be of variable significance. The problem of leukemogenesis is further complicated by results such as those obtained with strain C3H mice which were susceptible to the induction of leukemia with estrogens but not with methylcholanthrene.[8,9]

*Summary.* Irradiation, when used in conjunction with methylcholanthrene, failed to increase the incidence or to accelerate the onset of carcinogen-induced leukemia in strain Dba and strain F mice.

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## Simplified Procedures for Ascertaining Concentration of and Bacterial Susceptibility to Penicillin.

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The simplified procedures for determining the concentration of and bacterial resistance to penicillin presented herein, utilize only such equipment and supplies as are already available in all bacteriological laboratories and the results obtained are well within the limits of accuracy reported for the "serial dilution" and "cup assay" technics.

The method is based on the following principles and observations:

1. If a stipulated volume, or concentration, of penicillin solution is placed on the surface of an agar plate previously seeded with a susceptible organism, a circular growth-inhibition zone (area) is produced which is *directly* proportional to the concentration, or volume, respectively, of penicillin applied.

2. If a definite volume (0.01 or 0.02 cc) of a stipulated concentration of penicillin is employed, the growth-inhibition zone (area) will be inversely proportional to the resistance of the test organism.

3. A 4 mm (22-23 gauge) platinum loop was found to deliver a sufficiently constant quantity, from a solution containing 1 to 10 Oxford units of penicillin per cc, to warrant its employment for determination of bacterial resistance to and concentration of penicillin within the limits of accuracy required for routine clinical purposes. The inhibition zones produced by application of such a loop were generally perfectly circular, if care were taken to keep the petri dish flat during incubation.

4. Penicillin is sufficiently stable, in standard culture media stored in a refrigerator, to permit the use of such media for quickly ascertaining the relative susceptibility of bacteria (such as are encountered in lesions and exudates) to this bacteriostatic agent.

The technic is briefly as follows: To 15 cc melted nutrient agar cooled to about 45°C (2-3% blood is added when the test organism is a hemolytic streptococcus) is added 0.05 cc of a 20-24 hr 37°C broth culture of the test organism. The contents are then poured into a Petri dish, allowed to harden and placed in the refrigerator until ready for use (1-4 hours).

To determine the resistance of an organism, 4 mm loops of one or more concentrations of penicillin, in M/50 phosphate buffer at pH 7.2, are placed on the surface of the seeded agar plate (within a few minutes after it is removed from the refrigerator) which is then immediately incubated at 37°C, care being taken to see that the plates are horizontal in order to insure that the inhibition zones will be circular. The diameters of the zones of growth-inhibition are measured the following day (18-22 hr incubation) to the nearest 0.5 mm.

The authors naturally entertained some misgivings as to the constancy and duplicability of the inhibition zones which would be obtained with this loop technic. Repeated observations have demonstrated, however,

that the results are readily duplicable—the diameters of the inhibition zones produced by a series of loops from a given concentration of penicillin rarely differed by more than 1 mm while generally the error was not greater than ±0.5 mm—and assays performed simultaneously by different observers disclosed coefficients of variability of 2.5 to 4% in the diameters of the growth-inhibition zones which correspond to a variability of 10-16% in the assay of penicillin potency. Such errors are well within the limits of 15-25% claimed for the "serial dilution" and "cup assay" procedures. It is suggested that development of capillary pipettes which will deliver 0.015 or 0.02 cc would further enhance the accuracy of the technic presented.

TABLE I.
Relation of Concentration of Penicillin to Zone of Growth-Inhibition.

| Conc. of penicillin (Oxford units per ml) | Zone of inhibition on seeded agar* (Diameter in mm) |
|---|---|
| 10.2 | 29.8 ± 0.3 |
| 5.1 | 25.7 ± 0.3 |
| 2.55 | 20.2 ± 0.6 |
| 1.28 | 16.8 ± 0.3 |
| 0.64 | 12.7 ± 0.6 |
| 0.32 | 8.0 ± 0.4 |

* Produced by 4 mm loop employing the Oxford strain *Staphylococcus aureus* as inoculum.

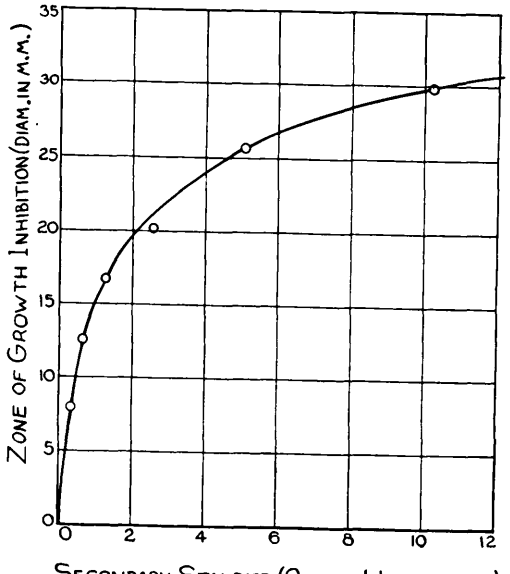


FIG. 1.
Effect of concentration of penicillin (4 mm loop) on zone of inhibition of Oxford strain of Staphylococcus.

