AVILAMYCIN, AN INHIBITOR OF THE 30 S RIBOSOMAL SUBUNITS FUNCTION*

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1. Introduction

Avilamycin, a chlorine-containing antibiotic produced by *Streptomyces viridochromogenes*, primarily inhibits the growth of Gram-positive bacteria. Isolation and characterization have been described [2]. The structure of avilamycin, a polysaccharide antibiotic bearing a curacin end group, is similar to that of curamycin and the everninomycins [2, 3]. However, nothing is known about the mode of action of these compounds.

This paper describes the effects of avilamycin on the protein and nucleic acid synthesis in *Bacillus brevis* cells, as well as on the polypeptide synthesis in the cell-free systems of *B. brevis* and *E. coli*.

2. Materials and methods

2.1. *Materials*

Ribosomes, 30 S and 50 S ribosomal subunits, and supernatant enzymes (S-100 fraction) from *E. coli* A19 [4], a RNAase I-less mutant, and *B. brevis* ATCC 9999 were prepared as described [5]. After high-speed centrifugation, a fraction of supernatant fluid was dialyzed with 10 mM Tris–HCl buffer pH 7.6 containing 10 mM magnesium acetate and 6 mM 2-mercaptoethanol. The ribosomes were suspended in 10 mM magnesium acetate, the ribosomal subunits however, in 1 mM magnesium acetate. All collected enzymes and ribosomal particles were kept until use at –65°C (see sect. 2.2).

Formyl-[^{3}H]methionyl-tRNA was prepared from *E. coli* MRE 600 tRNA (Boehringer, Mannheim) and [^{3}H]methionine according to the procedure of Hershey and Thach [6]. Crude initiation factors were isolated as described [7].

[^{3}H]methionine (9 Ci/mM), [^{14}C]isoleucine (10 mCi/mM), and [^{14}C]phenylalanine (492 mCi/mM) were purchased from the Radiochemical Centre, Amersham, [^{14}C]phenylalanyl-tRNA, 0.134 μCi/mg) from the New England Nuclear Corporation, and [^{3}H]poly U (81.6 μCi/μMP) from the Miles Laboratories. Crystalline avilamycin was isolated as described [2].

2.2. *Protein synthesis*

Experiments for polyphenylalanine synthesis were carried out by a method similar to that of Traub et al. [8]. Each incubation mixture (50 μl) contained: 10 10 mM Tris–HCl pH 7.8; 30 mM NH_4Cl; 10 mM magnesium acetate, 6 mM 2-mercaptoethanol; 1 mM ATP; 0.03 mM GTP; 0.05 mM 19 amino acids except phenylalanine; 5 mM phosphoenolpyruvic acid; 1 μg pyruvate kinase; 0.02 μCi [^{14}C]phenylalanine; 10 μg poly U; 5 μl enzyme fraction (S-100); 0.2 A_{260} unit of 70 S ribosomes or 50 S and 30 S ribosomal subunits and 1 μl of antibiotic solution (avilamycin was dissolved in ethanol).

The reaction mixture was incubated for 45 min at 30°C. The incorporation of radioactivity into hot 5% trichloroacetic acid-precipitable material was measured by assaying a sample (40 μl) of the reaction mixture by the paper disk method [9].

The assay for protein synthesis directed by natural mRNA was performed using the same reaction mixture as that described above, except that poly U is replaced by $Q\beta$ RNA (Miles Laboratories). The incubation mixture also contained 0.5 μg leucovorin.

* Metabolic products of microorganisms, 121; for preceding publication see [1].

2.3. *Determination of aminoacyl-tRNA binding to ribosomal particles*

The binding of [^{14}C]phenylalanyl-tRNA to ribosomes was assayed as described [10]. Fifty μl binding buffer (100 mM Tris–acetate, pH 7.2, 20 mM magnesium acetate, 50 mM KCl) contained 2 A_{260} units of washed ribosomes, 20 μg poly U, and 0.01 μCi [^{14}C]-phenylalanyl-tRNA.

The reaction mixture (50 μl) for enzymatic binding of formyl-[^{3}H]methionyl-tRNA to 30 S ribosomal subunits was similar to that of Hershey et al. [7]: 50 mM Tris–HCl pH 7.4; 100 mM NH_4Cl; 5 mM magnesium acetate; 6 mM 2-mercaptoethanol; 1 mM GTP; 0.2 mM AUG (Boehringer, Mannheim); 0.02 μCi formyl-[^{3}H]methionyl-tRNA, 1 A_{260} unit of 30 S ribosomal subunits, and 20 μg crude initiation factors.

