Mechanism of Bactericidal Action of Aminoglycosides

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INTRODUCTION

Since the discovery of the first aminoglycoside antibiotic, streptomycin (Str), in 1944, the mechanism of action of this group has been studied more extensively, and in more laboratories, than that of any other. The results have revealed a remarkably pleiotropic set of effects (reviewed in references 20, 27, 70, and 74), of which four appear to be especially important: ribosomal blockade, misreading in translation, membrane damage, and irreversible uptake of the antibiotic. In contrast to our understanding of the action of the β-lactams and many other antibiotics, investigators of the aminoglycosides have struggled with a succession of hypotheses that could account for some observations but were contradicted by others. In particular, the link between action on the ribosome and membrane damage was obscure.

About 10 years ago this problem became accessible to study, with the demonstration that bacteria, like eucaryotic cells, possess membrane-bound ribosomes which are involved in protein translocation into or across the membrane (19, 60, 65). Nevertheless, the relation of this process to membrane damage by aminoglycosides was ignored until very recently, when it was proposed that incorporation of misread protein in the membrane impairs its integrity, derived its support from its ability to explain a large number of already known features of aminoglycoside action. This review will now examine, in greater detail than was appropriate in the preceding experimental paper, the ability of the proposed model to accommodate all major features of aminoglycoside action.

The path to this model was exceptionally tortuous, and it involved a feedback to a set of findings that had been virtually buried 25 years earlier. It therefore seems of historical and philosophical interest to review the discovery of various aspects of aminoglycoside action and the resulting hypotheses and their problems, and also to discuss possible reasons for the delay in recognizing that membrane damage is central and is related to misreading. I will also review evidence for a surprising additional conclusion that emerged from the model: the release of incomplete proteins by puromycin, though not lethal, affects membrane integrity in the same way as random misreading induced by Str. Finally, I will consider further possible tests for the model and will discuss its implications for the structural requirements of normal membrane proteins.

The voluminous literature describes many additional effects of aminoglycosides, including alterations in the synthesis of deoxyribonucleic acid, ribonucleic acid (RNA), and polyamines and in the levels of cyclic adenosine monophosphate and guanosine tetraphosphate. However, such changes might be expected as secondary consequences of the several major metabolic perturbations caused by Str: inhibition of protein synthesis (and consequent accumulation of ribosomal RNA), accumulation of misread protein, altered membrane composition and permeability, and resulting alterations in ionic composition. Since there does not appear to be evidence that any of the observed additional effects are primary responses to the antibiotics, I will not review this literature. However, it should be noted that a recent review


FIG. 1. Uptake of \(^{14}C\)Str (SM). Labeled Str (40 \(\mu g/ml\)) was added to an exponentially growing shaking culture in minimal medium and to two similar cultures (labeled CE) to which chloramphenicol (20 \(\mu g/ml\)) had been added 2 min earlier. At the time indicated by the arrow, one CE culture received 0.02 ml of toluene per ml of culture. From reference 4, with permission.

of aminoglycoside action (J. E. Davies, ASM News 52: 620–624, 1986) differs markedly from this one in its selection of key effects.

Since the present review covers such a long history, the references are largely restricted to those papers that initially reported each phenomenon or clarified it substantially. Such a selection is inevitably somewhat subjective, and it has no doubt omitted a number of significant contributions. In addition, there is a growing literature on the molecular interactions of aminoglycosides with ribosomes and even more on aminoglycoside uptake (which is comprehensively reviewed elsewhere \([32, 33]\) and in H. W. Taber, manuscript submitted), but these topics will not be reviewed, except insofar as they contribute to understanding the membrane damage and the bactericidal process.

The various aminoglycosides will not be treated separately, because all appear to have the same fundamental mechanism of action, though they vary in certain respects (the number of molecules bound per ribosome, specificity of ribosomal resistance mutations, and susceptibility to inactivation by various resistance enzymes). Moreover, though the aminoglycoside antibiotics are also classified chemically as aminocyclitol, it is not useful to include in the aminoglycoside family of antibiotics those aminocyclitols (e.g., spectinomycin) that do not cause misreading and are not bactericidal.

### PROTEIN SYNTHESIS AND MEMBRANE DAMAGE

The earliest studies on Str showed that it is bactericidal, like penicillin, but instead of causing cell lysis it halts protein synthesis rapidly and irreversibly and leaves the cell grossly intact. Paradoxically, this irreversible action was found to require protein synthesis, since it was prevented by a reversible inhibitor, chloramphenicol (41).

