STUDIES ON THE MODE OF ACTION OF ALTHIOMYCIN

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Among syntheses of DNA, RNA and protein in intact cells of *E. coli* B, althiomycin exhibited the strongest inhibition against protein synthesis. In the cell-free system althiomycin was confirmed to inhibit protein synthesis. On the other hand, it did not inhibit protein synthesis of reticulocyte either in the intact cells or in the cell-free system. By further studies in *E. coli* B systems the antibiotic was shown to inhibit the puromycin reaction, without inhibiting aminoacyl-tRNA synthesis and binding of aminoacyl-tRNA to ribosomes.

Althiomycin is produced by *Streptomyces althioticus*[1,2], and its chemical structure has been reported by CRAM *et al.*[3]. The antibiotic inhibits the growth of both Gram-positive and negative bacteria. It is an interesting antibiotic in its low toxicity and its strong protective effect against infection with sensitive organisms. The mode of action of this antibiotic is described in this paper.

Materials and Methods

A sample of althiomycin was prepared by MAEDA, National Institute of Health, Tokyo, Japan from a culture filtrate of *S. althioticus*. Its minimum inhibitory concentration against growth of *E. coli* B examined by a broth dilution method was 1 μg/ml (1.4 μM).

^{14}C-Thymidine, ^{14}C-uracil and ^{14}C-amino acids were purchased from Daiichi Pure Chemicals Co. ^{14}C-Amino acid mixture used was acid hydrolysate of ^{14}C-labelled chlorella proteins supplied by our institute.

Poly U, poly A and poly C were products of Miles Laboratories, Inc.

Cells of *E. coli* B grown in a nutrient broth (1 % peptone, 1 % meat extract, 0.5 % NaCl and 0.3 % glucose, pH 7.4) under shaking at 37°C were collected at the early log phase, washed twice with saline and suspended in Tris buffer (pH 7.4, containing 1 g KH_2PO_4, 0.7 g $MgSO_4 \cdot 7H_2O$, 1 g NaCl, 4 g glutamate, 0.03 g $FeSO_4 \cdot 7H_2O$ and 5 g glucose in a liter with 0.05 M Tris-HCl) to make 3×10^8 cells per ml. This cell suspension was used for experiments testing incorporation of ^{14}C-thymidine, ^{14}C-uracil and ^{14}C-leucine into the acid-insoluble fraction.

Incorporation of ^{14}C-amino acids into protein in the cell-free system was studied by methods generally used as described by TANAKA *et al.*[4].

Rabbit reticulocytes were obtained by the method of ALLEN and SCHWEET[5]. Reticulocyte lysate was prepared and its protein synthesis assayed by usual methods as described by NISHIMURA[6].

The effect on aminoacyl-tRNA synthesis was studied by usual methods[4], collecting tRNA precipitated with 5 % TCA on Millipore filter. Binding of ^{14}C-phenylalanyl-tRNA

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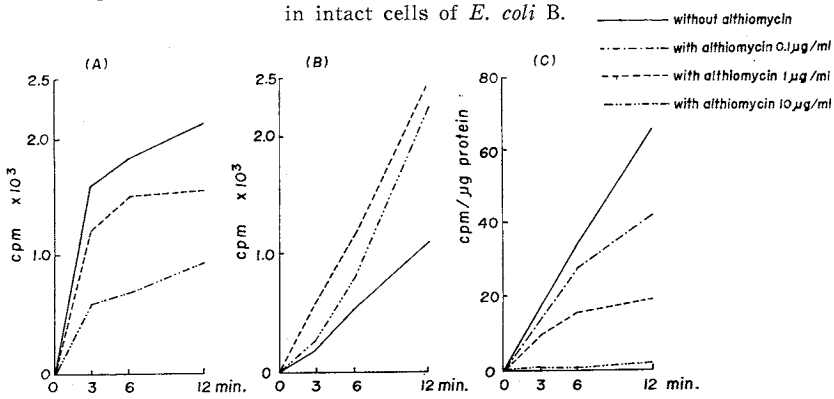
to ribosomes in response to poly U was examined by the method of NIRENBERG and MATTHAEI[7]. The puromycin reaction was assayed by measuring the formation of N-acetyl-^{14}C-phenylalanyl-puromycin according to the method of LEDER and BURSITYN[8].

