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DIFFERENTIAL EFFECT OF NEOMYCIN ON DNA DEPENDENT -DNA AND -RNA SYNTHESIS IN VITRO

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Summary: Neomycin inhibits in vitro DNA dependent DNA and RNA synthesis catalyzed by DNA polymerase I and RNA polymerase from E.coli. The effect of the antibiotic is more pronounced towards DNA synthesis. The inhibition of DNA synthesis is competitive with template DNA, does not reverse with excess deoxynucleoside triphosphate, Mg^{2+} or enzyme E.coli DNA polymerase I. Neomycin does not reduce the number of potential 3' -OH end or primer. It seems to shorten the size of the newly formed polynucleotide.

INTRODUCTION

Aminoglycoside antibiotics viz. streptomycin, kanamycin and neomycin are similar both in their structure and antibacterial action. They are known to have binding affinity for DNA (1-2) and produce translational ambiguity at the level of 30s ribosomal subunit (3). Neomycin enables single stranded DNA to bind with ribosomes and thereby stimulates amino acid incorporation (4-5). The antibiotic also inhibits in vitro DNA synthesis by eukaryotic DNA polymerase (6). The present investigation suggests that although neomycin inhibits in vitro DNA and RNA synthesis catalyzed respectively by DNA polymerase I and RNA polymerase from E.coli the antibiotic has a more pronounced effect on DNA synthesis. The study indicates that the antibiotic may act by interacting with DNA.

MATERIALS AND METHODS

Radioactive deoxynucleoside triphosphates and ribonucleoside triphosphates were purchased from, New England Nuclear Corporation

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or Radiochemical Centre, Amersham. Unlabelled deoxynucleoside triphosphates and ribonucleoside triphosphates were obtained from Calbiochem and polynucleotides and oligonucleotides were from P-L Biochemicals. E.coli DNA polymerase I and E.coli RNA polymerase were kind gifts from Dr. L.A. Loeb, University of Washington, Seattle, USA. E.coli DNA polymerase I and RNA polymerase were purified to homogeneity according to the procedures of Jovin et al. (8) and Springgate and Loeb (9) respectively. Poly d(A-T) was prepared by the de novo reaction using E.coli DNA polymerase I as described elsewhere (10). Maximally "activated" calf thymus DNA was prepared by digestion of native DNA with pancreatic DNAase according to the method of Loeb (11). Neomycin sulfate was purchased from Mayfair and Croydon.

DNA polymerase assay In vitro DNA synthesis using "activated" calf thymus DNA or native or denatured calf thymus DNA as templates were measured in a reaction mixture (total volume 0.05 ml) which contained 50 mM Tris-HCl (pH 7.4), 20 µM each of the four deoxyribonucleoside triphosphates containing either (^{3}H) dATP or (^{3}H)dTTP (specific activity as indicated in the legends to the Tables and Figures), 20 µg of template DNA (unless otherwise mentioned), 5 mM $MgCl_2$ and 8 nM E.coli DNA polymerase I. Incubations were carried out in duplicate for 30 minutes at 37°C. Incorporation of the radioactive deoxynucleotides into acid insoluble precipitate was determined in liquid scintillation spectrometer as described by Dube and Loeb (12).

In vitro DNA synthesis using polyd(A-T) as the template was measured in a reaction mixture (total volume 0.05 ml) containing 50 mM Tris-HCl (pH 7.4), 20 µM dTTP, 20 µM (^{3}H)-dATP (specific activity as indicated in the legends to the Tables and Figures), 1 µg ply d(A-T), 1 mM $MgCl_2$ and 8 nM E.coli DNA polymerase I. The assay mixtures were incubated for 15 minutes at 37°C. Termination of reaction and determination of radioactivity into acid insoluble material were done in the same manner as stated above.