Surprisingly, deprivation of various required amino acids, in auxotrophic mutants, failed to afford the same protection (B. D. Davis, unpublished data; see also references 64 and 89), though it does prevent the lethal action of \(\beta\)-lactams. However, the explanation is now clear. Amino acid deprival stimulates protein breakdown, which provides a trickle of amino acids. The misreading induced by Str contributes to continuing protein synthesis even more, by allowing incorrect aminoaacryl-transfer RNAs to replace the missing correct one. The first of these theoretically predictable effects has been demonstrated in cells in which polysomes continued to turn over during amino acid starvation (15, 61). For the second, in vitro studies have shown a large effect of Str in vitro: when protein synthesis was halted by supplying only 15 of the 20 required amino acids (20), or by using extracts with a temperature-sensitive aminoaacryl-transfer RNA synthetase at the nonpermissive temperature (71), Str restored chain elongation almost to normal. The failure of amino acid deprival to protect against the lethal action of Str can thus be reconciled with a requirement of protein synthesis for that action.

In 1960 Anand et al. (4) discovered a second, very different effect of Str: membrane damage. They observed that uptake of \(^{14}C\)Str by growing cells of Escherichia coli, measured after washing the filtered cells with water, was biphasic (Fig. 1). The primary uptake, which was very rapid, evidently consisted of molecules adsorbed to the cell surface, since it could be largely washed away with salt solution. It was followed, after a lag of some minutes, by a rapidly increasing, larger secondary uptake, which could not be washed out. Further work showed that the quantities observed in the two phases depend on the technique used for recovering and washing the cells (58). (In this paper I will use the term uptake to refer to the secondary uptake, into the cell.)

Treatment with toluene caused an immediate increase in cell-asssociated Str (Fig. 1), which suggested that Str might not enter the cell readily until the membrane was damaged. A variety of further observations then showed that Str itself caused membrane damage, which could evidently account for its delayed uptake. Thus, Str-treated cells leaked out nucleotides (63) and K\(^+\) (23). In addition, they exhibited hydrolysis of a \(\beta\)-galactoside (in a strain that lacked the corresponding transport system), utilization of citrate (which E. coli normally excludes), leakage of amino acids, and impaired ability to concentrate \(^{14}C\)valine (2). (Because these multiple effects all pointed to the same conclusion, they were reported without detailed documentation, though in retrospect a fuller description would have been in order.) The increased permeability to small molecules was clearly nonspecific, since it was exhibited by cations, anions, and uncharged molecules and in both directions. It evidently did not allow release of macromolecules, since the turbidity of the cultures did not decrease significantly, and the cells retained those enzymes that were tested.

Chloramphenicol, known to prevent killing by Str, also prevented its uptake (Fig. 1) and the small molecule leakage (3). This important finding led to the conclusion that Str does not damage existing membrane but affects some component in the "growing membrane" (4). Moreover, a high-level resistant mutant (Str\(^R\), later designated strA) exhibited only the initial adsorption and no uptake; hence, the mutation prevented both the irreversible inhibition of protein synthesis and the membrane damage by Str.

Among the effects of Str on sensitive cells the leakage of K\(^+\) could be detected just as early as the inhibition of protein synthesis (22). The membrane damage therefore appeared to be part of the bactericidal mechanism and not simply a secondary change in a killed cell; these kinetics led to the incorrect conclusion that the action on the membrane was
direct. Whether the bactericidal action resulted directly from this damage or from the subsequent penetration of Str into the cytoplasm was left open (4). The latter mechanism was favored by the report, by Erdos and Ullmann (25), that Str partly inhibited protein synthesis in extracts from sensitive Mycobacterium tuberculosis, but not in those from a resistant mutant.

THE RIBOSOME AS THE SITE OF ACTION

In 1961 Spotts and Stanier (69) approached the problem of identifying the site of action of Str indirectly on the basis of the known allelism of Str dependence with sensitivity and resistance in E. coli. They found that dependent cells deprived of Str accumulated RNA in excess over protein (as had been observed with sensitive cells treated with Str [2, 3]), and, believing that the additional RNA was located in ribosomal particles, they inferred that Str acts on the ribosome. However, in light of current knowledge of the regulation of ribosome synthesis, this conclusion was an inspired guess. This RNA clearly was located in what were later called chloramphenicol particles (ribosomal RNA synthesized without ribosomal proteins and hence nonspecifically associated with other proteins [44]), and interference with other components of the protein-synthesizing system could also have caused its accumulation.

