Kinetics and Mechanisms of Action of Antibiotics on Microorganisms III

Inhibitory Action of Tetracycline and Chloramphenicol on *Escherichia coli* Established by Total and Viable Counts

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The generation rate constants, k from $N = N_0e^{kt}$, $k > 0$, for viable counts of *Escherichia coli* growth have been shown to be linearly dependent on the concentration, A, of the antibiotics tetracycline and/or chloramphenicol, $k = k_0 - k_AA$. Alternative mechanisms of action are by inhibition of *E. coli* generation or by kill superimposed on an invariant generation rate. Methods and techniques have been developed to obtain reproducible total counts by the Coulter counter and have been statistically evaluated. For the case where $k > 0$, total and viable counts are equivalent in the presence of the antibiotics studied, and the mode of action is inhibitory. The dependence of *E. coli* generation rate constants on antibiotic concentration has been shown to be independent of the inoculum size over a range of 10^3 microorganisms/ml. Studies in media of double and half the nutrients, metal-free, reduced, and increased phosphate concentrations demonstrated no significant effects on *E. coli* generation rates.

VIABLE COUNT techniques have been developed to quantify the decrease in the exponential rates of *Escherichia coli* generation as a function of tetracycline and/or chloramphenicol concentrations (1, 2). A comparison of total microorganism counts (viable and nonviable) and colony counts (viable) is necessary to differentiate between two possible modes of action, either inhibition of generation rates or "kill" superimposed on generation. The term "kill" is operationally defined as a reduction in the number of cells capable of colony formation under the stated conditions. The Coulter counter (Coulter Electronics, Hialeah, Fla.) is a convenient tool for the determination of total cell counts (3–6).

The purposes of this paper are to define the action of tetracycline and chloramphenicol as either inhibitory or kill in defined concentration ranges, to establish the statistical reliability of the experimental techniques, and to determine the effect of inoculum size on the established kinetic equations. Also, since growth occurs in a medium with a wide range of natural nutrients, there is the possibility of interactions between constituents and antibiotic effect. These potential interactions are evaluated.

EXPERIMENTAL

Generation Rates.—*E. coli* strain B/r[1] was the test organism, and replicate slants were used for each experiment. The details of the experimental procedure have been described previously (1). A broth culture was allowed to grow for 12 hr. at 37.5°, then diluted into fresh broth and the growth rate determined by turbidimetric measurements with a Klett-Summerson colorimeter. When exponential growth had been established, samples were diluted into replicate 20-ml. volumes of fresh broth in 125-ml. loosely capped conical flasks. In general, sufficient inoculum to achieve a concentration of 10^6 *E. coli* /ml. was used, except when the effect of variable *E. coli* concentrations (10^3, 10^4, 10^5) was studied. The replicate cultures were maintained at 37.5° in a 50-gal. constant temperature water bath equipped with a shaker. After 90 min., sufficient antibiotic was added to the cultures to achieve concentrations of 0.0, 1.04, 2.08, 3.12, or 4.16×10^{-7} M tetracycline or 1.17, 2.33, 3.50, or 4.66×10^{-6} M chloramphenicol. The number of *E. coli* /ml., N, present in the cultures was determined by a viable (plate) count method and/or a total (Coulter) count method at appropriately spaced time intervals. Apparent generation rate constants, k in sec.$^{-1}$, were obtained from the least-squares slopes of a plot of log N *versus* time in accordance with the apparent first-order expression

$$\ln N = kt + \ln N_0 \qquad \text{(Eq. 1)}$$

where N_0 is the number of *E. coli* /ml. at $t = 0$, and t is in seconds.

Viable Count Method.—One milliliter of appropriately diluted (with 0.85% saline) 0.5-ml. aliquots of the culture was pipeted onto each of five replicate agar plates. The plates were incubated for 48 hr. at 37.5°, and the colonies were counted.

Total Count Method.—One-half-milliliter aliquots of culture were diluted with 0.85% saline which had been filtered previously through a double thickness of filter paper (type HA Millipore). A drop of formaldehyde was added, and the total number of organisms was counted with a model A Coulter counter equipped with a 30-μ orifice. The conditions were an aperture current of 5, a gain of 6, and a threshold setting of 10. When necessary, formalde-

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[1] In the prior papers (1, 2) this was erroneously referred to as E/r.

