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Streptomycin Accumulation in Susceptible and Resistant Strains of *Escherichia coli* and *Pseudomonas aeruginosa*

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Streptomycin accumulation by susceptible strains of *Escherichia coli* and *Pseudomonas aeruginosa* has been shown to be prevented or inhibited by inhibitors of electron transport, sulfhydryl groups and protein synthesis, and agents that uncouple oxidative phosphorylation. Streptomycin is recovered from cells in an unchanged form and is intracellularly concentrated above extracellular concentrations. Accumulation kinetics are multiphasic; an initial phase which cannot be prevented by the above inhibitors is unable to cause inhibition of cell growth or loss of cell viability. Prevention of further phases of uptake does prevent these events. Inhibitor-susceptible accumulation is time dependent and begins almost immediately upon exposure of cells to streptomycin. Streptomycin accumulation remains energy dependent even when cells are losing acid-soluble [^{3}H]adenine, presumably through loss of permeability control. These results demonstrate that streptomycin accumulation necessary for inhibition of cell growth or cell death requires energy and is not a process of diffusion or secondary to membrane leakage. Streptomycin accumulation in ribosomally resistant mutants of *E. coli* and *P. aeruginosa* is similar in that both energy-independent and energy-dependent accumulation can be demonstrated. The total energy-dependent accumulation is, however, significantly lower than that in streptomycin-susceptible cells due to the absence of an additional energy-dependent phase of accumulation, which seems dependent on ribosomal binding of streptomycin. Ribosomally resistant strains can be shown to concentrate streptomycin accumulated by the energy-dependent process above the external concentration in nutrient broth but not in Trypticase soy broth. The energy-dependent accumulation can be saturated in the Str^r strain of *E. coli* in nutrient broth, implying limited accumulation sites.

Entry of aminoglycoside antibiotics into bacterial cells is not well understood despite repeated investigation of the problem. Hancock showed that uptake of streptomycin into *Bacillus megaterium* was linear with time and could be greatly reduced by chloramphenicol, low temperature, and anaerobic conditions (12). Streptomycin uptake into *Bacillus subtilis* was inhibited by the respiratory antagonist 2-*n*-heptyl-4-hydroxyquinoline at concentrations of the latter that did not affect the growth rate of the organisms (11). Anand et al. (2) had earlier demonstrated that streptomycin uptake into *Escherichia coli* strain W consisted of two phases, an observation not detected in *B. megaterium*. The initial primary phase of streptomycin uptake into *E. coli* W was rapid, being essentially complete at the time of the first sample. The magnitude of primary uptake was shown to be directly related to the external drug concentration (13). A secondary phase of uptake has been repeatedly shown to occur after primary uptake. However, considerable variation in the kinetics of this phase of uptake has been reported. A lag preceding secondary uptake has been reported in several studies (2, 9, 16) but has been minimal or absent in other reports (12, 13). It has been frequently assumed that primary streptomycin uptake represents ionic binding to the cell surface and that initial entry into cells is by diffusion (9, 10). It has been proposed that secondary uptake could be due to streptomycin-mediated damage of the membrane, which permits entry of the drug. This interpretation of secondary uptake has been supported by the studies of Anand et al. (1, 2) and Dubin et al. (9), who demonstrated that secondary-phase uptake was associated with several observations, indicating a loss of membrane permeability control including increased permeability to a β-galactoside and leakage of nucleotides.

Hurwitz and Rosano showed that [^{14}C]- streptomycin accumulation occurred in three parts,

which they interpreted to indicate to be surface adsorbtion, accumulation at the time of loss of viability, and accumulation by killed bacterial cells (13). They also proposed that the function of chloramphenicol was to prevent the synthesis of streptomycin permease. Several studies have reported that inhibitors of oxygen-dependent electron transport inhibit the action or uptake of streptomycin. These inhibitors include anaerobiosis (12, 15) and various inhibitors of electron transport and agents that uncouple oxidative phosphorylation. (3, 11, 12, 18; L. E. Bryan, M. S. Shahrabadi, and H. M. Van Den Elzen, Prog. Abstr. Intersci. Conf. Antimicrob. Agents Chemother., 14th, San Francisco, Calif., Abstr. 166, 1974). The results of these studies suggest that accumulation of streptomycin is an active process.

Many strains of *Pseudomonas aeruginosa* are resistant to the aminoglycoside antibiotics streptomycin and gentamicin but do not possess known inactivating enzyme or "resistant ribosomes." These strains have been shown to accumulate little or no streptomycin or gentamicin until external drug concentrations are approximately those that inhibit the growth of these bacteria (5, 18). Such results suggest that the mechanism of resistance is an impairment of permeability to these drugs. To investigate these strains further, a better understanding of the uptake of aminoglycosides is necessary. Therefore, we undertook the study reported here to assess the requirement for active uptake of streptomycin in *E. coli*, to develop a model system for the study of uptake of other aminoglycosides, and to examine uptake in *P. aeruginosa*, since impaired permeability of aminoglycosides seems an important cause of antibiotic resistance.

MATERIALS AND METHODS

Bacterial strains. *E. coli* K-12 SA1306 was obtained from K. E. Sanderson, University of Calgary. *P. aeruginosa* 280 *met*$^-$ is a methionine auxotroph of a strain isolated from the clinical laboratory of the University of Alberta Hospital, Edmonton, Alberta (7). Streptomycin-resistant mutants (Str^r) were selected from susceptible strains as colonies growing on Trypticase soy agar containing 2,000 μg of streptomycin per ml after overnight incubation at 37 C.

Chemicals. All chemicals used were of analytical grade wherever available. Sources of reagents were potassium cyanide (KCN) from Fisher Scientific Co., sodium azide (NaN_3) from J. T. Baker Chemical Co., and sodium amytal from Eli Lilly & Co. Ltd.; all others were from Sigma Chemical Co.