In vitro DNA synthesis using polydA.oligodT and polyA.oligodT as templates were measured in an incubation system (total volume 0.025 ml) which contained 20 µM (^{3}H) dTTP (specific activity 167 cpm/pmole), 5 mM $MgCl_2$, 50 mM Tris-HCl buffer (pH 7.4), 2 µg template and 8 nM E.coli DNA polymerase I. The incubation was carried out for 15 minutes at 37°C in duplicate. Termination of reaction and determination of radioactivity into acid insoluble material were done as stated above.

In vitro.DNA synthesis using ∅ x DNA template and calf thymus DNA primer was measured in an incubation system (total volume 0.025 ml) containing 50 µM each of dATP, dGTP, dCTP and (^{3}H) dTTP (specific activity 238 cpm/pmole), 50 mM Tris/HCl (pH 7.4), 10 mM $MgCl_2$, 0.1 M KCl, 10 µg ∅ x DNA, 0.3 µg primer and 12.5 µg enzyme protein. The primer was prepared by DNAase digestion of denatured calf thymus DNA as described by Poddar & Sinsheimer (13).

RNA polymerase assay.RNA polymerase activity was measured according to the method of Springgate and Loeb (9). The reaction mixture is a total volume of 0.05 ml contained 50 mM Tri-HCl (pH 8.0), 0.1 mM DTT, 10 mM $MgCl_2$, 2 µM each of ATP, GTP, CTP and (^{3}H) UTP (specific activity is given in the legends to the corresponding Tables and Figures) and 2.5 µg of enzyme protein. Incubations were carried out in duplicate at 37°C for 40 minutes. Incorporation of radioactivity into acid insoluble material was determined in a liquid scintillation spectrometer as described earlier.

Table 1. Effect of neomycin on DNA dependent DNA synthesis by E.coli DNA polymerase I and DNA dependent RNA synthesis by E.coli RNA polymerase.

20 µg of "activated" DNA was used in both cases. Complete assay system has been described in the Materials and Methods section. Specific activity of (^{3}H) dATP was 606 cpm/pmole.

Additions	% Activity	
	DNA dependent - DNA synthesis	DNA dependent - RNA synthesis
None	100[a]	100[b]
Neomycin (1 mM)	21	75.5
Neomycin (2 mM)	17	76
Neomycin (5 mM)	13	16

[a]100% activity is 47 pmoles
[b]100% activity is 188 pmoles

Determination of 3'-OH groups. The number of 3'-OH groups present in poly d(A-T) molecule (prepared by the de novo reaction using E.coli DNA polymerase I) was determined by the method described by McClure and Jovin (14).

Agarose gel electrophoresis. Slab gels containing 0.5% agarose and 1.5% acrylamide as described by Travaglini et al (15) were used. The labelled deoxynucleoside triphosphate (^{3}H) dATP (specific activity 332 cpm/pmole). Acid insoluble material from the total reaction mixture was dissolved in 0.05 ml of 0.1 N NaOH solution and 0.04 ml of this solution containing approximately 17000 cpm in control and 7000 cpm in neomycin treated samples was mixed with sucrose and subjected to electrophoresis at 167 v/cm for 6 hours. Gel slices (2 mm in thickness) were dissolved in 0.1 ml of 0.4 M sodium perchlorate solution. Radioactive counts were taken with 10 ml of aqua sol as stated earlier.

RESULTS AND DISCUSSION

Data given in Table 1 show that neomycin inhibits both DNA dependent-DNA and -RNA synthesis on "activated" DNA template by E.coli DNA polymerase I and E.coli RNA polymerase respectively. The drug is evidently more active against DNA dependent-DNA synthesis. Neomycin at 1 mM concentration inhibits 79% of in vitro DNA synthesis. However, the 84% inhibition of RNA synthesis requires 5 mM concentration of the drug for the same amount of "activated" DNA template.

