STUDIES PERTAINING TO THE ANTIBACTERIAL ACTIVITY OF SULFATHIAZOLE AND ITS METHYL DERIVATIVE*

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INTRODUCTION

The present report represents a more detailed and extended presentation of experimental data pertaining to the antibacterial activity of the thiazole compounds and their possible mode of action than those previously published from this laboratory (Muir, Shamleffer, and Jones, 1940, 1941a, and 1941b). In the present instance it is our purpose (*a*) to show that synthetic media are distinctly superior to the conventionally employed peptone-containing media for such studies, (*b*) to present a detailed appraisal of the inhibitory effect of the thiazole drugs on cellular proliferation, (*c*) to demonstrate the drug-antagonistic effect of *p*-aminobenzoic acid and other substances in a strictly synthetic medium, and (*d*) to compare the antibacterial effect of the thiazole compounds as observed *in vivo*.

The organism employed in the *in vitro* studies was a strain of *Salmonella enteritidis*[1] recovered from an epizootic in a mouse colony, and upon isolation exhibited a fair degree of virulence, since a dose of 75 to 100 million organisms was sufficient to kill mice within 24 hours when administered by the intra-peritoneal route. In preparing inocula of this organism for the cultural studies, 24-hour meat-extract broth cultures were employed. The cells were sedimented by centrifuging, and washed twice with saline solution before being finally suspended in a volume equal to that of the original broth. The inocula consisted of 0.1 ml. amounts of 1×10^{-6} dilution of the final saline suspension and contained approximately 100 viable cells, as determined by plate counts. Since the inocula were prepared in this manner only very minimal amounts, if any, of the original culture medium were conveyed to the test cultures.

The meat-extract peptone broth used in these studies contained 0.3 per cent Bacto-Beef extract, 1 per cent Bacto peptone, and 0.5 per cent sodium chloride. These ingredients were dissolved in distilled water without the aid of heat, adjusted to pH 6.8, and sterilized by filtration through Berkefeld "N" candles. The drugs to be tested were incorporated in this medium prior to filtration and when necessary a minimal amount of heat was employed to facilitate solution.

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[1] We are indebted to Dr. P. R. Edwards for determining the serological character of this organism.

The synthetic medium employed contains the following ingredients:

Magnesium sulfate	0.2 gram
Ammonium phosphate, prim	1.0 gram
Dipotassium phosphate	1.0 gram
Sodium citrate	2.0 grams
Sodium chloride	5.0 grams
Water, twice distilled	1000.0 ml.

The above ingredients are in the same proportion as are contained in Simmon's citrate agar and when dissolved in twice-distilled water constitute a medium quite suitable for the cultivation of this organism even though very small inocula are employed.

TABLE 1

Comparison of sulfathiazole (S.T.) and sulfamethylthiazole (S.M.T.) in synthetic medium and in nutrient broth

DRUG	INCUBATION PERIOD	SYNTHETIC MEDIUM*						NUTRIENT BROTH*					
		20 .0008	10 .0004	5 .0002	2.5 .0001	1.25 .00005	0 0	20 .0008	10 .0004	5 .0002	2.5 .0001	1.25 .00005	0 0
	hrs.												
S.T.	24 hours	−	−	−	−	−	−	−	−	−	++	++++	++++
	48 hours	−	−	−	−	−	+++	++	+++	++++	++++	++++	++++
	72 hours	−	−	−	−	−	++++	+++	+++	++++	++++	++++	++++
	2 weeks	−	−	−	−	−	++++	++++	++++	++++	++++	++++	++++
S.M.T.	24 hours	−	−	−	−	−	−	−	−	−	++	++++	++++
	48 hours	−	−	−	−	−	+++	++	+++	++++	++++	++++	++++
	72 hours	−	−	−	−	−	++++	+++	+++	++++	++++	++++	++++
	2 weeks	−	−	−	−	−	++++	++++	++++	++++	++++	++++	++++

* Concentration of drug in mgm. per cent (upper figures) and molarity (lower figures).

BACTERIOSTASIS OF S. ENTERITIDIS BY SULFATHIAZOLE AND SULFAMETHYLTHIAZOLE IN NUTRIENT BROTH AND IN SYNTHETIC MEDIUM

In order to compare the relative effectiveness of sulfathiazole and sulfamethylthiazole against *S. enteritidis* in ordinary extract broth, these drugs were incorporated in this medium in the amounts given in table 1. The drug-containing media were then distributed in tubes in 5 ml. quantities and inoculated with approximately 180 organisms per tube. After incubation at 37°C. for various time intervals, the turbidity was estimated by visual inspection of the cultures and recorded on a scale of − to ++++.

The results, recorded in table 1, indicate that sulfathiazole and sulfamethylthiazole under these conditions do not differ materially in their inhibitory effects. In both cases growth is restrained during the earlier part of the observation period in those tubes containing the higher concentrations of drugs but eventually becomes maximal. Since we have not observed any evidence of developing "roughness" in such cultures, it is conceivable that the failure of the drugs to prevent growth under these conditions may be due to the rôle played by certain substances contained in the nutrient broth.

On the other hand, when the synthetic medium was employed in similar manner it was observed that the drugs manifested a much greater inhibitory effect, as is evident in the data recorded in table 1, where it will be seen that in synthetic medium a concentration of 1.25 mg. per cent of sulfathiazole completely inhibits growth whereas this result is not accomplished in nutrient broth by as much as 20 mg. per cent.[2] This superiority of the synthetic medium is further exemplified in identical experiments (not here recorded) wherein the inoculum was increased ten times (i. e., 1800 organisms per tube) and similar inhibition on the part of the drugs was manifested in that medium. With reference to the relative effectiveness of sulfathiazole and sulfamethylthiazole, no difference was observed in the concentrations studied, i. e., from 20 mg. per cent to 1.25 mg. per cent. That this remarkable inhibitory effect of the drugs was bacteriostatic in nature rather than bactericidal was revealed by successful subculture from those tubes showing no visual evidence of growth. A few workers in this field (Woods 1940, Kalmanson 1940, Keltch et al. 1941, and Spink and Jermsta 1941) have employed media of known chemical composition but in no case has any direct comparison been offered between results obtained in synthetic and non-synthetic media.

