MICROBIOLOGICAL ASPECTS OF PENICILLIN

I. Methods of Assay

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Available publications on penicillin (Fleming, 1929; Chain, *et al.*, 1940; Abraham, *et al.*, 1941, 1942; Hobby, *et al.*, 1942; McKee and Rake, 1942; Catch, *et al.*, 1942, Meyer, *et al.*, 1942) indicate that it has excellent prospects as a chemotherapeutic agent and that its microbiological production and chemical purification and isolation are formidable problems. Aside from the report of the Oxford group (Abraham, *et al.*, 1941), little information pertaining to the microbiological aspects of penicillin has appeared. Comprehensive investigations on various phases of the problem have been carried out in this laboratory and considerable experience has accumulated which is worth reporting at this time.

The matter of establishing with some degree of certainty and accuracy the potency of penicillin solutions is one of the most pressing issues confronting workers engaged in penicillin studies. Among various laboratories there are wide divergencies in penicillin assay methods, which, in a good many instances, make it virtually impossible to interpret findings from one laboratory in terms of those from another. The critical studies outlined in this paper are based on experience with all the different types of assays and the conclusions presented are the result of the daily handling for approximately a year of 50 to 100 assays under practical working conditions. Fleming (1942) has already emphasized some features of certain procedures used in penicillin testing, but these are largely qualitative in nature and do not deal with the quantitative aspects considered in the present paper.

METHODS OF ASSAY

Requirements of a penicillin assay

These may vary according to the particular interest and need for accuracy of the individual worker. The main objective is to determine, as accurately as required, the antibacterial activity of a penicillin sample. Depending on the particular objective and the nature of the experiment under way, a high degree of accuracy may not be necessary, and thus many factors conducive to precision are dispensable. On the other hand, detection of relatively small differences in potencies is often required and precautions must be taken to insure this order of sensitivity.

In certain types of biological experiments, such as in measuring blood levels and excretion and pharmacological studies, values accurate to within 100 per cent may be satisfactory. Studies on chemical fractionations, stabilities, purification, and recovery yields from large scale production batches create,

however, an urgent need for the "ideal" assay. A 20 per cent variation may mean quite a difference in the interpretation of a fractionation or extraction process or of a stability experiment.

The results on any given preparation must be reproducible in the same and different laboratories, and, of course, a common language for expressing the activity of penicillin preparations must be used.

Samples should not require pre-treatment to make them suitable for assay as pre-treatment becomes a great handicap where numbers of assays are run routinely.

Any method finally selected as filling the above requirements must be amenable to the running of large numbers of tests in routine.

Results should be available in as short a time as possible after the assay is set up, and the reading should be unequivocal.

The distinctive characteristic of penicillin is its ability to inhibit the normal growth of certain bacteria. At present, the only criterion of potency is the magnitude of the bacterial growth inhibition caused by penicillin preparations. The growth inhibition in certain media is proportional to the concentration of penicillin, and since no competitive phenomenon analagous to para-aminobenzoic acid reversal of sulfonamide action is known, all methods of assay center around determining the smallest amount of penicillin which will cause an arbitrarily established degree of inhibition of the growth of a susceptible test bacterium. In essence, all the proposed methods differ in the mechanics used to measure the inhibition.

Entirely fallacious values may result in the assay of penicillin by any method unless it is shown that the total antibacterial activity of the sample is due exclusively to true penicillin. This means first establishing that true penicillin is present and that it is the only antibacterial substance present. The significance of this point cannot be overemphasized since it is now known that cultures of *Penicillium notatum* may contain at least two antibacterial substances (Kocholaty, 1942; Coulthard, *et al.*, 1942; Waksman and Woodruff, 1942; Roberts, *et al.*, 1943). One of these, notatin (Coulthard, *et al.*, 1942) [also called coli factor (Waksman and Woodruff, 1942), penatin (Kocholaty, 1942), and penicillin B (Roberts, *et al.*, 1943)] has antibacterial activity only in the presence of glucose. The assay medium for penicillin contains no glucose, but culture filtrates of the mold invariably contain residual carbohydrate, and the amount incorporated into the assay medium with the sample being tested often is sufficient for the action of notatin.

The solubility of penicillin in organic solvents makes it important to consider the combined effect of solvent and penicillin. Thus, table 1, part A, shows the lethal concentrations of various solvents on the growth of *Staphylococcus aureus* H under the assay conditions (plate method, see below). Part B shows that sub-inhibitory doses of solvents have an additive effect on penicillin which renders the penicillin potency apparently higher than it actually is. This discrepancy is great enough to yield misleading results. It is obvious that penicillin samples for assay should be in aqueous solution containing, if possible, no solvents.

Designations of activity

Numerous terms have been used to express penicillin activity. These include the dilution, the number of micrograms and the number of Florey units causing inhibition.

