ON THE MODE OF ACTION OF MULTHIOMYCIN

I. EFFECTS OF MULTHIOMYCIN ON MACROMOLECULAR SYNTHESES

TERUO TANAKA, KENJI SAKAGUCHI*

and HIROSHI YONEHARA

Institute of Applied Microbiology, The University of Tokyo,
Tokyo, Japan
* Noda Institute for Scientific Research,
Noda, Chiba Prefecture, Japan

Muthiomycin, a sulphur-containing antibiotic, inhibited protein synthesis in whole cells of *Bacillus subtilis* and in *Escherichia coli* lamelloplast. It did not inhibit transfer of phenylalanine to tRNA or attachment of poly U to ribosomes, and the inhibition of polyphenylalanine synthesis was reversed by increasing amount of ribosomes.

Multhiomycin, a sulphur-containing antibiotic, was recently isolated by TANAKA *et al.*[1] as a potent inhibitor of the growth of *B. subtilis* during the course of screening for inhibitors of nucleic acid synthesis[2]. On acid hydrolysis, it gave chromophoric substances, threonine, and one or two ninhydrin-positive spots on thin-layer chromatography. Among the antibiotics containing threonine and a high content of sulphur in their molecules, siomysin was reported to inhibit protein biosynthesis[3]. In view of these facts, it seemed interesting to investigate the mode of action of multhiomycin.

The present communication describes the effect of multhiomycin on macromolecular syntheses in whole cells of *B. subtilis* and in *E. coli* lamelloplast, and on polypeptide synthesis in the cell-free system from *E. coli*.

Materials and Methods

Radioactive materials

[14]C-Amino acid mixture was an acid hydrolysate of *Chlorella* protein obtained in our institute (7.92 mC/mMC). ^{14}C-Uracil (21.0 mC/mM) was purchased from Daiichi Chemicals. and Industry Co., Ltd. ^{3}H-Thymine (14.0 C/mM), ^{3}H-uridine (25.2 C/mM), ^{3}H-thymidine (5.0 C/mM) and ^{14}C-phenylalanine (475 mC/mM) were from Radio Chemical Center. ^{3}H-Polyuridylic acid (22.5 mC/mM) was from Miles Laboratories.

Materials

Transfer RNA was prepared by the method of AVITAL *et al.*[4] ^{14}C-Phe-tRNA was prepared after NATHANS and LIPMAN[5] except that the un-labelled amino acids were omitted. Polyuridylic acid was from Miles Chemical Co. Puromycin was a gift from Dr. T. KINOSHITA in our institute. Crystalline multhiomycin was prepared as described previously[1] and dissolved in dimethyl formamide (DMF) at a concentration of 10 mg/ml, which was stored at −20°C. In the cell-free system, the reaction mixture contained 0.5 % DMF throughout

this investigation because multhiomycin is not soluble in water at above 10 μg/ml. This concentration of the solvent did not have any inhibitory effects in the system used.

Conditions for determining macromolecular syntheses

Cells were inoculated from a slant of *Bacillus subtilis* 168 thymine indole to Bacto antibiotic medium No. 3 (50 ml) and incubated at 37°C with shaking until optical density at 660 mμ reached 0.3. The cells were centrifuged, washed with minimal medium M9, and resuspended in the original volume of M9 supplemented with 10 μg/ml of casamino acids and 1 μg/ml of thymine (medium A). The cell suspension, 0.2 ml, was transferred to 1.3 ml of medium A containing 2 μC/ml of ³H-thymine, 0.02 μC/ml of ¹⁴C-uracil, 0.1 μC/ml of ¹⁴C-amino acid mixture and an adequate amount of the antibiotic. The reaction was carried out at 37°C and stopped at various time intervals by the addition of 1.5 ml of cold 10 % TCA. The cells which incorporated ³H-thymine or ¹⁴C-uracil were filtered through Millipore filter (0.45 μ pore size), and washed 3 times with cold 5 % TCA. The cells which incorporated ¹⁴C-amino acids were heated for 15 minutes at 90°C and filtered. Filters were dried and counted with a gas flow counter (Nihon Musen Co., Tokyo).

