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Mechanism of Action of Habekacin, a Novel Amino Acid-Containing Aminoglycoside Antibiotic

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The molecular basis for activity of habekacin was studied by using *Escherichia coli* Q-13. Electron microscopic studies revealed that numerous blebs, derived from the outer membrane, were formed on cells treated with habekacin. Cytoplasmic contents leaked into the lumina of blebs, and the membrane of some enlarged blebs was disrupted. In a cell-free system, habekacin interfered with polypeptide synthesis, caused codon misreading, and inhibited translocation of *N*-acetylphenylalanyl-tRNA from the acceptor site to the donor site on ribosomes. [^{3}H]habekacin bound to both 50S and 30S ribosomal subunits. The current experiments indicated that the mechanism of action of habekacin is similar to that of 2-deoxystreptamine-containing aminoglycoside antibiotics such as dibekacin, kanamycin, gentamicin, and related substances. The relationship of membrane damage to inhibition of ribosomal functions remains to be determined

Habekacin, or 1-*N*-[(S)-4-amino-2-hydroxybutyryl]dibekacin (Fig. 1), is a new amino acid-containing aminoglycoside antibiotic with a broad antibacterial spectrum, including *Pseudomonas aeruginosa* and some kanamycin-, gentamicin-, and tobramycin-resistant organisms (4, 5). Habekacin is effective against experimental corneal ulceration due to *P. aeruginosa*. The activity is comparable to those of gentamicin and tobramycin (17). Clinical investigations on habekacin are now in progress in Japan.

Antibiotics of the aminoglycoside group show different mechanisms of action (for reviews, see references 14 and 18). Kasugamycin binds to the 30S ribosomal subunit and selectively inhibits initiation of protein synthesis (9, 10). Codon misreading is caused by streptomycin, kanamycin, gentamicin, and related aminoglycosides, but not by kasugamycin (16). Translocation of peptidyl-tRNA from the acceptor site to the donor site on the ribosome is blocked by kanamycin, gentamicin, and other 2-deoxystreptamine-containing aminoglycosides but is not significantly blocked by streptomycin (6). Kanamycin prevents translocation by fixing peptidyl-tRNA to the acceptor site but not to the donor site (7). Streptomycin selectively binds to the 30S ribosomal subunit (18), but kanamycin, neomycin, and gentamicin bind to both 30S and 50S ribosomal subunits (6). In some kanamycin-resistant mutants, the resistance is due to mutational changes of the 30S ribosomal subunit; in others, it is due to change in the 50S subunit (1), but streptomycin resistance is attributed exclusively to alteration of the 30S subunit (18).

Since the mode of action of amino acid-containing aminoglycoside antibiotics, such as amikacin, fortimicin, and related substances, has been not well investigated, we have studied the mechanism of action of habekacin, which might differ from that of other aminoglycosides. The results are presented in this paper.

MATERIALS AND METHODS

Habekacin, dibekacin, and [^{3}H]habekacin (44.4 mCi/mmol) were generously given us by Meiji Seika, Kaisha, Ltd., Tokyo. [^{14}C]phenylalanine (496 mCi/mmol) and [^{14}C]isoleucine (360 mCi/mmol) were purchased from Amersham, Tokyo, Japan. [^{14}C]leucine (340 mCi/mmol) was purchased from New England Nuclear Corp., Boston, Mass. GMPP(NH)P was from Boehringer, Mannheim, West Germany. All the other reagents were of the highest grade commercially available.

Washed ribosomes, ribosomal subunits, EF-G, and *N*-acetyl-[^{14}C]phenylalanyl-tRNA were prepared from *Escherichia coli* Q-13 as described previously (6, 7). The binding of [^{3}H]habekacin to ribosomes and ribosomal subunits was determined by equilibrium dialysis (6, 7).

Electron microscopic studies were carried out as previously reported (12) with some modifications. The cells of *E. coli* Q-13 were fixed by glutaraldehyde-OsO_4 fixation. A culture (10 ml) was poured into 1.1 ml of 3% glutaraldehyde in 0.05 M phosphate buffer (pH