The dilution terminology, while a more traditional means of expression, has a serious disadvantage in that where a high degree of accuracy is required, the values obtained are not sufficiently rigorous to be accepted without reservation,

TABLE 1
*Influence of solvents on penicillin assay**
Part A. Effect of solvents alone

	ML. SOLVENT PER 10 ML. AGAR		
Amyl acetate	0.033 +	0.1 +	0.3 −
Ethyl acetate	0.1 +	0.33 +	1.0 −
Acetone	0.1 +	0.33 −	1.0 −
Chloroform	0.1 ±	0.33 −	1.0 −
Ethyl alcohol	0.063 +	0.2 +	0.6 +
$NaHCO_3$ (sat. sol'n.)	0.1 +	0.33 +	1.0 −
Ether	0.1 +	0.33 +	1.0 +

Part B. Effect of solvents with penicillin

SOLVENT PER 10 ML. AGAR	PENICILLIN† (MICROGRAMS PER 10 ML. AGAR)						
	0	8	10	12	14	16	17
No solvent	+	+	+	+	+	±	−
0.05 ml. amyl acetate	+	±	+	+	−	−	−
0.10 ml. amyl acetate	+	±	−	±	−	−	−
0.05 ml. chloroform	+	+	+	±	±	−	−
0.10 ml. chloroform	+	−	−	−	−	−	−

* +, growth; −, complete inhibition.
† Containing 6 Florey units per mgm.

due chiefly to variables in the assay. These may cause a significant deviation from day to day in the value of the same sample. Also, dilution results express only a range of values between which the real endpoint lies (see below).

Standard penicillin preparations

To eliminate the day-to-day deviation, the Oxford group introduced the concept of the penicillin unit and the use of a standard penicillin preparation. The potency of any penicillin sample is obtained by direct comparison with the primary standard or indirectly against secondary standards whose potencies

have been previously established by the primary Oxford standard. First called the Oxford unit, it has lately come to be known as the Florey unit after Professor Florey of Oxford University. Use of a standard run daily, side by side with the unknown test solutions, minimizes variations caused by slight changes in media, condition of and amount of inoculum, incubation time, etc., since the standards and unknowns presumably are affected alike.

Although the Oxford group explained the origin of the penicillin unit, it is not permissible for other laboratories to establish in the same way the unitage of their own preparations. The idea of Florey unitage today connotes a *comparison either directly or indirectly against the original Oxford standard of known unitage.* On account of the deviations caused by uncontrollable factors, attempts to evaluate the potency of samples after the manner of the Oxford definition give at best only an approximate value since the Florey unit applies *specifically and only* to the original standard set apart by the British workers and designated by them to contain so many units per milligram. The fact that they named the unit that amount[1] of penicillin which under the conditions of the cup assay gives an inhibition zone 24 mm. in diameter does not mean that every laboratory will arrive at the same value for the unit, even though each follows faithfully the definition laid down by Abraham, *et al.* (1941). From the experience in this laboratory values obtained were found to vary considerably from the true Florey unit. This laboratory will be glad to supply, without cost, small quantities of secondary penicillin standards which have been accurately standardized indirectly against the authentic Oxford standard.

Preparation of standards

Because of the tendency of some penicillin samples to deteriorate rapidly upon standing, the first requisite of a preparation to be chosen as a standard is that it shall be of *proven stability*. The criteria for the stability are obvious; the selected samples must be shown to have lost no activity during storage over a considerable time. In these laboratories, fairly large samples (1 to 2 grams) of four different penicillin preparations are stored in dry ice for a period of not less than two months, during which time they are tested once or twice each week. If they show no loss of activity, they are acceptable as standards. Dry ice storage is maintained as an extra precaution for stability. Refrigerator temperatures probably are satisfactory. Besides establishing stability, the weekly assays over a 2- to 3-month period serve to give a Florey unitage which is the average of several assays on different days and on different weighed samples. Table 2 shows the data establishing the stability and potency of a secondary standard. The primary standard in this case was obtained from Oxford.

Test bacteria

For the sake of uniformity and standardization, it would seem most desirable to employ the strain of *Staphylococcus aureus* H originally used by the Oxford

[1] Originally, this amount, when dissolved in 50 ml. of meat extract broth just inhibited completely the growth of the test strain of *S. aureus*. Thus, material containing one unit per mgm. just inhibits the growth of *S. aureus* at 1:50,000.

