Mutagenicity of Resorcinol Formed by the Reaction of *m*-Phenylenediamine with Sodium Nitrite

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In the present study, the mutagenicity of the reaction product of *m*-phenylenediamine with sodium nitrite against a streptomycin-dependent strain (SD 510) of *Salmonella typhimurium* TA 98 and *Escherichia coli* B/r WP2 trp⁻hcr⁻ has been examined. The reaction product separated by HPLC was identified as resorcinol (1,3-dihydroxybenzene). Resorcinol was mutagenic against both of these test strains in the presence of S-9 mix. The mutagenicity was highest at pH 3.0 under the pH conditions examined and found to decrease with increase in pH.

INTRODUCTION

The occurrence and formation of *N*-nitroso compounds in foods has generated considerable research attention throughout the world over the past few decades because of their unusual biological activities like carcinogenicity (Hotchkiss, 1987). *N*-Nitroso compounds are produced by the reaction of primary and secondary amines with nitrite under acidic conditions. They can be divided into the *N*-nitrosamines and the *N*-nitrosamides. *N*-Nitrosamines are *N*-nitroso derivatives of secondary amines, and the *N*-nitrosamides are amides, ureas, carbamates, or guanidines (Lijinsky, 1974; Mirvish, 1975; Rao and Krishna, 1975).

High mutagenicities and carcinogenicities have been reported for many *N*-nitrosamines (Hotchkiss, 1987). However, little is known about mutagenicities and carcinogenicities of *N*-nitroso compounds, especially those produced by the reaction of primary amines with sodium nitrite, because of their unstable chemical properties. A recent publication has also indicated that *m*-phenylenediamine, a component of hair dye formulations, has significant mutagenic activity under a *Salmonella*/mammalian microsome assay system (Clemmensen and Lam, 1984). Hence, the present study was initiated to find the mutagenicity of a derivative of a diazonium salt obtained by the reaction of *m*-phenylenediamine with sodium nitrite.

MATERIALS AND METHODS

Preparation of *m*-Phenylenediamine and Sodium Nitrite Solutions. *m*-Phenylenediamine and sodium nitrite were procured from Nacalai Tesque, Inc., Kyoto. Three-tenths of a mole of *m*-phenylenediamine and 0.1 mol of sodium nitrite solutions were prepared by using McIlvaine buffer solution, pH 4.2, as solvent.

Reaction of *m*-Phenylenediamine with Sodium Nitrite. Five hundred microliters of the *m*-phenylenediamine solution was reacted with the same volume of sodium nitrite at 0 °C for 3 h.

High-Performance Liquid Chromatography. The reaction product was quantified by high-performance liquid chromatography (HPLC) in a Shimadzu Model LC-6A equipped with a UV monitor (Model SPD-6A) and a reverse-phase column (Shimpack CLC-ODS, 6 mm × 15 cm, Shimadzu Co., Ltd., Kyoto). A mobile phase of acetonitrile–H_2O–triethylamine (50:50:0.05) (mobile phase A) or 35:65:0.05 (mobile phase B) was used at a flow rate of 1 mL/min. Absorbency at 273 nm was recorded with an integrator (Shimadzu Chromatopack C-R6A).

NMR Spectrometry. The ^1H NMR spectrum of the reaction product, which was separated by HPLC, was measured with a Bruker AC (^1H, 250 MHz) instrument with tetramethylsilane as an internal standard.

Mutagenicity Assay. The streptomycin-dependent strain, SD 510 of *Salmonella typhimurium* TA 98, and *Escherichia coli* B/r WP2 trp⁻hcr⁻ were used as test strains. SD 510 of *S. typhimurium* was cultured at 36 °C and maintained in SM 20 agar as reported previously (Hosono et al., 1986a).

One hundred microliters of the reaction product separated from the reaction mixture by HPLC analysis and 300 μL of S-9 mix were mixed and poured into a Nutrient Broth 2 (OXOID)–agar plate previously covered with a lawn of 100 μL of SD 510 prepared from an overnight culture suitably diluted in 0.05 M phosphate buffer of pH 6.8. For the control, 100 μL of the mobile phase solution was used instead of 100 μL of the test sample solution.