Radioactivity of ³H-thymine, ¹⁴C-uracil, and ¹⁴C-amino acids incorporated into the cells was taken as an amount of DNA, RNA and protein synthesized during the incubation respectively.

E. coli lamelloplast (an active membrane system) was prepared from *E. coli* K12 as described by NAGATA et al.[6,7] One ml of the reaction mixture contained 0.5 ml of lamelloplast fraction together with 0.5 mg of casamino acids supplemented with L-tryptophan (0.05 mg), 5 μmoles of MgCl₂, 50 μmoles of Tris-HCl buffer (pH 7.6), 0.5 μC of ³H-uridine, 1 μC of ³H-thymidine, and 0.5 μC of ¹⁴C-amino acid mixture. Reaction was carried out for 15 minutes at 30°C and terminated by the addition of 1 ml of 10 % cold TCA.

Cell wall synthesis of *B. subtilis*

Ten ml of the cell suspension in medium A was added to 10 ml of fresh medium containing 0.5 μC of ¹⁴C-amino acid mixture and incubated at 37°C. Aliquots of 5 ml were removed as indicated in Fig. 2 and added to 5 ml of 10 % TCA. The cells that had been precipitated with TCA were fractionated by the procedure of PARK and HANCOCK[8]. After trypsin digestion of the 75 % ethanol-insoluble and hot TCA-precipitable fraction, the degraded material was centrifuged (3,000 × g, 15 minutes). The radioactivity in supernatant and precipitate was taken as an amount of protein and cell wall fraction respectively.

Preparation of cell-free protein-synthesizing system

E. coli B was grown in a medium containing 0.3 % glucose, 0.5 % NaCl, 1 % beef extract, and 1 % peptone at pH 8 with vigorous shaking at 30°C. The cells were harvested in early log phase, washed once in standard buffer containing Tris-HCl 0.01 M (pH 7.4), KCl 0.05 M, Mg(CH₃COO)₂ 0.01 M, and β-mercaptoethanol 0.006 M and stored at −20°C. Cells were disrupted by grinding with twice their wet weight of sea sand and S-30 fraction was prepared after NIRENBERG and MATTHAEI[9]. The fraction was further dialyzed overnight in a standard buffer at 4°C. Ribosomes and S-105 fraction were obtained from dialyzed S-30 fraction by centrifugation at 105,000 × g for 2 hours and ribosomes were further washed with 0.5 M NH₄Cl by the method of NISHIZUKA and LIPMAN[10]. Protein was determined by the method of LOWRY et al.[11]

Sucrose density gradient centrifugation

Linear sucrose gradients (5~20 %) were made in a volume of 4.5 ml in 0.05 M Tris-HCl (pH 7.4), containing 0.01 M Mg(CH₃COO)₂ and 0.05 M KCl. The tubes were centrifuged for 90 minutes at 35,000 rpm in RPS 40 rotor (Hitachi Ltd.) at 4°C. Two-drop fractions were collected by piercing the bottom of the tube and alternate samples were used to measure absorbancy at 260 mμ. The contents of the other tubes were precipitated with 2 ml of cold 10 % TCA, and filtered through Millipore filters. After filters were dried, they were counted with a gas flow counter.

Results

Effect of Multhiomycin on the Syntheses of DNA, RNA and Protein
in *Bacillus subtilis* 168 and *E. coli* K12 Lamelloplast

Cells of *B. subtilis* 168 were grown at 37°C and subjected to the analysis of macromolecular synthesis as described in methods. Fig. 1 shows that multhiomycin slightly reduced DNA synthesis 20 minutes after the onset of the reaction. The synthesis of RNA was not blocked during the time of incubation, while protein synthesis was completely inhibited immediately after the addition of the antibiotic.

Fig. 1. Effect of multhiomycin on the incorporations of ^{3}H-thymine, ^{14}C-uracil and ^{14}C-amino acids into *B. subtilis* 168.

