A SIMPLE METHOD FOR THE DETERMINATION OF BACTERIAL SENSITIVITY TO SULPHONAMIDES BY THE USE OF BLOTTING-PAPER DISKS

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(With Plate 15)

Since the introduction of the sulphonamide drugs difficulty has been experienced in obtaining a reliable indication of their action on bacteria *in vitro*. This has been mainly due to the presence in most ordinary laboratory media of substances, known and unknown, which neutralize the inhibitory powers against bacteria which the sulphonamides possess *in vivo*.

To overcome this difficulty synthetic media have been introduced from time to time, but most of them are too expensive for routine use.

In 1945, Harper & Cawston described a method of removing the 'sulphonamide-antagonizers' from media containing peptone broth by the preliminary incubation of the broth with lysed horse red blood cells.

Various workers have suggested the use of disks of blotting paper, or of other absorbent material, impregnated with penicillin solutions as an alternative to the cup method for the detection of bacterial sensitivity or for the assay of solutions (Vincent & Vincent, 1944; Heatley, 1944; Epstein, Foley, Perrine & Lee, 1944). Disks impregnated with sulphonamides have seldom been suggested; Morley (1945) reported the use of disks containing sulphathiazole but he stated that the implications of a negative result had not been worked out.

Kokko (1947) described a method for the determination of bacterial sensitivity to sulphonamides using a synthetic medium, and showed that good results could be obtained if the impregnated blotting-paper disks were applied to the surface of the plate for several hours and were then removed before its inoculation and subsequent incubation.

I was able to confirm Kokko's findings that 5 hr. is the optimum period for the disk to be left in contact with the medium and that during this time satisfactory diffusion of the sulphonamide will take place at room temperature.

In the work which forms the basis of this paper, Kokko's suggestions have been developed and simplified by the use of ordinary nutrient media freed from sulphonamide antagonizers by the method of Harper & Cawston.

PREPARATION OF MATERIALS

(1) *Solutions of sulphonamides*

Solutions of the required sulphonamides were made up to a concentration of 200 mg./100 ml. As many sulphonamides are very insoluble unless in an alkaline solution, the final pH should be 8. An excess of sodium hydroxide was added to a part of the distilled water to be used; the sulphonamide dissolved readily in the alkaline solution and the pH was then brought to 8 by the addition of acid, the required volume being finally adjusted by the addition of further distilled water. The solution was then Seitz-filtered and stored in a sterile container.

(2) *Blotting-paper disks*

The disks were cut with a 14 mm. cork borer from cards of Ford's thick commercial blotting paper. After they had been dry-sterilized in a hot air oven, 0·05 ml. of the sulphonamide solution was applied to each by means of a sterile graduated pipette and, using an aseptic technique, the disk was then placed on its edge in a rack—improvised from glass rods—in a large Petri dish. After a suitable number of disks had been treated in this way the dish, with its contained rack and with its lid supported on sterile corks, was placed overnight in a desiccator over anhydrous calcium chloride.

After drying, the disks were stored in an ordinary Petri dish which did not need to be sealed and which was kept at room temperature. Full activity of the disks has been demonstrated after 10 months of such storage.

(3) *Preparation of the medium*

The medium was controlled with an organism of predetermined sulphonamide sensitivity which ensured the complete removal of sulphonamide antagonizers.

For this purpose I used a coliform bacillus the sensitivity of which to sulphathiazole had been measured by a method used by Garrod (personal communication) in which Seitz-filtered urine is used as a medium for serial dilutions of sulphathiazole.

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MacLeod (1940) has shown that human urine is free from active sulphonamide antagonizers.

Growth of the test organism used was inhibited by 0·4 mg. of sulphathiazole per 100 ml., and this finding did not vary over a period of a year during which time the organism was subcultured monthly on nutrient agar.

Approximately 6% of lysed horse blood was added to ordinary heart-infusion peptone broth. This amount of horse blood is suitable for the treatment of most samples of peptone broth, but the quantity was varied either way in the light of the results obtained with the control organism. The red cells were lysed with saponin or by alternate freezing and thawing after replacement of the serum by distilled water.

The broth, with the added horse blood, was incubated overnight at 37° C. and then steamed for 30 min. to precipitate the blood proteins. When cool, it was filtered through paper pulp and the resulting clear broth was sterilized in the normal way. From each batch a sample was withdrawn and checked for the absence of sulphonamide antagonizers by using it for the preparation of serial dilutions of sulphathiazole and noting the concentration of the drug necessary to prevent the growth of a small inoculum of the control organism.

When the broth had been passed as satisfactory it was used to make up any of the usual laboratory media, as it is unaffected by the addition of further blood, serum, or other enrichment which may be necessary for nutritive purposes, with the obvious exceptions of para-aminobenzoic acid and peptone-like substances.

(4) *Preparation of the plates*

The depth of the medium was not less than 6–7 mm. so that unintended variations in the diameter of the zones of inhibition were avoided (Hayes, 1945). When the agar had hardened, a disk impregnated with the selected sulphonamide was placed firmly on the surface of the medium where it was allowed to remain for 5 hr. at room temperature. During this time the sulphonamide diffused into the medium. After 5 hr. the disk was removed.

