MICROBIOLOGICAL ASPECTS OF PENICILLIN[1]

VI. Procedure for the Cup Assay for Penicillin

JACKSON W. FOSTER and H. BOYD WOODRUFF

Research Laboratories, Merck & Co., Inc., Rahway, N. J.

Received for publication November 10, 1943

Publication of this paper was prompted by the numerous inquiries pertaining to details of the cup assay procedure following the appearance of our recent article (Foster and Woodruff, 1943a) discussing the principles, advantages and disadvantages of the various methods of assay for penicillin. This surprisingly wide-spread interest in the cup assay, shown by military and civilian investigators alike, emphasized the realization that (a) numerous features of the test which are taken for granted by workers experienced with this method are quite unknown to the great majority of people who assay penicillin, and (b) there is no source in the literature where details of the complete procedure are available. This paper is intended to provide such information. It contains also a number of points of practical efficiency value which have evolved in this laboratory as a result of the handling of 100 to 400 cup assays in quadruplicate per day. The principle of the assay is that originally described by the Oxford group (Abraham *et al.*, 1941), but the procedure has since undergone substantial modification (Foster and Woodruff, 1943b).

TEST BACTERIA

Staphylococcus aureus (brought to this country from Oxford University by Dr. N. G. Heatley and hence called the "H" strain), first proposed by the Oxford group for use in the cup assay, has, in our experience, a number of features which make it considerably less suitable for this purpose than the strain of *Bacillus subtilis* already in use (Foster and Woodruff, 1943b).

Theoretically, any bacterium sensitive to penicillin can be used in the cup assay since the determination is based on the inhibitory power of the unknown sample as compared to the inhibitory power of a standard penicillin preparation of known potency against the same organism under identical conditions. Practically, the selection of a specific test organism is almost mandatory because of certain cultural characteristics of the different organisms. Our observations about variabilities in the biological properties of *S. aureus* under the conditions prevailing at different steps in the assay have been confirmed in other laboratories (private communications). Probably any rather sensitive spore-forming bacterium could be used similar to *B. subtilis*, but for general uniformity it obviously is best to employ one organism whose behavior characteristics already

[1] Papers number IV and V of this series are, for obvious reasons, being withheld from publication until after the war.

are well known and which has worked satisfactorily. Cultures of *B. subtilis* and *S. aureus* H are available from this laboratory without cost.

EVALUATION OF S. AUREUS AND B. SUBTILIS FOR THE CUP ASSAY

Use of *S. aureus* has a number of disadvantages, which do not apply to *B. subtilis*. For inoculum, young broth cultures must be used, necessitating daily transfer of the organism for overnight incubation in preparation for the next day's assays. This frequently means that inoculum must be prepared during inconvenient times, such as on weekends and holidays, if penicillin assays are to be run the following day. From day to day the physiological state (as well as the cultural appearance) of these broth cultures is apt to vary considerably. Inexplicably, different amounts of growth are obtained, and on one day the growth may consist principally of sediment, whereas on another it may be entirely suspended. Stock slant cultures have to be transferred at frequent intervals to keep them alive.

Preparatory to seeding the melted agar in the assay, it has to be carefully cooled to about 42–45°C. so that the cells of *S. aureus* are not destroyed by the heat. For a large number of assays where a considerable amount of agar is required, two consequences arise: (a) Prolonged exposure to this temperature gradually kills off *S. aureus*, so that, depending on how long it takes to dispense the agar into Petri dishes, the inoculum concentration becomes gradually diminished. Cooling ½ to 2 liters of agar carefully to 42–45° is awkward. (b) This temperature is so near the solidification point of the agar that the danger of hardening before all the plates are prepared always exists.

Optimum incubation of *S. aureus* is at 37°C. and at this temperature, especially in a humidified incubator, excess water condenses on the inside of the plate cover. Frequently during handling, drops of this condensate fall to the surface of the agar where they can cause the sharp edges of the inhibition zones to be less clear. The *S. aureus* plates can be incubated at 30° but full growth takes 20 to 30 hours as compared to 10 to 16 hours at 37°. Also, the opacity of *S. aureus* growth is not very great and it does not stand out in excellent contrast to the clear zones of inhibition on the plates.

At times *S. aureus* exhibits a peculiar behavior which is seriously disadvantageous in the assay and which has not been encountered with *B. subtilis*. Irregularly, but frequently, the inhibition zone edges are quite indistinct and exact delineation of the zone is difficult if not impossible to establish accurately. Instead of being sharply inhibited, growth grades progressively from complete inhibition to full growth. Sometimes there are concentric rings of turbidity over several millimeters in which there are alternate zones of growth and partial inhibition, resembling Liesegang rings. This was particularly noticeable with the F.D.A. strain of *S. aureus* in the few experiments tried with it. These phenomena are due to progressive lysis of the cells under penicillin inhibition. The tendency for full grown *S. aureus* cultures to lyse in the presence of bacteriostatic concentrations of penicillin has been observed repeatedly, and particularly under the conditions of the plate assay. This behavior is more pronounced with

some preparations than others and seems to be more frequent with lyophilized cultures of *Staphylococcus*. An example of progressive lysis is obtained by allowing *S. aureus* assay plates to incubate several hours beyond the normal 16-hour period, recording the zone sizes at intervals. The following tabulation shows the zone size increase with *S. aureus* due to lysis. There was no change with *B. subtilis*.

