The bactericidal action of streptomycin: membrane permeabilization caused by the insertion of mistranslated proteins into the cytoplasmic membrane of *Escherichia coli* and subsequent caging of the antibiotic inside the cells due to degradation of these proteins

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The mechanism by which the aminoglycoside antibiotic streptomycin permeabilizes the cytoplasmic membrane of *Escherichia coli* cells was reinvestigated. For this purpose, the extent of streptomycin-induced K⁺ loss from cells growing at low external K⁺ concentrations was taken as a measure of membrane permeabilization. Experiments with different K⁺-uptake mutants showed that the antibiotic specifically increased the passive permeability of the cell membrane to K⁺ and other ions. These permeability changes were small and the membrane potential of the treated cells remained high. The membrane permeabilization was not due to a direct interaction of the antibiotic with the cell membrane, since cells that carry an *rpsL* mutation and synthesize proteins in a streptomycin-insensitive way did not lose K⁺ after the addition of the antibiotic. Due to misreading and premature termination of translation the cells synthesized aberrant proteins under the conditions where membrane permeabilization occurred. Two conditions are described under which the cells both degraded these mistranslated proteins rapidly and reaccumulated K⁺, lending support to the hypothesis that membrane permeabilization is due to the presence of the mistranslated proteins in the cell membrane. Evidence is presented that the irreversibility of (dihydro)streptomycin uptake by cells washed free from the antibiotic might also be due to rapid degradation of the mistranslated proteins, leading to 'caging' of the antibiotic inside the cells.

Introduction

Aminoglycoside antibiotics primarily inhibit the process of translation in prokaryotes (for reviews see Davis *et al.*, 1974; Hancock, 1981*a*; Davis, 1987). The molecular basis for this inhibition differs from compound to compound, and is often the consequence of a number of pleiotropic effects, such as interaction with the 16S RNA, formation of an unstable initiation complex, inhibition of elongation, prevention of A-site occupation and misreading during translation (Nierhaus & Wittmann, 1980; Brimacombe, 1988; Hausner *et al.*, 1988; Wittmann, 1989). However, none of these effects explains why aminoglycosides are lethal to bacterial cells: the binding of streptomycin to the ribosome is

reversible (Chang & Flaks, 1972; Andry & Bockrath, 1974), and one would therefore expect efflux of the antibiotic from the cells to occur after the suspension has been diluted for plating, leading to bacteriostatic rather than bactericidal effects. Hence, it has been proposed that streptomycin and other bactericidal aminoglycosides must have a second site of action, causing the lethal event (Hancock, 1981*a, b*; Davis, 1987).

However, the uptake of dihydrostreptomycin by *Escherichia coli* is irreversible (Muir *et al.*, 1984; Nichols & Young, 1985). Davis (1987) has therefore proposed that the drug does not leave the cells during plating, leading to prolonged inhibition of protein synthesis, which prevents the formation of colonies on the plates. Apart from the fact that it is unclear why streptomycin uptake is irreversible (Nichols, 1989), this mechanism does not explain why aminoglycosides like streptomycin, gentamycin or kanamycin are bactericidal, whereas two compounds with a closely related structure, the aminocyclitols spectinomycin and kasugamycin, only exert bacteriostatic effects (Hancock, 1981*a*; Davis, 1987).

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Abbreviations: TPMP⁺, triphenylmethylphosphonium cation; TPP⁺, tetraphenylphosphonium cation.

The difference between these two groups of compounds is that the aminoglycosides induce misreading of protein synthesis, whereas the two aminocyclitols do not (Hancock, 1981*a, b*; Piepersberg, 1985; Davis *et al.*, 1986; Bakker, 1992).

Hancock (1981*b*) has proposed that the aminoglycoside-induced lethality might be a membrane event. It has been known for over 25 years that the release of K^+ is one of the earliest events after the addition of streptomycin to bacterial cells (Dubin & Davis, 1962; Dubin *et al.*, 1963; Hancock, 1964). Davis (1987, 1988) has proposed that the permeability changes caused by bactericidal aminoglycosides play an important role in the bactericidal action of these compounds. His hypothesis contains the following elements: (i) the insertion of aberrant proteins into the cell membrane causes an increase in the permeability of this membrane (see Hancock, 1964); (ii) the antibiotic can permeate through these 'channels', leading to enhanced streptomycin uptake (see Höltje, 1978, 1979); (iii) hence misreading increases and more channels are formed; and (iv) blockade of initiating ribosomes occurs. It is, however, controversial whether misreading (Gorini, 1974) or even protein synthesis (Stern *et al.*, 1966) are necessary for streptomycin to cause lethality (Hancock, 1981*b*; Hausner *et al.*, 1988). Moreover, the Davis mechanism does not explain why streptomycin uptake by cells is irreversible (Muir *et al.*, 1984; Nichols & Young, 1985; Nichols, 1989). Tanaka and coworkers have come forward with an alternative mechanism for the bactericidal action of aminoglycosides. They attribute habekacin-induced lethality to inhibition of the initiation of DNA replication due to the formation of an *oriC*-membrane complex being prevented (Tanaka *et al.*, 1984; Matsunaga *et al.*, 1986).

