STUDY ON FUNGICIDES (XV)

EFFECT OF THE COMBINATION OF AMPHOTERICIN B AND TETRACYCLINE ON MYCELIAL GROWTH OF FUNGI

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Tetracycline (TC), suppresses the growth of bacteria, but has been found to have no fungicidal effect. However, it is expected that fungal growth would be inhibited by elevating the TC induced permeability of the eucaryotic cell membrane. Namely, *Cochliobolus miyabeanus* belonging to the Ascomycetes cell membrane is first broken with amphotericin B (AMPH B) so as to induce leakage and then the cell is treated with TC. The results obtained were as follows.

1) 0.08 μg/ml of AMPH B or 137 μg/ml of TC were required to suppress 50% (ED_{50}) of mycelial growth.

2) 50% of fungal growth was inhibited when 45 μg/ml of TC was added in the presence of 0.04 μg/ml of AMPH B. This inhibition was not as remarkable as when AMPH B was used alone.

3) The incorporation of ^{14}C-amino acid into cells was largest when the cells were incubated with 0.04 μg/ml of AMPH B and after washing further incubated with 10 μg/ml of TC.

4) High molecular protein (more than 30,000 m. w.) in mycelial cells inactivated the activity of TC against growth of *E. coli*.

Key words : Tetracycline, Amphotericin B, Mycelia

Amphotericin B (AMPH B) was discovered by Gold et al. in 1956[1] and has been applied to the treatment of profunda mycosis and sublimits mycosis[2], because it has a wide fungicidal spectrum and effects. Furthermore, resistance to AMPH B is acquired slowly. However, AMPH B has some disadvantages : it is toxic for some organs, including the kidney, is only with difficulty soluble in water and thus scarcely migrates into the blood when orally administered[3]. The acute toxicity of AMPH B in mouse is relatively high. When it is intravenously injected in mice, the ED_{50} is 11 to 15 mg/kg[4]. When intravenously injected in human subjects, vomiting, chill or hematuria have been observed. Accordingly, the required dose should be as low as possible so as to reduce its toxicity. Tetracycline (TC) suppresses bacterial growth by inhibiting binding to the 70 S ribosome or 30 S subunit of aminoacyl-t-RNA, though it has been thought to have no antifungicidal effect[5]. However, FRANKLIN et al. clarified that TC suppresses protein synthesis by fungi consisting of eucaryotic cells[6]. Thus we hypothesized that mycelial growth could be suppressed by elevating the permeability of the eucaryotic cell membrane by the synergistic effect of combined AMPH B and TC, each at a low concentration. Namely, the fungal cell membrane is first broken with AMPH B so as to induce leakage of intracellular components and then the cell is treated by TC. This study attempts to clarify this phenomenon.

MATERIALS AND METHODS

1. Materials

(i) The effects of the antifungal agent were

evaluated using *Cochliobolus miyabeanus* F-01 (Ascomycetes spp. provided by Laboratory of Microbiology, Kobe Women's College of Pharmacy) which had been subcultured in 2% sucrose containing a potato liquid medium.

(ii) Major reagents employed in this study were : amphotericin B (AMPH B 60 mg titer, containing 45 mg of sodium deoxycholate as a solvent, Sankyo Co.), tetracycline hydrochloride (TC, Lederle Japan, Ltd.) ; and bovine albumin (Sigma Co.).

2. Evaluation of antifungal activity

A potato sucrose medium was pipetted in 100 ml portions into shaking flasks (300 ml) and sterilized. Then AMPH B or TC was added at a definite concentration. The growing mycelial tip of the F-10 stain, which had been preliminarily incubated in a plate medium, or a spore suspension of this fungus was inoculated to the medium and incubated, either statically or under shaking, in the dark at 28°C for a definite period. After completion of the incubation, growing mycelia were harvested, dried at 110°C and weighed. The antifungal activity was evaluated by comparing the weight with that of a control. Separately, an agar plate medium containing definite amounts of the active compounds was inoculated with fungal spores which were then incubated in the dark at 28°C for a definite period. Then the diameter of the growing mycelial colony thus formed was measured.