INCUBATION	ZONE SIZE (MM.)*	
	S. aureus	*B. subtilis*
hours		
17	24.5	17.3
21	25.0	—
46	25.8	17.2

* Averages of quintuplet readings.

The advantages of *B. subtilis* stand in distinct contrast to the defects of *S. aureus*. The troublesome daily preparation of fresh inoculum is eliminated; one spore preparation can be used indefinitely, or as long as the liquid culture lasts. In our laboratory, one flask of spore inoculum prepared as described below was used daily for six months. This eliminates variation in the amount and condition of the inoculum cells and thereby achieves practically perfect standardization of inoculum. Kept cold in a refrigerator, the spores in the suspension can be preserved indefinitely[2] and the suspension is used when needed, much like a laboratory reagent. Aseptic precautions with the suspension, while desirable, are hardly necessary since chance contaminants could not develop at refrigerator temperatures, and when such contaminants find their way into the finished assay plate, they cannot compete with the massive *B. subtilis* inoculum and at best would develop only as isolated colonies.

Preservation of stock cultures of this spore-former requires very little attention. The temperature of the agar at time of seeding can be 55–60°C. since the spores are unaffected, thereby eliminating the requirement for cautious cooling to 42°C. The ability to work with agar at higher temperatures minimizes the chances of hardening of the agar; this is an important feature where large numbers of assays are run, requiring liter quantities of agar to be seeded at one time.

Incubation temperature with *B. subtilis* is 30°C. which does not allow excessive condensation of moisture on the dish top. Also, possibly the penicillin is more stable during the incubation than it would be at 37°C. Growth of *B. subtilis* in a propertly seeded plate is very opaque and provides a very good contrast to the inhibition zones (Fig. 1). The edges of the inhibition zones are almost without exception knife-sharp, and the lysis property described for

[2] After standing 5 months at 4°C. plate counts for viable cells before and after pasteurization (20 min. at 60°C.) showed 324 and 278 millions per ml., respectively. Thus, 85.8 per cent of the total spore count had persisted as viable spores during this period.

S. aureus has never been observed. Prolonged incubation of a *B. subtilis* assay plate for several hours does not change significantly the zone diameters.

Finally, *B. subtilis* is non-pathogenic and plates and cups need not be sterilized before cleaning. In fact, plates have been used for the assay without any previous sterilization. Thus, *B. subtilis* is almost an ideal test organism for the cup assay of penicillin.

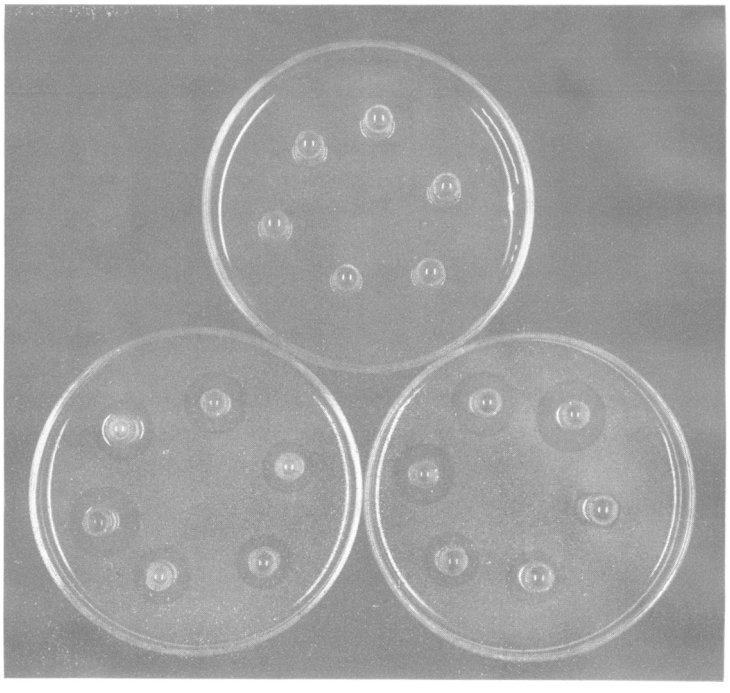

FIG. 1. B. SUBTILIS CUP ASSAY FOR PENICILLIN

Top plate shows cups on seeded agar ready for incubation. Lower right plate shows inhibition zones for a standard curve after 16 hours incubation at 30°C. Lower left plate shows inhibition zones produced by some unknown samples. (The smaller zones on the standard plate are obscured due to handling preparatory to making this photograph.)

