Lack of Accumulation of Exogenous Adenylyl Dihydrostreptomycin by Whole Cells or Spheroplasts of *Escherichia coli*

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Accumulation of purified adenylylated dihydrostreptomycin (DHS-AMP) was examined in two strains of *Escherichia coli*. *E. coli* JSRO-N was plasmid free and aminoglycoside (AG) susceptible; *E. coli* JSRO-N(pSAD1) contained a plasmid-encoded AG adenylyltransferase which modifies DHS and streptomycin and confers resistance to both of these drugs. Although both whole cells and spheroplasts of JSRO-N accumulated free DHS, we were not able to demonstrate uptake of DHS-AMP by this strain. Whole cells and spheroplasts of JSRO-N(pSAD1) accumulated DHS at a much slower rate than that observed in JSRO-N. This was presumably due to the activity of the adenylyltransferase in JSRO-N(pSAD1). However, this low rate of accumulation of DHS was still higher than the uptake of DHS-AMP by either JSRO-N or JSRO-N(pSAD1). Thus, the rate of accumulation of DHS-AMP was even lower than that of DHS during the slow, initial, energy-dependent phase of AG uptake seen in JSRO-N(pSAD1). We also found that when either JSRO-N or JSRO-N(pSAD1) was incubated with barely inhibitory or subinhibitory concentrations of DHS, rapid uptake of DHS could be stimulated by the addition of an inhibitory concentration of another AG, such as amikacin. Uptake of DHS-AMP could not be similarly enhanced by the addition of amikacin. Our results indicate that DHS-AMP is not accumulated by whole cells or spheroplasts of *E. coli*. These results are consistent with the postulated intracellular location of AG-modifying enzymes.

Among clinical isolates of aerobic gram-negative bacteria, the two most important mechanisms of resistance to aminoglycoside (AG) antibiotics are reduced uptake and enzymatic modification of these drugs. The current conception of AG uptake has been described principally by Bryan and colleagues (1-5) and Dickie et al. (8). AG resistance in clinical isolates that lack AG-modifying enzymes has been presumed to be due to a reduction in the amount of drug that can bind to the ribosome as a result of diminished rate of AG uptake into the cytoplasm (1). Such resistance is often low level and generally affects all AGs equivalently. Although the rate of AG uptake determines the intrinsic level of AG susceptibility, a more important role in AG resistance may be played by AG-modifying enzymes (7). These enzymes confer high-level resistance to AG substrates by converting them to derivatives which are inactive in inhibiting ribosomal protein synthesis (14). Unlike the nonspecific low-level resistance due to decreased AG uptake, high-level resistance due to AG modification is usually transferable because it is encoded on plasmids or transposable elements.

For a bacterial strain containing an AG-modifying enzyme, Bryan (1) and Bryan and Kwan (3) formulated a model which described the level of resistance to AGs as the result of a competition between the rate of AG uptake into the bacterium and the rate of enzymatic modification of the AG. According to this "rate competition" model, if the rate of modification is sufficiently greater than the rate of uptake, then all of the drug is modified before it can reach the ribosome and thus the bacterium is resistant. If, on the other hand, uptake is sufficiently rapid, some free drug escapes inactivation and is able to bind to the ribosomes. When

enough free drug has bound to the ribosomes, the bactericidal sequelae of drug action follow. The factors affecting the rate of modification include the K_m and V_{max} of the enzyme for the given AG substrate, the total activity per cell, and, presumably, the cellular location of the AG-modifying enzyme.

Bryan's earlier statement of the model (1) considered that the AG-modifying enzymes lie on the external surface of the cytoplasmic membrane and are thus exposed to the concentration of AGs in the external medium. However, Perlin and Lerner found that at least one AG-modifying enzyme, AG 3'-phosphotransferase II, resides intracellularly (12). Since modified AGs have been found in resistant cells, the hypothesis that AG-modifying enzymes are extracellular also led to the suggestion (1) that modified AGs are actively taken up into the cell. To test this hypothesis, we decided to investigate whether a modified AG can be accumulated by bacteria. We therefore examined uptake of adenylylated dihydrostreptomycin (DHS-AMP) by spheroplasts of an AG-susceptible *Escherichia coli* strain.

