

STUDY ON FUNGICIDES (XVI)

COMBINED EFFECT OF TETRACYCLINE AND POLYMYXIN B ON MYCELIAL GROWTH

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(Received, July 13, 1989 · Accepted October 25, 1989)

We studied the combined effect of tetracycline (Tc) and polymyxin B (PLB) against the mycelial growth of *Cochliobolus miyabeanus*, an *Ascomycetes* sp., and obtained the following results.

(1) The concentration of Tc and PLB required to inhibit 50% of mycelial growth (ED_{50}) was 140 $\mu\text{g/ml}$ and 7.5 $\mu\text{g/ml}$.

(2) Compared with the addition of either Tc or PLB, simultaneous addition of both drugs showed 3 ~ 8 times stronger inhibition. (17.5 $\mu\text{g/ml}$ Tc and 1.0 $\mu\text{g/ml}$ PLB).

(3) The incorporation of ^{14}C -Tc into cells increased about three-fold when Tc and PLB were added, compared to Tc alone, suggesting that PLB damages the cytoplasmic membrane.

(4) The protein content of growing mycelia was examined and was found to be lowest in the presence of both PLB and Tc, namely, about one-fourth of the control, while it was only about half of the control when either Tc or PLB was added.

These results suggest that the inhibition of mycelial growth is caused by the inhibition of protein synthesis by Tc in cells after PLB-induced damage to the cytoplasmic membrane.

Key words : Tetracycline, Polymyxin B, Mycelial growth, Protein synthesis

Though the antibacterial antibiotic, tetracycline (Tc), has no antifungal activity¹⁾, it inhibits protein synthesis in a cell-free system of fungi²⁾. We have also reported how protein synthesis in a fungus was inhibited by Tc when the fungal membrane was damaged by amphotericin B (AMPH-B) treatment³⁾. Like the polyene antibiotics, peptide antibiotics damage the cytoplasmic membrane. Since the damaging mechanism differs in each type of antibiotic⁴⁾, we studied the combined effect of Tc and a peptide antibiotic, polymyxin B (PLB), and obtained several results which differed from those for the combined effect of Tc and AMPH-B.

Materials and Methods

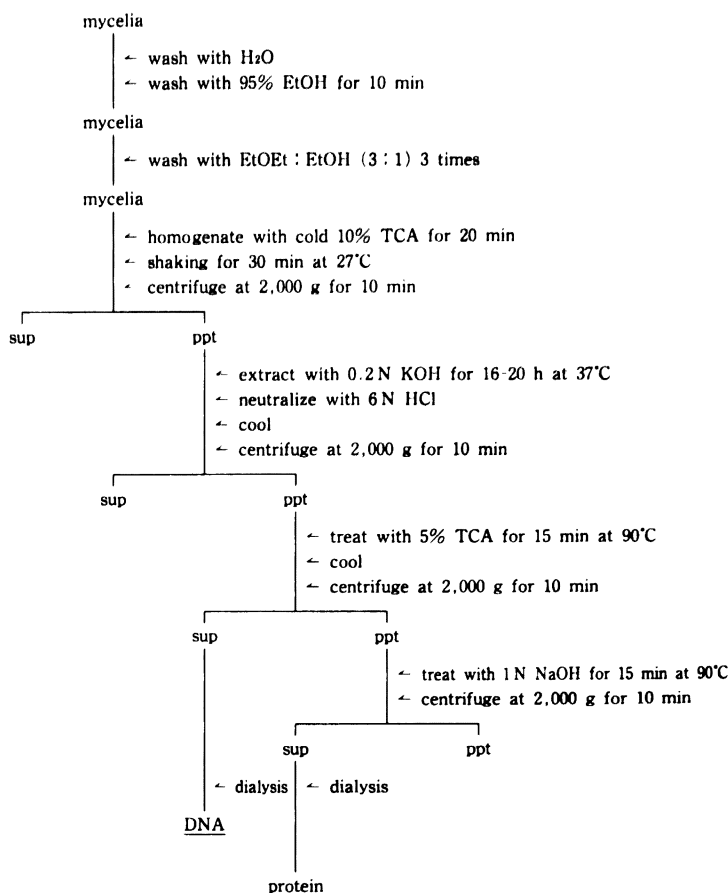
1. Materials

1) Microorganisms. *Cochliobolus miyabeanus* (*Ascomycetes* sp., a stock culture, strain F-01, from the Department of Microbiology of Kobe Women's College of Pharmacy) was subcultured at 27°C on slants of a 2% sucrose potato agar medium and cultured mycelia of the strain were used to study the effects of antifungal agents.

