THE SYNERGIC ACTION OF PENICILLIN AND SULPHATHIAZOLE ON *S. TYPHI*

BY JOHN C. THOMAS AND WILLIAM HAYES

From the Central Military Pathological Laboratory, India Command

It is becoming increasingly recognized that a true synergism exists in the action of penicillin and the sulphonamide series of drugs against a number of bacteria. Ungar (1943) using sulphapyridine, Bigger (1944*a*) using sulphathiazole and Chain & Duthie (1945) using sulphamethazine have all shown its existence against *Staph. aureus*. Tung (1944) found a similar synergism with the *Brucella* group which was most marked with *Br. suis* and least with *Br. melitensis*. The action on *Br. abortus* was intermediate and the observation of this variable degree of synergism in the action of the two drugs on different species of the same genus is important. Bigger (1946) demonstrated a degree of synergism against *S. typhi* which brought the critical effective concentration of the two drugs into the field of practical therapeutics. Following this, McSweeney (1946) has reported favourable clinical results from combined penicillin and sulphathiazole therapy in six severe cases of typhoid fever in Dublin, and the combination is receiving a large-scale field trial in the 1946 outbreak of the disease in Aberystwyth.

Acting on a suggestion of Prof. J. W. Bigger to one of us in 1945 we planned the experiments described here to investigate this synergic action against *S. typhi* in greater detail, to determine the conditions under which it is effective and to find if there is a variation in synergic action against the salmonellas such as Tung found amongst the *Brucella* genus. Most of the experiments were made at the Central Military Pathological Laboratory, India, and were reported in substance at the Annual Conference of Pathologists, Southern Army, India Command (Thomas, 1945).

METHODS

The great majority of the experiments were made with a strain of *S. typhi*, T455/45, chosen at random from the many sent to the laboratory for confirmation; our observations with this strain were subsequently confirmed with fifteen other random strains, with the old laboratory stock strain 901O, and with Bhatnagar's Vi(1) strain. Strain T455/45 was originally maintained on nutrient agar at room temperature; it was found that a spontaneous variation occurred under these conditions with the development of sulphathiazole-resistant variants, and all our strains were later maintained on Dorset's egg medium in the cold room. They were seeded when required into 5 ml. vol. of Lemco broth and incubated overnight. Viable counts by the method of Miles & Misra (1938) showed that the resulting bacterial density was of the order of 220×10^6 bacteria/ml.

Other salmonellas investigated were *S. paratyphi* A (strains 113/44 and Beamish/46), *S. paratyphi* B (strains 8006/Kauffmann and TCD), *S. enteritidis* var. *chaco* (strains 511/44, 570/44 and 562/45), *S. enteritidis* var. *jena* (strain 12316/JT) and *S. typhimurium* (strain WH77/46).

Sulphathiazole solutions were prepared from powdered tablets after due allowance for the excipient, as the pure chemical could not originally be obtained. A stock solution containing 40 mg./100 ml. corresponding to saturation at 20° C. was prepared in distilled water and autoclaved. Reid & Anderson (1946) have shown that there is no appreciable loss of sulphathiazole when it is autoclaved in solution either in distilled water or in broth media. The results were later confirmed with the pure reagent in this country.

Penicillin solutions were prepared from commercial preparations in distilled water buffered to *p*H 6·8 with *M*/15 phosphate buffer. Immediately after preparation each was assayed by the deep-plate method (Hayes, 1945) against the 1945 Provisional British Standard (Ca salt; 166 u./mg.) using the standard N.C.T.C. 6571 strain of *Staph. aureus*; the solutions were adjusted to 1000 units/ml. and stored in the cold. Appropriate working dilutions were prepared as required from this stock solution.

Both drugs were tested in a simple Lemco broth (Lab. Lemco, 1%; peptone (Fairchild's 310302), 1%; NaCl, 0·5%) and in a sulphonamide-inhibitor-free broth prepared from this by absorption with 5% lysed horse blood (Harper & Cawston, 1945). Our standard method was to make serial, doubling dilutions of the drug to be tested in 2·5 ml. vol. of the appropriate medium using 20 ml. neutral glass penicillin vials of the squat type. The use of empty penicillin bottles for bacteriological work offers many advantages over the use of tubes and plugs especially when a large series of tests has to be set up. The vials are of high quality neutral glass, they

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stand firmly and can be sterilized in bulk in trays so that individual racks are not required, and by using their slip-on metal caps instead of plugs the amount of manipulation and consequent risk of contamination is greatly decreased with a concomitant increase in ease and speed of working. Capped bottles, sterilized and left on the bench, remain sterile for long periods and we experienced no trouble from contamination amongst the many thousands we used. In setting up the titrations the same pipette, with a single graduation at 2·5 ml., was used throughout, being washed and sterilized at appropriate intervals in boiling water. By using a rubber tube and mouthpiece for suction it was unnecessary to move the bottle from its position in the series; all manipulations could be carried out at speed by simply lifting off the metal cap.

The action of the two drugs together was tested by setting up serial dilutions of double strength penicillin and adding to each 2·5 ml. of 5 mg./100 ml. sulphathiazole, giving a final correct concentration of penicillin reacting in the presence of 2·5 mg./100 ml. sulphathiazole. Double the inoculum for 2·5 ml. vol. was added to each bottle containing 5 ml. A control series of experiments showed that the increased volume of the synergism titrations did not affect the results, and that it is the concentration of penicillin rather than the total number of units present which is the important factor.

A dropping pipette, adjusted to deliver 40 drops/ml. was used for seeding. One drop of an overnight culture of the test organism was added to 15 ml. broth and well mixed. The pipette was well rinsed in boiling water and one drop of this dilute culture was used as the test inoculum, the total number of bacteria introduced being of the order of 9000.

$$(220 \times 10^6/40 \times 15 \times 40.)$$

The series of bottles were incubated for 24 hr. at 37° C. when the bacteriostatic concentration was judged by the absence of visible growth; visible growth was compared with the control (+) and graded as Tr. (trace of visible growth only), ± (definite reduction in the degree of growth) and + (full growth equal to the control). Sufficient *p*-aminobenzoic acid (PABA; one drop of a sterile 0·4% solution = 10 mg.) and penicillinase (one drop of a preparation with a titre of 10,000+ u./ml. = 250+ u.) were then added to each dilution and the tubes were reincubated for a further 24 hr.; the absence of growth at this time was taken as evidence of killing. Adequate controls were set up at each stage. The penicillinase used was a Seitz-filtered 10-day Lemco broth culture of *B. subtilis* N.C.T.C. 6346 to which penicillin had been added on the 4th and 6th days (Duthie, 1944). In all, over 600 titration experiments were made.

I. THE ACTION OF SULPHATHIAZOLE ON *S. TYPHI* T455/45

(1) *The effect of sulphonamide-inhibitors in broth*

Parallel titrations of sulphathiazole were set up in Lemco broth and in absorbed inhibitor-free broth under standard conditions and each seeded with the standard inoculum of T455/45. In Lemco broth the bacteriostatic concentration of sulphathiazole was normally found to be between 1·6 and 3·1 mg./100 ml.; in inhibitor-free broth it was between 0·2 and 0·4 mg./100 ml., approximately an eight-fold reduction. The addition of PABA, however, after 24-hr. incubation showed that there was no sterilization and that growth was resumed throughout following the inhibition of sulphathiazole action. At 25 mg./100 ml. there was often a diminution of growth after PABA in the inhibitor-free broth (Table 1).

