MECHANISM OF ACTION OF NEOTHRAMYCIN
I. THE EFFECT ON MACROMOLECULAR SYNTHESES

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Neothramycin was observed to prevent growth of mouse lymphoblastoma L5178Y and HeLa cells at the concentration of 0.5~1.0 μg/ml and exhibited a lethal effect at 5.0 μg/ml. Approximately 50% growth inhibition of *E. coli* was found at the concentration of 37 μg/ml. The effect of neothramycin on macromolecular syntheses in the mammalian and bacterial cells was investigated. The antibiotic produced a preferential inhibition of RNA over DNA synthesis in the intact cells of lymphoblastoma L5178Y: *i.e.* approximately 50% inhibition of the former was observed at the antibiotic concentration of 1.4 μg/ml, and that of the latter at 12.0 μg/ml. Protein synthesis was not significantly affected.

Contrary to the effect in the mammalian cells, thymine uptake into DNA was more markedly blocked than uridine incorporation into RNA by the antibiotic in the intact cells of *E. coli*. However, neothramycin was observed to cause a significant degradation of DNA, and the apparent inhibition of thymine uptake seemed to be due to degradation of DNA but not to the inhibition of net DNA synthesis. In *E. coli* the antibiotic prevented RNA synthesis more profoundly than DNA synthesis as in the mammalian cells. DNA synthesis with toluene-treated cells of *E. coli* polA$^-$ was not significantly affected by neothramycin, while RNA synthesis with the same system was markedly blocked by the antibiotic.

Neothramycin was demonstrated to prevent DNA-dependent RNA and DNA polymerase reactions, using *E. coli* enzymes and calf thymus DNA as a template. RNA polymerase reaction was more profoundly inhibited than DNA polymerase I: *i.e.* approximately 50% inhibition of RNA polymerase reaction was observed at the antibiotic concentration of 11 μg/ml, and that of DNA polymerase I at 100 μg/ml. The inhibition degree of DNA polymerase I was reversed by the increasing concentration of template DNA but not by that of the enzyme, suggesting the direct interaction of the antibiotic with DNA. The degree of inhibition of the polymerase reactions depended upon the period of preincubation of template DNA and neothramycin, increasing gradually until the preincubation reached 60 minutes. It suggested that the interaction of DNA and the antibiotic might need a certain time.

Neothramycin[1,2] belongs to the group of pyrrolo(1,4)benzodiazepine antitumor antibiotics, which includes anthramycin, tomaymycin, and sibiromycin (*cf.* reviews[3,4]). The two antibiotics A and B, which are stereoisomers and interconvertible in aqueous solution, have been isolated from the culture filtrate of a strain of cycloheximide-producing *Streptomyces*; and found to exhibit a marked inhibitory effect on mouse leukemia L1210 and YOSHIDA rat sarcoma as well as a weak antimicrobial activity and lower toxicity in mice than the other pyrrolo(1,4)benzodiazepine antibiotics[1].

The mechanism of action of neothramycin has been studied as one of the basic investigations for clinical application. It has been observed that the antibiotic blocks RNA and DNA syntheses by interacting with template DNA *in vivo* as well as *in vitro*. The former is about 10-times more sensitive to neothramycin than the latter. DNA degradation is also caused by the antibiotic, particularly in the intact cells of bacteria. The mode of action is principally similar to those of the other pyrrolo(1,4)-benzodiazepine antibiotics. The results with macromolecular syntheses will be presented in this publi-

cation, and those concerning the interaction with DNA in the following papers.

Materials and Methods

[^{3}H]Uridine (44.9 Ci/mmole), [^{3}H]thymine (13.2 Ci/mmole), [^{3}H]thymidine (49.8 Ci/mmole), L-[^{14}C]leucine (354 mCi/mmole), L-[^{14}C]valine (285 mCi/mmole), [^{3}H]GTP (13.2 Ci/mmole), and [^{14}C]-dATP (500 mCi/mmole) were purchased from New England Nuclear, Boston, Mass. [^{3}H]UTP (40 Ci/mmole) was obtained from Radiochemical Centre, Amersham, England. Ribonucleoside triphosphates and deoxyribonucleoside triphosphates were products of Boehringer Mannheim, Germany. Sepharose 6B (Pharmacia), DEAE-Sephadex (Pharmacia), DEAE-cellulose (Whatman DE-52), and phosphocellulose (Whatman P-11) were pretreated according to the manufacturer's directions.

