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# Kinetics and Mechanisms of Drug Action on Microorganisms. 13. Comparative Studies on Action of Lincomycin, Clindamycin, and U 24729A against *Escherichia coli*

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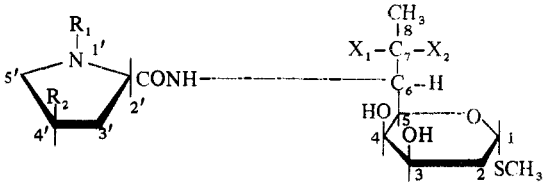
*Escherichia coli* cultures in broth affected by sub-completely-inhibitory concns of lincomycin (I) exhibit 2 phases of steady-state generation while those affected by the 7(S)-halogenated compounds, clindamycin (II) and U 24729A (III), have only one phase of steady-state generation. The generation rate constants of II- or III-affected cultures have the same functional dependencies on drug concentrations as lincomycin-affected cultures in phase I but are different from those of I in phase II generation. The possibility that lincomycin blocks 2 separate sites in a metabolic sequence whereas the 7(S)-halogenated compds block only the latter in the sequential biochemical process, can rationalize these phenomena. II is 6.6 times and III is 28.5 times as active as I calcd on the basis of molar equivalency. The effects of changes in pH on the activity of I, II, and III against *E. coli* suggest that the unprotonated fraction of the drug concn contributes to the activity. The combined actions of II and III are not antagonistic at any level of activity and can be quantitatively predicted from the separate equivalent dose-response curves of either drug. However, combinations of II or III with I show antagonistic effects which depend on the level of activity. These can be rationalized by the one (*e.g.*, lincomycin) allosterically modifying the receptor site for the other lincosaminide antibiotic.

Lincomycin (I) is an antibiotic produced by *Streptomyces lincolnensis.*[1,2] It has an antibacterial spectrum similar to that of erythromycin and was claimed to be superior[3,4] because of its effectiveness against both erythromycin-susceptible and -resistant strains of Gram-positive coccal organisms. However, it was subsequently found[5,6] that there was a "dissociated type" of cross-resistance in *Staphylococcus aureus* between erythromycin and lincomycin. Lincomycin was therefore modified[7] chemically to serve as a basis for the understanding of structure-activity relations. Thus, analogs of enhanced potency and broadened antibacterial spectrum were prepared.

The main structural effects claimed for *in vitro* activity[7] were (a) the variation of the alkyl substituent at the N′ atom of the pyrrolidine nucleus which changed the antibacterial spectrum of activity; (b) the increase in size of the alkyl group at the C-4′ in the pyrrolidine nucleus which increased lipophilicity and the activity; (c) halogen substitution of the 7-(S) configuration of lincomycin molecule which potentiated antibacterial effects; and (d) the need of



I, lincomycin
$R_1 = CH_3$
$R_2 = C_3H_7$
$X_1 = OH$
$X_2 = H$

II, clindamycin [7(*S*)-chloro-7-deoxylincomycin]
$R_1 = CH_3$
$R_2 = C_3H_7$
$X_1 = H$
$X_2 = Cl$

II. U 24729A [1′-demethyl-4′-depropyl-4′(*R*) and (*S*)-*n*-pentylclindamycin]
$R_1 = H$
$R_2 = C_5H_{11}$
$X_1 = H$
$X_2 = Cl$

maintaining the α configuration of the thioglycoside to maximize antibacterial activity. Clindamycin (II) and U 24729A (III) are 7(*S*)-Cl analogs which are claimed to be more than 4 times as active as the parent antibiotic (I)[8] against a variety of Gram-positive and Gram-negative organisms.[7,8]

Lincomycin is an inhibitor of bacterial protein synthesis.[9] The sequence of events occurring during protein synthesis[10] and the steps which might be inhibited by antibiotics have been discussed by Cundliffe and McQuillen.[11] I has been reported to inhibit peptide bond formation by competitive binding on a ribosomal site, possibly peptidyl "transferase" which appears to be an integral part of the 50S ribosomal subunit and is also the binding site for the amino acyl-tRNA.[12-14] In contrast, other workers[15,16] have claimed that I binds to "translocase," a factor which promotes the movement of a peptidyl tRNA elongated by a single amino acid residue from its acceptor site to a donor site. Thus the translocation of tRNA from one ribosomal site to the other is presumed to be inhibited by I. The apparent conflict may be due to an attempt to correlate the results of two distinctly different experimental tests:[16] (a) the puromycin reaction which is specific for detecting an inhibition of peptide bond formation and (b) the guanosine triphosphate (GTP) dependent G-factor catalyzed reaction for determining release of tRNA. An inhibition of the latter reaction does not necessarily imply inhibition of the former reaction *per se*, but would cause peptidyl tRNA to remain in the acceptor site from which the peptide moiety could not be removed by puromycin. Mielck and Garrett[17] observed from their study of I action by microbial kinetics that I, in fact, possessed 2 modes of action which they attributed to (a) an impairment in the functioning of the tRNA by binding of the drug to the 50S ribosomal site and (b) a possible interference in the synthesis and utilization of a stored metabolite.

The similarity in chemical structure of I, II, and III implies that the latter 2 analogs should have mechanisms of action similar to I but with differences in intrinsic biological activities, so that the combined action of I with such analogs should be quantifiable on a kinetically equivalent basis as has been shown for chloramphenicols[18] and sulfonamides.[19] Of course, it is possible that the enhanced antibacterial activity[7] obtained by halosubstitution at the 7-(S) configuration of I in the order Cl < Br < I may be associated with stereoselective binding at a different site. If this were the case, it is possible that the action of I and the 7-(S) halo analogs might show kinetic parameters of different functional dependencies from that of either drug alone. This communication presents the results of testing this hypothesis by microbial kinetics.

## Experimental Section

Organism. Replicate slants of *E. coli* ATCC 12407 (referred to as strain B/r in previous publications[18-21] were used in all experiments. The slants had been prepared from a single colony and were stored in a refrigerator at 4°.

Culture Media. Bacto Antibiotic Medium 3 (Difco Laboratories, Detroit, Mich.) 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 5.1–8.2, various amounts of Millipore-filtered 1.7 *N* HCl and 2 *N* NaOH, respectively, were added to the culture media aseptically before the sterilization.

Antibiotic. Assayed samples of I·HCl (895 μg of base equiv/mg), II·HCl (838 μg of base equiv/mg), and III·HCl (1000 μg of base equiv/mg) were supplied by courtesy of Dr. G. B. Whitfield, Jr., of The Upjohn Co., Kalamazoo, Mich. The references to concns 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 dild 100-fold into fresh medium. The generation of the culture was followed up to 2 × $10^7$ *E. coli*/ml. An aliquot of this culture (*i.e.*, 1 ml/100 ml of broth) was added to a bulk amount of broth contd in a Pyrex flask fitted with a 49.5-ml Calab pourer. The "seeded" broth was kept in an incubator at 37.5° for 20 min with intermittent shaking. Aliquots (49.5 ml) of the "seeded" broth were then aseptically transferred from the Calab pourer into replicate loosely capped erlenmeyer flasks. The flasks were maintained at 37.5° ± 0.1° in a 50-gal, constant temp water bath equipped with a shaker.

**Total Count Method.** This method has been previously described.[20] Samples of 1.00 ml were withdrawn at 20-min intervals from the cultures. They were dild to obtain counts within a range of 10,000–30,000 counts per 50 μl on the Coulter Counter, Model B (Coulter Electronics Co., Hialeah, Florida). The diluent used was a Millipore 0.45 μ HA-filtered aq soln of 0.85% NaCl and 1% $CH_2O$. The instrument was equipped with a 30-μ orifice. The settings were: aperture current of 5, amplification of 8, gain of 10, lower threshold of 13, and upper threshold at maximum. The total counts were corrected for the background count of the particular batch of media used, and dild in the same way as the sample. The background counts in general did not exceed 1000 counts per 50 μl. The coincidence of total (Coulter count) and viable (colony count) numbers of *E. coli*/ml in drug-free and in sub-completely-inhibitory, lincomycin-treated cultures had been previously demonstrated.[17] There was no significant evidence of kill superimposed on normal inhibition of generation in the presence of sub-completely-inhibitory concns of the drug.

**Effect of Antibiotic Concentration on Generation Rates.** Fresh solns of the respective antibiotics were aseptically prepd for each experiment. They were sufficiently dild so that aliquots of 0.5 ml added to 49.5-ml culture vols yielded the desired drug concns (Table I). The solns were added to the cultures generating at 37.5° in the log phase at an organism population of about 1.0 × $10^6$ *E. coli*/ml. Samples were withdrawn every 20–30 min and counted by

**Table I.** Apparent First-Order Generation Rate Constants, $k_{app}$ in $sec^{-1}$, and Other Derived Constants for Generation of *E. coli* in Various Concentrations of Lincomycin and Its Analogs at pH 7.05 at 37.5°.

| Lincomycin·HCl | | | Clindamycin·HCl | | III·HCl | |
|---|---|---|---|---|---|---|
| | $10^5 k_{app}$ | | | | | |
| μg/ml | Phase I | Phase II | μg/ml | $10^5 k_{app}$ | μg/ml | $10^5 k_{app}$ |
| 0 | 62.37 | | 0 | 61.55 | 0 | 62.04 |
| 30 | 53.64 | | 5 | 53.97 | 1.0 | 54.50 |
| 40 | 51.10 | | 10 | 45.07 | 2.0 | 49.20 |
| 60 | 46.21 | 33.70 | 20 | 32.26 | 2.5 | 44.06 |
| 80 | 39.80 | 28.00 | 30 | 20.58 | 3.0 | 40.72 |
| 100 | 34.18 | 24.01 | 40 | 14.69 | 5.0 | 29.39 |
| 150 | 24.66 | 15.87 | 50 | 10.32 | 7.0 | 20.24 |
| 200 | 17.11 | 10.33 | 60 | 8.61 | 10.0 | 14.23 |
| 250 | 13.06 | 7.82 | 70 | 8.06 | 13.0 | 9.50 |
| 300 | 10.30 | 3.55 | 80 | 6.62 | 15.0 | 8.50 |
| 350 | 9.34 | 2.57 | 100 | 6.15 | | |
| $10^5 k_c$[a] | 0.28 | | 1.62 | | 7.00 | |
| $10^5 k_a$[b] | 0.63 | 0.77 | 3.75 | | 15.63 | |
| $10^2 k_b$[c] | 0.91 | 1.03 | 5.43 | | 22.66 | |
| $10^5 k_a/k_b$[d] | 68.96 | 74.74 | 68.96 | | 68.96 | |

