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Colistin, sulphamethoxazole, and trimethoprim in synergy against Gram-negative bacteria

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SYNOPSIS The antibacterial activity of the four possible combinations of the three drugs, colistin, sulphamethoxazole, and trimethoprim, has been investigated with Gram-negative bacteria. All of the strains examined, with the exception of the strains of Proteus, were sensitive to colistin.

The combination of colistin and sulphamethoxazole was synergic against all 141 sulphamethoxazole-sensitive bacteria out of a total of 164 organisms against which it was tested. The sensitive strains comprised 27 of the 37 *Esch. coli*, 51 of the 54 *Ps. aeruginosa*, 24 of the 30 *Kl. aerogenes*, eight of the 12 shigellae, and all 21 Proteus and 10 salmonellae tested. The combined effect was indifference against the remaining 23 organisms which were resistant to sulphamethoxazole.

The combination of colistin and trimethoprim was synergic against all 72 organisms against which it was tested, which comprised 10 *Esch. coli*, 14 *Ps. aeruginosa*, 14 *Kl. aerogenes*, 12 Proteus spp, 10 salmonellae, and 12 shigellae. The combination of sulphamethoxazole and trimethoprim was synergic against 61 of the same 72 organisms; the exceptions were three *Esch. coli*, four *Kl. aerogenes*, and four shigellae, all of which were sulphamethoxazole resistant.

The combination of all three drugs—colistin, sulphamethoxazole, and trimethoprim—was more active than combinations of any two against 66 of the 72 organisms. The exceptions were three strains of *Esch. coli*, two of *Kl. aerogenes*, and one shigella, all of which were sulphamethoxazole resistant.

With some species of bacteria it has been established that drugs of the polymyxin group can be combined with either a sulphonamide or trimethoprim to produce an enhanced antibacterial effect. Garrod and Waterworth (1962), using the cellophane transfer technique, showed that the combination of polymyxin B and trimethoprim was lethal to *Proteus mirabilis* although trimethoprim alone was only bacteriostatic and polymyxin B had no effect; later this phenomenon was reported to have been demonstrated with all species of Proteus (Noall, Sewards, and Waterworth, 1962). Synergy between colistin (polymyxin E) and the sulphonamides has been demonstrated with Proteus species and *Ps. aeruginosa* (Russell, 1963; Simmons and McGillicuddy, 1969).

Furthermore, it is now clear that combinations of trimethoprim and the sulphonamides with each other are usually synergic (Darrell, Garrod, and Waterworth, 1968). The investigations described here were initiated to make a more detailed study than had previously been carried out of the activity *in vitro* of the combinations of colistin and the sulphonamide, sulphamethoxazole, and colistin and trimethoprim. A variety of Gram-negative bacteria was used, including

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not only Proteus species and *Pseudomonas aeruginosa*, which had been investigated before, but also salmonellae, shigellae, *Escherischia coli*, and *Klebsiella aerogenes*, which had not. When both these drug combinations were found to be synergic in most cases, the effect of combining all three drugs together—sulphamethoxazole, colistin, and trimethoprim—was studied.

Materials and Methods

INVESTIGATION OF COLISTIN/SULPHAMETHOXAZOLE COMBINATION

One hundred and sixty-four strains of Gram-negative bacteria were investigated. The distribution of the species is shown in Table I. The 37 strains of *Esch. coli*, 21 of Proteus (19 *Pr. mirabilis*, one *Pr. vulgaris*, and one *Pr. morganii*), 10 of 30 *Kl. aerogenes*, and nine of 54 *Ps. aeruginosa* were isolated at Chase Farm Hospital from patients with urinary tract infection. The other 20 strains of *Kl. aerogenes* were isolated from patients with urinary tract infection in the Dudley Road Hospital, Birmingham. The remaining 45 of *Ps. aeruginosa* and the 10 salmonellae and 12 shigellae were supplied by the Central Public Health Laboratory, Colindale. The strains of *Ps. aeruginosa* were selected as epidemiologically distinct. The salmonellae comprised one of each of the following bacterial species: *S. typhimurium*, *S. panama*, *S. infantis*, *S. heidelberg*, *S. enteritidis*, *S. dublin*, *S. anatum*, *S. senftenberg*, *S. newport*, and *S. indiana*. The 12 strains of Shigella comprised seven *Sh. flexneri* (two type 4, two type 5, three type 6), three *Sh. boydii* (one type 4, one type 5, one type 6), and two *Sh. sonnei*.

