LIST OF PUBLICATIONS

1. "Mitochondrial DNA synthesis : Effect of nitrofurantoin on the process", Dipak K. Dube, Swati Palit and Purabi Sinha. (Abstract) Proceedings of 47th Ann. meeting of Society of Biological Chemists (India, p. 73, 1978.

2. "Effects of nitrosoguanidine on *in vitro* mitochondrial DNA synthesis", Purabi Sinha, D.K. Dube, R.K. Chaudhuri. Presented at the Symposium on Chromosome Research, Calcutta. (Abs. No.25), Feb, 1979.

3. "Differential effects of aminoglycosidae antibiotics on *in vitro* DNA synthesis", Swati Palit, Purabi Sinha, Gobinda Sarkar, Dipak K. Dube. Curr. Sci., 48 (21), 936, 1979.

4. "Inhibition of plant mitochondrial protein and ribonucleic acid synthesis by N-methyl-N'-nitro-N-nitrosoguanidine", P. Sinha, S. Palit, G. Sarkar and D.K. Dube, Ind. J. Exp. Biol. (~~Communicated~~). January '81 issue Vol 19, No.1, 73-76, 1981

5. "Biochemical studies on Environmental Catcinogens : A general survey of the effects of N-nitrosocompounds on mitochondrial protein and ribonucleic acid synthesis", P. Sinha, S. Palit, G. Sarkar and D.K. Dube, Ind. J. Environ. Health (Communicated).

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DIFFERENTIAL EFFECTS OF AMINOGLYCOSIDE ANTIBIOTICS ON THE *IN VITRO* DNA SYNTHESIS

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ABSTRACT

Neomycin, Streptomycin and Kanamycin, the three well-known aminoglycoside antibiotics have been found to inhibit the *in vitro* DNA dependent DNA synthesis by the purified *E. coli* DNA polymerase I. Of the three, Neomycin is the most potent inhibitor for DNA polymerase activity. These three antibiotics also show similar differential effects on terminal deoxynucleotidyl transferase, a DNA polymerizing enzyme which requires only an oligonucleotide primer for non-specific DNA chain elongation. This pronounced inhibitory effect of Neomycin may be due to its stronger binding affinity towards DNA.

INTRODUCTION

HE aminoglycoside antibiotics have a broad antibacterial spectrum, including many gram-positive well as most gram-negative organisms. The antiotics streptomycin, neomycin, kanamycin produced Streptomyces have been shown to cause misreading protein synthesis both *in vivo* and *in vitro*[1, 2]. It is w well established that different aminoglycoside antiotics induce different specific type of ambiguity[1]. gain, these antibiotics have a very strong affinity for NA[3]. McCarthy and Holland first pointed out that presence of certain aminoglycoside antibiotics, natured DNA stimulated the incorporation of aminoids by the bacterial cell-free protein synthesizing stem[4]. Among them, neomycin was found to be ost effective which was subsequently confirmed by organ *et al.*[5] In this communication we now report at the aminoglycoside antibiotics inhibit the *in vitro* NA synthesis by the homogeneous *E. coli* DNA olymerase I and also by the terminal deoxyriboucleotidyl transferase (DNA deoxynucleotidyl exoansferase, EC. 2·7·7·31) from calf-thymus. Amongst e three aminoglycoside antibiotics tested, *viz.*, streptoycin, neomycin and kanamycin, neomycin has been und to be the most potent inhibitor for *in vitro* DNA nthesis.

MATERIALS AND METHODS

Radioactive deoxynucleotide triphosphates were purased from New England Nuclear Corporation or marsham Inc., unlabeled deoxynucleoside triphosphates were obtained from Calbiochem and oligo G_{12-18} from P-L Biochemicals. *E. coli* DNA polyerase I is a kind gift from Dr. L. A. Loeb, University f Washington, Seattle, U.S.A. *E. coli* DNA polyerase I was purified to homogeneity according to ie procedure of Jovin *et al.*[6] or by the modified method Springgate *et al.*[7] Poly *d*(A-T) was prepared by the *de novo* reaction using *E. coli* DNA polymerase I as described elsewhere[8]. Calf-thymus terminal deoxynucleotidyl transferase was obtained from S. Salser (University of California, Los Angeles). Maximally activated calf-thymus DNA was prepared by digestion with pancreatic DNase according to the method of Lobe[9].

Kanamycin sulfate was obtained from Sigma Chemicals Company, U.S.A. and also a kind gift from Prof. S. K. Majumder, Jadavpur University. Neomycin sulfate was purchased from Mayfair and Croydon, England. This was supplied also by Dr. S. N. Sinha, IJIRA, Calcutta. Streptomycin sulfate was purchased from Merck-Sharp and Dohme, India.

