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Reduction of p-Nitrobenzenesulfonamide and of Azobenzene-4,4'-disulfonamide by Animal Tissues.*

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The fate in the animal body of p-nitrobenzenesulfonamide, the nitro-compound corresponding to sulfanilamide, is of interest because of the sensitivity of microörganisms to this substance. Mayer and Oechslin[1] found it to have high antistreptococcal activity *in vivo*, and recently Burton, McLeod, McLeod and Mayr-Harting[2] have reported marked sensitivity of organisms of the Neisseria, Bacterium and Clostridium groups to it *in vitro*. The question arises whether the nitro-compound is effective as such or in consequence of its reduction to sulfanilamide or intermediate products.

As a contribution to this question we have studied the reduction of p-nitrobenzenesulfonamide *in vitro* by rat liver, and the extent of its reduction *in vivo* by normal rats. The reduction of azobenzene-4,4'-disulfonamide, an oxidation product of sulfanilamide, has also been investigated.

In vitro experiments. Fresh liver was ground with sand and water and squeezed through cheese cloth. To this suspension was added an equal volume of M/10 PO_4 buffer, pH 7.5, in which p-nitrobenzenesulfonamide had been dissolved. Each 5 cc of the liver suspension, as incubated, contained 2.5 mg of p-nitrobenzene sulfonamide and represented 1.25 g of liver. Two drops of toluene were added as a preservative. Aerobic incubation at 37.5°C was carried out in 25 cc Erlenmeyer flasks with cotton stoppers. Anaerobic incubation was carried out in Thunberg tubes immersed in a water bath at 37.5°C after evacuation with a Cenco Hyvac pump. Separate samples were used for analyses at various time intervals. Proteins were precipitated with 10% trichloracetic acid and the amounts of

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1 Mayer, R. L., and Oechslin, C., *Compt. Rend.*, 1937, **205**, 181.

2 Burton, H., McLeod, J. W., McLeod, T. S., and Mayr-Harting, A., *Brit. J. Exp. Path.*, 1940, **21**, 288.

TABLE I.
Reduction of p-Nitrobenzenesulfonamide to Diazotizable Compounds by Liver Suspension.

Exp. No.	% reduction							
	Aerobic				Anaerobic			
	2 hr	4 hr	6 hr	8 hr	2 hr	4 hr	6 hr	8 hr
1		30.1		35.0		43.7		51.7
2	10.2		17.2		13.7		23.0	
3	21.2	27.6		28.5	22.5	32.4		42.8

diazotizable material in the filtrates were determined by Marshall's method.[3]

Reduction of p-nitrobenzenesulfonamide by rat liver brei proceeds rapidly as shown in Table I. Aerobic reduction was somewhat slower than reduction in the absence of air by the same liver brei. The rate and extent of reduction varied in different experiments but as much as one-fifth (0.4 mg per g of liver) was reduced in 2 hours, and up to one-half (1.0 mg per g) in 8 hours' incubation.

In vivo experiments. The p-nitrobenzenesulfonamide, suspended in 5% acacia, was administered by stomach tube to normal rats weighing about 300 g. The animals were in individual cages and the urine was collected under toluene. Urine samples collected at intervals were analyzed for free and conjugated amine by Marshall's method.[3] Urine samples were analyzed for total amine after reduction with zinc and hydrochloric acid. This determination includes unchanged p-nitrobenzenesulfonamide and possible intermediates such as nitroso-, hydroxylamino-, hydrazo-, azoxy-, or azo-compounds in the urine. To 10 cc of diluted urine were added 2 cc of 4N- HCl and 200 mg of granulated zinc. The sample was heated in boiling water until the zinc had dissolved. This required over an hour; more acid was added if necessary. After adjusting the pH to 1 or 2, the sample was brought back to volume and analyzed by Marshall's method. Similar treatment of known amounts of p-nitrobenzenesulfonamide gave from 94 to 104% of the theoretical yield of sulfanilamide. Corrections were made for the small amount of diazotizable material present in the urine of normal rats, about 0.1 mg per day, calculated as sulfanilamide.

