GENTAMICIN ACCUMULATION BY SENSITIVE STRAINS OF *ESCHERICHIA COLI* AND *PSEUDOMONAS AERUGINOSA*

L.E. BRYAN* and H.M. VAN DEN ELZEN

Department of Medical Bacteriology, University of Alberta
Edmonton, Alberta, Canada

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Gentamicin accumulation with time shows multiphasic kinetics in strains of *Escherichia coli* and *Pseudomonas aeruginosa*. All but first phase accumulation may be prevented or reduced by inhibitors of electron transport, by a sulfhydryl poison, by agents which uncouple electron transport and oxidative phosphorylation and by an inhibitor of protein synthesis. The phases of accumulation which are sensitive to these inhibitors are required for loss of cell viability. Gentamicin can be extracted from cells in an unchanged form as judged by paper chromatography and is concentrated 4 to 250 times over extracellular concentrations within the bacterial cell. Gentamicin accumulation has been shown to occur before there is any evidence of release of acid-soluble ^{3}H-adenine from cells. These data demonstrate that productive gentamicin accumulation capable of causing cell death is by active transport.

Recent studies have established that the aminoglycoside antimicrobial agent streptomycin is accumulated by cells of *Escherichia coli* and *Pseudomonas aeruginosa* by active transport[2]. Cellular entry of other aminoglycosides has not been examined in detail. Although streptomycin shares with other aminoglycosides the capability to inhibit protein synthesis there are marked differences in structure among this group of drugs. Aminoglycosides have been classified structurally into those which contain either streptidine or deoxystreptamine. The latter group may be subdivided into those with the substituents on the deoxystreptamine ring attached either at adjacent positions or on nonadjacent hydroxyl groups. We have selected gentamicin for study of cell entry because it is a widely used deoxystreptamine-containing aminoglycoside antibiotic in contrast to streptomycin which contains streptidine. Initial studies have been directed to determine the nature of gentamicin accumulation and to compare that to the characteristics of streptomycin accumulation. We have examined gentamicin accumulation both in *E. coli* and *P. aeruginosa* as representative bacteria which are frequently treated with gentamicin in clinical medicine.

Materials and Methods

Bacterial strains.

E. coli K12 SA 1306 was obtained from Dr. K. E. SANDERSON, University of Calgary. *P. aeruginosa* 280 met$^-$ is a methionine auxotroph of a clinical isolate from the University of Alberta Hospital, Edmonton[3]. Streptomycin resistant mutants (*E. coli* K12 SA 1306 Str^r and *P. aeruginosa* 280 Str^r) were isolated as described[2].

Chemicals.

All chemicals were of analytical grade whenever available. Reagent sources were: potas-

* To whom correspondence should be sent.

sium cyanide (KCN)—Fisher Scientific Co., sodium azide (NaN_3)—J. T. Baker Chemical Co., sodium amytal—Eli Lilly & Co. Ltd., all others—Sigma Chemical Co.

Media.

Media used were trypticase soy broth (TSB), trypticase soy agar (TSA) and nutrient broth (NB) obtained from Baltimore Biological Laboratories (BBL). The medium used for anaerobic growth studies was prepared using the following ingredients (g/liter): Bacto-yeast extract, 5; Bacto-tryptone, 15; Bacto-dextrose, 5; L-cysteine, 0.25; NaCl, 2.5; and sodium thioglycollate, 0.3. The control medium for aerobic growth in these experiments was the same medium lacking thioglycollate and cysteine.

Radioactive gentamicin.

^{14}C-Gentamicin (specific activity, 1.75 dpm/ng) was kindly supplied by Schering Corp., Bloomfield, N.J. ^{3}H-Gentamicin was prepared as follows: gentamicin sulfate (Schering Corp., potency 588 μg/mg) was subjected to tritium exchange by Amersham-Searle. The product was purified by Sephadex G-10 (Pharmacia, Uppsala, Sweden) chromatography as described[6]. The final purity of Sephadex G-10 purified material (as judged by paper chromatography[7]) varied from batch to batch. Considerable labelled impurities not identifiable as gentamicin C_1, C_{1a} or C_2 were present in preparations stored in water at 4°C for 14 or more days. For this reason all ^{3}H-gentamicin used in uptake studies was further purified by paper chromatography[7]. The spots containing gentamicin C_1, C_{1a} and C_2 were cut out and the gentamicin eluted in water at 20°C. These preparations were centrifuged three or more times to remove residual paper and were subsequently stored at 4°C. Purified ^{3}H-gentamicin was used within 10 days of preparation.

