Studies on the Antibiotic Action of the Bacterial Pigment, Iodinin

III. Antibiotic Action of Iodinin and 1,6-Phenazine-diol on the Molds

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Introduction

In the former reports,[1,2] the mode of the action of iodinin on bacteria was investigated. Iodinin, 1,6-phenazine-diol-5,10-di-N-oxide, was found to have an inhibitory effect on the growth of *Bacillus* at a concentration of 1 to 6 μg/m*l*, while 1,6-phenazine-diol showed little antibiotic action on the bacterial strains employed. Several mold-strains employed were as susceptible to 1,6-phenazine-diol as to iodinin, or more susceptible to it than to iodinin. Further investigation has been carried on for the application of these pigments to a fungicide.

This report describes the mode of antibiotic action of iodinin and 1,6-phenazine-diol on the molds, including some phytopathogenic molds.

Materials and Methods

Organisms. Sixteen strains of *Aspergillus*, 6 strains of *Penicillium*, and other 13 strains employed in this investigation were supplied from the Institute of Applied Microbiology, University of Tokyo (Abbreviation: IAM), and from the Institute for Fermentation, Osaka (Abbreviation: IFO). Five phytopathogenic strains were supplied from the Laboratory of Plant Pathology, in this University. They are all shown in Table 1.

Preparation of iodinin and 1,6-phenazine-diol. The pigments were prepared by the same method as was shown in the former report[3].

Antibiotic spectra. One milliliter of the pigment solution was added to 4 m*l* of melting potato glucose agar in a test tube to give the final pigment concentrations of 1, 10, and 100 μg/m*l*. It was inoculated with 0.1 m*l* of a spore suspension in 0.1 per cent agar solution, mixed, and solidified into a slant. After an incubation at 30°C, inhibitory effect of the pigment was determined, comparing growth on a pigment slant with that on a control slant, inoculated with a spore suspension and including no pigment. The concentration, at which mold growth was completely inhibited, was taken to be minimum inhibitory concentration.

Inhibitory effect on a spore and spore germination. A drop of a spore suspension was

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mounted on a film of potato glucose agar, containing a pigment at a concentration of 10 μg/ml over a hole slide glass. After incubation at 30°C in a Petri dish with a filter paper, flooded over with 20 per cent glycerol solution, a ratio of spore germination was determined, according to the following formula, under a microscope by the period of 24 hours:

A ratio of spore germination (%)

$$= \frac{\text{a number of germinating spores}}{\text{total spores}} \times 100$$

Fifty milliliters of potato glucose medium, containing the pigment at a concentration of 10 μg/ml, was inoculated with 1 ml of spore-suspension or germinating spore-suspension, and shaked on a reciprocal shaker at 30°C. Dry weights were given by weighing a mycelium, collected on a filter paper by suction by 24 hours and dried in a desicator at a reduced pressure for 12 hours. Germinating spore-suspension was prepared from a culture of spore in potato glucose medium, shaked for 8 hours at 30°C. In this case, more than 90 per cent of spore were germinating, and most of hyphae from a spore were 8 to 10 μm long.

Inhibitory effect on growth of mycelial mat of mold. Mycelial mat was collected from a 4-day culture in 5 ml of potato glucose medium in a test tube, inclined in order to give a wider surface for mold-growth, and then was added to 50 ml of potato glucose medium, including the pigment at a concentration of 10 μg/ml, followed by cultivation on a shaker at 30°C and determination of dry weights by 3 hours.

Inhibitory effect on growing hyphae. Potato glucose agar blocks (10×5×0.5 mm) were mounted on the slide glass: with a block containing a pigment at a cencentration of 10 μg/ml, and a block containing no pigment, adjacent to each other, and was covered by one cover glass. Only a block containing no pigment was inoculated with spores. A slide glass was put on a U-glass rod in a Petri dish, in which 20 per cent of glycerol solution was flooded to prevent the drying of medium blocks, and then incubated at 30°C for 3 to 14 days.

Leakage of cell substances. Spore-suspension was prepared as follows: Spores were collected by flooding quarter-strength Ringer's solution over a colony on a potato glucose agar plate, followed by being washed once by a centrifugation at 2500 rpm for 10 min., and then were re-suspended in 20 ml of quarter-strength Ringer's solution. Five milliliters of a spore-suspension in a test tube containing a pigment at a concentration of 10 μg/ml, was shaken at 30°C. Sampled by 1 hour, a certain volume of the culture was centrifuged at 3000 rpm for 20 min. A supernatant liquid served for analyses of UV-absorbing substances, nucleic acid and protein, by Hitachi recording spectrophotometer and by phenol-Lowry method, respectively.

Mycelial mat, collected from a 6-day stationary culture of mold in 20 ml of potato glucose medium at 30°C, was washed twice with quarter-strength Ringer's solution by means of a centrifugation at 3500 rpm for 10 min., and was re-suspended in 50 ml of a pigment solution at a concentration of 10 μg/ml. After 4-hour cultivation, a supernatant was centrifuged at 3500 rpm for 20 min. and served for analyses.

Inhibitory effect on respiration of mold. Spores were collected by flooding over a plate-culture of mold with a sterile water, and were re-suspended in potato glucose broth. After incubation on a shaker at 30° C for 12 hours, germinating spores were collected by means of centrifugation at 3000 rpm for 10 min., washed, and suspended in

Table 1. Reaction mixture.

	Control	Pigment	Endogenous respiration
main compartment	Pgl-agar 2 m*l* cell suspension 0.2 m*l*	Pgl-agar 2 m*l* cell suspension 0.2 m*l* pigment 2 μg	1.5% agar soln. 2 m*l* cell suspension 0.2 m*l*
center well	20% KOH	20% KOH	20% KOH
side arm	—	—	—

* Two m*l* of water, employed for thermobarometer.

a phosphate buffer (pH 7.0). Two milliliters of potato glucose agar in a Warburg flask was flooded with 0.2 m*l* of germinating spore-suspension. Oxygen uptake by germinating spores was measured by 5 to 10 min. Reaction-mixture was as shown in Table 1.