2) Reagents. Antibiotics and reagents used in this study were: tetracycline hydrochloride (Tc, Lederle (Japan), Ltd.), polymyxin B sulphate (PLB, Sigma Chemical Company), ^3H -tetracycline ($[\text{}^3\text{H}(\text{N})]$ -Tc, 0.2 μCi) and ^{14}C -leucine (0.5 μCi , New England Nuclear Corp.).

2. Antifungal effects

An amount of the drug was added aseptically to a flask (300 ml capacity) containing 100 ml of



Scheme 1. Method for extracting protein and DNA from mycelial cells

Czapeck's medium and a spore suspension of the test strain was added to the medium. After shaking the flask at 28°C in the dark, growing mycelia were collected by filtration and heated at 110°C to measure their dry weights. The growth inhibition rate (%) was calculated against the control (dry weight of mycelia grown in a medium with no added drug).

3. Leakage of K⁺ from PLB-treated mycelial cells

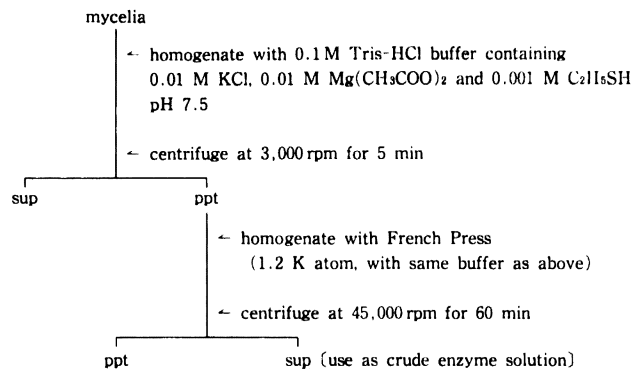
Mycelia of *C. miyabeanus*, which had been cultured at 28°C for 7 days in Czapeck's medium, were cultured again using a sucrose medium containing 0, 5.5, 6.0, 6.5 or 7.0 µg PLB/ml at 28°C for 60 min and the concentration of K⁺ in the

cultured broth was measured using a potassium ion meter (CD-35 MII, M&S Instruments Trading Co. Inc.) as the K⁺ leaked from the cells.

4. Electron microscopic observation

The antibiotics were added to 100 ml of Czapeck's medium with a final concentration of:

(i) Tc, 140 µg/ml; (ii) PLB, 7.4 µg/ml; and (iii) Tc, 70 µg/ml and PLB, 3.7 µg/ml. After adding mycelia of *C. miyabeanus* to these media and culturing at 28°C for 5 days, the grown mycelia were collected by filtration and fixed at 4°C for 60 min with a 2% potassium permanganate solution (0.1 M phosphate buffer, pH 7.4). The fixed mycelia were dehydrated using ethanol in step-wise increasing concentrations (60, 70, 80,



Scheme 2. Method for preparing crude enzyme solution from mycelia

90, 95 and 99%), with 2 ~ 3 repetitions at each concentration, and then dried with acetone. The samples were next stained with gold and carbon in the usual way and examined with a scanning electron microscope (Hitachi SSM-II).

5. Incorporation of ^3H -Tc into cells

Ninety ml of the potato-sucrose medium was transferred to a flask (300 ml capacity) and a needle attached to the bottom of its cork stopper was inoculated with a 3 mm piece of the mycelial tip of *C. miyabeanus* which had been grown on the potato-sucrose agar medium. To each flask was added (i) 0.2 ml of 0.2 $\mu\text{Ci/ml}$ ^3H -Tc (final concentration, 140 $\mu\text{g/ml}$), and (ii) 3.7 μg PLB/ml and 0.1 ml of 0.2 $\mu\text{Ci/ml}$ ^3H -Tc (final Tc concentration, 70 $\mu\text{g/ml}$). The resulting mixture was incubated at 28°C for 96 h. A flask without antibiotics was cultured under the same dark conditions and used as a control. Mycelial growth in each of these media was collected and rinsed in water. The radioactivity in cell-free extract obtained by treating the mycelia with 1 N NaOH at 100°C for 3 h was measured using a liquid scintillation counter (Fujitsu, EA-11) to detect the amount of Tc incorporated into the mycelial cells.

