Authors are alone responsible for the statements made and the opinions expressed in their papers.

Journal of the Royal Army Medical Corps.

Original Communications.

PENICILLIN ASSAY METHODS
By Captain GEORGE D. LUMB, M.B., B.S.,
Royal Army Medical Corps,
AND
Sergeant J. M. WILSON,
Royal Army Medical Corps.
From Central Pathological Laboratory, M.E.F.
[Received March 14, 1945.]

In presenting this paper we are setting out the methods used in this Laboratory for the routine investigations associated with penicillin therapy. Now that supplies of penicillin become more plentiful, many laboratories which previously have not had the opportunity of performing sensitivity tests and carrying out penicillin assay will be called upon to do so. The literature dealing with the technique of these tests is scattered throughout the medical journals, and it occurs to us that it would be helpful to embody in one short article a detailed account of the methods employed in a laboratory which has been continuously engaged in such estimations since 1942 (Pulvertaft, 1943). The present account outlines techniques employed whilst studying the cases under treatment in the hospital and also in experimental work on which we have been employed. We have attempted to outline a group of tests which can be used in any laboratory with limited apparatus and which at the same time will make it possible to control all cases undergoing penicillin therapy. These methods represent the modifications we have finally adopted after considerable experience of their use.

ORGANISM SENSITIVITY TO PENICILLIN.

Our first routine work was in assessing the sensitivity to penicillin of organisms isolated from patients. This we have done by means of the gutter plate (Fleming, 1929, 1942). The original description was of nutrient agar, but we have used a ditch 2 cm. wide cut in the diameter of a blood agar plate, and filled with nutrient agar at 50° C. containing 2·5 units of penicillin per c.c. When this is set the organism under test is smeared across the plate, a control streak being made at the same time of Oxford Staphylococcus aureus. The penicillin diffuses out of the gutter into the surrounding blood agar and according to the
sensitivity of the organism under test it will fail to grow for a varying distance on either side of the gutter. Its degree of sensitivity can be judged by the relative zone of inhibition of the Oxford staphylococcus and the test organism. Plates are placed on wooden trays when in the incubator in order to prevent condensation on the lid of the Petri dish. For the purpose of comparison we express our result as a factor against the standard staphylococcus. Thus, if Oxford staphylococcus is inhibited for 12 mm. on either side of the ditch and the test organism is inhibited for 14 mm. the factor is $\frac{14}{12} = 1.16$. It is not suggested that one organism is $1.16$ times as sensitive as the other, but this has proved a useful method of assessing possible intensity and duration of treatment in many cases. Those organisms with a low factor have required a greater amount of the drug to eradicate them from the patient.

If it is necessary to provide a result quickly the pus, sputum or other material containing the organism, may be streaked on the plate, but obviously, if time allows, more clear-cut readings will be obtained by isolating the predominant organism. In any case preliminary findings must be confirmed using a pure growth of the organism.

As a substitute for penicillin in the ditch, urine from patients receiving the drug may be used. The actual concentration of penicillin in the gutter is not of great importance as any inhibition can be checked against the control staphylococcus which should always be included on each plate. As many as ten tests may be performed on each plate.

The Staph. aureus used as a standard in this laboratory is a subculture from the original Oxford organism. Maintenance is on refrigerated agar slopes. Broth subcultures are made weekly and, after twenty-four hours incubation, fresh agar slopes are inoculated, incubated, and returned to the refrigerator.

Double Ditch.—We have found a useful variation of the above to be a method originally described by Fleming (1929)—a double gutter plate. Gutters are cut on either side of a blood agar plate, one being filled with penicillin agar, as previously described, and the other filled with agar containing one of the sulphonamide series of drugs, the one most frequently used being sulphadiazine.
Penicillin Assay Methods

We have tried out but discarded the Agar cup method (Fleming, 1942) as the results are not as clear cut and it is not possible to test such a large number of organisms on one plate.

