

## Effects of co-administration of butylated hydroxytoluene, butylated hydroxyanisole and flavonoids on the activation of mutagens and drug-metabolizing enzymes in mice

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Received 31 October 1996; accepted 11 April 1997

### Abstract

Effects of co-administration of food additives and naturally occurring food components were studied on the activation of mutagens. Male mice (ddY) were given diets containing butylated hydroxytoluene (BHT) or butylated hydroxyanisole (BHA) and flavone or flavanone (2,3-dihydroflavone) for two weeks and the ability of hepatic microsomes to activate aflatoxin B<sub>1</sub>, benzo[a]pyrene and *N*-nitrosodimethylamine was determined by the mutagenicity test. Co-administration of an antioxidant (0.1% BHT or 0.2% BHA in diet) and a flavonoid (0.1% flavone or 0.1% flavanone) resulted in additive effects on the activation of aflatoxin B<sub>1</sub> and benzo[a]pyrene, while the activation of *N*-nitrosodimethylamine was not elevated significantly by the co-administration. To understand the mechanism for the additive effects, induction of specific isozymes of cytochrome P450 involved in the activation of the mutagens was studied. Co-administration of BHT (0.1%) and flavone (0.1%) increased markedly the levels of proteins and the activities of the enzymes related to the isozymes of CYP2A and CYP2B, while co-administration of BHA (0.2%) and flavanone (0.1%) elevated those related to CYP1A. Further, the activation of aflatoxin B<sub>1</sub> and benzo[a]pyrene in hepatic microsomes was inhibited by the antibodies against these isozymes, which suggested that the enhanced activation of the mutagens by the co-administration might be mediated by the induction of these isozymes. © 1997 Elsevier Science Ireland Ltd.

**Keywords:** BHA; BHT; Flavonoids; Co-administration; Mutagens; P450

**Abbreviations:** ADI, acceptable daily intake; AHH, aryl hydrocarbon hydroxylase; BHA, butylated hydroxyanisole; B[a]P, benzo[a]pyrene; BHT, butylated hydroxytoluene; BROD, 7-benzoyloxyresorufin *O*-dealkylase; DMN, *N*-nitrosodimethylamine; ECOD, 7-ethoxycoumarin *O*-deethylase; ERMD, erythromycin *N*-demethylase; EROD, ethoxyresorufin *O*-dealkylase; GST, glutathione *S*-transferase; NPH, *p*-nitrophenol hydroxylase; PROD, 7-pentoxeresorufin *O*-dealkylase; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; UDP-GT, UDP-glucuronyl transferase.

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## 1. Introduction

A number of chemicals are ingested through diet, which includes food additives, naturally occurring food components and food contaminants. Of these, food additives are normally subjected to toxicity tests using animals before the usage and an acceptable daily intake (ADI) is determined according to the data obtained. However, toxicity tests are usually done by administration of one food additive alone. To evaluate the risk that may occur by ingesting various chemicals at the same time, it would be important to study whether co-administration of food additives, naturally occurring food components and food contaminants would produce any synergistic, additive, or antagonistic effects on the toxicity of chemicals.

It is well known that some chemicals might alter chemical carcinogenesis by modulating drug-metabolizing enzymes that are capable of activating or detoxifying carcinogens (Parke and Ioannides, 1981). A number of compounds in foods are known to modulate these enzyme activities (Guengerich, 1984). Of naturally occurring food components, flavonoids could modify the drug-metabolizing enzymes (Siess et al., 1992) and exert their effects on the metabolism of other drugs and chemicals (Buening et al., 1981). For example, it was reported that the intake of grapefruit juice together with drugs altered the metabolism of a variety of drugs and this interaction might be mediated by the modification of cytochrome P450 enzymes by the flavonoids in grapefruit juice (Fuhr et al., 1993; Bailey et al., 1994; Merkel et al., 1994).

Of the food additives, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are known to affect drug-metabolizing enzymes. The activities of the enzymes were inhibited in vitro by BHA and BHT in rats (Yang et al., 1974), while these were generally induced by in vivo treatment with BHA in rats and mice (Cha and Bueding, 1979; Hayes et al., 1991; Iwata et al., 1993; Buetler et al., 1995) and with BHT in rats and mice (Gilbert and Golberg, 1965; Kawano et al., 1980; Powell et al., 1986) and hamsters (Sun et al., 1995).

These findings indicated that BHA, BHT and flavonoids might modify the toxicity of other chemicals by affecting the drug-metabolizing enzyme activities. In fact, it was first reported by Wattenberg et al. (1976) that carcinogenicity of chemicals was modified by administration with BHA or BHT. A number of studies have been done on the interference of BHT and BHA with mutagens and carcinogens, which was reviewed by Kahl (1984), Ito and Hirose (1989) and Wattenberg (1993).

The present study was conducted to determine if concomitant intake of the antioxidants and naturally occurring food components could exert any synergistic, additive or antagonistic effects on the activation of mutagens. For this, the effects of dietary co-administration of BHT or BHA with flavone or flavanone were studied on the activation of aflatoxin B<sub>1</sub>, benzo[a]pyrene (B[a]P) and *N*-nitrosodimethylamine (DMN) which are known to be ingested with foods. The mutagenic activation was studied, as BHT and BHA are known to modify the activation of aflatoxin B<sub>1</sub> and B[a]P both in vitro and in vivo experiments (Shelef and Chin, 1980; Salocks et al., 1981; Fukayama and Hsieh, 1984; Kahl, 1984; Williams et al., 1986; Manson et al., 1987). Further, the mechanism by which co-administration of these chemicals affects the activation of the mutagens was studied with respect to the modification of the drug-metabolizing enzymes that are involved in the activation and detoxication of the mutagens.

## 2. Materials and methods.

### 2.1. Chemicals.

BHT, BHA, flavone, flavanone (2,3-dihydroflavone), L-histidine, (+)-biotin, *p*-nitrophenol and glutathione were purchased from Wako (Osaka, Japan). B[a]P, 7-ethoxycoumarin, 7-pentoxoresorufin, resorufin, 7-benzoyloxyresorufin, uridine 5-diphosphoglucuronic acid, 1-chloro-2'-4-dinitrobenzene were obtained from Sigma (St. Louis, MO). Aflatoxin B<sub>1</sub> was obtained from Aldrich (Milwaukee, WI). DMN was from Nacalai Tesque (Kyoto, Japan). Glucose 6-phos-

phate and glucose 6-phosphate dehydrogenase were purchased from Boehringer-Mannheim (Mannheim, Germany). Antibodies against cytochrome P450 isozymes, rat CYP1A2, hamster CYP2A and hamster CYP3A, were prepared in our laboratory. Antibody against rat CYP2B1 was a kind gift from Dr Omata Y. (School of Medicine, University of Kurume, Kurume, Japan). These antibodies recognized the proteins corresponding to the respective cytochrome P450 isozymes in hepatic microsomes in rats. Rabbit peroxidase anti-peroxidase was obtained from Organon Teknika (Durham, NC). *Salmonella typhimurium* TA98, TA100 and TA1535 were kindly donated by Dr Nohmi T. (Division of Genetics and Mutagenesis, Biological Safety Research Centre, National Institute of Health Sciences, Tokyo, Japan).

