THE PAPER-DISC AGAR-PLATE METHOD FOR THE ASSAY OF ANTIBIOTIC SUBSTANCES[1]

EDWIN J. DE BEER AND MARION B. SHERWOOD

The Wellcome Research Laboratories, Tuckahoe, New York

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This article has a twofold purpose. In part I a simplified modification of the Oxford plate method is described; and in part II some of the more important factors affecting the assay are discussed.

I

In brief, the new modification consists in substituting paper discs, cut from ordinary filter paper, for glass cylinders which serve as reservoirs from which the antibiotic substance diffuses into the agar medium. Preliminary reports on paper disc methods have been published (Vincent and Vincent, 1944; Epstein, Foley, Perrine, and Lee, 1944; Sherwood, Falco, and de Beer, 1944). The method possesses several practical advantages: (1) The measurement of volumes is accomplished simply by touching the edge of the paper disc to the material to be assayed, because discs of the same size absorb surprisingly uniform amounts of solution. (2) Only small amounts of solution are necessary, 0.02 ml per disc. (3) Quantities of penicillin as low as 0.009 Florey units per disc give measurable zones of inhibition. (4) The labor of washing and caring for the small cylinders and pipettes is eliminated.

Description of the Paper Disc Assay Method

Nutrient agar. Nutrient agar (final pH approximately 7.0) is prepared according to the *U. S. Department of Agriculture Circular* 198 (Ruehle and Brewer, 1931).

Bacillus subtilis spore suspension. A susceptible culture of *B. subtilis* (Foster and Woodruff, 1943) is grown on nutrient agar of the specified composition for 10 to 14 days at 37 C until sporulation appears to be at a maximum. The growth is uniformly suspended in sterile distilled water. The suspension is filtered through sterile absorbent cotton and heated at 80 to 85 C to destroy vegetative forms. The number of viable organisms is counted. Such a suspension may be stored in a refrigerator for at least 9 months.

Preparation of test plates. Insofar as possible, flat-bottomed petri dishes are selected. Sufficient *B. subtilis* spore suspension is added to the nutrient agar at a temperature of 42 to 45 C to ensure approximately 2.5×10^7 viable organisms per 100 ml. (In order to avoid delay in germination of the spores, the suspension used for inoculation is allowed to come to room temperature

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before use.) Twenty ml of this medium are measured into each petri dish (90 × 15 mm, inner dimensions). As soon as the agar has gelled, the plates are inverted and stored in the refrigerator for at least 16 hours so that the surface water will disappear. Storage for more than 96 hours is inadvisable because of the gradual diminution in the number of viable organisms.

Paper discs. Paper discs approximately 1 cm in diameter are carefully cut from multiple layers (16) of thin filter paper (E. & D. no. 615) with a sharp cork borer. If sterilization is required, the discs may be autoclaved and allowed to dry before use. Hot-air sterilization reduces the absorptive capacity of the paper, but drying for 1 hour at 100 C has little effect.

Preparation of the standard and the test dilutions. For the assay of penicillin preparations, the stronger solution of the standard should contain from 50 to 300 Florey units per ml. A portion of this standard solution is added to sufficient buffer or other diluent to make a 1:4 dilution. A 1:4 dilution of the "unknown" is similarly prepared.

Performance of the test. A paper disc is grasped with finely pointed forceps, and its lower edge is touched to the surface of the test solution. As soon as the upper edge of the disc appears dampened, it is removed from contact with the solution. Excess liquid is drained from the lower edge of the disc by touching it to the side of the container. Clean forceps are used for each solution, or the forceps are flamed and cooled between dilutions. The discs are spaced upon the agar to allow adequate room for the development of the zones of inhibition. When 90 × 15 mm petri dishes are used, the two discs used for the standard (one for each dilution) are placed in adjacent quadrants and the two for the "unknown" are set in the remaining quadrants so that the larger doses (standard and "unknown") are opposite each other. Reference marks on the outside of the petri dishes identify the discs. At least 4 replicate plates are prepared for each assay.

Measurement of results. Growth is occasionally rapid enough at 37 C so that the edges of the zones of inhibition are sufficiently defined to permit measurement of the zone diameter within 4 hours. However, incubation for 5 or 6 hours gives sharper definition. The diameter of the inhibition zone may be measured to the nearest $\frac{1}{4}$ mm by means of direct light and a millimeter rule. If it is desirable to increase the precision of the assay, measuring calipers or projection apparatus should be used since the error of the assay (other things being equal) depends upon the accuracy of the measurements.