Penicillin Assay in Solution.

Various methods of assaying the penicillin content of solutions have been employed. For relatively large concentrations the best results have been obtained with the Oxford Assay Cup Method (Abraham et al., 1941). Our technique is as follows. Nutrient agar is used, mixed with a twelve-hour Staph. aureus broth at 50° C., the proportions being 1 c.c. of a 1:10 dilution of broth with 100 c.c. of agar. This has been found to give much more clear-cut readings than the originally described method of pouring the broth over the surface of the agar. It also obviates the necessity for special arrangements of samples on the plates as growth is quite uniform. The sterile cup which is a small porcelain cylinder of standard size is then placed on the agar after preliminary heating to produce a seal, and the test fluid is poured in so that the cup is just filled. Zones of inhibition are read after twelve hours incubation. If the culture is incubated for long periods the zone is found to diminish gradually and isolated colonies begin to appear in the clear zone. The diameter of the zone is always used for the reading. The measurement is taken with dividers against a dark background. In order to obtain uniformity, readings have throughout been read by one of us (G.D.L.).

In place of the assay cylinder, we have used filter paper cut in circles and soaked in the test fluid before placing on the agar. This has been found to be a most satisfactory method. The first reference to it seems to have been by Foster and Woodruff (1943) quoting Dowdy, Vincent and Vincent.

In our hands it has been found that Chardin filter paper gives clearer and somewhat larger zones than other filter papers and various types of blotting paper which were tried out. The diameter of the circle used has been 14 mm., the size of our largest cork borer, which instrument, with its cutting edge sharpened somewhat, has been found to be a very convenient piece of apparatus for making the filter paper circles. We have experimented with all sizes of circles from 7 mm. to 22 mm. in diameter. The smaller sizes fail to demonstrate lower dilutions of penicillin, whilst those larger than 14 mm. do not demonstrate any inhibitory action in solutions weaker than those showing zones with the 14 mm. ring. Using this latter size, solutions containing 0.1 unit of penicillin per c.c. give a small but well defined zone. This concentration we consider to be the lowest which can be detected by this method.

Florey and his collaborators have always recommended the use of four cylinders for each test whilst certain American workers have suggested using two. We have recently adopted the method of setting up one cylinder and one filter paper each time. It has been found that occasionally one or other method may give equivocal results which may be checked in this way. Also, especially if ranges of dilutions are to be observed, the two parallel scales, of cylinder and filter paper, serve as a check on the one from the other. It is suggested that in laboratories where Oxford Assay Cylinders are unobtainable the filter paper method provides an accurate and thoroughly reliable alternative.

In our hands the filter paper method gives somewhat more constant readings than the cylinders, but in making a determination, if a standard range of penicillin is set up by both techniques and curves plotted of the results, a reasonably accurate assay can be made (see graph).

When performing penicillin estimations by the above methods it is always necessary to put up a standard range using a series of solutions containing a known number of units. In preparing such solutions it is better to use an accurate standard powder, but if that is not obtainable penicillin from the same batch as that under test should be used. It is common knowledge that when solutions exceed a certain strength a maximum zone of inhibition is reached. Therefore it is necessary when dealing with strong solutions to dilute these to a suitable range. The best for normal use is between 0.25 and 5 units per c.c.

When titrating urine from patients receiving penicillin therapy, it has been our custom to
dilute the specimens 1:5, 1:10, and 1:20—one sample of each dilution being assayed as described above. We have always used as diluent the same type of fluid of a similar pH as that under test or, if this has not been possible, saline of the same pH. If a standard series ranging from 0.25 to 5 units is put up with these one of the dilutions will fall in this scale and the penicillin concentration in the urine can then be calculated. It is considered important to make fresh standard curves for each batch of determinations performed.

In any of the above techniques it has been found that refrigeration of plates after inoculation for three hours before incubation produces more clear-cut results and, at the same time, larger zones.

SERIAL DILUTION METHODS OF ASSAY.

