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Enhancement of antibacterial activity by *p*-aminobenzoic acid and sulphadiazine

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Summary

Subinhibitory concentrations of combinations of the sulphonamide antagonist *p*-aminobenzoic acid with either carbenicillin, dibromopropamidine isethionate, or polymyxin B were shown to have synergistic antibacterial activity against *Pseudomonas aeruginosa, Enterobacter cloacae, Proteus mirabilis* and *Staphylococcus aureus*. Sulphadiazine was compared with *p*-aminobenzoic acid for its ability to enhance the killing times of dibromopropamidine isethionate, polymyxin B and carbenicillin. *p*-Aminobenzoic acid enhanced the activity of all the antibacterials and sulphadiazine enhanced the activity of dibromopropamidine isethionate and polymyxin B against all the bacteria. These findings indicate a potential usefulness for both *p*-aminobenzoic acid and sulphadiazine in enhancing the antibacterial activity of antibiotics and primary antibacterial agents in both topical and systemic use. The findings also indicate that *p*-aminobenzoic acid and sulphadiazine have distinctly different mechanisms of action. The antibacterial enhancing activity of sulphadiazine is not unique to its use in combination with trimethoprim.

Introduction

p-Aminobenzoic acid has been reported to inhibit *Pseudomonas aeruginosa* and *Escherichia coli* (Eagon and McManus, 1989, 1990) and to damage the outer membrane of *P. aeruginosa* causing an increased uptake and thus an increased activity of dibromopropamidine isethionate present in the medium/substrate (Richards and Xing, 1992).

The objectives of the present study were to investigate the enhancement by the sulphonamide antagonist *p*-aminobenzoic acid of the activity of additional antibiotics/antibacterials against a wide selection of bacteria and to compare that enhancing activity with the antibacterial enhancing activity of sulphadiazine.

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Materials and Methods

Pseudomonas aeruginosa NCTC 6750, *Enterobacter cloacae* NCTC 10005, *Proteus mirabilis* NCTC 60 and *Staphylococcus aureus* NCTC 10788 were obtained from the National Collection of Type Cultures, Colindale, London. Isosensitest broth and nutrient broth were obtained from Oxoid, Basingstoke, U.K. Inactivating recovery

medium was either made with nutrient broth, Tween 80 (3.0% w/v) and lecithin (0.25% w/v) or with isosensitest broth plus lecithin (0.125% w/v).

p-Aminobenzoic acid, polymyxin B sulphate, carbenicillin and sulphadiazine were all obtained from Sigma, Poole, U.K. Dibromopropamidine isethionate was a gift from May and Baker, Dagenham, U.K. Lecithin was obtained from BDH, Poole, U.K. and Tween 80 was purchased from ICI, Leatherhead, U.K.

Checkerboard MIC determinations

Checkerboard MIC estimates with two chemical combinations were based on the method of Sabbath (1968). *P. aeruginosa, E. cloacae, P. mirabilis* and *S. aureus* were the test organisms. A 10×10 checkerboard of test-tubes was prepared. Each test-tube contained isosensitest broth and was inoculated to give 5×10^3 cells/ml. MICs were determined for each antibacterial combination after 24 h incubation at 37°C and isobolograms plotted. The tests were carried out in duplicate.

Determination of killing times

Killing times were determined by a method similar to that previously described (Richards and McBride, 1973; Richards, 1975; Richards and Xing, 1991). The four test bacterial cells were grown in isosensitest broth for 18 h at 37°C and then centrifuged ($6000 \times g$, 10 min, 4°C). The cell pellets were washed in 0.9% w/v sodium chloride, centrifuged again and the pellets resuspended in 0.9% w/v sodium chloride. The cell concentration was adjusted to approx. 5×10^8 cells/ml. Duplicate tubes containing 9.9 ml of the antibacterial solutions under test were equilibrated in a water bath at 37°C. Then 0.1 ml of the above cell suspension was added to give a final inoculum of approx. 5×10^6 cells/ml. At intervals of 15, 30, 45, 60, 90, 120, 150, 180, 240 and 300 min after inoculation, 0.5-ml samples were aseptically transferred to 9.5 ml of inactivating recovery medium (nutrient broth containing Tween 80 (3.0% w/v) and lecithin (0.25% w/v)) and incubated at 37°C for 72 h. Tween 80 enhances the activity of polymyxin B (Brown and Richards, 1964). This necessitated the use of a different inactivating recovery medium for polymyxin B treated cells. Isosensitest broth plus lecithin (0.125% w/v) was found to be suitable. However, the autoclaved broth was cloudy, making bacterial growth difficult to detect. In order to overcome this difficulty, 0.5 ml of each inactivated sample was inoculated separately onto agar plates which were incubated for 24 h at 37°C and observed for growth or no growth.

