JOINT ACTION OF SOME ANTIMICROBIAL AGENTS

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ABSTRACT

The effect of ampicillin combined with penicillin or cephalosporin against ten staphylococcal strains and eighteen coliform bacteria was investigated. Eight penicillins and four cephalosporins were employed in the above experiment. It was found that, when these antibiotics were combined with ampicillin against penicillin-sensitive organisms, at least an additive effect and, often synergism, was observed. When the antibiotic combinations were tested against penicillin-resistant staphylococci, there was no interference observed in most cases. Antagonism was demonstrated between ampicillin and penicillinase-resistant penicillins in three cases. Synergism was also observed in three instances with the above combinations against resistant staphylococci. It was also found that cephalosporins showed synergism with ampicillin against three resistant staphylococcal strains and the coliform bacteria.

In vivo mouse protection tests were carried out with antibiotics that showed synergism with ampicillin *in vitro* against *S. aureus* 209P, 20137, *E. coli* 4007 and Klebsiella-Aerobacter sp. 4341. The antibiotics did not interfere with the action of ampicillin.

The action of four penicillinases (produced by *S. aureus* 20137, Pseudomonas sp. 3895, *Bacillus subtilis*, and a commercial penicillinase - Bacto-Penase, Difco.) on the ampicillin and B-lactam antibiotic combination was tested, using *S. aureus* 209P as the indicator organism. It was found that the antibiotics did not protect ampicillin from enzymic hydrolysis.

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CHAPTER I

INTRODUCTION

Bacterial antagonism or antibiosis was first observed by Pasteur and Joubert in 1877 and later by Fleming in 1929, however, the significance of these observations was not made popular until Chain et al (1940) and Abrahams et al (1941) investigated the use of penicillin as a therapeutic agent. Since the introduction of pencillin, many new antibiotics have been reported in the last fifteen years. However, penicillin still remains the drug of choice in many instances because of its relative non-toxic properties, its high activity against many sensitive bacteria and its readily attainable high serum levels. The indiscriminate use of this agent has resulted in a gradual evolvement of penicillin-resistant bacterial strains. This problem stimulated an extensive search for new semi-synthetic penicillins. Other investigators searched for antibiotic combinations that would elicit a synergistic effect on the resistant organisms.

Since the isolation of 6-amino-penicillanic acid by Batchelor et al (1959) many semi-synthetic penicillins have been produced. The new penicillins are the derivatives of 6-amino-penicillanic acid (6-APA). It is possible to synthesize penicillins with a variety of acid side chains, and as a result different physico-chemical and biological properties may be found among them.

Stedman et al (1964) described the effect of major side chain modifications on the biological properties of 2-biphenylyl-penicillin and have elucidated the structural features responsible for its high activity against both susceptible and resistant staphylococci. Price

et al (1966) described the relationship between the structure of the side chain and the biological activity of penicillin. He showed that the side chain at the 6-position of a penicillin molecule affected biological as well as other properties of penicillin.

There are numerous reports in the literature of combined antibiotic therapy. In most cases, penicillin was combined with other antibiotics, e.g., streptomycin, sulfonaminde, etc. (Hunter, 1946; Eagle and Fleischman, 1948; Gunnison et al, 1951; Gronroos , 1964; Strans and Fleming, 1964; and Wallace et al, 1965). Few reports, however, are found where two penicillin analogues were combined (Gourevitch et al, 1963; Acred and Sutherland, 1966; Bach et al, 1966; Kasik et al, 1966; Sabath et al, 1966). In the study reported here, the in vitro activity of ampicillin (6-(D(-)-α-aminophenyl-acetamido) Penicillanic acid or α-aminobenzyl penicillin) in combination with other penicillins and cephalosporins was investigated against various bacterial strains. Mouse protection tests were carried out with ampicillin and penicillins or cephalosporins that showed synergism in their in vitro combinations. The effect of penicillins and cephalosporins on the hydrolysis of ampicillin by four penicillinases was also studied.

CHAPTER II

REVIEW OF THE LITERATURE

Klein and Kimmelman in 1947 reported the synergistic combination of penicillin and streptomycin against S. aureus. This was followed by the successful treatment of syphilis in a rabbit with the combination of penicillin and bacitracin by Eagle and Fleischman in 1948. Hunter (1946) discovered that streptomycin acted synergistically with penicillin in treating patients suffering from enterococcic endocarditis. Jawetz and Gunnison in 1952, published a paper formulating a rough guide for the use of antibiotic combinations. Jawetz was of the opinion that fixed synergistic or antagonistic pairs of drugs do not exist. In fact, a combination of two antibiotics may be synergistic against one strain, indifferent to another and antagonistic against the third. Therefore, there is no sure way of predicting how an antibiotic pair would act on a particular bacterial strain unless an actual test is performed. However, the Jawetz scheme did provide a generalized guide line for the action of antibiotic combinations. He divided the commonly used antibiotics into two groups:

Group I: Bactericidal group: penicillin, streptomycin, bacitracin and neomycin.

Group 2: Bacteriostatic group: aureomycin, chloramphenicol and terramycin.

He put forward following formulae for in vitro conditions: -

1. Drugs from group 1 were often synergistic to each other,

occasionally indifferent and never antagonistic.

2. Group 2 drugs were not synergistic with, or antagonistic to each other, but were often additive.
3. Drugs from group 2 were capable of antagonizing those from group 1 when acting against bacteria sensitive to group 1.
4. Group 2 drugs might be synergistic with group 1 drugs when acting on group 1-resistant organisms.

In the above series of studies, according to Jawetz, synergism in vitro between two antibiotics was defined as a marked increase in the rate of bactericidal action within the twenty-four hours of exposure as compared to the rate with either drug alone. Antagonism between two antibiotics was defined as a decrease in the bactericidal rate as compared to the more active drug. Addition was referred to as an arithmetic summation of drug effects. Manten and Wisse (1961) confirmed the conclusions of Jawetz and Gunnison. In their experiments, bactericidal action was used as a criterion of antibacterial potency. The criterion was applied by means of replica tests. They experimented on dozens of strains of Staphylococci, Enterococci, Salmonellae and coliform bacteria, and as a result, proposed a more up-to-date scheme, showing the presence and absence of drug antagonism in pairs of antibacterial substances. They divided the antibiotics into four groups: -

1. Antibiotics that were bactericidal to resting bacteria.
2. Antibiotics that were bactericidal only to growing bacteria.
3. Agents that achieved their bacteriostatic effect rapidly.

4. Agents that required a long lag period before they caused bacteriostasis.

With the above as a basis of division, the following generalizations were drawn: -

1. Antibiotics of the same group could be combined with one another without any danger of antagonism.
2. When a group 1 agent was combined with one of either group 2, 3 or 4 agent, the activity of the bactericidal agent generally predominated. Synergism was sometimes observed, e.g., streptomycin and penicillin.
3. When agents from group 2 and group 3 were combined, the weaker bacteriostatic drug dominated and showed antagonism towards the other.
4. When agents from group 2 and group 4 were combined, no antagonism was observed. The bactericidal drug dominated and, at times, synergism was observed, e.g., penicillin and some sulfonamides.

Penicillin, belonging to group 2 could only act on bacteria in a growing stage while group 3 antibiotics could achieve bacteriostatic effect rapidly. Hence, a group 3 antibiotic would render a group 2 antibiotic (e.g., penicillin) ineffective by stopping the growth of bacteria.

Group 4 drugs, however, required a comparatively long lapse of time before they could cause any bacteriostatic effect. So, the quick

acting penicillin (group 2) in combination could kill the organism in growing stage. Hence, no antagonism would result.

Bactericidal activity of group 1 antibiotics were independent of the growth of bacteria. Thus, bacteriostasis caused by group 3 or 4 agents had no adverse effect on group 1 antibiotics.

I. ACTION OF PENICILLIN IN COMBINATION WITH BACTERIOSTATIC AGENTS:

A. Penicillin and Chloramphenicol:

Jawetz et al (1951) demonstrated the interference of chloramphenicol with the bactericidal action of penicillin. Antagonism could only be shown when penicillin was mixed in an effective concentration with a sublethal amount of chloramphenicol. Interference could not be demonstrated *in vitro* when the concentration of chloramphenicol was either too high or too low to be bacteriostatic. Similarly, antagonism was greatly diminished or could not be demonstrated when penicillin was present, either in a large excess or in an amount too small to have a significant bactericidal effect. Bagdasarian (1960) introduced a subinhibitory quantity of either chloramphenicol or streptomycin into a culture of *B. cereus* that was capable of high penicillinase production and which was also resistant to both of these antibiotics. He noted that, though the antibiotics did not interfere with the growth and multiplication of bacteria in the subinhibitory concentration, they reduced penicillinase production in the medium to one-seventh of the capacity. This finding may justify further experimentation on the activity of penicillin in association with either of the antibiotics against penicillinase-producing

staphylococci. Brock (1961) reported that chloramphenicol antagonized the action of those antibiotics which acted on growing cells (e.g., penicillin and streptomycin) but, its activity was additive with those that inhibited protein synthesis, such as the tetracyclines. This, is in accordance with the Jawetz's postulate. Strans and Fleming (1964) studied in vitro effect of ampicillin alone and in combination with chloramphenicol or streptomycin against Proteus mirabilis. He found that the combination of ampicillin and streptomycin greatly enhanced the antimicrobial activity of ampicillin. However, the concentration of streptomycin below one-third of the minimal inhibitory concentration (M.I.C.) had no effect on ampicillin. Chloramphenicol was found to be antagonistic with ampicillin only in suitable concentrations. A concentration of chloramphenicol less than one-sixth of M.I.C. usually had no effect on ampicillin. Streptomycin enhanced the bactericidal activity of ampicillin in serum, but chloramphenicol did not interfere with ampicillin in vivo. Wallace et al (1965) demonstrated antagonism between chloramphenicol and penicillin in experimental pneumococcal meningitis in dogs. He found that with penicillin alone or when penicillin therapy preceded chloramphenicol, there was a prompt killing of bacteria evident from the decreased counts of viable bacteria in the cerebrospinal fluid samples. With chloramphenicol alone or when chloramphenicol was given prior to penicillin, a markedly lowered bactericidal effect was observed. Simultaneous administration of the two agents resulted in an intermediary degree of killing. He concluded that this type of antagonism might be of clinical importance.

B. Penicillin and Tetracyclines:

Seligman and Hewitt (1963) demonstrated antagonism between ampicillin and oxytetracycline while working on E. coli. As can be predicted, the magnitude of the interference on the bactericidal activity of penicillin by the tetracyclines would depend largely on the extent to which the tetracycline could inhibit intracellular growth before a penicillin-induced defect in cell wall growth was accomplished. In their experiments, they found that in prolonging the period of prior tetracycline contact, the degree of subsequent penicillin bactericidal effect was lessened. They were not able to demonstrate antagonism in vivo if penicillin was given prior to oxytetracycline, however, when the drugs were administered in a reverse order, varying results were observed.

C. Penicillin and Erythromycin:

Waterworth (1963) showed that a marked increase in activity against resistant staphylococci was achieved with the combination of erythromycin and penicillin. Gronroos (1964) and Oswald et al (1964) demonstrated that the combination of erythromycin and penicillin G, penicillin V or phenethicillin exerted at least an additive action on staphylococci. It was also shown that in vivo, the two antibiotics did not interfere with each other's activity. Robert et al (1962) explained that the synergistic effect was probably due to the decreased rate of penicillin inactivation, resulting from the inhibitory effect of a small amount of erythromycin on bacterial penicillinase production. As a result, all the four penicillins (penicillin G, penicillin V, phenethicillin

and ampicillin) instead of being rapidly inactivated by penicillinase, were benefited by the protective effect of erythromycin. On the other hand, the penicillinase-resistant penicillins did not seem to be benefited by combining with erythromycin. The innoculum size was important in determining whether erythromycin would enhance the effect of penicillins in combination, as one of the actions of erythromycin was to exert an inhibitory effect on penicillinase production.

D. Penicillin and Sulfonamides:

Early reports on the combined effect of sulfonamides and penicillin on bacteria are conflicting, (Ungar, 1943; Bigger, 1946; T'ung, 1944; Stewart, 1947; Price et al, 1949; Hobby and Dawson, 1946, Klein and Kalter, 1946). The discrepancy among these reports was mainly due to the differences in technique and definitions of synergism and antagonism employed by different authors. Gunnison et al (1951) claimed that, sulfonamide-induced bacteriostasis only occurred after four to five hours, with a maximal effect after eight hours of incubation. They stated that, during the period of bacteriostasis, sulfonamides were capable of interfering with the bactericidal effect of penicillin. The death rate of bacteria was slower in the presence of sulfadiazine and penicillin than with penicillin alone from the sixth to tenth hour of incubation. This delay in antagonism by sulfadiazine appeared to be different from that observed with other bacteriostatic antibiotics such as terramycin. The latter showed antagonism with penicillin almost immediately. If the concentration of penicillin was such that it was rapidly bactericidal, the

presence of sulfadiazine would have no significant effect on the penicillin action. On the other hand, when a low dose of penicillin was used, the resistant bacteria that survived the penicillin action might grow into a new population if sulfadiazine was not present. Thus, it could be said that sulfadiazine exhibited an additive and perhaps, even synergistic effect if ultimate sterilization was used as the criterion of synergism, even though it might be antagonistic to penicillin in the early stage of exposure. Gunnison also reported that when K. pneumoniae was exposed for four hours to sulfadiazine before adding penicillin, interference was demonstrated without delay. In vivo experiments on mice with S. pyogenes showed that: -

1. When sulfadiazine was administered simultaneously or two hours before penicillin treatment, neither an antagonistic nor a synergistic effect was demonstrated.
2. Marked antagonism occurred when sulfadiazine was administered, five to seven hours prior to penicillin. On the whole, antagonism was only demonstrated when an effective concentration of penicillin either in vitro or in vivo were combined with a relatively non-effective amount of interfering sulfonamide. Manten and Terr (1964) found that antagonism between penicillin and the other bacteriostatic substances (chloramphenicol, tetracyclines or erythromycin) was not appreciably influenced by the absolute concentration of the drugs nor by their concentration ratio.

II. ACTION OF PENICILLIN IN COMBINATION WITH OTHER BACTERICIDAL AGENTS:

The antimicrobial activity of penicillin combined with one of the bactericidal agents can very well be predicted from Jawetz's schema. Perhaps the synergistic effect of the penicillin and streptomycin combination in the treatment of bacterial endocarditis is one of the best known examples. In general, penicillin-bacitracin, bacitracin-streptomycin, penicillin-kanamycin and other similar combinations are all reported to have synergistic effect on various organisms.

A. Penicillin, Streptomycin and Tetracycline:

Richardson and Holt (1962) found that streptomycin acted synergistically with penicillin and tetracycline to inhibit the growth of Brucella abortus within bovine cell cultures. Within certain limits, the synergistic effect of streptomycin and tetracycline did not appear to be a function of concentration. To achieve synergism of streptomycin with tetracycline on intracellular brucellae in bovine tissue culture, it required five to ten times the effective concentration of streptomycin against the same organisms outside the bovine cells. Penicillin and tetracycline on the other hand, would act effectively at the same concentration both on extracellular and intracellular brucellae.

B. Penicillin, Streptomycin and Kanamycin:

The report of Hewitt et al (1966) on the kinetics of the synergism of penicillin-streptomycin (Pn/St) and penicillin-kanamycin (Pn/Kn) for enterococci might be of some interest. Both streptomycin and kana-

mycin were reported to enhance the bactericidal effect of penicillin, wherein the Pn/Kn pair was superior to the Pn/St pair. The enhancement of the bactericidal effect of penicillin occurred immediately with kanamycin, but a lag period of about one hour was required by streptomycin to exercise its action on penicillin. The mechanism by which the two systems worked was obviously different. Both kanamycin and streptomycin were thought to act on bacteria whose cell wall had been damaged by penicillin. Kanamycin acted synergistically on the bacteria which were pre-exposed to penicillin. It was found that, when penicillinase was added to the penicillin pre-exposed bacteria and kanamycin, the synergistic activity persisted. The bactericidal effect of streptomycin in the presence of penicillin varied with different enterococcal strains while no such variation with kanamycin and penicillin was observed.

C. Penicillin and Isoniazid:

Vaichulis (1961) using the antibiotic disc method, found that isoniazid (Isonicotinic Acid hydrazide) acted synergistically with penicillin against Mycobacterium smegmatis. When the concentration of isoniazid remained constant, the zone of inhibition increased as the concentration of penicillin increased. Penicillin was thought to have potentiated the action of isoniazid. Vaichulis et al (1962) further reported that the activity of penicillin isoniazid combination was at least additive and possibly synergistic against isoniazid-suseptible as well as resistant strains of Mycobacterium tuberculosis. The activity of penicillin against either isoniazid susceptible or resistant Mycobacterium tuberculosis was

constant which would indicate that penicillin has a different mode of action from that of isoniazid against Mycobacterium tuberculosis.

