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30S Ribosomal Subunit Assembly is a Target for Inhibition by Aminoglycoside Antibiotics in *Escherichia coli*

A thesis
presented to
the faculty of the Department of Biochemistry
East Tennessee State University

In partial fulfillment
of the requirements for the degree
Master of Science in Biomedical Science

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by
Roopal Mehta
May 2002

Dr. W. Scott Champney, Chair
Dr. Mitchell Robinson
Dr. John Laffan

Keywords: 30S Ribosomal Subunit, Paromomycin, Neomycin, Protein Synthesis, *E. coli*

ABSTRACT

30S Ribosomal Subunit Assembly is a Target for Inhibition by Aminoglycoside Antibiotics in *Escherichia coli*

by

Roopal Mehta

Antibacterial agents specific for the 50S ribosomal subunit not only inhibit translation but also prevent assembly of that subunit. I examined the 30S ribosomal subunit in growing *Escherichia coli* cells to see if antibiotics specific for that subunit also had a second inhibitory effect. I used the aminoglycoside antibiotics paromomycin and neomycin, which bind specifically to the 30S ribosomal subunit. Both antibiotics inhibited the growth rate, viable cell number, and protein synthesis. I used a ^{3}H-uridine pulse and chase assay to examine the kinetics of ribosome subunit assembly in the presence and absence of each antibiotic. Analysis revealed a concentration dependent inhibition of 30S subunit formation in the presence of each antibiotic. Sucrose gradient profiles of cell lysates showed the accumulation of an intermediate 21S translational particle. Taken together this data gives the first demonstration that 30S ribosomal subunit inhibitors can also prevent assembly of the small subunit.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. W. Scott Champney, and my committee members, Dr. Mitchell Robinson and Dr. John Laffan, for their guidance during the past couple of years. Their advice and expertise offered has made this a worthwhile educational adventure. I also wish to thank Beverly Sherwood. Your unconditional friendship and ample supply of chocolate made coming into the lab every day a joy and a pleasure.

I would also like to thank my loving family. Dad, Mom, and Rajiv, you have continuously supported me even when you didn't know what I was really doing. While at times I failed to show my appreciation, I never forgot those who were standing in my corner—I love you. Finally, a special note to David B. Marlow: over the past two years, you have watched me scale every emotional tower regarding school and life, your strength and support have been a positive driving force in my life. I don't believe I can ever put into words how much your friendship has meant, the only thing I can say is THANK YOU!

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ABBREVIATIONS

C-6’	Carbon at the 6 position of molecule
21S	Particle with sedimentation coefficient of 21S
30S	Small subunit of ribosome
50S	Large subunit of ribosome
aa-tRNA	Amino acyl- transfer RNA
CPM	Counts per minute
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
IC_{50}	50% inhibitory concentration
MIC	Minimal inhibitory concentration
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
S-buffer	Subunit buffer
tRNA	Transfer RNA
TSB	Tryptic soy broth
VCC	Viable cell count

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CHAPTER 1

INTRODUCTION

Antibiotic chemotherapy is an important defense against infectious agents that target the human body. However, many infectious microorganisms have acquired resistance to clinically used antibiotics and now pose a major health threat (Amabile-Cuevas et al.1995; Gold and Moellering 1996). For this reason, it is increasingly important to identify both new drugs and also new cellular targets for anti-microbial agents (Chu et al. 1996).

Paromomycin and neomycin are aminoglycoside antibiotics that share many structural and functional properties (Kuryloowicz 1976). Both antibiotics are effective against a number of aerobic and facultative anaerobic gram-negative bacilli as well as gram positive *Staphylococci* (Vazquez 1979; Mingeot-Leclercq et al. 1999). Chemically, paromomycin and neomycin are four membered ring compounds consisting of a 2-deoxystreptamine ring linked to several amino sugars and hydroxyl- functional groups (Kotra et al. 2000) (Figure 1). Although the basic structure of the two antibiotics is similar, the two compounds differ in the functional group attached at carbon-6 on ring I (Kotra et al. 2000). Paromomycin possesses a hydroxyl group at the C-6' position while neomycin possesses an amino group (see Figure 1). The polarity and hydrophilic properties of their structure enable them to penetrate the cell's outer membrane and function as effective anti-microbial agents (Mingiot-Leclercq et al.1999).

Paromomycin and neomycin specifically bind to the 30S ribosomal subunit (Vazquez 1979). The high positive charge of each antibiotic gives them a high affinity for the negative charge of the rRNA within the cell (Mingeot-Leclercq et al.1999; Fourmy et al.1999). The antibiotics interact with the 16S rRNA of the 30S subunit causing a conformational change, which results in impaired proofreading, translocation (Moazed and Noller 1987; Recht et al.1999; Schroeder et al. 2000; Ogle et al. 2001) and subunit assembly.

Paromomycin I
R1 = CH2NH2,
R2 = H

Paromomycin II
R1 = H
R2 = CH2NH2

Neomycin
R1 = H
R2 = CH2NH2

Figure 1. Structures of Aminoglycoside Antibiotics Paromomycin and Neomycin.

Paromomycin and neomycin bind the A-site of the decoding region of the 30S ribosomal subunit (Ogle et al. 2001). The decoding site is the region of the ribosome that discriminates between the cognate and non-cognate tRNAs (Recht et al. 1999). Under standard conditions, there exists a codon/ anti-codon recognition step that facilitates discrimination between cognate, near-cognate and non-cognate aa-tRNA. This discrimination enables correct addition of amino acids and, therefore, a correct amino acid sequence (Ogle et al. 2001). The non-cognate and most near-cognate tRNAs are rejected by the ribosome during the proofreading and selection step. However, the accuracy of proofreading and selection is altered once paromomycin or neomycin binds to the A-site of the decoding. Binding to the decoding region results in a conformational change of the conserved bases in the loop of the A-site. This change facilitates high affinity binding between the 16S rRNA and the internal loop and rings I and II of the aminoglycoside antibiotics (Fourmy et al. 1996; Fourmy et al. 1998; Schroeder et al. 2000; Ogle et al. 2001). The tightly bound antibiotic contributes to codon misreading and mistranslation of mRNA.