[a] Calcd as the slope of a plot of $k_{app}$ *vs.* concn, *C* in accordance with the equation: $k_{app} = k_o - k_c C$, where *C* is from 0 to 100 μg/ml of I·HCl during phase I, 0 to 20 μg/ml of II·HCl, and 0 to 4 μg/ml of III·HCl, and where $k_c$ is in ml/μg sec. [b] Reciprocal of the intercept of a plot of $C/(k_o - k_{app})$ *vs.* *C* in accordance with the equation: $C/(k_o - k_{app}) = 1/k_a + k_b/k_a(C)$ for $C > 100$ μg/ml of I·HCl during phase I and for $0 < C < 350$ μg/ml of I during phase II, or for $C > 16.67$ μg/ml of II·HCl or for $C > 25$ μg/ml III·HCl; where $k_a$ is in ml/μg sec. [c] The quotient of slope and intercept of such plot within the limits of footnote *b* in ml/μg, or the product of drug partition constant, $K_1$, and drug affinity constant, $K_2$, in accordance with derivations for eq 3. [d] Reciprocal of the slope of such a plot within the limits of footnote *b* in $sec^{-1}$.

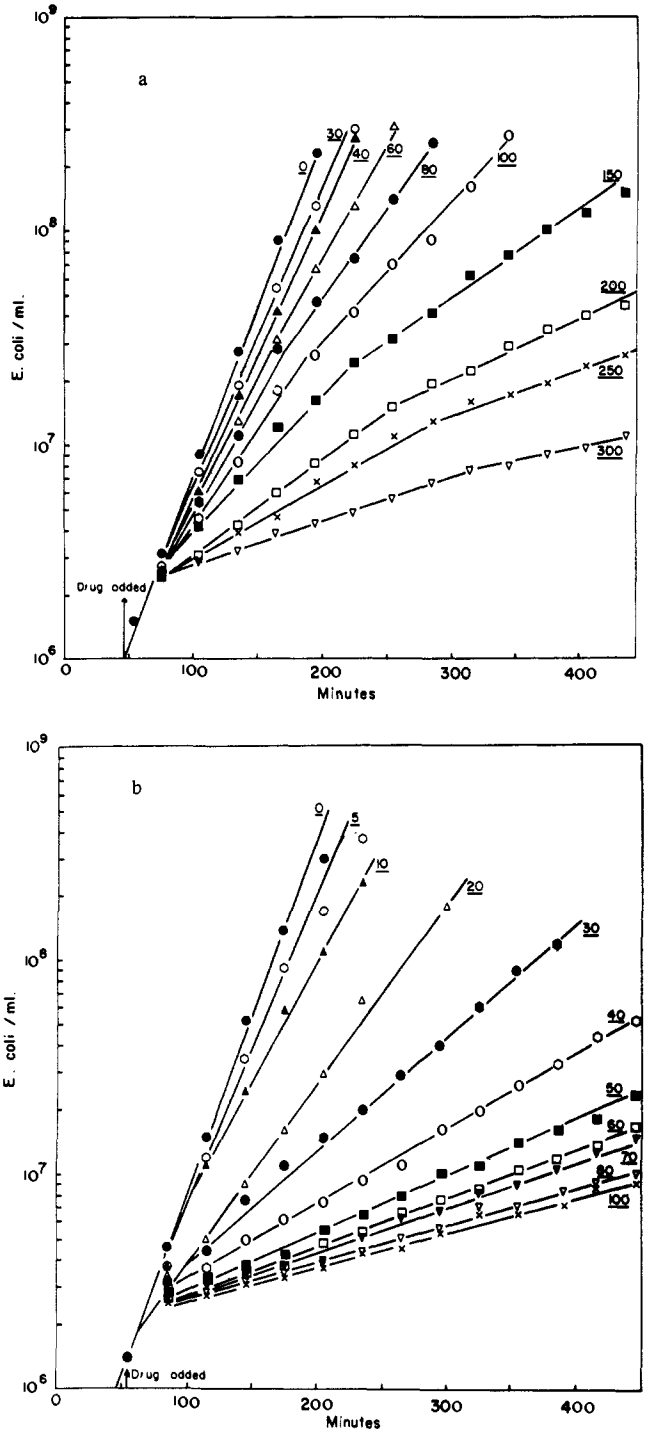



Figure 1. Generation rate curves of *E. coli* at 37.5° and pH 7.05 in the absence and presence of graded (a) lincomycin·HCl and (b) clindamycin·HCl concns. The curves are labeled according to the drug concn in μg/ml.

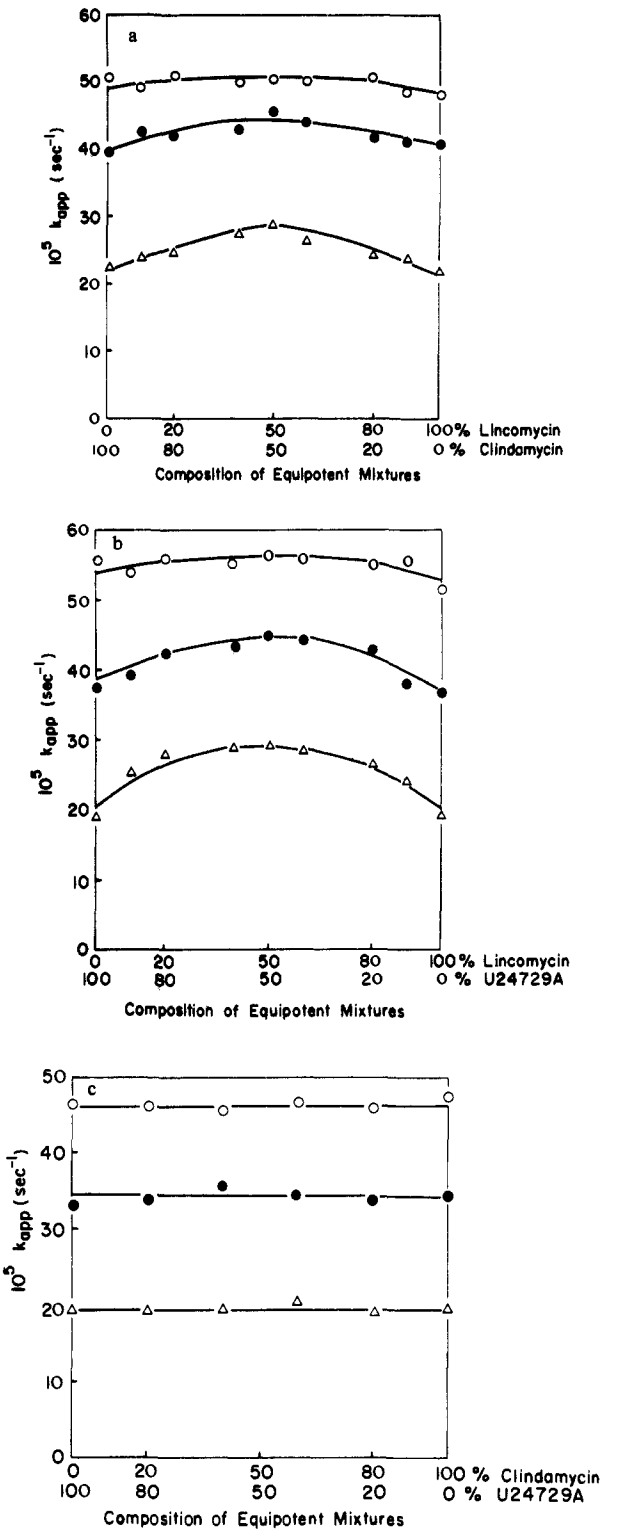



Figure 2. Effect of varied (a) clindamycin·HCl and lincomycin·HCl fractions, (b) III·HCl and I·HCl fractions, and (c) III·HCl and II·HCl fractions in equipotent mixts at 3 different potency levels on the apparent generation rate constants, $k_{app}$ (sec$^{-1}$) of *E. coli* at pH 7.05 and 37.5°. (a) II·HCl is presumed to be 6 times as potent as I·HCl (phase I) on a wt/wt basis. (b) III·HCl is presumed to be 25 times as potent as I·HCl (phase I) on a wt/wt basis. (c) III·HCl is presumed to be 4.16 times as potent as II·HCl on a wt/wt basis.

the Coulter method. One culture without drug was studied in each experiment as control to obtain the generation rate constant ($k_0$) in absence of drug. The generation curves for 0–300 μg/ml of I·HCl at pH 7.05 were obtd (Figure 1a). Similar experiments were performed for 0–100 μg/ml of II·HCl and 0–13 μg/ml of III·HCl.

**Effect of pH on Drug-Affected Generation Rates.** Sufficient amts of 1 *N* HCl and 2 *N* NaOH were added to broth to obtain pH values 6.10–8.20; 6 replicate 49.5-ml vols of each pH constant broth, inoculated with *E. coli* culture in the log phase of generation, were maintd at 37.5° until the organism population had reached $10^6$ *E. coli*/ml. Drug solns (0.5 ml) were added to replicate cultures to achieve the desired concns of antibiotics. The 6th replicate in each set contd no drug. Coulter counts were obtd from samples withdrawn every 20–30 min.

