9. G. H. Blekkenhorst, L. Eales and N. R. Pimstone, *Clin. Sci. molec. Med.* **55**, 461 (1978).
10. B. Schoene, R. A. Fleischmann, H. Remmer and H. F. von Oldershausen, *Eur. J. clin. Pharmac.* **4**, 65 (1972).
11. H. Bonkowsky, J. Pomeroy, J. Healey, P. Sinclair and J. Sinclair, *Gastroenterology* **80**, 1328 (1981).
12. H. Bonkowsky, P. Sinclair, J. Bement, J. Healey, J. Sinclair and S. Shedlofsky, *Hepatology* **2**, 732 (1982).
13. J. F. Sinclair, P. R. Sinclair, J. F. Healey, E. L. Smith and H. L. Bonkowsky, *Biochem. J.* **204**, 103 (1982).
14. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
15. H. L. Bonkowsky and J. S. Pomeroy, *Clin. Sci. molec. Med.* **52**, 509 (1977).
16. B. Grandchamp, J. C. Deybach, M. Grelier, H. de Verneuil and Y. Nordmann, *Biochim. biophys. Acta* **629**, 577 (1980).
17. K. E. Anderson, A. P. Alvares, S. Sassa and A. Kappas, *Clin. Pharmac. Ther.* **19**, 47 (1976).
18. B. B. Brodie, J. Axelrod, R. Soberman and B. B. Levy, *J. biol. Chem.* **179**, 25 (1949).
19. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
20. J. E. Falk, *Porphyrins and Metalloporphyrins*, p. 236. Elsevier, Amsterdam (1964).
21. M. Barry and S. Sherlock, *Lancet* **i**, 100 (1971).
22. P. Raj, E. R. Nelson and R. W. Estabrook, *Chem. Biol. Interact.* **3**, 303 (1971).
23. J. F. Sinclair, P. R. Sinclair and H. L. Bonkowsky, *Biochem. biophys. Res. Commun.* **86**, 710 (1979).
24. P. Sinclair, J. Bement, J. Sinclair, H. Bonkowsky, G. Dannan and S. Aust, *Toxicologist* **1**, 71 (1981).
25. D. Mauzerall, *J. Am. chem. Soc.* **84**, 2437 (1962).

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Preventive effect of cysteine on butylated hydroxytoluene-induced pulmonary toxicity in mice

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Butylated hydroxytoluene (BHT)* is widely used as an antioxidant in processed foods and petroleum products. Though generally considered to be safe at the concentration present in foods (the acceptable daily intake of BHT for man is 0.5 mg/kg [1]), high doses of this compound cause pulmonary hypertrophy, hyperplasia and general disorganization of the cellular components in mice [2–5]. The mechanism by which BHT causes such damage has not yet been elucidated, but it has been presumed that such damage is due to the interaction between reactive metabolites and some cellular components.

We have been studying the effect of this compound on cellular macromolecules [6–11]. Previous studies have demonstrated that (a) BHT is converted to highly reactive intermediates by a cytochrome P-450-linked monooxygenase system in the microsomes [6, 7], and (b) some of the activated metabolites, BHT-quinone methide and BHT-alcohol, specifically bind to a sulfhydryl group of cellular protein [9, 11]. In an *in vitro* study [7, 11], we found that cysteine and reduced glutathione significantly decreased the binding of BHT to cellular protein.

Based on previous *in vivo* and *in vitro* results, we investigated whether or not dietary cysteine could prevent pulmonary enlargement induced by BHT *in vivo*.

Methods and Materials

Animals. Four-week-old male mice of the BALB/cAn strain were obtained from Charles River Japan Inc. (Atsugi, Japan). The animals were housed in plastic cages on hard-wood laboratory bedding (Beta Chip, Northeastern Products Corp., Warrensburg, NY). Since Malkinson [12] reported that BHT-produced pulmonary damage was prevented by cedar terpenes derived from shavings used for cage bedding, the hard-wood laboratory bedding was employed in all experiments.

Chemicals. BHT (toluene[*methyl*-^{14}C]) (specific radioactivity, 0.485 μCi/μmole) was purchased from the New England Nuclear Corp. (Boston, MA). The radiochemical purity of the compound was rechecked by thin-layer chromatography and found to be more than 99%. Non-radioactive BHT was obtained from the Wako Pure Chemicals Co. (Osaka, Japan), and all other chemicals used were of the highest obtainable purity.