Nevertheless, the inferred action of Str on ribosomes was soon directly confirmed. Several laboratories, studying polypeptide synthesis with polyuridylic acid, found that Str caused partial inhibition with extracts of sensitive cells of E. coli but not with extracts of resistant mutants, confirming the earlier finding with endogenous synthesis in extracts of M. tuberculosis; in addition, the resistance was localized in the ribosomes (26, 47, 67). The site of this resistance was later traced to the S12 protein in the 30S subunit.

Although Str might well act on both the ribosome and the membrane, it was very unlikely that a single mutation to resistance, known to alter the ribosome, would also directly alter the membrane. Accordingly, the membrane damage was evidently not a direct effect of Str but instead resulted from its action on sensitive ribosomes. At that time there was no basis, either conceptual or experimental, for exploring the nature of the connection between ribosomes and membrane. Moreover, the elegant and influential Spotts and Stanier paper (68) emphasized an attractive philosophical theme: the importance of distinguishing the key action of an antibiotic from the “epiphenomena.” Since the ribosome was obviously directly involved, all investigators in the field soon dismissed the membrane damage, despite its early appearance, as an incidental change, and this effect received no further attention for about two decades.

MISREADING IN TRANSLATION

A few years later Gorini and Kataja discovered an additional important effect of Str: at sublethal levels it can cause phenotypic suppression of auxotrophic mutations in cells (30), and study with polyuridylic acid in extracts soon confirmed the inference that the effect was due to increased misreading (17). Moreover, this phenotypic alteration led to the isolation of rum (ribosomal ambiguity) mutations, in which genotypic alteration of the ribosome has a similar effect (62).

Misreading was of great scientific interest: it showed that alteration of the shape of the ribosome, by interaction with a small molecule or by mutation, can affect not only its catalytic activity but also its specificity; this variation in accuracy encouraged attention to the problem of fidelity of information transfer, in translation as well as in replication and transcription (29). In addition, since misreading implied a distortion of codon-anticodon interaction on the ribosome, the puzzle of Str dependence could be explained as a compensatory balance between two opposing distortions, by the antibiotic and by the dependence mutation. (However, a later finding, that growing cells can incorporate several molecules of Str into each ribosome [37], suggested that the corrective action of Str on dependent ribosomes also includes effects on their assembly.)

Nevertheless, it was uncertain whether the misreading had any relation to the chemotherapeutic action of the drug. Moreover, the mechanism by which different concentrations of Str could exert two incompatible effects on the ribosome, misreading and blockade, prevented a problem. Gorini and colleagues reported that free ribosomes in vitro bind two molecules of Str (8, 28), and the double effect could be easily explained if Str bound to two sites, with different affinities and different effects. However, a more thorough examination (14) showed that ribosomes bind only one molecule tightly, with $K_d$ of $10^{-4}$; the affinity of the nonspecific binding of additional molecules was too low ($K_d > 10^{-4}$) to be significant.

DIFFERENT ACTIONS ON INITIATING AND ON CHAIN-ELONGATING RIBOSOMES

The paradox of misreading versus blockade was resolved when Tai et al. (72) developed a method for sharply distinguishing effects of antibiotics on ribosomes in two different states: engaged in initiating protein synthesis (washed ribosomes, translating viral RNA as messenger) or engaged only in chain elongation (purified polysomes, free of initiation factors). Several antibiotics (spectinomycin, erythromycin, and kasugamycin) were found to have no effect on chain elongation but to block initiating ribosomes completely (20), allowing initiation complexes to form but then preventing their continuation into chain elongation and causing their breakdown after a few minutes (20, 70). Str (and other aminoglycosides) similarly blocks the initiation complex (5, 20, 70) and not chain-elongating ribosomes, but it does act on the latter, decreasing both the rate (75) and the accuracy (20, 70, 71) of translation. Since either system exhibits its characteristic response over a wide range of concentrations, the difference clearly arises from the state of the ribosome and not directly from the concentration of the antibiotic.

The alternative effects of high or low Str concentrations on cells could now be explained. At low concentrations Str encounters mostly the predominating polysomal ribosomes, and so it causes misreading, while at higher concentrations it reaches all of the ribosomes, including those engaged in initiation, and so it stops protein synthesis (20, 73). In addition, the initiation complexes blocked by Str are not stably bound to the messenger but fall off, with a half-life of about 5 min (46, 50). This finding explained the dominance of Str on both Str in heterozygotes (45), which contain both sensitive and resistant ribosomes (66): the blocked sensitive ribosomes not only exclude the resistant ribosomes from the existing messenger (which is meanwhile decaying), but by slowly recycling are also able to block the initiation sites on any newly formed messenger (73).

