Autostimulation of Dihydrostreptomycin Uptake in *Bacillus subtilis*

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In *Bacillus subtilis* it was shown that the membrane potential ($\Delta\psi$) has to reach a threshold value of -180 to -190 mV for efficient uptake of dihydrostreptomycin to occur. The magnitude of $\Delta\psi$ is raised above this threshold, and dihydrostreptomycin uptake greatly enhanced, not only by dihydrostreptomycin itself (autostimulation) and by other miscoding aminoglycoside antibiotics, but also by puromycin, bacitracin and *N,N'*-dicyclohexylcarbodiimide. Stimulation of uptake by dihydrostreptomycin or puromycin was dependent on a specific interference with ongoing protein synthesis. Thus, chloramphenicol prevented the stimulating effect of puromycin by lowering the magnitude of $\Delta\psi$. Although normally severely antagonizing streptomycin accumulation, K^+ , as well as spermidine and putrescine, which are known to stabilize ribosomes, consequently enhanced autostimulation of dihydrostreptomycin uptake in a K^+ -retention mutant with impaired protein synthesis. It is suggested that miscoding aminoglycosides and puromycin both enhance dihydrostreptomycin uptake by increasing $\Delta\psi$ due to ion fluxes, which are themselves caused by a dramatic stimulation of intracellular proteolysis of faulty proteins.

INTRODUCTION

Efficient killing of bacteria by streptomycin correlates well with the rate of its accumulation inside the cell (Hancock, 1962; Bryan *et al.*, 1976, 1979; Taber & Halfenger, 1976; Miller *et al.*, 1980; Eisenberg *et al.*, 1984). The factors which determine the uptake of streptomycin are thus of particular importance for its therapeutic efficiency.

In many aerobic bacterial strains [3H]dihydrostreptomycin uptake is autostimulated resulting in a biphasic uptake process (Anand *et al.*, 1960; Hurwitz & Rosano, 1962; Bryan & Van den Elzen, 1976). An early energy-dependent phase (EDPI) with a low rate of uptake is followed by a second greatly enhanced energy-dependent phase (EDPII) of uptake (Bryan & Van den Elzen, 1977). Self-enhancement of dihydrostreptomycin accumulation, a prerequisite for its antibacterial action (Hancock, 1981), has been suggested to depend on codon-misreading during protein synthesis (Hurwitz & Rosano, 1962; Höltje, 1978; Davis *et al.*, 1986). With *Escherichia coli* it has been shown that preincubation with streptomycin not only increases dihydrostreptomycin accumulation but also the uptake of the polyamines putrescine and spermidine (Höltje, 1978). However, the molecular basis that triggers streptomycin uptake with increased rate (EDPII) remains obscure.

A recent model proposes the creation of membrane channels by misread proteins to be responsible for the increase in uptake (Davis *et al.*, 1986). While the model indeed can account for a number of previously unexplained observations it fails to explain convincingly the energy

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; TPMP⁺, triphenylmethylphosphonium ion; EDPI, EDPII, energy-dependent phases I and II; $\Delta\psi$, transmembrane potential difference ('membrane potential'); ΔpH , transmembrane pH difference; Δp , transmembrane proton electrochemical difference ('protonmotive force').

dependence of uptake as well as the specificity of stimulated uptake. Furthermore, it cannot explain a number of findings described in the present communication.

It is now well established that the uptake of dihydrostreptomycin and other aminoglycoside antibiotics into bacteria is energized by the electrical component ($\Delta\psi$) (interior of cell negative) rather than the chemical component (ΔpH) of the proton electrochemical difference (Δp) across the cytoplasmic membrane (Damper & Epstein, 1981; Mates *et al.*, 1982; Bryan & Kwan, 1983; Eisenberg *et al.*, 1984; Arrow & Taber, 1986). It was, therefore, of interest to follow the membrane potential during the course of self-enhancement of dihydrostreptomycin accumulation. The method most used to estimate $\Delta\psi$ determines the partition of a permeable lipophilic cation, like tetraphenylmethylphosphonium (TPMP⁺), inside and outside the cell. Being a trivalent cation dihydrostreptomycin interferes with this technique, which therefore cannot be used in this case.

