

# PENICILLIN: ITS ANTIBACTERIAL EFFECT IN WHOLE BLOOD AND SERUM FOR THE HEMOLYTIC STREPTOCOCCUS AND STAPHYLOCOCCUS AUREUS<sup>1</sup>

By CHARLES H. RAMMELKAMP AND CHESTER S. KEEFER

(From the Evans Memorial, Massachusetts Memorial Hospitals, and the Department of Medicine, Boston University School of Medicine, Boston)

(Received for publication December 28, 1942)

The value of sulfanamide therapy in hemolytic streptococcal infections is now well established. In most instances, staphylococcal infections are much more resistant. In previous communications (1 to 3), it has been demonstrated that by the addition of the sulfonamide compounds to whole defibrinated blood *in vitro* or by their administration to normal subjects, an increase in antistreptococcal and antistaphylococcal activity of the whole blood is produced. In view of certain therapeutic results obtained with the antibiotic agent, penicillin, in the treatment of streptococcal and staphylococcal infections (4), it has seemed desirable to study the effect of penicillin on the bactericidal power of whole defibrinated blood.

## METHODS

The strain of hemolytic streptococcus used in this study was obtained from the blood stream of a patient with erysipelas. It belonged to Lancefield's Group A and was virulent for rabbits (5). The organism was stored on blood agar slants at 5° C. An 18-hour broth culture of this organism was used in all experiments.

When penicillin was added to whole blood *in vitro*, a standard solution containing 100 Florey units per cc., in 0.85 per cent sodium chloride, was used.

The penicillin<sup>2</sup> administered to normal subjects was in the form of the sodium salt and contained about 120 Florey units per mgm. In general, it was administered in a solution of 0.85 per cent sodium chloride, in concentrations of 1000 Florey units per cc. The methods used to investigate the bactericidal power of whole blood and to determine the concentration of penicillin in body fluids were the same as described previously (1, 3, 6). In brief, blood was withdrawn from normal subjects before, and at varying intervals after, the administration of penicillin and was defibrinated. In each of 7 pyrex tubes, 0.5 cc. of the sample to be tested was placed, and 0.1 cc. of various broth dilutions of the 18-hour

culture of hemolytic streptococcus was added. The tubes were then sealed and rotated in an incubator for 24 hours. At the end of this time, they were examined for hemolysis, which indicates full growth of the culture.<sup>3</sup> Those tubes showing no hemolysis were plated out and the colonies were counted.

The technique for determining the bactericidal power of serum was the same as that for whole blood.

## RESULTS

### I. Effect of penicillin on the hemolytic streptococcus

A. When added *in vitro* to whole blood. Known amounts of penicillin were added *in vitro* to samples of defibrinated whole blood obtained from normal subjects. The various concentrations required were made by dilution with a homologous sample of blood. From each dilution, an aliquot was removed and the serum separated by centrifugalization. The various dilutions of blood and serum were then used as media in the bactericidal test.

Table I records the results obtained in one such experiment. Whole blood, containing no penicillin, was able to kill 17 hemolytic streptococci per cc., whereas a similar inoculum in the sample of serum was not killed. This demonstrated the effect of the phagocytic cells in the normal mechanism of bactericidal action. The whole blood containing 0.3 Florey unit of penicillin per cc., and also the serum separated from it, exhibited an amazing antistreptococcal effect. Both the whole blood and the serum killed 17,000,000 organisms per cc. When the concentration was decreased to 0.03 Florey unit per cc., the killing effect of the whole blood was not so great; however, 170,000 organisms were killed. The antibacterial action of the serum appeared to be even greater than that of the whole blood, indicating that the cellular

<sup>1</sup> Supported by a grant from the Johnson Research Foundation, New Brunswick, New Jersey.

<sup>2</sup> The penicillin used in this study was supplied through the courtesy of Dr. George A. Harrop, Squibb Institute for Medical Research, New Brunswick, New Jersey.

<sup>3</sup> Blood showing hemolysis invariably contains from 10<sup>6</sup> to 10<sup>8</sup> organisms per cc.

TABLE I

*Effect on hemolytic streptococci of penicillin added in vitro to whole blood*

Florey units per cc. of whole blood	Culture medium	Dilution of culture						
		10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>	10 <sup>-7</sup>
0	Whole blood	+	+	+	+	+	+	0
	Serum	+	+	+	+	+	+	+
0.3	Whole blood	0	0	0	0	0	0	0
	Serum	0	0	0	0	0	0	0
0.03	Whole blood	+	+	0	200	0	0	0
	Serum	20	200	450	250	50	0	0
0.015	Whole blood	+	+	+	+	+	+	+
	Serum	+	+	+	+	500	0	0
0.003	Whole blood	+	+	+	+	+	+	0
	Serum	+	+	+	+	+	+	+

Explanation: Total number of organisms inoculated into 10<sup>-7</sup> dilution was 17.