Cells were grown as described in Materials and Methods. At zero time 0.05 μg/ml of multhiomycin was added and samples were taken at the time indicated. They were assayed for DNA, RNA and protein as described in Materials and Methods.
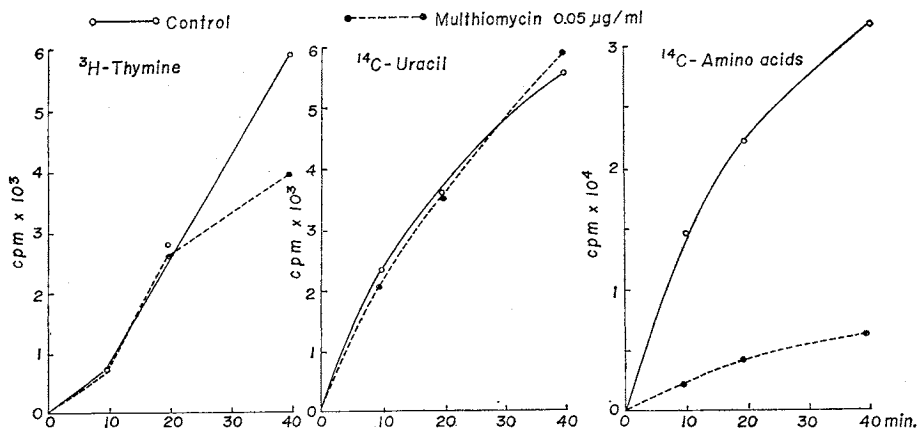
Because multhiomycin, as reported previously[1], does not affect the gowth of *E. coli* even at a concentration of 100 μg/ml, lamelloplast of *E. coli* was used to avoid the permeability barrier. In this active membrane system, multhiomycin similarly inhibited protein synthesis but not DNA and RNA syntheses (Table 1). Sixty percent inhibition of protein synthesis was observed at a concentration of 1 μg/ml.

Table 1. Effect of multhiomycin on ^{14}C-amino acids, ^{3}H-thymidine and ^{3}H-uridine incorporations into *E. coli* K12 lamelloplast.

|	Incorporation (cts/min/ml)		
	Hot TCA precipitable ^{14}C-amino acids	Cold TCA precipitable	
		^{3}H-thymidine	^{3}H-uridine
Control	1,068	3,107	6,785
+Multhiomycin 1 μg/ml	480	3,257	6,910
5 μg/ml	440	3,162	6,785

One ml reaction mixture contained 0.5 ml of lamelloplast together with 0.5 mg of casamino acids supplemented with L-tryptophan (0.05 mg), 5 μmoles of MgCl$_2$, 50 μmoles of Tris-HCl (pH 7.4), 0.5 μC of ^{3}H-uridine, 1 μC of ^{3}H-thymidine and 0.5 μC of ^{14}C-amino acids mixture. Reaction was carried out for 15 minutes at 30°C and terminated by the addition of 1 ml of cold 10 % TCA. Lammelloplast that had incorporated ^{3}H-uridine or ^{3}H-thymidine was filtered through a Millipore filter (0.45 μ, pore size). Lamelloplast that had incorporated ^{14}C-amino acid mixture with heated for 15 minutes at 90°C and filtered. Filters were washed with cold 5 % TCA, dried and counted with a gas flow counter.

Effect of Multhiomycin on the Incorporation of ^{14}C-Amino Acids
into the Protein and Cell-wall Fraction of *B. subtilis*

Since amino acids are incorporated into the protein and cell wall fraction, the incorporation of the labelled amino acids into the trypsin-resistant fraction of *B. subtilis* was used as a criterion for cell wall synthesis. Fig. 2 shows that 0.02 μg/ml of multhiomycin inhibited the ^{14}C-amino acids incorporation into protein fraction of *B. subtilis*, while uptake of these amino acids into cell wall fraction was not affected.

Fig. 2. Effect of multhiomycin on incorporation of ^{14}C-amino acid mixture into protein (2-a) and cell wall fraction (2-b) of *B. subtilis*.

