Kinetics and Mechanisms of Drug Action on Microorganisms XX: Integrated Receptor Site Model Rationalizing Observed Microbial Rate Dependencies on Drug Concentration and Microbial Kinetics Affected by Sodium Fusidate

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Abstract □ The steady-state generation of *Escherichia coli* (ATCC 12407) was inhibited by the antibiotic sodium fusidate in the drug concentration range of 0–100 μg/ml. There was no significant change in drug activity when the initial inoculum size was varied between 5×10^5 and 2×10^6 *E. coli*/ml. The drug activity was unaffected by varying the concentrations of ammonium ions, magnesium ions, amino acids, dextrose, or citrate components of the nutrient broth in Anton's medium over a fourfold concentration range and did not implicate binding to the components. The variation of drug activity with pH could be assigned completely to the action of the unionized species. Sodium fusidate was bacteriostatic up to 500 μg/ml, about five times the minimum inhibitory concentration, and the action of the drug was readily reversible on dilution. Resistant mutants of *E. coli* appeared after exposure of the organisms to sodium fusidate, and the numbers of such resistant organisms appeared to be a function of the inoculum size at the time of drug addition. No significant inactivation of the drug by resistant organisms was observed. The dependence of the apparent generation constant, k_{app}, on drug concentration in the medium is an S-shaped curve, which can be rationalized on the premise of a saturable enzymatic degradation of drug in the biophase of the microorganism. The same model, which considers the steady-state microbial generation as a consequence of rates of drug permeation into the biophase of the organism, the saturable biophasic degradation of the drug, and the reaction of drug with saturable receptor sites that leads to generation inhibition, can serve as a proper rationalization of all previously observed various generation rate dependencies on drug concentrations for different drugs. The action of sodium fusidate in combination with tetracycline, clindamycin, and 1′-demethyl-4′-depropyl-4′-[*(R)* and *(S)*]-*n*-pentylclindamycin is indifferent; with erythromycin and oleandomycin, it is mildly synergistic; and with novobiocin, it is antagonistic.

Keyphrases □ Sodium fusidate—quantitative kinetic effects on microbial generation of *E. coli*, broth constituents, broth pH, and inoculum size considerations □ *Escherichia coli* generation—quantitative kinetic inhibition by sodium fusidate, broth constituents, broth pH, and inoculum size considerations, theory of bacteriostatic inhibition developed □ Antibiotic activity—kinetics and mechanisms of sodium fusidate inhibition of *E. coli* generation □ Microorganisms, inhibition of *E. coli*—sodium fusidate, kinetics and mechanism

Sodium fusidate is a clinically effective antibiotic (1–5) that inhibits protein synthesis (6–9) and binds extensively to plasma proteins (1).

"Synergism" was reported for sodium fusidate (1) in combinations with erythromycin and in combination with penicillin against penicillinase-producing strains of *Staphylococcus aureus*.

This paper reports on the quantitative kinetic effects of sodium fusidate on the microbial generation of *Escherichia coli* (ATCC 12407). The effects of broth constituents, broth pH, and inoculum size on the drug activity were measured. Combinations of sodium fusidate and several other bacteriostatic drugs were studied by the method of equipotent mixtures (10) to gain insight into the possible mechanism of action of such combinations on bacterial growth rates.

Previously (10), the steady-state microbial generation due to the inhibition of protein synthesis by such bacteriostatic drugs was rationalized by the postulation of equilibration of drug between the medium and the biophase wherein the drug competes for receptor sites and the degree of inhibition was presumed to be related to the fraction of occupied sites. However, the steady-state generation may not be a consequence of true equilibration but of conditions where the rate of enzymatic drug inactivation in the biophase comes into balance with the rate of drug permeation into the biophase. Such a phenomenon, when saturable, may explain the observed unusual S-shaped dependency of the apparent generation rate constant, k_{app}, on drug concentration for sodium fusidate in the medium. Equations are developed to manifest this possible mechanism and to permit the analysis of the data for the pharmacokinetic constants of such processes.

EXPERIMENTAL

Test Organism—Replicate slants of *E. coli* (ATCC 12407), previously referred to as strain B/r (11), were used in all experiments. This nonclumping strain is particularly well suited for colony counts and for particle counts[1]. The slants were prepared from a single colony and were stored in the refrigerator at 4°.

Culture Medium—Only Anton's medium (12) was used. It has a pH of 6.9 and consists of 7.00 g K_2HPO_4, 3.00 g KH_2PO_4, 2.00 g dextrose, 1.00 g $(NH_4)_2SO_4$, 0.5 g sodium citrate, 0.10 g $MgSO_4 \cdot 7H_2O$, and 10.00 g casamino acid[2] plus distilled water to make 1 liter of solution. Since there is no protein in the broth, kinetic effects were studied without the added variable of protein binding. The broth was filtered through a 0.45-μm filter[3], autoclaved at 120° for 12 min, and then stored in an incubator overnight at 37.5° before using to ensure the absence of contaminating microorganisms. The pH of the broth was varied by changing the ratio of K_2HPO_4 to KH_2PO_4 while maintaining a total weight of 10 g/liter of phosphates. The effects of magnesium ions (Mg^{+2}), ammonium ions (NH_4^+), citrate, dextrose, and casamino acid concentrations were studied by first doubling and then halving these constituents. Dextrose and casamino acids were also studied at one-fourth the normal concentration in Anton's medium.

Antibiotics—The antibiotics used in combination experiments were all assayed bacteriostatic drugs and included sodium fusid-

[1] Coulter counter monitoring.
[2] Difco Laboratories, Detroit, Mich.
[3] HA Millipore.

ate[4], pure; erythromycin lactobionate[5], 670 μg/mg; chloramphenicol[6], purity grade; tetracycline hydrochloride[7] USP; novobiocin[7], 951 μg/mg; 1′-demethyl-4′-depropyl-4′-[(*R*) and (*S*)]-*n*-pentylclindamycin hydrochloride[7], pure; clindamycin hydrochloride[7], 860 μg/mg; lincomycin hydrochloride[7], 895 μg/mg; and oleandomycin phosphate[8], 775 μg/mg. The purities are given in terms of base equivalents.

Bacterial Generation and Monitoring—Methods used in this laboratory for bacterial culture, total count monitoring, viable counting, and size frequency distribution studies were previously described (13–15). The average background counts[9] have since been reduced to about 200/50 μl sample. The coincidence count corrections were made with a program on a calculator[10] in accordance with the coincidence correction chart supplied by the manufacturer[11].

Reversibility of Drug Action—Reversibility of drug action can be shown by taking aliquots of organism cultures containing about 10^7 *E. coli*/ml during the drug-affected steady-state growth at 37.5° and diluting into fresh broth with no added drug and also into fresh broth containing predetermined drug concentrations (14). Reversibility of drug action is established if the diluted cultures grow at the control rate in the fresh broth and at the predicted rates when drug is present in the diluent. Reversibility was studied at 0, 40, and 80 μg/ml of sodium fusidate at pH 6.9 and also at 0, 10, and 20 μg/ml of sodium fusidate at pH 6.4.

Effect of Equipotent Mixtures of Sodium Fusidate and Other Growth Inhibitors—Replicate 49.5-ml volumes of cultures containing 5×10^5 *E. coli*/ml, generating in the logarithmic phase at pH 6.9 and 37.5°, were treated with aliquots (0.5 ml) of equipotent mixtures of antibiotics (10, 14). The mixtures consisted of 100, 80, 60, 40, 20, and 0% of the equipotent sodium fusidate and the residual percentage of the other antibiotic to maintain equipotency at two different dosage levels. If the drugs precipitated on mixing, the mixture was diluted with Anton's medium until the precipitate dissolved; the appropriate volume was withdrawn from each culture flask before the addition of the drug solutions.

Prolonged Exposure of *E. coli* to Sodium Fusidate—*E. coli* was grown for 24 hr at 37.5° in Anton's medium with 100 μg/ml of sodium fusidate added. An aliquot of the culture was then diluted 1:100 into a second volume of broth with 100 μg/ml of sodium fusidate added. Following an additional 24 hr of culture, the organisms were grown as usual in the presence of graded sodium fusidate concentrations. To determine whether the sodium fusidate would be degraded significantly in the medium, the culture with 50 μg/ml of sodium fusidate was filtered after 200 min of growth on a 0.45-μm filter[3]. The bacteria were resuspended into broth and cultured, and the filtrate was seeded with *E. coli* that had not been exposed to sodium fusidate.

RESULTS

Effect of Drug Concentration on Generation Rates—The generation rates of *E. coli* in graded sodium fusidate concentrations, measured by the total count and the viable count methods in Anton's medium at pH 6.9, were coincident (Fig. 1); no bactericidal activity was observed up to 500 μg/ml of sodium fusidate, about five times the minimum inhibitory concentration (MIC). Thus, the use of total counts to determine the generation rates of organisms affected by subinhibitory concentrations of sodium fusidate is justified. Significant time elapsed before a logarithmic growth phase appeared and indicated a slow rate of equilibration of drug between the medium and the biophase of the organism. The apparent generation rate constants, k_{app}, in accordance with $\log N = k_{app}t$ + constant, where N is the number of organisms at time t, were obtained from the slopes of the terminal linear plots that characterized steady-state generation (10, 11) (Fig. 1). Plots of k_{app} against concentrations of sodium fusidate (Fig. 2) resulted in a sigmoid curve. Daily variability in generation rates could be

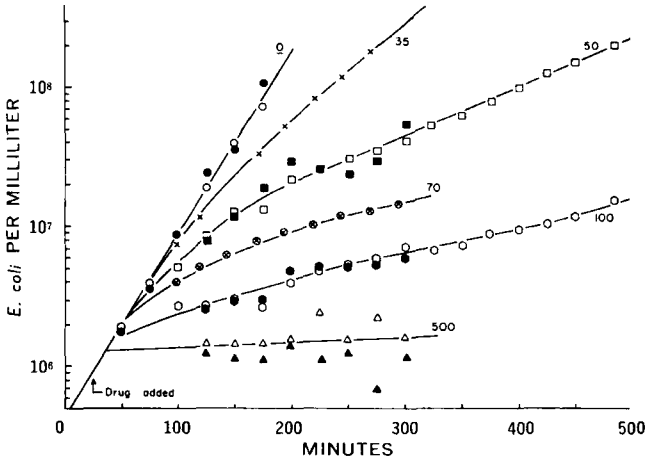


Figure 1—*Typical generation curves of* E. coli *in Anton's medium in the absence and presence of various concentrations of sodium fusidate, obtained by total (open symbols) and viable (dark symbols) counts. The curves are labeled according to the sodium fusidate concentrations in micrograms per milliliter.*

minimized by plotting $(k_0 - k_{app})$ against concentration (Fig. 3), where k_0 was the generation rate constant in the absence of drug.

