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Kinetics and Mechanisms of Action of Drugs on Microorganisms XI: Effect of Erythromycin and Its Supposed Antagonism with Lincomycin on the Microbial Growth of *Escherichia coli*

EDWARD R. GARRETT, SAMUEL M. HEMAN-ACKAH, and GEORGE L. PERRY

Abstract □ The steady-state growth of *Escherichia coli* in broth cultures is inhibited by erythromycin with a new steady-state growth-rate constant ($k_{app.}$), which is linearly related to drug concentrations in the range 0–10.0 mcg. ml.$^{-1}$ as $k_{app.} = k_0 - k_E E$, where k_E is the inhibitory-rate constant for drug concentration E of drug-free rate constant k_0. The $k_{app.}$ at $E > 10.0$ mcg. ml.$^{-1}$ adheres to a kinetic model, which implies the saturation of a limited number of receptor sites in accordance with the equation $k_{app.} = k_0 - k_a E/(1 + k_b E)$, where k_a and k_b are constants of proportionality related to drug concentration partitioned into the biophase and its affinity for available receptor sites. The dependence of *E. coli* growth rate on drug concentrations is invariant with the organism population or broth concentrations. However, values for k_a increase 10-fold as the pH of broth is increased from 6.80 to 7.80 while k_0 remains constant. This indicates that the unprotonated fraction of the drug concentration contributes to the activity. Lincomycin in Phase I-affected growth has the same formal dependency on concentration as does erythromycin with a potency ratio of 6.68:1, erythromycin base to lincomycin base, on a weight basis. The combined effects of erythromycin and lincomycin in Phase I of its effect are not antagonistic on the growth rate of *E. coli* in the subinhibitory range and can be predicted on the basis of adding equivalent amounts in accordance with the coincident response–dose curves of erythromycin and lincomycin (Phase I).

Keyphrases □ Erythromycin effect—*Escherichia coli* steady-state growth □ Lincomycin growth, Phase I—erythromycin effect □ Reversibility—erythromycin antimicrobial activity □ Microbiological analysis—erythromycin action on *E. coli* □ Kinetics—erythromycin action on *E. coli*.

The mode of action of the macrolide erythromycin is generally ascribed to inhibition of protein synthesis (1–9). Erythromycin binds exclusively to the 50 S subunit of ribosomes from *Escherichia coli* (1, 4, 10), *Staphylococcus aureus* (11), *Bacillus subtilis* (6, 8, 12), *Bacillus megaterium* (2, 13), and *Bacillus stearothermophilus* (2) in reconstituted cell-free systems to inhibit polypeptide synthesis which has been stated to be a rapid and reversible process (8). Tanaka *et al.* (4) observed maximum binding of the 50 S ribosomal subunit of *E. coli* Q. 13 at very low concentrations of erythromycin (~0.6 mcg. ml.$^{-1}$). Only about one-tenth of that amount was found to bind to the ribosomes of resistant mutants.

It has been speculated (8) that erythromycin competes with the transport RNA carrying the peptidyl radical involved in amino acid polymerization for a common binding site on the 50 S subunit of ribosomes. An alternative proposed model (12) for erythromycin's protein inhibition is that it inhibits a "translocase" factor which is necessary for the transfer of the peptidyl t-RNA elongated by a single aminoacyl residue from a ribosomal acceptor site to a donor site.

Conflicting views have been expressed about the modes of action of those antibiotics (*e.g.*, erythromycin, lincomycin, and chloramphenicol), which are supposed to have the common 50 S ribosomal binding site (7, 8, 10, 12). In fact, combinations of erythromycin and lincomycin have been claimed to be antagonistic (2, 3, 8, 10, 12), which would not necessarily follow from an assumption of similar modes of action.

The application of microbial kinetics to the quantification and prediction of antimicrobial action has been demonstrated (14–22). The effects of subinhibitory concentrations of drugs on the growth of bacteria have been studied in simple reproducible systems with *E. coli* strain B/r as the test organism to derive kinetic parameters that may characterize antibacterial action and drug–receptor interaction and to provide insight into the possible mechanisms of drug action.

This paper presents the results of such studies on the action of erythromycin on the growth of *E. coli*. It considers the formal dependence of the kinetic constants of growth inhibition on antibiotic concentration as affected by inoculum size, composition and pH of the media, and reversibility of the erythromycin concentrations. In addition, the actions of combinations of erythromycin and lincomycin on microbial growth, as compared with their *a priori* expectation, are considered.

EXPERIMENTAL

Organism—Replicate slants of *E. coli* strain B/r were used in all experiments. The slants had been prepared from a single colony and were stored in a refrigerator at 4°.[1]

Culture Media—Bacto Antibiotic Medium 3[2] was rehydrated according to the specifications of the manufacturer to peptone broth USP. The media were filtered twice through Millipore 0.45-μ HA filters and autoclaved at 120° for 15 min. The pH of the media was 7.05 ± 0.05, with the exception of those that were used to study the antibacterial activity as a function of pH. To obtain media with a pH in the range of 6.8–7.8, various amounts of Millipore-filtered 1.7 *N* HCl and 2 *N* NaOH, respectively, were added to the culture media aseptically after the sterilization.

Antibiotic—An assayed sample of erythromycin lactobionate[3] (670 mcg. base eq. mg.$^{-1}$) was used and will be referred to here as erythromycin I. An assayed sample of lincomycin hydrochloride[4] (895 mcg. base eq. mg.$^{-1}$) was also used. The references to concentrations of drugs throughout this paper refer to these samples of antibiotics.

Bacterial Cultures—An aliquot (5 ml.) of culture medium was inoculated from a fresh slant, and the culture was allowed to grow for 12 hr. at 37.5° in an incubator. A sample of 0.5 ml. was then diluted 100-fold into fresh medium. The growth of the culture was followed up to 2 × 10^7 *E. coli* ml.$^{-1}$. Samples of 0.5 ml. of this culture (undiluted or suitably diluted in broth) were finally added to replicate volumes of 49.0 ml. broth in loosely capped 125-ml. conical flasks. The growth of culture was followed to a desired inoculum size, and 0.5 ml. of drug was added. The cultures were maintained at 37.5 ± 0.1° in a 50-gal. constant-temperature water bath equipped with a shaker. All pipets and media used for the dilutions of the cultures were prewarmed to protect the organisms from temperature shocks.

Total Count Method—The method has been previously described (16). Samples of 1.00 ml. were withdrawn at 20-min. intervals from the cultures. They were diluted to obtain counts within a range of 10,000–30,000 counts/50 μl. on the Coulter counter, model B.[5] The diluent used was a Millipore 0.45-μ HA-filtered aqueous solution of 0.85% NaCl and 1% formaldehyde. The instrument was equipped with a 30-μ orifice. The settings were: aperture current, 5; amplification, 8; gain, 10; lower threshold, 13; and upper threshold, maximum. The total counts were corrected for the background count of the particular batch of medium used and diluted in the same way as the sample. The background counts in general did not exceed 1000 counts/50 μl.

Viable Count Method—Samples of 0.50 ml. were withdrawn from the cultures and appropriately diluted into sterilized 0.85% saline solution so that 50–150 colonies per plate would result. From these dilutions, aliquots of 1.00 ml. were pipeted onto each of three replicate agar plates. The plates were incubated for 48 hr. at 37.5°, and the colonies were counted on a Colony counter, model C-110.[6]

Effect of Antibiotic Concentration on Growth Rates—Fresh solutions of the respective antibiotics were aseptically prepared for each experiment. They were sufficiently diluted so that aliquots of 0.5 ml. added to 49.5 ml. culture volumes yielded the desired drug concentrations (Table I). The solutions were added to the cultures growing at 37.5° in the logarithmic phase at an organism population of 1.3 × 10^6 *E. coli* ml.$^{-1}$. Samples were withdrawn every 20 min. and counted by both the viable and total cell count methods. One culture without drug was studied in each experiment as the control to obtain the growth-rate constant in absence of the drug. The growth curves for 0–250 mcg. ml.$^{-1}$ at pH 7.05 were obtained (Fig. 1). A similar experiment was performed for 0–50 mcg. ml.$^{-1}$ erythromycin I, but counts were obtained by only the total cell count method (Fig. 2).