Media. Media used were Trypticase soy broth (TSB), Trypticase soy agar, and nutrient broth (NB) obtained from Baltimore Biological Laboratories (BBL). The medium used for anaerobic growth studies was prepared using the following ingredients (grams per liter): yeast extract (Difco), 5; tryptone (Difco), 15; dextrose (Difco), 5; L-cysteine, 0.25; NaCl, 2.5; and sodium thioglycolate, 0.3. The control medium for aerobic growth in these experiments was the same medium lacking thioglycolate and cysteine. Incubation and sampling was performed under a continuous stream of oxygen-free nitrogen.

Streptomycin uptake. Bacterial strains were grown at 37 C in TSB or NB until early to midlog growth phase using a New Brunswick reciprocal water bath shaker set at speed setting 5. Erlenmeyer flasks (125 ml) contained a maximum of 15 ml of culture per flask. Cell cultures were adjusted to 0.2 to 0.5 absorbancy units at 600 nm (A_{600}) (Beckman DBG spectrophotometer) per ml, and a mixture of [^{3}H]dihydrostreptomycin (Amersham/Searle, 3 Ci/mmol) with unlabeled streptomycin (BDH Pharmaceuticals, Toronto) was added to obtain various final concentrations of streptomycin. Trial experiments demonstrated no differences in results if streptomycin or dihydrostreptomycin was used as the unlabeled component. Final specific activities were from 40 to 100 dpm/ng of total streptomycin. Samples (1.2 ml) were removed and filtered through a 0.2-μm membrane filter (Sartorius, Göttingen, West Germany). Membrane filters were pretreated by filtering 2.5 ml of a solution of streptomycin (2,500 μg/ml). Pretreatment prevented variation due to different degrees of filter binding of extracellular unincorporated [^{3}H]streptomycin. Filters were washed with 20 ml of 3% (wt/vol) NaCl, dried at 60 C, placed in counting vials containing toluene counting solution (4 g of Omnifluor [New England Nuclear Corp.] per liter of toluene), and counted in a Beckman model LS-250 liquid scintillation counter. All determinations of accumulation represent total accumulation except that shown in Fig. 9, in which energy-independent accumulation of streptomycin has been subtracted.

Absorbance readings of uptake mixtures were monitored throughout uptake periods. Cell mass was determined using a predetermined relationship between absorbance and dry weight in which 1 A_{600} unit represents 0.33 mg (dry weight) of cells.

Cell viability. Cell viability after treatment of *P. aeruginosa* 280 or *E. coli* K-12 SA1306 in TSB with streptomycin plus or minus sodium azide or under anerobic conditions was determined in two ways. Bacterial suspensions were centrifuged at 10,000 $\times$ *g* (4 C) for 10 min, washed once with TSB, and resuspended to an A_{600} of 0.5, followed by dilution into TSB and the plating of 0.1-ml volumes on Trypticase soy agar in quintuplicate. Alternatively, cells were directly diluted into TSB, and 0.1 ml of various dilutions was plated onto TSB. Survival values were determined by determining the average viability for each of the experimental conditions.

Release of acid-soluble [^{3}H]adenine. *E. coli* K-12 SA1306 and *P. aeruginosa* 280 were labeled by adding [^{3}H]adenine to actively growing cells (A_{600} = 0.500) in TSB to a specific activity of 0.5 μCi/ml, and the suspensions were incubated at 37 C for 6 min in a New Brunswick reciprocal water bath shaker (speed

setting 5). Cells were removed and cooled rapidly to 4 C by swirling in an ice-water bath. Suspensions were centrifuged at 10,000 × *g* at 4 C for 10 min. Cells were washed once with TSB under the same conditions and subsequently resuspended to twice the original cell density in TSB (A_{600} about 1). Equal portions of resuspended cells were rapidly warmed to 37 C in separate flasks. Streptomycin (50 μg/ml) was added to one flask. A second flask without streptomycin served as a control. Aliquots (300 μl) were removed at specific times and centrifuged in a Beckman microfuge for 1 min, and 200 μl of the supernatant was removed to tubes held in ice containing 150 μl of 10% trichloracetic acid and 50 μl of 10 mg of bovine serum albumin per ml. After 10 min or more on ice, the tubes were centrifuged in a Beckman microfuge for 1 min, and 200 μl of the supernatant was removed to scintillation vials containing 10 ml of scintillation fluid (4). Counting was done in a Beckman model LS-250 liquid scintillation system.

RESULTS

Accumulation of streptomycin in susceptible *E. coli* and *P. aeruginosa*. Figure 1 and Figure 2 illustrate the kinetics of total streptomycin accumulation with time for *E. coli* K-12 SA1306 and *P. aeruginosa* 280 in TSB at concentrations of 10 and 50 μg of streptomycin per ml. Three phases of accumulation occur in streptomycin-susceptible strains under these conditions. The initial phase is characterized by an association of streptomycin with bacterial cells at the time the first sample (zero time) is taken. This phase is presumed to represent mainly ionic binding to the cell. The next uptake phase (second-phase uptake) varies in duration and rate depending on the streptomycin concentration. The rate of third-phase uptake is more rapid than that of the second. Second-phase uptake at 10 μg of streptomycin per ml and third-phase uptake at 10 and 50 μg of streptomycin per ml demonstrate linearity between accumulation of streptomycin and time. However, after long incubation times the rate of third-phase uptake declines and is no longer linear with time. At 50 μg of streptomycin per ml such a leveling of rate occurs in 30 to 45 min in TSB.

