Aminoglycoside uptake and mode of action—with special reference to streptomycin and gentamicin

II. Effects of aminoglycosides on cells

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Effects of aminoglycosides on cells

Introduction

Target theory and the lethal event. In considering the mode of action of aminoglycosides like streptomycin and gentamicin on bacterial cells, it is first worthwhile to describe exactly what is meant when one describes them as bactericidal. According to the target theory of drug action proposed by Paul Ehrlich (see Setlow & Pollard, 1962 for discussion), survival represents the total escape of a critical target within the cell. Thus, if $10^5$ streptomycin molecules are inside the cell at the time of loss of colony-forming ability there may be possibly only a single critical target site. Alternatively, there may be $10^5$ target sites each of which must be inactivated for cell death to occur. Thus, one can define a 'lethal event' at which time the cell loses colony-forming ability. This in itself does not imply that the cells are unable to increase their mass or undergo RNA, DNA and protein synthesis as well as many cellular metabolic processes. In fact, I will discuss evidence below that sensitive cells treated with streptomycin lose colony-forming ability before many of the observed effects on metabolism. This implies in all of these situations that while streptomycin
binding to the critical target site(s) might give rise to multiple pleiotropic effects, these resultant changes in cell metabolism do not necessarily constitute the lethal event.

A large group of aminoglycosides which are similar to streptomycin are all bactericidal despite different ribosomal binding sites, etc. Other aminocyclitols that differ substantially from streptomycin, such as spectinomycin, and kasugomycin, are bacteriostatic. Furthermore, only true aminoglycosides like streptomycin induce the initiation of EDPII (Energy Dependent Phase II of uptake; see Figure 1 for schematic representation of aminoglycoside uptake) (Höltje, 1978), an event that appears to be closely related to the onset of lethality (see below). In fact, a number of different aminoglycosides can induce EDPII for streptomycin, even in streptomycin-resistant mutants (Höltje, 1979a). These observations are highly instructive. It is the thesis of this review that there is a common critical target site and lethal event for streptomycin and related aminoglycosides. Furthermore, the evidence presented below, in my opinion, convincingly eliminates most current theories concerning the mode of action of aminoglycosides. I shall attempt to provide other suggestions consistent with the available evidence and present knowledge of bacterial physiology.

Sequence of events. It becomes important, when describing the sequence of events resulting from aminoglycoside action on cells, to consider first the relationship of the lethal event to the onset of the second energized phase of uptake, EDPII. This is made difficult by two factors. Firstly, papers on the simultaneous determinations of aminoglycoside uptake and colony forming ability at various times after aminoglycoside addition, report uptake on a linear scale and survival on a logarithmic scale, making comparisons difficult. Secondly, washing ionically bound aminoglycoside from cells can be difficult and is highly dependent on the washing medium (Plotz, Dubin & Davis, 1961). Thus, apparent uptake and even protein synthesis inhibition (Hurwitz, Landau & Doppell, 1962) can vary at least fivefold depending on the methodology used for washing or diluting the cells. While the washing procedure does not influence the apparent time of initiation of EDPII (but can alter the apparent amount of uptake), it is possible that this ionically bound streptomycin, if not removed, could contribute markedly to subsequent lethality. In this case, cells would become committed to die (i.e. suffer a lethal event) after the sample was taken. The results of Plotz et al. (1961), suggest that ionically bound streptomycin enters the cell within 15 sec after dilution of cells into water; the extent of post-sample uptake being related to the composition of the medium. While it is clear that great care is usually taken in washing cells for uptake assays, with some exceptions often less care is taken in the assay of viable cells. Bearing these two points in mind, the evidence below suggests that most or all of EDPII uptake is performed by cells that have already suffered a lethal event (although they may increase in mass for some time after it. In other words, the lethal event either precedes (gives rise to?) or is coincident with the onset of EDPII. Some of the evidence favouring this statement is:

(i) Cells rendered inviable with non-radioactive streptomycin (Höltje, 1978; Hurwitz & Rosano, 1962a) prior to the addition of radioactive streptomycin exhibit uptake rates and profiles similar to EDPII. No lag prior to the onset of EDPII, as normally seen for untreated cells is observed.

(ii) Cells treated with high (probably lethal) concentrations of kanamycin and gentamicin take up streptomycin at EDPII rates (Höltje, 1978). In contrast, cells treated with twenty-fold higher concentrations of the bacteriostatic
aminoglycosides spectinomycin and kasugomycin do not show enhanced streptomycin uptake.

(iii) Streptomycin resistant (rpsL) cells pretreated with a lethal concentration of kanamycin demonstrate considerably enhanced uptake, despite the fact that rpsL cells do not normally have an EDPII phase of uptake (Holtje, 1979a).

(iv) The addition of chloramphenicol to cells prevents initiation of streptomycin killing (Plotz & Davis, 1962), reduces EDPI uptake and prevents EDPII from occurring (Bryan & van den Elzen, 1976). Addition of chloramphenicol to cells during EDPIII immediately shuts off cell killing (Plotz & Davis, 1962) but has a relatively small effect on uptake (Andry & Bockrath, 1974; Bryan & van den Elzen, 1975; Holtje, 1978; Hurwitz & Rosano, 1962a). Thus, cells that have already lost colony forming ability (usually >99.9% at the time of chloramphenicol addition) continue to take up streptomycin at a high rate and only cells that have not yet initiated streptomycin uptake are rescued.

