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Antibacterial Activity of Substances Related to *p*-Aminobenzoic Acid

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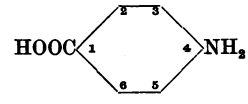
The current view of the mode of action of the sulphanilamide drugs is that these substances interfere with the growth of bacteria by virtue of a competitive inhibition of *p*-aminobenzoic acid, or some substance related thereto, which serves as an essential metabolite for the organisms. Further, this competitive inhibition is probably connected with a similarity in the spatial arrangement of the substituent groupings within the molecule of the growth-factor and growth-inhibitor, which provides them with an affinity for the same hypothetical enzyme system. It was, on this view, likely that substances which antagonize *p*-aminobenzoic acid might be devised from the parent structure, leaving the existing substituent groups intact, by introducing further substituents into the benzene nucleus. This has proved to be the case in certain instances.

While this paper was being prepared the results were published of similar investigations by Wyss, Rubin & Strandskov (1943) and by Johnson, Green & Pauli (1944), who have examined many of the compounds listed below. Nevertheless the present work has included compounds not used by the American workers and in addition more attention has been paid to *in vivo* experiments.

Derivatives of p-*aminobenzoic acid employed*

The substances shown in Tables 1 and 2, many of which are known compounds, were employed in the experiments. Table 1 contains the nuclear substituted derivatives and Table 2 a number of miscellaneous derivatives which were included in the investigation. The preparation of the new compounds will be described elsewhere.

Table 1. *Nuclear substituted* 4-*aminobenzoic acids*



Compound	Substituent(s)
I*	3-Chloro
II* †	2-Chloro
III*	3-Iodo
IV* †	3-Methyl
V*	2-Methyl
VI*	3-Hydroxy
VII* †	3-Methoxy
VIII*	2-Methoxy
IX‡	3-Ethoxy
X*	3-Amino
XI*	3-Carboxy
XII‡	3-Methylmercapto
XIII‡	3-Ethylmercapto
XIV‡	3-Methylsulphonyl
XV‡	3-Ethylsulphonyl
XVI	3:5-Dichloro
XVII	3:6-Dichloro
XVIII	3-Chloro-6-amino
XIX	3-Chloro-6-acetylamino
XX	3:5-Dibromo
XXI‡	3:6-Dimethyl
XXII‡	3:6-Dimethoxy
XXIII‡	2-Methoxy-5-methyl
XXIV‡	3-Methoxy-6-methyl

* Compounds used by Wyss, Rubin & Strandskov (1943).
† Compounds used by Johnson, Green & Pauli (1944).
‡ New compounds.

Behaviour towards streptococci in vitro

The general plan of all the experiments was the same. The organism used was *Streptococcus pyogenes*, 'Kruger' strain (Lancefield Group A), maintained by weekly subculture on agar. All tests were carried

Table 2. *Miscellaneous substances related to 4-aminobenzoic acid*

Compound	Constitution
XXV	2:2′-Diaminodiphenyldisulphide-5:5′-dicarboxylic acid
XXVI*	3-Chloro-4-aminobenzamide
XXVII*	2-(3′-Chloro-4′-aminobenzamido)-pyridine
XXVIII*	4:6-Dimethyl-2-(4′-aminobenzamido)-pyrimidine
XXIX	Ethyl-4-aminobenzoate
XXX	4-Benzamidobenzoic acid
XXXI*	4-(4′-Aminobenzamido)-benzoic acid
XXXII	2-Amino-benzthiazole-6-carboxylic acid
XXXIII	3-Amino-4-hydroxybenzoic acid

* New compounds.

out in Wright's broth to which 5% sterile defibrinated horse blood was added. Sufficient blood-broth to complete all the tubes in one test was infected with a 24 hr. culture of the test organism in plain broth to give a count of 200–500 colonies per ml. in the diluted culture. 0·1 ml. amounts of solutions of the drugs to be examined were placed in $3 \times \frac{1}{2}$ in. test-tubes in such concentrations that, when 1·0 ml. of the infected blood-broth was added to each, a range of concentrations 1 : 330, 1 : 1000, 1 : 3000, 1 : 9000, etc., was produced. The tubes were incubated for 24 hr. at 40°, inspected for haemolysis, and one loopful from each plated on blood-agar. A series of tubes containing sulphanilamide was always included as a standard.

The first compounds to be examined were the 2-methyl- (V), 3-methyl- (IV), 2-chloro- (II) and 3-chloro- (I) derivatives of 4-aminobenzoic acid. These derivatives were chosen as being the simplest representatives having typical non-polar and polar substituents in both possible positions in the benzene ring. Compounds II, IV, V did not inhibit streptococcal growth at concentrations of 1 : 330, but compound I partially inhibited growth at a concentration of 1 : 3000. A similar degree of inhibition was exerted by sulphanilamide in the same test at a concentration of 1 : 81,000. Thus compound I was about one twenty-seventh as effective as sulphanilamide in inhibiting streptococcal growth.