PREPARATION OF SPORE INOCULUM

Standard nutrient broth of the following composition is used: Peptone (Difco), 1 per cent; Beef extract (Difco), 0.3 per cent; NaCl, 0.5 per cent; Distilled water.

The pH after sterilization should be 6.0 to 7.0.

After sterilization of 150 ml. in a 250 ml. flask, the medium is inoculated from a slant culture of *B. subtilis* and incubated at 30° or 37°C. in a manner which prevents formation of the surface pellicle typical of aerobic spore-formers. This is done by maintaining the liquid continuously in a state of agitation, which insures development of the cells as a uniform suspension. Growth, also, is greatly accelerated because the limitations of diffusion rates are minimized. The best way to obtain the agitation is to secure the flask to any type of a

shaking machine, rotary or reciprocal. The degree of agitation is immaterial. Ordinary Kahn shaking machines can be easily adapted by any device which will secure the flask. An alternative, though less satisfactory method, is to aerate the culture continuously by passage of a stream of sterile humidified air rather vigorously through the liquid. The incubation is allowed to proceed until maximum sporulation occurs, usually 4 to 8 days. Microscopic examination of the fully grown culture confirms the abundant presence of spores. Suspensions prepared from growth on agar surfaces are just as satisfactory.

The whole flask culture or suspension is then pasteurized at 60°C. for 30 minutes to destroy the remaining viable vegetative cells. In effect, what is left is a rather heavy spore suspension of the organism which, stored cold, can be used repeatedly as inoculum for the penicillin assays until the supply is depleted.

The optimum density of inoculum is in the range of 15,000,000 spores per liter of melted agar. The spore count is established for each new batch of spore inoculum and that amount containing 15 million spores is used so long as that particular batch lasts. This level just gives a dense bacterial turbidity throughout the agar but still does not cause the individual minute colonies to be confluent with one another. Such a level normally shows a definite zone with 0.2 unit penicillin per ml. Zone sizes are somewhat larger for any given concentration of penicillin if the inoculum is lighter and *vice versa*. However, the amount of inoculum specified above provides good optical density for distinguishing the zones, without significantly reducing the sensitivity of the measurement.

PREPARING THE PLATES

The required amount of cooled melted agar[3] (50–60°C.) is seeded with the appropriate amount of spore suspension as established above and shaken thoroughly. The agar required for all the assays covering a week or more can be prepared in one batch, subdivided into convenient amounts, autoclaved and the melted sterile agar stored in a liquid state in an oven at 55–60°C. until used. This eliminates daily preparation of and melting of sterile agar medium which has solidified. Thirteen ml. portions (± 0.5 ml.) of the melted seeded agar are pipetted into sterile standard (100 x 15 mm.) Petri dishes by means of wide-mouth pipettes or an automatic pipetting machine. Regular 10 ml. graduated pipettes with the tips cut off to make an opening of about 3 to 4 mm. are satisfactory; the 13 ml. level is marked with red wax pencil. Accurate quantitative measurement of the agar is not essential. The plates are shaken gently to distribute the agar evenly over the entire surface and they are then allowed to harden. At this stage it is preferable that they be stored in the refrigerator for an hour or more. They can be stored cold for at least 24 hours before use (Foster and Woodruff, 1943a). The seeded plates never should be allowed to stand for more than several minutes at room temperature between the time

[3] 2 per cent agar added to the medium described above. If necessary, the agar should be clarified by filtration through absorbent cotton; clear agar makes a better contrast between the inhibition zone and the bacterial turbidity which ultimately develops on the plate.

they are prepared and the time they are treated with the penicillin samples and incubated (see below). The cooling prevents the bacteria from developing too soon and also renders the agar better for the next step which comprises sealing the glass cups to the surface.

SETTING UP THE CUPS

The cups are small glass cylinders cut uniformly from plain pyrex glass tubing (8 mm. outside diameter; 1 mm. wall thickness) by means of a motor-driven glass-cutting disc. The cylinders should be about 12 mm. in length, but they