Inhibitory effect on lactic acid fermentation. A synthetic culture medium for lactic acid fermentation of *Rhizopus oryzae* IFO 4070 was as follows: Glucose, 5 gm; $NH_4H_2PO_4$, 0.2 gm; KH_2PO_4, 0.2 gm; KCl, 0.05 gm; $MgSO_4 \cdot 7H_2O$, 0.05 gm; $FeSO_4 \cdot 7H_2O$, 0.001 gm; $ZnSO_4 \cdot 7H_2O$, 0.04 mg; distilled water, 100 m*l*; pH, 6.0[4]. A preculture, shaken at 30°C for 4 days, was served for main culture. Main culture consists of: a synthetic medium, 45 m*l*; preculture, 5 m*l*; 1,6-phenazine-diol, 5 mg (at a concentration of 100 μg/m*l*). It was shaken at 30°C, and after 5 mg of 1,6-phenazine-diol was added again in 24 hours, shaken for 24 hours more. Hyphae filtered off, the filtrate was served for analyses. Consumed sugar was determined by the modification of SOMOGYI method[5], neutral volatiles, as alcohol by KOLTHOFF's method, volatile acids, as acetic acid by titration with 0.1 N NaOH, ether extract, as lactic acid by titration with 0.1 N NaOH and paper chromatography with a solvent system: supernatant of a mixture of butanol, formic acid, and distilled water (4 : 1.5 : 1). Only a slight amount of lactic acid was produced in 48-hour cultivation, but within these hours, most of 1,6-phenazine-diol had been transformed into compound A.

Compound A production. Mycelial mat and 5 mg of 1,6-phenazine-diol were added to 60 m*l* of a synthetic medium in a 300 m*l*-Erlenmeyer flask. A flask was shaken at 30°C, and 5 m*l* of a culture was served for analysis by 2 hours. Spore-suspension and 0.5 mg of 1,6-phenazine-diol were added to 5 m*l* of a synthetic medium in a test tube, which, shaken at 30°C, was served for analysis by 3 hours. A zinc-free synthetic medium, from which zinc sulfate was removed, or a phosphate buffer, which would not allow *Rhizopus oryzae* to grow, was served for a control of compound A production.

Isotope experiments. A 2-day culture of *Brevibacterium stationis var. iodininofaciens* Po-363-21 was centrifuged for 10 min. at 400 rpm (ca. 27×G) to remove iodinin crystals formed extracellularly. The culture, from which iodinin crystals were removed was re-centrifuged at 3000 rpm for 10min. Iodinin crystal-free cells were washed twice with phosphate buffer (pH 7.0), and then were suspended in the same buffer, so that one fiftieth optical density at 660 nm might be adjusted to 0.1 to 0.2. To 50 m*l* of this cell-suspension were added glucose, to give the desired concentration of 0.03 M, sodium L-glutamate or ammonium sulfate, of 0.01 M, and 1 m*l* of uniformly labelled ^{14}C-glucose solution with a radioactivity of 1 μCi/m*l*. ^{14}C-iodinin was extracted with chloroform from 4-day shaking culture, and then was reduced into ^{14}C-1,6-phenazine-diol with

sodium hydrosulfite. ^{14}C-1,6-phenazine-diol was transformed into ^{14}C-compound A with a culture of *Rh. oryzae*, of which methanol solution was served for the measurement of radioactivity.

In order to investigate the incorporation of glucose-carbon into compound A, 1 m*l* of ^{14}C-glucose solution with a radioactivity of 1 μCi/m*l* was added to a culture of *Rh. oryzae* on transformation of 1,6-phenazine-diol into compound A.

Aloka-P. C. C. type 307 was employed for the measurement of radioactivity, and 1 m*l* of sample was served for the measurement.

Antibiotic activity of compound A. Determination of antibiotic activity was carried out in the same way as those of iodinin and 1,6-phenazine-diol were carried out.

Results and Discussion

Antibiotic spectra

Antibiotic spectra of iodinin and 1,6-phenazine-diol against molds were shown in Table 2.

Most strains of the genus *Aspergillus* employed seemed to suffer from growth-inhibition by neither of two pigments at a concentration of 1 μg/m*l*, slightly suffering at a concentration of 10 or 100 μg/m*l*. A considerable growth-inhibition of *Asp. sojae* was observed with both of the pigments at a concentration of 1 μg/m*l*. Excepting *Pen. nigricans* and *Pen. chrysogenum*, growth of the most strains of the genus *Penicillium* employed was inhibited. Growth of *Pen. funiculosum* and *Pen. roqueforti* was completely inhibited with both of the pigments at a concentration of 10 μg/m*l*, which showed one-half inhibition even at a concentration of 1 μg/m*l*.

Both of the pigments were completely inhibitory to the growth of *Neurospora crassa*, *Mortierella pusilla*, and both strains of *Polystictus* at a concentration of 1 to 10 μg/m*l* ; while they were hardly inhibitory to the growth of strains of *Fusarium*, *Rhizopus*, and *Mucor* at a concentration of less than 100 μg/m*l*.

All the strains of phytopathogenic molds were inhibited by iodinin at a concentration of 10 μg/m*l*, especially *Pellicularia sasakii* (*Hypochnus sasakii*) was completely inhibited at a concentration of 10 μg/m*l*, while *Helminthosporium sigmoideum* (*Sclerotium oryzae*) and *Piricularia oryzae* were strongly inhibited by 1,6-phenazine-diol.

All strains of *Rhizopus* and *Mucor* employed were inhibited by neither of these two pigments, and they transformed 1,6-phenazine-diol into yellow needle-shaped crystal, compound A, insoluble in chloroform and extractable from mycelial pellets with a diluted alkali solution.