(v) Several authors have shown experiments in which a population of cells have lost 60–90% of their viability prior to the initiation of EDPII (Carlson & Bockrath, 1970; Dubin, Hancock & Davis, 1963; Heller et al., 1980; Hurwitz & Rosano, 1962a) while many others have revealed that less than 1% of the cells will form colonies when less than 30–50% of EDPII uptake has been completed (Carlson & Bockrath, 1970; Heller et al., 1980; Miller et al., 1980; Nielsen, 1978).

It should be stressed that while the initiation of EDPII is normally associated with the lethal event, this association is not obligate. Puromycin can induce EDPIII-like uptake of streptomycin in rpsL cells without immediate effects on viability (Hurwitz, Braun & Rosano, 1981). This strongly suggests that, in wild type cells, the initiation of EDPII uptake is not the sole cause of cell death although it may contribute to it. With this in mind, I have attempted to analyse the sequence of events occurring in cells treated with streptomycin (Table I). The timing of events 11–15 effectively eliminates them from consideration as the lethal event. I shall attempt to eliminate certain other effects as being responsible for aminoglycoside lethality.
Table I. Sequence of initiation of effects arising from streptomycin addition to cells

<table>
<thead>
<tr>
<th>Effect</th>
<th>Time of initiation of effect</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ionic binding</td>
<td>Immediate</td>
<td>Plotz et al. (1961)</td>
</tr>
<tr>
<td>2. SM crosses the outer membrane</td>
<td>Early EDPI</td>
<td>Hancock et al. (1981)</td>
</tr>
<tr>
<td>3. CM sensitive step</td>
<td>Pre-EDPII initiation, pre-lethal event</td>
<td>Bryan &amp; van den Elzen (1975); Plotz &amp; Davis (1962)</td>
</tr>
<tr>
<td>4. K⁺ efflux</td>
<td>Middle EDPI, pre-lethal event</td>
<td>Dubin et al. (1963); Hancock (1964)</td>
</tr>
<tr>
<td>5. rpsL (strA) gene product involvement</td>
<td>Pre-EDPII initiation, pre-lethal event</td>
<td>Bryan &amp; van den Elzen (1976)</td>
</tr>
<tr>
<td>6. β-Galactosidase induction inhibition</td>
<td>Prior to bulk protein synthesis inhibition</td>
<td>Artman et al. (1972a); Pinkett &amp; Brownstein (1974)</td>
</tr>
<tr>
<td>7. RNA synthesis stimulated transiently</td>
<td>Around time of lethal event</td>
<td>Sakai &amp; Cohen (1975)</td>
</tr>
<tr>
<td>9. Protein synthesis inhibition</td>
<td>Around time of lethal event</td>
<td>Around time of EDPII initiation</td>
</tr>
<tr>
<td>10. Cell division arrested</td>
<td>Around time of lethal event</td>
<td>Hurwitz et al. (1962)</td>
</tr>
<tr>
<td>11. Respiration impaired</td>
<td>During EDPII b; after lethal event</td>
<td>Dubin et al. (1963)</td>
</tr>
<tr>
<td>12. β-Galactosidase excretion</td>
<td>During EDPII b; after lethal event</td>
<td>Dubin et al. (1963)</td>
</tr>
<tr>
<td>14. Turbidity (mass) stops increasing</td>
<td>During EDPII b; after lethal event</td>
<td>Dubin et al. (1963); Nielsen (1978)</td>
</tr>
<tr>
<td>15. RNA synthesis inhibition</td>
<td>After lethal event</td>
<td>Dubin et al. (1963)</td>
</tr>
</tbody>
</table>

Abbreviations: SM, streptomycin; CM, chloramphenicol.

*Effects 11–15 could be shown to occur at least 10 min after EDPII initiation in some experiments.

Ionic binding

The initial ionic binding of streptomycin has been studied by a number of authors. It is essentially complete a few seconds after streptomycin addition and is a linear function of the streptomycin concentration (Hurwitz & Rosano, 1962a). The apparent level of ionic binding is not influenced by inhibitors of energized uptake but can be markedly reduced by washing cells with solutions of various salts, particularly high concentrations of monovalent or lower concentrations of divalent cations (Plotz et al., 1961; Bryan & van den Elzen, 1977). Anions also apparently affect ionic binding (Plotz et al., 1961) suggesting that this is one locus of inhibition of uptake by ions (Hancock, 1981, p. 250. The binding of aminoglycosides occurs primarily at the cell surface, is readily reversible (McQuillen, 1951; Morris &
Jennings, 1975), and can neutralize 82–100% of the net negative charge on the cell surface (Morris & Jennings, 1975). The amount of streptomycin bound to cells can be significantly enhanced by destroying the permeability barrier with toluene or by heat killing of cells (Hurwitz & Rosano, 1962a).