Interestingly, among the many protein synthesis inhibitors besides aminoglycosides causing misreading, only puromycin also stimulates streptomycin uptake. The mechanism of puromycin-induced enhancement of uptake may be considered an analogous situation to the autostimulation phenomenon since both antibiotics cause ribosomes to synthesize non-functional proteins. Therefore, an attempt was made to follow $\Delta\psi$ during puromycin-stimulated dihydrostreptomycin uptake. Based on the experimental results a model was developed proposing a common basis for the mechanism of autostimulation and puromycin-enhanced dihydrostreptomycin uptake.

METHODS

Bacterial strains and growth conditions. All strains were grown routinely in 1% Tryptone broth (Bacto, Difco) with shaking at 37 °C. This medium contained approximately 11.5 mM-Na⁺, 0.8 mM-K⁺ and 0.19 mM-Mg²⁺. *Bacillus subtilis* 60015 (*trpC metC*) was a gift from Dr E. Freese (NIMH, NIH, Bethesda, Md., USA). The K⁺-retention mutant 168KL was kindly provided by Dr K. Willecke (Westdeutsches Tumorzentrum, Essen, FRG) and the ribosomally streptomycin-resistant strain SRB15 (*trpC lys3 metB strA*) was obtained from Dr G. H. Chambliss (University of Wisconsin, Madison, Wis., USA).

Uptake experiments. Uptake measurements were started by adding appropriate concentrations of the respective radioactive solute (³H]dihydrostreptomycin, 6.6 µM; 37 kBq ml⁻¹; or [¹⁴C]spermidine, 6.6 µM; 4.625 kBq ml⁻¹) to cells growing in the mid-exponential phase of growth at an OD₅₇₈ of about 0.2. At the indicated times 0.5 ml samples were withdrawn and collected by filtration through cellulose nitrate membranes (pore size 0.45 µm; Sartorius) which had been pre-soaked in 0.1 M-LiCl. The filters were immediately rinsed twice with 3 ml 0.1 M-LiCl, placed into scintillation vials and dried at 70 °C for 30 min. After the addition of 5 ml scintillation fluid (Quickscint 401; W. Zinsser) the radioactivity was determined in a liquid scintillation counter (mark II; Nuclear Chicago Corp.). The results were corrected for adsorption of radioactive material to membrane filters, non-specific binding to the bacterial cell surface and uptake by passive diffusion. For this, controls were prepared either lacking cells or with cells pre-treated with carbonyl cyanide *m*-chlorophenylhydrazine (CCCP). Further control samples were quickly cooled to 4 °C before the addition of the respective solute. Growth of the cells throughout the uptake experiment was monitored and the results were corrected for changes in the optical density of the culture. The relationship between OD₅₇₈ and cell titre was determined using the viable count method; dry weight and intracellular water space were calculated on the basis of the values published by Shioi *et al.* (1980).

