MECHANISM OF ACTION OF NEGAMYCIN IN *ESCHERICHIA COLI* K12

II. MISCODING ACTIVITY IN POLYPEPTIDE SYNTHESIS DIRECTED BY SYNTHETIC POLYNUCLEOTIDE

SATOSHI MIZUNO, KAZUO NITTA and HAMAO UMEZAWA

Department of Antibiotics, National Institute of Health, Shinagawa-ku, Tokyo, Japan

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The effect of negamycin was studied on polypeptide synthesis directed by synthetic polynucleotides in *Escherichia coli* K12 extracts.

1. Negamycin caused misreading of genetic codes of the mRNA in the extracts: (1) Incorporation of phenylalanine normally coded for by poly U was inhibited, while that of isoleucine, serine, or leucine, not normally coded for by the polymer, was stimulated. (2) Poly A-directed lysine or glutamic acid incorporation and poly C-directed proline or leucine incorporation were both stimulated by the drug.

2. Binding of phenylalanyl-tRNA to ribosomes-poly U complex was weakly inhibited by negamycin. On the other hand, that of isoleucyl-tRNA to the complex was about threefold stimulated by the drug.

3. Puromycin reaction, an analogue of peptide bond forming reaction, with N-acetyl-phenylalanyl-tRNA was not affected by negamycin.

4. In rat liver extracts, the incorporation of phenylalanine directed by poly U was much less affected by negamycin.

In a preceding paper on mechanism of action of negamycin in *Escherichia coli* K12, we reported that the primary action of the drug is the inhibition of protein synthesis and the inhibited step in the protein synthesis is peptide chain initiation[1].

In this paper, studies are reported on the effect of negamycin on polypeptide synthesis directed by synthetic polynucleotide in *E. coli* K12 extracts. The results indicate that negamycin induces misreading of the genetic codes of the synthetic mRNAs.

Methods and Materials

1. *E. coli* K12 extracts: *E. coli* K12 extracts were prepared as described in a preceding paper[1]. Incubated S30 was used for preparation of ribosomes and S100. Washed ribosomes were prepared by washing the ribosomes with a buffer containing 0.5 M NH_4Cl, 0.05 M KCl, 0.1 M Tris-HCl (pH 7.8), 0.01 M $Mg(CH_3COO)_2$, and 0.01 M β-mercaptoethanol, for three times and stored at $-20°C$.

2. Rat liver extracts: Rat liver ribosomes and pH 5 fraction which contains aminoacyl-tRNA synthetases were prepared as described in a previous paper[2]. Transfer RNA was isolated from the pH 5 fraction by phenol method.

3. Preparation of ^{14}C-aminoacyl-tRNA and N-acetyl-^{14}C-phenylalanyl-tRNA: ^{14}C-phenylalanyl-tRNA and ^{14}C-isoleucyl-tRNA were prepared by incubating *E. coli* K12 tRNA

(stripped) with S100, ATP and ^{14}C-phenylalanine and ^{14}C-isoleucine, respectively, as described by NATHANS and LIPMANN[3], and isolated by phenol method. N-Acetyl-^{14}C-phenylalanyl-tRNA was prepared according to HAENNI and CHAPEVILLE[4]. The acetylated products were examined by paper chromatography (butanol - acetic acid - water ; 4 : 1 : 2) and shown to be free of phenylalanyl-tRNA.

4. Assay of polypeptide synthesis: The reaction mixtures for polypeptide synthesis were described in the legend of each figure and table in the text. Incubation was stopped by adding equal volume of 10 % trichloroacetic acid (TCA) and the mixtures were washed with 5 % TCA for three times followed by heating at 90°C for 15 minutes in 5 % TCA. The hot TCA-insoluble radioactivity was counted with a windowless gas-flow counter.

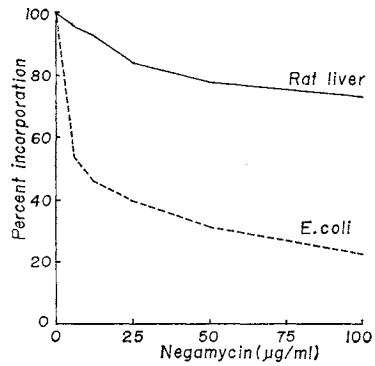
5. Assay of aminoacyl-tRNA binding: Assay of ^{14}C-aminoacyl-tRNA binding to ribosomes-mRNA complex was performed according to the method of NIRENBERG and LEDER[5]. A Millipore filter (22 mm diameter, 0.45 μ pore size) retainining ^{14}C-aminoacyl-tRNA was dried in a planchet and the radioactivity was counted with a windowless gas-flow counter.