Because of the controversy about the relevance of misreading and membrane permeabilization for the bactericidal action of the aminoglycosides, we have reinvestigated the mechanism by which these compounds cause K^+ loss. In this communication we present evidence (i) that streptomycin causes permeability changes for other inorganic cations in addition to K^+ ; (ii) that these permeability changes are sufficiently small that the treated cells still maintain a high membrane potential; (iii) that the increased membrane permeability occurs only under conditions where mistranslated proteins are present in the cell; (iv) that there exist conditions under which the cells degrade their mistranslated proteins rapidly; and (v) that this proteolysis might explain why streptomycin becomes 'caged' inside the cells. In the following paper it is shown that except for hygromycin B all the bactericidal aminoglycosides tested cause misreading and membrane permeabilization of the treated cells (Bakker, 1992).

Methods

Bacterial strains. The strains used are all derived from *E. coli* K12. Strains FRAG-1 (wild-type; Epstein & Kim, 1971), FRAG-5 $\Delta kdpABC5$ (Epstein & Kim, 1971), TK1001 $\Delta kdpABC5 trkD1$ (Rhoads *et al.*, 1976), TK1110 $\Delta kdpABC5 trkA405$ (Rhoads *et al.*, 1976), TK2240 *nagA trkA405 trkD1* (Epstein *et al.*, 1978) and TK2242 *nagA kdp42 trkA405 trkD1* (Epstein *et al.*, 1978) are all $F^- thi lacZ rha$ and were obtained from Dr W. Epstein, University of Chicago, Chicago, IL., USA. Strain AS-1 $F^- acrA$ (Imae, 1968) was from Dr Y. Imae, University of Nagoya, Japan.

Growth conditions. For most of the experiments cells were grown overnight at 37 °C under aerobic conditions in a minimal medium containing 46 mM- Na_2HPO_4 , 23 mM- NaH_2PO_4 , 8 mM- $(NH_4)_2SO_4$, 1 mM-sodium citrate, 0.4 mM- $MgSO_4$, 6 μ M- $FeSO_4$, 1 mM-KCl and 10 mM-glucose (Epstein & Kim, 1971). When required, thiamin was also present, at 1 mg l^{-1} . Next day the stationary-phase cells were diluted to an OD_{578} of about 0.1 in fresh growth medium in which the KCl concentration was reduced to 0.5 mM ('K0.5 medium'). The minimal growth medium free of Na^+ contained 119 mM-choline chloride, 10 mM-bis-trispropane, 20 mM- $(NH_4)_2H_2PO_2$ and the same KCl, $FeSO_4$, $MgSO_4$, glucose and thiamin concentrations as the minimal medium described above (Giffard *et al.*, 1985). Choline chloride was omitted from the medium that contained neither Na^+ nor choline.

Streptomycin treatment. The antibiotic was added to a concentration of 50 μ g per ml cell culture when the OD_{578} of the suspension had reached a value between 0.7 and 0.8. The OD_{578} of the suspension and the parameter of interest (e.g. cell K^+ content, cell viability) were then measured as a function of time both in control and in streptomycin-treated cells.

Ion content, membrane potential and internal pH. For these assays 1.0 ml cell samples were taken at different time points and centrifuged through silicone oil (AR200 oil, relative density ~ 1.04 ; Wacker Chemie) in a Beckman B minicentrifuge (Bakker & Mangerich, 1981). The K^+ and Na^+ content of the cell pellet were determined by flame photometry (Michels & Bakker, 1987). The membrane potential of growing cells of strain AS-1 (Hirota *et al.*, 1981) was determined with [^{14}C]TPP at a concentration of 20 nCi ml^{-1} (740 Bq ml^{-1}) and a TPP chloride concentration of 1 μ M. No correction was made for the binding of TPP to cellular components. Internal pH of the growing cells was determined with [^{14}C]benzoic acid and 3H_2O (Kashket & Barker, 1977) as described by (Dinnbier *et al.* (1988).

[3H]Dihydrostreptomycin transport. [3H]Dihydrostreptomycin uptake by washed cells and the efflux of the labelled compound from cells that had previously been loaded with the radioactively labelled antibiotic were determined as described by Nichols & Young, 1985.

Viable cell count. Samples were taken from the cell cultures and diluted into buffer consisting of the sodium phosphates and $MgSO_4$ of the minimal growth medium. The cells were then plated on 1.5% agar containing (l^{-1}): 10 g bactotryptone (Difco), 5 g yeast extract and 10 g KCl. The concentration of viable cells for each sample was calculated from the number of colonies observed after overnight incubation of the plates at 37 °C.

