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THE BACTERICIDAL ACTION OF PENICILLIN ON STAPHYLOCOCCUS PYOGENES.

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THIS paper records the results of a number of experiments in which Staphylococcus pyogenes was submitted to the action of penicillin, discusses the effect of penicillin on bacteria, advances a hypothesis to explain why such an apparently active bactericidal substance frequently fails to sterilise suspensions of susceptible micro-organisms and makes suggestions for improvement in penicillin therapy.

PART I.

The Killing of Staphylococci by Penicillin.

EXPERIMENT 1.—To 4 c.cs. of broth in a tube were added 1 c.c. of a solution of penicillin containing 1 unit per c.c., and 1 loopful of a broth culture of Staph pyogenes. The broth, which was quite clear after inoculation, was incubated for 24 hours; it was then found to have remained clear. To determine whether this result was due to the killing of the staphylococci which had been added (bactericidal action) or to inhibition of their growth (bacteriostatic action), 1 loopful of the broth was spread on an agar slope both before and after it had been incubated. On the first slope there were present, after incubation, several hundred colonies of staphylococci while, on the second, there was no growth. The first slope showed that the amount of penicillin carried to the agar with the loop was too small to prevent the growth of staphylococci. The second slope proved that 1/5 of a unit of penicillin per c.c. of broth kills staphylococci.

Bacteriologists who still believe the oft-repeated statement that penicillin is bacteriostatic but not bactericidal are recommended to repeat this simple experiment and to demonstrate it to their medical and surgical friends. If it is argued that what happens in broth may not happen in body fluids, the experiment may be repeated using serum in place of broth: the result will be the same.

EXPERIMENT 2.—The object of this experiment was to determine the rate at which different concentrations of penicillin kill staphylococci.

Dilutions of penicillin were made in 5.0 c.c. volumes of broth in tubes. Each tube was inoculated with 1 loopful of a 1 : 60 dilution of a broth culture of Staph. pyogenes and the tubes were incubated. One loopful of each was spread on an agar slope immediately after inoculation and further slopes were similarly inoculated at intervals of 3 hours up to 12 hours and also at 24 hours.

TABLE I.

(EXPERIMENT 2)

Number of colonies growing on agar from 1 loopful of broth (5 c.cs.) incubated for various times with penicillin and Staph. pyogenes.

Tube No.	Penicillin (unit per c.c.)	Time (hours)					
		0	3	6	9	12	24
1	1/4	43	22	1	0	0	0
2	1/8	63	44	1	0	0	0
3	1/16	66	61	16	2	1	0
4	1/32	56	61	91	++	+++	+++
5	1/64	44	56	104	++	+++	+++
6	1/128	79	120	++	+++	+++	+++
7	1/256	54	83	++	+++	+++	+++
8	0	75	105	++	+++	+++	+++

++ = large number of discrete colonies.
+++ = confluent growth.

There is no difference in the killing time of ¼ and ⅛ unit per c.c. but with 1/16 unit per c.c. the time required to reduce a population of 66 per loop to 0 is longer.

The results might be taken to show that penicillin, in concentrations too low to kill (e.g., in tubes 4 and 5), has a bacteriostatic effect; this is possibly true, but an alternative theory deserves consideration. In a bacterial population each of the very large number of individuals present has its particular degree of susceptibility to penicillin. The more susceptible are killed rapidly or by low concentrations, the less susceptible slowly or only by high concentrations. At concentrations of 1/32 and 1/64 unit per c.c., two processes may be occurring simultaneously, viz., dying of the more susceptible individuals and multiplication of the less. The combination of the two is capable, by yielding practically identical living counts at various intervals, of simulating bacteriostasis.

Another factor must also be considered. Broth at 37°C. is destructive of penicillin. The rate of destruction, which varies with different batches of broth even when these are prepared in the same way, is much slower than in serum, but cannot be completely ignored. In tube 4, 1/32 unit per c.c. was just insufficient to kill the staphylococci. At 3 hours it still seemed to have a chance of success, but by 6 hours it had obviously failed. During this time its strength had been decreasing. If it had been maintained at 1/32 unit per c.c. it might just have succeeded.

EXPERIMENT 3.—Tubes of broth (volume 5 c.cs.) containing more closely spaced decreasing concentrations of penicillin (1, 2/3, 1/2, 1/3, 1/4, 1/6, etc., unit per c.c.) were inoculated each with 1 loopful of a broth culture of Staph. pyogenes. After 24 hours' incubation, 1 loopful of each was spread on agar. All agars up to and including the one inoculated with the broth containing 1/24 unit per c.c. were devoid of growth after incubation. That inoculated with the broth containing 1/32 unit per c.c; and later tubes in the series gave extensive growth.

EXPERIMENT 4.—Thirty tubes of broth (6 c.c. volumes) were prepared. Ten contained 1/20 unit of penicillin per c.c., ten 1/25 unit per c.c., and ten 1/30 unit per c.c. All were inoculated with 400,000 Staph. pyogenes per c.c. After 4 days' incubation, none of the tubes containing 1/20 unit per c.c. gave growth on agar from 1 loopful. Of the ten tubes containing 1/25 unit per c.c., 3 gave growth on agar from 1 loop inoculation, 7 did not. Every one of the 10 containing 1/30 unit per c.c. gave growth on agars inoculated with 1 loopful.

