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Mechanism of Sulfonamide Action. III. Biological Action of Substituted *p*-Aminobenzoic Acids.*

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Ever since the identification by Woods[1] of *p*-aminobenzoic acid as a specific antagonist for sulfonamides, and particularly since it was found by Rubbo and Gillespie[2] that *p*-aminobenzoic acid is an essential nutrilite for *Clostridium acetobutylicum*, the action of sulfonamides in inhibiting bacterial growth has been interpreted as essentially one of "enzyme blockage." The structural similarity of sulfanilamide and *p*-aminobenzoic acid supports this interpretation. Other examples of bacteriostasis brought about by enzyme blockage with agents structurally patterned after natural metabolites have recently been reviewed by McIlwain.[3]

For some time we have been interested in the inactivation of sulfonamide inhibitors, particularly of *p*-aminobenzoic acid. It has been shown[4-6] that oxidizing agents, among them N,N'-Dichloroazodicarbonamidine, could be used for this purpose. In following up the mechanism of this particular inactivation it occurred to us that a chlorinated *p*-aminobenzoic acid might be formed, and that through this chemical change the biological function of *p*-aminobenzoic acid is abolished. We, therefore, investigated the properties of various halogenated *p*-aminobenzoic acids and *p*-aminobenzoic acids containing other substituents.†

Recently Hirsch[7] reported antibacterial action of the amide of *p*-aminobenzoic acid and Auhagen[8] reported *in vitro* and *in vivo* activity of *p*-aminophenylketones. Activities in both cases were considerably less than sulfanilamide.

Methods. The methods and the cultures of *Escherichia coli* used are those reported by us[9] previously.

The *Clostridium acetobutylicum* culture is No. 3625 of the American Type Culture Collection and the *Neurospora crassa* is the "aminobenzoicless" mutant developed and described by Tatum‡ and Beadle.[10]

The substituted *p*-aminobenzoic acids were prepared in our laboratory by procedures which rigidly exclude any use of *p*-aminobenzoic acid as starting material. We have found this precaution necessary since as little as one part in ten thousand of *p*-aminobenzoic acid in the final compound would have a significant effect upon the biological activity.

Results. The examination of the many derivatives of *p*-aminobenzoic acid that were studied revealed that 2-F-PAB showed consid-

* This work was initiated under the leadership of the late Dr. Franz C. Schmelkes.

1 Woods, D. D., *Brit. J. Exp. Path.*, 1940, **21**, 74.

2 Rubbo, S. D., and Gillespie, J. M., *Nature*, 1940, **146**, 838.

3 McIlwain, Henry, *Lancet*, 1942, **1**, 412.

4 Neter, E., *J. Pharm. and Exp. Therap.*, 1942, **74**, 52.

5 Goldberger, H. A., *Am. J. Surg.*, 1942, **56**, 353.

6 Schmelkes, F. C., and Wyss, O., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 263.

7 Hirsch, J., *Science*, 1942, **96**, 139.

† For the sake of brevity, we will refer to the various substituted *p*-aminobenzoic acids by prefixing the conventional symbol PAB with the numbers and chemical symbols for substituents in accordance with the nomenclature of *Chemical Abstracts*. For instance, 2-Cl-PAB meaning 2-chloro-4-aminobenzoic acid, or 3-5-di-Cl-PAB meaning 3-5-dichloro-4-aminobenzoic acid. The details of the preparation of these compounds will be reported in a subsequent publication.

8 Auhagen, E., *Z. physiol. Chem.*, 1942, **274**, 48.

9 Wyss, O., Grubaugh, K. K., and Schmelkes, F. C., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 618.

10 Tatum, E. L., and Beadle, G. W., *Proc. Nat. Acad. Sci.*, 1942, **28**, 234.

‡ The authors are indebted to Dr. Tatum for the organism and for advice as to its use.

TABLE I.
Relative Effectiveness of *p*-Aminobenzoic Acid and Its Substituted Derivatives as Growth Factors and as Antisulfonamide Agents.

	Amount required to give action equal to PAB					
Type of action	PAB	2-F-PAB	2-Br-PAB	2-I-PAB	3-COOH-PAB	*p*-Amino phenyl acetic acid§
Antisulfonamide	1	3	200	1500	2000	>1000
Growth Factor	1	3	150	1000	1000	1000

Antisulfonamide action determined against sulfanilamide with *E. coli*. Growth factor Activity determined with "aminobenzicless" mutant of Neurospora and with *Cl. acetobutylicum*.

TABLE II.
Bacteriostatic Activity of Substituted *p*-Aminobenzoic Acids.‖

	Growth of *E. coli* at pH 7.2 as % of control					
mg%	2-Cl-PAB	3-Cl-PAB	3-F-PAB	2-NH_2-PAB	3-NH_2-PAB	SA
.3	40	100	100	100	100	100
.5	0	100	100	100	100	100
1.0	0	100	100	100	85	80
2.0	0	100	70	100	30	50
5.0	0	100	25	50	0	0
10.0	0	70	15	35	0	0
25.0	0	0	0	25	0	0
50.0	0	0	0	25	0	0

erable PAB action in that it can be substituted for that factor in the nutrition of *Clostridium acetobutylicum* and the "aminobenzoicless" mutant of *Neurospora crassa*. It also reverses the action of sulfanilamide on *E. coli*. Three other derivatives show some action. The data are presented in Table I.