[1] The strain B/r of *E. coli* is an "in house" strain originally obtained from the Bacteriology Department of the University of Maine (Dr. D. B. Pratt) and has been used in both the Departments of Bacteriology and Pharmaceutics of this university for the last 10 years. In previous publications on "Kinetics and Mechanisms of Action of Drugs on Microorganisms I–X" (14–22), the authors have referred to this *E. coli* as strain B/r. A sample of this strain is being sent to the American Type Culture Collection for appropriate "official" numbering.

[2] Difco Laboratories, Detroit, Mich.

[3] Supplied by Abbott Laboratories, North Chicago, Ill.

[4] Supplied by The Upjohn Co., Kalamazoo, Mich.

[5] Coulter Electronics Co., Hialeah, Fla.

[6] New Brunswick Scientific Co., New Brunswick, N. J.

Table I—Growth-Rate Constants ($k_{app.}$ in sec.$^{-1}$) and Calculated Parameters for Erythromycin-Affected and Lincomycin-Affected *E. coli* in Broth at 37.5° and pH 7.05

Drug Concentrations, mcg. ml.$^{-1}$ × n^a	10^5 $k_{app.}$ Erythromycin I	10^5 $k_{app.}$ Lincomycin HCl, Phase I	10^5 $k_{app.}$ Lincomycin HCl, Phase II
0	60.1	60.1	60.1
2.5	56.9	56.9	—
5.0	54.6	53.6	—
7.5	50.6	50.0	41.7
10.0	45.6	47.5	35.1
20.0	36.9	34.8	26.2
30.0	25.5	24.8	14.5
40.0	18.0	18.6	8.4
50.0	13.4	14.3	5.7
10^5 k_E (ml. mcg.$^{-1}$ sec.$^{-1}$)[b]	1.50	0.27	—
10^5 k_a (ml. mcg.$^{-1}$ sec.$^{-1}$)[c]	1.64	0.35	0.63
10^5 k_a/k_b (sec.$^{-1}$)	117.65	117.65	66.7
10^2 k_b (ml. mcg.$^{-1}$)	1.39	0.28	0.93

[a] n = 1 for erythromycin; n = 0.2 for lincomycin, *i.e.*, actual concentration of lincomycin is five times that stated. [b] Calculated from the slope of the plot of $k_{app.}$ *versus* concentration from 0 to 10 mcg. ml.$^{-1}$ erythromycin I or 0 to 100 mcg. ml.$^{-1}$ lincomycin HCl. [c] k_a, k_b, and k_a/k_b are estimated from the slopes and intercepts of $D/(k_0 - k_{app.})$ *versus* D, where D is concentration of drug and k_0 is $k_{app.}$ at D = 0 for the expression $D/(k_0 - k_{app.}) = D(k_b/k_a) + 1/k_a$ for erythromycin I > 10 mcg. ml.$^{-1}$ and lincomycin HCl in the steady-state Phase I > 100 mcg. ml. and for all studied lincomycin concentrations in the second steady-state Phase II that occurs after several generations of lincomycin-affected growth (22).

Effect of Broth Concentration on Drug-Affected Growth Rates—Various strengths of peptone broth USP, buffered at pH 7, were prepared so that the concentrations of broth ingredients were

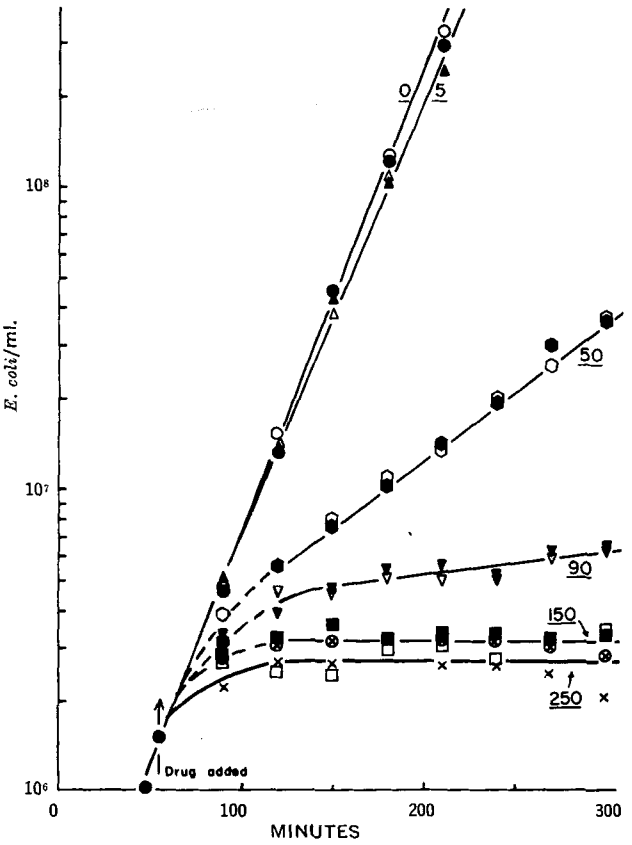

Figure 1—*Semilogarithmic plot of* E. coli/*ml. against time after broth inoculation in the presence of erythromycin I. Each curve is labeled in terms of micrograms per milliliter of erythromycin I. The open symbols are total counts obtained by the Coulter counter. The solid symbols are viable counts obtained by colony counts.*

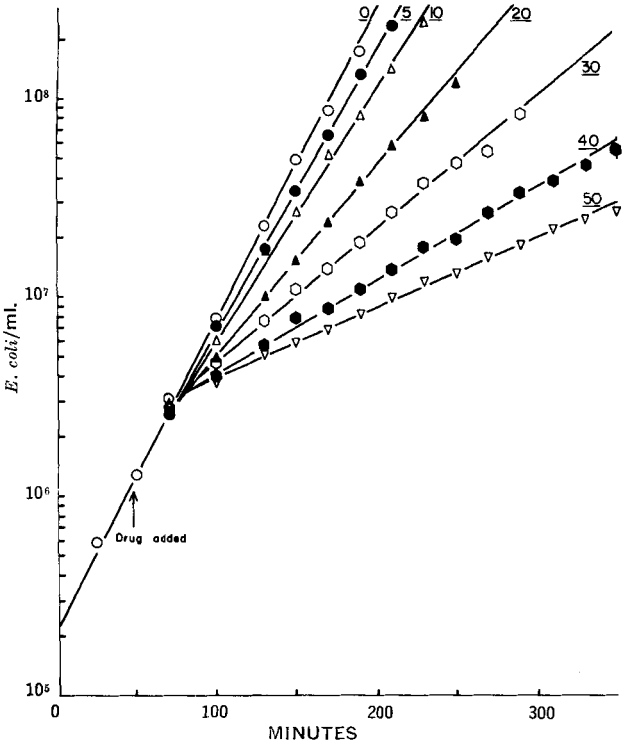

Figure 2—*Typical semilogarithmic plots of* E. coli *growth by total counts (Coulter) in the presence of the labeled concentrations of erythromycin I in micrograms per milliliter at 37.5° and pH 7.05.*

normal, halved, and doubled. Six replicate 49.5-ml. volumes of each kind were inoculated with 0.5 ml. culture containing 2×10^7 *E. coli* ml.$^{-1}$ in the logarithmic growth. They were maintained at 37.5°; when the cell concentration reached 10^6 ml.$^{-1}$, 0.5 ml. of drug solutions was added to achieve erythromycin I concentrations ranging from 5–80 mcg. ml.$^{-1}$ (Table II). The sixth replicate contained no drug. Coulter counts were obtained from samples withdrawn every 20 min.