## 2.2. Animals and treatment.

Male ddY mice (8 week-old) obtained from Nippon SLC (Shizuoka, Japan) were housed in plastic cages on wiremesh at constant temperature ( $24 \pm 2^\circ\text{C}$ ) and humidity ( $55 \pm 20\%$ ) with a 12h light/dark cycle. Mice were divided into 10 groups, each consisting of five animals. The animals were fed for 2 weeks either BHT-, BHA-, flavone-, flavanone-, BHT/flavone-, BHT/flavanone-, BHA/flavone-, BHA/flavanone- or BHT/BHA-containing diets. The chemicals were incorporated into the standard powder diet (F-2, Funabashi, Tokyo, Japan) at a dietary concentration of 0.1% for BHT, flavone and flavanone and 0.2% for BHA. Food and drinking water were provided ad libitum. The amounts of daily food intake were determined and the amounts of the intake of antioxidants and flavonoids were calculated.

## 2.3. Preparation of hepatic cytosols and microsomes

Mice were killed by decapitation and livers were quickly removed, weighed and washed with ice-cold 0.9%-NaCl, then homogenized with 4 volumes of 0.154 M KCl/50 mM Tris/HCl (pH 7.4) containing 1 mM EDTA. Homogenates were cen-

trifuged at  $9,000 \times g$  for 20 min and the supernatants were further centrifuged at  $105,000 \times g$  for 1 h. The resultant supernatants were designated as the cytosolic fraction. The pellets were washed once again and resuspended in the same buffer used for homogenization but with 20%-glycerol and the obtained fraction was designated as microsomal fraction. These preparations were stored at  $-80^\circ\text{C}$  until use.

## 2.4. Assays

Mutagenicity test was carried out principally by a modification of the Ames test (Yahagi et al., 1977). Briefly, hepatic microsomes were incubated at  $37^\circ\text{C}$  for 20 min in the presence of co-factors, a tester microbe and a mutagen (0.5  $\mu\text{g}$  of aflatoxin B<sub>1</sub>, 5  $\mu\text{g}$  of B[a]P or 50  $\mu\text{g}$  of DMN) in a total volume of 0.7 ml. As the tester strain, *Salmonella typhimurium* TA100 was used for aflatoxin B<sub>1</sub>, *Salmonella typhimurium* TA98 for B[a]P and *Salmonella typhimurium* TA1535 for DMN, respectively. Plates were incubated for further 48 h at  $37^\circ\text{C}$  before the counting of the revertants.

Microsomal and cytosolic proteins were quantified by the method of Lowry et al. (1951). Cytochrome P450 and b<sub>5</sub> contents in microsomes were determined according to the method of Omura and Sato (1964). Cytosolic glutathione S-transferase (GST) was assayed using 1-chloro-2'-4-dinitrobenzene as substrate as described by Habig et al. (1974). Microsomal UDP-glucuronyl transferase (UDP-GT) activity was determined by the method of Mulder and Van Doorn (1975) with *p*-nitrophenol as substrate. The activities of aryl hydrocarbon hydroxylase (AHH) (Dehnen et al., 1973), ethoxyresorufin *O*-dealkylase (EROD) (Burke and Mayer, 1974), 7-ethoxycoumarin *O*-deethylase (ECOD) (Greenlee and Poland, 1978), 7-pentoxoresorufin *O*-dealkylase (PROD) (Lubet et al., 1985), 7-benzoyloxyresorufin *O*-dealkylase (BROD) (Burke et al., 1985), erythromycin *N*-demethylase (ERMD) (Wrighton et al., 1985) and *p*-nitrophenol hydroxylase (NPH) (Peinke and Moyer, 1985) were also measured on the microsomal fractions.

Table 1  
Effects of feeding on the body weight gain and weights of body and liver

Treatment	Ingestion of chemicals <sup>a</sup> (mg/kg-body wt./14 day)	Body wt. gain (g/animal/14 days)	Liver wt./body wt. (%)
Control	0	2.8 ± 1.0 <sup>b</sup>	4.8 ± 0.2
BHT	1.8	4.2 ± 1.0	6.1 ± 0.8 <sup>c</sup>
BHA	3.7	2.4 ± 1.2	5.7 ± 0.3 <sup>c</sup>
Flavone	1.7	2.9 ± 0.8	5.8 ± 0.4 <sup>c</sup>
Flavanone	1.8	4.4 ± 0.5	5.4 ± 0.3
BHT/Flavone	1.6/1.6	1.9 ± 0.7	5.6 ± 0.6
BHT/Flavanone	1.8/1.8	3.5 ± 1.1	5.6 ± 0.4 <sup>c</sup>
BHA/Flavone	3.6/1.8	3.4 ± 0.6	5.7 ± 0.2 <sup>c</sup>
BHA/Flavanone	3.9/1.9	3.3 ± 1.6	5.0 ± 0.3
BHT/BHA	1.8/3.6	4.1 ± 1.5	6.0 ± 0.3 <sup>c</sup>

<sup>a</sup> The amounts of ingested chemicals calculated from the food consumption for 14 days of feeding.

<sup>b</sup> Values represent the means ± S.E. of five animals.

<sup>c</sup> Statistical significances of the differences vs. control with  $P < 0.05$ .

### 2.5. Immunoblot analysis

Hepatic microsomes were submitted to SDS-PAGE according to the method of Laemmli (Laemmli, 1970). The microsomes containing 5 µg proteins were subjected to SDS-PAGE using 10%-acrylamide gels. Immunoblotting was done as described by Guengerich et al. (1982). Namely, after SDS-PAGE, dissolved proteins were transferred onto nitrocellulose sheets, which were incubated for 1 h with the antibodies against cytochrome P450 isozymes, then with anti-rabbit IgG for 1 h and finally with rabbit peroxidase anti-peroxidase complex for 1 h. The bands were visualized using peroxidase-diaminobenzidine-tetrahydrochloride. The intensity of the bands was quantified by a densitometer, AE-6920WFNS (Atto, Tokyo, Japan).