The reaction mixture was incubated for 15 min at 24°C, than diluted by addition of 3 ml of ice-cold binding buffer, and filtered through a Millipore filter (HA 0.45 μm pore size). The filter was dried, and the radioactivity measured.

3. Results and discussion

The effect of avilamycin on the incorporation of [^{14}C]thymidine, [^{14}C]uracil, and [^{14}C]isoleucine by intact cells of *B. brevis* was determined. Avilamycin inhibited isoleucine incorporation within less than 3 min after addition, whereas the rate of thymidine and uracil incorporation remained essentially unchanged for 30 min after addition of the antibiotic (fig. 1). We inferred from these data that avilamycin acts as a specific inhibitor of protein synthesis in *B. brevis* cells. This conclusion was further substantiated by *in vitro* experiments.

As shown in fig. 2, avilamycin inhibited the poly U-directed polyphenylalanine synthesis in the *in vitro* system of *B. brevis* to the extent of approximately 68%.

Peptide synthesis in the *in vitro* system of *E. coli* was also inhibited, whereas the inhibition of growing cells was not observed (fig. 2). Thus the difference in susceptibility of *B. brevis* and *E. coli* may be due to a difference in factors other than those contained in the protein-synthesizing system.

When the protein synthesis was directed by natural mRNA, complete inhibition was observed with 1.43×10^{-5} M of avilamycin, while partial inhibition was observed, when the same incorporation system was used, except that the natural mRNA was replaced by poly U (fig. 2). These results suggest that avilamycin mainly interferes with the formation of the initiation complex.

In a further experiment the effect of avilamycin on the formation of [^{14}C]phenylalanyl-tRNA was examined. As shown in table 1, this reaction was not at all affected by the antibiotic. The attachment of

Table 1
Effect of avilamycin on the formation of [^{14}C]phenylalanyl-tRNA (*E. coli*).

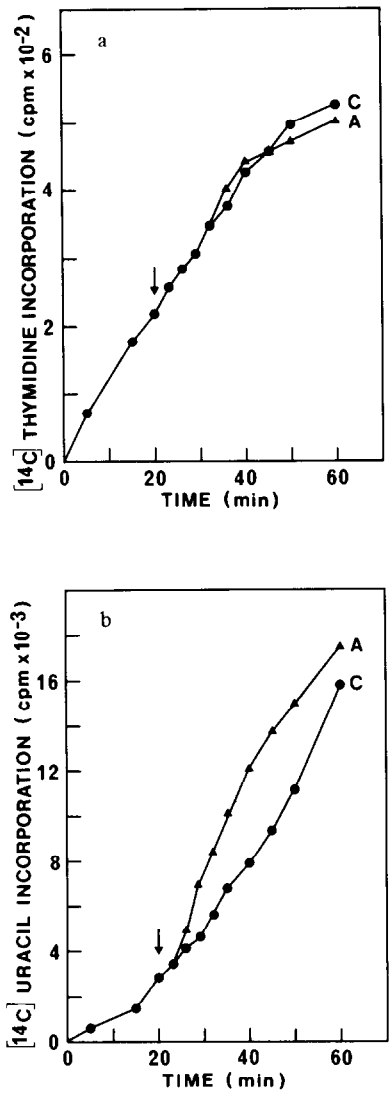
Avilamycin added (M)	[^{14}C]phenylalanyl-tRNA (cpm)	Synthesized (%)
Control	2 930	100
1.43×10^{-6}	2 880	98
1.43×10^{-5}	2 810	96
1.43×10^{-4}	2 800	96

The assay was performed using the incubation mixture for polyphenylalanine synthesis without ribosomal particles. The incorporation of radioactivity into cold 5% trichloroacetic acid-precipitate material was measured.

Table 2
Effect of avilamycin on poly U-directed incorporation of [^{14}C]isoleucine (*E. coli*).

Antibiotic added (M)	Polyisoleucine synthesized (cpm)
Control	80
Avilamycin	
1.43×10^{-7}	32
1.43×10^{-6}	26
1.43×10^{-5}	30
1.43×10^{-4}	18
Streptomycin	
1.37×10^{-7}	465
1.37×10^{-6}	436
1.37×10^{-5}	333
1.37×10^{-4}	331

The samples contained the standard reaction mixture except that 0.01 μCi [^{14}C]isoleucine was substituted for [^{14}C]-phenylalanine.



