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STUDIES ON MUTAGENICITY AND DESMUTAGENICITY OF OXIDIZED AND DIMERIZED DERIVATIVES OF BUTYLATED HYDROXYANISOLE

ブチルヒドロキシアニソールの酸化誘導体及び二量化誘導体の変異原性及び脱変異原性に関する研究

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CONTENTS

I GENERAL INTRODUCTION

Human cancer is closely related to the daily foods and life styles as shown by epidemiological studies [1]. The incidence of stomach and colon cancers among Japanese immigrants in the second generation shifted away from the pattern in Japan to that in the country of residence [2]; this implicates the importance of food habits in inducing cancers.

Human diet contains many helpful natural components such as anticarcinogens and antimutagens [3,4], and but it also includes a great variety of natural or artificial chemical carcinogens and mutagens [5-8]. They are (A) naturally occurring carcinogens and mutagens, such as pyrrolidine alkaloids [9,10], flavonoids [11,12] and anthraquinone [13,14] (especially in edible plants or spices), (B) the nitrosamines and nitrosamides formed from nitrosation reaction of amines and amides with nitrite while cooking or food processing, or digesting in the stomach [15-17], (C) food additives such as furylfuramide and sorbic acid [18-20] which are used as a disinfectant and a food preservative, respectively, (D) mycotoxins such as aflatoxin, produced by the contaminating fungi in foods

[21], (E) heterocyclic amines and polycyclic aromatic hydrocarbons generated by pyrolysis of amino acids in proteins (L-tryptophan, L-glutamic acid and so on) and food components [22-24], (F) some dicarbonyl compounds which are pyrolysates or fermentation products of carbohydrates [25], and (G) browning reaction (amino-carbonyl reaction) products [26]. The carcinogens are confirmed to induce the cancer by a clinical test, and the mutagens are experimentally proved to cause the mutation on a gene of bacteria. McCann *et al.* [27], Sugimura *et al.* [28], and Purchase *et al.* [29], had individually tested many carcinogens and mutagens, and they had concluded that most carcinogens (90%) can be detected experimentally as the mutagens by the Ames test. Therefore, it is easily understood that various food components directly participate in the human cancer.

There is another possibility on carcinogenesis as well as on anticarcinogenesis: Some food components produce the carcinogens or mutagens by an interacting with the other components in the animal digestive tracts such as stomach, but they *per se* are not toxicants. Beet, celery, lettuce, spinach, and radish contain about 200 mg of nitrate per 100 g [30].

Nitrate is endogenously reduced to nitrite by oral bacteria in saliva during digestion, and later nitrite occurs in the human stomach in large quantities. Appreciable amount of nitrite is also induced into the stomach by intake of dietary meat products. Under gastric acidity, nitrite easily reacts with amines [31], amino acids [32-35], phenols [36], mercaptans [37], and ascorbic acid [38], and frequently formed nitroso- or nitro-compounds. Three kinds of products are known as N-, C-, and S-nitroso or nitro compounds, most of which are carcinogens or mutagens. N-nitro or N-nitrosamines and their amides are formed from secondary amines and alkylamides. These compounds have their overall potency and versatility as animal carcinogens [39,40]. For example, N-methyl-N'-nitro-N-nitrosoguanidine is a potent carcinogen and mutagen. On the contrary, when nitrite reacts with primary aromatic amines such as the pyrolysate of tryptophan in gastric juices, nitrite converts the pyrolysate into its hydroxy derivative with little or no mutagenic activity [41]. On the other hand, C-nitro or C-nitroso compounds are also derived by an interaction of nitrite with phenolic compounds [42]. Thus, nitrite has a multiple reactivity under gastrically acidic

condition.

A phenolic antioxidant, butylated hydroxyanisole (BHA), is used widely as a food additive [43]. BHA has the multiplicity in function, which also exhibits both carcinogenicity and anticarcinogenicity. BHA has been shown to produce squamous cell carcinomas of the forestomach of F344 rats when fed at high concentrations for prolonged periods [44,45] and to have a promoting activity for urinary bladder carcinogenesis in F344 rats initiated by N-butyl-N-(4-hydroxybutyl) nitrosamine [46]. On the contrary, it has been shown that dietary administration of BHA inhibits the carcinogenesis caused by chemical carcinogens such as benzo[*a*]pyrene, dimethylaminoazobenzene and fluorenylacetamide in rodent tissues [47-50]. The administration of BHA stimulates the microsomal NADPH oxidase activity concomitant with inhibition of monooxygenase activity. Oral or intraperitoneal injection of BHA changes the cytochrome P-450-dependent metabolism of benzo[*a*]pyrene as well as the metabolism of the other carcinogens in a lung and forestomach [51-53]. Recent studies have demonstrated that metabolites of BHA inhibit electron transfer from NADPH-cytochrome P-450 reductase to its terminal monooxygenase of cytochrome

P-450 system, *in vivo* [54]. The dietary administration of BHA increased the activities of various enzymes involving in the disposition of foreign compounds, such as glutathione S-transferase, UDP-glucuronyltransferase, and epoxide hydrolase [55]. The inhibition *in vivo* by BHA against the chemical carcinogens comes to be fairly clear enzymatically. However, the mechanism of interaction of BHA with the various enzymes are still unclear, and the carcinogenic mechanism of BHA is also unclear even though the anticarcinogenicity of BHA can be explained with these enzymatic analyses. Thus, the multiplicity of the BHA function *in vivo* is unknown. I have assumed that the biological effects of BHA are due to not BHA itself but to the carcinogenic or anticarcinogenic activity of the derivatives of BHA, because BHA is easily converted into the various derivatives by interaction with food components such as nitrite under gastrically acidic condition.

The mutagenicity can be conventionally detected by the Ames test, and recent works on the mutagenicities of chemical carcinogens have demonstrated a close relationship between carcinogenicity and mutagenicity [56-58]. Ames' group developed a simple and short-term method for detecting carcinogens as mutagens, and

demonstrated to be effective in screening large numbers of carcinogens. A desmutagenicity of compound, an inhibiting effect on the mutagenicity, can be also detected by this method.

In this study, the mutagenicity and desmutagenicity of BHA derivatives, which were produced by interaction of BHA with sodium nitrite under acidic condition, were determined by the Ames test, and the bio-active derivatives of BHA were characterized by instrumental structural analyses. Two mutagens derived from BHA were identified as 2-*tert*-butyl-*p*-quinone and 3,3'-di-*tert*-butyl-biphenyldiquinone-(2,5,2',5') in Chapter II. One desmutagen derived from BHA was identified as 2,6-di-*tert*-butyl-8-hydroxy-dibenzofuran-1,4-quinone in Chapter III and the formation mechanism of the desmutagen was also described. In Chapter IV, the desmutagenicity of 2,6-di-*tert*-butyl-8-hydroxy-dibenzofuran-1,4-quinone toward a pyrolysate of tryptophan was described. In Chapter V, suppressing effects of food juices on the mutation caused by 2-*tert*-butyl-*p*-quinone were examined. Finally, in Chapter VI, the production mechanism of these derivatives of BHA was investigated.

REFERENCES

[1] R. Doll and R. Peto, *J. Natl. Cancer. Inst.*, 66, 1192 (1981).

[2] A. Kagan, B.R. Harris, W. Winkelstein, K.G. Johnson, H. Kato, S.L. Syme, G.G. Rhoads, M.L. Gay, M.Z. Nichaman, H.B. Hamilton and J. Tillotson, *J. Chron. Dis.*, 27, 345 (1974).

[3] L.W. Wattenberg, *Cancer Res.*, **43**, 2448 (1983).

[4] L.W. Wattenberg, *Cancer Res.*, **48**, 1 (1985).

[5] S.J. Jadhave, R.P. Sharma and D.K. Salunkle, *CRC Cri. Rev. Toxicol.*, **9**, 21 (1981).

[6] N. Katsui, F. Yagishita, A. Murai and T. Masamune, *Bull. Chem. Soc. Jpn.*, **55**, 2424 (1982).

[7] H. Griesebach and J. Ebel, *Angew. Chem. Int. Ed. Engl.*, **17**, 635 (1978).

[8] R.M. Bostock, R.A. Laine and J.A. Kuc, *Plant Phisiol.*, **70**, 1417 (1982).

[9] R.J. Huxtable, *Perspect. Biol. Med.*, **24**, 1 (1980).

[10] R. Schoental, *Toxicol. Lett.*, **10**, 323 (1982).

[11] A.M. Pamukcu, S. Yalciner, J.F. Hatcher and G.T. Bryan, *Cancer Res.*, **40**, 3468 (1980).

[12] G. Tamura, C. Gold, A. Ferro-Luzzi and B.N. Ames, *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 4961 (1980).

[13] J.P. Brown, *Mutation Res.*, **75**, 243 (1980).

[14] A.Ya. Potapenko, M.V. Moshnin, A.A. Krasnovsky, Jr. and V.L. Sukhorukov, *Z. Naturforsch.*, **37**, 70 (1982).

[15] M. Greenblatt, S.S. Mirvish and B.T. So, *J. Natl. Cancer Inst.*, **46**, 1029 (1971).

[16] P.N. Magee, *Fd. Cosmet. Toxicol.*, **9**, 207 (1971).

[17] H. Druckrey, *Gann Monogr.*, **17**, 107 (1975).

[18] Y. Kito and M. Namiki, *Tetrahedron*, **34**, 505 (1978).

[19] M. Namiki, S. Udaka, T. Osawa, K. Tsuji and T. Kada, *Mutation Res.*, **73**, 21 (1980).

[20] T. Osawa and M. Namiki, *Agric. Biol. Chem.*, **46**, 2299 (1982).

[21] G.N. Wogan, "*Mycotoxins in Foodstuffs*", MIT Press, Cambridge, Mass., 1965, pp. 1-291.

[22] T. Sugimura, T. Kawachi, M. Nagao, T. Yahagi, Y. Seino, T. Okamoto, K. Shudo, T. Kosuge, K. Tsuji, K. Wakabayashi, Y. Iitaka and A. Itai, *Proc. Jpn. Acad.*, **53**, 58 (1977).

[23] T. Kosuge, K. Tsuji, K. Wakabayashi, T. Okamoto, K. Shudo, Y. Iitaka, A. Itai, T. Sugimura, T. Kawachi, M. Nagao, T. Yahagi and Y. Seino, *Chem. Pharm. Bull.*, **26**, 611 (1978).

[24] T. Yamamoto, K. Tsuji, T. Kosuge, T. Okamoto, K. Shudo, K. Takeda, K. Yamaguchi, Y. Seino, T. Yahagi, M. Nagao and T. Sugimura, *Proc. Jpn. Acad.*, **54**, 248 (1978).

[25] C. Furihata, Y. Sato, T. Matsushima and M. Tatematsu, *Carcinogenesis*, **6**, 91 (1985).

[26] K. Shinohara, R.-T. Wu, N. Jahan, M. Tanaka, N. Morinaga, H. Murakami and H. Omura, *Agric. Biol. Chem.*, **44**, 671 (1980).

[27] J. McCann, E. Choi, E. Yamasaki and B.N. Ames, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 5135 (1975).

[28] T. Sugimura, S. Sato, M. Nagao, T. Yahagi, T. Matsushima, Y. Seino, M. Takeuchi and T. Kawachi, "*Overlapping of Carcinogens and Mutagens*", ed. by P.N. Magee, University of Tokyo Press, Tokyo, 1976, pp.191-215.

[29] I.F.H. Purchase, E. Longstaff, J. Ashby, J.A. Styles, D. Anderson, P.A. Lefevre and F.R. Westwood, *Nature*, **264**, 624 (1976).

[30] P.E. Hartman, *Environ. Mutagen*, 5, 111 (1983).

[31] S.S. Mirvish, *Toxicol. Appl. Pharmacol.*, **31**, 325 (1975).

[32] T. Ohta, M. Isa, Y. Suzuki, N. Yamahata, S. Suzuki and T. Kurechi, *Biochem. Biophys. Res. Commun.*,

100, 52 (1981).

[33] T. Ohta and S. Suzuki, *Agric. Biol. Chem.*, **47**, 405 (1983).

[34] A. Ohara, M. Mizuno, G. Danno, K. kanazawa and M. Natake, *Mem. Grad. School Sci. Technol., Kobe Univ.*, **4**, 47 (1986).

[35] A. Ohara, M. Mizuno, G. Danno, K. Kanazawa, T. Yoshioka and M. Natake, *Mutation Res.*, **206**, 65 (1988).

[36] M.E. Knowles, J. Gilbert and J. McWeeny, *Nature*, **249**, 672 (1974).

[37] G. Kubberod, *J. Food Sci.*, **39**, 1228 (1974).

[38] S.S. Mirvish, L. Wallcave, M. Egan and P. Shubik, *Science*, **177**, 65 (1972).

[39] B.S. Alam, J.B. Saporoschetz and S.S. Epstein, *Nature*, **232**, 116 (1971).

[40] W. Lijinsky and H.W. Taylor, *Fd. Cosmet. Toxicol.*, **15**, 269 (1977).

[41] D. Yoshida and T. Matsumoto, *Mutation Res.*, **58**, 35 (1978).

[42] J. Gilbert, M.E. Knowles and D.J. McWeeny, *J. Sci. Food Agric.*, **26**, 1785 (1975).

[43] B.N. Stuckey, "*Handbook of Food Additives*", ed. by T.E. Furia, Chemical Rubber Company, Cleveland,

OH., 1968, pp. 209-245.

[44] N. Ito, A. Hagiwara, M. Shibata, T. Ogiso and S. Fukushima, *Gann*, **73**, 132 (1982).

[45] N. Ito, S. Fukushima, A. Hagiwara, M. Shibata and T. Ogiso, *J. Natl. Cancer Inst.*, **70**, 343 (1983).

[46] K. Imaida, S. Fukushima, T. Shirai, M. Ohtani, K. Nakanishi and N. Ito, *Carcinogenesis*, **4**, 895 (1983).

[47] T.J. Slaga and W.M. Barcken, *Cancer Res.*, **37**, 1631 (1977).

[48] L.W. Wattenberg, *J. Natl. Cancer Inst.*, **48**, 1425 (1972).

[49] L.W. Wattenberg, *Adv. Cancer Res.*, **26**, 197 (1977).

[50] L.W. Wattenberg, *J. Environ. Pathol. Toxicol.*, **3**, 35 (1980).

[51] J.L. Speier and L.W. Wattenberg, *J. Natl. Cancer Inst.*, **55**, 469 (1975).

[52] L.K.T. Lam, A.V. Fladmoe, J.B. Hochalter and L.W. Wattenberg, *Cancer Res.*, **40**, 2824 (1980).

[53] M.W. Anderson, M. Boroujerdi and A.G.E. Wilson, *Cancer Res.*, **41**, 4309 (1981).

[54] Y.-N. Cha and H.S. Heine, *Cancer Res.*, **42**, 2609 (1982).

[55] S.W. Cummings and R.A. Prough, *J. Biol. Chem.*,

258, 12315 (1983).

[56] B.N. Ames, W.E. Durston, E. Yamasaki and F.D. Lee, *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 2281 (1973).

[57] J. McCann and B.N. Ames, *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 950 (1976).

[58] B.N. Ames and J. McCann, *Cancer Res.*, **41**, 4192 (1981).

II MUTAGENS DERIVED FROM BUTYLATED HYDROXYANISOLE WHEN REACTED WITH NITRITE UNDER GASTRICALLY ACIDIC CONDITION

II-1 INTRODUCTION

Prof. Natake *et al.* have shown that 2-*tert*-butyl-*p*-quinone (BQ) is one of the reaction products of butylated hydroxyanisole (BHA) and sodium nitrite. They found out that BQ had a high DNA-damaging activity [1,2]. Ishizaki *et al.* have also reported that the DNA-damaging activity arose by ultra violet light irradiation of the mixture of BHA and nitrite under a neutral pH condition [3]. The other many investigations also indicate that the reaction between BHA and nitrite forms a few kinds of products having DNA-damaging activity. One of them has been identified as BQ, the other are still unknown.

In this chapter, two derivatives of BHA were characterized by instrumental analyses, and determined as mutagens by the Ames test and the Rec-assay.

II-2 MATERIALS AND METHODS

Chemicals. BHA and sodium nitrite were purchased from Wako Pure Chemical Industries Ltd., Osaka, Japan. Agar, beef extract and yeast extract powder for the culture medium of microorganisms used in the Ames test and the Rec-assay were supplied from Difco Laboratory, Detroit, MI., U.S.A. Silica gel (Kiesel gel 60 PF_{254}) for thin layer chromatography (TLC) was obtained from Merck Co., Darmstadt, West Germany. Water and organic solvents were distilled before use.

The bacterial tester strains. *Salmonella typhimurium* TA 100 and TA 98 which require histidine for the growth are adopted for the mutagenicity tests. These tester strains contain each different type of mutation in the histidine operon, *hisG46* in TA 100 and *hisD3052* in TA 98. *HisG46* is present in a *hisG* gene coding for the first enzyme of histidine biosynthesis, and *hisD3052* mutation is present in a *hisD* gene coding for histidinol dehydrogenase [4]. TA 100 can detect mutagens that cause base-pair substitutions, primarily at one of these Guanine-Cytosine pairs. TA 98 can detect various frameshift mutagens. Since the frameshift mutagens attack N-atom of the bases and bound to them, frameshift mutagens can stabilize the

shifted pairing that often occurs in the respective sequences or "hot spot" of the DNA, resulting in a frameshift mutation which restores the correct reading frame for histidine synthesis. Both tester strains, in addition to the histidine mutation, contain other mutation that greatly increase their ability to detect mutagens. One mutation is located on *rfa* gene cluster, and causes partial loss of the lipopolysaccharide barrier that coats the surface of the bacteria and increases permeability to large molecules such as benzo[*a*]pyrene which do not penetrate the normal cell wall [5]. The other mutation (*uvrB*) is a deletion of a gene coding for the sensitivity in detecting many mutagens [5]. In both strains, moreover, the R-factor, pKM 101 is inserted. This plasmid increases chemical and spontaneous mutagenesis by enhancing an error-prone DNA repair system [6,7].

The other bacterial strain, Marburg strains of *Bacillus subtilis* was used for the screening of DNA-damaging activity. Strain H17 Rec^+ is a wild one and has a recombination capacity of the DNA repair system. Strain M45 Rec^- dose not have a recombination capacity [8] and is obtained by a treatment of H17 with N-methyl-N'-nitro-N-nitrosoguanidine.

Ames test [9,10]. *Salmonella typhimurium* strains were cultured overnight at 37°C in Difco nutrient broth medium. The mixture containing 0.7% agar and 0.6% sodium chloride was prepared as top agar and autoclaved. A sterile solution (10 ml) of 0.5 mM L-histidine and 0.5 mM biotin was added to 100 ml of the molten top agar. The culture strains (0.1 ml) were mixed thoroughly with test-substance (0.1 ml) and 0.1 M phosphate buffer (pH 7.4) or S-9 mix (0.5 ml) and incubated for 20 min at 37°C, and then, 2 ml of the molten top agar were added to the mixture. The mixture is poured onto minimal-glucose agar-medium, which contained 1.5% agar and 2% glucose in Vogel-Bonner medium E containing magnesium sulfate (2 g), citric acid monohydrate (20 g), potassium phosphate, dibasic (anhydrous) (100 g) and sodium ammonium phosphate (35 g) per 1 l medium. After incubation at 37°C for 2 days, revertant colonies were counted. Only the mutated bacteria can develop the colony on this medium.

Induced mutation frequency (IMF) method [11]. When the test-substance (mutagen) causes a lethal mutation, the IMF test should be used to remove an

experimental error due to the death of the test bacteria. The Ames cell was treated with a test-substance for 30 min at 37°C and then washed with phosphate buffer by centrifugation at 3,500 rpm for 10 min. After resuspension, the treated cell was poured onto the minimal medium, incubated for 2 days, and then the number of induced-His^+ colonies appearing on the medium was counted. Another part of the treated cell was also washed, diluted to an optimal concentration, and cultured on the minimal medium together with top agar containing a 5 mM excess of histidine. Then, the number of surviving colonies (His^-) was counted. The IMF value was estimated by dividing the number of revertant colonies by that of the surviving colonies.

In the case of the bio-antimutagenicity of the test-substance, the IMF test was also done, using a small different medium. Histidine and biotin were removed from the top agar, and a semi-enrich agar medium (MBB agar) was used in place of the minimal-glucose agar-medium. MBB agar was prepared by the additions of 4 g of glucose, 15 g of agar, 80 mg of Difco nutrient broth powder including histidine and 0.1 mg of biotin to 1 l of the Vogel-Bonner medium, which consist of 1 g of ammonium sulfate, 10 g of potassium

phosphate, monobasic, 0.1 g of magnesium sulfate, heptahydrate and 0.5 g of trisodium citrate per 1 l water. The revertant colony number was determined on the MBB agar and the surviving colony number was also counted after the dilution of the cell of the optimum concentration [12].

Rec-assay [8]. Rec^- and Rec^+ strains were cultured overnight in a basal synthetic (BS) medium supplemented with 0.1% casamino acid and 0.2% yeast extract. The cultured strains were diluted 10 fold with the BS medium, and streaked onto the surface of a broth agar plate. A paper disc, 8 mm in diameter, impregnated with 50 µl of solution of a test-substance was placed on the plate so as to cover the beginning of the bacterial streaks. The incubation for 24 h at 37°C visualized the bacteria that had grown except those in the inhibition zone, whose length was depending on the extent of toxicity of test-substance and on a variety of strain. A difference between the length of the inhibition zone of Rec^- and that of Rec^+ is proportionally related to the intensity of DNA-damaging activity.

Preparation of S-9 mixture [9]. The S-9 fraction was prepared from a liver of Sprague-Dawley rats as follows. The rats were given polychlorinated biphenyls at a dose of 500 mg/kg body weight and were sacrificed after 5 days. The liver was removed and perfused with cold 1.15% KCl solution, then weighed and homogenized with 3 volumes of the KCl solution using a Potter-type homogenizer. The homogenate was centrifuged at 9,000 x g, and supernatant was referred to as an S-9 fraction. The S-9 fraction was stored at -80^{o}C until use. The S-9 mix is composed of 0.3 ml of the S-9 fraction, 4 µmol NADPH, 2.5 µmol glucose 6-phosphate, 0.5 units glucose-6-phosphate dehydrogenase, 8 µmol $MgCl_2$, 33 µmol KCl and 100 µmol sodium phosphate buffer (pH 7.4), and the final volume was made up to 1 ml with water.

The reaction of BHA with sodium nitrite. Thirty millimoles of BHA were added to 300 ml of 70% ethanol containing 60 mmoles of sodium nitrite. The pH of mixture was adjusted to 2 with 2N HCl and allowed to stand for 1 h at an ambient temperature. The reaction mixture was extracted 3 times with a 300 ml portion of chloroform. The extract was washed with water, dried

over anhydrous sodium sulfate, and condensed *in vacuo* to 300 ml. A five milliliter aliquot of the condensed extract was allowed to dry up under nitrogen stream. The dried materials was dissolved in 1 ml of dimethyl sulfoxide (DMSO) and subjected to the determination of the mutagenic activity. Another portion of dried materials was dissolved in 1 ml of diethyl ether, subjected to TLC or gas chromatography (GC) (Shimadzu GC-6AM) using a ϕ 3 mm x 1 m column packed with 1.5% Silicon OV-1 (80-100 mesh) and FID detector. The analytical conditions were an increase in temperature from 80°C to 260°C at 5°C per min, a flow rate of 40 ml/min of nitrogen gas, and the injection temperature of 270°C.

