

## Effects of Kanamycin and Streptomycin on the Macromolecular Composition of Streptomycin-Sensitive and Resistant *Escherichia coli* Strains

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**Kanamycin and streptomycin are both aminoglycoside antibiotics that bind to the 30S ribosomal subunit and inhibit protein translation. Kanamycin may have potential secondary targets in *Escherichia coli* other than the protein synthesis machinery. The aim of this study was to find evidence against the existence of secondary targets by subjecting kanamycin-resistant and kanamycin-sensitive *E. coli* strains to the antibiotic and examining the resulting impact on the macromolecular composition of the cells. The concentrations of antibiotic used were too low to inhibit growth and an unexpected increase in the protein/carbohydrate content of sensitive cells was observed. We believe this increase is due to an accumulation of a large number of non-functional polypeptides in addition to the normal protein constituent required for growth. It is likely that numerous non-functional proteins did result from growth in the presence of a non-inhibitory concentration of these aminoglycosides. Only the protein/carbohydrate fraction of the *E. coli* was dramatically altered by the presence of an aminoglycoside. Although no evidence of kanamycin secondary effects were detected, due to experimental difficulties we are unable to say that they are non-existent.**

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Kanamycin is an aminoglycoside that exhibits bactericidal effects. The molecule irreversibly binds to the 16S rRNA of the 30S ribosomal subunit resulting in inhibition of the translation process by preventing protein elongation [2]. Resistance to kanamycin can occur through a variety of different mechanisms. Specific plasmid-encoded cytoplasmic phosphotransferases can inactivate the antibiotic by phosphorylating the 3'-OH group of kanamycin to produce kanamycin 3'-phosphate. The resulting detoxified product is no longer able to bind to the ribosome. Another resistance mechanism involves single step mutations in ribosomal proteins so that kanamycin can no longer recognize or bind to its target [1]. Streptomycin, another aminoglycoside antibiotic, is very similar to kanamycin in both structure and function. It is a protein synthesis inhibitor and also functions by binding to the 30S subunit of the ribosome. Both kanamycin and streptomycin can halt the 30S initiation complex (30S-mRNA-tRNA) to block further initiation of translation. They can also affect translation that has already been initiated and cause the mRNA to be misread.

The purpose of this study was to investigate the possibility that kanamycin may have secondary effects on *E. coli* that extend beyond inhibition of protein synthesis as was suggested in Experiment A1a [3]. In the source experiment, the proportion of radioactive <sup>14</sup>C detected in the fraction containing cell proteins was comparable in both the control and the kanamycin-treated sample. Fractions containing other macromolecules of which proportions were not expected to be affected by the presence of kanamycin had been distorted with respect to the control. This pattern suggested that kanamycin might have had secondary effects on the cell in addition to the direct inhibition of protein translation.

We attempted to acquire evidence indicating no occurrence of secondary effects, and that these aminoglycosides only affect translation machinery. The initial investigation involved growing kanamycin-resistant (C584) and kanamycin-sensitive (DH5α) *E. coli* strains in M9 minimal media supplemented with 0.2% glycerol and <sup>14</sup>C-labeled glucose. However, the kanamycin-resistant strain C584 did not grow in the media. The experimental protocol was subsequently modified, using streptomycin-resistant (VC10) and streptomycin-sensitive (B23) *E. coli* strains. A chromosomal mutation resulting in a modified 30S ribosomal subunit confers streptomycin-resistance in the *E. coli* strain VC10. Due to structural and target similarity between streptomycin and kanamycin, we hypothesized that this resistance mechanism would also confer kanamycin resistance. The two strains were grown in M9 minimal media supplemented with 0.2% glycerol, <sup>14</sup>C-glucose, and either 20 μl/ml kanamycin, 20 μl/ml streptomycin, or no antibiotic. The macromolecular incorporation of <sup>14</sup>C into various classes of macromolecules was examined.

Comparisons between the six treatments should indicate if secondary effects of either streptomycin or kanamycin treatment exist.

