NOTE

The Preparation of 3-Aminophthalhydrazide for Use in the Demonstration of Chemiluminescence

By Ernest H. Huntress, Lester N. Stanley and Almon S. Parker

Most of the important illustrations of chemiluminescence produced by the oxidation of definite organic compounds involve unpleasant, dangerous, or inconvenient reagents and are not well suited for demonstration purposes. In contrast to the classical cases of the oxidation of pyrogallol–formalin solutions with Perhydrol, the various oxidations of Grignard compounds, or the alcoholic potash treatment of lophine (or amarine), however, the oxidation of 3-aminophthalhydrazide can be carried out in exceedingly dilute aqueous alkaline solution with the mildest and easily accessible oxidants, while the brilliance of the effects obtained far surpasses any of the other recorded cases. Although the fact that light is evolved during the oxidation of this substance has been on record for some years, the reaction is apparently unfamiliar to most scientific workers, perhaps due to the hitherto difficult accessibility of the necessary reagent. It is the purpose of the present note to offer an improved method for the synthesis of this material, which in the interest of simplicity we have long referred to as "Luminol." Precise directions for the demonstration of the intense chemiluminescence obtained on its oxidation will be published in another journal.[1]

The following method of preparation involves first the isolation of 3-nitrophthalhydrazide and then its subsequent reduction to 3-aminophthalhydrazide by means of ammonium sulfide. The method thus avoids difficultly accessible raw materials and starts directly with 3-nitrophthalic acid. It avoids the excess of expensive hydrazine required by early investigators, and uses (in the form of the sulfate) only that quantity actually needed for the heterocyclic ring. By substituting sulfide as the reducing agent it avoids the difficulties attendant upon the separation of an amphoteric product from a stannous solution. By utilizing the enolic properties of the cyclic hydrazide the product may be purified and separated as the sodium salt.

The Preparation of 3-Nitrophthalhydrazide.—Hydrazine sulfate (130 g. or 1 mole) and crystallized sodium acetate (272 g. or 2 moles) are dissolved in 400 ml. of hot water and the clear solution added to solid 3-nitrophthalic acid (211 g. or 1 mole) contained in a 20 cm. porcelain evaporating dish. The resultant solution is evaporated over a free flame as rapidly as possible with constant stirring to avoid bumping or decomposition. This operation requires about one and one-half hours and is best conducted at a hood to facilitate removal of the acetic acid vapors. The residual dry solid is then ground to a fine powder, placed in a wide-mouthed flask or beaker, and heated for at least three hours at a temperature of 160 ± 10° in a suitable oil-bath. During this operation the solid should be stirred frequently and if there is any tendency toward caking in the earlier part of the baking it may be necessary to remove and powder the material before continuing. It is advisable to measure the temperature in the heated powder rather than externally and care must be taken not to overheat the mass. The escape of steam may also be facilitated by passing a gentle stream of air through the flask.

When the heating is finished, remove the solid, powder, and extract twice with 350 ml. of hot water to remove sodium sulfate. The residual solid is then dried at 105° to constant weight: 206 g. of 3-nitrophthalhydrazide (99.5%). The product so obtained is free from sulfate, melts 297–300° uncorr.,[2] and is sufficiently pure for reduction without further treatment.

The Reduction of 3-Nitrophthalhydrazide to 3-Aminophthalhydrazide ("Luminol").—The crude 3-nitrophthalhydrazide (*e. g.*, 192.5 g. or 0.93 mole) is then gradually added in small increments to 1 liter of 6 *N* ammonium sulfide solution. This may be prepared by saturating 200 ml. of concentrated ammonium hydroxide (sp. gr. 0.90) with hydrogen sulfide in the cold, adding 200 ml. more of ammonium hydroxide, and diluting to 1 liter. Vigorous spontaneous reduction occurs and the flask may require considerable external cooling. After all the solid nitrohydrazide has been added the resultant solution or suspension is kept at the boiling point for an hour while additional hydrogen sulfide is passed into the mixture. During the reduction the original nitro compound finally completely dissolves; later, however, the precipitation of the resultant "Luminol" begins, and these two processes may sometimes overlap in such a way that there is always some solid present in the flask. After the discontinuance of the hydrogen sulfide the solution is boiled for an hour more to complete the reaction and then allowed to stand until cold. The resultant yellow precipitate of mixed sulfur and "Luminol" is filtered with suction, thoroughly washed with water, and dried. In some runs all the sulfur remains in the excess sulfide solution and as high as 64.5% yield of very pure "Luminol" has been obtained directly at this point.