EXPERIMENT 5.—This experiment compared the killing effect of concentrations of 2 units and 1/10 unit of penicillin per c.c. in 5 c.cs. of broth.

An initial inoculum of 200,000 Staph. pyogenes per c.c. fell to 6 and 10 colonies per loopful in the 2 and 1/10 unit tubes respectively in 14 hours, and to 0 in both tubes in 16 hours. When the inoculum was 25 millions per c.c., the colonies per loopful were fewer in the 2 unit tube than in the 1/10 unit tube in 12 hours although both were uncountable. In 24 hours the agar count of the 2 unit tube was 0 and of the 1/10 unit tube 5.

Sampling by platinum loop is simple and economical, but the more usual method of colony counting in agar plates inoculated with 1 c.c. of the fluid or of decimal dilutions of it gives information not afforded by the loop method.

EXPERIMENT 6.—Plate counts were performed in this experiment in which 50 c.c. volumes of broth were used.

TABLE II.

(EXPERIMENT 6)

Number of Staph. pyogenes per c.c. surviving contact with penicillin in broth for various times.

Time (hours)	Penicillin	
	1 unit per c.c.	1/8 unit per c.c.
0	55,000	55,000
3	3,600	17,000
6	22	300
9	4	57
12	2	2
24	0	0

Killing was, at first, more rapid in the higher concentration, but sterility in 1 c.c. volumes was attained in 24 hours in both.

The experiments recorded, which are supported by many others essentially similar, justify the conclusion that, when the number of staphylococci is moderate (not exceeding half a million per c.c.), the rate of killing is very little affected by the concentration of penicillin provided that the amount does not, throughout the period of the experiment, fall below a minimum which is about 1/10 unit per c.c. With lower concentrations the rate of killing is slower, and with still lower killing does not occur.

EXPERIMENT 7.—Dilutions of a broth culture of Staph. pyogenes were prepared in broth, the volume in each tube being 5 c.cs. and the concentration of penicillin 1 unit per c.c. The broths were sampled daily, a loopful of each being spread on an agar slope. Sterility in 1 loopful was attained in the tube containing 25 million staphylococci per c.c. in 18 hours, in the 50 million tube in 40 hours, in the 100 million tube in 64 hours, but in the tube containing 200 million per c.c. it was not until incubation had been prolonged for 184 hours that 1 loop sterility was reached. It should, however, be realised that in this last tube the penicillin was, during the period of incubation, decreasing in strength. Sterility would probably have been attained earlier if the concentration of penicillin had been maintained constant at 1 unit per c.c.

The foregoing experiments prove conclusively that penicillin kills staphylococci. Some workers who admit that penicillin is lethal argue

that this is true only of concentrations impracticable of achievement in the human body.

Penicillin intended for treatment is usually assayed by the plate and cylinder method, while the amount in the serum of a patient is estimated by some modification of the slide cell technique. As the available literature did not correlate the two methods, several attempts were made to establish such a correlation. The results were not absolutely constant owing mainly, it is believed, to differences in the rate of destruction of penicillin by different samples of serum. When 10 per cent. normal serum in saline was used as diluent and readings were made after 18 hours' incubation, complete inhibition of growth of Staph. pyogenes was always given by serum containing 1 unit per c.c. in a dilution of 1 :8, almost always in a dilution of 1 :16, and very rarely in a dilution of 1 :32, at which dilution there was usually either partial or no inhibition. In practical work it may be taken, without grave error, that complete inhibition in a dilution of 1 in 16 corresponds to 1 unit per c.c. in the serum, in a dilution of 1 in 8 to ½ unit per c.c., in a dilution of 1 in 4 to ¼ unit per c.c. and so on. Such levels have often been observed in the sera of patients undergoing treatment with penicillin and the experiments recorded have shown many examples of the killing effect of penicillin in concentrations of 1/8 unit, 1/16 unit or even less per c.c.

We must, therefore, abandon the idea that penicillin in the human body merely prevents the multiplication of staphylococci and that their destruction is effected by polymorphonuclear leucocytes. This is undoubtedly the main, if not the only, mode of action of the sulphonamides in concentrations attainable in the body, but penicillin differs from the sulphonamides in that it, unaided by either cellular or humoral immunity, kills the invading bacteria.

PART II.

Sterilisation by Penicillin.

When it was realised that penicillin was capable of killing large numbers of staphylococci, no difficulty was foreseen in sterilising, that is killing all the bacteria in, any given volume of broth to which had been added staphylococci and an adequate amount of penicillin.

Sampling either by spreading loopfuls on agar or by plating 1 c.c. volumes in agar, is not a satisfactory method of judging the sterility of a volume of broth, as a negative result cannot be taken to imply more than the absence of living bacteria in the volume examined. The addition of penicillinase affords a method of stopping the action of penicillin in broth when desired, and of permitting the unrestrained growth of any bacteria which have survived up to the time of the addition.

