INHIBITION OF TRANSCRIPTION BY PLURAMYCIN AND BLEOMYCIN

NOBUO TANAKA*

Institute of Microbiology, Rutgers, The State University of New Jersey, New Brunswick, N. J., U. S. A.

(Received for publication September 12, 1970)

The effects of neopluramycin and bleomycins A_2 and A_5, functioning as antibiotics by binding to DNA, on the transcription of T4 phage were studied. Neopluramycin was observed to inhibit both *in vivo* and *in vitro* transcription of T4 phage. It also interfered with the development of infectious phage particles. Although bleomycins A_2 and A_5 did not significantly affect *in vivo* transcription of T4 phage, they inhibited the *in vitro* transcription, when the template phage DNA was incubated with the antibiotics for 10 minutes prior to the addition of RNA polymerase and nucleoside triphosphates. The RNAs synthesized in the presence of the antibiotics were smaller and seemed to contain larger ratio of pre-early RNA than the RNA synthesized in the absence of antibiotics. The results were in accordance with the assumption that the antibiotics inhibit RNA synthesis by binding to the template DNA and interfering with the elongation step of the RNA polymerase reaction. The RNA polymerase reaction, using calf thymus DNA as a template, was also inhibited by the antibiotics.

Bleomycin and pluramycin have been demonstrated to function as antibiotics by binding with DNA. They exhibit antimicrobial and antitumor activity[1~10]. Neopluramycin is a new antibiotic of pluramycin group[11].

Bleomycin causes decrease of Tm of DNA and scissions of DNA strands in the presence of sulfhydryl compounds or hydrogen peroxide[4~7]. On the other hand, pluramycin causes increase of Tm of DNA[9,10]. It indicates that the detailed modes of interaction of the two group antibiotics with DNA may be different. Therefore it is of interest to compare the effects on phage RNA synthesis. The effects of bleomycins A_2 and A_5 and neopluramycin on transcription have been studied in the T4 phage system. The results are presented in this publication.

Materials and Methods

The samples of neopluramycin and bleomycins A_2 and A_5 were kindly supplied by Prof. HAMAO UMEZAWA, Institute of Microbial Chemistry, Tokyo.

^{3}H-UTP (tetralithium salt, 17.1 Ci/mmole) and uridine-5-^{3}H (24.4 Ci/mmole) were products of Schwarz Bioresearch, Inc.

The media or buffers used were: PGAFe (Na_2HPO_4 50 mM, KH_2PO_4 22 mM, glucose 27.5 mM, NH_4Cl 18.6 mM, $MgSO_4$ 2.1 mM, $FeCl_3$ 0.001 mM), TB (Bacto-tryptone 1 %, NaCl

* Present address: Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan. This work was performed during the tenure of a National Science Foundation Senior Foreign Scientist Fellowship.

0.5 %), WF ($MgCl_2$ 1 mM, NaCl 17 mM, Tris 10 mM, pH 7.4), DNA buffer (Tris 10 mM, pH 7.4, EDTA 1 mM), and SSC (sodium citrate 15 mM, NaCl 150 mM).

Coliphage T4 was grown on *E. coli* B in PGAFe medium. At a bacterial concentration of 5×10^8/ml, T4 wild type phage was added at a multiplicity of infection of 0.1, and DL-tryptophan 2.5 μg/ml. The culture was grown for 8 hours at 37°C with aeration. Upon addition of $CHCl_3$, the culture was vigorously shaken and cooled. After removing bacterial debris by centrifugation, the phage was pelleted by centrifugation for 2 hours at 60,000 g, washed, suspended in washing fluid (WF), treated with DNase, and resuspended in WF at about 10^{12} particles/ml.

T4 DNA was extracted from the phage by gentle rotation (60 rpm) with freshly distilled buffered phenol (pH adjusted to 7.0 with 1 N NaOH and saturated with 0.1 M Tris, pH 7.2) for 30 minutes. The phenol extraction was repeated once more, followed by dialysis against DNA buffer.

