PENICILLIN

I. Methods of Assay[1]

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I. INTRODUCTION

Investigations involving the evaluation of bactericidal and bacteriostatic substances are dependent upon rapid and reliable assay methods. The cup plate method and the serial dilution method are the two procedures most commonly employed. Techniques for assaying penicillin have been developed by Foster (1942), Rammelkamp (1942), and Abraham, Chain, *et al.* (1941), the latter having devised the cylinder plate procedure which is a modification of the cup plate method. This modified method has been used extensively by the authors during recent months, during which time several pertinent observations and refinements have resulted. It is the purpose of this paper to present these features, as well as to describe a serial dilution procedure which has also been found useful in penicillin assaying.

II. CYLINDER PLATE METHOD

The cylinder plate procedure can only be used to test those substances which readily diffuse through agar. The method is a modification of the well-known cup plate method, yet it is unique and different in that glass or porcelain cylinders which have been ground perfectly smooth at one end, with outside edge beveled, are placed on top of an inoculated agar surface and then filled with the solution to be tested. The diameter of the zone of inhibition about the cylinder is a function of the bacteriostatic or bactericidal strength.

Many who are interested in this method may not find the paper by Abraham, Chain, *et al.* (1941) available to them; therefore, a brief description of the cylinders will be given. These are 1 cm. long and have an outside diameter of 7.9 $\pm$ 0.1 mm. (These dimensions are slightly different from those originally given by the British workers.) One end of the cylinder is beveled on the outside, and the opposite end is colored with a porcelain ink to help distinguish between the two ends. The cylinders may be made from Pyrex glass tubing, or preferably from procelain tubing if it is available. The glass cylinders have proved very satisfactory, although they chip more easily than the porcelain. The 1 cm.

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lengths are first cut, using a glass cutting saw, and then beveled; the outside edge is beveled by holding the tube at a 50°–60° angle against the side of the saw. The function of the bevel is merely to remove the sharp edge and to decrease the possibility of chipping. After the beveling, the end surface is ground perfectly flat using a fine grade of abrasive.

When Abraham, Chain, *et al.* first studied penicillin, they prepared a standard sample which could be used as a reference to evaluate new preparations. They established what is now called the Oxford unit,[3] which is defined by them as follows: "*that amount of penicillin which when dissolved in 1 c. cm. of water gives the same inhibition as this standard.*" The standard to which they referred was a solution of penicillin made up in dilute phosphate buffer which was saturated with ether and kept in the refrigerator. This solution gave a zone of inhibition having an average diameter of 24 mm.

In this Laboratory it has been found that the above definition is of little value in determining the Oxford unit; so many factors affect the size of the circle or zone of inhibition that it is meaningless to define a unit in terms of the diameter of the zone without simultaneously specifying many other conditions. The standard penicillin solution which is currently used in this Laboratory is prepared each week from a dry powdered penicillin[4] which has been assayed in terms of Oxford units per mg. This solution is not kept saturated with ether as recommended by the British workers since it has not been found necessary. When the procedure to be described is used, the Oxford unit gives zones of inhibition of a diameter of about 23 mm. rather than 24 mm.

The nutrient agar medium used for the assay plates must be clear, must support good growth, and must be easily duplicated from batch to batch. Medium I has given very satisfactory results.

Medium I

Bacto peptone	5.0 gms.
Bacto yeast extract	3.0 gms.
Commercial hydrated glucose (cerelose)	1.0 gms.
Bacto agar	15.0 gms.
Distilled water to make	1000 ml.

The medium is dispensed in 500 ml. quantities into one-liter Erlenmeyer flasks and sterilized at 15 lbs. for 20 minutes. The sterilized medium need not be kept refrigerated if it is made fresh every few days. Agar plates are poured about 24 hours before they are to be used. Large mouth pipettes are used to dispense 22 ml. of agar to each plate. The Petri dish used is somewhat deeper than that ordinarily employed, being 100 x 20 mm. for the bottom and 100 x 15 mm. for the top.

[3] We have been advised by the English workers that the term "Oxford Unit" should be used instead of the term "Florey Unit" which has appeared in some American publications.

[4] This material was supplied to us through the courtesy of Merck and Co. It was assayed in their laboratory against an authentic Oxford standard in terms of Oxford units per mg. of dry material. It was also compared in this Laboratory with a Burroughs Wellcome preparation.

