STUDIES ON PENICILLIN IN BLOOD

I. The Effects of Serum and Serum Filtrates upon the Growth of Staphylococcus aureus

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At the present time there exists no satisfactory procedure suited to routine application for the quantitative determination of penicillin in blood. The numerous methods which have been described for the assay of penicillin in body fluids are generally deficient in two respects: (1) visual estimation of inhibition is relied upon, and whether tube or serial dilutions are used this procedure results in considerable error (25 to 100 per cent); and (2) since the unknowns are compared with a standard which contains no serum, the questionable assumption that the two series are directly comparable is necessary. For most clinical procedures, these features are not serious objections. In certain instances, however, a quantitative procedure would be very useful. A case in point is the study of the relative values of various modes of penicillin administration.

The usual plate or cup methods of penicillin assays are generally inapplicable to this problem. The amounts of penicillin required to produce inhibition in the former, or measurable zones in the latter, are relatively high, considerably in excess of those known to be therapeutically effective. Recently, however, Cooke (1945) has described a modification of the plate method suitable for application to blood. This method is, however, only semiquantitative.

A number of broth methods have been proposed (Rammelkamp, 1942; Rosenblatt *et al.*, 1944; Kirby and Rantz, 1944; Fleming, 1945). In all of these methods, the concentration of penicillin is determined by estimation of the amount of fluid required to inhibit either the growth or the hemolytic activity of a hemolytic streptococcus.

Kirby and Rantz (1944) have suggested a modification of the Foster (1942) turbidimetric procedure for the determination of penicillin in serum. Nonspecific growth stimulation is avoided by the use of complementary volumes of the patient's serum, obtained prior to penicillin administration. The latter requirement seriously limits the application of the procedure described.

In attempting to devise a satisfactory turbidimetric assay for penicillin in blood, a considerable body of information relating to the effect of serum, plasma, and their globulin-free filtrates upon the growth in broth culture of *Staphylococcus aureus* has been obtained. This paper contains a summary of these data, together with a consideration of their relationship to the problem of such an assay.

EXPERIMENTAL METHODS AND RESULTS

In some of the earlier experiments, 1.0-ml volumes of the solutions to be tested, suitably diluted when necessary, were mixed with 5.0 ml of broth. Later it was

found more convenient to reduce the total volume to 3.5 ml, using 0.5 ml of the material being tested. The tubes were dry-sterilized before use, and the components were added aseptically. For most of the runs reported here, the sterile medium was mixed with 2 per cent by volume of an overnight (12 to 20 hours) culture immediately before use, although dropwise addition of the inoculum to each tube was also satisfactory. After mixing, the tubes were immersed in a water bath at 37.0 ± 0.1 C. At the end of the specified time interval, the racks were removed, and one drop of formalin was added to each tube to stop further growth. Growth levels were then measured with a Klett-Summerson photoelectric colorimeter, using the red (no. 66) filter. This was chosen in preference to the green (no. 54) filter despite the fact that somewhat higher readings were obtained with the latter. The instrument appears to be more sensitive at the longer wave length, and the red filter also minimizes the corrections which must be applied when hemolyzed blood samples are used. The instrument is set at zero with an uninoculated sample prepared at the same time as the other solutions. (This also provides a check upon the sterility of the solutions.)

The first penicillin assays made in this laboratory, done in connection with studies on the diffusion constant (Frieden, 1945), employed as the test organism a strain of *Staphylocccus aureus* H supplied by Mr. C. E. Lankford. Using a modified proteose peptone medium (yeast extract and glucose added), satisfactory growth levels were reached in 7 hours. One-half maximum inhibition was routinely observed at penicillin concentrations of 0.016 to 0.020 Oxford units per ml. Repeated checks upon the precision and accuracy of the method indicated that both were within 5 to 10 per cent.

For high concentrations of penicillin in blood (> 1.0 unit per ml) the turbidimetric assay of Folin-Wu (tungstic acid) filtrates is very satisfactory. Provided that care is taken to ensure a neutral filtrate, the filtrate alone is without effect upon the growth of staphylococcus under the conditions employed. Recoveries of added penicillin (from either serum, plasma, or whole blood) range from 85 to 100 per cent, depending upon the concentration. Apparently some penicillin is lost by adsorption. The loss is rather constant and is calculable. As might be expected, recoveries from plasma or serum are higher than from whole blood.