The mechanism of the two alternative effects on the ribosome is not known in molecular detail. Since a single mutation in protein S12 eliminates both responses, they
These garbled protein damage (344DAVI) and over, the ability site. These differences could explain the more drastic effect of Str on initiation than on chain elongation (70). In addition, they suggested, along with the observed inhibitory activity of the recycled Str-ribosome complexes released from blocked initiation complexes, that the binding of Str to the initiating ribosome might be irreversible (73). However, the irreversibility of the uptake of Str (55) can also explain why its action on the cell is irreversible.

The possibility was then still open that the accumulation of garbled protein in the cell might be responsible for the bactericidal action of Str, though the limited evidence had not favored this mechanism. Thus, 

\textit{ram} mutants are viable, despite their misreading, and in Str cells high concentrations of Str block protein synthesis so rapidly that there would be little time for accumulation of misread proteins. However, the demonstration of an effectively irreversible inhibition of initiating ribosomes made the hypothesis of lethal misreading seemed superficial. Misreading therefore seemed to be an interesting effect quite separate from the bactericidal action.

Though killing by ribosomal blockade thus seemed to be a key mechanism, it still left hanging some major problems: the mechanism of membrane damage, its possible role in uptake and in killing, and the reason that membrane damage, and killing, require protein synthesis.

**CONNECTION BETWEEN RIBOSOMES AND MEMBRANE DAMAGE**

The uptake of Str is prevented not only by chloramphenicol and mutations to ribosomal resistance, as we have noted, but also by plasmid-coded resistance (21), in which an enzyme (believed to reside in the membrane) inactivates the drug as it enters. It is thus clear that the phase of detectable uptake requires preceding interaction of active antibiotic with sensitive ribosomes engaged in protein synthesis.

Nevertheless, it was not obvious what kind of interaction could promote aminoglycoside uptake. The discovery of membrane-bound ribosomes in bacteria led to an ingenious and logical suggestion (if one ignores the earlier evidence for membrane damage): that membrane-bound Str-sensitive ribosomes engaged in protein synthesis play a direct role in uptake, accepting the antibiotic at the membrane surface and transferring it to the cytoplasm (11, 12). However, apart from the lack of any obvious mechanistic basis, two important findings decisively eliminated this hypothesis. First, when cells with Str-resistant ribosomes are treated with another aminoglycoside, to which they are sensitive, they can then take up Str (35, 36). Second, when addition of chloramphenicol to sensitive cells is delayed until after secondary uptake of Str has begun, it does not block continuation of the uptake (6, 35, 36, 53). Clearly, the ribosomes actively synthesizing protein in the presence of an aminoglycoside do not promote uptake by a direct participation in the process of entry: they must have an effect on the membrane during the lag; this effect then persists even when the ribosomal activity has ceased.

This persistent effect on the membrane was obviously the damage that had been described many years earlier. To try to explain why it requires ribosomal activity, another ingenious hypothesis was proposed: that it results from some kind of physical interaction of the active antibiotic-ribosome complex with the membrane (10). But while this hypothesis was logically consistent with the observations, no concrete mechanism for such a persistent effect could be specified.

What finally led to a more satisfactory explanation was the reinterpretation of another influence on aminoglycoside uptake, observed earlier: 

\textit{ram} mutations shorten the lag (62). It was initially assumed that they did so by adding their misreading to that induced by Str, resulting in a lethal accumulation of garbled protein, but we have seen several reasons to consider this mechanism of lethality unlikely. An alternative suggestion was that 

\textit{ram} mutations, which alter the ribosome, exert their potentiating effect by increasing its affinity for Str (9), but the observed increase was very small. Finally, a more credible possibility emerged from coupling the misreading effect of the 

\textit{ram} mutation with the renewed evidence that membrane damage is important and depends on active protein synthesis by sensitive ribosomes. In this proposal the stimulation of Str uptake by 

\textit{ram}, and the autocatalytic effect of Str on its own uptake, can both be explained by assuming that the membrane is damaged by the incorporation of misread proteins (18).

This proposal led to a multiphase model for the bactericidal action of aminoglycosides in which the increasing uptake leads first to membrane damage and then to ribosomal blockade. The sequence of events would be as follows:

(i) A small amount of antibiotic penetrates, by an unknown mechanism, into the cell, where its contact with chain-elongating ribosomes causes misreading.

(ii) Some of the misread protein is incorporated into the membrane, creating channels that permit influx of antibiotic and thus initiating an autocatalytic process of increasing influx, misreading, and channel formation.

(iii) The intracellular antibiotic eventually reaches a concentration that blocks all initiating ribosomes, thus preventing further protein synthesis.

