

## COLOURING AGENTS FOR USE IN DISC-ANTIBIOTIC SENSITIVITY TESTS

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Not only are the number of sensitivity determinations increasing in most laboratories but also the occasions when it is necessary to test pathogens against several antibiotics at the same time. In general, the practical value of sensitivity determinations depends upon the rapidity with which the clinician can be advised as to the most suitable antibiotic for any particular case. Using the disc diffusion technique this work can be expeditiously performed on a quantitative basis without a great deal of additional material or labour (Gould and Bowie, 1952).

The presence of several similar discs on a single plate necessitates some means whereby each antibiotic may be identified. Fairbrother, Martyn, and Parker (1951) have advocated the use of paper discs of a different shape for each antibiotic; this procedure is impracticable in quantitative sensitivity determinations. The identities of the discs may be written on the back of the petri dish, but the letters may be accidentally erased. Coloured labels may be attached, but this involves additional labour and facilitates confusion. A particular order may be adhered to when placing the discs on the plates, but this method does not eliminate the possibility of error. A satisfactory solution to the problem exists in the use of self-coloured discs. Originally Morley (1945) used blue and white blotting-paper discs to distinguish between sulphonamides and penicillin on agar plates. With the increased number of chemotherapeutic agents available, the problem has become more pressing and there is need of a wide range of distinctive colours.

A brief note without experimental data by Cook (1951) on the use in sensitivity tests of discs cut from Ford's coloured blotterettes encouraged us to communicate with the firm. They presented us with the following dyes in powder form: Ford red ("Clayton" aniline tolamine pink), Ford yellow ("durazol" yellow I.C.I. G.R. 200), Ford blue ("chlorazol" sky blue I.C.I. F.F. 200), Ford

orange ("durazol" fast orange I.C.I. R. 150), and Ford scarlet ("durazol" scarlet I.C.I. 4B 150).

After preliminary work with varying concentrations of these dyes in aqueous solution it was found that discs impregnated with dye solutions of 2.5 mg./ml. had a sufficiently distinctive colour; this persisted over any reasonable period of incubation on any of the common solid media, with the exception of MacConkey's medium, surface seeded with any of the bacteria normally isolated in a routine bacteriological laboratory.

The dyes are fast and consequently have no colouring effect whatever on solid media. For purposes of identification they are unaffected by any constituent of the common solid media incubated aerobically, anaerobically, or with carbon dioxide. From a practical point of view for sensitivity tests, the dyes are not affected by any of the enzymes formed during bacterial growth and they do not affect the adsorptive capacity of the discs. After exhaustive tests with all the organisms likely to be identified in a routine hospital laboratory, it was concluded that they had no inhibitory effect on organisms which would grow on the surface of solid media.

Since the dyes possessed all these negative characteristics essential for our purposes, we carried out further experimental work to determine their effect on penicillin, streptomycin, chloromycetin, terramycin, and sulphonamides.

As a colouring agent for discs in sensitivity tests, it is essential that the dye should have practically no effect on the antibiotic and vice versa. At the start it was found that the dyes were precipitated by streptomycin; we therefore use uncoloured discs for this antibiotic.

To investigate the stability of a mixture of the dyes and antibiotics, dilutions of each antibiotic were prepared so that the dyed and undyed solutions contained the same initial concentration of antibiotic. The dyes selected for each antibiotic are detailed in Table I.

TABLE I  
THE COLOURS USED FOR IDENTIFICATION OF ANTIBIOTICS

Antibiotic	Dye	Colour
Penicillin	Ford red	Red
Chloromycetin	3:1 mixture of Ford yellow and Ford blue	Green Yellow
Aureomycin	Ford yellow	Yellow
Terramycin	1:1 mixture of Ford orange and Ford scarlet	Terracotta
Sulphonamide	Ford blue	Blue

Guided by our preliminary work, the final concentrations of the dyes selected were 0.5, 1, and 2.5 mg./ml. Therefore for each antibiotic there was one undyed solution and three dyed, each containing identical initial concentrations of the antibiotic. At intervals over a period of three months each solution was assayed in quintuplicate. The results of this experiment (Table II) indicate

TABLE II  
COMPARISONS IN ACTIVITY BETWEEN DYED AND UNDYED ANTIBIOTIC SOLUTIONS

Initial Concentration of Antibiotic	Antibiotic	No. of Tests	New Assay Values as % of Initial Concentration			
			Within 24 Hrs.	Months		
				1	2	3
100 units/ml.	Penicillin	50	100	95	95	90
	Penicillin coloured	100	100	95	90	90
2,500 µg./ml.	Chloromycetin	50	100	90	85	80
	Chloromycetin coloured	100	100	90	80	75
5,000 µg./ml.	Aureomycin	50	100	90	85	75
	Aureomycin coloured	100	100	90	80	80
1,000 µg./ml.	Terramycin	50	100	60	50	40
	Terramycin coloured	100	100	50	50	50
1,000 µg./ml.	Sulphonamide	50	100	90	85	80
	Sulphonamide coloured	100	100	90	85	80

that dye concentrations between 0.5 and 2.5 mg./ml. do not enhance or diminish the potency of the antibiotics over a period of three months.