### 2.6. Inhibition of the activation of mutagens by antibodies

The inhibition of the microsomal activation of aflatoxin B<sub>1</sub> and B[a]P by the antibodies against cytochrome P450 isozymes was examined by the mutagenicity test. The mutagenicity test was done in the same manner as described above, except that the reaction mixtures consisting of the hepatic microsomes (3 µg protein) from BHT/flavone-treated mice and various amounts of the

antibodies, were preincubated for 20 min at 37°C before the addition of the mutagens.

### 2.7. Statistical analysis

A one way analysis of variance (ANOVA) of the results indicated large differences in variance among the treated groups, so that significances in the differences were evaluated using a parametric ANOVA between the control and the treated groups or between the groups treated with one chemical and those with two chemicals.

## 3. Results

### 3.1. General remarks on the animals

In the animals fed a diet containing BHT, BHA, flavone or flavanone for 2 weeks, there were no overt signs of toxicity except for a slight depression in the body weight gain in the mice fed 0.1% BHT/0.1% flavone-diet (Table 1). Food consumption was not affected by the incorporation of these chemicals into diet. Feeding of the chemicals elevated liver weights in most of the treated groups, except for flavanone-, BHT/flavone- and BHA/flavanone-diet groups. In general, the elevation of liver weight did not differ significantly between the groups fed two chemicals together and the groups fed one chemical separately.

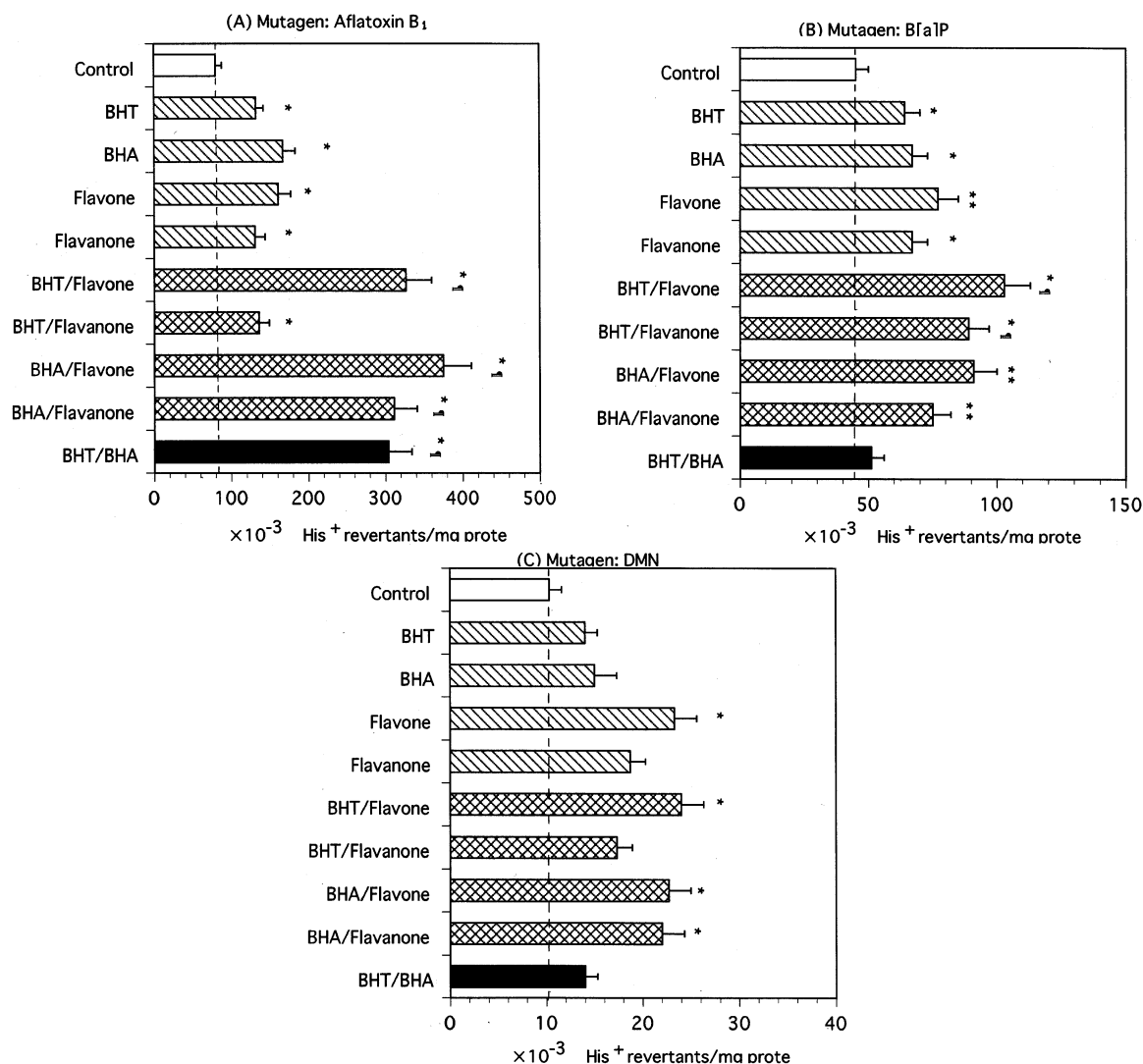


Fig. 1. Effects of co-administration of food additives and flavonoids on the activation of aflatoxin B<sub>1</sub>, B[a]P and DMN: The assay was done in the presence of hepatic microsomes and (A) aflatoxin B<sub>1</sub> (0.5 μg) and *S. typhimurium* TA100, (B) B[a]P (5 μg) and *S. typhimurium* TA98, or (C) DMN (50 μg) and *S. typhimurium* TA1535. Hepatic microsomes used were pooled from five animals of a group. Values represent means ± S.E. for triplicate analyses. Statistical significances of the differences vs. controls are indicated with  $P < 0.05$  (\*) and differences between the co-administration groups and the groups administered with one respective chemical with  $P < 0.05$  (\*).