#### METHODS AND MATERIALS

The methods and materials employed in our investigation are described in *Microbiology 421: Manual of Experimental Microbiology*, Experiment A1a, pp.1-4 [3], with the following changes to the protocol: we used two strains of *Escherichia coli*: B23, which is sensitive to streptomycin; and streptomycin-resistant VC10, which was derived from the W3110-1 wild type.

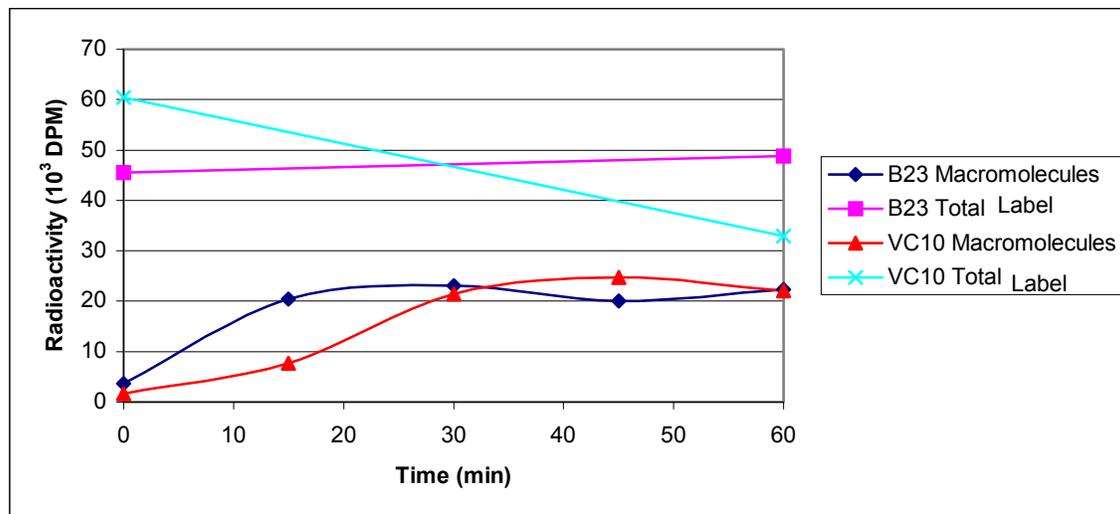
The two strains were prepared as follows: both strains were inoculated and propagated to mid-exponential growth phase in 25 ml of M9 minimal media containing 0.2% glycerol by incubating in a 37°C water bath for 4h. From each culture, 6.0 ml was transferred into three separate 125 ml Erlenmeyer flasks. For each strain, one culture was treated with 20 µg/ml kanamycin, another with 20 µg/ml streptomycin, and the third remained without antibiotic (control). The cultures were incubated in a 37°C water bath for 5 min, at which time 1.0 ml of <sup>14</sup>C-labeled glucose was added. The cultures were then incubated for 1h. During the 1h incubation, following the addition of <sup>14</sup>C-glucose, 100 µl samples from each control culture were taken at 15 minute intervals and transferred onto filter paper discs, as follows: 0 min, two samples from each were removed (one air dried, the other washed in cold TCA); 15 min, one sample from each was removed (cold TCA); 30 min, one sample from each was removed (cold TCA); 45 min, one sample from each was removed (cold TCA); 60 min, two samples from each were removed (one air dried, the other washed in cold TCA). After incubation, each culture was transferred to 30 ml Oakridge tubes and centrifuged at 9000 rpm for 10 min. The supernatant was then decanted and the pellet was resuspended in 0.5 ml cold saline and then transferred to 1.5 ml microfuge tubes.

A modified Park-Hancock fractionation protocol described in the laboratory manual [3] was followed to separate the various macromolecular fractions. Note that while performing the fractionation, we discovered that three of the microfuge tubes containing samples VC10 control, B23 control, and B23 with streptomycin had defective cracks. This problem resulted in the loss of supernatant during centrifugation. To compensate, an extra 75 µl of ethanol was added to these tubes during the lipid extraction.

