IN VITRO STUDIES CONCERNING THE ACTION OF PENICILLIN ON THE VIRIDANS STREPTOCOCCI, INCLUDING OBSERVATIONS ON THE SO-CALLED SYNERGISTIC EFFECT OF SULFONAMIDE DRUGS

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This investigation was stimulated by our interest in the treatment of subacute bacterial endocarditis with penicillin. We were anxious to learn something of the mode of action of penicillin on the viridans streptococci, the more common causative agents of this disease, but, after reviewing the relevant medical literature, we were left confused by conflicting opinions and seemingly contradictory statements. Therefore for purposes of clarification certain *in vitro* experiments were conducted and are reported herewith. The particular object of these studies concerning the mode of action of penicillin was the determination of the effects, if any, of (1) variations in the number of organisms exposed, (2) variations in the concentration of penicillin, and (3) the presence of sulfonamide drugs.

MATERIALS AND METHODS

Broth. All broth used in the experiments described in this report is the same as that used for many years at the House of the Good Samaritan for the preparation of streptolysin. It is essentially the same as the beef heart infusion described by Swift and Hodge (1933) except that the buffer has been modified so as to contain 3 grams of glucose instead of 2 grams, and 2 grams of $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$ instead of 1 gram, for every liter of infusion (Mote, unpublished).

Organisms. All organisms used for tests in these experiments have been strains of viridans streptococci isolated from the blood of patients ill with subacute bacterial endocarditis. Some of the strains were kindly supplied by Dr. Maxwell Finland of the Boston City Hospital. Most of the organisms have been subcultured several times or more in the course of the studies; others have been kept in the frozen and dried state until needed for the tests. In all experiments a freshly made overnight subculture in broth has been used. All dilutions, as indicated in each experiment, have likewise been made in broth.

Penicillin. Commercial preparations containing 100,000 units of the sodium salt of penicillin have been used for the making of stock solutions. The dry penicillin powder was dissolved in 100 ml of normal saline so as to make a concentration of 1,000 units per ml. This solution was passed through a Seitz filter and stored in the refrigerator. Studies showed that when kept in this way the penicillin solution maintained its strength for at least 3 months. The stock penicillin solutions were titrated from time to time by testing serial dilutions in broth with a stock strain of hemolytic streptococcus of known sensitivity. In all experiments broth was used for making dilutions of the stock penicillin solution.

Sulfonamide drugs. Stock solutions of the sulfonamide drugs were prepared by weighing out 20 mg of the dry powder and adding it to 100 ml of broth in which it was readily soluble. Sterilization was insured by passage through a Seitz filter.

The number of organisms in broth cultures and in broth solutions of penicillin or sulfonamide drugs was determined by making serial dilutions in broth from 10^{-1} to 10^{-7} , or in some instances, when necessary, out to as far as 10^{-12} . Exactly 0.1 ml of each dilution was added to a blood agar plate and thoroughly streaked over the surface with a wire loop. After incubation at 37 C for 48 hours, colony counts were made of those plates on which the colonies were not too crowded. From the number of colonies and the dilutions, the number of organisms per ml in the original solution or culture could be readily calculated. This method of surface streaking was found to be technically easier and just as accurate as pour plate methods. When repeated calculations are made by these methods of the number of organisms in a single culture, the results have shown a 10-fold (10^1) and sometimes even a 100-fold (10^2) variation. However, the relative error in any one experiment can be kept at a minimum by choosing blood plates on which the numbers of colonies are of about the same order. In general we have tried to choose blood plates with 200 to 1,000 colonies. When the number of organisms is being determined in cultures containing penicillin, it is important to make counts only from those dilutions in which the penicillin content has been reduced below the minimal inhibitory concentration. Excessive amounts of penicillin may interfere with the growth of the bacteria on the blood plates.

EFFECT OF VARIATIONS IN THE NUMBER OF EXPOSED ORGANISMS

Abraham *et al.* (1941), in referring to the characteristics of the antibacterial action of penicillin, stated that its action was influenced only to a minor extent by the number of bacteria to be inhibited. Hobby, Meyer, and Chaffee (1942), on the other hand, stated that with a given concentration of penicillin the rate of killing of a given strain of hemolytic streptococcus decreased as the number of organisms at zero hour increased. Fisher (1943) determined the minimal dilution of penicillin which caused inhibition, and with one strain of staphylococcus obtained the same end point with 8 billion organisms as with 17 million. Yet with a relatively resistant strain of staphylococcus he failed to get inhibition when the culture was diluted 1:1, but did get inhibition when the culture was diluted 100 times. He felt that the failure with the more concentrated culture was due to the lack of active multiplication of the organisms and pointed out that this was in accord with the views of Hobby, Meyer, and Chaffee (1942). The experience of Rantz and Kirby (1944) apparently was similar to that of Fisher. They found that very large numbers of susceptible organisms could be killed if the concentration of penicillin was large enough, but the retardation of growth of a resistant organism was more marked if the inoculum was small, and under these circumstances the cultures could be sterilized. We have investigated this problem in several different ways.

