MECHANISM OF ACTION OF NEGAMYCIN IN *ESCHERICHIA COLI* K12

I. INHIBITION OF INITIATION OF PROTEIN SYNTHESIS

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The mechanism of action of a new antibiotic, negamycin, was studied in *Escherichia coli* K12.

1. Negamycin exhibited a bacteriocidal action, and in macromolecular synthesis in negamycin-treated cells inhibition of protein synthesis was first demonstrated and then a decline of RNA synthesis followed with a little delay. The synthesis of DNA and cell wall was hardly impaired. These results indicate that protein synthesis is the primary site of inhibition by negamycin of the growing cells.

2. Negamycin inhibited *in vitro* protein synthesis directed by phage MS2 RNA as exogenous natural messenger RNA, and when the drug was added at the start of incubation the inhibition was marked during the first 5 or 10 minutes of the incubation and progressively decreased with the time. Furthermore, less inhibition was shown if the drug was added after protein synthesis had begun.

3. On the other hand, *in vitro* protein synthesis directed by endogenous natural mRNA was not inhibited but was rather stimulated by negamycin.

4. Negamycin inhibited binding of formylated methionyl-tRNA to ribosomes directed by MS2 RNA.

5. From these results negamycin seems to be a specific inhibitor of peptide chain initiation in natural mRNA directed protein synthesis and not of peptide chain elongation.

Negamycin is a new antibiotic with low toxicity, effective *in vitro* and *in vivo* against Gram-negative and Gram-positive bacteria[1].

In the present work, to elucidate the biochemical mechanism of its antibacterial action and its selective toxicity, we studied the effect of negamycin on macromolecular synthesis in *Escherichia coli* K12. We found that negamycin is a specific inhibitor of initiation of protein synthesis.

Methods and Materials

1. Examination of *in vivo* effect of negamycin: For examination of effect of negamycin on viability and synthesis of macromolecules in growing *E. coli* K12, the cells were grown in an M-9 synthetic medium: 30 % glucose 13.6 ml, 0.01 M $CaCl_2$ 10 ml, 0.1 M $MgSO_4$ 10 ml, 0.01 M $FeCl_3$ 1.0 ml, 1 % gelatine 1 ml and 10×M9 salt 100 ml in 870 ml water (pH 7.4). 10×M9 salt: $Na_2HPO_4 \cdot 12H_2O$ 14.7 g, KH_2PO_4 3 g, NaCl 0.5 g and NH_4Cl 1.0 g in 100 ml water. The detail of the experiments was described in the legend of each figure in the text.

2. Preparation of *E. coli* extracts: *E. coli* K12 was grown in a glucose broth (0.3 % glucose, 1 % meat extract, 1 % Polypeptone and 0.2 % NaCl) to late logarithmic phase and harvested. The extracts (S30, S100, ribosomes and incubated S30) were prepared according to the method of NIRENBERG and MATTHAEI[2] except the cells were disrupted through a French pressure cell.

3. Preparation of MS2 phage RNA: RNA bacteriophage MS2 and its host, *E. coli* K12 W3110 F^+, were supplied by Dr. MIYAKE, Keio University, Tokyo. MS2 phage and its RNA were prepared according to the method of GESTELAND and BOEDTKER[3]. The purification of the phage by centrifugation in CsCl was omitted and bentonite was replaced by polyvinylalcohol sulfate (10 μg/ml).

4. Assay of ^{14}C-amino acid incorporation by *E. coli* extracts: The reaction mixtures for assay of ^{14}C-amino acid incorporation into hot trichloroacetic acid (TCA)-insoluble materials by MS2 RNA or endogenous *E. coli* mRNA in the extracts, were described in the legend of each figure or table in the text. Incubation was terminated by addition of 2 ml of 5 % TCA. After the mixtures were washed with 5 % TCA and heated at 90°C for 15 minutes, hot TCA-insoluble material was dissolved in 2 N NH_4OH. Aliquots were taken, dried and the radioactivity was determined with a windowless gas-flow counter.