Table 1. *Action of sulphathiazole on* S. typhi *T*455/45 (*standard conditions*).
(1). *Effect of sulphonamide inhibitors in broth*

Sulphathiazole (mg./100 ml.)		25	12·5	6·25	3·1	1·6	0·8	0·4	0·2	0
Lemco broth	24 hr.	−	−	−	−	±	+	+	+	+
	+PABA	+	+	+	+	+	+	+	+	+
Inhibitor-free broth	24 hr.	−	−	−	−	−	−	−	Tr.	+
	+PABA	±	+	+	+	+	+	+	+	+

(2) *Bacterial variation and the development of sulphonamide-resistant variants*

The T455 strain was originally maintained on nutrient agar at room-temperature and at the outset of this work inhibitory levels of the order shown in Table 1 could repeatedly be obtained in inhibitor-free broths. When retested after some weeks, however, the inhibitory concentration was found to have risen and whereas previously there had only been a trace of growth in 0·2 mg./100 ml. of sulphathiazole, growth now occurred with as much as 3·1 mg./100 ml. The reagents were all checked and a number of fresh broths and sulphathiazole solutions prepared but the same high level was manifest in all. It was then thought that bacterial variation had occurred in the stock culture with the development of sulphathiazole-resistant variants.

To test this a sweep of the stock culture was seeded to broth, plated on to agar after 8-hr. incubation and ten colonies were picked at random into broth. These were then tested against sulphathiazole under standard conditions in inhibitor-free broth (Table 2).

It was immediately apparent that such a variation had occurred, one colony, T455/I, growing in the presence of 3·1 mg./100 ml. sulphathiazole, a second, T455/V, showing but the merest trace of growth at 0·2 mg./100 ml., and the remainder being inhibited by intermediate concentrations of the drug. The resistant (I) and sensitive (V) colonies were subcultured on to Dorset's egg and stored at 4° C. After 4 weeks, sweep cultures of each were seeded to broth, plated on agar and a further ten colonies from each picked and tested under standard conditions. No further significant variation occurred with either, each maintaining within a one-tube range its sensitivity or resistance, and under these conditions of storage this state has been maintained for over a year without further subculture.

This variation, with the development of sulphathiazole-resistant variants, is not similar to that produced by serial subculture in subinhibitory concentrations of the drug; it appears as a natural mutation occurring under certain conditions of storage. It seems important to recognize that such a variation can occur and to guard against it in work of this sort.

Table 2. *Action of sulphathiazole on* S. typhi *T*455/45 *(standard conditions).* (2) *Effect of bacterial variation on sulphathiazole resistance*

Sulphathiazole (mg./100 ml.)	25	12·5	6·25	3·1	1·6	0·8	0·4	0·2	0·1	0
Colony T455/I	−	−	−	±	+	+	+	+	+	+
T455/II	−	−	−	−	+	+	+	+	+	+
T455/III	−	−	−	−	−	−	+	+	+	+
T455/IV	−	−	−	−	−	−	±	+	+	+
T455/V	−	−	−	−	−	−	−	Tr.	+	+
T455/VI	−	−	−	−	−	−	+	+	+	+
T455/VII	−	−	−	−	−	−	−	+	+	+
T455/VIII	−	−	−	−	−	+	+	+	+	+
T455/IX	−	−	−	−	−	−	+	+	+	+
T455/X	−	−	−	−	−	+	+	+	+	+

Table 3. *Action of sulphathiazole on* S. typhi *T*455/45. (3) *Effect of size of inoculum*

Sulphathiazole (mg./100 ml.)	25	12·5	6·25	3·1	1·6	0·8	0·4	0·2	0·1	0·05
Inoculum:* 5,000,000	+	+	+	+	+	+	+	+	+	+
500,000	Tr.	±	+	+	+	+	+	+	+	+
50,000	−	−	−	−	−	±	+	+	+	+
5,000	−	−	−	−	−	−	−	−	+	+
500	−	−	−	−	−	−	−	−	−	+
50	−	−	−	−	−	−	−	−	−	−
5	−	−	−	−	−	−	−	−	−	−

* In each case the inoculum grew in a control tube containing no sulphathiazole.

(3) *Effect of size of the inoculum*

The number of bacteria inoculated into each tube is a matter of importance, and many of the divergences in bacteriostatic and bactericidal concentrations of various drugs reported in the literature are due to the use of widely differing inocula. The effect of varying the number of bacteria used for the test was examined with the T455/V variant. Seven sets of sulphathiazole dilutions in inhibitor-free broth were set up and inoculated with numbers of bacteria increasing logarithmically from 5 to 5×10^6 (Table 3). With inocula of 500,000 or more bacteria, growth was not inhibited or was only partially inhibited by 25 mg./100 ml. With smaller inocula there was an increasing degree of inhibition until, with five or fifty bacteria, growth was inhibited by as little as 0·05 mg./100 ml.

(4) *Effect of incubation time*

Under our standard conditions of test the bacteriostatic concentration of the drugs is judged by the absence of visible growth after 24-hr. incubation, and in all our sulphathiazole experiments the addition of PABA at that time has always shown that no sterilization occurs, further incubation resulting in growth in all concentrations.

To determine whether sterilization occurred after longer contact with the drug, two parallel titrations were set up in inhibitor-free broth under standard conditions. PABA was added to one series

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after 24 hr. while the other was observed daily for 5 days before adding PABA (Table 4). With prolonged incubation there is a progressive growth in the lower concentrations. At 24 hr. growth is inhibited by 0·2 mg./100 ml. as usual, but eventually growth appears in four times this concentration with the standard inoculum of 9000 bacteria. This is presumably due to a temporary inhibition of all the organisms, but with time the more hardy develop the power to grow and divide in the presence of increasing amounts of the drug. The addition of PABA after 5-days' contact with the drug, however, shows that there is a clearly marked killing effect and growth is not resumed with concentrations of 3·1 mg./100 ml. and above. This is of practical importance from the clinical aspect.

Table 4. *Action of sulphathiazole on* S. typhi *T*455/45 (*standard conditions*).
(4) *Effect of prolonged incubation*

Sulphathiazole (mg./100 ml.)	25	12·5	6·25	3·1	1·6	0·8	0·4	0·2	0·1	0
Incubated for 24 hr.	−	−	−	−	−	−	−	−	+	+
+PABA	+	+	+	+	+	+	+	+	+	+
48 hr.	−	−	−	−	−	−	−	+	+	+
72 hr.	−	−	−	−	−	−	Tr.	+	+	+
96 hr.	−	−	−	−	−	+	+	+	+	+
120 hr.	−	−	−	−	−	+	+	+	+	+
+PABA	−	−	−	−	±	+	+	+	+	+

II. THE ACTION OF PENICILLIN ON *S. TYPHI*

S. typhi is usually considered to be resistant to the action of penicillin. Thomas & Levine (1945), investigating the action of penicillin on the intestinal flora, showed that it was inhibited by 50 u./ml. but not by 20 u./ml., while *S. paratyphi* C was inhibited by as little as 2 u./ml.; Jordon & Jones (1945), using a ditch-plate technique, found slight inhibition at 5 u./ml., almost complete inhibition at 30 u./ml. and complete inhibition at 100 u./ml.; Bigger (1946) states that concentrations of 8 u./ml. reduce but do not completely inhibit growth of the typhoid bacillus in broth. With our strains and technique we have found very much lower inhibitory levels and the addition of penicillinase after 24-hr. incubation has shown that there is, in addition, a true killing effect.