TABLE I.—COMPOSITION OF CULTURE BROTHS USED TO IDENTIFY *E. coli*–BROTH–ANTIBIOTIC INTERACTIONS

Ingredients[a]	Peptone Broth[b] U.S.P.	One-Half Nutrients	Doubled Nutrients	Increased Phosphate	Decreased Phosphate
Beef extract[b]	1.5	0.75	3.0	1.5	1.5
Yeast extract[b]	1.5	0.75	3.0	1.5	1.5
Peptone[b]	5.0	2.50	10.0	5.0	5.0
Dextrose[c]	1.0	0.50	2.0	1.0	1.0
NaCl	3.5	4.40	0.37	2.96	4.07
K_2HPO_4	3.68	3.68	3.68	4.58	2.79
KH_2PO_4	1.32	1.32	1.32	1.64	1.00

[a] The ingredients are given in grams/liter of broth. [b] Difco Laboratories. [c] J. T. Baker Co.

TABLE II.—ANALYSIS OF VARIANCE OF PLATE COUNTS ON DILUTED SAMPLES STORED AT ROOM TEMPERATURE

Sources of Variation	Degrees of Freedom	Sum of Squares	Mean Square[a]
Plating time, *P*	7	125,287	17,878.1
Sample, *S*[b]	2	18,989	9,494.5
Operator, *O*	2	225	112.5
P × *S*	14	15,125	1,080.4
P × *O*	14	4,815	343.9
S × *O*	4	1,020	255.0
P × *S* × *O*	28	4,103	146.5
Error, *E*	288	12,311	42.7
Total	359	181,853	

[a] *F* test: *P* × *S* × *O*:*E* = 3.43; 1% *F* = 1.70 significant. *S* × *O*:*P* × *S* × *O* = 2.74; 5% *F* = 2.71 nonsignificant. *P* × *O*:*P* × *S* × *O* = 2.35; 5% *F* = 2.04 significant. *P* × *S*:*P* × *SO* = 7.37; 1% *F* = 2.75 significant. *O*:*P* × *O* = 1 nonsignificant. *S*:*P* × *S* = 8.19; 1% *F* = 6.51 significant. *P*:*P* × *S* = 16.57; 1% *F* = 4.28 significant. It is of interest to note the high significance, *F* = 8.79, of the sample mean square, which is attributable to the use of an inaccurate pipet in obtaining sample 2 and is not thought to be a factor in other studies.

hyde-inhibited culture aliquots were stored in the freezer for periods not longer than 4 hr.

Effect of Sample Storage on Viable Counts.—Three culture flasks were inoculated with 10^6 *E. coli*/ml. in the exponential growth phase. After 40 min. growth at 37.5°, three aliquots were removed from each of the flasks. An operator assigned to the aliquots from each flask made the appropriate dilutions and five replicate plates after 0, 15, 30, 45, 60, 120, 180, and 240 min. had elapsed.

Effect of Sample Treatment on Total Counts.—One culture flask, 4.16 × 10^{-7} *M* in tetracycline, was inoculated with 10^6 *E. coli*/ml. in the exponential growth phase. Five samples were removed after 20, 50, and 80 min. of growth at 37.5°. One sample at each growth time was appropriately diluted, and the number of *E. coli* present was determined immediately with the Coulter counter. The remaining samples were treated with 1 drop of formaldehyde and stored in a freezer for 80, 120, 185, and 240 min., respectively, at which times the total numbers