INFLUENCE OF THIAZOLE DRUGS UPON VIABLE POPULATION COUNTS OF THE ORGANISM IN SYNTHETIC MEDIUM AND IN NUTRIENT BROTH CULTURES

In order to develop more exact information regarding the quantitative aspects of the observed bacteriostasis, growth-curves were developed. This involved the preparation of control cultures containing no drug and test cultures containing sulfathiazole or sulfamethylthiazole in the amounts indicated in figures 1 and 2. The inoculum for all tests was prepared in the manner previously described and was of the order of 100 cells per tube containing 5 ml. of the medium. The cultures were incubated at 37°C. and viable populations of different tubes were determined by plate counts at designated intervals. The logarithms of these viable cell counts are plotted in figures 1 and 2.

In preliminary studies, when the growth curves of the organism in the presence of sulfathizaole and sulfamethylthiazole were compared, it was noted that there was no detectable difference in the activities of these two drugs. Accordingly, only the growth curves in sulfathiazole-containing media are incorporated in this report.

In figure 1 are recorded the growth curves of the organism in meat extract peptone broth, without drug, and with 5 and 10 mg. per cent sulfathiazole. One of the effects of the drug is seen as an alteration of the general character of the logarithmic phase of multiplication. From the termination of the short lag period up to almost the fourth hour of growth, cell reproduction seems to occur as a geometric progression at an equal rate in each of the three series and with a generation time of about 17 minutes. It is of interest to point out that during this time interval three or four generative cycles are completed. Subsequently,

[2] On other occasions a quantity as small as 0.3 mg. per cent has prevented growth in the former medium.

multiplication in the control culture continues to occur at a constant rate until the termination of the logarithmic phase (about 10 hours); in the cultures containing sulfathiazole, however, the apparent initial rate of growth is not main-

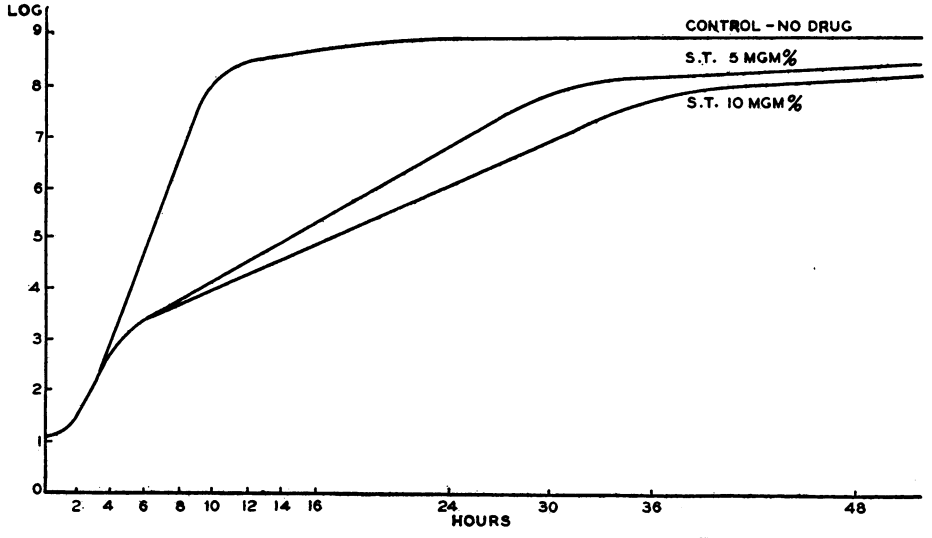

FIG. 1. VIABLE CELL COUNTS OF S. ENTERITIDIS IN MEAT-EXTRACT PEPTONE BROTH WITH AND WITHOUT SULFATHIAZOLE

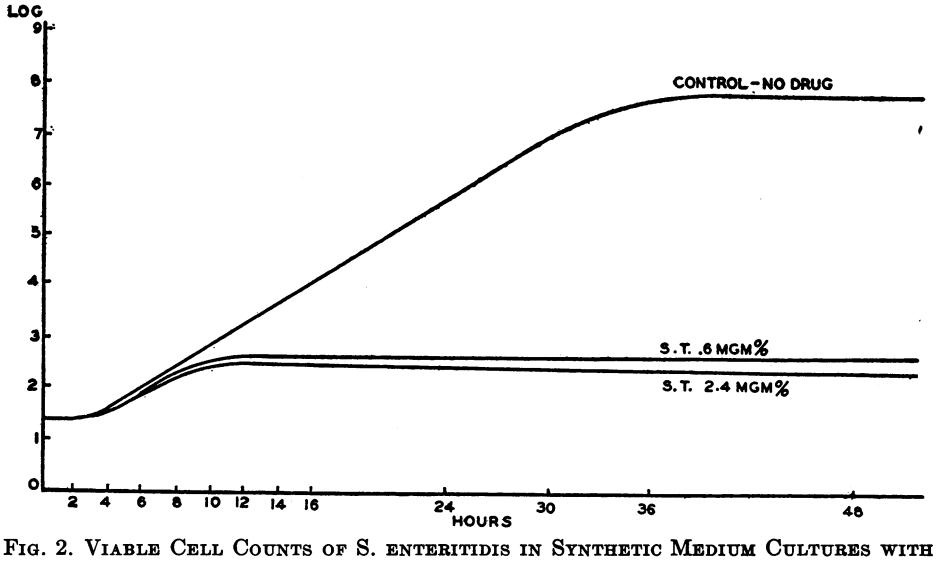

FIG. 2. VIABLE CELL COUNTS OF S. ENTERITIDIS IN SYNTHETIC MEDIUM CULTURES WITH AND WITHOUT SULFATHIAZOLE

tained but decreases significantly and loses its logarithmic character for an interval of about two hours. Following this period there ensues a prolonged interval (from about the sixth to the thirtieth and thirty-sixth hour) of log-

