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Lowell A. Rantz; ... et. al

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THE ACTION OF PENICILLIN ON THE STAPHYLOCOCCUS IN VITRO

LOWELL A. RANTZ AND WILLIAM M. M. KIRBY
WITH THE TECHNICAL ASSISTANCE OF ELIZABETH RANDALL

From the Department of Medicine, Stanford University School of Medicine, San Francisco, California

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Penicillin, a growth product of the fungus, *Penicillium notatum*, has become established as an effective agent in the treatment of infections caused by various organisms, particularly the pyogenic cocci. Little information is available in regard to its mode of action other than that minute amounts inhibit the growth of, or kill, large numbers of bacteria. It is also known that pneumococci, gonococci, meningococci, and hemolytic streptococci are very much more sensitive to the action of this material than are staphylococci (1, 2).

There is general agreement that multiplication of the organism is necessary for the establishment of the penicillin effect (3). Lysis of staphylococci by penicillin has been observed, but this phenomenon has usually been stated not to occur (4).

One of the most important uses of penicillin clinically has been the treatment of severe staphylococcal infections, many of which are not favorably influenced by the administration of sulfonamides. Several studies have been made of the *in vitro* effects of penicillin on these organisms in broth and in human blood and serum (2, 5). These have indicated that staphylococci are readily killed by low concentrations of penicillin without the assistance of antibodies or of leucocytes. If large initial inocula are used, a few organisms survive prolonged incubation in the presence of high concentrations of penicillin. There appears to be relatively little variation in the resistance of different strains to the action of the agent but organisms may become insensitive to the action of penicillin if permitted to multiply in its presence over a prolonged period (10).

Because previous reports (6, 7) have established the usefulness of turbidimetric methods for the measurement of the rate of bacterial growth in the study of sulfonamide bacteriostasis, it seemed likely that similar technics might be profitably applied in the determination of the mode of action of penicillin. This paper describes the results of a series of observations on the effect of penicillin on staphylococci in which these methods were applied.

METHODS

Organisms. Various strains of staphylococci were studied. Most of the experiments were conducted with Strain "O", a coagulase-positive, pigment-forming organism isolated from the circulating blood of a patient with a severe septic infection.

Medium. In many experiments a synthetic medium, identical with that previously described (6), was used to which 5 milligrams per liter of nicotinic acid and

of thiamin chloride had been added. This supported good but not optimal growth of staphylococci. Frequently this medium was augmented by the addition of various concentrations of peptone (Difco). In a few experiments, ordinary beef infusion broth was substituted for the synthetic medium.

Penicillin. The sodium salt of penicillin,[1] obtained as a powder, dehydrated and sealed in vacuum, was dissolved in a 0.9 per cent solution of sodium chloride in a concentration of 100 Oxford units per milliliter and sterilized by Seitz filtration. Appropriate dilutions were made from this solution.

Methods. In general the methods for the measurement of bacterial growth were similar to those previously described with one important modification. In earlier studies on sulfonamide bacteriostasis, only the rate of growth was of interest and an initial inoculum was used which was too small to be demonstrable photometrically. Since it was now desirable to observe the entire phenomenon of bacterial growth in the presence of penicillin, an inoculum was used which produced a faint initial turbidity. This was readily obtained by permitting the organisms to grow for 18 hours in a small amount of the basal medium which was then, at the time of the experiment, diluted with 4 to 5 volumes of fresh medium. Plate counts indicated that approximately 10,000,000 organisms per ml were present as the initial inoculum in these experiments.

Growth curves were readily obtained by frequent determinations of the turbidity of the various preparations during incubation at 37 C in a water-bath.

RESULTS

General observations. The growth of staphylococci in the synthetic medium plus 0.5 per cent peptone, and in broth, in the presence of various concentrations of penicillin is graphically portrayed in figure 1. The results obtained in the two media are similar. It will be observed that an initial period occurred during which the drug-treated and control organisms appeared to multiply at the same rate. This interval was quite long in the presence of 0.1 units per milliliter of penicillin and very short in the presence of 10 units per milliliter. After the induction phase there was another period of varying length in which the organisms appeared to multiply more slowly than the control, followed by a complete cessation of bacterial growth. Then, quite soon in the synthetic medium and more slowly in broth, the turbidity began to decrease. The organisms were undergoing lysis, more rapidly and completely in the higher concentrations of penicillin and in the synthetic medium. This phenomenon has been observed in every instance in which a sensitive staphylococcus has been subjected to the action of penicillin. Streptococci do not appear to exhibit this effect (3, 8).