Pulse-chase experiments with [^{35}S]methionine. These were done by labelling the proteins synthesized by 6 ml of streptomycin-treated cells with 25–50 μ Ci of radioactivity (specific activity ~ 1000 Ci $mmol^{-1}$; ~ 37 TBq $mmol^{-1}$). After 2 min any further incorporation of radioactivity into proteins was stopped by the addition of either 10 μ M-L-methionine or 10 μ M-L-methionine plus 100 μ g chloramphenicol ml^{-1} .

At different timepoints 1.0 ml samples of the cell suspension were mixed with 0.1 ml 50% (w/v) trichloroacetic acid. The samples were transferred to 1.5 ml microcentrifugation vials and incubated for 30 min at 4 °C. The denatured protein was pelleted by centrifugation, and the sediments were washed twice with 1 ml 10 mM-Tris/acetate, 0.1 mM-phenylmethylsulphonyl fluoride pH 7.5, followed each time by centrifugation. The pellets were solubilized in 0.1 ml of electrophoresis sample buffer. After the sample had been boiled for 5 min, the proteins were separated by SDS-PAGE on a gel prepared at 13% (w/v) acrylamide (Lugtenberg *et al.*, 1975). Total and radioactively labelled proteins were visualized by staining with Coomassie blue and by autoradiography of the dried gel with Kodak XAR-5 X-ray film, respectively.

Materials. Streptomycin sulphate, dihydrostreptomycin sesquisulphate, spectinomycin dihydrochloride, tetracycline and puromycin were from Sigma. Hygromycin B was a gift from Professor Dr A. Böck, University of Munich, FRG. Chloramphenicol was from Serva. [³H]Dihydrostreptomycin sesquisulphate (18.3 Ci g⁻¹; 677 GBq g⁻¹), tetra[¹⁴C]phenylphosphonium bromide (31.4 Ci mol⁻¹; 1.16 TBq mol⁻¹), [³⁵S]methionine (about 1000 Ci mmol⁻¹; about 37 TBq mmol⁻¹), ³H₂O and ⁸⁶Rb⁺ were from Amersham-Buchler. [¹⁴C]Benzoic acid (19.3 Ci mol⁻¹; 714 GBq mol⁻¹) was from Dupont-NEN.

Results

K⁺ efflux from K⁺-uptake mutants

Dubin *et al.* (1963) showed that the streptomycin-induced loss of K⁺ from cells is an early event that slightly precedes lethality. These authors also presented evidence that K⁺ loss is due to a stimulation of K⁺ efflux rather than to an inhibition of K⁺ uptake. Their experiments were, however, not carried out under well-defined conditions, since they were done at the low concentration of added K⁺ of 0.1 mM and during the course of the experiment the cells may have taken up as much as 80% of the added K⁺. Moreover, it is now known that at low K⁺_{out} *E. coli* derepresses the high-

affinity K⁺-uptake system, Kdp (for a review see Walderhaug *et al.*, 1987). The derepression of Kdp during the experiment may also have influenced the result.

Since 1963 mutants have been described that are impaired in K⁺ transport via the different K⁺-uptake systems (Table 1). These systems are the inducible high-affinity Kdp system (see above), the constitutive high-rate systems TrkG and TrkH, and the constitutive low-rate system Kup, which is the gene product of the *trkD* gene (Rhoads *et al.*, 1976; Bossemeyer *et al.*, 1989a, b; Dosch *et al.*, 1991). We tested the effect of 50 µg streptomycin ml⁻¹ on the K⁺ content of K⁺-uptake mutants growing at 0.5 mM-K⁺. This K⁺ concentration is sufficiently high that all the mutants still grew with similar rates, which enabled us to make a direct comparison of the effect of the antibiotic on the different strains (Fig. 1a). Moreover, at 0.5 mM-K⁺ the external K⁺ concentration decreased by only 30% during cell growth, which should not have influenced the level of *kdp* expression during the experiment to a great extent.

Fig. 1(a) shows that the less well the cells are able to pump K⁺, the faster was the onset of K⁺ efflux caused by streptomycin. Thus, with cells that are wild-type for K⁺-uptake systems (strain FRAG-1 and presumably strain AS-1; Table 1) it took about 20 min before streptomycin exerted any effect (open diamonds and open circles, respectively). For cells lacking functional Trk systems or lacking Kdp this time lag decreased to 15 min [strains FRAG-5 ($\Delta kdpABC$) and TK2240 (*trkA trkD*); open and filled squares, respectively]. The time lag was reduced to less than 10 min for cells that lacked functional Kdp and Kup systems (strain TK1001, filled circles). A lag time of approximately 5 min was observed for TK2242, which only contains a Kdp system with a reduced affinity for K⁺ uptake (Epstein *et al.*, 1978; Fig. 1, open triangles). The fastest onset of K⁺ loss was

Table 1. *K⁺-uptake systems in E. coli and their properties*

System	Mode of expression	K ⁺ -transport parameters†‡		K ⁺ /Rb ⁺ specificity†	Strain(s) in which system present‡
		K _m §	V _{max} §		
Kdp	Derepressible	0.002	50–150	≥1000	FRAG-1, TK2240, AS-1
Kdp*	Derepressible	~1	50–150	ND	TK2242
TrkG, TrkH	Constitutive	~1	200–500	~10	FRAG-5, TK1001, AS-1
Kup	Constitutive	~0.5	30–50	~1	FRAG-5, TK1110, AS-1

ND, Not determined.

