ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 1977, p. 743-749

Vol. 11, No. 4
Printed in U.S.A.

Evaluation of the In Vitro Antifungal Activity of Allicin

YASUO YAMADA* AND KEIZŌ AZUMA

Department of Microbiology, Fujita-Gakuen University School of Medical Technology, Toyoake, Aichi 470-11, Japan

Received for publication 4 December 1976

Allicin was effective in vitro against *Candida, Cryptococcus, Trichophyton, Epidermophyton,* and *Microsporum*. The minimal inhibitory concentrations (MICs) of allicin against these organisms were 3.13 to 6.25 μg/ml by the agar dilution method and 1.57 to 6.25 μg/ml by the broth dilution method, using Sabouraud glucose (SG) medium. However decreased activity was demonstrated against *Aspergillus*. The MIC of allicin against various pathogenic fungi was affected considerably by differences in the experimental conditions, e.g., incubation time, inoculum size, type of medium, and medium pH. The MIC of allicin against *Candida, Cryptococcus,* and *Aspergillus* remained constant after more than 3 days of incubation, and that against *Dermatophytes* remained constant after more than 10 days of incubation. Decreasing the inoculum size increased the susceptibility to allicin. The antifungal activity of allicin was stronger on SG agar medium with a pH of 5.6 than on the same medium with a pH of 6.0 or higher. By microscopical observation, allicin induced morphological abnormalities in hyphae of *Trichophyton mentagrophytes* Morita. Percent germination of spores of the Morita strain at 24 h in SG agar medium was greatly decreased with an allicin concentration of 3.13 μg/ml, and the lethal dose for the spores was about four times higher than the fungistatic concentration. These results suggest that allicin inhibits both germination of spores and growth of hyphae.

An investigation of the literature showed that *Allium sativum,* the common garlic, has been endowed with therapeutic virtues both in legend and in scientific publications. Several investigators have observed the antibacterial activity of garlic extracts. In 1944, an extract of oil from garlic was obtained by steam distillation and by fractional distillation of the crushed cloves and named allicin; this substance possessed potent antibacterial activity against both gram-positive and gram-negative bacteria (1). It is a colorless oil that is approximately 2.5% soluble in water and relatively unstable; it has been assigned the structure: allyl-S-S-allyl (2). ‖ O

In this report, the in vitro antifungal activity of allicin will be described.

MATERIALS AND METHODS

Organisms. *Candida albicans* 381 and Nakamura, *Cryptococcus neoformans* Duke, M-1, and 1814, *Aspergillus fumigatus* Kuboyama, N-2, and IFO 5840, *Trichophyton mentagrophytes* Morita, T-N-2, and T-N-4, *Trichophyton ferrugineum* T-N-1, *Trichophyton rubrum* Ishida and Suzuki, *Microsporum gypseum* Suzuki, and *Epidermophyton floccosum* Suzuki were used.

Stock cultures of *Candida, Cryptococcus,* and *Aspergillus* were grown at 37°C for 3 days on Sabouraud glucose (SG) agar medium, and those of *Trichophyton, Microsporum,* and *Epidermophyton* were grown at 25°C for 15 days on SG agar medium. They were maintained at room temperature.

Viable-cell suspensions of *Candida* or *Cryptococcus* were prepared by suspending viable cells harvested from 3-day-old SG agar slants in sterile saline.

Spore suspensions of *Aspergillus* or *Dermatophytes* were prepared by rubbing on the surface of 3- or 15-day-old SG agar slants with a loop after the addition of a solution of 0.5% (wt/vol) carboxymethylcellulose in sterile saline and filtered through two layers of gauze.

Cell or spore counts were made in a hemocytometer, and the suspension was adjusted to the desired strength by adding more sterile saline or 0.5% carboxymethylcellulose saline. A viable-cell suspension with 10^7 *Candida* or *Cryptococcus* cells per ml and spore suspensions with 10^7 *Aspergillus* or *Trichophyton* spores per ml, 10^6 *Microsporum* spores per ml, and 10^5 *Epidermophyton* spores per ml were used as standard suspensions in all experiments, unless stated otherwise.

Allicin. Allicin was extracted from the crushed cloves of *A. sativum* (4). The preparation used here was pure and had a d^{20} of 1.112, and n_D^{20} 1.561.