(iv) Lethality results from the irreversibility of this blockade, due to irreversible uptake and possibly also to irreversible binding to initiating ribosomes.

Let us now examine the ability of this model to explain other features of aminoglycoside action.

**EFFECT OF Str ON PROTEIN EXPORT**

The proposed mechanism has two new elements: misread protein is incorporated into membrane, and its poor fit there would make the membrane leaky to small molecules.

The first point, arising as a virtually self-evident prediction, is supported by the experimental demonstration that aminoglycosides grossly distort membrane composition. This evidence had in fact been obtained several years earlier, in tests of a rather different hypothesis: that misreading might be the cause of killing by aminoglycosides, after all, as a result of the incorporation of garbled protein in the membrane. This idea arose when Beckwith and co-workers (7) observed that certain genetic fusions of β-galactosidase with a periplasmic protein become stuck in the membrane and block the secretion of other proteins, resulting in cell death.

To test for a similar possibility with misread proteins, we used pulse-labeling with [35S]methionine to examine the distribution of the protein that is newly formed after partial inhibition of protein synthesis by Str. The results showed that the pretreatment with Str decreased by 40 to 50% the proportion of the labeled protein reaching the periplasm (18).
Moreover, it increased the proportion present in a “residual,” particulate fraction of low solubility in nonionic detergent; and this residual fraction included an insoluble form of a normally periplasmic protein, alkaline phosphatase (detected by its serological reactivity and its $M_t$). While this insoluble enzyme might have been present in aggregates, which have been seen with other kinds of abnormal proteins in the cytoplasm (59), it was evidently present in the membrane, since it had been processed to the size of the mature alkaline phosphatase, and this processing takes place on the periplasmic surface of the membrane. (We have not tested for an altered distribution of integral membrane proteins, but since E. coli contains a larger amount of these than of periplasmic proteins, their misreading may contribute even more to the membrane damage.)

Though these findings clearly demonstrated that misreading has an effect on the structure of the membrane, this effect was not sufficiently extensive or progressive to seem likely to be the direct cause of cell death, and so the results were not then published. Several years later, however, it became clear that they fitted very well an alternative hypothesis: that the alterations in the membrane might contribute to cell death indirectly by making the membrane permeable to Str (18).

Nevertheless, the main evidence for the second feature of the model, a role of altered membrane proteins in the leakiness, remains the ability of this model to explain all major features of Str action. Having described above certain of these features, which suggested the model, I will now review the others.

**UPTAKE OF AMINOGLYCOSIDES**

**Lag and Initial Entry**

Ever since the first studies on Str uptake, it has been widely observed that the duration of the lag before the secondary uptake in E. coli decreases with increasing Str concentration over a wide range. This pattern suggests that the initial, nondetectable entry of the antibiotic during the lag occurs by diffusion through an aqueous channel or by low affinity for a transport system (i.e., use of the early, linear portion of its concentration-rate curve). The outer membrane may also contribute to the lag, but it cannot be the major barrier altered by Str, since the leakiness that has been demonstrated after Str treatment (2, 23, 63) involves metabolites that cannot normally pass the cytoplasmic membrane (except by specific transport). In Bacillus megaterium the uptake of Str is reported to lack a significant lag (31), suggesting that this organism is more permeable to Str than E. coli.

Though this laboratory had initially reported a plateau of adsorption during the lag before secondary uptake in E. coli (Fig. 1), Bryan and Van den Elzen (11, 12) have described a slow linear uptake, called energy-dependent phase I, during that lag (and also in resistant cells, which do not develop secondary uptake). The subsequent rapid uptake in sensitive cells was called energy-dependent phase II. However, because the large background of adsorption on the outer surface of the cell (Fig. 1) would obscure even enough entry (1% of the total) to equal the number of ribosomes, the apparently linear phase I uptake may well be simply an expansion of the adsorption, proportionate to the continued growth of the cells. How large the initial entry must be to trigger the autocatalytic uptake is not known.

**Kinetics of Uptake through Nonspecific Channels**

Like the rate of overcoming the lag, the rate of uptake of Str in the secondary phase, after the lag, is also roughly proportional to its concentration over a wide range (53). This is the pattern that would be expected of diffusion (or electrophoresis) through nonspecific aqueous channels created by membrane damage. The role of nonspecific channels is further demonstrated by the asymmetric synergism between aminoglycosides and β-lactams: nonlethal pretreatment with the latter, causing damage to the cell envelope, accelerated the lethal action (56) and the uptake (51) of aminoglycosides, while exposure in the reverse order was not synergistic.

