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Effect of Enzymatic Adenylylation on Dihydrostreptomycin Accumulation in *Escherichia coli* Carrying an R-Factor: Model Explaining Aminoglycoside Resistance by Inactivating Mechanisms

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Strains of *Escherichia coli* carrying R-factor R71(a), which codes for a streptomycin-spectinomycin adenylyltransferase, have elevated levels of resistance to dihydrostreptomycin (DHS) compared with isogenic R^- bacteria. DHS accumulated by whole cells and spheroplasts of R^+ bacteria is lower than that observed for R^- strains, a result of the absence of the second and more rapid of the two energy-dependent phases of DHS uptake seen in susceptible *E. coli*. A mutant of R^+ *E. coli* with reduced DHS resistance has been shown to have reduced levels of streptomycin-spectinomycin adenylyltransferase activity as well as enhanced drug accumulation. Actively accumulated DHS was recovered from R^+ cells as the adenylylated derivative. Neither was inactivated antibiotic detected in culture filtrates, nor was actively accumulated drug lost from R^+ cells under normal conditions. The cellular distribution of actively accumulated DHS in R^+ and R^- cells was found to be the same. Membranes isolated from these cells retained only a small fraction ($\simeq$1%) of the total cell-associated drug. The R^+ derivative of a mutant with defective energy transduction (*E. coli* NR-70) and reduced ability to transport aminoglycosides has a significantly higher minimal inhibitory concentration of DHS than its R^+ parent (strain 7). Streptomycin-spectinomycin adenylyltransferase activity, from comparisons of K_m values and total activities of enzyme, was the same in both strains. The enzyme has been localized to the exterior surface of the bacterial inner membrane, although isolated membranes lacked detectable enzyme activity. The preceding observations are consistent with the proposal that the level of R71(a)-mediated DHS resistance is the outcome of competition between the rate of adenylylation and the rate of the first energy-dependent phase of DHS transport. When the rate of adenylylation exceeds the first energy-dependent phase, adenylylated DHS is accumulated, apparently in a manner identical to the accumulation of DHS. Unlike DHS, adenylylated DHS does not interact with ribosomes, and, consequently, there is a failure to initiate ribosomally dependent sequelae such as the second energy-dependent phase of accumulation, inhibition of protein synthesis, and/or misreading of mRNA.

R-factor-specified enzymatic modification of aminoglycoside antibiotics has been shown repeatedly to be a clinically important mechanism of resistance to aminoglycosides in bacteria. However, the precise mechanism by which enzymatic modification causes resistance is unknown. It has been shown in certain instances that modified aminoglycosides are no longer as effective at inhibiting protein synthesis or producing misreading of mRNA (23). These observations fail to explain aminoglycoside resistance because it has been demonstrated that only a very small fraction (less than 1%) of the total available, active aminoglycoside in the bacterial growth medium is detoxified. Thus, bacteria possessing aminoglycoside-inactivating enzymes are able to multiply in a milieu of almost entirely active drug. Another observation reported is that R^+ bacteria which possess inactivating enzymes accumulate less aminoglycoside antibiotic than do isogenic R^- bacteria (L. E. Bryan and H. M. Van Den Elzen, Progr. Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 15th, Washington, D.C., Abstr. no. 168, 1975). There-

fore, it appears that resistance in such R^+ bacteria results from a combination of reduced drug accumulation and drug inactivation

Aminoglycoside-inactivating enzymes are generally considered to occupy a site at the cytoplasmic membrane level, a position ideally suited to a function of intercepting and inactivating antibiotic during transit to the target site (7). This study was designed to define more exactly the inactivating enzyme's cellular location, to study drug accumulation kinetics in R^+ bacteria, and to determine the fate of inactivated drug. The results, together with observations of the effect that mutations which decrease aminoglycoside cell entry have on R-factor-specified resistance levels, have led to what is believed to be a realistic and comprehensive understanding of the mechanism of aminoglycoside resistance in bacteria possessing inactivating enzymes.

The study undertaken employed as a model system *E. coli* strains clinically resistant to dihydrostreptomycin (DHS) due to the presence of an R-factor with the genetic determinants for a streptomycin-spectinomycin adenylyltransferase [AAD-(3″)].

MATERIALS AND METHODS

Bacterial strains and selective techniques. *E. coli* K-12 J5 F^- *pro met* azide^r (5) was the source of R-factor R71(a). Recipients for R71(a) were rifampin resistant derivatives (Rif^r; selected for resistance to 100 μg of rifampin per ml) of *E. coli* K-12 SA1306 (4), *E. coli* ML308.225 *lacA lacZ* obtained from M. Pickard (University of Alberta, Edmonton, Alberta), *E. coli* K-12 strain 7 Cavalli Hfr (18), and *E. coli* K-12 NR-70 ATPase$^-$ Unc$^-$, a mutant derivative of strain 7 derived by B. Rosen (18). Conjugal transfer was accomplished by a filter mating technique (13). Transconjugants were picked from Trypticase soy agar (Baltimore Biological Laboratory) plates supplemented with 100 μg of rifampin and 15 μg of DHS per ml after overnight incubation at 37°C. DHS concentrations were increased to 50 μg/ml for the selection of NR-70 R71(a) strains. *strA* mutant derivatives (Str^r) of *E. coli* K-12 SA1306 (Rif^r) and ML308.225 (Rif^r) were selected by single-step resistance to 2,000 and 250 μg of DHS per ml, respectively, on Trypticase soy agar (Baltimore Biological Laboratory) plates. Methanesulfonic acid ethyl ester mutagenesis was done according to Osborn et al. (16).

Chemicals. 4-Acetamino-4′-isothiocyanotostilbene-2,2′-disulfonic acid (SITS) was obtained from Serva Feinbiochemica, Heidelberg, West Germany. All other chemicals were of reagent grade or better.

DHS uptakes in whole cells. The procedure for measuring [^{3}H]DHS (Amersham/Searle, 3 Ci/mmol) uptake has previously been described (3). The same technique was applied here except that cells were grown and accumulated drug was measured in nutrient broth (Baltimore Biological Laboratory). [^{3}H]DHS efflux was monitored after the filtration of cells prelabeled with [^{3}H]DHS under normal uptake conditions. Filtered cells were washed with and resuspended into nutrient broth at 37°C containing unlabeled DHS at concentrations used for prelabeling. Resuspended cells were incubated at 37°C, and determinations of cell-associated label were made at the specified times after resuspension.