Minimum inhibitory concentration

Minimum inhibitory concentration of iodinin, and 1,6-phenazine-diol against several mold-strains employed was shown in Table 3. 1,6-Phenazine-diol is slightly soluble in water, and iodinin, insoluble, though 1,6-phenazine-diol and iodinin are soluble in an alkali solution at pH more than 10, and at pH more than 12, respectively. Both of the pigments were found to have a tendency to remove to lecithine globules in emulsion from a water solution at the acid pH, and likely to cell lipids. Accordingly, a minimum inhibitory concentration of iodinin and 1,6-phenazine-diol is considered to be of significance.

Inhibitory effect on a spore and spore-germination

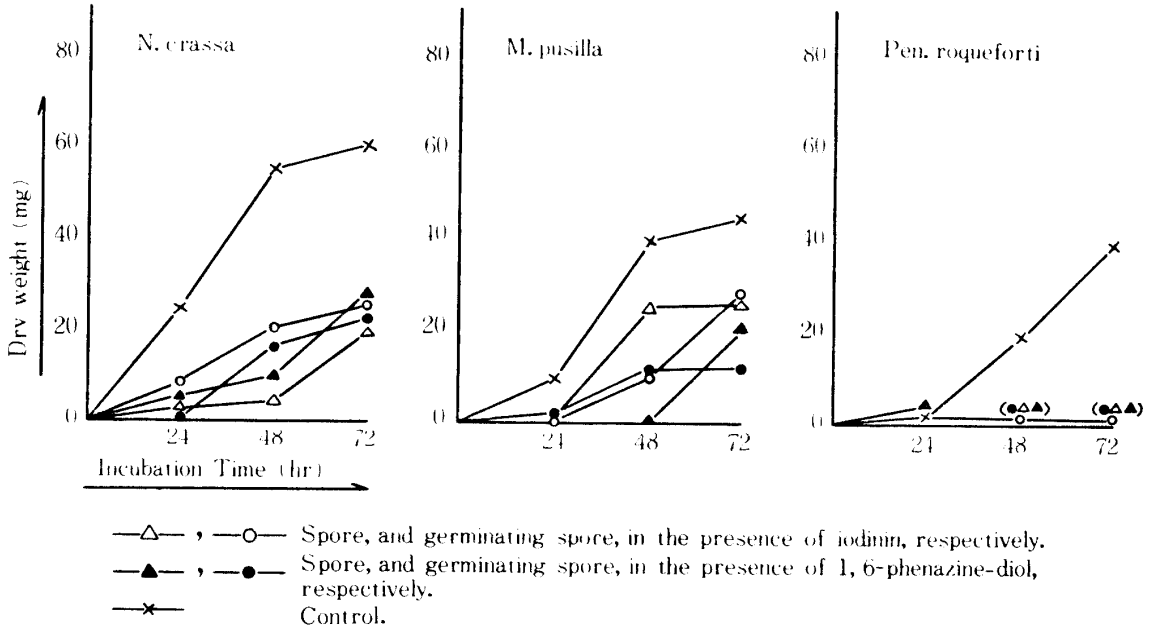
Table 2. Antibiotic Spectra.

Organism	iodinin			1, 6-phenazine-diol.		
	1 μg	10 μg	100 μg	1 μg	10 μg	100 μg
(*Aspergillus*)						
Asp. niger ATCC 6275	−	+	++	−	−	−
Asp. flavus IFO 4053	−	+	++	−	+	+
Asp. awamori IFO 4314	−	+	+	−	+	+
Asp. awamori var. fumeus IFO 4122	−	+	+	−	+	+
Asp. nidulans IAM 2006	−	+	+	+	+	+
Asp. luchuensis IFO 4281	−	+	+	−	+	+
Asp. oryzae IFO 4214	−	−	+	−	+	+
Asp. oryzae var. magnasporus IFO 4050	±	+	++	−	−	+
Asp. oryzae var. globosus IFO 4214	−	−	+	−	−	−
Asp. usamii IFO 4388	−	−	++	−	−	−
Asp. usamii var. shirousami IFO 6082	−	−	+	−	−	−
Asp. chevalieri IFO 4298	−	+	+	−	+	+
Asp. clavatus IAM 2002	−	−	−	−	−	−
Asp. tamarii IFO 4099	−	+	+	−	−	−
Asp. kawachii IFO 4308	−	+	+	−	−	−
Asp. sojae IFO 4244	+	+	+	++	++	++
(*Penicillium*)						
Pen. chrysogenum IFO 4626	+	+	+	−	−	−
Pen. claviforme IFO 5740	+	++	+++	−	+	+
Pen. decumbens IFO 6093	+	++	+++	+	++	++
Pen. funiculosum IFO 5857	++	+++	+++	++	+++	+++
Pen. roqueforti IFO 4022	+	+++	+++	+	+++	+++
Pen. nigricans IFO 6103	−	−	−	−	−	−
(*Others*)						
Mortierella pusilla IFO 6223	++	+++	+++	++	+++	+++
Neurospora crassa IAM 6660	+	+++	+++	+	+++	+++
Tilachlidium humicola IAM 5025	−	+	++	−	−	++
Fusarium moniliforme IAM 5062	−	−	−	−	−	−
Pullularia pullulans IAM 5055	−	+	+	−	+	+
Botrytis cinerea IAM 5126	−	+	++	−	−	+
Cephalosporium acremonium IAM 5027				−	−	+
Hormodendrum pedrosoi IAM 17-1	−	+	+	−	−	++
Rhizopus oryzae IFO 4070	−	−	−	−	−	−
Rhizopus japonicus IFO 5441	−	−	−	−	−	−
Mucor javanicus IFO 6087	−	−	+	−	−	−
Polystictus versicolor IAM 9018	+++	+++	+++	+++	+++	+++
Polystictus sanguineus IAM 9005	+++	+++	+++	+++	+++	+++
(*Phytopathogenic molds*)						
Cochliobolus miyabeanus H-1	−	++	+++	−	+	+
Cochliobolus miyabeanus	−	++	+++	−	+	+
Pellicularia sasaki	+	+++	+++	−	+	+
Helminthosporium sigmoideum	+	+++	+++	+++	+++	+++
Pyricularia oryzae	−	+	+++	−	++	+++

* +++ no Growth
++ 50% Inhibition
\+ 30% Inhibition
− no Inhibition

Table 3. Minimum Inhibitory Concentration.

mold	iodinin	diol
Polystictus sanguineus IAM 9005	2.5 μg/ml	2.5 μg/ml
Polystictus versicolor IAM 9018	2.5	5.0
Pen. roqueforti IFO 4022	10	2.5
Pen. funiculosum IFO 5857	10	2.5
Neurospora crassa IAM 6660	2.5	2.5
Mortierella pusilla IFO 6223	10	10



—△— , —○— Spore, and germinating spore, in the presence of iodinin, respectively.
—▲— , —●— Spore, and germinating spore, in the presence of 1, 6-phenazine-diol, respectively.
—×— Control.