### 3.2. Effects on the activation of mutagens

Comparison of the ability of hepatic microsomes for the activation of the mutagens is presented in Fig. 1. The values were obtained in the assays in which the linearity between the values of the mutagen activation and protein concentra-

tions was obtained. The effects of the chemicals on the ability to activate mutagens varied among the mutagens tested. A significant elevation of the aflatoxin B<sub>1</sub> activation was obtained in the treated animals compared to the control group. Especially in the animals co-administered with two chemicals, the elevation rate was much higher

Table 2

Effects on cytochrome P450 and b<sub>5</sub> contents and the activities of phase II enzymes in the liver

Treatment	Cytochrome P450 (nmol/mg protein)	Cytochrome b <sub>5</sub> (nmol/mg protein)	GST <sup>a</sup> (μmol/min per mg)	UDP-GT (nmol/min per mg)
Control	1.05 ± 0.06 <sup>b</sup>	0.29 ± 0.03	1.57 ± 0.22	18.8 ± 1.5
BHT	1.35 ± 0.06 <sup>c</sup>	0.32 ± 0.07	1.84 ± 0.27	25.7 ± 4.0 <sup>c</sup>
BHA	1.06 ± 0.04	0.29 ± 0.04	2.33 ± 0.09 <sup>c</sup>	27.7 ± 2.7 <sup>c</sup>
Flavone	1.23 ± 0.04 <sup>c</sup>	0.34 ± 0.02 <sup>c</sup>	2.50 ± 0.30 <sup>c</sup>	29.6 ± 1.7 <sup>c</sup>
Flavanone	0.90 ± 0.09	0.28 ± 0.03	1.92 ± 0.33	27.5 ± 5.8 <sup>c</sup>
BHT/Flavone	1.30 ± 0.12 <sup>c</sup>	0.40 ± 0.04 <sup>c</sup>	2.31 ± 0.09 <sup>c</sup>	28.9 ± 4.2 <sup>c</sup>
BHT/Flavanone	1.35 ± 0.12 <sup>c</sup>	0.31 ± 0.02	2.26 ± 0.27 <sup>c</sup>	27.6 ± 2.7 <sup>c</sup>
BHA/Flavone	1.58 ± 0.09 <sup>c,d</sup>	0.39 ± 0.02 <sup>c</sup>	2.82 ± 0.23 <sup>c</sup>	31.9 ± 3.8 <sup>c</sup>
BHA/Flavanone	1.31 ± 0.09 <sup>c</sup>	0.34 ± 0.01 <sup>c</sup>	2.85 ± 0.42 <sup>c</sup>	31.4 ± 3.8 <sup>c</sup>
BHT/BHA	1.38 ± 0.19 <sup>c</sup>	0.32 ± 0.06	2.29 ± 0.25 <sup>c</sup>	35.5 ± 7.1 <sup>c,d</sup>

<sup>a</sup> GST, glutathione *S*-transferase; UDP-GT, UDP-glucuronyl transferase.<sup>b</sup> Values represent the means ± S.E. of five animals.<sup>c</sup> Statistical significances of the differences vs. control with *P* < 0.05.<sup>d</sup> Statistical significances of the differences between the groups co-administered with two chemicals and those treated with one chemical with *P* < 0.05.

than the sum of elevation degree obtained with administration of one chemical. For example, treatment with BHT or flavone separately elevated the activation by 65% and 101%, respectively, while the co-administration of the two chemicals elevated the activation by 308% over the control value. Similarly, the elevation rate was higher in the co-administration of BHA/flavone, BHA/flavanone and BHT/BHA than that in the administration of one chemical. These suggested that the effects of the co-administration seemed to be synergistic rather than additive. However, statistical significance that indicate synergism of co-administration was not obtained. As for B[a]P (Fig. 1B), the activation of B[a]P was increased in most of the treated groups, but the rate of elevation was mostly equal to the sum of the elevation degree obtained by the administration of one chemical. For example, treatment with BHT or flavone alone elevated the activation by 42% and 71%, respectively, while the co-administration of BHT and flavone elevated by 119%. Similar elevation degree was obtained in the co-administration with BHT/flavanone, BHA/flavone and BHA/flavanone. These suggested that in case of B[a]P, the co-administration resulted in additive effects. As shown in Fig. 1C, the increase in the activation of DMN was obtained in most of the groups

treated with two chemicals but the effects of co-administration were not so marked as compared to those on aflatoxin B<sub>1</sub> and B[a]P.

### 3.3. Effect on the activity of drug-metabolizing enzymes

To elucidate the mechanism by which the elevation of the mutagenic activation occurred by these chemicals, we studied first the changes in the activities of drug-metabolizing enzymes. As shown in Table 2, the contents of cytochrome P450 were increased in most of the treated groups but those of cytochrome b<sub>5</sub> increased only in flavone, BHT/flavone, BHA/flavone and BHA/flavanone-treated groups. As for the phase II enzymes, the activity of GST was significantly increased in most of the treated groups except for BHT- and flavanone-treated groups. UDP-GT activity was also enhanced in all the treated groups, but the increase rate of the activities of these enzymes by co-administration was not additive.

As shown in Table 3, the changes in the activity of enzymes related to specific isozymes of cytochrome P450 varied depending on the enzymes. The activity of AHH specific to CYP1A1 was elevated by feeding with either BHT- or flavone-containing diet except for BHT/BHA group. The

Table 3  
Effects on the activities of phase I drug-metabolizing enzymes in the liver

