MECHANISMS OF ACTION OF ANTIBIOTICS

I. Additive Action of Chloramphenicol and Tetracyclines on the Growth of *Escherichia coli*

JENNIE CIAK AND FRED E. HAHN

Department of Rickettsial Diseases, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D. C.

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The present study is concerned with the relationships between the actions of chloramphenicol and of members of the tetracycline group on bacterial growth.

The antimicrobial spectra of these drugs are similar as expressed by the collective *nomen*, broad-spectrum antibiotics. Chloramphenicol and tetracyclines can produce cross resistance in bacteria (Pansy *et al.*, 1950, Fusillo and Romansky, 1951). The actions of the drugs on susceptible microorganisms are primarily bacteriostatic. Finally, chloramphenicol, chlortetracycline and oxytetracycline specifically block the biosynthesis of bacterial proteins, while other synthetic processes, notably the formation of nucleic acids, continue in the presence of these antibiotics (Gale and Folkes, 1953, Wisseman *et al.*, 1954).

While the mechanisms of action of these drugs remain unknown on the molecular level, it might be inferred that the modes of action of chloramphenicol and of the tetracyclines are closely related, if not identical.

The differences between the chemical structure of chloramphenicol and that of tetracycline, however, render unlikely the idea that these antibiotics exert their actions through an identical biochemical mechanism.

The question, therefore, arises how inhibitors which elicit similar secondary responses in a variety of complex test systems can produce these effects through primarily dissimilar mechanisms. We have tried to resolve this seeming paradox by experiments which were based on the following considerations.

In 1928 Loewe introduced a graphical procedure into pharmacology which permits an evaluation of the combined effects of drugs in pairs by means of *isobolograms*. The applications of this procedure have been recently reviewed by Loewe (1957). Elion *et al.* (1954) have employed this type of experimental design in a study of growth inhibitors in pairs. The method affords quantitative criteria for defining interference, potentiation or simple additivity of growth inhibitory effects. Certain relationships can be inferred to exist between the actions of growth inhibitors once the type of their combined effect has been established.

The present work has shown that the actions of chloramphenicol, chlortetracycline, and oxytetracycline on the growth of *Escherichia coli* strain B are additive.

MATERIALS AND METHODS

Chloramphenicol, chlortetracycline hydrochloride, and oxytetracycline hydrochloride were standardized preparations, obtained from the Food and Drug Administration, U. S. Department of Health, Education, and Welfare. Crystalline potassium penicillin G was a commercial preparation. Stock solutions of antibiotics in distilled water were prepared which contained 2.0 mg per ml of chloramphenicol or of the hydrochlorides of oxytetracycline or chlortetracycline, or 200 u per ml of penicillin. These solutions were sterilized by passage through ultrafine sintered glass filters, dispensed into vaccine bottles, and stored at −20 C. For each experiment the contents of a freshly thawed bottle were used to prepare final dilutions in synthetic medium immediately before use, and the excess stock solutions were discarded.

The test organism, *Escherichia coli* strain B, was obtained from Dr. Mark Adams. A series of slants were prepared from a culture of this bacterium in brain heart infusion broth (Difco), and for each experiment one of these slants was used as a source of the original inoculum, the slant subsequently being discarded. This precaution was taken in order to eliminate changes in growth characteristics which frequently occur upon serial passages of

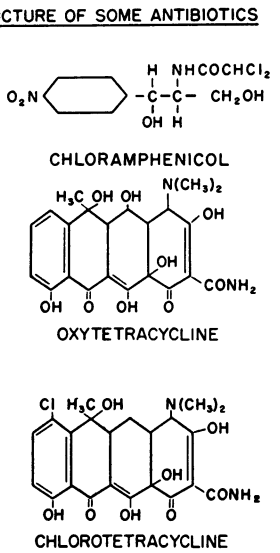

Figure 1. Chemical structures of chloramphenicol, oxytetracycline and chlortetracycline.

a bacterial strain during the course of an extended study.