Gentamicin accumulation.

The filtration method used was identical to that described for streptomycin accumulation[2] except that filter pre-treatment was with 2.5ml of a gentamicin solution (200 μg/ml). Accumulation studies were performed using specific activities of 1.75 dpm/ng gentamicin for ^{14}C-gentamicin or 100~1,000 dpm/ng gentamicin for ^{3}H-gentamicin. No significant differences in accumulation patterns were determined using either ^{3}H or ^{14}C-gentamicins except that the sensitivity of ^{3}H-gentamicin uptake was much greater at low concentrations of gentamicin (<2 μg/ml).

Cell viability.

Cell viability after treatment of *P. aeruginosa* 280 with gentamicin in the presence or absence of sodium azide or of *E. coli* K12 SA 1306 with gentamicin either under aerobic or anaerobic growth conditions was as described[2].

Release of acid-soluble ^{3}H-adenine.

Measurement of acid-soluble ^{3}H-adenine released from strains either treated or not treated with gentamicin was as described[2].

Results

Accumulation of Gentamicin

Fig. 1 illustrates gentamicin accumulation by *E. coli* K12 SA 1306 at 5 μg/ml gentamicin in TSB. Three phases of accumulation are present. The initial or first phase occurs very rapidly and is not prevented by metabolic inhibitors. The magniture of first phase accumulation is very similar to that obtained if cells are exposed to gentamicin at 4°C or in the presence of 0.1% sodium azide. The amount of first phase accumulation is directly related to concentration up to at least 100 μg/ml gentamicin in TSB. A significantly greater proportion of first phase uptake is removed by washing with 3% (w/v) NaCl as compared to water. Thus charateristics of this phase of uptake are those of an ionic or VAN DER WAALS interaction of gentamicin with the cell. The portion of the cell involved is presumably superficial in that initial

Fig. 1. Accumulation of ^{3}H-gentamicin with time and cellular release of acid-soluble ^{3}H-adenine by *E. coli* K12 SA 1306 into the culture supernatant using TSB at 37°C

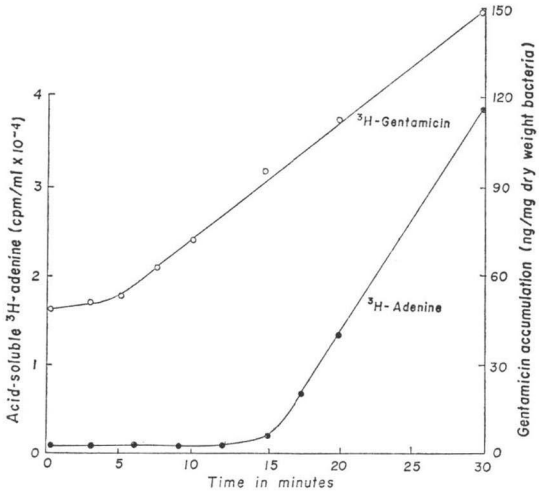
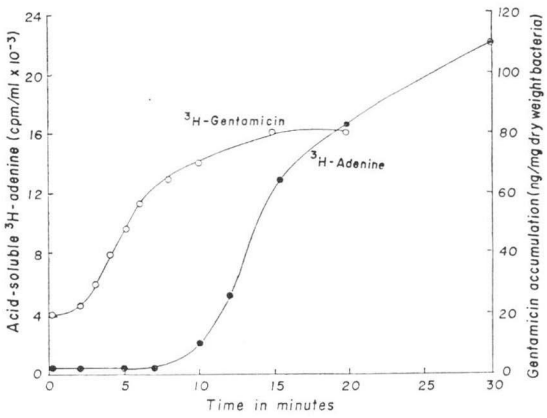


Fig. 2. Accumulation of ^{3}H-gentamicin with time and cellular release of acid-soluble ^{3}H-adenine into the culture supernant by *P. aeruginosa* 280 using TSB at 37°C



phase uptake is not associated with inhibition of cell growth or cell death (see below).

