JCI The Journal of Clinical Investigation

Interference with the Mannose Binding and Epithelial Cell Adherence of *Escherichia coli* by Sublethal Concentrations of Streptomycin

Barry I. Eisenstein, … , Itzhak Ofek, Edwin H. Beachey

J Clin Invest. 1979;63(6):1219-1228. https://doi.org/10.1172/JCI109417.

Research Article

When *Escherichia coli* was grown in sublethal concentrations of streptomycin, mannose binding activity and epithelial cell adherence of the *E. coli* cultures at stationary phase were significantly reduced in the drug-grown organisms. In a strain whose minimal inhibitory concentrations was 30 μg/ml, the percentage of reduction in mannose binding activity was dose related over a range of concentrations between 0.5 and 10 μg/ml streptomycin. Concomitant with the drug-induced suppression of mannose binding activity, antigenic and ultrastructural alterations on the surface of the drug-grown organisms were observed by agglutination tests and electron microscopy, respectively.

The streptomycin effect was reversible, required actively growing organisms, and was most apparent in the early log-phase of growth. High doses of antibiotic were ineffective when added to cultures which had acquired mannose binding activity. An isogenic derivative with high-level resistance to streptomycin was obtained as a single-step mutation from the test *E. coli* strain. Whereas the isogenic mutant possessed mannose binding activity and adhering ability similar to the parent strain, it was resistant to the streptomycin-induced suppression of the two activities at enormous concentrations (up to 10,000 μg/ml) of streptomycin. Taken together the results suggest that the suppression of epithelial cell adherence and mannose binding activity of *E. coli* grown in sublethal concentrations of streptomycin is a result of classic mechanisms of drug action upon the […]

Interference with the Mannose Binding and Epithelial Cell Adherence of *Escherichia coli* by Sublethal Concentrations of Streptomycin

BARRY I. EISENSTEIN, ITZHAK OFEK, and EDWIN H. BEACHEY, *Departments of Medicine and Microbiology, University of Tennessee and the Veterans Administration Hospital, Memphis, Tennessee 38163*

ABSTRACT When *Escherichia coli* was grown in sublethal concentrations of streptomycin, mannose binding activity and epithelial cell adherence of the *E. coli* cultures at stationary phase were significantly reduced in the drug-grown organisms. In a strain whose minimal inhibitory concentrations was 30 μg/ml, the percentage of reduction in mannose binding activity was dose related over a range of concentrations between 0.5 and 10 μg/ml streptomycin. Concomitant with the drug-induced suppression of mannose binding activity, antigenic and ultrastructural alterations on the surface of the drug-grown organisms were observed by agglutination tests and electron microscopy, respectively.

The streptomycin effect was reversible, required actively growing organisms, and was most apparent in the early log-phase of growth. High doses of antibiotic were ineffective when added to cultures which had acquired mannose binding activity. An isogenic derivative with high-level resistance to streptomycin was obtained as a single-step mutation from the test *E. coli* strain. Whereas the isogenic mutant possessed mannose binding activity and adhering ability similar to the parent strain, it was resistant to the streptomycin-induced suppression of the two activities at enormous concentrations (up to 10,000 μg/ml) of streptomycin. Taken together the results suggest that the suppression of epithelial cell adherence and mannose binding activity of *E. coli* grown in sublethal concentrations of streptomycin is a result of classic mechanisms of drug action upon the bacterial ribosome. The results support the possibility that antibiotics may act through mechanisms other than inhibition of growth and bacterial killing to eradicate bacteria from mucosal surfaces.

INTRODUCTION

The clinically important effect of antibiotics on bacterial isolates classically has been considered to be the relative ability of the antibiotic to either kill or completely inhibit the growth of the organism. The measurement of these abilities is referred to as minimal bactericidal concentration (MBC)[1] or minimal inhibitory concentration (MIC), respectively. At concentrations below the lethal or inhibitory levels, there are still demonstrable effects, such as decreased rate of growth or altered morphology. Several investigators have used the term "minimal active concentration" and defined it as the minimal drug concentration producing either a change in morphology seen by light or electron microscopy, or growth inhibition of at least one logarithm compared to control cultures without drug (1).

Little is known about the effect of sublethal concentrations of antibiotics on the expression or production of specific determinants of pathogenicity, which might impair the infectivity, rather than the replication, of the parasite. The infectious process is considered to be a chain of multiple steps initiated in most cases by bacterial colonization of mucosal surfaces (2). This first step in tissue invasion or damage most likely begins with the adherence of bacteria to epithelial cells of the host mucosa (3). We recently reported that penicillin caused nongrowing (stationary phase) streptococci to

Dr. Beachey is the recipient of a Medical Investigatorship Award from the U. S. Veterans Administration.

Received for publication 17 July 1978 and in revised form 16 February 1979.

[1] *Abbreviations used in this paper*: BHI, brain heart infusion; CFU, colony-forming units; MBC, minimal bactericidal concentration; MIC, minimal inhibitory concentration; α-MM, methyl α-D-mannopyranoside; mRNA, messenger RNA; PBS, phosphate-buffered saline; Str^R, streptomycin-resistant; Str^S, streptomycin-sensitive; YAP, yeast agglutinating power.

lose cell wall lipoteichoic acid, a mediator of adherence of streptococci to epithelial cells (4); the loss of lipoteichoic acid resulted in a loss of adhering ability.

Sugar residues on the surface of the epithelial cells have been shown to serve as receptors for the binding of certain bacteria (5). In the case of *Escherichia coli*, it has been shown that the organisms contain a lectin-like substance which binds the bacteria specifically to mannose residues on epithelial cells (6). Recently we found that mannose binding activity of intact organisms could be monitored by aggregometry employing mannan-containing yeast cells. The degree of aggregation was shown to correlate with the ability of the organisms to adhere to mannose residues on epithelial cells (7).

The purpose of this investigation was to analyze the sublethal effects of streptomycin on the ability of growing *E. coli* to bind mannose residues and adhere to human epithelial cells. Streptomycin was chosen as a prototype aminoglycoside antibiotic because its mode of action on ribosomal translation of messenger RNA (mRNA) is the best studied of all inhibitors of protein synthesis in susceptible bacteria (8–10). We were able to quantitate a novel effect of sublethal levels of streptomycin on *E. coli* as manifested by loss of mannose-mediated adherence to human epithelial cells, and to correlate this effect with antigenic and anatomic alterations of the bacterial surface. Furthermore, this effect was shown to follow the classical mechanism of streptomycin action (8–10) in that antibiotic-sensitive cells in the stationary phase or mutant resistant cells in all phases of growth were impervious to the effect.

