A MICROBIOLOGICAL METHOD FOR THE ASSAY OF SUBTILIN

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A turbidimetric method for the assay of subtilin has recently been reported by Lewis *et al.* (1947). In our laboratory a microbiological method, based on the principles described by Vincent and Vincent (1944) utilizing filter paper disks on agar plates, has served as a satisfactory assay for subtilin. The details of this method and the effect of various physical and chemical factors on the results obtained from it are described below.

PROCEDURE

Medium and preparation of plates. The medium used in the assay is composed of Difco peptone 0.5 per cent, beef extract 0.2 per cent, yeast extract 0.3 per cent, NaCl 2 per cent, and agar 1.5 per cent. These ingredients are dissolved in distilled water, and the pH is adjusted to 6.4 with HCl. After sterilization at 15 pounds' pressure for 15 minutes the medium is allowed to cool to 50 C, and 20-ml amounts are distributed to petri plates. A 20-ml syringe equipped with an automatic double valve (BD no. 470V) has been found to be convenient for this operation. After the agar solidifies, each plate is layered with 5 ml of the same agar seeded with the test organism. The plates are stored in the refrigerator as soon as they solidify.

Inconsistent results are obtained when plates are used that have been seeded for more than 4 days. If assays are to be run over a longer period, a sufficient number of plates containing the initial 20 ml of agar is prepared and the plates are then seeded as needed. These plates are stored in the refrigerator. More uniform seeding of the plates occurs if they are allowed to come to room temperature before seeding. Plates seeded at two different times should not be mixed in running assays.

Test organism. Stock spore suspensions of *Bacillus cereus* 247 are prepared by growing the organisms on the surface of Difco nutrient agar for 4 days at 34 C. The organisms are washed twice in distilled water, heated at 50 C for 30 minutes to kill the vegetative cells, and the suspensions stored in the refrigerator. The agar overlay should contain 300,000 spores per ml.

Preparation of samples. A solution of the reference standard which is twice the maximal concentration used to obtain the standard curve[2] is prepared by

[1] With the technical assistance of A. S. Herring.

[2] With the sample of subtilin used in our work this maximal concentration was 1,000 μg per ml. Since there seems to be no generally accepted unit, and since each sample will vary in potency, it is impossible to indicate the maximal concentration. It is necessary, therefore, to determine this concentration with each sample of subtilin by preliminary experiments.

The subtilin used in this work was a single lot (no. 118-T dated Aug. 19, 1946) furnished

dissolving the dry preparation in 1 N acetic acid. This solution is stored in an amber glass bottle in the refrigerator, since subtilin has been shown to decrease in potency in the presence of light (Jansen and Hirschmann, 1944). It is thus necessary to limit exposure of both the unknown and the standard. On the day of the assay a portion of the stock solution is brought to pH 5. with 1 N NaOH, and sufficient 0.2 M Soerensen's phosphate buffer (pH 5.8) is added to give a final subtilin concentration equal to that of the most concentrated sample to be used on the standard curve. This results in a buffer concentration of approximately 0.1 M. Further dilutions of the standard are made with 0.1 M buffer. It is recommended that five concentrations be run, spaced over the 10-fold concentration range of the assay. All unknowns are prepared so that the final buffer concentration is 0.1 M and the pH is 5.8. Alterations in the procedure for assaying subtilin in blood or serum are discussed in a later section.

Sterility is not required in the preparation of the unknowns or in the dilutions of the standard.

Setting up the assay. It is recommended that each dilution of the standard and the unknown be run in triplicate. The assay is set up by placing 3 filter paper disks,[3] flat side down, on 3 agar plates and pipetting a 0.10-ml aliquot of the sample onto each one as rapidly as possible. Only 3 disks are used on each plate because of the size of the zones obtained with high concentrations. A standard curve is run each time that unknowns are assayed.

Incubation of the plates. Plates are incubated at 30 C for 18 to 24 hours, and the diameters of the zones of inhibition are read to the nearest 0.5 mm. Shorter periods of incubation at 37 C are satisfactory, but the zones of inhibition are smaller than on plates incubated at 30 C.

Estimation of potency and its error. To illustrate the manipulation of results obtained in determining the estimate of potency of an unknown and its error, an example is given below.

Figure 1 shows the relationship between zone diameter and log of subtilin concentration under the experimental conditions proposed above for routine assay. This is a typical standard curve, except that 11 dilutions each in 6 replicates were run. The points on the curve represent the averages of the replicates. By plotting zone diameters versus log concentration the relationship becomes linear over the range of concentrations indicated. The line in figure 1 was computed by the method of least squares (y on x). For rigorous statistical treatment of the data this method is necessary, but for practical purposes it was found that the graphical method (using transparent ruler) suffices, provided the one who draws it has some experience with the method. Transforming the data to give linear regression[4] has the following advantages: (1) the best estimate of the correlation

by Dr. H. D. Lightbody, Western Regional Research Laboratories of the U.S.D.A., Albany, California. On the basis of a unit which has been proposed by Salle and Jann (1946) this sample contained about 1 unit per 100 μg dry weight.