(1) *General action of penicillin on* S. typhi

Eighteen strains were tested against commercial penicillin preparations under standard conditions, each penicillin solution being assayed to an accuracy of ± 10 % against the Provisional British Standard before use. Sixteen, including T455/45, were local strains isolated from cases of the disease, the others being the stock 901O and Vi(1) strains. The bacteriostatic level was judged by inhibition of growth after 24-hr. incubation in broth; excess penicillinase was then added and the bactericidal level similarly judged after a further 24-hr. incubation.

Of the eighteen strains, the growth of 15 (83·5 %) was inhibited by 5 u./ml. or less; 6·25 u./ml. sterilized the standard inoculum of 9 (50 %) of the strains, while 10 u./ml. sterilized 17 (94·5 %) strains. It is interesting to note that the inhibitory and lethal concentrations were the same in five cases. The stock 901O strain was one of these, being inhibited and subsequently killed by 6·25 u./ml.; Bhatnagar's Vi(1) strain was inhibited by 1·6 and killed by 6·25 u./ml. (Table 5).

These concentrations are the initial concentrations to which the organisms were exposed. Penicillin deteriorates rapidly in broth at 37° C., so that there is actually a constantly decreasing amount of penicillin present in the test systems. The rate of deterioration in our broth was determined by keeping a penicillin solution (Pfizer 2534) in broth at 37° C. for a week and making daily assays against the Provisional British Standard. The initial assay was 9·75 u./ml.; the subsequent daily concentrations were 6·3, 4·6, 4·4, 2·6, 1·3 and less than 1 u./ml. A control in buffered distilled water stored at 4° C. showed no significant loss over this period. During the 24-hr. incubation involved in our standard method, therefore, there is a 35 % loss of penicillin.

Table 5. *The action of penicillin on* S. typhi
(1) *Bacteriostatic and bactericidal concentrations against eighteen strains*

Penicillin (u./ml.)		
Bacteriostatic concentration	Bactericidal concentration	No. of strains
1·6	3·1	1
1·6	6·25	2
2·5	5	1
3·1	3·1	3
5	10	8
6·25	6·25	2
6·25	12·5	1

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(2) *Action of different penicillins on* S. typhi T455/45

The laboratory where the bulk of this work was done carried on the routine assay of considerable numbers of commercial batches of penicillin, for which one of us was responsible. Since the supply of standard penicillin was restricted, it was considered that samples of commercial penicillin, accurately assayed against Provisional British Standard Penicillin by Staphylococcus N.C.T.C. 6571, should be used instead in the investigation. When several samples of sodium penicillin prepared by various manufacturers had been tested against T455 it was found that results were inconsistent. Reassay showed that the variation was not due to sensitive to one than to the other of two or more fractions against which the *Staphylococcus* was equally sensitive. The only penicillin fraction then available to us was the pure triethylamine salt of penicillin II and, since T455 was repeatedly shown to be less sensitive to preparations of this than to those of Abbott (G404 A035) and Lederle (782H 131A), it was assumed that the marked efficacy of the two latter was due to the presence in them of some fraction other than II. We have not yet been able to obtain pure preparations of penicillins I, II, III and IV but samples of the sodium salt of II, and of IV in a fair state of purity, have recently been tested. Results are shown in Table 7.

Table 6. *The action of penicillin on* S. typhi *T*455/45. (2) *The inhibitory and lethal action of different penicillin preparations*

		Penicillin (u./ml.)						
Maker and batch no.	u./mg.	25	12·5	6·25	3·1	1·6	0·8	0
Abbott (G404 A035)	290	–	–	–	–	Tr.	+	+
		–	**–**	**–**	**Tr.**	**+**	**+**	**+**
Pfizer (2534)	284	–	–	–	+	+	+	+
		–	**–**	**+**	**+**	**+**	**+**	**+**
Commercial Solvents	415	–	–	–	+	+	+	+
Corp. (44093001)		**–**	**–**	**±**	**+**	**+**	**+**	**+**
Lederle (782 H131A)	—	–	–	–	–	–	+	+
		–	**–**	**–**	**–**	**±**	**+**	**+**
Squibb (32881)	—	–	–	–	+	+	+	+
		–	**–**	**±**	**+**	**+**	**+**	**+**
Squibb (32811)	—	–	–	–	+	+	+	+
		–	**–**	**+**	**+**	**+**	**+**	**+**
Glaxo (A4)	1435	–	–	Tr.	+	+	+	+
		–	**–**	**+**	**+**	**+**	**+**	**+**
Haffkine Institute	—	–	–	–	+	+	+	+
		–	**–**	**+**	**+**	**+**	**+**	**+**
Penicillin II (pure)	1300	–	–	–	–	±	+	+
(triethylamine salt)	approx.	**–**	**–**	**–**	**+**	**+**	**+**	**+**
Provisional British	166	–	–	–	Tr.	+	+	+
Standard (Ca salt)		**–**	**–**	**–**	**+**	**+**	**+**	**+**

Heavy type denotes readings after addition of penicillinase and reincubation.

error from this cause. It became apparent that T/455 was showing considerable variation in sensitivity to different batches of penicillin which were of equivalent unitage when tested against the *Staphylococcus*. Many commercial and other batches of penicillin were therefore tested, after initial standardization, against T455, with the results shown in Table 6.

A similar though more marked variation was given by two strains of *S. enteritidis* var. *chaco* when tested against selected samples of penicillin from the above series. It was thought, at the time these experiments were performed, that this variation might be due to varying proportions of the fractions of penicillin in different batches, T455 being more

Table 7 confirms the fact that pure penicillin II is less effective against *S. typhi* than Abbott (G404 A035) and also demonstrates the relative inefficacy of penicillin IV. With one exception (see Table 6), this batch of Abbott penicillin proved more potent against *S. typhi* than any other of the considerable number of penicillins tested. Its keeping properties are also worthy of note. Prior to the expiry date of the batch (November 1944) the samples used by us had been kept for about 6 months at room-temperature in Delhi during the hot weather when the shade temperature reaches as high as 120° F. and the nights are warm. Nevertheless, samples assayed at approximately 1 and 2 years after the date of expiry showed little deterioration

in potency. Details of this batch of penicillin have kindly been supplied to us by Dr R. D. Coghill, Director of Research, Abbott Research Laboratories. It was manufactured in April 1944 by the surface culture of *Penicillium notatum* 1249 B2L in litre bottles, corn steep liquor-lactose medium being used. Dr Coghill states: 'Judging from the experience we have had with this organism and medium, this particular sample may have contained as much as 10 to 20% of penicillin X (III), the remainder being a mixture of penicillins F and G (I and II).' We cannot go further than to state that the efficacy of this penicillin is almost certainly attributable to its content of either penicillin I or III.