**Outer membrane permeabilization**

In *Pseudomonas aeruginosa*, the interaction of streptomycin and gentamicin with cells resulted in permeabilization of outer membranes to lysozyme (a 14,000 dalton protein which degrades the peptidoglycan thus lysing the cell) and to nitrocefin (a chromogenic substrate of periplasmic \( \beta \)-lactamase) (Hancock, Raffle & Nicas, 1981). This permeabilization was very rapid, occurring within the first 30 seconds and was unaffected by the strA mutation, protein synthesis inhibitors, and uncouplers and inhibitors of electron transport demonstrating that it occurred prior to EDPI or EDPII transport. It was inhibited by low concentrations of Mg\(^{2+}\) and other divalent cations and by higher concentrations of phosphate (Raffle & Hancock, unpublished results). The data suggests that the interaction of aminoglycosides with the Mg\(^{2+}\) binding site on the outer membrane of *Ps. aeruginosa* (Figure 2) permeabilizes the outer membrane to the aminoglycoside, and that this event occurs very early in aminoglycoside uptake. The mechanism for aminoglycoside-mediated outer membrane permeabilization is possibly localized distortion of the bilayer, as has been postulated for polymyxin (Hartman, Galla & Sackman, 1978). Evidence for this is the cross resistance of outer membrane altered mutants with a decreased concentration of Mg\(^{2+}\) binding sites on the cell surface, to polymyxin and aminoglycosides. We have been unable to demonstrate permeabilization of outer membranes in *Escherichia coli*, and believe that aminoglycosides probably use the hydrophilic (porin protein-mediated) uptake pathway (Nikaido, 1976; see also Hancock, 1981, p. 269) in other bacteria. Iida & Koike (1974) have demonstrated surface blebbing within 10 min of addition of streptomycin or kanamycin to *E. coli* B and *Ps. aeruginosa* cells. However, since these alterations are inhibited by protein synthesis inhibitors and occur with other aminocyclitols like spectinomycin and kasugamycin, they are probably a non-specific result of inhibition of protein synthesis.

**Cytoplasmic membrane effects**

The leakage of \( \beta \)-galactosidase and of adenine nucleotides from cells after the initiation of EDPII (Table I), demonstrates that after the streptomycin lethal event, cytoplasmic membranes become leaky, possibly due to the enormous amount of streptomycin interacting with and presumably crossing the cytoplasmic membrane (the amount of streptomycin in the cell can increase to 1–8 \( \mu \)g/mg cell dry weight within 30 min of the start of EDPII—see Fig. 16, Bryan & van den Elzen, 1976). The mechanism for such leakage could conceivably be similar to the one postulated above for outer membrane permeabilization. Similarly, respiration is impaired after the lethal event (Table I), and cells might become at least partially uncoupled, although electron transport and the protonmotive force must be sufficient to allow the observed levels of aminoglycoside uptake. This would appear to rule out effects on the energy generating components as being the lethal event in aminoglycoside action, since critical damage to these components would prevent EDPI and II uptake (Hancock, 1981, pp. 252–4).
Figure 2. Model illustrating the proposed site of uptake of aminoglycosides through *P. aeruginosa* outer membranes (O.M.) (R core-the heptose, KDO, rough core region of the LPS; (P)_k-the polyphosphate portion of the *P. aeruginosa* LPS, this phosphate is negatively charged at neutral pH and is attached to the KDO-heptose region of the LPS and may consist of as many as 12-15 moles of phosphate/mole LPS). It is proposed that aminoglycosides act to replace Mg^{2+} at its LPS binding site and cause distortion of the outer membrane bilayer. This then permits passage of other molecules of aminoglycoside through this region of the outer membrane into the periplasm.

Other cytoplasmic membrane effects are putrescine and K^+ excretion, both of which occur before or near the onset of streptomycin-induced lethality. The effect on putrescine excretion is specific to this polyamine since spermidine levels are not affected until some time later (Raina & Cohen, 1966). Since bacteriostatic agents like levorphanol also cause putrescine excretion, this cannot be the cause of lethality. Putrescine excretion does not occur in the presence of chloramphenicol, and therefore, it probably results from the interaction of streptomycin with ribosomes. The leakage of K^+ does not occur in streptomycin-resistant cells of *Bacillus megaterium* (Hancock, 1964). This would seem to indicate either that interaction with ribosomes is required for K^+ leakage or that the apparent uptake of streptomycin in *strA* cells (Bryan & van den Elzen, 1976) is different in nature to the EDPI uptake in sensitive cells. An alternative explanation for K^+ release could be either involvement of K^+ in an antiport mechanism of streptomycin uptake (i.e. that streptomycin uptake is coupled to K^+ release; see Harold, 1977) or non-specific cation-mediated release of K^+ (Bernheim, 1978).

An additional permeability effect occurs during the initiation of EDPII. This results in an increase in uptake of related aminoglycosides (Höltje, 1978). The induction of EDPII does not result in a generally permeable membrane since the uptake of glucose, glucosamine, maltose, lactose, adenosine and deoxyadenosine is unaffected, although spermidine and putrescine uptake is considerably enhanced. Höltje (1978) has demonstrated that the enhanced uptake of polyamines involves a site common to the streptomycin uptake system operating during EDPII, since streptomycin competitively inhibits polyamine uptake. One possible explanation is that streptomycin action induces the EDPII uptake system (see p. 440) which can be utilized by polyamines and which has a higher affinity for polyamines than the normal cellular polyamine uptake system.

