Kinetics and Mechanisms of Action of Drugs on Microorganisms XVI: Effect of Oleandomycin and Its Combinations with Tetracycline, Chloramphenicol, Erythromycin, Lincomycin, and 1′-Demethyl-4′-depropyl-4′-(R and S)-*n*-pentylclindamycin on Microbial Generation of *Escherichia coli*

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Abstract □ The steady-state generation of *Escherichia coli* culture is inhibited by oleandomycin to a new initial steady state with a new generation rate constant, k_{app}, which is linearly related to drug concentrations in the low concentration range. At higher concentrations, a saturation model is implied in which drug molecules bind to a limited number of receptor sites and the k_{app} asymptotically approaches zero with infinite concentration. Oleandomycin demonstrates another subsequent action in that cell division is inhibited and the oleandomycin-affected cells show increased size with generation rates that are less than the initial effect. The antimicrobial activity of oleandomycin decreases with the nutrient concentrations of Medium 3 USP or casamino acid–glucose–buffer salt medium (Anton's medium), probably because of the binding of drug molecules to the proteins and casamino acids in the broth media. The activity of the drug increases with the pH of the media. This is consistent with the postulate that only the unprotonated fraction of the drug concentration contributes to the activity. Combinations of oleandomycin or erythromycin with tetracycline are synergistic while those with chloramphenicol, erythromycin, or lincomycin are indifferent. These phenomena are evidenced by the decreased or invariant k_{app} values of *a priori* equipotent concentrations.

Keyphrases □ Antimicrobial activity—effect of oleandomycin and combinations with other antibiotics on *Escherichia coli* generation, kinetics □ Oleandomycin and combinations with other antibiotics—effect on *Escherichia coli* generation, kinetics □ *Escherichia coli* generation—effect of subinhibitory oleandomycin and combinations with other antibiotics, kinetics □ Antibiotics—effect of oleandomycin and combinations with other antibiotics on *Escherichia coli* generation, kinetics

Oleandomycin (1) is a medium-spectrum antibiotic with an antibacterial spectrum similar to that of erythromycin and other macrolides (1, 2). Its action is predominantly bacteriostatic (3) and, like other macrolide antibiotics, it inhibits the binding of radioactive chloramphenicol to bacterial ribosomes *in vitro* (4), which suggests that the antibiotic also acts on the 50S ribosomal subunit. Oleandomycin, like erythromycin and lincomycin, shows the same differential effect as chloramphenicol on proline, lysine, and phenylalanine incorporation in cell-free systems (5). This implies that its action in protein synthesis may be at the same site as chloramphenicol.

Oleandomycin has been extensively used as a 1:2 mixture with tetracycline, and synergism has been claimed for this combination (6–8). However, this opinion has been denied by others (9–13).

The effects of drugs can be quantified and insight into their mechanisms of action can be gained by monitoring the generation rate of microorganisms in the presence of varied subinhibitory concentrations (14). This paper presents the quantitative kinetic expressions for microbial generation when inhibited by oleandomycin as functions of drug concentrations, inoculum size, pH, and compositions of the nutrient media. Microbial kinetics have served as a proper tool for the appropriate classification of the actions of drug combinations (14). This paper also presents studies on the evaluation of drug combinations containing oleandomycin.

EXPERIMENTAL

Organism—Replicate slants of *Escherichia coli* (ATCC 12407) were used in all experiments. The slants were prepared from a single colony and were stored in a refrigerator at 4°.

Culture Media—Bacto Antibiotic Medium 3[1] was rehydrated to Medium 3 USP according to the specifications of the manufacturer. The media were filtered and autoclaved as described previously (14). The pH of the media was 7.05 ± 0.05 with the exception of those used to study the antimicrobial activity as a function of pH. To obtain media with a pH in the range of 6.30–7.55, various amounts of concentrated hydrochloric acid or sodium hydroxide solutions were added aseptically to the culture media. To study the effects of various components of culture medium, Anton's medium (15), which had 10% acid-hydrolyzed casein (Bacto Casamino Acids[1]) in Davis's medium, was also used.

Antibiotics—The references to concentrations of oleandomycin phosphate[2] (775 mcg. base eq. mg.$^{-1}$) throughout this paper refer to this phosphate salt. Samples of tetracycline hydrochloride USP[3], lincomycin hydrochloride[3] (895 μg. base eq. mg.$^{-1}$), 1′-demethyl-4′-depropyl-4′-(R and S)-*n*-pentylclindamycin hydrochloride[3,4] (I) (pure), erythromycin lactobionate[5] (670 μg. base eq. mg.$^{-1}$), and chloramphenicol[6] (purity grade) were also used.

Inoculation and Generation—An aliquot (5 ml.) of culture medium was inoculated from a fresh slant, and the culture was allowed to generate for 15 hr. at 37.5° in an incubator. A sample of 0.5 ml. was then diluted into 49.5 ml. broth. The generation of the culture was followed up to 2×10^7 *E. coli*/ml. This culture was then finally diluted 100-fold into a bulk amount of broth. The inoculated broth, contained in a flask fitted with 49.5-ml. delivery head, was then shaken and kept in an incubator at 37.5° for 15 min. Aliquots (49.5 ml.) of the inoculated broth were then aseptically transferred into replicate 125-ml., loosely capped conical flasks. The cultures were maintained at 37.5 ± 0.01° in a 189.27-l. (50-gal.) constant-temperature water bath with constant shaking. All pipets and media used for the dilutions of the cultures were prewarmed to prevent temperature shocks on the organisms.

