Biochemical Pharmacology, Vol. 34, No. 12, pp. 2109–2114, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

FORMATION OF A GLUTATHIONE CONJUGATE FROM BUTYLATED HYDROXYTOLUENE BY RAT LIVER MICROSOMES

KAZUO TAJIMA,*† KENJI YAMAMOTO* and TAMIO MIZUTANI‡

*Department of Chemistry, School of Pharmacy, Hokuriku University, Kanazawa 920-11, Japan; and ‡Laboratory of Environmental Health and Toxicology, Department of Food Science, Kyoto Prefectural University, Sakyo-ku, Kyoto 606, Japan

(*Received* 23 *April* 1984; *accepted* 30 *October* 1984)

Abstract—Butylated hydroxytoluene (BHT) was converted to *S*-(3,5-di-*tert*-butyl-4-hydroxybenzyl)-glutathione (BHT-glutathione) by rat liver microsomes in the presence of NADPH, molecular oxygen, and glutathione. NADH was far less effective than NADPH and exhibited little synergistic effect when used together with NADPH. Cytochrome P-450 inhibitors, such as SKF 525-A, α-naphthoflavone, metyrapone, and carbon monoxide, significantly inhibited BHT-glutathione formation. Liver microsomes from phenobarbital-treated rats catalyzed the formation of BHT-glutathione at a rate that was nine times the rate of adduct formation by control microsomes. No stimulation of BHT-glutathione formation was observed with the addition of liver cytosol fraction to the microsomal incubation mixtures even at low glutathione concentrations. These results support the view that BHT is converted by the cytochrome P-450 monooxygenases to a chemically reactive metabolite, possibly BHT-quinone methide, which forms BHT-glutathione by nonenzymatic conjugation with glutathione.

Butylated hydroxytoluene (BHT, 2,6-di-*tert*-butyl-4-methylphenol) is an antioxidant used widely in foods and petroleum products. The metabolism of BHT has been studied extensively, and the predominant metabolic pathway involves the oxidation of the 4-methyl group [1–7]. The major metabolites are 3,5-di-*tert*-butyl-4-hydroxybenzoic acid, both free and as a glucuronide, and *S*-(3,5-di-*tert*-butyl-4-hydroxybenzyl)-*N*-acetylcysteine with minor amounts of BHT-alcohol§ and 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde [2, 4]. We previously identified BHQ, 3,5-di-*tert*-butyl-*p*-benzoquinone, and 2,6-di-*tert*-butyl-4-[(methylthio)methyl]phenol as minor metabolites in the urine of rats [5]. Moreover, BHT-quinone methide, a reactive metabolite, was identified in the liver [6] and bile [7] of rats. Recently, we identified *S*-(3,5-di-*tert*-butyl-4-hydroxybenzyl)glutathione (BHT-glutathione) and BHQ glucuronide as biliary metabolites after administration of BHT to rats [8].

It has been known that BHT causes hemorrhagic death in rats [9] and lung damage in mice [10, 11]. Based on the structure–lung toxicity relationships of BHT analogs [12] and the isotope effects on the metabolism and lung toxicity of BHT by deuteration of the 4-methyl group [13], we previously proposed that BHT-quinone methide may play a role in producing lung damage in mice given BHT. Recently, we demonstrated that the concentration of glutathione in rat liver and in mouse lung decreases to about 50 and 80%, respectively, of control 4–12 hr after i.p. administration of BHT [14]. This depletion of glutathione and the *in vivo* formation of BHT-glutathione [8] suggested that BHT is metabolically activated and the resulting intermediate, possibly BHT-quinone methide, conjugates subsequently with glutathione.

The present study deals with the *in vitro* transformation of BHT to BHT-glutathione during hepatic microsomal metabolism in the presence of glutathione and provides evidence for the involvement of cytochrome P-450 in this metabolic reaction.

