STUDIES ON ANTIBIOTIC SYNERGISM AND ANTAGONISM: THE EFFECT IN VITRO OF COMBINATIONS OF ANTIBIOTICS ON BACTERIA OF VARYING RESISTANCE TO SINGLE ANTIBIOTICS[1]

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Earlier studies on combined antibiotic action from this laboratory have resulted in the formulation of a scheme for combined antibiotic action. Antibiotics have been divided tentatively into two groups: I. penicillin, streptomycin, bacitracin, and neomycin; II. chloramphenicol, aureomycin, terramycin (Jawetz and Gunnison, 1952*a*,*b*; Jawetz *et al.*, 1952). Mixtures of group I drugs often resulted in synergism, never in antagonism. Mixtures of group II drugs showed no combined effects beyond simple addition. The mixture of an antibiotic of group I with a drug of group II resulted in either synergism or antagonism apparently depending in part upon the sensitivity of the test bacteria to the group I drug. Thus, if the bacteria were highly sensitive to the group I antibiotic, the addition of a group II drug often interfered with the early bactericidal action of the first drug (antagonism). On the other hand, if the organisms were relatively resistant to the group I drug, in some instances the addition of a group II drug resulted in synergism, i.e., in a marked increase of early bactericidal action and the killing of greater numbers of bacteria than could be expected from simple summation of single drug effects. Most of the earlier tests were carried out with bacteria isolated from random clinical infections.

Since the relative susceptibility of microorganisms to group I drugs appeared to influence the observed end result of group I + group II combined antibiotic action, it was deemed important to compare the behavior of bacterial variants of graded resistance to group I drugs when exposed to group I + group II combinations. The present paper reports such a study.

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Bacteria were selected which were highly sensitive to group I drugs and which were killed more rapidly when exposed to a group I drug alone than when a group II drug was added. Resistant variants were obtained then from these cultures and subjected to tests with antibiotics singly and in combination. It was thought possible that although a given pair of group I + group II antibiotics acted antagonistically against the originally sensitive culture they might act synergistically against the more resistant variants derived from it.

METHODS AND MATERIALS

Definitions. "Synergism" *in vitro* has been defined as the ability of two drugs to produce a marked increase in the bactericidal rate within the first 24 hours of exposure as compared with the rate with either drug alone and the killing of greater numbers of bacteria than could be expected from simple summation of single drug effects. The term "synergism" has been reserved for combined action unequivocally in excess of simple algebraic summation, and the term "addition" has been used for other instances of positive summation. "Antagonism" *in vitro* has been defined as a marked decrease in the early bactericidal rate as compared with that of the more active single drug even though the combination after prolonged incubation may kill more organisms than either drug alone. These definitions have been chosen because they seem to correlate well with results in the treatment of experimental infections in mice and with certain clinical evidence (Jawetz and Gunnison, 1952*a*,*b*).

Antibiotics. Stock solutions in sterile buffered 0.85 per cent salt solution were stored in the refrigerator, and final dilutions were prepared in broth immediately before use. Commercial crystalline preparations of potassium penicillin G and streptomycin sulfate were used. Bacitracin

(lot no. B-480420) was supplied by Dr. L. Smith; terramycin hydrochloride (lot no. WB507019), by Dr. G. L. Hobby; and chloramphenicol (lot no. 134006), by Dr. G. Rieveschl.

Media. Proteose no. 3 agar (Difco) and broth of similar composition were used. For *Streptococcus pyogenes*, one per cent sheep blood was added to the agar.

Bacteria. The following organisms and variants derived from them were studied: *Micrococcus pyogenes* var. *aureus*, strain H; *Micrococcus pyogenes* var. *albus*, strain M, isolated from a case of subacute bacterial endocarditis, and strain L, isolated from a case of osteomyelitis; *Streptococcus pyogenes*, strain C203; and *Klebsiella pneumoniae*, strain A-D.

