The Reaction of Butylated Hydroxyanisole and Its Metabolites with Some Arylamines: Investigations of Product Mutagenicity

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We examined \(t\)-butylhydroquinone (\(t\)-BHQ) and \(t\)-butylquinone (\(t\)-BuQ), two of the major microsomal metabolites of the synthetic antioxidant butylated hydroxyanisole (BHA), for their ability to react with the xenobiotic arylamines aniline and \(N\)-methylaniline. A number of substances were isolated by thin-layer chromatography. The main products were quantitatively evaluated and their structures assigned. BHA and \(t\)-BHQ yielded reaction products with anilines only in the presence of an oxidant such as iodate (KIO\(_3\)). We used the Salomonella/microsome mutagenicity assay to test the new compounds for mutagenic activity. The reaction products gave no evidence of mutagenicity in the \(S.\ typhimurium\) strains TA98 and TA100, with or without metabolic activation. In some instances the substituted quinone products are less toxic than \(t\)-BuQ alone. Key words: butylated hydroxyanisole, mutagenicity testing, structural assignment, substituted anilines, \(t\)-butylhydroquinone, \(t\)-butylquinone. Environ Health Perspect 102:96-99 (1994)

Reports in the literature on the breakdown pathways of phenolic antioxidants and interactions of their metabolites with foodstuff components are scarce. We previously reported that butylated hydroxyanisole (3-BHA; E320; CAS No.25013-16-5), used widely to prevent oxidative degradation in oils, fats, and shortenings, and 2-\(t\)-tert-butylhydroquinone (\(t\)-BHQ) are readily oxidized to 2-\(t\)-tert-butylquinone (\(t\)-BuQ). The latter reacts with amines to form red addition products (I). Recently Soma and Soma reported on the reaction of anilines with the quinone ring of humate by Michael addition, followed by oxidation to form stable amino-substituted quinones (2). By way of multiple transformations, for example, in the biosphere or in the diet, new, more hazardous substances may be formed. Combination effects are therefore of particular practical interest; however, investigation of this problem has been limited.

A wide variety of primary amines is found in foods, particularly foods that are produced by or subject to microbial fermentation, such as cheese, meat, and fish (3). The only two arylamines that seem to be widespread are aniline and \(N\)-methylaniline. According to Neurath (4), aniline is found primarily in rapeseed, a source of edible oil and protein (120 ppm) and carrots (31 ppm), whereas \(N\)-methylaniline is found in cheese (38 ppm). Other sources are given by Shepherd et al. (5). In addition, substituted anilines and halogenated derivatives, which are intermediates in the environmental degradation of many pesticides and other industrial chemicals, have different migration capacities, toxicities, and other properties. The ability of such pollutants to enter and accumulate in tissues may contaminate the entire food chain (6,7). Furthermore, field observations and experiments showed that plants readily take up aniline residues derived from phenoxyalkyl herbicides through their root systems and translocate it (8). Another source of contamination is water, because large quantities may be used to wash, heat (direct steam injection), or reconstitute foods. Unfortunately, assuring that the water is not contaminated via industrial pollution does not guarantee the absence of petrochemicals in marine animals (9). Other aquatic organisms, either grown in the water or serving as food for other marine animals, are frequently the source of anilines (10).

Due to the complex nature of food, the analysis of potential contamination or irreversible fixation of xenobiotics to food constituents is a massive task. Thus, model reactions are frequently used and have contributed much to the understanding of interactions of organic pollutants with food constituents. For our studies we chose the substances BHA, its accessible metabolites \(t\)-butylquinone and \(t\)-butylhydroquinone, and the arylamines aniline and \(N\)-methylaniline as representative model substances. The present study was undertaken to identify the reaction products and to assess their potential to act as mutagens in the Salmonella typhimurium assay (11). This was a first step in elucidating the biological and toxicological properties of these new products.

Materials and Methods

Aniline and \(N\)-methylaniline (NMA; Merck, Darmstadt, Germany) were used only after redistillation. \(^{15}\)N-enriched aniline-hydrochloride (96.0%) was purchased from VEB-Berlin-Chemie and used without further purification. The commercial preparation of BHA is a mixture of two isomers. The major isomer is 3-\(t\)-butyl-4-hydroxyanisole (BHA) and the minor one is 2-\(t\)-butyl-4-hydroxyanisole. BHA was obtained in >99% purity by fractional crystallization of the commercial sample (Carl Roth, Karlsruhe, Germany) from hot hexane containing 3% acetone. \(t\)-BHQ was obtained from Fluka (Neu-Ulm, Germany); \(t\)-BuQ was synthesized via oxidation of BHA as described previously (1). For preparative thin-layer chromatography (TLC), silica gel-60 F-254 plates (thickness 2, 0.25 mm; Merck, Darmstadt, Germany) were used. All other chemicals were used in the highest purity commercially available.

