be attributed to its excessive age and prolonged storage (31 years) preceding analysis, failure to detect toxins in the more recent collection No. B1 must be due to natural variablility. Block *et al.* (3) report similar results from their study of toxin-containing *Amanita* species in which several specimens identified as *A. verna* produced no toxic symptoms in mice nor could toxins be detected in them chromatographically. It is not clear whether the differences observed by Block *et al.* and by the authors are due to environmental conditions, ontogenetic considerations, or genetic factors, but it must be concluded that the amanita-toxin content of *A. verna* carpophores is extremely variable and, in general, appreciably less than that of *A. bisporigera* or *A. phalloides*.

No amanitins were detected in one sample of *A. virosa* (Tla), but another carpophore (Tlb) collected at the same time from the same site contained a very small amount of α-amanitin (<0.1 mg./Gm.), as did another, more recent collection (T4). Although standard reference works (15, 16) all refer to *A. virosa* as a deadly poisonous species, apparently the only previous experimental work on the subject was that of Ford (17). He found an extract to be toxic to guinea pigs and concluded that the toxins of the species were identical to those of *A. phalloides*. Identification of small amounts of α-amanitin in two carpophores from different collections confirms this earlier finding. However, as in the case of *A. verna*, the effects of environment, ontogenesis, and genetics must all be investigated before a definite explanation can be given for the irregular low-level occurrence of toxins in *A. virosa*.

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Kinetics and Mechanisms of Action of Antibiotics on Microorganisms V

Chloramphenicol and Tetracycline Affected *Escherichia coli* Generation Rates

By EDWARD R. GARRETT, GEORGE H. MILLER, and M. R. W. BROWN*

Total and viable count methods were used to study *Escherichia coli* generation rates at various temperatures and tetracycline and chloramphenicol concentrations. The negative dependence of apparent first-order generation rate constants on concentrations of these antibiotics was determined for all temperatures. The coincidence of the heat of activation for *E. coli* growth in antibiotic-free media and for the inhibitory rate constants was observed. The facile reversibility of antibiotic effects to predictable rates of *E. coli* generation in the subinhibitory concentration ranges of these antibiotics was demonstrated. A quantitative model consistent with the observed concentration dependencies and observed reversibilities is proposed which relates antibiotic partitioning from the media, a critical value for protein synthesis that results in microbial generation, and the present concept that these antibiotics compete for ribosomal binding sites and so inhibit the function of messenger RNA in protein synthesis.

THE CLINICAL importance of chloramphenicol and tetracycline has resulted in many investigations of their biochemical mode of action (1–4) and is generally ascribed to the inhibition of protein synthesis which has been principally observed under conditions of complete inhibition of growth. A more complete understanding of the action of these antibiotics could be realized if the kinetics of bacterial generation was more completely elucidated in antibiotic concentrations less than those that result in complete growth inhibition, *i.e.*, subinhibitory concentrations.

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* Present address: School of Pharmacy, Bristol College of Science and Technology, Ashley Down, Bristol, England.

Previous reports from this laboratory (5–8) have established methods for the determination of population growth rates of *Escherichia coli* by viable and/or total cell count methods in the presence of these antibiotics either alone or in combination and have demonstrated the linear dependency of such growth rates upon antibiotic concentration. This was a general inhibition of growth rate at 37.5° rather than kill superimposed on an uninhibited growth at subinhibitory antibiotic concentrations. At higher antibiotic concentrations a definite organism kill occurred where kill was defined as a loss of the ability to produce colonies when a diluted sample was incubated on an agar plate.

Fassin *et al.* (9) have reported some work on the temperature dependency of chloramphenicol action using inhibitory and subinhibitory antibiotic concentrations. A more complete kinetic knowledge of the effect of temperature on the kinetics of antibiotic inhibited *E. coli* growth and the derived apparent heat of activation, ΔH, of inhibition are needed to gain insight into the energy requirements of such inhibition.

A lag period in *E. coli* growth after removal of the bacteria from a medium containing inhibitory concentrations of chloramphenicol has been indicated (10). Since the subinhibitory concentrations of chloramphenicol are thought to stimulate RNA synthesis more than higher concentrations (11, 12), a more detailed knowledge of the reversibility of the inhibition of population growth rates at these concentrations is necessary for the formulation of a kinetic mechanism of action.

EXPERIMENTAL

Organism.—All experiments were carried out with *E. coli* strain B/r, a strain that had been employed in previous experiments in this laboratory (5–8).

Medium.—Peptone broth (U.S.P. XVII) was used as the culture broth and combined with agar for colony counts.

Materials.—Assayed samples of tetracycline hydrochloride were supplied by courtesy of The Upjohn Co., Kalamazoo, Mich., and chloramphenicol by courtesy of Parke, Davis and Co., Detroit, Mich.