After 30 min of uptake the concentration of streptomycin accumulated by either type of bacteria is in excess of the extracellular concentration. Using a value of 2.7 μl of cell water per mg (dry weight) (19), the total cellular accumulations after 30 min of incubation for the *E. coli* strain in Fig. 1 are 44 and 666 ng/μl and, with zero time values subtracted, 41 and 629 ng/μl, respectively, compared to external concentrations of 10 and 50 ng/μl. For *P. aeruginosa* 280 with an external concentration of 10 ng of streptomycin per ml (Fig. 2a), the values are 66 ng/μl without zero time accumulation removed

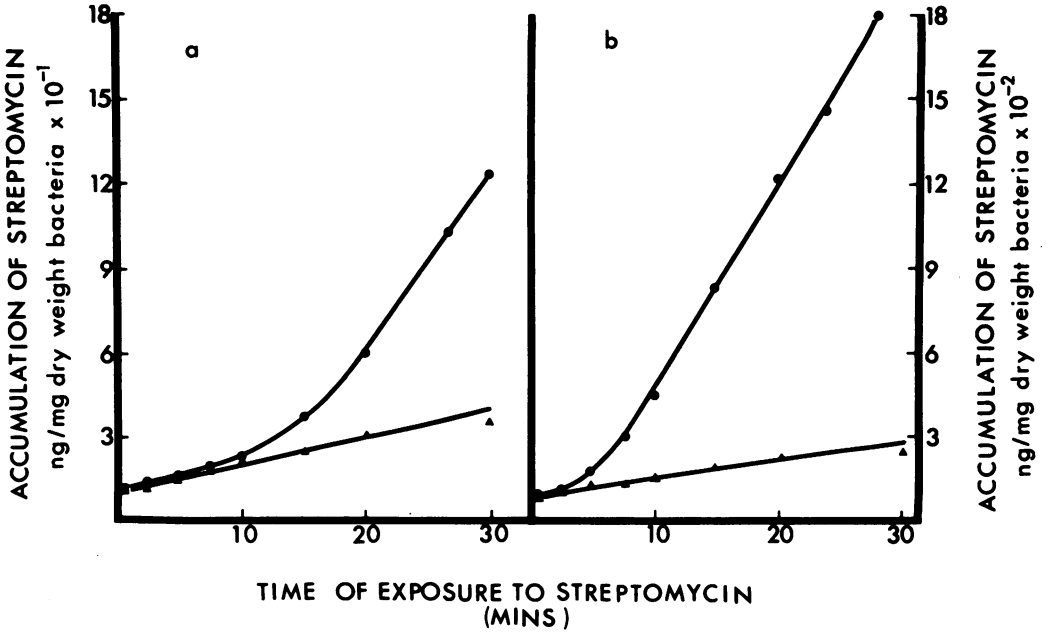

FIG. 1. *Streptomycin accumulation with time by E. coli K-12 SA1306 (●) at 10 μg (a) and 50 μg (b) of streptomycin per ml and by E. coli K-12 SA1306 Str^r (▲) at 10 μg (a) and 50 μg (1) of streptomycin per ml in TSB.*

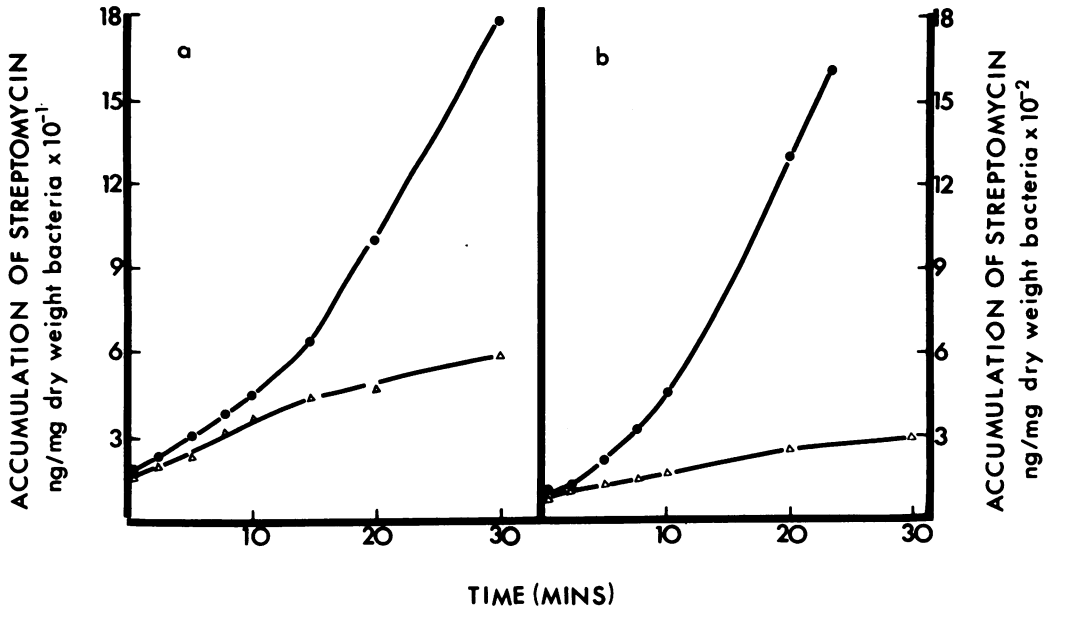

FIG. 2. *Streptomycin accumulation with time by P. aeruginosa 280 (●) at 10 μg (a) and 50 μg (b) of streptomycin per ml and by P. aeruginosa 280 Str^r (△) at 10 μg (a) and 50 μg (b) of streptomycin per ml in TSB.*

and 61 ng/μl with zero time subtracted. Intracellular concentrations 300 to 400 times the extracellular concentration can be obtained by using growth medium such as NB (BBL), which contains less magnesium and calcium than TSB.

Eighty to ninety percent of accumulated streptomycin can be extracted from cells by washing them with 10% trichloracetic acid at 20 C. Chromatography using Sephadex G-10 (Pharmacia, Uppsala, Sweden) demonstrates that extracted streptomycin emerges in the same fraction as a control preparation of streptomycin. This result indicates that the cell-associated streptomycin is recoverable in a form that has a molecular weight near to or identical to that of a control preparation of streptomycin. Extracted streptomycin has also been chromatographed using Dowex-50 by a method previously described (17) and shows no difference in elution pattern from a control preparation of streptomycin. The latter method has been used to differentiate streptomycin-phosphate and adenylyl-streptomycin from streptomycin (18). Thus, these data confirm that, within the limits of the methods used, the cell-associated streptomycin is identical to that of control streptomycin.