The DNA polymerase activity of *E. coli* P3478 (polA$^-$, thy$^-$) was assayed immediately before the experiment and found to be approximately 1% of that of the parent strain W3110 (thy$^-$).

Macromolecular syntheses in the intact cells of *E. coli*

E. coli was grown to the mid-logarithmic phase in DAVIS' minimal medium, supplemented with 0.1% casamino acids and 2 μg/ml thymine. Radiolabelled precursors were incorporated for 60 minutes at 37°C with or without 60 minutes preincubation with neothramycin, and the radioactivity of 5% cold TCA-insoluble materials was determined in a liquid scintillation counter. As radioactive precursors, 5 μl of 50 μCi/ml [^{3}H]uridine and 10 μl of 1 mg/ml unlabelled uridine, 15 μl of 100 μCi/ml [^{3}H]-thymine, or 4 μl of 50 μCi/ml [^{14}C]valine was added to 1 ml of the culture.

Degradation of DNA by neothramycin in the intact cells of *E. coli*

[^{3}H]Thymine was incorporated into DNA of the intact cells of *E. coli* for 2 hours at 37°C as described above, then 100-fold excess of unlabelled thymine (200 μg) with or without 39.2 μg of neothramycin was added to 1 ml of the culture, and the radioactivity of TCA-insoluble materials was chased in 2.5 hours.

Macromolecular syntheses in the intact cells of mouse lymphoblastoma L5178Y cells

Mouse lymphoblastoma L5178Y cells were grown in FISCHER's medium (Gibco) containing 10% horse serum (Flow Lab.) in test tubes tightly sealed with rubber stoppers. The cell number was determined with a COULTER counter. For investigations on RNA and DNA syntheses, 2.0 ml of the cell suspension (1.2×10^5 cells) was incubated with neothramycin for 60 minutes, prior to the addition of 10 μl of [^{3}H]uridine (100 μCi/ml) or [^{3}H]thymidine (100 μCi/ml). The radiolabelled precursor was incorporated for 60 minutes at 37°C, and the cold 10% TCA-insoluble radioactivity was determined in a liquid scintillation counter. Protein synthesis was assayed by the uptake of [^{14}C]leucine into hot 10% TCA-insoluble fraction.

DNA and RNA syntheses with toluene-treated cells of *E. coli* P3478 (polA$^-$)

DNA and RNA polymerase reactions with toluene-treated cells of *E. coli* P3478 followed the procedures of MOSES & RICHARDSON[9] and PETERSON *et al.*,[10] respectively.

The cells of *E. coli* P3478, grown to mid-logarithmic phase in Penassay medium (Difco) supplemented with 20 μg/ml of thymine, were concentrated 10-fold in 0.05 M phosphate buffer (pH 7.5) for DNA polymerase or in 0.05 M Tris-HCl (pH 7.5) for RNA polymerase. The cells were treated with 1% toluene with occasional shaking, centrifuged, suspended in the same volume of the buffer, and used immediately for polymerase reactions. The reaction for DNA polymerase, in 0.3 ml, contained: 70 mM potassium phosphate buffer, pH 7.5, 13 mM $MgCl_2$, 1.3 mM ATP, 0.033 mM each dATP, dCTP and dTTP, and 1 μCi [^{3}H]dGTP. The mixture for RNA polymerase, in 0.3 ml, contained: 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 80 mM KCl, 0.1 mM dithiothreitol, 1 mM each ATP, CTP and GTP, and 1 μCi [^{3}H]UTP. Toluene-treated cell suspension of 0.15 ml was added to each reaction mixture. The polymerase reaction was performed for 10 minutes at 37°C and was terminated by addition of cold 10% TCA containing 1% sodium pyrrophosphate and the insoluble radioactivity on glass filter was counted in a liquid scintillation counter.

Purification of enzymes

DNA-dependent RNA polymerase was prepared from *E. coli* Q13 by the method of KAMEYAMA

et al.,[5] except that DNase I treatment of cell extract was employed instead of protamine precipitation, followed by ammonium sulfate fractionation and chromatography on Sepharose 6B and DEAE-Sephadex A50 columns. DNA-dependent DNA polymerase I was purified from the same bacteria according to the technique of JOVIN *et al.*,[6] except that the final step, *i.e.* Sephadex G100 chromatography, was omitted, including streptomycin precipitation, autolysis, ammonium sulfate fractionation and chromatography on DEAE-cellulose and phosphocellulose.