MATERIALS AND METHODS

Bacterial strains. *E. coli* JSRO-N (11) is susceptible to streptomycin (SM) (MIC, 3.12 µg/ml or 5 µM), DHS, and amikacin (AK) (MIC, 3.12 µg/ml or 5 µM). *Pseudomonas aeruginosa* 83-37, a clinical isolate obtained from the Clinical Microbiology Laboratory of the University of Chicago Hospitals, had been shown to possess SM adenylylating activity. *E. coli* JSRO-N(pSAD1) is a transformant of JSRO-N which possesses the plasmid and SM adenylylating activity of *P. aeruginosa* 83-87. JSRO-N(pSAD1) is susceptible to AK (MIC, 3.12 µg/ml or 5 µM) and resistant to SM (MIC, 200 µg/ml or 0.34 mM).

Chemicals and reagents. DHS was purchased from Pfizer, Inc., New York, N.Y.; streptomycin sulfate from Sigma Chemical Co., St. Louis, Mo.; ATP from P-L Biochemicals, Inc., Milwaukee, Wis.; [³H]dihydrostreptomycin sesquisul-

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fate ($[^3\text{H}]\text{DHS}$; 1.8 Ci/mmol) and $[^{14}\text{C}]\text{ATP}$ (55 mCi/mmol) from Amersham Corp., Arlington Heights, Ill.; Sephadex G-10 from Pharmacia Fine Chemicals, Piscataway, N.J.; phosphocellulose powder from ICN Pharmaceuticals, Inc., Cleveland, Ohio; phosphocellulose filters (P81) from Whatman Ltd., London, England; and DNase I (1,588 U/mg) from Millipore Corp., Freehold, N.J.

Preparation of streptomycin adenyltransferase. *P. aeruginosa* 83-37 cells were grown at 37°C in brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.) with shaking and were harvested in the late logarithmic phase of growth. Sonic extracts were made as described previously (11). The supernatant was stored at -80°C.

Enzymatic formation of DHS-AMP. The reaction mixture for the generation of $[^3\text{H}]\text{DHS-AMP}$ included in 1.0 ml: 40 mM Tris-hydrochloride (pH 8.0), 9 mM MgCl_2 , 3 mM dithiothreitol, 2 mM $[^3\text{H}]\text{DHS}$ (specific activity, 12.5 $\mu\text{Ci}/\mu\text{mol}$), and 20 mM ATP. A sonic extract of *P. aeruginosa* 83-37 was added to a final protein concentration of ca. 2 mg/ml, and the reaction was carried out at 37°C. To obtain DHS- $[^{14}\text{C}]\text{AMP}$, we carried out the same reaction, but 2 mM DHS and 20 mM $[^{14}\text{C}]\text{ATP}$ (specific activity, 1.16 $\mu\text{Ci}/\mu\text{mol}$) were substituted for $[^3\text{H}]\text{DHS}$ and ATP, respectively.

The production of $[^3\text{H}]\text{DHS-AMP}$ was monitored by radioenzymatic assay (REA) (11) of decreasing residual-free DHS; a sonic extract of *P. aeruginosa* 83-37 and 2 mM $[^{14}\text{C}]\text{ATP}$ (specific activity, 8 $\mu\text{Ci}/\mu\text{mol}$) were used. The production of DHS- $[^{14}\text{C}]\text{AMP}$ was monitored by counting the radioactivity adsorbed to phosphocellulose filter disks after applying aliquots of the reaction mixture and washing them; this was similar to the procedure for REA.

Purification of DHS-AMP. A phosphocellulose column (75 cm by 2.8 cm²) was equilibrated with 0.1 M sodium cacodylate (pH 7.0). The entire reaction mixture for the formation of $[^3\text{H}]\text{DHS-AMP}$ or DHS- $[^{14}\text{C}]\text{AMP}$ was applied directly to the phosphocellulose column. This was followed by a linear gradient of 0.5 to 3 M NaCl in 0.1 M sodium cacodylate (pH 7.0) in a total volume of 170 ml. Fractions (1 ml) were collected at a flow rate of 0.25 ml/min, monitored for radioactivity, and assayed for free DHS by REA. We were thereby able to identify peaks of $[^3\text{H}]\text{DHS-AMP}$ and DHS- $[^{14}\text{C}]\text{AMP}$ separately from peaks of excess $[^3\text{H}]\text{DHS}$ and unlabeled DHS. The fractions of each peak were pooled, concentrated by lyophilization, and redissolved in a small volume of distilled water. Samples were then passed through a Sephadex G-10 column to remove NaCl.