Fig. 1. Effect of iodinin and 1,6-phenazine-diol on a sopre and a germinating spore.

A time-course of the change in cell dry weight of mold culture, in which iodinin, or 1,6-phenazine-diol was added, is shown in Fig. 1. This indicates that iodinin and 1,6-phenazine-diol are inhibitory to both spore and geminating spore. They could not grow on an agar medium, containing a pigment at a concentration of 10 μg/ml, while their growth were observed in a broth-medium, after being shaken, to make the pigment-crystals wrapped in mold pellets, and to make it difficult for them to come into contact with mold-cells.

A ratio of spore germination of molds from starting to 15 hours is shown in Table 4. 1,6-Phenazine-diol completely inhibited spore-germination of all the strains, excepting *Polystictus sanguineus*, of which hyphal fragments were employed, because of its little production of spore; although iodinin might extend a lag-time for spore-germination, it was not observed to stop spore-germination.

Inhibitory effect on growth of mycelial mat of mold

Effect of iodinin and 1,6-phenazine-diol on a dry weight of mycelial mat is shown in Fig. 2. Inhibition of growth of mycelial mat by a pigment appeared lower than that of spore did. Increase in dry weight of mycelial mat of *Neurospora crassa* is

Table 4. Effects of iodinin and 1,6-phenazine-diol on a ratio of spore germination.

strain	time (hr)	a ratio of spore germination		
		control	iodinin	diol
Neurospora crassa	7	40 (%)	0 (%)	0 (%)
	9	60	0	0
	12	70	10	0
	15	100	20	0
Polystictus sanguineus	7	60	0	0
	9	100	40	40
	12		50	50
	15		100	80
Mortierella pusilla	7	100	0	0
	9		20	0
	12		80	0
	15		100	0
Penicillium funiculosum	7	0	0	0
	9	50	0	0
	12	100	70	0
	15		70	0
Penicillium roqueforti	7	0	0	0
	9	50	0	0
	12	100	50	0
	15		50	0

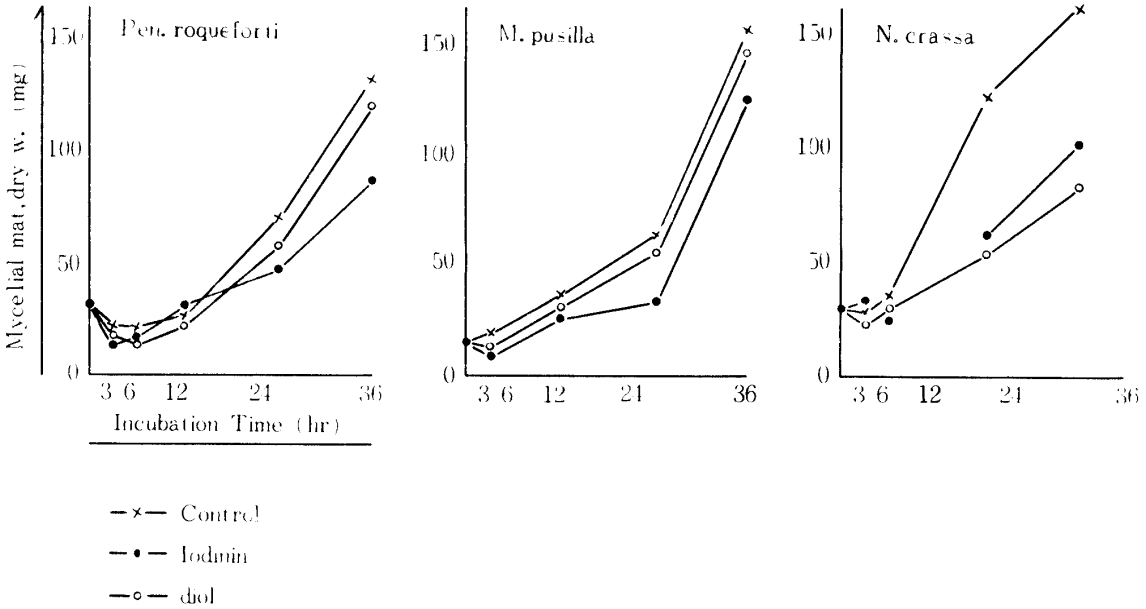
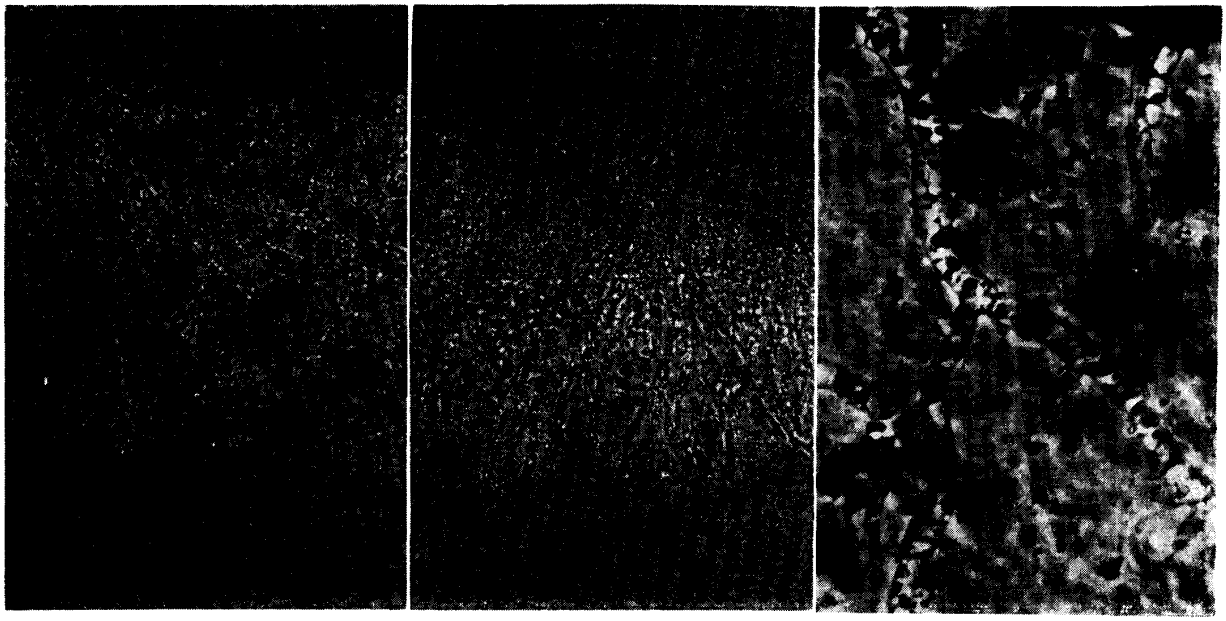