Pencillinase was obtained by filtering through a Seitz filter a broth culture of one of the coliform bacilli isolated by Harper (1943). Sufficient of the filtrate, which was usually diluted with broth for use, was added to a tube or bottle to neutralise at least five times the amount of penicillin which had originally been present. Culture was continued for at least three days after the addition of penicillinase, and broths

showing turbidity were recorded as positive. In doubtful cases, films and sub-cultures were made to ascertain if the turbidity was due to the presence of staphylococci.

EXPERIMENT 8.—Six bottles of broth containing, in a total volume of 50 c.cs., 1, ½, or ¼ unit of penicillin per c.c. and either 50,000 or 5,000 staphylococci per c.c. were incubated. Samples were removed for testing every three hours for twenty four hours. A loopful was spread on agar and 0·5 c.c. was added to broth containing penicillinase.

TABLE III.

(EXPERIMENT 8)

Progression towards sterility as judged by three methods in 50 c.c. volumes of broth containing Staph. pyogenes and penicillin.

BOTTLE	A	B	C	D	E	F
Staphylococci per c.c.	50,000	50,000	50,000	5,000	5,000	5,000
Penicillin (unit per c.c.)	1	½	¼	1	½	¼
Sterility in 1 loop, attained at (hours)	9	9	9	6	9	12
Sterility in 0·5 c.c. attained at (hours)	21	not at 21	not at 21	15	15	15
Number of tubes + out of 5 containing 1 c.c. amounts to which penicillinase was added at 24 hours	0	2	3	0	0	1
Number of tubes + out of 8 containing 5 c.c. amounts to which penicillinase was added at 24 hours	0	6	8	0	1	1

Table III shows when sterility in one loop and in 0·5 c.c. was attained. At the end of 24 hours the residue (45 c.cs.) in each bottle was dispersed into 13 tubes, 1 c.c. volumes in 5 and 5 c.c volumes in 8. Penicillinase was added to these tubes which were incubated. The number showing turbidity is given in the table.

In this experiment, 1 unit per c.c. of penicillin sterilised 45 c.cs. of broth containing 50,000 staphylococci per c.c. in 24 hours. Lower concentrations failed to achieve sterility. The number of survivors was greater with low concentrations of penicillin than with high and with high densities of bacteria than with low.

EXPERIMENT 9.—In this experiment, the volume tested was fixed at 6 c.cs. and the time of action of penicillin at 48 hours. Four concentrations of penicillin and 8 densities of staphylococci were tested, three tubes being used for each combination. Incubation was continued for 3 days after the addition of penicillinase.

The results given in Table IV show the value of high concentrations of penicillin, particularly when dealing with high densities of staphylococci. Attention is called to the occasional irregular and unexpected results (e.g., F, 1 unit per c.c.; G, ½ unit per c.c.; and F, ¼ unit per c.c.). These are such a recurring feature of all similar experiments, when every effort has been made to avoid errors in the dosage of penicillin and staphylococci, that they cannot, as might at first be suggested, be due to careless work or bad technique.

TABLE IV.

(EXPERIMENT 9)

Three tubes each containing 6 c.cs. of broth with penicillin and Staph. pyogenes tested for the presence of surviving cocci after 48 hours' incubation.

	Number of tubes (out of 3) positive							
Series	A	B	C	D	E	F	G	H
Staphylococci per c.c.	5 million	2·5 million	1 million	500,000	250,000	125,000	50,000	25,000
Penicillin (unit per c.c.)								
1	1	0	0	0	0	1	0	0
½	3	3	1	0	0	0	2	0
¼	3	3	0	0	0	1	0	0
⅛	3	3	3	3	1	2	0	0

EXPERIMENT 10.—This experiment was similar to experiment 9 but covered a restricted range of densities of staphylococci. Five tubes, each containing 6 c.cs. were used for each combination. The period of action of penicillin was 44 hours. The results are given in Table V.

TABLE V.

(EXPERIMENT 10)

Five tubes, each containing 6 c.cs. of broth with penicillin and Staph. pyogenes, tested for the presence of surviving cocci after 48 hours' incubation.

	Number of tubes (out of 5) positive			
Series	A	B	C	D
Staphylococci (millions per c.c.)	10	5	2	1
Penicillin (unit per c.c.)				
1	2	0	0	0
½	2	1	0	0
¼	1	0	0	1
⅛	1	0	0	0

Although the same strain of staphylococcus was used as in experiment 9, sterility was much more commonly attained in this experiment. It was found more difficult to attain sterility when the number of staphylococci was high. Irregular results again occurred. It is highly improbable that either ¼ or ⅛ unit per c.c. is more effective than 1 or ½ unit per c.c., or that it is easier to sterilise 2 million staphylococci per c.c. than 1 million.