3. Incorporation of amino acid into mycelial cells grown in the presence of AMPH B and TC

Ninety millilitre of potato liquid medium was pipetted into a Meyer flask (300 ml) provided with a needle on the bottom and a ^{14}C-amino acid mixture was added. With the tip of the needle, 3 mm of the mycelial tip of the F-10 stain, which had been preliminarily grown on an agar plate, was inoculated and statically incubated at 28°C in the dark under the following conditions (a) to (d).

(a) 0.4 mg/ml of AMPH B, corresponding to 1/2 of the 50% inhibition dose, was added and incubated for 96 hours.

(b) 0.04 mg/ml of AMPH B and 10 mg/ml of TC were added and incubated for 96 hours.

(c) The incubation was conducted for 48 hours in the presence of 0.04 mg/ml of AMPH B. Then the growing mycelia were washed with sterilized water and collected by filtration. The mycelia were then incubated in a medium containing 10 mg/ml of TC for 48 hours.

(d) Neither AMPH B nor TC was added and incubation was conducted for 96 hours (as control).

Mycelia growing under those conditions (a) to (d) were harvested, washed with water, dried and weighed. The mycelia were then treated with 1 N NaOH at 100°C for 3 hours and radioactivity incorporated into the soluble fraction was measured with a liquid scintillation counter.

4. Effect of AMPH B and TC on the incorporation of amino acid into mycelia protein fraction

Mycelia obtained by incubating for 72 hours under shaking were thoroughly washed and treated with a homogenizer for 10 seconds. Then the mycelia were cut into pieces to give a sample. This sample was divided into four lots and each lot was treated in the following manner.

(I) Cells were suspended in a 1% glucose solution containing 0.04 mg/ml of AMPH B and incubated at 27°C for 120 minutes followed by thorough washing with water. The cells were suspended again in a 1% glucose solution containing 10 mg/ml of TC and a ^{14}C-amino acid mixture and incubated at 27°C for 30 minutes and 60 minutes.

(II) Cells incubated in the same medium containing AMPH B as that used in (I) were washed and further incubated in a glucose solution containing 2 mg/ml of cyclohexide (CH) and a ^{14}C-amino acid mixture for additional 30 minutes and 60 minutes.

(III) Cells incubated in the presence of AMPH B as described in (I) were further incubated in a 1% glucose solution containing a ^{14}C-amino acid mixture at 27°C for 30 minutes and 60 minutes.

(IV) Cells were incubated in a 1% glucose solution at 27°C for 120 minutes and then further incubated in a 1% glucose solution containing a ^{14}C-amino acid mixture as the control.

Each sample thus obtained was thoroughly washed with water and centrifuged at 3,500 rpm for 5 minutes. The precipitate was divided into two fractions. One of these was extracted with 5% TCA at 100°C for 10 minutes. After completion of the extraction, the residue was collected by centrifuging at 3,500 rpm for 5 minutes and alkali soluble was heat-extracted with 1 N NaOH. The radioactivity of the amino acid incorporated into the intracellular protein was measured with a liquid scintillation counter.

5. TC inactivation

The minimum inhibitory concentrations (MIC) against *E. coli* in a nutrient broth medium were determined with the following systems.

(i) 0.1, 1, 5 or 10 mg/ml of TC alone was added.

(ii) 0.04 mg/ml of AMPH B was simultaneously added to the amounts in (i).

(iii) 0.04 mg/ml portions of TC and AMPH B and 0.5 g of mycelial pieces were added.

(iv) Mycelia were grown in the presence of 0.04 mg/ml of AMPH B for 72 hours. To the filtrate thus obtained, TC in the amounts defined in (i) was added.