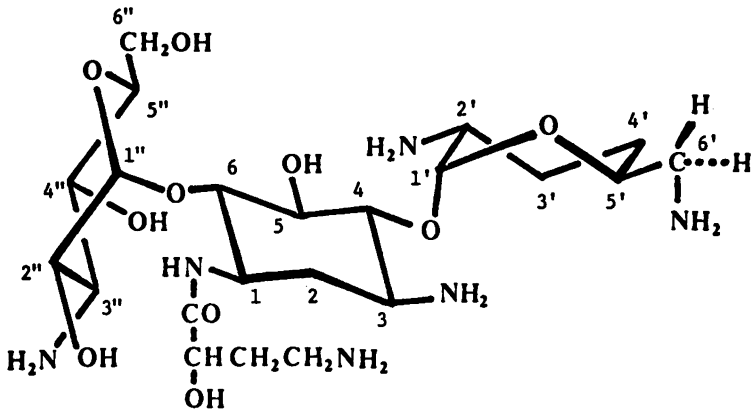

FIG. 1. Structure of habekacin.

7.0), and the cells were collected by sedimentation, transferred to fresh 3% glutaraldehyde, and kept for 2 h at room temperature, followed by washing several times in 0.05 M phosphate buffer. The cells were further fixed in 1% OsO_4 in the same buffer overnight in a refrigerator and were washed several times in distilled water. After being stained with 0.5% uranyl acetate for 2 h, the cells were embedded in a 2% agar

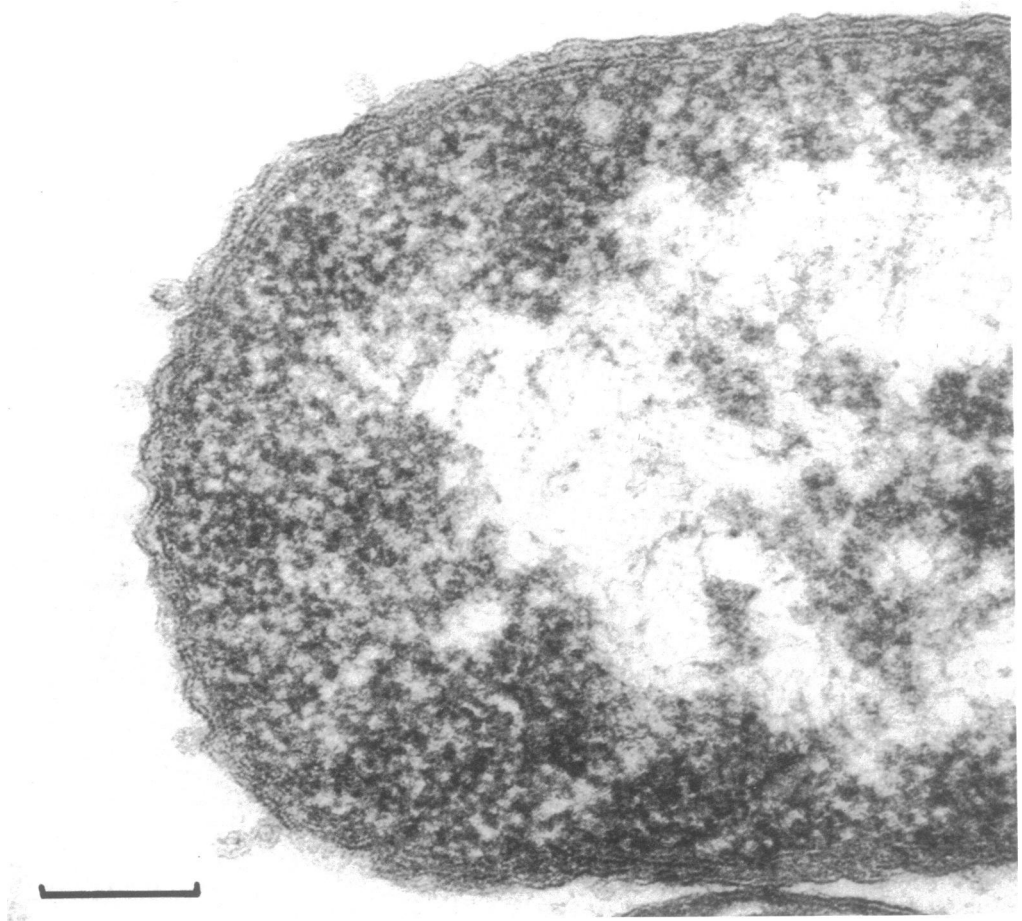

FIG. 2. Microphotograph of an ultrathin section of *E. coli* Q-13 treated with habekacin (25 μg/ml) for 2 h. Numerous blebs are seen on the cell surface. Bar, 0.2 μm.

