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Microbial Kinetics and Dependencies of Individual and Combined Antibiotic Inhibitors of Protein Biosynthesis

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The generation rate constants for the steady-state growth of antibiotic-inhibited *Escherichia coli* have the same formal dependency on concentration for deoxylincomycin, lincomycin (phase I), erythromycin, clindamycin, and U24729A. They may be kinetically classified as a group A, in which the first three compounds comprise a subgroup A_1 and the latter two a subgroup A_2. Generation rate constants initially decrease linearly with concentration but asymptotically approach zero at higher concentrations. With tetracycline or chloramphenicol, the generation rate decreases linearly with all concentrations, and these compounds may be kinetically classified as group B. Combining an antibiotic from group A with one from group B gives a response equal to that obtained with equivalent amounts of each antibiotic alone, and there are no significant effects from the order of antibiotic addition. However, combinations of an A_1 with an A_2 antibiotic are antagonistic, and there are significant effects from the order of addition. The dependencies of generation rate constants in the presence of these antibiotics can be rationalized by a receptor site model that considers varying degrees of the rate of drug transfer and drug inactivation in the organism.

Although chloramphenicol and tetracycline may bind differently on the ribosomal complex (7, 22, 24, 25, 30), they have been shown (12, 27) to be "kinetically equivalent" in their growth-inhibitory action against *Escherichia coli*. Erythromycin and the lincosaminide antibiotics are also presumed to be different in the mechanistic details of their action at the ribosomal site (3, 31), yet they demonstrate "kinetic equivalence" except where allosteric effects at the binding sites may yield antagonistic reactions (13, 20, 21, 27).

This paper presents the results of studies to determine the effects of combinations chosen from tetracycline, chloramphenicol, erythromycin, and the lincosaminides on the generation rate of *E. coli* and rationalizes the microbial kinetic dependencies on the concentrations of the separate and combined antibiotics. The microbial kinetics of deoxylincomycin have been studied in detail.

MATERIALS AND METHODS

Growth medium. Antibiotic medium 3 (Difco Laboratories) was used as the growth medium. The medium was clarified by filtration through 22-μm membrane filters (HA type; Millipore Corp.) followed by 0.45-μm membrane filters, and then autoclaved. The pH was 7.05 ± 0.05.

Bacteria and growth conditions. *E. coli* ATCC 12407, referred to as strain B/r in previous publications (12–15, 17, 18, 20, 21, 26), was used in all experiments. Slants were prepared from single-colony isolates and stored in a refrigerator at 4 C.

Overnight cultures, grown aerobically at 37.5 C in a shaker, were diluted 1,000-fold into fresh medium and brought to exponential growth. Further dilutions were made to provide replicate exponential cultures of 50 ml with a cell density of about 10^8/ml at the time that antibiotics were added.

Antibiotics. Assayed samples of lincomycin hydrochloride (895 μg base eq/mg), deoxylincomycin hydrochloride (893.4 μg base eq/mg), clindamycin hydrochloride (860 μg base eq/mg), U24729 A as the hydrochloride (906.1 μg base eq/mg), erythromycin lactobionate (670 μg base eq/mg), and tetracycline hydrochloride (USP) were used. The concentrations employed in all experiments refer to the salts of these antibiotics. Stock solutions were aseptically prepared by membrane filtration and stored at 4 C.

Total-count method. Total counts of bacterial cultures were determined with a Coulter counter (14). Samples of 1 ml were withdrawn at 20- to 30-min intervals and diluted in Formol-saline to be within a range of 10,000 to 30,000 counts per 50 μliters on a

model B Coulter counter. The Formol-saline was a membrane-filtered solution of 0.85% NaCl and 1.00% formaldehyde which arrested microbial growth instantaneously without cell lysis. The instrument settings were: aperture current, 5; amplification, 8; gain, 10; lower threshold, 13; and upper threshold, maximum. Only particles of 30 μm or less were counted, and the counts were corrected for the background count of the particular batch of medium diluted similarly to the sample. The background counts did not exceed 500 counts per 50 μliters.

RESULTS

Suitability of Coulter method for determining generation rates. The coincidence of plots for the total count (Coulter method) and the viable count (poured plate method) for exponential growth of *E. coli* in antibiotic-free and treated media has been demonstrated for chloramphenicol and tetracycline (14, 15), lincomycin (26), and erythromycin (13). There is no significant killing superimposed on normal inhibition of microbial generation in the presence of concentrations of the antibiotics used. The method has the advantages that it is relatively fast, efficient, and highly reproducible to a precision of $\pm 3\%$.

Effects of antibiotic concentration on generation rates. The addition of graded concentrations of antibiotics to growing balanced cultures of *E. coli* demonstrated a linear semilogarithmic plot shortly after addition of the antibiotic, in accordance with:

$$\log N = k_{app}t/2.303 + \text{intercept} \quad (1)$$

where N is the number of *E. coli*/ml obtained by Coulter count at time t, and k_{app} is the apparent first-order generation rate constant. The intercept is the logarithm of the organism concentration, N_0, at some time taken as zero time at which the antibiotic initially manifests a steady-state action on microbial generation (21).

Such plots are shown for graded concentrations of deoxylincomycin (Fig. 1) and lincomycin (Fig. 2). Deoxylincomycin-affected cultures showed one phase of steady-state generation similar to those for chloramphenicol, tetracycline (12, 14), erythromycin (13), clindamycin, and U24729A (20). Lincomycin-affected cultures exhibited the characteristic biphasic steady-state generation curves previously reported (13, 20, 26), i.e., an initial (phase I) steady-state growth, followed by an enhanced inhibition (phase II) with a lower steady-state growth rate after three to five generations.