Isolation of mutagens. The above reaction products of BHA with nitrite were dissolved in diethyl ether and applied to TLC with a solvent system of hexane-diethyl ether (7:3, v/v). Each product was extracted from silica gel of TLC with diethyl ether and the extracts were dried under nitrogen stream. After the dried materials were dissolved in 1 ml of DMSO, the mutagenic activity of each product was checked by the Ames test in the presence or absence of S-9 mix and by

the Rec-assay. The vacuum distillation method as described by Natake *et al.* [2] was carried out to obtain a mutagen in a large amount. On the other hand, one of the mutagens found with the above analyses was reprepared by the different procedure as follows: BHA (30 mmoles) was dissolved in 300 ml 70% ethanol solution of sodium nitrite (60 mmoles), the pH of solution was adjusted to 5 with 2N HCl, and allowed to stand for 1 h at ambient temperature. With time, light brown precipitate was produced, and was collected by filtration. After the 1 g precipitate was suspended in a mixed solvent of ethanol-diethyl ether-water (70:15:15), sodium nitrite (2 g) was added to the suspension again. The pH of the suspension was adjusted to 2 with 2N HCl and allowed to stand for 3 h at the ambient temperature and for the additional 30 min on ice. Yellow precipitate occurred was collected by the filtration.

Instrumental analyses. In order to elucidate structure of the isolated products, infrared spectrum (IR), ultraviolet spectrum (UV), nuclear magnetic resonance spectrum (NMR), gas chromatography-mass spectrometry (GC-MS) and high resolution mass spectrom-

etry were measured using a Hitachi EPI-S2, Hitachi 200-10 spectrometer, Varian XL-200 NMR spectrometer, Hitachi RMU-6MG and JEOL JMS-D300, respectively.

II-3 RESULTS

Mutagenicity of BHA derivatives separated by TLC

BHA was reacted with sodium nitrite at pH 2 and the reaction mixture was subjected to TLC (Fig. II-1). Nine products were obtained and subjected to the Ames test and the Rec-assay (Table II-1). Two products from Spot No. 2 (*Rf* 0.70) and Spot No. 3 (*Rf* 0.63) exhibited very high mutagenicities toward TA 100 strain in the absence of S-9 mix and DNA-damaging activities on the Rec-assay.

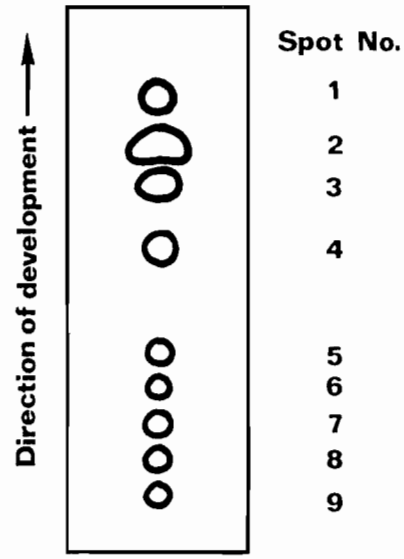

Fig. II-1. TLC of the reaction mixture of BHA and sodium nitrite.

Identification of two isolated mutagens

In order to obtain the above two mutagens in large amounts, these products were prepared by the vacuum

Table II-1

Mutagenic and DNA-damaging activity of the reaction products of BHA and nitrite separated on TLC

Spot No. on TLC	*Rf* value on TLC	Number of revertants per plate* on Ames test	Zone length of inhibition on Rec-assay** (mm)
Control***	-	123	0
1	0.80	124	0
2	0.70	10009	2.0
3	0.63	3745	1.7
4	0.50	128	0
5	0.28	123	0
6	0.22	125	0
7	0.14	122	0
8	0.07	127	0
9	0	125	0

* Mutagenic activity was measured using TA 100 without S-9 mix.

** DNA-damaging activity in Rec-assay was showed as the length of inhibition zone of Rec^- over that of Rec^+.

*** The sample-free solvent (DMSO) was used as a control of the Ames test and the Rec-assay.

distillation method and by the reaction of BHA with nitrite at pH 5 as described in MATERIALS AND METHODS. The product from Spot No. 2 was found to be identified with BQ, which was prepared by the vacuum distillation method, from the results of TLC, IR (1650 cm^{-1}), GC-MS (164, M^+) and UV (λ_{max} 248 nm). Its structure is elucidated as given in Fig. II-2.

The recrystallization from diethyl ether-hexane of the compound from Spot No. 3 gave the fine needles of melting point 170-171°C, which gave a molecular ion peak at *m/z* 326, and fragment ion peaks at *m/z* 311 (M^+-CH_3) and 298 (M^+-CO) in GC-MS spectrum (Fig. II-3). Its fragment ions

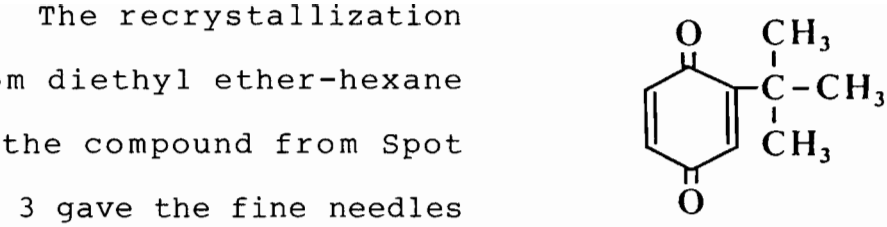

Fig. II-2. The structure of 2-*tert*-butyl-*p*-quinone (BQ) as one of the product mutagens derived from BHA.

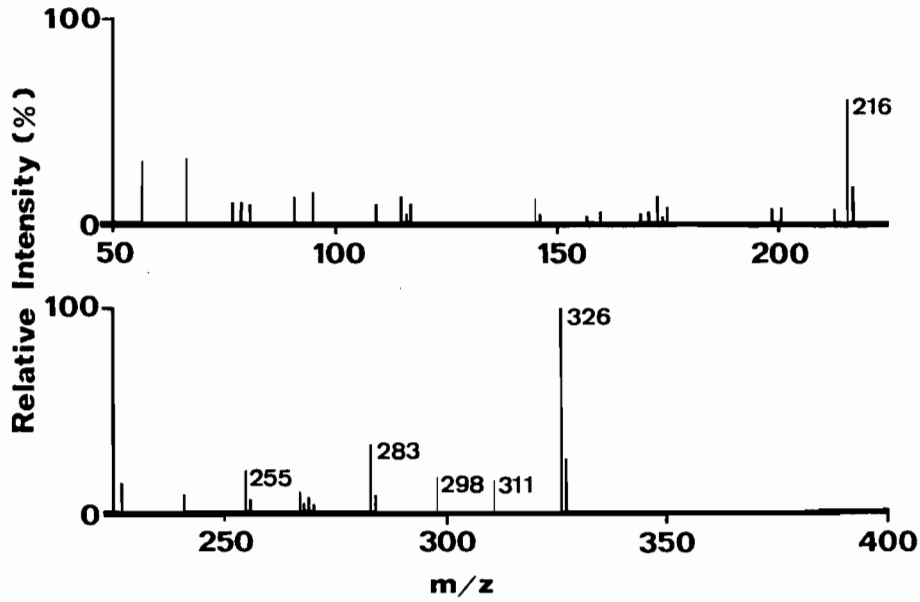

Fig. II-3. Mass spectrum of the isolated compound from Spot No. 3.

at *m/z* 298 and 216 [13,14] are typical of quinone, and the M^+-43 (*m/z* 283) fragment ion was ascribed to the rearrangement ion of *tert*-butyl group. A mechanism of

the fragmentation on this compound is summarized in Fig. II-4. The high resolution mass spectrum of this

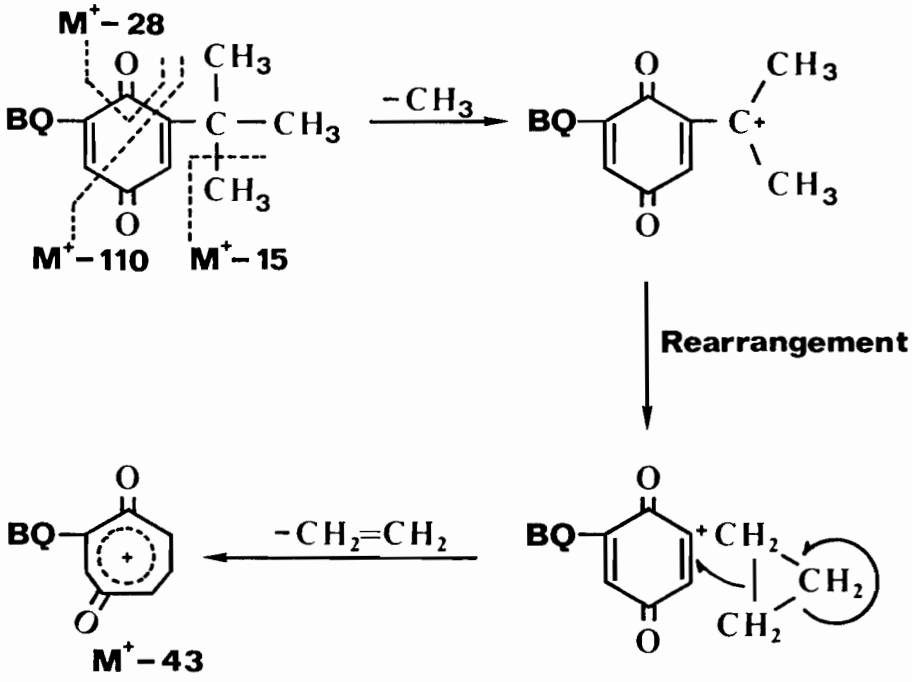

Fig. II-4. The scheme of fragmentation of the isolated compound from Spot No. 3.

compound showed *m/z* 326.1482 and indicated a formula of $C_{20}H_{22}O_4$. The elemental analysis; Found, C 73.68%, H 6.81%, O 19.51%, Calcd. for $C_{20}H_{22}O_4$, C 73.60%, H 6.80%, O 19.60%. The IR spectrum obtained as a KBr disc (Fig. II-5) showed bands at 2920 and 2850 (νCH), 1650 (νCO), 1390 and 1360 (δCH), and 1250 cm^{-1} ($(CH_3)_3$-C). The UV spectrum of this compound in methanol solution showed the maxima at 256nm (ε=18400), and

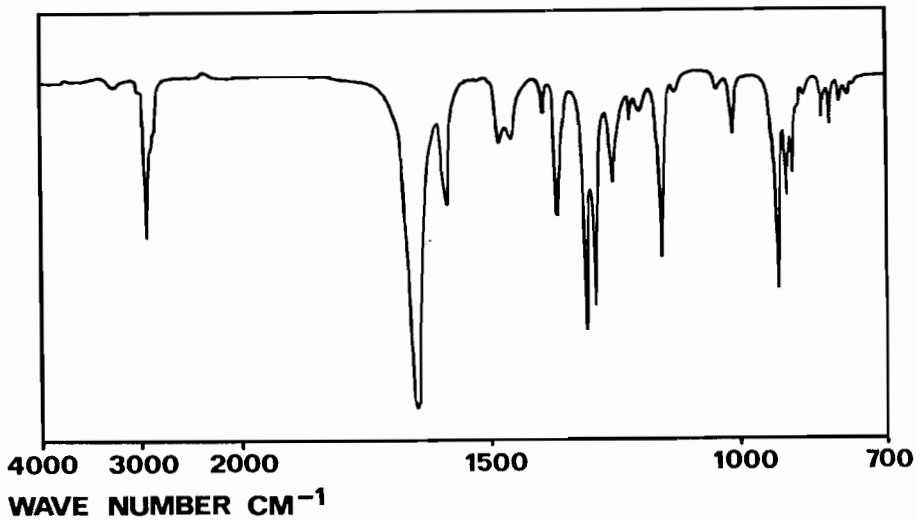

Fig. II-5. The IR spectrum of the isolated compound from Spot No. 3.

indicated a quinone skeleton. The ^{1}H-NMR spectrum in $CDCl_3$ (Fig. II-6) exhibited aromatic protons at δ6.70 (doublet, 2H, J=2.5Hz) and 6.73 (doublet, 2H, J=2.5Hz) and *tert*-butyl protons at δ1.32 (singlet, 18H). The coupling constant of aromatic protons indicated *meta*-coupling to each other. In the ^{13}C-NMR spectrum in DMSO-d_6, the signals at δ29.13 (quartet), 35.57 (singlet), 131.60 (doublet), 133.30 (doublet), 144.38 (singlet), 156.75 (singlet), 185.28 (singlet) and 187.02 (singlet) were assigned to C-8, 9, 10, 8', 9', 10', C-7, 7', C-4, 4', C-6, 6', C-3, 3', C-1, 1', C-2, 2' and C-5, 5', respectively (Fig. II-7). On the basis of these instrumental analyses, the structure of

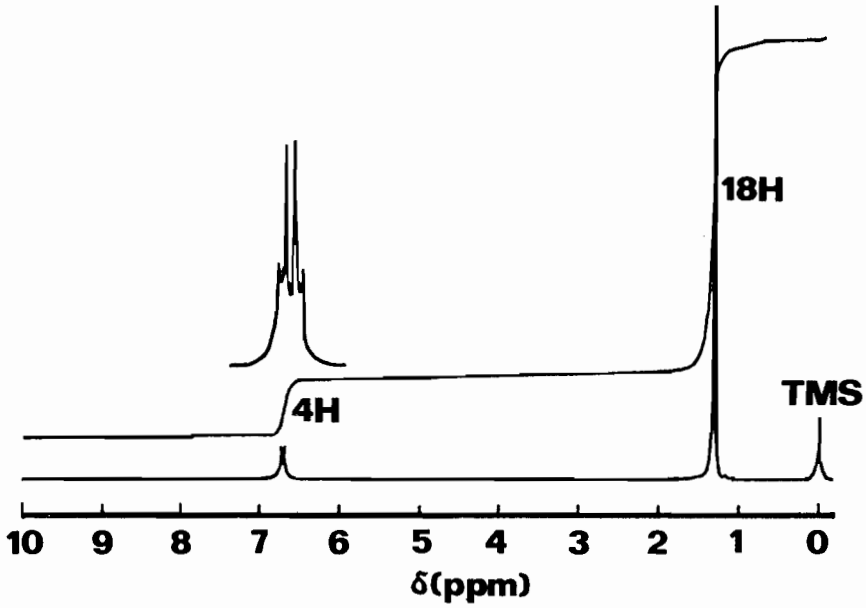

Fig. II-6. The ^{1}H-NMR spectrum of the isolated compound from Spot No. 3 in $CDCl_3$.

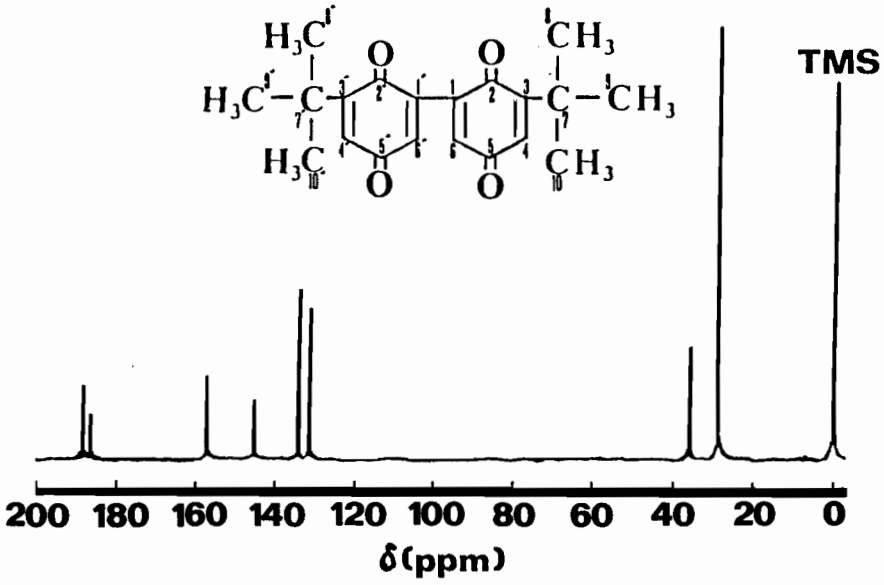

Fig. II-7. The ^{13}C-NMR spectrum of the isolated compound from Spot No. 3 in DMSO-d_6 (Decoupling complete).

compound from Spot No. 3 was determined to be 3,3'-di-*tert*-butyl-biphenyldiquinone-(2,5,2',5') (BBDQ, Fig. II-8).

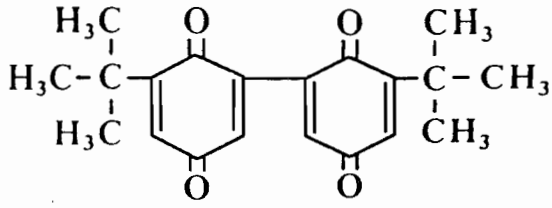

Fig. II-8. The structure of BBDQ derived from BHA.

The mutagenic activities of isolated BQ and BBDQ

The mutagenic activities of BQ and BBDQ were quantitatively determined at the various dose levels by the IMF method test without S-9 mix (Fig. II-9). With TA 100 strain, the IMF (from His^- to His^+) value of BQ was about 1.9×10^{-4} at the dose of 52 μg per plate. On the other hand, the surviving colony number was decreased with increasing dose levels of BQ from 41 (2 μg of BQ/plate) to 10 (52 μg of BQ/plate), and test bacteria did not grow at the dose of more than 104 μg of BQ/plate. This shows that BQ has not only mutagenic activity but also causes lethal mutations. Similarly, the IMF value with TA 98 strain was about 9.4×10^{-5}, and surviving colony number was decreased from 56 (2 μg/plate) to 3 (52 μg/plate) while the

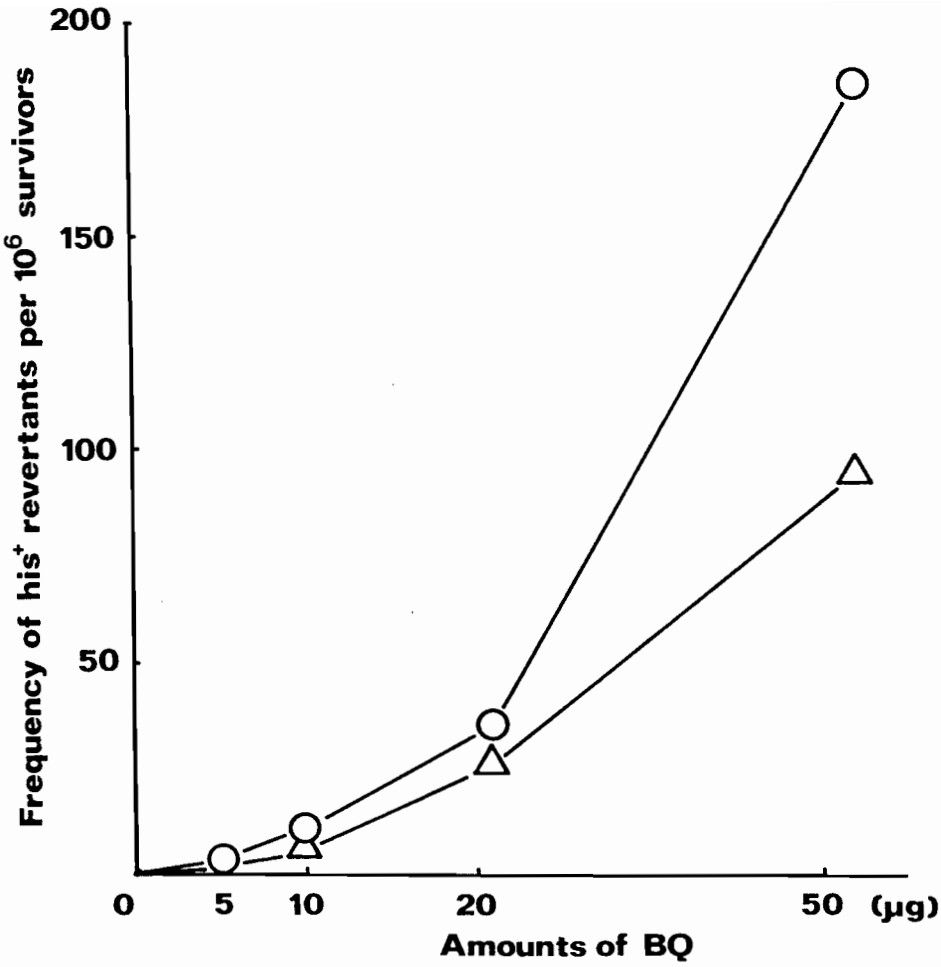

Fig. II-9. Mutagenicity for TA 100 (○) and TA 98 (△) of isolated BQ. Treated bacterial suspensions (0.1 ml) were plated for the observation of mutation induction. From the mean number of induced colonies appearing per plate, the mean number of spontaneous mutant colonies per plate (157 for TA 100 and 28 for TA 98) was subtracted, and the frequencies of mutations were calculated as followed; (mean number of induced His^+ colony - that of spontaneous mutant colony number) /that of surviving (His^-) colony number after 10^6 dilution.

strain did not grow at the dose of more than 104 μg of BQ/plate.

The IMF value of BBDQ with TA 100 was about 8.3 x 10^{-5} at the dose of 95 μg (Fig. II-10) and was higher

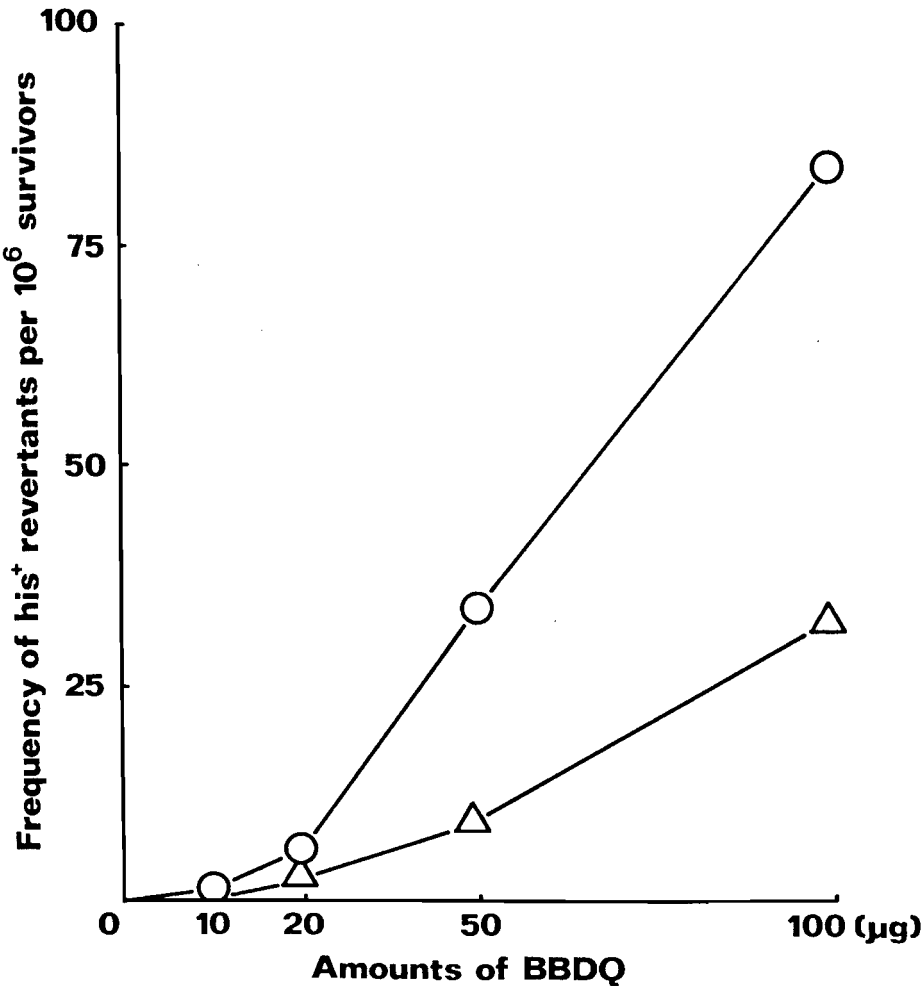

Fig. II-10. Mutagenicity for TA 100 (○) and TA 98 (△) of isolated BBDQ. Treated bacterial suspensions (0.1 ml) were plated for the observation of mutation induction. From the mean number of induced colony appearing per plate, the mean number of spontaneous mutant colony per plate (137 for TA 100 and 28 for TA 98) was subtracted, and the frequency was calculated by the same equation as noted in Fig. II-9.

than that with TA 98, as well as in the case of BQ. Surviving colony number decreased from 41 (5 μg/plate) to 23 (95 μg/plate). Thus, BBDQ also had the lethal mutagenic activity, but its magnitude was not so high as that of BQ. The mutagenic activity of BQ was 20 times as strong as that of BBDQ.

