6163

Rôle of the NH_2, OH and As = As Groups in Parasitotoxic Action of Arsphenamine Derivatives.

L. REINER AND C. S. LEONARD.

From the Experimental Research Laboratories, Burroughs Wellcome Co., Tuckahoe, N. Y.

Some of the derivatives of arsphenamine differ from one another considerably in their *in vitro* toxicity towards trypanosomes but are more alike in their chemotherapeutic activity. The reasons for this are probably manifold. The chemotherapeutically active compounds may be of the RAsO type (Ehrlich,[1] Voegtlin and Smith[2]) and the rates of formation of the substituted arsenious oxides from the RAs = AsR forms may differ, Mayer[3] and Maschmann[4] have suggested that the *in vitro* toxicity is due mainly to the other groups substituted on the benzol ring.

In order to draw conclusions as to the rôle of single groups in the toxicity, we have compared the toxic action of neoarsphenamine on *Trypanosoma equiperdum,* and also the color reaction of the drug with osmic acid, with the same properties of compounds of the RAsO type, lacking one or both of the substituted groups, OH and NH_2 possessed by the arsenious oxide corresponding to arsphenamine.

The reaction of neoarsphenamine with osmic acid (Hiramatsu[5]). To attempt to determine the amount of unchanged neoarsphenamine in a solution of the same exposed to the air, we used Hiramatsu's osmic acid colorimetric method, claimed by the author to determine quantitatively small amounts of neoarsphenamine even in the presence of protein. That a purple color is given by unoxidized solutions of neoarsphenamine or of 3-amino-4-hydroxyphenylarsenious oxide ("Arsenoxide") was confirmed. It was further observed that the reaction can be prevented by the addition of sufficient sodium thioglycollate (a few drops of M/20 $NaOOCCH_2SH$ in about 2 cc. of M/2000 neoarsphenamine or "Arsenoxide"). Although the color is probably due to the formation of colloidal osmium after reduction of osmic acid by the arsenical, the inhibition of reaction by

[1] Ehrlich, P., *Berichte,* 1909, **42**, 17.

[2] Voegtlin, C., and Smith, H. W., *J. Pharmacol.,* 1920, **15**, 475.

[3] Mayer, R. L., *Klin. Woch.,* 1926, **5**, 1699.

[4] Maschmann, E., *Berichte,* 1926, **59**, 1142, 1148.

[5] Hiramatsu, T., *Sei-i-Kwai Med. J.,* 1929, **48**, 133; *Chem. Absts.,* 1930, **24**, 392.

thioglycollate was first interpreted as an action on the —As = As— or the —AsO groups (formation of thioarsenites, Cohen, King and Strangeways[6]). Tests showed that neither p-aminophenylarsenious oxide nor phenylarsenious oxide* give the Hiramatsu reaction, while p-aminophenol *gives* the reaction. Furthermore, the development of color with the latter reagent can also be inhibited by the addition of thioglycollate. Sodium sulfite and sodium thiosulfate inhibited the osmic acid reaction of neoarsphenamine, of "Arsenoxide" and of p-aminophenol.

We conclude that Hiramatsu's test is *not specific* for trivalent arsenic bound to benzol, but rather is due to the joint presence of the amino and hydroxyl groups on the ring (hydroxyaminophenyl grouping) whether arsenic is present or not. Hence the state of oxidation of the arsenic will not be indicated by this test, nor should it be affected by splitting off the arsenic if the hydroxyaminophenyl grouping is unchanged. Since Hiramatsu found the blood of humans to give a positive reaction even several weeks after administration of neoarsphenamine, a considerable part of the injected dose must, at this late time, still bear unchanged $OH.NH_2\phi$ grouping.

Action of quinoid compounds on trypanosomes. Since thiol compounds inhibit the osmic acid reaction of aminophenol (probably an oxidation of the aminophenol) thiol compounds might interfere with the toxicity of aminophenol to trypanosomes, which appears dependent on auto-oxidation of the aminophenol. If commercial preparations of p-aminophenol hydrochloride (as a rule slightly colored) are, after careful neutralization, mixed with trypanosomes suspended in fresh plasma almost immediate parasiticidal action is observed at concentrations above 1%. Purified p-aminophenol, recrystallized as the hydrochloride, is distinctly less toxic to the parasites (Table I). Solutions exposed to the air for several days are more toxic than fresh solutions. Apparently p-aminophenol becomes toxic only as it is oxidized, probably to an iminoquinone. Oxidized p-aminophenol solutions are detoxified by thiol compounds. As we find that p-benzoquinone is highly toxic to trypanosomes, it seems likely that the quinoid configuration is concerned in the toxicity of oxidized aminophenol. The toxic action of quinone is also

[6] Cohen, A., King, H., and Strangeways, W. I., *J. Chem. Soc.*, 1931, 3043, 3236.