METHODS

Microorganisms. A streptomycin-sensitive (Str^S) strain of *E. coli*, M-Str^S, was obtained from a clinical specimen and purified by serial clonal selection (MIC of 30 μg/ml). A streptomycin-resistant (Str^R) mutant of the clinical strain was selected by spreading 10^8 colony-forming units (CFU) of M-Str^S on each of several agar plates containing 20 ml Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.) with 200 μg/ml streptomycin (Sigma Chemical Co., St. Louis, Mo.). After 72 h incubation at 37°C, several single colonies appeared, one of which was purified and ascertained to be a high-level Str^R mutant by standard methods. This derivative, M-Str^R, and the parent, M-Str^R, were stored on deep agar slants and in refrigerated nutrient broth at 4°C (brain heart infusion [BHI], Difco Laboratories). They were periodically checked for purity and identity by standard methods.

Candida albicans organisms were maintained on slants of Sabouraud's dextrose agar (Difco Laboratories) and periodically subcultured. A standard suspension was obtained by subculturing the organisms in nutrient broth for 24 h at 37°C with constant shaking.

Cultural conditions and growth studies. E. coli strains were subcultured 1:100 from stationary phase broth cultures to 100 ml of fresh nutrient broth and incubated at 37°C to early visible growth, typically 2 h, or for shorter periods of time. Aliquots of 0.05 ml were then further subcultured in a total of 5 ml broth with increasing concentrations of streptomycin, or without drug as a control. Experiments were typically run in duplicate or triplicate and included samples with sufficient antibiotic levels to determine the MIC. In several experiments, minimal media Davis (Difco Laboratories) replaced the nutrient broth.

Cultures were incubated in a water bath at 37°C for 48 h. Growth was monitored every 15 min to 1 h for the 1st 5 h and then at 24 and 48 h either by absorbance on a junior Coleman spectrophotometer (Coleman Systems, Irvine, Calif.) at 550 nm or by optical density on a Klett-Summerson colorimeter (Klett Manufacturing Co., New York) with No. 540 filter. In some experiments organisms were harvested by centrifugation, washed three times in 0.02 M phosphate-0.15 NaCl, pH 7.2 phosphate-buffered saline (PBS), and resuspended in the same buffer. The concentration of bacteria in the suspensions were determined by direct counts in a Petroff-Hausser chamber (C. A. Hausser and Son, Philadelphia, Pa.), by optical density measurements, or by viable counts by standard dilution plating technique. For the *E. coli* used in these experiments, 10^9 CFU were equivalent to a Klett of 100 or an OD of 0.3 at 550 nm.

Determination of protein and DNA synthesis. E. coli M-Str^S was subcultured as above in 125 ml nutrient broth, grown for 90 min at 37°C, and then supplemented with 0.1 mCi of [^{3}H]thymidine (New England Nuclear, Boston, Mass.; sp act 28.5 Ci/mmol) and 0.01 mCi of [^{14}C]leucine (New England Nuclear; sp act 54.4 mCi/mmol). After 20 min further growth at 37°C, 15-ml aliquots in replicate were distributed to screw-topped test tubes to reach a total volume per tube of 20 ml at streptomycin concentrations of: 0, 0.5, 2, and 10 μg/ml.

Yeast cell aggregation tests. Candida yeast cells harvested from 2 liters of broth were washed twice with PBS and resuspended in 100 ml PBS containing 0.1% gluteraldehyde. The mixture was stirred for 1 h at room temperature, sedimented, and washed again with PBS, resuspended in 100 ml PBS containing 0.1 M glycine, and stirred for 30 min at 23°C. The cells were then harvested, washed twice; and resuspended in PBS to a concentration of 2×10^7 yeast cells/ml, using a hemocytometer for counting. After adding sodium azide (0.02%) as preservative, the yeast cell suspension was stored at 4°C for up to 2 mo before use.

Aggregation of yeast cells was monitored in a Payton Aggregometer model 300A (Payton Associates, Buffalo, N. Y.) according to a turbidometric method (11). Aliquots (0.5 ml) of the standard yeast cell suspension were pipetted into 8 × 45-mm glass cuvettes and stirred at a constant speed of 800 rpm at room temperature. The aggregometer was adjusted to read 30% transmission for the yeast cells suspension and 90% for PBS. After establishing a stable base line, test organisms, 15×10^7 bacteria in 10-μl vol, were added. As aggregation proceeded, the change in percentage of transmission was continuously recorded as a function of time using a Goertz (Gelman Instruments Co., Ann Arbor, Mich.) recorder. Recently we have shown that the velocity of aggregation is proportional to the number of bacteria added and to the ability of these organisms to adhere to epithelial cells (7).

Slide agglutination tests. Glass slide agglutination was performed by mixing equal volumes (10 μl) of standard yeast cell suspension and dilutions of bacterial suspensions on a glass slide and manually rotating the slide gently. A positive reaction would give course clumping, visible to the naked eye, within 60 s. Dilutions of bacterial suspensions were prepared by pipetting 20 μl of each test suspension, with known bacterial counts, to the first row of wells in U-bottom clear microtiter trays (Cooke Laboratory Products, Alexandria, Va.), adding 20 μl PBS in wells in each row, and serially (1:2) diluting and mixing bacterial suspensions. The yeast agglutinating power (YAP) of a test suspension was calculated according to a modification of the method of Duguid and

Gillies (12) as 10^{11} divided by the minimal concentration of bacteria producing yeast cell agglutination. Minimal concentrations of bacteria were computed by dividing the bacterial concentration of the original suspension by the higher dilution (titer) capable of giving positive yeast cell agglutination. No difference in YAP was noted when dilutions were made directly from cultures or from prewashed bacteria.

Tests of reversibility of mannose binding activity. Organisms were grown in 5 ml BHI in routine fashion with the following streptomycin concentrations: 0, 2, 5, and 10 μg/ml. The YAP of each culture was determined after 48 h. The samples were then washed thrice in PBS, resuspended in 5 ml BHI to $\cong 10^7$ CFU/ml, and incubated for an additional 48-h growth (first subculture). Each of these subcultures was tested for YAP, subcultured 1:100 to fresh BHI (second subculture), and grown 48 h. The YAPs of the second subculture were then determined. Relative YAP was defined as: (YAP of 48-h antibiotic-grown organisms/YAP of 48-h organisms grown without antibiotic) × 100. After each of the two serial subcultures to drug-free broth, the above numerator was changed to: YAP of 48-h subcultured organisms derived from the initial antibiotic-containing culture.

