Studies on Helicobasidin, a Pigment Isolated from *Helicobasidium mompa* TANAKA, and its Toxic Action to Some Higher Plants and Microorganisms.

By

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CONTENTS

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Introduction

Recently many kinds of fungal products have been isolated and their bioactivity has been actively discussed. As to metabolic products of *Helicobasidium mompa* TANAKA causing violet root rot disease of plants, TANAKA's report[59] assumed the oxalic acid production was the first. About twenty years later, MIYAKE[26] found some organic acids other than oxalate. In the culture of the fungus, ITO[18] proved calcium oxalate and ascribed pH depression of the medium to its production. Recently ARAKI *et al.*[4], reported the production of itaconic acid and they however, assumed that the cause of pH depression of the medium at the primary stage of culture might be mainly due to the production of this acid.

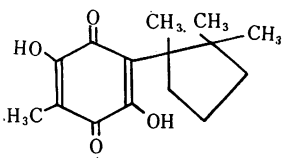
Besides these acids, ITÔ (l. c.) already found the formation of "mars orange amorphous bodies" on the culture medium, and even on the glass wall, a little distant from the hyphae of the fungus, and he recognized a phenolic property on this substance. The author being much interested in this substance tried to isolate it perfectly, and he obtained a cadmium orange pigment, m. p. 193° and gave it the molecular formula, $C_{15}H_{20}O_4$[53].

In the course of this study, the author was aware of a noticeable fact from the paper of SUZUKI *et al.*[50]. It has been written that NISHIKAWA isolated two kinds of pigments from mycelia of the fungus, named an orange yellow water-insoluble pigment "helicobasidin" and another purple, water-soluble pigment "mompain". However, the author has been unable to find any original article describing on these pigments. Further, he thought that his substance seemed closely similar to "helicobasidin" with its colour and in solubility in water. Then, he made a comparison between two substances. As a result of that, his substance decidedly seemed identical with "helicobasidin", therefore gave it a tentative name, helicobasidin, following NISHIKAWA's naming.

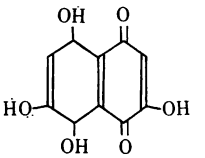
A year later, NISHIKAWA[29] reported the isolation of two colouring matters: helicobasidin, orange prisms m.p. 194°, $C_{16}H_{22}O_4$; purple needles, m.p.>300°, $C_{10}H_6O_6$ and of three colourless

substances: hemolic acid, m.p. 224°; licobasin, m.p. 136°; D-arabitol from mycelium of the fungus grown on malt decoction. Recently, NATORI *et al.*[27] have given helicobasidin the amended molecular formula $C_{15}H_{20}O_4$ which corresponds with the author's, and proved the structure of helicobasidin to be (S)-3-methyl-2,5-dihydroxy-6-(1,2,2-trimethyl cyclopentyl)-benzoquinone (I). From its structure, the possibility that helicobasidin may be concerned with the redox system in the fungus cells can be considered. As a matter of fact, OZAWA *et al.*[36] reported ubiquinone-like (coenzyme Q) activity in helicobasidin and suggested that it may play a certain important role in the electron transfer system.

Lately, NATORI *et al.*[28] have also determined the structure of mompain, finding it to be 2,5,7,8-tetrahydroxy-1,4-naphthoquinone (II).



(I)



(II)

In the course of the investigation the author thought that since it was isolated from the plant pathogenic fungus, helicobasidin must naturally have an effect upon organisms even though it might be toxic or non-toxic. According to MILLER's handbook[24], numberless products of fungi, as a matter of fact, are reported as toxicants or antibiotics. Further, with reference to the relationship between toxin from the plant pathogenic fungus and its host, general consideration has been given by BRAUN and PRINGLE[9], LUDWIG[23] and SUZUKI[51].

In the preliminary experiment, the author found that helicobasidin indicated toxic action against higher plants and microbes. Then, to approach the significance of helicobasidin in biology, extensive studies on the production of helicobasidin, the inhibition to plants and microbes and mechanisms of inhibitory action have been carried out.

In this paper the author deals with the results of the studies which have been finished up to now.

Here the author wishes to express his sincere thanks to Dr. Kazuo ITO, Director of the Forest Protection Division, Government Forest Experiment Station, for his helpful suggestions and instructive criticism. He also expresses his heartiest thanks to Professor Dr. Shigeyasu AKAI, Kyoto University, for useful suggestions and kindness in reading the manuscript. Further, he gratefully acknowledges the encouragement and advice of Mr. Rokuya IMAZEKI, formerly Director of the Forest Protection Division, Government Forest Experiment Station, throughout the progress of these studies. To Dr. Makoto MIYAZAKI and Mr. Moritami YASUE, Government Forest Experiment Station, who have given most useful advice during the course of isolation and have kindly prepared derivatives of helicobasidin, he wishes to express gratitude. Thanks are also due to Dr. Shinsaku NATORI, National Institute of Hygenic Sciences, for his useful suggestions and valuable cooperation in the giving of helicobasidin analogues. Grateful acknowledgement is made to Dr. Osamu CHIBA, Head of the Forest Disease Section, and to the members of the Laboratory of Forest Pathology, Government Forest Experiment Station, for their suggestions and help.

Experimental

Chapter I. Isolation of helicobasidin

At the suggestion of Dr. ITO, Government Forest Experiment Station, the author has repeated the isolation of metabolic products of the fungus and obtained crude products m.p. 176～187° form aqueous $NaHCO_3$ extract and m.p. 178～187° from aqueous Na_2CO_3 extract from the steam distillate of the culture, respectively (Fig. 1.). Both products were similar in colour, orange chrome, and showed no depression in the admixed melting point test. Further, the sublimed orange chrome needles, m.p. 189～190° on the glass wall of the culture flask also showed no depression in the same test with the above compounds. Thus, these three products were conclusively determined to be identical with each other. The author considered that the phenolic "mars orange amorphous bodies" on the culture noted by ITO[18] may be the same as the present compound.

This compound showed positive results for the phenolic group tests with $FeCl_3$, ARNOW's and MILLON's reagents, and also gave a positive result for the quinone group test with magnesium acetate. The empirical formula for the purified compound was revealed as $C_{15}H_{20}O_4$. Its ultraviolet absorption curve exhibited a maximum at λ 296 mμ (logε, 4.20) in ethanol and showed a shift by +27 mμ for λ_{max} in N/50 KOH alkaline solution (Fig. 2). Later, NATORI

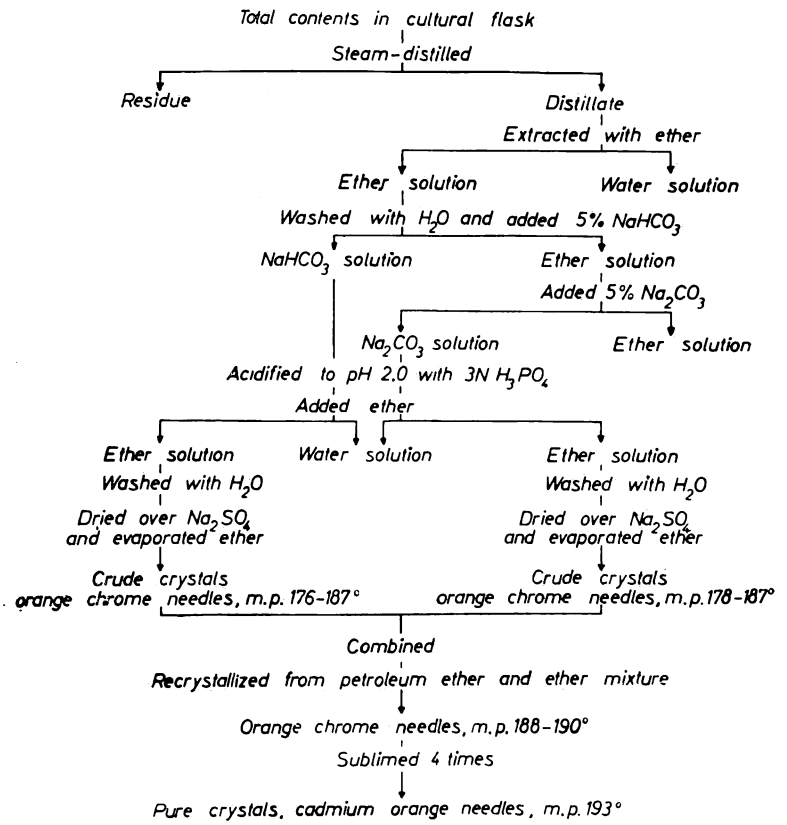


Fig. 1. The procedure of isolation of helicobasidin from *Helicobasidium mompa*.

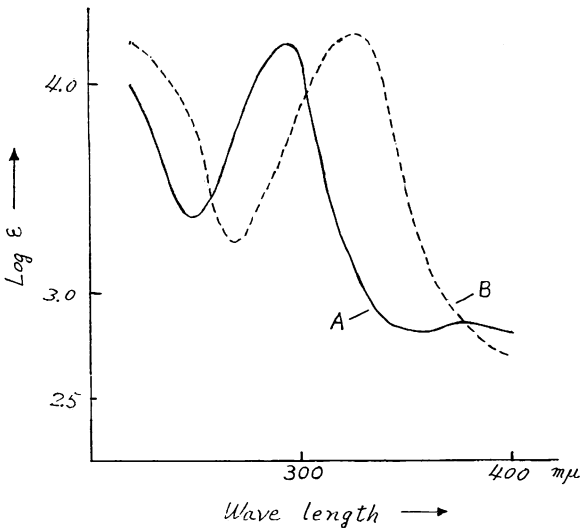


Fig. 2. Ultraviolet absorption spectra of helicobasidin.
A: in ethanol, B: in N/50 KOH alkaline ethanol.

et al.[27] added detailed data to the authors. According to these observations, this compound should possess a phenolic group in its molecule. This was supported by its infrared absorption spectra (Fig. 3) which showed the probable presence of phenolic OH (λ^{KBr}_{max} μ: 3.00, 7.71, $\lambda^{CCl_4}_{max}$ μ: 3.04, 7.36). These spectra also suggest the presence of conjugated C=O, characteristic for the quinone group (λ^{KBr}_{max} μ: 6.13, $\lambda^{CCl_4}_{max}$ μ: 6.08).

As the absorption of refined helicobasidin at λ 296 mμ was proportional to the range at least 3 to 11 μg per ml concentration, BEER's law was followed within this range (Fig. 4 A). Thus, it may suggest that spectrophotometrical determination for helicobasidin is possible.

Further, in order to examine the possibility of applying this method to the steam distillate, ultraviolet absorption spectra of the residue of ether extraction of the alkaline solution resulting from the steam distillate were taken (Fig. 5). It showed a lack of characteristic absorption for helicobasidin at λ 296 mμ. Thus, measurement of ultraviolet absorption of helicobasidin in the ether extract of the steam distillate at λ 296 mμ may be not really disturbed. The calibration curve for the ether extract of the steam distillate showed obedience to BEER's law within the range of the concentration giving absorbance as much as 0.400 to 0.800 at least (Fig. 4B). In view of the above facts it may be concluded that the amount of helicobasidin in the ether extract of the steam distillate resulting from the culture of the fungus can be quantitatively determined.

One-dimensional paper chromatography of helicobasidin was carried out (Table 1), but highly satisfactory results were not obtained. Several solvent systems for ascending technique, namely: benzene-acetone-water (5:5:3.5), Rf 0.96; petroleum ether saturated with methanol, Rf 0.87; and Xylene-ethanol-water (6:2:1), Rf 0.93 were available.

This compound seemed to be very similar to "helicobasidin" as mentioned above, especially, in its solubility in water and the original source. In order to directly compare this compound

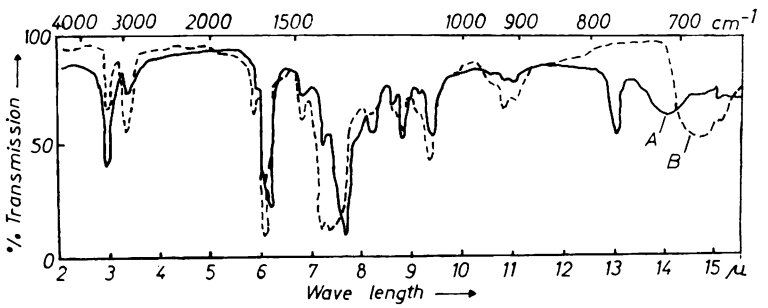


Fig. 3. Infrared absorption spectra of helicobasidin. A: KBr, B: CCl_4.

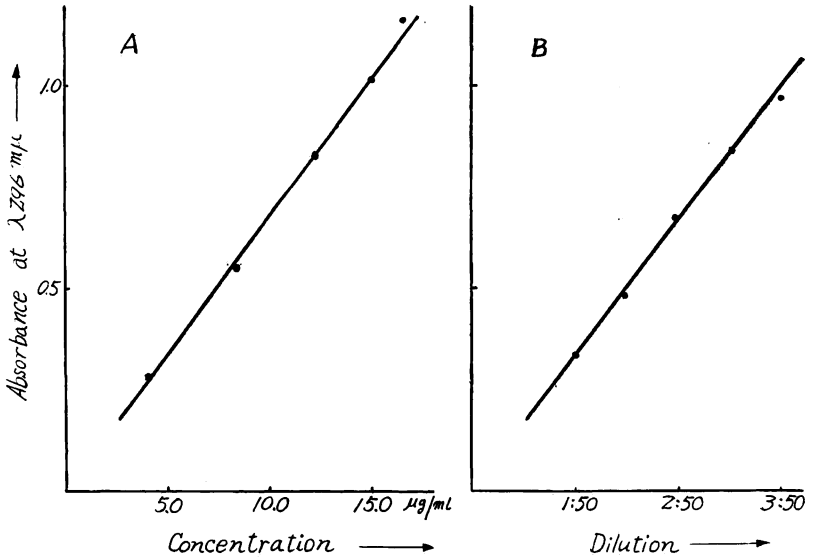


Fig. 4. Relationship between ultraviolet absorption and concentration of helicobasidin. A: refined helicobasidin, B: ether extract of the steam distillate resulting from the culture of the fungus.

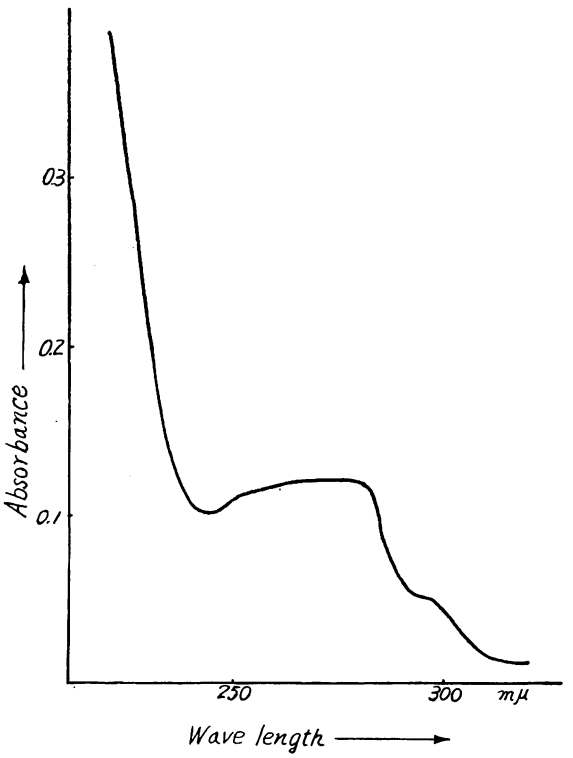


Fig. 5. Ultraviolet absorption spectra of the residue of ether extraction of the alkaline solution resulting from the steam distillate of the culture of the fungus.

with "helicobasidin", the author requested Dr. N. SUZUKI, formerly National Institute of Agricultural Sciences, for an authentic sample, and the author expresses his thanks to him for kindly cooperating. Determination of the admixed melting point giving no depression and observation of correspondence with λ_{max} 296 mμ in ultraviolet absorption spectra showed that the author's compound might be identical with "helicobasidin". This being so, the author decided to name this compound tentatively as helicobasidin following HISHIKAWA's naming.

Owing to the lack of detailed data on helicobasidin, the author thought that it would be appropriate to report some of its properties in this paper.

Most part of this chapter were published already[53].

Table 1. Rf values of helicobasidin with different solvents, by the ascending technique on Toyo-Roshi No. 50.

Solvent	Rf value	Developing reagent
Benzene·acetone·water (5:5:3.5)	0.96	1% $FeCl_3$: greyish brown spot
Buthanol sat. with 5 N NH_4OH	0.04–0.50	Sat. Mg $(OAc)_2$ ethanol solution: pinkish purple
Petroleum ether sat. with methanol	0.87	
Ligroin·ethanol·water (6:2:1)	0.93	
Xylene·ethanol·water (6:2:1)	0.93	

Experimental (The m.ps. given are uncorrected)

i) Organisms

Helicobasidium mompa TANAKA, strain Hm-7 (M-1), which was isolated by ITO[18] from the root of the sweet potato, was used in this experiment.

Preparation of the inoculum. For inoculation the culture was grown on a potato sucrose agar plate for 7 days. A small square cut from young colonies of the fungus was placed on the medium.

ii) Medium

Potato decoction containing two per cent sucrose was used. Vitamins consisting of 10 mg of thiamine, 3 mg of riboflavin, 3 mg of pyridoxine, 5 mg of calcium panthothenate and 10 μg of biotion were added to 1,000 ml of the basal medium. Two hundred ml of the medium in a 500 ml volume ERLENMYER's flask, adjusting pH to 6.0 with NaOH was autoclaved for 15 minutes at 10 lb pressure.

The culture was harvested after 90 days incubation at 25°C. The pH value of the culture medium shifted from 6.0 to 3.8 during incubation.

iii) Culture characteristics

On the production of the "mars orange amorphous bodies", ITO[18] observed that it was produced not only on culture media, but also on the mycelium, and on the glass wall, a little distant from the creeping hyphae of the fungus. In the present experiment the author obtained almost the same result on the metabolic production (Plate 1 B). On the basis of microscopic observation the author assumed that the substance could have originated on the mycelium of the fungus and, then, sublimed out from the mycelium (Plate 1 A). However, an attempt at its intracellular detection failed.

iv) Isolation of helicobasidin

The contents in the flasks were combined, distillated with steam and the resulting distillate was exhaustively extracted with ether. The chrome yellow ether extract was shaken with freshly prepared aqeous 5% $NaHCO_3$ and 5% Na_2CO_3 solutions, respectively. Colours of these alkaline extracts were pale purple and reddish purple, respectively. Both extracts were acidified with 3 N H_3PO_4 to pH 2.0 and extracted with ether. The upper layer was washed with water,

dried over Na_2SO_4 and evaporated, to afford orange chrome crude products from both alkaline extracts. The product from $NaHCO_3$ extract, m.p. 176～187°, and another from Na_2CO_3 extract, m.p. 178～187°, gave no depression in the melting point test (176～187°). Thus, both compounds were determined to be identical and combined.

Next, the orange chrome compound of the glass wall of the culture flasks was gathered with the spatula and sublimed. The resulting sublimate, orange chrome needles, m.p. 189–190°, showed no depression in the admixed melting point test with the above products (188～190°). Therefore, this was considered to be the same one as the alkaline products. Thus, these three compounds were conclusively proved to be identical with each other.

The yields of the crude compound were 140 mg from 10 flask cultures of the fungus containing 200 ml of sucrose-potato decoction.

v) Purification of helicobasidin

The crude compound from alkaline extracts was twice recrystallized from a mixed solvent of petroleum ether and ether, and the resulting compound was obtained as orange chrome needles m.p. 188～190° (Plate 2 A).

Further purification by successive sublimations four times gave fine cadmium orange needles, m.p. 193°.

vi) Determination of helicobasidin

The relationship between concentration and ultraviolet absorption of helicobasidin at λ_{max} 296 mμ was examined (Fig. 4 A). The refined helicobasidin ethanol solution, 16 μg per ml, was serially diluted with ethanol and each ultraviolet absorbance at λ 296 mμ was taken. Ultraviolet absorbance showed it to be proportional to the range of at least 3 to 11 μg per ml of helicobasidin.

Lack of a characteristic absorption of helicobasidin at λ 296 mμ for the residual fraction which resulted from ether extraction of the acidified alkaline solution showed the possibility of spectrophotometrical determination for helicobasidin in the steam distillate. Fifty ml of ether extract of steam distillate resulting from the whole culture of three flasks grown on KASAI's solution was made up. 0.2 ml of ether extract and 0.8 ml of ether were added into 4.0 ml of ethanol, and then serial dilution was made. Ultraviolet absorbance at least as much as 0.400 to 0.800 showed itself to be proportional to the corresponding dilution (Fig. 4 B).