Effect of Organism Population on Drug-Affected Generation Rates—In preliminary experiments on the effects of sodium fusidate on *E. coli* generation, the drug was added when the bacterial count was $1\text{–}3 \times 10^6$ *E. coli*/ml. In general, after these generation curves achieved steady state, they remained linear for about 250 min but, subsequently, the drug-uninhibited generation of resistant strains was observed since the apparent generation rate increased. This effect was eliminated when the drug was added at a lower organism population to the cultures—*viz.*, less than 5×10^5/ml.

No significant variations were observed (Fig. 2) in the drug-affected steady-state generation rate constants, k_{app}, for the addition of drug to bacterial populations that varied within $10^5\text{–}10^6$ *E. coli*/ml.

Effect of pH and Nutrient Broth Components on Drug-Affected Generation Rates—The generation rates and rate constants, k_{app}, of *E. coli* in the presence of equal concentrations of sodium fusidate were markedly reduced with decreased pH values of the medium (Fig. 3). When the total concentrations of sodium fusidate at all pH values (pKa 5.35) (1) were converted to con-

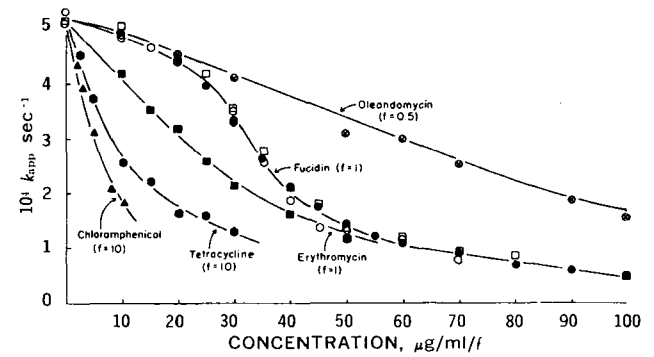


Figure 2—*The dependency of the apparent generation rate constant,* k_{app}, *of* E. coli *on drug concentration, plotted against concentration in micrograms per milliliter/*f *where* f = *10 for chloramphenicol* (C), f = *10 for tetracycline hydrochloride* (T), f = *1 for erythromycin lactobionate,* f = *1 for sodium fusidate (fucidin sodium)* (F) *where the data for several complete studies are given, and* f = *0.5 for oleandomycin phosphate* (O). *The lines drawn through the data are calculated on the premise of adherence to the saturable enzymic degradation of drug and receptor site action of drug in the biophase during the drug-affected steady state in accordance with Scheme I, the parameters of Table I, and Eqs. 59 and 61.*

[4] Leo Pharmaceuticals, Copenhagen, Denmark.
[5] Abbott Laboratories, North Chicago, Ill.
[6] Thomae GMBH, Germany.
[7] The Upjohn Co., Kalamazoo, Mich.
[8] Pfizer Inc., Brooklyn, N.Y.
[9] In the Coulter counter.
[10] Wang 700.
[11] Coulter counter model ZBI in conjunction with Channelyzer and *X–Y* plotter, Coulter Electronics, Inc., Hialeah, Fla.

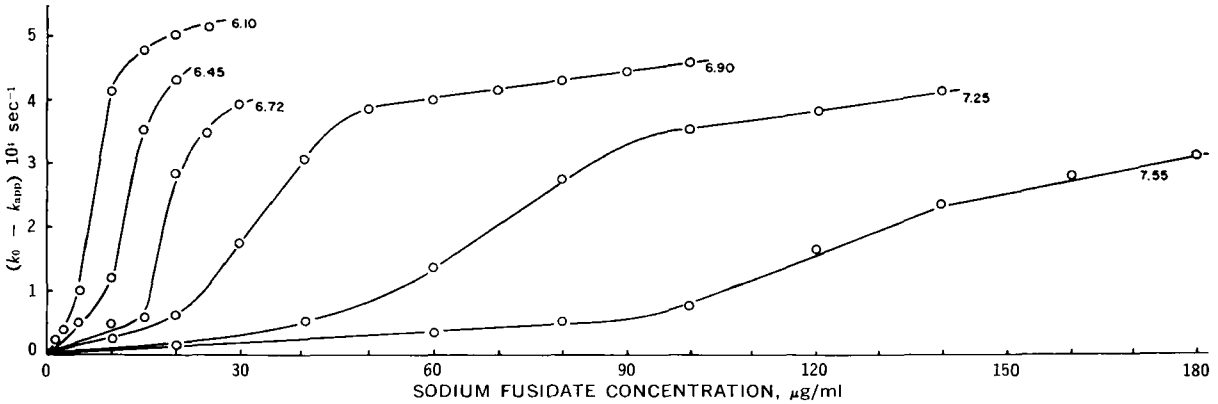


Figure 3—*Plots of the differences between the generation rate constants of* E. coli *in the absence of drug,* k_0, *and the apparent generation rate constants,* k_{app}, *at a given sodium fusidate concentration for the different pH values of the medium specified.*

centrations of unionized drug by multiplying by the fraction of total drug unionized at a given hydrogen-ion concentration (10, 11), $[H^+]/([H^+] + K_a)$, the plots of $(k_0 - k_{app})$ against the unionized drug concentrations showed that all data were coincident (Fig. 4). It may be concluded that only the unionized fraction of sodium fusidate penetrates the cell wall of *E. coli* to inhibit growth and that the MIC of unionized drug is about 2 μg/ml against *E. coli*. Such effects have been observed frequently (10).

The studied variations of the other components of Anton's broth medium had no observable effect on sodium fusidate-affected generation rate constants of *E. coli*.

Reversibility of Drug Action—The effects of sodium fusidate on *E. coli* generation were reversible after exposure of the organisms to 80 μg/ml of drug for 300 min at pH 6.9 and 6.4. When aliquots of medium were diluted 10-fold into fresh broth containing 0, 40, and 80 μg/ml of drug, the generation rates obtained were those for the new drug levels with fresh organisms (Fig. 2).

Prolonged exposure to sodium fusidate, *e.g.*, 100 μg/ml for 48 hr, permitted the emergence of resistant organisms. The generation rate of the filtered bacteria approached the generation rate in the absence of drug even when 100 μg/ml of sodium fusidate was present. When this resistant strain was grown for 200 min in the presence of 50 μg/ml of sodium fusidate and the organisms filtered from the medium, the *E. coli* showed continued resistance to 50 μg/ml but the filtrate inoculated with naive *E. coli* grew at the rate predicted (Fig. 2). Sodium fusidate, therefore, was not significantly degraded to inactive metabolite(s) by exposure to resistant *E. coli*.

Drug Effects on Size of *E. coli*—There was no significant difference between the cell size–frequency distribution of generating *E. coli* untreated with drug (14, 15) and of generating organisms treated with sodium fusidate concentrations up to 90 μg/ml at pH 6.9 at 37.5°. However, at and above the apparent MIC under these conditions of 100 μg/ml, the median size of the drug-affected cells throughout the experiment was twice that of the *E. coli* generating in the absence of sodium fusidate.

Effects of Drug Combinations with Sodium Fusidate—The observed k_{app} values for various combinations of sodium fusidate with other drugs are plotted in Fig. 5 against the volume fraction of sodium fusidate in the mixtures of equipotent concentrations of sodium fusidate and another drug.

THEORETICAL

Models (Schemes I and II) consistent with the variously observed dependencies (10) of the steady-state rate constants, k_{app}, for microbial generation on the concentration of a bacteriostatic drug in the nutrient medium can be constructed. They would be based on the premise that the drug of concentration C in the nutrient medium permeates the cell membrane into the biophase of drug concentration C' which competes with the reaction of a metabolite precursor, M, for those receptor sites, R. This latter process can result in a product, G, vital for microbial generation. Steady-state generation can result when the concentration C' of effective drug in the biophase is a constant. The accompanying postulates are that the loss of drug by binding to the receptor sites or inactivated by saturable or nonsaturable processes in the biophase does not significantly deplete the total concentration, C or C_T, in the nutrient medium.

These models differ from those given previously (10) in that they do not make it necessary to assume true equilibrium between the drug concentrations in the medium and biophase. They also afford a proper explanation for the observed differences in microbial sensitivity among Gram-positive and Gram-negative

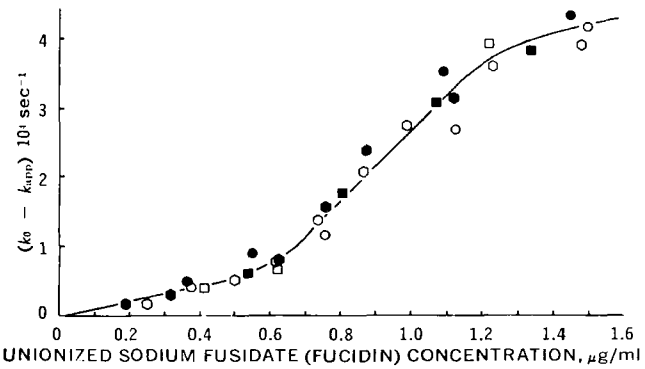
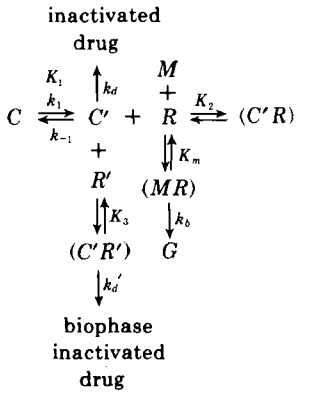


Figure 4—*Plots of the differences between the generation rate constants of* E. coli *in the absence of drug,* k_0, *and the apparent generation rate constants,* k_{app}, *against the micrograms per milliliter concentration of unionized sodium fusidate (fucidin) (sodium fusidate concentration in micrograms per milliliter times fraction of drug that is unionized). The data were obtained at different pH values of the media ranging between 6.10 and 7.55.*



Scheme I—Steady-state model for permeation of drug of concentration C *in nutrient medium into the biophase of the microorganism of concentration* C′ *with simultaneous saturable enzymic,* R′, *inactivation,* k_d', *and first-order inactivation,* k_d, *where the competition of drug* C′ *with substrate,* M, *for receptor sites,* R, *results in the observed inhibition of microbial generation rates, which is postulated to be proportional to the production of* G

microorganisms, which would not be anticipated if steady-state microbial generation is solely a consequence of true equilibrium. It follows that, in addition to the possible variable affinities of the biophase receptor sites of such organisms to a given drug and to the possible differences in metabolic processes, the differences in drug activities among microbial species may be assigned to wide differences in the interacting processes of rates of permeation into, and rates of degradation in, the biophase.

