

Synergism between sulphonamide drugs and antibiotics of the polymyxin group against *Proteus sp. in vitro*

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SYNOPSIS Interesting synergism *in vitro* between a recently introduced antibiotic, colistin methane sulphonate, and sulphonamide drugs is described. The observations were extended to polymyxin B sulphate and sulphonamides with essentially similar results. The combinations are bactericidal at therapeutic levels, the ratio of drugs to one another is not critical, and the effect is present over a fairly wide pH range.

Since the introduction of colistin methane sulphonate (Colomycin) it has been reiterated that it is inactive at therapeutic levels against *Proteus sp.* (Carroll and Malette, 1961; Taylor and Allison, 1962).

Synergism *in vitro* between colistin and sulphafurazole was easily observed on plate sensitivity tests in this laboratory against 23 out of 25 consecutively isolated strains of *Proteus* (Figs. 1 and 2). The first eight strains isolated conformed in biochemical characteristics to the species *Proteus mirabilis*, one being a non-spreading variant. Subsequent isolates were assigned to the genus purely on their odour and ability to spread on blood agar.

The synergistic effect was first noticed between a 100 µg. sulphafurazole disc (Oxoid) and a 300 µg. Colomycin disc (Mast). The synergism was generally as obvious on ordinary blood agar as on sensitivity test agar (Oxoid). Ordinary wire-loop inoculation of plates was used throughout. The discs were placed $\frac{3}{8}$ in. apart and zones of inhibition varied as in Figure 3.

Although the method of inoculation was crude, in view of the nature of the zones and concentration of the drugs used it was obvious that colistin was potentiating the effect of sulphafurazole.

On preliminary testing four of the isolated strains were apparently sensitive to sulphafurazole, chloramphenicol, and streptomycin, 12 to chloramphenicol and streptomycin, five to streptomycin only, and four to chloramphenicol only. (Kanamycin and ampicillin were not being used routinely at this time.)

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Five strains showing well-marked zones of inhibition were selected and used to test sulphafurazole in combination with each of the following antibiotics on blood agar plates: penicillin, streptomycin, tetracycline, chloramphenicol, and erythromycin. No synergism was noted. Colistin was tested in combination with the same antibiotics. Again no synergism was noted.

The exact relationship of colistin to members of the polymyxin group, to polymyxin E in particular, is not yet settled (Cuthbert, 1962; Stewart, 1962) but there appears to be a close similarity. Twenty-two of the original isolates, together with a further three isolates which showed the synergistic effect, making 24 showing synerism between colistin and sulphafurazole and one which did not, were tested on blood agar for synergism between sulphafurazole (200 µg. (Mast)) and filter paper discs impregnated with approximately 200 µg. polymyxin B sulphate. The same 24 isolates showed synergism between polymyxin B and sulphafurazole, the other isolate again being resistant to the combination. Zones of inhibition were smaller than when using colistin methane sulphonate and sulphafurazole.

EXPERIMENTAL

Three isolates, two showing the synergistic effect on plates and one not showing susceptibility, were subjected to the following tube dilution tests. Colistin methane sulphonate (80 mg.), polymyxin B sulphate (83.3 mg.), and sulphafurazole (0.5 g.) were dissolved or suspended in known volumes of physiological saline. Glucose peptone water without serum was made up containing (a) 400 µg. colistin methane sulphonate per ml.; (b)

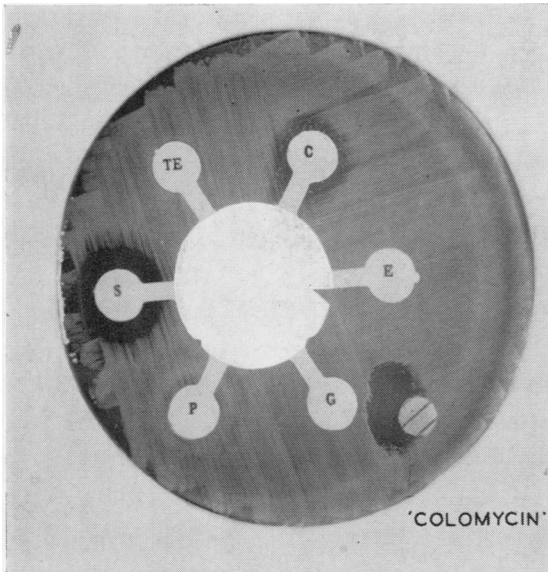


FIG. 1. Synergism between Colomycin and sulphafurazole against a strain of *Proteus*.