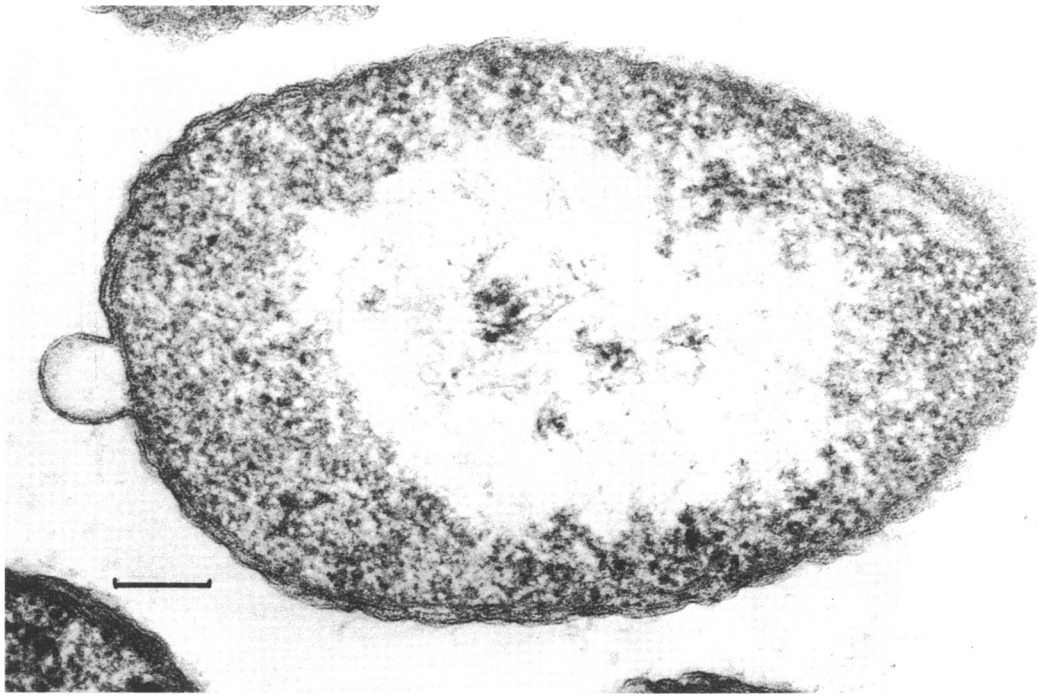

FIG. 3. Large bleb on the cell surface. The bleb membrane is derived from the outer membrane. Bar, 0.2 μm.