Beef extract and agar used to culture microorganisms in the \(S.\ typhimurium\) mutagenicity assay (11) were purchased from Difco Laboratory, Detroit, Michigan. Detailed preparation procedures and positive control experiments are described elsewhere (12).

Melting points, measured with a Büchi melting-point apparatus, were left uncorrected. We determined the UV-VIS spectra using a Perkin-Elmer Lambda 2 spectrophotometer. Infrared spectra were recorded on a Perkin-Elmer Infrared spectrophotometer model 881 in KBr. The NMR spectra were measured using Bruker AC-200 and AM-400 instruments in CDCl\(_3\). Mass spectra were obtained on a Finnigan MAT 4000 using 70 eV ionizing electrons.

Preparation of Arylamine Adducts of BHA and Derivatives

We dissolved aniline (5 mM, 93.12 mg) and \(t\)-BuQ (0.5 mM, 82.10 mg) in 30 ml ethanol and allowed it to stand for 15 hr at 21 C in the dark. The reaction mixture was extracted twice with 20 ml chloroform. The combined organic fraction was treated with anhydrous sodium sulfate and, after filtration and concentration, subjected to preparative TLC (thickness 2.0 mm), using light petroleum and acetone 8:2 v/v as eluent. Two red zones were observed on the TLC plate. Each of them was extracted with chloroform and rechromatographed on silica-gel layers (0.25 mm). We removed the red zones with \(R_t\) 0.47 (major; I) and 0.55 (II), respectively, and eluted them with 20 ml acetone. The

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eluates were then evaporated to dryness on a rotary evaporator under reduced pressure to give red powders, which were recrystallized from hot methanol/water. The crystalline red solids were dried in a vacuum desiccator. Their melting points were 133–134°C for I and 101–102°C for II.

To support our structure assignments, we also carried out the above synthesis using 15N-aniline. The reaction products were separated and collected in the same manner as described above. Recrystallized and vacuum dried material was investigated by mass spectrometry.

The reaction between NMA (5 mM, 107.16 mg) and t-BuQ (0.5 mM, 82.10 mg) was carried out as described above. Two red zones with Rf 0.39 (major, III) and 0.52 (IV), respectively, were eluted with 20 ml acetone and evaporated to dryness on a rotary evaporator under reduced pressure. The products were recrystallized from hot methanol/water to give red solids. We then analyzed the vacuum-dried products using the methods described above. Their melting points were 88°C for III and 119°C for IV. None of the compounds I–IV were found reactive toward nitrite in high excess and at low pH.

**Structural Determination of the Main Reaction Products**

**Compound I.** Mass spectral and elemental analyses indicated the molecular formula C16H13NO2. Analysis found: C, 74.98%; H, 6.50%; N, 5.40%. Calculated: C, 75.27%; H, 6.71%; N, 5.49%. The mass spectrum (Fig. 1) showed a molecular ion peak M⁺ at m/z 255, a strong fragment at 212 (M-C2H5), 240 (M-CH3), 77 (C6H5⁺) and other low-mass ion peaks: λmax (EtOH) 262 and 499 nm (18053 and 4233). The UV spectrum showed a strong bathochromic shift on addition of alkali (pH > 8) suggesting the presence of an N–H group in ortho position to a C=O group of the quinone ring. The most characteristic IR (KBr) absorption bands appeared at 3250 cm⁻¹ (NH-stretching), 2950 cm⁻¹ (CH3-stretching) and 1650 cm⁻¹ (C=O stretching), due to the quinoid ring.

The 15N-labeled compound followed the same general pattern as set out above and showed a molecular ion M⁺ at m/z 256 and a major fragment ion at m/z 213, supporting the presence of a single N atom in this adduct. Further evidence for the structural characteristics was obtained from 1H-NMR spectroscopy. One t-buty1 group was observed at 1.31 (s, 9H) and a typical set of two doublets at 6.52 (d, 1H) and 6.12 (d, 1H), both J = 2.4 Hz. With 15N the signal of the proton in position to the Ph–NH group was split into the two doublets at 6.12 (dd, 1H, J₁ = 2.4 Hz; J₂ = 1.0 Hz). Five aromatic protons and an NH were observed in the range 7.6–7.4 (m, 5H) and NH (d, 1H, J = 92.5 Hz). All of these data indicate the structure of the main product as that shown in Figure 1.

