

## STUDIES ON THE ANTIBACTERIAL ACTION OF THE SULFONAMIDE DRUGS

### I. THE RELATION OF *p*-AMINO BENZOIC ACID TO THE MECHANISM OF BACTERIOSTASIS\*

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The mechanism whereby sulfanilamide and its derivatives prevent bacterial growth is not known. None of the various theories advanced (1-3) has been conclusively substantiated by direct experimental proof. The most important recent contribution to the problem has been that of Woods (4), who demonstrated that *p*-aminobenzoic acid "blocks" the bacteriostatic effect of sulfanilamide *in vitro*. Following the isolation by Stamp (5) and Green (6) of bacterial extracts which antagonize the action of sulfanilamide, Woods obtained from yeast a potent antisulfanilamide factor which appeared to have chemical properties similar to *p*-aminobenzoic acid but which could not be definitely identified as such. *p*-Aminobenzoic acid itself was found to annul the bacteriostatic effect of sulfanilamide, and Woods predicted that this simple organic acid would eventually be shown to be a metabolite essential for bacterial growth.<sup>1</sup> He also formulated the theory that sulfanilamide causes bacteriostasis by inhibiting specifically the enzymatic reaction involved in the utilization of *p*-aminobenzoic acid and attributed the inhibition of this reaction to the chemical similarity of the drug and the essential metabolite.

Important evidence supporting Woods' hypothesis was published in the same year by Rubbo and Gillespie (8). They reported the chemical isolation from

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<sup>1</sup> To prove a substance essential to bacterial metabolism it is necessary first to demonstrate that it is a growth factor for at least one bacterial species. A growth factor may be defined as an essential metabolite which cannot be synthesized by the bacterial cell and which, therefore, must be added to the culture medium to promote optimal growth. According to views first introduced by Fildes (7), substances known to be bacterial growth factors for one or more species of organism may be considered to be metabolites essential for the growth of many other bacteria and probably of bacteria in general.

yeast of the benzoyl derivative of *p*-aminobenzoic acid and stated in addition that *p*-aminobenzoic acid stimulated the growth of *Clostridium acetobutylicum* in a synthetic medium.<sup>2</sup> Their observations led them to conclude that *p*-aminobenzoic acid is a bacterial growth factor and, therefore, an essential metabolite. They pointed out, however, that in their experiments with *Cl. acetobutylicum* one molecule of *p*-aminobenzoic acid antagonized 23,000 molecules of sulfanilamide. This tremendous disproportion between the antagonistic substances was interpreted as strong evidence against Wood's theory that the drug and the essential metabolite compete for the same receptor site on the organism.

The data reported in the present paper concern the quantitative aspects of the antagonistic action of *p*-aminobenzoic acid and the more commonly used sulfonamide drugs. A detailed study of the quantitative relationship between *p*-aminobenzoic acid and the bacteriostatic effect of sulfanilamide, sulfapyridine, sulfathiazole, sulfadiazine, sulfaguanidine, and diaminodiphenylsulfone has been carried out under rigidly controlled conditions in an attempt to ascertain the manner in which *p*-aminobenzoic acid interacts with these different sulfonamide drugs. It was hoped that knowledge of this interaction might lead to a clearer understanding of the mechanism of bacteriostasis and might possibly suggest an explanation for the marked differences in bacteriostatic potency exhibited by the various derivatives of sulfanilamide.

#### Materials and Methods

MacLeod (11) has demonstrated the widespread occurrence of sulfonamide inhibitors in ordinary bacteriological media, and has stressed the importance of using inhibitor-free media in studying the effect of sulfonamide drugs upon bacteria *in vitro*. *Bacterium coli* was used in the present studies since it will grow luxuriantly in an inhibitor-free medium containing only asparagine as a source of nitrogen, glucose as a source of carbon, and inorganic salts. The addition of *p*-aminobenzoic acid to the medium in concentrations corresponding to those used in the following experiments had no effect upon the growth of the organism as tested by repeated growth curves. This fact is of primary importance since the antibacteriostatic effect of compounds which stimulate growth cannot be attributed to a specific action upon the drug; their effect may be due entirely to a non-specific stimulation of the growth of the organism.<sup>3</sup>

<sup>2</sup> In attempting to confirm the important findings of Rubbo and Gillespie, both Lampen and Peterson (9) and Park and Wood (10) demonstrated independently that *p*-aminobenzoic acid will function as a growth factor for *Cl. acetobutylicum* only in the presence of biotin.

