Journal of Experimental Microbiology and Immunology (JEMI) Vol. 9:31-38

Effects of kanamycin on the Macromolecular Composition of kanamycin Sensitive *Escherichia coli* DH5α Strain

REZA FARAJI, ASHKAN PARSA, BAHAR TORABI, AND TODD WITHROW
Department of Microbiology and Immunology, UBC

Aminoglycosides such as kanamycin, bind to the 16S rRNA and cause misreading during translation. Other aminoglycosides have been shown to act on secondary targets other than the protein synthesis machinery. The effects of these secondary targets include cell membrane damage and inhibition of DNA and RNA synthesis. Results from our experiment indicate that addition of kanamycin to an *E. coli* DH5α culture results in similar secondary effects. Nucleic acid synthesis was inhibited and membrane structure may have been altered.

Bactericidal effect of the aminglycoside kanamycin has been extensively investigated revealing that it irreversibly binds to the 16S rRNA of the 30S ribosomal subunit [5]. kanamycin binds the decoding region at the 3' end of rRNA which may prevent tRNA from binding to the ribosomal A site [5]. This results in inhibition of protein translation by preventing protein elongation. In addition to the inhibition of translation, aminoglycoside antibiotics have been shown to have many other effects on the macromolecular composition of the cells. Anand was the first to show that aminoglycosides cause membrane damage [1]. Moreover, aminoglycosides have exhibited inhibition of DNA synthesis [2,11] and RNA synthesis [2] as well as misreading in translation [3,4,16]. It was because of the pleiotropic effects of these antibiotics that scientists had trouble with an explanation of their mode of action that encompassed all of these observations. However, some of the basic principles of aminoglycoside antibiotics have been discerned. Although several hypotheses have been proposed for the mechanism involved in aminoglycoside action, the hypothesis of Davis *et al* [3,4] has been backed up by some experimental evidence [3,17]. Davis' mechanism centered on the misreading during translation and he broke it into four steps:

i) The antibiotic enters the cell by an unknown mechanism, later discovered to be via the oligopeptide binding protein OppA [7,8], and causes a small amount of misreading.

ii) Misread proteins are incorporated into the cell membrane where they create non-specific channels that allow more antibiotic into the cell.

iii) The process of entry, misreading and insertion continues until the cell's internal concentration of antiobiotic is high enough to block translation at all ribosomes, preventing further translation.

iv) Lethality is due to the irreversibility of the binding to and blockade of initiating ribosomes, and due to irreversible uptake due to membrane damage. However, Matsanuga *et al*. showed that lethality may also be due to inhibition of DNA replication initiation [11].

Due to the limited availability of radioactive isotopes of aminoglycosides, the work done to characterize the effects of aminoglycosides was mostly performed using streptomycin, gentimicin and trobramycin. However, the effects of misreading, membrane damage, and nucleic acid synthesis inhibition have been assigned to all aminoglycosides [16]. Taber *et al.* pointed out the folly of this thinking when they observed that conditions that prevent the entry of streptomycin sometimes have no effect on gentamicin [16]. It is with this in mind that we chose to explore the mechanism of action of kanamycin. We hypothesized that kanamycin does have secondary effects on *E. coli* in addition to the direct inhibition of protein synthesis, as previously suggested in Experiment A1a[13].

To explore the pleiotropic effects of kanamycin on the cell, we assayed the incorporation of radioactive [^{14}C]glucose and radioactive [^{14}C]leucine in the kanamycin sensitive *E. coli* DH5α strain grown in M9 minimal media supplemented with 0.1% glucose and (1 ug/ml) thiamine. We originally attempted to include the kanamycin resistant *E. coli* strain C584 in our experiment; however, the strain failed to grow in this media. Previous studies had shown that the *E. coli* C584 strain was incapable of growth in M9 minimal media [10], consequently, the experimental protocol was modified so that the DH5α strain would serve as both the control and the strain under investigation. The modified Park-Hancock fractionation procedure was used to show the incorporation of radioactive [^{14}C]glucose and [^{14}C]leucine within the different classes of macromolecules in an attempt to discover secondary targets of kanamycin.

METHODS AND MATERIALS

Bacterial strains. *E. coli* DH5α was used as the kanamycin sensitive strain and *E. coli* C584 was chosen as the resistant strain. *E. coli* C584 is derived from the DH5α strain and contains the pMOB3 plasmid. This plasmid confers resistance to chloramphenicol and kanamycin [10]. The strains were obtained from the frozen laboratory stock of the University of British Columbia, Department of Microbiology and Immunology.

