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Bacterial Uptake of Aminoglycoside Antibiotics

HARRY W. TABER,[1]* JOHN P. MUELLER,[1] PAUL F. MILLER,[1]† AND AMY S. ARROW[2]

Department of Microbiology and Immunology, Albany Medical College of Union University, Albany, New York 12208,[1] and Biotix, Inc., Danbury, Connecticut 06810-4130[2]

INTRODUCTION

The uptake or accumulation of aminoglycoside antibiotics by bacterial cells is a research area that is now more than 25 years old, yet the fundamental mechanisms by which these compounds find their way across the procaryotic cell envelope to their ribosomal targets are still obscure. In this manuscript, we try to present a critical overview of the subject and address a number of unresolved issues about the overall process of aminoglycoside uptake to suggest directions for future research. While not attempting to be comprehensive, we hope to cite the most critical papers and discuss them in a way that will be helpful to the uninitiated. The discussion represents a somewhat personal view in a field undergoing considerable reexamination.

Some excellent reviews of antibiotic uptake by bacteria have appeared over the past several years. Chopra and Ball (26) have provided a thorough discussion which encompasses all groups of antibiotics about which information on uptake is available. Because of the breadth of coverage, Chopra and Ball could devote only limited space to aminoglycosides. Hancock addressed both aminoglycoside uptake and mode of action in two articles (50, 51), reviewing the literature thoroughly up to 1981 and integrating the available information and ideas in these closely related areas. Bryan (13), in a very effective monograph, treated aminoglycoside uptake as part of a broader discussion of bacterial resistance to antibiotics; in addition, he edited and contributed to a comprehensive volume on drug resistance (see references

* Corresponding author.
† Present address: Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, Bethesda, MD 20892.

13, 45, and 54). The present review concentrates on more recent results directly associated with the uptake phenomenon itself, and we suggest that the above-mentioned publications be consulted for a detailed discussion of related topics. In general, we do not present detailed discussions of topics covered comprehensively in previous reviews, unless our interpretations differ from those of the authors or more recent data have forced reexamination of the topic.

There are two other current reviews of aminoglycoside uptake. One is concerned largely with the molecular events following aminoglycoside binding to ribosomes, such that cellular properties are changed to allow rapid entry of the antibiotic (32). The other analyzes the various mechanistic pathways by which aminoglycosides might cross the cytoplasmic membrane, using concepts from transport biophysics (W. W. Nichols, Biochim. Biophys. Acta, in press). The present communication, on the other hand, has as one of its principal goals a placement of the bioenergetic basis of aminoglycoside uptake within the currently understood phenomenology and the considerable variety of experimental systems used by different investigators. These three current reviews are, then, largely complementary, and the reader is urged to consult all of them to gain a balanced view of the topic.

Aminoglycoside uptake is an energy-requiring process; bacterial cells can accumulate aminoglycosides against a concentration gradient. Considerable controversy surrounds the question of how energy is generated for use in the uptake system. Both the electrochemical potential across the cytoplasmic membrane and electron flow through membrane-bound respiratory chains have been shown to be important, and considerable effort has been expended in distinguishing between their contributions to the energy requirement.

The extent to which the uptake system possesses substrate specificity is not clear, since transport carriers have not been identified, and the uptake system is not substrate saturable. Aminoglycoside accumulation also is unusual in having an induction phase following exposure of cells to antibiotic, with binding to sensitive ribosomes necessary for development of the rapid uptake phase associated with lethality. One hypothesis for the induction period suggests that aminoglycoside-stimulated misreading during protein synthesis causes changes in the cytoplasmic membrane that permit rapid uptake to occur. Another hypothesis envisions an accumulation of runoff ribosomes providing a pool of binding sites for irreversible uptake.

The biological significance of aminoglycoside uptake resides in two areas. First, plasmid-borne resistance to these antibiotics is based on enzymatic inactivation, and modified aminoglycoside molecules do not act as inducers of the rapid uptake phase necessary for cell lethality. Second, aminoglycoside-synthesizing bacteria, particularly high-level producers developed in the industrial setting, have modifications in their uptake mechanisms to prevent rapid entry of antibiotic from the surrounding milieu.

METHODOLOGY AND TERMINOLOGY

Experimental Systems

Much of the research on aminoglycoside uptake has concentrated on only two compounds, streptomycin and gentamicin. This concentration has been due entirely to the limited availability of radioactively labeled aminoglycosides having sufficiently high specific activities to carry out uptake experiments (to illustrate the frustrations felt by workers in the field, tritiated tobramycin is now available, but gentamicin has been discontinued). Although some studies have been carried out with unlabeled compounds, e.g., kanamycin (see, e.g., reference 113), such procedures are cumbersome and the data obtained are not very satisfactory. The hazards of concentrating on a limited selection of aminoglycosides are illustrated by the finding that certain conditions limiting uptake of streptomycin may have no effect on gentamicin uptake (A. Arrow, Ph.D. thesis, Albany Medical College, Albany, N.Y., 1983). We would suggest that, in the future, each aminoglycoside be treated individually in terms of mechanism and energetics of uptake and that extrapolations from one compound to another not be made without regard for the significant differences already known to exist among them.

Experimental systems for the study of aminoglycoside uptake have included intact cells, modified cells (such as spheroplasts), and membrane vesicles. Most workers have used intact cells because of their convenience and because of persistent difficulties in obtaining vesicle preparations (made by the Kaback [63] procedure) capable of accumulating aminoglycosides at appreciable rates. By modifying the procedure to incorporate protein synthesis components into vesicles, Bryan and Kwan (17) obtained preparations from *Escherichia coli* that accumulate gentamicin with kinetics analogous to intact cells. There are two reports to date of apparently ribosome-free vesicle preparations exhibiting concentrative uptake of streptomycin. One report is from Eagon's group (117) describing the use of a preparation from *Pseudomonas putida* (there was no success in attempts to prepare transport-active vesicles from *Pseudomonas aeruginosa*). The second report concerns *E. coli* membrane vesicles made by the Kaback technique (28), apparently with no modifications. It is somewhat difficult to assess these uptake experiments (the use of membrane vesicles in aminoglycoside uptake protocols are treated in detail in a subsequent section). Thus far, there has been no systematic effort to compare preparations from different bacterial species, despite variability in results as described above. Such studies will eventually need to be done, to complement the comparative results with intact cells that already are available, and to explore the possibility that a given aminoglycoside may enter cells of different species by somewhat different routes.

Experimental measurements of drug uptake have usually relied on filtration through glass fiber, cellulose nitrate, or polycarbonate filters. The polycationic nature of aminoglycosides presents problems of nonspecific adherence, usually solved by washing filtered cells with various salt solutions and by careful attention to sample handling. Moreau et al. (86) described procedures for measuring antibiotic uptake by bacteria, using phase partition between two polymer solutions. Dihydrostreptomycin was the one aminoglycoside tested, and results were obtained by the phase partition method that were comparable to those obtained by filtration. Phase partition appears to be particularly useful when rapid kinetics need to be measured, e.g., sampling every 10 to 15 s.

Uptake, Accumulation, and Transport

In the above discussion, the term uptake has been used instead of transport. Most of the literature on aminoglycosides has used either uptake or accumulation, because the doubt still persists that the movement of aminoglycosides into bacterial cells has either the specificity or the satur-

ability usually associated with transport. Certainly, aminoglycosides can be accumulated against a concentration gradient, and substantial evidence now exists that this accumulation is not simply due to sensitive ribosomes acting as an intracellular sink, binding aminoglycosides irreversibly (see below).

Intracellular Binding of Antibiotic

Aside from in vitro data on rapidly reversible binding by dihydrostreptomycin to ribosomes (24), there are several reports on cellular loss of radiolabeled aminoglycosides following treatment of cells with organic solvents (toluene or butan-1-ol) to render their cytoplasmic membranes permeable to small molecules (see, e.g., references 6, 32, and 97). It is, then, justified to speak of active accumulation or active uptake of aminoglycosides. The requirements of specificity and saturability are more difficult ones to satisfy, and this may be a task for the future; there are indications that these conditions are being met, at least in some systems.

The essential role of antibiotic-sensitive ribosomes in aminoglycoside uptake has been clear for at least the past 10 years, yet the mechanism is quite unclear. Although the nature of aminoglycoside-ribosome interaction is beyond the scope of this discussion (32), it is important to stress that active protein synthesis, sensitive to aminoglycoside action, is necessary for lethal uptake to occur. This is true for intact cells or for in vitro membrane vesicle preparations (except for the one report previously mentioned).

PHASES OF UPTAKE BY INTACT BACTERIAL CELLS

Several aspects of aminoglycoside uptake kinetics remain ambiguous despite recent work in a number of laboratories. These include the bearing of kinetic measurements on the mechanism by which aminoglycosides cross the cytoplasmic membrane and whether uptake is a result of or coincident with the lethal event of aminoglycoside action. Several investigators have demonstrated that accumulation of streptomycin and gentamicin by an aerobically growing culture of bacteria occurs in three consecutive phases (6, 20, 32, 78). An initial ionic binding to cells is followed by two energy-dependent phases termed energy-dependent phase I (EDPI) and energy-dependent phase II (EDPII). EDPI represents a slow rate of energized uptake; its duration and the rate of uptake during EDPI are dependent on the concentration of aminoglycoside presented to the cell. EDPI can be completely abolished by using high concentrations of aminoglycoside (>30 μg of base per ml). The rapid energy-dependent accumulation of aminoglycoside following EDPI is referred to as EDPII.

Ionic Binding

When an aminoglycoside is added to a suspension of bacteria, electrostatic binding to anionic sites distributed on the cell surface occurs almost instantaneously (6, 20). This ionic binding is reversible, concentration dependent, and not influenced by inhibitors of energized uptake. Polycations have been demonstrated in several laboratories to antagonize significantly this initial adsorption phase. In gram-negative bacteria, the anionic binding sites include lipopolysaccharide, polar heads of phospholipids, and outer membrane proteins. In gram-positive bacteria, phospholipids and teichoic acids are utilized as the initial binding sites. This ionic binding represents the energy-independent phase of uptake kinetics. Aminoglycosides may reach the periplasm and ultimately the cytoplasmic membrane of gram-negative bacteria via a hydrophilic diffusion pathway through pores generated by outer membrane porin proteins or, as shown for *P. aeruginosa*, via a hydrophilic pathway utilizing a "self-promoted" transport mechanism. Similar mechanisms may function in other gram-negative organisms; however, this has not been extensively investigated.

The relationship of these pathways to the kinetics of binding and the transition to subsequent uptake phases is quite unclear; it is not known, for example, if any components of the periplasm bind to aminoglycosides. It seems likely that this additional barrier could reduce the effective antibiotic concentration presented to the cytoplasmic membrane.