arithmic increase at a rate (generation times of about 90 minutes and 120 minutes) which is significantly below that observed in the early hours of incubation. Thus, logarithmic increase in nutrient broth containing sulfathiazole seems to occur during each of two separate phases. The stationary phase is reached much earlier in control cultures than in drug-containing cultures. The ultimate number of cells developing in the latter media is about one-fourth the number found in the controls.

In figure 2 are recorded curves similarly developed from cultures in synthetic medium. In a comparison of the control culture and sulfathiazole cultures, it will be noted that the rate of multiplication does not appear to be markedly inhibited during the first 6 or 8 hours, a circumstance closely resembling that already noted in the case of the non-synthetic medium. Furthermore, the data from which the curves were plotted afford the indication that here again, 3 or 4 generative cycles have been completed before the sharp divergence occurs between the curves representing the growth in the control and the drug-containing cultures. Subsequent to this period while the control culture continues to increase logarithmically for the next 24 hours, the sulfathiazole cultures tend to remain stationary.[3] In the sulfathiazole cultures, during the interval from the fourth to the eighth hour, there is an ill-defined period of probable logarithmic increase. In the stationary phases the control culture populations were about 150,000 times those observed in the sulfathiazole cultures.

In a comparison of figures 1 and 2, it will be noted that with peptone broth, 5 and 10 mg. per cent concentrations of sulfathiazole were employed whereas 0.6 and 2.4 mg. per cent concentrations were used in the synthetic medium. In the control cultures (without drug) of each series, the generation time in nutrient broth is 17 minutes during the logarithmic phase, which terminates at about the tenth hour, whereas, in the synthetic medium, the generation time is 100 minutes and the logarithmic phase ends at about the thirty-sixth hour. The maximal number of viable cells developing in nutrient broth is 1,000,000,000 per ml., while in the synthetic medium the maximal number is 95,000,000 per ml.

In a comparison of those curves in figures 1 and 2 which pertain to the growth of bacteria in the presence of sulfathiazole, it will also be noted that in figure 1 there are two separate phases of logarithmic increase which differ sharply in their rate and in duration; whereas, in synthetic medium only one logarithmic phase of multiplication occurs. The stationary phase is reached in nutrient broth in 36 and 40 hours of incubation while it is reached in synthetic medium in about 12 hours. In peptone broth, the presence of sulfathiazole reduces bacterial proliferation by about 75 per cent; whereas, a much smaller quantity of sulfathiazole in synthetic medium reduces bacterial proliferation by more than 99.9 per cent. From these figures it becomes evident that sulfathiazole is approximately ten times as effective in our medium of known composition as it is in meat-extract peptone broth.

[3] The populations in these cultures have been found to remain static for 70 hours longer than is shown in figure 2, i.e., for a total of 120 hours.

INHIBITION OF SULFATHIAZOLE ACTIVITY BY COMMONLY USED INGREDIENTS OF CULTURE MEDIA

The significance of bacteriological peptone as an inhibiting agent in the bacteriostatic activity of sulfanilamide was reported by Lockwood (1938) and later confirmed by Fleming (1940). In order to ascertain the anti-sulfathiazole activity of a variety of bacteriological peptones and other substances commonly incorporated in culture media, an experiment was designed wherein these substances were added in varying amounts to our basal synthetic medium and to the same medium containing graded amounts of sulfathiazole. These cultures were examined for visible turbidity after 24, 48 and 72 hours, and after 6 days of incubation. The data thus secured are presented in table 2. It will be noted in the series of control cultures that a 4 plus degree of growth is maximum and is attained only after 72 hours of incubation, and further, that those tubes containing even the smallest amount of sulfathiazole employed (i. e., 2.5 mg. per cent) failed to show growth during the 6-day observation period.

With this concept of the control series in mind, a comparison of the results obtained when a variety of commercial bacteriological peptones are added, reveals in all cases a growth-stimulating effect of even the smallest amounts (0.005%) of these nutrient substances. On the other hand, the antisulfathiazole activity of these materials is very striking in each instance. The hydrolysates, Aminoids[4] and Casamino Acids,[5] which are of simpler chemical composition presumably, also display sulfathiazole-inhibiting activity; the former almost to the same degree as the various peptones, the latter being much less active. Both of these substances serve to promote growth appreciably when added to the basal medium. Each of the two brands of meat-extract investigated was found to stimulate growth and to interfere with the maximal manifestation of drug activity. The degree of interference, however, was not as great as that displayed by the peptones. Since agar, as made available from commercial sources, may contain growth factors, as for example biotin, reported by Robbins (1941), the possible anti-sulfathiazole activity of the former substance was investigated. It was found that agar exerted no detectable antagonistic effect against the drug and displayed only negligible growth-promoting properties. Beef serum, on the other hand, stimulated growth of the organisms to a slight extent but failed to counteract the bacteriostasis of the drug. The latter affords confirmation under controlled conditions of the earlier reports of Lockwood (1938) and Fleming (1940), regarding the failure of serum to affect sulfanilamide bacteriostasis. The addition of glucose to our basal medium brought about some enhancement of growth and did not interfere with the bacteriostatic activity of sulfathiazole.