Protein synthesis effects

Much of the evidence concerning the effects of aminoglycosides on protein synthesis has been obtained *in vitro*. It should be stressed that *in-vitro* protein-synthesizing
systems do not mimic precisely in-vivo synthesis (see Brock, 1966). In particular, the ionic strength ($\mu = 0.4$), $K^+ (0.15 \text{ m})$ and $\text{Mg}^{2+} (4 \text{ mM})$ concentrations found in E. coli can markedly effect levels of misreading and total protein synthesis both in the presence and absence of streptomycin (Sander, 1979; Tai, Wallace & Davis, 1978). Other factors such as temperature, pH, concentration of ribosomes and whether mRNA is pre- or post-treated with streptomycin prior to ribosome addition, all have large effects on the results obtained. It should also be stressed that in-vitro protein-synthesizing systems have a high natural rate of misreading (see e.g. Tai et al., 1978) and that the magnitude of the effects in vitro are consistently greater than those in vivo. As an example of this, streptomycin in in-vitro protein-synthesizing systems results in a stimulation of ppGpp formation (Pederson, Land & Kjeldgaard, 1973) while in-vivo streptomycin depresses ppGpp synthesis (Sakai & Cohen, 1975). I have thus attempted to restrict my arguments largely to in-vivo protein synthesis experiments.

While streptomycin undoubtedly inhibits protein synthesis, the major evidence for its role in the lethal event comes from the isolation of high level streptomycin resistant (rpsL or strA) mutants with altered ribosomal protein S12 (Hancock, 1981, p. 258). I will discuss this further below (see pp. 440 and 442) but it should be pointed out that similar mutants have not been obtained for other aminoglycosides. One can, however, rule out a gross effect on protein synthesis as being the bactericidal effect. For example:

(i) other inhibitors of protein synthesis which are considerably more efficient and faster acting are bacteriostatic at all concentrations used. These include two aminocyclitols that interact with ribosomes, kasugomycin and spectinomycin. It should be noted that both of these, like streptomycin (Davis, Tai & Wallace, 1974) inhibit initiation of protein synthesis (Okuyama et al., 1972; Reusser, 1976). The effect of these bacteriostatic antibiotics is very rapid (Davies, Anderson & Davis, 1965) and reversible upon dilution (Davis et al., 1974). The postulated mode of action of spectinomycin bears a large number of similarities to streptomycin (Davis et al., 1974).

(ii) The binding of streptomycin to ribosomes is reversible (Chang & Flaks, 1972) and does not irreversibly inactivate the ribosomes (although it may influence subsequent in-vitro misreading assays—Garvin, Rosset & Gorini, 1973). This rules out a mechanism of lasting ribosomal damage such as seen for virginiamycin M, an antibiotic that binds reversibly to ribosomes and leaves them inactive even after removal (Parfait & Cocito, 1980). Since binding of streptomycin is reversible and its action on protein synthesis dependent on its presence, if protein synthesis was the target, streptomycin should be bacteriostatic.

(iii) Chloramphenicol, spectinomycin and other protein synthesis inhibitors antagonize aminoglycoside killing, apparently at the level of transport (Hancock, 1981, p. 255). This has been rationalized by some authors as evidence for a role of protein synthesis in aminoglycoside killing (e.g. Davis et al., 1974). However, as noted above, spectinomycin has a very similar mode of inhibition of protein synthesis to streptomycin, and does not inhibit streptomycin binding to ribosomes (Chang & Flaks, 1972), and yet prevents killing by streptomycin (Davies et al., 1965). Furthermore, there is evidence that streptomycin, kanamycin and neomycin can kill cells in the virtual
(iv) The apparent level of in-vivo protein synthesis inhibition observed after streptomycin addition is made far more dramatic by two factors. Firstly, there is an increase of proteolysis around the time of cell growth inhibition by streptomycin (up to 40% of proteins made during streptomycin treatment; Hipkiss & Kogut, 1973). This effect is not specific since it occurs for bacteriostatic protein synthesis inhibitors like chloramphenicol (Pine, 1967; Pinkett & Brownstein, 1974). In addition, very early cyclic AMP mediated responses to streptomycin addition (see p. 437) cause selective inhibition of the synthesis of certain proteins (Artman, Werthamer & Golb, 1972a).

(v) Freda & Cohen (1966) showed that streptomycin treated cells could still support phage T6 infections and that the influence of streptomycin on, for example, histidine incorporation into phage protein was quite minor even at high (60 mg/l) levels of streptomycin. Furthermore, two phage enzymes were produced at just slightly less than normal concentrations and the phage yield was proportional to the phage DNA (rather than the protein) concentration. Phage T6 uses bacterial ribosomes in protein synthesis.

(vi) Streptomycin can inhibit growth well before in-vivo changes in the ribosome patterns can be observed (Kogut & Prizant, 1975).

Thus, while there is ample evidence for streptomycin inhibiting protein synthesis both in vitro (rapidly) and in vivo (after a considerable delay), the above arguments suggest that inhibition of protein synthesis and/or irreversible inactivation of ribosomes are not per se the lethal events of aminoglycoside action. They may however contribute (see p. 442). The observed inhibition of protein synthesis may result from any of a number of pleiotropic effects of streptomycin (as demonstrated in vitro) including inhibition of initiating ribosomes, destabilization of the initiation complex, partial inhibition of preformed polysomes, increased misreading on these polysomal ribosomes (see below), impaired dissociation of free ribosomes into subunits and increased rigidity of the ribosome (see Davis et al., 1974 for review). It should be noted however that to the casual reader in the field, it seems somewhat contradictory that the in-vitro effects of streptomycin on protein synthesis are often observed much better under non-physiological conditions (see above) and with very high levels of streptomycin which often cause only partial effects. The listed pleiotropic effects of streptomycin on in-vitro protein synthesis may or may not be of relevance in vivo.