Relationship between release of acid-soluble [^{3}H]adenine and accumulation of streptomycin. A loss of permeability control for various metabolites such as amino acids and nucleotides has been reported after treatment of *E. coli* with streptomycin (1). To determine if this is related to the accumulation of streptomycin, the relationship between these two functions was examined. Figure 3 illustrates the results of these studies. Streptomycin accumulation is well advanced prior to detection of release of acid-soluble [^{3}H]adenine. From these data and the observation that streptomycin accumulation is energy dependent as late as 15 min, it seems unlikely that loss of permeability control for acid-soluble products containing [^{3}H]adenine accounts for the accumulation of a significant quantity of streptomycin.

Effect of metabolic inhibitors on accumulation of streptomycin. Table 1 gives the results of the addition to uptake mixtures at zero time of various inhibitors of electron transport, of agents which uncouple electron transport and oxidative phosphorylation, and a sulfhydryl group poison on the accumulation of streptomycin. Clearly, streptomycin accumulation is inhibited to various degrees by a variety of these agents. The results for the two types of bacteria are similar except for the effect of sodium amytal, which is a more effective inhibitor with *E. coli* K-12 SA1306 than *P. aeruginosa* 280. The results shown here are in keeping with our earlier reports on the effects of various inhibitors (18; Bryan et al., Prog. Abstr. Intersci.

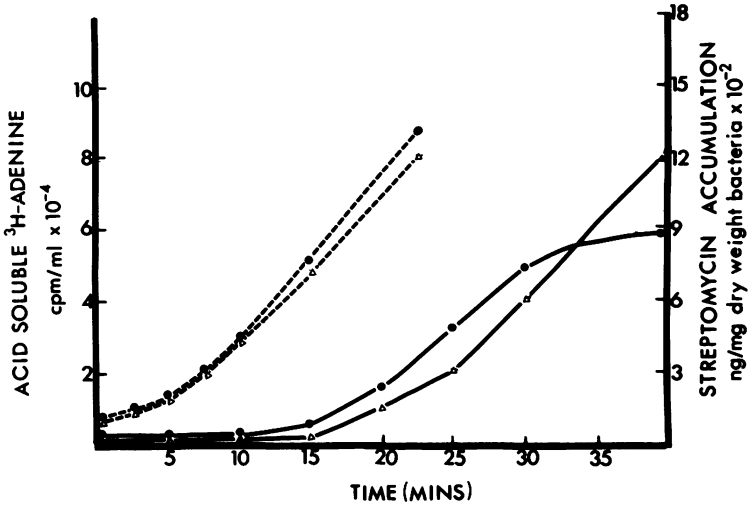

FIG. 3. *Release of acid-soluble [^{3}H]adenine to culture supernatant by E. coli K-12 SA1306 (Δ——Δ) and P. aeruginosa 280 (●——●) and corresponding accumulation of streptomycin by the E. coli (Δ- - -Δ) and P. aeruginosa (●- - -●) strains at 50 μg of streptomycin per ml in TSB at 37 C.*

TABLE 1. *Effect of metabolic inhibitors on streptomycin accumulation*[a]

Bacteria	Inhibitor[b]	Concn (mM)	Inhibition (%)	
			1[c]	2[c]
P. aeruginosa 280	4 C		58	90
	Oxamic acid	5	9	14
	Sodium amytal	5	4	6
	KCN	1	70	100
	NaN_3	0.1%	69	98
	DNP	2	34	46
	Sodium arsenate	50	50	75
	NEM	1	70	100
E. coli K-12 SA1306	4 C		74	98
	Oxamic acid	5	0	0
	Sodium amytal	5	45	60
	KCN	1	75	100
	NaN_3	0.1%	74	99
	Anaerobiosis			98
	DNP	2	70	94
	Sodium arsenate	10	65	87
	NEM	1	75	100

[a] Streptomycin concentration, 10 μg/ml; accumulation period, 15 min in TSB.
[b] DNP, 2,4-Dinitrophenol; NEM, *N*-ethylmaleimide.
[c] 1, Percentage of total uptake including zero time; 2, percentage of uptake after zero time value has been subtracted.

Conf. Antimicrob. Agents Chemother., 14th, San Francisco, Calif., Abstr. 166, 1974). These results demonstrate that streptomycin accumulation requires energy as well as a sulfhydryl group.

A requirement for a protein(s) or a functioning ribosomal cycle in streptomycin accumulation is demonstrated by the effect of chloramphenicol on streptomycin transport. It has been previously shown that chloramphenicol added before or at the time streptomycin was added to uptake mixtures would markedly reduce that streptomycin accumulation occurring in *E. coli* (13) after zero time (i.e., initial phase uptake is not prevented). We have confirmed this to be true for the *E. coli* strain used in these studies. However, using an NB uptake system in which streptomycin uptake is much increased (L. E. Bryan, manuscript in preparation) and thus is a more sensitive measure of streptomycin ac-

cumulation, it is possible to demonstrate that a low rate of streptomycin accumulation still occurs in cells to which chloramphenicol has been added either 10 min prior to or at the time of the addition of streptomycin (Fig. 4). The combination of the effects on streptomycin accumulation of metabolic inhibitors, *N*-ethylmaleimide, and chloramphenicol is similar to that seen with compounds like lactose, which are actively transported into bacterial cells.