Adherence tests. The experiments were performed by methods described previously (13). Briefly, 0.5 ml of a suspension of epithelial cells (2×10^5 cells/ml in PBS) from the buccal mucosa of one of us (Dr. Ofek) was mixed with 0.5 ml of washed *E. coli* adjusted to $1-2 \times 10^8$ bacteria/ml. The mixture was rotated end-over-end for 30 min at room temperature. The epithelial cells were separated from nonadherent bacteria by repeated differential centrifugation, resuspended in 0.05 ml PBS, and smeared on a glass microscope slide. After drying, the preparation was stained with gentian violet and examined with a bright field microscope. Slides containing more than four nonadherent bacteria per oil immersion field were omitted from analysis. Adherence was recorded as the average number of bacteria (±SE) per epithelial cell of 20 counted cells. A control sample of epithelial cells without added bacteria was always counted concurrently. Results of adherence tests were omitted when the background counts exceeded five bacteria/epithelial cell.

Preparation of rabbit antisera. *E. coli* M-Str^S was harvested from a 48-h culture in nutrient broth without added antibiotic, washed thrice, and resuspended in PBS. One rabbit was injected intravenously with 0.5 ml of the *E. coli* suspension (2×10^9 bacteria/ml) thrice weekly for 2 consecutive wk. Immune serum was collected at 4 wk and diluted 1:20 in PBS. An aliquot of immune serum was serially absorbed with washed *E. coli* M-Str^S grown in the presence of 10 μg/ml streptomycin until it no longer agglutinated drug-treated bacteria. The preimmune, unabsorbed, and absorbed antisera were treated with papain by the method of Porter (14) except that no attempt was made to separate the Fab from Fc fragments.

Inhibition of epithelial cell adherence by papain-treated sera. 0.05-ml aliquots of papain-cleaved sera were added at the desired dilution to 0.025 ml of drug-free-grown bacteria (2×10^8 bacteria/ml in PBS). After 10 min incubation at 22°C, 0.025 ml of a suspension of epithelial cells (10^6 cells/ml in PBS) was added. The remainder of the procedure to evaluate adherence was as described above except that only one differential centrifugation was performed. As controls, methyl α-D-mannopyranoside (α-MM) at a final concentration of 2.5% or PBS alone were used instead of sera.

Ultrastructural studies of bacterial cell walls and piliation by electron microscopy. Thin-section studies of the variously treated *E. coli* were prepared and examined according to previously described methods (15). Briefly, cells were fixed for 4 h in ice-cold 2.5% gluteraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4, washed twice in the same buffer without gluteraldehyde, post-fixed for 1 h in 1% osmium tetroxide, and dehydrated in stepwise increasing concentrations of ethanol. They were then suspended in Spurr low-viscosity embedding media (PolyScience Inc., Warrington, Pa.) and finally embedded in Spurr in Beem capsules. Ultrathin sections (Ultramicrotome; DuPont Instruments-Sorvall, Du Pont Co., Newtown, Conn.) were placed on naked copper grids, stained for 20 min with 4% uranyl acetate in water and for 5 min in 0.3% lead citrate in 0.1 N NaOH, and examined with an AEI, EM6B electron microscope (AEI Scientific Apparatus, Inc., Elmsford, N. Y.). In addition bacterial suspensions were negatively stained with phosphotungstic acid and examined by electron microscopy by methods previously described (16). Pili were enumerated the method of Novotny et al. (17) and as previously described in detail (7). To correlate degree of piliation with mannose binding activity, pili were enumerated in aliquots of the culture samples used in the mannose binding assays.

Preparation of pili. A partially purified preparation of pili from *E. coli* in drug-free broth was prepared as previously described (18). Briefly washed bacteria from a 15-liter broth culture were blended for 2 min, the depiliated bacteria were centrifuged out, and the supernate was subjected to serial cycles of 10% ammonium sulfate precipitation. The final pili precipitate was dissolved in PBS, dialyzed against distilled water, and lyophilized.

Hemagglutination. Hemagglutination of guinea pig erythrocytes by *E. coli* pili was performed by the method of Salit and Gotschlich (18). Inhibition of hemagglutination was done as follows: 15 μl of 1 mg/ml of pili was mixed with 15 μl of either PBS, 2.5% mannose solution, or serial dilutions of pre- or postimmune sera and incubated for 30 min at 22°C in microtiter wells. 10 μl of washed guinea pig erythrocytes was added to all wells and the mixtures were gently agitated and incubated for an additional 2–3 h at which time hemagglutination was recorded. All solutions and dilutions were prepared in PBS.

RESULTS

Growth effects induced by sublethal concentrations of streptomycin. During the logarithmic phase of growth, bacterial protein synthesis ([^{14}C]leucine incorporation) closely paralleled total bacterial mass (turbidity), and was preceded by DNA synthesis ([^{3}H]-thymidine incorporation) for both drug-free and 2 μg/ml streptomycin-grown bacteria (Fig. 1). Viable counts, at 24 and 48 h, were proportional to total bacterial mass for the cultures grown without drug or with 2 μg/ml of streptomycin, but only 25–50% of that predicted by turbidity for the cultures grown with 10 μg/ml of the drug. Total suppression of bacterial growth occurred at 30 μg/ml streptomycin (MIC). We employ the term sublethal concentration to denote any dose of streptomycin below the MBC or MIC (30 μg/ml), though death of a significant proportion of the cell population still occurred at 10 μg/ml. Slight growth suppression without bacterial killing occurred at 2 μg/ml antibiotic. MICs remained stable throughout these studies.

Streptomycin suppression of acquisition of mannose binding activity by growing bacteria. Aliquots of bacteria obtained at 6, 24, and 48 h from the cultures