Misreading during protein synthesis

Gorini (1974) has provided an excellent review of the evidence that, both in vivo and in vitro, bactericidal aminoglycosides cause misreading of the genetic code, i.e. amino acid substitutions during protein synthesis. Streptomycin-induced misreading has been observed (Tai et al., 1978) in both wild type and rpsL mutants. Gorini and colleagues have suggested that the build-up of faulty proteins inside the cell is the cause of the lethal event in streptomycin action. However, this proposition has a number of difficulties including:

(i) It does not explain the lethal action of streptomycin, kanamycin or neomycin in the absence of protein synthesis (Sakai & Cohen, 1975; Stern et al., 1966),
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nor the synergy between puromycin and streptomycin (Yamaki & Tanaka, 1963).

(ii) Freda et al. (1968) have demonstrated that cells infected with T6 phage at the
time of streptomycin addition show some quantitative but no gross
qualitative changes (i.e. no obvious high levels of misreading).

(iii) While some revertants of rpsL mutants that have increased sensitivity to
streptomycin, contain mutations that enhance misreading (Rosset & Gorini,
1972), others have no effect on misreading (Dabbs, 1980).

(iv) Measurement of the error frequency introduced by streptomycin suggested
that it increased slowly over the course of four generations of growth to a
maximum level of around 1–2% (i.e. 20-fold higher than normal) at levels of
streptomycin causing 40% growth inhibition (Edelmann & Gallant, 1977; see
also Garvin et al., 1973). The error frequency was reduced to normal levels
within two generations of resuspending cells in the absence of streptomycin
(Edelmann & Gallant, 1977) suggesting that the effects of misreading, unlike
the lethal event, are reversible. A maximum rate of misreading has also been
observed in vitro (Tai et al., 1978).

(v) Amino acid analogues which cause even greater misreading than strepto-
mycin did not significantly increase the rate of killing or the time of initiation
of EDPII uptake (Ahmad, Rechenmacher & Böck, 1980). Pine (1978) demon-
strated that ethionine caused a drastic effect on the activity (i.e. a 95%
inactivation) of β-galactosidase in 7 min but only started killing after a 2-h
delay suggesting that even gross misreading will not necessarily kill cells.

(vi) Misreading caused in vitro by streptomycin is barely observable at physio-
logical K+ (200 mM) and Mg2+ (4 mM) concentrations (Sander, 1979).

(vii) There is good evidence that mechanisms exist for the selective proteolysis
of nonfunctional (misread) proteins resulting from streptomycin action (Pinkett
& Brownstein, 1974).

Although the evidence does not favour misreading as the lethal event, there are
intriguing correlations between the level of misreading and the lethal action of
streptomycin (see e.g. Zimmermann, Rossett & Gorini, 1971). These correlations
appear to hold for streptomycin dependent cells as well as for sensitive cells.

Metabolic regulation

As shown by Pinkett & Brownstein (1974), streptomycin inhibits the production of
the β-galactosidase protein well before the initiation of bulk protein synthesis
inhibition. It is known that β galactosidase production is catabolite repressible, in
that its structural gene (lacZ) requires cyclic AMP and catabolite gene activator
protein for its transcription. Artman et al. (1972a) demonstrated that the preferential
inhibition by streptomycin of β-galactosidase synthesis could be reversed by the
addition of exogenous cyclic AMP. A similar effect was seen with 5-fluorouracil or
with subinhibitory levels of chloramphenicol instead of streptomycin. This
suggested that these agents reduce the cellular level of cyclic AMP prior to the
inhibition of bulk protein synthesis. Thus, this is one of the earliest known events in
streptomycin action (see Table I). Since it is known that cyclic AMP levels are
generally regulated at the level of the membrane (Saier, 1979), the effect may well be
related to EDPII aminoglycoside uptake. It is tempting, in the light of the known
stimulation of streptomycin action by cyclic AMP addition to cya mutants
(Hancock, 1981, p. 268), to postulate a key role for catabolic regulation or a catabolite repressible product in streptomycin action. However, cya and crp mutants, which are unable to support transcription of catabolite repressible genes, are only partially resistant to streptomycin (Alper & Ames, 1978). Despite this, one can conclude that the level of cyclic AMP in the cell may be involved in sensitivity to streptomycin in a contributory but non-essential fashion. Since the cyclic AMP requirement may well be at the level of transport (Höltje, 1978), it is interesting that streptomycin seems to reduce cellular cyclic AMP levels (see above). One reason for the long delay prior to initiation of streptomycin killing and EDPII uptake may be the fact that its action on cellular cyclic AMP levels limits uptake rates.

Another early manifestation of the interaction of streptomycin with cellular metabolic regulatory systems is the early enhancement of ribosomal RNA synthesis (Table I). Other studies have demonstrated that ribosomal RNA synthesis is dramatically and inversely affected by guanosine-5'-diphosphate-3'-diphosphate (ppGpp) levels (Nierlich, 1978). With this in mind, Sakai & Cohen (1975) studied the effect of streptomycin on ppGpp metabolism and ribosomal RNA synthesis. They concluded that the stimulation of ribosomal RNA synthesis occurred only in cells in which streptomycin influenced (i.e. suppressed) the ppGpp levels. However, they were able to demonstrate a specific strain and set of conditions in which cell death could occur despite the lack of effect of streptomycin on ppGpp levels and consequent absence of streptomycin stimulated ribosomal RNA synthesis. This implied that stimulation of RNA synthesis is not causally related to the lethal event.

The influence of streptomycin on cyclic AMP and ppGpp levels could explain some of the many pleiotropic effects of streptomycin (Table I), since the cellular levels of these two molecules can influence a wide range of cellular processes (Nierlich, 1978; Saier, 1978).