Spheroplast uptakes and spheroplast preparation. Measurement of [^{3}H]DHS uptake by spheroplasts was carried out in nutrient broth with 20% sucrose containing sufficient cells to produce an optical density at 600 nm (OD_{600}) of 0.5 under conditions otherwise identical for whole cells.

Spheroplasts were prepared by the method reported by Weiss (22) except that cells were originally grown in a medium containing (per liter): K_2HPO_4, 10.5 g; KH_2PO_4, 4.5 g; $(NH_4)_2SO_4$, 1.0 g; sodium citrate·$2H_2O$, 500 mg; $MgSO_4 \cdot 7H_2O$, 250 mg; glucose, 2 g; and thiamine hydrochloride, 5 mg. Spheroplasts were collected at 3,600 × *g* for 20 min and then gently resuspended in 5 mM tris(hydroxymethyl)aminomethane (Tris)-chloride, pH 8, with 20% sucrose to which was added deoxyribonuclease, ribonuclease, and $MgSO_4$ to 100 μg/ml, 30 μg/ml, and 10^{-3} M, respectively. The mixture was incubated at 37°C for 30 min, swirling throughout. Portions of these preparations, when diluted 1:10 into water, decreased in OD_{600} at least 10 times more than when diluted 1:10 in 20% sucrose. Spheroplasts obtained from cells exposed to [^{3}H]DHS were prepared as described above from cells washed twice with 5 mM Tris-chloride, pH 8, with 0.5 M NaCl.

Enzyme assays. β-Galactosidase was assayed by orthonitrophenyl-β-D-galactoside hydrolysis (14). Uridine phosphorylase was assayed according to Pardee and Watanabe (17). The assay mixture of alkaline phosphatase contained 0.85 ml of 1 M Tris-chloride (pH 8), 0.05 ml of *p*-nitrophenylphosphate (4 mg/ml), and 0.1 ml of enzyme preparation. The mixture was incubated at 30°C until the desired time, when the reaction was terminated by the addition of 4 ml of 0.1 M Na_3PO_4. Controls lacked enzyme. The generation of nitrophenol was measured as absorbance at 420 nm (A_{420}). Soluble lactate dehydrogenase was measured by pyruvate reduction at 25°C. The assay mixture contained 0.85 ml of 0.05 M Tris-chloride (pH 7.6), 0.05 ml of 3 mM reduced nicotinamide adenine dinucleotide, 0.05 ml of 0.5 M sodium pyruvate, and 0.05 ml of enzyme preparation. Initial rates of reduced nicotinamide adenine dinucleotide oxidation were determined from changes in A_{340}. AAD-(3″) was assayed by the method of Benveniste et al. (1) except that reaction mixtures contained (per 70 μl): 3 μmol of Tris-chloride (pH 8.3), 0.6 μmol of $MgCl_2$, 70 nmol of dithiothreitol, 10 nmol of DHS, 60 nmol of [^{14}C]adenosine 5′-phosphate (2 μCi/μmol; Amersham/Searle), and 20 μl of enzyme preparation.

By definition 1 enzyme unit of activity produced 1 μmol of produce per h except for lactate dehydrogenase, where 1 unit oxidized 1 μmol of reduced nicotinamide adenine dinucleotide per h, and uridine phosphorylase, where 1 unit of activity produced an A_{295} change of 0.001/s. All enzyme assays were carried out on extracts dialyzed overnight at 4°C against 500 volumes of 10 mM Tris-chloride (pH 7.8), 10 mM $MgCl_2$, and 1 mM dithiothreitol (buffer A).

Cell fractionations. Osmotic shocking of cells

grown in Trypticase soy broth (Baltimore Biological Laboratory) was performed according to Benveniste et al. (1). Unless specified otherwise, extracts of sonically disrupted cells were made from cells grown and washed as for osmotic shocking and resuspended in small volumes of buffer A. Cells were sonically disrupted with the intermediate-sized probe at setting 55 (Biosonik III, Bronwill Scientific Inc.) for 2 min at 4°C. Supernatant fluids obtained by centrifuging broken cells for 30 min at 40,000 × *g* were termed sonic extracts. Cells incubated in the presence of [^{3}H]DHS were washed twice with 5 mM Tris-chloride (pH 8) and 0.5 M NaCl before sonication.

Crude membranes of osmotically or freeze-thaw-disrupted cells were collected by centrifugation at 40,000 × *g* for 30 min after the removal of whole cells and debris by repeated centrifugations (two to four times) at 800 × *g* for 30 min. For the detection of enzyme activity, membranes were first solubilized in 0.1 M Tris-chloride (pH 8), 1% Triton X-100, and 10% sucrose for 15 min at 0 to 5°C. For the detection of membrane-bound [^{3}H]DHS, membranes were collected as described above after the lysis of spheroplasts prepared from cells grown in the presence of [^{3}H]DHS. Spheroplasts were lyzed by freezing in liquid nitrogen and thawing slowly under warm, running tap water in the presence of 100 μg of deoxyribonuclease per ml and 20 mM $MgSO_4$. Isolated membranes were rapidly diluted in 50 volumes of water before the quantitation of membrane-associated label, which was done by spotting concentrated portions onto nitrocellulose filters, then drying and counting.

SITS and trypsin treatment of spheroplasts. SITS and trypsin treatments of spheroplasts were carried out in 0.025 M sodium phosphate buffer, pH 8, with 20% sucrose. Exposure to 5 mM SITS was at 25°C for 60 min. Trypsin (9,000 BAEE [*N*-benzoyl-L-arginine ethyl ester] U/mg) was added to 20 μg/ml, and the suspension was incubated for 30 min at 37°C. Reactions were terminated in the former case by washing spheroplasts with the phosphate buffer and sucrose and in the latter case by chilling plus the addition of 50 μg of soybean inhibitor per ml (1 mg inhibited 1.5 mg of trypsin or 10,000 BAEE U/mg). Preparations were sonically disrupted in 0.025 M sodium phosphate buffer, pH 8. Controls included untreated samples, treated sonic extracts, sonic extracts plus soybean inhibitor (50 μg/ml), and sonic extracts treated with preformed inhibitor-trypsin complex to identical final concentrations (five parts inhibitor to two parts enzyme mixed on ice for 15 min).

