THE ACTION OF PENICILLIN ON STAPHYLOCOCCUS: FURTHER OBSERVATIONS ON THE EFFECT OF A SHORT EXPOSURE

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When a culture of *Staphylococcus* in the logarithmic period of growth is exposed to penicillin in appropriate concentration, growth of the bacteria is stopped promptly. If within a short time the penicillin is removed, no decrease in population of viable cells occurs, but, after a lag period, growth is resumed (Parker and Marsh, 1946). In earlier work it was shown that this effect occurred even though there was no detectable killing of organisms; it was not a peculiarity of the metabolic state of "persisters" (Bigger, 1944) in the culture. In the experiments to be detailed in this report, the observations have been extended to cover a total of 29 strains of *Staphylococcus* in order to determine whether the phenomenon was unique in the strain first observed or whether it was generally distributed. The strains were isolated from clinical material in a hospital laboratory and exhibited a wide range of penicillin sensitivity.

Source and selection of cultures. In the course of a study of the relation between size of the test inoculum and apparent resistance to penicillin, all strains of *Staphylococcus aureus* isolated from clinical material in the routine laboratory serving Lakeside Hospital were subjected to tests of penicillin sensitivity (Parker, 1946). From the 169 cultures so studied, 29 were chosen, covering the range of sensitivity exhibited by the series.

Test of sensitivity. The sensitivity test used was that described previously (Parker and Marsh, 1946; Parker, 1946). Serial dilutions of penicillin were prepared in nutrient broth; each was inoculated with 0.5 ml of a 10^{-6} dilution of an overnight broth culture. The tubes were incubated overnight, and the tube was then identified which contained just enough penicillin to prevent visible growth of bacteria. The final volume of the test was 1 ml. This was designated as the "penicillin sensitivity (small inoculum)" of the strain. Another test was made simultaneously, which differed only in that the inoculum contained 10,000 times as many organisms, and the inhibitory penicillin concentration identified in this test was designated as "penicillin sensitivity (large inoculum)."

Medium. The medium used was the tryptose broth supplied by the Digestive Ferments Laboratories; the pH after sterilization adjusted to 7.4 to 7.6.

EXPERIMENTAL RESULTS

In the first studies on this subject it was found, as it had been by others (Rantz and Kirby, 1944; Hobby and Dawson, 1944), that at very low concentrations of penicillin there was a definite interval between the time of addition of penicillin

[1] With the technical assistance of Helen Ferguson.

and the first demonstrable decrease in the number of viable bacterial cells, multiplication apparently continuing for a short time in the presence of the low concentration of penicillin. With higher concentrations, multiplication was apparently checked promptly, and replaced by a logarithmic decline in bacterial numbers. In the present experiments the duration of the initial stationary period of the culture on inoculation of an 18-hour culture into a new medium was determined, and also the time after inoculation when the population had increased by about 100-fold. Then, in order to determine the effect of brief exposure to penicillin, a culture was prepared that was in the logarithmic phase of growth and that had a population of about 10^4 viable bacteria per ml. A predetermined amount of penicillin was added. After 15 minutes the bacteria were

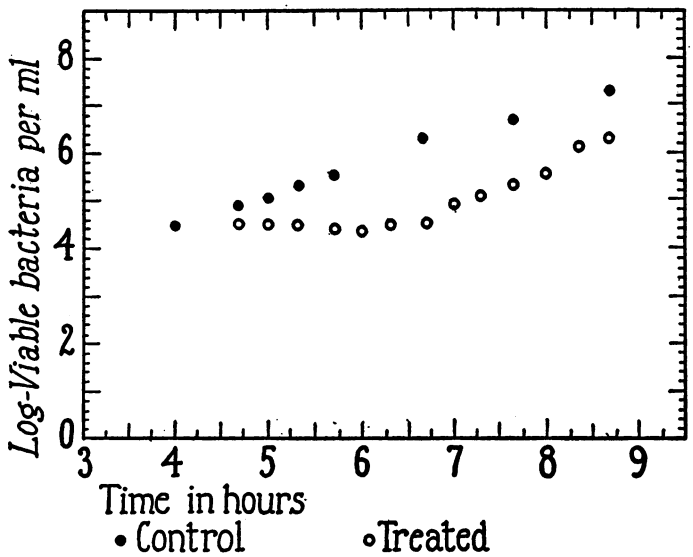

Figure 1. Effect of short exposure of *Staphylococcus* to penicillin. A 4-hour culture, exposed to penicillin for 15 minutes, then centrifuged and cells transferred to penicillin-free broth.

