Kinetics and Mechanisms of Action of Antibiotics on Microorganisms I

Reproducibility of *Escherichia coli* Growth Curves and Dependence Upon Tetracycline Concentration

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A method which minimizes the lag period of measuring growth rates of bacterial cultures using plate counts has been devised. The standard deviation, expressed as a percentage of the mean among replicate growth rate determinations for this strain of *Escherichia coli*, was 3.7 per cent. It has been shown that the apparent growth rate constants in the absence of antibiotic were independent of inoculum size. Growth rates and rates of kill have been obtained with cells in the constant growth phase in the presence of graded concentrations of tetracycline hydrochloride. Although low concentrations of antibiotic merely reduced the growth rate, increased concentrations had a definite bactericidal effect. After the initial kill with bactericidal concentrations of tetracycline, survivors multiplied at rates which appeared to be a function of the antibiotic concentration. The growth rate of the numbers of viables, X, can be quantitatively expressed as a function of tetracycline concentration, D, and the growth rate constant, k_o, so that $dx/dt = (k_o - kD^n)X$. For a net increase in viables, the rate is a function of the first power of the antibiotic concentration, whereas a dependence upon the concentration to the one-third power best describes the initial rate of change of numbers of viables when the bactericidal activity is experimentally manifested.

THE ELUCIDATION of the effect of antibiotics on the growth rate of microorganisms is complicated by the classical procedure of introducing organisms from steady state cultures into solutions of antibiotic in broth (1). The growth process undergoes an induction period when a degree of cell maturation precedes the logarithmic growth phase characterized by

$$X = X_o e^{kt} \qquad \text{(Eq. 1)}$$

where X_o is the cell concentration at some time $t = t_o$ during this growth phase, and X is the number of cells per unit volume at time t. The growth rate is characterized by the apparent first-order rate constant, k (2).

A systematic approach to the effect of an antibiotic in graded concentrations would be to analyze the induction and log growth phases separately. The latter can readily be studied by combining antibiotic with logarithmic phase cells. It can then be assumed that on a statistical basis each viable cell is reproducing at the same rate as the next (3, 4). There are two convenient procedures: (*a*) the introduction of cells from a master culture in the exponential growth phase into a broth solution of antibiotic, and (*b*) the introduction of antibiotic into a culture of log phase cells.

In these circumstances, any change in the net rate of growth characterized by the constant k can be assigned to the effect of the antibiotic. This can be deduced by the change in slope of the logarithmic transformation of Eq. 1 as a function of antibiotic concentration

$$\log X = kt/2.303 + \log X_o \qquad \text{(Eq. 2)}$$

It is apparent that for any net increase in the number of viables the diminution of the rate of growth compared to the rate in the absence of antibiotic can be assigned to actual "kill" of the microorganism and/or to a reduction in the generation rate. For any measured decrease in colony count from the time of antibiotic addition, the validity of the term "kill" is unquestioned, since it is operationally defined as a reduction in the numbers of cells capable of colony formation under the stated conditions.

It could be postulated *a priori* that if the decrease in the apparent growth rate constant is a function of antibiotic concentration for all log viable *versus* time plots for all antibiotic dosage, then "kill" by antibiotic as defined is valid, even for a positive but lessened growth rate. However, if the nature of the functional dependence of growth rate on antibiotic dosage varies with antibiotic dosage, then the interpretation is necessarily uncertain, and only when the apparent $k<0$ can "kill" be concluded.

It is difficult to deduce the relative contribu-

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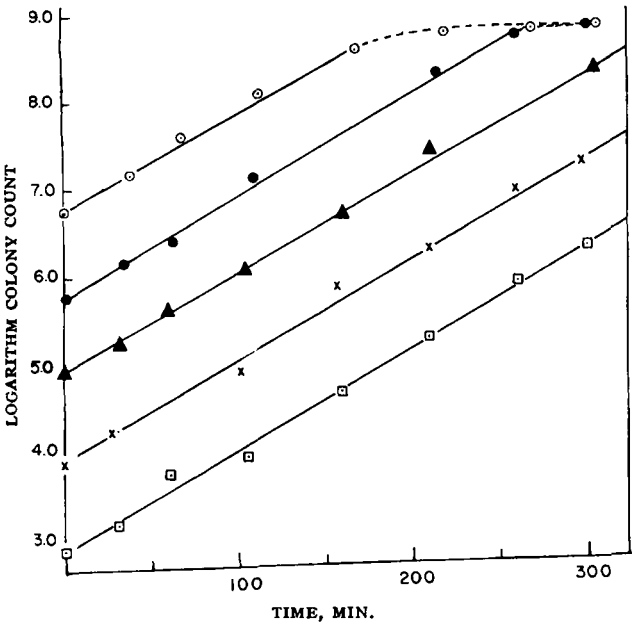