The lethal mutagenic activity of toxicants can be estimated by measuring the DNA-damaging activity by the Rec-assay. The difference of the length between the inhibition zones of Rec^- and that of Rec^+ were 3.8 mm for BQ and 1.5 mm for BBDQ per nmol, respectively, indicating that BQ had stronger DNA-damaging activities than BBDQ.

II-4 DISCUSSION

Knowles *et al.* [15] have reported that the reaction of phenols and nitrite formed C-nitro and C-nitroso compounds, most of which were mutagens. BQ and BBDQ are the reaction products of BHA with sodium nitrite under acidic condition, but are neither nitroso- nor nitro-compounds. They are the oxidation products and the strong mutagens of BHA. These are novel and noticeable findings in the field of food

chemistry.

It has been reported by Natake *et al.* [2] that BQ had DNA-damaging activity while it did not have mutagenic activity at its low dose level (5 μg BQ/plate). In the present study, it was found that BQ exhibited the activity at a limited concentration range (10 μg to 50 μg per plate) even though in the absence of S-9 mix. BBDQ was a dimer of BQ and was also mutagenic, although its mutagenic activity was weaker than that of BQ.

BBDQ was unstable, because it changed from a yellow to a red product under sunlight, especially under UV ray. Since it is predicted that this reaction occurred during the operation of the Ames test, the photolytic change of BBDQ may be partially responsible for the weaker activity of BBDQ than that of BQ in the Ames test. The photolytic product of BBDQ may not have any activities in the Ames test. The bio-activity of the photolytic product will be described in Chapter III.

There is a significant debate on the safety of BHA which popularly used as an antioxidant. Ito *et al.* reported that a large dose of BHA induced squamous cell carcinoma of forestomach in F344 rats. However, they

also found that Wistar rats and the other experimental animals having no forestomach did not get cancer in the glandular stomach corresponding to the human stomach [16,17]. Wattenberg [18-20], moreover, reported that administration of normal levels of BHA could suppress carcinogenesis caused by carcinogens such as benzo[*a*]pyrene. These information indicate that BHA has the complicated activities *in vivo*. The results obtained by the *in vitro* experiments suggested that the mutagens such as BQ and BBDQ might occur together with unmutagenic derivatives in the human stomach, when he took the foods containing the considerable amounts of BHA. Some derivatives from the reaction of BHA with nitrite have mutagenic activity but the others may have such opposed activity as des- or antimutagenicity. The desmutagenicity of some BHA derivatives will be also described in the following chapter.

REFERENCES

[1] M. Natake, G. Danno, T. Maeda, K. Kawamura and K. Kanazawa, *J. Nutr. Sci. Vitaminol.*, **25**, 317 (1979).

[2] M. Natake, K. Kawamura, G. Danno and K. Kanazawa,

Agric. Biol. Chem., **46**, 1083 (1982).

[3] M. Ishizaki, N. Oyamada, S. Ueno, K. Katsumura and Y. Hosogai, *J. Food Hyg. Soc. Jpn.*, **20**, 143 (1979).

[4] B.N. Ames, "*Chemical Mutagens, Principles and Methods of Their Detection*", ed. by A. Hollaender, Plenum, New York, Vol. 1, 1971, pp. 267-282.

[5] B.N. Ames, F.D. Lee and W.E. Durston, *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 782 (1973).

[6] J. McCann, N.E. Springarn, J. Kobori and B.N. Ames, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 979 (1975).

[7] D.E. Levin, E. Yamasaki and B.N. Ames, *Mutation Res.*, **94**, 315 (1982).

[8] T. Kada, K. Tutikawa and Y. Sadaie, *Mutation Res.*, **16**, 165 (1972).

[9] B.N. Ames, J. McCann and E. Yamasaki, *Mutation Res.*, **31**, 347 (1975).

[10] T. Yahagi, M. Nagao, Y. Seino, T. Matsushima, T. Sugimura and M. Okada, *Mutation Res.*, **48**, 121 (1977).

[11] T. Kada, H. Mochizuki and K. Miyao, *Mutation Res.*, **125**, 145 (1984).

[12] T. Kada, E. Brun and H. Marcovich, *Ann. Inst.*

Pasteur, **99**, 547 (1960).

[13] J.H. Bowie, D.W. Cameron and D.H. Williams, *J. Amer. Chem. Soc.*, **87**, 5094 (1965).

[14] J.H. Bowie, D.W. Cameron, R.G.F. Giles and D.H. Williams, *J. Chem. Soc. (B)*, 335 (1966).

[15] M.E. Knowles, J. Gilbert and D.J. McWeeny, *J. Sci. Food Agric.*, **26**, 267 (1975).

[16] N. Ito, A. Hagiwara, M. Shibata, T. Ogiso and S. Fukushima, *Gann*, **73**, 132 (1982).

[17] N. Ito, S. Fukushima, A. Hagiwara, M. Shibata and T. Ogiso, *J. Natl. Cancer Inst.*, **70**, 343 (1983).

[18] L.W. Wattenberg, *J. Natl. Cancer Inst.*, **48**, 1425 (1972).

[19] L.W. Wattenberg, *Adv. Cancer Res.*, **26**, 197 (1977).

[20] L.W. Wattenberg, *J. Environ. Pathol. Toxicol.*, **3**, 35 (1980).

III PHOTOLYSIS OF A MUTAGENIC 3,3'-di-*tert*-BUTYL-BIPHENYLDIQUINONE-(2,5,2',5') THAT INDUCES THE INHIBITION EFFECTS ON THE MUTAGENICITIES OF Trp-P-1 AND Trp-P-2

III-1 INTRODUCTION

I have conducted the experiments as to the mutagens formed in the reaction mixture of butylated hydroxyanisole (BHA) and nitrite under gastrically acidic conditions. In Chapter II, I found that two mutagens, 2-*tert*-butyl-*p*-quinone (BQ) and 3,3'-di-*tert*-butyl-biphenyldiquinone-(2,5,2',5') (BBDQ), were formed by the reaction of BHA with sodium nitrite. On the other hand, several other compounds having no mutagenicity were also isolated from this reaction mixture. It must be determined whether or not these compounds have some other bio-activities such as desmutagenicity. One of them was easily derived from BBDQ by a sunlight irradiation and showed a desmutagnic activity. I was interested in that one of the derivatives of BHA had a desmutagenic activity. The present chapter focussed on a formation mechanism of the desmutagen from BBDQ by the photolysis.

3-Amino-1,4-dimethyl-5*H*-pyrido[4,3-b]indole (Trp-P-1) and 3-amino-1-methyl-5*H*-pyrido[4,3-b]indole (Trp-P-2) are potent mutagens [1] and carcinogens [2,3], while the magnitude of mutagenicity of Trp-P-2 is slightly greater than that of Trp-P-1. Aflatoxin B1 is one of the strongest carcinogen among the chemical mutagens naturally occurring in foodstuffs as known well. The mutagenicity of Trp-P-2 is extremely high and comparable to that of aflatoxin B1.

On the other hand, Tsuda *et al.* [4] reported that Trp-P-1, Trp-P-2 and 2-amino-6-methyl-dipyrido-[1,2-*a*:3',2'-*d*]-imidazole (Glu-P-1) were readily converted into extremely weak or non-mutagenic deaminated compounds when treated with nitrite in an acidic solution. Yamaguchi [5] has reported that biologically active quinones such as menadione, anthraquinone and naphthoquinone had a desmutagenic activity toward Trp-P-2.

BBDQ is a quinone dimer and its photolytic product may have a quinonic structure. In this chapter, it was proved that the photolytic product of BBDQ had the desmutagenicity against Trp-P-1 and Trp-P-2, and the product was structurally elucidated by the instrumental analyses, and then the photolytic mechanism of BBDQ was proposed.

III-2 MATERIALS AND METHODS

Chemicals. BBDQ was isolated as yellow crystal from a reaction mixture of BHA and sodium nitrite as described in Chapter II. Trp-P-1 and Trp-P-2 (acetic acid salts) were purchased from Wako Pure Chemical Industries Ltd., Osaka, Japan.

Isolation of photolytic product from BBDQ. BBDQ was dissolved in diethyl ether at a concentration of 0.5 g/ml and irradiated with sunlight for 5 days. After the diethyl ether was evaporated *in vacuo*, the dried sample was recrystallized from ethanol. The obtained photolytic product was red needles, and its melting point was 236-237°C.

Acetylation. The red needle crystal was dissolved in anhydrous pyridine, followed by adding the equivalent volume of acetic anhydride, and allowed to stand for 1 day at ambient temperature. The acetylation product was extracted with chloroform 3 times after adding the distilled water. After dried over anhydrous sodium sulfate, the chloroform extract was concentrated by evaporation just before a yellow

powder appeared. The yellow fine crystal of acetylated product was obtained by standing the concentrated solution on ice.

Instruments for structural elucidation. In order to identify the photolytic product and its acetylated one, the analyses of IR, UV, NMR, electron ionization mass spectrometry (EI-MS) and field desorption mass spectrometry (FD-MS) were done using a Hitachi EPI-S2 instrument, Hitachi 200-10 spectrometer, Varian XL-200 NMR spectrometer, JEOL JMS-D300 and JEOL JMS-01SG-2, respectively.

Bacterial strain. *Salmonella typhimurium* TA 98 [6] was adopted as the bacterial tester strain, because Trp-P-1 and Trp-P-2 were more sensitive to this strain than to TA 100. The protocol for the treatment of this strain was described in detail in Chapter II.

Preparation of S-9 mix. An S-9 mix was prepared from male Sprague-Dawley rats according to the method described in Chapter II.

Desmutagenicity test of the photolytic product.

The reversion of *Salmonella typhimurium* TA98 by treatment with the mutagens was detected by the Ames test modified as follows. The order to mix the components in the test system was the mutagen (Trp-P-1 or Trp-P-2), the test-substance, S-9 mix (7.5 μl of the S-9 fraction per plate) and the test bacteria, and the total volume was 0.7 ml. After preincubation of the mixture at 37°C for 20 min, molten top agar (2 ml) was added to it, and the whole mixture was poured onto a minimal-glucose agar-medium. The revertant colonies were counted after 2 days incubation at 37°C. At least two independent experiments were done for each set of the reactions, and only the colonies on the surface of a lawn of non-mutated bacteria were counted microscopically as mutant colonies. The desmutagenicity (%) of BHDQ was calculated from an equation of (A - B) x 100/A, where A is the mean of revertant numbers on the plates treated only with mutagen, and B is the mean of revertant numbers on the plates treated with both BHDQ and the mutagen.

Free radical detection. In order to analyze the photolytic mechanism of BBDQ, electron spin resonance (ESR) spectra were measured for a 3% BBDQ benzene

solution irradiated with UV light under aerobic and anaerobic (degassed) conditions, using a JEOL FE-2XC.

III-3 RESULTS

Identification of the photolytic product from BBDQ

A fine red needle crystal of the photolytic product was obtained by the recrystallization from ethanol. The high-resolution EI-MS of the crystal showed *m/z* 326.1524 (M^+) and 311.1266 (M^+-CH_3), which indicated a formula of $C_{20}H_{22}O_4$ and $C_{19}H_{19}O_4$, respectively. The IR spectrum (Fig. III-1), obtained as a KBr disc, gave bands at 3400 (νOH), 2925 and 2850 (νCH), and 1640 cm^{-1} (νCO). The UV spectrum in

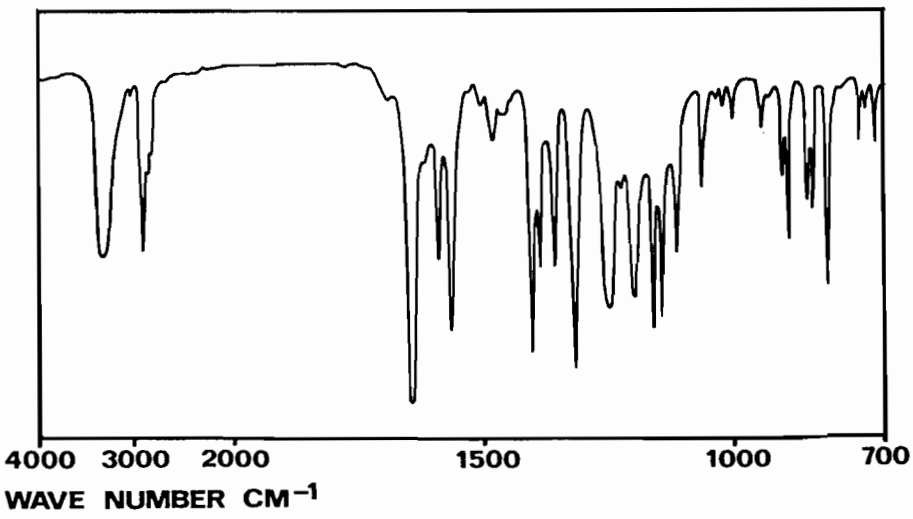

Fig. III-1. The IR spectrum of the photolytic product from BBDQ.

methanol (Fig. III-2) gave the maxima at 243 nm (ε=29900), indicating a skeleton of quinone, and at 287 nm (ε=7600), indicating a skeleton of phenol. The ^{1}H-NMR data in $CDCl_3$ (Fig. III-3) showed signals at δ1.38 (singlet, 9H), 1.50 (singlet, 9H), 4.94 (singlet, 1H, OH) which disappeared by adding D_2O, 6.64 (singlet, 1H), 7.02 (doublet, 1H, J=2.5 Hz) and 7.48 (doublet, 1H, J=2.5 Hz). These signals were assigned to protons on C-12, C-13, C-8, C-3, C-7 and C-9, respectively, which are numbered on the structure depicted in Fig.

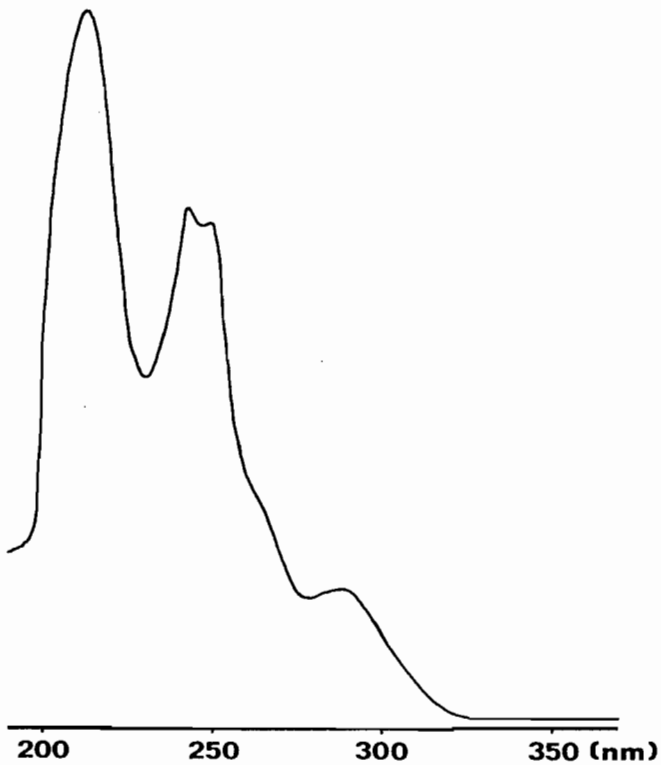

Fig. III-2. The UV spectrum of the photolytic product from BBDQ.

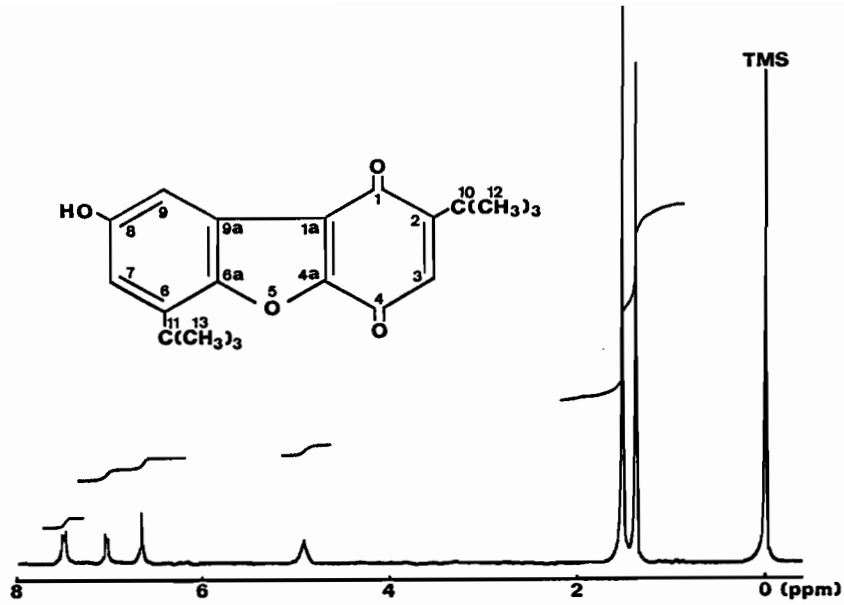

Fig. III-3. The ^{1}H-NMR spectrum of the photolytic product from BBDQ in $CDCl_3$.

III-3. The ^{13}C-NMR data in DMSO-d_6 exhibited signals at δ29.28 (quartet), 29.37 (quartet), 34.03 (singlet), 35.09 (singlet), 103.57 (doublet), 115.58 (doublet), 122.18 (singlet), 123.73 (singlet), 130.60 (doublet), 136.70 (singlet), 148.22 (singlet), 150.68 (singlet), 155.72 (singlet), 158.88 (singlet), 176.91 (singlet) and 183.93 (singlet). These spectra are shown in Fig. III-4 and assigned to carbons of C-12, C-13, C-10, C-11, C-9, C-7, C-2, C-6, C-3, C-8, C-9a, C-1a, C-6a, C-4a, C-4 and C-1, respectively. From these instrumental analyses, the structure of the photolytic product was presumed to be 2,6-di-*tert*-butyl-8-hydroxy-

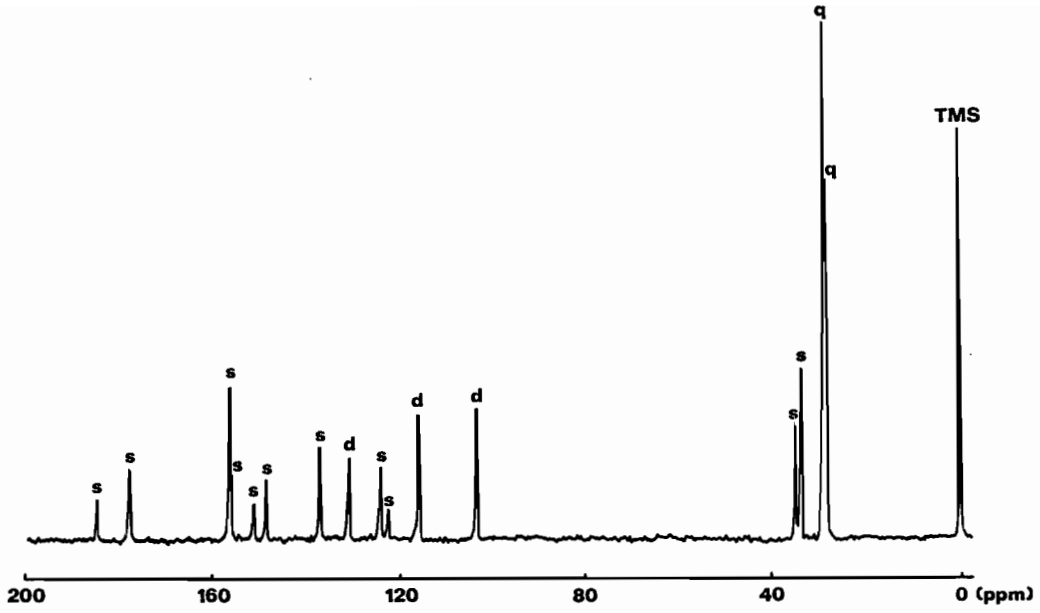

Fig. III-4. The ^{13}C-NMR spectrum of the photolytic product from BBDQ in DMSO-d_6 (decoupling complete).

dibenzofuran-1,4-quinone (BHDQ). In order to confirm the structure of this compound, the acetylated compound was prepared and subjected to the instrumental analyses. The high-resolution FD-MS of the acetylated compound showed *m/z* 368.1565 (M^+) and 325.1373 (M^+-CH_3CO), indicating a formula of $C_{22}H_{24}O_5$ and $C_{20}H_{21}O_4$, respectively. The IR spectrum (Fig. III-5) indicated the presence of no hydroxy group, acetyl group (1760 cm^{-1}) and quinone skeleton (1670 and 1660 cm^{-1}). The ^{1}H-NMR spectrum in $CDCl_3$ (Fig. III-6) gave a similar value to the signal due to BHDQ (Fig. III-3) except that the proton of the hydroxy group at δ4.94 of BHDQ

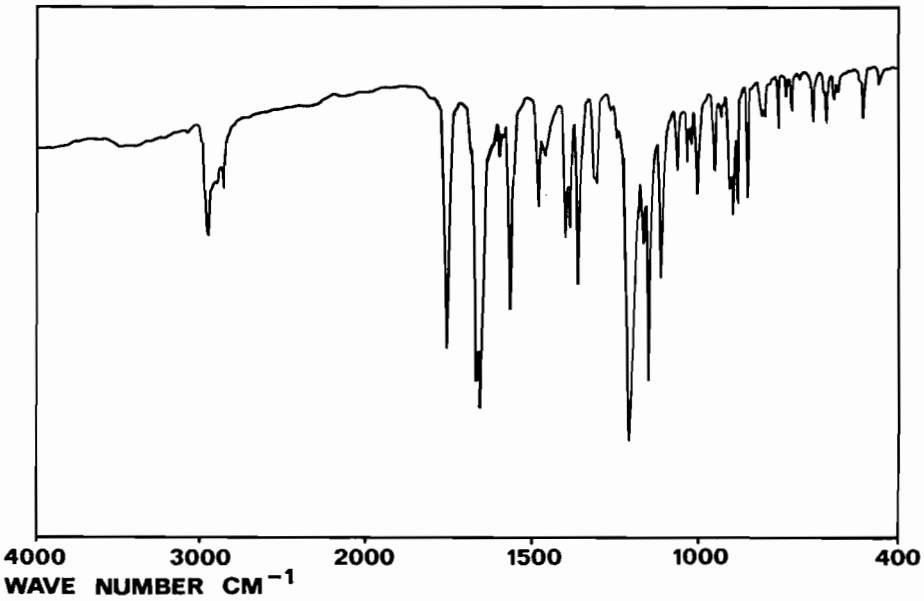

Fig. III-5. The IR spectrum of acetylated BHDQ.