DNA Polymerase Assay

In vitro DNA synthesis using "activated" calf-thymus DNA as the template was measured in a reaction (total volume 0·05 ml) which contained 100 mM *Tris*-HCl (pH 7·4), 20 μM dATP, dGTP and dCTP, 20 μM [^{3}H]-dATP (Sp activity is given in the legends of Tables and Figures) 10 μg "activated" calf-thymus DNA, 10 mM $MgCl_2$ and 8 nM *E. coli* DNA polymerase I. Assays were incubated for 30 min at 37° C. Incorporation of the radioactive deoxynucleotide into an acid-insoluble precipitate was determined in Liquid Scintillation Spectrometer as described by Dube and Loeb[10].

Poly d(A-T) directed DNA synthesis

In vitro DNA synthesis using poly *d*(A-T) as the template was measured in a reaction (total volume 0·05 ml) which contained 100 mM *Tris*-HCl (pH 7·4), 20 μM *dTTP*; 20 μM [^{3}H] dATP (400-500 cpm/p mole); 1 μg poly *d*(A-T) ; 8 nM *E. coli* DNA polymerase I and 1 mM $MgCl_2$. The assays were incubated for 15 min at 37° C. Termination of reaction and determination of radioactivity into acid-insoluble material were the same as stated above.

Assay of 3′–5′ exonuclease activity[11]

Exonuclease activity of *E. coli* polymerase I was determined by measuring the rate of radioactivity rendered acid-insoluble using heat denatured "activated" calf-thymus DNA labeled with *d*[(^{3}H) T] at the 3′-primer terminus. The incubation mixture (total volume 0·025 ml) contained 50 mM *Tris*-HCl (pH 7·4), 1 mM $MgCl_2$, 10 mM KCl and heat denatured radioactive DNA as the substrate. Incubation was carried out for different time periods as indicated in the figure[2] at 37° C. The reaction was stopped by the addition of 0·3 ml of 1·0 N perchloric acid and 100 μg of heat denatured calf-thymus DNA (1·0 mg/ml). The solution was mixed thoroughly, allowed to stand for 30 min at 0° C, and then centrifuged at 5000 × g for 20 min. The supernatant from each tube (100 μl) was transferred to a scintillation vial; 5 ml aquasol were added and the acid-soluble radioactivity was determined.

Terminal transferase assay

Terminal deoxynucleotidyl transferase[12,14] was assayed in reaction mixtures (total volume of 0·05 ml) containing the following: 50 mM *Tris*-HCl buffer (pH 7·4); 50 mM KCl; 0·1 mM $MnCl_2$; 0·1 μg of oligo dG_{12-18}; 20 μM [^{3}H]-dATP (1250–1400 p mole); 5 mM dithiothreitol and 0·1 unit of highly purified calf-thymus deoxynucleotidyl terminal transferase (6000 units/mg). Reactions were performed in duplicate for 60 min at 37° C and acid-insoluble radioactivity was determined as described by Dube and Loeb[10].

Results and Discussion

It is borne out from the data given in Table I that the *in vitro* DNA synthesis catalysed by homogeneous *E. coli* DNA polymerase I is inhibited by some aminoglycoside antibiotics, *viz.*, streptomycin, kanamycin and neomycin of which neomycin is the most potent. At only 2 mM concentration in the assay system neomycin inhibits 83% of the incorporation of [^{3}H]-dATP into acid-insoluble material. Inhibition by kanamycin or streptomycin on the other hand is only 55% at a concentration 5 times higher.

The inhibition of DNA synthesis by neomycin is released with increasing concentration of template DNA (Fig. 1). This release of inhibition indicates that neomycin acts either by binding with the template DNA (Lazarus and Kitron[13]) or by competing with template DNA for the same site of the enzyme. However the increase in the amount of enzyme does not show any reduction of inhibition (data not given). So the binding of the antibiotic with enzyme protein is not the cause of the inhibitory effect. Increase in the concentration of deoxynucleoside triphosphates in the assay system (from 20 to 100 μM) does not reduce the extent of inhibition significantly (data not

Table I

Effects of different aminoglycoside antibiotics on in vi *DNA synthesis by* E. coli *DNA Polymerase I*

Additions	Incorporation (*p* miles/Incubation system)		% Acti
None	47	(28, 308 cpm)[a]	100
+ Neomycin (2 mM)	8	(4670 cpm)	17
+ Neomycin (5 mM)	6	(3724 cpm)	13
+ Neomycin (10 mM)	2	(975 cpm)	4·2
Streptomycin (2 mM)	46	(27,764 cpm)	98
Streptomycin (5 mM)	22·4	(13,571 cpm)	48
Streptomycin (10 mM)	21·6	(13,122 cpm)	45
None	40	(17,354 cpm)[b]	100
Kanamycin (2 mM)	28	(12, 102 cpm)	70
Kanamycin (5 mM)	25	(11, 024 cpm)	62·2
Kanamycin (10 mM)	18	(7, 739 cpm)	45

Complete assay system is as indicated in the mate and method section.