EXPERIMENT 11.—This experiment was carried out to determine the maximum density of staphylococci which could be sterilised by penicillin acting for a limited time. A broth culture of Staph. pyogenes was diluted with broth containing penicillin in such a way as to obtain a series of tubes, each with 5 c.cs. total volume, containing 1 unit of penicillin per c.c., and the following densities of staphylococci—120, 60, 30, 15, 7·5, 3·75, and 1·8 millions per c.c. After three days' incubation, penicillinase was added and incubation continued. The first two tubes gave growth but none of the others. In this experiment, 5 c.cs. of broth containing a total of 150 million staphylococci were sterilised by 1 unit per c.c. of penicillin acting for 3 days.

The experiments in tubes had proved that it was possible, by the use of penicillin, to sterilise large numbers of staphylococci in broth.

The possibility of sterilising larger volumes than had been used in the tube experiments was next explored.

EXPERIMENT 12.—Three bottles, each containing 50 c.cs. of broth with ½ unit per c.c. of penicillin and 50,000 Staph. pyogenes per c.c., were sterilised in 72 hours.

EXPERIMENT 13.—Two bottles, each containing 50 c.cs. of broth with 1 unit per c.c. of penicillin and 200,000 Staph. pyogenes per c.c., were sterilised in 48 hours.

As the preliminary experiments were encouraging, experiments on a larger scale were attempted.

EXPERIMENT 14.—Twenty bottles were used. Each contained 50 c.cs. of broth with 1 million Staph. pyogenes per c.c. The initial concentration of penicillin in 10 was 1 unit per c.c. and in 10, ½ unit per c.c. Half the number with each concentration had added to them daily sufficient penicillin to balance the loss which occurs in broth at body temperature. A test of the penicillin content in two of the bottles, on the last day of the experiment, showed that the strength of penicillin had, in this way, been well maintained. One bottle from each series had penicillinase added to it daily.

TABLE VI.

(EXPERIMENT 14)

Attempts to sterilise 50 c.cs. of broth, containing 1 million Staph. pyogenes per c.c., by different concentrations of penicillin acting for various times.

Series	A	B	C	D
Penicillin (unit per c.c.)	1 (Single dose)	1 (maintained)	½ (Single dose)	½ (maintained)
Time of action of penicillin (days)				
1	+	—	+	—
2	+	+	—	+
3	—	+	+	+
4	—	+	+	+
5	+	—	—	—

\+ = growth after addition of penicillinase.
— = no growth after addition of penicillinase.

Prolonged study of the results, which are given in Table VI, failed to reveal any underlying law. Positive results are scattered indiscriminately with no relation to the concentration of penicillin or the duration of its action.

EXPERIMENT 15.—This experiment was planned to study the effect, in 50 c.c. volumes of broth in bottles, of variations in the density of staphylococci, the concentration of penicillin and its time of action.

Four sets of 7 bottles were used. The bottles in two sets contained 5 million staphylococci per c.c. and those in the other two sets 200,000 per c.c. The concentrations of penicillin were 1 unit per c.c. in one set of each density of staphylococci and ½ unit per c.c. in the other.

Penicillinase was added to one bottle of each set after 1, 3 and 4 days' incubation and to 2 bottles of each set after 2 and 5 days' incubation. Incubation was continued for 4 days, when the readings shown in Table VII were recorded.

TABLE VII.

(EXPERIMENT 15)

Result of the action of penicillin in two concentrations on two densities of Staph. pyogenes in 50 c.cs. of broth for various times.

Series	A	B	C	D
Staphylococci per c.c.	5 millions	5 millions	200,000	200,000
Penicillin (unit per c.c.)	1	¼	1	¼
Period of action (days)				
1	+	+	+	+
2	—	+	—	+
2	+	+	—	+
3	+	—	—	+
4	+	+	—	+
5	—	+	+	—
5	+	+	—	+

+ = Growth.
— = Sterility.

This experiment shows the irregularities characteristic of the bactericidal action of penicillin on staphylococci. Divergent results are recorded for the duplicate bottles examined on the second and fifth days. In only 9 of the 28 bottles was sterility attained.

The only deductions which can legitimately be made from these results are that sterility is more likely to be achieved by the action of 1 unit per c.c. than of ¼ unit per c.c. and against 200,000 staphylococci per c.c than against 5 million per c.c.

A number of experiments had suggested that it was more difficult to attain sterility in bottles with 50 c.cs. of broth than in tubes with 10 c.cs.

In order to determine if this was so and, if it was, to investigate the cause, several experiments were performed. Two variables—concentration of penicillin and duration of action—were eliminated by stabilising the former at 1 unit per c.c. and the latter at 72 hours. It was realised that the broth culture which formed the inoculum might also be a variable, but attempts to stabilise it by always using a culture 18 to 20 hours old were not entirely successful, and it is certain that some cultures yielded a higher rate of survivors than others.

It would be tedious to give full details of all the experiments and so the results of 5 have been given in a consolidated table (VIII). In all these experiments, the broth containing penicillin was at air temperature when inoculated. Despite slight difference in technique and the fact that the experiments were performed on different days, it is believed that the results given in the composite table are reasonably comparable.

Before considering the problems which the table presents, two observations of practical importance will be mentioned.