Other methods. Protein was measured by a modified Lowry method (8). Paper chromatographic separation of modified and unmodified [^{3}H]DHS was accomplished by a reported method (method 3 [21]). Chromatograms were dried, cut into 1-cm fractions, and counted in toluene counting solution (Omnifluor [New England Nuclear Corp.]) in a Beckman model LS-250 liquid scintillation counter. Dry weights were determined for exponentially growing cells by filtering portions through preweighed filters (Millipore Corp.), washing with 2 volumes of isotonic saline, and drying to a constant weight at 150°C. The dry weights for *E. coli* K-12 SA1306 and ML308.225 were 0.18 and 0.16 mg/OD_{600} per ml, respectively. Crude ribosomes were prepared according to Modollel (15). The assay for ribosomal binding of DHS has been described by Chang and Flaks (6).

Autoradiography. For autoradiography, cells were incubated in NB with 1.0 μg of [^{3}H]DHS (3 Ci/mmol) per ml at 37°C. After 30 min the cultures (10 ml volumes) were chilled on ice and washed twice with cold 0.05 M sodium phosphate buffer (pH 8) and 0.5 M NaCl. Fixing and embedding of cells and subsequent developing was achieved by a procedure described by Shahrabadi et al. (20). Distances from the center of grains to the midpoint of the nearest segment of the cell envelope were measured from photographs of nonoverlapping sections at a final magnification of × 12,936.

Determination of antibiotic susceptibilities of bacterial strains. Minimum inhibitory concentrations (MICs) for DHS and spectinomycin and disk susceptibilities were performed as described previously (4), with the exception that MICs were determined in nutrient broth.

RESULTS

DHS sensitivity and accumulation in R^+ and R^- whole cells and spheroplasts. The presence of R-factor R71(a) in recipient *E. coli* strains resulted in a several-fold increase of MIC of DHS (Table 1). Streptomycin adenylyltransferase activity was detectable in cell-free extracts of the R^+ organisms, whereas recipient bacteria had no such activity. R^+ bacteria also acquired resistance to tetracycline, chloramphenicol, sulfonamides, and ampicillin as determined by disk sensitivity tests. The DHS resistance produced by R71(a) was much lower than that due to a *strA* ribosomal mutation. As shown in Table 1, ribosomes from R^+ and R^- bacteria bound DHS, whereas those from *strA* cells did not.

Mutants of R^+ bacteria with reduced MICs of DHS and spectinomycin have a corresponding decrease in adenylyltransferase activity compared with the R^- parent (Table 1). A similar relationship has previously been described by Benveniste et al. (1). These mutants were isolated after methanesulfonic acid ethyl ester mutagenesis by selecting clones with impaired growth on medium selective for R^+ strains containing 15 μg of DHS per ml. Mutants retained normal levels of resistance to the other four antibiotics, and the reduced MICs of DHS and spectinomycin were stable properties. The mutation is most likely a single-point mutation because reversion to usual MICs for both drugs occurred in about 1 in 10^5 clones. Also, methanesulfonic ethyl ester mutagenesis has a high probability of producing point transitions (16). Mutant SR15 was selected to study the significance of adenylylating activity on DHS transport.

The kinetic profile for DHS accumulation by

susceptible *E. coli* is represented in Fig. 1. Uptake occurs in three stages. These have been described in detail elsewhere (4) and are designated, in the order of their occurrence, the energy-independent phase, the first energy-dependent phase (EDP-I), and the second energy-dependent phase (EDP-II). The energy dependence of the latter two phases is apparent from their absence in cells treated with the electron transport inhibitor potassium cyanide. The energy-independent phase, essentially complete before the taking of the first reading, is the only phase present in KCN-treated cells (R^+ or R^-).

R^+ cells differ from R^- cells in accumulating less drug at the same drug concentrations after 30-min incubation periods (Fig. 1 and 2a). The difference can be accounted for by the absence in R^+ bacteria of EDP-II. Active accumulation occurs but only at the lower rate of EDP-I. The uptake pattern is very similar to that demonstrated by *strA* bacteria for which EDP-II is absent if DHS accumulation is measured at concentrations below the MIC of DHS (3). In susceptible cells the onset of EDP-II corresponds with the onset of inhibition of protein synthesis. These events are thought to follow the binding of drug by the ribosome because they are absent in *strA* bacteria (4). In the R^+ strain with reduced AAD-(3″) activity (SR15) EDP-II is observed, but it is delayed in onset and reduced in rate compared to the R^- strain. This is consistent with an MIC for DHS intermediate to those of the susceptible and R^+ *E. coli* K-12 SA1306. Consequently, DHS adenylylation appears directly linked to a reduction in drug accumulation specifically associated with a change affecting EDP-II.

DHS accumulation by spheroplasts of R^+ and R^- *E. coli* is similar to that seen with whole cells (Fig. 2). The difference in total uptake between spheroplasts of R^- and R^+ bacteria is also attributable to the absence of EDP-II in R^+ spheroplasts. EDP-I is indistinguishable in rate between R^- and R^+ cells. Thus, even in the absence of a cell wall, the R^+ derivative retains the effects on DHS accumulation found in whole cells. In susceptible cells, the absence of a cell wall has not affected the rate or duration of EDP-I. This suggests that the cell wall is not a significant barrier to transport of the antibiotic.

Reductions in OD_{600} of DHS-treated R^- spheroplast suspensions relative to untreated controls were noticeable during uptake experiments. After 30 min a reduction of 30% was common. The reduction was initiated after the onset of EDP-II, suggesting that during this time DHS has a lytic action on spheroplasts. R^+ cells did not show this effect.

Strain ML308.225 was used for preparation of spheroplasts as this strain has been shown to produce spheroplasts with little cell wall contamination. Normally, >99% of the cells became spherical 20 to 30 min after the addition of ethylenediaminetetraacetic acid. Electron microscopic examination of negatively stained thin sections of the spheroplasts confirmed the absence of cell wall contamination in the form of polar caps or fragments of outer membrane.

