

SULFANILAMIDE BACTERIOSTASIS IN THE PRESENCE OF MERCURIC CHLORIDE AND p-AMINOBENZOIC ACID

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The fact that urethane, a structurally unrelated compound, can inhibit sulfanilamide action has caused Johnson (1942) to question the competitive metabolite hypothesis developed by McIntosh and Whitby (1939), Stamp (1939), Fildes (1940), Green (1940), Woods (1940) and Woods and Fildes (1940). He would explain the antagonism of p-aminobenzoic acid on the basis of the well-known fact that narcotics in low concentration may be stimulative. Johnson has demonstrated stimulation and inhibition by p-aminobenzoic acid on the luminescence and growth of the luminous bacteria. Sulfanilamide itself is capable of stimulating bacterial growth (Finklestone-Sayliss *et al.*, 1937; Johnson, 1942; Green *et al.*, 1942; Lamanna, 1942) and in this property is related to narcotics in general. McIlwain (1942) has reported that the antagonism of p-aminobenzoic acid and urethane is not comparable in all respects and that the competition with essential metabolite hypothesis remains, therefore, fundamentally unchallenged.

The present study presents relevant data describing the influence of p-aminobenzoic acid on the growth-stimulating concentration of sulfanilamide, and the mutual antagonism of mercuric chloride and sulfanilamide. A strain of *Escherichia coli* was used which gave abundant growth on the synthetic medium employed. The medium was copied after McLeod (1940).

I

In the first series of experiments a modified agar-cup-plate wet-filter-paper technique was utilized with the purpose of introducing into a petri plate of actively proliferating microorganisms test materials at such distances apart that their zones of inhibition and stimulation, when present, would coincide. Any additive or antagonistic effect could be determined by contrasting the growth with plates where each narcotic material was employed alone. The correct distances to place the narcotics from one another were determined by trial and error. The general method follows.

Large sterile petri dishes (15 cm. in diameter) were marked for placing the test materials. Sulfanilamide was added as a 0.325 gram tablet and placed in the dishes before adding the synthetic agar medium. The agar medium was inoculated with 0.5 ml. of a 24-hour-old broth culture grown at 37°C., and

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poured in 100 ml. quantities into each plate. When the plates solidified, two superimposed discs (6 mm. diameter) of No. 2 Whatman filter paper were placed on the agar surface in designated areas and 0.01 ml. of the sterile test narcotic solution was pipetted upon the discs. Bichloride of mercury was employed as an aqueous solution saturated at room temperature. The p-aminobenzoic acid used was a one percent aqueous solution pipetted immediately after taking it from a steam bath where it was placed in order to get all of the crystals into solution. In some experiments a 10 mg. percent solution of indole-3-acetic acid was employed. But this latter compound had no demonstrable effect under the conditions of the experiment. The plates were poured in duplicate and observed from five to six days.

The results consistently observed with this type of experiment are illustrated in the four accompanying photographs. Photo A illustrates the growth obtained in the presence of sulfanilamide alone. The sulfanilamide tablet is surrounded by a zone of no growth which is bounded by a ring of heavy growth, in turn surrounded by the lighter normal growth. Photo B pictures a similar situation with filter paper wet with mercuric chloride. In this case the zone of stimulation is seen to be of lesser width but of greater intensity than with sulfanilamide. In plate C, starting at the top of the plate and reading clockwise, are located a tablet of sulfanilamide, filter paper wet with p-aminobenzoic acid, mercuric chloride, and indole-3-acetic acid. Both the sulfanilamide and bichloride of mercury are surrounded by a zone of inhibited growth and a zone of stimulated growth. The indole-3-acetic acid exhibits no influence on the growth. The p-aminobenzoic acid does not influence the mercuric chloride but quite definitely shows an inhibition of sulfanilamide bacteriostasis. On the side of the sulfanilamide tablet facing the p-aminobenzoic acid the zone of inhibited growth is reduced in diameter. The growth surrounding the p-aminobenzoic acid is neither greater nor less than in its absence. Consequently the anti-sulfanilamide action is not dependent on growth stimulation. Of greater interest is the fact that on the side next to the p-aminobenzoic acid the zone of stimulated growth caused by a low concentration of sulfanilamide is pushed closer to the sulfanilamide tablet. This effect of p-aminobenzoic acid on the sulfanilamide zone of stimulation is explained readily and simply by the competition hypothesis. At relatively high concentrations of p-aminobenzoic acid and low concentration of sulfanilamide the p-amino benzoic acid molecules present in sufficient number compete favorably and thus antagonize the sulfanilamide bacteriostasis. In these plates the concentration of sulfanilamide increases as the p-aminobenzoic acid concentration decreases. At the edge of the inhibiting zone of sulfanilamide an area exists in which sulfanilamide molecules are present in sufficient numbers to compete with the p-aminobenzoic acid, and in excess in the concentration ordinarily capable of causing stimulation.

