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II. The Diffusion Methods

JOHN J. GAVIN

Food Research Laboratories, Inc., Long Island City, New York

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I. Principle. In these methods the substance to be assayed is allowed to diffuse through solid, inoculated culture medium. If the substance being assayed is a bacteriostatic or bactericidal agent, a zone of inhibition results. If the substance is a growth factor, a zone of growth (zone of exhibition [Bacharach and Cuthbertson, 1948]) develops. The size of the zone, either of inhibition or growth, is a function of the concentration or in certain circumstances, the amount (Heatley, 1948) of the substance being assayed. This function can be expressed as a linear relationship between the size of the zone and the logarithm of the concentration of the substance (Bliss, 1944; Davies, 1945–1946; Bacharach and Cuthbertson, 1948). By measuring the distance the substance diffuses, as evidenced by growth or lack of growth of the test organism, and comparing it with that of a known standard preparation, the potency of the sample may be calculated.

II. Types of Diffusion. There are two types of diffusion, vertical (linear diffusion) and horizontal (radial diffusion).

A. Vertical (linear) diffusion. In vertical diffusion methods, a given volume of the solution to be assayed is placed on top of a column of inoculated agar and allowed to diffuse down the column. The length of the column, in which the inhibition or exhibition takes place, is measured and the concentration of substance corresponding to this distance is read from a standard curve. The standard curve is prepared from a similar series of columns of known concentrations of the substance being assayed. This method is seldom used. Some Japanese workers (Torii et al., 1947) use it routinely, and several other investigators have developed workable methods (Florey et al., 1949; Davis and Parke, 1950: Davis et al., 1950). Its main disadvantages are:

1. Must necessarily use a facultative anaerobe.
2. Test solutions must be sterile.
3. Difficulty in observing the end point.
4. There is a relatively narrow spread of readings of the zone lengths.
5. The method is cumbersome. There is a lengthy time period involved in cleaning and setting up the apparatus.

One major advantage of this method (Davis and Parke, 1950) is the ability to better define and control the geometry of the diffusion system. To gain this one advantage, it is necessary to sacrifice others such as time, ease of handling, and so forth. From a practical standpoint, the increase in accuracy by better control of diffusion is not worth the sacrifice. The literature does not indicate the use of this method for the assay of substances other than antibiotics.

B. Horizontal (radial) diffusion. There are several

[References and further details would follow in the full text, but are not included here.]
methods employed using the horizontal type of diffusion. These may be divided into two groups (Heatley, 1948), first, those methods in which the diameter of the zone of inhibition or growth depends upon the concentration of the substance being tested, and second, those methods in which the zone is dependent upon the amount of the substance.

1. Zones dependent upon concentration. Two methods are included in this group, the cylinder and the cup plate methods. The two are essentially the same. The substance being assayed diffuses from a central source. The diameter of the zone of exhibition is measured and, as in vertical diffusion, compared to a series of standard concentrations of the substance being assayed. In the cylinder method the solution is contained within a cylinder. In the cup plate method a depression to hold the solution is made in the agar.

(a) Cylindrical method. The cylinders used in this assay may be of pyrex glass, glazed porcelain, aluminum, or stainless steel. They should have the following approximate dimensions, an outside diameter of 8 ± 0.1 mm, an inside diameter of 6 ± 0.1 mm, and a length of 10 ± 0.1 mm, with a thickness not exceeding 1 mm. If made of glass, porcelain, or aluminum, they should be beveled internally. The object of the bevel is to reduce the area of contact with the agar and so increase the weight per unit area of contact, thus giving a more effective seal (Florey et al., 1949). The beveled end, which is placed in contact with the agar, should be flat and free from chips or scratches. The bevel is unnecessary when the heavier stainless steel cylinders are used. Cylinders may be placed on the agar cold or they may be warmed before being applied. Warming the cylinder will give a more effective seal. Control of the depth that the cylinders are imbedded in the agar is important. If the cylinders penetrate too far, the zone size is reduced (Brownlee et al., 1948). Differences in depth between cylinders will lead to high experimental error as there will be a corresponding variation in zone size. In order to control this factor, the heating of the cylinders should be uniform. This can be done by using a hot plate.