Calculation of results. A simple method, involving only a single equation, has been devised for calculating the results of any assay of this design (Sherwood, Falco, and de Beer, 1944). In the following equation, *2* is the factor for converting to per cent and d is the log of the ratio of the stronger concentration to the weaker. This ratio between dilutions must be the same for both the standard penicillin and the "unknown" being assayed. The terms S_1, S_2, U_1, and U_2 are the sums indicated in the example cited. The same calculation, when readings are taken to the nearest 0.5 mm, gave a figure of 21.3 per cent. The true percentage, according to the dilutions made, was 20 per cent.

Bliss (1944a, 1944b) has described a simplified method of calculation applicable to this type of data that gives both the potency and its associated error. Knudsen (1945) has devised a graphic method for making the computations. Factorial analyses of typical assays, performed by the method under discussion and read to the nearest ½ mm, indicate an experimental error of approximately ± 8 per cent. This error can be readily decreased by increasing the number of plates

TABLE 1

Assay of an unknown penicillin-containing solution

PLATE NO.	DIAMETER (IN MM) OF ZONES OF INHIBITION			
	Standard		Unknown	
	1:4	Undiluted	1:4	Undiluted
1	23.0	27.0	17.0	22.5
2	23.75	27.75	18.5	22.75
3	23.25	27.0	18.0	22.75
4	22.5	26.75	18.75	22.0
Sums............	$S_1 = 92.5$	$S_2 = 108.5$	$U_1 = 72.25$	$U_2 = 90.0$

$$\text{Potency} = \text{Antilog}\left[2 + d\left(\frac{(U_2 + U_1) - (S_2 + S_1)}{(U_2 - U_1) + (S_2 - S_1)}\right)\right]$$

$$\text{Potency} = \text{Antilog}\left[2 + 0.602\left(\frac{(90.0 + 72.25) - (108.5 + 92.5)}{(90.0 - 72.25) + (108.5 - 92.5)}\right)\right]$$

$$\text{Potency} = \text{Antilog } 1.3088 = 20.36\%$$

TABLE 2

The results of assays of known penicillin solutions

TRUE CONCENTRATION*	CONCENTRATION FOUND BY ACTUAL ASSAY				
	Purified penicillin	*P. notatum* filtrate		Infranatant solution	
	M. B. S.	E. D. S.	M. B. S.	E. D. S.	M. B. S.
80	76.5	85.2	85.2	76.6	75.5
60	57.3	64.3	60.6	66.5	61.0
40	40.1	41.4	45.7		
20	21.0	20.1	21.3	19.5	19.1

* Expressed as per cent of the original, undiluted solutions.

used and by measuring the diameters of the zones of inhibition more precisely than to the nearest ½ mm.

Typical Assay Results

Some idea of the accuracy obtainable by the paper disc method, when tests are read to the nearest ½ mm, can be gained from table 2, which gives the results of assays of penicillin solutions of known relative concentration. Such dilutions

of penicillin were prepared and designated as the "unknowns." The original undiluted material served as the standard. The table shows also that different assayists (M. B. S. and E. D. S.) obtained results which agreed closely.

II

The factors which contribute to the precision of the assay method described are (1) the uniformity of the volume absorbed per disc; (2) a constant depth of agar through which the antibiotic diffuses; (3) a uniform seed; and (4) a linear log dose-response curve covering a wide range. Although the method is applicable both qualitatively and quantitatively to other antibiotics and other susceptible organisms, it should be stated that there are definite limitations to its use, e.g., the size or structure of the molecule of the antibiotic or its solubility may hinder or even prevent its diffusion through agar.

TABLE 3

Uniformity of imbibition of 9.9-mm discs

	DISC WEIGHT	WATER IMBIBED
	mg	*mg*
	6.40	18.85
	5.60	17.00
	5.60	16.50
	6.00	18.75
	6.10	19.40
	6.25	17.10
	6.80	19.20
	6.20	19.15
	6.50	20.10
	6.65	19.45
Mean weight:	6.21	18.55
Standard deviation:	0.127	1.227