<sup>3</sup> The non-specific effect of growth-stimulating substances is well illustrated by experiments reported in the next paper (12).

*Culture Medium.*—The synthetic medium used was prepared in the following manner (13). 5 gm. of NaCl, 2 gm. of  $K_2HPO_4$ , 0.5 gm.  $MgSO_3$ , 2 gm. of ammonium citrate, and 3.5 gm. of asparagine were added to 1000 cc. of distilled water which had been brought to a boil. After being cooled the mixture was adjusted to a pH of 7.6 with sodium hydroxide (3 to 5 cc. of 2 normal NaOH), and was sterilized in the autoclave. 0.1 cc. of a 10 per cent solution of dextrose was added to the 10 cc. of basal medium in each tube before inoculation. The dextrose solution was sterilized by being heated to boiling for 5 minutes. Both the dextrose solution and the basal medium were stored in the ice box.

*Strain of B. coli.*—The strain of *B. coli communior* employed in the present experiments was obtained through the courtesy of Dr. Eleanor A. Bliss of the Department of Preventive Medicine of the Johns Hopkins Medical School. The organism was originally isolated from the urine of a patient with pyelitis. Daily subcultures were made in synthetic medium and these cultures were used as the source of organism in each experiment. The growth obtained in the synthetic medium was remarkably constant.

*Inoculum.*—In all experiments the inoculum employed was 0.1 cc. of a 1:10,000 dilution of a 24 hour culture of *B. coli* in synthetic broth. The inoculum, as estimated by plate count, contained approximately 10,000 viable organisms.

*Preparation of Stock Solutions of Sulfonamide Drugs and p-Aminobenzoic Acid.*—In 200 cc. lots of sterile basal medium sulfanilamide and sulfaguanidine were dissolved in concentrations of 0.01 molar, and sulfapyridine, sulfathiazole, sulfadiazine, and diaminodiphenylsulfone were prepared in 0.001 molar concentrations. The powdered drug was added in each case to boiling medium and the solution was allowed to cool slowly. The maximum concentration of each drug used was limited by its solubility in the basal medium. 0.001 molar *p*-aminobenzoic acid in synthetic broth was prepared by the same procedure.

*Method of Titration.*—The antagonistic action of *p*-aminobenzoic acid to each sulfonamide drug was studied quantitatively by titrating the acid against the drug, the appearance of bacterial growth being used as an arbitrary end point. The desired concentrations of the antagonistic substances were attained by adding appropriate amounts of the stock solution of each to plain synthetic broth to make a final volume of 10 cc. 0.1 cc. of 10 per cent glucose was added to each tube just before it was inoculated. The tubes were examined at daily intervals for macroscopic evidence of bacterial growth and the final readings were made at the end of 5 days. All cultures were incubated at 37°C.

#### RESULTS

Data from a typical titration experiment are recorded in Fig. 1. It will be noted that over a relatively wide range of sulfapyridine concentrations the minimum amount of *p*-aminobenzoic acid needed to prevent bacteriostasis at the end of 5 days was such that the ratio of *p*-aminobenzoic acid to sulfapyridine was approximately constant.<sup>4</sup> As the concentration of drug was decreased,

<sup>4</sup> A constant ratio of *p*-aminobenzoic acid to each sulfonamide drug was noted also at end points determined after 48, 72, and 96 hours as well as at the end of 5 days.

however, a point was finally reached where the ratio changed and rapidly approached zero. This sudden bend in the titration curve was obviously caused by the drug level's approach to the minimum concentration of sulfapyridine needed to cause bacteriostasis in the basal medium, and it in no way