Thin-layer chromatography. Chromatography of material from concentrated peaks was carried out on silica gel plates, as described previously (10). Migration of DHS and DHS-AMP was visualized by staining the plates with 0.25% ninhydrin in pyridine-acetone (1:1) and heating them at 105°C for several minutes. Each lane was examined for radioactivity by liquid scintillation counting of material scraped from visible spots and other selected sections.

Preparation of spheroplasts. *E. coli* JSRO-N was grown in PA salts minimal medium (11) supplemented with proline (50 $\mu\text{g}/\text{ml}$) and methionine (50 $\mu\text{g}/\text{ml}$). The method for preparation of spheroplasts was described previously (12). Spheroplast suspensions (>90% spheroplasts) were kept at 4°C and used on the day they were prepared.

Uptake of radiolabeled DHS and DHS-AMP. *E. coli* JSRO-N was grown in Trypticase soy broth (BBL) in side-arm flasks at 37°C in a shaker water bath until the turbidity of the culture reached 60 Klett units. $[^3\text{H}]\text{DHS}$ (specific activity, 60 $\mu\text{Ci}/\mu\text{mol}$), $[^3\text{H}]\text{DHS-AMP}$ (specific activity, 12.5 $\mu\text{Ci}/\mu\text{mol}$), or DHS- $[^{14}\text{C}]\text{AMP}$ (specific activity, 1.16 $\mu\text{Ci}/\mu\text{mol}$) was

added to the culture to a final concentration of 0.04 or 0.10 mM. Samples of 1.2 ml taken at 1, 5, 10, 15, 25, and 34 min were filtered through 25-mm-diameter Millipore filters (pore size, 0.45 μm) which had been prewashed with 5 mM DHS. After filtration of the culture aliquots, the filters were washed with 20 ml of 3% sodium chloride and dried, and the radioactivity on them was counted in a scintillation counter. When spheroplasts were tested for uptake, they were suspended in nutrient broth (BBL) containing 20% sucrose. Suspensions of whole cells and spheroplasts were examined microscopically in a Petrott-Hausser and Helber counting chamber, so that we could adjust the number of spheroplasts per milliliter to be equivalent to the number of whole cells per milliliter at 60 Klett units. After addition of radioactive DHS or DHS-AMP, aliquots were filtered through Millipore filters (pore size, 0.22 μm). The filters were washed and dried, and the radioactivity was counted, as for whole cells.

RESULTS

Generation of DHS-AMP. After 24 h of incubation, 78% of the initial DHS was converted to the ^{14}C -adenylylated form. A similar percentage (80%) of $[^3\text{H}]\text{DHS}$ was no longer detectable by REA after 25 h of incubation, presumably because of conversion to $[^3\text{H}]\text{DHS-AMP}$.

Purification of DHS-AMP. Phosphocellulose chromatography of the reaction mixture for the generation of $[^3\text{H}]\text{DHS-AMP}$ revealed two peaks of radioactivity eluted by the linear gradient of salt. The second peak was shown by REA to contain $[^3\text{H}]\text{DHS}$, but there was no detectable DHS in the first peak. Similar results were obtained with phosphocellulose chromatography of the reaction mixture for the generation of DHS- $[^{14}\text{C}]\text{AMP}$. Thin-layer chromatography (Fig. 1) shows the results from the formation of DHS-AMP. Lanes 1 and 6 are $[^{14}\text{C}]\text{ATP}$ (20 mM, 1.16 $\mu\text{Ci}/\mu\text{mol}$) and $[^3\text{H}]\text{DHS}$ (2 mM, 12.5 $\mu\text{Ci}/\mu\text{mol}$), respectively. DHS had an R_f of 0.69. The majority of ATP counts remained at the origin; the remainder trailed off to an R_f of ca. 0.31. Lane 2 shows the sample from the reaction used to generate ^{14}C -adenylylated DHS. The larger ninhydrin spot corresponds to DHS- $[^{14}\text{C}]\text{AMP}$. The smaller ninhydrin spot corresponds to free DHS and had only background radioactivity. A large amount of radioactivity remained at the origin; this probably corresponds to residual excess $[^{14}\text{C}]\text{ATP}$. Lane 3 shows material obtained after the purification of the DHS- $[^{14}\text{C}]\text{AMP}$. Values are similar to those in lane 2, except that we note the elimination of radioactivity in ATP at the origin. Lane 4 shows an aliquot from the reaction used to generate $[^3\text{H}]\text{DHS-AMP}$, and lane 5 shows $[^3\text{H}]\text{DHS-AMP}$ obtained after the first step of purification. The results for lanes 4 and 5 showed similar yield and purification of DHS-AMP as in lanes 2 and 3, respectively.