The Uniformity of the Volume Absorbed per Disc

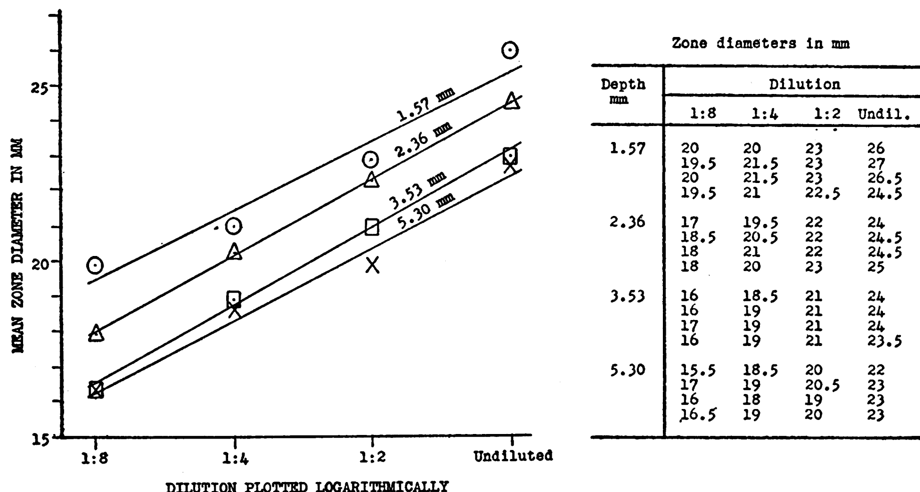
The paper discs absorb surprisingly uniform amounts of solution. This is shown in table 3 which gives the weights of 10 paper discs selected at random and the amounts of water absorbed by each disc. This is of fundamental importance, since uniform responses could not be expected with widely variable volumes.

The small amount of solution required for each disc, approximately 0.02 ml, is of advantage when limited amounts of material are available for assay purposes.

Agar Depth

Since the Oxford plate assay depends, fundamentally, on the diffusion of the antibiotic through the agar medium, any factor which tends to modify the diffusion process might significantly affect the results. One such factor is the

agar depth. To investigate this possibility, 4 sets of 4 plates each were poured. The first set contained 10 ml of medium per plate, and each successive set contained 50 per cent more medium. The actual depths were 1.57 mm, 2.36 mm, 3.53 mm, and 5.30 mm, respectively, for the 4 sets. Four concentrations of penicillin were placed on each plate so that dose-response curves (each



Zone diameters in mm

Depth mm	Dilution			
	1:8	1:4	1:2	Undil.
1.57	20	20	23	26
	19.5	21.5	23	27
	20	21.5	23	26.5
	19.5	21	22.5	24.5
2.36	17	19.5	22	24
	18.5	20.5	22	24.5
	18	21	22	24.5
	18	20	23	25
3.53	16	18.5	21	24
	16	19	21	24
	17	19	21	24
	16	19	21	23.5
5.30	15.5	18.5	20	22
	17	19	20.5	23
	16	18	19	23
	16.5	19	20	23

FIG. 1. THE INFLUENCE OF AGAR DEPTH ON THE DOSE-RESPONSE CURVE

TABLE 4

Analysis of variance of agar-depth data

SOURCE	DEGREES OF FREEDOM	MEAN SQUARES	VARIANCE RATIO (F)
Linear effect of depth upon mean diameter	1	85.078	149.26†
Curvature in the relation between depth and mean diameter	2	0.789	1.38
Between plates within depths	12	0.570	1.00
Average slope of dose-response curve (all depths)	1	389.403	1927.73†
Slope × depth	3	0.830	4.11*
Curvature on conc. (1.57 mm depth)	2	1.797	8.90†
Curvature on conc. (2.36 mm depth)	2	0.047	0.23
Curvature on conc. (3.54 mm depth)	2	0.188	0.93
Curvature on conc. (5.31 mm depth)	2	0.881	4.36*
Variation within sets	36	0.202	1.00

The word *curvature* signifies the combined quadratic and cubic terms.

* Significant.

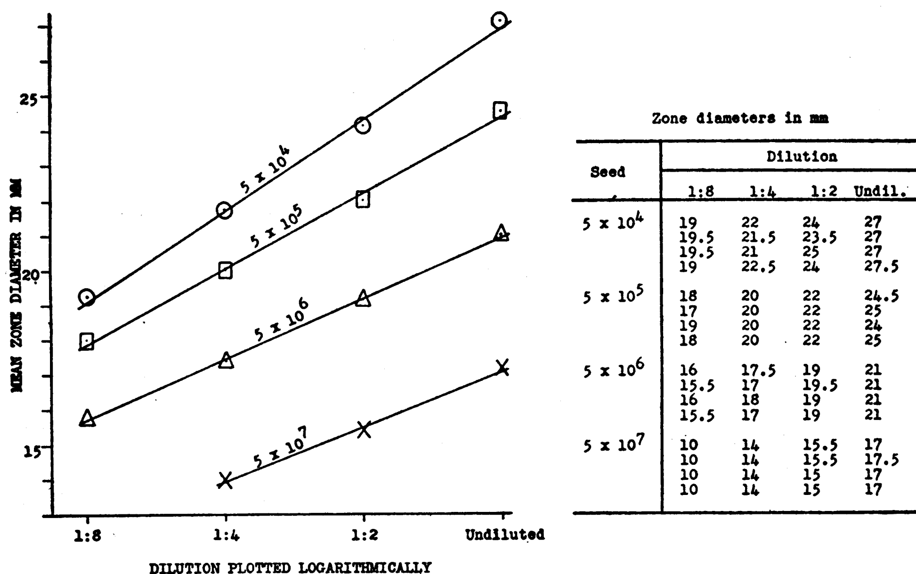
† Highly significant.

concentration in quadruplicate) could be obtained for each set. The results are plotted in figure 1, and it will be seen that definitely greater zones of inhibition were obtained when the agar was thin.