Temperature Studies.—A broth culture was allowed to grow for 12 hr. at 37.5°, diluted into fresh broth, and the growth rate determined by turbidimetric measurements with a Klett-Summerson colorimeter. When exponential growth had been established, sufficient inoculum to achieve a concentration of 10^6 *E. coli*/ml. was added to fresh broth to make 20-ml. replicate cultures. The cultures were maintained at either 25, 30, 31, 34, 35, 37, 37.5, 41, 43, or 45° on different days in constant-temperature water baths equipped with a shaker. Sufficient antibiotic was added after 90 min. 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 (colony) count method and/or a total count method at appropriately spaced time intervals (5–8). For total counts, the Coulter counter (Coulter Electronics, Hialeah, Fla.) was used. Apparent specific growth rate constants, $k_{app.}$ 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,

$$\log N = (k_{app.}/2.303)t + \log N_0 \quad \text{(Eq. 1)}$$

where N_0 is the number of *E. coli*/ml. at $t = 0$, and t is in sec. Regression analysis was performed by an IBM 720 digital computer.

Reversibility Studies.—Three replicate culture flasks were inoculated with sufficient *E. coli* to yield initial concentrations of 10^6 *E. coli*/ml.

Sufficient antibiotic to achieve concentrations of 0.00 and 4.16×10^{-7} *M* tetracycline and 4.66×10^{-6} *M* chloramphenicol was added to the cultures after 90 min. of growth. After 200 min. of growth, a tenfold dilution of the cultures was achieved by adding 2 ml. of the cultures to 18 ml. of fresh broth. The number of *E. coli* present in the cultures was determined by total counts at approximately equally spaced time intervals.

Three replicate culture flasks were inoculated with sufficient *E. coli* to yield an initial concentration of 10^6 *E. coli*/ml. Sufficient antibiotic to achieve concentrations of 0.00 and 1.04×10^{-7} *M* tetracycline and 1.17×10^{-6} *M* chloramphenicol was added after 90 min. of growth. Final concentrations of 0.00 and 1.04×10^{-7} *M* tetracycline and 4.16×10^{-6} *M* chloramphenicol were achieved with the addition of more antibiotic after 150 min. of growth. The number of *E. coli* present in the cultures was determined at appropriately spaced time intervals by total counts.

Viable Count Method.—One milliliter of appropriately diluted (with 0.85% saline) 0.5-ml. samples of the culture was pipeted onto each of 5 replicate agar plates within 15 min. of sampling. Previous experiments (7) have shown the necessity of completing plating within this time interval.

Total Count Method.—One-half milliliter samples of culture were diluted with 0.86% saline which had been previously filtered through a double thickness of type HA Millipore filter paper. 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, formaldehyde-inhibited culture aliquots were stored in the freezer for periods not longer than 4 hr. before counting. This procedure does not materially affect total counts (7).

RESULTS

Temperature Studies.—Apparent first-order generation rate constants as per Eq. 1 for the growth of *E. coli* in the presence of tetracycline and chloramphenicol at various temperatures are given in Table I. When the tetracycline concentration is

TABLE I.—APPARENT FIRST-ORDER RATE CONSTANTS (10^4 k IN sec.$^{-1}$) FOR *E. coli* GROWTH AT VARIOUS TEMPERATURES

	10^6 [Chloramphenicol]					10^7 [Tetracycline]			
° C.	0.00	1.17	2.33	3.50	4.66	1.04	2.08	3.12	4.16
25.0[a]	1.18	1.13	0.91	0.70	0.52	1.13	0.80	0.56	0.42
28.0[b]	1.74	1.69	1.31	0.90	0.63	1.78	1.18	0.88	0.63
30.0[a]	2.37	2.18	...	1.32	0.96	2.08	1.44	1.14	0.68
31.0[b]	2.39	2.18	1.57	1.12	0.80	2.17	1.48	1.09	0.74
34.0[b]	3.11	2.69	1.93	1.24	0.53	2.79	1.95	1.38	0.68
37.5[c]	4.88	3.62	2.60	1.46	0.64	3.95	3.01	2.24	1.58
39.0[a]	5.36[d]	4.10[d]	2.53	1.94	1.84	5.14[d]	2.99[d]	2.50[d]	...
41.0[b]	4.60	3.97	2.60	1.35	0.72	3.85	3.06[d]	...	2.01
41.0[a]	5.03	...	2.87	1.53	...	4.19	3.15[d]	3.10[d]	2.09[d]
43.0[b]	4.08	3.54	2.52[d]	1.40	0.62	3.87	3.04	2.49	1.96
43.0[a]	4.89[d]	4.23[d]	2.70[d]	1.55[d]	0.75[d]	4.43[d]	3.45[d]	3.04[d]	2.19[d]
45.0[b]	2.07	1.40	1.16	0.66	0.52	1.56	1.63	1.48	1.15
45.0[a]	2.19	1.56	1.16	0.89	0.47	1.80	1.95	1.57	0.88

[a] Derived from viable counts by colony counter. [b] Derived from total counts by Coulter counter. [c] Averaged from several total and viable studies. [d] The 95% confidence limits of these k values were ±0.06–0.10. All others were ±0.02–0.03.

below 4.16×10^{-7} M (0.2 mcg./ml.) or the chloramphenicol concentration is below 4.66×10^{-6} M (1.5 mcg./ml.), a net growth is observed; *i.e.*, $k > 0$. A coincidence of total and viable cell counts was observed during this reduced but exponential growth of the population in the presence of chloramphenicol. A typical example of such plots is given in Fig. 1. The mode of action at 37.5° has been shown to be an inhibition of population growth rate rather than kill superimposed on growth in similar experiments for tetracycline (7). Thus, the mode of action appears to be independent of the temperature range studied and similar for both chloramphenicol and tetracycline (Table I).

