Roles of Ribosomal Binding, Membrane Potential, and Electron Transport in Bacterial Uptake of Streptomycin and Gentamicin

L. E. BRYAN* AND S. KWAN

Department of Microbiology and Infectious Diseases, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada T2N 4N1

Received 12 January 1983/Accepted 31 March 1983

The effects of a set of conditions on aminoglycoside uptake were determined. Membrane vesicles either with a membrane potential (Δψ) of −125 mV (adequate to drive lysine uptake) or with succinate, lactate, or phenazine methosulfate did not accumulate gentamicin unless components of protein synthesis were included. Ribosomally resistant (rpsL) Escherichia coli cells demonstrated energy-dependent phase II uptake similar to that of a streptomycin-susceptible strain of E. coli when treated with 100 μg of puromycin per ml. Puromycin (100 μg/ml) also increased the uptake of the cationic compounds polyamine and arginine. These studies support a role of protein synthesis in aminoglycoside uptake and in the development of energy-dependent phase II. Δψ of cells did not increase either at the initiation of or during energy-dependent phase II, showing that energy-dependent phase II is not due to an elevation of Δψ. In a Bacillus subtilis system, significant streptomycin uptake requires a threshold value of Δψ which varies depending upon the concentration of streptomycin used. At 25 μg/ml, the uptake of streptomycin reached maximal levels after exceeding the threshold value, whereas at 100 μg/ml there was a gradual increase of the uptake to the maximal after the threshold value was exceeded. Several studies supported the view that electron transport has a specific role other than its requirement to produce the cellular Δψ. The uptake of gentamicin was stimulated to a greater extent by phenazine methosulfate-ascorbate than by the ionophore nigericin in strains of E. coli, although nigericin stimulated Δψ to a greater degree. Cells with 25% of the normal quinone concentration had Δψ values identical to cells with the normal quinone concentration, but the quinone-deficient cells had a significantly lower rate of gentamicin uptake. KCN prevented gentamicin uptake but did not prevent the development of Δψ. The effects of ubiquinone depletion in an E. coli strain were more evident on gentamicin uptake than on ATP-driven glutamine transport or proton motive force-driven proline transport, consistent with a specific requirement for quinones in aminoglycoside uptake. A detailed explanation of the mechanism of accumulation of streptomycin and gentamicin and a proposed mechanism for killing bacterial cells by these agents have been provided.

The uptake of the aminoglycoside antibiotics streptomycin and gentamicin has been shown to be influenced by a complex set of conditions. It has been shown that the kinetics of uptake involve an initial energy-independent phase associated with ionic binding to the cell surface and cytoplasmic membrane. This is followed by two energy-dependent phases, a slow initial rate of uptake termed energy-dependent phase I (EDP-I) and a second accelerated rate termed energy-dependent phase II (EDP-II). Initiation of the latter phase requires binding to ribosomes (reviewed in references 2 and 11).

The most effective energy source has been demonstrated to be electron transport involving quinone oxidation-reduction cycles. Evidence has been provided that the bacterial transmembranous electrical potential (Δψ) (interior of cell, negative) is the driving force (3, 4, 8, 9) for aminoglycoside entry. A model coordinating these events and explaining the role of anaerobiosis and the effects of aminoglycoside modification by aminoglycoside-modifying enzymes has been developed by our group (2–4, 10). The studies reported here were designed to test the model further and to provide more information to help answer several remaining important questions. Those questions
include the relationship of aminoglycoside uptake to the size of \( \Delta u \), greater specificity as to the role of electron transport, further information on the transporter species used by aminoglycosides, and an explanation for EDP-II. We have used the results obtained in this study to coordinate the roles of aminoglycoside uptake and perturbation of the ribosomal cycle and protein synthesis in our model to provide an explanation for aminoglycoside lethality.

**MATERIALS AND METHODS**

**Strains.** Strains used were _Escherichia coli_ AN384 _ubiA420_ menA401 rpsL, AN387 rpsL, and SA1306 pro met nal.

Media. Media used were tryptic soy broth (GIBCO Laboratories), nutrient broth (BBL Microbiology Systems), minimal salts medium (25), and gelsate-succinate aminoglycoside uptake medium consisting of 0.5% gelsate (BBL), 0.05 M Tris, and 10 mM sodium succinate (final pH, 7.0).