Cellular localization of adenylyltransferase activity. Release of enzyme activity was monitored during the fractionation of R^+ cells by osmotic shock or sonic oscillation. Adenylyltransferase activity is almost quantitatively released by osmotic shocking (Table 2). The pattern of release is similar to that for the periplasmic marker enzyme alkaline phosphatase. The procedure is associated with release of about 40% of β-galactosidase activity used as internal marker enzyme. Nonetheless, a clear demarcation of periplasmic and intracellular locations exists. The conversion of whole cells to spheroplasts allows a differentiation between the cellular localization of alkaline phosphatase and adenylyltransferase (Table 3). Whereas alkaline

TABLE 1. *MICs of DHS and spectinomycin; DHS ribosomal binding affinities and adenylylating activities of E. coli strains*

Strain	MIC (μg/ml)		Molecules of DHS/ribosome[a]	AAD-(3″) (EU/mg of protein[b])
	DHS	Spectinomycin		
K-12 SA1306	0.25	4.0	0.57	0
SA1306 R71(a)	15.6	250	0.65	6.4×10^{-2}
SA1306 R71(a) SR15	2.0	32		3.1×10^{-2}
SA1306 Str^r	250	4.0	0	0
ML308.225	0.5		0.51	
308.225 R71(a)	2.0		0.83	
308.225 Str^r	62.5		0	

[a] As determined from in vitro assays with crude ribosomal preparations.
[b] Enzyme units (EU) determined from osmotic shock extracts.

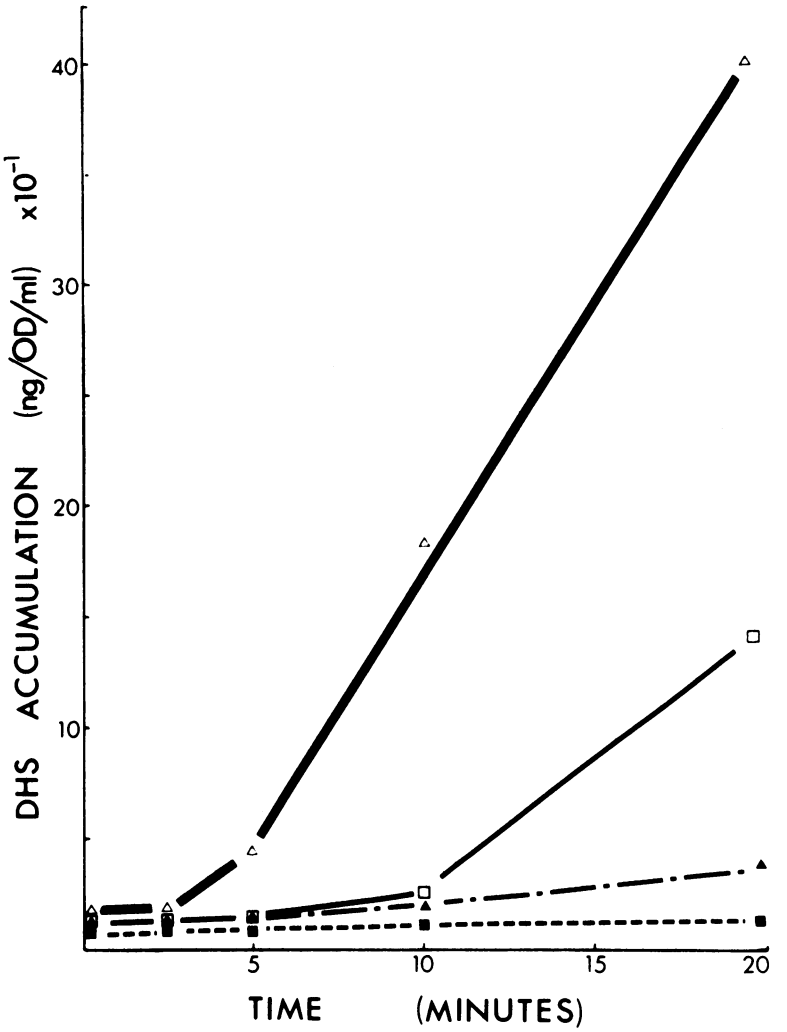

FIG. 1. *Accumulation of DHS at 37°C with time at 2.0 μg of DHS per ml by E. coli K-12 strains SA1306 (△——△), SA1306 in the presence of 1.0 mM KCN (■- - -■), SA1306 R71(a) (▲— · —▲) and SA1306 R71(a) SR15 (□——□).*

phosphatase is readily lost to the suspending fluid, adenylyltransferase is retained by spheroplasts (Table 3). This is consistent with the reduced DHS accumulation observed in spheroplasts of R^+ cells.

The preceding results suggest that the AAD-(3″) is associated with the bacterial cell membrane. A crude membrane fraction of osmotically shocked cells was prepared and assayed for residual enzyme activity. As shown in Table 2, no more than contaminating levels of enzyme were detected. Membrane vesicles prepared as described by Kaback (10) from ML308-225 R71(a) and suspended to 2.3 mg of protein per ml also had no activity when compared with membranes from R^- bacteria. Solubilizing buffer had no significant effect on measured levels of enzyme activity in cell-free extract controls.

To assess the localization of AAD-(3″) further, reagents capable of enzymatic inactivation but unable to penetrate the intact cytoplasmic membrane were used to determine the degree to which the enzyme was inactivated in intact spheroplasts. The proteolytic enzyme trypsin and the reagent dye SITS were used. SITS reacts with amino, histidyl, and guanidyl groups of peptides (12).

According to the results presented in Table 4 neither AAD-(3″) nor the internal marker enzyme lactate dehydrogenase is sensitive to in-

activation by SITS in intact spheroplasts. Each is sensitive, to different degrees, after the sonic disruption of the spheroplasts. This indicates either that the enzymes as a whole are inaccessible or that sites critical to inactivation are not available to interact with SITS. Trypsin, on the other hand, reduces spheroplast-bound AAD-(3″) activity almost 50% under conditions where the activities of cytoplasmic enzymes are unaltered. In cell-free extracts AAD-(3″) activity can be reduced to 10% of controls by tryptic digestion under similar conditions. That adenylyltransferase activity is not reduced in intact spheroplasts to the extent that it is in cell-free extracts suggests that the in situ position offers a limited degree of protection, most likely resulting from a membrane association.

