

Effect of Novobiocin and Its Combination with Tetracycline, Chloramphenicol, Erythromycin, and Lincomycin on the Microbial Generation of *Escherichia coli*

EDWARD R. GARRETT AND CHONG MIN WON

College of Pharmacy, University of Florida, Gainesville, Florida 32610

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Inhibition of the steady-state generation of *Escherichia coli* by the bacteriostatic antibiotic novobiocin is linearly related to drug concentration in the range of 0 to 30 $\mu\text{g/ml}$. Increased cell sizes result because the drug inhibits cell division. The generation rate dependence on drug concentration depends on the nonionized fraction of novobiocin and is invariant with inoculum size or medium composition. However, the antibacterial activity of novobiocin decreases as the concentration of nutrients and Mg^{2+} increases, although the inhibitory action of novobiocin on generation rate remains unchanged for concentrations of Mg^{2+} above 8.1×10^{-4} M. Novobiocin is synergistic in combinations with tetracycline in broth, but not when the Mg^{2+} was maintained at 4.05×10^{-3} M. Combinations of novobiocin with the 50S ribosomal subunit inhibitors chloramphenicol, erythromycin, or lincomycin are antagonistic, and the degree of growth inhibition is determined only by that component of the binary combination that would have the greater potency if it were acting alone.

The clinically useful antibiotic, novobiocin (27, 43, 45), is active against gram-positive and some gram-negative microorganisms and has an antibacterial spectrum similar to that of erythromycin. It has been suggested that novobiocin induces intracellular magnesium deficiency (6-9), impairs membrane integrity (1, 11, 30, 32, 33, 41, 42), and inhibits cell wall (44) and nucleic acid synthesis (41, 42). The primary mode of action remains undecided (19, 31).

The application of microbial kinetics to the quantification and prediction of antimicrobial action has been well demonstrated (16). This present study of the inhibitory effects of novobiocin on generation rates of *Escherichia coli* in defined concentration ranges evaluates quantitatively the effects of inoculum size, nutrient concentrations, and pH of the culture medium on drug activity.

Numerous studies have been made on combinations of novobiocin and other antibiotics, especially with tetracycline. Some authors (4, 5, 15, 29, 39) have claimed that novobiocin and tetracycline act synergistically in vitro and in vivo. However, others have stated (11, 13, 18, 19, 21, 22) that there was no suggestion of

synergism and that the combination was not even additive in some cases. Recent controversies (36) on the desirability of fixed antibiotic combinations demand critical and rigorous tests of the action of such combinations. This paper presents the results of kinetic studies of microbial generation with combinations of novobiocin and other growth inhibitors.

MATERIALS AND METHODS

The strain of *E. coli* used, the methods of preparing bacterial cultures, and the growth assays by Coulter counting are given in the previous paper (17).

Culture media. Two different media were used. One was antibiotic medium 3 (Difco) rehydrated to peptone broth (USP). The other, Anton medium (2), consisted of K_2HPO_4 , 7 g; KH_2PO_4 , 3 g; glucose, 2 g; $(\text{NH}_4)_2\text{SO}_4$, 1 g; sodium citrate, 0.5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g; Casamino Acids (Difco), 10 g per liter of distilled water. Both media were filtered through membrane filters (0.45 μm , HA, Millipore Corp.) and autoclaved. The pH of Anton medium was modified by varying the ratio of KH_2PO_4 and K_2HPO_4 to obtain different pH values between 6.00 and 7.45, maintaining the same phosphate molarity.

Various strengths of peptone broth (USP) and Anton medium were prepared so that the concentra-

tion of ingredients was normal, halved, and doubled. Also, the composition of Anton medium was varied with different amounts of Casamino Acids, phosphates, or magnesium sulfate.

Antibiotics. The assayed sample of novobiocin, sodium salt (918 $\mu\text{g}/\text{mg}$), was supplied by courtesy of the Upjohn Co. Throughout this paper, concentrations of the drug refer to this sample in terms of the free acid. Samples of tetracycline hydrochloride (USP) and lincomycin hydrochloride (865 $\mu\text{g}/\text{mg}$) were supplied by The Upjohn Co., erythromycin lactobionate (670 $\mu\text{g}/\text{mg}$) was supplied by Abbott Laboratories, and chloramphenicol (purity grade) was supplied by Thomae GMBH, Germany.

