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

I. Antagonists and mutants

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'If history repeats itself, streptomycin has a few more tricks up its sleeve for us'
—T. D. Brock

Introduction

Since its isolation in 1944 by Schatz, Bugie and Waksman, the mode of action and transport of streptomycin (and more recently, gentamicin) have invoked considerable interest and a great deal of controversy. The killing of susceptible bacterial cells by these aminoglycosides is accompanied by a wide range of pleiotropic effects and can be antagonized by compounds known to affect a variety of cellular metabolic functions. Further confusion has been added by the phenotypic diversity of mutants

*The author wishes to point out that he is not related to Ronald Hancock, who did some of the early pioneering work on streptomycin uptake.

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with altered susceptibility to streptomycin and gentamicin. It is the purpose of this review to attempt to explain this wealth of information in terms of currently-accepted bacterial physiology.

The timing of the events occurring during aminoglycoside killing of cells varies considerably according to the dose of aminoglycoside used. As a point of reference in this review, I will attempt to relate these events to known events in aminoglycoside transport. Much of our current understanding of aminoglycoside uptake owes itself to the work of Dr L. E. Bryan and colleagues. They have demonstrated that streptomycin and gentamicin are taken up in three phases: an initial ionic binding to cells, followed by two energy-dependent phases (called EDPI and EDPII) in which there is an apparent energized uptake of aminoglycosides at a slow rate (EDPI) followed by a very rapid energy-dependent accumulation of the aminoglycoside (EDPII). A typical aminoglycoside uptake experiment is illustrated in Figure 1. It is one of the propositions of this review that EDPII uptake is a result of or is coincident with the lethal event in aminoglycoside action [see Hancock, 1981, section (A) (b)]. Therefore, where possible, I will attempt to relate the site and mode of action of inhibitors and mutations to the two uptake phases EDPI and EDPII. Part II of this review will deal with the effects of aminoglycosides on cells and will discuss potential mechanisms for aminoglycoside uptake and cell killing.

Antagonists of aminoglycoside transport and cell killing

Perhaps one of the major areas of difficulty in aminoglycoside therapy in recent years has been created by the wide range of antagonists of aminoglycosides. Such antagonists lower the effective concentration of aminoglycosides and can result in failure of therapy. The mode of antagonism and effects on aminoglycoside action of such compounds is summarized in Table I and discussed in more detail below.

Cations and anions

The antagonism of streptomycin by salts has been known almost since its discovery. Donovick *et al.* (1948) studied the effects of a variety of salts and concluded that Mg^{2+} and Ca^{2+} caused the greatest interference while Na^+, Li^+ and K^+ had little effect. By keeping the cations constant and varying the anions they concluded that acetate and pyruvate were not antagonistic while other anions could be ranked in order of decreasing efficiency of antagonism nitrate > chloride > lactate > phosphate > tartrate > citrate > sulphate. A number of authors subsequently pursued the phosphate effect but their studies were not definitive. Phosphate, nitrate and fumarate could also be shown to inhibit streptomycin uptake (Campbell & Kadner, 1980; T. Nicas & R. E. W. Hancock, unpublished results) although the mechanism of inhibition remains unclear. Possibly these anions can compete with anionic groups on the cell surface for streptomycin binding. The results of Campbell & Kadner (1980) for *Escherichia coli* suggested that various salts which inhibited streptomycin uptake could be ranked spermidine^{3+}, $MgCl_2$, streptomycin^{3+}, $CaCl_2$ > putrescine^{2+} > cadaverine^{2+} > KCl > NaCl > sodium phosphate in order of decreasing effectiveness of antagonism. Since the ionic strengths of the 50% inhibitory concentrations varied from $1{\cdot}35 \times 10^{-4}$ for $MgCl_2$ to 47×10^{-4} for NaCl (i.e. 35-fold), it is unlikely that this is merely an ionic strength effect, but must reflect competition for binding sites. While various authors have described a non-specific

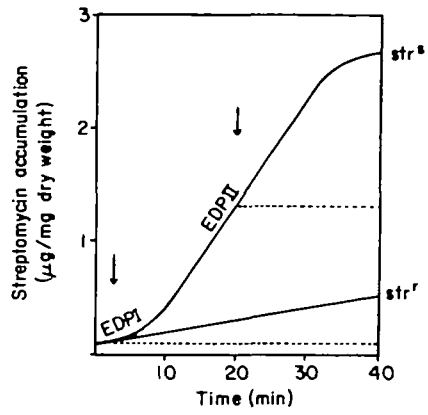

Figure 1. Typical aminoglycoside uptake curve: the result shown is for dihydrostreptomycin uptake in a sensitive *Ps. aeruginosa* strain (str^S) and its streptomycin resistant (str^r, *rpsL*) mutant. However uptake profiles essentially similar to the str^S uptake curve are seen for gentamicin, sisomicin and streptomycin uptake into *E. coli*, for gentamicin and sisomicin uptake into *Ps. aeruginosa*, and for gentamicin and sisomicin uptake into *Staph. aureus* and *K. pneumoniae* (Bryan & van den Elzen, 1976; Lee *et al.*, 1977; Miller *et al.*, 1980). The energy dependent uptake phases EDPI and EDPII are indicated. The initiation of the EDPII uptake phase is considered in this review to be the first time point at which uptake becomes linear and rapid (i.e. at 9 min in the above example). The arrows indicate the time of addition of inhibitor (for example CN^-) and the resultant effect on uptake is shown by the dotted lines.

ionic strength effect on gentamicin and streptomycin killing in *E. coli, Pseudomonas aeruginosa, Mycobacterium smegmatis,* etc. (Beggs & Andrew, 1976*a, b*; Heller, Spector & Aalyson, 1980; Medeiros *et al.*, 1971; Zimelis & Jackson, 1973), only a limited range of salts have been tested and usually complex media with high initial concentrations of salts have been used (see Medeiros *et al.*, 1971 for the concentrations of salts in various media). Nevertheless, the following trends have become clear. Divalent cations such as Mg^{2+}, Ca^{2+} and Sr^{2+} seem to have a preferential effect over monovalent cations in antagonism of aminoglycoside action in *Ps. aeruginosa* and *Myco. smegmatis* (Beggs & Andrews, 1976*a, b*; Medeiros *et al.*, 1971). This antagonism of aminoglycoside killing is caused by an inhibition of both ionic binding to the cell surface and energized uptake (Beggs & Andrews, 1976*a*; Bryan & van den Elzen, 1977; Campbell & Kadner, 1980). A similar preferential effect of divalent cations has been observed for streptomycin uptake in *E. coli* and *Staphylococcus aureus* (Bryan & van den Elzen, 1977; Campbell & Kadner, 1980) although the effect on the MIC is less clear (see e.g. Beggs & Andrews, 1976; Medeiros *et al.*, 1971) for *E. coli*. One site of action of divalent cation inhibition seems to be the cytoplasmic membrane since in the Gram-positive organisms *Staph. aureus* and *Mycobacterium*, significant divalent cation antagonism of uptake and killing by streptomycin was observed (Beggs & Andrews, 1976*a*; Bryan & van den Elzen, 1977). In addition, Ca^{2+} inhibited uptake in *E. coli* spheroplasts with permeabilized outer membranes.

With regard to its site of action, Ca^{2+} (at a concentration of 25 mM) caused immediate arrest of the bactericidal effect of gentamicin on *Ps. aeruginosa*, even when added after the cell viability was already reduced 10^5-fold (Zimelis & Jackson, 1973). This demonstrates that at least one of the sites of divalent cation antagonism is freely accessible to Ca^{2+} and that binding of aminoglycosides at this site is required for cell killing. We have identified an outer membrane Mg^{2+} binding site (the

lipopolysaccharide) which is also involved in divalent cation antagonism of streptomycin and gentamicin killing and uptake in *Ps. aeruginosa* (Hancock, Raffle & Nicas, 1981; Nicas & Hancock, 1980). The existence of such a binding site on the cell surface of *Ps. aeruginosa* probably explains the greater antagonism of aminoglycoside action by divalent cations in *Ps. aeruginosa* when compared to *E. coli* (Beggs & Andrews, 1976*b*; Medeiros *et al.*, 1971). It is perhaps worth noting that this site is also involved in killing by polymyxin B and EDTA, two other agents to which *Ps. aeruginosa* is unusually sensitive (Nicas & Hancock, 1980).