On the basis of Schemes I and II, it can be stated that:

$$dG/dt = k_b(MR) = k_bK_mRM \qquad \text{(Eq. 1)}$$

If the concentration of the necessary metabolite, M, and the total numbers of receptor sites, R_T, whether reacted or unreacted with drug, are invariant, then:

$$dG/dt = k_bK_mMR_t(1-\theta) = k_m(1-\theta) \qquad \text{(Eq. 2)}$$

where $k_m = k_bK_mMR_t$, and θ is the fraction of these sites reacted with drug. Since the equilibrium constant, K_2, for the drug-site interaction of Schemes I and II may be defined by:

$$K_2 = \frac{k_2}{k_{-2}} = \frac{(C'R)}{(R)(C')} = \frac{(C'R)}{[R_t-(C'R)](C')} \qquad \text{(Eq. 3)}$$

where $(C'R)$ is the number of drug-reacted sites, the fraction $\theta = (C'R)/R_t$ of sites reacted with drug may be obtained from rearrangement of Eq. 3:

$$\theta = (C'R)/R_t = K_2C'/(1+K_2C') \qquad \text{(Eq. 4)}$$

Thus, Eq. 2 on substitution of Eq. 4 becomes:

$$dG/dt = k_m - k_mK_2C'/(1+K_2C') \qquad \text{(Eq. 5)}$$

If it is postulated that the rate of population increase of N organisms is proportional to this rate of metabolism or product formation and to the number of organisms, N, in the balanced growth culture, it follows that:

$$dN/dt = q(dG/dt)N \qquad \text{(Eq. 6)}$$

$$dN/dt = \left[qk_m - \frac{qk_mK_2C'}{1+K_2C'}\right]N = k_{app}N \qquad \text{(Eq. 7)}$$

and if qk_m is defined as k_0, the generation rate constant in the absence of drug concentration in the biophase:

$$(k_0-k_{app})/k_{app} = K_2C' \qquad \text{(Eq. 8)}$$

where k_{app} is the apparent first-order steady-state generation rate constant in the presence of a constant drug concentration, C', in the biophase. The necessary prerequisite for the validity of a steady-state generation of rate constant, k_{app}, for the models of Schemes I and II is that ln N against time is linear and of a constant slope, k_{app}, at some time after drug addition.

Presence of Saturable and Nonsaturable Degradation of Drug in Biophase for Drug Saturable Receptor Sites (Scheme I)—When the steady-state generation characterized by Eqs. 7 and 8 holds, it must be postulated that the biophase concentration, C', of drug unbound to the receptor site in Scheme I must be constant in accordance with:

$$dC'/dt = k_1C - [k_{-1}+k_d+k_d'(1-\theta')]C' = 0, C' = \frac{k_1C}{k_{-1}+k_d+k_d'(1-\theta')} \qquad \text{(Eq. 9)}$$

where the concentration of the biophase, C', tends to increase at a rate proportional to the concentration—*viz.*, k_1C, where it is presumed that the concentration, C, or unbound drug C_u, in the nutrient medium is constant throughout the steady state, and tends to decrease by the back-diffusion and the nonsaturable and saturable degradation in the biophase characterized by the rate constants k_{-1}, k_d, and k_d', respectively. The fraction of drug-unreacted enzyme is $1-\theta'$, and the constant $k_d' = (k_d')_0R_T'$ is the product of the intrinsic rate of drug-enzyme degradation, $(k_d')_0$, and the total number of enzymes, R_T'.

The value of $\theta' = (C'R')/R_t'$ can be estimated from the rearrangement of:

$$K_3 = \frac{(C'R')}{(R')(C')} = \frac{(C'R')}{[R_t'-(C'R')]C'} \qquad \text{(Eq. 10)}$$

where $(C'R')$ is the steady-state constant amount of drug-reacted enzyme in the biophase drug and, analogous to Eq. 4:

$$\theta' = K_3C'/(1+K_3C') \qquad \text{(Eq. 11)}$$

so that:

$$(1-\theta') = 1/(1+K_3C') \qquad \text{(Eq. 12)}$$

Substitution of Eq. 12 into Eq. 9 results in:

$$C' = \frac{k_1C}{k_{-1}+k_d+k_d'/(1+K_3C')} \qquad \text{(Eq. 13)}$$

where, from Eqs. 7 and 13:

$$k_{app} = k_0 - \frac{k_0k_1K_2C/[k_{-1}+k_d+k_d'/(1+K_3C')]}{1+k_1K_2C/[k_{-1}+k_d+k_d'/(1+K_3C')]} = k_0 - \frac{k_0K_2C/[1/K_1'+k_d'/k_1(1+K_3C')]}{1+K_2C/[1/K_1'+k_d'/k_1(1+K_3C')]} \qquad \text{(Eq. 14)}$$

and from Eqs. 8 and 13:

$$(k_0-k_{app})/k_{app} = k_1K_2C/[k_{-1}+k_d+k_d'/(1+K_3C'] = K_2C/[1/K_1'+k_d'/k_1(1+K_3C')] \qquad \text{(Eq. 15)}$$

Rearrangement of Eq. 13 permits the determination of an explicit solution for C':

$$C' = \frac{k_1K_3C-(k_d+k_{-1}+k_d')\pm\sqrt{k_1^2K_3^2C^2+(k_{-1}-k_d'+k_d)2k_1K_3C+(k_d+k_{-1}+k_d')^2}}{2(k_{-1}+k_d)K_3} \qquad \text{(Eq. 16)}$$

At low values of C and C', it is apparent from Eq. 13 that:

$$\lim_{C'\to 0} C' = \frac{k_1C}{k_{-1}+k_d+k_d'} = K_1''C \qquad \text{(Eq. 17)}$$

and apparent from Eq. 15 that:

$$\lim_{C'\to 0} (k_0-k_{app})/k_{app} = K_1''K_2C \qquad \text{(Eq. 18)}$$

At very high values, when unity is very much less than K_3C' and when the degradation of C' is essentially zero order:

$$\lim_{C'\to\infty} C' = \frac{k_1C}{k_{-1}+k_d} - \frac{k_d'}{K_3(k_{-1}+k_d)} = K_1'C - \frac{k_d'}{K_3(k_{-1}+k_d)} \qquad \text{(Eq. 19)}$$

and:

$$\lim_{C'\to\infty} (k_0-k_{app})/k_{app} = K_1'K_2C - \frac{k_d'K_2}{K_3(k_{-1}+k_d)} \qquad \text{(Eq. 20)}$$

and $K_1'' < K_1'$.

In the particular case of nonsaturable inactivation of drug in the biophase, where the inactivation process is first order for all C' values, $k_d' = 0$ in Eq. 15 and:

$$(k_0-k_{app})/k_{app} = K_1'K_2C \qquad \text{(Eq. 21)}$$

where $K_1' = k_1/(k_{-1}+k_d)$. The $K_1'K_2$ values may be determined from the slope, S_2, of $(k_0-k_{app})/k_{app}$ against concentration, C, in accordance with Eq. 20 (Fig. 1*b*, curve A), where $k_d' = 0$ and thus K_1'' reduces to K_1'.

If there is no biophase inactivation in the model of Scheme I, $k_d = 0$ and K_1' reduces to K_1 where the slope, S_2, of such a plot is K_1K_2, where $K_1 = k_1/k_{-1}$ as was discussed previously (10).