Sensitivity tests. One of the commonly used methods for determining the sen-

sitivity of an organism involves exposing it to decreasing concentrations of penicillin for a fixed period of time such as 24 hours. The lowest concentration of penicillin (measured in units per ml) which causes inhibition represents the sensitivity of the organism. The exact way in which sensitivity tests have been performed by various investigators has varied considerably. Thus Rammelkamp and Maxon (1942), in performing tests with staphylococci, used a bacterial inoculum so that the final number of organisms was between 1,000 and 30,000 per 0.5 ml, and they reported the sensitivity of the strains tested as the minimal concentration of penicillin which produced complete killing. That is, they chose as the end point the greatest dilution of penicillin that was sterile after incubation for 18 to 24 hours. On the other hand, Dawson, Hobby, and Lipman (1944) tested the sensitivity of nonhemolytic streptococci by making a 10^{-2} or 10^{-1} dilution of the organisms in serial dilutions of penicillin. This usually produced a concentration of between 2,500,000 and 3,000,000 organisms per ml. Absence of turbidity after incubation was used as the index of inhibition. The authors pointed out that the contents of the tubes remaining clear were not necessarily sterile. Control tests were always set up with a standard stock strain of hemolytic streptococcus.

It seemed possible that the results of sensitivity tests might be influenced by the size of the inoculum and by the criteria chosen to represent "inhibition." Therefore, in order to determine this sensitivity tests were performed with several representative strains of viridans streptococci in the following way:

Experiment 1

An overnight (approximately 18 hours) broth culture was diluted serially in broth from 10^{-1} to 10^{-6} or to 10^{-8} . The number of organisms per ml in each dilution was calculated by the method already described. The 10^{-6} dilution usually was found to have between 500 and 2,000 organisms in 0.1 ml. Penicillin dilutions were prepared in broth, and 1 ml of each dilution was pipetted into each of six or seven tubes. In this way six or seven identical series of penicillin dilutions were prepared. Exactly 0.1 ml of the undiluted broth culture was added to each tube of the first series of penicillin dilutions. Then 0.1 ml of the 10^{-1} bacterial dilution was added to each tube of the second series of penicillin dilutions. This process was repeated successively with each of the bacterial dilutions so that there were obtained combinations of every bacterial dilution with every concentration of penicillin. The number of organisms per ml and the concentration of penicillin in units per ml in each tube at the start of the experiment were readily calculated since 0.1 ml of broth with a known bacterial content was added to 1 ml of a known penicillin concentration. After incubation at 37 C for between 18 and 24 hours the tubes were shaken and examined for the presence or absence of turbidity. A loop streak, or in some instances a 0.1-ml streak, on a blood agar plate was made of the contents of all tubes that remained clear in order to determine whether or not sterility had been produced. The plates were examined after 48 hours of incubation at 37 C. In this way it was possible to determine the minimal concentration of penicillin that prevented appreciable multiplication of the organisms (bacteriostasis) and the minimal concentration which completely or nearly completely killed the organisms (bactericidal effect).

In table 1A there are given the results of this experiment using the Torrice strain of viridans streptococci, which was found by preliminary tests to be relatively sensitive to the action of penicillin. The penicillin concentrations to

which the organisms were exposed varied from 1.0 down to 0.01 unit per ml. It is evident that the concentration of penicillin necessary to produce sterility

TABLE 1A

Effect of variation in the number of exposed organisms on the penicillin sensitivity of a relatively susceptible strain of viridans streptococci

DILUTION OF OVERNIGHT CULTURE USED FOR INOCULATION	CALCULATED NUMBER OF ORGANISMS PER ML AT START OF EXPERIMENT	AFTER OVERNIGHT INCUBATION AT 37 C	
		Lowest concentration of penicillin remaining clear	Lowest concentration of penicillin showing none or very few living organisms
Undiluted	5×10^7	0.06	0.06
10^{-1}	5×10^6	0.04	0.04
10^{-2}	5×10^5	0.04	0.04
10^{-3}	5×10^4	0.02	0.04
10^{-4}	5×10^3	0.02	0.02
10^{-5}	5×10^2	0.02	0.02

In this experiment 0.1 ml of each of the indicated bacterial dilutions of the Torrice strain of viridans streptococci was added to 1 ml of the following penicillin concentrations: 1.0, 0.8, 0.6, 0.4, 0.2, 0.1, 0.08, 0.06, 0.04, 0.02, and 0.01 units per ml. A control tube of 1 ml of broth without penicillin was also inoculated. Since each ml of penicillin solution was further diluted by the addition of 0.1 ml of a broth culture, the concentration of penicillin was actually about 9 per cent less than the figures shown above.