Each fraction was inoculated with 0.02 ml of an *E. coli* suspension, which had been preliminarily incubated at 37°C for 24 hours. After incubating the strain at 37°C for 48 hours, the minimum inhibitory concentration was determined by a turbidity test.

6. TC-inactivating substance

(1) Mycelia which had been preliminarily incubated in a potato sucrose medium for 4 days were further incubated in two 2% glucose solutions, one containing 0.08 mg/ml of AMPH B and the other without AMPH B, at 28°C for 48 hours. The cells were removed by centrifugation at 1,500 rpm for 2 minutes and the obtained supernatant was dialyzed (MW cut-off 1000) and concentrated *in vacuo*, To 0.4 ml of the obtained solution, TC was added so as to give a final concentration of 1.0 mg/ml. Then the mixture was allowed to stand at 28°C for 0, 60, 90 and 120 minutes. Subsequently the reaction mixture was added to 1.5 ml of a nutrient broth medium which was then inoculated with *E. coli*. After incubating at 38°C for 48 hours, the TC activity was measured.

(2) After removing glucose from the mycelial culture filtrate which was obtained as described in (1), it was concentrated and chromatogramed with the Sephadex G-50 column (coarse type, 2×4 cm). Thus it was divided into a high molecular weight (HF) of MW 2,000 or above, and a low molecular weight fraction (LF) of MW less than 2,000. To 0.4 ml portions of the HF and LF, TC was added so as to give a final concentration of 10 mg/ml. Each mixture was allowed to stand at 28°C for 120 minutes, then immediately added to a nutrient broth medium which was then inoculated with *E. coli*. The growth of this bacterium was observed.

(3) To 0.4 ml portions of a bovine albumin solution and an ovoalbumin solution, each prepared so as to give a final concentration of 20,000, 10,000, 5,000 or 2,000 μg/ml, TC was added so as to give a final concentration of 1.0 mg/ml. After incubating at 28°C for 120 minutes, a nutrient broth medium was added to each mixture and the growth of *E. coli* was monitored.

(4) To 0.4 ml portions of an $MgCl_2$ solution and a KCl solution, each prepared so as to give a final concentration of 0.4 M, TC was added so as to give a final concentration of 1.0 mg/ml. After incubating at 28°C for 120 minutes, a nutrient broth medium was added to each mixture and the growth of *E. coli* was measured.

RESULTS AND DISCUSSION

1. Stability of AMPH B

The stability of AMPH B at 28°C is shown in Fig. 1. A potato liquid or agar medium containing AMPH B was inoculated with *Cochliobolus miyabeanus* mycelia and incubated at 28°C in the dark and the growth of the mycelia was observed. In each case, mycelial growth was suppressed to a degree of 80% or more by AMPH B. It was assumed that this chemical showed no decrease in titer until the fourth day of incubation. The following experiments were conducted based on this assumption.

2. Effects of AMPH B or TC on mycelial growth

Potato sucrose liquid and agar plate media containing AMPH B or TC at various concentrations were inoculated with conidia of *C. miyabeanus*. After incubating at 28°C for 4 days, the growing weight or colonial size of the growing fungi was