Methods. A sensitive strain was obtained from each culture by repeated selection of single colonies from drug-free agar. Resistant variants were selected by serial transfer in gradually increasing concentrations of antibiotic in broth or in agar, by inoculation of large numbers (10^9) on various concentrations of antibiotic in agar, or by the gradient plate method (Szybalski and Bryson, 1952). Single colonies were isolated from a plate with a given concentration of antibiotic and reisolated 3 to 6 times on antibiotic containing agar, picking a single colony each time. All strains were preserved by freezing in broth containing the highest concentration of antibiotic that would permit growth at a normal rate and were stored at −20 C. For the final tests, the frozen cultures were inoculated on agar containing antibiotics in the highest amount permitting growth at a normal rate and from this into 100 ml of drug-free broth to be used as the inoculum.

Plate counts of the inoculum were made on agar containing antibiotic as well as on drug-free agar to determine the proportion of resistant cells. In all instances, at least 80 per cent of the cells were resistant to the selected concentration of antibiotic.

For the tests proper, broth containing antibiotics was inoculated with the 18 hour culture to give a concentration of $10^{6.5}$ to $10^{7.5}$ organisms per ml in a total volume of 15 ml. Samples of 0.5 ml were removed at intervals during incubation at 37 C, and the number of viable bacteria was estimated by plate counts. Details of the methods and their interpretation have been reported previously (Gunnison *et al.*, 1950*b*).

RESULTS

The first organism studied, *M. pyogenes* var. *albus*, strain M, was killed rapidly by bacitracin in a concentration of 10 μg per ml. A variant strain was obtained which was resistant to this concentration of bacitracin. The results of comparative tests of the resistant and sensitive strains exposed to bacitracin and terramycin alone and in combination are shown in figures 1 and 2. With the sensitive strain, the addition of terramycin (1 μg per ml) to bacitracin (10 μg per ml) resulted in antagonism; but with the resistant variant, the two drugs in these concentrations had an additive effect. This suggested that antagonism might be converted into synergism as the resistance of the culture to the group I drug increased. However, when a larger amount of bacitracin (100 μg per ml) was used which was bactericidal for the more resistant variant, the terramycin again reduced the rate of killing just as it had done with the sensitive strain at the lower level of bacitracin.

Six additional bacitracin resistant variants of this micrococcus with different degrees of resistance were tested. With those variants which were inhibited but not killed by a given dose of bacitracin, the combination of that dose with terramycin had an additive effect; with these which were rapidly killed by that dose of bacitracin, the combination had an antagonistic effect. Each variant was tested with bacteriostatic and bactericidal amounts of bacitracin alone and combined with terramycin. In each instance, terramycin showed addition when mixed with bacteriostatic doses of bacitracin but interfered with actively bactericidal doses.

In view of these results, the sensitive strain was tested further with a wide range of concentrations of bacitracin. Again it was found that static doses of bacitracin in a narrow range showed addition when combined with terramycin, whereas this group II drug interfered with bactericidal doses of bacitracin over a much wider range (figures 1 and 2). There was no essential difference in the pattern of behavior of the sensitive strain and the resistant variants derived from the same original culture. With the resistant forms, the levels of bacitracin concentrations at which addition or antagonism occurred were higher as the resistance increased, but the same sequence of events occurred. In no instance did a dose of bacitracin that was completely ineffective

against the strain tested show a joint effect, either additive or antagonistic.

The next organism studied was *M. pyogenes* var. *aureus*, strain H, against which terramycin was synergistic with bacitracin but antagonistic when acting alone; but whenever actively bactericidal doses of penicillin were used, the terramycin interfered. These findings applied not only to a sensitive strain but also to the variants at higher levels of penicillin concentration, except those against which penicillin was altogether ineffective. In one instance, with a culture resistant to 15 units of penicillin per ml no additive range at all could be demonstrated (figure 3).