Treatment	AHH <sup>a</sup> (nmol/min) <sup>b</sup>	EROD (nmol/min)	ECOD (nmol/min)	PROD (pmol/min)	BROD (nmol/min)	NPH (nmol/min)	ERDM (nmol/min)
Control	2.05 ± 0.33 <sup>c</sup>	161 ± 21	55 ± 7	<1 <sup>d</sup>	13 ± 2	0.62 ± 0.14	2.91 ± 0.38
BHT	3.20 ± 0.53 <sup>c</sup>	276 ± 25 <sup>e</sup>	70 ± 9	51 ± 9 <sup>e</sup>	96 ± 18 <sup>e</sup>	1.08 ± 0.20 <sup>e</sup>	4.41 ± 0.78 <sup>e</sup>
BHA	1.72 ± 0.26	147 ± 20	69 ± 4	<1	24 ± 6	0.88 ± 0.18	2.28 ± 0.39
Flavone	3.33 ± 0.27 <sup>e</sup>	145 ± 13	72 ± 8	277 ± 44 <sup>f</sup>	225 ± 48 <sup>f</sup>	0.89 ± 0.27	3.45 ± 0.50
Flavanone	2.07 ± 0.16	124 ± 6	50 ± 3	<1	40 ± 7	0.56 ± 0.08	2.33 ± 0.45
BHT/Flavone	4.80 ± 0.57 <sup>e,f</sup>	153 ± 19	55 ± 4	238 ± 25 <sup>g</sup>	306 ± 21 <sup>e,f</sup>	0.67 ± 0.11	3.77 ± 0.15 <sup>e</sup>
BHT/Flavanone	2.86 ± 0.36 <sup>e</sup>	206 ± 26	80 ± 11 <sup>e</sup>	80 ± 24 <sup>e</sup>	129 ± 19 <sup>e</sup>	0.62 ± 0.25	3.41 ± 0.39
BHA/Flavone	4.81 ± 0.68 <sup>e,f</sup>	383 ± 28 <sup>e,f</sup>	118 ± 12 <sup>e,f</sup>	304 ± 10 <sup>e</sup>	301 ± 24 <sup>e,f</sup>	0.82 ± 0.16	2.28 ± 0.32
BHA/Flavanone	2.33 ± 0.48	159 ± 27	112 ± 16 <sup>e,f</sup>	<1	39 ± 11 <sup>e</sup>	0.70 ± 0.12	2.30 ± 0.30
BHT/BHA	2.44 ± 0.54	(n.d.) <sup>j</sup>	80 ± 11 <sup>e</sup>	80 ± 13 <sup>i</sup>	(n.d.)	(n.d.)	3.63 ± 0.50

<sup>a</sup> AHH, arylhydrocarbon hydroxylase; EROD, ethoxyresorufin *O*-dealkylase; ECOD, 7-ethoxycoumarin *O*-deethylase; PROD, 7-pentoxoresorufin *O*-dealkylase; BROD, 7-benzoyloxyresorufin *O*-dealkylase; NPH, *p*-nitrophenol hydroxylase; ERDM, erythromycin *N*-demethylase.

<sup>b</sup> The enzyme activities are expressed as U/mg protein.

<sup>c</sup> Values represent means ± S.E. of five animals.

<sup>d</sup> Under the levels of detection.

<sup>e</sup> Statistical significances of the differences vs. control with  $P < 0.05$ .

<sup>f</sup> Statistical significances of the differences of the groups co-administered with two chemicals vs. those with one chemical with  $P < 0.05$ .

<sup>j</sup> Not determined.

effect of co-administration was additive in the activity of AHH, since that the increase rate by co-administration was almost equal to the sum of the increase rate of the administration of one chemical. EROD activity specific to CYP1A2 was increased only in BHT- and BHA/flavone-treated groups and the increase rate by co-administration was much higher compared to that by the administration of one chemical in BHA/flavone-treated group. ECOD activity specific to CYP2A and CYP1A2 was induced notably in the co-administration of BHA with other chemicals. Treatment with either BHT or flavone alone or with other chemicals induced significantly the PROD and BROD activities that are related to CYP2B1, while the PROD activity was not detected in the control and in the groups fed either with BHA or flavanone alone. NPH activity did not show any significant changes, except for the increase in the treatment with BHT. ERDM activity specific to CYP3A was enhanced only by BHT- and BHT/flavone treatment.

#### 3.4. Immunoblot analysis of cytochrome P450 isozymes

To know further the induction of specific isozymes of cytochrome P450, we analyzed hepatic microsomes by Western blotting. In Fig. 2 are shown the Western blots with the relative values of the intensity of the bands quantified by a densitometer. The antibodies against CYP1A2 (Fig. 2A) detected a band in the hepatic microsomes from all the groups and the intensity of the bands increased in the animals fed either BHT, BHA or flavanone, while the increase was not so marked in the animals fed flavone. The antibody against CYP2A (Fig. 2B) detected two bands in most of the groups. The upper band increased markedly by feeding the diets containing BHT alone or the diets containing two chemicals, while the lower band was not significantly changed. Two or three bands related to CYP2B1 (Fig. 2C) were detected in most of the groups. The upper band, probably Cyp2b10 as deduced from its molecular weight, was increased markedly by feeding the diets containing either BHT or flavone, namely in BHT-, flavone-, BHT/flavone-, BHT/flavanone-, BHA/flavone- and BHT/BHA-treated groups. The middle and lower

bands, possibly Cyp2b9 and Cyp2b13, varied depending on the treatment but the changes were less marked compared with those of Cyp2b10. The antibody against CYP3A detected weak bands which changed slightly by feeding the chemicals (data not shown).

#### 3.5. Inhibition of the activation of mutagens by antibodies

In order to identify the cytochrome P450 isozymes involved in the activation of the mutagens, we studied the inhibition of the activation

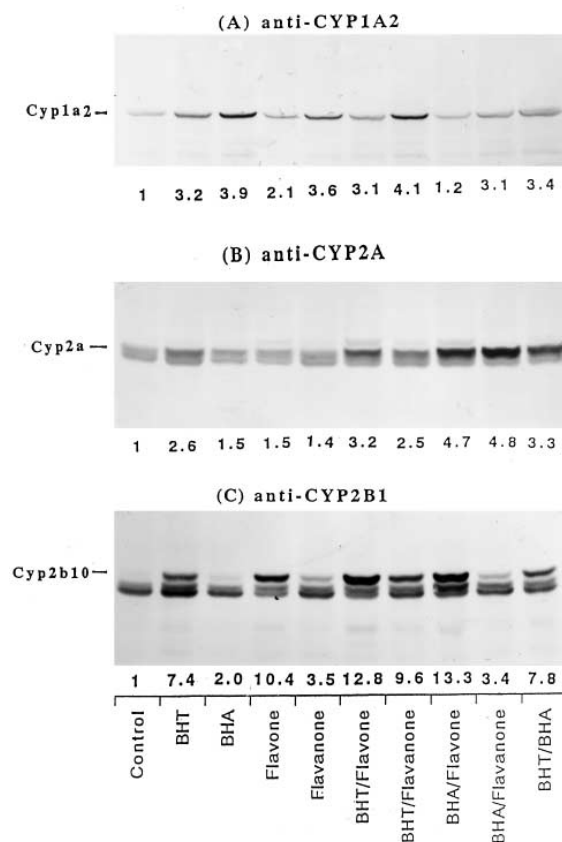


Fig. 2. Western blots of cytochrome P450 isozymes in hepatic microsomes: Hepatic microsomes (5  $\mu$ g of proteins) were subjected to SDS-PAGE and protein bands were detected with the antibodies against (A) CYP1A2, (B) CYP2A and (C) CYP2B1. The relative values of the intensity of the bands quantified by a densitometer are given below each lane (the control = 1).



