

RESEARCH PAPERS

THE COMBINED EFFECT OF PENICILLIN AND OF SULPHONAMIDES IN INFECTIONS WITH GRAM-NEGATIVE ORGANISMS

PARTS I AND II.

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PART I

INTRODUCTORY

THE synergism between penicillin and various sulphonamide compounds against Gram-positive organisms *in vitro* and *in vivo* was demonstrated by several workers¹⁻⁹. Regarding the Gram-negative bacilli, very little was done to show how they are affected by the combined action of penicillin and the sulphonamides. Tung¹⁰ demonstrated a synergistic action between penicillin and sulphathiazole against *Brucella melitensis in vitro*; Bigger¹¹ reported the same phenomenon against *Salmonella typhi in vivo* and Kolmer showed a similar effect against *Salm. typhi* infection in mice.

The object of this work was to demonstrate on some common pathogenic Gram-negative bacilli, the effect of penicillin when used in combination with different sulphonamides, compared with the effect of either alone. The organisms tested were *Bacterium coli*, *Salm. typhi*, *Salm. enteritidis*, *Shigella flexneri* and *Proteus vulgaris*.

MATERIAL AND METHOD

A. *In vitro experiments.* The sulphonamide compounds used were sulphathiazole, sulphadiazine, sulphamerazine, sulphamezathine and sulphapyrazine. A 1/1000 solution of each was made in a meat extract nutrient broth* which was used as culture medium. The sodium salt of crystalline penicillin was used, it was dissolved in sterile saline solution and diluted to the required concentration at the time of the experiment. The test inoculum was added to the culture medium in a volume of 0.1 ml. after suitable dilution of a 24-hours culture. The susceptibility of the particular strain tested to penicillin and to the different sulphonamides was first estimated. To determine the effect of the two chemotherapeutic substances together, a series of test tubes with the culture medium were prepared, and an inhibitory concentration of penicillin was made up in 9 ml. of culture medium in the first tube. 4.5 ml. from this tube was then transferred to the second tube in the series which contained the same volume of plain broth, thus getting half the penicillin concentration of the first tube. 4.5 ml. from the second tube was transferred to the third and the process was repeated to give serial dilutions of penicillin until such a concentration was reached as was known to be

* As supplied by Burroughs Wellcome and Co.

non-inhibitory to the inoculum used. One tube in each series was left without penicillin for control. A second series of tubes were prepared in which the sulphonamide compound was added in a concentration which did not inhibit the growth of the organism completely. Serial dilutions of penicillin were then made up in the same way as in the first series. The inoculum was then added to each tube in 0.1 ml. of broth. The tubes were incubated at 37°C. for 24 hours. The results were then recorded by comparing the turbidity of the culture medium in each tube caused by the growth of the organism with that of the control tube. For convenience, numbers were given to indicate different grades of turbidity, thus indicating the degree of growth; 4 to indicate full growth i.e., as in the control tube, 0 to indicate a clear medium i.e., no growth, and 1, 2 and 3 to indicate increasing grades of turbidity corresponding to increasing degrees of growth.

B. *In vivo experiments.* White mice with an average weight of 20 g. were used. The drugs were given each in one dose at the time of infection; penicillin was given subcutaneously in 0.1 ml. of saline solution and the sulphonamides were given by mouth in 1 ml. of mucilage of tragacanth. The animals were infected by the intraperitoneal injection of a multiple of the average lethal dose of the organisms suspended in 0.5 ml. of mucin. One batch of animals was always left without treatment for control, other batches received penicillin alone, the sulphonamides alone, and the last received both chemotherapeutic substances together. The doses of penicillin and of the sulphonamides used were those shown to be only partially protective in the preliminary experiments.

Recording of the results. The number of mice surviving in each group was recorded daily up to 7 days, when the experiment was ended. The relative value of the different lines of treatment was estimated by comparing the average survival period per mouse in each group, obtained by adding together the number of days survived by each mouse and dividing the sum by the number of mice in the group.

THE COMBINED EFFECT ON *BACT. COLI*.

A. *In vitro experiments.* The result of the combined effect of various concentrations of penicillin and of 0.1 mg./ml. of each of the sulphonamide compounds is shown in Table I.

From Table I it is seen that these sulphonamides produce different effects. Sulphadiazine, sulphapyrazine and sulphathiazole were equally effective and were all more potent than sulphamezathine, while sulphamerazine was the least effective of all the drugs used. When they were used in combination with penicillin, a much more pronounced antibacterial action resulted, more with sulphadiazine and with sulphapyrazine than with the others. Thus 50 units of penicillin/ml. were required for complete inhibition of this inoculum of *Bact. coli*, while only 3.125 units/ml., i.e., 1/16th of the original concentration, were required to inhibit the growth of the same inoculum when sulphadiazine or sulphapyrazine was present in the medium as well.