RNA polymerase was purified from *E. coli* B by a modified method of BURGESS[12], including disruption of cells, DNase treatment, streptomycin precipitation, ammonium sulfate fractionation, polyethylene glycol fractionation, DEAE cellulose chromatography, DNA-cellulose chromatography, and agarose gel filtration. Its specific activity was *ca.* 2,200 units/mg. One unit of RNA polymerase catalyzes the conversion of one mμmole of UTP into the TCA precipitable for 10 minutes under the assay conditions described below.

The RNA polymerase reaction was performed in the following reaction mixture (per ml): T4 DNA 50 μg, *E. coli* RNA polymerase 40 μg, ATP, CTP, and GTP 200 mμmoles each, UTP 2.5 μCi/200 mμmoles, bovine serum albumin 0.5 mg, KCl 0.15 M, $MgCl_2$ 0.01 M, 2-mercaptoethanol 0.01 M, EDTA 0.001 M, and Tris 0.04 M, pH 7.9. The reaction was carried out at 37°C. It was terminated by addition of cold 5 % TCA, and 150 μg of herring sperm DNA was added as a carrier. The acid precipitates were collected on glass fiber filters, washed, dried and counted in a liquid scintillation counter, using a toluene base scintillator.

Results

Effects of Antibiotics on Development of T4 Phage

Neopluramycin completely inhibited the production of infectious phage particles at the concentration of 0.2 μg/ml (Fig. 1). Bleomycin A_2 was observed to exert no significant inhibitory effect on phage development at the concentration of 50 μg/ml. Bleomycin A_5 slightly inhibited it at 50 μg/ml. At these concentrations of the antibiotics, approximately 50 % inhibition of growth of the host cells was observed in 60 minutes in a parallel experiment (the data are not shown).

Effects of Antibiotics on *in vivo* Transcription of T4 Phage

E. coli B was infected with T4 phage (moi 5). At 2 minutes after infection, when the host RNA synthesis was presumably arrested and switched to the phage RNA synthesis, the antibiotics were introduced to the infected culture for 5 minutes and then uridine-5-^{3}H was allowed to be incorporated for 2 minutes. By the method employed, neopluramycin markedly inhibited the RNA synthesis, most of which was presumably the phage transcription. However, it was not significantly affected by bleomycins A_2 and A_5. The results are summarized in Table 1.

Effects of Antibiotics on *in vitro* Transcription on T4 Phage DNA

Neopluramycin markedly inhibited the RNA polymerase reaction, but bleomycins A_2 and A_5 did not significantly affect it, when the antibiotics were introduced into

the reaction mixture at the beginning of the reaction. T4 phage DNA was used as a template for the RNA polymerase reaction and *E. coli* as an enzyme source. The results are presented in Fig. 2. Approximately 50 % inhibition was observed at the cocentration of 0.2 μg/ml of neopluramycin (Fig. 3).

If T4 phage DNA and bleomycin were incubated for 10 minutes prior to the addition of RNA polymerase and nucleoside triphosphates, bleomycins A_2 and A_5 were observed to inhibit the *in vitro* transcription of T4 phage DNA. The results are presented in Fig. 4. Approximately 50 % inhibition was demonstrated at the concentration of 4 μg/ml of bleomycin A_2 and at 0.3 μg/ml of bleomycin A_5 (Fig. 5).

The sedimentation profiles of the RNAs transcribed *in vitro* for 30 minutes at 37°C in the presence and absence of the antibiotics are shown in Fig. 6. T4 DNA was incubated with bleomycins for 10 minutes prior to the addition of RNA polymerase and nucleoside triphosphates. The sedimentation coefficients were compared with those of 23S, 16S and 4S *E. coli* RNAs, measured in a separate tube. The RNAs formed in the presence of neopluramycin (2 μg/ml) and bleomycins A_2 and A_5 (each 80 μg/ml) were observed to show much lower sedimentation velocities than that of the RNA transcribed in the absence of antibiotics. By the method employed, the average size of the RNA transcribed with neopluramycin was shorter than those with bleomycins A_2 and A_5. Since the antibiotics cause no significant scissions of RNA strands (data not shown), the results indicate that bleomycins and neopluramycin may

Fig. 1. Effects of antibiotics on T4 phage development.