6. Determination of protein and DNA contents in mycelial growth in the PLB- and/or Tc-containing media

The mycelia of *C. miyabeanus* used for the extraction experiments were cultured at 28°C for 5 days in Czapeck's medium containing final conc-

entrations of: (i) PLB, 7.5 $\mu\text{g/ml}$ (equivalent to ED_{50}), (ii) Tc, 140 $\mu\text{g/ml}$ (equivalent to ED_{50}), and (iii) PLB, 3.7 $\mu\text{g/ml}$ and Tc, 70 $\mu\text{g/ml}$. In addition (iv), the mycelia were first cultured for 2 days using the medium containing PLB (3.7 $\mu\text{g/ml}$), the grown mycelia were washed with sterilized water and then cultured again for 3 days in the presence of Tc (70 $\mu\text{g/ml}$). Protein and DNA were extracted from growing mycelia in these four media according to the method shown in Scheme 1. Quantitative analysis of protein and DNA was performed according to Lowry's method⁵⁾ (standard, bovine serum albumin) and the indole method⁶⁾ (standard, salmon sperm DNA).

7. Incorporation of ^{14}C -leucine in an *in vitro* protein synthesis system

(1) Preparation of intracellular enzyme solution

Mycelia were cultured at 28°C for 5 days in Czapeck's medium, the growing mycelia were disrupted according to Scheme 2 and centrifuged and the supernatant fluid thus obtained was used as a crude intracellular enzyme solution. All procedures for the extraction of enzyme were performed at below 4°C.

(2) Inhibition of protein synthesis and incorporation of ^{14}C -leucine by Tc in the intracellular enzyme solution

A reaction mixture was prepared as shown in Table 1, the mixture was shaken at 28°C in an incubator and samples were collected for the extraction of protein, whose radioactivity was

Table 1. Reaction mixture for biosynthesis of protein

100	$\mu\text{mol/ml}$	Tris buffer pH 7.5
50	$\mu\text{mol/ml}$	KCl
20	$\mu\text{mol/ml}$	$\text{Mg}(\text{CH}_3\text{COO})_2$
16	$\mu\text{mol/ml}$	$\text{C}_2\text{H}_5\text{SH}$
2	$\mu\text{mol/ml}$	Glutathione
0.5	$\mu\text{mol/ml}$	GTP
2	$\mu\text{mol/ml}$	ATP
5	$\mu\text{mol/ml}$	PEP
20	$\mu\text{g/ml}$	Pyruvate kinase
0.25	$\mu\text{mol/ml}$	Amino acids (Phe, Asp, Gly, Cys, Pro, Ala)
600	$\mu\text{g/ml}$	t RNA
0.5	$\mu\text{Ci/ml}$	^{14}C Leucin
1	$\mu\text{mol/ml}$	Phenyl methyl sulfonyl fluoride

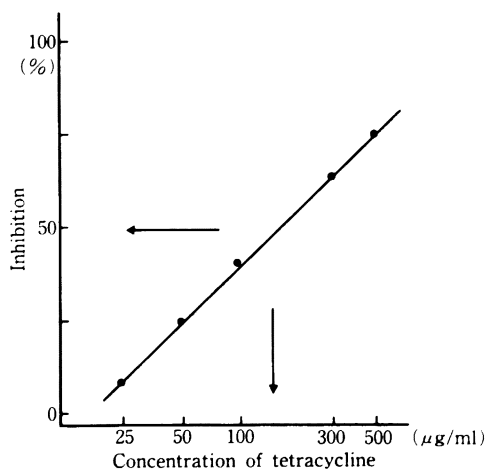


Fig. 1. Effect of tetracycline on mycelial growth

measured by liquid scintillation counter (Fujitsu, EA-118). In order to examine the inhibition of ^{14}C -leucine incorporation by Tc, to the reaction mixture shown in Table 1 was added a final concentration of : (i) Tc, 140 $\mu\text{g/ml}$, or (ii) Tc, 70 $\mu\text{g/ml}$ and PLB, 3.7 $\mu\text{g/ml}$.