Miscellaneous effects

Although these effects are grouped under the heading 'miscellaneous effects', I do not wish to minimize their importance. In fact, as will be discussed on p. 442, one of these effects could well be the key to discovering the true nature of the lethal event in aminoglycoside killing.

It has frequently been observed in the literature that after aminoglycoside addition, the turbidity of cells increases for some time after the viable count has begun to fall precipitously (e.g. Dubin et al., 1963; Miller et al., 1980; Nielsen, 1978). This implies that the cells are able to undergo considerable metabolism and mass increase after the lethal event. Normally protein which makes up 50% of the mass of cells is the major contributor to mass increase. However, it would seem that given the effects of streptomycin on protein synthesis, most of the mass increase must involve RNA metabolism (Dubin et al., 1963) and cell membrane and wall growth. The available evidence suggests that cells, once killed by streptomycin, are unable to undergo even one cell division (Hurwitz et al., 1962a). With respect to RNA synthesis, the effects seemed to be highly medium dependent, with almost no early effect of streptomycin on RNA metabolism in a low phosphate (Hancock, 1964), or low K+ medium (Dubin et al., 1963), compared to a variety of effects in high phosphate medium (Hancock, 1964; Stern et al., 1966).

Finally, I would like to draw attention to an extremely interesting and perhaps important observation that seems to have received scant attention. Freda et al.
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(1968) examined thin sections of phage-infected and uninfected streptomycin-treated E. coli cells. The appearance of the DNA (which is less electron dense than other cytoplasmic components in thin sections) was unusual, forming rounded 'nucleoids' occupying a central position in the cell. This observation will be discussed further below (see p. 442).

Mechanism of transport

The significance of transport in aminoglycoside action is illustrated by the data reviewed in my previous article (Hancock, 1981). All well-studied antagonists of aminoglycosides and all mutations altering aminoglycoside susceptibility influence aminoglycoside action at the level of uptake. In fact only one type of mutation, the high-level streptomycin resistance (rpsL, strA) mutation is known to abolish EDPII uptake without influencing the initial rate of streptomycin uptake during EDPI (Bryan & van den Elzen, 1976). This mutation is critical when one considers the possible mechanism of transport and mode of action of streptomycin. Bryan & van den Elzen (1977) have described a useful model for the energization and possible carriers involved in aminoglycoside uptake. I wish to enlarge upon this model and make four major points: (i) that there is no stringent proof to date that the EDPI actually represents uptake, (ii) from consideration of the uptake characteristics of strA mutants, the EDPI can be separated into two components only one of which is dependent on ribosomal affinity, (iii) that while the evidence favours a role of reduced quinones in streptomycin uptake, it seems unlikely that the quinones themselves are the streptomycin carrier and (iv) that permeabilization of the cytoplasmic membrane to further aminoglycoside uptake, which is initiated at the start of the EDPII may be related to the lethal event in aminoglycoside action.

It should be stressed that aminoglycoside uptake studies are technically extremely difficult to perform. Streptomycin, for example, is known to have quite high affinity for a wide range of compounds and can precipitate a number of these and adsorb to others (see Brock, 1966 for review). Among these are the cell surface (see p. 433), DNA, RNA, ribosomes, serum proteins, phosphatidyl ethanolamine, casein, cellulose and nitrocellulose filters. Washing techniques remove much of this non-specifically bound aminoglycoside but one can never be certain that the washing procedures are totally efficient (see e.g. Beggs & Andrews, 1976; Plotz et al., 1961 for some of the potential pitfalls).

Bryan & van den Elzen (1976) have demonstrated that energy-dependent streptomycin uptake by streptomycin-resistant (rpsL) E. coli cells is a time-dependent saturable process. From their data, one can calculate an apparent $V_{\text{max}}$ of approximately $5 \times 10^9$ molecules of streptomycin/bacterium/10 min (assuming $3 \times 10^9$ bacteria per mg dry weight) and an apparent affinity constant (= substrate concentration at half $V_{\text{max}}$) $K_m = 0.5 \text{ nm}$. This $K_m$ is much higher (i.e. the affinity of the uptake system for streptomycin is much lower) than for most other energized transport systems. It is clear that some streptomycin must penetrate streptomycin resistant (and dependent) cells since Gorini and colleagues have demonstrated phenotypic suppression by streptomycin in such cells while others have demonstrated that these cells can be killed by streptomycin (Hancock, 1981, p. 442). However, it is not at all clear whether the apparent uptake in these cells represents uptake or binding. For example, similar time-dependent, saturable uptake
characteristics are observed for hormones binding to their receptors (e.g. Brumbaugh & Hausler, 1974; Giorgio, Johnson & Blecher, 1974), and similar binding reactions can require a membrane potential (Kessler & Toggenburger, 1979). The stringent proof of whether this uptake in streptomycin resistant cells represents actual translocation across the membrane or binding to a cytoplasmic membrane receptor is as yet lacking. Such studies should include the ability of excess non-radioactive aminoglycosides and of energy and protein synthesis inhibitors to compete with radioactive aminoglycoside after a given period of uptake (see also Kessler & Toggenburger, 1979 for other possible methods).