By calculation, analysis of allicin showed (for $C_6H_{10}OS_2$): molecular weight, 162; C, 44,44; H, 6.17; S, 39.51. Composition as found was: C, 44.35; H,

6.25; S, 39.70%. Before use, allian was diluted with water to the desired concentration and sterilized by filtering through a 0.22-μm-pore size membrane filter (Millipore Corp.).

Medium. SG agar medium and SG broth medium were used. The pH was 5.6.

Recording of radial growth of colonies. A 0.002-ml standard spore suspension of *T. mentagrophytes* Morita was inoculated by stabbing with a wire needle into the center of an SG agar plate containing the different concentrations of allicin, and the resultant colonies were measured along two diameters, crossing at right angles, marked on the back of the plates, and the average diameters of colonies were calculated. To determine the growth rate, this measurement was repeated at various intervals of time.

Germination test and morphological observation. A twofold-dilution series of allicin in molten SG agar medium at 45°C was prepared in 20-ml volumes in petri dishes, immediately added to a 0.1-ml standard spore suspension of *T. mentagrophytes* Morita, mixed well, and solidified in plates. The final concentration of allicin ranged from 0 to 12.5 μg/ml. After 24 h of incubation, square samples (10 by 10 mm) cut off from the plates were mounted in lactophenol cotton blue and examined microscopically. The number of germination of spores in a total of 500 was counted, and the percentage of germination was determined. A spore was considered to be germinated when the germ tube was as long as it was wide.

Morphological changes were observed at various intervals of time for 15 days of incubation.

Minimal inhibitory concentration (MIC). The agar dilution method used was as follows: a 0.01-ml viable-cell suspension of *Candida* or *Cryptococcus* was inoculated by making a 2-cm-long streak with a loop on an SG agar plate containing the different concentrations of allicin, and the plate was incubated at 37°C for 5 days. A 0.002-ml spore suspension of *Aspergillus* or *Dermatophytes* was inoculated by stabbing with a wire needle in the center of an SG agar plate containing the different concentrations of allicin, and the plate was incubated at 37 or 25°C for 5 or 15 days, respectively. The minimal concentration at which a colony did not grow after incubation at a suitable temperature for a set time was taken as the MIC. The broth dilution method was as follows: a twofold-dilution series of allicin in SG broth medium was prepared in 10-ml volumes in test tubes, inoculated with 0.05 ml of viable-cell or spore suspension, and incubated at a suitable temperature. The minimal concentration which caused complete inhibition of growth after a set time was taken as the MIC. The concentrations of allicin used were as follows: 0, 0.05, 0.1, 0.2, 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, and 25.0 μg/ml.

Effect of pH on activity. The MIC of allicin against fungi on medium with different pH values was determined by the agar dilution method on SG agar medium adjusted to a final pH value of 5.6, 6.0, 6.5, and 7.5 with inoculum sizes of 0.01 or 0.002 ml of a standard viable-cell or spore suspension.

Effect of inoculum size on activity. The MIC of allicin against fungi with different inoculum sizes was determined by agar dilution method on SG agar medium (pH 5.6) with an inoculum size of 0.01 or 0.002 ml of 10^4, 10^5, 10^6, and 10^7 viable cells or spores per ml.

Fungicidal activity test. A series of test tubes containing 10 ml of SG broth medium incorporating from 0 to 25 μg of allicin per ml was prepared, inoculated with 0.1 ml of a standard spore suspension of *T. mentagrophytes* Morita, and incubated at 25°C for 10 days. Samples (10 ml) were taken after 10 days of exposure to the drug, washed twice with large volumes of sterile saline to remove unabsorbed allicin, and resuspended in 0.5 ml of sterile saline; 0.2-ml samples of this suspension were spread over each of the SG agar slants, and the survival or death of the spores was determined by the presence or absence of colonies that developed within 15 days of incubation.