The cylinders may be placed on the agar by hand or by the use of automatic dispensing machines. Devices for the automatic placement of cylinders have been described by Reeves and Schmidt (1945), Oswald and Randall (1945), Beadle et al. (1945), and Chandler and Shaw (1946). When a machine is used for the placement of cylinders, they are applied cold.

The standard and sample solutions are pipetted into the cylinders. The cylinders should be filled completely with solution to avoid error. Grenfell et al. (1947) found an 18 per cent range in zone size when they varied the volume of solution in the cylinders from 0.1 to 0.3 ml.

Care should be taken when moving the plates to avoid breaking the seal between the cylinder and the agar, or spilling the solution contained in the cylinder. The advantages of the cylinder plate assay are:

1. Solution being assayed need not be sterile.
2. Adaptable to rapid assay.
3. More sensitive to low dilutions than the paper disc assay.

The disadvantages are:

1. Extreme care must be taken in the handling of plates to avoid breaking seal or spilling the contents.
2. Handling, cleaning, and sterilizing the cylinders.
3. Filling of cups is slow and tedious.
4. Suspended material interferes with diffusion of liquid from the cylinder.

(b) Cup plate method. The depression, which is cut in the agar, can be made in either of two ways.

1. A sterile rubber stopper, test tube, marble, or something similar, is placed in the molten agar. When the agar solidifies, the object is removed leaving a depression.

or

2. The agar is allowed to harden and a plug is removed by means of a sterile cork borer. The size of the cork borer is optional. It has been found that there is no difference in results using cork borers from 9 1/2 to 13 mm (Harris and Ruger, 1953).

In using this method, there are several factors which are important if accurate and reproducible results are to be obtained (Vesterdal, 1946).

These factors are:

1. Cups must be cut vertically.
2. Cups must be completely filled with the solution being assayed.
3. Optimum thickness of the agar is 5 to 6 mm.

The zones obtained using this method may be sources of error. If the agar on the edge of the cup is lifted away from the glass, a false large zone will result from the seepage of the solution between the agar and the bottom of the plate. When a single layer of agar is used, a double zone effect may occur. If the same edge of each zone is read, the error will be negligible (Brownlee et al., 1948). The curve obtained
with this test and the accuracy of the test are the same as for the cylinder plate method. The advantages of the cup plate method are:

1. Handling of cylinders is eliminated.
2. It is more sensitive to low dilutions than is the paper disc assay.
3. Suspended material does not interfere with diffusion of the solution.
4. Variation in the size of the cylinder is avoided (Brownlee et al., 1948).
5. It can be used as a screening test for substances elaborated by microorganisms (Harris and Ruger, 1953; Raper et al., 1944; Wilkens and Harris, 1944).
6. It is adaptable to rapid assay.

The disadvantages are:

1. Cutting of cups is a laborious operation.
2. Some care must be taken in handling plates to avoid spilling the solution.

2. Zones dependent upon amount. Two methods are also included in this group, the drop plate method and the paper disc method. These two methods are, in a sense, as similar in comparison with each other as the two methods described previously. In the drop plate method, a drop of the substance to be tested is placed directly on a plate and diffuses into the agar. A zone of inhibition or exhibition results. In the paper disc method, the drops are indirectly placed on the agar by means of a filter paper disc, with subsequent diffusion and the resultant zones.

(a) Drop plate method. Either of two techniques may be employed with this method. The first involves placing a drop of the solution to be assayed on the surface of a seeded agar plate ( Forgacs and Kuchera, 1946; Thomas et al., 1944). A loop, a pipette, or a syringe may be used to apply the drop. This method is more adaptable for a qualitative than quantitative assay. The second technique is that of White (1945–1946). It is claimed that by this method a difference of 5 per cent in potency can be determined. The dilutions to be tested are made up in broth. Each dilution is inoculated with a suitable microorganism. One drop of each dilution is placed on nutrient agar which is incubated. The results are based on the appearance of the bacterial growth between dilutions.

This method has a "restricted application, being most suitable for the accurate assay of a few samples of approximately known potency" ( Heatley, 1948). It is obvious that neither of these two methods would be the method of choice, except in some special application.