After 24 hours of incubation of a sensitive strain in the presence of penicillin, the medium appeared perfectly clear. Cultures, however, always revealed the presence of living staphylococci in large numbers. Further incubation resulted

[1] The penicillin was provided by the Office of Scientific Research and Development from supplies assigned by the Committee on Medical Research for clinical investigations recommended by the Committee on Chemotherapeutic and Other Agents of the National Research Council.

in a progressive decrease in the colony count, this effect occurring most rapidly in the synthetic medium and in the higher concentrations of penicillin. The number of viable organisms at the end of 120 hours of incubation in a typical experiment is presented in table 1.

Complete sterilization occurred only in synthetic medium in the presence of 10 units per milliliter of penicillin.

The viable organisms after prolonged incubation were repeatedly tested for sensitivity to the action of penicillin and were invariably discovered to be as readily lysed and killed by this material as was the parent strain.

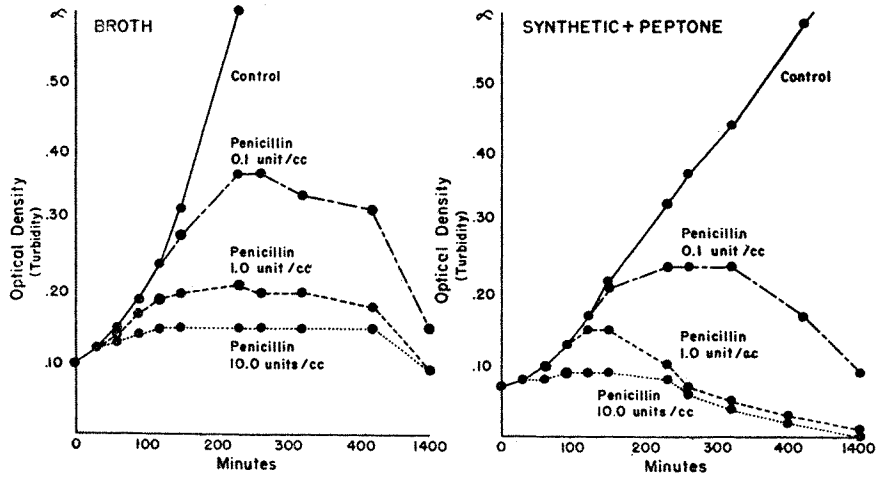

FIG. 1. GROWTH CURVES DETERMINED BY TURBIDIMETRIC METHODS ILLUSTRATING THE EFFECT OF VARIOUS CONCENTRATIONS OF PENICILLIN ON STAPHYLOCOCCUS AUREUS IN BROTH AND IN A SEMI-SYNTHETIC MEDIUM PLUS PEPTONE

TABLE 1

Number of viable staphylococci after 120 hours of incubation in the presence of penicillin

MEDIUM	SYNTHETIC + 0.5% PEPTONE		BROTH	
Penicillin units per milliliter	1.0	10.0	1.0	10.0
Viable staphylococci per milliliter	20	0	More than 1,000	500

Concentration of penicillin. The observations just described indicate that increasing concentrations of penicillin are more actively lytic and bactericidal. When similar experiments were conducted with concentrations of penicillin from 0.02 to 0.10 units per milliliter, the results obtained were similar to those just described and are presented in figure 2. It will be observed that even the smallest concentration caused some lysis in 24 hours and that the degree of clearing of the medium varied roughly with the amount of penicillin present. Furthermore, if the turbidity at 240 minutes be plotted against penicillin concentration, a straight line is obtained, confirming the previous observation (9) that,

under certain circumstances, the bacteriostatic activity of penicillin is proportional to its concentration.

Inoculum. The technics described in this report have utilized an inoculum many times larger than that usually used in the study of penicillin bacteriostasis. Several strains of staphylococci were discovered during the study that grew as well in the presence of penicillin in a concentration of 1.0 unit per milliliter and 90 per cent as well in the presence of 10 units per milliliter as did the controls. Some of these organisms were studied in the manner previously described, using the synthetic medium plus 0.5 per cent peptone, except that the initial concentration of organisms was serially decreased. The turbidity of the various tubes was