The mineral medium used was that of Fisher and Armstrong (1947) in which glucose was substituted for glycerol and to which the bacteria were adapted by two serial transfers prior to each growth assay. The spectrophotometric procedure for determining bacterial growth rates in the presence of sub-bacteriostatic concentrations of antibiotics has been described previously (Hopps *et al.*, 1956). The spectrophotometer used was a Coleman Junior Model 6A instrument, and turbidity of cultures was read at a wave length of 450 mμ.

Growth rates of cultures in the early logarithmic phase were determined: (1) in the absence of antibiotics, (2) in the presence of sub-bacteriostatic concentrations of single antibiotics in appropriate dilution series, and (3) in the presence of paired combinations of antibiotics in serial dilutions, employing a factorial type of experimental design.

The logarithmic growth rate of the antibiotic-free control cultures was defined as 100 per cent growth, and rates which were decreased owing to the action of antibiotics, were expressed as per cent of the growth rate of the control culture. Growth inhibition in per cent was expressed as the difference between per cent growth and 100 per cent. Plotting per cent inhibition against antibiotic concentration yielded conventional dosage-response curves (Treffers, 1956) that were transformed into nearly straight lines by a probit transformation (Finney, 1952). This transformation facilitated visual curve fitting and permitted the application of the method of the least squares to arithmetic curve fitting. In this procedure all probit values entered with equal weight while in formal probit analysis different weighting coefficients are employed. The present, less formal method was used because the probit values furthest from 5.0 usually did not significantly deviate from the linear course of the dose response curve when an appropriate range of antibiotic concentrations was employed.

Fifty per cent inhibitory concentrations of the individual antibiotics and of each series of paired antibiotics were determined from the dose response curves. These concentrations were defined as ED_{50}s, i. e. the particular dose which caused a fifty per cent decrease in the growth rate of a culture.

Finally, "fractional inhibitory concentrations" of both antibiotics for each pair, representing one ED_{50}, were calculated as described by Elion *et al.* (1954). Plotting such fractional concentrations of two paired antibiotics against each other afforded a simple indication of the type of combined action of the respective drugs. Additive action was verified more rigorously by calculating the sums of the fractional concentrations. In additive action, these sums are unity by definition.

RESULTS

The presence of graded sub-bacteriostatic concentrations of chloramphenicol, oxytetracycline, or chlortetracycline in growing cultures of *E. coli*

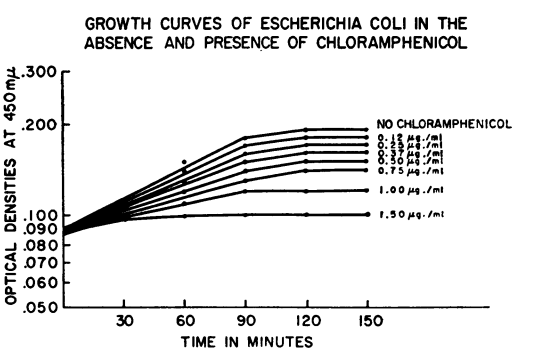

Figure 2. Influence of sub-bacteriostatic concentrations of chloramphenicol on the growth curve of *Escherichia coli* strain B.

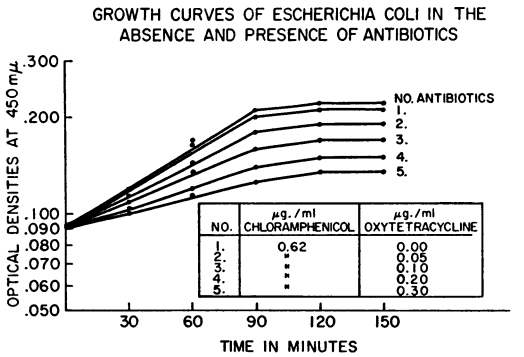

Figure 3. Influence of sub-bacteriostatic concentrations of chloramphenicol and oxytetracycline in combination on the growth curve of *Escherichia coli* strain B.

resulted in a reduction of the growth rates, depending upon the antibiotic concentrations. Figure 2 shows a typical family of growth curves of partly inhibited cultures.