Viable count assay. Samples (0.50 ml) of the culture were withdrawn and appropriately diluted into sterilized 0.85% saline solution. Diluted samples (1 ml) were spread onto each of three replicate agar plates, the plates were incubated for 48 h at 37.5 C, and colonies were counted on a colony counter (model C-110, New Brunswick Scientific Co.).

Cell size analyses. The size distribution of bacteria in logarithmic growth in the absence or presence of 40 and 100 μg of novobiocin per ml was determined with a model ZBI counter in conjunction with a channelyzer and plotter (Coulter Electronics, Inc.). The instruments were calibrated with polyvinyl toluene latex beads of 3.49- μm mean diameter.

RESULTS

Effects of novobiocin concentration on growth rates. The coincidence of growth inhibition of *E. coli* obtained by total and viable cell counts of cultures in Anton medium (Fig. 1) showed no significant killing of bacteria to occur between 0 and 40 μg of the antibiotic per ml. Thus, the use of total cell counts to determine the growth inhibition of the organism is justified. At completely bacteriostatic concentrations of 100 $\mu\text{g}/\text{ml}$ or greater, the numbers of viable cell counts were less than the total, even though both counts remained constant.

The typical generation curves for *E. coli* in Anton medium in the presence of novobiocin (Fig. 1 and 2) showed that a lower steady-state growth rate was established 20 min after the drug was added to the exponential cultures. This new steady state was apparently altered after 200 min (Fig. 1), at which time there was a definite change in cell size distribution.

Reversibility of novobiocin action. The steady-state generation (Fig. 2) of *E. coli* inhibited by 30 μg of novobiocin per ml (curve B) reverted to a new steady state when diluted 10-fold at 200 min (curve D) and also at 90 min. The diluted culture inhibited by 3 μg of novobiocin per ml (curve D) was further inhibited by the addition of more drug to a final concentration of 30 $\mu\text{g}/\text{ml}$ (curve E). Similarly, 10-fold dilution of the culture of curve B into fresh medium containing restored (curve F) or dou-

bled concentrations of the drug produced a new steady-state generation that was the same as when the same drug concentration had been added to a fresh culture.

Effect of novobiocin on cell size. Size frequency distributions (curve C, Fig. 3a; curves A-C, Fig. 3b) showed that cell size increased significantly after 150 min in the presence of novobiocin. When this culture was diluted 10-fold, the generation reverted to a new steady state, and the oversized cells reverted to a more normal size frequency distribution (curve A, Fig. 3a). It may be concluded that the antibacterial effect by sub-inhibitory concentrations of novobiocin on generation and the perturbation of size frequency distribution is reversible.

Generation rate dependency on novobiocin concentration, pH, and inoculum size. The apparent first order generation rate constants, k_{app} , were obtained from the slopes of the first linear portion of semilogarithmic plots after drug addition, in accordance with:

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

where N is the number of organisms at time t , and N_0 is the organism concentration at some time zero after the antibiotic manifests a new

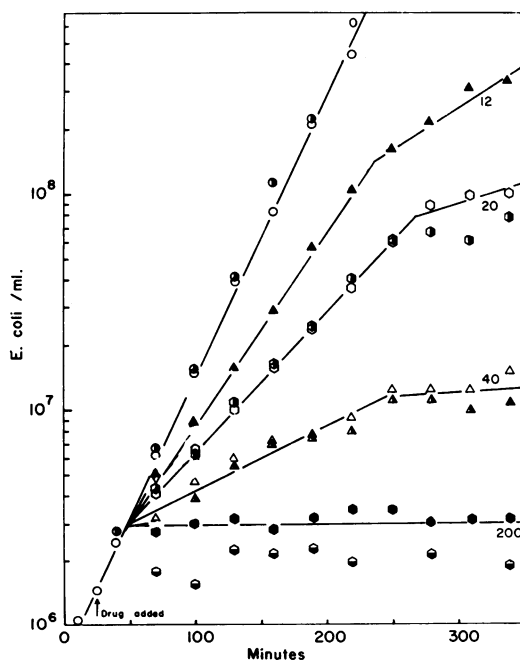


FIG. 1. Growth inhibition of *E. coli* by novobiocin in Anton medium at pH 6.90 and 37.5 C. Total cell counts (O, Δ , \square , \triangle , \bullet) and viable cell counts (\circ , \odot , \blacktriangle , \ominus). The curves are labeled according to the novobiocin concentration in micrograms per milliliter.