Previous work with a number of structurally different inhibitors of 50S subunit function has demonstrated that these antibiotics also inhibit 50S particle formation (reviewed in Champney (Champney 2001)). These antibiotics halt 50S subunit assembly and cause accumulation of a precursor particle, which later becomes degraded by cellular ribonucleases (Usary and Champney 2001). Inhibition of assembly is equivalent to inhibition of translation for most of these drugs (Champney and Burdine 1996; Champney and Burdine 1998; Champney and Tober 2001). The present investigation examines the effect of paromomycin and neomycin on growing *Escherichia coli* cells. The results indicate that these aminoglycoside antibiotics also have 2 inhibitory effects, preventing protein synthesis and preventing 30S particle assembly in cells.

CHAPTER 2

MATERIALS AND METHODS

Materials

Escherichia coli strain SK901 has been described previously (Champney 1979). Paromomycin sulfate and neomycin sulfate were purchased from Sigma Chemical Corporation. Stock solutions of the antibiotics paromomycin and neomycin were made at 10 mg/ml in de-ionized sterile water. Lysozyme and RNAse free-DNAse were purchased from Sigma Chemical Corporation. Tryptic soy broth (TSB), sucrose, and ScintiSafe Gel, scintillation fluid were purchased from Fisher. Subunit buffer was made using 10 mM TRIS-HCl (pH 8.0), 50 mM NH_4Cl and 0.5 mM Mg Acetate. A 5X solution of A-salts was made using 52.5g K_2HPO_2, 22.5g KH_2PO_4, 5.0g $(NH_4)_2SO_4$, 2.5g Na citrate and 1ml 20% $MgSO_4$(Miller 1972). ^{3}H-uridine (44.50 Ci/ mmol) and ^{35}S-methionine and cysteine (TRANS^{35}S-LABEL 1175 Ci/ mmol) was purchased from ICN Pharmaceuticals.

All calculations regarding gradient profile, subunit assembly, protein synthesis, and minimal inhibitory concentration curves were performed using computer software package, Cricket Graph III. Structures were drawn using the computer software package CS ChemDraw Net.

Measurements of Cell Growth

Cell cultures were grown at either 37^0 C or 27^0 C in TSB in the presence and absence of each antibiotic after the method of Champney and Burdine 1998. Growth was initiated by adding 8.0 X 10^7 cells/ml to TSB growth media. Growth rates were measured by following the increase in cell density in a Klett-Summerson colorimeter. Cells were monitored for at least 2 doublings. A Klett of 20 corresponds to 1.6 X 10^8 cells/ ml and a Klett of 80 equals 5.4 X 10^8

cells/ ml. The growth rate of the cells was based upon the time required for 1 cell doubling in the presence or absence of an antibiotic treatment.

Measurements of Ribosomal Subunit Assembly To examine ribosomal subunit assembly, cell culture growth was initiated by adding cells to a start flask containing 30 ml of TSB. Cell density was monitored from a Klett of 10 to a Klett of 20. At a Klett of 20, the start flask was split into 6- 5 ml flasks, using sterile techniques. Once separated, 1 flask received no antibiotic while the other 5 flasks received different concentrations of 1 antibiotic. Thirty-minutes after the cell culture was split and treated, cells were labeled with ^{3}H-uridine (1 μCi/ ml; 1 μg/ ml) and allowed to grow for 2 doublings each in the presence or absence of the drug. Isotope incorporation was halted by adding an excess of cold uridine to a final concentration of 50 μg/ ml followed by a thirty-minute chase period. After the thirty-minute chase, cells were collected in 15 ml Fisher tubes and centrifuged in a Beckman low speed centrifuge (J2-21) at 6,000 rpm for 15 min in a JA21 rotor. Once pelleted, the radioactive media was discarded into isotope waste. The pelleted cells were re-suspended and washed with sterile S-Buffer. Once re-suspended, the tubes were centrifuged at 6,000 rpm for 15 min. The excess buffer was discarded and the cell pellets were stored in the -70^0C freezer.

In order to determine subunit assembly, cells were lysed and centrifuged in a linear sucrose gradient. Harvested cell cultures were removed from the deep freezer and allowed for thaw on ice. Once the cells were completely thawed, 200 μl of S-buffer was added to each cell pellet and mixed using a vortex mixer. After mixing, 5 μl of a 10mg/ml solution of lysozyme was added to each culture. Each tube was then hand mixed and warmed at room temperature. After a 15-minute incubation period, the cell culture began to get thicker and more opaque indicating that the cells had begun to lyse. Once it was apparent that the cells had lysed, the cells underwent a freeze-thaw process 3 times. During this processes, the cells were taken to the -70^0C freezer and frozen for 5 min and then removed and thawed at room temperature. This procedure ensured that the cells had indeed lysed. Once the lysis process was complete, 1-2

units of RNase free-DNase I was added to each culture. Once the DNA was degraded, the tubes were centrifuged at 6,000 rpm for 15 min as before.

The supernatant from the low speed centrifugation was loaded onto a 5-20 % linear sucrose gradient in S-buffer. The gradients were then loaded into a SW40 rotor and centrifuged in a Beckman LE80K Ultracentrifuge at 39,000 rpm for 4.25 hours or at 20,000 rpm for 16 hrs. For some experiments, centrifugation times were increased to 5.25 hours (39,000 rpm) or to 16 hrs (23,000 rpm) to better observe the 30S precursor region of the gradients. Gradients were pumped through an ISCO gradient fraction and the absorbance at 254 nm for each gradient was detected with an ISCO Model UA-5 absorbance monitor. Gradient fractions were mixed with 3 ml of ScintiSafe Gel and the incorporation of ^{3}H-uridine into RNA was measured by liquid scintillation counting (Beckman LS 3801).