Methods of titrating dependent on obtaining bacteriostasis of a test organism in a dilution of broth have been tried in many ways. The simplest is to make serial dilutions of the penicillin in broth to which is added a drop of a Staph. aureus culture. The dilution in which bacteriostasis occurs gives an end-point. By this method one finds that growth of Oxford

Staph. aureus is usually inhibited in the presence of 0.03 unit of penicillin. Any serial dilution method is obviously open to 100 per cent error which can only be reduced by the tedious method of making many intermediate dilutions.

We have attempted many modifications of the dilution method which have proved unsatisfactory—such as the use of indicators for producing end-points; the decolorization of methylene blue. One technique which seemed at first satisfactory but which we have not yet been able to perfect was to make use of the plasma-coagulating power of Staph. aureus. Another broth serial dilution method which we have tried is that devised by Rammelkamp (1942) in which he uses a sensitive hemolytic streptococcus as the test organism. We discarded this method as the end-point, depending on presence or absence of hemolysis, was not always easy to read and the method was still open to the objection of 100 per cent error because of the serial dilution.

Whilst attempting to devise a method dependent on the addition of specific volumes rather than serial dilutions, our attention was drawn to a personal communication of Hoogerheide to Foster and Wilker (1943). The modification (described below) of his technique
which we have employed appears to be a most accurate method of penicillin assay. Our routine is as follows:

HOOGERHEIDE'S METHOD (MODIFIED).

220 c.c. of 0.25 per cent glucose broth is inoculated with 22 drops of a six-hour Staph. Oxford broth culture. This is then tubed off in 2 c.c. amounts, 22 tubes arranged in two rows of 11 being sufficient for one complete test.

Method.—With a micro pipette the following amounts of a standard normal saline solution containing 0.50 units of penicillin per c.c. are added to one row of tubes: 0.10 c.c., 0.09 c.c., 0.07 c.c., 0.065 c.c., 0.06 c.c., 0.055 c.c., 0.05 c.c., 0.04 c.c., 0.035 c.c., 0.03 c.c., the eleventh tube being used as positive control. Similar amounts of the unknown solution are added to the other series, having been previously diluted at a guess to contain also about 0.50 units per c.c. The tubes are incubated for twelve hours, and the end-point is shown, not by the tube with complete inhibition, but in that containing a clear supernatant fluid with sediment.

The method of inoculating the glucose broth seems to be important. In the original method the Oxford staphylococcus was added to the 2 c.c. amounts of glucose broth. We found that by adding the staphylococcus to the stock broth a more even suspension of the organism was obtained with less chance of contamination.

The most suitable type of tube for this test was found to be 2½ by ½ inches.

Better results were obtained by leaving the inoculated broth three hours in the refrigerator before incubation.

The above method was found to be very accurate in the assay of penicillin solutions and urines but it appeared that in the presence of protein-containing fluids, such as serum, some change had occurred which produced inconsistent results.

We therefore discarded this method for serum estimations in favour of the following technique which provides more accurate results.

PENICILLIN IN BLOOD.

For titrating penicillin in blood various modifications of a slide cell technique have been suggested (Fleming, 1943; Colebrook, Storer and Wright, 1923; Bigger, Thomas and Caldwell, 1944), and so far this seems to be the best method. It is dependent on showing that a given serum produces bacteriostasis of a test organism—usually Oxford Staph. aureus—in certain dilutions.

The modifications we have adopted, which we find infinitely simpler to carry out than any method yet described, is as follows:

The test is carried out in the bottom of an empty sterile Petri dish.