**Uptake of streptomycin (dihydrostreptomycin), gentamicin, glutamine, proline, spermidine, and arginine.** The uptake of \(^{3}H\)dihydrostreptomycin (3.2 Ci/mmole; Amesham Corp.) and \(^{3}H\)gentamicin (508 mCi/mmole; Amesham Corp.) was determined as previously described (5, 6, 10), using nutrient broth or nutrient broth supplemented with 1 mM 4-hydroxybenzoate or gelsate-succinate medium with added NaCl or KCl or both. Uptake of other compounds was determined as previously described (4). Specific activities and initial concentrations of \(^{3}H\)-labeled compounds were: proline, 283 mCi/mmole; 0.91 \( \mu \)M; glutamine, 42 mCi/mmole; 2.59 \( \mu \)M; spermidine, 85 mCi/mmole; 2.95 \( \mu \)M; arginine, 340 mCi/mmole; 1.75 \( \mu \)M. For determination of streptomycin uptake in the presence of puromycin, streptomycin uptake was measured as previously described (6). In those preparations to which puromycin was added, the drug was added at zero time with streptomycin. Final concentrations of puromycin used were 50, 100, 200, 500, and 1,000 \( \mu \)g/ml. Puromycin at 100 \( \mu \)g/ml produced maximal streptomycin uptake. Growth inhibitory effects of puromycin were followed by a comparison of growth rates of _E. coli_ AN384 and AN387 in the presence and absence of puromycin at 100 \( \mu \)g/ml. Puromycin produced clearly detectable deviations from logarithmic growth under these conditions for both _E. coli_ AN384 and AN387. The effect of puromycin uptake on spermidine and arginine was also determined by adding puromycin at zero time.

**Formation of membrane vesicles and measurement of uptake into vesicles.** The spheroplast formation method described by Weiss (27) was used for the preparation of spheroplasts, and the method described by Kabeck (17) was used for the preparation of membrane vesicles. Potassium-loaded vesicles were formed as described by Hirata et al. (13). Membrane vesicles containing components of protein synthesis were formed by suspension of membrane preparations into a solution of 0.1 M phosphate buffer (pH 6.6) containing 10 mM EDTA at a protein concentration of 22 mg/ml. Ribosome fractions and the 30,000 \( \times \) g supernatant were prepared as described by Modolell (20), using a buffer consisting of 50 mM Tris-hydrochloride (pH 7.8), 60 mM NH4Cl, 15 mM magnesium acetate, and 6 mM 2-mercaptoethanol. The final protein concentration of the ribosomal preparation and the 30,000 \( \times \) g supernatant was 10 mg/ml. The 30,000 \( \times \) g supernatant was enriched by the addition of 5 mM creatine phosphate, 50 \( \mu \)g of creatine kinase per ml, 0.8 mg of _E. coli_ K-12 transfer RNA per ml, 0.1% Casamino Acids (if added), 6.6 \( \times \)10^{-2} M ATP, 10^{-3} M GTP, and 100 \( \mu \)g of polyuridylic acid per ml. In some experiments, a 2\( \times \)-concentrated ribosomal preparation was added to the 2\( \times \)-concentrated, enriched 30,000 \( \times \) g supernatant to obtain a final concentration of 10 mg of protein per ml of 30,000 \( \times \) g supernatant and ribosomes. Equal amounts of the membrane preparation and either the ribosomal preparation or enriched 30,000 \( \times \) g supernatant or the combined enriched 30,000 \( \times \) g supernatant and ribosomal preparation or the enriched 30,000 \( \times \) g supernatant minus Casamino Acids were mixed. Vesicles were allowed to form under these conditions at 48°C for 10 min. Preparations were chilled in ice, centrifuged at 30,000 \( \times \) g for 10 min, and washed one time with 0.4 M sucrose–5 mM MgSO4–7H2O. Vesicles were further suspended and uptake was determined as described previously (16, 19), using a final concentration of 100 or 200 \( \mu \)g of gentamicin per ml and modifying the procedure to wash vesicles on membrane filters as described for dihydrostreptomycin and gentamicin uptake (5, 6).