Results and Discussion

1. Effects of Tc and PLB on mycelial growth

A spore suspension of *C. miyabeanus* was inoculated into Czapeck's medium supplemented with different concentrations of Tc or PLB, the weight of mycelia grown after 7 days' culturing,

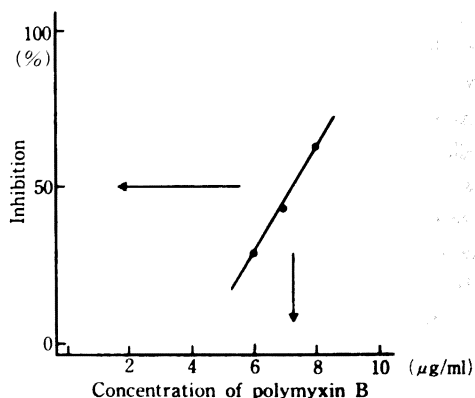


Fig. 2. Effect of polymyxin B on mycelial growth

the inhibition rate of mycelial growth was obtained in terms of the drug concentration. As shown in Figs. 1 and 2, the concentration of Tc and PLB required to inhibit 50% of the mycelial growth (ED_{50}) was 140 $\mu\text{g/ml}$ and 7.5 $\mu\text{g/ml}$, respectively. The ED_{50} value of PLB was about 100-times that of amphotericin B (AMPH-B, 0.08 $\mu\text{g/ml}$). The difference may be explained as follows. Though the reaction mechanisms of PLB and AMPH-B are the same in terms of cytoplasmic membrane damage, the peptide antibiotic, PLB, activates phospholipase and causes hydrolysis of phospholipids by attaching to the outer membrane lipopolysaccharides and inner

Table 2. Inhibition rates of mycelial growth by polymyxin B or tetracycline

Added component ($\mu\text{g/ml}$)		Inhibition (%)				
PLB	Tc	Treatment				
		none	PLB only	Tc only	PLB+Tc	PLB→Tc
7.5	70.0	0.0	41.7	26.3	100.0	81.3
3.8	70.0	0.0	20.3	24.5	91.9	68.8
1.9	35.0	0.0	15.2	13.0	84.6	65.2
1.0	17.5	0.0	12.2	9.5	73.2	62.0

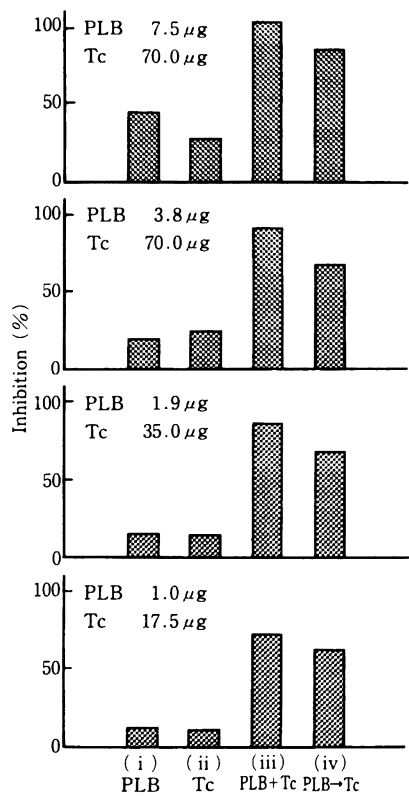


Fig. 3. Inhibition rates of mycelia growth by PLB or Tc

tion damage, though it does not affect bacterial membranes without steroidal structures⁸⁻¹². Consequently, the difference between these drugs in their ability to inhibit fungal growth is thought to be due to the fact that PLB damages the bacterial cytoplasmic membrane, though its effect against fungal membranes is weak, while AMPH-B affects membranes of fungi and protozoa, but not of bacteria.

2. Combined effect of Tc and PLB on the inhibition of mycelial growth

Any Tc antibiotic enters easily into bacterial cells, attaches to the 30S subunit of ribosome and inhibits attachment of aminoacyl-t-RNA to ribosomes, but scarcely inhibits fungal cell growth. FRANKLIN²⁾ has reported, however, that inhibition of protein synthesis occurred even in fungal cells when Tc was applied to a cell-free system. We have reported elsewhere that Tc showed an inhibitory effect on fungal growth when used in combination with AMPH-B, which has a membrane-damaging function³⁾. Consequently, the effect of Tc in combination with PLB, whose reaction mechanism differs from that of AMPH-B but also damages the cytoplasmic membrane, was examined under the following 5 conditions: (i) five-day culture in PLB-added medium, (ii) five-day culture in Tc-added medium, (iii) five-day culture in a medium containing both PLB and Tc, (iv) two-day culture in PLB-added medium and subsequent three-day culture of growing mycelia after washing in a Tc-added medium and (v) five-day culture in a medium with no drugs. The inhibition rates (%)

membrane acid phospholipids of Gram-negative bacteria. Regarding phospholipids, remarkable hydrolysis of phosphatidyl ethanolamine and phosphatidyl glycerol have been reported⁷⁾. On the other hand, the polyene antibiotic, AMPH-B is known attach to steroids in the cell membranes of fungi and animals and to cause membrane func-