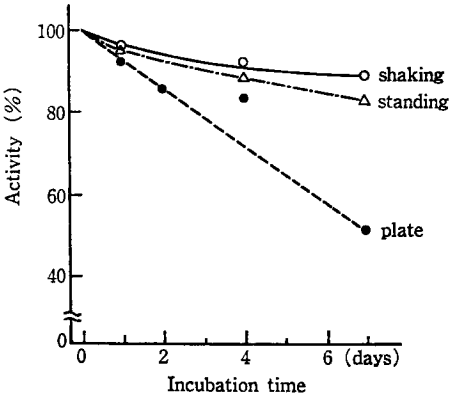


Fig. 1. Stability of amphotericin B in potato liquid medium at 28°C

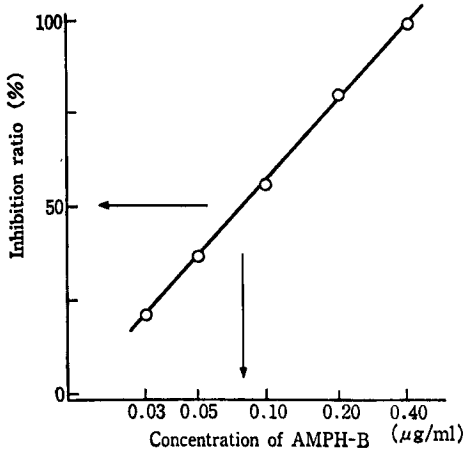


Fig. 2. Inhibitory effect of amphotericin B on mycelial growth by shaking culture

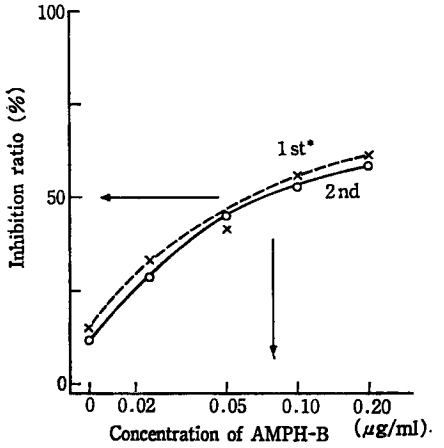


Fig. 3. Inhibitory effect of amphotericin B on mycelial growth by plate agar culture
* experimental No.

determined. Results (Figs. 2~4) showed that 0.08 μg/ml of AMPH B or 137 μg/ml of TC were required to suppress mycelial growth at a rate of 50% (ED_{50}). In Table 1, the ED_{50} of AMPH B and TC are compared with those of cyclohexymide (CH), griseofulvin (GRF), erythromycin (EM) and thiamphenicol (TP) on mycelial growth. Antifungal agents AMPH B, CH and GF suppressed fungal growth each at a low concentration. Among these compounds, AMPH B in particular showed a remarkable effect. In contrast, antibacterial agents TC, EM and TP required a high concentration.

3. Effects of the combined use of AMPH B and TC on mycelial growth

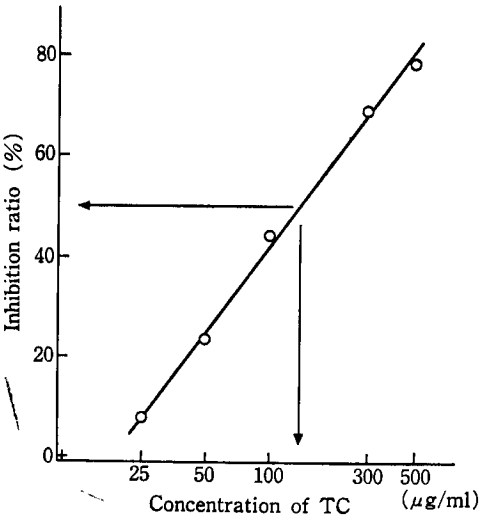


Fig. 4. Inhibitory effect of tetracyclin on mycelial growth by plate agar culture

Table 1. ED_{50} of various drugs against mycelial growth

	ED_{50}
Amphotericin B (AMPH B)	0.08 μg/ml
Tetracycline (TC)	137.0
Cyclohexide (CH)	4.5
Griseofulvin (GRF)	1.5
Erythromycin (EM)	2,000.0
Thiamphenicol (TP)	3,600.0