[3] Schleicher and Schuell no. 740 E $\frac{1}{2}$-inch filter paper disks.

[4] One-cycle semilogarithmic paper can be used, thus eliminating the necessity of transforming concentrations into logarithms.

line is available, and (2) the error of the method can be calculated. The broken lines in figure 1 parallel to the solid line represent confidence limits for single zones spaced 2 standard errors of the estimate from the solid line. This means that 19 times out of 20 the zone size obtained by any specific concentration x should fall within these limits. The confidence limits are actually parabolic, but in this case the error introduced by assuming them to be linear is very small.

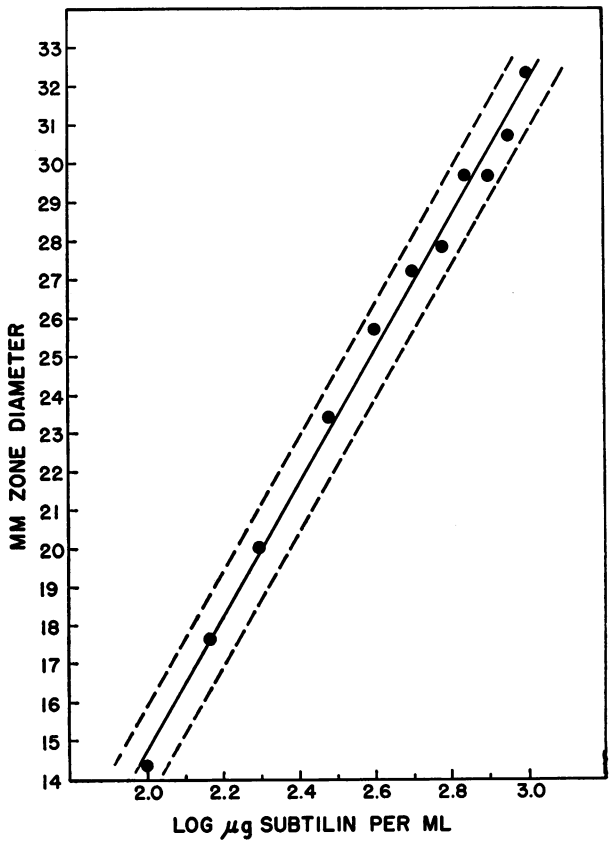

FIG. 1. STANDARD CURVE FOR THE DISK ASSAY OF SUBTILIN

The variance around the line over the range of concentrations indicated was found to be homogeneous by Bartlett's Chi-square test (Snedecor, 1946).

Taking figure 1 as the standard curve, an unknown (1 disk) falling within the range will have an error of ± 17 per cent.[5] It is proposed as standard procedure in this assay to run all dilutions of the antibiotic in triplicate. The diameters of the zones are averaged, and the estimate of concentration is taken from the aver-

[5] Error limits are given as ± 2 standard errors, which means that 19 times out of 20 the true values will be within these limits. The upper and lower confidence limits are not exactly equal when converted to antilogs, but for practical purposes they are made equal by taking their average.

age. If one dilution falls within the range, the error is ±10 per cent. When more than one dilution falls within the range, an estimate of concentration is obtained from each dilution and the estimates are averaged. The errors for 2, 3, 4, and 5 dilutions falling within the range are ±7, 5.7, 4.9, and 4.4 per cent, respectively.

Although the error of the method should not vary too much if the technique is well controlled, it is advisable that each laboratory determine its own error for the method and perhaps check it occasionally.

EFFECT OF VARIABLES

pH of the medium. In the early portion of the work NaCl was not included in the assay medium. During this period some experiments were done to deter-

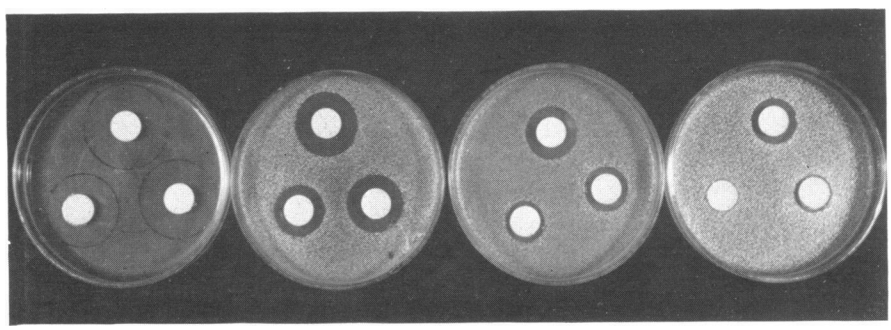

FIG. 2. EFFECT OF pH OF THE MEDIUM ON THE ZONE OF INHIBITION

pH of medium in plates, left to right, is 5.9, 6.2, 6.4, and 7.2, respectively. Top disk on each plate moistened with 0.10 ml subtilin containing 1,000 μg per ml, right disk 500 μg per ml, left disk 333 μg per ml. Zones of inhibition obtained at pH 5.9 are outlined in ink; the poor growth at this pH is not visible on the photographic plate. No NaCl in the medium.

