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The *In Vitro* Effects upon Sulfonamides of Local Anesthetics Derived from N-Substituted *p*-Aminobenzoic Acids.*

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Following the discovery by Woods[1] that the antibacterial effects of the sulfonamides could be blocked by small amounts of *p*-aminobenzoic acid, it was found that diethylaminoethyl *p*-aminobenzoate hydrochloride (procaine hydrochloride, U. S. P.) and other local anesthetics which are esters of *p*-aminobenzoic acid also have antisulfonamide action.[2] Peterson and Finland[3] recently demonstrated that procaine, in amounts ordinarily employed for local anesthesia, may be absorbed into the circulation in sufficient concentration to exert a definite inhibitory effect on the action of sulfonamide drugs that may be present in the blood. They furthermore noted that in spite of the presence in the body of bacteriostatic concentrations of sulfonamides, an infection may become locally established in an area which has been infiltrated with procaine.

In studies with hemolytic streptococcal infections in mice, De Waal *et al.*[4] observed that the antagonistic effect of procaine against sulfanilamide is most marked when large doses are given early in the course of sulfanilamide treatment. They noted, moreover, that although repeated small doses of the anesthetic reduced the efficacy of sulfanilamide therapy, occasional small doses had no antagonistic effect and occasional large doses had no lasting antagonistic effect. This may possibly be explained by Legge and Durie's[5] observation that in the mouse injected procaine is hydrolyzed to *p*-aminobenzoic acid, some of which is then acetylated to *p*-acetylaminobenzoic acid. Acetylation of the amino group of *p*-aminobenzoic acid leads to a 10,000-fold decrease in its antisulfonamide activity.[1]

On the basis of these findings it appeared worth while to determine whether a similar substitution of an acetyl group on the aromatic nitrogen of procaine would correspondingly affect the antisulfonamide action of the local anesthetic. In the course of these investigations derivatives of procaine were studied in which substitutions of alkyl groups were also made in the position mentioned. Dimethylaminoethyl *p*-butylaminobenzoate hydrochloride (tetracaine hydrochloride, U. S. P.), an anesthetic derived from *p*-butylaminobenzoic acid, and the acetyl derivative of α,β-dimethyl-γ-dimethylaminopropyl *p*-aminobenzoate hydrochloride (butamin, N. N. R.) were included among the compounds tested for antisulfonamide action.

Methods and Results. 1. *Tests in Nutrient Broth Medium.* A series of dilutions of the local anesthetics and their derivatives ranging from 1:100 to 1:1,000,000 were prepared in distilled water. 1.0 cc of each dilution was added to 8.7 cc of veal-infusion-dextrose broth containing 20 mg % of one of the sulfonamides. The tubes were autoclaved at 10 pounds for 10 min., and upon cooling 0.1 cc normal horse serum was added to each, followed by 0.2 cc of a 1:1,000 dilution of a 24-hour broth culture of the β-hemolytic streptococcus C-203. This gave a final series of dilutions of the local anesthetics ranging from 1:1,000 to 1:10,000,000. The tubes were incubated at 37° C and examined after 24 hours for visible growth. The results obtained with sulfanilamide, sulfathiazole, sulfadiazine, and sulfa-

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

2 Keltch, A. K., Baker, L. A., Krahl, M. E., and Clowes, G. H. A., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 533.

3 Peterson, O. L., and Finland, M., *Am. J. M. Sc.*, 1944, **207**, 166.

4 De Waal, H. L., Kanaar, A. C., and McNaughton, J., *Lancet*, 1942, **2**, 724.

5 Legge, J. W., and Durie, E. B., *M. J. Australia*, 1942, **2**, 561.

TABLE I.
Limiting Dilutions Affecting Sulfonamide Action Against Beta Hemolytic Streptococcus.

Anesthetic	Sulfonamide—20 mg %			
	Sulfanilamide	Sulfadiazine	Sulfathiazole	Sulfapyridine
Procaine hydrochloride—U.S.P.	$10^{-7}>$	10^{-6}	10^{-6}	10^{-6}
Procaine hydrochloride Ethyl derivative	10^{-5}	2×10^{-5}	10^{-3}	10^{-4}
Procaine hydrochloride Butyl derivative	2×10^{-5}	10^{-4}	$<2\times10^{-4}$	$<2\times10^{-4}$
Procaine hydrochloride Acetyl derivative	10^{-3}	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$
Butamin, N. N. R.	10^{-5}	10^{-5}	2×10^{-4}	10^{-4}
Butamin Acetyl derivative	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$
Tetracaine hydrochloride—U.S.P.	$<2\times10^{-4}$	$<2\times10^{-4}$	$<2\times10^{-4}$	$<2\times10^{-4}$

$10^{-7}>$ indicates highest dilution studied proved antagonistic to sulfanilamide; $<2\times10^{-4}$ indicates no antagonistic effect in this concentration, furthermore, the greater concentration (10^{-3}) proved to be bacteriostatic in presence or absence of sulfonamides; $<10^{-3}$ indicates greatest concentration studied had no antisulfonamide action.