The action mechanism of TC comprises the inhibition of the binding of bacteria to the codon-dependent 70 S ribosome or the 30 S subunit of aminoacyl-t-RNA[5]. Tetracycline shows scarcely any antifungal effect, namely on eucaryotes. FRANKLIN reported that addition of TC to eucaryotic cells does not affect protein synthesis but that protein synthesis of eucaryotic cells in a cell-free system is inhibited by this chemical[6]. Therefore we expected that TC would exert a satisfactory antifungal effect if able to permeate through the fungicidal cell membrane and enter into cells. Amphotericin B, on the other hand, induces leakage of intracellular components by binding to ergosterol so as to damage the fungal cell membrane. Therefore we studied the potential synergistic effects of combined AMPH B and TC. The results are shown in Figs. 5, 6 and Table 2. Fig. 5 shows the inhibition rate (%) of fungal growth determined by varying the amount of TC while

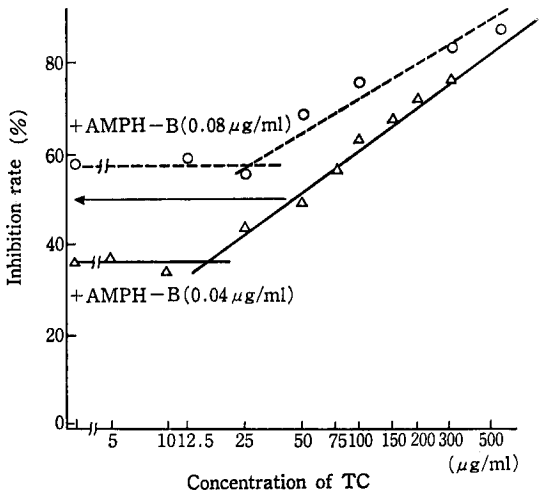


Fig. 5. Effects of combined use of amphotericin B and tetracycline on mycelial growth by shaking culture

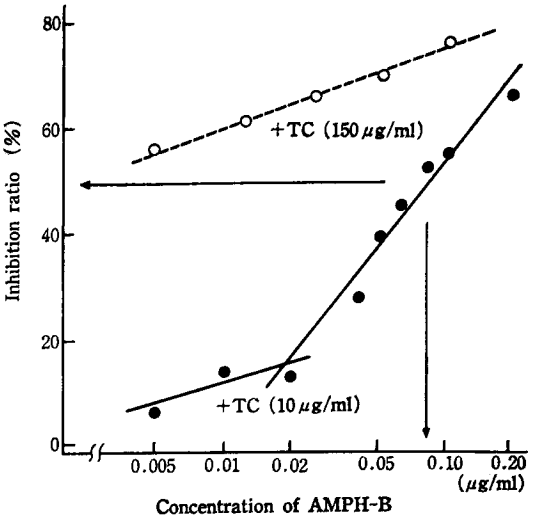


Fig. 6. Effects of combined use of amphotericin B and tetracycline on mycelial growth by plate agar culture

keeping the amount of AMPH B constant, and Fig. 6 shows the inhibition rate (%) determined by varying the amount of AMPH B while keeping the amount of TC constant. As shown in Figs. 5 and 6, 60% of the fungal growth was inhibited when 12 μg/ml of TC was added in the presence of 0.08 μg/ml of AMPH B. When the amount of AMPH B was 0.04 μg/ml, 45 μg/ml of TC was required in order to achieve an inhibition rate of 50%. On the other hand, fungal growth was inhibited at a rate of 60% by adding 0.05 μg/ml of AMPH B in the presence of 150 μg/ml of TC, though this inhibition was not so remarkable as that observed when 150 μg/ml of TC was added alone. These results showed scarcely any synergistic effect, unexpectedly, when TC and AMPH B were added simultaneously. In contrast, Table 2 shows the inhibition rate of mycelial growth calculated from cell weight and colonial size and the results of the determination of ^{14}C-amino acid incorporated into cells in the following experimental systems (i to iv).

(i) Cells were incubated in a medium containing 0.04 μg/ml of AMPH B for 96 hours.

(ii) Cells were incubated in a medium containing 0.04 μg/ml of AMPH B and 10 μg/ml of TC for 96 hours.