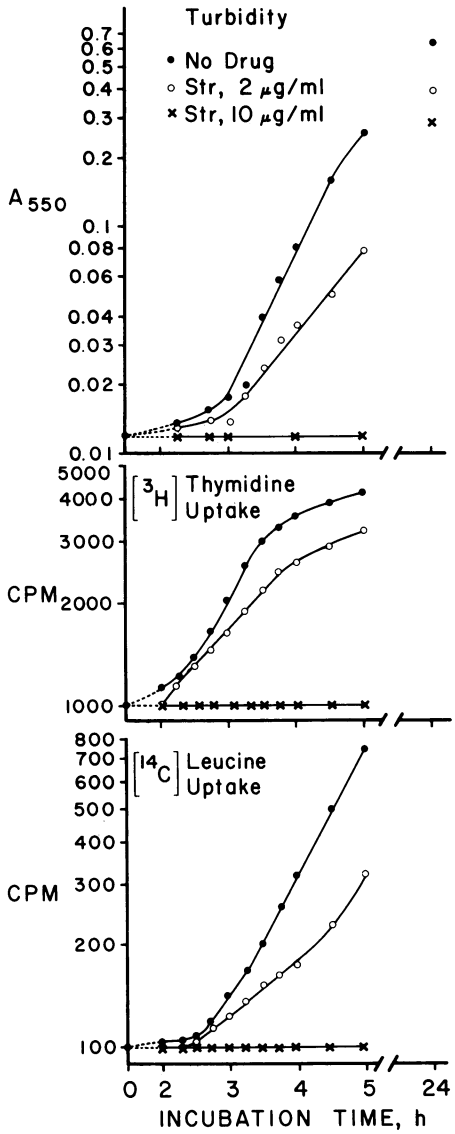

FIGURE 1 Turbidity, [^{3}H]thymidine uptake and [^{14}C]leucine uptake during the growth of *E. coli* in nutrient broth containing no drug (●), 2 μg/ml (○), and 10 μg/ml. (x) of streptomycin. 1-ml aliquots were removed from the growing cultures at times indicated for measurement of turbidity and for counting in a liquid scintillation counter (Nuclear-Chicago, Des Plaines, Ill.) of radiolabel associated with bacteria after washing on a nitrocellulose filter (Millipore Corp., Bedford, Mass., pore size 0.45 μm).

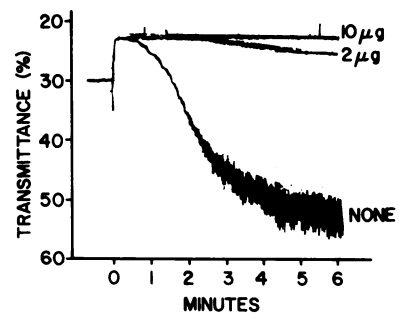

FIGURE 2 Aggregation of mannan-containing yeast cells by *E. coli* grown in concentrations of streptomycin: 0, 2, and 10 μg/ml. A base line of percentage of transmittance of a yeast cell suspension (30) was established as described in Methods. 15×10^7 bacteria in 10-μl vol were added at 0 time as indicated by an abrupt small decrease in transmittance. The bottom curve (none) shows aggregation by *E. coli* grown without antibiotic as demonstrated by an increase in transmittance to 50%. The curves on the top demonstrate the diminished ability of antibiotic-grown bacteria to cause aggregation.

grown with and without streptomycin were assayed for mannose binding activity. Compared to the control organisms, *E. coli* grown in sublethal amounts of streptomycin lost their mannose binding activity, as indicated by both rate (Fig. 2) and magnitude (Table I) of aggregation of mannan-containing yeast cells. When these experiments were repeated multiple times, we found a marked dose-dependent loss of mannose binding ability in the antibiotic-grown *E. coli* (Fig. 3). The lowest concentration at which a reproducible effect was noted was 0.5 μg/ml streptomycin, an amount that produced no detectable suppression of viability, bacterial mass, or protein or DNA synthesis (data not shown). At any given dose there was an increase in mannose binding activity over time: 48 h > 24 h >>>6 h (Table I). Once acquired, mannose binding ability was unmodified by subsequent exposure of the organism to lethal levels of streptomycin (10,000 μg/ml) or to sodium azide. Culture filtrates from stationary phase organisms grown in the absence or in the presence of the various drug concentrations were devoid of any detectable mannose binding activity or ability to inhibit yeast cell aggregation induced by *E. coli*. These results indicate that the development of mannose binding activity is not due to the direct action of antibiotics on formed ligand but rather on ligand synthesis.

Relationship between piliation and mannose binding activity. Because other investigators have shown that binding of type 1 pili to eukaryotic cells is mannose sensitive (18), we examined *E. coli* M-Str^S for the relationship between degree of piliation and mannose binding activity under various cultural conditions. In the first set of experiments, we analyzed piliation of bacteria grown without antibiotic at various times of growth. There was a time-dependent increase in the proportion of piliated organisms: at 6 h growth the mean percentage was 58; at 24 h, 91; and at 48 h, 97%. The acquisition of pili paralleled the time-dependent increase of mannose binding activity (Table I). When these studies were repeated multiple times, there was a strong positive correlation between degree of piliation and mannose binding activity ($r = 0.75$, $n = 17$, P

TABLE I

Loss of Yeast Cell Agglutinating Activity by E. coli (M-Str^S) Grown in Subinhibitory Concentrations of Streptomycin

Hours of growth	YAP* (±SE) of *E. coli* grown with streptomycin			
	None	2 μg/ml	10 μg/ml	30 μg/ml
h				
6	<100	<100	No growth	No growth
24	6,050±30	150±2	<100	No growth
48	9,700±830	510±140	<100	No growth

* See Methods for derivation.

< 0.001). Of note was an apparent threshold requirement of 50–60% piliation before the development of measurable mannose binding activity.

Cultures grown in the presence of streptomycin for 48 h had a reduction in piliation that was positively correlated with reduced mannose binding activity ($r = 0.35$, $n = 52$, $P < 0.05$).

Reduced ability of streptomycin-grown E. coli to adhere to human epithelial cells. Adherence to epithelial cells of *E. coli* M-Str^S was found to be mannose-sensitive: 51±7, 7±4, and 56±8 bacteria/epithelial cell in the presence of PBS, 2.5% α-MM, and 2.5% methyl α-D-glucopyranoside, respectively, indicating that epithelial cell adherence of this strain is mediated by mannose residues on epithelial cells (6). We found that the reduced ability of bacteria grown in sublethal concentrations of streptomycin to adhere to human epithelial cells paralleled the antibiotic-induced reduction of mannose binding activity (Fig. 4).

Epithelial adherence, mannose binding activity, and piliation in a streptomycin-resistant derivative. All previously known consequences of streptomycin action require streptomycin receptors on bacterial ribosomes and single-step, highly resistant mutants are believed to lack these receptors (19). To determine whether the sublethal antibiotic effect required the classic streptomycin target site, we employed an Str^R isogenic mutant of *E. coli* M-Str^S. This highly resistant derivate was readily selected from a large population of the cloned, sensitive M-Str^S by growing the parent population on agar plates containing a concentration of streptomycin greatly in excess of the MIC. The purified, cloned derivative, M-Str^R, possessed a similar degree of piliation, yeast cell agglutinating (mannose binding), and epithelial cell adhering ability as the parent strain, M-Str^S. We found that enormous concentrations of streptomycin (up to 10,000 μg/ml) caused minimal loss of epithelial cell adherence and mannose binding