Plate D illustrates the conditions that resulted when the tablet of sulfanilamide (on the reader's left side) was placed 3.10 cm. from filter paper discs wet with mercuric chloride. Each narcotic is surrounded by a zone of inhibited and stimulated growth. Where the zones of stimulation should coincide, rather than

the growth appearing greatest, which would result from an additive effect, there is actually no growth. The horse-shoe-shaped inhibitive zone of mercuric chloride extends into the sulfanilamide zone of no growth. Thus, stimulating concentrations of sulfanilamide and mercuric chloride added together yield a toxic effect. Of significance is the observation that the growth surrounding the mercuric chloride is closed in the *sulfanilamide toxic zone* by a line of slight growth indented toward the sulfanilamide. This is indicative that a concentration of mercuric chloride less than stimulating, a concentration that does not seemingly effect normal growth, has the capacity to neutralize the toxicity of a certain harmful concentration of sulfanilamide.

Of other organisms that would grow in the synthetic medium, *Aerobacter aerogenes* gave the same results as *E. coli*. *Pseudomonas aeruginosa* gave the same results except that the toxic coinciding stimulation zones were not closed by a line of growth. *Proteus vulgaris* was like *P. aeruginosa* in this respect.

II

The second series of experiments had as their purpose the determination of the exact concentrations of sulfanilamide antagonized by mercuric chloride, and the revelation of any antagonism that sulfanilamide might have for mercuric chloride, an effect not observed with the growth on agar plates. Total growth of *Escherichia coli* in the presence of various concentrations of sulfanilamide and mercuric chloride was compared with growth in mixtures of various combinations of these same concentrations of the two toxic agents. The concentrations employed were arrived at by trial and error in a few preliminary experiments.

The mercuric chloride was weighed out and diluted through the medium to the concentrations desired. The necessary amounts of sulfanilamide were weighed out directly. Total growth was determined for the medium alone, and with the addition of 11 different concentrations of mercuric chloride, six of sulfanilamide and the 66 possible combinations of these concentrations. A total of 84 different batches of medium had to be prepared. The media were tubed in five milliliter quantities and autoclaved at 15 pounds pressure for 20 minutes.

Before the start of an experiment all materials were incubated overnight at 37°C. The inoculations were carried out in a room incubator at the same temperature. As inoculum, 0.01 ml. of a 1:100 dilution of a ten-hour broth culture was pipetted into each tube of medium. Diluent for the inoculum was a modified Ringer-Locke's solution. A plate count using nutrient agar was made of the ten-hour-inoculum culture. The data to be reported were collected in four experiments in which three tubes of each medium were inoculated. Thus, a total of 12 separate counts were taken for each concentration and combination of the drugs. A total of 83,500 organisms were present in the inoculum of the first experiment, 82,000 in the second, 88,000 in the third, and 125,000 in the fourth.