Four drops of diluent, each of 0.4 c.c., measured with a sterile 0.2 c.c. pipette graduated in 1/1,000th, are placed in the dish in positions B, C, D, E (see diagram). One drop (again of 0.04 c.c.) of the test serum is placed at position A and another mixed with drop B. A serial dilution is then made from B to C and then to D, leaving E as a control. One loopful of a twelve-hour broth of Oxford Staph. aureus diluted 1 : 1,000 is mixed with each broth. Each one is then covered with a sterile ½ inch coverslip and vaselined into position. We have found that, using the above quantities with this size of coverslip, an even smear is left without any leakage around the edges. The plate is incubated at 37° C. for twelve hours, at the end of which time the results can be read naked eye or with the aid of a hand lens as:

Complete inhibition = no growth
Partial inhibition = partial growth
No inhibition = growth equal to that in control

The diluent used in our first experiment was normal human serum and we still feel that this gives a closer approximation to conditions in the body than any other fluid. We have found that if the serum is diluted 1 : 10 to 1 : 100 in saline somewhat higher readings are obtained. Similarly if broth is used the same higher readings result. Using serum or a dilution of serum as diluent small clear-cut colonies are easily visible. With broth, however, there is a greater tendency to turbidity in the drops when growth is occurring and for this
reason we have discarded this method. It may be noted that in mixtures where partial
inhibition has occurred, not only are the colonies fewer in number but they are somewhat larger.

Bigger (Lancet, 1944) has reported that if serum is allowed to stand, more particularly
in the incubator, lower titres will result. He accounts for this as being evidence of inhibition
of penicillin by the blood serum and therefore suggests that the diluent should always be
serum diluted 1 : 10 and that incubation should never be longer than twelve hours.

We have found that if the Petri dish cells are placed in the refrigerator for three or more
hours before incubation there is no appreciable change in the results.

We have tried an identical method using nutrient agar and blood agar instead of the empty
plate, but the organisms tend to grow very rapidly and results are difficult to read. It
would therefore seem that the most suitable diluent is serum, either diluted 1 : 10, as suggested
by Bigger, or used undiluted, which would seem more nearly to approach body conditions.

ASSAY IN EXUDATES AND BODY FLUIDS OTHER THAN BLOOD.

The body fluids other than urine and blood which may be assayed to advantage are
sputum, pus, wound exudates, blister fluids, pleural fluid, joint fluid and cerebro-spinal
fluid. It is interesting to note that whereas, in serum, the penicillin only rises to a concentra­
tion which can be detected by the filter paper method for very short periods, i.e. fifteen to
thirty minutes after an intramuscular injection, levels which can be detected by such a
method are frequently found in pus, sputum, etc., and persist for hours after the penicillin
has disappeared from the blood. When dealing with substances of a sticky consistency,
such as sputum or pus, we have noticed that the filter paper is the method of choice. It
would appear that the composition of the sputum or pus prevents diffusion out of the cup in
some cases, whereas this has never occurred when using filter papers.

DISCUSSION.

In a normal laboratory the use of three of the above tests can give all the information
required regarding a case being treated with penicillin. Thus a swab from a patient who
it is felt should be treated with penicillin is tested by means of the gutter plate, and the sensi­
tivity of the organisms together with a factor is determined. It should be emphasized that
treatment should be controlled by bacteriological examinations in order to determine when
organisms disappear. Pus, urine, sputum, etc., may be tested during treatment by means
of assay cylinders and filter papers in order to determine penicillin concentration. Blood
serum levels may be obtained using the Petri dish cell technique.

SUMMARY.

1) Modifications of penicillin assay methods used by us in routine investigations are
described.

2) It is considered that an account such as this will prove useful to pathologists carrying
out investigations on patients under treatment with the drug.

We wish to express our thanks to Lieutenant-Colonel C. J. Harwood Little, O.B.E.,
R.A.M.C., the Officer Commanding this Laboratory, for his help throughout this work,
and to Colonel H. J. Findlay for permission to forward this paper. We also wish to
record our appreciation of the photographic work which was done by Private P. Wood,
R.A.M.C.

REFERENCES.

Abraham, E. P., Fletcher, C. M., Florey, H. W., Gardner, A. D., Healney, N. G., Jennings, M. A.