Similar differential behaviour of neomycin is also observed when poly d(A-T) template is used instead of activated DNA. Fig. I depicts

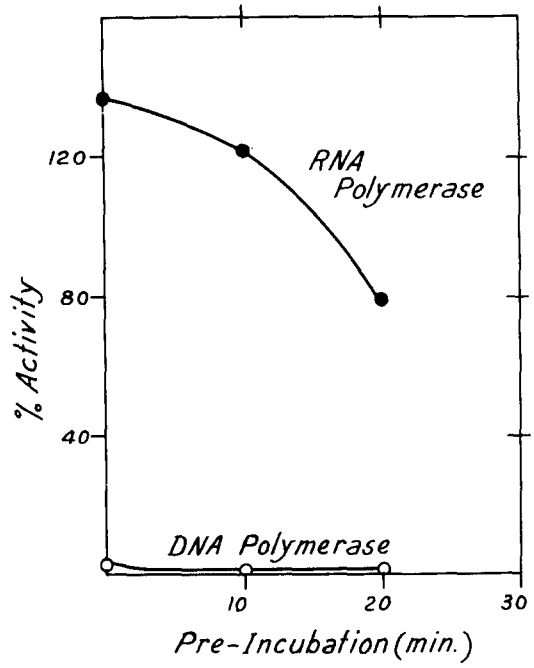

Fig. 1. In vitro DNA and RNA synthesis using poly d(A-T) template preincubated with neomycin.

O, DNA synthesis; ●, RNA synthesis.

Pretreatment of poly d(A-T) with neomycin was carried out by incubating 0.7 µg of poly (A-T) with 1 mM neomycin in 50 mM Tris/HCl (pH 8.0) at 30°C. Specific activity (^{3}H) dTTP was 122 cpm/pmole and that of (^{3}H) UTP was 807 cpm/pmole.

the % activities of DNA polymerase I and RNA polymerase from E.coli when poly d(A-T) template is preincubated with neomycin. Preincubation with 1 mM neomycin appears to facilitate the inhibition of in vitro DNA dependent RNA synthesis. However, inhibition of in vitro DNA synthesis catalyzed by E.coli DNA polymerase I at the said concentration of neomycin, does not require any pretreatment.

Inhibitions of in vitro DNA dependent-DNA and -RNA synthesis catalyzed by E.coli DNA polymerase I and E.coli RNA polymerase respectively with neomycin are reduced by the addition of excess template. Kinetic studies with these in vitro DNA and RNA synthesizing systems indicate that the nature of inhibition by neomycin in both cases is competitive with

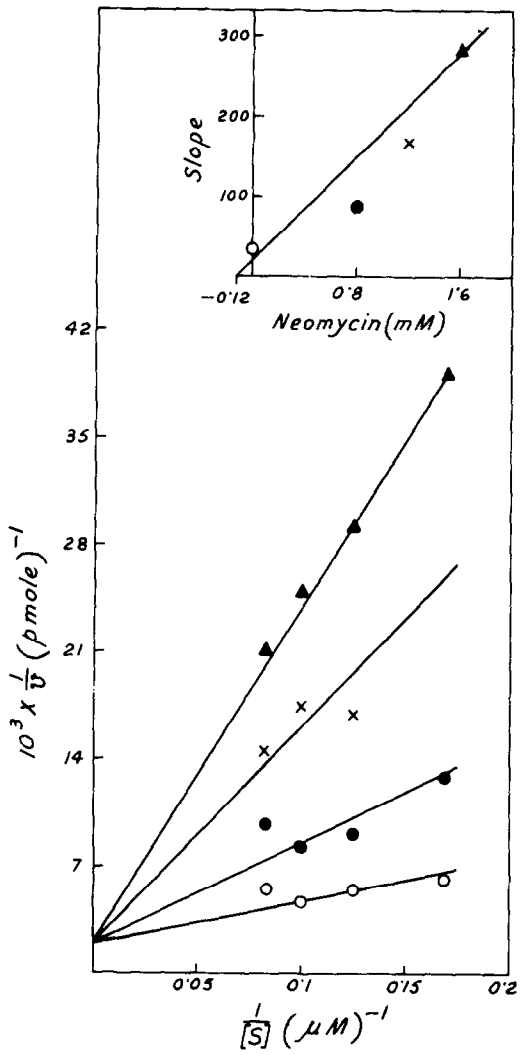

Fig. 2. Determination of Ki of neomycin with DNA polymerase I using activated DNA as template - primer.