It has been demonstrated that EDTA, a chelator of divalent cations, and aminoglycosides act synergistically against bacterial cells (Davis & Iannetta, 1972). This probably results from removal of antagonistic divalent cations by chelation, but may also involve facilitation of aminoglycoside movement across the outer membrane of Gram-negative bacteria since EDTA has been shown to permeabilize outer membranes (Leive, 1965).

Many workers have studied the effects of NaCl on aminoglycoside action. Heller *et al.* (1980) demonstrated that NaCl influences cell viability at the level of transport. The work of Donovick *et al.* (1948, see above) suggests that it is the Cl^- ion rather than the Na^+ ion which influences aminoglycoside action, although others have argued that ionic strength is the determining factor.

Electron transport inhibitors and anaerobiosis

One of the major areas in which our knowledge of aminoglycoside uptake and mode of action has increased in recent years is in the energetics of uptake. An outline of the chemiosmotic hypothesis of cellular energetics, and the mode of action of certain inhibitors, is presented in Figure 2 as a basis for the following discussion.

It was first demonstrated by Hancock (1962) that the uptake and lethality of streptomycin required respiration (electron transport) since it was antagonized by anaerobic conditions, by carbon monoxide, an inhibitor of cytochrome oxidase, and by 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HOQNO), now recognized as an electron transport inhibitor. Since this time, a number of authors have demonstrated essentially similar results using the electron transport inhibitors potassium cyanide, oxamic acid, sodium azide, sodium amytal, *N*-ethylmaleimide, and parachloromercuribenzoate (Andry & Bockrath, 1974; Bryan & van den Elzen, 1975, 1976; Höltje, 1978; Miller *et al.*, 1980). The effect was observable for both gentamicin and streptomycin uptake and killing in *Ps. aeruginosa, E. coli* (Bryan & van den Elzen, 1975, 1976) and *Staph. aureus* (Miller *et al.*, 1980). Both the EDPI and EDPII phases of uptake, but not the primary ionic binding to cells, were inhibited (see Figure 1). In those cases where uptake and/or killing were not totally antagonized by electron transport inhibitors (e.g. Nielsen, 1978), the lack of controls demonstrating the degree of inhibition of respiration make interpretation of such experiments difficult. Although the original demonstration by Hancock (1962) that anaerobiosis prevented streptomycin uptake and killing was taken by some authors to indicate a requirement for oxygen in streptomycin action, this conclusion has since been disproved. Under anaerobic conditions, the alternate terminal electron acceptor nitrate allows electron transport and consequent aminoglycoside uptake and killing of cells (Bryan *et al.*, 1980; Campbell & Kadner, 1980) although nitrate itself partially inhibits aerobic streptomycin uptake probably via an ionic mechanism. In agreement with this, strictly anaerobic bacteria such as *Clostridium perfringens* and

irreversible damage of electron transport is responsible for the observed antagonism of aminoglycoside uptake and killing by electron transport inhibitors.

Uncouplers and other inhibitors of energetics

E. coli cells treated with cyanide and similar electron transport inhibitors are still able to maintain a protonmotive force on an appropriate carbon source by the hydrolysis, using the Ca^{2+}, Mg^{2+}-stimulated ATPase, of ATP derived from glycolysis (Figure 2). However, uncouplers such as 2,4 dinitrophenol (DNP) and carbonyl cyanide m-chlorophenol hydrazone (CCCP) dissipate the protonmotive force (Harold, 1977), thus inhibiting transport systems dependent on the protonmotive force. These uncouplers also inhibit streptomycin and gentamicin uptake (Andry & Bockrath, 1974; Bryan & van den Elzen, 1975, 1976; Höltje, 1978; Muir & Wallace, 1979; Miller *et al.*, 1980; Campbell & Kadner, 1980). The protonmotive force is considered to consist of both an electrical potential gradient ($\Delta\psi$) and a pH gradient (ΔpH) across the cytoplasmic membrane with the cytoplasm being both negatively charged and alkaline relative to the periplasm or medium. At a medium pH greater than 7·5, the pH gradient does not contribute effectively to the protonmotive force. Since both streptomycin and gentamicin are highly effective at medium pH values greater than 7·5 (Donovick *et al.*, 1948) and due to their cationic character, it would seem likely that the electrical potential gradient (interor negative) provides the driving force for aminoglycoside uptake across the cytoplasmic membrane (Bryan *et al.*, 1980).

While aminoglycoside uptake is antagonized by inhibitors of both the electron transport chain and the protonmotive force, not all inhibitors of energetics were effective antagonists. *N,N'*-dicyclohexylcarbodiimide (DCCD), an inhibitor of the Ca^{2+}, Mg^{2+}-stimulated ATPase (Figure 2), did not inhibit, and in fact stimulated gentamicin uptake in *Staphylococcus aureus*, provided cells were able to maintain a protonmotive force via electron transport (Miller *et al.*, 1980).

Media effects

Carlson & Bockrath (1970) investigated the effect of the suspending medium on resistance to streptomycin and found that phosphate-based minimal medium protected cells from streptomycin at concentrations lethal to cells suspended in nutrient broth, by virtue of decreased efficiency of streptomycin accumulation. The rapid alteration in streptomycin susceptibility on shifting from one medium to the other suggested that this was primarily an effect of the medium composition. In agreement with this, the data of Medeiros *et al.* (1977) demonstrated correlations between the MIC of gentamicin and streptomycin and the ionic composition of the medium as judged by osmolality and conductivity measurements. In particular, nutrient broth had an 8–10-fold lower osmolality and conductivity than trypticase soy broth and MICs for gentamicin and streptomycin of cells grown in nutrient broth were 15–100 fold lower. Bryan & van den Elzen (1976) subsequently showed that this effect manifested itself at the level of uptake. Thus, the effects described above are due to the inhibition of aminoglycoside uptake by ions in the various media. Similarly, antagonism of aminoglycosides by serum (Davis & Iannetta, 1972; Ramirez-Ronda, Holmes & Sandford, 1975; Zimelis & Jackson, 1973) almost certainly results from the high concentration of divalent cations in serum.

Conrad, Wulff & Clay (1979) demonstrated that a variety of carbon sources had little effect on MICs of gentamicin. In contrast, glucose causes a large inhibition of streptomycin killing (as well as trimethoprim and sulphamethoxazole killing) of *E. coli* by suppressing the level of cyclic AMP (Dahloff, 1979) which is required for optimal streptomycin killing and transport [Artman & Werthamer, 1974; Höltje, 1978; see also p. 268]. This result implies that other carbohydrates which can cause catabolite repression by lowering cyclic AMP levels should also inhibit streptomycin uptake and killing. Other common media components such as aromatic amino acids, but no other amino acids (Dahloff, 1979), and the terminal electron acceptors fumarate and nitrate (Campbell & Kadner, 1980) also antagonized streptomycin killing (see above).

The influence of the pH of the medium on streptomycin action was studied in detail by Donovick *et al.* (1948) who found that streptomycin MICs were reduced (i.e. killing was enhanced) as the medium pH was raised from 6·0 to 9·0. This effect was separated from the effect of phosphate ions on streptomycin killing but the actual locus of the effect is uncertain. The magnitude of the electrical potential gradient $\Delta\psi$ (which may energize aminoglycoside translocation—see p. 254 increases between external pH of 5 and 7 but does not rise significantly above a medium pH of 7·5 (Felle *et al.*, 1980; Kaback, 1975). Therefore it seems unlikely that the pH effect is related to the energization of uptake. Conceivably, the pH effect may be related to the ionization of groups either on the streptomycin molecule itself or on the cell surface.