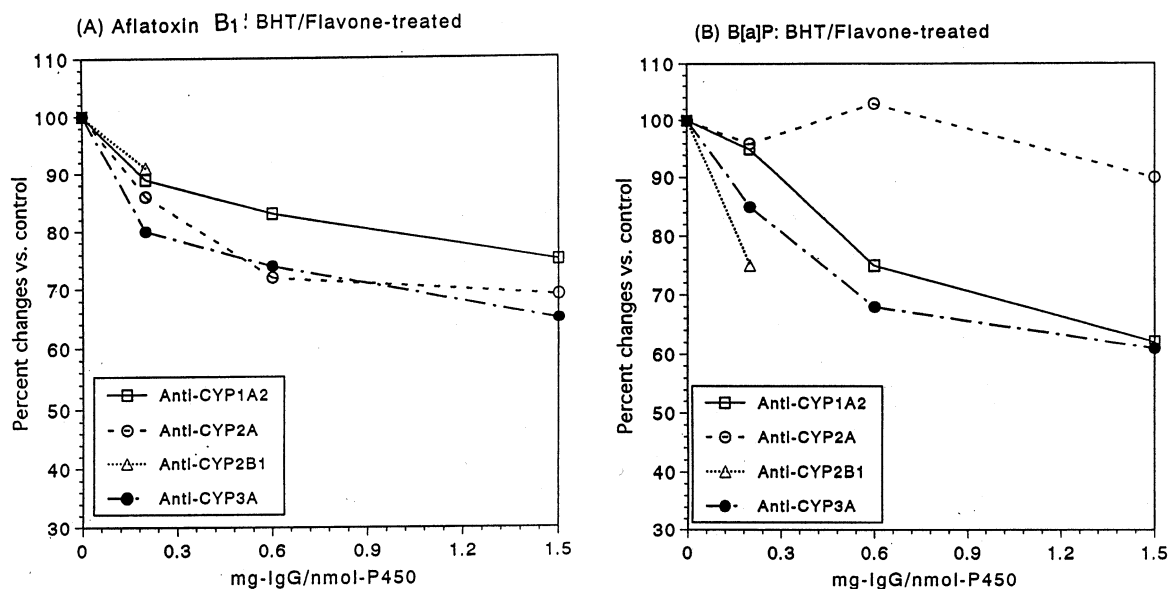


Fig. 3. Inhibition by antibodies of the activation of aflatoxin B<sub>1</sub> and B[a]P by hepatic microsomes from BHT/flavone-treated mice. Hepatic microsomes (3  $\mu$ g protein) were incubated for 20 min with various amounts of antibodies against cytochrome P450 isozymes prior to the mutagenicity assay. The assay was done in the presence of (A) aflatoxin B<sub>1</sub> (0.5  $\mu$ g) and (B) B[a]P (5  $\mu$ g). For the controls, IgG from normal rabbits was used. Values given are the percent inhibition vs. control values with the mean of two determinations. The control values at the concentration of 0.15 mg IgG/nmol P450 was (A) 240 His<sup>+</sup> revertants/plate and (B) 135 His<sup>+</sup> revertants/plate, respectively.

by the antibodies against the isozymes. A representative result is presented in Fig. 3 in which hepatic microsomes from the BHT/flavone-treated animals that showed a marked increase in the activation of aflatoxin B<sub>1</sub> and B[a]P were used. The activation of aflatoxin B<sub>1</sub> by the microsomes (Fig. 3A) was inhibited markedly by anti-CYP2A and anti-CYP3A and in a lesser degree by anti-CYP1A2 and anti-CYP2B1. For the activation of B[a]P (Fig. 3B), anti-CYP2B1 inhibited markedly, and anti-CYP3A and anti-CYP1A2 moderately the activation by the microsomes, while anti-CYP2A had no effects on the activation.

#### 4. Discussion

Although a number of studies have been done on the effects of antioxidants on mutagens and carcinogens (Weisburger et al., 1977; Kahl, 1984; Iverson et al., 1987; Ito and Hirose, 1989), relatively few works have been reported on the co-ad-

ministration of antioxidants with other chemicals found in foods. Brown et al. (1959) studied the effects of co-feeding of BHT or BHA with fats for two years and found expression of toxic effects in feeding with BHT together with lards (20% in diet). Graham and Grice (1955) did not find any toxic effects in the co-administration of BHA with other food additives for 32 weeks. Ito and Hirose (1989) reported that BHA modified the carcinogenicity of a number of carcinogens, and Kahl (1984) indicated that the mechanism for the interference of the chemicals with other factors involved the modulation of cytochrome P450-related enzymes.

The present study demonstrated that co-administration of food additives with naturally occurring food components elevated significantly the activation of aflatoxin B<sub>1</sub> and B[a]P in the mouse liver. The co-administration of antioxidants and flavonoids produced additive effects on the activation of the mutagens. The mechanism by which the chemicals exerted these effects might involve the induction of specific cytochrome P450

isozymes. Aflatoxin B<sub>1</sub> and B[a]P are not mutagenic per se but require to be metabolized to epoxides by drug-metabolizing enzymes to induce genetic damage. Hence, the elevated activation of the mutagens by these chemicals might be mediated by the induction of cytochrome P450 isozymes which are capable to convert aflatoxin B<sub>1</sub> and B[a]P into active metabolites. Induction of the specific isozymes by co-administration was demonstrated by the elevated activities of AHH, EROD, ECOD, PROD and BROD, which are related to cytochrome P450 isozymes, such as CYP1A, CYP2A and CYP2B subfamilies. Further, Western blot analysis demonstrated an increase in the protein levels of the isozymes related to CYP1A2, CYP2A and CYP2B. In addition, the role of these isozymes was demonstrated by the inhibition study which showed reduction of aflatoxin B<sub>1</sub> activation by the antibodies against CYP2A and CYP3A, and reduction of B[a]P activation by the antibodies against CYP1A2, CYP2B1 and CYP3A. In some cases, the degrees of induction of the enzyme activity and inhibition by antibodies were not parallel, which might be partly due to the different catalytic ability of the isozymes towards the mutagens and due to the contribution of constitutive forms of the isozymes which might play a role in the activation of the mutagens.

