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The Butylated Hydroxyanisole-Nitrite Reaction: Effects on N-Nitrosodimethylamine Formation in Model Systems

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Butylated hydroxyanisole (BHA) prevented the formation of N-nitrosodimethylamine from dimethylamine and nitrite in a heterogeneous emulsion system. The reaction between BHA and nitrite was shown to yield 8 compounds, including 1-hydroxy-2-*t*-butyl-4-methoxy-6-nitrobenzene (I) as a major product.

Keywords——antioxidant; butylated hydroxyanisole; N-nitrosodimethylamine; nitrite; 1-hydroxy-2-*t*-butyl-4-methoxy-6-nitrobenzene; Sústane

The reaction of nitrite with secondary amines produces carcinogenic nitrosamines[2] under acidic conditions[3] and in the human stomach[4]. Several compounds, such as ascorbic acid,[5] polyphenols,[6,7] α-tocopherol,[6,8] propyl gallate[6] and sorbic acid,[9] are known to inhibit this reaction. Lipid-containing foods such as cow's milk, mayonnaise and egg yolk[10] have also been shown to prevent the reaction.

The present paper deals with the effect of butylated hydroxyanisole (BHA), an antioxidant which is widely used in Japan, on nitrosamine formation in a heterogeneous emulsion system, and with the characteristics of its reaction with nitrite.

Experimental

Sústane emulsion A, containing 10% BHA, 30% vegetable oil and 13% *Gum Arabic*, and the control emulsion, composed of 30% vegetable oil and 13% *Gum Arabic*, were kindly supplied by Nikki-Universal Company, Ltd. Butylated hydroxyanisole (BHA) was obtained by recrystallization of Sústane (Nikki-Universal Company, Ltd.) from petroleum ether to remove the 3-isomer.[11] N-Nitrosodimethylamine (standard compound for gas chromatography) was a product of Wako Pure Chemical Industries, Ltd.

Gas chromatography was carried out with a Yanaco G80 gas chromatograph equipped with a hydriogen flame ionization detector. Nuclear magnetic resonance spectra were taken with a JEOL CP-100 machine. A Hitachi 101 spectrophotometer was used for the measurement of absorbance. Thin-layer chromatography was performed with Kieselgel DF-5 (Chemie-Erzeugnisse und Adsorptionstechnik AG, CAMAG). Silica gel (100 mesh, Kanto Chemical Company, Inc.) was used for column chromatography.

Effect of Sústane Emulsion on Nitrosamine Formation——First, 3.0 ml of 4 M $NaNO_2$, 3.0 ml of 1 M dimethylamine hydrochloride and 7.2 g of either Sústane emulsion A or the control emulsion were added to 50 ml of 0.1 M sodium citrate. The mixtures were adjusted to the desired pH's and made up to 60 ml with water. The mixtures were incubated in stoppered 100 ml flasks at 37° for 3.5 hr. The mixtures turned brown during incubation. Portions of 10 ml were removed from the reaction mixtures and extracted with 40 ml of $CHCl_3$ in the presence of 2.0 g of NaCl and 5 ml of 5 N NaOH. Control experiments without the emulsions were carried out simultaneously. N-Nitrosodimethylamine in the $CHCl_3$ extracts (5 μl) was

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determined by gas chromatography. The chromatograph, using a glass column (3 mm i.d. ×3 m) of polyethyleneglycol 6000 (25%) on 80—100 mesh Chromosorb W AW, was operated under the following conditions: column at 130°, injector and detector at 150° and nitrogen gas flow at 20 ml/min. The amount of nitrosamine in the extract was determined by comparing the peak area of a sample with that of authentic standard solution in $CHCl_3$ (5 μl, 0.40 mg/ml).

Analysis and Isolation of the Reaction Products Formed from BHA and Nitrite——For isolation of the reaction products, 0.70 g (10.1 mmol) of $NaNO_2$ and 50 ml of ethanol containing 0.45 g (2.5 mmol) of BHA were added to 50 ml of 0.1 M sodium citrate. The solution was adjusted to pH 3.0 and incubated at 37° for 4 hr. The yellow reaction mixture was concentrated to about 40 ml and then extracted twice with 50 ml of benzene. The combined yellow extracts were washed with water and dried over anhydrous Na_2SO_4.

The products in the extract were analyzed by gas chromatography and thin-layer chromatography. The gas chromatograph, equipped with a glass column (3 mm i.d.×2 m) of silicon OV-17 on 60—80 mesh Chromosorb W AW, was operated under the following conditions: column temperature, linearly increased at 10°/min from 120° to 250°; nitrogen gas flow at 15 ml/min. A representative chromatogram is shown in Fig. 1. It gave 7 peaks besides the peak corresponding to BHA. The peak with a retention time of 9.6 min corresponded to the major product (I) and that with a retention time of 20.5 min corresponded to authentic 2,2′-dihydroxy-5,5′-dimethoxy-3,3′-di-*t*-butylbiphenyl (II).[11] Thin-layer chromatography, developing with *n*-hexane–dioxane (4: 1), revealed 7 orange spots and 2 spots positive to 2,6-dichloroquinone chloroimide reagent (Fig. 2). The orange spot having the highest R*f* value (0.6) corresponded to the major product (I). Two chloroimide-positive spots corresponded to BHA and II.