[4] Aminoids, trade name of biuret-free material used as a constituent of culture media, and marketed by the Arlington Chemical Co.

[5] Casamino Acids, a complete acid hydrolysate of casein, kindly supplied by the Digestive Ferments Co.

TABLE 2

The anti-sulfathiazole activity of peptones and other commonly used ingredients of culture media when added to the synthetic medium

S.T. TIME	CONTROL				GLUCOSE											
					0.005%				0.05%				0.5%			
	0	2½	5	10	0	2½	5	10	0	2½	5	10	0	2½	5	10
24 hours	—	—	—	—	1	—	—	—	2	—	—	—	2	—	—	—
48 hours	2	—	—	—	3	—	—	—	3	—	—	—	3	—	—	—
72 hours	4	—	—	—	4	—	—	—	4	—	—	—	3	—	—	—
6 days	4	—	—	—	4	—	—	—	4	—	—	—	3	—	—	—

S.T. TIME	BACTO-PEPTONE												NEOPEPTONE												PROTEOSE-PEPTONE											
	0.005%				0.05%				0.5%				0.005%				0.05%				0.5%				0.005%				0.05%				0.5%			
	0	2½	5	10	0	2½	5	10	0	2½	5	10	0	2½	5	10	0	2½	5	10	0	2½	5	10	0	2½	5	10	0	2½	5	10	0	2½	5	10
24 hours	3	—	—	—	4	3	2	—	5	4	3	—	—	—	—	—	4	3	2	—	5	3	2	—	—	—	—	—	4	4	3	—	5	5	5	—
48 hours	3	—	—	—	4	4	4	—	5	5	5	3	4	—	—	—	4	4	4	1	5	5	5	2	4	1	1	—	4	4	4	3	5	5	5	5
72 hours	5	—	—	—	5	4	4	2	5	5	5	4	5	2	1	—	5	4	4	3	5	5	5	4	5	2	1	—	5	4	4	3	5	5	5	5
6 days	5	—	—	—	5	4	4	3	5	5	5	4	5	3	3	—	5	4	4	4	5	5	5	5	5	3	3	—	5	5	5	4	5	5	5	5

S.T. TIME	PROTEOSE-PEPTONE NO. 3												BACTO-TRYPTOSE												PEPTONE FAIRCHILD											
	0.005%				0.05%				0.5%				0.005%				0.05%				0.5%				0.005%				0.05%				0.5%			
	0	2½	5	10	0	2½	5	10	0	2½	5	10	0	2½	5	10	0	2½	5	10	0	2½	5	10	0	2½	5	10	0	2½	5	10	0	2½	5	10
24 hours	3	—	—	—	4	3	—	—	4	4	4	—	3	—	—	—	4	3	2	—	5	4	4	—	3	—	—	—	4	2	2	—	5	5	5	—
48 hours	3	—	—	—	4	4	4	1	5	5	5	2	4	—	—	—	4	4	3	—	5	5	5	3	4	—	—	—	4	4	4	—	5	5	5	3
72 hours	5	—	—	—	5	4	4	2	5	5	5	3	5	—	—	—	5	4	4	1	5	5	5	4	5	1	1	—	5	5	5	—	5	5	5	4
6 days	5	3	3	—	5	4	4	3	5	5	5	4	5	2	3	—	5	4	5	3	5	5	5	4	5	3	3	—	5	5	5	3	5	5	5	4

S.T. TIME	AMINOIDS (ARLINGTON)												CASAMINO ACIDS (DIFCO)												BACTO-AGAR											
	0.005%				0.05%				0.5%				0.005%				0.05%				0.5%				0.005%				0.05%				0.5%			
	0	2½	5	10	0	2½	5	10	0	2½	5	10	0	2½	5	10	0	2½	5	10	0	2½	5	10	0	2½	5	10	0	2½	5	10	0	2½	5	10
24 hours	3	—	—	—	4	3	2	—	5	4	3	—	2	—	—	—	4	—	—	—	5	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—
48 hours	4	—	—	—	4	3	3	—	5	4	4	2	4	—	—	—	4	—	—	—	5	4	—	—	2	—	—	—	3	—	—	—	4	—	—	—
72 hours	5	—	—	—	5	4	4	—	5	5	5	3	4	—	—	—	5	—	—	—	5	4	2	—	3	—	—	—	3	—	—	—	4	—	—	—
6 days	5	3	3	—	5	4	4	2	5	5	5	4	4	—	—	—	5	3	1	—	5	4	4	—	3	—	—	—	4	—	—	—	4	—	—	—

S.T. TIME	BEEF EXTRACT WILSON												BACTO-BEEF EXTRACT												BEEF SERUM											
	0.005%				0.05%				0.5%				0.005%				0.05%				0.5%				0.005%				0.05%				0.5%			
	0	2½	5	10	0	2½	5	10	0	2½	5	10	0	2½	5	10	0	2½	5	10	0	2½	5	10	0	2½	5	10	0	2½	5	10	0	2½	5	10
24 hours	3	—	—	—	4	1	—	—	4	3	2	—	3	—	—	—	4	1	—	—	4	3	—	—	—	—	—	—	—	—	—	—	3	—	—	—
48 hours	4	—	—	—	4	1	—	—	5	4	4	—	4	—	—	—	4	1	—	—	5	4	3	—	3	—	—	—	4	—	—	—	4	—	—	—
72 hours	5	—	—	—	5	3	—	—	5	4	4	—	5	—	—	—	5	2	—	—	5	4	3	—	3	—	—	—	4	—	—	—	4	—	—	—
6 days	5	—	—	—	5	3	3	—	5	4	5	3	5	—	—	—	5	3	—	—	5	4	4	—	4	—	—	—	4	—	—	—	4	—	—	—

Estimates of bacterial growth are based upon visible turbidity and are recorded on a scale of — to 5 S.T. = Sulfathiazole, the values given are in mgms. per cent; Inoculum: 130 *S. enteritidis* cells (washed) per tube.