Inasmuch as the preparation of a protein-free filtrate involves a tenfold dilution of the sample, that procedure is useless except for certain specialized research problems. It is, of course, inapplicable to routine blood level studies. Attempts to prepare protein-free filtrates in such a way as to avoid high dilutions failed; in each case very high losses occurred.

Attention was therefore directed to the use of whole plasma or serum. For a turbidimetric assay, a coagulase-negative organism is required. A culture of the Oxford strain of *Staphylcoccus aureus* was obtained from Professor C. M. Pomerat. Besides being coagulase-negative,[1] this strain has the added advantage of high

[1] It has been observed that a coagulaselike action is obtained with samples of serum or plasma which have been stored for some time, although fresh samples are unaffected. Repeated subculturings of the parent strain do not change the sensitivity to penicillin although pigment production is diminished.

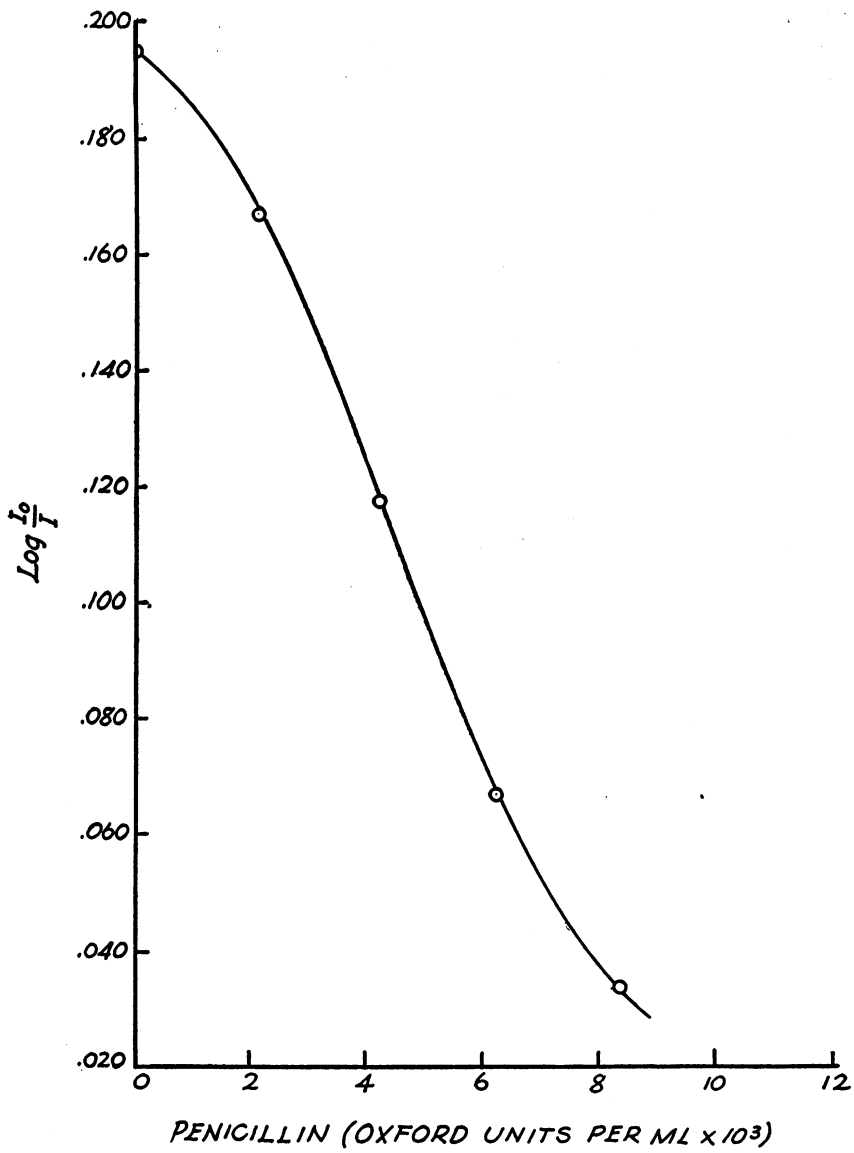

FIG. 1. EFFECT OF PENICILLIN UPON THE GROWTH OF STAPHYLOCOCCUS AUREUS (OXFORD STRAIN)