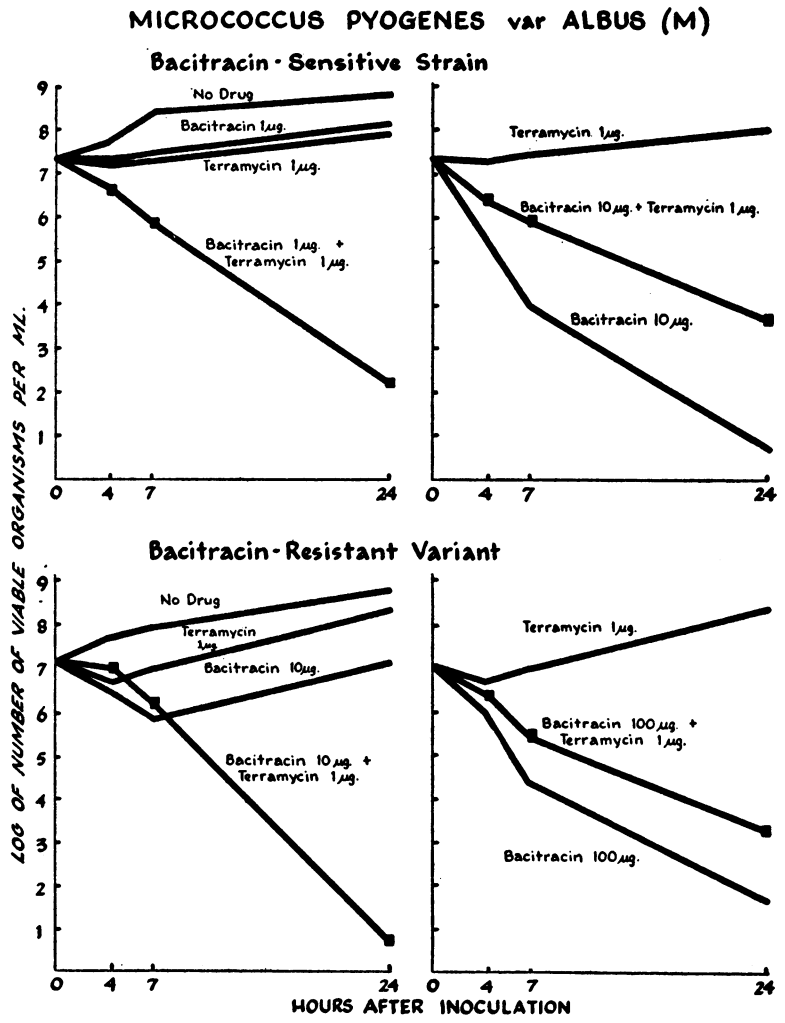

Figure 1. Combined effect of bacitracin and terramycin on strains of *Micrococcus pyogenes* var. *albus*, strain M, sensitive or resistant to bacitracin. With the bacitracin sensitive strain, bacitracin 10 µg + terramycin 1 µg result in antagonism. With the bacitracin resistant variant, this same drug combination results in addition. However, when the drugs are tested over wide ranges, antagonism and addition are demonstrated with both strains.

to penicillin (Jawetz *et al.*, 1952). Eight variants at different levels of penicillin resistance were selected for the tests summarized in table 1 and figures 2 and 3. Again, addition was shown in a narrow range of penicillin concentrations approximately equal to its bacteriostatic range

A phenomenon rarely encountered in previous tests was noted with certain of these resistant

variants but not with the sensitive strain; namely, antagonism of penicillin to bactericidal doses of terramycin. With rapidly bactericidal doses of both drugs, the interference was mutual; i.e., the mixture was less effective than either drug alone (table 1). However, this observation was the exception rather than the rule.

bacitracin resistant variant was obtained and comparative tests made as shown in table 2 and figure 2. Again, as with the cultures of micrococci, an increase in resistance of the culture did not change an antagonistic relationship of the group I and II drugs into a synergistic one but merely raised the level of the doses of bacitracin at which

