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4-Hydroxylaminobenzenesulfonamide, its Acetyl Derivatives and Diazotization Reaction

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As a result of the work of Mayer,[1] who proposed that products formed by oxidation of the amino group were responsible for the mechanism of action of sulfanilamide, considerable study has been devoted to 4-hydroxylaminobenzenesulfonamide.[2] An attempt was made[2a] to develop a colorimetric method for the detection of 4-hydroxylaminobenzenesulfonamide based upon its unusual behavior to diazotization following acetylation. It was found that a diazo reaction could be obtained under certain conditions even after acetylation. In this respect it could be differentiated from sulfanilamide, although later work showed that this procedure was not specific.[3] In this paper the isolation and the properties of the substance responsible for the diazo reaction are described.

The action of nitrite upon hydroxylamines in acid solution was investigated by Bamberger,[4] who showed that the action of one mole of nitrite upon phenylhydroxylamine produces N-nitrosophenylhydroxylamine. This compound decomposes easily either in dry state or in solution with formation of various products, one of them being diazobenzene nitrate. The diazo compound was formed more readily by action of sodium nitrite upon nitrosophenylhydroxylamine in glacial acetic acid solution[4b]

$$C_6H_5NHOH + HNO_2 \longrightarrow C_6H_5N(NO)OH + H_2O \quad (1)$$

$$C_6H_5N(NO)OH + HNO_2 \longrightarrow C_6H_5{-}N_2{-}NO_3 + H_2O \quad (2)$$

The N-nitroso derivative of 2-hydroxylaminobenzoic acid which was obtained only in solution,[5] gave the diazo reaction with an excess of nitrite.

Our study of the 4-hydroxylaminobenzenesulfonamide and of the 4-hydroxylaminobenzoic acid confirms and supplements the observations of Bamberger.

By action of nitrous acid on 4-hydroxylaminobenzenesulfonamide, the N-nitroso-4-hydroxylaminobenzenesulfonamide, $H_2NSO_2C_6H_4N(NO)$-OH, is formed as a white crystalline, sparingly soluble precipitate, while diazotization occurs to a small extent only. The nitrosohydroxylaminobenzenesulfonamide is fairly stable in dry state and can be recrystallized by dissolving in ethyl acetate with subsequent addition of petroleum ether. However, it behaves differently in glacial acetic acid solution. This solution, without showing any sign of decomposition, contains diazobenzenesulfonamide which can readily be detected by coupling with N-(1-naphthyl)-ethylenediamine.[6] Colorimetric determinations of the purple dye, using sulfanilamide as a standard, showed that about 34% of the nitrosohydroxylamine was changed to the diazo compound.

For comparison, 4-hydroxylaminobenzoic acid,[7] which has not hitherto been isolated, and its N-nitroso derivative were prepared. The latter, when dissolved in glacial acetic acid and compared with an equivalent solution of 4-aminobenzoic acid, formed about 15% of diazo compound.

N-Nitrosophenylhydroxylamine behaved in the same way. About 18% of diazo compound was found, using aniline for comparison.

With all three nitrosohydroxylamines, the amount of diazo compound spontaneously formed in glacial acetic acid solution was only slightly changed by addition of nitrite or acetic anhydride (see below).

The spontaneous formation of a diazo compound from a nitrosohydroxylaminobenzene derivative requires the simultaneous formation of oxidation products. If we assume that a nitrosobenzene derivative is the main oxidation product, the following equation shows that three moles of nitrosohydroxylamine form two moles of diazo compound

$$3R{-}N\begin{matrix}NO\\OH\end{matrix} \longrightarrow 2R{-}N{=}N{-}OH + RNO + HNO_2 \quad (3)$$

The amount of diazo compound actually found was less than required by this equation.

To a small extent, formation of a diazo compound occurs also, as previously mentioned, with the preparation of the nitrosohydroxylaminobenzene sulfonamide. A larger amount of the diazo compound is formed by treating hydroxylaminobenzenesulfonamide in dilute acid solution with an excess of nitrite. The yield of diazo compound was considerably increased by treating the hydroxylaminobenzenesulfonamide with acetic an-

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(3) S. M. Rosenthal, *J. Am. Med. Assoc.*, **113**, 2169 (1939).

(4) (a) E. Bamberger, *Ber.*, **27**, 1548 (1894); (b) **31**, 574 (1898).

(5) E. Bamberger and F. L. Pyman, *ibid.*, **42**, 2297 (1909).