The single test antibacterials were as follows: for *P. aeruginosa*, *p*-aminobenzoic acid (600 μg/ml), sulphadiazine (400 μg/ml), carbenicillin (1500 μg/ml), polymyxin B sulphate (1.5 μg/ml) and dibromopropamidine (250 μg/ml); for *P. mirabilis*, *p*-aminobenzoic acid (200 μg/ml), sulphadiazine (400 μg/ml), carbenicillin (1500 μg/ml), polymyxin B sulphate (1.5 μg/ml) and dibromopropamidine (10 μg/ml); for *E. cloacae* and *S. aureus*, *p*-aminobenzoic acid (400 μg/ml), sulphadiazine (400 μg/ml), carbenicillin (750 μg/ml), polymyxin B sulphate (1.0 μg/ml) and dibromopropamidine (10 μg/ml). The antibacterial combinations were either *p*-aminobenzoic acid or sulphadiazine plus one of the following; carbenicillin, polymyxin B sulphate, or dibromopropamidine isethionate. Positive growth controls to demonstrate adequate inactivation were prepared by adding 5×10^3 cells from each cell suspension above to duplicate tubes of inactivator medium containing either *p*-aminobenzoic acid (1000 μg/ml), sulphadiazine (600 μg/ml), carbenicillin (2000 μg/ml), dibromopropamidine (500 μg/ml) or polymyxin B sulphate (5 μg/ml).

Results and Discussion

The isobolograms constructed from the checkerboard MIC data are shown in Figs 1–11.

p-Aminobenzoic acid plus either carbenicillin or dibromopropamidine isethionate at subinhibitory concentrations both showed synergism against the three Gram-negative organisms and the Gram-positive organism tested. (Figs 1–7). *p*-Aminobenzoic acid plus polymyxin B sulphate showed marked synergism at subinhibitory concentrations against the Gram-negative organisms

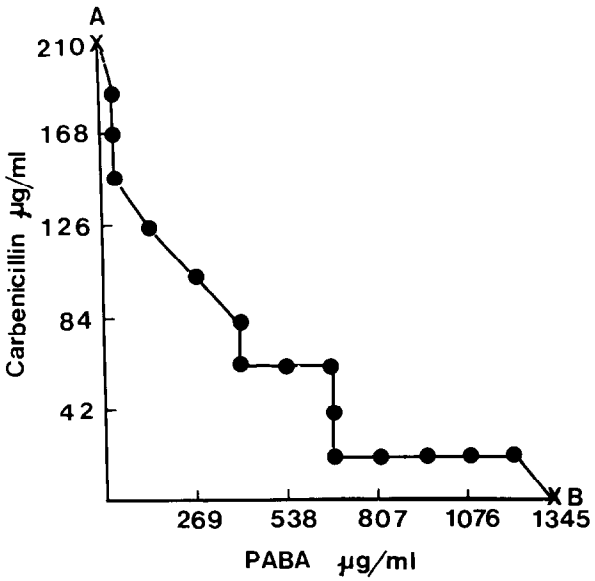

Fig. 1. Isobologram constructed from checkerboard MIC data showing combinations of carbenicillin with *p*-aminobenzoic acid (PABA) against *P. aeruginosa* in isosensitest broth. MIC of carbenicillin = 210 μg/ml; MIC of PABA = 1345 μg/ml.