Results

Effect of Althiomycin on the Syntheses of DNA, RNA and Protein in Intact Cells of *E. coli* B

Effects of althiomycin on incorporation of ^{14}C-thymidine into DNA, ^{14}C-uracil into RNA and ^{14}C-leucine into protein in growing cells of *E. coli* B were studied. As illustrated in Fig. 1, althiomycin was observed to inhibit incorporation of ^{14}C-thymidine and even more of ^{14}C-leucine, and to stimulate incorporation of ^{14}-uracil. This result suggested that inhibition of protein synthesis would be the primary action of the antibiotic.

Fig. 1. Effect of althiomycin on DNA, RNA and protein synthesis in intact cells of *E. coli* B.



After 20 minutes of preincubation of the *E. coli* B cell suspension under shaking of 30℃, althiomycin and (A) ^{14}C-thymidine (0.1 μCi/ml), (B) ^{14}C-uracil (0.2 μCi/ml) or (C) ^{14}C-leucine (0.2 μCi/ml) were added. Incorporation at 30℃ was terminated at 3, 6 and 12 minutes by adding 0.5 ml of 10% TCA to 0.5 ml of the reaction mixture. The precipitate was collected on Millipore filter and washed three times with 5 ml of 5% TCA and the radioactivities were determined by a windowless gas flow counter.

Inhibition by Althiomycin of Polypeptide Synthesis in a Ribosome System with Native mRNA

The effect on protein synthesis with native messengers in S 30 fraction which was prepared by 30,000 *g* centrifugation of *E. coli* B homogenate was studied. As shown in Table 1, the antibiotic inhibited protein synthesis in an *E. coli* B cell-free system. At the concentration of 1 μg/ml and 10 μg/ml, inhibition percents after 10 minutes incubation were 47 and 70 respectively.

Table 1. Effects of althiomycin on protein synthesis in *E. coli* B S 30 fraction with native messengers.

	Incorporation of ^{14}C-amino acids (c.p.m./mg protein) Incubation time at 37°C		
	5 min.	10 min.	20 min.
Control	3,520 (100)	6,080 (100)	9,140 (100)
+Althiomycin 0.1 μg/ml	3,540 (102)	5,670 (93)	7,230 (79)
1 μg/ml	2,050 (58)	3,230 (53)	5,050 (55)
10 μg/ml	1,150 (33)	1,840 (30)	2,370 (26)
+Blasticidin S 20 μg/ml	200 (6)	440 (7)	730 (8)

Reaction mixture; (in 0.2 ml) 0.2 mg protein of S 30 fraction (supernatant of 30,000 *g* centrifugation containing ribosomes and S 100 fraction), 0.2 μmoles ATP, 0.04 μmoles GTP, 0.8 μmoles phosphoenol-pyruvate, 10 μg pyruvate kinase, 60 μg tRNA, 10 μmoles Tris (pH 7.8), 20 μmoles KCl, 2 μmoles $MgCl_2$, 0.2 μmoles DTT (dithiothreitol) and 0.04 μCi ^{14}C-amino acid mixture.

Hot 5% TCA insoluble part was collected on Millipore filter, washed and counted by a windowless gas flow counter.

Effects of Althiomycin on Polypeptide Synthesis with Synthetic Polynucleotides as Messengers

Effects of althiomycin on the incorporation of ^{14}C-phenylalanine, ^{14}C-lysine and ^{14}C-proline in response to poly U, poly A and poly C were examined. In these experiments, ribosomes were separated from S 100 fraction by centrifugation at 105,000g for 2 hours. The results are presented in Table 2. Strong inhibition by the antibiotic was observed with poly C and poly U, and the inhibition percent was larger with the former. However, almost no inhibition was observed with poly A.

Table 2. Effects of the althiomycin on polypeptide synthesis dependent on added polynucleotide in cell-free systems of *E. coli* B.