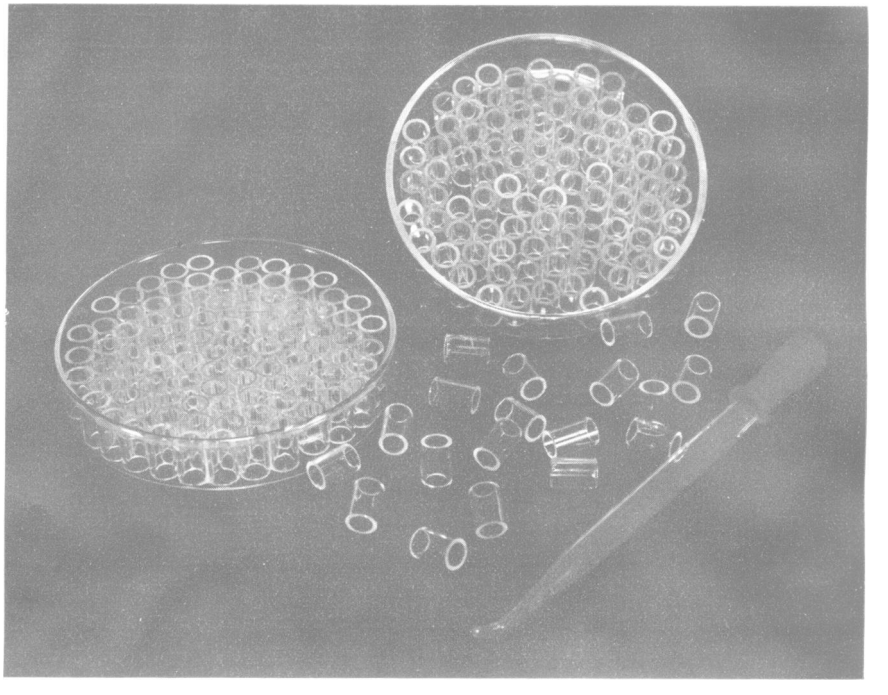

FIG. 2. GLASS CYLINDERS USED IN THE CUP ASSAY AND PETRI DISHES FULL READY FOR STERILIZATION

The type of medicine dropper used for filling the cups is also shown

all must be of uniform size. The bevelled edge originally recommended by the Oxford group for making a water- and bacteria-tight seal is not essential with the heating procedure described below. The bevelled cylinders are difficult to make and are not generally available in this country, whereas in the course of a day any suitably equipped glass-blowing shop can turn out thousands of the straight edge type. A few cups are available as samples from this laboratory. Perfectly flat edges are desirable; chipped or uneven cylinders are discarded. The cylinders are sterilized with dry heat in an upright position in Petri dishes. Figure 2 shows some of the glass cylinders and the manner of sterilizing them.

One end of each cylinder is warmed and then set lightly on the surface of the seeded agar in the prepared plates so that the pressure on the agar comes only from the weight of the cylinder. The warm edge slightly melts the agar locally which hardens again almost immediately, thereby effecting a seal between the agar and the glass cylinder (making a "cup"). Six cups are spaced equidistantly around each plate. Until recently, the heating was done by passing momentarily the end of each cylinder through a Bunsen flame by means of forceps. Considerable improvement in this technique has been achieved by heating all the cylinders (one or more Petri dishes full) simultaneously on an electric hot plate and setting them individually on the agar with forceps. This eliminates an important variable which can affect the assay, due to differences in the depths to which the warm cylinders melt the agar. Caution should be taken that the cups are not warm enough to sink into the agar. Removal of sealed cups should leave only a slight rim indentation in the agar. The correct hot plate temperature can be established by trial assays. If the cups are not warm enough, the seal gradually gives way on standing for several hours filled with liquid. Properly sealed cups do not permit any free liquid to escape from the cup during the whole incubation period. Inverting the plates tests whether the cups are sealed securely. They should remain attached. Often, leaky cups can be traced to unevenness of the cylinder edge.

In practice, all the plates planned for use in any one day should be seeded in one batch, placed quickly in the refrigerator, withdrawn in small batches for setting up the cylinders and then all stored again in the refrigerator until used.

SETTING UP THE SAMPLES

Unknown penicillin solutions are diluted with sterile M/50 phosphate buffer at pH 7.0 so that on the basis of predicted potency the final dilution contains approximately 0.5 to 1.0 Oxford unit per ml. (See Foster and Woodruff, 1943a for discussion of Oxford unit.) With solids, 2 to 5 mg. are weighed to the nearest 0.1 mg. in a tared small test tube and sterile buffer added so that the ml. of buffer equals numerically the mg. of sample. This gives a solution of 1 mg. per ml. If, for example, the sample is expected to contain about 250 units per mg., this first solution is then diluted 1 part plus 24 of buffer and this in turn 1 part plus 9 of buffer, giving finally a dilution of 1:250; thus, the final solution ready for assay contains approximately 1 unit per ml. Although the range is missed occasionally, workers soon become experienced in estimating the potency of solutions or solid products. Significant amounts of extraneous materials, such as solids, reagents and solvents, should not, of course, be present.

It is essential to keep penicillin solutions cold at all times, especially where large numbers of samples are being assayed with consequent long delay before the last samples are finished. All samples should be diluted first, and then filled into the cups at one time later. One person can assay about 150 previously diluted samples per day. Glassware used for penicillin solutions should be scrupulously clean.

The final dilution of each sample is filled by means of a medicine dropper[4] (see Fig. 2) of about 2 ml. capacity (with outlet at 90° angle) into one cup on each of four different plates. Cups should be filled to the top, but extreme accuracy in this is not essential, minor variations in the amounts of liquid having little effect on zone size. For the next sample, the same medicine dropper is used after rinsing briefly in 5% phenol, followed by a wash in sterile distilled water and finally by one rinse with the next solution to be tested. This prevents carrying over of contamination to subsequent samples if one sample should happen to be contaminated with penicillin-destroying bacteria (Abraham and Chain, 1940). The same medicine dropper is used for all the samples, thereby obviating the requirement for countless pipettes.