Total Count Method—Samples of 1.00 or 0.50 ml. were withdrawn at 25-min. intervals and diluted into an appropriate amount (24 or 49 ml.) of diluent in such a way that the obtained counts were less than 100,000 counts/50 μl. on the Coulter counter[7], which was

[1] Difco Laboratories, Detroit, Mich.
[2] Pfizer Inc., Brooklyn, N. Y.
[3] The Upjohn Co., Kalamazoo, Mich.
[4] Referred to in previous manuscripts as U-24,729A.
[5] Abbott Laboratories, North Chicago, Ill.
[6] Thomae GMBH, Germany.
[7] Model ZBI, Coulter Electronics Inc., Hialeah, Fla.

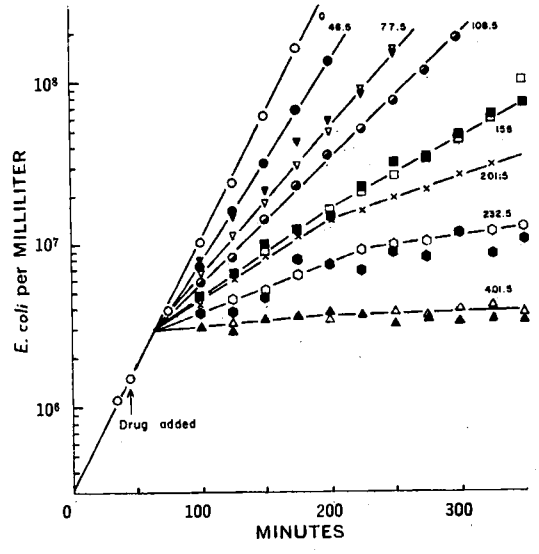

Figure 1—*Typical generation curves of* E. coli *in Medium 3 USP at pH 7.05 and 37.5° in the absence and presence of various concentrations of oleandomycin phosphate, obtained by total (○●▽○□×○△) and viable (▼■○▲) counts. The curves are labeled according to the oleandomycin phosphate concentration in micrograms per milliliter.*

equipped with a 30-μ orifice. The coincidence corrections for the counter were made by programming in a calculator[8] in accordance with the coincidence correction chart supplied in the manufacturer's manual. The diluent used was a filtered[9] aqueous solution of 0.85% NaCl and 1% formaldehyde. The total counts were also corrected for the background count of the particular batch of medium used, diluted in the same way as the sample. The average background counts were normally 400/50 μl. Semilogarithmic plots of *E. coli* per milliliter total counts against time for various oleandomycin phosphate concentrations in Medium 3 USP are given in Fig. 1.

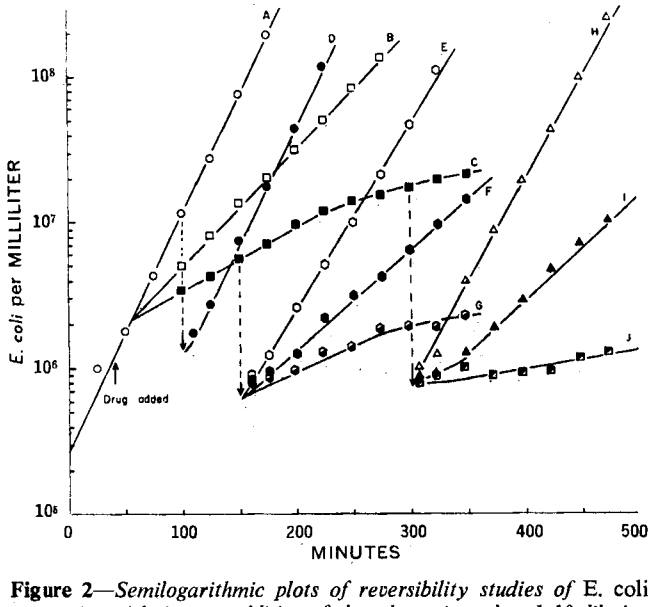

Figure 2—*Semilogarithmic plots of reversibility studies of* E. coli *generation with time on addition of oleandomycin and on 1:10 dilution of culture with broth. Curve A is drug-free culture. Curves B and C are after addition of oleandomycin phosphate to achieve final concentrations of 101 and 202 mcg./ml., respectively. Curve D is after 1:10 dilution of curve A culture. Curves E, F, and G and curves H, I, and J are after 1:10 dilution of curve C at 150 and 300 min., respectively, with broth containing oleandomycin. Curves E and H, curves F and I, and curves G and J had final concentrations of 21, 101, and 202 mcg./ml. oleandomycin phosphate, respectively.*