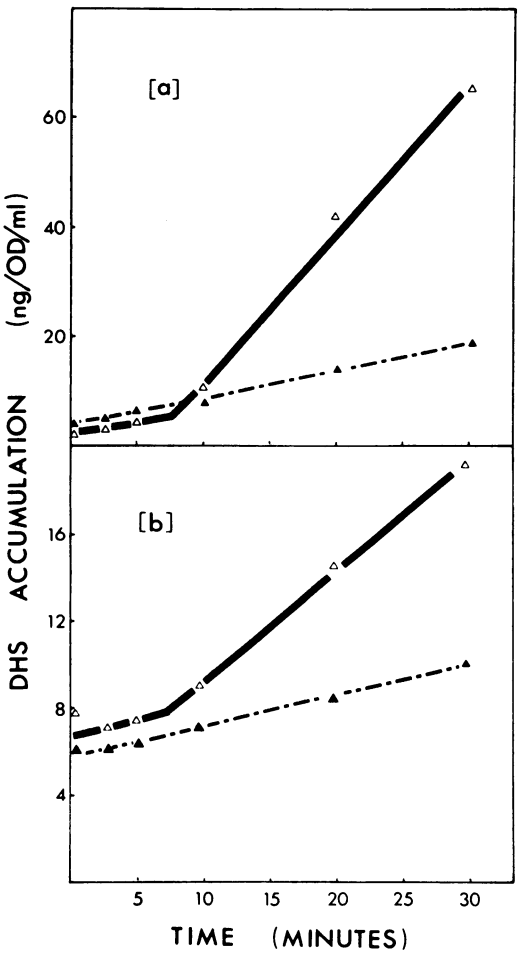

FIG. 2. *Accumulation of DHS at 37°C with time at 0.5 μg of DHS per ml by (a) E. coli strains ML308.225 (△——△) and ML308.225 R71(a) (▲-·-·-▲) and by (b) spheroplasts of E. coli strains ML308.225 (△——△) and ML308.225 R71(a) (▲-·-·-▲).*

Fate of accumulated DHS. It has been repeatedly demonstrated that bacteria with aminoglycoside-inactivating functions fail to detoxify significantly the drug medium within which they grow. We did not detect adenylylated DHS in culture filtrates of growth medium of R^+ *E. coli* after 4 and 16 h of incubation in medium containing concentrations of DHS below MIC levels. Filtrates were examined by a paper chromatography procedure capable of detecting modification of 1% of the DHS present. Efflux of actively accumulated drug from R^+ cells growing in the presence of DHS was also not detected. In the experiments of Fig. 3, KCN-treated and untreated R^+ cells preincubated with [^{3}H]DHS were diluted, washed, and resuspended into NB broth with cold DHS. A rapid loss of label was detected immediately. This label is considered to be loss of [^{3}H]DHS ionically bound to cell surfaces or filters. From 2 min onward there is no significant loss of label from untreated R^+ cells. During this period drug accumulation continues (top curve) by a process shown to be sensitive to KCN poisoning. Those cells treated with KCN continue to lose label at a slow rate through the duration of the experiment. Because this is not seen in KCN-untreated cells, it indicates that the drug bound at these sites is irreversibly accumulated when respiration is permitted.

In the absence of drug efflux the reduced amount of drug accumulated by R^+ cells relative to R^- cells can be directly related to a reduced

TABLE 2. *Enzyme release during the osmotic shocking of E. coli K-12 SA1306 R71(a)*

Cell fraction[a]	Enzyme activity (U/g [dry wt] of cells)		
	AAD-(3″)	Alkaline phosphatase	Beta-galactosidase
Whole-cell sonic extract	22.5	30.1	90.1
Sucrose-EDTA supernatant	1.4	2.9	0.0
Shock fluid	20.7	24.0	35.8
Shocked cells	2.1	1.2	50.1
Crude membranes of shocked cells	0.2	0.6	0.4

[a] Enzyme was released from shocked cells by sonic disruption, and membranes were collected from the same preparation; otherwise, see text. EDTA, Ethylenediaminetetraacetic acid.

TABLE 3. *Enzyme release during conversion of E. coli M308.225 R71(a) to spheroplasts*

Cell fraction[a]	Enzyme activity (U/g [dry wt] of cells)		
	AAD-(3″)	Alkaline phosphatase	Lactate dehydrogenase
Whole-cell sonic extract	16.7	16.5	651
Spheroplast supernatant	1.9	16.9	44
Nuclease digest supernatant	3.0	0.5	132
Sonic extract of spheroplasts	10.3	0.9	432

[a] See text.

TABLE 4. *Enzyme inactivation by SITS and trypsin in spheroplasts of E. coli ML308.225 R71(a)*

Reagent	Spheroplast treatment[a]	Enzyme activity (U/g [dry wt] of cells)		
		AAD-(3″)	Lactate dehydrogenase	Uridine phosphorylase
SITS	A	4.2	4.4	ND[b]
	B	6.5	212	ND
	C	7.0	212	ND
Trypsin	A	1.2	242	309
	B	7.8	343	611
	C	13.5	320	556
	C+inh	12.6	320	570
	C+inh.enz	12.6	306	574

[a] A, Treatment after sonic disruption; B, treatment before sonic disruption; C, untreated control; C+inh, control plus inhibitor alone; C+inh.enz, control plus enzyme-inhibitor complex alone.
[b] ND, Not determined.

influx. The nature of that DHS actively accumulated by R^+ cells was examined. R^+ cells were incubated with [^{3}H]DHS for 15 and 30 min. Under these conditions DHS accumulation is by EDP-I kinetics only. These cells were washed, sonically disrupted, and centrifuged (see above). Supernatant fluids of sonically disrupted samples were chromatographed. R^- cells were included as a control. As shown in Fig. 4, after 15 min about one-quarter of the drug accumulated is adenylylated. After 30 min there is a greater increase in modified drug so that it represents about 40% of the total. These values correlate well with the fraction of cell-associated drug accumulated energetically after 15 and 30 min. The proportion of DHS represented by unmodified DHS is likely drug accumulated during the energy-independent phase. This is probable because the amount of drug accumulated per unit mass of cells by the energy-independent process is stable with time (see Fig. 1), whereas there is continuing accumulation by an energy-dependent process. Thus, the increasing fraction represents drug accumulated by the energy-dependent process. The absence of detectable adenylylated DHS in the growth medium also supports the concept that modified drug represents most if not all of the actively, irreversibly accumulated fraction of the cell-associated drug. Table 5 shows that the vast majority of the label has been released into the supernatant fluid chromatographed, indicating that the ratio expressed above is representative of the population of cell-associated drug as a whole.

The results of the previous experiment indicated that modified drug does not have high affinity for the bacterial cell membrane. To attempt to clarify this, membranes isolated from ML308.225 R71(a) having actively accumulated [^{3}H]DHS (at 0.5 μg/ml for 30 min) during EDP-I only were assayed for radioactivity. Little drug was retained by membrane fractions obtained after the fractionation of cells as described above. All but 1% of the cell-associated drug could be traced to the supernatant fluid. The distribution in R^- cells was identical.