EDPI

The second phase of uptake, EDPI, varies in duration and rate depending on the external concentration of aminoglycosides (21). Muir et al. (90) recently showed that the duration of EDPI for streptomycin accumulation in chemostat cultures of *E. coli* also is dependent on the growth rate prior to addition of streptomycin. EDPI precedes the loss of viability and inhibition of protein synthesis, and uptake during EDPI can be blocked by inhibitors of electron transport (e.g., cyanide and anaerobiosis) and oxidative phosphorylation (e.g., dinitrophenol [DNP] and carbonyl cyanide-*m*-chlorophenyl-hydrazone [CCCP]). The initial energy-independent phase still occurs, but EDPI is abolished (90). Bryan and colleagues have suggested that during EDPI polycationic aminoglycosides cross the cytoplasmic membrane in response to the membrane potential ($\Delta\psi$; internal negative) of the cell (21, 30). This process requires a threshold $\Delta\psi$ (the magnitude of which depends on the aminoglycoside concentration) before significant uptake occurs (17, 43). The mechanism of transport of aminoglycosides across the cytoplasmic membrane during EDPI may involve quinone-linked redox energy or additional components of the electron transport chain or both. EDPI may also represent slow uptake through nonspecific membrane channels created by low-level intrinsic misreading or slight imperfections in the cytoplasmic membrane, particularly in zones of growth which may permit low concentrations of aminoglycosides to penetrate into the cell (31). Whether EDPI represents translocation of aminoglycoside across the cytoplasmic membrane or progressive binding of drug to the outside of the cytoplasmic membrane remains unclear (97). Some authors have concluded that no cytoplasmic uptake occurs during this phase (97), and others concluded that slow respiration-dependent uptake occurs (20, 21). Nichols and Young (97) have suggested that significant uptake of dihydrostreptomycin does not occur during the second phase of uptake prior to the onset of EDPII. When the second phase of uptake was assumed to be a plateau, the plateau was indistinguishable in several experiments from the value for the plateau observed under anaerobic conditions, in which only the energy-independent phase of uptake was observed (97). Therefore, EDPI may represent increased adsorption of aminoglycosides to the outer surfaces of the cell proportional to the continued growth of the cells. If in fact EDPI does represent a lag period during which no further net uptake of dihydrostreptomycin occurs, the nature of energy coupling to this process remains obscure.

EDPII

An understanding of the third phase of aminoglycoside uptake, EDPII, likewise remains elusive. The kinetics of streptomycin and gentamicin uptake under the usual conditions of measurement are not representative of saturable transport systems. Instead, the transport system, though clearly energy dependent, exhibits diffusion kinetics (21). Competition studies with solutes transported by known carrier systems have ruled out "borrowed" solute carrier systems as the mechanism ("illicit transport") by which streptomycin and gentamicin are translocated across the cytoplasmic membrane (18, 21). Uptake experiments have not ruled out the alternative of low-affinity multiple carrier systems, each to some extent contributing to the total uptake of aminoglycosides. In general, EDPII represents an accelerated linear rate of aminoglycoside transport across the cytoplasmic membrane by a process that uses energy from electron transport and possibly from adenosine 5'-triphosphate (ATP) hydrolysis, since uptake during EDPII can be blocked by cyanide, sulfhydryl reagents, and uncouplers of oxidative phosphorylation (20).

Effects of protein synthesis inhibitors. EDPII also can be reduced or abolished by certain inhibitors of protein synthesis. Addition of chloramphenicol (an inhibitor of peptidyl transfer) either prior to or simultaneously with the addition of streptomycin inhibits EDPII, suggesting that protein synthesis is a prerequisite for the induction of EDPII (61). Muir et al. showed that addition of chloramphenicol together with streptomycin inhibited EDPII, but not EDPI (90). This group also found that protein synthesis was required for both initiation and continuation of EDPII. When the addition of chloramphenicol to cultures containing streptomycin was delayed until EDPII had commenced, further accumulation was reduced but not abolished. The events that lead to the initiation of EDPII seem to be dependent on a complex set of conditions. Unquestionably, the induction of EDPII requires aminoglycoside-sensitive ribosomes engaged in protein synthesis. Several studies have demonstrated that ribosomal binding contributes significantly to total uptake of streptomycin and gentamicin (2, 21, 61). The development of EDPII following exposure to streptomycin is presented in *rpsL* mutants of *E. coli* (21), and EDPII is reduced or abolished in bacteria that contain streptomycin- or gentamicin-modifying enzymes. Either alteration of ribosomal binding sites or covalent modification of aminoglycosides during transport reduces the affinity of the drugs for the ribosome; in either case, only the energy-independent phase and EDPI are observed.

In wild-type cells, the initiation of EDPII is not the sole cause of cell death, although it may contribute. Despite having ribosomal binding sites different from streptomycin, other aminoglycosides have been shown to induce the initiation of EDPII for streptomycin, even in streptomycin-resistant (*rpsL*) mutants (58, 60). Membrane vesicles prepared from *E. coli* cells pretreated with dihydrostreptomycin were shown to be impaired in their ability to maintain a proton gradient and transport proline (37), and it was suggested that vesicle membranes were sufficiently disordered to enhance permeability. However, the effect of this treatment on the uptake of dihydrostreptomycin itself was not examined. Grisé-Miron and Brakier-Gingras (48) demonstrated that neomycin can increase the binding affinity of dihydrostreptomycin for the ribosome. Although preincubation of *E. coli* with neomycin induces only a minor enhancement of streptomycin uptake (60), kanamycin and gentamicin may also increase the affinity of streptomycin for the ribosome above that observed for neomycin. Further studies are needed to clarify enhancement of aminoglycoside affinity for the ribosome as a mechanism of EDPII induction.

Unlike chloramphenicol, puromycin (which acts on translation by causing premature release of polypeptide chains) at 50 to 100 μg/ml does not block the uptake of streptomycin but induces an accelerated rate of uptake and killing of *E. coli* (61). Puromycin can induce EDPII-like uptake of streptomycin in *rpsL* cells without immediate effects on viability (61). An interesting exception is the inhibition of the puromycin-induced EDPII uptake in an *E. coli* ubiquinone-deficient mutant (17). Ubiquinone-deficient mutants of *E. coli* generate sufficient $\Delta\psi$ to transport aminoglycosides; however, *ubi* mutants are respiration deficient (17), suggesting that puromycin induction of streptomycin uptake may be respiration dependent. Several groups have shown that energy inhibitors and sulfhydryl reagents block further uptake during EDPII. Hurwitz et al. (61) showed that the puromycin-induced EDPII-like kinetics are inhibited by *N*-ethylmaleimide. This result further suggests that puromycin-induced uptake may be energy dependent; however, cyanide and protonophores have not been analyzed. An unusual paradox of the puromycin-induced streptomycin uptake is that, while moderate concentrations (50 to 100 μg/ml) of puromycin induce rapid uptake, high concentrations (>500 μg/ml) decrease the uptake of streptomycin. It has been proposed that low concentrations of puromycin, by liberating nascent chains, could increase the frequency of binding of streptomycin to ribosomes (61). High concentrations of puromycin, on the other hand, might decrease the effective affinity constants of various aminoglycosides for the ribosome. A direct effect of puromycin on ribosomal binding of aminoglycosides is only speculative, since experimental evidence is limited in this area, and the ability of puromycin to induce accelerated streptomycin uptake in *rpsL* mutants suggests that alterations of ribosome-binding constants may be minor. The implications of the puromycin paradox will also be considered in light of a model for EDPII uptake kinetics presented below.

Studies with membrane vesicle preparations. Aminoglycoside transport studies with whole cells have been difficult due to binding to the outer membrane and to ribosomes. Increasing outer membrane permeability with ethylenediaminetetraacetic acid may induce changes in the inner membrane, affecting interpretation of the data. To avoid potential ambiguities encountered when using whole cells in transport studies, several laboratories have attempted to prepare transport-active "Kaback" vesicles from *E. coli*. For reasons that are unclear, uptake of streptomycin or gentamicin with energized vesicles capable of transporting proline has not been successful. The inability to demonstrate aminoglycoside transport in membrane vesicles has delayed the dissection of specific energy requirements and transport systems potentially involved in translocation of streptomycin and gentamicin across the cytoplasmic membrane.

Bryan and Kwan (17) demonstrated that membrane vesicles derived from *E. coli* did not accumulate gentamicin unless both an energy source and all components needed to establish protein synthesis were present. Under these conditions, gentamicin uptake was respiration dependent, and addition of 1.0 mM KCN prevented gentamicin uptake. It was not established whether streptomycin (in addition to gentamicin) accumulation or protein synthesis could occur under these circumstances. When these vesicles were loaded with potassium ions and subsequently treated with the

ionophore valinomycin, a diffusion potential was created that was well above the threshold value known to be necessary for aminoglycoside uptake by intact cells; despite this, no gentamicin accumulation was observed (17). Nichols and Young (97) independently showed that energized membrane vesicles prepared from *E. coli* did not take up dihydrostreptomycin under conditions that stimulate proline transport. Whether or not protein synthesis by appropriately prepared membrane vesicles is required to induce gentamicin uptake is still unknown; nevertheless, experimental manipulation of vesicle systems for kinetic studies of aminoglycoside transport finally may be possible.

In contrast to the findings described above, a recent report of aminoglycoside uptake in *E. coli* membrane vesicles (prepared by the Kaback procedure) has appeared in the literature (28). Accumulation of nine aminoglycosides was observed in right-side-out vesicles prepared from aerobically grown *E. coli*. Unfortunately, the methodology utilized to measure aminoglycoside transport was not described in detail, and there was limited reference to previously documented requirements necessary for uptake (e.g., an exogenous energy source). Thompson et al. (117) provided evidence that dihydrostreptomycin can be accumulated by isolated cytoplasmic membrane vesicles of *P. putida* by an active transport mechanism. Uptake exhibited saturation kinetics and was inhibited by the proton ionophore CCCP. Inhibition of electron transport and subsequent effects on uptake were not examined. The authors suggested that the observed kinetics were those of the EDPI component of aminoglycoside uptake, since the induction of EDPII requires sensitive ribosomes which were not present in the vesicle preparations. This demonstration of dihydrostreptomycin uptake in membrane vesicles of *P. putida* (but not in *E. coli* [17, 97] or *P. aeruginosa* [117]) may represent fundamental differences in aminoglycoside uptake among gram-negative bacteria. Exploration of physiological and metabolic differences may be quite important to an understanding of the mechanism of streptomycin uptake, and studies with these transport-active membrane vesicle preparations from *P. putida* should be vigorously pursued.

Models for the initiation of EDPII and the uptake process. Although the events leading to the initiation of EDPII are poorly understood, the collective data suggest that interactions of aminoglycosides with ribosomes during the initial phase of uptake is a prerequisite to EDPII. In the following discussion, we attempt to present existing models in the context of an overall theme of aminoglycoside uptake, eschewing topics such as specific "transporter" molecules or the energetics of uptake. These topics are covered in later sections. Early proposals suggested that EDPII may be due to an increase in the membrane electrical potential, $\Delta\psi$, as a result of the initial effects of streptomycin and gentamicin. During the onset of EDPII, cells show a marked loss of K^+ from cells, which in theory could increase the magnitude of $\Delta\psi$. However, Bryan and Kwan (17) provided evidence that EDPII is not due to an increase in $\Delta\psi$. Damper and Epstein (30) proposed that uptake during EDPII involves a system that allows aminoglycosides, acting as polycations, to diffuse across the cytoplasmic membrane in response to the $\Delta\psi$. Nichols and Young (96, 97, 122) attempted to determine whether aminoglycoside uptake is a passive (uniport) process, which would allow exchange diffusion between intra- and extracellular streptomycin. They were unable to detect any exchange of intracellular [^{3}H]dihydrostreptomycin for unlabeled extracellular dihydrostreptomycin during EDPII. Efflux of dihydrostreptomycin was also not observed by inhibiting respiration anaerobically or by adding a proton ionophore. These workers concluded that uptake of dihydrostreptomycin is irreversible and therefore likely to be mediated by a single transport system. This irreversibility may be directly associated with the lethal action of streptomycin and related aminoglycosides, although the mechanism of irreversible uptake is not at all understood.