Kanamycin resistance tests. To confirm that the DH5α strain was kanamycin sensitive and the C584 strain was kanamycin resistant, plates of Luria-Bertani medium (LB) with and without kanamycin (Cat.# 11815-024, Life Technologies) were prepared by pour plating. The LB media consisted of 1% (w/v) casein hydrolysate, 0.5% yeast extract, 0.5% NaCl, and 1.5% agar. The kanamycin was obtained form the laboratory stock at the University of British Columbia, Department of Microbiology and Immunology, mixed with water to give a concentration of 5 mg/ml, and then filter sterilized. Two plates were prepared without kanamycin and two plates with kanamycin at a concentration of 50 ug/ml. Each strain was streaked onto one plate containing kanamycin and one plate without kanamycin. Plates were incubated overnight, aerobically, in the dark at 37ºC.

Culture conditions. Thiamine hydroxide (Cat.# T-4625, Sigma Chemical Company) obtained from the laboratory stock of the University of British Columbia, Department of Microbiology and Immunology, was mixed with distilled water to give a final concentration of 1 mg/ml, and then filter sterilized. Minimal M9 media was prepared with 1 ug/ml thiamine and 0.2% glycerol instead of 0.4% glycerol as the carbohydrate carbon source [14]. Two separate 125 ml Erlenmeyer flasks containing 25 ml of minimal M9 media with 0.2% glycerol and 1 ug/ml thiamine were inoculated and incubated at 37ºC in the shaking water bath. For the DH5α strain, a pure colony from the corresponding kanamycin minus, LB plate was used to inoculate the overnight culture. For the C584 strain, a pure colony from the corresponding kanamycin containing LB plate was used to inoculate the overnight culture.

Growth culture for labeling. Thirty milliliters of fresh minimal M9 media supplemented with 0.2% glycerol and 1 ug/ml thiamine was inoculated with 10 ml of the DH5α overnight culture. This inoculum volume was calculated based on the measured turbidity of the overnight culture at an OD_{460nm}, taken at the start of the growth/labeling experiment. This culture was incubated at 37ºC in the shaking water bath for an hour with OD_{460nm} readings taken at 15 min intervals to confirm growth.

Transfer of DH5α cells to glycerol free media. When the OD_{460nm} reached 0.194, the culture was transferred into a 250 ml plastic centrifuge bottle, loaded into a JA-14 rotor on the Beckman J-21 high-speed centrifuge, balanced with another 250 ml plastic centrifuge bottle filled with water, and spun at 1000 x g for 15 minutes. The supernatant was carefully removed using a 25 ml glass pipette. The pellet was resuspended in glycerol free minimal M9 media containing 1 ug/ml thiamine. This suspension was transferred to a new 125 ml Erlenmeyer flask and placed in the 37ºC water bath for 1 minute.

Addition of carrier glucose and leucine. To the 125 ml Erlenmeyer flask containing the cell suspension, 4.4 ml of 1% (w/v) cold carrier glucose solution (Cat.# G-5000, Sigma Chemical Company) and 22 ul of 5 mg/ml stock cold carrier leucine were added. The stock glucose and leucine were obtained from the University of British Columbia, Department of Microbiology and Immunology. To mix the contents, this flask was placed back in the 37ºC shaking water bath for 1 minute.

Monitoring growth. An 8 ml sample was taken from this flask and placed into a 125 ml Erlenmeyer flask containing 32 ul of 5mg/ml stock kanamycin. Another 8 ml sample was taken and placed into a 125 ml Erlenmeyer flask without any kanamycin. These flasks were incubated in the 37ºC shaking water bath for 2 hours, during which OD_{460nm} readings were taken at 15 min intervals to monitor growth.

Monitoring incorporation of [^{14}C]glucose and [^{14}C]leucine. Four 6 ml samples were taken from the culture flask containing carrier glucose and carrier leucine in glycerol free M9 and added to four 25 ml Erlenmeyer flasks as follows: two flasks with 2 uCi radiolabeled leucine (Cat.# not available, Radiochemical Center), one of which contained kanamycin at 20 ug/ml; and two flasks with 10 uCi radiolabeled glucose (Cat.# 3770155, New England Nuclear), one of which contained kanamycin at 20 ug/ml. The radiolabeled leucine and radiolabeled glucose were obtained from the University of British Columbia, Department of Microbiology and Immunology. All flasks were returned to the water bath and incubated for 2 hours, during which incorporation was monitored. At time 0, a filter paper disc (Cat.#: 1003323, Whatman International Ltd.) was placed in 200 ml of cold 5% Trichloroacetic acid (TCA) to serve as the blank. From then on, 50 ul samples were extracted from each culture at 15 min intervals for 2 hours, poured onto filter paper discs, and placed in a flask containing 5% cold TCA. Note that at t = 1 min and at t = 120 min, duplicate 50 ul samples were poured onto filter paper discs, one was air-dried and the other was placed in the 5% cold TCA flask. All filter disks were transferred to 95% Ethanol after TCA treatment and then baked in the STABIL-THERM Gravity oven overnight at 125ºC.

