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Accumulation in Gram-positive and Gram-negative Bacteria as a Mechanism of Resistance to Erythromycin

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Erythromycin was recovered in high yield after incubation with gram-negative bacteria. The cell-free protein-synthesizing preparation from gram-negative bacteria is equally as susceptible to the antibiotic as is that from gram-positive bacteria. Thus, neither destruction of erythromycin nor the absence of the step susceptible to the antibiotic plays an important role in the resistance mechanism of gram-negative bacteria. A 100-fold difference in accumulation of erythromycin between gram-positive and gram-negative bacteria was observed. This alone explains the resistance of gram-negative bacteria to erythromycin. Furthermore, data showed that the inhibition of growth is closely related to the accumulation of erythromycin. The concentration of intracellular erythromycin in gram-positive bacteria was found to be 44- to 90-fold greater than that of the extracellular medium. However, the antibiotic did not accumulate on the cell walls, nor was the accumulation energy-dependent. It is proposed that it takes place by the binding of erythromycin to the bacterial ribosomes, forming a very stable complex. The dissociation constants of erythromycin-*Staphylococcus aureus* complex and erythromycin-*Bacillus subtilis* complex were determined to be 1.1×10^{-7} and 3.4×11^{-7} M, respectively.

The mechanisms for bacterial resistance to antibiotics can be classified into three categories (8): (i) destruction of the antibiotic, (ii) absence of the step susceptible to the antibiotic, and (iii) impermeability to the drug. No systematic study of resistance mechanisms of gram-negative bacteria to erythromycin has been reported. Although Haight and Finland (2) showed that erythromycin incubated with *Escherichia coli* and *Proteus vulgaris* still was biologically active, no attempt was made to quantitate the recovery or to identify the recovered compound. As to resistance mechanism (ii), it is generally accepted that erythromycin inhibits protein synthesis (13; Mao, Abstr. Interscience Conference on Antimicrobial Agents and Chemotherapy, 6th, p. 43, 1966) by binding to ribosomes (6, 12). There is evidence (9, 15) that protein synthesis in *E. coli* cell-free preparations can be inhibited by erythromycin, and Tanaka et al. (10) have reported that erythromycin can bind to *E. coli* ribosomes. Relative to mechanism (iii), Taubeneck (11) found that a stable L form of a *P. mirabilis* strain was susceptible to erythromycin, but the parent culture with the intact cell wall was 1,000-fold more resistant. These reports indicate that the erythromycin resistance in gram-negative bacteria probably is due to the impermeability of the cell to the antibiotic.

To extend and validate the previous observations, a comparison was made between two gram-negative (*E. coli*, *P. vulgaris*) and two gram-positive (*Staphylococcus aureus*, *Bacillus subtilis*) bacteria, considering all three resistance mechanisms. The accumulation of erythromycin in gram-positive bacteria was further investigated.

MATERIALS AND METHODS

Materials. *N*-^{14}C-methyl-erythromycin was synthesized by reductive methylation of des-*N*-methyl-erythromycin (1), and purified by countercurrent distribution. The product was chromatographically pure on silica gel thin-layer plates in three solvent systems (7), had a specific activity of 15 mc/mmole, and had antibacterial activity of 730 units/μmole.

^{14}C-phenylalanine (375 mc/mmole), ^{14}C-lysine (240 mc/mmole), and ^{14}C-algal protein hydrolysate (1.5 μc/mg) were purchased from New England Nuclear Corp., Boston, Mass. Triphosphates of adenosine, cytidine, guanosine, and uridine (ATP, CTP, GTP, UTP, respectively), phosphoenolpyruvate, pyruvate kinase, polyuridylic acid (poly U), polyadenylic acid (poly A), and dithiothreitol were obtained from Calbiochem, Los Angeles, Calif. Cell

walls of *S. aureus* and *B. subtilis* were kindly donated by J. L. Strominger, University of Wisconsin.

Cultures. E. coli ATCC 11775, *P. vulgaris* ATCC 7897, *S. aureus* 209P, and *B. subtilis* ATCC 10707 were grown in Erlenmeyer flasks overnight at 34 C in 3.7% Brain Heart Infusion broth (Difco). The 50% inhibition concentration of erythromycin for these microorganisms is 2×10^{-5}, 5×10^{-5}, 3×10^{-7}, and 2×10^{-7} M, respectively, under our experimental conditions. Samples of 1 ml were inoculated into flasks containing 125 ml of fresh medium and grown until the optical density at 550 mμ reached 0.2 to 0.3. Most of the experiments were conducted in this early log phase. One optical density unit is approximately equivalent to 3×10^8 bacteria per ml.