Second phase uptake (in Fig. 1 from zero time to about 7 or 8 minutes) is the lowest rate of accumulation. The duration and rate of this phase changes slightly with concentration of gentamicin. Third phase uptake begins in Fig. 1 at about 7 or 8 minutes and is linear with time for 20 or more minutes.

Fig. 2 illustrates gentamicin accumulation in *P. aeruginosa* 280. Accumulation differs from that seen with *E. coli* K12 SA 1306 in that second and third phase uptake periods are shortened. Third phase uptake levels off in about 8 minutes after which the accumulation rate reaches a plateau. Third phase accumulation is prolonged when higher concentrations of gentamicin are used in the uptake mixture. The properties noted above for first phase uptake in *E. coli* K12 SA 1306 apply to that phase in the *Pseudomonas* strains. We have examined several other *Pseudomonas* strains and they have all shown a similar accumulation profile although in many instances post-first phase uptake is not initiated until a higher gentamicin concentration than 5 μg/ml in TSB.

The amount of gentamicin accumulated in second and third phases is dependent on the concentration of gentamicin in uptake mixtures and on the growth medium. The most important variable in the latter appears to be Ca^{++} and Mg^{++} concentration (manuscript in preparation).

The intracellular concentration of gentamicin accumulated during second and third phase uptake (*i.e.* with first phase uptake subtracted) in 30 minutes by *E. coli* K12 SA 1306 and in 20 minutes by *P. aeruginosa* 280 is between 4 and 7 times the extra-cellular gentamicin concentration. These values are based on a value of 2.7 μl cell water per mg dry weight of bacteria[9] Accumulation of gentamicin reaches 150~250 times the external concentration when gentamicin accumulation is measured in NB (BBL). (see control curves Fig. 3 and Fig. 4).

Metabolic Inhibitors and Gentamicin Accumulation

A variety of inhibitors reduce or prevent second and third phase uptake in both *E. coli* and *P. aeruginosa* (Table 1). In no instance is first phase accumulation prevented. Inhibitors of electron transport and sulfhydryl groups and agents which uncouple oxidative phosphorylation and electron transport reduce gentamicin accumulation.

Table I. The effect of metabolic inhibitors on gentamicin accumulation*

Bacterial species	Inhibitors	Concentration	Inhibition (%)	
			(1)**	(2)**
Pseudomonas aeruginosa 280	4°C		58	86
	Oxamic acid	5 mM	10	15
	Na amytal	5 mM	8	12
	KCN	1 mM	67	100
	Na azide	0.1%	64	96
	DNP***	2 mM	31	54
	Sodium arsenate	50 mM	50	75
	NEM***	1 mM	67	100
Escherichia coli K12 SA 1306	4°C		73	93
	Oxamic acid	5 mM	0	0
	Na amytal	5 mM	42	57
	KCN	1 mM	74	100
	Anaerobic conditions		70	95
	Na azide	0.1%	70	95
	Sodium arsenate	10 mM	64	86
	DNP	2 mM	62	84
	NEM	1 mM	74	100

* Gentamicin concentation 2 μg/ml in TSB
** 1: % of total uptake 2: % of uptake after zero time
*** DNP: 2, 4-dinitrophenol, NEM: N-ethylmaleimide

The effects are similar for both types of bacteria except that sodium amytal is a more effective inhibitor in *E. coli*. In general, agents effective on terminal electron transport are more potent inhibitors than agents effective at earlier parts of the transport chain.