E. coli B/r WP2 trp⁻hcr⁻ was cultured overnight in B2 medium as reported previously (Hosono et al., 1986b). Cells in the stationary phase were harvested from the medium. One-tenth of a milliliter of cell suspension (ca. 1.2×10^{10}/mL) together with 100 μL of the test sample solution and 300 μL of S-9 mix was added to 3 mL of molten soft agar prepared by dissolving 6 g of NaCl and 6 g of agar in 1000 mL of distilled water. They were mixed well and poured onto a MB plate (Hosono et al., 1986b). The plates thus prepared were incubated at 36 °C in the dark for 3 days. Induced trp⁺ revertant colonies were counted and compared with a control plate, where 100 μL of the mobile phase solution was used in place of the test sample.

All of the experiments were carried out in duplicate.

Metabolic Activation. Microsomal fraction and S-9 preparation were prepared according to the procedure of Hosono et al. (1986b).

RESULTS

Identification of Reaction Product Obtained by Reaction of *m*-Phenylenediamine with Sodium Nitrite. In the first step to isolate and identify the reaction product of *m*-phenylenediamine with sodium nitrite, the reaction mixture was quantified by HPLC, and the chromatogram obtained is shown in Figure 1. The reaction product (peak C) was collected, concentrated, and lyophilized. The NMR spectrum ($CDCl_3$ + MeOH) δ_H of the lyophilized sample is shown in Figure 2A, which is identical with that of resorcinol (1,3-dihydroxybenzene) as shown in Figure 2B. On the other hand, parts A–C of Figure 3 show the HPLC chromatograms of the lyophilized sample (10 μg/5 μL of MeOH), authentic resorcinol (10 μg/5 μL of MeOH), and a mixture of the lyophilized sample (5

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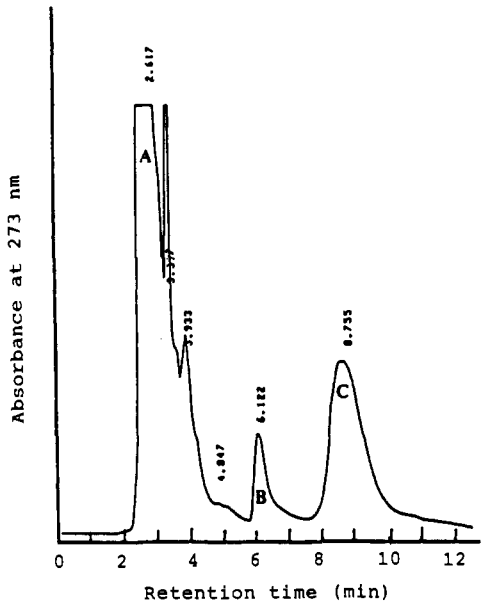


Figure 1. HPLC of the mixture resulting from the reaction of *m*-phenylenediamine (0.3 M) with sodium nitrite (0.1 M). (A) Medium nitrite; (B) *m*-phenylenediamine; (C) reaction product.

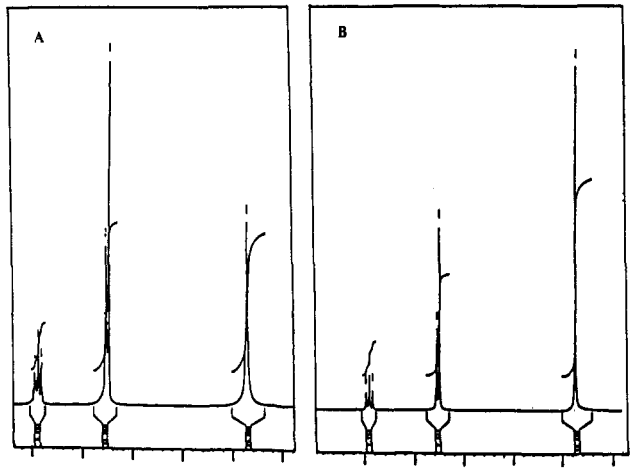


Figure 2. 250-MHz ^1H NMR spectra of the reaction product (A) and resorcinol (B).