Furthermore, the data obtained with the greatest and the least depths of agar fit the calculated straight lines poorly, and the slopes of these lines vary since

they are not parallel. The analysis of variance[2] shown in table 4 confirms these observations.

The highly significant variance ratio of 149.26 shows that the diameter of the zone of inhibition is a linear function of the log agar depth. The nonsignificance of all other types of curvature as shown by an F value of 1.38 emphasizes that the relationship is almost exclusively linear. Analysis of the four dose-response curves showed that the combined quadratic and cubic terms were significant for the thinnest and thickest agar. This means that, in this experiment, these two dose-response curves departed significantly from a straight line. The diameters of the zones of inhibition which were used in plotting these curves were harder to measure than were those obtained for the two intermediate thicknesses of agar. This may, in part, explain the divergence.



Zone diameters in mm

Seed	Dilution			
	1:8	1:4	1:2	Undil.
5×10^4	19	22	24	27
	19.5	21.5	23.5	27
	19.5	21	25	27
	19	22.5	24	27.5
5×10^5	18	20	22	24.5
	17	20	22	25
	19	20	22	24
	18	20	22	25
5×10^6	16	17.5	19	21
	15.5	17	19.5	21
	16	18	19	21
	15.5	17	19	21
5×10^7	10	14	15.5	17
	10	14	15.5	17.5
	10	14	15	17
	10	14	15	17

FIG. 2. EFFECT OF QUANTITY OF ORGANISMS ON THE DOSE-RESPONSE CURVE

This experiment indicates the care that must be taken to maintain uniform agar thickness for all plates by means of such precautions as selecting petri dishes of uniform size with flat bottoms and by measuring equal volumes of medium into each plate. Extremely thin or extremely thick plates should not be prepared for routine work since the log dose-response curves obtained may not be straight lines in such cases.

Number of Test Organisms

Another problem of possible importance to the assayist is the selection of the optimum number of test organisms. To determine this factor, an experiment

[2] The analysis of variance is described in standard texts such as G. W. Snedecor's *Statistical Methods*, 1940, Iowa State College Press, Ames, Iowa, or R. A. Fisher's *Statistical Methods for Research Workers*, 8th ed., 1941, Oliver and Boyd, Edinburgh and London.

was designed along the same pattern as the previous one, except that this time the agar depth was kept constant and the number of *B. subtilis* spores per plate was varied.

Figure 2 shows that the greatest response was obtained with the smallest amount of seed. Heavy seeding tended to overcome the action of the penicillin; in fact, no measurable zone was obtained with the heaviest seed and the weakest concentration. Consequently, the balance of the experimental design was destroyed. Separate analyses of variance were calculated for each dose-response curve (table 5). The highly significant variance ratios for the linear

TABLE 5

Effect of quantity of seed on dose-response curve

SEED	ANALYSIS OF VARIANCE				s	b	λ
	Source	D.F.	M.S.	F			
5×10^4	Linear	1	135.200	564.27*	0.4895	8.638	0.0567
	Curvatures	2	0.181	0.76			
	Plates and error	12	0.240	1.00			
5×10^5	Linear	1	95.703	427.25*	0.4733	7.267	0.0651
	Curvatures	2	0.234	1.07			
	Plates and error	12	0.224	1.00			
5×10^6	Linear	1	61.250	653.33*	0.3062	5.814	0.0527
	Curvatures	2	0.031	0.33			
	Plates and error	12	0.094	1.00			
5×10^7	Linear	1	19.531	468.37*	0.2041	5.191	0.0393
	Curvatures	1	0.094	2.25			
	Plates and error	9	0.042	1.00			