A uniform inoculation of the assay plates depends on using a culture which does not contain stringy or granular growth. Experience in handling the test organism, *Staphylococcus aureus*,[5] has shown that a medium containing approximately 0.8 per cent salts is necessary to provide a uniformly turbid culture. Medium II, which contains 0.85 per cent salts, has given excellent results.

Medium II

Bacto peptone	5.0 gms.
Bacto yeast extract	1.5 gms.
Bacto beef extract	1.5 gms.
Commercial hydrated glucose (cerelose)	1.0 gms.
NaCl	3.5 gms.
1 per cent phosphate buffer pH 7.0	500 ml.
Distilled water to make	1000 ml.

Dispense 10 ml. per tube and sterilize at 15 lbs. pressure for 15 minutes.

1% phosphate buffer

This buffer solution is prepared by dissolving 2.63 grams of KH_2PO_4 and 7.36 grams of K_2HPO_4 in distilled water and bringing to a final volume of one liter. The pH should be between 6.9 and 7.1. It is advisable to heat-sterilize the buffer and keep it from bacterial and fungal contaminants. As a further insurance against contamination, this solution is kept saturated with toluene when it is used to dilute penicillin solutions.

Preparation of plates

The Petri plates containing 22 ml. of agar are flooded with 3 ml. of Medium I which has been melted and cooled to 48–50°C. and then inoculated with 1 per cent inoculum (1 ml. of a 24-hour broth culture per 100 ml. of agar). When the inoculated agar has been introduced into the dish, the plate must be tilted and rotated to give an even inoculation over the entire surface. The plates are placed on a level surface for 5–10 minutes while the agar is hardening. Following this, the cylinders are dropped from a height of approximately $\frac{1}{8}$ inch onto the agar surface. Five cylinders are placed on each plate, each cylinder being equidistant from the center. The center of the plate should not be used since the agar is usually thinnest at that point (see part III). The plates so prepared are placed on wooden boards which hold four dishes. The glass covers are now replaced with unglazed porcelain tops and the dishes are allowed to stand at room temperature for 45 to 60 minutes. If the room temperature is quite high and growth takes place readily, resulting in a hazy edge of inhibition, the inoculum may be reduced or the standing time may be reduced. On the other hand, if the zones are extra large, the inoculum may be increased or the plates may be incubated at 37° for a short time. Handling of the plates must be adjusted to conditions in the laboratory.

[5] This is the standard test organism used by the Food and Drug Administration, which is known as their strain 209.

Two cylinders on each plate are filled (to the top) with a standard penicillin solution containing 1 unit per ml., and the other three cylinders are filled with the test sample. After the cylinders are filled, the plates are carefully placed in the 37° C. incubator where they remain for 15 to 16 hours. It has been our experience that each worker is able to prepare about seventy plates per day.

Measuring the circle of inhibition

The diameter of each circle of inhibition (Plate 1, Figs. 1 and 2) is measured to the nearest 0.25 mm.; a magnifying glass has proved to be a great aid in this operation. Readings for the standards and the test samples are averaged, unless it is obvious that a cylinder has been jarred or that it leaks badly. (Zones around such cylinders are often abnormally large or not perfectly circular.) Porcelain covers which do not fit tightly may also cause variations in the size of the zones by removing more moisture at one portion of the plate than at another.

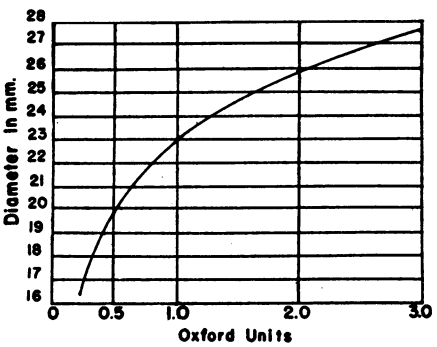

FIG. 1. A TYPICAL STANDARD CURVE

The diameter of the one-unit standard characterizes the particular curve to be used in converting the diameters of the sample to Oxford units. Variations from day to day or even from plate to plate necessitate that standards be placed on every plate and that calculations be based on the diameter of the standard for each particular plate.

The standard curve

A standard curve is prepared by determining the diameter of the circle of inhibition produced by solutions of known Oxford unit value within the range of 0.25 to 3.0 Oxford units per ml. (see fig. 1). The standard curve depicted in figure 1, in common with all other curves presented in this paper, was derived from average values of numerous determinations.