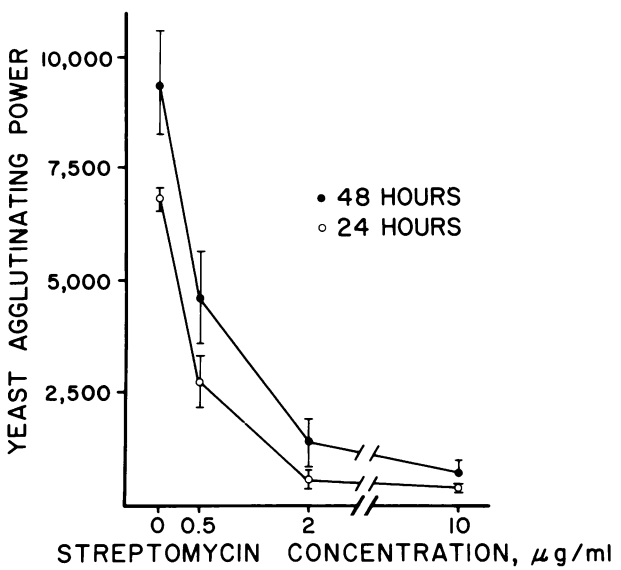

FIGURE 3 Agglutinating activity of *E. coli* grown in increasing concentrations of streptomycin. Aliquots of *E. coli* M-Str^S were harvested after a 24- and 48-h growth period and their YAP (±SE) were determined and expressed as described in Methods.

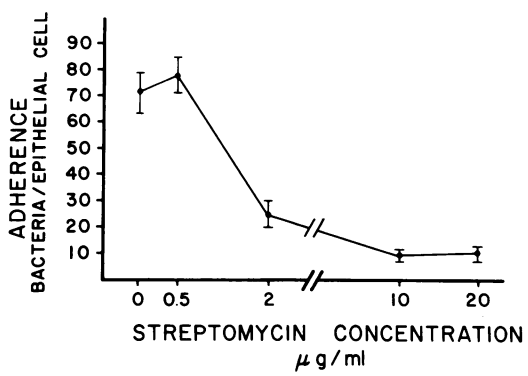

FIGURE 4 Adherence to human epithelial cells by *E. coli* grown in increasing concentrations of streptomycin. Aliquots of *E. coli* M-Str^S were harvested after 48 h growth, washed, adjusted to contain 1 × 10^8 bacteria/0.5 ml, and mixed with 0.5 ml of epithelial cells. After 30 min incubation at room temperature, the mean number of bacteria adherent per epithelial cell (±SE) was calculated and plotted against the concentration of streptomycin in the growth medium.

TABLE II

Effects of Streptomycin on Adherence and Yeast Agglutination of a Streptomycin-Resistant Strain of E. coli (M-Str^R) Grown for 48 h with or without Antibiotic

Streptomycin concentration	Adherence (±SE) bacteria/epithelial cell	YAP
μg/ml		
0	106±13	7,000
5,000	113±7	8,200
10,000	35±5	4,900
20,000	No growth	No growth

activity of the resistant strain (M-Str^R) (Table II). Although pili were present it was difficult to quantitate accurately the degree of piliation when bacteria were grown in high drug concentrations (≥5 μg/ml) due to large electron dense deposits on bacterial surfaces.

Effects of altered growth conditions on streptomycin action. The timing of the addition of streptomycin in a growing culture determined the magnitude of its effect; the earlier the addition, the greater the effect. As much as 10 μg/ml antibiotic, when added to growing bacteria after the culture had passed midlog phase (about 5×10^8 CFU/ml), did not prevent the subsequent acquisition of mannose binding activity. The sublethal effects of streptomycin also were observed when *E. coli* were grown in minimal medium instead of BHI.

Reversal of sublethal effects upon subculture to antibiotic-free broth. To determine the reversibility of the streptomycin-induced suppression of mannose binding activity, we subcultured serially the antibiotic-treated samples into drug-free media. All cultures recovered full activity; the rapidity of the recovery process was inversely proportional to the concentration of drug used initially (Table III). Piliation returned to ≥90% in all drug-treated cultures after their first 48-h subculture to drug-free medium.

Antigenic alterations induced by sublethal streptomycin. Rabbit antiserum, raised by immunization with *E. coli* M-Str^S grown without antibiotic, was serially absorbed with washed *E. coli* M-Str^S grown in the presence of 10 μg/ml streptomycin. The drug-grown bacteria used for absorption were only 11% piliated. The absorbed serum lost all agglutinating activity against the absorbing phenotype (antibiotic-grown M-Str^S), but maintained a high titer of activity against the immunizing phenotype (drug-free-grown M-Str^S) (Fig. 5). Apparently, growth under antibiotic pressure caused M-Str^S to lose a reactive antigen(s) concomitant with the organism's loss of mannose binding activity.

TABLE III

Reversibility of Streptomycin-Induced Suppression of Mannose Binding Activity: Relative YAP of E. coli (M-Str^S) Grown in Streptomycin and Serially Subcultured to Fresh Broth without Drug*

	Drug concentration of initial growth			
Serial culture	None	2 μg/ml	5 μg/ml	10 μg/ml
Initial drug culture	100	5–10	5–10	<1
First drug-free subculture	100	100	30	15
Second drug-free subculture	100	100	100	100

* See Methods for derivation.

Further experiments were conducted to shed light on the nature of the antigen(s) which was lost or not expressed in drug-grown organisms. Antiserum absorbed with drug-grown organisms retained antipilus antibodies as demonstrated by the ability of the serum to inhibit a well known biological activity of pili, namely the mediation of mannose-sensitive hemagglutination

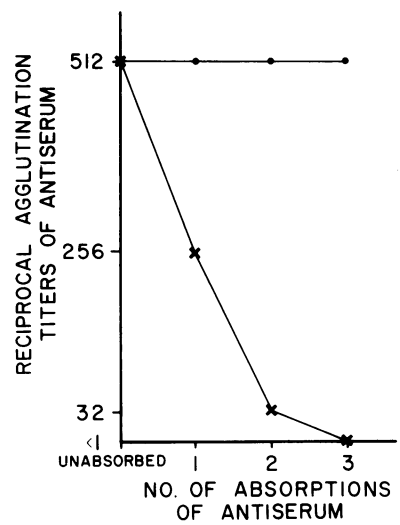

FIGURE 5 Antigenic alteration induced by bacterial growth in sublethal concentration of streptomycin. *E. coli* M-Str^S grown in 10 μg/ml streptomycin or in drug-free media was harvested after 48 h growth, washed, and resuspended in PBS. 1 ml of rabbit antiserum raised against whole cells of M-Str^S grown in drug-free media (immunizing phenotype) was absorbed with 10^{10} whole cells of M-Str^S grown in 10 μg/ml streptomycin (absorbing phenotype). Agglutination of bacteria was performed by mixing on a glass slide equal volumes (10 μl) of serially diluted antiserum and either immunizing phenotype (●) or absorbing phenotype (x) each at 2×10^9 CFU/ml PBS. The reciprocal of the highest dilution (titer) of antiserum giving visible agglutination was plotted against the number of absorptions of antiserum with the streptomycin-grown phenotype.