MATERIALS AND METHODS

Materials. BHT was obtained from Nakarai Chemicals, Ltd. (Kyoto, Japan). Glutathione was purchased from P-L Biochemicals, Inc. (Milwaukee, WI); NADP, NADH, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were from the Sigma Chemical Co. (St. Louis, MO); NADPH and NAD were from the Yamanouchi Pharmaceutical Co. Ltd. (Tokyo, Japan); and BHT-alcohol, α-naphthoflavone (7,8-benzoflavone), metyrapone [2-methyl-1,2-di-(3-pyridyl)-1-propanone], and DMPO were from the Aldrich Chemical Co. (Milwaukee, WI). SKF 525-A was a gift from Smith Kline & French Laboratories (Philadelphia, PA). BHT-glutathione was synthesized by the method described previously [8]. Other materials used were of reagent grade.

Treatment of animals. Male Wistar rats weighing 160–200 g were used throughout this study. The rats were injected i.p. with phenobarbital (80 mg/kg in

† Address all correspondence to: Kazuo Tajima, School of Pharmacy, Hokuriku University, Kanagawa-machi, Kanazawa 920-11, Japan.

§ Abbreviations: BHT-alcohol, 3,5-di-*tert*-butyl-4-hydroxybenzyl alcohol; BHQ, 3,5-di-*tert*-butylhydroquinone; BHT-quinone methide, 2,6-di-*tert*-butyl-4-methylene-2,5-cyclohexadienone; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; and SKF 525-A, 2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride.

isotonic saline) or 3-methylcholanthrene (20 mg/kg in olive oil) once a day for 3 days; control rats received olive oil.

Preparation of microsomes. The rats were starved overnight before decapitation. The isolated livers were perfused with ice-cold phosphate buffer (0.01 M phosphate buffer containing 0.15 M KCl, pH 7.4) and homogenized in 3 vol. of the same buffer. The homogenate was centrifuged at 10,000 *g* for 20 min. The supernatant fraction was centrifuged at 105,000 *g* for 60 min. After removing floating lipid particles, the soluble supernatant fraction was carefully separated. The microsomal pellets were washed twice and resuspended in 0.1 M phosphate buffer (pH 7.4). Protein was determined by the method of Lowry *et al.* [15] with bovine serum albumin as a standard.

Preparation of cytosol fraction. The liver cytosol fraction obtained from the 105,000 *g* centrifugation was subjected to a Sephadex G-25 (Pharmacia, Sweden) column (3.2 × 47 cm) equilibrated with 0.05 M phosphate buffer, pH 7.4, at 4°. The protein band eluting from the column was collected. Glutathione in the cytosol fraction was determined by the method of Ellman [16], and the cytosol fraction free of glutathione was prepared. The denatured cytosol fraction was obtained by immersion of the native cytosol fraction in a boiling water bath for 10 min. The activity of cytosolic glutathione-*S*-transferases was assayed according to the method of Habig *et al.* [17], using 1-chloro-2,4-dinitrobenzene as the substrate, and was 2.21 μmoles/mg protein/min. The protein content of the cytosol fraction was measured as described above.

Incubation conditions. The incubation mixtures contained the following in a final volume of 2 ml: 5 mM glutathione, 2.5 mM NADP, 25 mM glucose-6-phosphate, 5 units glucose-6-phosphate dehydrogenase, 5 mM $MgCl_2$, 50 mM phosphate buffer, 2 mg microsomal protein, and native or denatured cytosol fraction (6 mg protein). For studies on the cofactor requirements, the NADPH-generating system was replaced by NADPH, NADH, NADP, or NAD (2.5 mM each). The reactions were initiated by the addition of BHT or BHT-alcohol (2.5 μmoles in acetone, 20 μl) and performed at 37°. For the termination of the reaction, the vessel was chilled and immediately shaken twice with 3 vol. of ice-cold ether to remove the unchanged substrate and the ether-soluble metabolites. After the separation of the ether layer by centrifugation for 5 min, nitrogen gas was bubbled through the aqueous layer to remove dissolved ether. Methanol (4.0 ml) was added to the aqueous layer, and the mixture was sonicated and centrifuged to remove precipitated protein. The methanol fraction was evaporated *in vacuo*. The residues were dissolved in 20% methanol, and BHT-glutathione formed was analyzed by high performance liquid chromatography (HPLC).