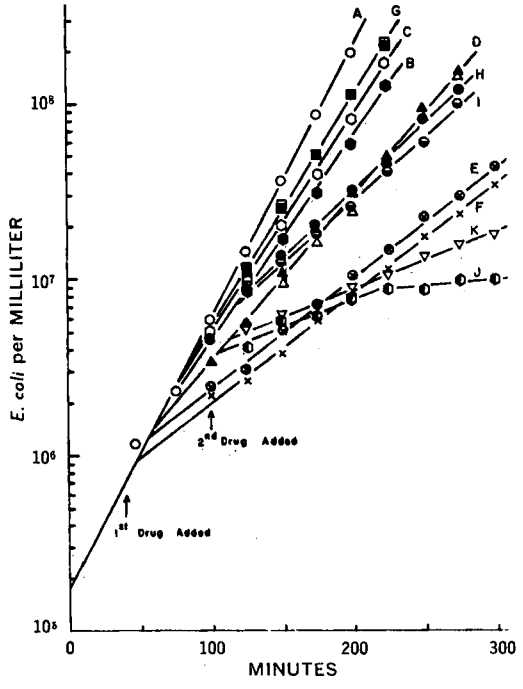

Figure 3—*Effects of order of addition of equipotent oleandomycin and tetracycline on generation rates of* E. coli *in Medium 3 USP at 37.5°. Curve A is for the generation of* E. coli *in the absence of drug. Curves B and C are for the generation in the presence of 38 mcg./ml. oleandomycin phosphate and 0.034 mcg./ml. tetracycline, respectively. Curve G is when equipotent tetracycline is added (■) to the oleandomycin-affected culture or oleandomycin is added (□) to the tetracycline-affected culture so that the final concentrations are equipotent with those of curves B and C. Curve D is for the generation in the presence of 86 mcg./ml. oleandomycin phosphate (△) or 0.076 mcg./ml. tetracycline (▲). Curves H and I are when equipotent tetracycline is added to the oleandomycin-affected culture and equipotent oleandomycin is added to the tetracycline-affected culture, respectively, so that the final concentrations are equipotent with that of curve D. Curves E and F are for the generation in the presence of 152 mcg./ml. oleandomycin phosphate and 0.15 mcg./ml. tetracycline, respectively. Curves J and K are when equipotent tetracycline is added to the oleandomycin-affected culture and equipotent oleandomycin is added to the tetracycline-affected culture, respectively, so that the final concentrations are equipotent with those of curves E and F.*

Viable Count Method—Samples of 0.50 ml. were withdrawn from the cultures and appropriately diluted into sterilized 0.85% saline solution in accordance with a preplanned dilution scheme so that 60–80 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[10]. Semilogarithmic plots of *E. coli* per milliliter viable counts for various oleandomycin phosphate concentrations in Medium 3 USP are given in Fig. 1.

Cell Size Analyses—The size-frequency distributions of the cells of *E. coli* in the absence or presence of oleandomycin were studied using the Coulter counter[7] in conjunction with a Channelyzer[11] and plotter[11]. The instruments were calibrated with polyvinyl toluene latex beads of 3.49 mean diameter. Two replicate 49.5-ml. samples of culture generating in logarithmic phase were treated with oleandomycin phosphate solution to give final concentrations of 31 and 77.5 mcg./ml. Samples were taken every 30 min., and size distributions as well as total counts were obtained. A culture generated in the absence of the drug was studied as a control.

Effect of Antibiotic Concentrations on Generation Rates—Fresh solutions of the respective antibiotics were aseptically prepared for

[8] Wang 700.
[9] Millipore 0.45-μ HA.

[10] Model C-110, New Brunswick Scientific Co., New Brunswick, N. J.
[11] Coulter Electronics Inc., Hialiah, Fla.

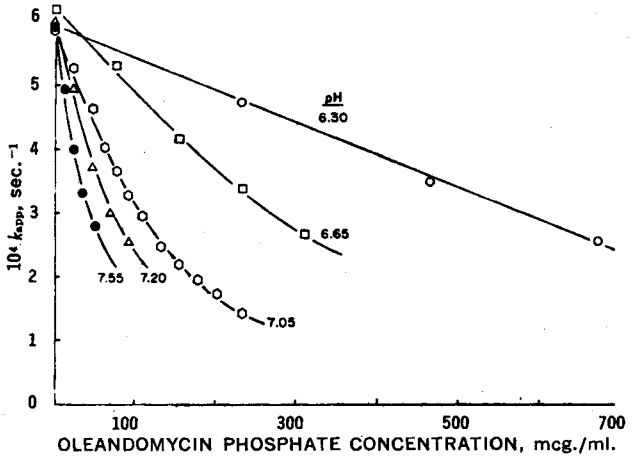

Figure 4—*Dependence of the apparent first-order generation rate constant,* k_{app}, *for* E. coli *in Medium 3 USP on oleandomycin phosphate concentration at various pH values at 37.5°.*

each experiment. They were sufficiently diluted so that aliquots of 0.50 ml. added to the 49.5-ml. culture volumes yielded the desired oleandomycin phosphate concentrations. The drug solutions were added to the cultures generating at 37.5° in the logarithmic phase at predetermined organism concentrations. Samples were withdrawn every 25 min. and counted by the Coulter counter. One culture without drug was studied in each experiment to obtain a control generation rate constant.