A similar family of curves was obtained when a series of concentrations of a second antibiotic was added to a constant concentration of the first antibiotic, as shown in figure 3. The data for one complete experiment consisted of as many such diagrams as the number of concentration levels of the first antibiotic employed.

The pairs chloramphenicol-chlortetracycline, chloramphenicol-oxytetracycline, and chlortetracycline-oxytetracycline were studied and found to give an identical type of response in the assay procedure.

When the fractional inhibitory concentrations of both antibiotics for each pair, representing one ED_{50}, were calculated and plotted against each other, a linear function was obtained which intersected with the abscissa at an angle of 135°. This type of response indicates additive action (Elion *et al.*, 1954).

Additive action was quantitatively demonstrated by calculating the sums of the fractional inhibitory concentrations. Table 1 lists the actual partial antibiotic concentrations, the corresponding fractional inhibitory concentrations and their sums for a typical experiment with the pair chloramphenicol-oxytetracycline. These sums were close to 1.0, indicating additive action with a high degree of precision.

Essentially the same results were obtained also with the pairs chloramphenicol-chlortetracycline and oxytetracycline-chlortetracycline.

It will be recognized that the fractional inhibitory concentrations are abstract numbers which provide information on combined antibiotic action only relative to the ED_{50}s of the individual antibiotics. A knowledge of the molar concentrations, representing one ED_{50}, and a comparison of these values for the three antibiotics were of further interest.

The ED_{50}s for chloramphenicol, chlortetracycline, and oxytetracycline were, therefore, determined very accurately in repeated multiple series of growth inhibition experiments and fitting of the dose-response curves by the method of the least squares. The values for the ED_{50}s were then divided by the molecular weights of the respective antibiotics to give the molar concentrations, representing one ED_{50}. For the purpose of comparison, the molar concentrations were all divided by that for oxytetracycline in order to show the molar ratios between the ED_{50}s of this and of the other two antibiotics. The values of the ED_{50}s, the respective molar concentrations, and the molar ratios relative to oxytetracycline are given in table 2. Molecule for molecule the growth inhibiting potencies of oxytetracycline and chlortetracycline were nearly identical while it took approximately 5.5 times the molar concentration of chloramphenicol to inhibit the growth rate of the test organism by 50 per cent.

Originally it had been intended to include penicillin in this study. The response of this drug in the present test system, however, differed basically from that of the broad-spectrum antibiotics. Low concentrations of penicillin, up to 6 units per ml effected only slight changes in the

TABLE 1

Concentrations of chloramphenicol and oxytetracycline in combinations producing 50 per cent growth inhibition of Escherichia coli

Chloramphenicol		Oxytetracycline		Sum of Fractional Inhibitory Conc
Conc	Fractional inhibitory conc	Conc	Fractional inhibitory conc	
μg/ml		*μg/ml*		
0	0	0.17	1.00	1.00
0.06	0.07	0.16	0.94	1.01
0.12	0.14	0.16	0.94	1.08
0.25	0.29	0.13	0.76	1.05
0.50	0.57	0.09	0.52	1.09
0.80	0.92	0.02	0.12	1.04
0.87	1.00	0	0	1.00

TABLE 2

50 Per cent growth inhibitory concentrations of antibiotics and their molar ratios

Antibiotic	Molecular Weight (Calculated)	ED_{50} (*Escherichia coli* B)		Molar Ratio Based on Oxytetracycline
		μg/ml	μmoles/10^8 ml	
Oxytetracycline HCl	496.7	0.21	0.42	1:1
Chlortetracycline HCl	515.2	0.19	0.37	0.9:1
Chloramphenicol	323.1	0.75	2.32	5.5:1