TABLE 2

Potency and stability of a secondary standard

SOLUTION FOR ASSAY MADE UP ON	DATE OF ASSAY ON SOLUTION	ASSAYED BY	METHOD	FLOREY UNITS PER MGM.
4/28/42	4/28	B. L. W.	Turbidimetric	61.7
	4/28	B. L. W.	Turbidimetric	59.8
	4/30	B. L. W.	Turbidimetric	74
	4/30	N. G. H.	Cup	48
	5/4	B. L. W.	Turbidimetric	85*
5/4	5/4	B. L. W.	Turbidimetric	69.5
5/5	5/5	B. L. W.	Turbidimetric	52.5
5/12	5/12	B. L. W.	Turbidimetric	63.2
	5/12	H. B. W.	Cup	58
5/12	5/20	H. B. W.	Cup	59
	5/20	H. B. W.	Cup	60
6/22	6/22	B. L. W.	Turbidimetric	61.6
	6/22	B. L. W.	Turbidimetric	50
	6/22	H. B. W.	Cup	62
	6/23	H. B. W.	Cup	56
	6/24	H. B. W.	Cup	46.8
	6/25	H. B. W.	Cup	61
	6/25	H. B. W.	Cup	67
	6/25	B. L. W.	Turbidimetric	68
	6/25	B. L. W.	Turbidimetric	61.7
	6/25	B. L. W.	Turbidimetric	60
6/24	6/24	H. B. W.	Cup	64.2
	6/25	H. B. W.	Cup	81.5
	6/25	H. B. W.	Cup	66.0
6/29	6/29	B. L. W.	Turbidimetric	56.2
	6/29	B. L. W.	Turbidimetric	58.6
	6/29	B. L. W.	Turbidimetric	58.6
	7/2	H. B. W.	Turbidimetric	55.4
	7/2	H. B. W.	Turbidimetric	61.7
	7/2	H. B. W.	Turbidimetric	63.0

Average values

SAMPLE	AVERAGE OF INDIVIDUAL ASSAYS ON EACH SOLUTION	REMARKS
4/28	60.9	Value of 85 excluded from averages
5/4	61.0	
5/12	60.6	
5/20	59.5	
6/22	60.8	
6/29	58.9	
Grand average..............	60.3	

* Obviously too high, discarded.

group to assay penicillin. Subcultures are available from these laboratories upon request. Use of a penicillin standard tends to reduce the importance of the authentic *S. aureus* H culture; however, when a standard is not employed,

it is imperative to use the H strain. It is virtually meaningless to use any strain of *S. aureus* for a quantitative method because different strains may vary markedly in their sensitivity. This was already noted for certain other bacteria in the Abraham, *et al.* paper (1941). Table 3 compares the sensitivities of a number of different strains of *S. aureus* and stresses the desirability of using a standard strain for penicillin assays.

Tables 4 and 5 bring out the variations in the sensitivity of *S. aureus* H when assayed at different times and on different media. It is obvious that a significant variation in sensitivity may occur from time to time and from medium to medium. The only explanation at present is the empirical claim for "variation".

TABLE 3

Sensitivity of different strains of S. aureus

Figures represent Florey units per ml. of brain-heart agar causing inhibition of a streak of the organism. Where two figures are given, the first did not inhibit and the second did.

STRAIN	FLOREY UNITS
1	0.0096–0.108
2	.024 - .030
3	.030
4	.024
5	.030
6	.010 - .012
7	.030 - .060
8	.60
9	.03 - .10
10	.03 - .10
11	.01 - .03

The following four strains were tested in liquid nutrient broth by the dilution method:

STRAIN	INHIBITING DILUTION OF PENICILLIN BROTH
A	50
B	166–250
C	166–250
D	50–100

Further to minimize the variation due to condition of inoculum, the following procedure for handling the test organism has been adopted. Stock nutrient agar slants are stored in a refrigerator. At monthly intervals or so, subculture slants are made from them, which, in turn, are kept cold after overnight growth at 37°C. Overnight broth cultures are made each day from a slant, the same slant being kept cold and used for about a week. Serial transfer in broth has at times led to abnormalities such as development of resistant cells and of cells subject to lysis during penicillin inhibition.

Individual methods of assay

Serial dilution. This procedure consists of adding by suitable dilution procedures different amounts of penicillin to either liquid or solid media.

a. Liquid broth assays: Fleming, in 1929, first utilized the serial dilution procedure for penicillin, and many workers consider it, or modifications thereof the most useful for routine assays. But again it is important to specify the order of accuracy required. As pointed out above, without a penicillin standard the results cannot be comparable to those of other laboratories unless the standard strain of *S. aureus* H is employed as the test organism. Even then the results may be taken only as an order of magnitude of potency owing to uncontrollable daily fluctuations. The precision of this method depends on the steps in dilution, and in our experience a clear-cut plus and minus endpoint occurs only where the dilutions differ by a factor of at least 50%. This accuracy is tolerable only in those cases where real quantitative results are not the main object.

Sterile samples and aseptic technique are required. In many cases the samples must be Seitz-filtered before use. With high potency samples the high

TABLE 4

Variations in sensitivity of S. aureus H on different media

Figures represent Florey units per ml. of agar causing inhibition of a streak of the organism. Where two figures are given, the first did not inhibit and the second did.

DATE	MEDIUM	FLOREY UNITS
7/15/42	Blood agar	0.005–.0075
9/ 3/42	Brain-heart	0.014–.024
10/ 8/42	Nutrient	0.10 –.30
10/ 8/42	Brain-heart	0.010–.030
10/13/42	Brain-heart	0.010–.030
10/13/42	Nutrient	0.030
11/12/42	Brain-heart	0.013–.014
1/ 6/43	Yeast-extract glucose	0.090–.012
1/ 6/43	Yeast-extract	0.015

dilution required to reach the endpoint usually eliminates contaminations. With small amounts of material filtration losses may be significant, either mechanically or possibly through adsorption. In general, Seitz filtration may be accomplished without loss of potency. Often samples which are too contaminated for use directly may be pasteurized (60°C. for 30 minutes). However, a definite though variable deterioration usually results from pasteurization, ranging from 5 to 30 per cent of the activity.