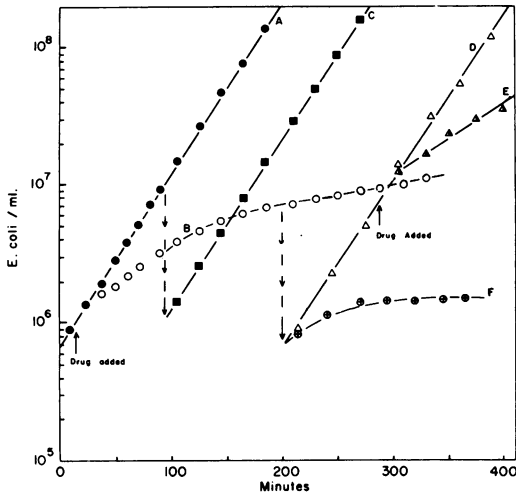


FIG. 2. Reversibility of the novobiocin inhibition. Curve A, a drug-free culture; curve B, a culture with 30 μg of novobiocin per ml; curve C, a 1:10 dilution of the curve A culture at the arrow (\downarrow); curve D, a 1:10 dilution of the curve B culture at 200 min, with a final concentration of 3 μg of novobiocin per ml. Drug was added to the 1:10 dilution of curve B after curve D established steady-state growth to give curve E. Curve F is a dilution at 200 min with a final concentration of 30 $\mu\text{g}/\text{ml}$.

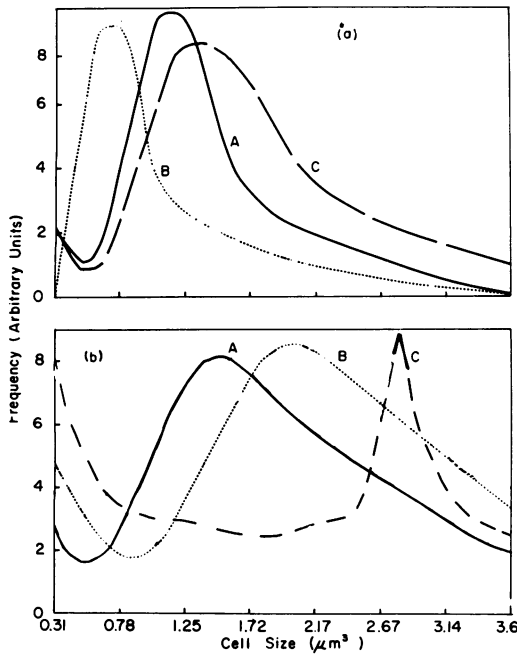


FIG. 3. Representative size frequency distributions of novobiocin-treated cultures. (a) Control culture of *E. coli* in peptone broth (USP) 30 to 140 min (curve A) and 170 to 200 min (curve B) after inoculation; a culture treated with 40 μg of novobiocin per ml (curve

steady state on microbial generation. The k_{app} values are linearly dependent on drug concentration up to 30 $\mu\text{g}/\text{ml}$ (Fig. 4), or up to 80% inhibition of the generation rate of the drug-free culture, in accordance with the expression:

$$k_{app} = k_o - k_A A \quad (2)$$

where k_o is the generation rate constant in sec^{-1} second for drug-free culture, k_A is the inhibitory constant for novobiocin in milliliters per microgram per second, and A is the concentration of novobiocin in micrograms per milliliter.

There were no significant differences among the k_{app} values for different bacterial densities over a 50-fold range for any drug concentration studied (Fig. 4). The inhibitory constants, k_A , of equation 2, were obtained from the slopes of the linear segments of the plots in Fig. 4 and increased markedly with pH. The drug-free generation rate constants, k_o , were not significantly affected by pH in the range studied. (In peptone broth [USP], the pH values and the k_o [$\times 10^4$] values were, respectively: 8.40, 6.07; 8.20, 6.17; 8.00, 6.14; 7.80, 6.37; 7.60, 6.09; 7.40, 6.18; 7.20, 6.15; 7.00, 6.22; 6.80, 6.17; 6.70, 6.34; 6.40, 6.21; and 6.10, 6.15.) The pH values in all of the cultures were constant throughout the time intervals studied. The inhibitory constants, k_A , are plotted semi-logarithmically