The filtrate from this primary precipitate is then acidified with a slight excess of glacial acetic acid and the resultant precipitate of mixed sulfur and "Luminol" is filtered with

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[1] Huntress Stanley and Parker, *J. Chem. Ed.*, March, 1934.

[2] Although Bogert and Boroschek, This Journal, **23**, 750 (1901), state that 3-nitrophthalhydrazide melts with decomposition at about 320°, we have consistently observed values close to 300° uncorr. Radulesca and Alexa, *Z. physik. Chem.*, **B8**, 393 (1930), give m. p. 310–311° but do not state whether corrected or not.

suction, washed with water, and dried. In order to free the aminophthalhydrazide from sulfur, the crude mixture is stirred up with that amount of 5% sodium hydroxide solution which would just correspond to the assumption that the dry solid contained no sulfur. After stirring and very slight warming the solution is filtered from undissolved sulfur, cooled to 0°, and stirred and scratched. Presently precipitation of the monosodium salt[3] of 3-aminophthalhydrazide begins and increases for some time. Finally the solid is filtered with suction, pressed as dry as possible on the filter, then washed sparingly with absolute alcohol or ether. It may then be dried in the air if it is to be preserved as the sodium salt. If, however, it is desired to reconvert the substance to "Luminol," the original solid is not washed with alcohol but is redissolved in water and reprecipitated by adding a slight excess of glacial acetic acid. The voluminous flocculent precipitate is again filtered with suction, washed free from sodium acetate with water, and dried. The product so obtained is free from sulfur and melts at 319–320° uncorr. (using a 360° melting point thermometer in a Berl and Kuhlmann copper block). The color of the final 3-aminophthalhydrazide appears to vary according to the mode of precipitation from almost white to quite deep yellow.

Anal. Calcd. for $C_8H_7N_3O_2$: C, 54.22; H, 3.98; N, 23.73. Found: C, 54.20. 54.31; H, 4.48, 4.62; N, 24.20, 24.02. *Neut. equiv.* Calcd., 177. Found: 175.

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[3] Samples of the sodium salt (dried to constant weight at 105°) were analyzed by igniting in porcelain crucibles and converting the ash to sodium sulfate. Calcd. for $C_8H_6N_3O_2Na$: Na, 11.55. Found: Na, 11.77, 11.99.

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COMMUNICATIONS TO THE EDITOR

AN INTERMEDIATE OXIDATION PRODUCT OF CYSTINE

Sir:

Previous work, reported at the April, 1933, Meeting of the American Society of Biological Chemists by Toennies and Lavine, but not completely included in the abstract [*J. Biol. Chem.*, **100,** XCI (1933)], has shown that the oxidation of cystine in non-aqueous solution by perbenzoic acid at low temperature consumed a maximum of four atoms of oxygen per molecule of cystine. The first two atoms of oxygen reacted more rapidly than the second two, and following the first stage and during the further oxidation there separated a precipitate whose composition accorded with that of the disulfone ($+{}^1/_2\,HClO_4$). This product, after hydrolysis by 95% methyl alcohol containing hydrogen chloride, and addition of chloroform, yielded a crystalline precipitate, whose properties and analytical values were those of a mixture of cysteic acid and the corresponding sulfinic acid. To permit step by step study of the course of the oxidation there was developed an analytical method involving rapid removal of perbenzoic acid from the reaction mixture, and application of a semi-quantitative form of the cyanide–nitroprusside test following acid–iodide treatment. It was observed that response to this test reached a maximum during the first stage of the oxidation (between one and two atoms of oxygen consumed), becoming negative toward the end, thus indicating the test to be associated with an oxide, or oxides, lower than the trioxide [*cf. J. Biol. Chem.*, **100,** 464 (1933)].

On the basis of these studies experiments aimed at the isolation of such intermediate oxidation products of cystine have been made. When cystine was oxidized to the stage at which somewhat more than two atoms of oxygen were consumed (with formation of only a little of the insoluble final oxidation product, and with only traces of unoxidized cystine left), immediate neutralization of the filtered liquid by addition of pyridine produced a precipitate. Sparing extraction of the filtered precipitate with water left a white residue which crystallized in the manner of an ampholyte, and which responded neither to the direct cyanide-nitroprusside test for —S—S—, nor to the nitroprusside test for —SH. After reduction of this substance with a large excess of 3 *N* hydriodic acid, the cyanide–nitroprusside test was as strong as for an equal amount of cystine. The amount of iodine liberated during the acid–iodide treatment corresponded to the requirements for reduction of the disulfoxide to cystine. The optical rotation of the reduced solution indicated an almost quantitative regeneration of cystine, and by neutralization of the solution cystine was precipitated.