*Biochemical Pharmacology*, Vol. 36, No. 4, pp. 441–446, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00
Pergamon Journals Ltd.

# HEPATOTOXICITY OF PRECOCENE I IN RATS

## ROLE OF METABOLIC ACTIVATION *IN VIVO*

VIJAYALAKSHMI RAVINDRANATH, MICHAEL R. BOYD and DONALD M. JERINA*†

Laboratory of Experimental Therapeutics and Metabolism, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892; and * Section on Oxidation Mechanisms, Laboratory of Bioorganic Chemistry, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, U.S.A.

(*Received* 29 *July* 1985; *accepted* 30 *June* 1986)

**Abstract**—The mechanism of the hepatotoxicity of precocene I has been investigated in male, Sprague–Dawley rats. Administration of a single dose of precocene I caused a large depletion of liver glutathione (GSH) levels that was both time and dose dependent. Concomitant with the decrease of liver GSH, there was an increase in serum glutamic pyruvic transaminase (GPT) levels which was also time and dose dependent. Administration of a single dose of [4-$^3$H]precocene I resulted in extensive covalent binding of the radiolabel to liver proteins and DNA in the liver; the extent of binding increased with increasing dose. Treatment of the rats with the mixed-function oxidase inhibitor piperonyl butoxide, before administration of precocene I, significantly decreased the proportion of the radiolabel bound covalently to proteins and DNA, although the total radioactivity (bound and unbound) in the liver remained the same. Piperonyl butoxide pretreatment limited both the liver GSH depletion and the hepatic necrosis normally caused by precocene I. These results are consistent with the view that the hepatotoxicity of precocene I is due to reactive metabolites formed through cytochrome P-450 mediated metabolism of precocene I.

Precocene I (7-methoxy-2,2-dimethyl-2*H*-benzo[*b*]pyran) and precocene II (6,7-dimethoxy-2,2-dimethyl-2*H*-benzo[*b*]pyran) are naturally occurring insect growth regulators isolated from the plant *Ageratum houstonianum*. They inhibit the production of juvenile hormone by direct action on the corpora allata of various insect species [1]. The corpora allata is an important endocrine gland in insects and is necessary for the production of juvenile hormone, which is essential for the normal metamorphosis and certain reproductive processes of insects. Precocene I and precocene II, by their actions on the corpora allata, cause precocious metamorphosis of larvae to sterile adults [1] and, hence, have been considered for use as fourth generation insecticides. Indirect evidence has shown that precocene I and precocene II may be metabolically activated to highly reactive epoxides *in situ* in the corpora allata itself [2, 3], analogous to the mammalian bioactivation of polycyclic aromatic hydrocarbons to reactive epoxides that alkylate cellular macromolecules [4]. Precocene I is metabolized *in vitro* by rat liver microsomes to *cis* and *trans* 3,4-diols, suggesting the initial formation of a reactive epoxide [5]. Halpin *et al.* also observed that a single dose of 300 mg/kg body weight of precocene I causes hepatic centrilobular necrosis in rats. In view of the potential use of precocene I as an insecticide, study of the *in vivo* metabolism of precocene I in the rat was undertaken. The present study describes the *in vivo* bioactivation of precocene I to reactive metabolite(s) that binds covalently to tissue macromolecules. The generation and subsequent covalent binding of the reactive metabolite(s) correlated with the observed tissue damage.

† All correspondence should be sent to: Dr. D. M. Jerina, Bldg. 8, Rm. 1A-11, National Institutes of Health, Bethesda, MD 20892.

### MATERIALS AND METHODS

*Animals*. Male, Sprague–Dawley rats weighing 150–200 g were obtained from Charles River Laboratories (Kingston, NY). Animals were given a laboratory diet *ad lib*. and were not fasted prior to use.

