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RELATION OF AEROBIOSIS AND IONIC STRENGTH TO THE UPTAKE OF DIHYDROSTREPTOMYCIN IN *ESCHERICHIA COLI*

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Summary

Aminoglycoside antibiotics exhibit a markedly reduced antibacterial activity under anaerobic conditions. Anaerobiosis or inhibitors of electron transport produced an extensive decrease in the uptake of dihydrostreptomycin in *Escherichia coli* K-12. Uptake of proline or putrescine were only slightly impaired under anaerobic conditions in the presence of glucose. Both the susceptibility to and the uptake of dihydrostreptomycin under anaerobic conditions were partially restored by addition of the alternative electron acceptor, nitrate. This stimulation required functional nitrate reductase activity. Abolition of uptake by 2,4-dinitrophenol under both aerobic and anaerobic conditions indicates that streptomycin uptake requires electron transport as well as a sufficient membrane potential. In addition, the initial rate of dihydrostreptomycin uptake was competitively and reversibly inhibited by added salts. The inhibition was relatively nonspecific with respect to the identity of salt added, being approximately dependent on the ionic strength. Although dihydrostreptomycin and polyamines mutually inhibited each other's uptake, several conditions (polyamine limitation, streptomycin uptake-deficient mutants) were found in which uptake of these two substrates was oppositely affected. Aminoglycosides thus do not appear to enter on one of the usual cellular transport systems, but perhaps utilize a component of the electron transport system.

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

Introduction

Antibiotic entry into bacterial cells represents an interesting but complicated subject. Entry of any hydrophilic antibiotic must be mediated by some cytoplasmic membrane constituent. Selective pressures demand that any carrier must not be involved solely in antibiotic uptake, but must play some important role in cellular metabolism. Some antimicrobial agents are analogues of normal cellular constituents and are accumulated by the transport system for that nutrient [1—3]. Other macromolecular lethal agents, such as phages or colicins, enter by means of cell surface receptors which are structural or transport components of obvious physiological function [4,5].

Of particular interest is the uptake of streptomycin and other aminoglycoside antibiotics, which is a complex process exhibiting several unusual characteristics. Bryan and van den Elzen [6] separated the uptake of dihydrostreptomycin by sensitive cells into three phases. Energy-independent binding of the drug to the cell surface was followed by energy-dependent transport processes and then by binding of drug to sensitive ribosomes. The latter phase comprised the most rapid and extensive accumulation of the drug. Certain unusual features of bacterial susceptibility to aminoglycosides were reflected or caused by the properties of these transport processes. Both the lethality and the rapid phase of dihydrostreptomycin uptake behave as if inducible, in that both require brief exposure of cells to an aminoglycoside under conditions permissive for protein synthesis; rapid uptake did not occur in mutant strains with streptomyin-insensitive ribosomes [6—8]. Furthermore, killing by aminoglycosides is dependent on aerobic conditions and is strongly influenced by the composition of the growth medium [9—12]. Transport of dihydrostreptomycin is severely depressed under anaerobic conditions or in the presence of inhibitors of electron transport [6,9] and is also inhibited by divalent cations [6,13] or polyamines [14].

There have been two recent suggestions for the identity of the streptomycin carrier in *Escherichia coli*. Based on the transport activities of mutants blocked in different steps of aerobic energy metabolism, Bryan and van den Elzen [13] proposed that aminoglycosides bind to a membrane complex involved in maintaining the high-energy membrane state, possibly ubiquinones. Ubiquinone-deficient mutants exhibit reduced rates of streptomycin uptake, and the remaining activity was insensitive to inhibition by divalent cations. In contrast, Höltje [14] proposed that streptomycin enters by means of a polyamine transport system which is only induced by the presence of aminoglycosides. Evidence for this proposal was the mutual competitive inhibitions of uptake of aminoglycosides and polyamines.

This paper examines the apparent dependence of dihydrostreptomycin uptake on aerobic conditions and the effect of cations. It will be shown that the requirement for oxygen can be replaced by an alternate electron acceptor, nitrate. Furthermore, both polyamines and divalent cations inhibit dihydrostreptomycin uptake, probably through the same mechanism. The transport properties of mutants with depressed streptomycin uptake and of strains starved for polyamines demonstrated the non-parallel response of polyamine and dihydrostreptomycin uptake. The suggestion will be made that the aminoglycoside carrier is a component of the electron transport system.