Recovery of ^{14}C-erythromycin from cultures. ^{14}C-erythromycin (100 μg) was added to 100 ml of bacterial culture in Brain Heart Infusion broth. After incubation for 30 min at 34 C, the culture was chilled in an ice bath, and the erythromycin was extracted with ethyl ether at *p*H 9.0 (four times, 100 ml each time). A 1-ml amount of the ether extract was evaporated in a counting vial, and 10 ml of scintillation fluid was added. The radioactivity was determined by use of a Packard Scintillation Spectrometer with a counting efficiency of about 78% for ^{14}C. The remaining ether extract was dried over anhydrous sodium sulfate and evaporated to 0.5 ml. This sample was applied to three silica gel G thin layer plates and developed in three solvent systems (6).

Assay for polypeptide synthesis. S. aureus and *E. coli* cell-free extracts (S-30) were prepared and assayed for polypeptide synthesis as described previously (5). The standard reaction mixture contained the following, in a volume of 0.5 ml: 0.1 M tris(hydroxymethyl)-aminomethane (Tris)-acetate (*p*H 7.6), 0.016 M magnesium acetate, 0.05 M ammonium acetate, 0.0001 M dithiothreitol, 0.001 M ATP, 5×10^{-5} M GTP, CTP, and UTP, 0.005 M phosphoenolpyruvate, 0.02 mg of pyruvate kinase, 0.25 μc of ^{14}C-labeled amino acid, and 6×10^{-5} M of each of the remaining amino acids. The assay was usually started by adding 0.1 ml of the S-30 fraction and was carried out at 34 C for 30 min. In the studies of ^{14}C-phenylalanine and ^{14}C-lysine incorporation, 50 μg of poly U and 100 μg of poly A, respectively, were added.

^{14}C-erythromycin uptake by bacteria. The uptake of ^{14}C-erythromycin from the extracellular medium by bacteria was measured by filtering a 5-ml sample of the bacterial suspension through a glass-fiber filter (Reeve Angel, 984H Ultra, 24-mm diameter). The cells were filtered free from medium by suction, and were washed four times with 5 ml of nonradioactive erythromycin solution (10 μg/ml). The radioactivity became constant after three washes. The bacterial cells washed with Brain Heart Infusion broth containing 10 μg of cold erythromycin per ml gave similar results. The absorption of ^{14}C-erythromycin on the filter could be minimized by washing the filter with 5 ml of nonradioactive erythromycin solution before the filtration of bacteria. A filter absorption curve with various concentrations of ^{14}C-erythromycin was constructed to determine the background absorption. It was found that 2.8×10^{-12} moles of ^{14}C-erythromycin was absorbed by the filter when 5 ml of 1.36×10^{-6} M ^{14}C-erythromycin solution was filtered. The absorption of ^{14}C-erythromycin by a membrane filter (HA, 0.45-μ; Millipore Corp., Bedford, Mass.) was about four times higher than by the glass-fiber filter. Adsorption of ^{14}C-erythromycin on isolated cell walls of *S. aureus* and *B. subtilis* was determined either by the filtration method described above, or, alternatively, after incubation with ^{14}C-erythromycin, the cell walls were recovered by centrifugation and resuspended in a nonradioactive erythromycin solution four times.

Formation of the erythromycin-ribosome complex. The reaction mixture contained 120 μμmoles of *S. aureus* or *E. coli* ribosomes and various amounts of ^{14}C-erythromycin in 0.5 ml of buffer solution (0.01 M Tris-acetate, *p*H 7.6, 0.016 M magnesium acetate, 0.05 M ammonium acetate, and 0.0001 M dithiothreitol). The reaction mixtures were incubated at 34 C for 30 min, diluted with 3 ml of the cold buffer solution, filtered through a nitrocellulose membrane filter (B-6, 25 mm; Schleicher & Schuell Co., Keene, N.H.), and washed with three 3-ml portions of cold buffer solution. A filter adsorption curve was constructed at various concentrations of ^{14}C-erythromycin and used to calculate the net binding of ^{14}C-erythromycin to the ribosomes.