Effect of Organism Population on Drug-Affected Growth Rates—Each flask of three sets of six replicate 49.5-ml. volumes of culture medium was inoculated with 0.5 ml. of appropriately diluted culture growing in the logarithmic phase. The three sets were allowed to grow at 37.5° to cell concentrations of 1.6×10^4, 1.3×10^5, and 1.1×10^6 *E. coli* ml.$^{-1}$, respectively. Aliquots (0.5 ml.) of drug solutions were added to achieve erythromycin I concentrations from 2.5–12.5 mcg. ml.$^{-1}$ (Table III). One culture in each set contained no drug. Coulter counts were obtained from samples withdrawn every 20 min.

Reversibility of Drug Action—A 49.5-ml. volume of broth was inoculated at zero time with *E. coli* to a cell concentration of 2×10^5 ml.$^{-1}$ (Figs. 3 and 4). The culture was allowed to grow at 37.5°

Table II—Growth-Rate Constants ($k_{app.}$) for Erythromycin-Affected *E. coli* in Various Broth[a] Concentrations at pH 7.05 and 37.5°

Erythromycin I Concentration, mcg./ml.$^{-1}$	10^5 $k_{app.}$, sec.$^{-1}$ Peptone (USP) Broth[a]	Doubled Nutrients	One-Half Nutrients
0	61.1	57.2	58.6
5	54.3	52.3	52.4
10	45.7	45.8	45.4
20	37.5	38.9	34.0
40	22.7	24.8	26.4
80	5.9	7.5	5.4

[a] The normal composition in grams per liter of broth was: beef extract, 1.5; yeast extract, 1.5; peptone, 5.0; and dextrose, 1.0. The K_2HPO_4 of 3.68 g./l. and KH_2PO_4 of 1.32 g./l. were maintained for all three broths. However the NaCl was 3.5 g./l. for the normal, 0.37 for the doubled, and 4.40 for the halved nutrients prepared from Bacto Antibiotic Medium 3 (Difco Laboratories, Detroit, Mich.).

Table III—Growth-Rate Constants ($k_{app.}$) for Erythromycin-Affected *E. coli* of Various Organism Populations at the Time of Drug Addition

Erythomycin I Concentration, mcg. ml.$^{-1}$	10^5 $k_{app.}$ sec.$^{-1}$ —*E. coli*/ml. at Time of Drug Addition— 9.0×10^3	1.0×10^6	1.10×10^6
0	61.5	61.0	59.5
2.5	59.5	55.8	56.0
5.0	51.8	52.1	49.9
7.5	46.2	45.4	46.8
10.0	42.0	41.2	41.6
12.5	34.6	35.0	37.9

(Curves A). At 50 min., when the growth was in the logarithmic phase and the cell concentration was 10^6 *E. coli* ml.$^{-1}$, 0.5 ml. of a sufficiently diluted erythromycin I solution was added to achieve a final concentration of 50 mcg. ml.$^{-1}$ (Curves B). At 125 min., after the culture of Curve B had settled into a new steady-phase growth, aliquots of 5 and 0.5 ml. were added to 45 and 49.5 ml. fresh broths, respectively, so that both organisms and drug concentrations were diluted 10-fold (Curve C, Fig. 3) and 100-fold (Curve C, Fig. 4). The drug-free culture of Curve A was likewise diluted 10-fold (Curve D, Fig. 3) and 100-fold (Curve D, Fig. 4). At the same time, aliquots of the cultures of Curve B (Figs. 3 and 4) were diluted 10-fold (Curve E, Fig. 3) and 100-fold (Curve E, Fig. 4), respectively, in broths containing enough erythromycin I so that the drug concentration was restored to 50 mcg. ml.$^{-1}$. Dilutions of the cultures of Curve B were also made 10-fold (Fig. 3) and 100-fold (Fig. 4) in broths (Curves F) containing enough erythromycin I to increase the drug concentration about 2-fold (*i.e.*, 100 mcg. ml.$^{-1}$).

At 200 min., when the cultures of Curves C containing 5 mcg. ml.$^{-1}$ (Fig. 3) and 0.5 mcg. ml.$^{-1}$ (Fig. 4) of erythromycin I had emerged from the lag phase, 0.5-ml. aliquots of solutions containing enough erythromycin I were added to reestablish both drug concentrations to 50 mcg. ml.$^{-1}$ (Curves G, Figs. 3 and 4).

Whenever any variation was effected, samples of the broth culture were taken and counted on the Coulter every 10 min. for the first 30 min. and thereafter at intervals of 30 min.

Effect of pH on Drug-Affected Growth Rates—Sufficient amounts of 1 *N* HCl and 2 *N* NaOH were added to the broth to obtain pH values of 6.80, 7.05, 7.20, 7.60, 8.20, and 8.40 (Fig. 5). Six replicate 49.5-ml. volumes of each broth were inoculated with 0.5 ml. of appropriately diluted *E. coli* culture in the logarithmic phase of growth. They were maintained at 37.5° until the organism population reached 10^6 *E. coli* ml.$^{-1}$. Drug solutions (0.5 ml.) were added to five replicates to achieve the desired concentrations of antibiotic shown in Fig. 4. The sixth replicate in each set contained no drug. Coulter counts were obtained from samples withdrawn every 20 min.

Comparison of Growth Rates of Erythromycin-Affected Organisms with Lincomycin-Affected Organisms—Solutions (0.5 ml.) of erythromycin I were added to five replicate 49.5-ml. volumes of broth inoculated with *E. coli* in the logarithmic phase of growth and containing an organism population of 10^6 ml.$^{-1}$ at 37.5°. The final concentration of the drug achieved in the broths ranged from 2.5 to 50 mcg. ml.$^{-1}$ erythromycin I. This was repeated for concentrations of lincomycin HCl, from 12.5 to 250 mcg. ml.$^{-1}$ (Table I). Counts were obtained from samples withdrawn every 20 min. The relationships between the apparent growth-rate constants, $k_{app.}$, and drug concentrations are shown in Fig. 6.

Action of Erythromycin and Lincomycin Combinations—Replicate 49-ml. samples of cultures in the steady-state growth (Curve A, Fig. 7) with 10^6 ml.$^{-1}$ *E. coli* were treated with antibiotic solutions. Aliquots (0.5 ml.) of appropriately diluted erythromycin I or lincomycin HCl solutions were added to separate cultures of Curve A 50 min. after inoculation. The resultant plots are given as Curve B in Fig. 7 for final concentrations of 15.8 mcg. ml.$^{-1}$ of erythromycin I or 60 mcg. ml.$^{-1}$ of lincomycin HCl or as Curve D for final concentrations of 31.6 mcg. ml^{-1} of erythromycin I or 120 mcg. ml.$^{-1}$ of lincomycin HCl. Seventy-five minutes after the addition of the drug, when the culture had settled to a new steady-state growth, aliquots (0.5 ml.) of appropriately diluted lincomycin HCl or erythromycin I solutions were added to the replicate solution that had been treated previously with the alternate drug. The resultant curve