For the detection of BHT-quinone methide, the microsomal incubation of BHT described above was carried out on a preparative scale without glutathione. After incubation for 30 min, the incubation mixtures were extracted with 2 vol. of hexane. The hexane layer was evaporated *in vacuo* under nitrogen gas. The residues were analyzed by gas chromatography-mass spectrometry as described previously [7].

HPLC analysis. HPLC was carried out on a Hitachi model 638-30 liquid chromatograph equipped with a u.v. spectrophotometer (280 nm). Each sample was injected onto a NUCLEOSIL 10 C_{18} column (30 cm × 4.0 mm) with a flow rate of 1.0 ml/min. The mobile phase consisted of 60% methanol containing 0.1 M $NH_4H_2PO_4$. The amount of BHT-glutathione formed was determined from a standard curve of peak area for the authentic sample.

RESULTS

Formation of BHT-glutathione. BHT was incubated with rat liver microsomes in the presence of an NADPH-generating system and glutathione. BHT-glutathione formed in the incubation mixtures was

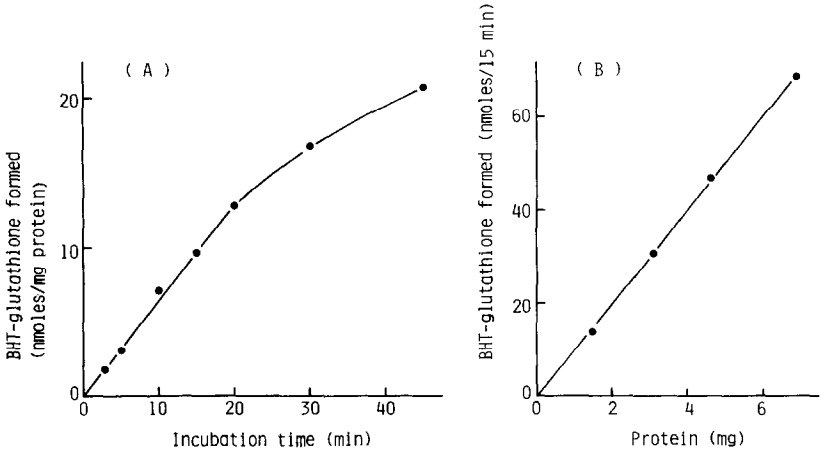

Fig. 1. Effects of incubation time and microsomal protein concentration on the formation of BHT-glutathione from BHT by rat liver microsomes. BHT (2.5 μmoles) was incubated with 2 mg of microsomal protein for the time indicated (A) and with the protein concentration indicated (B) for 15 min. Each point represents the mean of at least three determinations. Incubation conditions are described in Materials and Methods.

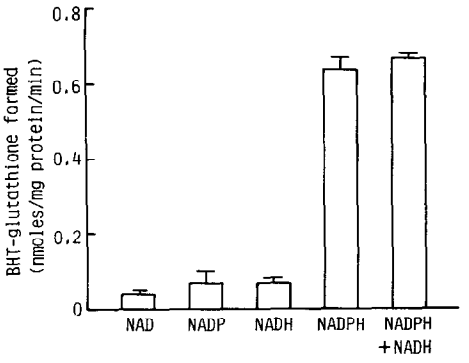

Fig. 2. Cofactor requirements for BHT-glutathione formation by rat liver microsomes. Incubation conditions are given in Materials and Methods. Each bar represents the means ± S.E. of three determinations.