Measurement of $\Delta\psi$. This was done by measuring the distribution of the lipophilic cation [¹⁴C]TPMP⁺ at a final concentration of 8.4 µM (1.48 kBq ml⁻¹) as described by Shioi *et al.* (1980). The lipophilic anion tetraphenylboron was added to the cell suspension at a final concentration of 2 µM 0.5 min before TPMP⁺ addition to enhance TPMP⁺ uptake. Because TPMP⁺ adsorbs strongly to cellulose nitrate, in these experiments cellulose acetate membrane filters (pore size 0.45 µm; Schleicher and Schüll) were used. The filters were pre-soaked with 8.4 µM unlabelled TPMP⁺ in 0.1 M-LiCl solution. After collection of the cells on the filters they were rinsed twice with 3 ml 0.1 M-LiCl containing 8.4 µM unlabelled TPMP⁺. $\Delta\psi$ was calculated from the Nernst equation: $\Delta\psi = (2.3 RT/F) \log([TPMP^+]_{out}/[TPMP^+]_{in})$. At 37 °C, the value of 2.3 RT/F is about 61.25 mV. TPMP⁺ uptake was usually complete in less than 1 min and remained constant for about 1 to 6 min. The mean of the values measured within that time range (normally three values per individual experiment) was taken to calculate the momentary $\Delta\psi$ of the cells at zero time, i.e. the addition of [³H]TPMP⁺ to the cell suspension. The results were corrected for adsorption of TPMP⁺ to the filters. Controls for non-specific binding of TPMP⁺ to the cells were done as stated for dihydrostreptomycin uptake measurements but with toluenized cells. This procedure showed that there was no substantial non-specific binding of TPMP⁺ to the cells.

Fig. 1. Effect of various inhibitors on the uptake of dihydrostreptomycin by *B. subtilis* 60015. Cells were grown in complex medium at 37 °C to an OD_{578} of about 0.2 and pre-incubated for 3 min in the presence of 10 μ g actinomycin ml^{-1} (▲), 10 μ g bacitracin ml^{-1} (○), 10 μ g DCCD ml^{-1} (△), 8 μ g puromycin ml^{-1} (□) or for 5 min in the presence of 100 μ g chloramphenicol ml^{-1} (■). One culture received no inhibitor and served as a control (●). [3H]Dihydrostreptomycin was added at time zero to give a final concentration of 5 μ g ml^{-1} (37 kBq ml^{-1}); samples were withdrawn at the indicated times and accumulation of dihydrostreptomycin was determined as described in Methods. The arrows indicate the times of inhibitor addition after initiation of dihydrostreptomycin uptake.

Chemicals. [3H]Dihydrostreptomycin (66.6 GBq $mmol^{-1}$; 37 MBq ml^{-1}); [^{14}C]spermidine (740 MBq $mmol^{-1}$; 1.85 MBq ml^{-1}) and [^{14}C]TPMP iodide (177.6 MBq $mmol^{-1}$; 3.7 MBq ml^{-1}) were obtained from Amersham. Puromycin, bacitracin, actinomycin, chloramphenicol, streptomycin, kanamycin and gentamicin were purchased from Serva. Tetraphenylboron and DCCD were bought from Sigma and putrescine, spermidine and valinomycin from Calbiochem. All other chemicals used were reagent grade and obtained from various commercial sources.

RESULTS

Correlation between dihydrostreptomycin uptake and the magnitude of $\Delta\psi$

Inhibiting or stimulating effects of various antimetabolites on the rate of uptake of dihydrostreptomycin were found to correlate well with changes in $\Delta\psi$. Cells in the exponential phase of growth were either pre-incubated with the effectors before the addition of [3H]dihydrostreptomycin or the agents were added after the onset of dihydrostreptomycin uptake. As shown in Fig. 1 pre-incubation with bacitracin, DCCD and puromycin resulted in a pronounced enhancement of dihydrostreptomycin uptake as well as an increase in the magnitude of $\Delta\psi$ (Table 1). The peculiar biphasic character of uptake was abolished in the stimulated uptake curves, that is no further autostimulation was observed. The EDPI phase of uptake seemed to be skipped and dihydrostreptomycin accumulation occurred from the very beginning at a rate characteristic of the EDPII phase of uptake. In contrast to these agents, chloramphenicol and actinomycin impaired dihydrostreptomycin uptake and decreased ($-\Delta\psi$), as compared with the control, when added to cells taking up dihydrostreptomycin or when added before [3H]dihydrostreptomycin (Fig. 1; Table 1).