ANTI-SULFATHIAZOLE ACTIVITY OF *p*-AMINOBENZOIC ACID

It was shown by Woods (1940) that very small quantities of *p*-aminobenzoic acid inhibit the antibacterial activity of sulfamilamide for streptococci in ordinary culture media and for *Escherichia coli* in a medium of known chemical composition. It was reported by Muir, Shamleffer, and Jones (1941a) and Weis and Jones (1941) that *p*-aminobenzoic acid exercises a blocking effect against sulfathiazole and this has been confirmed since by Landy and Wyeno (1941), Strauss, Lowell and Finland (1941) and in a recent report by Spink and Jermsta (1941) who employed the synthetic medium of Gladstone for propagating the

TABLE 3

BACTERIOSTASIS BY SULFATHIAZOLE IN SYNTHETIC MEDIUM			INHIBITION OF SULFATHIAZOLE-BACTERIOSTASIS BY p-AMINOBENZOIC ACID IN SYNTHETIC MEDIUM (MOLAR CONCENTRATION OF p-AMINOBENZOIC ACID)							
Sulfathiazole	Hours	Control (medium + drug)	0.000,004	0.000,002	0.000,001	0.000,000,5	0.000,000,25	0.000,000,125	0.000,000,062	0.000,000,031
0.0002M (5mg%)	48	−	+	−	−	−	−	−	−	−
	72	−	+++	−	−	−	−	−	−	−
	120	−	++++	+	−	−	−	−	−	−
0.0001M (2.5mg%)	48	−	++	+	−	−	−	−	−	−
	72	−	++++	+++	+	−	−	−	−	−
	120	−	++++	++++	++	−	−	−	−	−
0.00005M (1.25mg%)	48	−	++	++	+	−	−	−	−	−
	72	−	++++	++++	+++	+	−	−	−	−
	120	−	++++	++++	++++	+++	−	−	−	−
0.000025M (0.625mg%)	48	−	++	++	++	+	−	−	−	−
	72	−	++++	++++	++++	+++	+++	+	−	−
	120	−	++++	++++	++++	++++	++++	+	−	−
0.0000125M (0.312mg%)	48	−	++	++	++	++	+	+	−	−
	72	−	++++	++++	++++	++++	+++	+++	++	−
	120	−	++++	++++	++++	++++	++++	++++	++++	−
Control without drug	48	++	++	++	++	++	++	++	++	++
	72	++++	++++	++++	++++	++++	++++	++++	++++	++++
	120	++++	++++	++++	++++	++++	++++	++++	++++	++++

Inoculum: 100 *S. enteritidis* cells (washed) per tube.

test organism, staphylococcus. In table 3 are shown the effect of sulfathiazole upon cultures of *S. enteritidis* in synthetic medium and the blocking of the inhibitory activity of the drug by small amounts of *p*-aminobenzoic acid.[6] It appears that the molar quantity of *p*-aminobenzoic acid required to neutralize the antibacterial effect of sulfathiazole is approximately one one-hundredth of the amount of sulfathiazole involved, when tested under these standarized conditions. This ratio is clearly manifested at each of the three higher concentrations of sulfathiazole; and while it decreases to 1 to 200 in the lower con-

[6] The *p*-aminobenzoic acid used was purchased from the Research Laboratory of the Eastman Kodak Company and was warranted only in regard to identity and quality.

centrations, we consider that this decline may be a reflection of experimental error involved in the extensive dilution of both the drug and the inhibitor and particularly, since in other experiments (not herein reported), the ratio of 1 to 100 has been repeatedly observed. This quantitative relationship differs somewhat from that observed by Woods, who also made use of an entirely synthetic basal medium in his experiments with *E. coli* and sulfanilamide. He found a greater activity on the part of *p*-aminobenzoic acid than our data seem to indicate. In this connection, however, the results reported by Strauss, Lowell, and Finland (1941) likewise indicate a lesser degree of effectiveness of *p*-aminobenzoic acid when tested against sulfathiazole than that reported by Woods. In connection with the possible growth-stimulating effect of *p*-aminobenzoic acid upon *S. enteritidis* in our synthetic medium, it will be noted (table 3) that in the quantities used (3.1×10^{-8} to 4×10^{-6} M) *p*-aminobenzoic acid appears to be devoid of any such properties.

INHIBITION OF SULFATHIAZOLE BY COMPOUNDS RELATED TO PARA-AMINOBENZOIC ACID

Woods (1940) reported upon the anti-sulfanilamide activity of a number of substances related to *p*-aminobenzoic acid. Studies in this general field have been reported more recently by Landy and Wyeno (1941), Boroff, Cooper, and Bullowa (1941), and Keltch *et al.* (1941). In the present investigation, we have studied the anti-sulfathiazole activity of a number of substances having some degree of chemical similarity to *p*-aminobenzoic acid. The results are given in table 4 wherein it will be noted that of the various substances tested, novocaine, *p*-nitrobenzoic acid, sulfanilic acid and acetyl *p*-aminobenzoic acid exert a distinct inhibitory effect upon the antibacterial activity of sulfathiazole. In addition to the substances listed in table 4, we have attempted to study the anti-sulfathiazole activity of hydroquinone (a substance possessing neither an amino nor a carboxylic group) and have found it to possess rather remarkable antibacterial activity, so extensive, in fact, that the possibility of testing its anti-sulfathiazole activity was precluded. A further investigation revealed that its antibacterial activity even exceeds that of sulfathiazole when the two compounds are quantitatively compared on a molar basis and that in contrast to sulfathiazole this substance may, under equivalent conditions, manifest a bactericidal effect. This antibacterial effect of hydroquinone is not neutralized to any significant extent by *p*-aminobenzoic acid.