0 = no growth. + = complete hemolysis or 10<sup>6</sup> to 10<sup>8</sup> organisms per cc.

Figures = number of organisms per cc. after 24 hours' incubation.

In each instance, the serum used for culture medium was separated from the whole blood sample after the penicillin had been added. It is to be noted that the concentration of penicillin in Tables I and II is expressed as Florey units per cc. of whole blood, which is in contrast to the rest of the experiments.

component is not a necessary part of the killing effect of penicillin in whole blood. A concentration of 0.015 Florey unit of penicillin per cc. of whole blood did not exhibit an increased bactericidal effect, but the serum separated from this sample did show a definite increase in antibacterial action.

This study, then, demonstrated that the addition of penicillin, even in small amounts to whole blood *in vitro*, resulted in a marked increase in the bactericidal power of both the whole blood and the separated serum. That this action was not dependent on phagocytosis was indicated by the fact that the serum displayed as great or even greater antibacterial action than did the whole blood. In general, a concentration of between 0.03 and 0.3 Florey unit per cc. of whole blood was necessary to cause maximal killing of the hemolytic streptococcus.

B. *When administered to normal subjects.* It having been demonstrated that penicillin increased the bactericidal power of whole blood and serum when added *in vitro*, it was next necessary to determine whether similar results could be obtained in man. In numerous experiments following the administration of penicillin by the oral,