Medium: 2 per cent proteose peptone no. 3, 0.5 per cent yeast extract, 0.5 per cent glucose, 0.5 per cent NaCl, and 0.5 per cent Na_2HPO_4. Incubation for 6 hours at 37 C.

sensitivity to penicillin. Figure 1 represents a typical standard curve obtained after 7 hours' incubation in proteose peptone medium with added yeast extract and glucose.[2]

[2] Foster and Wilker (1943) have reported that irregularities appear in penicillin inhibition curves when glucose is added to the basal medium. No difficulties of this kind have been encountered in the work described here.

Kirby and Rantz (1944) have reported that the addition of serum to brain heart infusion cultures of a hemolytic streptococcus causes very marked nonspecific growth stimulation. We have investigated the effects of serum and plasma addition upon staphylococcus growth in a series of media. Typical results are shown in figure 2.

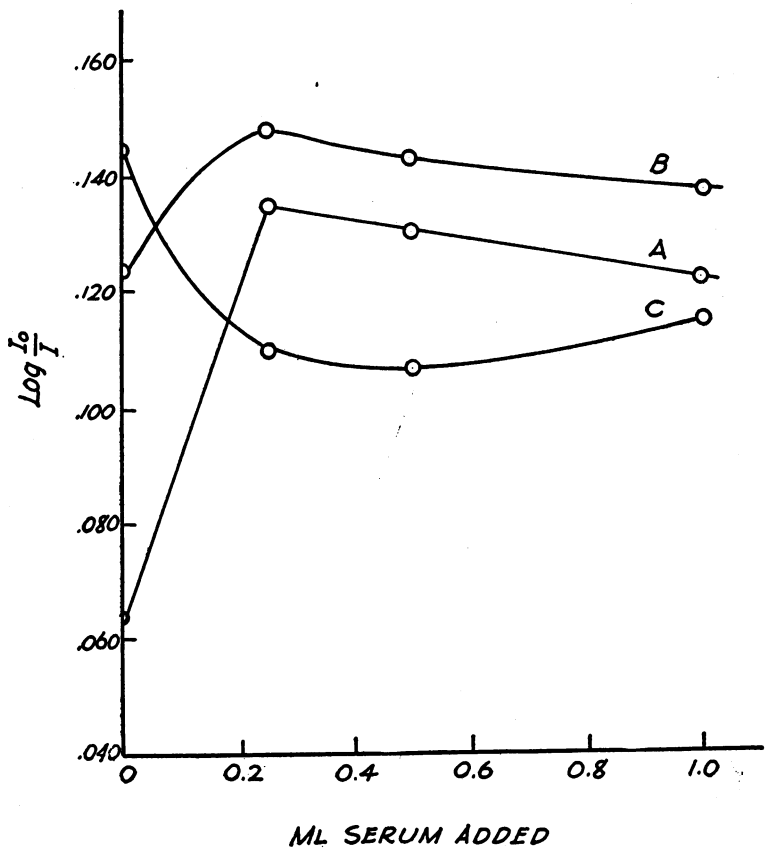

FIG. 2. EFFECT OF ADDED SERUM UPON GROWTH OF STAPHYLOCOCCUS AUREUS (OXFORD STRAIN) IN VARIOUS MEDIA

A: 2 per cent nutrient broth, 0.5 per cent yeast extract, and 0.5 per cent glucose. B: 2 per cent proteose peptone no. 3, 0.5 per cent yeast extract, and 0.5 per cent glucose. C: 4 per cent proteose peptone no. 3, 1 per cent yeast extract, and 1 per cent glucose. Incubation for 7 hours at 37 C.

It may be observed that, although stimulation occurs when relatively poor media are used, this effect is eliminated by the use of a richer substrate. Whereas sufficient enrichment can be afforded by increasing the concentration of the basal ingredients, similar results are also obtained by the addition of a hydrolyzed whole blood or plasma supplement to the medium used (curve B, figure 2).