(b) Paper disc method. The disc, rather than the cylinder or cup, is used as the reservoir for the substance being assayed by this method. The solution to be assayed is placed on the disc either by use of a pipette or loop, or the disc may be dipped into the solution. As indicated previously, the major difference between the cylinder or cup method and the paper disc method is that the amount of substance, rather than the concentration, determines the size of the zone. This factor allows the discs to be placed on the seeded agar either wet or dry. Because of this it is also possible to assay materials containing organic solvents (Stansly, 1952). The disc can be wet with the substance to be assayed and then the solvent allowed to evaporate before the disc is placed on the agar. It has been found that 60 per cent methanol, 40 per cent ethanol, 40 per cent acetone, 10 per cent dioxane, or 5 per cent pyridine does not interfere with the assay of streptomycin (Loo et al., 1945). This same factor has been responsible for the success of the commercially available antibiotic sensitivity discs which are used for diagnostic purposes. A modification of this method has been used for the differential assay of the penicillins. Paper chromatography is used to separate various penicillins and the strips are then placed on agar (Glister and Grainger, 1950; Goodall and Levi, 1947). Zones of inhibition appear at intervals along the strip depending upon the location of the particular type of penicillin.

The curve obtained is a straight line and the accuracy and reproducibility of results are the same as for cylinder plate and cup plate assays.

The advantages of the paper disc method over other diffusion methods are:

1. Simplicity. It does not require the tedious work of filling cylinders or cutting out plugs of agar.
2. Convenience. It does not require handling, cleaning and sterilizing of cylinders. This decreases the over-all time required for assay and allows more samples to be handled during any given period of time.
3. Rapidity. The discs can be handled rapidly. As in (2) above, the over-all time for assay is decreased and more samples can be handled.
4. Ease of handling plates. There is no need for the special handling of assay plates as with other diffusion methods.
5. Accuracy. There are more consistent zones of inhibition due to improved diffusion as there is better contact of disc with agar. Variable diffusion due to leaks and chips
and inconsistently applied cylinders is avoided.

(6) Ability to assay solutions containing relatively high concentrations of organic solvents. High and erratic results are obtained with other methods.

(7) Only small amounts of solution are necessary to saturate the discs. This advantage is valuable when a limited sample of low concentration is assayed. [Direct assay of blood (Sherwood and de Beer, 1947).]

The disadvantage of the paper disc method is that it is less sensitive to low dilutions than either the cylinder or cup plate method.

III. FACTORS THAT INFLUENCE DIFFUSION ASSAYS. In any assay method, there are factors which are peculiar to that type of assay. Control of these factors will result in accurate and reproducible assays. The factors to be discussed are the main conditions that need control in the diffusion type assay. There are undoubtedly many others, for each substance to be assayed, and the form in which it is available, presents new and different problems. Some of these factors are applicable only to the diffusion assay, while others might have broader application.

Although the lack of control on some of these conditions will lead to significant errors, variation of a factor or factors could improve an assay. For example, decrease in depth of agar increases the sensitivity. This might be a desirable effect.

A. Agar. The diffusion assay is fundamentally dependent upon the diffusion of the substance to be assayed through the agar. Thus the agar becomes one of the most important factors in this type of assay. In the following discussion, the nutrient value of the agar, which is considered elsewhere to a greater extent, is not evaluated. The concern here is for those factors which materially affect the physical diffusion through the agar of the substance to be assayed.

1. Depth of the agar. Agar has been placed in containers in a variety of depths ranging from the “microfilm” method of Forgaes and Kuchera (1946) to the 8-mm thickness layer of Hayes (1945). A thin layer will give larger zones than thicker layers thus increasing the sensitivity of the assay. Most authors recommend a layer of 3 to 5 mm. In a statistical analysis of this problem, de Beer and Sherwood (1945) found that the best results were found with layers of 2 to 4 mm. The analysis of variance indicated that extremely thick or thin plates should not be used as the log dose-response curve obtained may not be a straight line. Hayes (1945) in his statistical evaluation, indicated that an 8-mm layer of agar gave the most reproducible results. Other workers (Higgins et al., 1953; Kersey and Leghorn, 1953; Larkin and Stuckey, 1951) have had better results with thinner layers.