The cells of *E. coli* B were grown in medium PGAFe to 5×10^8/ml, and infected with 0.1 T4 phage/ml after addition of 2.5 μg/ml DL-tryptophan. The antibiotics were introduced to the infected culture immediately after infection to give final concentrations of 50 μg/ml bleomycins and 2 μg/ml neopluramycin. At 5 minutes after infection, 0.2 ml of each infected culture was transferred to 19.8 ml of prewarmed PGAFe medium with or without antibiotics. At 7, 20, 25, 30 and 40 minutes after infection, 0.1 ml of each infected culture was diluted with 0.9 ml TB containing several drops of $CHCl_3$. All the incubation was performed at 37°C. They were further diluted in TB and seeded on agar, which consisted of 2.5 ml of soft agar (Bacto-agar, Difco 8 g, Bacto-tryptone, Difco 8 g, NaCl 5 g per liter) in the upper layer and 15 ml of hard agar (Bacto-agar, Difco 13 g, Bacto-tryptone, Difco 10 g, NaCl 5 g, sodium citrate 2 g, glucose 1.3 g per liter) in the lower layer. The number of phage plaques was counted, after incubation at 37°C for 18 hours.

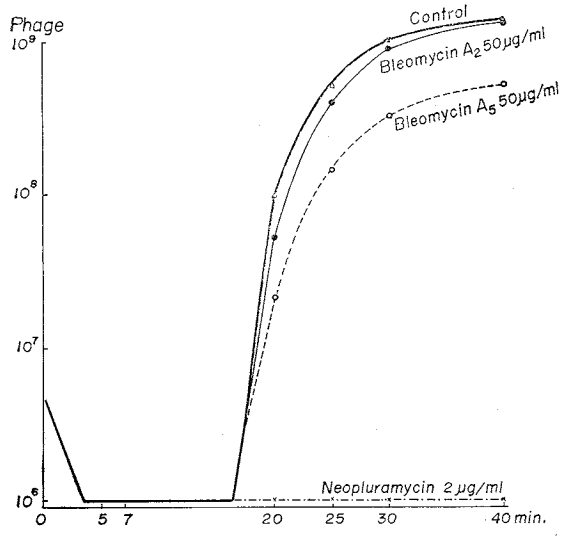


Table 1. Effects of antibiotics on incorporation of ^{3}H-uridine after T4 infection of *E. coli* B.

Antibiotics	^{3}H-Uridine incorporation	
	cpm	%
None	278,200	100
Bleomycin A_2 100 μg/ml	273,900	98.4
Bleomycin A_5 100 μg/ml	274,400	98.7
Neopluramycin 5 μg/ml	77,000	27.6

The cells of *E. coli* B were grown in medium PGAFe to 5×10^8/ml and infected with 5 T4 phage/cell, after 2.5 μg/ml DL-tryptophan were added to the medium. At 2 minutes after infection, 1 ml of antibiotic solution was added to 9 ml of the infected culture to give final concentrations of 100 μg/ml of bleomycins and 5 μg/ml of neopluramycin. At 7 minutes after infection, 20 μCi uridine-5-^{3}H was introduced to the infected culture with or without antibiotics and 2 minutes later 12 ml of 10% TCA were added. The incubation was performed at 37°C. The acid precipitates were collected on glass fiber filters, washed, dried and counted in a liquid scintillation counter using a toluene base scintillator.

Fig. 2. Effects of antibiotics on RNA polymerase reaction.

The RNA polymerase reaction was performed, as described in the text, using T4 phage DNA as a template and *E. coli* RNA polymerase. The antibiotics were added to the reaction mixture at the beginning. The final concentrations used were 40 μg/ml of bleomycins A_2 and A_5 and 10 μg/ml of neopluramycin.