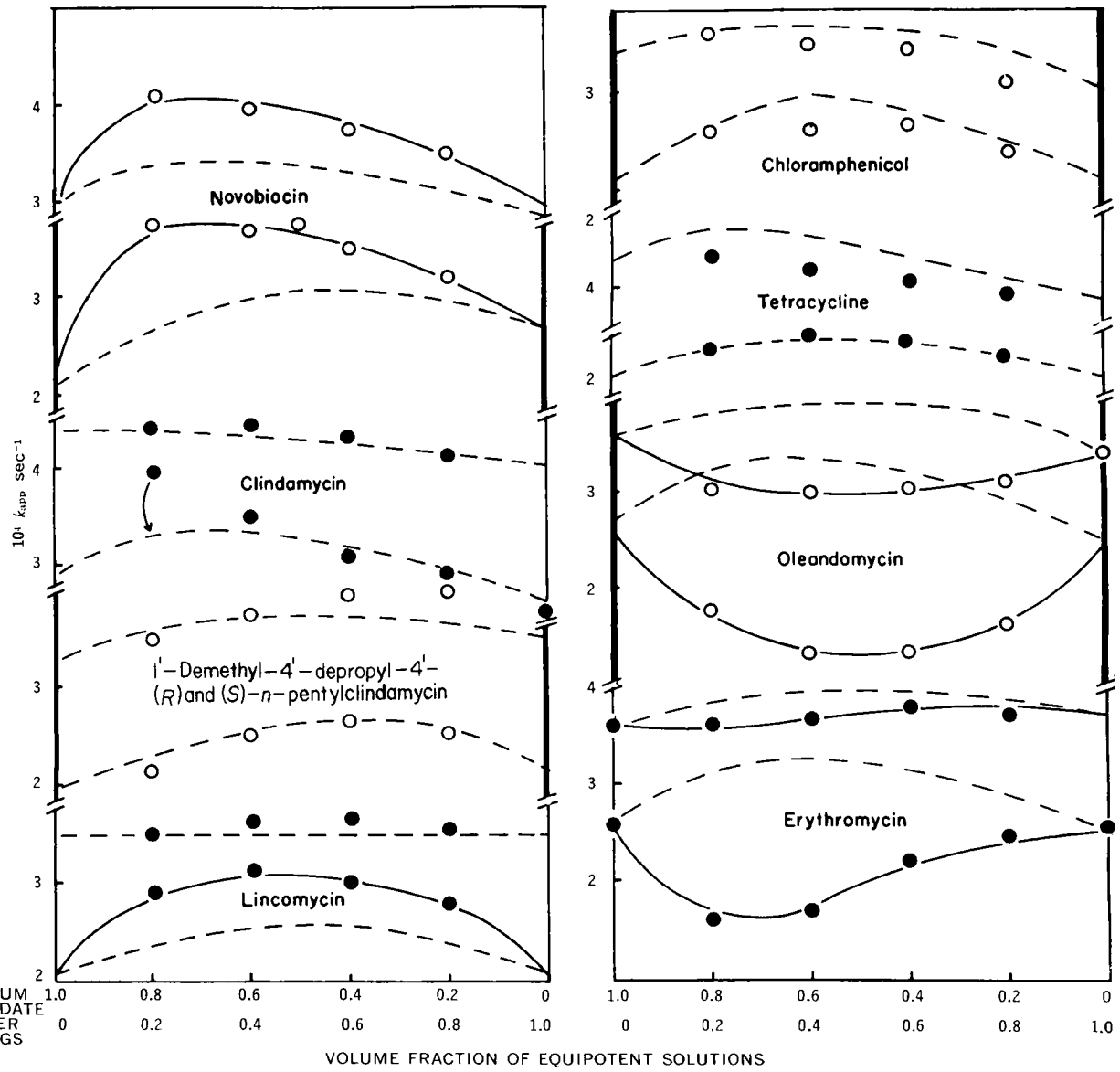


Figure 5—*Effect of varied volume fraction of sodium fusidate solution in various mixtures of sodium fusidate solution and equipotent solution of the specified drug on the apparent generation rate constant,* k_{app}, *observed for the combination at two different potency levels. Because of the nonsuperimposability of the* $(k_{app})_D$ versus *concentration curves (Fig. 2) for all of these drugs with that for sodium fusidate,* $(k_{app})_F$, *the mixtures are not expected to give the same* k_{app} *values at all volume fractions of the sodium fusidate solution. The expected* k_{app} *values for the combinations on the basis of the additivity of actions of both drugs is* $k_0 - (k_{app})_F - (k_{app})_D$ *and are indicated by the dotted lines.*

However, for both cases where there is or is not first-order biophase inactivation, the plot of k_{app} against drug concentration, C, shows a continuously decreasing rate of change of k_{app} with concentration (Fig. 6*a*, curve A). However, there is no *kinetic* method of determining the existence of a first-order inactivation process of constant k_d or of separating the quotients $K_1'K_2$ and K_1K_2. Plots in accordance with Eq. 21 (Fig. 6*b*, curve A) are completely linear under these circumstances.

In the more general case of a saturable process of degradation of drug in the biophase (Eqs. 14 and 15), the rate of decrease of k_{app} with concentration initially increases with increasing saturation of the degrading enzyme or catalyst. Subsequently, as the receptor sites necessary for microbial generation are lessened, the rate of decrease of k_{app} decreases with concentration. An S-shaped curve of k_{app} against concentration, C, would be anticipated (Fig. 6*a*, curve B, and Fig. 7*a*).

At low values of C or C', C' can approach the values expressed by Eq. 17, so that from Eq. 18, the initial linear slope, S_1, of $(k_0 - k_{app})/k_{app}$ against C (Fig. 7*b*, insert) estimates $K_1''K_2$. With increasing concentrations, C, of drug, the inactivation of C' in the biophase is essentially zero order and of maximal rate k_d'/K_3, and C' can be expressed explicitly by Eq. 19 so that Eq. 20 holds at high values of C or C', and the terminal linear slope, S_2, of $(k_0 - k_{app})/k_{app}$ against C (Fig. 6*b*, curve B, and Fig. 7*b*) estimates $K_1'K_2$, and the extrapolated ordinate intercept I of the terminal straight line of such plots (Fig. 6*b*, curve B, and Fig. 7*b*) estimates $-[k_d'K_2/K_3(k_{-1}+k_d)]$.

Concentration C_1 exists (the abscissa intercept of the terminal straight line as shown in Figs. 6*b* and 7*b*) where Eq. 20 equals zero, $C = C_1$, and:

$$C_1 = k_d'/K_1'K_3(k_{-1}+k_d) = k_d'/k_1K_3 \qquad \text{(Eq. 22)}$$

where k_d'/K_3 is the maximal zero-order rate of drug degradation in the biophase.

If the affinity constant, K_3, of drug for enzyme in the biophase is extremely high, K_3C' in Eq. 13 is very much greater than unity and C' can be defined explicitly as:

$$C' = \frac{k_1C}{k_{-1}+k_d} - \frac{k_d'}{K_3(k_{-1}+k_d)} = K_1'C - \frac{k_d'}{K_3(k_{-1}+k_d)} \qquad \text{(Eq. 23)}$$

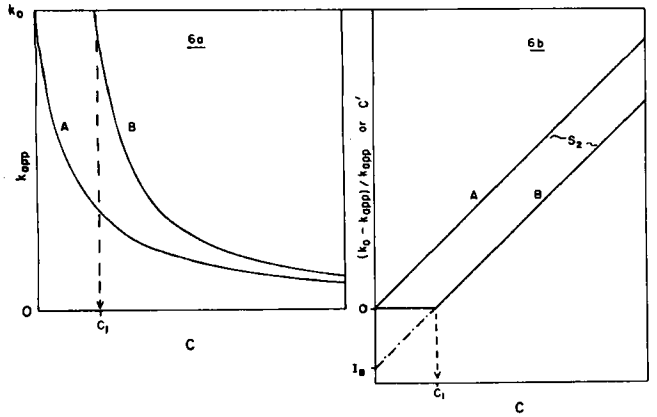
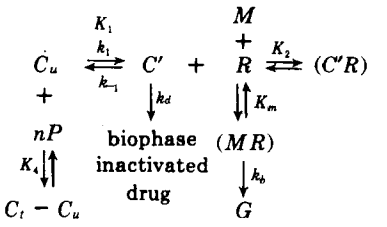


Figure 6—*Plots for the cases for a saturable receptor site model where the degree of inhibition of microbial generation depends on the fraction of receptor sites in the microbial biophase reacted with drug.* (a) *Anticipated shapes of plots of the steady-state drug-affected microbial generation rate constants,* k_{app}, *against drug concentration,* C, *in the medium for the case (curve A) when the drug may or may not be degraded by a first-order process in the biophase and for the case (curve B) when either the drug has an additional zero-order degradation in the biophase or is tightly bound to saturable binding sites in the nutrient medium, where* k_0 *is the generation rate constant in the absence of drug.* (b) *Anticipated dependencies for these cases of the concentration of free unbound drug in the biophase,* C′, *and the function* $(k_0 - k_{app})/k_{app}$ *on the concentration of drug in the medium,* C. *For* C′ versus C, *the slopes* S_2 *are* $K_1' = k_1/(k_{-1} + k_d)$, *where* k_1 *and* k_{-1} *are the rate constants for inflow and outflow with respect to the biophase, respectively, and* k_d *is the first-order rate constant for degradation in the biophase. For* $(k_0 - k_{app})/k_{app}$ versus C, *the slopes* S_2 *are* $K_1'K_2$, *where* K_2 *is the affinity constant of drug for receptor sites. In the case where there are no saturable binding sites or a saturable degradation process (curve A), the intercept is zero. In the cases of an additional saturable degradation or inactivation process of high affinity of drug for degrading enzyme or binding sites in medium, where either the rate of degradation in the biophase is constant and apparent zero order or the drug is effectively unavailable as* C′ *in the biophase, there is no apparent* C′ *or change in* k_{app} *and* $(k_0 - k_{app})/k_{app}$ *until* $C = C_1'$. *In the case of* C′ versus C *and for a saturable enzyme degradation of drug in the biophase, the extrapolated intercept* I_B *of curve B is* $-k_d'/K_3(k_{-1} + k_d) = -K_1'C_1$, *where* k_d *is the first-order rate constant for drug degradation that is nonsaturable,* k_d' *is the first-order rate constant for the saturable degradation of enzyme-reacted drug, and* k_d'/K_3 *is the maximal zero-order rate of drug degradation by this process where* K_3 *is the affinity constant of drug for enzyme; for a saturable binding in the medium, the extrapolated intercept* I_B *is* $-K_1'n[P] = -K_1'C_1$, *where the concentration of binding molecules is* [P] *with* n *sites per molecule. In the case of* $(k_0 - k_{app})/k_{app}$ versus C *and for a saturable enzyme degradation of drug in the biophase, the extrapolated intercept* I_B *is* $-k_d'K_2/K_3(k_{-1} + k_d) = -K_1'K_2C_1$; *for a saturable binding in the medium, the extrapolated intercept* I_B *is* $-K_1'K_2n[P] = -K_1'K_2C_1$.

so that from Eq. 8 at all concentrations of C:

$$(k_0 - k_{app})/k_{app} = K_1'K_2C - \frac{k_d'K_2}{K_3(k_{-1}+k_d)} \qquad \text{(Eq. 24)}$$

Thus, there is essentially a zero-order degradation of drug in the biophase and k_{app} remains constant at k_0 (Fig. 6*a*, curve B) and $(k_0 - k_{app})/k_{app}$ is zero (Fig. 6*b*, curve B) for an initial range of drug concentration, C, from 0 to C_1 since the rate of degradation, k_d'/K_3, exceeds the rate of diffusion, k_1C, of drug into the biophase for that concentration range. Subsequent to this concentration, C_1, k_{app} decreases and the rate of decrease decreases with concentration as the availability of the number of receptor sites necessary for microbial generation is lessened.