Table 7. *The action of penicillin on* S. typhi *T* 455/45. (3) *The inhibitory action of different penicillin preparations**

Fraction	Purity %	Unitage/mg.	Maker	Penicillin (u./ml.) 40	20	10	5·0	2·5	1·25	0
II	100	1600	British St. penicillin	−	−	−	±	+	+	+
II	100	1600	Glaxo	−	−	−	±	+	+	+
IV (+II)	66	1200	Glaxo	−	+	+	+	+	+	+
Unknown	—	290	Abbott (G 404 A 035)	−	−	−	−	+	+	+

* The inhibitory levels here are approximately double those shown in Table 6 which were evaluated about a year previously. This is probably due to variation in sensitivity of T 455. This culture of the strain has never been subjected to colony selection but it must be remembered that a culture appears as sensitive as the most resistant organism in the inoculum, so that variation in the direction of resistance by only a minute proportion of the bacterial population would affect the apparent sensitivity of the culture as a whole.

(3) *Action of penicillin on colonial variants of* S. typhi *T* 455/45

In view of the development of spontaneous sulphathiazole-resistant variants in this strain, the possibility of a similar mutation with regard to penicillin had to be considered. The ten colonies previously tested for sulphathiazole sensitivity were therefore tested against penicillin. The penicillin used (C.S.C. 44093001) inhibited the original culture at 6·25 and killed at 12·5 u./ml. The bacteriostatic concentrations for these ten variants were: 6·25 u./ml. − 1; 12·5 u./ml., with only a trace of growth at 6·25 u./ml. − 5; 12·5 u./ml., with partial inhibition (±) at 6·25 u./ml. − 2; 12·5 u./ml. with full growth at 6·25 u./ml. − 2. Six were killed by 12·5 u./ml., and the remainder by 25 u./ml. There is, therefore, some slight variation, but it is not of the same order as that shown against sulphathiazole.

(4) *Effect of size of inoculum on the action of penicillin*

This was determined in the same way as for sulphathiazole using similar logarithmic inocula of T 455/V, and the more potent Abbott (G 404 A 035) penicillin. The bacteriostatic concentrations were found to be much the same irrespective of the number of bacteria in the infecting dose; 1·6 u./ml. inhibited the growth of 5,000,000 organisms and 0·8 u./ml. were required to inhibit 5. There was, however, a striking effect in the concentrations required to sterilize the inoculum. Thus, while 12·5 u./ml. did not kill 500,000 bacteria, 3·2 u./ml. killed 50,000 and 5000, 1·6 u./ml. killed 500 and 50 and 0·8 u./ml. effectively sterilized an inoculum of 5 typhoid bacilli.

III. THE ACTION OF PENICILLIN AND SULPHATHIAZOLE ON *S. TYPHI*

(1) *Synergic action on* S. typhi *T* 455/45

The synergic action of penicillin and sulphathiazole was investigated by setting up parallel penicillin titrations in inhibitor-free broth, one with penicillin alone and the other with the addition of 2·5 mg./100 ml. sulphathiazole, infecting each with the standard inoculum of T 455/45, determining the bacteriostatic concentration of penicillin after 24-hr. incubation (no growth appears in the sulphathiazole series at this time as the amount of sulphathiazole is itself inhibitory), adding excess PABA and penicillinase, reincubating for a further 24 hr. and determining the final bactericidal concentrations with and without sulphathiazole. All our experiments showed that there is constantly a reduction in the amount of penicillin required to sterilize the test inoculum when it is allowed to act in the presence of small amounts of sulphathiazole. It should be emphasized that sulphathiazole alone did not kill the inoculum under the experimental conditions used. A typical experimental protocol is given in Table 8. This confirms Bigger's work in which he showed that 1 u./ml. of penicillin sterilized 7000 *S. typhi* when acting in the presence of sulphathiazole.

On one occasion a colony variant has been found against which no synergic action could be demonstrated, but normally the synergism varied from a two- to eightfold reduction in the bactericidal concentration of penicillin.

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(2) *Synergism with other strains of* S. typhi

With the exception of the colonial variant already mentioned, this synergic action was demonstrated on every occasion with all the eighteen strains we tested. Eleven strains showed at least a twofold reduction, six a fourfold and one an eightfold reduction in the amount of penicillin required to kill the test dose of typhoid bacilli.

(3) *Bacterial variation in relation to synergism*

When the ten colonies previously tested for resistant variants were retested against penicillin in the presence of sulphathiazole, a considerable variation in the degree of synergism exhibited was found, but this was not related to the degree of sulphathiazole-resistance shown by the individual gism varies from nil (the single case in which no synergism could be demonstrated) to an eightfold reduction in the bactericidal concentration of penicillin. The penicillin used in these tests was not very potent (see Table 6), and the resistant and sensitive variants (I and V) were retested against the triethylamine salt of penicillin II. In both cases with penicillin alone the inhibitory and lethal concentrations were 3·1 and 6·25 u./ml. respectively, and the bactericidal concentration in the presence of sulphathiazole was 1·6 u./ml. In this experiment, the resistant variant showed only a trace of growth at 0·8 u./ml.

It appears, therefore, that this synergic action is independent of the sensitivity of the organism to sulphathiazole *per se*.

Table 8. *Synergic action of penicillin and sulphathiazole on* S. typhi *T*455/45. *Bactericidal concentrations of pure triethylamine salt of penicillin II under standard conditions*

Penicillin (u./ml.)	12·5	6·25	3·1	1·6	0·8	0·4	0·2	0
Penicillin alone	−	−	+	+	+	+	+	+
Penicillin + 2·5 mg./100 ml. sulphathiazole	−	−	−	−	Tr.	+	+	+

Table 9. *Effect of colony variation on penicillin sulphathiazole synergism against* S. typhi *T*455/45 (*standard conditions*)

Colony	Bacteriostatic conc. of sulphathiazole (mg./100 ml.)	Penicillin alone (C.S.C. 44093001) Bacteriostatic conc. (u./ml.)	Penicillin alone (C.S.C. 44093001) Bactericidal conc. (u./ml.)	Penicillin + sulphathiazole. Bactericidal conc. (u./ml.)	Synergism
T455/I	6·25	12·5	12·5	6·25	× 2
T455/II	3·1	12·5	25	12·5	× 2
T455/III	0·8	6·25	12·5	6·25	× 2
T455/IV	0·8	12·5	25	6·25	× 4
T455/V	0·4	12·5	25	6·25	× 4
T455/VI	0·8	12·5	12·5	6·25	× 2
T455/VII	0·4	12·5	25	3·1	× 8
T455/VIII	1·6	12·5	12·5	6·25	× 2
T455/IX	0·8	12·5	12·5	6·25	× 2
T455/X	1·6	12·5	12·5	12·5	0

variant. Thus the resistant variant, T455/I, exhibited a twofold synergism, and with this colony it is interesting to note that there was not only a synergism of bactericidal action but also a remarkable synergism of bacteriostasis. We had previously shown that this variant grew in the presence of 3·1 mg./100 ml. sulphathiazole, so that the 2·5 mg. present in the test series was not bacteriostatic. Similarly, there was a partial reduction of growth in the presence of 6·25 u./ml. of penicillin alone. Yet in the combined series there was complete bacteriostasis at 24 hr. down to 0·8 u./ml. of penicillin in the presence of the subinhibitory concentration of sulphathiazole. The bacteriostatic and bactericidal concentrations against these ten variants are given in Table 9.