O, control; ●, 0.8 mM neomycin; X, 1.2 mM neomycin; ▲ 1.6 mM neomycin.

v, incorporation of (^{3}H)-dAMP; S, concentration of activated DNA.

The complete incubation system in a total volume of 50 µl contained 100 µM (^{3}H) dATP (specific activity 483 cpm/pmole), 0.1 m KCl, 5 mM Mg^{2+}, 50 mM Tris-HCl (pH 7.4) and 8 nM E.coli DNA polymerase I. Incubations were carried out in duplicate for 20 minutes at 37°C.

template DNA (Figs. 2 & 3). The inhibitory constant (Ki) of neomycin for in vitro DNA dependent -DNA synthesis determined from Fig. 2 (inset Fig. 2) is 0.12 mM and that for in vitro RNA synthesis by E.coli RNA

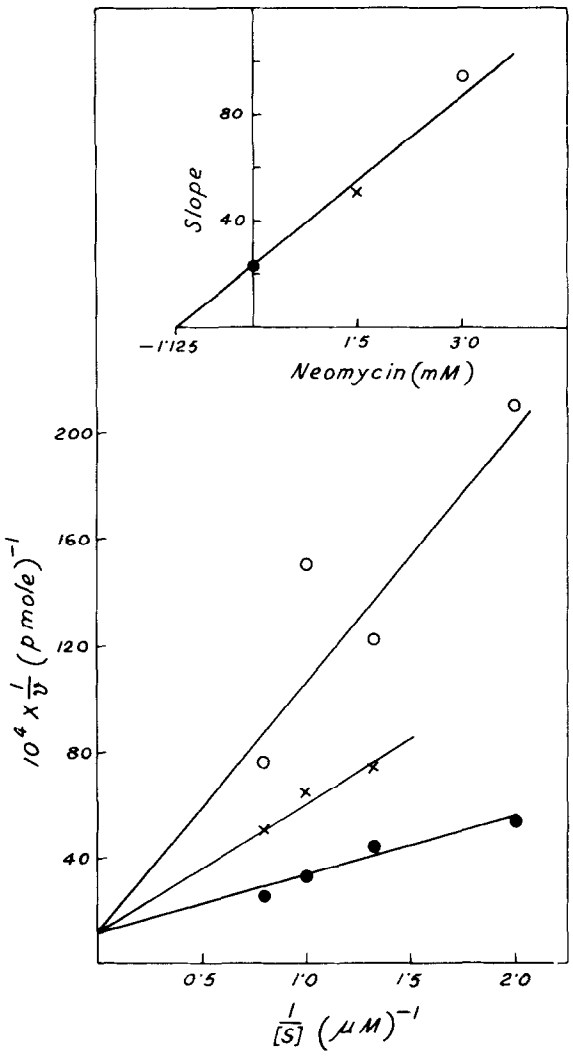

Fig. 3. Determination of Ki of neomycin with E.coli RNA polymerase using activated DNA as template.

●, control; X, 1.5 mM neomycin; O, 3 mM neomycin.

v, incorporation of (^{3}H) UPM; S, concentration of activated DNA. Complete incubation system and reaction conditions were same as described in the Materials and Methods section. Specific activity of (^{3}H) UTP was 30.5 cpm/pmole.

polymerase is 1.125 mM (inset Fig. 3). The values are in agreement with the observation that neomycin is more active against DNA synthesis than against RNA synthesis in vitro

Table 2. Effect of neomycin on *in vitro* DNA synthesis by *E.coli* DNA polymerase I using different template - primer.