Application of BEER's law is obviously possible for the correlation beween concentration and absorbance over the range of 3 to 11 μg per ml of either refined or unrefined helicobasidin.

vii) Paper chromatography of helicobasidin

Additional Rf values of helicobasidin for one-dimensional paper chromatography by the ascending technique were as follows: value, 0.96 for benzene·acetone·water (5 : 5 : 3.5), 0.04～0.50 for buthanol saturated with 5N NH_4OH, 0.87 for petroleum ether saturated with methanol, 0.93 for ligroin·ethanol·water (6 : 2 : 1). As colouring reagents, 1% $FeCl_3$ ethanol solution and saturated magnesium-acetate-ethanol solution were available and gave greyish brown and pinkish spots, respectively (Table 1).

viii) Comparison of this compound with "helicobasidin"

The direct comparison of "helicobasidin", m.p. 186–188°, kindly sent by SUZUKI, with this

compound, m.p. 193°, isolated by the author, was carried out through the admixed melting piont test and the ultraviolet absorption spectra. No significant depression of the melting point (187～190°) and no difference in ultraviolet spectra were observed. $\lambda_{max}^{Ethanol}$ 296 mμ and $\lambda_{min}^{Ethanol}$ 250 mμ corresponded well.

Based on the above tests, the author's sample is probably identical with that was recognized to be isolated by NISHIKAWA.

ix) General properties of helicobasidin

Helicobasidin: $C_{15}H_{20}O_4$ (Found: C, 68.03, 68.29: H, 7.37, 7.82: N, S, Cl, nil: M, 253 (Rast): requires C, 68.16: H, 7.63: O, 24.21: M, 264.3), cadmium orange needles, m.p. 193°, most readily soluble in ether and pyridine, readily soluble in chloroform, dioxane and methyl acetate, soluble in acetone, benzene, n-hexane, ligroine, petroleum ether, and petroleum benzene mixture, and insoluble in water. Solutions are intensely yellow to orange in colour with the exception of the pink pyridine solution. It dissolves in cold aqueous $NaHCO_3$ and Na_2CO_3 solutions giving pale reddish solutions respectively from which it is regenerated on acidification. Its ethanolic solution gives a reddish brown to purple brown with ethanolic $FeCl_3$ pale yellow with ARNOW's reagent, a yellow with MILLON's reagent, and a pinkish with magnesium acetate. But its colour reaction with diazotized reagent is indistinct owing to its sensitive colouration with Na_2CO_3. The ultraviolet absorption spectra of helicobasidin were as follows: $\lambda_{max}^{Ethanol}$ mμ (logε): 296 (4.20), 377 (2.87), $\lambda_{max}^{N/50KOH-ethanol}$ mμ (logε): 323 (4.25), showing the presence of phenolic OH (Fig. 2).

The infrared absorption spectra of sublimed helicobasidin were taken at the Research Laboratories, Sankyo Co., Ltd. through their kind cooperation: λ_{max}^{KBr} μ: 3.00, 7.71, $\lambda_{max}^{CCl_4}$ μ: 3.04, 7.36 (phenolic OH), λ_{max}^{KBr} μ: 6.13, $\lambda_{max}^{CCl_4}$ 4 μ: 6.08 (conjugated C=O) (Fig.3).

Chapter II. Factors affecting helicobasidin production

Ascertaining factors affecting production of a certain metabolite of the organism is very important for elucidating the problems of its physiological significance. JOHNSON and GOULD[19] reported the production of two pigments, auroglaucin and flavoglaucin by *Aspergillus glaucus* and they made clear that pigment production was markedly promoted by glycerol as a sole carbon source and further, that glycerol was effectively metabolied to these pigments through mannitol. SANDHU[40], and STOLL and RENZ[48], and STOLL *et al.*[49] presented that their works extensively dealt with the biogenesis of fusaric acid and dehydrofusaric acid by *Fusarium lycopersici* and *Gibberella fujikuroi*. On another main product of *F. oxysporum* f. *lycopersici*, lycomarasmine, DIMOND and WAGGONER[11] reported a fine relationship between mycelial growth of the fungus and lycomarasmine production. They also made clear that lycomarasmine was an autolytic product of mycelium of the fungus.

With reference to the C/N ratio of culture medium for pigmentation by the fungus, HAMADA[15] reported that *Armillaria mellea* depends on the N/C ratio of the medium containing peptone and glucose as a nitrogen and carbon source, respectively. SANWAL[41] also noted that *Fusarium lycopersici* required a C/N ratio 5, for the production of fusaric acid, and CARBONE and JOHNSON[10] reported that pigment production of *Aspergillus umbrosus* does not depend on an increase of nitrogen concentration.

ITO[18] experimented extensively for pigmentation in the mycelium and the culture medium of *Helicobasidium mompa*; however, it was not performed in connection with production of the

substance which was revealed to be helicobasidin by the author later, KASAI and ARAKI[20] created a better synthetic medium for culture investigation of *Helicobasidium mompa*. In spite of reviewing investigations associated with the fungus, the author has been unable to find any description about helicobasidin production. Therefore, wishing to give some data on this subject, the author reports the results of the experiments which were undertaken to determine the factors affecting helicobasidin production. The abstract of this work was presented previously[55].

1. Materials and methods

The strain Hm-7 (M-1) of *Helicobasidium mompa* TANAKA was used basically. Three kinds of plant tissue decoction containing two per cent sucrose were employed as natural media. The decoction was prepared at the rate of 200 g of sweet potato tubers and 500 g of carrot roots per 1,000 ml of water, respectively. WAKSMAN's solution as a semisynthetic medium, CZAPEK's solution, RICHARDS' solution and KASAI's solution[20] as synthetic media were used respectively. Mainly KASAI's solution was generally taken as a basal solution for most cultural tests. The initial pH of the medium was adjusted to 6.0 in all cases.

Vitamins consisting of 10 mg of thiamine, 3 mg of riboflavin, 3 mg of pyridoxine, 5 mg of calcium panthothenate and 10 μg of biotin were added to 1,000 ml of every basal medium. Fifty ml of medium per 200 ml volume of ERLENMYER's flask was taken basically except in the case of employing a bigger ERLENMYER's flask or a test tube. As a rule, sterilization of the medium followed autoclaving for 15 minutes at 10 lb pressure excepting that pasturization was employed for sterilization of a medium containing pectin.

For the inoculation, the culture was grown on a potato sucrose agar plate for 7 days at 25°C. A small square cut from the young colonies of the fungus was placed on the medium. Six flasks culture was prepared for every plot, as a rule. The culture of the fungus was submitted to stationary culture excepting that the culture was shaken by the reciprocal shaker set at 110 rpm in the test with nitrogen source.

At desired intervals during growth, six flasks were harvested. Three of these six flasks were used for measurement of mycelial growth of the fungus and helicobasidin production, respectively. Measurement of mycelial growth was made by weighing the dry mycelium of the fungus.

Determination of helicobasidin production was worked up spectrophotometrically. A steam distillate of the whole culture was extracted with ether. The chrome yellow-coloured ether solution was diluted with ethanol to contain 6~11 μg of helicobasidin per ml for measurement of ultraviolet absorption at $\lambda_{max}^{Ethanol}$ 296 mμ.

From the calibration curve (Fig. 4A) μg of helicobasidin per ml corresponding with the measured absorbance was obtained. The total amount of helicobasidin was calculated from the concentration of the diluted sample for measurement. For comparison with the ability of helicobasidin production, the total amount of helicobasidin which was produced by the fungus was essentially insufficient, because a favourable medium for mycelial growth of the fungus is not always favourable for helicobasidin production. In other words, production of mycelium might not be parallel to production of helicobasidin sometimes. Thus, for this course, a specific yield of helicobasidin could be more suitably defined as mg of helicobasidin produced per mg of dry weight of the fungus mycelium.

2. Results

i) Tests on the favourable medium for helicobasidin production

Comparison among three kinds of natural medium, a kind of semisynthetic medium, and three kinds of synthetic medium were used for these tests. Within natural and semisynthetic media, the highest yield of helicobasidin was obtained from potato decoction. Carrot decoction was also favourable, but WAKSMAN's medium was extremely unfavourable (Table 2). Among three synthetic media RICHARDS' was best and CZAPEK's was poor (Table 3). Concerning specific yield of helicobasidin, RICHARDS' medium was almost equivalent to potato decoction. KASAI's showed less value than RICHARDS', but it still may be considered favourable.

Table 2. Effect of natural and semisynthetic media on helicobasidin production.

Medium	pH	Helicobasidin (mg)
Carrot decoction	3. 9	9. 300
Potato decoction	5. 2	9. 867
Sweet potato decoction	3. 0	3. 533
WAKSMAN's solution	4. 5	0. 199
KASAI's solution*	7. 5	4. 725

Volume, 80 ml of medium per 300 ml volume of ERLENMYER's flask. Temperature, 25°C. Time, 77 days.
*: for a comparison.

Mycelial growth of the fungus on these media did not show a large difference. A noticeable feature is that RICHARDS' medium in which the C/N ratio was smallest, supported the highest production of helcobasidin, but CZAPEK's in which the C/N ratio was largest, resulted in the lowest production. The relationship between the C/N ratio and helicobasidin production will be examined and described in more detail below.

Table 3. Effect of synthetic media on helicobasidin production.

Medium	C/N ratio	pH	Mycelial growth (M) (mg/flask)	Helicobasidin production (H) (mg/flask)	Specific yield H/M (%)
CZAPEK's sol.	38. 3	3. 8	172. 9	0. 907	0. 53
RICHARDS' sol.	15. 0	3. 3	197. 6	3. 173	1. 62
KASAI's sol.	16. 7	4. 7	200. 9	1. 930	0. 97
Potato decoction*	undetermined	3. 3	197. 8	3. 597	1. 83

Volume, 50 ml of medium per 200 ml volume of ERLENMYER's flask. Temperature, 25°C. Time, 77 days.
*: for a comparison.

ii) The test on nitrogen source

KASAI's medium, the basic mineral medium used, was modified by substituting six nitrogen-containing compounds for L-asparagine. Compounds were so substituted that the equivalent weight of nitrogen per liter of each medium was constant. Four tested compounds, sodium aspartate, sodium glutamate, L-phenylalanine and sodium nitrate supported production of helicobasidin, while two other compounds, L-proline and L-tyrosine were not available (Table 4). Sodium nitrate, however, was poor for helicobasidin production. L-Phenylalanine showed a highly abundant yield of helicobasidin. It was visually evident that phenylalanine did not support sufficient mycelial growth of the fungus. Thus, it seems reasonable to assume that L-phenylalanine is not favourable for mycelial growth of the fungus, but makedly excellent for helicobasidin production.

Table 4. Effect of various nitrogen sources on helicobasidin production.

Nitrogen source	pH	Helicobasidin (mg)
Sodium aspartate	4.7	1.699
Sodium glutamate	4.9	1.129
L-Phenylalanine	4.5	5.517
L-Proline	5.0	0
L-Tyrosine	6.2	0
Sodium nitrate	4.9	0.633

Volume, 100 ml of medium per 500 ml volume of the shaking flask. Shake culture. Temperature, 28°C. Time, 60 days.

Chrome coloured steam distillates were obtained from the fungal culture given L-proline and L-tyrosine, respectively. But these distillates lacked characteristic absorption at λ 296 mμ for helicobasidin, indicating no evidence of helicobasidin production. It might be concluded that these compounds did not support production of helicobasidin; alternatively, they produced only a slight quantity of helicobasidin and/or the product was already destroyed.

iii) The tests on the addition of pectin

The above-mentioned data showed that carrot decoction originally containing plenty of pectic substance was a good substratum for helicobasidin production. YAMAZAKI[65] reported that the fungus has a specially strong pectinase system. In view of these points it might be considered that pection could have an effect on helicobasidin production. In order to determine whether pection could support a high production of helicobasidin or not, experiments were carried out.

Addition of one per cent citrus pectin resulted in an increase of both mycelial growth and helicobasidin production in all media used (Table 5). Modified KASAI's medium in which equivalent nitrogen of L-phenylalanine for L-asparagine was substituted, most highly increased the yield of helicobasidin by about 50 per cent. This increase is three to four times the specific yield of the other media. The qualitative difference between phenylalanine and asparagine resulted in a great difference in helicobasidin production. While potato decoction showed a high specific yield by itself, the additional effect of pectin was not remarkable.

iv) The test on the C/N ratio

From the results of tests obtained already, it is assumed that the C/N ratio, as well as the

Table 5. Effect of pection on helicobasidin production.

Medium	pH	Mycelial growth (M) (mg/flask)	Helicobasidin (H) (mg/flask)	Sp. yield (H/M, %)	% increase in H/M
Potato decoction	4.8	71.2	1.932	2.26	
————+ pectin	4.9	84.5	2.459	2.96	
Difference		13.3	0.527	0.33	11.15
KASAI's solution	5.4	45.5	0.947	2.04	
————+ pectin	6.5	74.6	1.576	2.41	
Difference		29.1	0.629	0.37	15.35
Modified KASAI's solution (N : L-phenylalanine)	4.2	29.9	0.378	1.24	
————+ pectin	3.0	55.0	1.448	2.46	
Difference		25.1	1.070	1.22	49.59

Volume, 15 ml of medium per 2.5 cm diam. test tube. Temperature, 25°C. Time, 106 days.

quality of nitrogen source, may play an important role in helicobasidin production. Accordingly tests were quantitatively conducted to determine the relationship between helicobasidin production and the C/N ratio of the medium.

Sucrose and L-asparagine were used as carbon and nitrogen sources, respectively. The carbon skelton of asparagine, however, was considered to be a part of the carbon source. While keeping one source at the standard level another source was varied. On these tests 20 g of sucrose per liter as a carbon source and 3 g of L-asparagine per liter as a nitrogen source were defined as the standard level. Mycelial growth and helicobasidin production were markedly influenced by the C/N ratio (Table 6, Fig. 6).

When the amount of sucrose was varied, growth was the greatest at the highest carbon concentration, and the rapid increase of growth was almost linear. A maximium specific yield of helicobasidin was obtained at the standard, the specific yield in either case was reduced.

Table 6. Effect of C/N ratio on helicobasidin production.

Sucrose (g/l)	L-Asparagine (g/l)	C/N ratio	pH	Mycelial growth (M) (mg/flask)	Helicobasidin production (H) (mg/flask)	Specific yield (H/M, %)
20	12	4.6	6.1	282.2	2.965	1.06
20	9	5.7	5.0	294.6	2.064	0.65
20	6	7.9	4.3	367.3	1.546	0.69
10	3	7.9	4.3	112.6	0.099	0.07
20	3	14.4	4.3	209.7	1.058	0.50
20	1.5	27.6	4.3	148.5	0.717	0.36
40	3	27.6	3.5	443.9	1.231	0.30
60	3	53.9	3.5	635.0	1.682	0.28

Medium, KASAI's solution. Volume, 50 ml of medium per 200 ml volume ERLENMYER's flask. Temperature, 25°C. Time, 77 days.

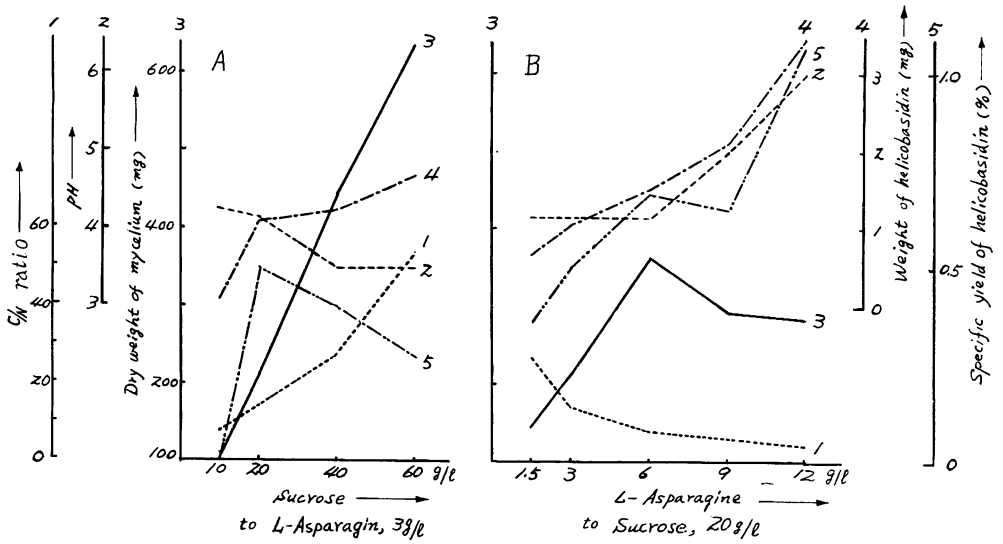


Fig. 6. Effect of varied quantity of carbon and nitrogen sources on mycelial growth of the fungus and helicobasidin production.

Moreover, the negative effect of varying the carbon source on helicobasidin production was greater at the lower carbon level (Fig. 6 A). Increasing the amount of asparagine ranging from 1.5 to 12.8 g per liter tended to increase specific yield of helicabasidin, while the growth decreased at the concentration more than 6 g per liter (Fig. 6 B).

The pH shift of the culture filtrate inclined towards dropping with increasing carbon concentration and contrarily inclined towards rising with increasing nitrogen concentration (Fig. 6, Curve 2).

As shown in Table 6, specific yield of helicobasidin differed widely even though the same C/N ratio was given. In these experiments two pairs of the same C/N ratio were taken. At the lower C/N ratio, the carbon level under the standard gave much less specific yield of helicobasidin than the standard, whereas at the higher C/N ratio, the carbon level above standard showed hardly any change in specific yield of helicobasidin.

v) The test on the phases of helicobasidin production

It seems that data on the phases of helicobasidin production give an approach to the physiological significance of helicobasidin to the fungus. Experiments were made to determine the correlation of the phases of helicobasidin production to that of mycelial growth of the fungus and the pH shift of culture filtrate. The production of helicobasidin began earlier than at least 65 days after inoculation (Table 7, Fig. 7).

Table 7. Relationship among pH of culture filtrate, mycelial growth of the fungus and helicobasidin production.

Days	pH	Mycelial growth (M) (mg/flask)	Helicobasidin production (H) (mg/flask)	Specific yield (H/M, %)
65	3.9	162.3	2.000	1.23
70	4.0	192.3	2.263	1.18
75	4.1	215.8	2.243	1.04
80	4.2	222.9	2.528	1.13
85	4.8	219.8	3.500	1.59
90	4.9	215.1	3.625	1.69
95	4.7	246.1	4.097	1.66
100	5.2	240.0	2.778	1.03
105	5.2	200.4	4.000	2.00
110	5.3	221.5	1.050	0.68
115	5.0	219.3	3.197	1.46
120	5.2	218.3	3.525	1.61
125	5.9	228.3	2.261	0.99
130	6.0	222.3	2.545	1.14
135	6.2	222.9	0.795	0.35
140	6.4	243.8	1.550	0.64
145	—	—	—	—
150	6.5	231.7	1.581	0.68
155	6.7	209.9	1.896	0.90

Medium, KASAI's solution. Vloume, 50 ml of medium per 250 ml volume ERLENMYER's flask. Temperature, 25°C.

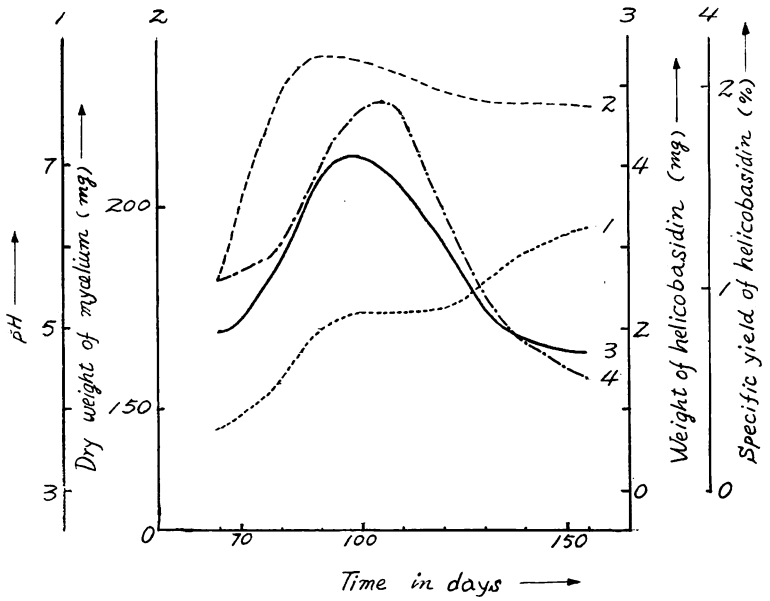


Fig. 7. Estimated relationship among pH of culture filtrate, mycelial growth of the fungus and helicobasidin production.

The highest yield of both helicobasidin and growth of the fungus was shown for 95 days culture (Fig. 7, Curve 2, 3). However, the peak of specific yield of heilcobasidin was reached after approximately 105 days (Fig. 7, Curve 4). Decrease of mycelial weight after passing the peak was so mild that autolysis was not apparent within 155 days, while rapid decrease in specific yield of helicobasidin was remarkable. This means that helicobasidin began to disappear. The pH shift of the culture filtrate which ranged from approximately 4 to 7 was continuous (Fig. 7, Curve 1). Generally, it seemed that the pH shift has three conspicuous phases which consist of a rapid rise during approximately linear growth to attain the maximum, an almost negligible one from the time when the peak growth appeared to the time when helicobasidin began to markedly decrease, and a rise during the successive decline of helicobasidin at the last.

vi) The tests on various strains of the fungus

These tests were undertaken to see if helicobasidin could be a common product in all strains of *H. mompa* and *H. purpureum* (TUL.) PAT. which closely resembles *H. mompa*. Eight strains of *H. mompa* and a strain of *H. purpureum* were tested (Table 8).