The apparent first-order generation rate constants (k_{app} in s^{-1}), obtained from the slopes of the semilog plots in Fig. 1 and 2, are plotted against antibiotic concentration in Fig. 3 and are linearly dependent on antibiotic concentration for the entire range 0 to 1.8 μg of chloramphenicol per ml and 0 to 0.24 μg of tetracycline per ml (Fig. 3), in accordance with the expression:

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

where k_0 is the generation rate constant for antibiotic-free culture, k_{app} is the generation rate constant for a culture treated with a concentration of C (μg/ml) of antibiotic, and k_a is the specific inhibitory rate constant (ml/μg-s) for the antibiotic. However, k_{app} is linearly dependent on antibiotic concentration in accordance with equation 2 only over a limited concentration range for the other antibiotics, viz., U24729A, 0 to 4 μg/ml: clindamycin, 0 to 16.67 μg/ml; erythromycin, 0 to 20 μg/ml; lincomycin, 0 to 100 μg/ml; and deoxylincomycin, 0 to 250 μg/ml. Above these concentration ranges, k_{app} asymptotically approaches zero. In the case of lincomycin phase II action, a nonlinear decrease of k_{app} with increasing concentration of the antibiotic is observed throughout the range studied (13, 20, 26). The relative potency of the antibiotics on a weight basis, in the low concentration region where the k_{app} dependence is linear for all antibiotics in accordance with equation 2, is approximately of the order 0.4:1:5:6:25:125:846 for deoxylincomycin-

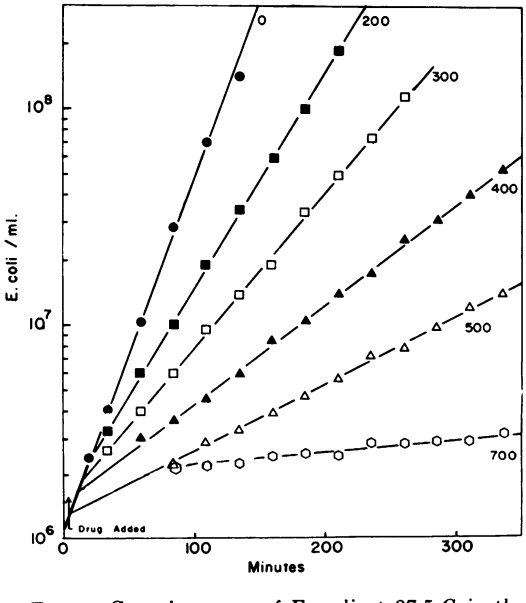


FIG. 1. *Growth curves of E. coli at 37.5 C in the absence and presence of graded concentrations of deoxylincomycin. Numbers indicate the drug concentration in micrograms per milliliter.*

lincomycin (phase I)-erythromycin-U24729A-chloramphenicol-tetracycline. Thus tetracycline, the most potent of the antibiotics, is more than 2,000 times as active as deoxylincomycin, the least potent antibiotic. When k_{app} values are plotted against corresponding antibiotic concentrations multiplied by the potency factors, coincidence of plots are obtained at the lower antibiotic concentrations (Fig. 3).

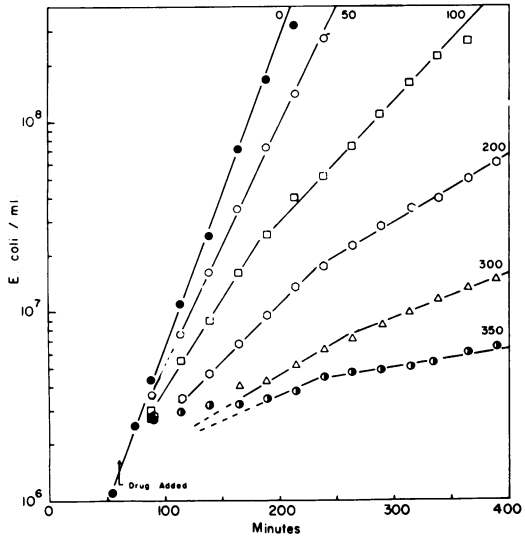


FIG. 2. *Growth curves of E. coli at 37.5 C in the absence and presence of graded concentrations of lincomycin. Numbers indicate drug concentration in micrograms per milliliter.*

The plots of $C/(k_0 - k_{app})$ versus C from the data of Fig. 3 are given in Fig. 4. These plots are in accordance with a previously derived saturable receptor site model (17, 26)

$$C/(k_0 - k_{app}) = C(k_b/k_a) + 1/k_a \quad (3)$$

where k_a and k_b are constants of proportionality related to the antibiotic partition through cell membranes and its affinity for the receptor sites in the biophase.

Adherence to the saturation equation 3 is observed from linear plots in Fig. 4 obtained from concentrations of antibiotics greater than the following: deoxylincomycin, 250 μg/ml; lincomycin, 100 μg/ml (phase I); erythromycin, 20 μg/ml; clindamycin, 16.67 μg/ml; and U24729A, 4 μg/ml. Deviations occur, in all cases, in the lower concentration ranges of these antibiotics and for the entire concentration ranges of chloramphenicol and tetracycline studied.

Effect of the order of addition of two antibiotics on microbial growth. There appeared to be three general classifications of effects of order of addition of two antibiotics.