RNA and DNA polymerase reactions

The reactions were assayed by conversion of radiolabelled precursors into acid-insoluble products. The reaction mixture for RNA polymerase, in 0.25 ml, contained: 50 mM Tris-HCl, pH 7.9, 5 mM $MgCl_2$, 2 mM $MnCl_2$, 0.1 mM dithiothreitol, 0.4 mM each ATP, UTP and CTP, 0.08 mM (2 μCi/ml) [^{3}H]GTP, 100 μg/ml calf thymus DNA, and 27.2 μg/ml enzyme. The incorporation was carried out for 10 minutes at 37°C, and cold 5% TCA-insoluble radioactivity, collected on Millipore filter, was measured in a liquid scintillation counter. The reaction mixture for DNA polymerase, in 0.3 ml, contained: 66.7 mM potassium phosphate buffer, pH 7.4, 6.7 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.035 mM each dCTP, dTTP and dGTP, 0.014 mM (0.083 μCi/ml) [^{14}C]dATP, 100 μg/ml calf thymus DNA, and 35 μg/ml enzyme. It was incubated for 30 minutes at 37°C, and cold 1 N perchloric acid-insoluble radioactivity, collected on glass filter (Whatman GF/C), was determined as above. Neothramycin was incubated with template DNA at 37°C for a certain periods, prior to the polymerase reaction, when indicated.

Results

Inhibition by Neothramycin of Growth of Mouse Lymphoblastoma L5178Y and HeLa Cells, and that of *E. coli*

Neothramycin was added to the culture of L5178Y or HeLa cells grown to the logarithmic phase, and the effect on the growth was observed by counting the cell number. The antibiotic prevented growth of L5178Y cells at the concentration of 0.5 and 1.0 μg/ml. The number of cells decreased at 5.0 μg/ml, indicating the lethal effect. The partial inhibition was found in the concentration range of 0.2 to 0.5 μg/ml. The growth was not significantly affected at the antibiotic concentrations less than 0.04 μg/ml. The results are illustrated in Fig. 1. The same tendency of the degree of growth inhibition was demonstrated with HeLa cells.

Fig. 1. Effects of neothramycin on growth of mouse lymphoblastoma L5178Y cells.

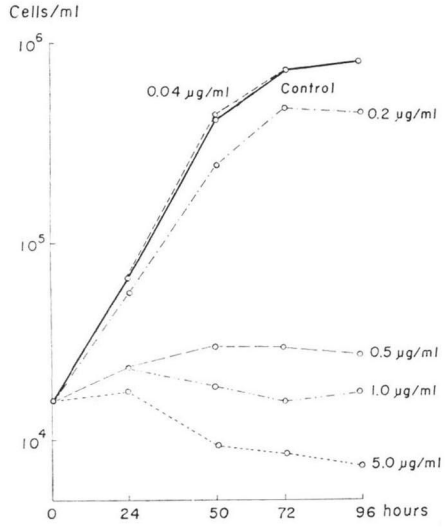
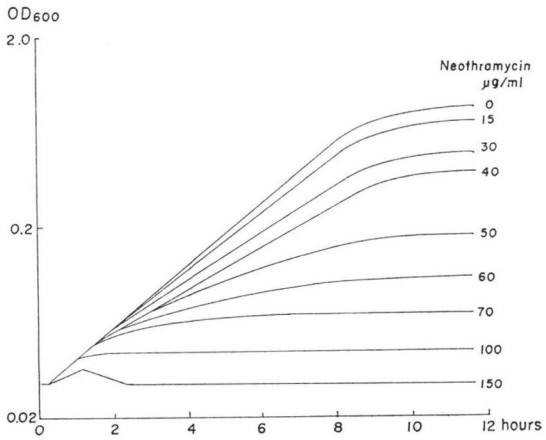


Fig. 2. Effects of neothramycin on growth of *E. coli*.



Neothramycin was observed by optical density method to block growth of *E. coli* P3478 at the concentrations higher than 15 μg/ml in DAVIS' minimal medium supplemented with 0.1% casamino acids and 20 μg/ml thymine (Fig. 2). Approximately 50% inhibition was observed at the antibiotic concentration of 37 μg/ml. Slightly greater inhibition was found in L-broth supplemented with 40 μg/ml of thymine.