Two methods were used to investigate the effect on the organisms of this drug combination, a standard procedure and a replica plate method.

In the standard procedure, which was employed with all of the 164 strains, the surface of a colony of an overnight culture on blood agar was touched with the tip of a straight wire which was then agitated in 2·5 ml Oxoid sensitivity test broth. A small drop of the resulting suspension was transferred with a wire loop 3 mm in diameter to a 9 cm diameter Petri dish containing Oxoid diagnostic sensitivity test agar (DSTA) and spread over the surface with a cottonwool swab. A filter paper disc containing 50 μg colistin sulphomethate sodium was placed on the surface with its margin 1·5 cm from the margin of a disc containing 200 μg sulphamethoxazole. The plate was incubated at 37°C for 18 hours, examined, reincubated for a further 24 hours, and examined again.

The sensitivity of organisms to each drug acting alone was read after the first 18 hours, and, if an organism was sensitive to one of the drugs and resistant to the other, the combined action could also be determined at this time. If the zone around the effective drug was an undistorted circle the combined effect was considered to be indifference, but if the zone was distorted so that it extended towards the other disc the combined action was considered to be synergic (Fig. 1).

Where organisms were sensitive to both drugs the combined effect could not usually be determined after the first 18 hours as the zones of inhibition around the discs merged into each other. However, 24 hours later survivors around the sulphamethoxazole disc had grown and any eccentricity of the zone around the colistin disc indicating potentiation of this antibiotic by the sulphonamide was clearly demonstrable (Fig. 1C). With three sulphamethoxazole-sensitive strains of *Esch. coli* and seven sensitive of shigella the survivors did not appear even after the additional 24 hours' incubation. With these few organisms the test was repeated on blood agar employing discs containing 2 μg or 20 μg sulphamethoxazole instead of 200 μg and then survivors grew.

By this standard method it was possible to demonstrate synergy, but not whether a combined inhibitory effect was bactericidal or merely bacteriostatic, for viable bacteria which had simply failed to grow may have been present on the surface of the agar in zones of apparently total inhibition. Therefore, in order to determine whether inhibitory effects were bactericidal or bacteriostatic, 31 of the 164 organisms which had already been tested by the standard procedure were examined by the replica plate method (Elek, Hilson, and Jewell, 1953; Elek and Hilson, 1954). Twenty-two of these organisms were *Esch. coli*, four *Kl. aerogenes*, four *Pr. mirabilis*, and one was *Pr. morganii*. Primary plates were prepared in the same way as in the standard procedure and incubated for 18 hours. The discs were then removed, a sterile velvet stamp was pressed evenly on the surface of a primary plate and then pressed similarly on the surface of a sterile 'replica' plate which was incubated overnight. In this way viable bacteria within zones of inhibition on the primary plate were transferred to the replica plate where, removed from the influence of the drugs, they could multiply, and areas in which there was no growth on the replica plate indicated zones on the primary plate within which the drugs were exerting a lethal effect (Fig. 2).

INVESTIGATION OF COLISTIN/TRIMETHOPRIM COMBINATION

Seventy-two organisms, whose response to the sulphamethoxazole/colistin combination was known, were studied (Table IV). The 14 strains of *Ps. aeruginosa* were selected from 20 organisms the characteristics of which have been described elsewhere (Simmons and McGillicuddy, 1969). The 12 strains of Proteus (10 *Pr. mirabilis*, one *Pr. vulgaris*, one *Pr. morganii*), the 10 of *Esch. coli*, 14 of *Klebsiella aerogenes*, 10 of salmonella, and 12 of shigella were chosen from the organisms