Preparation for modified Park-Hancock fractionation. The remainder of the radioactive cultures were poured into 10ml Oakridge tubes and centrifuged at 6750 x g for 10 minutes in the IEC Centra-4B Centrifuge. Due to unstable pellets, the majority of the supernatant was drawn off with Pasteur pipettes, the pellets were re-suspended in a small volume of the supernatant, and transferred to 1.5 ml microfuge tubes. These tubes were microfuged at 7500 x g for 5 minutes, the supernatants were removed and the pellets were frozen for 6 days before the modified Park-Hancock fractionation was performed on them.

Modified Park-Hancock protocol. This protocol was carried out as described previously [13] to fractionate the cells of the four cultures containing radiolabeled cells into proteins and carbohydrates, lipids and hydrophobic molecules, and nucleic acid fractions. Note change to protocol: 150 ul of the supernatant of each fraction was sampled onto a filter paper disc instead of 50 ul. This was done to maximize the available radioactivity in the fractions taken for the kanamycin treated cultures. The first sample, taken before the fractionation was actually carried out, remained 50 ul as mentioned in the modified protocol [13].

Puromycin pretreatment experiment. An over-night culture of DH5α in minimal M9 media supplemented with 0.2% glycerol and 1 ug/ml thiamine was established and used the next day to inoculate a new culture. The new culture was then split into two 16 ml cultures, where one was pretreated with 50 ug/ml of puromycin (Cat.# P-7255, Sigma Chemical Company) and the other one served as the control. The puromycin was obtained from the laboratory stock at the University of British Columbia, Department of Microbiology and Immunology, mixed with water to give a concentration of 1 mg/ml, and then filter sterilized. After treatment with puromycin, the culture was centrifuged for 10 minutes at 7500 x g in an IEC Centra-4B Centrifuge and the supernatant was discarded. The pelleted cells were then re-suspended in another 16 ml of minimal M9 media with 0.2% glycerol and 1 ug/ml thiamine. Each 12 ml culture was further split into two 6 ml cultulres, resulting in four 6 ml cultures, two of which had been pretreated with puromycin. One of the 6ml cultures that had not been pretreated with puromcyin, was treated with 100 ug/ml kanamycin. The second 6 ml culture that had not been pretreated with puromycin was used as the control. Of the two 6 ml cultures that had been pretreated with puromycin, one was also treated with 100 ug/ml kanamycin. Growth of the cultures was monitored for two hours by taking turbidity readings at OD_{460nm} at 10 minute intervals for 100 minutes.

RESULTS

The control culture lacking kanamycin (kan^-) exhibited a steady increase in culture concentration up to the 100 minute mark and leveled off. The culture had

an initial concentration of 0.155 OD_{460nm} and grew at a constant rate of about $2.4x10^{-3}OD_{460nm}$/min to a final culture concentration of 0.200 OD_{460nm} at 100 minutes. With regards to the culture containing kanamycin (kan^+), the initial concentration (T=0min) was 0.130 OD_{460nm}. The turbidity of the culture increased to a maximum of 0.151 at the 20 minute mark, and from then on decreased at a rate of $1.8x10^{-3}$ OD_{460nm}/min to a concentration of 0.109 OD_{460nm} at the end of the 2 hours.

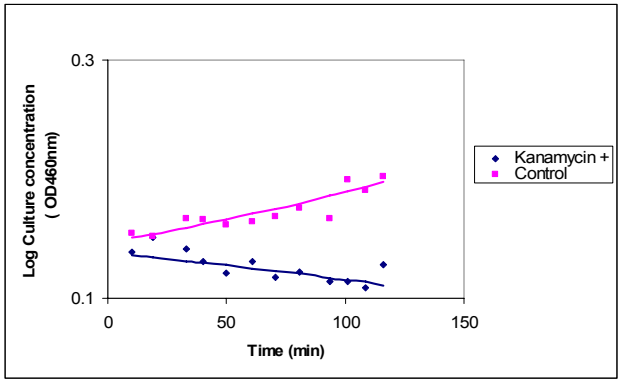

FIG.1 Effects of kanamycin treatment on growth of *E.coli* DH5α cultured cells.

The trend displayed in Figure 2 for the incorporation of radioactive glucose in the kan^-culture follows the general trend of the growth curve (Fig.1). Incorporation increased with time at a rate of about 0.002 KBq/ml/min up to the 100 minute mark and then decreased. Incorporation of radiolabeled glucose in the Kan^+ culture gradually increased throughout the 2 hours, but at a slower rate (0.0007 KBq/ml/min) compared to the control.