* Kdp* is a Kdp system with reduced affinity for K⁺ uptake (Epstein *et al.*, 1978).

† Data from Rhoads *et al.* (1977), Bakker (1983), Bossemeyer *et al.* (1989a).

‡ Data from Rhoads *et al.* (1976), Epstein *et al.* (1978), Dosch *et al.* (1991).

§ K_m in mM, V_{max} in nmol min⁻¹ (mg cell dry wt)⁻¹.

|| It is not known which K⁺-uptake systems are made by strain AS-1.

Fig. 1. K^+ efflux (a) and loss of viability (b) of K^+ uptake mutants treated with streptomycin. At zero time the antibiotic was added at a concentration of $50 \mu g\ ml^{-1}$ to cells growing in K0.5 medium. The K^+ content of the cells and the number of viable cells ml^{-1} were determined as a function of time as described in Methods. $\diamond$, Strain FRAG-1; $\circ$, strain AS-1; $\square$, strain FRAG-5; $\bullet$, strain TK1001; $\blacksquare$, strain TK2240; $\triangle$, strain TK2242; $\blacktriangle$, strain TK1110.

observed with strain TK1110 (filled triangles), which possesses a functional Kup system only. Strain TK1110 was employed for most of the experiments described below.

Fig. 1(b) shows that poorly K^+ -pumping cells also started to lose their viability earlier. Below we will argue that these differential effects of the antibiotic on cells with different K^+ -uptake systems may be due to faster uptake of the antibiotic by poorly K^+ -pumping cells.

The effects depicted in Fig. 1 did not occur in cells of an *rpsL* strain, in which protein synthesis is insensitive to streptomycin (results not shown; cf. the results of Hancock, 1964, with *Bacillus cereus*), indicating that K^+ efflux from the cells is not the result of a direct interaction of the antibiotic with the cytoplasmic membrane.

$^{86}Rb^+-Rb^+$ exchange

The Kup system transports K^+ and Rb^+ with similar rates and affinities (Bossemeyer *et al.*, 1989b). Thus, we were able to use $^{86}Rb^+$ to mimic K^+ transport in strain TK1110. Streptomycin caused Rb^+ loss from Rb^+ -loaded cells with a time course almost identical to that of K^+ loss

Fig. 2. Streptomycin specifically stimulates Rb^+ efflux from cells growing at low K^+ (Rb^+) concentrations. Cells of strain TK1110 were grown in three identical portions in K0.5 medium. At $t = -120$ min two cultures received $0.1\ mM$ - $RbCl$ and $20\ nCi\ ^{86}Rb^+\ ml^{-1}$ ($\circ$, $\bullet$). At zero time $50 \mu g$ of streptomycin ml^{-1} was added to one of these cultures ($\bullet$). The third culture received at $t = -120$ min only $0.1\ mM$ - $RbCl$ and at zero time $50 \mu g$ of streptomycin ml^{-1} . From this culture 5 ml of cells were transferred to Erlenmeyer flasks at different timepoints and incubated with $20\ nCi\ ^{86}Rb^+\ ml^{-1}$ ($\square$, $\blacksquare$, $\blacktriangle$, $\blacklozenge$, $\blacktriangledown$). The $^{86}Rb^+$ content of the cells was determined as a function of time. The 100% value of the $^{86}Rb^+$ content of the cells amounts to about $80\ nmol\ (mg\ dry\ wt)^{-1}$ ($\sim 55\ mM$).

(not shown). The unidirectional influx of the isotope remained the same during the period that the cells lost net Rb^+ (Fig. 2). Hence, streptomycin stimulates Rb^+ (K^+) efflux rather than inhibiting the influx of these cations. The same conclusion was drawn by Dubin *et al.* (1963).

At high K^+ streptomycin increases the K^+ content of the cells

At increasing external K^+ concentrations the time lag between the addition of the antibiotic and its effects on cell K^+ content and cell viability increased (Fig. 3). Moreover, at 30 or 115 mM - K^+ the antibiotic caused net uptake instead of net loss of K^+ (Fig. 3a), indicating that the antibiotic increases the passive permeability of the cell membrane to K^+ . The retardation of the action of streptomycin by high K^+ may be due to a slower uptake of the antibiotic by K^+ -replete than by K^+ -depleted cells (see below).