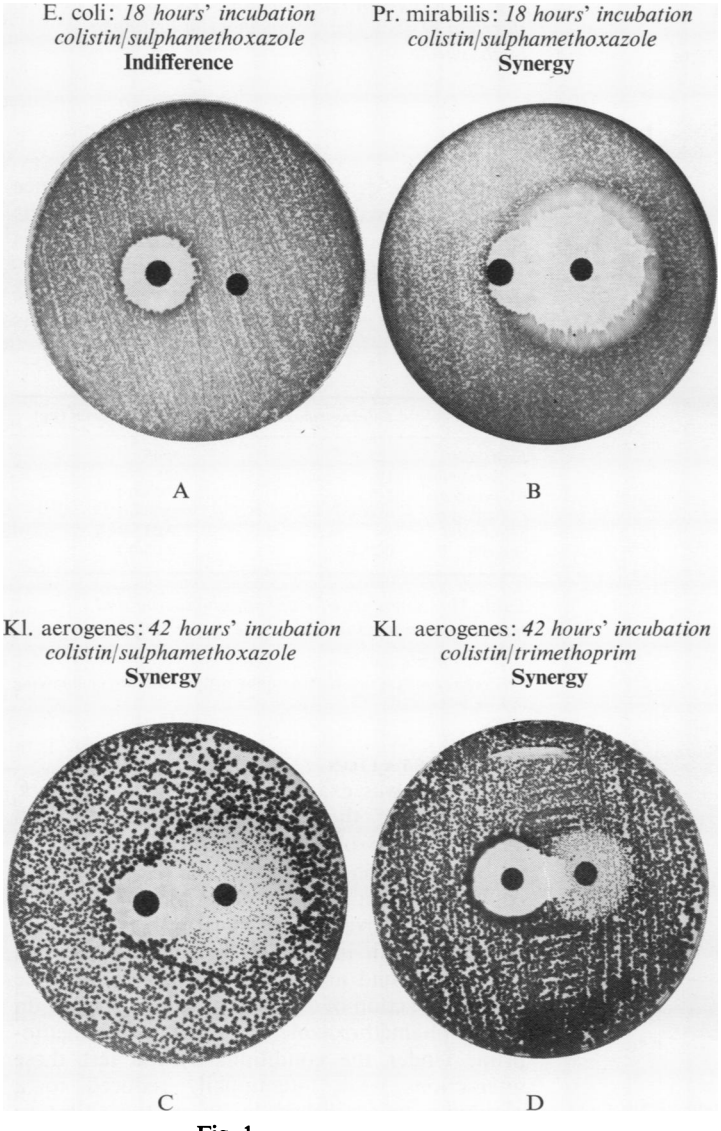

Fig. 1.

Fig. 1 *Results with the standard method. Colistin discs on the left of each plate, trimethoprim or sulphamethoxazole on the right.*

used in the colistin/sulphamethoxazole investigation and are described above.

The two methods used to investigate the colistin/trimethoprim combination, a standard technique and replica plating, were similar to those used to investigate the colistin/sulphamethoxazole combination.

In the standard procedure which was used with all 72 organisms a trimethoprim disc was substituted for the sulphamethoxazole disc. The amount of trimethoprim per disc was usually 2 μg but with *Ps. aeruginosa* it was increased to 20 μg and with a few shigellae it was reduced to 0·5 μg. The distance between the colistin and trimethoprim discs was usually 1·5 cm, but in tests with *Ps. aeruginosa* and Proteus it was reduced to 0·5 cm and with a few shigellae to 1 cm. The plates were examined after 18 and 42 hours' incubation and the interpretation of the results was similar to the interpretation of the colistin/sulphamethoxazole tests. The sensitivity to each drug acting alone was read after the first 18 hours, as was the combined effect upon organisms which were sensitive to one of the drugs and resistant to the other. Again, where organisms were sensitive to both drugs it was often difficult to determine combined action after 18 hours as zones of inhibition merged into each other, but, after a further 24 hours, reading the results was usually facilitated by the multiplication of survivors around the trimethoprim disc (Fig. 1D).

Twenty of the 72 organisms were examined by the replica plating technique: six of them were *Ps. aeruginosa*, five *Kl. aerogenes*, five *Esch. coli*, three *Pr. mirabilis*, and one *Pr. vulgaris*.