Fig. 1.—Growth curves for *E. coli* using graded inocula concentrations.

Table I.—Apparent First-Order Growth Rate Constants for Replicate *E. coli* Cultures on Different Days

Replicate Growth Rates		
Day 1	Day 2	Day 3
0.01202	0.01153	0.01178
0.01165	0.01110	0.01145
0.01193	0.01121	0.01147
0.01129	0.01113	0.01130
0.01124	0.01118	0.01169

EXPERIMENTAL

Methods.—The test organism was *Escherichia coli* strain E/r. A broth culture from an isolated colony was used to inoculate replicate agar slants using Bacto antibiotic medium 3;[1] these slants were then stored frozen. This medium was also used for broth cultures and plate counts. Dilutions for plate counts were made using distilled water containing 0.85% sodium chloride. Plates were incubated for at least 48 hours before counting, and subsequently re-examined for appearance of new colonies after further incubation. Assayed tetracycline hydrochloride (995 mcg./mg.) was supplied by courtesy of The Upjohn Co. Antibiotic solutions in distilled water containing 2 mg./milliliter were sterilized by filtration, then stored frozen. Fresh slants and antibiotic solution were used for each experiment.

Reproducibility of Growth Rate Measurements.—Growth rates were measured with a Klett-Summerson colorimeter and also by plate counts. The colorimeter was used mainly as a screening device prior to viable count experiments. A measured volume of an overnight broth culture was diluted into broth

Table II.—Analysis of Variance of Apparent First-Order Growth Rate Constants for Replicate *E. coli* Cultures Between and Within Days

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Components of Variance	Variance Ratio (F)
Between days	2	5.0×10^{-7}	2.5×10^{-7}	$5\sigma_B^2 + \sigma_W^2$	4.3
Within days	12	7.0×10^{-7}	5.8×10^{-8}	σ_W^2	
Totals	14	12×10^{-7}			

σ_W^2 = variance within days = 5.8×10^{-8}
σ_B^2 = variance between days = 3.8×10^{-8}
$\sigma^2 = \sigma_W^2 + \sigma_B^2 = 9.6 \times 10^{-8}$
$\sigma = 3.1 \times 10^{-4}$

S.D.: % of Mean = 2.7

Tabulated value of (F) at 0.05 Significance level = 3.9

tions of cells whose generation rate has been reduced (perhaps to zero), cells which have lost the capacity to form colonies, and cells which continue to reproduce at an unchanged rate. All of these types of cells may contribute to an observed reduction in colony count with time.

This paper presents initial results of an investigation of these concepts. The effect of inoculum size upon the exponential growth rate and its reproducibility has been tested. Attempts have been made to fit a functional dependence of rate upon antibiotic dosage.

[1] Difco Laboratories, Detroit, Mich.

TABLE III.—APPARENT FIRST-ORDER GROWTH RATE CONSTANTS FOR *E. coli* USING INOCULA OF GRADED CONCENTRATION

Initial Concn., approx.	Rate Constant
10^3	0.0118
10^4	0.0115
10^5	0.0117
10^6	0.0115
10^7	0.0113