Even under conditions at which streptomycin caused net K^+ uptake the drug was bactericidal (Fig. 3, open and filled squares). Moreover, addition of $10\ mM$ - K^+ to cells previously treated with streptomycin at $0.5\ mM$ - K^+ led to reaccumulation of K^+ , but did not retard the rate with which the cells lost their viability (Fig. 4). Thus, loss of K^+ is not a prerequisite for the bactericidal action of streptomycin.

Fig. 3. K^+ content (a) and viability (b) of cells treated with streptomycin at different K^+ concentrations. Cells of *E. coli* TK1110 were grown at the following K^+ concentrations: 0.5 mM ($\circ$); 10 mM ($\bullet$); 30 mM ($\square$) and 115 mM ($\blacksquare$). Streptomycin ($50\ \mu g\ ml^{-1}$) was added at zero time. The K^+ content of the cells and number of viable cells of the suspension were determined as a function of time.

Permeability changes for other ions

Under the conditions where the cells lost K^+ , they took up 20% more Na^+ than they lost K^+ (Fig. 5a). Thus, streptomycin not only affects the transmembrane concentration gradient of K^+ and Rb^+ , but also that of another inorganic cation. In the experiment of Fig. 5(a), part of the Na^+ taken up in excess of K^+ was electrically compensated for by the efflux of H^+ , since the internal pH of the cells increased by 0.15 units (Fig. 5b). Sodium was not required for K^+ loss, since streptomycin-treated cells grown in the absence of Na^+ lost K^+ with a time course and to an extent similar to those of cells grown in the presence of Na^+ (Fig. 5c, e). Cells grown in the absence of Na^+ did not exchange K^+ for H^+ after streptomycin treatment, since the internal pH of these cells increased rather than decreased (Fig. 5d). This indicates that under these conditions the uptake of unidentified cations (possibly choline) or the exit of unidentified anions accompanies K^+ loss. Omission of choline and Na^+ from the medium led to a slightly different behaviour: K^+ loss became transient (Fig. 5e) and the internal pH of the treated cells was only initially

Fig. 4. The addition of an excess of K^+ to cells treated with streptomycin reverses K^+ loss (a) but does not affect the rate with which cells lose their viability (b). Two cultures of *E. coli* TK1110 were grown in K0.5 medium. At zero time $50\ \mu g$ streptomycin ml^{-1} was added to the two cell suspensions. At $t = 9$ min one culture received 10 mM-KCl ($\bullet$). The second culture served as the control ($\circ$). The K^+ content of the cells and the number of viable cells were determined as a function of time.

higher than that of control cells (Fig. 5f). We will return to the phenomenon of transient K^+ loss below.

Membrane potential

The effects of streptomycin on cell membrane permeability are not large since streptomycin-treated cells still maintained substantial concentration gradients of K^+ (Rb^+) and H^+ across their cytoplasmic membranes (Figs 1–5). To obtain more information on this point we measured the membrane potential of cells of strain AS-1, in which this potential can be measured without pretreatment with EDTA (Hirota *et al.*, 1981). Remarkably, streptomycin caused hyperpolarization during the period that the cells lost K^+ (Fig. 6). This effect may be partially due to the inhibition of protein synthesis by streptomycin since chloramphenicol, added at a concentration at which it blocks protein synthesis completely, caused an even larger hyperpolarization (Fig. 6a). This

Fig. 5. K^+ and Na^+ content and internal pH of streptomycin-treated cells grown in media of different composition. *E. coli* TK1110 cells were grown in K0.5 medium, which contains 115 mM- Na^+ (a, b), in medium without Na^+ (c, d) or in medium without Na^+ and choline (e, f). The K^+ concentration was in all cases 0.5 mM. The K^+ ($\circ$, $\bullet$) and Na^+ content ($\triangle$, $\blacktriangle$) and cytoplasmic pH ($\square$, $\blacksquare$) of the cells were determined as a function of time. Open symbols, control cells; filled symbols, cells treated with 50 μ g streptomycin ml^{-1} at zero time.

Fig. 6. Membrane potential (a) and K^+ content (b) of streptomycin-treated cells of *E. coli* strain AS-1. [^{14}C]TPP chloride (1 μ M, 20 nCi ml^{-1}) was added at $t = 30$ min to cells growing in K0.5 medium. $\circ$, Control cells; $\bullet$, control cells to which 100 μ g chloramphenicol (CAM) ml^{-1} was added at $t = 57$ min. $\triangle$, Cells that were treated with 50 μ g streptomycin ml^{-1} at zero time; $\blacktriangle$, streptomycin-treated cells that also received 100 μ g chloramphenicol ml^{-1} at $t = 57$ min.

hyperpolarization is probably the result of the decreased ATP demand of cells in which protein synthesis is inhibited.