Effects of Neothramycin on Macromolecular Syntheses in the Intact Cells of Mouse Lymphoblastoma L5178Y and *E. coli*

Neothramycin was demonstrated to block the uptake of [^{3}H]uridine and [^{3}H]thymidine into cold acid-insoluble fraction of the intact cells of L5178Y. The results are summarized in Table 1. By the

Table 1. Effects of neothramycin on macromolecular syntheses in the intact cells of mouse lymphoblastoma L5178Y cells.

Neothramycin	Incorporation of		
	[^{3}H]uridine	[^{3}H]thymidine	[^{14}C]leucine
0 μg/ml	2,370 (100)	12,700 (100)	4,750 (100)
0.2	1,820 (77)	12,070 (95)	4,810 (101)
1.0	1,590 (70)	12,300 (97)	4,430 (94)
5.0	219 (9)	10,200 (80)	4,410 (93)
20.0	78 (3)	3,600 (28)	3,880 (82)

The number represents cpm/1.2×10^5 cells, and that in the bracket relative incorporation.

Table 2. Effects of neothramycin on macromolecular syntheses in the intact cells of *E. coli*.

E. coli strain	Neothramycin	Incorporation of		
		[^{3}H]thymine	[^{3}H]uridine	[^{14}C]valine
W3110 (without preincubation)	0 μg/ml	30,300 (100)	25,790 (100)	38,300 (100)
	5	27,320 (90)	26,230 (101)	39,000 (102)
	10	25,690 (85)	26,750 (103)	39,550 (103)
	20	21,510 (71)	25,690 (99)	40,180 (105)
	40	16,820 (56)	23,390 (90)	39,730 (104)
	80	9,520 (31)	18,140 (70)	34,940 (91)
P3478 (without preincubation)	0	29,630 (100)	27,300 (100)	
	10	18,890 (64)	25,080 (92)	
	20	8,330 (28)	17,310 (63)	
	40	2,310 (8)	8,961 (33)	
	60	1,470 (5)	7,550 (28)	
	80	1,090 (4)	5,830 (21)	
P3478 (with 60 min. preincubation)	0	25,020 (100)	21,710 (100)	24,599 (100)
	2	22,410 (90)	21,850 (101)	26,080 (107)
	4	20,070 (80)	21,680 (100)	28,320 (116)
	6	17,970 (72)	19,760 (91)	27,310 (112)
	8	10,400 (42)	13,500 (62)	22,890 (93)
	10	7,020 (28)	10,650 (49)	19,300 (79)
	12	2,960 (12)	5,820 (27)	12,270 (50)

The number represents cpm/ml of the culture, and that in the bracket relative incorporation.

method employed, 50% inhibition of RNA and DNA syntheses was observed at the antibiotic concentration of 1.4 μg/ml and 12.0 μg/ml, respectively. The antibiotic slightly depressed the incorporation of [^{14}C]leucine into hot acid-insoluble fraction at high concentrations. The results indicated that neothramycin produced a preferential inhibition of RNA synthesis to DNA synthesis, and did not significantly affect protein synthesis in L5178Y cells.

Contrary to the effects in the lymphoblastoma cells, the antibiotic was found to block [^{3}H]thymine uptake more markedly than [^{3}H]uridine uptake in the intact cells of *E. coli* (Table 2). Approximately 50% inhibition of thymine incorporation was observed at the antibiotic concentration of 13 μg/ml in *E. coli* P3478 and 45 μg/ml in *E. coli* W3110; and that of uridine incorporation at 25 μg/ml in P3478 and higher than 80 μg/ml in W3110. Protein synthesis was not significantly affected. The degree of inhibition of nucleic acid syntheses increased, and protein synthesis was slightly suppressed, when neothramycin was preincubated with the intact cells of *E. coli* P3478 (Table 2).

Degradation of DNA by Neothramycin in the Intact Cells of *E. coli*

For the purpose of elucidating the reason for the discrepancy of the apparent inhibition degrees of RNA and DNA syntheses in L 5178Y cells and *E. coli*, degradation of DNA by neothramycin was studied with the intact cells of *E. coli*. It was observed by the procedure described in "Materials and Methods" that the antibiotic induced marked degradation of [^{3}H]thymine-labelled DNA in an hour (Fig. 3). The results suggested that the inhibition of [^{3}H]thymine uptake by neothramycin was not mainly due to that of DNA synthesis but attributed to the degradation of DNA.