Scheme II—Steady-state model for permeation of drug of concentration C *in nutrient medium into the biophase of the microorganism of concentration* C′, *where significant binding occurs to components of the medium, and the net result is the observed inhibition of the microbial generation rate, which is postulated to be proportional to the production of* G

If the special conditions are considered for a saturable process of enzymatic degradation (Scheme I) so that the drug concentration in the biophase, C', is exceedingly small when the rate of saturable degradation is extremely large and the reactivity, K_2, of the drug for the receptor site that results in inhibition of microbial generation is great, Eq. 13 may be simplified to:

$$C' = \frac{k_1C}{k_d'/(1+K_3C')} \qquad \text{(Eq. 25)}$$

since $k_d'C'/(1 + K_3C')$ may be considered very much greater than $(k_d + k_{-1})C'$. Thus, C' can be defined explicitly as:

$$\lim_{C'\to 0} C' = k_1C/(k_d' - k_1K_3C) \qquad \text{(Eq. 26)}$$

when C is small and C' is negligible. Equations 19 and 20 can still hold as C' and C approach infinity.

However, from Eqs. 8 and 26:

$$\lim_{C'\to 0} (k_0 - k_{app})/k_{app} = K_2C' = k_1K_2C/(k_d' - k_1K_3C) \qquad \text{(Eq. 27)}$$

Then:

$$\lim_{\substack{C'\to 0 \\ 1/C\to\infty}} \frac{k_{app}}{k_0 - k_{app}} = \frac{k_d'}{k_1K_2}\frac{1}{C} - \frac{K_3}{K_2} \qquad \text{(Eq. 28)}$$

Thus, at high values of $1/C$, the slope, S_2', of the terminal straight line should estimate k_d'/k_1K_2 and the extrapolated ordinate intercept, I', of the terminal line should estimate $-K_3/K_2$.

Of course, the same information can be obtained from the slopes and intercepts of the appropriately modified Eq. 28:

$$\lim_{C'\to 0} \frac{k_{app}C}{k_0 - k_{app}} = \frac{k_d'}{k_1K_2} - \frac{K_3}{K_2}C \qquad \text{(Eq. 29)}$$

Presence of Saturable Drug Binding Sites in Medium for Drug Saturable Receptor Sites in Biophase—If the antibacterial agent binds to components of the medium, the effective concentration in the medium that establishes the concentration gradient into the biophase is that which is unbound, C_u. It is related to the total concentration in the medium by:

$$C_u = C_t\Big/\left[1 + \frac{n[P]}{K_4 + C_u}\right] \qquad \text{(Eq. 30)}$$

where n is the number of binding sites per molecule of binding agent P, and K_4 is the dissociation constant of the complex. Such values can be determined from separate binding studies (16). The explicit definition of the concentration of free drug is for $C = C_t$:

$$C_u = \frac{C - K_4 - n[P] \pm \sqrt{(K_4 + C + n[P])^2 - 4n[P]/C}}{2} = \frac{C - K_4 - n[P] \pm \sqrt{(K_4 + C - n[P])^2 + 4n[P]K_4}}{2} \qquad \text{(Eq. 31)}$$

A general expression relating the potential saturation of receptor

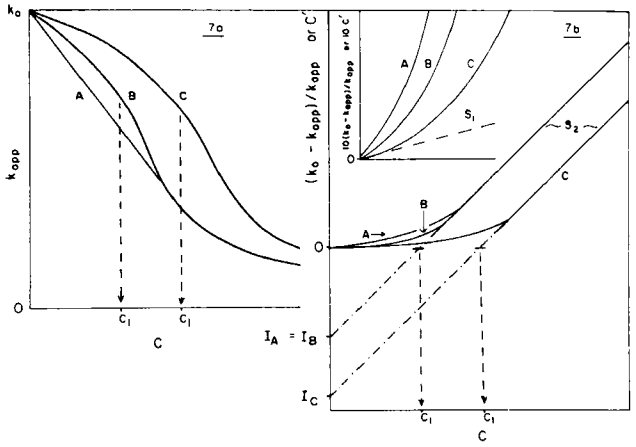


Figure 7—*Plots for the cases for a saturable receptor site model where the degree of inhibition of microbial generation depends on the fraction of receptor sites in the microbial biophase reacted with drug and the drug is degraded in the biophase by a saturable enzyme system or is bound to saturable binding sites in the nutrient medium.* (a) *Anticipated shapes of plots of the steady-state drug-affected microbial generation constants,* k_{app}, *against drug concentration,* C, *in the medium when the diffusion constants* k_1 *and* k_{-1}, *additional first-order degradation constant* k_d, *and the affinity constant* K_2 *for drug–receptor site interaction are the same. Curves A and B are drawn for the case when the maximal zero-order rate of degradation in the biophase,* k_d'/K_3, *as evaluated from the extrapolated intercepts of Fig. 7b, is the same. The first-order rate constant for the rate of degradation of enzyme-reacted drug,* k_d', *is smaller for the case of curve A than for the case of curve B. It follows that the affinity constant,* K_3, *is smaller for the case of curve A. Curves B and C are drawn for the case when the maximal zero-order rates of degradation in the biophase,* $k_d'/K_3 = (k_d')_0 R_T'/K_3$, *differ. The specific rates of degradation of an enzyme-reacted drug complex,* $(k_d')_0$, *may be the same but the amount of enzyme in the biophase,* R_T', *would be greater in the case of curve C. The first-order rate constant for the rate of degradation of enzyme-reacted drug,* k_d', *thus would differ and be greater in the case of curve C, but the affinity constant* K_3 *of the enzyme for the drug may be the same. Alternative explanations for these cases are that the drug is saturably bound to binding sites in the medium and, in the case of curves A and B, the total numbers of binding sites are equal but the dissociation constant,* K_4, *for binding in the medium are smaller in the case of curve B. Curves B and C can represent the case when the dissociation constants of the drug-bound complexes are equal, but the total numbers of binding sites are less in the case of curve B.* (b) *Anticipated dependencies for these cases of the concentration of free unbound drug in the biophase,* C′, *and the function* $(k_0 - k_{app})/k_{app}$ *on the concentration of drug in the medium,* C. *The significances of the slopes,* S_2, *of the terminal lines, and of the extrapolated intercepts of these lines were given in detail for Fig. 6b. The insert of Fig. 7b has a 10-fold expansion of the ordinate values. In the case of saturable enzymic degradation of drug in the biophase, the initial slopes,* S_1, *of these plots of* C′ versus C *approach* $K_1'' = k_1/(k_{-1} + k_d + k_d')$ *and of* $(k_0 - k_{app})/k_{app}$ versus C *approach* $K_1''K_2$. *In the case of saturable drug binding in the medium, the initial slopes,* S_1, *of these plots of* C′ versus C *approach* $k_1/(k_{-1} + k_d)\ (1 + n[P]/K_4) = K_1'/(1 + n[P]/K_4)$ *and the initial slopes of these plots of* $(k_0 - k_{app})/k_{app}$ versus C *approach* $K_1'K_2/(1 + n[P]/K_4)$.

sites and concentration C_u of diffusible drug in the medium in the absence of a saturable degradation in the biophase is, from Eq. 21:

$$(k_0 - k_{app})/k_{app} = K_1'K_2C_u \qquad \text{(Eq. 32)}$$

In the special case when drug concentration is large enough to saturate the available binding sites and there is a high affinity of drug for binding sites, K_4 is reasonably small and $C_u \gg K_4$. Thus, the latter may be neglected in Eq. 29 and:

$$\lim_{C_u \to \infty} C_u = C_t - n[P] \qquad \text{(Eq. 33)}$$

Thus, from Eqs. 32 and 33:

$$\lim_{C_u \to \infty} (k_0 - k_{app})/k_{app} = K_1'K_2C_t - K_1'K_2n[P] \qquad \text{(Eq. 34)}$$

In the general case when the concentration of drug is small and/or there is a low affinity of drug for binding sites, K_4 is reasonably large and $K_4 \gg C_u$. Thus, the latter may be neglected in Eq. 30 and:

$$\lim_{C_u \to 0} C_u = C_t/(1 + n[P]/K_4) \qquad \text{(Eq. 35)}$$

Thus, from Eqs. 32 and 35:

$$\lim_{C_u \to 0} (k_0 - k_{app})/k_{app} = \frac{K_1'K_2C_t}{1 + n[P]/K_4} \qquad \text{(Eq. 36)}$$

It follows that a plot of k_{app} against concentration C will be an S-shaped curve where the initial rate of change will increase as a greater fraction of the added drug becomes available to diffuse into the organism's biophase as the medium binding has approached saturation (Fig. 6*a*, curve B, and Fig. 7*a*).

Thus, plots of $(k_0 - k_{app})/k_{app}$ against C would show an initial slope, S_1 (Fig. 7*b*, insert), at low values of C that approaches $K_1'K_2/(1 + n[P]/K_4)$. This slope is essentially zero if the affinity of drug for binding sites, $1/K_4$, is extremely high (Fig. 6*b*, curve B). At high values of C, the slope, S_2, approached by the plot should estimate $K_1'K_2$ and the extrapolated intercept, I (Fig. 6*b*, curve B, and Fig. 7*b*), of the terminal line should be $-K_1'K_2n[P]$.

Concentration C_1 exists (the abscissa intercept of the extrapolated terminal straight line as shown in Figs. 6*b* and 7*b*) where Eq. 33 equals zero, $C = C_1 = n[P]$.

Both the binding of drug to saturable binding sites in the medium and the saturable enzymatic degradation of drug in the biophase can give the same S-shaped curve for plots of k_{app} against drug concentrations and similarly shaped plots for $(k_0 - k_{app})/k_{app}$ against concentration. The former process may be denied if there are no significant changes in plots when concentrations of nutrients in the medium are varied and when the inoculum size of organisms is varied.