(i) Indifferent action. Coincident generation curves are obtained for the action of 16.67 μg of clindamycin per ml in curve B, and the equipotent concentration of 0.12 μg of tetracycline per

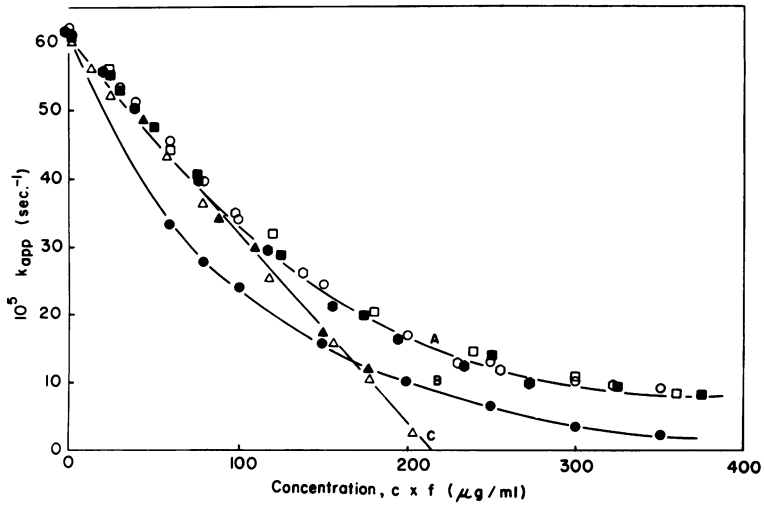


FIG. 3. *Coincidence of the dependence of apparent generation rate constants, k_{app} for E. coli on equipotent concentrations, C, in micrograms of antibiotic per milliliter. Curve A, dependence for deoxylincomycin (●); lincomycin, phase I (○); erythromycin (◇); clindamycin (□); U264729 (■); chloramphenicol (▲); and tetracycline (△), with f values of 0.4, 1, 5, 6, 25, 125, and 846, respectively. Curve B, dependence of the k_{app} of lincomycin hydrochloride, phase II (●). Curve C, linear dependency of k_{app} on concentration for deoxylincomycin, lincomycin, erythromycin, clindamycin, and U24729A in the concentration range 0 to (100 × 1/f) μg/ml and of chloramphenicol and tetracycline at $0 < C < (200 \times 1/f)$ μg/ml.*

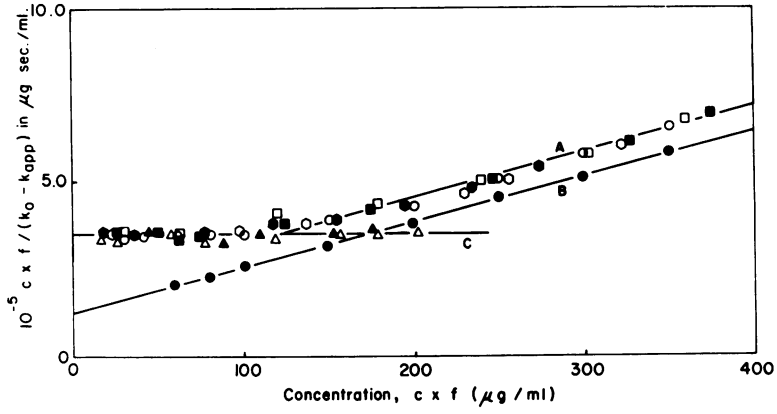


FIG. 4. *Applicability of saturation kinetics to the action of the antibiotics at higher concentrations. Curve A, represents this application for deoxylincomycin > 230 μg/ml (●), lincomycin (phase I) > 100 μg/ml (○), erythromycin > 21.5 μg/ml (◇), clindamycin > 16.67 μg/ml (□), and U24729A > 4 μg/ml (■). Curve B, represents the application to lincomycin (phase II) at 0 < C < 350 μg/ml (●). Curve C demonstrates the nonadherence for the action of chloramphenicol (▲) and tetracycline (△) at 0 < C < (200 × 1/f) μg/ml and for deoxylincomycin, lincomycin (phase I), erythromycin, clindamycin, and U24729A in the concentration range 0 to (100 × 1/f) μg/ml, where the f values are as indicated in the legend to Fig. 3.*

ml in curve C (Fig. 5), as was expected from the equipotency factors given in Fig. 3. There are also no significant differences in the effective generation inhibition produced by a mixture of clindamycin at 16.67 μg/ml and tetracycline at 0.12 μg/ml (curve D) and the equipotent concentration of either drug alone, i.e., clindamycin at 33.34 μg/ml (curve E) or tetracycline at 0.16 μg/ml (the latter gave a curve of the same slope as curves D and E). There are no significant differences in the effective generation inhibition produced by an equipotent amount of tetracycline added after 50 min to the clindamycin-affected culture of curve B, or that which is produced by an equipotent amount of clindamycin added after 50 min to the tetracycline-affected culture of curve C. The semilogarithmic plots of these studies have the same slopes as curves D and E. Thus the order of addition of the two antibiotics produces no significant change on the ultimate generation inhibition. This pattern of response has been observed for equipotent combinations of clindamycin and U24729A (20) and was demonstrated in these present studies for equipotent combinations of tetracycline (0.11 μg/ml)-chloramphenicol (0.9 μg/ml), tetracycline (0.1 μg/ml)-erythromycin (21.5 μg/ml), tetracycline (0.1 μg/ml)-deoxylincomycin (230 μg/ml), chloramphenicol (0.8 μg/ml)-erythromycin (21.5 μg/ml), chloramphenicol (0.8 μg/ml)-deoxylincomycin (230 μg/ml), erythromycin (21.5 μg/ml)-deoxylincomycin (230 μg/ml), and lincomycin (100 μg/ml)-deoxylincomycin (230 μg/ml).

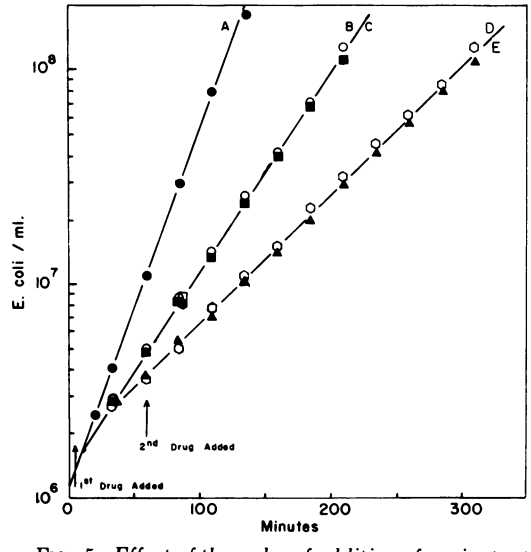


FIG. 5. *Effect of the order of addition of equipotent clindamycin and tetracycline on the growth of E. coli. A, Culture growth in the absence of drugs; B, a culture in the presence of 16.67 μg of clindamycin per ml as antibiotic I; coincident curve C, a culture in the presence of 0.12 μg of tetracycline per ml as antibiotic II; D, a mixture of equipotent concentrations of clindamycin (16.67 μg/ml) and tetracycline (0.12 μg/ml); E, 33.34 μg of clindamycin per ml.*