Analytical procedures are described in Materials and Methods.
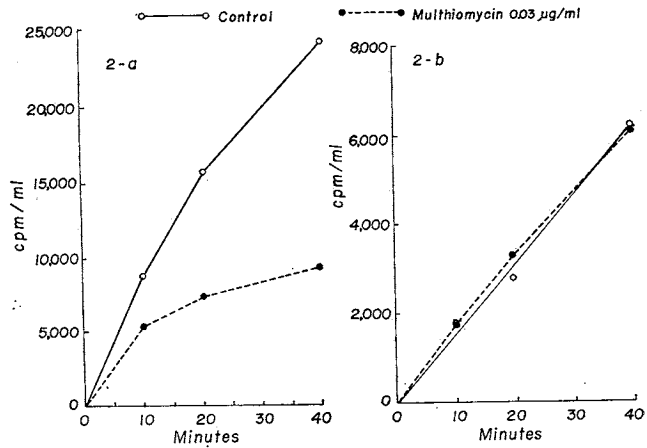
Effect of Multhiomycin on Polypeptide Synthesis in Cell-free System from *E. coli*

Table 2 shows that multhiomycin inhibited the incorporation of ^{14}C-phenylalanine into polypeptide directed by endogenous messenger or by polyuridylic acid. Almost the same extent of inhibition was observed with either system, whether at 50 μg/ml or at 10 μg/ml multhiomycin. Fig. 3 illustrates the time course of polyphenylalanine synthesis in the presence of 50 μg/ml multhiomycin. Inhibition was immediately after the addition of the antibiotic.

Fig. 3. Time course of polyphenylalanine synthesis in the presence of 50 μg/ml of multhiomycin.

Conditions are the same with Table 2 except that the reaction mixture was 0.7 ml. Portions of 0.1 ml were removed and assayed for polyphenylalanine synthesis.

Table 2. Effect of multhiomycin on polypeptide synthesis directed by endogenous messenger and polyuridylic acid.

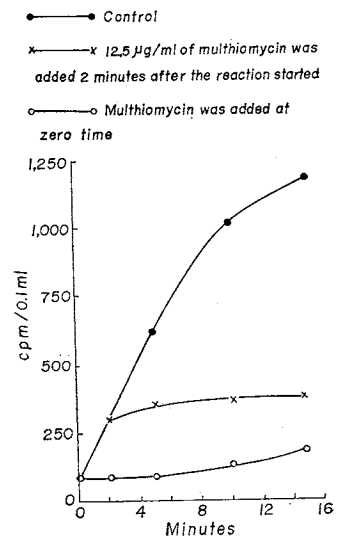|	^{14}C-Phenylalanine incorporation			
	endogenous *m*RNA		polyuridylic acid	
	cpm/0.25 ml	% of control	cpm/0.25 ml	% of control
Control	686	100	3,128	100
Puromycin 5 μg/ml	248	36	3,068	97
Multhiomycin				
50 μg/ml	258	38	810	26
10 μg/ml	590	87	2,900	92
5 μg/ml	700	—	3,252	—

The reaction mixture in 0.25 ml contained 1.25 μl of DMF, 12.5 μmoles of Tris–HCl (pH 7.4), 2.5 μmoles of Mg(CH₃COO)₂, 12.5 μmoles of KCl, 1.5 μmoles of β-mercaptoethanol, 0.25 μmoles of ATP, 0.1 μmole of GTP, 1.25 μmoles of creatine phosphate, 5 μg of creatine kinase, 0.05 μC of ^{14}C-phenylalanine, 10 μg of poly U where indicated and 0.6 mg of S-30 protein.

The reaction mixture was incubated for 15 minutes at 37℃, terminated by the addition of 2 ml of 10 % TCA and heated for 15 minutes at 90℃. The resulting precipitate was collected on a Millipore filter, and washed with 5 % TCA.

Effect of Multhiomycin on ¹⁴C–Phenylalanyl–tRNA Formation

The effect of multhiomycin on charging tRNA with phenylalanine was studied using *E. coli* 105,000×*g* supernatant. As shown in Table 3, 50 μg/ml of multhiomycin had no effect on the transfer of ¹⁴C–phenylalanine to ¹⁴C–phenylalanyl–tRNA. Fig. 4 represents the extent of inhibition of poly U–directed polyphenylalanine synthesis by varying amounts of the antibiotic. Fifty percent inhibition was observed at a concentration of 12 μg/ml.
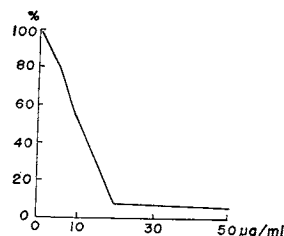
Fig. 4. Effect of concentrations of multhiomycin on polyphenyl-alanine synthesis.