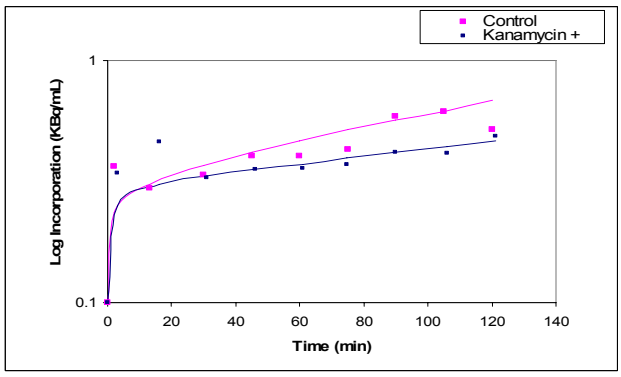

FIG.2 The effect of inhibition by kanamycin on incorporation of labeled glucose into *E.coli* DH5α cultured cells.

In Figure 3, the kan^- culture incorporated radiolabeled leucine at a constant rate of 0.007 KBq/ml/min throughout the 2 hour period. It had a final total incorporation of 0.71 KBq/ml. The incorporation of leucine in the kan^+ culture increased to 0.095 KBq/ml in 31 minutes, then steadily decreased at 0.002 KBq/ml/min to almost 0 KBq/ml at 120 minutes.

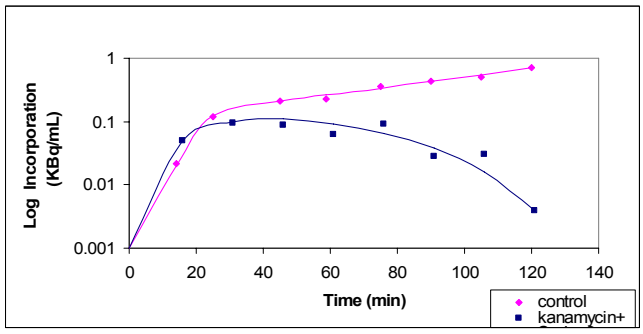

FIG.3 The effect of inhibition of kanamycin on incorporation of labelled leucine into *E.coli* DH5α cultured cells.

Comparing the four fractions containing cellular macromolecules labeled by [^{14}C]glucose (Table I), the ethanol soluble fraction, containing lipids and other hydrophobic macromolecules, exhibited the highest total radioactivity and percentage distribution of radioactivity in both the kan^+ culture (76%) and Kan^- culture (63%). The ribonuclease soluble fraction containing RNA and the hot TCA soluble fraction containing DNA and RNA complexes, had almost no traces of radioactivity. The hot TCA insoluble fraction, which holds carbohydrates and proteins, had the second highest incorporation of radioactive glucose in both the kan^+ and kan^- cultures with 24% and 31%, respectively. The albumin suspension should contain the initial radioactivity of 100% and the cold TCA soluble sample, containing low molecular weight compounds, should exhibit 0% of the radioactivity [9].

Table II displayed similar results to Table I when comparing the four fractions containing cellular macromolecules. The ethanol soluble fraction had the highest total radioactivity, with 100% distribution in the kan^+ sample and 93% in the control sample. However, the hot TCA insoluble fraction was as low as the ribonuclease soluble and hot TCA soluble fractions, with close to 0% distribution of radioactivity. The total label recovery in the kan^+ culture was 17%, compared to 64% in the control culture.

The total final radioactivity in the air-dried samples, taken at 120 minutes, increased in all four cultures, with the greatest increase in the two glucose cultures (Table III). The net incorporation, measured from the TCA samples of each culture at 120 minutes, was slightly lower in the glucose kan^+ culture than in the glucose control culture (0.4871 KBq/ml vs 0.5178 KBq/ml). On the other hand, the net incorporation in the leucine kan^+ culture was noticeably lower than in its control culture (0.0039 KBq/ml vs 0.7067 KBq/ml).

Table 1 Distribution of Incorporated Radioactive Glucose in the *E.coli* DH5α control culture and the *E.coli* DH5α kanamycin- treated culture.

Fraction of Sample	Net fraction volume (ml)	Concentration of radioactivity (KBq/ml)	Total radioactivity (KBq)	Distribution of radioactivity in the macromolecules (%)
Albumin control Suspension kanamycin+	0.35 0.35	3.51 0.16	1.23 0.02	100 100
Cold TCA control Soluble kanamycin+	0.7 0.7	0.28 0	0.19 0	16 0
Ethanol control Soluble kanamycin+	0.2 0.2	1.49 0.08	0.29 0.01	63 76
Ribonuclease control Soluble kanamycin+	0.4 0.4	0 0	0 0	0 0
Hot TCA control Soluble kanamycin+	0.2 0.2	0.13 0	0.03 0	5 0
Hot TCA control Insoluble kanamycin+	0.2 0.2	0.74 0.02	0.15 0.00	31 24