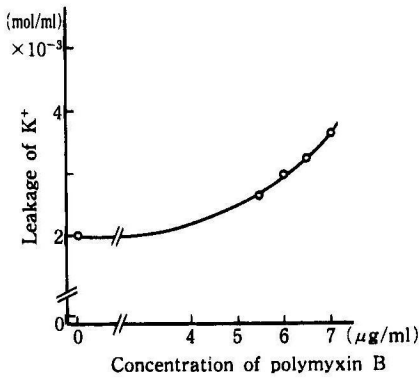


Fig. 4. Amount of K⁺ leakage from mycelia after treatment with polymyxine B

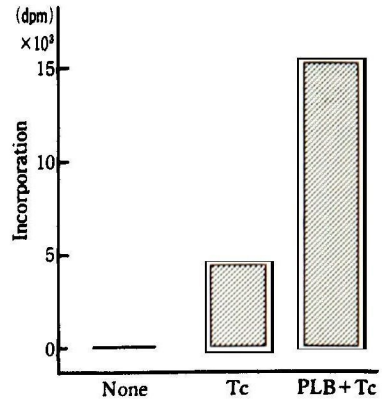
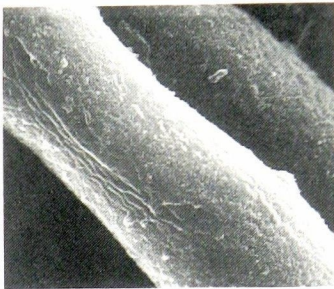
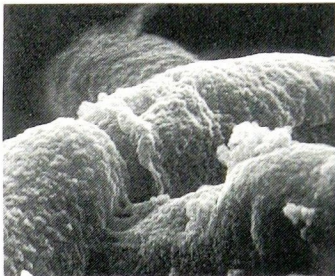


Fig. 6. Amount of ³H-tetracycline incorporated into mycelia

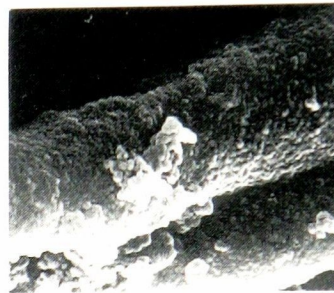
(1)



(3)



(2)



(4)

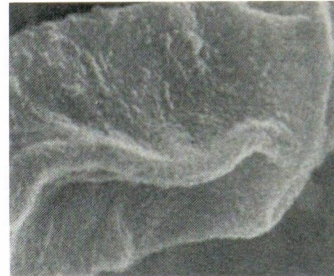


Fig. 5. Morphological observations of mycelia after treatment by drugs

(1) non-treatment (2) Tc only
(3) PLB only (4) Tc+PLB

obtained by measuring the weights of the growing mycelia are shown in Table 2 and Fig. 3. In three of the experimental condition (i, ii and iii), the growth inhibition effect increased in proportion to the amount of drug added. Compared to the addition of one drug (i and ii), simultaneous addition of both drugs (iii) showed inhibition 3 ~ 8 times stronger than that of Tc, even when the

amounts of Tc and PLB were as low as 17.5 μg/ml and 1.0 μg/ml, equivalent to about one-eighth of their respective ED₅₀ values. In the case of condition (iv), in which membranes were first damaged by PLB and then exposed to Tc, the inhibitory effect was not so remarkable as when both drugs were added simultaneously, but about 2 ~ 4 times stronger than when only one drug was

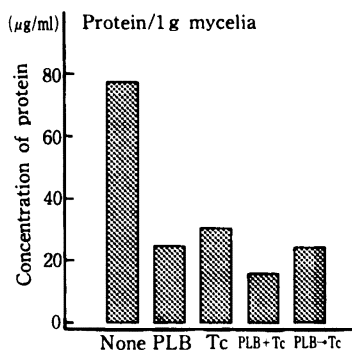


Fig. 7. Amounts of protein in mycelia after treatment with various drugs

added. These results indicate clearly that growth inhibition is effected by the incorporation of Tc into mycelial cells through the damaged membranes.