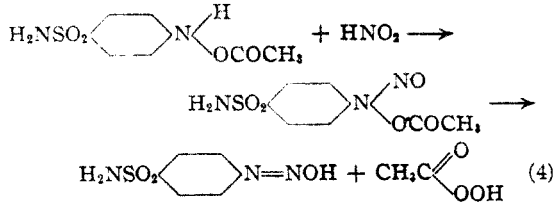
(6) A. C. Bratton and E. K. Marshall, Jr., *J. Biol. Chem.*, **128**, 537 (1939).

(7) F. J. Alway, *Am. Chem. J.*, **32**, 389 (1904); H. Goldschmidt and H. Larsen, *Z. physik. Chem.*, **71**, 437 (1910).

hydride before adding sodium nitrite. The amount of diazo compound formed was increased from 23 to 48% with 4-hydroxylaminobenzenesulfonamide, from 45 to 63% with 4-hydroxylaminobenzoic acid, from a trace to 10% with phenylhydroxylamine.

It was found that acetylation of 4-hydroxylaminobenzenesulfonamide in dilute aqueous solution yields two derivatives, one of which can be diazotized, the other cannot. As already pointed out by Bamberger,[8] for the 2-hydroxylaminobenzoic acid, the non-diazotizable product contains the acetyl group linked to the nitrogen. If acetylation is carried out in very dilute aqueous solution, little of the N-acetyl derivative is formed. The mother liquor contains the diazotizable product which can be extracted with ether or ethyl acetate. This isomer, which was not obtained in a pure state, apparently contains the acetyl group linked to the oxygen of the hydroxylamino group. Upon diazotization, about 65% was converted to the diazo compound; not more than 66.7% could be expected, according to equation 3.

The presence of the oxygen-bound acetyl group facilitates the formation of the diazo compound. It may be assumed that, in the first stage of reaction, an acetylated nitrosohydroxylamine is formed which is easily hydrolyzed in dilute aqueous solution with formation of peracetic acid (see equation 4). The latter could not be detected inasmuch as it is consumed immediately as an oxidizing agent.



(4)

As mentioned above, the diazotization reaction of the acetylated 4-hydroxylaminobenzenesulfonamide can be applied to the detection of this compound in body fluids in presence of sulfanilamide,[2a] although positive results must be confirmed by other methods because of the lack of specificity of this reaction. Other conjugation products of sulfanilamide which give a diazo reaction similar to 4-hydroxylaminobenzenesulfonamide may possibly be formed in the body. While acetylsulfanilamide cannot be diazotized, those conjugation products which are split by nitrous acid with formation of the diazo compound cannot be differentiated from the hydroxylamine. For example, a glucoside of sulfanilamide, tested with the acetylation–diazotization reaction, gave a positive result.[3]

Experimental

4-Hydroxylaminobenzenesulfonamide, $H_2NSO_2C_6H_4NHOH$.—The procedure for the preparation of 4-hydroxylaminobenzenesulfonamide differs in details from the method used by Bratton, White and Marshall,[2b] and in the present work the mother liquor was employed for the preparation of nitrosobenzenesulfonamide.

To a warm solution of 6 g. of 4-nitrobenzenesulfonamide[9] in 200 cc. of alcohol a solution of 10 g. of ammonium chloride in 100 cc. of water was added. With vigorous stirring, 8 g. of zinc dust was added gradually while the temperature was kept between 45 and 52°. After fifteen minutes stirring, the reaction mixture was kept in ice, for ninety minutes. The zinc sludge was filtered by suction and washed with alcohol. The yellow filtrate was concentrated under diminished pressure at 35°, while passing through a stream of nitrogen. When crystals separated, the distillation flask was cooled in an ice–salt mixture for two hours. The yellowish crystals of the hydroxylamine which separated, were filtered and washed twice with ice water. The yield varied between 3.5 (63% of the calcd.) and 4.95 g. (88.5% of the calcd.), the melting point of the crude product varied between 135 and 140°.

For further purification, the substance was dissolved in ethyl acetate, and petroleum ether was added. The colorless crystals thus obtained melted at 143–144° to a liquid which, on further heating, became yellow and decomposed between 148–158° to an orange solid.

Anal. Calcd. for $C_6H_8O_3N_2S$: C, 38.27; H, 4.29; S, 17.04. Found: C, 38.56; H, 4.40; S, 17.20.

Bratton, *et al.*,[2b] give the m. p. 139.5–140.5°. Formation of a substance of m. p. 163–164°, as obtained by Burton,[2g] consisting of a complex of two molecules of the hydroxylamine and one molecule of sulfanilamide, according to Sevag,[2i] was not observed when using the method described above.