Experimental protocol. In the initial experiments, mice were fed a commercial stock diet (CLEA CE-2, CLEA Japan Inc., Tokyo) for 3 weeks. They were then fed a diet supplemented with L-cysteine or another L-amino acid, which was added to the CE-2 diet, for 3 days. After the 3 days, each mouse was given a solution of BHT dissolved in corn oil (0.1 ml/20 g body wt) by intraperitoneal (i.p.) injection each day for 4 days. During this time they were maintained on the supplemented diet. The corresponding control animals received an equivalent volume of corn oil. Twenty-four hours after the last BHT dose, mice were decapitated under anesthesia with ether, and the lungs were removed and weighed. Trapped blood was not apparent in the tissues of the decapitated animals. Measurement of lung weight was used in most experiments to monitor whether pulmonary damage had occurred [3, 12].

In a second series of experiments, after feeding a commercial stock diet for 3 weeks, mice were fed a 1% cysteine-supplemented diet for 3 days. Then the animals received 200 mg/kg of BHT containing [^{14}C]BHT dissolved in corn oil (0.1 ml/20 g body wt, approximately 1.5 to 1.6 μCi/animal). Mice were decapitated 6 hr after i.p. injection of [^{14}C]BHT. The lungs and liver were perfused with saline and homogenized with 1.15% KCl in a Polytron homogenizer (Kinematica GmbH, Switzerland). Radioactivity covalently bound to tissue macromolecules was determined by the procedure described previously [6, 8].

Other methods. Radioactivity was measured by a Beckman scintillation spectrometer, model LS-355, and compared with external standard. The scintillation medium used consisted of 2 vol. toluene phospher (4 g PPO and 100 mg dimethyl-POPOP per 1000 ml of toluene) and 1 vol. Triton X-100 [13]. Protein content was measured by the biuret method [14].

Results and Discussion

Figure 1 shows the effect of dietary cysteine on pulmonary enlargement induced by BHT in mice. The relative

* Abbreviations: BHT, 3,5-di-*tert*-butyl-4-hydroxytoluene; BHT-alcohol, 2,6-di-*tert*-butyl-4-hydroxymethylphenol; BHT-quinone methide, 2,6-di-*tert*-butyl-4-methylene-2,5-cyclohexadienone; PPO, 2,5-diphenyloxazole; and POPOP, 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]benzene.

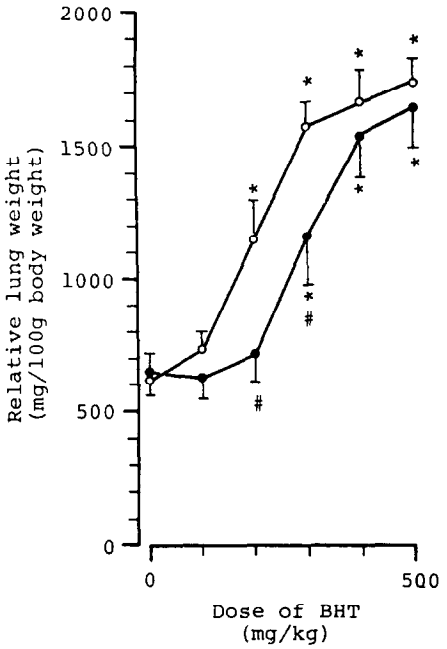

Fig. 1. Effect of cysteine-supplemented diet on pulmonary enlargement induced by BHT in mice. After feeding 1% cysteine-added diet (●) or control diet (○) for 3 days, a solution of BHT (0–500 mg/kg) dissolved in corn oil was given to each mouse by i.p. injection each day for 4 days accompanied by the diets; the corresponding control animals received an equivalent volume of corn oil. Twenty-four hours after the last BHT dose, the lung and body weights were determined. Each value represents the mean ± S.D. from five individual animals. Key: (*) significantly higher than control animals ($P < 0.01$, Student's t-test); and (#) significantly lower than cysteine-untreated animals at each dose of BHT ($P < 0.01$).

weights of lung (lung wt/100 g body wt) increased with increasing doses of BHT. The increase in lung weight induced by BHT at a dose of 200 mg/kg or less was completely prevented by a 1% cysteine-supplemented diet. The mean intake of cysteine calculated from food intake was about 1500 mg/kg/day, in groups of BHT dose of 200 mg/kg or less, and all animals in the groups, regardless of cysteine and/or BHT treatment, gained weight at the same rate during the experimental period. At a dose of 400 mg/kg or greater, however, a complete inhibition of increase in lung weight could not be obtained by 1% cysteine.