From these measurements it can be concluded that wild-type *B. subtilis* 60015 cells, which during the exponential phase of growth have a standard $\Delta\psi$ of -168 mV when grown in Tryptone broth at 37 °C, seem to have to exceed a certain threshold of $\Delta\psi$ (about -180 to -190 mV) to be able to take up dihydrostreptomycin at the rate of EDPII. This is clearly seen in a plot of dihydrostreptomycin uptake rate against $\Delta\psi$ (Fig. 2). Threshold values for dihydrostreptomycin uptake in *B. subtilis* have been reported and shown to vary depending on dihydrostreptomycin as well as K^+ concentration (Bryan & Kwan, 1983). At a concentration of 25 μ g dihydrostreptomycin ml^{-1} and 3 mM- K^+ a threshold $\Delta\psi$ of -119 mV was found. Since we determined $\Delta\psi$ at a dihydrostreptomycin concentration of 5 μ g ml^{-1} and in the presence of 0.8 mM- K^+ the values are consistent with each other.

Table 1. *Effect of various inhibitors on the membrane potential ($\Delta\psi$) in *B. subtilis*.* $\Delta\psi$ was determined from [^3H]TPMP⁺ measurements as described in Methods.

Strain	Medium supplement	Pre-treatment		$\Delta\psi^*$ (mV)
		Inhibitor	Concn ($\mu\text{g ml}^{-1}$)	
60015 (wild type)		–	–	-168 ± 4 (29)
		Chloramphenicol	100	-145 ± 20 (9)
		Puromycin	8	-190 ± 4 (12)
		Bacitracin	10	-182 ± 2 (4)
		DCCD	10	-199 ± 3 (15)
		Chloramphenicol	100	
		+ DCCD	10	-182 ± 7 (6)
		Chloramphenicol	100	
SRB15 (ribosomally streptomycin-resistant)		+ puromycin	8	-153 ± 6 (6)
		–	–	-138 ± 9 (6)
168KL (defective in K^+ -retention)	3 mM- K^+	DCCD	10	-187 ± 5 (6)
		–	–	-184 ± 1 (3)
	6 mM- K^+	–	–	-163 ± 4 (9)
		DCCD	10	-184 ± 3 (6)
	30 mM- K^+	–	–	-171 ± 3 (3)
		DCCD	10	-179 ± 5 (3)
	10 μM -spermidine	–	–	-160 ± 3 (3)
		DCCD	10	-177 ± 8 (3)
				-184 ± 3 (6)

* Mean value $\pm$ SD; no. of determinations in parentheses.

Interestingly, the reduction in the magnitude of $\Delta\psi$ caused by pre-incubation in the presence of chloramphenicol was overcome by DCCD but not by puromycin (Table 1), although in neither case was the obligate threshold value reached.

*Dihydrostreptomycin and polyamine uptake in the ribosomally streptomycin-resistant mutant *B. subtilis* SRB15*

Autostimulation of dihydrostreptomycin uptake does not take place in ribosomally streptomycin-resistant strains, although other miscoding aminoglycosides do enhance dihydrostreptomycin uptake in such mutants (Höltje, 1979). This was confirmed for *B. subtilis* SRB15 (Fig. 3). Besides kanamycin and gentamicin puromycin and DCCD also stimulated dihydrostreptomycin uptake (Fig. 3) by increasing the magnitude of $\Delta\psi$ (shown for DCCD in Table 1). Generally, dihydrostreptomycin accumulation in this mutant was lower than in the wild-type. This parallels the finding that the magnitude of the standard $\Delta\psi$ as well as the elevated potential provoked by DCCD pre-incubation is lower in *B. subtilis* SRB15.

A ribosomally streptomycin-resistant mutant offers the opportunity of measuring dihydrostreptomycin uptake under conditions where the miscoding effect of dihydrostreptomycin and its resulting autostimulation of uptake is eliminated. Under these conditions, the trivalent cation spermidine is taken up by DCCD-stimulated mutant cells to the same extent as dihydrostreptomycin when added in equimolar concentrations (Fig. 4), indicating a non-specific electrogenic transport of these polycations. Because the pK' of the secondary amine group of dihydrostreptomycin is about 7.6 and the pH of the assay medium is 7.2, dihydrostreptomycin is likely to be taken up in its trivalent cation form.