Fig. 3. Degradation of DNA, caused by neothramycin, in the intact cells of *E. coli*.
Arrow shows the time, at which neothramycin (39.2 μg/ml) was added to the culture.
A: control without addition of unlabelled excess thymine
B: control with addition of unlabelled excess thymine
C: the same as B in the presence of neothramycin

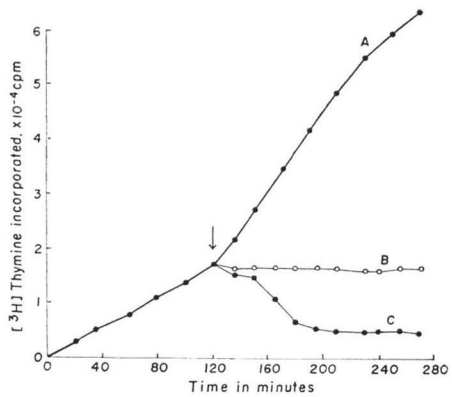


Table 3. Effects of neothramycin on DNA and RNA polymerase reactions in toluene-treated cells of *E. coli* P3478 (polA^{-}).

System	DNA polymerase [^{3}H]dGMP incorp.	RNA polymerase [^{3}H]UMP incorp.
Complete	3,623 (100)	10,977 (100)
−ATP	218 (6)	
−dNTPs or −NTPs	0 (0)	1,534 (14)
+Neothramycin		
6.3 μg/ml	3,602 (99)	10,088 (89)
12.5	3,757 (104)	9,014 (82)
25.0	3,684 (102)	8,213 (75)
50.0	3,527 (97)	7,390 (67)
100.0	3,393 (94)	5,963 (54)
+NEM 1.5 mM	652 (18)	

The number represents cpm/tube, and that in the bracket relative incorporation.
NEM: N-ethylmaleimide.

Effects of Neothramycin on DNA and RNA Polymerase Reactions in Toluene-treated Cells of *E. coli* (polA^{-})

As shown in Table 3, DNA polymerase reaction in toluenized cells of *E. coli* P3478 (polA^{-}) was highly dependent on ATP and strongly inhibited by addition of N-ethylmaleimide, indicating that

this reaction reflected DNA replication *in vivo*. Neothramycin did not significantly affect the DNA polymerase reaction in the toluene-treated cells at the antibiotic concentration up to 100 μg/ml. On the other hand, RNA polymerase reaction was blocked by the antibiotic; about 50% inhibition at 100 μg/ml and 10% even at 6.3 μg/ml of neothramycin. The results showed that the antibiotic inhibited RNA polymerase reaction but did not significantly affect DNA replication which involved DNA polymerases II and III.

Effects of Neothramycin on DNA-dependent RNA and DNA Polymerase Reactions with Isolated Enzymes

Neothramycin was found to prevent RNA and DNA polymerase reactions, using *E. coli* enzymes and calf thymus DNA as a template. The results of the experiments, in which template DNA and the antibiotic were incubated for 60 minutes at 37°C prior to the polymerase reactions, are illustrated in Fig. 4. The activities of RNA polymerase and DNA polymerase I were blocked by approximately 50% at the antibiotic concentrations of 11.0 μg/ml and 100 μg/ml, respectively, in the reaction mixtures containing 100 μg/ml of DNA. RNA polymerase reaction was more markedly affected than DNA polymerase I reaction.

Fig. 4. Inhibition of DNA-dependent RNA or DNA polymerase reaction by neothramycin.

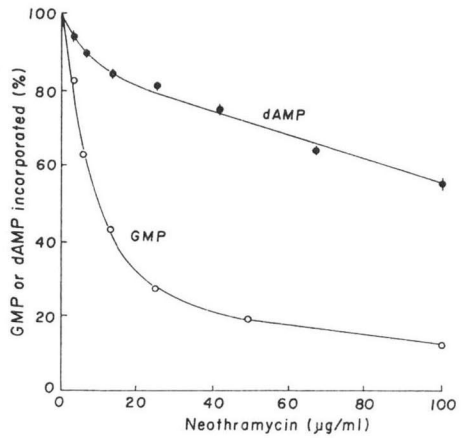


Fig. 5. Inhibition by neothramycin of DNA polymerase reaction at different concentrations of primer DNA.