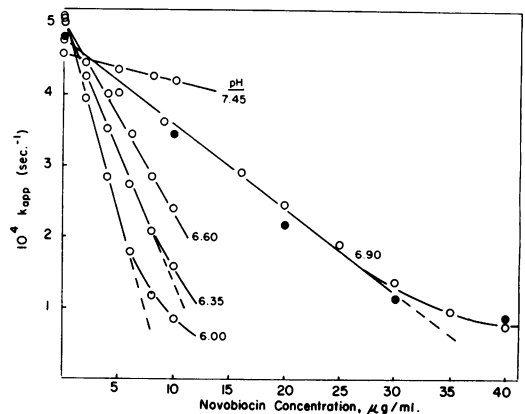


FIG. 4. Dependence of the apparent first order generation rate constant, k_{app} , on novobiocin concentration at various pH values and inoculum sizes at 37.5 C. The inoculum sizes are 4.3×10^8 (O) and 2.0×10^7 per ml (●). The cultures were grown in Anton medium at the pH values indicated.

C) at all times after the drug addition. (b) A culture treated with 100 μg of novobiocin per ml, 110 (curve A), 140 (curve B), and 200 to 350 (curve C) min after time of inoculation. The size frequency distribution 75 min after this dose was the same as curve A in (a).

(Fig. 5) as a function of pH and demonstrate significant decreases with increasing pH values.

Effect of media composition on novobiocin sensitivity. The k_{app} values for different concentrations of nutrients in peptone broth (USP) and Anton medium are given as a function of novobiocin concentration (Fig. 6). There were significant differences among the rate constants for different nutrient concentrations in peptone broth (USP), but the differences in Anton medium were relatively slight. The differences in k_{app} values for variations in Anton medium were not due to Casamino Acids or phosphates present. However, Mg^{2+} concentration had a significant effect. At comparable drug concentrations, k_{app} increased with increasing Mg^{2+} concentrations in Anton medium (Fig. 7). Thus, larger amounts of drug are required for the same degree of inhibition of microbial growth with increases in Mg^{2+} concentrations. The k_A values were not linearly dependent on Mg^{2+} concentration and approached a constant value at 8.1×10^{-4} M Mg^{2+} , i.e., at 0.02% salt concentration.

Effects of combinations of novobiocin and other antibiotics. The k_{app} values for cultures treated with equipotent mixtures of novobiocin with either tetracycline, chloramphenicol, erythromycin, or lincomycin (phase I) are shown in Fig. 8. The equipotent combination of novobiocin and tetracycline showed increased activity (lower k_{app}) over that of the expected activity or of 100% of either antibiotic alone except at the lowest concentration (Fig. 8a, line

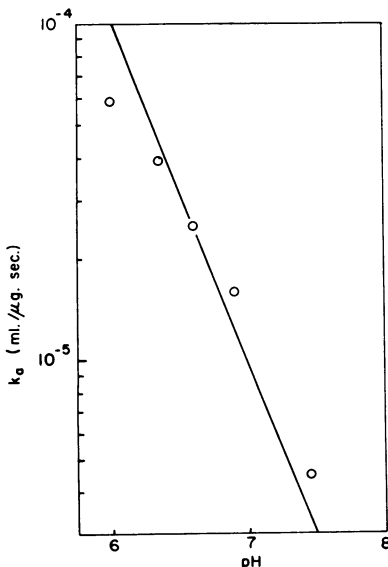


FIG. 5. Dependence of the logarithm of the inhibitory constant, k_A , on pH. The values of k_A are taken from the slopes of the curves in Fig. 4.

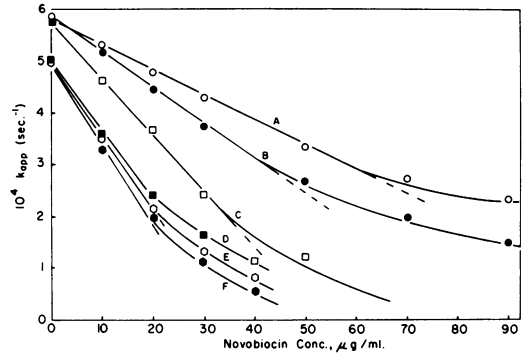


FIG. 6. Dependence of k_{app} on novobiocin concentration with various growth media. Multiples of the stated nutrient concentrations of peptone broth (USP) are: A, 2.0; B, 1.0; C, 0.5. Multiples of the stated nutrient concentrations of Anton medium are: D, 2.0; E, 1.0; F, 0.5. Values for k_A (10^6) in milliliters per microgram per second for the curves are: A, 0.475; B, 0.68; C, 1.07; D, 1.32; E, 1.40; F, 1.52.