**Induction of \( \Delta \psi \) into membrane vesicles.** Potassium-loaded vesicles were formed as described previously (13). Resuspended vesicles were treated by the addition of 10 \( \mu \)l of valinomycin dissolved in 95% ethanol (200 or 400 \( \mu \)g/ml) to obtain a final concentration of 2 or 4 \( \mu \)g/ml. The valinomycin–ethanol mixture or 10 \( \mu \)l of 95% ethanol (control) was added to the vesicle preparation at the times indicated in Fig. 4. Thereafter, lysine and gentamicin uptake in the vesicles was measured as described previously (16, 19) except that in the case of gentamicin uptake the washing procedure was that described for gentamicin uptake (5).

**TPP uptake and determination of \( \Delta \psi \).** Tetraphenylphosphonium (TPP) uptake was measured and the \( \Delta \psi \) values were determined as described by Kashket (18), using \(^{3}H\)tetraphenylphosphonium ([\( ^{3}H\)TPP]) bromide at a final concentration in 10 \( \mu \)M and a specific activity of 4.3 Ci/mmole. Background binding of TPP was determined by using toluene-treated cells as described by Kashket (18). TPP uptake was continued until steady-state accumulation was reached. Only the first steady-state value is shown in most cases in Fig. 6, 7, and 9. Cell water was 1.6 \( \mu \)l/mg (dry weight). For the \( \Delta \psi \) values determined at 30°C, a value of Z of 60 mV was used and for the uptake at 37°C a value of 61.7 mV was used. All \( \Delta \psi \) values are the average of three separate determinations. Phthalamine methosulfate (PMS), sodium ascorbate, and nigericin were used at final concentrations of 0.1 mM, 20 mM, and 10 \( \mu \)M, respectively. To determine the influence of PMS-ascorbate or nigericin on gentamicin or TPP uptake, cells were suspended in nutrient broth at absorbance readings at 600 nm (\( A_{500} \)) of 0.4 for gentamicin or streptomycin uptake and about 2 for TPP uptake (1 \( A_{500} \) unit = 0.35 mg [dry weight]). A final concentration of 10 mM EDTA was added to this preparation and mixed at the temperature of uptake for 2 min. Cells were washed with nutrient broth by centrifugation at 3,000 \( \times \) g and suspended at the same cell
concentration in nutrient broth containing a final concentration of 0.5 mM EDTA. For aminoglycoside uptake, the aminoglycoside was added simultaneously with either nigericin or the preparation of PMS-ascorbate. For TPP uptake, TPP was added immediately before the addition of nigericin or PMS-ascorbate. For quinone-sufficient cells, growth and uptake were carried out in nutrient broth containing 1 mM 4-hydroxybenzoate. Quinone-deficient cells were allowed to undergo two cell generations at 37°C in nutrient broth. Under these circumstances, cells contained approximately 25% of the normal content of quinone.

To determine the effect of gentamicin on Δψ, the following protocol was used. E. coli AN384 was grown in nutrient broth supplemented with 4-hydroxybenzoate from a starting A_{600} reading of 0.1 to a final reading of 0.4. Cells were centrifuged at 3,000 × g and suspended in nutrient broth at an A_{600} reading of about 0.4. These cells were treated for 2 min at 30°C with EDTA at a final concentration of 10 mM. Cells were washed with nutrient broth and suspended to the original optical density. Preparations were divided into a control and a gentamicin pretreatment sample. The control was incubated in nutrient broth containing 0.5 mM EDTA for 20 min at 30°C. Cells were washed with nutrient broth and suspended to an optical density of about 2 for TPP uptakes. The gentamicin pretreatment sample was suspended in nutrient broth containing 0.5 mM EDTA and 10 μg of gentamicin per ml and incubated under identical circumstances. These cells were washed and suspended to a similar A_{600} reading to carry out TPP uptake. The second method for determination of the effect of gentamicin on Δψ was by measurement of Δψ in the presence of gentamicin. Cells were treated for 2 min at 30°C in a preparation of nutrient broth containing 10 mM EDTA. Cells were washed and suspended in nutrient broth containing a final concentration of 0.5 mM EDTA at an A_{600} reading of about 0.4. This preparation was divided into three portions. To the first was added [3H]TPP at a final concentration of 10 μM; to the second [3H]TPP (10 μM) and a final concentration of gentamicin of 10 μg/ml were added; to the third a final concentration of 10 μg of tritiated gentamicin per ml was added. TPP uptake was measured from the first and second preparations, and gentamicin uptake was measured from the third preparation.