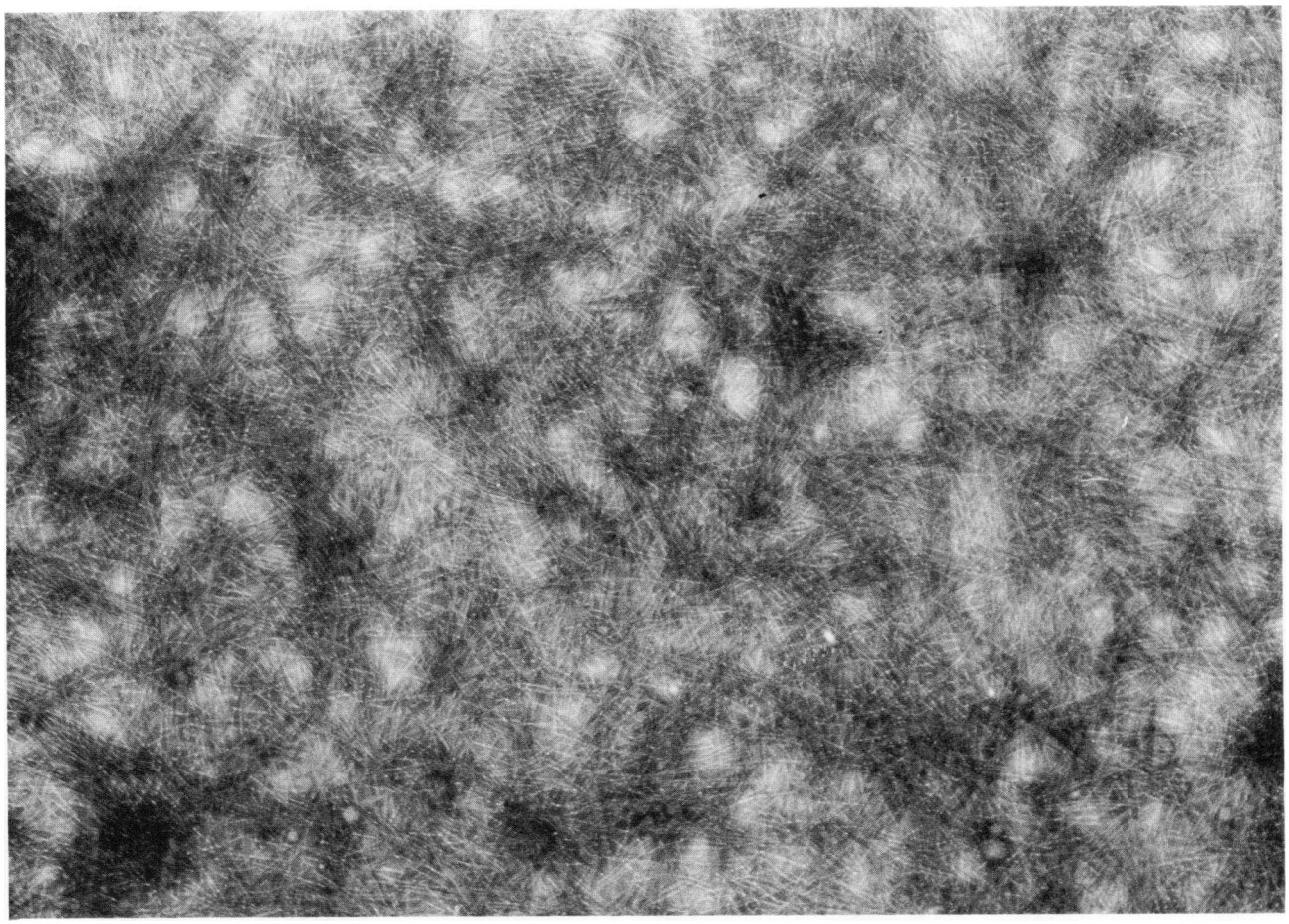

FIGURE 6 Pilus preparation. Negatively stained with phosphotungstic acid. ×60,000.

(12, 18). The pili preparation (shown in Fig. 6) caused hemagglutination which was inhibited by mannose. The absorbed but not the preimmune serum, when preincubated with the pilus preparation, inhibited the pilus-mediated hemagglutination up to a dilution of 1:100. The papain-cleaved antisera (absorbed and unabsorbed) lost their ability to agglutinate drug-free-grown bacteria but were able, at a dilution of 1:10, to inhibit agglutination of these bacteria by the non-papain-cleaved antisera indicating that there were Fab anti-*E. coli* fragments in the enzyme-treated sera. Significant inhibition of *E. coli* adherence, comparable to that obtained by α-MM, was obtained in the presence of papain-cleaved absorbed and unabsorbed, but not preimmune, antisera (Table IV). Taken together the results indicate that epithelial adherence was lost in the drug-grown organisms concomitant with loss of pilus antigen and mannose binding activity.

Cell wall alterations induced by growth in streptomycin. Ultrastructural studies of thin sections of *E. coli* revealed two apparent alterations in the drug-grown organisms: (*a*) the cell cytoplasm was somewhat retracted from the overlying membrane, and (*b*) the outline of the inner membrane was less distinct than that of the drug-free-grown strain (Fig. 7). Whether or not these changes might reflect changes in the bacterial location responsible for synthesis of ligand (e.g., pili), requires further investigation. In some samples (not shown), blebs were seen on the cell surface, similar to previous observations by Iida and Koike (20).

DISCUSSION

In this study we have demonstrated that streptomycin can alter *E. coli* adhering ability at drug concentrations significantly below the MIC. We previously showed that adherence of several laboratory strains (6) and most clinical isolates (7) of *E. coli* to human epithelial cells is mannose sensitive and mediated by the interaction of mannose-bearing receptors on epithelial cells with a lectin-like protein, specific for mannose, on the bacterial surface. In this investigation we demonstrated that the epithelial cell adherence of the strain employed also was mannose sensitive and that

TABLE IV
Inhibition of Epithelial Cell Adherence by Mannose and Papain-Treated Antisera

Inhibitor	Final dilution	Percentage of inhibition*	*P* value‡
		%	
α-MM	2.5	74	<0.001
Cleaved preimmune serum	1:2	18	—
Cleaved unabsorbed antiserum	1:2	78	
	1:10	78	<0.001
	1:20	77	
Cleaved absorbed antiserum	1:2	71	<0.001
	1:10	59	<0.01
	1:20	58	<0.02

* Calculated as compared to PBS control. For the purpose of this evaluation, a background of four bacteria per epithelial cell was subtracted from all adherence values.
‡ Derived from two-tailed Student's *t* test comparing adherence of bacteria in test inhibitor to adherence of bacteria in cleaved preimmune serum. There was no significant difference between adherence in cleaved preimmune serum and adherence in PBS.

sublethal levels of antibiotic suppressed the strain's ability to bind both to human epithelial cells and to mannose residues on yeast cells. We conclude that the suppression of adherence to human epithelial cells was due to the inability of antibiotic-grown organisms to express their mannose-specific ligand. The effect was seen at streptomycin concentrations 50-fold less than the MIC, at which alterations in bacterial growth, as measured by viability, protein and DNA synthesis, and net bacterial mass, were undetectable.