**Action of Equipotent Mixtures Composed of Different Fractions of Lincomycin and Its Analogs.** Replicate 49.5-ml samples of cultures contg $10^6$/ml *E. coli* in steady-state generation were treated

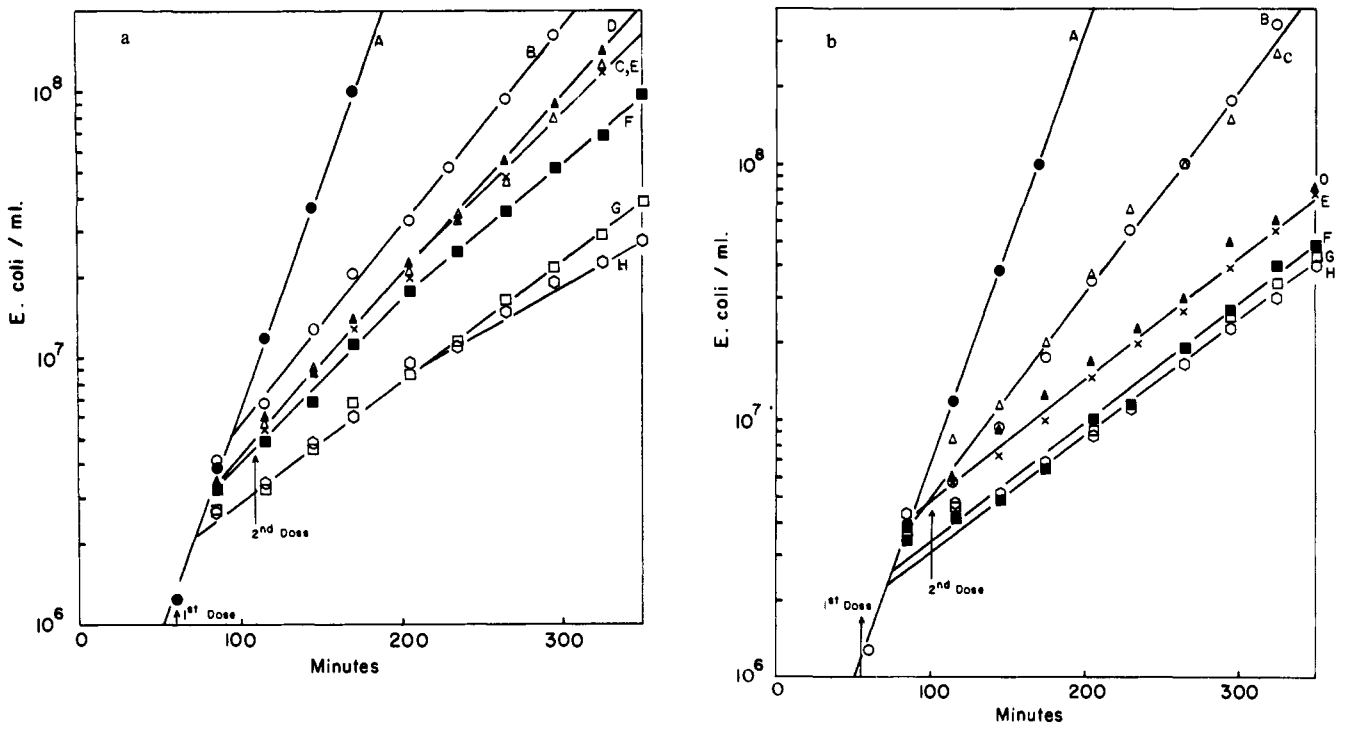



Figure 3. Effects of order of addn of equipotent (a) clindamycin·HCl and lincomycin·HCl and (b) of equipotent III·HCl and II·HCl on generation rates of *E. coli*. (a) Curve A is for generation of culture in the absence of drug. Curve B is for generation of culture in the presence of 16.67 μg/ml of II·HCl and curve C is generated when in the presence of equipotent 100 μg/ml of I·HCl. Curve D is generated when equipotent I·HCl (100 μg/ml) is added to the clindamycin-affected culture of curve B and curve E is generated when equipotent II·HCl (16.67 μg/ml) is added to the phase I lincomycin-affected culture of curve C. Curve F is generated for a mixt of equipotent concns of II·HCl (16.67 μg/ml) and I·HCl (100 μg/ml), added to the culture of curve A. Curve G is generated when 33.34 μg/ml of II·HCl is added to the culture of curve A and curve H is generated when equipotent 200 μg/ml of I·HCl is added to the culture of curve A. (b) Curve A is for generation of culture in the absence of drug. Curve B is for generation of culture in the presence of 4 μg/ml of II·HCl or the coincident curve C is for generation of culture when in the presence of equipotent 16.67 μg/ml of II·HCl. Curve D is generated when equipotent II·HCl (16.67 μg/ml) is added to the III-affected culture of curve B or the coincident curve E is generated when equipotent III·HCl (4 μg/ml) is added to the clindamycin-affected culture of curve C. Curve F is generated for a mixt of equipotent concns of III·HCl (4 μg/ml) and II·HCl (16.67 μg/ml) added to the culture of curve A. Curve G is generated when 8 μg/ml of III·HCl is added to the culture of curve A or the coincident curve H is generated when equipotent 33.34 μg/ml of II·HCl is added to the culture of curve A.

with aliquots (0.5 ml) of equipotent mixts of antibiotics. The mixts consisted of 100, 90, 80, 60, 50, 40, 20, 10, and 0% concn of 1 antibiotic at 1 dose level and the residual percentage of equipotent concn of the other antibiotic to maintain the anticipated equipotency at that dose level. The equipotent concns were considered as 100 μg/ml of I·HCl, 16.67 μg/ml of II·HCl, and 4 μg/ml of III·HCl, respectively. These studies were repeated at levels of activity corresponding to the equipotencies of 50, 100, and 200 μg/ml of I. Cultures were treated with equipotent mixts of I and II (Figure 2a), I and III (Figure 2b), II and III (Figure 2c). Coulter counts were obtained from samples of cultures withdrawn every 20–30 min and apparent generation rate constants ($k_{app}$) detd.

**Effect of the Order of Addition of Lincomycin and Its Analogs in Combination on Microbial Generation.** Replicate 49.5-ml samples of cultures contg $10^6$/ml of *E. coli* in steady-state generation (curve A in Figure 3a) were treated with aliquots (0.5 ml) of equipotent solns of I and its analogs. Equipotency of action is shown by coincident or parallel generation curves of the drug-affected cultures having the same generation rate constant. The resultant generation curves for the action of equipotent concns of 16.67 μg/ml of II·HCl and 100 μg/ml of I·HCl (phase I) are given as curves B and C, respectively. Replicate cultures of curve A were also treated with aliquots of a mixt of equal parts of the equipotent concns of II and I (curve F) which was prepd to be *a priori* as equipotent as the corresponding II (curve G) or I (curve H) alone.

Fifty min after the clindamycin-affected culture of curve B had settled to a new steady-state generation, an equipotent amount of I was further added. The resultant generation is given as curve D. A similar treatment of the lincomycin-affected culture of curve C with equipotent amount of II resulted in ultimate generation given as curve E.

The experiment was repeated in like manner for equipotent concns of 4 μg/ml of III·HCl and 100 μg/ml of I·HCl or for equipotent concns of 16.67 μg/ml of II·HCl and 4 μg/ml of III·HCl (Figure 3b).

Coulter counts were obtd from samples of the cultures withdrawn every 20–30 min.

**Effects of Clindamycin and III on Lincomycin-Affected Cultures in Phase I and Phase II Generation.** Replicate 49.5-ml samples of cultures contg $10^6$/ml of *E. coli* in steady-state generation (curve A in Figure 4) were treated with aliquots (0.5 ml) of solns of I and its analogs and in combinations thereof. The separate effects for equipotent concns of II and I (phase I) on the generation of cultures are shown, respectively, as curves B and C in Figure 4. Curve N is for the effect of a mixt of equal parts of the equipotent concns of II and I which was prepared to be *a priori* as equipotent as the corresponding II (curve O) or I (curve P) alone.

When the lincomycin-affected cultures of curve C had settled to steady-state phase I generation, *i.e.*, 80 min after addn of I in Figure 4, aliquots (0.5 ml) of graded concns of II were added to each of 5 replicates so that the total antibiotic concn maintd in the cultures would be *a priori* 1.0, 2.0, 2.2, 3.0, and 3.5 times as equipotent as the concn of the I initially present in the culture (Table II). The resultant generation curves are given as D, E, F, G, and H, respectively.

Again, when the cultures of curve C had entered into steady-state generation phase II, *i.e.*, 175 min after the addn of I in Figure 5 5 other replicates were treated with similar concns of the clindamycin (curves I, J, K, L, and M). Coulter counts were obtd on samples of the cultures withdrawn every 20–30 min.

The effects of III on lincomycin-affected cultures in phase I and phase II generation were detd in a similar manner and sequence (Table II).

**Determination of the $pK_a'$ Value of II and III.** The $pK_a'$ values of II and III were detd by titration in $H_2O$ with NaOH at an ionic strength of 0.122 *M* and temp of 37.5° on a Radiometer automatic titrator Model III Lc-SBR 2c-SBUI.