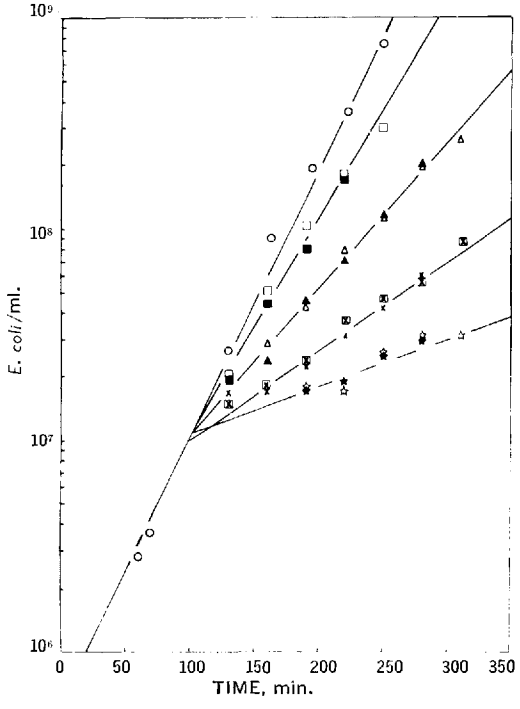

Fig. 1.—Example of coincidence of viable (open symbols) and total (solid symbols) counts for the logarithmic growth of *E. coli* in the presence of various concentrations of chloramphenicol at 41°. Key: ○, 0.00×10^{-6} M chloramphenicol; ■ and □, 1.17×10^{-6} M chloramphenicol; ▲ and △, 2.33×10^{-6} M chloramphenicol; × and ⊠, 3.50×10^{-6} M chloramphenicol; ★ and ☆, 4.66×10^{-6} M chloramphenicol.

It has been shown (5–8) that the specific growth rate constants obtained at 37.5° are linearly dependent upon the concentrations of tetracycline or chloramphenicol, A,

$$k_{app.} = k_0 - k_a A,\ k > 0 \qquad \text{(Eq. 2)}$$

where k_0 is the population growth rate constant in the absence of antibiotic. The inhibitory coefficient, k_a, may be obtained from plots of generation rate constants *versus* antibiotic concentration (Fig. 2). This linear relationship was found to hold over the entire temperature range studied, and the inhibitory coefficients are summarized in Table II.

The Arrhenius equation for the dependence of reaction rate constants upon temperature is

$$\log k = \log P - (\Delta H/2.303R)\ (1/T) \qquad \text{(Eq. 3)}$$

where R is 1.987 cal./mole and T is in degrees Kelvin. A typical Arrhenius plot for *E. coli* growth in the absence of antibiotics is shown in Fig. 3. The heat of activation is 20.8 ± 1.6 Kcal./mole for the linear portion. A similar temperature dependence is exhibited by the inhibitory coefficients, k_a, of Eq. 2. The heats of activation for k_a are 20.6 ± 1.5 Kcal./mole for tetracycline HCl and 23.7 ± 0.4 Kcal./

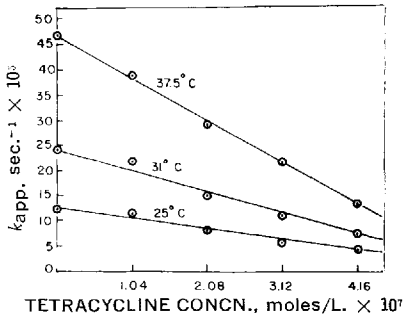

Fig. 2.—Typical example of the dependence of the apparent first-order generation rate constant for *E. coli* growth on antibiotic concentrations.

TABLE II.—APPARENT INHIBITORY CONSTANTS, k_a, FOR *E. coli* AT VARIOUS TEMPERATURES, k IN sec.$^{-1}$

° C.	25.0[a]	28.0[b]	30.0[a]	31.0[b]	34.0[b]	37.5[c]	41.0[b]	41.0[a]	43.0[b]	43.0[a]	45.0[b]	45.0[a]
Chloramphenicol	15	26	32	36	57	85	89	89	83	84	27	32
Tetracycline	180	280	330	390	550	790	660	660	600	640	200	310

[a] Derived from viable counts by colony counter. [b] Derived from total counts by Coulter counter. [c] Averaged from several total and viable studies.