Addition of chloramphenicol to cultures at different stages of killing by Str provided further evidence, briefly noted above, on the kinetics of channel formation. At each stage the uptake of Str continues (6, 35, 36, 53), but the viable cell count is immediately stabilized (57). It therefore appears that without further protein synthesis the killed cells continue to take up the antibiotic in amounts far larger than those responsible for killing, but those cells that are still viable have not yet made enough channels for visible uptake.

This pattern suggests that at a lethal concentration of Str the autocatalytic process of entry, misreading, and increased entry results in a rapid transition between virtual impermeability and a lethal uptake. The uptake required for killing is small, relative to what can be measured: approximately 1% of the maximal uptake matches the number of ribosomes. However, there is evidently a lag between a lethal uptake and its complete blockade of protein synthesis, since studies (summarized above) on the effect of Str on the distribution of newly synthesized protein (18) showed significant synthesis of abnormal protein during the period when Str was causing progressive cell death. The stabilization of the viability count by chloramphenicol suggests that this synthesis is occurring largely in cells already condemned to death.

**Proton Motive Force and Irreversible Uptake**

Studies with various mutants and inhibitors have shown that the proton motive force has a strong influence on uptake and is probably required (1, 6, 11–13, 34). Moreover, its Δ$\phi$ component is more important than the ΔpH (16, 24, 48, 49), perhaps producing an electrophoresis of the drug through aqueous channels. In further studies along these lines it will be important to determine whether the initial entry through the normal membrane and the later entry through the damaged membrane differ in their dependence on membrane potential.

It is not clear why the entry of Str, clearly through nonspecific channels, is irreversible (53, 55). The attainment of a huge excess over the number of ribosomes excludes irreversible attachment to ribosomes as the reason. While the importance of the membrane potential suggests entry of the antibiotic by electrophoresis (rather than a free diffusion) through the channels, it is not washed out in buffer (which would not sustain a potential), even though a nonspecific aqueous channel should be equally permeable to Str in either direction. This difference between entry and exit could be explained if the channel is not fixed but is gated in response to the presence or absence of a potential. The large uptake after permeabilization of the cell by toluene (Fig. 1) suggests a simple explanation: loose, ionic binding of the polycationic antibiotic to anionic macromolecules and surfaces within the cell, like the extensive adsorption to the exterior.
puromycin would allow many chains to become long enough to enter, and so misreading could affect membrane integrity. (Why modest concentrations even stimulate Str uptake is discussed below.) Moreover, this predicted difference in average chain length has been experimentally confirmed by measurement of the rate of polysome decay at these different puromycin concentrations (38).

Since the paradoxical interactions with puromycin have been a particularly refractory obstacle to all previous theories of aminoglycoside action, the ability to provide a simple, clear explanation is probably the strongest single argument for the proposed mechanism, relating misreading to membrane damage.

**PUROMYCIN ALSO CAUSES MEMBRANE DAMAGE**

As a by-product of this explanation for the paradoxical action of puromycin, it now appears that membrane damage by incorporation of abnormal proteins is not confined to the aminoglycosides. This conclusion was suggested by a surprising observation encountered during the study of the interaction of puromycin and Str just described (38): in the presence of appropriate concentrations of puromycin, not only Str-sensitive cells but also Str-resistant cells took up Str (Fig. 2). This uptake, which has been confirmed (10), must require membrane damage: yet it is difficult to ascribe the damage to the Str, since at the concentration used it would not ordinarily act on resistant ribosomes. An alternative possibility, that puromycin might cause resistant ribosomes to respond to Str, is very unlikely, since the site of action of puromycin, at the aminolar terminus of transfer RNA, is far from the codon-anticodon site. Moreover, polyanines, which have multiple cationic groups like the aminoglycosides, do not cause misreading; yet puromycin similarly accelerated their uptake in both Str-sensitive and Str-resistant cells (38).

The proposed explanation for membrane damage by Str suggested a similar explanation for this facilitating action of puromycin in resistant cells (18): polypeptide chains prematurely released by puromycin, without other alteration of sequences, can evidently create membrane channels, like the misread chains formed in the presence of Str. Indeed, the extent of the uptake of Str in puromycin-treated resistant cells (Fig. 2a) suggests that puromycin can make the membrane fully as leaky as Str does. This finding raises the question of how much of the effect of Str-induced damage to the membrane is due to misreading within a sequence and how much is due to the premature termination that is also part of Str-induced misreading.

This explanation for the action of puromycin is now supported by direct evidence for damage to the membrane (P. C. Tai and E. O'Leary, personal communication). At 50 \( \mu \)g/ml, puromycin caused leakage of nucleotides from growing *E. coli* cells, like that observed earlier with Str (63), while high concentrations had no such effect.

