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Comparison of Certain Pharmacological and Antibacterial Properties of p-Hydroxaminobenzenesulfonamide and Sulfanilamide.*

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Mayer[1, 2, 3] advanced the hypothesis that p-hydroxaminobenzenesulfonamide was responsible for the antibacterial action of sulfanilamide. In support of this theory, Mayer found (1) that the hydroxylamine‡ *in vitro* was 100 times more bactericidal than sulfanilamide and acted without a latent period; (2) that it converted hemoglobin to methemoglobin, thus explaining the frequent appearance of this pigment in the blood of patients treated with sulfanilamide, and (3) that para-nitrobenzene sulfonamide was inactive *in vitro* but 5-6 times more active than sulfanilamide *in vivo* (the nitro-compound being presumed to yield hydroxylamine more readily than the amine). The hydroxylamine was found to be no more active than sulfanilamide in the mouse infected with streptococci, but Mayer considered that this was due to its rapid decomposition by the blood pigment and that conditions were quite different when hydroxylamine was formed from the amino- or nitro-compound in the immediate vicinity of the bacteria.

Recently, considerable interest has been manifested in the hydroxylamine theory of sulfanilamide action.[4, 5, 6] Methods of preparation and properties of the hydroxylamine and certain of its derivatives, a method for its analysis, the stability of its aqueous solutions, its activity *in vitro*, and its behavior in animals are presented in this communication.

Preparation and Properties. Since the method of preparation and

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† Lalor Foundation Fellow.

1 Mayer and Oechslin, *Compt. rend. Acad. d. sc.*, 1937, **205**, 181.

2 Mayer, *Bull. Acad. de méd.*, Paris, 1937, **117**, 727.

3 Mayer, *Biol. Med.* (supplement), 1937, **27**, 74.

‡ For the sake of brevity, p-hydroxaminobenzenesulfonamide will be referred to as the hydroxylamine.

4 Locke, Main and Mellon, *Science*, 1938, **88**, 620.

5 Shaffer, *Science*, 1939, **89**, 547.

6 Rosenthal and Bauer, *Public Health Reports*, 1939, **54**, 1880.

properties of p-hydroxaminobenzenesulfonamide have not been described, they are included here. Starting with p-nitrochlorobenzene, p,p'-dinitro-diphenyldisulfide was prepared,[7] using 2 moles of sodium disulfide. This disulfide may be converted to p-nitrobenzenesulfonamide by either of 2 methods: (a) oxidation to ammonium p-nitrobenzenesulfonate[8] with subsequent formation of the acid chloride, then the amide by the usual procedure;[9] (b) direct conversion of the disulfide to the acid chloride by chlorine in the presence of nitric acid,[10, 11, 12] followed by conversion to the amide. The yield of amide by method (a), based on p-nitrochlorobenzene, was 47%; by method (b), 35%.

Ten grams of p-nitrobenzenesulfonamide was ground intimately with 7.2 g of zinc dust (90%). This was added during 10 minutes with moderate stirring and appropriate cooling to 100 cc of water maintained at 65°C, containing 2.7 g of ammonium chloride. Stirring was continued 10 minutes longer. The mixture was quickly filtered by suction, and the zinc oxide cake washed with two 15 cc portions of hot water, the washings being received in the filtrate. Thirty grams of C.P. sodium chloride was added to each 100 cc of filtrate, the flask evacuated, and rotated in a tilted position in an ice bath for 45 minutes. The crude hydroxylamine was filtered by suction onto a 12 cm Büchner funnel and sucked as dry as possible, using rubber dam on the funnel to minimize oxidation. The precipitate was dried overnight over calcium chloride in a well-evacuated desiccator in the ice chest. The crude hydroxylamine was boiled with 3 successive 400 cc portions of anhydrous C.P. ether, filtering each extract into an equal volume of petroleum ether. After chilling, the precipitates were collected separately and dried *in vacuo*. Yield, 1.0-1.5 g (10-16%).

The hydroxylamine is a white powder, which under the microscope appears as colorless irregular prisms. It melts at 139.5-140.5°C with slight decomposition.§ Its solubility at 25° in various solvents is: water, about 2%; absolute alcohol, about 5%; absolute ether, about 0.2%. It is soluble in alkali and dioxane, moderately soluble in acetone, slightly soluble in ethyl acetate, very slightly solu-

7 *Organic Syntheses*, 1928, **8**, 64, John Wiley & Sons, New York.