Effect of Inoculum Size on Drug-Affected Generation Rates—An aliquot of a culture in logarithmic phase containing 2×10^7 *E. coli*/ml. was diluted 1000-fold into a bulk amount of broth. The inoculated bulk broth was then shaken and kept in an incubator at 37.5° for 15 min. Aliquots (49.5 ml.) of the inoculated broth were aseptically transferred into one control and three sets of five replicate flasks. The drug solutions were added to each set when the organism concentrations in the control flask reached 1.8×10^6, 1.8×10^6, and 1.1×10^7/ml.

Effect of Broth Variations on Drug-Affected Generation Rates—Various strengths of Medium 3 USP and Anton's medium were prepared so that the concentrations of broth ingredients were normal, halved, and doubled. The ingredients of Anton's medium were varied in several ways to study the effects of each ingredient on the drug-affected generation rates. The ingredients studied were phosphates, magnesium sulfate, and casamino acids.

Effect of pH on Drug-Affected Generation Rates—The preparation of Medium 3 USP with different pH values was described previously (14), and the drug-affected generation rates were studied by the total count method.

Reversibility of Drug Action at 37.5°—An aliquot (5.0 ml.) of a drug-free culture generating in the logarithmic phase (curve A in Fig. 2) was added to 49.5 ml. of fresh broth so that the organism population was diluted 11-fold (curve D). Two flasks with 49.5 ml. broth were treated with 0.5 ml. drug solutions to achieve final concentrations of 101 (curve B) and 202 mcg./ml. (curve C). When the drug-affected culture of curve C was in a steady-state generation, an aliquot (5.0 ml.) of the culture was added to 49.5 ml. of fresh medium (curve E). At the same time, aliquots (5.0 ml.) of the culture of curve C were added to 50 ml. volume broth which contained enough drug solution so that the drug concentrations were restored to 101 (curve F) and 202 (curve G) mcg./ml. Similar dilutions were made after the drug-affected culture of curve C had deviated from the semilogarithmic linearity. They were made with fresh medium (curve H) and to broth containing enough drug solution to reestablish final concentrations of 101 (curve I) and 202 (curve J) mcg./ml.

Effect of Equipotent Combinations of Oleandomycin with Other Antibiotics—Equipotent mixtures of 100, 90, 80, 60, 50, 40, 20, 10, and 0% of oleandomycin and the residual percentage of tetracycline, chloramphenicol, erythromycin, lincomycin, or I were prepared. Aliquots (0.50 or 1.00 ml.) of the equipotent mixtures of drug solutions were added to replicate 49.5-ml. volumes of culture generating in logarithmic phase in Medium 3 USP. Samples were withdrawn every 25 min., and the bacterial cells were counted by the total

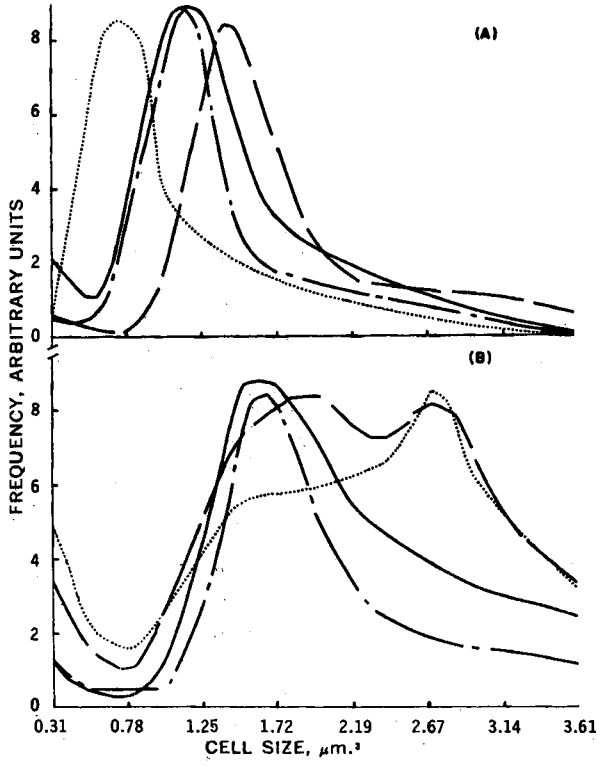

Figure 5—*(A) Size-frequency distribution of the control organisms of* E. coli *in Medium 3 USP 30–140 (——) and 170–200 (- - -) min. after inoculation and of generating organism treated with 132 mcg./ml. oleandomycin phosphate 75–230 (— —) and 260–350 (— - —) min. after the time of inoculation (see Fig. 1). (B) Size-frequency distribution of organisms treated with 233 mcg./ml. oleandomycin phosphate 75–230 (— - —), 260 (——), 290–320 (— —), and 350 (- - -) min. after the time of inoculation (see Fig. 1).*

count method. The combination studies of oleandomycin with tetracycline were repeated in Anton's medium with 0.01 and 0.1% $MgSO_4 \cdot 7H_2O$. They were also repeated in Anton's medium with the normal concentration of nutrients and with half the normal concentration.