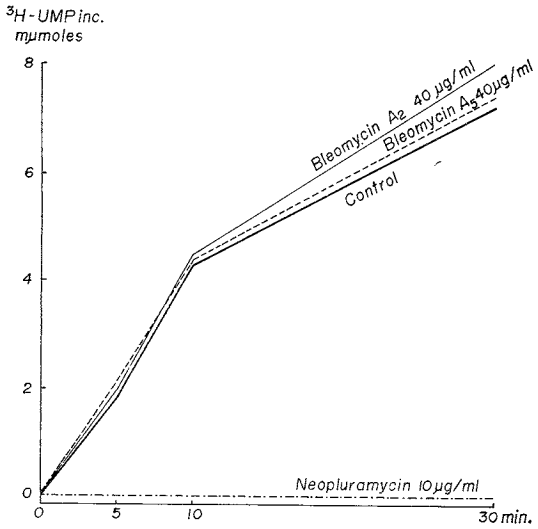


Fig. 3. Effect of concentration of neopluramycin on the RNA polymerase reaction.

The assay conditions are described in the text. The polymerase reaction was terminated in 15 minutes.

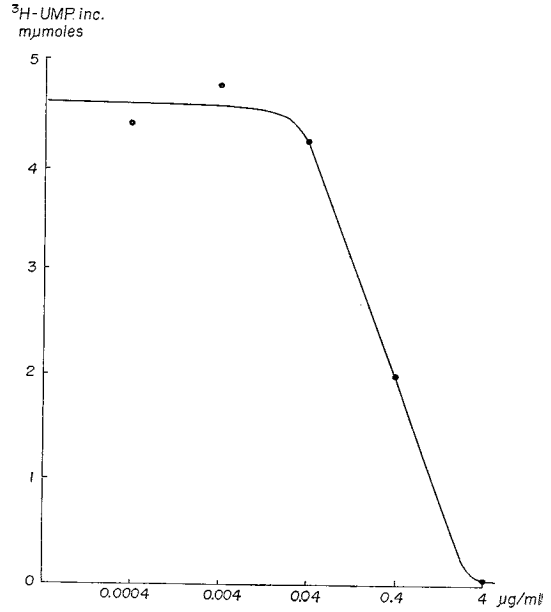


Fig. 4. Effects of bleomycins A_2 and A_5 on the RNA polymerase reaction.

The assay method is described in the text. The DNA and bleomycin were incubated for 10 minutes at 37°C prior to the addition of RNA polymerase and nucleoside triphosphates. The concentration of the antibiotics employed was 100 μg/ml.

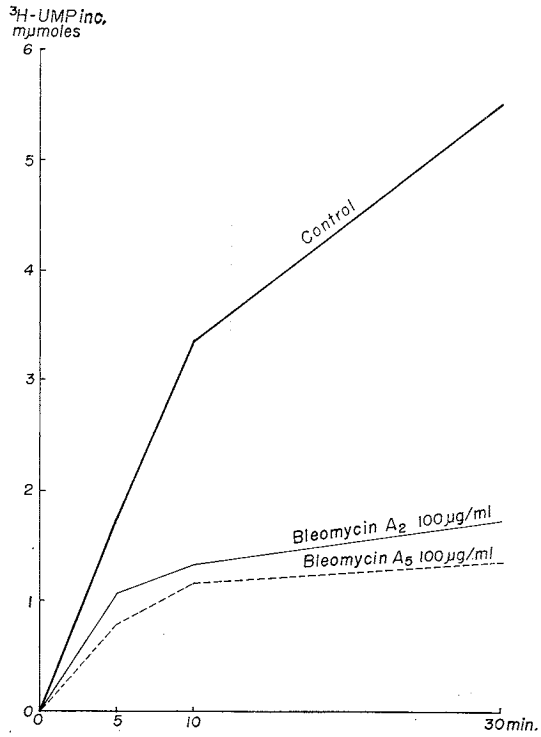


Fig. 5. Effects of concentrations of bleomycins A_2 and A_5 on the RNA polymerase reaction.

The assay conditions are described in the text. The reaction was carried out for 15 minutes.