*Radiochemical*. [4-$^3$H]Precocene I was synthesized as described [5]. Radiochromatographic purity as determined by high performance liquid chromatography was greater than 97%. [4-$^3$H]Precocene I was diluted with unlabeled precocene I (Aldrich Chemical Co., Milwaukee, WI) to the specific activity of 0.1 mCi/mmole. Precocene I solutions were prepared in sesame oil such that 0.5 ml/100 g body weight was administered i.p. to yield the desired dose.

Rats were administered various doses of precocene I ranging from 200 to 600 mg/kg body weight, i.p. Eight hours after administration of precocene I, a sample of blood (400 $\mu$l) was taken from the retro-orbital sinus, and the serum was separated and analyzed for glutamic pyruvic transaminase (GPT) activity [6]. Rats were killed by cervical dislocation, and a sample of tissue was removed from the main lobe of the liver and frozen immediately in liquid nitrogen. Total liver glutathione (GSH) levels were determined by the method described by Akerboom and Sies [7].

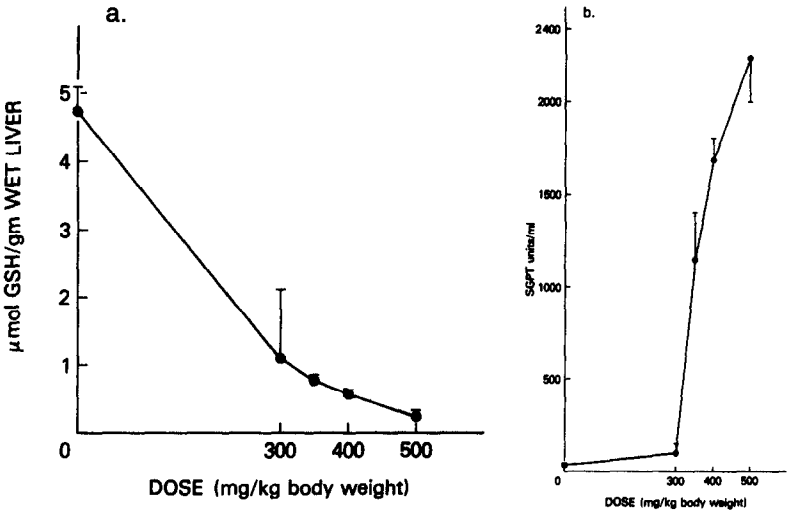


Fig. 1. (a) Depletion of liver GSH, and (b) serum GPT activity after a single i.p. administration of precocene I. Rats were killed 8 hr after dosage. Values are represented as mean ± SD (N = 4).

For the time–course study, 400 mg/kg body weight of precocene I was administered to rats, i.p., and groups of four rats were killed at 0.5, 1, 2, 4, 6, 8 and 12 hr after dosage. Liver GSH levels and serum GPT activity were assayed as above.

Covalent binding studies were conducted with two sets of rats; one set received [4-$^3$H]precocene I alone and the other set of rats was treated with piperonyl butoxide 1 hr prior to administration of precocene I. Piperonyl butoxide was administered neat, at a dose of 1600 mg/kg body weight, s.c. For the dose–response study, two sets of control and piperonyl butoxide-treated rats were given 200–600 mg/kg body weight of [4-$^3$H]precocene I, i.p., and groups of four rats were killed 4 hr later by cervical dislocation. Before sacrifice, a sample of blood (400 μl) was collected by retro-orbital puncture and analyzed for serum GPT levels, after separation of serum [6]. Two samples of the main lobe of the liver were taken and frozen separately in liquid nitrogen. Samples were stored at −70° prior to analysis. One sample was analyzed for liver GSH levels [7], and the other was used to determine the covalent binding of the label derived from [4-$^3$H]precocene I to proteins and DNA as described [8]. For the time–course study, the control and piperonyl butoxide pretreated groups were administered 400 mg/kg body weight of [4-$^3$H]precocene I, i.p., and were killed in groups of four rats at 1, 2, 4 and 8 hr after dosage. Blood and tissues were collected and processed as described for the dose–response study.