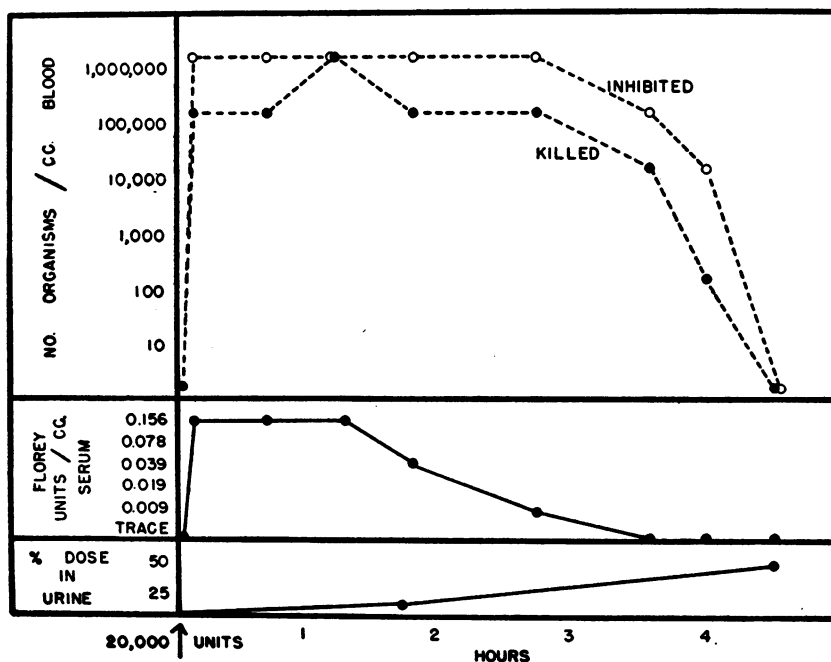


FIG. 1. EFFECT OF BLOOD ON HEMOLYTIC STREPTOCOCCUS AFTER INTRAMUSCULAR PENICILLIN

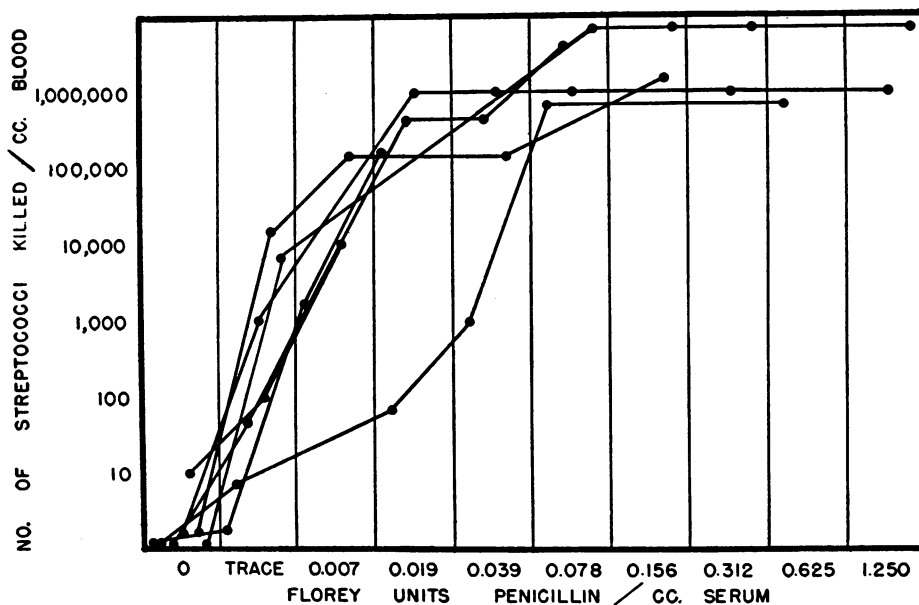


FIG. 2. BACTERICIDAL EFFECT OF WHOLE BLOOD FOLLOWING ADMINISTRATION OF PENICILLIN

intraduodenal, rectal, intravenous, subcutaneous, intramuscular, intra-articular, intrabursal, intrapleural, and intrathecal routes, an increased bactericidal power of the blood was noted.

Figure 1 shows the results obtained after a single intramuscular injection of 20,000 Florey units. Ten minutes after the injection, the blood serum was found to contain 0.156 Florey unit per cc., and a 1 cc. sample of whole blood killed 200,000 hemolytic streptococci. When 2,000,000 organisms were added to 1 cc. of blood, there was a marked inhibition of growth; the actual number of streptococci present after 24 hours' incubation was 40.

For a period of 4 hours after the injection, the whole blood exhibited a marked antistreptococcal effect. It is interesting to note that this effect persisted for at least 25 minutes after penicillin had disappeared from the blood serum.<sup>4</sup> In general, bacteriostasis was of the same order as the bactericidal effect of the whole blood against the hemolytic streptococcus. In this study, as well as in the *in vitro* experiments, the serum exhibited as great or greater bactericidal power than the whole blood.

In Figure 2 are recorded the results obtained after the administration of various amounts of

penicillin. This study was undertaken in order to determine the concentration of penicillin in the serum necessary to maintain maximal bactericidal action against hemolytic streptococci. In all, a total of 7 observations were made on 7 different subjects. The blood obtained before the penicillin was administered killed less than 10 hemolytic streptococci per cc.

As soon as a trace of penicillin was detected, 1 cc. of blood was able to kill from 2 to 20,000 organisms. As the concentration increased, there was a rapid rise in the bactericidal power of the blood, so that maximal action was obtained with concentrations between 0.019 and 0.156 Florey unit per cc. of serum. These studies indicate, then, that concentrations of this order should be maintained in the treatment of hemolytic streptococcal infections.

*C. Comparison of sulfadiazine and penicillin.* Since the foregoing observations indicated that penicillin increased the antistreptococcal action of whole blood, a study of the comparative effect of sulfadiazine and penicillin was undertaken.

In preliminary studies, sulfadiazine was added to whole defibrinated blood *in vitro* so that the final concentration was 3.4 mgm. per 100 cc. This blood was then compared to a homologous sample containing varying amounts of penicillin. The blood containing 0.03 unit or more of peni-

<sup>4</sup> It is impossible to detect quantities smaller than 0.003 Florey unit per cc. of serum (estimated).