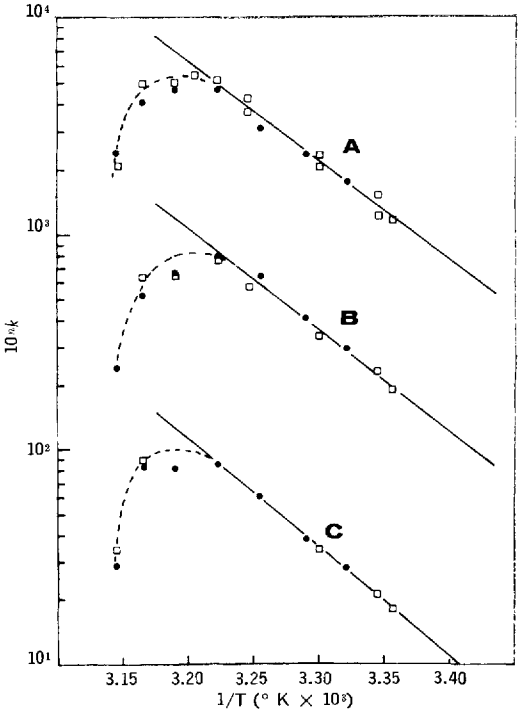

Fig. 3.—Arrhenius plots for the apparent first-order growth of *E. coli* and the inhibitory rate constants of such growth by tetracycline and chloramphenicol as determined from both total (solid symbols) and viable (open symbols) counts. Key: A, antibiotic free, $n = -8.0$; B, tetracycline inhibited, $n = 0$; C, chloramphenicol inhibited, $n = 0$.

mole for chloramphenicol. The coincidence of total and viable count methods are clearly demonstrated in the plots.

Reversibility.—Evidence that the inhibition of growth rates caused by these antibiotics is reversible in the subinhibitory range is shown in Fig. 4 for the chloramphenicol case. Cultures inhibited by the antibiotics, chloramphenicol and tetracycline, revert to population growth rates coincident with those found in the presence of very small concentrations of these antibiotics when they are diluted into fresh broth (Table III). Alternatively (Fig. 5), cultures inhibited by low concentrations of these antibiotics may be further inhibited by the addition of more antibiotic to give growth rates with the predictable rate constants (Table IV). Therefore, the inhibition of the growth rates caused by these antibiotics in the subinhibitory concentrations under study is reversible, at least to the same extent as for cultures in antibiotic-free media on dilution.

DISCUSSION

An operational model for the interference of antibiotic with protein synthesis in the individual cell can be constructed

$$A \rightleftarrows A' + R \underset{k_2}{\overset{k_1}{\rightleftarrows}} (A'R) \qquad \text{(Eq. 4)}$$
$$K_1 \qquad K_2$$

A basic postulate is that the concentration of the antibiotic A in the media is readily partitioned into the cell with an equilibrium constant K_1, where A′ is the antibiotic concentration within the cell. There are R unreacted receptor sites within the cell

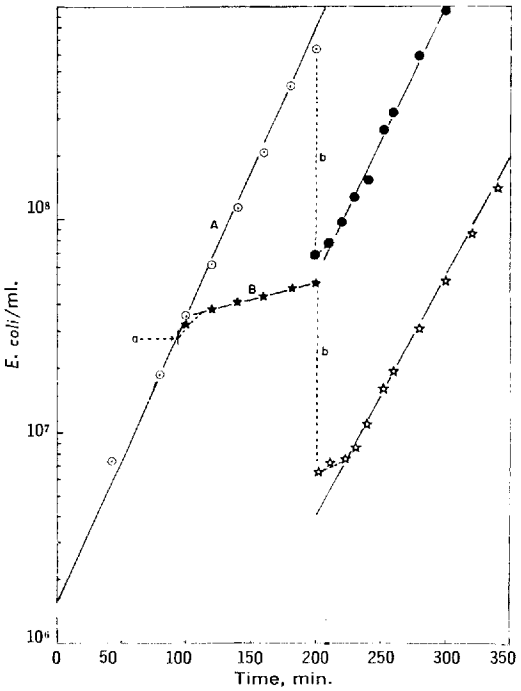

Fig. 4.—Example of reversibility of *E. coli* growth rate on dilution of chloramphenicol concentration. Curve A represents the logarithmic growth phase of an antibiotic-free culture. At time *a*, 4.66×10^{-6} moles/L. chloramphenicol are added to maintain the apparent first-order growth rate represented by line B. At time b, both the antibiotic-free culture, A, and the chloramphenicol culture, B, are diluted tenfold to demonstrate rapid reversibility of culture growth rates on dilution of antibiotic concentration in the subinhibitory antibiotic concentration range. Key: ⊙ = 0, chloramphenicol; ● = 0, chloramphenicol; ★ = 4.66×10^{-6} moles/L. chloramphenicol; ☆ = 4.66×10^{-7} moles/L. chloramphenicol.