TABLE 1B

Effect of variation in the number of exposed organisms on the penicillin sensitivity of a moderately resistant strain of viridans streptococci

DILUTION OF OVERNIGHT CULTURE USED FOR INOCULATION	CALCULATED NUMBER OF ORGANISMS PER ML AT START OF EXPERIMENT	AFTER OVERNIGHT INCUBATION AT 37 C	
		Lowest concentration of penicillin remaining clear	Lowest concentration of penicillin showing none or very few living organisms
Undiluted	2×10^8	0.6	Many organisms in all tubes
10^{-1}	2×10^7	0.6	Many organisms in all tubes
10^{-2}	2×10^6	0.6	Many organisms in all tubes
10^{-3}	2×10^5	0.4	Many organisms in all tubes
10^{-4}	2×10^4	0.4	0.4
10^{-5}	2×10^3	0.4	0.4
10^{-6}	2×10^2	0.4	0.4

In this experiment 0.1 ml of each of the indicated bacterial dilutions of the Abizaid strain of viridans streptococci was added to 1 ml of the following penicillin concentrations: 100, 75, 50, 25, 10, 5, 2, 1, 0.8, 0.6, 0.4, 0.2, 0.1, and 0.05 units per ml. A control tube of 1 ml of broth without penicillin was also inoculated. Since each ml of penicillin solution was further diluted by the addition of 0.1 ml of a broth culture, the concentration of penicillin was actually about 9 per cent less than the figures shown above.

was very small and the same or nearly the same as that needed for bacteriostasis. Furthermore, it is also apparent that increasing the number of exposed organisms from 500 to 50,000,000 per ml caused only a slight increase in the minimal concentration of penicillin required for either a bacteriostatic or a bactericidal effect (0.02 to 0.06 units per ml).

When the same experiment was repeated with the Abizaïd strain, a somewhat more resistant viridans streptococcus, the results were not quite the same (table 1B). In this experiment the concentrations of penicillin varied from 100 down to 0.05 units per ml. When the number of exposed organisms was 200, both a bacteriostatic and a bactericidal effect were obtained with 0.4 units of penicillin. This minimal concentration was not appreciably altered by increasing the number of organisms to 20,000, but, when the number of organisms was increased beyond this, numerous bacteria were still alive after exposure to even 100 units of penicillin. Nevertheless, the minimal concentration of penicillin required for bacteriostasis was only increased from 0.4 to 0.6 units per ml when the number of organisms was increased from as few as 200 to as many as 200,000,000 per ml. Similar tests performed with other strains of viridans streptococci have yielded comparable findings.

This experiment therefore suggests that a very susceptible strain of viridans streptococci is not only prevented from multiplying but actually killed by very low concentrations of penicillin even when the number of exposed organisms is very large. With less susceptible strains bacteriostasis can likewise be effected even when large numbers of organisms are exposed, the minimal effective concentration of penicillin, of course, being larger than that for a more susceptible strain. On the other hand, with the less susceptible strains it is difficult to produce complete sterilization even with very concentrated solutions of penicillin, especially when the number of exposed organisms is large.

Survival curves. In order to obtain more detailed information concerning the difference in behavior of a very susceptible strain and a less susceptible strain of viridans streptococci when exposed to an excess of penicillin, the following experiment was performed with the same two organisms used for the sensitivity tests just reported:

Experiment 2

In this experiment 0.4 ml of an undiluted overnight broth culture of the strain to be tested was added to 3.6 ml of a penicillin solution in broth, and then the mixture was incubated at 37 C for 48 hours. The penicillin solution was prepared so that after the addition of 0.4 ml of broth culture the final concentration was 1.0 unit per ml. Immediately after the bacteria were added to the penicillin and at various intervals thereafter for a period of 48 hours, 0.1 ml was removed and the number of organisms present was determined by the serial dilution method previously described.

Figure 1 is a graphic representation of the results of this experiment when performed with each of the strains of viridans streptococci (Torrice and Abizaïd) used for the sensitivity tests. For purposes of comparison there is also shown in figure 1 the growth curve of one of the strains (Abizaïd) in the absence of penicillin. The logarithm of the number of surviving organisms per ml has been plotted against the time of exposure to penicillin in hours.

From the curve of the less susceptible strain (Abizaïd) it is evident that the number of surviving organisms gradually became less, and at an approximately constant rate. At the end of 48 hours there were still a good many viable

bacteria present. The more susceptible strain (Torrice) decreased much more rapidly, so that within 12 hours the original concentration of 10,000,000 bacteria per ml was reduced to 70 viable organisms per ml. Up to the twelfth hour the rate of decrease was more or less constant, but after the twelfth hour the few remaining organisms disappeared more slowly. However, by 24 hours there were

FIG. 1. SURVIVAL CURVES OF A VERY SUSCEPTIBLE AND A MORE RESISTANT STRAIN OF VIRIDANS STREPTOCOCCI IN THE PRESENCE OF 2 UNITS OF PENICILLIN

The number of organisms per ml have been charted logarithmically. The penicillin sensitivity of the Abizaid strain was about 0.35 units, and that of the Torrice strain about 0.035 units. Both strains were exposed to about 2 units of penicillin in broth. For comparison there is also shown the growth curve of the Abizaid strain in broth without penicillin.

only 10 viable organisms per ml, and by 48 hours the penicillin solution was completely sterile.