Amongst these ten colonies the degree of syner-

(4) *Effect of varying sulphathiazole concentrations on synergism*

The sulphathiazole-sensitive variant, T455/V, was found to give partial growth in inhibitor-free broth containing 0·2 mg./100 ml. sulphathiazole and full growth at half this concentration. The probable inhibitory concentration is therefore about 0·25 mg./100 ml. A series of four synergism titrations were set up using Abbott (G 404 A 035) penicillin, and varying amounts of sulphathiazole were used in each. Accepting 0·25 mg./100 ml. as the probable inhibitory concentration, 0·5, 0·25 and 0·125 mg./100 ml. were added to three of the sets, while 2·5 mg./100 ml. was added to the fourth as a standard technique control. Tested against penicillin alone, the bactericidal concentration was 1·6 u./ml. In each of the four titrations in the

presence of sulphathiazole this was reduced to 0·8 u./ml. The synergic action can, therefore, be demonstrated with very small and with subinhibitory concentrations of sulphathiazole. Additional proof of this is afforded by the exhibition of synergism in the presence of sulphonamide inhibitors.

(5) *Effect of size of inoculum on synergism*

As the number of bacteria inoculated into the test systems is so important in determining the being in the level at which it is exhibited. That it does exist even with 5,000,000 bacteria is shown by the fact that there is full growth at 12·5 u./ml. of penicillin alone, but only a trace of growth at that concentration in the presence of sulphathiazole. These results again confirm Bigger's demonstration that 1 u./ml. of penicillin in the presence of sulphathiazole will sterilize an inoculum of 7000 typhoid bacilli, partially inhibit one of 70,000 and is without effect on one of 700,000.

Table 10. *Synergic action of penicillin and sulphathiazole on* S. typhi *T* 455/45. *Effect of size of inoculum (standard conditions)*

No. of bacteria inoculated	Penicillin alone: Bacteriostatic conc. (u./ml.)	Penicillin alone: Bactericidal conc. (u./ml.)	Penicillin + sulphathiazole. Bactericidal conc. (u./ml.)	Synergism
5,000,000	1·6	12·5+	*	Present
500,000	0·8	12·5+	6·25	Present
50,000	0·8	3·1	1·6†	×2+
5,000	0·8	3·1	0·8	×4
500	0·8	1·6	0·8	×2
50	0·8	1·6	0·4	×4
5	0·8	0·8	0·4	×2

* Tr. at 12·5 u./ml. † Tr. only at 0·8 u./ml.

Table 11. *Synergic action of penicillin and sulphathiazole on* S. typhi *T* 455/45. *Effect of different penicillins (standard conditions)*

Maker and batch no.	Bactericidal concentration (u./ml.): Penicillin alone	Bactericidal concentration (u./ml.): Penicillin + sulphathiazole	Synergism
Abbott (G 404 A 035)	3·1	0·8	×4
Pfizer (2534)	12·5	6·25	×2
C.S.C. (44093001)	12·5	3·1	×4
Squibb (32881)	12·5	6·25	×2
Squibb (32811)	12·5	6·25	×2
Lederle (782 H 131 A)	3·1	0·8	×4
Glaxo (A 4)	12·5	6·25	×2
Haffkine Institute	12·5	3·1	×4
Provisional British Standard	6·25	3·1	×2
Pure penicillin II (triethylamine salt)	6·25	1·6*	×4+

* Tr. at 0·8 u./ml.

extent of action of both penicillin and sulphathiazole, it is to be expected that it would also effect the degree of synergism exhibited. To examine this, seven paired sets of falling dilutions of penicillin (Abbott G 404 A 035) in inhibitor-free broth were set up, one of each pair with penicillin alone and the other with the addition of 2·5 mg./100 ml. of sulphathiazole. Each pair was then seeded with numbers of T 455/V ranging logarithmically from 5 to 5×10^6 (Table 10). Taking the bactericidal concentrations after the addition of PABA and penicillinase, the synergic action can be demonstrated with all the inocula, the only difference

(6) *Effect of different penicillins on synergism*

Synergism has been demonstrated with every penicillin preparation which we have tested, the difference between them being largely in the level at which they are effective. Thus a number were only bactericidal in concentrations of 12·5 u./ml., the addition of sulphathiazole reducing the lethal concentration to one-half or one-quarter of this; others were effective in lower concentrations such as 3·1 u./ml. alone which was still further reduced in the presence of sulphathiazole (Table 11). Similar differences in the degree of synergism

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exhibited by different penicillins against T455/45 were demonstrated with other strains. Thus strain T606 was killed by 12·5 u./ml. of each of two different penicillins; with sulphathiazole the bactericidal concentration of one was reduced to 6·25 u./ml. and to 1·56 u./ml. for the other, a two- and eightfold synergism respectively. It is important to note that pure penicillin II, which is largely present in the modern deep-brew penicillins, exerts at least a fourfold synergism. The stock T455/45 strain was killed by 6·25 u./ml. of this penicillin; with sulphathiazole, 1·6 u./ml. effectively sterilized the test inoculum, while only a trace of growth appeared with 0·8 u./ml.

(7) *Effect of sulphonamide inhibitors on synergism*

The mechanism whereby this synergism occurs is a matter of considerable interest. It is not a simple additive process in which one reactant kills those bacteria which are not killed by the other, for we have shown that it occurs with concentrations of each which are, by themselves, ineffective against small inocula. It appeared important, therefore, to determine whether this synergism was manifested in the presence of substances which directly inhibit the normal action of sulphathiazole.

(*a*) *Synergism in unabsorbed Lemco broth*

The Lemco broth used in this experiment allowed sulphathiazole to inhibit the growth of T455 in a concentration of 3·1 mg./100 ml. When absorbed with lysed horse blood, the sulphonamide inhibitors were removed and the inhibitory concentration of sulphathiazole was reduced to 0·2 mg./100 ml. Thus, in this broth, the 2·5 mg./100 ml. of sulphathiazole used in testing synergism is subinhibitory. The test inoculum of T455 was killed in this broth by 12·5 u./ml. of penicillin (Pfizer 2534); in the presence of sulphathiazole the bactericidal concentration was reduced to 3·1 u./ml.

(*b*) *Synergism in casein hydrolysate*

Large quantities of casein hydrolysate prepared by the Bengal Immunity Co. for use in cases of famine oedema were available, and a simple 1/20 dilution of this in distilled water was found to support a prolific growth of *S. typhi*. In this, sulphathiazole failed to inhibit the growth of T455 at a concentration of 25 mg./100 ml. A control titration in inhibitor-free broth showed complete inhibition at 0·4 and only a trace of growth at 0·2 mg./100 ml. In the casein hydrolysate medium, penicillin (C.S.C. 44093001) killed the test dose of T455 at 25 u./ml.; this was reduced to 6·25 u./ml. by the addition of 2·5 mg./100 ml. of sulphathiazole. In the inhibitor-free broth, the respective concentrations were 12·5 and 3·1 u./ml., so that, in both cases, there was a fourfold synergism.