Streptomycin, the prototype of aminoglycoside antibiotics, inhibits or distorts protein synthesis by binding to the S12 protein of sensitive ribosomes (19), thereby interfering with normal translation of mRNA (10). The suppression of mannose-binding activity by streptomycin was probably mediated by this classical mechanism because the effect was: (*a*) reversible and therefore probably not dependent on selection of a mutant, (*b*) seen only in growing organisms, and (*c*) not seen with the antibiotic-resistant mutant. Although the suppression of ligand was deteced at doses of antibiotic below that which caused noticeable effect on growth, the degree of ligand suppression was greater as the streptomycin dose increased to affect the growth rate. Moreover, there was no direct effect on ligand by antibiotic: even high doses of streptomycin were ineffective once mannose-binding activity was present and no liberation of ligand could be detected in the media of drug-treated cultures. Taken together the results indicate that suppression of mannose binding activity was due to either inhibition or distortion of mRNA translation, but do not distinguish clearly between the two streptomycin actions on bacterial ribosomes. Nevertheless, we infer that distortion rather than inhibition of protein synthesis was responsible for the streptomycin effect on mannose binding activity. This notion is supported by previous studies (21–23) of the effects of streptomycin on protein synthesis, both with intact *E. coli* and in a cell-free system of protein synthesis. Gorini and Kataja (21) showed that a mutant strain of *E. coli* grown with antibiotic under conditions where no killing occurred demonstrated a dose-dependent formation of abnormal proteins in the cell with limitation of further growth. Bissell (22) showed that wild-type *E. coli* B, when grown in streptomycin at low concentration (2 μg/ml), synthesized aberrent enzymes. Davies et al. (23) reported that when antibiotic was added to synthetic messenger in a cell-free system of protein synthesis, the drug caused extensive misreading, i.e., incorporation of incorrect amino acids. These effects have been interpreted to be the consequence of streptomycin-induced distortion of the fiedlity of the translation of mRNA. Like the antibiotic effect observed in this study, the misreading effect was obtained at sublethal concentrations of antibiotic and was reversible, and total protein synthesis was unaffected.

Definitive proof that sublethal amounts of streptomycin caused misreading of mRNA responsible for ligand synthesis would require isolation and comparison of the lectin-like substance from drug-free bacteria with its anatomic counterpart on the drug-treated phenotype which is deficient in ligand activity. Recent evidence suggests that in *E. coli* the mannose-sensitive ligand is a protein and resides on the type 1 pilus (18). Our ultrastructural studies of pili on bacteria grown in streptomycin demonstrated moderate, but incomplete loss of pili without evidence of structural alteration. The suppression of pilus formation was proportional to drug concentration and may have accounted for loss of ligand. Nevertheless results were not conclusive enough to exclude an additional factor(s) such as altered (nonfunctional) pili or suppressed ligand not present on the pilus per se as the method of Novotny et al. of counting pili (17) may be insufficiently quantitative. Concomitant with functional ligand suppression, alterations in surface membrane ultrastructure and antigenic determinants followed growth in sublethal levels of drug. Thus the drug-grown *E. coli* were unable to express fully pilus and ligand antigen(s) because immune serum absorbed with drug-grown *E. coli* still possessed antipilus activity, as detected by inhibition of pilus-induced