* 3-amino-4-hydroxyphenylarsenious oxide was kindly furnished us by Dr. Carl Voegtlin of the National Institute of Health, Washington, D. C., to whom the writers desire to express their gratitude. For the preparation of the other arsenious oxides used in this work, the writers are indebted to Dr. J. S. Buck of these laboratories. Our strain of *Tr. equiperdum* was kindly furnished 3 years ago by Dr. George McCoy, Director of the National Institute of Health.

TABLE 1.
Action of p-aminophenol on trypanosomes and its inhibition by sodium thioglycollate.

No.	Agent	Motility after 15 min. at dilutions* cited: 1/3	1/6	1/12	1/24	1/48	1/96	1/192	Control
1	A = p-aminophenol	—	—	—	—	+	++	++	++
2	A + Na thioglycollate	—	+	++	++	++	++	++	++
3	B = p-aminophenol,recrystallized	—	+	++	++	++	++	++	++
4	B + Na thioglycollate	+	++	++	++	++	++	++	++

Legend: — = not motile, + = slightly motile, ++ = fully motile.

* Technique: Trypanosome emulsion (1) obtained by centrifuging citrated blood of infected rat. p-aminophenol solution (2) = 50 mg. in 3 cc. H_2O, warmed, neutralized with 0.12 cc. 2N NaOH, made to 5 cc. with 0.85% NaCl with which dilutions were also made. Na thioglycollate solution (3) = 0.20 cc. of thioglycollic acid neutralized with 1.60 cc. 2N NaOH, made to 20 cc. with 0.85% NaCl, Exps. 1 and 3: 0.25 cc. of dilutions of (1) +0.25 cc. rat serum + 0.25 cc. of (2) were mixed and motility observed under the microscope, after 15 min. In experiments 2 and 4, 0.25 cc. of (3) were added. Controls the same, without A or B.

TABLE II.
Action of quinone on trypanosomes and its inhibition by sodium thioglycollate.

No.	Agent	Motility at* 5 min.	15 min.	60 min.
5	C = Quinone	—	—	—
6	C + Na thioglycollate	++	++	++

* Technique: Trypanosome emulsion (1) and thioglycollate solution (2) as in Table 1. Quinone solution (3) 20 mg. quinone in 20 cc. 0.85% NaCl solution (4). Locke solution (5) with 0.5% glucose. Exp. 5 = 0.6 cc. (5) + 0.1 cc. (3) + 0.2 cc. (1) + 0.1 cc. (4). After 10 min. 0.2 cc. rabbit plasma. Exp. 6 like Exp. 5 but 0.1 cc. (2) in place of 0.1 cc. (4).

hindered by sodium thioglycollate (Table II). One might think that part or all of the inhibitory effect of thiol compounds on arsphenamine and arsenoxides is due to inhibition of oxidation of the $OH.NH_2\phi$ grouping. But the fact that the toxic action of benzoquinone imine can also be inhibited by thiosulfate and sulfite (Table III), whereas the toxicity of neoarsphenamine is inhibited only by thiol compounds, indicates that part of the toxicity of arsphenamine arises from other than the $OH.NH_2\phi$ grouping. That a part of the toxicity is contributed by this grouping may account for the differences in *in vitro* toxicity between derivatives of arsphenamine with chemically substituted or "covered" OH or NH_2 groups and that of arsphenamine itself in which the groups are free. On the other hand, that *in vivo* many compounds of arsphenamine type behave like the parent substance may be due to ready formation from them all of one and the same "uncovered" arsenious oxide. The positive Hiramatsu test which is due to the presence of $OH.NH_2\phi$ groups, and which is given by neoarsphenamine, supports this view.

TABLE III.
Action of p-aminophenol in the presence of $Na_2S_2O_5$ and $Na_2S_2O_3$.

No.	Agent	Motility after 20 min. at dilutions: 1/8	1/16	1/32	1/64	1/128	1/256	1/512	Control
7	A (Table I)	—	—	—	—	—	++	++	++
8	A + $Na_2S_2O_5$	—	—	+	++	++	++	++	
9	A + $Na_2S_2O_3$*	—	—	+	+	+	+	+	
10	B (Table I)	—	—	—	+	++	++	++	++
11	B + $Na_2S_2O_5$	—	+	++	++	++	++	++	
12	B + $Na_2S_2O_3$	—	+	+	+	+	+	+	

* $Na_2S_2O_3$ solutions are somewhat toxic alone.

Technique: Trypanosome emulsion (1) and solutions (2) of A and B as in Table I. $Na_2S_2O_5$ solution (3) 95 mg. in 10 cc. 0.85% NaCl solution. $Na_2S_2O_3$ solution (4) = N/20.