Total recovery: control: 54%
kanamycin + : 100%

Table 2 Distribution of Incorporated Radioactive Leucine in the *E.coli* DH5α control culture and the *E.coli* DH5α kanamycin- treated culture

Fraction of Sample	Net fraction volume (ml)	Concentration of radioactivity (KBq/ml)	Total radioactivity (KBq)	Distribution of radioactivity in the macromolecules (%)
Albumin control Suspension kanamycin+	0.35 0.35	8.08 0.07	2.82 0.02	100 100
Cold TCA control Soluble kanamycin+	0.7 0.7	0.01 0.00	0.00 0.00	35 0
Ethanol control Soluble kanamycin+	0.2 0.2	2.52 0.02	0.50 0.00	93 100
Ribonuclease control Soluble kanamycin+	0.4 0.4	0.00 0.00	0.00 0.00	0 0
Hot TCA control Soluble kanamycin+	0.2 0.2	0.02 0.00	0.01 0.00	2 0
Hot TCA control Insoluble kanamycin+	0.2 0.2	1.46 0.00	0.03 0.00	5 0

Total recovery: control: 64%
kanamycin + :17%

Table 3. Total Recovery and Incorporation of the Radioactivity

Labeled precursor	Culture Treatment	Observed radioactivity (KBq/ml)		
		Total Initial	Total Final	Net Incorporated
Glucose	Control	28.75	30.97	0.52
	kanamycin	33.85	35.67	0.49
Leucine	Control	4.30	4.91	0.71
	kanamycin	5.14	5.20	0.00

Figure 4 displays distribution of radioactive glucose and leucine among the various fractions of *E. coli* cells. Within the cold TCA soluble fraction, the kan$^-$ culture containing radioactive glucose displayed 8% of the total radioactivity; 42% in the alcohol soluble fraction, 4% in the hot TCA soluble fraction and 21% in the hot TCA insoluble fraction. Whereas the kan$^+$ culture with radiolabeled glucose displayed 52% of total radioactivity incorporated into the ethanol soluble fraction and 16% in the hot TCA insoluble fraction. The radiolabeled leucine kan$^-$ culture showed about 1% of its total radioactivity in each of the cold TCA soluble and hot TCA soluble fraction, 31% in the ethanol soluble fraction and 18% in the hot TCA insoluble fraction. The kan$^+$ culture with radiolabeled leucine exhibited incorporation only in the ethanol soluble fraction, with a value of 30%.

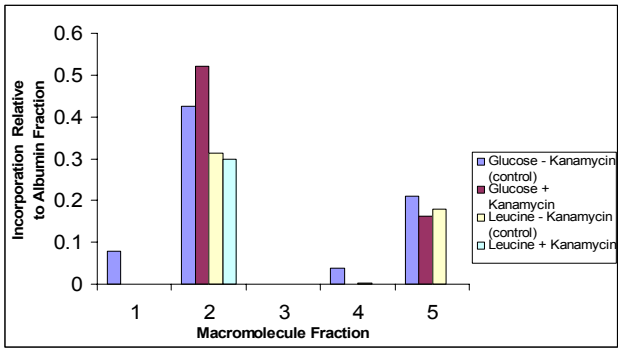

FIG.4. The effect of kanamycin on the incorporation of labeled glucose and leucine in various fractions of *E.coli* DH5α cultured cells. (1)Cold TCA soluble fraction; (2)Ethanol soluble fraction;(3) ribonuclease soluble fraction; (4)hot TCA soluble fraction; (5)hot TCA insoluble fraction.

For the glucose control culture, over 60% of the radiolabeled glucose was incorporated into the ethanol soluble fraction, 5% was incorporated into the hot TCA soluble fraction and 30% was incorporated into the hot TCA insoluble fraction (Fig.5). For the kan$^+$ glucose culture, 75% of the radiolabeled glucose was incorporated into the ethanol soluble fraction and the remaining 25% was incorporated into the hot TCA soluble fraction. For the leucine control culture, over 90% of the radiolabeled leucine was incorporated into the ethanol soluble fraction, with the remaining 10% incorporated into the hot TCA soluble fraction and the hot TCA insoluble fraction. In the kan$^+$ leucine culture, all of the radiolabeled leucine was incorporated into the ethanol soluble fraction. The discrepancy in the percentages obtained from Figures 4 and 5 is because Figure 4 displays percentages relative to the albumin fraction, while Figure 5 takes into account the volume of each fraction sample.