RESULTS

Antimicrobial spectrum. The MIC of allicin against *Candida, Cryptococcus, Aspergillus, Trichophyton, Epidermophyton,* and *Microsporum* was measured by the broth and agar dilution methods with an inoculum of a standard viable-cell or spore suspension incubated for 5 or 15 days. The results are given in Table 1. The MIC value against *Candida, Cryptococcus, Trichophyton, Epidermophyton,* and *Microsporum* was as low as 3.13 to 6.25 μg/ml on SG agar medium, and as low as 1.57 to 6.25 μg/ml in SG broth medium, even after either 5 to 15 days of incubation. The MIC value against *Aspergillus* was 12.5 to 25 μg/ml by both broth and agar dilution methods after 5 days of incubation. The antimicrobial activity of allicin against *A. fumigatus* Koboyama and *Dermatophytes* was stronger on agar medium than in broth medium. The MIC value of allicin against fungi changed significantly between 1 and 15 days of incubation. However, the MIC against *Candida* and *Cryptococcus* (except *C. neoformans* Duke) by the broth dilution method and the MIC against *Aspergillus* by both broth and agar dilution methods remained constant after 3 days of incubation. The MIC value against dermatophytes by both broth and agar dilution methods remained constant after 10 days of incubation.

Effect of inoculum size on activity. The MIC of allicin against *Candida, Cryptococcus, Aspergillus, Trichophyton, Microsporum,* and *Epidermophyton* on SG agar medium (pH 5.6) with different inoculum sizes was determined by the agar dilution method (Tables 1 and 2). The activity of allicin against these fungi, except *C. albicans* 381, *T. mentagrophytes* Morita, T-N-2, and T-N-4, and *T. ferrugineum* T-N-1 increased as inoculum size decreased from 10^7

TABLE 1. *MICs of allicin against various pathogenic fungi*

Organism[a]	Medium[b]	MIC (μg/ml)				
		1 day	3 days	5 days	10 days	15 days
Candida albicans 381	A	3.13	6.25	6.25		
	B	3.13	6.25	6.25		
C. albicans Nakamura	A	1.57	1.57	3.13		
	B	0.20	3.13	3.13		
Cryptococcus neoformans Duke	A	–[c]	1.57	3.13		
	B	–	1.57	3.13		
C. neoformans 1814	A	–	1.57	3.13		
	B	–	3.13	3.13		
C. neoformans M-1	A	–	0.79	3.13		
	B	–	1.57	1.57		
Aspergillus fumigatus Kuboyama	A	6.25	12.5	12.5		
	B	12.5	25.0	25.0		
A. fumigatus N-2	A	3.13	25.0	25.0		
	B	3.13	25.0	25.0		
A. fumigatus IFO 5840	A	25.0	25.0	25.0		
	B	12.5	25.0	25.0		
Trichophyton mentagrophytes Morita	A	–	1.57	3.13	3.13	3.13
	B	–	3.13	6.25	6.25	6.25
T. mentagrophytes T-N-2	A	–	1.57	3.13	3.13	3.13
	B	–	1.57	3.13	3.13	3.13
T. mentagrophytes T-N-4	A	–	1.57	3.13	3.13	3.13
	B	–	0.79	3.13	6.25	6.25
T. ferrugineum T-N-1	A	–	0.79	3.13	3.13	3.13
	B	–	3.13	6.25	6.25	6.25
T. rubrum Suzuki	A	–	0.79	1.57	3.13	3.13
	B	–	3.13	6.25	6.25	6.25
T. rubrum Ishida	A	–	–	0.78	1.57	3.13
	B	–	1.57	1.57	3.13	3.13
Microsporum gypseum Suzuki	A	–	1.57	1.57	3.13	3.13
	B	–	3.13	6.25	6.25	6.25
Epidermophyton floccosum Suzuki	A	–	–	0.78	3.13	3.13
	B	–	1.57	3.13	6.25	6.25

[a] Inoculum size was 0.01 or 0.002 ml of a suspension of 10^7 viable cells or spores per ml, except for *Microsporum* and *Epidermophyton*: inoculum size was 0.002 ml of suspensions containing 10^6 and 10^5 spores per ml, respectively.

[b] A, SG agar medium, pH 5.6; B, SG broth medium, pH 5.6.

[c] –, No growth.

to 10^4 viable cells or spores per ml.