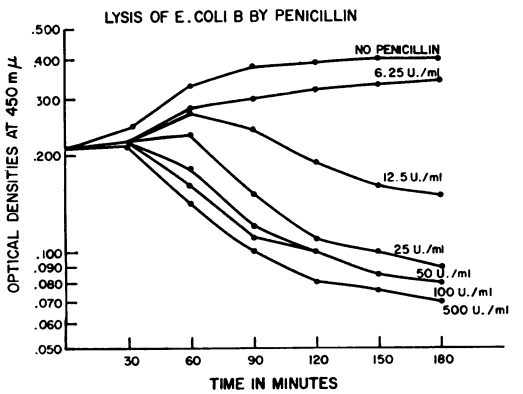

Figure 4. Lysis of *Escherichia coli* strain B induced by penicillin.

growth rates of the test organism, while concentrations above 12.5 units per ml caused lysis of the bacteria, as shown in figure 4. A study of this phenomenon has been published elsewhere (Hahn and Ciak, 1957).

DISCUSSION

The present study has shown that the reductions in the growth rate of *E. coli* strain B that are caused by chloramphenicol, chlortetracycline, and oxytetracycline are additive.

Determination of bacterial growth rates is considered a type of bioassay which is superior to the estimations of certain other parameters of growth (Monod, 1949). The merits of this procedure have been discussed in detail in a previous publication (Hopps *et al.*, 1956). It will be recognized, however, that this assay procedure is limited to the investigation of substances which cause a depression of bacterial growth rates at sub-bacteriostatic concentrations. Thus, the procedure did not lend itself to the study of penicillin which elicits a different type of response (Hahn and Ciak, 1957).

The principal result of the present investigation, the additive growth-inhibitory action of chloramphenicol and tetracyclines, has been anticipated by Jawetz and Gunnison (1952) on the basis of a less rigorous experimental and theoretical treatment of the problem, aimed at selecting useful combinations of antibiotics against pathogenic bacteria. In contrast, the present study is concerned with theoretical aspects of antibiotic action.

Two hypotheses can be offered to explain the additive growth-inhibitory actions of chloramphenicol, oxytetracycline, and chlortetracycline: (1) the mechanisms of action of these antibiotics are identical and based on the reaction of the drugs with the same biological site; (2) the mechanisms of action are dissimilar but affect anabolic pathways which contribute to a common synthetic end product, a type of action named "concurrent blocking" by Elion and her associates (1954).

The close structural relationship between oxytetracycline and chlortetracycline which have the ring structure and nine functional groups in common, and the finding that equimolar concentrations of these antibiotics elicited identical responses in the present test system, strongly suggest that the two drugs possess identical mechanisms of action through reaction with an identical biological site.

The structural discrepancy between chloramphenicol and the tetracyclines and the fact that molecule for molecule more than five times the amount of chloramphenicol was needed to equal the growth inhibitory potency of oxytetracycline or chlortetracycline, renders unlikely the idea that chloramphenicol and the tetracyclines possess identical mechanisms of action through reaction with an identical biological site.

We interpret the additive action of chloramphenicol and tetracyclines as concurrent blocking of different anabolic pathways which jointly contribute to protein synthesis. This interpretation is in accord with the highly specific action of these drugs on protein formation in bacteria (Gale and Folkes, 1953, Wisseman *et al.*, 1954) and on processes such as the induced synthesis of enzymes (Hahn and Wisseman, 1951) or the multiplication of bacteriophages (Altenbern, 1953; Bozeman *et al.*, 1954) which involve the biosynthesis of specific proteins.

SUMMARY

The actions of chloramphenicol, chlortetracycline, and oxytetracycline in sub-bacteriostatic concentrations on the growth rate of *Escherichia coli* B were found to be additive. Equimolar concentrations of chlortetracycline and oxytetracycline elicited nearly identical growth-inhibitory effects while, molecule for molecule, chloramphenicol was approximately five times less active.

The mechanisms of action of the two tetracyclines are considered to be identical. The additive effect of chloramphenicol and tetracyclines is interpreted as concurrent blocking of different metabolic pathways which contribute to protein synthesis.

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