**Compound II.** The purified minor product (II) also yielded the same molecular formula, C16H13NO2, as compound I. Its mass spectra showed M⁺ at m/z 255 and a strong fragment at m/z 212; λmax (EtOH) 262 and 495 nm (ε 19545 and 3409). The addition of alkali (pH > 8) also resulted in a bathochromic shift from 498 to 510 nm. The NMR data show one t-buty1 group at 1.25 (s, 9H) and a typical set of two singlets at 6.48 (s, 1H) and 6.02 (s, 1H). The five aromatic protons and an NH were observed in the range 7.6–6.9 (m, 5H) and at 7.5 (s, 1H). The IR spectrum was similar to that of compound I. All these findings indicate the structure of II to be the isomeric anilino-quinone shown in Figure 2.

**Compound III.** The major product obtained from the reaction of t-BuQ with N-methylaniline gave a molecular formula of C17H19NO2, which was supported by its mass spectrum (Figure 1), giving M⁺ at m/z 269 and strong fragment ions at m/z 254 (M-CH3); 226 (M-C2H5); 77 (C6H5⁺). Analysis found: C, 76.00%; H, 7.13%; N, 5.20%. Calculated: C, 76.10%; H, 7.11%; N, 5.20%; λmax (EtOH) 475 and 257 nm (16809 and 3822). Its IR spectrum showed the loss of the NH absorbance at 3250 cm⁻¹. Further characteristic bands appeared at 2950 cm⁻¹ (asymmetric methyl stretching vibration), 2870 cm⁻¹ (symmetric methyl stretching vibration), 1500 cm⁻¹ (C=O stretch) and 1650 cm⁻¹ (C–N stretch).

**Figure 1.** Mass spectra of the quinone adducts isolated from reaction of BHA with aniline, 15N-aniline and N-methylaniline as described in Materials and Methods.

**Figure 2.** Possible mechanism for the formation of quinone adducts initiated by interactions of BHA with aniline (I, II; R=H) and N-methylaniline (III, IV; R=Me).
(symmetric stretching), 1260 and 1210 cm⁻¹ (tertiary butyl vibration). The NMR data show the t-butyl group at 1.01 (s, 9H) and methyl protons at 3.21 (s, 3H). A typical set of doublets at 5.79 (d, 1H; J = 2.8 Hz) and 6.41 (d, 1H; J = 2.5 Hz) together with the aromatic protons in the range of 6.88 – 7.33 (m, 5H) were good evidence for the assigned structure (III; Figure 1).

**Compound IV.** The mass spectrum produced the same characteristic fragmentations as shown in the assigned structure (III); \( m_{\text{max}} (\text{EtOH}) \) 254 (shoulder) and 492 nm (3040 sh). The NMR data [8: 1.24 (s, 9H), 3.25, (s, 3H), 5.70 (s, 1H), 6.27 (s, 1H), 6.90–7.39 (m, 5H)] indicate the structure of the compound (IV; Fig. 2).

**Reaction Yields and Mutagenicity**

After completion of the structural identification of the major products, we attempted to determine their yields in reactions run at various physiological pH values. Observations during workup indicated the rates of appearance of some of the products depended on pH. In general, the initial reaction conditions and separations were as already described. The substances were removed from the TLC plates, dissolved in chloroform, evaporated to dryness, and the residues dissolved in ethanol for UV spectrophotometric analysis. The yields are shown in Table 1.

Mutagenicity was assayed according to the original protocol of Ames et al. (11). We used the histidine auxotrophic strains TA98 and TA100 of *S. typhimurium* as test organisms. Tester strains were maintained and checked for retention of properties as recommended by Maron and Ames (13). We prepared hepatic post-mitochondrial fractions (S9) from three pooled livers of male Sprague-Dawley rats treated with Aroclor-1254. The S9 fraction was used at a concentration of 0.1 ml/mL of S9-mix.