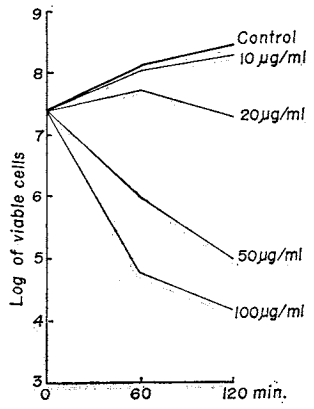
5. Preparation of washed ribosomes and initiation factors: For the preparation of washed ribosomes and crude initiation factors, incubated S30 of *E. coli* K12 was used. The preparation was performed according to the method described by HERSHEY and THACH[4]. The ammonium sulfate precipitate (0.8 saturated) of the wash fluid was directly used for the initiation factors after overnight dialysis.

6. Preparation of formylated ^{14}C-methionyl-tRNA and its binding assay: Formylated ^{14}C-methionyl-tRNA was prepared by incubating *E. coli* K12 tRNA (stripped) with S100 which contains aminoacyl-tRNA synthetases and methionyl-tRNA transformylase, in the presence of ^{14}C-methionine, formyltetrahydrofolate and ATP, as reported by HERSHEY and THACH[4] and isolated by phenol extraction. The binding of formylated ^{14}C-methionyl-tRNA to ribosomes was assayed as described in the legend of Table 4.

7. Chemicals: Adenine-8-^{14}C (22 mc/mM) and ^{14}C-lysine (U) (214 mc/mM) were obtained from Daiichikagakuyakuhin, Tokyo. Methionine-^{14}C(U) (187 mc/mM) was from New England Nuclear Corp. ^{14}C-Amino acids mixture (chlorella protein acid hydrolysate) was from the Institute of Applied Microbiology, University of Tokyo. D-Glucosamine-1-^{14}C (55.6 mc/mM) from The Radiochemical Centre, Amersham, England. *E. coli* K12 tRNA (stripped) and formyltetrahydrofolate from General Biochemicals. Negamycin was given by Dr. S. KONDO, Institute of Microbial Chemistry, Tokyo.

Fig. 1. Bacteriocidal action of negamycin.

E. coli K12 cultures growing exponentially in M-9 synthetic medium were treated with different amounts of negamycin. Samples were withdrawn at the time indicated and after suitable dilutions, plated on agar containing the M-9 medium.



Results

1. Bacteriocidal Action of Negamycin

Negamycin showed complete inhibition of growth of *E. coli* K12 in an M-9 synthetic medium at 16 μg/ml (inoculum size: 1.4×10^6/ml). It was examined whether negamycin has a bacteriocidal action or a bacteriostatic action on growing *E. coli* K12. The cells exponentially growing in the medium were treated with different amounts of the drug and after 60 and 120 minutes treatments samples were taken, diluted and immediately

plated. Negamycin showed a killing action at the minimal inhibitory concentration and at higher concentrations, and the rate of the killing action depended on the concentration of the drug (Fig. 1). This indicates that the action of negamycin is irreversible.