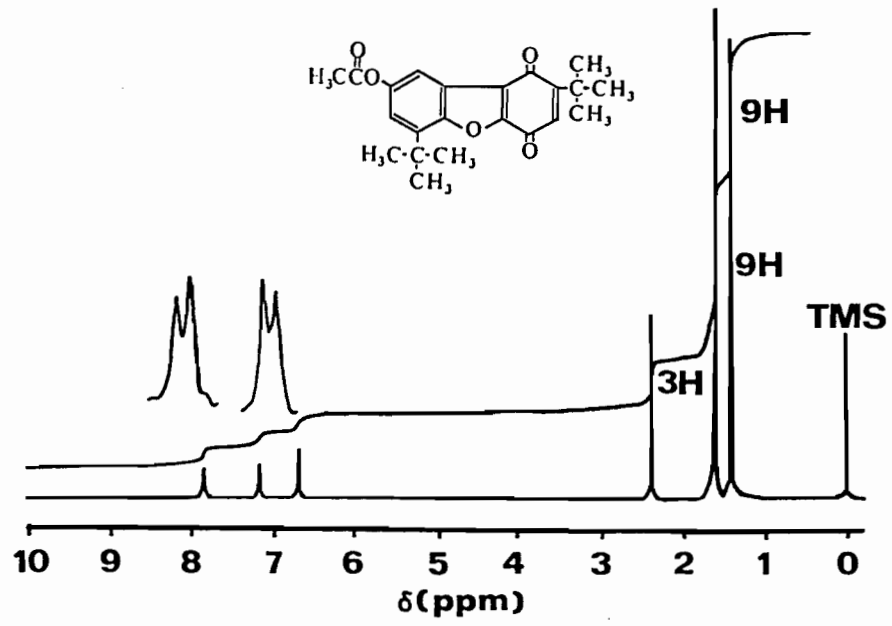

Fig. III-6. The ^{1}H-NMR spectrum of acetylated BHDQ in $CDCl_3$.

(Fig. III-3) was replaced methyl-protons of the acetyl group at δ2.36 (singlet, 3H). Then, the signals due to the acetylated compound were assigned to protons on C-12 (1.38 ppm), C-13 (1.52 ppm), C-15 (2.36 ppm), C-3 (6.67 ppm), C-7 (7.15 ppm) and C-9 (7.85 ppm). The ^{13}C-NMR data in $CDCl_3$ (Fig. III-7) was assigned to carbons of C-15, C-12 and 13, C-10, C-11, C-9, C-7, C-2, C-6, C-3, C-8, C-9a, C-1a, C-6a, C-4a, C-14, C-4 and C-1 appearing from the high-magnetic field. The analytical data of the acetylated compound indicated that BHDQ had one hydroxy group. Thus, the photolytic product from BBDQ was identified to be BHDQ.

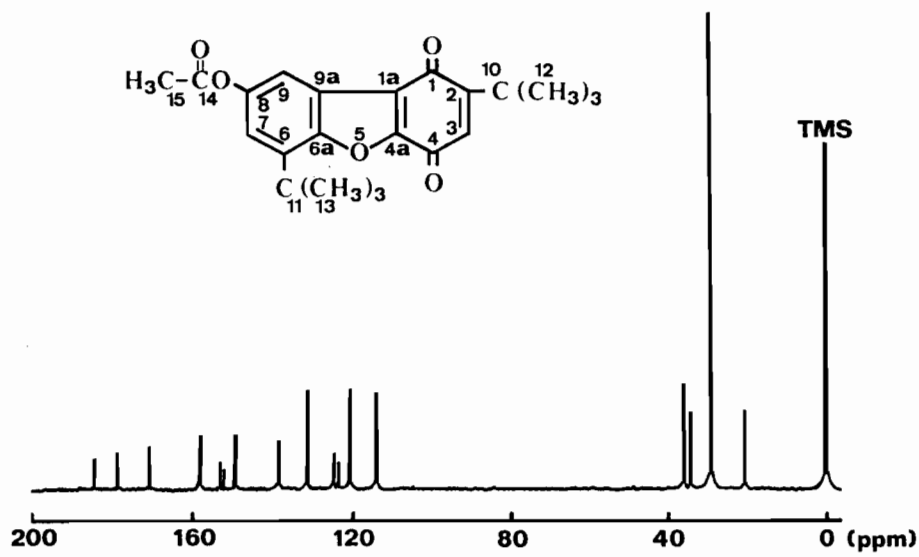

Fig. III-7. The ^{13}C-NMR spectrum of acetylated BHDQ in $CDCl_3$.

Free radicals formed during the UV irradiation

The ESR spectra of the BBDQ solution being UV-irradiated under both the aerobic and anaerobic conditions were measured. Under aerobic condition (Fig. III-8), only one signal of free radical was detected, while under anaerobic condition (Fig. III-9), at least two species of radicals were detected. It was observed that under anaerobic condition the other signal overlapped on the signal formed under aerobic condition (shown with arrow). With the elapse of irradiation time, the arrowed signal virtually remained unchanged in intensity. On the contrary, the intensity of the other signal increased with the elapse of irradiation time. Thus, one radical was formed in

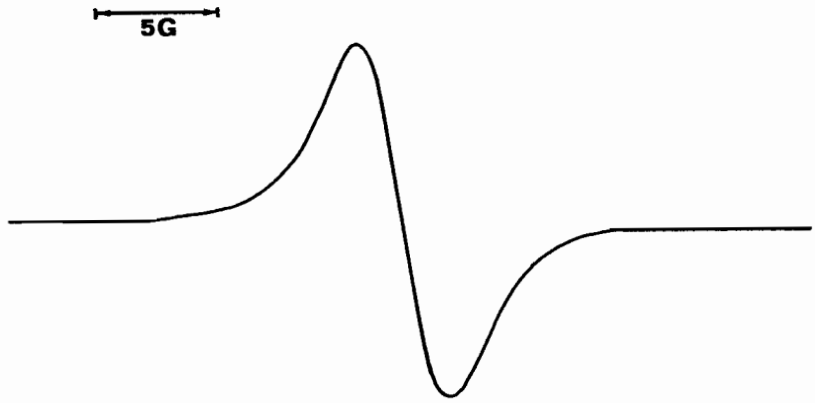

Fig. III-8. ESR spectrum of BBDQ while UV-irradiating under aerobic condition. Spectrometer setting: 20 mW microwave power, 100 KHz modulation frequency, 2.5 mG modulation amplitude.

both the absence and presence of oxygen, but the other radical was not formed in the presence of oxygen. It is considered that the two radicals arose during the photolysis of BBDQ, and that one radical was responsible to oxygen and the other was not.

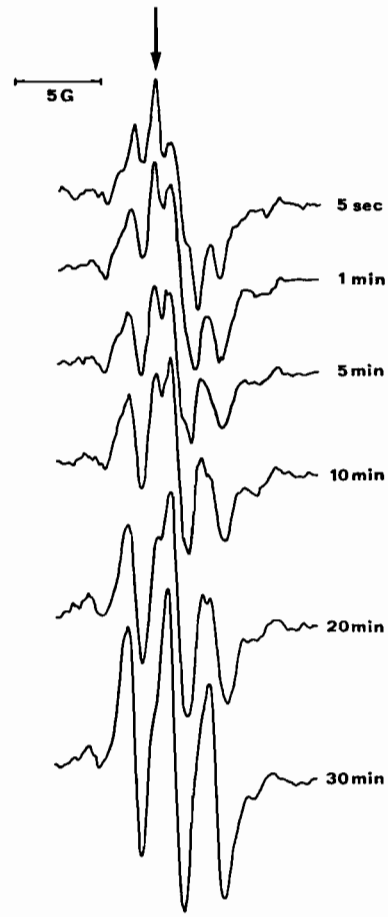

Fig. III-9. ESR spectrum of BBDQ while UV-irradiating under anaerobic condition at each indicated time. Spectrometer setting: 20 mW microwave power, 100 KHz modulation frequency, 2.5 mG modulation amplitude.

Inhibition effects of BHDQ on the mutagenicities of Trp-P-1 and Trp-P-2

Table III-1 shows the effect of BHDQ on the mutagenicity of Trp-P-1. The number of His^+ revertant colonies caused by Trp-P-1 was apparently reduced by the addition of BHDQ. BHDQ behaved as a desmutagen against Trp-P-1, depending on its dose levels ranging from 0.25 to 2.5 μg/plate.

BHDQ showed an inhibition effect on the mutage-

nicity of Trp-P-2, too. The effect of BHDQ against Trp-P-2 was exhibited in the dose-response manner as well, ranging from 0.1 to 1.0 μg/plate of dose level. BHDQ acted as a stronger desmutagen against Trp-P-2 than against Trp-P-1.

Table III-1

Effect of BHDQ on the Mutagenicities of Trp-P-1 and Trp-P-2

The mean number of spontaneous revertants showed 35 in each dose level of BHDQ ranging from 0 to 5.00 μg/plate. The number thus obtained are shown in the Table, along with desmutagenicity % in parentheses.

Amount of BHDQ (μg)	Number of His^+ colonies per plate (% desmutagenicity)	
	Trp-P-1 (1μg/ml)	Trp-P-2 (0.5μg/ml)
0	583	1044
0.1	-	1043 (0)
0.25	412(29)	730 (30)
0.50	381(35)	415 (60)
1.00	321(45)	265 (75)
2.50	237(59)	246 (76)
5.00	303(48)	-

III-4 DISCUSSION

In Chapter II, I isolated the yellow mutagen, BBDQ, from the acidic reaction products of BHA with sodium

nitrite. In this chapter, I found that the yellow colored BBDQ was easily converted into a non-mutagenic and red colored product when allowed to stand under sunlight.

I proved that the photolytic product was BHDQ by the instrumental analyses. BHDQ had the same molecular weight of 326 as that of BBDQ, but the chemical structure of BHDQ was asymmetrical (Fig. III-3). In the ^{1}H-NMR data of BHDQ, the signals of protons due to two *tert*-butyl groups were indicated at δ1.38 (singlet, 9H) and 1.50 (singlet, 9H), whereas those of BBDQ were at δ1.32 indicating its symmetrical structure (Chapter II). During the photolytic process of BBDQ to BHDQ, two distinct free radicals were produced. One radical was responsible to oxygen and the other was not (Fig. III-9). The former radical may be an oxygen anion radical of semiquinone, because the semiquinone radical is known to be unstable to oxygen. The other one appeared in a relatively constant quantity regardless of oxygen (Figs. III-8 and 9), and hereby, this radical was probably a carbonium ion radical in quinone skeleton but not semiquinone radical.

The radical reaction mechanism of BHDQ formation from BBDQ during irradiation could be proposed as shown

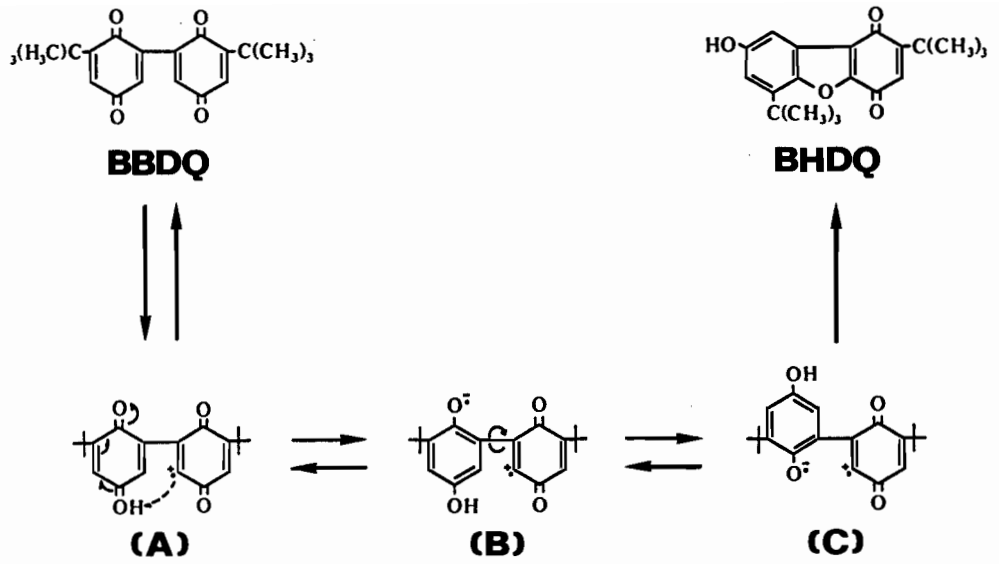

Fig. III-10. Proposed pathway for the electron transformation to BHDQ from BBDQ during the irradiation with sunlight.

in Fig. III-10. I explain the mechanism as follows: the carbonyl oxygen atom in one quinone moiety of BBDQ is activated by irradiation energy and attracts an electron of the quinone skeleton. The inductive effect of carbonyl group pulls out one proton from another quinone moiety and forms the semiquinone (A). Then, both radicals of semiquinone and carbonium ion are intermediately produced (B). One of the aromatic skeletons is rotated and subsequently the two radicals come close to each other (C). In the intramolecular termination reaction of the radicals, the semiquinone radical combines with the carbonium radical to produce

BHDQ. I consider that the termination reaction is irreversible, because of stability of BHDQ.

The conversion of BBDQ to BHDQ *in vitro* was facilitated with ray energy, and assumed to be also occurred during the experiment of the Ames test (Table III-1). BBDQ acted as the mutagen even though in the absence of S-9 mix, but its photolytic product, BHDQ, had no mutagenicity even though in the presence of S-9 mix. It is well known that several quinones as naturally-occurring pigments in a variety of plants and fungi are carcinogens and that some of them are clinically important antitumor drugs [7]. The opposed functions *in vivo* of these quinones may be also explained by the analogy of the conversion mechanism of BBDQ to BHDQ as shown in Fig. III-10. They can undergo either one- or two-electron reduction *in vivo*. One-electron reduction of quinones is catalyzed by microsomal NADPH-cytochrome P-450 reductase or mitochondrial NADH-ubiquinone oxidoreductase [8,9]. The mutagenicity of quinones may be exhibited *via* the formation of semiquinone radicals, which is resemble (B) of Fig. III-10, and is formed through the microsomal redox system. The semiquinone radicals autoxidize to form active oxygen species such as the superoxide

anion radical, which is well known to be carcinogen [10,11]. Two-electron reduction of quinones such as BHDQ, contrary, induces an antitumor function, and is catalyzed by NAD(P)H-quinone reductase, known as DT-diaphorase present in the S-9 fraction [12]. Two-electron reduction forms quinol compounds but not the semiquinone radical [13,14]. BHDQ does not return to the semiquinone radical because the photolytic reaction is irreversible. Therefore, BHDQ can be converted into its quinol by DT-diaphorase *in vivo*, but can not be converted into a mutagenic semiquinone radical either spontaneously or enzymatically.

Thus, BHDQ may be a precursor of an anticarcinogenic quinol. BHDQ apparently showed the inhibition effects on Trp-P-1 and Trp-P-2 in the presence of S-9 mix by the Ames test (Table III-1). The inhibition effects of BHDQ will be described in detail in the next chapter.

REFERENCES

[1] T. Sugimura, T. Kawachi, M. Nagao, T. Yahagi, Y. Seino, T. Okamoto, K. Shudo, K. Tsuji, K. Wakabayashi, Y. Iitaka and A. Itai, *Proc. Jap.*

Acad., **53**, 58 (1977).

[2] S. Takayama, T. Hirakawa and T. Sugimura, *Proc. Jpn. Acad.*, **54**, 418 (1978).

[3] T. Sugimura, *Mutation Res.*, **150**, 33 (1985).

[4] M. Tsuda, Y. Takahashi, M. Nagao, T. Hirayama and T. Sugimura, *Mutation Res.*, **78**, 331 (1980).

[5] T. Yamaguchi, *Agric. Biol. Chem.*, **46**, 2327 (1982).

[6] B.N. Ames, J. McCann and E. Yamasaki, *Mutation Res.*, **31**, 347 (1975).

[7] J. S. Driscoll, G. F. Hazard, H. B. Wood and A. Golden, *Cancer Chemother. Rep. (Part 2)*, **4**, 1 (1974).

[8] T. Iyanagi and I. Yamazaki, *Biochim. Biophys. Acta*, **216**, 282 (1980).

[9] G. Powis and R.L. Appel, *Biochem. Pharmacol.*, **29**, 2567 (1980).

[10] L. Rossi, G.A. Moore, S. Orrenius and P.J. O'Brien, *Arch. Biochem. Biophys.*, **251**, 25 (1986).

[11] K.B. Patel and R.L. Willson, *J. Chem. Soc. Faraday Tran.*, **69**, 814 (1973).

[12] C. Lind, P. Hochstein and L. Ernster, *Arch. Biochem. Biophys.*, **216**, 178 (1982).

[13] S.K. Carter, *J. Natl. Cancer Inst.*, **55**, 1265 (1975).

[14] H. Thor, M.T. Smith, P. Hartzell, G. Bellomo, S.A. Jewell and S. Orrenius, *J. Biol. Chem.*, **257**, 12419 (1982).

IV DESMUTAGENICITY OF 2,6-di-*tert*-BUTYL-8-HYDROXY-DIBENZOFURAN-1,4-QUINONE TOWARD THE MUTAGENICITY OF Trp-P-2

IV-1 INTRODUCTION

As described in Chapter III, 2,6-di-*tert*-butyl-8-hydroxy-dibenzofuran-1,4-quinone (BHDQ) was assumed to be a precursor of desmutagenic quinol compound, and this compound showed the inhibition effects on the mutagenicities of tryptophan pyrolysates, Trp-P-2 and Trp-P-1. It is very important to know the inhibition mechanism of BHDQ to the chemical mutagens.

It is well known that various mutagenic and carcinogenic compounds are formed by the pyrolysis of such amino acids as L-tryptophan [1], L-glutamic acid [2] and L-lysine [3], and by roasting protein-containing foods such as fish and meat. Especially, the pyrolytic products of L-tryptophan, 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-b]indole (Trp-P-1) and 3-amino-1-methyl-5*H*-pyrido[4,3-b]indole (Trp-P-2), are strong mutagens and their mutagenic activities are reportedly higher than that of benzo[*a*]pyrene [4,5]. Trp-P-1 and Trp-P-2 have also been proved to be carcinogens [6,7].

The carcinogenicity and the mutagenicity of the Trp-P's have been ascribed to the modification of DNA with the metabolites which are generated in the activation process by hepatic microsomal enzymes [8]. The metabolic activation mechanism of Trp-P-2 is also made clear with *in vitro* experiments. Trp-P-2 is converted into 2-hydroxyamino derivative (N-OH-Trp-P-2) *in vitro* [9,10] by cytochrome P-448, which was an inducible enzyme in the liver. N-OH-Trp-P-2 reacts with DNA only after its *O*-acetylation, and produces an adduct with guanine, 3-(C^8-guanyl)amino-1-methyl-5*H*-pyrido[4,3-b]indole [9].

Cytochrome P-448 can be induced when rat was treated with polychlorinated biphenyl or 3-methylcholanthrene [11-13], and obtained in the microsomal S-9 fraction. In this chapter, Trp-P's and other chemical mutagens were activated by the S-9 mix, and then the suppressive effect of BHDQ on their mutagenic activities was examined.

IV-2 MATERIALS AND METHODS

Chemicals. BHDQ was prepared from BBDQ as described in Chapter III. Trp-P-2 and Trp-P-1 (acetic acid salts) were purchased from Wako Pure Chemical

Industries Ltd., Osaka, Japan. Benzo[*a*]pyrene (B[*a*]P), 1-nitropyrene (1-NP), acetylaminofluorene (AAF) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), were purchased from Aldrich Chemical Company, Inc., Wisc., U.S.A.

Bacterial strain. *Salmonella typhimurium* TA 98 and TA 100 were used in this experiment.

Preparation of S-9 mix. The S-9 fraction was prepared as shown in Chapter II.

Preparation of a mixture containing activated metabolites of Trp-P's. Around 0.2 nmol of Trp-P-2 or Trp-P-1 was dissolved in 0.1 ml water, mixed with 0.5 ml of the S-9 mix (contained 2.4 µg protein), and incubated at 37°C for 20 min while shaking. The reaction was stopped by the addition of 0.6 ml cold acetone and the mixture was allowed to stand on ice for 15 min. After centrifugation of the mixture at 3,500 rpm for 10 min, the supernatant was evaporated *in vacuo*. The resultant residue consisted the activated metabolites of Trp-P's (N-OH-Trp-P-2 or N-OH-Trp-P-1) and their intact forms, as detected on a high perfor-

mance liquid chromatogram (HPLC) [9]. The activated metabolites are very unstable and its half-life is ca. 30 min [14]. Here, the residue was dissolved again in water and immediately subjected to the following experiments.

Desmutagenic assay of BHDQ. The desmutagenicity of BHDQ was determined by counting the decreasing numbers of revertants of *Salmonella typhimurium* TA 98 caused by the mutagens, Trp-P's and their activated metabolites. The reversion (from His^- to His^+) of *Salmonella typhimurium* TA 98 was detected by the Ames test, as described in detail in Chapter II and III. Trp-P's (0.2 nmol) in 0.1 ml water was mixed in the following order with various concentrations of BHDQ in 0.1 ml dimethyl sulfoxide, 0.5 ml S-9 mix, and 0.1 ml TA 98 medium-suspension. When the activated metabolites were used as mutagen, the same volume of 0.1 M phosphate buffer at pH 7.0 was added in replace of the S-9 mix. After incubation at 37°C for 20 min, 2 ml of molten top agar was added to the mixture and then poured onto an agar medium of minimal glucose. These plates were cultured for 2 days at 37°C in dark and the number of colonies developed on a surface of lawn of

non-mutated bacteria was counted as the revertants under a microscope. The desmutagenicity (%) of BHDQ was calculated using the same equation as in Chapter III.

Assay of bio-antimutagenicity. TA 98 bacteria were grown at 37°C overnight in a liquid broth medium, washed with 1/15 M phosphate buffer 3 times, and then resuspended in the same buffer. In order to induce the mutation on the bacteria, 5 ml of bacterial suspension was mixed with 5 ml solution containing the activated metabolite of Trp-P-2 and incubated at 37°C for 30 min. The mixture was centrifuged at 3,500 rpm for 10 min and the bacterial cells were washed with the buffer 3 times to remove the remaining mutagen. The bacterial cells of mutagen free were suspended in 5 ml of the phosphate buffer. An aliquot, 0.1 ml, of the bacterial suspension was mixed with various concentrations of 0.1 ml BHDQ solution. The mixture was placed on 3 ml of molten top agar while shaking and poured onto a semi-enriched agar medium (MBB agar). The above bacterial suspension of mutagen free was also subjected to the determination of survival colony number. The suspension was diluted to 10^6 and 0.1 ml

aliquot was poured onto the MBB plate together with 3 ml of molten top agar. After incubation at 37°C for 4 days, the numbers of both colonies of revertant (His^+) and survival (His^-) were counted. The bio-antimutagenicity of BHDQ was determined by calculating the IMF value as described in detail in Chapter II.