RESULTS

Recovery and identification of erythromycin from gram-negative bacterial cultures. ^{14}C-erythromycin incubated with *E. coli* and *P. vulgaris* for 30 min at 34 C was recovered in yields of 97 and 93%, respectively. The recovered compound had R_F values identical to erythromycin in three solvent systems, it had antibacterial activity, and the radioactivity was concurrent with the spot test for erythromycin (7).

Comparison of sensitivity to erythromycin of E. coli and S. aureus in cell-free protein-synthesizing preparations. The effect of erythromycin on the endogenous messenger ribonucleic acid (mRNA)-directed incorporation of ^{14}C-amino acids, poly A-directed incorporation of ^{14}C-lysine, and poly U-directed incorporation of ^{14}C-phenylalanine was tested with the cell-free extracts from *E. coli* and *S. aureus*. The results (Fig. 1) indicate that there is no significant difference between *E. coli* and *S. aureus* extracts.

Uptake of ^{14}C-erythromycin by gram-positive and gram-negative bacteria. In a culture with increasing concentrations of erythromycin, the uptake of the antibiotic by gram-positive bacteria rapidly reached a saturation concentration of 0.5×10^{-6} M. The uptake of erythromycin by gram-negative bacteria at any concentration was negligible (Fig. 2).

The time course of erythromycin uptake by bacteria in a medium containing 1.36×10^{-6} M ^{14}C-erythromycin is shown in Fig. 3. The two

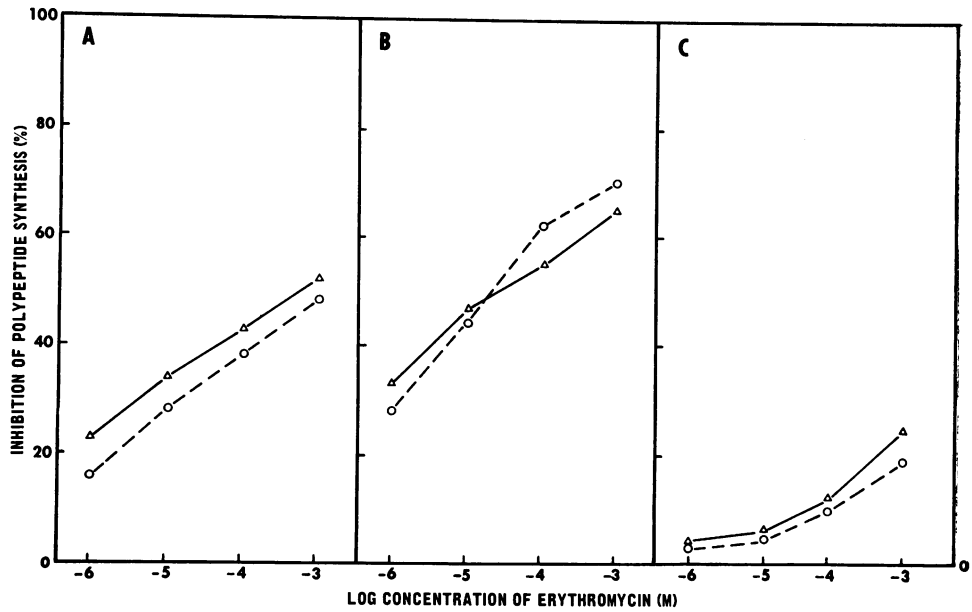

FIG. 1. *Inhibition of polypeptide synthesis in cell-free extracts from Staphylococcus aureus (△) and from Escherichia coli (○). (A) endogenous mRNA-directed synthesis of protein; (B) poly A-directed synthesis of polylysine; (C) poly U-directed synthesis of polyphenylalanine. The polypeptide-synthesizing activities in the cell-free extracts of E. coli and S. aureus were similar. The total incorporation of labeled amino acids in controls (without antibiotics) of the endogenous system, the poly A system, and the poly U system of a representative experiment were 8,500, 20,000, and 88,000 counts per min, respectively, per mg of protein. The zero-time samples were about 450 counts per min.*