It should be noted that uptake rates in streptomycin-resistant and -sensitive cells are initially identical (Bryan & van den Elzen, 1976; Campbell & Kadner, 1980) but begin to diverge before the start of EDPII rapid uptake (Ahmad et al., 1980; Bryan & van den Elzen, 1976). There is in fact a period of accelerating uptake for some time towards the end of EDPI (see Figure 1). As shown by Ahmad et al. (1980), this uptake appears to be more rapid in mutants with enhanced ribosomal affinity for streptomycin. This, together with results obtained with protein synthesis inhibitors may suggest a role for ribosomal affinity in late-EDPI uptake (providing this really is uptake). Alternatively, this accelerating uptake may represent a more sensitive subpopulation of cells, although there is no particular evidence for the existence of such a subpopulation at present.

As suggested by Bryan & van den Elzen (1977), there is a growing body of evidence which implicates reduced quinones in the uptake of aminoglycosides. However, it should be pointed out that a simple consideration of the structure of quinones together with the preferential inhibition of streptomycin uptake by divalent cations, would appear to rule out a direct role for quinones in uptake of streptomycin. Since quinones can interact with a large number of different integral membrane and membrane-associated components of the electron transport chain (Singh & Bragg, 1976; Wallace & Young, 1977), it is possible that it is the interaction of reduced quinones with one of these proteins which provides the binding site and/or facilitator of uptake of streptomycin.

The EDPII provides one of the real mysteries associated with aminoglycoside uptake. I have summarized the data which suggests that the initiation of EDPII uptake occurs at or around the time of killing. However, the events that lead to the initiation of EDPII are unclear. Certainly one cannot explain the specific effect of enhancement of uptake of aminoglycosides and polyamines in the absence of gross membrane damage, as seen at the initiation of EDPII, by postulating that misreading or inhibition of protein synthesis somehow damages the membrane. The proof of this is that EDPII initiation is apparently not delayed significantly (i.e. enough time for protein synthesis effects to occur) when high enough concentrations of aminoglycoside are used in uptake assays (as seen for some uptake curves in Bryan & van den Elzen, 1977; Bryan, Haraphongse & van den Elzen, 1976; Miller et al., 1980; Muir & Wallace, 1979; Nielsen, 1978). There is good evidence that EDPII involves specific energized uptake (Bryan & van den Elzen, 1977; Höltje, 1978). The aminoglycoside taken up during the EDPII is not tightly bound to cytoplasmic components since it can be almost totally released by toluene (Andry & Bockrath, 1974) or sonication (Dickie, Bryan & Pickard, 1978). A number of authors have attempted to explain these and other observations on the basis of induction of a specific permease for a transport system (e.g. Hurwitz & Rosano, 1965; Höltje, 1978). However, to date
no such transport system or inducible permease has been identified (see p. 434 and Hancock, 1981, p. 251). Indeed, it seems unlikely that cytoplasmic membrane should have specific permeases capable of taking up such non-physiological molecules as aminoglycosides (i.e. tetracationic trisaccharides). Nevertheless, equally unusual antibiotics do find their way into the cell. A further difficulty is provided by the observation that the aminoglycoside uptake can be utilized by such different molecules as polyamines (Höltje, 1978) and adenylated or acetylated aminoglycosides (Dickie et al., 1978; Höltje, 1979a). In the absence of any identified transport system, it seems reasonable to propose that a self-promoted transport mechanism, similar to the one we have described for outer membranes (Hancock, Raffles & Nicas, 1981) may operate for the cytoplasmic membrane.

A number of authors have demonstrated a linear correlation between the apparent amount of streptomycin taken up within a given time period and the MIC (Hancock, 1962), growth inhibition (Kogut, Lightbrown & Isaacson, 1966) and killing (Carlson & Bockrath, 1970; Heller et al., 1980). This implies that there is a critical level of aminoglycoside uptake required for cell killing. Estimates of the number of streptomycin molecules required for an individual lethal event vary between 20,000 and 100,000 per cell (Ahmad et al., 1980; Carlson & Bockrath, 1970; Kogut et al., 1966). However, due to the technical difficulties involved in aminoglycoside assays, these must be treated as approximate.

The following model therefore attempts to account for the wide range of data present in the literature. Aminoglycosides bind ionically to the negatively charged outer surface of the cell. In Gram-negative organisms, the aminoglycosides then pass through the outer membrane either by interacting with and disrupting a Mg$^{2+}$-binding site (the LPS) or passing through the hydrophilic channels of porin proteins (see p. 433). The aminoglycosides then bind to a cytoplasmic membrane site by a process that requires active involvement from oxidized quinones and possibly also an energized membrane (Hancock, 1981, p. 267). After binding, translocation across the cytoplasmic membrane occurs. The reaction involved in translocation is relatively slow, dependent on external aminoglycoside concentrations and perhaps also on the transmembrane electrical potential gradient, and is kinetically unfavourable in the absence of intracellular binding sites. The progressive binding of aminoglycoside to the high affinity ribosomal binding site results in the acceleration of uptake seen towards the end of EDPI (see Figure 1). Upon saturation, by the aminoglycoside, of the target sites (which probably include the ribosome, thus affecting protein synthesis, as well as a membrane-bound protein), it is postulated that an event occurs at the level of the membrane which contributes to but is not alone sufficient to cause lethality (see below for details). This event results in initiation of the rapid uptake phase (EDPII), and contributes to inactivation of a critical cellular function. The rapid influx of aminoglycoside causes progressively increasing membrane damage resulting eventually in leakage of cytoplasmic components (Table I). It is further proposed that the slow uptake observed in streptomycin resistant cells either represents binding to the outside of the cytoplasmic membrane or reflects the lack of high affinity ribosomal binding sites but presence of other binding sites of lower affinity.