All tested strains of *H. mompa* produced helicobasidin, but the strain of *H. purpureum* did not produced it at all. The ability to produce helicobasidin among the tested strains extended over a wide range. However, the strain Hm-7 showed the highest ability of all the strains tested.

3. Discussion

The data obtained in the course of these nutritional investigations indicate that all the tested media supported helicobasidin production. They are almost the same as the results on the production of "mars orange amorphous bodies" reported by ITO[18].

L-Phenylalanine was a clearly excellent nitrogen source for helicobasidin production. Besides, addition of pectin to phenylalanine considerably increased the production of helicobasidin. On

Table 8. Comparison of the ability to produce helicobasidin among various strains of *Helicobasidium mompa*.

Strains	pH	Dry wt. of mycelium (M) (mg)	Helicobasidin (H) (mg)	H/M (%)	Origin
Hm-11 (1)	3.7	370.7	0.949	0.27	Root of mulberry
Hm- 5 (2)	4.4	454.8	0.814	0.17	Root of sweet potato
Hm- 7 (3)	4.1	221.9	7.712	3.21	do.
Hm-11 (2)	3.4	319.2	2.567	0.80	Root of asparagus
Hm-12 (3)	4.7	328.9	1.149	0.43	Root of *Quercus glauca*
Hm-13 (2)	5.8	287.5	0.808	0.27	Root of apple tree
Hm-14 (3)	5.6	214.5	7.074	3.19	Root of *Pinus strobus*
Hm-15 (3)	3.5	265.9	2.296	0.98	Root of *Cryptomeria japonica*
H. purpureum (4)	5.6	678.9	0	0	unknown

Medium, potato decoction. Volume, 50 ml of medium per 200 ml volume ERLENMYER's flask. Temperature, 25°C. Time, 77 days.

(1) Sericultural Experiment Station (Tokyo). The strain was kindly given by Dr. K. AOKI.

(2) National Institute of Agricultural Sciences (Tokyo). These strains were kindly given by Dr. N. SUZUKI.

(3) Government Forest Experiment Station (Tokyo).

(4) Botany School, University of Cambridge (Cambridge). The strain was kindly given by Professor Dr. S. D. GARRETT.

the contrary, L-proline and L-tyrosine showed negative effect. Orginally, such aromatic amino acids as phenylalanine and tyrosine are not likely to serve as a carbon source. Even tyrosine which often acts toxicically on fungi was more or less utilized as a sole nitrogen source by the fungus. Proline, however, is one of the amino acids which are readily ulitized by fungi, in general. There must be three possibilities about helicobasidin production concerned with these two ineffective amino acids, proline and tyrosine. In the first place it was not produced at all: secondly, so little was produced it may not be detected: thirdly, it was slightly produced but thoroughly decomposed for unknown reasons. Examining the final pH value of the culture filtrate in these cases, 5.0 for that of proline and 6.2 for that of tyrosine, seems unlikely that helicobasidin suffered from decided autodecomposition, because the pH value of these lies at the acidic side more stable than at the alkaline side.

PACKTER and GLOVER[37] found that phenylalanine and tyrosine added to the culture medium produced a slight increase in the ubiquinone content in mycelia of *Aspergillus fumigatus* above the control level. These slight increases in the ubiquinone production, however, are very significant because other amino acids such as leucine and methionine have the capacity to cause the production to be doubled. According to OLSON *et al.*[32][33] the only aromatic substance so far examined and found to be partially successful in substitution required for the benzoquinone nucleus of ubiquinone 9 to enter the ubiquinone biosynthetic scheme in rat liver, were phenylalanine and tyrosine, the latter being slightly better than the former.

Because of the very close similarity of helicobasidin to ubiquinone, these observations are taken into consideration in the present case. Whether phenylalanine acted as the only nutritional source or as a precursor of helicobasidin is indefinite at this stage; nevertheless, in accordance with the view of the above mentioned workers it might be reasonable to presume that phenyl-

alanine possibly behaved as a precursor of benzoquinone nucleus of helicobasidin. Negative effect of tyrosine on helicobasidin production seems to be discordant with these views. The conclusive elucidation for this fact, however, should be made after perfomance of further necessary experiments in this respect.

As for the precursor of helicobasidin, on the contrary, NATORI *et al.*[27] pointed out regarding to the biogenesis of the side-chain of helicobasidin, that the origin of the trimethylcyclopentyl side-chain is apparently mevalonic acid. However, the author has not yet experimented in respect to this view.

Additional effects of pectin would give some suggestions. When pectin was added to asparagine, its effect was clearly apparent, whereas when it was added to phenylalanine its effect was extremely remarkable. It might be considered that phenylalanine is not utilized enough unless some supplementary factors such as pectin are given. Its addition to potato decoction was not particulary effective. It may mean that potato decoction could be almost perfect for both growth and helicobsidin production.

ITO[18] has examined the effects of the C/N ratio on hyphal growth and pigmentation of the fungus. However, there was no description about formation of " mars orange amorphous bodies " with reference to the C/N ratio. The C/N ratio apparently affected helicobasidin production. The carbon source may play a role in maintenance of mycelial growth of the fungus, but may not so greatly contribute to helicobasidin production on a wide range. Further, the nitrogen source may more greatly contribute to helicobasidin production rather than mycelial growth. Then it seems reasonable to conclude that within the limit of deficiency and toxic excess, an increase of the C/N ratio induces an increase of mycelial growth of the fungus, but induces an increase of helicobasidin production and a pH rise.

ITO[18] and ARAKI *et al.*[4] respectively reported that pH depression of the culture filtrate is attributed to production of dicarboxilic acids, namely oxalic acid and a great amount of itaconic acid at the early stage of culture. It may suggest that a lower pH of the filtrate would indicate more active growth of the fungus on the normal medium. As a matter of fact, data which were obtained in these tests concerning the phases of helicobasidin production, showed a continuous rise within the lower pH range during the active growth before reaching the peak. Consequently a resulting lower pH at the greater C/N ratio may indicate that there must be still continued active growth. A resulting higher pH at the C/N ratio, however, may indicate that mycelial growth has either reached or passed the maximum. In other words, at that time, mycelium may be still unmatured at the greater C/N ratio and already matured at the smaller C/N ratio.

Essential carbon concentration for helicobasidin production must be considered. Two per cent of sucrose was best (Fig. 6 A, Curve 5). Even at the same C/N ratio, two per cent sucrose supported good development of helicobasidin, while one per cent sucrose did not. On the basis of these facts, it is reasonable to assume that a two per cent may be the minimum carbon concentration necessary to keep normal growth which supports higher helicobasidin production. Data obtained from tests on the phases of helicobasidin production were accompanied by some variance. The reason for inconsistent values at every measurement is found in the method by which mycelial growth and helicobasidin production were measured with separated flasks. Due to helicobasidin tending to sublimate, it was impossible to avoid sublimation of helicobasidin during the course of desiccation of mycelium. Thus it was necessary to measure the growth of the fungus and helicobasidin production with separated flasks. Consequently, variance among

flasks measured made absolutely accurate results impossible. Because some errors cannot be entirely eliminated, evaluation of the results had to be made somewhat roughly. On the basis of the experimental data, summarized relationship among pH of the filtrate, mycelial growth and helicobasidin production is given in Fig. 7.

Data concerned with the phases of helicobasidin production showed a difference between mycelial growth of the fungus and the specific yield of helicobasidin. The maximum of specific yield of helicobasidin appeared behind the maximum of mycelial growth.

The pH shift of the filtrate seems to be closely related to growth and helicobasidin production. A continuous rise appeared during active growth and another continuous rise also appeared during marked destruction of helicobasidin. Moreover, a negligible shift of pH around the maximum specific yield of helicobasidin production laid between two rising phases of pH. In regard to the developing stage of the mycelium it seems reasonable to conclude that mycelium attained maturity at this stage. However, helicobasidin production was not initiated at the mature stage, but was initiated at the early stage. It may be concluded from the results of these nutritional investigations that helicobasidin is not an autolytic product and maturation of mycelium is closely related to its production. We face the question of why already produced helicobasidin is destroyed in spite of negligible autolysis of mycelium. The answer is not yet available, but in addition to the change of the extracellular condition, the intracellular condition should be regarded as important. Originally, phenolic compound is unstable under alkaline condition. So it might be assumed that as the pH of the filtrate approaches the alkaline side, destruction of helicobasidin would gradually proceed.

All strains of *H. mompa* tested produced helicobasidin. It is likely though, that helicobasidin is a common product of *H. mompa*. However, the tested strain of *H. purpureum* did not produce it. It would be very interesting to know if helicobasidin is produced by other strains of *H. purpureum*.

Chapter III. Toxic effect of helicobasidin on higher plants

With regard to the presence of a toxic principle in the staled culture medium of *H. mompa*, ITO[18] obtained positive data with 15 species of higher plants. YAMAZAKI[65] confirmed markedly strong pectolytic action in the fungus. It can be also considered as a kind of toxic agent to plants.

Due to its insolubility in water, it may be thought that helicobasidin could not be easily concerned with the toxic action as revealed by ITO (l.c). Still, some kinds of its toxic effect on plants would be naturally presumed because it is a product of a plant pathogen which widely attacks a large number of higher plants, both woody and herbaceous. Furthermore, its chemical structure would emphasize this presumption. According to NATORI *et al.*[27] it is a kind of benzoquinone, and as to the benzoquinone structure, expectation of its bioactivity, whether toxic or non-toxic, would naturally occur.

The present investigation was undertaken to discover the toxic effect of helicobasidin on higher plants. The outline of these results has been presented in the previous papers[53][54].

1. Materials and methods

Six kinds of higher plants, camphor (*Cinnamomum camphora* SIEB.), mulberry (*Morus bombycis* KOIDZ.), false indigo (*Amorpha fructicosa* L.), soy bean (*Glycine max* MERRILL),

tomato (*Lycopersicon esculentum* MILL.) and broadbean (*Vicia faba* L.) were used. The treating methods for assay were as follows: absorption of the test solution for cuttings of plants and its leaf treatment for all tested plants (except broadbean) and radicle treatment for broadbean. Cuttings of tested plants were suspended in short test tubes containing the test solution and absorption proceeded under a bell jar. Excised leaves were placed in moistened PETRI dishes and contacted with the test solution. A small mass of absorbent cotton immersed in the tested solution was placed on either a wounded or a non-wounded area on the leaf. The wound on the leaf was made by cutting with a surgeon's knife. These experiments were carried out under room temperature in August to September. Broadbean radicles grown to a length 5～10 mm were tied round with absorbent cotton to which a drop of the test solution was given. Then, broadbean seedlings were incubated on the filter paper which was put in the PETRI dish and was wetted with two per cent aqueous ethanol, at 25°C. Due to its insolubility in water, helicobasidin was tested in the form of sodium salt. Adding aqueous NaOH solution to helicobasidin-2% ethanol solution, a neutral test solution was made up.

Evaluation of the toxic effect was made by comparison with the intensity of wilting, necrosis of foliage and the broadbean radicle.

2. Results

Toxicity of helicobasidin to higher plants is apparantly shown (Table 9 and Plate 2 B-D, 3). Remarkable wilting action especially on both tomato foliages and cuttings, and camphor cuttings was observed. For example, tomato foliages and cuttings suffered from wilting and necrosis during 20 hours and completely collapsed in 48 hours when they were treated with the concentration of helicobasidin more than 1/8,000 (5.2×10^{-4}): and further, vein necrosis and chlorosis appeared with the concentration more than 1/24,000 of helicobasidin solution during 48 hours. Microscopic observation of the section of the affected stem gave no sign of browning in its vascular bundles though. False indigo cuttings were apparently somewhat affected also, while mulberry cuttings were less affected.

Table 9. Toxicity of helicobasidin to higher plants.

Symptom	Plants	Wound	1/2,000	1/4,000	1/6,000	1/8,000	1/12,000	1/16,000	1/24,000	1/32,000	1/64,000	0
Wilting of cuttings	Camphor		##	##	##	##						—
	Mulberry		#	#		#						—
	False indigo			##	#	#		#		—		—
	Soybean			##	#	#		#		—		—
	Tomato[a)]		##	##	##	##	#	#	+	+		—
Necrosis on leaves or radicles	Camphor*	+	#	+	+	+						—
		—	—	—	—	—						—
	Mulberry*	+	##	#		+						—
		—	+	—		—						—
	Tomato*	+	##	##	#	#	+	+	—			—
		—	—	—	—	—	—	—	—			—
	Broadbean**		##	##	##	##	##	##	##	##	#	—

##: severe wilting or necrosis, #: light wilting or necrosis,
+: partial wilting or necrosis, —: no reaction.
*: excised leaf, **: radicle of seedling.
a): represents the data for both foliages and cuttings.

On the other hand, broadbean radicles directly produced clear necrosis at the area treated with helicobasidin up to 1/64,000 in concentration during 24 hours (Plate 2 D). Observation under the microscope of the section of a radicle was made and its photograph is given in Plate 1 D, E. A conspicuous browning layer in cortex was observed (the arrow in Plate 2 B). Such a browning layer was never found in the untreated radicle. Decidedly, it was a symptom of the results of helicobasidin treatment. The affection probably might extend from outside to the browning layer. Further microscopic observation has not been carried out as yet.

3. Discussion

The tomato foliages and cutting and broadbean radicle were most severely affected among the plants assayed. The camphor cutting, although it is a woody plant, wilted heavily. However, the direct treatment with leaves did not produce such apparent symptom as the cuttings. Besides, it showed that helicobasidin could not produce symptoms on leaves unless taken through vascular bundles or wounds. On the contrary, the broadbean radicle showed a high sensitivity presenting a brown ring in the cortex.

There have been a great number of papers on wilt toxins produced by plant pathogens. Good reviews of this subject with a complete bibliography were given by GÄUMANN[13], BRAUN, and PRINGLE[9], LUDWIG[23] and SUZUKI[51], therefore it is deemed unnecessary to include such a review in this paper. Helicobasidin possesses dihydroxy benzoqinone structure. So far as the author has ascertained, there have been no articles about toxicity of benzoquinone naturally occurring in the vegetable kingdom, especially in fungi, to higher plants.

ALLEN[3] noticed the fact that there are many kinds of quinones or unsaturated laktone among antibiotics produced by fungi, and they commonly have α, β-unsaturated ketone as a functional group. He assumed that the common respiratory increase in diseased plant tissues may be caused by such compounds which inhibit SH enzyme groups.

DIMOND and WAGGONER[12] have given a decided definition to a vivotoxin. They have defined it as a substance produced in the infected host by the pathogen and/or its host, and which functions in the production of disease but is not itself the initial inciting agent of disease. The author thinks that it may be still too early to give a conclusion about helicobasidin as to whether it is a vivotoxin or not, because an examination of its presence in diseased tissues and its role of disease incidence has not yet been made. To add certain data to this problem of the significance of helicobasidin in the host-parasite relationship may be essential at the present stage.

Chapter IV. Antibiotic action of helicobasidin

There are a number of benzoquinone produced by microbes. However, when we look for compounds with a similar structure as helicobasidin among them, fumigatin, spinulosin, phoenosin, oosporein and iso-oosporein can be found[24]. These commonly possess both methyl and hydroxyl radicales at the position 2, 6 or 3, 5. In view of its similar function, ubiquinone could be cited. As a matter of fact, OZAWA *et al.*[36] have confirmed that helicobasidin has a remarkable ubiquinone-like function. Further, among these examples, fumigatin and spinulosin were proved to have noticeable antibiotic properties[34][35].

In the preliminary experiment the author noticed that helicobasidin inhibited sporulation of *Aspergillus niger* and growth of several bacteria. So, it was thought that helicobasidin may be

a kind of antibiotic. One of the main purposes of this work was to give the spectrum of antibiotic activity of helicobasidin *in vitro*. WHIFFEN[62] has given a widely extended antibiotic spectrum of cycloheximide to fungi. It seems to be advisable to assay as extensively as possible. Thus, in these tests, organisms were selected as widely and systematically as possible for assay. The outline of this work has been presented previously[58].

The author wishes to express his heartfelt thanks to Professor Dr. Hiroshi IIZUKA and Assistant Professor Dr. Sakuzo FUKUI, Institute of Applied Microbiology, Tokyo University, Dr. Hiroshi KURATA, National Institute of Hygenic Sciences, Dr. Yoshiro OKAMI, formerly National Institute of Health, Dr. Tokito TOMINAGA, National Institute of Agricultural Sciences, and Dr. Koichi YAMAMOTO, Institute of Fermantation, for their useful suggestion on assaying microbes and their kind donation of culture.

1. Materials and methods

To determine the critical concentration completely inhibiting the growth of microbes and the degree of inhibition mainly against fungal sporulation, a two-fold agar streak dilution method and a paper disk plate method were employed, respectively.

i) The agar streak dilution method: Weighed crystallin helicobasidin was primarily dissolved with dimethylformamide finally to give two per cent concentration of the solvent. And, then, in order to give solubility in water a few drops of N NaOH aqueous solution were added into the basal solution until its pH reached 7.0. Finally, a definite volume of water with 0.01 per cent Tween 20 was added, then the mother solution was made up. The mother solution was diluted two-fold with the same concentration of the solvent as it. Three ml of each test solution and 12 ml of molt agar medium adjusted pH to 7.0 were thoroughly mixed in the PETRI dish. The water used was all deionized. Glucose nutrient agar for some bacteria and potato sucrose agar for other bacteria and for fungi, were employed as test media. As inoculum, spore or mycelial fragment suspension was used. Spores were obtained from the agar culture incubated for 7～14 days and a mycelial fragment was made of a liquid shake culture for 2～5 days. A loopful of the suspension was spotted on the area of the plate blocked at the outside of the bottom of the PETRI dish by a glass pencil. The concentration at which there was complete inhibition of growth was recorded after some intervals of incubation at 30°C for most bacteria and at 25°C for other bacteria and fungi.

ii) The paper disk plate method: The method of DE BEER and SHEERWOOD[5] was used to assay the ability to inhibit fungal sporulation. A paper disk, 8 mm in diameter and 1.2 mm in thickness, was employed. The helicobasidin suspension to be tested was prepared in the following manner. Weighed crystallin helicobasidin was primarily dissolved with dimethylformamide to give two per cent final concentration of solvent. Then, 0.01 per cent Tween 20 deionized aqueous solution with 1/4 part of MCILVAIN's buffer solution (pH 4.0) was added into the basal solution to give the final concentration of helicobasidin, 132 μg per ml. Helicobasidin which was dissolved in dimethylformamide suspended in the solvent as fine particles by addition of water. The mother liquid was two-fold serially diluted to 16 μg per ml. A seeded PETRI dish with paper-disk procedure with helicobasidin was incubated at 25°C except *Sclerotinia mali*. The culture of *S. mali* was incubated at 17°C, being irradiated by the fluorescent lamp, 15 w in power, 30 cm distant. This procedure was essential for its conidial formation. Potato sucrose agar medium was basically employed as a seeding medium. However, a specific medium for sporulation of

two different fungi was needed. HARA and ITO's medium[16] for *Guignardia laricina* and MISATO's agar medium[25] for *Piricularia oryzae* were used. After some periods of incubation the diameter of the produced zone of inhibition was measured. When the zone was not visually apparent the affect of the treatment on sporulation was determined microscopically.

2. Results

The assays applied by the modified agar streak method and the paper disk method were carried out on 23 species of bacteria and 78 species of fungi.

i) The test on bacteria

Among tested bacteria, 5 sensitive species, 5 comparatively sensitive species, respectively to helicobasidin, are shown (Table 10). The tested species in Enterobacteriaceae were considerably resistant to helicobasidin except *Erwinia milletiae* which gave somewhat lower resistance. Tested species in four families other than Enterobacteriaceae in the order Eubacteriales showed more or less sensitivity. The tested two species belonging to the genus *Mycobacterium* in the order Actinomycetes also seemed to be rather sensitive.

Table 10. Effect of helicobasidin on growth of bacteria (by the agar streak dilution method).