D. Ampicillin and Other Broad Spectrum Antibiotics:

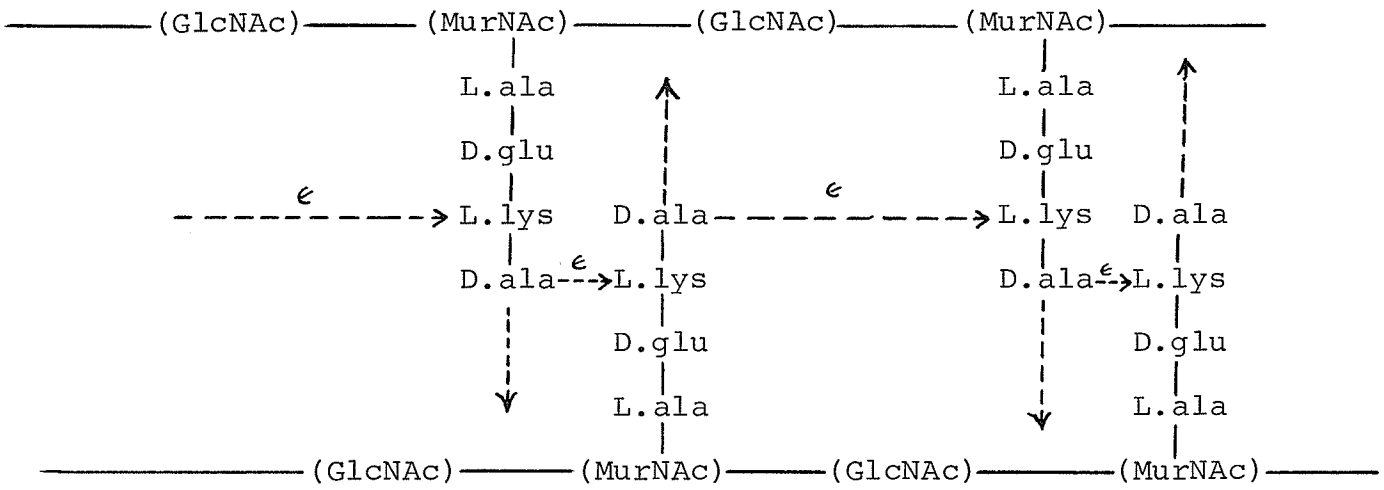
Bulger and Kirby (1963) found that gentamicin/ampicillin combination was very effective against Proteus mirabilis. Combinations such as penicillin G/gentamicin, ampicillin/kanamycin and kanamycin/gentamicin also gave a synergistic effect in most instances. The ampicillin and gentamicin combination was more effective against indole positive proteus. Colistin/gentamicin was found to be the most effective combination against Klebsiella-Aerobacter group as well as Pseudomonas aerogenosa.

III. PENICILLINS AND CEPHALOSPORINS:

A. Mode of Action of Penicillin:

It had long been suspected that penicillin is a highly specific inhibitor of bacterial cell wall synthesis. Rogers and Jeljaszewicz (1961), Rogers and Mandeistam (1962) confirmed this hypothesis by direct isotopic measurements of cell wall synthesis carried out with several penicillins in both gram negative and gram positive bacteria. Meadow et al (1964) and Anderson et al (1965) showed that the reactions which led to the synthesis of the linear cell wall glycopeptide from uridine-diphosphoacetylmuramyl L-ala.D-glu. L-lys.D-ala.D-ala (UDP-MurNAc-Pentapeptide), uridine diphosphoacetyl glucosamine (UDP-GlcNAc) and other required substrates were insensitive to penicillin. However, the natural glycopeptide is cross-linked by peptide bridges, and Martin (1964) suggested that penicillin blocked the formation of these peptide cross-

links. Tipper and Strominger (1965) suggested that, in cell wall synthesis, the terminal reaction might be a transpeptidation in which linear glycopeptides were cross-linked to form a three dimensional network (Figure 1). Based on these molecular models, it is now generally accepted that penicillin is a structural analogue of acyl-D-alanyl-D-alanine in the linear glycopeptide. It inhibits transpeptidation by reacting preferentially with the enzyme binding site of a transpeptidase for this substrate. A facile acylation of the transfer site occurs with the opening of the B-lactam ring forming a penicilloyl-enzyme complex. The highly reactive amide bond of the B-lactam ring in the penicillin molecule is considered equivalent to the peptide bond of D-alanyl-D-alanine. The integrity of the B-lactam ring is essential for penicillin activity (Price et al, 1966). Erlanger and Goode (1967) approached the problem of penicillin activity from the enzymological point of view. Penicillin was assumed to be a potent competitive inhibitor of an enzyme that participated in some phase of cell wall synthesis. They suggested that the high activity of penicillin was due to its rigidly constrained structure. The penicillin molecule can, then, be divided into two parts - the part that resembles the substrate and that part that holds the molecule in the proper conformation.(Figure 2). In the case of penicillin G, the substrate analogue is phenacetylglycyl-D-valine. The acylated glycyl-D-valine or the related derivatives were found to be biologically inactive because they are not structurally constrained.



GlcNAc - N-acetyl glucosamine

MurNAc - 3-0-D-carboxy ethyl N-acetyl glucosamine
or N-acetyl muramic acid

L.ala - L -alanine

D.glu - D-glutamic acid

L.lys - L-lysine

D.ala - D-alanine

Fig. 1 General Structure for Cell Wall Mucopeptide

B. The Penicillin Molecule:

The penicillins are acyl derivatives of 6-aminopenicillanic acid (6-APA). With the variation of the side chain R (Figure 2), hundreds of different semi-synthetic penicillins, with different biological, pharmacological and physiochemical properties can be synthesized.

Price et al (1966) in his discussion on the structure activity relationships of semi-synthetic penicillins, pointed out that the antimicrobial activity of penicillin depended on the integrity of the B-lactam ring in the penicillin nucleus. The side chain R, however, contributed a great deal to the antimicrobial potency and other important properties of individual penicillin. It was also found that the elimination of the S-atom from the thiazolidine ring resulted in the complete loss of antimicrobial activity of penicillin. The side chain structure was not only responsible for the high or low antimicrobial activity, the acid stability and the antimicrobial spectrum of the penicillin, but also contributed to its stability in the presence of B-lactamase.

The Cephalosporin Molecule:

The cephalosporin nucleus has a B-lactam-dihydrothiazine ring instead of a B-lactam thiazolidine ring in the penicillin nucleus (Figure 3). The various possible side chain structures for R_1 and R_2 also confer different properties to the 7-aminocephalosporanic acid nucleus (7-ASA) as in the case of the 6-APA. The structure-activity relationships among 7-acylamido-cephalosporanic acids were carefully studied by Chauvette et al (1962) and the biological and chemical properties of the cephal-

-------- CONSTRAINING BONDS

———— SUBSTRATE BONDS

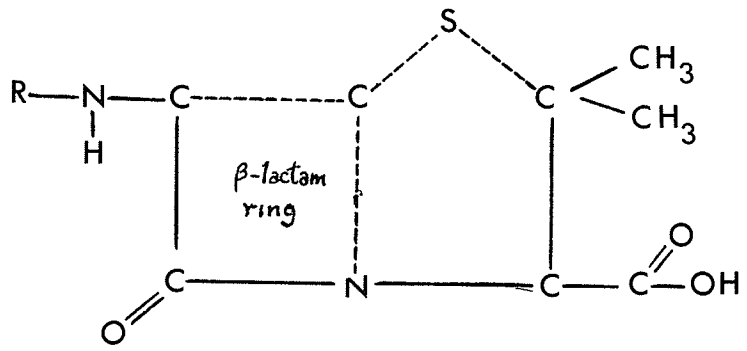


Fig. 2 THE PENICILLIN MOLECULE

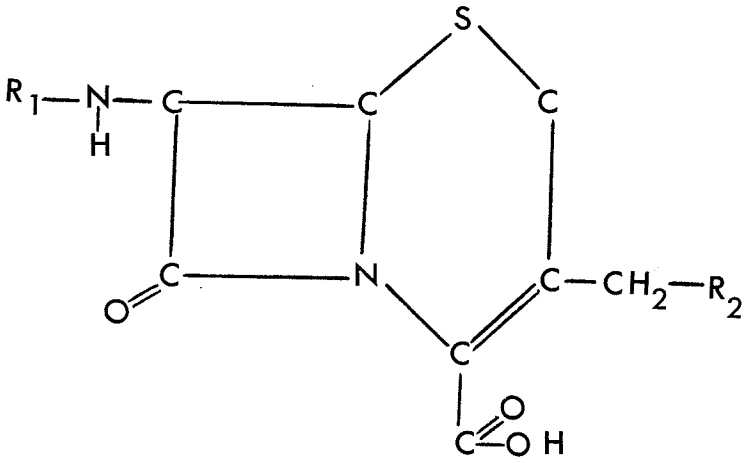


Fig.3 THE CEPHALOSPORIN MOLECULE

osporins were described by Flynn (1966). It was found that the activity of the cephalosporins again depended on the integrity of the B-lactam ring. The acylation of the carboxyl group, however, also resulted in the loss of activity.

C. B-lactamase and Bacterial Resistance to Penicillins:

Seligman (1966) reported two types of bacterial resistance to penicillin: -

1. Methicillin resistance (or hetero-resistance with respect to B-lactam antibiotic according to Seligman).
2. Drug destruction subsequent to penicillinase production.

1. Methicillin Resistance:

Methicillin is much less susceptible to the hydrolytic activity of the B-lactamase because (1) of its low affinity for B-lactamase and (2) its bulky groups attached to the carbon in the side chain. However, methicillin-resistant strains of Staphylococcus aureus were also encountered (Jevons, 1961). The amount of penicillinase produced by methicillin-resistant organisms was not more than that produced by methicillin sensitive bacteria (Ayliffe and Barber, 1963; Richmond and John, 1964). This data suggests some intrinsic factors are responsible for the methicillin resistance. Dyke et al (1966) selected penicillinase-negative variants from the methicillin-resistant staphylococcal cultures and found that they were resistant to both methicillin and benzylpenicillin. He concluded that this was a type of resistance which was different from

that resulting from penicillinase production. Seligman (1966) pointed out that, colonies which grew in high concentrations of methicillin were mutants that possessed a high intrinsic resistance to methicillin. The parent culture, however, showed marked heterogeneity in its resistance to methicillin. He called those strains heteroresistant staphylococci.

2. Penicillinase and Resistance to Penicillin:

Pollock (1964) stated that there were three kinds of enzymes produced by organisms which reduced the antibacterial property of penicillins and cephalosporins.

a. the acylesterase which significantly reduces the activity of cephalosporins.

b. the amidases which hydrolyses the amide bond of the side chain of penicillin or cephalosporin forming the inactive derivatives 6-APA or 7-ACA respectively.

c. the B-lactamases which hydrolyses the amide bond in the B-lactam ring to give penicilloic acid or cephalosporoic acid. The antimicrobial activity is completely destroyed with the breakdown of the B-lactam ring.

B-lactamase or penicillinase production is, by far, the most significant type of penicillin resistance. B-lactamase probably has a wide distribution among bacteria. Only Gram positive species liberate this enzyme into the environment to a significant extent and it appears to be markedly inducible. B-lactamases produced by the Gram negative bacteria appear to be different from those produced by the Gram positive

bacteria (Smith and Hamilton-Miller, 1963 a,b). They are noninducible and cell bound. Sabath et al (1965) reported that the hydrolysis of penicillins and cephalosporins by an induced enzyme preparation from Pseudomonas pyocyanea was due to a single B-lactamase. Pollock (1964) believed that only one type of enzyme existed in his system that was capable of hydrolysing the penicillins as well as the cephalosporins though, the ratio of activity of the enzyme on the two varied greatly. Chang and Weinstein (1963) studied the B-lactamase activity of the culture supernatant of a Herellea strain and concluded that a single enzyme was responsible for the much higher activity of the supernatant against cephalothin in comparison to benzyl-penicillin G. Richmond (1963) confirmed that, one type of B-lactamase could hydrolyse different penicillin and cephalosporin derivatives. The fact that B-lactamase has a wide spectrum of activity does not preclude the existence of more than one B-lactamase in a pure bacterial culture. Pollock (1965) demonstrated the coexistence of two immunologically distant B-lactamases in the culture of Bacillus cereus, one being extracellular and the other, bound to the cells. A similar situation was observed by Ishimoto (1963) in cultures of B. subtilis T98 where the cell bound enzyme differed from the exoenzyme. The exo-penicillinase of B.cereus was observed to precipitate with B. subtilis penicillinase antiserum.

D. Side Chain Structure and Susceptibility to Penicillinase:

Penicillins differ from each other only in the structure of the side chain, hence, a comparative study of penicillins as substrates for a B-lactamase may be regarded as a study of the side chain on the

rate of hydrolysis by an enzyme preparation. Henry and Housewright (1947) first noticed that B. cereus B-lactamase hydrolysed benzylpenicillin and P-hydroxybenzylpenicillin at about the same rate but, $\triangle^2$-pentenylpenicillin and n-heptylpenicillin were hydrolysed slowly. Behrens and Kingkade, in the following year, also observed a different rate of hydrolysis by a B-lactamase on eighteen penicillins with different side chains. Gourevitch et al (1962) studied the effect of staphylococcal penicillinase on the side chains of penicillins. They found that penicillinase-sensitive penicillins had low Km (Michaelis constant) and high Vmax (maximum velocity). The resistant penicillins had either moderately large Km and low Vmax or very large Km. Depue et al (1964) pointed out that, very little variation in Vmax and Km was found with phenyl, benzyl and aliphatic penicillin series. A positively charged nitrogen adjacent to the amide linkage in the side chain, however, caused a marked increase in Km. Km and Vmax of Staphylococcal penicillinase with phenyl penicillins varied with the ionization constants of their parent side chain acids. Km increased with increasing acid strength while Vmax increased with decreasing acid strength of the parent side chain acids. The kinetic constants of penicillinase exhibited relatively large changes with penicillins having large stericly hindered side chains (Doyle et al, 1961). Depue et al (1964) also pointed out that the high degree of resistance of methicillin to hydrolysis was a result of a very weak binding of the compound to the enzyme (increased Km). The bulky phenyl ring projecting upward from the plane of the penicillanic acid ring system, could have distorted the conformation of the enzyme and

thus, the enzyme was not reactive.

The effect of side chain structure has also been studied in cephalosporins. Although the information here is much more limited, the general picture is similar to that obtained with the penicillins.

E. Competitive Inhibition of Penicillinase by Penicillinase-Resistant Penicillins or Cephalosporins:

Abraham and Newton (1956) showed that, in the presence of penicillinase, the penicillinase-resistant cephalosporin C, (which is closely related structurally to penicillin) competed with the Penicillin for the active sites on the enzyme. The hydrolysis of penicillin was reduced in the presence of cephalosporin C. Gourevitch et al (1962 a) found that, when penicillin G was mixed with Methicillin in the presence of B-lactamase, no competitive inhibition of penicillin G hydrolysis was evident. The benzyl-penicillin component was decomposed at the same rate as if it was the only penicillin present and its decomposition did not destroy the enzyme. When all benzylpenicillin had been degraded, the methicillin started to inactivate the enzyme and the reaction rate decreased. A similar result was obtained when oxacillin was used in place of methicillin. Since the Michaelis constant (Km) of oxacillin and methicillin is 200 to 1000 times greater than that of the sensitive penicillins (Gourevitch et al, 1962 a, 1963), enzyme inactivation does not take place in the presence of sensitive penicillins to which the enzyme binds preferentially. Gourevitch also showed that methicillin and oxacillin alone inactivated penicillinase. Thus, their resistance to penicill-

inase was due, not only to their resistance to hydrolysis by the enzyme but, also by virtue of their ability to inactivate it (Gourevitch et al, 1962 a; Citri et al, 1962 a.b.). Gourevitch et al (1963) emphasized that the minimal inhibitory concentration of a mixture of penicillinase-sensitive and penicillinase-resistant penicillins against penicillinase producing Staphylococcus aureus strains depended upon the order of addition of the penicillins. The penicillin-resistant penicillins acted as enzyme inactivators only if they were introduced prior to the penicillinase-sensitive penicillin. The difference in the affinity of the various substrates for the enzyme was responsible for the above activity. In in vivo experiments, similar results were obtained. When oxacillin was administered to mice thirty to sixty minutes prior to a challenge dose and penicillin G given subsequently (120 minutes post-challenge), the survival rate was slightly increased.

Selzer and Wright (1964), when comparing the effectiveness of the different penicillinase-resistant penicillins to the action of penicillinase produced by B. cereus, found that nafcillin was superior to methicillin, oxacillin and diphenicillin in protecting penicillin G. In contrast to their ability to inhibit the action of B. cereus penicillinase, the semi-synthetic penicillins did not interfere with the action of staphylococcal penicillinase on penicillin G. This may be due to the fundamental structural difference between the two enzymes, or to the fact that the B. cereus penicillinase is a free extracellular enzyme whereas, staphylococcal penicillinase is intracellular. This presumably, may have some bearing on the accessibility of the penicillin G molecules

to the enzyme. In the case of the intracellular penicillinase, it is possible that the penicillin G molecules pass through the cell wall more readily than the semi-synthetic penicillins. The intracellular enzyme may thus, be comparatively shielded from the inhibitory effect of the semi-synthetic penicillins. Hamilton-Miller (1963), Hamilton-Miller and Smith (1964), Hamilton-Miller et al (1964) and Sutherland and Batchelor (1964), reported that methicillin and cloxacillin were inhibitors of penicillinase produced, not only by Ps. pyocyanea but also, by a number of other gram negative bacteria. These inhibitors acted synergistically with benzylpenicillin G or ampicillin against several gram negative bacteria.