To assess the assumption that the streptomycin sensitive marker in VC10 would confer resistance to other amino glycosides, we inoculated four Luria plates with a cotton swab to produce a confluent lawn growth (two for each strain). Each plate was divided into four sections: gentamicin, streptomycin, kanamycin, and control (H<sub>2</sub>O). 10 µl of each treatment were transferred onto small filter paper discs and placed on corresponding sections of each plate. Each strain was tested with 20 µg/ml of antibiotics on one plate, and 100 µg/ml on the other. The plates were incubated at 37°C for 24h. Observations and halo diameters were recorded.

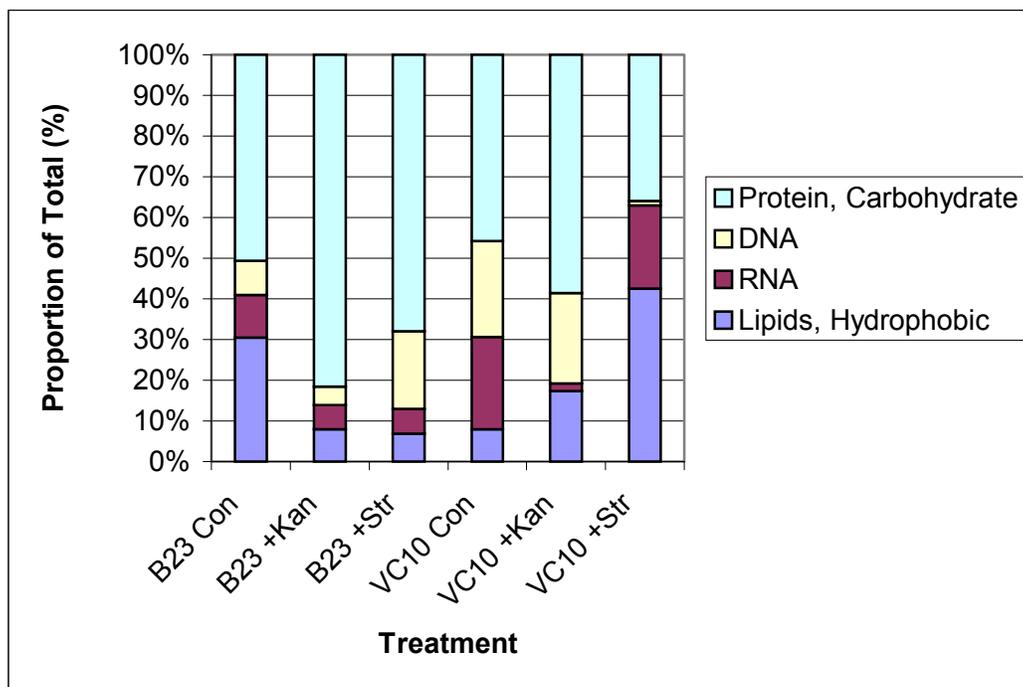
#### RESULTS

By sampling the B23 and VC10 *E. coli* cultures in 15-minute intervals during incubation in the presence of <sup>14</sup>C-glucose, we followed the rate of incorporation of <sup>14</sup>C into macromolecules within the cells. While both cultures began incorporation of <sup>14</sup>C immediately following the addition of <sup>14</sup>C-glucose, as indicated by the total radioactivity of the intracellular macromolecules, total incorporation in the B23 culture appeared to plateau after approximately 15 minutes; whereas the total incorporation in the VC10 culture continued for 30 minutes (Fig. 1). This is likely due to running out of usable <sup>14</sup>C label. The total combined radioactivity of the B23 culture including both cells and media was determined to have remained constant during incubation, as expected. However, the total combined radioactivity of the VC10 culture appeared to have decreased (Fig. 1).



**Figure 1.** The incorporated macromolecular radioactivity from 0.1 ml samples of the B23 and VC10 *E. coli* cultures during incubation in M9 minimal media with 0.2% glycerol and <sup>14</sup>C-glucose. Also shown is the total radioactivity present in both the cells and in the media of each culture.

The patterns of  $^{14}\text{C}$ -glucose incorporation among the various classes of macromolecules differed between the kanamycin and streptomycin treatments, and the control (Fig.2). After treatment with either kanamycin or streptomycin, relative to the control, the B23 cells more favored the incorporation of  $^{14}\text{C}$  into proteins and carbohydrates fraction, while less favoring the lipid fraction. However, similar treatments resulted in the opposite effect with VC10 cells, as the lipid fraction was more favored.



