THE DETERMINATION OF BACTERIAL SENSITIVITY TO ANTIBIOTICS

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INTRODUCTION

THE need for a simple, expeditious and reliable *in vitro* technique for the determination of the sensitivity of bacteria to antibiotics becomes more essential with the ever-extending use of these drugs.

Technical simplicity is essential since requests for these tests are becoming numerically formidable and their value to the clinician depends greatly upon the rapidity with which they can be performed.

Realising four years ago that one of the main factors in successful chemotherapy is speed in preliminary bacteriological investigations, one of us (J. C. G.) began to use impregnated filter paper discs implanted on inoculated culture plates as suggested by Morley (1945) and Kolmer (1947). The method is now widely used and many modifications have been developed.

The purpose of this paper is to describe the techniques evolved in this department to cover all the antibiotic work required in the hospital. We believe the results are adequate for clinical purposes. In the preliminary bacteriological reports emphasis is laid upon sensitivity rather than species identification, and with most specimens a statement of the organisms present and their sensitivity, relative resistance, or resistance can be made within twenty-four hours. Technical procedures have been simplified and standardised as far as possible so that the results are reliable, easily reproducible, and tedious daily controls are eliminated. Moreover, the results are unaffected by minor variations in technique.

The use of absorbent paper as a means of carrying penicillin solutions on the surface of solid media for diffusion purposes appears to have been first suggested by Pope in 1940 (Heatley, 1944). A few years later when methods of assaying penicillin were being explored a number of investigators adopted absorbent paper discs in place of formerly used methods (Lamanna and Shapiro, 1943, Foster and Woodruff, 1943, De Beer and Sherwood, 1944, Sherwood, 1944, Epstein, 1944, and Vincent and Vincent, 1944).

Filter paper is the most convenient form of absorbent paper likely to be of uniform quality and for this reason has been used by most workers. Uniform quality is most important because the great advantage of paper discs is that each absorbs the same amount of fluid if they are of the same size.

As used both in sensitivity determination and in assay, the filter-paper disc method is simply a form of diffusion technique (Sherwood,

1944). As with the other standard diffusion techniques such as the "cup-plate," "ditch-plate" and "cylinder-plate" methods it is affected by certain factors (Vincent and Vincent, 1944, Loo, 1944, Schmidt, 1944, Hobby, 1942) such as the *p*H, the thickness and state of hydration of the medium and by the lag phase of the test organism. However, the disc method appears to be less subject to these variable factors than the other methods (De Beer and Sherwood, 1944; Sherwood, 1944; Vincent and Vincent, 1944; Sherwood, 1947) and undoubtedly has definite advantages over them in the ease with which the discs can be prepared and stored, the increase in accuracy due to the amount of fluid which each will absorb, the even and constant contact of the disc with the medium, and the simplicity of the technique which allows replicate tests to be rapidly set up; also the convenience with which the plates can be handled.

Materials

Preparation of the Paper Discs.—Throughout this work, Whatman No. 1 filter paper was used. The discs were cut out of sheets of the filter fabric with a paper hole-puncher having a diametral size of 7 mm. The discs so produced are fractionally less than 7 mm. in diameter and vary insignificantly in size as long as the instrument is sharp and single thicknesses of paper are punched; single sheet punching also ensures that the discs remain discrete and are more easily handled.

The discs are counted accurately into lots of 100, and put into 1 oz. screw-capped wide-mouthed containers. These bottles are then sterilised in the hot-air oven at 150° C. for one hour.

Preparation of Antibiotic Solutions and Impregnation of the Discs.—The solutions are prepared with sterile distilled water and commercial preparations of high standard issued for clinical use. One ml. of the required solution is then added to each bottle of 100 discs, and as the entire volume is absorbed we may assume that each disc takes up approximately 0·01 ml. Thus dilutions of the antibiotics are prepared to contain, in 1 ml., 100 times the quantity required in each disc. The amount of each antibiotic per disc is as follows :—

TABLE I

Amount of Antibiotic per Disc

Penicillin.	Streptomycin.	Terramycin.	Chloromycetin.	Aureomycin.
1 unit (0·66 μg.)	10 μg.	10 μg.	25 μg.	50 μg.

The discs are used wet. The bottles may be stored in the refrigerator at 5° C. and the antibiotics will retain their potency for at least three months. Before use the bottle should be shaken to distribute the discs around the walls of the container and this allows them to be picked up more easily with forceps.

The discs are transferred to the inoculated plates with a pair of fine-pointed tweezers. The tweezers may be kept with their tips immersed in 70 per cent. alcohol which is flamed off before use. The tips of the instrument should be flame-sterilised between each transference to prevent contamination, but even without this precaution the contamination rate of the discs is surprisingly low. When it does occur organisms of the *Proteus* Group are the common offenders.

The Standard Organism.—The standard organism used for all the assays and for the preparation of the standard graphs for each antibiotic was a Staphylococcus aureus (S5). This organism has the following sensitivity values as calculated by the recognised procedures :—

TABLE II

Minimum Concentration per ml. of antibiotic required to inhibit the Standard Staphylococcus

Penicillin.	Streptomycin.	Chloromycetin.	Aureomycin.	Terramycin.
0·03 units (0·02 μg.)	0·5 μg.	1·5 μg.	0·25 μg.	0·25μg.

The staphylococcus was grown on nutrient agar and before use was subcultured in nutrient broth at 37° C. for eighteen-twenty hours. This culture was diluted five times, and the average number of cells per ml. was then about 100 million by opacity standards.

Preparation of the Standard Graphs.—The preparation of the standard graph for penicillin will be described in detail. Graphs for the other antibiotics are prepared in a similar manner and do not require description.

Solutions of penicillin in distilled water were prepared to give the following range of concentrations per disc :—0·001, 0·005, 0·01 0·05, 0·1, 0·2, 0·5, 1·0, 2·0, 5·0 and 10 units. The discs were impregnated as previously described. Petri dishes containing nutrient agar or blood agar were surface-sown with the standard staphylococcus. The nutrient broth culture containing 100 million organisms per ml. was flooded on to the surface of the plates with a capillary pipette, the plate tilted and the excess pipetted off. The plates were then inverted and dried in the incubator for half an hour at 37° C.