TABLE II.
Effects of Local Anesthetics upon Sulfathiazole Activity Against *E. coli*.

Molar conc. of sulfathiazole	Molar Concentrations of Anesthetics									
	Procaine					Ethyl Procaine				
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	none	10^{-3}	10^{-4}	10^{-5}	10^{-6}	none
10^{-3}	4	0	0	0	0	0	0	0	0	0
4×10^{-3}	4	4	1	0	0	0	0	0	0	0
1.6×10^{-4}	4	4	4	0	0	1	0	0	0	0
6.4×10^{-4}	4	4	4	4	4	3	2	1	1	1
None	4	4	4	4	4	4	4	4	4	4
	Butyl Procaine					Acetyl Procaine				
10^{-3}	0	0	0	0	0	0	0	0	0	0
4×10^{-3}	0	0	0	0	0	0	0	0	0	0
1.6×10^{-4}	0	0	0	0	0	0	0	0	0	0
6.4×10^{-4}	1	0	0	0	0	0	0	0	0	2
None	4	4	4	4	4	4	4	4	4	4
	Butamin					Acetyl Butamin				
10^{-3}	0	0	0	0	0	0	0	0	0	0
4×10^{-3}	2	0	0	0	0	0	0	0	0	0
1.6×10^{-4}	4	3	0	0	0	0	0	0	0	0
6.4×10^{-4}	4	4	4	0	2	0	0	0	0	0
None	4	4	4	4	4	4	4	4	4	4

The anesthetics were used in the form of the hydrochlorides. 0 = no antisulfathiazole activity; 1-3 = increasing degrees of antisulfathiazole activity; 4 = complete antisulfathiazole activity, or growth equivalent to control.

pyridine are given in Table I.

Results of tests in broth medium. From the data presented in the table it is apparent that procaine exhibits a high degree of antisulfonamide activity against all the sulfonamides tested. With the substitution of an ethyl group on the aromatic nitrogen, the antisulfonamide action of the local anesthetic is markedly decreased. A butyl group substituted in this position further decreases this effect, and with the substitution of an acetyl group, the antisulfonamide activity is practically eliminated. Butamin, which is also a derivative of *p*-aminobenzoic acid, has approximately half as much antisulfonamide activity as procaine. This may be due to the fact that the former is a substituted secondary alkyl ester which would be expected to hydrolyze more slowly to *p*-aminobenzoic acid than does procaine. Acetylation of butamin destroys its antagonistic reaction toward the sulfonamides. Tetracaine, the derivative of *p*-butylaminobenzoic acid, failed to give any indication of antisulfonamide action. Although not given

in the table, other local anesthetics which are not derivatives of *p*-aminobenzoic acid were also tested and found to have no effect upon sulfonamides. This group included N,N'-*p*-ethoxy phenylacetamidine hydrochloride monohydrate (phenacaine hydrochloride, U. S. P.), β-diethylaminoethylamide of 2-butoxy-cinchoninic acid hydrochloride 2-butoxy-4-(β-diethylaminoethylamido) carboxy quinoline hydrochloride (dibucaine, N. N. R.), and 2-benzoxy-2-dimethylaminomethyl-1-dimethylaminobutane hydrochloride (amydricaine, N. N. R.).

2. *Tests in Synthetic Medium.* In this series of tests the most active sulfonamide studied, sulfathiazole, was mixed in varying concentrations with a series of dilutions of the local anesthetics. A synthetic medium described by Kalmanson and Bronfenbrenner[6] was used with *Escherichia coli* as the test organism. A 10^{-2} molar concentration of each local anesthetic was prepared by dissolving the compound in distilled water. The solution was sterilized by boiling for 2 to 3 min., following which it was cooled rapidly to room temperature. Subsequent dilutions of 10^{-3}, 10^{-4}, and 10^{-5} molar concentrations were made aseptically from the initial dilution in sterile distilled water. In a similar manner a 10^{-2} molar concentration of sulfathiazole was prepared and then diluted further to give molar concentrations of 4×10^{-2}, 1.6×10^{-3}, and 6.4×10^{-3}.

Each dilution of an anesthetic was combined with each concentration of sulfathiazole by aseptically pipetting sterile solutions, per tube, as follows:

	cc
Synthetic medium	6.0
Local anesthetic solution	1.0
Sulfathiazole "	1.0
5% dextrose "	0.4
Distilled water	1.5
	9.9

This resulted in a final molar concentration of 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} of the anesthetic and 10^{-3}, 4×10^{-3}, 1.6×10^{-4}, and 6.4×10^{-4} of sulfathiazole. The inoculum consisted of a 0.1 cc of a 24-hour growth of *E. coli* in the synthetic medium, diluted 1:10 with the same medium. Controls were maintained by omitting in one instance the local anesthetic, in another sulfathiazole, and a final control lacking both these ingredients. All tubes were incubated at 37°C and examined after 48 hours. The results of these tests are given in Table II.