The addition of KCN, sodium azide, or *N*-ethylmaleimide after 15 min of streptomycin accumulation stops further accumulation (Fig. 5). These results demonstrate that energy is required even after 15 min of streptomycin uptake at a time when a loss of permeability control for acid-soluble [^{3}H]adenine-containing metabolites is occurring. These results substantiate the observation noted above that streptomycin enters by an energy-dependent process at a time when permeability control of acid-soluble [^{3}H]adenine has been altered. The use of metabolic inhibitors does not eliminate initial phase uptake even at very high inhibitor concentrations. Table 2 shows that increasing concentrations of sodium azide up to 0.1% results in a reduction of streptomycin uptake. The amount of streptomycin associated with bacterial cells at 0.1% azide corresponds to values obtained if the initial sample ("zero time" sample) is taken as rapidly as possible after addition of streptomycin in the absence of azide. Thus, "zero time" samples appear to represent initial energy-independent streptomycin accumulation.

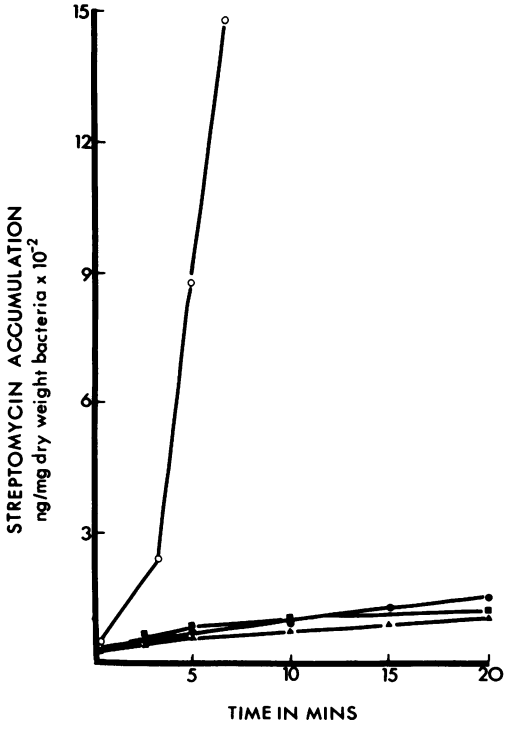

FIG. 4. *Streptomycin accumulation by E. coli K-12 SA1306 with time in NB (37 C) with streptomycin added at 0 min at a concentration of 10 μg/ml. Symbols: Streptomycin only (○); streptomycin + chloramphenicol (CM), both added at 0 min (50 μg of CM per ml, ●) (100 μg of CM per ml, ■); and streptomycin + CM, streptomycin added at 0 min and CM (100 μg/ml) at 10 min prior to zero (▲).*

Streptomycin accumulation and loss of cell viability or inhibition of cell growth. Sodium

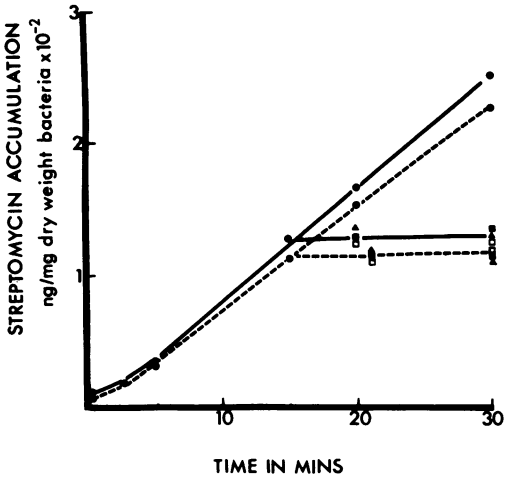

FIG. 5. *Streptomycin accumulation in TSB at a streptomycin concentration of 10 μg/ml by P. aeruginosa 280 (●——●) and E. coli K-12 SA1306 (●- - -●), with no additions or with 1 mM KCN (□——□, □- - -□), 1 mM N-ethylmaleimide (▲——▲, ▲- - -▲), or 0.1% NaN_3 (■——■, ■-----■) added after 15 min of streptomycin accumulation.*

TABLE 2. *Inhibition of streptomycin accumulation by sodium azide*[a]

Concn (%, wt/vol)	Cell-associated streptomycin (ng/mg, dry weight)
0.01	18.3
0.025	16.5
0.05	15.9
0.1	14.4
0.25	13.8
0.5	14.4
0.0, zero time[b]	15.0

[a] Strain used was *P. aeruginosa* 280.

[b] Sample was taken immediately after addition of streptomycin (10 μg/ml) to the uptake mixture held at 37 C.

azide treatment of *P. aeruginosa* 280 was used to determine the relationship of that portion of streptomycin accumulation requiring metabolic energy to loss of cell viability. Duplicate flasks of cells were preincubated at 37 C in TSB with 0.1% (wt/vol) sodium azide for 10 min. At this time streptomycin (200 μg/ml) was added to one of the azide-treated sets of cells and to another flask containing a cell count identical to that of azide-treated cells but that had not been treated with sodium azide. After an additional 60 min of incubation at 37 C, samples were taken from the three sets of cells and cell viability was determined as described above. Results given in Table 3 show that sodium azide prevented cell multiplication and that 50 to 67% of cells recovered from the treatment with sodium azide. Streptomycin treatment without sodium azide caused a 10^6-fold reduction in cell count, which was almost totally prevented by combining sodium azide with the streptomycin treatment. Similar studies were performed with *E. coli* K-12 SA1306, except that anaerobic conditions were used to inhibit oxygen dependent electron transport (Table 3). Cells treated with streptomycin (200 μg/ml) under anaerobic conditions retained nearly full viability, whereas the same streptomycin concentration under anaerobic conditions caused marked loss of viability. Figure 6 illustrates anaerobic protection against growth inhibition of the *E. coli* strain by lower streptomycin concentrations than those used in the experiments shown in Table 3. The corresponding streptomycin accumulation for each of the conditions is noted in brackets on Fig. 6. The protection of *E. coli* and *P. aeruginosa* against streptomycin induced lethality by these metabolic inhibitors, and the associated failure to accumulate streptomycin strongly suggests the protection is due to a failure to transport streptomycin. Kogut et al. have previously shown that streptomycin is active in inhibiting *E. coli* anaerobically if cells were initially exposed to streptomycin aerobi-