2. Inhibition of Protein Synthesis in the Growing *E. coli* Cells

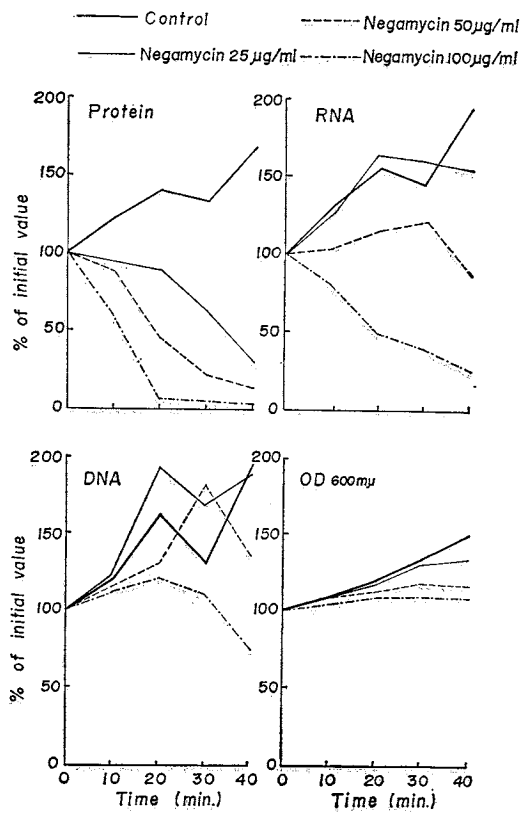
The effect of negamycin was studied on macromolecular synthesis in growing *E. coli* K12. The cells exponentially growing in the synthetic medium were treated with different amounts of negamycin and aliquots of the culture were taken at intervals and pulse-labeled for one minute with ^{14}C-amino acids and ^{14}C-adenine, separately. As seen in Fig. 2, in the treated cells amino acid incorporation into protein was inhibited first, and the decrease of adenine incorporation into RNA followed with little delay. On the other hand, the non-treated cells increasingly incorporated the precursors during the incubation owing to the growth of the cells. In the cells treated with 25 μg/ml of negamycin for 30 minutes, protein synthesis was decreased to 46 % of that in the non-treated cells, while RNA and DNA syntheses were little affected. With 50 μg/ml of nagamycin for 20 minutes, the syntheses of protein, RNA and DNA were 32, 74 and 81 % of those in the non-treated cells, respectively, and with 100 μg/ml of the drug for 20 miuntes, those were 6, 32 and 75 %, respectively. The incorporation of ^{14}C-glucosamine, a component of the cell wall glycopeptide, into the cells was not affected during 30-minute treatment with 50 μg/ml of negamycin (data not shown).

In vitro RNA synthesis by the isolated DNA-dependent RNA polymerase was not inhibited by negamycin, assayed by incorporation of ^{3}H-CMP into TCA-insoluble fraction in the presence of other three kinds of nucleotide triphosphate and template DNA.

We concluded from these results that the primary action of negamycin is the inhibition of protein synthesis in the cells and the decline of RNA synthesis is rather

Fig. 2. Effect of negamycin on protein and nucleic acid syntheses in growing *E. coli* K12.

E. coli K12 exponentially growing in M-9 medium was treated with different amounts of negamycin. After the treatment for the time indicated, one ml aliquots of the cultures were taken and pulse-labeled for one minute with ^{14}C-amino acid mixture (chlorella protein acid hydrolysate, 8 mc/mmC) 0.4 μc and ^{14}C-adenine (22 mc/mM) 0.5 μc, separately, at 37°C. For amino acid incorporation into protein, hot TCA-insoluble radioactivity was determined and for adenine incorporation into RNA and DNA, cold TCA-insoluble materials were fractionated according to the method of SCHMIDT and THANNHAUSER[5]. The radioactivity was counted with a windowless gas-flow counter. The incorporated amounts were indicated by percentage of initial value of control at time zero. The growth of the cells were also measured by absorption at 600 mμ. It was 0.27 at time zero.



a secondary effect of the drug owing to its inhibition of protein synthesis.

3. Lack of Inhibition of Protein Synthesis Directed by Endogenous mRNA in *E. coli* Extracts

A cell-free extract (ribosomes and S100) was prepared for *in vitro* protein synthesis according to the method of NIRENBERG and MATTHAEI[2], and the effect of negamycin was studied on ^{14}C-amino acids incorporation directed by endogenous mRNA in the extract. In this system negamycin unexpectedly did not inhibit the incorporation of amino acids but showed rather a weak stimulation of it, contrary to the rapid inhibition of *in vivo* protein synthesis in the growing cells (Table 1 and Fig. 3). A change of Mg^{2+} concentration from 2 mM to 14 mM in the reaction mixtures did not affect the result (data not shown).

Fig. 3. Time course of amino acid incorporation directed by endogenous mRNA in *E. coli* extracts.

Reaction mixtures were the same as described in the legend of Table 1. Incubation at 35℃.