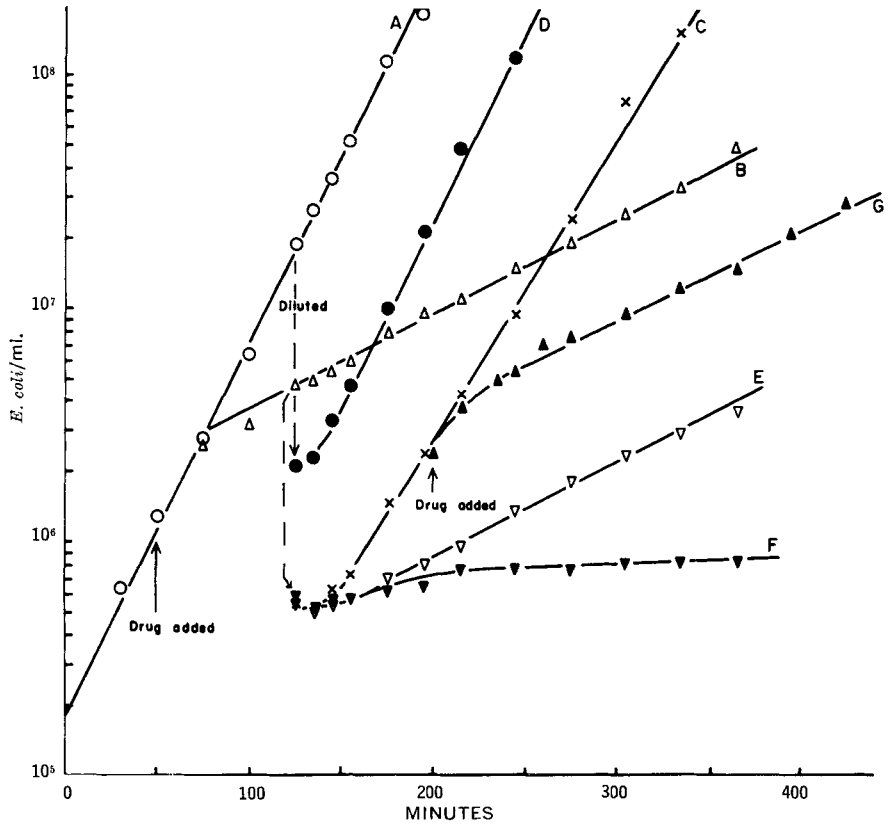

Figure 3—*Semilogarithmic plots of reversibility studies of* E. coli *growth with time on addition of erythromycin I and 1:10 dilution of culture with broth. Curve A is without drug. Curve B is after addition of 50 mcg./ml. Curves C, E, F, and G are after 1:10 dilution of the Curve B culture and have final drug concentrations of 5, 50, 100, and 50 mcg./ml., respectively. The dilution broth contained the erythromycin I in the cases of Curves E and F. There was no drug in the diluent in the case of Curve C, and drug was added at 200 min. for Curve G. Curve D is after 1:10 dilution of the culture of Curve A with no drug added.*

for both replicates, independent of the order of addition, was Curve C, Fig. 7. The final concentrations of the drugs in the cultures, represented by Curve C, were 15.8 mcg. ml.$^{-1}$ erythromycin I and 60.0 mcg. ml.$^{-1}$ lincomycin HCl. Coulter counts were obtained on samples that were withdrawn from the treated cultures every 20 min.

Effect of Mixtures of Graded Equipotent Concentrations of Erythromycin and Lincomycin—Equipotent solutions of 42.55 mcg. ml.$^{-1}$ erythromycin I and 200 mcg. ml.$^{-1}$ lincomycin HCl were prepared. They were then mixed in graded proportions using 100–0% of the lincomycin solution and the residual percentage of the erythromycin solution (Fig. 8). This was repeated to give nine graded equipotent mixtures for each of three antibiotic equivalent concentrations. Replicate 49.5-ml. cultures in steady-state growth that contained 10^6 *E. coli* ml.$^{-1}$ were each inoculated with an aliquot (0.5 ml.) of one of the antibiotic mixtures. Coulter counts were obtained for samples withdrawn every 20 min.

RESULTS

The addition of graded concentrations of erythromycin to growing balanced cultures of *E. coli* strain B/r demonstrated a linear semilogarithmic plot (Figs. 1–4) shortly after the addition or dilution of the antibiotic concentration in accordance with Eq 1:

$$\ln N = k_{app.}t + \text{intercept} \qquad \text{(Eq. 1)}$$

where N is the number of organisms, t is time, and $k_{app.}$ is obtained from the slope of the appropriate plots in the steady-state growth. In the absence of antibiotic, $k_{app.}$ is k_0 and the intercept is the natural logarithm of the inoculum size, ln N_0, at time zero.

Coincidence of Total and Viable Counts of Organisms—The coincidence of total (Coulter count) and viable (colony count) numbers of *E. coli* ml.$^{-1}$ in drug-free and in erythromycin-treated growing balanced cultures could not be denied for all erythromycin concentrations up to 150 mcg. ml.$^{-1}$ (Fig. 1). There is no significant evidence of kill in the time intervals studied at doses above the inhibitory concentrations. This evidence of the bacteriostatic action of erythromycin permits the use of total counts to determine the growth rates of organisms affected by subinhibitory concentrations of erythromycin.

Effect of Drug Concentrations on Growth Rates—The derived apparent growth-rate constants, $k_{app.}$ (Eq. 1), for various concentrations of erythromycin I were obtained from the slopes of the plots of Fig. 2 and are listed in Table I.

The extent of inhibition is directly proportional to the erythromycin I concentration, E, in the range 0–10 mcg. ml.$^{-1}$ in accordance with the expression:

$$k_{app.} = k_0 - k_E E \qquad \text{(Eq. 2)}$$

where k_E is defined as the specific inhibitory-rate constant. The $k_{app.}$ is not a linear function of increasing drug concentration when $E > 10$ mcg. ml.$^{-1}$, but it asymptotically approaches zero (Figs. 5 and 6). A plot of $E/(k_0 - k_{app.})$ *versus* E is reasonably linear for those concentrations >10 mcg. ml.$^{-1}$ but shows deviations at concentrations of 0–10 mcg. ml.$^{-1}$. This implies quantitative adherence of the action of erythromycin I on *E. coli* to a previously derived kinetic model (20, 22):

$$\frac{E}{(k_0 - k_{app.})} = E\left(\frac{k_b}{k_a}\right) + \frac{1}{k_a} \qquad \text{(Eq. 3)}$$

at erythromycin I concentrations, $E > 10$ mcg. ml.$^{-1}$. The k_a and k_b are constants of proportionality, which may be related to drug availability in the biophase and drug affinity to receptor or binding sites. The calculated values for the constants k_a and k_b were obtained from the slope and intercept of the linear portion of such a plot in accordance with Eq. 3. Typical plots are given for the data of Fig. 5 in Fig. 9.

Analysis of Culture Broth Variations—The apparent growth-rate constants, $k_{app.}$, obtained at different concentrations of erythromycin I at different concentrations of nutrients in broth are given in Table II. Variation of these growth-rate constants among the different broths is not significantly different from that observed in daily variation in organism growth rates. There is no significant inactivation or binding of the erythromycin by broth constituents at the nutrient concentrations studied.

Effect of Organism on Drug-Affected Growth Rates—The apparent growth-rate constants, $k_{app.}$, for *E. coli* obtained at various concentrations of erythromycin I for three different organism concentrations at the time of drug addition are given in Table III. There are no significant differences among the growth rates for the different organism concentrations at any drug concentration in the range studied. In the absence of any evidence for systematic influence of organism population on growth-rate constants, it is