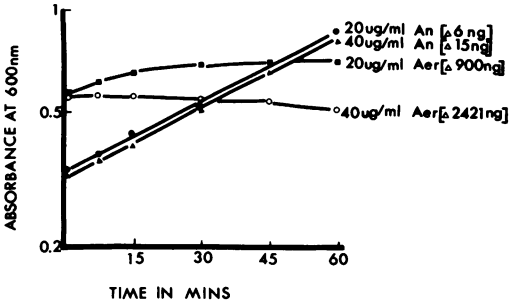

FIG. 6. *Growth of E. coli in the presence of streptomycin (20 or 40 μg/ml) under anaerobic (An) or aerobic (Aer) growth conditions and the amount of streptomycin accumulated in 60 min under each set of conditions (nanograms of bacteria per milligram [dry weight]).*

TABLE 3. *Viability of P. aeruginosa 280 and E. coli K-12 SA1306 after treatment with streptomycin in the presence and absence of sodium azide or under anaerobic growth conditions*

Bacteria	Conditions of treatment[a]	Cell counts/ml
P. aeruginosa 280	No additives, zero time	3×10^8
	1. 0.1% NaN_3, washed	1.5×10^8
	0.1% NaN_3, diluted	2×10^8
	2. Streptomycin, 200 μg/ml, washed	2×10^2
	Streptomycin, 200 μg/ml, diluted	2×10^2
	3. 0.1% NaN_3, streptomycin, 200 μg/ml, washed	1×10^8
	0.1% NaN_3, streptomycin, 200 μg/ml diluted	1.2×10^8
E. coli K-12 SA1306	Zero time, aerobic or anaerobic	2×10^8
	Anaerobic only	5×10^8
	Aerobic only	6×10^8
	Streptomycin, 200 μg/ml, aerobic	1.5×10^2
	Streptomycin, 200 μg/ml, anaerobic	4.7×10^8

[a] *P. aeruginosa* 280 was grown in TSB, 3×10^8 cells/ml (zero time). Cultures were divided into three equal portions and held at 4 C. Sodium azide (0.1%) was added to two portions (1 and 3), and these were incubated at 37 C for 10 min. Streptomycin was then added to portions 2 and 3, and all three preparations were incubated at 37 C for 60 min. Samples were removed, and viable counts were performed after washing with TSB or by direct dilution into TSB (see text). *E. coli* K-12 SA1306 was grown to 2×10^8 cells/ml in thioglycolate broth (anaerobic) or the same broth minus thioglycolate and cysteine (aerobic). Streptomycin was added to a set each of anaerobic and aerobic growth tubes. Control cultures for each growth situation contained no antibiotic. Additions to anaerobic cultures were performed using a continuous flow of oxygen-free nitrogen. The growth period was 60 min at 37 C. Viable counts were determined after dilution (aerobic broth) using the aerobic growth medium with 1.5% agar added and incubated in air at 37 C.

cally for short periods of time (15). In view of the recognized capability of *E. coli* cells to carry out protein synthesis anaerobically, it seems extremely unlikely that the protection by the above inhibitors results from an interference with the action of streptomycin on the ribosome.

The studies also demonstrate that initial phase uptake observed in zero time samples that is not eliminated by azide or anerobic conditions is insufficient to cause bacterial death. Energy-dependent uptake is necessary for cell death and is thus a measure of productive cellular streptomycin accumulation.

Streptomycin accumulation by ribosomally resistant strains. Streptomycin-resistant mutants of *E. coli* K-12 SA1306 and *P. aeruginosa* 280 were selected by growth on Trypticase soy agar containing 2,000 μg of streptomycin per ml. *P. aeruginosa* 280 Str^r was further characterized by demonstrating that an in vitro R17 ribonucleic acid-directed, amino acid-incorporating system, using ribosomes isolated from the mutant and the remainder of the bacterial components from the streptomycin-susceptible parent (for method, see reference 5), was unsusceptible to inhibition by streptomycin (100 μg/ml). Ribosomes from the resistant mutant bound significantly less [^{3}H]dihydrostreptomycin than those from the parent using an equilibrium dialysis system with polyuridylic acid as messenger ribonucleic acid, which has been previously described (14). The minimal inhibitory concentrations of streptomycin for both mutants in TSB were greater than 4,000 μg/ml.

The streptomycin-resistant mutants of either species accumulate significantly less streptomycin than the susceptible parents (Fig. 1 and 2). First- and second-phase uptake occurs in the resistant mutants, but there is no evidence of third-phase uptake. At 10 μg of streptomycin per ml, the rates of second-phase uptake for the parents and corresponding mutant are similar. This observation also seems true at 50 μg/ml, although for a much shorter period of time. Our studies have shown that the concentrations of magnesium and calcium in the uptake medium exert a marked effect on streptomycin uptake (manuscript in preparation). Uptake of streptomycin is enhanced in NB due to a low concentration (compared to other complex media) of these cations. The use of NB provides a more sensitive system for the measurement of streptomycin accumulation in the ribosomally resistant mutants and more clearly illustrates the effects of various conditions noted subsequently. A study of streptomycin uptake by the two ribosomally resistant mutants using NB established that even in the resistant strain streptomycin is concentrated within the cell. Figure 7 and Figure 8 illustrate the streptomycin uptake at 10 ng of streptomycin per μl using NB. After 30 min of streptomycin accumulation, the cell-associated streptomycin with zero time values subtracted is 12 and 28 times the external concentration of streptomycin for *E. coli K-12 SA1306 and P. aeruginosa* 280, respectively. Thus, intracellular concentration of the antibiotic does not appear dependent on the presence of "susceptible" ribosomes and the associated streptomycin binding sites. The concentration of antibiotic within Str^r strains is, however, reduced by a factor of 10 or 20 compared to the streptomycin-susceptible strains.