Effect of medium pH on activity. The MIC of allicin against *Candida, Cryptococcus, Aspergillus, Trichophyton, Epidermophyton,* and *Microsporum* on SG agar medium with a pH of 5.6 to 7.5 was determined by the agar dilution method with an inoculum consisting of a standard viable-cell or spore suspension (Tables 1 and 2). The MIC of allicin against these fungi, except *C. albicans* Nakamura, *A. fumigatus* N-2 and IFO 5840, and *T. rubrum* Suzuki, was higher on the medium with a pH of 6.0 or above than on that with a pH of 5.6.

Radial growth of colony. The effects of various additions of allicin on radial growth of colonies of *T. mentagrophytes* Morita on SG agar medium are shown in Fig. 1. From the growth curve drawn for each concentration of allicin, the length of the lag phase was determined: at 0.78 or 1.57 μg/ml, the lag phase increased by 1 or 2 days over that of the controls, and the radial growth of colonies at subinhibitory concentrations was considerably restricted in comparison with the controls.

Effect of inoculum size on radial growth of colony. The effect of inoculum size on radial growth of colonies of *T. mentagrophytes* Morita after 15 days of incubation on SG agar medium containing 0 to 3.13 μg of allicin per ml is shown in Fig. 2. The size of the colonies decreased both when inoculum size was decreased from 10^7 to 10^5 spores per ml and when allicin concentration was increased from 0 to 3.13 μg/ml.

Effect of allicin on germination. The percent germination of spores of *T. mentagrophytes* Morita after 24 h in the presence of allicin (incorporated into SG agar medium) was

TABLE 2. *Effect of inoculum size and medium pH on the MIC of allicin against various pathogenic fungi*

Organism	MIC (μg/ml)[a]						
	Inoculum size[b] (viable cells or spores/ml)			pH of SG agar medium[c]			
	10^4	10^5	10^6	6.0	6.5	7.0	7.5
Candida albicans 381	6.25	–[d]	6.25	6.25	–	6.25	12.5
C. albicans Nakamura	1.57	–	3.13	3.13	–	3.13	3.13
Cryptococcus neoformans Duke	1.57	–	3.13	3.13	–	3.13	6.25
C. neoformans 1814	1.57	–	3.13	3.13	–	6.25	12.5
C. neoformans M-1	0.78	–	3.13	3.13	–	6.25	12.5
Aspergillus fumigatus Kuboyama	1.57	–	3.13	12.5	–	12.5	25.0
A. fumigatus N-2	3.13	–	12.5	25.0	–	25.0	25.0
A. fumigatus IFO 5840	1.57	–	25.0	25.0	–	25.0	25.0
Trichophyton mentagrophytes Morita	–	3.13	3.13	6.25	6.25	12.5	–
T. mentagrophytes T-N-2	–	3.13	3.13	3.13	3.13	6.25	–
T. mentagrophytes T-N-4	–	3.13	3.13	6.25	6.25	12.5	–
T. ferrugineum T-N-1	–	3.13	3.13	6.25	6.25	12.5	–
T. rubrum Suzuki	–	1.57	3.13	3.13	3.13	3.13	–
T. rubrum Ishida	–	1.57	3.57	3.13	3.13	6.25	–
Microsporum gypseum Suzuki	1.57	3.13	1.13	3.13	3.13	6.25	–
Epidermophyton floccosum Suzuki	1.57	3.13	–	3.13	3.13	6.25	–

[a] Expressed as a value determined after 5 days of incubation against *Candida, Cryptococcus,* and *Aspergillus,* and as that after 15 days of incubation against *Dermatophytes.*

[b] Inoculum size was 0.01 or 0.002 ml of suspensions containing 10^4, 10^5, and 10^6 viable cells or spores per ml on SG agar medium (pH 5.6).

[c] Inoculum size was 0.01 or 0.002 ml of a suspension containing 10^7 viable cells or spores per ml, except for *Microsporum* and *Epidermophyton*: inoculum size was 0.002 ml of suspensions containing 10^6 and 10^5 spores per ml, respectively.

[d] –, Not tested.