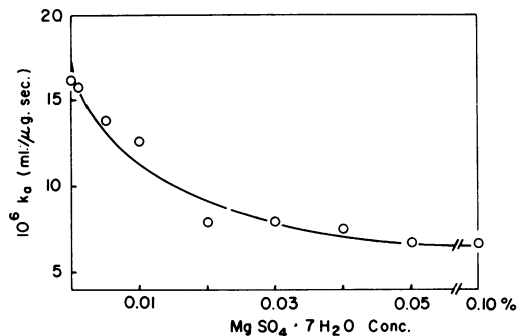


FIG. 7. Dependence of the inhibitory constant, k_A , on the magnesium salt concentration. Growth was in Anton medium, pH 6.90, at 37.5 C with the indicated Mg^{2+} concentrations.

A). However, this apparent synergism disappeared at 4.05×10^{-3} M Mg^{2+} . The equipotent combinations of novobiocin with either chloramphenicol, erythromycin, or lincomycin, on the contrary, exhibited decreased activity (higher k_{app}) over that of the sum of activity with either novobiocin and the other antibiotic alone.

The addition of an equipotent amount, at three different dosage levels, of either chloramphenicol or novobiocin 60 min after the cultures were treated with the other drug did not change the generation rates. Thus, the sequential addition of the second antibiotic had no significant effect on the activity of the first. Similar results were observed for combinations of novobiocin and lincomycin at the three different dosage levels studied.