Chloramphenicol also prevents gentamicin accumulation in *E. coli* (Table 2). The addition of chloramphenicol (100 μg/ml) at either the same time as gentamicin or 10 minutes prior to the gentamicin almost totally prevents

Table 2. The effect of addition of chloramphenicol on gentamicin accumulation by *E. coli* K12 SA 1306*

Time of addition of CM*	Gentamicin accumulation (ng/mg dry weight) at various times		
	0 mins.	7.5 mins.	15 mins.
No CM added	5.1	55.5	152
minus 10 mins.	8.1	9.4	10.2
0 min.	8.1	8.4	8.5
7.5 mins.	5.1	55.5	84.3

* Accumulation was determined in nutrient broth (BBL) at 0.75μg/ml gentamicin. CM (chloramphenicol) concentration was 100 μg/ml. CM was not added in the control and was added 10 minutes prior to or at the same time or 7.5 minutes after the addition of gentamicin in other preparations.

any gentamicin accumulation. However if chloramphenicol is added 7.5 minutes after gentamicin there is a significant reduction in gentamicin accumulation but an appreciable amount of uptake occurs. These data suggest that protein synthesis is required for gentamicin accumulation even after 7.5 minutes of gentamicin uptake. However at 7.5 minutes a fraction (about one-third in this experiment) of gentamicin accumulation is independent of further protein synthesis. Presumably at 7.5 minutes a fraction of the protein or proteins necessary for transport is already synthesized and available for continuing transport. The residual rate of transport may also be reduced by a direct inhibitory effect of chloramphenicol on transport independent of its function as an inhibitor of protein synthesis. The latter conclusion is suggested by the observation that chloramphenicol succinate which does not inhibit bacterial growth does reduce gentamicin accumulation by 20~30% if added at zero time to accumulation mixtures.

These results indicate that transport of gentamicin is an active process which requires newly synthesized protein and functional sulfhydryl groups. These are characteristics exhibited by materials known to enter cells by active transport (*e.g.* lactose).

Gentamicin Accumulation and Release of Acid-soluble ^{3}H-Adenine from Cells

Streptomycin is known to alter control of membrane permeability to materials such as amino acids and oligo-nucleotides or nucleotides[1]. This effect of streptomycin on cell membranes which had been previously considered to account for post-first phase (*i.e.* inhibitor-sensitive) uptake of streptomycin has recently been shown not to be the cause of this phase of uptake. Gentamicin, like streptomycin causes release of ^{3}H-adenine-containing materials from cells. Figs. 1 and 2 illustrate the release of acid-soluble ^{3}H-adenine in relation to uptake of gentamicin. Both types of bacteria release ^{3}H-adenine after 10~15 minutes of treatment with gentamicin but accumulation of gentamicin is well underway prior to any detectable release of adenine.

Fig. 3. Accumulation of ^{3}H-gentamicin in NB at 37°C by *E. coli* K12 SA 1306 without added inhibitor or with inhibitors added 7.5 minutes after the addition of gentamicin; KCN, NaN_3, NEM

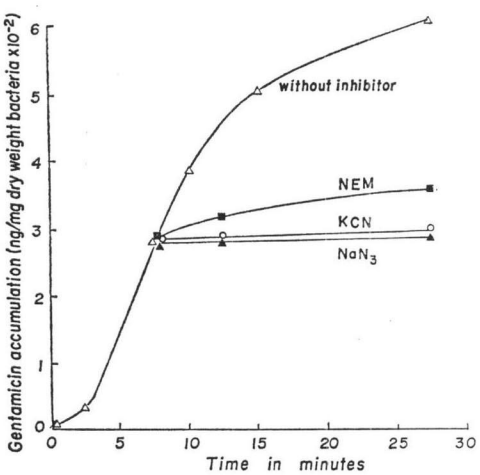
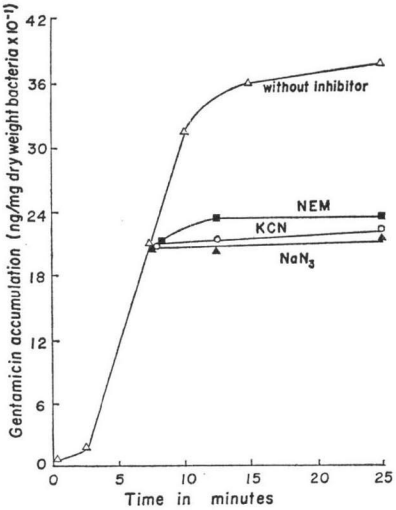