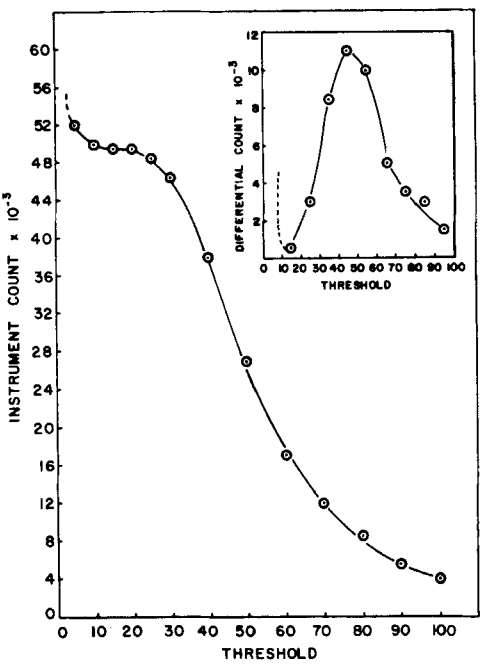

Fig. 1.—The dependence of instrument count upon threshold setting of the Coulter counter. The insert shows the size distribution of *E. coli* in terms of threshold setting. Total counts were obtained at a threshold setting of 10.

TABLE III.—AVERAGE TOTAL COUNT × 10^{-7} AFTER SAMPLE STORAGE AT THREE GROWTH TIMES

Treatment: Formaldehyde	Treatment: Time in Freezer, min.	Growth Time, min.: 20	50	80
0	0	1.62	1.71	1.80
+	70	1.46	1.74	1.78
+	130	1.43	1.66	1.83
+	180	1.55	1.66	1.83
+	240	1.57	1.71	1.91

TABLE IV.—ANALYSIS OF VARIANCE OF TABLE III DATA

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square[a]
Sample storage, *S*	4	8.35 × 10^{12}	2.08 × 10^{12}
Growth time, *G*	2	119.73 × 10^{12}	59.87 × 10^{12}
S × *G*	8	12.98 × 10^{12}	1.62 × 10^{12}
Replicates	4	0.01 × 10^{12}	0.25 × 10^{10}
Error, *E*	56	2.92 × 10^{12}	0.05 × 10^{12}
Total	74	143.99 × 10^{12}	

[a] *F* test: *S* × *G*:*E* = 32.4; 1% *F* = 2.82 significant. *G*:*S* × *G* = 36.96; 1% *F* = 8.65 significant. *S*:*S* × *G* = 1.28; 5% *F* = 3.84 nonsignificant.

TABLE V.—APPARENT FIRST-ORDER GENERATION RATE CONSTANTS FOR *E. coli* GROWTH IN ALTERED ENVIRONMENTS DETERMINED BY TOTAL CELL COUNTS AT 37.5°

Antibiotic 10^7 [Tetracycline HCl]	Peptone Broth[a] U.S.P.	One-Half Nutrients	Doubled Nutrients	Increased Phosphate	Decreased Phosphate	Reduced Metals
0.00	4.88	4.63	4.71	4.67	4.79	4.53
1.04	3.95	3.68	3.86	4.09	3.97	3.91
2.08	3.01	2.80	2.88	3.02	3.03	2.86
3.12	2.24	2.17	2.01	2.21	2.19	2.09
4.16	1.58	1.45	1.28	1.23	1.51	1.05
10^6 [Chloramphenicol]						
1.17	3.62	3.55	3.15	...	3.97	3.83
2.33	2.60	2.57	2.44	2.91	2.79	2.63
3.50	1.46	1.58	0.99	1.44	1.67	1.59
4.66	0.64	0.67	...	0.50	0.67	0.59

[a] Average values of several experiments, total and viable counts.

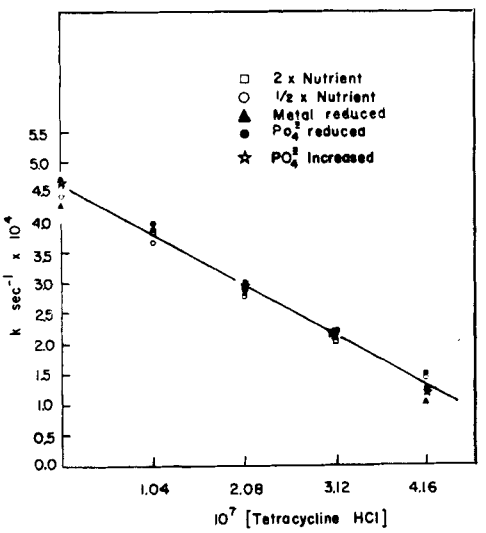

Fig. 2.—Constancy of linearity for growth in modified media of generation rate constants, k, as a function of [tetracycline]. The single slope, k_A, is $7.9 \times 10^{-2}\ M^{-1}$ sec.$^{-1}$.