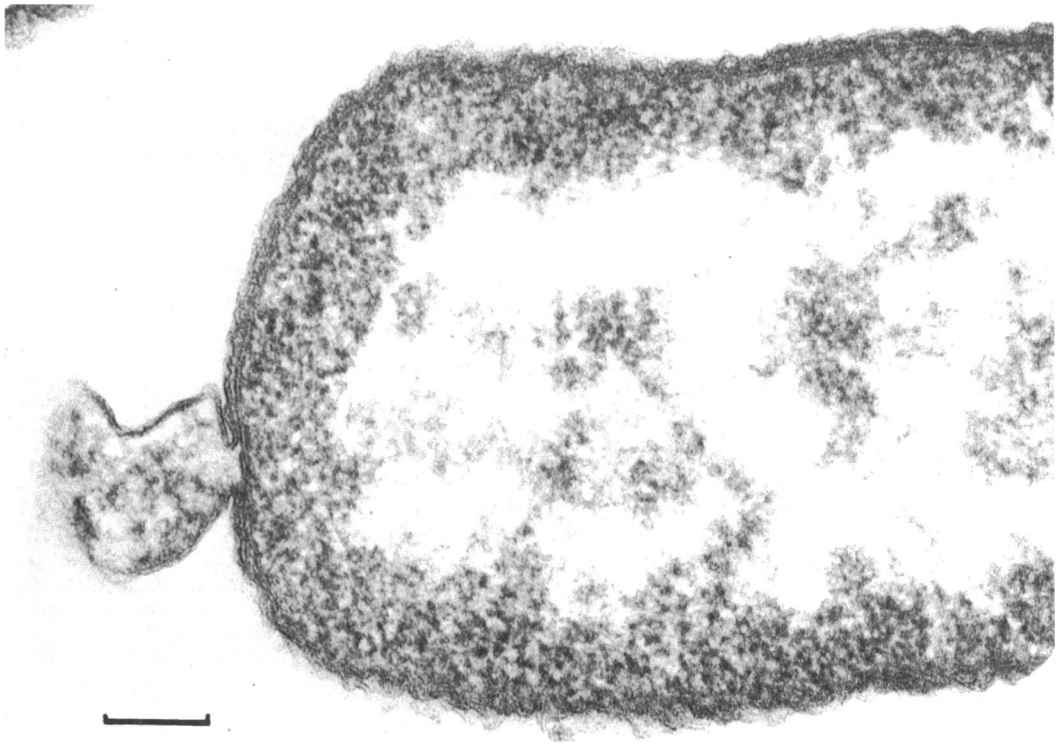

FIG. 4. Enlarged bleb. The bleb membrane is derived from the outer membrane, and the lumina is filled with cytoplasmic contents. Bar, 0.2 μm.

FIG. 5. Disruption of the membrane of an enlarged bleb. Bar, 0.2 μm.

block, dehydrated in graded solutions of ethanol and acetone, and embedded in Spurr resin (13). Ultrathin sections were cut with a DuPont diamond knife on a Porter-Blum MT-2 ultramicrotome, picked up on Formvar-coated single-slot copper grids, and stained with uranyl acetate and lead citrate (11). All sections were examined in a JEOL 200 CX electron microscope at 100 kV.

RESULTS

Morphological changes of *E. coli* induced by habekacin. Electron microscopic studies revealed that the most prominent morphological alteration of *E. coli* due to the action of habekacin was the appearance of blebs on the cell surface. The untreated cell, grown to exponential phase in heart infusion broth, was surrounded by an undulated cell wall with a smooth surface, and the cytoplasm was filled with ribosomes and a less electron-dense area of fibrous nuclear material (12). Some blebs appeared in 30 min, and numerous blebs were observed in 2 h on cells treated with habekacin (25 μg/ml). The membranes of the blebs were derived from the outer membrane, and cytoplasmic contents leaked into the lumina of blebs (Fig. 2 through 4). The membrane was disrupted in some enlarged blebs (Fig. 5). The results showed that habekacin causes membrane damage of bacteria, resulting in cell lysis.

Effect on polypeptide synthesis and codon misreading activity. The effect of habekacin on polypeptide synthesis was examined in comparison with dibekacin, using the *E. coli* ribosomal system. The results are presented in Table 1. Habekacin was observed to inhibit polyphenyl-

TABLE 1. Effect of antibiotics on polyuridylic acid-dependent polypeptide synthesis in cell extract obtained from *E. coli* Q-13[a]

Antibiotic	Concn (μM)	Relative incorporation (%)[b]		
		Phenylalanine	Isoleucine	Leucine
None		100	0.2	4.1
Habekacin	0.1	92	0.3	6.0
	1	39	1.2	7.9
	10	19	2.0	9.4
	100	24	5.4	17
Dibakacin	0.1	90	0.1	4.8
	1	49	0.5	5.1
	10	17	1.1	6.7
	100	16	1.7	9.7

[a] The reaction mixture contained, in 200 μl: ATP, 2 mM; phosphoenolpyruvate, 5 mM; pyruvate kinase, 4 μg; GTP, 0.2 mM; polyuridylic acid, 20 μg; tRNA, 20 μg; ribosome, 20 pmol; S-100 fraction, 100 μg of protein; ^{14}C-amino acid, 0.08 μCi; Tris-hydrochloride (pH 7.8), 50 mM; NH_4Cl, 80 mM magnesium acetate, 8 mM; 2-mercaptoethanol, 6 mM; and antibiotic, 0.1 to 100 μM, as shown. The mixture was incubated at 37°C for 20 min. The hot trichloroacetic acid-insoluble radioactivity, collected on a glass filter, was assayed with correction for values without messenger.

[b] Relative incorporation of ^{14}C-amino acid; 100% = 80 pmol per tube.

TABLE 2. Effect of antibiotics on *N*-acetylphenylalanyl-puromycin synthesis in the absence or presence of EF-G and GMPP(NH)P[a]

Antibiotic	Concn (μM)	*N*-acetyl-[^{14}C]phenylalanyl-puromycin formed (%)	
		Without EF-G and GMPP(NH)P[b]	Enhanced by EF-G and GMPP(NH)P[c]
None		100	100
Habekacin	0.1	97	97
	1	105	47
	10	102	15
	100	97	10
Dibekacin	0.1	105	102
	1	93	63
	10	95	19
	100	98	14