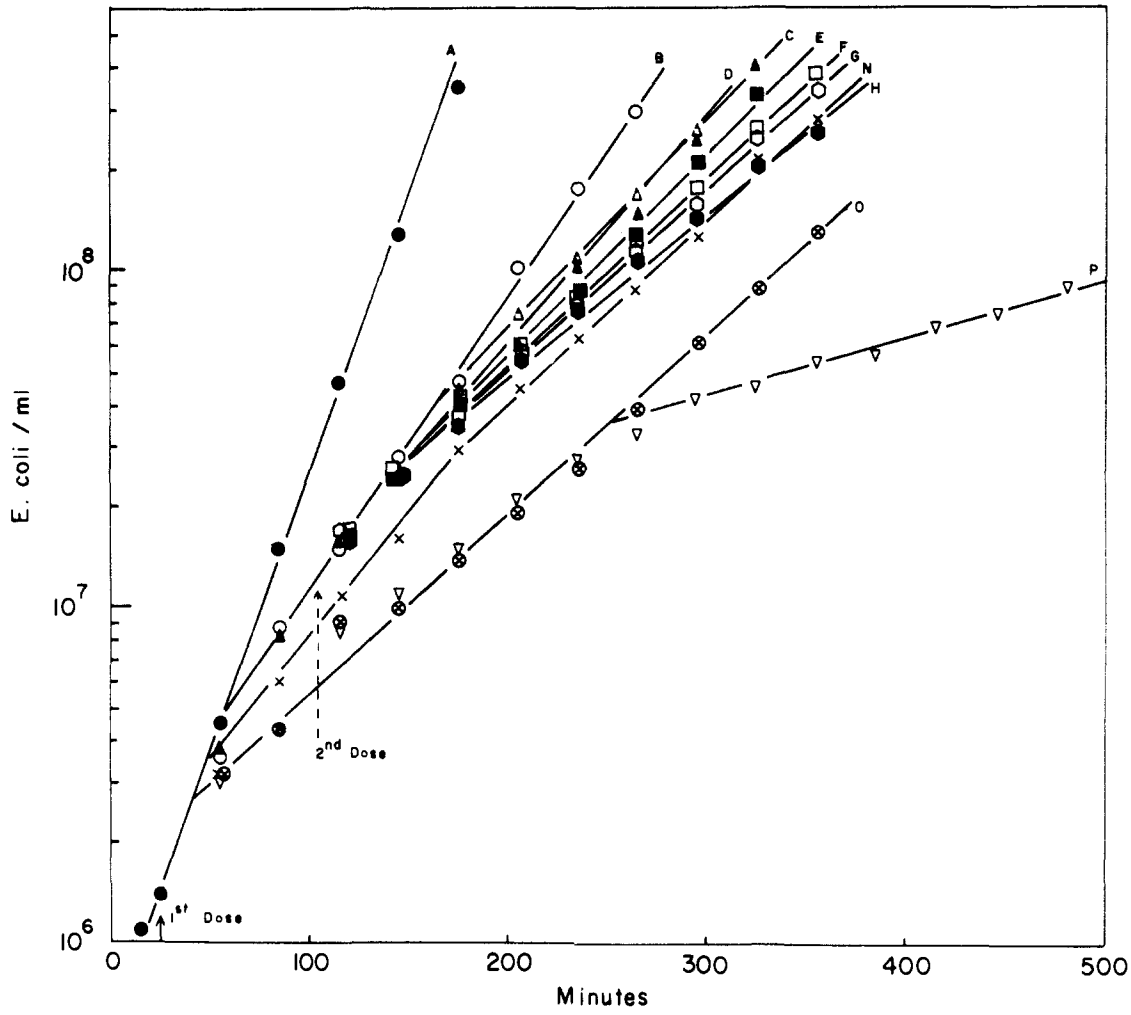



Figure 4. Demonstration of antagonistic action of II on phase I generation of lincomycin-affected cultures. Curve A is for generation of culture in the absence of drug. Curve B is for generation of culture in the presence of 16.67 μg/ml of II·HCl and curve C is for generation when in the presence of equipotent 100 μg/ml of I·HCl. Curves D, E, F, G, and H are for generations of phase I lincomycin-affected cultures of curve C, when concns of II·HCl: 16.67, 20.0, 25.0, 33.34, and 41.67 μg/ml are, respectively, added after 80 min of initial addn of the I. Curve N is for a mixt of equipotent concns of II·HCl (16.67 μg/ml) and I·HCl (100 μg/ml) added to the culture of curve A. Curve O is generated when 33.34 μg/ml of II·HCl is added to the culture of curve A and curve P is generated when equipotent 200 μg/ml of I·HCl is added to the culture of curve A.

**Reversibility of Action of I and Its Analogs.** Aliquots (5 and 0.5 ml) of a drug-free culture in the log phase of generation and contg $10^7$ *E. coli*/ml (curve A in Figure 6) were added to 45 and 49.5 ml of fresh broth, respectively; so that the organism population was dild 10-fold (curve A' in Figure 6) and 100-fold (curve A'' in Figure 6).

A 49.5-ml vol of broth contg $10^6$ *E. coli*/ml in log phase generation (curve A in Figure 6) was treated with 0.5 ml of a soln of II·HCl to achieve a final concn of 33.34 μg/ml (curve B in Figure 6). When the clindamycin-affected culture of curve B was in a steady-state generation and had reached an organism population of $10^7$ *E. coli*/ml, aliquots (5 and 0.5 ml) were added to 45 and 49.5 ml of fresh broth, respectively, so that both the organism and drug concns were dild 10-fold (curve C in Figure 6) and 100-fold (curve D in Figure 6), respectively. At the same time, aliquots of the culture of curve B were dild 10-fold (curve E in Figure 6) and 100-fold (curve F in Figure 6), respectively, in broths contg enough I·HCl so that drug concn was restored to 16.17 μg/ml.

The experiment was repeated in like manner and sequence for cultures affected with equipotent concns of 200 μg/ml of I·HCl and 8 μg/ml of III·HCl. Coulter counts were obtd on samples withdrawn every 15 min.

## Results

**Shape of Generation Curves for Drug-Affected Organisms.** The plots of log of numbers of *E. coli*/ml *vs.* time, obtained from coulter counts, are given for graded concns of I (Figure 1a), II (Figure 1b), and III. The curves for this latter compound were similar to those for clindamycin. Lincomycin exhibits the characteristic 2 phases of steady-state generation previously reported[17] while II and III have only 1 phase of steady-state generation. The apparent first-order generation rate constants ($k_{app}$ in $sec^{-1}$) are obtd from the slopes of the linear portions of the plot of log of numbers, $N$ of *E. coli* per milliliter against time, $t$, in accordance with the equation

$$\ln N = k_{app} t + \ln N_0 \quad (1)$$

**Effect of Antibiotic Concns on Generation Rates.** A plot of $k_{app}$ *vs.* concn (Table I) is given in Figure 7. The $k_{app}$ values are linearly dependent on drug concns up to 100 μg/ml of I·HCl (phase I growth), 16 μg/ml of II·HCl, and 4.0 μg/ml of III·HCl respectively, in accordance with the expression

$$k_{app} = k_0 - k_c C \quad (2)$$

where $k_0$ is the generation rate constant for the drug-free culture, $k_{app}$ is the generation rate constant for culture af-

fected with drug concn, $C$, and $k_c$ (in ml/μg sec) is the inhibitory rate constant for the drug. Above these concn ranges, the curves slowly approach asymptotes. On the other hand, nonlinear decrease of the $k_{app}$ with drug concn is observed for I in phase II generation throughout the concn range studied. The relative potency of the drugs on a weight basis, estimated in accordance with eq 2 is approximately of the order 1:6:25 as I·HCl (phase I)-II·HCl-III·HCl. Thus coincidence of the plots of $k_{app}$ *vs.* concn of the drugs (Figure 7) is obtained by multiplying the actual concns of II·HCl and III·HCl, in the data of Table I by factors of 6 and 25, respectively.

**Applicability of Saturable Receptor Site Model to the Action of I, II, and III.** Figure 8 gives a plot of $C/(k_o - k_{app})$ *vs.* $C$ in accordance with a previously[17-19] derived saturable receptor site model

$$C/(k_o - k_{app}) = C(k_b/k_a) + 1/k_a \quad (3)$$

where $k_a$ and $k_b$ are constants of proportionality related to drug availability in the biophase and drug affinity for receptor or binding sites. Similar consideration of the potency estimates for I·HCl (phase I), II·HCl, and III·HCl in the plots of Figure 8 in accordance with eq 3 makes the plots coincident. Adherence to the model is observed from the

**Table II.** Concentrations of Clindamycin·HCl or III·HCl Added to Phase I and Phase II Generations of Cultures Affected by 100 μg/ml of Lincomycin·HCl

| Reference curves[a] | | Concn (μg/ml) of analogs added to phase I[b] and phase II[c] lincomycin-affected cultures | | Final relative equipotent concn[d] of total antibiotics added |
|---|---|---|---|---|
| a | b | Clindamycin | III | |
| C | C | | | 1.0 |
| D | I | 16.67 | 4.0 | 2.0 |
| E | J | 20.00 | 4.8 | 2.2 |
| F | K | 25.00 | 6.0 | 2.5 |
| G | L | 33.34 | 8.0 | 3.0 |
| H | M | 41.67 | 10.0 | 3.5 |

[a] As indicated in Figures 4 and 5. [b] At 80 min after the initial addition of I. [c] At 175 min after the initial addn of the I. [d] 100 μg/ml I·HCl = 16.67 μg/ml of II·HCl = 4.0 μg/ml of III·HCl).