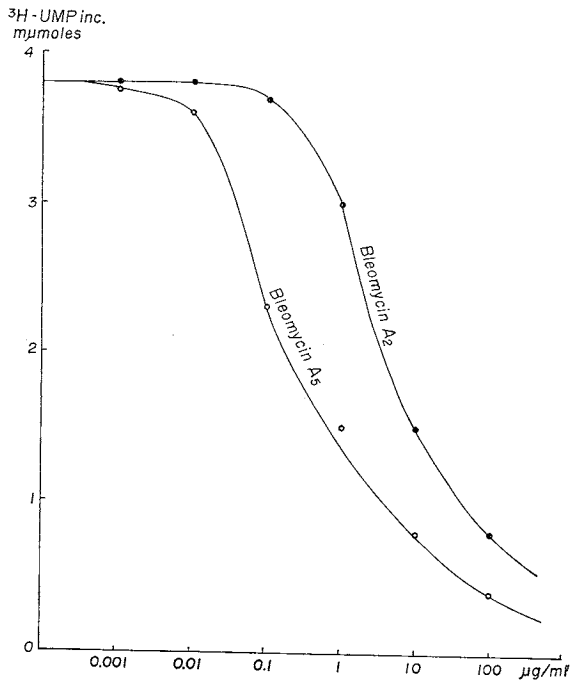
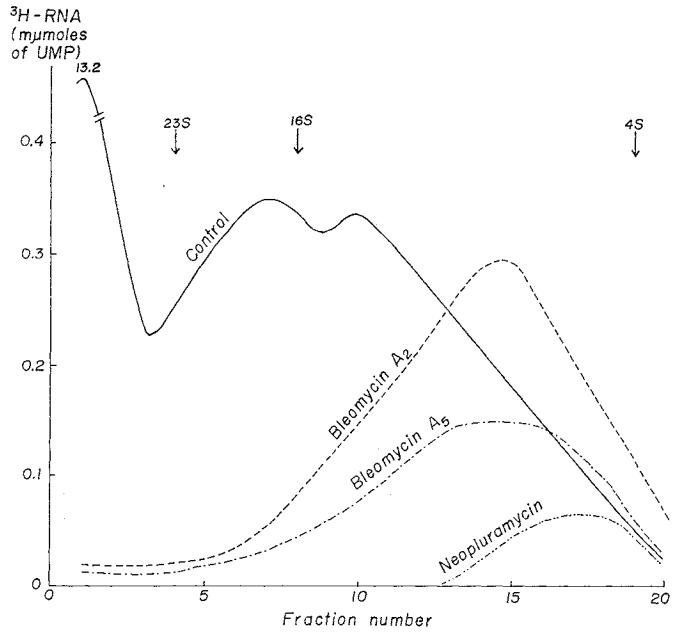


Fig. 6. Sucrose gradient sedimentation of T4 RNA transcribed *in vitro* in the absence and presence of antibiotics.

T4 DNA and bleomycin were incubated at 37℃ for 10 minutes in the buffer, prior to the addition of RNA polymerase and nucleoside triphosphates. The reaction mixture was the same as described in the text. It was incubated further for 30 minutes at 37℃. The final concentrations of antibiotics were 80 μg/ml for bleomycins A_2 and A_5, and 2 μg/ml for neopluramycin. Then 125 μl samples were added to 150 μl of 1% sodium dodecyl sulfate and 0.01 M EDTA, pH 8, and were allowed to stand at room temperature for an hour. The EDTA stopped the polymerase reaction by removing Mg^{++} and the sodium dodecyl sulfate dissociated the RNA from T4 template by denaturing the polymerase. The samples were then sedimented along 12 ml of 5 to 20% w/v sucrose gradients containing 0.1 M KCl, 0.01 M Tris, pH 8, 0.001 M EDTA. Sucrose was the density gradient grade, RNase-free product from Mann Research Corporation. The centrifugation was carried out for 16 hours at 33,000 rpm using a Beckman 41 rotor. *E. coli* RNAs, extracted by the phenol method[16], were employed as the reference.



decrease the rate of RNA chain elongation and terminate the transcription during the chain propagation process by binding to the template T4 DNA.

