycin for 1 minute before incubation, a concentration of 0.1 μg/ml was enough to cause complete inhibition of thymidine-2-^{14}C incorporation into DNA. Inhibition of RNA synthesis required over 10 minutes preincubation. Another test microorganism, *E. coil*, was not so sensitive as *B. subtilis* against the antibiotic. Thymidine-2-^{14}C incorporation into DNA of *E. coli* was not influenced by bihoromycin even at 10 μg/ml.

Table 2. Effect of pre-incubation periods on the inhibition of nucleic acid syntheses by bihoromycin

Nucleic acids	Bihoromycin (μg/ml)	Pre-incubation period			
		1 min.	10 min.	20 min.	30 min.
RNA	0	6,130*	5,700	5,100	4,650
	0.1	4,920	484	352	408
		(19.8)**	(91.5)	(93.1)	(91.2)
DNA	0	2,070	2,270	2.260	2,150
	0.1	354	16	16	43
		(82.9)	(99.2)	(99.2)	(98.0)

Conditions were as in Fig. 3 except that incubation was carried out for 30 minutes.
* specific radioactivity (cpm/OD at 260 mμ). ** inhibition rate.

Fig. 3. Effect of bihoromycin on incorporation of uracil-2-^{14}C into RNA (A) and thymidine-2-^{14}C into DNA (B)

Cell suspension was fractionated by 5-ml portions into L tubes. After 15 minutes preincubation with antibiotic, 0.5 μc uracil-2-^{14}C or thymidine-2-^{14}C was added and incubated at 30°C in Monoshin shaker.

RNA or DNA fraction was prepared by the method of SCHNEIDER *et al.*, and each content was determined from the value of optical density at 260 mμ.

The radioactivity was counted with an Aloka gas flow counter.

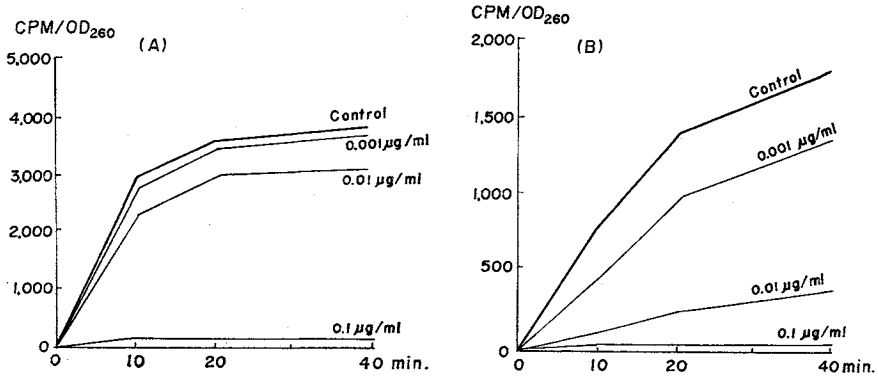


Table 3. Effect of bihoromycin on DNA synthesis with enzyme extracts from *B. subtilis* and *E. coli*

Conditions	*B. subtilis*	*E. coli*
Control	437 cpm	772 cpm
0 time	28	34
– DNA*	187	429
Bihoromycin		
1.5×10^{-3} μg/ml	392	758
1.5×10^{-2} μg/ml	327	657
1.5×10^{-1} μg/ml	215	595

* salmon sperm DNA as a primer.

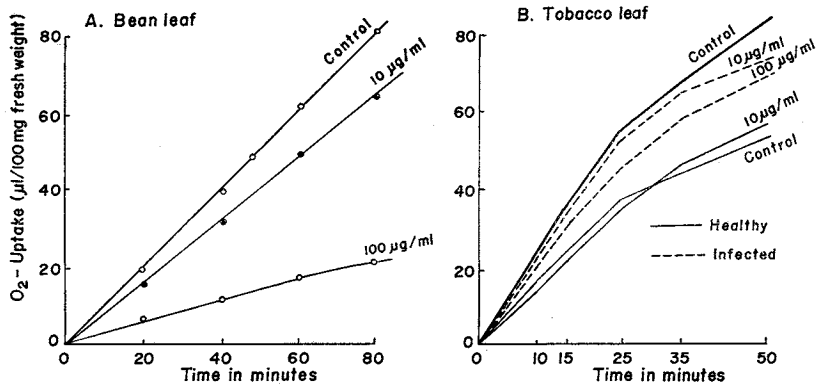
All these results seemed to suggest that bihoromycin acted as an inhibitor of DNA synthesis of sensitive microbes.

Further experiments were performed to confirm whether bihoromycin would inhibit DNA polymerase reaction by cell-free extracts from *B. subtilis* or *E. coli* (Table 3). The DNA polymerase reaction, using salmon sperm DNA as a primer, was not so affected by bihoromycin.

3. Effect of Bihoromycin on Host Plants of TMV

Bihoromycin inhibited specifically local lesion formation by TMV on the leaf of pinto-bean, and was less effective on TMV multiplication in tobacco leaf tissues, and symptom development. Because of difference, the specificity of bean and tobacco leaves to bihoromycin was examined. As

Fig. 4. Effect of bihoromycin on the O_2-uptake in the tissue of bean leaf and tobacco leaf



shown in Fig, 4, O_2 uptake of pinto-bean in the presence of the antibiotic was markedly inhibited, while both TMV inoculated and healthy tobacco leaves were not so influenced by bihoromycin.

Discussion

Our results indicate that bihoromycin inhibited the syntheses of macromolecules of *P. oryzae*, especially nucleic acid synthesis. It is without a strong effect on protein synthesis and, without any effect on the respiration of the organism. Further experiments demonstrated that thymidine-2-^{14}C incorporation into DNA was prevented by bihoromycin more profoundly than uracil-2-^{14}C incorporation into RNA in *B. subtilis*. It had no effect on DNA synthesis of *E. coli* intact cells.

Although bihoromycin showed marked inhibitory effect on DNA synthesis of *B. subtilis in vivo*, DNA polymerase reaction assayed by using the crude enzyme extracts was not so significantly affected by the antibiotic. These results suggest that the primary action of bihoromycin is not the inhibition of DNA polymerase activity. Effects of bihoromycin on the thermal denaturation or renaturation curves of DNA were not observed. (Data are not shown.)

From all these results, it is obvious that though bihoromycin inhibits DNA synthesis in intact cells of sensitive microbes, the primary site of action of the antibiotic is apparently different from those of actinomycin D, mitomycin C or bleomycin[11]. Bihoromycin might act on certain earlier stages of DNA synthesis or on some site on cell membranes essentially related to DNA synthesis.

Bihoromycin showed a marked inhibition on the local lesion formation caused by TMV on the leaf of pinto bean, while TMV multiplication in tobacco leaf tissues was not affected.

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