

Factors Which Influence Synergism by Neomycin and Oxytetracycline

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Six strains of enteropathogenic gram-negative bacteria were tested for susceptibility to neomycin or oxytetracycline alone and combined in fixed ratios. The minimal inhibitory concentration for the combination was less than one-half of that expected if the antibiotic activities were simply additive. Neomycin alone was more effective against bacteria multiplying in the presence of abundant oxygen, whereas oxytetracycline alone was more effective against bacteria multiplying in relatively anaerobic environments; when combined, the antibiotics complemented each other by their opposing optima for activity. Oxygen concentration, pH, and neomycin activity are related, and the depression of acid production by oxytetracycline is believed to be partially responsible for the synergistic activity of this pair of antibiotics.

Antibiotic combinations are frequently employed clinically when the etiology of an infection is unknown or uncertain. Although the indiscriminate use of a few combinations has occasionally resulted in antibiotic antagonism with undesirable effects (10), the judicious use of others has resulted in synergism with beneficial effects (1, 4, 5). Synergistic action by neomycin and oxytetracycline has been observed in vitro by Jawetz et al. (6, 7) and in vivo by Milberg et al. (11). In addition to these well-controlled experiments, enhanced prophylactic effects were observed clinically when this combination was used as a preoperative treatment for intestinal surgery (1, 2).

Because synergism by bactericidal antibiotics such as neomycin combined with bacteriostatic antibiotics such as oxytetracycline is infrequently observed, experiments were designed to determine the effects of the neomycin-oxytetracycline combination upon selected strains of bacteria which are known to be virulent pathogens for poultry or swine, or both, and to determine some of the factors which are responsible for the phenomenon.

MATERIALS AND METHODS

Test bacteria. *Salmonella typhimurium*, *S. typhimurium* var. Copenhagen, *S. gallinarum*, *Escherichia coli*, *S. choleraesuis* var. Kunzendorf, and *Paracolobacterium arizona* (7:1,2,6) were employed for the study. All of these strains were isolated from infected chickens, turkeys, or swine, and the virulence of each was confirmed by challenging susceptible host

animals. Each strain is susceptible in vitro to both neomycin and oxytetracycline. All test bacteria were characterized by standard methods (14) and classified by the nomenclature of *Bergey's Manual* (7th ed., 1957). The identities of the salmonellae and *P. arizona* were confirmed serologically by the National Animal Disease Laboratory, Ames, Iowa.

Tests in surface-seeded agar plates. Whatman no. 2 filter paper was cut into 1-cm strips and saturated with aqueous solutions containing 400 μ g of oxytetracycline hydrochloride per ml or 400 μ g of neomycin base per ml (calculated from the sulfate). The antibiotic-impregnated strips were then air-dried at room temperature. Tryptose agar plates were inoculated with *S. typhimurium*, *S. gallinarum*, *E. coli*, *S. choleraesuis* var. Kunzendorf, or *P. arizona* (7:1,2,6) by spreading broth suspensions uniformly over their surfaces with cotton-tipped applicators. Neomycin-impregnated paper strips were placed centrally upon the surfaces of the seeded agar plates, and an oxytetracycline- or neomycin-saturated strip was placed adjacent and perpendicular to each forming a "cross." Parallel plates were prepared in the same manner except that the arrangement of the antibiotic-impregnated strips was reversed. The cultures were incubated for 48 hr at 37 C, and the sizes and shapes of the inhibition zones which resulted were photographed (Fig. 2).

Tests in deep agar media. A basal medium consisting of 0.5% glucose and 1.5% agar in phenol red broth base (Difco) was prepared. The medium was autoclaved and cooled to 45 C; then 8 μ g of oxytetracycline hydrochloride per ml, 8 μ g of neomycin base per ml, or 4 μ g of oxytetracycline per ml plus 4 μ g of neomycin base per ml was added to separate portions. Media were inoculated with 1% (v/v) of a 16-hr culture of *S. typhimurium* var. Copenhagen. Inoculated media

TABLE 1. Effects of medium volume on the antibiotic activity of neomycin or oxytetracycline, or both, against *Salmonella typhimurium* var. *Copenhagen*