PROTECTIVE EFFECTS OF THE THIAZOLE COMPOUNDS AND PARA-NITROBENZOIC ACID ON EXPERIMENTAL INFECTIONS IN MICE

The protective effect of sulfamethylthiazole against *S. enteritidis* infection in mice was reported in a previous publication of Muir, Shamleffer, and Jones (1940). In those experiments, sulfamethylthiazole was administered *per os* on a dosage basis of two grams per kilogram of body weight, two such doses each day. Two hours after the initial medication, the animals were inoculated intraperitoneally with approximately 100 million cells of the same strain of

S. enteritidis as was employed in the foregoing cultural experiments. In four consecutive experiments wherein control groups (i.e., non-medicated animals) exhibited average survival times of 14, 14, 15 and 16 hours, the medicated groups on the other hand, survived an average of 92, 96, 96 and 89 hours respectively. While the protective effect was definite, it was manifested only by an increase in average survival time since all of the medicated animals eventually succumbed to the infection.

TABLE 4

The anti-sulfathiazole activity of para-aminobenzoic acid and related compounds as demonstrated in synthetic medium

SUBSTANCE TESTED	ACTIVE AT M. CONC.*
Concentration of sulfathiazole = 2.5×10^{-5} M.	
p-Aminobenzoic acid	$1.25 - 5.00 \times 10^{-7}$
b-Diethylaminoethyl *p*-aminobenzoate HCl (novocaine)	$8.00 - 16.00 \times 10^{-6}$
p-Nitrobenzoic acid	3.1×10^{-5}
Acetyl *p*-aminobenzoic acid	1.25×10^{-4}
Sulfanilic acid	$2.50 - 5.00 \times 10^{-3}$
The following compounds displayed no activity in the amounts indicated;* larger amounts interfered directly with growth:	
Acetyl salicylic acid	1.6×10^{-4}
Aniline	1.0×10^{-2}
Benzidine	5.0×10^{-4}
Benzoic acid	2.5×10^{-3}
Butesin	1.8×10^{-6}
Ethyl *p*-aminobenzoic acid	1.0×10^{-3}
p-Amino phenol	1.0×10^{-6}
p-Amino sodium cinnamate†	5.0×10^{-4}
p-Phenylenediamine HCl	3.7×10^{-5}
Dimethyl *p*-phenylenediamine HCl	9.5×10^{-6}
Tetramethyl *p*-phenylenediamine HCl	9.5×10^{-6}
Phenol	5.0×10^{-3}

* The molarities recorded are the lowest ones at which there was some degree of interference with sulfathiazole activity at the time when control cultures reached maximal growth.

† Synthesized and kindly supplied by the Department of Chemistry of Saint Louis University.

In order to extend the scope of our experimental observations regarding the effectiveness of the thiazole compounds in protecting mice, more recently, experiments have been made wherein a mouse-virulent organism[7] provisionally classified as belonging to the genus *Klebsiella*, was used as the infective agent and sulfathiazole, sulfamethylthiazole and *p*-nitrobenzoic acid[8] were used as protective agents.

[7] In most of its cultural characteristics, this organism resembled *Klebsiella ozaenae* as described in Bergey's Manual of Determinative Bacteriology, 5th ed.

[8] Kindly supplied by the Department of Medical Research of the Winthrop Chemical Co.

In table 5, are listed the results of one of these experiments, wherein all medicated animals received the indicated drug in the manner and dosage recorded for a period of five days, following which the animals were kept under observation for an additional five days before terminating the experiment. Those animals which died during the course of the experiment were autopsied and cultures made from the heart blood invariably revealed the presence of the organism. On the other hand, in mice surviving the 10-day period, the

TABLE 5

Protective effect of sulfathiazole, sulfamethylthiazole and p-nitrobenzoic acid in mice infected intraperitoneally with 1600 organisms of a Klebsiella sp.

SURVIVAL TIME (HRS.) AFTER INOCULATION			
Controls, no medication	Sulfathiazole, medicated	Sulfamethylthiazole, medicated	p-Nitrobenzoic acid, medicated
40	87	**158**	29
40	**158**	**182**	29
40	**158**	**189**	33
40	**158**	**197**	35
40	**168**	**197**	40
48	Survived*	**197**	40
48	Survived—10 days*	**220**	40
51	Survived—10 days*	**220**	40
51	Survived—10 days*	**231**	40
63	Survived—10 days*	Survived—10 days*	40
63	Survived—10 days*	Survived—10 days*	40
63	Survived—10 days*	Survived—10 days*	48
74	Survived—10 days*	Survived—10 days*	51
76	Survived—10 days*	Survived—10 days*	57
87	Survived—10 days*	Survived—10 days*	63
Av. 54.9 hours			Av. 41.6 hours

* At necropsy, 10 days after the start of the experiment, cultures from heart blood and from spleen were negative.

Medication with sulfathiazole and sulfamethylthiazole involved *per os* administration of 2 grams per kg., per dose, two doses per day; p-nitrobenzoic acid 0.3 grams per kg., per dose, two doses per day.