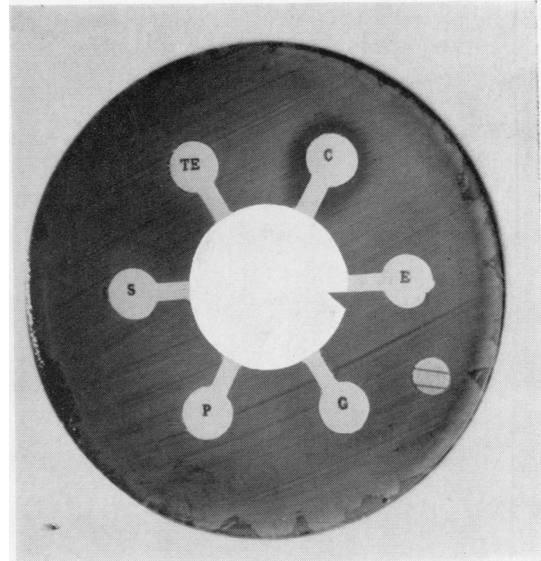


FIG. 2. A different strain of *Proteus* resistant to the combination of drugs.

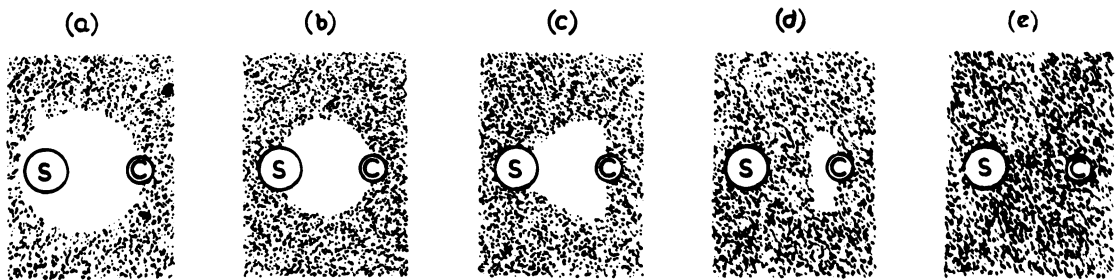


FIG. 3. Varying zones of inhibition of *Proteus* sp. observed. C = Colomycin disc (300 $\mu\text{g.}$); S = sulphafurazole disc (100 $\mu\text{g.}$).

100 $\mu\text{g.}$ sulphafurazole per ml.; and (c) 400 $\mu\text{g.}$ colistin methane sulphonate + 100 $\mu\text{g.}$ sulphafurazole per ml. Eight doubling dilutions were made and the dilutions distributed in 1 ml. amounts in Bijou bottles. This procedure was subsequently repeated using 400 $\mu\text{g.}$ of polymyxin B sulphate per ml., 100 $\mu\text{g.}$ sulphafurazole per ml., and 400 $\mu\text{g.}$ polymyxin B sulphate per ml. + 100 $\mu\text{g.}$ sulphafurazole per ml. as initial concentrations. In every test two drops of an overnight broth culture were added to about 3 ml. broth, and one drop of this dilution was used to inoculate each bottle. This, a fairly heavy inoculum, gave semiconfluent growth on MacConkey agar; viable counts were not performed. Bacteriostasis was assessed by acid production after overnight incubation, and bactericidal action after appropriate subculture onto MacConkey agar.