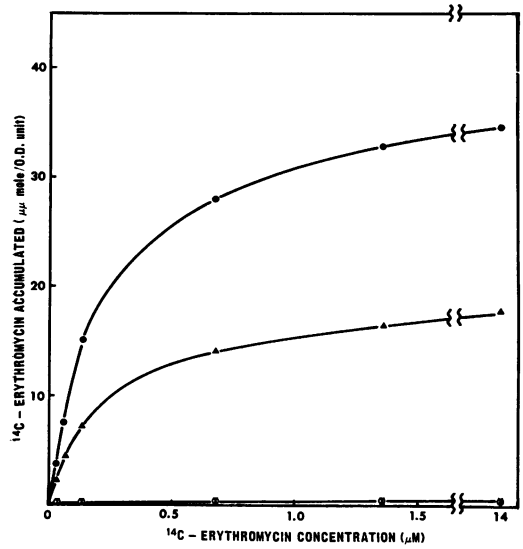

FIG. 2. *Uptake of erythromycin by bacteria after 30 min of incubation, at various concentrations of ^{14}C-erythromycin. Symbols: ●, Bacillus subtilis; ▲ Staphylococcus aureus; ○, Escherichia coli; ×, Proteus vulgaris.*

gram-positive bacteria accumulated erythromycin rapidly. Maximal accumulation occurred in 5 to 7 min, followed by a slight release, until an equilibrium level was attained. However, no significant accumulation of erythromycin by gram-negative bacteria was observed even after prolonged incubation.

Cell wall adsorption of ^{14}C-erythromycin. Isolated cell walls (0.3 mg per sample) of *S. aureus* and *B. subtilis* incubated with ^{14}C-erythromycin showed no adsorption of radioactive material (Fig. 3).

Relation of erythromycin accumulation to growth inhibition of B. subtilis. The amount of ^{14}C-erythromycin accumulation at various concentrations of the antibiotic and the inhibition of growth rate at these concentrations were determined at the end of 30 min of incubation (Fig. 4). The results indicate that the inhibition of gram-positive bacteria is a function of erythromycin accumulation.

Effect of uncoupling agents of oxidative-phosphorylation on the uptake of ^{14}C-erythromycin by S. aureus. Growth of *S. aureus* was inhibited by potassium cyanide (5×10^{-3} M) completely, and about 20% by sodium azide (2×10^{-2} M)

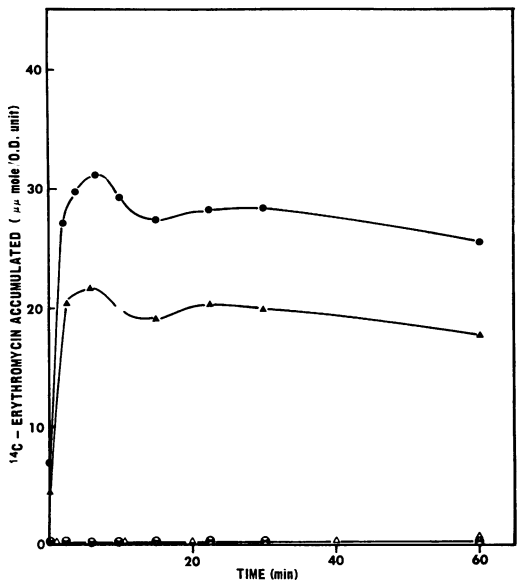

FIG. 3. *Time course of erythromycin uptake by bacteria and bacterial cell walls. Symbols*: ●, *Bacillus subtilis*; ▲, *Staphylococcus aureus*; ○, *Escherichia coli*; △, *Proteus vulgaris; and* ×, *cell walls of B. subtilis and S. aureus.*