It is not important that an exact numerical value is obtained for the depth of an optimum layer, but rather there is need for a constant thickness for a given series of plates or assays. To obtain a constant thickness it is necessary to control the volume of agar added to each plate and select dishes that have flat bottoms and are of uniform size. Many types of containers have been used: Petri dishes, flat bottom baking dishes (Epstein et al., 1944), flat pyrex plates with pyrex rims (Kersey and Leghorn, 1953) and window glass, with wooden or aluminum rims (Brownlee et al., 1948). It is advantageous to obtain as many zones as possible from one plate. Environmental conditions are more apt to be similar for an assay conducted on one plate than for an assay using several plates. This was statistically proved by Brownlee et al. (1948) using large plates containing 64 cups.

2. Dryness of agar. Insufficient dryness of the agar may cause streakiness and indistinct, obscure zones. Some workers feel that drying is essential while others have dispensed with drying techniques and have obtained excellent results. Partial drying is desirable if streakiness is to be avoided (Florey et al., 1949). While drying the plates, care should be taken to avoid aerial contamination. This may be done by covering the plates with several thicknesses of cheesecloth (Florey et al., 1949), using porcelain covers, or by inverting the plates. Drying may be done by leaving the plates at room temperature or by placing them in an incubator at elevated temperatures. The length of time required is a function of temperature and humidity, but the operator’s experience is the best guide as to the extent the plates must be dried before using. Plates may be stored in a refrigerator for several days after seeding, with no adverse effects.

3. Amount of agar. There are reports in the literature that the sensitivity of the diffusion method can be increased by decreasing the agar content of the medium. The results of Grady and Williams (1953) are difficult to interpret in this regard, as they varied the depth of the agar at the same time that they varied the concentration of agar. It is probable that the amount of agar used will affect the assay. This would be dependent, however, on the quality and origin of the agar.

B. pH of agar and assay solutions. The prime requisite for the pH of the agar is that the value should be in the ranges that are suitable for the optimum growth of the test organism. On the other hand, the
main requirement for the pH of the assay solution is that it be in a range that is favorable for both the activity and stability of the substance being assayed, the ideal situation being that the pH of the assay solution is as close as possible to the optimum pH of the test organism.

Substances vary in both activity and stability with changes in pH. Buffer solutions are recommended as diluting agents to prevent decomposition of pH-sensitive substances. Foster and Woodruff (1943) found that the activity of penicillin increased with decreased pH values of the medium. Eagle et al. (1952) report that the antibacterial action of basic substances decreases. Vitamin \( B_3 \) was unstable in alkaline solutions used for diffusion assay (Larkin and Stuckey, 1951).

The zone diameter will change when the pH of the agar is varied (Grenfell et al., 1947) (table 1). With polymyxin, Stansly and Schlosser (1947) changed the pH of the seed layer and found that at pH 5.0 no zone developed, while at pH 9.0 the zones were smaller than those at pH 7.0.

The zone sizes also change with the pH of the assay solution (Larkin and Stuckey, 1951; Loo et al., 1945). The optimum pH for the maximum activity of streptomycin against *Bacillus subtilis* is about pH 8.5. This is high for culture media, so Loo et al. (1945) used a value of pH 7.9 ± 0.1. The Food and Drug Administration (1953) uses a buffer solution of pH 7.8 to 8.0. Smith et al. (1950) in the assay of choramphenicol in body fluids and tissues, found that changes between 4.5 and 9.0 in the pH of the assay solution showed no difference in activity, but the pH of the agar had to be held close to 7.0.

There are numerous combinations of pH values for agar and assay solutions. The most suitable for a particular assay must be decided empirically. Again, an optimum numerical value is hard to obtain. Solution and agar pH values should be as similar as possible. The emphasis must be placed on establishing a workable set of conditions. When this is accomplished, the established conditions should be adhered to rigidly.