	Incorporation of ^{14}C-amino acid (c. p. m.)		
	poly U-phe.	poly A-lys.	poly C-pro.
Complete	5,132 (100)	9,620 (100)	3,150 (100)
—Polynucleotide	559	1,301	312
+Althiomycin 0.1 μg/ml	5,242 (102)	9,809 (102)	2,135 (74)
1 μg/ml	4,130 (83)	9,482 (99)	1,533 (49)
10 μg/ml	2,169 (42)	8,940 (93)	833 (28)
+Blasticidin S 20 μg/ml	259 (5)	2,502 (26)	673 (21)

Complete reaction mixture (in 0.2 ml) ; 0.2 mg ribosomes, 0.2 mg protein of S 100 fraction, 0.2 mg tRNA, 0.2 μmoles ATP, 0.02 μmoles GTP, 0.8 μmoles phosphoenol-pyruvate, 10 μg pyruvate kinase, 0.2 μmoles DTT, 10 μmoles Tris (pH 7.8), 20 μmoles KCl, 2 μmoles $MgCl_2$ and 0.04 μCi ^{14}C-amino acid. Incubation ; at 37℃ for 10 minutes.

Experimental precedures were the same as in Table 1 except that 5% TCA containing 0.1% phosphotungstic acid in the case of incorporation of lysine and 15% TCA in the case of that of proline were employed instead of 5% TCA.

Effects of Althiomycin on Protein Synthesis in Rabbit Reticulocyte Systems

To investigate the effect of althiomycin on protein synthesis of mammalian cells, studies were carried out on rabbit reticulocytes. As shown in Table 3, the antibiotic did not show any substantial inhibition of protein synthesis either in the intact cells or in the cell-free system, just like chloramphenicol, which was tested for comparison.

Effects of Althiomycin on Aminoacyl-tRNA Synthesis, Binding of Aminoacyl-tRNA to Ribosomes and Puromycin Reaction

Studies were continued to localize the site of action of althiomycin in the process of bacterial protein synthesis. Effects of the antibiotic on aminoacyl-tRNA synthesis,

Table 3. Effects of althiomycin on protein synthesis of intact reticulocytes and their cell-free system.

(a) Intact reticulocytes

	Incorporation of ^{14}C-valine (c. p. m.) Incubation time at 37℃	
	10 min.	20 min.
Control	2,304 (100)	4,495 (100)
+Althiomycin 1 μg/ml	2,081 (87)	3,974 (88)
10 μg/ml	1,900 (82)	3,420 (76)
100 μg/ml	2,452 (110)	4,438 (99)
+Chloramphenicol 20 μg/ml	1,763 (77)	3,278 (73)
+Puromycin 20 μg/ml	235 (10)	232 (5)

(a) Reaction mixture ; 0.4 ml cell suspension (containing 1.7 mg protein), 0.025 μCi ^{14}C-valine (168 mCi/mM) and drug in 0.5 ml.

Incorporation of ^{14}C-valine into hot 5% TCA insoluble fraction was determined by the same method as in Fig. 1.

(b) Lysate

	^{14}C-valine incorporated (c. p. m.)
Control	1,258 (100)
+Althiomycin 10 μg/ml	1,147 (91)
100 μg/ml	1,122 (89)
+Chloramphenicol 100 μg/ml	1,061 (84)
+Puromycin 20 μg/ml	366 (29)

(b) Reaction mixture (in 0.5 ml) ; 0.3 ml lysate (1.8 mg protein), 150 μg tRNA, 0.5 μmoles ATP, 0.5 μmoles GTP, 0.2 μmoles phosphoenol-pyruvate, 25 μg pyruvate kinase, 0.5 μmoles DTT, 15 μmoles Tris-HCl (pH 7.8), 25 μmoles KCl, 2 μmoles $MgCl_2$ and 0.2 μCi ^{14}C-valine. Incubation ; at 37℃ for 20 minutes.

Table 4. Effect of althiomycin on aminoacyl-tRNA synthesis.