Materials and Methods

Bacterial strains and growth

Escherichia coli K-12 strain W1485F⁻ was employed in most experiments. From it were derived mutants resistant to either high levels of streptomycin (>200 μg/ml), low levels of streptomycin (20 μg/ml), low levels of streptomycin and neomycin (each 20 μg/ml), or chlorate. The *hemA* strain RK3303 was a neomycin-resistant mutant which required addition of δ-aminolevulinic acid (20 μg/ml) for growth with succinate as sole carbon source [15]. The polyamine-requiring strain MA255 (*thi thr leu rpsL speB speC*) was provided by W.K. Maas. All strains were stored in 20% glycerol at −70°C, then tested for their appropriate growth responses before use.

Media employed for growth of cells included nutrient broth (Difco), L broth [16], or minimal medium A of Davis and Mingioli [17], supplemented with glucose (0.5%) and required supplements. Amino acids and putrescine were added to 100 μg/ml; δ-aminolevulinic acid to 20 μg/ml. Cells were grown aerobically in nephelometer flasks at 37°C with vigorous aeration. Anaerobic cultures were grown with constant bubbling with sterile N_2. Cells were harvested by centrifugation and were washed twice and suspended in the specified medium. Washed cells were kept at 4°C for no more than 2 h prior to assay.

Effect of streptomycin on viability

Washed cells were suspended in the specified medium to a density of 5×10^8/ml and incubated at 37°C under a stream of either O_2 or N_2 for 10 min. Streptomycin was added to final concentrations ranging from 0.1 to 100 μg/ml. At intervals, samples were withdrawn, diluted in L broth, and spread on L agar plates. Viable cells were enumerated after overnight incubation at 37°C.

Transport assays

Washed cells, suspended in one of the media listed above or in 10 mM Hepes buffer (pH 7.0), were stored on ice. Cells were equilibrated for 10 min at 37°C. either in open flasks or, when specified, under a stream of O_2 or N_2. Any additions were present during this incubation period. Then, [^{3}H]dihydrostreptomycin (Amersham/Searle) was added to the specified final concentration (1 μCi/ml; 29.2 mCi/mmol). The substrate concentration in the routine assay was 33 μM. Portions (0.20 ml) were removed at intervals (usually 0.3, 3, 5, 10 and 20 min) and were filtered on membrane filters (0.45 μm pore size; Millipore, Co.). The filters were washed with 5 ml of Hepes buffer, immediately removed from the filtration apparatus, and dried. Radioactivity retained on the filters was measured in a scintillation spectrometer with toluene-Omnifluor (New England Nuclear, Inc.). Correction was made for substrate bound to filters in the absence of cells. Similar procedures were employed to measure the uptake rates of [^{14}C]proline (4 μM, 0.2 μCi/ml, final) and [^{14}C]putrescine (10 μM, 0.5 μCi/ml, final). Uptake data are expressed in terms of pmol of substrate accumulated per μl of cell water. A value of 2.5 μl of cell water per mg dry weight had been previously determined.

Results

Requirement for electron transport

Decreased killing by and uptake of aminoglycosides under anaerobic conditions has been reported [6,9,11]. This reduction in streptomycin uptake under anaerobic conditions was partially overcome by the addition of potassium nitrate; about 50% of the aerobic uptake rate occurred in the presence of 2.5 mM KNO_3 (Fig. 1A). Higher concentrations of nitrate resulted in reduced rates of dihydrostreptomycin uptake under either aerobic or anaerobic conditions. In these experiments, glucose was present to provide a fermentative energy source. The rates of uptake of proline and putrescine were depressed less than 10% during anaerobic incubation under these conditions.

Stimulation of dihydrostreptomycin uptake by nitrate required prior growth of the cells both under anaerobic conditions and in the presence of nitrate (usually 0.5%), in order to induce formation of nitrate reductase activity. Less than 10% stimulation by nitrate occurred with uninduced cells. A chlorate-resistant mutant lacking nitrate reductase activity did not exhibit nitrate-stimulated anaerobic dihydrostreptomycin uptake after growth under inducing conditions (Fig. 1B). Another electron acceptor, fumarate (0.5%), did not stimulate anaerobic dihydrostreptomycin uptake in cells grown anaerobically in the presence of fumarate; however, aerobic dihydrostreptomycin uptake was also strongly inhibited by this concentration of fumarate. Under all conditions, dihydrostreptomycin uptake was totally blocked by the uncoupler, 2,4-dinitrophenol (1 mM).

