THE ACTION OF TETRACYCLINE AND CHLORAMPHENICOL ALONE AND IN ADMIXTURE ON THE GROWTH OF *ESCHERICHIA COLI**

BY EDWARD R. GARRETT AND M. R. W. BROWN†

From the College of Pharmacy, University of Florida, Gainesville, Florida

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The dependence of *E. coli* growth rates upon chloramphenicol concentration has been established. Chloramphenicol and tetracycline are equivalent in their action on *E. coli* and their combined effects on growth rate are additive. The decrease in rate of growth is a function of the first power of concentration of either antibiotic or combinations of them. A new mechanism of action is implied when the growth rate decreases to negative values: at this stage the rate of decrease is a function of a fractional power of the concentration.

IN a previous paper the dependence of *Escherichia coli* growth rates upon tetracycline concentration and also the reproducibility of the methods was demonstrated (Brown and Garrett, 1963). It was found that where there was a positive but decreased growth rate in the presence of tetracycline the inhibitory effect was a function of the first power of the tetracycline concentration. It is intended to set up kinetic dependencies for separate antibiotics and to predict from these the rate of change of viables in the presence of combinations of them. A theoretical basis for this approach, including classification and definitions of combined antibiotic activity has been published previously (Garrett, 1958).

Using turbidimetric techniques based on the principles previously defined by Elion, Singer and Hitchings (1954), Ciak and Hahn (1958), showed that the action of chloramphenicol and tetracycline on the growth of *E. coli* was additive in the sense that equipotent units of these antibiotics when added to each other gave the same activity as an activity-equivalent amount of each antibiotic alone. These antibiotics are thus convenient to use in an initial venture into the prediction of combined antibiotic action, since it would seem possible on the basis of separate growth rate studies to establish an equipotency factor.

The purpose of this paper is to present results of investigations to ascertain the validity of these *à priori* predictions.

METHODS

E. coli strain E/r was the test organism and replicate slopes were used for each experiment. Details of experimental procedures have been described previously (Brown and Garrett, 1963). Bacto Antibiotic Medium 3, buffered at pH 7 was used for broth cultures and plate counts.

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† Present address: School of Pharmacy, Bristol College of Science and Technology, Ashley Down, Bristol 7, England.

Assayed samples of tetracycline hydrochloride (995 μg./mg.) were supplied by courtesy of the Upjohn Company and chloramphenicol by courtesy of Parke, Davis and Company. Antibiotic solutions were sterilised by filtration and replicate samples stored frozen. Fresh samples were used for each experiment.

Growth Rate Measurements

An overnight broth culture was diluted into broth at 37·5° and the growth rate followed by means of a Klett-Summerson colorimeter. At a predetermined optical density, such that the bacterial cells were known to be in the logarithmic phase, samples were further diluted into the required number of replicate volumes of broth maintained at 37·5°. This procedure reduces any lag phase to a minimum (Brown and Garrett, 1963). Dilutions of the replicate cultures were then plated out at intervals.

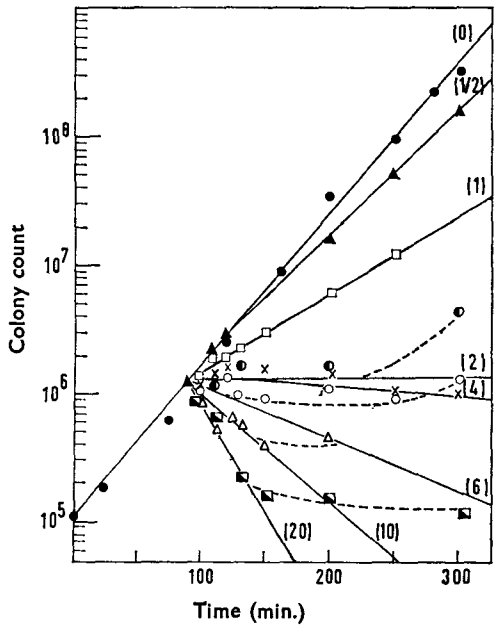

FIG. 1. Growth curves for *E. coli* in the presence of graded concentrations of chloramphenicol (μg./ml.).

Effect of Antibiotic Concentration

Eight replicate cultures of logarithmic phase cells were allowed to grow at 37·5° and after a predetermined interval, seven were inoculated with chloramphenicol solution. The cultures then contained 20, 10, 6, 4, 2, 1, 0·5 and zero μg./ml. of antibiotic. Colony counts were made at intervals and the results are illustrated in Fig. 1. Similar experiments for tetracycline have already been reported in greater detail (Brown and Garrett, 1963).