Uptake of DHS and DHS-AMP. Figure 2 illustrates the uptake of DHS and DHS-AMP at 0.04 mM by whole cells of *E. coli* JSRO-N. Fig. 2a resembles the pattern of uptake reported by several workers (1, 5, 9) for DHS. Fig. 2b and c show the results of experiments which test for uptake of $[^3\text{H}]\text{DHS-AMP}$ and DHS- $[^{14}\text{C}]\text{AMP}$, respectively. Despite fluctuations, we see that there is no progressive net uptake of either of these two modified AGs by whole cells of JSRO-N. Figure 3 shows the similar uptake experiments with spheroplasts of *E. coli* JSRO-N. DHS (Fig. 3a) was taken up quickly, reaching ca. 0.40 nmol/ml of the spheroplast suspension. There was little, if any, accumulation of DHS-AMP by spheroplasts over time (Fig. 3b and c). Although the points from 10 to 35 min in Fig. 3b may reflect

FIG. 1. Schematic representation of thin-layer chromatograms. All samples were spotted at the origin. Lanes: 1, 20 mM [^{14}C]ATP (specific activity, 1.16 $\mu\text{Ci}/\mu\text{mol}$) (5 μl); 2, from the [^{14}C]adenylation reaction (1 μl); 3, DHS- ^{14}C AMP after the phosphocellulose column step (20 μl); 4, from the reaction of [^3H]DHS adenylation (1 μl); 5, [^3H]DHS-AMP after the phosphocellulose column step (20 μl); 6, 2 mM [^3H]DHS (specific activity, 12.5 $\mu\text{Ci}/\mu\text{mol}$) (5 μl). Speckled areas show ninhydrin spots. Outlined areas were scraped off and counted for radioactivity; adjacent numbers indicate counts per minute.

uptake, the rate was considerably less than that found with DHS (Fig. 3a). The amount of modified drug associated with the spheroplasts never exceeded the level of the initial, energy-independent phase which corresponds to ionic binding of AGs to the cytoplasmic membrane. To determine the rate of DHS uptake in the absence of ribosomal binding, we also measured uptake of [^3H]DHS by whole cells and spheroplasts of *E. coli* JSRO-N(pSAD1), a transconjugant of JSRO-N which bears an enzyme which adenylates DHS in vitro. Both in whole cells and in spheroplasts of JSRO-N(pSAD1), respectively, [^3H]DHS was taken up at substantially lower rates than in whole cells and spheroplasts of JSRO-N (Table 1). However, the rates of uptake of [^3H]DHS-

AMP by whole cells and spheroplasts of JSRO-N were even lower than the corresponding rates for the uptake of [^3H]DHS by JSRO-N(pSAD1). There was no demonstrable uptake of [^3H]DHS-AMP by spheroplasts of JSRO-N(pSAD1).

The fate of [^3H]DHS incubated with JSRO-N(pSAD1) is illustrated schematically by the thin-layer chromatogram in Fig. 4. Lanes 1 and 2 are, respectively, [^3H]DHS and unlabeled DHS (12.5 μg). Lane 4 is purified [^3H]DHS-AMP (10 μl). Lane 3 is from a sonic extract of whole cells of JSRO-N(pSAD1) which were incubated with [^3H]DHS (0.10 mM, 30 $\mu\text{Ci}/\mu\text{mol}$) for 45 min. We see that the bulk of the radioactivity in lane 3 is associated with a spot which corresponds to purified DHS-AMP (lane 4), confirming that DHS-AMP is found internally in cells with an adenylating enzyme that are exposed to external DHS (8, 12).