| Reactants mole ratio | % Yield<sup>a</sup> | \( \text{An} \) | \( \text{t-BuQ} \) | pH | I | II |
|----------------------|----------------------|-----------------|-----------------|-----|-----|-----|
| 1 1<sup>b</sup>      | 7.0                  | 17.01 ± 6.8     | 1.4 ± 1.0       |
| 1 1                  | 7.0                  | 10.0 ± 1.3      | <1.0            |
| 2 1<sup>c</sup>      | 7.0                  | 36.50 ± 9       | 19.2 ± 3.1      |
| 1 1<sup>d</sup>      | 2.0                  | 1.03 ± 4        | <0.1            |
| 1 2                  | 7.0                  | 18.6 ± 1.9      | 1.02 ± 2.9      |
| 1 2<sup>e</sup>      | 2.0                  | 2.6 ± 2.4       | <1.0            |

| NMA | \( \text{t-BuQ} \) | III | IV |
|-----|-----------------|-----|-----|
| 1   | 7.0             | 3.2 ± 0.5 | 1.1 ± 0.2 |
| 2   | 7.0             | 6.0 ± 1.3 | 1.7 ± 0.4 |

<sup>a</sup>Expressed as percentage of the theoretical reaction; values are means (SD; n = 2).

<sup>b</sup>Performed in ethanol solution.

Test samples were dissolved in dimethyl sulfoxide. All the experiments were performed in two separate assays with triplicate plates.

**N-methylaniline caused no increase in the number of revertants per plate in S. typhimurium strains TA98 and TA100 at concentrations up to 2.0 mg/ml plate with or without S9 mix. Because of the low yield of compound IV, insufficient material was available to conduct testing.

**Results and Discussion**

The fate of available aniline residues in food constituents is difficult to characterize because, in general, foodstuffs are so heterogeneous and complex. However, the chemical bonding of aniline to food additives can be characterized through models that allow the determination of suspicious products. In this report we examined the chemical reactions initiated by the food additive BHA and its main metabolites with some xenobiotic food constituents like aniline and NMA, which are also known to be breakdown products of some pesticides.

Under the mild reaction conditions described, both aniline and NMA yielded red products with t-BuQ. No visible change was observed in individual solutions of the two phenolic antioxidants BHA and t-BHQ in aniline or NMA during the same time period. The results of the investigations are summarized in Table 1. The maximum yield of the anilinoquinones depended on the combination of concentrations of the substrates and on the initial pH value.

Based on our model experiments with \(^{15}\)N-labeled aniline, we suggest that the reaction proceeds basically as in Figure 2. This type of mechanism is often postulated to explain the binding of substituted anilines to phenolic humus constituents by way of enzymatic oxidation (14, 15). Thus it seems likely that the phenols will be first oxidized by oxidants like \( O_3 \) and \( MnO_2 \) (pyrolusite) or the food additives \( NaNO_2 \) and \( KIO_3 \) to the corresponding quinones (16). The oxidation reactions were also observed by incubating BHA with liver microsomes (17). The quinone is an obvious oxidation product of both BHA and t-BHQ. Recent work by Bergmann et al. has indicated the participation of the \( \alpha \)-butyl- semiquinone anion radical during the microsomal incubation of t-BuQ and \( \alpha \)-BHQ (18).

The studies described above, as well as those cited by Warner et al. (19) demonstrate that phenolic antioxidants undergo a sequence of reactions in food. In a subsequent step the attack of the nucleophilic amine occurs at the less sterically hindered ring position of the quinone. It was therefore not unexpected that only one additional isomeric product was observed in low yields.

It is generally accepted that univalent reduction of quinones is a major cause of their intrinsic toxicity (20). Data on the toxicity of t-BuQ is not available. However, a mutagenic compound arising from BHA upon treatment with nitrite under acidic conditions has been identified as \( t\)-BuQ (21). In recent studies, no mutagenicity was detected in the ROS-sensitive tests.

**Table 2. Mutagenicity testing of the reaction products on *S. typhimurium* TA98 and TA100 in the absence and presence of S9-mix**