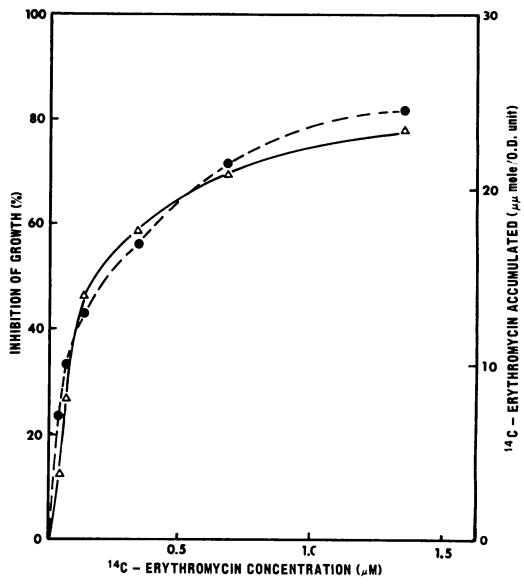

FIG. 4. *Relationship between erythromycin uptake and growth inhibition of Bacillus subtilis. Symbols*: ●, *amount of erythromycin accumulated;* △, *per cent growth inhibited. Various amounts of* ^{14}*C-erythromycin were added to B. subtilis cultures. After 30 min of incubation at 34 C, 5 ml of the culture was filtered, washed, and counted. The optical density of the remaining sample was measured immediately at the end of 30 min. The per cent inhibition of growth rate was calculated from the optical density with the nontreated sample as the control.*

and 2,4-dinitrophenol (4×10^{-3} M). Yet, the accumulation of erythromycin (at extracellular concentrations of 1.36×10^{-6}) increased (Fig. 5).

Dissociation constants of the bacteria-erythromycin complexes. The accumulation of ^{14}C-erythromycin by *S. aureus* and *B. subtilis* after 30 min of exposure to increasing external concentrations of erythromycin follows a Langmuir isotherm (Fig. 6). The dissociation constants of the bacteria-erythromycin complexes were calculated from the plots to be 1.1×10^{-7} and 3.4×10^{-7} M, respectively. The amounts of ^{14}C-erythromycin accumulated by the gram-negative bacteria were too small to calculate the dissociation constants.

Dissociation constants of the ribosome-erythromycin complexes. Formation of the ribosome-erythromycin complexes was determined by the nitrocellulose membrane-filter method. The binding of erythromycin to ribosomes was complete after 10 min at 34 C, and further incubation up to 60 min had no effect on the complexes. The complexes were very stable, as they remained intact after three washings with 3 ml of cold buffer. The ribosomes isolated from *E. coli* behaved comparably to those isolated from *S. aureus*. The dissociation constants of *S. aureus* and *E. coli* ribosome-erythromycin complexes calculated by the double reciprocal plot (Fig. 7) were 2.8×10^{-6} and 3.6×10^{-6} M, respectively.

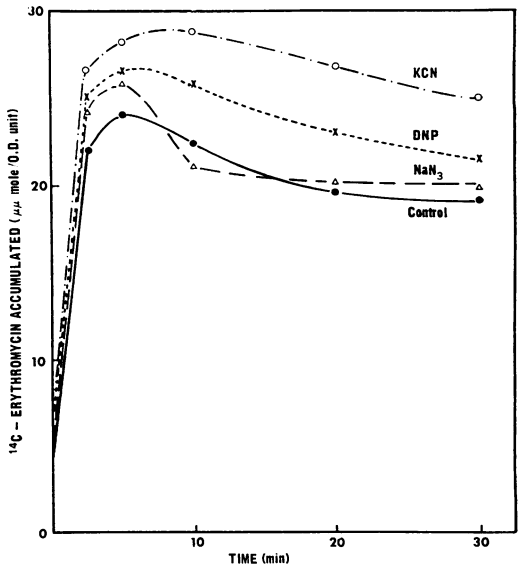

FIG. 5. *Effect of uncoupling agents on erythromycin uptake by Staphylococcus aureus. Symbols*: ●, *without uncoupling agent;* ○, 5×10^{-3} M *KCN;* ×, 4×10^{-3} M *dinitrophenol;* △, 2×10^{-2} M *sodium azide.*

Effect of heat or toluene on the uptake of erythromycin. E. coli and *P. vulgaris* treated with toluene or heat did not show any uptake of erythromycin. *S. aureus* treated with toluene or heat in the same manner as *E. coli* completely lost the ability to accumulate erythromycin.