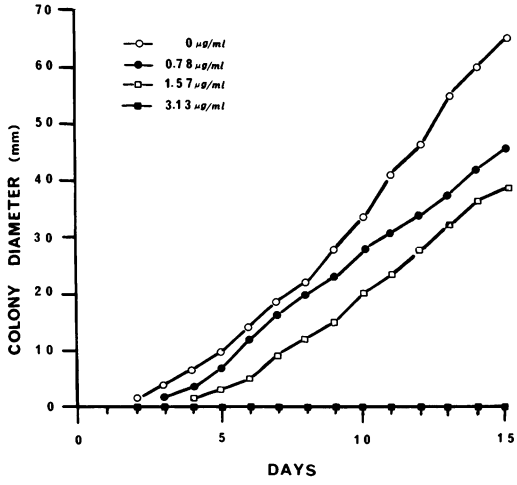

FIG. 1. *Effect of allicin on radial growth of colonies of Trichophyton mentagrophytes Morita on SG agar medium.*

determined by counting the number of germinating spores microscopically. The results are plotted in Fig. 3. Allicin markedly inhibited swelling and germination of spores at concentrations of 3.13 μg or more per ml. The numbers that were germinated decreased with increasing allicin concentration, until a concentration was reached at which no swelling of the spores occurred (12.5 μg/ml). After 24 h of incubation, the numbers of spores that germinated at subinhibitory concentrations of allicin increased, and a few germinated at a concentration that had been inhibitory for 24 h. This was probably due to the normal decomposition of allicin, but the small numbers of spores that germinated at high concentrations could not continue to develop into normal hyphae.

Effect of allicin on hyphae. Morphological effect of allicin on hyphae of *T. mentagrophytes* Morita was observed as follows: on normal SG agar medium, the germ tubes grew rapidly; they were long and regularly branched, and their tips appeared normal. But on SG agar medium containing 3.13 μg of allicin per ml, spherical hyphae or bamboo-joint-like hyphae occurred after 8 or 15 days of incubation, respectively (Fig. 4). These abnormal hyphae were shorter and thicker than those in the controls. They resulted from the noticeable inhibition of longitudinal extension of hyphae by allicin.

Fungicidal activity of allicin. At low concentrations, nystatin is fungistatic to *Aspergillus*

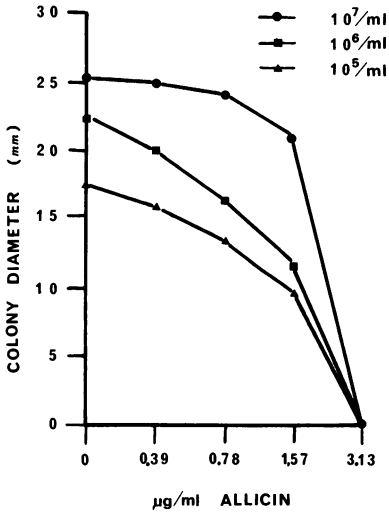

FIG. 2. *Effect of inoculum size on radial growth of colonies of Trichophyton mentagrophytes Morita at 15 days on SG agar medium containing various amounts of allicin. The inoculum size was 0.002 ml of a suspension containing 10^7 to 10^5 spores per ml.*

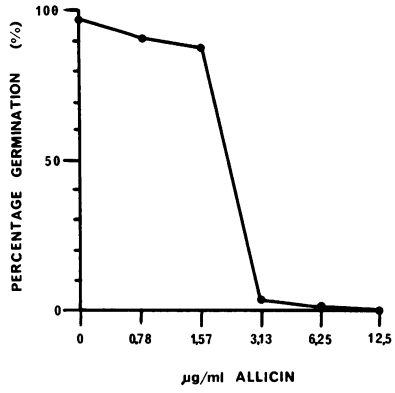

FIG. 3. *Percentage of germination of spores of Trichophyton mentagrophytes Morita after 24 h in SG agar medium containing various amounts of allicin.*

species, but at high concentrations it becomes fungicidal (5). Therefore, the following experiment was performed to determine the concentration of allicin at which this occurred with *T. mentagrophytes* Morita. After exposure to allicin concentrations ranging from 0 to 25 µg/ml in SG broth medium at 25°C for 10 days, the spores were removed from unabsorbed allicin by washing with sterile saline, inoculated onto each of SG agar slants, and incubated. The survival or death of the spores was determined by the presence or absence of colonies that developed on each of the slants within 15 days of incubation. The results are given in Table 3. No significant fungicidal action was noted at allicin concentrations ranging from 1.56 to 12.5 µg/ml, although growth on the slants was delayed from 1 to 4 days in comparison with the controls, but at concentration of 25 µg/ml no spores survived. Since the MIC of allicin against the Morita strain was 6.25 µg/ml in SG broth medium, we conclude that the fungicidal effect occurred at a concentration of allicin about four times higher than the fungistatic concentration.