The higher bactericidal concentration of penicillin in casein hydrolysate is of interest and confirms Schwartzman's (1945*a*) observation that more penicillin was required to inhibit the salmonellas in a synthetic medium to which casein hydrolysate had been added than in the medium alone. Casein hydrolysate exerts, therefore, an antipenicillin action as well as being a marked sulphonamide-inhibitor. The fact that synergism can be demonstrated in the presence of such a medium again suggests that it is not the normal mechanism of sulphonamide action which is concerned in its production.

(*c*) *Synergism in thioglycollic acid broth*

The addition of thioglycollic acid to broth in 0·1 % amounts was found to increase the effective inhibitory concentration of sulphathiazole very considerably. A full degree of synergism was demonstrated, however, in this broth against both T455 and 901O strains.

(*d*) *Synergism in* p-*aminobenzoic acid broth*

Repeated attempts have been made to demonstrate synergism in the presence of *p*-aminobenzoic acid. On one occasion each of us, independently, has encountered a series containing *p*-aminobenzoic acid which appeared to show a twofold synergism in bactericidal action against T455. On the other hand, a fourfold synergism in bacteriostatic effect which occurred with subinhibitory amounts of sulphathiazole was regularly abolished by the addition of *p*-aminobenzoic acid to the series, even when the amount added was such as only just to neutralize the amount of sulphathiazole present. Similarly, no synergic inhibitory effect could be demonstrated when an amount of *p*-aminobenzoic acid just sufficient to allow growth in the presence of 2·5 mg. sulphathiazole per 100 ml. had been added to inhibitor-free broth. Moreover, not only was there usually a complete inhibition of synergic killing effect, but, in one series, a higher concentration of penicillin was required to kill T455 in the presence of *p*-aminobenzoic acid and sulphathiazole than when the penicillin acted alone.

It would therefore appear that *p*-aminobenzoic acid, unlike those sulphonamide-inhibitors present in nutrient broth and casein hydrolysate, is capable of abolishing the synergic effect of sulphathiazole on penicillin. The few results obtained which suggested the contrary are probably of a similar nature to those departures from the usually observed killing effect which have occasionally been noticed in other series of experiments and which may be due to variations in the inoculum or to the presence in it of small numbers of 'persisters' (Bigger, 1944*b*, *c*)

IV. THE ACTION OF PENICILLIN AND SULPHATHIAZOLE AGAINST OTHER SALMONELLAS

In addition to *S. typhi*, two strains each of *S. paratyphi* A and B, three strains of *S. enteritidis* var. *chaco* and one strain each of *S. enteritidis* var. *jena* and *S. typhimurium* have been tested for sensitivity to penicillin, alone and in the presence of sulphathiazole. Crystalline sodium penicillin II, a standard concentration of 2·5 mg. sulphathiazole/100 ml. and an inoculum of approximately 10,000 organisms/2·5 ml. broth were employed. Untreated broth, in which the standard amount of sulphathiazole did not prevent growth, was used in order that the inhibitory synergic effect, which is thought to be was evident. The wide differences which may be encountered between the sensitivities of different strains of the same type is amply demonstrated by the behaviour of the two strains each of *S. paratyphi* A and B.

DISCUSSION AND SUMMARY

Due to earlier reports of the relative resistance of *S. typhi* and other members of the salmonella group to penicillin, and to the prevalent preoccupation of most workers with those organisms against which treatment with penicillin was more obviously applicable, little attention was payed to the chemotherapy of enteric fever until Bigger (1946) published his findings on the synergic action of

Table 12. *Action of penicillin and sulphathiazole on various salmonella types. Bacteriostatic and bactericidal concentrations against nine strains (tested in treated and untreated broth)*

	Untreated broth Bacteriostatic concentration (u./ml.)		Treated broth Bactericidal concentration (u./ml.)		
Type and strain	Penicillin alone (pure II)	Penicillin + sulphathiazole (2·5 mg./100 ml.)	Penicillin alone (pure II)	Penicillin + sulphathiazole (2·5 mg./100 ml.)	Bacteriostatic synergism only
S. paratyphi A (113/44)	6·25	1·6	6·25	12·5	× 2
S. paratyphi A (Beamish/46)	25	12·5*	50+	25	Less than × 2
S. paratyphi B (8006/Kauffmann)	25	12·5	50+	50+	× 2
S. paratyphi B (TCD)	0·8*	Less than 0·8	6·25	3·1	Present
S. enteritidis (511/44/*chaco*)	25	12·5	50+	50+	× 2
S. enteritidis (570/44/*chaco*)	25	12·5	50+	50+	× 2
S. enteritidis (562/45/*chaco*)	12·5	6·25	50+	50+	× 2
S. enteritidis (12316/JT/*jena*)	25	12·5	50+	50	× 2
S. typhimurium (WH 77/46)†	—	—	25	12·5	—

* Tr. growth in these tubes.

† This strain was tested in inhibitor-free broth only. In this broth its growth was not inhibited by 20 mg. sulphathiazole/100 ml. Glaxo (A 4) penicillin was used.

a more reliable guide to the occurrence of synergism than sterilization, might be demonstrated. In conformity with the previous results, however, treated broth was also used to demonstrate synergic killing effect. Results are given in Table 12.

T 455, when tested under similar conditions, was inhibited by 6·25 u./ml. penicillin and showed a twofold inhibitory synergism in the presence of sulphathiazole. It will be seen that all the strains tested showed some degree of inhibitory synergism but that, in the majority, the concentration required to inhibit growth was considerably higher than in the case of *S. typhi*. With the majority of strains the concentration of penicillin required to destroy the inoculum in 24 hr. at 37° C. was greater than 25 u./ml., even in the presence of sulphathiazole, while in one case no bactericidal synergism at all

penicillin and sulphathiazole against *S. typhi*. Although, therefore, much work has been published concerning the synergic action of the sulphonamide group on penicillin activity in general, little information is available on the *in vitro* behaviour of the salmonellas in this respect despite the present wide, though tentative, application of the principle in the treatment of enteric fever. The experiments presented above were undertaken with the object of confirming those findings hitherto reported and of studying in detail the effects of sulphathiazole and penicillin, and of combinations of the two, on the salmonella group, with special reference to *S. typhi*.

For purposes of discussion it is convenient to summarize our results under the principal headings used in the text.

