THE ACTION OF PENICILLIN ON STAPHYLOCOCCUS[1]

R. F. PARKER AND HARRIET C. MARSH

Department of Medicine, Western Reserve University, and Lakeside Hospital, Cleveland 6, Ohio

Received for publication October 3, 1945

Bacteria exposed to one of the active members of the sulfonamide group of drugs continue for a time to grow at a normal rate; growth is then interrupted and bacterial death begins (Hirsch, 1944). Some of the earlier work with penicillin indicated that, in contrast to this, penicillin causes the death of a certain proportion of the dividing cells of a growing culture without any appreciable lag period (Hobby *et al.*, 1942; Lee *et al.*, 1944). Rantz and Kirby (1944), however, have shown that at limiting dilutions of penicillin there may be a definite interval between the addition of penicillin and the onset of growth inhibition, as evidenced by turbidimetric determinations. From this, as well as from other studies of the action of penicillin, it has been concluded that the substance acts only on dividing cells, leaving nondividing cells unaffected. Hobby and Dawson (1944) likewise demonstrated that bacteria continued to multiply for a time after being planted in broth containing small concentrations of penicillin. According to these experiments, however, a similar lag in the onset of effective action of penicillin was also apparent when higher concentrations were used. The present studies were undertaken in order to gain more precise knowledge on this point and to determine how long the influence of penicillin on bacteria might last after its complete removal from the culture medium.

A commercial preparation of penicillin was used, the manufacturer's assay of its potency being accepted. The *Staphylococcus* employed was designated as strain Mx by Julianelle and Wieghard (1935) and is their type B. It is inhibited by 0.06, but not by 0.03, units of penicillin per ml and is thus somewhat less sensitive to penicillin than the Oxford strain. Tryptose phosphate broth and tryptose phosphate agar, manufactured by the Digestive Ferments Company, were used as culture media. Viable bacteria were determined by plate count after 24 hours' incubation. When samples were taken from the growing culture, they were chilled to 0 C in an ice bath after being withdrawn and held there until dilutions could be prepared for plating.

An initial series of experiments was set up to determine the effect of adding penicillin to a growing culture, according to the following pattern: To 10 ml of a 4-hour broth culture, penicillin solution was added in small volume and in amount sufficient to produce the desired concentration. At intervals thereafter a 0.5-ml portion was removed, immediately diluted to 5 ml with ice-cold saline solution, and centrifuged for 5 minutes at approximately 2,000 times gravity. The fluid was decanted, and the bacteria were resuspended in a second portion of saline solution (5 ml) and centrifuged. The organisms were then suspended in cold saline solution and held briefly until dilutions could be prepared for plating. Preliminary experiments had indicated the necessity for

[1] This work was supported in part by a grant from the Ben Venue Laboratories, Inc.

removing the penicillin in this way, even though the final dilution in the plate might be well beyond the theoretically active concentration. The results are presented in a series of graphs (figure 1).

It is readily apparent that whereas the ultimate lethal effect of penicillin on the staphylococcal cells is uniform, the time of onset of the effect is distinctly influenced by the concentration of the drug. When the concentration is high (1.0 unit per ml), logarithmic growth is apparently replaced at once by logarithmic death. When the concentration is low (0.03 unit per ml), an increase in the population continues during the period of observation, but at a rate which is lower than normal. When the concentration causing "complete inhibition" in the overnight test was doubled, the phase of logarithmic death was preceded by a 30-minute period of apparently uninterrupted growth. These experiments