Daily employment of a penicillin standard puts the dilution assay on a sound basis, but, nevertheless, the factor of poor sensitivity is not eliminated. As mentioned previously, this requirement depends on the experimental objectives. Steps in dilution less than 100 per cent as a rule give progressive degrees of inhibition which make a decision as to the real endpoint difficult. Furthermore, on penicillin samples of unknown potency, use of smaller steps in dilution necessitates an excessive number of dilutions to insure the end-point falling within the range selected. The alternative would be to cover a wide range with larger intervals between dilutions, thereby establishing the approximate value, and

then, on a second assay, run it down within a limited range. In any case, extra work is involved, and where large numbers of assays are run routinely, such procedures are excessive. The act of inoculating each dilution tube separately is itself an undesirable routine. The endpoint obtained by this method may be anywhere between that dilution which inhibits completely and that which does not. For many kinds of chemical experiments on penicillin a specific value is desirable and such accuracy is inadequate.

Rammelkamp's (1942) modification of the tube dilution method is designed to meet the requirements of the clinician who is faced with the problem of having only small amounts of blood or body fluid available for assay. Very low levels of penicillin, such as would be contained in a small amount of blood must be measured. This was achieved with a strain of *Streptococcus hemolyticus*, selected

TABLE 5

Variability in daily plate assays on a sample of penicillin

(This solution contained 0.24 mg. crude penicillin per ml.)

DATE ASSAYED	ML. PER 10 ML. AGAR REQUIRED TO INHIBIT S. AUREUS
5/14/42	0.001
5/15	.0009
5/16	.0009
5/18	.0011
5/19	less than .0008
5/20	greater than .0011
5/21	.0009–.001
5/22	less than .0008
5/23	.0011
5/25	less than .0008
5/26	.0011
5/27	greater than .0011
5/28	.001–.002

because of its high sensitivity to penicillin. Thus, levels and amounts of penicillin as low as 0.0039 Florey unit in 0.2 ml. [see also (Foster, 1942)] can be measured in this way by comparison with a standard. The sensitivity undoubtedly could be further increased with a more susceptible test organism. The presence of 1% erythrocytes in the veal infusion medium facilitates reading the endpoint resulting through hemolysis accompanying growth. Although this method permits a useful sensitivity, its accuracy is good only to 100 per cent since the dilutions differ by factors of 2, and in certain cases only partial inhibition is obtained. While undoubtedly of value to the clinician, this order of accuracy is quite unsuited for chemical studies.

Through the courtesy of Dr. J. C. Hoogerheide of the Squibb Biological Laboratories, the details of a modified broth dilution method devised in those laboratories are available. An accuracy of 15 per cent is stated. Two ml. amounts of beef-heart infusion broth containing 0.25% glucose and inoculated with a 1 to 10 dilution of a 6-hour culture of *S. aureus* H are distributed in a

series of ten small tubes. To one tube each is added respectively with a micro pipette, 0.10, .09, .08, .07, .06, .05, .045, .040, .035, and .030 ml. of a standard made up to contain 0.60 Florey units per ml. Similar amounts of the unknowns diluted to 0.60 units per ml. on the basis of predicted potency are added to other series of tubes. After overnight incubation, the endpoint is stated to be reproducibly clear-cut between two tubes. The endpoint is not necessarily that dilution which inhibits growth completely but, rather, that dilution in which growth is so retarded that the organism fails to develop a uniform turbidity throughout the broth, forming, instead, a sediment in the bottom of the tube leaving a clear supernatant. The endpoint on the unknown contains that amount of penicillin corresponding to the endpoint in the standard series. Samples would have to be reasonably free from contaminating bacteria. Aside from the tedium of making a large number of accurately measured pipettings, this would appear to be one of the most promising methods for practical application.

b. Plate assays: Since these are serial dilution methods, the same comments outlined above on expression of activities and numbers of dilutions required also apply here. Different amounts of the appropriate dilution of penicillin are mixed with 10 ml. of melted agar in plates, and after solidification, the surface of the plate is streaked with the test organism and the point of complete inhibition obtained after overnight incubation. An incidental advantage here is that as many as six different test organisms may be tested on each plate, i.e., at each dilution, but this is unnecessary for routine assays. One feature of dubious advantage is that fairly heavily contaminated samples can still be read, but this loses importance because even lightly contaminated samples, especially in solution form, can be partially inactivated by penicillase-producing bacterial contaminants (Abraham and Chain, 1940).

The cooled melted (nutrient or brain-heart) agar may be seeded with the test organism before distributing the agar, thereby eliminating streaking. About 0.1 ml. of a 20 hour nutrient broth culture is used per 100 ml. agar. Plates remaining clear after overnight incubation have inhibited the test organism. While the vast majority of cells may be inhibited, a few isolated colonies persistently develop over two or three successive dilutions. These resistant colonies usually are insignificant in numbers and may be disregarded for practical purposes. In many instances the inhibition is quite gradual in a series of close dilutions, making the endpoint difficult to establish.