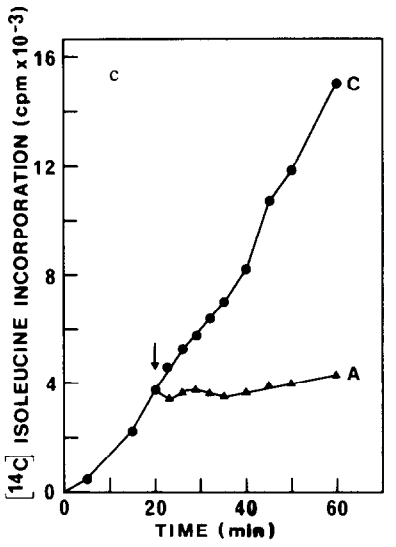


Fig. 1. Effect of avilamycin on the incorporation of a) [^{14}C]-thymidine, b) [^{14}C]uracil, and c) [^{14}C]isoleucine by intact cells of *B. brevis* (time of addition of the antibiotic is indicated by the arrow): C) control; A) avilamycin 0.7 μg/ml. Experimental conditions were identical to those described previously [11].

[^{3}H]poly U to ribosomes was studied in the presence and absence of avilamycin by zone sedimentation on linear density gradients. Messenger RNA bound to ribosomes sediments more rapidly than free mRNA and is found in the gradient at a position close to that of the particle to which it is attached. The data of these binding studies indicated that avilamycin did not change the binding capacity of ribosomes to poly U (fig. 3).

Avilamycin was also tested in an assay system, where streptomycin increases poly U-directed incorporation of [^{14}C]isoleucine. As shown in table 2, avilamycin did not promote mistakes in the translation of the genetic code like streptomycin does.

The binding of [^{14}C]phenylalanyl-tRNA to washed ribosomes directed by poly U was assayed employing the filter technique of Nirenberg and Leder [10]. The results obtained indicated that 2.86×10^{-5} M of avilamycin inhibits this reaction to the extent of approximately 50% (table 3). Higher antibiotic con-

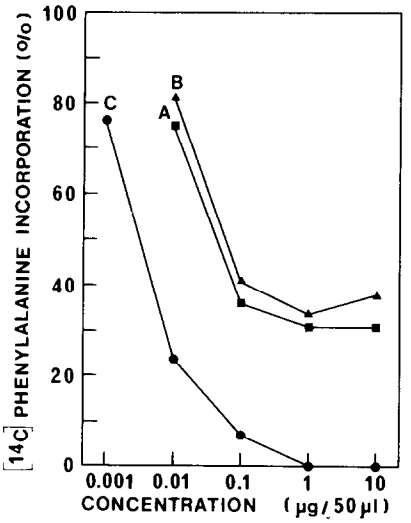


Fig. 2. Effect of concentrations of avilamycin on the polypeptide synthesis directed by poly U (A, B) or by the RNA of phage Qβ (C) in the cell-free systems of *B. brevis* (A) and *E. coli* (B, C).

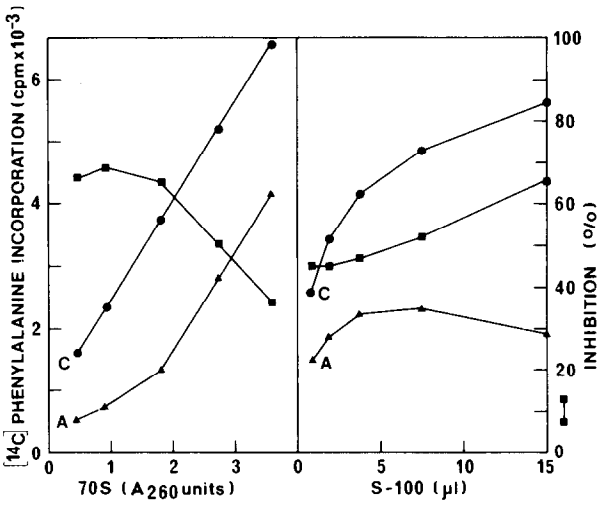


Fig. 4. Effect of concentrations of high-speed supernatant (S-100) and ribosomes (70 S) on the extent of inhibition of the poly U-directed polyphenylalanine synthesis (*E. coli*) by avilamycin 0.1 μg/50 μl.

centrations did not result in a significant further increase of inhibition. The fact, that avilamycin inhibited the binding of phenylalanyl-tRNA to ribosomes by no more than about 50% suggested that this antibiotic blocked one of the two binding sites for aminoacyl-tRNA.