Four plates with empty cups are withdrawn at a time from the refrigerator and these accommodate six samples each in quadruplicate. Triplicates could be used with perhaps somewhat less assurance of accuracy. Only one dilution is run for each sample unless the potency of the sample is entirely unknown. As soon as the four plates have been filled with six samples, they are set in the 30° incubator and the next set withdrawn for filling from the refrigerator. This precaution of not allowing the plates to stand at room temperature before or after filling the cups is particularly important when large numbers of samples are being handled since some plates would stand longer than others. The inhibition zones finally obtained are essentially the resultant between the rate of diffusion of the penicillin from the solution down and out radially through the agar and the rate of growth of the test bacterium. The longer the interval between diffusion and growth at the optimum temperature, the further the penicillin diffuses out into the agar before the bacterial development appears and is delineated at the points where the penicillin concentration is sufficient for inhibition. In the manner described, the zero time for the diffusion process is standardized at the minimum by incubating at 30°C. each set of plates immediately after filling, regardless of the time of setting up different samples.

Similarly, if the seeded plates stand at room temperature for as little as 1 to 2 hours *before* filling the cups, *B. subtilis* starts to grow. Consequently, after the cups are filled and penicillin diffusion commences, the bacterial turbidity will have appeared before diffusion proceeded to the extent it would have otherwise, and the inhibition zones are, therefore, smaller.

On the other hand, several hours difference in total incubation time after the *B. subtilis* has developed is inconsequential, so that even if several hours elapse between the time the first sample is set up and the last sample, they may all be read at the same time the following morning. Actually, full bacterial growth and zone development takes place in about 12 hours. Plates may be removed from the incubator after 12 to 16 hours and stored in a refrigerator for one or more days before reading them, without affecting significantly the results. This

[4] Use of this instrument was demonstrated to us by Dr. N. G. Heatley of Oxford University, England.

is of great aid over weekends when it may be inconvenient to read plates on schedule.

STANDARD CURVE

Penicillin assays have precise quantitative meaning only if a standard is run daily side by side with the unknowns and the values of the unknowns calculated from a standard curve. The principles of a standard and details of its preparation are discussed elsewhere (Foster and Woodruff, 1943a). Fig. 3 shows a typical standard curve.

The dry standard[5] (and solutions made from it), whose unitage has been carefully calibrated directly or indirectly against the original Oxford standard

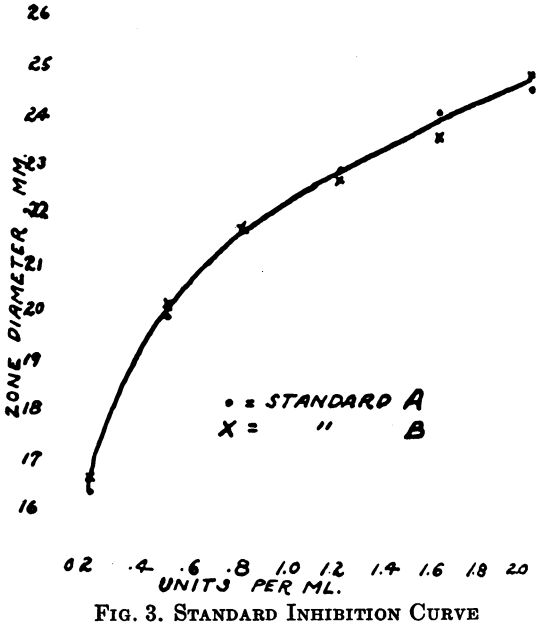

FIG. 3. STANDARD INHIBITION CURVE

should be stored at refrigerator temperature at all times. The dry product is quite hygroscopic and rapidly loses activity after picking up moisture from the atmosphere. The vessel containing the dry product should be allowed to come to room temperature in a desiccator before opening. For a stock solution, two to ten mg. are accurately weighed out as rapidly as possible on an analytical balance. The stock solution is made to contain 32 Oxford units per ml. with sterile M/50 phosphate buffer at pH 7.0. It is best to keep this stock solution frozen because even at refrigerator temperatures it tends to lose activity slowly over a period of a few weeks. Freezing can be done in dry ice or in the

[5] Small amounts for purposes of standardization are available from this laboratory without cost.

freezing compartment of a refrigerator. Each day 0.5 ml. of this stock solution is diluted with 7.5 ml. buffer to give 2.0 units per ml.

The following tabulation shows how subsequent dilutions are made to give the desired final concentrations used for the standard curve.

ML. OF SOLUTION CONTAINING 2.0 UNITS/ML.	BUFFER	FINAL CONCENTRATION
	ml.	*units/ml.*
2.0	0	2.0
1.6	0.4	1.6
1.2	0.8	1.2
0.8	1.2	0.8
0.5	1.5	0.5
0.2	1.8	0.2

Extreme care must be taken with the cleanliness of the glassware coming in contact with the standard penicillin solution.