The inoculated tubes were observed for turbidity at hourly intervals for the first five to twelve hours, then every 12 hours to the 48th hour, and finally at 24-hour intervals up to 120 hours. At the end of this time the tubes were re-

moved from the incubator and proper dilutions were made where necessary so that turbidity readings could be made with a Klett-Summerson photoelectric colorimeter using a 660 (23, 30) millimicron filter.

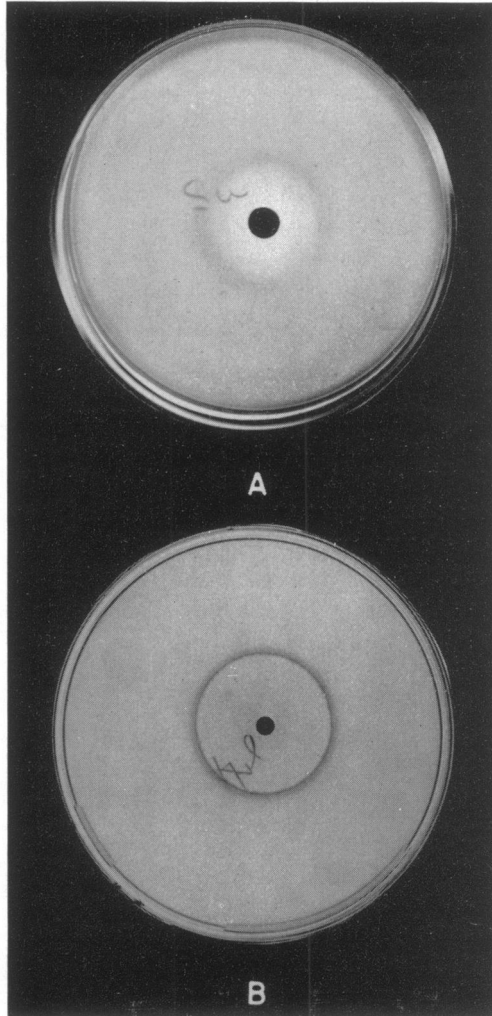


FIG. A. SULFANILAMIDE TABLET SURROUNDED BY ZONES OF INHIBITED, STIMULATED AND NORMAL GROWTH
 FIG. B. FILTER PAPER WET WITH MERCURIC CHLORIDE PLACED IN CENTER OF PLATE

To determine whether the differences in the means of the final number of cells were significant an analysis of variance was run.²

Table 1(a) records the data obtained except for concentrations of 3.7×10^{-5} and 2.9×10^{-5} molar mercuric chloride which invariably gave no growth as

² G. W. Snedecor Statistical methods applied to experiments in agriculture and biology, Ames, Iowa, 1940.

indicated by lack of visible turbidity both in the absence and presence of sulfanilamide. The difference that must exist between the treatment means for statisti-