$$\sigma^2 = \Sigma \frac{(x - \bar{x})^2}{n - 1} = 5.4 \times 10^{-8}$$

at 37.5°, the growth rate followed turbidimetrically at 500–570 mμ and at a predetermined turbidity, so that the cells known to be in the logarithmic phase were diluted into five replicate volumes of broth at 37.5°. This procedure of diluting logarithmic phase cells into warm broth reduces any lag phase to a minimum (Fig. 1). Dilutions of the replicate cultures were plated out at intervals; these intervals were chosen after preliminary experiments had indicated which were suitable for determination of growth rates. These growth rate determinations for five replicate cultures were repeated on each of 3 days. The correlation coefficients were calculated for the logarithm of viable count against time for the part of the curves preceding th tionary phase. *In every* case the correlatior highly significant ($P > 0.99$); Table I lists th culated apparent first-order growth rate cons obtained on each of the 3 days. Table II pre the results of an analysis of variance of the gr rate constants between and within days. The minations of reproducibility of viable counts made with cultures in Klett-Summerson colori tubes since the reproducibility of the colorir determinations was established concurr Erlenmeyer flasks (250-ml.) were used conta 20 ml. of culture.

Effect of Inoculum Size.—Graded concentra of logarithmic phase cells were used to inoc broth previously warmed to 37.5°, and the gr curves of the resulting cultures were deter by plate count (Fig. 1). Table III lists the gr rate constants obtained for the cultures in Fig

Effect of Tetracycline Concentration.—Fo purposes of this study it is necessary to adc arithmic phase cells to solutions of the antibic prewarmed broth or to add antibiotic to logari phase cells (4). The former procedure was initially in this work, and the results are illust in Fig. 2. Subsequently, it was decided to add biotic to cells in the logarithmic phase as the s

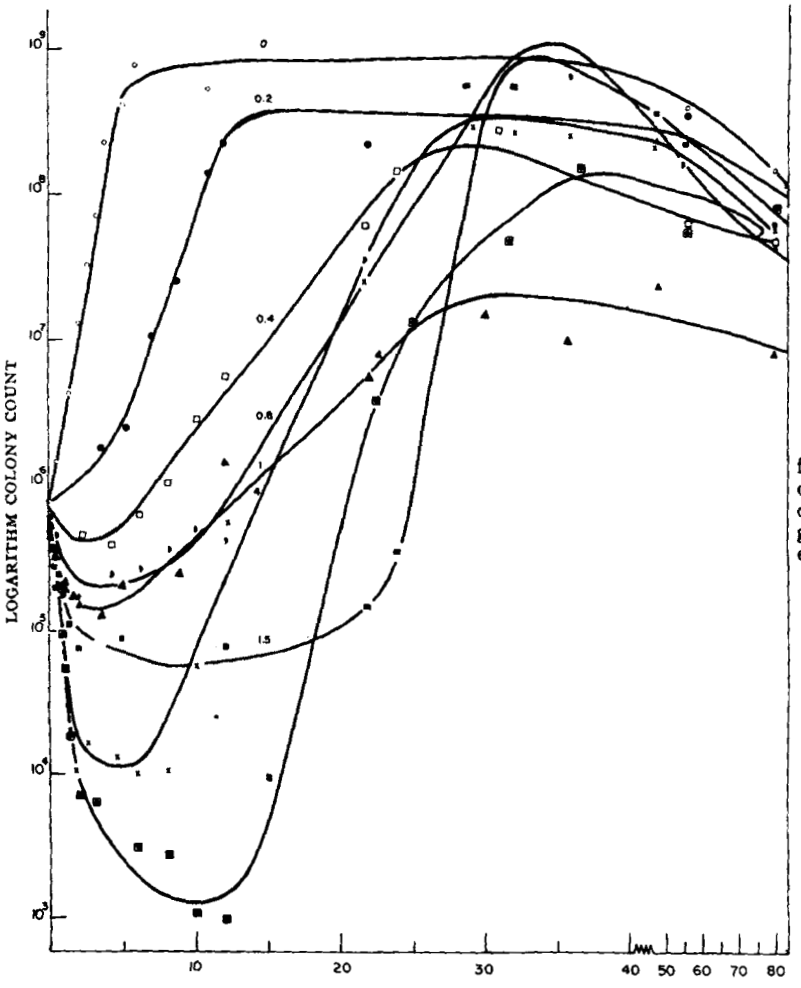

Fig. 2.—Growth for *E. coli* in the pr of graded concentr of tetracycline (grams per millilite extended periods.