We are currently developing methods to improve the resolution of autoradiography so that whole-cell distribution of [^{3}H]DHS can be accurately determined. Results from our observations to this time will be briefly described. *E. coli* K-12 SA1306 (R^+ and R^-) were incubated in NB with 1.0 μg of [^{3}H]DHS per ml for 30 min and then were prepared for autoradiographic analysis (see above). No significant difference existed between the distribution patterns established for identically treated R^- and R^+ cells, although a greater number of grains was associated with R^- cells. The majority of the accumulated label appeared to be associated with cell membrane or envelope, with a slight skewing toward the cells' interior evident in each case.

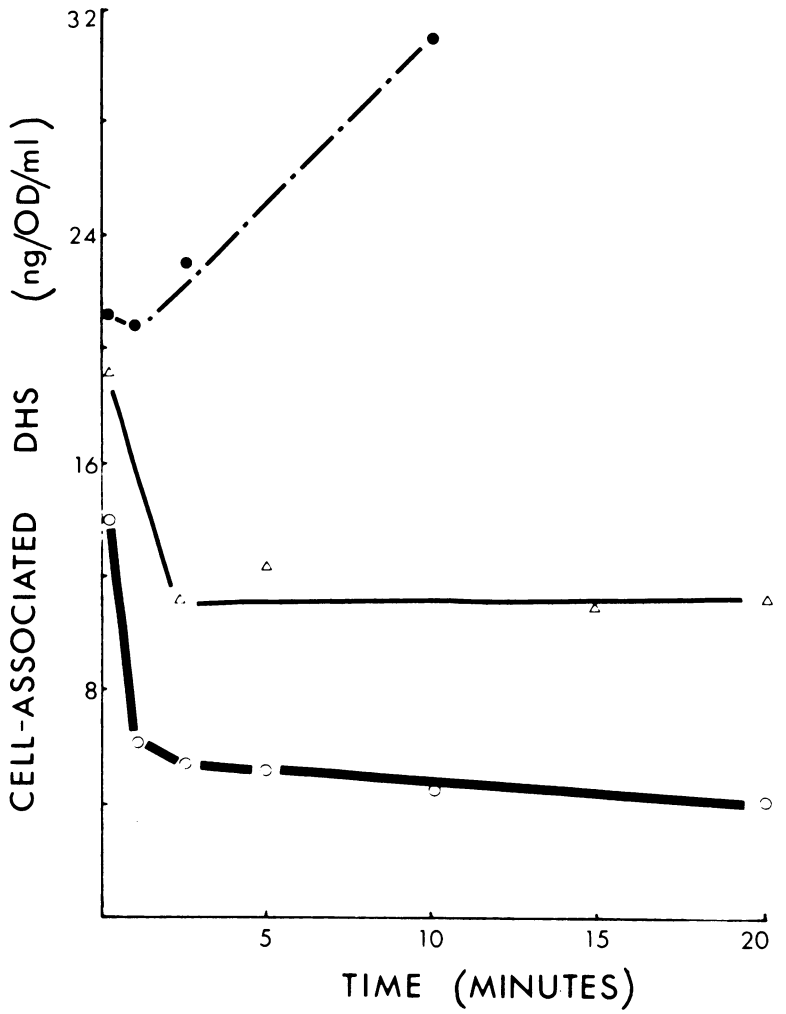

FIG. 3. *Release of [^{3}H]DHS with time from E. coli K-12 SA1306 R71(a), grown for 12 min at 37°C with 2.0 μg of [^{3}H]DHS per ml, after resuspension into broth with 2.0 μg of unlabeled DHS per ml* (△——△). *Controls include cells resuspended into 2.0 μg of [^{3}H]DHS per ml* (●-·— ·—●) *and cells grown in the presence of 1 mM KCN before and after resuspension* (●——●).

Effect of transport mutations on DHS sensitivity in R$^+$ bacteria. It has been shown that NR-70, an Unc$^-$ ATPase$^-$ mutant of *E. coli* strain 7, has a reduced capability to transport aminoglycosides (4) probably due to an impaired protonmotive force (19). In turn, NR-70 has reduced susceptibility to various aminoglycosides including streptomycin when compared with strain 7. The effect of this reduced capability to transport [^{3}H]DHS by NR-70 on R$^+$-mediated DHS resistance was determined. R71(a) was introduced into NR-70 and strain 7, and MICs were determined. As shown in Table 6, the MIC of DHS for R$^+$ NR-70 was several times higher than for R$^+$ strain 7. To confirm that the AAD-(3″) activity was unchanged in strain NR-70, K_m values determined from Lineweaver and Burk plots and total activity of the enzyme from NR-70 and strain 7 were compared. The results in Table 6 demonstrate that enzyme activity was identical and that the differential resistance of the two strains was due to the differences in DHS transport between NR-70 and strain 7 previously demonstrated (4).

DISCUSSION

Resistance achieved by inactivation of less than 1% of the total amount of DHS or other aminoglycosides present in the growth medium by R-factor-specified aminoglycoside-modifying

enzymes suggests that these enzymes interfere with drug transport. DHS accumulation has been shown in this study to be significantly reduced in R^+ cells compared to R^- cells. This reduction could be due to blocking further accumulation, to increased efflux, or to a change in influx kinetics independent of blocked transport sites. Our results demonstrate that efflux of either modified or unmodified actively accumulated drug does not occur, and, thus, that explanation can be eliminated. Our data do not provide evidence for blocked transport but rather are consistent with an alternative mechanism which is discussed below.