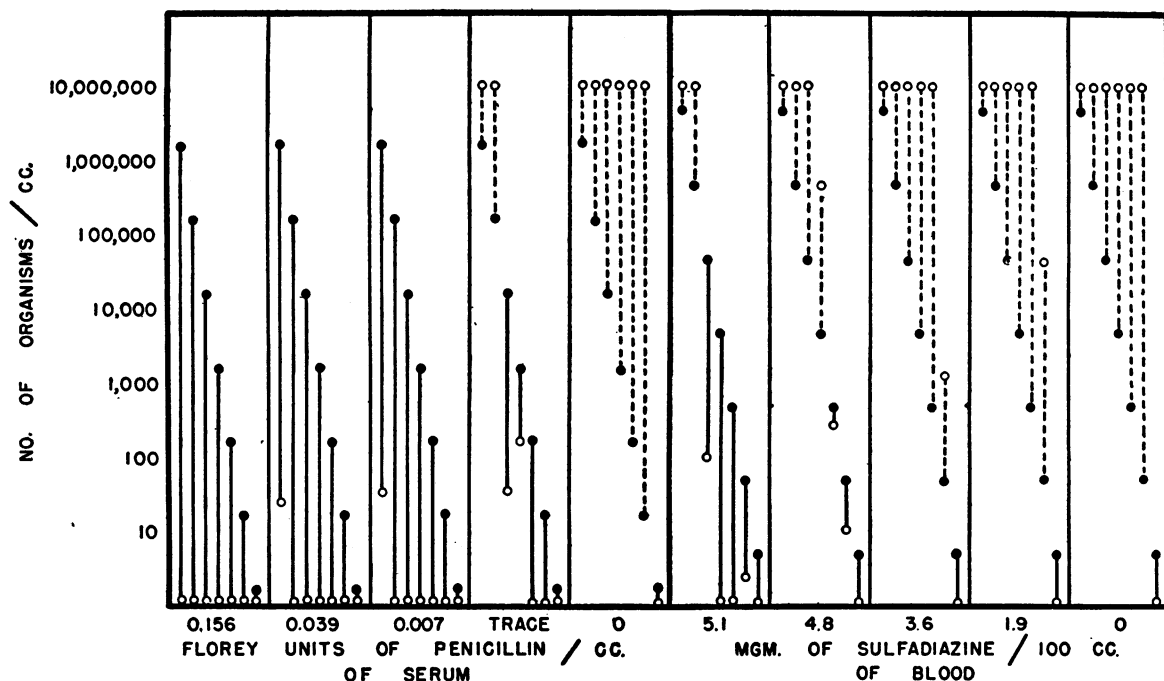


FIG. 3. COMPARATIVE EFFECT OF PENICILLIN AND SULFADIAZINE ON HEMOLYTIC STREPTOCOCCUS

Solid dots represent original inoculum per cc. of *Streptococcus hemolyticus*. Circles represent final number of organisms per cc. at the end of 24 hours incubation. Solid lines indicate killing; broken lines, growth.

illin per cc. of whole blood exhibited a much greater antibacterial effect than did that containing sulfadiazine.

In another experiment, penicillin was administered to a normal subject and samples of blood were withdrawn at various intervals. The bactericidal power of the whole blood and the concentration of penicillin in the serum were determined. Twenty-four hours later, the same subject was given 5 grams of sulfadiazine and the bactericidal power and blood concentrations were determined on several specimens of blood (Figure 3). A concentration of 1.9 mgm. of sulfadiazine per 100 cc. was found to cause only slight inhibition of growth, and it was not until the concentration reached 5.1 mgm. that there was a marked increase in the bactericidal power of the blood.

When the results with the blood obtained after penicillin administration were compared with the antibacterial action of sulfadiazine, the difference was most remarkable. A trace of penicillin in the serum was sufficient to cause a bactericidal effect as great as that produced by 5.1 mgm. of sulfadiazine per 100 cc. of blood. When the concen-

tration of penicillin was greater than 0.007 Florey unit per cc. of serum, killing was observed in all tubes, and complete sterilization occurred with a concentration of 0.156 Florey unit.

These observations, therefore, show that the antibacterial action of whole blood containing 0.007 Florey unit of penicillin per cc. of serum is much greater than that of whole blood containing 5.1 mgm. of sulfadiazine per 100 cc. of blood, both *in vitro* and *in vivo*.

#### COMMENT

It has been demonstrated previously (7) that penicillin has a marked antibacterial effect against the hemolytic streptococcus. In view of the fact that the several preparations of this antibiotic agent vary in potency, it is necessary to express all antibacterial effects in terms of Florey units. We have found, in general, that various strains of hemolytic streptococci are killed by concentrations of 0.0009 and 0.0156 Florey unit per cc. of veal infusion broth (4).

In the treatment of clinical infections caused by the hemolytic streptococcus, it is well to maintain concentrations of at least 0.019 Florey unit per

cc. of serum. In general, it is necessary to administer penicillin, either intramuscularly or intravenously, in doses of 10,000 to 20,000 Florey units every 3 to 4 hours in order to maintain this concentration (8). Such therapy often results in sterilization and dramatic healing of streptococcal infections (4).

## II. Effect of penicillin on *Staphylococcus aureus*

The five strains of *Staphylococcus aureus* used in this study were obtained from subjects with active infections. A 12-hour broth culture was used in all tests. The methods employed were similar to those described above, except that the blood containing the staphylococci was rotated for 48 hours in the incubator instead of 24 hours.

A. *When added in vitro to whole blood.* Penicillin was added *in vitro* to whole defibrinated blood and the proper dilutions were made with a penicillin-free sample of homologous blood. Each dilution was divided into 2 samples, from one of which the serum was separated. The serum and the whole blood were then used in the bactericidal test.

TABLE II

Effect on *Staphylococcus aureus* of penicillin added in vitro to whole blood

Florey units per cc. of whole blood	Culture medium	Dilution of culture						
		10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>	10 <sup>-7</sup>
0	Whole blood Serum	+	+	+	+	+	+	0
		+	+	+	+	+	+	+
3	Whole blood Serum	1000	130	0	0	0	0	0
		0	0	0	0	0	0	0
0.3	Whole blood Serum	340	330	0	0	0	0	0
		0	0	0	0	0	0	0
0.03	Whole blood Serum	+	+	+	1200	230,000	0	0
		+	+	+	+	+	+	+
0.015	Whole blood Serum	+	+	+	+	+	0	0
		+	+	+	+	+	+	+

Total number of organisms inoculated into 10<sup>-7</sup> dilution was 5. For explanation, see Table I.