TABLE III.—THE EFFECT OF DILUTION ON THE APPARENT GENERATION CONSTANTS, $10^4\ k$, OF *E. coli* CULTURES INHIBITED BY ANTIBIOTICS AT 37.5°

Before Dilution			After Tenfold Dilution		
$10^7\ M$ Antibiotic	Calcd. $10^4\ k^a$	Actual $10^4\ k^b$	$10^7\ M$ Antibiotic	Calcd. $10^4\ k^a$	Actual $10^4\ k^b$
0.0	4.88	4.90 ± 0.06	0.0	4.88	4.88 ± 0.10
46.6 (chloramphenicol)	0.92	0.73 ± 0.09	4.66 (chloramphenicol)	4.48	4.18 ± 0.03
4.16 (tetracycline)	1.60	0.80 ± 0.04	0.42 (tetracycline)	4.55	4.82 ± 0.03

[a] The calculations from $k = k_0 - k_a$ [antibiotic]. [b] 95% confidence limits of rate constant included.

that when interacted with the antibiotic in a reversible equilibrium, K_2, inhibit protein synthesis.

The subsequent development will consider that the rate of protein synthesis is proportional to unbound receptor sites. In order to account for the lack of direct proportionality between the rates of protein synthesis and the rates of reproduction of microorganisms, it will be necessary to introduce the concept that a minimum rate of protein synthesis is necessary for microbial generation. The rate of population increase will then be considered as proportional to this net rate of protein synthesis and to the number of organisms in the balanced-growth growth culture. Although total protein synthesis will be considered in the model, it is plausible that the diminution in reproductive rates may involve selective inhibition in the synthesis of specific proteins. The development will then be evaluated against the experimental information of this paper and the data available in the literature.

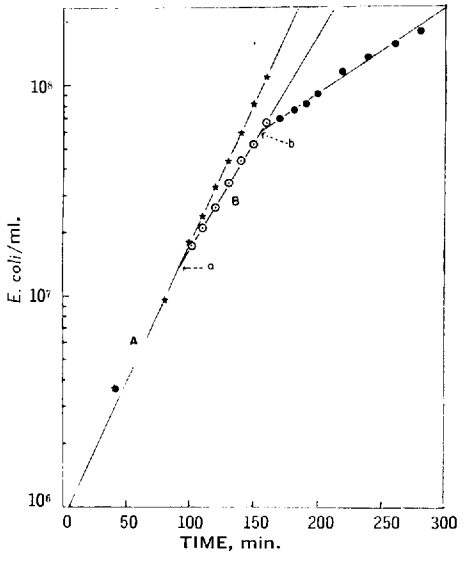

Fig. 5.—Example of rapid dependence of apparent generation rate constants of *E. coli* growth on increased amounts of antibiotic concentration. At times a and b, amounts of tetracycline were added to adjust the antibiotic concentrations to subinhibitory antibiotic concentrations specified. Key: ★ = 0, tetracycline; ⊙ = 1.04×10^{-7} moles/L.; ● = 4.16×10^{-7} moles/L.

The simplest postulate to make is that if Θ is the fraction of receptor sites reacted with antibiotic, the rate of protein synthesis, dP/dt, in a bacterium is proportional to the fraction, $1 - \Theta$, of receptor sites that are free.

$$dP/dt = k_P\,(1 - \Theta) \qquad \text{(Eq. 5)}$$

An implicit assumption in this postulate is that the numbers of receptor sites in a single bacterium are constant and independent of the mass of the cell or a time dependency of protein synthesis. Possible cases where this is not assumed are considered in the *Appendix*.

The equilibrium constant, K_2, for the antibiotic-site interaction of Eq. 4 may be defined by

$$K_2 = k_2/k_1 = (A'R)/[R_T - (A'R)](A') = (A'R)/(R)(A') \qquad \text{(Eq. 6)}$$

where (A'R) is the number of reacted sites, R is the number of unreacted sites, and R_T is the total number of sites whether reacted or unreacted. Thus, one may define the fraction, Θ, of sites reacted with antibiotic on appropriate rearrangement of Eq. 6 as

$$\Theta = (A'R)/R_T = K_2A'/(1 + K_2A') \qquad \text{(Eq. 7)}$$

Thus, Eq. 5 becomes

$$dP/dt = k_p - k_pK_2A'/(1 + K_2A') \qquad \text{(Eq. 8)}$$

where k_p is the steady-state rate of protein synthesis in the absence of antibiotic.

The concentration of antibiotic, A', in the cell may be related to the concentration, A, in the media by

$$A' = K_1A \qquad \text{(Eq. 9)}$$

It follows that the rate of protein synthesis by a

TABLE IV.—THE EFFECT OF ANTIBIOTIC ADDITION ON APPARENT GENERATION RATE CONSTANTS OF *E. coli* CULTURES INHIBITED BY ANTIBIOTICS AT 37.5°

Before Addition			After Addition		
$10^7\ M$ Antibiotic	Calcd. $10^4\ k^a$	Actual $10^4\ k^b$	$10^7\ M$ Antibiotic	Calcd. $10^4\ k^a$	Actual $10^4\ k^b$
0.00	4.88	4.90 ± 0.06	0.00	4.88	...
11.7 (chloramphenicol)	3.87	3.98 ± 0.11	46.6 (chloramphenicol)	0.92	1.05 ± 0.03
1.04 (tetracycline)	4.06	3.80 ± 0.07	41.6 (tetracycline)	1.60	1.48 ± 0.05

[a] Calculated from $k = k_0 - k_a$ [antibiotic]. [b] 95% confidence limits of rate constant included.

single bacterium is related to the antibiotic concentration A in the media by

$$dP/dt = k_p - k_pK_1K_2A/(1 + K_1K_2A) \quad \text{(Eq. 10)}$$

This rate of protein synthesis is associated with the generation time for a single organism, but not necessarily directly since it is reasonable to assume that a large fraction of the protein synthesized is consumed in sustaining processes for the maintenance of vitality and that a certain excess is necessary for reproduction.