Effect of Order of Addition of Oleandomycin–Tetracycline Combinations on Microbial Generation—Replicate 49.5-ml. volumes of a culture containing 7×10^5 *E. coli*/ml. generating in the logarithmic phase (curve A in Fig. 3) were treated with 1.0 ml. of either oleandomycin or equipotent tetracycline solution to give three different concentrations in the final Medium 3 USP media: 152 mcg./ml. oleandomycin phosphate (curve E) or 0.15 mcg./ml. tetracycline (curve F), 86 mcg./ml. oleandomycin phosphate (curve D) or 0.076 mcg./ml. tetracycline (curve D), and 38 mcg./ml. oleandomycin phosphate (curve B) or 0.034 mcg./ml. tetracycline (curve C). The cultures to which 0.5 ml. of the above tetracycline solutions had been added were treated with 0.5 ml. of the oleandomycin solutions 60 min. after the first drug addition at three different dose levels so that the mixtures and the solutions of either drug alone were equipotent *a priori*. Similarly, 0.5-ml. amounts of tetracycline were added to the cultures to which oleandomycin had been added initially.

RESULTS

In the logarithmic generation phase, the bacterial generation rate, dN/dt, is proportional to the number, N, of organisms per unit volume, so that:

$$\log N = (k_{app}/2.303)t + \log N_0 \qquad \text{(Eq. 1)}$$

where N_0 is the number of organisms per unit volume at some zero time when the new steady-state generation is achieved, and the k_{app} determined from the slopes of appropriate plots is the apparent first-order generation rate constant. The addition of graded concentrations of oleandomycin to the culture decreases the generation

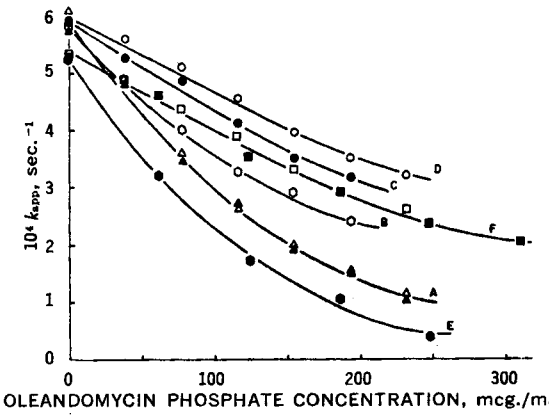

Figure 6—*Dependence of the apparent first-order generation rate constant,* k_{app}, *for* E. coli *on oleandomycin phosphate concentrations at various nutrient concentrations of Medium 3 USP (A, B, C, and D) and of Anton's medium [E and F (■)] and at various phosphate concentrations in Anton's medium [F (■) and F (□)]. The curves, the multiples of the stated concentrations, and the* 10^6 k_A*'s are: A (▲), 0.5, 3.2; A (△), 1.0, 3.2; B, 1.3, 2.3; C, 1.6, 1.5; D, 2.0, 1.3; E, 0.5, 3.4; F (■), 1.0, 1.3; and F (□), 0.5, 1.3.*

rate as a function of antibiotic concentrations, and the semilogarithmic plots of cell numbers against time show a linear relation with slopes that decrease with increasing drug concentrations (Fig. 1). Plots of the determined k_{app} values against various drug concentrations, A, of oleandomycin are given in Fig. 4. At the lower drug concentrations, the plot conforms to the expression:

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

where k_0 is the generation rate constant in the absence of drug and k_A is defined as the inhibitory constant.

Since the numbers of viable and total cells are coincident (Fig. 1), no kill or death of microorganisms occurs up to the highest drug concentration studied and the use of total counts to determine the generation rates of organism affected by subinhibitory concentrations of oleandomycin is justified. Prolonged contact of microorganisms to higher drug concentrations made the cells grow in size (Fig. 5) and appeared to be related to the deviation from the semilogarithmic linearity of the initial drug-affected steady state. The k_{app} values used were calculated from this initial steady state.

Culture Broth Variations—The concentrations of nutrients in the culture media, both Medium 3 USP and Anton's medium (Fig. 6), have significant effect on the drug-affected generation rate constants. There were no significant differences in drug-affected generation rate constants when the concentrations of phosphates were varied

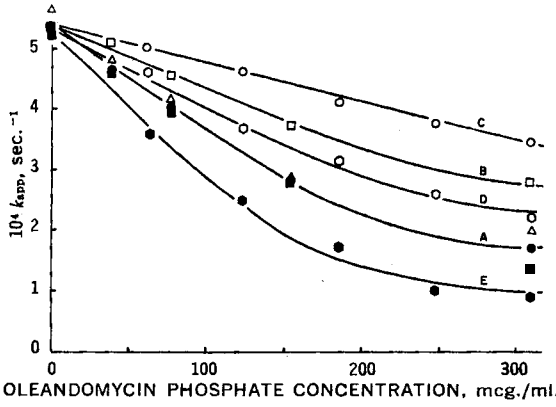

Figure 7—*Dependence of the apparent first-order generation rate constant,* k_{app}, *for* E. coli *on oleandomycin phosphate at various magnesium salt (A and B) and casamino acid (C, D, and E) concentrations in Anton's medium. The curves, the percent* $MgSO_4 \cdot 7H_2O$ *concentrations or multiples of the stated casamino acid concentrations, and the* 10^6 k_A*'s are: A (■), 0.001, 2.2; A (●), 0.005, 2.2; A (▲), 0.02, 2.2; B, 0.1, 1.1; C, 2.0, 0.65; D, 1.0, 1.4; and E, 0.5, 2.5.*