TABLE I

Sulphonamide 0.1 mg./ml.	Penicillin concentration in units/ml.							
	100	50	25	12.5	6.25	3.125	1.56	0
.....	0	0	3	4	4	4	4	4
Sulphadiazine	0	0	0	0	0	0	1	2
Sulphapyrazine	0	0	0	0	0	0	1	2
Sulphathiazole	0	0	0	0	0	1	2	2
Sulphamezathine	0	0	0	0	0	2	3	3
Sulphamerazine	0	0	0	0	0	3	4	4

Sulphathiazole used in the same concentration with penicillin showed the same phenomenon, although to a less marked degree; the inhibitory penicillin concentration being reduced from 50 units to 6.25 units/ml. In the 6th tube of this series, although growth was not completely inhibited, it was definitely much less than in the corresponding tube which contained penicillin only (3.125 units/ml.).

It is more interesting to observe the effect of the combination of sulphamezathine or of sulphamerazine with penicillin. Sulphamezathine was only slightly effective by itself and sulphamerazine exerted no bacteriostatic action, but when these drugs were present with a non-inhibitory concentration of penicillin, growth was completely inhibited. This effect was observed in the tubes containing 25, 12.5 and 6.25 units of penicillin/ml.

The advantage of this combination was also observed when these sulphonamides were used in a smaller concentration, 0.05 mg. per ml., this latter concentration was still less effective by itself on the growth of the inoculum. Table II shows the result of an experiment similar to the previous one, where the sulphonamides were used in this lower concentration.

TABLE II

Sulphonamide 0.05 mg./ml.	Penicillin concentration in units/ml.							
	100	50	25	12.5	6.25	3.125	1.56	0
.....	0	0	3	4	4	4	4	4
Sulphadiazine	0	0	0	0	0	2	2	3
Sulphapyrazine	0	0	0	0	0	2	3	3
Sulphathiazole	0	0	0	0	0	2	3	3
Sulphamezathine	0	0	0	3	4	4	4	4
Sulphamerazine	0	0	0	0	3	4	4	4

Sulphadiazine, sulphapyrazine and sulphathiazole were the best drugs for showing this "synergistic" effect with penicillin. In the case of the tubes containing penicillin alone in concentrations of 12.5 and 6.25

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units/ml., maximum growth occurred in 24 hours as in the control tube. When sulphadiazine, sulphapyrazine or sulphathiazole was added to these tubes, growth was completely inhibited, although the sulphonamide concentrations alone was only slightly inhibitory. Sulphamerazine and sulphamezathine on the other hand, did not exert any bacteriostatic effect on this inoculum when used in a concentration of 0.05 mg./ml. Nevertheless, a combination of either with an equally ineffective concentration of penicillin resulted in complete inhibition of the growth. From these observations it could be concluded that penicillin and any of these sulphonamide compounds acting together, exerted a greater antibacterial effect than either alone. Moreover, concentrations of either substance which, when used alone, were non-inhibitory or only slightly inhibitory, resulted, when used together, in complete inhibition of the growth.

B. *In vivo experiments.* Penicillin in a comparatively big dose, 2,000 units, did not show an appreciable therapeutic value against *Bact. coli* infections in mice. The different sulphonamides, however, showed variable protective effects against this infection; sulphadiazine and sulphapyrazine being the best, then sulphathiazole, sulphamerazine and sulphamezathine. The value of the combined use of doses of penicillin and of the different sulphonamide compounds is demonstrated in Table III where the average survival periods of several groups of animals under different schemes of treatment are recorded.

TABLE III

	Treatment		Number of Mice Used	Average Survival Period Days
	Penicillin	Sulphonamide		
2000 units	...	Sulphadiazine 0.1 mg.	18	1.7
		Sulphadiazine 0.1 mg.	12	4.1
2000 units	...	Sulphapyrazine 0.1 mg.	12	1.7
		Sulphapyrazine 0.1 mg.	12	5.0
2000 units	...	Sulphathiazole 0.5 mg.	30	2.3
		Sulphathiazole 0.5 mg.	24	5.7
2000 units	...	Sulphamerazine 0.5 mg.	12	3.1
		Sulphamerazine 0.5 mg.	6	5.8
2000 units	...	Sulphamezathine 0.5 mg.	6	1.7
		Sulphamezathine 0.5 mg.	6	5.5
2000 units	60	1.4
Untreated controls	60	0.4

From the results in Table III it is seen that the average survival period per mouse was 1.7 days in the groups treated with sulphadiazine or with sulphapyrazine or with sulphamezathine; when each of these drugs was combined with penicillin, the survival times were increased by: 2.4, 3.0 and 3.2 times respectively. Similarly, the average survival time was increased by 2.5 times when sulphathiazole was used with penicillin than when the same dose of sulphathiazole was used alone.

It is important to emphasise here that the marked increase in the average survival period was not the only improvement observed in those

animals having the combined therapy, but the general condition of the mice as judged by their activity and food consumption showed a very noticeable difference; the mice which received combined therapy and survived the experiment were very active and appeared quite normal; on the other hand the survivors from the groups receiving single treatment were very ill and much less active. This difference does not appear from the Tables as all these latter were counted as survivors.