The extract was concentrated *in vacuo* and the resulting oil was applied to a silica gel column (28 mm i.d.×12 cm). The column was eluted with *n*-hexane-dioxane (4: 1), and the first 60 ml was evaporated to dryness *in vacuo*. The residue was crystallized from petroleum ether to afford 130 mg of 1-hydroxy-2-*t*-butyl-4-methoxy-6-nitrobenzene (I) (yield, 23%). Recrystallization of I from ethanol gave yellow needles, mp 92.5—93.5° (lit.[12]: 87.5—89°). Nuclear magnetic resonance spectrum (d_6-dimethylsulfoxide; internal standard, tetramethylsilane) δ, ppm: 1.42 (9H, s, $-C(CH_3)_3$), 3.78 (3H, s, $-OCH_3$), 7.20 (1H, d, H_3 or H_5, $J_{3,5}$=3 Hz), 7.36 (1H, d, H_3 or H_5, $J_{3,5}$=3 Hz), 10.70 (1H, bs, OH). *Anal.* Calcd for $C_{11}H_{15}NO_4$: C, 58.67; H, 6.67; N, 6.22%. Found: C, 58.51; H, 6.94; N, 6.20%.

Results and Discussion

The nitrite consumptions by fat-soluble antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and dl-α-tocopherol (Toc) were measured in 10% ethanolic buffered solution (pH 3.0). When 0.5 mM nitrite was treated with 2.0 mM of the antioxidants at 37°, the color of each reaction mixture turned yellow or orange, and the nitrite concentrations, in terms of the production of the azo-dye from sulfanilamide-naphtylethylenediamine were reduced to 45, 72, 65 and 82% by BHA, BHT, PG and Toc, respectively, after incubation for 2 hr. The decrease of nitrite concentration by BHA was dependent on pH, and was maximum at pH 3.0 (between pH 3.0 to 5.0).

Table I. Inhibitory Effects of Sústane Emulsion A on the Formation of N-Nitrosodimethylamine

	pH	Concentration of the nitrosamine formed (M)	Formation ratio
Control	3.0	0.049	1.00
Control emulsion	3.0	0.020	0.41
Sústane emulsion A	3.0	0.001	0.02
Control	4.0	0.027	0.55
Control emulsion	4.0	0.015	0.31
Sústane emulsion A	4.0	0.006	0.12
Control	5.0	0.004	0.08
Control emulsion	5.0	0.003	0.06
Sústane emulsion A	5.0	0.003	0.06

A mixture of 0.2 M $NaNO_2$, 0.05 M dimethylamine and the emulsion (12%) was incubated at 37° for 3.5 hours. Sústane emulsion A (12%) contained 0.07 M BHA.

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Sústane emulsion A, composed of BHA, vegetable oil and *Gum Arabic*, is considered to be one of the best models for foodstuffs rich in fats, and is used as an antioxidant preparation in Japan. The effects of Sústane emulsion A on nitrosamine formation were investigated, and compared with the effects of the control emulsion system, lacking BHA (Table I). When 0.2 M nitrite and 0.05 M dimethylamine were incubated at pH 3.0, 4.0 and 5.0 and at 37° for 3.5 hours in the presence of 12% of either control or Sústane emulsion, all the heterogeneous emulsion mixtures gradully turned yellow. The control emulsion retarded the formation of N-nitrosodimethylamine by 59% at pH 3.0, 44% at pH 4.0 and 25% at pH 5.0. Sústane emulsion prevented the reaction by 98% at pH 3.0, 78% at pH 4.0 and 25% at pH 5.0. The inhibitory effect of the control emulsion on the formation of N-nitrosodimethylamine may be due to the unsaturated fatty acid residues of the vegetable oil,[10] to tocopherols contained in it, or to differences in the partition of the reactants, nitrite and dimethylamine, between the oil and water phases. Inhibitory effects due to BHA were demonstrated at pH 3.0 and 4.0.