Presence of Saturable and Nonsaturable Degradation of Drug in Biophase for Nonsaturable Drug Binding to Receptor Sites—It has been shown in the cases of tetracycline and chloramphenicol that k_{app} is linearly related to drug concentration. This can be rationalized on the premise of a receptor site model (10) where: *(a)* a drug-bound receptor inhibits or blocks a substrate attack on adjacent receptors so that a single drug-bound receptor inactivates multiple receptors from being effective in microbial generation, *(b)* there are multiple binding sites of equivalent affinity where the attack of a drug molecule on any one of them inhibits the reaction of substrate with a receptor site that results in microbial generation, or (*c*) it is only necessary to react the drug with a small fraction of the total number of receptor sites to obtain complete inhibition of microbial generation. If any of these factors are extent, the k_{app} implicitly defined in Eq. 7 may be approximated by:

$$k_{app} = k_0 - k_0K_2C' \qquad \text{(Eq. 37)}$$

and:

$$(k_0 - k_{app})/k_0 = K_2C' \qquad \text{(Eq. 38)}$$

since $K_2C' \ll 1$.

If there is first-order degradation in the biophase, $C' = K_1'C$ and plots of steady-state generation rate constants k_{app} or of $(k_0 - k_{app})/k_0$ against concentration C are linear with slopes of $-k_0K_1'K_2$ and $K_1'K_2$ and intercepts of k_0 and zero, respectively. Plots of $1/(k_0 - k_{app})$ against $1/C$ are linear with slopes of $1/k_0K_1'K_2$ and intercepts of zero, and plots of $C/(k_0 - k_{app}) = 1/k_0K_1'K_2$ against C are invariant.

If there is an additional degradation in the biophase that is saturable, the explicit value of C' is obtainable from Eq. 16 and the rate of decrease of k_{app} with concentration initially increases to

result ultimately in a constant rate where the initial slopes, S_1, from Eq. 17 for plots of k_{app} and $(k_0 - k_{app})/k_0$ approximate $-k_0K_1''K_2$ and $K_1''K_2$, respectively, since:

$$\lim_{C'\to 0} k_{app} = k_0 - k_0K_1''K_2C \qquad \text{(Eq. 39)}$$

$$\lim_{C'\to 0} (k_0 - k_{app})/k_0 = K_1''K_2C \qquad \text{(Eq. 40)}$$

and the slopes of the terminal straight lines approximate $k_0K_1'K_2$ (from Eq. 19) and $K_1'K_2$, respectively, in accordance with:

$$\lim_{C'\to \infty} k_{app} = k_0 - k_0K_1'K_2C + k_0K_2k_d'/K_3(k_{-1} + k_d) \qquad \text{(Eq. 41)}$$

and:

$$\lim_{C'\to \infty} (k_0 - k_{app})/k_0 = K_1'K_2C - K_2k_d'/K_3(k_{-1} + k_d) \qquad \text{(Eq. 42)}$$

with the extrapolated intercepts of $k_0 + k_0K_2k_d'/K_3(k_{-1} + k_d)$ and $k_0K_2k_d'/K_3(k_{-1} + k_d)$, respectively.

If the special conditions are considered for a saturable process of enzymatic degradation where the drug concentration in the biophase, C', is exceedingly small with an extremely large degradation rate constant, k_d', of saturable degradation and an extremely large drug affinity, K_2, for receptor sites, Eqs. 25, 26, and 38 hold and:

$$\lim_{C\to 0} (k_0 - k_{app})/k_0 = k_1K_2C/(k_d' - k_1K_3C) \qquad \text{(Eq. 43)}$$

Then the following expression is linear:

$$\lim_{C\to 0} \frac{k_0}{k_{app} - k_0} = \frac{k_d'}{k_1K_2}\frac{1}{C} - \frac{K_3}{K_2} \qquad \text{(Eq. 44)}$$

where the slope, S_1', of the plot of the left-hand function against the reciprocal of the concentration is k_d'/k_1K_2 and the negative of the intercept, $-I$, is K_3/K_2.

If there is saturable binding of drug to the components of the medium, Eqs. 30, 31, 33, 34, 37, and 38 hold and $C' = K_1'K_2C_u$. Thus:

$$\lim_{C\to 0} (k_0 - k_{app})/k_0 = K_1'K_2C/(1 + n[P]/K_4) \qquad \text{(Eq. 45)}$$

and the initial slope is $K_1'K_2/(1 + n[P]/K_4)$ and:

$$\lim_{C\to \infty} (k_0 - k_{app})/k_0 = K_1'K_2C - K_1'K_2n[P] \qquad \text{(Eq. 46)}$$

and the terminal slope is $K_1'K_2$ with an extrapolated intercept of the terminal line of $-K_1'K_2n[P]$.

DISCUSSION

The antibiotic sodium fusidate is bacteriostatic in its action on *E. coli* (ATCC 12407) but does permit the emergence of resistant strains that do not significantly degrade the drug in the medium. There does not appear to be any significant effect of inoculum size or concentration of the components of the nutrient medium used on the bacteriostatic action of sodium fusidate in the limited ranges studied. However, the addition of sodium fusidate to larger inoculum sizes does give rise to a greater number of resistant organisms than when the inoculum size is smaller.

There is a marked effect of pH on drug action, which is readily explained by the fact that the effective species in the medium is nonionized. The MIC of nonionized drug, which has a pKa' = 5.35 (1), is one-fiftieth of that at pH 6.9.

It has been shown (Figs. 6 and 7) that the S-shaped curve obtained (Fig. 2) for the dependence of the apparent generation rate constant, k_{app}, on sodium fusidate concentration in the medium can be assigned to saturable binding to the components of the medium (Scheme II) or to a saturable degradation of the drug in the biophase of the organism (Scheme I), where the drug action is postulated to be effective through saturable receptor site binding.

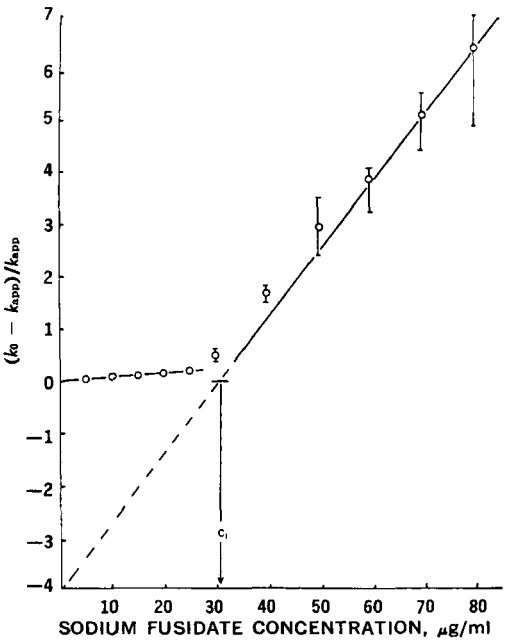


Figure 8—*Plot of* $(k_0 - k_{app})/k_{app}$ *against sodium fusidate concentration in the medium in accordance with Eqs. 15, 30, and 32 where the asymptotic terminal slope,* S_2, *is an estimate of* $K_1'K_2 = 0.13$ *ml/μg; the asymptotic initial slope,* S_1, *is an estimate of* $K_1'K_2/(1 + n[P]/K_4)$ *or* $K_1''K_2 = 0.00375$ *ml/μg; and the* C_1 *intercept on the abscissa is an estimate of* $n[P]$ *or* $k_d'/k_1K_3 = 31$ *μg/ml.*

Rationalization of Observed Generation Rate Constant, k_{app}, Dependence on Sodium Fusidate Concentration for Saturable Binding of Drug to Components of Medium—It has been shown (Figs. 6 and 7), when other evidence is lacking to the contrary, that the S-shaped curve obtained (Fig. 2) for the dependence of the apparent generation rate constant, k_{app}, on sodium fusidate concentration in the medium can be assigned to saturable binding to the components of the medium (Scheme II).

If this postulate is valid, when the data for sodium fusidate at pH 6.9 and 37.5° are plotted as $(k_0 - k_{app})/k_{app}$ against concentration C (Fig. 8) and if Eq. 32 holds, Eq. 36 may be applicable at low drug concentrations and Eq. 34 at high. The reasonable linearity of the terminal data at high sodium fusidate concentrations permits the estimation from the terminal slope, S_2, of:

$$S_2 = K_1'K_2 = 0.13 \text{ ml}/\mu\text{g} \qquad \text{(Eq. 47)}$$

The intercept of the extrapolated terminal line on the abscissa estimates:

$$C_1 = n[P] = 31 \,\mu\text{g/ml} \qquad \text{(Eq. 48)}$$

The intercept on the ordinate from Eq. 34 is:

$$I = -K_1'K_2n[P] = -4.0 \qquad \text{(Eq. 49)}$$

The initial slope, S_1, of the plot of Fig. 8 at low concentrations of drug permits the preliminary estimation, in accordance with Eq. 36, of:

$$S_1 = K_1'K_2/(1 + n[P]/K_4) = 0.00375 \text{ ml}/\mu\text{g} \qquad \text{(Eq. 50)}$$

It follows from the quotient of Eqs. 47 and 50 that:

$$S_2/S_1 = 1 + n[P]/K_4 = 34.7 \qquad \text{(Eq. 51)}$$

so that $n[P]/K_4 = 33.7$.

It also follows from Eqs. 48 and 51 that:

$$\frac{n[P]/K_4}{C_1} = 1/K_4 = 1.09 \text{ ml}/\mu\text{g} \qquad \text{(Eq. 52)}$$

or $K_4 = 0.917$ μg/ml.