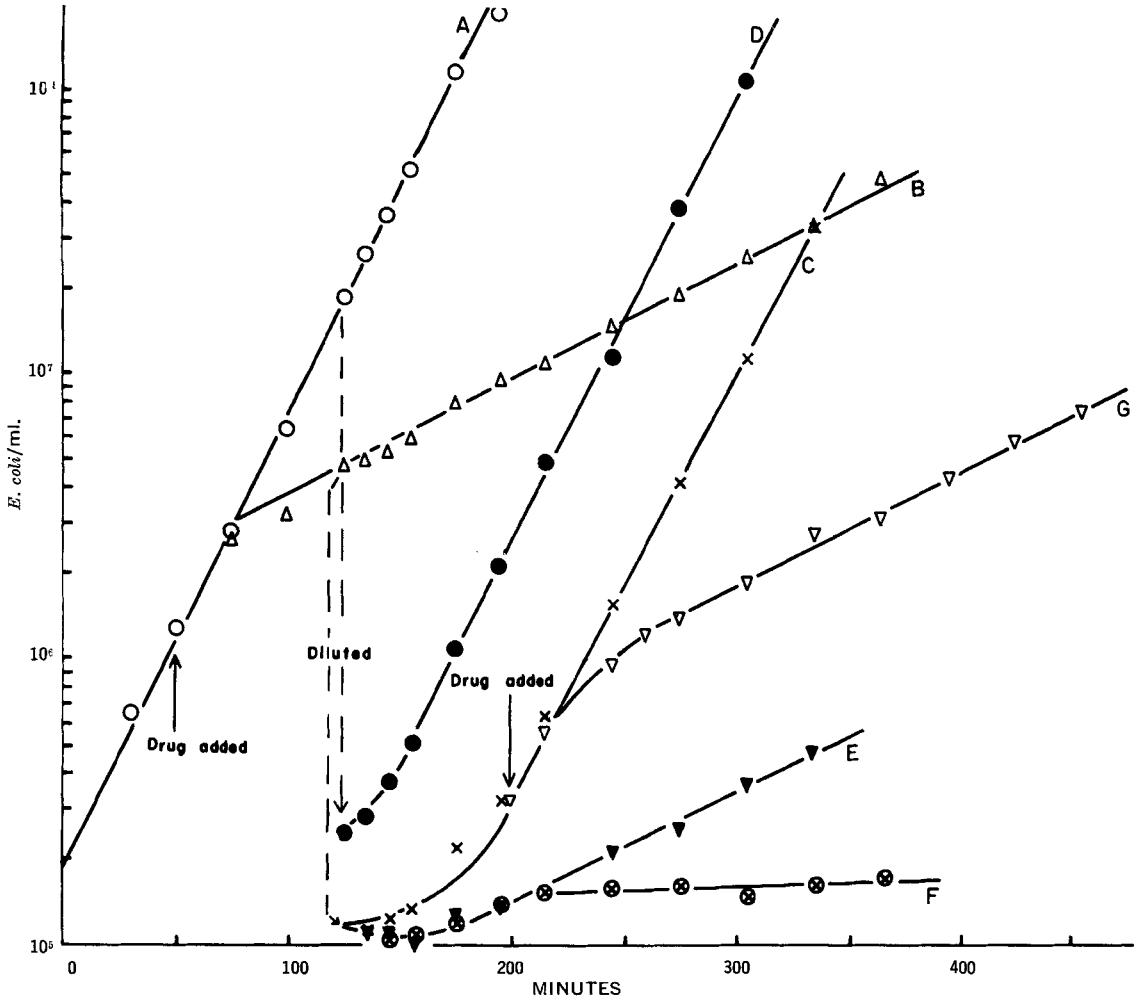

Figure 4—*Semilogarithmic plots of reversibility studies of* E. coli *growth with time on addition of erythromycin l and 1:100 dilution of culture with broth. Curve A is without drug. Curve B is after addition of 50 mcg./ml. Curves C, E, F, and G are after 1:100 dilution of the Curve B culture and have final concentrations of 0.5, 50, 100, and 50 mcg./ml., respectively. The dilution broth contained the erythromycin l in the cases of Curves E and F. There was no drug in the diluent in the case of Curve C, and drug was added at 200 min. for Curve G. Curve D is after 1:100 dilution of the culture of Curve A with no drug added.*

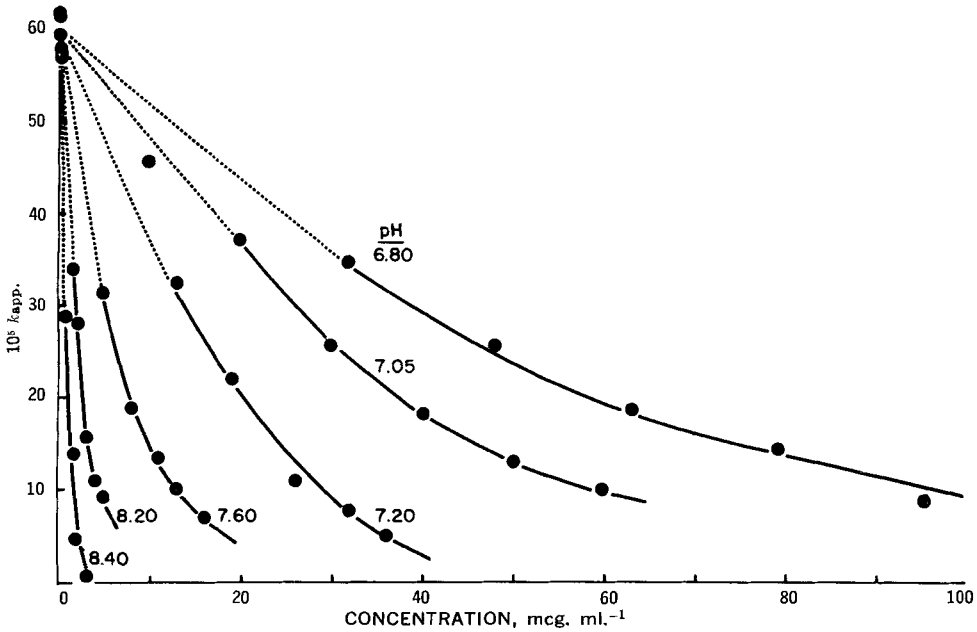

Figure 5—*Dependence of the apparent growth-rate constant,* $k_{app.}$ *in sec.*$^{-1}$, *for* E. coli *on erythromycin concentration at various pH values and at 37.5°.*

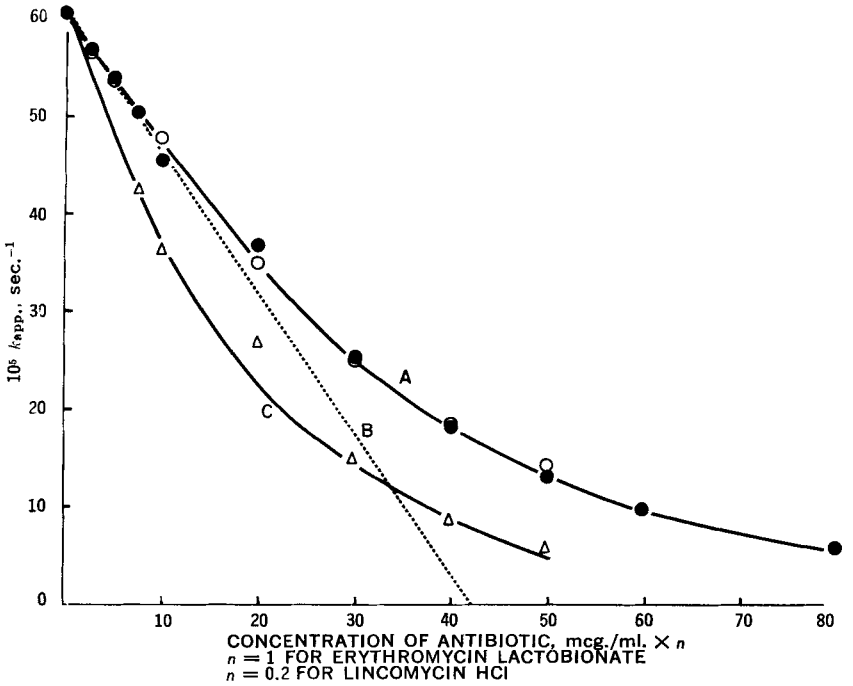

Figure 6—*Demonstration of the coincidence of the dependencies of the apparent growth-rate constants,* $k_{app.}$ *in sec.*$^{-1}$, *for* E. coli *at 37.5° and pH 7.05 on equipotent concentrations of erythromycin l and lincomycin HCl. Curve A represents this dependence, where the closed symbols are for erythromycin l and the open symbols for lincomycin HCl in its Phase 1 growth and where the actual concentrations of lincomycin HCl are five times the stated values. The dashed line, Curve B, demonstrates the linear dependency of* $k_{app.}$ *on drug concentration at low drug concentrations. Curve C represents the* $k_{app.}$ *dependence for Phase II steady state of lincomycin-affected microbial growth.*

concluded that the drug is neither metabolized by the organisms nor depleted in the medium as a result of adsorption to cellular components, inactivation by excretory products of metabolism, and other interactions which may be functions of organism numbers. The effective drug concentrations may, therefore, be assumed to remain constant over the period of study.