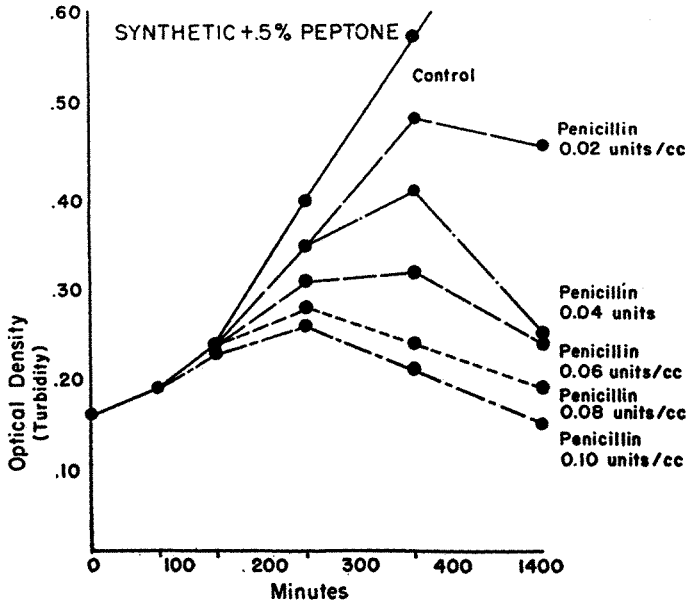

FIG. 2. FURTHER GROWTH CURVES DETERMINED BY TURBIDIMETRIC METHODS ILLUSTRATING THE EFFECT OF VARIOUS CONCENTRATIONS OF PENICILLIN ON STAPHYLOCOCCUS AUREUS IN A SEMI-SYNTHETIC MEDIUM PLUS PEPTONE

determined at 24, 48, and 72 hours. Those that were clear at 48 hours were cultured. The results of two typical experiments are presented in table 2.

It will be observed that the growth with the drug and the control was identical when the inoculum was 1 million bacteria per milliliter. As the inoculum decreased, the drug appeared to become more effective, since progressively longer intervals were required for growth to appear. With 10,000 and 1,000 organisms per milliliter, visible growth never appeared in the presence of 10 units per milliliter of penicillin and when the tubes were cultured those containing 10 units per milliliter and 1,000 initial organisms were sterile.

Medium. Studies of the mode of action of the sulfonamides have indicated that materials which improve the ability of the culture medium to support bacterial growth decrease the degree of sulfonamide bacteriostasis (7). Studies

on penicillin have suggested that this effect was probably not demonstrable with this agent (3).

Evidence previously presented in the first section of this report partially confirms these observations, but it was pointed out that lysis was more rapid and the bactericidal effect more complete in the synthetic medium plus 0.5 per cent peptone than in broth which was approximately twice as effective a medium.

TABLE 2

The effect of various initial inocula on penicillin activity in vitro

CONCENTRATION OF PENICILLIN	STRAIN 27828			STRAIN 27558		
	Hours of incubation					
	24	48	72	24	48	72
Initial inoculum 1,000,000 organisms per milliliter						
units/milliliter						
10.0	0.85			0.83		
1.0	0.85			0.83		
0	0.85			0.85		
Initial inoculum 100,000 organisms per milliliter						
10.0	0.03	0.04+	0.77	0.02	0.74	
1.0	0.66	0.70	0.77	0.72	0.74	
0	0.77	0.77	0.77	0.74	0.74	
Initial inoculum 10,000 organisms per milliliter						
10.0	0.04	0.04+	0.04	0.03	0.04+	0.04
1.0	0.02	0.74	0.74	0.02	0.74	0.74
0	0.72	0.74	0.74	0.70	0.74	0.74
Initial inoculum 1,000 organisms per milliliter						
10.0	0.02	0.03−	0.04	0.03	0.04−	0.04
1.0	0.02	0.03+	0.47	0.03	0.04+	0.66
0	0.70	0.70	0.70	0.70	0.70	0.70

+, culture positive.

−, culture sterile.

The figures represent the optical densities of the cultures after varying periods of incubation.

In other experiments the growth rate of the control organisms was enhanced twofold by the addition of increasing concentrations of peptone to the synthetic medium up to 1.5 per cent without decrease in penicillin activity.

Penicillin resistance. Naturally occurring strains of staphylococci resistant to the action of penicillin have been briefly mentioned but, in the only extensive study, 29 strains were all found by Rammelkamp and Maxon (10) to be killed by concentrations of penicillin of 0.02–0.35 unit per milliliter, an approximate

15-fold variation in sensitivity. These workers used an initial inoculum of 1,000 to 30,000 organisms.