Measurements of Translation Rates The rate of protein synthesis was determined by treating the cells using the method described above. Cells were grown for 2 doublings in the presence or absence of the antibiotic. At a Klett reading of 75, ^{35}S-methionine and cysteine (TRAN3^{35}S-LABEL) was added to each culture to a final concentration of 1 μCi/ ml. After adding the label, 3 samples of 0.2 ml were collected at 5 min intervals and precipitated with 10% TCA at room temperature. The TCA precepitible material was collected on glass fiber filters (Whatman GF/A) and measured by liquid scintillation counting.

Measurements of Viable Cells To measure the number of viable cells, cells were grown for 2 doublings in the presence or absence of the drug as described previously. Once the cells reached a Klett value of 80, 10 μl of sample was removed and added to 1 ml of A-salts (Miller 1972). A serial dilution was performed to achieve a final dilution of 10^{-4} and 10^{-5} in A-salts (Miller 1972). Once the dilution procedure was complete, 10 μl of the 10^{-4} and 10^{-5} dilution was

plated on square TSB agar plates after the procedure of (Jett et al.1997). Colonies were counted after 24 hr incubation at 37^{0}C.

Uridine Pulse and Chase Labeling To examine ribosome subunit assembly kinetics, 12 ml of TSB media were inoculated as described above. Each tube either received no antibiotic, paromomycin at a final concentration of 2.5µg/ ml or neomycin at a final concentration of 3.0µg/ ml. The culture was grown to a Klett of 40. At this cell density, the cells were pulse labeled with ^{3}H-uridine (1µCi/ ml) for 90 sec and then chased with uridine at 25µg/ ml. At 5, 10, 15, 20, 30, and 60-minute intervals, samples of 2 ml were removed, collected in 15ml Corex tubes and centrifuged in Beckman low speed centrifuge at 6,000 rpm for 15 min. Once pelleted, the radioactive media was discarded into isotope waste. The pelleted cells were re-suspended and washed with sterile S-Buffer. Once re-suspended, the tubes were centrifuged at 6,000 rpm for 15 min. The excess buffer was discarded and the cells were stored in the –70^{0}C freezer.

Subunit assembly was examined using the procedure descibed above.

MIC Determination The minimal inhibitory concentration (MIC) for paromomycin and neomycin was determined by a dilution method as described (Champney and Burdine 1998). Six tubes each containing 1 ml of TSB received 25 µl of an overnight culture of *E. coli* cells and one antibiotic over a concentration range. The tubes were incubated at 37^{0}C for 24 hr and the absorbance at 600 nm was read in an Eppendorf Biophotometer and recorded.

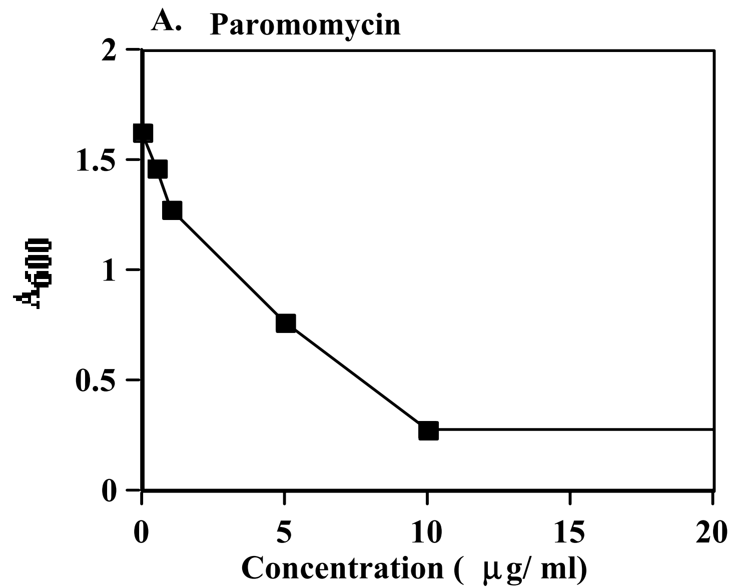
CHAPTER 3

RESULTS

Paromomycin and neomycin are structurally similar aminoglycoside antibiotics. Each compound consists of a 2-deoxystreptamine ring linked to several amino sugars with hydroxyl functional groups (Vazquez 1979). Paromomycin and neomycin differ in chemical structure on the functional group attached to the C'6 of ring 1. Paromomycin has a hydroxyl group at this position while neomycin possesses and amino group. The chemical structure of each antibiotic is shown in figure 1.

The MIC for each antibiotic in *E. coli* cells growing in TSB was determined by measuring the absorbance of light, which passes through each 1 ml sample, depending on cell density. Once each absorbance value was measured and recorded, the values were plotted against the respective concentration value. The resulting graph demonstrates a relationship between increasing concentration of antibiotic and decreasing number of cells (Figure 2). The MIC value is the value that represents the minimum concentration of antibiotic, which will affect the growth of the cells. Paromomycin had an MIC of 10μg/ ml and neomycin had an MIC of 15 μg/ml.

After establishing the inhibitory concentration of antibiotic, I used sub-inhibitory concentrations of antibiotic in growing *E. coli* cells to investigate their effects on cell growth and protein synthesis. Each antibiotic decreased the growth rate and reduced the cell viability in a similar way. Figure 3A and 3B show the concentration dependent inhibition of the growth rate and viable cell number by each drug. The IC_{50} values for paromomycin and neomycin inhibition are listed in Table 1. Both antibiotics had similar 50% inhibitory concentration values for effects on growth rate and cell viability.