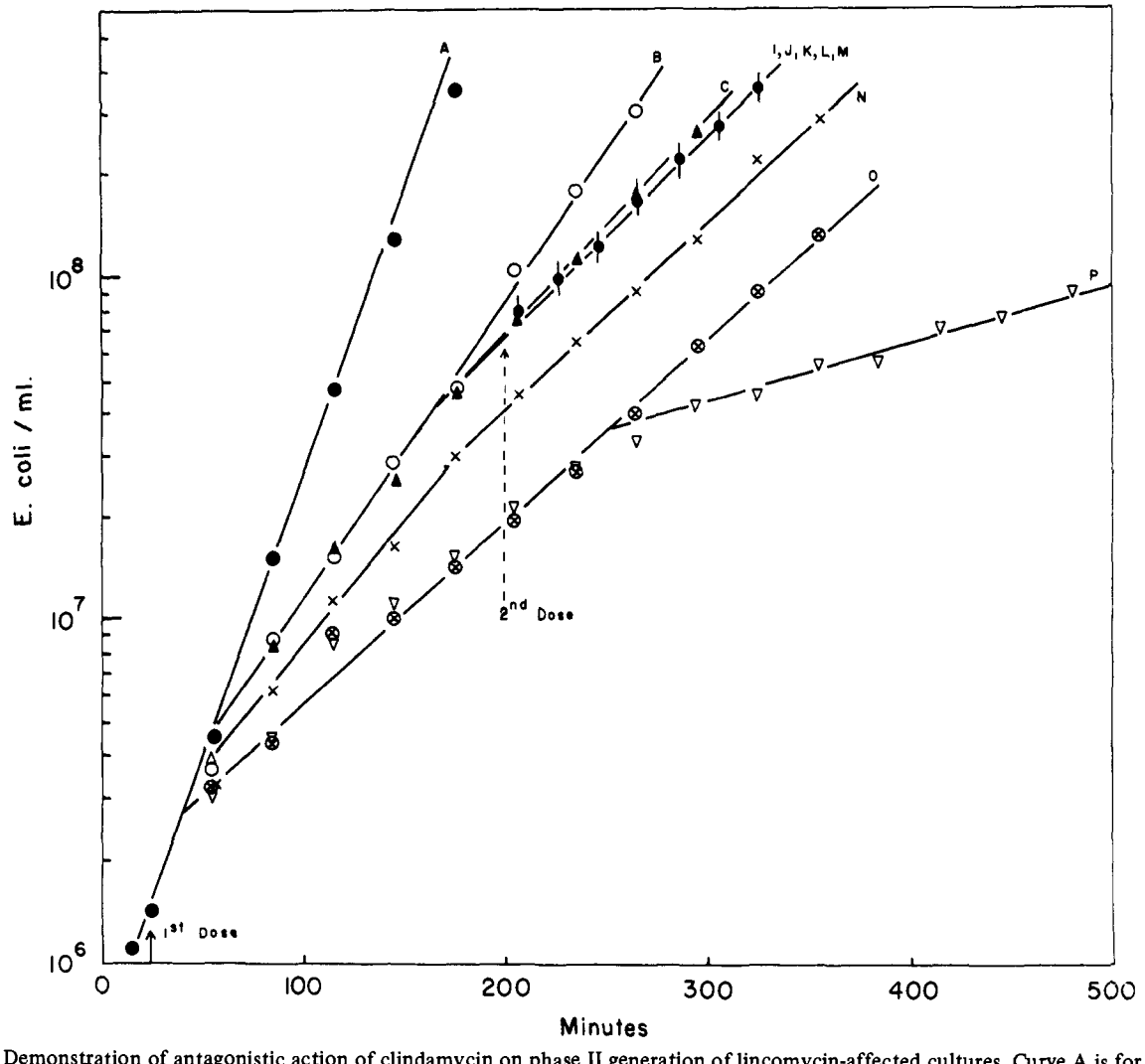



Figure 5. Demonstration of antagonistic action of clindamycin on phase II generation of lincomycin-affected cultures. Curve A is for generation of culture in the absence of drug. Curve B is for generation of culture in the presence of 16.67 μg/ml of II·HCl and curve C for generation of culture in the presence of equipotent 100 μg/ml of I·HCl. Curves I, J, K, L, and M are for generations of phase II lincomycin-affected cultures of curve C when concns of II·HCl: 16.67, 20.0, 25.0, 33.34, and 41.67 μg/ml are, respectively, added after 175 min of initial addn of the lincomycin. Curve N is for a mixt of equipotent concns of II·HCl (16.67 μg/ml) and I·HCl (100 μg/ml) added to the culture of curve A. Curve O is generated when 33.34 μg/ml of II·HCl is added to the culture of curve A, and curve P is generated when equipotent 200 μg/ml of I·HCl is added to the culture of curve A.

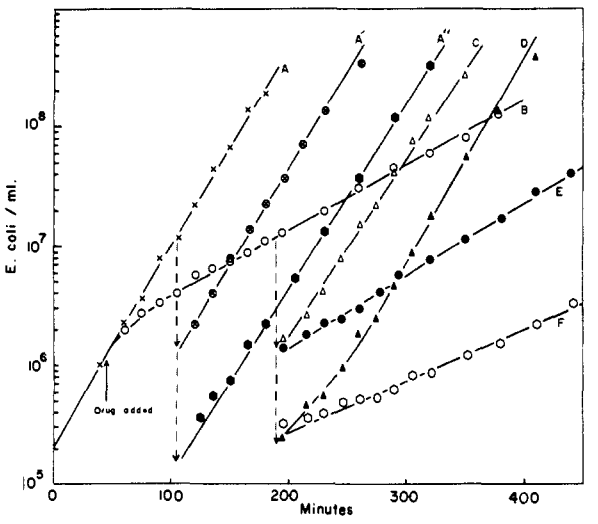



Figure 6. Semilog plots of reversibility studies of *E. coli* generation with time on addition of II·HCl and diln of the cultures with broth. Curve A is without drug. Curve B is after addn of II·HCl to the culture of curve A to a final concn of 33.34 μg/ml. Curves A' and A'' are after diln of the culture of curve A, 1:10 and 1:100 with broth, respectively. Curves C and D are after diln of the culture of curve B, 1:10 and 1:100 with broth to final drug concns 3.34 and 0.34 μg/ml of II·HCl, respectively. Curves E and F are after diln of the culture of curve B, 1:10 and 1:1000 with broth contg sufficient amts of II·HCl, respectively, so that the final drug concn is restored to 33.34 μg/ml of II·HCl.

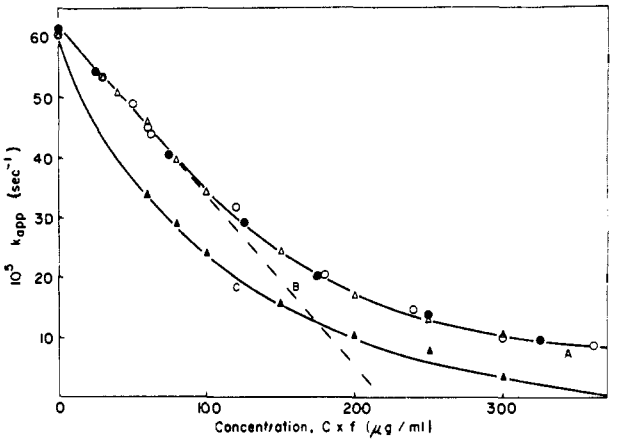



Figure 7. Demonstration of the coincidence of the dependencies of the apparent generation rate constants, $k_{app}$ for *E. coli* cultures at 37.5° and pH 7.05 on equipotent concns, *C*, of I·HCl, II·HCl, and III·HCl. Curve A represents this dependence, where the open triangles are for I·HCl (phase I), the open circles are for II·HCl, and the closed circles are for III·HCl, and where $f = 1.0$ for I·HCl, $f = 6.0$ for II·HCl, and $f = 25.0$ for III·HCl. The dashed line, curve B, demonstrates the linear dependency of $k_{app}$, on drug concn at low drug concns. Curve C represents the dependence of the $k_{app}$ on concns, *C*, of I·HCl (phase II) at $0 < C < 350$ μg/ml.

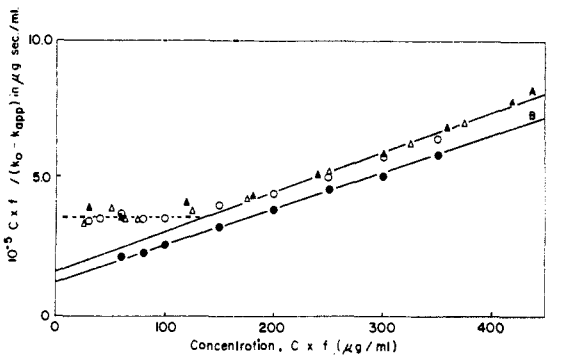



Figure 8. Application of saturation kinetics to the action of I·HCl, II·HCl, and III·HCl at higher concns, *C*, on the apparent generation rate constants, $k_{app}$ ($sec^{-1}$) of *E. coli* at 37.5°. Curve A represents this application, where the open circles are for concns of I·HCl (phase I) > 100 μg/ml, the closed triangles are for concns of II·HCl > 16.67 μg/ml, and the open triangles are for concns of III·HCl > 4 μg/ml, and where $f = 1.0$ for I·HCl, $f = 6$ for II·HCl, and $f = 25.0$ for III·HCl. Curve B represents the application to concns of I·HCl (phase II) at $0 < C < 350$ μg/ml. The curves are plotted from the data of Table I according to eq 3.

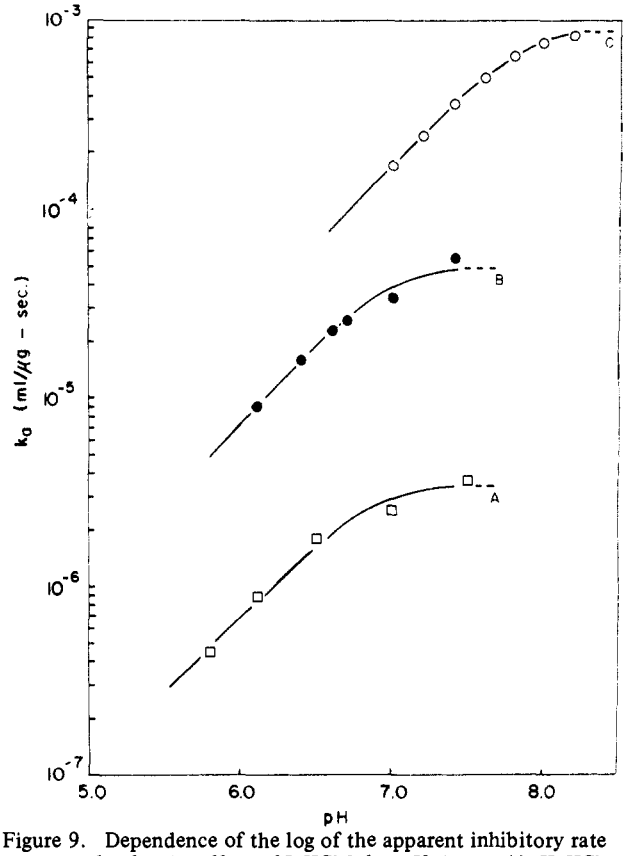



Figure 9. Dependence of the log of the apparent inhibitory rate constant, $k_a$, for the effect of I·HCl [phase I] (curve A), II·HCl (curve B), and III·HCl (curve C) on the growth of *E. coli* at 37.5°, on the pH of the broth medium. The drawn lines are consistent with the expression: $k_a = k_a^* K_a'/(K_a' + [H^+])$, where $k_a^*$ is the intrinsic inhibitory rate constant of the unprotonated drug and $K_a'$ is the dissociation constant for the protonated drug concn, *C*. The $k_a$ is obtd from the expression: $k_{app} = k_0 - k_a C/(1 + k_b C)$.

linear plots obtained for concns of drugs greater than 100 μg/ml of I·HCl (phase I), 16.0 μg/ml of II·HCl, and 4 μg/ml of III·HCl, respectively. Deviations occur, in all cases, in the lower concn ranges. I (phase II) adheres to eq 3 at all concns studied. The values of the constants $k_a$ and $k_b$ calcd from the slopes and intercepts of such plots are given in Table I.