Bryan and colleagues elaborated a comprehensive model to explain aminoglycoside accumulation and lethality (17). In this model, the progressive transfer of aminoglycosides to ribosomes (transfer event) results in polysome degradation, accumulation of runoff ribosomes, and inhibition of protein synthesis, with polysomes serving as a binding sink for aminoglycosides. EDPII results from an accelerated rate of transfer events (possibly due to polysome decay), and this accumulation of transfer events compromises cytoplasmic membrane integrity and function, leading to cell death. The process is unfavorable either in the absence of the intracellular ribosomal binding site (e.g., in a *rpsL* mutant) or when polysome degradation is inhibited by the addition of chloramphenicol (61). The model presented by Bryan provides an explanation for the majority of the experimental results and suggests how aminoglycosides may interact with bacterial cells at several levels. However, the mechanism of the transfer event remains vague; that is, how aminoglycoside molecules on the outside of the cytoplasmic membrane can interact with (membrane-bound) ribosomes on the inside of the cytoplasmic membrane during EDPII at an accelerated, nonsaturable rate is not specified. In addition, the number of aminoglycoside molecules taken up during EDPII exceeds the number of ribosomes in the cell, suggesting that the existence of a binding sink is unlikely.

Davis and co-workers (32, 33) recently presented a stimulating hypothesis for the initiation and maintenance of EDPII. H. J. Busse and E. P. Bakker (Abstr. 9th Int. Cong. Microbiol., no. B23, 1986) independently postulated a similar model to explain the loss of permeability control (particularly for K^+) in aminoglycoside-treated cells of *E. coli*. The model suggests that EDPII results from the incorporation of mistranslated proteins into the cytoplasmic membrane, with these aberrantly structured proteins functioning as nonspecific channels to facilitate translocation of aminoglycosides. According to this model, the linear accelerated rate of uptake during EDPII results from an autocatalytic cycle of increasing aminoglycoside flux, misreading, and channel formation. As the intracellular concentrations of aminoglycoside rise, all initiating ribosomes become blocked, preventing protein synthesis; lethality results from the irreversible nature of the uptake process (122). However, irreversible uptake through nonspecific channels created by insertion of faulty proteins into the membrane is problematic and may overstep the laws of thermodynamics (W. Nichols, personal communication) unless such channels are nonspecifically "gated" by an unknown mechanism (38, 122).

Davis et al. (33) provided some direct experimental evidence for insertion of misread proteins: alkaline phosphatase, which normally is secreted through the cytoplasmic membrane into the periplasm of *E. coli*, was seen to accumulate instead in a "residual fraction." This fraction may contain both membrane material and aggregated cytoplasmic protein, but was shown to contain polypeptides of appropriate M_r precipitable by anti-alkaline phosphatase antibody (33). This seems to be an attractive and testable hypothesis. For example, experiments with aminoglycosides such as spectinomycin or kasugamycin that do not cause translational misreading might show whether the latter mechanism

is obligatory for rapid antibiotic entry. The model does not deal with the entry of aminoglycosides during the EDPI uptake phase, which presumably occurs in the absence of defective proteins, nor does it explain the electron transport component-dependent uptake specificity exhibited by *Bacillus subtilis* (7, 77). There remains also the troubling matter of uptake irreversibility, at least for streptomycin (90, 97), and of restriction of uptake by decreases in lipophilic quinones that form part of the electron transport chain (21, 113).

A model involving misreading and artificial channel formation does, however, help to explain effects of chloramphenicol and other protein synthesis inhibitors. These inhibitors could halt the synthesis of aberrant proteins, and the short lifetimes of these proteins in bacteria might allow membrane integrity to be restored. Puromycin-stimulated streptomycin uptake by *rpsL*$^+$ and *rpsL* strains of *E. coli* could be explained as follows: polypeptide chains prematurely released by puromycin could create membrane channels, analogous to those formed by the misread proteins synthesized after addition of streptomycin to Str^s cells. High concentrations of puromycin (100 to 500 μg/ml), however, would cause early release of nascent chains, the majority of which would not have become long enough to enter the membrane and induce EDPII. The reduction in streptomycin uptake observed under these circumstances may occur by a secondary effect on cell physiology or metabolism or both. Growth and protein synthesis are inhibited >90% by 500 μg of puromycin per ml, a concentration that inhibits streptomycin uptake in both sensitive and resistant cells (61). Puromycin-induced streptomycin uptake has not been experimentally tested in energized membrane vesicles of *E. coli* containing protein synthesis machinery (17); if protein synthesis does occur in this system, the mechanism of puromycin induction of streptomycin uptake could be addressed.

As previously mentioned, puromycin-induced EDPII is abolished in a ubiquinone-deficient mutant of *E. coli* (17). Bryan and Kwan (17) have suggested that the enhanced uptake produced by puromycin requires redox-active quinones, or it may depend on efficient electron transport. However, puromycin induction should be reexamined in chemostat cultures, since in this experimental system rates of accumulation of streptomycin for two ubiquinone-deficient *E. coli* mutants and for the parent strain have been shown to be identical (88). Muir et al. (90) recently proposed that the rate of streptomycin uptake is related to the number of streptomycin-sensitive ribosomes actively engaged in protein synthesis, and the inability of puromycin to induce EDPII in ubiquinone-deficient strains may be accounted for by differences in growth rates between *ubi*$^+$ and *ubi* strains. If, however, quinones are involved in the formation of nonspecific channels in the cytoplasmic membrane, it may be that inhibitors of electron transport (e.g., cyanide) may inhibit further uptake during puromycin-induced EDPII. Cyanide has been shown by several groups to inhibit further uptake by streptomycin-sensitive cells during EDPI (20). Hurwitz et al. (61) have shown that *N*-ethylmaleimide immediately blocks puromycin-induced uptake of dihydrostreptomycin in both resistant and sensitive cells. Whether *N*-ethylmaleimide affects the ability of misread proteins to form channels or inhibits electron transport redox reactions is unknown. It has been proposed that ATP is an efficient energy source for protein insertion and translocation (25); if we assume that the insertion of nonspecific channels into the cytoplasmic membrane requires energy, electron transport may be necessary to generate the ATP necessary for misread or prematurely released nascent proteins to be inserted into the membrane.

Refinement of the models presented above for the mechanism of aminoglycoside uptake (and lethality) should provide a suitable framework for future conceptualization and experimentation. The initiation of aminoglycoside transport across the cytoplasmic membrane, if (and by what mechanism) faulty proteins create channels, and the irreversible nature of aminoglycoside uptake are among the unresolved questions that must be accounted for in useful models for aminoglycoside uptake.

EFFECTS OF CELL ENVELOPE STRUCTURE ON AMINOGLYCOSIDE UPTAKE

The outer layers of the bacterial cell envelope form the initial barrier encountered by aminoglycosides. These structures include capsules and exopolysaccharides, the outer membrane of gram-negative species, and peptidoglycan layers. Influences of each layer on aminoglycoside uptake are considered, together with recent information on the effects of envelope modification.

Capsules and Exopolysaccharide Layers

In gram-negative and -positive species, capsules or exopolysaccharide layers or both may occur and hinder the inward movement of antibiotic molecules (reviewed by Godfrey and Bryan [45]). Slack and Nichols (110) developed a mathematical model from which they concluded that polysaccharide layers surrounding single cells will not be rate limiting for entry. Thus, it is possible that these most superficial layers decrease the free diffusion of aminoglycosides to the underlying cell envelope (cf. reference 109), but the hindrance is minor compared with the more substantial blockage by such structures as the outer membrane and the cytoplasmic membrane. This conclusion is supported by recent experimental data on diffusion of tobramycin through alginate (Nichols et al., submitted for publication). A further question, somewhat difficult (it would seem) to address, is whether or not extensive exopolysaccharide networks (as, for example, those produced by oral flora) might decrease antibiotic availability by impeding bulk fluid flow in the vicinity of the bacterial cell.

Outer Membrane of Gram-Negative Bacteria

Permeability of the outer membrane to aminoglycosides has been studied extensively by a number of investigators, with *E. coli* and *P. aeruginosa* receiving most attention. The reviews by Bryan and co-workers (13, 14, 45) and by Hancock (51, 52) should be consulted for general discussions of antibiotic permeability and modifications of envelope permeability. Nikaido and Vaara (98) have provided a detailed discussion of the molecular structures underlying outer membrane permeability and evaluated methodologies for measuring diffusion rates of different molecular species.

There are two possible routes for entry of antibiotics through the outer membrane (14, 45): (i) the hydrophilic pathway requiring functional nonspecific diffusion channels ("pores") formed by porin proteins, and (ii) the hydrophobic pathway involving antibiotic solubilization in the lipid bilayer of the outer membrane. In *E. coli* K-12, pores formed from OmpC or OmpF proteins comprise the hydrophilic pathway; Nakae and Nakae (92) have measured diffusion rates of several aminoglycosides into proteoliposomes re-

constituted from phospholipids and purified porins. Surprisingly, the aminoglycosides diffuse more rapidly (by comparison with hydrophilic uncharged sugars) than would be expected on the basis of their molecular weights. The use of reconstituted proteoliposomes for diffusion measurements on charged molecules has been questioned (98) because of the formation of membrane potentials induced by differential diffusion of ions, which in turn causes complex movements of buffer and other ions. However, if the experiments are carried out in high salt concentrations (e.g., 100 mM NaCl), counterion diffusion effects should be negligible (Nichols, personal communication). On balance, it may be premature to conclude that the polycationic aminoglycosides enter *E. coli* via the nonspecific hydrophilic pathway represented by OmpF and OmpC porin trimers.

With *P. aeruginosa*, the interaction of aminoglycosides with the outer membrane has been studied extensively, particularly by Hancock and his co-workers (51, 52, 55, 70, 85, 94, 95, 102), frequently as part of broader investigations into the structure and permeability of the *Pseudomonas* cell envelope. *P. aeruginosa*, in contrast to *E. coli*, appears to have a single constitutive porin, protein F (see references in reference 94). Measurements of pore size by permeability to uncharged saccharides or polysaccharides gave an exclusion limit of M_r 3,000 to 7,000 (34, 53), considerably in excess of the M_r-500 to -600 exclusion limit generally accepted for enteric gram-negative species. However, most of these pores appear to be nonfunctional, resulting in poor permeability of the *P. aeruginosa* outer membrane to hydrophilic antibiotics such as aminoglycosides. Recently, Caulcott et al. (23) and Yoneyama and Nakae (121) reexamined this question and obtained evidence for functional diffusion pores smaller than those found in enteric species. Whatever the exact nature of the hydrophilic pathway in *P. aeruginosa*, it seems clear that it is not used for significant aminoglycoside entry. Instead, as proposed by Hancock et al. (55; see also references 51 and 52), aminoglycosides cross the outer membrane via a self-promoted pathway, involving disruption of lipopolysaccharide (LPS)-Mg^{2+} cross bridges. This disruption depends on the cationic nature of aminoglycosides and can be brought about by other polycations such as polymyxin and protamine. Indeed, gentamicin and polymyxin may share binding sites with one another (84) as well as with the naturally stabilizing cations Mg^{2+} and polyamine (84, 93, 95; see also reference 1). The fluorescent probe 1-*N*-phenylnaphthylamine has been used (70) to show that exposure of the hydrophobic outer membrane lipid bilayer can occur in the presence of aminoglycosides. In dissecting the process further, Peterson et al. (102) measured antibiotic binding to LPS by displacement of a cationic electron spin resonance probe and correlated the relative affinities of antibiotics for LPS with their ability to increase outer membrane permeability. Relative affinities of several polyamines, on the other hand, were correlated with their ability to stabilize outer membrane structure. The authors proposed that polycationic antibiotics such as aminoglycosides bind to LPS and rearrange its packing, resulting in the formation of "cracks" in the membrane structure. These artificially induced channels must be hydrophilic to accommodate charged molecules such as aminoglycosides.