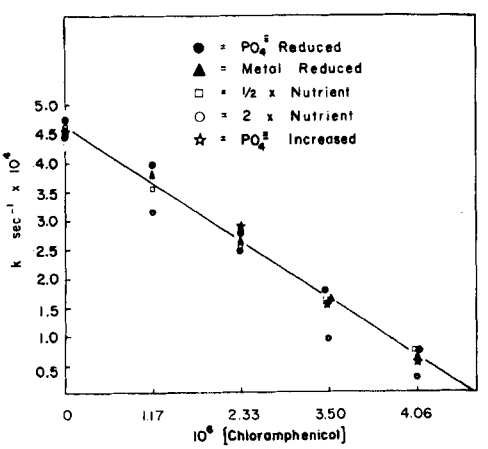

Fig. 3.—Constancy of linearity for growth in modified media of generation rate constants, k, as a function of [chloramphenicol]. The single slope, k_A, is $8.5 \times 10^{-1}\ M^{-1}$ sec.$^{-1}$.

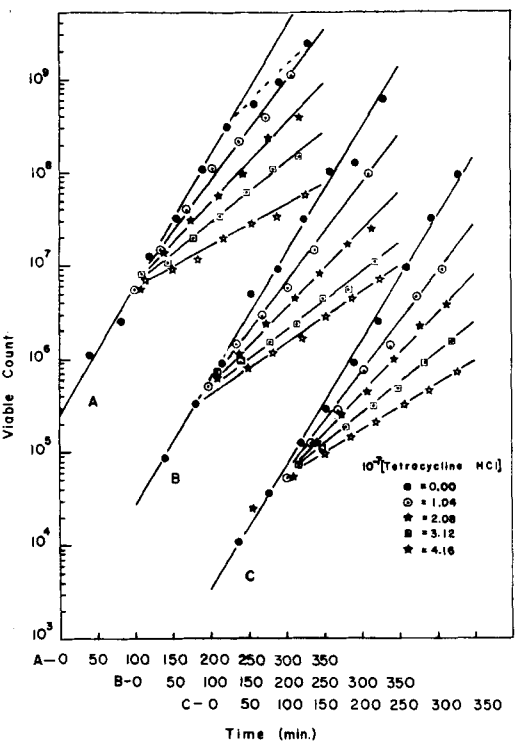

Fig. 4.—Generation curves for *E. coli* in the presence of graded concentrations of tetracycline at three initial *E. coli* concentrations. Lines are fitted to experimental points by the method of least squares.

of *E. coli* present were counted. A similar procedure was used in the absence of tetracycline HCl.

Culture Broth Variations.—Peptone broth, U.S.P.,[2] buffered at pH 7 and filtered through a double thickness of filter paper (type HA Millipore), was used for broth cultures and combined with agar for plate counts. The composition of the media was varied in the several ways listed in Table I. In addition, media was treated to remove di- and trivalent metal ions. This was accomplished by shaking broth with dithizone solution U.S.P. until

[2] Available as Bacto Antibiotic Medium 3, Difco Laboratories, Detroit, Mich.

TABLE VI.—APPARENT FIRST-ORDER GENERATION RATE CONSTANTS[a] SEC.$^{-1}$ × 10^4 OBTAINED BY TWO COUNTING METHODS FROM SEVERAL INITIAL *E. coli* CONCENTRATIONS

Initial *E. coli*/ml.	Counting Method	10^7 [Tetracycline] 0.00	1.04	2.08	3.12	4.16
3 × 10^5	Total	4.83	3.95	2.88	2.25	1.63
3 × 10^5	Viable	5.21	4.11	3.19	2.46	1.69
3 × 10^4	Viable	5.14	4.01	3.12	2.42	2.09
3 × 10^3	Viable	5.21	4.09	3.33	2.30	1.90

[a] The standard error of rate constants varies from 0.03 to 0.12 × 10^{-4}.

the dithizone retained its original color. Excess dithizone was removed by centrifugation and evaporation of the broth to dryness. The dry broth was redissolved in redistilled water.