In Table I are shown the diameters of the inhibition zones obtained when 4 mm loops of various concentrations of a standard penicillin (kindly furnished by Dr. J. W. Foster[1] of Merck & Co.) were placed on nutrient agar which had been seeded with the Oxford strain *Staph. aureus*. The accompanying photograph (Plate 1) illustrates the appearance of inhibition zones produced against *Staph. aureus* by a 4 mm loop of various concentrations of penicillin.

It will be noted from Fig. 1, where the inhibition zones are plotted against the concentrations of penicillin applied, that the points fall along a smooth curve which will be employed as a curve of reference. Penicillin, as employed therapeutically, is generally prepared so as to contain 5000 Oxford units per cc. If this solution is further diluted 500 (or 1000) fold in M/50 phosphate buffer at pH 7.2, a 4 mm loop of the resulting solution (presumably containing 10 (or 5) units per cc) placed on the surface of agar seeded with the Oxford strain of *Staph. aureus* and the plate incubated at 37°C for 20 hours, the inhibition zone produced, when projected on to the curve in Fig. 1, will show the concentration of the penicillin being employed.

This reference curve is particularly useful for studies on the stability, or rate of destruction of penicillin. With an initial concentration of approximately 10 (or 100) Oxford units of penicillin per cc, the rate of decrease in potency may be easily followed by (1) placing a 4 mm loop of the test solution (diluted 1 to 10 if the initial concentration was 100 units) at desired intervals, on to seeded agar, (2) measuring the diameter of the growth-inhibition zones after 18-22 hours incubation at 37°C and (3) reading off from the reference curve the corresponding penicillin concentrations. A single seeded agar plate may be employed for 4 to 6 determinations and preparation of an extensive series of dilutions of the material under observation (such as would be required for either the

[1] Foster, J. W., and Woodruff, H. Boyd, *J. Bact.*, 1943, **46**, 187.

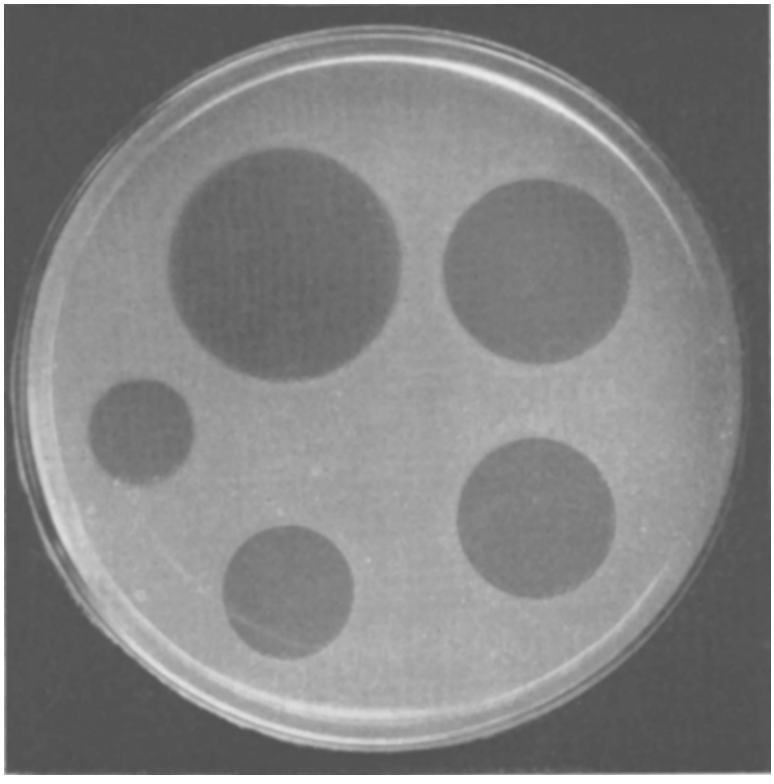

PLATE 1.
Inhibition zones produced by 4 mm loops of penicillin solutions containing 10, 5, 2.5, 1.25, and 0.63 Oxford units per cc, placed on the surface of agar seeded with the Oxford strain of *Staph. aureus*.

TABLE II.
Inhibition Zones Produced by 4 mm Loop of 10 Units of Penicillin on Agar Seeded with Staphylococci of Various Degrees of Resistance to Penicillin in Broth.

| No. of Obs. | Conc. of penicillin (Oxford units per ml) | | Zone of inhibition on seeded agar (Diam. in mm) |
|---|---|---|---|
| | Permitting growth | Preventing growth | |
| 16 | 0.02 | 0.04 | 28.9 ± 1.2 |
| 9 | 0.04 | 0.08 | 24.6 ± 1.3 |
| 7 | 0.08 | 0.16 | 18.9 ± 1.0 |
| 4 | 0.16 | 0.32 | 17.1 ± 0.3 |
| 5 | 0.32 | 0.64 | 14.0 ± 0.8 |

serial dilution or cup assay technics) is eliminated.