(ii) Apparent antagonism of action as a consequence of two phases of antibiotic action. There are no significant differences between the growth inhibition produced by 230 μg of deoxylincomycin per ml (curve B) and the equipotent concentration of 100 μg of lincomycin per

ml (phase I; curve C, Fig. 6), since the initial portion of curve C is coincident with curve B. Also, there are no significant differences in the effective growth inhibition produced by a mixture of 230 μg of deoxylincomycin per ml with 100 μg of lincomycin per ml, in phase I action (curve F), and the equipotent concentration of either antibiotic alone, i.e., 460 μg of deoxylincomycin per ml (curve G) or 200 μg of lincomycin per ml in phase I action (curve H). The initial portions of curves F and H are coincident with curve G. There are no significant differences in growth inhibition (curve D) produced by an equipotent amount of deoxylincomycin added after 50 min to the lincomycin-affected culture of curve C, or that which is produced (curve E) by an equipotent amount of lincomycin (phase I) added after 50 min to the deoxylincomycin-affected culture of curve B. The initial portions of curves D and E are coincident and parallel to those of curves F, G, and H. This indicates that the order of addition of the antibiotics produces no significant change on the ultimate generation inhibition in phase I action of the mixtures.

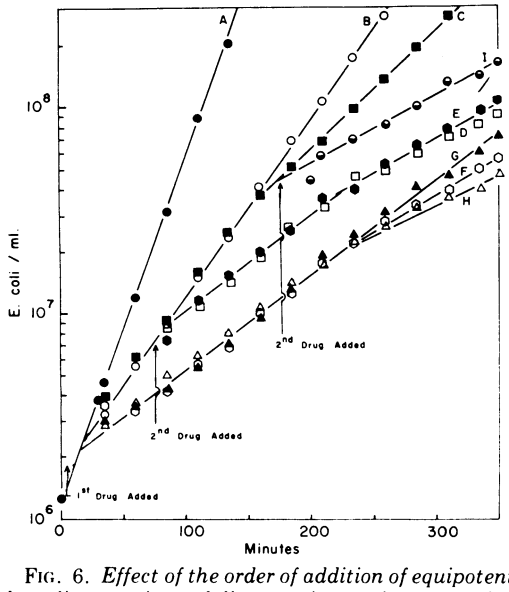


FIG. 6. *Effect of the order of addition of equipotent deoxylincomycin and lincomycin on the generation rate of E. coli. A, Culture in the absence of drug; B, a culture in the presence of 230 μg of deoxylincomycin per ml; C, a culture with 100 μg of lincomycin per ml; D, a culture with equipotent deoxylincomycin (230 μg/ml) added to the lincomycin-affected culture of curve C in phase I generation at 80 min; coincident curve E, equipotent lincomycin (100 μg/ml) added to the deoxylincomycin-affected culture of curve B at 80 min; F, a mixture of equipotent concentrations of deoxylincomycin (230 μg/ml) and lincomycin (100 μg/ml) added to the culture of curve A; G, 460 μg of deoxylincomycin per ml added to the culture of curve A; H, equipotent 200 μg of lincomycin per ml added; I, equipotent deoxylincomycin (230 μg/ml) added to the lincomycin-affected culture of curve C in phase II generation.*

There are, however, significant differences in the growth inhibition during phase II action of the mixtures. An equipotent amount of deoxylincomycin added at 150 min to the lincomycin-affected cultures of curve C gives the same inhibition (curve I) as those produced by the phase II action of equipotent mixtures as in curves D, E, and F. Curves D, E, F, and I have the same slope in the phase II steady-state generation. However, combinations with lincomycin show that the phase II response characteristic of this antibiotic is dominant in such mixtures, and give an apparent antagonism of effects due to dilution of the phase II lincomycin action by the presence of deoxylincomycin, which does not possess such action. The same pattern has been observed for equipotent combinations of lincomycin and erythromycin (21) and, in the present studies, for lincomycin (100 μg/ml) with chloramphenicol (0.8 μg/ml) and lincomycin (100 μg/ml) with tetracycline (0.1 μg/ml).

(iii) True antagonism of action. There are no significant differences between the growth inhibition produced by deoxylincomycin at 230 μg/ml (curve B) and clindamycin at 16.67 μg/ml (curve C), since curves B and C are coincident (Fig. 7). However, significant differences in the effective growth inhibition are produced by the action of a mixture of deoxylincomycin at 230 μg/ml and clindamycin at 16.67 μg/ml (curve F), and that of the equipotent concentration of either antibiotic alone, i.e., deoxylincomycin at 460 μg/ml (curve G) and clindamycin at 33.34 μg/ml (curve H). The slope of curve F is greater than the coincident curves G and H, and demonstrates antagonism between deoxylincomycin and clindamycin. Also, an equipotent amount of clindamycin, added after 50 min to deoxylincomycin-affected cultures (curve B), produces an ultimate steady-state generation (curve E) whose slope is greater than that produced (curve D) when an equipotent amount of deoxylincomycin is added after 50 min to the clindamycin-affected culture of curve C. Thus the order of addition of the antibiotics produces significant effects on the ultimate growth inhibition and indicates antagonism of effects between clindamycin and deoxylincomycin. This pattern of response has been observed previously for combinations of clindamycin and lincomycin (phase I), and U24729A and lincomycin (phase I) (20), and has been demon-