Nitrate also stimulated the rate of killing by streptomycin of anaerobic cells. For example, exposure of strain W1485F$^-$ to 10 μg of streptomycin per ml of nutrient broth for 10 min resulted in the decrease in viability to 0.002%. There was at least 10% survival under anaerobic conditions. Survival decreased to

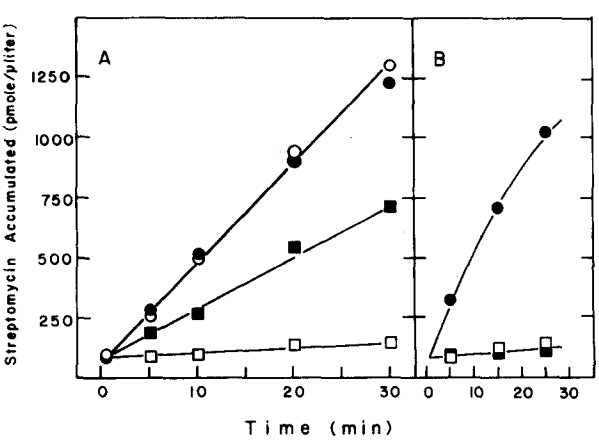

Fig. 1. Effect of nitrate and anaerobiosis on uptake of dihydrostreptomycin. Cells were grown in L broth into mid-log phase anaerobically in the presence of 0.05% KNO_3. Cells were washed and suspended in 10 mM Hepes buffer with 0.5% glucose. Cells were incubated for 10 min at 37° C under a stream of either O_2 (circles) or N_2 (squares), with (filled symbols) or without (open symbols) 0.025% KNO_3, before addition of labeled dihydrostreptomycin. Panel A, wild-type strain W1485F$^-$; Panel B, chlorate-resistant derivative.

0.7% when nitrate-induced anaerobic cells were incubated with nitrate (2.5 mM). Thus, effective streptomycin uptake was dependent not on aerobic conditions, but on the occurrence of electron transport, in addition to a sufficient protonmotive force.

Bryan and van den Elzen [13] found that *hemA* mutants, blocked in the synthesis of the heme precursor, δ-aminolevulinic acid, were unable to accumulate dihydrostreptomycin when grown in the absence of δ-aminolevulinic acid for many generations. This apparent requirement for electron transport was studied further. The *hemA* strain RK3303 was grown aerobically in nutrient broth with δ-aminolevulinic acid. Washed cells were then inoculated into duplicate flasks with nutrient broth and glucose, one containing δ-aminolevulinic acid, the other not. Both cells maintained the same growth rate for 2.5 generations, after which growth in the unsupplemented culture slowed markedly (Fig. 2). Prior to the onset of growth limitation, cells from both cultures were competent for dihydrostreptomycin accumulation. By the time cells became limited for heme, uptake activity was almost completely abolished, even though the cells were still capable of considerable further growth. We had previously shown that δ-aminolevulinic acid-starved cells of this strain were capable of proline uptake at a rate 40% of that of 5-aminolevulinic acid-supplemented aerobic cells [15]. The results obtained were consistent with a strict dependence of dihydrostreptomycin uptake on electron transport.

Effect of assay medium

Numerous previous reports had shown that aminoglycoside uptake and killing are strongly influenced by the composition of the assay medium. Accordingly, most previous transport assays had been carried out in nutrient broth

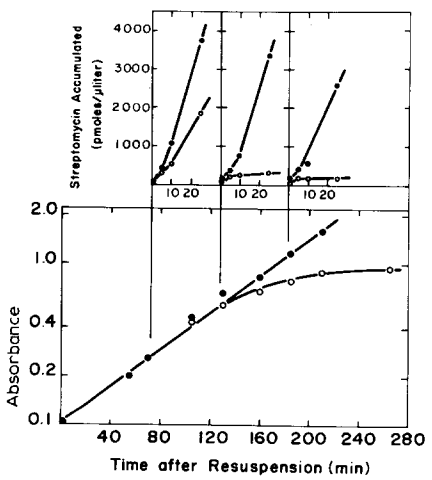

Fig. 2. Effect of heme limitation on streptomycin uptake. Strain RK3303 (*hemA*) was grown overnight and then into log phase in L broth containing 20 μg of δ-aminolevulinic acid per ml. Cells were washed and suspended in L broth with (filled symbols) or without (open symbols) δ-aminolevulinic acid. Aeration was continued at 37°C. The lower panel shows the absorbance of the cultures. At the indicated times, portions of the cells were removed, washed, suspended in Hepes-glucose buffer, and assayed for uptake of dihydrostreptomycin, shown in the upper panel at the same time scale and with the same symbols.