For all values of C, C_u can be calculated from Eq. 31 from the evaluated parameters K_4 and $n[P]$. Thus, the values of $(k_0 -$

Table I—Evaluated Parameters at pH 6.9 and 37.5° that Fit the Observed Dependency of the Apparent Generation Rate Constant, k_{app}, on Drug Concentration for the Model of Scheme I that Includes Saturable Biophasic Enzymic Inactivation and Saturable Receptor Site Binding of Drug

Drug	Tetracycline Hydrochloride[a]	Chloramphenicol	Sodium Fusidate	Erythromycin Lactobionate	Oleandomycin Phosphate
$K_1'K_2$					
ml/μg of total drug	11.0	2.5	0.13	0.13	0.03
liters/mole of total drug	5.3×10^6	8.1×10^5	7.0×10^4	1.4×10^5	2.4×10^4
liters/mole of uncharged drug	6.1×10^6	8.1×10^5	2.6×10^6	1.13×10^7	0.96×10^6
$K_1''K_2$					
ml/μg of total drug	5.5	0.5	0.00375	0.02	0.003
liters/mole of total drug	2.6×10^6	1.61×10^5	2.0×10^3	2.2×10^4	2.40×10^3
liters/mole of uncharged drug	3.0×10^6	1.61×10^5	7.5×10^4	1.81×10^6	0.96×10^5
$C_1 = k_d'/k_1K_3$					
μg/ml (total drug)	0.15	0.28	31	31	160
mole/liter (total drug)	3.1×10^{-7}	8.7×10^{-7}	5.8×10^{-5}	2.85×10^{-5}	2.0×10^{-4}
mole/liter (uncharged drug)	2.7×10^{-7}	8.7×10^{-7}	1.6×10^{-6}	3.42×10^{-7}	5.0×10^{-6}
$K_1'K_3$					
ml/μg of total drug	66.7	14.3	1.10	0.18	0.056
liters/mole of total drug	3.2×10^7	4.6×10^6	5.9×10^5	1.96×10^5	4.38×10^4
liters/mole of uncharged drug	3.7×10^7	4.6×10^6	2.2×10^7	1.63×10^7	1.79×10^5
K_1'/K_1''	2.0	5.0	34.7	6.5	10
$k_d'/(k_{-1} + k_d)$	1.0	4.0	33.7	5.5	9
$k_d'K_2/K_3(k_{-1} + k_d) = k_d'K_1'K_2/k_1K_3$	0.20	3.8	4.0	4.6	5.6
K_2/K_3	0.20	0.95	0.12	0.84	0.62
pKa	7.7	...	5.35	8.8	8.5
Fraction uncharged at pH[a] 6.9	0.86	1.00	0.027	0.012	0.0245

[a] The pH dependency of tetracycline action is not assured. However the pKa was one chosen on the assumption that this represents the loss of a proton from the neutral zwitterion.

$k_{app})/k_{app}$ of Eq. 32 and the corresponding values of k_{app} can be calculated for all values of C from the known $K_1'K_2$ and the values of C_u estimated from Eq. 31.

In actuality, the curve of Fig. 2 for k_{app} against concentration was best fitted using the values given in Eqs. 47–52 to demonstrate that the data are consistent with the model of Scheme II for a saturable binding of drug to the components of the medium.

Although this model for saturable binding to components of the medium can be excluded in the case of sodium fusidate since varying the concentrations of the components of Anton's medium had no apparent effect on sodium fusidate activity, this procedure clearly demonstrates the applicability of microbial kinetics to the determination of the binding constants of antibacterial bacteriostatic agents to various compounds for saturable binding of drug to receptor sites. Analogous procedures can be developed to estimate the saturable binding constants to components of the medium for nonsaturable binding to receptor sites from Eqs. 45 and 46 and should be applicable to spectinomycin (17) where such a phenomenon was observed.

Rationalizations of Observed Generation Rate Constant, k_{app}, Dependence on Sodium Fusidate Concentration for Enzymic Saturable Degradation in Biophase—On the postulation of the validity of this premise for k_{app} dependence, the data for sodium fusidate at pH 6.9 and 37.5° have been plotted in Fig. 8 as $(k_0 - k_{app})/k_{app}$ against concentration C and, if Eq. 15 holds, Eq. 18 may be applicable at low drug concentrations and Eq. 20 at high. The reasonable linearity of the terminal data in accordance with Eq. 20 at high sodium fusidate concentrations permits the estimation from the terminal slope, S_2, of:

$$S_2 = K_1'K_2 = 0.13\ \text{ml}/\mu\text{g}\ (7.0 \times 10^4\ \text{liters/mole}) \quad \text{(Eq. 53)}$$

at pH 6.9. In terms of unionized drug, the value of S_2 would be 4.83 ml/μg (2.6×10^6 liters/mole). The intercept on the abscissa estimates:

$$C_1 = k_d'/K_1'K_3(k_{-1} + k_d) = k_d'/k_1K_3 = 31\ \mu\text{g/ml}\ (5.8 \times 10^{-5}\ M) \quad \text{(Eq. 54)}$$

or, for unionized drug, $C_1 = 0.84$ μg/ml (1.56×10^{-6} M), where k_d'/K_3 is the maximal rate of saturable enzymic degradation in the biophase. The value of C_1 also may be considered as the ratio of maximal zero-order degradation within the biophase to the first-order rate constant for permeation from the medium.

The intercept on the ordinate from Eq. 20 is:

$$I = -k_d'K_2/K_3(k_{-1} + k_d) = -k_d'K_1'K_2/k_1K_3 = -4.0 \quad \text{(Eq. 55)}$$

The initial slope, S_1, of the plot of Fig. 8 at low concentrations of drug permits the preliminary estimation in accordance with Eq. 18 of:

$$S_1 = K_1''K_2 = 0.00375\ \text{ml}/\mu\text{g}\ (2.02 \times 10^3\ \text{liters/mole}) \quad \text{(Eq. 56)}$$

In terms of unionized drug, the value of S_1 would be 0.139 ml/μg (7.47×10^4 liters/mole).

It follows from the quotient of Eqs. 53 and 56 that:

$$S_2/S_1 = K_1'/K_1'' = \left(\frac{k_1}{k_{-1}+k_d}\right)\Big/\left(\frac{k_1}{k_{-1}+k_d+k_d'}\right) = 1 + k_d'/(k_{-1} + k_d) = 34.7 \quad \text{(Eq. 57)}$$

so that $k_d'/(k_{-1} + k_d) = 33.7$.

It also follows from Eqs. 54 and 57 that:

$$\frac{k_d'/(k_{-1}+k_d)}{C_1} = \frac{k_d'/(k_{-1}+k_d)}{k_d'/K_1'K_3(k_{-1}+k_d)} = K_1'K_3 = 1.09\ \text{ml}/\mu\text{g}\ (5.9 \times 10^5\ \text{liters/mole}) \quad \text{(Eq. 58)}$$

In terms of unionized drug, the value of $K_1'K_3$ would be 4.04 ml/μg (2.2×10^7 liters/mole).

If the numerator and denominator of Eq. 16 are multiplied by k_1K_1' and it is realized that $K_1' = k_1/(k_{-1} + k_d)$ and $K_1'' = k_1/(k_{-1} + k_d + k_d')$, it can be shown that:

$$K_3C' = \frac{K_1'K_3C - K_1'/K_1'' \pm \sqrt{(K_1'K_3C)^2 + 2K_1'K_3C - 2K_1'K_3Ck_d'/(k_{-1}+k_d) + (K_1'/K_1'')^2}}{2} \quad \text{(Eq. 59)}$$

so that K_3C' can be calculated for all values of C from the evaluated parameters $K_1'K_3 = 1.09$ ml/μg, $k_d'/(k_{-1} + k_d) = 33.7$, and $K_1'/K_1'' = 34.7$.

If the numerator and denominator of Eq. 15 are multiplied by K_1', it can be shown that:

$$\frac{k_0 - k_{app}}{k_{app}} = \frac{K_1'K_2C}{1 + [k_d'/(k_1 + k_d)][1/(1 + K_3C')]} \quad \text{(Eq. 60)}$$

so that this function can be calculated for all values of C from the

evaluated parameters $K_1'K_2$ = 0.13 ml/μg and $k_d'/(k_{-1} + k_d)$ = 33.7 and the values of K_3C' estimated from Eq. 59.

Similarly, it can be shown from Eq. 14 that:

$$k_{app} = k_0 - \frac{k_0K_1'K_2C/\{1+[k_d'/(k_{-1}+k_d)][1/(1+K_3C')]\}}{1+K_1'K_2C/\{1+[k_d'/(k_{-1}+k_d)][1/(1+K_3C')]\}} \quad \text{(Eq. 61)}$$

In actual practice, the values given in Eqs. 53–56 were those finally resolved to give the best fit of k_{app} against C (Fig. 2) consistent with the model of Scheme I for a saturable degradation process in the biophase and saturable binding to receptor sites. The actual procedure was to vary the initial estimates of S_2 (Eq. 53), C_1 (Eq. 54), I (Eq. 55), and S_1 (Eq. 56) obtained from the plot of Fig. 8 and used to estimate $k_d'/(k_{-1} + k_d)$ from Eq. 57 and $K_1'K_3$ from Eq. 58.

Analogous procedures can be developed to estimate the parameters of a saturable degradation process in the biophase for nonsaturable binding to receptor sites from Eqs. 37–42.

If these premises are accepted, it is possible to conclude that the greater activity of sodium fusidate against Gram-positive organisms than against Gram-negative organisms such as *E. coli* (1) is due to the former's lack of a saturable degradation process in the biophase and/or a more active transport process of sodium fusidate into the biophase with lessened biophase degradation, and/or the receptor sites in the biophase of Gram-positive organisms having a higher affinity for the drug than those of Gram-negative organisms.

Compatability of Diverse k_{app} Dependencies on Drug Concentration for Various Drugs on Premise of Drug-Affected Steady-State Generation with Enzymic Saturable Degradation in Biophase—In the fluorouracil and sulfonamide cases, the saturable receptor site model appears to hold for all concentrations (10) and implies no saturable enzymic degradation in the biophase for these compounds. The form of the k_{app} dependency on drug concentration in the medium is different for several classes of drugs (10), as shown in Fig. 2. Such plots for tetracycline and chloramphenicol are usually remarkably linear (10), whereas for the macrolides and lincosaminides they are initially linear at low concentrations and the rate of change of k_{app} subsequently decreases with concentration.