3. Damage of cytoplasmic membrane by PLB

Since it is known that extracellular secretion of K^+ , proenzyme, etc., can be found at a relatively early stage when cytoplasmic membranes are damaged⁴⁾, we studied the secretion of K^+ . That is, mycelia were grown in the presence of PLB (5.5 ~ 7.5 $\mu\text{g/ml}$) and the amount of leaked K^+ outside the cells was measured by the electrode method. As shown in Fig. 4, the amount of leaked K^+ increased in proportion to the concentration of PLB, indicating that PLB had damaged the cytoplasmic membrane of *C. miyabeanus*. We then attempted to observe the damage to the membranes using an electron microscope. Fig. 5 shows electron microphotographs of growing mycelia in medium containing 140 μg Tc/ml (equivalent to ED_{50}), 7.4 μg PLB/ml (ED_{50}) or both Tc (70 $\mu\text{g/ml}$) and PLB (3.7 $\mu\text{g/ml}$). Compared to the normal mycelia (Fig. 5-1) no remarkable differences were found in the electron microscopic observation of mycelia grown in the presence of Tc or PLB (Figs. 5-2 and 5-3), except for a rough surface. In the case of mycelia grown in the presence of both Tc and PLB (Fig. 5-4), significant flattening of the mycelium and formation of a rough surface were observed. These results indicated that the inhibition of mycelial growth occurs only in the presence of both Tc and PLB. In consequence, we concluded that the damage

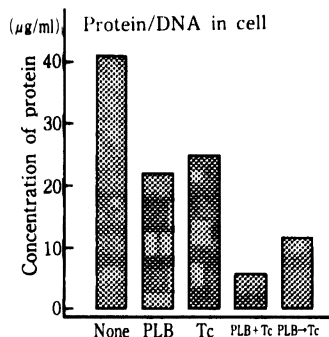


Fig. 8. Amounts of protein/DNA in cell after treatment with drugs

PLB-induced membrane made possible the incorporation of Tc into the cell, thereby inhibiting mycelial growth, since the leakage of K^+ increased in the presence of PLB and abnormal changes were found in the appearance of mycelia grown in the presence of both Tc and PLB.

4. Incorporation of Tc into fungal cells

We studied the changes in the incorporation of Tc into fungal cells caused by PLB-induced damage to the cellular membrane, by measuring the amount of ^{14}C -Tc in mycelia grown in the presence of 140 μg Tc/ml (ED_{50}) or both Tc (70 $\mu\text{g/ml}$) and PLB (3.7 $\mu\text{g/ml}$). As shown in Fig. 6, the incorporation of ^{14}C -Tc into the cells increased about three-fold in the presence of both Tc and PLB, compared to Tc alone, that PLB damages the cytoplasmic membrane, and enables Tc to enter the cell easily.

5. Protein contents in growing mycelia in the presence of PLB and Tc

Protein contents were measured in mycelial cells grown under the following five different conditions: (i) single addition of PLB to the medium, (ii) single addition of Tc, (iii) simultaneous addition of PLB and Tc, (iv) culture in the presence of PLB and subsequent culture of the grown mycelia in the presence of Tc and (v) control medium without drugs. As shown in Figs. 7 and 8, the amount of protein was lowest in the mycelial cells cultured in the presence of both PLB and Tc (iii), namely about one-fourth of the control (v). In the case of PLB (i) or Tc (ii) alone, on the other hand, protein decreased

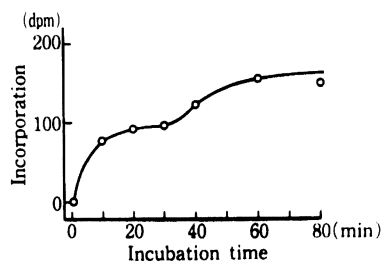


Fig. 9. Volume of ^{14}C -Leucin incorporated in protein (pH 7.5)

to about half that of the control. When the cellular membranes were first damaged by the PLB-treatment and then exposed to Tc (iv), almost the same degree of inhibition of protein synthesis as in the presence of both drugs was observed. These results indicated inhibition of protein synthesis by the incorporation of Tc into the cells after PLB-induced damage to the cytoplasmic membranes of the eucaryotic cells.