The results summarized in Table III represent one experiment designed to investigate the suitability of the dyes for use in disc sensitivity tests. In this instance the organism was *Staphylococcus aureus* and the medium was blood agar. The final concentrations of the dyes in the dye-antibiotic mixtures added to the discs were 0.5, 1, 2.5, and 10.0 mg. per ml. In each case the assays were performed aerobically in statistically significant numbers.

The readings obtained in this experiment indicate that the dyes do not interfere with the

TABLE III  
COMPARISON OF COLOURED AND UNCOLOURED DISCS IMPREGNATED WITH ANTIBIOTICS

Discs	No. of Tests	Diameter of Zones of Inhibition (mm.)			
		Within 24 Hrs.	Months		
			1	2	3
Penicillin	100	31	30	30	29
Penicillin coloured	100	30.5	30	29.5	29
Chloromycetin	100	28	28	27	26
Chloromycetin coloured	100	28	27	27	26
Aureomycin	100	19	19	19	19
Aureomycin coloured	100	19	18	18	18
Terramycin	100	25	24	23	23
Terramycin coloured	100	25	23.5	23	22
Sulphonamide	100	25	25	24	23
Sulphonamide coloured	100	25	25	23	23

diffusion of the antibiotic through the medium, that they have no inhibitory or enhancing effect on the antibiotics, and that they do not increase the rapidity with which the potency of the impregnated discs is lost.

### Preparation of the Coloured Antibiotic Discs

To colour the discs for quantitative sensitivity tests (Gould and Bowie, 1952) discs are cut from sheets of Whatman's No. 1 filter paper with a hole puncher having a hole-diameter of 6.3 mm.; they are autoclaved or sterilized by dry heat in lots of 100 in 1-oz. universal containers. Solutions of the antibiotics twice the potency per millilitre required for the discs are made by adding sterile distilled water to commercial preparations of the various antibiotics: penicillin 200 units per ml.; chloromycetin 5,000 µg. per ml.; aureomycin 10,000 µg. per ml.; terramycin 2,000 µg. per ml.; sulphathiazole 100 mg. per ml. Streptomycin precipitates the dyes and is therefore used in uncoloured discs, 10 µg. per disc.

Double-strength solutions of the dyes (5 mg./ml. is adequate) are made in the same way and sterilized by steaming for one hour.

Equal parts of the antibiotic and dye solution are mixed and 1 ml. of the mixture added to each bottle of 100 discs; the disc antibiotic content is therefore 1 unit in the penicillin bottles; 25 µg. with chloromycetin, 50 µg. with aureomycin, and 10 µg. with terramycin. The discs may be stored at 4°C. and will retain their potency for several months in the wet state.

The discs are applied to the surface of plates inoculated either for primary isolation or for subculture. The diameter of the zones of inhibition of growth are measured and referred to standard graphs for each particular antibiotic, prepared with a standard organism of known sensitivity.

### Other Colouring Agents

With regard to the use of other colouring agents, we investigated several groups of dyes such as representatives of the triphenyl-methane and acrid-

dine series, the oxidation-reduction, and the pH indicator dyes. As expected on theoretical grounds, these proved to be unsatisfactory for the purpose; the antibacterial effects of the first and second interfered with our tests, and with all the dyes the colours altered even during normal periods of incubation. Many were not fast.

The use of cellulose or cotton-fast dyes appeared to be the answer and we were able to show that they had no undesirable side-effects.

### Summary

The preparation of discs coloured with Ford dyes for use in routine quantitative sensitivity tests is detailed. These dyes provide a full range of stable colours which enable the different antibiotic discs to be easily recognized.

At a concentration of 2.5 mg./ml. the dyes coloured the discs distinctly so that they were recognizable over normal incubation periods on all common media, MacConkey's medium excepted, seeded with any of the bacteria encountered in

routine bacteriology. The colours are fast, are not affected by the atmospheres of incubation, and have no effect upon the inhibitory activity of antibiotics. Also, the antibiotics have no effect upon the dyes, with the exception of streptomycin, which precipitates all the dyes and is therefore used with uncoloured discs.

The dyes have no effect on the diffusion of the antibiotic through agar media, and, as far as sensitivity tests are concerned, the coloured discs are as stable as the uncoloured discs.

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