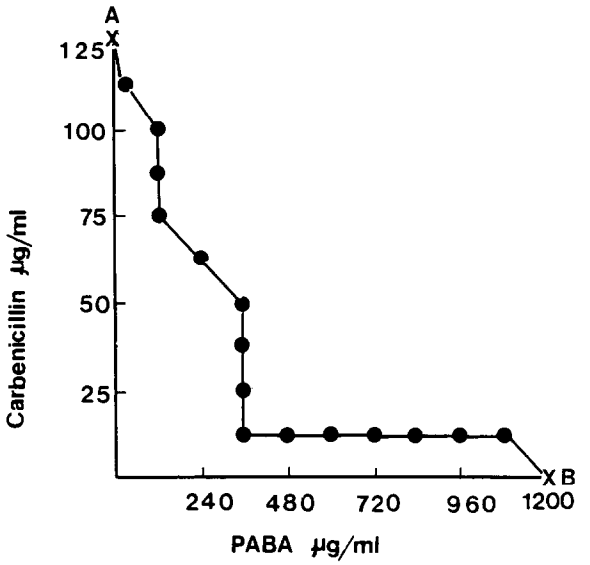

Fig. 2. Isobologram constructed from checkerboard MIC data showing combinations of carbenicillin with *p*-aminobenzoic acid (PABA) against *E. cloacae* in isosensitest broth. MIC of carbenicillin = 125 μg/ml; MIC of PABA = 1200 μg/ml.

(Figs 8–10) but only a small area of borderline synergism or addition against *S. aureus* (Fig. 11).

The fractional inhibitory concentration index (FIC index) was also calculated using the following equation:

$$\text{FIC index} = \frac{\text{concentration A in MIC A + B}}{\text{MIC A used alone}} + \frac{\text{concentration B in MIC A + B}}{\text{MIC B used alone}}$$

The FIC indices for the combinations used are given in Table 1. If the FIC index is 0.8 or below, a synergistic interaction is concluded whilst for values greater than 1.2 an antagonistic interaction is concluded. FIC index values between 0.8 and 1.2 are taken to represent addition (Richards and Xing, 1991). Therefore, the results listed in Table 1 help quantify the antibacterial activity of the antibacterial combinations. Previous results indicated that *p*-aminobenzoic acid exerted an effect on cell envelope permeability which enabled lysozyme to penetrate to the peptidoglycan layer

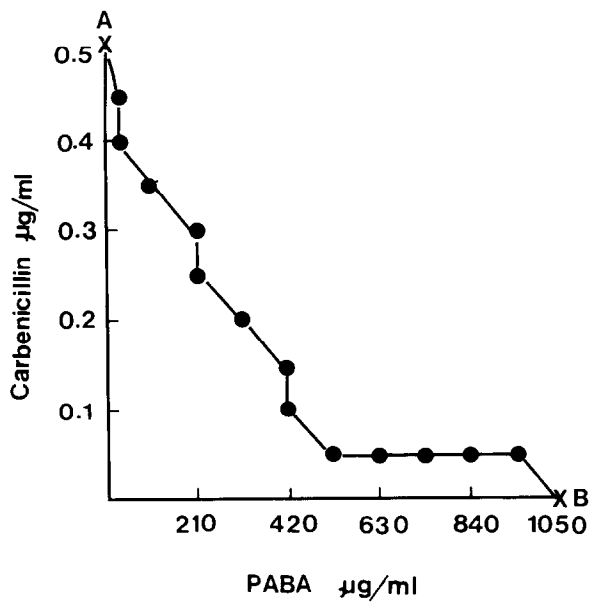

Fig. 3. Isobologram constructed from checkerboard MIC data showing combinations of carbenicillin with *p*-aminobenzoic acid (PABA) against *P. mirabilis* in isosensitest broth. MIC of carbenicillin = 0.5 μg/ml; MIC of PABA = 1050 μg/ml.