Organism	Gram staining	Medium	Temp. (°C)	μg/ml completely inhibiting growth 15	40	60 hr
Class Schizomycetes						
Order Pseudomonadales						
Family Pseudomonaceae						
Pseudomonas aeruginosa	—	GN	30	>832		
Ps. caronafaciens	—	GN	25		63	63
Ps. maculicola	—	GN	25		>500	
Ps. tabaci	—	GN	25		125	125
Xanthomonas citri	—	P	25		500	500
X. oryzae	—	P	25			63 (96 hr)
X. phaseoli	—	P	25	8	63	63
Order Eubacteriales						
Family Rhizobiaceae						
Agrobacterium tumefaciens	—	GN	25		125	125
Family Enterobacteriaceae						
Eschericia coli NIHJ	—	GN	30	832	>832	
Es. coli PO 1495	—	GN	30	>832		
Erwinia aroideae	—	GN	25		>500	
Er. carotovora	—	GN	25		>500	
Er. milletiae	—	P	25	125	250	500
Salmonella entridis	—	GN	30	832		
Shigella dystenteriae	—	GN	25		500	500
Family Micrococcuceae						
Micrococcus flavus	±	GN	25		125	125
Staphylococcus aureus	+	GN	30	104	104	104
Sarcina lutea	+	GN	25		63	125

Organism	Gram staining	Medium	Temp. (℃)	μg/ml completely inhibiting growth 15	40	60 hr
Family Corynebacteriaceae						
Corynebacterium sepedonicum	+	P	25			32 (105 hr)
Family Bacillaceae						
Bacillus cereus	+	GN	25		63	63
B. subtilis	+	GN	25		250	250
Order Actinomycetales						
Family Mycobacteriaceae						
Mycobacterium phelei	+	GN	25		125	250
M. sp. 607	+	GN	30	104	104	104

GN: glucose nutrient agar, P: potato decoction agar

With reference to the Gram staining character of the tested bacteria, almost all of Gram positive species were more or less sensitive, in general; on the other hand, Gram negative species did not exhibit any definite tendency, that is, although species representing the family Enterobacteriaceae showed resistance, *Agrobacterium* in the same order was less sensitive. Also two different genus in the order Pseudomonales included three resistant species *Pseudomonas aeruginosa*, *Ps. maculicola* and *Xanthomonas citri*, three sensitive species *Ps. caronafaciens*, *X. oryzae* and *X. phaseoli*, and lower sensitive species *Ps. tabaci*.

ii) Tests on fungi

The assay by the modified agar streak dilution method was applied 78 species and that by the paper disk plate method was applied to 35 of the 78 species. Eight species, sensitive to helicobasidin and 4 species rather stimulated fructification appear in Table 11, 12 and Plate 4. *Endothia parasitica*, *Septotinia populiperda*, *Ascochyta pisi*, *Haprosporella* sp., *Stagonospora cryptomeriae*, *Cercospora cryptomeriae*, *Helminthosporium sativum* and *Isariopsis* sp. reacted sensitively. On the contrary, zygospore formation for *Cunninghamella blakesleeana*, conidial formation for *Curvularia lunata* and *Botrytis* sp. were stimulated, respectively. There was no taxonomically related tendency in reaction to helicobasidin except for *Aspergilli*. Inhibition to sporulation was not obvious either, except for some species of *Aspergillus*.

However, stimulation of perithecial formation of *Neurospora* sp. at a higher concentration was remarkable. It visually appeared as a brown zone around the paper disk (Plate 4 A). Confirmation of ascospore formation failed. Zygospores of *Cunninghamella blakesleeana* were abundantly produced around the paper disk (Plate 4 B). It obviously showed a stimulation effect. *Botrytis* sp. was stimulated in its conidial formation on the medium with 63～125 μg per ml concentration of helicobasidin. The conidia of *Curvularia lunata* were abundantly produced around the paper disk although they did not appear macroscopically as a contrasting zone. Inhibition to conidial formation was visually apparent in *Trichoderma viride* (Plate 4 C).

iii) Tests on *Aspergillus*

Twenty-two species of *Aspergillus* were assayed. Inhibition of helicobasidin to growth was extremely weak, in general. However, inhibition to sporulation was evident in some species. Tested 6 species representing *A. niger* group were all inhibited to sporulation (Table 13, Plate 5).

Table 11. Effect of helicobasidin on fungi—I.

Organism	Inoculum	μg/ml completely inhibiting growth (by the agar streak dilution method) 24	48	72 hr	Response in the assay by the paper disk plate method
Class Phycomycetes					
Order Mucorales					
Cunninghamella blakesleeana	S	500	500	500	—
Mucor javanicus	S	500	500	500	—
Rhizopus formosaensis	S	500			—
R. nigricans	S	500	500	500	—
Class Ascomycetes					
Order Saccharomycetales					
Saccharomyces cerevisae	S	208 (15 hr)	416 (24 hr)		—
Order Sphaeriales					
Cochliobolus miyabeanus	S		63	500	—
Mycosphaerella sojae	M			125	
Ceratocystis fimbriata	S			125	—
Thielaviopsis basicola	S		63	125	—
Neurospora sp.	S	250		500	+
Guignardia laricina	S			500	—
Endothia parasitica	S			32	—
Diaporthe conorum	M		32	250	
Order Pezizales					
Sclerotinia mali	S				—
Sclerotinia sp.	M			125	—
Septotinia populiperda	S		63	63	
Order Hypocreales					
Neocosmospora vasinfecta	S	63	500	500	—
Clss Basidiomycetes					
Order Polyporaceae					
Laetiporus sulphureus	S		500		—
Pycnoporus coccineus	S		63	125	
Fungi Imperfecti					
Order Sphaeropsidales					
Ascochyta pisi	S		32	63	
Diplodia pinea	M	500			
Haprosporella sp.	S		16	32	
Phomopsis sp.	S			125	—
Stagonospora cryptomeriae	S		16 (6 days)	16 (7 days)	
Chaetomella sp.	S			125	
Order Melanconiales					
Colletotrichum gloeosporioides			125	250	+
Pestalotia sp. PeC-9	S		125	500	+
P. sp. PeB-3	S				—
P. sp. 4290	S				—

Table 11. Effect of helicobasidin on fungi—continued.

Organism	Inoculum	μg/ml completely inhibiting growth (by the agar streak dilution method) 24	48	72 hr	Response in the assay by the paper disk plate method
Order Moniliales					
Candida albicans	S	832			—
C. tropicalis	S	832			
Arthrobotrys sp.	M		125	250	
Aspergilli (Table 13)					
Botrytis sp.	S	250	500	500	
Penicillium chrysogenum	S		500		+
Piricularia oryzae	S		125	250	—
P. zingiberi			125	250	+
Trichoderma viride	S	500 (15 hr)			+
Trichothecium roseum	S		250	500	—
Trichophyton radians	M	208		208	
Alternaria longipes			32	250	—
Cercospora cruenta	S		500		
C. cryptomeriae	S	32 (4 days)		63 (7 days)	
C. kikuchii	M			500	
Cladosporium colocasiae	S			125	—
Corynespora cassiicola	M			125	
Curvularia lunata	S		250	500	+
Helminthosporium sativum	S		63	63	—
Heterosporium phelei	S			125	+
Stemphylium sarcinaehorme	S			125	—
Torula rubra	S	416 (15 hr)			—
Isariopsis sp.	S			63	—
Fusarium oxysporum	S		500	500	
F. solani	S		500	500	—
F. sp.	S		500	500	
Order Mycelia Sterilia					
Sclerotium bataticola	M	250	500		

S: spore, M: mycelial fragment.

On the contrary, 16 species belonging groups other than *A. niger* group were not influenced to sporulation without exception. It was a clear contrast. Effect of helicobasidin on development in the conidial formation of *A. niger* was traced under the microscope (Table 14, Plate 6). Growth of conidiophore in *A. niger* was gradually arrested as the concentration of helicobasidin increased. However, the results of spaced observations suggested that no concentration of helicobasidin keeps conidiophore development at a particular stage, and after some retardation it will bear conidia.

Table 12. Effect of helicobasidin on fungi—II.
(by the paper disk agar method)—from the last column of Table 11.

Organism	Type of response	Effect	Helicobasidin (μg/ml)	Diameter of reacted zone (mm)
Cunninghamella blakesleeana	Zygospore formation	S	132, 66, 33, 16	22. 5, 30, 30, 23
Neurospora sp.	Perithecial formation	S	1056, 528, 264, 132	20. 5, 18. 2, 16. 2. 11. 7
Phomopsis sp.	Growth	I	1056, 528, 264, 132	21. 1, 18. 5, 17. 1, 14. 9
Colletotrichum gloeosporioides	Conidial formation	I	132, 66, 33, 16	slight
Pestalotia sp. Pe C-9	Pycnidial formation	I	do.	11. 8, 9. 4, +, —
Piricularia zingiberi	Growth	I	do.	16. 4, 11. 9, —, —
Trichoderma viride	Conidial formation	I	do.	12. 3, —, —, —
Curvularia lunata	do.	S	do.	slight
Heterosporium phelei	Growth	I	do.	15. 1, 13. 1, 9. 0, —
Botrytis sp.	Conidial formanion	S	125, 63	by the agar streak dilution method

I: inhibition, S: stimulation, +: slight response, —: no response.

Table 13. Comparison with the inhibition of helicobasidin to sporulation among different groups of the genus *Aspergillus*.

Organism	Group no.	μg/ml completely inhibiting growth* (24 hr)	Diameter of inhibition zone of sporulation** (60 hr) (mm)			
			132	66	33	16 (μg/ml)
A. oryzae	1	> 500	—	—	—	—
A. clavatus	2	> 500	—	—	—	—
A. giganteus	2	> 500	—	—	—	—
*A. amstelodami***	3	> 500	—	—	—	—
*A. chevalieri***	3	250	—	—	—	—
A. fumigatus	4	> 500	—	—	—	—
A. nidulans	5	> 500	—	—	—	—
A. versicolor	6	> 500	—	—	—	—
A. cinamomeus	7	> 500	—	—	—	—
A. terreus	7	> 500	—	—	—	—
A. ustus	8	> 500	—	—	—	—
A. flavipes	9	> 500	—	—	—	—
A. candidus	10	> 500	—	—	—	—
A. ochlaceus	11	> 500	—	—	—	—
A. tamarii	12	> 500	—	—	—	—
A. awamori	13	> 500	18. 2	16. 4	13. 0	12. 2
A. batatae	13	> 500	22. 5	20. 6	17. 9	15. 8
A. carbonarius	13	> 500	21. 2	17. 6	15. 8	10. 8
A. japonicus	13	> 500	14. 7	13. 7	12. 0	8. 8
A. niger	13	> 500	22. 1	22. 0	19. 7	16. 5
A. usamii	13	> 500	14. 8	12. 9	11. 4	11. 8

Organism	Group no.	μg/ml completely inhibiting growth* (24 hr)	Diameter of inhibition zone of sporulation** (60 hr) (mm)			
			132	66	33	16 (μg/ml)
A. wentii	14	63	—	—	—	—
		500 (48 hr)				

1: *A. flavus-oryzae* group　8: *A. ustus* group
2: *A. clavatus* group　9: *A. flavipes* group
3: *A. glaucus* group　10: *A. candidus* group
4: *A. fumigatus* group　11: *A. ochlaceus* group
5: *A. nidulans* group　12: *A. tamarii* group
6: *A. versicolor* group　13: *A. niger* group
7: *A. terreus* group　14: *A. wentii* group
(after grouping of THOM and RAPER[60])
*: by the agar streak dilution method.
**: by the paper disk plate method.
***: on the medium with 20% sugar.

Table 14. Effect of the concentration of helicobasidin on development of the conidial apparatus of *A. niger*.

μg/ml	Development of the conidial apparatus
500	Foot cell partially bearing initial conidiophore.
250	Partially developing conidiophore.
125	Developing conidiophore.
63	Developing of vesicle by swelling of the terminal portion of the conidiophore, partially, young primary sterigmata.
32	Early developing of primary sterigmata.
16	Later stage in developing of primary sterigmata.
0	Developing of matured conidia.

Time, 5 days. Temperature, 25°C.

3. Discussion

The antibiotic spectra of helicobasidin with 23 species of bacteria and 78 species of fungi have been given. Bacteria used were selected within a set for practical screening test of antibiotics and within six genera of phytopathogenic bacteria. Fungi assayed are extended to orders from Phycomycetes to Basidiomycetes and Fungi Imperfecti. The spectrum showed that helicobasidin was not so effective at first on either bacteria or fungi in the means of growth inhibition. Secondly, it was highly selective, with the effect on sporulation of fungi or with activity against growth of microbes. The effects of helicobasidin on microbes were mainly observed on inhibition to growth and fructification, and stimulation to fructification. The critical concentration on each effect differed with each organism, and inhibition to growth was more apparent in bacteria than fungi, in general.

However, among bacteria tested there have been noticed some species that are highly resistant to the toxic effect of helicobasidin. The resistant species are members belonging to the families Enterobacteriaceae and Pseudomonaceae. The species of the order Eubacteriales, except the organisms belonging to the family Enterobacteriaceae, were rather sensitive to helicobasidin.

This suggests that there have been some tendencies in the response to helicobasidin from the taxonomical point of view.

Gram positive bacteria, generally speaking, seem much more sensitive to helicobasidin than Gram negative ones. This character resulted in making a clear taxonomical contrast among the families in the order Eubacteriales: that is, all tested Gram negative bacteria which represent the family Enterobacteriaceae showed the resistance to helicobasidin, even though there was an exception that a Gram negative species which represents the family Rhizobaceae responded rather sensitively. On the contrary, all tested Gram positive species which represent another order Actinomycetales, not to mention the order Eubacteriales, were more or less sensitive. With bacteria in the order Pseudomonales, however, a variable tendency was observed in spite of their same Gram negative property. Some of them were resistant and the others were sensitive in various degrees. It may be a clear tendency, however, the author feels he must refrain from giving any final conclusion on this subject, because samples for the test were insufficient for submitting to the discussion.

On the other hand, fungi are more selective to helicobasidin. No definable tendency has been found in the spectra excepting that the inhibition to sporulation of the genus *Aspergillus* is restricted to the *A. niger* group. The effect upon fructification of fungi may be a characteristic property of helicobasidin. In addition to inhibition, helicobasidin showed some stimulating effects on fructification of fungi: on the perithecial formation of *Neurospora* sp., the zygospore formation of *Cunninghamella blakesleeana*, and the conidial formation of *Curvularia lunata* and *Botrytis* sp. It appears to be evident that these fungi are tolerable to inhibition. However, it is noteworthy that *Neurospora* sp. promoted its perithecial formation even at as a high concentration as 1,056 μg per ml. LEE[22] reported that perithecial formation of *Neurospora crassa* which is highly infertile was greatly increased by the presence of kinetin, a kind of growth regulator. If a further investigation with helicobasidin is made, it might be possible to apply it as a stimulator for this type of fungi.

BEHAL and EAKIN[6] reported that 6-hydroxy-2-mercaptopurine prevents the development of the conidiophore, 6-ethylthiopurine inhibits the formation of spores at the top of sterigmata, and 6-hydroxy-2-mercapto-8-azapurine also inhibits pigmentation of spores of *Aspergillus niger*. And then they concluded that the specific chemical inhibits the specific stage of sporulation including conidiophore development at the specific time of treatment, as the result of testing 20 analogues of purine and pyrimidine at various times of incubation. It must be noted that the action of helicobasidin to *A. niger* is very similar in the point of inhibition to spore formation but not inhibition to growth. It is not yet decidedly confirmed whether helicobasidin inhibits a particular stage of development of the organism or not, as has been mentioned. However, microscopic observation found that helicobasidin gradually inhibits the developing process according to the concentration. On the other hand, the fact that inhibition of sporulation is specific in the *A. niger* group bearing black conidia is certainly interesting. It seems that the metabolic differences with sporulation, between the *A. niger* group and other groups in the genus *Aspergillus* produce differences in response to helicobasidin.

According to STEINBERG[46),47] *A. niger* essentially requires Fe, Zn, Cu and Mn for normal sporulation. Moreover, Cu, especially, is a limiting factor for giving black colour to conidia. As cited above, the findings reported by BEHAL and EAKIN that 6-hydroxy-2-mercapto-8-azapurine causes production of colourless conidia of *A. niger* inhibiting pigmentation is also

very interesting in this respect. In fact, grouping species in *Aspergillus* depending upon conidial colour distinguishes the *A. niger* group from others by the characteristic colour, black. Helicobasidin highly selectively inhibited sporulation of *A. niger* group fungi. This suggests that its inhibition is strongly related to a certain particular metabolism bound to sporulation of the *A. niger* group. So far, helicobasidin has been considered as a typical chelator as will be mentioned in the following chapter. Thus, in view of this fact, it may be assumed that inhibition is caused by an inactivation of essential metals for organisms by helicobasidin. Standing at this assumption, metabolic differences between the fungi in *A. niger* group and the fungi in other groups in *Aspergillus* should be regarded as important. A strong requirement for metallic ions in *A. neger* as asserted by STEINBERG holds a certain inference in this problem. Further, some comparative studies concerned with metallic requirement for sporulation between *A. niger* group fungi and the fungi in other groups in *Aspergillus* may provide an approach to problems on the mechanism of inhibition to sporulation by helicobasidin. Reversely, it is to say that helicobasidin which induces different responses to different fungi may be thought to be a useful tool for comparative studies on a metabolic difference among fungi, especially, between the fungi producing black conidia and other fungi in *Aspergillus* such as combination in this experiment.

Thus, from these characteristics it may be considered that one of the possible modes of inhibition of sporulation is a chelation of essential metals by helicobasidin. Otherwise, the point that helicobasidin has one of the ubiquinone-like function must be considered. If the presence of some ubiquinone in the fungi of *A. niger* group might be assumed, a possibility of antimetabolic function in helicobasidin would be naturally considered. The fact that helicobasidin showed not only highly selective inhibition, but conversely also much selective stimulation, might have some bearing on this presumption, because a highly selective effect is a feature of antimetabolic action.

Chapter V. A possible mode of toxic action of helicobasidin

There are numerous toxins isolated from plant pathogens. As already cited GÄUMANN[13], BRAUN and PRINGLE[9], LUDWIG[23], and SUZUKI[51] presented reviews of wilt toxins produced by plant pathogens and explained mechanisms of their toxic action. HORSFALL[17], in addition, gave an excellent review in which lots of suggestions on the mechanisms of toxic action of the fungal metabolites against fungi were presented.

Most of the toxic metabolites of plant pathogens have an ability to chelate and the chelation is associated with their toxicity. This view, however, is not always able to explain an ultimate mode of toxic action. It is often pointed out that chelation may play only an intermediate role in poisoning. The mode of toxic action of oxine (8-hydroxyquinoline), a typical chelator, against microbes has been widely studied. RUBBO *et al.*[39] and ALBERT *et al.*[2] demonstrated the startling fact that oxine owes its toxic effect on bacteria to metal chelation alone, and if a medium is depleted of Fe and Cu, oxine is no longer inhibitory. With respect to phytotoxin a similar fact was shown by GÄUMANN[13] at the same time. Lycomarasmine, a wilt toxin against tomato plants, is toxic only as the complex with Fe, so its toxicity should decrease when Cu ions which are more affinitive to it are introduced into a system with Fe and a limited amount of lycomarasmine[21].

The fact that the Cu, Ni, Cd and Ag salts of oxine, which are saturated with respect to metal, however, have a high fungistatic activity according to SEXTON[44] who indicated that chelation

was not the basis for the toxicity but that phenolic properties of oxine caused it. ALBART[1] proposed that oxine removes "guardian metals" such as Co, which protects cell thiol groups, and then, Fe or Cu of the chelate promotes oxidation of the essential thiol. TOYODA[61] presented the facts that Cu-oxine gave a much stronger inhibition of spore germination, mycelial-, homogenate-, and mitochondrial respiration of *Alternaria kikuchiana* than either $CuSO_4$ or oxine, and the presence of oxine together with $CuSO_4$ stimulated the penetration of Cu ions into the mycelial cell of the fungus. And then, supporting the view of ALBERT *et al.*[2] he concluded that oxine plays the role of a vehicle which transfers Cu ion from outside into inside of intact cells through cell membrane, therefore, metal poisoning is the ultimate mode of toxic action.

On the other hand, many examples to indicate reduction of toxicity owing to chelation can be shown. OKU and NAKANISHI[31] demonstrated, for example, that ascochytine, a metabolic product of *Ascochyta fabae*, produced necrotic spots on coleoptiles of broadbean and its toxicity is antagonized by ferric Fe. SCHLÖSSER[42], and SCHLÖSSER and STEGEMANN[43] also showed a similar fact that a yellow-coloured-Cercospora-substance (GF) in which a tropolon nucleus was assumed, reduced its antibacterial activity due to chelation.

As already mentioned, BEHAL[8], and BEHAL and EAKIN[6][7] fully reported that some purine and pyrimidine analogues inhibit the sporulation, but not the growth of *Aspergillus niger*. Furthermore, they demonstrated an apparent dependence of spore formation on functioning of the citric acid cycle and the methionine metabolism. NISHIMURA[30] reported that phytonivein, a wilt toxin produced by *Fusarium niveum*, inhibits some metal enzymes of water melon. The concept of antimetabolism was proposed by WOOD[63]. He discovered that sulfanilamide is an "antimetabolite" for p-aminobenzoic acid. This discovery was made by reversing the toxicity of sulfanilamide with p-aminobenzoic acid. Later, WOOLLEY[64] recongnized that antimicrobial action of dichlone was reversed by addition of vitamine K which is associated with the electron transport system.

In the course of the isolation of helicobasidin the author presumed the possibility of chelation from its U.V. and I.R. absorption spectra showing the presence of phenolic OH and adjacent conjugated C=O. Then, tests on toxic action of helicobasidin on higher plants were made. From the results of the tests the author assumed that helicobasidin obviously forms chelates with metallic ions. Due to its unknown structure, however, a conclusive decision could not be given. The abstract of the preliminary experiments on this problem were presented by the author[56] in 1962. Two years later, NATORI *et al.*[27] proposed its structure as (S)-3-methyl-2, 5-dihydroxy-6-(1, 2, 2-trimethylcyclopentyl)-benzoquinone for helicobasidin. The author knew that his assumption was not wrong, and then studies were extended to the structual function of helicobasidin.

This work was undertaken to determine the possible mode of toxic action of helicobasidin to both higher plants and microbes. The outline of the work was preliminarily presented[57].