RESULTS AND DISCUSSION

Analysis of Viable Count Techniques.—Previously (1), the reproducibility of generation rate constants among and within days and for various initial *E. coli* concentrations in the absence of antibiotic had been evaluated. The standard deviation within days for the same and different initial *E. coli* concentrations was 2.1% of the mean rate constant, and its was 2.7% among days.

In many of the studies, the large numbers of samples required make it impossible to plate immediately after sampling. Thus, it is important to determine the effect of storage of diluted samples on obtained values. Table II presents the results of an analysis of variance of plate counts made by three operators on three diluted samples stored at room temperature for various times. The time a sample is allowed to stand before plating is highly significant. Inspection of the data revealed that the difference between 0 and 15 min. of storage is less than 2.5%, whereas between 0 and 30 min. it is 10%. Therefore, it was considered necessary to design all future experiments so that plating was completed within 15 min. of sampling.

Analysis of Total Count Techniques.—The Coulter counter measures the number and size of voltage pulses produced when a suspension of particles passes through an aperture separating two electrodes. Its use for the determination of the total number of *E. coli* present in cultures has been described (3–8). The proper threshold setting for total counts of *E. coli* can be obtained from a plot of instrument count *versus* threshold (Fig. 1). The threshold chosen is one at which electrical noise is minimum, and the numbers of organisms counted is maximum. It was not always possible to count immediately after sampling. For this reason, culture aliquots with added formaldehyde were stored in a freezer for periods of time not greater than 4 hr. Table III lists the results of a study made to determine the effect of this procedure on total cell counts. The results of an analysis of variance (Table IV) of this data indicate that this treatment of samples does not significantly affect total cell counts.

Analysis of Culture Broth Variations.—Table V lists apparent first-order generation rate constants obtained from the growth of the test organism in several environments. The results of analysis of variance of this data indicate that the variation among generation rate constants is not significantly greater than that to be expected from day-to-day variation in organism growth rates, with the possible exception of the case where the nutrients were doubled in the presence of chloramphenicol.

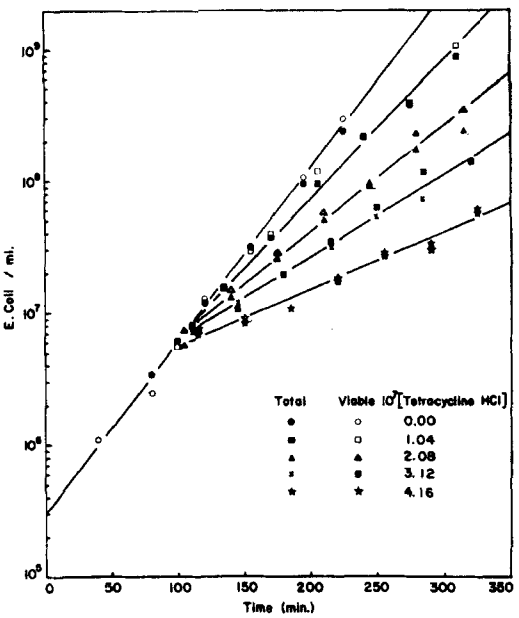

Fig. 5.—Coincidence of generation curves for *E. coli* obtained by total and viable counts in the presence of graded concentrations of tetracycline.

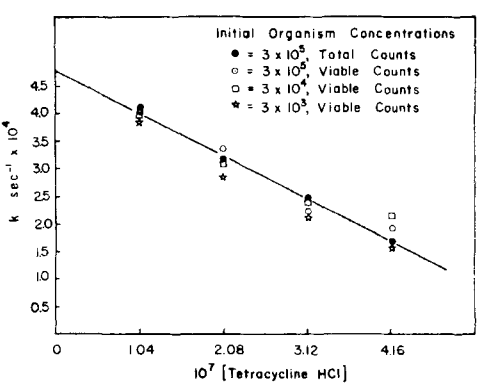

Fig. 6.—Constancy of linearity for growth at several initial *E. coli* concentrations of generation rate constants, k, as a function of [tetracycline]. The single slope, k_A, is 7.7 × 10^{-2} M^{-1} sec.$^{-1}$.