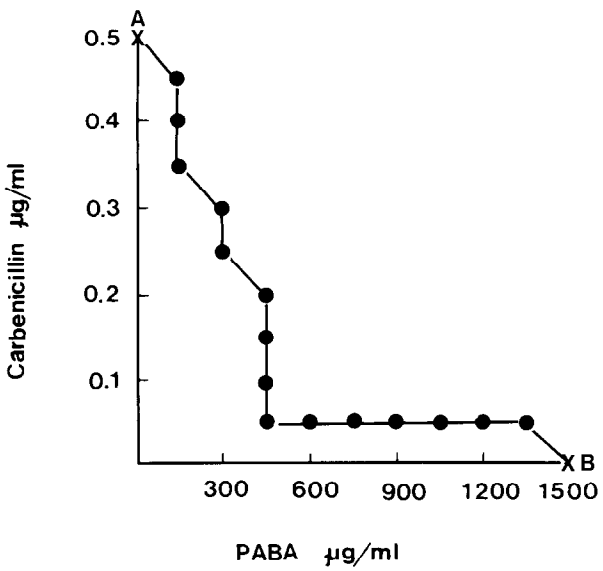

Fig. 4. Isobologram constructed from checkerboard MIC data showing combinations of carbenicillin with *p*-aminobenzoic acid (PABA) against *S. aureus* in isosensitest broth. MIC of carbenicillin = 0.5 μg/ml; MIC of PABA = 1500 μg/ml.

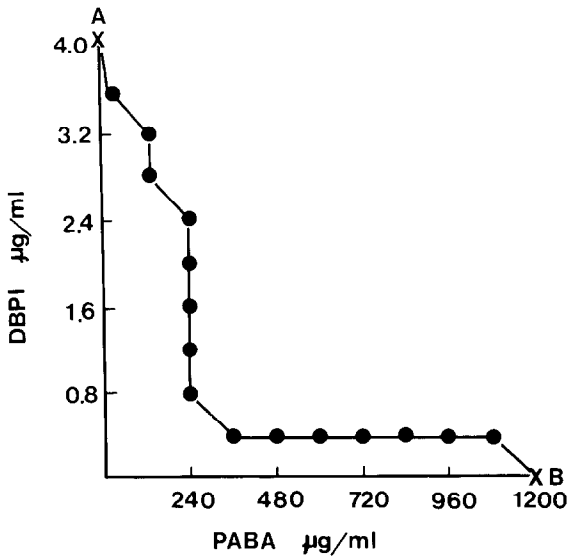

Fig. 5. Isobologram constructed from checkerboard MIC data showing combinations of dibromopropamidine isethionate (DBPI) with *p*-aminobenzoic acid (PABA) against *E. cloacae* in isosensitest broth. MIC of DBPI = 4.0 μg/ml; MIC of PABA = 1200 μg/ml.

of *P. aeruginosa* cells. (Richards and Xing, 1992). It has also been reported that *p*-aminobenzoic acid enhanced the antibacterial activity of phosphanilic acid against *P. aeruginosa* (Eagon and McManus, 1989). In addition, combinations of *p*-aminobenzoic acid and dibromopropamidine isethionate were shown to be synergistic against *P. aeruginosa* (Richards and Xing, 1992). This was related to an increased uptake of a second antibacterial present in the medium (Richards et al., 1991a; Richards and Xing, 1992). In the present work, this synergistic effect was also demonstrated against two more Gram-negative bacteria, *E. cloacae* and *P. mirabilis* (Figs 5 and 6) and against the Gram-positive *S. aureus* (Fig. 7). The fact that *p*-aminobenzoic acid can modify cell envelope permeability had prompted the hypothesis that *p*-aminobenzoic acid may enhance the activity of other antibiotics/antibacterials against *P. aeruginosa, E. cloacae* and *S. aureus* and possibly against other organisms. Figs 1–4 and Figs 8–10 indicate that the hypothesis is confirmed for carbenicillin and polymyxin against the test organisms. The synergism between both carbenicillin and polymyxin B in combination with *p*-

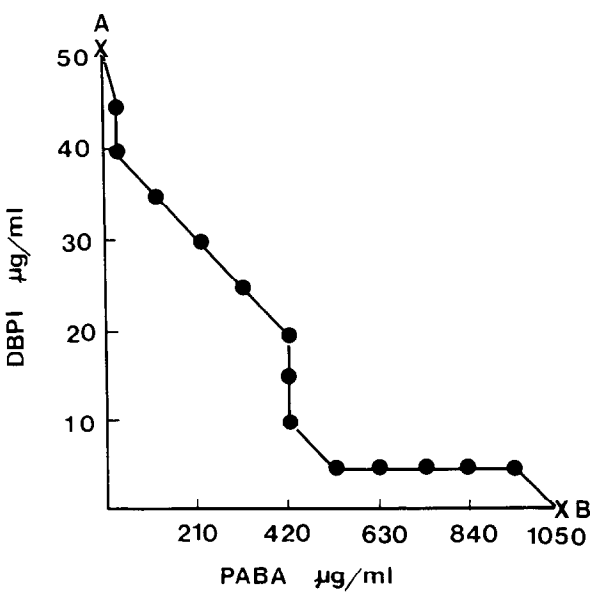

Fig. 6. Isobologram constructed from checkerboard MIC data showing combinations of dibromopropamidine isethionate (DBPI) with *p*-aminobenzoic acid (PABA) against *P. mirabilis* in isosensitest broth. MIC of DBPI = 50 μg/ml; MIC of PABA = 1050 μg/ml.