Protein synthesis inhibitors

A number of authors have demonstrated that streptomycin (and gentamicin) uptake and cell killing is antagonized by protein synthesis inhibitors such as chloramphenicol (Anand, Rechenmacher, 1960; Bryan & van den Elzen, 1975, 1976; Hurwitz & Rosano, 1962*a*, *b*; Plotz & Davis, 1962), tetracycline, erythromycin (Yamaki & Tanaka, 1963) and spectinomycin (Davies, Anderson & Davies, 1965; Campbell & Kadner, 1980). The addition of chloramphenicol in concentrations that inhibit protein synthesis, results in immediate cessation of killing by streptomycin during all uptake phases (Plotz & Davis, 1962). Cells pretreated with chloramphenicol bind but do not take up streptomycin and gentamicin (Bryan & van den Elzen, 1975, 1976). However in cells treated with chloramphenicol at the same time as streptomycin, EDPI and EDPII of aminoglycoside uptake are not totally inhibited (Bryan & van den Elzen, 1976; Nielsen, 1978), while addition of chloramphenicol during EDPII has a relatively small effect on uptake (Andry & Bockrath, 1974; Bryan & van den Elzen, 1975; Höltje, 1978; Hurwitz & Rosano, 1962*a*) although as pointed out above it prevents further loss of viability. The conclusion one can make from this data is that chloramphenicol is only effective if added before EDPII. Its addition to a given population of cells after the onset of rapid loss of viability and rapid uptake (EDPII) prevents further loss of viability but does not influence those bacterial cells which have already lost viability and started EDPII (see Hancock, 1981, section 1 (A) (b)). This suggests that chloramphenicol antagonizes aminoglycoside action by influencing the events required for EDPI. Hurwitz & Rosano (1962*b*) also provided evidence for a chloramphenicol sensitive and a chloramphenicol insensitive phase and argued that the chloramphenicol sensitive phase suggested a requirement for *de novo* synthesis of an induced streptomycin permease.

This argument was further developed by Höltje (1978, 1979) who proposed that streptomycin used the putative polyamine permease. However, there is now substantial evidence in the literature that the polyamine and aminoglycoside uptake systems are not identical (Bryan *et al.*, 1980; Campbell & Kadner, 1980). Ahmad *et al.* (1980) were unable to demonstrate an induced permease protein using a highly sensitive technique, two dimensional SDS polyacrylamide gel electrophoresis. Furthermore, a requirement for mRNA synthesis has not been clearly established since only pleiotropic inhibitors have been tested (see e.g. Hurwitz & Rosano, 1965 and below). In addition, amino acid starvation, which reduces the protein synthetic capacity of cells to less than 0·5%, still allows the killing of cells by streptomycin, kanamycin and neomycin (Sakai & Cohen, 1975; Stern, Barner & Cohen, 1966). Thus, while induction of a streptomycin permease is superficially an attractive hypothesis, the data tend to argue against this possibility.

Another inhibitor of protein synthesis, puromycin, under appropriate conditions actually stimulates killing by streptomycin, gentamicin, kanamycin and similar aminoglycosides (White & White, 1964; Yamaki & Tanaka, 1963). It was recently demonstrated by Hurwitz, Braun & Rosano (1961) that this effect is due to a stimulation of aminoglycoside uptake. They also observed stimulation by puromycin in *rpsL* (formerly called *strA*) mutants, although the kinetics of the stimulated uptake, the basal level of puromycin required to stimulate uptake in the mutants, and the lack of immediate cell killing of the mutants provided significant differences from the situation observed for wild type strains. The authors suggested, like other authors before them (Brock, 1966; Davis, Tai & Wallace, 1974; White & White, 1964), that the mode of action of puromycin was to cause a decrease in polysomes and an increase in free ribosomes which could act as receptors for concentrating streptomycin in cells. While this explanation is in agreement with the known mode of action of puromycin, it does not really fit all of the available data, since:

(i) It is hard to see how a decrease in polysome numbers from 58% at zero time (see Table 1, Hurwitz *et al.*, 1981; Wallace & Davis, 1973) to 40% at 20 min (see Fig. 2, Hurwitz *et al.*, 1981) can result in the uptake of 5 μg of streptomycin/10^{10} cells (i.e. 5×10^5 molecules/cell) at 20 min given that there are probably only about 10^4 total ribosomes in the cell and one high affinity binding site per ribosome (Chang & Flaks, 1972).

(ii) In sensitive cells it required at least 500 mg/l of puromycin to inhibit more than 90% of growth, *in-vivo* protein synthesis and induced enzyme synthesis. These effects probably resulted from a tenfold reduction in cellular polysomes within 2 min at this concentration of puromycin (since protein synthesis is usually mediated by polysomes rather than individual 70S ribosomes). In contrast, only 10 mg/l of puromycin caused maximal stimulation of streptomycin uptake while 500 mg/l of puromycin severely depressed it (Hurwitz *et al.*, 1981). White & White (1964) showed that length of time of pretreatment with puromycin was critical to observation of the synergistic effect. For instance, 60 min pretreatment with puromycin resulted in antagonism of gentamicin and streptomycin action, while addition of puromycin together with streptomycin gave synergistic effects.

(iii) Hydroxylamine causes severe depression of polysome levels within two minutes of addition to cells (Wallace & Davis, 1973) but, in contrast to

puromycin, hydroxylamine is a strong antagonist of streptomycin action (Rosenbranz, 1964).

(iv) The proposed mode of puromycin stimulation of aminoglycoside uptake assumes that polysomes are unable to bind streptomycin whereas free 70S ribosomes efficiently bind streptomycin. However there is no evidence that this is true, in fact the addition of mRNA to 70S monosomes actually stimulated the rate of streptomycin binding but did not affect the binding affinity (Chang & Flaks, 1972). In addition, since *rpsL* mutants lack the high affinity ribosomal binding site for streptomycin it is hard to explain on the basis of ribosomal binding effects the very similar levels (although different kinetics) of stimulation of streptomycin uptake by 50, 100, 250 and 500 mg/l of puromycin in both wild type and *rpsL* mutants (Hurwitz *et al.*, 1981).

Therefore it seems most likely that puromycin under the appropriate conditions acts directly to stimulate the initiation of the EDPII. The results of Hurwitz *et al.* (1981) do make a critical point. The observed stimulation by puromycin of EDPII uptake in resistant (*rpsL*) cells, in the absence of an immediate effect on cell viability, demonstrates that EDPII uptake is not itself the actual cause of cell death, although in sensitive cells it may be involved in the lethal event (see Hancock, 1981).