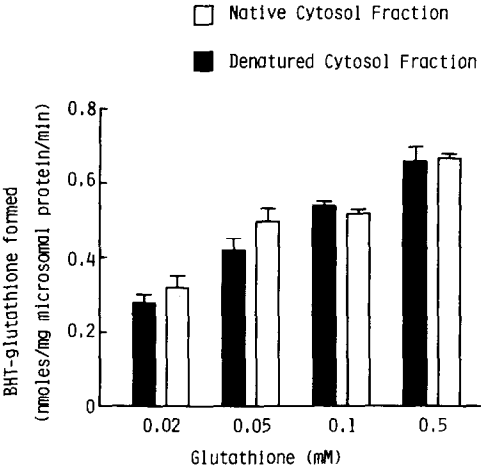

Fig. 3. Effect of cytosol fraction on BHT-glutathione formation. Incubation mixtures contained rat liver microsomes (2 mg protein), BHT (1 mM), an NADPH-generating system, glutathione (0.02, 0.05, 0.1, and 0.5 mM), and native or denatured cytosol fraction (6 mg protein). Other conditions are described in Materials and Methods. Each bar represents the means ± S.E. of three determinations.

isolated and identified by TLC and HPLC comparisons with the authentic sample as described in the previous paper [8].

The data in Fig. 1 indicate that the formation of BHT-glutathione was linear with time for 20 min and with microsomal protein concentrations up to 3.45 mg/ml. The maximum rate of this reaction was observed in the pH range from 7.4 to 8.0. On the basis of these results, incubations were performed for 15 min, at 37° and pH 7.4 with 2 mg of microsomal protein.

The microsomal incubation of BHT-alcohol as a substrate in the presence of glutathione produced no BHT-glutathione.

Various conditions of BHT-glutathione formation. As shown in Fig. 2, the formation of BHT-glutathione from BHT required NADPH. NADH was far less effective than NADPH and exhibited little synergistic effect on the formation of BHT-glutathione when used together with NADPH. When glutathione or microsomes were omitted from the incubation medium, almost no BHT-glutathione was produced (Table 1). Similarly, when the incubation was carried out with denatured microsomes (treated at 70–80° for 10 min) or with cytosol fraction instead of microsomes, no BHT-glutathione was produced. The dependence of BHT-glutathione formation on glutathione concentration is shown in Fig. 3. The rate of BHT-glutathione formation increased with the increasing concentrations of glutathione in the incubation medium containing either native or denatured cytosol fraction. Figure 3 also shows that the addition of native cytosol fraction did not increase significantly the rate of BHT-glutathione formation at any glutathione concentrations tested, when compared to the addition of denatured cytosol fraction.

Effects of various inhibitors on BHT-glutathione formation. The formation of BHT-glutathione from BHT required molecular oxygen, and it was inhibited markedly by carbon monoxide (Table 2). The addition of typical cytochrome P-450 inhibitors such as SKF 525-A, α-naphthoflavone, and metyrapone [18, 19] markedly inhibited the formation of BHT-

Table 1. *In vitro* formation of BHT-glutathione from BHT by rat liver microsomes*

Incubation conditions	BHT-glutathione formed (nmoles/mg microsomal protein/min)
Complete system	0.65 ± 0.01
Minus glutathione	0.03 ± 0.01
Minus NADPH-generating system	0.01 ± 0.01
Minus microsomes	ND†
Minus microsomes plus cytosol	0.01 ± 0.01‡
Denatured microsomes§	ND

* Incubation was carried out with 2.5 μmoles BHT, 5 mM glutathione, 2 mg microsomal protein, 5 mM $MgCl_2$, and an NADPH-generating system (2.5 mM NADP, 25 mM glucose-6-phosphate, 5 units glucose-6-phosphate dehydrogenase) in phosphate buffer (pH 7.4) at 37° for 15 min. Values are the means ± S.E. of at least three determinations.

† Not detectable.

‡ Expressed in nmoles/mg cytosolic protein/min.

§ Microsomes were treated at 70–80° for 10 min.

Table 2. Effects of various inhibitors on BHT-glutathione formation from BHT by rat liver microsomes*

Inhibitor	Concentration	BHT-glutathione formed† (nmoles/mg protein/min)	% of Control activity
None		0.65 ± 0.03	100
SKF 525-A	0.5 mM	0.21 ± 0.01	32
α-Naphthoflavone	0.5 mM	0.28 ± 0.02	43
Metyrapone	1.0 mM	0.32 ± 0.02	49
KCN	1.0 mM	0.55 ± 0.01	85
NaN_3	1.0 mM	0.59 ± 0.03	91
Nitrogen‡	100%	0.06 ± 0.01	9
Carbon monoxide‡	$CO/O_2 = 4$	0.19 ± 0.01	29
DMPO	75 mM	0.49 ± 0.03	75
Ascorbic acid	5.0 mM	0.50 ± 0.02	77

* The incubation mixture contained 2 mg of microsomal protein and each concentration of inhibitor in a total volume of 2 ml. Other conditions are the same as described in Table 1.