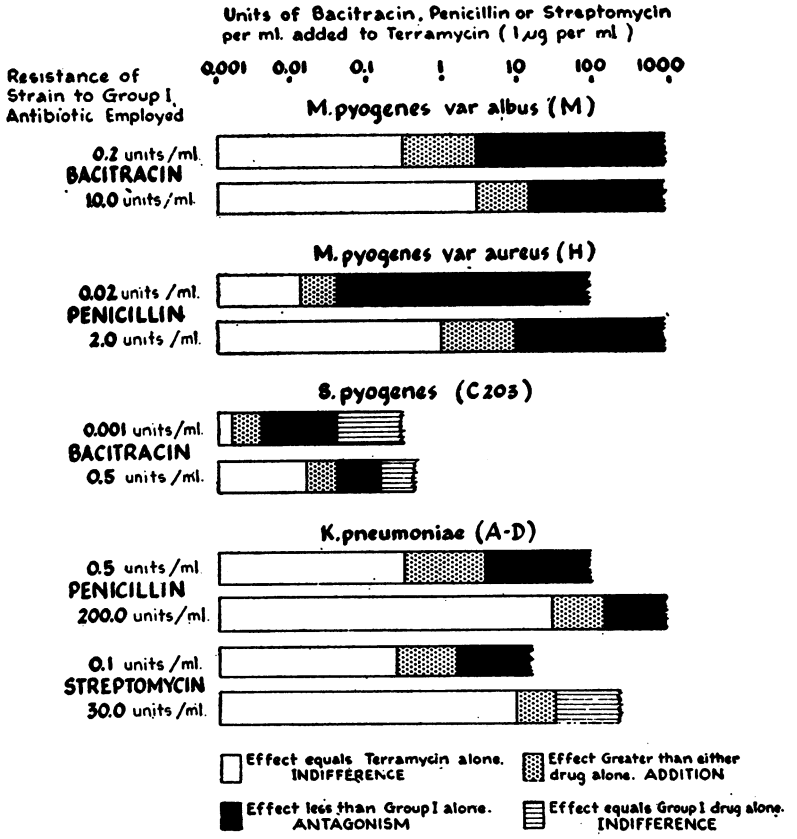

Figure 2. Spectra of combined effect of increasing concentrations of bacitracin, penicillin, or streptomycin (group I drugs) plus a constant amount of terramycin (group II drug) on strains of varying resistance to the group I drugs. The resistance to the group I drug used is expressed as that concentration of antibiotic incorporated in agar which permitted growth of at least 80 per cent of the cells of the test culture. (Spectra suggested by those of Marshall and Hrenoff (1937) for single disinfectants.)

Another culture of *M. pyogenes* var. *albus*, strain L, and penicillin resistant variants derived from it were exposed to mixtures of penicillin and chloramphenicol with essentially the same results as those described above.

Streptococcus pyogenes, strain C203, was known to be highly sensitive to both penicillin and bacitracin. Terramycin had been shown to interfere with the activity of these group I drugs (Gunnison *et al.*, 1950*a*; Speck *et al.*, 1951). A relatively antagonism was demonstrable about 10-fold. Mutual antagonism occasionally resulted when bactericidal concentrations of both drugs were used. Similar findings were obtained with a relatively penicillin resistant strain.

Klebsiella pneumoniae, strain A-D, was studied, and variants resistant to terramycin, penicillin, or streptomycin were isolated. It had been established that terramycin interfered with the activity of both penicillin and streptomycin

TABLE 1

Combined effect in vitro of penicillin and terramycin on a penicillin sensitive strain and a penicillin resistant variant of Micrococcus pyogenes var. aureus, strain H

PENICILLIN (UNITS/ML)	EFFECT OF		
	Penicillin alone	Penicillin + terramycin (1 μg/ml)	Penicillin + terramycin (100 μg/ml)
Penicillin sensitive strain			
0.01	inactive	= terramycin alone	= terramycin alone
0.02	static	addition	= terramycin alone
0.05	slowly cidal	addition	= terramycin alone
0.1–100	rapidly cidal	antagonism to penicillin	= penicillin alone
Penicillin resistant variant			
1.0	inactive	= terramycin alone	= terramycin alone
5.0	static	addition	addition
20.0	slowly cidal	addition	antagonism to terramycin
100.0	rapidly cidal	antagonism to penicillin	mutual antagonism

Static = growth inhibited for 24 hours.
Slowly cidal = 99% killed in 24 hours.
Rapidly cidal = 99.99% killed in less than 24 hours.
Terramycin 1 μg/ml alone = bacteriostatic.
Terramycin 100 μg/ml alone = rapidly cidal.