The first of these (experiment 17) dealt with the number of cocci which survived in 50 c.c. volumes of broth in which 250 million Staph. pyogenes were acted upon by 1 unit per c.c. of penicillin for 72 hours. From each bottle, 10 1 c.c. volumes were transferred to separate tubes to which penicillinase was added, as it also was to the residues in the bottles. The

TABLE VIII.

(EXPERIMENTS 16, 17, 18, 19, and 20)

Consolidated results of 5 experiments in which 1 unit of penicillin per c.c. acted for 72 hours on 50 or 10 c.cs. of broth containing various numbers of Staph. pyogenes.

Total number of staphylococci added	50 c.c. volumes					10 c.c. volumes				
	Density (Staphylococci per c.c.)	Number of tests	+	—	% +	Density (Staphylococci per c.c.)	Number of tests	+	—	% +
250 million	5 million	75	69	6	92	25 million	45	26	19	60
50 million	1 million	20	16	4	80	5 million	20	3	17	15
10 million	200,000	15	11	4	73	1 million	20	1	19	5
2 million	40,000	10	5	5	50	200,000	10	0	10	0

number of tubes which developed turbidity enabled the total number of survivors per 50 c.cs. to be calculated. The distribution of the total numbers in the 55 bottles investigated is given in Table IX.

TABLE IX.

(EXPERIMENT 17)

Distribution in 55 tests of the estimated numbers of cocci which survived the action of 1 unit per c.c. of penicillin on 250 million Staph. pyogenes in 50 c.cs. of broth for 72 hours.

Number of 1 c.c. volumes + out of 10 examined	Result in residue (40 c.cs.) of broth	Estimated number of cocci per 50 c.cs.	Number of bottles giving each result
10	+	?	18
9	+	115 ± 47	5
8	+	80 ± 32	0
7	+	60 ± 24	1
6	+	46 ± 19	3
5	+	35 ± 16	3
4	+	26 ± 13	5
3	+	18 ± 10	7
2	+	11 ± 8	1
1	+	5 ± 5	7
0	+	?	1
0	—	0	4
			55

The finding that the action of what is considered to be an adequate concentration of penicillin acting for an adequate time produced sterility in only 7 per cent. of tests is mitigated by the discovery that, in 25 per cent. of tests less than 11 cocci, in 50 per cent. less than 35 cocci, and in 67 per cent. less than 115 cocci, survived out of the 250 million cocci originally present.

The second observation was made in experiments 16, 17, 18, and 19. In previous experiments in which the action of penicillin was stopped by the addition of penicillinase and the tubes or bottles were incubated, it had been observed that not all which ultimately developed turbidity,

indicating lack of sterility, did so after one day's incubation. In some cases turbidity was not observed until the second or, occasionally, the third day. In most experiments, incubation was continued only for 3 or 4 days, when the results were recorded. The bottles used in these four experiments had all originally contained 50 c.cs. of broth in which 1 unit per c.c. of penicillin had acted for 72 hours on various numbers of staphylococci. From some of them samples of 5 or 10 c.cs. had been removed to enable the number of cocci which had survived to be estimated. Penicillinase was added to the broth (50, 45 or 40 c.cs.) in the bottles which were incubated for 14 days, those showing turbidity being recorded daily. The results are given in Table X.

TABLE X.

(EXPERIMENTS 16, 17, 18, 19)

Distribution of 101 bottles according to day on which turbidity was first observed after destruction of penicillin by penicillinase.

Number of days of incubation after addition of penicillinase	Number of bottles first observed to be turbid on each day
1	64
2	29
3	4
4	1
5	1
6	1
7	0
8	0
9	1
10—14	0
	101

Before considering these results it is necessary to point out that the destruction of penicillin by penicillinase requires some hours for its completion and also that, when a very few staphylococci commence to grow in a large volume of broth, a considerable time is necessary before the broth becomes definitely turbid. It is, therefore, probable that, in the majority, if not in all, of the bottles in which turbidity was recorded as being first observed on the second day, that is 48 hours after the addition of the penicillinase, growth had already commenced at the end of 24 hours' incubation when they were first examined and recorded as showing no turbidity.

Two important conclusions are to be drawn from these observations. The first is that, in most cases, growth commenced very soon after the destruction of penicillin. This strongly suggests that, in these cases, the penicillin had acted bacteriostatically and that it was its presence which prevented the occurrence of growth and not dormancy of the surviving cocci.

The second conclusion is that, in a few cases, there was considerable delay before cocci, when freed from the restraining action of penicillin, commenced to multiply. Such a period as 9 days before the development of definite turbidity is the longest recorded, but instances of 6, 7 and 8 days were observed in experiments in tubes.

These conclusions, which are regarded as very important, may be summarised in this way :—

In broth which was not sterilised by penicillin, the majority of the cocci multiplied very soon after the penicillin, which had held them in check, was destroyed, but in some the cocci were in a dormant condition and did not commence to multiply for more than a week.

We may now revert to the problems presented in Table VIII, which may be set out thus :—

1. Why is the proportion of 50 c.c. volumes which remain unsterilised by penicillin almost independent of the number of staphylococci originally present?