An interesting feature of the data of figure 2 is the fact that an actual inhibition of growth occurs in rich media when even small amounts of serum are added. A number of experiments have indicated that all samples of human serum show

more or less inhibition, although considerable variation is observed.[3] Destruction of the complement by heating the serum at 56 C for one-half hour has no effect upon the inhibition phenomenon. The use of brain heart infusion broth instead of proteose peptone resulted in less abundant growth, without changing the essential effect of serum addition. In the hope that a sufficiently high plasma concentration would minimize the effect of serum addition, a supplemented medium containing aseptically added sterile plasma was tried. Aside from a general depression of growth, the general characteristics of the curves were retained.

A characteristic feature of the growth of staphylococcus in the presence of serum or plasma is the pronounced flocculation of the organism that is apparent after about 6 hours. This flocculation is easily distinguishable from the coagulation occurring with other strains, as well as the slight sedimentation which sometimes occurs in tubes containing an abundant growth in the absence of plasma. It seemed reasonable to suppose that the flocculation was associated with the presence of agglutinins, and also likely that the latter were perhaps concerned in the apparent growth inhibition. Removal of the agglutinating fraction might therefore be expected to eliminate the inhibition and possibly yield more consistent results. Since agglutinins are known to be part of the globulin fraction, the effect of precipitation of serum and plasma with one-half saturated ammonium sulfate was investigated.

It was first necessary to establish that adequate growth of the organism would occur in the presence of ammonium sulfate, and also that the salt concentrations used would not seriously affect the sensitivity to penicillin. Preliminary experiments indicated that in 6 to 7 hours a satisfactory growth level was reached in the presence of fairly high concentrations (up to 5 per cent) of ammonium sulfate. The addition of 0.5 ml of one-half saturated ammonium sulfate to 3.0 ml of medium results in a final concentration of about 4.1 per cent. No significant difference in sensitivity of the organism is apparent in the presence of this concentration of the salt.

Another matter requiring investigation is the effect of ammonium sulfate solutions and plasma or serum filtrates upon the pH of the medium, since significant pH differences might render invalid a comparison of solutions containing filtrates and those containing penicillin in ammonium sulfate alone. Reference to table 1 shows that nearly identical final pH values are obtained, although the pH of one-half saturated ammonium sulfate is considerably lower than that of the plasma filtrate. The medium thus appears to have a very substantial buffering capacity.

Figure 3 shows the effects upon growth levels observed with plasma filtrates prepared with different ammonium sulfate concentrations. In each case the filtrate was prepared by adding to 4.0 ml of plasma the quantity of saturated

[3] Similar experiments with rabbit serum have indicated that whereas inhibition similar to that described above sometimes occurs, the effect is rarely as pronounced as that obtained with human samples.

ammonium sulfate calculated to give the designated final concentration. After centrifugation, the supernatants were used in the quantities indicated. To each

TABLE 1

Effect of ammonium sulfate solutions upon the pH of the medium

SOLUTION	pH
1. Undiluted medium (4% proteose peptone, 1% yeast, 1% glucose)	6.90
2. Plasma filtrate (prepared by adding 3.0 ml satd. $(NH_4)_2SO_4$ to 3.0 ml plasma and filtering)	6.46
3. One-half saturated $(NH_4)_2SO_4$	3.76
4. 3.0 ml medium plus 0.5 soln. 3	6.66
5. 3.0 ml medium plus 0.5 soln. 2	6.68