4-Hydroxylaminobenzenesulfonamide is soluble in water, alcohol, ether, ethyl acetate, sparingly soluble in benzene, insoluble in petroleum ether. A water solution, saturated at 22.5°, contained 2.7 g. in 100 cc. Fehling solution and silver nitrate solution are immediately reduced at room temperature.

When titrated with 0.1 *N* sodium nitrite in dilute acetic acid solution, one mole of nitrite was used for one mole of 4-hydroxylaminobenzenesulfonamide, forming the nitroso derivative. The assay varied between 98 and 102%.

Reduction of the hydroxylamine with stannous chloride, following the procedure of Limpricht,[10] gave an assay of 94%.

N-Nitroso-4-hydroxylaminobenzenesulfonamide, $H_2NSO_2C_6H_4N(NO)OH$.—To an ice-cold solution of 1.2 g. of 4-hydroxylaminobenzenesulfonamide in 60 cc. of water and 7 cc. of 2 *N* hydrochloric acid, 6 cc. of *N* sodium nitrite solution were added at once. The colorless needles of the nitroso derivative which separated immediately, were filtered after half an hour standing and washed with ice water; 1.04 g. of the nitroso compound (75% of the calcd. amount) was obtained which melted with violent decomposition at 117°. It is soluble in alcohol or ethyl acetate, slightly soluble in water, not soluble in ether, benzene and petroleum ether. When recrystallized by dissolving in cold ethyl acetate and adding petroleum ether, the substance melted at 120°. It gives a beautiful Liebermann nitroso reaction; an intense blue color is formed with diphenylamine sulfuric acid reagent, a red color with ferric chloride. When dissolved in glacial acetic acid, the nitroso compound immediately forms diazobenzenesulfonamide; about 34% were found by coupling with N-(naphthyl)-ethylenediamine[6] (Table II).

Analysis. For nitrogen determination, the Kjeldahl method was modified in the following way: about 120 to 150 mg. of substance was dissolved in 10 cc. of water with addition of 3 drops of 10 *N* sodium hydroxide. 1 g. of sodium dithionite was added and the mixture was gently heated for one-half hour. After addition of 10 cc. of 2 *N* sulfuric acid the heating was continued for one more hour. Then 10 cc. of concentrated sulfuric acid was added, the water was evaporated and the digestion finished in the

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(10) H. Limpricht, *Ber.*, **11**, 35 (1878).

TABLE I

FORMATION OF DIAZO COMPOUND FROM HYDROXYLAMINES

	Compared with diazotized	Diazotized found, %	Treated with acetic anhydride and diazotized found, %
4-Hydroxylaminobenzenesulfonamide	Sulfanilamide	23	48
4-Hydroxylaminobenzoic acid	4-Aminobenzoic acid	45	63
Phenylhydroxylamine	Aniline	Trace	10
O-Acetylhydroxylaminobenzenesulfonamide	Sulfanilamide	63–67	..

TABLE II

SPONTANEOUS FORMATION OF DIAZO COMPOUND FROM N-NITROSOHYDROXYLAMINES IN GLACIAL ACETIC ACID SOLUTION

	Dissolved in glacial acetic acid, found, %	Dissolved in glacial acetic acid with addition of acetic anhydride, found, %	Dissolved in glacial acetic acid with addition of sodium nitrite, found, %
N-Nitroso-4-hydroxylaminobenzenesulfonamide	34	33	35–36
N-Nitroso-4-hydroxylaminobenzoic acid	15	15–16	22
N-Nitrosophenylhydroxylamine	17–18	23–24	18

usual manner. The results obtained were about one per cent. low.

Anal. Calcd. for $C_6H_7O_4N_3S$: N, 19.35; S, 14.76. Found: N, 18.46; S, 15.04.

N-Acetyl-4-hydroxylaminobenzenesulfonamide.—To 1 g. of 4-hydroxylaminobenzenesulfonamide 10 cc. of acetic anhydride was added. The substance changed its appearance but did not dissolve, while the mixture became warm. After standing for several hours, the mixture was diluted with ethyl acetate. The crystalline substance was filtered and washed with ethyl acetate. It melted at 228°, showed no diazo reaction, and gave a red color with ferric chloride.[3] No diacetyl derivative was formed, as is the case with phenylhydroxylamine.[3]

Anal. Calcd. for $C_8H_{10}N_2O_4S$: S, 13.93. Found: S, 14.21.