Acred and Sutherland (1966) found that cloxacillin improved the broad spectrum activity of ampicillin against penicillinase-producing staphylococci and most strains of gram negative bacteria. No antagonism was observed and synergism was demonstrated both in vitro and in vivo against certain penicillinase-producing gram negative bacteria. The synergism was shown to be due to an inhibition of the action of penicillinases produced by these bacteria by cloxacillin and a subsequent reduction of the inactivation of ampicillin. Bach et al (1966) also demonstrated the in vitro and in vivo synergism of ampicillin and B-lactamase-resistant penicillins against gram negative organisms. Dicloxacillin was the most effective compound found to produce a synergistic effect with ampicillin. Cloxacillin and oxacillin were almost as effective, whereas, nafcillin and methicillin were less likely to act as synergists than the three isoxazole penicillins. Cephalothin and propicillin were essent-

ially nonsynergistic. All organisms undergoing such synergistic effects, produced a B-lactamase which could be inhibited by dicloxacillin in the presence of ampicillin. Farrar et al (1966) pointed out that, the penicillinase-resistant penicillins were ineffective against gram negative bacteria but, they might act as penicillinase inhibitors and render these organism more susceptible to penicillin G and related antibiotics. Penicillin G, ampicillin and cephalothin were tested in combination with penicillinase inhibitors (methicillin, oxacillin or nafcillin) against fifty-seven strains of gram negative bacteria that were highly resistant to penicillins or cephalosporins or both. The penicillin and inhibitor combinations showed synergism in 79% of the strains. They thought that combined therapy might be a valuable approach to the treatment of urinary tract infections. Sabath et al (1966) used the penicillins-inhibitor combination in human urinary tract infection caused by Pseudomonas aeruginosa, Proteus sp., Escherichia coli or members of the Klebsiella-Aerobactor group. An effective synergistic level in the urine was attained by the drugs but, never in serum. Recurrence was found to be common after ceasation of therapy. Klebsiella-Aerobacter organisms were not affected much by this combined therapy. Sabath and Abraham (1964) demonstrated synergistic antimicrobial action against Pseudomonas pyocyanea (NCTC8203) by a B-lactamase inhibitor (methicillin or cloxacillin) with any one of several derivatives of 6-APA or 7-ACA which were susceptible to enzymatic hydrolysis. A similar finding was reported by Shirley and Moore (1965) on the synergistic combination of methicillin and benzylpenicillin in treating a urinary tract infection caused by Pseudomonas aeroginosa.

They also evaluated the factors involved in competitive inhibition of penicillinase. They were convinced that a strong affinity for an accessible penicillinase compared to that for an antibacterial substrate (enzyme for cell wall synthesis) was one of the properties required of a competitive inhibitor to protect B-lactamase susceptible antibiotics. A second requirement was an affinity for the enzyme (penicillinase) which should be high in relation to the antibacterial activity of the inhibitor. Cromptom et al (1962) had illustrated that N-phenylacetyl-7ACA (Cephaloram) had a strong affinity for staphylococcal penicillinase and would be expected to show a significant synergistic effect with benzylpenicillin by inhibiting enzymatic destruction of the latter. However, with the concentration of such an inhibitor which was lower than that at which the inhibitor alone was bactericidal against S. aureus R1 (about 0.5μ/ml), the inhibitor was not able to reduce the rate of inactivation of a penicillinase sensitive substance to less than about 10% of the original value. A third property was the ability of the inhibitor to reach the enzyme, especially when it was located intracellularly. Hamilton-Miller et al (1965) reported that cephaloridine could enter freely into the gram negative bacterial cell wall and that it was five times more readily destroyed by B-lactamase than was ampicillin. However, the cephaloridine hydrolysis was more susceptible to inhibition than was penicillin G hydrolysis by penicillinase in the presence of methicillin, cloxacillin or quinacillin. Many gram negative bacteria produce B-lactamase that would inactivate cephalosporins more readily than penicillins. In the presence of penicillinase-resistant penicillins, such as cloxacillin, cephalo-

sporins can be protected from being hydrolysed by the enzymes. O'Callaghan et al (1966) found that some of the cephalosporin analogues were resistant to B-lactamase. These analogues could be used as enzyme inhibitors similar to cloxacillin. They found that the B-lactamase-resistant cephalosporins could protect the enzyme-sensitive analogues from being hydrolysed. The combination of a B-lactamase-sensitive with a resistant cephalosporin showed a lowering of M.I.C. against Proteus morgani even though the individual antibiotic alone was not effective. Mice also, could be protected against experimental infections with such antibiotic combinations.

Kasik (1964) reported that B-lactamase type of enzyme produced by R_1R_v strain of Mycobacterium tuberculosis was inhibited by several B-lactamase-resistant penicillins including oxacillin, methicillin, cloxacillin and dicloxacillin. The combinations of penicillin G and oxacillin or penicillin G with dicloxacillin were synergistic against this strain of mycobacterium in vitro. Kasik et al (1966) demonstrated in vivo synergism by dicloxacillin and penicillin G against murine tuberculosis. Methicillin was not effective with penicillin G against M. tuberculosis in vivo . Though methicillin inhibited mycobacterial penicillinase, it was less active against this enzyme than dicloxacillin. It was also shown that the in vitro activity of methicillin alone against the R_1R_v organisms was much less than that of dicloxacillin.

The competitive inhibition of the action of B-lactamase by resistant penicillins or cephalosporins is usually reversible. However, irreversible destruction of the enzyme by methicillin and other B-lactamase-resistant antibiotics are also reported (Citri and Garber, 1962a,b;

Gourevitch et al, 1962a). In the search for therapeutic agents against penicillin-resistant bacteria, it is worthwhile to look for a competent inhibitor that can act synergistically in combination with an active but B-lactamase-sensitive antibiotic.

IV. METHODS OF TESTING COMBINED ANTIBIOTIC ACTIVITY:

Generally, there are two conventional methods in testing combined antibiotic activity:

A. In a liquid medium with subculturing.

B. By agar plate transfer methods.

A. Test in Liquid Medium With Subculturing:

Martin et al (1952) adapted the conventional tube method of testing the combined effect of antibiotics. He simplified it by using a fixed antibiotic concentration. Antibiotics were added singly and in combination to tubes with constant volume of broth and were incubated at 37°C. overnight with the test organism. The cultures were plated out for viable counts after a suitable period of incubation. Similarly, the test system could be subcultured at hourly intervals or at some appropriate time during incubation at 37°C. to determine the rate of killing. The turbidimetric method rather than that of viable counting, was used by Thomas and Hayes (1947) and Burnell and Kirby (1951) to estimate the growth from surviving bacteria. This enables a rapid testing of combined antibiotic effect on a large scale.

B. Transfer Methods:

1. Lederberg's replica plate method: - The replica plate method was designed by Lederberg and Lederberg (1952). The method was later modified and described by Elek, Hilson and Jewell (1953), Elek and Hilson (1954) and Manten (1954, 1956). In this method, antibiotic impregnated discs were placed on a uniformly inoculated plate. After incubating the plates, zones of inhibition were produced around the discs containing antibiotics. A replica plate, inoculated by a velvet pad transfer from the original plate, indicated whether the zones of inhibition contained any survivors. The combined action of antibiotics was determined either by placing the discs of different antibiotics near each other or by including both antibiotics in one disc. Although the replica method is simple and gives significant results, one disadvantage of it is that, the velvet pad only transfers about 1% of the bacteria from the primary plate.

2. Cellophane transfer method: - This method was originally devised by Chabbert (1957). Chabbert and Patte (1960) and Garrod and Waterworth (1962) used it for testing the bactericidal action of antibiotic combinations. The method, as described by Chabbert, consists of applying strips of blotting paper impregnated with different antibiotics on an agar plate. The paper strips were arranged at right angles to each other to form a rectangle. The paper strips were then allowed to stand for a short time on the agar surface to enable the antibiotics to diffuse into the agar. They were then removed and a cellophane "tambour"

was applied (the inner surface of which was heavily inoculated with the test organism). Both nutrients and antibiotics diffused through the tambour. After a preliminary incubation of six to eighteen hours on the original plate, the tambour was transferred to an agar plate. The surviving bacteria in the antibiotic impregnated areas would then form colonies. In this method, the disadvantage described in the replica plate technique is eliminated as all procedures are carried out on the same surface. There is an additional advantage in that, the action of the individual antibiotic alone and, in combination, can be observed and compared at the same time. In the critical area where the two separate antibiotics meet at different points of their diffusion gradients, various degrees of antagonism or synergism between the antibiotics can be observed. However, the antibiotics carried over by the cellophane tambour might cause inhibition of bacterial growth. Chabbert and Waterworth (1965) studied the "carry-over" of antibiotics by using the cellophane transfer technique. They found that it was important to have a sufficient depth of agar in order that any carry-over of antibiotic may be diluted out. It was also found that polymyxin B and colistin were heavily adsorbed by cellophane. Thus, the cellophane transfer method is not recommended for use with polypeptide antibiotics.

In the tube tests, it is difficult to differentiate between bactericidal or bacteriostatic action, unless the test samples are either diluted to allow the survivors to grow or are plated out on nutrient agar plates. The replica plate and cellophane transfer techniques are suitable for testing the bactericidal effect of antibiotics.

CHAPTER III

MATERIALS AND METHODS

A. MATERIALS:

1. Antibiotics -

a. Penicillins - A total of nine penicillins were used: Penicillin G Sodium Glaxo (Crystapen) obtained from Glaxo-Allenbury (Canada) Ltd., Potassium Phenoxymethyl Penicillin tablets (V-Cillin K) obtained from Eli-Lilly & Co. Ltd., Toronto, Canada; Potassium Phenethicillin, Sodium Methicillin, Ampicillin Trihydrate, and Sodium Oxacillin obtained from Bristol Laboratories, Syracuse, N.Y.; Octacillin obtained from Hindustan Antibiotics Ltd., India; Cloxacillin Sodium and Ampicillin Sodium (Penbritin - 100 injectable used for mouse protection tests) obtained from Ayerst Laboratories, Canada; Sodium Nafcillin (Unipen) obtained from John Wyeth and Brother (Canada) Ltd., Ontario, Canada.

b. Cephalosporins - Four cephalosporins were used: Cephalothin Sodium, Cephaloridine, Sodium Cephalosporin C and Cephaloglycin obtained from Eli-Lilly and Company, Indianapolis, U.S.A.

2. Strains of Bacteria Employed -

a. Staphylococci - A total of ten strains of staphylococci were used in the investigation. All are clinical isolates obtained from the Bacteriology Department of the Winnipeg General Hospital, with the exception of Staphylococcus aureus Oxford 209P.

b. Coliform bacteria - Four strains each of Escherichia coli and Proteus sp. and five strains each of Klebsiella-Aerobacter sp. and Pseudomonas sp. were used. All of them were clinical isolates obtained from the Department of Bacteriology, Winnipeg General Hospital.

On receipt, all staphylococcal strains were plated out immediately on blood agar plates (B.A.P.) McConky agar plates (M.A.P.) were used for coliform bacteria. All plates were incubated at 37°C overnight. Colonies were picked from the overnight B.A.P. or M.A.P. cultures and inoculated in 20 mls. of Brain Heart infusion broth (B.H.I.), incubated at 37°C for eighteen hours on a rotary shaker at 120 revolutions per minute, then streaked on nutrient agar slants. The purity of the cultures was checked by a Gram's stain. The slant cultures were then incubated overnight and stored at 4°C as stock cultures.

3. Liquid Media for "in vitro" tests -

Brain heart infusion broth (B.H.I.) (Difco Laboratories, Detroit, Michigan, U.S.A.) was used for culturing the bacteria.

Trypticase soy broth (T.S.B.) (Baltimore Biological Laboratories, Baltimore, Maryland, U.S.A.) was used as the medium for all in vitro tests. In our study on combined antibiotic activity, a liquid medium was preferred to a solid medium for two reasons: 1. various concentrations of two antibiotics could readily be accomplished with little variation in the final concentrations. This was desirable when experiments were to be repeated several times.

2. the experimental results could be read within twenty-four hours. Reproducible results were obtained with the liquid medium by keeping the inoculum,

incubation time and temperature constant while using the same concentrations of antibiotics.

The media were rehydrated according to the instruction of the manufacturer, the pH of the media was checked to be at 7.4.

4. Mice -

C.F.W. Swiss Albino female mice were supplied by Canadian Breeding Laboratories, St. Constant, La Prairie Co., Quebec. The mice were kept on a regular diet of Victor Fox cubes and unlimited water. When used for the experiments, they were five and one-half weeks old (± one-half week) and weighed between 17 to 22 grams. The environmental temperature was kept at 72 to 74°F.

5. Mucin Additive for Mouse Protection Test -

Wilson's granular mucin type 1701 W 5% w/v was prepared by homogenizing five grams of the mucin in 100 mls. of distilled water in a Waring blendor for half an hour. It was freshly prepared for each experiment, autoclaved and the pH adjusted to 7.2 to 7.4.

6. Medium for Penicillinase Production -

Peptone water was prepared by dissolving ten grams of peptone and five grams of sodium chloride in one litre of 0.2 M phosphate buffer at pH 7.4 with gentle warming. The medium was distributed in volumes of 200 mls. per flask and autoclaved at fifteen pounds for fifteen minutes. The final pH was adjusted to 7.4.

7. Penicillinases -

Penicillinases were prepared from Staphylococcus aureus 20137,

Pseudomonas sp. 3895 and Bacillus subtilis. A commercial penicillinase, Bacto-Penase, Difco, was also employed.

B. METHODS:

1. Standard Broth Culture -

One loopful of bacteria from the nutrient slant stock culture was inoculated in 20 mls. of brain-heart infusion broth. After inoculation, the same loop was streaked on a blood agar plate (B.A.P.) to check the purity of the culture. The broth was incubated at 37°C on a rotary shaker for eighteen hours at 120 revolutions per minute.

2. Viable Counts -

Ten-fold serial dilutions were made from an eighteen hour standard broth culture in sterile 0.85% saline. Four drops were delivered from each dilution onto four quadrants of a B.A.P. by a standard Teflon dropper pipette. The volume of each drop was 0.025 ml. The plates were dried and incubated for eighteen hours at 37°C. The colonies from each drop were counted and the sum of the four drops represented the number of bacteria in 0.1 ml. of that dilution. The result multiplied by the reciprocal of the dilution was taken as the number of bacteria in 0.1 ml. of the original broth culture. The mean average of three experiments was taken as the result and is recorded in the Tables.

3. Tube Dilution Test -

The individual antibiotic was weighed out in small amounts and made into a solution of desired concentration by dissolving in an appropriate amount of sterile distilled water. Small amounts (3 mls.) were

dispensed into Bijou bottles and were kept frozen as stock solution for sets of experiments.

a. Rough Minimal Inhibitory Concentration (rough M.I.C.)-

The rough estimation of the M.I.C. of an antibiotic against any strain of bacteria was determined by the serial dilution method. Ten-fold serial dilutions of an antibiotic solution were made in trypticase soy broth (T.S.B.). One drop (0.025 ml.) of an eighteen hour standard broth culture was added to each dilution. The contents were mixed with a Vortex mixer (model K 500 J, Scientific Industries, Inc., Queens Village, New York) and incubated at $37^{o}C$ for eighteen hours.

b. Final minimal inhibitory concentration (final M.I.C.) -

The final M.I.C. was determined from the rough M.I.C. titration by taking the highest dilution of antibiotic that inhibited growth and making a series of two fold dilutions to the dilution that showed no inhibition.

4. Checkerboard Method of Antibiotic Combination:

The M.I.C.s of the thirteen antibiotics against all the bacterial strains employed were determined. In order to measure combined activity of two antibiotics, half M.I.C. and concentrations below half M.I.C. of each antibiotic were set up against half M.I.C. and concentrations below half M.I.C. of ampicillin in a "checkerboard" manner as shown in Figure 4. M.I.C. control of each antibiotic; broth control for its sterility and a positive culture control were included. To each tube in the test, with the exception of the broth sterility control, one drop of the eighteen hour standard broth culture was added. The content of

the tubes was mixed with a Vortex mixer, and incubated at 37°C for eighteen hours. The tubes were checked for visible growth after incubation.