| Compound | Concentration (mg/plate) | His<sup>+</sup> revertant colonies/plate<sup>a</sup> |
|----------|--------------------------|-----------------------------------------------|
|          | Strain TA98              | \(-S9\) | \(+S9\) |
|          | Strain TA100             | \(-S9\) | \(+S9\) |
| \( t\)-BuQ | 0 (control)             | 20 ± 3.1 | 39 ± 6.3 | 105 ± 6.2 | 102 ± 10.7 |
|          | 10                      | 19 ± 5.5 | 38 ± 5.7 | 96 ± 11.2 | 105 ± 4.5 |
|          | 20                      | 18 ± 2.9 | 35 ± 6.0 | 58 ± 10.2 | 87 ± 7.2 |
|          | 30                      | Toxic     | 31 ± 9.1 | 13 ± 7.9 | 89 ± 7.3 |
|          | 40                      | Toxic     | 34 ± 4.3 | 15 ± 4.6 | 75 ± 6.8 |
|          | 100                     | Toxic     | 27 ± 6.5 | Toxic     | 73 ± 7.0 |
| \( \text{AnQ} (I) \) | 0 (control)             | 14 ± 3.7 | 316 ± 9 | 102 ± 6.2 | 101 ± 5.3 |
|          | 10                      | 16 ± 4.2 | 296 ± 8 | 97 ± 11.1 | 98 ± 8.0 |
|          | 100                     | 18 ± 5.2 | 286 ± 1 | 101 ± 12.1 | 96 ± 11.2 |
|          | 150                     | 14 ± 4.6 | 245 ± 5 | 85 ± 12.0 | 98 ± 11.7 |
|          | 1000                    | 12 ± 7.6 | 227 ± 9 | 84 ± 16.1 | 90 ± 1.7 |
| \( \text{AnQ} (II) \) | 0 (control)             | 12 ± 4.2 | 37 ± 8.0 | 104 ± 4.5 | 103 ± 3.0 |
|          | 10                      | 14 ± 3.2 | 27 ± 2.4 | 94 ± 5.5 | 94 ± 7.3 |
|          | 100                     | 15 ± 2.7 | 22 ± 3.1 | 81 ± 6.1 | 96 ± 1.0 |
|          | 500                     | 15 ± 6.6 | Toxic     | 17 ± 1.5 | 5 ± 0.8 |
|          | 1000                    | Toxic     | Toxic     | Toxic     | Toxic     |
| \( \text{NMA-Q (III)} \) | 0 (control)             | 16 ± 3.9 | 315 ± 6 | 106 ± 5.9 | 103 ± 8.6 |
|          | 10                      | 18 ± 2.0 | 283 ± 5 | 1016 ± 17.9 | 97 ± 12.7 |
|          | 100                     | 14 ± 2.1 | 284 ± 1 | 90 ± 13.8 | 94 ± 10.0 |
|          | 500                     | 16 ± 2.7 | 203 ± 2 | 72 ± 12.2 | 73 ± 7.5 |
|          | 1000                    | 13 ± 4.3 | 203.5 | 91 ± 2.7 | 28 ± 5.6 |

Abbreviations: \( t\)-BuQ, \( \alpha \)-butylquinone; \( \text{AnQ} \), anilinoquinone; NMA, \( N \)-methylanilinoquinone.

*The results are averages of two independent experiments each using three plates; means ± SD.*
tester strains *S. typhimurium* TA102 and TA104 (22). Our results, summarized in Table 2, demonstrate that t-BuQ showed no mutagenic response at two nontoxic doses in the standard *Salmonella*/microsome test. With increasing concentration of the quinone a cytotoxic effect was observed, apparent in the decreasing yield of macroscopically visible His' revertant colonies and in the thinning of the background lawn. This observation is in agreement with the investigations of Kabl et al. (23); It was, moreover, partly reduced by the addition of S9-mix. The thinning of the background lawn was not quantitative determined, hence we may make no statement concerning the proportionality to the His' revertants. We are looking into potential antigenotoxic effects using eukaryotic systems and will report these studies later. The toxicity was completely abolished in the adduct (I) prepared by the reaction of t-BuQ with aniline. In contrast to compound I, the isomeric compound II was cytotoxic at high concentrations. The main product in the reaction of t-BuQ with NMA (III) was cytotoxic in the *S. typhimurium* assay. All of the investigated products failed to induce any increases in revertant numbers in either of the strains, with or without S9-mix.

**Conclusions**

*N*-nitroso-*N*-methylaniline is a potent esophageal carcinogen (24). Spinach and many pickled vegetables (cucumber, celery, and others) are known to be sources of one of its precursors, the NMA used in this study. From our work it seems possible that BHA and its quinoid metabolites could exert a protective effect against the formation of *N*-nitroso-*N*-methylaniline. This might involve diversion of both the aniline and nitrite to form the innocuous nitrite-inactive compounds found in this study. This conclusion is important in connection with Kato's recent observation of the transformation of arylamines into direct-acting mutagens by the action of nitrite (25). Furthermore, a diminution in the superoxide anion (18) may occur as the metabolites are transformed into anilino adducts. Clearly, the availability of these reagents and the relative kinetics of their reactions with each other ('additive cocktail') will determine the importance of these effects. Delimitation of the conditions favoring protection in such cases will be the subject of further work.

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