In Table II are shown the inhibition zones produced when 4 mm loops of M/50 phosphate buffer (pH 7.2) containing 10 units of penicillin per cc, were placed on agar seeded with staphylococci of various degrees of resistance to penicillin. By plotting the diameters of the inhibition zones against the concentrations of penicillin permitting (or preventing) growth in broth, a curve is obtained which may be utilized to convert growth-inhibition zones produced on agar seeded with a test organism into the probable concentrations of penicillin which would permit (or prevent) growth of the organism in broth. In Table III, which is derived from such a curve, are shown the probable inhibiting concentrations of penicillin in broth corresponding to growth-inhibition zones (at intervals of 2 mm) obtained on seeded agar.

In practice, a knowledge of the exact resistance of an organism to penicillin is not as significant as is the information whether a suspected organism is so susceptible (or resistant) that the concentration attainable in the blood stream may reasonably be expected

TABLE III.
Relation of Inhibition Zone Produced* on Agar Seeded with Staphylococci to Probable Concentration of Penicillin Which Will Permit (or Prevent) Growth in Broth.

| Inhibition zone on seeded agar (Diameter in mm) | Probable conc. (Oxford units per cc) in beef extr. broth | |
|---|---|---|
| | Permitting growth | Preventing growth |
| 29 | 0.02 | 0.04 |
| 27 | 0.03 | 0.06 |
| 25 | 0.04 | 0.08 |
| 23 | 0.05 | 0.10 |
| 21 | 0.07 | 0.14 |
| 19 | 0.09 | 0.18 |
| 17 | 0.14 | 0.28 |
| 15 | 0.21 | 0.42 |

* When a 4 mm loop of 10 units of a penicillin solution in M/50 phosphate buffer (pH 7.2) is placed on the surface of chilled seeded agar.

to be effective (or ineffective) against the organism in question. If growth in 0.2 units were taken as the limit of resistance of staphylococci or other organisms amenable to penicillin treatment, then, if inhibition zones less than 15 mm in diameter were obtained by the procedure described above, the organisms in question would be considered too resistant to warrant satisfactory results when dealing with systemic infections.

It has been observed that penicillin, when placed in bacterial culture media, such as thioglycollate broth or blood agar, deteriorates quite rapidly at 37°C, but when stored at ice-box temperature the decrease in potency was insignificant, even after a period of several weeks. This would indicate that it should be feasible to employ blood agar or other suitable media containing penicillin, if stored in a refrigerator, to ascertain, *simultaneously*, the nature of the organisms present in a lesion or exudate and, within desired limits, the probable resistance of these organisms to penicillin. Observations with several strains of *Staph. aureus* disclosed that inhibiting concentrations of penicillin in broth were practically the same as those obtained when penicillin was incorporated into blood agar media.

It has been the practice in the laboratory of the Brooke General Hospital for several months to suspend exudates in small quantities (0.2 to 0.3 cc) of physiological salt solution (or M/50 phosphate buffer at pH 7.2) and streak out a loop of this suspension on blood agar and blood agar containing 0.04, 0.1, and 0.25 units of penicillin per cc of media. The types of organisms growing on the media containing various concentrations of penicillin were then allocated tentatively, with respect to penicillin susceptibility, as shown in Table IV. When the various types of colonies developing on the blood agar without penicillin were fished and examined for penicillin resistance by the dilution and inhibition-zone technics, the results correlated well with the tentative allocations of the organisms as respects their susceptibility to penicillin and with the clinical observations.

*Summary.* Technics requiring only such equipment and supplies as are already available in any bacteriological laboratory are presented (1) for developing reference curves and tables which may be used (a) for determination of penicillin concentration of solutions employed therapeutically and (b) for following readily the rate of decrease of penicillin potency, (2) for converting growth inhibition zones (for staphylococci) on seeded agar to probable inhibiting concentrations of penicillin in broth, and (3) which make feasible the tentative allocation of bacteria, as respects their susceptibility to penicillin, simultaneously with the determination of the types of organisms encountered, in lesions and exudates, within 15-18 hours after collection of specimens.

TABLE IV.
Relation of Growth of Organisms from Exudates on Blood Agar Containing Penicillin to Anticipated Therapeutic Response.

| Susceptibility to penicillin | Conc. penicillin in blood agar (Oxford units per cc) None | 0.04 | 0.1 | 0.25 | Anticipated response to therapy* |
|---|---|---|---|---|---|
| | Growth on blood agar (24 hrs 37°C) | | | | |
| Very high | + | — | — | — | Satisfactory |
| High | + | + | — | — | " |
| Moderate | + | + | + | — | Questionable |
| Resistant | + | + | + | + | Unsatisfactory |

* As respects amenability to systemic infection.