Figure 2 shows the dose-relationship for the preventive effect of cysteine on the pulmonary enlargement induced by BHT. The increment of lung weight was inhibited by cysteine in a dose-dependent manner. Complete protection from this dose of BHT (200 mg/kg) required at least 1% cysteine. The results in Figs. 1 and 2 indicate that 1% cysteine-supplemented diet prevented pulmonary enlargement induced by BHT at doses near the threshold (200 mg/kg or less) for toxicity.

In addition, we examined whether BHT-induced pulmonary enlargement could be inhibited by other amino acids or glutathione (Table 1). The amino acids used were chosen on the basis of their structure and chemical properties. All animals, regardless of treatment, gained weight at the same rate during the experimental period. All the other amino acids tested were ineffective. Glutathione at a concentration of 1% significantly depressed the pulmonary enlargement induced by BHT. The prevention by glutathione was slightly less than that of cysteine. This may be due to a difference of absorption into tissues between cysteine and glutathione, since it is known that cysteine enters cells more rapidly and that glutathione is rapidly hydrolyzed in the systemic circulation [15, 16]. Furthermore, this may be due to a difference of amount of free sulfhydryl group between the 1% glutathione- and 1% cysteine-added diets. The data in Table 1 suggest that the preventive effect is due to a free sulfhydryl group.

It has been reported that i.p. injection of BHT to mice produces a pulmonary injury which is followed by extensive hyperplasia and hypertrophy [2–5]. Although the mechanism by which BHT causes the pulmonary damage has not yet been elucidated, several studies have suggested a relationship between the cytochrome P-450-mediated formation of activated BHT metabolites and the damage [5, 12]. To investigate the mechanism by which cysteine prevents the pulmonary damage, [^{14}C]BHT bound to lung macromolecules was determined in an *in vivo* experiment, as shown in Table 2. Since it has been reported that the maximum concentration of BHT is present in the lung between 4 and 8 hr after i.p. injection [4], we determined radioactivity in mice 6 hr after [^{14}C]BHT administration. The content of [^{14}C]BHT-equivalents in the lung of cysteine-pretreated mice was not significantly different than that of untreated mice. However, a significant decrease (about 50% of untreated mice) in the amount of bound [^{14}C]BHT-equivalents to lung macromolecules was found in the cysteine-treated mice. This phenomenon was also found in the liver. However, the amount of bound [^{14}C]BHT-equivalents (per mg protein) in the lung was much greater than that of the liver in cysteine-untreated mice. This may be an important factor in the pulmonary toxicity induced by BHT, since it has been reported that there is a relationship between the amount of bound metabolites and the extent of injury produced by some hepatotoxins [17] as well as pulmonary toxicants [18].

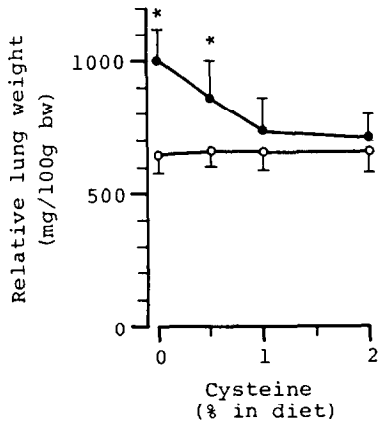

Fig. 2. Effect of cysteine dose on lung weight increase induced by BHT at a dose of 200 mg/kg. After feeding 0 to 2.0% cysteine-added diet for 3 days, a solution of BHT dissolved in corn oil was given to each mouse (●) by i.p. injection each day for 4 days accompanied by the diet; the control (BHT-untreated) animals (○) received an equivalent volume of corn oil. Lung and body weights were determined 24 hr after the last dose of BHT. Each value represents the mean ± S.D. from five individual animals. Key: (*) significantly higher than control mice ($P < 0.01$).

Table 1. Effects of amino acids and glutathione on BHT-induced pulmonary enlargement in mice*

Treatment	Relative lung wt	
	mg/100 g body wt	% of BHT-treated†
Untreated	637 ± 64	55
BHT	1149 ± 52	100†
BHT + alanine	1034 ± 77	90
+ arginine	1163 ± 37	101
+ cysteine	673 ± 90‡	59
+ glutamic acid	1027 ± 221	89
+ methionine	1108 ± 148	96
+ phenylalanine	1149 ± 255	100
+ tryptophan	1481 ± 86	129
+ glutathione	853 ± 75‡	74

* After feeding a diet supplemented with 1% amino acids or glutathione for 3 days, BHT (200 mg/kg) was given by i.p. injection each day for 4 days accompanied by the supplemented diet; corresponding control (untreated) mice received an equivalent volume of corn oil. Lung and body weights were determined 24 hr after the last dose of BHT. Each value represents the mean ± S.D. from five individual animals.