The values of the slopes (S_1), for the initial straight line part of the curves for the plot of logarithm colony count against time were determined: this represents a pseudo first order rate constant for *E. coli* growth. The error of estimate in these cases was of the order of 10 per cent for the data (Fig. 1) obtained within an hour after the addition of the antibiotic to the microorganisms in the logarithmic growth phase. The variation was greatest in those instances where a net increase of viables was not observed. Each value of S_1 was subtracted from the value of the slope in the absence of any antibiotic (S_0). Logarithm ($S_0 - S_1$) was plotted against logarithm antibiotic concentration for both tetracycline and chloramphenicol (Fig. 2).

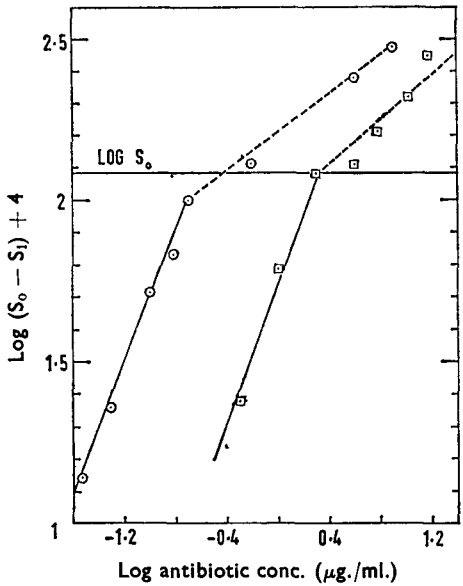

FIG. 2. Relationship between log ($S_0 - S_1$) and log concentration of tetracycline or chloramphenicol for *E. coli*. S_0 and S_1 are growth rates in the presence of zero and i^{th} concentrations of antibiotic. ○ Tetracycline. □ Chloramphenicol.

When positive values of S_1 were plotted against antibiotic concentration for both antibiotics it was observed that the slope of the straight line for tetracycline was greater than that for chloramphenicol by a factor of 7·5. This implies a difference in potency over this range. The phenomenon is illustrated in Fig. 3 by reducing the actual chloramphenicol dose by a factor of 7·5. Then the data for the two antibiotics can be accurately represented by one straight line.

Effect of Combinations of Antibiotics

Equipotent solutions of each antibiotic were made. These were then mixed in graded proportions using 100, 85, 65, 50, 35, 15 and zero per cent tetracycline solution and the residual percentage of chloramphenicol solution. This process was repeated to give seven graded, equipotent

mixtures for each of five different antibiotic equivalent concentrations. Thirty-six replicate cultures of logarithmic phase cells were incubated for a predetermined time at 37·5° when thirty-five of them were inoculated with one of the antibiotic mixtures. The slopes for the initial straight line part of the curves for the plot of logarithm colony count against time for each of the antibiotic mixtures are illustrated in Fig. 4 where the value for $(S_o - S_1)$ is plotted against the percentage of antibiotic in the combination.

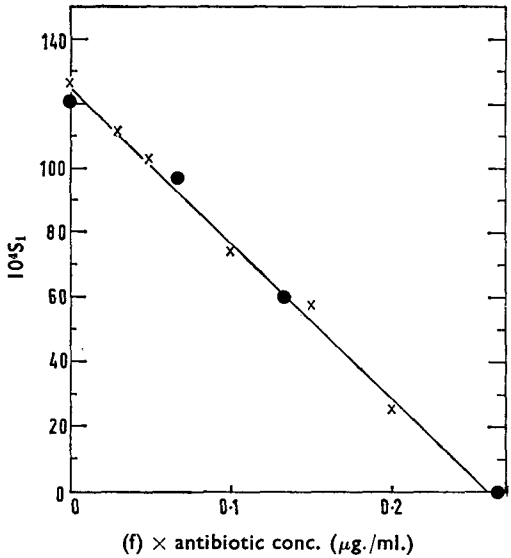

FIG. 3. Effect of tetracycline or chloramphenicol concentration upon the pseudo first order rate constant (S_1) for growth of *E. coli*. ×—Tetracycline (f) = 1. ●—Chloramphenicol (f) = 1/7·5.