**Figure 2.** The distribution of  $^{14}\text{C}$  incorporated within various classes of macromolecules in B23 and VC10 *E. coli* treated with either 20  $\mu\text{g/ml}$  kanamycin (+Kan), 20  $\mu\text{g/ml}$  streptomycin (+Str), or no antibiotics (Con).

The partial loss of some fractions due to cracked tubes has likely affected our results with respect to the determined total radioactivity of the various fractions. Despite the fact that only some of the fractions were affected, the change might have significantly distorted Figure 2, which is a consequence of displaying the data as a percent distribution. To ensure the data remains independent of one another, the relative  $^{14}\text{C}$ -glucose incorporation of each fraction was calculated by dividing the total radioactivity of each fraction in a particular treatment by the total radioactivity of the albumen fraction. The value for the albumen fraction is a reliable reference value because we are certain that there was no unexpected loss in that fraction.

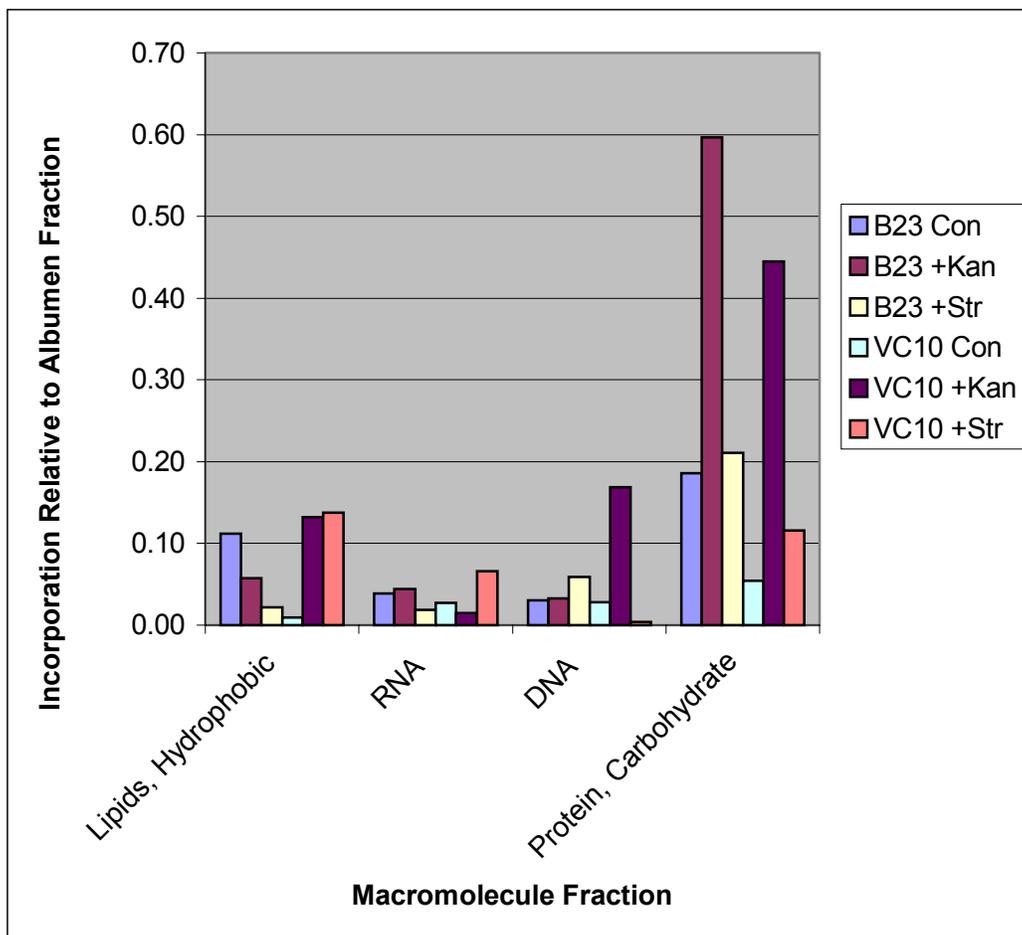
Figure 3 clearly shows the increase in relative  $^{14}\text{C}$  incorporation into lipids for B23 cells treated with kanamycin or streptomycin. Conversely, the VC10 cells showed a decrease in relative incorporation into lipids following exposure to the antibiotics. For both B23 and VC10, there is no significant change in the relative incorporation into RNA. The exposure of B23 and VC10 cells to kanamycin has caused a significant increase in the incorporation of  $^{14}\text{C}$ -glucose into proteins and carbohydrates. This change was not as pronounced with the streptomycin treatment.