Plate methods, requiring numbers of plates and large volumes of agar, are at best very tedious and on account of the disadvantages mentioned above are not to be preferred. A standard is imperative for best accuracy. The variability of results obtained from day to day on a stable sample without a standard is exemplified in table 5.

The results show the frequency of "greater than" or "less than" values, which, of course, require repetition of the assay. Where two values are given, partial inhibition was observed over that range.

The pH of the assay medium has an unexpected effect on penicillin activity in

the plate method. Nutrient agar was adjusted in lots to pH 5.5, 6.0, 6.5, and 7.0, respectively, and the plate assay run as described with *S. aureus* H. Table 6 shows that the activity of penicillin is increased with lowered pH at least down to pH 5.5 and that a difference of 0.5 pH unit definitely influences the results. A sensitivity effect at pH 5.5 greater than 3-fold that at 7.0 was obtained in this experiment. The same type of effect was noted with other sensitive bacteria, including *S. hemolyticus*, *Micrococcus lysodeikticus*, *Salmonella paratyphi* A, and *Staphylococcus albus*. The interpretation of this effect is not clear at present. Indeed, when this experiment was repeated in liquid media with the turbidimetric method discussed below, no significant difference in penicillin action at the various pH levels could be detected. The details of this experiment together with a discussion will be given in the next paper of this series.

Turbidimetric method. This method has already been described by this laboratory (Foster, 1942), and the details will not be repeated here. In our experience it is the most accurate method available, but to attain this high

TABLE 6

Effect of pH of test medium on inhibition of S. aureus in plate assay

PENICILLIN (MICROGRAMS PER ML. AGAR)	pH			
	5.5	6.0	6.5	7.0
0	+	+	+	+
.08	−	+	+	+
.12	−	−	+	+
.16	−	−	±	+
.20	−	−	−	+
.24	−	−	−	±

+, growth; −, inhibition.

precision, the special and fastidious techniques required render it rather cumbersome for the handling of many assays. An accuracy of ±10 to 15 per cent is obtainable. For special purposes, such as, for example, in stability experiments, molecular weight studies by diffusion, etc., it has proved most valuable.

The method is based on the proportional inhibition of growth of *S. aureus* H in liquid media as a function of penicillin concentration. Growth measurements are made with a photoelectric colorimeter. A standard curve is run daily with the unknowns, which themselves are run at 3 to 5 different dilutions, depending on how many can be predicted to fall on the central three-quarters region of the standard curve. Potencies of the unknowns are computed after comparison with the standard curve which covers accurately about an 8-fold range in values. Rigid observance of technique, accuracy, and the need for aseptic precautions detract from its usefulness as a general method. In addition, operations attendant to reading the turbidities are time-consuming.

Turbidimetry offers a means of securing results in a time shorter than the 16 or 20 hours required at present. For routine work little is gained by setting up 20 to 50 assays and having the results in the same working day since probably little further experimental work could be done that day on the basis of those

results. In certain instances, however, a real need exists for a short time assay. For example, it is crucial to determine when a large scale production batch of penicillin is ready for extraction. Maximum accumulation of penicillin in cultures of *Penicillium notatum* frequently persists for only a brief period, after which the potency may drop off sharply. To know the strength of a production batch within a few hours of the time of sampling would aid greatly in eliminating such losses. The time required for the assay can, theoretically, be reduced by increasing the sensitivity of the method used to measure growth. With the sensitivity that the regular Evelyn photoelectric colorimeter affords, a satisfactory growth spread can be obtained in about 4 hours by accelerating the rate of growth by use of a heavy inoculum. Figure 1 shows the effect of different amounts of a 20 hour nutrient broth culture as inoculum on the growth curve of *S. aureus* hourly for 5 hours. The lag phase seems appreciable only below 0.4 ml. inoculum, and the maximum rate of growth is constant for this and the higher levels. In 4 hours the 0.4 ml. inoculum made the greatest increase in turbidity at a constant rate and, hence, is the most suitable level for an assay of 4 hours' duration. Figure 2 shows the penicillin inhibition in nutrient broth after a short time. The inset shows the type of standard curve obtained in 16 hours' incubation. Only the first half of the short time curves are useful for assay purposes. Beyond that, the turbidity change with increments of penicillin is too small. Before reading short time turbidities, all tubes are placed in cold water to stop growth. Samples that have been assayed by the short time method agree with the regular overnight method, but results generally are less reproducible in the former. Nevertheless, this method has found valuable application in our laboratories.