Addition of 100 mM- K^+ to streptomycin-treated cells depolarized the cells only partially (not shown). Together with the results of Figs 1–6 this result indicates that streptomycin increases the permeability of the cell membrane for more ions than K^+ alone, and that these effects are small, and balance each other in such a way that the membrane potential remains high. The actual value of the membrane potential of streptomycin-treated cells will be determined both by the extent of the permeability changes and by the decreased energy demand of the cells due to inhibition of protein synthesis.

Reversal of permeabilization by a second inhibitor of protein synthesis

Figs 6(a) and 7 show that the addition of 100 μ g chloramphenicol ml^{-1} reversed K^+ loss from cells previously treated with streptomycin. Under these conditions chloramphenicol prevents further loss of viability of streptomycin-treated cells, but does not reverse it (Plotz & Davis, 1961). The addition of 50 μ g hygromycin B or spectinomycin ml^{-1} also reversed K^+ loss from streptomycin-treated cells. Other inhibitors of protein synthesis were less effective in this process: tetracycline (15 μ g ml^{-1}) reversed K^+ loss, but with a slower rate than did chloramphenicol. Puromycin (200 μ g ml^{-1}) merely prevented further K^+ efflux (results not shown).

Fig. 7: Reversal of K^+ loss from streptomycin-treated cells by chloramphenicol. Four cultures of cells of strain TK1110 were grown in K0.5 medium. One culture ($\circ$) served as a control. Streptomycin ($50 \mu\text{g ml}^{-1}$) was added to the other three cultures at zero time. To two of these cultures $100 \mu\text{g}$ chloramphenicol (CAM) ml^{-1} was added at $t = 21$ ($\blacktriangle$) and $t = 36$ min ($\blacksquare$). The fourth culture did not receive any further addition ($\bullet$). The K^+ content of the cells was determined as a function of time.

Reaccumulation of K^+ due to degradation of mistranslated proteins

The streptomycin-induced permeability changes of the cell membrane may arise from mistranslated proteins present in this membrane (Hancock, 1964; Davis, 1987). If this hypothesis is correct, one would expect that streptomycin-treated cells that reaccumulate K^+ (Figs 5e, 6 and 7) have degraded their mistranslated proteins. Fig. 8 shows that this is indeed the situation. In this experiment streptomycin-treated cells were pulse-labelled with [^{35}S]methionine. The autoradiogram of the synthesized proteins separated by SDS-PAGE showed diffuse bands (Fig. 8, lanes 1 and 7), due to the errors in protein synthesis (Piepersberg, 1985; Davis *et al.*, 1986; Bakker, 1992). During a subsequent chase with an excess of non-radioactive methionine plus chloramphenicol the labelled proteins were degraded during the time that the cells reaccumulated K^+ (Fig. 8, lanes 2–6). Under these conditions cell proteins synthesized before streptomycin addition were stable (results not shown), indicating that only the mistranslated proteins were hydrolysed. A control experiment in which chloramphenicol was omitted during the chase showed neither degradation of mistranslated proteins nor reaccumulation of K^+ (Fig. 8, lanes 7–12). Cells growing in the medium without Na^+ and choline (i.e. the conditions of Fig. 5e, f) also degraded the proteins synthesized after the addition of streptomycin (not shown). Thus, reversal of K^+ efflux appears to be due to enhanced rate of proteolysis of the mistranslated proteins.

Fig. 8. Chloramphenicol induces proteolysis of the aberrant proteins synthesized by streptomycin-treated cells. Two cultures of TK1110 cells were grown in K0.5 medium. At $t = -9$ min both cultures received $50 \mu\text{g}$ streptomycin ml^{-1} . To 6 ml of each culture $25 \mu\text{Ci}$ [^{35}S]methionine ($1000 \text{ Ci/mmol}^{-1}$) was added at zero time. Culture (a) received at $t = 2$ min 10^{-5} M -methionine plus $100 \mu\text{g}$ chloramphenicol ml^{-1} ('chase'). Culture (b) was 'chased' with 10^{-5} M -methionine alone. At various timepoints cells were denatured with trichloroacetic acid and the pattern of radioactively labelled proteins was determined as described in Methods (upper part of the figure). In a parallel experiment the K^+ content of the cells was determined at the different timepoints after the 'chase' (lower part).

The irreversibility of (dihydro)streptomycin uptake

We confirmed the results of Nichols & Young (1985) that dihydrostreptomycin uptake is irreversible. Moreover, even when cells were washed free of external antibiotic and were subsequently completely depolarized by the addition of 5 mM of the protonophore 2,4-dinitrophenol, they did not lose their previously accumulated dihydrostreptomycin (not shown, cf. Fig. 2 of Nichols & Young, 1985). We then argued that the