Occasionally all the samples on any one day give unexpectedly high values indicating that the potency of the stock standard penicillin solution was lower than it was assumed to be. It seems likely that this is due to rapid inactivation of the penicillin by impurities left in the flasks and pipettes used to handle the solution. However, direct proof of this has not been obtained.

Glassware for use in penicillin assays should not be cleaned with regular H_2SO_4-$K_2Cr_2O_7$ cleaning solution. Slight traces of residual chromium ion may partially inactivate low concentrations of penicillin. Heating to 80–90°C. in concentrated H_2SO_4 to which is added a few crystals of KNO_3 daily is recommended. Penicillin in solution is well known to be extremely unstable, particularly at room temperatures and in the presence of certain metallic ions.

In this laboratory, to eliminate the possibility of ruining a whole day's assays and also as a check on the daily standard solutions, two different sets of standard curves are made up independently from different penicillin standards and run daily. Usually the curves are virtually superimposable (see Fig. 3). If one is significantly lower than the other, this is a good indication of loss of activity and the samples for that day are read from the good curve. On several occasions these extra precautions have justified themselves. All standard solutions should be kept cold up until the few minutes before they are placed into the cups. Each of these six standard solutions is put into one cup of each of quintuplet plates in the manner described for the unknown samples. Thus, each plate has on it an entire range of concentrations for a standard curve. These plates are incubated immediately at 30°C. for 12 to 16 hours.

MEASUREMENT OF INHIBITION ZONES

After incubation, different sizes of clear circular zones of inhibition are observed depending on the concentration of penicillin in the solutions in the cups (Fig. 1). Within limits, the diameter of the inhibition zones, the edges of which almost without exception are knife-sharp, is measured in any convenient

manner. A good system is to set a pair of mechanical drawing dividers for the diameter limits and then measure the span against mm. lines on printed graph paper or on a mm. rule. Readings are made to the nearest half mm.

Where large numbers of assay plates are to be read, a handy device is one in which the plates are set on an exposed blank lantern slide with mm. lines cut into the gelatin with a dull razor. This side is counter-sunk in a wooden block and a small microscope lamp[6] shining up through the ruled scale provides a good optical contrast for the measurement of the zones. A piece of white paper set under the scale diffuses the light and facilitates the reading of the plates. The zones are in clear contrast against the black background. A magnifying glass fixed on a ring stand eases strain on the eyes. The construction of the

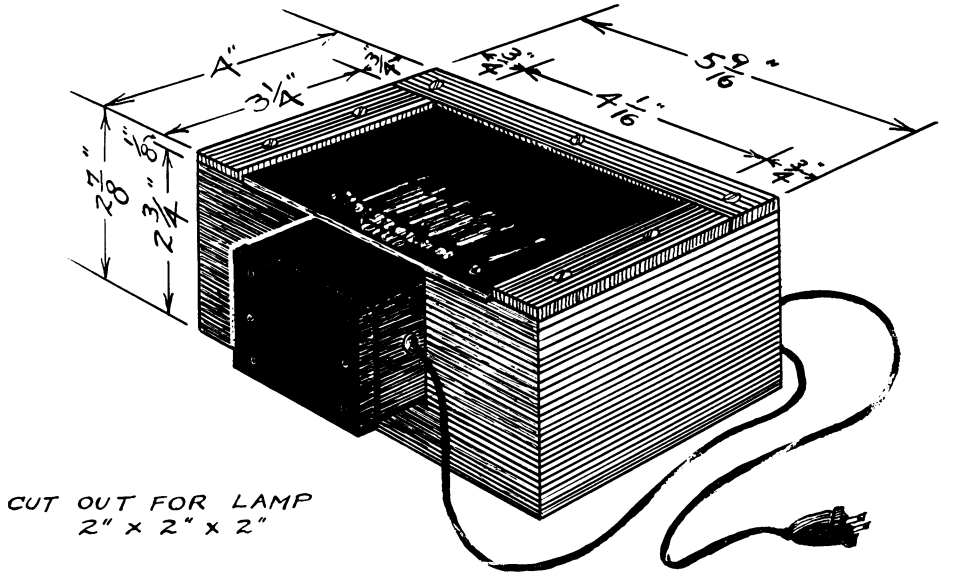

FIG. 4. READING BLOCK FOR MEASURING INHIBITION ZONES

reading block is illustrated in Fig. 4, and the setup under laboratory conditions is shown in Fig. 5.

The replicate zone diameter values of unknowns and standard are averaged separately. In practise, all six zones on one plate are read in sequence. A simplified system of identifying the samples and their respective replicates is to number the plates only with a wax pencil. The individual cups need not be marked if a vertical wax pencil mark is made on the side of the Petri dish bottom opposite one of the cups. Beginning with that cup, the others are identified by clockwise sequence. Thus, one needs only to have a list of the samples and to read the plates in order and record the values beside the respective sample numbers on the list.