5. Mouse Protection Test:

The penicillins or cephalosporins that showed synergism with ampicillin against Staphylococcus aureus 209 P in vitro were tested for their combined protective effect in mice against experimental infection.

a. Randomization - Mice were randomized by numbers. Each cage was assigned a number and there was a fixed number of cards corresponding to the cage. When a card was drawn, a mouse was picked at random and put into the cage corresponding to the number of the card picked.

b. Standardization of the Culture - A blood agar plate was seeded with S. aureus 209 P to give a confluent growth. After eighteen hours of incubation, the culture was harvested with 2 mls. of 0.85% saline. An even suspension was made with a pasteur pipette. The suspension was standardized with a Klett/summerson photometer, using a No. 42 filter, to give an absorbance of 300 Klett units. A saline blank was used for the zero adjustment.

c. Dose Response Curve - Groups of ten mice each, were given various challenge doses of standardized bacterial suspension intraperitoneally. The animals were kept for seven days for observation. The lowest dosage that gave a 100% mortality was taken as "the Challenge dose" for subsequent experiments. The experiment was repeated three times.

P. 0 A. 0	P. 0 A. ½	P. 0 A. ¼	P. 0 A. ⅛	P. 0 A. 1/16
P. ½ A. 0	P. ½ A. ½	P. ½ A. ¼	P. ½ A. ⅛	P. ½ A. 1/16
P. ¼ A. 0	P. ¼ A. ½	P. ¼ A. ¼	P. ¼ A. ⅛	P. ¼ A. 1/16
P. ⅛ A. 0	P. ⅛ A. ½	P. ⅛ A. ¼	P. ⅛ A. ⅛	P. ⅛ A. 1/16
P. 1/16 A. 0	P. 1/16 A. ½	P. 1/16 A. ¼	P. 1/16 A. ⅛	P. 1/16 A. 1/16
		BROTH ALONE	M.I.C. A.	M.I.C. P.

THE FIGURES REPRESENT FRACTIONS OF THE M.I.C. USED

A Ampicillin

P Penicillin or Cephalosporin

Fig. 4 Checkerboard Method

d. Pilot Tests - Various concentrations of ampicillin and a penicillin were prepared by dissolving calculated amounts of each antibiotic in appropriate volumes of sterile distilled water. Five mice each, were used for each antibiotic concentration. The drugs were given intramuscularly in the thigh. Immediately after the drug treatment, the animals were challenged intraperitoneally with the standardized "challenge dose". Controls included a group of mice challenged with bacteria only and, groups of mice given each of the antibiotics alone. The mice were kept under observation for seven days and the number of deaths was recorded daily. The dosage of antibiotic that gave protection to two or three mice out of five was tested again, using ten mice in a group. The pilot test was repeated at least twice, in order to establish a guide line for choosing the correct drug concentrations in the experiments on combined drug therapy.

e. The Final Mouse Protection Test with S. Aureus 209 P As Challenge Organism - Mice were randomized and kept in groups of ten. Penicillin and ampicillin were administered intramuscularly at appropriate dosages both singly and in combination in 0.5 ml. amounts. Immediately after the administration of the drugs, a challenge dose of S. aureus 209 P was given intraperitoneally. A control group of mice with a challenge dose alone, was included. The whole procedure was completed within one hour. The mice were kept under observation for seven days.

f. S. aureus 20137 with Mucin Additive as Challenge Organism - S. Aureus 20137 was less virulent to the strain of mice used than the S. aureus 209 P culture. In order to keep the standardized

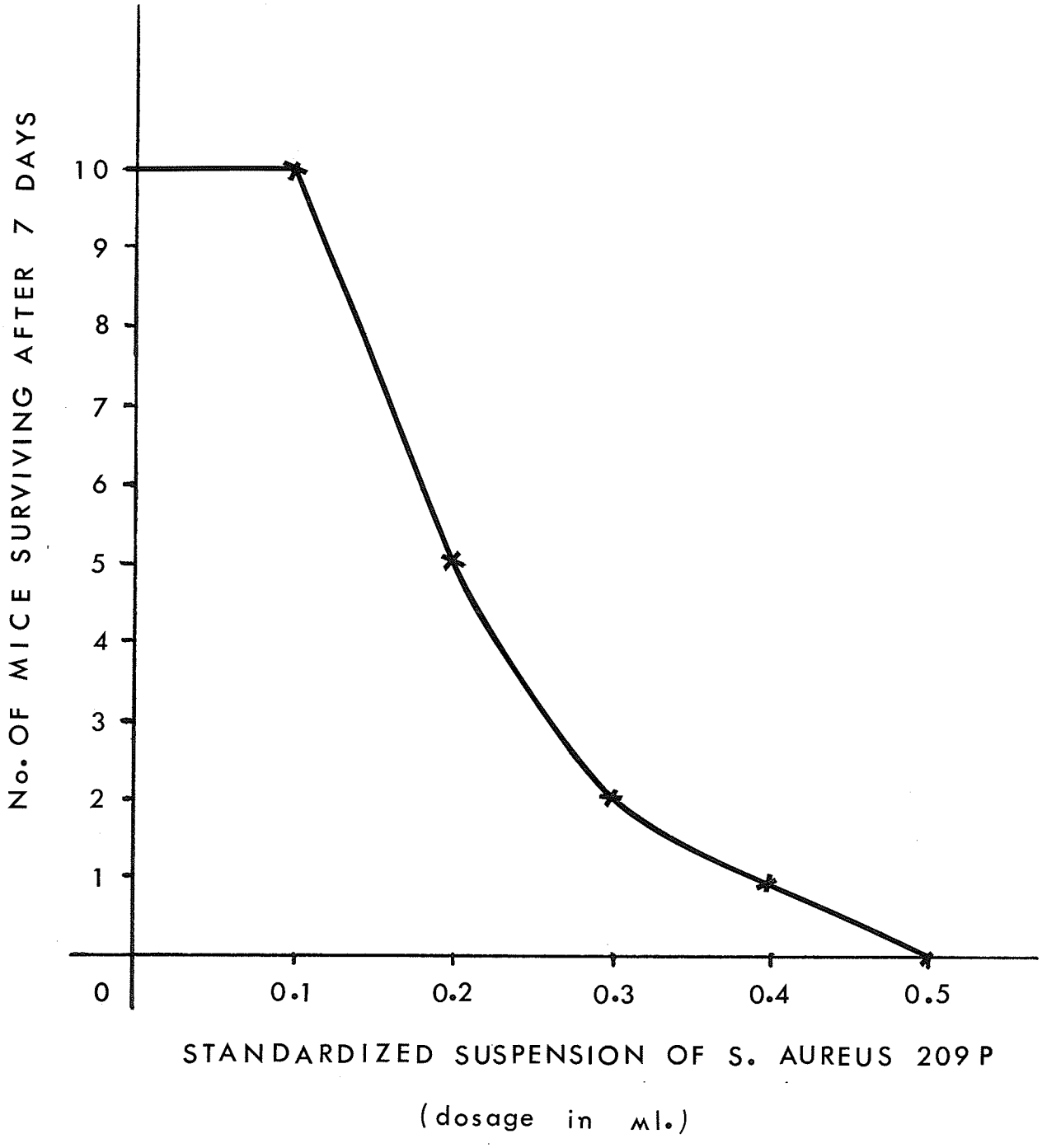


Fig. 5 DOSE RESPONSE CURVE

"challenge dose" within the limits of accuracy with the Klett Summerson photometer, mucin was used to enhance the virulence of this strain of staphylococci. An overnight B.A. culture was harvested and standardized in the same way as S. aureus 209 P culture. A No. 42 filter was used and the culture was adjusted to 350 Klett units. Five per cent (w/v) mucin was prepared and diluted 1:2 with equal volume of 0.85% saline. An equal volume of diluted mucin and bacterial suspension (350 Klett units) was mixed and was ready for use. In this experiment, an additional mucin control was included where the mucin was diluted 1:4 in saline and was given alone intraperitoneally to a group of ten mice.

g. Other Bacterial Strains as Challenge Organisms - E. coli 4007 and Klebsiella-Aerobacter sp. 4341 were also used for the mouse protection test. The challenge dose of E. coli 4007 was standardized to 280 and that of Klebsiella-Aerobacter sp. 4341 to 180 units in the Klett machine. Both of these strains were used without a mucin additive.

6. Experiments With Penicillinases:

a. Production of Penicillinases:

Bacillus cereus spores were obtained from the Cadham Public Health Laboratories, Winnipeg. The spores were allowed to germinate in brain heart infusion broth (B.H.I.). The broth culture was plated out on a blood agar plate. Colonies were picked and inoculated into 20 mls. of B.H.I. broth. One loopful each of the slant stock cultures of Pseudomonas sp. 3895 and Staphylococcus aureus 20137 was also inoculated

in 20 mls. of B.H.I. broth in separate flasks. The cultures were incubated at 37°C for eighteen hours with constant agitation. Four mls. of each of the above three broth cultures were inoculated into three flasks containing 200 mls. of buffered peptone water. The flasks were shaken for eighteen hours at room temperature. Benzylpenicillin G was added to each flask to give a concentration of one unit per ml. and the flasks were shaken for another twenty-four hours at room temperature. Penicillin was then added to give a concentration of five units per ml. After a further twenty-four hours incubation final additions of penicillin were made in following steps. An increase of five units per ml. of penicillin was made a hourly intervals until the final concentration of twenty-five units per ml. was reached. Then, a single addition of penicillin was made to bring the final concentration of penicillin to fifty units per ml. The cultures were then allowed to grow for another twenty-four hours, after which, they were centrifugated at 35,000 r.p.m. in a Spinco model L preparative Ultracentrifuge for fifteen minutes. The supernatants were passed through a Millipore filter with pore size of 0.45/μ. The filtrates were kept at 4°C and labelled as "crude preparation of penicillinases". One ml. of the crude penicillinase was added to nine mls. of T.S.B. and incubated at 37°C overnight, in order to check the sterility of the preparations.

b. Penicillinase Titration - One ml. of the crude penicillinase preparation was added to tubes containing various amounts of benzylpenicillin. The contents of the tubes were mixed and incubated at 37°C for half an hour. One drop of the eighteen hour broth culture

of S. aureus 209 P (M.I.C. < 1 unit/ml. of penicillin G) was then added to each tube. The potency of the penicillinases was calculated from the corresponding amount of penicillin G destroyed. The tubes that showed a visible growth after an eighteen hour incubation period at 37°C would indicate that the penicillinase had destroyed penicillin to an ineffective level. The above experiment was carried out with the three penicillinase preparations and the commercial penicillinase, Bacto-Penase, Difco.

7. Testing the Effect of Penicillinases on Ampicillin in Combination with Penicillins or Cephalosporins:

Penicillinases were diluted in sterile distilled water to give a concentration of approximately two units per ml. (penicillinase in the test system was always in a slight excess). Ampicillin was tested alone and in combination with a B-lactam antibiotic for penicillinase susceptibility using S. aureus 209 P as the indicator strain. Controls included were penicillin G alone, penicillin G plus penicillinase, penicillinase alone, broth alone, heated penicillinase plus penicillin G and heated penicillinase plus ampicillin (Penicillin G and ampicillin were in M.I.C.) The penicillinases were heated by autoclaving at 250°F for twenty minutes. The final volume in the tubes was ten mls. The test organism (one drop of eighteen hour broth culture of S. aureus 209 P), was added to the test system (except the penicillinase and broth sterility controls) after the penicillinase was allowed to react with the penicillins at 37°C for half an hour. The test was read for visible growth after an eighteen hour incubation period at 37°C. The above experiment was carried out with penicillin G and other penicillins and cephalosporins.

CHAPTER IV

EXPERIMENTAL RESULTS

I. IN VITRO TESTS

A. The Minimal Inhibitory Concentrations (M.I.Cs.):

1. Against Staphylococcus aureus - Thirteen B-lactam antibiotics were tested against ten strains of S. aureus. Only three strains were found to be sensitive to less than one microgram per ml. of the penicillinase-sensitive penicillins used. The minimal inhibitory concentrations of the penicillins against these three strains (209 P, 35667, and 37650) are shown in Table I (page 44). All other strains of S. aureus (19993, 20137, 20165, 21312, 21313, 11965 and 28628) showed resistance to more than one hundred micrograms per ml. of all the penicillinase-sensitive penicillins used. The M.I.Cs. of the penicillinase-resistant penicillins (oxacillin, cloxacillin, methicillin and nafcillin) against all the staphylococcal strains tested are shown in Table II (page 45). It was found that, the penicillinase-resistant penicillins were, in general, less potent against the sensitive strains of staphylococci than the penicillinase-sensitive penicillins. Of the four cephalosporin antibiotics tested, only cephalothin and cephaloridine were active against the staphylococci. The M.I.Cs. of the four cephalosporins against the ten strains of S. aureus are shown in Table III, (page 47).

2. Against Gram Negative Bacilli - Thirteen B-lactam antibiotics were tested against five strains each of Klebsiella-Aerobacter sp.

TABLE I

MINIMAL INHIBITORY CONCENTRATIONS OF PENICILLINS AGAINST SENSITIVE STAPHYLOCOCCUS AUREUS STRAINS

(Concentration in μ/ml.)

Strain \ Penicillin	PG	PV	PHEN	AMP	OCT	OXA	CLOX	METH	NAF
209 P	0.06	0.04	0.08	0.12	0.08	0.4	0.4	1.4	0.4
35667	0.04	0.04	0.06	0.06	0.08	0.2	0.4	0.8	0.2
37650	0.4	0.4	0.1	0.2	0.1	0.6	0.6	4	0.4

PG	Benzylpenicillin G	OXA	Oxacillin
PV	Phenoxymethyl penicillin	CLOX	Cloxacillin
PHEN	Phenethicillin	METH	Methicillin
AMP	Ampicillin	NAF	Nafcillin
OCT	Octacillin		

TABLE II

MINIMAL INHIBITORY CONCENTRATIONS OF PENICILLINASE-RESISTANT PENICILLINS AGAINST STAPHYLOCOCCUS AUREUS STRAINS

(Concentration in μ/ml.)

STRAIN	OXACILLIN	CLOXACILLIN	METHICILLIN	NAFCILLIN
209 P	0.4	0.4	1.4	0.4
35667	0.2	0.4	0.8	0.2
37650	0.6	0.6	4.0	0.4
19993	1.0	0.8	3.0	0.4
20137	0.8	0.4	1.0	0.4
20165	1.0	0.8	4.0	0.4
21312	0.8	0.8	4.0	0.4
21313	100	100	100	3.0
21965	2.0	0.8	4.0	0.4
28628	2.0	1.0	4.0	0.6

TABLE III

MINIMAL INHIBITORY CONCENTRATIONS OF CEPHALOSPORINS AGAINST STAPHYLOCOCCUS AUREUS STRAINS

STRAIN	CEPH. C	-GLYCIN	CEPH.	-IDINE
209 P	100	100	0.2	0.06
35667	100	2.0	0.2	0.02
37650	80	6.0	0.4	0.1
19993	100	100	2.0	20
20137	100	100	0.8	100
20165	100	100	2.0	100
21312	100	100	0.8	100
21313	100	100	100	100
21965	100	100	2.0	100
28628	100	100	2.0	20

Ceph. C.	Cephalosporin C.	-glycine	Cephaloglycin
Ceph.	Cephalothin	-idine	Cephaloridine

(4341, 4293, 8268, 8956 and sp. II) and Pseudomonas sp. (4133, 3895, 8059, 8151 and 7682); and four strains each of Proteus sp. (8422, 8479, 8544 and 8237) and Escherichia coli (4007, 4009, 3893 and 4166). All the Pseudomonas and Klebsiella-Aerobacter strains (except 4341 and 4293) were resistant to more than five hundred micrograms of all the thirteen antibiotics used. Among the penicillins, benzylpenicillin G and ampicillin were more active against gram negative bacteria. Phenoxymethyl penicillin (penicillin V), phenethicillin, octacillin and the penicillinase-resistant penicillins were not active against any strain of the gram negative bacteria tested even at a concentration as high as five hundred micrograms per ml. All the four Proteus strains were resistant to the penicillins. Cephalosporin antibiotics were more active than penicillins against gram negative bacteria, especially against the Proteus sp. and the E. coli strains. The M.I.Cs. of penicillin G, ampicillin and the cephalosporins against the sensitive strains of Klebsiella-Aerobacter sp., E. coli and Proteus sp. are shown in Table IV.

B. Definitions:

1. Synergism is defined as the condition in which no visible growth is observed when less than one-half M.I.C. of one antibiotic is combined with one-half or less than one-half M.I.C. of ampicillin.
2. Antagonism is defined as the condition in which visible growth is observed when one-half or more than one-half of the M.I.C. of the two antibiotics are combined.
3. Additive action is defined as the condition where no visible growth is observed when one-half M.I.C. of an antibiotic is

combined with one-half M.I.C. of ampicillin.

4. "Not interfering" is defined as the condition where reduced bacterial growth occurs when a fixed concentration (50/μ/ml.) of an antibiotic (the M.I.C. of which is more than 100/μ and was not determined) is added to one-half or less than one-half M.I.C. of another antibiotic.

C. Antibiotics in Combination with Ampicillin:

1. Against Penicillin-sensitive Staphylococci:

The results of the antibiotics in combination with ampicillin against the sensitive strains of Staphylococcus aureus 209 P, 35667, and 37650 are presented in Tables V, VI and VII respectively. In seventy-five percent of the cases, synergism was observed.