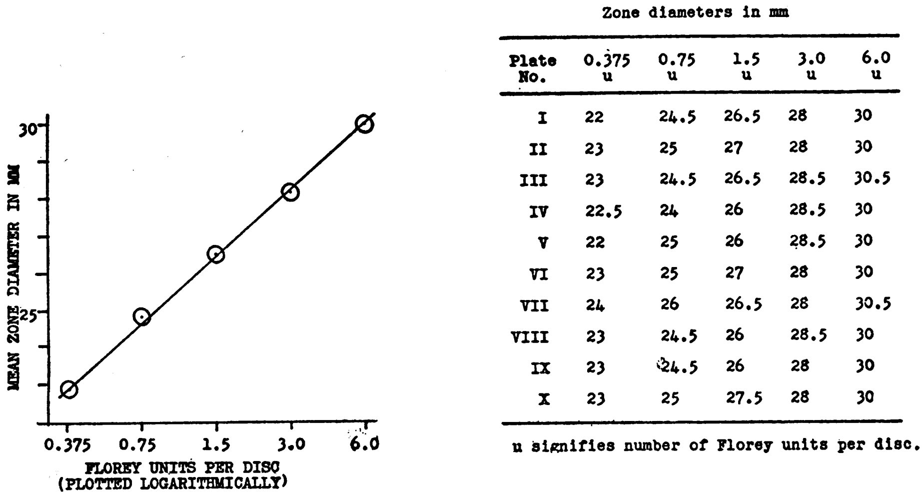
D. F. = degrees of freedom.
M. S. = mean squares.
F = variance ratio.
s = standard deviation.
b = slope.
λ = ratio of the standard deviation to the slope.
* Highly significant.

terms show that, in each case, the dose-response curve was a straight line and that the assayist would not have to fear that variations in the amount of seed would affect the shape of the dose-response curve. The precision of the assay is directly proportional to the standard deviation and inversely proportional to the slope. Therefore, it is desirable to have a small standard deviation and a large slope. The standard deviation, which is chiefly a measure of the reproducibility of the response for any single dose, tended to become smaller as the quantity of seed increased, but this was, in turn, offset by changes in slope so that the over-all precision was approximately the same for the first three seed levels. With the fourth level, practical difficulties in reading the responses

were encountered. It may be concluded that changes in seed have little effect on precision, although they may account for some of the changes in slope occasionally experienced.

Dose-Response Curve

Under controlled conditions, excellent dose-response curves result. The curve presented in figure 3 was obtained as follows: Five penicillin dilutions



Zone diameters in mm

Plate No.	0.375 u	0.75 u	1.5 u	3.0 u	6.0 u
I	22	24.5	26.5	28	30
II	23	25	27	28	30
III	23	24.5	26.5	28.5	30.5
IV	22.5	24	26	28.5	30
V	22	25	26	28.5	30
VI	23	25	27	28	30
VII	24	26	26.5	28	30.5
VIII	23	24.5	26	28.5	30
IX	23	24.5	26	28	30
X	23	25	27.5	28	30

u signifies number of Florey units per disc.

FIG. 3. DOSE-RESPONSE CURVE FOR PENICILLIN

Additional data indicate that 0.009 Florey units per disc give a measurable zone of inhibition and that the log dose-response curve is linear between 0.009 and 6.0 Florey units per disc when *B. subtilis*, strain 3R 8788 (Merck), is used.

TABLE 6

Analysis of variance of the dose-response curve

SOURCE	DEGREES OF FREEDOM	MEAN SQUARE	VARIANCE RATIO (F)
Linear	1	320.4100	1866.104*
Quadratic	1	0.0072	0.004
Other curvatures	2	0.1015	0.591
Between plates	9	0.3272	1.715
Error	36	0.1717	

* Highly significant.

were prepared, each solution being half as concentrated as the preceding one. Separate discs were dipped into each solution and placed on an agar plate so that each plate had 5 discs—each disc representing a different concentration. Ten such plates were prepared. *B. subtilis* served as the test organism. The concentration of seed was 5×10^6 spores per plate. The agar depth was 3.2 mm. Plates were incubated at 37 C for 6 hours. These data are incorporated in figure 3.

The analysis of variance given in table 6 reveals a favorable picture. The large variance ratio of 1,866 for the linear term confirms the graphic impression that the data fit a straight line unusually well. The quadratic and other types of divergence from a straight line have negligible F values. The variation between plates was not significant, as is shown by the small F term. This confirms, in another way, the conclusion drawn from table 2 that the discs absorb uniform amounts of solution.

The excellence of the log dose-response curve and its linearity over a wide range of concentrations warrants the use of the paper-disc agar-plate method for the assay of penicillin.

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SUMMARY

A simplified modification of the Oxford plate method for the assay of antibiotic substances has been described.

A study has been made of the effects of the following factors upon the precision of the assay: (a) method of measuring dose volumes, (b) depth of agar, and (c) number of test organisms per plate.

The log dose-response curve was found to be linear.

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