removed to fresh penicillin-free broth, and the culture was observed until growth was resumed. The details of technique were as described below.

To 50 ml of tryptose broth, previously brought to 37 C, enough of an overnight (18-hour) culture was added to give a final count of about 100 cells per ml. One-ml samples were withdrawn at 20-minute intervals thereafter for 6 hours, and plate counts of viable cells were made. From the data so obtained the time was determined at which the culture would be expected to contain 10,000 cells per ml, and a similarly prepared culture of this age was used in subsequent experiments. The exact time varied but was usually about 4 hours from the time of first inoculation of the flask.

The effect on the organism of continuous exposure to penicillin was next determined. To the growing culture (4 hours after inoculation), penicillin was added in a quantity adequate to produce as a final concentration a small multiple of the

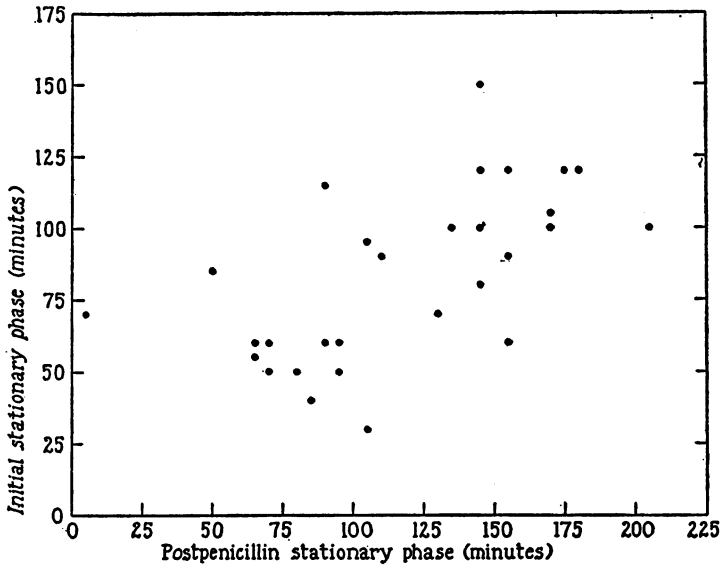

Figure 2. Scatter diagram. The relation between initial stationary phase and postpenicillin stationary phase of growth, both in minutes.