6. The effect of Tc against the incorporation of ^{14}C -leucine at the time of protein synthesis by intracellular enzymes

Since Tc is known to inhibit protein synthesis specific to ribosome function, we devised a system for *in vitro* protein synthesis, as shown in Table 1, and examined the effect of Tc on protein synthesis in fungal cells. A mycelial cell suspension in Tris buffer was disrupted with a French Press, centrifuged at 45,000 rpm and the supernatant fluid obtained was used as a crude enzyme solution, after addition of ^{14}C -leucine. Samples were collected at certain intervals (from 0 to 80 min), and protein radio activity extracted and measured. As shown in Fig. 9, the incorporation of ^{14}C -leucine became constant after 60 min of the reaction, with the maximum incorporation at pH 7.5. After fixing these conditions, further incorporation experiments were performed in the presence of Tc (i), Tc and PLB (ii) and without drugs (iii). As shown in Table 3, inhibition of ^{14}C -leucine incorporation was observed both in the case of single addition of Tc (i) and of simultaneous addition of Tc and PLB (ii). Tc is generally known to inhibit protein synthesis in procaryotic bacteria but not in eucaryotic cells. Recently, however, inhibition of protein synthesis

Table 3. Amount of ^{14}C -Leucin incorporated in protein after treatment with drugs

Addition	dpm
None	1,002.6
Tetracycline	90.4
Tetracycline + polymyxin B	21.7

(reaction time : 60 min)

by Tc in a cell-free system of a eucaryote has been reported by FRANKLIN²¹. We recently obtained similar results using an *in vitro* system²². These findings indicate that incorporation of Tc into fungal cells in some way makes possible the inhibition of protein synthesis and subsequent inhibition of mycelial growth.

In a previous report²³, we studied the inhibition of fungal mycelial growth by Tc in combination with AMPH-B, a polyene antibiotic which, like PLB, makes a damages to the cytoplasmic membrane. Using this system, remarkable inhibition of mycelial growth was observed, not in the presence of Tc and AMPH-B, but when washed mycelia grown in the presence of AMPH-B were re-cultured in a Tc-containing medium. Study of this phenomenon revealed that the activity of Tc is inhibited by means of its bonding with a polymer substance excreted from the cells of *C. miyabeanus*²⁴. On the other hand, no inhibition of Tc activity was observed when it was used simultaneously with PLB. In this case, we hypothesize that the activity of Tc is not inhibited because of the bonding of PLB with a protein secreted from the cells, since serum protein can combine with PLB¹³, though no reports are available so far on the bonding of AMPH-B with any protein.

In this study, we found a synergistic inhibitory effect of Tc and PLB on mycelial growth. Since only a few antifungal agents are available for clinical use, the authors wish to continue studies in this field.

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抗糸状菌剤に関する研究（第16報）

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

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Ascomycetes に属する *Cochliobolus miyabeanus* の菌糸生育におよぼす tetracycline (TC) と polymyxin B (PLB) の併用効果について検討し、次の結果を得た。

(1) 菌糸の生育を 50% 抑制するのに必要な TC と PLB の濃度 (ED_{50}) は、各々 $140 \mu\text{g/ml}$ および $7.5 \mu\text{g/ml}$ であった。

(2) TC と PLB を併用した場合、すなわち TC $17.5 \mu\text{g/ml}$ と PLB $1.0 \mu\text{g/ml}$ の共存下で生育させた場合には、各々単独添加の場合に比べ菌糸生育の阻害効果は 3 ~ 8 倍増強される。

(3) TC と PLB 共存下での細胞中への ^{14}C -TC のとりこみ量を調べたところ、TC の単独の場合に比べてその量は約 3 倍に増加した。この結果は、PLB が細胞質膜に障害を与えたことを示唆するものである。

(4) PLB と TC の共存下で生育させた菌糸中のタンパク質について調べたところ、PLB と TC の共存下で生育させた場合が最も少なく、その量は対象群の約 1/4 量であった。なお、PLB あるいは TC 単独添加で生育させた場合にも菌体内タンパク量は少なく無処理の場合の約 1/2 量であった。

以上の結果は、PLB が菌糸の細胞質膜を障害し、TC が細胞内へ侵入し易くなったためにタンパク合成が阻害されたことを推定させるものであった。

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