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1. Materials and methods

Tests were made with tomato cuttings, broadbean radicles, and *Aspergillus niger*. The helicobasidin preparation employed was made in the same manner described in Chapter III and IV. The aqueous solution of sodium salt of helicobasidin for the assay by tomato cuttings and the suspension of its fine particles for the assay by broadbean radicles and *A. niger* were used,

respectively. Sources of various metallic ion solutions are given below: $CoSO_4 \cdot 7H_2O$ for Co^{++}, $CuSO_4 \cdot 5H_2O$ for Cu^{++}, $FeSO_4 \cdot 7H_2O$ for Fe^{++}, $Fe_2(SO_4)_3 \cdot XH_2O$ for Fe^{+++}, $MgSO_4 \cdot 7H_2O$ for Mg^{++} $MnSO_4 \cdot 4H_2O$ for Mn^{++} and $ZnSO_4 \cdot 7H_2O$ for Zn^{++}.

The final concentration of chemicals was $5/2 \times 10^{-4}$M for the tomato cutting assay and $5/4 \times 10^{-4}$ for the assay by broadbean radicle and *A. niger*, respectively, as a rule. The helicobasidin and metal complex was prepared by the equimolar mixing, basically. The chelators added to helicobasidin and metal complex were ethylendiaminetetraacetic acid disodium salt (EDTA) and 8-hydroxyquinoline (oxine), and they were submitted to reaction with helicobasidin and metal complex at equimol. L-Cysteine solution was added to helicobasidin at the equimolar concentration. For control of pH of the test liquid McIlvaine's buffer solution was employed. To confirm the release of helicobasidin from its metal complex the spectrophotometrical technique was applied in the same fashion as described in Chapter II.

The assay for the biotoxicity was carried out by three methods: The assay on tomato cuttings described in Chapter III was based on evaluation of change in toxic symptoms, namely, wilting of the foliage and collapse of the stem. The tomato cutting was obtained from a young seedling bearing 2 or 3 leaves besides cotyledons. The assay on broadbean radicles also as described in Chapter III was based on evaluation of change in intensity of necrosis. The microbial assay on *A. niger* was made by the paper disk plate method mentioned in Chapter IV. And to evaluate the effect of treatment the diameter of the inhibiting zone for sporulation was measured. Spores obtained from the agar slant culture of the fungus incubated for 7 days at 25°C were used as inoculum in the paper disk plate method. Spores were seeded in potato sucrose agar, at pH 5.6, in the rate of one slant of the culture per 100 ml of the medium.

2. Results

A remarkable difference in toxicity was produced in each experiment, especially with higher plants. In fact, the same procedure often yielded different intensities of toxic symptoms. However, the correlation between intensities in different treatments was almost unchanging. Then, to avoid mistaken evaluation, more than three repetitions were made. The preliminary experiment showed that the concentration of metallic ion solutions employed were not harmful to either plants or *A. niger*. In evaluation of the experiments, a change in the response was noticed rather than intensity of the response.

i) Effects of metallic ions on toxicity of helicobasidin

A) Tests on tomato cuttings

As mentioned above, toxic symptoms in the tomato cutting produced by helicobasidin were mainly wilting and necrosis in foliage and collapse of the stem. A light symptom was chlorosis at the leaf margins. In the tests, wilting and collapse were marked because they produced a clear difference by every treatment. Addition of metallic ions to helicobasidin induced some changes in comparison with initial toxicity of helicobasidin (Table 15).

With the wilting action of helicobasidin addition of Co^{++} and Mg^{++} caused marked reduction, and that of Fe^{++}, Fe^{+++} and Zn^{++} caused apparent reduction, respectively. In contrast to these facts, addition of Cu^{++} caused a marked increase in the wilting action. In other words, Co^{++}, Fe^{++}, Fe^{+++}, Mn^{++} and Zn^{++} acted more or less inhibitorily aagingt helicobasidin and Cu^{++} acted sinergistically. On the other hand, with collapse of the stem, addition of metallic ions increased the toxic action without exception.

To see if the chelation between helicobasidin and metal principally causes change of toxicity,

Table 15. Change in toxicity to tomato cuttings induced by addition of metallic ions to helicobasidin.

Plot	Wilting Intensity (Example)			Frequency R	N	I	Result	Collapse of stem Intensity (Example)			Result
H+H_2O	++ ++	++ +++	+++ +++					— —	— —		
H+Co^{++}	+ +	± ±	— —	4	1	0	RR	+ ++	— —		N
H+Cu^{++}	++ +++	+++ +++	+++ +++	0	0	6	II	+++ +++	++ +++		II
H+Fe^{++}	± ±	— —	— —	3	0	1	R	+++ +++	+++ +++		II
H+Fe^{+++}	— ±	— —	— —	3	0	1	R	+++ +++	+++ +++		II
H+Mg^{++}	+++ +++	+ +	++ ++	3	0	2	N	++ +++	— +++		II
H+Mn^{++}	— —	— —	— ±	5	0	0	RR	+++ +++	++ +++		II
H+Zn^{++}	± +	++ ++	— ±	2	2	0	N～R	++ ++			I
H+Cys.	+ ++	+ ++	±	2	1	0	R	— —	++ ++		I

H: helicobasidin, Cys.: L-cysteine, I, II: relative increase of toxicity, N: no change, R, RR: relative reduction of toxicity. Concentration, $5/2\times10^{-4}$M with every component.

two kinds of known chelators were added to the helicobasidin-metallic ion solution. These chelators, EDTA and oxine brought about successive changes (Table 16). As a result of the addition of EDTA to helicobasidin-metal complex (it was assumed that the formation of helicobasidin-metal complex was due to the colour change of solution) a reduction of toxicity was shown with helicobasidin-Co, ferrous Fe and Mn complexes. The addition of oxine modified the toxicity in a manner different from that of EDTA in some cases. For example, the initial toxicity of helicobasidin and ferrous Fe complex was increased by the presence of EDTA, but it was markedly reduced by the presence of oxine. Although the change of toxicity of helicobasidin-Zn complex was negligible by addition of EDTA, it was considerably increased by the addition of oxine. With collapse of the tomato stem, a contrasting effect on changes of toxicity was produced between addition of EDTA and that of oxine.

B) Tests on broadbean radicles

Table 16. Change in toxicity to tomato cuttings induced by addition of chelators to helicobasidin and metal complex.

Plot	Addition of EDTA W	C	Addition of oxine W	C
H+Co^{++}	I	I	I	I
H+Cu^{++}	R	R	R	N
H+Fe^{++}	I	N	RR	N
H+Fe^{+++}	I	I	I	N
H+Mg^{++}	N	R	II	R
H+Mn^{++}	I	R	N	N
H+Zn^{++}	N	I	II	R
H+H_2O	N	N	II	N
H_2O	N	N	N	N

W: wilting, C: collapse. Concentration, $5/2\times10^{-4}$M with every component

To determine the relationship between the pH of helicobasidin preparation and its toxicity, the assay was made using broadbean radicles. It was evident that toxicity was not affected by a pH value at acidic side but it might be reduced at alkaline side to some degree (Table 17).

Additional effect of metallic ions on its toxicity of helicobasidin is shown in Table 18. Co^{++} and Fe^{++} caused remarkable reduction of toxicity. Cu^{++} and Fe^{+++} also apparently reduced toxicity, Zn^{++} did not affect it and L-cysteine did not either. There was no ion associated with an increase of toxicity. The addition of two kinds of chelators to helicobasidin metal complex showed interesting responses (Table 19). EDTA and oxine recovered the reduced toxicity of helicobasidin-Cu complex up to the initial toxicity of free helicobasidin. With reference to additional changes in the toxicity of helicobasidin-Mg complex, EDTA did not affect any change but oxine caused a marked reduction. The toxicity of helicobasidin-ferrous Fe and helicobasidin-Zn complexes was additionally increased by EDTA, but was reduced by oxine. The toxicity of helicobasidin-Co complex was never changed by addition of either chelator. With addition of a chelator to free helicobasidin solution, EDTA gave no change in toxicity and oxine gave a slight reduction in toxicity. However, neither chelators showed any visible toxicity by themselves.

From these data it seems to be reasonable to assume that helicobasidin may form chelation with various metals, and the principle of toxic action may not be its metal complex but free helicobasidin. To further prove the hypothesis, ultraviolet absorption technique was applied. All the mixtures of helicobasidin and six kinds of metallic ion solutions indicated a shift for λ_{max} of helicobasidin (Table 20). Further, addition of EDTA to helicobasidin-Cu chelate which gave a shift for the absorption at λ_{max} 296 mμ for helicobasidin to λ_{max} 397 mμ caused apparent recovery of the absorption at λ_{max} 296 mμ and showed release of helicobasidin from its Cu

Table 17. Effect of pH on toxicity of helicobasidin to broadbean radicles.

Plot	pH 2	3	4	5	6	7	8
Helicobasidin	＋＋＋	＋＋＋	＋＋＋	＋＋＋	＋＋＋	＋＋＋	＋＋～＋＋＋

Concentration, $5/4\times10^{-4}$M. Buffered by McIlvaine's solution.

Table 18. Change in toxicity to broadbean radicles induced by addition of metallic ions to helicobasidin.

Plot	Toxicity (Example)									Frequency R	N	I	Result
H+H_2O	＋＋＋	＋＋＋	＋＋＋	＋＋	＋＋＋	＋＋＋	＋＋	＋＋＋	＋＋＋				
H+Co^{++}	±	±	＋	−	＋	＋	−	＋	＋	8	0	0	RRR
H+Cu^{++}	±	＋＋	＋＋	＋	＋	＋＋	±	±	＋＋	6	1	1	RR
H+Fe^{++}	＋	＋＋	＋＋	±	＋	＋	±	＋	＋	7	1	0	RRR
H+Fe^{+++}	＋	＋＋	＋＋	−	−	±	−	＋	＋＋	5	2	1	RR
H+Mg^{++}	＋＋＋	＋＋＋	＋＋＋	＋＋＋	＋＋＋	＋＋＋	＋＋＋	＋＋＋	＋＋＋	1	4	3	N～I
H+Mn^{++}	±	＋	＋＋	＋	＋＋	＋＋	＋＋	＋＋	＋＋	3	5	0	R
H+Zn^{++}	＋＋	＋＋＋	＋＋＋	＋＋	＋＋	＋＋＋	＋＋＋	＋＋＋	＋＋＋	4	3	1	R～N
H+Cys.	−	＋＋	＋＋＋	＋＋	＋＋	＋＋＋	＋＋＋	＋＋＋	＋＋＋	3	3	2	N

Concentration, $5/4\times10^{-4}$M with every component.

Table 19. Change in toxicity to broadbean radicles induced by addition of chelators to helicobasidin and metal complex.

Plot	Addition of EDTA		Addition of oxine	
	Change	Result	Change	Result
H+Co^{++}	± ± ±	N	± ± +	N
H+Cu^{++}	++ ++ ++	II	+ ++ ++	II
H+Fe^{++}	+ + ++	I	− − ±	R
H+Fe^{+++}	− − ++	N	± ± +	N
H+Mg^{++}	± ± ±	N	= = =	RR
H+Mn^{++}	± ± ±	N	− ± ±	N
H+Zn^{++}	+ ++ ++	I	= − ±	R
H+H_2O	− − ++	N	− − −	R
H_2O	± ± ±	N	± ± ±	N

= : Remarkable reduction. Concentration, $5/2\times10^{-4}$M with every component

Table 20. Shift for λ_{max}, of helicobasidin after mixing with metallic ion solutions.

Complex	λ_{max}^{EtOH**} mμ (log ε)		
Helicobasidin	296 (4.20),	377 (2.87)	
Helicobasidin-Co	316 (2.80)		
Helicobasidin-Cu	397 (2.97)		
Helicobasidin-Fe*	309 (2.80),	405 (3.04)	
Helicobasidin-Mg	302 (2.82),	373 (2.67),	502 (1.72)
Helicobasidin-Mn	296 (2.69),	370 (2.64)	
Helicobasidin-Zn	301 (2.74),	360 (2.70),	502 (1.91)

*: ferric Fe, **: 60% ethanol
pH; 6.0 (The spectrum of the complex with ferrous Fe could not be obtained because of its insolubility in the solvent).

chelate. The reason why oxine was omitted at the spectrophotometrical work for chelation was that oxine has an apparent absorption at λ_{max} 309 mμ and it therefore influences determination for helicobasidin at λ_{max} 296 mμ in practice. Besides colour reaction with various metallic solutions, the results of the spectrophotometrical examination may strongly support the possibility of chelation between helicobasidin and metals.

C) Tests on *Aspergillus niger*

The effect of pH of helicobasidin preparation on inhibition of sporulation on agar media has been examined. Inhibition of helicobasidin preparation was not much dependent on a pH on the acidic side, but was reduced at the neutral or the alkaline side (Table 21 and Plate 7 A-B). This tendency was almost entirely the same as in the case of broadbean radicles. The maximum inhibition appeared at pH 4.0.

For determination of effect of metallic ion on helicobasidin inhibition to fungi, an assay was made (Table 22). Preparation of free helicobasidin, helicobasidin-metal complex added were assayed. Helicobasidin-metal complex seemed to increase inhibition on potato sucrose agar.

Table 21. Effect of pH on inhibition of sporulation by helicobasidin to *A. niger*

pH	2	3	4	5	6	7	8
Diameter of inhibition zone (mm)	22.1	22.2	23.3	22.3	22.0	20.1	17.8

Concentration, $5/4\times10^{-4}$M.

Table 22. Additional effect of metallic ions to helicobasidin on inhibition of sporulation to *A. niger*.

Plot	Diameter of inhibition zone (mm)							
	Ca^{++}	Co^{++}	Cu^{++}	Fe^{++}	Fe^{+++}	Mg^{++}	Mn^{++}	Zn^{++}
H	25.0	21.3	24.4	20.3	26.2	23.3	27.9	24.9
H+M	25.7	23.1	25.7	21.4	28.1	23.3	28.6	28.3
H+M+E	25.5	19.7	25.0	21.0	29.3	24.2	29.6	26.0
H+M+O	25.2	25.2	25.4	19.7	29.8	23.6	25.7	26.6
M	—	—	—	—	—	—	—	—

E: EDTA, O: oxine, M: metallic ion, Concentration, $5/4\times10^{-4}$M with every component.

Additional effect of the chelator on inhibition was not very evident. Potato decoction originally contains various kinds of chelators. Therefore, the environmental factors around the fungus on the medium may be so complicated that the analysis of the result would be very difficult. To ascertain the possible form of helicobasidin present in potato sucrose solution, ultraviolet absorption determination was applied. It revealed that potato sucrose solution evidently but partially released helicobasidin from its metal complex. Further, the same medium where conidia of the fungus germed also released helicobasidin from the complex (Fig. 9).

If chelation between quinone group and hydroxyl group is performed at two places in the

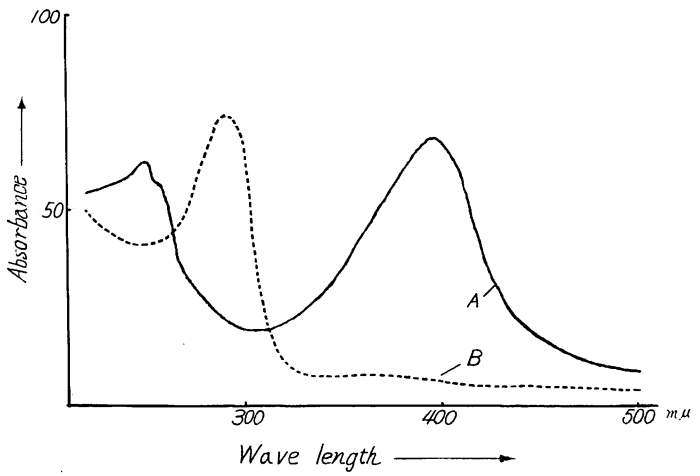


Fig. 8. Shift of ultraviolet absorption spectra of helicobasidin-Cu complex induced by addition of EDTA.
A: helicobasidin-Cu complex. B: addition of EDTA to helicobasidin-Cu complex.
Concentration: helicobasidin and $CuSO_4$; 10^{-4}M, EDTA; 1.2×10^{-4}M.

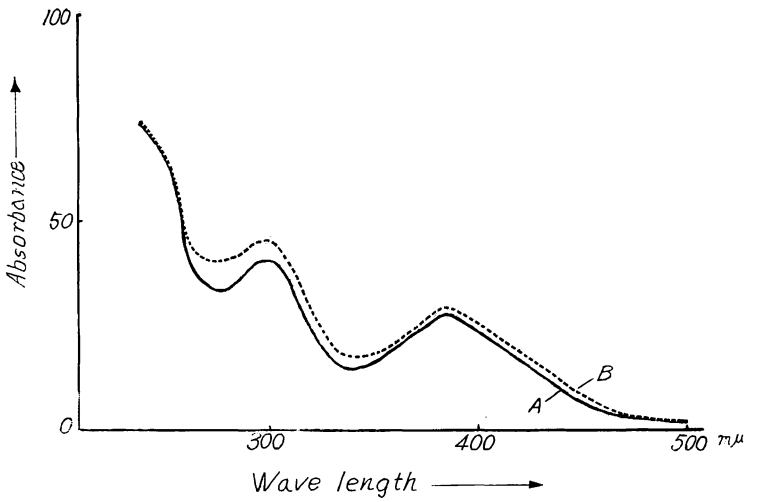


Fig. 9. Release of helicobasidin from its Cu complex by potato sucrose medium. A: addition of potato sucrose medium, B: addition of potato sucrose medium where conidia of *A. niger* germed.

helicobasidin molecule, addition of equimolar metallic ion would be insufficient on logical grounds. Accordingly, to see if an amount of metal ion added to helicobasidin changes inhibition, the combination of helicobasidin and Cu^{++} at various ratios was tested on the effect on *A. niger*, however, the data failed to give any significant difference (Table 23).

A summarized scheme of toxic change induced by metallic ions and succesive change induced by further addition of chelator is given in Figure 10.

ii) Effect of derivatives and analogues of helicobasidin on toxic action

It was revealed that toxic action of helicobasidin might be closely related to its chelating ability to the metal. And if it is, the question of what the structural group of helicobasidin attributes to toxic action is raised. To determine what structural group is active and in what way, two kinds of the derivative and six kinds of analogue of helicobasidin were tested. Structure of compounds tested are given in Table 24. Derivatives of helicobasidin were kindly prepared by Mr. YASUE, and analogous compounds of helicobasidin were kindly given by Dr. S. NATORI.

Table 23. Effect of helicobasidin: Cu ratio on inhibition of sporulation to *A. niger*

Ratio of component		Diameter of inhibition zone (mm)
Helicobasidin	$CuSO_4$	
1	1	21.8
1	1/2	22.2
1	1/4	21.2
1	2	21.5
1	4	22.8
0	4	—

Concentration, $5/4\times10^{-4}$M basically.

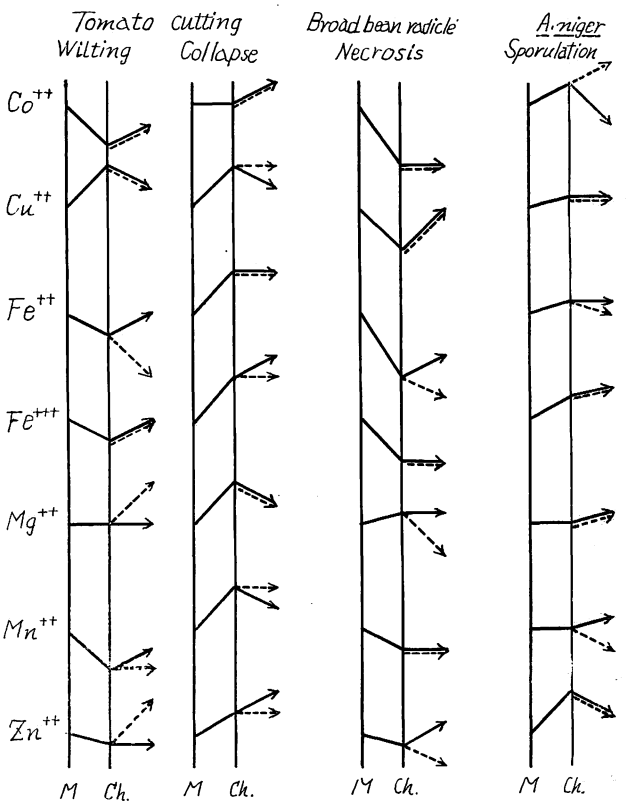


Fig. 10. Summarized scheme of changes in toxicity induced by metallic ions and chelators.

M: addition of metallic ions, Ch.: addition of chelators, →: EDTA, ⇢: oxine.

The lines upward, level and downward indicate an increase, no change and a reduction of toxicity, respectively.

With reference to the functional group, helicobasidin possesses both quinone and phenolic groups. While a partially acetylated product, helicobasidin diacetate, possesses only quinone group, further, a completely acetylated product, helicobasidin leucotetraacetate no longer possesses either quinone or phenolic OH group. Toxicity of these derivatives on broadbean radicles was different with each chemical. Helicobasidin diacetate indicated that it has still apparent toxicity; further, its toxicity was reversed by addition of equimolar L-cysteine. However, helicobasidin leucotetraacetate did not indicate any toxicity. Within the analogues maesaquinone and dihydromaesaquinone, which are the same as helicobasidin except for the side chain, gave a fairly toxic response, but analogues other than these gave scarcely any toxic symptom (Table 25).