DISCUSSION

Allicin was found to be highly effective in vitro against *Candida, Cryptococcus, Trichophyton, Epidermophyton,* and *Microsporum.* However, less activity was demonstrated against *Aspergillus.* The MIC value of allicin against these organisms was considerably affected by inoculum size, medium pH, type of medium, and incubation time; also, there were some differences in the MIC according to whether solid or liquid medium was used; i.e., more allicin was required to inhibit growth in liquid than in solid medium. This may have happened because allicin deteriorated more rapidly in liquid than in solid medium. Heavy inocula are known to be far less susceptible to fungicides than light inocula (3). The decreased susceptibility to allicin in medium with a pH of more than 5.6 may be partly a consequence of the further degradation of allicin at a pH value higher than 5.6 and partly a consequence of the decrease of acidity of medium. It is desirable to express the MIC of fungicides as a value determined after 5 days of incubation against *Candida, Cryptococcus,* and *Aspergillus* and as determined after 15 days of incubation against *Dermatophytes.*

Allicin affected germination of spores of *T. mentagrophytes* Morita and the morphology of the hyphae. The lengthening in the lag phase at concentrations approaching the inhibitory level resulted from the delay in swelling and germination of spores, and the restriction of the radial growth of fungal colony on SG agar medium containing allicin resulted from the noticeable inhibition of longitudinal extension of hyphae. The level of allicin necessary to inhibit spore germination was the same as that needed to inhibit hyphal growth. The small numbers of spores that germinated at the inhibitory concentration after 24 h developed into the hyphae with abnormal morphology and could not grow any longer during 15 days of incubation. This suggests a possible fungicidal effect.