This experiment, therefore, suggests that the less susceptible strain of viridans streptococci (Abizaid) differed from the more susceptible strain (Torrice), not only in that the minimal concentration of penicillin necessary for bacteriostasis was greater, but also in that, even in the presence of an excess of penicillin, the

rate at which the organisms were killed was slower. We have also determined the survival curves of other strains of viridans streptococci and have found several moderately resistant ones to behave similarly to the Abizaid strain. However, of four susceptible strains whose bacteriostatic sensitivities fell within the range of 0.04 to 0.08 units, only one showed a rate of decrease which was at all comparable to that of the Torrice strain. From our data, therefore, we are not yet able to determine whether the rate of decrease of organisms in the presence of an excess of penicillin is correlated in any way with the minimal concentration of penicillin necessary to prevent multiplication (bacteriostatic sensitivity).

Experiment 2 also clarifies the results of the previously described sensitivity tests (experiment 1). The rate with which the Abizaid strain is killed even in the presence of an excess of penicillin is too slow to produce sterilization at the end of 24 hours unless very few organisms are present at the start. On the other hand, the rapid rate with which the Torrice strain is killed by an excess of penicillin makes understandable the fact that a culture containing even large numbers of organisms can be completely or nearly completely sterilized within 24 hours.

Of interest is the change in the slope of the curve for the Torrice strain after all but 70 organisms per ml had been killed (figure 1). This type of effect has previously been reported by Hobby, Meyer, and Chaffee (1942), who concluded that the number of survivors decreased by geometric units as the time increased by arithmetic units. Thus they found that the log of the number of survivors when plotted against time followed a straight line until 99 per cent of the organisms were killed. It should be noted that even with the more resistant Abizaid strain the number of viable organisms had been reduced from 7,000,000,000 at zero hour to 1,500,000 at the end of 24 hours. Although this represents a reduction of well over 99 per cent and is therefore confirmatory of the observations reported by these investigators, nevertheless the absolute number of survivors is large.

The small number of organisms of even a susceptible strain that fail to get killed readily are referred to by Bigger (1944b) as "persisters." However, these remaining organisms cannot multiply so long as they are exposed to the action of penicillin, and eventually they may die of their own accord. On the other hand, if the penicillin is destroyed or for some other reason is reduced below the minimal concentration necessary for inhibition, even a few remaining viable organisms may rapidly multiply again. This is of some practical importance if the *in vivo* action of penicillin is at all comparable to that observed *in vitro*.

Effect of variations in concentration of the organisms on the rate of decrease. From figure 1 it is evident that the less susceptible Abizaid strain of viridans streptococci, even in the presence of an excess amount of penicillin, was killed relatively slowly. In this particular experiment a fairly large inoculum was used so that at zero hour there were 7×10^9 organisms per ml. In order to determine whether or not the number of organisms influenced the rate of killing the following type of experiment was performed:

Experiment 3

A solution of penicillin in broth was prepared so that after 0.2 ml of an undiluted overnight broth culture of the Abizaid strain had been added to 1.8 ml of the solution, the final concentration of penicillin was 2 units per ml. By making appropriate serial dilutions of this with broth containing 2 units of penicillin per ml, we obtained a series of ten tubes, each of which contained a total volume of 1 ml, 2 units of penicillin, and in sequence the following dilutions of the original overnight broth culture: 10^{-1} , 0.5×10^{-1} , 10^{-2} , 0.5×10^{-2} , 10^{-3} , 0.5×10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , and 10^{-7} . The number of organisms present in each dilution immediately after preparation was determined by the method previously described. The tubes were then placed in the incubator at 37 C, and after 24 hours the number of viable organisms per ml in each tube was again determined. As a control a duplicate set of tubes was prepared with broth not containing penicillin and incubated simultaneously.

In all of the control tubes that did not contain penicillin, the number of viable organisms at the end of 24 hours had increased to approximately the same final concentration, the range of variation being between 1.5×10^{11} and 5×10^{12} organisms per ml.

The results of the tests with the penicillin broth cultures are shown by the graph on the left-hand side of figure 2, where the calculated number of organisms per ml at zero hour and the number after 24 hours' incubation in the presence of 2 units of penicillin are recorded logarithmically. The corresponding points for each tube have been connected by straight lines. The slope of the line for each tube represents the rate of decrease of viable organisms. It should be borne in mind that these rates are necessarily only approximate because of the relatively large chance for error in calculating the number of organisms per ml by the dilution method.

For purposes of confirmation, the same type of experiment was repeated with the Miller strain of viridans streptococci, which was found by previous tests to have a sensitivity of 0.4 units per ml, a value similar to that for the Abizaid strain. The tests with the Miller strain were performed with 1 unit of penicillin per ml and seven different dilutions of the overnight broth culture. The number of organisms per ml at zero hour and again after 24 hours' incubation at 37 C are also shown graphically in figure 2.