We can now readily understand why moderate concentrations of puromycin not only permitted but accelerated Str uptake and killing in sensitive cells, as noted in the preceding section: the puromycin itself was contributing to membrane damage and hence to the entry of Str. In an additional possible effect, premature release of nascent chains should accelerate ribosomal reinitiation and hence might increase the number of chains available to enter the membrane, including those misread in the presence of Str.

Since puromycin causes membrane damage without the complicating secondary consequences associated with kill-
ing, and since it can be used in eucaryotic as well as procaryotic cells, it may be a more useful experimental tool than aminoglycosides for studying the physiological consequences of increased permeability to small molecules. In addition, as has been noted (18), in the widespread use of this antibiotic to inhibit protein synthesis, its indirect action on the membrane might well be responsible for some of the effects that have been observed.

The effect of Str plus puromycin on resistant cells rigorously confirms two features of the mechanism of aminoglycoside action. First, since sensitive and resistant cells differ only in their ribosomes, the failure of Str to kill the latter, after puromycin has facilitated its entry (38), rigorously proves that its lethal action must result from its interaction with the ribosomes and not with other components that are accessible after entry. Second, that treatment with puromycin alone is not lethal provides particularly strong evidence that the membrane damage from Str does not contribute directly to cell death.

**METHODOLOGICAL CONSIDERATIONS AND THE ROLE OF PREDICTION**

Since the integration of the pleiotropic actions of aminoglycosides into a coherent, multistep mechanism now seems quite obvious, it may be of interest to speculate about possible reasons for the long inattention to membrane damage and for the later delay in recognizing misreading as a possible source of that damage.

One probable reason for the abandonment of interest in membrane damage was technical: the large background of adsorption of Str on the bacterial surface, and its variation with experimental conditions (58), concealed the very low level of uptake occurring during the lag and hence prevented analysis of the autocatalytic transition to the secondary uptake. (We should recall that 1% of the maximal uptake would be sufficient to saturate the ribosomes.) Probably a more important factor was philosophical: the persistent effort to identify a single key mechanism, uncluttered by epiphenomena, as advocated in the early paper of Spotts and Stanier (68). This approach has usefully sharpened the focus of much research, but in this case it can be seen to have been misleading.

The order of appearance of the discoveries probably also contributed to establishing a persistent and limiting conceptual framework. Thus, a reaction to the incorrect theory of direct action of Str on the membrane discouraged further attention to the correct finding of a reproducible and early effect. Moreover, the subsequently discovered effects on the ribosome were more accessible to investigation, and by the time the translocation of proteins into the membrane became accessible to experimental study, the unexplained ancient observations on membrane damage were essentially buried (even though our laboratory became engaged in study of protein translocation). If, on the other hand, membrane damage had been discovered after misreading (and even more, after protein export became an active field of investigation), its connection to the ribosome might have been recognized much more easily.

The methodological assumptions of this review also deserve comment. I have presented the proposed multistep mechanism not as a tentative hypothesis, but as an explanation that is firmly established by its consistency with all known features of aminoglycoside action. One might object that this conclusion is too strong, since the proposal has not led to crucial predictions that were then tested. We should note, however, that in principle this model could have been formulated as soon as we had recognized, in 1973, the basis for the double effect of Str on ribosomes: misreading and blockade. The model could then have led to many predictions, including the several puzzling aspects of the uptake of Str, discovered later, that are presented above as the basis for inferring the model. In particular, the paradoxical effect of puromycin on Str uptake (shown in 1981) could also have been predicted.

Today we can make a further prediction that would directly test for the inferred effect of misread or truncated proteins on membrane integrity: appropriate genetic alterations in major proteins of the membrane should have the same effect. Their further utility, in detailed exploration of the mechanism of membrane damage, is discussed below.

**MECHANISTIC CHALLENGES**

Among the several steps in aminoglycoside action, the misreading and the blocking effects on the ribosome are quite well understood in principle, and we can expect studies on ribosomal conformation to clarify the molecular mechanisms. This paper raises another challenge: the molecular structure of the channels created by misread or truncated proteins in the membrane.

At present we can only speculate about this problem. Altered folding might make these abnormal proteins porous, i.e., create transmembrane aqueous channels within them. This possibility is supported by the loose folding observed with synthetic polypeptides of arbitrary sequence, in contrast to evolved natural proteins. Alternatively, channels might arise along a series of polar residues on the predominantly hydrophobic embedded surface of transmembrane proteins. In a third possibility, transmembrane proteins that normally contain gated channels might have lost the ability to close the gates. This alternative seems less likely, because the known gated channels in bacteria are not open to molecules as large as Str (581 daltons).