Fig. 4. Accumulation of ^{3}H-gentamicin in NB at 37°C by *P. aeruginosa* 280 without added inhibitor or with inhibitors added 7.5 minutes after the addition of gentamicin; KCN, NaN_3, NEM



A similar conclusion is reached by treating the *E. coli* and *P. aeruginosa* strains with 0.1% NaN_3 or 1mM KCN after third phase accumulation is well underway. Figs. 3 and 4 illustrate the effects of these reagents on gentamicin accumulation in NB (BBL). Gentamicin accumulation in NB is much more active due to the relatively low concentrations of Ca^{++} and Mg^{++} present (manuscript in preparation) and is a sensitive uptake system. In the results shown in Fig. 3 and Fig. 4 although the gentamicin concentration is lower than used for the experiments of Fig. 1 and Fig. 2 (0.75 μg/ml *vs* 5 μg/ml) the actual uptake is greater. However the general kinetics are similar. Uptake in NB is of value to measure low rates of accumulation and thus was selected to show that 1 mM KCN and 0.1% NaN_3 totally stop further gentamicin accumulation. However a small residual rate of accumulation persists with N-ethylmaleimide (NEM). In NB release of acid-soluble ^{3}H-adenine is already detectable at 7.5 minutes. Thus gentamicin accumulation remains an energy-dependent process at a time when ^{3}H-adenine is "leaking" from cells.

Gentamicin Accumulation and Loss of Cell Viability

Table 3 demonstrates that the effect of the addition of 0.1% NaN_3 to cells of *P. aeruginosa* is almost fully reversible (about 10% loss of viability compared to the untreated control). Gentamicin used alone causes extensive cell death but that cell death is markedly reduced by the presence of 0.1% NaN_3. Thus that component of gentamicin uptake susceptible to inhibition

Table 3. Viability of *P. aeruginosa* 280 and *E. coli* K12 SA 1306 after treatment with gentamicin in the presence or absence of sodium azide or under anaerobic growth conditions

Bacteria	Conditions of treatment*	Cell counts/ml
P. aeruginosa 280	no additives, zero time	5×10^8
	1. 0.1% NaN_3 (washed)	3×10^8
	0.1% NaN_3 (diluted)	3.5×10^8
	2. gentamicin 10 μg/ml (washed)	<10
	gentamicin 10 μg/ml (diluted)	<10
	3. 0.1% NaN_3., gentamicin 10μg/ml (washed)	2×10^8
	0.1% NaN_3., gentamicin 10μg/ml (diluted)	3×10^8
E. coli K12 SA 1306	zero time—aerobic and anaerobic	2×10^8
	anaerobic growth	5×10^8
	aerobic growth	6×10^8
	gentamicin 10μg/ml, aerobic growth	<10
	gentamicin 10μg/ml, anaerobic growth	4.2×10^8

* *P. aeruginosa* 280 was grown to 5×10^5 cells/ml in TSB (zero time). The culture was divided into 3 equal portions and held briefly at 4°C. Sodium azide (0.1%, w/v) was added to portions 1 and 3 and these were incubated at 37°C for 10 minutes. Gentamicin was then added to portions 2 and 3 and all tubes were incubated for 60 minutes at 37°C. Samples were removed and cell counts performed after washing with TSB or dilution into TSB.