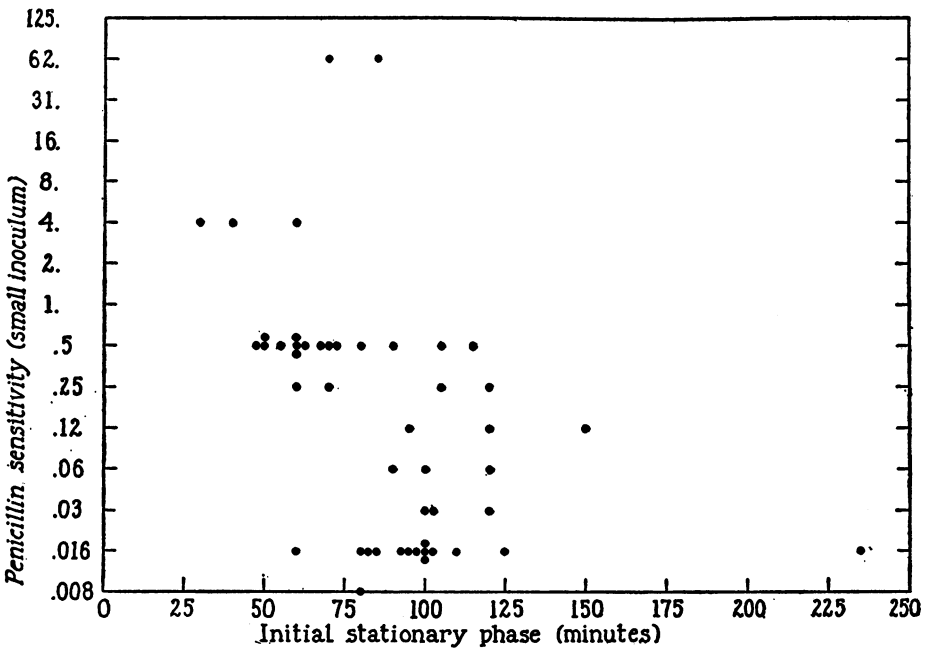

Figure 3. Scatter diagram. The relation between penicillin sensitivity (small inoculum) and initial stationary phase of growth in minutes.

previously determined inhibitory concentration (small inoculum). At 20-minute intervals thereafter aliquot portions were withdrawn, the cells centrifuged down

and resuspended in fresh medium in order to remove the penicillin, and dilutions made for plate counts. The lowest concentration that served to check multiplication promptly, initiating a progressive fall in numbers of viable bacteria, was designated as the "minimal bactericidal concentration" and used in the short exposure experiments.

TABLE 1

Effect of short exposure to penicillin on Staphylococcus

CULTURE NO.	SENSITIVITY, SMALL INOCULUM*	SENSITIVITY, LARGE INOCULUM*	"BACTERICIDAL CONCENTRATIONS"†	INITIAL STATIONARY PERIOD	SECONDARY STATIONARY PERIOD
	units/ml	*units/ml*	*units/ml*	*minutes*	*minutes*
1	0.06	0.06	8	100	145
2	62	250	400	70	5
8	0.25	500	8	105	170
9	0.03	0.03	0.5	120	155
16	0.25	500	8	120	175
25	0.06	0.06	1	120	180
31	0.5	250	32	90	110
37	62	125	400	85	50
48	0.25	2	3	60	155
54	0.016	0.25	0.5	100	205
68	0.5	125	32	115	90
70	0.12	500	2	150	145
76	0.016	0.016	0.25	100	170
81	4	8	250	40	85
85	4	8	250	30	105
91	0.12	500	2	120	145
114	0.5	31	32	80	145
126	0.25	125	32	70	130
136	0.06	8	0.5	90	155
140	0.125	0.125	8	95	105
142	4	8	250	60	95
146	0.016	0.06	0.125	100	135
124	0.5	125	4	50	70
101	0.5	125	4	50	95
107	0.5	125	4	55	65
113	0.5	250	2	60	90
115	0.5	125	2	60	70
116	0.5	250	2	60	65
131	0.5	125	2	50	80

* Sensitivity to penicillin inhibition in overnight test. "Small inoculum" consists of 0.5 ml of 10^{-6} dilution of culture. "Large inoculum" is a 10^{-3} dilution.

† Concentration of penicillin inducing a prompt fall in population when added to a growing culture.

In determining the effect upon *Staphylococcus* of short exposure to penicillin, a culture was prepared as described above. At the predetermined time after inoculation, when the concentration of viable cells would be expected to be about 10,000 per ml, penicillin was added to one portion of the culture, in a quantity sufficient to give the "minimum bactericidal concentration." A second portion was reserved for control. (These manipulations were conducted in a 37 C room.)

After 15 minutes both treated and control cultures were removed from the warm room and centrifuged for 5 minutes, the supernatant was replaced with warm penicillin-free broth, and the cultures were again centrifuged. The supernatant was removed and the sedimented cells were suspended in a volume of warm broth equal to the original and replaced in the 37 C room. At intervals samples were taken from each and plate counts made to determine the number of viable cells.