O-Acetyl-4-hydroxylaminobenzenesulfonamide, $H_2NSO_2C_6H_4NH(OCOCH_3)$.—A 1% aqueous solution of the hydroxylamine was shaken with acetic anhydride and repeatedly extracted with ethyl acetate. The combined extracts were concentrated under diminished pressure and petroleum ether was added. The precipitate thus formed was washed with alcohol and further purified by dissolving in a little methyl alcohol and mixing with petroleum ether. The latter was separated from the alcoholic layer and yielded yellowish crystals of m. p. 138°.

Anal. Calcd. for $C_8H_{10}O_4N_2S$: S, 13.93. Found: S, 13.40.

A solution in aqueous acetic acid is readily diazotized by addition of sodium nitrite; 63–67% was converted to the diazo compound. The calculated value according to equation 3 is 66.7%.

4-Nitrosobenzenesulfonamide.—After separation of the 4-hydroxylaminobenzenesulfonamide a considerable amount of this compound remained in the mother liquor. It was oxidized by addition of an excess of 10% ferric chloride solution. The white precipitate of the nitroso compound thus produced was filtered and crystallized from alcohol; the alcoholic solution was green. The compound did not show a melting point; decomposition started at 155° and was complete at 268°. With diphenylamine–sulfuric acid reagent a vivid red color was formed.

Anal. Calcd. for $C_6H_6O_3N_2S$: S, 17.23. Found: S, 17.45.

4-Hydroxylaminobenzoic Acid.—Seventeen grams of 4-nitrobenzoic acid was dissolved in 350 cc. of water and 10 cc. of 10 *N* sodium hydroxide. After addition of 20 g. of ammonium chloride, the mixture was cooled to 15°, and 15 g. of zinc dust was added gradually with stirring. The temperature rose to 20°. After one-half hour stirring, the zinc sludge was filtered off. To the filtrate hydrochloric acid (sp. gr. 1.12) was added slowly. Amorphous flakes, which separated while the reaction was still neutral, were removed by filtration. Upon addition of more hydrochloric acid to the filtrate, white needles separated; yield 4.8 g. (31% of the calculated amount). The substance did not melt up to 300°, became dark at about 240°; sparingly soluble in cold water; readily in hot water; soluble in alcohol, ether, and ethyl acetate; not soluble in petroleum ether. Fehling solution is reduced immediately at room temperature.

Anal. Calcd. for $C_7H_7NO_3$: N, 9.15. Found: N, 9.15.

N-Acetyl-4-hydroxylaminobenzoic Acid.—To a solution of 1 g. of 4-hydroxylaminobenzoic acid in ether, acetic anhydride was added: colorless crystals separated, 0.6 g.: melted at 210° with darkening and decomposition; gave an intense red color with ferric chloride.

Anal. Calcd. for $C_9H_9O_4N$: N, 7.18. Found: N, 7.11.

N-Nitroso-4-hydroxylaminobenzoic Acid.—To an ice-cold solution of 1.5 g. of 4-hydroxylaminobenzoic acid in 400 cc. of water, 10.5 cc. of 2 *N* hydrochloric acid and 9 cc. of *N* sodium nitrite solution were added. A white, amorphous precipitate separated, 1.3 g. It did not melt up to 270°, but decomposed gradually with blackening. With ferric chloride solution a red color was produced. When dissolved in glacial acetic acid and coupled with Marshall's reagent,[5] about 15% of diazo compound was formed (Table II).

Coupling of Hydroxylamines.—For colorimetric determination of diazo compound formed by diazotization of hydroxylamines, the procedure of Bratton and Marshall[6] was used with slight modifications. Treatment of hydroxylamines with acetic anhydride before diazotizing increases the amount of diazo compound formed (Table I). N-Nitrosohydroxylamines, when dissolved in glacial acetic acid, spontaneously form diazo compounds (Table II).

Summary

Nitrous acid acts upon 4-hydroxylaminobenzenesulfonamide with formation of N-nitroso-4-hydroxylaminobenzenesulfonamide. The latter, when dissolved in glacial acetic acid, is partly transformed to diazobenzenesulfonamide. 4-Hydroxylaminobenzoic acid and phenylhydroxylamine behave the same way. To some degree, dilute solutions of hydroxylamines form the corresponding diazo derivative with nitrous acid. By treating dilute solutions of hydroxylamines with acetic anhydride before diazotization, an increase of diazo compound was obtained. It was found that acetylation yields two derivatives. The

derivative which gives the diazo reaction was isolated. It contains the acetyl group presumably linked to the oxygen of the hydroxylamino group. The other derivative is acetylated on the nitrogen and cannot be diazotized. The application of this reaction to the detection of hydroxylaminobenzenesulfonamide in body fluids is discussed. The preparation of 4-nitrosobenzenesulfonamide is described.