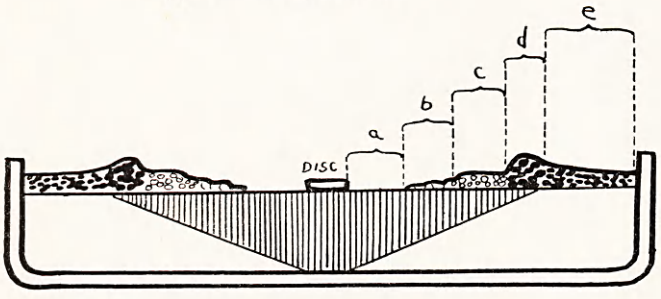
Ten blood agar plates were taken for each concentration of disc, that was, a total of 100 plates, and a minimum of ten estimations for each concentration were carried out. The discs may be applied to the centre of the surface or eccentrically, in which case more than one disc can be placed on each plate.

Each disc was applied carefully to the surface of the agar without lateral movement once the surface had been touched. Where necessary they were flattened down with the points of the forceps.

The plates were then incubated for eighteen-twenty hours at 37° C. ; the resulting zones of inhibition were measured and plotted on graph-paper.

Zones of inhibition.—As often described with sensitive organisms, there are several well-defined zones apparent from within outwards around a source of antibiotic on inoculated solid media.

They are (Fig. 1) (*a*) complete inhibition of growth,
(*b*) delayed growth,
(*c*) lysis, where the shadows of bacterial colonies are present,
(*d*) stimulation of growth, and
(*e*) normal growth.



(*a*) Zone of complete inhibition. (*b*) Zone of delayed growth.
(*c*) Zone of lysis. (*d*) Zone of stimulated growth.
(*e*) Zone of normal growth.

FIG. 1.—Petri-dish in section showing in diagrammatic form the theoretical concentration gradient of antibiotic after diffusion from the disc. Also diagrammatically represented are the various zones of inhibition and growth that may occur.

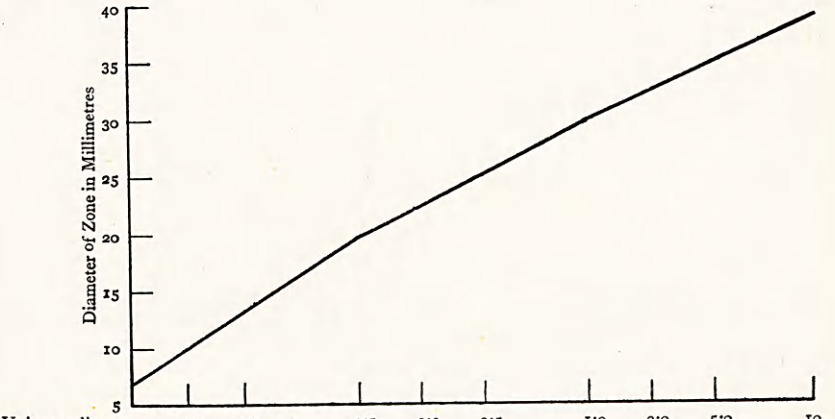
One or more of these zones may not be apparent, depending upon the antibiotic, the sensitivity of the organism and the time of incubation. Measurement is best carried out with dividers in a strong reflected light, and we found it most satisfactory to include the zone of lysis, when present, in our measurements. The diameter of the zone of inhibition is taken in preference to the radius or the distance from the margin of the disc. The results given in Table III are typical of the readings obtained in many experiments.

TABLE III

Zones of Inhibition with Different Concentrations of Penicillin per Disc

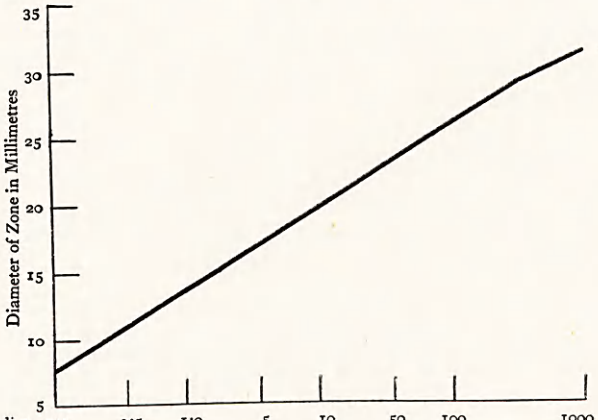
Concentration of Penicillin per Disc (units).	Zone of Inhibition (m.m.)
0·001	0
0·005	0
0·01	7·0
0·05	12·8
0·1	19·6
0·2	22·7
0·5	25·5
1·0	30·5
2·0	33·0
5·0	35·1
10·0	38·8

The graph is constructed by plotting the zone of inhibition in mm. against the logarithm of the concentration in microgrammes or units. Over a wide range of concentration the result is a straight line. Specimen graphs for penicillin and the other antibiotics are given in Figs. 2-6.



Units per disc	·01	·02	·05	0·1	0·2	0·5	1·0	2·0	5·0	10
Units per ml.	1	2	5	10	20	50	100	200	500	1000
Coefficient of resistance	100	50	20	10	5	2	1	0·5	0·2	0·1
Sensitivity units per ml.	3·0	1·5	0·6	0·3	0·15	0·06	0·03	0·015	0.006	·003

FIG. 2.—Standard Graph for Penicillin.



μg per disc	0·1	0·5	1·0	5	10	50	100	1000
μg per ml.	10	50	100	500	1000		10 mgm.	
Coefficient of resistance	100	20	10	2	1	0·2	0·1	0·01
Sensitivity μg per ml.	50	10	5	1	0·5	0·1	0·5	·005

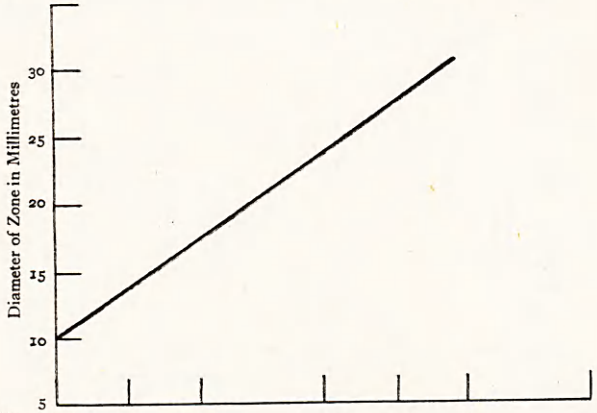
FIG. 3.—Standard Graph for Streptomycin.