Much experience and a large amount of data have indicated that although the curve is almost constant in shape, it is lowered or raised on the Y-axis depending upon various factors which affect the diameter of the circle of inhibition. By using a family of curves it is possible to construct a table relating the standard on each plate to the samples on the same plate. Factors affecting the size

of the zone on each plate affect both sample and standard alike, therefore, if the unit value of each sample is calculated in terms of its respective standard no error is introduced.

III. FACTORS AFFECTING THE CYLINDER PLATE METHOD

Depth of the agar medium

It has been found that the depth of the agar plays an important part in determining the size of the circle of inhibition and in defining the edge of the circle. As the depth of the agar is increased, the size of the zone of inhibition is decreased and the amount of bacterial pigment is increased. The bright orange pigment of the organism helps define the edge of the zone of inhibition. When the standard quantity of 25 ml. of medium per plate is used, the edge of the

TABLE 1

The effect of pH on the activity of penicillin

Cylinder plate method

pH OF AGAR MEDIUM	MATERIAL TESTED	DIAMETERS OF ZONES OF INHIBITION	AVERAGE
6.0	2 Oxford units	24.2, 25, 25, 25, 24.2, 24, 24.5, 25, 24.7, 24.2	24.6
7.05	2 Oxford units	23, 23, 22.7, 22.2, 23, 23.2, 23, 23.2, 23.7, 24	23.1
8.0	2 Oxford units	22.2, 22.5, 22.7, 23, 22.5, 23, 23, 22.2, 23, 22.2	22.6

Serial dilution method

pH OF BROTH*	MATERIAL TESTED	DILUTIONS SHOWING INHIBITION (−) AND GROWTH (+)
6.0	pn 254-2	1:1500− 1:1600+
7.0	pn 254-2	1: 600− 1: 700+
7.6	pn 254-2	1: 600− 1: 700+

* Broth contains 0.5% phosphate salts (KH_2PO_4 + K_2HPO_4) to give desired pH.

circle of inhibition is distinct and clear; however, if only 15 ml. of medium are used, the edge of the zone of inhibition becomes indistinct.

pH of the agar medium

It has been well established that penicillin is more active against susceptible bacteria in an acid than an alkaline environment. This pH effect is more pronounced in the serial dilution method than in the cylinder plate method. It will be noted in table 1 that the activity of penicillin toward the test organism is not greatly decreased, however, when the pH of the medium is raised from 7 to 8, although the effect between pH 6.0 and 7.0 is quite marked.

In the cylinder plate method the effect of the low pH (pH 6.0) is not only shown by the increased diameter of the zone of inhibition, but by the very clear-cut edge of the zone. As the pH of the agar is increased the edge of the zone becomes more indistinct. Because of this pH effect it was considered

eminently desirable to choose arbitrarily a pH value at which to carry out all assays so that values from different cultures of different pH values would be comparable. The neutral point, pH 7.0, was chosen. As was mentioned previously, all samples are diluted with a buffer of pH 7.0.

The stock culture

The stock culture of *Staphylococcus aureus* is kept on an agar slant of medium I. Inoculum for the broth culture is taken from such slants. It has been noted that serial transfer from broth to broth results in decreased pigment formation.

Concentration of the inoculum

The number of organisms distributed over the surface of a plate determine to a certain extent the size of the circle of inhibition. Figure 2 illustrates the fact that the heavier the inoculation the smaller the circle of inhibition. When the inoculation is too heavy, the circles are not uniform and consistent. On

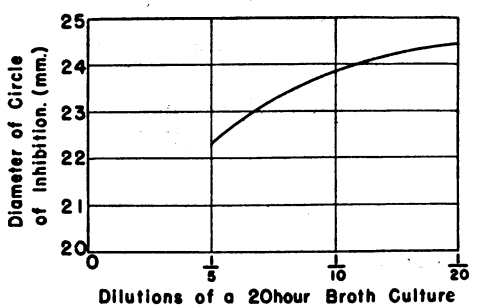

FIG. 2. THE EFFECT OF THE CONCENTRATION OF THE INOCULUM ON THE SIZE OF THE CIRCLE OF INHIBITION OF A 2-UNIT STANDARD

the other hand, if the inoculation is too light, the circle becomes very large and the edges are ill-defined.