† Values are the means ± S.E. of at least three determinations.

‡ Gas was bubbled through the mixture for 1–5 min prior to the start of incubation in a sealed vessel.

glutathione. Both DMPO and ascorbic acid, free radical traps, slightly decreased the rate of BHT-glutathione formation. On the other hand, KCN and NaN_3 had little effect on the formation of BHT-glutathione.

Effects of in vivo *treatment with inducers on BHT-glutathione formation.* The effects of pretreatment of rats with enzyme inducers on BHT-glutathione formation are shown in Table 3. Phenobarbital-treated rat liver microsomes catalyzed the formation of BHT-glutathione at nine times the rate of control microsomes while microsomes from 3-methylcholanthrene-pretreated animals formed BHT-glutathione at a rate that was similar to control microsomes.

Formation of BHT-quinone methide. Gas chromatographic and mass spectral analyses of the metabolites produced by microsomal incubation of BHT without glutathione were carried out by essentially the same method as described previously [7]. A peak found near that of BHT on the gas chromatogram of incubation extracts showed a molecular ion (M^+) at *m/e* 218 with fragment ions at *m/e* 203 ($M^+ - CH_3$), 175 ($M^+ - CH_3 - C_2H_4$), and 161 ($M^+ - C_4H_9$). These data were consistent with the mass fragment pattern of authentic BHT-quinone methide. This result demonstrated the occurrence of BHT-quinone methide in the incubation mixture. The amount of BHT-quinone methide formed could not be determined quantitatively, because the recovery of BHT-quinone methide from the incubation mixtures was low and variable. This is probably due to the accompanying formation of dimeric products of BHT-quinone methide in the course of isolation and concentration process [7]. When the incubation was carried out in the presence of glutathione, no BHT-quinone methide was detected.

Table 3. Effects of pretreatment with phenobarbital and 3-methylcholanthrene on BHT-glutathione formation from BHT by rat liver microsomes*

Treatment	BHT-glutathione formed (nmoles/mg protein/min)
Olive oil	0.62 ± 0.02
Phenobarbital	5.58 ± 0.21
3-Methylcholanthrene	0.66 ± 0.01

* Microsomes were prepared from rats treated as described in Materials and Methods. Values are the means ± S.E. of three determinations.

DISCUSSION

Glutathione conjugation of chemically reactive metabolites arising from the cytochrome P-450 mediated metabolism of a number of inert chemicals has been shown to be an important detoxication pathway in mammalian species [20]. The present study has demonstrated that BHT is metabolized to BHT-glutathione by rat liver microsomes in the presence of an NADPH-generating system, molecular oxygen, and glutathione as summarized in Fig. 4. The formation of BHT-glutathione was inhibited markedly by cytochrome P-450 inhibitors such as SKF 525-A, α-naphthoflavone, metyrapone, and carbon monoxide (Table 2). These results suggest that a cytochrome P-450 monooxygenase system mediates the formation of BHT-glutathione from BHT. This interpretation was substantiated by the results obtained by employing differential inducers of the oxidative pathway; that is, microsomes from rats treated with phenobarbital formed BHT-glutathione nine times more rapidly than did normal microsomes, whereas pretreatment with 3-methylcholanthrene had little effect on BHT-glutathione formation (Table 3).

In the present study, the occurrence of BHT-quinone methide during the microsomal metabolism of BHT was confirmed when the incubation was carried out in the absence of glutathione, whereas the incubation in the presence of glutathione produced BHT-glutathione but not BHT-quinone methide. This supports the suggestion that BHT-quinone methide is a metabolic intermediate responsible for BHT-glutathione formation [8].