It should be noted that the smallest zone of inhibition that can be measured is that which is just visibly greater than the disc, about 7·0 mm. in diameter.

As can be visualised from Fig. 1 the concentration of the antibiotic constantly decreases with distance from the disc. Some antibiotics

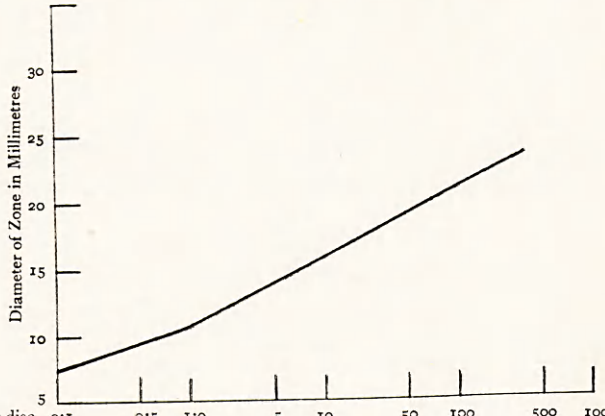
Note.—Those wishing to follow the detailed technique described in Figs. 1 to 6 should transcribe and enlarge the graphs on sectional paper.

diffuse more rapidly than others. If the organism is inoculated before applying the disc to the medium, diffusion must take place rapidly enough to produce clear-cut zones of inhibition. If conditions are identical in repeated tests, the rate and extent of diffusion will remain constant, and identical zones of inhibition will be obtained.



μg per disc	1·0	2	5	10	20	50	100
μg per ml.	100	200	500	1000		5 mgm.	10 mgm.
Coefficient of resistance	25	12·5	5	2·5	1·25	0·5	
Sensitivity μg per ml.	37·5	19	7·5	3·75	1·9	0·75	

FIG. 4.—Standard Graph for Chloromycetin.



μg per disc	0·1	0·5	1·0	5	10	50	100	500	1000
μg per ml.	10	50	100	500	1000	10 mgm.			100 mgm.
Coefficient of resistance	500	100	50	10	5	1	0·5	0·1	0·05
Sensitivity μg per ml.	125	25	12·5	2·5	1·25	0·25	·125	·025	

FIG. 5.—Standard Graph for Aureomycin.

Absolutely constant conditions are impractical in routine work; we have investigated the variable factors to determine their effect on the results and to decide upon the latitude which may be allowed without significant alteration in the zones of inhibition. As a result of these experiments we have been able to adopt certain standards and procedures which constitute our modifications of the original method.

EXPERIMENT 1. *Variation in the Size of Disc.*—Different sizes of disc were prepared of diameter ranging from 4 to 12 mm. Using 1·0 ml.

of fluid the larger discs are not uniformly saturated as 100 will absorb more than this amount. The smaller discs are uniformly saturated but do not absorb all the fluid. The results obtained over a number of experiments with discs of different sizes is shown in Table IV. The

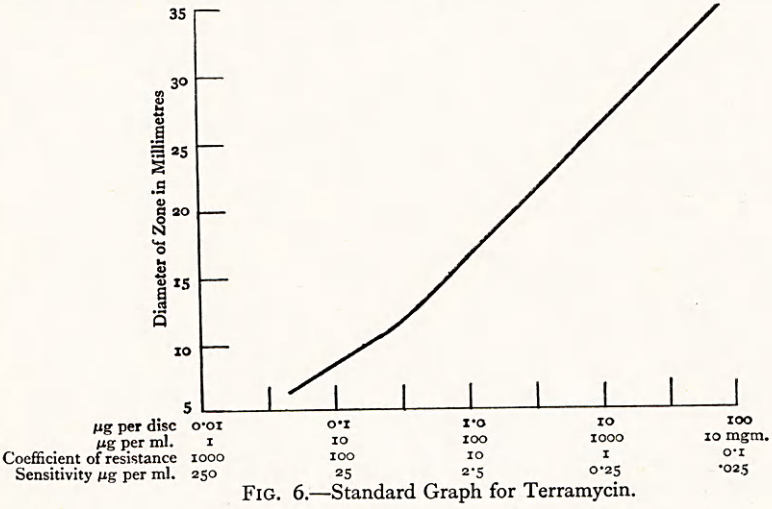


FIG. 6.—Standard Graph for Terramycin.

size of disc, 100 of which will absorb 1·0 ml. of fluid, is about 7·0 mm. The disc of 6·8 mm., being slightly smaller, ensures that each disc is fully saturated when 100 are mixed with 1·0 ml. of solution and the reduction of antibiotic content is insignificant. For this reason the 6·8 mm. disc was adopted as our standard.

EXPERIMENT 2. *Variation in Type of Paper.*—6·8 mm. discs of Whatman No. 1, No. 2 and No. 40 and of thick and thin blotting paper were used under conditions similar to those in Experiment 1. There

TABLE IV

Zones of Inhibition with Different Sizes of Disc

Size of Disc.	Number of Tests.	100 Discs to 1·0 ml. Solution (zones in mm.).	Discs Individually Saturated (zones in mm.).
12·0	50	35	34
8·0	50	33	32
6·8	50	30	30
5·0	50	27	27

was little variation between the zones obtained with the filter papers but the blotting paper gave unsatisfactory results since a disc of this size absorbs more than 0·01 ml. The results then depend upon the amount of fluid that the paper is capable of absorbing.

EXPERIMENT 3. *Conditions of Storage.*—Discs prepared in the way already described were stored under different conditions and then tested for potency as shown by the zones of inhibition. As can be seen from Table V the discs retained their potency for a longer period in the

refrigerator than at room temperature, and longer when wet than when dry. The variation in zone diameter was also less when wet than when dry.