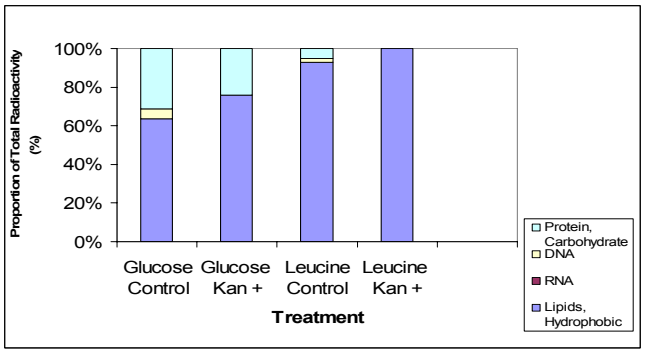

FIG.5. The distribution of labeled glucose and leucine within various classes of macromolecules in DH5α *E.coli* control cultures and kanamycin-treated cultures.

From Figure 6, it is observed that the puromycin pretreatment had little effect on subsequent kanamycin treatment since the culture lacking treatment with puromycin displayed a similar growth curve. Both the puromycin pretreated and untreated cultures showed reduction of cell concentration at approximately the same time, after 30 minutes.

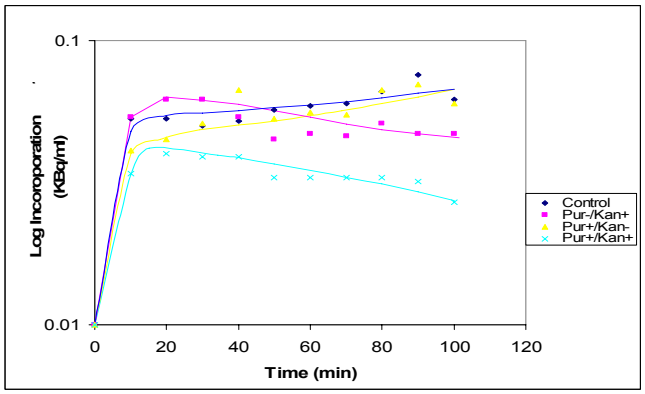

FIG.6. The effects of puromycin and kanamycin on the growth of *E.coli* DH5α cultured cells.

DISCUSSION

The *E. coli* C584 strain did not grow in minimal M9 Media supplemented with glycerol and thiamine. The chloramphenicol acetyl transferase gene on the pMOB3 plasmid has been found to be responsible for inhibiting the growth of this strain in this media [6]. The protocol was therefore modified to only include the *E. coli*

DH5α strain. There was a lower rate of incorporation of radiolabeled glucose in the kan^+ culture compared to the control culture. This observation is in agreement with the growth curve in Figure 1 where the kan^+ culture displayed a net decrease in culture concentration, implying net death of cells. The incorporation of radiolabeled glucose in the control culture appeared to increase at a constant rate up to 120minutes, which is consistent with Figure 1 where the culture turbidity also increases in this time interval. The control also exhibited a constant positive rate of leucine incorporation, similar in trend to the culture growth and glucose incorporation.

Initially, the kan^+ culture displayed a positive rate of leucine incorporation (Fig.3) until 30 minutes, then decreased sharply afterwards. This decrease is unique to leucine incorporation, suggesting an increased turnover rate of cellular proteins but not of material labelled with glucose. Glucose, unlike leucine, is a central metabolite and is involved in the synthesis of all cellular components. And so the increased breakdown of proteins does not have a significant effect on the glucose incorporation compared to the leucine incorporation. The increase in breakdown of proteins is caused by an increase in mistranslated and misfolded proteins due to the action of kanamycin [3,4,17].
This is consistent with Davis *et al.* who hypothesized that misread protein synthesis was important for creating nonspecific membrane channels in the membrane that allowed increased entry of aminoglycosides [3,4]. The internal cellular concentration of the antibiotic eventually reaches the level where it blocks translation initiation at all ribosomes [3,4], as seen in Figure 3.

In the cold TCA soluble fraction, some incorporation of radiolabeled glucose was observed for the control treatment. This could be explained by the low molecular weight cellular components such as nucleic acid bases, hydrolyzed lipids, free amino acids, and metabolic intermediates [15]. A very small amount of leucine incorporation was observed in the control treatment and this represents the free leucine amino acids [15]. It has been shown that membrane damage caused by aminoglycosides results in a leaky membrane [3]. Therefore, nucleotides and amino acids leaked across the membrane in the kan^+ cultures before the fractionation procedure was carried out and were lost when the cells were pelleted and media was removed. This explains the observed lack of incorporation of leucine or glucose in the kan^+ treatments.