Antibiotic concn	Depth of sterile zones (mm) in various volumes of medium				
	10.0 ml	7.5 ml	5.0 ml	2.5 ml	1.25 ml
Neomycin (8 μ g/ml)	4 (AG) ^a	3 (AG)	3 (AG)	4 (AG)	4 (AG)
Oxytetracycline (8 μ g/ml)	0 (A)	0 (A)	0 (A)	0 (A)	0 (A)
Neomycin (4 μ g/ml), oxytetracycline (4 μ g/ml)	12 (A)	13 (A)	12 (A)	12 (A)	10 ^b
Positive control (no antibiotic)	0 (AG)	0 (AG)	0 (AG)	0 (AG)	0 (AG)
Negative control (no inoculum)	0	0	0	0	0

^a A = acid; G = gas.

^b Acid or gas not produced. Medium only 10 mm deep.

were mixed by gentle rotation, and 1.25, 2.5, 5.0, 10.0, or 15 ml was immediately dispensed into sterile test tubes of uniform diameter and cooled to room temperature. Seven replicates of each volume of medium and concentration of antibiotic(s) were prepared. Controls which contained identical volumes of sterile basal medium were prepared for comparison. A basal medium containing no antibiotic was inoculated and dispensed as above to serve as positive controls. Cultures were incubated for 96 hr at 37 C, and the production of acid and gas was determined from the color change of the phenol red and the presence of gas bubbles in the agar medium, respectively. The depths of the upper strata of media in which the bacteria failed to grow were measured (Table 1 and Fig. 1). The antibiotics employed for this and all subsequent tests were NF grade oxytetracycline hydrochloride or USP grade neomycin sulfate (Pfizer Inc., New York, N.Y.).

Determination of minimum inhibitory concentrations in broth. Twofold serial dilutions of neomycin, oxytetracycline, and a combination of the two in a 1:1 ratio were prepared in Tryptose phosphate broth. Concentrations of each antibiotic ranged from 40 to 0.312 μ g/ml. Five milliliters of medium containing each antibiotic alone or in combination was inoculated with one 4-mm loopful (approximately 0.01 ml) of a 24-hr broth culture of *S. typhimurium*, *S. gallinarum*, *S. choleraesuis* var. *Kunzendorf*, *E. coli*, or *P. arizonae* (7:1,2,6). Cultures were incubated for 48 hr at 37 C and visually observed for turbidity. The lowest concentration in which turbidity failed to develop was considered the minimum inhibitory concentration (MIC) for each antibiotic. All cultures were stained by the Gram method, examined microscopically, and subcultured to check turbid media for the presence of contaminants or to determine whether the bacteria were dead or dormant in the nonturbid media, or both. This experiment was repeated with all of the above species of bacteria, except that *S. choleraesuis* var. *Kunzendorf* was included in the second experiment only.

Comparison of theoretical antibiotic activities with those observed. One MIC of neomycin or oxytetracycline, based upon the results of previous experiments, was prepared in Tryptose phosphate broth. These were then diluted by increments of 0.1 MIC to

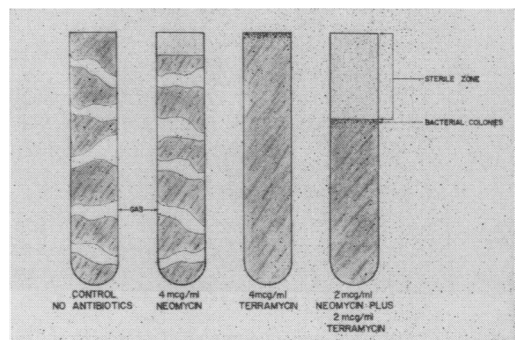


FIG. 1. Effects of oxytetracycline and neomycin alone and combined upon the fermentation and reproduction of *Salmonella typhimurium* in a semisolid medium.

obtain concentrations ranging from 1.0 to 0.1 MIC for each species tested. One-half of a MIC of each antibiotic was added together to broth and similarly diluted by increments of 0.1 for the combination. Five milliliters of each concentration was inoculated, incubated, and examined as before. All tests were duplicated.