Survival times appearing in bold-faced type represent those deaths occurring after completion of the 5 day medication period.

cultures from heart blood and spleen without exception failed to reveal the presence of the *Klebsiella* organism. In the control group of 15 animals (receiving no medication), none survived and the deaths occurred between the 40th and 87th hour post inoculation, with an average survival time of 54.9 hours. In a similar group of animals medicated with sulfathiazole, however, only 5 animals succumbed to the infection during the experiment and of these only 1 died during the 5-day medication period (87 hours); whereas the other 4 died during the succeeding 5-day observation period (158 to 168 hours). The re-

maining 10 animals were sacrificed at the end of the experiment, at which time cultures from the heart blood and spleen were negative. This affords some indication of the ability of the medicated animals to become free of the infective agent. It also becomes apparent that sulfathiazole is a remarkably effective agent in combating this type of experimental infection in the mouse. Sulfamethylthiazole likewise exhibited a considerable protective effect in the present experiment since only 9 of the 15 animals succumbed to the infection and the first of these deaths did not occur until the 158th hour or after the end of the medication program. Again, necropsy cultures from the six surviving animals were negative. It was deemed of interest to investigate similarly any possible protective effect that *p*-nitrobenzoic acid might display under these experimental conditions. Para-nitrobenzoic acid is a compound which, broadly speaking, is chemically related to certain of the sulfonamide drugs and to *p*-aminobenzoic acid. In this experiment, we were mindful of the possibility of significant amounts of *p*-nitrobenzoic acid being transformed into *p*-aminobenzoic acid during body metabolism, a circumstance which might serve to lessen the protective effect of the former compound either by diminution of the amount of *p*-nitrobenzoic acid or by the possibility that *p*-aminobenzoic acid might counteract the effectiveness of the nitro compound. In our experiment, all of the 15 animals treated with *p*-nitrobenzoic acid in the manner indicated in table 5 died between 29th and 63rd hour post-inoculation, with an average survival time of 41.6 hours. These results indicate a lack of effectiveness of this compound in protecting animals at the dosage level and under the experimental conditions employed.

Since it has been established (Muir, Shamleffer and Jones 1941a) that *p*-aminobenzoic acid exerts a pronounced inhibitory effect upon the antibacterial activity of sulfathiazole *in vitro*, an attempt was made to ascertain whether a similar effect might be observed *in vivo*. Accordingly, an experiment was undertaken in which 30 mice were injected intraperitoneally with 30,000 of the *Klebsiella* organisms. Ten of these animals served as controls and received no subsequent treatment; 10 were medicated with sulfathiazole in the manner previously described and the remaining 10 received the same amount of sulfathiazole plus simultaneous *per os* administration of 0.2 gram per kg., per dose of *p*-aminobenzoic acid, a quantity well below that postulated by Selbie (1941) as being able *per se* to enhance infectivity in the experimental animal. All of the animals eventually succumbed to the infection induced by the relatively large inoculum employed. The average survival time of the control group was 29.5 hours; whereas animals receiving sulfathiazole alone survived 127.7 hours. On the other hand, those animals in the test group receiving both sulfathiazole and *p*-aminobenzoic acid survived for an average time of only 80.4 hours. Thus, it becomes evident that *p*-aminobenzoic acid inhibits the protective action of sulfathiazole in the animal body.

DISCUSSION

In our experience, no appreciable difference in the antibacterial activities of sulfathiazole and sulfamethylthiazole has been encountered when these com-

pounds are compared in culture experiments, using *S. enteritidis* as the test organism, and when the relative inhibiting qualities of the two compounds are carefully investigated by plotting viable population counts. Likewise in animal protection experiments wherein a *Klebsiella sp.* was the test organism, such differences as were observed were considered to be rather insignificant. Thus, it appears that methylation of the sulfathiazole compound does not alter the effectiveness of the drug when tested under these *in vitro* and *in vivo* conditions.

A definite and well controlled demonstration of the superiority of synthetic media over ordinary peptone-containing media in the determination of the antibacterial activity of the sulfonamide drugs is afforded by our experimental results. This is strengthened by the observation that the addition of 0.005 per cent peptone interferes significantly with drug activity (table 3). An analysis of the growth curves also substantiates the superiority of synthetic medium since 99.9 per cent suppression of growth is accomplished by 0.6 mg. per cent of the drug, as compared with only 75 per cent suppression brought about by 5 mg. per cent in ordinary broth. We are mindful that the superiority of synthetic media may rest upon one or the other or both of two possible bases. First, it is clearly recognized that the synthetic medium is devoid of such substances as we have shown (table 2) to be capable of interfering with the action of sulfathiazole. Obviously, the presence of such substances in a medium would serve to obscure the complete potential manifestation of the drug's activity and thus lead to error in the evaluation of its effectiveness. Second, we recogize the fact that a synthetic medium does not permit as rapid and as extensive growth as does a nutrient broth containing peptone or meat extract or both.

In a detailed consideration of the events during the early hours of growth in synthetic medium, with and without sulfathiazole, (fig. 2), it should be pointed out that cell proliferation is not significantly impeded by either concentration of the drug during the time interval up to the 8th hour. While it is difficult to offer an explanation for this, it seems probable that the bacterial cells in the inoculum, despite their washing, paucity, and dilution may carry with them certain materials derived from the original culture medium, or therein synthetisized, which permit multiplication to occur in presence of the drug. Such adsorbed substances might serve to (1) inhibit the drug until, through cell multiplication, they become progressively reduced below the limit of their effectiveness and (2) supply the cell with a required substrate enabling it to multiply through the successive generations which we have demonstrated above as occurring between the second and eighth hours. Subsequent to this time, these substances may be present in only sub-minimal amounts, so that regardless of their exact mode of action, circumstances become favorable for the manifestation of drug activity. An observation which favors the contention that such adsorbed substances serve to affect cell proliferation favorably (rather than to specifically inhibit drug activity) is afforded by the fact that little or no difference in antibacterial activity is seen between cultures containing 0.6 mg. per cent of sulfathiazole and those containing 4 times this amount (i.e., 2.4 mg. per cent). Alternate explanations for the delay in thiazole activity seen during the 2nd to the 8th hour, might be (1) the necessity for activation of the