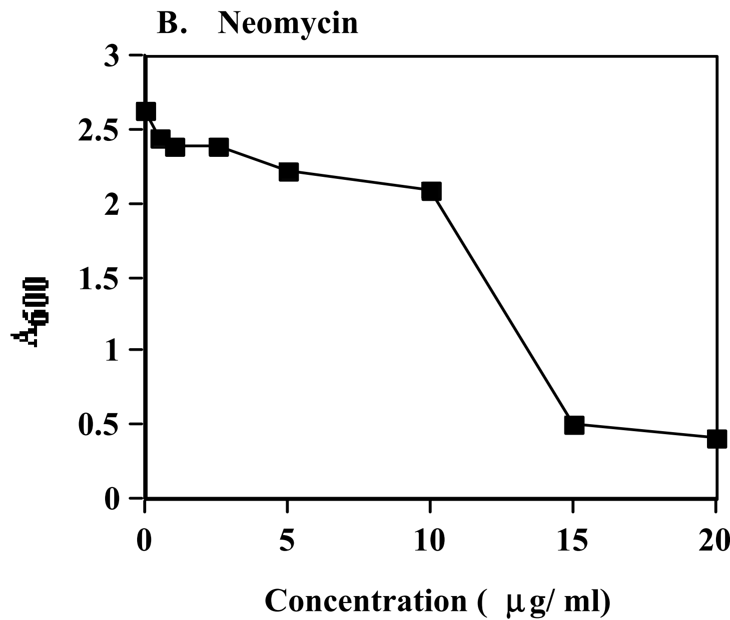
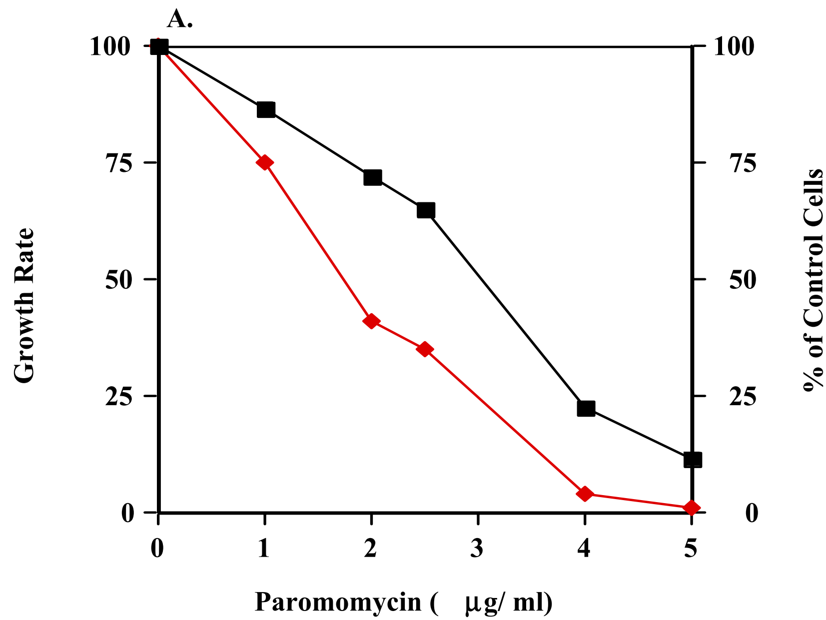


Figure 2. Minimal Inhibition Concentration Assay for the Antibiotic Paromomycin and Neomycin.

Increasing concentrations of antibiotic were added to 25 μl of *E. coli* cells. The cells were grown overnight at 37^0C. The amount of growth was measured in a spectrophotometer at a wavelength 600 nm.



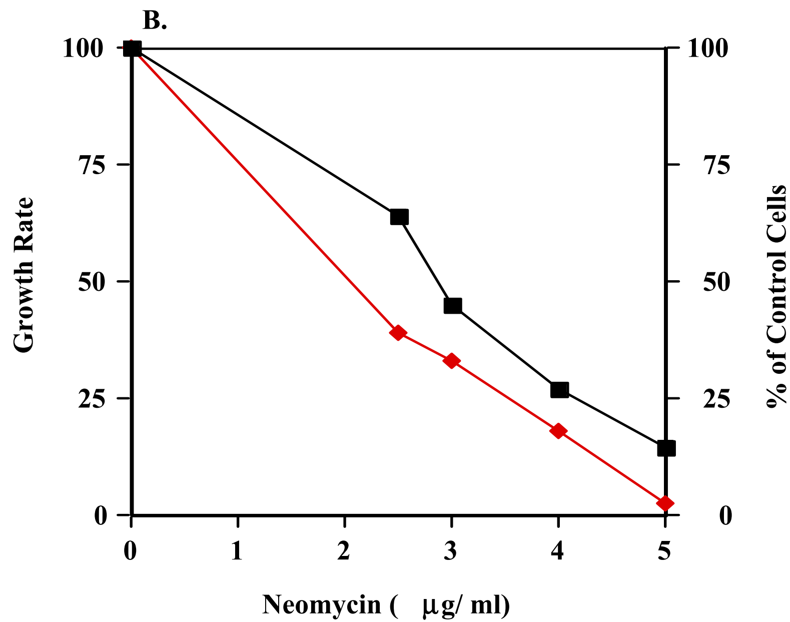


Fig. 3. Inhibition of Growth Rate and Viable Cell Number by Increasing Concentrations of Paromomycin and Neomycin in Cells Growing at 37^0C.

(A). Inhibitory effect of paromomycin on growth rate (***black***) and cell number (***red***). (B). Inhibitory effect of neomycin on growth rate (***black***) and cell number (***red***). Results are the means of duplicate experiments within a S.E. of +/- 13%.

Table 1. IC_{50} values (μg/ ml) for paromomycin and neomycin inhibition of growth rate, viable cell number, protein synthesis rate and 30S subunit assembly.

Table 1.

50% Inhibition Concentration (IC_{50})

	Paromomycin (μg/ml)	Neomycin (μg/ml)
Viable Cell Count	**1.6**	**1.8**
Growth Rate	**3.2**	**3.6**
Protein Synthesis	**3.2**	**3.6**
Ribosomal Assembly	**6.4**	**7.2**

The rate of translation by growing *E. coli* cells was examined by measuring the incorporation of ^{35}S amino acids into cellular proteins. Figures 4A and 4C show the effect of increasing concentrations of each drug on the rate of protein synthesis. As the concentration of antibiotic increases, the amount of protein synthesized decreases respectively. The relationship between increasing drug concentrations and protein synthesis inhibition is displayed in Figures 4B and 4D. The IC_{50} for the inhibition of protein synthesis by each drug is shown in Table 1. Paromomycin and neomycin had similar IC_{50} values (3.2 and 3.6 μg/ ml).