without added salt. The previously mentioned inhibition of aerobic dihydrostreptomycin uptake by nitrate and fumarate confirmed this effect of the medium. Uptake in L broth or in minimal medium A was barely detectable. Cells suspended in 10 mM Hepes buffer or in medium A diluted 10-fold with water had uptake rates at least as high as those measured in nutrient broth. Addition of glucose stimulated uptake to a level approximately twice that seen in nutrient broth. Accumulation of dihydrostreptomycin by cells in Hepes-glucose buffer required concomitant protein synthesis since exposure of the cells to spectinomycin (100 μg/ml) for 10 min before addition of labeled dihydrostreptomycin completely abolished uptake. The effect of assay medium was immediate and reversible; uptake activity was characteristic of the assay medium and independent of the medium in which the cells had been grown or suspended prior to assay.

Effect of ionic strength

Based on these observations, the effect of added salts on the initial rate of dihydrostreptomycin uptake in Hepes-glucose buffer was measured (Fig. 3, Table I). As previously shown by Höltje [14], the polyamines spermidine, putrescine, and cadaverine were effective competitive inhibitors of dihydrostreptomycin uptake. Divalent inorganic cations were also potent inhibitors. Much higher concentrations of monovalent ions, such as KCl or NaCl, were needed for comparable inhibition. Some inorganic cations (Zn(II), Co(II), and CU(II) and Fe(III)) inhibited dihydrostreptomycin uptake at concentrations below 0.1 mM, but also inhibited proline uptake and are likely to be non-specific inhibitors. In the same concentration range, both polyamines and divalent cations ($CaCl_2$, $MgCl_2$) inhibited putrescine uptake. Both streptomycin and KCl also inhibited putrescine uptake at concentrations much lower than were effective at inhibiting proline uptake (half-inhibition in the range of 50 to 100 mM) (Table I).

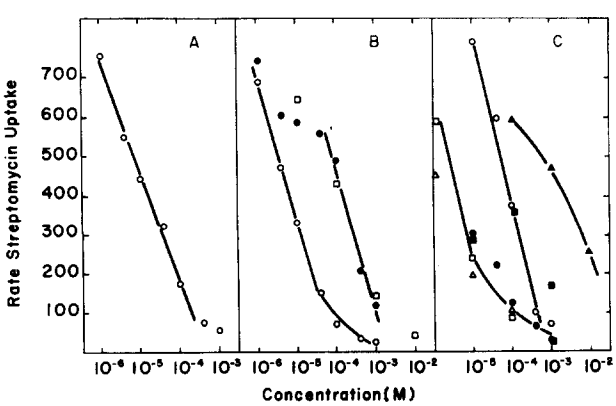

Fig. 3. Effect of added salts on rate of uptake of dihydrostreptomycin. Cells of strain W1485F⁻ were grown in L broth with aeration, then washed and suspended in Hepes-glucose medium at 37° C. Portions were added to a mixture of [^{3}H]dihydrostreptomycin (34 μM) and the addition at the indicated concentration. Uptake of label was measured over the initial 5 min period and is plotted in terms of pmol/μl per min. Panel A: streptomycin (○). Panel B: spermidine (○); putrescine (●); cadaverine (□). Panel C; $MgCl_2$ (○); $CaCl_2$ (●); cobaltous acetate (□); cupric acetate (■); zinc acetate (△); KCl (▲).

TABLE I

INHIBITION BY SALTS OF UPTAKE OF DIHYDROSTREPTOMYCIN AND PUTRESCINE

Strain W1485F⁻ was grown in medium A with glucose, then washed and suspended in Hepes-glucose. The initial rates of uptake of dihydrostreptomycin (34 μM) and putrescine (10 μM) were determined over the 5 min after addition of substrate along with the indicated competitor at concentrations from 10 μM to 50 mM. The degree of inhibition in at least two experiments was plotted against concentration of inhibitor, as in Fig. 3, and the concentration giving 50% inhibition extrapolated from the plot. n.d., not done.