### Table 1. Effect of various factors on the assay of penicillin by the cup assay

<table>
<thead>
<tr>
<th>Factor Varied</th>
<th>Normal</th>
<th>Experimental</th>
<th>Average Change in Zone Diameter (% of Normal)</th>
<th>No. of Zones Measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of inoculum (hr)</td>
<td>16</td>
<td>22.5</td>
<td>+4.4</td>
<td>156</td>
</tr>
<tr>
<td>Temperature of inoculation (degrees)</td>
<td>47</td>
<td>56</td>
<td>+10.5</td>
<td>262</td>
</tr>
<tr>
<td>pH of agar</td>
<td>6.9</td>
<td>6.35</td>
<td>+7.3</td>
<td>265</td>
</tr>
<tr>
<td>Delay before incubating plates after filling cups (min)</td>
<td>30</td>
<td>60</td>
<td>+4.5</td>
<td>105</td>
</tr>
<tr>
<td>Volume in cups (ml)</td>
<td>0.2</td>
<td>0.1</td>
<td>−11.0</td>
<td>79</td>
</tr>
</tbody>
</table>

* Grenfell et al., 1947.

C. Incubation. The time and temperature of incubation required and the combination of these factors which gives sufficient growth or inhibition to determine zone size vary from organism to organism. The requirements of a particular situation will determine what combination of factors should be selected. In some cases, the substance being assayed will influence primarily the choice of temperature of incubation. For example, chlortetracycline is not only slow in diffusing through agar but is unstable at 37°C. When this occurs, an organism which is sensitive to the substance and which will grow at lower temperatures is chosen. The use of some organisms will necessitate more rigid temperature control than others. Some lactobacilli are affected when the incubation temperature varies from 2 to 4°C (Borek and Waelsch, 1951; Price and Graves, 1944). This variation in temperature can be controlled by the use of incubators having adequate air circulation and thermostatic temperature regulation.

Some fast-growing organisms will give adequate zones in as little as 3 hr while others need as long as 30 hr. In other instances, the growth of a single organism may be increased or decreased at the convenience of the analyst and without loss of accuracy by changing the temperature at which the assay is run. A strain of *B. subtilis* will give results which can be read after 31/2 hr incubation at 37°C or results which may be read after 18 hr if incubated at 25 to 28°C (Florey et al., 1949). For most purposes, over-night incubation is satisfactory.

With some organisms a prolonged incubation time might cause a change in zone size, while with others it has no effect (Waksman and Lechevalier, 1951; Harrison et al., 1951; Harris and Ruger, 1953). There are cases where a combination of incubation temperatures has been used. In order to get sharper zones while using *Escherichia coli* as a test organism for polymyxin assay, Stansly and Schlosser (1947) incubated plates for 18 hr at 25°C and then for 6 hr at 37°C. This combination of incubation times and temperatures was chosen because *E. coli* grows quite rapidly while polymyxin diffuses slowly. Another technique is available to overcome slow diffusion. The elapsed time between placing the samples on the plates and incubating the plates can be varied. The rate of diffusion of the substance into the agar determines the zone size. This is correlated with the growth rate of the test organism. The activity of the substance is measured only to the extent of the diffusion of this material before multiplication of the test organism begins. If large zones are desired, then the lag phase of the growth of the organism can be altered.
This is done by refrigerating the plates or allowing them to remain at room temperature after the samples have been placed on them, to prolong the lag phase of the growth and thus obtain large zones. Schmidt and Moyer (1944) using four different concentrations of penicillin, showed an increase in the size of the zone of inhibition when plates were refrigerated from 1.5 to 7.5 hr. Cuthbertson et al. (1951) obtained similar increases in the zone of inhibition, in the assay of vitamin B₁₂, when plates were allowed to stand at room temperature before incubation (table 2).

To reduce the lag phase with resultant smaller zones, the plates are incubated before the samples are placed on them. In the assay of vitamin B₁₂, Harrison et al. (1951) found that deviation from normal assay treatment, that is, plating out of dilutions and immediate incubation, either by refrigerating after plating out or incubation before plating out had considerable effect on the size of the zone of inhibition. They concluded that for accurate assay comparisons an obvious gain in precision will be obtained if pre-refrigeration of the assay plate between plating out and incubation is performed.

If plates are refrigerated to allow diffusion to proceed before growth starts, it is important not to put them directly into a warm incubator. Moisture might condense on the lid and ruin the plates. This is especially true if the cylinder or cup plate method is used, since the assay plates can not be inverted.