RESULTS

The data presented in Table 1 and schematically illustrated by Fig. 1 indicate that neomycin alone completely inhibited bacterial multiplication in the upper strata of the media but was ineffective in the lower strata. This phenomenon suggested that neomycin was more effective when the bacteria were growing in an abundance of oxygen.

Conversely, colonies were larger and more abundant upon and near the surfaces of media which contained oxytetracycline alone and the bacteria produced very little or no acid or gas in the lower strata (Fig. 1). These data are consistent with those of others (8, 9, 13; H. A. Lechevalier, Ph.D. Thesis, Rutgers Univ., New

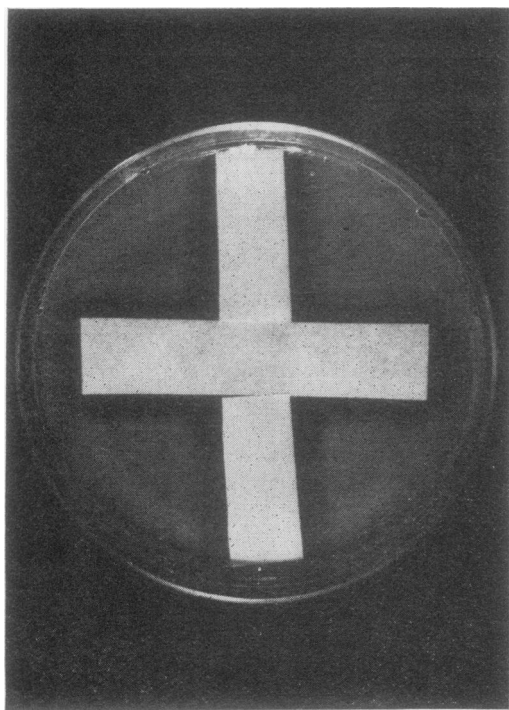


FIG. 2. Location and geometrical pattern of the inhibition zones produced by the antibiotics diffusing against each other in agar. Horizontal and top vertical paper strips contain neomycin. The lower vertical paper strip contains oxytetracycline. The test bacterium is a field isolate of pathogenic *Escherichia coli*.

Brunswick, N.J., 1951) and suggest that the oxygen concentration in which susceptible bacteria are multiplying is critical for activity by each of the antibiotics alone and that the two complement one another by their opposing optima.

Figure 2 shows the location and geometrical pattern of the inhibition zones produced by the antibiotics diffusing against each other in agar. Zones are larger and curve outward near the intersection of paper strips impregnated with unlike antibiotics but remain identical in size and shape near the intersection of paper strips impregnated with like antibiotics. This phenomenon was observed for all bacterial species tested. Tables 2 and 3 illustrate the magnitude of the differences between the observed and expected MIC or minimum bactericidal concentrations (MBC), or both, of the combined antibiotics for each of the five species of bacteria tested. The observed MIC or MBC (or both) was lower for each species than expected if the antibiotic activity had been simply additive.

DISCUSSION

The preponderance of the data reported here and elsewhere (6) indicates that a true synergistic effect occurs *in vitro* when susceptible bacteria are exposed to adequate concentrations of neomycin and oxytetracycline.

The greater depths of sterile zones which were observed in agar cultures containing both anti-

TABLE 2. Antibacterial activity of neomycin or oxytetracycline, or both, against specific enteropathogenic bacteria

Determination ^a	<i>Escherichia coli</i>	<i>Salmonella typhimurium</i>	<i>S. gallinarum</i>	<i>Paracolonobacterium arizona</i>	<i>S. choleraesuis</i>
MIC ($\mu\text{g/ml}$), neomycin	10	20	20	20	10
MIC ($\mu\text{g/ml}$), oxytetracycline	10	20	20	>40	20
MIC ($\mu\text{g/ml}$), neomycin + oxytetracycline	2.5	5	5	10	5
Theoretical MIC, neomycin + oxytetracycline	10	20	20	>30	15
Ratio of MIC (theory/observed)	4.0	4.0	4.0	>3.0	3.0
MBC ($\mu\text{g/ml}$), neomycin	10	20	20	20	10
MBC ($\mu\text{g/ml}$), oxytetracycline	>40	>40	>40	>40	>40
MBC ($\mu\text{g/ml}$), neomycin + oxytetracycline	5	10	5	10	10
Theoretical MBC (neomycin + oxytetracycline)	>25	>30	>30	>30	>25
Ratio of MBC (theory/observed)	>5.0	>3.0	>6.0	>3.0	>2.5

^a MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration.