6. Chemicals: Poly U, poly A and poly C were obtained from Sigma Chemicals. *E. coli* K12 tRNA (stripped) was obtained from General Biochemicals. ^{14}C-Amino acid (Phe, Ileu, Ser, Leu, Pro and Lys) was obtained from Radiochemical Centre. ^{14}C-Amino acid mixture (*Chlorella* protein acid hydrolysate) was supplied from the Institute of Applied Microbiology, the University of Tokyo. Negamycin and its degraded products were given by Dr. S. KONDO, the Institute of Microbial Chemistry, Tokyo.

Fig. 1. Inhibition by negamycin of polyphenylalanine synthesis directed by poly U

E. coli reaction mixtures (0.3 ml) containing 50 mM Tris-HCl, pH 7.8, 60 mM KCl, 10 mM Mg-acetate, 10 mM β-mercaptoethanol, 1 mM ATP, 0.1 mM GTP, 5 mM creatine phosphate, creatine kinase 15 μg, tRNA (*E. coli*) 85 μg, poly U 20 μg, ^{14}C-phenylalanine 0.2 μc (0.68 mμmole), S100 0.6 mg protein and ribosomes 0.6 mg protein and negamycin of indicated amount, were incubated at 35°C for 60 minutes and hot TCA-insoluble radioactivity was determined. Control without negamycin incorporated 1.09×10^5 cpm, which represented 100 % incorporation and incorporation without poly U was 7.3 % of that of the control.

Rat liver reaction mixtures (0.2 ml) containing 50 mM Tris-HCl, pH 7.6, 25 mM KCl, 5 mM Mg-acetate, 50 mM sucrose, 1 mM ATP, 0.1 mM GTP, 5 mM creatine phophate, creatine kinase 10 μg, ribosomes 0.2 mg, and pH 5 fraction containing aminoacyl-sRNA synthetases, 1.2 mg, were pre-incubated at 35°C for 20 minutes and to the mixtures were added poly U 20 μg, tRNA (Rat liver) 140 μg, ^{14}C-phenylalanine 0.1 μc (0.34 mμmole) and negamycin, and the mixtures (0.4 ml) were further incubated for 60 minutes. Control without negamycin incorporated 5.2×10^4 cpm and incorporation without poly U was 2.6 % of that of the control.



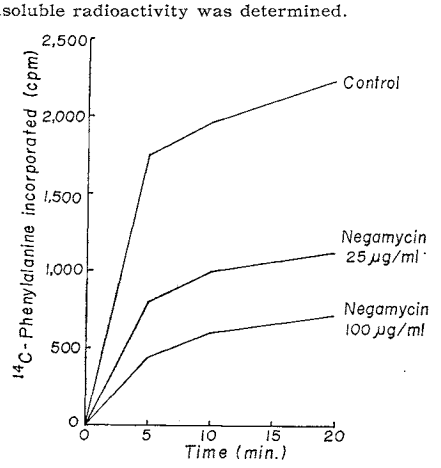
Results and Discussion

1. Inhibition of Polyphenylalanine Synthesis Directed by Poly U

Effect of negamycin was studied on polyphenylalanine synthesis directed by poly U in *E. coli* K12 extracts. The incorporation of phenylalanine into hot TCA-

Fig. 2. Inhibition by negamycin of phenylalanine-transfer from phenylalanyl-tRNA to polyphenylalanine

Reaction mixtures (0.3 ml) contained 50 mM Tris-HCl, pH 7.8, 60 mM KCl, 10 mM Mg-acetate, 10 mM β-mercaptoethanol, 0.05 mM GTP, poly U 20 μg, ^{14}C-phenylalanyl-tRNA 8,200 cpm, S100 0.3 mg and ribosomes 0.4 mg. Incubation was at 30°C and hot TCA-insoluble radioactivity was determined.



insoluble fraction was inhibited by negamycin: Fifty percent inhibition was obtained by 10 μg/ml of the drug (Fig. 1).