[a] Specific activity of [^{3}H]-dATP is 606 cpm/p m

[b] Specific activity of [^{3}H] dATP is 435 cpm/p mo

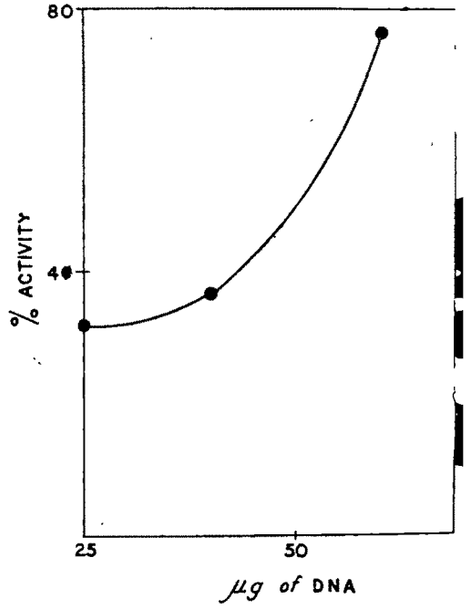

Fig. 1. Effect of increased amount of activa DNA on activity of DNA Polymerase I in prese of 1 mM neomycin. The complete assay system is discussed in the material and method section. ; activity of [^{3}H]-dATP is 400–600 cpm/p mole.

given). So the inhibition of DNA synthesis by neomycin does not involve the competition of the antibiotic with deoxynucleoside triphosphates for a common binding site on the enzyme. Neomycin mediated inhibition of *in vitro* DNA synthesis by Pol I may be the result of chelation of the essential bivalent metal cation by the antibiotic. This possibility can be ruled out as the extent of inhibition in poly d (A–T) directed DNA synthesis by Pol I with neomycin (1 mM) is independent of Mg^{2+} concentration (data not given). Neomycin, not only inhibits the polymerase activity of Pol I but has also been found to inhibit the associated $3' \rightarrow 5'$ exonuclease activity ("proof-reading" activity) of the enzyme (probably by interaction with single-stranded DNA used as the substrate) (Fig. 2).

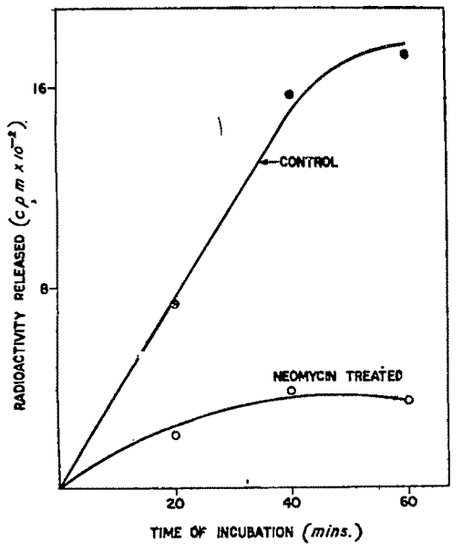

FIG. 2. Inhibition of DNA Polymerase I associated $3' \rightarrow 5'$ exonuclease activity in presence of 1 mM neomycin. Complete assay systems are as described in material and method section.

The idea that neomycin alter the specificity of DNA in various enzymatic reactions is further substantiated by the fact that neomycin also inhibits the terminal transferase activity *in vitro*. This DNA polymerizing enzyme requires only single-stranded DNA or oligonucleotide as the primer[12]. The data given in Table II again show the differential effects of different aminoglycoside antibiotics on the terminal transferase activity. Neomycin at 5 mM concentration inhibits 80% of the enzyme activity. On the contrary, only 60% inhibition was observed either with streptomycin or kanamycin even at greater concentration (10 mM).

TABLE II

Effect of different aminoglycoside antibiotics on polynucleotide synthesis by terminal deoxynucleotidyl transferase from calf-thymus

Addition	Incorporation (*p* moles/incubation system)	% Activity
None	23·5 (29, 856 cpm)[1]	100
Neomycin (2 mM)	15 (18, 864 cpm)	63·8
Neomycin (5 mM)	4 (4, 668 cpm)	17
Neomycin (10 mM)	0·85 (1084 cpm)	3·6
None	9 (11318 cpm)[2]	100
Streptomycin (2 mM)	5 (6310 cpm)	56
Streptomycin (5 mM)	4 (5250 cpm)	45
Streptomycin (10 mM)	3 (3802 cpm)	33
None	26 (35, 204 cpm)[3]	100
Kanamycin (2 mM)	16 (21, 678 cpm)	61·5
Kanamycin (5 mM)	14·5 (19838 cpm)	55·8
Kanamycin (10 mM)	11·5 (15, 675 cpm)	44

Complete assay system is as indicated in the material and method section.

[1] Specific activity of [^{3}H]-dATP is 1267 cpm/p mol.
[2] Specific activity of [^{3}H]-dATP is 1258 cpm/p mol.
[3] Specific activity of [^{3}H]-dATP is 1367 cpm/p mol.

From the present investigations it may be concluded that amongst the three aminoglycoside antibiotics, neomycin is the most potent inhibitor for DNA polymerizing enzymes. The marked difference in the inhibitory effect of the different aminoglycoside antibiotics may reflect the stronger binding affinity of neomycin with DNA.

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