What then is the mode of antagonism or stimulation by protein synthesis inhibitors? There would appear to be at least four possibilities: (i) that the protein synthesis inhibitors alter the affinity of the ribosome for aminoglycosides; (ii) that the protein synthesis inhibitors affect the ratio of polysomes to free ribosomes, with the latter preferentially binding aminoglycosides; (iii) that these inhibitors compete with aminoglycosides during uptake, and (iv) that the known pleiotropy of most antibiotics somehow accounts for the varying effects of the above inhibitors on aminoglycoside action. Of these possibilities, a direct effect on ribosomal binding affinity would seem unlikely. For example, chloramphenicol has been shown to antagonize the action of kanamycin (Yamaki & Tanaka, 1963), but it does not influence the ribosomal binding of kanamycin (Misumi *et al.*, 1978). Similarly, spectinomycin antagonizes streptomycin uptake (Campbell & Kadner, 1980) but does not affect streptomycin binding to ribosomes (Chang & Flaks, 1972). In addition puromycin stimulates streptomycin uptake, but does not enhance the affinity of 70S ribosomes for streptomycin (Hurwitz *et al.*, 1981). The second possibility is the one most favoured in the literature (Brock, 1961; Davis *et al.*, 1974), although it depends on the assumption that free ribosomes have a higher affinity for streptomycin than polysomes. As described above this idea is not supported by available evidence. In order to demonstrate conclusively that possibility (ii) is valid, it would be necessary to eliminate possibilities (iii) and (iv) above. Certainly for spectinomycin, direct ionic inhibition of streptomycin uptake would be a probable mechanism of antagonism, given that other polycations are also inhibitory (see p. 250). However, in view of other evidence summarized in the following article (Hancock, 1981), effects on ribosomal affinity remain the most likely mode of action of at least some of the protein synthesis inhibitors on aminoglycoside killing and transport. The implications of this for the mechanism of EDPI uptake will be discussed (Hancock, 1981).

Other inhibitors

There are few, if any, unambiguous reports in the literature about the role of RNA synthesis in aminoglycoside action. Proflavine and borate, bacteriostatic agents which block RNA synthesis but not protein synthesis, prevent streptomycin killing (Hurwitz & Rosano, 1965). However, at the concentration of borate used 0·2 M), ionic effects cannot be ruled out, while the mode of action of proflavine is known to involve its cationic character and thus it may compete with streptomycin for binding sites during uptake.

Another inhibitor shown to cause a significant decrease in streptomycin and gentamicin uptake is arsenate at a concentration of 10 mM (Andry & Bockrath, 1974; Bryan & van den Elzen, 1975, 1976). Normally, this ion is used to deplete cellular ATP levels through its action as a phosphate analogue, but in view of other data on energy inhibitors (pp. 253–5) and mutants (p. 266), it seems likely that arsenate at this concentration has an anionic effect similar to other anions studied (Donovick *et al.*, 1948).

Aminoglycoside resistant and supersensitive mutants

A large variety of mutants with altered susceptibility to aminoglycosides have been isolated. As discussed below, the study of the phenotypic characteristics of these mutants has provided a considerable body of information about aminoglycoside uptake and mode of action. I have attempted to restrict discussion to those mutants and phenotypic properties which have been studied in detail (Table II).

Ribosomal

Types of mutants. Selection of mutants resistant to high levels of streptomycin yields both streptomycin resistant and dependent mutants at a low frequency (10^{-9} to 4×10^{-14}, La Coste, Lacaille & Brakier-Gingras, 1977). These mutants are altered in a single ribosomal protein, S12, located in the 30S ribosomal subunit (Ozaki, Mizushima & Nomura, Birge & Kurland, 1969). Genetic mapping reveals only two possible sites for resistant (Breckenridge & Gorini, 1970) and three for dependent mutants (Momose & Gorini, 1971). These sites within the *rps*L gene correlate with certain phenotypic characteristics of the mutants. Selection of streptomycin-resistant mutants by ethyl methane-sulphonate mutagenesis selects double mutants which have two amino acid alterations in the protein S12 product of the *rps*L gene (La Coste *et al.*, 1977), in contrast to the classical spontaneous streptomycin-resistant mutants (mentioned above) which have single amino acid alterations at either positions 42 or 87 in the S12 protein (Funatsu & Wittman, 1972). The *rps*L mutation to streptomycin resistance reduces the streptomycin binding affinity (Ka) of the ribosome from $1 \cdot 1$–$2 \cdot 5 \times 10^{7}$ M^{-1} for streptomycin sensitive strains to 6×10^{3} M^{-1} for an *rps*L mutant (Chang & Flaks, 1972). It was concluded by Chang & Flaks (1972) that *rps*L mutants had lost the single high affinity binding site on the 30S ribosomal subunit for streptomycin, but retained the low affinity binding sites (with a Ka of 10^{4} M^{-1}) which are also present on wild type ribosomes.

Protein S12 does not represent the actual binding site for streptomycin although the results with *rps*L mutants clearly demonstrate its involvement. Lelong *et al.* (1974) demonstrated inhibition of streptomycin binding to 30S ribosomal subunits

Table I. Antagonists and enhancers of aminoglycoside transport and cell killing

Antagonist or enhancer	Known modes of action	Effect on transport of aminoglycoside	Effect on killing by aminoglycoside
Monovalent cations, e.g. Na^+, K^+, etc.	Ionic—nonspecifically inhibit binding	Slightly reduced	Weakly antagonized
Di- and trivalent cations, e.g. Mg^{2+}, Ca^{2+}, etc.	Ionic—specifically compete for cell binding sites	Strongly reduced	Strongly antagonized
EDTA, etc.	Divalent cation chelator-makes outer membrane permeable	Not studied	Enhanced
Anions, e.g. $PO_4^=$ Cl^-, citrate, etc.	Ionic—mechanism unknown	Reduced	Antagonized
Salts, e.g. $MgSO_4$, $CaCl_2$, NaCl, etc.	Ionic—combination of cation and anion effects	Reduced	Antagonized
Increasing pH	Ionization of cell surface molecules and of aminoglycosides	Not studied	Enhanced
Cyanide, azide, etc.	Electron transport inhibitors; little effect on protonmotive force	Energy dependent phases prevented	Prevented
Anaerobic conditions	Prevents aerobic electron transport	Energy dependent phases prevented	Prevented
Anaerobic conditions in the presence of nitrate, etc.	Allows anaerobic electron transport	Permits energy dependent uptake	Permitted
Uncouplers, e.g. DNP, CCCP, etc.	Uncouple ATP synthesis from electron transport by dissipating the protonmotive force	Energy dependent phases prevented	Prevented
DCCD	Specifically inhibits Ca^{2+}, Mg^{2+} ATPase	No effect	No effect
Chloramphenicol, tetracycline, erythromycin, spectinomycin	Protein synthesis inhibitors (ionic effects?)	Highly dependent on time of addition	Prevented or antagonized
High levels of puromycin	Protein synthesis inhibitor	Dependent on time of addition	Prevented or antagonized
Low levels of puromycin	Not known	Enhanced	Enhanced
Glucose (in *E. coli*)	Lowers cellular cyclic AMP levels	Reduced	Antagonized

using antibodies against 12 of the 21 30S subunits proteins, but interestingly anti S12 antiserum did not block streptomycin binding. In addition, *ram* (for *r*ibosomal *am*biguity, now called *rps*D) mutants which contain alterations in ribosomal proteins S4 and S5 not only make cells hypersensitive to streptomycin, but can suppress *rps*L mutations (Rosset & Gorini, 1969). This suggests that S4 and S5 are also involved in the ribosome binding site for streptomycin. Further evidence for the

Table II. Aminoglycoside resistant and supersensitive mutants

Mutant designation[a]	Bacterium	Aminoglycoside susceptibility[b]			Change in MIC for SM or GM[c]	Effect on aminoglycoside uptake	Other properties
		Resistant	Normally susceptible	Super sensitive			
*rps*L (formerly *str*A)	many	SM	GM,TM,KM,NM		100+ ↑	Blocks EDPII	Altered 30S ribosomal protein S12 (see Table III)
*rps*D	*E. coli*		GM,KM,NM	SM	2 ↓	Enhances	Altered 30S ribosomal protein S4, makes protein synthesis less accurate
*rps*E	*E. coli*		GM,KM,NM	SM	5 ↓	Enhances	Altered 30S ribosomal protein S5, makes protein synthesis less accurate
*rpl*F	*E. coli*	GM,KM,NM (SM)			5 ↑	Inhibits	Altered 50S ribosomal protein L6
*hem*A	*E. coli*	SM,GM			4 ↑	Inhibits	Cytochrome deficient—no electron transport
*ubi*D	*E. coli*	SM,GM			4 ↑	Inhibits	Reduced levels of ubiquinone, an electron transport component