As reported in our preceding paper[1], negamycin inhibited initiation of protein synthesis by phage MS2 RNA, a natural messenger RNA. The most critical difference between the natural and the artificial mRNA is known to be that the natural mRNA initiates protein synthesis by a mechanism requiring formylated methionyl-tRNA for the corresponding AUG initiation codon at low magnesium concentration (7~8 mM)[6]. In contrast, poly U has no natural initiation codon and initiates the polypeptide synthesis by a mechanism that seems to require distortion of ribosomal binding site which occurs at high magnesium concentration (10 mM)[7]. Both the reactions were inhibited by negamycin as described above and in a preceding paper. These results suggested that the poly U-driected polyphenylalanine synthesis was affected by a mechanism which seemed to be different from that observed in protein synthesis by the natural mRNA.

Table 1. Effect of negamycin on poly U coded binding of ^{14}C-phenylalanyl-tRNA and N-acetyl-phenylalanyl-tRNA to ribosomes

	CPM retained on filter
^{14}C-Phenylalanyl-tRNA	
Complete	2,291
〃 −Poly U	152
〃 −Ribosomes	21
〃 +Negamycin 25 μg/ml	1,855(19.1)
〃 +Negamycin 50 μg/ml	1,718(25.3)
〃 +Negamycin 100 μg/ml	1,531(33.5)
N-Acetyl-^{14}C-phenylalanyl-tRNA	
Complete	381
〃 −Poly U	22
〃 +Negamycin 200 μg/ml	410

The number in parentheses represents % inhibition.

Complete reaction mixtures (0.1 ml) for binding of ^{14}C-phenylalanyl-tRNA contained 0.1 M Tris-HCl, pH 7.1, 0.05 M KCl, 0.02 M Mg-acetate, poly U 30 μg, washed ribosomes 1.0 mg and ^{14}C-phenylalanyl-tRNA 7,700 cpm. The mixtures were incubated at 25℃ for 20 minutes and diluted by 3 ml of an ice-cold buffer (0.1 M Tris-HCl, pH 7.1, 0.05 M KCl, and 0.02 M Mg-acetate) and the amount of bound ^{14}C-phenylalanyl-tRNA was determined according to the method of Millipore filter technique of NIRENBERG and LEDER[5].

Reaction mixtures (0.1 ml) for binding of N-acetyl-^{14}C-phenylalanyl-tRNA contained 0.1 M Tris-HCl, pH 7.1, 0.05 M KCl, 0.01 M Mg-acetate, poly U 10 μg, washed ribosomes 1.0 mg and N-acetyl-^{14}C-phenylalanyl-tRNA 1,220 cpm. The mixtures were incubated at 35℃ for 10 minutes and assayed as above. Radioactivity retained on filter was counted with a windowless gas-flow counter.

Polyphenylalanine snythesis in rat liver extract was much less inhibited by negamycin (Fig. 1). This different sensitivity between *E. coli* extract and rat liver extract towards negamycin would be directly related to different affinity of the drug for ribosomes of both the cells, and it would be a biochemical basis for the selective toxicity of the drug against bacteria.

Phenylalanyl-tRNA synthetase activity in *E. coli* extract was not inhibited by negamycin (data not shown).

Effect of negamycin on the formation of ribosome-poly U complex was examined. ^{14}C-Poly U (10^4 cpm/20 μg, Miles Chemicals) was mixed with ribosomes of *E. coli* K12 at 0°C and the mixture was filtered on a Millipore membrane filter and

Table 2. Effect of negamycin on puromycin reaction with N-acetyl-^{14}C-phenylalanyl-tRNA

	CPM retained on filter	CPM released
Complete	409	
〃 +Puromycin	179	230
〃 +Negamycin	492	
〃 +Puromycin+Negamycin	228	264

Reaction mixtures (0.1 ml), containing 0.1 M Tris-HCl, pH 7.1, 0.05 M KCl, 0.01 M Mg-acetate, poly U 10 μg, washed ribosomes 1.0 mg and N-acetyl-^{14}C-phenylalanyl-tRNA 1,220 cpm, were incubated at 35℃ for 15 minutes with or without puromycin 50 μg and/or negamycin 20 μg. After the mixtures were diluted, the amount of N-acetyl-^{14}C-phenylalanyl-tRNA bound to ribosomes was assayed by the method of NIRENBERG and LEDER[5]. Released radioactivity by puromycin was obtained by subtracting the retained cpm determined with puromycin from that without puromycin.

the filter was washed with a cold buffer (0.1 M Tris-HCl, pH 7.1, 20 mM magnesium acetate and 50 mM KCl). The radioactivity of ^{14}C-poly U bound to the ribosomes and retained on the filter was determined with a windowless gas-flow counter. Negamycin did not show any inhibitory activity on the formation of the complex (data not shown).