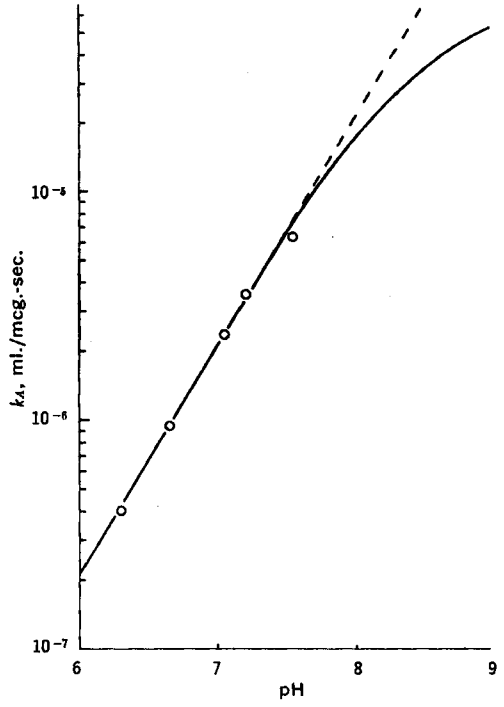

Figure 8—*Dependence of the logarithm of the inhibitory constant,* k_A, *for the action of oleandomycin phosphate on* E. coli *in Medium 3 USP at 37.5° on the pH value. The points are experimental values and the solid line is the theoretical value calculated on the basis that only the unprotonated fraction of the drug concentration contributes to the activity.*

(Fig. 6). Magnesium ion had a minor antagonistic effect only when its concentration was 10 times greater than its normal concentration in Anton's medium (Fig. 7). However, there were significant differences in k_{app} values at the same drug concentrations when the concentrations of casamino acids in Anton's medium were varied; the generation rate constants decreased with increasing nutrient strengths (Fig. 7). The drug may be bound to the casamino acids of Anton's medium and to the proteins in Medium 3 USP to decrease the amount of drug available for antibacterial activity.

Effect of Inoculum Size on Drug-Affected Generation Rates—The apparent generation rate constants were obtained at various concentrations of oleandomycin over almost a 100-fold range in inoculum size at the time of drug addition and were not significantly different. Thus, it cannot be concluded that the drug is metabolized by the organisms, adsorbed to the cells, or inactivated by excretory substances which may be functions of organism population.

Effect of pH on Drug-Affected Generation Rates—The apparent generation rate constants, k_{app}, obtained in broth at pH values 6.30–7.55 in the absence and presence of graded oleandomycin concentrations are plotted in Fig. 4. The drug-free generation rate constants were not significantly affected, but the drug-affected generation rate constants decreased markedly with increasing pH at comparable drug concentrations. The logarithms of the inhibitory constants, k_A, obtained from the slopes of the initial linear portion of plots similar to Fig. 1 appear to be a linear function of the pH of the medium in the range studied (Fig. 8).

Reversibility of Drug Action (Fig. 2)—The culture attained a new steady-state generation 20–25 min. after the drug addition to the culture generation in logarithmic phase. The reasonably short lag time would predict a reversibility of the action of oleandomycin within short time intervals. The steady-state generation affected by 202 mcg./ml. oleandomycin phosphate (curve C) reverted to a new steady state when diluted 11-fold (curve E), and the new steady state was as readily achieved as the drug-free culture (curve A to curve D). There were no significant differences between magnitudes of rate constants obtained from the generation (curve B) attained directly by addition of drug solution to the culture to give a final concentration of 101 mcg./ml. and that attained by diluting the culture of curve C into fresh medium containing enough drug

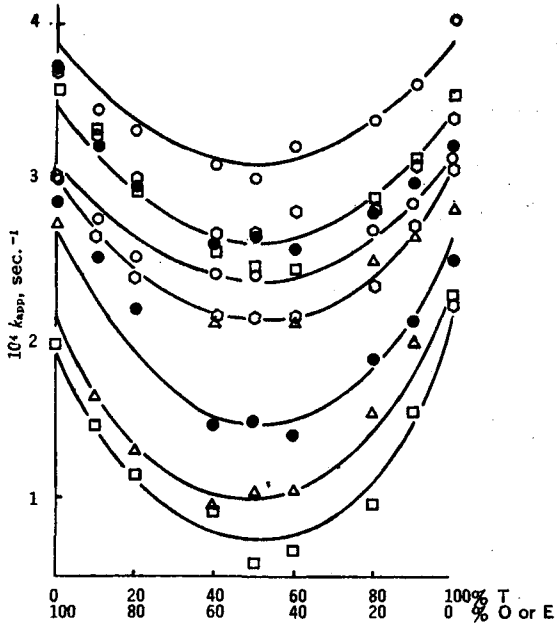

Figure 9—*Effect of varied oleandomycin (O) and tetracycline (T) fractions of* a priori *equipotent combinations at various potency levels in Medium 3 USP (□) and in Anton's medium with a normal concentration of nutrients, 0.01% $MgSO_4 \cdot 7H_2O$ (○), with half the normal concentration (△), and with a normal concentration except for 0.1% $MgSO_4 \cdot 7H_2O$ (○). The effect of varied erythromycin (E) and tetracycline fractions in Anton's medium (●) at 37.5° is also shown.*