By contrast with *P. aeruginosa*, the increasingly important opportunistic pathogen *Pseudomonas cepacia* was found to be resistant to the outer membrane-permeabilizing effects of aminoglycosides or polymyxin (85). This correlates with the resistance of clinical isolates to these antibiotics. Purified LPS from *P. cepacia*, however, had binding properties for dansyl-polymyxin very similar to those of *P. aeruginosa* LPS. The authors suggest that *P. cepacia* does not use the self-promoted pathway for aminoglycoside entry, apparently because the cation-binding sites on its LPS are protected by other constituents of the outer membrane. These findings emphasize the importance of the self-promoted pathway for successful entry of aminoglycosides in at least some gram-negative species.

Martin and Beveridge (73) recently examined gentamicin-treated *P. aeruginosa* by electron microscopy, using negative-contrast staining, thin sectioning, and freeze-fracture techniques. They found that as early as 1 min after exposure to low concentrations (5 to 25 μg/ml) of the antibiotic, large increases in numbers of membrane blebs were seen on the cell surface by negative-contrast staining or thin sectioning. Changes in surface organization were confirmed by freeze-fracture studies; furthermore, the surface disruption occurred even in the presence of KCN or NaN_3, suggesting that it was independent of intracellular metabolism or macromolecular synthesis. These morphological studies appear to correlate with chemical and biophysical measurements of aminoglycoside interaction with outer membrane structures and support the self-promoted pathway as the entry mechanism for aminoglycosides, at least in *P. aeruginosa*. It seems likely that a similar mechanism, perhaps differing in molecular details, functions in enteric species, since blebbing can be observed when *E. coli* K-12 is treated with gentamicin (unpublished data quoted in reference 73). However, the situation in enteric bacteria is clouded by the possibility that entry may also occur through functional pores, since their exclusion limit is approximately the same as the molecular weight of many aminoglycosides.

Peptidoglycan and Teichoic Acid Wall Structures of Gram-Positive Bacteria

Interactions of aminoglycosides with envelope structures from gram-positive bacterial cells have not been studied extensively, presumably because the absence of an outer membrane is thought to allow free access of aminoglycosides (and other classes of antibiotics) to the cytoplasmic membrane. This may be true for many gram-positive species, but enough exceptions exist to suggest caution. *Streptococcus faecalis*, for example, binds but shows no time-dependent accumulation of streptomycin (83). While the multilayered peptidoglycan structure which comprises the bulk of the gram-positive cell wall excludes molecules only of >100,000 daltons, a number of other polymers with a variety of binding sites occur in the wall. In the enterococci there are group- and type-specific antigens (the chemical nature and biophysical properties of which are not known in detail), and in many gram-positive species there are both wall teichoic acids (covalently linked to peptidoglycan) and lipoteichoic acids (noncovalently embedded in the cytoplasmic membrane and often extending through the entire thickness of the wall; cf. reference 119).

Kusser et al. (69) have in fact studied the binding of aminoglycosides to both lipoteichoic and wall teichoic acids from a number of gram-positive species. Teichoic acid-depleted cell wall preparations bound very little [^{3}H]dihydrostreptomycin, demonstrating that these polymers are likely to be the sites of initial binding in intact cells. Cooperative binding of dihydrostreptomycin to purified teichoic acids was observed, and competition between dihydrostreptomycin and other aminoglycosides, Mg^{2+}, and spermidine for the same or closely spaced binding sites was

seen. This behavior is similar to that characteristic of LPS-aminoglycoside interactions and raises questions about the structural modification of gram-positive cell walls as they bind aminoglycosides. Dihydrostreptomycin readily displaces Mg^{2+} from teichoic acids in vitro (69), yet Mg^{2+} is a normal counterion associated with the envelope of gram-positive bacterial cells and contributes to its stability. Perhaps more attention ought to be paid to interaction of aminoglycosides with cell walls and wall components of gram-positive species, especially those exhibiting envelope-based intrinsic resistance to these antibiotics.

Modification of Envelope Structure by Antibiotics

Another poorly explored mechanistic area is the apparent increase in cell envelope permeability to aminoglycosides caused by antibiotics with envelope-modifying activity, particularly the beta-lactams. Bactericidal synergism between these two classes of antibiotics has been noted for many years (see references 3 and 31) and has been used in treatment of some pathogens. In the study on *S. faecalis* cited above (83), sublethal concentrations of penicillin markedly increased uptake (and bactericidal activity) of streptomycin, suggesting that modest modifications in cell envelope polymer structure can have profound effects on the integrity of the envelope as a barrier to aminoglycosides. The enhanced entry of streptomycin into *E. coli* in the presence of penicillin had been shown many years before by Plotz and Davis (104) and was proposed as a mechanism for some antibiotic synergisms. There are alternative explanations for intrinsic resistance to aminoglycosides among streptococci; Bryan (14) has suggested that a decrease in the magnitude of the proton electrochemical potential (Δp) compared with many other species may account for this resistance. However, it is not immediately apparent how beta-lactams could increase Δp across the cytoplasmic membrane.

Recently, Miller and his colleagues have begun a systematic reexamination of beta-lactam effects on streptomycin uptake by *Staphylococcus aureus* (123), viridans and enterococcal streptococci (79), and *P. aeruginosa* (80). With *Staphylococcus aureus*, bactericidal synergism and enhanced uptake of streptomycin was seen in the presence of oxacillin, if streptomycin was below its minimal inhibitory concentration (MIC) and oxacillin was at or above its MIC. If, however, streptomycin was above its MIC, synergism and enhanced uptake were lost. Oxacillin-induced enhancement of uptake (at sub-MIC streptomycin concentrations) was observed in an Str^r (*rpsL*) mutant, suggesting that ribosomal binding of the aminoglycoside was not necessary for the stimulation of uptake. In studies on penicillin enhancement of streptomycin uptake by streptococci (79), *S. faecalis* was found to show such enhancement, in confirmation of previous studies (83). However, the viridans streptococci (*Streptococcus sanguis*, *Streptococcus mitis*) that were examined did not show enhanced uptake. Results on *Staphylococcus aureus* and on streptococci have been brought together by Miller et al. under a model for beta-lactam effects on aminoglycoside uptake. They suggest that, at low streptomycin concentrations, lipo- and wall teichoic acids bind antibiotic and prevent access to the cytoplasmic membrane. Treatment with a beta-lactam would cause loss of teichoic acid and peptidoglycan from the cell wall, reducing the importance of this obstacle to aminoglycoside entry. The hypothesis benefits from its testability, perhaps by using mutants blocked in various steps in teichoic acid biosynthesis and using the physiological manipulations that have been devised to regulate cellular teichoic acid content. Since gram-negative bacteria lack teichoic acids, this model cannot be extended to include such species as *P. aeruginosa*, which Miller et al. (80) have shown (confirming Plotz and Davis [104] for *E. coli*) also responds to beta-lactams with enhanced tobramycin and streptomycin uptake.

TRANSPORT AND RIBOSOMAL BINDING OF POLYAMINES AS THEY AFFECT AMINOGLYCOSIDE UPTAKE

Since Höltje first reported the uptake of streptomycin by an inducible polyamine transport system (58), several other investigators have generated conflicting evidence, and it is now accepted that aminoglycoside accumulation is not related to specific polyamine transport (15, 22). Nonetheless, an interesting relationship between polyamines and aminoglycosides exists. It is clear that aminoglycosides exhibit their effects as a consequence of their interaction with ribosomal targets (see reference 101). Polyamines stimulate protein synthesis and stabilize 70S particles (115), and this role in protein synthesis appears to be necessary for streptomycin function. Goldemberg and Algranati (46) demonstrated that streptomycin-induced inhibition of protein synthesis in a polyamine auxotroph occurred only if the culture was supplemented with polyamines. Neomycin, kanamycin, and kasugamycin behaved similarly to streptomycin, while spectinomycin and gentamicin inhibited protein synthesis regardless of the state of polyamine sufficiency. Also, these investigators showed that small ribosomal subunits from polyamine-supplemented cells exhibited an altered conformation when exposed to streptomycin. In contrast, no antibiotic-induced changes were observed with ribosomes from polyamine-starved cells. Streptomycin was shown to bind only to low-affinity sites on subunits from starved cells, while polyamine-sufficient subunits displayed an additional high-affinity site (47). Taken together, it seems likely that streptomycin requires ribosomal targets of the appropriate conformation to induce subsequent misreading events involved in drug-mediated lethality. This effect may be specific for streptomycin, and it is important to remember that all aminoglycosides, while having the ribosome as a common general target, may not interact with it in the same way. Gentamicin, for example, appears to be capable of inhibiting protein synthesis in either the presence or the absence of normal levels of endogenous polyamines. However, polyamines added to a preparation of ribosomes in vitro displace gentamicin from the ribosome (87). This is in contrast to the streptomycin situation described above.

ENERGETICS OF UPTAKE

Proton Electrochemical Gradient

The chemiosmotic hypothesis of Mitchell (81, 82) states that the proton motive force (Δp) is the driving force for energy-requiring processes such as solute transport and ATP synthesis. Electrical and chemical parameters contribute to Δp according to the following relationship: $\Delta p = \Delta\psi - (Z\,\Delta pH)$, where $\Delta\psi$ represents the electrical potential across the membrane and ΔpH is the chemical difference in proton concentration across the membrane ($Z = 58.8$ at room temperature).

In aerobically growing *E. coli*, Δp is generated via a membrane-bound respiratory chain with oxygen as the terminal electron acceptor. For bacterial cells, vectorial

translocation of protons occurs such that protons are extruded to the external environment, creating an electrical potential and pH gradient across the cytoplasmic membrane which constitute the proton motive force (interior negative and alkaline). The movement of protons back into the cell can be coupled to ATP synthesis via the membrane-bound H^+-adenosine triphosphatase (ATPase). Hydrolysis of ATP by the enzyme can cause secretion of protons and generate a proton motive force. In the presence of nonfermentable carbon sources (i.e., succinate and lactate), only electron transport can be used to generate Δp and ATP. Under anaerobic conditions, in the absence of alternate electron acceptors (e.g., nitrate or fumarate), ATP is produced solely by substrate-level phosphorylation, and a portion of this ATP is hydrolyzed to maintain Δp.