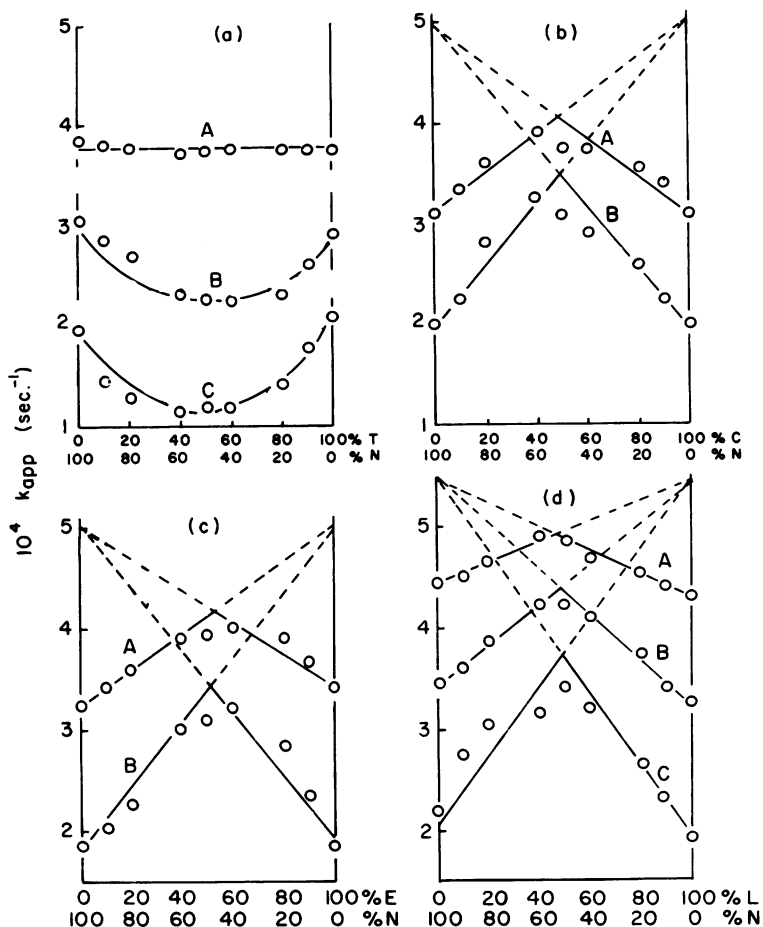


FIG. 8. Effects of tetracycline (T), chloramphenicol (C), erythromycin (E), or lincomycin (L) in combination with novobiocin (N). (a) Novobiocin in combination with tetracycline; (b) novobiocin and chloramphenicol; (c) novobiocin and erythromycin; (d) novobiocin and lincomycin. All of the experiments were carried out in Anton medium at pH 6.9 and 37.5 C. The 100% antibiotic concentrations in micrograms per milliliter are as follows: (a) A, 8.0 N, 0.035 T; B, 16.0 N, 0.070 T; C, 23.0 N, 0.130 T; (b) A, 11.5 N, 0.80 C; B, 23.0 N, 1.60 C; (c) A, 11.5 N, 11.5 E; B, 23.0 N, 23.0 E; (d) A, 10.0 N, 75 L; B, 20.0 N, 150 L; C, 40.0 N, 300 L. To prevent precipitation on mixing, the stock solution of novobiocin (12.5 mg/ml) was diluted with an appropriate amount of Anton medium before mixing with erythromycin (5 mg/ml) or lincomycin (25 mg/ml) stock solutions. The dashed lines in the figures represent the effect on k_{app} for each component of the combination alone.

DISCUSSION

The fact that no significant changes were observed in generation rate constants for antibiotic-free culture when the nutrient concentrations in a given medium were varied (Fig. 4 and 6) or when the pH was changed (Fig. 4) implies that the differences in the rate constants observed in the presence of novobiocin were most likely related to novobiocin-medium interactions. The magnitude of the effect of Mg^{2+} concentration on novobiocin activity (Fig. 7) can not explain the large differences in novobiocin activity observed between the two media (Fig. 6), where the degree of inhibition in Anton

medium is much larger than in peptone broth (USP) at equivalent novobiocin concentrations. The differences in k_A among various strengths of peptone broth (USP) (Fig. 6) are also much larger than those that can be ascribed to the Mg^{2+} effect (Fig. 7). A plausible explanation is that novobiocin is reversibly bound to protein or peptide components of the broth medium. The binding of novobiocin to proteins is well documented (12, 26, 28), and such binding of drugs to macromolecules can effectively reduce their antibacterial activity (3, 16).

The coincidence of viable and total counts in the sub-inhibitory range ($< 50 \mu\text{g/ml}$) of novobiocin demonstrates that the action at these

concentrations is bacteriostatic. There is no indication of significant bactericidal activity, even up to the highest dosage of 200 $\mu\text{g}/\text{ml}$.

The linearity of the k_{app} with drug concentration for novobiocin is similar to the concentration dependence found with chloramphenicol, tetracycline, and spectinomycin (16) and can be explained as described in the previous paper in this series (17).

The antimicrobial activity of novobiocin is pH dependent and increases in acidic medium (20, 24, 37, and Fig. 7). Novobiocin has two acidic groups, a weakly acidic phenol (pK_a 9.1) and a more strongly acidic enol on the coumarin ring (pK_a 4.3) (23). The monoanion is the predominant species over the pH range studied. If the monoanion were the active species, the activity of novobiocin should be pH independent, provided that the effective concentration of reactive receptors did not change. The enhanced antimicrobial action observed at lower pH values may be due to an enhanced concentration of the membrane-diffusible undissociated fraction, f , of the total drug in the medium, as has been shown for many antimicrobial agents (16).

It has been shown that, for dissociable acids with a given K'_a , the inhibitory constant can be defined as:

$$k_A = k^* f \\ = k^*_A [\text{H}^+]/(K'_a + [\text{H}^+]) \sim k^*_A [\text{H}^+]/K'_a \quad (3)$$

Since $K'_a \ll [\text{H}^+]$ in the studied pH region between 6.0 and 7.5, it follows that the last term of equation 3 is a proper simplification of the more exact previous term, where k^*_A is defined as the intrinsic inhibitory constant of the undissociated species. Thus,

$$\log k_A = \log k^*_A/K'_a - \text{pH} \quad (4)$$

and a plot of $\log k_A$ versus pH should give a straight line with a slope of unity. The intrinsic inhibitory constant, k^*_A , is calculated from the intercept of such a plot (Fig. 5) and is 5×10^{-3} ml per $\mu\text{g}\cdot\text{s}$.

Brock (6-10) proposed that an intracellular Mg^{2+} deficiency, caused by formation of a novobiocin- Mg^{2+} complex, was the basis for the primary action of novobiocin. However, Niebergall et al. (38) and Morris et al. (35) were unable to find any evidence for complex formation. If novobiocin were bound to bacterial cells to the extent proposed by Brock (9), a considerable effect of inoculum size on the antibacterial activity of novobiocin would be anticipated. The fact that there are only negligible effects (Fig. 4) contradicts this premise. Furthermore, the novobiocin-induced morphological and bio-

chemical effects, specifically filament formation in *E. coli* (10, 31, 34, 43), are not necessarily caused only by Mg^{2+} deficiency; they can be the secondary consequences of other effects (31). The ancillary action of novobiocin that produces filaments or monstrous cells may be due to attack on the specific subcellular location of the template enzyme complex, which is thought to be in the bacterial cell membrane (14).