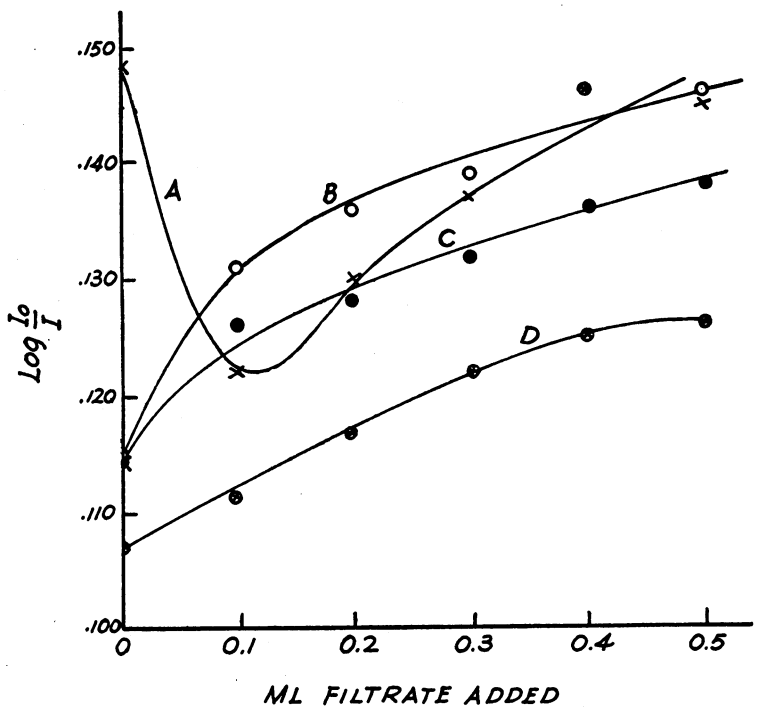

FIG. 3. EFFECTS OF SERUM FILTRATES PREPARED IN DIFFERENT WAYS UPON THE GROWTH OF STAPHYLOCOCCUS AUREUS (OXFORD STRAIN)

A: Precipitated with 0.25 satd. $(NH_4)_2SO_4$. B: Precipitated with 0.33 satd. $(NH_4)_2SO_4$. C: Precipitated with 0.43 satd. $(NH_4)_2SO_4$. D: Precipitated with 0.50 satd. $(NH_4)_2SO_4$. Medium: 2 per cent proteose peptone no. 3, 0.5 per cent yeast extract, 0.5 per cent glucose and 0.5 per cent Na_2HPO_4. Incubation for 6 hours at 37 C.

solution was added sufficient ammonium sulfate to maintain a constant salt concentration for each set of experiments.

It may be observed that the inhibition of growth persists in the presence of 0.25 saturated salt, but disappears when higher concentrations are used, being replaced by a definite stimulation. The disappearance of the inhibition phe-

nomenon occurs simultaneously with the disappearance of the flocculation mentioned previously.

In subsequent investigations of the effects of filtrates, the following procedure was used: To a known volume of plasma or serum was added an equivalent volume of saturated ammonium sulfate. The solutions were mixed thoroughly and

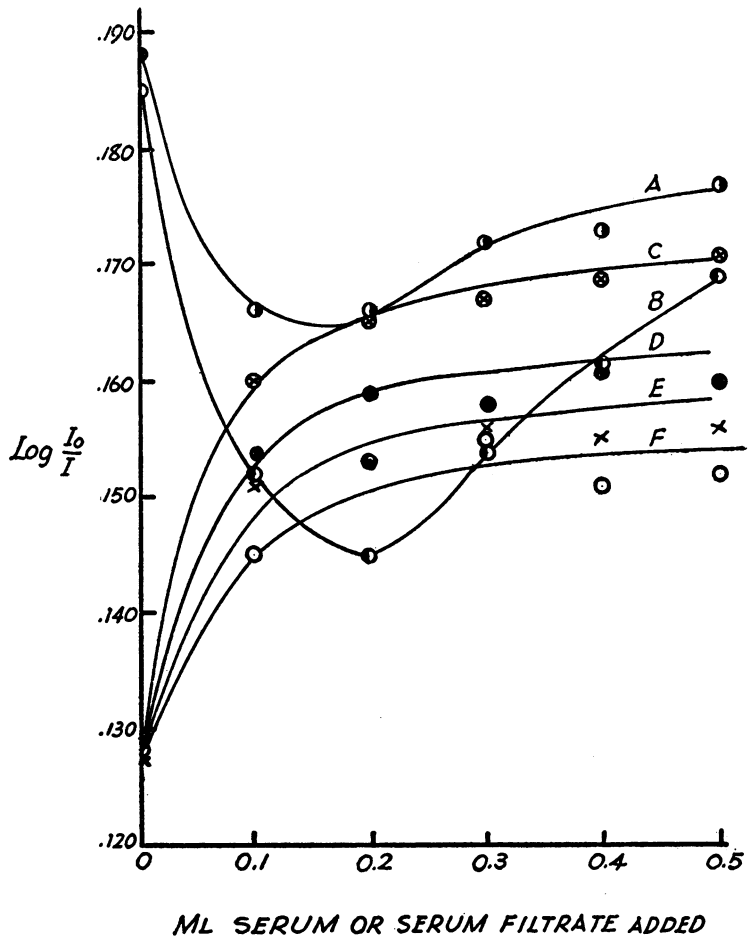

FIG. 4. EFFECTS OF TREATED AND UNTREATED SERA UPON THE GROWTH OF STAPHYLOCOCCUS AUREUS (OXFORD STRAIN)