‡ Significantly different from mice that received BHT alone ($P < 0.01$).

Table 2. Effect of cysteine on [^{14}C]BHT binding to lung and liver macromolecules in mice*

Treatment	Body wt (g)	Lung wt (mg)	Liver wt (g)	Bound radioactivity (nmoles/mg protein)		Total radioactivity (nmoles/100 mg tissue)	
				Lung	Liver	Lung	Liver
Untreated	28.9 ± 0.6	172 ± 9	1.49 ± 0.07	0.365 ± 0.051	0.193 ± 0.008	7.78 ± 1.31	12.5 ± 4.0
1% Cysteine	28.9 ± 0.3	180 ± 6	1.47 ± 0.10	0.192 ± 0.059†	0.156 ± 0.007†	6.93 ± 1.68	11.3 ± 4.4
% of Untreated	100	106	99	53	81	89	90

* After feeding 1% cysteine-supplemented diet for 3 days, the animals received 200 mg/kg of BHT containing [^{14}C]BHT dissolved in corn oil (approximately 1.5 to 1.6 μCi/animal. Radioactivity bound to tissue macromolecules was determined 6 hr after i.p. injection. Each value represents the mean ± S.D. from four individual animals.

† Significantly different from untreated mice ($P < 0.01$).

The results in Table 2 indicate that a cysteine-supplemented diet has an inhibitory effect on the covalent binding of reactive BHT metabolites to tissue macromolecules *in vivo*. Our previous studies have demonstrated that the activated metabolites of BHT specifically covalently bind to sulfhydryl groups of protein, cysteine and glutathione [9, 11]. Further, we found that the sulfhydryl compounds remarkably decreased the binding of BHT to cellular protein [7, 9]. This study, therefore, suggests that the activated metabolites of BHT in tissues are trapped by nucleophilic sulfhydryl groups such as those of cysteine or glutathione derived from the supplemented diet. Kehrer and Witschi [5] have reported that the administration of the drug metabolism inhibitors SKF 525-A and piperonyl butoxide completely prevented BHT-induced pulmonary damage in mice. In addition, Malkinson [12] reported that young mice, which have very low levels of cytochrome P-450, are nonresponsive to the pulmonary toxicity of BHT. Consequently, the results presented support the concept that BHT-induced pulmonary damage in mice is due to the interaction between reactive metabolites of BHT and some cellular macromolecules.

In conclusion, we have demonstrated that cysteine-supplemented diet completely prevented pulmonary hypertrophy induced by repeated BHT treatment, at doses near the threshold for toxicity, in mice. Furthermore, the dietary cysteine significantly decreased the amount of BHT bound to lung macromolecules.

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REFERENCES

1. *WHO Food Addit. Ser.* (Eds. Joint FAO/WHO Expert Committee on Food Additives), No. 15, p. 106 (1980).
2. A. A. Marino and J. T. Mitchell, *Proc. Soc. exp. Biol. Med.* **140**, 122 (1972).
3. W. Saheb and H. Witschi, *Toxic. appl. Pharmac.* **33**, 309 (1975).
4. D. Williamson, P. Esterez and H. Witschi, *Toxic. appl. Pharmac.* **43**, 577 (1978).
5. J. P. Kehrer and H. Witschi, *Toxic. appl. Pharmac.* **53**, 333 (1980).
6. Y. Nakagawa, K. Hiraga and T. Suga, *Chem. pharm. Bull., Tokyo* **27**, 442 (1979).
7. Y. Nakagawa, K. Hiraga and T. Suga, *Chem. pharm. Bull., Tokyo* **27**, 480 (1979).
8. Y. Nakagawa, K. Hiraga and T. Suga, *Biochem. Pharmac.* **29**, 1304 (1980).
9. Y. Nakagawa, K. Hiraga and T. Suga, *Biochem. Pharmac.* **30**, 887 (1981).
10. Y. Nakagawa, K. Hiraga and T. Suga, *Biochem. Pharmac.* **30**, 3132 (1981).
11. Y. Nakagawa, K. Hiraga and T. Suga, *Biochem. Pharmac.* **32**, 1417 (1983).
12. A. M. Malkinson, *Toxic. appl. Pharmac.* **49**, 551 (1979).
13. M. S. Patterson and R. C. Green, *Analyt. Chem.* **37**, 854 (1965).
14. A. G. Gornall, C. S. Bardawill and M. M. David, *J. biol. Chem.* **177**, 751 (1949).
15. R. Hahn, A. Wendel and L. Flohe, *Biochim. biophys. Acta* **639**, 324 (1978).
16. N. Takeishi, T. Higashi, A. Naruse, K. Nakashima, H. Shiozaki and Y. Sakamoto, *J. Biochem., Tokyo* **82**, 117 (1977).
17. J. R. Gillette, *Biochem. Pharmac.* **23**, 2785 (1974).
18. M. R. Boyd, *CRC Crit. Rev. Toxic.* **7**, 103 (1980).

