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Reduction of Isomeric Nitrobenzoic Acids by Rats.*

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Mayer and Oechslin[1] demonstrated that p-nitrobenzoic acid has a bacteriostatic effect upon pneumococci. p-Aminobenzoic acid, the reduction product of the nitro-compound, has been shown by Woods,[2] Selbie,[3] Rubbo and Gillespie,[4] Janeway,[5] and others to inhibit the bacteriostatic effect of sulfanilamide. It is of interest that Miller found in experiments with *Streptococcus viridans* that p-aminobenzoic acid also inhibits the bacteriostatic effect of p-nitrobenzoic acid.[6] Sherwin and Hynes[7] investigated the metabolism of the isomeric nitrobenzaldehydes in man, dog and rabbit, and recovered crystalline compounds from the urine. They concluded that the aldehydes were oxidized to the corresponding acids but found no reduction of the nitro- group.

We have studied the reduction of the isomeric nitrobenzoic acids *in vitro* by liver and kidney tissues. We have also investigated the reduction of these compounds *in vivo* by rats after oral or intraperitoneal administration.

In Vitro Experiments. Preparation and incubation of tissue suspensions were carried out as in previous experiments on the reduction of p-nitrobenzenesulfonamide.[8] Solutions of the isomeric nitrobenzoic acids were neutralized with NaOH. Each 10 cc of brei, corresponding to 2.5 g of tissue, contained 2 mg or 5 mg of substrate and M/10 PO_4 buffer, pH 7.5. Aliquot portions of the suspension were removed at intervals during the incubation period for analysis. Proteins were precipitated with 10% trichloracetic

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1 Mayer, R. L., and Oechslin, C., *Arch. intern. pharmacodynamie*, 1939, **62**, 211.

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

3 Selbie, F. R., *Brit. J. Exp. Path.*, 1940, **21**, 90.

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

5 Janeway, C. A., *J. Am. Med. Assn.*, 1941, **116**, 941.

6 Miller, J. K., *J. Pharm. and Exp. Ther.*, 1941, **71**, 14.

7 Sherwin, C. P., and Hynes, W. A., *J. Biol. Chem.*, 1921, **47**, 297.

8 Flynn, L. M., and Kohl, M. F. F., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 466.

acid and the amounts of diazotizable material in the filtrates were determined by the use of the Marshall sulfanilamide test reagents.[9] The ortho-isomer coupled so slowly that readings for this compound were made three hours after addition of the reagents. Full color development required an hour with the meta-isomer. Colorimetric readings could be made fifteen minutes after addition of the reagents to the para-isomer.

Results of typical tissue experiments are shown in Table I. The reduction of ortho-nitrobenzoic acid to diazotizable, free amino-compound is accomplished to only a small extent by tissue suspensions. Meta- and para-nitrobenzoic acids are reduced readily to diazotizable compounds by suspensions of rat or mouse livers and by rat kidney.

In Vivo Experiments. The isomeric nitrobenzoic acids were administered orally or intraperitoneally as aqueous solutions, neutralized with NaOH, to rats that weighed about 300 g. The animals were kept in individual cages, and the urine was collected under toluene. Urine samples collected at intervals were analyzed for free and conjugated amine, by the use of Marshall's reagents, as described earlier in this paper. Urine samples were analyzed for total amino-compounds after reduction with zinc and hydrochloric acid by methods previously described.[8] This determination included unchanged nitro-compound and possible intermediate reduction products. Similar treatment of known amounts of the isomeric nitrobenzoic acids gave the following yields of diazotizable compounds: o-nitrobenzoic acid, 50% to 70% of the theoretical yield; m-nitrobenzoic acid, 84% theoretical; p-nitrobenzoic acid, 94% to 102% theoretical. In all

TABLE I.
Reduction of Isomeric Nitrobenzoic Acids to Diazotizable Compounds by Tissue Suspensions.

Exp. No.	Isomer tested	Substrate concentration mg%	Tissue	% substrate changed				
				2 hr	4 hr	6 hr	7 hr	8 hr
1	Ortho	50	Rat liver	0.65	0.97		1.43	
2	"	20	Mouse liver	0.86	2.83	2.83		
1	Meta	50	Rat liver	4.8	12.4		30.3	
2	"	20	Mouse liver	7.0	12.2	23.5		
3	"	20	Rat kidney	5.2	12.0		20.4	
1	Para	50	Rat liver	9.1	14.7		28.2	
2	"	20	Mouse liver	9.9	18.0	23.2		
4	"	50	Rat kidney	3.0				10.6

[9] Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, **128**, 537.

experiments corrections were made for the small amount of diazotizable material excreted in the urine of normal rats.