*Dihydrostreptomycin uptake in the K^+ -retention mutant *B. subtilis* 168KL*

Cations and monovalent ions (in particular K^+) antagonize uptake and killing of bacteria by aminoglycoside antibiotics (Campbell & Kadner, 1980; Hancock, 1981). On the other hand, the first measurable alteration in the physiology of the target cell caused by dihydrostreptomycin is

Fig. 2. Dependence of the rate of dihydrostreptomycin uptake on $\Delta\psi$. Different values of $\Delta\psi$ were obtained by pre-treatment of *B. subtilis* 60015 as indicated (see Fig. 1 and Methods). [^3H]Dihydrostreptomycin uptake was measured over a 3 min time interval.

Fig. 3

Fig. 4

Fig. 3. Uptake of dihydrostreptomycin by the ribosomally streptomycin-resistant mutant *B. subtilis* SRB15. Cells were grown in complex medium at 37 °C to an OD_{578} of about 0.2 and pre-incubated for 5 min in the presence of 5 $\mu\text{g ml}^{-1}$ of streptomycin (●), gentamicin (□) or kanamycin (△), or in the presence of 8 μg puromycin ml^{-1} (■) or in the presence of 10 μg DCCD ml^{-1} (▲) before addition of [^3H]dihydrostreptomycin (5 $\mu\text{g ml}^{-1}$; 37 kBq ml^{-1}); one batch served as a control (○). Samples were withdrawn as indicated and dihydrostreptomycin accumulation was determined as described in Methods.

Fig. 4. Comparison of spermidine and dihydrostreptomycin uptake by DCCD-stimulated *B. subtilis* SRB15. Cells were grown at 37 °C in complex medium to an OD_{578} of about 0.2 and incubated for 3 min in the presence of 10 μg DCCD ml^{-1} . The culture was divided into two subcultures: to one 6.6 μM -[^3H]dihydrostreptomycin (37 kBq ml^{-1}) was added (○); to the second 6.6 μM -[^{14}C]spermidine (4.625 kBq) was added (△). Samples were withdrawn at the indicated times and the accumulation of spermidine or dihydrostreptomycin was determined as described in Methods.

Fig. 5. Effect of valinomycin on uptake of dihydrostreptomycin by *B. subtilis* 60015. Cells were grown in complex medium at 37 °C to an OD₅₇₈ of about 0.2. The culture was divided into two subcultures: one was pre-incubated for 3 min in the presence of 10 μM DCCD ml^{-1} (----) before addition of [^3H]dihydrostreptomycin (5 $\mu\text{g ml}^{-1}$; 37 kBq ml^{-1}) to both subcultures. Valinomycin (5 $\mu\text{g ml}^{-1}$) was added (arrowed) at 0.75 min (Δ) or 1.25 min ($\square$) after initiation of dihydrostreptomycin uptake; one batch received no valinomycin and served as a control ($\bullet$). Dihydrostreptomycin accumulation was determined as described in Methods.

an efflux of K^+ (Dubin *et al.*, 1963), which coincides with the onset of the stimulated phase of dihydrostreptomycin uptake. In the light of $\Delta\psi$ being the driving force for aminoglycoside accumulation it is a reasonable assumption that efflux of K^+ increases ($-\Delta\psi$) and thereby stimulates dihydrostreptomycin uptake (EDPII). It was, therefore, of interest to study the effect of the K^+ -specific ionophore valinomycin on dihydrostreptomycin uptake.