The difference in ribosomal binding between DHS and DHS-AMP, and hence their differential stimulation of subsequent AG uptake, makes it difficult to compare the uptake of these compounds into *E. coli* JSRO-N, especially when DHS is at a higher inhibitory concentration. To circumvent this problem, we also examined above the uptake of these compounds into *E. coli* JSRO-N(pSAD1), in which neither the exogenous DHS-AMP nor the DHS, which is converted to DHS-AMP in the cell, can bind to the ribosomes and stimulate AG uptake. In another approach to reduce the influence on uptake of the differential binding of DHS and DHS-AMP to ribosomes, we examined the uptake of these compounds in the presence of an inhibitory concentration of AK, which should bind to the ribosomes and stimulate AG uptake. We incubated spheroplasts of JSRO-N with either [^3H]DHS (5 μM , 120 $\mu\text{Ci}/\text{mol}$) or [^3H]DHS-AMP (0.19 mM, 12.5 $\mu\text{Ci}/\mu\text{mol}$) and measured the uptake until 20 min after addition. Then we added AK to each suspension to a final concentration of 50 $\mu\text{g}/\text{ml}$ (0.08 mM) and continued uptake measurements. In addition, uptake of [^3H]DHS (0.19 mM, 17.5 $\mu\text{Ci}/\mu\text{mol}$) by JSRO-N(pSAD1) was measured both before and after addition of AK (final concentration, 50 $\mu\text{g}/\text{ml}$ or 5 μM). Figures 5 and 6 show that, although uptake of DHS was enhanced by exposure of both JSRO-N and JSRO-N(pSAD1) to an inhibitory concentration of AK, the rate of uptake of DHS-AMP into JSRO-N was not similarly enhanced.

DISCUSSION

We have found that whole cells and spheroplasts of *E. coli* JSRO-N accumulate [^3H]DHS but are unable to take up the adenylated DHS derivative. We examined uptake in spheroplasts to avoid possible interference with DHS-AMP uptake by an intact outer membrane and cell wall. The modified DHS was prepared with two different radioactive labels, in the DHA or the AMP moiety. This allowed us alternative

FIG. 2. Uptake of free DHS and DHS-AMP by whole cells of *E. coli* JSRO-N. (a) [^3H]DHS; (b) [^3H]DHS-AMP; (c) DHS- ^{14}C AMP.

FIG. 3. Uptake of free DHS and DHS-AMP by spheroplasts of *E. coli* JSRO-N. (a) $[^3\text{H}]\text{DHS}$; (b) $[^3\text{H}]\text{DHS-AMP}$; (c) $\text{DHS-}[^{14}\text{C}]\text{AMP}$.

means of following the production, purification, and eventual use of DHS-AMP in uptake experiments.

Our chromatographic results indicate that we were able to purify DHS-AMP from contaminating ATP and free DHS. Thus, we were able to ensure that uptake results for DHS-AMP were not complicated by the presence of radiolabeled ATP or DHS.

Bryan describes the kinetics of AG uptake by whole bacterial cells as multiphasic (1). The first, very rapid phase is ionic binding, which does not require energy. The next phase is energy dependent (EDPI) and represents AG transfer across the cytoplasmic membrane to the ribosome. After ribosome binding of the AG, uptake shifts to the more rapid energy-dependent phase (EDPII). For both EDPI and EDPII, electron transport to oxygen is the most effective energy source (1).

Uptake data in this study showed that whole cells and, to a greater extent, spheroplasts take up free DHS. EDPI was never clearly observed; the concentration of DHS in the uptake mixture was much higher than the MIC, so the shift to EDPII may have been so rapid that EDPI was missed. In contrast to the rapid uptake of DHS by whole cells, there was no appreciable uptake of DHS-AMP by these cells. Although spheroplasts did not appear to take up increased amounts of DHS-AMP with time, they initially bound half as much DHS-AMP as the final amount of DHS that was accumulated. The apparent increase in radioactivity associated with spheroplasts in uptake experiments with both DHS and DHS-AMP may reflect elimination of interference with their penetration to the plasma membrane binding sites by removal of the cell envelope. Since DHS-AMP does not bind to ribosomes (14), there could be no shift to EDPII. To establish comparable conditions for the uptake of DHS, we measured its uptake in *E. coli* JSRO-N(pSAD1), a strain

bearing an adenylating enzyme which modifies DHS. The only measurable uptake in such a strain should be equivalent to EDPI. This rate was still higher than the very low rates we obtained for the uptake of DHS-AMP by *E. coli* JSRO-N, the plasmid-free strain. In addition, if DHS-AMP could be taken up, we should have been able to stimulate its uptake into JSRO-N by the addition of inhibitory concentrations of another AG, such as AK, which was not susceptible to modification by the adenylating enzyme and would therefore bind to the ribosomes. However, we were unable to stimulate the uptake of DHS-AMP by the addition of AK, even though AK enhanced the uptake into both JSRO-N and JSRO-N(pSAD1) of free DHS at concentrations that were at or below the respective MICs of SM for those strains.