A and B: Untreated sera nos. 1 and 2, respectively. C, D, E, F: Sera nos. 3, 4, 2, and 1 (precipitated with half-saturated $(NH_4)_2SO_4$). Medium: 4 per cent proteose peptone no. 3, 1 per cent yeast extract, 1 per cent glucose, and 1 per cent Na_2HPO_4. Incubation for 6 hours at 37 C.

the precipitate separated by centrifugation. The effect of the supernatant upon the growth of staphylococcus was studied by adding 0.5 ml or less to each tube. Complementary volumes of one-half saturated ammonium sulfate were added to all tubes to maintain a constant salt concentration.

Figure 4 shows the effect of ammonium sulfate precipitation upon five different sera. The curves for two untreated sera are included for comparison.

The data offer evidence that serum or plasma filtrates prepared as described contain an accessory growth factor (or factors) which leads, in 6 to 7 hours, to an increase of growth over that of controls. The use of a medium supplemented with plasma hydrolyzate did not eliminate the difference. Experiments in which the various components of the medium were varied singly and in combination were likewise unsuccessful in eliminating the difference. Increasing the proteose peptone concentration to 6 per cent uniformly increased the levels of growth, without affecting the stimulatory effect of filtrates. When the yeast extract concentration was raised beyond 1 per cent, repression of growth was observed for all samples. Changes in glucose concentration affected the curves in like manner; 4 per cent glucose was definitely repressive.

The possibility exists that globulin precipitation introduces a change in ammonium sulfate concentration by preferential salt adsorption. Since, in preparing the solutions for study, the assumption is made that the supernatant is one-half saturated in ammonium sulfate, the apparent stimulation might actually be a reflection of changes in salt concentration. This possibility appears unlikely in view of the fact that the stimulation produced by 0.1 ml of filtrate is nearly as great as that produced by five times this volume. Appropriate experiments indicated that in order to show an effect of the magnitude of that observed, the salt concentration would have to be reduced to 60 per cent of its original value, a possibility which can be neglected.

Figure 5 represents the results of an experiment performed to determine the effect of an extended incubation period upon the observed stimulation. The upper curve (A) represents the rate of growth of staphylococcus in the presence of 0.5 ml of plasma filtrate. The lower curve (B) represents the rate when the filtrate is replaced by an equal volume of half-saturated ammonium sulfate. It is evident that the accessory factor present in filtrates makes its presence felt primarily in the lag phase. More important for present considerations, however, is the fact that the two curves coincide after about 10 hours. Accordingly, the effects of serum filtrates were determined by using a growth period of 12 hours. Within limits of experimental error, the final growth levels reached during this period are independent of filtrate volume, and have been observed to be the same for a considerable number of filtrates from different human sources.

It is apparent, provided no other effects are involved, that these observations offer a means of directly comparing penicillin concentrations in plasma or serum filtrates with standards prepared in ammonium sulfate solutions. To test the applicability of this procedure, penicillin inhibition curves in six different serum filtrates were determined. Part of each filtrate was used to prepare a penicillin solution of the required concentration (about 0.01 u/ml), and aliquot portions of each solution were added to a series of tubes. Complementary volumes of filtrate containing no penicillin were added to each tube to maintain a constant filtrate concentration. These were compared with similar curves in one-half saturated ammonium sulfate. Figure 6 is representative of the data obtained. Apparently, in the presence of serum filtrate, penicillin shows an enhanced inhibitory action.