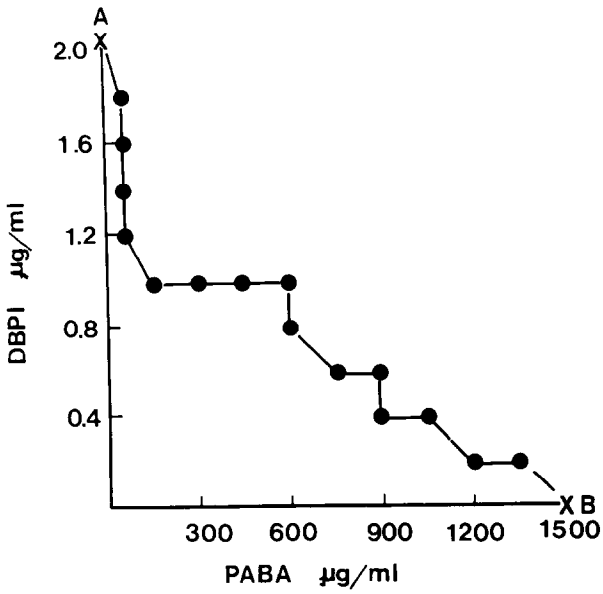

Fig. 7. Isobologram constructed from checkerboard MIC data showing combinations of dibromopropamidine isethionate (DBPI) with *p*-aminobenzoic acid (PABA) against *S. aureus* in isosensitest broth. MIC of DBPI = 2 µg/ml; MIC of PABA = 1500 µg/ml.

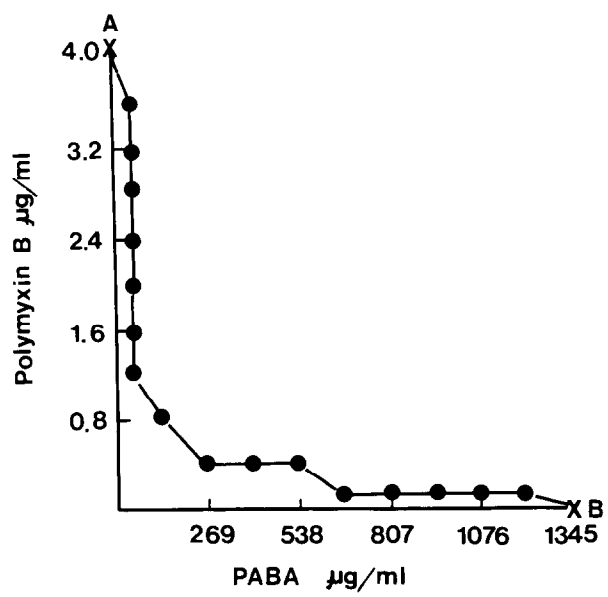

Fig. 8. Isobologram constructed from checkerboard MIC data showing combinations of polymyxin B with *p*-aminobenzoic acid (PABA) against *P. aeruginosa* in isosensitest broth. MIC of polymyxin B = 4 µg/ml; MIC of PABA = 1345 µg/ml.