INVESTIGATION OF SULPHAMETHOXAZOLE/TRIMETHOPRIM COMBINATION AND OF THE TRIPLE COMBINATION COLISTIN/SULPHAMETHOXAZOLE/TRIMETHOPRIM

The same 72 strains used in the colistin/trimethoprim investigation were studied and each of them was investigated in two steps. In the first step the largest amount of sulphamethoxazole, which, when incorporated into a disc, produced little or no inhibition of growth, was determined. Discs were prepared which contained 200, 100, 50, 20, 10, 5, 2, 1, 0·5, and 0·25 μg sulphamethoxazole, and these were placed on the surface of diagnostic sensitivity test agar plates which had been inoculated in the same way as in the previous investigations. They were examined after 18 hours' incubation and the disc containing the greatest amount of sulphamethoxazole producing little or no inhibition of growth was found (Fig. 3). The quantity of trimethoprim producing the same effect was also determined using discs containing 2, 1, 0·5, 0·25, 0·1, 0·05, and 0·025 μg of that substance.

In the second step of the investigation three discs each containing 50 μg colistin sulphomethate sodium were placed on the surface of an inoculated Oxoid plate in a straight line. Three other discs were then placed on the plate opposite them in a parallel line (Fig. 3). One, which contained the subinhibitory amount of sulphamethoxazole, was placed opposite the colistin disc at one end; another, which contained the subinhibitory amount of trimethoprim, was placed opposite the colistin disc at the other end; and the third, which contained the subinhibitory quantities of both sulphamethoxazole and trimethoprim, was placed

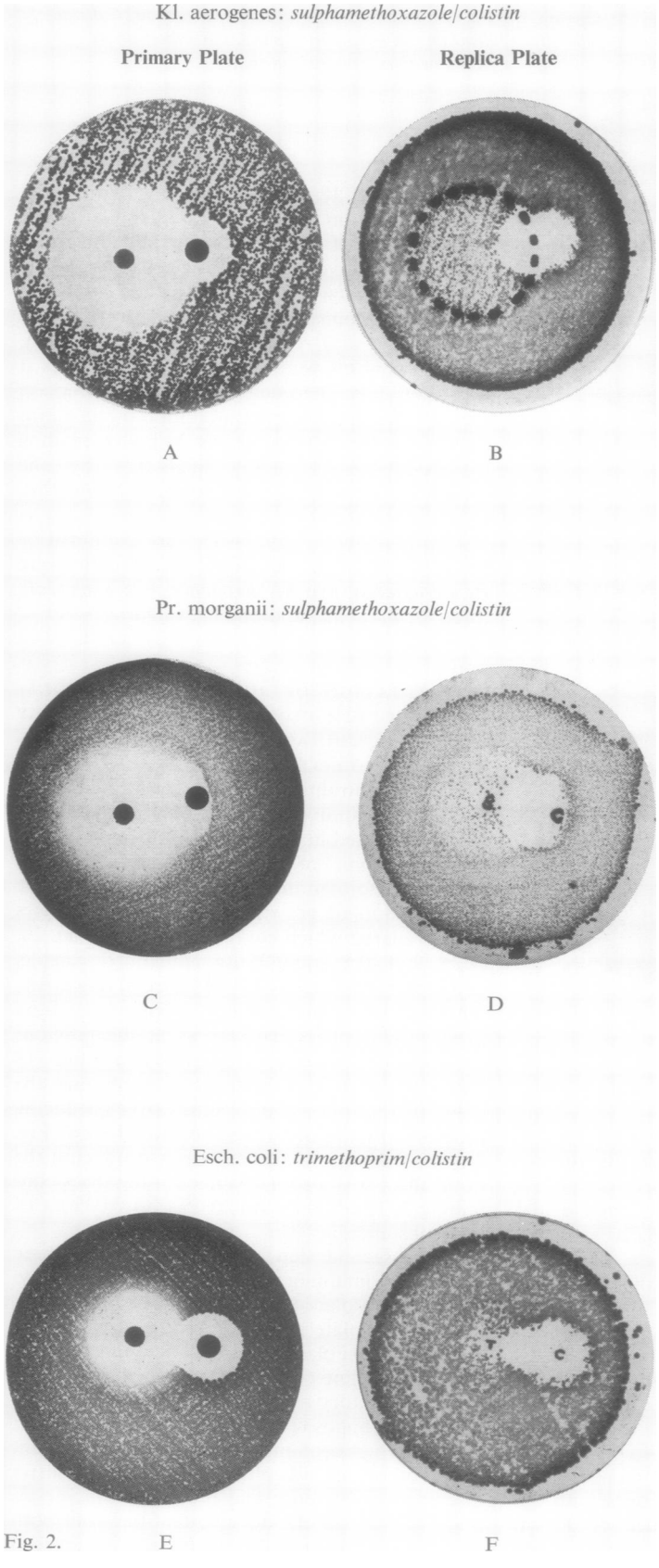

Fig. 2. *Results of replica plating. Colistin discs on the right of each plate, trimethoprim or sulphamethoxazole on the left.*