Fig. 2. Effect of iodinin and 1,6-phenazine-diol on growth of mycelial mat.



a. b. c.

Photo 1. Hyphae of *Mortierella pusilla var. vinacea* IFO 6223.
a. Hyphae, entering into an iodinin agar.
b. Hyphae, hardly entering into a 1, 6-phenazine-diol agar.
c. A great many of vacuoles were observed in hyphae, entering into an iodinin agar.

Photo 2. Compound A, crystallized out around hyphae of *Rhizopus oryzae* IFO 4707.

restricted only by the pigmemts.

Inhibitory effect on growing hyphae

Effect on growing hyphae varied to the genus, as shown in Photo 1 and 2.

Aspergillus: Inhibitory effect of both of the pigments on growth of mycelium and conidia-formation of strains of the genus *Aspergillus* employed was not observed. Iodinin caused a little decrease in an amount of conidial head of such strains, as *Asp. flavus* IFO 4053 and *Asp. oryzae* IFO 5239; a considerable decrease in an amount of conidial head and substratal hyphae of such strains, as *Asp. sojae* IFO 4244 and *Asp. tamarii* IFO 4099. Inhibitory effect on growing hyphae of such strains, as *Asp. clavatus* IAM 2002, *Asp. niger* ATCC 6275, and *Asp. usamii* IFO 4388, was not observed.

Penicillium: A significant effect of 1,6-phenazine-diol on strains of the genus *Penicillium* employed was observed. Strains of the genus *Penicillium* employed are apt to produce coralloid hyphae. *Pen. decumbens* IFO 6093 and *Pen. funiculosum* IFO 5857 were completely inhibited, by 1,6-phenazine-diol and *Pen. roqueforti* IFO 4022 was considerably inhibited, while *Pen. nigricans* IFO 6103 was not inhibited.

Cochliobolus: *C. miyabeanus* H-1 was completely inhibited by both the pigments, and hyphae grown on a control agar block were not able to enter into a pigment agar block.

Fusarium: *F. moniliforme* IAM 5062 is apt to produce coralloid hyphae, and is slightly inhibited by iodinin.

Mortierella: *Mor. pusilla var. vinacea* IFO 6223 had a tendency to produce coralloid hyphae and yeast-like cells in a slide culture. No difference was observed between morphology of hyphae on a 1,6-phenazine-diol agar block and that of hyphae in and over a control agar block, but hyphae on a 1,6-phenazine-diol agar block could hardly enter into this agar block. Hyphae on an iodinin agar block were observed to have entered into this agar block, in which a great number of vacuoles were found. The fact indicates that iodinin may differ in the mode of antibiotic action on *Mortierella* from 1,6-phenazine-diol.

Mucor and *Rhizopus*: There is no significant difference in growth and conidia formation of *Mucor javanicus* IAM 6087 and *Rhizopus oryzae* IFO 4707 between a pigment agar block and a control agar block. 1,6-Phenazine-diol crystals about hyphae in a 1,6-phenazine-diol agar block might dissolve and be incorporated into hyphal cytoplasm to be transformed into compound A. Compound A, yellow crystals, was observed around hyphae in a block.

Neurospora: *N. crassa* IAM 6660 was inhibited by neither of two pigments, showing a slight conidia-formation on a pigment agar block as well as in a control agar block.

Polystictus: *P. sanguineus* IAM 9005 was completely inhibited by both of the pigments, but in a iodinin agar block, a slight invasion of hyphae and a normal clamp formation were observed.

Leakage of cell substances

Absorption spectra and protein analyses of supernatant liquid are shown in Table 5. Neither absorption-band of a nucleic acid nor that of protein were observed in absorption spectra of supernatant liquids of spores and mycelial mat, a peak at 270 nm in spectra proving to be that of 1,6-phenazine-diol. No evidence for leakage of protein from spores and mycelial mat was given by means of colorimetric determination.

Inhibitory effects on respiration of mold

Table 5. Analysis of protein*

i) Spore suspension

strain	iodinin	diol	control
Pen. roqueforti	0.072	0.060	0.064
M. pusilla	0.034	0.037	0.062
N. crassa	0.086	0.086	0.035

ii) Mycelial mat

strain	iodinin	diol	control
Pen. roqueforti	0.010	0.023	0.020
M. pusilla	0.034	0.044	0.010
N. crassa	0.044	0.078	0.064

* Optical density at 770 nm.