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Differential inhibition of human placental monooxygenase activity: evidence for multiple forms of 7-ethoxycoumarin O-deethylase

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Preparations of human placental microsomes or homogenates from maternal smokers have been shown to catalyse the *in vitro* hydroxylation of zoxazolamine [1], benzo(α)pyrene [2] and 2,5-diphenyloxazole [3], the *N*-demethylation of aminoazo dyes [4] and the *O*-deethylation of 7-ethoxycoumarin [5] and 7-ethoxyphenoxazone [6]. Of these activities only ethoxycoumarin *O*-deethylation has been observed to any significant extent in placentas from non-smokers [2, 5], suggesting the possible existence of both a basal (constitutive) and an induced form of deethylase activity in the placenta. Multiple forms of cytochrome P-450 have been found in various animal tissues [7–10] and have been distinguished by their sensitivity to various inhibitors of monooxygenase activity. In this study we investigated qualitative differences between basal and induced human placental ethoxycoumarin *O*-deethylase activities using α-naphthoflavone, metyrapone and dimethylsulphoxide as selective monooxygenase inhibitors. Metyrapone and dimethylsulphoxide are known to inhibit one form of cytochrome P-450, α-naphthoflavone having little or no effect, whilst the reverse situation occurs with a different form of cytochrome P-450. Samples of placental homogenates were obtained from a number of maternal smokers and non-smokers and also from an epileptic receiving phenytoin anticonvulsant therapy throughout her pregnancy.

Materials and Methods

Placentas were obtained from three non-smoking women, three women who smoked between 15 and 30 cigarettes per day during their pregnancy and from a non-smoking epileptic woman who had received phenytoin (350 mg/day) throughout her pregnancy; her plasma concentration of phenytoin at delivery was found to be 14.5 μg/ml. All patients delivered at term after a normal pregnancy and labour. The placentas were immediately stored at −20° after delivery until required for assay when they were thawed out overnight at 4° before being minced using a Bauknecht tissue mincer after first removing the membranes and umbilical cord. A portion of the mince was homogenised (14,500 rpm, 1 min) in ice-cold 50 mM Tris–HCl buffer (pH 7.5) using a Silverson homogeniser to produce a 20% w/v homogenate.

Placental 7-ethoxycoumarin *O*-deethylase activity was measured using a modification of the method of Greenlee and Poland [11]. The enzyme reaction was initiated by adding 1.0 ml of a mixture of NADPH (2.4 mM) and 7-ethoxycoumarin (0.67 mM) in 50 mM Tris–HCl buffer (pH 7.5) to placental homogenate (0.5 ml). After incubation (10 min, 37°) the reaction was stopped by addition of 4.5 ml of chloroform : acetone (9 : 1 v/v). The tubes were vortex-mixed (15 sec), centrifuged (1500 *g*, 5 min) and the lower, solvent layer back-extracted into 1.0 ml of 50 mM Tris buffer (pH 9.8) by vortex mixing (15 sec). After centrifugation (1500 *g*, 5 min) the fluorescence of the upper aqueous layer was determined in an Aminco-Bowman spectrophotofluorometer at uncorrected excitation and emission wavelengths of 368 and 456 nm respectively. The mean recovery of metabolic product (7-hydroxycoumarin) was found to be 50.0 ± 1.9% [12]. The effect of inhibitors upon 7-ethoxycoumarin deethylase activity was determined by adding appropriate amounts of each of the inhibitors to the corresponding assay medium. Dimethylsulphoxide and metyrapone inhibitors were dissolved in 50 mM Tris–HCl buffer (pH 7.5) and α-naphthoflavone was dissolved in methanol. Inhibitor concentrations used were: α-naphthoflavone (0.1–2.0 μm), metyrapone (0.05–1.0 mM) and dimethylsulphoxide (10–200 mM).

Results

The optimum assay conditions required for the determination of 7-ethoxycoumarin deethylase activity in homogenate preparations from induced and non-induced placentas were found to be virtually identical. Incubation of