Similar tendencies were also observed in the case of inhibition of sporulation to *A. niger*. Helicobasidin diacetate apparently showed inhibition and, in contrast, helicobasidin leucotetraacetate did not show any toxicity any longer (Plate 4 D). Of the analogues only 2-methyl-3-hydroxy-5-octyl-benzoquinone gave very slight inhibition. Moreover, maesaquinone and dihydromaesaquinone did not show any inhibition to *A. niger* (Table 26 and Plate 7 C-F). There

Table 24. Chemical structure of derivatives and analogues of helicobasidin.

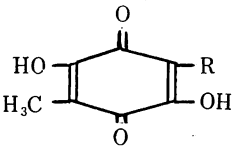
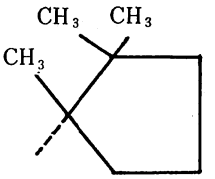
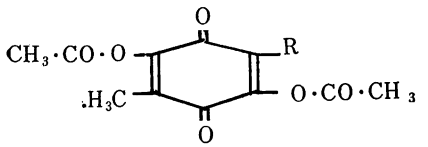
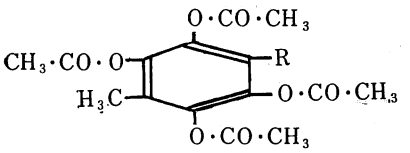
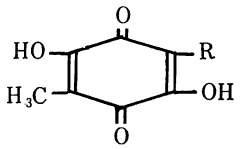
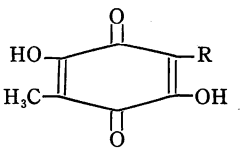
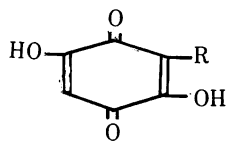
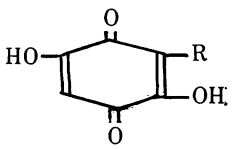
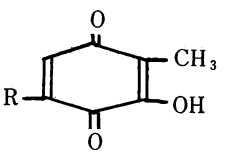
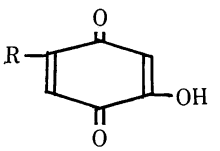
Structure	R	Name
		Helicobasidin
		Helicobasidin diacetate
		Helicobasidin leucotetraacetate
	$(CH_2)_{13}CH=CH(CH_2)_3CH_3$	Maesaquinone
	$(CH_2)_{18}CH_3$	Dihydromaesaquinone
	$(CH_2)_{10}CH_3$	Embelin
	$(CH_2)_{12}CH_3$	Rapanone
	$(CH_2)_7CH_3$	2-Methyl-3-hydroxy-5-octyl-benzoquinone
	$(CH_2)_{15}CH_3$	2-Hexadecyl-5-hydroxy-benzoquinone

Table 25. Toxicity of helicobasidin derivatives and analogues to broadbean radicles.

Chemical	Toxicity
Helicobasidin	##
Helicobasidin diacetate	#～##
Helicobasidin diacetate+cys.	+
Helicobasidin leucotetraacetate	—
Maesaquinone	#
Dihydromaesaquinone	#
Embelin	—
Rapanone	—
2-Methyl-3-hydroxy-5-octyl-benzoquinone	—
2-Hexadecyl-5-hydroxy-benzoquinone	±

Concentration, $5/4\times10^{-4}$M.

Table 26. Inhibition of helicobasidin derivatives and analogues to sporulation of *A. niger*.

Chemical	Diam. of inhibition zone (mm)	Concentration (10^{-4}M) 5	5/2	5/4	5/8
Helicobasidin		19.1	18.1	16.1	15.3
Helicobasidin diacetate		11.3	10.6	—	—
Helicobasidin leucotetraacetate		—	—	—	—
Maesaquinone		—	—	—	—
Dihydromaesaquinone		—	—	—	—
Embelin		—	—	—	—
Rapanone		—	—	—	—
2-Methyl-3-hydroxy-5-octyl-benzoquinone		+	+	—	—
2-Hexadecyl-5-hydroxy-benzoquinone		—	—	—	—

+: slight inhibition, —: no effect.

Table 27. Effect of concentration of helicobasidin and its diacetate on inhibition to sporulation of *A. niger*.

Chemical	X / Y	Concentration ($\times10^{-4}$M.) 5	5/2	5/4	5/8	5/16	5/32
Helicobasidin	Diameter of inhibition zone (mm)	19.8	18.9	17.9	17.3	15.3	12.3
Helicobasidin diacetate		18.9	16.7	11.9	—	—	—

may be a marked difference in the range of inhibitory concentration between helicobasidin and its diacetate (Table 27, Plate 4 E-F). Further, the outline of the inhibition zone was not clear, and halo was not produced at the margin of the zone of inhibition in helicobasidin diacetate as in helicobasidin. To ascertain the significance of this difference statistical analysis was applied. If the relationship between the logarithm of the reciprocal of concentration of the chemical (x) and logarithm of the zone diameter of inhibition (y) is drawn, it would be linear (Fig. 11), and each relationship is given as in the following formula.

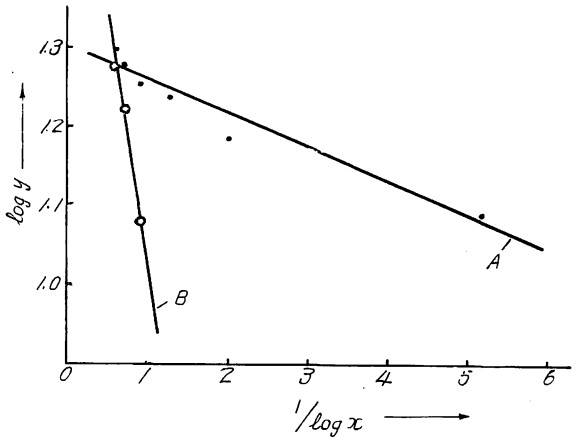


Fig. 11. Correlation between concentration of chemicals and diameter of inhibition zone of *A. niger*.
x: concentration, y: diameter of inhibition zone, A: helicobasidin, B: helicobasidin diacetate.

$$\log y = 1.29874 - \frac{0.04231}{\log x} \qquad \text{for helicobasidin (A)}$$

$$\log y = 1.65940 - \frac{0.63328}{\log x} \qquad \text{for helicobasidin diacetate (B)}$$

Furthermore, the tests of difference between the regression constants and the regression coefficients of these chemicals showed the evidently significant difference, respectively. Thus, the result may support the view that there is a basical difference in the mode of the inhibitory action of those chemicals.

3. Discussion

It must be recognized that to act against living tissues a chemical should infiltrate cell wall or some layer surrounding the active center in the intact cell. In permeation, lipoid solubility is an important factor, because the lipoid membrane surrouning cell wall or the active center such as mitochondria in the cell regulates the passage of substances. Helicobasidin, in fact, possesses lipoid solubility as indicated by its higher solubility in chloroform. Therefore, it seems to be reasonable to assume that helicobasidin possibly penetrate into cell.

Owing to the simplicity of the environmental factors the results of the experiments using broadbean radicles may be considered to give the clearest tendency with chelation. Evaluation of the toxicity with tomato cuttings seems to involve some errors because the chelate complex produced a precipite; further, helicobasidin was released from the metal complex suspended in the test liquid as fine particles, thus conduction of tomato cuttings might be interfered with in some degree. The results of tests with *A. niger* were not so good as those with broadbean radicles. It may be true that the environmental factors around fungi on the medium were too complicated, because potato sucrose agar medium employed must contain various kinds of chelators. Then, if the chelate complexes are given to a system they would be dissociated by another naturally occurring chelator in the medium in some cases, as shown by the spectrophotometrical determination. To analyse the results, therefore, may be difficult regarding change of inhibition by the treatment. Thus, for the reason mentioned above, the results obtained by

the broadbean assay are the most reliable as regards chelation.

Helicobasidin is a kind of dihydroxy-bezoquinone. Thus, it can be expected to form chelate complexes. As a matter of fact, the results of the present experiments gave evidence in three ways: confirmation of reduction of toxicity caused by addition of metals, that of recovery of reduced toxicity caused by the addition of another chelator, and the spectrophotometrical detection of released helicobasidin from its Cu-complex. Helicobasidin was considerably reduced in toxicity by the addition of Co, Cu, ferrous and ferric Fe, with reference to broadbean radicles. The agent of toxic action, however, may not be any metallic complex but free helicobasidin. All tested metallic ions acted as antagonists to helicobasidin. Then, the toxicity of the complex with Cu was raised up to the initial toxicity of helicobasidin by further addition of EDTA and oxine. This fact would suggest that Cu may be more affinitive to these chelators rather than helicobasidin. The fact that Co-complex did not increase the toxicity in spite of addition of other chelators probably means that Co forms a more stable complex with helicobasidin.

The data concerning alteration of toxicity of helicobasidin by addition of metallic ions and further addition of chelators in regard to the case of broadbean radicle may indicate that chelation is closely associated with a mode of toxic action. On the contrary, helicobasidin-metal complex did not cause a significant reduction in inhibition to sporulation of *A. niger* as observed in the case of broadbean assay.

STEINBERG[46)47)] revealed that *A. niger* essentially requires Fe, Zn, Cu and Mn for sporulation and Cu is a limiting factor of pigmentation of conidia. RICH and HORSFALL[38)] had confirmed the fact that dimethylglyoxine, a chelating agent for metal and other chelating oxims reduced the production and colouration of conidia in *A. niger*. Similarly, helicobasidin showed a considerable inhibition on sporulation in the same fungus, and its inhibition was not extended to hyphal growth. Although not dealing with chelating ability of tested chemicals, this tendency is also very similar to the report by BEHAL and EAKIN[6)]. In addition to its property to easily chelate with metal, the strong metal requirement for sporulation of the fungus may lead us to the assumption that helicobasidin may freeze essential metals for sporulation from the medium so that the fungus can not absorb them and/or it may enter the cell and freezes metals there; thus, the metabolism specific for sporulation is inhibited. Due to the chelate ability of helicobasidin it might be advisable to examine inhibition to metal enzymes which contain Fe or Cu.

The shift of toxicity accompanied by modification of the structure of helicobasidin may explain what and where the potential groups are. For chelation, the quinone group and hydroxyl group adjacent to the quinone group may be essential, basically. When only the hydroxyl groups are lost by acetylation, the remaining quinone group still keeps toxicity. It was evident in the cases of both broadbean radicles and *A. niger*. The fact that the response curve of sporulation of *A. niger* to helicobasidin and its diacetate were essentially different and the addition of cysteine induced a reduction of toxicity only with helicobasidin diacetate in the broadbean assay, may evidently mean a change of mode of toxic action. The additional reaction between quinone and thiol compounds has been fully established by SNELL and WEISBERGER.[45)] Further, GEIGER[14)] revealed that the activity of quinone is suppressed by equimolar addition of thiol compounds. In view of this point, it can be assumed that another mode of toxic action as quinone under very limited conditions might be possible with helicobasidin.

From the tests on the analogues of helicobasidin, there is reason for concluding that the presence of the side chain, 1, 2, 2-trimethylcyclopentyl group is essential for sporulation of *A.*

niger at the present stage. However, the positive toxicity due to maesaquinone and dihydromaesaquinone to broadbean radicles is not consistent with the case of the *A. niger* assay. This could mean that there are some differences in the mode of toxic action between broadbean radicle and *A. niger*.

By way of summarizing the aspects of a possible mode of toxic action of helicobasidin to higher plants and microorganisms as obtained in these present experiments, we may temporarily conclude that chelating ability of helicobasidin is surely associated with its toxicity to organisms; however, it is not yet firmly related to the ultimate mode of toxic action. The results of the tests on broadbean, as a matter of fact, seemed to support this aspect, but experiments on *A. niger* suggest that the mechanism of toxic action is unlikely to be explained only by such a simple factor, chelation, at least in microorganisms. Highly selective effect of helicobosidin to microorganisms would not be sufficiently supported by the chelation hypothesis. Therefore, a speculation about some delicate regulation at any site in the system would be raised on the assumption that helicobasidin is good enough for penetration into the cell. It could be supported by the fact of the presence of marked ubiquinone-like activity in helicobasidin as cited above and the conception of antimetabolism. If so, it would follow that the regulation toward inhibition or stimulation must be carried on at the site of mitochondria. On this basis, to get information on regulation at the mitochondrial site in an appropriate biological system in response to helicobasidin might be a key to making an approach to the ultimate mode of toxic action.

Summary

I. Isolation of helicobasidin

A mould pigment, helicobasidin was isolated from the whole culture of *Helicobasidium mompa* TANAKA grown on potato decoction with sucrose. It was found not only on the culture medium, but also on the mycelium, and on the glass wall, a little distant from the creeping hyphae of the fungus.

1. The products in and on the mycelium, on the culture medium and on the glass wall were entirely the same. This finding and the subliming character of helicobasidin combined to give a suggestion that helicobasidin probably sublimed out from inside to outside of the fungal mycelium.

2. Solubility of helicobasidin in various solvent was given. It was particularly noticed that helicobasidin is readily soluble in chloroform and insoluble in water.

3. The empirical formula for helicobasidin was determined as $C_{15}H_{20}O_4$. It consists of cadmium orange needles from petrolium ether and ether mixture, and melts at 193°C.

4. Positive results for the phenolic and quinone group tests with some reagents on helicobasidin, and ultraviolet and infrared absorption spectra of helicobasidin suggested the probable presence of phenolic OH and quinone groups in its molecule.

5. An available method for spectrophotometrical determination of helicobasidin was shown. It was based on its obedience to BEER's law within the range 3–11 μg per ml for the absorbance at λ_{max} 296 mμ characteristic for helicobasidin. Further, a practical application of this technique to helicobasidin determination in steamed distillate resulting from the culture of the fungus was also added.

6. Available solvent systems for one dimensional ascending paper chromatography of helico-

basidin was given.

7. In a comparison of the authors sample with that of NISHIKAWA, an examination for ultraviolet absorption spectra and admixed melting point tests provided the results to support a perfect consistence between them.

II. Factors affecting helicobasidin production

Factors affecting helicobasidin production have been examined, basically using the strain Hm-7 (M-1) of *Helicobasidium mompa* TANAKA.

1. To determine the favourable medium for helicobasidin production three kinds of natural medium, a semisynthetic medium and three kinds of synthetic medium have been tested. All tested media supported helicobasidin production. Among them the natural medium was generally more favourable, though RICHARDS' solution was as favourable as potato decoction. KASAI's solution was not unfavourable either.

2. Six nitrogen containing compounds have been examined as a nitrogen source. L-Phenylalanine was not always favourable for growth but extremely favourable for helicobasidin production. While, L-tyrosine and L-proline did not support helicobasidin production.

3. Addition of one per cent citrus pectin to the medium increased both mycelial growth and helicobasidin with all tested media. The medium containing L-phenylalanine increased it by approximately 50 per cent. From these facts, it appeared that phenylalanine was a specially favourable source for helicobasidin production.

4. The C/N ratio of the medium is an important factor for helicobasidin production. The smaller the C/N ratio was, the greater was the specific yield of helicobasidin. Even at the same C/N ratio, its effect was quite different for two distinct concentrations. Two per cent sucrose may be the minimum concentration of carbon source for helicobasidin production. Increased nitrogen concentration parallelly increased helicobasidin production and reversely decreased mycelial growth of the fungus.

5. The phase of helicobasidin production and its association with the growth phase have been made clear. Helicobasidin was produced during active growth. Therefore, it is evidently not an autolytic product of the fungal mycelium. The fact that the peak of helicobasidin production appeared after reaching the peak of mycelial growth of the fungus, may suggest that maturation of the mycelium is a factor for production of helicobasidin. A pH shift may rationally associate with helicobasidin production and mycelial growth of the fungus. Further, a smaller C/N ratio may cause earlier maturity of the mycelium of the fungus.

6. Eight strains of *Helicobasidium mompa* and one strain of *H. purpureum* have been tested. All strains of *H. mompa* produced helicobasidin while the strain of *H. purpureum* tested did not produce it at all. Specific yield of helicobasidin differed among the strains.

III. Toxic effect of helicobasidin on higher plants

Bioassay elucidating for toxic action of helicobasidin was carried out on six kinds of higher plants.

1. Absorption of sodium salt of helicobasidin by cuttings produced wilting in all five kinds of plants tested. In tomato cutting heavy wilting or collapse occurred at the concentration more than 1/8,000 (5.2×10^{-4}M) of sodium salt of helicobasidin, and vein necrosis and chlorosis of leaves at the concentration of more than 1/24,000, respectively.

2. The direct leaf treatment did not produce any apparent symptom; only the treatment to the wounded area on the leaf produced necrosis.

3. Broadbean radicles produced clear necrosis at the concentration of 1/64,000 or less of helicobasidin sodium salt at 25°C during 48 hours.

4. In microscopical examination for the section of the broadbean radicle treated with helicobasidin, a necrotic layer in cortex surrounding the central cylinder was found.

IV. Antibiotic action of helicobasidin

Antibiotic action of helicobasidin on microbes was extensively tested applying an agar streak dilution method and paper disk plate method. Twenty-three species of bacteria from three taxonomical orders and 78 species of fungi extended from Phycomycetes to Basidiomycetes and Fungi Imperfecti have been tested. As responses, not only inhibition of growth and sporulation but also stimulation of fructification were observed.

1. The action of helicobasidin was highly selective with all tested organisms and responses particularly with fungi.

2. Bacteria were more sensitive to helicobasidin than fungi, in general. And limited correlation between response and the taxonomical position of microbes was observed on bacteria and *Aspergillus*.

3. In tested bacteria, Gram positive bacteria showed a tendency to be more sensitive to helicobasidin than Gram positive ones.

4. Marked inhibition of sporulation was observed on a certain group of *Aspergillus*. It was specific in *A. niger* group. It might lead to some helpful suggestions on problems concerning the mode of inhibitory action of helicobasidin.

5. The stage of inhibited fructification of *A. niger* was examined. Inhibition of every stage of development and the concentration of helicobasidin was correlated.

6. Stimulation in formation of perithecia, conidia and zygospores was observed on *Neurospora* sp., *Botrytis* sp. and *Cunninghamella blakesleeana*, respectively. Slight inhibition to conidial and pycnidial formation was observed on *Colletotrichum gloeosporioides*, *Trichoderma viride* and *Pestalotia* sp.. No effect was observed on sclerotium formation of *Scleratinia* sp..

V. A possible mode of toxic action of helicobasidin

To confirm the possible mode of toxic action of helicobasidin, bioassays using tomato cuttings, broadbean radicles and *Aspergillus niger* have been made. In most of the assays changes in toxicity based on the addition of metallic ions and successive additions of chelators were traced. Further, the relationship between toxicity and the chemical structure of helicobasidin was given.

1. Effect of chelate formation of helicabasidin with metals on toxicity was demonstrated. Helicobasidin forms chelate with metals and, then the initial toxicity is reduced. Reduction of toxicity was considerable in chelation with Co, Cu and ferrous Fe.

2. It was demonstrated that chelated helicobasidin can be released by other chelators.

3. It was concluded that free helicobasidin, not the chelated form, may be an agent of toxic action and chelation with essential metals for organism assumed to be closely associated with the mechanism of inhibition at the present stage.

4. With reference to the chemical structure of helicobasidin quinone group and phenolic hydroxyl group adjacent to the quinone group may be essential for inhibition. In addition to that the presence of (S)-1, 2, 2-trimethylcyclopentyl group might be necessary. On the other hand the quinone group only may be capable of causing inhibition. The difference in form of action between free helicobasidin and helicobasidin diacetate gave a suggestion as to a possible mode of toxic action.

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Explanation of plates

Plate 1.

A–B: Sublimated helicobasidin,

A: on the mycelium of the fungus (arrow).

B: on the culture medium in the right flask (arrow).

Plate 2.

A: Pure crystals of helicobasidin.

B–D: Necrotic symptoms on the broadbean radicle induced by helicobasidin,

B–C: transversal section of the radicle,

B: treated with 1/8,000 concentration of helicobasidin (arrow).

C: untreated.

D: response to various concentrations of helicobasidin,

1/4,000, 1/6,000, 1/8,000, 1/12,000, 1/16,000, 1/24,000, 1/32,000, 1/64,000, 0 and 0 showing the way of the treatment. (from left to right).

Plate 3.

A–B: Wilting responses of higher plant cuttings to various concentrations of helicobasidin,

A: camphor cutting.

1/2,000, 1/4,000, 1/6,000, 1/8,000 and 0.

B: false indigo cutting.

0, 1/64,000, 1/32,000, 1/16,000, 1/8,000, 1/6,000 and 1/4,000.

C: tomato foliage,

1/2,000, 1/4,000, 1/6,000 1/8,000 and 0.

(from left to right respectively).

D–E: Necrotic symptom on the wounded and non-wounded mulberry leaf induced by 1/2,000 concentration of helicobasidin,

D: treated.

E: untreated.

Plate 4–5 and 7.

Paper disks were arranged in a circle from the top towards the right in the order of the description with the concentration or with the names of chemicals used.

Plate 4.

Effect of helicobasidin on fructification of fungi.

A–C: Application of paper disk plate method,

A: stimulation of perithecial formation of *Neurospora* sp.

1,056, 528, 264 and 132 μg/ml.

B: stimulation of zygospore formation of *Cunninghamella blakesleeana*.

C: inhibition of conidial formation of *Trichoderma viride*.

132, 66, 33 and 16 μg/m*l*, common in B and C.

D–F: Inhibition of sporulation to *Aspergillus niger*.

D: Derivatives of helicobasidin,

helicobasidin, helicobasidin diacetate and helicobasidin leucoteraacetate ($5/4\times10^{-4}$M).