In the present study, 70 strains of staphylococci derived from various clinical sources have been subjected to the action of penicillin by the methods described in this paper, using the synthetic medium plus 0.5 per cent peptone, and an initial inoculum of approximately 10,000,000 organisms. A strain was determined to be resistant to a given concentration of penicillin when growth in the presence of the drug was 90 per cent or more of that in the control tube.

The results are presented in table 3, the strains being divided on the basis of coagulase activity which is believed to be of greater clinical importance than is pigment formation.

It will be observed that 75 per cent of the coagulase-positive and an equal number of the coagulase-negative strains were actively killed and lysed by 0.1 units of penicillin per milliliter. 21 per cent of the coagulase-positive, but none of the coagulase-negative, organisms grew freely in the presence of 10.0 units per milliliter of penicillin. A few strains of both types grew well in the presence of the lower dilutions of penicillin.

TABLE 3

Resistance of strains of staphylococci to penicillin

COAGULASE TEST	NUMBER OF STRAINS	NUMBER OF STRAINS RESISTANT TO PENICILLIN			NUMBER OF STRAINS LYSED BY 0.1 UNIT/ML
		10 units per ml	1.0 units per ml	0.1 units per ml	
Positive	43	9	9	11	32
Negative	27	0	2	6	21

These results, therefore, indicate that the application of the method described in this report permits the demonstration of at least a hundred-fold difference in sensitivity to penicillin between strains of staphylococci. It was not possible to discover any relationship between the source of the strain and its sensitivity to penicillin. The colonial form of all of the resistant strains was identical with that of the non-resistant ones.

DISCUSSION

Certain facts in regard to the action of penicillin on staphylococci *in vitro* emerge from the results of the experiments just described and from a consideration of previous studies by others.

Penicillin is actively bactericidal for the staphylococcus and, during the course of its action, lysis of the organism occurs. This has not been demonstrated for the hemolytic streptococcus. Whether this dissolution of the organism is the result of the direct action of penicillin on the cell membrane or is caused by autolytic enzymes present in the bacterial cell that become active as the result of interference with vital bacterial metabolic processes by penicillin, cannot now be determined. The latter hypothesis is, perhaps, the more probable since it is

known that staphylococci will, under certain circumstances, undergo spontaneous lysis (11).

In order to establish the penicillin effect the organism must, apparently, divide. This is clearly shown in the experiments described above when small amounts of penicillin were used. Under these circumstances an induction period occurred. With a large concentration of penicillin, it appeared to be very short. These observations must, however, be interpreted in relationship to the fact that bacteriolysis was occurring simultaneously. It is likely that, when high concentrations of penicillin are used, lysis begins as soon as cell division has taken place so that the turbidity does not increase, whereas with lower concentrations the lytic process is more gradual.

In spite of the ease with which enormous numbers of staphylococci may be killed and lysed by small amounts of penicillin, many organisms remain alive even after prolonged exposure to this chemical. The remaining viable bacteria on retesting may be shown to be as sensitive to the action of penicillin as was the parent strain, so that their survival is not the result of artificially induced penicillin resistance. It has been suggested (5) that these organisms have ceased to divide and are, therefore, insusceptible to the action of the chemical. If this situation also prevails in infected tissues, the sterilization of abscess cavities will be difficult unless the clearing mechanisms of the body can be brought into play to remove the residual surviving organisms. That this is so has been borne out by certain difficulties that have arisen in the sterilization of abscesses in the bones in instances of staphylococcus osteomyelitis (12).

It has been previously demonstrated that a relationship exists between the bactericidal action of penicillin and its concentration and that above certain levels the activity is not enhanced by increasing amounts of the drug. This point varies with the inoculum and the strain of staphylococcus and would appear to be in the neighborhood of 1 unit $\pm$ 0.8 unit per milliliter (3, 5). Below this critical upper level there is correlation between the activity of penicillin and its concentration and it has been proposed that this fact be utilized in the biological estimation of penicillin by turbidimetric measurements of bacterial growth (9). The presence in the blood and tissues of 0.1–0.2 unit of penicillin per milliliter would, on the basis of the observations recorded in this report and those of Rammelkamp, in which staphylococci were exposed to the action of penicillin in whole blood and serum, seem to be adequate for the therapy of most clinical infections.