BETHESDA, MD. RECEIVED JANUARY 6, 1944

[CONTRIBUTION FROM THE BAKER LABORATORY OF CHEMISTRY AT CORNELL UNIVERSITY]

Gliotoxin, the Antibiotic Principle of *Gliocladium fimbriatum*. II. General Chemical Behavior and Crystalline Derivatives[1]

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In a preliminary examination of the chemical behavior of gliotoxin, Weindling[4] has observed that in spite of its nitrogen content, gliotoxin has no basic properties. By prolonged heating with acid, it was slowly converted to a gum from which none of the original material could be recovered. In a partition between ether and 1% potassium hydroxide, practically none of the gliotoxin was dissolved in the alkaline layer. This showed the non-acidic nature of the material. It was, however, rapidly altered by treatment with alkali. Boiling with 5% potassium hydroxide rapidly eliminated sulfur and, even in the ether partition, a large proportion of the material was altered. Gliotoxin was found strongly reducing toward permanganate.

To extend these observations, a quantitative study of the effect of alkali on gliotoxin seemed desirable. By connecting a trap containing 0.1 *N* hydrochloric acid with a refluxing alkaline suspension of gliotoxin, evolution of a volatile base was readily demonstrated. On evaporation of the acid solution, a hydrochloride and from it a chloroplatinate were prepared which showed that the volatile base was methylamine, and in an amount which accounted for about half the nitrogen in the sample. The amount of sulfur liberated as sulfide by the action of alkali was then determined by acidification and steam distillation of the solution which remained. From 40–60% of the total sulfur was liberated as hydrogen sulfide, and some as elementary sulfur. In the non-volatile portion, a red amorphous solid remained, insoluble in acid, but soluble in alkali, containing nitrogen and sulfur. By using barium hydroxide in place of sodium hydroxide, the same volatile products were observed, but the residue was not as highly colored. On purification by sublimation *in vacuo*, it yielded a small amount of crystalline solid. This solid, slightly soluble in water, dissolved readily in sodium bicarbonate solution, and formed a silver salt, methyl and ethyl esters through which it was identified as indole-2-carboxylic acid.

Gliotoxin proved inert toward phenyl isocyanate, either when refluxed in benzene solution or on long standing at ordinary temperature: the original material was recovered unchanged. With diazomethane, methyl iodide and dimethyl sulfate, some reaction may have occurred since none of the original material was recovered; only gums and sirups were obtained.

A Zeisel determination showed the absence of methoxyl or ethoxyl groups. The N-methyl determination[5] was positive in agreement with the isolation of methylamine. No reaction occurred with the carbonyl reagents hydroxylamine, semicarbazide or 2,4-dinitrophenylhydrazine. The Ehrlich reaction, pine splint test, Keller's indole reaction and the ninhydrin reaction were all negative. Clowes' test for $CH_2\begin{smallmatrix}O\\O\end{smallmatrix}$ or $CH_2\begin{smallmatrix}S\\S\end{smallmatrix}$ using phloroglucinol and 50% sulfuric acid,[6] was also negative. Ammoniacal silver nitrate (Tollens reagent) and phosphotungstic acid (Folin reagent) in the presence of sulfite were reduced. These reactions and a positive nitroprusside reaction could be attributed to the products, particularly sulfide, formed as a result of the lability of gliotoxin in alkaline media.

Oxidizing agents such as permanganate, bromine water and sodium hypochlorite rapidly converted the sulfur of gliotoxin to sulfate. Reducing agents, including sodium sulfite, stannous chloride, hydriodic acid, aluminum amalgam, zinc or tin and acid, reduced the sulfur in gliotoxin to hydrogen sulfide. Certain of these procedures will be considered in detail in later papers. By treatment of gliotoxin with mercuric acetate or silver nitrate, *one* sulfur atom was removed. Cupric sulfate, lead acetate, and barium chloride gave no reaction.

A Zerewitinoff determination of active hydrogen in gliotoxin dissolved in pyridine gave somewhat uncertain results, for a rather large blank from the pyridine was observed, and while the blank was constant under given conditions, it was variable with time. It was necessary to use pyridine because gliotoxin is insoluble in the isoamyl ether

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(4) Weindling, *Phytopathology*, **26**, 1068 (1936).

(5) Herzig and Meyer, *Monatsh.*, **18**, 379 (1897).

(6) Clowes, *Ber.*, **32**, 2841 (1903).