Table II shows the results obtained in one experiment. The maximum number of organisms killed by 1 cc. of the control sample of blood was 5. The addition of penicillin to the whole blood sample in concentrations of 3 Florey units per cc. increased greatly the bactericidal power of the whole

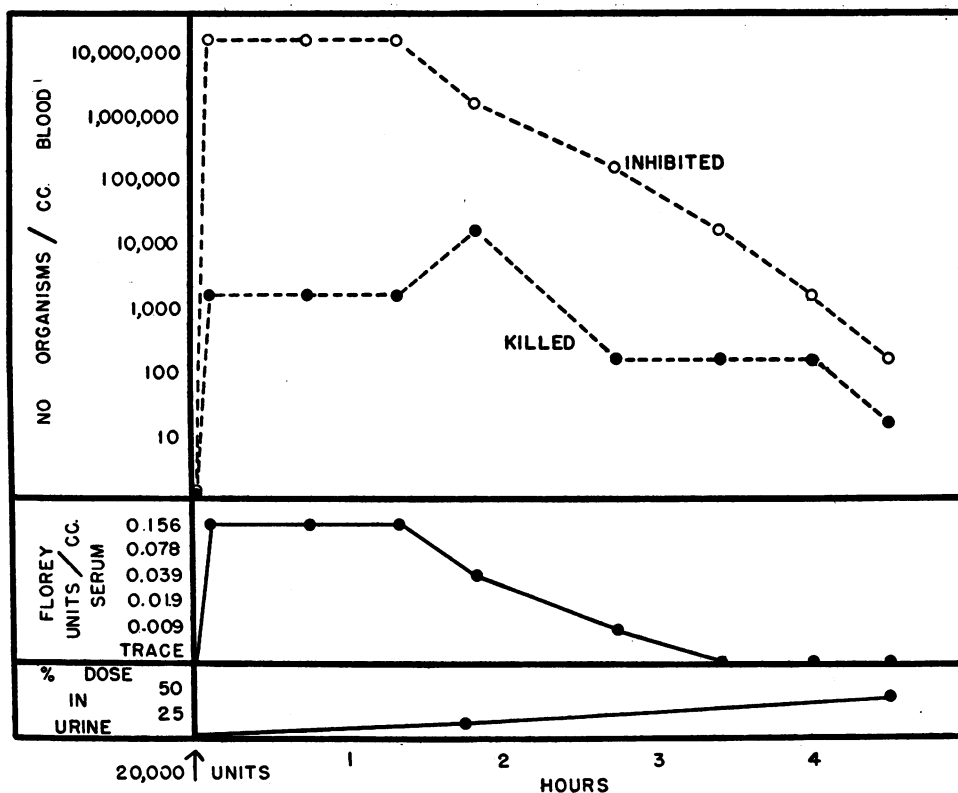
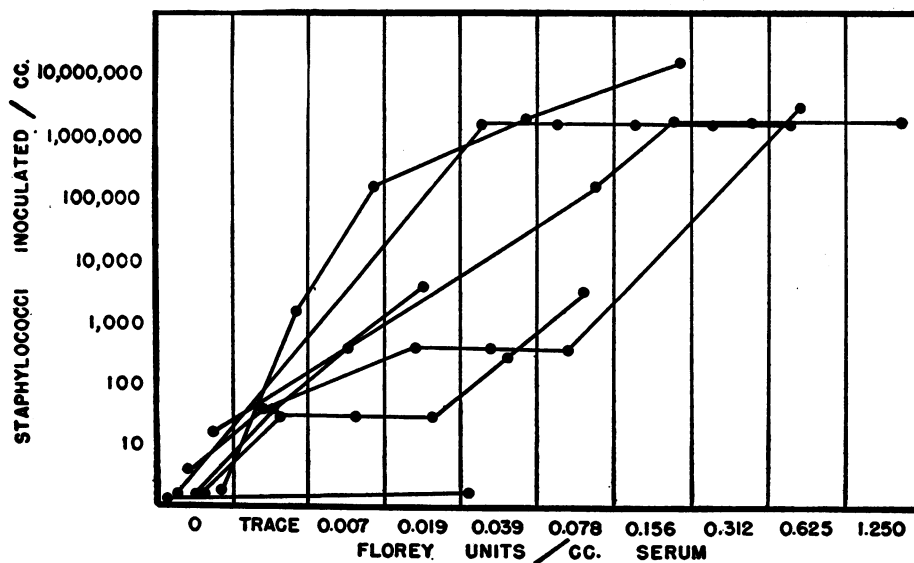
blood and the separated serum sample. Practically identical results were obtained when the concentration was decreased to 0.3 Florey unit. When the concentration in the whole blood reached 0.03 Florey unit, only a slight antibacterial effect was noted; the separated serum displayed somewhat less antistaphylococcal action than did the whole blood.<sup>5</sup>

B. *When administered to normal subjects.* The bactericidal and bacteriostatic properties of the whole blood were studied, after the administration of penicillin, by various routes, to normal subjects. In one subject who received an intramuscular injection of 20,000 Florey units (Figure 4), the maximum concentration of penicillin in the blood serum was 0.156 Florey unit per cc. This level was maintained for 75 minutes, during which time the bactericidal power had increased so that 1 cc. of the blood would kill 2300 staphylococci. Marked inhibition of growth was noted in those tubes containing an inoculum of 23,000,000 organisms per cc., as evidenced by lack of hemolysis. The colony count on the blood from these tubes, however, showed only 1000 to 2000 staphylococci, which indicates an actual bactericidal effect.