Results of the antibiotic tests on Luria plates indicated that the growth of B23 and VC10 strains showed similar zones of inhibition at 100  $\mu\text{g/ml}$  of kanamycin (10 mm and 11 mm halo, respectively) and gentamicin (14 mm and 15 mm, respectively). However, VC10 was resistant to 100  $\mu\text{g/ml}$  of streptomycin while B23 was sensitive (7 mm). The 20  $\mu\text{g/ml}$  concentrations of kanamycin and streptomycin were insufficient to inhibit the growth of either *E. coli* strain.

## DISCUSSION

Due to the inability of C584 and DH5 $\alpha$  to proliferate in the M9 minimal media, we used B23 (streptomycin-sensitive) and VC10 (streptomycin-resistant) *E. coli* strains for our studies. Since the mechanism of action of

kanamycin and streptomycin are similar, we hypothesized that the VC10 strain would also be resistant to kanamycin. However, our Luria agar plates indicated that at 100 ug/ml, the growth of VC10 was inhibited by kanamycin, but not streptomycin. This result suggested that VC10 was sensitive to kanamycin. At 20 ug/ml, the growth of VC10 was not inhibited by any of the antibiotics. The B23 strain was inhibited by both kanamycin and streptomycin at a concentration of 100 ug/ml, as expected. However, at 20 ug/ml, no growth inhibition was observed. The results suggested that an antibiotic concentration of 20 ug/ml is insufficient to inhibit the growth of B23 and VC10. However, there may be unobservable metabolic effects caused by the antibiotics. Gentamicin was used as a positive control and did inhibit growth of both the streptomycin sensitive and resistant strains at both 20 ug/ml and 100 ug/ml. Our assumption that streptomycin-resistant VC10 would also be kanamycin-resistant was flawed. Due to different sites of action of the antibiotics on the ribosomes, kanamycin-resistant bacteria are also resistant to streptomycin, but streptomycin-resistant bacteria are susceptible to kanamycin and gentamicin [4].



**Figure 3.** A comparison of the macromolecular incorporation of  $^{14}\text{C}$ -glucose relative to the albumen fraction in B23 and VC10 *E. coli* treated with either kanamycin (+Kan), 20  $\mu\text{g}/\text{ml}$  streptomycin (+Str), or no antibiotics (Con).

Due to the cracked microfuges tubes, the supernatant of some fractions was lost. The loss of volume was originally thought to be due to pipetting error when initially realized during the separation of fraction 2. Additional ethanol was added to the VC10-control, B23-control, and B23-streptomycin treated samples to replace the lost volume. This diluted the fraction, resulting in underestimated radioactivity data. Without the problematic dilution, the lipid fractions would possibly contain a greater proportion of the total radioactivity than shown in Figure 2, shrinking the proportion of the other fractions relatively. Our error may have proportionately affected Figure 2 to hide the possibility that both kanamycin samples (resistant and sensitive) had a greater proportion of protein and carbohydrate production than the other samples. This observation was seen in the control experiment results [3],

where kanamycin had protein and carbohydrate production similar to that of the control, but far more than chloramphenicol or tetracyclin.