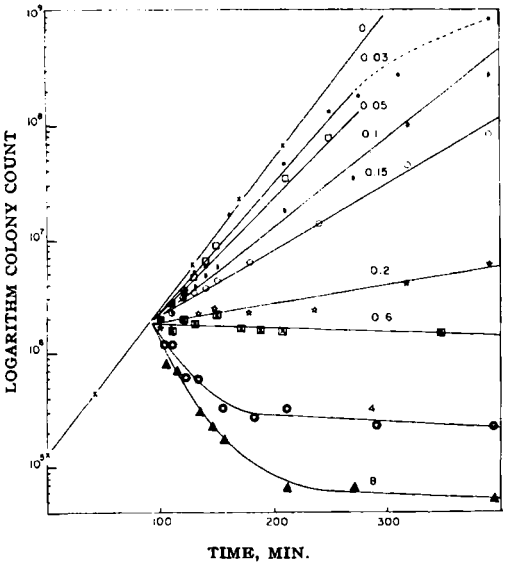

Fig. 3.—Initial growth curves for *E. coli* in the presence of graded concentrations of tetracycline (micrograms per milliliter).

ard method. The procedure was as follows. Nine replicate cultures of logarithmic phase cells were allowed to grow at 37.5° for a predetermined period so that the concentration increased from about 10^5 cells/ml. to about 10^6/ml.; eight of the cultures were then inoculated with tetracycline hydrochloride solution. The cultures then contained 0, 0.03, 0.05, 0.1, 0.15, 0.2, 0.6, 4, and 8 mcg./ml. of antibiotic. Viable counts were made at intervals; Fig. 3 illustrates the results. The growth rate constant was calculated for the initial straight line part of each curve.

DISCUSSION

The results of the analysis of variance of the apparent first-order growth rate constants for replicate *E. coli* cultures show that the between days variance is significantly greater than the within days variance (F test, Table II). The apparent rate constants that were used were uncorrected for the transformation to decadic logarithms, *i.e.*, $S = k/2.303$ for the slope of Eq. 2. The mean value for the rate constants, S, is 0.01146, and the standard deviation for the several studies within days $\sigma_W = 2.4 \times 10^{-4}$ and among days is $\sigma_D = 1.95 \times 10^{-4}$, so that the standard deviation of an S value among and within days is $\sigma = \sqrt{\sigma_W^2 + \sigma_D^2} = 3.1 \times 10^{-4}$ or 2.7% of the mean value.

The procedures used have resulted in an apparent elimination of the lag period of growth for each of the initial concentrations tested (Fig. 1). The variance, $\sigma^2 = 5.4 \times 10^{-8}$ (Table III and Fig. 1) among rate constants for the cultures with various initial inocula concentrations is not significantly different from the variance among rate constants within days in Table II ($\sigma^2 = 5.8 \times 10^{-8}$) for cultures with replicate inocula.

The rates of growth of *E. coli* in the presence of tetracycline are decreased as functions of the concentrations of the antibiotic but are still consistent with Eq. 1. The log X *versus* time plots of Fig. 3 in the presence of antibiotic are consistent with the expectation of Eq. 2, which describes the exponential growth of X_o microorganisms from the time of antibiotic addition. A more general logarithmic expression, in the presence of an ith concentration of tetracycline is

$$\log X = S_i t + \log X_o \qquad \text{(Eq. 3)}$$

where $S_i = k/2.303$, k in minutes^{-1}. The apparent first-order growth rate constant, S_i, is a linear function of the antibiotic concentration, D, in micrograms per milliliter for all positive values of S_i (Fig. 4)

$$S_i = S_o - KD \qquad \text{(Eq. 4)}$$

where $S_o = 1.20 \times 10^{-2}$ is 2.303 times the rate constant $k = k_o$ (minutes^{-1}) for the growth of *E. coli* in the absence of tetracycline, $D = 0$. The specific rate constant for tetracycline concentration effect is

$$K = 4.81 \times 10^{-2}$$

At $S_i = 0$ or less, after an initial period of reasonable linearity, a nonlinear relationship develops between log viables and time. This could be due to consumption or degradation of antibiotic, development of resistant organisms, and/or the presence of antibiotic-resistant strains in the original culture.