The antisulfonamide action parallels the growth factor activity. When high concentrations are required to reverse the action of the sulfonamide they may exert some slight additional bacteriostatic action, introducing the discrepancies noted in Table I. Because of this, *p*-aminophenylacetic acid did not act as an antisulfonamide in any concentration used.

Five of the derivatives, 2-Cl-PAB, 3-Cl-PAB, 3-F-PAB, 2-NH_2-PAB and 3-NH_2-PAB, were bacteriostatic in the concentrations used. Tables II and III show the extent of their activity as well as the inhibition of this action by unsubstituted PAB.

Although the 2-NH_2 compound gives considerable inhibition in low concentrations, growth is not completely inhibited even by the highest concentrations used. In the presence of high concentrations of the 3-Cl derivative growth is never completely restored by any concentration of *p*-aminobenzoic acid. Thus, the compound is shown to have a toxic effect on the bacteria in addition to its sulfonamide action.

The most active of these 5 derivatives is the 2-Cl-PAB. In a simple medium at pH 7.0 it is almost as active as sulfapyridine against *E. coli*. However, it was found to be completely inactive against *Staphylococcus aureus* (F.D.A. Strain No. 209) in the amino acid medium of Gladstone and Knight. This inactivation was due to methionine. Reversion of sulfonamide activity by methionine has first been reported by Bliss and Long[11] using *E. coli* as the test organism. It has also been analyzed by Harris and Kohn.[12]

§ This inactive compound was included since high activity has been reported. Our findings are essentially in agreement with Peterson and Lampen (personal communication).

‖ The authors are grateful to Dr. B. J. Ludwig for assistance in the preparation of some of these compounds.

11 Bliss, E. A., and Long, Perrin H., *Bull. Johns Hopkins Hosp.*, 1941, **69**, 14.

12 Harris, J. S., and Kohn, Henry I., *J. Pharm. and Exp. Therap.*, 1941, **73**, 383.

TABLE III.
Inhibition of the Bacteriostatic Activity of Substituted *p*-Aminobenzoic Acids and Sulfanilamide (SA) by PAB.

	Growth of *E. coli* at pH 7.2 as % of control in the presence of 25 mg % of					
PAB mg%	2-Cl-PAB	3-Cl-PAB	3-F-PAB	2-NH_2-PAB	3-NH_2-PAB	SA
0	0	0	0	20	0	0
.001	0	15	15	20	0	0
.003	0	50	15	20	0	0
.010	20		50	20	0	50
.030	50			50	100	100
.100	100	55	60		100	100
.200	100		100	100	100	100
.300	100	60	100	100	100	100

TABLE IV.
Effect of Methionine on the Inhibition of *E. coli* by 2-Cl-PAB and Sulfanilamide.

		Turbidity units		
Drug, 10 mg %	Methionine mg%	16 hr	42 hr	70 hr
None	0	.086	.119	.136
2-Cl-PAB	0	0	0	.142
	0.3	.086	.108	.133
	0.1	.026	.060	.105
	0.03	.013	.025	.154
	0.01	0	0	.161
Sulfanilamide	0	0	0	.015
	100	0	.011	.012
	50	0	.012	.099
	25	0	.020	.047
	10	0	0	0

As can be seen from Table IV, the effect of methionine on 2-Cl-PAB differs strikingly from its action on sulfanilamide. In the first case, methionine reversion occurs early in the growth period, whereas with sulfanilamide the reversion is delayed and incomplete. Also, on prolonged incubation full growth takes place with the 2-Cl-PAB whereas with sulfanilamide it does not. The data indicate that the organisms eventually synthesize an inhibitor for 2-Cl-PAB. Since the other 4 substituted PAB's are entirely unaffected by methionine, it is hoped that further study may help throw some additional light upon the role of this interesting amino acid.

TABLE V.
Summary of Activity of Substituted *p*-Aminobenzoic Acids.

	Positions		
Substituent	2	3	3-5
Cl	SA	SA*	D
F	PAB*	SA*	NT
Br	PAB	D	I
I	PAB	I	I
NH_2	SA	SA	NT
NO_2	D	I	NT
CH_3	I	I	NT
COOH	I	PAB	NT
OH	I	I	NT
CH_3O	D	I	NT

PAB = Reverses sulfonamide bacteriostasis.
SA = Produces sulfonamide type bacteriostasis. (Reversible by *p*-aminobenzoic acid).
I = Inactive at 50 mg % or limit of solution.
D = Doubtful action—some slight bacteriostasis at highest concentration.
NT = No test.
*First synthesized in these laboratories.

Chemotherapeutic Experiments. Experiments on mice infected with a virulent strain of type I pneumococci (Strain SV-1, obtained from Dr. Colin M. MacLeod) revealed that these derivatives have little chemotherapeutic activity under such conditions. This is due in part to the fact that high blood levels cannot be obtained. For example, when fed at a level of 3% of the ration the 2-chloro derivative produced a blood level of only 1 mg %.

Summary. The biological action of substituted *p*-aminobenzoic acids studied in this

series of experiments is summarized in Table V.

Substitutions on the ring of *p*-aminobenzoic acid resulted in compounds which were either inactive or had varying degrees of sulfonamide or antisulfonamide action. Although the compounds with sulfonamide action are relatively non-toxic, their value in systemic chemotherapy has not been demonstrated.