Data from typical experiments are shown in Table II. Percentages of injected or ingested drugs recovered in the urine were as follows: ortho-isomer, 72%; meta-isomer, 63%; para-isomer, 89-94%. Excretion of the para-isomer was complete in 48 to 72 hours. Small traces of the ortho- and meta-compounds were still present in the urine after 96 hours. 88% of the total ortho-compound recovered was excreted in the first 24 hours; 80% was excreted within 8 hours. 21% of the recovered ortho-compound was excreted as free amine; amounts of conjugated amine and higher oxidation products could not be determined without altering the method used. 84% of the total meta-compound recovered was excreted in the first 24 hours; 80% was excreted within 8 hours. Of the total amount of meta-isomer recovered 8% was free amine, 22% was conjugated amine, and 70% was excreted in a state of oxidation above the amine. With the para-compound 94% of the amount recovered was excreted during the first 24 hours; 80% was excreted within the first 8 hours. Of the total amount of the para-isomer recovered 2.5% to 4% was free amine, 11% to 20% was conjugated amine, and 76% to 87% was excreted in a state of oxidation above the amine.

The excretion by the rats of 76% to 87% of the p-nitrobenzoic acid in a state of oxidation above the amine is in decided contrast to our results in similar tests with p-nitrobenzenesulfonamide. Only

TABLE II.
Reduction of Isomeric Nitrobenzoic Acids to Diazotizable Compounds by Rats.

Isomer	Dose,* mg	Route	% of dose recovered	Free amine, mg	Total amine after hydrolysis, mg	Total amine after reduction, mg	Distribution of compounds recovered: Free amine, %	Conjugated amine, %	Not reduced to amine in animal body, %
Ortho	20	Intraperitoneal	72.1†	2.45		11.84†	20.6		
Meta	20	"	62.6	0.83	3.07	10.27	8.1	21.8	70.1
Para	20	"	89.4	0.58	3.51	14.67	3.9	20.0	76.1
"	20	Oral	93.4	0.39	2.06	15.33	2.5	10.9	86.6
"	20	"	94.0	0.39	3.52	15.43	2.5	20.3	77.2

*20 mg nitrobenzoic acid is equivalent to 16.41 mg of aminobenzoic acid.

†The formation of the undiazotizable anhydride, anthranil, during the heating of o-aminobenzoic acid at an acid pH makes these values too low.

5% to 16% of the p-nitrobenzenesulfonamide is excreted in a state of oxidation above the amine.[8]

Conclusions. p-Nitrobenzoic and m-nitrobenzoic acids are readily reduced to amino-compounds by tissue suspensions. o-Nitrobenzoic acid, in contrast, is reduced to only a small extent by tissue suspensions.

When p-nitrobenzoic acid or m-nitrobenzoic acid is administered to rats only a small fraction of the dose is excreted in the urine in the reduced form, the remainder is present in a state of oxidation above the amine. The reduced fraction is conjugated to a high degree. o-Nitrobenzoic acid is also reduced by rats; an appreciable amount of free amine is recoverable from the urine.

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Occurrence of Hydrogenase in Nitrogen-Fixing Organisms.*

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Several years ago this laboratory[1] demonstrated that molecular H_2 specifically inhibits the assimilation of atmospheric N_2 by red clover plants inoculated with *Rhizobium trifolii.* In an effort to determine the mechanism of this unusual type of inhibition, cultures of the bacteria were tested for *hydrogenase,* the enzyme which Stephenson and associates[2] found in several species of heterotrophic organisms catalyzing the reversible reaction: $H_2 \rightleftarrows 2H$. Attempts to find the enzyme in cultures of *Rh. trifolii* failed. Recently Wyss and Wilson[3] showed that H_2 likewise inhibits nitrogen fixation by the free-living *Azotobacter.* Accordingly, species of this organism were tested for *hydrogenase,* and in contrast with the earlier results using the root nodule bacteria the azotobacter species were found to possess an active enzyme. This report presents the evidence proving the existence of the enzyme; a future paper will discuss its properties and possible significance for the mechanism of biological nitrogen fixation.

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1 Wilson, P. W., *Biochemistry of Symbiotic Nitrogen Fixation,* Madison, Wis., 1940.

2 Stephenson, M. S., *Bacterial Metabolism,* London, 1939.

3 Wyss, O., and Wilson, P. W., *Nat. Acad. Sci., Proc.,* 1941, **27**, 162.