	^{14}C-amino acids rendered TCA insoluble (c.p.m.) Incubation time at 37°C		
	5 min.	10 min.	20 min.
Control	8,562	10,054	11,191
+Althiomycin 10 μg/ml	9,239	8,424	10,386

Reaction mixture (in 0.2 ml); 0.2 mg tRNA, 0.2 mg protein of S 100 fraction, 0.4 μmoles ATP, 10 μmoles Tris (pH 7.8), 20 μmoles KCl, 2 μmoles $MgCl_2$ and 0.04 μCi ^{14}C-amino acid mixture.

Table 5. Effect of althiomycin on binding of aminoacyl-tRNA to ribosomes.

	^{14}C-Phenylalanyl-tRNA bound to ribosomes (c.p.m.)	
Complete	952	(100%)
−Poly U	52	
+Althiomycin 0.1 μg/ml	1,160	(122)
1 μg/ml	1,156	(122)
10 μg/ml	1,120	(118)
+Tetracycline 100 μg/ml	224	(24)

Complete reaction mixture (in 0.2 ml); 0.36 mg ribosomes, 10 μg poly U, 48 μg ^{14}C-phenylalanyl-tRNA, 10 μmoles Tris (pH 7.6), 20 μmoles KCl and 4 μmoles $MgCl_2$. Incubation; at 30°C for 10 minutes.

Table 6. Effect of althiomycin on puromycin reactions.

	N-Acetyl-^{14}C-phenylalanyl-puromycin formed c.p.m. (% inhibition)	
Control	636	
−Puromycin	100	
+Althiomycin 0.1 μg/ml	604	(5)
1 μg/ml	500	(21)
10 μg/ml	271	(57)
+Blasticidin S 10 μg/ml	161	(75)
+Mikamycin A 2 μg/ml	322	(49)
+Chloramphenicol 100 μg/ml	247	(61)

Reaction mixture (in 0.2 ml); 0.2 mg ribosomes, 10 μg poly U, 60 μg N-acetyl-^{14}C-phenylalanyl-tRNA, 0.2 μmoles DTT, 10 μmoles Tris (pH 7.6), 20 μmoles KCl, 2 μmoles $MgCl_2$ and 4 μg puromycin.

After the mixture without puromycin was preincubated at 30°C for 25 minutes, puromyin and an inhibitor were added and incubated at 30°C for 10 minutes.

binding of aminoacyl-tRNA to ribosomes, and the puromycin reaction were examined with *E. coli* B systems. The results are shown in Tables 4, 5 and 6. It was demonstrated that the antibiotic inhibited the puromycin reaction without showing any inhibition against aminoacyl-tRNA synthesis and binding of aminoacyl-tRNA to ribosomes, and suggested that althiomycin interfered with peptide bond formation.

Discussion

In this study althiomycin was demonstrated to inhibit protein synthesis of *E. coli* B, and the site of its action was suggested by the inhibition of the puromycin reaction to be located in peptide bond formation. On the other hand, the antibiotic did not practically affect protein synthesis of reticulocytes. This result parallels the low toxicity of the antibiotic for the host.

Althiomycin, which contains sulfur, has no structural relation with puromycin, chloramphenicol, lincomycin, mikamycin A (ostreogrycin A) and blasticidin S, which are known to interfere with peptide bond formation. Peptide bond formation is catalyzed by 50 S ribosomal subunits. Considering that various antibiotics with diverse structures interfere with the reaction, the structure of 50 S ribosomal subunits seems to be complex.

The antibiotic was observed to stimulate RNA synthesis in intact cells of *E. coli* B. A similar effect is well known in chloramphenicol.

Inhibition by althiomycin of polypeptide synthesis on a synthetic polynucleotide was strong in the cases of poly C-proline and poly U-phenylalanine but was weak in poly A-lysine. Similar differences have been observed with chloramphencol, bottromycin, macrolide antibiotics and mikamycin B. However, relative strength of inhibition on these polynucleotides are not same, for instance, chloramphenicol inhibits most strongly polyproline synthesis, and most weakly polyphenylalanine synthesis. In these cases, no structural relationship can not be seen among the antibiotics, the polynucleotides and the amino aicds.

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