μg/2.5 μL of MeOH) and authentic resorcinol (5 μg/2.5 μL of MeOH), respectively. From the retention time of HPLC shown in this figure, the identity of the reaction product with resorcinol was also recognized.

Mutagenicity of Reaction Products. In the present study the mutagenicity was found in the reaction mix beginning both with *m*-phenylenediamine and with authentic resorcinol.

Figure 4 shows the mutagenicity of the reaction product obtained by the reaction of 0.3 M *m*-phenylenediamine with 0.1 M sodium nitrite against SD 510 of *S. typhimurium* TA 98 and *E. coli* B/r WP2 trp⁻hcr⁻. The reaction product was mutagenic against both of these test strains in the presence of S-9 mix, and higher mutagenicity against the SD 510 strain was observed.

The mutagenicity of authentic resorcinol against SD 510 of *S. typhimurium* and *E. coli* B/r WP2 trp⁻hcr⁻ under various pH conditions is shown in Figure 5. Highest mutagenicity was observed at pH 3.0 against both test strains under the pH conditions examined. However, the mutagenicity was found to decrease with an increase in pH, and little or no mutagenicity was observed above pH 5.0.

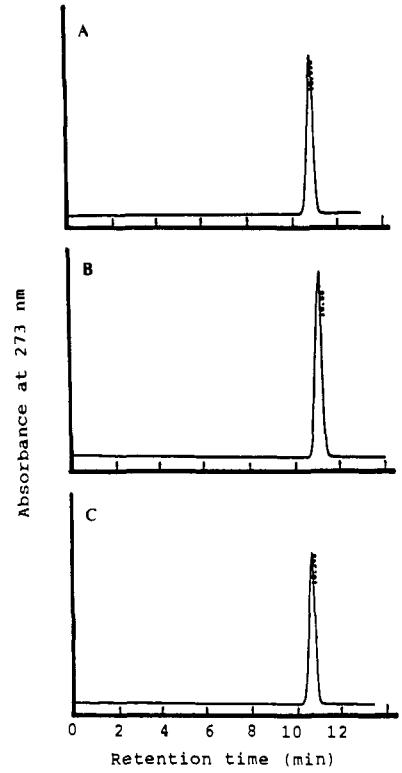


Figure 3. HPLC of authentic resorcinol (A), the reaction product of *m*-phenylenediamine with sodium nitrite (B), and a mixture of (A) and (B) (C).

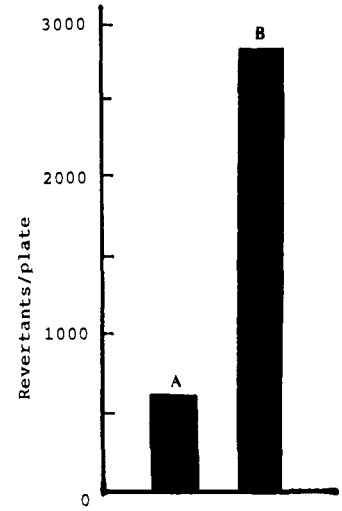


Figure 4. Mutagenicity of the reaction product obtained by the reaction of 0.3 M *m*-phenylenediamine with 0.1 M sodium nitrite against the SD 510 strain of *S. typhimurium* TA 98 (A) and *E. coli* B/r WP2 trp⁻hcr⁻ (B). Mutagenicity assay was carried out in the presence of S-9 mix.

DISCUSSION

The reaction of aromatic amines with nitrite at low pH to yield a diazo compound and a phenol is classic chemistry. Thus, the finding of resorcinol is quite expected. In fact, the reaction product of *m*-phenylenediamine with sodium nitrite was identified as resorcinol. Production of resorcinol is assumed to have taken place by the mechanism shown in Scheme I, because production of N_2 gas was observed when 1 M *m*-phenylenediamine was reacted with 2 M sodium nitrite at −5 °C (data not shown). However, none of the diazonium salts (I–III) shown in Scheme I were identified by HPLC analysis, probably due to their unstable nature.