Reversibility of Drug Action—The equilibrium of drug between the nutrient medium and the biophase in the microorganism is readily achieved. It took about 30–50 min. after drug addition for the culture to attain a new steady-state phase of growth (Figs. 1–3). The steady-state growth of *E. coli* inhibited by 50 mcg. ml.$^{-1}$ erythromycin l (Curves B in Figs. 3 and 4) reverted to a new steady state when diluted 10-fold and 100-fold, respectively (Curves C in Figs. 3 and 4). The new growth-rate constants were coincident with those found in the presence of the resultant concentrations of the drug, *i.e.*, 5 and 0.5 mcg. ml.$^{-1}$, respectively. The new steady state was attained after 20–30 min. of an apparent initial lag phase. Both drug-free (Curves D in Figs. 3 and 4) and drug-affected cultures (Curves C in Figs. 3 and 4) showed this initial lag period on dilution into fresh broth, so that this may be attributed to a possible need for cell rejuvenation before reestablishment of the new steady state or to a consequence of the shock of dilution.

Cultures inhibited by low concentrations of the drug, *i.e.*, 5 and 0.5 mcg. ml.$^{-1}$ erythromycin l, were further inhibited by addition of more drug to a final concentration of 50 mcg. ml.$^{-1}$, with predictable growth-rate constants (Curves G in Figs. 3 and 4). The time for equilibration of drug between the medium and biophase was also 20–30 min. Similarly, 10-fold and 100-fold dilutions of the culture of Curve B by fresh medium with the same concentration of drug (Curves E of Figs. 3 and 4) achieved similar steady-state growth rates after the same initial lag phase periods of 20–30 min. There were no significant changes in growth-rate constants. A similar dilution of the culture of Curve B into fresh medium containing high concentrations of the drug to increase the final concentration twofold (Curves F of Figs. 3 and 4) produced further inhibition and new steady-state growth rates were attained after 20–30 min. with predictable rate constants.

Thus it was concluded that the action of subinhibitory concentrations of erythromycin on intact *E. coli* cells is readily reversed in finite time intervals. Time lags introduced by dilution effects were the same in the presence or absence of erythromycin.

Effect of pH on Drug-Affected Growth Rates—The apparent growth-rate constants, $k_{app.}$, obtained for *E. coli* in broth at pH values 6.80–8.40 in the absence and presence of graded erythromycin l concentrations are plotted in Fig. 5. Growth-rate constants for drug-free cultures are not significantly affected by pH; the values for drug-affected cultures are significantly decreased at higher pH values for the same erythromycin concentrations (Fig. 5). Since it has been shown (22) that numbers of microorganisms in the

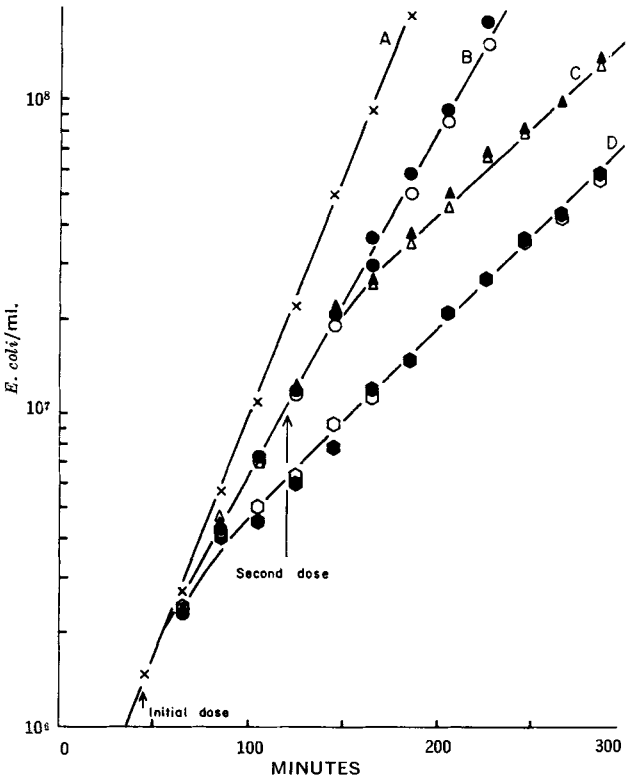

Figure 7—*Nonsignificant effects of order of addition of equipotent erythromycin l and lincomycin HCl on growth rates of* E. coli. *Curve A is for growth of* E. coli *in the absence of drug. Curve B is for growth of* E. coli *in the presence of 15.8 mcg./ml. erythromycin l (closed symbols) or equipotent 60 mcg./ml. lincomycin HCl (open symbols). Curve C is when equipotent lincomycin HCl (60 mcg./ml.) is added to the erythromycin-affected culture of Curve B (closed symbols), or when equipotent erythromycin l (15.8 mcg./ml.) is added to the lincomycin-affected culture of Curve B (open symbols). Curve D is when 200 mcg./ml. lincomycin HCl (open symbols) and 31.6 mcg./ml. erythromycin l (closed symbols) are added to the culture of Curve A.*

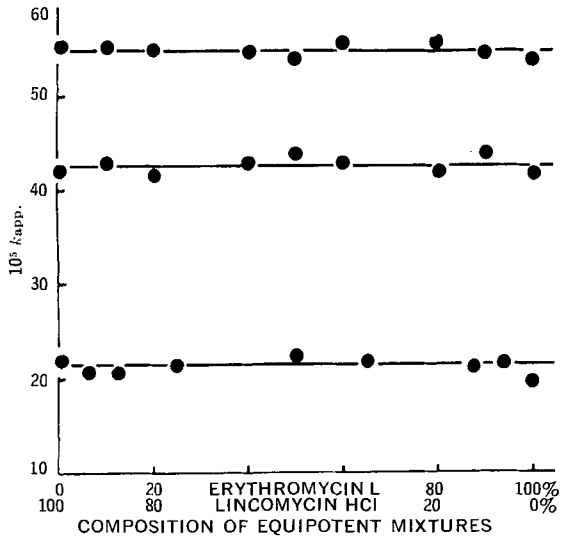

Figure 8—*Effect of continuously varied erythromycin l and lincomycin HCl fractions in equipotent mixtures at three different potency levels on the apparent growth-rate constants,* $k_{app.}$ *in sec.*$^{-1}$, *of* E. coli *at pH 7.05 and 37.5°. Erythromycin l is presumed to be five times as potent as lincomycin HCl on a weight-to-weight basis.*

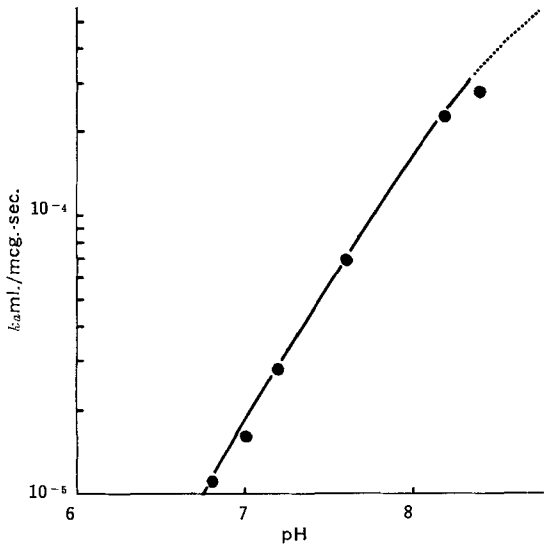

Figure 10—*Semilogarithmic plot of the apparent inhibitory-rate constant,* k_a, *for the effect of erythromycin l on the growth of* E. coli *at 37.5°. The drawn line is consistent with the expression* $k_a = k_a^*(K_s/(K_a + [H^+])$, *where* $k_a^* = 1.11 \times 10^{-3}$ *ml./mcg.-sec. is the intrinsic inhibitory-rate constant for the nonprotonated erythromycin l of pKa' 8.8, and* k_a *is obtained from* $k_{app.} = k_0 - k_aE/(1 + k_bE)$.