It would appear from figure 2 that the number of organisms at zero hour, at least within the range of the bacterial concentrations tested, does not influence appreciably the rate of bactericidal activity of an excess of penicillin. The slopes of the lowest two curves for both the Abizaid strain and the Miller strain suggest that the rate of killing may diminish slightly when the number of exposed organisms becomes relatively small. These conclusions have been borne out by our experience on many other occasions with these same two strains (Abizaid and Miller) and by tests of several other relatively resistant strains.

RELATION OF THE BACTERICIDAL ACTIVITY OF PENICILLIN TO ITS CONCENTRATION

Abraham and his associates (1941), in referring to the plate method for assaying penicillin, showed that the diameter of the zone of inhibition did not increase appreciably after the concentration of penicillin was increased above a certain amount. Hobby, Meyer, and Chaffee (1942) likewise demonstrated in ex-

periments with hemolytic streptococci that with a given number of organisms the rate of killing increased within limits as the concentration of penicillin was increased, but there was a point beyond which increases in concentration of penicillin no longer increased the rate of killing. On the other hand, Fisher (1943) stated that the speed and degree of action of penicillin depended on its strength.

FIG. 2. EFFECT OF VARIATIONS IN THE NUMBER OF ORGANISMS ON THE BACTERICIDAL ACTION OF PENICILLIN

The number of organisms are charted logarithmically. Determinations were made at zero hour and again after incubation for 24 hours. The zero and 24-hour points have been connected by straight lines. The Abizaid strain was exposed to 2 units of penicillin per ml and the Miller strain was exposed to 1 unit.

Miller, Green, and Kitchen (1945) also found that inhibition was directly related to the penicillin concentration. Rantz and Kirby (1944) in the body of their paper stated that above a certain concentration the activity of penicillin was not enhanced by increasing amounts of the drug. Yet in the summary of this same paper they said that "there is a close correlation between the concentration of penicillin and its activity."

From the sensitivity tests of experiment 1 (table 1A) and from the survival curve shown in figure 1, it is apparent that even a very heavy inoculum of a very

sensitive organism, such as the Torrice strain, can be almost completely destroyed within a relatively short period. When such bacteria are concerned, it is obvious that all one needs to be sure of is that the penicillin be maintained above the minimal concentration necessary for inhibition. On the other hand, when one is dealing with organisms such as the Abizaid and Miller strains, which are not killed so rapidly, a question which might be of some practical importance is whether the rate of destruction or the number of organisms destroyed can be enhanced by increasing the concentration of penicillin above the minimal concentration needed to prevent growth. Since an unequivocal answer could not be

TABLE 2
Effect of variation in the concentration of penicillin on its bactericidal activity

PENICILLIN UNITS PER ML	NUMBER OF ORGANISMS PER ML	
	At zero hour	At 24 hours
7.3	1×10^9	2.3×10^6
3.6	1×10^9	1.9×10^6
1.8	1×10^9	1×10^6
0.9	1×10^9	1.2×10^6
0.46	1×10^9	1.2×10^6
0.23	1×10^9	2×10^9
0	1×10^9	1.5×10^{12}

Calculations of the concentration of penicillin and the number of organisms at zero hour take into consideration the fact that 0.2-ml amounts of a 10^{-1} dilution of a broth culture were added to 2-ml volumes of penicillin in broth. The Abizaid strain which was used in this experiment had previously been determined to have a sensitivity of about 0.35 units.

obtained from a review of the medical literature, the following experiment was performed:

Experiment 4

In a series of tubes 2-ml volumes of broth were prepared so that they contained the following concentrations of penicillin: 8, 4, 2, 1, 0.5, 0.25, and 0 units per ml. Each tube was inoculated with 0.2 ml of a 10^{-1} dilution of an overnight broth culture of the Abizaid strain of viridans streptococci. It is apparent that the concentration of penicillin in each tube was reduced by approximately 9 per cent by the addition of the broth culture, so that the final penicillin concentrations were 7.3, 3.6, 1.8, 0.9, 0.46, 0.23, and 0 units per ml. The number of organisms in the inoculum, and hence the number of organisms per ml in each tube at zero hour, were calculated by the serial dilution method. The tubes were placed in an incubator at 37 C, and the number of viable organisms per ml in each tube was determined at the end of 24 hours. Previous experiments had shown the Abizaid strain to have a penicillin sensitivity of about 0.35 units.

The results of this experiment are shown in table 2. With 0.23 units of penicillin, a concentration slightly under the "sensitivity" of the Abizaid strain, there seems to be some bacteriostasis but no appreciable killing. On the other hand, with concentrations of 0.46 units of penicillin and higher there is a definite reduction in the number of viable organisms during the 24-hour period. It is

evident that the degree of reduction is about the same in all of the first five tubes. Hence it appears that above a critical concentration even a 16-fold increase in the concentration of penicillin (0.46 to 7.3 units) caused no increase in the number of organisms killed. Many similar experiments with this same strain of viridans streptococci and with other strains have yielded similar results.