Accumulation under Anaerobic Conditions and in the Presence of Electron Transport Inhibitors

R. Hancock originally demonstrated that uptake and lethality of streptomycin required respiration, since anaerobiosis and an electron transport inhibitor, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide, reduced uptake and subsequent killing by streptomycin (49). (Ronald Hancock was a pioneer in aminoglycoside uptake, and should not be confused with a more recent major contributor to the field, R. E. W. Hancock.) In the two decades since this report, several investigators have obtained similar results by using the electron transport inhibitors potassium cyanide, sodium azide, and *N*-ethylmaleimide (6, 20, 78). Agents such as cyanide completely block respiration in *E. coli* with only a slight decrease in $\Delta\psi$ at a pH of >7.5 as long as a fermentable carbon source is present to allow ATP generation by substrate-level phosphorylation. Electron transport inhibitors abolish uptake and killing by streptomycin and gentamicin in a variety of bacteria, including *P. aeruginosa*, *E. coli* (20), and *Staphylococcus aureus* (78).

Although growth of bacteria anaerobically generally results in the inhibition of aminoglycoside uptake, alternate terminal electron acceptors (nitrate and to a lesser extent fumarate) promote electron transport and aminoglycoside uptake and killing (15, 22). Nitrate-induced uptake can be abolished with the uncoupler DNP (22). These results are in general agreement with the finding that, in unsupplemented media, the strict anaerobes *Clostridium perfringens* and *Bacteroides fragilis* are unable to take up streptomycin and gentamicin (18). However, when *Bacteroides fragilis* is provided with hemin, menadione, and fumarate, streptomycin uptake and killing occur as a consequence of anaerobic electron transport (18). Fermentative bacteria (e.g., *Streptococcus* spp.) display low-level resistance due to incomplete electron transport chains (13). Dissociation of anaerobic electron transport from Δp as the driving force for antibiotic uptake is complicated when anaerobic cultures are supplemented with respiratory chain precursors or alternate terminal electron acceptors. Whether or not strictly anaerobic bacteria develop a $\Delta\psi$ adequate to exceed the threshold required for aminoglycoside uptake is still unclear. In most cases, $\Delta\psi$ values were not recorded or uncouplers were not utilized to inhibit anaerobic aminoglycoside uptake. Unfortunately, attempts to induce an artificial potassium-valinomycin diffusion potential in anaerobically grown cells (e.g., *E. coli*) have not appeared in the literature. Bryan and Kwan (15) observed limited aminoglycoside uptake by *C. perfringens* with the artificial redox compound phenazine methosulfate and ascorbate. In either aerobic or anaerobic *E. coli* cultures, ascorbate/phenazine methosulfate-stimulated uptake was not cyanide sensitive. They suggested that electron flow through only a limited portion of a respiratory chain is necessary for uptake and that ascorbate/phenazine methosulfate might even replace redox quinones in the naturally quinone-deficient *C. perfringens*.

Involvement of Respiratory Chain Components in Aminoglycoside Uptake

Mutants having modified uptake characteristics. The evidence summarized above and in succeeding sections provide strong indications that electron flow through at least some segments of the respiratory chain is necessary to drive aminoglycoside uptake by bacterial cells. It seems possible, in theory, to identify these segments by isolation of mutants deficient in one or another of the components making up these segments (see reference 50). Biochemically, respiratory chains in (nonphotosynthetic) bacteria are composed of flavoprotein dehydrogenases, iron-sulfur proteins, lipophilic quinones (ubiquinone or menaquinone or both), and the hemoprotein cytochromes. Mutations in either the structural genes for the protein components of the chain or the biosynthetic enzymes for quinones or heme could be expected to reduce or abolish aminoglycoside uptake. This of course presumes that such mutations, if not compensated for, are compatible with growth of the cell; for example, mutations affecting flavin biosynthesis would have widespread metabolic effects. However, mutational blocks in heme, ubiquinone, or menaquinone biosynthesis should cause loss of functionality only in the respiratory chain, as should mutations in structural or controlling genes for the cytochromes, flavoproteins, iron-sulfur proteins, or quinone-binding proteins.

Ubiquinone-deficient mutants of *E. coli* have been studied the most extensively for their aminoglycoside uptake properties (see reference 50). Bryan and Van den Elzen (21) showed that a *ubiD* mutant accumulated gentamicin and streptomycin poorly; Bryan and Kwan (17), using a *ubiA menA* double mutant, demonstrated that gentamicin uptake could be completely eliminated while maintaining normal transport of glutamine and proline and a normal $\Delta\psi$. Utilizing selection for low-level resistance to streptomycin, Muir et al. (89) isolated and characterized a *ubiF* mutant that was deficient in uptake of streptomycin and gentamicin, but not in proline transport. This strain had impaired respiration and could not grow on succinate as a carbon source (Suc^-); transductants to Suc^+ lost aminoglycoside resistance. However, a mutation altered in succinate dehydrogenase (*sdh*) was not aminoglycoside resistant (89); this suggests that electron transfer through ubiquinone, rather than overall succinate oxidation, may be associated with aminoglycoside uptake. Bryan's group (see references 7 and 21) has developed a model for aminoglycoside accumulation by *E. coli* that envisions a direct involvement of ubiquinone molecules in aminoglycoside movement across the cytoplasmic membrane, perhaps acting, in the ionized hydroquinol form, as negatively charged binding sites.

The strategy of Muir et al. (89), to select mutants blocked in aminoglycoside uptake by resistance to low levels of the antibiotic, has been successful in other systems. This approach is, however, likely to yield mutants altered in ribosomal binding sites, especially if resistance to streptomycin is sought (see Hancock [50] for a discussion of ribosomal Str^r mutations). Among small-colony *B. subtilis* mutants resistant to low concentrations of the aminoglycoside kanamycin

was found an *aroD* mutant conditionally deficient in the lipophilic electron transport component menaquinone (112). The Men^- and Kan^r phenotypes could be reversed by addition of shikimic acid (the product of the *aroD* enzyme in the common *aro* pathway). Accumulation of kanamycin (113) and gentamicin (114) was deficient in this strain when made Men^- by deprivation of shikimic acid. Interestingly, only 10% of the normally regulated menaquinone level (112) was sufficient to restore aminoglycoside accumulation rates and aminoglycoside sensitivity to that of $aroD^+$ (wild-type) strains (114). Miller and his colleagues have isolated a number of classes of gentamicin-resistant, small-colony mutants of *Staphylococcus aureus* (78). One mutant appeared to be a menaquinone auxotroph, since its growth and resistance phenotypes could be reversed with the menaquinone analog menadione. Again, resistance was correlated with deficiency in gentamicin accumulation, and uptake was restored by menadione supplementation. These results and those with *B. subtilis* suggest that, in gram-positive species (which do not possess ubiquinone), menaquinone may have a function in aminoglycoside uptake similar to that of ubiquinone in gram-negative species. However, experiments relating quinone content to respiration, $\Delta\psi$, and aminoglycoside uptake have not been carried out with the *Staphylococcus aureus* Men^- strain. In addition, the *B. subtilis aroD* (Men^-) strain (112–114) exhibits pleiotropic cytochrome deficiencies when made sufficiently Men^- to compromise aminoglycoside uptake. Such properties make detailed analysis difficult.

Over the past 30 years several reports have appeared on the use of aminoglycoside resistance to isolate hemin deficiency (*hem*) mutations in a variety of bacteria; these have included *E. coli* (8, 108), *Staphylococcus aureus* (118), and *B. subtilis* (5, 10). When deprived of hemin or δ-aminolevulinic acid, *E. coli hemA* mutants become generally cytochrome deficient and exhibit decreased capacity for aminoglycoside uptake (21, 22); *hem* mutants of *Staphylococcus aureus* and *B. subtilis* have not yet been studied for their antibiotic uptake properties.

As was pointed out by Hancock (50), the observed increase in MIC of streptomycin due to a *hemA* mutation (21) seemed modest compared with the complete lack of energy-dependent uptake. However, uptake by the *hemA* mutant was measured at a streptomycin concentration (4 μg/ml) below the MIC (8 μg/ml) in the absence of δ-aminolevulinic acid (i.e., Hem^-), but above the MIC (2 μg/ml) in the presence of δ-aminolevulinic acid (Hem^+). When Hem^-, this strain was resistant to growth inhibition by a number of other aminoglycosides, and when gentamicin uptake was measured at antibiotic concentrations above the MIC for the Hem^- state, no uptake was observed. Campbell and Kadner (22) measured streptomycin uptake by an independently isolated *hemA* mutant and found that uptake depended on a Hem^+ phenotype. In this study, the external streptomycin concentration was 33 μM (70 μg/ml); presumably, this value exceeded the MIC, although no data were given. In their report on the relationship of $\Delta\psi$ to aminoglycoside resistance, Damper and Epstein (30) reported that a third independently isolated *hemA* mutant had (when Hem^-) a 10-fold-higher MIC compared with its parent strain, although $\Delta\psi$ decreased only modestly, from −142 to −106 mV. These three studies illustrate several principles: (i) the MIC, although a meaningful clinical value, is not a useful endpoint when making inferences about cellular mechanisms of aminoglycoside uptake; (ii) a common set of mutants should be used to facilitate correlation of results from different laboratories; (iii) measurements of proton motive force and uptake should be made as part of the same set of experiments; (iv) even when some apparently essential components of the respiratory chain (e.g., in the above case, cytochromes) have been eliminated by mutational means, there appear to be mechanisms for maintaining $\Delta\psi$ and perhaps additional, parallel pathways for movement of aminoglycosides across the cytoplasmic membrane.

Experiments with *hem* mutants can provide information on the involvement of bacterial heme proteins in aminoglycoside uptake, but cannot specify which of the several cytochromes in an electron transfer pathway is crucial to the uptake process. To approach this question, mutants lacking individual cytochromes have been isolated by resistance to aminoglycosides. Using ethyl methane sulfonate mutagenesis, Bryan and his colleagues obtained *P. aeruginosa* strains multiply resistant to aminoglycosides (but not to other antibiotics) lacking either cytochrome c_{552} and nitrate reductase (19) or cytochrome *d* and nitrite reductase (16). Both types of mutants readily reverted to parental phenotype, suggesting involvement of single genes in the mutant phenotypes. In addition, the mutation causing cytochrome c_{552}/nitrate reductase deficiency was transducible and defined a locus, *aglA*, in *P. aeruginosa*. Uptake of gentamicin and streptomycin was impaired in the *aglA* mutant, but transport of several metabolites (amino acids, glucose, and spermidine) was normal. However, in the cytochrome *d*/nitrate reductase-deficient strain, reduced uptake of aminoglycosides was accompanied by decreased transport of cationic compounds such as lysine, arginine, and spermidine, as well as proline and glucose. Interestingly, glutamine transport was enhanced in this type of mutant.

In the gram-positive bacterium *B. subtilis*, mutations at the *strC* locus (111) give rise to nonribosomal resistance to streptomycin, without increased resistance to other aminoglycosides. *strC* strains are deficient in cytochrome aa_3 oxidase (112) and have been shown to accumulate streptomycin poorly (77). These mutants are not deficient in other cytochromes, although they exhibit elevated levels of cytochrome *d*, which presumably can function as a terminal oxidase in *B. subtilis*. Measurements of the membrane potential in intact cells showed that normal $\Delta\psi$ and oxygen consumption values were maintained in the face of the cytochrome aa_3 deficiency associated with *strC* (7; Arrow, Ph.D. thesis). Furthermore, transport of proline and glutamate was normal. The tentative conclusions drawn from these results were twofold: (i) rapid entry of streptomycin into *B. subtilis* requires the presence of functional cytochrome aa_3 in the cytoplasmic membrane; and (ii) in the absence of cytochrome aa_3, a normal $\Delta\psi$ can be established by electron flow to other terminal oxidases (cytochromes *o* and *d*), but $\Delta\psi$ by itself is insufficient to promote streptomycin accumulation.