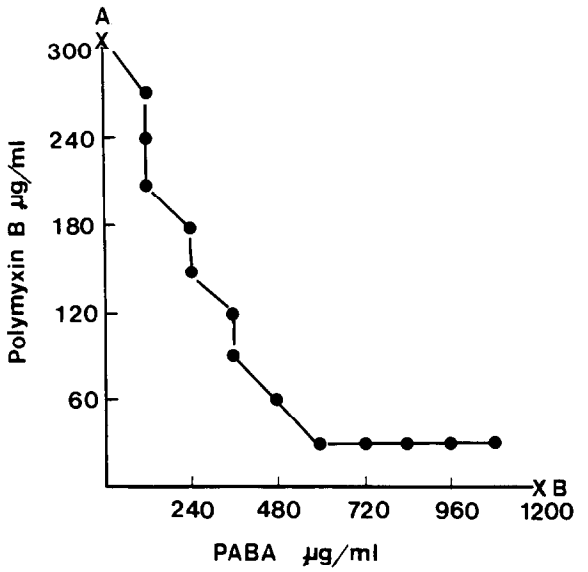

Fig. 9. Isobologram constructed from checkerboard MIC data showing combinations of polymyxin B with *p*-aminobenzoic acid (PABA) against *E. cloacae* in isosensitest broth. MIC of polymyxin B = 300 µg/ml; MIC of PABA = 1200 µg/ml.

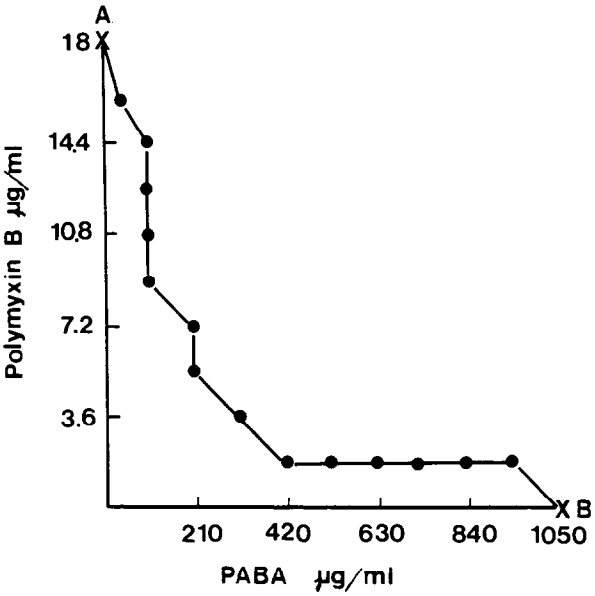

Fig. 10. Isobologram constructed from checkerboard MIC data showing combinations of polymyxin B with *p*-aminobenzoic acid (PABA) against *P. mirabilis* in isosensitest broth. MIC of polymyxin B = 18 µg/ml; MIC of PABA = 1050 µg/ml.

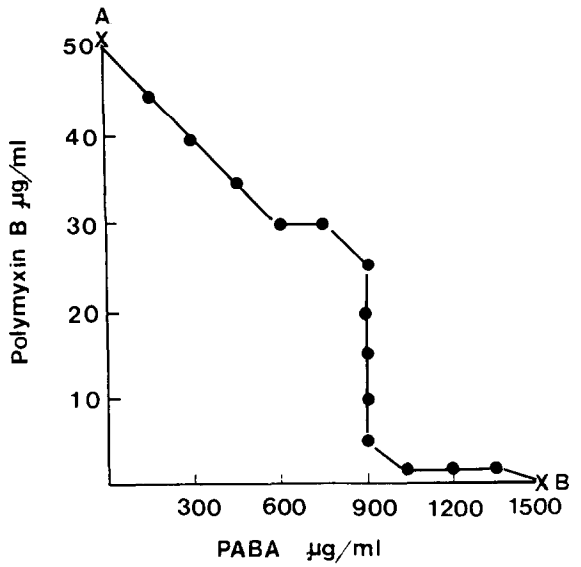

Fig. 11. Isobologram constructed from checkerboard MIC data showing combinations of polymyxin B with *p*-aminobenzoic acid (PABA) against *S. aureus* in isosensitest broth. MIC of polymyxin B = 50 μg/ml; MIC of PABA = 1500 μg/ml.

aminobenzoic acid may be explained as follows. Penicillin and polymyxin both affect cell envelope structure but in different ways. The additional effect of *p*-aminobenzoic acid on cell envelope structure could be expected to magnify the consequences of the damage caused by either of the other two agents. In the case of carbenicillin, the action of *p*-aminobenzoic acid could in addition increase the uptake of carbenicillin to its active sites within the cells.