(iii) Cells were incubated in a medium containing 0.04 μg/ml of AMPH B for 48 hours. After washing, these cells were further incubated in a medium containing 10 μg/ml of TC for 48 hours.

(iv) Cells were incubated in a medium containing neither AMPH B nor TC for 96 hours.

As shown in Table 2, the growth of cells incubated in a medium containing AMPH B alone (i)

Table 2. Effects of AMPH B and TC against incorporation of amino acid into mycelia

	Inhibition (%)		Incorporation (dpm) (ratio %)
	diameter	weight	dpm/ml protein
(i) AMPH*→AMPH	21.0	24.9	21,238 (109.6)
(ii) AMPH+TC	18.0	28.8	19,296 (99.6)
(iii) AMPH→TC	44.8	48.5	22,162 (114.4)
(iv) Control	0.0	0.0	19,372 (100.0)

* AMPH B

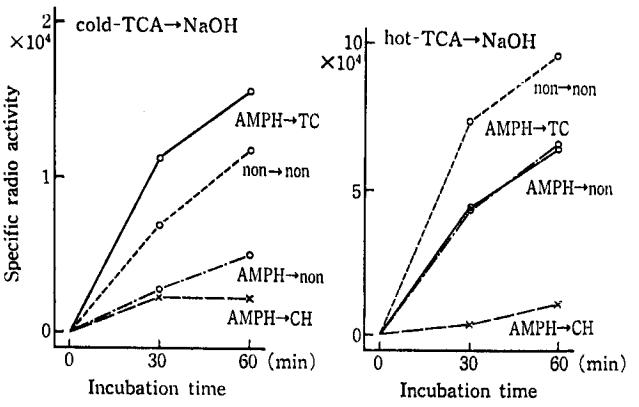


Fig. 7. Effects of amphotericin B and tetracycline on incorporation of amino acid into intact cells

Table 3. Effects of AMPH B and TC on incorporation of amino acid into mycelial protein

Treatment	Extracted with	
	Cold-TCA	Hot-TCA
(i) AMPH→TC	130.17%	68.73%
(ii) AMPH→non	—	63.17
(iii) Control	100.00	100.00

and in one containing AMPH B and TC (ii) was inhibited at a rate of 20 to 25%, compared with the control (iv). No significant difference was observed between systems (i) and (ii). In contrast, the growth of the cells in system (iii), in which the cells were first treated with AMPH B, washed with saline and then treated with TC, was inhibited at a rate of 45 to 50%. System (iii) showed the largest amount of amino acid incorporated into cells, while systems (i) and (ii) showed scarcely any difference (cf. Table 2). These results suggest that the expected synergistic effects can be achieved by treating cells with AMPH B and with TC, rather than by treating cells in the coexistence of AMPH B and TC.

4. Effects of AMPH B and TC on the incorporation of amino acid into intact cells

The fact that the largest amount of amino acid was incorporated in the cells whose growth was the most seriously inhibited by AMPH B and TC was examined. Growing mycelia were cut into fine pieces and further allowed to grow in the presence of AMPH B. Subsequently mycelia were suspended again in a medium containing TC (i) and in a TC-free medium (ii). As shown in Fig. 7, ^{14}C-amino acid was then incorporated in these cells. The mycelia were washed with cold-TCA and then treated with hot-KOH. The RI activity of the extract thus obtained was measured. It was confirmed that the amount of incorporated amino acid increased with the addition of TC (i), as shown in the results. When these mycelia were washed with hot-TCA, however, no difference was observed in the incorporation of (i) and (ii) (Table 3). When the amino acid incorporation in the control system was referred to 100%, that of the system cells incubated in the presence of TC were washed with cold-TCA, showed an increase to 130%, while the other system in which cells were washed with hot-TCA showed a decrease to 69%. TC binds to the 30 S ribosome of a eucaryotic cell so as to inhibit specific binding of the 30 S ribosome of aminoacyl-t-RNA to the m-RNA ribosome complex at A site. As described, FRANKLIN reported that TC allowed protein synthesis in a eucaryotic cell to proceed up to the stage of binding of t-RNA to amino acid but inhibited subsequent synthesis in a cell-free system. Thus TC-induced inhibition causes the accumulation of aminoacyl-t-RNA in cells. In this study, free amino acid was exclusively extracted from cells washed with cold-TCA, while not only free amino acid but also nucleic acid were extracted with hot-TCA. Accordingly, the radio activity of the NaOH-treated fraction was comparable to that of the control, since t-RNA binding to amino acid was extracted in the former case.