BHA was reacted with a 4-fold molar excess of nitrite in 50% ethanolic citrate buffer (pH 3.0) at 37° for 4 hours. The reaction mixture was extracted with benzene, and the extract was then analyzed by gas chromatography (Fig. 1) and thin-layer chromatography (Fig. 2). The reaction products comprised at least 8 compounds. The major product (I) observed in both chromatograms was isolated in a yield of 23% after purification by silica gel column chromatography. It was identified as the known 1-hydroxy-2-*t*-butyl-4-methoxy-6-nitro-

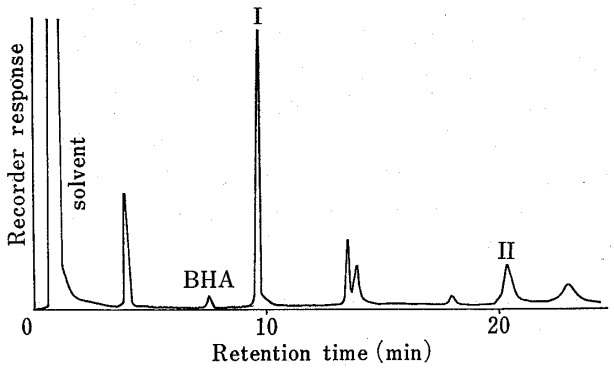


Fig. 1. Gas Chromatogram of an Extract of the Reaction Mixture of BHA and Nitrite

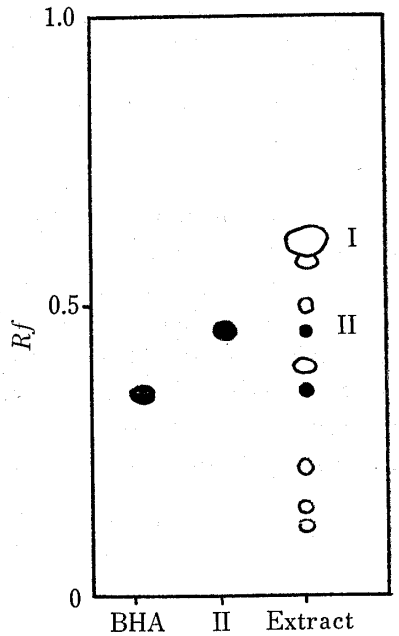
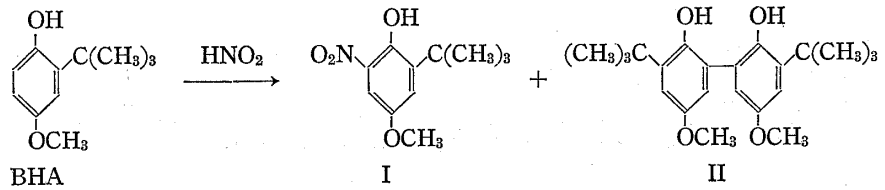


Fig. 2. Thin-Layer Chromatogram of an Exatract of the Reaction Mixture of BHA and Nitrite

○: Spots of yellow or orange color, and ●: spots positive to 2,6-dichloroquinone monochloroimide reagent.



+ unidentified products

Chart 1

benzene.[12] Among the minor products, 2,2′-dihydroxy-5,5′-dimethoxy-3,3′-di-*t*-butylbiphenyl (II) was identified by comparison of the retention time and *Rf* value with those of an authentic specimen.[11]

Monophenolic compounds are known to undergo nitrosation by nitrite under acidic conditions. Phenol,[13] *p*-cresol[14] and sesamol[15] are nitrosated at the *o*- or *p*-position. Tocopherol is oxidized to the corresponding quinone by nitrogen dioxide.[16] The profile of the reaction of BHA with nitrite was different from those of the above phenols. Ready transformation of BHA into the nitrophenol (I) by nitrite under mild acidic conditions prevented the formation of the nitrosamine in the reaction between dimethylamine and nitrite. Although I has no mutagenicity in rec-assay,[17] it might be metabolically transformed into toxic substances, such as hydroxylamine derivatives.

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A Molecular Orbital Study on the Approach of Hydride Ion to NAD^+ as a Coenzyme

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An *ab initio* molecular orbital study on the approach of a hydride ion (H^-) to NAD^+ as a coenzyme was performed. The nicotinamide ring (NA) of NAD^+ is attacked by H^- at the 4-position in an enzyme such as lactate dehydrogenase. It was found that the order of the electrophilic reactivity was 4-position>2-position>6-position, considering the total π electron densities and the frontier electron densities. Thus, it appears that the reactivity of the 4-position of NA of NAD^+ may be due to its electronic nature rather than to steric factors involving amino acid residues of the enzyme.

Keywords——MO; structure; molecular orbital; *ab initio*; hydride ion; NAD; NADH; nicotinamide; coenzyme; electronic structure

The mechanism of reduction of NAD^+ to NADH has been studied by many researchers. It has been shown that hydrogen is transferred to the nicotinamide ring (NA) of NAD^+ as a hydride ion in experiments using model compounds of NAD^+.[2–4] When NAD^+ is reduced by hydrosulfate, NADH is formed by the addition of H^- at the 4-position of NA.[5] In relation to the reactivity of the 4-position of NA, CN^- reacts with NAD^+ only at this position.[6] How-

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