Fig. 9. 'Caging' of dihydrostreptomycin inside cells due to degradation of the mistranslated proteins. The protocol of this experiment was closely similar to that of Fig. 2 of Nichols & Young (1985). Cells of strain TK1110 were suspended at $0.18 \text{ mg dry wt ml}^{-1}$ in 50 mM -sodium HEPES, pH 7.0. The suspension was shaken at 37°C . At zero time 10 mM -glucose and $15 \mu\text{g}$ dihydrostreptomycin ml^{-1} were added to the suspension. At $t = 15 \text{ min}$ 10 ml of this suspension was labelled with $25 \mu\text{Ci}$ [^{35}S]methionine. At $t = 20 \text{ min}$ any further incorporation of radioactivity into protein was prevented by the addition of 10^{-5} M -methionine. Lane 1 represents the pattern of radioactively labelled proteins from 1 ml of cells taken immediately after the latter addition. The remainder of the suspension was shaken for 120 min . Lanes 2 and 3 show the pattern of radioactively labelled proteins from 1 ml of cell samples taken after 50 and 90 min of shaking, respectively. After 120 min the remaining cells were collected by centrifugation, and washed free of external antibiotic and methionine by two subsequent centrifugations. The cells were then suspended in 5 ml of the sodium HEPES/glucose buffer described above and again shaken at 37°C . Lanes 4 and 5 show the pattern of radioactively labelled proteins from 1 ml of cells taken immediately after resuspension in buffer and after 20 min of shaking, respectively.

irreversibility of streptomycin uptake might also be due to rapid degradation of the mistranslated proteins. Fig. 9 shows that under the conditions used by Nichols & Young (1985) for their experiments the aberrant proteins that were synthesized after dihydrostreptomycin addition were indeed degraded during the subsequent incubation and washing procedure.

Discussion

The data presented in the first part of this paper indicate that treatment with streptomycin increases the passive permeability of the *E. coli* cell membrane to several small ions (Figs 1–6). The changes in these permeabilities are relatively small and the membrane potential of the treated cells remains high (Fig. 6). Our data therefore argue against the notion that streptomycin treatment leads to formation of transmembrane channels (Davis, 1987) in the sense of aqueous pores, since the presence of such channels would increase the permeability of the cell membrane to one or more ions by several orders of magnitude.

Due to the increase of the passive K^+ permeability of the cell membrane, the transmembrane K^+ gradient of the treated cells will have a stronger tendency to equilibrate with the high membrane potential. Thus at low K_{out}^+ (i.e. at $61.5 \log([K^+]_{\text{in}}/[K^+]_{\text{out}}) \geq \Delta\psi$) the treated cells will lose some of their K^+ (Figs 1–8), whereas at high K_{out}^+ (i.e. at $61.5 \log([K^+]_{\text{in}}/[K^+]_{\text{out}}) \leq \Delta\psi$) the treated cells will take up some additional K^+ (Figs 3 and 4).

Streptomycin-induced lethality was not prevented by high K_{out}^+ (Figs 3 and 4), indicating that lethality is not the consequence of net K^+ loss from the cells. This situation is different from that of lethality caused by membrane-active bacteriocins. These compounds form channels in the cell membrane (for a review see Cramer *et al.*, 1983) and lethality is at least partly due to the complete loss of K^+ via the channel, since plating of colicin A-treated cells at high K_{out}^+ leads to survival of the cells (Kopecky *et al.*, 1975).

The inverse correlation observed between the onset of streptomycin action and the ability of the cells to pump K^+ (Fig. 1a) may be related to the K^+ content of the cells and their membrane potentials: cells that pump K^+ poorly at 0.5 mM - K^+ contained relatively little K^+ (Fig. 1a). K^+ -depleted cells develop higher membrane potentials than do K^+ -replete cells (Kashket & Barker, 1977; Bakker & Mangerich, 1981; Kroll & Booth, 1981). Since the rate at which the polycation streptomycin is taken up by the cells is a steep function of the membrane potential (Damper & Epstein, 1981; Bryan & Kwan, 1983; Emling & Hölte, 1987), K^+ -depleted cells will take up streptomycin faster and will be affected by the antibiotic earlier (Figs 1 and 3). Hence, our data indirectly support the notion that the magnitude of the membrane potential determines the rate at which streptomycin is taken up by the cells (Damper & Epstein, 1981; Bryan & Kwan, 1983; Taber *et al.*, 1987; Emling & Hölte, 1987).

Different groups have reported different effects of some antibiotics on the membrane potential of *E. coli* or *Bacillus subtilis*: gentamicin depolarizes *B. subtilis*