E–E: Concentration of helicobasidin and helicobasidin diacetate,

E: helicobasidin.

F: helicobasidin diacetate,
5×10^{-4}M, $5/2\times10^{-4}$M, $5/4\times10^{-4}$M, $5/8\times10^{-4}$M, $5/16\times10^{-4}$M and $5/32\times10^{-4}$M, common in E and F.

Plate 5.

Inhibition of sporulation to *Aspergillus niger* group fungi induced by helicobasidin.

A: *A. awamori.*
B: *A. batatae.*
C: *A. carbonarius.*
D: *A. japonicus.*
E: *A. niger.*
F: *A. usamii*
132, 66, 33 and 16 μg/ml, commonly.

Plate 6.

Effect of the concentration of helicobasidin on development of the conidial apparatus of *Aspergillus niger.*

A: 500 μg/ml.
B: 250 μg/ml.
C: 125 μg/ml.
D: 32 μg/ml.
E: 16 μg/ml.
F: 0.

Plate 7.

Inhibition of sporulation to *Aspergillus niger.*

A–B: Effect of pH on inhibition of helicobasidin.
A: pH; 2, 3, 4 and 5.
B: pH; 6, 7 and 8.

C–F: Effect of derivatives and analogues of helicobasidin on inhibition of sporulation, helicobasidin, helicobasidin diacetate, embelin, rapanone, 2-mehtyl-3-hydroxy-5-octyl-benzoquinone and 2-hexadecyl-5-hydroxy-benzoquinone.
C: 5×10^{-4}M.
D: $5/2\times10^{-4}$M.
E: $5/4\times10^{-4}$M.
F: maesaquinone, 5×10^{-4}M, $5/2\times10^{-4}$M, $5/4\times10^{-4}$M, $5/8\times10^{-4}$M and solvent control.

むらさきもんぱ病菌 (*Helicobasidium mompa* TANAKA) より単離したヘリコバシジンおよび高等植物，微生物に対するその毒性に関する研究

摘　　要

高　井　省　三(1)

微生物代謝物に対する学理的・応用的関心は，ペニシリンの *Penicillium chrysogenum* よりの発見単離とその劃期的な医学的応用以来，日ごとにたかまっている。菌類の代謝物だけに限っても今日までにおびただしい数のものが単離され，それらの理化学性だけでなく生物活性に関しても活発な論議が展開されつつある。

現今，寄生範囲のもっとも広いものの1つと考えられているむらさきもんぱ病菌 *Helicobasidium mompa* TANAKA の代謝物に関する研究の歴史も決して浅くはない。田中[59]はすでに1891年本菌がシュウ酸を代謝物として産生すると推定したが，その後伊藤[18]はシュウ酸石灰の形でこの酸の産生を確認した。また三宅[26]はシュウ酸以外の酸の産生を推定したが，近年になって荒木ら[4]はイタコン酸を本菌の代表的代謝物として単離同定するとともにその生理的意義につき報告している。これより先，伊藤[18]は本菌培養の際，菌体上はもとより培地上もしくは培養容器の器壁に橙黄色不定形物質がかなりけん著に産生することに着目し，その物質がフェノール性のものであろうと推定した。その後筆者は伊藤のすすめに応じてこの物質の純粋単離を試みた結果，水に不溶性の橙黄色針状 m.p. 193°，$C_{15}H_{20}O_4$ を得てヘリコバシジンと命名した。U. V. および R. I. 吸収よりヘリコバシジンがフェノール性 OH 基および共軛性 C=O 基を有するとともに試薬による定性反応結果とをあわせ考えて，このものがハイドロキシ・ベンゾキノンようのものであろうと推論した。

一方筆者とは別に西川[29]は水に不溶性の橙色針状色素 "ヘリコバシジン"，m.p. 194°，$C_{16}H_{22}O_4$，水可溶性紫色針状色素モンパイン，m.p. > 300°，$C_{10}H_6O_6$，のほか無色のヘモール酸，m.p. 224°，リコバシン m.p. 136° および D-アラビトールの5種を本菌菌体より単離し，筆者よりおくれて印刷公表した。筆者はこの研究の発表に際し，西川の単離がすでに行なわれていたにもかかわらず未発表のまま経過していることを知り検討の結果，筆者の単離物質は西川が単離命名したと伝えられる "ヘリコバシジン" と全く同一物であることを確認した。このため混乱をさけるためこの色素をヘリコバシジンと命名して公表した。

名取ら[27][28]はヘリコバシジンおよびモンパインの構造式を決定し，それぞれ (S)-3-メチル-2,5-ヂヒドロキシ-6-(1,2,2-トリメチルシクロペンチル)-ベンゾキノンおよび 2,5,7,8-テトラヒドロキシ-1,4-ナフトキノンを提唱するとともに西川により発表されたヘリコバシジンの分子式を筆者の提出した $C_{15}H_{20}O_4$ に訂正した。これと前後して小沢らはヘリコバシジンが明らかなユビキノンよう活性を有することを報告し，

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この物質が生体内の電子伝達系に関与しうる可能性を暗示した。

以上の沿革よりわかるように，ヘリコバシジンの生物活性に関する研究は小沢ら[36]のものを除いては未公表であるので，筆者の今日までに実施した研究を取りまとめて報告した。

I．ヘリコバシジンの単離

H. mompa (Hm-7) の菌体をふくむジャガイモせん汁培地上での培養を水蒸気蒸留してえた留分のアルカリ抽出物より水に不溶，昇華性の橙黄色針状 m.p. 193° をえて $C_{15}H_{20}O_4$ の分子式を与えた。また器壁に晶出している橙黄色色素もこの物質と同一物であることを確認した。

この物質はエーテル，クロロホルム，ピリジンなどに易溶であるがベンゼン，n-ヘキサン，リグロイン，石油エーテルなどにはよく溶けず水には不溶である。$FeCl_3$ 反応，MILLON 反応，ARNOW 反応（以上フェノール反応）および酢酸マグネシウム反応（キノンの反応）はともに陽性であった。この物質に関し，

U. V. 吸収，

$\lambda_{max}^{Ethanol}$ $m\mu$ (log ε)：296 (4.20), 377 (2.87)
$\lambda_{max}^{N/50\ KOH-ethanol}$ $m\mu$ (log ε)：324 (4.25)
} フェノール性 OH

I. R. 吸収，

λ_{max}^{KBr} μ：3.00, 7.71
$\lambda_{max}^{CCl_4}$ μ：3.04, 7.36
} フェノール性 OH

λ_{max}^{KBr} μ：6.13,
$\lambda_{max}^{CCl_4}$ μ：6.08
} 共軛性 C=O

がえられた。

以上のことからヘリコバシジンがフェノール性 OH 基をもったキノンであることを推定した。

精製されたヘリコバシジンの U. V. 分光分析法による定量法を確立し，さらに水蒸気蒸留分のエーテル抽出物中のヘリコバシジンの簡易定量法を案出し，ヘリコバシジンの産生条件検討の基礎を作った。またヘリコバシジンの一次元ペーパークロマトにつき検討を加え利用できる展開溶媒系，発色剤等を示した。

西川の単離したと伝えられる，いわゆる“ヘリコバシジン”と筆者の単離した物質との異同につき，U. V. 吸収，混融法により検討を加えた結果，両者が完全に同一物であることを確認し，混乱をさけるため筆者の分離物質をもヘリコバシジンと呼称することとした。

II．ヘリコバシジンの産生条件

ヘリコバシジンの産生条件を培地の種類，組成，培養の時期，菌株などにつき比較検討した。

供試した培地のうちでは一般に天然培地の方が合成培地よりもヘリコバシジンの産生に好適なようである。ヘリコバシジンがベンゼン核を有すると推定されたので，ベンゼン核の前駆的役割を考慮しながら笠井処法による合成培地の窒素源を変じて産生を比較した。この結果，芳香族アミノ酸である L-フェニールアラニンがヘリコバシジンの産生にきわめて好適であることが示されたが，同じくベンゼン核をもつL-チロシンはほとんど産生に寄与しなかった。

また本菌は強力なペクチン分解酵素を産生するので培地にペクチンを添加して産生能に対する影響を検討した。この結果ジャガイモせん汁および L-アスパラギンを窒素源とする笠井培地ではあまり大きな産生能の向上はみられなかったが窒素源に L-フェニールアラニンを置換した笠井培地では約 50% の産生能の向上がみられた。このことはフェニールアラニンがヘリコバシジンのベンゼン核生合成に前駆体的寄与を

しているのではないかとの暗示を与えるように思われる。

培地の C/N 比を変えてヘリコバシジン産生能を比較すると，炭素（C）源は 2% 以下ではヘリコバシジン菌体双方の生産に好適ではなく，炭素源の増加はヘリコバシジン産生能にはほとんど寄与せず菌体生産への寄与が大きい。これに反し窒素源はヘリコバシジン産生への寄与は大きく，その量の増加と平行して産生能を向上させた。これらの結果を要約すると，過剰の害のない範囲では C/N 比は小さい方がヘリコバシジン産生に好適であるといえる。

ヘリコバシジンの経時的産生の傾向からヘリコバシジンは菌体の分解産物ではないこと，菌体の最大生産時期（95 日）よりもヘリコバシジン産生能の極大期（105 日）がおくれることから，ヘリコバシジンの産生には菌体の熟度が関係していることが想像された。またいったん産生されたヘリコバシジンは培地の pH の上昇につれて減少しているがこのことはフェノール系物質はアルカリ性条件下では不安定であるため，分解されつつあると考えるべきであろう。

H. mompa 8 菌株とこれとの異同につき議論されている，きわめて近縁の *H. purpureum* 1 菌株につき産生能を比較した結果 *H. mompa* の 8 菌株はすべて，多かれ少なかれヘリコバシジンを産生した。したがってヘリコバシジンは *H. mompa* に関しては種間共通の代謝物ではないかと想像された。しかし，*H. purpureum* はヘリコバシジンを全然産生しなかった。この種についてはさらに多数の菌株を供試してみないと，ヘリコバシジンがこの種において産生されるかどうかにつき結論づけることはできない。

III. ヘリコバシジンの高等植物に対する毒性

H. mompa はきわめて多犯性の植物病原菌であるのでこの代謝物も寄主植物に対して毒性をもつかもしれないことが予想された。そこでクス，イタチハギ，クワ，ダイズ，トマトおよびソラマメの 6 種植物につき毒性の検定を実施した。このうち，ソラマメは発芽した幼根にヘリコバシジンを処理したが，他のものについては，切枝をヘリコバシジン溶液にさしていちょう（萎凋）の発生の有無を，葉に処理してえ死に至る変化を調べた。切枝におけるいちょうはいずれの植物においても発生したが，クスおよびトマトにおいて比較的強く現われた。葉においても有傷処理の場合は明らかなえ死およびクロロシスを発生した。ソラマメ幼根における毒性の発現はかなり鋭敏で 1/64,000 (6.5×10^{-5} M) の濃度においても明らかな褐変えい死が観察された。処理部の切片を顕微鏡下で観察すると周皮において明らかな褐変層が中心柱を囲んで環状に生じているのが確認されヘリコバシジンが組織内に浸透して阻害的に作用していることが明らかになった。

IV. ヘリコバシジンの抗菌作用

ヘリコバシジンの構造的特性であるデヒドロキシ・ベンゾキノンの骨格からこのものが微生物に対しても何らかの作用を及ぼすであろうことは容易に想像される。そこで微生物に対するヘリコバシジンの影響を調査した。

検定菌の選択に当たっては，それらが微生物の分類体系を代表するように特に留意し，細菌 23 種，糸状菌 78 種を検定菌として抽出した。

ヘリコバシジンの微生物に対する作用にはきわめて著しい選択性が認められた。その作用は，観察した範囲では細菌に対しては阻害的であったが，糸状菌に対しては種によっては阻害的で，他の種に対しては逆に刺激的であったりした。そして細菌および *Aspergillus* 属内ではヘリコバシジンに対する反応と分類体系との間に比較的高い相関が認められたが，*Aspergillus* 属菌を除いた糸状菌の間では何ら一定の傾向

はえられなかった。ヘリコバシジンに対しては，一般に糸状菌よりも細菌の方が感受性が強かった。

供試した細菌の中では，グラム陽性菌の方がグラム陰性菌よりもヘリコバシジンに対し耐性が低い傾向が認められた。しかし，比較的耐性の高いグラム陰性菌であってもかなり感受性の菌が認められた。

一方糸状菌のうち *Aspergillus* 属では *A. niger* 群の全供試菌に特異的な胞子形成阻害が認められたが，同属の他の群の菌には阻害が認められなかった。*A. niger* 群は黒色の分生胞子の色を他群との類別の根拠としているが，この群にのみ特異的な阻害の認められたことは興味深い傾向である。

供試糸状菌は一般にヘリコバシジンに対して耐性が高かったが，*Endothia parasitica*, *Septotinia populiperda*, *Ascochyta pisi*, *Haprosporella* sp., *Stagonospora cryptomeriae*, *Cercospora cryptomeriae*, *Helminthosporium sativum* および *Isariopsis* sp., などは比較的強い発育阻害を受けた。またヘリコバシジンは胞子形成に対し全く選択的な影響を示した。*Aspergillus niger* に対する胞子形成阻害のほかにはあまりけん著ではないが数種の菌の胞子形成を阻害した。逆に子実体形成を刺激的に促進することが *Cunninghamella blakesleeana*（接合胞子形成），*Neurospora* sp.（子のう殻形成），*Botrytis* sp. および *Curvularia lunata*（分生胞子形成）などにおいて観察された。

A. niger の分生胞子形成に対するヘリコバシジンの阻害と濃度との関係を顕微鏡下で観察した結果，16～500 μg/ml の範囲では分生胞子の形成にいたるまでの分化過程に対し濃度の増加に応じて段階的に阻害をましていることを確認した。しかし BEHAL ら[6]のいうような，特定濃度の薬剤が分生胞子形成に達する特定の分化過程にとどめるような阻害の様式があるかどうかについては明らかにしえなかった。

以上のような微生物によるヘリコバシジンの生物検定の結果はヘリコバシジンの阻害機構に関する考察に対し有力な示唆を与えた。

V．ヘリコバシジンの阻害機構

ヘリコバシジンの毒性発現の機構を考察するために，主としてソラマメ幼根に対する毒性の発現と *Aspergillus niger* に対する胞子形成阻害に関して実験を行なった。

ヘリコバシジンが生体に影響を及ぼすためには，生体外で生体に必須な条件を左右するような何らかの作用を演ずる（間接的作用）か生体内に到達して生体に作用する（直接的作用）の2とおりが考えられる。後者の場合にはまずヘリコバシジンは生体と外界，生体内の作用中心と周囲とをへだてる膜もしくは層を通過して終局的には作用中心に到達しなければならぬ。この選択的透過を調節するものはリポイドであるから，ある物質がこれらの膜もしくは層を通過するためにはリポイド可溶性が要求される。ある物質のクロロホルムに対する溶解性はその物質のリポイド可溶性を推察する目安とされている。ヘリコバシジンはクロロホルムに易溶であるので一応この条件はみたされるのではないかと思われる。

ヘリコバシジンはその構造より推定されるように金属と容易にキレートを生成する。ヘリコバシジンのキレートはソラマメ幼根に対する毒性をいちじるしく低下させるとともに，そのキレート溶液に EDTA やオキシンなどのキレート滴定剤を添加するとおのおののキレートに固有な毒性の変化を起こす。すなわち Cu キレートでは毒性がもとのヘリコバシジンのそれまで回復するが，Co キレートでは低下した毒性の回復は認められなかった。Cu-キレートにつき U. V. 吸収で EDTA 添加の効果を検討するとヘリコバシジン-Cu キレートは親和性の高い EDTA に Cu が結合してヘリコバシジンが解離され，そのために毒性の回復が生じたことが確認された。

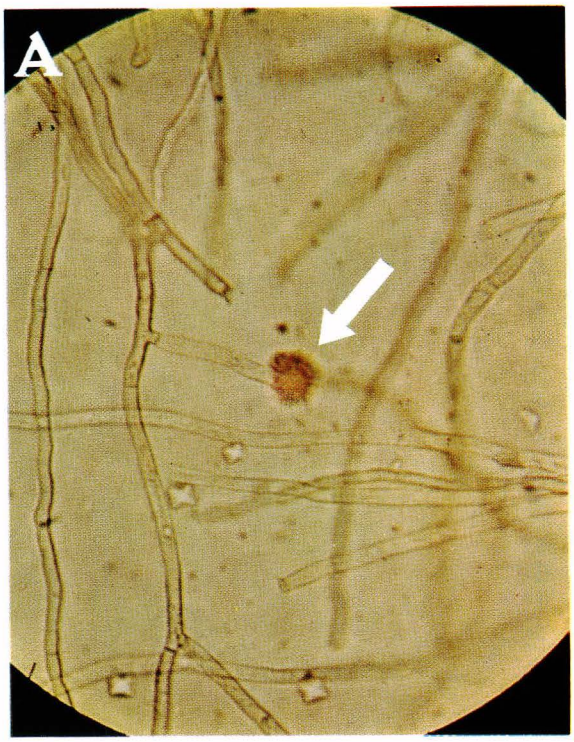
以上の事実は毒性の本源は遊離のヘリコバシジンにあるのであって，そのキレートにあるものではない

こと，またキレート生成は生体内外の系におけるその生体に必須の金属を凍結して利用できないようにするとも考えうるのでその結果阻害が発生するのではないかという推論を可能にするようである。しかし，環境因子の単純なソラマメ幼根による実験系ではこのような結果をえられたが，*A. niger* の実験系ではキレートによる阻害の生起を明らかに示すことができなかった。これはこの系が，多数の天然キレーターをふくみ，U. V. 吸収で一部確認したように与えられたヘリコバシジンのキレートを変化させてしまったためと思われる。

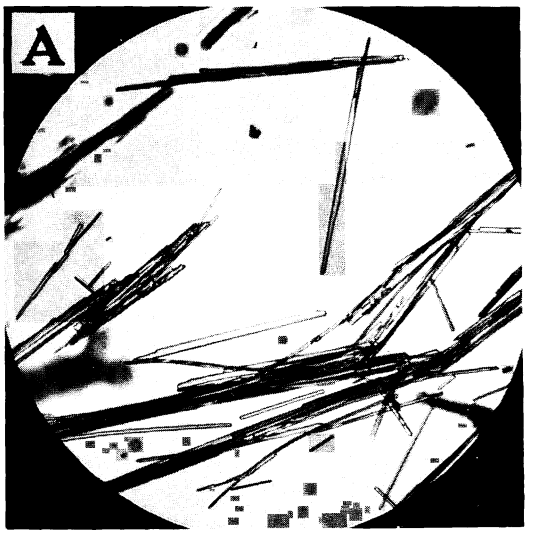
さらにヘリコバシジンの化学構造と毒性との関係を，ヘリコバシジンの誘導体および類似物質について検討した。ヘリコバシジンの毒性はベンゼン母核中のフェノール性 OH 基と共軛性 C=O（つまりキノン基）により支えられているもののようであり，部分的アセチル化によりえられた OH 基をもたないヘリコバシジン・ジアセテートでは単にキノンとしての毒性を示すに過ぎない。このことは L-システインによるヘリコバシジン・ジアセテートの解毒，*A. niger* の胞子形成阻害と濃度との関係が両者において全く異なることなどからヘリコバシジンとは異質の阻害作用であることが推定された。さらに C=O をもふくめて完全にアセチル化したヘリコバシジン・ロイコテトラアセテートでは毒性は全く消失する。

また類似物の毒性の比較の結果，ベンゼン母核に仮りに OH，C=O を有しキレート形成能をもっていてもヘリコバシジンに匹敵する毒性は認められない。さらにベンゼン母核に関してはヘリコバシジンと全く同一であるが同じ位置につく側鎖が直列構造のメサキノンもしくはジヒドロメサキノンでもソラマメ幼根に対する毒性はかなり劣り，*A. niger* では全く認められないという事実は，ヘリコバシジンの側鎖の環状構造が重要な意味をもつように思われる。この構造が膜透過性を高めているものであるか，あるいはキレート生成機構に影響しているものであるかについては不明である。

以上のような結果を総合考察するとき，毒性の原因をキレートにのみ求めるのには，なお慎重を要するように思われる。なぜならば，菌の胞子形成に対するヘリコバシジンの作用はきわめて選択性が強く，ある菌に対しては阻害的に働くが，他の菌に対してはかえって促進的に働いている事実は，キレート説では容易に説明づけられないからである。このような，対照的な選択作用の説明には，むしろ代謝拮抗の概念の導入が必要となろう。小沢ら[36]により示されたようにヘリコバシジンはかなり著しいユビキノンよう作用を有する。このことは，ミトコンドリヤにおける電子伝達系の作動におけるその生体固有のユビキノンの寄与に対しヘリコバシジンが拮抗的に働いた場合は阻害，逆に同調的に働いた場合は阻害を生ずることなく，ある場合には促進がおこりうると考えることが可能なようであるが，この推論の真否については今後の実験により確かめたい。