An objection is made to the refrigeration procedure by Sykes (1952) in a publication of the Council of the Pharmaceutical Society of Great Britain. It is claimed that there is difficulty insuring absolute uniformity of cooling and subsequent warming in a group of plates. This objection may be valid, but excellent results have been obtained using this procedure.

### D. Application of the organism

The organism can be applied to the plates in a variety of ways.

The three main ways are:

1. By flooding the surface of the agar with a suspension of the test organism and then removing the excess fluid. Variations in zone diameters result from an uneven distribution of the culture.

2. By seeding the agar in bulk. This insures complete uniformity of medium and inoculum.

3. By pouring a layer of inoculated agar (seed layer) on a nutrient agar plate (base layer). This method has the advantage of sharpness of zones, but involves more time than the second method.

There are modifications of all these methods. Instead of flooding the surface of the agar, spraying has been suggested (Wilska, 1947). Seeding may be done in each Petri dish rather than in bulk before the plates are poured. Reilly and Sobers (1952) recommended the use of plain agar as the base layer, when using the third method, for increasing sensitivity of the paper disc assay. Esposito and Williams (1952) and Grady and Williams (1953) increased the sensitivity by use of a single layer as in the second method.

In applying the microorganism to the plate, the temperature of the molten agar may affect the assay. If it is too warm, some of the test organisms will be killed, while if it is too cold, it will solidify and become unworkable. The best temperature for inoculating molten agar is 50°C. Larger zones of inhibition resulted when bulk inoculation was done at 56°C than at 47°C.

### E. Substance to be assayed

1. **Requirements.** Before a substance can be assayed, there are some intrinsic characteristics that the material must have. If the diffusion method is to be used, the material must:
   
   (1) Have growth stimulatory or inhibitory properties towards some microorganism.

   (2) Be soluble in a solvent that will not interfere with the assay in the concentration used.

   (3) Be diffusible through agar.

   If these conditions are not met, another type of assay method must be chosen.

2. **Dosage levels.** The choice of dosage levels is dependent upon the activity of the substance upon any given microorganism. There is another difference between substances which must be considered. When antimicrobial agents are assayed by this method, diffusion takes place with no loss or utilization of the substance by the test microorganism. But when growth factors are assayed, the nutrient is utilized to the extent that it stimulates the growth of the microorganisms. As diffusion and utilization proceed in an
outward direction, the rate of increase in the diameter of the zone and the intensity of microbial growth are affected, resulting in a gradual loss of sharpness in the zone boundary.

This means that the dosage levels of the growth factor have to be higher than those used for inhibition assays. Using higher levels of growth factor will result in sharper zones with responses high enough for accurate measurement. In either case, the actual concentration of the substance should be such that the response is within the workable range of the dose response curve.

IV. Zones of Inhibition and Exhibition

A. Types of zones obtained. Various degrees of growth or inhibition may occur and may affect the accurate reading of the zones obtained. The range is from the ideal zone, with well defined edges that are distinct and easily read, to zones that are blurred, diffuse and with or without halos and concentric rings.

A variety of factors cause ill defined zones. The organism itself may be at fault. When B. subtilis is used as the test organism for penicillin assay, the zones are usually quite clear and distinct. When Micrococcus pyogenes var. aureus is used, the zones are not so well defined. An even finer distinction can be made between strains of M. aureus (Cholden, 1944). In addition to the test organism itself, a number of degrees of sharpness can be obtained by variation of the density or age of the inoculum. The effectiveness of a substance to stimulate or inhibit growth may vary with the age of the culture used. In some cases, the constituents of the culture medium may cause lack of definition at the zone edges. Sugars markedly affect this definition (Bond, 1952). Peptones and yeast extracts (Goodall and Levi, 1947) modify the type of zone obtained.