*Statistics.* Mean values for each treatment group were compared to the appropriate controls with the multiple $t$ statistic of Dunnett [9]. The respective mean values were considered statistically different from control when $P \leq 0.01$.

## RESULTS

Administration of a single i.p. dose of precocene I to rats led to considerable depletion of liver GSH levels, which was dose dependent (Fig. 1a). After a single dose of 350 mg/kg body weight of precocene I, nearly 85% of the liver GSH was depleted within 8 hr. Nearly 95% of the liver GSH was depleted in 8 hr after a single dose of 500 mg/kg of precocene I.

Concomitant with the decreasing levels of liver GSH, the serum GPT levels were elevated following precocene I administration. Although a single dose of 300 mg/kg body weight of precocene I did not cause significant elevation of serum GPT, measured 8 hr after dosage, a dose of 350 mg/kg caused a very large increase in the serum enzyme levels (Fig. 1b), which increased further with increasing doses up to the largest dose of 500 mg/kg body weight of precocene I that was administered. These results indicated that the precocene I was hepatotoxic when administered to rats in doses above 350 mg/kg body weight, consistent with histological data obtained at similar doses in a previous study [5].

The time–course of GSH depletion was monitored over the period of 0.5 to 12 hr after administration of a single i.p. dose of 400 mg/kg body weight of precocene I. Liver GSH levels decreased by 10% within a half-hour after dosage (Fig. 2b). However, at the end of 1 hr, liver GSH had decreased by 25% as compared to rats that received vehicle alone. Maximal depletion was observed 2–3 hr after dosage; liver GSH was only 10% of that in controls. Twelve hours after dosage, liver GSH levels were 50% of controls and continued to increase up to 24 hr (data not shown). However, at the end of 24 hr, three out of five animals that had been dosed with precocene I died.

Serum GPT activity did not show any elevation up to 2 hr after a single i.p. dose of 400 mg/kg body weight of precocene I (Fig. 2a). By the end of 4 hr the enzyme levels were three times those of animals treated with vehicle alone. However, 8 hr after administration of precocene I, the serum GPT levels were thirty times those of the untreated control rats. There was no further increase in serum GPT up to 12 hr after dosage.

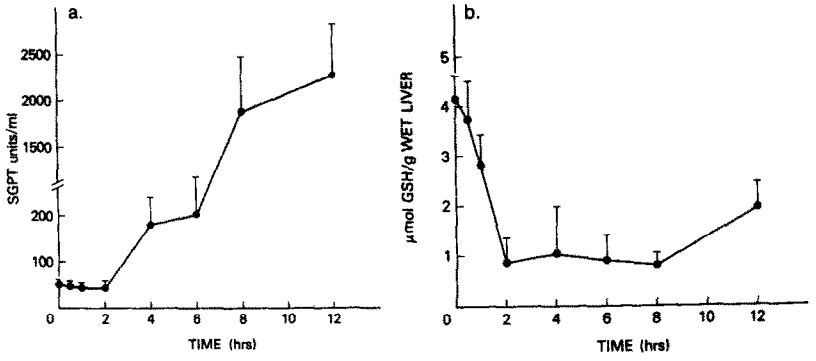


Fig. 2. (a) Serum GPT levels and (b) liver GSH levels following a single dose of 400 mg/kg body weight of precocene I. Values are expressed as mean ± SD (N = 5).