Previous studies had revealed that antibiotics that target the 50S ribosomal subunit for translational inhibition also prevented that subunit from being assembled (Champney, 2001). Paromomycin and neomycin were examined to see if they had a similar effect upon the 30S subunit. Ribosome subunit assembly was examined by a ^{3}H-uridine pulse and chase label procedure performed on cells growing at 27^{0}C. Ribosome assembly times are proportional to the growth rate of cells (Michaels, 1972; Schlessinger, 1974). Previous studies showed that subunit assembly kinetics can be seen most clearly in cells growing at a reduced rate at 27^{0}C (Usary and Champney, 2001). The reduced temperature increases the doubling time of cells allowing slowed subunit assembly. Figure 5 shows the sucrose gradient profiles for cells grown at 27^{0}C in the absence of drug (Figure 5A), in the presence of paromomycin (Figure 5C), or neomycin (Figure 5E). The gradients of figure 5 (A, C and E) represent a 90 second ^{3}H-uridine pulse followed by a 30-minute cold uridine chase. At this point in the chase period, there is reduction in accumulation of 30S subunit compared to the control.

Figure 5 (B, D and F) also shows the kinetics of subunit assembly for the control (Figure 5B), and for paromomycin (Figure 5D) and neomycin treated cells (Figure 5F). In the absence of the antibiotics, 30S subunit formation was completed in 15 min and 50S particle synthesis was finished in 30 min (Figure 5B). When the cells were treated with either paromomycin or

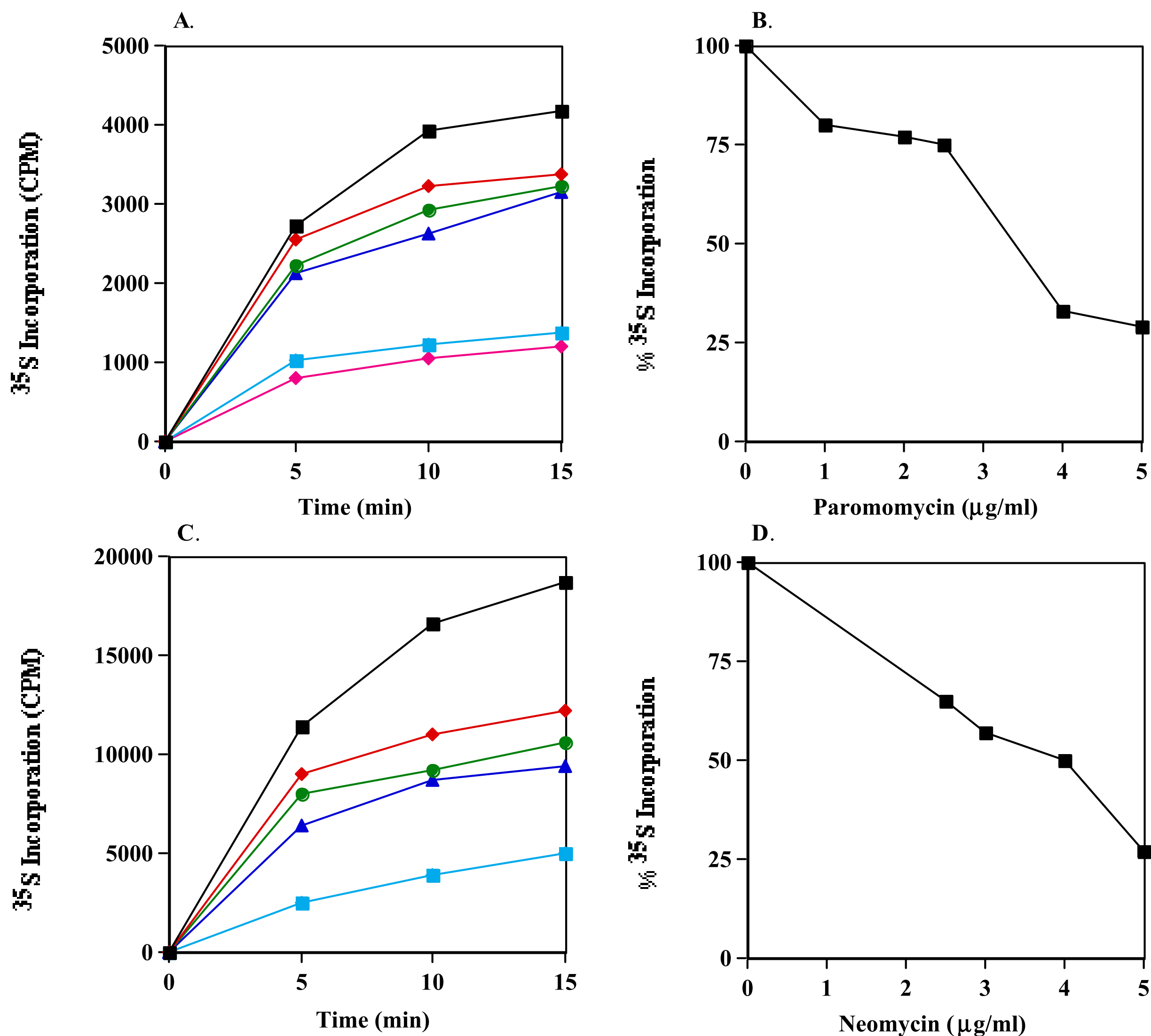
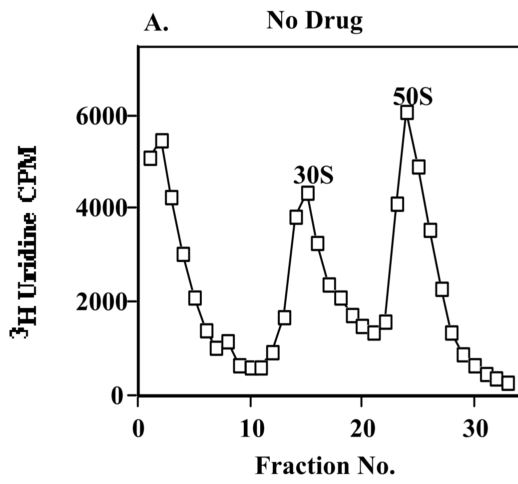
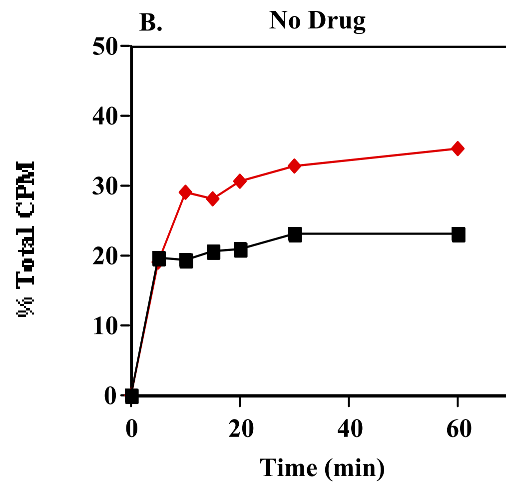
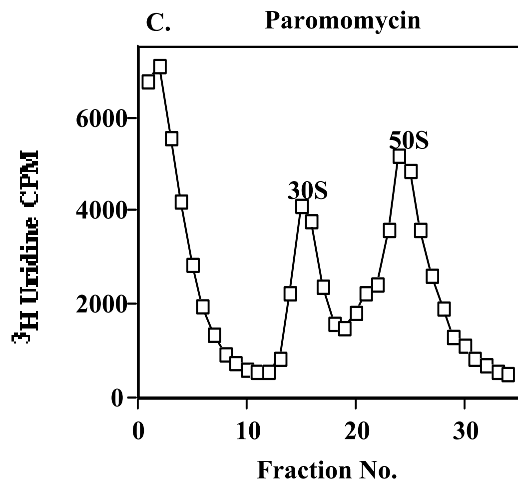