The complete incubation system and reaction conditions have been described in the Materials and Methods section.

Template	Additions	Incorporation (pmole/assay system)
"Activated" DNA	None	360[a]
"	Neomycin (1 mM)	76
Denatured DNA	None	95.7[b]
"	Neomycin (1 mM)	60.8
"	Neomycin (2 mM)	4.3
Native DNA	None	51.2
"	Neomycin (1 mM)	22
"	Neomycin (2 mM)	3.94
Poly d(A-T)	None	21.2[c]
"	Neomycin (1 mM)	2.7
Poly dA.oligo dT	None	60.5[d]
"	Neomycin (0.2 mM)	42
"	Neomycin (0.5 mM)	8.5
"	Neomycin (1 mM)	0.6
"	Neomycin (5 mM)	0.3
Poly A.oligo dT	None	61.7
"	Neomycin (0.2 mM)	3.75
"	Neomycin (0.5 mM)	0
"	Neomycin (1 mM)	0
"	Neomycin (5 mM)	0
Ø x DNA template with calf thymus DNA primer	None	17.75[e]
"	Neomycin (1 mM)	3.7
"	Neomycin (2 mM)	0.5

a) Specific of (^{3}H)dATP 1 897 cpm/pmole
b) Specific activity of (^{3}H)dATP is 201 cpm/pmole
c) Specific activity of (^{3}H)dATP = 1615 cpm/pmole
d) Specific activity of (^{3}H)dTTP 167 cpm/pmole
e) Specific activity of (^{3}H)dTTP is 238 cpm/pmole.

The competitive nature of the kinetic curve (Fig. 2) gives rise to the possibility that neomycin may act either by binding with the template DNA or by competing with the template DNA for the same site of the enzyme.

To see whether neomycin has any specificity for templates the effect of neomycin on *in vitro* DNA synthesis was tested using various templates. Data given in Table 2 show that neomycin inhibits *in vitro*

Table 3. Effect of increase in enzyme concentration on neomycin induced inhibition of in vitro DNA synthesis.

The complete incubation system and reaction conditions have been described in the "materials and methods" section. Poly d(A-T) was used as template. Incubations were carried out for 10 minutes at 37°C. Specific activity of (^{3}H) dTTP was 728 cpm/pmole.

Concentration of enzyme (nM)	Neomycin concentration (mM)	% Inhibition
0.917	0.15	58.00
1.8	"	57.30
3.669	"	42.30
5.5	"	49.05
8.256	"	56.74
"	"	49.22

DNA synthesis by E.coli DNA polymerase I on all the templates used. However, the extent of inhibition is markedly high in the case of poly dA.oligo dT and poly A.oligo dT. templates.

An increase in the amount of the enzyme E.coli DNA polymerase I from a concentration of 0.917 nM-13.8 nM does not show any appreciable decrease in the extent of inhibition of in vitro DNA synthesis using poly d(A-T) as template - primer (Table 3). Neomycin, therefore, does not appear to bind with the enzyme in exerting its inhibitory role. Unpublished results show that poly d(A-T) directed DNA synthesis by E.coli DNA polymerase I requires about 2 mM Mg^{2+} for its optimum activity. To find out whether neomycin chelates out this essential bivalent metal cation, Mg^{2+}, from the reaction mixture the concentration of Mg^{2+} was raised to 5 mM in an incubation system containing 1 mM neomycin. This increase in Mg^{2+} concentration does not diminish the inhibition (results not given). Also, increase in deoxynucleoside triphosphate concentrations from 20 to 100 µM does not release the inhibition by 1 mM neomycin (percentage of inhibition shown in Table 1 and Fig. 2 is nearly the same).