Within the ethanol soluble fraction, reside the lipids and hydrophobic molecules [13,15]. The kan^+ treatment has more glucose incorporation into this fraction than the control treatment. An explanation for this is that the cell was trying to make more lipids to stabilize the leaky membrane caused by the kanamycin treatment [1,3,4,17].
The kan^+ treatment displayed approximately the same leucine incorporation into this fraction as the control treatment. This is consistent with the hypothesis that protein synthesis is important for kanamycin action [3,4,17] and is consistent with Figure 3. Misread membrane proteins could still be inserted into the membrane [17], taken out with the lipid fraction, and result in similar incorporation as the control culture.
Within the ribonuclease soluble fraction, resides the RNA [13,15]. For this fraction, we observed no incorporation of leucine or glucose for kan^+ treatments or control treatments. Because leucine is an amino acid and does not incorporate into RNA, this is expected. The observed lack of incorporation of glucose could be due to the low carbon content of nucleic acids, the low half-life of mRNA, and possibly poor separation of this fraction during the protocol as some RNA complexes are removed with the Hot-TCA soluble fraction [15].

Within the hot-TCA soluble fraction, reside the DNA and RNA complexes [13,15]. In this fraction, the control shows that glucose is incorporated into nucleic acids, corresponding to the sugar component of their backbone. DNA constitutes approximately 3% of E. coli dry weight and therefore, it is expected that it would have a small percentage of the total carbon content [15]. We observed no incorporation of glucose or leucine for the kan^+ treatments. Because aminoglycosides have been shown to inhibit DNA replication initiation and RNA transcription [2,11], it is expected that there would be no incorporation of glucose into these nucleic acids. Since leucine is an amino acid, it is expected that no incorporation would take place into the DNA fraction. This is what was seen in the control where there was little incorporation of leucine into this fraction, possibly representing DNA associated proteins.

Within the hot-TCA insoluble fraction, reside protein and carbohydrate polymers [13]. In the control treatments, incorporation of glucose and leucine is observed as expected. For the Kan^+ treatments, slightly lower glucose incorporation is seen as well as no leucine incorporation. The lower glucose incorporation could be due to the slower growth rate of this culture as seen in Figure 1. The observed lack of leucine incorporation could be due to the inhibition of protein synthesis by kanamycin. As seen in Figure 3, samples taken during labeling of the cell with radiolabeled leucine exhibited low level of incorporation at the start and the incorporation rate decreased throughout the experiment. With each successive fraction in the Park-Hancock fractionation protocol, we observed that the pellet became less and less stable. This could result in loss of molecules with the supernatant, some of which could have been protein. Their absence would explain

the lower leucine incorporation into the protein and carbohydrate fraction compared to the lipids and hydrophobic fraction in the control cultures. The loss of pellet throughout the fractionation could also explain why the recovery in the lipid and hydrophobic compound fraction was significantly higher than the later fractions (TableII and Fig.4).

Compared to the source experiment by Kestell *et al.*, incorporation of glucose into the lipids and hydrophobic fraction of our kan^+ and control cultures was very high [9]. It should be noted that due to cracked microfuge tubes, some of their fractions were lost and they concluded that the lipids and hydrophobic fraction should have contained a higher proportion of the total radioactivity. This is in accordance with our results (Fig.4).

To test our hypothesis about kanamycin's effect on membrane stability due to misread proteins, the puromycin pretreatment experiment was performed. Based on previous experiments of puromycin pretreatment followed by aminoglycosides such as streptomycin, due to membrane damage by puromycin, it was expected that puromycin would enhance the uptake of kanamycin [3,17]. The result of this would have been quicker killing rate by kanamycin [17]. This was not observed in our experiment because the turbidity assay was inadequately sensitive to detect cell death at such low turbidity readings. All cultures in this assay started out at a low absorbance reading and this reading did not change significantly for the puromycin pre-treated or untreated cultures. In addition, the puromycin concentration might have been lower than the minimum inhibitory concentration, thus the expected results were not observed. Therefore, we were unable to reproduce such an experiment and to extend these conclusions to kanamycin.

The aminoglycoside kanamycin acts primarily on is the 30S ribosomal subunit resulting in prevention of protein elongation. Our results coincide with the above, in that the radioactive distribution of leucine within the protein and carbohydrate fraction was significantly reduced in the Kan^+ culture compared to the control. In addition to targeting the protein synthesis machinery, kanamycin also inhibited the synthesis of DNA and targeted the cellular membrane composition. The increase in the incorporation of glucose into the lipids and hydrophobic fraction showed that cellular membrane was also damaged by the kanamycin treatment. This is all consistent with previous studies on the secondary effects of other aminoglycosides.

FUTURE EXPERIMENTS

Our results showed that DNA synthesis inhibition could be one of the secondary targets of kanamycin. However, the exact mechanism of replication inhibition by aminoglycosides has not yet been elucidated. Barmada *et al.* suggested that it may be due to nonspecific binding of aminoglycosides to DNA [2], whereas Matsunaga *et al.* showed that it may have been due to the interruption of the *oriC*-membrane complex [11]. Future experiments should focus on these two mechanisms and determine which of the two actually occurs.