Discs saturated with aqueous solutions of the other antibiotics were

TABLE V

The Effect of Storage and Time on Potency of Discs

Penicillin 1 Unit Discs.		Time. (Zones of inhibition in mm.).				
		1 Week.	4 Weeks.	2 Months.	3 Months.	4 Months.
Wet	Room temperature .	30	29	29	28	28
	4° C.	30	30	30	30	29
Dry	Room temperature .	29	29	28	27	26
	4° C.	29	28	27	27	27

TABLE VI

Showing the Effect of Time on the Potency of Discs

Antibiotic μg./Disc.	Time in Months. (Zones of inhibition in mm.).									
	1.	2.	3.	4.	5.	6.	9.	12.	18.	24.
Streptomycin 10	18	18	18	18	18	18	17	17	16	15
Chloromycetin 25	30	30	30	30	30	30	27	25	...	...
Aureomycin 50	21	21	20	20	20	18	...	...	...	...
Terramycin 10	27	26	23	23	...	...	...	...	...	...

TABLE VII

Effect of the Amount Inoculum on Zone of Inhibition

Inoculum (orgs./ml.).	Penicillin Discs Containing 1·0 Units.		
	Number of Tests.	Zone of Inhibition in mm.	Remarks.
50,000	50	37·5	Individual colonies on plate
500,000	50	30·5	Almost confluent growth
5,000,000	50	30·5	Confluent growth
50,000,000	50	30·0	Confluent growth
100,000,000	50	30·5	Confluent growth
200,000,000	25	30·0	Confluent growth
500,000,000	25	29·0	Confluent growth
1,000,000,000	10	28·5	Confluent growth

stored in the wet state and tested for potency after lengthening periods of time. The results are given in Table VI.

EXPERIMENT 4. *Amount of the Inoculum.*—In the standard procedure already described the culture plates are seeded with broth containing approximately 100 million staphylococcal cells per mm. The effect of varying this inoculum was tried and as will be seen from Table VII, within fairly broad limits, there is no great variation in the zones of inhibition produced by discs of the same potency.

That this does not hold true for all antibiotics, particularly Terramycin, is shown in Table VIII where the effect of a ten-fold variation in inoculum is shown for each agent.

EXPERIMENT 5. *Age of the Inoculum.*—The older the culture, the smaller the number of viable cells it will contain ; the lag period for

TABLE VIII

The Effect of Inoculum on Zones of Inhibition

Antibiotic.	Inoculum (cells/ml.).	Zone of Inhibition (mm.).
Streptomycin .	20,000,000	18
	200,000,000	17
Chloromycetin .	20,000,000	30
	200,000,000	28
Aureomycin . .	20,000,000	21
	200,000,000	18
Terramycin . .	20,000,000	27
	200,000,000	21

these cells will be longer than for those used in the logarithmic period of growth, as in experiment 4. However, in practice the age of the cells of the inoculum has an insignificant effect on the zone diameter.

EXPERIMENT 6. *Variation in the Method of Inoculation.*—Pour plates of nutrient agar previously seeded with the staphylococcus are not recommended because the zones of inhibition are more difficult to read and their preparation is more complicated. Surface seeding gives the best results when the distribution of the organisms is reasonably

TABLE IX

Showing Effect of Different Methods of Inoculation Plates

Method of Inoculation.	Inoculum (orgs./ml.).	Zone of Inhibition (mm.).
Pour plates . . .	100,000,000	28
Fixed volume of inoculum	100,000,000	30
Flooded plate . .	100,000,000	30

uniform. A small amount of broth culture may be spread over the surface of the medium with a suitable instrument and allowed to dry ; if carefully done this results in a uniform growth. However it is easier to pour an excess of the diluted broth over the surface of the medium, drain off the excess, and dry in the incubator.

The zones of inhibition obtained by these methods are similar (Table IX).

The time of drying the plates in the incubator at 37° C. does not

affect the zones of inhibition provided that it does not exceed two hours. After two hours the plates become dehydrated to some extent and colony growth begins ; both factors influence test readings.

EXPERIMENT 7. *Type of Medium Used.*—The *p*H of the medium has been shown to have a marked effect on the zones of inhibition of growth of penicillin (Schmidt, 1944), streptomycin (Loo, 1944) and aureomycin (Pratt and Dufrenoy, 1948, and Ingram, 1950). We did not pursue this as, with few exceptions, the routine laboratory media have a *p*H around 7·2-7·6. Variation within these limits has insignificant effect upon the zones obtained with any of the antibiotics.

The agar base of the medium must be homogeneous ; otherwise there will be irregularities in diffusion.

The effect of the concentration of agar, and the addition of other materials such as blood (blood agar medium) and bile-salts (McConkey's

TABLE X

Showing the Effect of Concentration of Agar and of Other Ingredients in the Medium

Concentration of Agar.	Medium.	Zones of Inhibition in mm.				
		Penicillin.	Strepto-mycin.	Chloro-mycetin.	Arueomycin.	Terramycin.
1 per cent.	Nutrient agar	29	18	30	20	26
2 ,,	,,	30	19	29	20	25
3 ,,	,,	30	19	29	18	25
4 ,,	,,	29	18	29	18	25
6 ,,	,,	23	16	25	16	24
2 ,,	Blood agar	30	19	30	19	24
2 ,,	McConkey	30	20	30	19	24

medium) were investigated. The more concentrated agar (Table X) retards diffusion of penicillin since the zones of inhibition are smaller but there is no significant difference within the range of concentrations usually used in preparing laboratory media (1·5-3·0 per cent.). The zones of inhibition obtained on blood agar medium and on McConkey's medium are the same as those obtained on nutrient agar medium.

EXPERIMENT 8. *Thickness of the Medium.*—Other conditions being equal, it is the depth of the medium which determines the extent of diffusion of the antibiotic (De Beer and Sherwood, 1944) ; the thicker the agar medium the smaller the zone of inhibition. When the medium is very thin there is the additional factor of indistinct margins to the zones of inhibition, making exact measurement difficult. Using the plates poured daily for routine use in the laboratory, we found no significant variation on this account.