If the minimum rate of protein synthesis necessary for reproduction division is k_p', the net rate of protein synthesis that results in reproductive division of a single cell is

$$dP'/dt = dP/dt - k_p' \quad \text{(Eq. 11)}$$

which in the absence of antibiotic is $k_p - k_p'$.

If it is postulated that the rate of population increase of N organisms is proportional to this net rate of protein synthesis and to the number of organisms in the balanced growth culture, it follows that

$$dN/dt = q(dP/dt - k_p')N \quad \text{(Eq. 12)}$$

where the proportionality constant q has dimensions of bacteria per unit of protein. Equation 10 may be substituted into Eq. 12.

$$dN/dt = [q(k_p - k_p') - qk_pK_1K_2A/(1 + K_1K_2A)]N \quad \text{(Eq. 13}a\text{)}$$

$$= [k_0 - k_aA/(1 + k_bA)]N \quad \text{(Eq. 13}b\text{)}$$

$$= k_{app.}N \quad \text{(Eq. 13}c\text{)}$$

where

$$k_a = k_b(k_0 + qk'_p) \quad \text{(Eq. 14)}$$

where the apparent first-order generation constant, $k_{app.}$, is observed in the presence of a constant antibiotic concentration, A. The inconsistency of derived equations with experiment when the need for a net rate of protein synthesis is ignored is considered in the *Appendix*.

When

$$k_bA \ll 1 \quad \text{(Eq. 15)}$$

i.e., when complete inhibition of microbial generation is effected by reaction of only a small fraction of total receptor sites, Eq. 13*b* reduces to

$$dN/dt = [k_0 - k_aA]N = k_{app.}N \quad \text{(Eq. 16)}$$

or

$$N = N_0e^{k_{app.}t} \quad \text{(Eq. 17)}$$

or

$$\log N = \log N_0 + (k_{app.}/2.303)t \quad \text{(Eq. 18)}$$

where

$$k_{app.} = k_0 - k_aA \quad \text{(Eq. 19)}$$

which is equivalent to Eq. 2.

In accordance with Eq. 18, the plots of the logarithm of numbers of organisms against time for any concentration of antibiotic that permits a net growth should give a straight line and is confirmed in the typical data for chloramphenicol plotted in Fig. 1.

The apparent first-order generation rate constants, $k_{app.}$, are obtainable from the slopes of such linear plots and are given in Table I for the several temperatures studied and can be obtained with a high order of precision.

If inhibition of the population growth rate is the mechanism for subinhibitory concentrations of the antibiotic rather than kill superimposed on normal growth, viable and total counts of *E. coli* with time should be coincident and the derived rate constants, $k_{app.}$, should be coincident for both total and viable count data. This has been shown to be true in the case of tetracycline (7) at one temperature and is now shown to be so for chloramphenicol and tetracycline for the several temperatures (Fig. 3 and Tables I and II). The coincidence of the plots of total and viable counts for *E. coli* population growth in the presence of graded concentrations of chloramphenicol are apparent from Fig. 1. The coincidence of the calculated $k_{app.}$ values from both count methods at various temperatures is apparent from Table I.

If the contingency of Eq. 15 is valid, it is predicted that a plot of the apparent first-order population growth rate constant in the presence of the antibiotics tetracycline and chloramphenicol should be reasonably linear when plotted against the concentration of the antibiotics. This has been shown to be the case for tetracycline and chloramphenicol (5) at one temperature and is demonstrated by the typical plots in Fig. 2 for several temperatures. The data of Table II include the apparent k_a values obtained from the slopes of such plots.

The proposed model is subject to test by the consistency of experiment with the several hypotheses. The reversible equilibria K_1 and K_2 of Eq. 4 should be quickly established. Literature evidence is confirmatory but has not been obtained at subinhibitory concentrations. The evidence in Fig. 4 and Tables III and IV clearly shows that addition and dilution of chloramphenicol concentrations in media containing a reproducing *E. coli* culture causes rapid changes in apparent population growth rates that are consistent with their linear dependence on antibiotic concentration (Eqs. 2 and 19). The rate transitions are no slower than that which occurs in diluting a control without antibiotic. Similar studies (Fig. 5, Tables III and IV) demonstrate the same phenomena for tetracycline.

Binding should have small energy requirements since it does not involve covalent bonding. The heat of activation, ΔH_a, of the population growth of *E. coli* can be estimated from the negative slope of logarithm of the generation rate constant, $\log k_0$, in the absence of antibiotic against the reciprocal of the absolute temperature, $1/T$ (Fig. 3) in accordance with the logarithmic Arrhenius expression of Eq. 3. The temperature for maximum *E. coli* growth rates can be noted from the maxima of the curves in Fig. 3.