Electron transport and energy coupling. Oxygen consumption as a measure of electron transport was determined as previously described (4). Cytochrome spectra and quinone contents were determined as described previously (4, 22).

Δψ and potassium concentration in Bacillus subtilis. Shioi et al. (24) previously described a method of manipulating the Δψ and ΔpH components of the proton motive force. We modified this method so that there was a linear relationship between the extracellular potassium concentration and Δψ at a lower ionic strength than described by Shioi et al. (24). The medium used was the gellyate-succinate medium containing 10 mM succinate. To this, different combinations of potassium chloride and sodium chloride were added to obtain a final concentration of 25 mM potassium or sodium. TPP uptake and Δψ were determined as described above. Dihydrostreptomycin uptake was determined at concentrations of 25 and 100 μg/ml as described above.

**RESULTS**

Components of protein synthesis and aminoglycoside uptake. Previous studies have shown that the development of EDP-II of streptomycin transport can be prevented by the use of rpsL mutants (6). R-factors which specify streptomycin adenylylation and reduce ribosomal affinity for streptomycin are associated with absent or reduced EDP-II transport (10). Similar findings occur with gentamicin acetylation (L. E. Bryan, unpublished data). These studies demonstrated that ribosomal binding contributes significantly to the total uptake of streptomycin or gentamicin.

Figure 1 illustrates the uptake of gentamicin, using membrane vesicles derived from E. coli formed in the presence of components of protein synthesis. Vesicles did not accumulate gentamicin unless both an energy source and all of the components needed to establish protein synthesis were present. We did not establish whether protein synthesis actually occurred under these circumstances. Removal of amino acids, the ribosome preparation, or the 30,000 × g supernatant or the addition of 1 mM KCN prevented gentamicin uptake.

Membrane vesicles (without protein synthesis components) with succinate, lactate, or PMS as energy sources accumulated proline and lysine

![FIG. 1. Uptake of gentamicin at 37°C into sodium membrane vesicles with ribosomes, amino acids and 30,000 x g supernatant or 200μg/ml (\(\Delta\)) plus 1mM KCN](http://aac.asm.org/DownloadedFrom/aac.asm.org)
Puromycin was equally effective at inhibiting cell growth in ubiquinone-deficient or -sufficient strains, demonstrating that quinone deficiency did not alter puromycin entry.

The effect of puromycin was not limited to streptomycin. The rates of accumulation of the polyamine spermidine and the cationic amino acid arginine were also increased by treatment of cells with 100 μg of puromycin per ml (Fig. 3).

Δψ and gentamicin uptake. Evidence has been provided that the electrically negative Δψ of bacterial cells is the driving force for gentamicin and streptomycin entry (3, 9). Figure 4 shows the results of uptake of gentamicin and the cationic amino acid lysine into K⁺-rich vesicles treated with valinomycin to induce Δψ. As shown, there was a rapid influx of lysine but no detectable uptake of gentamicin. Uptake of [³H]TPP demonstrated that Δψ of −125 mV was achieved under these circumstances (in excess of the threshold value of Δψ needed for streptomycin uptake in whole cells [see below]). This experiment demonstrated that Δψ alone cannot induce gentamicin accumulation into membrane vesicles, whereas it can for another cation, lysine.