Neomycin was found to inhibit DNA polymerase I associated 3'→ 5' exonuclease activity as also the deoxynucleotidyl terminal transferase

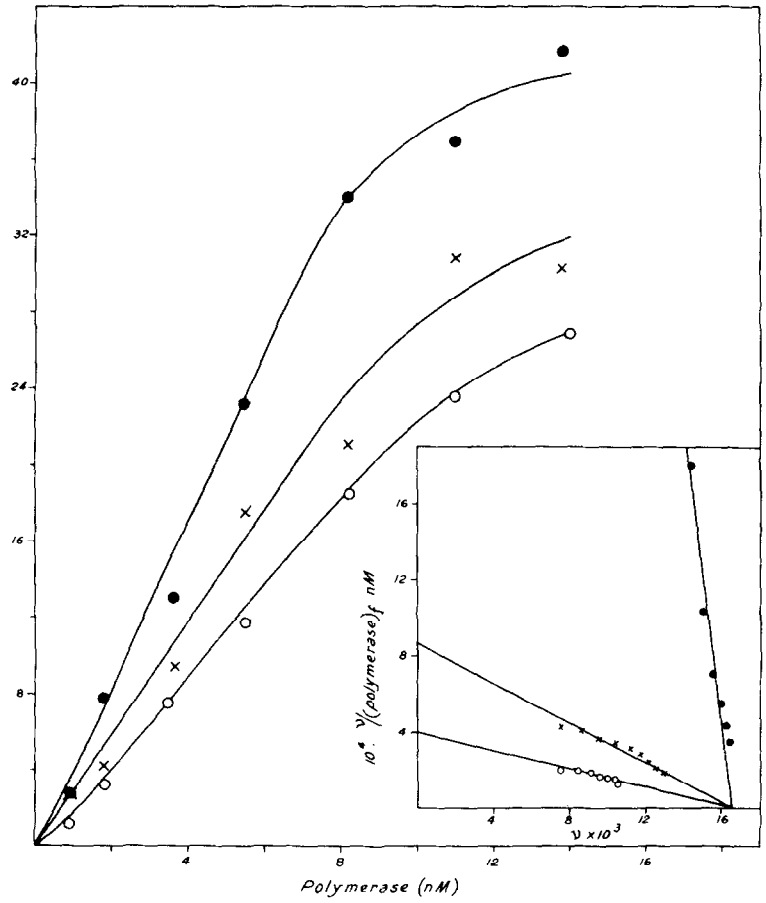

Fig. 4. Determination of number of 3' - OH groups in poly d(A-T) template in presence and absence of neomycin.

O, Control; X, 0.1 mM neomycin; ●, 0.15 mM neomycin.

ν = (Enzyme template complex formed): (Total polymer nucleotide). To determine the amount of enzyme complex a fixed concentration (570 nM) of poly d(A-T) was saturated by gradually increasing the concentration of the enzyme in a reaction mixture (total volume 0.05 ml) containing 20 μM dATP, 20 μM (^{3}H) dTTP (specific activity 474 cpm/pmole), 50 mM Tris/HCl (pH 7.4) and 1 mM $MgCl_2$. Incubations were carried out in duplicate for 5 minutes at 37°C. The velocity of the reaction (Vo), that is, the number of phosphodiester bond formed per minute, was then plotted against the concentration of the enzyme. The amount of enzyme complexed with the template and also that remained free in the system was determined from linear portion of the curve.

activity (7). So it was of interest to see if neomycin interacts at 3'-OH position of the primer where initiation of DNA synthesis occurs. The number of 3'-OH groups in the presence and absence of neomycin was determined by the method described in the Materials and Methods section. Fig. 4 indicates that neomycin does not cause reduction of the number of