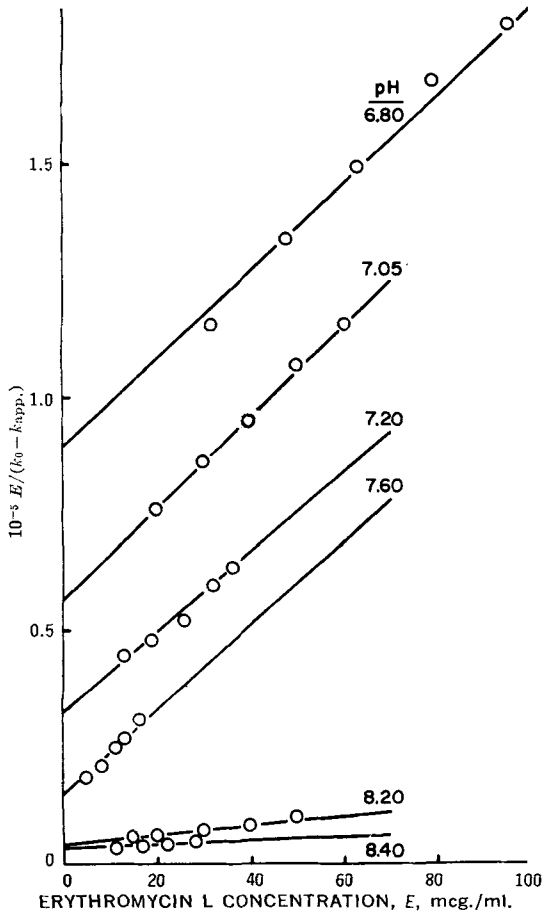

Figure 9—*Demonstration of saturation kinetics for the action of erythromycin l at higher concentrations,* E, *on the apparent growth-rate constants,* $k_{app.}$, *of* E. coli *at 37.5°. The curves are labeled as to the pH values of the media and are plotted in accordance with the expression* $E/(k_0 - k_{app.}) = (k_b/k_a)E + 1/k_a$ *where* k_b/k_a *and* $1/k_a$ *are the slopes and intercepts, respectively, and* k_0 *is the rate constant in the absence of drug.*

growing culture have no significant effect on the pH of the medium up to 10^8 ml.$^{-1}$ organisms, the variations in growth-rate constants are attributed to variations in the initial pH values of the broths. The calculated values of k_a obtained from the intercepts of the plots of Fig. 9 in accordance with Eq. 3 are plotted as a function of pH in Fig. 10. The values of k_a increased about 10-fold for a unit increase in the pH over the range 6.80–7.60. The slope of the log k_a *versus* pH plot tended to lessen above that pH value.

Effect of Order of Addition of Lincomycin and Erythromycin on Microbial Growth—The dependence of the growth-rate constants of erythromycin-affected cultures on drug concentration is similar to that of lincomycin-affected cultures in Phase I of growth (22). The plot of $k_{app.}$ *versus* concentration (Fig. 6) for erythromycin l is coincident with that for lincomycin HCl when the actual lincomycin concentration is reduced by an average factor of 5.0. This implies that 5.0 weight units of lincomycin HCl are equipotent to 1.0 weight unit of erythromycin l, *i.e.*, there is a molar potency ratio of 11.9. Aqueous solutions of erythromycin l lose potency during storage at refrigerator or room temperature. Consequently, the potency ratio between erythromycin l and lincomycin HCl may be far less than the figure quoted, unless freshly prepared aqueous solutions are compared.

There were no significant differences among the growth-rate inhibitions produced by 31.6 mcg. ml.$^{-1}$ of erythromycin l (Curve D, Fig. 7), equipotent 120 mcg. ml.$^{-1}$ lincomycin HCl (Curve D, Fig. 7), and equipotent combinations of 15.8 mcg. ml.$^{-1}$ erythromycin l and 60.0 mcg. ml.$^{-1}$ lincomycin HCl (Curve C, Fig. 7) where Curves C and D had the same slopes. Also there was no significant difference in the effective inhibition by the combinations (Curve C, Fig. 7) when the equipotent amount of erythromycin l was added after 75 min. of lincomycin-affected growth (Curve B, Fig. 7) or when the equipotent lincomycin HCl was added after 75 min. of erythromcyin-affected growth (also Curve B, Fig. 7). Thus, the order of addition of the antibiotics showed no significant difference in ultimate growth inhibition.

Effect of Mixtures of Graded Equipotent Concentrations of Erythromycin and Lincomyin—The growth-rate constants of cultures affected by mixtures of erythromycin and lincomycin are plotted in Fig. 8. The mixtures contained 100–0% erythromycin l and the residual percentage of equipotent lincomycin HCl solution. The mixtures were prepared so as to be *a priori* equipotent in their combined action on *E. coli* growth, in accordance with Fig. 6, at three different levels of action. The null slopes of the plots of the $k_{app.}$ for all the *a priori* equipotent mixtures demonstrate the lack of any significant bacteriostatic antagonism or synergism (23) in the subinhibitory range.

DISCUSSION

The steady growth rates of *E. coli*, as affected by concentrations of erythromycin, are unaffected by significant changes in the contituents of the nutritive media (Table II) and by organism population (Table III). The extent of inhibition of growth ($k_0 - k_{app.}$) is not directly proportional to drug concentration over the complete range (Fig. 6) in accordance with Eq. 2 and suggests that some saturable process, such as the binding of the drug to a limited number of receptor sites, may become rate determining at higher drug concentrations.

An operative kinetic model, similar to that which defined the kinetics of lincomycin action (22) on *E. coli*, may be applied:

$$E \overset{K_1}{\rightleftharpoons} E_1 + R \overset{K_2}{\rightleftharpoons} E_1R \qquad \text{(Eq. 4)}$$

where E, the erythromycin concentration in the culture medium, is in equilibrium with E_1, the concentration within the cell or biophase, which reversibly binds to a free receptor site R to form a drug–receptor complex E_1R. On the basis of the assumption that the rate of increase in microbial numbers in a balanced culture is proportional to a net rate of protein synthesis above a minimum rate required for life-sustaining processes and is proportional to the number of organisms initially present in the culture, the following expression has been derived (18) to quantify the extent of inhibition of growth produced by the action of the drug:

$$k_0 - k_{app.} = \frac{k_aE}{1 + k_bE} \qquad \text{(Eq. 5)}$$

where k_0 is the growth-rate constant of the drug-free culture; $k_{app.}$ is the growth-rate constant of culture affected by the concentration, E, of erythromycin; $k_b = K_1K_2$, the product of the partition coefficient, K_1, and the affinity constant, K_2, of Eq. 4; and $k_a = qK_1K_2$, where q is a proportionality constant. It may be that at low drug concentrations (0–10 mcg. ml.$^{-1}$), the inhibition of microbial growth is affected by reaction of the drug with only a small fraction of the available receptor sites. Thus K_2, k_a, and k_b are small with $k_bE << 1$; Eq. 5 simplifies to Eq. 2, which is an expression for the observed linear dependence of the growth-rate constant on the drug concentration in this concentration range (Fig. 6 and Table I).

At higher drug concentrations, >10 mcg. ml.$^{-1}$, it is possible that the already complexed receptor sites either reduce the availability of remaining sites by steric effects, configurational changes, protective colloid action, *etc.*, or transform them to another type of reduced but constant affinity. Whatever the mechanism, it takes progressively greater concentrations of the drug to bind the remaining sites. Thus, the $k_{app.}$ does not remain linearly dependent on erythromycin concentrations. The arithmetic transformation of Eq. 5 is Eq. 3, so that the linear plots of $E/(k_0 - k_{app.})$ *versus* E for concentrations >10 mcg. ml.$^{-1}$ (Fig. 9) demonstrate adherence of the data to Eq. 5. The calculated value of $k_a/k_b = 117.25 \times 10^{-6}$ from such a plot (Table I) is approximately 2 k_0, and it is the same with lincomycin (22).

The addition of the drug to a balanced culture results in inhibition of growth, with a new steady state of growth attained after 30–50 min. (Figs. 1–4). Dilution of drug-affected organisms into drug-free or drug-containing media established a new steady-state growth of the culture within similar time intervals. The rate transitions were no slower than those occurring on dilution of the drug-free culture into fresh media. Cultures inhibited by erythromycin reverted to growth rates coincident with those found in the presence of very small concentrations of the antibiotic when they were diluted into fresh broths. Conversely, cultures inhibited by low concentrations of the antibiotic were further and quickly inhibited by addition of more antibiotics to predictable growth-rate constants (Figs. 3–6). It is concluded that the action of erythromycin on bacterial culture is readily reversible.