(5) *Inoculation of the plate*

The aim in the inoculation of the plate was to produce a culture in which the colonies remained discrete. The plate was sown either direct with the material under investigation or with a suspension of the organism if it had been isolated from primary culture.

In the former case the size of the inoculum was arrived at after consideration of a stained film. Alternatively, if the organism had been isolated, an overnight culture was made in 'treated' broth, to which whole blood was added if necessary, and about 2 ml. of a suitable dilution of this culture was poured over the surface of the plate. After the surplus had been poured off, the surface of the medium was dried by placing the plate, with its lid tilted, in the incubator for about half an hour.

The density of the bacterial suspension used varied inversely as the size of the colony and the rate of growth of the organims. An overnight culture of *Streptococcus pyogenes*, *Str. pneumoniae*, or *Haemophilus influenzae* was diluted about 1 in 500, while the dilution of a similar culture of a coliform bacillus was of the order of 1 in 60,000.

INTERPRETATION OF FINDINGS

If the organism under test was sensitive to the selected sulphonamide, its growth was partly or completely inhibited in a zone centred on the area of the plate previously occupied by the blotting-paper disk.

The diameter of this zone of inhibition varied with the sulphonamide sensitivity of the organism.

In this investigation, so as to correlate the diameter of the zone of inhibition with the actual sensitivity of the organism, a parallel series of plates of 'treated' agar, in which the sulphonamide had been incorporated in a range varying from 10 mg. to 0·04 mg. %, was inoculated with the same bacterial suspension as the plate to which the blotting-paper disk had been applied. In this way the minimum concentration of the sulphonamide required to inhibit the growth of the organism was determined. Similar tests were made with several sulphonamides and with different batches of medium.

With each of the sulphonamides used, a given diameter of inhibition zone corresponded to the same concentration of the drug in serial dilution. The findings by this method for the sensitivity of an organism to various drugs of the sulphonamide series were similar to those of other workers (Frisk, 1943).

Table 1

Organism	Diameter of zone of inhibition (mm.)	Concentration of sulphathiazole required to inhibit growth (mg./100 ml.)
(1) *Staph. aureus*	Nil	10·0
(2) *Bact. coli*	14	5·0
(3) *Str. pneumoniae*	28	0·6
(4) *Bact. coli*	28	0·6
(5) *Bact. coli*	30	0·3
(6) *Str. pneumoniae*	35	0·15
(7) *Str. pyogenes*	40	0·08
(8) *Str. pneumoniae*	55	0·0025

Table 1 records the findings in a series of experiments in which a number of organisms were tested against sulphathiazole. From this it will be seen that

no inhibition zone occurred unless growth of the organism was prevented by, at the most, 5 mg. of the drug per 100 ml. One strain of *Str. pneumoniae* was exceptionally sensitive to sulphathiazole, and in this case it was necessary to extend the normal range of plates containing the drug incorporated in the medium.

EFFECT OF SIZE OF INOCULUM

It is well established that the inoculum must be small if the sulphonamide sensitivity of an organism under test is not to be masked. It has been found, however, that in this method a rather wider latitude is permissible on this point than in some other *in vitro* tests.

Whether the inhibition zone was free of bacterial colonies or whether it was only represented by a relative thinning of the carpet of growth, appeared to depend upon the size of the inoculum and the rate of growth of the organism, but in both cases the diameter of the zone was approximately the same.

To demonstrate this, five dilutions of an overnight culture of a coliform bacillus were made ranging from 1 in 1000 to 1 in 120,000 and plates were inoculated with these suspensions. The former gave a continuous carpet of growth with only a relative thinning in the zone of inhibition while the latter gave rise to no growth in this zone and to widely separated colonies over the rest of the plate, but in each of the five plates the diameter of the zone was 30 mm.

CLINICAL APPLICATION

The sensitivity of an organism to more than one sulphonamide can be determined simultaneously by applying a number of disks impregnated with the selected sulphonamides to the surface of the plate at the same time. Pl. 15, fig. 1, shows the effect of five sulphonamides on a coliform bacillus isolated from a case of urinary infection. No inhibition zones occurred where disks bearing sulphanilamide and sulphacetamide had been placed and this result agrees with other tests which indicate that more than 5 mg. % of either of these drugs is needed to inhibit growth.

In cases of infection of the urinary tract, an indication by this method that the organism is resistant to 5 mg. % of a particular sulphonamide may be followed by the use of serial dilutions of the drug in 'treated' broth up to a concentration equivalent to that attainable with safety in the urine.

Pl. 15, fig. 2, shows a primary culture on 'chocolate' agar of a sputum in which *Haemophilus influenzae*, *Neisseria catarrhalis*, and *Staphylococcus aureus* were the predominant organisms. Colonies of *Staph. aureus* can be seen growing apparently unaffected in a zone where the Neisseria has been completely inhibited and where only a few colonies of the *Haemophilus* are present.