8 Wohlfahrt, *J. prakt. Chem.*, 1902, (2) **66**, 553.

9 Obermiller, *J. prakt. Chem.*, 1914, (2) **89**, 85.

10 Fierz, Schlittler, and Waldmann, *Helv. chim. Acta*, 1929, **12**, 667.

11 *Organic Syntheses*, 1935, **15**, 55, John Wiley and Sons, New York.

12 Schreiber and Shriner, *J. Am. Chem. Soc.*, 1934, **56**, 115.

§ Mayer gives m.p. 161°, solubility in water 0.12 g per 100 cc.

ble in benzene and chloroform. The solid product may be kept in the ice chest for at least 2 months without decomposition.

Analysis. Calculated for $C_6H_8N_2SO_3$: C, 38.29; H, 4.28; N, 14.89; S, 17.04. Found: C, 38.08; H, 4.37; N, 14.40; S, 16.84.

Since the elementary analysis would not show the presence of small amounts of sulfanilamide or the corresponding azoxy compound, a series of preparations of the hydroxylamine was evaluated by the dye reduction method given below. Eight preparations which appeared to be of high purity by melting point gave reduction values in 20 mg % solution between 52 to 53.8%.‖ On diazotization and coupling, as used for determining sulfanilamide,[13] first a yellow, then an orange, and in 2-3 minutes a pink color develops which is, however, only 3-4% of the color obtained from an equivalent of sulfanilamide. When a 0.3 mg % solution was treated with acetic anhydride according to the method of Rosenthal and Bauer,[6] 46-50% of the color expected from an equivalent of untreated sulfanilamide was obtained.

To establish further the structure of the hydroxylamine, the monoacetyl derivative was prepared by acetylation in aqueous solution with acetic anhydride. The product p-(N-hydroxyacetamido)benzenesulfonamide separated from 50% alcohol in glistening white plates, melting point 227-229.5 (dec).

Analysis. Calculated for $C_8H_{10}N_2SO_4$: C, 41.73; H, 4.38; N, 12.17; S, 13.92. Found: C, 41.73; H, 4.33; N, 11.85; S, 13.99. No color was produced on diazotization and coupling,[13] and the product gives a blood-red color with ferric chloride, which establishes the position of the acetyl group on the nitrogen.

On oxidation of the hydroxylamine by oxygen in alkaline solution, p,p'-azoxybisbenzenesulfonamide resulted. The product crystallized from 25% dioxane in 95% alcohol as light yellow irregular prisms, melting point 301-2°C (dec)¶

Analysis. Calculated for $C_{12}H_{12}N_4S_2O_5$: C, 40.44; H, 3.40; N, 15.72; S, 17.99. Found: C, 40.56; H, 3.52; N, 15.85; S, 17.95. It is soluble in alkali, but of low solubility in water and the usual organic solvents.

Method of Analysis and Stability. Owing to the extreme instability of the hydroxylamine under certain conditions, some method of determining actual concentrations in solutions as well as of assay-

‖ A preparation of the hydroxylamine made by Dr. Hugo Bauer and kindly sent us gave a reduction value of 51.5%.

[13] Bratton and Marshall, *J. Biol. Chem.*, 1939, **128**, 537.

¶ Mayer gives m.p. 300°C.

ing solid preparations is necessary. The reduction of a standard solution of 2-6 dichlorophenolindophenol was utilized for this purpose. The reduction is not stoichiometric and varies with different aromatic hydroxylamines; an empirical calibration with the pure hydroxylamine was used.

A solution of 2-6 dichlorophenolindophenol of a concentration of about 10 mg % is standardized against a solution of pure ferrous ammonium sulfate.[14] An amount of this solution calculated to contain 4 mg of dye and 100 cc of an M/15 phosphate buffer of pH 7.0 are diluted to 200 cc. Distilled water which has been boiled and cooled to expel most of the dissolved air is used in preparation of the above solutions. To determine hydroxylamine in a solution, 2 cc of the solution (containing from 2 to 20 mg%) is added to 20 cc of the dye-buffer-mixture and the resulting color compared in a colorimeter against 20 cc of the mixture to which 2 cc of water has been added. The comparison should be made in 1 or 2 minutes. The percentage reduction of the dye is now calculated and the hydroxylamine content of the unknown solution found by reference to an empirically determined calibration curve.**