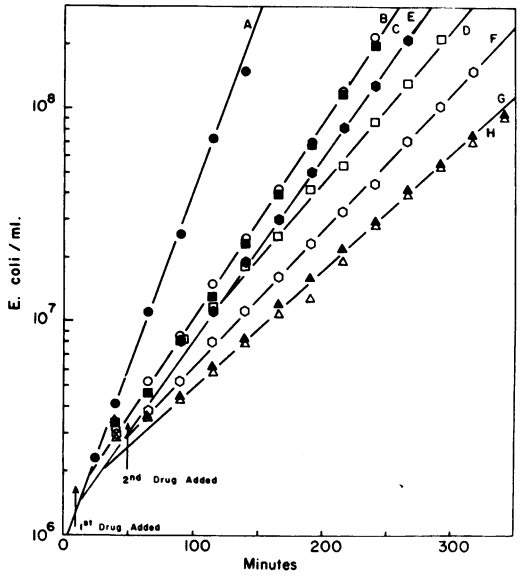


FIG. 7. *Effect of the order of addition of equipotent deoxylincomycin and clindamycin on the generation rate of E. coli. A, Culture in the absence of drug; B, culture with 230 μg of deoxylincomycin per ml; C, culture with 16.67 μg of clindamycin per ml; D, culture with equipotent deoxylincomycin (230 μg/ml) added to the culture of curve C; E, equipotent clindamycin (16.67 μg/ml) added to the culture of curve B; F, culture with a mixture of equipotent concentrations of deoxylincomycin (230 μg/ml) and clindamycin (16.67 μg/ml); G, a culture with 460 μg of deoxylincomycin per ml; and coincident curve H, equipotent clindamycin (33.34 μg/ml) present.*

strated in these studies for U24729A-erythromycin and clindamycin-erythromycin combinations.

Action of equipotent mixtures composed of different fractions of the antibiotics. The generation rate constants of cultures affected by mixtures of erythromycin and chloramphenicol are plotted in Fig. 8. The mixtures contained from 0 to 100% erythromycin with the residual percentage of equipotent chloramphenicol. The mixtures were prepared so as to be equipotent in their combined action in accordance with the functional dependencies of k_{app} on antibiotic concentrations (Fig. 3). The null slopes of the plots of k_{app} for all the mixtures at three different levels of action demonstrate the lack of any significant antagonism or synergism (10). These results were obtained also for combinations of chloramphenicol-lincomycin (phase I), chloramphenicol-clindamycin, tetracycline-lincomycin (phase I), and tetracycline-clindamycin. Similar indifference effects were reported for the action of combinations of chloramphenicol and tetracycline (12). However, unequivocal antagonistic effects (20) were demonstrated for combinations of lincomycin (phase I) and clindamycin or U24729A.

Effects of graded concentrations of chloramphenicol on the inhibitory action of lincomycin and erythromycin. Coincident generation curves were obtained for the action of 21.5 μg of erythromycin per ml or 0.8 μg of chloramphenicol per ml (curve B) on *E. coli* cultures (Fig. 9). However, significant differences in the effective inhibition were produced by twice the respective equipotent antibiotic concentrations, i.e., erythromycin at 43 μg/ml (curve C) and chloramphenicol at 1.6 μg/ml (curve F).

The addition of 0.6 μg of chloramphenicol or 34 μg of erythromycin per ml to the erythromycin-affected cultures of curve B, at 50 min after initial addition of erythromycin, results in coincident generation curves (curve D) which are parallel to that of the culture of curve F (1.6 μg of chloramphenicol per ml). Similarly, the addition of 0.2 μg of chloramphenicol per ml to the erythromycin-affected cultures of curve C at 50 min after initial addition or erythromycin results in an ultimate generation curve E which is parallel to that of the culture of curve F. Therefore, there are no significant differences in the effective generation inhibition produced by the action of a combination of 21.5 μg of erythromycin and 0.6 μg of chloramphenicol per ml, and a combination of 43 μg of erythromycin and 0.2 μg of chloramphenicol per ml, which should be equipotent to 55.5 μg of erythromycin lactobionate or 1.4 μg of chloramphenicol per ml alone.

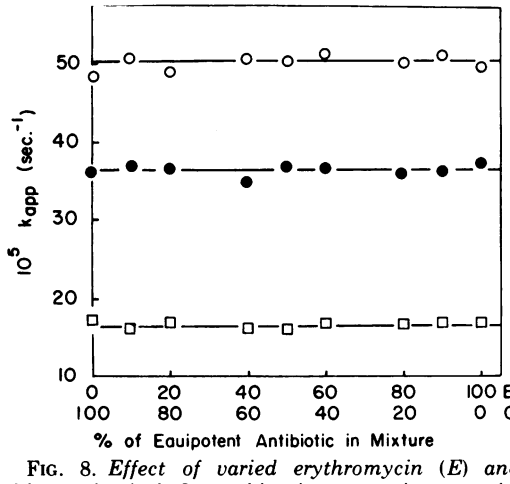


FIG. 8. *Effect of varied erythromycin (E) and chloramphenicol (C) combinations as equipotent mixtures at three levels of activity on the apparent generation rate constants of E. coli in terms of volume percentage of the solutions of equipotent concentration.*