Co-administration of BHT and BHA has been reported to modify the activities of the phase II drug-metabolizing enzymes, notably GST which is thought to have protective activity against carcinogens (Kahl, 1984; Ito and Hirose, 1989). In the present study, we had no evidences as to the role of the elevated activities of GST, since that the mutagenicity of aflatoxin B<sub>1</sub> and B[a]P was determined in vitro using hepatic microsomes, which excluded the role of the cytosolic GST in the activation of the mutagens. Since that the activities of GST and UDP-GT were increased by co-administration (Table 2), the in vivo effect of co-administration of BHT and BHA with flavonoids on the carcinogenicity of chemicals would be less than that predicted by the in vitro experiments as was done in the present study. Further, the other phase II enzymes that were not studied in the present study would play protective effects against the carcinogenicity of chemicals.

The ADI of BHT and BHA is 0.5 mg/kg body weight as proposed by FAO/WHO Expert Committee on Food Additives. In the present study, the amounts taken by the animals is much more than the ADI of BHT and BHA, but the effects at these doses could not be neglected as species differences exists in the toxicity and in the induction rate of drug-metabolizing enzymes as was observed in case of BHT (Kawano et al., 1980; Sun et al., 1995). Further, according to the regulation, the usage of BHT and BHA for fish conservation is admitted to a maximum of 0.1% (1 g/kg foods) in Japan. On the other hand, flavonoids are found in many plants, such as green tea leaves and grapefruit juice which contain flavonoids at a level of 16% and 10% of the dry weight (Guengerich and Kim, 1990; Obermeier et al., 1995). This suggests that in some cases, it is very likely to take foods containing BHT, BHA and flavonoids at the levels used in the present study. It is also likely that the ingestion of foods containing these chemicals together with other chemicals might produce toxicity, because synergistic or additive effects might occur by the chemicals that would not induce any toxicity when these are administered separately.

The present study demonstrated that co-administration of food additives and naturally occurring food components could exert additive effects on the activation of mutagens and that the toxicity study on the co-administration of chemicals is important when two chemicals modify the drug-metabolizing enzymes. When the chemicals induce the same cytochrome P450 isozymes, co-administration might result in the synergistic or additive effects on the toxicity of chemicals. Further studies should be done to evaluate the risk of co-administration of more than two chemicals which are ingested daily with foods and are capable to modify the activity of drug-metabolizing enzymes.

### **Acknowledgements**

This work was supported by the grants from Japan Health Sciences Foundation and the Ministry of Health and Welfare of Japan. B.S. is an awardee of the fellowship from the Foundation for Public Health Promotion of Japan.

## References

- Bailey, D.G., Arnold, J.M.O., Spence, J.D., 1994. Grapefruit juice and drugs: how significant is the interaction? *Clin. Pharmacokinet.* 26, 91–98.
- Brown, W.D., Johnson, A.R., O'Halloran, M.W., 1959. The effect of the level of dietary fat on the toxicity of phenolic antioxidants. *Aust. J. Exp. Biol. Med. Sci.* 37, 533–548.
- Buening, M.K., Chang, R.L., Huang, M.T., Fortner, J.G., Wood, A.W., Conney, A.H., 1981. Activation and inhibition of benzo[a]pyrene and aflatoxin B<sub>1</sub> metabolism in human liver microsomes by naturally occurring flavonoids. *Cancer Res.* 41, 67–72.
- Buetler, T.M., Gallagher, E.P., Wang, C., Stahl, D.L., Hayes, J.D., Eaton, D.L., 1995. Induction of phase I and phase II drug-metabolizing enzyme, mRNA, protein, and activity by BHA, ethoxyquin, and oltipraz. *Toxicol. Appl. Pharmacol.* 135, 45–57.
- Burke, M.D., Mayer, R.T., 1974. Ethoxyresorufin: direct fluorimetric assay of a microsomal *O*-dealkylation which is preferentially inducible by 3-methylcholanthrene. *Drug Metab. Disp.* 2, 583–588.
- Burke, M.D., Thompson, S., Elcombe, C.R., Halpert, J., Haaparanta, T., Mayer, R.T., 1985. Ethoxy-, pentoxy- and benzyloxyphenoxazones and homologues: a series of substrates to distinguish between different induced cytochromes P-450. *Biochem. Pharmacol.* 34, 3337–3345.
- Cha, Y.N., Bueding, E., 1979. Effect of 2(3)-tert-butyl-4-hydroxyanisole administration on the activities of several hepatic microsomal and cytoplasmic enzymes in mice. *Biochem. Pharmacol.* 28, 1917–1921.
- Dehnen, W., Tomingas, R., Roos, J., 1973. A modified method for the assay of benzo[a]pyrene hydroxylase. *Analyt. Biochem.* 53, 373–383.
- Fuhr, U., Klittich, K., Staib, A.H., 1993. Inhibitory effect of grapefruit juice and the active component, naringenin, on CYP1A2 dependent metabolism of caffeine. *Brit. J. Clin. Pharmacol.* 35, 431–436.
- Fukayama, M.Y., Hsieh, D.P.H., 1984. The effects of butylated hydroxy-toluene on the in vitro metabolism, DNA binding and mutagenicity of aflatoxin B<sub>1</sub> in the rat. *Food Chem. Toxicol.* 22, 355–360.
- Gilbert, D., Golberg, L., 1965. Liver response tests. III. Liver enlargement and stimulation of microsomal processing enzyme activity. *Food Cosmet. Toxicol.* 3, 417–432.
- Graham, W.D., Grice, H.C., 1955. Chronic toxicity of bread additives to rats Part II. *J. Pharmacy Pharmacol.* 7, 126–134.
- Greenlee, R.C., Poland, A., 1978. An improved assay of 7-ethoxycoumarin *O*-deethylation activity; induction of hepatic enzyme activity in C57BL/6J and DBA/2J mice by phenobarbital, 3-methylcholanthrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *J. Pharmacol. Exp. Ther.* 205, 596–605.
- Guengerich, F.P., 1984. Effects of nutritive factors on metabolic processes involving bioactivation and detoxication of chemicals. *Ann. Rev. Nutr.* 4, 207–231.
- Guengerich, F.P., Kim, D.H., 1990. In vitro inhibition of dihydropyridine oxidation and aflatoxin B<sub>1</sub> activation in human liver microsomes by naringenin and other flavonoids. *Carcinogenesis* 11, 2275–2279.
- Guengerich, F.P., Wang, P., Davidson, N.K., 1982. Estimation of isozymes of microsomal cytochrome P-450 in rats, rabbits, and humans using immunochemical staining coupled with sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Biochemistry* 21, 1698–1706.
- Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione *S*-transferase. The first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* 249, 7130–7139.
- Hayes, J.D., Kerr, L.A., Peacock, S.D., Cronshaw, A.D., McLellan, L.I., 1991. Hepatic glutathione *S*-transferases in mice fed on a diet containing the anticarcinogenic antioxidant butylated hydroxyanisole. Isolation of mouse glutathione *S*-transferase heterodimers by gradient elution of the glutathione-Sepharose affinity matrix. *Biochem. J.* 277, 501–512.
- Ito, N., Hirose, M., 1989. Antioxidants-carcinogenic and chemopreventive properties. *Adv. Cancer Res.* 53, 247–302.
- Iverson, F., Campbell, J., Clayton, D., Hierlihy, S., Labossiere, E., Hayward, S., 1987. Effects of antioxidants on aflatoxin-induced hepatic tumors in rats. *Cancer Lett.* 34, 139–144.
- Iwata, N., Suzuki, K., Minegishi, K., Kawanishi, T., Hara, S., Endo, T., Takahashi, A., 1993. Induction of cytochrome P450 1A2 by musk analogues and other inducing agents in rat liver. *Europ. J. Pharmacol.* 248, 243–250.
- Kahl, R., 1984. Synthetic antioxidants: Biochemical actions and interference with radiation, toxic compounds, chemical mutagens and chemical carcinogens. *Toxicology* 33, 185–228.
- Kawano, S., Nakao, T., Hiraga, K., 1980. Species and strain differences in the butylated hydroxytoluene (BHT)-producing induction of hepatic drug oxidation enzymes. *Jp. J. Pharmacol.* 30, 861–870.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.G., 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265–275.
- Lubet, R.A., Mayer, R.T., Cameron, J.W., Nim, R.W., Burke, M.D., Wolf, R.T., Guengerich, F.P., 1985. Dealkylation of pentoxyresorufin: a rapid and sensitive assay for measuring induction of xenobiotics in rat. *Arch. Biochem. Biophys.* 238, 43–48.
- Manson, M.M., Green, J.A., Driver, H.E., 1987. Ethoxyquin alone induces preneoplastic changes in rat kidney whilst preventing induction of such lesions in liver by aflatoxin B<sub>1</sub>. *Carcinogenesis* 8, 723–728.
- Merkel, U., Sigusch, H., Hoffmann, A., 1994. Grapefruit juice inhibits 7-hydroxylation of coumarin in healthy volunteers. *Eur. J. Clin. Pharmacol.* 46, 175–177.