2. Why is it more difficult to sterilise a given number of staphylococci in 50 c.cs. of broth than in 10 c.cs.?

It was obvious that the two problems were closely related and might, indeed, be merely two aspects of the same problem.

It appeared that, in the case of 10 c.c. volumes, the main factor in determining the number of survivors and hence the percentage of bottles remaining unsterilised was the number of cocci introduced but that, in the case of 50 c.c. volumes, this factor was overshadowed by others, the nature of which could not even be guessed.

At first it was thought that the factor which decided the survival rate was the density of cocci present, and that 50 c.cs. of broth containing a total of 250 million cocci should be as easily sterilised as 10 c.cs. of broth containing a total of 50 million, since the density in each was 5 million per c.c. Table VIII shows a better (although by no means good) agreement between 50 c.c. and 10 c.c. results when total numbers are used as a basis of comparison, than when densities are compared, and reflection showed that this should have been expected.

The time required to kill staphylococci depends (as experiments 5, 7, and 8 showed) on their density, but if the concentration of penicillin and its time of action are adequate, as they are believed to have been in all the experiments here considerd, densities may be ignored. The factor which controls the success or failure of sterilisation should be the total number of cocci present, as it actually is in the case of 10 c.c. volumes, and would be in the case of 50 c.c. volumes if it were not for the introduction of complicating factors.

The frequent failure to sterilise broth containing moderately large numbers of staphylococci cannot be due to the inability of penicillin to cope with these numbers. As has been shown (Table IX) in the majority of bottles containing 50 c.cs. of broth and a total of 250 million staphylococci, the survivors, which are responsible for lack of sterility, number less than 100. If these cocci survived merely because the penicillin present was insufficient to kill the last 100 organisms out of 250 million originally present, sterility should always be produced with an inoculum of 249 million, but is not, nor is it even when the inoculum is as small as 10 million. The survivors must, therefore, differ in some important respect from those which succumb. The nature of this difference will be dealt with in the next part of the paper.

On the question of the attainment of sterility we are now able to clarify our ideas a little. The only hypothesis which is believed to explain the

facts is this. In a population of staphylococci there are a few cocci—rarely more than 1 in each million—which differ from the majority in their resistance to penicillin, so that they survive a concentration sufficient, in the time used, to kill all the other cocci. If the inoculum is very large, one or more of these cocci is certain to be present in it and sterility will not be attained. As the size of the inoculum is reduced, the proportion of non-sterile broths decreases until only an occasional one fails to attain sterility. These special cocci, which I have called " persisters ", as this term does not imply acceptance of any theory of why they survive as does the name " resister ", are distributed in a random manner in a population of staphylococci. This explains the apparently freakish distribution of positive and negative results in experiments 14 and 15. A possible explanation of the relative lack of success in sterilising a given number of staphylococci in 50 c.cs. as compared with 10 c.cs. was thought to be the development of persisters in the large volume but not in the small, and experiments designed to test this theory and to investigate the factors operating were carried out. Many experiments were performed and many culs-de-sac which it was hoped might be avenues were explored before the theory received experimental support and a factor was revealed. It would be neither profitable nor creditable to describe these experiments which were embarked on hopefully and which now seem rather futile. Signposts which pointed the way were afforded by differences in results obtained in identical preparations in different types and sizes of vessels and also by the effect of different positions in the incubator on the result. These suggested the probability of temperature being a factor. Preliminary experiments confirmed this but only the crucial experiments need be described.

EXPERIMENT 19.—Four sets, each of 10 bottles, containing 50 c.cs. of broth with 1 unit of penicillin per c.c., were used. The inoculum was, in each case, 50 million Staph. pyogenes in broth at 37°. Bottles of set A were incubated before and after the addition of penicillin and were removed from the incubator for the minimum time required for their inoculation. Bottles of set B were kept at air temperature until they had been inoculated with staphylococci, after which they were immediately incubated. Bottles of sets C and D were kept at air temperature for 5 hours after inoculation. They differed only in that penicillin was added to bottles of set C at the beginning of the 5 hours and to those of set D at the end. Immediately after set D had received penicillin, the bottles of the two sets were transferred to a 37° water bath for 15 minutes to warm their contents rapidly and then to the incubator without delay.

The experiment demonstrates convincingly the effect of temperature on the production of persisters. When staphylococci in warm broth are added to warm broth containing penicillin, there are among them a certain number of cocci destined to become persisters and to resist the lethal action of penicillin. When they are added to cold broth containing penicillin, despite the immediate incubation of the bottle, fresh persisters develop during the time required for the temperature of the broth to rise from that of the air to 37°. In this experiment, the time required for bottles of series B to warm must have been much shorter than in previous experiments of this type, as most of the bottles in the incubator were already at 37°, whereas, in other experiments, the incubator was tightly packed with bottles all containing cold broth.

Set C differed from set B only in that the bottles remained at air temperature for 5 hours before being warmed to 37°. During this time there was a considerable further increase in the number of persisters.

TABLE XI.

(EXPERIMENT 19)

Investigation of different factors involved in the sterilisation of 50 c.cs. of broth containing 50 million Staph. pyogenes and 1 unit per c.c. penicillin.