Mutations at the *unc* locus in *E. coli*, which cause defects in the membrane-spanning H^+-ATPase, also have been associated with alterations in aminoglycoside susceptibility and rates of uptake. Both susceptibility and resistance have been associated with unique *unc* mutations (91), but as yet there is no hint of the mechanism by which modification of the various subunits of H^+-ATPase might change uptake rates. The review by Hancock (50) should be consulted for a summary of *unc* mutants and their aminoglycoside susceptibility phenotypes. Finally, *E. coli eup* (energy-uncoupled phenotype) mutants should be mentioned, since they are resistant to aminoglycosides (103). These mutants have normal electron transport and ATP activities and undimin-

ished Δp. Kinetic studies revealed that the normal *eup* gene product functions in regulating lactose carrier activity and proline transport under deenergized conditions (103), and *eup* mutations appear to mediate an aberrant form of the proposed regulation (103). Since Δp is required for aminoglycoside uptake, Plate and co-workers have postulated that *eup* regulation transcends transport function and applies generally to Δp-coupled processes. If indeed aminoglycoside uptake is "coupled" to Δp, this *eup* system may provide new insight into the mechanism of the energy-transducing process which regulates aminoglycoside uptake.

Physiological modification of the respiratory chain. In *strC*$^+$ (i.e., wild-type) *B. subtilis* strains, cytochrome aa_3 concentrations can be altered in a systematic way by manipulation of the growth medium (7, 77). Cultivation of *strC*$^+$ strains in minimal salts medium with glucose as carbon source induces a nonmutational StrC$^-$ phenotype; i.e., the cells become cytochrome aa_3 deficient and resistant to growth inhibition by streptomycin and exhibit decreased cellular accumulation of streptomycin (77). This phenotype can be reversed by supplementation with amino acid mixtures (e.g., vitamin-free Casamino Acids [Difco Laboratories]). As with the cytochrome aa_3 deficiency in *strC*$^-$ strains, this physiologically induced StrC$^-$ phenotype does not cause a decrease in Δψ (7), yet streptomycin is not accumulated efficiently in the absence of cytochrome aa_3. These findings support the proposal of Bryan and Kwan (17) that electron flow through specific segments or components of the respiratory chain is necessary for aminoglycoside accumulation, in addition to development of a sufficient Δψ across the cytoplasmic membrane. The data from studies on cytochrome aa_3 in *B. subtilis* suggest that the uptake role played by molecular components of the electron transport chain may be specific to a particular aminoglycoside or structural class of aminoglycosides. They also stress the importance of careful definition of the physiological state of bacterial cell preparations that are to be used in aminoglycoside uptake experiments, since regulation of formation of electron transport pathways is highly responsive to growth medium composition and other physiological parameters.

Comparison of Aminoglycoside Uptake and Other Transport Systems

The requirement by an uptake system for a functional electron transport chain is unusual. Transport systems can be energized by Δp, they can be ATP dependent, or they can depend on group translocation mechanisms (56). None of the transport systems energized by these means are as sensitive to inhibitors of oxidative metabolism as is aminoglycoside uptake, despite the fact that Δp and ATP formation often require electron transfer reactions. The proposal (referred to in several places above) that a component of the electron transport chain itself may function as a carrier or as a facilitator in the transmembrane movement of aminoglycosides is reminiscent of the early model by Kaback and Barnes for solute transport in *E. coli* (64). According to this model, electron transfer leads to changes in oxidation state of a redox group in the carrier, which in turn causes conformational and affinity changes at solute-binding sites. In the oxidized form, the carrier has a high affinity for its solute; in the reduced form, it has low affinity. As applied to aminoglycoside uptake, it is possible that quinones sense the redox environment, altering the oxidation-reduction state of the remaining components of the electron transport chain. One or more components may then act in a direct or indirect role in aminoglycoside uptake, either as a carrier or by activating a carrier, respectively. The model need not specify whether quinones function as "translocators" or "facilitators" in aminoglycoside translocation. The redox state of the carrier or facilitator of transport may be determined by the Δp across the cytoplasmic membrane and the redox potential of the environment. In the absence of Δp (or more properly, Δψ), binding affinity would be low, and in the presence of Δp (above the threshold necessary to initiate aminoglycoside uptake) binding affinity would be high. Perhaps aminoglycoside uptake requires the quinone-linked redox reactions within the respiratory chain, but these may themselves be regulated by the magnitude of Δψ. Inhibition of electron transport or dissipation of Δψ may disrupt the local redox environment of the electron transfer chain, blocking uptake of aminoglycoside.

The lack of direct evidence that electron transfer components or their redox intermediates act as aminoglycoside transporters (or create an environment suitable for transport), together with evidence pointing to chemiosmotic energy coupling, does not favor the model discussed above. Furthermore, although the role of Δp as a driving force for solute transport in bacteria is thought to be well established, recent results have been presented that cannot readily be placed in the chemiosmotic context. These indicate that other mechanisms of energy coupling could be involved in solute transport (40, 42). Konings and colleagues provided evidence that a direct interaction can exist between solute carriers and the electron transfer system and that redox transitions in the lactose carrier protein take place during the solute translocation step (39, 40); this proposal is a modification of the original hypothesis outlined by Kaback and Barnes (64). Elferink et al. (40) have suggested a role for sulfhydryl groups during this process; it would be of interest to determine whether alteration of sulfhydryl redox state could modify aminoglycoside transport in respiration-inhibited cells. The implications of these findings to aminoglycoside uptake are unclear; however, alternatives outside of the chemiosmotic paradigm may be applicable. The development of vesicle systems functional in aminoglycoside uptake (17, 117) should provide the means to address nonchemiosmotic alternatives, as has been necessary in the critical and detailed analysis of the energetics of lactose accumulation in *E. coli* (40, 42).

Relationships between Δψ and Uptake

Protonophores such as DNP and CCCP dissipate (or "short circuit") the proton motive force by providing a transport pathway for protons to leak across the membrane, thus disrupting the normal proton-impermeable state of the membrane. Consequently, uncouplers inhibit transport systems coupled to Δp, but have little or no effect on cell respiration. Based on the inhibition of streptomycin and gentamicin uptake by DNP and CCCP, Δp has been proposed as the driving force for aminoglycoside uptake (6, 20, 22, 78). Which component (Δψ or ΔpH) contributes to uptake? At a medium pH of >7.5, ΔpH does not contribute effectively to Δp, but since aminoglycosides are highly effective inhibitors at pH values of >7.5, it has been suggested that the Δψ (interior negative) component of Δp provides the driving force (22). In intact bacteria, a requirement for an energized membrane initially was suggested by the observation that a proton uncoupler blocked aminoglycoside uptake and killing (6). Strong correlations between the magnitude of the Δψ component of Δp and aminoglyco-

side susceptibility have been experimentally documented under a variety of conditions (22). The cumulative data suggest that a reduction of $\Delta\psi$ diminishes aminoglycoside uptake, resulting in the manifestation of an aminoglycoside-resistant phenotype. Several groups have demonstrated that effective aminoglycoside uptake is related to the magnitude of $\Delta\psi$ in *Staphylococcus aureus* (43, 78), *E. coli* (17, 30), and *B. subtilis* (17, 77); however, specific electron transport components may provide a more direct role in aminoglycoside uptake under conditions of high $\Delta\psi$ as discussed in the preceding section. Under certain conditions or in specific mutants, $\Delta\psi$ may be the major determinant of aminoglycoside resistance.

In a series of elegant and detailed studies, Miller and co-workers have provided a strong foundation for a relationship between aminoglycoside uptake and $\Delta\psi$ in *Staphylococcus aureus* (38, 71, 74). Inhibition of aminoglycoside uptake by CCCP in wild-type *Staphylococcus aureus* suggested that a proton motive force is required for aminoglycoside uptake (78). Under conditions of acidic pH, ΔpH across the plasma membrane of *Staphylococcus aureus* could be increased to about –100 mV; in response to this, $\Delta\psi$ declined to approximately −90 mV. Mates et al. (74) observed that, by reducing the pH of the medium, gentamicin uptake and killing were abolished, despite the maintenance of a significant $\Delta\psi$.

Ionophores were utilized to manipulate the contributions of ΔpH and $\Delta\psi$ to Δp. The antibiotic ionophore nigericin catalyzes the electrically neutral exchange of H^+ for K^+ (Na^+), collapsing ΔpH with a compensatory increase in $\Delta\psi$ under certain conditions (106). Addition of nigericin to *Staphylococcus aureus* cells at pH 5.0 resulted in a dramatic increase in the rate of gentamicin uptake, a decrease in cell viability and an increase in $\Delta\psi$ (71, 74). Under these conditions, aminoglycoside uptake was comparable to that at pH 6.0; however, killing was far less than at higher pH values. The ionophore valinomycin specifically increases membrane permeability to K^+, resulting in the collapse of $\Delta\psi$ in the presence of adequate K^+ (106). Under conditions of neutral pH and 50 to 100 mM K^+, addition of valinomycin to *Staphylococcus aureus* abolished $\Delta\psi$, and with it gentamicin uptake and killing (43, 74). The studies from Miller's laboratory imply that a threshold $\Delta\psi$ of −80 to −90 mV is required to initiate aminoglycoside uptake; similar results have been obtained with *E. coli* and *B. subtilis* (17). Gilman and Saunders (43) confirmed the findings of Mates et al. (74) with *Staphylococcus aureus* and also showed that the threshold $\Delta\psi$ required to initiate uptake varies with the gentamicin concentration.

An interesting observation during the initial stages of the studies conducted by Miller and his colleagues was that 20 to 120 μM *N,N'*-dicyclohexylcarbodiimide (DCCD), which nonspecifically inactivates the ATPase complex as well as other proton pumps, did not inhibit but increased gentamicin uptake in wild-type strains of *Staphylococcus aureus* (80). It has been suggested that DCCD increases gentamicin uptake and killing by increasing the magnitude of $\Delta\psi$ (43, 71) (it is of note that the aminoglycoside-hypersensitive *E. coli unc* mutants do not have elevated $\Delta\psi$ values, but do have enhanced respiratory activity and uptake [91]). The mechanism by which DCCD might enhance $\Delta\psi$ and aminoglycoside uptake in *Staphylococcus aureus* is unknown. One explanation proposes that, in the presence of DCCD, energy normally utilized for ATP synthesis may be available for gentamicin uptake (38, 43). Eisenberg et al. (38) exploited the "DCCD effect" to establish a quantitative association between $\Delta\psi$ and gentamicin uptake and killing in *Staphylococcus aureus*. This strategy has provided supporting evidence that the initiation of aminoglycoside uptake is directly dependent on the magnitude of $\Delta\psi$. Under anaerobic conditions or in the presence of 0.2 mM KCN, DCCD shows no stimulation of gentamicin uptake or bactericidal effect (88). It is intriguing that the effects of DCCD in increasing $\Delta\psi$ and gentamicin uptake are unique to *Staphylococcus aureus*. This may again suggest that physiological and architectural features cause important differences in the mechanisms of aminoglycoside uptake among different genera of bacteria.