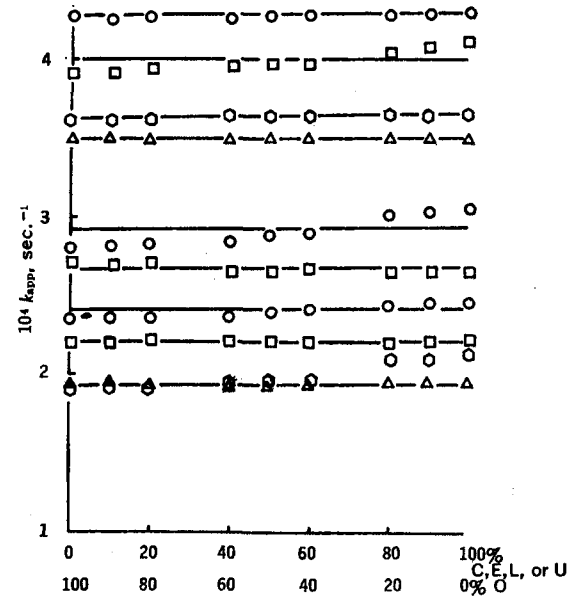

Figure 10—*Effect of varied fractions of oleandomycin with chloramphenicol [C (○)], erythromycin [E (○)], lincomycin, [L (□)], and I [U (△)] in* a priori *equipotent combinations at various potency levels on the apparent generation rate constant of* E. coli *in Medium 3 USP at pH 7.05 and 37.5°.*

solution to give the same final drug concentration (curve F). The dilution of curve C to the restored drug concentration (curve G) had no significant effect on the magnitude of the generation rate constant nor on the fact that the prolonged contact enlarges the cell size. Similarly, the 11-fold dilution of the culture of oversized cells (later portions of curve C) into fresh medium containing halved (curve I) or restored (curve J) concentrations of the drug produced new steady-state generations with predictable rate constants after longer lag periods.

Effect of Equipotent Mixtures of Oleandomycin and Other Antibiotics—The generation rate constants, k_{app}, of cultures affected by equipotent combinations of oleandomycin and tetracycline, erythromycin, lincomycin hydrochloride, chloramphenicol, or I in Medium 3 USP are given in Fig. 9. The mixtures contained 0–100% oleandomycin and the residual percentage of an equipotent amount of the other antibiotic. The mixtures were prepared so as to be *a priori* equipotent in their combined action at two or three different potency levels. The mixtures of oleandomycin and tetracycline exhibited increased activity, as indicated by lower k_{app} values than the equipotent antibiotic used alone. This unequivocally demonstrates synergism (14) between oleandomycin and tetracycline in their combined action against the generation of *E. coli*. The synergism was also observed in Anton's medium with a normal concentration (0.01%) and with a higher concentration (0.1%) of $MgSO_4 \cdot 7H_2O$. It was observed also with half the normal concentration of ingredients (Fig. 9). A similar synergism was observed with the antibiotic pair erythromycin and tetracycline (Fig. 9). However, the null slopes of plots of the k_{app} values for all of the other *a priori* equipotent mixtures demonstrate the lack of any antagonism or synergism in the subinhibitory concentration range (Fig. 10).

Effect of Order of Addition of Oleandomycin and Tetracycline in Combination on Microbial Generation—There were no significant differences between the generation inhibitions produced by the action of 152 mcg./ml. oleandomycin phosphate (curve E in Fig. 3) or 0.15 mcg./ml. tetracycline (curve F), between the inhibitions by 86 mcg./ml. oleandomycin phosphate or 0.076 mcg./ml. tetracycline (curve D), and between the inhibitions by 38 mcg./ml. oleandomycin phosphate (curve B) or 0.034 mcg./ml. tetracycline (curve C).

The addition of either 19 mcg./ml. oleandomycin phosphate (curve G) to the 0.017-mcg./ml. tetracycline-affected culture 60 min. after the first drug addition or 0.017 mcg./ml. tetracycline to the 19-mcg./ml. oleandomycin phosphate-affected culture did not inhibit the generation rate any more than the equipotent amounts of either drug alone did (curves B and C). The combined action is indifferent at the lowest dose level studied.

The addition of either 43 mcg./ml. oleandomycin phosphate (curve I) to the 0.038-mcg./ml. tetracycline-affected culture or 0.038 mcg./ml. tetracycline (curve H) to the 43-mcg./ml. oleandomycin phosphate-affected culture inhibited the generation rate more than did the equipotent amounts of either drug alone (curve D). This demonstrates that the combined action of oleandomycin with tetracycline is synergistic at higher dose levels. This pattern of response was likewise observed for combinations of 76 mcg./ml. oleandomycin phosphate with equipotent concentrations of 0.075 mcg./ml. of tetracycline, since the slopes of curve K and initial portions of curve J are lower than those of curves E and F. In both cases the sequence of addition of the antibiotics produced no significant effects on the synergistic action, since the slopes of curve I and curve K and those of curve H and the initial portions of curve J are the same, respectively.