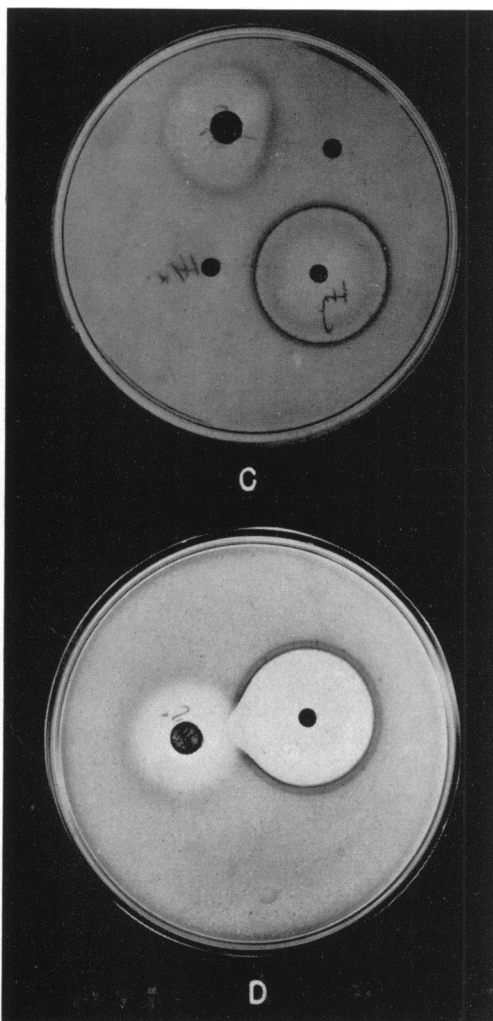


FIG. C. TABLET OF SULFANILAMIDE, FILTER PAPERS WET WITH p-AMINOBENZOIC ACID, MERCURIC CHLORIDE, INDOL-3-ACETIC ACID
(Read from top clockwise)

FIG. D. TABLET OF SULFANILAMIDE (ON LEFT) MERCURIC CHLORIDE (ON RIGHT) PLACED SO THAT ZONES OF STIMULATION COINCIDE

Note break in stimulation zones, indicating toxicity. Note thin line of growth in sulfanilamide toxic zone on side next to mercuric chloride, indicating anti-sulfanilamide activity by mercuric chloride.

cal significance is calculated and recorded in table 1(b). The statistic is known as the minimum significant difference (M.S.D.) and is calculated for both the five and one percent levels.

The concentrations that are significantly antibacterial are: 8.7×10^{-4} , 5.8×10^{-4} M sulfanilamide; 2.2×10^{-5} M mercuric chloride.

TABLE 1

(a)

Average of 12 determinations of total growth after 120 hours expressed as the number of bacteria $\times 10^8$ per ml.

HgCl ₂ MOLAR CONC.	SULFANILAMIDE MOLAR CONCENTRATION							ALL HgCl ₂ AVERAGE
	0	8.7×10^{-4}	5.8×10^{-4}	4.65×10^{-4}	3.5×10^{-4}	2.3×10^{-4}	1.15×10^{-4}	
0	22.583*	6.902	16.196	23.696	24.833	24.392	20.992	19.9419
2.2×10^{-5}	6.225	0†	13.700‡	3.517	14.750‡	17.917‡	6.021	8.8756
1.5×10^{-5}	23.308	0§	4.275§	7.117¶	9.608¶	0¶	16.525	8.6905
7.4×10^{-6}	23.508	7.522	16.092	21.458	21.800	19.458	23.383	19.0318
3.7×10^{-6}	18.883	8.592	21.187	24.400	21.908	21.917	23.742	20.0899
1.8×10^{-6}	22.942	0§	16.467	22.658	24.933	20.058	21.592	18.3786
3.7×10^{-7}	22.900	9.288	16.713	24.000	24.850	24.608	24.092	20.9214
1.8×10^{-7}	23.083	2.612	13.592	22.825	24.250	24.517	23.975	19.2649
3.7×10^{-8}	22.317	8.137	22.767	22.483	23.425	21.842	23.458	20.6327
1.8×10^{-8}	22.758	7.335	17.221	23.933	24.383	24.183	23.683	20.4996
All sulfanila- amide aver- age.....	20.8508	5.0388	15.8208	19.6088	21.4742	19.8892	20.7462	17.63269**

* Normal growth.

† Additive effect of mixture of toxic concentrations of mercuric chloride and sulfanilamide.

‡ Anti-mercuric chloride activity by sulfanilamide.

§ Increase of sulfanilamide toxicity by addition of non-toxic concentrations of mercuric chloride.

¶ Toxicity by combination of non-inhibitory concentrations of sulfanilamide and mercuric chloride.

|| Anti-sulfanilamide activity by mercuric chloride.

** Grand average.

If the populations referred to vary from the [appropriate control by 4.3313×10^8 cells per ml. there is less than one chance in 20 that the difference is due to chance alone. If the difference is 5.6988×10^8 cells per ml. or more the probability is less than one in a 100 that chance alone is responsible.