We use 7 cm. Petri dishes for routine work and find 5 ml. of medium satisfactory. Table XI shows the zones of inhibition obtained with thicker and thinner layers of medium.

EXPERIMENT 9. *Time of Incubation.*—Penicillin and the other antibiotics diffuse rapidly outwards before any appreciable growth has taken place. We may thus disregard the effect of time on the diffusion of the active substance.

The standard organism grows appreciably in six-eight hours' time, and by then there are well-marked zones of inhibition of growth with penicillin. These are zones of absolute inhibition and correspond in size to the zone of absolute inhibition seen after twenty hours. The zone of lysis forms between ten-twenty hours and is included by us in the zone measured. However, there is no reason why the zone of absolute inhibition should not be used as the criterion and read at eight hours if desired; this is usually inconvenient unless the plates are inoculated first thing in the morning. Further, it will not be feasible

TABLE XI

Showing the Effect of the Thickness of the Medium

Size of Plate Diameter in cm.	Amount of Medium per Plate.	Thickness of Medium.	Zones in mm.				
			Penicillin.	Strepto-mycin.	Chloro-mycetin.	Aureomycin.	Terramycin.
7	3·0 ml.	0·8 mm.	33	...	...	...	...
7	5·0 ,,	1·3 ,,	30	20	29	18	27
7	7·5 ,,	2·0 ,,	28	...	...	...	...
7	10·0 ,,	2·6 ,,	26	20	27	17	25
7	15·0 ,,	4·0 ,,	22	...	...	...	...

in the sensitivity determination of a slow-growing organism. The zone of lysis does extend after twenty hours and is appreciably larger at forty-eight hours; for this reason readings should not be too long delayed.

EXPERIMENT 10. *Incubator Temperature.*—Variation within the usual range of incubator temperatures used in the laboratory produces little effect upon the zone of inhibition provided that growth actually takes place.

SUMMARY OF EXPERIMENTS 1-10

As a result of these experiments the following points are of importance in obtaining consistent results.

Petri-dishes containing a uniform thickness of medium are used. They are surface-sown with an over-night broth culture of the standard organism for assay purposes, or with the test organism, for sensitivity determination. The plates are then dried for thirty minutes at 37° C.; the discs are then applied. It is essential that the discs be of uniform size and material, and similar to those used in the preparation of the standard graphs. Measurements are made after eighteen-twenty hours' incubation.

Adequate results can be obtained with routine laboratory plates sown in a variety of ways; these are described below.

EXPERIMENT 11. *Test of the Reproductibility of Results.*—1000 penicillin 1 unit discs were taken from freshly prepared stock and used under the conditions just described. Table XII gives the results.

THE DETERMINATION OF THE SENSITIVITY OF AN ORGANISM

The exact identity of the organisms present is unnecessary for sensitivity determinations as long as the colonies are individually recognisable on the medium.

An initial sensitivity result can be obtained from the primary cultures by placing discs on suitably inoculated plates at the time of inoculation. The variety of media inoculated, and atmospheres of incubation, must be sufficiently wide to ensure growth of any species of micro-organism likely to be present. For example, discs must be placed on anærobic blood medium if anærobic streptococci are anticipated. This test may be regarded as a screening test.

The Screen Test.—This may be varied to suit different conditions.

TABLE XII

Results of 1000 *Consecutive Tests with* 1 *Unit Penicillin Discs*

Number of Tests.	Mean of Zones. mm.	Standard Deviation.	Standard Error of Mean.
1000	30·13	1·075	0·034

(1) Where the sensitivity to one antibiotic only is required. (*a*) Area of initial inoculation in primary cultures of the specimen is made larger than usual so that it covers about one-third of a $3\frac{1}{2}$ ins. Petri-dish and is spread as uniformly as possible. The disc containing the antibiotic is applied to the centre of the area. After incubation the zone of inhibition of growth is measured and in most cases will give a satisfactory assessment when compared with the more detailed test carried out at a later stage.

There will be a number of tests where the result cannot be assessed properly as (i) where more than one species are present, the growth of an insensitive organism occasionally masks the sensitivity of a sensitive organism, and (ii) where the inoculum is so sparse that a satisfactory zone of inhibition is not formed. (*b*) Alternatively, a plate may be inoculated especially for the discs by stroking out the material with a loop or swab across the whole area of the plate as uniformly as possible.

(2) Where the sensitivity to more than one antibiotic is required.

A large Petri-dish or several smaller dishes should be inoculated as described in (1) (*b*) and the individual discs applied at suitably spaced intervals. A 4 in. Petri-dish will accommodate five discs comfortably.

Method of Reading Results.—The diameter of the zone produced by the disc on the plate cultures of the organism is measured in mm.

From the standard graph prepared for the particular antibiotic, the concentration of the antibiotic to which the organism is sensitive, can be read off directly in figures together with its coefficient-of-resistance compared with the standard Oxford Staphylococcus.

The initial screening test will give information usually adequate for clinical purposes and no further sensitivity determination will be necessary. However, it is often desirable to carry out further tests in order to obtain a more exact estimation or to determine the sensitivity to additional antibiotic substances. In such cases we carry out the sensitivity determination on subcultures of the test organism with a standardised inoculum.

The Sensitivity Test on Subcultures of the Test Organism.—This can be carried out as soon as the organisms have been isolated. Subcultures are made in a suitable fluid medium and are usually incubated for eighteen hours, but often for less. The resulting cultures are diluted to give approximately 100 million cells per ml. by opacity standards, and the test plates are surface seeded. One plate is sufficient for each organism to be tested, but as many as desired may be inoculated as duplicates. The disc for each antibiotic is then applied and the zones of inhibition growth read after eighteen-twenty hours' incubation.

The sensitivity may be read off from the standard curve as before.

In a case where the result of a sensitivity test is urgently required and it has not been feasible to carry out the screening test, for example with a blood culture, a heated blood agar plate may be seeded with material from the primary culture in the usual way and the required disc applied. After six-ten hours the zone can be measured if the organism has grown sufficiently. This zone will differ from the zone that would be obtained after eighteen-twenty hours, and due allowance must be made in interpreting the sensitivity values.