Valinomycin, when added very early during the course of autostimulation, i.e. in the EDPI uptake phase, greatly enhanced uptake (Fig. 5) whereas when given during the second phase of uptake (EDPII) it inhibited dihydrostreptomycin uptake (not shown). Also, for cells which had been pre-treated with DCCD ('stimulated for uptake') a defined time period was found in which further stimulation of dihydrostreptomycin uptake by valinomycin could be achieved (Fig. 5).

Unfortunately, unlike in *E. coli*, only one K^+ -transport mutant of *B. subtilis* has been described so far. This mutant (*B. subtilis* 168KL) shows normal K^+ uptake but is unable to retain and accumulate K^+ (Willis & Ennis, 1968). For normal growth sufficient K^+ has to be supplied with the medium. In Tryptone broth containing 0.8 mM - K^+ the mutant grew slowly; uptake of dihydrostreptomycin was quite poor and no autostimulation took place (Fig. 6), although a $\Delta\psi$ value of -183 mV (Table 1) was determined. Addition of increasing amounts of K^+ to the growth medium (which stimulated growth only slightly) increased overall streptomycin accumulation but amplified the stimulatory effect on the rate of uptake caused by pre-treatment of the cells with DCCD (Fig. 6).

An even more dramatic enhancement of dihydrostreptomycin uptake in the K^+ -retention mutant was observed in the presence of a 10 μM concentration of the polyamines putrescine or spermidine, known to stabilize ribosomes and thus stimulate misreading induced by dihydrostreptomycin (Goldemberg & Algranati, 1981). It should be noted that the polyamines, unlike DCCD, did not increase ($-\Delta\psi$) but obviously enabled the cells to be autostimulated by dihydrostreptomycin as indicated by the biphasic character of dihydrostreptomycin accumulation (Fig. 7).

Fig. 6

Fig. 7

Fig. 6. Effect of K^+ on the uptake of dihydrostreptomycin by the K^+ -retention mutant *B. subtilis* 168KL. Cells were grown in complex medium at 37 °C in the presence of 0.8 mM ($\circ$), 3 mM ($\blacksquare$), 6 mM ($\blacktriangle$) or 30 mM ($\bullet$) K^+ . Each culture was divided into two and one of each pair of subcultures was pre-incubated for 3 min in the presence of 10 μ M DCCD ($----$) before addition of [3 H]dihydrostreptomycin (5 μ M; 37 kBq ml^{-1}). Samples were taken as indicated and treated as described in Methods.

Fig. 7. Effect of polyamines on the uptake of dihydrostreptomycin by the K^+ -retention mutant *B. subtilis* 168KL. Cells were grown in complex medium at 37 °C in the presence of either 10 μ M-spermidine ($\triangle$) or 10 μ M-putrescine ($\square$), or without any addition ($\circ$). Uptake of [3 H]dihydrostreptomycin (5 μ M; 37 kBq ml^{-1}) was determined as described in Methods.

DISCUSSION

The driving force of the energy-dependent accumulation of the aminoglycoside antibiotic dihydrostreptomycin is the electrical component, the $\Delta\psi$, of the protonmotive force, Δp , which is defined as $\Delta p = (\Delta\psi - 2.3 RT/F)/\Delta pH$ (Arrow & Taber, 1986; Damper & Epstein, 1981). This we confirmed and extended for the enhancement of dihydrostreptomycin uptake in *B. subtilis* by agents as diverse as bacitracin (inhibits murein synthesis), puromycin (inhibits protein synthesis) and DCCD (inhibits the membrane-bound H^+ -ATPase). The common effect of all three compounds was shown to be an increase in $(-\Delta\psi)$. We could estimate a threshold value for $\Delta\psi$ (under the experimental conditions about -180 to -190 mV) that was required in order to establish a high rate of uptake (EDPII).