Data from typical experiments are shown in Table II. From 82% to 83% of the nitrobenzenesulfonamide administered was recovered in the urine in 3 days, most of the excretion occurring during the first day. Of the total amount recovered 10%

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

was free amine, 74% to 85% was conjugated amine, and 16% to 5% was excreted in a state of oxidation above the amine. In contrast to these results with p-nitrobenzenesulfonamide, the excretion of the conjugated amine by rats after sulfanilamide dosage is comparatively low. Marshall found the average 8-hour urinary excretion of sulfanilamide by rats to be 48.6% of the oral dose. Only 25% of the excreted sulfanilamide was conjugated.[4] It seems very possible that when nitrobenzenesulfonamide is administered conjugation occurs before reduction has reached the stage of the free amine. It is of interest to note that acetylsulfanilamide, once formed, is deacetylated to only a small extent.[5-8]

It seemed of interest to compare the *in vivo* reduction of an azo-compound with that of the nitro-compound. Crystalline azobenzene-4,4'-disulfonamide was prepared in this laboratory by Mr. Louis Berger by oxidation of sulfanilamide with molybdicyanide. Because of the very low solubility of the azo-compound in water, doses were given as a fine aqueous suspension, injected subcutaneously.

Data from a typical experiment are given in Table III.

TABLE II.
Reduction of p-Nitrobenzenesulfonamide to Diazotizable Compounds by Rats.

Dose,* mg	% of dose recovered	Days	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, %
20	81.7	1	.985	8.26	10.15			
		2	.353	2.93	3.14			
		3	.054	0.48	0.62			
			1.392	11.67	13.91	10.0	73.9	16.1
10	82.5	1	.60	5.03	5.25			
		2	.11	1.4	1.5			
		3	.01	0.25	0.28			
			.72	6.68	7.03	10.2	84.8	5.0

* 10 mg of nitro-compound equivalent to 8.516 mg of amine.

4 Marshall, E. K., Jr., and Cutting, W. C., *Bull. Johns Hopkins Hosp.*, 1938, **63**, 328.

5 Marshall, E. K., Jr., Cutting, W. C., and Emerson, K., Jr., *J. Am. Med. Assn.*, 1938, **110**, 252.

6 Ockerblad, N. F., and Carlson, H. E., *J. Urol.*, 1939, **41**, 801.

7 Allen, J. G., *New Eng. J. Med.*, 1940, **222**, 1029.

8 Kohl, M. F. F., and Flynn, L. M., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 455.

TABLE III.
Reduction of Azobenzene-4,4′-disulfonamide to Diazotizable Compounds by Rats.

Dose,* mg	% of dose recovered	Days	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, %
10	14.5	1	.065	.155	.282			
		2	.020	.035	.104			
		3	.095	.093	.238			
		4	.110	.142	.260			
		5	.020	.020	.147			
		6	.110	.125	.440			
			.420	.570	1.471	28.5	10.2	61.3

* 10 mg of azobenzene-4,4′-disulfonamide equivalent to 10.12 mg sulfanilamide.

Only 14% of the azobenzene-4,4′-disulfonamide injected had been recovered when urine collections were discontinued at the end of 6 days. Probably the slow excretion paralleled slow absorption from subcutaneous tissues. Of the total amount recovered 29% was free amine, 10% conjugated amine and 61% was excreted in a state of oxidation above the amine.

Summary. p-Nitrobenzenesulfonamide is rapidly reduced by rat liver suspensions. After oral administration p-nitrobenzenesulfonamide is rapidly reduced by rats; a high percentage of the dose is excreted in the reduced form, and the extent of conjugation of the reduced form is large. A small amount of the drug is excreted in a state of oxidation above that of the amine. Azobenzene-4,4′-disulfonamide administered subcutaneously to rats is excreted slowly, reduced to only a small extent and conjugated to only a low degree.