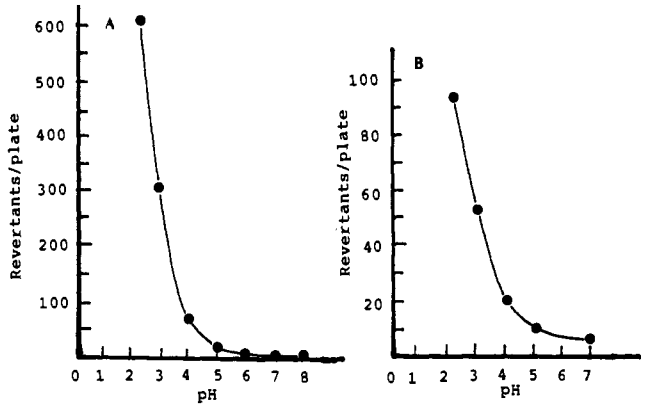
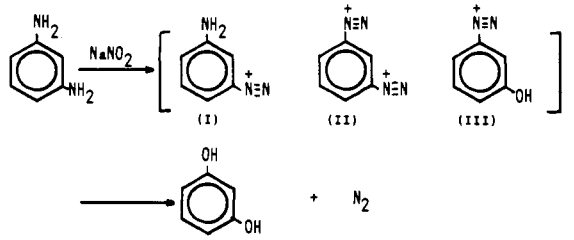


Figure 5. Effect of pH on the mutagenicity of resorcinol against the SD 510 strain of *S. typhimurium* TA 98 (A) and *E. coli* B/r WP2 trp⁻hcr⁻ (B).

Scheme I. Possible Course of the Reaction of *m*-Phenylenediamine with Sodium Nitrite



Phenols in the presence of nitrite yield nitroso and diazo compounds that are mutagenic without S-9 mix (Ohshima et al., 1989; Ochiai et al., 1984). However, the results obtained from the present study showed that resorcinol has mutagenic activity against the SD 510 strain of *S. typhimurium* TA 98 and *E. coli* B/r WP2 trp⁻hcr⁻ under the assay conditions with S-9 mix. There is little information on the mutagenicity of resorcinol. In fact, there was no mutagenicty by resorcinol against these test strains under the assay conditions without S-9 mix (data not shown). However, the mutagenicity of resorcinol with S-9 mix that demonstrated activity only at low pH is a most unusual finding. Induction of mutagenicity of the reaction product of *m*-phenylenediamine with sodium nitrite against the test strains at low pH can be attributed to the protonation of resorcinol, but no evidence has been currently established to support this hypothesis. In this context another interesting observation was that naturally occurring alkylresorcinols such as 1,3-dihydroxy-5-tridecylbenzene, 1,3-dihydroxy-5-pentadec-*cis*-8′-enylbenzene, and 1,3-dihydroxy-5-[14′-(3″,5″-dihydroxyphenyl)tetradec-*cis*-6′-enyl]benzene bind or cleave DNA in the presence of copper, which in turn leads to hydroxy radicals that can affect DNA (Scannel et al., 1988).

Though sufficient information about mutagenicities and carcinogenicities of *N*-nitrosoamides was not derived in the present study to evaluate their hazards in the digestive tract, it is very likely that we are exposed to a variety of *N*-nitrosamides formed in the digestive tract following the ingestion of primary amines, since nitrite is present in meat products (Woolford et al., 1976) and human saliva (Spiegelhalder et al., 1976; Ishiwata et al., 1975) at a relatively high concentration. In this context, it would be of interest to study desmutagenic activities of lactic acid bacteria against *N*-nitrosamides, as several species of lactic acid bacteria like *Leuconostoc paramesenteroides*, *Streptococcus lactis* subsp. *diacetylactis*, and *St. cremoris* are known to have inhibitory effects against the mutagenicities of volatile *N*-nitrosamines such as *N*-nitrosodiethylamine and *N*-nitrosodimethylamine (Hosono et al., 1990).

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