(b)

$$\text{Minimum significant difference (M.S.D.)} = \left[\sqrt{\frac{\text{error variance} \cdot 2}{\text{no. of variates in one of the means compared}}} \right] \cdot t \text{ value at 5 or 1\% level for the error degrees of freedom}$$

	M.S.D.	
	0.05 level	0.01 level
Sulfanilamide.....	1.3697	1.8021
Mercuric chloride.....	1.6370	2.1538
Sulfanilamide \times HgCl ₂	4.3314	5.6988

The toxicity of 8.7×10^{-4} M sulfanilamide is not antagonized by any of the concentrations of mercuric chloride studied. But the bacteriostatic power of

this concentration is increased by non-toxic 1.5×10^{-5} M and 1.8×10^{-6} M mercuric chloride. The opposite effect, that is, the enhancement of mercuric chloride toxicity by non-toxic concentrations of sulfanilamide was not observed. Unfortunately, whether this can take place is not answerable from our data. For not as wide ranges of non-toxic molar concentrations of sulfanilamide were employed as for the mercuric chloride.

The bacteriostasis due to 5.8×10^{-4} M sulfanilamide is not great and is completely eliminated by two non-toxic and non-stimulatory concentrations of mercuric chloride, 3.7×10^{-6} M and 3.7×10^{-8} M. Intermediate concentrations (1.8×10^{-6} M, 3.7×10^{-7} M, 1.8×10^{-7} M) of mercuric chloride were without effect. Thus, the antagonism of mercuric chloride for sulfanilamide resembles McIlwain's (1942) experience, who found that urethane antagonism was shown only toward low, just-toxic concentrations of sulfanilamide. It differs from the antagonism of p-aminobenzoic acid which acts over a relatively wide and continuous range of concentrations, and which can inhibit extremely toxic concentrations of sulfanilamide.

TABLE 2
Analysis of variance

SOURCE OF VARIATION	SUMS OF SQUARES	DEGREES OF FREEDOM	VARIANCE	CALCULATED F. VALUES	F _{0.05} LEVEL	F _{0.01} LEVEL
Sulfanilamide treatments . . .	24,683.06	6	4113.84	140.96	2.105	2.83
HgCl ₂ treatments	16,903.61	9	1878.18	64.36	1.890	2.43
Interreaction Su \times HgCl ₂ . .	8,986.88	54	166.42	5.70	1.360	1.56
Error	22,471.76	770	29.18			
Totals	73,045.31	839				

The data also reveal that the toxicity of 2.2×10^{-5} M mercuric chloride can be partially neutralized by 5.8×10^{-4} M, 3.5×10^{-4} M, and 2.3×10^{-4} M sulfanilamide. Growth in the mixtures containing the latter two concentrations are not as great as for growth with the sulfanilamide alone. The antagonism does not depend on the employment of stimulatory amounts of sulfanilamide, since antagonism is also expressed by 5.8×10^{-4} M sulfanilamide, a slightly toxic concentration.

Furthermore, the data demonstrate the possibility that two non-toxic concentrations when added together may exert a bacteriostatic effect. The combination of non-inhibitory 1.5×10^{-5} M mercuric chloride individually with non-toxic 4.65×10^{-4} M, 3.5×10^{-4} M, 2.3×10^{-4} M, and 1.15×10^{-4} M sulfanilamide results in bacteriostasis. The same concentration of mercuric chloride greatly enhanced the low toxicity of 5.8×10^{-4} M sulfanilamide.

The time of first appearance of turbidity in tubes could be correlated with the final counts obtained. Mixtures of antagonistic concentrations were characterized by the appearance of turbidity sooner than for the controls. Similarly, toxic concentrations and combinations of the drugs showed first appearance of turbidity later than the control tubes. Thus, the differences in end growth

would seem expressive of a difference in growth rate early in the cultures development, probably soon after coming out of the lag phase.