2. Against Penicillin-resistant Staphylococci:

The results of the antibiotics in combination with ampicillin against the resistant strains of S. aureus 19993, 20137, 20165, 21312, 21313 and 21965 and 28628 are presented in Tables VIII, IX, X, XI, XII, XIII and XIV respectively. In most cases, the penicillins and cephalosporins did not interfere with the activity of ampicillin in the system. Oxacillin was observed to have combined synergistically with ampicillin against S. aureus 21312, 21965 and 28628 while it acted antagonistically against S. aureus 20137. Antagonism was also observed in the case of methicillin and ampicillin combination against S. aureus 19993 and 20165. (Tables XV, SVI and XVII) Cephalothin was observed to act synergistically with ampicillin against S. aureus 21312 and 28628.

TABLE IV

MINIMAL INHIBITORY CONCENTRATIONS OF PENICILLINS AND CEPHALOSPORINS AGAINST COLIFORM BACTERIA

(Concentrated in μ/ml.)

BACTERIAL STRAINS		P.G.	AMP.	CEPH. C.	-CLYCINE	CEPH.	-IDINE
Escherichia Coli	4007	60	10	300	100	30	10
	4009	80	10	300	200	30	10
Klebsiella Aerobacter Sp.	4341	100	40	300	300	60	10
	4293	500	500	500	500	20	20
	sp. II	150	150	500	500	500	500
	8422	300	80	100	300	20	20
Proteus sp.	8479	300	160	50	300	200	80
	8544	200	160	100	300	100	40
	8237	200	160	50	300	20	40

P.G. = Benzylpenicillin G AMP = Ampicillin CEPH. C. = Cephalosporin C.

CEPH. = Cephalothin -IDINE = Cephaloridine -CLYCINE = Cephaloglycine

TABLE V

ANTIBIOTICS IN COMBINATION WITH AMPICILLIN AGAINST S. AUREUS 209 P

(Concentration in μ/ml.)

ANTIBIOTICS	M.I.C.	CONCENTRATIONS USED IN COMBINATIONS	COMBINATIONS THAT GIVE NO VISIBLE GROWTH	
A Penicillin G	0.06	0.005 to 0.03	0.06 0.03	
A Penicillin V	0.04	0.0025 to 0.02	0.02 0.02	0.05 0.01
A Phenethicillin	0.08	0.01 to 0.04	0.03 0.04	0.04 0.03
A Octacillin	0.08	0.01 to 0.05	0.02 0.04	0.03 0.03
A Oxacillin	0.4	0.1 to 0.4	0.02 0.2	
A Cloxacillin	0.4	0.15 to 0.3	0.04 0.2	
A Methicillin	1.4	0.1 to 2.0	0.03 1.0	0.05 0.5
A Nafcillin	0.4	0.1 to 0.4	0.03 0.2	
A Cephalothin	0.2	0.025 to 0.1	0.03 0.1	0.05 0.075
A Cephaloridine	0.06	0.01 to 0.04	0.06 0.02	

A= Ampicillin (M.I.C. - 0.12 . Concentrations used in combinations were 0.02 to 0.06).

TABLE VI

ANTIBIOTICS IN COMBINATION WITH AMPICILLIN AGAINST S. AUREUS 35667

(Concentration in μ/ml.)

ANTIBIOTICS	M.I.C.	CONCENTRATIONS USED IN COMBINATIONS	COMBINATIONS THAT GIVE NO VISIBLE GROWTH	
A Penicillin G	0.04	0.005 to 0.02	0.02 0.02	0.04 0.01
A Penicillin V	0.04	0.005 to 0.02	0.02 0.02	0.04 0.01
A Phenethicillin	0.06	0.005 to 0.03	0.03 0.03	0.04 0.02
A Octacillin	0.08	0.01 to 0.04	0.03 0.04	0.04 0.02
A Oxacillin	0.2	0.025 to 0.1	0.02 0.1	0.04 0.05
A Cloxacillin	0.4	0.05 to 0.2	0.2 0.2	0.1 0.1
A Methicillin	0.8	0.1 to 0.4	0.04 0.4	0.2 0.2
A Nafcillin	0.2	0.025 to 0.1	0.02 0.1	0.04 0.05
A Cephalothin	0.2	0.025 to 0.1	0.01 0.1	0.03 0.05
A Cephaloridine	0.02	0.0025 to 0.01	0.04 0.01	
A Cephaloglycin	2.0	0.25 to 1.0	0.01 1.0	0.03 0.5

A = Ampicillin (M.I.C. - 0.06μ/ml. Concentrations used in combinations were 0.01 to 0.04).

TABLE VII

ANTIBIOTICS IN COMBINATION WITH AMPICILLIN AGAINST S. AUREUS 37650

(Concentration in μ/ml.)

ANTIBIOTICS	M.I.C.	CONCENTRATIONS USED IN COMBINATIONS	COMBINATIONS THAT GIVE NO VISIBLE GROWTH	
A			0.05	0.1
Penicillin G	0.4	0.05 to 0.2	0.2	0.1
A			0.05	0.1
Penicillin V	0.4	0.05 to 0.2	0.2	0.1
A			0.075	0.1
Phenethicillin	0.1	0.02 to 0.05	0.05	0.03
A			0.1	
Octacillin	0.1	0.02 to 0.05	0.04	
A			0.075	0.1
Oxacillin	0.6	0.05 to 0.3	0.3	0.2
A			0.075	0.1
Cloxacillin	0.6	0.05 to 0.3	0.3	0.2
A			0.025	0.1
Methicillin	4.0	0.5 to 2.0	2.0	0.5
A			0.05	0.1
Nafcillin	0.4	0.05 to 0.2	0.2	0.1
A			0.025	0.1
Cephalothin	0.4	0.05 to 0.2	0.2	0.1
A			0.05	0.075
Cephaloridine	0.1	0.02 to 0.05	0.05	0.03
A			0.05	0.1
Cephaloglycin	6.0	0.5 to 3.0	3.0	0.5
A			0.05	0.1
Cephalosporin C	80.0	10.0 to 40.0	40.0	20.0

A = Ampicillin (M.I.C. - 0.2 μ/ml Concentrations used in combinations 0.025 to 0.1).

Cloxacillin and cephaloridine also acted synergistically with ampicillin against the 28628 strain.

3. Against Coliform Bacteria -

The results of the antibiotics in combination with ampicillin against E. coli strains are shown in Tables XVIII and XIX, against Klebsiella-Aerobacter sp. are shown in Table XX, and against Proteus sp. strains are shown in Tables XXI, XXII, XXIII and XXIV. In all but one instances (cephaloridine plus ampicillin against Proteus sp. 8479 in Table XXIV) the cephalosporins were found to act synergistically with ampicillin against the gram negative bacteria tested.

II. MOUSE PROTECTION TEST

A. Viable Counts:

Viable counts of the eighteen hour broth cultures of S. aureus 209 P, 19993, 20165 and 20137; E. coli 4007; Klebsiella-Aerobacter sp. 4143, and Pseudomonas sp. 3895 were carried out. Viable counts were also carried out with the "standardized challenge doses" of S. aureus 209 P and 20137; E. coli 4007 and Klebsiella-Aerobacter sp. 4341 to calculate the number of cells required for the challenge dose in the in vivo mouse protection test. The results are shown in Table XXV.

B. Combined Therapy:

The results of combined therapy on S. aureus 209 P experimental infection in mice are presented in Figure 5 to 9. In vitro combination of ampicillin and oxacillin showed antagonism against S. aureus 20137. The same antibiotic pair was tested in mice against S. aureus 20137

TABLE VIII

ANTIBIOTICS IN COMBINATION WITH AMPICILLIN AGAINST S. AUREUS 19993

(Concentration in μ/ml.)

ANTIBIOTICS	M.I.C.	CONCENTRATIONS USED IN COMBINATIONS	COMBINATION THAT GIVE NO VISIBLE GROWTH
A	100	50	G
Oxacillin	1.0	0.2 to 0.5	
A	100	50	G
Cloxacillin	0.8	0.1 to 0.4	
A	100	50	Antagonism
Methicillin	3.0	0.5 to 2.0	
A	100	50	G
Nafcillin	0.4	0.05 to 0.2	
A	100	50	G
Cephalothin	2.0	0.25 to 1.0	

A = Ampicillin

G = Visible Growth was observed

TABLE IX

ANTIBIOTICS IN COMBINATION WITH AMPICILLIN AGAINST S. AUREUS 20.37

(Concentration in μ/ml.)

ANTIBIOTICS	M.I.C.	CONCENTRATIONS USED IN COMBINATIONS	COMBINATION THAT GIVES NO VISIBLE GROWTH
A	100	50	Antagonism
Oxacillin	0.8	0.05 to 0.4	
A	100	50	G
Cloxacillin	0.4	0.05 to 0.2	
A	100	50	G
Methicillin	1.0	0.2 to 0.5	
A	100	50	G
Nafcillin	0.4	0.05 to 0.2	
A	100	50	G
Cephalothin	0.8	0.1 to 0.4	

A = Ampicillin

G = Visible growth was observed.

TABLE X

ANTIBIOTICS IN COMBINATION WITH AMPICILLIN AGAINST S. AUREUS 20165

(Concentration in μ/ml.)

ANTIBIOTICS	M.I.C.	CONCENTRATIONS USED IN COMBINATIONS	COMBINATIONS THAT GIVE NO VISIBLE GROWTH
A	100	50	G
Oxacillin	1.0	0.2 to 0.5	
A	100	50	G
Cloxacillin	0.8	0.1 to 0.4	
A	100	50	Antagonism
Methicillin	4.0	0.5 to 2.0	
A	100	50	G
Nafcillin	0.4	0.05 to 0.2	
A	100	50	G
Cephalothin	2.0	0.25 to 1.0	

A = Ampicillin

G = Visible Growth was observed.

TABLE XI

ANTIBIOTICS IN COMBINATION WITH AMPICILLIN AGAINST S. AUREUS 21312

(Concentration in μ/ml.)

ANTIBIOTICS	M.I.C.	CONCENTRATIONS USED IN COMBINATIONS	COMBINATIONS THAT GIVE NO VISIBLE GROWTH
A	100	50	50
Oxacillin	0.8	0.1 to 0.4	0.3
A	100	50	G
Cloxacillin	0.8	0.1 to 0.4	
A	100	50	G
Methicillin	4.0	0.5 to 2.0	
A	100	50	G
Nafcillin	0.4	0.05 to 0.2	
A	100	50	50
Cephalothin	0.8	0.1 to 0.4	0.4

A = Ampicillin

G = Visible growth was observed.

TABLE XII

ANTIBIOTICS IN COMBINATION WITH AMPICILLIN AGAINST S. AUREUS 21313

(Comcentration in μ/ml.)

ANTIBIOTICS	M.I.C.	CONCENTRATIONS USED IN COMBINATIONS	COMBINATIONS THAT GIVE NO VISIBLE GROWTH
A	100	50	G
Oxacillin	100	50	
A	100	50	G
Cloxacillin	100	50	
A	100	50	G
Methicillin	100	50	
A	100	50	G
Nafcillin	310	0.25 to 1.5	
A	100	50	G
Cephalothin	100	50	

A = Ampicillin

G = Visible growth was observed.

TABLE XIII

ANTIBIOTICS IN COMBINATION WITH AMPICILLIN AGAINST S. AUREUS 21965

(Concentration in μ/ml.)

ANTIBIOTICS	M.I.C.	CONCENTRATIONS USED IN COMBINATIONS	COMBINATIONS THAT GIVE NO VISIBLE GROWTH
A	100	50	50
Oxacillin	2.0	0.25 to 1.0	0.75
A	100	50	G
Cloxacillin	0.8	0.1 to 0.8	
A	100	50	G
Methicillin	4.0	0.5 to 2.0	
A	100	50	G
Nafcillin	0.4	0.05 to 0.2	
A	100	50	G
Cephalothin	2.0	0.25 to 1.0	

A = Ampicillin

G = Visible growth was observed.

TABLE XIV

ANTIBIOTICS IN COMBINATION WITH AMPICILLIN AGAINST S. AUREUS 28628

(Concentration in μ/ml.)

ANTIBIOTICS	M.I.C.	CONCENTRATIONS USED IN COMBINATIONS	COMBINATIONS THAT GIVE NO VISIBLE GROWTH
A	100	50	50
Oxacillin	2.0	0.25 to 1.0	1.0
A	100	50	50
Cloxacillin	1.0	0.2 to 0.5	0.5
A	100	50	G
Methicillin	4.0	0.5 to 2.0	
A	100	50	G
Nafcillin	0.6	0.05 to 0.3	
A	100	50	50
Cephalothin	2.0	0.4 to 1.0	1.75
A	100	50	50
Cephaloridine	20	4.0 to 10	10

A = Ampicillin

G = Visible growth was observed.

TABLE XV

THE COMBINATION OF AMPICILLIN WITH OXACILLIN AGAINST S. AUREUS 20137

(Concentration in μ/ml.)

OXACILLIN (Ox) ↓		0	Ox 0.2	Ox 0.4	Ox 0.5
AMPICILLIN (A) 0 →		+++	+	+	+
A	50	+++	++	++	++
A	100	+++	++	++	++
A	200	+++	++	++	++

\+ Visible Growth

++ Turbid Growth

+++ Heavy Growth

\- No visible Growth

Broth Sterility Control -

TABLE XVI

THE COMBINATION OF AMPICILLIN WITH METHICILLIN AGAINST S. AUREUS 19993

(Concentration in μ/ml.)

METHICILLIN (M.) ↓		0	M 1.5	M 3.0
AMPICILLIN (A.) 0 →		+++	++	-
A	50	+++	++	+
A	100	+++	++	+
A	200	+++	++	+

\+ Visible Growth

++ Turbid Growth

+++ Heavy Growth

\- No visible growth

Broth Sterility Control -

TABLE XVII

THE COMBINATION OF AMPICILLIN WITH METHICILLIN AGAINST S. AUREUS 20165

(Concentration in μ/ml.)

METHICILLIN (M) ↓		0	M 2.0	M 4.0
AMPICILLIN (A) 0 →		+++	+	-
A	50	+++	+	+
A	100	+++	+	+
A	200	+++	+	+

\+ Visible Growth

++ Turbid Growth

+++ Heavy Growth

\- No visible Growth

Broth Sterility Control -

TABLE XVIII

ANTIBIOTICS IN COMBINATION WITH AMPICILLIN AGAINST ESCHERICHIA COLI 4007

(Concentration in μ/ml.)

ANTIBIOTICS	M.I.C.	CONCENTRATIONS USED IN COMBINATIONS	COMBINATIONS THAT GIVE NO VISIBLE GROWTH	
A			5.0	
Penicillin G	60	5.0 to 30	30	
A			2.0	4.0
Cephalothin	30	2.5 to 15	15	10
A			2.0	5.0
Cephaloridine	10	2.0 to 5.0	5.0	2.0

A + Ampicillin (M.I.C. = 10μ/ml. Concentrations used in Combinations were 2.0μ to 5.0μ).

TABLE XIX

ANTIBIOTICS IN COMBINATION WITH AMPICILLIN AGAINST ESCHERICHIA COLI 4009

(Concentration in μ/ml.)

ANTIBIOTICS	M.I.C.	CONCENTRATIONS USED IN COMBINATIONS	COMBINATIONS THAT GIVE NO VISIBLE GROWTH	
A			5.0	
Penicillin G	80	10 to 40	40	
A			2.0	5.0
Cephalothin	30	2.5 to 15	15	10
A			2.0	5.0
Cephaloridine	10	2.0 to 5.0	5.0	2.0

A = Ampicillin (M.I.C. = 10μ/ml. Concentrations used in Combinations were 2.0 to 5.0).

TABLE XX

ANTIBIOTICS IN COMBINATION WITH AMPICILLIN AGAINST KLEBSIELLA-AEROBACTER SP.

(Concentration in μ/ml.)

STRAIN	ANTIBIOTICS	M.I.C.	CONCENTRATIONS USED IN COMBINATIONS	COMBINATION THAT GIVE NO VISIBLE GROWTH	
4293	A	500	50 to 300	100	
	Cephalothin	20	410 to 10	10	
4293	A	500	10 to 300	300	
	Cephaloridine	20	4.0 to 10	10	
4341	A	40	2.5 to 20	2.5	10
	Cephalothin	60	5.0 to 30	30	10
4341	A	40	2.5 to 20	2.5	
	Cephaloridine	10	1.0 to 5.0	4.0	
sp.II	A	150	20 to 80	40	80
	Penicillin G	150	20 to 60	60	40

A = Ampicillin

TABLE XXI

ANTIBIOTICS IN COMBINATION WITH AMPICILLIN AGAINST PROTEUS SP. 8544

(Concentration in μ/ml.)

ANTIBIOTICS	M.IC.	CONCENTRATIONS USED IN COMBINATIONS	CONCENTRATIONS THAT GIVE NO VISIBLE GROWTH	
A	200	10 to 40	G	
Penicillin G		10 to 80		
A	100	20 to 80	20	
Cephalothin		5.0 to 50	25	
A		20 to 80	20	80
Cephaloridine	40	2.5 to 20	20	2.5

A = Ampicillin

M.I.C. = 160μ/ml.

G = Visible growth was observed.