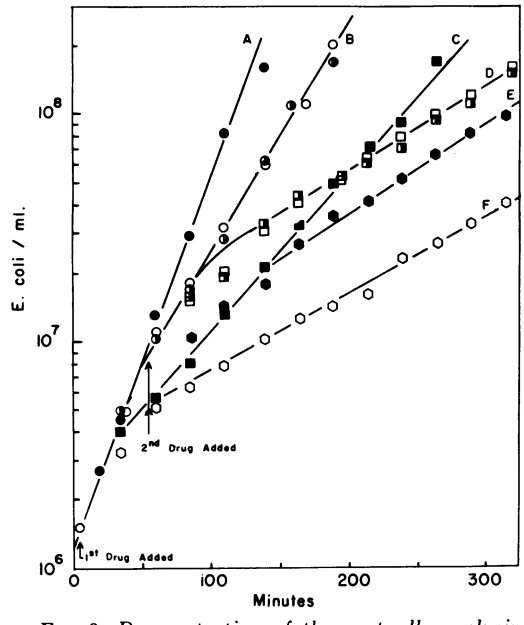


FIG. 9. *Demonstration of the mutually exclusive action of erythromycin and chloramphenicol on the generation rate of E. coli. A, Culture in the absence of drugs; B, coincident curves obtained with either 0.8 μg of chloramphenicol or 21.5 μg of erythromycin per ml present; C, 43 μg of erythromycin per ml present; D, coincident generation curve obtained with either 0.6 μg of chloramphenicol or 34 μg of erythromycin per ml added to the erythromycin-affected culture of curve B; E, 0.2 μg of chloramphenicol per ml added to the erythromycin-affected culture of curve C; F, 1.6 μg of chloramphenicol per ml present.*

Similarly, the coincidence of curve B and the initial portion of curve C (Fig. 10) shows equipotency for the action of chloramphenicol at 0.8 μg/ml and lincomycin at 100 μg/ml (phase I), respectively. There are, however, significant differences in the effective inhibition produced by the action of double the respective antibiotic concentrations, i.e., chloramphenicol at 1.6 μg/ml and lincomycin at 200 μg/ml in phase I action. This is clearly a consequence of the difference in the concentration dependencies of the k_{app} values for the two antibiotics (Fig. 3).

The addition of 0.6 μg of chloramphenicol per ml (curve E) or 170 μg of lincomycin per ml (curve F) to the lincomycin-affected culture of curve C in phase I, at 50 min after initial addition of the lincomycin, resulted in ultimate phase I generation curves which were parallel to that of the culture affected by the predicted equipotent 1.6 μg of chloramphenicol per ml. Later addition of chloramphenicol (0.2 μg/ml) to a lincomycin (200 μg/ml)-affected culture resulted in an ultimate phase I generation curve G parallel to that of the culture affected by the predicted equipotent 1.6 μg/ml of chloramphenicol. Thus, there seems to be no significant difference in the effective inhibition produced by the phase I action of combinations of lincomycin at 100 μg/ml and chloramphenicol at 0.6 μg/ml, or lincomycin hydrochloride at 200 μg/ml and chloramphenicol at 0.2 μg/ml, which are equipotent to lincomycin (270 μg/ml) or chloramphenicol (1.6 μg/ml) alone.

Addition of 170 μg of lincomycin per ml (curve D) to lincomycin-affected culture of curve C in phase II, at 150 min after initial addition of lincomycin, results in an ultimate phase II generation (curve D) which is parallel to that of curve F. Thus, effective inhibition is the same in both cases. However, the slopes of curves D and F are not as great as those in

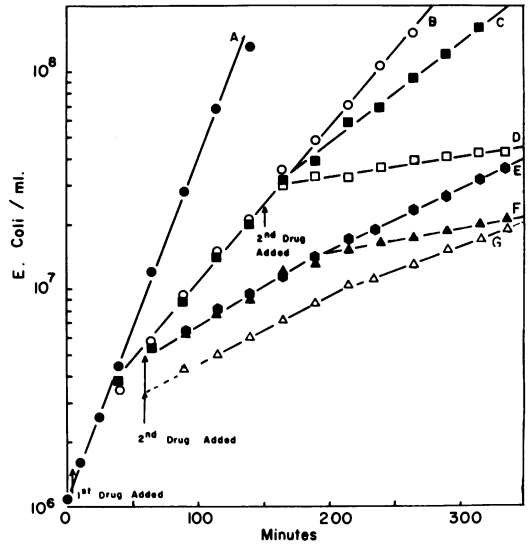


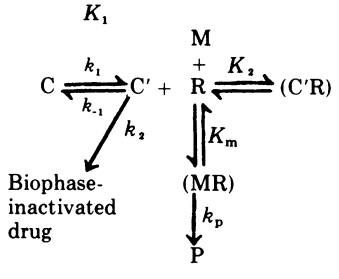
FIG. 10. *Demonstration of the mutually exclusive action of lincomycin and chloramphenicol on the generation rate of E. coli. A, culture in the absence of antibiotics; B, culture in the presence of 0.8 μg of chloramphenicol per ml; C, culture in the presence of 100 μg of lincomycin per ml; D, culture with 170 μg of lincomycin per ml added to the lincomycin-affected culture of curve C in phase II generation; E, 0.6 μg of chloramphenicol per ml added to the lincomycin-affected culture of curve C in phase I generation; F, 170 μg of lincomycin per ml added later to the lincomycin-affected culture of curve C in phase I generation; G, 0.2 μg of chloramphenicol per ml added later to a culture affected by 200 μg of lincomycin per ml in phase I generation. When 1.6 μg of chloramphenicol or 200 μg of lincomycin per ml was added alone at zero time, the generation curves were parallel to curves E, F, and G, at least for the phase I of lincomycin action.*

curves E and G, and the phase II response characteristic of lincomycin is dominant in such mixtures, although diluted by chloramphenicol.

DISCUSSION

Dependencies of the generation rate constant (k_{app}) of *E. coli* cultures on drug concentrations for deoxylincomycin, lincomycin (phase I), erythromycin, clindamycin, and U 26729A (Fig. 3) are the same and permit their grouping into one kinetic classification, group A. These similar kinetic dependencies suggest that these group A antibiotics have similar mechanisms of action.