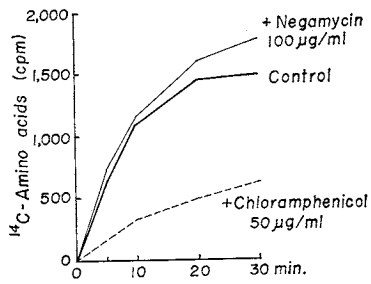


Table 1. Lack of inhibition by negamycin of ^{14}C-amino acid incorporation directed by endogenous messenger RNA in *E. coli* extracts

		CPM incorporated	Rate (%)
^{14}C-Amino acid mixture	Complete	1,750	100
	〃 +negamycin 10 μg/ml	1,924	110
	〃 + 〃 20	2,162	123
	〃 + 〃 50	2,292	131
	〃 + 〃 100	2,336	128
	〃 + 〃 200	2,403	137
^{14}C-Phenylalanine	Complete	5,510	100
	〃 +negamycin 25 μg/ml	6,545	119
	〃 + 〃 100	8,050	146
^{14}C-Lysine	Complete	816	100
	〃 +negamycin 6.25 μg/ml	1,862	228
	〃 + 〃 25	2,110	258
	〃 + 〃 100	2,502	306

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, ribosomes 0.64 mg protein, S100 1.0 mg and ^{14}C-amino acid (^{14}C-amino acid mixture 0.2 μc/25 mμmoles, ^{14}C-Phe 0.2 μc/0.64 mμmole or ^{14}C-Lys 0.12 μc/0.56 mμmole). Incubation was at 37℃ for 30 minutes.

For the preparation of the cell-free extract employed in the above experiments, the cells were disrupted in a French pressure cell and the extract was treated with a small amount of deoxyribonuclease (Worthington Biochemical Co.) and therefore, it can be said that no mRNA synthesis took place in the extract during the incubation of amino acids incorporation, and the incorporation of amino acids in the cell-free extract represented extension of pre-existing peptide chains directed by endogenous *E. coli* mRNA which was already attached to ribosomes and no initiation of new polypeptide chains occurred.

Thus, the result indicates that negamycin does not inhibit the step of peptide chain elongation in protein synthesis directed by natural mRNA but the drug rather weakly stimulates the chain elongation.

4. Inhibition of Protein Synthesis Directed by Exogenous Natural mRNA

Considering the inconsistent result with the effect of negamycin between *in vivo* protein synthesis and *in vitro* protein synthesis in the extract by endogenous mRNA, we next employed a cell-free system consisted of exogenous natural mRNA and

Fig. 4. Time course of inhibition by negamycin of MS2 RNA-directed protein synthesis in *E. coli* extracts.

Reaction mixtures (0.4 ml) contained 50 mM Tris-HCl, pH 7.8, 60 mM NH_4Cl, 8.6 mM Mg-acetate, 10 mM β-mercaptoethanol, 1 mM ATP, 0.1 mM GTP, 5 mM creatine phosphate, creatine kinase 20 μg, MS2 RNA 0.26 mg, S100 0.45 mg, incubated S30 1.3 mg, ^{14}C-amino acid mixture (chlorella protein acid hydrolysate) 0.3 μc, and Try, CySH, Glu(NH_2) and Asp(NH_2), 0.1 μmole each, which were not contained in the ^{14}C-amino acid mixture. The mixtuers were incubated at 35℃ with or without negamycin and at indicated times 0.1 ml aliquots were taken and hot TCA-insoluble radioactivity was determined.

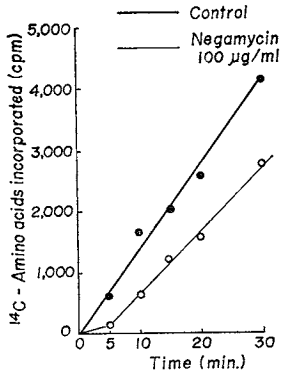


Fig. 5. Kinetics of negamycin inhibition of ^{14}C-lysine incorporation directed by MS2 RNA in *E. coli* extracts.