HPLC analysis of Trp-P-2 and its activated metabolite. Trp-P-2 (194 nmol) was treated with an S-9 system which contained various amounts of S-9 fraction and 1.5 mg NADPH in 1 ml of the final mixture under the condition described above. Trp-P-2 and its activated metabolite were extracted from the incubation mixture with an equivalent volume of cold acetonitrile, centrifuged, and the extract was subjected to the HPLC analysis with a Hitachi 655 Liquid Chromatograph. The conditions were as follows: column, ZORBAX ODS (4.6 mm x 15 cm); eluting solvent system, 30% acetonitrile in 20 mM potassium dihydrogenphosphate; flow rate, 0.7 ml/min; detection, at 254 nm, λ_{max} of N-OH-Trp-P-2. The amount of Trp-P-2 was calculated from the chromatogram using a calibration curve. The calibration curve for the determination of N-OH-Trp-P-2 was tentatively prepared using the ratio of the molecular extinction

coefficients at 254 nm, Trp-P-2 (4.3×10^7) *versus* N-OH-Trp-P-2 (4.7×10^7) [9].

IV-3 RESULTS

Desmutagenicities against Trp-P's and their activated metabolites

BHDQ markedly reduced the mutations caused by the activated metabolites of Trp-P's (Fig. IV-1). The number of His^+ revertant colony caused by Trp-P-2 (0.2 nmol) was proportionally decreased by the addition of less than 3 nmol of BHDQ (Fig. IV-1A). Trp-P-2 was

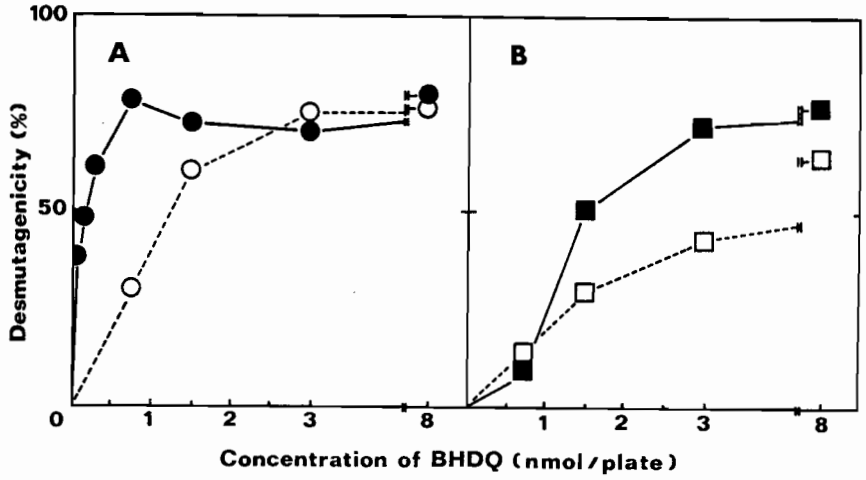

Fig. IV-1. The Desmutagenicity of BHDQ. (A); against the mutagenicities of Trp-P-2 (○) and against the mixture containing its activated metabolite (●), and (B); against those of Trp-P-1 (□) and against the mixture containing its activated metabolite (■).

partly converted into its activated metabolite by incubation with S-9 mix, and then the effect of BHDQ on the mutagenicity caused by the activated metabolite was measured. BHDQ suppressed the mutagenicity of the activated metabolite of Trp-P-2 depending proportionally on the concentration of BHDQ until 0.8 nmol. The decreases in the mutagenicities of Trp-P-2 and its activated form were 30% and 80%, respectively, when 0.8 nmol of BHDQ was added. Thus, the desmutagenicity of BHDQ against the activated metabolite was greater than that against Trp-P-2 itself. BHDQ also exhibited the concentration-dependent desmutagenicity against Trp-P-1 and its activated metabolite, and the extent of the desmutagenicity against the activated metabolite was greater than that against the intact form (Fig. IV-1B). On the other hand, when the desmutagenicities of BHDQ toward Trp-P-2 were compared with that toward Trp-P-1, the magnitudes at 1.5 nmol BHDQ against Trp-P-2 and Trp-P-1 were 60% and 30%, respectively. Thus, the effect of BHDQ appeared more conspicuously on Trp-P-2.

Bio-antimutagenicity of BHDQ

There is an insoluble problem, whether such suppressive effect of BHDQ against the mutagenicity of

Trp-P-2 is due to its bio-antimutagenicity or to its desmutagenicity. Then, BHDQ was added to the bacterial suspension containing the induced mutant (His^+ revertant) and the bio-antimutagenicity of BHDQ was measured according to the IMF method (Table IV-1). The number of the survival colonies (His^-) remained

Table

Bio-antimutagenicity

Added amount of BHDQ (nmol/plate)	Mean number of survival (His^-) colonies (x 10^6/plate)
0	140
1.5	123
3.0	141
7.5	153
15.0	145
30.7	138

* The treated bacterial suspension (0.1
induction. From the mean number of
the mean number of spontaneous mutant
** The induced mutation frequency was
column by that of the second column

constant and the number of the induced revertant colonies slightly decreased with increase in the amount of BHDQ. In the present case, the value of IMF is a ratio of the number of His^+ colony *versus* the number of His^- colony, and the decrease in the value signifies the recovery of His^+ revertant to His^-. The IMF value

IV-1

of BHDQ on mutated bacteria

Mean number of induced (His^+) colonies (Mean number/plate)*	Frequency of induced His^+ revertants/10^8 survivors**
7384	5300
6542	5300
6753	4800
6853	4500
6706	4600
5599	4000

ml) was plated for the observation of mutation
induced (His^+) colonies appearing per plate,
colonies per plate was subtracted.
calculated by dividing the number of the third
and multiplying the value by 100.

in the plate without BHDQ was 5,300 x 10^{-8}, which is an original induced frequency. The addition of 1.5 nmol BHDQ retained the frequency unchanged and the addition of 30.7 nmol BHDQ reduced it to 4,000 x 10^{-8}. Thus, the high concentration of BHDQ exhibited 25% of the bio-antimutagenicity, but BHDQ at such low levels as those used in Fig. IV-1A did not. It was considered that the suppressive effect of BHDQ on the mutation caused by Trp-P's was ascribed to its desmutagenicity.

The effect of BHDQ on the activated metabolite of Trp-P-2

Trp-P-2 is metabolically converted into N-OH-Trp-P-2 by the S-9 system. Here, the effect of BHDQ on the production of N-OH-Trp-P-2 from Trp-P-2 by the action of S-9 fraction was measured *in vitro* (Table IV-2). The remaining Trp-P-2 and the produced N-OH-Trp-P-2 in the reaction mixture were quantitatively quantified by HPLC. A typical HPLC pattern is shown in Fig. IV-2. With the increasing amount of BHDQ, the remaining amount of Trp-P-2 was increased and the production of N-OH-Trp-P-2 was decreased together with its mutagenicity (Table IV-2). Thus, BHDQ depressed the production of N-OH-Trp-P-2 by S-9 fraction.

Table IV-2

The effect of BHDQ on the production of N-OH-Trp-P-2 and its mutagenicity

Added amount of BHDQ (nmol)	Amount of Trp-P-2 (nmol)*	Amount of N-OH-Trp-P-2 (nmol)*	Mutagenicity (revertant number/plate)**
0 and without S-9	194	ND	23
0	62.3	41.4	2212
1	70.7	37.9	1631
6	118.2	28.8	1314
30	145.2	12.6	795

* The mixture of Trp-P-2, the indicated amount of BHDQ, and S-9 fraction was incubated, and then the remaining amount of Trp-P-2 and the produced amount of N-OH-Trp-P-2 were determined by the HPLC as described in the Text.

** The reaction mixture was diluted 1000-fold with 1/15 M phosphate buffer and was subjected to the Ames test.

The question arose whether or not BHDQ directly attacked and decomposed N-OH-Trp-P-2. Then, Trp-P-2 and N-OH-Trp-P-2 were extracted from the incubation mixture containing Trp-P-2 and S-9 fraction. BHDQ was added to the extracts and then incubated without S-9 fraction (Table IV-3). The amount of N-OH-Trp-P-2 was decreased with increasing amount BHDQ, while the amount of Trp-p-2 was concomitantly increased. When 60 nmol of BHDQ was added to the mixture, N-OH-Trp-P-2 was

disappeared. The sum of the amounts of N-OH-Trp-P-2 and Trp-P-2 was kept almost constant in each mixture. This result indicates that N-OH-Trp-P-2 was non-enzymatically converted into Trp-P-2 by BHDQ.

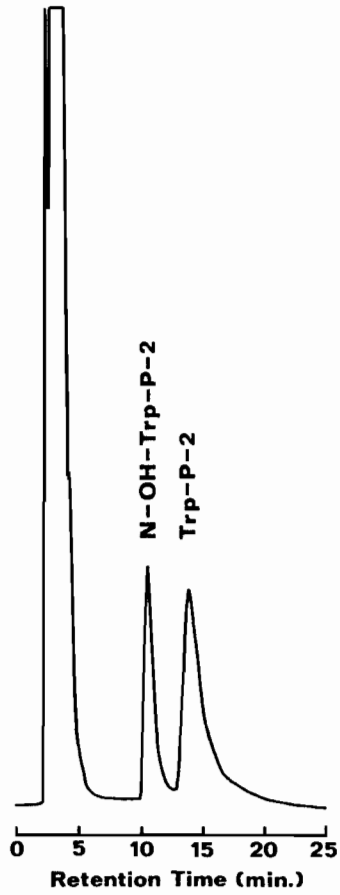

Fig. IV-2. HPLC of the products from Trp-P-2 incubated with S-9 system.

Desmutagenicities toward other mutagens

The desmutagenicity of BHDQ against B[*a*]P, 1-NP, AAF and MNNG was also determined by the Ames test using *Salmonella typhimurium* TA 98 and TA 100. In the absence of BHDQ, 100 nmol of B[*a*]P and 2 nmol of 1-NP produced 148 and 822 revertant colonies, respectively (Table IV-4). The addition of 30 nmol BHDQ reduced the numbers to 60 and 439, respectively. In short, its desmutagenicities were 60% and 42%, respectively. Thus, BHDQ exhibited the suppressive effects against the mutagenicities of B[*a*]P and 1-NP. However, the effects of BHDQ against

Table IV-3

The production of Trp-P-2 from N-OH-Trp-P-2 by BHDQ

Added amount of BHDQ	Amounts* of Trp-P-2	N-OH-Trp-P-2	Sum
	(nmol)		
0	94.6	24.5	119
1	98.2	24.4	123
3	103.7	13.8	118
6	105.5	8.6	114
12	107.3	6.5	114
30	110.5	2.3	113
60	117.0	ND	117

* BHDQ was incubated with the mixture of Trp-P-2 and N-OH-Trp-P-2 in the 50% acetonitrile solution at 37°C for 20 min, and then the amounts of Trp-P-2 and N-OH-Trp-P-2 in the incubated mixture were estimated by HPLC.

Table IV-4

Desmutagenicity of BHDQ against other chemical mutagens

	Tester strain			
	TA 98		TA 100	
Added amount of BHDQ (nmol)	100 nmol of B[*a*]P with S-9	2 nmol of 1-NP without	99 nmol of AAF with S-9	7 nmol of MNNG without
0	148	822	139	463
5	139	764	169	597
15	93	568	141	454
30	60	439	205	582

Abbreviations: B[*a*]P; benzo[*a*]pyrene, 1-NP; 1-nitropyrene, AAF; acetylaminofluorene, MNNG; N-methyl-N'-nitro-N-nitrosoguanidine.

mutagenicities of AAF and MNNG were not recognized at all.

IV-4 DISCUSSION

The present study demonstrated that the suppressive effect of BHDQ against the mutagenicity of Trp-P-2 was due to its desmutagenicity.

The bio-antimutagenic activity of BHDQ was negligible as compared to its desmutagenicity (Table IV-1). BHDQ markedly reduced the production of N-OH-Trp-P-2 from Trp-P-2 in the S-9 system (Table IV-2). The suppressive effect of BHDQ on the mutation was greater when the mutation was induced by the activated metabolites of Trp-P's than when induced during the metabolism of Trp-P's in the S-9 mix (Fig. IV-1). Therefore, BHDQ directly attacked the N-OH-forms of Trp-P's, but not the metabolic system of S-9 fraction. When N-OH-Trp-P-2 was incubated with BHDQ, N-OH-Trp-P-2 was returned to the original Trp-P-2 (Table IV-3). The sum of the amounts of N-OH-Trp-P-2 and Trp-P-2 was kept constant in the system without S-9 fraction (Table IV-3), but not in the system with S-9 fraction (Table IV-2). The addition of the large amount of BHDQ (30

nmol) to the mixture of Trp-P-2 and S-9 system retained Trp-P-2 almost unchanged, and the sum of the amounts of N-OH-Trp-P-2 and Trp-P-2 (158nmol) was not so differed from the initial amount of Trp-P-2 (194 nmol). On the contrary, no addition of BHDQ decreased the sum to around a half of the initial amount (Table IV-2). This seemed to indicate that the reaction of Trp-P-2 to N-OH-Trp-P-2 by S-9 mix involved at least one intermediate. Therefore, BHDQ may also return the intermediate to the original Trp-P-2. It was concluded that the desmutagenicity of BHDQ was ascribed to its non-enzymic effect which returned the activated metabolites (N-OH-Trp-P-2 or its intermediate) of Trp-P-2 to the inactive form.

BHDQ also exhibited the suppressive effects against the mutagenicities of B[*a*]P and 1-NP (Table IV-4). The magnitudes of desmutagenicities were about 60% and 50%, respectively. The activated forms of B[*a*]P and 1-NP are their monoxidized derivatives like N-OH-Trp-P's. B[*a*]P is metabolized to active B[*a*]P-4,5-epoxide or B[*a*]P-7,8-diol-9,10-epoxide by the hepatic P-450 enzymes [15]. 1-NP is active as a mutagen without being metabolized by P-450 enzymes, because nitro compound is easily converted into the

hydroxy form (N-OH-NP) by a bacterial reductase of TA 98 [16]. BHDQ had no desmutagenicities against AAF and MNNG, which are the mutagens of base-pair change-type and detectable with TA 100 strain. Thus, BHDQ showed the desmutagenicities only toward the mutagens whose activated forms were the hydroxy types and their mutagenicities were expressed as frame-shift mutations, which were able to detect with TA 98 but not with TA 100.

Recently, Armstrong and Wattenberg [17] reported that the dimer of BHA was formed in the rat liver microsome when BHA was orally administered. There is also a report that BQ was produced as one of the microsomal metabolites of BHA in cultured hepatocyte [18]. Therefore, the dimer of BQ, BBDQ, may also be formed *in vivo*. BHDQ is photolytically produced from BBDQ (quinone dimer) *via* a form of its semiquinone radical *in vitro*. It is generally believed that quinones are reduced *in vivo* through an one electron-reduction to their semiquinone radicals by the microsomal NADPH-cytochrome P-450 reductase or the mitochondrial NADH-ubiquinone oxidoreductase [19,20]. Since these enzymes do not have strict specificities for quinones [21], it may be a possible hypothesis that

BBDQ is also converted into BHDQ *in vivo*. Therefore, it seems likely that some of the mutagenic and desmutagenic derivatives of BHA are also formed in the hepatic microsome of animals.

REFERENCES

[1] T. Sugimura, T. Kawachi, M. Nagao, T. Yahagi, Y. Seino, T. Okamoto, K. Shudo, T. Kosuge, K. Tsuji, K. Wakabayashi, Y. Iitaka and A. Itai, *Proc. Jpn. Acad.*, **53**, 58 (1977).

[2] T. Yamamoto, K. Tsuji, T. Kosuge, T. Okamoto, K. Shudo, K. Takeda, K. Yamaguchi, Y. Seino, T. Yahagi, M. Nagao and T. Sugimura, *Proc. Jpn. Acad.*, **54**, 248 (1978).

[3] K. Wakabayashi, K. Tsuji, K. Kosuge, T. Takeda, K. Yamaguchi, Y. Seino, T. Yahagi, M. Nagao and T. Sugimura, *Proc. Jpn. Acad.*, **54**, 569 (1978).

[4] M. Nagao, M. Honda, Y. Seino, T. Yahagi and T. Sugimura, *Cancer Lett.*, **2**, 335 (1977).

[5] S. Mizusaki, T. Takashima and K. Tomaru, *Mutation Res.*, **48.**, 29 (1977).

[6] S. Takayama, T. Hirakawa and T. Sugimura, *Proc. Jpn. Acad.*, **54**, 418 (1978).

[7] T. Sugimura, *Mutation Res.*, **150**, 33 (1985).

[8] Y. Hashimoto, K. Takeda, K. Shudo, T. Okamoto, T. Sugimura and T. Kosuge, *Chem.-Biol. Interactions*, **23**, 137 (1978).

[9] Y. Hashimoto, K. Shudo and T. Okamoto, *Biochem. Biophys. Res. Commun.*, **96**, 335 (1980).

[10] Y. Yamazoe, K. Ishii, T. Kamataki, R. Kato and T. Sugimura, *Chem.-Biol. Interactions*, **30**, 125 (1980).

[11] K. Ishii, M. Ando, T. Kamataki, R. Kato and M. Nagao, *Cancer Lett.*, **9**, 271 (1980).

[12] J. Watanabe, K. Kawajiri, H. Yonekawa, M. Nagao and Y. Tagashira, *Biochem. Biophys. Res. Commun.*, **104**, 193 (1982).

[13] T. Niwa, Y. Yamazoe and R. Kato, *Mutation Res.*, **95**, 159 (1982).

[14] K. Saito, Y. Yamazoe, T. Kamataki and R. Sato, *Carcinogenesis*, **4**, 1551 (1983).

[15] M.K. Buening, P.G. Wislocki, W. Levin, H. Yagi, D.R. Thakker, H. Akagi, M. Koreeda, D.M. Jenina and A.H. Conney, *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 5358 (1978).

[16] P.C. Howard, R.H. Heflich, F.E. Evans and F.A. Beland, *Cancer Res.*, **43**, 2052 (1983).

[17] K.E. Armstrong and L.W. Wattenberg, *Cancer Res.*, **45**, 1507 (1985).

[18] S.W. Cummings, G.A. Ansari, F.P. Guengrich, L.S. Crouch and R.A. Prough, *Cancer Res.*, **45**, 5617 (1985).

[19] T. Iyanagi and I. Yamazaki, *Biochim. Biophys. Acta.*, **216**, 282 (1980).

[20] G. Powis and R.L. Appel, *Biochem. Pharmacol.*, **29**, 2567 (1980).

[21] P.L. Chesis, D.E. Levin, M.T. Smith, L. Ernster and B.N. Ames, *Proc. Natl. Acad. Sci. U.S.A.*, **81**, 1696 (1984).

V DESMUTAGENIC EFFECTS OF SULFHYDRYL COMPOUNDS ON 2-*tert*-BUTYL-*p*-QUINONE

V-1 INTRODUCTION

Recently, it has been found and become a topic that some foods have desmutagenic activities. Some foods contained such anti- or desmutagens as peroxidase [1], long-chain fatty acids [2], hemin [3], and retinoids [4]. Dietary fibers from vegetables reduced the mutagenic activities of Trp-P-1 and Trp-P-2 by adsorbing these mutagens [5]. In addition, some food components (ascorbic acid, cysteine and phenols) have been reported to react with nitrite, preventing the formation of nitroso amines *in vitro* as well as *in vivo* [6,7]. Kada *et al*. described that juices from vegetables such as cabbage, spinach, celery and sprouts suppressed the mutagenicity of pyrolysates of tryptophan, Trp-P-1 and Trp-P-2 [8]. However, they did not yet refer to the desmutagenic compounds present in these juices. Then, it is very interesting to examine whether the food components can reduce the mutagenicity of BQ, which is derived from a food additive, BHA.

The present chapter deals with the effects of vegetable and fruit juices on the mutagenicity of BQ. These juices suppressed the mutagenicity of BQ and it was revealed that the desmutagenic activities of the juices were ascribed to the sulfhydryl compounds contained in them. Then, in order to make the desmutagenic mechanism of BQ clear, the interaction of BQ with glutathione, one of the biologically important sulfhydryl compounds, was investigated.

V-2 MATERIALS AND METHODS

Chemicals. Glutathione (GSH), pantethine and dithiothreitol (DTT) were purchased from Wako Pure Chemical Industries Ltd., Osaka, Japan. Pantethine solution was reduced to pantetheine (PaSH) with sodium borohydride, and the pH was neutralized to 7 with hydrochloric acid before use. BHQ, 2-*tert*-butylhydroquinone, was purchased from Tokyo Kasei Kogyo Co., Ltd. Pure BQ was prepared by the reaction of BHA with sodium nitrite as described in Chapter II.

Preparation of juices from plant foods. Vegetables and fruits were purchased from markets in the city

of Kobe, Japan. They were washed well with tap water, and homogenized by a Waring blender with an equal volume of distilled water. The homogenates were each centrifuged at 9,000 x g for 30 min at 4°C, and the supernatants were stored at -80°C and used as sample juices.

Desmutagenic assay. Desmutagenicities of juices and chemicals were estimated by measuring the decrease in the histidine reversions of *Salmonella typhimurium* TA 100. The method for assaying the desmutagenic effects of vegetable and fruit juices or chemicals on the mutagenicity of BQ was as follows: BQ (5 μg) dissolved in 100 μl of dimethyl sulfoxide (DMSO) was mixed with 100 μl of each juice or 100 μg of each compound and 500 μl of 0.1 M phosphate buffer (pH 7.4). After incubating for 30 min at 37°C, the mixture was combined with 2 ml of soft agar (45°C) containing 100 μl of a bacterial culture of TA 100 and poured onto histidine-free minimal agar medium. All the plates were allowed to stand at 37°C for two days.

Induced mutation frequency (IMF) test. BQ and authentic sulfhydryl compounds may cause a lethal

mutation, which would lead to an experimental error. To avoid this risk, the desmutagenic activity of authentic sulfhydryl compounds toward BQ was measured by the IMF test as described in Chapter II. The Ames cells were treated with BQ in the presence and absence (control) of various amounts of sulfhydryl compounds for 30 min and then washed with phosphate buffer. The BQ-treated cell was poured onto the minimal medium of soft agar containing 1 mM histidine, incubated for 2 days, and then the number of induced-His^+ colonies appeared on the medium was counted. Another portion of BQ-treated cells was washed, diluted to an optimal concentration, and cultured on the same medium with soft agar containing 5 mM excess of histidine. Then, the number of surviving colonies was counted. The IMF value was estimated by dividing the number of revertant colony by that of the surviving colony. The IMF value due to sulfhydryl compounds was also determined with BQ-untreated cells.

Reaction of BQ with GSH. The reactivity of BQ to GSH was measured by the determination of the remaining amounts of sulfhydryl group by the method of Ellman [9]. Various concentrations of BQ (1.25, 2.5 and 5.0

mM) in 2 ml 30% acetonitrile (CH_3CN) was allowed to stand with 2.5 mM GSH at 37°C. Ten microliter aliquot of reaction mixture was taken to determine the residual amount of sulfhydryl group at 0, 2, 5, 15, 30, 60 and 120 min after incubation.

Identification of the reaction products of BQ with GSH. One of the two reaction products generated in the foregoing reaction mixture was extracted with diethyl ether for subsequent analyses. The extract was applied on high-performance liquid chromatography (HPLC, Hitachi 655 liquid chromatograph) and gas chromatography (GC, Shimadzu GC-6AM gas chromatograph). The conditions for HPLC were as follows: the column used for HPLC was a ZORBAX ODS (4.6 mm x 15 cm); the eluting solvent program was a linear gradient from 30% to 100% CH_3CN over 10 min; the flow rate was 1.0 ml per min; a UV detector was used at 280 nm. For GC, a glass column (2 mm x 1 m) packed with Silicon OV-1 (2%) was used; the carrier gas was nitrogen at a flow rate of 40 ml per min; temperatures of the column and injector were 120°C and 270°C, respectively. Then, a Hitachi RMU-6MG gas chromatograph-mass spectrometer was used to identify the product.