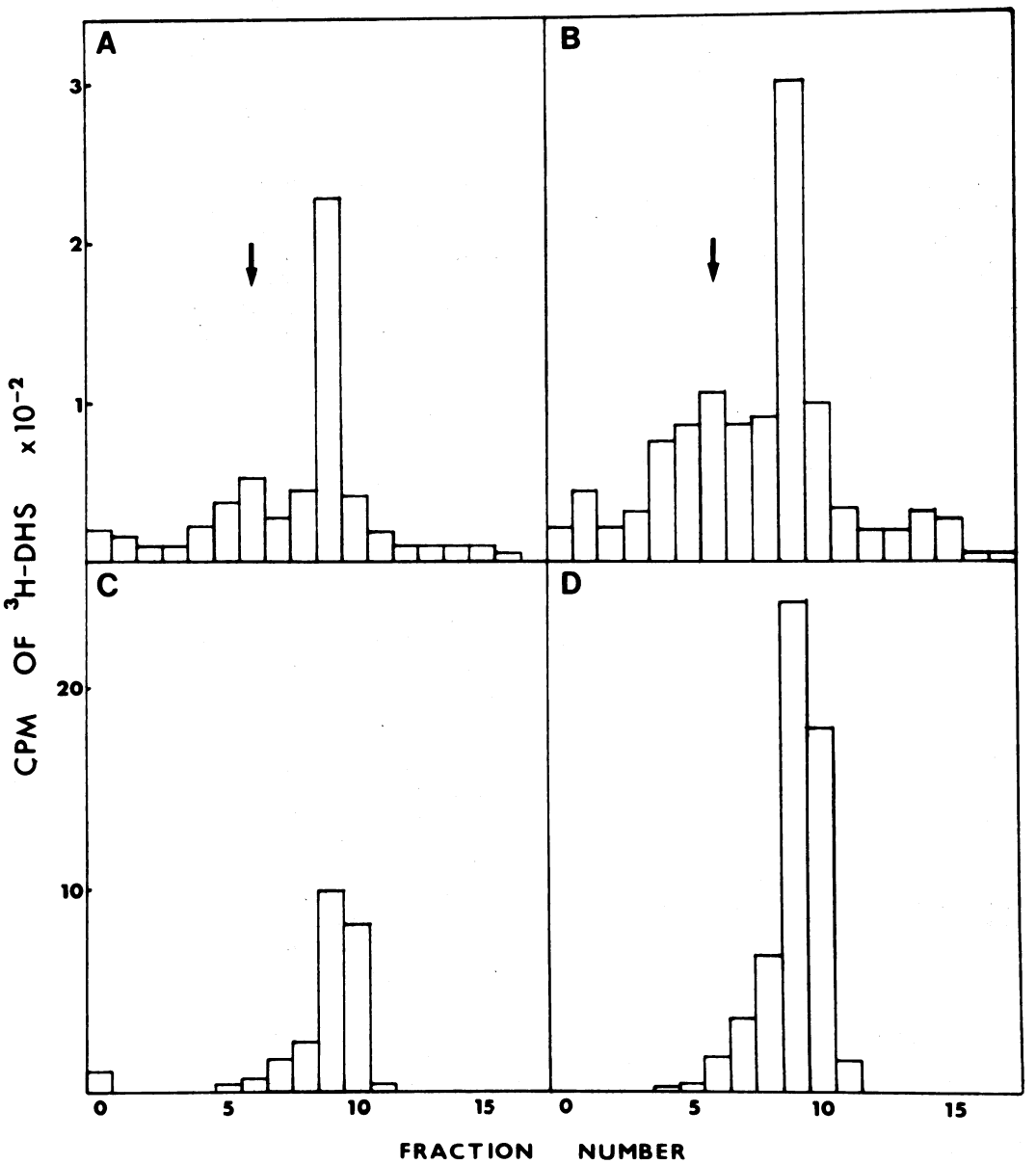

FIG. 4. *Paper chromatographic analysis of sonic extracts prepared from E. coli K-12 SA1306 R71(a) (A and B) (100 μl) and K-12 SA1306 (C and D) (20 μl) grown for 15 (A and C) and 30 (B and D) min at 37°C in nutrient broth with 1.0 μg of [^{3}H]DHS per ml. Fractions are 1-cm strips. 0 represents the origin. The arrows mark the peak migration of adenylylated DHS determined separately.*

The energetics and mechanics of aminoglycoside transport in susceptible bacteria have been studied and reported elsewhere (4). Accumulated evidence indicates that EDP-I represents the transport of drug across the cytoplasmic membrane to accessible ribosomal binding sites. Binding of drug by the ribosomes then initiates subsequent events such as enhanced transport (EDP-II), inhibition of protein synthesis, and the eventual onset of loss of cell viability. Recently, the enhanced transport has been proposed to be due to a polyamine transport system (9). All these events are absent in *strA* bacteria whose ribosomes fail to bind streptomycin. Our results confirm the absence of EDP-II in R^+ bacteria and the relationship of this effect and AAD-(3″) activity. Nonetheless, R^+ bacteria accumulate more drug during EDP-I than the amount required to initiate EDP-II in susceptible bacteria. Chromatographic analysis of cell-associated drug indicates that the proportion of DHS actively accumulated by R^+ bacteria is in the adenylylated form. If the only DHS transported and presented to ribosomes was adenylylated, this alone would explain DHS resistance because adenylylated DHS does not bind to ribosomes (23). It would also explain the reduction in DHS accumulation, because in the absence of ribosomal binding, just as in *strA* bacteria, EDP-II would not occur. If this is the sole factor causing resistance in these strains, it implies that the aminoglycoside transport mechanism in *E. coli* does not distinguish between DHS and its modified derivative and transport mechanisms are not otherwise affected. This last conclusion is in keeping with the previously described lack of specificity of initial EDP-I DHS transport. Experimental evidence from this study supports the above concept.

TABLE 5. *Release of DHS from E. coli strains after sonic disruption after periods of growth in NB with 1.0 μg of DNS per ml*

E. coli strains	Incubation time (min)	DHS (ng/OD_{600} per ml of whole cells)	
		Sonic extract[a]	Cell envelope
K-12 SA1306 R71(a)	15	5.6	1.0
	30	12.4	0.9
K-12 Sa1306	15	103	1.0
	30	266	2.1

[a] Sonic extracts and cell envelope fractions are the supernatant fluids and pellets, respectively, of 30-min, 40,000 × *g* centrifugations of disrupted cells. Sonic extracts were chromatographed as described in the legend to Fig. 4.

Comparisons were made between the distribution of drug accumulated after the EDP-I and EDP-II phases of transport in R^- bacteria and the EDP-I of R^+ bacteria. The data arising from cell fractionations and from autoradiography indicate no significant qualitative differences between the two cell populations. Fractionation of cells always resulted in the majority of drug being found in soluble fractions. Little could be traced to membrane fractions (~1%). Clearly there was no enrichment of R^+ membranes with drug of any form which would distinguish them from the membranes of R^- cells. Distribution patterns of drug in R^+ and R^- cells were the same in spite of differences observed in their accumulation kinetics profiles. Indeed the absence of EDP-II in R^+ bacteria was the only recognizable difference related to drug transport observed between the two strains in our studies. The rate of EDP-I in R^+ bacteria remained unchanged. This has been observed both in whole cells and spheroplasts. The results suggest two things: that the transport of drug by R^+ cells during EDP-I is similar to that in R^- cells and that the fate of the drug accumulated during the EDP-I and EDP-II phases is the same. Thus, it is likely that adenylylated drug is being actively transported to the ribosomes in R^+ bacteria during EDP-I.