The different effects of the combination of *p*-aminobenzoic acid observed with polymyxin B against Gram-positive and Gram-negative organisms, namely addition and synergism, respectively, can be explained by the different cell structures of Gram-positive and Gram-negative cells. Gram-positive cells lack the outer membrane which is a major site of activity for polymyxin against Gram-negative cells (Newton, 1954). The synergism seen with high concentrations of *p*-aminobenzoic acid and low concentrations of polymyxin (Fig. 11) indicates that *p*-aminobenzoic acid is enhancing the uptake of polymyxin to attack internal sites in *S. aureus*. These sites may be associated with the cytoplasmic membrane.

The killing times for *p*-aminobenzoic acid, sulphadiazine, carbenicillin, dibromopropamidine isethionate and polymyxin B either alone or in paired combinations against *P. aeruginosa, E. cloacae, P. mirabilis* and *S. aureus* cells are listed in Table 2. For the combinations of *p*-aminobenzoic acid plus either carbenicillin, dibromopropamidine isethionate or polymyxin B, killing times were greatly reduced compared with those for the single antibacterials against Gram-negative organisms. The increased activity was especially marked for *p*-aminobenzoic acid with carbenicillin. Carbenicillin was not effective within 24 h when used alone and *p*-aminobenzoic acid alone needed at least 6 h to be effective. The antibacterial combinations killed the four inocula within 15–120 min.

Carbenicillin would not be expected to have a bactericidal effect in this test system where the cells are not multiplying and it is surprising to observe an apparent enhanced activity when combined with *p*-aminobenzoic acid. The latter substance has previously been shown to be bactericidal against *P. aeruginosa* (Richards and Xing, 1992) and, at subinhibitory concentrations, to affect the outer membrane of *P. aeruginosa* cells and alter cell permeability (Richards and Xing, 1992). It is therefore hypothesised that the action of *p*-aminobenzoic acid is to facilitate the entry

TABLE 1

FIC indices for p-aminobenzoic acid (PABA) plus a second antibacterial determined in isosensitest broth against P. aeruginosa, E. cloacae, P. mirabilis and S. aureus

Organism	FIC index		
	PABA + carbenicillin	PABA + DBPI	PABA + polymyxin B
P. aeruginosa	0.6 synergism	0.6 synergism	0.3 synergism
E. cloacae	0.4 synergism	0.4 synergism	0.6 synergism
P. mirabilis	0.6 synergism	0.6 synergism	0.5 synergism
S. aureus	0.4 synergism	0.6 synergism	0.7 or 1.0 synergism or addition

of carbenicillin into the cell and to have an effect on cell metabolism even though the cells are not actively dividing.

A killing effect within 24 h was observed with neither sulphadiazine alone, nor the sulphadiazine plus carbenicillin combination. It can be concluded that in this test system *p*-aminobenzoic acid in combination with carbenicillin has a very different action from sulphadiazine plus carbenicillin.

Enhancement of antibacterial activity was detected with both sulphadiazine and polymyxin B and the sulphadiazine and dibromopropamidine isethionate combinations against the organisms tested. The action of polymyxin and dibromopropamidine isethionate, which are known to affect in different ways the permeability properties of the cell envelopes (Newton, 1954; Richards et al., 1991a,b), would appear to enable sulphadiazine to exert a damaging effect on cell metabolism even though the cells are not actively dividing.

Polymyxin in combination with either *p*-aminobenzoic acid or sulphadiazine showed increased activity against *S. aureus* when compared with the antibacterials used singly. Unexpectedly, polymyxin plus carbenicillin showed marked enhancement of activity against both *P. mirabilis* and *S. aureus*. Neither of these two organisms is very sensitive to polymyxin B alone and in addition the two antibacterials are known to be physically incompatible at higher concentrations which would also be expected to reduce their activity in combination.

These results with the cell suspensions may be regarded as controversial, since it is usually accepted that penicillins need actively dividing cells in order to exert a bactericidal effect and that sulphonamides require dividing cells in order to exert a bacteriostatic effect. Both *p*-aminobenzoic acid and sulphadiazine at subinhibitory concentrations can affect the cell envelope permeability of dividing cells. The sulphadiazine effect

TABLE 2

Killing times against approx. 5×10^6 P. aeruginosa, E. cloacae, P. mirabilis or S. aureus cells for p-aminobenzoic acid (PABA), carbenicillin, polymyxin B, sodium sulphadiazine and dibromopropamidine solutions either used alone or in the combinations indicated