TABLE 1. Uptake of $[^3\text{H}]\text{DHS}$ or $[^3\text{H}]\text{DHS-AMP}$

Strain and cell type	Uptake ^a of:	
	$[^3\text{H}]\text{DHS}$	$[^3\text{H}]\text{DHS-AMP}$
<i>E. coli</i> JSRO-N		
Whole cells	8.9	0.1
Spheroplasts	19.5	1.7
<i>E. coli</i> JSRO-N (pSAD1)		
Whole cells	1.0	ND ^b
Spheroplasts	6.7	0.1

^a Uptake was measured at a specific activity of 12.5 $\mu\text{Ci/mol}$ and 0.1 mM final concentration of labeled AG; rates of AG uptake are expressed as picomoles of AG taken up per milligram (dry weight) of bacteria per minute; rates were calculated from linear least-squares analyses of the data.

^b ND, Not done.

FIG. 4. Schematic representation of thin-layer chromatograms. All samples were spotted at the origin. Lanes: 1, $[^3\text{H}]\text{DHS}$ (specific activity, 26.1 Ci/mmol , 5 μl); 2, DHS (12.5 μg); 3, from a sonic extract of JSRO-N(pSAD1) incubated with 0.1 mM $[^3\text{H}]\text{DHS}$ (specific activity, 30 $\mu\text{Ci}/\mu\text{mol}$) at 37°C for 45 min (20 μl); 4, purified $[^3\text{H}]\text{DHS-AMP}$ (10 μl). Speckled areas show ninhydrin spots. Outlined areas were scraped off and counted for radioactivity; adjacent numbers indicate counts per minute.

The model for AG uptake described by Bryan and Kwan (1, 3) is a variation of the chemiosmotic hypothesis for transport described by Mitchell (10). The model described by Bryan proposes that AGs are driven across the cytoplasmic membrane by the cellular proton motive force, which is made up of the cross-membrane potential and pH gradient. Polycationic AGs are driven across the cytoplasmic membrane primarily by the magnitude of the cross-membrane potential. Bryan has proposed that membrane components of the electron transport chain such as quinones might serve as the "transporter" for AGs (1, 3). However, the evidence for such a direct role is inconclusive. The work of Damber and Epstein (6) more strongly suggests that electron transport components serve only an indirect role in AG transport, by creating and maintaining the cross-membrane potential, which serves as a driving force.

Perhaps the lack of DHS-AMP uptake is due to its lower net positive charge than that of DHS; DHS-AMP is thus less likely to be driven across the membrane by the cross-membrane potential. Alternatively, the DHS-AMP molecule may be poorly accommodated by the putative transport of DHS. One could argue that the transport of DHS-AMP (and, by analogy, other modified AGs) across the cytoplasmic membrane requires the assistance of the AG-modifying enzyme itself. If so, investigating the uptake of DHS-AMP in an AG-susceptible strain would not be appropriate. Therefore, we examined the uptake of DHS-AMP in a strain which adenylates DHS, *E. coli* JSRO-N(pSAD1). There was no demonstrable uptake of DHS-AMP by spheroplasts of this strain either.

We previously found that another AG-modifying enzyme, AG 3'-phosphotransferase II, was located inside spheroplasts (12). After exposure of such spheroplasts to an AG substrate of the enzyme, the modified AG was found within the spheroplasts. In addition to our evidence for an internal location of the enzyme, the finding of a modified AG within spheroplasts suggests that the free AG was modified after entry. It is not known whether the results on internal location of the AG phosphotransferase are applicable to AG adenylating enzymes. However, the data from the present study, which show the inability of both whole cells and

FIG. 5. Uptake of free DHS and DHS-AMP by *E. coli* JSRO-N after addition of AK (indicated by the arrows) to a final concentration of 50 μ g/ml (0.08 mM). JSRO-N was preincubated for 20 min with either (a) 5 μ M [3 H]DHS (specific activity, 120 μ Ci/ μ mol) or (b) 0.19 mM [3 H]DHS-AMP (specific activity, 12.5 μ Ci/ μ mol).