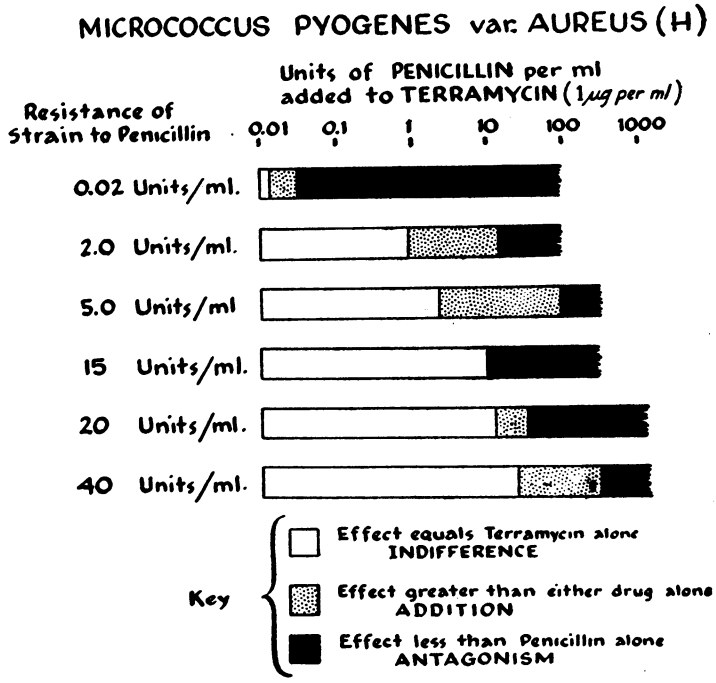

Figure 3. Spectra of combined effect of increasing concentrations of penicillin plus a constant amount of terramycin on strains of *Micrococcus pyogenes* var. *aureus*, strain H, of varying resistance to penicillin. The resistance to penicillin is expressed as that concentration incorporated in agar which permitted growth of at least 80 per cent of the cells of the test culture. One strain resistant to 15 units of penicillin per ml showed no additive effect.

against this organism (Gunnison *et al.*, 1950*a*; Jawetz *et al.*, 1951*a*). With the terramycin resistant variant, however, this drug no longer interfered with the action of streptomycin except for a slight, transitory antagonism when large, somewhat bacteriostatic doses of terramycin were used (100 μg per ml). This was not surprising because ineffective amounts of group II drugs never interfered with group I drugs. When terramycin was combined with penicillin, the results were less clear-cut as this terramycin resistant variant multiplied slowly and was therefore somewhat less sensitive to penicillin alone.

Tests with a sensitive strain showed, as with the cocci, that static or slowly bactericidal amounts of penicillin or of streptomycin gave an additive effect in a narrow zone when combined with terramycin or chloramphenicol even though the group II drugs were antagonistic to actively bactericidal amounts. The results with a penicillin resistant variant (figure 2) follow the same "spectrum" of activity as with the sensitive strain; i.e., a narrow range of additive action and a wide range of antagonistic effect as the amount of penicillin were increased. With the sensitive strain, 0.5 to 1.0 units of penicillin per ml showed an additive effect in the presence of terramycin, whereas with the resistant strain 200 to 400 units were required to give this effect.

Difficulty was encountered in obtaining streptomycin resistant strains which were not also streptomycin dependent, but a variant which was not dependent was isolated finally by the gradient plate method. This strain differed in behavior from the penicillin or bacitracin resistant variants of the organisms tested thus far in that no antagonism could be demonstrated (figure 2). Here, the lower doses of streptomycin when combined with terramycin showed the usual additive effect. Above this range, however, there was a sharp end point where the streptomycin alone was rapidly bactericidal. There was no antagonism whatever between terramycin and any concentration of streptomycin. Although here, too, antagonism was not converted into synergism as resistance to the group I drug developed, in this instance the antagonism was completely obliterated; whereas with strains resistant to penicillin or bacitracin it merely occurred at higher drug levels.