As shown in Fig. 3 and Fig. 4, a fall in oxygen up-take of mold-strains employed was observed, when iodinin, or 1, 6-phenazine-diol was added to a medium. The fact indicates that inhibitory effect of both the pigments on respiration of mold was confirmed.

Inhibitory effect on lactic acid fermentation of Rhizopus oryzae

Analyses of culture filtrate were shown in Table 6. No significant difference was observed in the amounts of consumed sugar, neutral volatiles, volatile acids, and non-volatile acids between a control culture and a culture, containing 1,6-phenazine-diol, in which most of 1,6-phenazine-diol added had been transformed into compound A. The fact indicates that addition of 1,6-phenazine-diol to a mold-culture might give no effect on lactic acid fermentation of mold, and production of compound A might not depend on lactic fermentation.

Compound A production

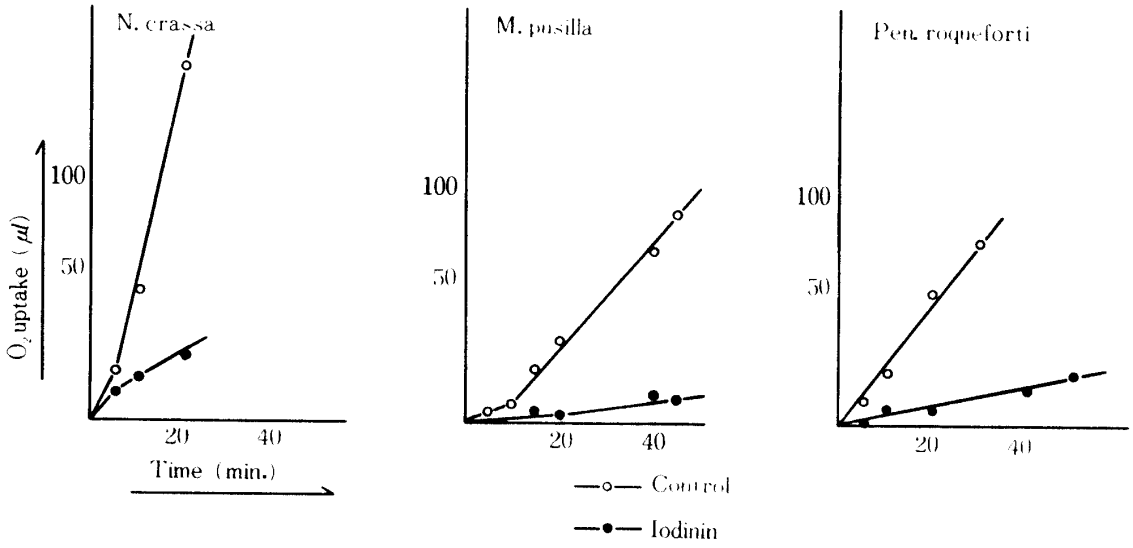


Fig. 3. Inhibitory effect of iodinin on respiration.

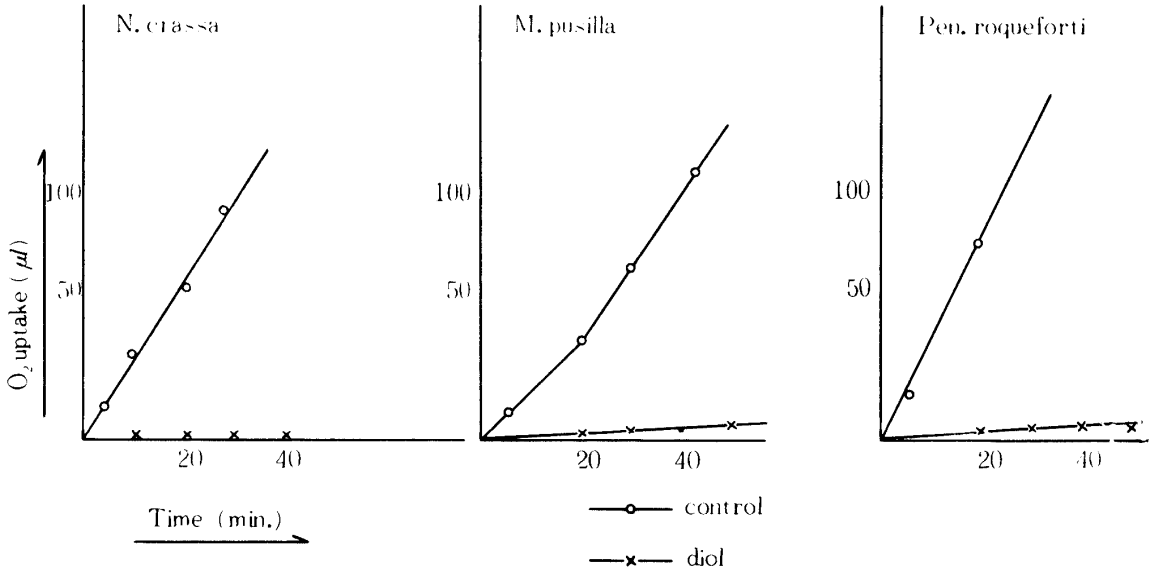


Fig. 4. Inhibitory effect of 1,6-phenazine-diol on respiration.

Toble 6. Analyses of culture filtrate of *Rh. oryzae*

	control	diol
consumed sugar	24.4 mg	23.0 mg
neutral volatiles	2.30	2.79
volatile acids (as acetic acid)	0.00	0.00
non-volatile acids (as lactic acid)	0.04	0.06

* in 1 m*l* of culture filtrate.