Inhibitor	Concentration (μM) for 50% inhibition of uptake of	
	Dihydrostreptomycin	Putrescine
Streptomycin	33	45
Spermidine	24	14
$MgCl_2$	30	200
$CaCl_2$	33	200
Putrescine	140	n.d.
Cadaverine	245	n.d.
KCl	4700	4500
NaCl	4700	n.d.
Potassium phosphate (pH 7.0)	5700	n.d.

Transport in streptomycin-resistant mutants

There were several classes of streptomycin-resistant mutants of strain W1485F⁻. Some were specifically resistant to even high levels of streptomycin (200 μg/ml), presumably as a result of altered ribosome binding of the drug (StrA). Others were resistant only to lower levels of streptomycin (20 μg/ml); some of these were also resistant to low concentrations of neomycin. These strains were fully sensitive to streptomycin at 200 μg/ml. The transport activities of several representatives of these classes of streptomycin-resistant mutants were determined (Table II). For all strains, the initial rate of dihydrostreptomycin uptake (measured at 5 min) was a linear function of the external dihydrostreptomycin concentration over the range from 1 to 70 μM. In Hepes-glucose medium, the StrA-type mutant had essentially the same initial rate of

TABLE II

TRANSPORT ACTIVITIES OF STREPTOMYCIN-RESISTANT MUTANTS

Uptake was measured in Hepes-glucose buffer during the first 5 min after addition of labeled substrate. All strains were derived from W1485F⁻; StrA is resistant to >200 μg of streptomcyin/ml; Str(20) is resistant to 20 μg of streptomycin/ml, but sensitive to 100 μg/ml; StrNeo(20) is resistant to 20 μg of streptomycin or neomycin/ml, but sensitive to 100 μg/ml of either. The substrate concentration were dihydrostreptomycin, 34 μM: putrescine, 10 μM; proline, 4 μM. Uptake activities are expressed as pmol of substrate accumulated per μl of cell water per min.

Strain	Initial rate of uptake		
	Dihydro-streptomycin	Putrescine	Proline
W1485F⁻ (Str^+)	620	680	500
StrA	610	660	510
Str(20)	135	1000	380
StrNeo(20)	135	860	290

dihydrostreptomycin uptake as the parental strain; at later times, the level of dihydrostreptomycin accumulated was lower than that in the parental strain. The mutants resistant only to low levels of streptomycin exhibited markedly lower rates of dihydrostreptomycin uptake, approx. 20% of that of the parental strain.

The level of general transport activity was assessed from the rate of proline uptake. Proline uptake was normal in the StrA-type mutant, but markedly reduced in the low-level resistant mutants; the degree of reduction was not as extreme as was that of dihydrostreptomycin uptake. In contrast, the rate of putrescine uptake was somewhat higher in the low-level resistant mutants than in either the parental or StrA-type strains. In all strains, putrescine uptake was completely inhibited in identical manner, i.e., 50% inhibition of uptake of 10 μM putrescine by streptomycin in the concentration range from 10 to 30 μM.

Transport in putrescine-starved cells

The effect of polyamine deprivation on dihydrostreptomycin uptake activity was determined in the polyamine auxotrophic strain MA255 of *E. coli*. Washed cells of this strain which had been grown overnight in the presence of putrescine were dispensed into duplicate flasks containing minimal medium without or with putrescine. The cells in the unsupplemented culture stopped growth after 6 h, having gone through three doublings. At this time, cells from both cultures were washed and suspended in Hepes-glucose buffer for assay of uptake activity. Relative to the cells from the supplemented culture, dihydrostreptomycin uptake in the putrescine-limited cells was reduced by 40 to 50%; proline uptake was depressed by 30%. In contrast, putrescine uptake was elevated by at least 150%. Uptake of streptomycin in both cultures was almost completely blocked by 0.1 M KCl, showing that the response of dihydrostreptomycin uptake to high salt was not mediated through cellular polyamines. Uptake of putrescine was 90% inhibited by 0.1 M KCl in putrescine-supplemented cells, but only 70% inhibited in polyamine-limited cells.