The relationship between the rate of diffusion of penicillin solution and growth of the test organism

The size of the circle of inhibition is in part dependent on the time allowed for diffusion before the test organism produces a substantial amount of growth. If a plate is completed and ready for incubation but is then refrigerated for an hour or two before incubation, abnormally large circles are obtained because refrigeration retards bacterial growth and permits a longer time for the diffusion of the penicillin. Figure 3 shows the effect of refrigeration on the size of the circle of inhibition.

Conversely, a prolonged incubation or drying period serves to reduce the size of the circle of inhibition as depicted in figure 4.

The most reliable results are obtained from those plates which are ready for incubation $1\frac{1}{4}$ to $1\frac{1}{2}$ hours after they are flooded. Occasionally, when this

schedule cannot be maintained for all plates, it will be found that those plates placed in the incubator last will have smaller circles of inhibition than the first ones. This difference in zones of inhibition necessitates that a standard be placed on every plate, and that the unit values for the unknown samples be calculated from their corresponding standards.

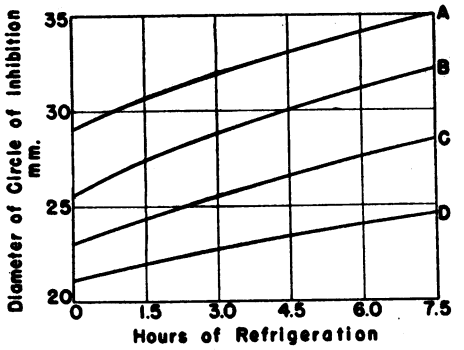

FIG. 3. THE EFFECT OF REFRIGERATION ON THE SIZE OF THE ZONE OF INHIBITION
Curves A, B, C, and D represent four different concentrations of penicillin

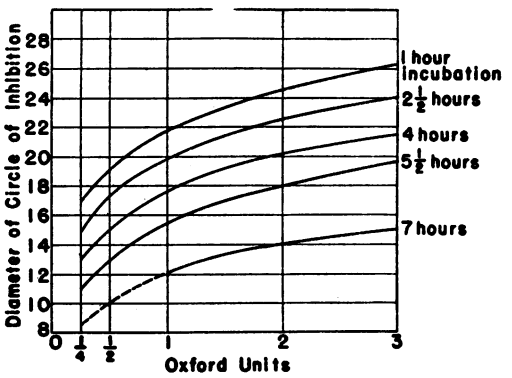

FIG. 4. THE EFFECT OF A PROLONGED INCUBATION-DRYING PERIOD ON THE SIZE OF THE ZONE OF INHIBITION

Condition of the ground glass surface of the cylinder

A small crack or chip on the surface of the cylinder which comes in contact with the agar causes leaks which lead to abnormally large and irregular zones. Heavy contamination may also result from faulty cylinders since dilutions of samples are not made up in sterile buffer nor is a sterile capillary pipette used for filling the cylinders. A ring of growth around the cylinder usually causes a circle to become too large as compared to its normal duplicate.

IV. THE SERIAL DILUTION METHOD

Concentrated ethereal solutions of penicillin and potent untreated culture liquor are usually assayed by the serial dilution method to confirm the values

obtained by the cylinder plate method. The procedure is simple and fast, requiring only about six minutes to prepare both blanks and make dilutions. Glass or aluminum caps which are used in place of cotton plugs greatly facilitate handling the tubes. The medium used is that referred to above as Medium II. It is dispensed in 200-ml. quantities into 500-ml. Erlenmeyer flasks, sterilized at 15 lbs. pressure for 15 minutes and then kept in the refrigerator. As each flask is used it is inoculated with 1 ml. of a 20-hour broth culture of *Staphylcoccus aureus*. The broth and inoculum are thoroughly mixed, and the broth is then immediately pipetted in various amounts to sterile test tubes.

The sample of penicillin which is being diluted must be substantially free from bacteria which are insensitive to penicillin. Solutions contaminated with bacteria may be filtered through a Seitz or sintered glass (grade 5 on 3) filter.