*Conc. Sulfapyridine (moles)*

$9 \times 10^{-4}$   $5 \times 10^{-4}$   $2 \times 10^{-4}$   $1 \times 10^{-4}$   $5 \times 10^{-5}$   $2 \times 10^{-5}$   $1 \times 10^{-5}$

<i>Conc. p-Aminobenzoic Acid (moles)</i>	$2 \times 10^{-5}$	+	+	+	+	+	+	+
	$1 \times 10^{-5}$	+	+	+	+	+	+	+
	$5 \times 10^{-6}$		+	+	+	+	+	+
	$2 \times 10^{-6}$			+	+	+	+	+
	$1 \times 10^{-6}$				+	+	+	+
	$5 \times 10^{-7}$					+	+	+
	$2 \times 10^{-7}$						+	+
	$1 \times 10^{-7}$							+
	$5 \times 10^{-8}$							
	$2 \times 10^{-8}$							

FIG. 1. Titration of *p*-aminobenzoic acid against sulfapyridine using bacterial growth as an arbitrary end point. + = visible growth of *B. coli* in 10 cc. of a synthetic medium within 5 days of inoculation. Standard inoculum employed was 10,000 organisms. Cultures incubated at 37°C.

invalidates the linear relationship exhibited at higher concentrations. In the subsequent discussion of titration curves, therefore, only those points will be considered at which the drug level is well above the minimum bacteriostatic concentration.

The data summarized in Table I reveal a similar linear relationship between *p*-aminobenzoic acid and each of the sulfonamide compounds studied, the ratio of *p*-aminobenzoic acid to drug remaining approximately constant over

TABLE I  
*Quantitative Study of the Antagonistic Action of p-Aminobenzoic Acid upon  
 Various Sulfonamide Drugs*