These data also demonstrate that *P. aeruginosa* 280 Str^r accumulates about four to five times as much streptomycin as *E. coli* K-12 SA1306 Str^r.

The results shown in Fig. 9 demonstrate that no significant increase in the amount of enery-dependent streptomycin accumulation by *E. coli* K-12 SA1306 Str^r occurs after the external streptomycin concentration reaches 1,000 μg/ml. The rate of increase of accumulated energy-dependent streptomycin is no longer linear with concentration after about a concentration of 250 μg of streptomycin per ml in the uptake mixture. Thus, at least some component of the streptomycin accumulation mechanism appears to be saturable. This is a property in

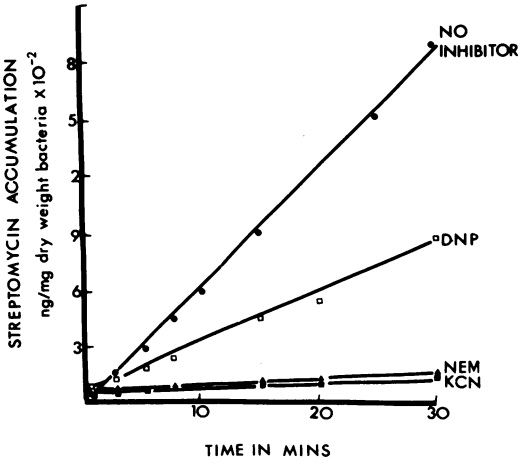

FIG. 7. *Accumulation of streptomycin in NB (BBL) at 37 C by P. aeruginosa 280 Str^r exposed to streptomycin alone (10 μg/ml) or with 2 mM 2,4-dinitrophenol(DNP; □), 1 mM KCN (■), or 1 mM N-ethylmaleimide (NEM; ▲). Streptomycin and inhibitors were added at 0 min.*

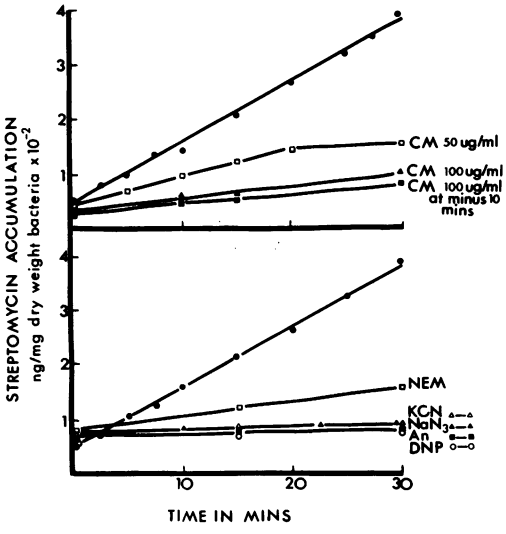

FIG. 8. *Accumulation of streptomycin in NB (BBL) at 37 C by E. coli K-12 SA1306 Str^r exposed to streptomycin alone (10 μg/ml) (●) or with one of the following: chloramphenicol (CM), 50 μg/ml (□) or 100 μg/ml (▲), added at zero time or 10 min prior to zero time (100 μg/ml, ■); 1 mM N-ethylmaleimide (NEM; □); 1 mM KCN (△); 0.1% NaN_3 (▲); 2 mM 2,4-dinitrophenol (DNP; ○) or exposed to streptomycin under anaerobic conditions (An, ■).*

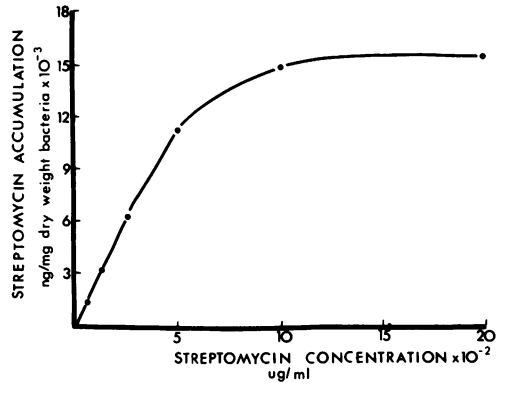

FIG. 9. *Energy-dependent accumulation of streptomycin by E. coli K-12 SA-1306 Str^r in NB (BBL) at 37 C at various streptomycin concentrations. Cells were exposed to various concentrations of streptomycin and [^{3}H]dihydrostreptomycin for 10 min in the presence and absence of 1 mM KCN. Energy-dependent accumulation was determined by subtraction of KCN values from those obtained without KCN after correction for accumulation per milligram of dry weight.*

keeping with a limited number of streptomycin transport sites. However, the external streptomycin concentrations required to produce this effect are high, and in TSB saturation is not obtained even at 5,000 μg of streptomycin per ml. This suggests a relatively low affinity transport mechanism in the streptomycin-resistant strain.

Second-phase streptomycin accumulation in *P. aeruginosa* 280 Str^r and *E. coli* K-12 SA1306 Str^r is inhibited by sodium azide, KCN, dinitrophenol, and *N*-ethylmaleimide (Fig. 7 and 8). Anaerobic conditions also prevented second-phase accumulation by *E. coli* strain SA1306 Str^r (Fig. 8). As with the susceptible strain, primary-phase uptake is not prevented by any of the inhibitors.

Chloramphenicol (50 μg/ml) also reduced uptake of streptomycin by *E. coli* K-12 SA1306 Str^r to about one-third that of the control (see Fig. 8). The amount of uptake is further reduced by a change in chloramphenicol concentration from 50 to 100 μg/ml. Preincubation of the strain with 100 μg of chloramphenicol per ml for 10 min prior to the addition of streptomycin causes a small further reduction in uptake.