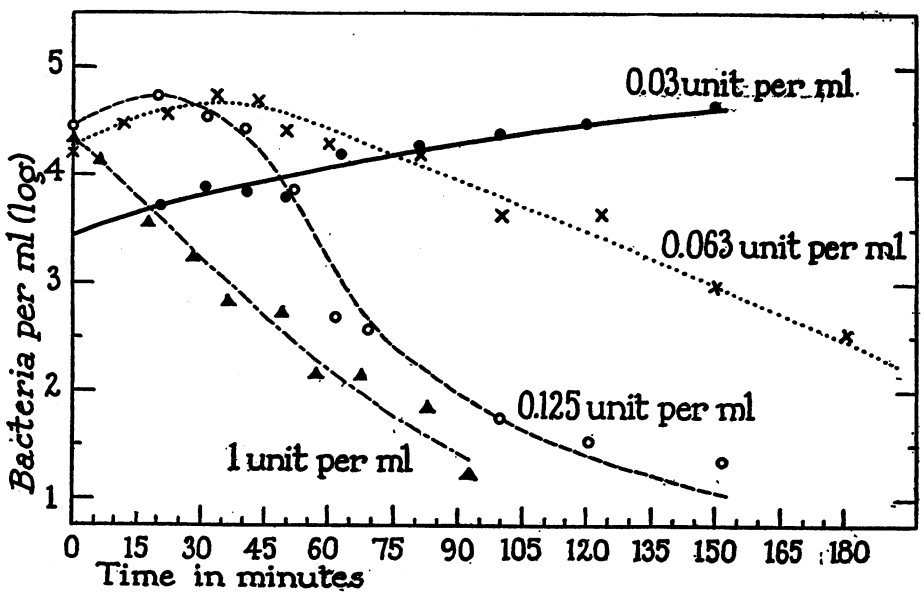

FIG. 1. CONTINUOUS EXPOSURE OF GROWING CULTURES TO 1.0, 0.125, 0.063, 0.03 UNITS OF PENICILLIN PER ML
Line drawn by inspection

were designed to follow the viable cell count only for the first period of penicillin action. In some experiments, evidence was obtained that the *rapid logarithmic* death rate continued only during the reduction of the population of the culture to 1 or 0.1 per cent of the maximum and that the rate was then reduced appreciably, in agreement with the findings of Hobby and Dawson (1944).

In a second series of experiments, penicillin was added to the liquid medium and after a predetermined time interval the bacterial cells were removed from the broth containing penicillin to a fresh, penicillin-free broth. The following experiment is typical of the series.

Fifty ml of broth (at 37 C) were seeded with 10^{-5} ml of an 18-hour broth culture of *Staphylococcus* Mx. After 4 hours a 12-ml sample was removed, penicillin was added to produce a concentration of 1.0 unit per ml, and incuba-

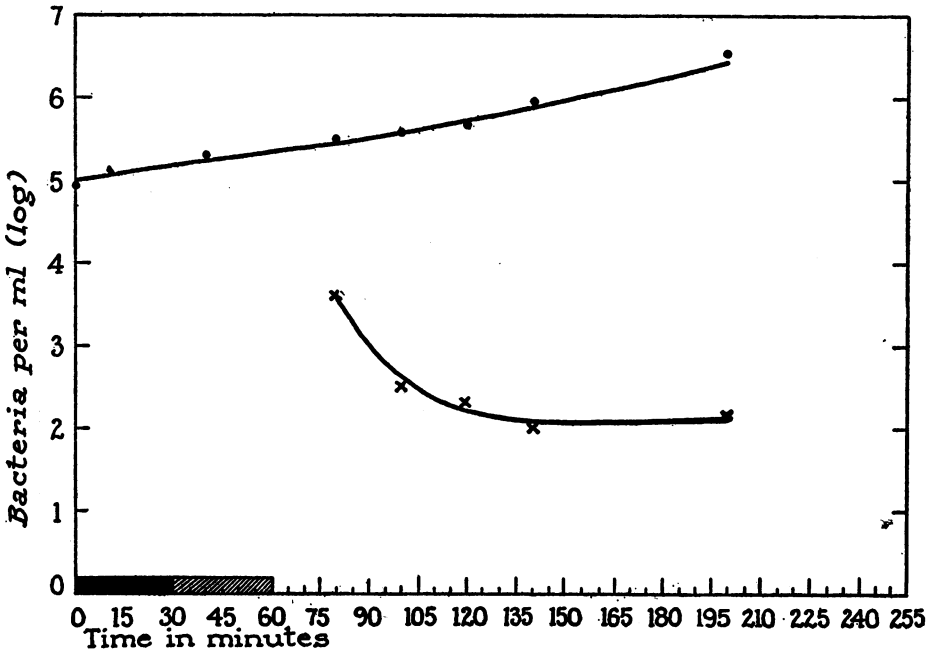

FIG. 2. EXPOSURE OF A GROWING CULTURE TO 1.0 UNIT PER ML OF PENICILLIN FOR 30 MINUTES

Upper curve, control culture. Lower curve, treated culture. Solid block indicates time of exposure; cross-hatched block, time of centrifugation and washing of culture. Lines were drawn by inspection.