(Bryan & Kwan, 1983) and *E. coli* (our unpublished results); by contrast, streptomycin hyperpolarizes these bacteria (Emling & Hölte, 1987; Fig. 6 of this study); in addition, chloramphenicol depolarizes *B. subtilis* (Emling & Hölte, 1987), but hyperpolarizes *E. coli* (Fig. 6). It is difficult to compare these results with each other, since (i) two organisms were treated with different antibiotics; (ii) the media in which the cells were suspended and the temperature were different; (iii) different probes were used to monitor the membrane potential [i.e. TPP⁺ by Bryan & Kwan (1983) and by us, and TPMP⁺ in the presence of a small concentration of tetraphenylboron by Emling & Hölte (1987)]; and (iv) the concentration of the probe was different. The latter point is important, since both *E. coli* and *B. subtilis* possess an efflux system for lipophilic cations like TPP⁺ and TPMP⁺ (e.g. Midgley, 1986; Bakker, 1990). The activity of this system disturbs membrane potential measurements at low probe concentrations (Midgley, 1986; Bakker, 1990). On the other hand, high concentrations of TPP⁺ or TPMP⁺ inhibit many cellular processes (e.g. Bakker, 1990). Thus, results on bacterial membrane potentials obtained with lipophilic cations like TPP⁺ have to be taken with extreme caution. Nevertheless, the following conclusions can be drawn: (i) gentamicin and streptomycin have opposite effects on the membrane potential (Bryan & Kwan, 1983; Emling & Hölte, 1987; Fig. 6, and our unpublished observations); (ii) hyperpolarization alone cannot be the cause of the acceleration of (dihydro)streptomycin uptake known as 'EDPII phase' (e.g. Hölte, 1979; Bryan & Kwan, 1983), since (a) chloramphenicol hyperpolarizes *E. coli* cells (Fig. 6), but inhibits (dihydro)streptomycin uptake, and (b) gentamicin depolarizes bacterial cells (Bryan & Kwan, 1983), but stimulates (dihydro)streptomycin uptake (Allen *et al.*, 1987). In addition, our data do not support the notion that the phenomena of K⁺ efflux and the phase of enhanced (dihydro)streptomycin uptake ('EDPII') are directly related to each other. First, the extent of K⁺ release is orders of magnitudes larger than that of streptomycin uptake, and second, and more importantly, during K⁺ release the cells take up more than an equivalent amount of Na⁺ ions (Fig. 5a). Rather, all of these data can be explained satisfactorily by – but do not prove – the hypothesis that aminoglycoside treatment leads to the insertion of aberrant proteins into the membrane of the treated bacteria (Davis, 1987), causing some permeabilization, the extent of which varies with the antibiotic used. Permeabilization then leads to K⁺ efflux, stimulation of uptake of aminoglycosides and Na⁺, and differential effects on the membrane potential.

Our data not only support but also extend the hypothesis of Davis (1987) about how streptomycin exerts lethality. First, we observed a correlation between

the presence of mistranslated proteins in the cell and the increase in the cell membrane permeability (Fig. 8), lending support to the notion that these permeability changes are the consequence of the presence of mistranslated proteins in this membrane (Hancock, 1964; Davis *et al.*, 1986; Davis, 1987). Secondly, the result that streptomycin-treated cells often degrade the mistranslated proteins rapidly (Figs 5, 7–9) may shed some light on the hitherto unexplained observation that streptomycin uptake by the cells is irreversible (Muir *et al.*, 1984; Nichols & Young, 1985; Nichols, 1989). Under the conditions used for measuring efflux of the antibiotic the cells had already degraded the majority of their aberrant proteins (Fig. 9). Thus, the excess of antibiotic that had been taken up by the cells during the period that the cells were permeabilized became 'caged' inside the cytoplasm. Moreover, the driving force for streptomycin efflux by de-energized cells is small, being a function of the cell membrane permeability multiplied by the concentration difference of the antibiotic across this membrane. By contrast, the driving force for uptake of the drug by energized cells is much larger, being a steep function of the membrane potential (Damper & Epstein, 1981; Bryan & Kwan, 1983; Emling & Hölte, 1987). Thus, it can be explained why cells in which membrane tightness has been restored lose streptomycin extremely slowly under conditions where the same cells when energized would still take up the antibiotic slowly. Thirdly, 'caged' antibiotic will cause prolonged inhibition of protein synthesis and may prevent colony formation in plating experiments (Davis, 1987, 1988).

Although we consider the hypothesis of Davis (1987) as reasonably satisfactory, we cannot exclude the possibility that lethality caused by streptomycin is due to a different, hitherto unknown effect of the antibiotic. It is for instance possible that misreading affects a cellular process in such a way that a vital process like cell division or DNA replication becomes impaired. In fact, evidence has already been presented that lethal aminoglycosides that do cause misreading also inhibit the initiation of DNA replication in a temperature-sensitive *dnaC* strain (Tanaka *et al.*, 1984; Matsunaga *et al.*, 1986; Bakker, 1992).

Finally, it is not clear why enhanced protease activity occurs only under the conditions of the experiments of Figs 5(e, f), 7 and 8 (lanes 1–6). Nevertheless, these results extend the observation that treatment with streptomycin increases the protease activity of the treated cells (Hipkiss & Kogut, 1973). In the following paper it is proposed that streptomycin treatment may initiate the general stress response of the cells (Bakker, 1992). Thus, conditions like those in the experiments of Fig. 5(e, f), or Figs 7 and 8 may stimulate the activity of the proteases among the stress proteins.

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