DISCUSSION

The rates of growth of *E. coli* in the presence of chloramphenicol (Fig. 1) and tetracycline (Brown and Garrett, 1963) are decreased as functions of the concentrations of the antibiotics. When there is a finite increase in viable organisms the plot of the logarithm of the number of viables by colony counting, log X, against time, t, is linear (Fig. 1) so that the expression

$$X = X_o e^{kt} \quad .. \quad .. \quad .. \quad .. \quad (1)$$

is fully descriptive of the exponential growth of X_o microorganisms from the initial time of inoculation or of antibiotic addition.

The corresponding logarithmic expression applicable to the plots of Fig. 1 is

$$\log X = S_1 t + \log X_o \quad .. \quad .. \quad .. \quad (2)$$

in the presence of an i^{th} concentration of antibiotic where $S_1 = k/2{\cdot}303$, k $min.^{-1}$.

The pseudo rate constant, S_1, is a linear function of the antibiotic concentration, D in μg./ml. for all positive values of S_1 (Fig. 1 and 3),

$$S_1 = S_o - k_A D_A \quad .. \quad .. \quad .. \quad (3)$$

where the subscripts represent the particular antibiotic A and where $S_o = 1{\cdot}20 \times 10^{-2}$ is 2·303 times the rate constant $k = k_o$ (min.$^{-1}$) for the growth of *E. coli* in the absence of antibiotic, $D_A = O$. The specific rate constants for antibiotic concentration effects are $k_T = 4{\cdot}81 \times 10^{-2}$ for tetracycline and $k_c = 6{\cdot}42 \times 10^{-3}$ for chloramphenicol.

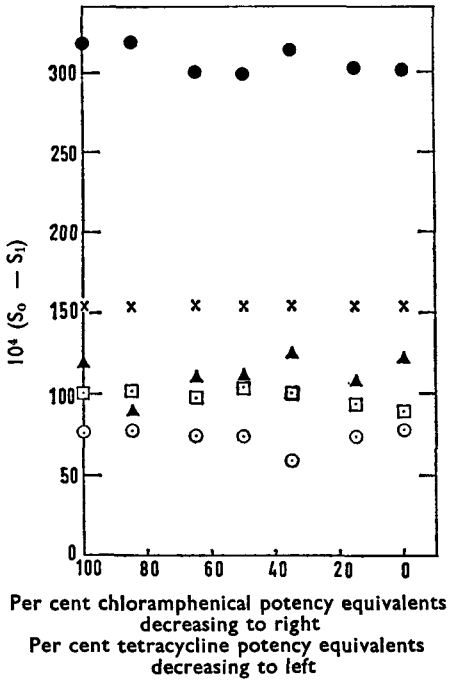

FIG. 4. Relationship between $(S_o - S_1)$ and proportion of antibiotic in a mixture of tetracycline and chloramphenicol for equivalent concentrations indicated.
(S_o and S_1) are growth rates in presence of zero and i^{th} concentration of antibiotic 7·5 μg. chloramphenicol has potency equivalent to 1 μg. tetracycline when measured separately against *E. coli*.) ●, 4 μg./ml. ×, 0·6 μg./ml. ▲, 0·2 μg./ml. □, 0·15 μg./ml. ○, 0·1 μg./ml.

If additivity of action of combined antibiotic concentrations on rates of *E. coli* growth are postulated it can be predicted for our system that

$$\begin{aligned} S_1 &= S_o - k_T D_T - k_c D_c \\ &= 1{\cdot}20 \times 10^{-2} - 4{\cdot}81 \times 10^{-2} D_T - 6{\cdot}42 \times 10^{-3} D_c \quad .. \quad (4) \end{aligned}$$

where D_c and D_T are the concentrations of chloramphenicol and tetracycline respectively in μg./ml.

The substitution of equation (4) into equations (1) or (2) permits the prediction of numbers of viables at any time in the presence of various

ratios of the combined antibiotics for an initial viable count of 10^6 organisms/ml. at 37·5°.

The invariance of the exponential change of *E. coli* viables with varying ratios of chloramphenicol to tetracycline, so calculated that the potency should be equivalent on the basis of equation (4) (see Fig. 4), is confirmation of the additivity of the antimicrobial effects for these two antibiotics. Some variation is evident but not sufficient to indicate a uniform decrease of growth rate as the proportion of any one antibiotic reduces. A reduction in the rate of growth of the organism in the presence of equipotent antibiotic mixtures when compared to the effect of either antibiotic alone would have indicated synergism. Conversely, an increase in rate with the mixtures as compared to that with either antibiotic alone would have indicated antagonism (Garrett, 1958).