The synergistic effect of serum filtrates upon penicillin activity may be related to the formation of a complex composed of penicillin and blood albumin, as

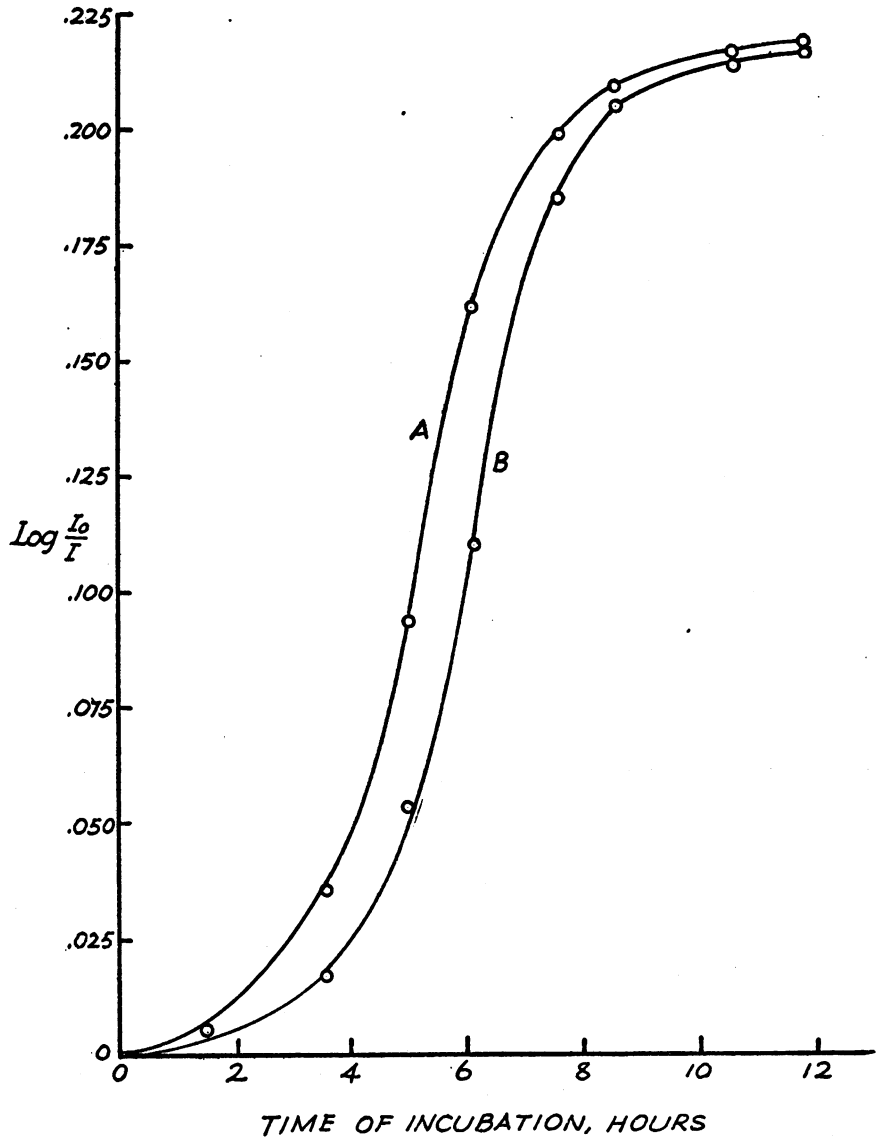

FIG. 5. EFFECT OF AN EXTENDED INCUBATION PERIOD UPON THE GROWTH OF STAPHYLOCOCCUS AUREUS (OXFORD STRAIN), WITH AND WITHOUT PLASMA FILTRATE

Medium: 4 per cent proteose peptone no. 3, 1 per cent yeast extract, 1 per cent glucose, and 1 per cent Na_2HPO_4. Incubated at 37 C.

postulated by Chow and McKee (1945). It is possible that the complex functions as a more effective antibiotic than does penicillin itself. A more plausible explanation, suggested by Dr. T. Tung, is that the association of penicillin with