**Effects of pH on Drug-Affected Generation Rates.** The apparent first-order generation rate constants ($k_{app}$) were obtained at different pH values (6.10-8.20) of the culture media in the absence and presence of graded concns of I, II, and III. The values for I were obtained by Mielck and Garrett[17] at similar pH values using the same organism and experimental technique. The drug-free generation rate constants were invariant with pH within the range studied. The drug-affected generation rate constants at a specified concn of each drug, decrease significantly with increased pH values. Thus, larger amounts of a drug are required for the same

fractional inhibition of microbial growth as the pH of the medium is decreased. The constants, $k_a$, derived from plots of $c/(k_0 - k_{app})$ *vs.* $C$ in accordance with eq 3 at different pH values are plotted as log $k_a$ against pH in Figure 9. The values of $k_a$ increase 10-fold for unit increase in the pH over the range 5.80–6.50 for I (phase I), 6.10–6.70 for II, and 7.00–7.60 for III, respectively. The slopes of the plots of log $k_a$ *vs.* pH however, tend to lessen, in all cases, as the pH approaches the respective $pK_a'$ value of the drug.

**Effect of Equipotent Mixtures Composed of Different Fractions of I and Its Analogs on Generation Rates.** The generation rate constants, ($k_{app}$) of cultures affected by equipotent mixtures of I and II are shown in Figure 2a, I and III in Figure 2b, and II and III in Figure 2c, respectively. The mixtures consisted of 100 to 0% of 1 antibiotic and the residual percentage of equipotent amount of the other antibiotic. The mixtures were prepared so as to be *a priori* equipotent in their combined action on *E. coli* generation in accordance with Figure 7 at 3 different levels of action. The null slopes of the plots of the $k_{app}$ for all the *a priori* equipotent mixtures of II and III (Figure 2c) demonstrate the indifference[22] of effects. I and II mixtures or I and III mixtures, on the other hand, exhibit lessened activity as indicated by higher $k_{app}$ values than the equipotent antibiotic used alone (Figures 2a and 2b). This is most readily manifested at the higher potency levels. The effect is less striking at the lowest concn studied. This unequivocally demonstrates antagonism[22] of effects between I and its analogs in their combined action against *E. coli*.

**Effect of the Order of Addition of I and Its Analogs on Microbial Generation.** There are no significant differences among the generation inhibition produced by the action of 100 μg/ml of I·HCl in phase I (curve C) and the equipotent concn 16.67 μg/ml of II·HCl (curve B) in Figure 3a since the initial portion of curve C has the same slope as curve B. There are however, significant differences in the effective inhibition of generation produced by the action of a mixture of 100 μg/ml of I·HCl and 16.67 μg/ml of II·HCl (curve F) and that of the *a priori* equipotent concn of either drug alone, *i.e.*, 200 μg/ml of I·HCl in phase I (curve H) or 33.34 μg/ml of II·HCl (curve G). The slope of curve F is higher than that of curve G or the initial portion of curve H. This demonstrates antagonism of effects between I (phase I) and II. Also, an equipotent amount of II, added after 50 min to a lincomycin-affected culture of curve C, produces an ultimate steady-state generation rate (curve E) which is the same as that of the culture affected initially with I. However, the addition of I after 50 min to a clindamycin-affected culture of curve B, produces an ultimate steady-state generation rate (curve D) which is the same as that of the culture affected initially with the II (curve B) but shows nothing of the I (phase II) effect.

This pattern of response is likewise observed for combinations of 100 μg/ml of I·HCl with an equipotent concn of 4 μg/ml of III·HCl. In both cases the order of addition of the antibiotics produces significant effects on the ultimate generation inhibition and shows antagonism of the action between I and II (or III).

There are however no significant differences in the effective inhibition of generation produced by the action of a mixture of 16.67 μg/ml of II·HCl and 4 μg/ml of III·HCl (curve F) in Figure 3b and that of the *a priori* equipotent concn of either drug alone, *i.e.*, 33.34 μg/ml of II·HCl (curve H) or 8 μg/ml of III·HCl (curve G), where curves F, G, and H have the same slopes. Also, there are no significant differences in the effective inhibition of generation, produced by an equipotent amount of the II added after 50 min to III-affected culture (curve D) or by an equipotent amount of III added after 50 min to II-affected culture (curve E). Curves D and E have the same slopes as curves F, G, and H. Thus, the order of addition of these 2 antibiotics produces no significant change on the ultimate generation inhibition.

**Antagonistic Action of II or III on Phases I and II Generation of Lincomycin-Affected Cultures.** The equipotency of action for II (curve B) and I (curve C) is indicated in Figure 4 by coincident or parallel growth curves of cultures affected with the drugs. A mixture of equipotent concns of II and I (curve N) is less active than the *a priori* equipotent concn of either the II (curve O) or I (curve P) alone since curve N has a higher slope than curve O or the initial part of curve P. Curve N possesses some elements of the biphasic characteristics observed for I action alone.

The addition of graded concns of II to I-affected cultures in phase I steady-state generation (curve C) results in graded responses as shown by decreasing slopes of growth curves with increasing concns of II (curves D, E, F, G, and H). The ultimate generation rates represented by the final slopes of curves D through H are less than would be expected *a priori* from the additivity of equipotent concns of either drug alone and shows antagonism. For example, the data of Table II show that the total drug concn in the culture of curve G is about 1.5 times as equipotent as the II in the culture of curve O or the I in the culture of curve P. However, curve G has about the same slope as curve O or the initial portion of curve P. This indicates that the combination of I and II in the culture of curve H has about 1/1.5 or 66.67% of its *a priori* potency.

On the other hand, the ultimate steady-state generation rates of lincomycin-affected cultures in phase II are not significantly changed by addition of graded concns of II (curves I, J, K, L, and M in Figure 5).

Mixtures of III with I produce patterns that are similar to the effects of II on lincomycin-affected cultures. In both cases the antagonism of effects produced by combinations of II or III with the I on the generation of cultures appear to be greater in phase II than in phase I of the lincomycin action.

**$pK_a'$ of Lincomycin and Its Analogs.** The determined $pK_a'$ values at 37.5° and ionic strength of 0.122 are 7.00 for II and 8.05 for III (Table III). The value of 7.76 has been reported for I.[17]

Table III. Values of $pK_a'$ and Intrinsic Inhibitory Rate Constants ($k_a$*) for Lincomycin and Its Analogs

| Drug | $10^5 k_a$*[a] (ml/μg sec) | Kinetically[b] determined $pK_a$ | Experimentally[c] determined $pK_a'$ | Cited literature values of $pK_a'$ |
|---|---|---|---|---|
| Lincomycin | 1.43 | 7.57[d] | 7.76[g] | 7.6[e] |
| Clindamycin | 7.14 | 6.94 | 7.00 | 7.6[f] |
| III | 125.00 | 7.79 | 8.05 | 8.0[f] |

[a] As detd from the slopes and intercepts of plots of $1/k_a$ *vs.* $[H^+]$ in accordance with the equation: $1/k_a = 1/k_a^* K_a'[H^+] + 1/k_a^*$, where $k_a$ is obtd from the equation fitting the apparent generation rate constant, $k_{app}$, of the drug-affected cultures at the higher concns, $C$ of drug, *i.e.*, $C/(k_0 - k_{app}) = C(k_b/k_a) + 1/k_a$. [b] Calcd from the quotient of the slope and intercept of the plot according to the equation in footnote *a*. [c] As detd by titration. [d] Based on $k_a$ values at pH 5.80 and 6.10, respectively, from data of Mielck and Garrett.[17] [e] Reported by Herr and Bergy.[2] [f] Personal communication from Dr. G. B. Whitfield, Jr., The Upjohn Co., Kalamazoo, Mich. [g] See ref 17.

**Reversibility of Drug Action.** The equilibration of I and its analogs between nutrient medium and biophase is readily achieved. It took 15-20 min for cultures affected with equipotent concns of 33.34 μg/ml of II·HCl (curve B in Figure 6), 200 μg/ml of I·HCl, and 8 μg/ml of III·HCl to attain a new steady-state phase of generation. These latter curves were practically coincident with those of II so they are not presented here. The steady-state generation of the drug-affected cultures reverted to new steady-state generation with predictable rate constants when diluted 10-fold and 100-fold, respectively (curves C and D, respectively in Figure 6). However, the new steady state was attained after an interval which was dependent on the dilution factor. For instance, it took 30-40 min for the 1:10 dilution, but 60-80 min for the 1:100 diluted II-affected cultures to attain the new steady states. Similar lag periods were observed for corresponding dilutions of I-affected and III-affected cultures. However, the correspondingly diluted drug-free cultures showed a very short lag period, *e.g.*, 5-10 min to revert to their new steady states.

If the time required for rejuvenation of the cells or consequence of shock on dilution is considered, the prolonged lag period observed for the drug-affected cultures to attain a new steady-state generation upon dilution suggests that either the rate of dissociation of drug-receptor complex in the biophase is less than the rate of formation of the complex and/or the rate of diffusion of drug from biophase to the broth medium into the biophase through the cell membranes.

Cultures inhibited by low concns of the drugs, *e.g.*, 1.67 and 0.167 μg/ml of II·HCl (curves E and F in Figure 6), 20 and 2 μg/ml of I·HCl, 0.8 and 0.08 μg/ml of III·HCl were further inhibited by addition of more drug to final concns of 16.67 μg/ml of II·HCl, 200 μg/ml of I·HCl, and 8 μg/ml of III·HCl, respectively, at the same relatively short equilibration time of 15-20 min.

Thus no significant difference is observed between the reversible action of I, II, and III against *E. coli*.