A time course of decrease of 1,6-phenazine-diol, added to the culture of *Rh. oryzae*, was shown in Fig. 5. Added to the mycelial mat suspension, 1,6-phenazine-diol decreased lineally with time, and disappeared in 12 hours. In spite of the disappearance of 1, 6-phenazine-diol, a culture remained yellow, resulting from production of compound A, which was proved by means of paper chromatography. In spore-suspension 1,6-phenazine-diol decreased lineally with time after a lag time of 6 hours, agreeing with a time required for a completion of spore-germination of *Rh. oryzae*, and disappeared in 9 hours after a lag time. Transformation of 1,6-phenazine-diol to compound A was not observed in a medium, from which zinc sulfate had been removed. These facts indicate that this transformation started after a completion of spore-germination, and it might require respiration, or growth of *Rh. oryzae*, or both. A slight amount of crystal of compound A was observed in a iodinin agar block on a slide, but in a shaking culture, containing iodinin, neither decrease in an amount of iodinin, nor production of compound A was observed in 24 hours. Iodinin added to a shaking culture, was absorbed to mold pellets, showing a violet color, as well as to lecithine globules in emulsion, showing a pink color, as shown in Photo 3.

Isotope experiments

Incorporation of ^{14}C to iodinin from ^{14}C-glucose by brevibacterium was as shown in Table 7. No significant difference was found between sodium L-glutamate and

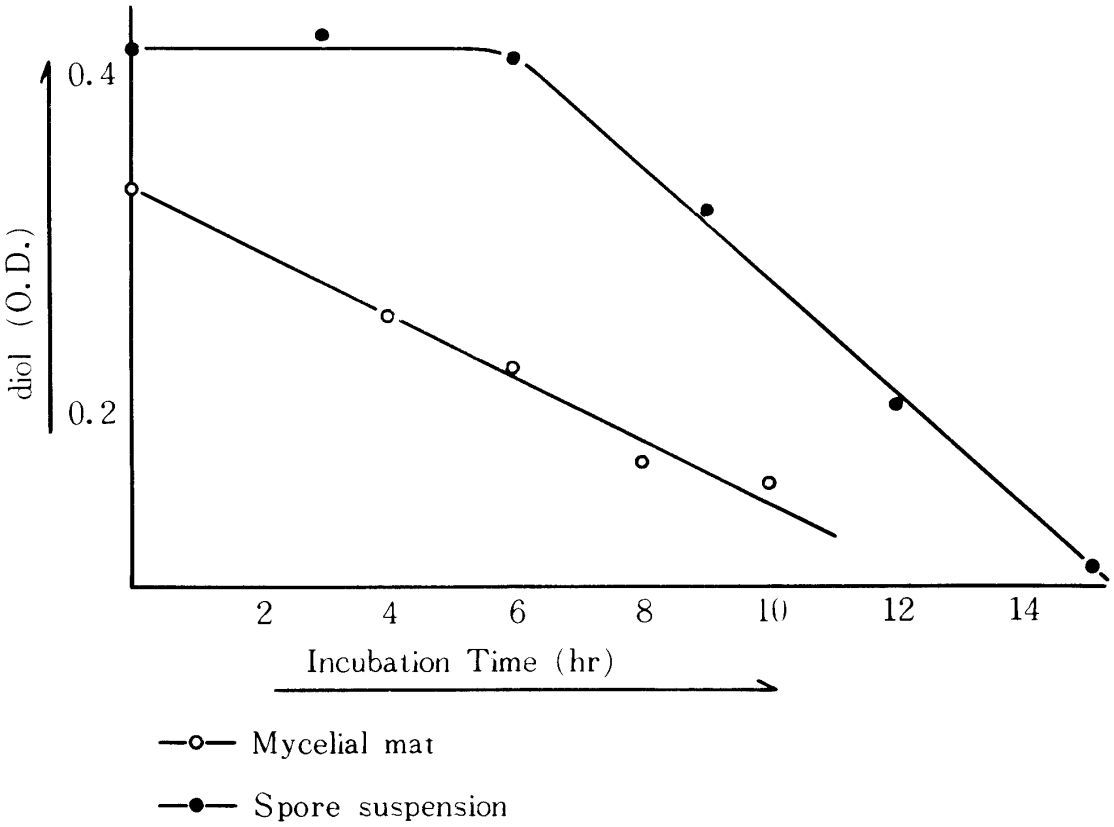


Fig. 5. Transformation of 1,6-phenazine-diol to compound A.

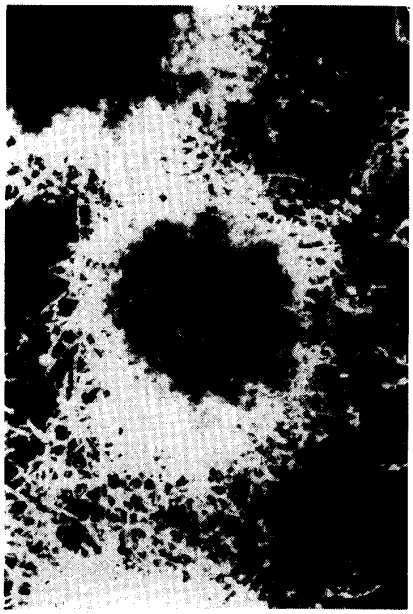


Photo 3. Pellets of *Rhizopus oryzae* IFO 4707, in which iodinin was wrapped, showing a violet color.

Table 7. Incorporation of ^{14}C to iodinin from ^{14}C-glucose.

	nitrogen sources	
	$(NH_4)_2SO_4$	Na-L-glutamic acid
iodinin*	0.037 mg	0.031 mg
radioactivity*, cell	347.4 cpm	415.4 cpm
culture broth	253.5 cpm	474.0 cpm
iodinin	48.0 cpm	58.5 cpm

* in 1m*l* of culture broth.

Table 8. Transformation of ^{14}C-iodinin to ^{14}C-phenazine-diol.

	iodinin, added	diol, produced
total amounts, mg	24.4	18.5
specific radioactivity, cpm/μmole	293.3	284.5
total radio activity, cpm	29325	24811

ammonium sulfate as a nitrogen source for iodinin production by resting brevibacterial cells. Less than 10 per cent of ^{14}C was incorporated into iodinin, and the rest in radioactivity was detected in a cell and a broth-sample.