Results obtained turbidimetrically check well with values obtained by the Oxford cup method. The following list compares the values on 7 samples:

SAMPLE	FLOREY UNITS PER MG.		PERCENTAGE DIFFERENCE
	Turbidimetric	Cup	
A	0.97	0.90	+10
B	15.4	14.7	+5
C	7.3	7.0	+4
D	9.7	11.2	−15
E	11.0	13.2	−17
F	26.0	26.4	−2
G	17.4	17.2	+1

Oxford cup method. Except for certain modifications, the published details (Abraham, *et al.*, 1941) of this procedure will not be repeated here. Small porcelain or glass tubes set on the surface of solidified inoculated agar plates are filled with penicillin solutions and incubated. Where the penicillin diffuses out into the agar, growth of the test organism is inhibited and a circular clear zone results. Within limits, the diameter of the zone is a function of penicillin concentration, and the unknowns can be calculated in terms of the standard curve of inhibition which is drawn for each day's run.

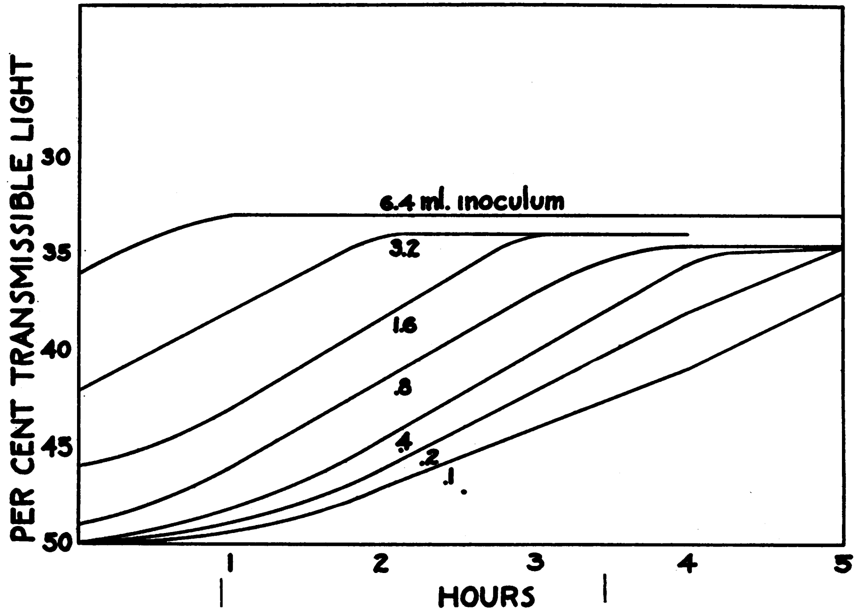

FIG. 1. INOCULUM SIZE AND S. AUREUS H GROWTH

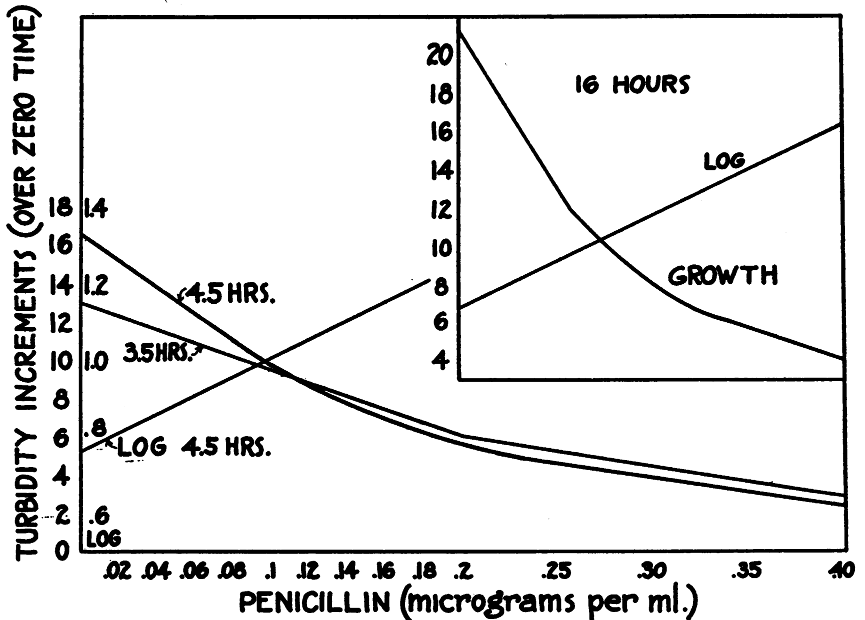

FIG. 2. PENICILLIN INHIBITION OF S. AUREUS WITH TIME
This penicillin contained 10 Florey units per mgm. Medium: nutrient broth.

In our experience the cup method is by far the most useful routine assay method. One person can assay at least twice as many samples with the cup method as by any other tried. If the potency of the sample can be predicted

approximately, only one dilution is required for assay; otherwise 2 and at most 3 would be needed. The standard curve covers about a 7-fold range (0.2 to 1.5 Florey units per ml.). A specific value is always obtained instead of the range characteristic of dilution methods. We average the quadruplicate values obtained on the final dilution predicted to contain one Florey unit per ml. At some sacrifice in accuracy, only two or three replicates may be run, but this accuracy probably would be well within the range of that of dilution methods. Our experience substantiates the ±25 per cent accuracy claimed for the method (Abraham *et al.*, 1941). In practice, the work involved in running extra replicates is practically negligible. Most laboratories which employ this method concur in regard to its simplicity and general usefulness. The procedure is not nearly so cumbersome as other methods.