**Mode of action**

The elusive mode of action of aminoglycosides has intrigued microbiologists for
nearly four decades. Over the years, a number of theories and counter-theories have arisen, fuelled by the many pleiotropic effects of aminoglycosides (Table I), the large variety of antagonists of aminoglycoside action, the annoying ability of these antibiotics to bind to a wide range of anionic biological molecules and the relative dearth of high level aminoglycoside resistant mutants. The existence of unique ribosomal mutants showing high level resistance to one aminoglycoside (streptomycin) and the pleiotropic effects of aminoglycosides on *in-vitro* and *in-vivo* protein synthesis have lead to the conclusion that they act at the level of protein synthesis. While this is certainly true, it is hardly a unique mode of action amongst antibiotics, including many bacteriostatic antibiotics. As pointed out in this review, a general inhibition of protein synthesis and an ability to cause misreading are almost certainly not the specific reason for the bactericidal nature of streptomycin and related aminoglycosides, although as pointed out below they might well contribute to it. The actual lethal event must remain speculative but I will attempt to express one or two possibilities consistent with the evidence to date.

Despite the high pleiotropy of effects caused by aminoglycosides like streptomycin, almost all theories have proposed a ‘one hit’ model for streptomycin action, i.e. the existence of a single critical target. It would in fact seem that a ‘two hit’ model would better fit the data. A ‘two hit’ model would require two separate targets; the inactivation of either of them being non lethal, but the inactivation of both of them resulting in cell death. The contributory role of protein synthesis could be expressed if one of the two targets was a labile protein which was inactivated after the fulfilment of its function, i.e. similar to the *tonB* protein (Bassford, Schnactman & Kadner, 1977), at least one protein involved in the initiation of DNA synthesis (Lark & Renger, 1969), or any of the number of proteins hypersensitive to proteolysis (Goldberg & St. John, 1976). Thus continuous protein synthesis would be required to allow maintenance of the function of this target protein. The ‘second target’ might be inactivated in a number of ways reflecting the unique properties of streptomycin-like aminoglycosides. For example, either irreversible binding of aminoglycosides (as occurs to superoxide dismutase, Britton & Fridovich, 1978) or competition for and disruption of a Mg$^{2+}$ or polyamine site important in the interaction of two molecules (as probably occurs in the outer membrane of *Ps. aeruginosa*, Hancock *et al.*, 1981), might well result in inactivation of the ‘second target’. The inactivation of these two targets would then result in irreversible loss of a key cellular function.

How then might aminoglycosides kill a bacterial cell? There would appear to be at least five potential mechanisms for bringing about rapid cell death. These include cell wall destruction leading to lysis, cytoplasmic membrane puncture, a lethal hit on an essential gene, crosslinking or double stranded breaks in the DNA, and disruption of the DNA-membrane attachment site. Since it is known that aminoglycosides cause a variety of membrane-associated disruptions, but do not result in lysis or general membrane puncture until well after the lethal event (see Table I), I would like to focus on the DNA-membrane attachment site as a potential target of action of streptomycin. The site of attachment of DNA to the membrane has been proposed to play an important role in segregation of newly-synthesized chromosomes into daughter cells, and in the process of DNA initiation (Matsushita & Kubitshek, 1975). Disruption of such a site would have the following effects: (i) the newly synthesized chromosomes would not separate and would tend to become entangled, (ii) the chromosome would detach from the cytoplasmic membrane and (iii) there
would probably be no immediate effects on the rate of DNA elongation although there would be no new initiation of DNA synthesis and little or no cell division. In fact, predictions (i) and (ii) have been observed to occur in streptomycin-treated cells (i.e. the 'pathological nucleoids' of Freda et al., 1968). I can find no definitive studies on DNA synthesis and streptomycin. However streptomycin killed cells do not undergo cell division (Hurwitz et al., 1962a), although their mass does increase for some time after the lethal event (Table I). In addition, it is known that there is a requirement for new protein synthesis (the first target?) prior to DNA initiation, although protein synthesis inhibition by the bacteriostatic antibiotic chloramphenicol causes only reversible inhibition of DNA initiation (Lark & Renger, 1969). The existence of a 'second target' for streptomycin could thus explain the bactericidal nature of streptomycin. In addition, the dominance of streptomycin sensitivity over resistance could be explained by the fact that the synthesis of the 'first target' apparently occurs during a short period of the cell cycle (Lark & Renger, 1969) and would thus be highly susceptible to even moderate protein synthesis inhibition/misreading. It should be noted that at the time of the lethal event the number of streptomycin molecules in the cell (20,000–100,000) are more than sufficient to allow binding to ribosomes as well as the putative 'second target'. An extension of this proposal would be that the disruption of the DNA-membrane attachment site would result in the EDPII. An alternative possibility might be the inactivation of the 'second target' as a consequence of the accelerated movement of aminoglycoside molecules across the cytoplasmic membrane immediately after the initiation of EDPII (as a response to the interaction of aminoglycosides with ribosomes or with the cytoplasmic membrane?). These models are offered tentatively. However, it is clear that despite the large body of research on aminoglycoside mode of action and transport, we have a long way to go before we understand the molecular mechanisms involved in aminoglycoside action. It is my hope that this review has highlighted some of the accessible problems.

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Additional References

(References given in part I (Hancock, 1981) are not repeated here)


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