EFFECT OF SULFONAMIDE DRUGS ON THE ACTION OF PENICILLIN

Ungar (1943) reported that the inhibitory effect of penicillin in the presence of small quantities of sulfapyridine, which per se were insufficient to show any effect, was at least double. The *in vitro* tests were made with staphylococci and streptococci, and the activity of the penicillin was determined by observing the highest dilution which caused inhibition of growth. Ungar also confirmed these conclusions by *in vivo* experiments with mice. Rammelkamp and Keefer (1943) observed that the addition of small amounts of penicillin, which in itself displayed no killing effect against staphylococci, seemed to enhance the effectiveness of sulfathiazole in whole defibrinated blood. An apparent synergism of sulfonamide drugs and penicillin was likewise found by T'Ung (1944) in experiments with sulfathiazole, penicillin, and *Brucella* organisms, and by Bigger (1944a) in experiments with staphylococci, penicillin, and various sulfonamide drugs. Bigger concluded that sulfathiazole was more effective as a synergistic agent against the staphylococcus than sulfanilamide and sulfapyridine, and he found that the degree in which the penicillin activity was enhanced by sulfathiazole varied with the strain. *In vivo* experiments on mice by Soo-Hoo and Schnitzer (1944) also suggested that sulfonamide drugs increased the effectiveness of penicillin as an antistreptococcal agent.

In contrast to these reports, Hobby and Dawson (1944a, 1944b, 1945) found no evidence of a synergistic action between sulfadiazine or sulfapyridine and penicillin. They pointed out that the activity of penicillin is decreased by conditions which slow the rate of multiplication of the organism. Furthermore, they observed that sulfonamides decreased the rate of multiplication and hence actually tended to decrease the rate at which penicillin acted. Kirby (1944) also conducted *in vitro* experiments on the bacteriostatic action of sulfonamide-penicillin mixtures. He concluded that the effect of penicillin plus sulfadiazine was equal to the effect of penicillin alone plus the effect of sulfadiazine alone. That is, there was no actual synergism or potentiation, but the effects were additive.

Because of these apparently contradictory findings regarding the effect of sulfonamides on the action of penicillin, experiments of the following type were performed:

Experiment 5

Serial dilutions of penicillin in 2-ml volumes were prepared in plain broth and in broth containing sulfamerazine.¹ The concentrations of penicillin were 1.0, 0.5, 0.25, 0.125, 0.06, and 0 units per ml. The final concentration of the sulfamerazine was 10 mg per 100 ml.

¹ The sulfamerazine was supplied through the courtesy of Dr. J. W. Crosson of the Sharp and Dohme Company.

Each of the 12 tubes was then inoculated with 0.2 ml of a 10^{-3} dilution of an overnight culture of a strain of viridans streptococcus, designated A2, the sensitivity of which had previously been found to be approximately 0.25 units. It is apparent that by adding 0.2 ml of broth culture to 2 ml of penicillin solution the concentration of penicillin in each tube was reduced by approximately 9 per cent. Thus the actual concentrations of penicillin after addition of the bacteria were 0.91, 0.45, 0.23, 0.11, 0.05, and 0 units of penicillin per ml. The actual sulfamerazine concentration was also reduced to approximately 9.1 mg per 100 ml. The number of organisms per ml in each tube was determined by the method previously described immediately after inoculation and again after incubation of the tubes for 24 hours at 37 C.

The results of this experiment are shown in table 3. It is evident that, in the absence of sulfamerazine, concentrations of penicillin below 0.23 units per ml had

TABLE 3

Bactericidal effect of various concentrations of penicillin in the presence and in the absence of sulfamerazine

PENICILLIN UNITS PER ML	SULFAMERAZINE MG PER 100 ML	NUMBER OF ORGANISMS PER ML	
		At zero hour	At 24 hours
0.91		1.2×10^6	7×10^3
0.45	0	1.1×10^6	4×10^3
0.23	0	1.0×10^6	1×10^3
0.11	0	0.8×10^6	3×10^3
0.05	0	0.8×10^6	2×10^3
0	0	0.8×10^6	1×10^{10}
0.91	9.1	0.8×10^6	5×10^3
0.45	9.1	0.8×10^6	2×10^3
0.23	9.1	0.7×10^6	1.5×10^3
0.11	9.1	0.8×10^6	2×10^3
0.05	9.1	0.8×10^6	2×10^4
0	9.1	0.8×10^6	2×10^3

Calculations of the concentration of penicillin and the concentration of sulfamerazine take into consideration the fact that 0.2-ml amounts of a 10^{-3} dilution of a broth culture were added to 2-ml volumes of penicillin in broth or to penicillin and sulfamerazine in broth. The strain used in this experiment had a sensitivity of about 0.25 units.

very little inhibitory effect on the growth of the bacteria. The organisms multiplied readily, although there is a suggestion that the rate of multiplication may have been slightly slower than in the control tube containing no penicillin. It is also evident that a fourfold increase in the concentration of penicillin from 0.23 units to 0.91 units per ml did not accelerate the rate of destruction of the bacteria. The latter finding is similar to that demonstrated by experiment 4, the results of which are shown in table 2.