Figure 3 isolates the data so that only the lipid fraction is affected by our dilution error. It is clearly visible that B23-kanamycin treated sample and VC10-kanamycin treated sample incorporated most of the  $^{14}\text{C}$ -label into the protein and carbohydrate fraction, as compared to the other samples. Since kanamycin inhibits protein translation, this result is intriguing. An explanation for this result may be that the 20 ug/ml concentration of kanamycin affected the protein elongation, but was not lethal. The kanamycin concentration was not high enough to bind all of the 16S rRNAs of the 30S ribosomal subunits, therefore enough proteins (incorporating  $^{14}\text{C}$ ) were made to allow cell survival. By interacting with the 30S ribosomal subunits, kanamycin can block initiation and elongation of peptide chains as well as misreading the genetic code [4]. Therefore, many of the cell's proteins may have been non-functional due to the misreading, while others may have been truncated due to kanamycin binding to the ribosome during translation. It is possible that most of the macromolecule synthesis was therefore centered on making functional protein, causing the high level of  $^{14}\text{C}$  incorporation in the protein fraction.

The loss of supernatant in the three samples also explains the low label recovery. Unexpectedly, the VC10-streptomycin also had low recovery despite no apparent leakage. This could have potentially resulted from a variety of experimental errors or from an unrepresentative sample.

Since we had a decently high recovery yield of the B23-kanamycin treated sample and VC10-kanamycin treated sample (78% and 85% respectively) we can attempt to compare these two samples using Figure 2. Since we have discovered that VC10 is not kanamycin resistant, we would expect the two samples to have similar distribution of radioactivity among the fractions. This is not apparent. Part of the reason this relationship is not clear is due to the recovery yield, which should be >90%. In addition, comparison is further hindered by the fact that two different strains were used, which may have different metabolic properties. In the original experimental design, kanamycin resistance and sensitive strains were to be the same *E. coli* strain, but with or without the pMOB3 plasmid. This would have been a better comparison. Comparison using Figure 2 is also vitally hindered by not having reliable controls.

Further experimentation should include recreating the above experiment with kanamycin sensitive and resistant strains of *E. coli*. Since we could not grow kanamycin-resistant (C584) and sensitive (DH5 $\alpha$ ) *E. coli* strains well in M9-glycerol minimal media, further experimentation should be conducted as to the reason they did not grow well, or to developing strains that do grow well in M9-glycerol minimal media.

#### REFERENCES

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## APPENDIX

### Sample Calculations

$$\text{Concentration of} = (\text{DPM} - \text{DPM of blank}) / [(2.2 \times 10^9 \text{ DPM/mCi}) \times 0.1 \text{ ml}]$$

$$\begin{aligned} \text{Radioactivity} &= (79633.73 \text{ DPM} - 1904.34 \text{ DPM}) / [(2.2 \times 10^9 \text{ DPM/mCi}) \times 0.1 \text{ ml}] \\ &= 3.53 \times 10^{-4} \text{ mCi/ml} \end{aligned}$$

$$\begin{aligned} \text{Total Radioactivity} &= (\text{Concentration of Radioactivity}) \times (\text{Fraction Volume}) \\ &= (3.53 \times 10^{-4} \text{ mCi/ml}) \times 0.5 \text{ ml} \\ &= 1.77 \times 10^{-4} \text{ mCi} \end{aligned}$$

$$\text{Distribution of} = [(\text{Total Radioactivity}) / (\text{Sum of Total Radioactivity of Fractions 2-5})] \times 100\%$$

$$\begin{aligned} \text{Macromolecules} &= [(1.97 \times 10^{-5} \text{ mCi}) / (6.49 \times 10^{-5} \text{ mCi})] \times 100\% \\ &= 30\% \end{aligned}$$

$$\begin{aligned} \text{Label Recovery} &= [(\text{Sum of Total Radioactivity of Fractions 1-5}) / (\text{Total Radioactivity of} \\ &\quad \text{Albumin Fraction})] \times 100\% \\ &= [(8.35 \times 10^{-5} \text{ mCi}) / (1.77 \times 10^{-4} \text{ mCi})] \times 100\% \\ &= 47\% \end{aligned}$$

$$\begin{aligned} \text{Relative Incorporation of} &= (\text{Total Radioactivity of Cold TCA Fraction}) / (\text{Total Radioactivity of} \\ \text{Cold TCA Fraction} \quad &\quad \text{Albumin Fraction}) \\ &= (1.86 \times 10^{-5} \text{ mCi}) / (1.77 \times 10^{-4} \text{ mCi}) \\ &= 0.11 \end{aligned}$$