Sulfonamide drug	Concentration (molar) Sulf. drug	Concentration (molar) P.a.b.a. needed to prevent bacteriostasis	Ratio: $\frac{\text{Molar conc. P.a.b.a.}}{\text{Molar conc. Sulf.}}$
Sulfanilamide	$9 \times 10^{-3}$	$6 \times 10^{-6}$	$6.7 \times 10^{-4}$
	$7 \times 10^{-3}$	$4 \times 10^{-6}$	$5.7 \times 10^{-4}$
	$5 \times 10^{-3}$	$3 \times 10^{-6}$	$6.0 \times 10^{-4}$
	$3 \times 10^{-3}$	$2 \times 10^{-6}$	$6.7 \times 10^{-4}$
	$2 \times 10^{-3}$	$1 \times 10^{-6}$	$5.0 \times 10^{-4}$
	$1 \times 10^{-3}$	$7 \times 10^{-7}$	$7.0 \times 10^{-4}$
Average ratio . . . . . $6.2 \times 10^{-4}$			
Diaminodiphenyl- sulfone	$9 \times 10^{-4}$	$6 \times 10^{-7}$	$6.7 \times 10^{-4}$
	$8 \times 10^{-4}$	$6 \times 10^{-7}$	$7.5 \times 10^{-4}$
	$7 \times 10^{-4}$	$5 \times 10^{-7}$	$7.1 \times 10^{-4}$
	$6 \times 10^{-4}$	$5 \times 10^{-7}$	$8.3 \times 10^{-4}$
	$5 \times 10^{-4}$	$3 \times 10^{-7}$	$6.0 \times 10^{-4}$
	$4 \times 10^{-4}$	$3 \times 10^{-7}$	$7.5 \times 10^{-4}$
Average ratio . . . . . $7.2 \times 10^{-4}$			
Sulfaguanidine	$9 \times 10^{-3}$	$1 \times 10^{-5}$	$1.1 \times 10^{-3}$
	$5 \times 10^{-3}$	$5 \times 10^{-6}$	$1.0 \times 10^{-3}$
	$2 \times 10^{-3}$	$2 \times 10^{-6}$	$1.0 \times 10^{-3}$
	$1 \times 10^{-3}$	$1 \times 10^{-6}$	$1.0 \times 10^{-3}$
	$5 \times 10^{-4}$	$5 \times 10^{-7}$	$1.0 \times 10^{-3}$
Average ratio . . . . . $1.0 \times 10^{-3}$			
Sulfapyridine	$9 \times 10^{-4}$	$1 \times 10^{-5}$	$1.1 \times 10^{-3}$
	$5 \times 10^{-4}$	$5 \times 10^{-6}$	$1.0 \times 10^{-3}$
	$2 \times 10^{-4}$	$2 \times 10^{-6}$	$1.0 \times 10^{-3}$
	$1 \times 10^{-4}$	$1 \times 10^{-6}$	$1.0 \times 10^{-3}$
	$5 \times 10^{-5}$	$5 \times 10^{-7}$	$1.0 \times 10^{-3}$
Average ratio . . . . . $1.0 \times 10^{-3}$			
Sulfadiazine	$9 \times 10^{-4}$	$1 \times 10^{-5}$	$1.1 \times 10^{-3}$
	$5 \times 10^{-4}$	$5 \times 10^{-6}$	$1.0 \times 10^{-3}$
	$2 \times 10^{-4}$	$2 \times 10^{-6}$	$1.0 \times 10^{-3}$
	$1 \times 10^{-4}$	$1 \times 10^{-6}$	$1.0 \times 10^{-3}$
	$5 \times 10^{-5}$	$5 \times 10^{-7}$	$1.0 \times 10^{-3}$
Average ratio . . . . . $1.0 \times 10^{-3}$			
Sulfathiazole	$9 \times 10^{-4}$	$3 \times 10^{-5}$	$3.0 \times 10^{-2}$
	$7 \times 10^{-4}$	$2 \times 10^{-5}$	$2.9 \times 10^{-2}$
	$5 \times 10^{-4}$	$1 \times 10^{-5}$	$2.0 \times 10^{-2}$
	$3 \times 10^{-4}$	$7 \times 10^{-6}$	$2.3 \times 10^{-2}$
	$2 \times 10^{-4}$	$5 \times 10^{-6}$	$2.5 \times 10^{-2}$
	$1 \times 10^{-4}$	$3 \times 10^{-6}$	$3.3 \times 10^{-2}$
	$8 \times 10^{-5}$	$3 \times 10^{-6}$	$3.7 \times 10^{-2}$
Average ratio . . . . . $2.8 \times 10^{-2}$			

Sulf.—sulfonamide.  
 P.a.b.a.—p-aminobenzoic acid.

the range of each titration experiment. This relationship may be expressed mathematically as follows:

$$(1) \quad \frac{(\text{P.a.b.a.})}{(\text{Sulf.})} = K$$

or

$$(2) \quad (\text{P.a.b.a.}) = K (\text{Sulf.})$$

in which (P.a.b.a.) = molar concentration of *p*-aminobenzoic acid.  
 (Sulf.) = molar concentration of sulfonamide drug.  
 $K$  = a constant.

Equation (2) plotted on Cartesian coordinates represents a straight line, with slope  $K$ , passing through the origin. Since the absolute values for  $K$  (as shown in Table I) are extremely small, the titration curves cannot be conveniently plotted arithmetically, their slopes being so small that they practically lie upon the  $x$  axis. The results of each titration experiment can, however, be graphed on a logarithmic scale as shown in Fig. 2. It will be noted that the data thus plotted fall on straight lines parallel to one another and running at an angle of  $45^\circ$  with the axis. These lines all approximate very closely the general equation:

$$(3) \quad \log (\text{P.a.b.a.}) = \log (\text{Sulf.}) + \log K$$

which will be recognized as the logarithmic variant of equation (2) expressing the same linear relationship between drug and *p*-aminobenzoic acid.

Two facts regarding the significance of the constant  $K$  deserve special emphasis:

(a) The constant is equal to the molecular ratio of *p*-aminobenzoic acid and sulfonamide drug at the arbitrary end point chosen for the titration experiments. Under standard conditions it defines the amount of *p*-aminobenzoic acid needed to nullify the antibacterial effect of a given quantity of drug.