Chemical	Killing time (min) [b], at 37°C [a]			
	P. aeruginosa	*P. mirabilis*	*E. cloacae*	*S. aureus*
PABA	360	9 h	7 h	> 10 h
Sulphadiazine	> 24 h	> 24 h	> 24 h	> 24 h
Carbenicillin	> 24 h	> 24 h	> 24 h	> 24 h
Dibromopropamidine	180	300	240	240
Polymyxin B	300	> 24 h	7 h	7 h
PABA + carbenicillin	45	120	60	< 15
PABA + dibromopropamidine	45	120	45	150
PABA + polymyxin B	60	150	90	300
NaSD + carbenicillin	> 24 h	> 24 h	> 24 h	> 24 h
NaSD + dibromopropamidine	30	150	90	150
NaSD + polymyxin B	120	90	60	300
Dibromopropamidine + carbenicillin	120	< 15	90	30
Polymyxin B + carbenicillin	240	90	240	< 15

[a] Duplicate determinations.

[b] Since different concentrations of chemical were used in most cases for the various organisms, the killing times are only directly related in the vertical columns for *P. aeruginosa* and *P. mirabilis*. *E. cloacae* and *S. aureus* are also comparable horizontally. Chemical concentrations (μg/ml) used in the test were: for *P. aeruginosa*, [PABA] = 600, [NaSD] = 400, [carbenicillin] = 1500, [dibromopropamidine] = 250, [polymyxin] = 1.5; for *P. mirabilis*, [PABA] = 200, [NaSD] = 400, [carbenicillin] = 1500, [dibromopropamidine] = 10, [polymyxin] = 1.5; for *E. cloacae* and *S. aureus*, [PABA] = 400, [NaSD] = 400, [carbenicillin] = 750, [dibromopropamidine] = 10, [polymyxin] = 1.0. NaSD, sodium sulphadiazine.

is via the folate synthetic pathway but that of *p*-aminobenzoic acid is very likely by some other mechanism (Richards et al., 1991a; Richards and Xing, 1992).

It is seen (Tables 1 and 2) that all of the killing time determinations for *p*-aminobenzoic acid plus a second antibacterial are consistent with the checkerboard MIC data presented here and support previous data showing that *p*-aminobenzoic acid increased the uptake of dibromopropamidine isethionate by bacteria (Richards and Xing, 1992). The killing times determined for sulphadiazine plus a second antibacterial are consistent with previous data obtained with checkerboard MIC determinations (Richards and Xing, 1991) and the finding that sulphonamides can increase the bacterial uptake of a second antibacterial (Richards et al., 1991a).

The findings with the antibiotics/antibacterials tested indicate a potential usefulness for *p*-aminobenzoic acid and the sulphonamides in enhancing the antibacterial activity of antibiotics and primary antibacterial agents in both topical and systemic use. In fact, contrary to what has been accepted, there may be little that is unique about the enhancing activity of the sulphonamide on the trimethoprim when they are used in combination, except that together they form a sequential blockade of the folate synthetic pathway. The mutual enhancement of bacterial uptake of the antibacterials may be the critical effect at subinhibitory concentrations and this can occur with sulphonamides plus many other antibacterials or trimethoprim plus other antibacterials (Richards et al., 1991a). The combination of sulphadiazine with polymyxin B against *P. mirabilis* was found in this study to be markedly synergistic, the single substances both being ineffective for a contact time of 24 h whereas the combination resulted in a killing time of 90 min. Previous work has confirmed the antibacterial effectiveness of either sulphadiazine or sulphamerazine in combination with dibromopropamidine and related this to a mutual increase in bacterial uptake of the components of the combination (Richards et al., 1991a). The same workers demonstrated the mutual increase in bacterial uptake of both components of the sulphadiazine plus trimethoprim combination. Although the sulphadiazine plus carbenicillin combination shows no enhancement of activity in killing time determinations, increased activity has been observed with sulphonamide and penicillin against growing cultures (Bigger, 1944; Weinstein et al., 1964).

p-Aminobenzoic acid has been used systemically at a dose of 1–4 g to treat rickettsial infections such as typhus and Rocky Mountain spotted fever (Martindale, 1977). It needs to be determined whether doses will provide sufficient *p*-aminobenzoic acid at the site of infection to enhance the effect of antibiotic treatment for appropriate infections.

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