opposite the centre colistin disc. The distance between the margin of each colistin disc and its sulphamethoxazole or trimethoprim partner was 1·3 cm. The actual amounts of sulphamethoxazole and trimethoprim which were found to be subinhibitory and were therefore used in this test are shown in Table II.

Organism	*Sulphamethoxazole*[1] (μg)	*Trimethoprim* (μg)
Esch. coli	2	0·05
Ps. aeruginosa	10	20
Kl. aerogenes	1-5	0·05-0·1
Proteus spp	0·25	0·05-0·1
Salmonella	5	0·05
Shigella	0·25-0·5	0·05-0·1

Table II *Amounts of sulphamethoxazole and trimethoprim used in discs in tests for 'triple enhancement'*

[1] If organisms were resistant to sulphamethoxazole discs containing 200 μg were used

INTERPRETATION

Each plate was examined after 18 or 42 hours' incubation. If the disc which contained both sulphamethoxazole and trimethoprim produced visible inhibition of growth while the discs which contained either of these drugs alone did not, it was taken as evidence of synergy between these two drugs. An interaction between the colistin disc at one end and its partner represented the combined action of a pair of drugs, either colistin and sulphamethoxazole or colistin and trimethoprim. Under the conditions of the test these interactions were intentionally reduced to a minimum or abolished by using subinhibitory discs of trimethoprim and sulphamethoxazole.

An interaction between the pair of discs in the centre represented the combined action of all three drugs—colistin, sulphamethoxazole, and trimethoprim. When this effect was clearly greater than any produced by either pair of discs at the ends it was taken as evidence that the combination of all three drugs was more active than the combination of any two and this was designated 'triple enhancement' (Fig. 3).

Results

COLISTIN/SULPHAMETHOXAZOLE COMBINATION (TABLES I AND III)

Proteus species

All 21 strains of Proteus were sensitive to sulphamethoxazole and resistant to colistin. With the