Set		A		B		C		D	
Volume of broth (c.cs.)		50		50		50		50	
Total number of staphylococci added (millions)		50		50		50		50	
Whether broth was hot (H) or cold (C) when inoculated		H		C		C		C	
Whether penicillin (P) or staphylococci (S) were added first		P		P		P		S	
Interval between addition of P and S to broth		1 hr.		1 hr.		1 hr.		—	
Interval between addition of S and P to broth		—		—		—		5 hrs.	
Temperature at which broth kept during interval		37°		air		air		air	
Interval between last addition and incubation		0		0		5 hrs.		0	
Number of bottles + out of 10 used.		6		8		10		10	
	Bottle No.	T	R	T	R	T	R	T	R
Number of tubes (T) + out of 5 each containing 1 c.c. broth and result in residue (R) (45 c.cs.) of broth.	1	0	—	0	—	5	+	5	+
	2	0	+	2	+	0	+	5	+
	3	0	+	0	—	5	+	5	+
	4	0	—	4	+	0	+	5	+
	5	0	—	0	+	5	+	4	+
	6	0	+	5	+	3	+	5	+
	7	0	+	5	+	4	+	5	+
	8	5	+	5	+	2	+	5	+
	9	0	—	0	+	4	+	5	+
	10	5	+	2	+	4	+	5	+

Sets C and D contrasted the effect of the addition of penicillin before and after a period of contact of staphylococci with cold broth. The number of persisters is much larger in set D than in set C and it is believed that this proves that it is the effect of cold broth, and not of penicillin, which is responsible for the great production of new persisters. Penicillin, indeed, seems to interfere with, but not completely to prevent, the development of persisters.

It was thought probable that the effect of chilling on the persister rate might explain the greater number of persisters developing from the same inoculum in 50 c.cs. than in 10 c.cs., because the larger volume requires a longer time in an incubator to reach 37° than the smaller volume. The results in set C (Table XI) show that even a short time is sufficient greatly to increase the number of persisters. The theory was tested in the next experiment which was carried out on the same day and with the same materials as experiment 19.

EXPERIMENT 20.—Three sets of 10 large bottles, each containing 50 c.cs. of broth, and three sets of 10 small bottles, each containing 10 c.cs. of broth, were used. The concentration of penicillin in each was 1 unit per c.c. and the time of action 72 hours. To bottles of the first pair of sets (large and small bottles), were added 250 million staphylococci, to those of the second pair of sets, 50 million, and to those of the third, 10 million. In every case the bottles were incubated before and after the addition of penicillin and were removed from the incubator for the minimum time required to enable the addition of cocci (the dilutions of which were prepared in warm broth) to be made. So, every effort was made to prevent the cocci being chilled.

TABLE XII.

(EXPERIMENT 20)

Comparison of the effect of penicillin acting on Staph. pyogenes in 50 and in 10 c.c. volumes of broth when the inoculated cocci are never chilled.

Set	A	B	C	D	E	F
Volume of broth (c.cs.)	50	10	50	10	50	10
Total number of staphylococci added (millions)	250	250	50	50	10	10
Density (number per c.c.) of staphylococci	5 million	25 million	1 million	5 million	1/5 million	1 million
Number of bottles + out of 10 used	10	9	6	2	3	1

A comparison of the results (Table XII) with those of previous experiments suggests that the broth culture used was particularly rich in persisters, but, despite this, the main aim of the experiment was achieved. As with experiments using the ordinary technique, the proportion of 10 c.c. volumes remaining unsterilised is roughly proportional to the number of cocci introduced, especially if we attribute the one positive bottle in set F to the operations of chance. The agreement between the number of cocci introduced and the number of 50 c.c. volumes remaining unsterilised is less good, but is much better than when the usual technique was employed (Table VIII). In this experiment, the proportion of unsterilised 50 c.c. volumes falls as the number of staphylococci introduced falls although the relationship is not direct.

From these results it may be deduced that chilling is a very important, but not the only, factor responsible for inducing the formation of persisters. It is suggested that mere dilution is a factor, as it is in prolonging the lag phase of bacterial growth, and probability is given to this suggestion by the higher rate of production of persisters in 50 c.c. volumes than in 10 c.c volumes and, in the case of 50 c.c. volumes, with small inocula than large.

We can now attempt to answer the two questions which we propounded. Lack of sterility is due to the presence of persisters. Some of these are in the persister phase when inoculated, but this phase may be induced in ordinary forms by their new environment. If the broth into which they are introduced is cold, fresh persisters develop, the numbers depending more on the duration of exposure to cold broth, than to the number of cocci present. Since 10 c.c. volumes warm more quickly in an incubator than 50 c.c. volumes, fewer persisters develop as the result of exposure to cold in 10 c.c. tubes or bottles than in 50 c.c. bottles. This is certainly not the only factor and it is suggested that another is dilution or dispersal of the cocci in their new environment. Other unknown factors may also be concerned, but more important than the revealing of these is the demonstration that failure to sterilise staphylococci in broth with penicillin is due to the presence of persisters, some of which were present in the inoculum, while some assumed that state as the result of exposure to a new environment.