The other product of the reaction of BQ with GSH was obtained by washing the reaction mixture 3 times with diethyl ether to remove the ether soluble product and lyophilized. The lyophilized material was subjected to ^{13}C-NMR (Varian XL-200) measurement.

Table. V-1

Desmutagenicity of vegetable and fruit juices and of chemicals against the mutagenicity of BQ

Juice		
Vegetable	Fruit	Chemicals
Tomato*	Orange*	Pantetheine*
Sweet pepper*	Lemon*	Glutathione(GSH)*
Burdock	Melon*	Dithiothreitol*
Radish	Kiwi fruit*	2-Mercaptoethanol*
Spinach	Strawberry*	Thiamine
Cabbage	Apple	Sorbic acid
Broccoli	Pineapple	Linoleic acid
Carrot	Banana	α-Tocopherol
Eggplant	Grape	β-Carotene
		Uric acid
		Ascorbic acid

* Remarkable action as a desmutagen against BQ.

V-3 RESULTS

Desmutagenicities of vegetable and fruit juices, and of chemicals against BQ

The desmutagenicities of the vegetable and fruit juices, and of chemicals against BQ were measured by the Ames test (Table V-1). Marked desmutagenicities were given by the juices of sweet pepper, lemon, tomato, orange, strawberry, melon, and kiwi fruit. Of the chemicals, GSH, PaSH, DTT and 2-mercaptoethanol also showed marked activities. This indicates that sulfhydryl group may have contributed to the desmutagenic effect. Then, the contents of sulfhydryl group present in juices were determined (Table V-

Table V-2

Relation of the sulfhydryl group contents in juices of vegetable and fruit to their desmutagenic activities against BQ

	Concentration of sulfhydryl group (μM)
*Sweet pepper	192
*Lemon	171
*Tomato	164
*Orange	122
*Strawberry	74
*Melon	65
*Kiwi fruit	61
Apple	0
Banana	0
Grape	0
Cabbage	52
Carrot	55

* Remarkable action as a desmutagen against BQ.

2). The potent desmutagenicities of the juices proportionally related to the content of sulfhydryl group.

Desmutagenicities of GSH, PaSH and DTT against BQ

GSH, PaSH and DTT indicated potent desmutagenicities toward BQ. Then, the desmutagenic activities of GSH, PaSH and DTT against BQ was determined by the method of IMF (Tables V-3, 4 and 5). One milligram of these compounds gave low IMF values in the absence of BQ, i. e., these compounds *per se* had no mutagenic activity. With increasing amount of GSH, PaSH, and DTT, the number of induced mutant colony (His^+ revertants) was significantly decreased (the fourth column in these Tables), whereas the number of surviving colony remained unchanged (the third column). In the absence of sulfhydryl compounds, the IMF value (from His^- to His^+) was in the level of 10^{-5} with 5 μg of BQ per plate. The IMF value was significantly decreased to the level of 10^{-7} by the addition of 100 ug of GSH, PaSH or DTT. Thus, the sulfhydryl compounds had the strong desmutagenicity against BQ.

Table V-3

Desmutagenic activity of GSH toward BQ

BQ (μg)	Amount of GSH added to each plate (μg)	Mean number of surviving (His^-) colonies /plate after 10^5 dilution	Mean number of induced His^+ colonies /plate	Frequency of induced His^+ revertants /10^8 survivors
0	0	559	85	-
0	1000	597	93	13
5	0	580	1445	2345
5	10	580	360	474
5	100	558	111	47
5	1000	633	98	21

Table V-4

Desmutagenic activity of PaSH toward BQ

BQ (μg)	Amount of PaSH added to each plate (μg)	Mean number of surviving (His^-) colonies /plate after 10^5 dilution	Mean number of induced His^+ colonies /plate	Frequency of induced His^+ revertants /10^8 survivors
0	0	559	85	-
0	1000	483	98	28
5	0	580	1445	2345
5	10	658	108	35
5	100	444	95	23
5	1000	469	92	15

Table V-5

Desmutagenic activity of DTT toward BQ

BQ (μg)	Amount of DTT added to each plate (μg)	Mean number of surviving (His^-) colonies /plate after 10^5 dilution	Mean number of induced His^+ colonies /plate	Frequency of induced His^+ revertants /10^8 survivors
0	0	816	76	-
0	1000	880	82	7
5	0	810	1441	1685
5	10	892	95	21
5	100	867	96	23
5	1000	868	93	20

Reduction of BQ by GSH

GSH is found in all mammalian cells in relatively high concentration, and particularly, the concentration in the hepatocyte is as high as 1-10 mM [10]. GSH may detoxify the toxin such as BQ, and then, the reactivity of GSH toward BQ was examined (Fig. V-1). Several concentrations of BQ was incubated with GSH (2.5 mM), and the amount of the sulfhydryl group was measured at described intervals. The content of the sulfhydryl group rapidly decreased 2 min after the incubation, and then reached a constant level in all the mixtures.

The level of the remaining sulfhydryl group after incubation was reversely proportional to the added level of BQ. When 2.5 mM or 1.25 mM of BQ was reacted with 2.5 mM of GSH, about 1 mM and 2 mM of the sulfhydryl group remained free, respectively. When a 2-fold quantity of BQ was added to GSH, the sulfhydryl group was completely consumed. From these results, it was stoichiometrically estimated that 2 mole of BQ were consumed by 1 mole of GSH.

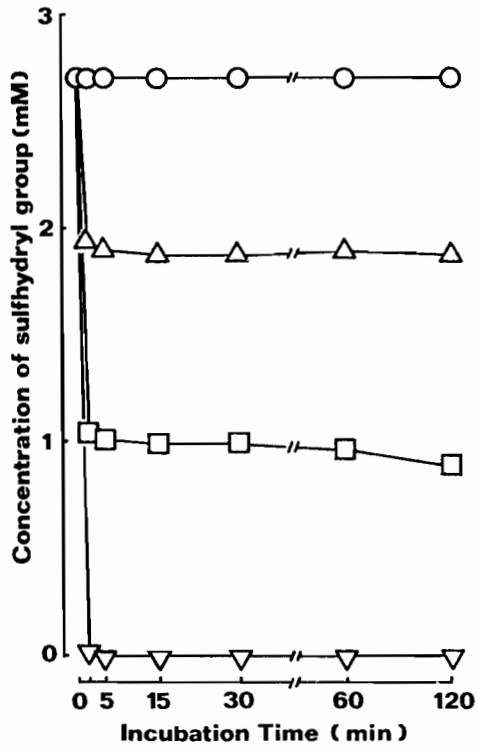

Fig. V-1. Decrease in the amount of the sulfhydryl group of GSH (2.5 mM) during incubation with various concentrations of BQ: free (○), 1.25 mM (△), 2.5 mM (□) and 5 mM (▽).

Identification of the reaction product of BQ with GSH

The reaction mixture of GSH with BQ was directly subjected to HPLC, and two peaks of products were detected (Fig. V-2). When an ether extract of the reaction mixture was subjected to HPLC, only one of them (the shaded line) was seen at a retention time of

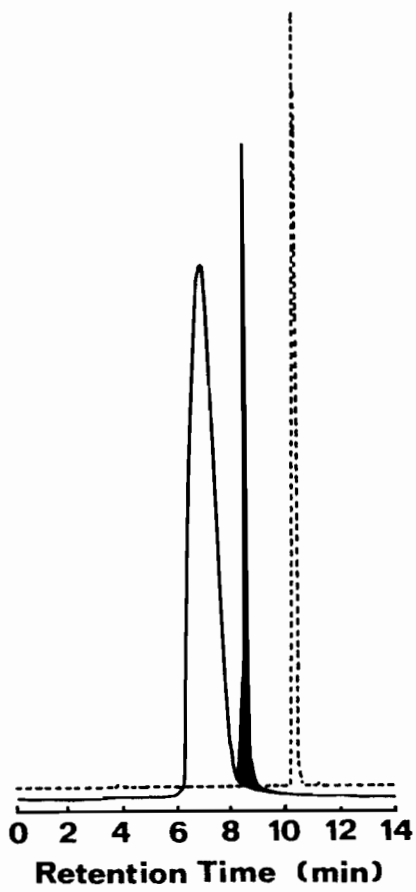

Fig. V-2. HPLC of the reaction mixture of 2.5 mM GSH with 5 mM BQ (———), and that of 5 mM BQ solution (-----).

8.6 min. The product of this peak was collected and subjected to GC and GC-MS. As shown in Fig. V-3, this product gave molecular ion peaks at *m/z* 166 (M^+), and fragment ion peaks at *m/z* 151 (M^+-CH_3), 136 ($M^+-(CH_3)_2$), 123 ($M^+-C_3H_7$) and 109 ($M^+-C_4H_9$). These fragment ions were identical to those of authentic 2-*tert*-butyl-hydroquinone (BHQ). Moreover, the retention times on HPLC and GC were also identical to those of authentic BHQ. In this way, one of the products from the reaction between GSH and BQ was found to be BHQ, whose structure is shown in Fig. V-3.

The other peak (the solid line) eluted at a retention time of 7.1 min (Fig. V-2), were pooled. The product of this peak was subjected to hydrolysis at pH 4.5 for 5 min. The hydrolysate was positive to the

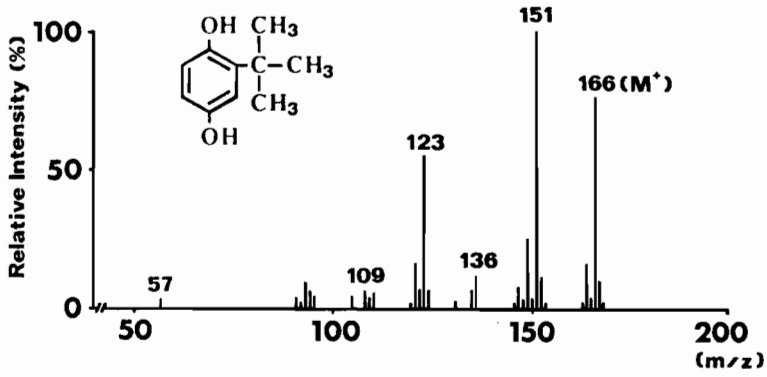

Fig. V-3. GC-MS of a product ether-extracted from the reaction mixture of GSH with BQ.

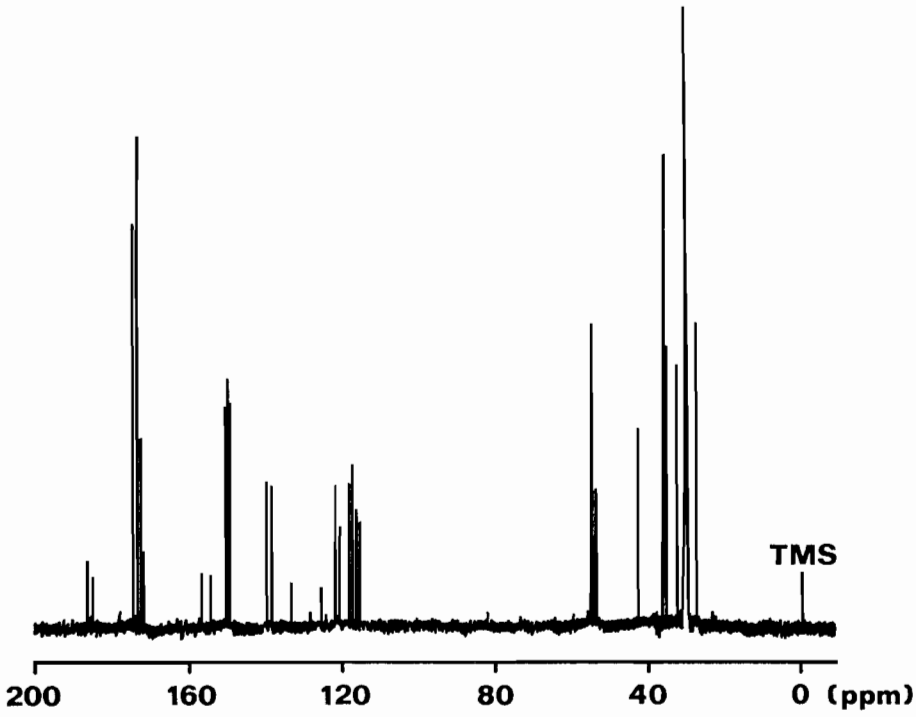

Fig. V-4. The ^{13}C-NMR spectrum of GSH-BQ conjugated compound(s) in DMSO-d_6.

Ellman test. The hydrolysate was extracted with diethyl ether, and the extract was subjected to GC and identified to contain BQ. Neither sulfhydryl group nor BQ were produced when the pooled product was kept at under pH 7.0. Thus, the product was able to be ascribed to a complex between BQ and GSH such as conjugated compound. In order to confirm its structure instrumentally, the lyophilized material was subjected to ^{13}C-NMR measurement. The ^{13}C-NMR spectrum in DMSO-d_6 (Fig. V-4) exhibited the signals of carbons in quinone skeleton at δ185.19 (singlet) and 186.48 (singlet). The ^{13}C-NMR data suggested that the lyophilized material contained the conjugated compound(s) of BQ with GSH, 2-*tert*-butyl-6-*S*-glutathionyl-*p*-quinone and/or 5-isomer.

V-4 DISCUSSION

BQ had a potent mutagenicity as shown in Chapter II. When screening studies on desmutagenic activity against BQ were performed, 11 of the 28 samples of foodstuffs and chemicals showed potent desmutagenic activities (Table V-1). Of the vegetable and fruit juices, those of sweet pepper, lemon, tomato, orange,

strawberry, melon, and kiwi fruit were able to reduce the mutagenic activity of BQ. Of the chemicals, such sulfhydryl compounds as GSH, PaSH and DTT had potent desmutagenicities against BQ (Tables V-3, 4 and 5). The juices having potent desmutagenicities contained a relatively higher content of sulfhydryl group (Table V-2).

Plural desmutagenic factors may be present in these juices, and sulfhydryl compounds are probably one of the desmutagenic factors. Especially, GSH occurs in all mammalian hepatocytes in high concentration. Such carcinogens and mutagens as aflatoxin B1 [11-13], benzo[*a*]pyrene (B[*a*]P) [14-18], benzo[*a*]anthracene [19,20], styrene [21] and cholesterol [22] have been reported to be inactivated by GSH. For example, B[*a*]P is oxidized to active B[*a*]P-4,5-epoxide or B[*a*]P-7,8-diol-9,10-epoxide by the hepatic P-450 enzymes [23]. These active epoxides are detoxicated by being transformed to inactive water-soluble compounds such as GSH-conjugates and then excreted through urine. Thus, GSH plays an important role in the detoxication of carcinogens and mutagens, probably *in vivo* also.

The inactivation mechanism of BQ by GSH was next investigated. GSH very easily reacted with BQ, and

one mole of GSH consumed two moles of BQ (Fig. V-1). Two products were formed during this reaction. One was BHQ (Fig. V-3) and the other was indicated to be a GSH-BQ conjugated compound (Fig. V-5), which is

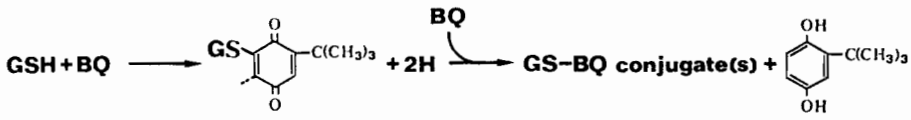

Fig. V-5. A proposed process mechanism for the overall conversion of BQ during the reaction with GSH. GSH-BQ conjugated compounds consist of two position isomers at C-5 and C-6.

simultaneously produced together with BHQ. The overall reaction is presumed to proceed as shown in Fig. V-5. GSH is a nucleophilic compound, which attacks to one mole of BQ, and forms the GSH-BQ conjugated compound and 2 protons. The two protons are readily consumed for the reduction of the other one mole of BQ. It is considered that GSH readily detoxify BQ because this reaction is very fast.

It is well known that quinones are highly toxic to the isolated hepatocytes of rat [24,25]. Cummings *et al.* reported that BQ was also produced by incubating BHA with the isolated hepatic microsome [26]. I suppose that BQ occurs in human body when BHA-

containing foods are ingested, and the sulfhydryl compounds, especially GSH, play an important role *in vivo* in reducing the mutagenicity of BQ.

REFERENCES

[1] T. Kada, K. Morita and T. Inoue, *Mutation Res.*, **53**, 351 (1978).

[2] S. Arimoto, Y. Ohara, T. Namba, T. Negishi and H. Hayatsu, *Biochem. Biophys. Res. Commun.*, **92**, 662 (1980).

[3] H. Hayatsu, S. Arimoto, K. Togawa and M. Makita, *Mutation Res.*, **81**, 287 (1981).

[4] S.R. Sirianni, H.H. Chen and C.C. Hung, *Mutation Res.*, **90**, 175 (1981).

[5] T. Kada, M. Kato, K. Aikawa and S. Kiriyama, *Mutation Res.*, **141**, 149 (1984).

[6] M. Mirvish, L. Wallcave, M. Egan and P. Shubic, *Science*, **177**, 65 (1972).

[7] J.I. Gray and C.J. Randall, *J. Food Protection*, **42**, 168 (1979).

[8] K. Morita, M. Hara and T. Kada, *Agric. Biol. Chem.*, **42**, 1235 (1978).

[9] G.L. Ellman, *Arch. Biochem. Biophys.*, **82**, 70

(1959).

[10] D.J. Reed and P.W. Beatty, *Rev. Biochem. Toxicol.*, **2**, 213 (1980).

[11] G.O. Emerole, N. Neskovic and R.L. Dixon, *Xenobiotica*, **9**, 737 (1979).

[12] E.J. Moss, D.J. Judah, M. Przybylski and G.E. Neal, *Biochem. J.*, **210**, 227 (1983).

[13] P.D. Lotlikar, E.C. Jhee, S.M. Insetta and M.S. Clearfield, *Carcinogenesis*, **5**, 269 (1984).

[14] B. Jernstroem, J.R. Babson, P. Moldeus, A. Holmgren and D. Reed, *Carcinogenesis*, **3**, 861 (1982).

[15] S. Hesses, B. Jernstroem, M. Martinez, P. Moldeus, L. Christodoulides and B. Ketterer, *Carcinogenesis*, **3**, 757 (1982).

[16] A. Cooper, A. Hewer, O. Riberio, P.L. Grover and P. Silms, *Carcinogenesis*, **1**, 1075 (1980).

[17] B. Jernstroem, R. Brigelius and H. Sies, *Chem.-Biol. Interactions*, **44**, 185 (1983).

[18] T. Watabe, A. Hiratsuka and T. Tsurumori, *Biochem. Pharmacol.*, **33**, 4051 (1984).

[19] D. Cobb, C. Boehlert, D. Lewis and R.N. Armstorong, *Biochemistry*, **22**, 805 (1982).

[20] H. Glatt, T. Friedberg, P.L. Grover, P. Sims and

F. Oesch, *Cancer Res.*, **43**, 5713 (1983).

[21] K. Yoshikawa, M. Isobe, T. Watabe and E. Takabatake, *Mutation Res.*, **78**, 219 (1980).

[22] T. Watabe, T. Sawahata and J. Horie, *Biochem. Biophys. Res. Commun.*, **87**, 469 (1979).

[23] M.K. Buening, P.G. Wislocki, W. Levin, H. Yagi, D.R. Thakker, H. Akagi, M. Koreeda, D. M. Jenina and A. H. Conney, *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 5358 (1978).

[24] L. Rossi, G.A. Moore, S. Orrenius and P.J. O'Brien, *Arch. Biochem. Biophys.*, **251**, 25 (1986).

[25] G.A. Moore, L. Rossi, P. Nicotera, S. Orrenius and P.J. O'Brien, *Arch. Biochem. Biophys.*, **259**, 283 (1987).

[26] S.W. Cummings, G.A.S. Ansari, F.P. Guengerich, L.S. Crouch and R.A. Prough, *Cancer Res.*, **45**, 5617 (1985).

VI PRODUCTION MECHANISM OF DERIVATIVES OF BUTYLATED HYDROXYANISOLE

VI-1 INTRODUCTION

I have been studying on the bio-activities of the products of the mutual reaction under acidic condition between the food additives, butylated hydroxyanisole (BHA) and sodium nitrite. I have so far found two mutagens, 2-*tert*-butyl-*p*-quinone (BQ) and 3,3'-di-*tert*-butyl-biphenyldiquinone-(2,5,2',5') (BBDQ), and one desmutagen 2,6-di-*tert*-butyl-8-hydroxy-dibenzofuran-1,4-quinone (BHDQ) from the reaction products. Two interesting problems still remain: by what mechanism the products are formed and how much amounts of these bio-active compounds are produced in the reaction of BHA with nitrite. Solution of these problems may explain the *in vivo* toxicity of BHA taken orally.

In Chapter II, I detected nine compounds from the reaction mixture of BHA and nitrite by a thin layer chromatography (TLC). Three of them were identified as described above. In this chapter, it was attempted to determine the chemical structures of the other four products. It is generally considered that the

reaction of phenols such as BHA with nitrite involves C-nitrosation which is independent of pH of the system ranging from 1 to 5 [1]. Then, the periodic yields of the reaction products were measured in two reaction systems of pH 2 and pH 5, and the reaction mechanism was studied to make clear. I discussed on a possibility of the *in vivo* generation of these products and on their safety, when BHA was orally ingested.

VI-2 MATERIALS AND METHODS

Chemicals. BHA, sodium nitrite, hypophosphorous acid and mannitol were purchased form Wako Pure Chemical Industries Ltd., Osaka, Japan. Methyl palmitate as an internal standard of gas chromatography (GC) was purchased from Sigma Chemical Company, MO., U.S.A.

The reaction of BHA with sodium nitrite. BHA (30 mmol) and sodium nitrite (60 mmol) were dissolved in 70% ethanol (300 ml) and the mixture was adjusted to pH 2 or 5. The reaction products were periodically extracted from 2 ml of the reaction mixtures with 2 ml ethyl acetate, after addition of 5 ml water. An aliquot (0.2 ml) of the extract was dried up under

nitrogen stream and was dissolved again into 2 ml of ethyl acetate containing a standard amount of methyl palmitate. This solution was subjected to GC to measure the yield of each reaction product. The GC analysis was carried out under the same condition as described in Chapter II. Each reaction product was identified by comparing *Rf* value on TLC with the results in Chapter II.

Isolation of the reaction products. The reaction products were extracted with diethyl ether from the reaction mixture of pH 2. The extract was applied to TLC and developed with a solvent system of hexane-diethyl ether (7:3, v/v). Two of the nine bands on the TLC was scraped off, and extracted with diethyl ether. The extract was dried up and purified by recrystallization from methanol. Two more reaction products were prepared as follows: after the reaction mixture of pH 5 was allowed to stand for a few hours, light brown precipitate was produced, and the amount increased with time. The precipitate was collected by filtration. The light brown precipitate (1 g) was suspended together with the additional 2 g of sodium nitrite into a solvent of ethanol-diethyl ether-water

(70:15:15) of pH 5. The precipitate was dissolved gradually and orange crystals were precipitated in the mixture and were collected by the filtration. Both the crystals of light brown and orange were purified by the recrystallization from methanol and subjected to instrumental determination of their structures.