We showed that $\Delta\psi$ not only drives uptake of the polycationic dihydrostreptomycin but also the accumulation of polycations like putrescine and spermidine. In the streptomycin-resistant mutant *B. subtilis* SRB15 [^{14}C]spermidine was accumulated at the same rate and extent as [3H]dihydrostreptomycin after raising $(-\Delta\psi)$ by pre-incubation with DCCD. Similarly, it has been reported that increasing the magnitude of $\Delta\psi$ by pre-incubation with puromycin in a ribosomally resistant mutant (rpsL) of *E. coli* increased the uptake of polyamines and arginine (Bryan & Kwan, 1983). Thus, by raising $(-\Delta\psi)$ above a certain threshold the uptake of polycations is stimulated. An earlier suggestion, however, that dihydrostreptomycin may enter the cell via an inducible polyamine uptake system, which was thought to be induced by the specific interaction of streptomycin itself with protein synthesis (Höltje, 1978), can be ruled out on the basis of several experimental results. Firstly, no indication of the appearance of a new transport protein together with enhancement of uptake could be obtained (Ahmad *et al.*, 1980). Secondly, a non-parallel response of polyamine and dihydrostreptomycin uptake has been demonstrated with streptomycin-transport mutants (Bryan *et al.*, 1980; Campbell & Kadner,

1980) and, after starvation of the cells, for polyamines (Campbell & Kadner, 1980). Mutual competitive inhibition of the uptake of dihydrostreptomycin and polyamines is easily explained by depolarization of the membrane (decreasing the magnitude of $\Delta\psi$) by both classes of polycationic compounds that seem to be taken up by electrogenic transport (Damper & Epstein, 1981). The carriers involved have not yet been identified but a strong dependence on a functional electron transport system indicates that a component of the electron transport chain may be involved directly in the uptake process. Whereas ubiquinones have been proposed to be responsible for gentamicin and dihydrostreptomycin uptake in *E. coli* (Bryan & Kwan, 1983), a requirement for cytochrome *aa₃*, besides an adequate $\Delta\psi$, has been demonstrated for the uptake of dihydrostreptomycin in *B. subtilis* (Arrow & Taber, 1986). High rates of cell growth (i.e. high rate of protein synthesis) are necessary for stimulated uptake of dihydrostreptomycin (Muir *et al.*, 1984, 1985).

Dependence of the second phase of energy-dependent uptake (EDPII) on protein synthesis, which was taken as an indication of the induction of an additional transport system (Höltje, 1978), is shown here to be the result of a specific interference of dihydrostreptomycin itself with protein synthesis that ultimately increases the magnitude of $\Delta\psi$. Polyamines as well as dihydrostreptomycin merely respond non-specifically to the increase in $(-\Delta\psi)$. The question to be answered is: how is the magnitude of $\Delta\psi$ increased by the interaction of streptomycin with ongoing protein synthesis?

The peculiar phenomenon of autostimulation of dihydrostreptomycin accumulation depends on codon misreading, since all miscoding aminoglycoside antibiotics similarly stimulate dihydrostreptomycin uptake, whereas kasugamycin and spectinomycin, which do not cause miscoding, are ineffective (Höltje, 1978). In contrast to the aminoglycosides most inhibitors of protein synthesis impair dihydrostreptomycin accumulation (Hancock, 1981). However, puromycin, within certain concentrations, stimulates uptake by increasing $(-\Delta\psi)$ as shown here. By analogy to dihydrostreptomycin the stimulatory effect of puromycin depends on a specific interaction with protein synthesis (Hurwitz *et al.*, 1981). Indeed, miscoding aminoglycosides and puromycin have a common effect on protein synthesis, namely to cause non-functional proteins to accumulate (Davies *et al.*, 1964). In the case of puromycin peptidyl puromycin fragments are released from the ribosomes (Pestka, 1971). In contrast to Davis *et al.* (1986), who proposed that the interference with normal protein synthesis by streptomycin or puromycin results in the incorporation of faulty proteins into the membrane which form non-specific membrane channels, we would like to emphasize another common consequence of the action of these antibiotics. The abnormal proteins synthesized are rapidly degraded by proteases (Goldberg, 1972; Hewitt & Kogut, 1977) at a rate that increases exponentially with time (Kogut & Carrier, 1980). This causes an osmotic problem for the bacterium by the sudden increase in the intracellular concentration of small peptides and amino acids. The cell would be expected to respond to this change by a net efflux of K^+ , which is known to be one of the most important mechanisms by which bacteria adapt to changes in the osmotic pressure (Epstein & Schultz, 1965). Indeed, efflux of K^+ has been described as one of the first effects of dihydrostreptomycin (Dubin *et al.*, 1963). Efflux of cations, however, would increase the magnitude of $\Delta\psi$. As shown in this report $(-\Delta\psi)$ does increase in the presence of puromycin and, very probably, is also increased by the action of dihydrostreptomycin during autostimulation.