Shioi et al. (24) have described a system in which B. subtilis is used that allows the manipulation of components of the proton motive force. We previously used this system to relate Δψ to gentamicin uptake (3). In those preliminary stud-

FIG. 2. Dihydrostreptomycin (streptomycin) uptake at 2 μg/ml in nutrient broth (37°C) by either a ribosomally streptomycin-susceptible E. coli K-12, SA1306 strain (Δ), or a ribosomally resistant strain of E. coli K-12, AN387, in the absence (○) or presence (●) of puromycin (Pm) at 100 μg/ml and dihydrostreptomycin uptake by a ribosomally resistant strain of E. coli, AN384, grown in nutrient broth in the absence of 4-hydroxybenzoate so that the strain has less than 1% of its normal quinone complement in either the presence (■) or the absence (□) of 100 μg of puromycin per ml.

but did not accumulate gentamicin (data not shown). This was so even for membranes obtained from cells preexposed to 10 μg of gentamicin or streptomycin per ml for either 10 or 15 min.

Hurwitz and colleagues have previously shown that E. coli rpsL mutants show accelerated uptake of streptomycin when treated with selected concentrations of puromycin (15). Results shown in Fig. 2 confirm that observation and that streptomycin uptake in a puromycin-treated rpsL mutant is very similar to that of a streptomycin-susceptible strain. Higher (200, 500, and 1,00 μg/ml) or lower (50 μg/ml) puromycin concentrations produced lower rates of streptomycin uptake. Also shown in Fig. 2 is the dependence of the enhanced uptake produced by puromycin on electron transport in that cells with <1% the ubiquinone content of the wild type did not show the effect of puromycin.

FIG. 3. Uptake by E. coli AN387 at 37°C of arginine in the presence of puromycin (Pm) at 100 μg/ml (Δ) or the absence of puromycin (Δ) and the uptake of spermidine (Spe) in the presence of puromycin at 100 μg/ml (●) or absence of puromycin (○).
ies, we found that a threshold of Δψ was required to produce gentamicin uptake with no increase in uptake with a further increase in Δψ. The disadvantage of the system was that the gentamicin uptake obtained was poor due to the high ionic strength used in the system. We report here the development of a modified system that allows the manipulation of Δψ at a much lower ionic strength, which was a condition favorable to aminoglycoside uptake.

Figure 5 shows that the values of Δψ are linearly related to K⁺ concentrations between 0 and 10 mM when the total ionic strength was constant. When streptomycin uptake was measured at 25 μg/ml, there was a clear threshold relationship with Δψ in that the uptake occurred at 3 mM K⁺ (−119 mV) but was greatly reduced at 6 mM K⁺ (−107 mV). We also measured the uptake at 4.5 mM K⁺ (−112 mV) and determined that the uptake is equal to that at 3 mM K⁺. Thus, a threshold value of −107 mV is needed to drive significant streptomycin uptake at 25 μg/ml under the conditions used. At 100 μg/ml, streptomycin uptake fell off less abruptly. The uptake was clearly decreased at 6 mM K⁺ but fell to a very low level at 10 mM K⁺. Streptomycin uptake at 100 μg of streptomycin per ml required a lower Δψ and increased gradually between 10 and 3 mM K⁺.

These studies demonstrate that a threshold Δψ is required for streptomycin uptake although this varies depending on the streptomycin concentration.

One of the possible explanations for the onset of EDP-II is an increase in Δψ due to initial effects of streptomycin or gentamicin. EDP-II is associated with increased rates of accumulation of polyamines (14) as well as the cationic amino acid arginine (4). These observations are also found with puromycin treatment of cells (Fig. 3). Data in Fig. 6 and 7 show that rather than an increase in the uptake of TPP associated with the onset of EDP-II for gentamicin, there was a decrease in Δψ whether TPP uptake was measured with gentamicin present (Fig. 6) or after pretreatment with gentamicin (Fig. 7). Thus, EDP-II cannot be caused by an increase in Δψ.

Is electron transport required for aminoglycoside uptake only to produce Δψ? It is possible that the requirement for electron transport needed for significant gentamicin uptake is due to its being the most effective means of producing Δψ.

To determine whether electron transport works entirely through creation of Δψ or whether it has a specific role in aminoglycoside transport, we determined gentamicin uptake with PMS-ascorbate. In these experiments, we compared the gentamicin uptake induced by PMS-ascorbate with the uptake produced by the ionophore nigericin, which produced greater values of Δψ than those produced by PMS-ascorbate.