The transfer reaction of phenylalanine from phenylalanyl-tRNA to polyphenylalanine on poly U-ribosomes complex was effectively inhibited by negamycin (Fig. 2).

Effect of negamycin was studied on binding of phenylalanyl-tRNA and N-acetylphenylalanyl-tRNA to ribosome-poly U complex according to the method of NIRENBERG and LEDER[5]. Negamycin weakly inhibited the phenylalanyl-tRNA binding (33.5% inhibition by 100 μg/ml of negamycin) (Table 1). The binding of acetylphenylalanyl-tRNA, however, was not inhibited but was rather slightly stimulated by negamycin (Table 1, see also Table 2).

The phenylalanyl-tRNA bound to the complex did not react with puromycin at 10 and 20 mM Mg^{2+} concentration (data not shown). However, acetylphenylalanyl-tRNA bound to the complex at 10 mM Mg^{2+}, of which binding was slightly increased by negamycin, reacted with puromycin and was released from the complex (Table 2). The puromycin reaction with acetylphenylalanyl-tRNA was not influenced by nagamycin (Table 2).

Since puromycin reaction can be taken as an analogue of peptide bond forming reaction by peptidyltransferase, a structural component of 50S subunit[8~11], it can be said that negamycin does not inhibit the step of peptide bond formation in chain elongation and not an inhibitor of 50S subunit. This is also supported by a fact that the drug, as reported in a preceding paper[1], does not inhibit peptide chain extension by endogenous mRNA. Thus the inhibition by negamycin of polyphenylalanine synthesis directed by poly U in *E. coli* extracts is supposed to be caused by the lowered binding of phenylalanyl-tRNA to ribosomes-poly U complex.

2. Miscoding Activity of Negamycin in Polypeptide Synthesis

The primary site of action of most aminoglycoside antibiotics including streptomycin and kanamycin is bacterial ribosomes and these antibiotics not only inhibit protein synthesis but also cause misreading of the genetic code[10]. Although negamycin is not an aminoglycoside antibiotic, it has the common basic property with amino groups in the molecule. In polypeptide synthesis directed by poly U in *E. coli* extracts, negamycin showed a remarkable stimulation of the incorporation of isoleucine, which is normally not coded for by the polymer (Table 3). About 15-fold stimulation of the incorporation was obtained by 20 μg/ml of negamycin, with the correction for the incorporation without poly U. In addition, binding of isoleucyl-tRNA to ribosome-poly U complex was 2.8-fold stimulated by 10 μg/ml of negamycin, contrary to its weak inhibition of phenylalanyl-tRNA binding (Table 4).

These results suggest that negamycin increased the binding of isoleucyl-tRNA to the ribosome-poly U complex and lowered the binding of phenylalanyl-tRNA, by inducing misreading of U as A in 5′-terminal of UUU triplet (the genetic code for Phe and Ileu is UU^{u}_{c} and AU^{u}_{c}, respectively).

Tabale 3. Stimulation by negamycin of ^{14}C-isoleucine-incorporation directed by poly U

	CPM incorporated
Complete system	165
〃 +Negamycin 20 μg/ml	1,382
〃 +Negamycin 50 μg/mi	1,325
〃 +Negamycin 100 μg/ml	1,567
〃 −Poly U	78
〃 −Poly U+Negamycin 100 μg/ml	73

Reaction mixtures (0.3 ml) contained 50 mM Tris-HCl, pH 7.8, 60 mM KCl, 10 mM Mg-acetate, 10 mM β-mercaptoethanol, 1 mM ATP, 0.1 mM GTP, 5 mM creatine phosphate, creatine kinase 15 μg, tRNA 200 μg, poly U 20 μg, ribosomes 0.2 mg, S100 0.3 mg, and ^{14}C-isoleucine 0.2 μc (20 mμmoles). The mixtures were incubated at 35℃ for 40 minutes and hot TCA-insoluble radioactivity was determined.