[a] The assay for peptidyltransferase reaction and translocation of peptidyl-tRNA was performed by acetylphenylalanyl-puromycin synthesis. The reaction mixture contained, in 0.2 ml: Tris-hydrochloride (pH 7.8), 50 mM; NH_4Cl, 150 mM; magnesium acetate, 20 mM; 2-mercaptoethanol, 6 mM; polyuridylic acid, 20 μg; ribosome, 50 pmol; and *N*-acetyl-[^{14}C]phenylalanyl-tRNA, 50 μg. It was incubated at 37°C for 40 min and cooled in an ice bath. Puromycin (0.2 mM) and the antibiotic were added to the mixture with or without 160 μg of EF-G and 10 mM GMPP(NH)P. The reaction was terminated by the addition of 1 ml of 0.1 M sodium acetate (pH 5.5) and was extracted with 1.5 ml of ethyl acetate. The radioactivity of the solvent layer was assayed in toluene–Triton X-100 scintillator.

[b] 100% = 0.9 pmol.

[c] 100% = 1.5 pmol.

alanine synthesis but to enhance incorporation of [^{14}C]isoleucine and [^{14}C]leucine in the presence of polyuridylic acid. The activity was comparable to that of dibekacin, showing that habekacin interferes with polypeptide synthesis and induces codon misreading in vitro.

Inhibition of translocation of *N*-acetylphenylalanyl-tRNA on the ribosome. *N*-Acetylphenylalanyl-puromycin synthesis by the ribosome with *N*-acetyl-[^{14}C]phenylalanyl-tRNA and puromycin in the absence of EF-G and GMPP(NH)P was employed as a model for the peptidyltransferase reaction, and the effects of antibiotics were examined (Table 2). The reaction was not significantly affected by habekacin or dibekacin at antibiotic concentrations up to 100 μM, suggesting that habekacin does not block peptidyltransferase reaction.

Translocation of *N*-acetylphenylalanyl-tRNA from the acceptor site to the donor site was observed by the puromycin reaction enhanced by the addition of EF-G and GMPP(NH)P (3, 7, 15). The stimulated reaction was markedly prevented by habekacin or dibekacin. Approximately 50% inhibition was observed at a habekacin concentration of 1 μM (Table 2). The results show that habekacin blocks translocation of peptidyl-tRNA on the ribosome.

Binding of [^{3}H]habekacin to ribosomes and ribosomal subunits. Equilibrium dialysis experiments for binding [^{3}H]habekacin to *E. coli* ribosomes and ribosomal subunits were performed over a range of concentrations of ^{3}H-labeled antibiotics, and the dependence on the concen-

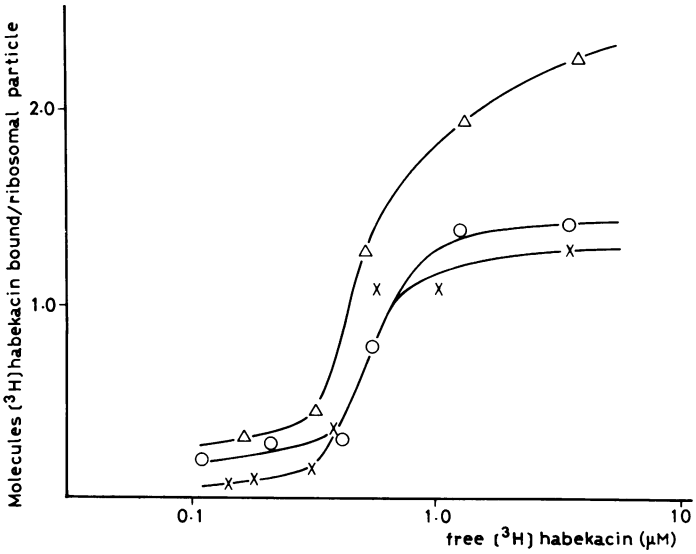

FIG. 6. Dependence on [^{3}H]habekacin concentration for binding to ribosomes and ribosomal subunits. Symbols: △, ribosome; ○, 50S subunit; ×, 30S subunit.

tration of [^{3}H]habekacin for its binding is illustrated in Fig. 6. [^{3}H]habekacin was demonstrated to bind to both 50S and 30S ribosomal subunits as well as ribosomes. Each subunit seemed to possess at least one binding site with an apparent association constant of ca. 10^6 M^{-1}.