DISCUSSION

The coincidence of viable and total counts in the subinhibitory range ($\leq$600 mcg./ml.) of oleandomycin phosphate-affected generation (Fig. 1) confirms that the action of oleandomycin is primarily bacteriostatic (3). The drug-affected generation rate constants, k_{app}, are not a linear function of oleandomycin phosphate concentration throughout the entire concentration range (Fig. 4). The shape of the curve indicates the presence of some saturable process in which drug molecules at the high concentrations bind to a limited number of receptor sites (14).

The fact that no significant changes in apparent generation rate constants were observed in the control culture when the concentrations of nutrients in Medium 3 USP or Anton's medium were varied (Fig. 6) implies that the differences in the oleandomycin-affected generation rate contants with different concentrations of Medium 3 USP and Anton's medium can be attributed to the reversible binding of oleandomycin molecules to both protein ingredients of Medium 3 USP and casamino acids in Anton's medium (Fig. 7).

The extent of generation inhibition by oleandomycin increases as the pH of the media increases (Figs. 4 and 8). Since oleandomycin has a pKa of 8.5 (16), the unprotonated fraction of the total drug concentration might be the only effective inhibitory species,

since the relative concentration of the unprotonated species increases as pH increases. The inhibitory constant k_A should be a function of the unprotonated fraction f of oleandomycin in accordance with the equation (14):

$$k_A = k_A^* f = k_A^* \left(\frac{K_a}{K_a + [H^+]}\right) \qquad \text{(Eq. 3)}$$

where k_A^* is the intrinsic inhibitory constant of the unprotonated species. The semilogarithmic plot of k_A against pH is given in Fig. 8. The intrinsic inhibitory constant k_A^* is calculated from the intercept of the plot when $[H^+] \gg K_a$; $k_A^* = 6.96 \times 10^{-5}$ ml./mcg.-sec., from which the antibacterial activity at any pH value can be estimated using Eq. 3.

At doses higher than 200 mcg./ml. of oleandomycin phosphate, a definite change in cell size distribution (Fig. 4) occurs subsequent to the inhibition of microbial generation by the drug. This results in the observed two phases of action (Fig. 1). This phenomenon was not observed with erythromycin-affected generation (17).

Both macrolides, oleandomycin and erythromycin, are synergistic in combined action with tetracycline against microbial generation (Fig. 9). Synergism of antimicrobial activity observed with a combination of two drugs (14) may be assigned to: (*a*) the allosteric modification of the receptor site of one drug by the other so that the affinity of the former increases, (*b*) sequential blocking (18) in which each drug acts on separate rate-determining steps in the sequential metabolic pathway (14) and both drugs show a saturable mechanism of binding to receptor sites, (*c*) the increase in transport to the biophase of one drug by another, and/or (*d*) the fact that one drug effectively eliminates or negates a compound or species in the nutrient media that normally acts as the inhibitor of the action of the other.

The first possible explanation is the most speculative. The second possible explanation cannot be readily reconciled with the linear dependence of k_{app} on tetracycline concentrations which demonstrates a nonsaturable process for the action that results from tetracycline binding to its receptor sites. On the basis of the similarity of structures and postulated equivalent modes of action of the macrolides, erythromycin and oleandomycin, the speculation that combinations of erythromycin and tetracycline would be also synergistic in their action was confirmed (Fig. 9).

The additivity effects for combinations of oleandomycin with chloramphenicol, erythromycin, lincomycin, or I (Fig. 10) may be expected since all of these antibiotics bind to the 50S ribosomal subunit and presumably act on a similar rate-determining step in protein biosynthesis. However, it does appear anomalous that oleandomycin is not antagonistic in combination with both lincomycin and I whereas erythromycin is antagonistic with but one (I) of the two.

The third possible explanation might lead to the demonstration of a significant variation in the degree of synergism with a change in the sequence of addition of oleandomycin and tetracycline; *i.e.*, a greater synergism may result if the antibiotic that increases the transport of the other is added first. However, there was no significant difference in the extent of synergism with a change in the sequence of addition (Fig. 3).

The fourth possible explanation is not valid with respect to inhibition by magnesium salt concentration (Fig. 9) since the degree of synergism is independent of this concentration. Thus, the explanation given for the synergistic action of novobiocin–tetracycline combinations (unpublished data) that the removal of significant amounts of magnesium ion by tetracycline chelation increased the antibacterial effect of novobiocin at low magnesium-ion concentrations is not applicable here. The demonstration of the same extent of synergism in half the normal concentration of nutrient media (Fig. 9) rejects the possibility of negation of the inhibitory action of another constituent of the media by one antibiotic of the combination.

The probable mode of action of the macrolides is on translocation (19) rather than on peptidyl transfer. Combinations of tetracycline with antibiotics such as the lincosaminides and chloramphenicol, which are presumed to act in such a manner (19), show no synergism. Thus, it is reasonable to project that these two different mechanisms of action may be manifested in combinations with tetracycline by the presence or absence of the synergism observed in these studies. It is also implied that the action of a macrolide is enhanced by tetracycline or, conversely, possibly by one increasing the affinity of the other to its respective receptor site or by one increasing the permeability of the other through the cell wall.

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