(b) The absolute value of  $K$  will vary with the type of organism, the size of the inoculum, the conditions under which the organism is grown, and the potency of the bacteriostatic agent employed. If all but the last of these variables is kept rigidly constant, as in the present experiments, the magnitude of  $K$  becomes an index of the relative bacteriostatic power of the drug tested. Under these conditions it is justifiable to refer to  $K$  as the "bacteriostatic constant."

As shown in Table II the bacteriostatic constants of the various sulfonamide compounds vary over a relatively wide range of values. It will be noted that the more potent the drug the greater is the value of its bacteriostatic constant  $K$ , and, likewise, the lower its *p*-aminobenzoic acid titration curve falls on the

logarithmic graph (Fig. 2). Thus the bacteriostatic constant of sulfathiazole, the most potent drug studied, is 0.028, whereas that of sulfanilamide, the least active, is only 0.00062.

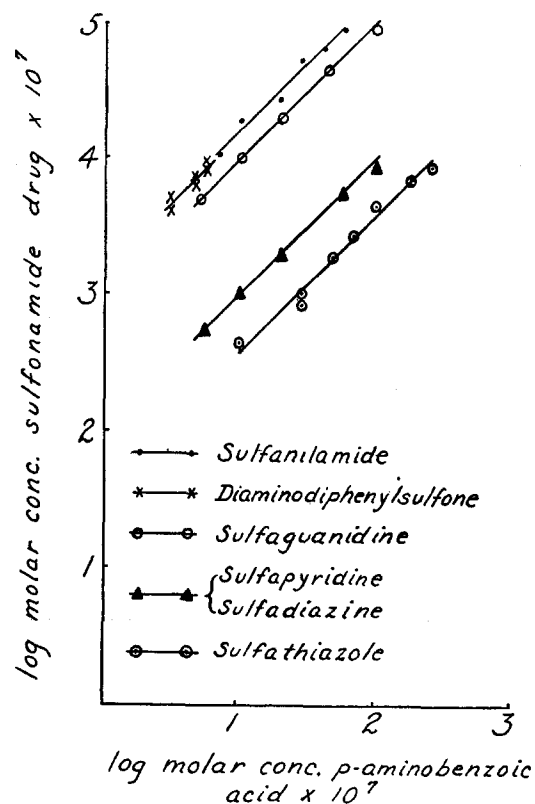


FIG. 2. *p*-Aminobenzoic acid titration curves of sulfonamide drugs. Visible growth of *B. coli* (in 10 cc. of a synthetic medium) used as arbitrary end point for each titration. Inoculum standardized at 10,000 organisms and final readings made at the end of 5 days incubation at 37°C. Curves plotted for convenience on logarithmic coordinates.

It is of interest to compare the relative values obtained for  $K$  with the bacteriostatic potency of the drugs measured by the usual method, *i.e.* by determining the minimum concentration needed to cause bacteriostasis in the absence of additional *p*-aminobenzoic acid. The two sets of values may best be compared by using sulfanilamide as a standard and determining the "sulfanilamide coefficient" as shown in Table II. The data reveal a remarkably close correlation between the sulfanilamide coefficients determined by the two

methods.<sup>5</sup> This close correlation is of considerable theoretical significance since it suggests that the bacteriostatic potency of each of the sulfonamide compounds is directly proportional to its ability to neutralize the action of the *p*-aminobenzoic acid added to the culture medium.

In summary it may be stated that a quantitative study of the interaction of various sulfonamide drugs with *p*-aminobenzoic acid has revealed the following: The antibacterial effect of each sulfonamide compound studied, regardless of chemical structure, was neutralized by the addition of *p*-aminobenzoic acid to the culture medium. The amount of *p*-aminobenzoic acid needed to prevent

TABLE II  
*Sulfanilamide Coefficients of Various Sulfonamide Drugs Determined Both by Measuring the Minimum Bacteriostatic Concentration and by Titrating with p-Aminobenzoic Acid*