Greatest reliability of results is obtained if the values for the unknown fall

[6] Bausch & Lomb Micro-lamp substage form. A. H. Thomas Cat. No. 6927-A.

within points on the curve corresponding to about 0.3 to 1.4 units. Points on the high region of the curve are apt to be only approximate, because small differences in zone measurements can mean relatively large differences in the penicillin value read, depending on how flat the standard curve is on any particular day. From day to day there are some changes in the shape of the curve, but usually this occurs in the upper region where it flattens out. Values on the curve below the 0.3 unit point also are not apt to give best accuracy but can be counted on within about 30 per cent. Where highest accuracy is desired,

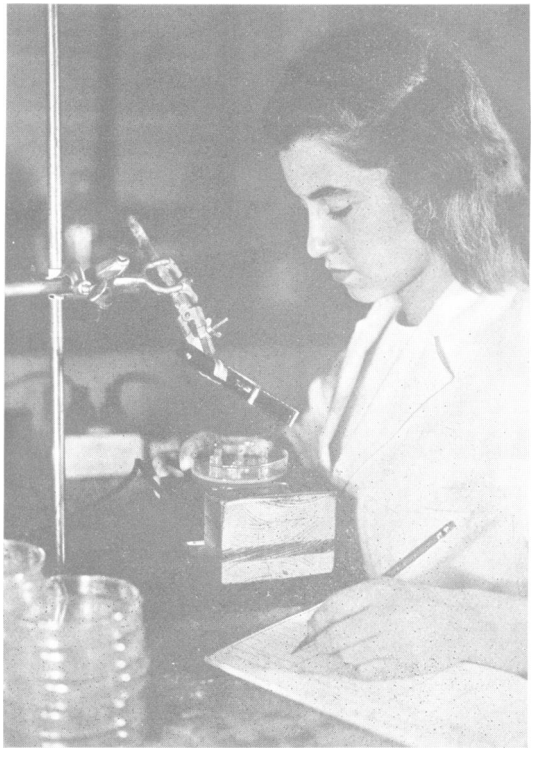

FIG. 5. COMPLETE ASSEMBLY FOR READING THE PLATES

samples with values falling outside the central region of the curve should be reassayed.

CALCULATION OF RESULTS

Generally, the individual zone diameters check within 1.5 mm. of the average, the arbitrarily established limit in this laboratory. Occasionally, for an unknown reason, all the diameter values on any one plate, i.e., one replicate for each of six samples, deviate significantly (usually downwards) from those on the rest of the plates. Experience has shown that such readings are generally invalid, and they may be excluded from the averages. With quadruplicates, however, inclusion of any one value which is suspiciously high or low does not change greatly the final average. The following are typical readings for stand-

ard curves from two different standards for one day's assays. The readings from four unknown samples are also given.

	DILUTION—UNITS/ML.					
	0.2	0.5	0.8	1.2	1.6	2.0
Standard curve using standard "A"						
Replicate inhibition zone diameters, mm.	15.5	19	22	23	24	25
	17	21	22	23	24.5	25
	17.5	20	21.5	23	24	24.5
	16	20	22	22.5	24	24.5
	16	19	21	23	24	24
Average.....	16.4	19.8	21.7	22.9	24.1	24.6
Standard curve using standard "B"						
Replicate inhibition zone diameters, mm.	17	21	22	23	24	25
	15	19	22	23	23	24
	17	20	21	22	22.5	25
	17	20	21	22.5	24	25
	17	20	22.5	23	24.5	25.5
Average.....	16.6	20	21.7	22.7	23.6	24.9

Unknown samples

	#1	#2	#3	#4
Replicate values for diameters of inhibition zones, mm.	19.5	19	20	22
	18	17	20	22
	18	18	21	23
	19	19	21	22
Average.....	18.6	18.3	20.5	22.3

A standard curve constructed from the above data is illustrated in Fig. 3. It is similar to that originally published by Abraham *et al.* (1941) except that it covers only the lower concentrations of penicillin and thereby increases the accuracy of the assay.

The zone diameter averages for the unknowns are projected from the ordinate horizontally to the curve, thence vertically to the abscissa, giving thereby that unitage of penicillin producing the same diameter of zone inhibition as the particular dilution of the unknown. Read against the standard curve in Fig. 3, the four unknowns contain, in order, 0.32, 0.31, 0.58 and 1.0 units per ml. Multiplication by the dilution factor gives the potency of the original solution.

ACCURACY

Abraham *et al.* (1941) claimed a ± 25 per cent precision for the cup method. It is probable that it is well within that figure and can be taken as nearer ± 15

per cent. Still better accuracy is obtainable if more than one dilution of the unknown is run and the values averaged.

The variation on different days generally is greater than that between samples run side by side on the same day. For that reason in experimental work it is always preferable to assay all the treatments and controls from any one experiment on the same day. For absolute precision in evaluating a particular sample, as for example in establishing a standard, it is imperative to assay it on a number of successive days and average the results or to assay different aliquots of the original sample. The following tabulation illustrates the order of variation in cup assay values on one solid preparation over a period of 14 months.