## Discussion

The data reported in this paper show that the generation rate constants ($k_{app}$) of *E. coli* cultures affected by I (phase I), II, and III have similar functional dependencies on drug concns (Figure 7). This suggests that the analogs have a mechanism of action similar to I (phase I). On this presumption, the ratio of the biological potencies of these drugs are 1:6:25 as I·HCl-II·HCl-III·HCl on a weight basis at pH 7.05. The corresponding molar ratio of potencies would be 1:6.6:28.5. These apparent differences in potency can be attributed to differences in degrees of partitioning across the bacterial cell membrane and/or to differences in the affinities of the drug for the receptor site, and/or to differences in the intrinsic efficacies of the drug bound to the site. The steady-state generation of lincomycin-affected culture changes after several generations of growth to a new steady state, *i.e.*, phase II (Figure 1a), which does not occur with cultures affected by the other two analogs (Figure 1b). The functional dependency of the $k_{app}$ on concn for I (phase II) suggests a different mechanism of I action (Figure 7) which dominates the latter stages of lincomycin-bacterial interaction.

**pH Effect on Activity of Lincosaminide Antibiotics.** The extent of generation inhibition by lincomycin (phase I), II, or III increases as some function of pH values of the broth medium. The constants $k_a$ (ml/μg sec) as defined in eq 3 for various pH values (Figure 9) adhered to the expression[17]

$$k_a = k_a^* f = k_a^* \frac{K_a'}{K_a' + [H^+]} \quad (4)$$

where, $k_a^*$ is the intrinsic inhibitory rate constant of the unprotonated drug, $f$ is the fraction of the drug concn unprotonated, $K_a'$ is the dissociation constant of the protonated base, and $[H^+]$ is the $H^+$ concn. The plot of $\log k_a$ *vs.* pH approaches a slope of unity when $[H^+] > K_a'$ and a slope of 0 when $K_a' > [H^+]$ (Figure 9). Arithmetical transformation of eq 4 yields

$$\frac{1}{k_a} = \frac{[H^+]}{k_a^* K_a'} + \frac{1}{k_a^*} \quad (5)$$

Values of $k_a^*$ and $pK_a'$ of the drugs derived from the slopes and intercepts of linear regressions obtained by plotting $1/k_a$ *vs.* $[H^+]$, are given in Table III. A reasonably good agreement is obtained between the experimentally determined $pK_a'$ values by potentiometric titrations for the proline rings of the drug species and the kinetically determined values from eq 5. It is therefore concluded that eq 4 holds and that it is the unprotonated fraction of the drug concn that is responsible for the antibacterial activity of I and its analogs. The relative intrinsic activity of the uncharged drugs on a weight basis is estimated to be approximately of the order of 1:5.0:87.4 as I·HCl-II·HCl-III·HCl. The ratios in terms of molar concns are for the uncharged drugs 1:5.2:94.4.

**Anomalous Antagonisms among Lincosamide Antibiotics.** Equipotent mixtures of II and III are equivalent in their action against *E. coli* (Figure 2c) and their effects can be quantified on the basis of additivity from the separate dose-response curves of either drug alone (Figure 7). This suggests a similar mode and locus of action for the 2 drugs.[22] Furthermore, the order of addition of the drugs in a combination has no significant effect on the ultimate steady-state generation of drug-affected cultures (Figure 3b), which therefore demonstrates the lack of any significant bacteriostatic antagonism or synergism (by the definitions of Garrett[22]) in the sub-completely-inhibitory range similar to that observed[21] for combinations of I (phase I) and erythromycin.

In contrast, equipotent mixtures of I (phase I) and II (Figure 2a) or I (phase I) and III (Figure 2b) demonstrate unequivocal antagonism, since the mixtures are less effective on the generation of *E. coli* cultures than the *a priori* equipotent concentration of either drug alone. In addition, the extent of generation inhibition produced by the combined action of I (phase I) with II (Figure 3a) or I (phase I) with III depends on the order of addition of the drugs to a culture. For instance, the ultimate generation inhibition produced by admixing an equipotent amount of I (phase I) with an equipotent amount of II or of I (phase I) with an equipotent amount of III, before addition of the mixture to a culture is greater than that obtained by adding an equipotent amount of one drug after an interval of time to a culture pretreated with an equipotent amount of the other drug. In the latter case, the I (phase II) action is eliminated, when I is added to a culture pretreated with II or III.

This antagonism of effects obtained by various combinations of I with II (or III) suggests, as one possibility, that I (phase I) may have a different binding site from that of II (or III) and hence their mechanisms of action[22] are not the same. Therefore, the similarity in functional dependencies of the $k_{app}$ for cultures affected with I (phase I), II, or III

on drug concns could be attributed to the fact that their binding sites, though different, nevertheless, are functionally linked. Such would be the case, if the separate sites were engaged in "sequential" or "convergent" metabolic processes[23] which lead to a common end product. However, on this presumption, the combined action of II (or III) with I (phase I) would either cause a "sequential blocking"[24] of the metabolic pathway to produce "synergism" or cause a "concurrent blocking"[25] to produce "additivity" (or equivalence) of effects. Hence, neither of these 2 mechanisms can explain the observed antagonism between I (phase I) and II or III.

Certainly, these phenomena would be more readily understood if the one drug inhibited the transport of the other to the receptor site where both acted. However, the fact that the separate action of each of these several antibiotics is relatively rapidly reversible on dilution denies any significant inability of either drug to reach or leave the biophase. This fact makes it difficult to accept the conjecture that the presence of a molecule of one drug may sterically block or selectively inhibit the entrance of a molecule of the other to the biophase and, in effect, change its apparent partition coefficient $K_1$ or apparent activity.

**A Possible Kinetic Model to Rationalize Antagonistic Action.** One possibility for rationalizing these antagonisms is that the presence of one drug quasipermanently modifies the allosteric configuration of the receptor site so that it diminishes the effective affinity constant of the other drug for that receptor site without significantly affecting the receptor site-substrate interaction necessary for microbial growth and generation.[26-28] This can be expressed schematically[29] by

$$\begin{array}{ccccccc} & & (B) & & (A) & & \\ & & \Updownarrow K_B & & \Updownarrow K_A & & \\ (B'R) & \underset{k_B + k_B'(A')}{\overset{k_{-B}}{\rightleftharpoons}} & (B') & + R + & (A') & \underset{k_A}{\overset{k_{-A}}{\rightleftharpoons}} & (A'R) \\ & & & & \downarrow k_m & & \\ & & K_B'[1 + (k_B'/k_B)(A')] & & \text{metabolites} & & K_A' \end{array} \qquad (6)$$

for the competition of 2 drugs, A and B for the same receptor site, R, where the steady state metabolic rate, $dM/dt$, that results in generation is proportional to the fraction of these unreacted receptor sites as per

$$dM/dt = k_m(1 - \theta_A - \theta_B) \qquad (7)$$

where $\theta_A$ and $\theta_B$ are the fractions of total receptor sites, $R_T$, occupied by drugs A and B, respectively. The (A′) and (B′) values are the drug concns in the biophase in equilibria with the respective extracellular (A) and (B) concns and the respective concns of bound receptors, *i.e.*, (A′R) and (B′R). The dissociation of one receptor-drug complex; *e.g.*, (B′R) is additionally catalyzed by the available concentration, (A′), of the other drug in the biophase. It should follow that the number of occupied receptor sites in the presence of $n$ equipotent units of a mixture of A and B will be less than the number in the presence of $n$ equipotent units of A or B alone. Thus, the metabolic rate which is porportional to a fraction of free receptor sites, eq 7, should be higher from the combination than would be predicted on the basis of drug equivalency. Thus, such allosteric modifications should result in antagonism for such a combination.

The equilibrium constant for the interaction of drug B with the free receptor site, R, can be implicitly defined in

$$\begin{aligned} B' \cdot R/(B'R) &= B'[R_T - (B'R) - (A'R)]/(B'R) \\ &= (k_B + k_B'(A'))/k_{-B} \\ &= 1 + (k_B'/k_B)(A')/K_B' \end{aligned} \qquad (8)$$

and similarly for drug A,

$$\begin{aligned} (A') \cdot R/(A'R) &= (A')[R_T - (B'R) - (A'R)]/(A'R) \\ &= k_A/k_{-A} = 1/K_A' \end{aligned} \qquad (9)$$

Equation 8 can be rearranged to explicitly define the reciprocal of the fraction of receptor sites occupied by the drug B as

$$1/\theta_B = R_T/(B'R) = 1/K_B'(B') + (k_B'(A'))/k_{-B}(B') + 1 + (A'R)/(B'R) \qquad (10)$$

and eq 9 can be similarly rearranged to define the reciprocal of the fraction of receptor sites occupied by the drug A as

$$1/\theta_A = R_T/(A'R) = 1/K_A'(A') + 1 + (B'R)/(A'R) \qquad (11)$$

Equivalent values may be obtained by solving eq 10 and 11 for R. When these two rearranged expressions are equated, the ratio (A′R)/(B′R) can be obtained and is

$$(A'R)/(B'R) = [1 + (k_B'/k_B)(A')]K_A'(A')/K_B'(B') \qquad (12)$$

This value, or its reciprocal, can be inserted into eq 10 and 11, respectively, and since

$$(A') = K_A(A) \qquad (13)$$

$$(B') = K_B(B) \qquad (14)$$

where (A) and (B) are the respective concns of the drugs in the media,

$$1/\theta_B = [1 + K_AK_A'(A) + (k_B'/k_B)K_A(A) + (k_B'/k_B)K_A'(K_A(A))^2 + K_BK_B'(B)]/K_BK_B'(B) \qquad (15)$$

and

$$1/\theta_A = [1 + K_AK_A'(A) + (k_B'/k_B)K_A(A) + (k_B'/k_B)K_A'(K_A(A))^2 + K_BK_B'(B)]/[1 + (k_B'/k_B)K_A(A)]K_AK_A'(A) \qquad (16)$$

Thus, the fraction of receptor sites, $f_R$, not reacted with either drug A or B is

$$\begin{aligned} f_R &= 1 - \theta_A - \theta_B \\ &= [1 + (k_B'/k_B)K_A(A)]/[1 + K_AK_A'(A) + K_BK_B'(B) + (k_B'/k_B)K_A(A)(1 + K_AK_A'(A))] \end{aligned} \qquad (17)$$

which equation, if the dissociation of the B′R complex is not catalyzed by the drug A, *i.e.*, $k_B' = 0$, is transformed to

$$f_R = 1 - \theta_A - \theta_B = 1/(1 + K_AK_A'(A) + K_BK_B'(B)) \qquad (18)$$

Thus the ratio of the fraction of free receptor sites, $f_R$, for the case when drug A catalyzes the dissociation of receptor site complex of drug B, to the fraction of free receptor sites, $f_R'$, when no such catalysis exists and A and B compete for such sites, *i.e.*, their inhibitory action differs only by a potency factor, is

$$f_R/f_R' = \frac{1 + K_AK_A'(A) + K_BK_B'(B) + (k_B'/k_B)K_A(A) + (k_B'/k_B)K_A'(K_A(A))^2 + (k_B'/k_B)K_BK_B'(B)}{1 + K_AK_A'(A) + K_BK_B'(B) + (k_B'/k_B)K_A(A) + (k_B'/k_B)K_A'(K_A(A))^2} > 1 \qquad (19)$$

Thus, such a model as eq 6 to represent the modification of an allosteric configuration of a receptor site for one drug by the presence of another results in an antagonism of antibacterial action; since less receptor sites are occupied by drug than would be anticipated on the basis of simple equivalency of action of each drug of the combination.[29] The developments of eq 6-19 are based only on drug A catalyzing the dissociation of the (B'R) complex. Similar expressions can be developed for the simultaneous catalysis of the dissociation of the (A'R) complex by the drug B.