The reaction mixture in 0.2 ml contained 1 μl of DMF, 10 μmoles of Tris–HCl (pH 7.4), 10 μmoles of KCl, 2 μmoles of Mg(CH₃COO)₂, 1.2 μmoles of β–mercaptoethanol, 10 μg of poly U, 0.2 mg of ribosome, 29 μg of ¹⁴C–phe–tRNA (8,700 cpm) and 0.25 mg of 105,000 ×*g* supernatant protein. Incubation was carried out for 15 minutes at 37℃ and terminated by the addition of 5 ml of 10 % TCA as described in Table 2.

Table 3. Effect of multhiomycin on the formation of ¹⁴C–phenylalanyl–tRNA.

	cpm/mg RNA	% inhibition
Control	46,450	—
Multhiomycin 50 μg/ml	46,020	1
10 μg/ml	49,900	—
–tRNA	1,640	97

The reaction mixture was the same as that described in Table 2 with the difference that poly U, S–30 were omitted and 0.2 mg of S–105 protein was added. Incubation was for 15 minutes at 37℃, terminated by the addition of 2 ml of cold 10 % TCA. The TCA precipitate was collected on the Millipore filter and washed with cold 5 % TCA.

Effect of Concentrations of 105,000×*g* Supernatant, Polyuridylic Acid, Ribosomes and Phenylalanyl–tRNA on the Inhibition of Polyphenylalanine Synthesis by Multhiomycin

The data presented in Fig. 5 a∼c show that the extent of inhibition in the presence of 12.5 μg/ml of the antibiotic was not altered by increasing the concentration of ¹⁴C–phe–tRNA, poly U, or 105,000×*g* supernatant, but was reduced by a higher concentration of ribosomes in the reaction mixture. These results suggest that the multhiomycin interacts with ribosomes.

Fig. 5. Effect of concentrations of 105,000×*g* supernatant, poly U, ribosomeand ¹⁴C–phe–tRNA on the extent of inhibition of polyphenylalanine synthesis by multhiomycin.

Conditions and procedures are the same with Fig. 4. Each panel shows the response of one additive in the presence of all the others.

●————● *Control*   ○————○ *Multhiomycin 12.5 μg/ml*   ○--------○ *Percent inhibition*
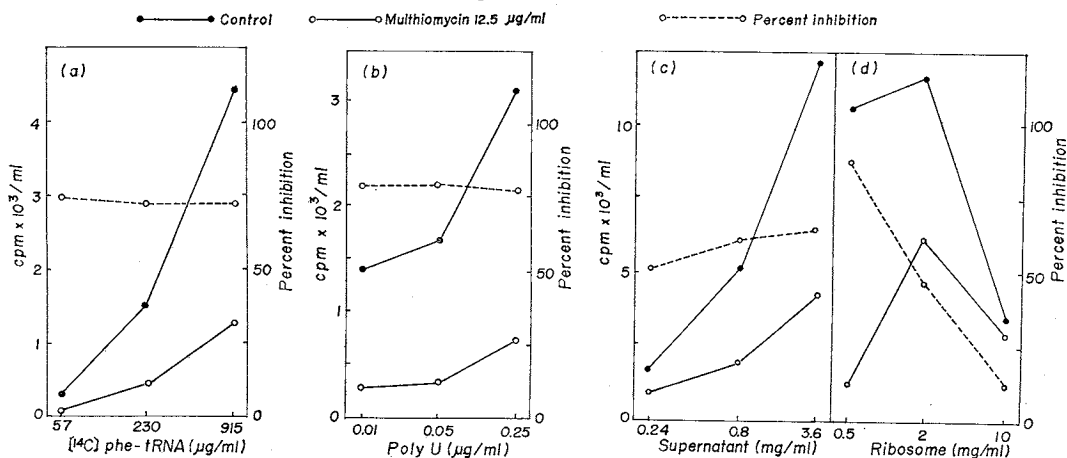
## Effect of Multhiomycin on ³H–Poly U Attachment to Ribosomes