The plots (Fig. 3) are coincident when the drug concentrations are multiplied by respective potency factors, *f*. The shape of the common curve, although initially linear, indicates that the increase in antimicrobial action is diminished with higher doses. Such a phenomenon can be assigned to a saturable process such as the binding of the drug to a limited number of receptor sites, where the generation rate of the drug-affected culture is proportional to, or dependent on, the number of free or unbound receptor sites. A kinetic model similar to that which defined the action of sulfonamides (17), lincomycin (26), and fluorouracil (16) against *E. coli* may be operative:



(4)

where C is the drug concentration in the broth medium equilibrated with the drug concentration C′ in the biophase. The drug in the biophase may bind reversibly to the receptor sites R to form a drug-receptor complex (C′R). K_1 may be considered as the drug-partition constant through cell membranes, dependent upon rate-constant ratio k_1/k_{-1}, and K_2 the drug affinity constant for the receptor sites. The metabolite or substrate concentration in the biophase M may be utilized by the free or unbound receptor sites to form an intermediate (MR) in the synthesis of an essential product P, which is necessary for microbial generation. K_m is the metabolite affinity constant and k_p is the rate constant for product formation. The drug therefore competes with the metabolite or substrate for the receptor site. This model differs from those previously proposed (11, 16, 17) in that a possible first-order-dependent inactivation of rate constant k_2 of the drug in the biophase is considered.

On the assumption that the rate of microbial generation, dN/dt, is proportional to the rate of product production, dP/dt, and thus to the fraction of free or unbound receptor sites, the following expression may be derived (11, 15, 17, 26) from the model in equation 4,

$$k_{app} = k_0 - k_aC/(1 + k_bC) = qk_m - \frac{qk_mK_2Ck_1/(k_{-1} + k_2)}{1 + K_2C_1k_1/(k_{-1} + k_2)} \quad (5)$$

where k_{app} is the generation rate constant of a culture affected with drug concentration C, $k_b = K_2k_1/(k_{-1} + k_2)$, and $k_a = qk_mK_2k_1/(k_{-1} + k_2)$, where q is a constant of proportionality and $k_m = k_pK_m$. The necessary prerequisite for the validity of a steady-state generation of rate constant k_{app} for the model of equation 4 is that ln N against time is linear and of constant slope k_{app} at some time after drug addition. This relationship of equation 5 is necessary to describe the non-linearity of k_{app} values plotted against drug concentration for sulfonamides (17) and fluorouracil (16) action throughout the entire range of their concentrations and for the higher concentrations of the group A antibiotics. The factor f (Fig. 3) for this group is $k'_a/k_a = k'_b/k_b$ when k_a and k_b are the microbial kinetic constants for the reference group A antibiotic lincomycin, and the primed values are for another in the group.

The linear dependency of k_{app} on drug concentrations for group A antibiotics in the low concentration range, 0 to 100 × f in μg/ml, and for all concentrations of tetracycline, chloramphenicol, and spectinomycin (11) which can be kinetically classified under group B, may be described by equation 2. This expression results when, in equation 5, $k_bC \ll 1$ or k_2 is large. This can be a consequence of a high rate of inactivation or metabolism of the antimicrobial agent in the biophase. It must be postulated that the biophase concentration C′ must be relatively constant in accordance with:

$$dC'/dt = k_1C - (k_{-1} + k_2)C' = 0; \quad C' = k_1C/(k_{-1} + k_2) \quad (6)$$

so that the pseudo-steady state of drug action be manifested by a constant rate of drug-affected microbial generation characterized by the k_{app} constant.

Equation 4 can also rationalize the two sequential steady-state phases of lincomycin-

affected microbial generation (Fig. 2). If the process of biophase inactivation of drug is terminable as by consumption of the inactivating agent, k_2 in equations 4 and 5 is negligible in the phase II of lincomycin action, and the dependency of k_{app} on concentration conforms to the saturable model characterized by equation 5 for the entire range of lincomycin concentration (Fig. 3 and 4).

The plots of k_{app} versus concentration, when multiplied by a potency factor f, are coincident in the linear ranges for group A and group B antibiotics (Fig. 3). The factor f (Fig. 3) is k'_a/k_a where k_a is the microbial kinetic constant for the reference antibiotic and k'_a for another. The k_a which we obtained from the initial linear segment of the plot for lincomycin (phase I; Fig. 3) was taken as the reference.

Strict adherence to an arithmetical transformation of equation 5 (equation 3) was observed (Fig. 4) for the action of group A antibiotics at concentrations greater than 100 × f and for lincomycin at all concentrations in phase II. An alternate explanation for the validity of equation 2 to conform with the model of equation 4, if the magnitude of biophase inactivation k_2 does not permit the simplification of equation 5, demands that the numbers of receptor sites available and necessary for microbial generation are in such excess that the amount of drug-receptor complex (C'R) is strictly proportional to drug concentration in the range 0 to 100 × f for group A antibiotics and over the entire concentration range for group B antibiotics.

Equipotent mixtures of any pair of the following antibiotics—deoxylincomycin, lincomycin (phase I) and erythromycin, which form subgroup A_1—demonstrate indifference or equivalence (10, 11) of action (Fig. 8), and their effects can be predicted from the separate dose-response curves (Fig. 3) of either drug alone. These activities are independent of the order of addition (Fig. 6; references 11, 13, 21). This suggests a similar mode and locus of action (10) for all in this subgroup.

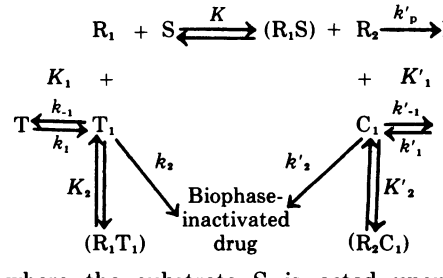
Similarly, equipotent mixtures of the 7(S)-chloro analogues of lincomycin, i.e., clindamycin and U24729A, which form a subgroup A_2, have been shown to demonstrate equivalence of action (20, 21). This suggests a similar mechanism and the same locus of action for all in this subgroup.