The effect of the size of the initial inoculum on the activity of penicillin on the staphylococcus *in vitro* is not clear. Very large numbers of susceptible organisms may be killed if the concentration is adequate, but the retardation of growth of a resistant organism is more marked if the inoculum is small and the cultures may become sterile.

It is generally stated that penicillin is not inhibited by serum, body fluids or peptones. The observations described here indicate that lysis occurs somewhat more slowly and that more organisms remain viable after prolonged exposure to penicillin if a rich broth be used rather than the relatively incomplete synthetic

medium. When peptones were added to the synthetic medium in increasing concentration, the control organism multiplied more rapidly but penicillin activity was unimpaired. This is in striking contrast with the action of the sulfonamides. In the latter experiments only the rate of growth of the organism was studied. It is possible that the constituents of the culture medium have no effect on the inhibitory phase of penicillin action on the staphylococcus but are concerned in the ease with which the agent may induce lysis and killing of the bacteria.

The action of penicillin upon various strains of staphylococci in a previous study suggested that no more than a fifteen-fold variation existed in their sensitivity to the chemical. A small inoculum was used in these experiments. When a very much larger inoculum was used in the study described here, the situation was different. 21 per cent of coagulase-positive, but none of the coagulase-negative strains grew freely in the presence of a concentration of penicillin 100 times greater than that required to kill and lyse the most sensitive organisms It has not yet been determined whether penicillin resistance as determined by this method may be correlated with the failure of clinical response to therapy in the infected human being. Such studies are in progress.

In conclusion it may be stated that penicillin is an extraordinarily potent agent which in minute amounts induces the death and lysis of staphylococci. That this effect is not complete and that viable organisms remain after prolonged exposure to the drug is unfortunate, and may explain certain clinical failures. There is a close correlation between the concentration of penicillin and its activity. By the methods described in this report, a very great natural variation in the sensitivity of strains of coagulase-positive staphylococci to the action of penicillin may be demonstrated, the clinical significance of which has not been evaluated.

SUMMARY AND CONCLUSIONS

1. The action of penicillin on staphylococci has been studied *in vitro* by photoelectric turbidimetric methods.

2. Penicillin is bactericidal for the staphylococcus and causes lysis of the organism.

3. There is a close correlation between the concentration of penicillin and its activity.

4. The number of organisms in the initial inoculum appears to affect the activity of penicillin but the constituents of the culture medium do not.

5. More than one hundred-fold variations in the sensitivity of strains of staphylococci to penicillin have been demonstrated.

REFERENCES

(1) FLEMING, A. 1929 The antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of B. influenzae. Brit. J. Exp. Path., **10**, 226–236.

(2) HOBBY, GLADYS L., MEYER, KARL, AND CHAFFEE, ELEANOR. 1942 Activity of penicillin in vitro. Proc. Soc. Exptl. Biol. Med., **50**, 277–280.

(3) HOBBY, GLADYS L., MEYER, KARL, AND CHAFFEE, ELEANOR. 1942 Observations on the mechanism of action of penicillin. Proc. Soc. Exptl. Biol. Med., **50,** 281–285.
(4) SMITH, L. D., AND HAY, T. 1942 The effect of penicillin on the growth and morphology of staphylococcus aureus. J. Franklin Inst., **233,** 598–602.
(5) RAMMELKAMP, C. H., AND KEEFER, C. S. 1942 Penicillin: Its antibacterial effect in whole blood and serum for the hemolytic streptococcus and staphylococcus aureus. J. Clin. Invest., **22,** 649–657.
(6) KIRBY, W. M. M., AND RANTZ, L. A. 1943 Quantitative studies of sulfonamide resistance. J. Exp. Med., **77,** 29–39.
(7) RANTZ, L. A., AND KIRBY, W. M. M. Quantitative Studies of sulfonamide inhibitors. J. Immun., Chemotherapy & Virus Research (In Press).
(8) KIRBY, W. M. M., AND RANTZ, L. A. To be published.
(9) FOSTER, JACKSON W. 1942 Quantitative estimation of penicillin. J. Biol. Chem., **144,** 285–286.
(10) RAMMELKAMP, CHARLES H., AND MAXON, THELMA. 1942 Resistance of Staphylococcus aureus to the action of penicillin. Proc. Soc. Exp. Biol. Med., **51,** 386–389.
(11) A system of bacteriology in relation to medicine. 1930 His Majesty's Stationery Office. Page 506.
(12) RANTZ, L. A., KIRBY, W. M. M., AND BLOOMFIELD, A. L. To be published.