The attachment of ³H–poly U to ribosomes was studied by sucrose density gradient. Fig. 6 shows that the radioactive peak, which is thought to be complexed ribosomes, sedimented slightly faster than 260 mμ absorbancy peak of 70 S ribosomes. When 50 μg/ml of multhiomycin was added 5 minutes before the addition of ³H–poly U, there was about a 2.5 times stimulation of radioactivity in the polysome region. Multhiomycin thus did not prevent the attachment of ³H–poly U to ribosomes.

Fig. 6. Effect of multhiomycin on the attachment of ³H–poly U to ribosomes.

The reaction mixture in 0.25 ml contained 1.25 μl of DMF, 12.5 μmoles of Tris-HCl (pH 7.4), 12.5 μmoles of KCl, 2.5 μmoles of Mg(CH₃COO)₂, 1.5 μmoles of β-mercaptoethanol, 0.25 mg of ribosomes and 0.05 μC of ³H–poly U.

The samples without ³H–poly U were preincubated in the presence or absence of 50 μg/ml of multhiomycin for 5 minutes at 37℃ and further incubated for 7 minutes at 37℃ with added ³H–poly U.

Aliquot of 0.2 ml was layered on top of a linear sucrose gradient (5∼20 %) and centrifuged. Analytical procedures are described in Materials and Methods.
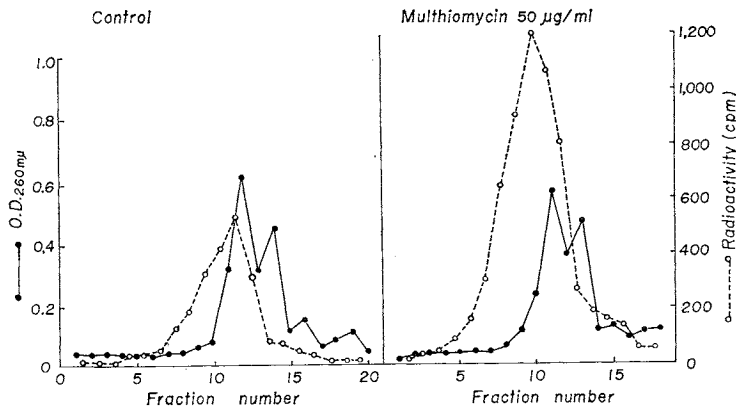
Discussion

Experiments with intact *B. subtilis* showed that multhiomycin inhibited ¹⁴C–amino acid incorporation into protein but had little or no effect on RNA and DNA syntheses. Cell wall synthesis, as measured by the uptake of ¹⁴C–amino acids by trypsin–resistant fraction, also continued. In an *E. coli* cell–free system, 20 μg/ml of multhiomycin completely inhibited the incorporation of ¹⁴C–phenylalanine into ¹⁴C–polyphenylalanine. However, intact cells of *E. coli* were insensitive even at a concentration of 100 μg/ml, suggesting that *E. coli* is impermeable to multhiomycin. The inhibition ratio of protein synthesis *in vitro* by multhiomycin was approximately the same either with endogenous messenger or with poly U. In the poly U–containing system, polyphenylalanine synthesis was stopped immediately after the addition of the antibiotic. The inhibition of polyphenylalanine synthesis was reversed by the addition of more ribosomes but was not affected by the addition of a high concentration of poly U, phe–tRNA and 105,000 × g supernatant fraction. This phenomenon is similar to the action of tetracycline[12] and bottromycin[13] which interact with the ribosome.

Multhiomycin had no inhibitory effect on the formation of ¹⁴C–phenylalanyl–tRNA or on attachment of ³H–poly U to ribosomes, and even stimulated the latter. The findings presented in this paper suggest that the site of action of multhiomycin is some step following the formation of a ribosome–messenger complex.

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