Our interpretation directly contradicts the attractive model presented by Bryan & Kwan (1983) to explain the various steps in the complex pathway from first binding, followed by uptake into the cell, to finally killing of the bacterial cell by aminoglycoside antibiotics. In this model the onset of aminoglycoside uptake with increased rate (EDPII) by autostimulation is neither paralleled nor triggered by an increase of $(-\Delta\psi)$; rather, binding of the aminoglycoside to ribosomes that are part of polysomes is claimed to initiate perturbation of the ribosomal cycle and thereby disturb the integrity of the cytoplasmic membrane. This part of the model is based on the experimental finding of a decrease in tetraphenylphosphonium ion accumulation in *E. coli* caused by gentamicin, which, in our opinion, must not be taken as evidence for a decrease of the membrane potential because of the depolarizing effect of the trivalent gentamicin cation. In addition the K^+ efflux that coincides with the onset of increased dihydrostreptomycin uptake

(EDPII) must contribute to an increase in the magnitude of $\Delta\psi$ (inside negative). The second set of experiments to determine $\Delta\psi$ during autostimulation, reported by Bryan & Kwan (1983), suffer from the fact that before addition of the lipophilic probe the cells had been centrifuged and washed to remove the aminoglycoside. We found that centrifugation and resuspension in fresh medium severely affected the transport activity of the cells. This has also been reported for *Bacillus licheniformis* (MacLeod *et al.*, 1973). Furthermore, the dramatic increase in the rate of uptake during the establishment of the EDPII can hardly be explained by increased binding to polysomes. The exponential increase of proteolysis (Kogut & Carrier, 1980), however, may very well explain the stimulation of uptake by a factor as high as 1000-fold. Also, toluene treatment of cells from EDPII released most of their accumulated dihydrostreptomycin, indicating that this portion is probably in a free form and not bound to ribosomal structures.

Besides DCCD, which increases ($-\Delta\psi$) at the expense of ΔpH by blocking the influx of protons via the ATPase, a major return route for protons, it was shown that valinomycin and bacitracin can also stimulate dihydrostreptomycin uptake. Valinomycin renders the cytoplasmic membrane specifically permeable to K^+ , stimulating influx or efflux depending on the difference in concentration between the inside and outside of the cell and on the existing $\Delta\psi$ (Shioi *et al.*, 1980). Added during EPDI it stimulated dihydrostreptomycin uptake, whereas applied later it impaired uptake, possibly due to the fact that the depolarizing effect of valinomycin became predominant. Bacitracin, which was shown here for the first time to enhance dihydrostreptomycin uptake, may similarly function as an effector of K^+ fluxes. Bacitracin, which inhibits murein synthesis by binding to the lipid carrier undecaprenyl pyrophosphate, surprisingly also has an effect on protoplasts of *B. megaterium*: this has been shown to be due to an efflux of K^+ caused by bacitracin (Hancock & Fitz-James, 1964). It can be concluded that, in general, changes in the fluxes of cations are likely to affect the uptake of aminoglycoside antibiotics and thereby their therapeutic efficacy.

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