The unwieldy procedure of inoculation described originally by the Oxford workers can be effectively replaced by seeding the cooled, melted agar before pouring the plates. One-tenth ml. of a 24-hour nutrient broth culture is used to seed 100 ml. of cooled, melted agar. The agar should be uniformly measured into each plate by apportioning 13 ml. amounts with an open tip pipette.

The sharp bevelled edge of the cups recommended by the Oxford group to obtain a seal between the tube and the surface of the agar is unnecessary. Difficulties in obtaining such bevelled tubes has tended to discourage their use. Uniform length tubes made from 9 mm. outside diameter (1 mm. wall thickness) glass tubing with a glass cutting machine work just as satisfactorily if, just before planting on the agar, one end of the tube is passed momentarily through a flame to warm it slightly. It melts the agar locally, which sets again practically immediately, making a good seal. This step requires very little extra time in setting up the plates for use. The tubes can be from 6 to 12 mm. in length, but those employed on any one assay run must all be of uniform size, and they must be filled uniformly to the top with the solution.

Serious irregularities encountered with large numbers of assays revealed that if as little as one hour elapses between the times that the standard penicillin solutions and unknown samples are put into the cups, a significant discrepancy in values occurs. Handling a large number of assays is time-consuming, and no matter when the standard is set up, some of the samples are likely to be set up and incubated two or more hours before or after. The following experiment illustrates this effect. The cups in seeded plates were filled with portions of one solution at four hourly intervals. All plates were left on the laboratory bench until the last one was completed and then incubated at 37°C. overnight. The results are listed as follows:

TIME OF ASSAY	AVERAGE DIAMETER OF INHIBITION ZONES	FLOREY UNITS	PER CENT DIFFERENCES FROM TRUE VALUE (4:00 P.M.)
	mm.		
1:00 p.m.	24.8	1.28	68
2:00	23.5	0.97	27
3:00	23.0	0.88	16
4:00	22.4	0.76	—

Thus, plates allowed to stand at room temperature for 1, 2 and 3 hours before incubation give proportionately higher values than the sample incubated at 37°C. immediately after filling. The differences are large enough to be serious. The explanation lies in the fact that during the intervals of 3, 2 and 1 hour, respectively, the penicillin diffuses out into the surrounding agar. Meanwhile, at room temperature the bacterial cells seeded in the agar make very little, if any, growth, but when placed at 37°C., the growth is rapid. Those plates standing at room temperature show larger zones because the penicillin has a longer time to diffuse before growth appears. In the case of incubation directly at 37°C., growth appears before diffusion is complete, hence the smaller zones. For uniform results it is apparent that the cells should make no growth from the time the agar is poured until the plates are placed at 37°C. Possibly this could be achieved by keeping the plates cold until diffusion is complete; this is being tested. To standardize diffusion with respect to growth, as short a time as possible should elapse between filling the cups and incubating at 37°C. Thus, there is an equal period of growth during the active diffusion for all plates from the time the samples are added. In our laboratories, four plates, each containing six cups, are handled at a time. These accommodate six samples on each plate. Plates and dilutions are prepared in advance and stored cold as below; filling the cups in four plates takes about ten minutes after which each set of plates is immediately placed at 37°C. When assaying low potency *Penicillium* filtrates extreme pH's of the samples should be neutralized.

To have a number of poured seeded plates on hand at one time and to prevent growth until the plates are used, a large number of seeded plates are made and stored in a refrigerator and withdrawn for use four at a time. This idea was tested by assaying a sample in 18 replicates on seeded plates that had been stored in a refrigerator for 0, 1.5 and 23 hours. The large number of replicates was taken to give an indication of the normal variation in zone size. The standard was run at zero time.

HOURS IN REFRIGERATOR	DIAMETER OF INHIBITION ZONES (MM.)						AVERAGE ZONE SIZE	FLOREY UNITS PER MGM. OF SAMPLE	DIFFERENCES FROM ZERO TIME
							mm.		*per cent*
	28.1	28.0	27.5	27.8	28.0	27.5			
0	28.5	28.0	28.0	28.0	27.5	28.0	28.0	144	
	28.0	28.2	27.5	28.0	28.0	28.0			
1½	27.5	28.0	28.1	28.5	28.0	28.2			
	29.0	28.0	28.3	27.3	29.0	28.0	28.1	147	2
	28.0	28.0	28.0	28.0	28.1	27.5			
23	29.0	29.0	27.3	27.0	29.0	29.0			
	28.5	28.2	28.2	27.5	28.0	28.0	28.2	150	4
	28.0	29.0	28.6	27.7	27.5	29.0			

It is evident that so long as growth is not permitted to commence until just after filling the cups, the seeded plates may be stored at refrigerator temperature at

least as long as 23 hours without influencing the assay results. The variation between replicates is small, but it is important to have at least two replicate cups, and preferably more. The best average value is obtained by having each cup of each sample on different plates.