DATE ASSAYED	OXFORD UNITS PER MG.
7/14/42	95
7/16/42	118
7/22/42	120
12/10/42	112
12/18/42	98
12/21/42	86
7/ 2/43	108
7/ 3/43	120
7/ 7/43	96
7/10/43	97
7/12/43	84
9/18/43	103
9/20/43	95
9/21/43	93
9/22/43	99
Average	102

Maximum deviation from the average is +18 and −18 per cent.

DISCUSSION

As emphasized in our earlier paper (Foster and Woodruff, 1943a), the type of assay used, the manner of executing the separate steps and the precautions taken all depend on the particular interest, objectives and requirements of different investigators or laboratories. The procedure outlined above has evolved from the handling of large numbers of assays per day. All operations are designed for efficiency without sacrifice in accuracy or reproducibility, hence they also are directly applicable for the running of small numbers of assays. However, in the latter case, some alternative steps or modifications are just as applicable since they do not become limiting factors until the turnover of assays reaches sizable proportions.

For example, with only small numbers of samples it probably is just as satisfactory to dispense with the glass cups entirely and use the filter paper disc modification of Vincent, Vincent and Dowdy described previously (Foster and Woodruff, 1943a). However, it appears that carefully measured amounts

of the samples must be added to the paper discs. Although satisfactory for small numbers of assays, these pipette measurements become a great handicap with large numbers. Also, bacterial contamination becomes much more of a problem with the disc method. Again, a higher order of accuracy is possible if more replicates or different levels of each sample are run and vice versa, but this is not feasible with a considerable number of samples.

Up to the present, the best application of the cup method is in the microbiological research and development and production of penicillin for clinical use. Numerous assays are required daily for continuous laboratory experimental studies, pilot plant fermentation studies and as controls and analyses for large scale fermentation batches. In addition, numerous assays come daily from the extraction process and in the evaluation of the finished product ready for ampule distribution. Meanwhile, there are continuous investigations on chemical purification and fractionation which can be studied only with assays. Invariably, in all these cases the original penicillin samples have to be diluted out so far that the final dilution offers no difficulties due to solids, the nature of solution, etc.

Assay of blood and body fluids. Up to now the cup assay has not been successfully adapted to the determination of penicillin in blood and other body fluids. Because of the low concentrations of penicillin it usually contains, blood usually has to be assayed undiluted or in low dilution. However desirable, in our opinion the need for an assay for clinical work giving the order of precision of the cup assay is not great. The main objective of blood level studies is to establish whether the blood contains sufficient penicillin at any given time to inhibit the development of the infecting organisms. The first requirement is to isolate in pure culture the organism and ascertain by streak or tube dilution methods in the presence of blood the concentration of penicillin effectively inhibiting its growth. For that particular organism, the inhibiting concentration (including the antibacterial power of blood itself) is the critical value in the blood, and blood levels below that value clinically are unimportant. The important thing is to determine whether the blood contains the critical concentration or more and how long it continues to exceed that level. Also, since the blood level changes so rapidly due to excretion, except where the continuous intravenous drip method of administration is employed, accuracy better than 50 to 100% is not particularly valuable. The tube serial dilution procedure of Rammelkamp (1942) is satisfactory for this purpose, whereas, for example, in studies on the chemical purification of penicillin it would not be suitable. Generally, the clinical studies on penicillin do not involve the assay of large numbers of blood or body fluid samples which makes the tube dilution method readily applicable.

After this manuscript was written, we learned that the idea of preparing a stock suspension of bacterial spores (*Bacillus metiens*) as a means of standardizing test bacterium inoculum over long periods of time was successfully employed in 1927 by Levine and coworkers in testing the efficiency of hypochlorite

solutions (see Rudolph, A. S., and Levine, M., Bulletin 150, Iowa Engineering Experiment Station, 1941).

SUMMARY

Complete details of the cup assay for penicillin are described including discussions of the principles involved.

REFERENCES

ABRAHAM, E. P., AND CHAIN, E. 1940 An enzyme from bacteria able to destroy penicillin. Nature, **146**, 837.

ABRAHAM, E. P., CHAIN, E., FLETCHER, C. M., FLOREY, H. W., GARDNER, A. D., HEATLEY, N. G., AND JENNINGS, M. A. 1941 Further observations on penicillin. Lancet, **241**, 177–89.

FOSTER, J. W., AND WOODRUFF, H. B. 1943(a) Microbiological aspects of penicillin. I. Methods of assay. J. Bact., **46**, 187–202.

FOSTER, J. W., AND WOODRUFF, H. B. 1943 (b) Improvements in the cup assay for penicillin. J. Biol. Chem., **148**, 723.

RAMMELKAMP, C. H. 1942 A method for determining the concentration of penicillin in body fluids and exudates. Proc. Soc. Exptl. Biol. Med., **51**, 95–97.