Instrumental analyses. In order to elucidate the structures of the purified four products, IR, NMR, EI-MS, and fast atom bombardment mass spectrometry (FAB-MS) analyses were performed using a Hitachi EPI-S2, Buruker AC250, JEOL JMS-D300 and JEOL JMS-HX110, respectively.

VI-3 RESULTS

Analyses on the structures of the purified four reaction products

Of nine compounds detected from the reaction mixture of BHA and nitrite, BQ, BBDQ and BHDQ had been identified (Chapters II and III). The remaining 6 compounds were still unknown in this chapter. Two of them were obtained by the preparative TLC, and the other two compounds were obtained as a light brown

crystal and a orange crystal from the reaction mixture as described in MATERIALS AND METHODS.

One of the compounds, with *Rf* value of 0.50 on TLC, obtained by the preparative TLC was identified as intact BHA by GC. The other one with *Rf* value of 0.80 on TLC was subjected to routine instrumental analyses. It gave a molecular ion peak at *m/z* 225 (M^+) and a fragment ion peak at *m/z* 210 (M^+-CH_3) (Fig. VI-1).

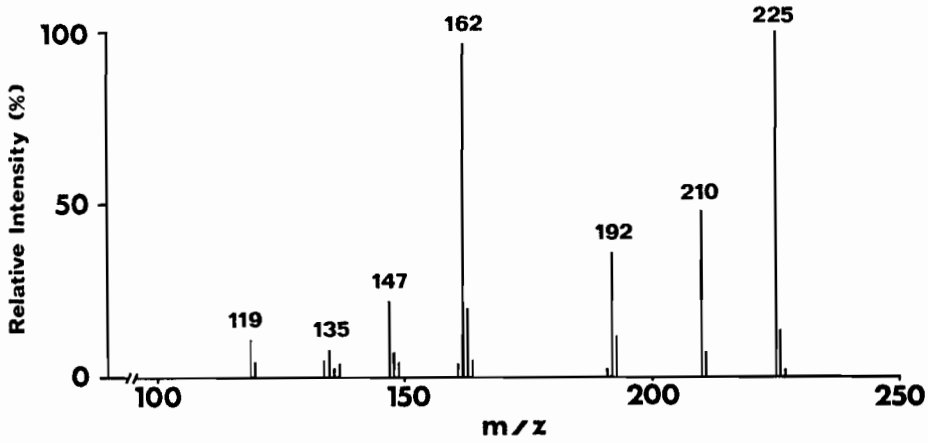

Fig. VI-1. EI-MS of one compound with *Rf* value of 0.80 on TLC.

The high-resolution EI-MS gave *m/z* 225.0987 and 210.0828, which indicated a formula of $C_{11}H_{15}O_4N$ and $C_{10}H_{12}O_4N$, respectively. The IR spectrum obtained as KBr disc gave bands at 3400 (νOH), 1540 and 1360 cm^{-1} (νNO_2). The ^{1}H-NMR spectrum in $CDCl_3$ (Fig. VI-2)

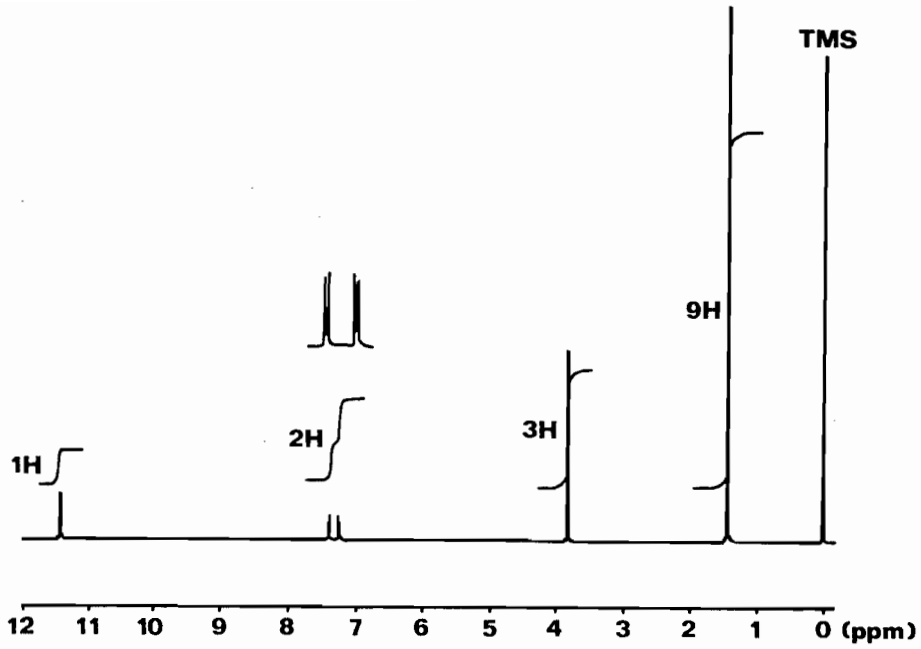

Fig. VI-2. The ^{1}H-NMR spectrum of one compound with *Rf* value of 0.80 on TLC.

exhibited a phenolic proton at δ11.42 (singlet, 1H) whose signal disappeared by adding D_2O, aromatic protons at δ7.25 (doublet, 1H, *J*=2.5 Hz) and 7.37 (doublet, 1H, *J*=2.5 Hz), methoxy protons at δ3.81 (singlet, 3H) and *tert*-butyl protons at δ1.43 (singlet, 9H). Signals of the aromatic protons and phenolic proton were shifted to the low-field as compared with that of BHA. This shift indicated that a strong electron withdrawing group such as nitro group was bound to the aromatic skeleton of BHA. The coupling

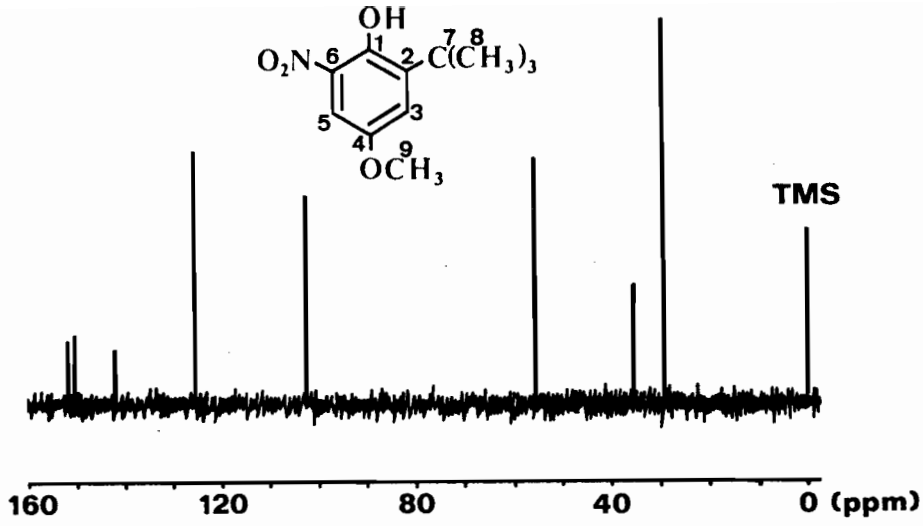

Fig. VI-3. The ^{13}C-NMR spectrum of one compound with *Rf* value of 0.80 on TLC.

constant of aromatic protons showed a *meta*-coupling to each other. In the ^{13}C-NMR spectrum in $CDCl_3$ (Fig. VI-3), the signals at δ29.21, 35.68, 55.80, 102.60, 125.56, 142.09, 150.64 and 151.50 were assigned to C-8, C-7, C-9, C-5, C-2 and 3, C-6, C-1 and C-4, respectively. On the basis of these analytical data, this compound with *Rf* value of 0.80 on TLC was determined to be 6-nitro-butylated hydroxyanisole (NO_2-BHA).

The EI-MS spectrum of the light brown product with *Rf* value of 0.28 on TLC (Fig. VI-4) showed the signals at *m/z* 358 (M^+), 343 (M^+-CH_3) and 315 ($M^+-C_3H_7$), which exhibited the similar fragment ions to those from BHA.

The IR spectrum (Fig. VI-5) obtained as KBr disc gave an absorption band of OH group at 3300 cm^{-1}. The ^{1}H-NMR data in DMSO-d_6 (Fig. VI-6) showed the signals at

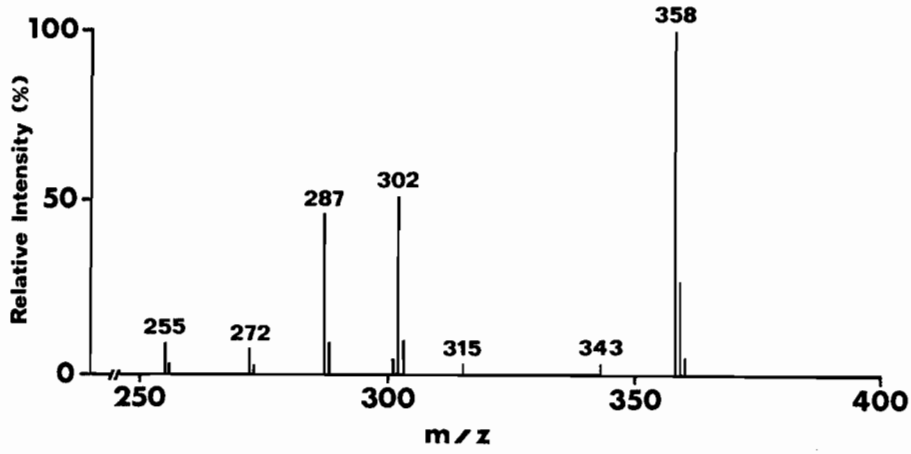

Fig. VI-4. EI-MS of the light brown crystal with *Rf* value of 0.28 on TLC.

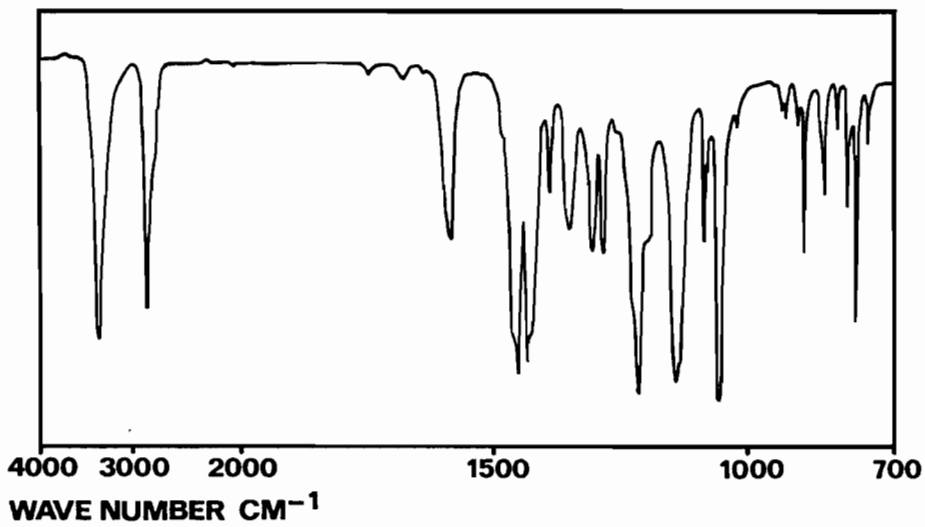

Fig. VI-5. The IR spectrum of the light brown crystal with *Rf* value of 0.28 on TLC.

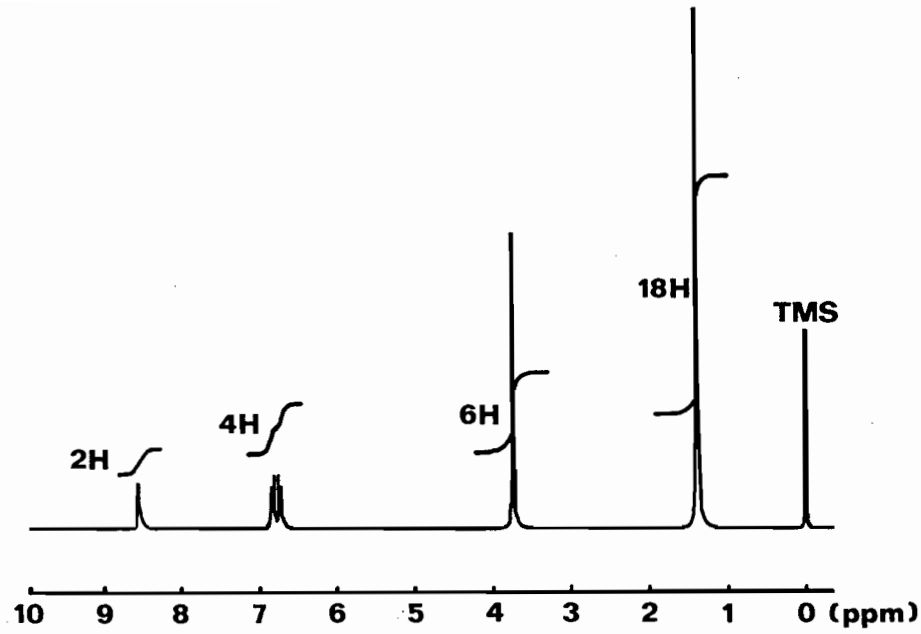

Fig. VI-6. The ^{1}H-NMR spectrum of the light brown crystal with *Rf* value of 0.28 on TLC.

δ1.39 (singlet, 18H, $(CH_3)_3$), 3.74 (singlet, 6H, OCH_3), 6.73 (doublet, 2H, *J*=2.5 Hz, aromatic protons) and 6.80 (doublet, 2H, *J*=2.5 Hz, aromatic protons), and 8.54 (singlet, 2H, OH) which disappeared by adding D_2O. These signals were assigned to protons on C-8, 8', C-9, 9', C-4, 4', C-6, 6' and C-2, 2' (see the numbering in Fig. VI-7), respectively. The ^{13}C-NMR data in DMSO-d_6 exhibited the signals at δ30.14, 35.16, 55.63, 113.37, 113.48, 131.14, 140.92, 145.51 and 153.52 (Fig. VI-7). These signals were assigned to carbons of C-8, 8', C-7.

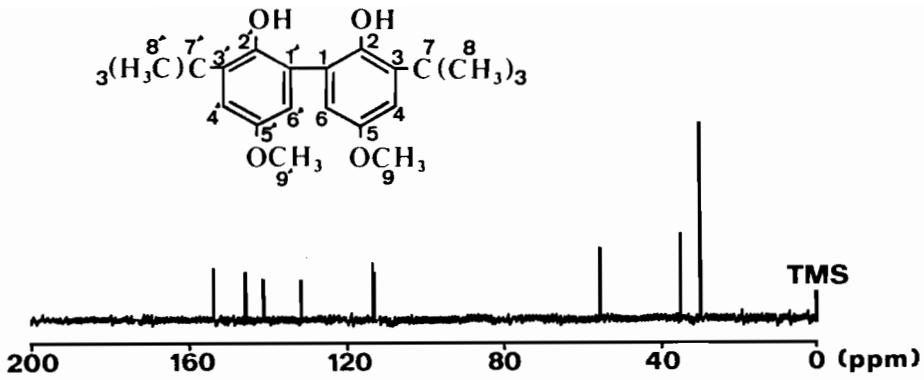

Fig. VI-7. The ^{13}C-NMR spectrum of the light brown crystal with *Rf* value of 0.28 on TLC.

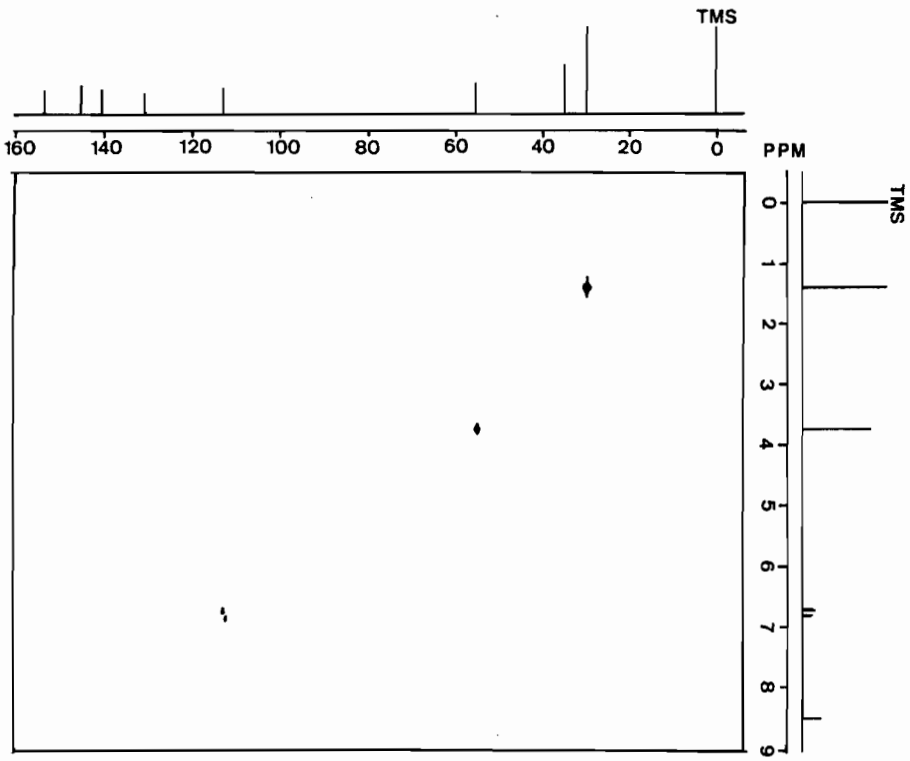

Fig. VI-8. The ^{13}C, ^{1}H cosy NMR of the light brown crystal with *Rf* value of 0.28 on TLC.

7', C-9, 9', C-4, 4', C-6, 6', C-3, 3', C-1, 1', C-5, 5' and C-2, 2', respectively. ^{13}C, ^{1}H cosy NMR spectra of this compound (Fig. VI-8) elucidated the mutual connection of *tert*-butyl carbon and protons at 29.73 ppm and 1.39 ppm, methoxy carbon and protons at 55.19 ppm and 3.73 ppm, and aromatic carbon and proton at 112.94 ppm and 6.80 ppm, and at 113.08 ppm and 6.72 ppm. From these data, the light brown crystal was identified to be 2,2'-dihydroxy-3,3'-di-*tert*-butyl-5,5'-dimethoxy-biphenyl (di-BHA).

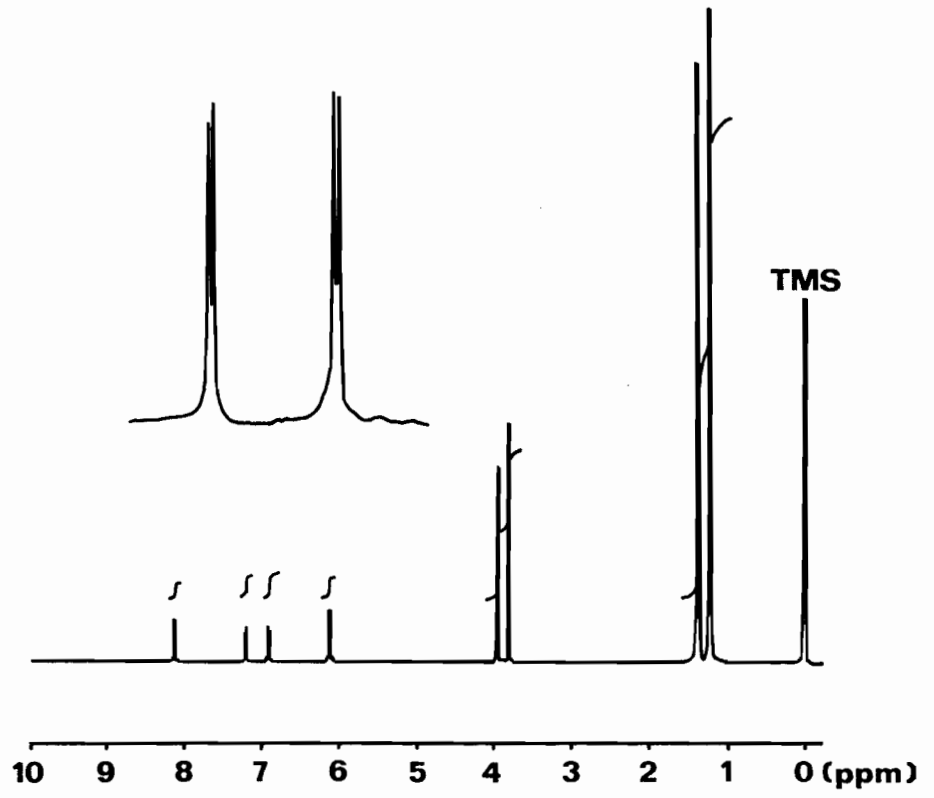

Fig. VI-9. The ^{1}H-NMR spectrum of the orange crystal with *Rf* value of 0.14 on TLC.

The ^{1}H-NMR spectrum of the orange product with *Rf* value of 0.14 on TLC in $CDCl_3$ (Fig. VI-9) showed the signals at δ1.20 (singlet, 9H), 1.38 (singlet, 9H), 3.81 (singlet, 3H), 3.94 (singlet, 3H), 6.12 (singlet, 1H), 6.90 (doublet, 1H, *J*=2.5 Hz), 7.90 (doublet, 1H, *J*=2.5 Hz) and 8.11 (singlet, 1H). The ^{13}C-NMR spectrum in $CDCl_3$ (Fig. VI-10) exhibited the signals at δ26.07, 29.33, 34.30, 44.26, 55.35, 55.61, 102.16, 107.74, 116.38, 122.24, 126.68, 130.71, 135.23, 155.58, 166.90 and 203.98. The IR spectrum had the absorption bands at 2950, 1760, 1555 and 1480 cm^{-1}. The FAB-MS

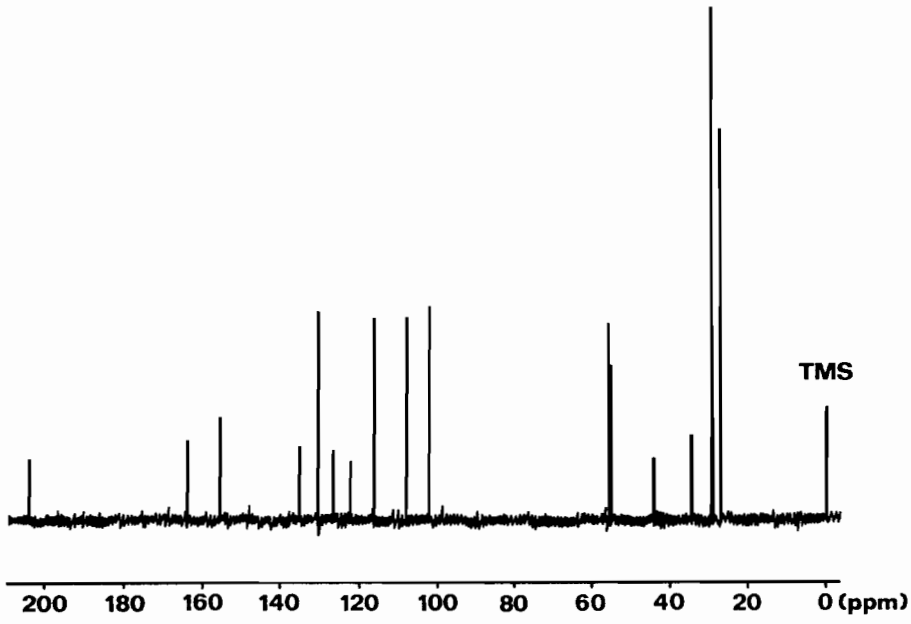

Fig. VI-10. The ^{13}C-NMR spectrum of the orange crystal with *Rf* value of 0.14 on TLC.

