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Effect of Ring Substitution on the Hemolytic Action of Arylhydrazines[1] (38409)

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Phenylhydrazine is oxidized by oxygen or potassium ferricyanide to phenyldiazene ($C_6H_5-N=NH$), an unstable compound which reacts with ferrihemoglobin to produce a compound with the visible absorption spectrum of a ferrihemochromogen (1, 2). Formation of the latter compound has been postulated to destabilize the protein molecule and contribute to the formation of Heinz bodies (2). Ring-substituted phenylhydrazines such as tolylhydrazine and hydrazinobenzoic acid are also oxidized to aryldiazenes (3). We have investigated the influence of substituents on the benzene ring on the effectiveness of arylhydrazines as hemolytic agents, on the one hand, and on the reactivity of corresponding aryldiazenes toward ferrihemoglobin on the other.

Materials and Methods. Phenylhydrazine HCl (Eastman) was recrystallized twice from ethanol. Tolylhydrazine (*o*-, *m*-, and *p*-) HCl and *p*-hydrazinobenzoic acid HCl (Aldrich) were recrystallized twice from methanol. Aqueous solutions of the arylhydrazine hydrochlorides were neutralized with NaOH and stored at 2° under nitrogen.

Female Sprague–Dawley rats (210 ± 15 g, *ad lib.* diet) were used. Blood was obtained by tail clipping and collected in heparinized capillary tubes for microhematocrit determinations. Usually two microhematocrits were obtained from each animal at each bleeding. Groups of three rats were injected subcutaneously with 41 μmoles of the test compound on Days 1, 2, and 4 of the experiment so that a total of 123 μmoles was administered over 4 days. On Days 5 and 7 microhematocrit determinations were obtained. An additional sample was obtained in a heparinized capillary tube on Day 7 from one animal in each group for a reticulocyte count. Five hundred cells or 100 reticulocytes were scored for each count.

Human ferrihemoglobin solution was prepared by oxidation of washed and centrifuged red blood cell lysates with potassium ferricyanide (1). Excess ferricyanide was removed by dialysis against phosphate buffer of pH 6.8, μ 0.2. Each of the arylhydrazines was oxidized to aryldiazene with potassium ferricyanide (2, 3), and the aryldiazene was reacted with ferrihemoglobin (1). Visible absorption spectra were taken of the ferrihemoglobin with the Cary Model 17 recording spectrophotometer before and after addition of aryldiazene.

Results. The effect of different substituents para to the hydrazino group are shown in Table I. *p*-Tolylhydrazine induced as much anemia and reticulocytosis as did unsubstituted phenylhydrazine. In contrast, *p*-hydrazinobenzoic acid had no significant effect on either parameter. The effect of a methyl group ortho, meta, or para to the hydrazino group was tested, and the results of Table II indicate that the position of the methyl group was without significant effect.

A difference in the effects of methyl and carboxy substituents was also found in the reactions of aryldiazenes with ferrihemoglobin. The tolyldiazenes ($H_3C-C_6H_4-N$ = NH) behaved like phenyldiazene in producing ferrihemochromogen spectra when reacted with ferrihemoglobin. On the other hand, *p*-

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TABLE I. Effect of Ring Substitution on Induction of Anemia and Reticulocytosis by Arylhydrazines.

Compound	Day	Hematocrit[a] 1	5	7	Reticulocytosis[b] 7
NaCl (saline)		46	47	43	1.3%
Phenylhydrazine		48	31	40	55.4%
p-Tolylhydrazine		45	31	41	52.3%
p-Hydrazinobenzoic acid		50	43	43	2.2%

[a] Each recorded hematocrit represents the average value from three rats; usually two microhematocrits were obtained from each sample.

[b] Each recorded reticulocyte count is a single determination from each group of animals.

diazenylbenzoic acid (HOOC–C_6H_4–*N* = NH) did not produce a ferrihemochromogen, but instead reduced ferrohemoglobin to ferrohemoglobin. Formation of ferrohemoglobin was confirmed by reaction of the product with carbon monoxide and nitrosobenzene to form CO–hemoglobin and C_6H_5NO–hemoglobin (4), respectively.

Discussion. Among the arylhydrazines of the present study, those that cause hemolysis, namely phenylhydrazine and the tolylhydrazines, are also the ones oxidized to aryldiazenes that convert ferrihemoglobin to ferrihemochromogen. Hydrazinobenzoic acid, which does not cause hemolysis, is oxidized to diazenylbenzoic acid, which reduces ferrihemoglobin to ferrohemoglobin. Thus, ability of aryldiazene to cause formation of ferrihemochromogen *in vitro* parallels the ability of the parent arylhydrazine to produce hemolysis *in vivo*. Other substituted phenylhydrazines are being investigated in order to determine the structural basis of this relationship. Chemical studies of the reaction of aryldiazenes with ferrihemoglobin will be described in detail elsewhere.[3]

Phenylhydrazine and its acetylated derivative 1-acetyl-2-phenylhydrazine have been used for many years to produce hemolysis and reticulocytosis; however, these compounds have been found to be toxic (5). A major end product in the oxidation of phenylhydrazine with oxygen is benzene (6), a highly toxic compound. On the other hand, toluene, the corresponding product of oxidation of tolylhydrazine, is much less injurious than benzene, especially to the hematopoietic system (7). The present study has shown that *o*-, *m*-, and *p*-tolylhydrazine are as effective as phenylhydrazine in causing hemolysis. The low toxicity of toluene suggests the use of tolylhydrazine and acetyltolylhydrazine for the production of hemolysis and reticulocytosis.

Summary. Hemolytic anemia and reticulocytosis result when phenylhydrazine or *o*-, *m*-, or *p*-tolylhydrazine is injected, but not when *p*-hydrazinobenzoic acid is injected. Phenyldiazene or tolyldiazene reacts with ferrihemoglobin to produce a compound with the visible

[3] Itano, H. A., and Mannen, S., manuscript in preparation.

TABLE II. Effect of Position of Methyl Group on Induction of Anemia and Reticulocytosis by Arylhydrazines.

Compound	Day	Hematocrit[a] 1	5	7	Reticulocytosis[b] 7
Phenylhydrazine		46	28	39	76.8%
p-Tolylhydrazine		45	27	38	56.6%
m-Tolylhydrazine		48	29	41	75.4%
o-Tolylhydrazine		44	30	36	64.3%

[a] Each recorded hematocrit represents the average value from three rats; usually two microhematocrits were obtained from each sample.

[b] Each recorded reticulocyte count is a single determination from each group of animals.

absorption spectrum of a ferrihemochromogen. On the other hand, *p*-diazenylbenzoic acid does not produce a ferrihemochromogen, but reduces ferrihemoglobin to ferrohemoglobin.

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