Following this initial examination the three compounds showing no antibacterial action were re-examined to see whether they retained the power of the parent compound, 4-aminobenzoic acid, to antagonize the action of sulphanilamide.

Five sets of tubes were prepared, containing medium and organisms as in the first experiment. Each series contained a range of sulphanilamide concentrations from 1 : 1000 to 1 : 243,000. The first set of tubes served as controls. The remaining tubes contained compounds II, IV and V each at a concentration of 1 : 1000. All tubes were incubated and examined as before, with the results shown in Table 3.

The 3-methyl- and 2-chloro-compounds are seen to resemble 4-aminobenzoic acid in antagonizing completely the growth-inhibitory action of sulphanilamide, whilst the 2-methyl-derivative is inert, neither restraining the growth of the organisms nor inhibiting the action of sulphanilamide upon them. No attempt was made to assess the relative sulphanilamide-antagonizing powers of 4-aminobenzoic acid and its 3-methyl- and 2-chloro-derivatives.

Some of the other compounds listed in Tables 1 and 2 were examined for antisulphanilamide activity; three only were found to possess this property, but only to a very slight degree. They were: 4-amino isophthalic acid (XI); 4-(4′-aminobenzamido)-benzoic acid (XXXI); ethyl 4-aminobenzoate (XXIX). Woods (1940) found the ester (XXIX) inactive. The absence of free acid from our sample was assured as far as possible by rigorous purification.

Two other compounds which showed marked antibacterial activity were 3:4-diaminobenzoic acid dihydrochloride (X) and 3-hydroxy-4-aminobenzoic acid (VI). The former had the same order of activity as the 3-chloro-compound, whilst compound VI was examined many times and found to be about one-third to one-ninth as inhibitory as sulphanilamide. This compound was the most active growth-inhibitor of the series examined. Table 4 gives the results of a typical experiment showing the relative inhibitory powers of these compounds.

Table 3. *Antagonism by 4-aminobenzoic acid and derivatives of inhibition of growth of* Strep. pyogenes *by sulphanilamide*

	Concentration of sulphanilamide, one part in						Control (no sulphanilamide)
	1,000	3,000	9,000	27,000	81,000	243,000	
Antagonist (1 : 1000 in each tube)	Effect on growth						
None	–	–	–	–	–	+	+
4-Aminobenzoic acid	+	+	+	+	+	+	+
Compound II	+	+	+	+	+	+	+
Compound IV	+	+	+	+	+	+	+
Compound V	–	–	–	–	–	+	+

– indicates no haemolysis in tubes after 24 hr. + indicates full haemolysis in tubes after 24 hr.

Table 4. *Antibacterial activity of compounds related to 4-aminobenzoic acid against* Strep. pyogenes

(The medium was 5% blood-broth; temperature of incubation 40°.)

Compound examined	Concentration, one part in: 1,000	3,000	9,000	27,000	87,000	243,000	729,000	Nil (control)
Sulphanilamide	−	−	−	−	+	+ +	+ + +	+ + +
Compound I	−	−	−	+	+ + +	+ + +	+ + +	+ + +
Compound VI	−	−	−	−	+ +	+ + +	+ + +	.
Compound X	−	−	−	+ +	+ + +	+ + +	+ + +	.

+ + + represents complete haemolysis.

It was observed that the antibacterial actions of 3-chloro- or 3-hydroxy-4-aminobenzoic acid were considerably decreased but not completely abolished by 4-aminobenzoic acid at a concentration of 1 : 1000 and that the antibacterial action of these compounds showed the delay in onset characteristic of sulphanilamide. This latter point was illustrated by the following experiment.

Three similar cultures of *Strep. pyogenes* in 5% blood-broth were prepared; one was kept as control and to the others was added either 1 : 1000 sulphanilamide or 1 : 1000 3-hydroxy-4-aminobenzoic acid. All the cultures were incubated at 37°. Samples were removed initially and after intervals of 3, 7 and 24 hr. These samples were plated in blood agar after suitable dilution. Table 5 shows the colony counts per ml. of culture.

Table 5. *Inhibition of growth of* Strep. pyogenes *by sulphanilamide and by 3-hydroxy-4-aminobenzoic acid*

Period of incubation (hr.)	Cultural conditions: medium plus: No addition	1 : 1000 sulphanilamide	1 : 1000 3-hydroxy-4-aminobenzoic acid
	Colony counts per ml. of culture		
0	2,000	2,000	2,000
3	100,000	50,000	50,000
7	10^8	130,000	100,000
24	$1{\cdot}5 \times 10^9$	6,000	12,000

Further evidence that the antibacterial action exerted by these compounds was essentially due to their relationship to 4-aminobenzoic acid was given by the observation in another experiment that neither benzoic acid nor 3-chlorobenzoic acid nor 3-amino-4-hydroxybenzoic acid (isomeric with VI) showed any antibacterial action at concentrations of 1 : 1000, whereas 3-chloro-4-aminobenzoic acid suppressed growth at 1 : 3000 in the same experiment.