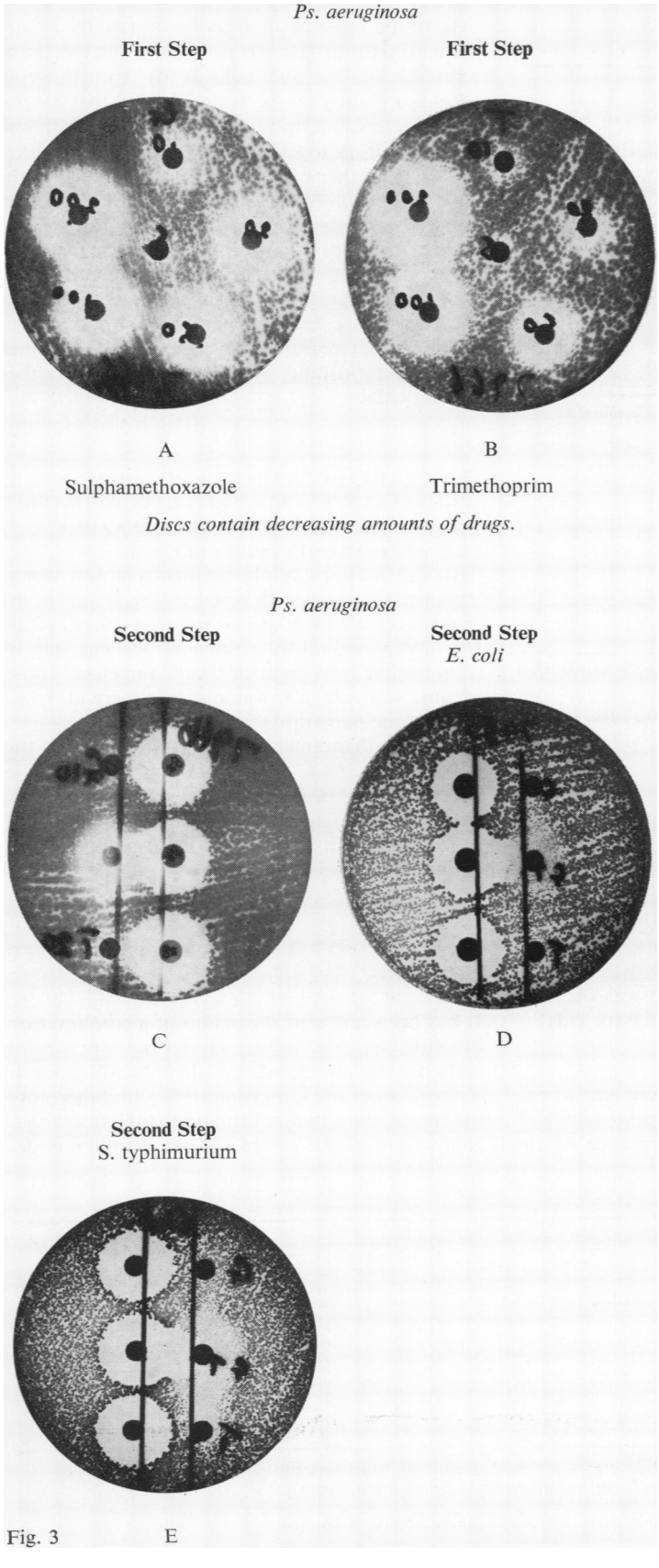

Fig. 3

Fig. 3 *'Triple enhancement.'* C *discs on left: upper sulphamethoxazole, lower trimethoprim, middle both drugs; discs on right, colistin.* D *and* E *discs on right: upper sulphamethoxazole, lower trimethoprim, middle both drugs; discs on left: colistin.*

standard procedure, after the first 18 hours' incubation with all strains the zone of sulphonamide inhibition was seen to extend towards the colistin disc and the combined action was therefore considered to be synergic. Replica plating showed that with four of the five strains tested, although sulphamethoxazole alone was only bacteriostatic and colistin had no effect, the two drugs together were bactericidal. With the remaining *Pr. mirabilis* colistin alone had no effect, but sulphamethoxazole alone and in combination with colistin was bactericidal.

Other species

All of the 143 organisms other than Proteus were sensitive to colistin. Twenty-seven of the 37 *Esch. coli*, 51 of the 54 *Ps. aeruginosa*, eight of the 12 Shigellae, 24 of the 30 *Kl. aerogenes*, and all 10 salmonella organisms were sensitive to sulphamethoxazole. With all of these sulphamethoxazole-sensitive strains the appearances of tests carried out by the standard procedure were similar to those shown in Figure 1C. After the full 42 hours' incubation the zone around the colistin disc was seen to extend towards the sulphamethoxazole, indicating potentiation of colistin by the sulphonamide. Replica plating was carried out on 22 of these sulphamethoxazole-sensitive strains. In the area where colistin was acting alone and where its activity was augmented by sulphamethoxazole a bactericidal effect was observed. Sulphamethoxazole on its own was only bacteriostatic.

With the 17 sulphamethoxazole-resistant organisms, which comprised 10 *Esch. coli*, three *Ps. aeruginosa*, and four shigellae (two *Sh. flexneri*, one type 4, one type 5, one *Sh. boydii* type 5, and one *Sh. sonnei*), in the standard procedure after 42 hours' incubation the zones around the colistin discs remained an undistorted circle and so the combined effect was classified as indifference. Replica plating, which was carried out on four sulphamethoxazole-resistant *Esch. coli*, confirmed that against these organisms the sulphonamide alone exhibited no antibacterial activity and did not enhance the activity of colistin which was bactericidal.

COLISTIN/TRIMETHOPRIM COMBINATION (TABLES IV AND V)

Proteus species

All 12 strains of Proteus were sensitive to trimethoprim and resistant to colistin. With the

Species	Total Tested	Sulphamethoxazole[1]		Colistin	
		Sensitive	Resistant	Sensitive	Resistant
Esch. coli	37	27	10	37	0
Ps. aeruginosa	54	51	3	54	0
Kl. aerogenes	30	24	6	30	0
Proteus spp	21	21	0	0	21
Salmonella spp	10	10	0	10	0
Shigella spp	12	8	4	12	0
Totals	164	141	23	143	21

Table I *Sensitivity of 164 strains of Gram-negative bacteria to sulphamethoxazole and colistin*

[1]Synergy between sulphamethoxazole and colistin was observed with all the sulphamethoxazole-sensitive strains. Indifference was observed with all sulphamethoxazole-resistant strains.