The results of such an experiment are portrayed graphically in figure 1. It will be seen that the manipulations are almost without effect on the control culture. In contrast, the culture exposed to penicillin enters a second stationary phase of growth which, after a period, is succeeded by a logarithmic period of multiplication. Pertinent data for all of the cultures studied are given in table 1, and diagrams illustrating the correlation of certain values are given in figures 2 and 3.

SUMMARY AND DISCUSSION

The present experiments are seen to confirm and extend the previous observations on the effect of brief exposure of *Staphylococcus* to penicillin. Exposure of a growing culture to an appropriate concentration for 15 minutes followed by removal of the penicillin is consistently followed by a period during which the population of viable cells is constant. This effect is not due to selective killing of dividing cells, leaving resting cells unaffected, for the effect is as apparent when there is no change in the viable cell count after penicillin treatment as when the population is moderately reduced. It was anticipated when the experiments were undertaken that there might be a correlation between the penicillin-induced lag period and the ability of the culture to dispose of penicillin with penicillinase. Other experiments (Gilson and Parker, 1948) have shown that no correlation exists between the duration of the postpenicillin lag period of a culture and its content of penicillinase. There is, however, a high degree of correlation between the duration of the postpenicillin lag period, on the one hand, and both the duration of the initial lag period (figure 2) and the resistance to penicillin (figure 3), on the other. The significance of these associations is not immediately apparent. It is seen from inspection of figure 4, portraying data from a larger number of strains, that a relation exists between the degree of penicillin sensitivity and the duration of the initial lag period. The degree of association between these two values for the 29 strains here reported, which include all of the highly sensitive and all of the highly resistant strains available, is much less than that existing between the penicillin sensitivity and the duration of the postpenicillin dormant period, but is nevertheless significant. The coefficient of correlation is 0.8, indicating that the probability that such an association as is seen here could occur by chance alone is much less than 0.01.

In the absence of complete knowledge of the metabolic basis for the initial lag period, and lacking precise information on the mechanism of action of penicillin, it may be fruitless to speculate on the significance of the lag period that is induced by brief exposure to this substance. Several hypotheses, however, suggest themselves. It may be that penicillin blocks the synthesis of some material essential

for the growth of the cell; Krampitz and Werkman (1947) have suggested that this is ribonucleic acid. If this be so, it may be that while synthesis of this substance is blocked, the metabolism of other materials proceeds, and the supply of some other essential metabolite is exhausted. With release of the penicillin inhibition, time would be required for the resynthesis of this other metabolite before growth could proceed. The time required for a cell to repair such drug-produced metabolic damage might be related to its ability to repair the damage produced by aging, which some believe to be responsible for the initial lag period. It is conceivable also that penicillin combines with or otherwise renders useless some essential metabolite. If this were true, the postpenicillin lag period would be accounted for as the time required to synthesize a new supply of the metabolite in question before growth could proceed. On the other hand, the relation be-

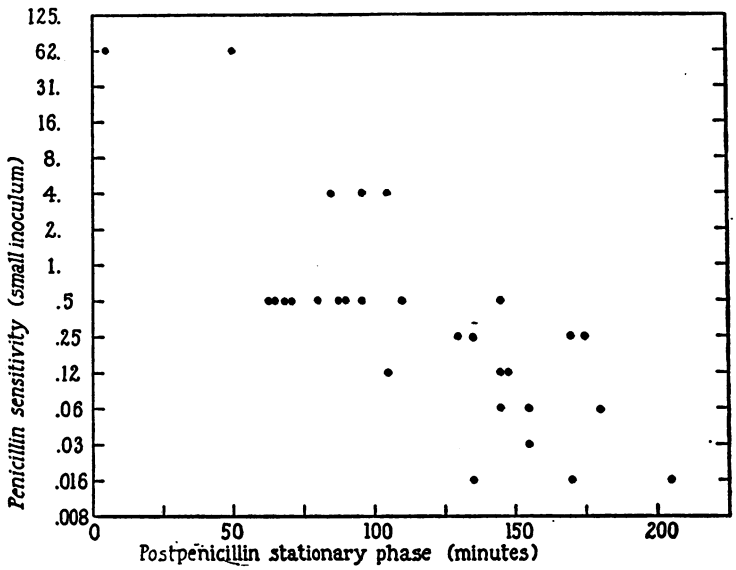

Figure 4. Scatter diagram. The relation between penicillin sensitivity (small inoculum) and postpenicillin stationary phase in minutes.