When the derived inhibitory coefficients, k_a (Eq. 19) as given in Table II, are similarly treated, the ΔH_a values for the tetracycline and chloramphenicol inhibitory coefficients are the same as for the population growth rate constants of *E. coli* as can be observed from the parallelism of the slopes of Fig. 3. The consistency of total and viable counts with the Arrhenius relation can be also observed in Fig. 3. The parallelism of the Arrhenius

plots for the inhibitory coefficients and the growth rate constant in the absence of antibiotics is strongly indicative that the k_a is related to the k_0 as is indicated in Eqs. 13*a–c* and 13*A–C*. For example, consider

$$\log k_a = \log K_1K_2k_0 = \log k_0 + \log K_1 + \log K_2 = \log \text{constant} - \{[(\Delta H_a)_{k_0} + (\Delta H_a)_{K_1} + (\Delta H_a)_{K_2}]/2.303R\}\,(1/T) \quad \text{(Eq. 20)}$$

since each of the logarithmic values can be described by an Arrhenius expression similar to Eq. 3. Since, the heat of activation for k_a is within 1–2 Kcal./mole of that for k_0, the energies of partition and binding for K_1 and K_2 must be extremely small as would be expected if this model reflected reality.

A linear equation can be derived for the general case of Eq. 13 where the simplifying premise of Eq. 15 is not necessary and

$$1/(k_0 - k_{\text{app.}}) = (1/k_a)(1/\text{A}) + k_b/k_a \quad \text{(Eq. 21)}$$

When the reciprocal of the experimentally observed differences between the generation rate constants in the absence, k_0, and presence, $k_{\text{app.}}$, of antibiotics is plotted against the reciprocal of the antibiotic concentration, A, for the various data of Table I and previous data (7), reasonable linearity is obtained with intercepts passing close to or through the origin. This is to be expected if Eq. 19 is true. In several cases, a finite intercept could be ascertained whose reciprocal was of the order of magnitude $k_a/k_b > 10k_0$.

If the assumption were valid that the population growth was directly proportional to the rate of protein synthesis (Eqs. 13*A–15a*, *Appendix*) k_a/k_b should equal k_0. (See Eq. 15*a*, *Appendix*, Eq. 18.) This is not so. The postulate that population growth rate is proportional to a net rate of protein synthesis (Eqs. 11–14) is consistent with the observed facts where

$$k_a/k_b = k_0 + qk_p' > k_0 \quad \text{(Eq. 22)}$$

The fact that $k_a/k_b > 10k_0$ implies that it is only necessary to affect less than about 10% of the available protein synthesizing sites to completely inhibit population increase.

The interesting observation that cell-free preparations demonstrate chloramphenicol inhibition of protein synthesis regardless of whether they are derived from chloramphenicol resistant microorganism strains (13) is strongly indicative that the basic difference among strains is in the process by which the compound reaches the site of action or in the permeability of the cells. In this model, this is directly related to the magnitude of K_1 in Eq. 4.

Most of the data available on substrate incorporation on bacterial synthesis are for superinhibitory concentrations of chloramphenicol and tetracycline (11–15) where this refers to concentrations in excess of those minima necessary for complete inhibition of population increase. There are some data that can be interpolated from the literature, however, that are confirmatory when one considers that in the authors' *E. coli*, B/r system about 2–2.5 mcg./ml. of chloramphenicol completely inhibits generation at 37.5°. Chloramphenicol at 100 mcg./ml. inhibits 20–60% of various amino acid incorporation in *Staphylococcus aureus* (11). For *E. coli*, strain B, there is 50% inhibition of $^{35}SO_4$ incorporation and no inhibition of [^{14}C] uracil incorporation at 2 mcg./ml. of chloramphenicol (15). There is less than 20% inhibition of ammonia assimilation and oxygen consumption in the presence of about 2.5 mcg./ml. of chloramphenicol (12).

In cell-free systems of several *E. coli*, 2.5 mcg./ml. of chloramphenicol inhibited amino acid incorporation 30–50% and at 1.3 mcg./ml., the inhibition was 18–35% (13). This is optimum efficiency since partition of chloramphenicol (K_1 in Eq. 4) into the microorganism does not completely reflect the concentration of the antibiotic in the media and the actual inhibition of protein syntheses in the bacteriostatic region of chloramphenicol action, *i.e.*, 1.5 mcg./ml., will be much less than this 35%. The only data available in the appropriate concentration region for tetracycline are the demonstrations of 10–25% inhibition of amino acid incorporation in cell-free systems for various strains of *E. coli* at 2.5 mcg./ml. (13). In this case the authors have complete bacteriostasis at less than 0.2 mcg./ml. of tetracycline which certainly would correspond to a very low value of inhibition of protein synthesis.

These values are consistent with the necessary model that only a small fraction of protein synthesis inhibition is concomitant with bacteriostasis.