The next experiment was designed to test if the prolonged action of

penicillin in high concentration could sterilise broth more heavily inoculated with Staph. pyogenes.

EXPERIMENT 22.—Ten identical bottles each contained, in 50 c.cs. of broth, 2 units per c.c. of penicillin and 10 million Staph. pyogenes per c.c. The bottles were incubated for 11 days when penicillinase was added, and incubation was continued for a further 4 days. Growth of staphylococci occurred in 2 of the bottles : 8 remained sterile.

It is probable from this result that, by prolonging the time of action of penicillin, the number of bottles, the contents of which become sterile, can be increased, but even with such a long period as 11 days, 2 of the 10 bottles were not sterilised.

All the experiments so far recorded were carried out with Staph. pyogenes, strain H. (N. C. T. C. No. 6571), the strain used in the School of Pathology, Oxford, for assaying penicillin. To determine if other strains behaved in the same way, eight, all isolated from human lesions, were examined.

It is sufficient to say that penicillin acted on these 8 strains as it did on strain H. In concentrations as low as 1/16 unit per c.c., it was lethal for all, but with them, as with strain H, complete sterility could not be attained with any regularity, although the experiments left the impression that it was less difficult to sterilise these strains with penicillin than strain H. In one experiment (No. 23), in which 4 strains were tested, the volume of broth was 100 c.cs., the concentration of penicillin $\frac{1}{2}$ unit per c.c. and the density of staphylococci 5 millions per c.c. In every case complete sterility was attained in 45 hours.

Serum, a medium more closely resembling the body fluids than broth, was used in tests of the action of penicillin on Staph. pyogenes.

EXPERIMENT 24.—Three bottles containing respectively 1, $\frac{1}{2}$ and $\frac{1}{4}$ unit of penicillin per c.c. and 5 million staphylococci (strain H) per c.c. in a total volume of 50 c.cs. of human serum were incubated for 45 hours. The contents of the bottle were dispersed into tubes in 5 c.c. amounts. To these penicillinase was added, after which they were incubated.

TABLE XIII.

(EXPERIMENT 24)

Number of tubes (5 c.cs.) positive out of 10 and number of cocci surviving out of 250 million present in 50 c.cs. of serum in which penicillin in various concentrations had acted for 45 hours.

Bottle	A	B	C
Penicillin (unit per c.c.)	1	$\frac{1}{2}$	$\frac{1}{4}$
Number of tubes + out of 10	2	4	6
Estimated number of cocci surviving per 50 c.cs.	$2{\cdot}2 \pm 1{\cdot}6$	$5{\cdot}1 \pm 2{\cdot}6$	$9{\cdot}2 \pm 2{\cdot}9$

The number of tubes remaining unsterilised and the number of cocci which survived increased as the concentration of penicillin was reduced.

The next experiment with serum as the culture medium was planned to test if, despite the prolonged action of an adequate dose of penicillin, staphylococci persisted as they did in broth.

EXPERIMENT 25.—Four identical bottles each contained, in a total volume of 50 c.cs. of serum, 1 unit per c.c. of penicillin and 10 million Staph. pyogenes (H) per c.c. To balance the destructive action of serum, the concentration of penicillin was maintained approximately constant by the addition, each morning and evening, of fresh penicillin. Incubation was continued for three days, at the end of which time the contents of each bottle were dispersed, in equal amounts, into 10 tubes which were incubated for a further three days without any addition of penicillin. Penicillinase was then added and the tubes were again incubated.

The ten tubes from each of two bottles remained sterile. One out of each of the ten from the other two bottles gave a growth of staphylococci, 9 in each case remaining sterile.

The many experiments recorded in this part of the paper have shown that penicillin is capable of sterilising a suspension of Staph. pyogenes, either in broth or in serum, but that, when the number of cocci present is large, success is rarer than failure. I attribute this to the presence of a special type of coccus to which I have given the name persister. Persisters are not killed by a concentration of penicillin acting for a sufficient time to kill the great majority of the cocci present.

(*Continued in December issue.*)

THE TREND IN TUBERCULOSIS.

By JOSEPH T. DANIEL.

IT is now almost a platitude to state that the mortality rate of tuberculosis has fallen steadily within the past fifty years. The following figures are so well known that they hardly need reproducing, yet their steady decline gives one a little hope for the future, hope in an ultimate victory over a scourge that has ravaged mankind for long ages past.

Death Rate	Ten year periods ending
4,300	1842
3,500	1855
3,500	1865
3,000	1875
2,600	1885
2,200	1895
1,800	1905
1,500	1915
1,500	1925
950	1935

Deaths, per million living, from all forms of Tuberculosis.—(Registrar-General's Reports, 1842–1935.)

This decline is, of course, noted only in the European white races and the people of North America. Amongst the coloured races, such as those of India, the morbidity and mortality rates are still very high and have changed but little. To what is this decline in mortality due, and how may it be reduced still further, till tuberculosis becomes a legend like the plague? Many reasons are given, such as, a steady decline in the virulence of the causative organism, or a steady increase