However, all of these plots can be fitted to Eq. 61, obtained from Eq. 14, showing k_{app} dependency on concentration in accordance with the model of Scheme I where a saturable enzymic degradation in the biophase may be postulated. Judicious choices of values for $K_1'K_2$ (Eq. 53), $C_1 = k_d'/k_1K_3$ (Eq. 54), and K_1'/K_1'' (Eq. 57) permitted the calculation of proper values of $k_d'/(k_{-1} + k_d) = (K_1'K_1'') - 1$, $K_1'K_3$ (Eq. 58), and K_3C' (Eq. 59), so that the lines of best fit based on these values could be plotted (Fig. 2) for data obtained for sodium fusidate, tetracycline, chloramphenicol, erythromycin, and oleandomycin in Anton's medium (12) in accordance with Eq. 61.

Values of these parameters that gave the excellent fits to the data plotted in Fig. 2 are given in Table I and are for antibiotics of widely different structure. The $k_d'/(k_{-1} + k_d)$ values show the ratios of the rate constant for the loss of drug by saturable enzyme inactivation in the biophase to the loss of drug from all first-order processes. The large value assigned to sodium fusidate indicates a significant amount of saturable enzyme inactivation of this drug. The values of C_1, the ratios of the maximal rate of saturable enzyme inactivation of drug (k_d'/K_3) to the first-order rate constant for permeation of the drug into the biophase (k_1), show the largest values for those drugs of the lowest potency against the Gram-negative *E. coli*—*viz.*, sodium fusidate, erythromycin, and oleandomycin. In the case of sodium fusidate, this loss of drug to saturable enzymic inactivation explains the unique S-shaped curve observed in Fig. 2, since at lower concentrations and thus lower rates of penetration into the biophase a larger fraction of the drug that permeates the cell wall is rendered ineffective. As this enzyme becomes saturated, a larger fraction of the sodium fusidate that penetrates is available to exercise inhibition.

The parameter $K_1'K_3$ may be written $K_3(k_1/k_{-1} + k_d)$; if it were assumed that k_d were zero (no nonsaturable enzyme degradation) and that different unionized drugs had similar partition constants ($K_1 = k_1/k_{-1}$) into the biophase, then the relative affinities of the various drugs for saturable inactivating enzymes could be obtained from the ratios of the $K_1'K_3$ values. Thus, relative to sodium fusidate and in terms of equivalents of unionized drug, such relative affinities were: tetracycline, 1.6; chloramphenicol, 0.21; erythromycin, 0.74; and oleandomycin, 0.086.

Comparative Effects of *a priori* Equipotent Combinations of Other Bacteriostatic Drugs with Sodium Fusidate—The effects of *a priori* equipotent combinations of sodium fusidate predicted on the basis of the observed k_{app} values of the separate bacteriostatic drugs are shown in Fig. 5. All drug combinations were studied in Anton's medium, pH 6.9, 37.5°, at two levels of potency in the k_{app} ranges of 2.0–2.5 and 3.0–4.0. If two drugs have the same functional dependency of k_{app} on concentration and one does not affect the action of the other in combination, the k_{app} is invariant with the change in the fraction of either drug in the *a priori* equipotent combination (10). This is not true if the functional dependencies of the two drugs differ, even though the presence of the one may not affect the action of the other in the combination. For example, if a drug with an S-shaped k_{app} *versus* concentration curve is combined with a drug having a linear k_{app} *versus* concentration curve, then a constant k_{app} is not expected for combinations of various fractions of *a priori* equipotent amounts. The expected k_{app} of the combination, $(k_{app})_{comb}$, on the basis of equivalency may be calculated from the apparent generation rate constants $(k_{app})_A$ and $(k_{app})_B$, which resulted when drugs A and B were used separately (14, 18, 19):

$$(k_{app})_{comb} = k_0 - (k_{app})_A - (k_{app})_B \quad \text{(Eq. 62)}$$

The predicted k_{app} dependencies for various combinations of sodium fusidate with another drug are given in Fig. 5. Significant increases in k_{app} values above these predictions can be considered as evidence for antagonism; a significant decrease below these predictions is evidence of synergism (10). Thus, sodium fusidate and novobiocin can be interpreted to be antagonistic, oleandomycin or erythromycin with sodium fusidate is synergistic, and combinations of sodium fusidate with tetracycline, chloramphenicol, 1′-demethyl-4′-depropyl-4′-[(*R*) and (*S*)]-*n*-pentylclindamycin are indifferent (10). The apparent mild antagonism of lincomycin with sodium fusidate is not definitive since inactive complexes may be formed in solution between these drugs. Sodium fusidate precipitates both lincomycin and clindamycin at pH 6.9, although the drug-affected bacterial growth rates were measured with no obvious precipitate in the growth medium.

Erythromycin is a protein synthesis inhibitor whose mode of action requires binding to the 50S ribosomes and, in addition, a distortion of peptidyl transferase with resultant inhibition of the translocation step of peptide synthesis (9). The similarity of structure between erythromycin and oleandomycin permits the assumption of the same mode of action, an assumption that is further strengthened by the synergy both drugs show in combination with sodium fusidate. The mode of action of sodium fusidate is also inhibition of protein synthesis, but the mechanism involves a binding to, and stabilization of, an energy-releasing complex, with subsequent inhibition of translocation (6–9). Reference to Fig. 2 shows that the three drugs all exhibit saturation of action against *E. coli* in the higher concentration range, when k_{app} approaches 1.5×10^{-4} sec^{-1}. The requirement for synergistic action is that drugs in combination act on saturable receptor sites and on separate steps in sequential processes (19). This requirement appears to be met by sodium fusidate and the macrolides in the sequential steps of the translocation process.

The antagonism of sodium fusidate and novobiocin is consistent with the observed antagonism of novobiocin with other 50S binding inhibitors of protein biosynthesis such as chloramphenicol, erythromycin, and lincomycin, which has been rationalized on the premise that novobiocin inhibits the production of messenger RNA necessary for the translocation of ribosomes (18). This latter process is inhibited by the 50S binding antibiotics, and the overall rate of protein biosynthesis (and thus the dependent rate of microbial generation) is determined by whichever process is the slower with the demonstration of an apparent antagonism.

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ACKNOWLEDGMENTS AND ADDRESSES

Received August 10, 1973, from the *College of Pharmacy, J. Hillis Miller Health Center, University of Florida, Gainesville, FL 32610*

Accepted for publication February 1, 1974.

Supported in part by Public Health Service Research Grant ROI AI 10058-03, National Institutes of Health, Bethesda, MD 20014

The authors thank Leo Pharmaceuticals, Copenhagen, Denmark, for generous gifts of sodium fusidate (fucidin sodium); Pfizer Inc., Brooklyn, N.Y., for oleandomycin phosphate; The Upjohn Co., Kalamazoo, Mich., for tetracycline hydrochloride, novobiocin, 1′-demethyl-4′-depropyl-4′-[*(R)* and *(S)*]-*n*-pentylclindamycin, clindamycin, and lincomycin; Thomae GMBH, Germany, for chloramphenicol; and Abbott Laboratories, North Chicago, Ill., for erythromycin lactobionate.

The authors are also indebted to Mr. George L. Perry, Sr., for excellent technical assistance.

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Kinetics and Mechanisms of Drug Action on Microorganisms XXI: Effect of Quinacrine on *Escherichia coli* and Its Possible Complexation with Components of Nutrient Growth Medium

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Abstract □ The steady-state generation of *Escherichia coli* (ATCC 12407) was inhibited by the antimalarial quinacrine in the drug concentration range of 0–100 μg/ml at pH 6.9 in Anton's medium, and the rate of decrease of generation constants increased with drug concentration. The drug activity was affected by the concentration of amino acids in the nutrient broth and indicated that the inactivation of quinacrine may be explained by its saturable complexation to the amino acids; methods were applied to estimate concentrations of complexing sites. Studies of generation inhibition at various pH values showed that the diprotonated quinacrine exerted no significant antibacterial activity. Quinacrine was bactericidal at concentrations above 120 μg/ml. Equipotent combinations of quinacrine with tetracycline or chloramphenicol were indifferent.

Keyphrases □ Quinacrine—inhibition of *E. coli* generation in Anton's medium, amino acid concentration effect on quinacrine inactivation □ *Escherichia coli* generation—inhibition by quinacrine, in Anton's medium, amino acid concentration effect on quinacrine inactivation □ Microorganisms, inhibition of *E. coli*—quinacrine in Anton's medium, amino acid concentration effect on quinacrine inactivation

The antimalarial (1) quinacrine, 6-chloro-9-[[4-(diethylamino)-1-methylbutyl]amino]-2-methoxyacridine, has been reported to be clinically effective and synergistic *in vivo* (2) and *in vitro* (3–5) in combinations with other antibiotics to delay or prevent the emergence of antibiotic-resistant strains of Gram-negative and Gram-positive bacteria. Quinacrine, a known nucleic acid intercalating agent, inhibits DNA replication and prevents the transfer of R-factors to result in (6, 7) antibacterial activity.

This paper reports on the quantitative microbial kinetics of the Gram-negative *Escherichia coli* (ATCC 12407) as affected by quinacrine. A general theory, recently developed (8) to rationalize the steady-state microbial generation of *E. coli* affected by bacteriostatic drugs, is applied to quinacrine-affected generation rates. Specifically, it is shown how microbial kinetics may be used to quantify the protein binding or nutrient complexation of a bacteriostatic agent when such binding is a saturable process.

EXPERIMENTAL

Test Organism—Replicate slants of *E. coli* (ATCC 12407) were used in all experiments. This nonclumping strain is well suited for colony counts and for small particle monitoring[1].

Culture Medium—Only Anton's medium (9) was used in this study. Its preparation and the variations of the broth employed were described previously (8).

Antibiotics—The antibiotics used in combination experiments were all assayed antibiotics and included quinacrine hydrochloride[2] USP (hereafter referred to as quinacrine), chloramphenicol[3] (purity grade), and tetracycline hydrochloride[4] USP.

[1] Coulter counter.
[2] Winthrop Laboratories, New York, N.Y.
[3] Thomae GMBH, Germany.
[4] The Upjohn Co., Kalamazoo, Mich.