The Reporting of Results

Only in special cases such as septicæmia and in research work is it necessary to know the exact level of sensitivity expressed in micrograms per ml., units or coefficient of resistance. An opinion expressed in terms such as " sensitive," " relatively resistant " and " resistant," should suffice for clinical purposes.

Since *in vivo* sensitivities cannot be accurately gauged from *in vitro* levels of sensitivity, mathematical expressions of these might even be misleading. We therefore use descriptive words, but relate them to two arbitrary ranges of *in vitro* concentrations ; one range (Table XIV) is used in reporting examinations of the urine, and the second (Table XIII) is used for all other specimens ; it is based on the average systemic levels of the antibiotics usually obtained during treatment.

In determining these ranges we have attempted to combine the experience of our clinical colleagues in Edinburgh with our reviews of follow-up bacteriological examinations.

The Presence and Detection of Resistant Colonies

Occasionally within the clear zone of inhibition, a few colonies may grow more or less right up to the disc. If these colonies are of the same species then they may be regarded as resistant variants. Alternatively they may be another species separated by the selective activity of the antibiotic.

The resistant colonies may be subcultured and the actual sensitivity

TABLE XIII

Correlation of Clinical Terms with Sensitivity Values for Usual Systemic Concentrations

Term for Clinical Use.		Antibiotic. *In vitro* Levels in μg./ml.				
		Penicillin.	Strepto-mycin.	Chloro-mycetin.	Aureomycin.	Terramycin.
Sensitive .	Up to . .	0·3	1·0	2·5	2·5	1·0
Relatively Resistant	Up to . .	1·0	10·0	10·0	10·0	5·0
Resistant .	Greater than .	1·0	10·0	10·0	10·0	5·0

TABLE XIV

Correlation of Clinical Terms with the Sensitivity Values for Usual Urinary Concentrations

Term for Clinical Use.		Antibiotic. *In vitro* Levels in μg./ml.				
		Penicillin.	Strepto-mycin.	Chloro-mycetin.	Aureomycin.	Terramycin.
Sensitive .	Up to . .	50	10	10	15	10
Relatively Resistant	Up to . .	100	100	40	100	25
Resistant .	Greater than .	200	100	40	100	25

determined. Their presence will usually weigh heavily against the use of that particular antibiotic.

A heavy inoculum of 1000 million organisms, or more per ml. may be used to seed the test plates where it is desired to determine the presence or absence of resistant variants in a culture. This is especially applicable to urinary tract pathogens. The heavier the inoculum, the greater the chance of isolating resistant variants. The practical use of this has already been shown in the case of Myco. tuberculosis by Tinne (1950).

Procedure with Antibiotics Other Than Penicillin

As we already said the procedure given is the same for all the

antibiotics in common use at present. Streptomycin and chloromycetin behave exactly like penicillin with regard to these tests ; further discussion may therefore be confined to aureomycin and terramycin.

(1) *Aureomycin.*—This antibiotic is susceptible to certain substances present in blood, and sometimes in peptones, used to make media (Herrell, 1950, Bliss and Chandler, 1948) so that it tends to be inactivated during the period of growth of the test organism. This is one of the chief reasons for the relatively small zones of inhibition with aureomycin and the tendency for the test organism to grow inwards towards the disc during the later hours of incubation. We can overcome this is three ways.

(*a*) By using medium with a *p*H of 5·5 (buffered) when the inactivating substances are rendered inert and the zones of inhibition are much larger and better defined. Unfortunately many bacteria to be tested will not grow at this *p*H level and also we must admit that conditions will be even further removed from those likely to be present *in vivo*.

(*b*) By adopting the policy, already referred to, of reading the zones of inhibition after eight hours' incubation.

(*c*) By incorporating reducing substances in the medium or incubating under anærobic conditions (Price, Randall and Welsh, 1948).

(2) *Terramycin.*—The zones of inhibition around a terramycin disc are clear cut in the early hours of incubation and we have adopted the technique of reading results after eight-ten hours' incubation.

There is a great difference between the bacteriostatic and bactericidal concentrations of terramycin for most organisms ; this would appear to be the reason for the diminished zone of inhibition during the later hours of incubation. This of course means that in accepting the eight-hour zones we are taking the " bacteriostatic " level ; the " bactericidal " level is related to the usually much smaller zone obtained after longer incubation. This reasoning is substantiated by parallel serial dilution tube tests where the wide gap between bacteriostatic and bactericidal levels is obvious.

The Determination of the Sensitivity to Streptomycin of Myco. Tuberculosis

The determination of the sensitivity of *M. tuberculosis* to antibiotics presents difficulties—among others, the particularly slow rate of growth of the organism in culture.

We have found that it is possible to use the disc diffusion technique for the sensitivity determination of this organism to streptomycin. So far we have carried out only a limited number of tests and are attempting to improve the technique ; we mention this merely to indicate that satisfactory zones of inhibition can be obtained.

Lowenstein-Jensen, or similar medium, is coagulated in Petri-

dishes so that the thickness of the medium is similar to that used for the routine sensitivity tests. Material containing the tubercle bacillus (this may be a concentrate of sputum, etc., known to contain the bacillus, or an emulsion of a culture of the organism) is spread over the surface of the medium as uniformly as possible, and allowed to dry. The discs of streptomycin (10 μg./disc) and Para-amino salicylic (200 μg./disc) are then applied to the surface of the medium. The space between the lid and the base of the Petri-dish is sealed with vaseline-paraffin wax mixture to prevent dessication, and the plates are incubated for the time required to produce visible growth. Aeration of the cultures is carried out at the usual intervals.

Using the standard H.37 Rv. strain of M. tuberculosis (kindly supplied by Professor C. P. Beattie) the zones of inhibition obtained with the streptomycin disc correspond fairly well with the calculated zone for an organism sensitive to 0·5 μg./ml. of streptomycin.

Satisfactory zones are frequently obtained within ten days, and as this is possible with primary cultures of sputa, the method may have some practical application.