*agl*A	*Ps. aeruginosa*	SM,GM,TM		4–15 ↑	Inhibits	Lacks cytochrome c_{552} a component of nitrate reductase
mar	*Staph. aureus*	GM,KM,NM		8–30 ↑	Inhibits	Menaquinone auxotroph
mar	*B. subtilis*	KM,GM,NM		–	Inhibits	Cytochrome or quinone deficiencies
*unc*A,B	*E. coli*		SM,GM	20 ↓	Enhances	Defective ATPase associated with a stimulation of respiratory activity
*unc*A,B	*E. coli*	SM,GM,NM,KM		4 ↑	Inhibits	Defective ATPase associated with a decreased protonmotive force
*ecf*B	*E. coli*	SM,GM,NM,KM		4 ↑	Inhibits	Generally defective in aerobic active transport
cya or *crp*	*Salm. typhimurium*	SM		4 ↑	Inhibits	Pleiotrophic effect on cyclic AMP dependent systems
Polymyxin resistant	*Ps. aeruginosa*	SM,GM,TM		4 ↑	Delays EDPII	Large increase in outer membrane protein H1. Polymyxin and EDTA resistant

[a]Where possible the genetic designation of the mutant has been presented. Mnemonics are: *rps*, ribosomal protein small subunit; *rpl*, ribosomal protein large subunit; *hem*, heme deficient; *ubi*, uniquinone deficiency; *agl*, aminoglycoside resistant; *mar*, multiple aminoglycoside resistant; *unc*, uncoupled for oxidative phosphorylation; *ecf*, deficient in a putative energy coupling factor; *cya*, adenylcyclase deficient; *crp*, cyclic AMP receptor protein deficient.

[b]Only results for those aminoglycosides of the group streptomycin (SM), tobramycin (TM), gentamicin (GM), kanamycin (KM), and neomycin (NM) are presented.

[c]↑, increase; ↓, decrease; –, MIC not determined.

complex nature of this site was provided by the isolation of a novel streptomycin-dependent mutant (Dabbs & Wittman, 1976). This mutant is altered in many ribosomal proteins, while revertants to streptomycin independence are altered in one of 28 different 30S and 50S ribosomal subunit proteins. The conclusion one can make from the above data is that the high affinity streptomycin binding site is a three-dimensional region of the 30S subunit which is influenced by or involves a number of different ribosomal proteins from both subunits. In agreement with this, Chang & Flaks (1972) have shown that the 70S ribosome has a 20-fold higher affinity for streptomycin than its 30S subunit. The actual ribosomal binding site may not be a protein. Gorini and colleagues have argued that the 16S ribosomal RNA of the 30S subunit contains the true ribosomal binding site, while the above mutations influence the availability of this site (see Garvin, Biswas & Goring, 1974).

Streptomycin-resistant (*rps*L) mutants are generally cross resistant to bluensomycin and partially resistant to hygromycin B, but unaltered in susceptibility to a wide range of other aminoglycosides including gentamicin, kanamycin, tobramycin, neomycin, paromomycin, kasugomycin and spectinomycin (Buckel *et al.*, 1977). They are not totally immune to the effects of streptomycin. As shown by Bryan & van den Elzen (1977), the MICs for four *rps*L mutants of *E. coli* were 64, 512, 2048, and >4096 mg/l in a specially designed aminoglycoside uptake medium. The difference in the MIC values depended on the other genetic mutations in the cells. Other authors have reported MICs in the range of 2–5 g/l (Piepersberg, Noseda & Böck, 1979; Sparling & Davis, 1972). As further evidence that streptomycin can affect *rps*L mutants, the suppression of certain auxotrophic mutants by streptomycin at subinhibitory concentrations was first demonstrated in *str*A (*rps*L) mutants (Davis, Gilbert & Gorini, 1964) and only subsequently in streptomycin-sensitive cells (Gorini & Kataja, 1965). Tai, Wallace & Davis (1978) have demonstrated that streptomycin can induce a moderate level of misreading *in vitro* using purified ribosomes from either streptomycin sensitive cells or *rps*L mutants, although 100-fold more streptomycin was required in the latter case.

Transport of streptomycin into *rps*L cells has also been shown to be defective. The initial rate of uptake of streptomycin (EDPI) for *rps*L cells is identical to that of wild type cells (Bryan & van den Elzen, 1976; Campbell & Kadner, 1980), although the latter part of the EDPI and the second energized phase of uptake, EDPII, is missing, at least at subinhibitory concentrations (Figure 1). This will be discussed in detail in the following article.

It is interesting that two early results by Joshua Lederberg have not been adequately explained. For example, he demonstrated that streptomycin resistance was genetically recessive in partial diploids, in that sensitivity was dominant to resistance (Lederberg, 1951). Similar results were obtained by Davis and colleagues (Sparling *et al.*, 1974) who suggested that the results could be explained on the basis of 'cyclic blockade of protein synthesis initiation sites'. Even given the 2 : 1 ratio of sensitive to resistant ribosomes (or even the 10 : 1 ratio found by Chang *et al.*, 1974), this explanation seems inadequate to explain why such partial diploids are killed (see Hancock, 1981). For example, spectinomycin and erythromycin also demonstrate such 'cyclic blockade of initiation sites' and mutants resistant to these inhibitors are also recessive to the sensitive allele in partial diploids (see Davis *et al.*, 1974 for review). However spectinomycin and erythromycin are bacteriostatic

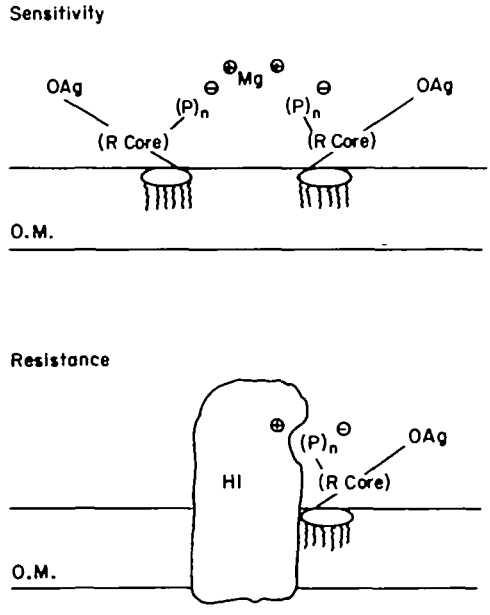

Figure 3. Model illustrating the proposed mechanism of resistance to aminoglycosides, polymyxin B and EDTA in *Ps. aeruginosa* strains with high levels of protein H1 (O.M., outer membrane; R, Core, the heptose; KDO, rough core region of the LPS; O Ag, the somatic antigen of *Ps. aeruginosa* LPS: $(P)_n$, the polyphosphate portion of the *Ps. aeruginosa* LPS; this phosphate is negatively charged at neutral pH and is attached to the KDO-Heptose region of the LPS and may be as many as 12–15 moles of phosphate/mole LPS; H1-major outer membrane protein H1 which appears in large amounts in the outer membranes of specific mutants and in strains grown in Mg^{2+} deficient media). It is proposed that the crosslinking of the negatively charged polyphosphate regions of the LPS by Mg^{2+} is important for outer membrane stability in sensitive cells. EDTA by removing Mg^{2+}, and the highly cationic antibiotics aminoglycosides and polymyxin B by displacing Mg^{2+}, lead to disruption of the outer membrane permeability barrier (see Hancock *et al.*, 1981; Nicas & Hancock, 1980). It is proposed that in cells with high levels of protein H1, this protein replaces Mg^{2+} in the outer membrane (an inverse correlation between the level of protein H1 in the outer membrane and the level of cell envelope Mg^{2+} has been demonstrated). Protein H1 thus protects the polyphosphate site on the LPS from attack by aminoglycosides, polymyxin B and EDTA and makes the cell relatively resistant to these agents.

rather than bactericidal and antagonize streptomycin killing (see pp. 256). In addition to this result, Lederberg & St. Clair (1958) showed that penicillin-induced osmotically-stabilized spheroplasts (L forms) of streptomycin-resistant cells became sensitive to 20 mg/l of streptomycin. Penicillin-streptomycin synergy was ruled out by another experiment. The removal of diaminopimelate, a peptidoglycan side-chain component, from the growing medium of diaminopimelate auxotrophs created osmotically-sensitive, penicillin-resistant L forms which appeared to be lacking cell walls. Such L forms in stabilizing medium, when derived from parent auxotrophs resistant to more than 1000 mg/l of streptomycin, became sensitive to 20 mg/l of streptomycin. One possible explanation of this data is that the streptomycin resistance mutation was affecting permeability of the drug rather than being the target.