The quantitative relationship between $\Delta\psi$ and gentamicin uptake in *Staphylococcus aureus* suggested that deficient uptake under anaerobic conditions was due to low $\Delta\psi$ (75). It is known that anaerobically growing *Staphylococcus aureus* cells generate a $\Delta\psi$ well above the threshold value necessary to initiate aminoglycoside uptake (65, 75). Under anaerobic conditions in the absence of alternate terminal electron acceptors, aminoglycoside uptake and killing are absent in *Staphylococcus aureus* (a situation that has been thought to obtain for *E. coli*, but see below). The $\Delta\psi$ in anaerobically growing *Staphylococcus aureus* remains at a constant level (−140 mV), while for *E. coli* the value is approximately −122 mV (67). As a technical note, when measuring $\Delta\psi$ by distribution of the lipophilic tetraphenylphosphonium cation, care must be taken to ensure that the probe reaches the cytoplasmic membrane. If the outer membrane is not disrupted by a sufficiently high concentration of ethylenediaminetetraacetic acid, $\Delta\psi$ may appear to be low or absent (27, 66). An additional caveat should be raised concerning experiments with anaerobic cultures of facultative species of bacteria. Muir et al. (88) discovered that anaerobic chemostat cultures of *E. coli* accumulated streptomycin at rates equivalent to aerobic cultures, but only after a 1.5-h induction period. Since CCCP abolished this anaerobic uptake, it appeared to be $\Delta\psi$ dependent. Most aminoglycoside uptake experiments are carried out in time periods shorter than that used by Muir et al., and some conclusions drawn from previous studies may need revision.

In an effort to increase $\Delta\psi$ and thereby aminoglycoside uptake, anaerobic *E. coli* and *Staphylococcus aureus* cells have been treated with nigericin. In *E. coli*, the ionophore caused an increase in $\Delta\psi$ without an accompanying increase in uptake (17), while in *Staphylococcus aureus* both $\Delta\psi$ and uptake increased (75). However, in the latter study the maximal anaerobic uptake of gentamicin after nigericin addition averaged only 18% of the maximal aerobic uptake despite a rise in anaerobic $\Delta\psi$ to match aerobic $\Delta\psi$ values. Thus, although $\Delta\psi$ is related to gentamicin uptake and killing, there must be additional factors that modify the relationship (75). This contention is supported by the observation that, although nitrate addition to anaerobically growing *E. coli* stimulates electron transport and induces aminoglycoside uptake, the level attained is substantially below that obtained under aerobic conditions (22). Aminoglycoside uptake may require not only a threshold $\Delta\psi$, but also a critical intracellular environment which is not established during anaerobic growth (75).

RELATIONSHIP OF ENZYMATIC INACTIVATION OF AMINOGLYCOSIDES TO THEIR UPTAKE

Clinically important aminoglycoside resistance has been examined in several organisms of medical significance and determined to result primarily from the enzymatic modification of these antibiotics (120). Modifying enzymes appear to consist of three classes: *N*-acetyltransferases, *O*-adenyl-

yltransferases, *O*-nucleotidyl transferases, and *O*-phosphotransferases (a detailed discussion of these enzymes and their nomenclature is given in a recent review by Bryan [14]). Genes for these enzymes frequently are carried on plasmids. Several different antibiotics may be modified by a single enzyme, and the distribution of resistance-bearing (R) plasmids is widespread. Recently, a bifunctional enzyme exhibiting both *N*-acetyl- and *O*-phosphotransferase activity in *S. faecalis* has been characterized (41).

Kinetic Characteristics of Aminoglycoside Uptake in Bacteria Carrying Resistance Plasmids

Enzymatic modification of aminoglycosides diminishes the affinity of these drugs for their ribosomal targets (9). Bryan and co-workers examined R-plasmid-carrying (R^+) strains of *E. coli* and demonstrated that these strains exhibit only type EDPI uptake kinetics, similar to those seen in strains bearing chromosomal mutations (*rpsL*) conferring ribosomal resistance (16, 20, 21, 36). The observation of Hurwitz et al. (61) that puromycin enhances the rate of streptomycin accumulation in both $rpsL^+$ and $rpsL^-$ strains was extended in studies with R^+ *Staphylococcus aureus*. As discussed earlier, Miller and co-workers demonstrated that DCCD could enhance gentamicin uptake in sensitive and resistant isolates, presumably due to an elevated $\Delta\psi$ (71). In both cases (*E. coli rpsL* and *Staphylococcus aureus* R^+ strains), increased rates of uptake were not associated with killing. Gilman and Saunders (44) confirmed the effects of DCCD on R^+ *Staphylococcus aureus* and also showed that puromycin can stimulate uptake of gentamicin in *Staphylococcus aureus*. These results demonstrate that the resistance phenotype of R^+ organisms can be dissociated from the reduced rate of antibiotic uptake and that artificial enhancement of uptake does not reverse the resistance phenotype.

The nature of the enzyme-substrate interaction is critical to determining whether or not a modifying enzyme will confer clinically significant resistance to an aminoglycoside (11, 12, 105). An *O*-2″-nucleotidyltransferase enzyme from *Serratia marcescens* was shown to confer resistance to gentamicin and tobramycin, while the purified enzyme was capable of modifying not only these aminoglycosides but also netilmicin. Susceptibility of this strain to netilmicin was the result of a significantly higher K_m of the enzyme for netilmicin than for gentamicin or tobramicin (35). Other workers have implicated the ratio K_m/V_{max} in deciding whether a modifying enzyme would yield significant resistance to a given antibiotic.

Model for Aminoglycoside Resistance in R^+ Strains

Based on their findings, Bryan and co-workers put forth a model to describe the interactions required for resistance (36). The model posits a competition between the rate of enzymatic inactivation of a substrate and the initial rate of uptake of that substrate during EDPI. As ribosome binding is required for EDPII, and modification of aminoglycoside targets renders these drugs incapable of binding to ribosomes, a high rate of modification would prevent the onset of EDPII. Conversely, enzymes with low affinities for a particular aminoglycoside, or a low velocity of modification, might not be able to confer resistance except at very low external drug concentrations (14, 36, 100). For therapeutic purposes, the ability to overcome enzymatic resistance requires an understanding of all phases of the interactions among aminoglycosides, cells, and the appropriate modifying enzymes, as well as of regulatory elements controlling R-gene expression, and the nature of the vectors that propagate and transmit these elements. Treatments that enhance the rate of EDPI would increase the likelihood of positive therapeutic results.

Recently, Perlin and Lerner (100) described a new and clinically relevant induction of amikacin resistance by a phosphotransferase enzyme, *O*-3′-phosphotransferase II, of *E. coli*. This enzyme is normally able to utilize amikacin poorly as a substrate. However, selection of a general aminoglycoside transport deficiency with gentamicin, a drug not recognized by *O*-3′-phosphotransferase-II, results in significant amikacin resistance. This appears to be the consequence of a reduction of transport to a level that now permits a higher level of drug modification by the enzyme. The authors correctly assess the importance of this finding, as amikacin is a drug that is used sparingly to prevent the selection of resistant isolates. Such isolates may now appear due to the use of other aminoglycosides in the absence of amikacin exposure.

DEPENDENCE OF AMINOGLYCOSIDE UPTAKE ON PHYSIOLOGICAL STATE OF THE CELL

It is possible to extrapolate from the findings (discussed above) on physiological regulation of cytochrome aa_3 biosynthesis in *B. subtilis* and its relationship to streptomycin uptake to suggest that general cellular regulatory systems will influence bacterial uptake of aminoglycosides. These controls might operate by controlling the concentration of a specific membrane component (such as cytochrome aa_3 or lipophilic quinones) or might act at several points in cellular metabolism to control the rate of growth of the cell. We consider the latter possibility first and then discuss the effects of global regulatory systems such as the stringent response and catabolite repression.

Growth Rate Effects

Bacterial cells must be growing for streptomycin to cause loss of viability; in fact, the higher the growth rate of cells at the time of antibiotic addition, the greater the growth-inhibitory effect (summarized in reference 90). Utilizing continuous-culture techniques, Muir et al. (90) examined the influence of growth rate on streptomycin accumulation. Uptake velocity was found to be proportional to growth rate at the time of antibiotic addition, for both *E. coli* and *Bacillus megaterium*. Although accumulation rates for both rapidly and slowly growing cultures increased with increasing extracellular streptomycin concentrations, the ratio of accumulation rates for the two growth rates remained approximately equal. Measurements of efflux rates from streptomycin-loaded cells showed very little effect of growth rate on this parameter; furthermore, studies with an *E. coli rpsL* mutant showed no EDPII uptake, confirming earlier work in batch culture (20) and demonstrating that antibiotic-sensitive ribosomes are required for rapid (EDPII) uptake.

In a subsequent publication, Muir et al. (88) described experiments that considered whether the higher respiration rates associated with rapidly growing, carbon-limited chemostat cultures could be responsible for the high rates of streptomycin uptake accompanying high dilution rates, as seen in the initial study (90). By growth of chemostat cultures under phosphate, rather than carbon, limitation, they were able to dissociate respiration rate from growth rate: a culture growing with a dilution rate (*D* value) of 0.25

h^{-1} had essentially the same respiration rate as a culture with a D value of 0.6 h^{-1}. Despite similar respiration rates, the more rapidly growing culture accumulated streptomycin at a rate of at least fourfold higher than the slowly growing culture. Furthermore, when *ubi*$^+$ and *ubi* strains growing at identical D values were compared for respiration and streptomycin uptake, the latter rates were identical, but respiration by the *ubi*$^+$ strain was 1.7 times that of the *ubi* strain. The experiments of Muir et al. (88, 90) raise several important issues. If the rate of streptomycin uptake is dependent on growth rate (or a property of the cell, such as ribosome content, that is proportional to growth rate) rather than on respiration rate, then the importance of electron flow through the respiratory chain would seem to be less than previously thought. Also, the decreased aminoglycoside accumulation associated with lipophilic quinone deficiency in *ubi* strains may be a consequence of the decreased rate of growth exhibited by these strains, rather than a direct effect of compromised electron flow through the quinone-specific portion of the respiratory chain.

Perhaps a reconciliation can be attempted in the following way. There are at least two important components to the aminoglycoside accumulation system: (i) sensitive ribosomes, which may interact with the antibiotic during EDPI to generate a product (mistranslated protein?) that increases the permeability of the cytoplasmic membrane to the aminoglycoside; and (ii) the membrane-bound respiratory chain, which may be important to both generate a minimum $\Delta\psi$ across the membrane and provide elements of a transmembrane pathway (a "transport system") for aminoglycoside entry to the cell. These two components (sensitive ribosomes and respiratory chain) have been identified qualitatively as the minimum requirements for aminoglycoside entry without significant kinetic lag. However, the quantitative contributions of the two components have not been assessed. For example, under the conditions used by Muir et al. (88), ribosome content and not respiration could have been rate limiting for accumulation, and only a portion of electron flow through the respiratory chain might have been utilized to drive uptake. Similarly, the correlation of rates of uptake by *ubi*$^+$ and *ubi* strains with growth rather than quinone content must be examined in light of the fact that the *ubi* strain had been made partially Ubi$^+$ by biosynthetic precursor feeding and had a ubiquinone content 12% of that of the wild type. If only a portion of the ubiquinone pool is involved in streptomycin accumulation, and this portion is relatively unaffected by quinone limitation down to a level of, perhaps, 10% (as is clearly the case of menaquinone in *B. subtilis* [114]), then under the growth conditions used by Muir et al. (80) ubiquinone was not rate limiting for streptomycin accumulation (but, presumably, ribosome content was limiting). Similarly, batch culture studies of *ubi* strains by other investigators (e.g., references 17 and 21), in which mutants were made partially Ubi$^-$ by removal of precursor for several generations and lost capacity for gentamicin uptake, could be interpreted to mean that during the slowed growth period there was a progressive dilution of ribosomes as well as of ubiquinone and that the former became rate limiting before the latter. However, there is a minimum ribosome content of bacterial cells that will be maintained under ubiquinone precursor limitation, while ubiquinone content can be diluted to a very low level and become rate limiting. This may be the condition achieved by Bryan and Kwan (17), in which growth of an *E. coli ubi men* strain for two generations without precursor allowed retention of gentamicin accumulation at 25% of normal, but precursor limitation for two additional generations completely eliminated accumulation.