Table 4. Stimulation by negamycin of poly U-coded ^{14}C-isoleucyl-tRNA binding to ribosomes

	CPM retained on filter
Complete system	52
〃 −Poly U	17
〃 +Negamycin 10 μg/ml	113
〃 +Negamycin 25 μg/ml	114

Reaction mixtures (0.2 ml) contained 0.1 M Tris-HCl, pH 7.1, 0.05 M KCl, 0.02 M Mg-acetate, poly U 20 μg, washed ribosomes 0.3 mg and ^{14}C-isoleucyl-tRNA 3,200 cpm (300 μg). The mixtures were incubated at 24℃ for 20 minutes and assayed by the Millipore filter method.

The effect of negamycin was examined on the incorporation of other amino acids directed by poly U, poly A and poly C. The results were summarized in Table 5. The incorporations of leucine, serine or an amino acid mixture with poly U, lysine or glutamic acid with poly A, and proline or leucine with poly C were all strongly or weakly stimulated by 10 μg/ml of the drug.

Table 5. Miscoding activity by negamycin in polypeptide syntheses directed by synthetic homopolynucleotides

Polynucleotide	and amino acid	CPM incorporated −NGM(A)*	CPM incorporated +NGM(B)*	B/A
Poly U	Phe	10,880	4,990	0.46
	Ileu	150	625	4.17
	Leu	4,221	7,520	1.78
	Ser	481	1,890	3.94
	Amino acid mixture**	1,956	3,760	1.92
Poly A	Lys	2,380	5,090	2.14
	Glu	127	264	2.08
Poly C	Pro	1,267	7,614	6.00
	Leu	621	1,501	2.42

* NGM : Negamycin 10 μg/ml.
** *Chlorella* protein acid hydrolysate.
Reaction mixtures (0.3 ml) contained 50 mM Tris-HCl, pH 7.8, 60 mM KCl, 10 mM Mg-acetate, 10 mM β-mercaptoethanol, 1 mM ATP, 0.1 mM GTP, 5 mM creatine phosphate, creatine kinase 15 μg, tRNA 300 μg, homopolynucleotides (poly U 20 μg, poly A 30 μg and poly C 30 μg), ribosomes 0.14 mg, S100 0.3 mg and each amino acid-^{14}C (Phe 0.1 μc/0.34 mμmole, Ileu 0.2 μc/20 mμmoles, Leu 0.2 μc/0.13 mμmole, Ser 0.3 μc/3 mμmoles, Lys 0.3 μc/1.4 mμmole, Glu 0.3 μc/18.7 mμmoles, Pro 0.3 μc/2.5 mμmoles, and amio acid mixture 0.1 μc/12.5 mμmoles C). The mixtures were incubated at 35℃ for 40 minutes with or without negamycin (10 μg/ml) and hot TCA-insoluble radioactivity was counted.

It could be concluded from the misreading of these polymers that negamycin caused misreading of U as A, A as G, and C as U, in both the 5′-terminal and the internal positions of codons. The pattern of the misreading by negamycin was the same as that by streptomycin[12,13]. Streptomycin is reported by CERVA *et al.*[14] and SUZUKI *et al.*[15] to inhibit puromycin reaction with bacterial 70S ribosomes, but MODOLELL and DAVIS[16] claim that streptomycin does not inhibit the reaction, while these investigators demonstrated the inhibition of peptide chain elongation with streptomycin in accordance with one another. Whereas, negamycin inhibited neither puromycin reaction nor peptide chain elongation as shown in this and a preceding papers[1].

Furthermore, negamycin completely inhibited the growth of a streptomycin-resistant strain of *E. coli* in the same amount as inhibited the sensitive strain. These results suggest that the precise binding site(s) of negamycin on 30S subunit would

not be the same as that of streptomycin. Studies on negamycin-binding to ribosomal subunits remain to be elucidated.

The study on the miscoding activity by degraded products of negamycin indicated that the whole molecular structure of the drug was required for the induction of misreading of the synthetic mRNAs (data not shown).

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