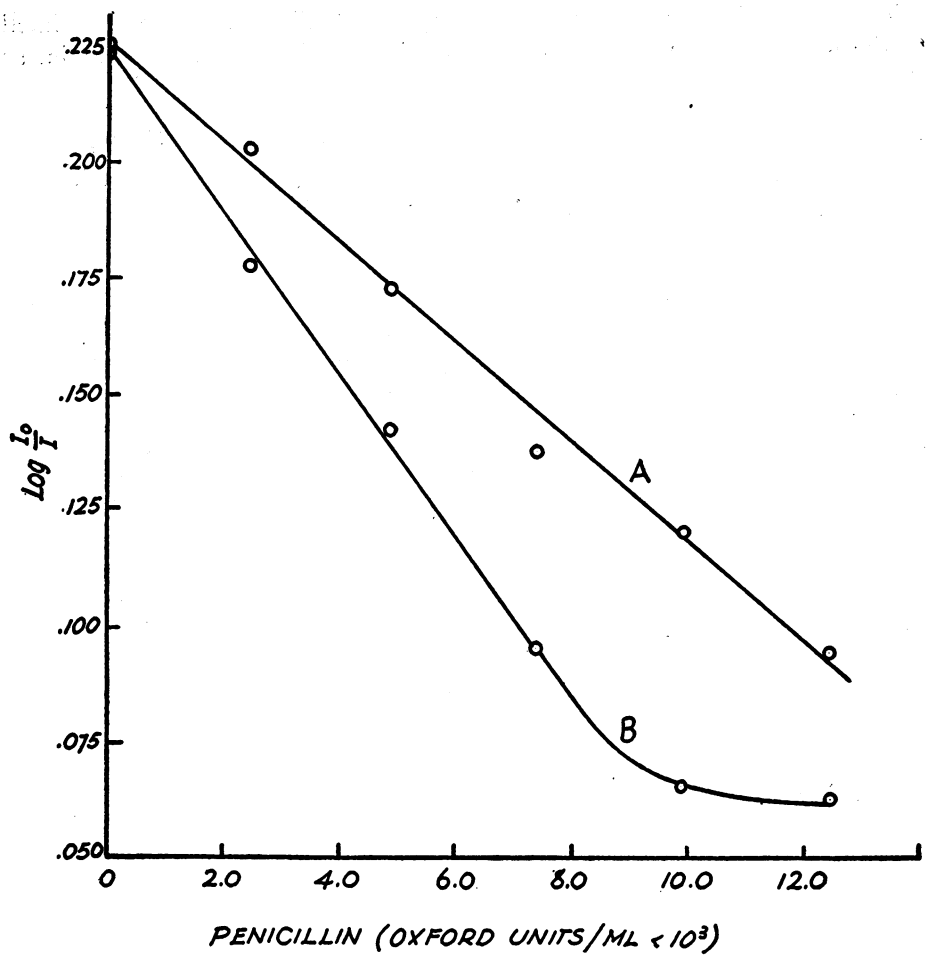

FIG. 6. PENICILLIN INHIBITION CURVES FOR STAPHYLOCOCCUS AUREUS (OXFORD STRAIN)
A: In ammonium sulfate. B: In serum filtrate. Medium: 4 per cent proteose peptone no. 3, 1 per cent yeast extract, 1 per cent glucose, and 1 per cent Na_2HPO_4. Incubation for 12 hours at 37 C.

TABLE 2

Zone diameters produced by solutions of penicillin

SOLVENT	PENICILLIN	ZONE DIAM.
	Oxford units per ml	*mm*
Half-saturated $(NH_4)_2SO_4$	1.0	19.0
Half-saturated $(NH_4)_2SO_4$	2.0	22.0
Plasma filtrate	1.0	26.5
Plasma filtrate	2.0	26.5

albumin stabilizes the material with respect to thermal inactivation. During a period of 12 hours at 37 C some decomposition undoubtedly occurs. An increased stability in the presence of filtrate would lead to the effects noted.

The enhancement of penicillin activity noted above has also been demonstrated by means of the cup assay method. Significantly larger zones of inhibition are found with solutions of penicillin in plasma filtrates than are obtained with equivalent concentrations in half-saturated ammonium sulfate. Typical data are given in table 2.

The synergistic effect of serum filtrate upon penicillin activity precludes the possibility of developing a method of assay based upon quantitative comparison of growth levels obtained in the presence of filtrates of unknown sera with standard curves prepared in ammonium sulfate solution. It is possible, however, that a method of stabilizing the standard curve can be developed to eliminate the differences which have been found. Further work on this phase of the problem is now in progress.

SUMMARY

The effects of plasma, serum, and their globulin-free filtrates upon the growth of *Staphylococcus aureus* have been studied. The inhibition of staphylococcus growth which occurs in the presence of plasma or serum when supplemented media are used can be abolished by removal of the globulin fraction of the serum proteins. Evidence for the presence in globulin-free filtrates of an accessory growth factor or factors has been presented.

It has been shown that the presence of plasma or serum filtrates enhances the bacteriostatic activity of penicillin. The mechanism of this effect has been discussed.

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