Sulfonamide drug	Minimum molar conc. of drug causing bacteriostasis	Bacteriostatic constant, <i>K</i> or Molar conc. P.a.b.a. Molar conc. Sulf. (P.a.b.a. titration)	Sulfanilamide coefficients	
			Min. bact. conc. S. Min. bact. conc. X.	$\frac{K_s}{K_x}$
Sulfanilamide . . . . .	0.0004	0.00062	1	1.0
Diaminodiphenylsulfone . . . . .	0.0002	0.00072	2	1.2
Sulfaguanidine . . . . .	0.0002	0.001	2	1.6
Sulfapyridine . . . . .	0.00002	0.01	20	16.1
Sulfadiazine . . . . .	0.00002	0.01	20	16.1
Sulfathiazole . . . . .	0.000008	0.028	50	45.2

*K*, bacteriostatic constant determined by titration with P.a.b.a. (See average ratios, Table 1.)

P.a.b.a., *p*-aminobenzoic acid.

Sulf., sulfonamide drug.

Min. bact. conc. S., minimum molar concentration of sulfanilamide causing bacteriostasis.

Min. bact. conc. X., minimum molar bacteriostatic concentration of sulfonamide drug being compared with sulfanilamide.

*K<sub>s</sub>*, bacteriostatic constant of sulfanilamide.

*K<sub>x</sub>*, bacteriostatic constant of sulfonamide drug being compared with sulfanilamide.

bacteriostasis was directly proportional to the relative bacteriostatic potency of the drug. Over a wide range of drug concentrations the minimum amount

<sup>5</sup> The *p*-aminobenzoic acid titration method is considered the more accurate of the two since the absolute value for *K* is obtained by averaging the numerous end points on the titration curve rather than by determining a single end point as in the simple bacteriostasis method. It should be emphasized, however, that neither of these *in vitro* methods can be relied upon at present to estimate the relative therapeutic effectiveness of untried sulfonamide compounds. The sulfanilamide coefficients listed above have been measured under highly specialized conditions, namely, in an inhibitor-free medium; it does not necessarily follow that the relative bacteriostatic potency of the drugs will be the same in a more complex culture medium or in the animal body.



of *p*-aminobenzoic acid needed to nullify the bacteriostasis was such that the ratio of *p*-aminobenzoic acid to the drug was constant. The *p*-aminobenzoic acid titration curve for each sulfonamide drug thus followed a linear relationship. The implications of these facts in relation to the possible mode of action of the sulfonamide drugs are considered significant and will be discussed below.

#### DISCUSSION

The important investigations of Woods on the mechanism of action of sulfanilamide were based on the working hypothesis that "antibacterial substances act by interfering with some substance essential to the bacterial cell." Having demonstrated that *p*-aminobenzoic acid in minute quantities neutralized the bacteriostatic effect of sulfanilamide and sulfapyridine, Woods suggested that the essential substance was *p*-aminobenzoic acid or a closely related compound. It is noteworthy, however, that at the time of his publication there was no direct experimental evidence that *p*-aminobenzoic acid was essential to bacterial metabolism, this evidence being supplied later by workers in other laboratories (8-10). Woods suggested further that sulfanilamide and sulfapyridine, because of their close chemical similarity to *p*-aminobenzoic acid, prevent bacterial growth by competing with the latter compound for the bacterial enzyme normally involved in its utilization. As evidence for this concept, he pointed out that the slightest deviation from the chemical structure of *p*-aminobenzoic acid greatly reduces the potency of the antisulfonamide effect, just as any change in the *p*-amino structure of sulfanilamide decreases its bacteriostatic properties. In addition he drew attention to other examples in enzyme chemistry of "competitive inhibition" due apparently to similarity of chemical structure. It should be emphasized that Woods' experimental data did not conclusively prove his theory which was presented only as a guide to further investigation.