DISCUSSION

The present experiments revealed that habekacin causes membrane damage and binds to both subunits of ribosomes, resulting in interference with protein synthesis, codon misreading, and inhibition of translocation. Since habekacin inhibits translocation and binds to both ribosomal subunits, the mechanism of action seems to differ from that of streptomycin but to be similar to that of 2-deoxystreptamine-containing aminoglycoside antibiotics such as kanamycin, gentamicin, and related substances (compare the reviews in references 14 and 18). The relationship of inhibition of ribosomal functions to membrane damage remains to be determined.

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LITERATURE CITED

1. **Choi, E. C., T. Nishimura, Y. Tanaka, and N. Tanaka.** 1980. Mutational alterations of either large or small ribosomal subunit for the kanamycin resistance. Biochem. Biophys. Res. Commun. **94**:755–762.
2. **Iida, K., and M. Koike.** 1974. Cell wall alteration of gram-negative bacteria by aminoglycoside antibiotics. Antimicrob. Agents Chemother. **5**:95–97.
3. **Kinoshita, T., Y.-Y. Liou, and N. Tanaka.** 1971. Inhibition by thiopeptin of ribosomal functions associated with T and G factors. Biochem. Biophys. Res. Commun. **44**:859–863.
4. **Kondo, S., K. Iinuma, H. Yamamoto, K. Maeda, and H. Umezawa.** 1973. Synthesis of 1-*N*-[(S)-4-amino-2-hydroxybutyryl]-kanamycin B and -3′,4′-dideoxykanamycin B active against kanamycin-resistant bacteria. J. Antibiot. **26**:412–415.
5. **Kondo, S., K. Iinuma, H. Yamamoto, K. Maeda, and H. Umezawa.** 1973. Syntheses of (S)-4-amino-2-hydroxybutyryl derivatives of 3′,4′-dideoxykanamycin B and their antibacterial activities. J. Antibiot. **26**:705–707.
6. **Misumi, M., T. Nishimura, T. Komai, and N. Tanaka.** 1978. Interaction of kanamycin and related antibiotics with the large subunit of ribosomes and the inhibition of translocation. Biochem. Biophys. Res. Commun. **84**:358–365.
7. **Misumi, M., and N. Tanaka.** 1980. Mechanism of inhibition of translocation by kanamycin and viomycin: a comparative study with fusidic acid. Biochem. Biophys. Res. Commun. **92**:647–654.
8. **Miyasaka, T., D. Ikeda, S. Kondo, and H. Umezawa.** 1980. Syntheses and properties of the 6″-deoxy or 4″,6″-dideoxy derivatives of the kanamycin antibiotics. J. Antibiot. **33**:527–532.
9. **Okuyama, A., N. Machiyama, T. Kinoshita, and N. Tanaka.** 1971. Inhibition by kasugamycin of initiation complex formation on 30S ribosomes. Biochem. Biophys. Res. Commun. **43**:196–199.
10. **Okuyama, A., N. Tanaka, and T. Komai.** 1975. The binding of kasugamycin to the *Escherichia coli* ribosomes. J. Antibiot. **28**:903–905.
11. **Reynolds, E. S.** 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. **17**:208–212.
12. **Someya, A., K. Tanaka, and N. Tanaka.** 1979. Morphological changes of *Escherichia coli* induced by bicyclomycin. Antimicrob. Agents Chemother. **16**:87–91.
13. **Spurr, A. R.** 1969. A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. **26**:31–43.
14. **Tanaka, N.** 1982. Mechanism of action of aminoglycoside antibiotics, p. 221–266. *In* H. Umezawa and I. R. Hooper (ed.), Aminoglycoside antibiotics. Springer-Verlag, Berlin.
15. **Tanaka, N., T. Kinoshita, and H. Masukawa.** 1968. Mechanism of protein synthesis inhibition by fusidic acid and related antibiotics. Biochem. Biophys. Res. Commun. **30**:278–283.
16. **Tanaka, N., H. Masukawa, and H. Umezawa.** 1967. Structural basis of kanamycin for miscoding activity. Biochem. Biophys. Res. Commun. **26**:544–549.
17. **Tanaka, Y.** 1981. Effects of habekacin, a novel aminoglycoside antibiotic, on experimental corneal ulceration due to *Pseudomonas aeruginosa*. J. Antibiot. **34**:892–897.
18. **Wallace, B. J., P. C. Tai, and B. D. Davis.** 1979. Streptomycin and related antibiotics, p. 272–303. *In* F. E. Hahn (ed.), Antibiotics, vol. 1: mechanism of action of antibacterial agents. Springer-Verlag, Berlin.