In further study of the nature of the channels, observations on their electrical properties, perhaps most conveniently with puromycin-treated eucaryotic cells, may be informative. For detailed molecular insights, specific genetic alterations will no doubt be more useful than the random changes induced by antibiotics, and prematurely released chains should be easier to simulate than those altered by misreading.

Indeed, studies on specific altered proteins in the membrane are already under way, though they have not focused on membrane integrity. A short C-terminal deletion has been reported to shift the *E. coli* β-lactamase from the periplasm to the membrane (43). While similar findings with the maltose-binding protein were thought to reflect attachment of aggregated protein to the membrane, rather than retention in the membrane (40), a truncated glycerophosphate phosphodiesterase seems definitely to be shifted from periplasm to membrane, since it interferes with the secretion of other periplasmic proteins (34). In such studies, it would be of interest to test for effects not only on protein export, but also on the integrity of the osmotic barrier.

In addition to not knowing how abnormal proteins create channels, we do not know how the first molecules of antibiotic enter the cell, before they have begun to induce the autocatalytic process of increasing misreading and channel formation. This initial entry might depend on channels created by the low level of intrinsic misreading. Alternatively, it is possible that inevitable imperfections in the
process of cell growth may occasionally produce channels (whether transient or permanent) between normal constituents, perhaps where membrane is attached to edges in the expanding cell wall, or at sites of protein secretion. Finally, Str might poach inefficiently on a transport system evolved for the uptake of some other compounds. Whether the path of initial entry differs significantly from the channels induced by Str remains to be seen.

We might briefly note an evolutionary and a practical implication of the complex bactericidal mechanism described here. First, it is not clear whether the aminoglycosides have been selected for the advantage of their antibiotic action to the organisms that produce them or whether they are secondary metabolites evolved for some other function; however, either way, it is not obvious how such an intricate bactericidal mechanism could have emerged. Second, while one hopes that understanding of the mechanism of action of a drug will provide a useful model for the rational design of related drugs, the action of the aminoglycosides seems too complex to serve that purpose. Another possibility is more encouraging: since even very few channels in a cell may accelerate initiation of the autocatalytic entry of aminoglycosides, it would seem worthwhile to explore the synergism of these antibiotics with low levels of puromycin.

**IMPLICATIONS FOR THE STRUCTURE OF NORMAL MEMBRANE PROTEINS**

In research on the extremely complex systems of living organisms, a discovery is more interesting if it can not only answer a question but also lead to new questions. The proposed model for aminoglycoside action initially seemed only to provide an answer to an old puzzle. However, it may also reveal an interesting feature of normal membrane proteins: they must have evolved sequences that fold tightly and fit in well, so that the cytoplasmic membrane is impermeable to small molecules, much smaller than Str. Since this is obviously not a universal property of proteins that can reside in the membrane, detailed molecular characterization of membrane proteins will have to be concerned with not only the properties that localize them in the membrane, but also the properties that prevent them from forming aqueous channels.

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Volume 51, no. 3, p. 341-350: This article focused on the role of nonspecific membrane damage in the uptake of aminoglycosides and on the role of misread proteins in creating that damage. It was assumed that after entry of the antibiotic its blockade of initiating ribosomes accounts for its lethal effect. Evidence for an additional lethal mechanism had inadvertently been overlooked (N. Tanaka, K. Matsanuga, H. Yamaki, and T. Nishimura, Biochem. Biophys. Res. Commun. 122:460–465, 1984; K. Matsanuga, H. Yamaki, T. Nishimura, and N. Tanaka, Antimicrob. Agents Chemother. 30:468–474, 1986). These workers used temperature-sensitive mutants of Escherichia coli, defective in different aspects of DNA replication [dnaC(Ts) or dnaE(Ts)], to show that aminoglycosides block the initiation but not the continuation of this process.

The aminoglycosides appear to do so by some mechanism other than simple interference with the synthesis of required new initiation proteins, since chloramphenicol did not block the initiation; moreover, aminoglycosides were shown to interfere with reconstitution of a DNA-membrane complex in vitro. This interference suggests that the antibiotic interacts specifically with a component(s) of the assembling initiation complex, just as it does in interfering with initiation in protein synthesis.

Presumably either the blockade of protein synthesis or the blockade of DNA synthesis could account for the lethal action of aminoglycosides; presumably both effects occur in the cell, but the interference with protein synthesis might begin earlier in most cells, because the cycle of DNA replication is longer than the macrocycles of translation.