In other experiments the effect of avilamycin on the AUG-directed enzymatic binding of formyl-[^{3}H]-methionyl-tRNA to the 30 S ribosomal subunits was tested. We have observed a complete inhibition of the reaction by the antibiotic (table 4).

Studies were continued to localize the binding site of the antibiotic. As shown in fig. 4, the inhibitory

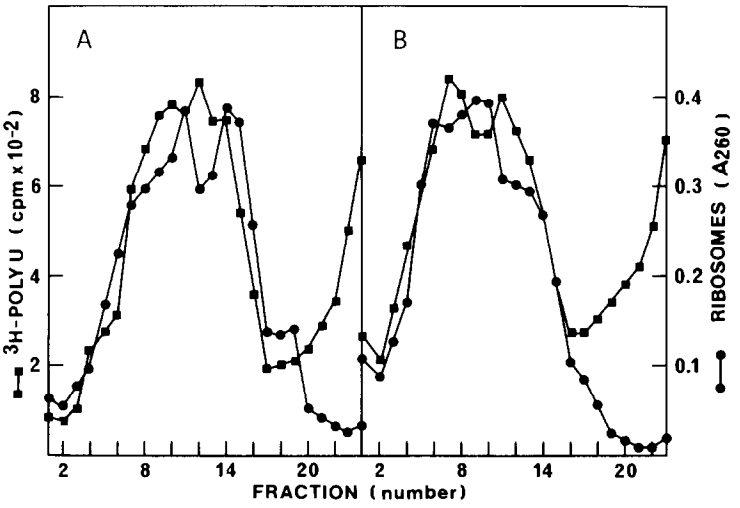


Fig. 3. Effect of avilamycin on the attachment of [^{3}H]poly U to ribosomes (*E. coli*): A) control; B) avilamycin 50 μg/250 μl. Experimental conditions were identical to those described [12].

Table 3
Effect of avilamycin on the binding of [^{14}C]phenylalanyl-tRNA to ribosomes (*E. coli*).

Antibiotic added (M)	[^{14}C]phenylalanyl-tRNA bound to ribosomes (cpm)	(%)
Control	1 256	100
Avilamycin		
2.86×10^{-8}	945	75
2.86×10^{-7}	874	70
2.86×10^{-6}	630	50
2.86×10^{-5}	786	63
2.86×10^{-4}	626	50
Tetracycline		
8.3×10^{-6}	846	67
8.3×10^{-5}	638	51
8.3×10^{-4}	742	59

effect of avilamycin was noticeably decreased by the addition of excess of ribosomes (70 S), but not by addition of supernatant enzymes (S-100 fraction).

Further experiments were carried out with separated subunits of *E. coli* ribosomes. As shown in table 5, the combination of avilamycin-treated 30 S subunits and control 50 S subunits showed a much lower peptide synthesizing activity than that of treated 50 S subunits and control 30 S subunits.

The data indicate that avilamycin binds with the 30 S subunits of *E. coli* ribosomes and interferes with the polypeptide-synthesizing function by affecting the attachment of aminoacyl-tRNA to the ribosomes.

Table 4
Effect of avilamycin on the binding of formyl-[^{3}H]methionyl-tRNA to the 30 S ribosomal subunits directed by AUG (*E. coli*).

Antibiotic added (M)	Formyl-[^{3}H]methionyl-tRNA bound to the 30 S ribosomal subunits (cpm)	(%)
Complete system	462	100
– AUG	80	0
Avilamycin		
1.43×10^{-7}	367	75
1.43×10^{-6}	143	16
1.43×10^{-5}	56	0
1.43×10^{-4}	88	2

Table 5
Effect of avilamycin-treated ribosomal subunits on the polyphenylalanine synthesis in the cell-free system of *E. coli*.

Ribosomal subunits added**		Concentration of avilamycin (M)	Polyphenylalanine synthesized (cpm)	(%)
30 S	–	–	819	11
30 S*	–	–	675	9
–	50 S	–	1 431	20
–	50 S*	–	1 022	14
30 S	50 S	–	7 314	100
30 S*	50 S*	–	3 894	53
30 S	50 S	1.43×10^{-5}	3 055	42
30 S*	50 S	–	4 027	*55*
30 S	50 S*	–	5 346	*73*

** Ribosomal subunits preincubated in the absence (30 S, 50 S) or presence (30 S*, 50 S*) of an excess of avilamycin (20 min at 30° C) and dialyzed at 4° C for 4 hr with 3 changes of standard buffer.

The samples contained the standard reaction mixture, however 0.3 A_{260} units 30 S and 0.6 A_{260} units 50 S particles were added per assay according to the data in the table.

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