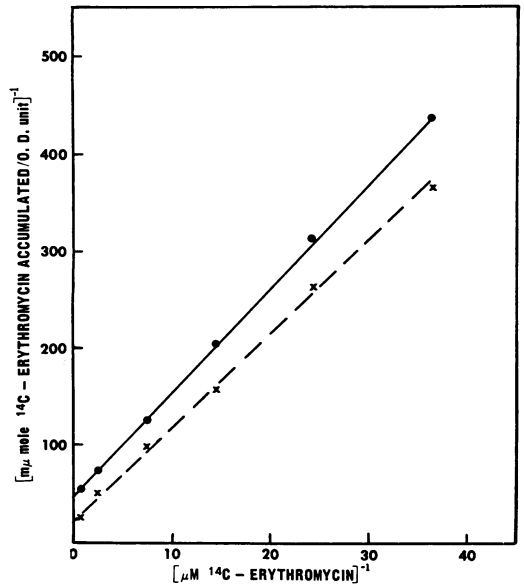

FIG. 6. *Langmuir adsorption isotherm plots of 30-min* ^{14}C*-erythromycin accumulation. Symbols*: ●, *Staphylococcus aureus;* ×, *Bacillus subtilis.*

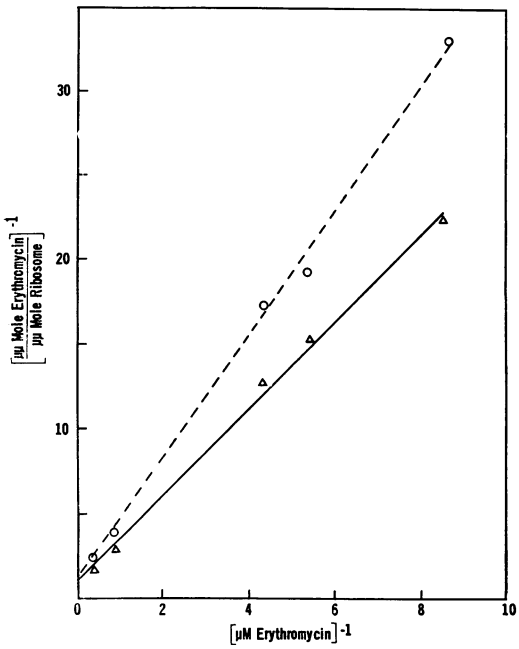

FIG. 7. *Evaluation of dissociation constants of ribosome-erythromycin complexes by the double reciprocal plot. Symbols*: ○, *Escherichia coli ribosome-erythromycin complex;* △, *Staphylococcus aureus ribosome-erythromycin complex.*

DISCUSSION

Since erythromycin can be recovered in high yields from cultures of *E. coli* and *B. subtilis*, degradation of erythromycin as the mechanism of resistance in these two gram-negative bacteria can be ruled out.

A study of cell-free protein-synthesizing preparations indicates that the *E. coli* system is equally as susceptible to erythromycin as the *S. aureus* system (Fig. 1). The small difference in susceptibility (about 5%) between *E. coli* and *S. aureus*, shown in Fig. 1, is not statistically significant, since the experimental error in the cell-free protein synthesis assay is about 10%. Preparations from both microorganisms show that poly A-directed synthesis is most sensitive to erythromycin, the endogenous mRNA-directed system is less sensitive, and the poly U-directed synthesis is least sensitive. Wolfe and Hahn (15) have shown that *E. coli* cell-free preparations require an extremely high concentration of erythromycin to inhibit polyphenylalanine synthesis. Our results show that this is due to the insensitivity of the poly U-directed cell-free system rather than to the *E. coli* cell-free system per se. Further evidence to support the thesis that the protein-synthesizing apparatus of gram-negative bacteria is equally as sensitive to erythromycin as that of the gram-positive bacteria is obtained by comparing the dissociation constants of the ribosome-erythromycin complexes of *S. aureus* and *E. coli*. The dissociation constant of the *E. coli* ribosome-erythromycin complex is 3.6×10^{-6} M, which is only slightly higher than that of the *S. aureus* ribosome-erythromycin complex (2.8×10^{-6} M). It should be noted in Fig. 7 that both lines intercept the ordinate at a value of the ribosome-erythromycin ratio of unity. This is the maximal binding capacity of the ribosomes. These data verify our earlier finding, by sucrose gradient centrifugation, that *S. aureus* ribosomes form a one to one complex with erythromycin (6).