Previous studies showed that radiolabel from

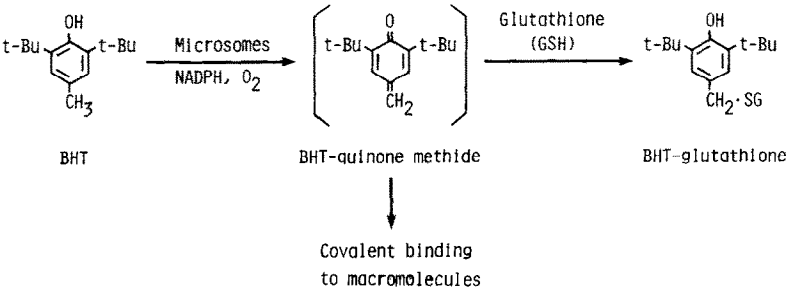

Fig. 4. Proposed pathways of BHT metabolism *in vitro*.

carbon 14 labeled BHT becomes covalently bound to liver, lung, and kidney macromolecules in both mice [21] and rats [21, 22] following its *in vivo* administration. Furthermore, Nakagawa *et al.* [23] reported the covalent binding of BHT metabolites to rat lung and liver microsomes *in vitro*. This covalent binding is apparently mediated by a cytochrome P-450-linked monooxygenase system, and it is prevented by the addition of thiol compounds such as glutathione and cysteine [23]. As mentioned above, the present study has demonstrated that the formation of BHT-glutathione from BHT in the presence of glutathione was also mediated by a cytochrome P-450 monooxygenase system. In view of this result and the observations presented by Nakagawa *et al.* [23], it is most likely that the same intermediate of BHT metabolism that is responsible for BHT-glutathione formation, BHT-quinone methide, is involved also in the covalent binding of BHT to macromolecules (Fig. 4).

There is a possibility that BHT-quinone methide is produced via the phenoxy radical of BHT [24]. To study whether such a radical is responsible for BHT-quinone methide formation and hence for BHT-glutathione formation, DMPO and ascorbic acid, which have been shown to trap hydroxy radicals and superoxide radicals [25–28], were added to the microsomal incubation mixtures. Although both agents inhibited the formation of BHT-glutathione, the magnitudes of the inhibition were relatively small (Table 2). However, this result does not necessarily rule out the possible involvement of the phenoxy radical as an intermediate, because the poor inhibitor effects of radical-trapping reagents could result if the phenoxy radical of BHT, a sterically hindered radical, failed to react well with these radical traps.

The glutathione *S*-transferases, a group of enzymes present mainly in the soluble fractions of homogenates of tissues such as liver, kidney, and lung, catalyze the conjugation of glutathione with a variety of electrophilic substrates [29]. If the cytosolic enzymes act as catalyzers in the reaction between BHT-quinone methide and glutathione, the stimulatory effect should be apparent when the liver supernatant fraction is added to the microsomal incubation medium at low glutathione concentrations. However, the cytosol fraction appeared to have no effect on the rate of BHT-glutathione formation even at low glutathione concentrations (Fig. 3). This result suggests that the conjugation of glutathione with the reactive metabolite of BHT proceeds nonenzymatically. Buckpitt and Boyd [30] also reported that cytosolic preparations from homogenates of rat lung and liver do not enhance the formation of the glutathione conjugates of 4-ipomeanol and suggested that glutathione-*S*-transferase activities do not play a major role in glutathione conjugate formation from reactive 4-ipomeanol metabolites.

When BHT-alcohol, an NADPH-generating system, and glutathione were incubated with rat liver microsomes, no BHT-glutathione was detected in the incubation mixtures. This finding suggested that microsomal enzymes do not mediate the formation of BHT-quinone methide from BHT-alcohol. On the other hand, our previous studies showed that BHT-quinone methide [7] and BHT-glutathione (unpublished data) were excreted in rat bile after i.p. administration of BHT-alcohol as well as BHT. At present, the reason for this discrepancy between the *in vitro* and the *in vivo* results is not clear.