In the fungicidal test, it was found that the

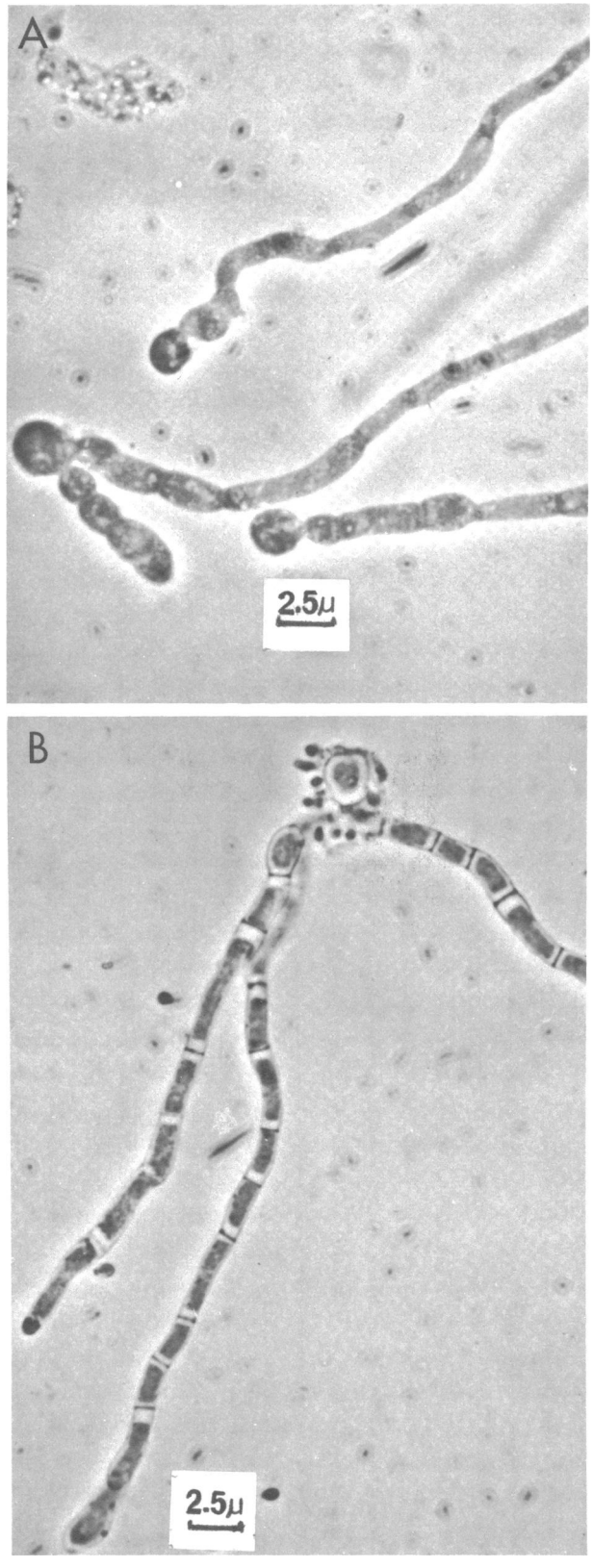

FIG. 4. *Abnormal hyphae of Trichophyton mentagrophytes Morita in SG agar medium containing 3.13 μg of allicin per ml. (×350) (A) Spherical hyphae, at 8 days; (B) bamboo-joint-like hyphae, at 15 days.*

TABLE 3. *Death of spores of Trichophyton mentagrophytes Morita caused by exposure to allicin in SG broth medium*[a]

Concn of allicin in SG broth medium (μg/ml)	Growth of colonies on SG agar slant[b] after days of incubation:														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
25.0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
12.5	−	−	−	−	+[c]	+	+	+	+	+	+	+	+	+	+
6.25	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+
3.13	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+
1.56	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+
0	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+

[a] Exposure was for 10 days at 25°C.

[b] Survival or death of the spores after exposure to allicin was determined by the presence or absence of colonies that developed on SG agar slant within 15 days.

[c] +, Colonies present; −, no colonies.

spores were killed by allicin at a concentration only four times greater than that which caused fungistasis.

Cavallito et al. (1, 2) reported that an allicin solution has a pH of approximately 6.5 and shows immediate inactivation upon addition of an excess of any of the standard alkalis, although dilute acids have no effect, and that nonalkaline, aqueous, or nonaqueous solutions or dry preparations undergo a chemical change after standing at room temperature, resulting in an inactive liquid usually within 2 days. Yet, the data obtained with the fungicidal test suggest that the allicin added to the SG broth medium retained its activity for at least 10 days. At present, it is difficult to explain this discrepancy concerning the stability of allicin, but it may have occurred because allicin is comparatively stable in the presence of SG medium as well as in the presence of blood and artificial gastric juice (4) or because the adsorbed allicin on the spore or cell is more stable than free allicin in the medium.

Studies on the mechanism of action of allicin are now in progress. The details of these investigations will be published elsewhere.

ACKNOWLEDGMENTS

We are grateful to K. Fujita for his encouragement throughout this work, and to S. Kuroki, Y. Hoshino, K. Yonemitsu, and K. Kinukawa for their technical assistance.

LITERATURE CITED

1. **Cavallito, C. J., and J. H. Brailey.** 1944. Allicin, the antibacterial principle of *Allium sativum*. I. Isolation, physical properties and antibacterial action. J. Am. Chem. Soc. 66:1950–1951.
2. **Cavallito, C. J., J. S. Buck, and C. M. Suter.** 1944. Allicin, the antibacterial principle of *Allium sativum*. II. Determination of the chemical structure. J. Am. Chem. Soc. 66:1952–1954.
3. **Horesfall, J. G.** 1945. Fungicides and their action. *In* F. Verdoon (ed.), Annales cryptogamici et photopathologici, vol. 2. Chronica Bontanica Co., Waltham, Mass.
4. **Raghunandana, R. R., R. S. Srinivasa, and P. R. Venkatarman.** 1946. Investigations on plant antibiotics. I. Studies on allicin, the antibacterial principle of *Allium sativum* (garlic). J. Sci. Ind. Res. 1B:31–35.
5. **Stanley, V. C., and M. P. English.** 1965. Some effects of nystatin on the growth of four *Aspergillus* species. J. Gen. Microbiol. 40:107–118.