As the concentration of penicillin in the serum decreased, there was a steady decline in the bacteriostatic power of the whole blood as shown by degree of hemolysis. The actual killing power decreased also, but here the results were not so regular. The total duration of increased antistaphylococcal action of the whole blood after the injection of 20,000 Florey units was 270 minutes. This was true, even though the concentration of penicillin in the serum was too low to detect during the last 55 minutes of the test period.

It was next necessary to determine the concentration of penicillin required to cause maximal antistaphylococcal activity of the blood. A total of 7 normal subjects received penicillin in this study. The largest inoculum which failed to show hemolysis in the bactericidal test was chosen as an index of penicillin activity against the staphylococcus since, in general, such blood showed less than 1000 organisms per cc. after the 48-hour pe-

<sup>5</sup> In the majority of experiments in normal subjects, the serum separated from the whole blood, in general, displayed a greater antistaphylococcal effect than did the whole blood.

FIG. 4. EFFECT OF BLOOD ON *Staphylococcus aureus* AFTER INTRAMUSCULAR PENICILLINFIG. 5. BACTERIOSTATIC EFFECT OF PENICILLIN ON *Staphylococcus aureus*

Dots represent the inocula rendered bacteriostatic in whole blood following administration of penicillin.

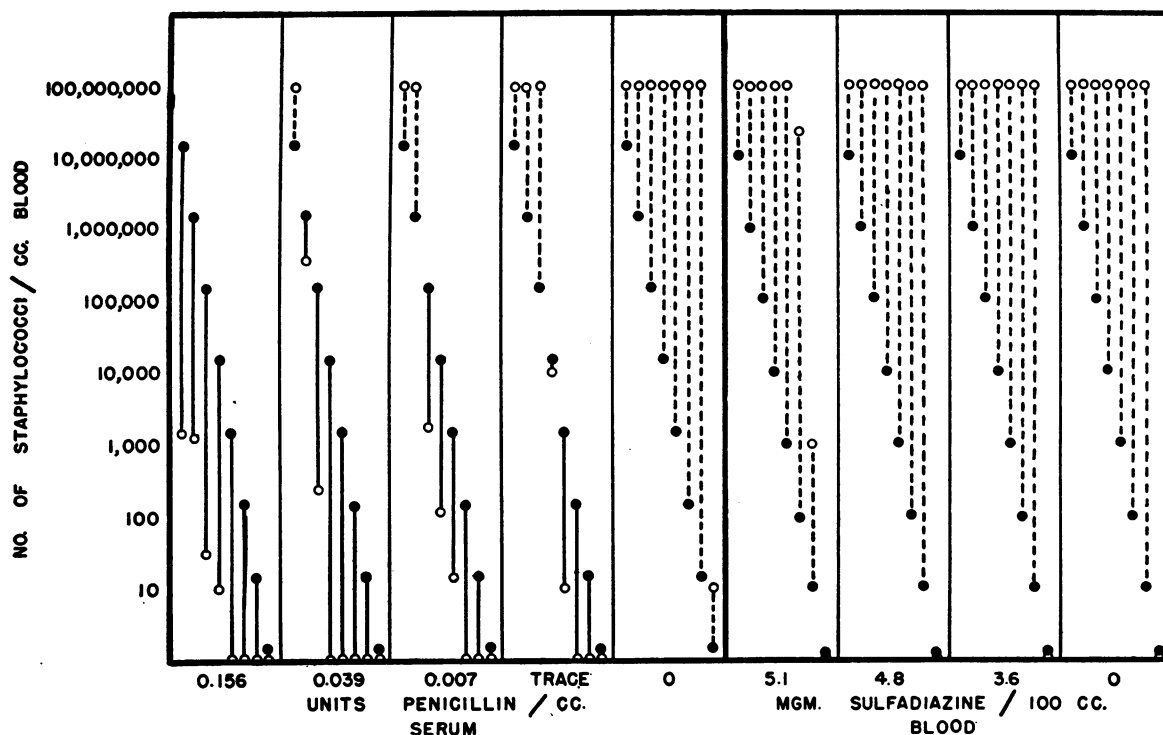


FIG. 6. COMPARATIVE EFFECT OF PENICILLIN AND SULFADIAZINE ON *Staphylococcus aureus*

Solid dots represent original inoculum per cc. of *Staphylococcus aureus*. Circles represent final number of organisms per cc. at the end of 48 hours incubation. Solid lines indicate killing; broken lines growth.

riod of incubation. The sterilization of the blood was not used as the index of activity, since the results were somewhat irregular and confusing, as is indicated by the increased killing effect when the concentration was falling, as observed in the subject illustrated in Figure 4.