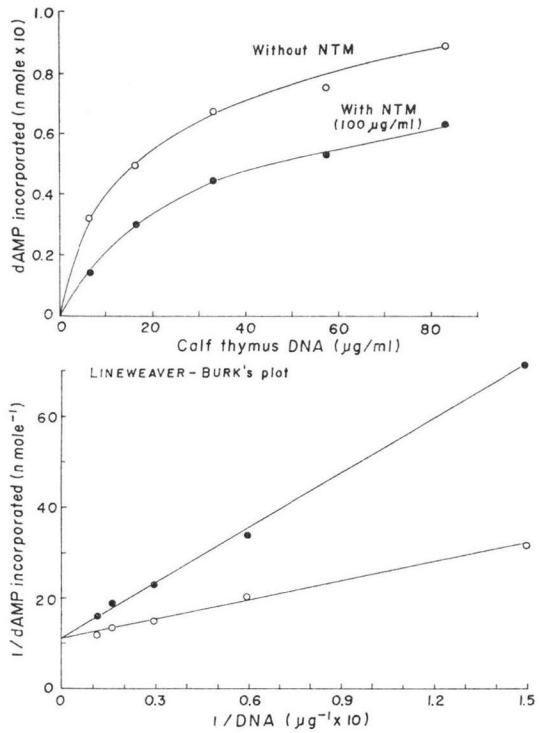
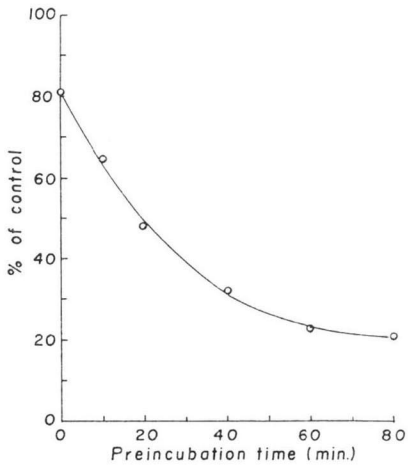


Fig. 6. Effects of preincubation of DNA and neothramycin on the inhibition of DNA-dependent RNA polymerase reaction.



The inhibition degrees of DNA polymerase I reaction was determined at various concentrations of template DNA or the enzyme. As shown in Fig. 5, the inhibition of DNA polymerase I activity was reversed by increasing amount of DNA. The LINEWEAVER-BURK's plot indicated that the reversion by DNA of neothramycin effect seemed to be competitive. On the contrary, the inhibition degree by the antibiotic of DNA polymerase I reaction did not significantly change at various concentrations of the enzyme (data are not shown). The results suggested that neothramycin directly interacted with DNA, resulting in the inhibition of DNA and RNA polymerase reactions.

The inhibition degree of RNA and DNA polymerase reactions depended upon the period of incubating the antibiotic with template DNA, prior to the addition of the other reaction reagents. It increased gradually until the preincubation time reached approximately 60 minutes in each reaction system. The results with RNA polymerase reaction at the antibiotic concentration of 50 μg/ml are illustrated in Fig. 6. It suggested that the binding of neothramycin to DNA needed a certain period, provided that the inhibition of polymerase reactions was caused by the interaction with DNA.

Discussion

Neothramycin exhibits stronger growth inhibition of L5178Y and HeLa cells than that of bacteria. The difference in degree of inhibition may be due to permeability barrier, metabolic system, and/or status of chemoreceptor.

The results, observed in the present experiments, suggest that the chemoreceptor of neothramycin may be DNA. It has been further confirmed by investigations on the interaction of DNA and the antibiotic, using circular dichroism and absorption spectra, thermal denaturation, and binding of [^{14}C]neothramycin to DNA. The results will be reported in the following paper.

The apparent inhibition by neothramycin of [^{3}H]thymine uptake in the bacterial cells may be mainly due to degradation of DNA caused by the antibiotic. Degradation of DNA is not so marked in the mammalian cells. However, the discrepancy in DNA degradation in the prokaryote and eukaryote, and the precise mechanism of DNA degradation remain to be determined.

Neothramycin blocks RNA synthesis more markedly than DNA synthesis in both mammalian and bacterial cells. The preferential inhibition of RNA to DNA synthesis has been also demonstrated in polymerase reactions with toluene-treated cells of *E. coli* (polA$^-$) and with isolated enzymes. It is generally accepted that DNA polymerase I is involved mainly in the repair system and toluenized cells of the DNA polymerase I-deficient mutant contain replication enzymes. Both *in vitro* systems of DNA synthesis are more resistant to the antibiotic than RNA polymerase reaction. The difference of inhibition degrees of DNA and RNA polymerase reactions may be attributed to the mode of binding of neothramycin with DNA. However, the precise mechanism of interaction with DNA remains to be determined.

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