To compare rates of electron transport and Δψ with strains of E. coli, it was necessary to determine an EDTA concentration that allowed access of nigericin and TPP to the cytoplasmic membrane but did not significantly alter gentamicin uptake. A final concentration of 0.5 mM EDTA in uptake mixtures was determined to meet these requirements.

Results (Fig. 8) demonstrate that PMS-ascorbate-stimulated uptake both in quinone-sufficient cells of E. coli AN384 and in cells depleted to about 25% of the usual quinone concentration (quinone-deficient cells) (cf. Fig. 8A with B). In contrast, although nigericin stimulated Δψ to values greater than those obtained with PMS-ascorbate (Fig. 8B and C), there was no stimulation of gentamicin uptake in quinone-sufficient or -deficient cells (Fig. 8A and C).

Quinone-sufficient and -deficient cells demonstrated similar Δψ values under all conditions examined but showed significantly different rates of gentamicin uptake (Fig. 8A, B, and C).

These results support a specific role of electron-transport distinct from the development of Δψ.

FIG. 4. Uptake of lysine and gentamicin (200 μg/ml) at 37°C by membrane vesicles of E. coli K-12 SA106 as described in the text. Lysine and gentamicin were added at zero time, and ethanol and valinomycin or ethanol alone was added at the times indicated by the arrows.
FIG. 5. (A) Magnitude of $\Delta \psi$ with different concentrations of potassium in whole cells of *B. subtilis* at 30°C (X) and the uptake of streptomycin at 25 µg/ml (B, lower panel) or 100 µg/ml (B, upper panel) at different potassium concentrations.

We examined the effect of 5 mM KCN on the uptake of TPP and gentamicin associated with the use of PMS-ascorbate (Fig. 9). KCN use inhibited almost all gentamicin uptake but was still associated with the uptake of TPP and the development of $\Delta \psi$. Unlike the pattern of TPP uptake seen in the absence of KCN, a loss of TPP occurred in later samples, suggesting a gradual loss of $\Delta \psi$. However, the inhibitory effect was much greater on gentamicin uptake than on $\Delta \psi$.

The relationship in *E. coli* AN384 among ubiquinone content, ATP-driven glutamine transport, proton motive force-driven proline transport, cytochrome content, O$_2$ consumption, and gentamicin uptake is given in Table 1. These data show that glutamine transport was independent of the quinone concentration to at least a reduction to 6% of that normally found. Proline transport was reduced at low quinone concentrations but is still about 70% of the rate seen with the normal amount of quinones. In contrast, gentamicin transport fell off markedly at 25% or lower quinone ratios. These findings illustrate the unique dependence of aminoglycoside uptake on quinone-associated electron transport in bacteria.

**DISCUSSION**

The results reported in this series of studies are consistent with results predicted by our model of aminoglycoside uptake (1, 2). An updated version of that model is explained in Fig. 10. Support for this comprehensive model is now strong. Nakae and Nakae recently provided good evidence that aminoglycosides penetrate the outer membrane of gram-negative bacteria such as *E. coli* through the aqueous pores formed by porin proteins (21). The only current exception to this seems to be *Pseudomonas aeruginosa*, where aminoglycosides likely disrupt the outer membrane as a mode of penetration (12).

Transmission across the cytoplasmic membrane is clearly dependent on $\Delta \psi$ (3, 9), as predicted in the model. However, some questions had not been fully answered. What is the relationship of the magnitude of $\Delta \psi$ and the rate of aminoglycoside transport? Does electron
transport have a direct role in aminoglycoside transport in addition to its role in the development of ΔΨ? What is the nature of the species acting as the membranous transporter for aminoglycosides? Why does the transport rate increase (EDP-II) after initial EDP-I transport?