5. TC activity in coexistence with AMPH B

Table 4. Effects of various treatments on antibacterial activity (against *E. coli*) of TC

Treatment	MIC (μg/ml)
1) TC	1.0>
2) TC+AMPH	0.1>
3) TC+AMPH+fungi	10.0<
4) TC+filtrate of (AMPH+fungi)	10.0<

Table 5. Growth inhibition of *E. coli* by AMPH B and TC

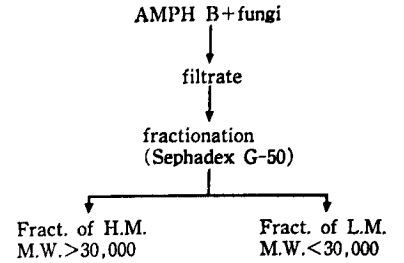
Exp. No.*	Time (min)			
	0	60	90	120
1	—	—	±	+
2	—	—	±	+
3	—	±	+	+

* No.1 medium+AMPH B → TC+medium
No.2 medium only → TC+medium
No.3 medium only → medium only

As shown in Table 2, no synergistic effect of TC and AMPH B on mycelial growth was observed in the medium containing both. In contrast, a remarkable inhibition effect was observed when mycelia grown in the presence of AMPH B were washed and then further grown in a medium containing TC. Thus it was attempted to clarify the reason for this. Table 4 shows the MICs of AMPH B and TC against the growth of *E. coli*. The MIC of TC was 1 μg/ml or below, while that of TC and AMPH B was 0.1 μg/ml. However no inhibition of the growth of *E. coli* was observed when TC was present together with AMPH B and mycelia or when mycelia were grown in the presence of AMPH B for 72 hours and then TC was added to the filtrate, even at a high TC concentration of 10 μg/ml or above. This might suggest that some substance capable of inactivating TC was formed in the incubation supernatant when mycelial cells were present together with AMPH B.

6. Detection of TC inactivating substance

Mycelia were grown in two 2% glucose solutions, one containing AMPH B and the other without for 48 hours. To each filtrate of incubation thus obtained, TC was added so as to give a concentration of 1.1 μg/ml. The mixture was allowed to stand at 25°C for 0, 60, 90 and 120 minutes. Then



Scheme 1. Fractionation of proteins from secreted materials in culture filtrate

Table 6. Effects of additive compound on TC activity

TC	Additive comp.	Growth
1.1*	fract. H.M. (protein 25.0*)	+**
1.1	fract. L.M. (protein 25.0*)	−
1.1	serum albumin (20,000)	−
1.1	ovo-albumin (20,000)	+
1.1	Mg^{2+} (0.4 mM as $MgCl_2$)	+
1.1	Ca^{2+} (0.4 mM as $CaCl_2$)	+
1.1	non	−