A second major class of streptomycin-resistant mutants mapping in the *rps*L gene, streptomycin-dependent cells, actually require streptomycin for growth. Three classes of streptomycin-dependent cells have been described (Momose & Gorini, 1971) based on (i) the numbers and types of drugs individually able to satisfy their dependence (e.g. some mutants can have their dependence satisfied by streptomycin

Table III. Pleiotrophy of high level streptomycin resistant (SM^R) and streptomycin dependent (SM^D) mutants mapping in the *rps*L (*str*A) gene

Mutation	Bacterium	Effects	Reference[a]
SM^R	*E. coli*	loss of phenotypic suppression by low levels of KM, SM and NM	A
SM^R	*E. coli*	streptomycin-dependent auxotrophy	B
SM^R	*E. coli*	made male strains (i.e. F^+, Hfr) permissive for phage T7	C
SM^R	*E. coli*	altered surface charge properties	D
SM^R	*Salm. typhimurium*	activity of *sup*U suppressor of UGA mutations abolished	E
SM^R	*E. coli*	suppression of RNA polymerase mutants	F
SM^R	*E. coli*	twofold reduction in efficiency of protein synthesis	G
SM^R	*E. coli*	decreased growth rates and poor support of phage growth	G,H
SM^R	*Ps. aeruginosa*	suppressed fluorophenylalanine resistance	I
SM^R	*Rhizobium trifolii*	loss of infectivity for plants, loss of ability to produce spheroplast-like form, acquisition of ability to grow at 38°C, alteration in phage sensitivity	J
SM^R	*Bacillus subtilis*	asporogeny	K,L
SM^R and SM^D	*E. coli*	reduced induction of λ phage mutant at high temperature, reduced growth rate, suppress restriction of phage λ by prophage P1	H
SM^D	*E. coli*	high alkaline phosphate levels	M
SM^D	*E. coli*	high cyclic AMP levels	N
SM^D	*E. coli*	cells made almost obligate aerobes	O
SM^D	*E. coli*	suppression of lethal mutations in protein, RNA, DNA or lipid synthesis	P
SM^D	*Staph. aureus* *B. cereus* *B. megaterium*	appearance of unusual compound in cell walls	Q

[a]References are A, Apirion & Schlessinger (1967); B, Chakrabarti & Maitra (1977); C, Chakrabarti & Gorini (1975); D, Pestka *et al.* (1977); E, Johnston & Roth (1980); F, Molholt (1976); G, Zengel *et al.* (1977); H, Couturier *et al.* (1964); I, Waltho & Holloway (1966); J, Zelasna-Kowalska (1977); K, Quan *et al.* (1979); L, Campbell & Chambliss (1977); M, Rosenkranz (1963); N, Polglase *et al.* (1978); O, Bryan (1980); P, Murgola & Adelberg (1970); Q, Lawrence & Scruggs (1966).

or paromomycin *or* ethanol, whereas others have dependences which can only be satisfied by one or two of these drugs); (ii) their resistance to streptomycin, paromomycin and kanamycin; (iii) their ability to suppress the phenotypes of certain auxotrophic mutations; (iv) their ability to revert to drug independence through secondary mutations; and (v) their genetic location within the *rps*L gene (the

mutants map separately from the two known streptomycin resistance loci in the *rps*L gene). The ribosomes from at least one streptomycin-dependent mutant bind streptomycin only at the low-affinity binding site (Chang & Flaks, 1972). The requirement of steptomycin-dependent mutants for streptomycin has been studied by a number of authors and it has been variously suggested that streptomycin is required either for cell division (Landman & Burchard, 1962; Viceps & Brownstein, 1975) or for the maintenance of a high level of protein synthesis (Spotts, 1962; Viceps & Brownstein, 1975) or for reversal of 'phenotypic masking', i.e. the suppression of a phenotype by the *rps*L mutation can be reversed by streptomycin (Zimmermann, Rossett & Gorini, 1971) or for the maintenance of the integrity of the electron transport system (Bragg & Polglase, 1963). At present, the available evidence does not favour any hypothesis to the exclusion of others.

Studies of revertants of *rps*L mutants have revealed that ribosomal alterations in 30S subunit proteins S4 (*rps*D) or S5 (*rps*E) are able to suppress *rps*L mutations resulting in either streptomycin dependence or resistance (Bjare & Gorini, 1971; Hasenbank *et al.*, 1973). In some, but not all cases, the suppression of streptomycin dependence is accompanied by a large increase in susceptibility to streptomycin (Bjare & Gorini, 1971). Although protein S5 mutants are often spectinomycin-resistant, the *rps*E revertants altered in protein S5 were not spectinomycin-resistant suggesting a different alteration was involved (Hasenbank *et al.*, 1973). These mutations, originally named *ram*, seem to mimic to some extent the phenotypic suppression (misreading) caused by streptomycin although they are not lethal like streptomycin. The *rps*D and *rps*E mutants appear to facilitate ribosomal binding of dihydrostreptomycin, are hypersensitive to dihydrostreptomycin and shown more rapid initiation of EDPII streptomycin uptake, at a given concentration, than the isogenic wild type strain (Ahmad *et al.*, 1980; Böck, Petzet & Piepersberg, 1979).

A class of streptomycin dependent (*rps*L1) mutants which did not revert spontaneously was isolated by Bjare & Gorini (1971) and used by Dabbs (1977, 1980) to select, by mutagenesis, unique classes of streptomycin independent mutants which he called *qui* mutants. These mutants mapped separately from the mutants described above. One class of mutants, *qui*A appeared to have a ribosomal protein alteration in one of proteins S14, S15 or S19 (Dabbs, 1980). Another class *qui*B mapped near the *ksg*A locus which contains a cluster of genes affecting ribosomal phenotypic characteristics. A third mutant mapping near the gene for RNA polymerase suppressed all streptomycin resistant and dependent alleles and was associated with the acquisition of a 25,000 dalton protein by the ribosome (Dabbs, 1977).

Single-step high-level gentamicin resistant mutants directly analogous to the *rps*L mutants have not yet been isolated (see Buckel *et al.*, 1977). However, mutants called *rpl*F, selected as resistant to low levels of gentamicin have an alteration in ribosomal protein L6 of the 50S ribosomal subunit. These mutants, described as 'partially' gentamicin-resistant, were resistant to five times as much gentamicin as the parent strain although the original isolates were double mutants with 50-fold higher resistance (Ahmad *et al.*, 1980). The *rpl*F mutants demonstrated little, if any, uptake of radioactive gentamicin although experiments were performed only at a single concentration of gentamicin (Ahmad *et al.*, 1980). The effects of the *rps*L and *rpl*F mutations on ribosomes could be differentiated both *in vivo* and *in vitro* (Kuhberger *et al.*, 1979). In agreement with the mutant studies, there is evidence

from competition binding studies that gentamicin and streptomycin have different binding sites on the ribosome (Le Goffic *et al.*, 1980) and corresponding differences in their effects on protein synthesis *in vitro* (Zierhut, Pipersberg & Böck, 1979).