Reaction mixtures (0.2 ml) were the same as those in Fig. 4 except that MS2 RNA 100 μg, S100 0.3 mg, S30 0.78 mg and ^{14}C-lysine 0.5 μc (2.35 mμmoles) and 19 kinds of amino acids except lysine, 25 mμmoles each, were uesd. Incubation was at 33℃. At indicated times 40 μl aliquots were taken and assayed.

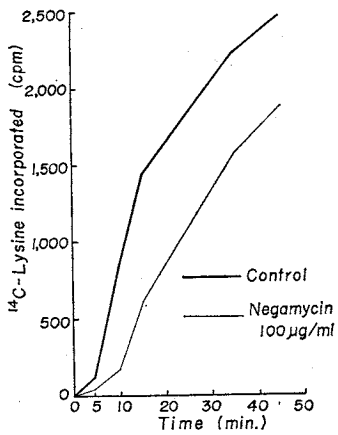


Table 2. Inhibition by different amounts of negamycin of ^{14}C-amino acid incorporation directed by MS2 RNA in *E. coli* extracts

	CPM incorporated	
	5 min.	30 min.
Complete sysem	684	3,564
〃 −MS2 RNA	53	204
〃 +Negamycin 25 μg/ml	390(43.0)	3,152(11.8)
〃 +Negamycin 50 μg/ml	240(64.8)	2,766(22.4)
〃 +Negamycin 100 μg/ml	145(78.8)	3,174(11.1)

The number in parentheses represents percent inhibition.
Reaction mixtures and incubation were the same as those in Fig. 4.

ribosomes free of endogenous mRNA. RNA of bacteriophage MS2 was used as the natural mRNA and *E. coli* K12 S30 extract was prepared and used after pre-incubation to free the ribosomes of endogenous mRNA. The incubated S30 extract was supplemented with S100 supernatant by which polysomes formation was promoted[6]. Protein synthesis directed by this viral mRNA consists of initiation of the peptide chains, subsequent elongation and termination of the chains, at relatively low Mg^{2+} concentration (7~8 mM). The initiation of the peptide chains specifically requires formylated methionyl-$tRNA_F$ for corresponding AUG initiation codon of the natural mRNA[7]. In this system negamycin indeed exhibited its inhibitory activity on the amino acids incorporation into hot TCA-insoluble materials (Table 2 and Fig. 4).

When negamycin (100 μg/ml) was added to the complete reaction mixture at the start of the incubation and time course of the inhibitory effect was examined, it was demonstrated that the negamycin-inhibition of the protein synthesis was remarkable within first 5 or 10 minutes of the incubation time and then progressively decreased with the time (Fig. 4): The inhibition by 100 μg/ml of negamycin was 77.4 % at 5 minutes and 62.5 % at 10 minutes, however, that became 40.0, 38.8 and 34.0 % at 15, 20 and 30 minutes, respectively. As shown in Table 2, the inhibition by different amounts of negamycin gave similar results.

Comparable results were also obtained with negamycin-inhibition of ^{14}C-lysine incorporation directed by the viral mRNA (Fig. 5): The

Fig. 6. Effet of time of addition of negamycin on MS2 RNA-directed protein synthesis.

Reaction mixtures (0.1 ml) contained 50 mM Tris-HCl, pH 7.8, 60 mM NH_4Cl, 9 mM Mg-acetate, 10 mM β-mercaptoethanol, 1 mM ATP, 0.1 mM GTP, 5 mM creatine phosphate, creatine kinase 5 μg, MS2 RNA 50 μg, S100 150 μg protein, incubated S30 260 μg and ^{14}C-amino acid mixture 0.05 μc with the four kinds of cold amino acids described in the legend of Fig. 4. Incubation was 35℃. The arrows indicate the times of negamycin-addition.