The Observation of the Presence or Absence of Synergistic Inhibitory Effect by Combinations of Antibiotics

The combination of two or more antibiotics is being more widely used in treatment with or without definite evidence that the pathogens are more sensitive to the combination than to one or other of the components of the mixture, alone. The effect of such a combination on bacteria can be shown when antibiotics are mixed in fluid media but has been more difficult to demonstrate with solid media using diffusion techniques. Placing a disc of antibiotic A on top of a disc of antibiotic B, or *vice versa* may not be very satisfactory as A may diffuse more rapidly and to a greater extent than B, so that all the inhibitory effect is due to A, B being present in effective concentration only within the zone of inhibition due to A.

To forecast the synergistic effect of combinations of antibiotics we use the disc-diffusion method after the manner of Lamanna and Shapiro (1943). The sensitivity of the test organism to antibiotics A and B of the intended combination is determined in the usual way. Next, a fresh culture plate is sown with the test organism, and discs of A and B are applied at a distance " X " mm. apart, where X equals $a+b$; a being the radius of the zone of inhibition to antibiotic A, and b the radius of the zone to B. After incubation the two zones of inhibition will just make contact and there will be a thin wedge of growth on either side of the point of contact of the zones. When there is appreciable additive or synergistic inhibitory effect (Kolmer, 1945) of individually sub-inhibitory concentrations of antibiotics A and B, the growth in the area of the wedges will be inhibited and can be seen without difficulty.

So far we have not attempted to interpret this in a quantitative manner.

Mutual interference of antibiotics may also be demonstrated by this method.

Comparison of the Disc Diffusion Method with Other Diffusion Methods and the Serial Dilution Tube Method

The " ditch " (Fleming, 1929) and " cylinder " (Foster and Woodruff, 1943, Abraham, 1941, Foster and Woodruff, 1944) plate techniques give results comparable with the disc modification but they are infinitely more tedious, less accurate and give less easily reproducible results.

The serial dilution tube method is very much more laborious than the disc-diffusion method. In routine work mistakes tend to occur more easily with this method, pipetting errors being especially common. The medium must adequately support the growth of the test organism otherwise very discrepant results may be obtained. When the disc-diffusion method was carried out in parallel with the serial dilution tube method, closely similar results were obtained.

In one experiment, 100 consecutive cases of staphylococcal infection were taken and the sensitivity of the infecting organism determined by the screen test we have described for primary cultures ; after subculture the sensitivity was again determined, both by the serial dilution tube method and by the diffusion method. There was satisfactory correlation between the results of the screen test and those of the serial dilution tube test ; and even closer agreement between the tests on subculture. 95 of the 100 cases gave sensitivity results which agreed in all three tests. Of the remaining 5 cases, 3 gave results in the screen test which placed them in the " relatively resistant " zone of the penicillin graph ; on subculture they proved to be fully resistant to 20 units by both the serial dilution tube method and by the disc method. A fourth organism was " sensitive " by the screen test, and sensitive to 1·0 unit/ml. by the tests on subculture. In the fifth case the organism, sensitive to 0·2 units by the screen test and diffusion test on subculture, was found to be resistant to 20 units by the serial dilution tube method.

This experiment is typical of our experience and shows that the diffusion technique is as accurate as the more complicated tube test for all purposes. In addition it has the advantage of simplicity, speed and easy reproductibility.

The Assay of Antibiotic Substances

The number of requests to assay antibiotics in body fluids is small in a routing hospital laboratory.

Paper discs may be used to assay these substances and, within the limitations of the method, provides a very simple and rapid procedure. The chief limitation is in the amount of antibiotic that can be detected.

This corresponds to the smallest amount which will give an appreciable zone of inhibition around the disc ; as the disc will only absorb 0·01 ml., the amount of antibiotic present in 1·0 ml. of the test fluid must be considerable. The practical lower limits assayable for each antibiotic with the standard staphylococcus are as follows :—

TABLE XV

Assayable Lower Limits ug./ml.

Penicillin.	Streptomycin.	Chloromycetin.	Aureomycin.	Terramycin.
2·0	15·0	75·0	10·0	10·0

The sensitivity of the test may be increased by layering discs, one on top of the other, up to a maximum of 4, and this has been found to give fairly satisfactory results. In this manner concentrations of penicillin of 0·5 units per ml. may be assayed.

The range of the test may also be increased by using a particularly sensitive organism for each antibiotic. This necessitates the construction of separate standard curves, and will not be considered here.

In our opinion the disc method is of value in assaying specimens of urine and other fluids where the concentration expected is high ; it is also useful for checking assays on laboratory standard solutions of antibiotics.

The Method of Assay.—The technique is the same as that described for determinations of the sensitivity of an organism except that the unknown quantity is the amount of antibiotic in the disc.

The standard staphylococcus in eighteen-hour broth culture, 100 million cells per ml., is surface seeded on nutrient agar plates which are then dried for thirty minutes. One ml. of the fluid for assay may be added to 100 sterile discs in a bottle, or alternatively, individual discs may be soaked with the fluid by dipping. The latter method is not quite so accurate but is more economical in discs. Care must be taken not to carry over an excess of fluid, and this is best avoided by draining the disc against the side of the container.

The zones are read after eighteen-twenty hours ; the concentration per disc or ml. can be read off from the standard curve for the particular antibiotic concerned.

Discussion

Clinical diagnosis is not a dependable guide to the microbial flora present and certainly not to their antibiotic sensitivity. Bacteriological data not only serve as guides to the drug of choice, and alternatives, but also as an indication of the adequate dose.

Clinicians agree in principle that in most bacterial infections the sensitivity of the causative organism should be determined before chemotherapy is begun, but inadequate laboratory resources, incon-

veniently situated laboratories or delay in the receipt of laboratory advice make this difficult or often impossible. Since progress has been made in the development of rapid laboratory techniques for the purpose, and the range of available antibiotics is still expanding—each with its own antimicrobial "spectrum"—it can be categorically stated that specimens should now be taken for bacteriological examination before these remedies are exhibited and that it is advisable to know the pre-treatment sensitivity of the causative organism. Of course this may be impossible if no material containing the causative organism is available, or if, as in an emergency, it is necessary to give an antibiotic before the result of laboratory data is to hand.