Results of tests in synthetic medium. The same general trend of antisulfonamide activity is again displayed by procaine and butamin as noted in the previous tests in broth medium. The efficacy of alkylation and acetylation of the anesthetics in reducing their antisulfathiazole activity is again apparent.

Discussion. The antagonistic action of *p*-aminobenzoic acid against the sulfonamides has been a subject of considerable interest during the past few years. It has recently been found that various esters of *p*-aminobenzoic acid exert this same antagonism in varying degrees. Among the esters of *p*-aminobenzoic acid are procaine, butamin, mono-*n*-amylaminoethyl-*p*-aminobenzoate hydrochloride (amylcaine hydrochloride, N. N. R.), γ-dibutylaminopropyl-*p*-aminobenzoate-N-sulfate (butacaine sulfate, U. S. P.), γ-diethylamino-β-β-dimethylpropyl-*p*-aminobenzoate hydrochloride (larocaine hydrochloride, N. N. R.), and *n*-butyl-*p*-aminobenzoate (normal butyl aminobenzoate, U. S. P.) Upon saponification of any of these anesthetics, the probable end-product is *p*-aminobenzoic acid.

Other local anesthetics which are not esters of *p*-aminobenzoic acid have failed to show evidence of any antisulfonamide activity. This latter group includes tetracaine, a derivative of *p*-butylaminobenzoic acid, which, contrary to our observations, was found by Keltch *et al.*[2] to have an antagonistic action to sulfonamides. This raised the question as to whether similar substitutions of alkyl groups in those anesthetics derived from *p*-aminobenzoic acid would also affect their antisulfonamide action. Furthermore, inasmuch as acetylation of *p*-aminobenzoic acid is known to markedly reduce its antagonism towards sulfonamides, it could be assumed that acetyla-

[6] Kalmanson, G., and Bronfenbrenner, J., *J. Gen. Physiol.*, 1939, **23**, 201.

tion of its esters would produce the same result. With this thought in mind, the series of alkyl and acetyl derivatives of procaine and the acetyl analogue of butamin were prepared and studied.†

Our findings confirmed the above assumption in that the N-substituted anesthetics markedly decreased or neutralized completely the antisulfonamide action of these compounds.

Summary. Studies are presented which indicate that substitution of an alkyl group on the aromatic nitrogen of procaine will markedly reduce or inactivate completely the antisulfonamide action of the local anesthetic. Substitution of an acetyl group in the position mentioned on procaine, as well as butamin, will likewise result in an inactivation of the antisulfonamide property of these compounds.

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Anomalous Offspring and Growth of Wistar Rats Maintained on a Deficient Diet.

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The nutritional deficiency of the maternal diet has been shown to influence the developmental anatomy of rats *in utero*. Gross congenital malformations were induced in approximately one-third of the offspring of rats of the Sprague-Dawley and Baltimore strains reared and maintained on a deficient diet.[1,2,3,4] However, Wistar rats similarly reared and maintained failed to produce anomalous young (of 21 Wistar female rats, few reached maturity, and only one female had a litter, consisting of one normal young.)[3] From these observations it was suggested that a strain difference exists in the reactivity of rats to the deficient diet. We have repeated the immature Wistar rat experiments of Warkany *et al.*[3] noted above, in an attempt to determine if Wistar rats might produce litters with either normal or abnormal offspring. In addition adult Wistar rats have been similarly maintained.

The data, presented below, indicate that adult Wistar rats maintained on the same diet can produce anomalous young and that immature Wistar rats reared and maintained on the same diet grow poorly and generally do not attain sexual maturity.

The experimental diet employed was the following: yellow corn meal, 76 parts; wheat gluten, 20 parts; calcium carbonate C. P., 3 parts; and iodized sodium chloride, 1 part.† At least 50 international units of vitamin D in cod liver oil were given each animal by pipette each week. This diet is definitely deficient in vitamins of the B_2 group and is probably deficient in inorganic elements and

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1 Warkany, J., and Nelson, R., *Anat. Rec.*, 1941, **79**, 83.

2 Warkany, J., and Nelson, R., *J. Nutrition*, 1942, **23**, 321.

3 Warkany, J., Nelson, R., and Schraffenberger, E., *Am. J. Dis. Child.*, **64**, 960.

4 Warkany, J., and Schraffenberger, E., *J. Nutrition*, 1944, **27**, 477. Riboflavin has been identified as the factor whose absence is responsible for the anomalous offspring.

† Diet is essentially the same as used by Warkany *et al.*[2,3,4] and Remington[5] with the exception that the former supplemented the diet with 60 international units of vitamin D as viosterol every tenth day and the latter supplemented the diet with .2% dried yeast.