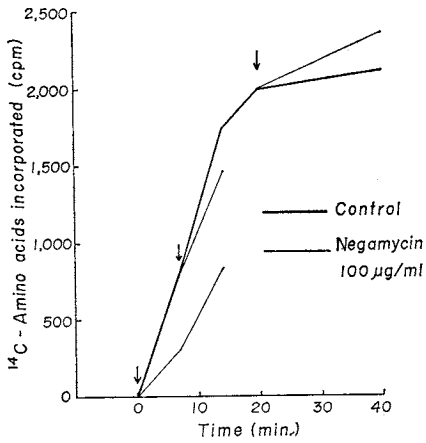


Table 3. Negamycin-stimulation of ^{14}C-amino acid incorporation dependent on added *E. coli* ribosomal RNA

	CPM incorporated	Rate
Control (−rRNA)	216	1.0
〃 +Negamycin 100 μg/ml	302	1.4
〃 +rRNA	466	2.2
〃 +rRNA+Negamycin 10 μg/ml	1,084	5.0
〃 +rRNA+Negamycin 25 μg/ml	1,400	6.5
〃 +rRNA+Negamycin 50 μg/ml	1,538	7.1
〃 +rRNA+Negamycin 100 μg/ml	1,604	7.4

Reaction mixture (0.4 ml) was the same as that of Fig. 4 except MS2 NRA was replaced by *E. coli* ribosomal RNA (1 mg) isolated by phenol extraction from the ribosomes prepared according to NIRENBERG and MATTHAEI[2]. Incubation was at 35℃ for 30 minutes.

percent inhibitions were 53.8, 77.8, 56.8, 29.5 and 24.1 % at 5, 10, 15, 35 and 45 minutes incubations, respectively. The percent inhibitions in Figs. 4 and 5 were not corrected for the residual incorporation at the corresponding time directed by endogenous mRNA in the extract. For the progressive decrease in the inhibition with time in these experiments might account for incomplete inhibition of the initiation at the employed concentration of negamycin, possible inactivation of the drug by some components of the mixture, or both.

The effect of time of addition of negamycin was examined on the protein synthesis by MS2 RNA. When negamycin was added at the start of the incubation, 62 and 52 % inhibitions were obtained in 7 and 14 minutes of the incubation, respectively (Fig. 6). However, when the drug was added at 7 minutes after the reaction had begun, only 29 % of the inhibition was shown in the subsequent 7 minutes incubation. Furthermore, when it was added at 20 minutes, a weak stimulation (111 % of the control) of the incorporation was demonstrated.

These experimental results that (1) when the drug was added before the start of the reaction, the inhibition was marked during the first 5 or 10 minutes and then progressively decreased with the time, and (2) less inhibition was demonstrated if the drug was added after the reaction had started, strongly suggested that negamycin is a specific inhibitor of initiation of protein synthesis and not of elongation of peptide chains.

Concerning the restoration of amino acid incorporation after the 5- or 10-minute incubation in the presence of negamycin, a possible explanation is that MS2 RNA on which normal initiation of protein synthesis is inhibited by the drug would be liable to be attacked by ribonuclease in the extracts and broken during the incubation and artificial fragments of MS2 RNA would be generated. Directed by the artificial MS2 RNA fragments, some polypeptide synthesis would take place in the presence of negamycin, consisting of abnormal initiation and subsequent elongation.

This was inferred from the result of the experiment indicated in Table 3.

E. coli K12 ribosomal RNA was isolated from the ribosomes by phenol extraction and added to pre-incubated S30 fraction together with different amounts of negamycin and the effect of the drug was examined on amino acid incorporation in the extracts. Whereas about twofold stimulation of the incorporation was obtained with the ribosomal RNA alone, five- to sevenfold stimulation was demonstrated in the presence of negamycin. The result suggested that some polypeptide synthesis took place directed by the ribosomal RNA and the presence of negamycin was required for the synthesis.

5. Inhibition of Formylmethionyl-tRNA Binding to Ribosomes Directed by MS2 RNA

In initiation of protein synthesis directed by natural mRNA in *E. coli* extracts, formylated methionyl-$tRNA_F$ binds to the mRNA-ribosome complex in the presence of GTP and initiation factors[8,9]. We studied the effect of negamycin on the binding of formylated ^{14}C-methionyl-$tRNA_F$ to ribosomes directed by MS2 RNA. The result was obtained indicating that negamycin reduced the amount of formylated ^{14}C-methionyl-$tRNA_F$ bound to ribosomes (approximately 56 % inhibition by 100 μg/ml of negamycin) (Table 4). Thus it was shown that the negamycin inhibition of protein synthesis directed by MS2 RNA in *E. coli* K12 extracts resulted from the inhibition by the drug of the formation of an initiation complex by the binding of formylated methionyl-$tRNA_F$ to ribosomes-mRNA complex.