CONCLUSIONS

With all the sulphonamides tested (sulphanilamide, sulphacetamide, sulphadiazine, sulphamezathine, and sulphathiazole) it was possible to obtain inhibition of the growth of sensitive organisms in zones the diameters of which bore an approximately constant relationship to the concentration of the drug necessary to prevent growth of the organism.

Zones of 15–25 mm. and 25–35 mm. in diameter were produced when organisms were inhibited by a concentration of the sulphonamide of between 5·0 and 1·0 mg. % and 1·0 and 0·1 mg. % respectively. No zone occurred if the organism would grow in the presence of 5 mg. % of the sulphonamide.

No significant variations were noted between the results obtained with different batches of the medium.

SUMMARY

A description is given of a simple method of determining the sensitivity of bacteria to sulphonamides by means of impregnated blotting-paper disks applied to nutrient agar prepared from broth freed from sulphonamide antagonists by incubation with lysed horse red blood cells.

With organisms sensitive to the selected sulphonamide, a zone of inhibition of growth will occur, the diameter of which is related to the concentration of the sulphonamide necessary to inhibit growth. Sensitivity to several sulphonamides can be determined simultaneously.

The impregnated disks can be stored without loss of potency.

It is a pleasure to acknowledge my indebtedness to Prof. L. P. Garrod, in whose laboratory this investigation was carried out, for his advice and criticism. I would also like to thank Dr R. A. Shooter for his assistance.

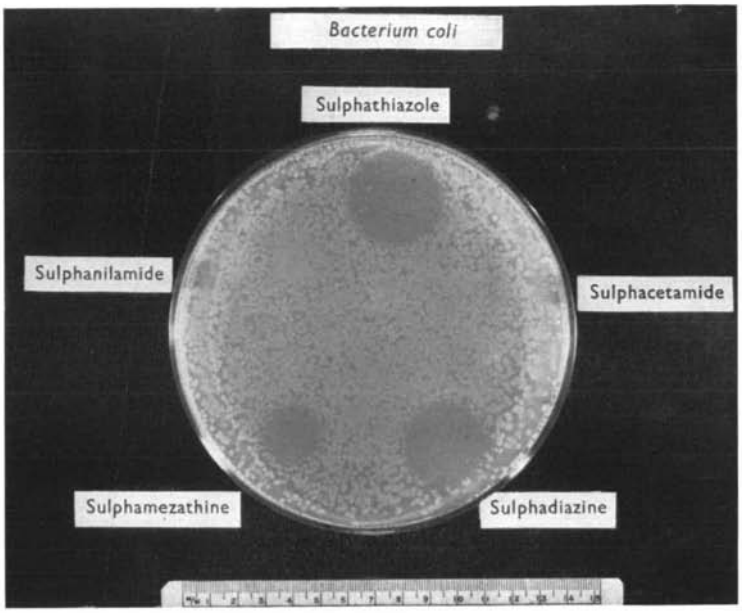

Fig. 1

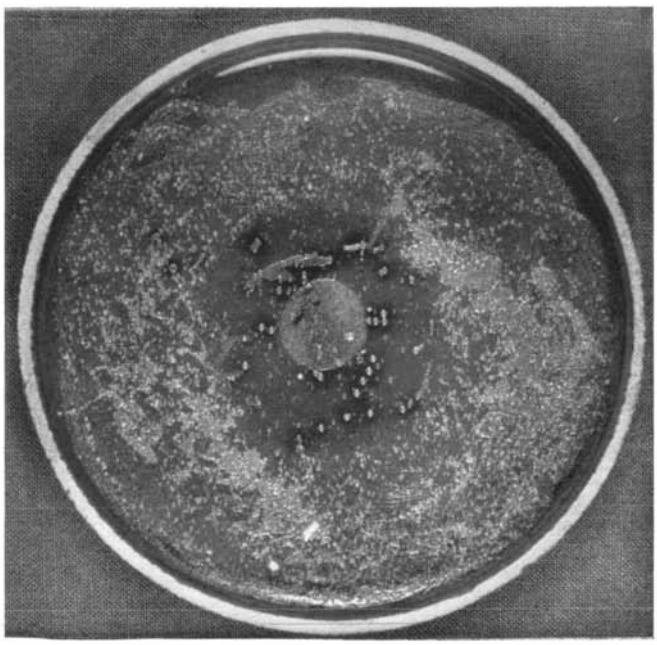

Fig. 2

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EXPLANATION OF PLATE 15

Fig. 1 shows the effect of five sulphonamides on a coliform bacillus. Inhibition zones occurred where disks bearing sulphathiazole, sulphadiazine, and sulphamezathine had been placed. Disks bearing sulphacetamide and sulphanilamide failed to cause inhibition of growth.

Fig. 2 shows a primary culture on 'chocolate' agar of a sputum in which *Haemophilus influenzae*, *Neisseria catarrhalis* and *Staphylococcus aureus* were the predominant organisms. Colonies of *S. aureus* can be seen growing apparently unaffected in a zone where *N. catarrhalis* has been completely inhibited and where only a few colonies of *H. influenzae* are present.

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