The most rational hypothesis to account for the action of chloramphenicol and tetracycline is that they inhibit the function of messenger RNA by blocking its attachment to ribosomes through competition for ribosomal binding sites (3). It has also been noted that the variation in chloramphenicol binding by ribosomes is in agreement with the ability of the antibiotic to inhibit protein synthesis in cell-free systems (16). These observations and data are consistent with the proposed model, the binding of the receptor sites on the ribosomes by these antibiotics to lower protein synthesis.

Although significant increases in both total and viable numbers of bacteria in cultures of *E. coli*, B/r, have been reported in the presence of "bacteriostatic concentrations" (these concentrations of 50 mcg./ml. are really superinhibitory) of chloramphenicol (17), no such significant increases were observed in the studies reported here in the subinhibitory concentration ranges of chloramphenicol up to the 2.5% mcg./ml. where total inhibition of population growth rate was observed. This was also true for tetracycline in comparable subinhibitory concentrations in these and previous studies (7). A change in the overt mechanism of action of these antibiotics in the sub- and superinhibitory ranges is indicated; from a truly bacteriostatic agent functionally dependent on the first power of chloramphenicol and tetracycline concentration where inhibition is additive on a kinetic basis (5, 8), to a bactericidal agent with a more complex functional dependency on these antibiotic concentrations (5, 6).

The proposed model explains the bacteriostatic effects and the functional dependencies on the premise of diminution of protein synthesis below the critical level necessary for generation; the bactericidal effects may be explained by the further inhibition of protein synthesis below that minimum necessary for survival and for regaining of generation capabilities.

APPENDIX

Variation of Receptor Sites with Time.—An implicit assumption in the postulated Eq. 5 is that the numbers of receptor sites in a single bacterium are constant and independent of the mass of the cell or a time dependency of protein synthesis. This is not necessarily so. The rate constant k_p of Eq. 5 includes the intrinsic synthesizing activity $(k_p)_0$ associated with each site and the numbers, R, of such sites so that

$$k_p = (k_p)_0 R \qquad \text{(Eq. 5}a\text{)}$$

If R at a time, t, is R_0, and the numbers of such sites exponentially increase with time with a rate constant, k_r, then

$$k_p = (k_p)_0 R_0 e^{k_r t} \qquad \text{(Eq. 5}b\text{)}$$

A possible simplifying assumption, is that proliferation of such synthesizing sites is associated with protein synthesis and proceeds at the same rate so that if $k_p \sim k_r$, the result is the transcendental equation

$$k_p = (k_p)_0 R_0 e^{k_p t} \qquad \text{(Eq. 5}c\text{)}$$

An alternate modification which was considered later in the development of the model was that a constant rate of protein synthesis is needed to sustain metabolic processes. Thus, only the excess rate characterized by $k_p - k_p'$ can be utilized in manufacturing synthesizing sites so that Eq. 5c may be modified to

$$k_p = (k_p)_0 R_0 e^{(k_p - k_p')t} \qquad \text{(Eq. 5}d\text{)}$$

where $k_p = (k_p)_0$ immediately after division at $t = 0$ of the generation time and k_p is a maximum at $t = t_{max.}$, the generation time for a single cell under the stated conditions. Since balanced growth cultures are being considered, a weighted mean k_p for the entire culture would be approximately constant averaged over all the organisms in the statistically distributed phases of growth between $t = 0$ and $t = t_{max.}$ If the increase in the numbers of sites is reasonably linear with time, Eq. 5b may be given as

$$k_p = (k_p)_0 (R_0 + k_r t) \qquad \text{(Eq. 5}B\text{)}$$

Nonpostulation of a Minimum Rate of Protein Synthesis for Reproduction Division.—If the hypothesis of a minimum rate of protein synthesis necessary for reproduction is not made, it is considered only that the rate of population increase of N organisms is directly proportional to the rate of protein synthesis in a single cell and the number of organisms. Then

$$dN/dt = [q'k_p - q'k_p K_1 K_2 A/(1 + K_1 K_2 A)]\ N \qquad \text{(Eq. 13}A\text{)}$$

$$= [k_0 - k_a A/(1 + k_b A)]N \qquad \text{(Eq. 13}B\text{)}$$

$$= k_{app.} N \qquad \text{(Eq. 13}C\text{)}$$

where

$$k_a = k_b k_0 \qquad \text{(Eq. 14}a\text{)}$$

It follows from Eqs. 13B, 13C, and 14a that

$$1/(k_0 - k_{app.}) = (1/k'_b k_0)(1/A) + 1/k_0 \qquad \text{(Eq. 15}a\text{)}$$

Thus, when the reciprocal of the experimentally observed difference between the generation rate constants in the absence, k_0, and presence, $k_{app.}$, of antibiotics are plotted against the reciprocal of the antibiotic concentration, A, the intercept of the presumed straight line should be $1/k_0$. It was shown that this is not the case, that the reciprocal of the intercept significantly exceeds the experimental k_0 value.

Thus, the hypothesis that the generation rate is proportional to a net rate of protein synthesis above that minimum necessary for maintenance of viability is preferable.

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