- Mulder, G.J., Van Doorn, A.B.D., 1975. A rapid NAD<sup>+</sup>-linked assay for microsomal uridine diphosphate glucuronyltransferase of rat liver and some observations on substrate specificity of the enzyme. *Biochem. J.* 151, 131–140.
- Obermeier, M.T., White, R.E., Yang, C.S., 1995. Effects of bioflavonoids on hepatic P450 activities. *Xenobiotica* 25, 575–584.
- Omura, T., Sato, R., 1964. The carbon monoxide-binding pigment of liver microsomes. 1. Evidence for its hemo-protein nature. *J. Biol. Chem.* 239, 2370–2378.
- Parke, D.V., Ioannides, C., 1981. The role of nutrition in toxicology. *Ann. Rev. Nutr.* 1, 207–234.
- Peinke, L.A., Moyer, M.J., 1985. *p*-Nitrophenol hydroxylation: a microsomal oxidation which is highly inducible by ethanol. *Drug Metab. Disp.* 13, 548–552.
- Powell, C.J., Connelly, J.C., Jones, S.M., Grasso, P., Bridges, J.W., 1986. Hepatic responses to the administration of high doses of BHT to the rat: Their relevance to hepatocarcinogenicity. *Food Chem. Toxicol.* 24, 1131–1143.
- Salocks, C.B., Hsieh, D.P.H., Byard, J.L., 1981. Butylated hydroxytoluene pretreatment protects against cytotoxicity and reduces covalent binding of aflatoxin B1 in primary hepatocytes cultures. *Toxicol. Appl. Pharmacol.* 59, 331–345.
- Shelef, L.A., Chin, B., 1980. Effect of phenolic antioxidants on the mutagenicity of aflatoxin B1. *Appl. Environ. Microbiol.* 40, 1039–1043.
- Siess, M.H., Bon, A.M.L., Suschetet, M., 1992. Dietary modification of drug-metabolizing enzyme activities: dose-response effect of flavonoids. *J. Toxicol. Environ. Hlth.* 35, 141–152.
- Sun, B., Fukuhara, M., Takanaka, A., 1995. Characterization of benzo[a]pyrene metabolism and related cytochrome P-450 isozymes in Syrian hamster livers. *J. Toxicol. Environ. Hlth.* 46, 47–55.
- Warttenberg, L., 1993. Chemoprevention of carcinogenesis by minor non-nutrients constituents of the diet. In: Parke, D.V., Ioannides, C., Walker, R. (Eds.), *Food, Nutrition and Chemical Toxicity*. Smith-Gordon Nishimura, London, pp. 287–300.
- Wattenberg, L.W., Loub, W.D., Lam, L.K.T., Speier, J.L., 1976. Dietary constituents altering the responses to chemical carcinogens. *Fed. Proc.* 35, 1327–1331.
- Weisburger, E.K., Evarts, R.P., Wenk, M.L., 1977. Inhibitory effect of butylated hydroxytoluene (BHT) on intestinal carcinogenesis in rats by azoxymethane. *Food Cosmet. Toxic.* 15, 139–141.
- Williams, G.M., Tanaka, T., Maeura, Y., 1986. Dose-related inhibition of aflatoxin B1 induced hepatocarcinogenesis by the phenolic antioxidants, butylated hydroxyanisole and butylated hydroxytoluene. *Carcinogenesis* 7, 1043–1050.
- Wrighton, S.A., Maurel, P., Schuetz, E.G., Watkins, P.B., Young, B., Guzelian, P.S., 1985. Identification of the cytochrome P-450 induced by macrolide antibiotics in rat liver as the glucocorticoid responsive cytochrome P-450p. *Biochemistry* 24, 2171–2178.
- Yahagi, T., Nagao, M., Seino, Y., Matsushima, T., Sugimura, T., Okada, M., 1977. Mutagenicities of *N*-nitrosamines on *Salmonella*. *Mutation Res.* 48, 121–130.
- Yang, C.S., Strickhart, F.S., Woo, G.K., 1974. Inhibition of the mono-oxygenase system by butylated hydroxyanisole and butylated hydroxytoluene. *Life Sci.* 15, 1497–1505.