At $S_1 = 0$ or less, a non-linearity in the plot of logarithm viables against time subsequently develops for the antibiotics separately (Fig. 1, Brown and Garrett, 1963) and in combination. This could be attributed to consumption of the antibiotic, the development of resistant mutants and/or the presence of antibiotic-resistant strains in the original inocula.

Notwithstanding this phenomena, the curves are initially linear and a slope can be obtained from a tangent to a curve at the time of antibiotic addition.

The chosen equipotency, 7·5 weight units of chloramphenicol equipotent to 1 weight unit of tetracycline hydrochloride was quite satisfactory for all values of S_1. This is evident from the fact that no significant variation in initial rate of microorganism increase or decrease occurs for any equipotent combination of antibiotics calculated on this premise (Fig. 4). On a molecular weight basis this means that tetracycline is about 11 times more active than chloramphenicol, molecule for molecule. This is not inconsistent with the molar potency ratio of 5·5 obtained by Ciak and Hahn (1958) when it is considered that they did not use tetracycline *per se*, they used a different strain of organism with a different medium and measurements were made turbidimetrically.

The rate of change of the number of viable organisms with time may be a function of the growth rate and the antibiotic concentration (Garrett, 1958), viz.,

$$(1/2{\cdot}303)\, dX/dt = S_1X = (S_o - k_cD_c^m - k_TD_T^n)X \quad .. \quad (5)$$

an equation which integrates to equations 1 and 2, a rational explanation therefore for the apparent first order growth rates of *E. coli* in the presence of these antibiotics is that normal generation rate, S_o, competes with the removal of viable microorganisms by a rate determining attack of antibiotic molecules on the microorganism. The dependence of the slope S_1 on the first power of the antibiotic concentrations (Fig. 3) for $S_1 > 0$ permits the assignment $m=n=1$ in Equation 5 and implies the inactivation of one microorganism by one molecule of antibiotic.

An alternative method of evaluation is to subtract the slope of the log viable — time plot, S_1, for any given i^{th} dose of antibiotic, from the slope S_o of the same system with no antibiotic. It follows from equation

(3) if the rate dependency on a power, m, of the antibiotic concentration is permitted, that

$$\log (S_o - S_1) = m \log D_A + \log k_A \quad .. \quad .. \quad (6)$$

and a plot of log $(S_o - S_1)$ against log D_A should be linear and of a slope of positive m. From such plots (Fig. 2) for both chloramphenicol and tetracycline it is apparent that the decrease in growth rate with antibiotic concentration is a function of the first power of the concentration of these antibiotics when there is a net increase in numbers of micro-organisms, $S_1 > 0$. When "kill" occurs with higher antibiotic concentrations, in the sense that there is a decrease in colony counts with time, the initial rates of such antimicrobial action are dependent on a fractional power of the antibiotic concentration.

The plots of Fig. 2 for log $(S_o - S_1) > \log S_o$; $S_1 < 0$ permit a reasonable estimate of the slope, m. Thus, the rate dependence for kill is to the 1/3 power of the concentrations of each antibiotic, although ignoring one value of the chloramphenicol data would permit m to approach 1/2 for this antibiotic.

The important conclusion, to be drawn, however, is that although diminution of positive growth rate is a function of the first power of the antibiotic concentration, the antibiotic is significantly more effective in terms of this postulated reduction in numbers of organisms capable of reproduction, i.e., "kill" in the system. This implies a difference in mechanism of action in that inhibition of growth rate may not necessarily "kill" in the same sense as with a net loss of viables. An investigation and comparison of total microorganism counts (viable and non-viable) with colony counts (viables) is needed to clarify these phenomena, to validate the basic postulates of equation 5 and to differentiate between the two possible modes of action in the change in microbial growth rates over the entire concentration ranges of these antibiotics.

The additive action of chloramphenicol and tetracylcine on *E. coli* as proposed by Ciak and Hahn (1958) on turbidimetric measurement is confirmed by our kinetic treatment of viable counting and is certainly consistent with their hypothesis of concurrent blocking of different anabolic pathways which jointly contribute to protein synthesis. However, on these kinetic grounds alone it is not possible to disregard the hypothesis that the mechanisms of action of these antibiotics are identical and based on the reaction of the drugs with the same biological site (Garrett, 1958).

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