The assay solution may be responsible, also. If the solution contains substances, in addition to the substance being assayed, that stimulate or inhibit growth, the type of zone will vary accordingly. In a B12 assay of materials which contained desoxyribosides in addition to B12, Cuthbertson et al. (1951) found two types of zones. One was caused by the B12 activity, the other by desoxyribosides. According to these workers, they could distinguish between the two types of the zone by the type of zone. Dufrenroy and Pratt (1947) attributed the growth increase which is seen in some antibiotic assays to the stimulatory effect of sub-bacteriostatic quantities of the antibiotic. Synergism between antibiotics or growth factors may also cause unexpected zone types. As mentioned previously, Brownlee et al. (1948) found a double zone effect in the cup plate assay of streptomycin. This double edge was due to a small zone at the glass agar interface and a larger zone on the upper surface of the agar. By choosing the edges of one of these zones and reading at that point for all zones, the undesirable effect of a double zone was eliminated.

B. Measurement of zone diameter. The diameter of the zones of inhibition or exhibition may be read using any convenient measuring device. Millimeter rulers, calipers, and specially designed equipment have been utilized. For ease of reading, zones have been magnified and projected on screens and several types of illumination boxes have been built. An excellent instrument1 for this purpose is the one developed by Davis et al. (1949).

V. Accuracy of Results. The limits of error have been estimated by several workers. Most estimates have ranged from ±15 per cent to ±20 per cent. The early literature in the antibiotic field indicates the error of assay to be in this range. Better precision has been obtained, in later years, by more rigid control of conditions including the use of a larger number of replicates. The British Pharmacopoeia (1948) gave the limits of error \( P = 0.99 \) as follows:

- when 10 cylinders, or cups, are used \( ±21\% \)
- 20 \( ±15\% \)
- 40 \( ±10.5\% \)
- 80 \( ±7.5\% \)

However, the latest edition (1953) included only the limits of error for 5 to 10 cylinders. Brownlee et al. (1948) obtained limits of error \( P = 0.95 \), including both internal and external errors, of ±5 per cent.

Investigators of vitamin assay methods (Bacharach and Cuthbertson, 1948; Cuthbertson et al., 1951; Larkin and Stuckey, 1951) found the limits \( P = 0.95 \) to be ±15 per cent. In the assay of riboflavin in malt (Bacharach and Cuthbertson, 1948), limits of error \( P = 0.95 \) were reported to be ±6 per cent.

Several factors are involved. Some are controllable, others are not.

The main factors are:

1. Skill of the individual operator.
2. Individual modification of technique.
3. Number of discs, cylinders or cups used for each dilution.

The human element is the source of some error. Operators can be trained, well instructed in the details of the assay and still obtain varied results. Tedium and lack of interest may attribute to discrepancies in technicians' results, but varied results are also obtained by interested operators.

The best way to overcome many of these errors is to use a large number of zones with a good balance between the standard and the sample zones. Generally, the best balance would appear to be obtained when the number of zones of standard and sample are the

same. It has been suggested (Wood, 1946) that for every n zone used for the sample, n is required for the standard.

Routinely, it appears advantageous to use large plates with several zones for each dilution in order to obtain satisfactory results. If greater accuracy is desired, replicate tests, on several consecutive days, can be run. Charts and nomographs can be prepared for these assays to calculate the error of each individual assay from internal evidence.

VI. ADVANTAGES AND DISADVANTAGES OF THE DIFFUSION TYPE ASSAY

A. The advantages of the diffusion type assay are the following:

1. The method is readily mechanized and adapted for large-scale routine analysis.
2. The method is simple and convenient. A variety of samples from different sources can be handled easily. Numerous dilutions of high potency material are not required.
3. The method allows for more rigid control of conditions, giving an assay of greater precision. The probable error can be readily calculated and the accuracy increased to any degree by the inclusion of sufficient replicates.
4. Samples need not be sterile. This prevents possible loss of activity from sterilization procedures.
5. The presence of high concentrations of organic solvents does not interfere with the assay.
6. The testing of several samples on the same culture surface increases uniformity.

B. The disadvantages of the diffusion type assay are the following:

1. The method is relatively insensitive when compared to other methods of assay (that is turbidimetric, acridimetric, and so forth).
2. The method is limited to those substances which diffuse readily in agar.
3. The activity of a substance is measured only to the extent of the diffusion before multiplication.
4. There is a lack of control and definition of zones due to the geometry of radial diffusion.
5. The method cannot be used for comparison of different substances, but is limited to the measurement of activity of only one substance.
6. The method cannot be used for unknown substances until a standard has been established.

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