Equipotent mixtures of any two antibiotics selected from groups A_1 and A_2, respectively, demonstrate unequivocal antagonism and dependencies of action on the order of addition (Fig. 7). Since the functional dependency of k_{app} on drug concentrations for group A_1 and A_2 antibiotics are the same (Fig. 3), it was rationalized (20) that members of the two subgroups may bind on different but functionally linked loci at the same receptor site, with the one quasipermanently allosterically modifying the receptor site for the reaction of the other.

Equipotent mixtures of the group B antibiotics chloramphenicol and tetracycline are equivalent in their action (12, 18). Our results show that equipotent mixtures consisting of a group A and a group B antibiotic also demonstrate equivalence of action (Fig. 8) and are independent of the order of addition (Fig. 9 and 10) even in the concentration ranges where k_{app} dependencies on concentrations are not the same. When lincomycin is present in the combinations, an "apparent antagonism" of effects is manifested in the phase II action (Fig. 6), but this is merely a trivial consequence of assuming equipotencies based on lincomycin phase I action (21).

It has been argued that, if two drugs operate on separate sites engaged in "sequential" or "convergent" metabolic processes which lead to a common end product (15, 20), it would lead to "synergism" (20, 23) rather than to an observed "equivalence" or "indifference" (10, 15). This has been clearly shown in the effect on the kinetics of microbial generation for combinations of trimethoprim and sulfonamides (6, 15, 28).

The proposed mechanisms of action of tetracycline and chloramphenicol, tetracycline and a lincosaminide, tetracycline and a macrolide, chloramphenicol and a macrolide imply that each antibiotic of these given pairs operates on a different step in the biosynthetic protein pathway (1, 3, 4, 5, 7-9, 22, 24, 29, 30). A typical model would be



(7)

where the substrate S is acted upon by a receptor site R_1 to produce a complex (R_1S) catalyzed by a receptor site R_2 that can be transformed into a product P. Biophasic processes for drug inactivations characterized by the rate constants k_2 and k'_2 may exist. The microbial generation rate may be formulated as

being proportional to the rate of product production;

$$dN/dt = q(dP/dt)N \quad (8)$$

The particular case of tetracycline and chloramphenicol combinations can be considered as an example. R_1 can be assumed to be synonymous to the donor site no. 1 and S the aminoacyl-transfer ribonucleic acid (tRNA) in the mechanisms proposed (2, 4, 8, 19, 30) for protein biosynthesis. The receptor site R_2 can be postulated to be the peptidyl transferase necessary for peptide bond formation to form an elongated peptidyl-tRNA, P, at site no. 1, which translocates to site no. 2, and T_1 and C_1 can represent tetracycline and chloramphenicol concentrations in the biophase, respectively.

The rate of product formation may be constructed as being proportional to the product of the fractions θ_T and θ_C of the total receptor sites $(R_1)_t$ and $(R_2)_t$ that are unreacted with the drugs of concentrations T_1 and C_1 in the biophase, respectively. Thus:

$$\begin{aligned} dP/dt &= k'_p(R_1S)R_2 = k'_pKR_1SR_2 \\ &= k'_pK(R_1)_tS(R_2)_t(1-\theta_T)(1-\theta_C) \quad (9) \\ &= k_m(1-\theta_T)(1-\theta_C) \\ &= k_m(1-\theta_T-\theta_C+\theta_T\theta_C) \end{aligned}$$

where $k_m = k'_pK(R_1)_tS(R_2)_t$, 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. Substitution of equation 9 into equation 8 leads to:

$$\begin{aligned} dN/dt &= qk_m(1-\theta_T)(1-\theta_C)N \\ &= qk_m(1-\theta_T-\theta_C+\theta_T\theta_C)N = k_{app}N \end{aligned} \quad (10)$$

For separate drug action on sequentially reacted receptor sites, equation 10 has been shown (11) to lead to an apparent generation rate constant k_{app}, for an equipotent combination of drugs C and T where both adhere separately to a saturated receptor site model, which is less than the apparent generation rate constant for such a combination where each drug competes for the same site. This would constitute synergism (10, 11).

Similar to the derivations of equation 5 based on the model of equation 4, an equation for k_{app} can be derived from the model of equation 7;

$$k_{app} = k_0 - k'_aC/(1+k'_bC) - k_aT_0(1+k_bT) + k_ak'_aCT/qk_m(1+k'_bC)(1+k_bT) \quad (11)$$

where the constants are defined implicitly in equations 4 and 5, $k_0 = qk_m$ and $k_m = k_pK_m$ where k_p and K_m are defined implicitly in equation 4.

However, if either or both rate constants for biophasic inactivation are relatively large (or if the fractions of available receptor sites occupied by one or both drugs are relatively small and yet result in large inhibition), it follows that the product $\theta_T\theta_C$ in equation 10 is also small, and equation 11 reduces to

$$\begin{aligned} k_{app} &= qk_m(1-\theta_T-\theta_C) \\ &= k_0 - k'_aC - k_aT/(1+k_bT) \end{aligned} \quad (12)$$

or, $$k_{app} = k_0 - k'_aC/(1-k'_bC) - k_aT \quad (13)$$

or $$k_{app} = k_0 - k'_aC - k_aT \quad (14)$$

Since equation 2 holds for both chloramphenicol and tetracycline, the nonsynergistic equation 14 which implies indifference for various equipotent mixtures of these antibiotics (11) is the predicted expression for the k_{app} in the presence of both compounds. Thus, synergism was not anticipated, nor did it occur, for such compounds with the dependencies of equation 2, even when they acted on different steps in a metabolic sequence such as that given in equation 7.

Similar response-equivalent or indifferent action would be predicted for equipotent combinations of tetracycline or chloramphenicol with the erythromycin or the lincosaminide antibiotics by equations 12, 13, and 14 when either equation 2 or 5 holds for this latter group if the latter antibiotics act at the same step or at a step subsequent to the action of the former. The data of this paper confirm this prediction.

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