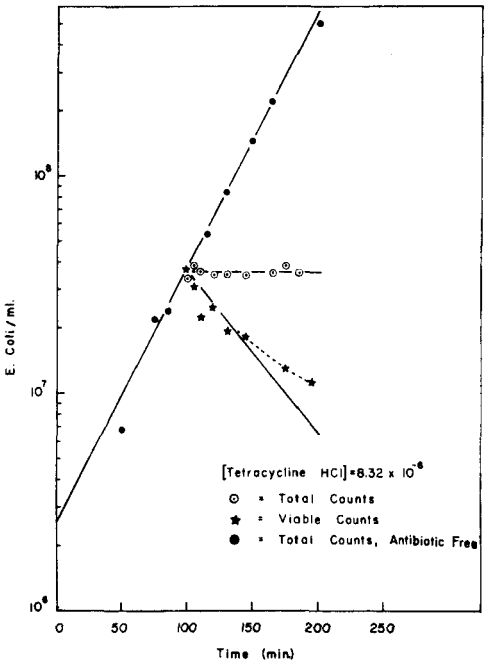

Fig. 7.—Generation curve for *E. coli* in the presence of a killing [tetracycline] obtained by total and viable counts.

Previous papers (1, 2, 9, 10) have shown that, in the concentration ranges under study, plots of apparent generation rate constants *versus* antibiotic concentration are linear with slopes of $8.8 \times 10^{-2}\ M^{-1}$ sec.$^{-1}$ and $8.9 \times 10^{-1}\ M^{-1}$ sec.$^{-1}$ for tetracycline and chloramphenicol, respectively. The slopes obtained from such plots (Figs. 2 and 3) of the data in Table V are in good agreement with the literature. One can conclude that none of the environmental factors (Tables I and V) interact with the effect of the antibiotics on *E. coli* in these studies.

Establishment of Inhibitory Action and Independence of Inoculum Size.—Apparent first-order generation rate constants were obtained for cultures illustrated in Fig. 4 and listed in Table VI. The coincidence of generation rates is attributed to the fact that at any given time the same *E. coli* concentration was obtained by the two counting methods (Fig. 5). Therefore, the decreased generation rates observed in the presence of tetracycline in the specified concentrations must be due to an inhibition of generation time rather than a kill of organisms. This mode of action is independent of the initial organism concentration (Fig. 6). Similar results have been obtained when chloramphenicol inhibited generation was studied (10).

Proof that the use of these two counting methods is capable of demonstrating a kill is shown in Fig. 7. After addition of tetracycline to an *E. coli* culture in a concentration 40 times greater than that used above, there is a decrease in the number of viable organisms. Allison *et al.* (11) in a similar experiment with chloramphenicol have reported a slight increase in total cell counts, but definitive kill was observed with a loss of viable cells.

The previously reported equation (1, 2, 9)

$$k = k_0 - k_A[A] \qquad \text{(Eq. 2)}$$

where k is the apparent generation rate constant in sec.$^{-1}$ (Eq. 1) in the presence of antibiotic, A, and k_0 is the generation rate constant in the absence of antibiotic holds for the present data (Figs. 2, 3, 6). The second-order rate constant, k_A, can now be considered as an inhibitory rate constant for the antibiotics tetracycline HCl and chloramphenicol.

SUMMARY AND CONCLUSIONS

When total counts of *E. coli* are to be obtained with the Coulter counter, the samples may be treated with a drop of formaldehyde and frozen for as long as 4 hr. without materially affecting the count. The time before plating a diluted aliquot of a culture is significant and must be minimized.

The concentrations of nutrients, phosphate buffers, and metal ions in the culture are not significant for the range of variations studied.

Under the conditions of these studies, where tetracycline concentration is below $4.16 \times 10^{-7}\ M$ and chloramphenicol concentration is below $4.66 \times 10^{-6}\ M$, apparent generation rate constants and the effect of the antibiotics are independent of the initial inoculum size. Also, under these conditions, coincidence of total cell counts and viable cell counts in the growth rate studies indicates that there is no measurable kill of the organisms by these antibiotics; therefore, the reduced generation rates must be ascribed to a general inhibition of generation time.

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