REFERENCES

1. A. S. Wright, D. A. A. Akintonwa, R. S. Crowne and D. E. Hathway, *Biochem. J.* **97**, 303 (1965).
2. J. W. Daniel, J. C. Gage and D. I. Jones, *Biochem. J.* **106**, 783 (1968).
3. G. M. Holder, A. J. Ryan, T. R. Watson and L. I. Wiebe, *J. Pharm. Pharmac.* **22**, 832 (1970).
4. A. L. Branen, *J. Am. Oil Chem. Soc.* **52**, 59 (1975).
5. K. Yamamoto, K. Tajima and T. Mizutani, *J. Pharmacobio-Dynamics* **2**, 164 (1979).
6. O. Takahashi and K. Hiraga, *Fd Cosmet. Toxic.* **17**, 451 (1979).
7. K. Tajima, K. Yamamoto and T. Mizutani, *Chem. pharm. Bull., Tokyo* **29**, 3738 (1981).
8. K. Tajima, K. Yamamoto and T. Mizutani, *Chem. pharm. Bull., Tokyo* **31**, 3671 (1983).
9. O. Takahashi and K. Hiraga, *Toxic. appl. Pharmac.* **43**, 399 (1978).
10. A. A. Marino and J. T. Mitchell, *Proc. Soc. exp. Biol. Med.* **140**, 122 (1972).
11. W. M. Haschek and H. Witschi, *Toxic. appl. Pharmac.* **51**, 475 (1979).
12. T. Mizutani, I. Ishida, K. Yamamoto and K. Tajima, *Toxic. appl. Pharmac.* **62**, 273 (1982).
13. T. Mizutani, K. Yamamoto and K. Tajima, *Toxic. appl. Pharmac.* **69**, 283 (1983).
14. K. Tajima, K. Yamamoto and T. Mizutani, *J. Pharmacobio-Dynamics* **7**, s-89 (1984).
15. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **192**, 265 (1951).
16. G. L. Ellman, *Archs Biochem. Biophys.* **82**, 70 (1959).
17. W. H. Habig, M. J. Pabst and W. B. Jakoby, *J. biol. Chem.* **249**, 7130 (1974).

18. F. M. Goujon, D. W. Nebert and J. E. Gielen, *Molec. Pharmac.* **8**, 667 (1972).
19. R. Kato, E. Chiesara and P. Vassanelli, *Biochem. Pharmac.* **13**, 69 (1964).
20. L. F. Chasseaud, *Adv. Cancer Res.* **29**, 175 (1979).
21. J. P. Kehrer and H. Witschi, *Toxic. appl. Pharmac.* **53**, 333 (1980).
22. Y. Nakagawa, K. Hiraga and T. Suga, *Chem. pharm. Bull., Tokyo* **27**, 442 (1979).
23. Y. Nakagawa, K. Hiraga and T. Suga, *Chem. pharm. Bull., Tokyo* **27**, 480 (1979).
24. H. U. Wagner and R. Gompper, in *The Chemistry of Quinonoid Compounds* (Ed. S. Patai), p. 1145. John Wiley, New York (1974).
25. E. Finkelstein, G. M. Rosen and E. J. Rauckman, *Archs Biochem. Biophys.* **200**, 1 (1980).
26. S. D. Nelson, D. C. Dahlin, E. J. Rauckman and G. M. Rosen, *Molec. Pharmac.* **20**, 195 (1981).
27. G. J. Mulder, L. E. Unruh, F. E. Evans, B. Ketterer and F. E. Kadlubar, *Chem. Biol. Interact.* **39**, 111 (1982).
28. T. Sawahata and R. A. Neal, *Molec. Pharmac.* **23**, 453 (1983).
29. D. M. Jerina and J. R. Bend, in *Biological Reactive Intermediates* (Eds. D. J. Jollow, J. J. Kocsis, R. Snyder and H. Vainio), p. 207. Plenum Press, New York (1977).
30. A. R. Buckpitt and M. R. Boyd, *J. Pharmac. exp. Ther.* **215**, 97 (1980).