tween the length of the lag period and the sensitivity of the strain to penicillin suggests that the explanation may be simpler. It has been shown that penicillin in aqueous solution is adsorbed by certain serum proteins, and it is possible that it might be similarly adsorbed by bacterial proteins. Donovick, Lapedes, and Pansy (1947) explain the results obtained by them in testing the effect of mixtures of penicillins as probably due in part to differential avidity of adsorption of the different compounds by the bacterial cell. If it be assumed that such adsorbed penicillin could act to interfere with some essential process such as ribonucleic acid synthesis, it would appear to be not unreasonable to predict that the sensitivity of the organism might vary with the firmness with which penicillin is bound, and that in turn the removal of penicillin on transfer to a penicillin-free medium would have an inverse correlation with sensitivity. The data of figure 4 indicate that among the other factors correlated with the resistance of an organ-

ism to penicillin is the ability of the "resting" or more properly the "aged" cell quickly to become a "growing" cell. Thus a cell in which these adjustments are made slowly is in general more sensitive to penicillin effect than one with a more rapidly acting mechanism. This explanation appears to be consistent with the data recorded above, and experiments designed to test it as well as other hypotheses are now in progress.

These experiments may also have a bearing on the theoretical basis for the practice of chemotherapeutics. It is widely held that best results in chemotherapy with penicillin are achieved when the penicillin concentration is held constant, and many ingenious devices have been invented to facilitate this. The experience of the clinic, however, is witness to the general effectiveness of intermittent injections, often given in doses that allow the concentration of penicillin to fall below theoretical inhibiting levels for part of the time. The data presented may provide a partial explanation. Reference to figure 3 reveals that when staphylococci having a penicillin sensitivity of 0.25 units per ml or less (87 per cent of our strains) are exposed for 15 minutes to a penicillin concentration of the order of 1 unit per ml *and the penicillin then removed*, no bacterial multiplication occurs for (on the average) $2\frac{1}{2}$ hours. Penicillin serum concentrations of this order may be maintained for 15 to 30 minutes after intramuscular injection of 50,000 units. Further, the penicillin is not then immediately removed, but its concentration falls slowly. If bacterial reactions *in vivo* are similar to those we have observed, at least part of the reason for the paradoxical efficiency of intermittent administration of penicillin may be at hand. In this connection it is to be remembered that all of the observations under discussion were made on *Staphylococcus*, and may not be transferable without modification to other genera.

CONCLUSIONS

Multiplication in a young culture of *Staphylococcus*, stopped by brief exposure to an appropriate concentration of penicillin, is resumed after a variable period of incubation in a penicillin-free medium.

The duration of the "postpenicillin stationary phase" is directly related to the initial lag period of growth, and to the penicillin sensitivity of the strain.

REFERENCES

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

DONOVICK, R., LAPEDES, D., AND PANSY, F. 1947 Studies on the quantitative differential analysis of mixtures of several essentially pure penicillin types. J. Bact., **54**, 425–442.

GILSON, BETTY, AND PARKER, R. F. 1948 *Unpublished observations.*

HOBBY, G. L., AND DAWSON, M. H. 1944 Bacteriostatic action of penicillin on hemolytic streptococcus *in vitro*. Proc. Soc. Exptl. Biol. Med., **56**, 78–81.

KRAMPITZ, L. O., AND WERKMAN, C. H. 1947 On the mode of action of penicillin. Arch. Biochem., **12**, 57–67.

PARKER, R. F. 1946 Action of penicillin on *Staphylococcus*. Effect of size of inoculum on the test for sensitivity. Proc. Soc. Exptl. Biol. Med., **63**, 443–446.

PARKER, R. F., AND MARSH, HARRIET C. 1946 The action of penicillin on *Staphylococcus*. J. Bact., **51**, 181–186.

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