TABLE XXII

ANTIBIOTICS IN COMBINATION WITH AMPICILLIN AGAINST PROTEUS SP. 8422

(Concentration in μ/ml.)

ANTIBIOTICS	M.I.C.	CONCENTRATIONS USED IN COMBINATIONS	CONCENTRATIONS THAT GIVE NO VISIBLE GROWTH	
A	100	10 to 40	10	20
Cephalosporin C		5.0 to 50	15	5.0
A		10 to 40	20	30
Cephalothin	20	1.0 to 10	10	5.0
A		10 to 40	20	40
Cephaloridine	20	1.0 to 10	10	5.0

A = Ampicillin

M.I.C. = 80 μ/ml.

TABLE XXIII

ANTIBIOTICS IN COMBINATION WITH AMPICILLIN AGAINST PROTEUS SP. 8237

(Concentration in μ/ml.)

ANTIBIOTICS	M.I.C.	CONCENTRATIONS USED IN COMBINATIONS	CONCENTRATIONS THAT GIVE NO VISIBLE GROWTH	
A		10 to 40	10	
Cephalosporin C	50	10 to 25	10	
A		10 to 40	10	
Cephalothin	20	1.0 to 10	10	
A		20 to 80	20	80
Cephaloridine	40	5.0 to 20	20	15

A = Ampicillin

M.I.C. = 160 μ/ml.

TABLE XXIV

ANTIBIOTICS IN COMBINATION WITH AMPICILLIN AGAINST PROTEUS SP. 8479

(Concentration in μ/ml.)

ANTIBIOTICS	M.I.C.	CONCENTRATIONS USED IN COMBINATIONS	CONCENTRATIONS THAT GIVE NO VISIBLE GROWTH
A	50	10 to 25	20
Cephalosporin C			10
A			80
Cephaloridine	80	1.0 to 40	40

A = Ampicillin

M.I.C. = 160μ/ml.

Concentrations used in combination: 20μ/ml to 80μ/ml.

(with mucin additive) and the result is shown in Figure 10. It can be seen that antagonism was not apparent in vivo. Ampicillin and cephaloridine were also used against experimental infections by E. coli 4007 and Klebsiella-Aerobacter sp. 4341. The results are shown in Figure 11 and 12 respectively. In no case, did penicillin or cephalosporin antagonize the activity of ampicillin. In 75% of the cases, the combination of a penicillin or cephalosporin with ampicillin protected the mice better than either antibiotic when used alone.

III. EFFECT OF PENICILLIN OR CEPHALOSPORIN ON AMPICILLIN WHEN THEY ARE EXPOSED TO PENICILLINASES:

The effect of four penicillinases (produced by S. aureus 20137, Pseudomonas sp. 3895, Bacillus subtilis and a commercial penicillinase, Bacto-Penase, Difco) on ampicillin when combined with penicillinase-sensitive penicillins, with penicillinase-resistant penicillins and with cephalosporins was tested, employing S. aureus 209 P as the test organism. The results are shown in Tables XXVI, XXVII, and XXVIII respectively. It was found that hydrolysis of ampicillin by the four penicillinases was not inhibited by the penicillins or cephalosporins.

TABLE XXV

VIABLE COUNTS IN STANDARDIZED BROTH CULTURES AND IN STANDARDIZED CHALLENGE DOSES:

CULTURES		NO. OF VIABLE UNITS PER ML. OF BROTH	NO. OF VIABLE UNITS PER ML. OF CHALLENGE DOSE
S. aureus	209 P	2.84×10^9	2.0×10^9
S aureus	19993	2.0×10^9	
S. aureus	20165	1.6×10^9	
S. aureus	20137	2.19×10^9	1.15×10^9
E. coli	4007	3.5×10^9	2.63×10^9
Kleb.-Aerob. sp.	4143	3.65×10^9	8.4×10^8
Pseudomonas sp.	3895	2.3×10^9	

(The data presented are the averages of three experiments)

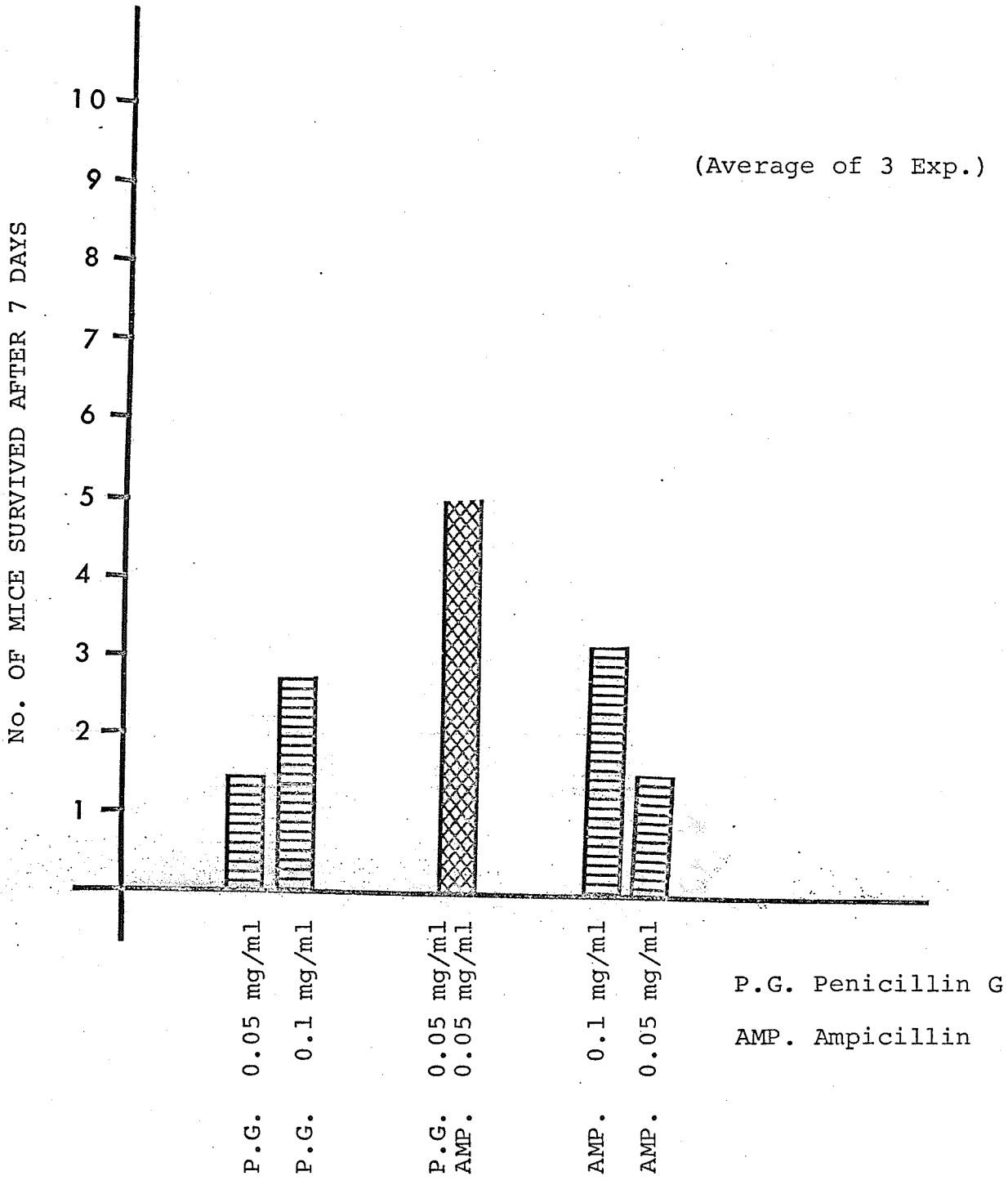
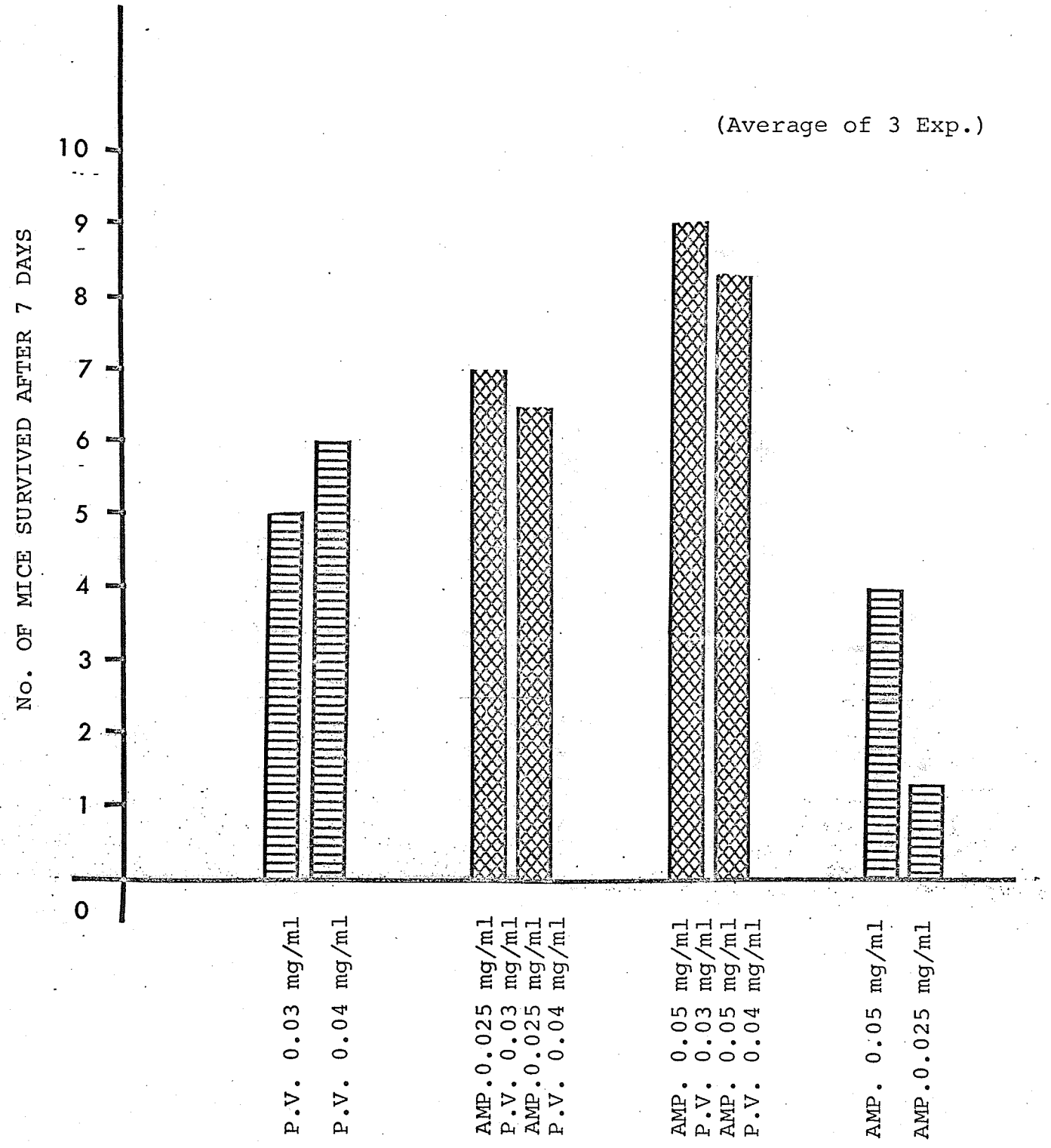