Because EDP-I drug transport appears unaffected by antibiotic inactivation (and represents DHS being transported into the cell) then resistance must be dependent upon the inactivation of drug which gains access to the ribosomes. Under these conditions, resistance would depend on the capability to inactivate exceeding the

TABLE 6. *AAD-(3″) activity in aminoglycoside transport-deficient E. coli K-12 NR-70 compared with parent strain 7: effect on MIC for DHS*

Strain	DHS MIC (μg/ml)	AAD-(3″) activity (U/mg of protein)	K_m (M)
K-12 strain 7	0.03	—[a]	—
K-12 NR-70	0.24	—	—
K-12 strain 7 R71(a)	3.9	4.1×10^{-2}	2.4×10^{-5}
K-12 NR-70 R71(a)	15.6	3.8×10^{-2}	2.4×10^{-5}

[a] —, No activity.

capability to transport DHS. A prediction of this proposal is that the R^+ resistance level should be increased if the rate of DHS transport declines. We have shown here that a mutation reducing the rate of transport (strain NR-70) acts cooperatively with inactivating enzymes. Lundbäck and Nordström made a similar observation with other *E. coli* mutants (11). Conversely, if the rate of inactivation was reduced, the resistance would be expected to be decreased. This has been shown for mutant SR15. Also, Biddlecome et al. (2) have reported that in *E. coli*, a gentamicin acetyl transferase, which inactivates both gentamicin and tobramycin, is resistant to gentamicin alone. The reason apparently lies in the fact that the K_m is 10× lower and the V_{max} is 6× higher for gentamicin than for tobramycin. Presumably the inactivation of tobramycin cannot compete with the rate of its transport. The capability of aminoglycoside inactivation in bacteria to cause clinically significant resistance appears dependent on the relatively low rate of transport characteristic of EDP-I. Under these conditions, inactivating enzymes, whose activities are reportedly low also, can be successfully competitive.

Data obtained with other R-factors specifying aminoglycoside modification in mutants of *E. coli* and *Pseudomonas aeruginosa* with reduced or enhanced aminoglycoside accumulation rates also support our conclusion of resistance being the outcome of competition between rates of inactivation and transport. We have, for example, examined various R-factors in mutants of *P. aeruginosa* PAO which initiate transport of aminoglycosides at reduced aminoglycoside concentrations and show faster transport at equivalent concentrations (compared with the parent). Resistance levels found with the R-factors in the mutants are 1/4 to 1/10 that seen with the R-factor in the parent.

Only that fraction of DHS transported during EDP-I need be inactivated to ensure resistance. EDP-I declines in rate after 30 to 60 min, and no further uptake occurs after 60 to 120 min (4). An equilibrium is eventually established apparently due to saturation of available transport and binding sites at any given drug concentration. Such an equilibrium is usually reached when between 0.5 and 1% of the total drug present has been accumulated. Thus, only 0.5 to 1% of all drug need be inactivated, and complete detoxification of the medium is not required to achieve resistance. Small amounts of detoxified drug can be detected after a 30-min exposure of whole cells to DHS (see Fig. 4). This fraction represents <0.5% of the total DHS in the medium and corresponds closely to the amount actively accumulated during EDP-I after 30 min.

A physical as well as a functional association of AAD-(3″) and the aminoglycoside transport system may exist. Davies and Benveniste have localized aminoglyside-inactivating enzymes in general to the cytoplasmic membrane. Our results are in agreement, at least for AAD-(3″) in *E. coli*, and suggest further that the association is with the outer surface of this membrane. Retention of AAD-(3″) activity by spheroplasts is not likely the result of the localization of enzyme with contaminating elements of cell wall material. The fact that the enzyme remains functionally active in spheroplasts supports the belief that it occupies an in situ position. Its partial accessibility to impermeable chemical reagents in spheroplasts demonstrates that it is external to the membrane permeability barrier and indicates an association with the outer membrane surface in whole cells. However, the enzyme can only be tenuously bound because isolated membrane vesicles are without activity. Its activity is not lipid dependent, and it is releasable during osmotic shocking, thus indicating a distinction from periplasmic and intrinsic membrane proteins.

It is now possible to propose a sequence of events occurring in R^+ cells growing in the presence of DHS. As in susceptible cells, drug is probably immediately bound electrostatically to anionic components of the cell wall and then to sites deeper in the cell envelope, including the cell membrane. Energy is then required for the active and irreversible accumulation of drug bound to these inner membrane sites. Inactivation of the antibiotic must follow the first step in the process of irreversible binding because modified drug is not found in detectable quantities in the environment. This first step probably involves the electrostatic binding of drug to membrane sites (probably polar heads of phospholipids) and as such is available for transport. When electron transport is functional, the drug is transported across the membrane at a rate exceeding its dissociation from these sites. If electron transport is inhibited by the addition of KCN, then this population of drug is slowly lost from the cells (see Fig. 3). As inactivation rates exceed the rate of transport during EDP-I, only inactivated drug is presented to the ribosome at the inner surface of the membrane. Failing to bind to ribosomes, the drug accumulates until available cellular sites are saturated: an equilibrium is reached between the external cell concentration and cell binding sites. Net transport and inactivation would then cease.

Our observations suggest that transport of aminoglycosides occurs normally in R^+ bacteria

and there is a failure to distinguish between modified and unmodified drug. Consistent with this, it has been shown that bacteria that modify gentamicin but not streptomycin when exposed to the two antibiotics simultaneously remain susceptible to streptomycin. Streptomycin transport is presumably not affected by the resistance mechanism active against gentamicin. The reverse situation also occurs in that R-factors modifying streptomycin like R71(a) do not affect susceptibility to gentamicin or other aminoglycosides. One important consideration from a clinical standpoint can be made at the present time. With the resistance mediated by inactivating functions being dependent on low rates of transport during EDP-I, means by which these rates can be increased would be advantageous in counteracting the resistance mechanism. For this reason a better understanding of the mechanisms of aminoglycoside transport in bacteria has much possible practical significance.

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