As is shown in Figure 5, the presence of a trace of penicillin in the serum of normal subjects increased the bacteriostatic power of the whole blood; as the concentration increased, the bacteriostatic power gradually became greater. In general, the maximal effect was not attained until a concentration of at least 0.156 Florey unit per cc. of serum was reached. Concentrations of this strength increased the bactericidal power so that an inoculum of from 2 to 200,000 organisms was killed by the 3 bloods tested, as shown in Figure 5.

C. *Comparison of penicillin with sulfadiazine.* In one subject, the bactericidal power of the whole blood against *Staphylococcus aureus* was studied after the administration of penicillin, and again, 24 hours later, after the administration of 5 grams of sulfadiazine. As seen in Figure 6, the

comparison showed striking differences. Sulfadiazine exhibited no effect with concentrations of 4.8 mgm. per 100 cc., and only a slight bacteriostatic effect with concentrations of 5.1 mgm. On the other hand, the blood obtained after penicillin administration showed definite killing of staphylococci with only a trace of penicillin, and marked antibacterial action at concentrations of 0.039 and 0.156 Florey unit per cc. of serum. It is to be noted that in the blood containing penicillin, a large inoculum was usually not completely sterilized but that the number of organisms decreased greatly during the period of the test.

#### COMMENT

Although *Staphylococcus aureus* is somewhat more resistant than the hemolytic streptococcus to the action of penicillin, it is, nevertheless, very susceptible. From *in vitro* studies, we have found that the lowest concentration of penicillin required to kill from 1000 to 30,000 organisms of each of 29 strains of *Staphylococcus aureus* is 0.02 to 0.35 Florey unit per cc. of veal infusion broth (9).

It is of some interest that *Staphylococcus albus* is equally susceptible to the action of penicillin (4).

The present studies have demonstrated that the addition of penicillin to whole defibrinated blood *in vitro* results in an increased bactericidal power of the blood and of serum separated from the blood sample. Similar results were obtained when penicillin was administered by various routes to normal subjects and the whole blood and serum were then tested against the staphylococcus. In general, blood containing a large inoculum of *Staphylococcus aureus* was not completely sterilized after 48 hours' incubation; however, the number of organisms was usually greatly decreased. This phenomenon was observed during the course of many bactericidal tests. The explanation of the rapid killing of 96 to 99 per cent of a large inoculum of *Staphylococcus aureus*, and the failure to kill the few remaining organisms, may be either that the staphylococci have ceased to multiply and therefore are not susceptible to the action of penicillin (10), or that the staphylococci have become relatively resistant to penicillin (9).

#### DISCUSSION

The antibiotic substance, penicillin, as shown above, exhibits a marked antistreptococcal and antistaphylococcal effect when added to whole defibrinated blood *in vitro*. Similar results were obtained when the whole blood or serum was tested after the administration of penicillin to normal subjects. The great increase in the antibacterial effect of the blood, noted after penicillin administration, indicates that this substance should prove to be an exceptionally effective agent in the treatment of staphylococcal and hemolytic streptococcal infections in man. In a limited number of patients with infections caused by the streptococcus, pneumococcus, or staphylococcus, treatment with penicillin has shown a dramatic therapeutic effect (4).

In general, blood or serum containing adequate amounts of penicillin killed all the organisms, in even the largest inoculum of hemolytic streptococci. This is in distinct contrast to the action of adequate amounts of penicillin on *Staphylococcus aureus*, since with this organism all the bacteria are not killed on the addition of a large inoculum to blood or serum. This observation may

have an important bearing on the use of penicillin in the treatment of staphylococcal infections in man, and suggests that adequate concentrations of the antibiotic substance should be maintained in the blood stream for a long period of time in order to ensure the complete killing of all staphylococci.

Hobby (10) has shown that penicillin exhibits a greater antibacterial effect than sulfathiazole when tested against the hemolytic streptococcus in broth cultures. The present studies have demonstrated that when penicillin is added to whole defibrinated blood, the antistaphylococcal and antistreptococcal effect is greater than when the sulfonamide drugs are added. Similar results were obtained on testing the blood of normal subjects after the administration of penicillin or sulfadiazine. It is important to point out, however, that the antibacterial action of blood after a single injection of penicillin in man is of relatively short duration, since it is excreted rapidly in the urine and a small amount is destroyed in the body (8, 11). In the treatment of clinical infections, then, penicillin must be given frequently and in adequate doses in order to maintain sufficient concentrations in the body to exert an antibacterial effect (8).