DISCUSSION

The results of this study demonstrate that accumulation of streptomycin capable of inducing growth inhibition or loss of cell viability (productive accumulation) in streptomycin-susceptible strains of *E. coli* and *P. aeruginosa* requires energy. We have concluded from these results that the requirement for energy from electron transport is to transport streptomycin across the cytoplasmic membrane to the ribosomal binding site and not for ribosomal binding. There are several observations that, in our opinion, strongly support this conclusion. *E. coli* cells growing anaerobically generate adenosine 5'-triphosphate by substrate phosphorylation and clearly carry out protein synthesis. Under anaerobic conditions the ribosomal binding site should be available to streptomycin, providing streptomycin crossed the membrane. The failure to accumulate streptomycin anaerobically thus seems due to a failure to supply aerobically generated energy to transport streptomycin. Similarly it is well recognized that energy is not required to bind streptomycin to isolated ribosomes. Strictly anaerobic bacteria such as *Clostridium welchii* do not carry out oxygen-dependent electron transport, are resistant to streptomycin, and have ribosomes susceptible to streptomycin-mediated inhibition of in vitro amino acid incorporation (L. E. Bryan, unpublished data). The resistance of anaerobic bacteria seems clearly in keeping with a requirement for an energy-dependent streptomycin transport system.

Results reported here clearly demonstrate an

accumulation system in streptomycin-resistant *E. coli* and *P. aeruginosa* that shows the same susceptibility to inhibitors as the accumulation mechanism in susceptible strains. Thus, bacteria that have been shown to fail to bind streptomycin to their ribosomes (*P. aeruginosa* 280) possess an energy dependent phase of accumulation. This observation supports a requirement for energy to transport streptomycin and not to bind it to ribosomes. Clearly, Str^r strains accumulate less streptomycin and do so because of a failure of a much enhanced rate of accumulation (third-phase kinetics) to appear. However, second-phase, energy-dependent kinetics are nearly identical in Str^r and streptomycin-susceptible strains, suggesting that transport is similar in both types of cells and that what differs is a perturbation of streptomycin accumulation dependent on ribosomal binding (third-phase kinetics). Since the third phase of accumulation has been shown to be energy dependent, it seems highly improbable that it is the result of passive passage of streptomycin through a "leaking membrane." It also seems unlikely that the third phase is secondary to an induction of a streptomycin permease (13) because of the failure of the Str^r strain to show the necessary induction phenomenon.

An adequate explanation of third-phase accumulation in streptomycin-susceptible bacteria is difficult with the information available from the studies reported here. It appears that ribosomal binding of streptomycin is able to alter some property or properties of the membrane transport mechanism, causing it to transport streptomycin more rapidly. A consideration of the various ways by which this may happen requires the addition of more information, and firm conclusions are not possible on the basis of results reported here.

We have made several other observations not reported in this study that lend further support to our conclusions that energy is required for transport and not for ribosomal binding. One of the most significant of these is the effect of cations on energy-dependent streptomycin accumulation. Cations enhance in vitro binding of dihydrostreptomycin to both 70S ribosomes and 30S ribosomal subunits up to a concentration of about 10 mM magnesium (8). However, our observation has been that cations (Mg^{2+}, Ca^{2+}, Mn^{2+}, or Co^{2+}) act to reduce energy-dependent accumulation at external concentrations in NB as low as 0.1 mM either in whole cells of *P. aeruginosa* or *E. coli* or with spheroplasts of *E. coli*. Thus, the effect is the reverse of that noted on ribosomal binding. The effect of cations is unlikely to be a structural effect on the "gram-negative" cell wall, since whole cells of *Staphylococcus aureus* also exhibit the effect and the inhibitory effect is apparent within seconds of the addition of cations to uptake mixture (L. E. Bryan and H. M. Van Den Elzen, manuscript submitted for publication) containing *E. coli* or *P. aeruginosa*.

We feel that there is no good evidence to support the proposal that energy is necessary for streptomycin ribosomal binding. The effect of the Str^r mutant on accumulation can just as readily be looked on as a failure of streptomycin binding and the consequences of that step to modify the transport process.

The source of energy for the transport process is interesting. The results of the effects of various inhibitors demonstrate that electron transport is required. Anaerobic growth by *E. coli* should allow membrane energization by adenosine 5'-triphosphate hydrolysis through the membrane adenosine triphosphatase complex. Our results suggest that this source of energy can not be effectively used for streptomycin transport. The failure of adenosine 5'-triphosphate hydrolysis to supply energy for streptomycin transport suggests the energy source for streptomycin transport (and gentamicin [5]) must be coupled to membrane structures intimately related to membrane energization by electron transport. Thus it seems a reasonable possibility that streptomycin (gentamicin) transport could be due to an integral structure of the membrane complex coupling electron transport to membrane energization. Although the accumulation of streptomycin superficially meets most of the criteria normally applied to active transport of a compound such as lactose, it is not possible to conclude that streptomycin is concentrated in the absence of intracellular binding. Even in Str^r strains binding to nucleic acid would be expected. Thus, rather than active transport it seems wiser to apply the term active accumulation for streptomycin. Although we have proposed that gentamicin is accumulated by active transport, we now believe the same reservation should be applied to that process as well.

The results provided in this study and on gentamicin (5) and those we have observed for *Clostridium welchii* offer a rational explanation for the general pattern of susceptibility of bacteria to aminoglycosides. Strictly anaerobic bacteria are resistant, as would be expected because of their lack of oxygen-dependent electron transport. Similarly, facultative bacteria growing anaerobically are generally much more resistant to aminoglycosides. Facultative bacteria such as streptococci, which have susceptible ribosomes (20) and incomplete electron transport chains and carry out only limited

oxidative phosphorylation, are relatively resistant to aminoglycosides. Strictly aerobic bacteria such as mycobacteria or pseudomonas are generally sensitive to aminoglycosides. Most *P. aeruginosa* strains are relatively resistant to streptomycin, but strains like 280 exist that are exquisitely susceptible to the drug.

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