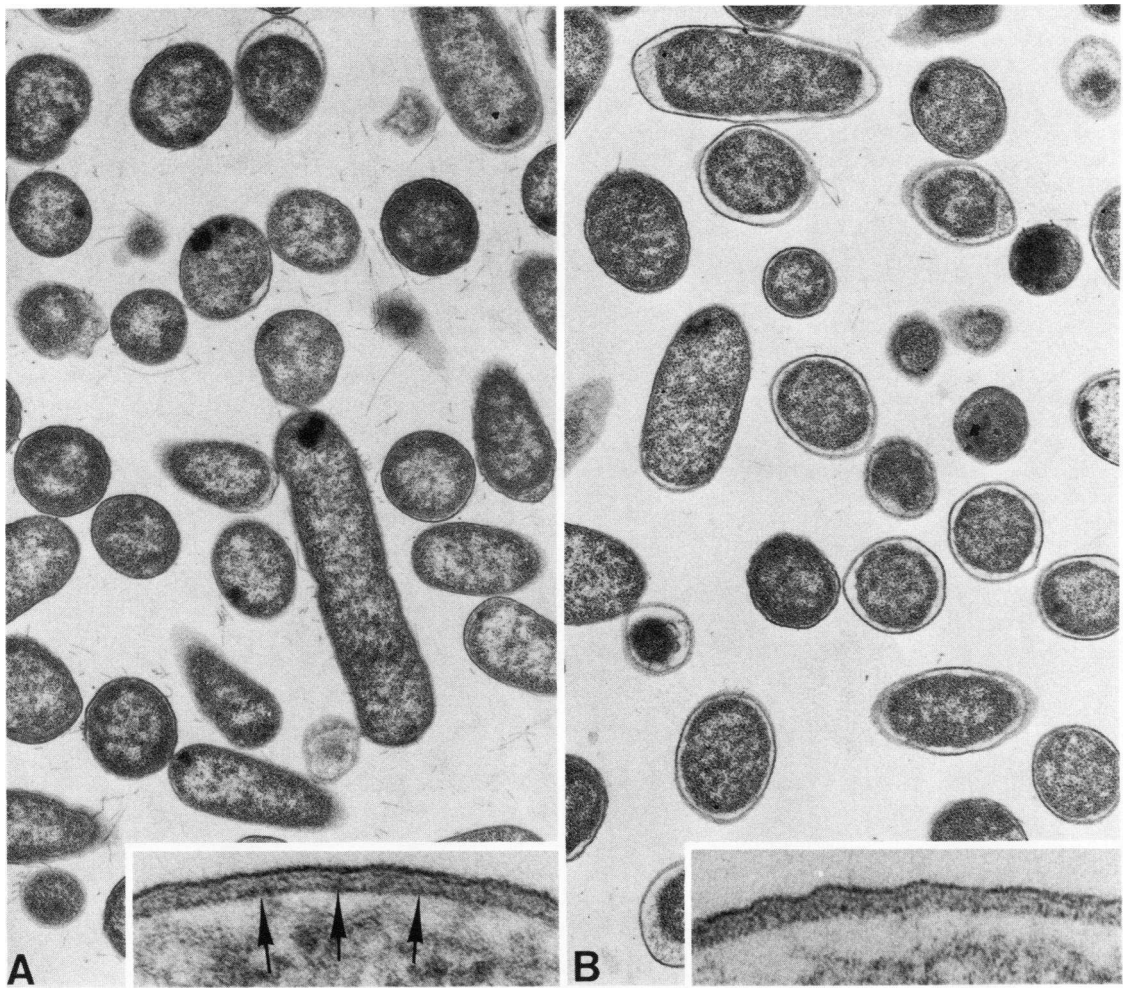

FIGURE 7 Thin-section ultrastructure of *E. coli* M-Str^S grown in drug-free media (A) and in 10 μg/ml streptomycin (B). ×10,000. Note plasmolysis (B) and loss of definition of inner membrane (inset, B) of drug-grown organisms. ×150,000. Appearance of inner membrane of *E. coli* grown in drug-free media, is indicated by arrows (inset, A). ×150,000.

hemagglutination, and antiligand activity, as measured by an adherence inhibition assay.

Two aspects of this investigation deserve mention. First, sublethal concentrations of streptomycin and related antibiotics may reach mucosal surfaces intermittently during a typical course of therapy and thereby interfere with the ability of *E. coli* to colonize these surfaces. Second, the use of inhibitors of the mannose-specific ligand, and possibly of other bacterial substances important for adherence, may provide a tool for the elucidation of the genetic and biochemical bases of bacterial factors responsible for mucosal colonization.

ACKNOWLEDGMENTS

We thank Valerie Long, Susan White, Harry Courtney, and Loretta Hatmaker for expert technical assistance.

This work was supported by program-directed research funds from the U. S. Veterans Administration and by research grants AI-13550 and AI-10085 from the U. S. Public Health Service.

REFERENCES

1. Lorian, V. 1975. Some effects of subinhibitory concentrations of antibiotics on bacteria. *Bull. N. Y. Acad. Med.* **51:** 1046–1055.
2. Savage, D. C. 1972. Survival on mucosal epithelia, epithelial penetration and growth in tissue of pathogenic bacteria. *In* Microbial Pathogenicity in Man and Animals. H. Smith and J. H. Pearce, editors. Cambridge University Press, New York. 25–51.
3. Gibbons, R. J. 1977. Adherence of bacteria to host tissue. *In* Microbiology. D. Schlessinger, editor. American Society for Microbiology, Washington, D. C. 395–406.
4. Alkan, M. L., and E. H. Beachey. 1978. Excretion of lipoteichoic acid by group A streptococci. Influence of penicil-

lin on excretion and loss of ability to adhere to human oral mucosal cells. *J. Clin. Invest.* **61:** 671–677.
5. Ofek, I., E. H. Beachey, and N. Sharon. 1978. Surface sugars of animals cells as determinants of recognition in bacterial adherence. *Trends Biochem. Sci.* **3:** 159–160.
6. Ofek, I., D. Mirelman, and N. Sharon. 1977. Adherence of *Escherichia coli* to human mucosal cells mediated by mannose receptors. *Nature (Lond.).* **265:** 623–625.
7. Ofek, I., and E. H. Beachey. 1978. Mannose binding and epithelial cell adherence of *Escherichia coli. Infect. Immun.* **22:** 247–254.
8. Peska, S. 1971. Inhibitors of ribosomal functions. *Annu. Rev. Microbiol.* **25:** 287–557.
9. Davis, B. D., P-C. Tai, and B. J. Wallace. 1974. Complex interactions of antibiotics with the ribosome. *In* Ribosomes. M. Nomura, A. Tissieres, and P. Lengyel, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y. 771–789.
10. Gorini, L. 1974. Streptomycin and misreading of the genetic code. *In* Ribosomes. M. Nomura, A. Tissieres, and P. Lengyel, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y. 791–803.
11. Baumgartner, H. R., and G. V. R. Born. 1968. Effects of 5-hydroxytrypatime on platelet aggregation. *Nature (Lond.).* **218:** 137–141.
12. Duguid, J. P., and R. R. Gillies. 1957. Fimbriae and adhesive properties in dysentery bacilli. *J. Pathol.* **74:** 397–411.
13. Ofek, I., E. H. Beachey, W. Jefferson, and G. L. Campbell. 1975. Cell membrane-binding properties of group A streptococcal lipoteichoic acid. *J. Exp. Med.* **141:** 990–1003.
14. Porter, R. R. 1959. The hydrolysis of rabbit gamma globulin and antibodies with crystalline papain. *Biochem. J.* **73:** 119–129.
15. Beachey, E. H., and I. Ofek. 1976. Epithelial cell binding of group A. streptococci by lipoteichoic acid on fimbriae denuded of M protein. *J. Exp. Med.* **143:** 759–771.
16. Ofek, I., E. H. Beachey, and A. L. Bisno. 1974. Resistance of *Neisseria gonorrhoeae* to phagocytosis: Relationship to colonial morphology and surface pili. *J. Infect. Dis.* **139:** 310–316.
17. Novotny, C., J. Carnaham, and C. C. Brinton, Jr. 1969. Mechanical removal of F pili, type I pili, and flagella from Hfr and TRF donor cells and the kinetics of their reappearance. *J. Bacteriol.* **98:** 1294–1306.
18. Salit, I. E., and E. C. Gotschlich. 1977. Hemagglutination by purified type I *Escherichia coli* pili. *J. Exp. Med.* **146:** 1169–1181.
19. Ozaki, M., S. Mizuchima, and M. Nomura. 1969. Identification and functional characterization of the protein controlled by the streptomycin-resistant locus in *E. coli. Nature (Lond.).* **222:** 333–339.
20. Iida, K., and M. Koike. 1974. Cell wall alterations of gram negative bacteria by aminoglycoside antibiotics. *Antimicrob. Agents Chemother.* **5:** 95–97.
21. Gorini, L., and E. Kataja. 1964. Streptomycin-induced oversuppression in *E. coli. Proc. Natl. Acad. Sci. U. S. A.* **51:** 995–1001.
22. Bissell, D. M. 1965. Formation of an altered enzyme by *Escherichia coli* in the presence of neomycin. *J. Mol. Biol.* **14:** 619–622.
23. Davies, J., W. Gilbert, and L. Gorini. 1964. Streptomycin, suppression, and the code. *Proc. Natl. Acad. Sci. U. S. A.* **51:** 883–890.