Previously it was proposed that the transporter responsible for EDP-II was a polyamine transport system (14). This proposal was based on the acceleration of polyamine transport associated with the onset of EDP-II. However, there is little evidence to support this proposal. Polyamines do not induce streptomycin (14) or gentamicin uptake (4), mutants exist with differential effects on polyamine and aminoglycoside uptake (7), and a new or increased quantity of protein induced by streptomycin cannot be demonstrated (1). These findings argue effectively against the polyamine transport system. Streptomycin and gentamicin are not saturable transport systems in normal bacteria, and the transport system has diffusion kinetics. Again, this argues against a specific carrier system being involved (but does not exclude multiple carrier systems).

Results in this study demonstrate that there is a role in aminoglycoside transport for electron transport outside of its requirement to develop ΔΨ. This has been shown by PMS-ascorbate being more effective than nigericin in stimulating aminoglycoside uptake despite a smaller change in ΔΨ. It has also been supported by the different extent of the effect of KCN on ΔΨ and on aminoglycoside uptake, by the failure of ΔΨ in vesicles to drive aminoglycoside uptake despite driving lysine uptake, by the reduction of aminoglycoside uptake seen in quinone-deficient cells compared with quinone-sufficient cells despite similar ΔΨ values in both types of cells, and to a lesser extent by the differential effect of quinone depletion on aminoglycoside uptake compared with proton motive force-driven proline uptake. Conversely, a direct relationship between aminoglycoside uptake and the size of ΔΨ has not

![FIG. 6. Uptake of TPP by E. coli SA1306 at 30°C in nutrient broth in the absence of gentamicin (●) or in the presence of 10 µg of gentamicin per ml (X) and the uptake of gentamicin by gentamicin (10 µg/ml)-treated cells (■).](http://aac.asm.org/)

![FIG. 7. Uptake of TPP by E. coli SA1360 cells preexposed to 10 µg of gentamicin per ml in nutrient broth at 30°C (●) or in cells not exposed to gentamicin (X).](http://aac.asm.org/)
FIG. 8. Uptake of gentamicin (10 μg/ml) and Δψ values at 30°C for quinone-sufficient and -deficient (see the text) cells of E. coli AN384 in preparations treated as controls (A), treated with PMS (0.1 mM) and sodium ascorbate (20 mM) (B), or treated with nigericin (10 μM) (C). Quinone-deficient cells (Ubi-') were grown for two generations in nutrient broth with no added 4-hydroxybenzoate, and quinone-sufficient (Ubi') cells were grown in nutrient broth with 1 mM 4-hydroxybenzoate. Uptakes were carried out in nutrient broth containing a final concentration of 0.5 mM EDTA. All cells were treated for 2 min before the uptake with 10 mM EDTA, washed, and suspended in the uptake medium.

been supported by the failure of nigericin to stimulate uptake in quinone-sufficient and -deficient E. coli, by the decrease in Δψ seen with the onset of EDP-II, and by the relationship of the size of Δψ to streptomycin uptake at 25 μg/ml in B. subtilis. The last studies show that a threshold for Δψ is needed but that additional uptake does not result with increasing Δψ. However, at 100 μg of streptomycin per ml, some additional uptake does occur with increasing Δψ. Damper and Epstein (9) have reported a relationship between Δψ and the minimal inhibitory concentration of streptomycin.

The direct role of electron transport clearly is related to quinone content. Our results provide further evidence for a direct role of quinones but do not confirm that they are the transporter. The pleiotropic effects of the reduction of the quinone content makes a final statement on quinones very difficult indeed.

Figure 10 is a diagrammatic representation of a model of the processes involved in aminoglycoside uptake and lethality. The following is an explanation of the processes proposed to account for the uptake and lethality.