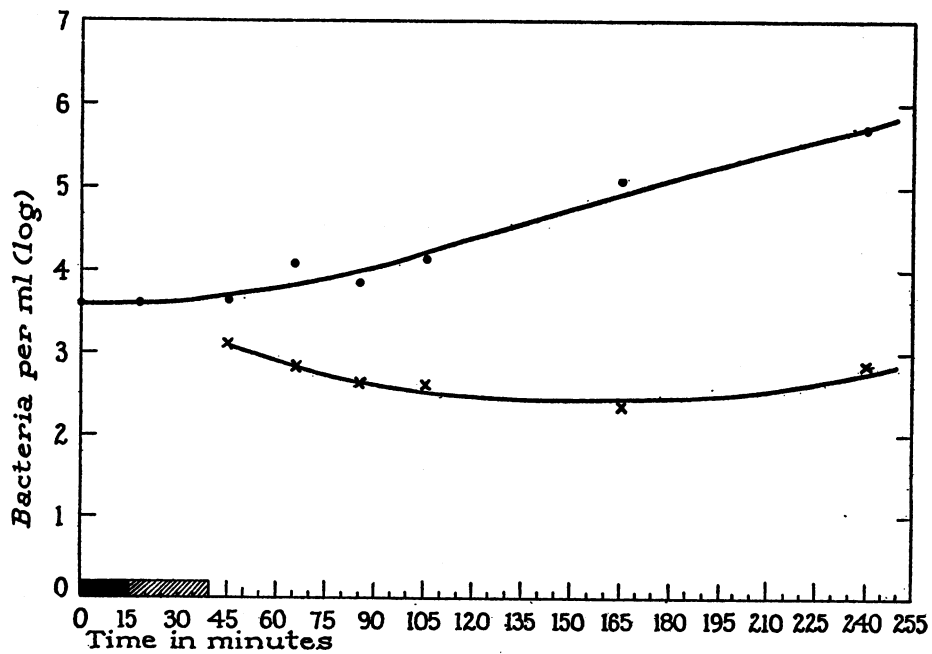

FIG. 3. EXPOSURE OF CULTURE TO 1.0 UNIT PER ML FOR 15 MINUTES

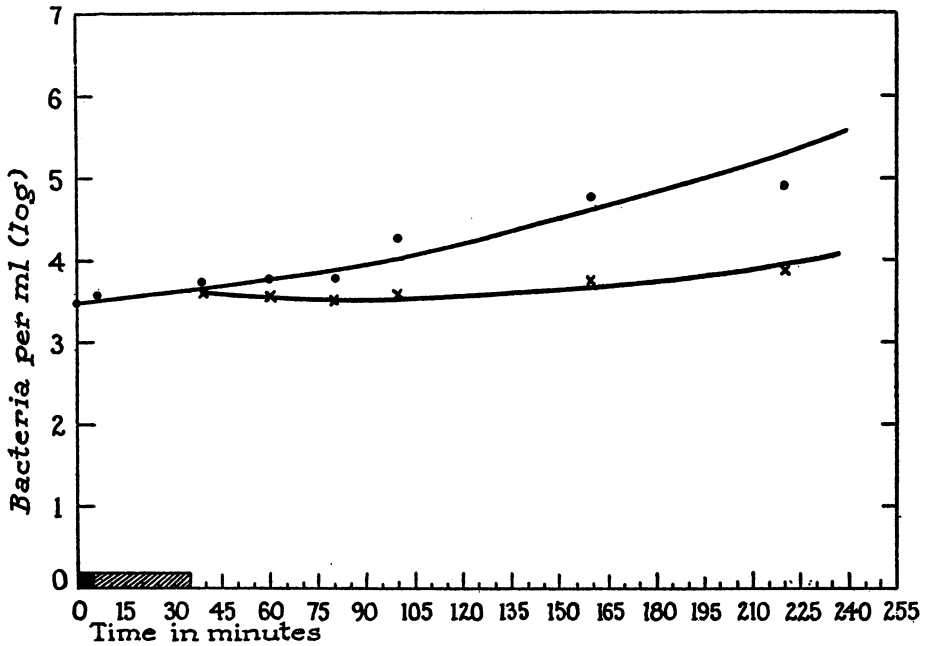

FIG. 4. EXPOSURE OF CULTURE TO 1.0 UNIT PER ML FOR 5 MINUTES

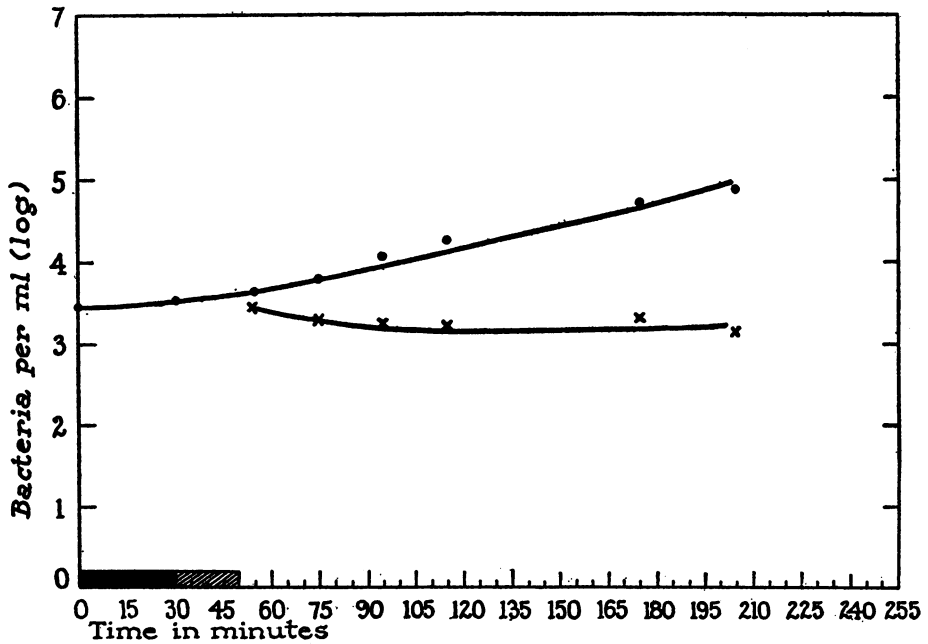

FIG. 5. EXPOSURE OF CULTURE TO 0.063 UNIT PER ML FOR 30 MINUTES

tion was continued. After the lapse of 30 minutes the penicillin-treated culture and an untreated control culture were centrifuged for 5 minutes. The supernatant was siphoned off, cells were immediately resuspended in warm broth,

centrifuged, resuspended in the original volume of warm broth, and returned to the incubator at 37 C. Samples were taken at intervals for counts of the viable cells. The results of a series of such experiments are presented in figures 2 to 5.

It will be noted that removal of growing cells from a culture medium by centrifugation and their transference to a fresh medium is accompanied by only a temporary reduction in the rate of reproduction. This is probably due to the reduction in temperature during the period of centrifugation at room temperature and the fact that during part of each run the packed cells were denied free access to the nutrients of the medium. This temporary effect on the rate of bacterial growth might have been predicted; however, the action of penicillin under these conditions was wholly unexpected. It will be observed that exposure of the growing culture to 1.0 unit of penicillin per ml led to a sharp drop in the number of viable cells, as in the first experiments. When, however, the cells were removed from a medium containing penicillin to one free of the drug, the decrease in the number of viable organisms continued for a time. Then the rate of death decreased until the viable cell count remained constant. This period was eventually followed by renewed growth, as has also been shown by other experiments. An explanation immediately suggested itself: the organisms present during this period of stationary population were the "persisters" described by Bigger (1944), organisms not in the proper physiologic state to be attacked by penicillin and therefore unaffected by it. Presumably, they were in a "resting," nonmultiplying stage. That this was not the whole explanation soon became apparent. When the period of exposure to penicillin was reduced to 15 minutes, the effect on the total number of viable cells was somewhat less, although there was a period of 3½ hours before growth began. When the time of exposure was reduced to 5 minutes, essentially no bacterial deaths occurred. But for 3 hours after the removal of penicillin no bacterial multiplication occurred. Subsequent experiments (figure 5) showed that the effect could also be produced by a longer exposure to a lower concentration (0.06 units per ml) of penicillin. (Reference to figure 1 reveals that when a culture is continuously exposed to 0.06 units of penicillin per ml, the population does not begin to fall for about an hour after penicillin is added.) Very short exposure to the lower concentration of penicillin produced no significant effect.