Certainly Mg^{2+} does antagonize novobiocin activity (Fig. 5), but growth inhibition by novobiocin is not completely antagonized and is practically unchanged at 40 to 50% of its maximum above Mg^{2+} concentrations of 8.1×10^{-4} M. The hypothesis of novobiocin-induced Mg^{2+} deficiency by chelation can not explain this concentration effect.

Combined drug action can be predicted on the basis of kinetic expressions for the generation of microorganisms in the presence of each separate drug (16). Deviation from such quantitative prediction may be either synergism or antagonism (16). Equipotent mixtures of novobiocin and tetracycline demonstrate an unequivocal synergism in normal Anton medium (Fig. 8). This synergism is completely eliminated at higher magnesium ion concentrations (Fig. 8). These phenomena can be explained by the fact that the tetracycline binding of metallic ions such as Mg^{2+} (25) lowers the concentration of free Mg^{2+} available to antagonize the activity of novobiocin in normal Anton medium. The lack of synergism in media with higher magnesium ion concentrations can be explained by the fact that novobiocin activity is not a function of Mg^{2+} concentrations above 8.1×10^{-4} M (Fig. 6).

The observed antagonism (Fig. 8) between novobiocin and chloramphenicol, lincomycin, or erythromycin appears to be irrefutable. All three of these latter antibiotics inhibit protein synthesis, with their primary mode of action involving specific binding on the 50S ribosomal subunit (46, 47). Tetracycline, in contrast, has a greater binding affinity for the 30S subunit (41), and its presumed mode of action is distinctly different from the 50S subunit inhibitors which share some commonality in function.

Tetracycline shows no antagonistic effects in binary combinations with chloramphenicol (16), erythromycin, or lincomycin (17), as well as with novobiocin (Fig. 8a, b). Tetracycline is presumed to bind to the 30S subunit, blocking the acceptor site on this subunit and thus preventing the binding of *N*-formylmethionyl-transfer ribonucleic acid (tRNA) and aminoacyl-tRNA (46). Antibiotics that attach to the 30S ribosome also may inhibit the attachment

and movement of messenger RNA (mRNA) (46). The indifferent effect (16) of tetracycline in combination with chloramphenicol, erythromycin, or lincomycin has been explained (16, 17).

The tetracycline may not only inhibit protein synthesis by blocking the acceptor site, but may also prevent the attachment of mRNA to the ribosome and/or interfere with the movement of the mRNA along the ribosome in such a manner that its action adds to that of novobiocin, an additivity not obtained with novobiocin combinations with 50S binding inhibitors of protein synthesis.

A possible cause of the observed antagonism between novobiocin and the 50S binding antibiotics is a modification of the cell membrane by novobiocin which would inhibit the transport of another antibiotic. With such a mechanism, a greater antagonism may occur if novobiocin is added before the other antibiotics, but none was observed.

A plausible explanation of the observed antagonism where the antibiotic of the higher potency in the combination has the only apparent effect (Fig. 8) is that the mechanism of action of novobiocin and that of chloramphenicol, erythromycin, or lincomycin are independent of each other but are functionally linked, like two meshed cogwheels. The similarity is that the rotation rate of both wheels would be determined by the wheel with the slower rate, so that the antibiotic in combination that has the greatest inhibitory action determines the extent of inhibition of microbial generation by the combination.

The observed antagonism can be explained by realizing that protein synthesis can only continue when adequate mRNA is provided for the translocation of ribosomes and the peptide bond formation and translocation of peptidyl tRNA permits the movements of ribosomes along the mRNA (40). The blockage of one ribosome near the terminus of the mRNA could, in effect, block the movement (and thereby the function) of all ribosomes proximal to it, whether they are inhibited or not (17). The slower process would determine the overall rate of protein biosynthesis and thus the dependent rate of microbial generation. These phenomena would be consistent with the cogwheel analogy.

The mechanism of novobiocin's inhibition of protein synthesis could be a consequence of the inhibition of DNA synthesis (41, 42), which would result in a reduction of the rate of mRNA production, or of the direct inhibition of the mRNA production.

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