* μg/ml
** growth of *E. coli*

the inhibition effect of TC on the growth of *E. coli* was observed. As shown in Table 5, the activity of TC disappeared after contacting TC with the filtrate for 120 minutes. It is assume that when AMPH B was added, TC was inactivated after 120 minutes by a substance which was leaked from the cells. Then mycelia were incubated in a glucose solution containing AMPH B and the filtrate was fractionated with a Sephadex G-50 column. Thus a high molecular weight fraction (FH) of MW 30,000 or above and a low molecular weight fraction (FL) of MW less than 30,000 were obtained. As shown in Scheme 1, 1.1 μg/ml portions of TC were added to these fractions and each mixture was allowed to stand at 25°C for 120 minutes. Then the inhibition effect of the growth of *E. coli* was examined. Results showed that the FH inactivated TC while the FL did not. It is reported that TC is generally inactivated by metal ions[7]. As shown in Table 6, the effect of TC on inhibiting bacterial growth was obviously inactivated by Mg^{2+} and Ca^{2+} ions. However no inactivation of TC was observed in the FL. These results suggest that the metal ions leaking from cells would inactivate TC. It is also reported that

TC is inactivated by serum protein[8]. We therefore assume that protein leaking from cells caused the inactivation of TC by the FH. A study on the polymer(s) capable of inactivating TC is now in progress.

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References

1) VINING L C : The polyene antifungal antibiotics. Hindustan Antibiotics Bull., 3, 37, 1960
2) TORTORA G J, FUNKE B R, CASE C L : Microbiology. An Introduction, p. 508, 1986, The Benjamin/Cummings Publishing Company Inc.
3) IRIE M, KURODA H, NANBA H : Yakugakusei no tameno biseibutugaku, Hirokawasyoten, p. 224, 1985
4) SUMIKI Y : Antibiotics, II, p. 87, Tokyo Univ. Press, 1973
5) KURODA H henn : Kiso seibutuyakuhinnkagaku dai 2 hann, Hirokawasyoten, p. 79, 1982
6) FRANKLIN T J : Biochem. J., 87, 449, 1963
7) LAMPEN J O : Biochemical Studies of Antimicrobial Drugs. (Sixteenth Symposium of the Society for General Microbiology), Univ. of Cambridge Press, p. 122～123, 1966
8) KUNIN C M, FINLAND M : Clin. Pharmacol., 2, 51, 1961

抗糸状菌剤に関する研究（第 15 報）

—真菌菌糸の生育におよぼす amphotericin B と tetracycline との併用効果について—

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抗細菌剤として臨床で使用されている tetracyclin (TC) は，抗真菌作用を示さないことが知られている。しかし，TC は無細胞系では真核細胞のタンパク合成を阻害することが明らかにされている。そこで，TC の真菌増殖抑制作用を，ascomycetes に属する *Cochliobolus miyabeanus* を用いて検討し，つぎの結果を得た。

1) Amphotericin B (AMPH B) は 0.08 μg/ml の濃度で，菌糸の生育を 50% 抑制したが，TC は 137 μg/ml を要した。

2) AMPH B 0.04μg/ml と TC 45μg/ml の両薬物を併用しても菌糸生育抑制は，AMPH B 単独 0.04 μg/ml 添加の場合と同じであった。すなわち，TC を AMPH B と共存させた培地で菌糸を培養させた時には，TC の抗菌性はまったく認められず，AMPH B のみの抑制効果しか得られなかった。

3) AMPH B 0.04μg/ml 存在下で菌糸をまず前培養させたのち，つぎに TC 10μg/ml 存在培地で生育させた場合は，2) に比べ生育抑制は約 2 倍であった。

4) AMPH B および TC 共存下での菌体内への ^{14}C-アミノ酸のとりこみは，AMPH B 単独添加および TC との併用添加画分では，薬物無添加の画分と比べても差は認められなかった。しかし，AMPH B 存在下で生育させたのち，TC 添加培地でさらに増殖させた画分では，とりこみ量の増加は認められた。

5) 本菌の培養ろ液から分子量 3,000 以上，およびそれ以下の 2 種のタンパク画分を精製したところ，高分子タンパクの添加により CT の活性は失なわれた。

以上の結果，AMPH B により，まず菌糸に傷害を与えたのち TC を添加すると，真菌の生育抑制がみられた。しかし，菌糸から分泌される分子量 3,000 以上のタンパクにより TC は不活化することが認められた。

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