RESULTS

The results are shown in Tables I to III. These

results show that a bactericidal action was achieved with 25 to 50 $\mu\text{g.}$ colistin methane sulphonate combined with 6.25 to 12.5 $\mu\text{g.}$ sulphafurazole and with 50 to 100 $\mu\text{g.}$ polymyxin B sulphate combined with 12.5 to 25 $\mu\text{g.}$ sulphafurazole.

Two further experiments, using nutrient broth without serum as diluent, were carried out in order to determine how critical the ratio of drugs to one another was, and to see if the pH of the medium (covering roughly a range which might be found in the lower urinary tract) greatly altered the results. In the first experiment, the technique of inoculation was as before and two different isolates were used, one with colistin and sulphafurazole, the other with polymyxin B and sulphafurazole. The results are in Table IV.

Although the ratio of drugs to one another is not critical, increasing the concentration of sulpha-

TABLE I

Strain	Drugs	Dilutions ¹								
		1	2	3	4	5	6	7	8	9
1	Colistin	+	+	+	+	+	+	+	+	+
	Sulphafurazole	-	-	-	-	+	+	+	+	+
	Both	-	-	-	-	-	-	-	+	+
2	Colistin	+	+	+	+	+	+	+	+	+
	Sulphafurazole	-	-	-	-	+	+	+	+	+
	Both	-	-	-	-	-	-	-	+	+
1	Polymyxin B	+	+	+	+	+	+	+	+	+
	Sulphafurazole	-	-	±	±	±	±	±	±	±
	Both	-	-	-	-	-	-	+	+	+
2	Polymyxin B	+	+	+	+	+	+	+	+	+
	Sulphafurazole	-	±	±	±	±	±	±	±	±
	Both	-	-	-	-	-	-	+	+	+

+ = acid production, *i.e.*, growth- = no acid, *i.e.*, no growth

± = very slight acid production

¹See text for amount of drug in the various dilutions.

Sulphafurazole alone inhibits both strains.

furazole over colistin or polymyxin B decreased the bactericidal effect in spite of a static concentration of the latter drugs (see also zone example in Fig. 3d).

Broth adjusted to pH 8.4, using N NaOH and phenol red as indicator, and to pH 5.2, using N HCl and bromocresol purple as indicator, was used as diluent in the next experiment using one isolate with both combinations of drugs. A weaker inoculum was used. Viable counts were not performed. The results are in Table V.

It can be seen that the synergistic effect is maintained over the pH range 5.2 to 8.4. It may be noted here that sulphonamide drugs are recommended for use at pH values in excess of 7 because of their greater solubility at this pH. It was noted in this experiment that a low pH value itself had an in-

TABLE III

Drug	Dilutions ¹								
	1	2	3	4	5	6	7	8	9
Colistin	+	+	+	+	+	+	+	+	+
Sulphafurazole	+	+	+	+	+	+	+	+	+
Both	+	+	+	+	+	+	+	+	+
Polymyxin B	+	+	+	+	+	+	+	+	+
Sulphafurazole	+	+	+	+	+	+	+	+	+
Both	+	+	+	+	+	+	+	+	+

+ = acid production, *i.e.*, growth

++++ = confluent growth

¹As in Table I.

Sulphafurazole alone has no action on this strain.

hibitory effect on growth, a fact also widely known and previously utilized in the treatment of urinary infections with ammonium chloride.

The apparently potentiating effect of the low pH has not been investigated further. It would be prudent to avoid undue acidity in treating a urinary infection with a sulphonamide drug.