The stability of solutions of the hydroxylamine depends apparently on the hydrogen ion concentration of the solution. In acid solution, the hydroxylamine is fairly stable and apparently goes only to the azoxy-compound, in neutral or alkaline solution, decomposition is

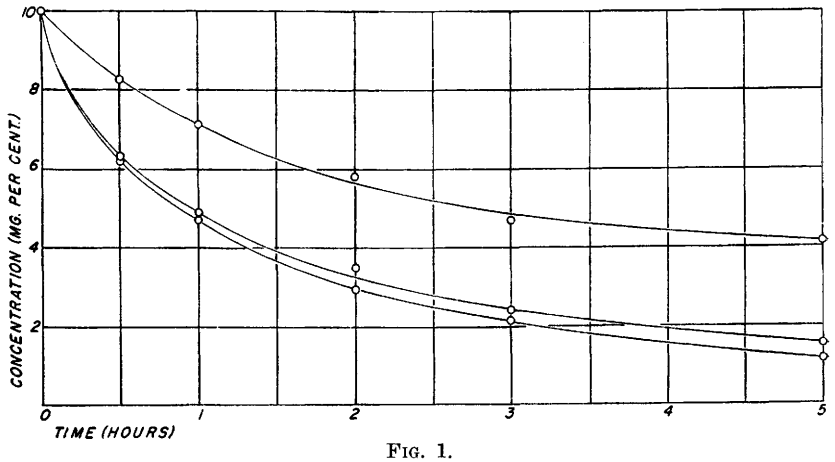

FIG. 1.

Decomposition of solutions of the hydroxylamine in peptone-glucose broth at pH 7.3. Upper curve 23°, middle curve 37°, lower curve 39.3°C.

[14] Lorenz and Arnold, *J. Ind. Eng. Chem.* (anal. ed.), 1938, **10**, 687.

** Reduction values for 20, 12.5, and 9.4 mg % were respectively 52.0, 40.7 and 34.2%.

accelerated and both azoxy-compound and sulfanilamide are formed.

Fig. I shows the rate of decomposition of 10 mg % solutions in broth at 23°, 37° and 39.3°C. The percentage conversion of the hydroxylamine to sulfanilamide after complete decomposition was quite variable (19-42). The precipitate which formed was identified by mixed melting points as the azoxy compound. Higher or lower concentrations than the above of the hydroxylamine (1 to 100 mg %) appeared to decompose at about the same relative rate, and the presence of an inoculum of β-hemolytic streptococci in the broth did not change the rate of disappearance of the hydroxylamine.

Even if 50 mg % of the hydroxylamine is added to dog's blood, none can be recovered in a trichloroacetic acid filtrate using the dye. When 5 mg % are added to rabbit's or dog's blood at 39°C, after 5 minutes, only 40% of the hydroxylamine is converted to sulfanilamide, in 3 hours 75%. In 24 hours dog's blood gave 75 and rabbit's blood 92% conversion.

Injection into Animals. The hydroxylamine is not extremely toxic for animals; no symptoms except methemoglobinemia were noted in dogs on intravenous injection of 20 mg per kg, while in rabbits no symptoms were noted with 30 to 50 mg per kg injected intravenously over 1½ hours. β-phenylhydroxylamine and β-(p-tolyl)-hydroxylamine are much more toxic: the lethal dose for dogs was 20 mg per kg or less. The increased toxicity of these compounds was confirmed on mice injected subcutaneously (the latter 2 hydroxylamines being at least 10 times as toxic as p-hydroxaminobenzenesulfonamide).

When injected into rabbits and dogs, a reaction for an arylamine (presumably sulfanilamide) is obtained in blood and urine. After injections of the hydroxylamine and of an equivalent amount of sulfanilamide in two dogs, the sulfanilamide content of blood and urine was determined. Both experiments gave similar results so that only one is described briefly.

Dog. 11.3 kg. Injected intravenously with 20 mg per kg of the hydroxylamine dissolved in 10 cc of water. Injected intravenously one week later with 18.8 mg per kg of sulfanilamide in 15 cc of water. Table I gives a summary of the results. The first half-hour

TABLE I.
Sulfanilamide Content of Blood and Urine.