A


B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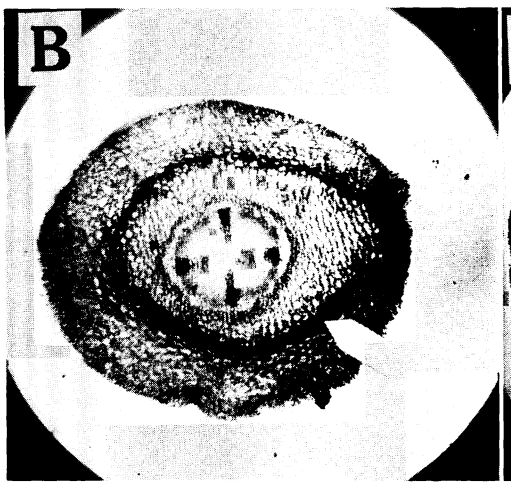
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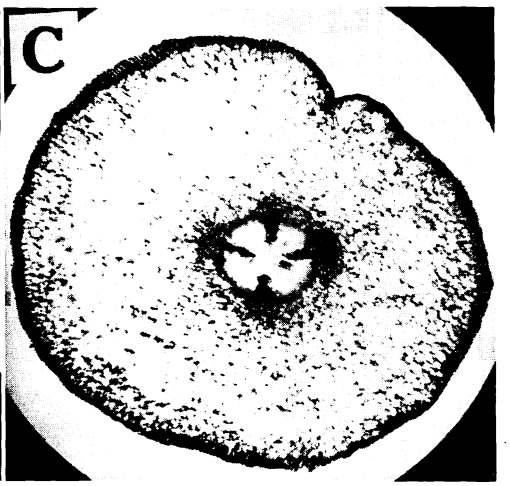
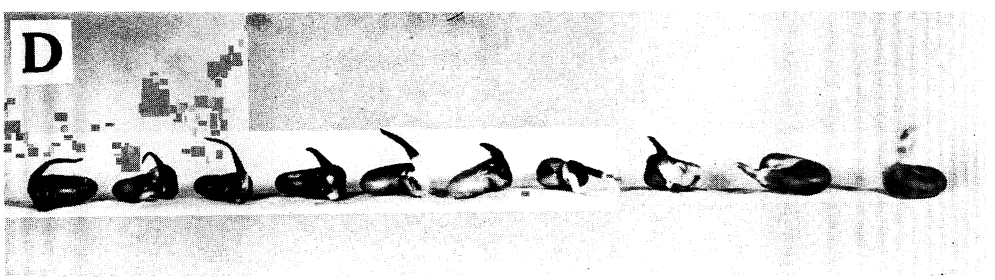

C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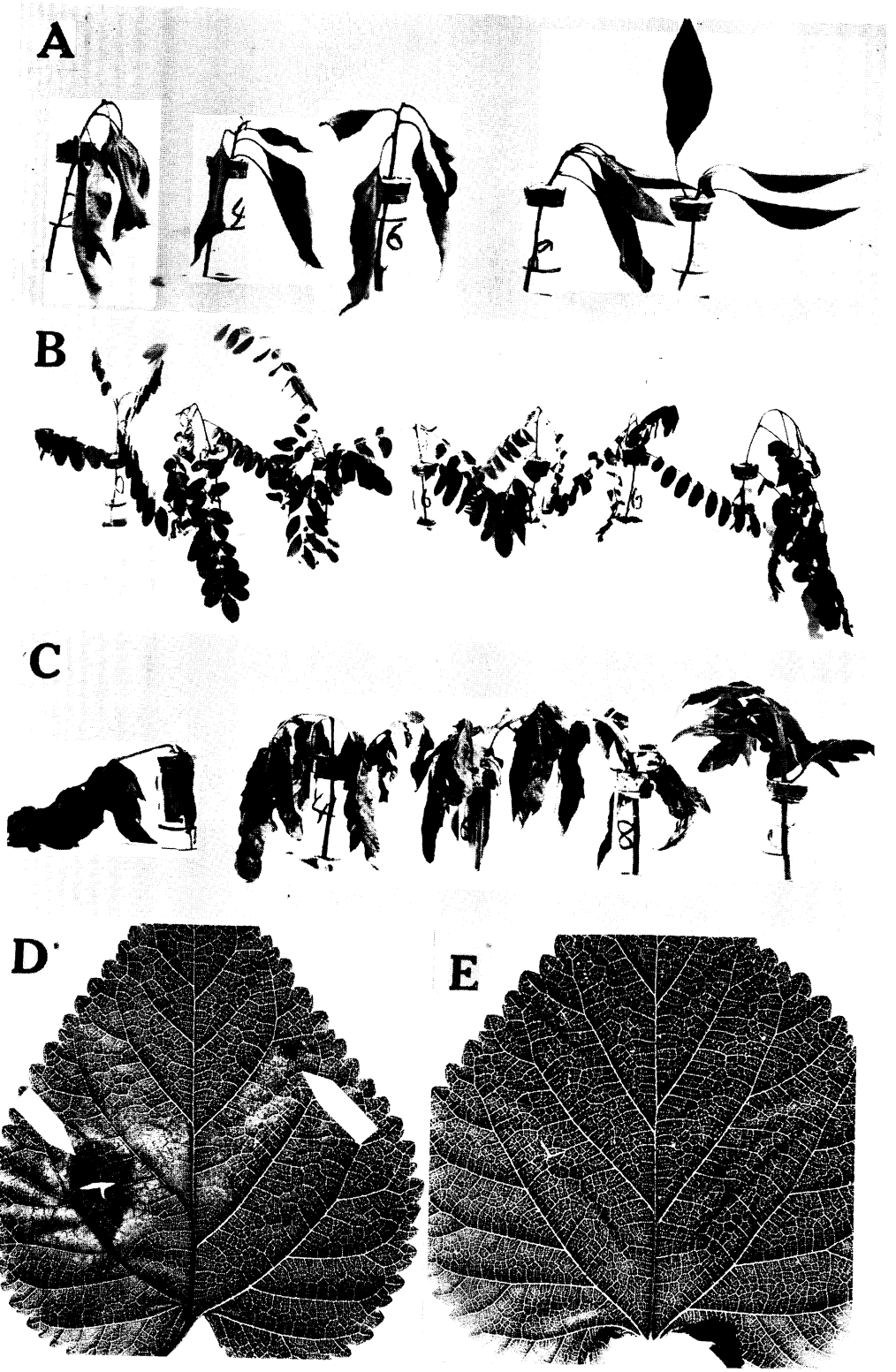
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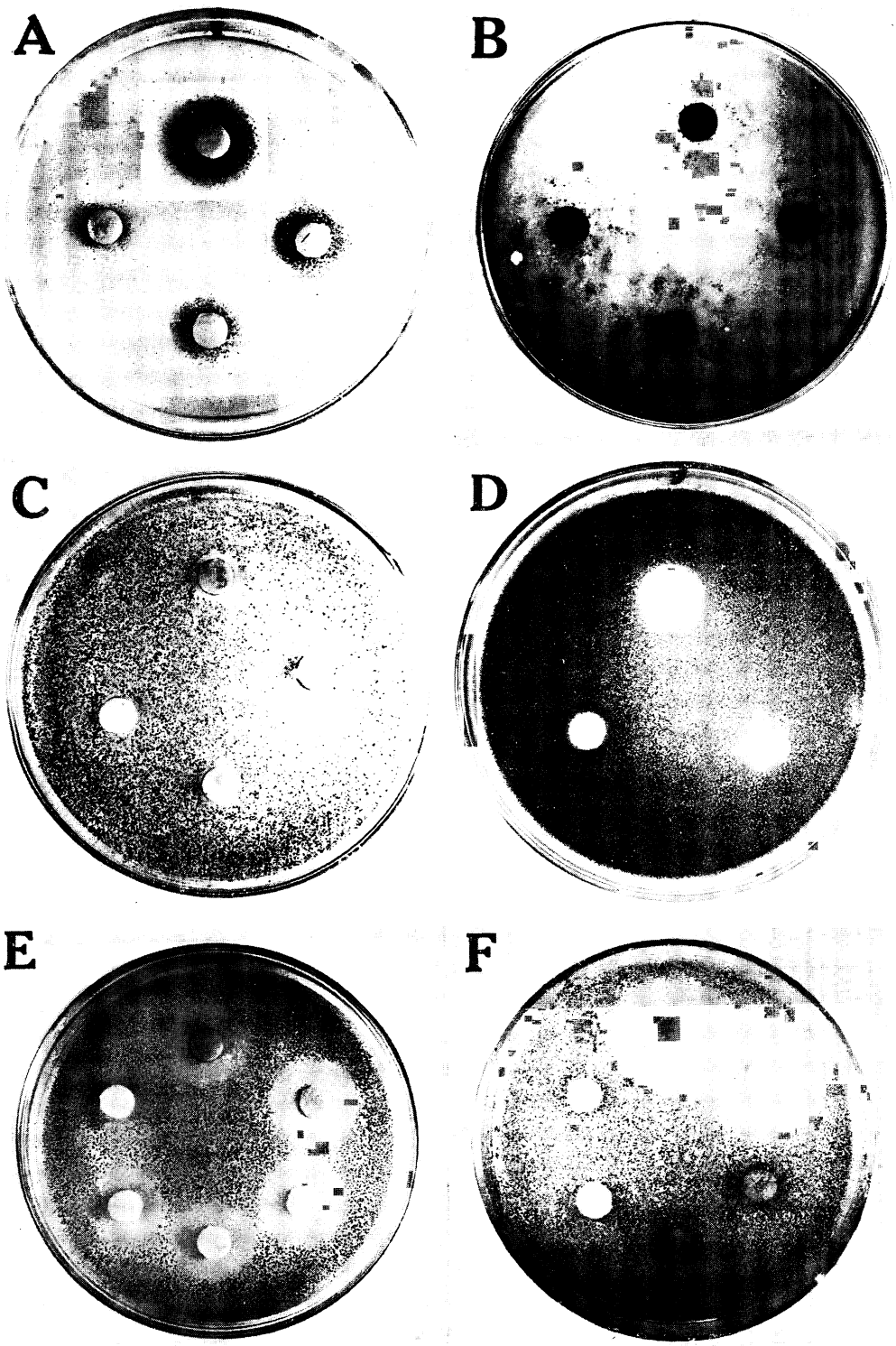
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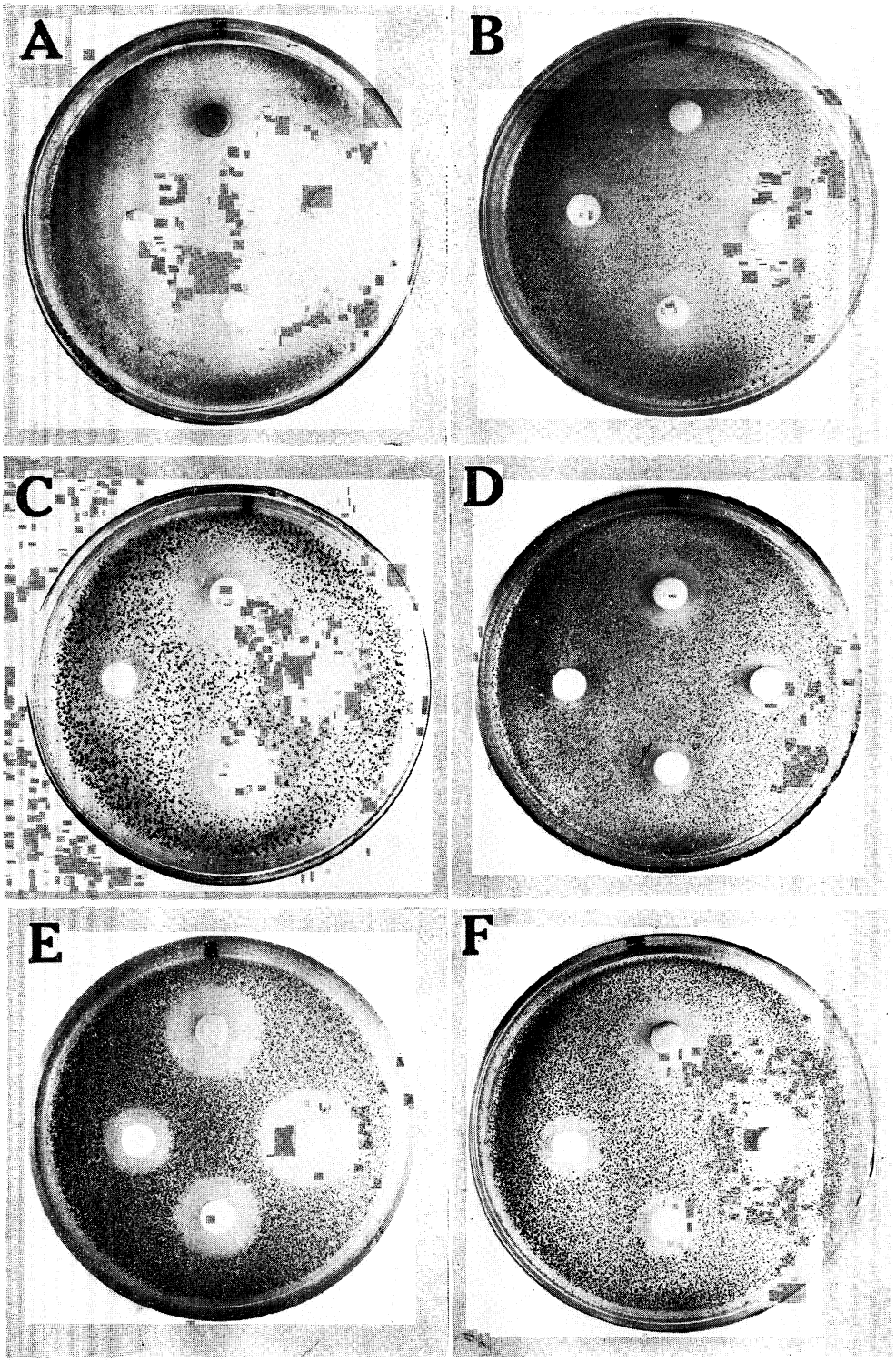
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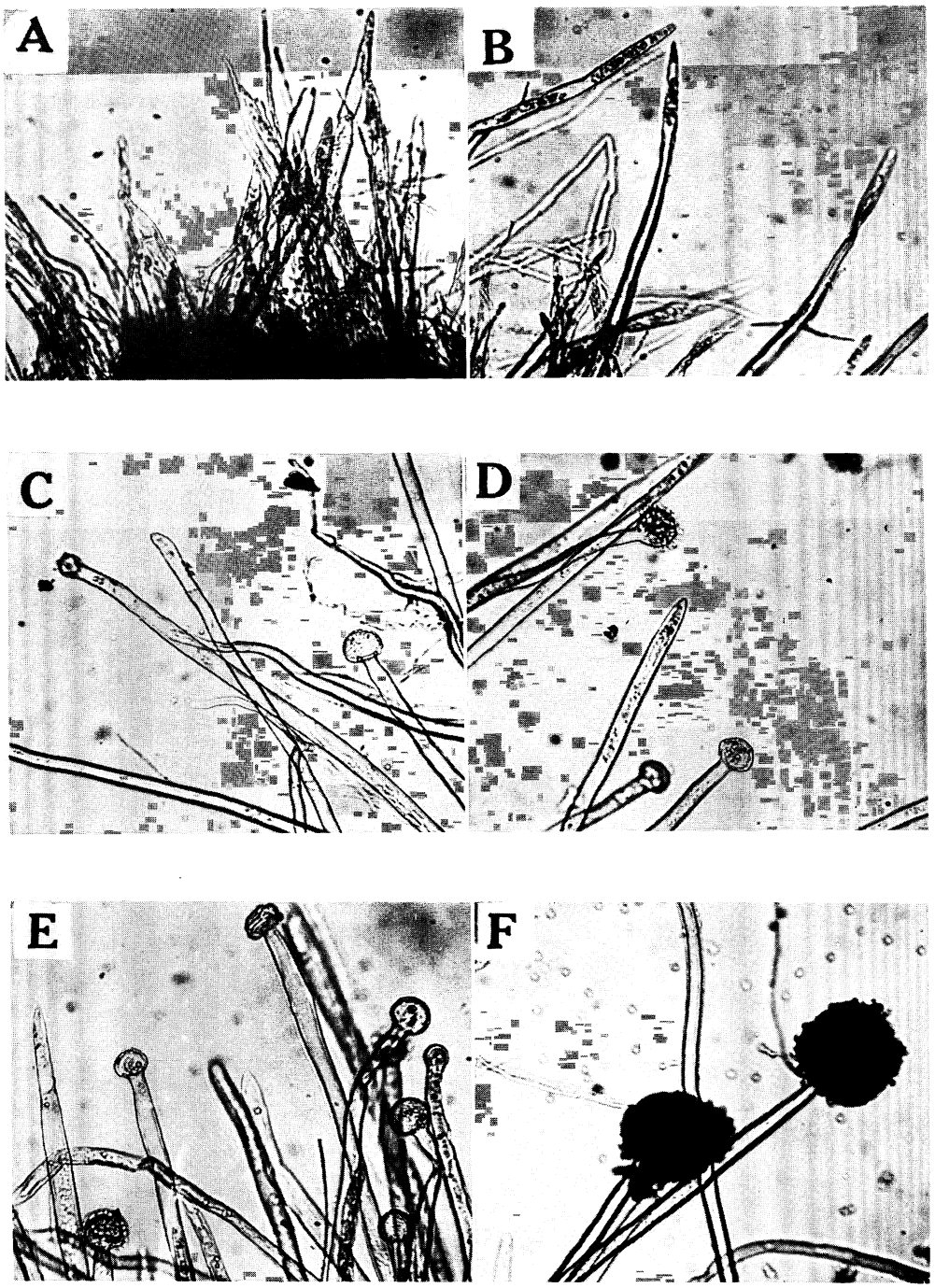
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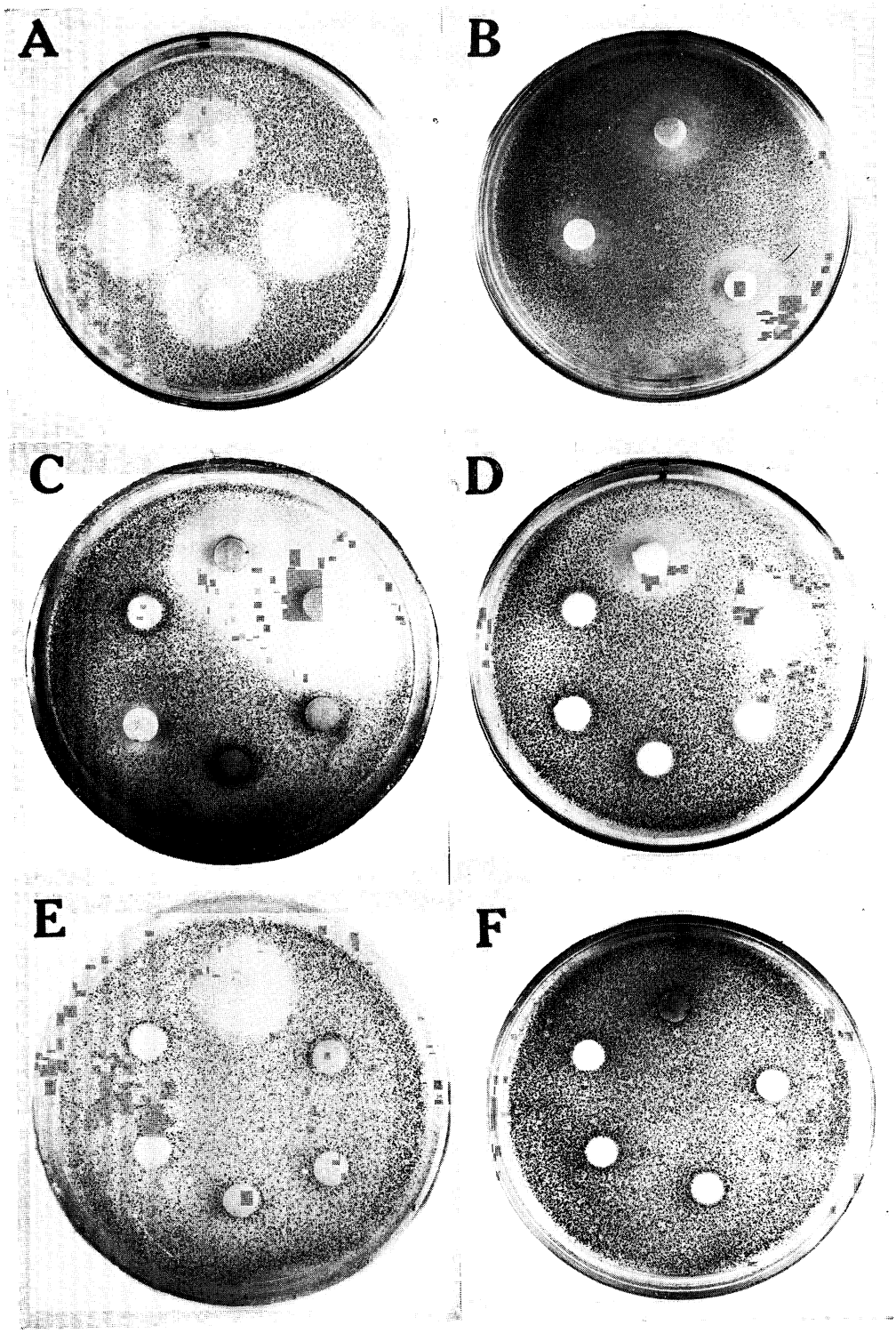
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