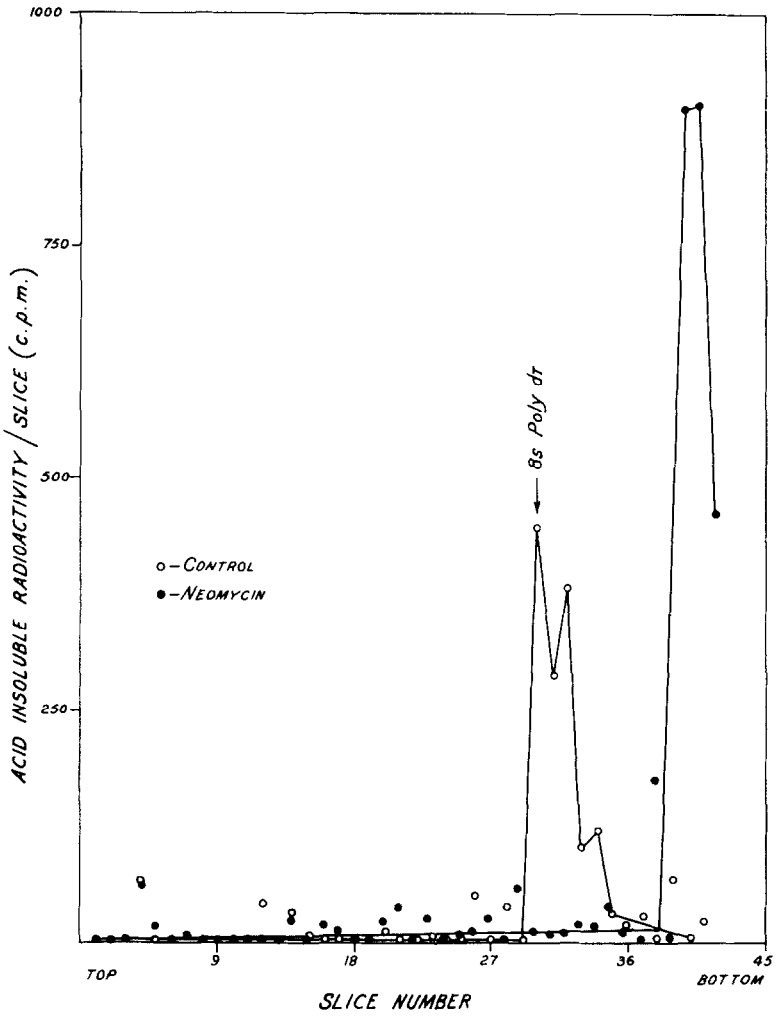

Fig. 5. Agarose gel electrophoretic analysis of the size of the product formed in a reaction catalyzed by E.coli DNA polymerase I using poly dT.oligo dA as template in presence of neomycin.

O, control; ●, 0.4 mM neomycin.

The complete assay system contained in a total volume of 0.15 ml 20 μM (^{3}H) dATP (specific activity 332 cpm/pmole, 5mM $MgCl_2$, 50 mM Tris/HCl (pH 8.0), 20 μg poly dT(85).oligo dA and 8 nM E.coli DNA polymerase I. The incubations were carried out in duplicate at 37°C for 20 minutes.

potential 3'-OH terminui during its action. It is likely, then, that the site of action of neomycin is elsewhere, on the template or on the primer.

In order to examine whether neomycin binds at the single stranded region of the DNA template and thus terminates the lengthening of the

primer, the size of the polynucleotide formed in the presence and absence of neomycin was analyzed electrophoretically. An examination of Fig. 5 indicates that the neomycin treatment gives rise to the production of polynucleotides of shorter length. These results suggest the possibility that neomycin may terminate the elongation of the primer by binding on the DNA template in single or double stranded region. This presumption can also explain the results presented in Table 2 which shows that the inhibition of DNA synthesis is more pronounced in the case of poly dA.oligo dT or poly A.oligo dT template than in the case of poly d(A-T), Ø x DNA template with calf thymus DNA initiator and activated DNA. It seems that the first set contains less initiating points than the second one. The template with multiple initiating points would require more neomycin molecules for complete inhibition.

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