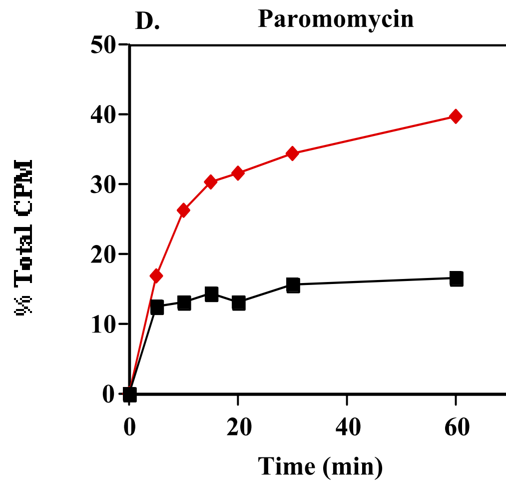
Figure 4. Inhibition of Protein Synthesis Rates by Different Concentrations of Paromomycin and Neomycin in Cells Growing at 37^{0}C

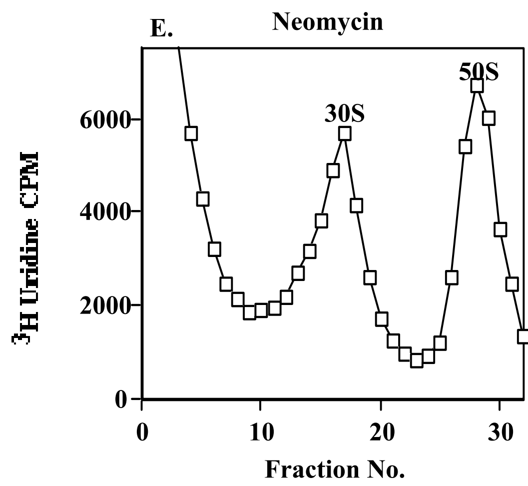
(A) Protein synthesis inhibition by paromomycin at 0 µg/ ml (***black***); 1 µg/ ml (***red***); 2 µg/ ml (***green***); 2.5 µg/ ml (***blue***); 4 µg/ ml (***cyan***) and 5 µg/ ml (***pink***). (B) Concentration dependence of paromomycin inhibition of translation. (C) Protein synthesis inhibition by neomycin at 0 µg/ml (***black***); 2.5 µg/ ml (***red***); 3 µg/ ml (***green***); 4 µg/ ml (***blue***); and 5 µg/ ml (***cyan***). (D) Concentration dependence of neomycin inhibition of translation. The standard error of the mean is within +/- 7.18, which is based upon duplicate experiments.


A.
No Drug
30S
50S
6000
4000
2000
0
0
10
20
30
^{3}H Uridine CPM
Fraction No.


B.
No Drug
50
40
30
20
10
0
0
20
40
60
% Total CPM
Time (min)


C.
Paromomycin
30S
50S
6000
4000
2000
0
0
10
20
30
^{3}H Uridine CPM
Fraction No.


D.
Paromomycin
50
40
30
20
10
0
0
20
40
60
% Total CPM
Time (min)


E.
Neomycin
30S
50S
6000
4000
2000
0
0
10
20
30
^{3}H Uridine CPM
Fraction No.

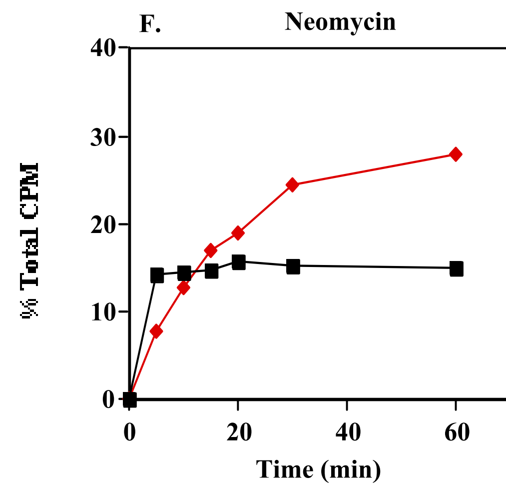

F.
Neomycin
40
30
20
10
0
0
20
40
60
% Total CPM
Time (min)

Figure 5. Sucrose Gradient Profiles and Pulse-Chase Labeling Kinetics for control and Cells Grown with Paromomycin or Neomycin at 27^0C.