Pleiotropy. There is considerable evidence in the literature to suggest that *rps*L mutants are highly pleiotropic. This is relevant to this review since it suggests at least some of the cellular processes in which ribosomal protein S12 plays a role. Amongst these cellular processes may be either the lethal target for streptomycin action or a process which contributes to the pleiotropic nature of streptomycin action [see Hancock, 1981, section (A) (b)]. A list of some of the pleiotropic effects of the *rps*L mutation is included in Table III. This pleiotrophy is not easily explained merely on the basis of an alteration in coding accuracy in *rps*L mutants (see e.g. Apirion & Schlessinger, 1967).

Energetics

Mutants in the *hem*A locus are unable to produce the electron transport chain components, cytochromes, unless provided with the precursor δ-amino-levulinic acid. These heme-deficient mutants are thus unable to respire aerobically or anaerobically (using nitrate as a terminal electron acceptor) in unsupplemented medium. Bryan & van den Elzen (1977) showed that in *hem*A mutants starved of δ-aminolevulinic acid, transport of and killing by streptomycin and gentamicin were strongly reduced. This was confirmed by Campbell & Kadner (1980), who demonstrated that as *hem*A mutants ran out of cytochromes, streptomycin transport was gradually abolished. While this is strong evidence for a direct role of electron transport in streptomycin uptake, especially when considered together with the inhibitor evidence (p. 252), the loss of cytochromes from the *hem*A mutant only caused a fourfold increase in MIC for streptomycin or gentamicin.

Another mutant in an electron transport component, the *ubi*D mutation, also results in a fourfold increase in MIC to gentamicin and streptomycin and a pronounced transport deficiency at all levels of streptomycin and gentamicin tried (Bryan & van den Elzen, 1977). This result is complicated by the leakiness of the mutation (*ubi*D mutants synthesize 20% of the level of ubiquinone present in the parent strain) and by the *str*A background in which the uptake studies were performed. However, there is other evidence in the literature suggesting a role for quinones in aminoglycoside uptake. From experiments with strains producing low levels of ubiquinone and no menaquinone, Muir (cited in Muir & Wallace, 1979) suggested that quinone concentrations, rather than overall electron transport rates, control the rates of streptomycin uptake in *E. coli*. Furthermore, a *mar* (for *m*ultiple *a*minoglycoside *r*esistant) mutant of *Staph. aureus* was shown to be a menadione (2-methyl-1,4-napthoquinone) auxotroph which was resistant to and defective in the transport of gentamicin. Supplementation of the growth medium with menadione resulted in restoration of sensitivity to gentamicin and reversed the transport defect (Miller *et al.*, 1980). Similarly the anaerobe *Bact. fragilis* was resistant to streptomycin unless supplemented with hemin, menadione and fumarate allowing anaerobic electron transport and consequent streptomycin uptake and killing (Bryan *et al.*, 1979).

The above results have provided circumstantial evidence for a role for quinones in aminoglycoside transport. The relatively small effect of removal of cytochromes by depriving *hem*A mutants of δ-aminolevulinic acid, can be explained on the basis of

an observation that quinones can still function in electron transport even in the absence of cytochromes (Singh & Bragg, 1976). It should be noted, however, that the inhibitor evidence suggests that the ubiquinones must be undergoing cycles of oxidation and reduction, as shown by the sensitivity of aminoglycoside uptake to cyanide which does not affect quinones but inhibits electron transport through its action on cytochromes. A further demonstration of the requirement for a functional electron transport chain came from the study of an *agl* A mutant of *Ps. aeruginosa* (Bryan *et al.*, 1980). This strain was impaired to gentamicin, tobramycin, streptomycin and amikacin killing and in streptomycin and gentamicin uptake. The defect was localized to a common component of the terminal cytochrome oxidase/nitrate reductase, possibly cytochrome 552. In addition, a number of multiple aminoglycoside resistant (*mar*) mutants have been described in both *B. subtilis* (Taber & Halfenger, 1976) and *Staphylococcus aureus* (see above) by selection on low levels of kanamycin and gentamicin, respectively. The *B. subtilis mar*-2 and *mar*-3 mutants had a variety of cytochrome deficiencies and consequent defects in respiration, while one of the strains had become a menaquinone auxotroph. Both strains were deficient in kanamycin uptake.

A series of other studies have been performed on *unc* mutants which affect the Ca^{2+}, Mg^{2+}-ATPase and therefore uncouple ATP production from electron transport and the protonmotive force (Harold, 1977). Thus, ATP cannot be made using the protonmotive force, nor can the hydrolysis of ATP generate a protonmotive force. Under aerobic conditions, *unc* mutants can generate a protonmotive force via the normally functioning electron transport chain. Mutants in both *unc*A and *unc*B loci have been described which cause hypersensitivity to streptomycin (Turncock *et al.*, 1972; Muir & Wallace, 1979), and a resultant enhancement of streptomycin uptake (Muir & Wallace, 1979). In these mutants, a stimulation of respiratory activity was observed. Other *unc* mutants have been isolated which show low level resistance to a wide range of aminoglycosides and are deficient in gentamicin and/or streptomycin uptake (Ahmad *et al.*, 1980; Bryan & van den Elzen, 1977; Kanner & Gutnick, 1972; Thorbjarnardotter *et al.*, 1978). This low level resistance may result from an apparent decrease in the protonmotive force in these particular *unc* mutants, possibly caused by leakiness of the cytoplasmic membrane to protons (Bryan & van den Elzen, 1977; Thorbjarnardotter *et al.*, 1978). It is interesting that both aminoglycoside supersensitivity and aminoglycoside resistance can be used in the selection of both *unc*A and *unc*B mutants (e.g. Kanner & Gutnick, 1972; Muir & Wallace, 1979).

Mutants with normal electron transport and Ca^{2+}, Mg^{2+}-ATPase activities, *ecf* (for *e*nergy *c*oupling *f*actor) mutants, but with general defects in aerobic active transport have been isolated. Such *ecf* mutants show an increased MIC for a range of aminoglycosides and enhanced streptomycin and gentamicin uptake (Bryan & van den Elzen, 1977; Thorbjarnardottir *et al.*, 1978). The role of the putative energy coupling factor (protein) has to date not been elucidated.

In summary, studies with mutants in energy transduction are by and large consistent with the results of inhibitors in suggesting a dual requirement of aminoglycoside transport for both the protonmotive force and electron transport.

Catabolite repression

The phenomenon of catabolite repression (see Rickenberg, 1974; Saier, 1979, for

reviews) involves the inhibition of transcription of certain genes (e.g. the lactose operon in *E. coli*) in the presence of a readily metabolized substrate (e.g. glucose in *E. coli*). Uptake of the readily metabolized substrate switches off the membrane-bound adenyl cyclase and causes excretion of cyclic AMP into the medium. This then prevents transcription of a wide range of genes requiring cyclic AMP and the cyclic AMP receptor protein for transcription. Catabolite repressible genes or phenomena are usually studied using adenyl cyclase deficient (*cya*) or cyclic AMP receptor protein (*crp*) deficient mutants. The fact that such strains are viable demonstrates that no indispensible processes in the cell absolutely require cyclic AMP. It has been observed by a number of authors that *cya* and *crp* mutants of *E. coli* and *Salm. typhimurium* show increased resistance to streptomycin (Alper & Ames, 1978; Artman & Werthamer, 1974; Höltje, 1978) and a deficiency in streptomycin uptake (Höltje, 1978). The defect, both in streptomycin uptake and other catabolite repressible systems is correctable in *cya* mutants but not *crp* mutants by the addition of exogenous cyclic AMP (Artman & Werthamer, 1974; Höltje, 1978). The role of cyclic AMP is further illustrated by the 'glucose effect' on growth inhibition by streptomycin, where glucose addition to acetate grown *E. coli* reduces both streptomycin killing and cellular cyclic AMP levels (Artman, Werthamer & Golb, 1972*b*). Since many different phenomena are affected by catabolite repression and by *cya* and *crp* mutants including oxidative phosphorylation (Hempfling & Beeman, 1971), the protonmotive force (Dills & Dobrogosz, 1977), cell envelope proteins (Aono, Yamasaki & Tamura, 1978), fatty acid composition, cytochrome content, and various dehydrogenases and transport systems (Dallas, Tseng & Dobrogosz, 1976), it is likely that one or more of these is the reason for the increased resistance to streptomycin. As yet unexplained, however, is the elevated level of cyclic AMP in glucose grown, streptomycin dependent cells (Polglase, Iwacha & Thomson, 1978). Perhaps the continuous interaction of streptomycin with the membranes in these cells stimulates the activity of adenylcylase.