The results of the studies reported in the present paper add important direct evidence in favor of Woods' theory. It was observed that *p*-aminobenzoic acid would neutralize the bacteriostatic properties of all of the six sulfonamide compounds studied, regardless of the differences in their chemical structure, and it was found also that the amount of *p*-aminobenzoic acid needed to prevent bacteriostasis was directly proportional to the bacteriostatic potency of the drug, provided all other variables were held constant. Both of these observations suggest that the bacteriostatic mechanism<sup>6</sup> of the sulfonamide

<sup>6</sup> It is of interest that the sulfonamide portion of the molecule rather than the *p*-amino nucleus, appears to be responsible for certain of the toxic reactions caused by these drugs. The  $-\text{SO}_2\text{NH}_2$  radical of sulfanilamide inactivates carbonic anhydrase (14), and this fact has been offered as an explanation for sulfanilamide acidosis (15). Patients becoming sensitive to one sulfonamide drug are often found insensitive to others (16), an observation suggesting that the sensitivity phenomena are caused by some part of the molecule other than the *p*-amino nucleus which is common to all of the compounds.

drugs works mainly, if not entirely, through the *p*-amino nucleus which is common to *p*-aminobenzoic acid and to all of the drugs tested. The fact that an organism made "drug fast" to sulfathiazole is found to be resistant to the action of other sulfonamide compounds (17) also substantiates this concept.

In addition, the quantitative study of the interaction of *p*-aminobenzoic acid and the various sulfonamide drugs in bacterial cultures revealed that over a wide range of drug concentrations the minimum amount of *p*-aminobenzoic acid needed to prevent bacteriostasis was such that the ratio of *p*-aminobenzoic acid to drug remained constant. Woods previously had observed, in similar experiments, a constant ratio of *p*-aminobenzoic acid to drug when sulfanilamide was added to cultures of *B. coli* or *Streptococcus hemolyticus*, and while the present work was in progress Rubbo and Gillespie reported the same observation with sulfanilamide and *Cl. acetobutylicum*. In none of those experiments, however, were drugs other than sulfanilamide investigated.<sup>7</sup>

The linear relationship between drug and *p*-aminobenzoic acid is of considerable theoretical significance since it suggests that bacteriostasis is accomplished through the "competitive inhibition" (19) of an essential enzyme reaction by a substance chemically related to the substrate. Assuming the substrate to be *p*-aminobenzoic acid (or a closely related compound) and the inhibitor to be the sulfonamide drug, an equation expressing the same straight-line relationship may be derived on purely theoretical grounds as follows:

If *p*-aminobenzoic acid (*P*) and sulfanilamide, or one of its derivatives (*S*), compete for the same bacterial enzyme (*E*), the interaction of *P* and *S* with *E* may be expressed by the equations:



Since the concentrations of *P* and *S* are infinitely large compared to that of *E*,<sup>8</sup> it may be assumed that practically all of the available enzyme is bound either by *P* or *S* and, therefore, may be considered to be equivalent to  $(PE) + (SE)$ . The amount of available enzyme is constant at the start of each experiment (constant inoculum of bacteria), so that

$$(3) \qquad (PE) + (SE) = k_1$$

<sup>7</sup> Strauss, Lowell, and Finland (18) studied sulfanilamide, sulfapyridine, and sulfathiazole, and although they concluded that a linear relationship existed between drug and inhibitor, their data do not bear out this conclusion, since the curve obtained for each drug is a straight line only when plotted on semilogarithmic coordinates.

<sup>8</sup> The concentration of *P* and *S* ranged from 1 to  $10^{-5}$  mg. per cc., whereas the concentration of bacteria introduced in the inoculum was 1,000 organisms (or approximately 10 to 12 mg.) per cc. Even if it is assumed that the entire bacterial cell functions as active enzyme and that the relatively small *p*-aminobenzoic acid and sulfonamide molecules combine weight for weight with the much larger enzyme molecule, there is still a tremendous discrepancy between the amount of available enzyme and the concentrations of *P* and *S*.