The polycationic aminoglycoside molecule ("Ag") binds to negative charges on the cell surface and passes through water-filled spaces (porin-formed pores in gram-negative bacteria (21); interstices of the cell wall in gram-positive bacteria) to reach and ionically bind to the cytoplasmic membrane. Cell surface binding groups are mainly phosphate residues of lipopolysaccharides and lipoteichoic acids; cytoplasmic binding sites are phosphate heads of phospholipids and possibly hydrophilic compo-

TABLE 1. Relationship of ubiquinone content, cytochrome content, and oxygen consumption with gentamicin, proline, and glutamine uptake in E. coli AN384

<table>
<thead>
<tr>
<th>Ubiquinone ratio*</th>
<th>Glutamine (Acpm/2 min × 10⁻³)</th>
<th>Proline (Acpm/2 min × 10⁻³)</th>
<th>Cytochrome content*</th>
<th>O₂ consumed (ratio)</th>
<th>Gentamicin (Acpm/A₅₀₀ at 30 min [ratio])</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>90</td>
<td>28</td>
<td>No change</td>
<td>1</td>
<td>800 (1)</td>
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<tr>
<td>5</td>
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<td>28</td>
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<tr>
<td>0.06</td>
<td>102</td>
<td>35</td>
<td>ND</td>
<td>0.08</td>
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</table>

* E. coli AN384 requires 4-hydroxybenzoate to form quinones and was grown in defined medium (25) for various times without 4-hydroxybenzoate (selective medium). Cells were recovered by centrifugation and suspended in (i) selective medium with glucose for O₂ consumed, (ii) selective medium without amino acids for amino acid uptake and cytochrome scans, and (iii) aminoglycoside uptake medium for gentamicin uptake at a gentamicin concentration of 15 μg/ml. The ubiquinone content in cells grown with 4-hydroxybenzoate was 0.25 pmol/mg (dry weight; ratio = 1).

* Rates were linear over the time period measured.

* 500 to 640 nm. ND, Not determined.
With each transfer event, it is proposed that the structure of the cytoplasmic membrane is disturbed (potential lethal events), resulting in an initial loss of permeability control for small molecules (K⁺) and later larger molecules (bases, nucleotides, small oligonucleotides, etc.) and still later for proteins (β-glucosidase) (5, 6, 11).

In bacteria which contain modifying enzymes, these enzymes are located at the outer (10) or inner (23) surface of the cytoplasmic membrane. The enzymes modify Ag⁺ in transport and reduce its affinity for the ribosome (10). Thus, transfer events do not occur or are markedly slowed, resulting in a failure of lethality for all or most cells. If the rate of transport exceeds the rate of inactivation, sufficient transfer events occur to produce lethality (10). In rpsL mutants, transfer events do not occur due to a loss of ribosomal affinity for Ag⁺ (streptomycin) (6).

Lethality in the preceding would result from the cumulation of the effects on the cytoplasmic membrane of the transfer of aminoglycosides to the binding sink. Any process that increases the rate of these transfer events (puromycin) would increase killing, and those processes that reduce it (rpsL mutation, aminoglycoside modification, anaerobiosis, inhibition of electron transport, collapse of Δψ, or others) would decrease killing. We propose that lethality results from a gradual disruption of membrane integrity and, subsequently, function due to the physical forces involved in the transfer events. This is supported by the long-recognized loss of permeability control due to aminoglycosides (11). It also explains the requirement for ribosomal binding and perturbation of protein synthesis to cause killing. The study of Hurwitz et al. (15) and our studies clearly show that EDP-II can be mimicked by puromycin, which produces at least some effects similar to streptomycin. We have also shown that EDP-II is not due to an increase in Δψ (this study) and that both the aminoglycoside- and puromycin-induced EDP-II require quinones (2; this study). We have also shown that in membrane vesicles aminoglycoside uptake did not occur in the presence of various energy sources or with Δψ adequate to direct whole-cell uptake. It appeared to require components needed for protein synthesis. These results are fully consistent with our previous studies showing that EDP-II is prevented (or reduced) by rpsL mutations or by aminoglycoside modification (6, 10).

To date, the vast majority of experimental results are consistent with all major aspects of our model explaining aminoglycoside accumulation and lethality. In our view, it provides a full understanding of the way that aminoglycosides work.
FIG. 10. Model for aminoglycoside (Ag) uptake and lethality. See the text for details. *Ag+, Polycationic aminoglycosides; Ag-S, enzymatically modified aminoglycoside.

ACKNOWLEDGMENTS

We gratefully acknowledge financial support by the Medical Research Council of Canada. We are also grateful for the typing of the manuscript by Joan Godfrey and Claire Kenno.

LITERATURE CITED