As illustrated in Fig. 7, the RNA synthesized *in vitro* by *E. coli* RNA polymerase on T4 DNA for 30 minutes at 37°C was competed with both by pre-early RNA synthesized *in vivo* during 2 minutes after infection in the presence of chloramphenicol and by *in vivo* early RNA, which was isolated at 5 minutes after infection in the absence of the antibiotic[18]. The RNAs synthesized *in vitro* with neopluramycin, bleomycin A_2 or A_5 were more effectively competed by the *in vivo* pre-early than the RNA formed without antibiotics was. It indicated that the RNAs synthesized in the presence of the antibiotics had higher ratio of pre-early RNA to early RNA than the control RNA. By the method employed, the RNA formed with neopluramycin seemed to contain relatively more pre-early RNA than those with bleomycins. It is in accordance with the above result that neopluramycin RNA was shorter than bleomycin RNAs. The hybridization efficiencies of the RNAs transcribed in the presence of the antibiotics were less than that of control RNA, as presented in the legend of Fig. 7.

Fig. 7. RNA-DNA hybridization competition of T4 ^{3}H-RNA synthesized in the presence and absence of antibiotics.

Cold competitor RNA was isolated from *E. coli* B cells harvested 2 minutes after infection in the presence of chloramphenicol (• *in vivo* pre-early RNA) and 5 minutes after infection in the absence of chloramphenicol (○ *in vivo* early RNA). Extraction was by the phenol method[16]. The reaction mixture for *in vitro* RNA synthesis was the same as described in the text. In the case of bleomycins, T4 DNA and bleomycin were incubated at 37℃ for 10 minutes, prior to the addition of RNA polymerase and nucleoside triphosphates. Futher incubation was for 30 minutes at 37℃. The concentrations of antibiotics were 80 μg/ml for bleomycins A_2 and A_5, and 4 μg/ml for neopluramycin. After addition of 1 mg phenolized *E. coli* NRA, the mixture was quickly frozen. The template was removed by treatment of DNase after heating at 90℃ for 1 minute. The RNA was then isolated by the phenol method in the presence of 0.5% sodium dodecyl sulfate[16]. The hybridization competition mixture contained in 0.4 ml of 2×SSC : 6 μg of denatured T4 DNA, *ca.* 7,000 cpm ^{3}H-RNA and increasing amounts of cold *in vivo* T4 RNA. The incubation was for 5 hours at 62℃ and DNA-RNA hybrids were collected on nitrocellulose filters[17]. Hybridization efficiencies were 18% for control, 8% for bleomycin A_2-RNA, 8% for bleomycin A_5-RNA and 0.5% for neopluramycin-RNA.

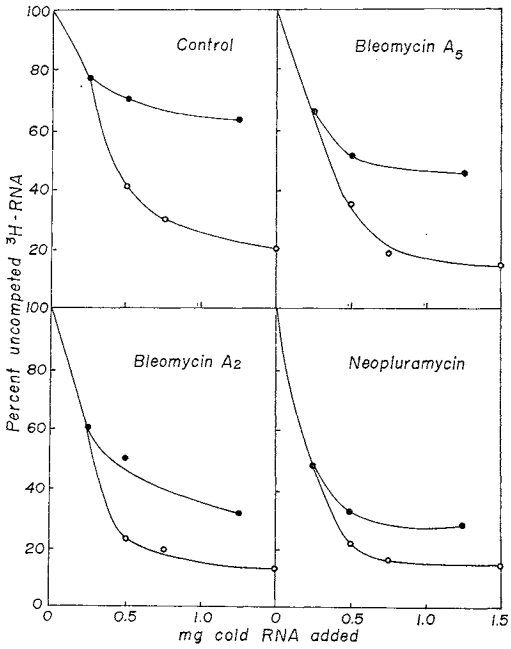
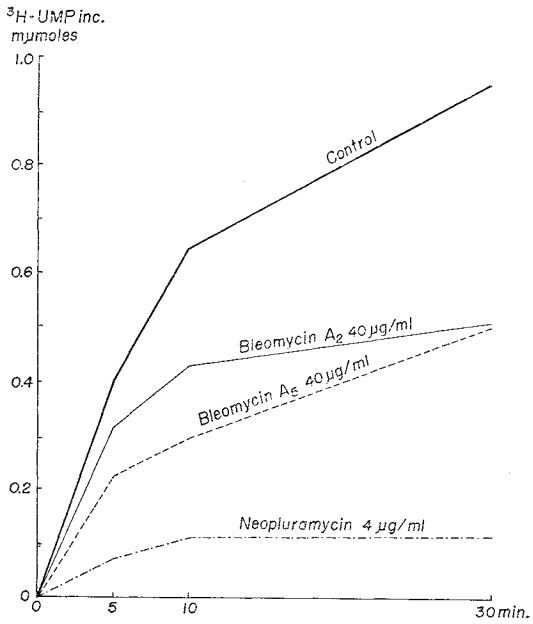