In view of the results reported here, all of the original cultures previously studied (Jawetz

TABLE 2

Combined effect in vitro of bacitracin and terramycin on a bacitracin sensitive and a bacitracin resistant variant of Streptococcus pyogenes, strain C203

BACITRACIN (UNITS/ML)	EFFECT OF		
	Bacitracin alone	Bacitracin + terramycin (1 μg/ml)	Bacitracin + terramycin (10 μg/ml)
Bacitracin sensitive strain			
0.002	inactive	= terramycin alone	= terramycin alone
0.005	slowly cidal	addition	antagonism to terramycin
0.01	rapidly cidal	antagonism to bacitracin	mutual antagonism
0.05	rapidly cidal	= bacitracin alone	= bacitracin alone
Bacitracin resistant variant			
0.01	inactive	= terramycin alone	= terramycin alone
0.02	static	addition	= terramycin alone
0.05	slowly cidal	addition	= terramycin alone
0.2	rapidly cidal	antagonism to bacitracin	mutual antagonism
0.5	rapidly cidal	= bacitracin alone	not tested

Static = growth inhibited for 24 hours.
Slowly cidal = 99% killed in 24 hours.
Rapidly cidal = 99.99% killed in less than 24 hours.
Terramycin 1 μg alone = slowly cidal.
Terramycin 10 μg alone = rapidly cidal.

et al., 1952) were retested over a wider range of concentrations to see whether similar patterns of behavior were shown. In particular it was necessary to determine whether any of the instances which had been reported as "synergism" were merely examples of the additive effect of bacteriostatic amounts of group I drugs when combined with group II drugs which might be antagonistic when higher bactericidal doses of the group I drug were used. With all pairs of drugs previously designated as antagonistic when acting on a given organism, there was a narrow zone of concentrations of the group I antibiotic in which the effect was additive, whereas the group II drug always interfered with larger doses. On the other hand, with all pairs that previously had been designated as synergistic against a given organism there was an additive effect with all concentrations of group I drug over a wide range, including bactericidal amounts, and no interference at any level. Furthermore, in these synergistic systems the combination was more effective than 2 to 10 times the dose of either drug acting alone.

DISCUSSION

The aims and results of the work reported in this paper are restated in the following questions and answers:

(1) Can both synergism and antagonism be demonstrated with a given pair of drugs acting on a single strain of bacteria, depending upon the relative proportion of drugs in the mixture? In earlier papers in this series it was stated that synergism could not be converted into antagonism nor *vice versa* within a given test system by simply altering the proportion of drugs present. The quantitative data of the present work support this conclusion. An additional fact is brought out, however. When the whole range of concentrations of an "antagonistic" pair of drugs is studied, a narrow range often can be discovered within which the drugs are additive in their action. With resistant bacterial variants, the additive zone tends to be somewhat wider than with the original sensitive strains. This additive range occurs when both drugs are present in bacteriostatic quantities. The magnitude of additive action within this range of concentrations is often small so that the death rate of bacteria exposed to the combination of drugs usually does not equal, and rarely exceeds, the death rate obtained by doubling the concentration of a single drug. While this effect does not fulfill the criteria for "synergism" used in our work, it well might account for the reports by others of both antagonistic and synergistic effects of a given drug pair against a single strain of microorganisms (Lankford and Lacy, 1949; Spicer, 1950; Bliss *et al.*, 1952).

The spectrum of combined action within a single "antagonistic" test system therefore may include the following consecutive zones as the concentration of the active, group I drug is raised progressively; (a) effect equal to that of the interfering drug, (b) additive effect (usually a narrow zone), (c) effect of more active agent diminished by interfering drug (zone of antagonism), and (d) effect equal to that of the more active agent alone (demonstrated only infrequently).

(2) Is antibiotic antagonism a unilateral phenomenon or can any drug interfere with any other drug? Earlier work had suggested that antagonism was unilateral in that group II drugs were capable of interfering with group I drugs, provided the microorganism was sensitive to the latter, but not *vice versa*. The additional studies reported in this paper indicate that, in general, these conclusions are justified. Under special circumstances, however, when high doses of group I drugs act upon bacteria relatively resistant to these drugs but for which a group II drug is rapidly lethal, the group I drug may antagonize the group II agent. The circumstances under which this event has been observed suggest that the mode of action may be distinct from that seen in the much more common antagonism of a group II agent to a group I drug.