Global Regulatory Mechanisms

The influence of the global cellular regulatory mechanisms catabolite repression and stringent regulation on aminoglycoside accumulation were discussed by Hancock (50, 51). We will reiterate the information available to 1981 only briefly and concentrate on more recently published data. Resistance of *Salmonella typhimurium cya* and *crp* mutants to streptomycin (and several other antibiotics) was used by Alper and Ames (4) in a positive selection scheme for mutant isolation and suggested that the transport systems for these compounds were under catabolite repression control. Höltje (58) used direct uptake measurements to show that streptomycin accumulation by *cya*$^+$ *E. coli* was decreased in the presence of glucose and that in a *cya* strain accumulation was limited even in the absence of glucose. This latter limitation could be lifted by addition of cyclic adenosine 5′-monophosphate. As discussed by Hancock (50), many energy-linked functions of the cell envelope are under catabolite repression, and it is difficult to identify which of these might be responsible for the uptake-repressed phenotype. Catabolite repression is exhibited even in bacterial species that do not have cyclic adenosine 3′, 5′-monophosphate; one such species is *B. subtilis*. In this organism, glucose has the effect (among others) of repressing the formation of cytochromes aa_3, *b*, and *c*. In view of the association of cytochrome aa_3 with streptomycin accumulation in *B. subtilis* (see above), it is not surprising that glucose-grown cells show depressed streptomycin uptake as well as increased resistance (77). Furthermore, cytochrome aa_3-deficient (*cta*) *B. subtilis* mutants can be isolated by selection on relatively low concentrations of streptomycin (J. P. Mueller and H. W. Taber, unpublished). At least in this organism, catabolite repression of aminoglycoside uptake may be mediated through specific effects on cytochrome formation.

A complex relationship between stringent regulation and streptomycin action was identified many years ago by Sakai and Cohen (107; see Hancock [50, 51]). Streptomycin blocked the stringent response in a *rel*$^+$ *E. coli* strain: guanosine-3′-diphosphate-5′-diphosphate (ppGpp) did not accumulate, nor was ribonucleic acid synthesis shut off following deprivation of a required amino acid. Also, an isogenic *rel* strain was 10-fold more susceptible than the *rel*$^+$ strain to killing by streptomycin under stringent conditions, and this was attributed to enhanced streptomycin uptake by the *rel* strain. Höltje (59) used the same pair of isogenic strains to confirm this result, but with additional experiments showed that the interpretation should be that uptake in the *rel*$^+$ strain was depressed by amino acid starvation; i.e., it is subject to stringent control. By contrast with the effects of streptomycin on *E. coli*, in *B. subtilis* the antibiotic had the effect of inducing, rather than blocking, a stringent response in a *rel*$^+$ strain (57), as measured by both cessation of ribonucleic acid synthesis and transient accumulation of ppGpp and guanosine-3′-diphosphate-5′-triphosphate (ppGppp). An isogenic *rel* strain did not show this response. Tetracycline did not induce the response in the *rel*$^+$ strain, but pretreatment with tetracycline blocked subsequent induction of the stringent response by streptomycin. Although no uptake data were included, this study underscores the complexity of aminoglycoside interaction with ribosomes and the range of metabolic and macromolecular effects that need to be considered to understand the series of amino-

glycoside-induced events that results in rapid uptake and eventual lethality.

CODA: BIOENERGETIC ENIGMA

Perhaps the key challenge to the field of aminoglycoside uptake research is the nature of the energy source driving movement of aminoglycosides across the cytoplasmic membrane. Whether a specific role exists for electron transport in the uptake of aminoglycosides beyond its requirement in the formation of $\Delta\psi$ is still controversial. A wealth of information with a variety of bacterial hosts supports a relationship between aminoglycoside uptake and the magnitude of $\Delta\psi$ at pH values where $\Delta p = \Delta\psi$. This conclusion is reinforced by studies utilizing conditions (changes in external pH and treatment with ionophores) that can change the energetic state of the plasma membrane. The initial experiments by several investigators utilizing reagents such as cyanide to block respiration and aminoglycoside uptake suggested that respiration was the energy source responsible for driving movement of aminoglycosides across the membrane. Whether electron transport chain components such as quinones or cytochromes act as carrier molecules or the chain acts indirectly by generating $\Delta\psi$ or as a link with a redox-sensitive transport system remains an unsolved problem. It is intriguing to consider that, despite decades of intensive work, the mechanism by which lactose transport in *E. coli* is energized has found no refuge from controversy. It is still unclear whether Δp, cyclic electron transfer, or a combination of both energizes this transport (cf. references 39 and 42). Questions about how a cell energizes lactose transport or aminoglycoside uptake continue to rely on the basic premises of the chemiosmotic theory, and the appropriateness of this reliance is unsure.

The proposal (30) that $\Delta\psi$ provides the driving force for aminoglycoside uptake developed out of electron transport inhibition studies carried out in several laboratories in the 1960s, during the period when the chemiosmotic theory was being elaborated. In an effort to dissociate electron transfer from the generation of Δp by electron transfer coupled to a functional H^+-ATPase, the effects of uncouplers were examined, and the results favored an association between loss of the membrane potential and inhibition of aminoglycoside uptake and killing. These studies implied that membrane energization associated with active aminoglycoside accumulation required electron transport exclusively for the generation of Δp. Surprisingly, if one measures the $\Delta\psi$ of *E. coli* cells treated with sufficient cyanide (~2 mM) to completely block respiration, $\Delta\psi$ values are maintained well above the documented threshold to initiate aminoglycoside uptake as determined by Bryan and Kwan (17). Despite an adequate $\Delta\psi$, cyanide completely blocks aminoglycoside uptake. The values of $\Delta\psi$ obtained under these conditions occur in a range (−120 to −150 mV) in which the measurement of tetraphenylphosphonium cation accumulation is precise (42). Unfortunately, measurements of $\Delta\psi$ have not been part of the experimental design in many studies examining the effect of respiratory inhibitors on aminoglycoside uptake. The use of uncouplers to decipher the energy requirements for aminoglycoside transport also occasions difficulties in interpretation. Uncouplers permeabilize the membrane to protons, functionally dissociating the H^+-ATPase from the electron transport chain; whether uncouplers have any undetected nonspecific effects on membrane topology or integrity is uncertain. CCCP, DCCD, and DNP diminish $\Delta\psi$ (Δp) to zero at pH 7.65. Under these conditions, aminoglycoside uptake is inhibited without affecting respiration. The results obtained with uncouplers immediately suggest that only $\Delta\psi$ is required to drive aminoglycoside uptake, and respiration is necessary for the sole purpose of generating a threshold $\Delta\psi$. Critical evaluation is limited in most analyses, since experiments demonstrating normal respiration in the presence of uncouplers often are not presented.

The paradox, then, appears to be the following: conditions that block respiration (e.g., cyanide) with little effect on $\Delta\psi$ inhibit aminoglycoside uptake and killing, while conditions that diminish $\Delta\psi$ (e.g., CCCP) with little effect on respiration also inhibit aminoglycoside uptake. The conclusion drawn previously, in the last critical review of this topic (50, 51), suggested a dual requirement in aminoglycoside uptake for both a threshold $\Delta\psi$ and electron transfer. The quantitative contribution of one or the other to aminoglycoside uptake may be exquisitely sensitive to the surrounding environment and to the hierarchy of physiological controls in a particular organism. Recent evidence, however, suggests an explanation amenable to experimental analysis: Kinoshita et al. (68) have provided preliminary evidence that *E. coli* can grow in the absence of a proton motive force in synthetic medium with glucose as an energy source at pH 7.5, where $\Delta p = \Delta\psi$. Addition of 50 μM CCCP to growing *E. coli* results in cessation of growth. After 30 min (depending on the strain), the cells resume exponential growth with an extended generation time and with a glucose dependence. In the presence of 50 μM CCCP, $\Delta\psi$ is dissipated almost completely, since cells growing in the presence of CCCP fail to accumulate proline. As stated by the authors, the results do not argue against the chemiosmotic theory, but only suggest that energization of transport systems by $\Delta\psi$ is not obligatory for growth of *E. coli*. Formally, this seems to require that *E. coli* have alternate means for energy transduction consistent with growth.

It is clear that a number of factors determine susceptibility to aminoglycosides. Miller and Dougherty (personal communication), have applied the work of Kinoshita et al. (68) to the analysis of energized aminoglycoside uptake. Miller and Dougherty have demonstrated that *E. coli* cells growing in an enriched broth containing CCCP lack a detectable $\Delta\psi$, but that aminoglycoside uptake and killing were observed after the lag period in growth following addition of CCCP. The ability of cyanide to inhibit uptake in CCCP-treated cells was not examined, although previous studies from this group have suggested that $\Delta\psi$ regulates uptake of aminoglycosides. Miller and Dougherty suggest that, when $\Delta\psi$ is dissipated with CCCP, *E. coli* has secondary pathways which permit growth and uptake of aminoglycosides. The role of electron transfer or ATP or both awaits scrutiny as potential alternate sources of energy responsible for driving aminoglycoside uptake in CCCP-treated *E. coli*. Aminoglycoside transport system(s) may be energized by ATP-dependent systems with additional involvement from the proton motive force. These observations provisionally explain the rapid dissipation of aminoglycoside uptake by *E. coli* after the addition of CCCP; *E. coli* seems to require an adaptive phase for activation of alternate pathways of energy transduction.

The uncharacteristic nature of the energetics of aminoglycoside uptake, in part, may be attributed to the variety of systems investigated or to difficulties involved in the measurement of the different parameters ($\Delta\psi$, respiration, and aminoglycoside uptake) or to both. To provide continuity in future analysis of the energetics of aminoglycoside uptake, $\Delta\psi$, respiration rate, and antibiotic uptake should be recorded simultaneously and continuously under conditions in

which Δp is composed only of $\Delta\psi$. For *E. coli*, Δp can be maintained in the absence of electron transfer (39). This allows one to separate the direct role of electron transfer from its indirect role via generation of $\Delta\psi$. Conditions which progressively inhibit respiration by the addition of increasing amounts of potassium cyanide, when compared with those which progressively titrate $\Delta\psi$ by controlled addition of protonophores, may provide insight into the unusual energetic requirements of aminoglycoside uptake. The ability to generate and vary a potassium/valinomycin-induced diffusion potential under anaerobic conditions also provides an opportunity to investigate the contribution of $\Delta\psi$ in the complete absence of electron transfer (34). Critical analyses of this nature will verify whether a unique (linear) relationship exists between aminoglycoside uptake and either $\Delta\psi$ or electron transfer. Such an approach has been extremely successful in evaluating the energetics of lactose accumulation in *E. coli* (40, 42).

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