In patients with staphylococcal bacteremia or localized infections caused by *Staphylococcus aureus*, the blood or local lesion may not be sterilized for several days after the institution of penicillin therapy (4). That such an observation should be made might have been predicted from the *in vitro* studies of the effect of penicillin in whole blood on a large inoculum of staphylococci. In view of the fact that Powell and Jamieson (12) and McKee and Rake (13) have demonstrated that sulfonamide-resistant pneumococci are readily susceptible to the action of penicillin, it would appear that the mechanism of antibacterial action of penicillin and that of the sulfonamide compounds are different. This suggests that a combination of sulfathiazole or sulfadiazine with penicillin might prove more effective than either compound alone in the treatment of staphylococcal infections. Preliminary observations in this laboratory indicate that the addition of small amounts of penicillin, which in itself displays no killing effect against staphylococci, will enhance the antistaphylococcal effect of sulfathiazole in whole defibrinated blood.



## SUMMARY

A study was undertaken to show the action of penicillin against the *Streptococcus hemolyticus* and *Staphylococcus aureus* in whole defibrinated blood and serum. The following facts emerged from the study:

The addition of penicillin to whole blood *in vitro* resulted in a marked increase in the antistreptococcal and antistaphylococcal activity. This antibacterial effect was not dependent on phagocytosis, since a similar action was noted in the serum separated from whole blood containing penicillin.

When penicillin was administered to normal subjects by various routes, the blood and serum exhibited a bactericidal and bacteriostatic effect against *Staphylococcus aureus* and *Streptococcus hemolyticus*. In general, the effect was more marked against the streptococcus.

The degree of antibacterial action observed in whole blood after the administration of penicillin was directly related to its concentration in the serum. Maximal bactericidal effects against the hemolytic streptococcus were produced by concentrations of 0.019 to 0.156 Florey unit per cc. of serum. Concentrations of at least 0.156 Florey unit were required for maximum bacteriostatic action against *Staphylococcus aureus*.

The antistaphylococcal and antistreptococcal effect produced by adding sulfathiazole or sulfadiazine to whole blood *in vitro* was less marked than when penicillin was added to a homologous sample of blood. Similar results were obtained when the antibacterial action of blood was tested after the administration of sulfadiazine and penicillin to normal subjects.

Miss Marjorie Jewell and Miss Thelma Maxon gave valuable assistance during the course of this study.

## BIBLIOGRAPHY

1. Rammelkamp, C. H., and Keefer, C. S., Sulfathiazole: Effect on *Staphylococcus aureus in vitro*. Proc. Soc. Exper. Biol. and Med., 1940, 43, 664.
2. Rammelkamp, C. H., and Keefer, C. S., Sulfathiazole therapy of *Staphylococcus aureus* bacteremia. New Eng. J. Med., 1940, 223, 877.
3. Keefer, C. S., and Rammelkamp, C. H. Sulfadiazine: A study of its effect on hemolytic streptococci. Tr. A. Am. Physicians, 1941, 56, 165.
4. Rammelkamp, C. H., and Keefer, C. S., Unpublished observations.
5. Rammelkamp, C. H., Tyrothricin therapy of experimental hemolytic streptococcal empyema. J. Infect. Dis., 1942, 71, 40.
6. Rammelkamp, C. H., A method for determining the concentration of penicillin in body fluids and exudates. Proc. Soc. Exper. Biol. and Med., 1942, 51, 95.
7. Florey, H. W., *et al.*, Further observations on penicillin. Lancet, 1941, 2, 177.
8. Rammelkamp, C. H., and Keefer, C. S., The absorption, excretion and distribution of penicillin. J. Clin. Invest., 1943, 22, 425.
9. Rammelkamp, C. H., and Maxon, T., Resistance of *Staphylococcus aureus* to the action of penicillin. Proc. Soc. Exper. Biol. and Med., In press.
10. Hobby, G. L., Meyer, K., and Chaffee, E., Observations on the mechanism of action of penicillin. Proc. Soc. Exper. Biol. and Med., 1942, 50, 281.
11. Rammelkamp, C. H., and Bradley, S., Excretion of penicillin in man. Proc. Soc. Exper. Biol. and Med., 1943, 53, 30.
12. Powell, H. M., and Jamieson, W. A., Response of sulfonamide-fast pneumococci to penicillin. Proc. Soc. Exper. Biol. and Med., 1942, 49, 387.
13. McKee, C. M., and Rake, G., Activity of penicillin against strains of pneumococci resistant to sulfonamide drugs. Proc. Soc. Exper. Biol. and Med., 1942, 51, 275.