The rate of change of viable organisms with time may be a function of the growth rate and the antibiotic concentration (4)

$$1/2.303\ (dX/dt) = S_i X = (S_o - KD^n)X \qquad \text{(Eq. 5)}$$

which integrates to Eq. 3. Consequently, the apparent first-order growth rate to *E. coli* in the presence of tetracycline may be explained on the assumption that the rate constant, S_o, characterizing growth, is in competition with the removal of viable microorganisms. This removal is caused by a rate determining attack of antibiotic molecules on the microorganism. The dependence of the slope S_i on the first power of the antibiotic concentration (Fig. 4) for $S_i > 0$ implies that $n = 1$ in Eq. 5, and that one microorganism may be inactivated by one molecule of antibiotic.

One may conclude from Eq. 4 that if the rate dependency on a power n of antibiotic concentration is permitted

$$\log (S_o - S_i) = n \log D + \log K \qquad \text{(Eq. 6)}$$

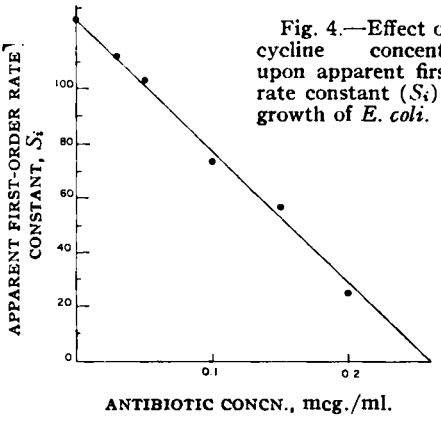

Fig. 4.—Effect of tetracycline concentrations upon apparent first-order rate constant (S_i) for the growth of *E. coli*.

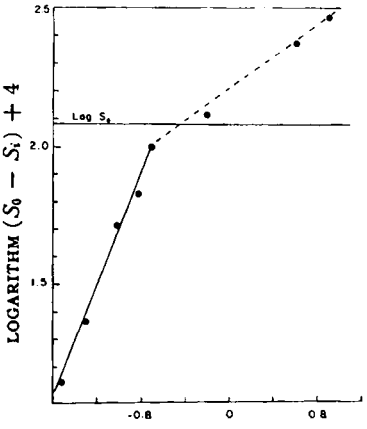

Fig. 5.—Relationship between log ($S_0 - S_i$) and log concentration of tetracycline for *E. coli*. S_0 and S_i are apparent growth rate constants in the absence and in the presence, respectively, of ith concentrations of antibiotic.

A plot of log ($S_o - S_i$) against log D should be linear and of slope positive n. Figure 5 shows that for tetracycline the decrease in growth rate with increased antibiotic concentration is a function of the first power of tetracycline concentration ($n = 1$) for $S_i > 0$. At concentrations of tetracycline sufficiently high to cause a reduction in the logarithm of colony count with time, the initial rates of "kill" are dependent on a fractional power of the antibiotic concentration. Estimates from Fig. 5 for log ($S_o - S_i$) > log S_o; $S_i < 0$ indicate that the rate dependence for "kill" is to the one-third power of antibiotic concentration. These two relationships for $S_i > 0$ and $S_i < 0$ have been reproduced by several replicate experiments made on different days.

The results in Fig. 2 indicate that after an initial "kill" at the higher antibiotic concentrations, the multiplication of the remaining viable organisms was sufficient to overcome the net decrease in colony count. The transition from a negative to a positive slope in Fig. 2 for the 8 mcg./ml. of tetracycline study is an example. On this premise, the survivors of the lower antibiotic concentrations (in the tetracycline concentration range that reduced the total number of viables) multiplied at slower rates than the survivors at the higher concentrations. The bacteria that survived concentrations of 4 and 8 mcg./ml. of tetracycline reproduced at rates approximately the same as those of cultures without antibiotic. The possible implications have been previously discussed, and experiments are in progress to evaluate the possible alternative explanations. A recent experiment with culture containing 8 mcg./ml. of tetracycline has shown more conclusively that the rate of growth of these survivors is not significantly different from that for an initial inoculum in the absence of antibiotic.

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