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I. *The action of sulphathiazole alone.* The presence of sulphonamide inhibitors in nutrient broth and their removal by treatment with 5% laked horse blood (Harper & Cawston, 1945) have been confirmed. In the case of both Lemco-peptone and horse meat infusion broths the inhibitory effect of sulphathiazole is increased about eightfold by treatment. The use of inhibitor-free broth is essential for the comparative *in vitro* estimation of the sensitivity of organisms to sulphonamide since the values obtained thereby are absolute, whereas those derived from tests in untreated broth may vary widely depending on the amount of inhibitor present. Results obtained in inhibitor-free broth, however, cannot afford any indication of the likely effect of sulphonamides in treatment since the body fluids themselves contain considerable amounts of inhibitor substances. In this connexion it is interesting to note that the treatment of broth by Harper & Cawston's method may not result in a constant decrease in the apparent amount of inhibitor removed when different organisms are tested against treated and untreated broth in the presence of varying amounts of sulphonamide. For example, with one meat infusion broth absorption with laked horse blood reduced the inhibitory concentration of sulphathiazole for T 455 from 1·25 to 0·16 mg./100 ml., that is, approximately eight times, while at the same time the reduction for two strains of *Str. dysenteriae* (Flexner) was about sixty-fold, from 50 to 0·8 mg./100 ml. The degree of sensitivity of *S. typhi* demonstrated by us is remarkably high, the growth of an inoculum of approximately 4000 organisms/ml. of our initial culture of strain T 455 being prevented by 3·1 mg. sulphathiazole/100 ml. in untreated and by 0·4 mg./100 ml. in treated broth. Bigger (1946) appears to have obtained much higher inhibitory levels since in his experiments he records the development of almost full turbidity in treated broth containing 10 mg. sulphathiazole/100 ml. when the inoculum was 700,000/ml. and only 'greatly reduced turbidity' with an inoculum as low as 7000/ml. The possibility of a variation towards sulphonamide resistance in cultures preserved on agar at room-temperature must be borne in mind. We have demonstrated that such variation may occur spontaneously, in the absence of previous contact with sulphonamide, and may result in as much as a sixteenfold increase in the apparent resistance of the culture. Sensitive and resistant subcultures obtained by colony selection appear to remain stable for long periods when preserved in the cold on egg medium. It is not clear whether such spontaneous variation is a property only of freshly isolated strains or is likely to occur at any time in laboratory cultures. It is obvious that it embodies a potent source of error in the assessment of sulphonamide resistance. Another source of error which has been confirmed for *S. typhi* is the effect of size of inoculum. There is a steady increase in the amount of sulphathiazole required to inhibit growth for each logarithmic increase in inoculum from 20 to 20,000 organisms/ml. A change in inoculum from 20,000 to 200,000/ml., however, increases the requisite inhibitory concentration of sulphathiazole thirty to sixty times. A relatively small variation in an inoculum within this range, therefore, may produce a disproportionately large alteration in the concentration of sulphathiazole necessary to prevent its growth, constituting an important source of experimental error.

Sulphonamides are often regarded as purely bacteriostatic agents. Colebrook & Cawston (1945), however, have demonstrated their lethal action against streptococci. Bigger (1946) has shown that 20 and 10 mg. sulphathiazole/100 ml. can sterilize an inoculum of seven *S. typhi*/ml. in 96 hr., though inocula of 70/ml. or more were not sterilized. In our experiments, 3·1 mg. sulphathiazole/100 ml. in inhibitor-free broth effectively sterilized the standard inoculum of approximately 9000 *S. typhi* strain T 455 after 120 hr., though 25 mg./100 ml. failed to produce sterility in 24 hr. During the 5-day period over which this series was observed, the growth extended one tube each day until, on the fourth day, it was present in four times the concentration of sulphathiazole required to suppress growth during the first 24 hr. This is probably due to the development of increasing degrees of sulphonamide resistance by adaptation of the survivors from the inoculum.

II. *The action of penicillin alone.* It has been mentioned that previous reports on the sensitivity to penicillin of *S. typhi* have differed widely. In order to obtain some absolute indication of sensitivity we tested eighteen strains against samples of commercial penicillin which had been carefully assayed against Provisional British Standard penicillin. Growth of all the strains was inhibited by 6·25 u./ml. and of three strains by as low a concentration as 1·6 u./ml. Ten u./ml. sufficed to sterilize the standard inoculum of all save one of the strains in 24 hr., the remaining strain being sterilized by 12·5 u./ml. These figures were based on results obtained with a batch of penicillin which subsequently proved slightly less potent for *S. typhi* than pure penicillin II which forms the present International Standard. Moreover, during the 24-hr. period over which the penicillin was allowed to act it deteriorated about 35% from its initial value so that our results tend rather to underestimate the sensitivity of the strains than otherwise. *S. typhi* strain 901, devoid of Vi antigen, and strain Vi(1) (Bhatnagar), which is a Vi-R variant, fell within the limits of sensitivity shown by recently isolated

strains so that sensitiveness to penicillin appears to be uncorrelated with antigenic structure. These results indicate a higher degree of sensitiveness than those reported elsewhere. Experiments in colony selection suggest that some degree of variation towards penicillin resistance may occur, though this is not nearly so marked as is the development of spontaneous sulphonamide resistance. It is not, perhaps, out of place here to mention a relevant observation on the behaviour of two freshly isolated strains of *Str. dysenteriae* (Flexner) received for examination on egg slopes. Growth of standard inocula from primary broth cultures of both strains was inhibited by 6·25 u./ml. of pure penicillin II. After a few serial subcultures in broth, however, 25 u./ml. of the same penicillin were required to inhibit both strains.

Bigger (1946) has drawn attention to the fact that in the presence of 10 mg. sulphathiazole/100 ml., the concentration of penicillin required to kill *S. typhi* depends mainly on their numbers. It is of interest, therefore, to examine the relationship between inoculum size and the inhibitory and killing effect of penicillin alone. We have confirmed the fact that inhibitory activity is largely independent of the numbers of bacteria involved, in one of our experiments the inhibitory concentration being only doubled for a millionfold increase in the inoculum of *S. typhi*. On the contrary, the ability of penicillin to kill, like that of sulphathiazole to inhibit growth, is largely a function of inoculum size. An increase in the numbers of *S. typhi* from 20,000 to 200,000/ml. requires a more than fourfold increase in penicillin concentration to effect sterilization while for logarithmically decreasing inocula below 20,000/ml. the effective penicillin concentration decreases though at a less rapid rate. Thus the most critical range over which small variations in numbers of organisms may result in large variations in drug effect is the same for penicillin sterilization as for sulphonamide inhibition. It is not yet clear why sterilization and the suppression of growth by penicillin should differ so markedly in their reaction to bacterial numbers. It would seem reasonable to suppose, *a priori*, that the interference with the vital processes of the cell by penicillin which prevented its multiplication would be the same as that resulting in its death and, therefore, that the ratio between the bacteriostatic and bactericidal concentrations of the drug would depend solely on the length of time for which it was allowed to act. In all the experiments presented above, sterility has been judged by the development of turbidity after the addition of penicillinase to the bottle, so that the subsequent absence of growth implied the killing of every organism introduced in the inoculum. In other experiments samples, usually about 0·05 ml., were transferred to tubes of broth after the addition of penicillinase as before. As a rule such samples were found to have been sterilized by much lower concentrations of penicillin than were required to sterilize the whole inoculum, indicating that the range of penicillin concentrations between the inhibitory and sterilizing doses were sufficient to kill the majority, but not all, of the organisms in the inoculum. Not infrequently, however, the presence or absence of growth in such sample cultures from bottles seeded with large inocula displayed a randomness highly suggestive of the presence of 'persisters' (Bigger 1944*b*, *c*). It has already been mentioned that similar results have occasionally been observed in standard sterility tests. It is not improbable that the dependence of penicillin sterilization on inoculum size is due, in some degree at least, to the increased chance of 'persisters' being present as the size of inocula increases. It has not yet been ascertained whether the bactericidal effect of penicillin depends on the total number of bacteria present or on their concentration.