With the minimal inhibitory penicillin concentration of 0.23 units and in concentrations above this, the presence of sulfamerazine did not appreciably increase or decrease the bactericidal activity of the penicillin, the decrease in the number of organisms being approximately the same in those tubes containing sulfamerazine as in those without the drug. However, in the presence of sul-

famerazine a concentration of 0.11 units of penicillin, which in the absence of sulfamerazine had been ineffective, caused almost as great destruction of bacteria as 0.23 units. Even 0.05 units had some bactericidal activity in the presence of sulfamerazine. It is evident that the sulfamerazine in the absence of penicillin had extremely slight if any inhibitory effect on the growth of this strain of viridans streptococci.

Thus it would appear that under the experimental conditions the presence of sulfamerazine caused a lowering of the minimal concentration of penicillin necessary to produce both bacteriostasis and destruction of the organisms. Other similar experiments with the same strain and with other strains have yielded practically identical results. However, we have encountered some strains of viridans streptococci for which this apparent synergism could not be demonstrated.

In table 4 we have compared the minimal bacteriostatic concentrations of penicillin for different strains of viridans streptococci in the presence and in the

TABLE 4

Penicillin sensitivity of different strains of viridans streptococci when tested with penicillin alone and with penicillin plus sulfonamides

STRAIN	MINIMAL CONCENTRATION OF PENICILLIN (IN UNITS PER ML) NECESSARY TO CAUSE A BACTERIOSTATIC EFFECT	
	In the absence of sulfonamides	In the presence of sulfonamides
Miller.....	0.40	0.20
Abizaïd.....	0.35	0.15
A2.....	0.23	0.05
W2.....	0.10	0.10
Torrice.....	0.035	0.025

absence of sulfonamides. It is evident that when sulfonamides enhanced the action of penicillin the minimal bacteriostatic concentration was reduced anywhere from about 25 to 75 per cent, depending on the strain.

We have compared this synergistic activity of sulfathiazole, sulfadiazine, and sulfamerazine and have found no significant difference. We have also performed tests with various concentrations of the sulfonamide drugs and have found a concentration of 5 mg per 100 ml to be nearly if not actually as effective as 10 mg per 100 ml, but concentration below 5 mg per 100 ml became decreasingly effective.

DISCUSSION AND CONCLUSIONS

The data presented in this report suggest that when the viridans streptococci are incubated in the presence of increasing concentrations of penicillin in broth a critical concentration is reached at which the bacteria fail to multiply. Further increases in the concentration of penicillin cause a diminution of the number of bacteria. These findings are consistent with the fact, now well established by many investigators, that penicillin produces a bactericidal as well as a bacteri-

ostatic effect. The critical concentration at which multiplication is prevented varies for different strains, and for any particular strain it is a measure of the organism's penicillin sensitivity.

Increases in the concentration of penicillin beyond that necessary for bacteriostasis produce increases in the rate at which the bacteria are killed until another critical concentration is reached beyond which further increases fail to manifest any greater bactericidal activity. These observations are confirmatory of those reported by Hobby, Meyer, and Chaffee (1942). Thus we have found that when a relatively resistant strain of streptococcus is involved, the rate at which the organisms are killed cannot be accelerated by exposing the bacteria to even very high concentrations of penicillin.

On the basis of our own observations as well as from data found in the medical literature, it seems likely that the difference between the minimal concentration necessary for bacteriostasis and the minimal concentration necessary for a maximum bactericidal effect is relatively small for most bacteria. However, our experience with at least one strain of viridans streptococci suggests that the difference may occasionally be relatively large, although we have made no attempt as yet to study this aspect of the problem in any great detail.

Our observations on strains of viridans streptococci incubated in the presence of bactericidal concentrations of penicillin confirm the statement of Hobby, Meyer, and Chaffee (1942) that the log of the number of survivors plotted against time follows a straight line until at least 99 per cent of the organisms are killed. The slope of this line that represents the rate of decrease varies for different strains. For all of the relatively resistant strains which we have studied (minimal bacteriostatic concentration of penicillin = 0.1 unit per ml or more), the slope of the logarithmic survival curves have been relatively shallow. Thus, one relatively resistant strain was reduced in a period of 24 hours from 1,000,000 to approximately 2,000 organisms per ml, and another relatively resistant strain was reduced from 4,000,000 to about 20,000 organisms per ml (figure 2). Although the number of survivors in each instance represents less than 1 per cent of the original inoculum, nevertheless the absolute number is still fairly large.

Some very susceptible strains (minimal bacteriostatic concentration of penicillin 0.04 units or less) have been found to be killed very rapidly in the presence of an excess of penicillin, and their logarithmic survival curves are steep. Thus in one particular instance an inoculum of 3,000,000 organisms per ml was reduced to 20 viable organisms in the course of 24 hours (Torrice strain in figure 1). However, several other strains whose bacteriostatic sensitivities ranged between 0.04 and 0.08 units of penicillin were found to be killed more slowly and at a rate which was comparable to that of the more resistant strains.