DISCUSSION

The anti-sulfanilamide action of structurally unrelated compounds such as urethane and mercuric chloride probably does not carry sufficient weight to neutralize the evidences of competition. The antagonism is not quantitatively comparable with what is observed for p-aminobenzoic acid (Wyss, 1941; Wood, 1942). But the existence of antagonism and even synergism by structurally unrelated compounds does mean that competition with an essential metabolite is not an all-inclusive explanation of all the ways in which sulfanilamide may act. Thus, *in vitro* inhibition of *Cypridina* luminescence by sulfanilamide and p-aminobenzoic acid, in which purified extracts of the luciferin-luciferase system are utilized, can hardly be related to p-aminobenzoic acid metabolism (Johnson and Chase, 1942). The authors remark that though luminescence inhibition is non-competitive sulfanilamide bacteriostasis may involve unrelated enzyme systems.

If sulfanilamide exerts growth-inhibiting powers because of interference with the metabolic role of p-aminobenzoic acid a large series of experimentally established facts are explained. But unexplained remain the anti-sulfanilamide effects of structurally dissimilar substances. In the case of the mercuric ion, toxicity is thought to be due to combination with free sulfhydryl groups (Fildes, 1940) which are probably necessary for activity of some enzymes (Hellerman, 1939; Bernheim and Bernheim, 1939). It is inconceivable that sulfanilamide can compete with mercuric ions for free sulfhydryl groups. However, by assuming that each acts on different enzymes and, dependent upon how the equilibria relating these enzymes are affected, one may postulate a variety of end results. For mercuric chloride and sulfanilamide there may exist additive inhibitory and antagonistic combinations as our data show. Nor need both added together in stimulative combinations still be stimulative. Stimulation of two separate enzyme systems simultaneously may so disturb the balance of reaction rates between them that the end result is affected adversely. A wide variety of dissimilar and unrelated compounds would show antagonism. The list for sulfanilamide already includes urethane, mercuric chloride, methionine (Bliss and Long, 1941; Kohn and Harris, 1941), and carbon tetrachloride (Leach and Forbes, 1941).

Sulfanilamide is easily adsorbed (Eyster, 1942). Davis (1942) found that sulfa drugs are "bound" to plasma proteins. Both consider that therapeutic efficacy of the various sulfa drugs may be related to differences in adsorptive capacity.

For isolated enzyme systems not dependent upon the participation of p-aminobenzoic acid, the influence of sulfanilamide would depend on adsorption alone, the exact nature of which Johnson, Brown and Marsland (1942) have begun to reveal. On this basis the inhibition of respiration reported by Sevag and Shelburne (1942) and confirmed by Wyss, Strandskov, and Schmelkes (1942) may be harmonized with the later authors' additional findings that the inhibition

of respiration could not be correlated with the bacteriostatic potency of sulfanilamide and its derivatives.

SUMMARY AND CONCLUSIONS

In the presence of p-aminobenzoic acid the growth-stimulating concentration of sulfanilamide is increased, a result compatible with the competition hypothesis. The anti-sulfanilamide activity of p-aminobenzoic acid is not dependent on its stimulating growth. Under the experimental conditions p-aminobenzoic acid does not influence bacteriostasis caused by mercuric chloride.

Mixtures of various concentrations of sulfanilamide and mercuric chloride reveal a number of effects on growth: 1) Mixtures of both in stimulating concentration are toxic. 2) Sulfanilamide may antagonize mercuric chloride bacteriostasis and the latter, sulfanilamide bacteriostasis. Neither result is dependent upon the growth-stimulating capacity of small quantities of the antagonist. 3) Addition of some non-inhibitory concentrations of mercuric chloride may enhance sulfanilamide toxicity. (4) Increased additive inhibition is observed in certain mixtures of toxic concentrations.

The view is supported that growth inhibition by sulfanilamide is chiefly the result of interference with p-aminobenzoic acid metabolism and secondarily "binding" of sulfanilamide by diverse enzyme systems.

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