Fig. 6 PENICILLIN G AND AMPICILLIN AGAINST EXPERIMENTAL INFECTION IN MICE WITH STAPHYLOCOCCUS AUREUS 209P



P.V. =Phenoxymethyl penicillin AMP = Ampicillin

Fig. 7 PHENOXYMETHYL PENICILLIN AND AMPICILLIN AGAINST EXPERIMENTAL INFECTION IN MICE WITH STAPHYLOCOCCUS AUREUS 209P

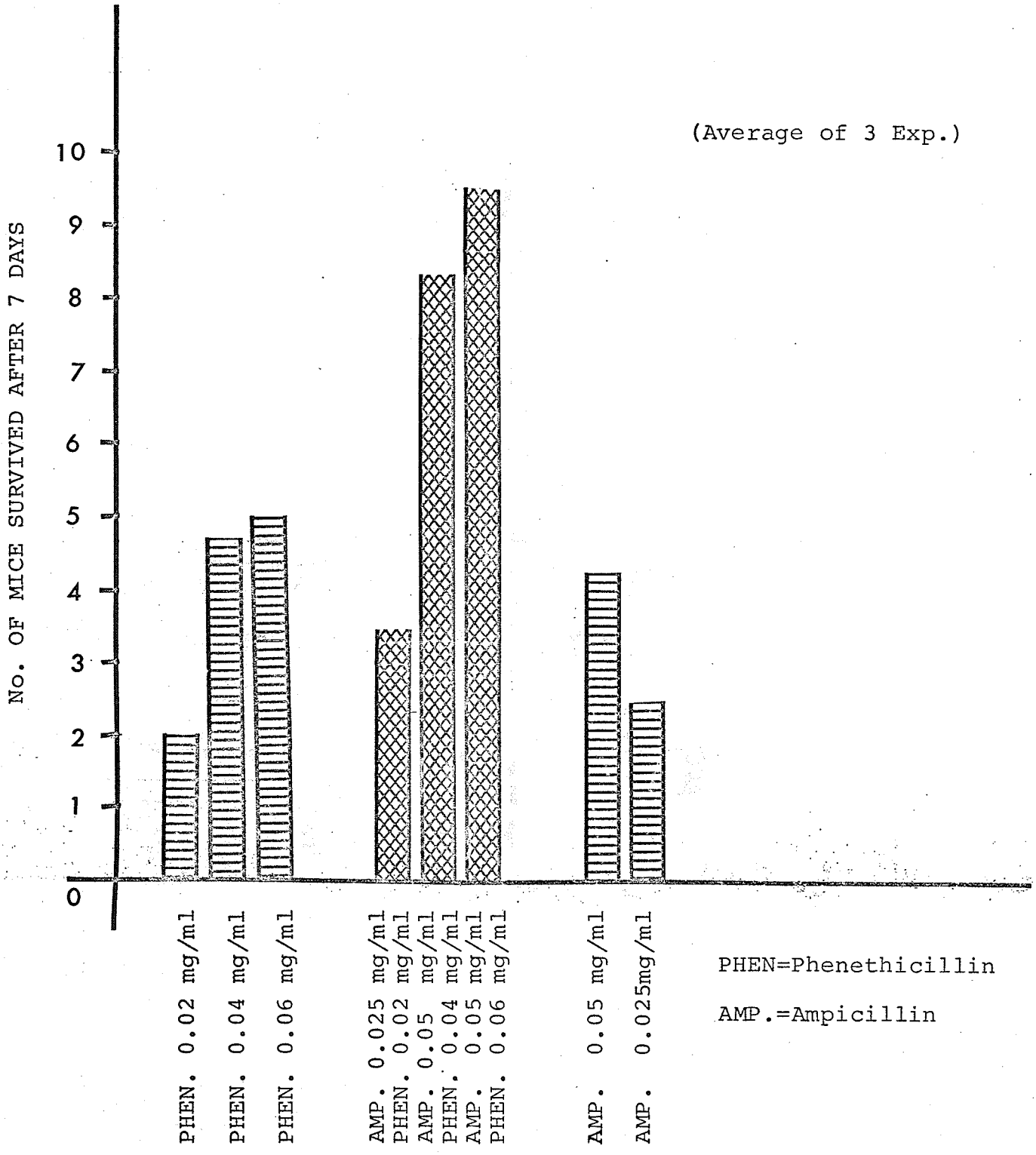


Fig. 8 PHENETHICILLIN AND AMPICILLIN AGAINST EXPERIMENTAL INFECTION IN MICE WITH STAPHYLOCOCCUS AUREUS 209 P

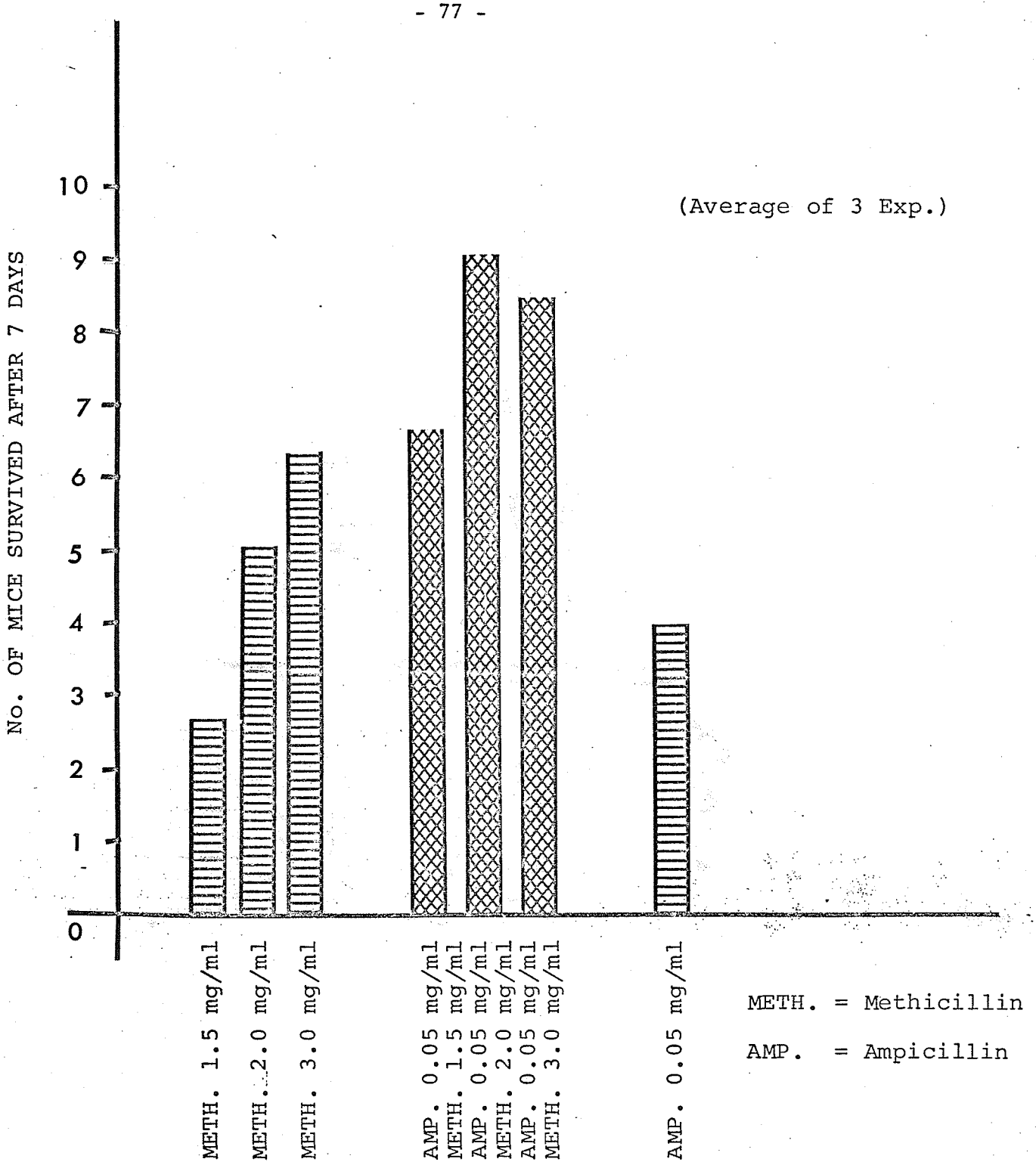


Fig. 9 METHICILLIN AND AMPICILLIN AGAINST EXPERIMENTAL INFECTION IN MICE WITH STAPHYLOCOCCUS AUREUS 209P.

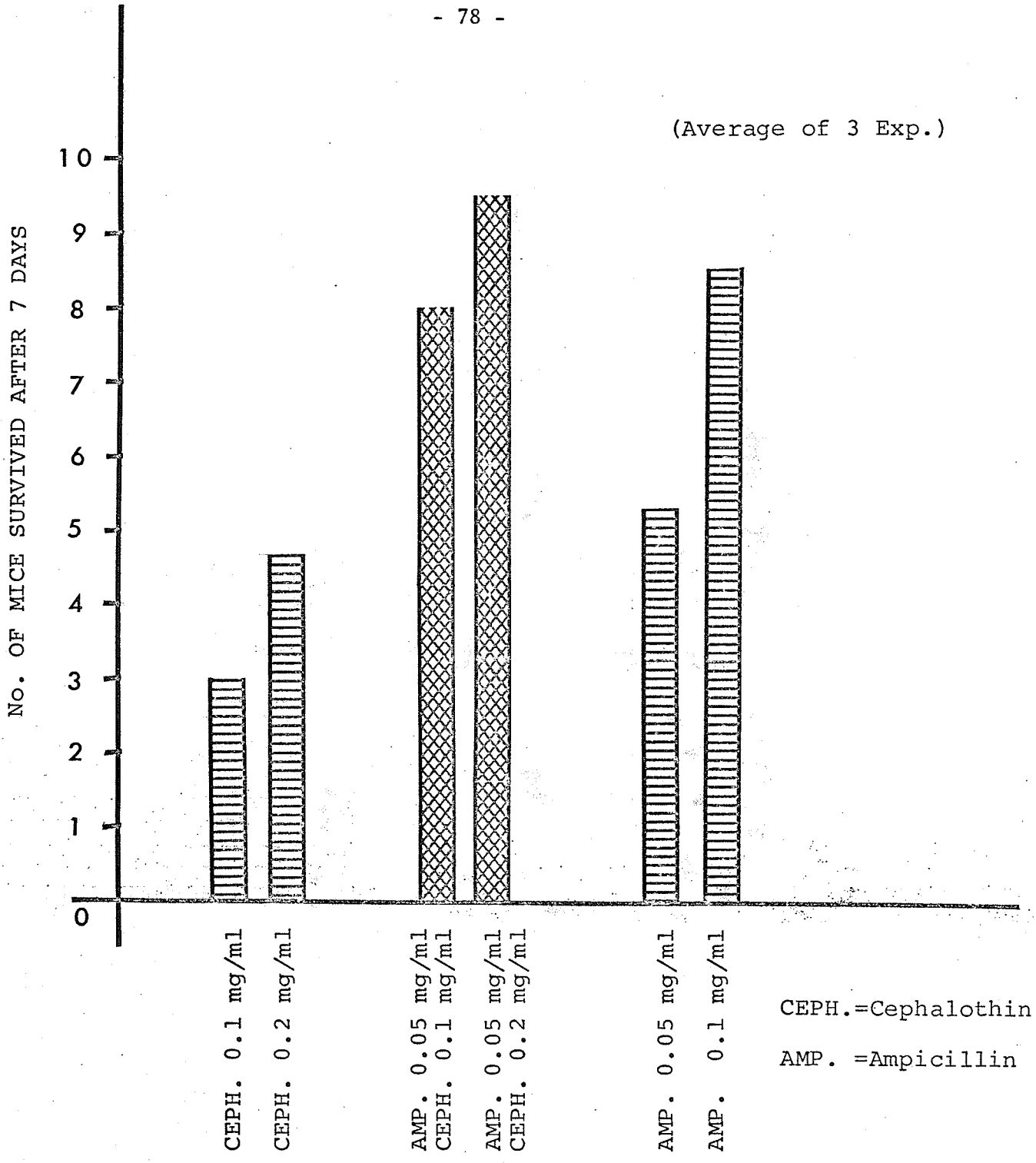


Fig. 10 CEPHALOTHIN AND AMPICILLIN AGAINST EXPERIMENTAL INFECTION IN MICE WITH STAPHYLOCOCCUS AUREUS 209P.

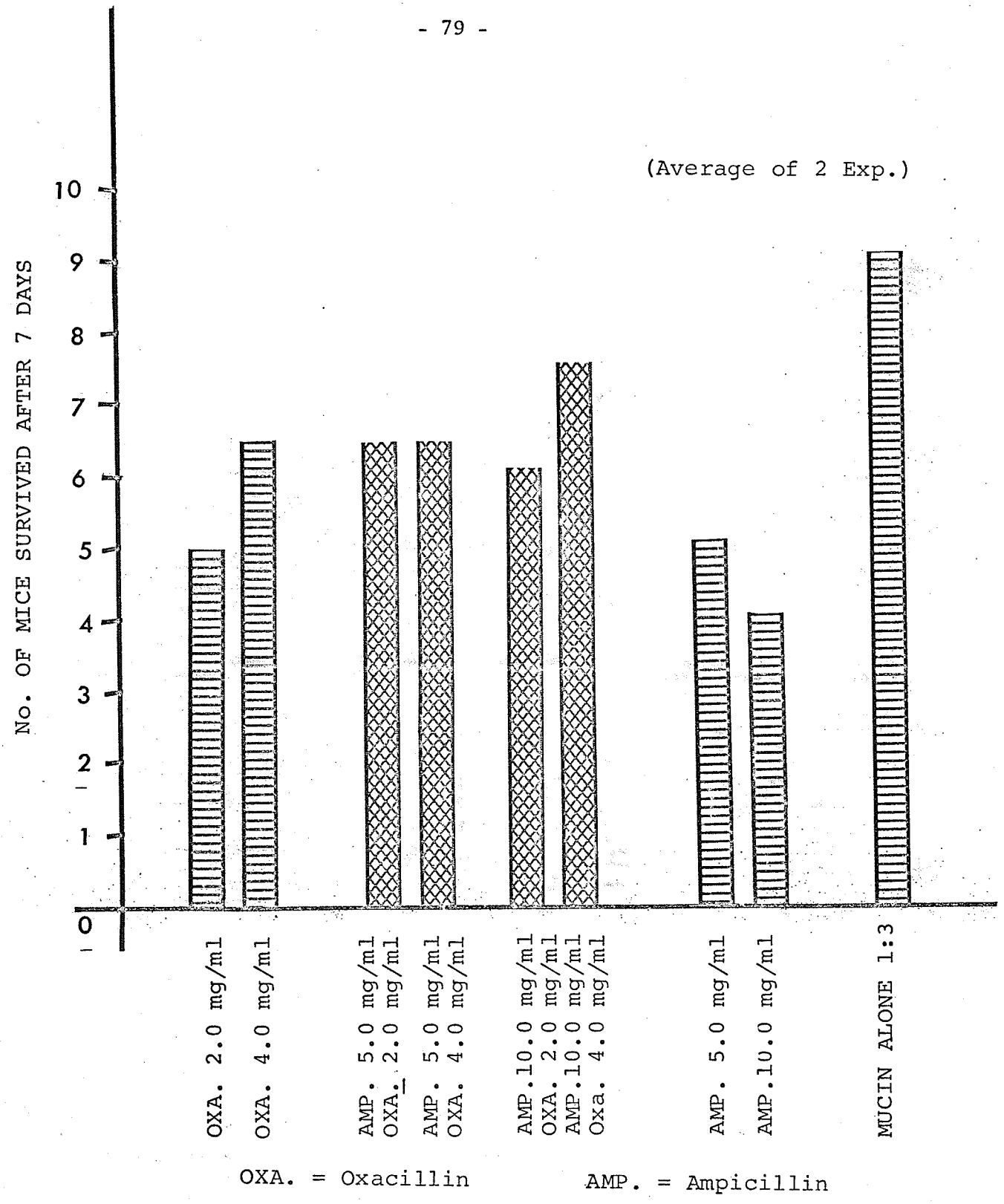


Fig.11 OXACILLIN AND AMPICILLIN AGAINST EXPERIMENTAL INFECTION IN MICE WITH STAPHYLOCOCCUS AUREUS 20137.

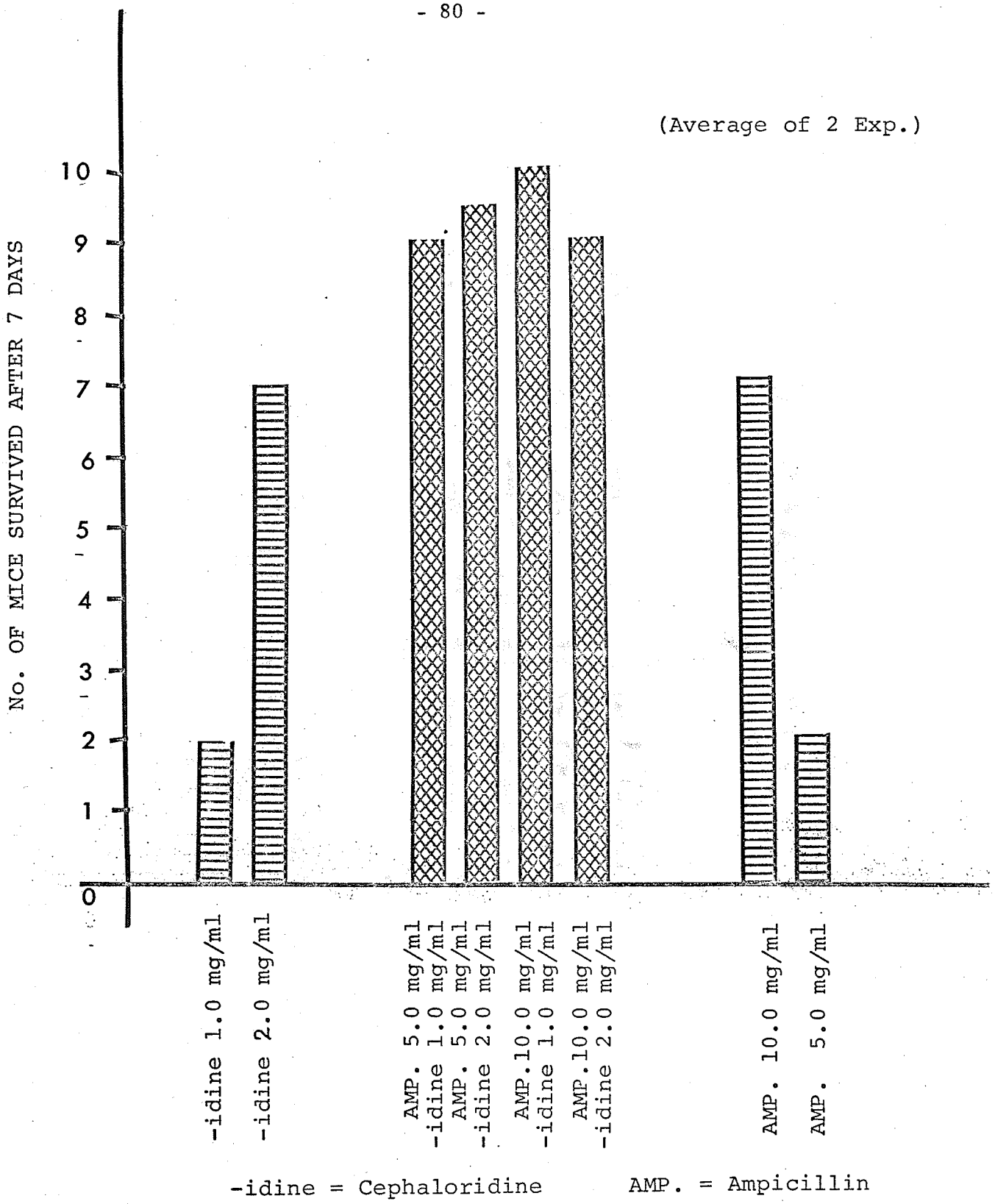


Fig. 12 CEPHALORIDINE AND AMPICILLIN AGAINST EXPERIMENTAL INFECTION IN MICE WITH ESCHERICHIA COLI 4007.

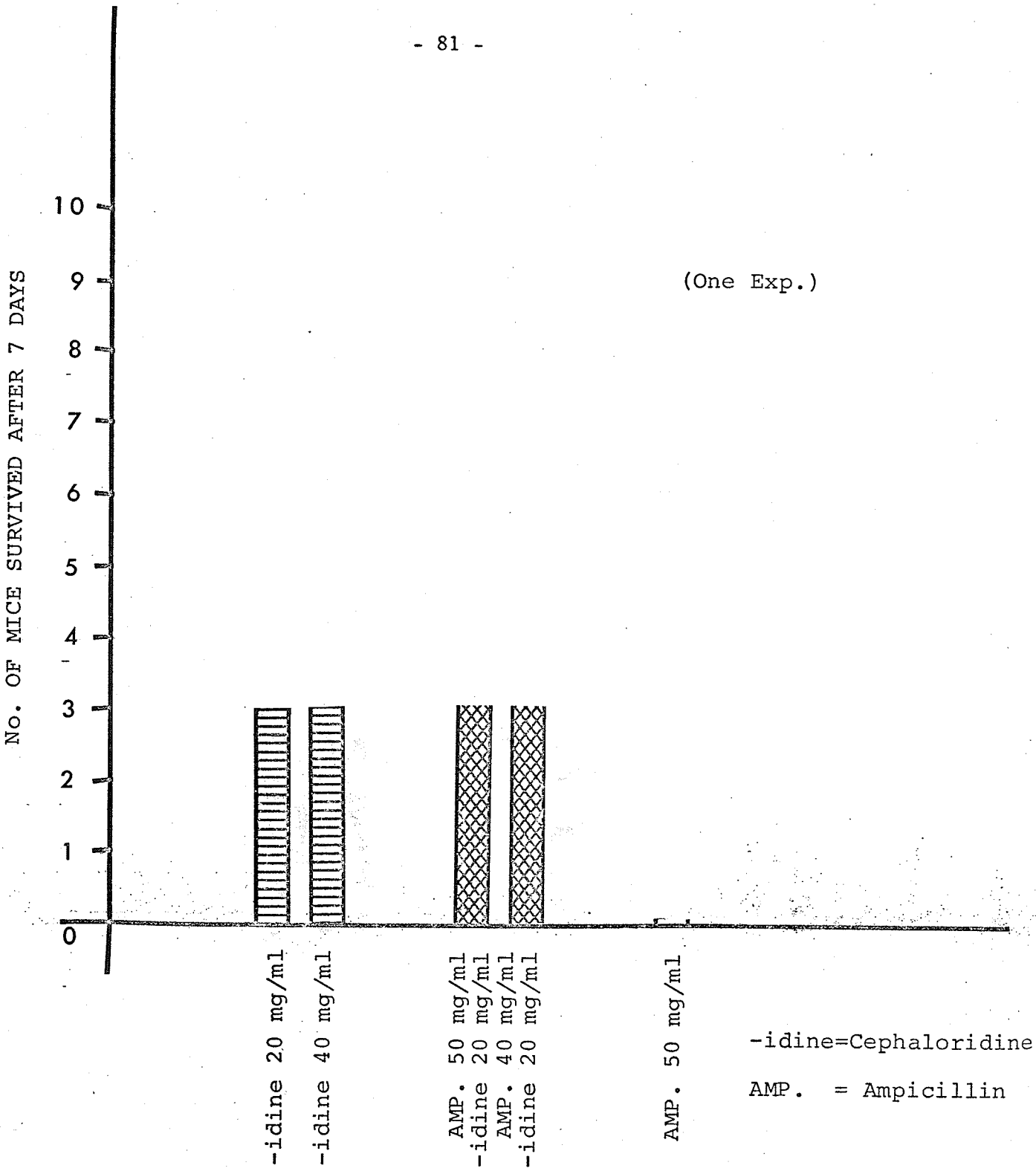


Fig. 13 CEPHALORIDINE AND AMPICILLIN AGAINST EXPERIMENTAL INFECTION IN MICE WITH KLEBSIELLA-AEROBACTER SP. 4341.