The fact that different batches of penicillin of equal potency for the Oxford *Staphylococcus* may differ widely in their effect on *S. typhi* is of considerable interest and importance. The method of deep cultivation now employed commercially in penicillin production gives a very high yield of penicillin II whereas the older method of surface cultivation in flasks yielded a greater proportion of penicillins I and III (Ory, Meads & Finland, 1945). The relative amounts of the four fractions is also a function of the strain of *P. notatum* used, strain Q 176, now widely employed because of its high yield, producing under certain conditions a considerable proportion of IV (see *Lancet*, 1946, **2**, 387). The four different penicillins differ quantitatively in their antibacterial activity, III, for example, being more effective than II against haemolytic streptococci but much less potent against staphylococci or *Tr. pallidum* while I, of equal potency with II against staphylococci, is less effective against haemolytic streptococci or *Tr. pallidum*. While IV gives the highest *in vitro* value of all the fractions against staphylococci and haemolytic streptococci, its effectiveness *in vivo* is considerably lower than that of II due, apparently, to its greater rate of destruction in the body (Eagle & Musselman, 1946). It appears probable from our results that the *in vitro* value of penicillin IV is less than one-quarter that of II against *S. typhi*, while the efficacy of II, although high, is lower than that of some other fraction not yet identified but probably either I or III. It is of importance, therefore, in the treatment of typhoid fever to select a penicillin free from fraction IV. Further research into the action of the different fractions on *S. typhi* is clearly indicated.

III. *The synergic action of penicillin and sulphathiazole.* Some degree of synergism of bactericidal action has been demonstrated with all eighteen strains of *S. typhi* tested and with nine out of ten colonies selected from strain T/455. With the majority of strains and of single colony cultures of T455 synergism was twofold. Synergism was observed with all batches of penicillin tested, the only significant difference between them being the level at which the effect occurred. Increasing the size of the inoculum raised the concentration of penicillin required to sterilize it but did not inhibit the synergic action of sulphathiazole even when 5×10^6 organisms, sufficient to produce full turbidity in ten times the concentration of sulphathiazole used, was added. That synergism is not dependent on inhibitory concentrations of sulphathiazole has been demonstrated in other ways as, for example, by the absence of correlation between extent of synergism and degree of sulphathiazole resistance of variant colonies of strain T455, variants capable of full growth in the standard amount of sulphathiazole giving the same result as highly sensitive variants whose growth was completely inhibited. Again, no variation in synergic activity was observed when one sensitive variant was tested in various concentrations of sulphathiazole on either side of the inhibitory. The most striking demonstration, however, was the occurrence of full synergism in the presence of sulphonamide inhibitors both in nutrient broth and, in more marked degree, in casein hydrolysate which also exerts some anti-penicillin effect. This is of considerable importance since, judging by the failure of sulphonamide alone in the treatment of typhoid fever, it is doubtful whether sulphathiazole itself has any inhibitory effect on *S. typhi* in the body. Such failure does not rule out the possibility of a useful synergic role for the drug in conjunction with penicillin. Experiments on synergism in the presence of plasma or whole blood are clearly indicated. Para-aminobenzoic acid, on the other hand, appears definitely to abolish synergism. It has been pointed out that, despite many contrary results, an apparent bactericidal synergic effect was twice obtained in the presence of this substance. In view of the many uncontrollable variables which have here been revealed in experiments involving penicillin sterilization, however, it is thought that undue significance should not be attached to aberrant results. Results of bacteriostasis experiments are much more uniform, and the consistent prevention by *p*-aminobenzoic acid of synergic inhibition of *S. typhi* is, in our opinion, almost conclusive evidence that it abolishes all manifestations of synergism. The bearing of these findings on the possible mechanism of synergism is more a matter for speculation than of fact. Duthie (1945), on the grounds that penicillin-resistant staphylococci produced penicillinase and that their sensitivity to penicillin was greatly increased in the presence of sulphamethazine, postulated that the synergic action of sulphonamides could be attributed to their inhibition of penicillinase production whether or not they prevented growth. The production of penicillinase by penicillin-resistant staphylococci has been confirmed by Kirby (1944). Woodruff & Foster (1945) have also demonstrated its presence in cultures of the Oxford *Staphylococcus*. The inhibition of penicillinase production by sulphonamides, however, has not, so far as we know, the backing of published experimental evidence. This theory seems inherently capable of explaining all the facts of synergism but is untenable in the absence of more supporting evidence. The effects of inoculum size on the bactericidal concentration of penicillin and on inhibitory sulphathiazole levels suggest an alternative theory. In the presence of sulphathiazole the numbers of *S. typhi* in the inoculum, after an initial rise, fall rapidly to a very low level. Since the lethal action of penicillin increases as the inoculum decreases it is clear that reduction in the numbers of organisms by sulphathiazole will bring them within the effective range of penicillin concentrations which alone were unable to cope with the original inoculum. Bactericidal synergism, in the presence of inhibitory amounts of sulphathiazole at least, is thus adequately explained. Again, if subinhibitory amounts of penicillin produced an initial fall in the numbers of the inoculum sufficient to bring them within the bacteriostatic range of amounts of sulphathiazole incapable of preventing growth of the original inoculum, synergic bacteriostasis would follow. A small number of experimental counts have so far failed to demonstrate such a fall. A mechanism of the kind postulated would inevitably be limited in its range and it is difficult to visualize its producing either the eightfold effect which has often been observed or any synergism at all in casein hydrolysate.

It may be mentioned that, in a few experiments, sulphamethazine has failed to show synergism against *S. typhi* and that we have been unable to confirm the synergic action of sulphathiazole against *Staphylococcus* N.C.T.C. 6571, using pure penicillin II.

The results obtained with other salmonella types, set out in Table 12, bring out three points. The first is the wide differences which may occur in the sensitivity to penicillin of different strains of the same type so that individual assessment of strains is essential. All the strains tested had been maintained for widely varying but considerable lengths of time in artificial culture so that the possibility of spontaneous increase in penicillin resistance must be remembered when assessing the divergences.

Secondly, the majority of strains and types showed a considerably greater degree of resistance to penicillin, and especially to its bactericidal action, than *S. typhi*. McKee, Rake & Menzel (1944) record that certain Gram-negative bacilli, in particular *S. enteritidis*, previously thought resistant to penicillin on the basis of tests with relatively crude preparations, had been shown to be susceptible to a considerable extent to more highly purified preparations. Our experience has been that *S. enteritidis* is definitely more resistant than is *S. typhi*. Thirdly, bacteriostatic synergism has been demonstrated with all strains and types tested though its degree has never been more than twofold within the limits of our experiments. It is improbable, therefore, that the promise shown by the combined penicillin-sulphathiazole treatment of typhoid fever will be repeated in the majority of other salmonella infections if our *in vitro* results reflect the behaviour of strains actually causing infection, though the possibilities of treatment in individual cases can only be assessed after examination of the infecting strain.

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