Blood agar base (Oxoid) containing colistin methane sulphonate was used to test isolates of *Proteus* against the following sulphonamides:— Sulphafurazole, sulphamethiazole, sulphadiazine, sulphadimidine, and sulphatriad. It was found that a concentration of 2.5 µg. colistin per ml. gave results comparable with sensitivity test agar (Oxoid) using a dilution technique of inoculation. If the concentration was raised to 25 µg. per ml. no dilution of

TABLE II

Strain	Drug	Dilutions ¹								
		1	2	3	4	5	6	7	8	9
1	Colistin	++++	+++	+++	+++	++++	+++	++++	++++	++++
	Sulphafurazole	+	+++	+++	+++	+++	+++	+++	+++	+++
	Both	-	-	-	++	++	+++	++++	++++	++++
2	Colistin	++++	+++	+++	+++	++++	++++	++++	++++	++++
	Sulphafurazole	+	+++	+++	+++	+++	+++	+++	+++	+++
	Both	-	-	+	-	++	+++	+++	+++	+++
1	Polymyxin B	++++	+++	+++	++++	+++	+++	+++	+++	+++
	Sulphafurazole	+	+++	+++	+++	+++	+++	+++	+++	+++
	Both	-	-	+	++	+++	+++	+++	+++	+++
2	Polymyxin B	++++	+++	+++	+++	+++	+++	+++	+++	+++
	Sulphafurazole	+	+++	+++	+++	+++	+++	+++	+++	+++
	Both	-	-	-	+++	+++	+++	+++	+++	+++

++++ = confluent growth; +++ = semiconfluent growth; ++ = > 10 colonies; + = 1-10 colonies; - = no growth
Subculture of a peptone control (without drug), receiving the same inoculum, before incubation yielded +++ growth.

¹As in Table I.

TABLE IV

EFFECT OF VARYING DRUG RATIOS

Drug	Concentration of Drug in Tube 1 (µg. per ml.)	Doubling Dilutions								
		1	2	3	4	5	6	7	8	9
<i>Strain 1</i>										
Sulphafurazole	100	+++	+++	+++	+++	+++	++++	++++	++++	++++
Sulphafurazole	100	-	-	-	++	+++	++	+++	++++	++++
Colistin	400									
Sulphafurazole	100	-	-	-	++	++	+++	+++	++++	++++
Colistin	100									
Sulphafurazole	400	-	-	++	++	+++	+++	+++	++++	++++
Colistin	100									
<i>Strain 2</i>										
Sulphafurazole	100	++	++	++	+++	++++	++++	++++	++++	++++
Sulphafurazole	100	-	-	-	-	-	+	+++	++++	++++
Polymyxin B	400									
Sulphafurazole	100	-	-	-	-	-	++	++++	++++	++++
Polymyxin B	100									
Sulphafurazole	400	-	-	-	-	+	+	++	+++	++++
Polymyxin B	100									

The inoculated controls gave ++ growth before incubation.

TABLE V

ACTION OF pH ON SYNERGISTIC EFFECT

pH of Medium	Drug	Concentration of Drug in Tube 1 (µg. per ml.)	Doubling Dilutions								
			1	2	3	4	5	6	7	8	9
8.4	Sulphafurazole	100	+	+	+	+++	+++	++++	++++	++++	++++
8.4	Sulphafurazole	100	-	-	-	-	-	-	+	++	++
	Colistin	400									
5.2	Sulphafurazole	100	-	-	-	-	-	-	+	++	++
5.2	Sulphafurazole	100	-	-	-	-	-	-	-	-	-
	Colistin	400									
8.4	Sulphafurazole	100	+	+	++	+++	++++	++++	++++	++++	++++
8.4	Sulphafurazole	100	-	-	-	-	-	-	++++	++++	++++
	Polymyxin B	400									
5.2	Sulphafurazole	100	-	-	-	-	-	-	++	++	+++
5.2	Sulphafurazole	100	-	-	-	-	-	-	-	-	+++
	Polymyxin B	400									

Inoculated controls gave + growth and ++++ growth after incubation in both alkaline and acid media but growth by inspection was obviously less in the incubated broths at the lower pH. Repetition of sulphafurazole alone at both pH levels serves as a control.

