10818

Anticatalase Activity of Sulfanilamide and Related Compounds. IV. Peroxide Accumulation and Growth Inhibition in Pneumococcus.

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Sulfanilamide and structurally related compounds which have a free amino group in the position para to the sulfonamido group have been shown to have anticatalase activity.[1] The bacteriostatic activity of the compounds appears to depend upon this property.[2, 3, 4] An anticatalase, added to cultures of bacteria which form hydrogen peroxide as a metabolic product, interferes with its destruction, normally brought about by catalase. As a result, the concentration of peroxide around the organism may become sufficiently high to retard further growth. That sulfanilamide acts only indirectly as a growth-retarding agent by permitting the accumulation of hydrogen peroxide has been demonstrated for the Type I pneumococcus.[5] In the presence of sulfanilamide in concentrations of 8 to 10 mg % the amount of peroxide accumulated was such that its concentration per unit of growth was several times greater than in controls not containing sulfanilamide. Further evidence that hydrogen peroxide is the actual growth-checking agent was shown by the fact that sulfanilamide is unable to check growth of pneumococci in the absence of oxygen essential to the production of hydrogen peroxide.[6]

The development of a quantitative method[7] for determination of hydrogen peroxide in bacterial cultures has made it possible to show a quantitative relation between peroxide concentration and growth. Table I summarizes results of 4 typical experiments in which the peroxide concentration and amount of growth in broth cultures of the pneumococcus were determined at hourly intervals. The cultural conditions were the same as described before.[6] Growth was estimated turbidimetrically with $BaSO_4$ standards. The percentages

1 Main, E. R., Shinn, L. E., and Mellon, R. R., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 272.

2 Locke, A., Main, E. R., and Mellon, R. R., *Science*, 1938, **88**, 620.

3 Locke, A., Main, E. R., and Mellon, R. R., *J. Immunol.*, 1939, **36**, 183.

4 Mellon, R. R., *Modern Hospital*, 1938, **51**, 53.

5 Shinn, L. E., Main, E. R., and Mellon, R. R., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 591.

6 Shinn, L. E., Main, E. R., and Mellon, R. R., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 640.

7 Main, E. R., and Shinn, L. E., *J. Biol. Chem.*, 1939, **128**, 417.

TABLE I.

Effect of Sulfanilamide on Growth and Hydrogen Peroxide Accumulation in Broth Cultures of Type I Pneumococcus.

Ten cc quantities of veal infusion broth containing 0.2% glucose were inoculated with 0.15 cc of 12-16-hour broth cultures. One cc samples were withdrawn at the intervals indicated, for estimations of growth and hydrogen peroxide content.

Exp. No.	5 hr			6 hr			7 hr			8 hr		
	G*	H_2O_2‖	A†	G	H_2O_2	A	G	H_2O_2	A	G	H_2O_2	A
a. C‡	18	10	0.6	26	15	0.6	70	20	0.3	70	21	0.3
a. S§	7	9	1.3	12	14	1.1	16	12	.8	21	23	1.1
b. C	15	11	.7	41	15	.4	80	21	.3	80	25	.3
b. S	7	<6	—	7	11	1.6	13	16	1.2	17	19	1.1
c. C	40	12	.3	70	18	.3	80	24	.3	80	26	.3
c. S	10	12	1.2	21	17	.8	21	22	1.0	21	34	1.6

*G = Growth. Growth was estimated by comparisons with $BaSO_4$ standards which had been calibrated against dilutions of a pneumococcus culture arbitrarily selected to represent 100% growth. The figures given are therefore growth units or percentages relative to the standard culture.

†A = Ratio, H_2O_2 concentration per unit of growth.

‡C = Control cultures containing no sulfanilamide.

§S = Cultures containing 10 mg% sulfanilamide added before inoculation as a 0.5% sterile solution in water.

‖H_2O_2 concentration in micrograms per cc.

reported in the table are in terms of a fully grown culture taken to represent 100% growth. Determinations of turbidities were made only after 5 hours' growth since the degree of turbidity developed before the fifth hour was too small to be accurately estimated.

For the estimation of hydrogen peroxide, 1 cc samples were withdrawn. To each was added 6 drops of a potato extract* and 2 drops of a 10% solution of *o*-tolidine in glacial acetic acid. The colors developed were compared with dilutions of a standard solution of malachite green oxalate and the peroxide concentrations were obtained by reference to a calibration curve made by plotting the dye concentrations against concentrations of peroxide which gave colors of equal intensity.

The amount of growth retardation is shown by a comparison of the growth, G, in the control cultures, C, with that in cultures, S, containing sulfanilamide. During the period of observation the growth was from 2 to 6 times greater in the controls than in the presence of sulfanilamide. Variations in the degree of stasis from experiment to experiment are to be expected, since the static action is readily affected by slight changes in the composition of the broth, temperature of incubation, pH, and size and age of the inoculum. The ratio,

* Made by extracting 120 g potato with a solution composed of equal volumes of glycerol and a pH 7.0 phosphate buffer.