E. coli K12 SA 1306 was grown to 2×10^5 cells/ml in thioglycollate broth (anaerobic) or the same broth minus thioglycollate and cysteine (aerobic). Gentamicin (10 μg/ml) was added to a set each of anaerobic and aerobic growth tubes. Control cultures were allowed to continue growth without the addition of gentamicin. Additions to anaerobic cultures were performed using a continuous flow of oxygen-free nitrogen. The growth period was 60 minutes at 37°C. Viable counts were determined following dilution in the aerobic broth and plated on that medium with 1.5% agar and incubated in air at 37°C.

by sodium azide is essential for cell death. An alternative explanation of these results is that sodium azide can prevent gentamicin-induced cell death by directly interfering with the intracellular process necessary for loss of cell viability. Loss of cell viability is currently considered due to either inhibition of protein synthesis or misreading of the amino acid code although the exact mechanism by which loss of viability occurs is unknown. Results of studies with *E. coli* argue strongly against the alternative explanation. Anaerobic conditions prevent second and third phase uptake and gentamicin-mediated cell death. *E. coli* clearly carries out protein synthesis anaerobically and would be susceptible to the inhibitory effects of gentamicin if the latter were transported to the ribosome. Thus from these studies it is clear that first phase (inhibitor-insensitive) uptake alone can not cause cell death and that productive uptake in terms of cell death is energy-dependent.

Gentamicin Extracted from Cells

Cells of *E. coli* K12 SA 1306 or *P. aeruginosa* 280 which had been exposed to ^{14}C-gentamicin for 60 minutes and washed once with 3% (w/v) NaCl were treated with 10% trichloracetic acid for 30 minutes at 22°C to extract gentamicin. This preparation was centrifuged for 15 minutes at 7,000×g and aliquots of the supernatant and cellular fractions were counted in a liquid scintillation system. Gentamicin recovered in the cell-free supernatant was 80~85% of the total original cell-associated radioactivity. Chromatography of the final supernant demonstrated about >95% of the activity was obtained within 3 peaks consistent with gentamicin C_1, C_{1a} or C_2. Thus the majority of cell-associated gentamicin can be recovered in an unchanged form.

Discussion

Accumulation of gentamicin by strains of *E. coli* and *P. aeruginosa* follows a pattern very similar to that found to exist for streptomycin accumulation by these bacteria. Gentamicin accumulation is reduced or prevented by a variety of inhibitors of electron transport, by agents which uncouple oxidative phosphorylation and electron transport, by a sulfhydryl poison and by an inhibitor of protein synthesis. The drug can be recovered after extraction from cells in an apparently unchanged form and is concentrated intracellularly over extra-cellular drug levels. Gentamicin accumulation therefore follows criteria normally assigned to accumulation of agents by active transport such as, for example, lactose.

As with streptomycin a phase of gentamicin accumulation exists which is insensitive to the preceeding inhibitors. However this gentamicin accumulation does not cause loss of cell viability and appears to be binding of gentamicin to the cell surface.

Gentamicin induces a loss of permeability control for acid-soluble ^{3}H-adenine, an effect also known to occur with streptomycin[1,4]. However active gentamicin transport (*i.e.* second and third phase uptake) is well underway before this "leaking of the cell membrane" is apparent and even at this time accumulation can be prevented by inhibitors of electron transport and markedly reduced by NEM. Thus there is no evidence for cellular entry of gentamicin by diffusion or by disorganization of the membrane as has been proposed for streptomycin entry[4,5]. This hypothesis has recently been shown to be incorrect for streptomycin entry as well[2].

The failure of cells of *E. coli* to accumulate gentamicin anaerobically strongly suggests that the inactivity of gentamicin under anaerobic growth conditions is due to a failure to transport the drug. This is supported by an examination of gentamicin transport in *Clostridium perfringens* (an obligate anaerobe) where the drug is not transported at concentrations as high

100 μg/ml. (BRYAN, L.E. unpublished results). Gentamicin and other aminoglycosides are not absorbed by the oral route which could be due to the anaerobic conditions of the gut. In addition we also have found that gentamicin transport declines markedly as the pH falls below 7 (BRYAN, L.E., unpublished results). Thus transport into cells of the stomach may be impaired on these grounds.

The actual mechanism by which gentamicin enters cells remains unclear. The effects of NEM and chloramphenicol suggest that a protein (or proteins) with reactive sulfhydryl groups is necessary. This is suggestive that a transport carrier is involved. Studies currently underway in our laboratory show that other aminoglycoside antibiotics compete with gentamicin for cell entry which suggest that a limited number of transport sites such as transport carriers exist for gentamicin.

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