Assessment of antistreptococcal activity in mice

Before carrying out therapeutic experiments with these compounds, some estimations were made of the blood concentrations in mice resulting from oral or subcutaneous dosings. Estimations were made by the method of Rose & Bevan (1944). The results are shown in Figs. 1 and 2 which are based on the concentrations found in pooled blood samples from groups of three mice given the drugs as dispersions in water.

It was obvious that absorption and elimination of these two compounds from the blood stream was extremely rapid, especially when compared with sulphanilamide. The most active compound, 3-hydroxy-4-aminobenzoic acid, being an *o*-hydroxy amine, did not readily undergo diazotization and coupling in the normal manner and hence could not be estimated. It is probable that it would disappear from the blood stream at least as rapidly as the other compounds.

In the therapeutic experiments conditions were so arranged that the drugs under examination had the greatest chance of exerting their action. A preliminary toxicity test indicated that doses of 10 mg./20 g. mouse, either of 3-chloro- or of 3-hydroxy-4-aminobenzoic acid given subcutaneously every 1–2 hr. during the daytime, were as much as the mice would tolerate without evident distress. The drugs were administered as relatively coarse suspensions, in order to prolong the period of absorption. The first dose was given at the time of infection. The mice were infected intraperitoneally with a relatively small number of organisms (0·25 ml. of a 1 : 10^6 dilution of an 18 hr. blood-broth culture of either *Streptococcus pyogenes* ('Kruger' strain) or *Diplococcus pneumoniae* (Type I). The treatments given are shown in Table 6.

The therapeutic results obtained in these experiments are not very marked, but are quite definite. Considering the limited *in vitro* activity of the compounds concerned and the rapidity with which they are removed from the blood stream, they are probably as good as can be expected.

An attempt was made to neutralize the limited therapeutic effect demonstrated by 3-hydroxy-4-aminobenzoic acid by the simultaneous administration of 4-aminobenzoic acid. This was not successful

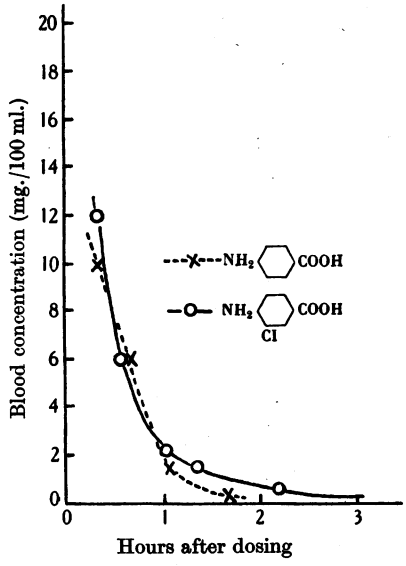


Fig. 1. Blood concentrations following single oral doses of 5 mg./20 g. mouse of 4-aminobenzoic acid and 3-chloro-4-aminobenzoic acid.

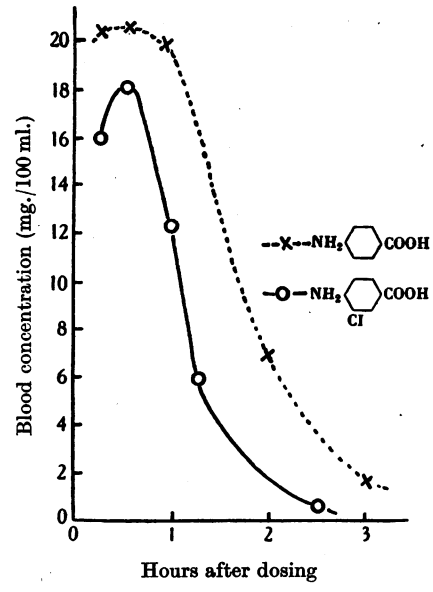


Fig. 2. Blood concentrations following single subcutaneous doses of 5 mg./20 g. mouse of 4-aminobenzoic acid and 3-chloro-4-aminobenzoic acid.

Table 6. *Therapeutic experiments, using two* p-*aminobenzoic acid derivatives against infection with* Strep. pyogenes *and* Diplococcus pneumoniae

(Infecting dose of organism: 0·25 ml. of 1:10^6 dilution of 18 hr. blood-broth culture.)
Dose of drug: 10 mg./20 g. mouse.