mine the effect of the pH of the medium on the activity of subtilin. The results of one such experiment are shown in figure 2. Maximal activity occurred at pH 5.9, and there was a gradual decrease in activity as the pH was increased to 7.2. The exceptionally large zones of inhibition observed at pH 5.9 were probably due largely to inhibition of growth of the test organism by the low pH. Above pH 6.2 there was no obvious change in density of growth. Although larger zones were obtained at pH 6.2 than 6.4, the latter pH was chosen for routine assay to permit a factor of safety in the adjustment of the pH of the medium.

NaCl concentration of the medium. Larger zones of inhibition were observed when NaCl was included in the test medium. In this series of experiments the medium was adjusted to pH 6.4 and incubated at 30 C overnight. Controls were run with no NaCl, and similar assays were done on media containing 0.85, 2, and 5 per cent NaCl. The test organism did not grow on the medium containing 5 per cent NaCl. The size of the zones varied directly with the concentration of NaCl (figure 3). On the basis of these results, 2 per cent NaCl was included in the media employed throughout the remainder of the work.

Assay of subtilin in certain body fluids. Larger zones of inhibition were ob-

served when subtilin was assayed in the presence of blood or serum, as compared with those obtained in the presence of phosphate buffer. It was found that within certain limits, in the presence of a constant amount of subtilin, the zone diameter was dependent on the amount of blood and serum present.

Consistent results were obtained in the presence of more than 10 per cent blood or more than 12 per cent serum. When 100 μg subtilin per ml were assayed in the presence of varying concentrations of serum (0.05 to 50 per cent), there was a difference of 10 mm in diameter of the zones over the range of 0.05 per cent to 12 per cent serum, and negligible differences in higher concentrations. These results could not be duplicated in the presence of human globulin or albumin alone or in combination.

In view of the foregoing results it is necessary, when assaying for the antibiotic

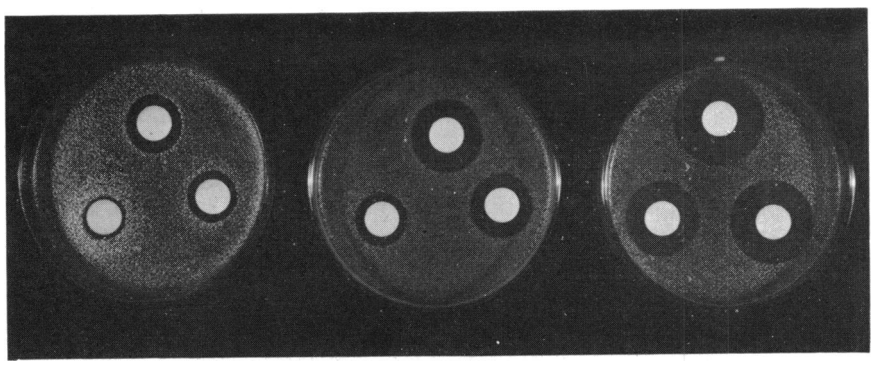

FIG. 3. EFFECT OF NaCl CONCENTRATION OF THE MEDIUM ON THE ZONE OF INHIBITION

NaCl concentration in the plates, left to right, is 0, 0.85, and 2 per cent, respectively. Top disk on each plate moistened with 0.10 ml of subtilin containing 1,000 μg per ml, right disk 500 μg per ml, left disk 333 μg per ml. pH of medium 6.4.

in blood or serum by this method, to dilute the unknown and the sample used for the standard curve with the concentrations of blood or serum mentioned above. The observation that the zone diameter was consistent when different blood or serum samples were used permits dilution of the unknown and standard with normal blood or serum.

The assay of subtilin is not affected by the presence of normal urine in concentrations up to 50 per cent. Assays of such samples are computed from the standard curve obtained from samples diluted with 0.1 M phosphate buffer at pH 6.4.

SUMMARY

A filter paper disk method for the microbiological assay of subtilin, using *Bacillus cereus* 247, has been described. This method also is applicable to the assay of subtilin in certain body fluids. With the proposed procedure the error of the assay is approximately ±5 to 10 per cent, depending on the number of dilutions of the unknown falling within the range of the standard curve.

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