One practiced person can conveniently assay in quadruplicate cups about 40 to 50 samples per day. Readings are facilitated by placing the Petri dish with zones over an exposed photographic plate with fine millimeter lines cut into the gelatin. This is set over an opening in a block with a diffuse lighting source shining through. The size of the zones is defined by the white lines against the dark background.

Through the kindness of Dr. A. H. Dowdy of the Strong Memorial Hospital, University of Rochester, we are able to reveal an unpublished novel simplification of the cup method which Helen and James Vincent of that institution have developed in collaboration with Dr. Dowdy. Instead of using cups, discs of filter paper saturated with the penicillin samples are used to produce the zones of inhibition. This feature should reduce materially the labor involved in setting up large numbers of assays, and, therefore, ultimately may find valuable application.

Miscellaneous Assay Methods

A number of different principles have been tested for possible use in measuring penicillin and especially for short time assays. For the most part, unpromising results have been obtained; in some cases the preparation and special technique are not conveniently adaptable. Following are some of the ideas tried without success.

1. Methylene-blue reduction by washed cells of a susceptible organism.
2. Inhibition of luminescence in cultures of luminescent bacteria.
3. Microscopic observation of cessation of motility of bacteria.
4. Microscopic observation of appearance of enlarged and involution cell shapes.
5. Titration of acid formed by lactic acid bacteria, *S. aureus*, etc. This may have application for overnight assay.

SUMMARY

The principles, merits and disadvantages of the different bacteriological methods used for the quantitative determination of penicillin are summarized and discussed. The numbers of assays to be run, the accuracy desired, and the facilities available govern the final choice of a method. A standard penicillin preparation of known Florey unitage to be run daily with unknowns is recommended to minimize uncontrollable fluctuations. Serial dilution methods, with liquid or solid media, are wanting in accuracy and do not yield specific values for penicillin activity. A modified broth method devised by the Squibb laboratories gives good sensitivity and is practical. The turbidimetric method has application in special types of investigations where high accuracy is required but is too fastidious for routine assays. The Oxford cup method (modified) is

an excellent all-around method for routine assays, especially for large numbers. Its accuracy is good, a specific value is obtained, and, from the standpoint of labor involved, it is quite the most rapid procedure where accuracy is required. A number of experiments characterizing the different methods are reported.

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.

Abraham, E. P., Chain, E., and Holiday, E. R. 1942 Purification and some physical and chemical properties of penicillin; with a note on the spectrographic examination of penicillin. Brit. J. Exptl. Path., **23**, 103–120.

Catch, J. R., Cook, A. H., and Heilbron, I. M. 1942 Purification and chemistry of penicillin. Nature, **150**, 633–634.

Chain, E., Florey, H. W., Gardner, A. D., Heatley, N. G., Jennings, M. A., Orr-Ewing, J., and Sanders, A. G. 1940 Penicillin as a chemotherapeutic agent. Lancet, **239**, 226–228.

Coulthard, C. E., Short, W. F., Skrimshire, F. H., Birkinshaw, J. H., Michealis, R., Sykes, G., Standfast, F. B., and Raistrick, H. 1942 Notatin: an anti-bacterial glucose-aerodehydrogenase from *Penicillium notatum* Westling. Nature, **150**, 634–635.

Fleming, A. 1929 An antibacterial action of cultures of *Penicillium* with special reference to their use in isolation of *B. influenzae*. Brit. J. Exptl. Path., **10**, 226–236.

Fleming, A. 1942 In-vitro tests of penicillin potency. Lancet, **242**, 732–740.

Foster, J. W. 1942 Quantitative estimation of penicillin. J. Biol. Chem., **144**, 285–286.

Hobby, G. L., Meyer, K., and Chaffee, E. 1942 Chemotherapeutic activity of penicillin. Proc. Soc. Exptl. Biol. Med., **50**, 285–288.

Kocholaty, W. 1942 Cultural characteristics of *Penicillium notatum* in relation to the production of antibacterial substances. Indication of the dual nature of the antibacterial substance. J. Bact., **44**, 469–477.

McKee, C. M., and Rake, G. 1942 Activity of penicillin against strains of pneumococci resistant to sulfonamide drugs. Proc. Soc. Exptl. Biol. Med., **51**, 275–278.

Meyer, K., Chaffee, E., Hobby, G. L., Dawson, M. H., Schwenk, E., and Fleischer, G. 1942 On penicillin. Science, **96**, 20–21.

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

Roberts, E. C., Cain, C. K., Muir, R. D., Reithel, F. J., Gaby, W. L., van Bruggen, J. T., Homan, D. M., Katzman, P. A., Jones, L. R., and Doisy, E. A. 1943 Penicillin B, an antibacterial substance from *Penicillium notatum*. J. Biol. Chem., **147**, 47–58.

Waksman, S. A., and Woodruff, H. B. 1942 Selective antibiotic action of various substances of microbial origin. J. Bact., **44**, 373–384.