TABLE II.

Growth Inhibition and Hydrogen Peroxide Accumulation in Broth Cultures of Type I Pneumococcus Containing Sulfanilamide and Related Compounds.

Ten cc quantities of veal infusion broth containing 0.2% glucose were inoculated with 0.15 cc of 12-16-hour cultures. Samples withdrawn at intervals of 1 hr were tested for growth and hydrogen peroxide content.

Compound added	H_2O_2 concentration per unit of growth at 6 hr	7 hr	8 hr	Relative therapeutic effectiveness*
None	0.4	0.3	0.2	
Sulfanilamide	(1.8)	1.7	2.0	+
4, 4'-Diaminobenzenesulfonanilide†	(4.6)	1.5	2.0	+
2-(*p*-Aminobenzenesulfonamido)-pyridine‡	2.0	1.3	2.0	+
p-Acetylaminobenzenesulfonamide†	.6	.2	.2	0
None	0.6	0.3	0.3	
Sulfanilamide	1.1	.8	1.1	+
2-(*p*-Acetylaminobenzenesulfonamido)-6-aminopyridine§	1.5	1.5	1.6	+
4-Aminobenzenesulfonyl-4'-hydroxyanilide†	1.0	1.4	2.2	+
Sodium sulfanilyl sulfanilate‖	1.5	1.2	2.2	
N-(*p*-aminobenzenesulfonyl)-piperazine†	.6	.3	.3	toxic
N-(*p*-aminobenzenesulfonyl)-salicylamide†	2.2	2.8	2.7	+
None	0.5	0.3	0.3	
Sulfanilamide	1.2	.8	.7	+
p-Aminobenzamide	.5	.2	.3	0
p-Nitrobenzamide	1.8	1.4	.7	toxic
2-(*p*-Aminobenzenesulfonamido)-thiazole§	1.0	1.1	1.0	+
2-(*p*-Aminobenzenesulfonamido)-4-methylthiazole§	.8	1.1	1.1	+

*Therapeutic assays of these compounds will be reported in detail from this laboratory at a later date.

These compounds were furnished through the courtesy of Monsanto Chemical Co. (†); Merck and Co. (‡); Maltbie Chemical Co. (§), and Calco Chemical Co. (‖).

A, peroxide concentration per unit of growth, is in every instance higher in the presence of sulfanilamide than in its absence, and in the experiments cited was from 2 to 5 times greater.

The study of bacteriostatic action was next extended to include some structurally related compounds, both active and inactive. The results are summarized in Table II. For simplification, only the ratios, peroxide concentration per unit of growth, are given. Two types of control were used for each group of compounds, the one a culture containing sulfanilamide and the other a culture containing no added compound. Values in parenthesis are doubtful because of the slight degree of turbidity at the time of observation.

In the first group of compounds, 4,4'-diaminobenzenesulfonanilide and 2-(*p*-aminobenzenesulfonamido)-pyridine (sulfapyridine) showed ratios equivalent to those of sulfanilamide. *p*-Acetylaminobenzenesulfonamide, which is recognized as therapeutically inactive, caused no bacteriostasis and no increase in peroxide accumulation.

N-(*p*-aminobenzenesulfonyl)-piperazine, in the third group of compounds, showed no bacteriostatic activity and gave the same ratio as the control containing no added compound. The other compounds of this group may be considered to be about equivalent to sulfanilamide. While some of the ratios seem to be somewhat higher than those of sulfanilamide, the difference is probably of no significance because of the rather wide variations resulting from slight differences in experimental conditions.

The bacteriostatic activity of the thiazole compounds in the third group appeared to be about equivalent to that of sulfanilamide. *p*-Aminobenzamide, the carboxylic acid derivative corresponding to sulfanilamide showed no bacteriostatic activity. *p*-Nitrobenzamide showed marked activity.

Compounds which have been found to have therapeutic activity in pneumococcus or streptococcus infection in mice are indicated by +, in the last column of the table. Of the compounds tested so far in this laboratory, all of those which had bacteriostatic action, associated with increased accumulation of peroxide, were therapeutically active. Two compounds were too toxic in therapeutic dosage to be used in animal experiments. While it may be concluded that compounds with bacteriostatic activity have therapeutic effect, no prediction as to degree of efficacy in animal infection can be made, since many other factors must be considered in evaluating their usefulness as therapeutic agents.

The results demonstrate that there is increased accumulation of hydrogen peroxide in cultures containing compounds with anticatalase activity and that this accumulation of peroxide is associated with retardation of growth. Measurements of peroxide content is limited to that which has diffused into the medium from the immediate environment of the organism, where it may be present in concentrations many times greater than in the medium.

Summary. The effect of sulfanilamide, and other compounds capable of inhibiting catalase, upon growth and accumulation of hydrogen peroxide in broth cultures of the pneumococcus has been subjected to quantitative measurement. The concentration of peroxide per unit of growth was from 2 to 10 times greater in the presence of compounds which caused bacteriostasis than in control cultures.