Experiments: 0.5 cc. of dilution of (2): 0.5 cc. of 0.85% NaCl, or (3) or (4): 0.5 cc. rat citrate plasma (containing about 2 parts of 3.5% sodium citrate solution in 5 parts) + 0.5 cc. (1). Observation after 20 min. Control the same but without A or B.

TABLE IV.
Lack of influence of $Na_2S_2O_5$ on action of 3-amino-4-hydroxyphenylarsenious chloride (D*) on trypanosomes.

No.	Agent	Motility after 20 min. 1/128	1/256	1/512	1/1024	1/2048	1/4096	Control
13	D	—	—	+	++	++	++	++
14	D + $Na_2S_2O_5$	—	—	+	++	++	++	++

Technique: Trypanosome emulsion (1) as in Table I. $Na_2S_2O_5$ (2) solution as in Table III. (3) Solution of D (about M/200) 12.5 mg. in 10 cc. of 0.85% NaCl solution. In Exp. 13, 0.5 cc. of the dilution of (3) + 0.5 cc. of 0.85% NaCl + 0.5 cc. of (1) in rabbit plasma. Exp. 14 the same except 0.5 cc. of (2) replaced the 0.85% NaCl. Control the same without D.

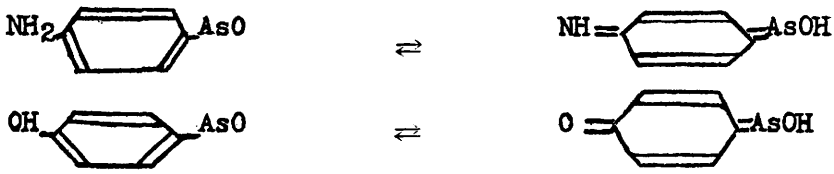
* This substance is assumed to hydrolyze in water giving a hydrate of the organic arsenious oxide.

We next studied the *in vitro* toxic action of arsphenamine derivatives on trypanosomes in the presence of reducing agents and found that they behave distinctly differently from p-aminophenol with respect to detoxification by these agents. Such experiments have been reported by Voegtlin and his coworkers[7, 8] and ourselves,[9] and the following experiments (Table IV) only confirm and complete this picture. Sulfite and thiosulfate were not found noticeably to decrease the toxic action. *In vitro* toxic action was seen even with arsenious oxides not containing both the OH and NH_2 groups. However, it might be assumed that these compounds could also exist in a quinoid modification and thus their activity be connected chiefly with this property. The following tautomerism may exist:

[7] Voegtlin, C., Dyer, H., and Leonard, C. S., *U. S. Public Health Reports*, 1923, **38**, 1882.

[8] Rosenthal, S. M., and Voegtlin, C., *J. Pharmacol.*, 1930, **39**, 347.

[9] Reiner, L., Leonard, C. S., and Chao, S. S., *Arch. intern. pharmacodynamie*, in press.



However, the fact that unsubstituted phenyl arsenious oxide is highly toxic to trypanosomes and that its toxicity is markedly inhibited by thioglycollate proves the essential toxicity of the trivalent arsenic attached to the benzol ring.† It also speaks for the detoxification mechanism by formation of thioarsenites,[6, 8] which we[9] have shown occurs in the medium, shielding the parasites from adsorption of the arsenic. That an additional toxicity due to OH and NH_2 groups may manifest itself when these groups are present is not excluded. Possibly a further contribution by these groups when present "uncovered" may be concerned with a catalysis by the iminoquinonyl-aminohydroxyphenyl oxidation-reduction system of the oxidation of the arsenogrouping to the toxic arsenious oxide stage.

Summary. 1. Hiramatsu's reaction is given by compounds possessing aminophenol groups and the presence of arsenic is not necessary. 2. p-Aminophenol is not toxic to trypanosomes but its autoxidation product, probably iminoquinone, is highly toxic. Reducing agents, such as sulfite, thiosulfate and thiol compounds, inhibit the toxic action. 3. Quinone is toxic to trypanosomes and its toxicity is inhibited by thioglycollate. 4. Various organic arsenious oxides, not capable of giving on oxidation iminoquinone structure, are more toxic to trypanosomes than are oxidized aminophenol and quinone. 5. The toxicity of arsphenamine derivatives to trypanosomes is due chiefly to the presence of trivalent arsenic and not chiefly to hydroxyaminophenyl groupings, although these may contribute to the *in vitro* toxicity.

† Phenyl arsenious oxide is very slightly soluble in water. The test was, therefore, carried out in the following way: 2 mg. were dissolved in 1 cc. of ethyl alcohol and this solution diluted 1/200 with a mixture of 3 parts of physiological salt solution and 1 part of rat plasma. Trypanosomes suspended in dilute rat plasma were killed almost immediately by this solution. Control emulsions containing equal amounts of ethyl alcohol per cc. could be kept for a few hours at room temperature.