Discussion

There are several interesting and unusual features of aminoglycoside uptake which make it difficult to postulate that these antibiotics enter bacterial cells by means of some nutrient uptake system. Uptake of dihydrostreptomycin appears to be dependent on the operation of the electron transport system. It is possible to maintain an adequate protonmotive force in cells under anaerobic conditions or in the presence of cyanide as long as glucose or another fermentative carbon source is present to allow ATP generation by substrate-level phosphorylation. This is evident from the only moderate reduction in proline or putrescine uptake activities under these conditions. However, these conditions resulted in the drastic reduction in the rate of dihydrostreptomycin uptake and in the lethality resulting from exposure to streptomycin. Certainly at least some component of the protonmotive force is also required for dihydrostreptomycin accumulation, as is evident from the inhibition produced by proton permeability of the cytoplasmic membrane [13].

The effect of anaerobiosis was not a specific response to oxygen, but rather to the inhibition of electron transport. This requirement for electron transport could be supplied by the alternate electron acceptor, nitrate, in cells capable of using nitrate as electron acceptor. Full restoration of dihydrostreptomycin uptake activity by nitrate under anaerobic conditions was not observed, nor was significant stimulation provided by fumerate. This probably resulted from the operation of two antagonistic actions of these additives, whereby increasing concentrations of these ions would stimulate electron transport but inhibit dihydrostreptomycin uptake through their effects on the ionic strength of the medium. Further evidence for the role of electron transport came from the results with the *hemA* mutant conditionally unable to make heme. Dihydrostreptomycin uptake activity declined in close proportion to the number of doublings of the cells in the absence of heme precursor, i.e., dihydrostreptomycin uptake rates appeared to be proportional to the amount of heme moieties present and, presumably, to the amount of electron transport occurring. Very few of the nutrient transport systems that have been studied are so sensitive to inhibitors of oxidative metabolism; one example is citrate uptake in *Salmonella* [18]. Perhaps dihydrostreptomycin uptake is mediated by some component of the electron transport chain and the mobility of this component to allow uptake requires the oxidized state or the cyclic oxidation and reduction of this component which would be blocked by inhibition of electron transport.

There is a relationship between aminoglycoside and polyamine uptake in that both inhibit each other's uptake, as shown by Höltje [14]. However, several conditions were found to affect the uptake of these two types of substrates in opposite directions, so that it is difficult to maintain that aminoglycosides enter on polyamine transport systems. As noted above, putrescine uptake activity was much less sensitive to anaerobiosis than was that of dihydrostreptomycin. Mutants insensitive to low but not to high levels of streptomycin were readily obtained. These were often affected in their response to other aminoglycosides and exhibited decreased uptake of dihydrostreptomycin. These mutants also had decreased rates of proline uptake, suggestive of a defect in maintenance of the protonmotive force. Surprisingly, they exhibited somewhat elevated putrescine uptake activities. The current inability to isolate mutants specifically defective in aminoglycoside uptake is consistent with an essential role of this uptake component in aerobic metabolism.

Not only did polyamines inhibit dihydrostreptomycin uptake, but so too did all other ions tested. Spermidine was the most effective inhibitor found; at higher concentrations, putrescine and cadaverine were equally inhibitory. Inorganic salts inhibited at concentrations far below those necessary to inhibit proline uptake. Sucrose was not inhibitory in this range of concentrations. This suggests that uptake of dihydrostreptomycin, and of the polyamines, is inhibited by elevated ionic strength. It should be noted that the salt concentrations needed for 50% inhibition of dihydrostreptomycin uptake varied over about a 100-fold range, much more than the 10-fold difference expected for the contribution of these compounds to the ionic strength of the medium. Thus, the observed competition between dihydrostreptomycin and polyamines for uptake need not be for binding to a carrier, but could be represented by compe-

tition for binding to nonspecific sites on the cell surface or simply by their contribution to the ionic strength.

The inhibitory effect of ionic strength on the uptake of dihydrostreptomycin and the polyamines could have several explanations. It is interesting to note that, whereas elevated ionic strength inhibits the uptake of these cationic substrates, the uptake of several anionic substrates, including glucose 6-phosphate [19], requires the presence of salt, the identity of which is relatively unimportant. Increasing ionic strength would both shield anionic groups on the cell surface and dissipate the Donnan equilibrium potential (30 mV, interior negative [20]) across the outer membrane. The dissipation of these charges and potentials by salt could have the effect of impeding uptake of cationic substrates and accelerating uptake of anionic substrates, as is observed.

It is apparent that aminoglycoside entry consists of several stages, the major one of which requires the occurrence of protein synthesis in the presence of an aminoglycoside. The mechanism whereby this period of protein synthesis under the influence of these drugs affects the membrane so as to allow increased uptake of aminoglycosides and of polyamines remains to be elucidated.

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