Time in min after injection of	Blood concentration, mg %							Urine content, mg	
	5	15	30	60	120	240	24 hr	4 hr	24 hr
Hydroxylamine	3.0	2.5	2.4	2.2	1.9	1.6	0.5	58	130
Sulfanilamide	2.6	2.5	2.4	2.2	2.1	1.7	0.5	44	125

sample of urine after injection of the hydroxylamine probably contained small amounts of this substance (not more than 2-3% of the amount injected) because it gave the acetic anhydride reaction of Rosenthal and Bauer[6] and gave greater reduction of the dye than samples of urines taken before or after this period. This phenomenon was not observed when sulfanilamide was injected.

Bactericidal Action. The relative bactericidal effect of sulfanilamide and the hydroxylamine has been determined at 37° and 39.3° with moderate and large initial concentrations of β-hemolytic streptococci (C 203). These experiments were performed by the test procedure described by one of us[15] for measuring the antibacterial activity of compounds *in vitro* with the following modifications. The peptone-glucose broth used as the test medium contained 2.0% of tryptose (Difco), 0.1% of peptone (Pfanstiehl), sodium chloride 0.5%, glucose 0.2%, and a mixture of phosphates (0.02 molar) to buffer it at pH 7.3. The hydroxylamine was added to broth immediately after seeding with bacteria, and the tubes rapidly brought to test temperature in the water bath. This insured test concentrations of the hydroxylamine within 10% of the calculated value. Table II summarizes the results. The only other information given by the data was that the hydroxylamine is much more inhibitory than sulfanilamide against a large inoculum at 39°. Against the moderate inoculum at this temperature and against both inocula at 37° the inhibitory ratios of the two drugs was similar to the ratios obtained from the bactericidal end points.

Summary. The preparation, properties, stability, and colorimetric

TABLE II.

Bactericidal activity of p-hydroxaminobenzenesulfonamide and sulfanilamide against β-hemolytic streptococcus strain C 203 in peptone-dextrose broth.

Test	Initial bacterial conc. per cc (plate count)	48-hr incubation temperature °C	Minimal bactericidal conc. mg %		Hydroxylamine sulfanilamide activity ratio
			Hydroxylamine	Sulfanilamide	
1	3,000	39.3	4	10	2.5
2	4,500	39.3	6	20	3.3
3	8,500	39.0	6	60	10.0
4	850,000	39.0	>100	400	< 4.0
5	3,000,000	39.3	>100	400	< 4.0
6	4,500,000	39.3	>100	400	< 4.0
7	3,000	37.0	>100	800	< 8.0
8	4,500	37.0	>100	600	< 6.0
9	8,500	37.0	>100	600	< 6.0
10	850,000	37.0	>100	800	< 8.0
11	3,000,000	37.0	>100	1000	<10.0
12	4,500,000	37.0	>100	1000	<10.0

[15] White, *J. Bact.*, 1939, **38**, 549.

analysis of p-hydroxaminobenzenesulfonamide are described. When injected into dogs, this substance appears to be completely converted to sulfanilamide within 5 minutes. *In vitro,* under the conditions of our experiments, it is no more than ten times as active as sulfanilamide.

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Vitamin K Deficiency and Prothrombin Levels. Effect of Vitamin K Administration.*

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In using the chick for the assay of vitamin K, certain workers have used the preventive technic,[1] and thus have given an indication of the basal need of the chick for this vitamin. Others have used the curative technic. Ansbacher[2] has used a curative period of 6 hours, and Thayer and coworkers[3] have lengthened the period to 18 hours. With periods longer than this,[4] the basal utilization becomes significant, we believe, and the assay represents a combination of preventive and curative technics.

Both the preventive and the curative technics supply incidental information concerning physiological problems of great importance. Unfortunately, those who have used brief curative periods have not supplied data on the prothrombin level, but have simply used the whole blood clotting time as a measure of deficiency and of response to treatment. It is our present purpose to give part of the data which are still lacking. It is hoped that these data will help to clarify the various assay procedures now in use, and at the same time provide a much needed quantitative correlation between the prothrombin level and the vitamin K supply.

Materials and Methods. White Leghorn chicks, newly hatched,

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1 Almquist, H. J., and Stokstad, E. L. R., *J. Nutrition,* 1937, **14**, 235.

2 Ansbacher, S., *J. Nutrition,* 1939, **17**, 303.

3 Thayer, S. A., McKee, R. W., Binkley, S. B., MacCorquodale, D. W., and Doisy, E. A., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 194.

4 Dam, H., and Glavind, J., *Biochem. J.,* 1938, **32**, 1018.