Gradient profiles are from cells chased with uridine for 30 min. (A). Sucrose gradient profile of ^{3}H-uridine labeled control cells. (B). Pulse-chase labeling kinetics of 30S (***black***) and 50S (***red***) subunits in control cells. (C). Sucrose gradient profile of ^{3}H-uridine labeled cells grown with paromomycin at 2.5 µg/ ml (D). Pulse-chase labeling kinetics of 30S (***black***) and 50S (***red***) subunits in cells grown with paromomycin. (E). Sucrose gradient profile of ^{3}H-uridine labeled cells grown with neomycin at 3 µg/ ml. (F). Pulse-chase labeling kinetics of 30S (***black***) and 50S (***red***) subunits in cells grown with neomycin. Results are the means of duplicate experiments with a S.E. of +/- 5%.

neomycin, 30S subunit formation was halted at approximately 50% of the control amount, while 50S ribosomal subunit assembly was little affected.

To further examine the inhibitory effects of paromomycin and neomycin on 30S assembly, the concentration dependence of subunit formation was tested on cells growing at 37^0C. The increased temperature allows examination of subunit assembly under standard conditions. Figure 6 shows a comparison of the sucrose gradient profiles of growing *E. coli* cells in the presence of paromomycin (Figure 6A) or neomycin (Figure 6B) at 5 μg/ml. These gradient profiles represent a ^{3}H-uridine label present over two cell doublings followed by a 30 min uridine chase. Such a labeling procedure enabled examination of ribosome assembly over a greater length of time in the presence or absence of antibiotic. Centrifugation was conducted for a more extended period of time allowing cell lysates to sediment further down the sucrose gradients allowing for the detection of 30S precursor particles. The profiles show that in the presence of each antibiotic there was an accumulation of a 21S particle, suggestive of the accumulation of a precursor to the 30S subunit.

Figure 6C and 6D show the inhibitory effects of increasing concentrations of each antibiotic on 30S ribosomal subunit assembly in growing *E. coli* cells. As the drug concentration increased, there was a decrease in 30S subunit amounts and the increased accumulation of a 21S particle. This relationship exists with both paromomycin (Figure 6C) and neomycin (Figure 6D). Table 1 shows the IC_{50} values for 30S assembly inhibition for both antibiotics. A decline in 50S subunit amounts was also observed. This was most apparent at higher concentrations of neomycin.