Infecting organism	Treatment	No. of mice	Observations at 24 hr.	30 hr.	48 hr.	72 hr.	Remarks
Streptococcus pyogenes	None (controls)	6	2 dead 2 very ill	6 dead	—	—	—
	3-Hydroxy-4-amino-benzoic acid: 7 doses on first day; 7 doses on second day; 2 doses on third day	6	All alive and well	All alive but slightly depressed	All alive and fairly well	6 dead	All control mice dead more than 18 hr. before first treated mouse died
Streptococcus pyogenes	None (controls)	12	None dead	10 dead	11 dead	11 dead	10 controls dead before first treated mouse died
	3-Hydroxy-4-amino-benzoic acid: 4 doses on first day	12	None dead	None dead	10 dead	11 dead	
Diplococcus pneumoniae (Type I)	None (controls)	12	4 dead	12 dead	—	—	18 hr. after all controls died, only 2 of treated group had died
	3-Hydroxy-4-amino-benzoic acid: 7 doses on first day; 5 doses on second day	12	None dead	None dead	2 dead	12 dead	
Streptococcus pyogenes	None (controls)	6	3 dead	6 dead	6 dead	—	Differences in death rates not significant, but treated group remained well for a longer period and died suddenly
	3-Chloro-4-amino-benzoic acid: 4 doses on first day; 3 doses on second day	6	1 dead	5 dead	6 dead	—	

because of the large doses it was necessary to use; the infected mice receiving both compounds died more quickly than those receiving 3-hydroxy-4-aminobenzoic acid alone. One of a group of control uninfected mice died after receiving the same treatment.

The above results suggest an explanation of the *in vivo* therapeutic action of 4-nitrobenzoic acid upon pneumococci which has been reported (Mayer & Oechslin, 1939). It is known that 4-nitrobenzoic acid is reduced in the animal body (Flynn & Kohl, 1941). It is possible that 4-hydroxylaminobenzoic acid arises during this process and may be rearranged to the active 3-hydroxy-4-aminobenzoic acid.

SUMMARY

1. Thirty-three substances related to 4-aminobenzoic acid have been examined for inhibitory action on the growth of streptococci.

2. Three of them, 3-hydroxy- and 3-chloro-4-aminobenzoic acid and 3:4-diaminobenzoic acid, were found to be inhibitory. The action of the first substance was approximately one-third to one-ninth that of sulphanilamide *in vitro*. This substance exhibited a slight therapeutic effect in mice infected with streptococci or pneumococci.

3. Five of the compounds examined antagonized the bacteriostatic effect of sulphanilamide on streptococci *in vitro*.

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The Colorimetric Estimation of Diethylstilboestrol, Hexoestrol and Dienoestrol

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The extensive use of synthetic oestrogens in both human and veterinary medicine has made the chemical assay of these substances a matter of great importance. Methods have already been described for the estimation of diethylstilboestrol (4:4′-dihydroxy-$\alpha\beta$-diethylstilbene) by absorption spectrophotometry (Elvidge, 1939), by volumetric analysis (Sondern & Burson, 1942), and by colorimetry (Dingemanse, 1940; Dechene, 1941; Tubis & Bloom, 1942; Huf & Widmann, 1942; Cocking, 1943; Dracass & Foster, 1943). Elvidge (1939) has also proposed absorption spectrophotometry as a suitable method for the estimation of hexoestrol (4:4′-dihydroxy-$\gamma\delta$-diphenyl-*n*-hexane), but otherwise no chemical methods are available for the assay of this substance, or of dienoestrol (4:4′-dihydroxy-$\gamma\delta$-diphenyl-$\Delta^{\beta\delta}$-hexadiene).

The methods described here differ from one another only in their detail, and are based on the recommendations of Stoughton (1936), who advised the use of acetic acid as a solvent in the estimation of non-water-soluble phenolic compounds and described the development of a yellow colour after treating such solutions with nitric acid and their subsequent neutralization.

METHODS

Standard solutions. Standard oestrogen solutions are prepared by dissolving the appropriate oestrogen in glacial acetic acid to give a final strength of 0·1% (w/v). It is necessary to store these solutions in the dark in order to prevent the formation of a yellow colour (diethylstilboestrol, hexoestrol) or to retard its development (dienoestrol). No loss of chemical activity could be detected in these solutions after 1 month's (diethylstilboestrol) or 6 months' (hexoestrol, dienoestrol) storing and the presence of a yellow colour in dienoestrol solutions after such a period was not correlated with any loss in activity as determined by chemical assay. Possible effects of storing on the biological activity of the oestrogens were not investigated.

Unknown solutions. For all estimations the oestrogen is required in solution in glacial acetic acid (minimum strength approx. 0·01% (w/v)). The procedure used for this purpose will depend upon the nature of the preparation to be investigated. Ether extraction of certain aqueous suspensions,