Organism	Total Tested	Effect of Drug Acting Alone						Combined Effect	
		Sulphamethoxazole			Colistin			Cidal (synergy)	Indifference
		Cidal	Static	None	Cidal	Static	None		
Sulphamethoxazole-sensitive *Esch. coli*	18	0	18	0	18	0	0	18	0
Sulphamethoxazole-resistant *Esch. coli*	4	0	0	4	4	0	0	0	4
Kl. aerogenes[1]	4	0	4	0	4	0	0	4	0
Pr. mirabilis[1]	4	1	3	0	0	0	4	4	0
Pr. morganii[1]	1	0	1	0	0	0	1	1	0

Table III *Effects of colistin/sulphamethoxazole after replica plating on 31 strains*

[1]These organisms were all sulphamethoxazole-sensitive.

standard procedure the appearances after the first 18 hours' incubation were similar with all strains. The zone of trimethoprim inhibition extended towards the colistin disc and the combined action was considered to be synergistic. The appearance of the replica plates was similar with all four strains tested in this way: colistin alone had no effect, trimethoprim was bacteriostatic, and the two drugs together were cidal.

Other organisms

All 14 strains of *Ps. aeruginosa* were sensitive to colistin and resistant to trimethoprim and with the standard procedure the appearance of the plates after incubation was similar with all of them; the zones of inhibition produced by colistin extended towards the trimethoprim disc indicating potentiation of the colistin by trimethoprim. Replica plating on six strains showed that colistin was bactericidal, trimethoprim had no effect, and the two drugs together were cidal.

All the other organisms, which comprised the 10 *Esch. coli*, 14 *Kl. aerogenes*, 10 salmonellae, and 12 shigellae, were sensitive to both colistin and trimethoprim. After 42 hours' incubation a synergic effect was seen with potentiation of the colistin by the trimethoprim (Fig. 1D). Replica plating of five *Esch. coli* and four *Kl. aerogenes* showed that colistin was bactericidal, trimethoprim bacteriostatic, and the two drugs together bactericidal.

SULPHAMETHOXAZOLE/TRIMETHOPRIM COMBINATION AND TRIPLE COMBINATION OF COLISTIN, SULPHAMETHOXAZOLE, AND TRIMETHOPRIM (TABLE IV)

Synergy between sulphamethoxazole and trimethoprim was seen with seven of the strains of 10 *Esch. coli*, all 14 of *Ps. aeruginosa*, 10 of the 14 *Kl. aerogenes*, all 12 strains of Proteus, all 10 salmonellae, and eight of the 12 shigellae. Those strains with which it was not seen were all resistant to sulphamethoxazole.

Triple enhancement was seen with seven of the 10 strains of *Esch. coli*, all 14 of *Ps. aeruginosa*, 12 of the 14 *Kl. aerogenes*, all 12 strains of Proteus, all 10 salmonellae, and 11 of the 12 shigellae. All the strains with which it was not seen were resistant to sulphamethoxazole.

Discussion

It has been said that the action of a combination of antibacterial drugs on a given organism cannot certainly be predicted on theoretical grounds alone (Jawetz and Gunnison, 1952), and therefore the observation that colistin and sulphamethoxazole were synergic against all the sulphonamide-

Species	Total[1]	Colistin Sensitive	Trimethoprim Sensitive	No. Showing Synergy		No. Showing Triple Enhancement with Colistin/Sulphamethoxazole/Trimethoprim
				Colistin/Trimethoprim	Sulphamethoxazole/Trimethoprim	
Esch. coli	10 (7)	10	10	10	7	7
Ps. aeruginosa	14 (13)	14	0	14	14	14
Kl. aerogenes	14 (8)	14	14	14	10	12
Proteus spp	12 (12)	0	12	12	12	12
Salmonella spp	10 (10)	10	10	10	10	10
Shigella spp	12 (8)	12	12	12	8	11
Total	72 (58)	60	58	72	61	66

Table IV *Results of investigations on 72 organisms with combinations of colistin/trimethoprim, sulphamethoxazole/trimethoprim, and colistin/ sulphamethoxazole/trimethoprim*

[1]The figures in parentheses refer to the number of strains which were sulphamethoxazole-sensitive.