Progressively larger amounts of erythromycin are needed to produce the same degree of inhibition of growth of *E. coli* as the pH is increased (Fig. 5). This confirms the literature (24, 25) that the minimum inhibitory concentration (M.I.C.) for erythromycin action on Gram-positive and Gram-negative organisms decreases with an increase in pH. Erythromycin base has a pKa = 8.8 (25), and the enhanced antibacterial action observed at higher pH values may be assigned to the unprotonated fraction, f, of the total drug which contributes to the activity. If it is postulated that only the unprotonated species partitions into the microorganism (26), the inhibitory-rate constant, k_a, of Eq. 5 should be a function of this unprotonated fraction, f, and then

$$k_a = k_a^*f = k_a^* \left(\frac{K_a}{K_a + [H^+]}\right) \qquad \text{(Eq. 6)}$$

where the intrinsic inhibitory-rate constant of the unprotonated species is $k_a^* = qK_1^*K_2$, K_1^* is the intrinsic partition coefficient between medium and microbial biophase for the uncharged species, qK_2 is invariant in that the biophase pH is considered constant, and f can be defined by the parenthetical expression of Eq. 6 (22).

A plot of log k_a *versus* pH must, therefore, approach a slope of unity when $[H^+] > K_a$, and the slope should tend to decrease as K_a becomes less than $[H^+]$, as in Fig. 10. The calculated value for k_a^* is $11.08 \pm 0.70 \times 10^{-4}$ (ml./mcg.-sec.), from which it is estimated that at pH 8.8 the activity of erythromycin will be 30-fold that at pH 7.0.

The coincidence of the plot of $k_{app.}$ against concentration for erythromycin I and lincomycin HCl in Phase I (22) (Fig. 6), when the latter is presumed to have one-fifth the potency of erythromycin on a weight basis, is not inconsistent with the same mechanisms of action for the two antibiotics. Antagonism would not be predicted for the action of combinations of these two antibiotics (23) on the premise of this same peculiar dependency of growth inhibition on dose (Fig. 6). This *a priori* prediction for *E. coli* is well verified by the fact that equipotent mixtures of lincomycin and erythromycin demonstrate the same inhibitory effect on the growth of *E. coli* at any magnitude of inhibition in the subinhibitory range (Fig. 8). Also, the sequence of addition of erythromycin and lincomycin produced no differences in growth-rate inhibition when the resultant drug combinations were equipotent (Fig. 7). For freshly prepared solutions, when the potencies of the antibiotic samples are taken into account, erythromycin base is 6.68 times as potent as the lincomycin base at 37.5° and pH 7.05 in the system against *E. coli*.

These observations are consistent with the facts that each of these compounds binds to the 50 S ribosomal subunit (2, 3, 7, 9, 10) in cell-free extracts and thus may inhibit polypeptide synthesis similarly in the intact living cell by interfering with the function of transfer RNA. If there is no peculiar or physicochemical interaction, the combined action of the two drugs should be predictable on the basis of the potencies determined from their individual dose–response relations (23) and assigned to their relative ability to compete for binding sites, where the relative potencies are functions of the products of their relative affinity for the receptor site, K_2, and their partition into the biophase, K_1, as per Eq. 4.

Since the *a priori* prediction and the kinetic results do not demonstrate antagonism, the problem is to reconcile this condition with the statements of supposed antagonism that exist in the literature (2, 3, 8, 10, 12). Most of these statements of antagonism are based on studies of protein synthesis in cell-free extracts, where the criteria of effect are degrees of amino acid incorporation (3, 8) where added erythromycin is supposed to reverse the inhibition of amino acid incorporation by lincomycin. It is apparent, however, that in balanced growth cultures of intact cells such as *E. coli*, no such antagonistic effects appear.

The present studies on such combinations were based on the Phase I lincomycin-affected steady state of *E. coli* growth (22). However, lincomycin has a second mode of action on a growing culture after prolonged contact with the cells. The $k_{app.}$ dependency on lincomycin concentration during this Phase II steady state of lincomycin-affected growth is different from that of the Phase I dependency and that of erythromycin-affected growth (Fig. 6). Thus, Phase II lincomycin effects may be expected to be different than erythromycin, and the observed cell-free antagonisms may be possible. The action of combinations of erythromycin and lincomycin in Phase II will be investigated further to test this hypothesis.

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Biosynthesis of Deuterated Benzylpenicillins I: Solvent Deuterium Oxide Participation

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Abstract □ The 53–414 strain of *Penicillium chrysogenum* was cultured in a defined medium containing glucose, acetate, lactate, and phenylacetic acid as carbon sources and 99.8% deuterium oxide as solvent. Partially deuterated benzylpenicillin was isolated from the culture. The extent of solvent participation in the biosynthesis of the penicillin molecule was determined by analysis of the proton magnetic resonance spectra. Incorporation of deuterium appears almost complete at two positions: the C-3 position of the thiazolidine ring and the C-6 position of the β-lactam ring. A partial incorporation of deuterium at the C-5 position is also observed. The deuterium atoms in the C-5 and C-6 positions apparently arise during biosynthesis of the amino acid cysteine. The deuterium atom at the C-3 position apparently arises either in the biosynthesis of the amino acid valine or in the closing of the thiazolidine ring.

Keyphrases □ Deuterated benzylpenicillin—biosynthesis □ *Penicillium chrysogenum* cultures—benzylpenicillin deuteration □ Biosynthesis, deuterated benzylpenicillin—*P. chrysogenum* cultures □ Proton magnetic resonance—analysis, structure □ IR spectrophotometry—structure

A variety of organisms has been successfully cultured in pure deuterium oxide (D_2O) (1); in several instances, pharmacologically active principles containing deuterium have been isolated and studied. Nona *et al.* (2–4) studied the effects of D_2O on the growth of *Penicillium janczewskii*. Deuterated griseofulvin was isolated and its antifungal activity evaluated. Mrtek *et al.* (5, 6) cultured a strain of *Claviceps purpurea* in D_2O and examined the biosynthesis of deuterated clavine alkaloids isolated from the culture. Katz and Crespi (7) reviewed the literature on isotope effects in biological systems.

Shaffer *et al.* (8) observed the effects of D_2O on the growth rate and morphology of *Penicillium notatum*, but penicillin production was not investigated. Mohammed *et al.* (9, 10) reported the effects of D_2O on the growth and antibiotic production of 13 low-producing strains of two species of *Penicillium* and three high-producing strains of *Penicillium chrysogenum*. Here again, deuterated antibiotic was not isolated. Behrens *et al.* (11) synthesized deuterophenylacetyl-^{15}N-DL-valine and found the deuterophenylacetyl moiety was incorporated into the benzylpenicillin molecule. Demain (12) used partially deuterated benzylpenicillin, which contained five deuterium atoms in the benzene ring, to study the stability of penicillin during fermentation. Laskar and Mrtek (13) synthesized and studied the activity of a benzylpenicillin which was fully deuterated in the benzyl moiety. A reduction in antibiotic activity was reported.

In the present study, *P. chrysogenum* 53-414, a mutant of the Wisconsin Q-176 strain, was cultured in a defined medium containing D_2O as the solvent. Partially deuterated benzylpenicillin was isolated, and the degree of solvent participation in its biosynthesis was determined by proton magnetic resonance (PMR) analysis of this isotope hybrid compound.[1]

EXPERIMENTAL

Preparation of Slants—*P. chrysogenum*, Wisconsin strain 53–414, was obtained from the American Type Culture Collection (number 12690) in the lyophilized form. Agar slants were prepared as described by Mohammed *et al.* (9). The lyophilized material was suspended in Difco nutrient broth, streaked on the surface of the agar

[1] "Isotope hybrid compound" refers to a compound that contains one type of atom but also more than trace amounts of one of its isotopes (14).