Transformation of ^{14}C-iodinin to ^{14}C-1,6-phenazine-diol was shown in Table 8. A yield of ^{14}C-phenazine-diol from ^{14}C-iodinin was 87.2 per cent of the theoretical amount. The theoretical value of total radioactivity of ^{14}C-1,6-phenazine-diol produced from ^{14}C-iodinin is 25571 cpm, agreeing well with its experimental value. Accordingly, phenazine-ring in 1,6-phenazine-diol should be derived from that of iodinin.

Transformation of ^{14}C-1,6-phenazine-diol into ^{14}C-compound A was shown in Table 9. Most of total radioactivity in ^{14}C-1,6-phenazine-diol was transferred to compound A. Transformation of 1,6-phenazine-diol to compound A, when ^{14}C-glucose was employed as a carbon source for respiration, was shown in Table 10. Most of total radioactivity of ^{14}C-glucose was found out in a culture-broth, and only a slight radioactivity was incorporated to compound A. These facts indicate that most of carbons in 1,6-phenazine-diol were transferred to compound A, and a little or no incorporation of carbon, excepting that of 1,6-phenazine-diol, to compound A was observed.

Table 9. Transformation of ^{14}C-1,6-phenazine-diol to ^{14}C-compound A.

	diol	culture broth	compound A
radioactivity, cpm/m*l*	760	3.2	231.6
Total radioactivity, cpm	38000	2156	34740

Table 10. Incorporation of ^{14}C from Glucose.

	culture broth	compound A
radioactivity, cpm/m*l*	1203	67.5
total radioactivity, cpm	649620	12825

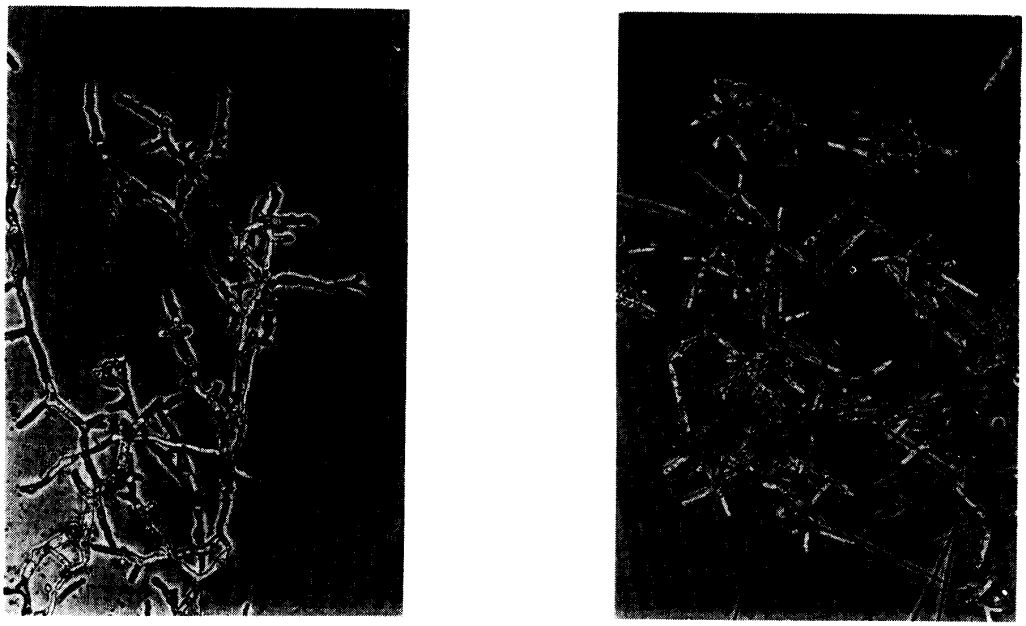


Photo 4. Spore germination of *Rhizopus oryzae* IFO 4707.
a. Hyphae, in the presence of iodinin, not differing from hyphae in no addition of pigment in elongation.
b. Hyphae, against which a slight inhibition of 1,6-phenazine-diol was observed, shorter than those of no pigment, or in the presence of iodinin.

Absorption spectra of compound A in the visible region and in the ultraviolet region are similar to these of 1,6-phenazine-diol, and accordingly, they might have many similar features in the structure.

Antibiotic activity of compound A

Compound A showed no growth-inhibition to all mold-strains employed at a concentration of 100 μg/m*l*.

As shown in Photo 4, a slight inhibition of 1, 6-phenazine-diol to spore germination or growth of germinating spore of *Rh. oryzae* was observed, as compared with a control. According to the fact, transformation of 1,6-phenazine-diol into compound A is considered as a neutralization of growth-inhibition of 1,6-phenazine-diol to members of the genera *Rhizopus* and *Mucor*.

Summary

Iodinin and 1,6-phenazine-diol almost completely inhibited the growths of most strains of *Penicillium*, *Neurospora crassa*, *Mortierella pusilla*, *Polystictus*, and phytopathogenic molds at concentrations of 1 to 10 μg/m*l*, while *Rhizopus* and *Mucor* were not inhibited at a concentration of 100 μg/m*l*, and iodinin had a slight inhibitory effect on the growth of *Aspergillus*. Inhibitory effects of iodinin and 1,6-phenazine-diol varied to the genus of mold. Iodinin had a slight suppressing effect on conidia-production of *Aspergillus*, while 1,6-phenazine-diol had larger effect on the growth of *Penicillium* than iodinin had. 1,6-Phenazine-diol suppressed an invasion of hyphae of *Mortierella pusilla* to agar medium, while iodinin brought about increase of the number of vacuole in its hyphae. Leakage of protein and nucleic acid was not observed with iodinin and

1,6-phenazine-diol at a concentration of 100 μg/m*l*. As a fall in oxygen uptake was observed with the pigments, both the pigments seemed to suppress respiration of molds, but inhibitory effect on lactic acid fermentation of *Rhizopus oryzae* was not observed.

Rh. oryzae transformed 1,6-phenazine-diol into compound A smoothly. As compound A showed no growth inhibition to all mold-strains at a concentration of 100 μg/m*l*, transformation of 1,6-phenazine-diol into compound A was considered to be a neutralization of growth-inhibition of 1,6-phenazine-diol.

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