DISCUSSION

Before any definite description of the mechanism of action of penicillin can be formulated, much more must be known than has been learned so far about the actual events which occur when bacteria are subjected to its action. As data become available, however, it is reasonable to examine currently held working hypotheses in the light of the new information. At the time of writing, the most widely accepted hypothesis as to the action of penicillin on bacteria holds that the substance acts on dividing cells only, and that cells escaping its action are either materially more resistant to penicillin than the average of the culture or are in an unsuitable physiologic state to be affected. With this hypothesis our experiments with relatively high concentrations of penicillin do not disagree. Destruction of each cell as it began or completed the process of division, or

death of a given percentage of the dividing cells, would produce the observed logarithmic death rate. As the concentration is reduced, however, a lag appears, and at the minimal inhibitory concentration it seems that a full generation is produced before bacterial death begins. Further, although the rate of growth may be lowered during this phase, it does not seem to be lowered significantly. When killing begins, it proceeds logarithmically for the period observed, although at a significantly lower rate than when the cells are exposed to a higher concentration of penicillin.

It might be presumed that the lag period of penicillin action during which there is no demonstrable effect on the bacteria is a period during which a critical concentration of the drug is being built up or the supply of some essential metabolite is being exhausted. On this basis, withdrawal of the drug before the lethal effect is apparent would be expected to allow prompt resumption of growth. In that event, however, a considerable period of time would be required for the bacteria to recover from the damage which had been done them, damage which was not yet evident at the time of removal of the penicillin. It may be important that there seems to be a time-concentration relationship in the production of this effect. Whether the interval between the removal of penicillin from the suspending medium and the resumption of growth is utilized by the bacteria in getting rid of the penicillin, repairing damaged enzyme systems, rebuilding stores of metabolites, or for some other purpose cannot be said at present.

CONCLUSIONS

At low penicillin concentrations, the lethal action of the antibiotic agent on *Staphylococcus* is preceded by a period of unaffected growth. At higher concentration the lethal action appears to begin without lag.

After removal of the bacterial cells to a penicillin-free medium, the effect of penicillin persists for a time. When *Staphylococcus* is exposed to 1 unit of penicillin per ml of culture medium, death of bacteria continues for a time after removal of the penicillin, and after a period of some 3 hours, growth is resumed. At lower concentrations of penicillin, exposure for a comparable time may lead to no deaths, but growth is inhibited for 3 hours after removal of the drug.

REFERENCES

BIGGER, J. W. 1944 Treatment of staphylococcal infections with penicillin by intermittent sterilization. Lancet, **2**, 497–500.

HIRSCH, J. 1944 The velocities of inhibition of bacterial growth by sulfonamide and of the antagonistic effect by *p*-amino-benzoic acid. J. Immunol., **48**, 199–201.

HOBBY, G. L., AND DAWSON, M. H. 1944 Bacteriostatic action of penicillin on hemolytic streptococcus *in vitro*. Proc. Soc. Exptl. Biol. Med., **56**, 178–181.

HOBBY, G. L., MEYER, K., AND CHAFFEE, E. 1942 Observations on the mechanism of action of penicillin. Proc. Soc. Exptl. Biol. Med., **50**, 281–285.

JULIANELLE, L. A., AND WIEGHARD, C. W. 1935 The immunological specificity of staphylococci. I. The occurrence of serological types. J. Exptl. Med., **62**, 11–21.

LEE, S. W., FOLEY, E. J., AND EPSTEIN, J. A. 1944 Mode of action of penicillin. I. Bacterial growth and penicillin activity. J. Bact., **48**, 393–399.

RANTZ, L. A., AND KIRBY, W. M. 1944 The action of penicillin on staphylococcus *in vitro*. J. Immunol., **48**, 335–343.