Fig. 8. Effects of antibiotics on RNA polymerase reaction using calf thymus DNA as a template.

The reaction mixture was the same as described in the text, except that T4 DNA was replaced by calf thymus DNA. The DNA and bleomycin were incubated for 10 minutes at 37℃, prior to the addition of RNA polymerase and nucleoside triphosphates. The concentrations of antibiotics were 40 μg/ml for bleomycins A_2 and A_5, and 4 μg/ml for neopluramycin.



The RNA polymerase used contained σ factor; and the transcription was presumably initiated at promotor sites of pre-early genes and continued to early gene regions *in vitro*. Therefore the results are consistent with the assumption that the antibiotics interfere with the RNA chain elongation by binding to the template DNA. The transcription may be inhibited at various sites of T4 DNA strands, and more pre-early genes may be transcribed than early genes.

The RNA polymerase reaction, using calf thymus DNA as a template, was also inhibited by neopluramycin and bleomycins A_2 and A_5. The DNA was incubated with bleomycins for 10 minutes prior to the start of the reaction. The results are presented in Fig. 8.

Discussion

The results reported here show that neopluramycin and bleomycins inhibit the transcription by binding to the template DNA and interfering with the elongation process of the

RNA polymerase reaction. Pluramycin and bleomycin function as antibiotics by interacting with DNA[4~10]. Since pluramycin causes an increase in Tm of DNA and a change in the difference spectrum with DNA, the mechanism of action of pluramycin seems to be similar to that of actinomycin, stabilizing the double strand structure. On the other hand, bleomycin causes a decrease in Tm of DNA and scissions of DNA strands. These observations indicate that the mechanism of action of bleomycin is different from that of actinomycin.

The mode of inhibition by pluramycin of transcription seems to be similar to that reported with actinomycin[14~16]. The main inhibitory effect resides in the propagation step of RNA chains on DNA strands, since shorter RNA chains are formed in the presence of the antibiotic and the transcribed RNA contains higher ratic of pre-early RNA to early RNA. The mode of inhibition by bleomycin of transcription seems to be similar to those of actinomycin and pluramycin in the sense that they all interact with the template DNA and inhibit the elongation of RNA chains, although the mechanisms of interaction with DNA are different.

The inhibition of *in vitro* transcription by bleomycins was significantly observed, only when the DNA was incubated with the antibiotics for 10 minutes prior to the addition of RNA polymerase and nucleoside triphosphates. It may be due to the fact that a certain period is needed for bleomycin to interact with DNA[4~7]. The failure of bleomycins to inhibit *in vivo* transcription may be due to the experimental conditions used, in which bleomycins may be unable to interact sufficiently with DNA prior to transcription.

Acknowledgement

The author wishes to express many thanks to Dr. E. K. F. BAUTZ for his cooperation during this study. This work was carried out in his laboratory and was supported by the grants from U. S. Public Health Service and National Science Foundation. The author appreciates the advice and cooperation of Dr. T. KASAI in this study.

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