Since the generation rate is proportional to the metabolic rate leading to generation and the number, $N$, of generating organisms, then from eq 7

$$dN/dt = q(dM/dt)N = qk_m(1 - \theta_A - \theta_B)N = k_{app}N \qquad (20)$$

and the apparent generation rate constant, $k_{app}$, will always be greater for such allosterically modifying components of combinations (Figures 2a and 2b) than can be predicted *a priori* on the premise of drug equivalence of the components (Figure 2c and eq 19).

Steric configuration may play an important role in this type of drug-receptor reaction, since it has been observed[7] that replacement of the 7(*R*)-OH group of I by a halogen substituent in the 7(R) configuration had little or no effect on *in vitro* antibacterial activity but a halogen substitution in the 7(S) configuration increased the activity 4- to 32-fold in the order Cl < Br < I. These facts may indicate that the 7(R) and 7(S) epimers of I bind differently on the same receptor site (or enzyme). One feasible proposition could be that 2 possible binding sites exist for one lincosaminide molecule, a "catalytically active" site and an "allosterically modifying site."[26-28]

The high activity of II (or III) relative to I (phase I) suggests the possibility that I (phase I) may exert an allosteric effect which modifies the affinity of itself and the others for the catalytically active site. The binding of I (phase I) at the "allosteric site" possibly may produce an extensive conformational change that causes an "unfavorable" configuration of the "active" site of the receptor and thereby decreases the net reactivity of the "catalytically active" site for all of these drugs. Of course, the "unfavorable" configuration for I may be induced by the other drugs; or both may induce such configurations for the other. The fact that I does have a second mode of action is of interest with regard to this hypothesis in that it may be speculated that the phase II of lincomycin action may be related to its possible ability to allosterically modify the receptor site for the other lincosaminide antibiotics.

**A Possible Kinetic Model to Rationalize the Biphasic Action of Lincomycin.** I (phase II) action may be rationalized as a different mechanism from that of I (phase I) and II (or III) action. It implies an action which is interdependent with the initial I (phase I) action, but which becomes effective only after a finite time of drug-bacteria interaction (Figure 1a). The data of Table I show that the extent of generation inhibition ($k_0 - k_{app}$) is greater for phase II than for the phase I action of I at each level of concn over the range 60-350 μg/ml of I·HCl.

An explanation that has been proposed[17] for the lincomycin (phase II) action was that I may block an additional receptor site engaged in the synthesis of an essential metabolite. The metabolite or its successors is possibly utilized by the enzyme (or receptor site) that is also the binding site for I (phase I) action, in a "sequential blocking"[24] of protein synthesis to enhance generation inhibition.[23] The depletion of this necessary metabolite or its precursors during successive microbial generations imposes a new rate-limiting factor and a new and lessened steady-state generation (phase II) is established. This model may be considered as similar to the synergistic action of sulfonamide-trimethoprin combinations on the tetrahydrofolate utilization sequence[29] where the prior sulfonamide action on folic acid synthesis has a 5.8 generation lag for its effect to be observed after drug addition, whereas the subsequent dihydrofolate reductase inhibition of trimethoprin has a relatively immediate effect on microbial generation. Consider the metabolic sequence

$$S + R_1 \overset{K_{R_1}}{\rightleftharpoons} (SR_1) \xrightarrow{k_1} M_1 + R_2 \overset{K_{R_2}}{\rightleftharpoons} (R_2M_1) \xrightarrow{k_2} M_2 \qquad (21)$$

where the substrate, S, is acted upon by the receptor site, $R_1$, to produce the metabolite $M_1$ necessary for the receptor site, $R_2$, to transpose it to the metabolite, $M_2$, which is vital for microbial growth and generation. If I competes with $M_1$ for the receptor site $R_2$ in phase I action and competes with S for $R_1$ in phase II action, then

$$\begin{aligned} dM_2/dt = k_2(R_2M_1) = k_2K_{R_2}R_2M_1 = k_1k_2K_{R_2}R_2(SR_1) = \\ = k_1k_2K_{R_1}K_{R_2}SR_1R_2 \\ = k_1k_2K_{E_1}K_{E_2}S(R_1)_T(R_2)_T(1 - \theta_1)(1 - \theta_2) \\ = k_m(1 - \theta_1)(1 - \theta_2) \end{aligned} \qquad (22)$$

where

$$k_m = k_1k_2K_{R_1}K_{R_2}S(E_1)_T(E_2)_T \qquad (23)$$

since it may be postulated that the amount of substrate, S, and the total amounts of receptor sites $(R_1)_T$ and $(R_2)_T$ are constant. The $\theta_1$ and $\theta_2$ are the respective fractions of these sites that are treated with I, respectively, and thus unavailable for reaction in the metabolic sequence of eq 21 leading to the $M_2$ vital for microbial growth and generation.

These fractions of receptor sites treated with I may be defined[29] as

$$\theta_1 = K_1K_{R_1}L/(1 + K_1K_{R_1}L) \qquad (24)$$

$$\theta_2 = K_1K_{R_2}L/(1 + K_1K_{R_2}L) = (k_0 - k_{appI})(k_a/k_b) \qquad (25)$$

where $k_{appI}$ is the apparent generation rate constant in I (phase I) action defined[29] by

$$\begin{aligned} k_{appI} = qk_m(1 - \theta_2) = k_0 - k_aL(1 + k_bL) \\ = k_0 - k_0K_1K_{R_2}L/(1 + K_1K_{R_2}L) \\ = k_0/(1 + K_1K_{R_2}L) \end{aligned} \qquad (26)$$

where $L$ is the I concn and $k_0 = qk_m = k_a/k_b$.

When phase II action comes into play, then, as in eq 18,

$$dN/dt = q\ dM/dt = qk_m(1 - \theta_1)(1 - \theta_2)N = k_{appII}N \qquad (27)$$

and when $k_0 = qk_m$ and eq 26 are considered, the new generation rate constant, becomes

$$\begin{aligned} k_{appII} = k_0(1 - \theta_1 - \theta_2 + \theta_1\theta_2) = k_{appI} - k_0\theta_1(1 - \theta_2) \\ = k_0/(1 + K_1K_{R_1}L)(1 + K_1K_{R_2}L) \\ = k_{appI}/(1 + K_1K_{R_2}L) \end{aligned} \qquad (28)$$

and thus the apparent generation rate constant $k'_{app}$ for phase II lincomycin action will always be less than the apparent generation rate constant, $k_{app}$, for phase I lincomycin action.

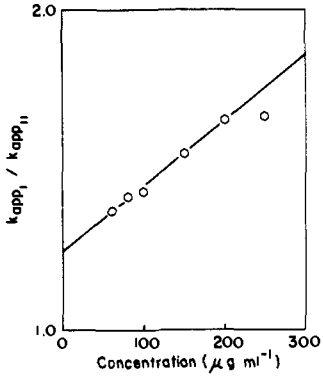



Figure 10. Linearity of the plot of the ratio $k_{app_I}/k_{app_{II}}$ *vs.* concn for lincomycin-affected *E. coli* cultures at 37.5° in accordance with the equation, $k_{app_I}/k_{app_{II}} = k_c L + b$, where $k_{app_I}$ is the generation rate constant for lincomycin-affected cultures in the phase I steady-state generation, $k_{app_{II}}$ is the generation rate constant for lincomycin-affected cultures in phase II steady-stage generation and where $k_c$ may be the product of the drug partition constant $K_1$ and the drug affinity constant $K_2$ for the lincomycin at the site of its phase II action. The parameter values are $k_i = 2.03 \times 10^{-3}$ ml/μg and $b = 1.25$.

The ratio of the apparent rate constants for phase I and phase II lincomycin action can be derived from eq 28

$$k_{app_I}/k_{app_{II}} = 1 + (K_1 K_{R_2})L \qquad (29)$$

and is shown to be reasonably valid by the plots of these ratios against I concn in Figure 10 which clearly demonstrates a slope of $K_1 K_{R_2}$. The intercept, however, is slightly greater than unity which may be attributed to the fact that $k_a/k_b$ of eq 27 and 28 may not be exactly equal to, although they are proportional to, $k_0$, the generation rate constant in the absence of drug.[29]

The precise physiological significance of these mechanisms cannot be ascertained from the available kinetic data. However, it can be conjectured that the common action of I (phase I) and II (or III) is related to the inhibition of the "peptide bond formation" reported by other workers[12-14] using cell-free extracts. In this case, I (phase II) action may be associated with the inhibition of a synthesis of some essential amino acid or derived peptide precursors not normally available in the broth medium. Alternatively, the common action of I (phase I) and II (or III) could be related to the inhibition of the "translocation" of peptidyl-tRNA in polypeptide synthesis and that of I (phase II) to the inhibition of "peptide bond formation.[12-14] Whatever the mechanisms of action, I does antagonize the action of its 7-(S) Cl analogs against *E. coli*.

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