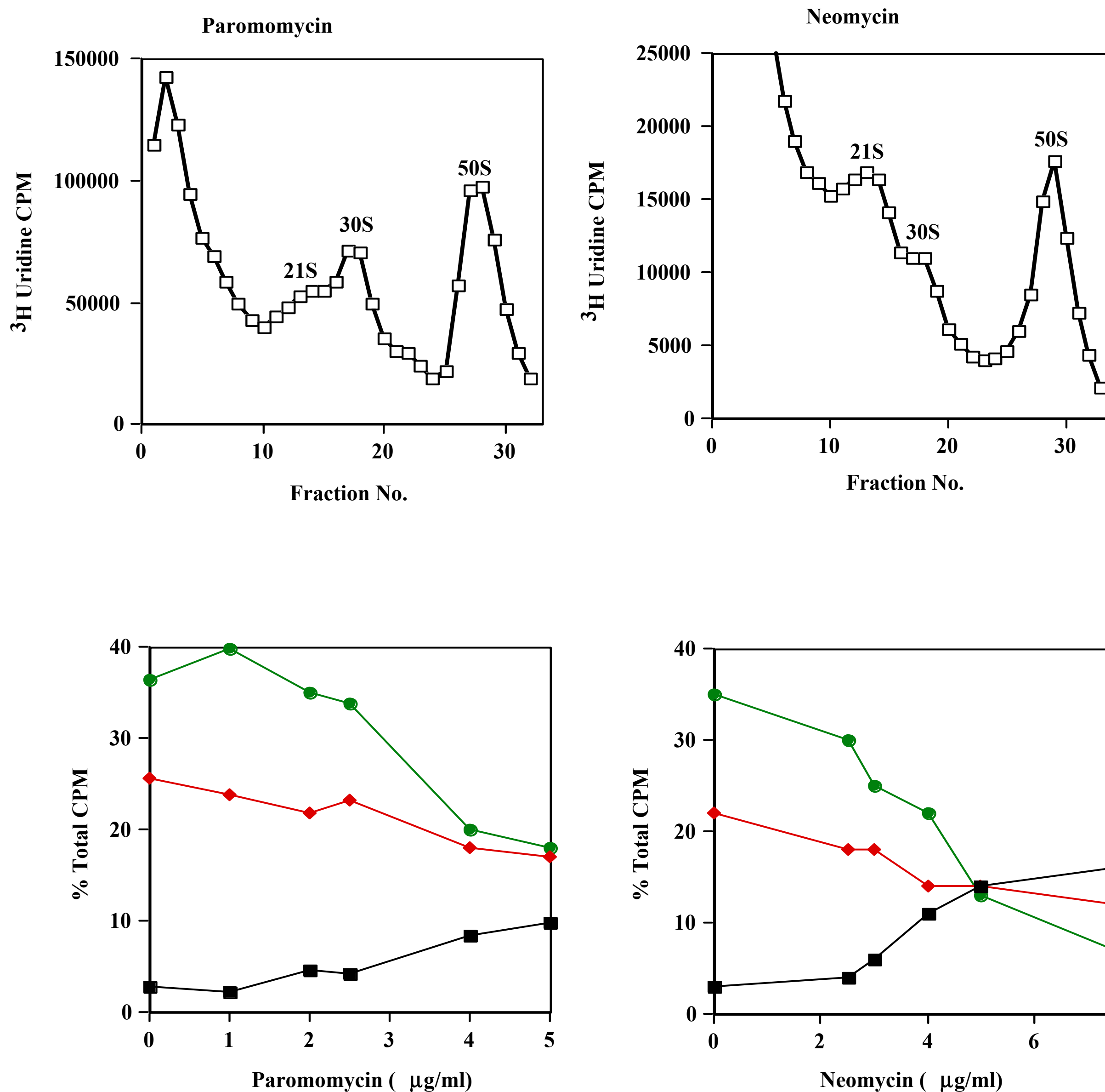


Figure 6. Concentration Dependence of Ribosomal Subunit Assembly Inhibition by Paromomycin and Neomycin in Cells Growing at 37^0C.

(A) Sucrose gradient profiles of cells grown with paromomycin at 5 µg/ ml. (B) Sucrose gradient profiles of cells grown with neomycin at 5 µg/ ml. (C) Inhibition of 30S (***red***) and 50S (***green***) subunit formation by increasing concentrations of paromomycin. Increase in 21S particle amounts (***black***). (D) Inhibition of 30S (***red***) and 50S (***green***) subunit formation by increasing

concentrations of neomycin. Increase in 21S particle amounts (***black***). Results are the means of duplicate experiments with a S.E. of +/- 3.5%.

CHAPTER 4

DISCUSSION

Previous studies have identified the formation of the 50S ribosomal subunit in bacterial cells as a novel target for inhibition by a variety of large subunit translational inhibitors (Champney 2001). These antibiotics bind to a precursor particle, which stalls the ribosome assembly sequence and permits ribonucleases to degrade the RNA in the incomplete subunit (Usary and Champney 2001). The present study suggests that two aminoglycoside antibiotics act in a similar fashion on the 30S ribosomal subunit.

Paromomycin and neomycin exhibited similar inhibitory effects within *E.coli* cells. They show equivalent IC_{50} values for inhibition of cell growth rates and cell number. These are expected results since these compounds differ in structure by only an amino (neomycin) or hydroxyl (paromomycin) group on the C'6 position of ring 1. The MIC and IC_{50} values for growth inhibition found for each compound in this study are comparable to those found by other investigators for effects in *E. coli* (Mingeot-Leclercq et al. 1999; Kotra et al. 2000)

For both antibiotics, the IC_{50} value for 30S particle formation is two-fold higher than the IC_{50} value for inhibition of translation (Table 1). When antibiotic is added at the IC_{50} values for the inhibition of translation, 50% of the cells are halted at the point of assembly due to the presence of the antibiotic. Of the remaining cells, 50% of those cells are halted in translation and are unable to make new protein for cell survival. The percentage of functional ribosomes remaining in the presence of the antibiotic is only 25% of a control cell culture. This is the expected outcome for antibiotics with equivalent inhibitory effects on the two processes. This suggests that the interactions with the target site on the precursor particle and the mature 30S subunit are similar. As documented with a number of 50S antibiotics, inhibition of translation and 50S formation are equivalent effects of these compounds (Champney and Burdine 1996; Champney and Burdine 1998; Champney and Tober 2001). Direct binding of erythromycin to a

50S precursor particle has been demonstrated (Usary and Champney 2001), binding of paromomycin and neomycin to a 30S precursor is predicted.

Inhibition of 50S particle assembly by 50S specific translational inhibitors does not affect 30S particle synthesis (Champney 2001). The opposite does not seem to be true. Paromomycin and neomycin are aminoglycoside antibiotics specific for the 30S subunit. In theory, the presence of this antibiotic should only affect the smaller ribosomal subunit. However, at higher concentrations of both paromomycin and neomycin, an inhibition of 50S particle formation was observed. This observation is likely due to a downstream effect of 30S particle assembly inhibition. Transcription of 16S rRNA precedes transcription of 23S and 5S rRNAs and concomitant 30S assembly precedes 50S particle formation (Nierhaus 1982; Nomura 1973). Stalling of 30S synthesis could have a non-specific downstream effect of slowing 50S synthesis without a direct effect of the antibiotic. Both aminoglycoside antibiotics tested bind with specificity only to the 30S particle, both in *vitro* and in *vivo*, and stimulate misreading on this subunit (Kotra et al. 2000).

Paromomycin has been documented to stimulate mistranslation by locking a particular conformation of the 30S particle so that base pair mismatches are stimulated in the presence of the antibiotic (Fourmy et al. 1996; Fourmy et al. 1998; Ogle et al. 2001). Presumably, neomycin functions in a similar fashion. This observation suggests that these antibiotics bind the 16S rRNA, limiting the number of ribosomal proteins associated with the rRNA and lock the 21S precursor particle in a non-functional conformation. This locked conformation prevents maturation of the 30S subunit and in turn prevents 50S subunit synthesis and maturation. Conformational changes and progression through precursor particles are required during particle assembly both in *vivo* and in *vitro* (Nomura, 1973) in order for cell survival. The accumulation and turnover of a stalled 30S precursor particle combined with translational inhibition could account for the bactericidal activity of these antibiotics in some cells (Vazques 1979).

In conclusion, this work represents the first demonstration of antibiotics that target the small ribosomal subunit and also function to inhibit its synthesis. These results extend observations made with 50S antibiotics to include inhibitors of 30S particle activity. This expands the generality of the effect of ribosomal antibiotics as having two modes of inhibitory activity in growing cells.

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