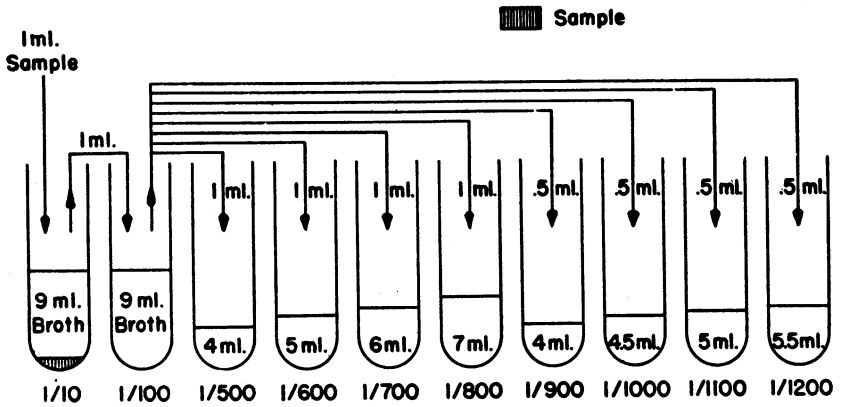

FIG. 5. SCHEME FOR SERIAL DILUTION

This method permits the concentration of inoculum to remain practically constant throughout every ml. of broth. The amount of broth varies considerably from tube to tube, but this does not apparently interfere with obtaining consistent and reliable results. Before incubation every tube must be thoroughly shaken to mix broth and penicillin.

Incubation

Although any period of incubation may be used providing a standard is assayed and an end point determined, it has been our practice to incubate at 37°C. for 18 or 40 hours, the latter interval being preferred.

18-hour reading

The calculations for converting dilutions to Oxford units are based on the highest dilution showing no growth. Assuming that the cylinder plate method gives an accurate value, we have found that, in serial dilution cultures, it takes approximately 0.045 Oxford units per ml. to inhibit growth of the organisms.

To convert dilution into units, the reciprocal of the dilution is multiplied by the factor 0.045.

Example: 18-hour reading

$$\begin{matrix}1/900-\\1/1000+\end{matrix} \quad 900 \times 0.045 = 40 \text{ units per ml. of sample}$$

40-hour reading

The 40-hour results are much easier to read and more definite. Usually in a series one tube will be very clear and the next very turbid. Calculations are based on the highest dilution which remains clear. Slight growth such as granules and stringiness are disregarded. When the incubation period is prolonged, the amount of penicillin needed to prevent growth also increases; numerous determinations indicate that in 40-hour cultures approximately 0.1 unit per ml. is required to inhibit growth.

Example: 40-hour reading

$$\begin{matrix}1/400-\\1/500+\end{matrix} \quad 400 \times 0.1 = 40 \text{ units per ml. of sample}$$

V. DISCUSSION

In the opinion of the authors, the cylinder plate method is the more practical procedure to use because of the following advantages it offers over the serial dilution method:

(1) It is easier to assay daily a large number of samples.
(2) Samples need not be sterile.
(3) Samples which may have a potency from 0 to 500 Oxford units involve relatively few dilutions compared to the number necessary for the serial dilution method.
(4) Antibiotic substances such as Penicillin B (Roberts, Cain, *et al.*, 1943), notatin (Coulthard, Short, et al., 1942), and penatin (Kocholaty, 1942) which are protein in nature, do not diffuse readily through agar; therefore these substances do not interfere with the penicillin assays.

A combination of the two methods is very useful when it is necessary to check accurately the strength of a penicillin solution. It is believed that the error in the serial dilution may be about 10 to 15 per cent, while the cylinder plate method may be in error 15 to 20 per cent.

VI. SUMMARY

1. Procedures are given for assaying penicillin by the cylinder plate and the serial dilution methods.

2. A number of different factors which affect the cylinder plate method are listed and discussed. The influence of the following factors is considered: (a) depth of agar, (b) stock culture of the test organism, (c) pH of the medium, (d) the relationship between the rate of diffusion of penicillin and growth of the test organism, (e) concentration of the inoculum, and (f) condition of the ground glass surface of the cylinder.

3. Under conditions described it has been determined that it takes approximately 0.045 Oxford unit per ml. of broth to inhibit growth of the test organism at 18 hours and 0.1 unit per ml. of broth at 40 hours.

ADDENDUM

Recent studies[6] on the stability of penicillin indicate that it is more stable at pH 6.0 than at 7.0. For that reason the pH value of the buffer has been lowered from 7.0 to 6.0. Solutions of KH_2PO_4 and K_2HPO_4, each containing 1 per cent salts, are mixed in such a proportion to give a final pH of 6.0. This buffer is used only for making dilutions and is not used in making Medium II. It is kept saturated with toluene and chloroform at all times.