The premature use of antibiotics for the alleviation of symptoms, *e.g.* pain or fever, or as an aid to diagnosis is to be deprecated. This practice masks symptoms, delays effective treatment, and complicates laboratory identification of the causative organism; the bacteria assume bizarre characters and growth may be temporarily reduced.

A working knowledge of the anti-microbial "spectrum" possessed by each of the five antibiotics in general use, is of advantage, but this has been perhaps over emphasised by those concerned in the production and distribution of these drugs. It should be remembered that all organisms of a particular group or species are alike in their sensitivity to one particular antibiotic; to give only a few examples, the antibiotic of choice for coagulase-positive staphylococci, streptococci other than those of Lancefield's Group A, and Gram-negative bacilli of wounds and of the urinary tract, can only be determined by a sensitivity test in each individual case.

In practice there is no reliable guide to the therapeutic use of antibiotics other than *in vitro* sensitivity allied to clinical experience. There are individual cases in which the taking of a pre-treatment specimen for bacteriological examination is inexpedient: there are others such as meningococcal meningitis in which the exhibition of a sulphonamide should not be delayed for sensitivity determination: but in general the choice and dose of antibiotic should be guided by the bacteriological findings in each individual case.

The object of the work presented in this paper has been the adaptation of tests in connection with antibiotics to routine bacteriological procedures in a hospital laboratory.

When the laboratory and clinical points of view are correlated there is no doubt that diffusion methods with solid media are the methods of choice; they show more clearly than other test methods the different ways in which an organism may react to antibiotics *in vitro*.

Of the diffusion methods, the impregnated disc technique is the most simple and flexible. We have adapted the technique to cover all antibiotic work likely to be required of a hospital laboratory and we have found the results adequate for practical purposes.

In most laboratories the staff engaged in preparing and setting up primary cultures could undertake the screen test on every specimen

sent for bacteriological examination—without excessive increase in the amount of work when the tests become routine. Subculture sensitivity tests are a different matter, but one worker can undertake more than 100 in a day if necessary, and the difference in time required to apply the separate discs for each additional antibiotic is small compared with the time taken in preparing and seeding the plates.

It is suggested that sensitivity levels be reported in the form of descriptive words correlated with levels known to exist in body fluids when the patient is under treatment. Owing to the higher levels of various antibiotics in the urine as compared with the concentration obtainable in the blood and tissue fluids, we use two scales for the interpretation of *in vitro* levels.

It is as well to remember that we are as yet at an elementary stage in correlating the clinical administration of antibiotics with *in vitro* sensitivity determinations designed to give some idea of the amount of antibiotic required.

It is possible that the sensitivity determination, using the primary culture, gives a result which is actually more nearly related to *in vivo* conditions, especially when multiple species of organisms are concerned. The mutual effect of the organisms on one another is coupled with the action of the antibiotic; so the test result may well differ from that obtained when the organisms are tested in pure subcultures.

If the discs are applied to primary culture plates, apparent anomalies may occasionally influence the reading of the result; the inhibition by a given antibiotic may differ under ærobic and anærobic conditions. It may be that the organism *in vivo* is subject to a reduced oxidation-reduction potential; in such a case the anærobic sensitivity level would perhaps have greater significance. As it is not possible to decide the actual conditions in the patient, it is our custom to report the lower level of sensitivity, where there is any clinical reason for so doing.

We believe that the study of the zone of inhibition surrounding the source of an antibiotic on a Petri-dish, can give a great deal of information about the action of the antibiotic on the organism under test, and that this information may be applicable to clinical work. The appearance of a few resistant colonies within the zone of inhibition of the majority is of significance. The merging of zones between two antibiotics is an indication of additive or synergistic inhibition. The difference between the bactericidal and bacteriostatic effects of an antibiotic can be very clearly seen and would appear to have great significance with agents such as terramycin. Using the serial dilution tube technique, visual end-points give sensitivity values which are lower than the bactericidal level determined by the recovery of viable cells from the apparently clear tubes. This can be seen on diffusion plates where the well-marked zone of inhibition, present during the early hours of incubation, is encroached upon during the later hours. Within the bactericidal concentration for the test organism, all cells on the surface of the medium are killed and at no time will there be

growth in the zone, no matter how long the plate is incubated. This zone of complete inhibition corresponds to the bactericidal level. Outside the zone of complete inhibition the concentration falls to the bacteriostatic level and within the zone, corresponding to this second concentration, the cells on the surface of the medium are not killed; the lag period of growth is increased. Once this lag period is over, growth is demonstrated by a fine haze over the surface of the medium spreading inwards to the periphery of the zone of complete inhibition. With terramycin this zone of delayed growth or bacteriostasis, is particularly large, varying of course with the particular organism concerned. Probably this has a distinct significance with regard to the outcome of treatment with this drug.

Summary

Details are given whereby the disc method may be incorporated in the procedures of any hospital laboratory for all antibiotic work.

The methods described include a screening sensitivity test on primary plate cultures, a sensitivity test on subcultures, a method by which antibiotic synergism against any particular organism may be observed, and lastly, a method for the assay of antibiotics in fluid.

The few simple materials required are available in any hospital laboratory and no special culture media are necessary. No tedious controls are involved and dilutions of antibiotics need be made only once in three months.

As a screening test the method may be allied with the usual procedures involved in the isolation and identification of micro-organisms in hospital laboratories; the few additional manipulations are extremely simple and may be performed within a few seconds. The incorporation of the technique in routine bacteriological examinations actually facilitates the isolation of micro-organisms present in many specimens; the organisms and their sensitivity to the various antibiotics can generally be reported from primary cultures—that is to say, within twenty-four hours of the time the specimen is received.

By merely measuring the diameter of the zone of inhibition the sensitivity level and coefficient of resistance, compared with the standard staphylococcus, may be obtained from standard graphs. These are included in the text for terramycin, penicillin, streptomycin, chloromycetin and aureomycin; assays may be performed with equal facility.

Experiments performed to test accuracy show that this is adequate for clinical purposes. In other respects it has practical advantages over other techniques.

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