TABLE VI

SULPHONAMIDE SENSITIVITIES ON ANTIBIOTIC-IMPREGNATED AGAR

No. of Strains	Sulphafurazole		Sulphamethiazole		Sulphadimidine		Sulphadiazine		Sulphatriad		Peptone
	Colistin	Polymyxin ¹	Colistin	Polymyxin	Colistin	Polymyxin	Colistin	Polymyxin	Colistin	Polymyxin	
1	-	-	-	-	-	-	-	-	-	-	Acid
4	±	±	±	±	±	±	±	±	±	±	Acid (weak)
20	+	+	+	+	+	+	+	+	+	+	No acid

Impregnated agar. - = resistance; + = sensitivity; ± = sensitivity of bulk of growth and resistant colonies within zones.

inoculum was required to produce clear-cut zones of inhibition with sulphonamides.

A blood agar base was prepared containing 25 µg. colistin methane sulphate per ml. and 25 µg. polymyxin B sulphate per ml. and dispensed in roughly 10 ml. amounts in plastic Petri dishes. The plates were then seeded in duplicate with the 25 isolates of *Proteus* under test (24 showing synergism) from overnight broth cultures. Discs (Mast),

each of 200 µg., of the five named sulphonamides were placed on each plate. A loopful of each broth culture was transferred to about 3 ml. nutrient broth, the loop flamed, and a loopful of the second broth used to charge 2 ml. of glucose peptone water containing Andrade's indicator and 200 µg. sulphafurazole per millilitre. Plates and peptone waters were incubated overnight and examined next morning. All the peptone waters were then subcultured on

to MacConkey agar to verify the presence of *Proteus*. The results are shown in Table VI.

No significant difference in the size of zones was noted in the series of plates containing colistin and those containing polymyxin B. Attempts to display the synergistic effect on plates from subcultures of colonies within the zones of inhibition failed. This is probably the result of mutation.

DISCUSSION

Many clinicians, especially those dealing frequently with urinary infections, feel that *Proteus* infections often respond to sulphonamide therapy in spite of pessimistic laboratory reports. These experiments confirm that one drawback of including sulphafurazole on standard Multodisk sets is that the bacteriostatic effect against *Proteus* is often underestimated, particularly with heavy infections and routine inoculation techniques.

The drugs which commonly inhibit *Proteus* species are chloramphenicol, streptomycin, kanamycin, ampicillin, and sulphonamides. It not infrequently happens that an isolate of *Proteus* is apparently sensitive to chloramphenicol and kanamycin only, and these simple experiments show that a large proportion of such strains are susceptible *in vitro* to a combination of a sulphonamide and colistin methane sulphonate or polymyxin B sulphate. Colistin methane sulphonate has been so far found free from serious toxic effects and from pain at the injection site (Edgar and Dickinson, 1962; McMillan, Price, McLaren, and Scott, 1962; Marsden and Hyde, 1962). Polymyxin B sulphate causes pain at the site of injection (Edgar and Dickinson, 1962).

Synergism between sulphonamides and both polymyxin B and colistin has been described before (Herman, 1959; 1961), and it is interesting that the synergism in this instance was first noted between sulphafurazole and the recently introduced colistin. This was due to the much higher concentration of colistin methane sulphonate in sensitivity discs as compared with polymyxin B sulphate (300 $\mu\text{g.}$:10 $\mu\text{g.}$)

which made the synergistic effect easily noticed on a routine plate.

It would appear that colistin is chemically so closely related to the known polymyxins as to merit inclusion in the group (Stewart, 1962). It has been noted that when used to treat infections with *Ps. pyocyanea* *Proteus* strains were not uncommonly isolated during and after treatment (McMillan *et al.*, 1962). Such cases seem to offer a clear indication for combined therapy.

The synergistic effect is easily observed by routine methods of inoculating plates. (This does not apply as described to polymyxin B, the routine discs containing only 10 $\mu\text{g.}$ of antibiotic.) The variation in zone size which was noted was probably due to a varying inoculum.

The ratio of drugs to one another, and the pH at which they act are not critical.

Mutant colonies were noted in four out of the 24 'sulphonamide-sensitive' strains, and it is unlikely that such strains would be eradicated by therapy.

It is interesting that the tube tests in such cases show a degree of resistance.

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