TABLE 3. Antibiotic activity of neomycin and oxytetracycline alone and combined in ratios of 0.5 MIC of each

Determination ^a	<i>Escherichia coli</i>	<i>Salmonella typhimurium</i>	<i>S. gallinarum</i>	<i>Paracolonobacterium arizona</i>	<i>S. choleraesuis</i>
MIC ($\mu\text{g/ml}$), neomycin	7	16	8	10	10
MIC ($\mu\text{g/ml}$), oxytetracycline	9	9	8	10	14
MIC ($\mu\text{g/ml}$), neomycin + oxytetracycline	4	6	<4	<4	3
Theoretical MIC, neomycin + oxytetracycline	8	12.5	8	10	12
Ratio MIC (theory/observed)	2	2.1	>2	>2.5	4

^a MIC, minimum inhibitory concentration.

biotics, the larger inhibition zones which curved outward near the intersection of paper strips containing unlike antibiotics, and the consistently lower MIC for the combination of antibiotics against all species of bacteria tested are characteristically synergistic phenomena (4, 12) and constitute imposing evidence that neomycin and oxytetracycline are truly synergistic in their activity upon certain species of enteric pathogens. The depths of the sterile zones observed in the upper strata of shallow agar media which contained neomycin were no larger than those in deep media of identical composition. Consequently, the ratio of surface area to volume of medium failed to affect significantly the activity of this antibiotic. This observation suggests that the three- to fourfold increase in depths of sterile zones seen in media which contained both antibiotics resulted from their combined activity and was not an experimental artifact resulting from chance differences in the amounts of oxygen in the head spaces of the test tubes.

Neomycin was more effective in the upper strata of semi-solid media, and others have reported oxygen to be a critical factor for its antimicrobial activity (13; H. A. Lechevalier, Ph.D. Thesis, Rutgers Univ., 1951). Therefore, it may be assumed that the oxygen concentration was the principal factor which limited the depths of the sterile zones observed in this study. However, neomycin is less active in acidic media (15), and all of the bacteria studied produce acid from glucose, which was an ingredient of all the media employed for the tests reported here. Consequently, the lack of oxygen per se may not have been the sole factor which limited neomycin activity in the relatively anaerobic regions of the test media. Acid production is usually inversely related to the oxygen concentrations of media in which bacteria are growing (e.g., the "Pasteur effect"). It appears likely, therefore, that some of the decreased neomycin activity which was observed in the deeper strata of the media resulted

from the more rapid acid production by the test bacteria growing anaerobically. Oxytetracycline alone suppressed acid production and thus maintained the pH of the medium more favorable for neomycin activity when the antibiotics were combined. Consequently, a relationship of each antibiotic with the oxygen concentration or pH, or both, at various depths of media appears to be the principal cause for the synergism observed in this study.

Another factor to consider is that the MBC of neomycin is directly related to the numbers of susceptible bacteria which are in contact with it. Because oxytetracycline is primarily bacteriostatic, fewer bacteria would be expected to develop near paper strips which contain this antibiotic than in the surrounding medium. Consequently, less neomycin should be required to kill the smaller numbers of bacteria which develop within the immediate vicinity of oxytetracycline-laden strips, as the antibiotics are diffusing through the agar. This appears to be one of the causes for the larger inhibition zones which developed on surface-seeded agar plates near the intersection of paper strips containing dissimilar antibiotics.

Oxytetracycline should theoretically aid in maintaining the alkalinity of the intestinal contents by suppressing the fermentation of carbohydrates by susceptible indigenous microflora. This should create an in vivo environment more favorable for neomycin activity and possibly accounts for the improved efficacy which is frequently observed when this combination of antibiotics is employed for the treatment of gastrointestinal disturbances caused by susceptible enteropathogenic bacteria.

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