Table 4. Inhibition by negamycin of formylated ^{14}C-methionyl-tRNA binding to ribosomes directed by MS2 RNA

	CPM retained on filter	
	10 min.	20 min.
Complete system	162	208
〃 +Negamycin 100 μg/ml	103	126
〃 −MS2 RNA	56	59

Reaction mixtures (0.55 ml) contained 50 mM Tris-HCl, pH 7.5, 60 mM NH_4Cl, 7.5 mM magnesium acetate, 10 mM β-mercaptoethanol, washed ribosomes 0.8 mg protein, initiation factors 0.25 mg, MS2 RNA 0.4 mg, GTP 0.05 mM and *E. coli* K12 tRNA containing formylmethionyl-$tRNA_F$ 1.0 mg (9,170 cpm). Incubation was at 30℃ and at 10 and 20 minutes 0.25 ml aliquots were taken, diluted and the amount of formylmethionyl-$tRNA_F$ bound to ribosomes was determined according to NIRENBERG and LEDER[10]. The radioactivity was counted in a Beckmann liquid scintillation spectrometer (in BRAY's solution).

Discussion

Negamycin showed its primary inhibition of protein synthesis in growing *E. coli* K12 and the inhibited step might be peptide chain initiation but not chain elongation.

Concerning the effect of negamycin on *in vivo* protein synthesis in the growing cells, when the cells were treated with 100 μg/ml of negamycin for 20 minutes in advance and then labeled for one minute with ^{14}C-amino acids, almost complete inhibition of the incorporation was observed, as shown in Fig. 2. However, when the labeled precursor and negamycin were added simultaneously to the culture at the start of the cumulative incorporation, only a weak inhibition (20 %) was obtained with 100 μg/ml of the drug in 20 minutes incubation (data not shown). This different inhibitory effect of negamycin between the two different methods of experiment is likely to indicate the characteristic mode of action of the drug in its inhibition of protein synthesis. The weak inhibition in the cumulative incorporation would be attributed to that amino acid incorporation for the peptide chain elongation which was already in progress when negamycin was added was not inhibited but might be stimulated by the drug until the peptide chains were completed and the cumulative amount incorporated covered the decrease of the

incorporation due to negamycin inhibition in peptide chain initiation in the net result. In addition, possibly slow onset of inhibition in cells due to slow entry might promote the net radioactivity incorporated, although this was not tested. On the other hand, in the case of pulse-labeling in cells treated with negamycin in advance, most peptide chain elongation was finished during the time (20 minutes) because initiation was suppressed by the drug, and number and size of polyribosomes in the cells decreased, and therefore, the amount of the radioactivity incorporated could be considered to express mainly the incorporation for chain initiation. It could be expected thus that in the negamycin-treated cells polyribosomes gradually decrease with time and ribosome monomer accumulates progressively in the cells as demonstrated by LUZZATTO *et al.*[11] in streptomycin-treated cells of *E. coli*.

Inhibition of RNA synthesis seems to be a secondary effect of negamycin resulting from the inhibition of initiation of protein synthesis. RNA synthesis in *E. coli* was suggested to be regulated by the initiation of protein synthesis since it was shown that trimethoprim which inhibited pepide chain initiation by preventing the formation of formylated methionyl-tRNA, also inhibited net RNA synthesis in *E. coli*[12].

As reported in our following paper[13], negamycin has an activity to induce misreading of genetic code. The stimulation of amino acid incorporation by negamycin observed in the step of chain elongation may be caused by the miscoding activity of the drug : The presence of the drug would permit the incorporation of any other amino acids than the normally corresponding one into protein, by distorting the ribosomes-mRNA complex.

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