PART II.

THE COMBINED EFFECT ON *SALMONELLA TYPHI*.

A. *In vitro experiments.* The combined effect of penicillin and of the different sulphonamides on *Salm. typhi* showed the same synergistic phenomenon as against *Bact. coli*. Table IV shows the result of the presence of the sulphonamides in a concentration of 0.2 mg./ml. in the culture medium together with various concentrations of penicillin, compared with the effect of either alone on the growth of the organisms.

TABLE IV

Sulphonamide 0.2 mg./ml.	Penicillin concentration in units/ml.							
	20	10	5	2.5	1.25	0.6	0.3	0
.....	0	0	4	4	4	4	4	4
Sulphathiazole	0	0	0	0	0	1	1	1
Sulphapyrazine	0	0	0	0	1	1	1	1
Sulphadiazine	0	0	0	0	2	2	2	2
Sulphamezathine	0	0	0	0	0	2	2	2
Sulphamerazine	0	0	0	0	1	1	3	3

Inoculum 20,000 organisms approximately. Tubes were incubated for 24 hours at 37°C.

Sulphathiazole and sulphapyrazine in this concentration were more active than the other sulphonamide compounds, and their combined effect with penicillin resulted in a greater degree of inhibition than when sulphadiazine, sulphamezathine or sulphamerazine were combined with penicillin. Although 10 units of penicillin/ml. were required to prevent the growth of this inoculum, only 1.25 units/ml. were sufficient to prevent the growth when sulphathiazole was present in the culture medium as well. A similar result was obtained when the same concentration of sulphamezathine was present instead of sulphathiazole. When sulphadiazine or sulphamerazine was present in the same concentration with penicillin, the inhibitory concentration of the latter was reduced from 10 to 2.5 units/ml.

When the sulphonamides were used in one half the previous concentration, although their individual bacteriostatic effect was weaker, they showed nearly the same inhibitory action when combined with penicillin (Table V).

Again, the inhibitory penicillin concentration was reduced from 10 units to 1.25 units/ml. when 0.1 mg. of sulphathiazole/ml. was present

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TABLE V

Sulphonamide 0.1 mg./ml.	Penicillin concentration in units/ml.							
	20	10	5	2.5	1.25	0.6	0.3	0
...	0	0	2	3	4	4	4	4
Sulphathiazole	0	0	0	0	0	2	2	2
Sulphapyrazine	0	0	0	0	2	2	2	2
Sulphadiazine	0	0	0	0	2	3	3	3
Sulphamezathine	0	0	0	0	1	2	3	3
Sulphamerazine	0	0	0	0	1	3	3	3

with penicillin. Sulphadiazine, sulphamerazine and sulphamezathine were less active than sulphathiazole, but they also showed a "synergistic" action with penicillin. Apart from the reduction in the inhibitory penicillin concentration, it is seen that in the tubes containing 1.25 units of penicillin/ml., although the growth was not completely inhibited, it was definitely less than in the corresponding tube containing penicillin or one of these sulphonamides alone.

Therefore, the presence of these sulphonamides in partially bacteriostatic concentrations, with penicillin in non-inhibitory concentrations, resulted in complete prevention of the growth of the organism. When comparatively small concentrations of penicillin are combined with the sulphonamides, growth may not be completely prevented, but it was less when both substances were present together than when either substance was present alone.

B. *In vivo experiments.* The mice were infected by an intraperitoneal injection of 100 times the minimal lethal dose of *S. typhi*. Different groups of animals were treated with penicillin, a sulphonamide compound, or a combination of both and the average survival period per mouse in these groups was compared. The results recorded in Table VI show clearly the advantage of the combined therapy.

TABLE VI

Treatment	Number of mice used	Average survival period days
2000 units	12	1.7
2000 units	12	6.3
2000 units	12	1.2
2000 units	12	6.1
2000 units	12	1.9
2000 units	12	6.0
2000 units	12	2.4
2000 units	12	6.5
2000 units	12	0.7
2000 units	12	6.1
2000 units	12	1.4
Untreated controls	30	0.2

In all the cases, the average survival period resulting from the combined therapy was 6 days or more out of a maximum of 7 days, whereas in no case was it as much as 2.5 days when similar sulphonamide doses were used alone. The increase in the survival time as a result of the combined therapy was 3.7 times in the case of sulphadiazine, 3.1 times with sulphamezathine and 2.7 times with sulphathiazole. A dose of 2 mg. of sulphapyrazine or of 5 mg. of sulphamerazine exerted a small protective action, less than that of 2,000 units of penicillin, but still when penicillin was given together with either of these sulphonamide doses, the average survival time of the mice was 4.3 times as long as that resulting from penicillin alone.

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Corrections

Page 960, figure 3. Replace 1:5:6-Trimethylbenziminazole by 5:6-Dimethylbenziminazole. Replace 5:6-Dimethylbenziminazole by 1:5:6-Trimethylbenziminazole.