Although other studies showed that puromycin pre-treatment enhanced the uptake of aminoglycosides, our data failed to show whether this was the case with kanamycin. However, our growth assay may not have been sensitive enough to detect this effect. We used a turbidity assay, while other studies used plate counts and we propose that the experiment should be repeated using plate counts. In addition, puromycin concentrations should be increased to the minimal inhibitory concentration in the cells.

ACKNOWLEDGEMENTS

This work was supported by the University of British Columbia, Department of Microbiology and Immunology. We would like to thank the teaching staff at the University of British Columbia, Department of Microbiology and Immunology: Dr. William Ramey, Jennifer Sibley, Karen Smith, and Dr. Gaye Sweet. Your patience, knowledge, support and assistance allowed for this experiment. Special thanks to Lando and all of the staff in the media room for providing necessary reagents and autoclaving required glassware and reagents.

REFERENCES

1. **Anand, N., and B. D. Davis.** 1960. Damage by streptomycin to the cell membrane of *Escherichia coli*. Nature (London). **185:**22-33.
2. **Barmada, S., S. Kohlhepp, J. Leggett, R. Dworkin, and D. Gilbert.** 1993. Correlation of tobramycin-induced inhibition of protein synthesis with postantibiotic effect in *Escherichia coli.* Antimicrob. Agents Chemother. **37:**2678-2683.
3. **Davis, B. D.** 1987. Mechanism of bacterial action of aminoglycosides. Microbiol. Rev. **51:**341-350
4. **Davis, B. D., L. Chen, and P. C. Tai.** 1986. Misread protein creates membrane channels: an essential step in the bactericidal action of aminoglycosides. Proc. Natl. Acad. Sci. USA **83:**6164-6168.
5. **Franklin, T. J., and G. A. Snow.** 2005. Biochemistry and molecular biology of antimicrobial drug action. 6th ed. Springer, Cheshire, England.
6. **Imperial, S., T. Lin, and V. Posarac**. 2005. Intracellular acetyl-CoA depletion by the *cat* gene is responsible for growth inhibition of *Escherichia coli* C584 on M9 minimal media. J. Exp. Microbiol. Immunol. **7:**49-56.
7. **Kashiwagi, K., A. Miyaji, S. Ikeda, T. Tobe, C. Sasakawa, and K. Igarashi.** 1992. Increase of sensitivity to aminoglycoside antibiotics by polyamine-induced protein (oligopeptide-binding protein) in *Escherichia coli*. J. Bacteriol. **174:**4331-4337.
8. **Kashiwagi, K., M. H. Tsuhako, K. Sakata, T. Saisho, A. Igarishi, S. O. Pinto da Costa, and K. Igarishi.** 1998. Relationship

between spontaneous aminoglycoside resistance in *Escherichia coli* and a decrease in oligopetide binding protein. J. Bacteriol. **180:**5484-5488.

9. **Kestell, D., S. Lai, G. Liang, S. Waters, and A. Wladichuk.** 2002. Effects of kanamycin and streptomycin on the macromolecular composition of streptomycin-sensitive and resistant *Escherichia coli* strains. J. Exp. Microbiol. Immunol. **2**:103-108.

10. **Lau, J., R. Grover, and D. Leung.** 2004. Effects of chloramphenicol acetyl transferase gene deletion from pMOB3 on the growth inhibition of *Escherichia coli* C584 in M9 minimal media. J. Exp. Microbiol. Immunol. **5:**95-100.

11. **Matsunaga, K., H. Yamaki, T. Nishimura, and N. Tanaka.** 1986. Inhibition of DNA replication initiation by aminoglycoside antibiotics. Antimicrob. Agents Chemother. **30:**468-474.

12. **Picon, A., MJ. Teixeira de Mattos, and PW. Postma.** 2005. Reducing the glucose uptake rate in *Escherichia coli* affects growth rate but not protein production. Biotechnol Bioeng. **90**(2): 191-200.

13. **Ramey, W. D. 2002.** Microbiology 421: Source Experiments for Experimental Projects. University of British Columbia, Vancouver, B.C.

14. **Ramey, W. D. 2005.** Microbiology 421: Manual of Experimental Microbiology. University of British Columbia, Vancouver, B.C.

15. **Roberts, R.B., D.B. Cowie, P.H. Abelson, E.T. Bolton, and R.J. Britten.** 1955. Studies of Biosynthesis in *Escherichia coli*. Carnegie, Washington, D.C.

16. **Taber, H.W., J.P. Mueller, P. F. Miller, and A. S. Arrow.** 1987. Bacterial uptake of aminoglycoside antibiotics. Microbiol. Rev. **51:**439-457.

17. **Wyka, M. A. and A. C. St. John.** 1990. Effects of production of abnormal proteins on the rate of killing of *Escherichia coli* by streptomycin. Antimicrob. Agents Chemother. **34:**534-538.