Stringent regulation

The stringent response is a cellular regulatory mechanism that apparently involves an unusual nucleotide guanosine-5′-disphosphate-3′-diphosphate (ppGpp) which accumulates during amino acid starvation in stringent strains (*rel*$^+$) but not in relaxed strains (*rel*$^-$) (see Nierlich, 1978 for review). In addition to this, a change from a good growth medium to a poor growth medium is accompanied by an increase in cellular ppGpp and cyclic AMP concentrations. This mechanism, called the carbon source downshift mechanism, may be regulated at the level of the membrane (Gallant, 1976; Pao & Gallant, 1978). An increase in cellular guanosine tetraphosphate levels can also be mediated by starvation for nitrogen, in *rel*A$^+$ but not *rel*A$^-$ strains or by carbon source, energy source, Mg^{2+}, K^{2+} PO_4'' or SO_4'' starvation in both *rel*A$^+$ and *rel*A$^-$ strains, the former through the stringent response acting at the level of the ribosome and the latter group via the carbon source downshift mechanism. The effects of this increase are extremely pleiotropic (Bridger & Paranchych, 1979; Höltje, 1979*b*; Nierlich, 1978) but the most pronounced effect is a dramatic decrease in rRNA synthesis at high cellular ppGpp levels (Nierlich, 1978). Sakai & Cohen (1975) showed that a *rel*A$^-$ strain starved of amino acids was 10 fold more susceptible to streptomycin than its amino acid-starved isogenic *rel*A$^+$

parent. The effect is mediated at the level of streptomycin uptake (Höltje, 1979*b*; Sakai & Cohen, 1975). These results would tend to indicate that a stringent response at the level of the ribosome, somehow contributes to streptomycin uptake. The above results are further discussed in the following article (Hancock, 1981).

Outer membrane mutants

We recently described mutants of *Ps. aeruginosa* PAO1 which contain high levels of a major outer membrane protein H1 (Nicas & Hancock, 1980). These mutants are resistant to killing by low levels of streptomycin, gentamicin and tobramycin and high levels of polymyxin B and EDTA. Growing wild type cells in medium containing low Mg^{2+} levels caused an adaptive increase in protein H1 and a corresponding resistance to killing by the above antibiotics, when killing was assayed in a common assay medium (Hancock *et al.*, 1981). Under all growth conditions, the amount of Mg^{2+} in the cell envelope was inversely related to the level of protein H1 in the outer membrane. These results were consistent with protein H1 replacing Mg^{2+} at an outer membrane Mg^{2+} binding site which streptomycin and gentamicin normally use to pass through the outer membrane (Fig. 3) (Hancock *et al.*, 1981; Nicas & Hancock, 1980). Consistent with this, the mutant caused a delay in the onset of the EDPII at all concentrations of streptomycin tested (Hancock *et al.*, 1981). Similar mutants have not been found in other bacteria, possibly due to the fact that other routes of outer membrane uptake occur in other Gram-negatives. Evidence for this is provided by the finding of Foulds & Chai (1978), who demonstrated that a porin Ia deficient mutant of *E. coli*, defective in the hydrophilic antibiotic uptake pathway was somewhat more resistant to kanamycin and gentamicin, although no quantitation was performed.

The observation of altered surface charge in certain *rpsL* mutants of *E. coli* (Pestka, Walter & Wayne, 1977) strongly suggests an outer membrane alteration, since the outer membrane is the major contributor to cell surface charge in Gram-negative bacteria. Other possible outer membrane mutants in the literature include the gentamicin-resistant clinical isolates of *Ps. aeruginosa* studied by Bryan, Haraphongse & van den Elzen (1976). These isolates demonstrate reduced gentamicin uptake but the defects have not as yet been localized.

R factor mediated resistance

R factor mediated resistance can be caused by a range of enzymes located in the periplasmic space or near the cell surface which modify the structure of aminoglycosides, thus inactivating them (Bryan, 1980). Such enzymatic modification results in a failure to induce EDPII (Dickie, Bryan & Pickard, 1978; Höltje, 1979; Lee *et al.*, 1977), although kanamycin can induce EDPII for modified streptomycin when cells are pretreated for 20 min with a concentration of kanamycin 20-fold higher than the MIC. Even at this higher rate of uptake, all streptomycin is modified and does not apparently contribute to cell death (Höltje, 1979*a*). A number of *Ps. aeruginosa* plasmids coding for non-enzymatic resistance have been isolated (Kono, Sei & O'Hara, 1977) and probably involve decreased permeability to streptomycin, kanamycin or gentamicin.

Clinical significance

It is perhaps relevant to discuss the clinical significance of the studies outlined above, since many of these studies were undertaken in an attempt to understand the reasons for success or lack of success of aminoglycoside therapy. In the late 1960s, researchers started to realize that laboratory MIC tests did not always accurately predict the outcome of aminoglycoside therapy for a given pathogenic strain. Such factors as the high divalent cation concentrations found in serum (Davis & Iannetta, 1972; Ramirez-Ronda *et al.*, 1975; Zimelis & Jackson, 1973), ionic strength (see p. 250), polyphosphates like DNA or RNA (Brock, 1966), mucopolysaccharides similar to those found in cystic fibrosis patients (Brock, 1966), pH (see p. 255), antagonists produced by the pathogen or other bacteria (e.g. HOQNO, see p. 252) and the ability of the pathogen to undergo aerobic or anaerobic electron transport (see p. 253) are important determinants of success in therapy, and have been the subject of detailed studies as described above. One result of such laboratory studies has been the development of media with defined divalent cation contents for aminoglycoside MIC determinations of clinical isolates (e.g. Medeiros *et al.*, 1971; Zimelis & Jackson, 1973). Such media provide a far more accurate, although by no means infallible method of assessing whether aminoglycosides will be therapeutically valuable. An example of a promising development arising from the above studies is in the laboratory usage of divalent cation chelators like EDTA to enhance cellular susceptibility to aminoglycosides (see p. 252). Only preliminary trials on experimental infections have been performed to date (e.g. Wilson, 1970) but this sort of approach has considerable potential.

Recent studies have suggested that the higher antibacterial activity of, for example, sisomicin compared to gentamicin (Lee *et al.*, 1978), results from its enhanced efficiency of transport rather than differing ribosomal affinities. Furthermore, our own studies (Hancock *et al.*, 1981) have suggested that the decreased ability of streptomycin to interact with an outer membrane Mg^{2+} binding site when compared to gentamicin is closely reflected by the lower efficacy of streptomycin against *Ps. aeruginosa*. These findings suggest that in the design of new aminoglycosides, enhancement of cellular uptake rates should be a major consideration. Future studies on the mechanism of action and uptake of aminoglycosides should provide further valuable information of clinical significance.

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