BIBLIOGRAPHY

ABRAHAM, E. P., CHAIN, E., FLETCHER, C. M., GARDNER, A. D., HEATLEY, N. G., JENNINGS, M. A., AND FLOREY, H. W. 1941 Further observations on penicillin. Lancet, **2**, 177–189.

COULTHARD, C. E., SHORT, W. F., SKRIMSHIRE, G. E. H., BIRKINSHAW, J. H., MICHAELIS, R., SYKES, G., STANDFAST, A. F. B., AND RAISTRICK, H. 1942 Notatin: an antibacterial glucose-aerodehydrogenase from *Penicillium notatum* Westling. Nature, **150**, 634–635.

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

KOCHOLATY, W. 1942 Microbial antagonism and *Brucella abortus*. J. Bact., **44**, 143.

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.

ROBERTS, E. C., CAIN, C. K., MUIR, R. D., REITHEL, F. J., GOBY, 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.

[6] Unpublished data.—W. H. Schmidt and R. G. Benedict.

PLATE 1

FIG. 1. Oblique view showing arrangement of cylinders.

FIG. 2. Perpendicular view showing various sized zones of inhibition.

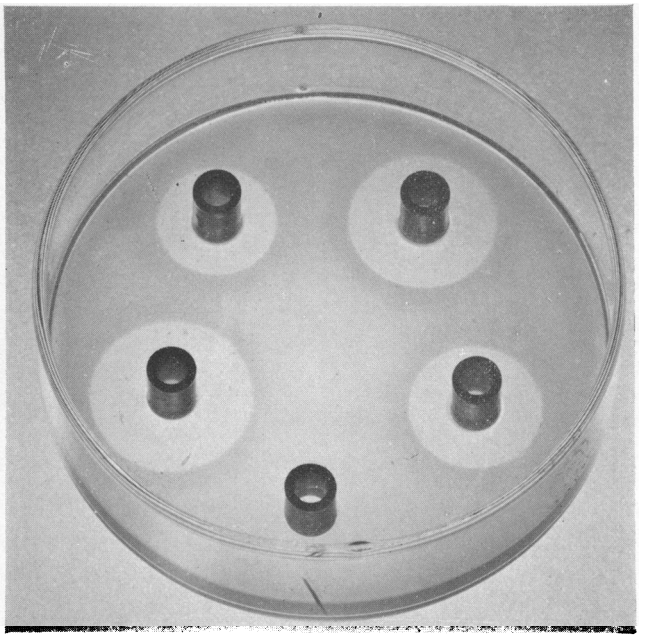

FIG. 1

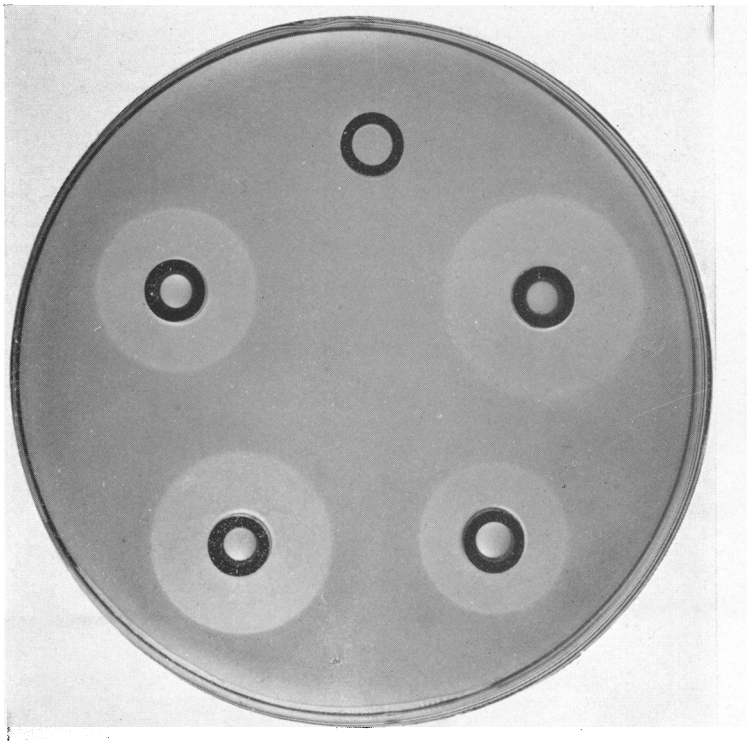

FIG. 2

(William H. Schmidt and Andrew J. Moyer: Penicillin)