Organism	*Total Tested*	*Effect of Drug Acting Alone*						*Combined Effect*	
		Trimethoprim			*Colistin*			*Cidal (synergy)*	*Indifference*
		Cidal	*Static*	*None*	*Cidal*	*Static*	*None*		
Esch. coli	5	0	5	0	5	0	0	5	0
Ps. aeruginosa	6	0	0	6	6	0	0	6	0
Klebsiella aerogenes	5	0	5	0	5	0	0	5	0
Proteus spp	4	0	4	0	0	0	4	4	0

Table V *Effects of colistin/trimethoprim after replica plating on 20 strains*

sensitive bacteria, and colistin and trimethoprim synergic against all the bacteria tested seems surprising. However, similar observations have been made before with Proteus species and *Ps. aeruginosa* (Garrod and Waterworth, 1962; Russell, 1963; Simmons and McGillicuddy, 1969) and this investigation demonstrated the same phenomenon with other species. Truant and Penn (1962 and 1964) investigated the effect of combinations of colistin sulphate and each of seven sulphonamides on a large number of Gram-negative bacteria. Although they observed synergy with many of the strains of Proteus and some Pseudomonas they failed to demonstrate any consistent effect with other species. The potency of individual sulphonamides is very different (Neipp, 1964), and a possible explanation for the difference between the results of Truant and Penn (1962 and 1964) and those described here is that none of their sulphonamides was as active as sulphamethoxazole which was used in this investigation. This suggestion is supported by their observation that combinations of colistin with some sulphonamides were much more active than others.

Jawetz and Gunnison (1952) suggested that where an organism was susceptible to a bactericidal and a bacteriostatic drug, combinations of the two would frequently be antagonistic, and at first sight the finding that the combinations of colistin with either trimethoprim or sulphamethoxazole were synergic appears to be in conflict with this hypothesis since colistin was bactericidal and the other two drugs were usually bacteriostatic. However, Manten and Wisse (1961) proposed that bactericidal antibiotics should be divided into classes: those, such as penicillin, which kill only growing bacteria and which are therefore antagonized by some static agents, and those, such as the polymyxins, which kill resting bacteria and which are not antagonized by static drugs. Their proposals are supported by the findings of Manten and de Nooy (1956 and 1959) who showed that polymyxin B, which is bactericidal, and chloramphenicol, which is bacteriostatic, act synergically on salmonellae. Their observations with their drug combinations are very similar to those described here with the combinations of colistin and either sulphamethoxazole or trimethoprim.

The explanation that is usually given for synergy between sulphamethoxazole and trimethoprim is that the two drugs block successive steps in the bacterial synthesis of folinic acid, and it has been suggested that since the polymyxins act synergically with both of these drugs they may exert an effect in the same metabolic sequence, presumably at a later stage, in purine synthesis or beyond (Garrod and O'Grady, 1968). Under these circumstances, although the combination of all three drugs—colistin, sulphamethoxazole, and trimethoprim—has apparently not been examined before, the finding that with 66 out of 72 organisms it was more active than the combination of any two ('triple enhancement') was not unexpected.

Only tests *in vitro* were carried out in this study and no attempt was made to assess the therapeutic value of the drug combinations. The combination of trimethoprim and sulphamethoxazole (Bactrim, Septrin) is known to be effective (Reeves, Faiers, Pursell, and Brumfit, 1969; Grüneberg and Kolbe, 1969) but a search of the literature revealed only two reports of combined treatment with colistin and one of the other drugs; Noall *et al* (1962) reported the successful treatment of a case of Proteus septicaemia with polymyxin B and trimethoprim, and Truant and Penn (1964) successful treatment of one case of Pseudomonas meningitis with colistin and sulphonamides. No reports of treatment with all three drugs together were found.

The results of the investigations *in vitro* described in this paper suggest that a trial of combined treatment with colistin and the other drugs would appear to be justifiable at least in patients with Gram-negative infections which other antibiotics have failed to eradicate.

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