We have found that the uptake of erythromycin by gram-positive bacteria is 100 times greater than the uptake by gram-negative bacteria (Fig. 2 and 3), while the minimal inhibitory concentrations of erythromycin for *E. coli* and *P. vulgaris* cultures are about 100 times greater than for *S. aureus* and *B. subtilis* cultures (2). This difference in accumulation of erythromycin between gram-positive and gram-negative bacteria is sufficient to explain the resistance mechanism of gram-negative bacteria. A close relationship

between accumulation of erythromycin and inhibition of growth in gram-positive bacteria has been established (Fig. 4).

Taubeneck (11) showed that the L form of a *P. mirabilis* strain is very susceptible to erythromycin, although the parent strain with the intact wall is resistant. Kagan et al. (4) observed that the L form of *S. aureus* is more sensitive to kanamycin, neomycin, polymyxin, lincomycin, and gentamycin than the intact cell. Thus, if the membranes of the intact cell and the L form are identical, then the transport barrier is probably the cell wall rather than the cell membrane. It has also been reported that exposing *E. coli* cells to toluene for 15 min or heating them to 70 C for 2 min increased their permeability to streptomycin (3). However, *E. coli* and *P. vulgaris* show no uptake of erythromycin after such treatment. This finding may be interpreted in either of two ways: that the barrier to erythromycin transport cannot be destroyed by heat or toluene; or that the barrier is destroyed but a component essential for the uptake is also destroyed. The latter interpretation seems more likely, since heat- or toluene-treated gram-positive bacteria lose the ability to accumulate erythromycin.

To ascertain that erythromycin is accumulated in gram-positive bacterial cells rather than merely adsorbed on the cell wall surface, the adsorption of erythromycin by isolate cell walls of *S. aureus* and *B. subtilis* was determined. No erythromycin was detected on the cell walls. There were 0.3 mg of cell walls per sample. Assuming 20% of the dry cell weight is cell wall, this is equivalent to 1.5 mg of dry cell per sample. In the other experiments conducted in this report, less than 1 mg of dry cells per sample was used.

At an extracellular erythromycin concentration of 1.36×10^{-6} M, *S. aureus* could accumulate 18×10^{-12} to 30×10^{-12} moles of erythromycin per 3×10^{8} cells, and *B. subtilis* could accumulate 20×10^{-12} to 37×10^{-12} moles. Assuming the cell volume of a bacterium is 10^{-12} ml, the total cell volume of 3×10^{8} cells is 3×10^{-4} ml. The 18×10^{-12} to 37×10^{-12} moles of erythromycin accumulated in this volume give an intracellular concentration of 60×10^{-6} to 123×10^{-6} M. This concentration of erythromycin in gram-positive bacteria is 44- to 90-fold greater than that of the medium.

Although the intracellular concentration of erythromycin is greater than that of the surrounding medium, active transport does not appear to be involved since uncoupling agents, such as cyanide, azide, and 2,4-dinitrophenol, do not inhibit the accumulation. The accumulation occurs only in viable cells; heat- or toluene-treated cells lose the ability to accumulate erythromycin. This can be explained by assuming a binding of erythromycin to ribosomes. It has been shown by sucrose gradient centrifugation or by column chromatography that erythromycin binds to ribosomes of *S. aureus* (6), *B. subtilis* (12), and *E. coli* (9). That the ribosome-erythromycin complex can survive prolonged centrifugation through a drug-free sucrose gradient or through a drug-free column indicates that the complex is very stable. The stability of the ribosome-erythromycin complex is quantitated by the dissociation constants of the complexes. The dissociation constants are indeed very small (2.8×10^{-6} and 3.6×10^{-6} M for *S. aureus* and *E. coli*, respectively). The dissociation constants of bacteria-erythromycin are 1.1×10^{-7} and 3.4×10^{-7} M for *S. aureus* and *B. subtilis*. The discrepancy between the ribosome-erythromycin dissociation constants and the bacteria-erythromycin constants probably reflects that in bacterial cells there is a small fraction of erythromycin in addition to that bound to ribosomes.

Tissières et al. (13) have estimated that one *E. coli* cell (cell volume about 10^{-12} ml) at the early log phase contains 90,000 ribosomes. Assuming that the density of ribosomes in *B. subtilis* and *S. aureus* is approximately the same as in *E. coli*, and that each ribosome binds one erythromycin molecule, the intracellular concentration can reach 0.15 mM. This agrees with the 0.06 to 0.12 mM concentration of erythromycin in *B. subtilis* and *S. aureus* observed in our studies.

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