drug regarded by some investigators as being essential, or (2) that the period of delay is a time interval during which combination of the drug with the organism, as suggested by Neter (1941), or with an essential metabolite, as suggested by Fildes (1940), is effected. In a meat-extract peptone broth, on the other hand, the initial lag phase is very brief, occupying only about one hour as is shown in figure 1. This is followed by a short period during which multiplication seems to proceed logarithmically and at an equal rate in the control and drug-containing cultures. Subsequently, a sharp divergence between the control and test curves is seen and the ensuing periods of logarithmic growth in the sulfathiazole cultures differ sharply from that seen in the control culture since they occupy a longer time interval and fail to reach as high a level, and, consequently, the slopes of the curves are less steep. These features are regarded as manifestations of drug activity. A comparison of the calculated generation times in control, 5 mg. per cent and 10 mg. per cent sulfathiazole cultures (17:90:120 minutes) seems to indicate that the drug acts in such a way as ultimately to retard the generative cycle. This retardation is not quantitatively related to the amount of sulfathiazole present, an apparently anomalous circumstance for which an adequate explanation is not available at the moment. This period of logarithmic increase in cultures of drug-containing peptone broth[9] is in sharp contrast to the prolonged period of stasis observed in the corresponding cultures in synthetic medium.

Having at hand a synthetic medium suitable for the growth of this organism has enabled us to carry forward under controllable and reproducible conditions quantitative studies pertaining to the antagonistic effects of a variety of substances against sulfathiazole. Among the more complex substances which we have attempted to assay in this manner are some of the more common constituents of culture media. Of these, the peptones (table 2) were found to interfere with the activity of the drug to a greater extent than any of the other substances examined. Aminoids reacted almost as strongly as did the peptones while Casamino Acids showed much less activity. Meat-extracts, agar, beef serum, and glucose showed very little or no anti-sulfathiazole activity. It is of particular interest to note that Aminoids is a biuret-free material, which forces recognition of the fact that the inhibiting material in peptones is not necessarily as complex in character as would be required to elicit the biuret reaction.

The use of a synthetic medium such as that employed in the present study permits of a more exact evaluation of the anti-sulfathiazole role played by *p*-aminobenzoic acid and other substances than is possible with a non-synthetic medium. In a detailed consideration of the data presented in table 4, it is of interest to point out that of the five compounds listed which are active in this respect all possess an amino or a nitro group (*p*-nitrobenzoic acid) in the para position to an intact carboxyl, substituted carboxyl group or in the case of sulfanilic acid, to a sulfo group. It will be noted that the compound (acetyl *p*-aminobenzoic acid) resulting from the acetylation of the amino group in *p*-

[9] The possibility exists of the organism undergoing adaptive variation, however, we have not observed any evidence which might support this view.

aminobenzoic acid is one thousand times less active against sulfathiazole. This is in accordance with the published results of Woods who found the acetyl derivative to be less active than *p*-aminobenzoic acid in anti-sulfanilamide activity, but fails to conform to the findings of Loomis, Hubbard and Neter (1941) who reported that the experimental acetylation of *p*-aminobenzoic acid destroys its anti-sulfanilamide activity. Substitution within the carboxyl group, as in novocaine, leads to a ten-fold decrease in anti-sulfathiazole activity. A conspicuous feature of *p*-nitrobenzoic acid, which also possesses anti-sulfathiazole activity, is the replacement of the amino by the nitro group. Replacement of the carboxyl group by the sulfo radical (SO_3H), as in sulfanilic acid, leads to a ten-thousand-fold decrease in anti-sulfathiazole activity. On the other hand, some of the inactive compounds studied, conforming to the above in the salient features of their chemical configuration (e.g., ethyl *p*-aminobenzoate and butyl *p*-aminobenzoate), failed to display any anti-sulfathiazole activity in concentrations less than those which directly interfered with growth. The remarkable inhibitory effect of hydroquinone on the organisms prevented the testing of this substance.

Our *in vivo* experiments involving a study of the thiazole drugs (table 5) indicate that a high degree of protection against the *Klebsiella* organism is afforded by these compounds. Para-nitrobenzoic acid on the other hand, failed to show any protective qualities when similarly tested. This finding regarding ineffectiveness of *p*-nitrobenzoic acid against this organism is of interest since Mayer and Oechslin (1939) found it to be protective against pneumococci in mice and Gruzhit (1940) found the drug to protect mice against *Streptococcus viridans*.

The anti-sulfanilamide activity of *p*-aminobenzoic acid *in vivo* was originally reported by Selbie (1940), and McCarty (1941) has shown its anti-sulfapyridine activity in penumococcus-infected mice. The ability of *p*-aminobenzoic acid to inhibit the protective action of sulfathiazole is demonstrated in our experiments wherein its use reduced the average survival time of medicated mice from 127.7 hours to 80.4 hours.

SUMMARY

Sulfathiazole and sulfamethylthiazole display practically identical bacteriostatic activities, *in vivo* and *in vitro*, under the conditions obtaining in these experiments. The antibacterial activity of the thiazole drugs is significantly greater in the synthetic medium herein employed than in an ordinary nutrient broth.

Viable cell counts made from cultures in synthetic media containing sulfathiazole reveal an initial lag phase in drug activity which is followed by a prolonged period of bacteriostasis. Similar cell counts made from nutrient broth cultures likewise exhibit an initial lag phase in drug action followed by a period of increase, logarithmic in character but reduced in tempo.

Para-aminobenzoic acid and a few closely related compounds as well as a variety of bacteriological peptones and other commonly used ingredients of

ordinary culture media were found to possess anti-sulfathiazole activity when tested under standardized conditions.

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