In rare instances, mutual antagonism was observed when moderately bactericidal doses of both drugs were employed. This was noted particularly with a drug sensitive hemolytic streptococcus. With this same organism, mutual antagonism had been seen as a chance observation *in vivo* (Jawetz and Speck, 1950) but later had been discounted because of its extreme rarity.

In contrast to the ordinarily unilateral nature of antibiotic antagonism, synergism is a mutual phenomenon and has never been changed to antagonism by changing the relative concentrations of drugs. Only one of the members of a synergistic drug pair needs to exhibit a biological

effect in the concentration entering into the combination (Jawetz, 1952). The *in vitro* study of the dosage relationships in such pairs of drugs is limited by the rapid killing effect of high drug concentrations, making the demonstration of additive phenomena impractical.

(3) To what extent does knowledge of the group I resistance of a microorganism permit prediction of the effect of a combination of group I + group II drugs? It had been suggested earlier that with a group I sensitive microorganism antagonism might result; with a group I resistant microorganism, synergism. The material presented in this paper indicates that such a statement is undoubtedly an oversimplification of the situation, though perhaps of some practical validity. The first requirement for the occurrence of antagonism, as defined in our studies, is the rapid bactericidal action of one drug which can be reduced by the addition of a second agent. In the present paper it has been demonstrated that even with moderately resistant bacterial variants, antagonism could occur, provided that sufficiently high concentrations of the group I drug were employed to result in rapid bactericidal action.

Antagonism is most likely to occur with organisms highly susceptible to the group I drug employed and, conversely, synergism would be most likely with organisms resistant to that drug. As shown in this paper, however, the relative degree of resistance as such is not the determining factor in combined action. The basic features which determine whether the action of a group I + group II combination on a microorganism will result in indifference, antagonism, or synergism must be more essential and unalterable cellular characteristics than the mere level of resistance to the group I drug.

(4) The *in vitro* demonstration of antibiotic antagonism has given rise to concern about its clinical occurrence although the phenomenon undoubtedly is rare in the treatment of patients (Lepper and Dowling, 1951). Can experimental evidence explain this apparent discrepancy? Antibiotic antagonism can be demonstrated readily in the treatment of experimental infections of animals. However, the phenomenon occurs only under special circumstances which rarely prevail in clinical practice. It has been pointed out repeatedly that antagonism is limited by time and dose relationships (Jawetz *et al.*, 1951*a*,*b*; Speck *et al.*, 1951; Ahern *et al.*, 1952; Ercoli and Carminati, 1952; Lepper *et al.*, 1952). An excess of either of the participating drugs frequently overcomes antagonism *in vivo* (Speck *et al.*, 1951; Ahern *et al.*, 1952), and this has been demonstrated *in vitro* in some instances. With the highly drug sensitive organisms, against which antagonism is most likely, the drug excess obtained with ordinary doses in therapy must be large so that demonstrable antagonism would not be expected. With drug resistant bacteria, on the other hand, it is unlikely that sufficiently high, bactericidal levels could be reached in the tissues to permit antagonism. These points add to previously summarized evidence in minimizing the likelihood of observable antibiotic antagonism in clinical situations.

SUMMARY

Bacterial variants of graded antibiotic resistance were tested for their behavior toward antibiotic combinations in order to investigate the role played by resistance toward one agent in the response to a mixture of drugs. It was found that antibiotic antagonism with a certain drug pair acting on an antibiotic sensitive bacterial strain was not converted to synergism when the same drugs acted on an antibiotic resistant variant. The type of combined action, whether synergism, indifference, or antagonism, was essentially stable within a given test system although wide variations in drug concentration were necessary to demonstrate this action against bacteria of varying resistance.

When "antagonistic" drug pairs were subjected to a detailed study of dosage relationships, a narrow additive zone was found often. This occurred in the range of bacteriostatic concentrations of both drugs and never had the magnitude of true synergism. Synergistic test systems did not contain areas of antagonism. The relationship of these results to earlier work is discussed, and factors limiting the occurrence of antibiotic antagonism in animal and human infections are summarized.

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