FIG. 6. Uptake of DHS by *E. coli* JSRO-N(pSAD1) after addition of AK (indicated by the arrow) to a final concentration of 50 μ g/ml (0.08 mM). JSRO-N(pSAD1) was preincubated with 0.19 mM [3 H]DHS (specific activity, 17.5 μ Ci/ μ mol) for 20 min.

spheroplasts to transport DHS-AMP, support the concept that this AG adenyltransferase, which is encoded by pSAD1, may be intracellular as well.

The "rate competition" model of AG resistance earlier proposed by Bryan (1) was revised (3) to accommodate the evidence (12) that AG 3'-phosphotransferase II is not at the external surface of the plasma membrane. Our current demonstration that DHS-AMP is not taken into cells or spheroplasts further supports the suggestion (3) that AG modification occurs after AG transport has been initiated. Thus, the amount of an AG that is accessible to an AG-modifying enzyme depends on the rate of AG uptake, which is a function not only of the external AG concentration but also of the uptake process.

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LITERATURE CITED

1. Bryan, L. E. 1982. Bacterial resistance and susceptibility to chemotherapeutic agents, p. 95, 146-159. Cambridge University Press, Cambridge.
2. Bryan, L. E., R. Haraphongse, and H. M. Van Den Elzen. 1976. Gentamicin resistance in clinical isolates of *Pseudomonas aeruginosa* associated with diminished gentamicin accumulation and no detectable enzymatic modification. *J. Antibiot.* 29:743-753.
3. Bryan, L. E., and S. Kwan. 1983. Roles of ribosomal binding, membrane potential, and electron transport in bacterial uptake of streptomycin and gentamicin. *Antimicrob. Agents Chemo-*

- ther. **23**:835-845.
4. Bryan, L. E., and H. M. Van Den Elzen. 1975. Gentamicin accumulation by sensitive strains of *Escherichia coli* and *Pseudomonas aeruginosa*. *J. Antibiot.* **28**:696-703.
 5. Bryan, L. E., and H. M. Van Den Elzen. 1977. Effects of membrane-energy mutations and cations on streptomycin and gentamicin accumulation by bacteria: a model for entry of streptomycin and gentamicin in susceptible and resistant bacteria. *Antimicrob. Agents Chemother.* **12**:163-177.
 6. Damper, P. D., and W. Epstein. 1981. Role of the membrane potential in bacterial resistance to aminoglycoside antibiotics. *Antimicrob. Agents Chemother.* **20**:803-808.
 7. Davies, J., and D. I. Smith. 1978. Plasmid-determined resistance to antimicrobial agents. *Annu. Rev. Microbiol.* **32**:469-518.
 8. Dickie, P., L. E. Bryan, and M. A. Pickard. 1978. Effect of enzymatic adenylation on dihydrostreptomycin accumulation in *Escherichia coli* carrying an R-factor: model explaining aminoglycoside resistance by inactivating mechanisms. *Antimicrob. Agents Chemother.* **14**:569-580.
 9. Høltje, J.-V. 1978. Streptomycin uptake via an inducible polyamine transport system in *Escherichia coli*. *Eur. J. Biochem.* **86**:345-351.
 10. Mitchell, P. 1966. Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. *Biol. Rev. Camb. Philos. Soc.* **41**:445-502.
 11. Perlman, M. H., and S. A. Lerner. 1979. Amikacin resistance associated with a plasmid-borne aminoglycoside phosphotransferase in *Escherichia coli*. *Antimicrob. Agents Chemother.* **16**:598-604.
 12. Perlman, M. H., and S. A. Lerner. 1981. Localization of an amikacin 3'-phosphotransferase in *Escherichia coli*. *J. Bacteriol.* **147**:320-325.
 13. Robinson, D. S. 1964. Oxidation of selected alkanes and related compounds by a *Pseudomonas* strain. *Ned. Tijdschr. Hyg. Microbiol. Serol.* **39**:303-316.
 14. Yamada, T., D. Tipper, and J. Davies. 1968. Enzymatic inactivation of streptomycin by R-factor-resistant *Escherichia coli*. *Nature (London)* **219**:288-291.