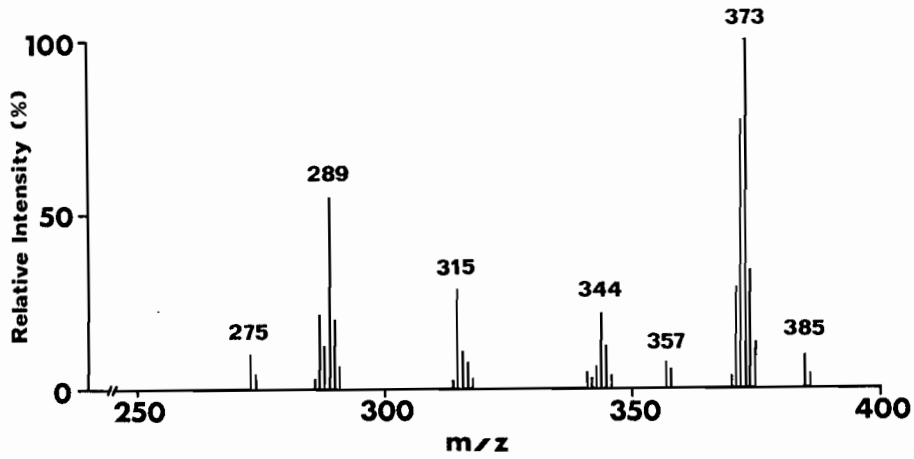

Fig. VI-11. FAB-MS of the orange crystal with *Rf* value of 0.14 on TLC.

(Fig. VI-11) gave a signal at *m/z* 385 (M^++1). These analytical data were complicated, and it was difficult to assign these information to the appropriate position on the chemical structure. The structure of the orange product was not able to be elucidated from these analyses.

Formation rates of toxic products

BHA was reacted with sodium nitrite at pH 2, and the reaction products were periodically measured. A main product in the reaction was BQ whose amount increased in inverse proportion to that of BHA until 60 min of incubation, and then reached at the level of over 50% of total amounts of the components in the

reaction mixture (Fig. VI-12A). BBDQ was also formed, but its content was smaller than that of BQ. The yields of these reaction products from the acidic

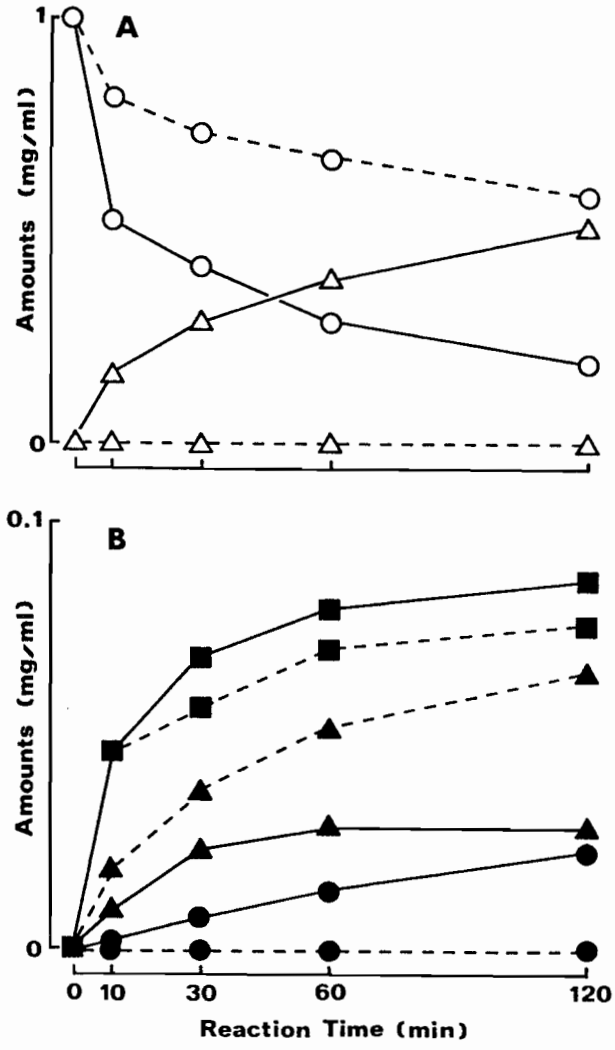

Fig. VI-12. The formation rates of BHA (○), BQ (△), di-BHA (▲), NO_2-BHA (■), and BBDQ (●) at pH 2 (————) or pH 5 (-------).

reaction mixture are shown in Table VI-1. When the consumed amount of BHA was taken as 100, the percentages of BQ, di-BHA, NO_2-BHA, and BBDQ were 71.4, 2.0, 8.6, and 1.4, respectively. Thus, BQ was a main product under the gastric and acidic condition (pH 2) and a considerable amount of NO_2-BHA was also formed.

On the other hand, when the pH of the reaction

Table VI-1

Yields of the reaction products

Product	Recovery amounts from reaction mixture (mmol)	Yield %*
(Consumed BHA)	(24.0)	-
BQ	17.1	71.4
NO_2-BHA	2.1	8.6
di-BHA	0.5	2.0
BBDQ	0.3	1.4
BHDQ	trace	-
Unknown	trace	-

* Determined by GC. When 30 mmol BHA was subjected to the reaction, 6 mmol BHA remained unchanged and 24 mmol BHA was consumed. The yield percentages of the products were calculated, when the consumed BHA was taken as 100.

mixture was 5, the consumption of BHA was decreased to the half of that at pH 2, and neither BQ nor BBDQ were detected (Fig. VI-12). Thus, under the near neutral condition, both the toxic products, BQ and BBDQ, were not formed. On the contrary, NO_2-BHA and di-BHA were produced at pH 5 as well as at pH 2 (Fig. VI-12B). The formation rate of NO_2-BHA remained almost unchanged, but the production of di-BHA increased to 2-fold of that at pH 2 after 120 min.

Inhibition test of the reaction between BHA and nitrite

In order to study the reaction mechanism of BHA with nitrite, the inhibition of the reaction by the addition of various reagents was examined. Nitrite easily forms nitrous acidium ion ($H_2NO_2^+$), nitrogen trioxide (N_2O_3), and nitroso radical ($NO^\cdot$) under acidic condition, and nitrite produces a diazonium compound in the reaction with phenols through a formation of nitroso compound under acidic condition [2,3]. Therefore, two promoters are generally expected for the polymerization of phenols by nitrite such as BHA to di-BHA under the acidic condition: nitrous acidium ion and $NO^\cdot$ radical. Then, hypophosphorous acid which is a quencher of azide, and mannitol which is a radical

scavenger, were added to the reaction mixture at pH 5, and the consumption of BHA and formation of di-BHA were measured by GC. The addition of hypophosphorous acid gave a slight increase in the formation rate of NO_2-BHA and a slight decrease in that of di-BHA, but clear result was not obtained because of the presence of water in this system. On the other hand, the formation rate of di-BHA almost unchanged regardless of the addition of mannitol (Figs. VI-12B and 13). Thus,

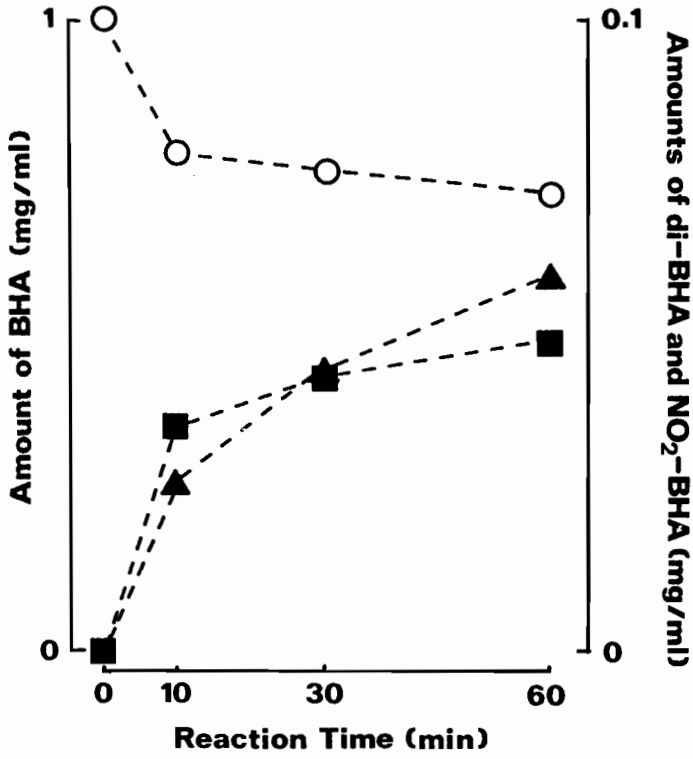

Fig. VI-13. The effects of mannitol (10 mM) on the consumption of BHA (○), and the formations of di-BHA (▲) and NO_2-BHA (■).

it was made clear that any radicals did not participate in the formation of di-BHA from BHA.

VI-4 DISCUSSION

When an antioxidant BHA was reacted with nitrite under acidic condition, nine compounds were produced as seen on TLC. The reaction products, BQ and BBDQ, have mutagenic activities (Chapter II), and the product, BHDQ, has desmutagenic activity (Chapter V). I will discuss here on the formation mechanism of these compounds. A compound with *Rf* value of 0.50 on TLC is the intact BHA. Two of the other 5 products were NO_2-BHA (Figs. VI-1, 2 and 3) and di-BHA (Figs. VI-4, 5, 6 and 7), and other one was not identified because of complicated date (Figs. VI-9, 10 and 11). The remaining two unknown compounds are considered to be the reaction intermediates, nitrosation and azide compounds of BHA.

Two mechanisms for the dimerization of phenol compounds caused by nitrite are proposed. One is a polymerization by radicals generated from nitrite [3] and the other is a polymerization reaction *via* the nitrosation and diazotization of phenols [4]. In the

latter reaction, nitro compound is formed from the nitrosation intermediate as one of by-products. Here, NO_2-BHA was detected in the reaction mixture as described above, and the yield of di-BHA remained almost unchanged regardless of the addition of a radical trapper, mannitol (Fig. VI-12B and 13). Moreover, when the pH of the reaction system was 5, the yield of di-BHA increased to around 2-fold. This result indicated that the formation of di-BHA was proceeded by a ionic mechanism but not by radical one. It is, therefore, considered that the formation of di-BHA from BHA was due to the nitrosation of BHA by nitrite. The formation mechanism of these compounds in the reaction of BHA with nitrite was illustrated in Fig. VI-14. Nitrite acted on BHA chiefly as an oxidant and formed two oxidative derivatives, BQ and BBDQ. The other significant action of nitrite was a dimerization of BHA to di-BHA *via* formations of nitroso and diazonium BHA. BBDQ was an oxidative product from di-BHA, since BBDQ was obtained from the reaction of the di-BHA with nitrite.

Under the gastric and acidic condition, BHA was oxidized by nitrite mainly to BQ, whose yield was around 70% (Table VI-1). The nitroso and diazonium

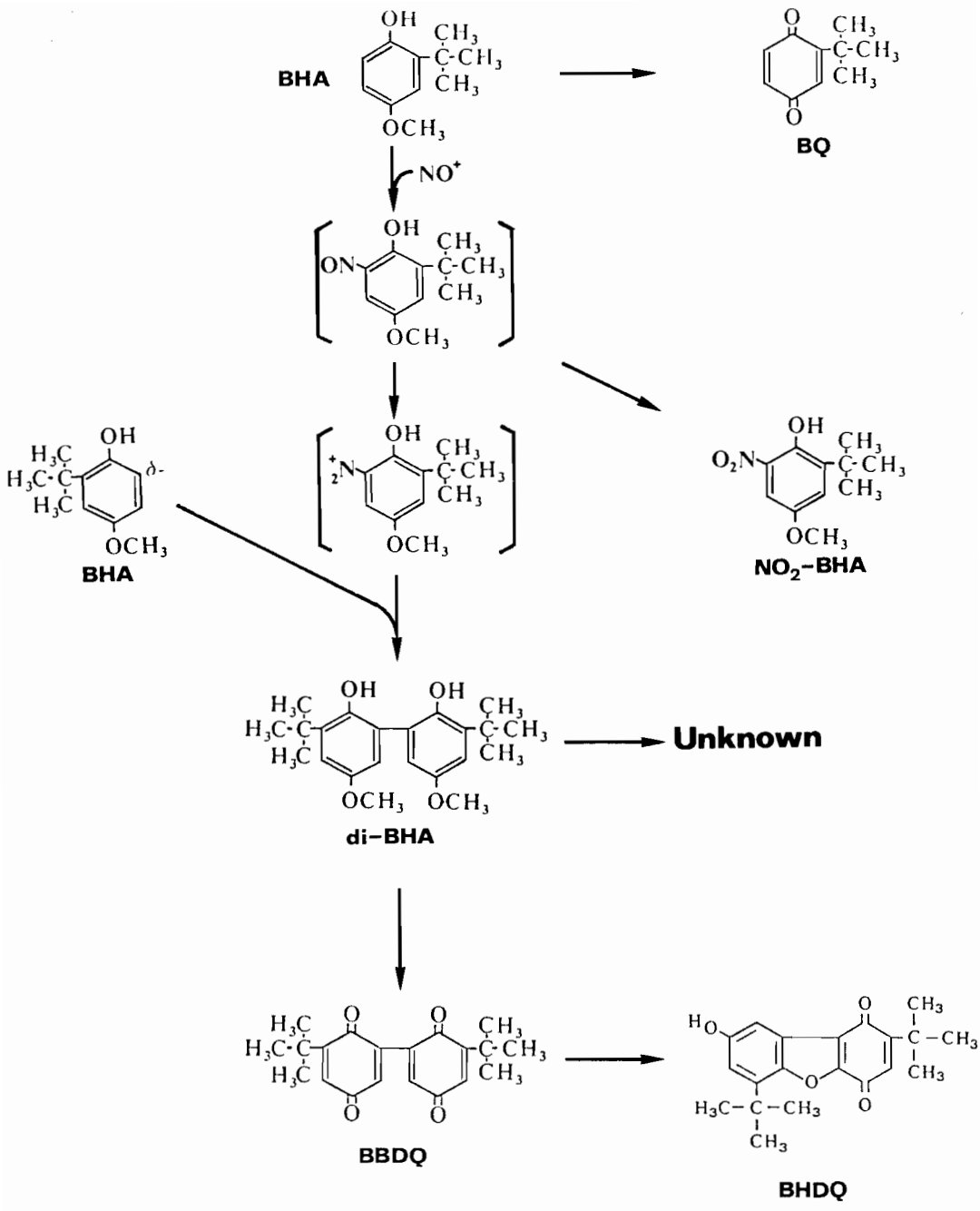

Fig. VI-14. The reaction mechanism of BHA with nitrite.

BHA may be involved in the formation pathway of di-BHA. The mutagenicity of the main product, BQ, was 20 times as strong as BBDQ which was produced by way of the nitrosation reaction (Chapter II). It is concluded that the toxicity caused by the BHA derivatives is ascribed to BQ under the gastrically acidic condition. On the contrary, both mutagens, BQ and BBDQ, were almost not formed under near neutral pH such as pH 5 (Fig. VI-12). I will speculate that these mutagens may be produced in the human stomach, but may not in the intestines as well as in the blood or liver. On the other hand, even when BQ is formed in the stomach of animals having only glandstomach such as human, the toxicity of BQ may be easily removed by sulfhydryl compounds present in gastro contents, food components, gastric juice, and pancreatic juice as described in Chapter V. While the animals having the forestomach such as F344 rat may accumulate BQ in the forestomach, which is normally empty and does not contain the sulfhydryl compounds. It is, therefore, reasonable that Ito *et al.* had detected cancer in the forestomach of F344 rats when the large amount of BHA was orally administered [5,6]. BQ produced in forestomach and glandstomach may be incorporated into blood through the

intestines. The incorporated BQ is also detoxified by the thiol compounds such as GSH because blood contained at high level. The detoxification mechanism of BQ with GSH was described in detail in Chapter V. It is concluded that BQ itself has no toxicity in the human body.

BBDQ is found also to be unstable *in vivo* as well as *in vitro* (Chapter III). BBDQ was easily converted into BHDQ *via* its semiquinone radical by the one-electron reduction by the hepatic P-450 system. BHDQ had a considerable desmutagenicity against the chemical mutagens such as Trp-P-2 (Chapter IV). BHDQ is expected also to be produced endogenously from the incorporated BHA by the hepatic P-450 system because di-BHA was formed in the liver [7,8] and converted into BBDQ by the P-450 system. I consider that the desmutagenicity of the orally administered BHA as reported by Wattenberg *et al.* is ascribed to BHDQ derived from BHA. On the basis of these observations, I finally conclude that BHA as one of food additives has a favorable effect for human being rather than its undesirable toxicity.

REFERENCES

[1] B.C. Challis and A. Lawson, *J. Chem. Soc. (B)*, 770 (1970).

[2] J. Gilbert, M.E. Knowls and D.J. McWeeny, *J. Sci. Food Agric.*, **26**, 1785 (1975).

[3] R.G. Cassens, M.L. Greaser, T. Ito and M. Lee, *Food Technology*, **33**, 46 (1979).

[4] M.E. Knowls, D.J. McWeeny, L. Couchman and M. Thorogood, *Nature*, **247**, 288 (1974).

[5] N. Ito, A. Hagiwara, M. Shibata, T. Ogiso and S. Fukushima, *Gann*, **73**, 132 (1982).

[6] N. Ito, S. Fokushima, A. Hagiwara, M. Shibata and T. Ogiso, *J. Natl. Cancer Inst.*, **70**, 343 (1983).

[7] A. Gauma, L.D. Corte, M.G. Giovanni, F. de Sarlo and G. Sgaragli, *Drug Metb. Dispos.*, **11**, 581 (1983).

[8] K.E. Armstrong and L.W. Wattenberg, *Cancer Res.*, **45**, 1507 (1985).

VII CONCLUSION

A food additive, butylated hydroxyanisole (BHA), has the complicated physiological activities. Some researchers have asserted that the oral administration of BHA causes a cancer, and the others have argued that BHA can reduce *in vivo* the risks of carcinogenesis induced by chemical carcinogens. This controversy may come to an end by this study.

I assumed that these complex activities of BHA orally taken were not only due to BHA *per se* but also some derivatives of BHA endogenously formed. BHA was easily converted into its derivatives under the gastric condition in the co-presence of the other food component such as nitrite. I have conducted a series of work for the analyses on the BHA derivatives. I isolated each product from the reaction mixture of BHA with nitrite, determined their chemical structures by instrumental analyses, and then examined their bioactivities, especially their mutagenicities and desmutagenicities using the Ames's strains of *Salmonella typhimurium* TA 98 and TA 100.

The reaction mixture of BHA and nitrite under the gastric and acidic condition contained nine compounds.

Two of them were mutagens and identified as 2-*tert*-butyl-*p*-quinone (BQ) and its dimer, 3,3'-di-*tert*-butyl-biphenyldiquinone-(2,5,2',5') (BBDQ) (Chapter II). When their mutagenicities were tested by the induced mutation frequency method, the magnitude of the mutagenicity of BQ was 1.9×10^{-4} at 52 μg/plate and that of BBDQ was 8.3×10^{-5} at 95 μg/plate, 1/20 smaller than that of BQ. The yields of BQ and BBDQ from the reaction mixture were 71.4% and 1.4% of the amounts of the consumed BHA, respectively (Chapter VI). BQ was the main product in this reaction, and hereby, the mutagenic toxicity of BHA orally administered was considered to be ascribed to BQ.

One of the other 7 products was identified as 2,6-di-*tert*-butyl-8-hydroxy-dibenzofuran-1,4-quinone (BHDQ) (Chapter III), which was derived from BBDQ by a photolytic one-electron reduction *via* its semiquinone radical (Chapter IV). BHDQ was a potent desmutagen, it suppressed the frameshift-type reverse mutations induced by the chemical mutagens such as Trp-P-1, Trp-P-2, B[*a*]P and 1-NP. For example, 1.5 nmol of BHDQ exhibited 60% desmutagenicity against the mutagenicity of 0.2 nmol Trp-P-2.

Thus, it has been found that three products from

the reaction of BHA with nitrite, BQ, BBDQ, and BHDQ, had the bio-activities. The other 6 products did not have any mutagenicities and desmutagenicities (Chapter II), and were also analyzed as to their chemical structures (Chapter VI). They were the intact BHA, 6-nitro-BHA (NO_2-BHA), 2,2'-dihydroxy-3,3'-di-*tert*-butyl-5,5'-dimethoxy-biphenyl (di-BHA) and unknown one, and the remaining two products were suggested to be the reaction intermediates through a pathway of the formation of BBDQ from BHA, i. e. nitroso BHA and diazonium BHA.

A model experiment on the detoxification of BQ was carried out from the view point of food chemistry (Chapter V). The juices of sweet pepper, lemon, tomato, orange, strawberry, melon, and kiwi fruits reduced the mutagenicity of BQ, and their desmutagenic activities were contributed to their components such as sulfhydryl compounds. The detoxification mechanism was made clear using authentic sulfhydryl compounds. One mole of glutathione reduced one mole of BQ to 2-*tert*-butyl-hydroquinone and simultaneously formed a conjugated compound with one mole of BQ.

Thus, BQ was a main product of the reaction between BHA and nitrite under the gastric and acidic

condition (pH 2), but BQ was not produced under near neutral pH of 5 (Chapter VI). I speculate that these mutagens may be produced in the human stomach, but may not in the intestines. The toxicity of BQ is easily removed by sulfhydryl compounds which present in gastro contents, such as food components, gastric juice, and pancreatic juice, of the animals having only glandstomach, besides the endogenous BQ is easily detoxified by serum GSH because blood contains it at a high level. It is, therefore, considered that BQ has no toxicity in the human body. BBDQ may also be unstable *in vivo* as well as *in vitro* (Chapter III), because it is easily converted into BHDQ which exhibits a considerable desmutagenicity (Chapter IV). On the basis of these observations, I conclude that BHA as one of the food additives has a favorable effect on human body and that the possible toxicity due to some derivatives of this compound is actually ineffective.

List of Publications

a. Mutagens Formed from Butylated Hydroxyanisole Treated with Nitrite under Acidic Conditions
Mutation Res., **176**, 179 (1987).

b. Photolysis of a Mutagenic Biphenyldiquinone Derivative that Induces a Desmutagen to Trp-P-1 and Trp-P-2
Agric. Biol. Chem., **52**, 2265 (1988).

c. Desmutagenic Effects of Sulfhydryl Compounds on a Mutagen Formed from Butylated Hydroxyanisole Reacted with Sodium Nitrite
Agric. Biol. Chem., **52**, 2843 (1988).

d. Desmutagenicity of Dibenzofuran-Quinone Derivative Toward the Mutagenicity of Trp-P-2
Agric. Biol. Chem., **53**, in press (1989).

e. Mutagenicity and Desmutagenicity of Oxidation Products of Butylated Hydroxyanisole
Proceeding of IInd International Conference on Mechanisms of Antimutagenesis and Anticarcinogenesis, Ohito Japan, December 4-9, 1988, p. 151.

f. Herb Water-Extracts Eloquently Suppress Mutagenicity of Trp-P-2
Agric. Biol. Chem., accepted.

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