The ability of the organism to multiply depends upon a certain minimum amount of *p*-aminobenzoic acid combining with enzyme to form the essential complex *PE*. At the selected titration end point only the minimum amount of *PE* needed to initiate growth is present at the start of the experiment, and, therefore, at this point (*PE*) may be considered a fixed quantity,  $k_2$ . It follows that

$$(4) \quad (PE) = k_2$$

and

$$(5) \quad (SE) = k_1 - k_2$$

At the titration end point the proportions of enzyme bound by *P* and *S* at the start of each experiment will be determined by two factors: (a) the relative affinities of *P* and *S* for the enzyme, *E*, and (b) the relative amounts of *P* and *S* in the medium. The affinities of *P* and *S* for the enzyme are determined by the chemical properties of the compounds and may be considered constant in any one experiment, although the enzyme affinities of the different sulfonamide drugs probably vary with their chemical structure (12). It follows then that the relative concentrations of *PE* and *SE* are directly proportional to the relative concentrations of *P* and *S*. That is,

$$(6) \quad \frac{(PE)}{(SE)} = k_3 \frac{(P)}{(S)}$$

and

$$(7) \quad \frac{(P)}{(S)} = \frac{k_2}{k_3(k_1 - k_2)}$$

Since (*PE*) and (*SE*) are infinitely small as compared with (*P*) and (*S*), the terms (*P*) and (*S*) may be considered equivalent to the total amounts of *p*-aminobenzoic acid (P.a.b.a.) and sulfonamide drug (Sulf.) added to the medium.

$$(8) \quad \frac{(\text{P.a.b.a.})}{(\text{Sulf.})} = K$$

The last equation given expresses precisely the linear relationship observed experimentally. The close agreement between the theoretical equation and the experimental data adds strong evidence in favor of the assumption that the sulfonamide drugs and *p*-aminobenzoic acid compete for the same enzyme site on the bacterial cell.<sup>9</sup>

If *p*-aminobenzoic acid is essential for bacterial growth, and the sulfonamide drugs, through their chemical similarity to this essential metabolite, succeed in blocking the enzyme system normally involved in its utilization, it obviously follows that the bacteria will not grow. The fact that one molecule of *p*-aminobenzoic acid will antagonize the action of several thousand molecules of drug

<sup>9</sup> Since the completion of this manuscript Wyss (20) has published similar experiments with *p*-aminobenzoic acid and sulfanilamide and has reached the same conclusion through a different type of mathematical analysis.

does not invalidate this theory as to the mechanism of bacteriostasis; it may only indicate that the essential metabolite has a far greater affinity for the enzyme than has the drug, in which case a great excess of drug will be required to block the essential metabolite from its bacterial enzyme. The well known lag in the bacteriostatic action of the drug (21) may be explained by assuming that the supply of *p*-aminobenzoic acid already in the medium (and possibly combined with bacterial enzyme) must become inadequate before the rate of bacterial growth will be noticeably affected.

In conclusion it should be emphasized that although the experiments reported in the present paper strongly substantiate Woods' theory as to the mechanism of action of the sulfonamide drugs, the final proof of the theory must await the identification and careful study of the enzyme system (or systems) involved in the utilization of *p*-aminobenzoic acid.

#### SUMMARY

The following observations have been made which substantiate the theory that the sulfonamide drugs used in the treatment of bacterial infections exert their bacteriostatic effect by competing with the essential metabolite, *p*-aminobenzoic acid, for an important enzyme site on the bacterial cell.

1. *p*-Aminobenzoic acid was shown to nullify the bacteriostatic effect of all of the six sulfonamide compounds studied even though the drugs exhibited marked differences in chemical structure.

2. The bacteriostatic potency of each sulfonamide drug was found to be directly proportional to its ability to counteract the antibacteriostatic action of *p*-aminobenzoic acid.

3. In the case of each drug tested over a wide range of concentrations the minimum amount of *p*-aminobenzoic acid needed to prevent bacteriostasis was such that the ratio of *p*-aminobenzoic acid to drug was constant.

4. The linear relationship between *p*-aminobenzoic acid and drug was interpreted as indicating the competitive inhibition of an essential enzyme reaction by a substance chemically related to the substrate. This interpretation was supported by the fact that the equation derived on purely theoretical grounds relating drug and acid expressed the same linear relationship as that observed experimentally.

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