We have not yet had the opportunity to study a sufficient number of strains of viridans streptococci in order to determine whether or not there is any relation between the maximum rate of killing (slope of the logarithmic survival curve) and the minimal concentration of penicillin necessary for bacteriostasis (sensitivity). Whatever might be the outcome of such observations, it seems to us that the maximum rate at which bacteria can be killed by penicillin is a quality

which may be of some importance, and which may have some bearing on the response of patients with subacute bacterial endocarditis to treatment with penicillin. It seems possible that some of the failures of penicillin therapy might be related to the fact that the causative organisms, though prevented from multiplying, were killed very slowly and hence survived for long periods of time even in the presence of concentrations of penicillin above the sensitivity level. This possibility, though only conjectural, deserves further investigation.

Our observations, in confirmation of the statement made by Abraham *et al.* (1941), suggest that the number of organisms do not alter appreciably the action of penicillin. When a very susceptible strain of viridans streptococci is involved, all of the organisms, or all but a very few "persisters," are killed within 24 hours, even when the original inoculum is large. When the strain under observation is killed more slowly, as is true of the more resistant strains which we have observed, the rate of decrease in the number of viable organisms in the presence of an effective concentration of penicillin is not appreciably altered by wide variations in the size of the inoculum. The number of viable organisms present after incubation will depend upon the size of the original inoculum. If the inoculum of such a strain is large, many viable organisms will still be present at the end of 24 hours. However, if the inoculum is very small, the rate of decrease may be sufficient to bring about complete sterility at the end of 24 hours, though usually the presence of at least a few viable organisms can still be detected.

These studies throw some light on the use and interpretation of sensitivity tests. If to a series of tubes containing different concentrations of penicillin there is added a bacterial inoculum of such size that the tubes still appear clear, then after incubation for 24 hours the presence or absence of turbidity can be used as the criteria for determining the end point. Those tubes that are turbid after shaking obviously contain concentrations of penicillin in which the organisms readily multiplied. The lowest concentration of penicillin remaining clear is the minimal concentration which prevents appreciable multiplication and therefore represents the bacteriostatic sensitivity of the organism. This is essentially the method used by Dawson, Hobby, and Lipman (1944) for determining the sensitivity of various strains of nonhemolytic streptococci.

The presence or absence of viable organisms in the clear tubes depends on the size of the original inoculum and the rate at which the organisms are killed. If the strain is one which is killed slowly and the original inoculum is relatively large, such as might be obtained with a 10^{-1} or a 10^{-2} dilution of the overnight culture, then it is likely that many viable organisms will still be present in all of the clear tubes. If the original inoculum of the same strain is small, such as might be obtained with a 10^{-5} or 10^{-6} dilution of the overnight culture, then the tubes remaining clear after incubation may be sterile or contain very few viable organisms. If the strain being tested is one which is killed rapidly by effective concentration of penicillin, then the tubes remaining clear after 24 hours of incubation will be either sterile or contain only a few viable organisms, and this will be so irrespective of the size of the original inoculum. Therefore it is ap-

parent that if one wishes to obtain satisfactory information regarding both the rate at which a particular strain can be killed and the minimal concentration of penicillin producing the maximum rate of killing, it is necessary to determine the number of organisms in the original inoculum and also in the tubes remaining clear after incubation.

Our *in vitro* studies with sulfonamides are confirmatory of the original observations of Ungar (1943). We have found that with many strains of viridans streptococci the minimal concentration of penicillin necessary to produce either a bacteriostatic or a bactericidal effect can be reduced 25 to 75 per cent by the presence of sulfonamide drugs. In other words, a subeffective concentration of penicillin in which the bacteria readily multiply may, in the presence of sulfonamides, cause just as rapid killing of the organisms as a more concentrated preparation of penicillin. However, if penicillin is present in a concentration well above that of the sensitivity of the strain of viridans streptococci being tested, sulfonamides will not alter appreciably the rate at which the organisms are killed.

SUMMARY

From studies of the *in vitro* action of penicillin on various strains of viridans streptococci the following observations have been made:

1. Concentrations of penicillin above the minimal concentration necessary for bacteriostasis have a bactericidal action. For any given strain there is a critical concentration of penicillin beyond which further increases in concentration have no increased bactericidal activity.
2. Strains of viridans streptococci differ not only in regard to the minimal concentration of penicillin necessary for bacteriostasis but also in regard to the rate at which they are killed in the presence of an excess of penicillin. There is need for further studies concerning the possible correlation of these two variables.
3. Within wide limits, variations in the number of exposed organisms do not appreciably alter the rate of bactericidal action of penicillin.
4. Sulfonamide drugs seem to have the following effect on the *in vitro* action of penicillin: (a) The minimal effective concentration of penicillin is reduced for many strains of viridans streptococci. (b) In the presence of penicillin in amounts above the minimal effective concentration, sulfonamides do not appreciably alter the bactericidal action of the penicillin.

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