TABLE XXVI

THE EFFECT OF PENICILLINASES ON AMPICILLIN IN COMBINATION WITH PENICILLINASE-SENSITIVE PENICILLINS AGAINST STAPHYLOCOCCUS AUREUS 209 P

(Concentration in μ/ml.)

ANTIBIOTICS	M.I.C.	AB + P-ASE				AB + H.P-ASE				TEST				COMB.	CON.
		B	P	S	C	B	P	S	C	B	P	S	C		
AMPICILLIN	0.12	+++	+	+++	+++	-	-	-	-						½ MIC ++
PENICILLIN G	0.06	+++	+++	+++	+++	-	-	-	-	+++	++	++	+++	-	0.03 +
PENICILLIN V	0.04	+++	+++	+++	+++	-	-	-	-	+++	++	+++	+++	-	0.02 +++
PHENETHICILLIN	0.08	+++	+	+	++	-	-	-	-	+++	+	+	+	-	0.03 +
OCTACILLIN	0.08	+++	+++	+++	+++	-	-	-	-	+++	+++	+++	+++	-	0.04 +

AB + P-ASE = antibiotics in M.I.C. + penicillinase

AB + H.P-ASE = antibiotics in M.I.C. + heated penicillinase

TEST = the minimal inhibitory combination + penicillinase

COMB = the minimal inhibitory combination

CON = controls

PENICILLINASE PRODUCED BY BACILLUS SUBTILIS B
PSEUDOMONAS SP P
STAPHYLOCOCCUS AUREUS S
OBTAINED FROM COMMERCIAL SOURCE C

- = no visible growth
+ = visible growth
++ = turbid growth
+++ = heavy growth

TABLE XXVII

THE EFFECT OF PENICILLINASES ON AMPICILLIN IN COMBINATION WITH PENICILLINASE RESISTANT PENICILLINS AGAINST

STAPHYLOCOCCUS AUREUS 209 P

(Concentration in μ/ml.)

ANTIBIOTICS	M.I.C.	AB + P-ASE				AB + H.P-ASE				TEST				COMB.	CON.
		B	P	S	C	B	P	S	C	B	P	S	C		
AMPICILLIN	0.12	+++	+	+++	+++	-	-	-	-						½ MIC ++
METHICILLIN	1.4	-	-	-	-	-	-	-	-	++	+	+	+++	-	0.5 +++
OXACILLIN	0.4	-	-	-	-	-	-	-	-	+	-	+	+	-	0.2 +
CLOXACILLIN	0.4	-	-	-	-	-	-	-	-	+	-	+	+	-	0.2 +
NAFCILLIN	0.4	+	+	-	+	+	+	-	+	+	+	+	+++	-	0.2 +

AB + P-ASE = antibiotics in M.I.C. + penicillinase

AB + H.P-ASE = antibiotics in M.I.C. + heated penicillinase

TEST = the minimal inhibitory combination + penicillinase

COMB = the minimal inhibitory combination

CON = controls

PENICILLINASE PRODUCED BY BACILLUS SUBTILIS B
PSEUDOMONAS SP P
STAPHYLOCOCCUS AUREUS S
OBTAINED FROM COMMERCIAL SOURCE C

- = no visible growth
+ = slight growth
++ = turbid growth
+++ = heavy growth

TABLE XXVIII

THE EFFECT OF PENICILLINASES ON AMPICILLIN IN COMBINATION WITH CEPHALOSPORINS AGAINST STAPHYLOCOCCUS AUREUS 209 P

(Concentration in μ/ml.)

ANTIBIOTICS	M.I.C.	AB + P-ASE				AB + H.P-ASE				TEST				COMB.	CON.
		B	P	S	C	B	P	S	C	B	P	S	C		
AMPICILLIN	0.12	+++	+	+++	+++	-	-	-	-						½ MIC ++
CEPHALOTHIN	0.2	-	+++	-	-	-	-	-	-	-	+++	+	+	-	0.1 +
CEPHALORIDINE	0.06	-	+	-	-	-	-	-	-	+	-	-	±	-	0.02 ++

AB + P-ASE = antibiotics in M.I.C. + penicillinase

AB + H.P-ASE = antibiotics in M.I.C. + heated penicillinase

TEST = the minimal inhibitory combination + penicillinase

COMB = the minimal inhibitory combination

CON = controls

PENICILLINASE PRODUCED BY BACILLUS SUBTILIS — B

PSEUDOMONAS SP — P

STAPHYLOCOCCUS AUREUS — S

OBTAINED FROM COMMERCIAL SOURCE — C

- = no visible growth

+ = slight growth

++ = turbid growth

+++ = heavy growth

CHAPTER V

DISCUSSION

It is well known that penicillins are generally more active against gram positive bacteria than against the gram negative ones. It is also known that penicillin resistance found among gram positive cocci is largely due to their ability to produce B=lactamases. B-lactamases have a very wide distribution among bacteria. Smith and Hamilton-Miller (1963a) showed that, gram positive bacteria produced B-lactamases with higher activation energies than those produced by gram negative bacteria. Thus, B-lactamases from gram negative organisms are more effective as catalytic agents than those from gram positive sources. Only the enzymes of gram positive species have been shown to be liberated physiologically into the environment to a significant extent and are markedly inducible. Enzymes from gram negative bacteria are cell bound and the disrupted cell preparations often show B-lactamase activity several times higher than the whole cell preparation. (Smith and Hamilton-Miller, 1963b). Pollock (1961) showed unequivocally that, B-lactamase is responsible exclusively for penicillin resistance among many gram positive bacterial species. In our investigation, among the ten staphylococcal strains tested, seven were penicillin-resistant strains. There is a great difference in the M.I.C. between the penicillin-sensitive and penicillin-resistant strains of staphylococci ($<1\mu/ml$ to $>100\mu$). The high M.I.Cs. of the resistant strains are mainly due to their ability to produce B-lactamases. Among the penicillin sensitive strains studied, it is evident that there are

individual differences in their resistance to penicillins and cephalosporins. The M.I.Cs. of the penicillinase-resistant penicillins were observed to be higher than the M.I.Cs. of the penicillinase-sensitive penicillins when tested against the penicillin-sensitive staphylococcal strains. It may be partly due to the difference in their molecular weights and also to the difference in side chain structures. The side chain structure of a penicillinase-resistant penicillin is responsible for the low affinity of the penicillin for the B-lactamases. The same structure may also confer to the penicillin a low affinity for the transpeptidases which are responsible for bacterial cell wall synthesis. Among the penicillinase-resistant penicillins, methicillin is the least active. Presumably, the two methoxy-groups on the benzene ring that provide a steric hindrance to the penicillinase also hinders its ability to combine with the transpeptidase. No correlation between penicillinase specificity and antibiotic potency is evident from our results, as the penicillins showed considerable variation in antibiotic potency against penicillin-sensitive organisms. Among the cephalosporins, cephalothin is resistant to penicillinase and it is interesting to note that its M.I.Cs. against the staphylococcal strains are similar to those obtained from the four penicillinase-resistant penicillins. Cephaloridine is as active as penicillin G against sensitive staphylococcal strains and it is inactive against resistant strains of staphylococci. Both cephalothin and cephaloridine show a higher activity against gram negative organisms than penicillin G or ampicillin (Table IV). It is evident that factors other than penicillinase production are responsible for penicillin re-

sistance in gram negative organisms. One may speculate that gram negative species produce "species-specific" penicillinases. For example, E. coli strains hydrolyse only benzylpenicillin and phenoxymethylpenicillin to an appreciable extent while A. aerogenes strains hydrolyse 6-aminopenicillanic acid rapidly and A. cloacae hydrolyses 5-methyl-3-phenyl-4-isoxazolylpenicillin rapidly. (Smith and Hamilton-Miller, 1963b).

The results of the combination of penicillins and cephalosporins with ampicillin against penicillin-sensitive staphylococcal strains show that an additive effect is achieved in all cases. The above results are expected as the organisms are sensitive to both antibiotics. Synergism was observed in many cases (Tables V to VII). It was observed that a pair of antibiotics (e.g., methicillin and ampicillin) showed synergism against S. aureus 209 P strain (Table V) while the same antibiotic pair demonstrated antagonism against S. aureus 19993 and 20165 (Tables VIII and X respectively). It is apparent from the above results that, a fixed synergistic or antagonistic pair of antibiotics does not exist for all bacterial strains. There was no significant difference demonstrated among the penicillinase-sensitive penicillins, penicillinase-resistant penicillins and the cephalosporins that would enable us to say which would give a better antimicrobial effect when combined with ampicillin against sensitive staphylococci. The results of the experiments with penicillinase added to the antibiotic pairs demonstrate that the association of a penicillin or cephalosporin with ampicillin does not improve the resistance of ampicillin

towards enzymic hydrolysis by the penicillinases. When these antibiotic pairs were tested against penicillinase-producing staphylococci, similar results were obtained (Table VIII to XV) with the exceptions of oxacillin and cephalothin (Tables XI, XIII and XIV). Neither the penicillinase-resistant penicillins nor the cephalosporins acted synergistically with ampicillin. The findings are in accordance with those published by Selzer and Wright (1964). Even oxacillin and cephalothin did not show synergism with ampicillin against all the resistant staphylococcal strains. This could be explained if it was known that there are fundamental differences in the permeability barrier in cell wall structure among bacterial strains. If oxacillin or cephalothin gained access to the bacterial cell more readily than ampicillin, they could act as an enzyme inhibitor or inactivator, thus freeing the ampicillin for its bactericidal action. Oxacillin however, antagonized ampicillin in one case (Table XV) and methicillin antagonized ampicillin in two cases (Tables XVI and XVII) in our experiments. It is known that penicillins highly resistant to penicillinase are found to inactivate the enzyme (Gourevitch et al, 1962a). However, penicillinase-resistant penicillins have a much smaller affinity for the enzyme than do the penicillinase-sensitive ones. (Gourevitch et al, 1963). As a result, enzyme inactivation does not take place in the presence of sensitive penicillins because the enzyme preferentially binds them. Methicillin is not expected to act synergistically with ampicillin when both of them are present at the same time. On the other hand, Sabath and Finland (1962) pointed out that, the so-called penicillinase-resistant penicillins

(ancillin, methicillin and oxacillin) denatured much more rapidly in a nutrient medium at 37°C when exposed to a moderately high concentration of the penicillinase-producing staphylococci (strain 60/1) than when incubated in the same medium without these organisms. Appreciable destruction of the antibiotics occurred when the drugs were exposed to a concentration of 10^8 viable units of Staphylococcus 60/1. However, the number of bacteria also declined until the level of the penicillinase-resistant drug was low enough to permit multiplication of the surviving organisms. Thus, when a staphylococcal culture produces extracellular B-lactamase in a significant amount, in addition to the cell bound enzyme and when its cell wall structure permits easy access for ampicillin, we may expect "antagonism" to occur. Ampicillin will be destroyed by the cell bound enzyme when it enters the bacterial cell wall. Since the penicillinase-resistant penicillins usually have bulky side chains and are less likely to gain access through the cell wall prior to ampicillin, they may fail to function as enzyme inhibitors. When methicillin is present in the culture medium, it is susceptible to enzymic hydrolysis by the free extracellular staphylococcal B-lactamase after the penicillinase-sensitive ampicillin is being destroyed rapidly both intracellularly and extracelluarly.

Penicillinase-resistant penicillins have found to be ineffective against gram negative bacteria used in our experiments. Farrar et al (1966) also came to the same conclusion. The experiments on combined antibiotic activity showed that, penicillin G exerted an additive effect on ampicillin against gram negative organisms that were

sensitive to both antibiotics. Cephalosporins were shown to act synergistically with ampicillin. The results are in accordance with that reported by O'Callaghan et al (1966). They showed that B-lactamase-resistant cephalosporins could exert a protective effect similar to penicillinase-resistant penicillins. It was also found that the penicillinase-sensitive cephaloridine was also synergistic to ampicillin in several gram negative species. Cephalothin was also found to be synergistic with ampicillin against some gram negative strains and the finding is contrary to what is reported by Bach et al (1966). The disagreement in results might be due to the use of different test organisms. O'Callaghan et al (1966) claimed that, the combination of some B-lactamase-sensitive and resistant cephalosporins protected mice from experimental infection by gram negative bacteria. It is obvious in the experiments carried out in this laboratory that, the antibiotics used in the mouse protection tests did not interfere with the action of ampicillin in vivo. In general, an additive or slightly better than additive effect on mouse protection was observed. The experimental data of in vivo tests are not enough to demonstrate synergism but, they give evidence that no antagonism is encountered when ampicillin and other semi-synthetic penicillins or cephalosporins are given together at the same time to experimentally infected mice.

SUMMARY:

The effect of the combination of ampicillin with each of the seven semi-synthetic penicillins (potassium phenoxymethyl penicillin, potassium phenethicillin, octacillin, sodium oxacillin, sodium cloxacillin, sodium methicillin and sodium nafcillin), four cephalosporins (sodium cephalosporin C, sodium cephalothin, cephaloglycin and cephaloridine) and sodium benzylpenicillin was tested against ten strains of staphylococci and eighteen strains of coliform bacteria. The minimal inhibitory concentrations of these antibiotics against the twenty-eight strains of bacteria were determined. It was found that the M.I.Cs. of the penicillinase-sensitive penicillins were generally lower than those of the penicillinase-resistant penicillins against penicillin-sensitive staphylococci. No significant difference in M.I.Cs. was observed in the case of penicillinase-resistant penicillins against either penicillin-sensitive or resistant staphylococcal strains. Among the cephalosporins, cephalothin was resistant to penicillinase and it was interesting to note that, its M.I.Cs. against the staphylococcal strains were similar to those of the four penicillinase-resistant penicillins. Cephaloridine was as active as penicillin G against penicillin-sensitive strains of staphylococci. Both cephalothin and cephaloridine showed higher activity against gram negative organisms in comparison to penicillin G or ampicillin. It was found that, the B-lactam antibiotics did not antagonize the effect of ampicillin against penicillin-sensitive staphylococci. An additive effect was always observed when ampicillin

was combined with each of the eleven antibiotics against penicillin sensitive staphylococci, and in 75% of the cases, synergism was observed. When penicillin-resistant organisms were used as test organisms, penicillins and cephalosporins did not interfere with the activity of ampicillin in all but four cases. Oxacillin in combination with ampicillin acted synergistically against S. aureus 21313, 21965 and 28628 while, it acted antagonistically against S. aureus 20137. Antagonism was also observed in the combination of methicillin and ampicillin against S. aureus 19993 and 20165. Cephalothin, cephaloridine and cloxacillin showed synergism with ampicillin against S. aureus 28628. Cephalothin also combined synergistically with ampicillin against S. aureus 21312. Only penicillin G, cephalothin, cephalosporin C and cephaloridine were used in combination with ampicillin against gram negative bacteria. It was found that penicillin G showed an additive effect while, the cephalosporins demonstrated synergism with ampicillin against coliform bacteria.

In vivo mouse protection tests were carried out with the antibiotics that showed definite synergism in vitro against S. aureus 209 P and 20137, E. coli 4007 and Klebsiella-Aerobacter sp. 4341. It was found that the activity of ampicillin was not interfered with by the penicillins or cephalosporins used. In most cases, an additive effect was observed.

The effect of three penicillinases produced by Bacillus subtilis, Staphylococcus aureus 20137 and Pseudomonas sp. 3895 and a commercial penicillinase preparation (Bacto-Penase) was tested on the combination of ampicillin with each of the penicillins and cephalo-

sporins against S. aureus 209 P. It was found that, hydrolysis of ampicillin by the four penicillinases was not inhibited by the penicillins or cephalosporins.

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