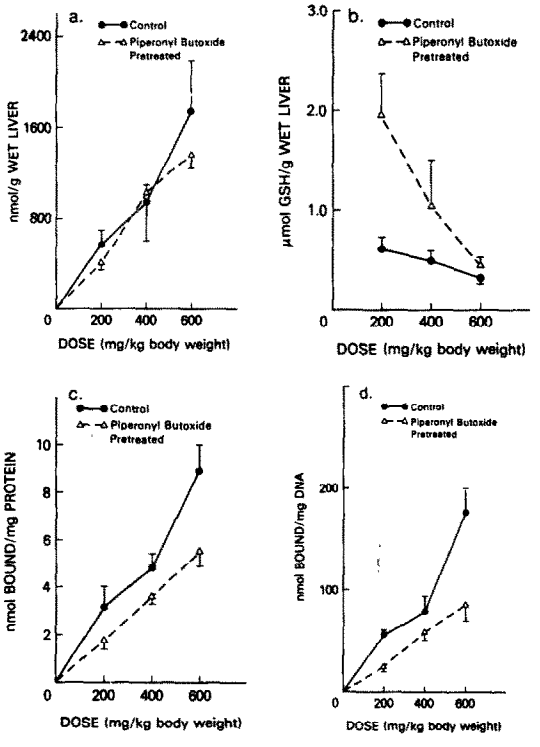


Fig. 3. (a) Total radioactivity and (b) GSH levels in liver, and (c) covalent binding to liver proteins and (d) covalent binding to liver DNA following a single i.p. dose of 400 mg/kg body weight of [4-$^3$H]precocene I. Rats were killed 4 hr after dosage. Values are expressed as mean ± SD (N = 4).

Total radioactivity was measured in liver 4 hr after single doses of 200–600 mg/kg body weight of [4-$^3$H]precocene I (Fig. 3a). At the lowest dose of 200 mg/kg body weight of [4-$^3$H]precocene I, piperonyl butoxide pretreated animals had slightly lower levels of total radioactivity in the liver 4 hr after dosage as compared to the animals that received [4-$^3$H]precocene I alone. With the increased doses of 400 and 600 mg/kg body weight of [4-$^3$H]precocene I, there was no significant difference in the total radioactivity in liver in piperonyl butoxide pretreated versus control groups that received [4-$^3$H]precocene I alone. A dose of 400 mg/kg body weight of [4-$^3$H]precocene I was hence administered to determine the covalent binding of [4-$^3$H]precocene I over a time period.

Although there was no difference from control in the amount of total radioactivity present in the liver after a dose of 400 and 600 mg/kg body weight of [4-$^3$H]precocene I to rats first treated with piperonyl butoxide, there was a significant reduction in the amount of tritium covalently bound to liver proteins (Fig. 3c). The difference was more marked at the highest dose examined. Covalent binding of tritium from [4-$^3$H]precocene I to DNA in liver was considerable (Fig. 3d). Prior treatment of the rats with piperonyl butoxide, however, reduced the amount of covalent binding to DNA at all doses examined.

Depletion of GSH in liver was also monitored 4 hr after a single i.p. dose of 200, 400 and 600 mg/kg [4-$^3$H]precocene I (Fig. 3b). While the GSH levels in livers of rats treated with precocene I alone were in the range of 0.3 to 0.6 μmole of GSH/g wet liver over a dose of 600, 400 and 200 mg/kg body weight of precocene I, the levels in the animals that received both piperonyl butoxide and precocene I were in the range of 0.45 to 1.95 μmoles of GSH/g wet liver (Fig. 3b). Prior administration of the mixed-function oxidase inhibitor piperonyl butoxide significantly lessened the depletion of liver GSH by precocene I at all doses examined.

The total radioactivity present in the liver was monitored at 1, 2, 4 and 8 hr after a single i.p. dose of 400 mg/kg body weight of [4-$^3$H]precocene I to control rats and to rats first treated with piperonyl butoxide (Fig. 4). Maximal levels of tritium in the liver were attained 1–2 hr after dosage, and the level remained constant up to 8 hr in both groups. Thus, piperonyl butoxide pretreatment did not seem to alter the distribution of [4-$^3$H]precocene I over the time period examined.

One hour after administration of [4-$^3$H]precocene I, there was no significant change in the covalent binding of the label to liver proteins between the groups of animals that received both piperonyl butoxide and [4-$^3$H]precocene I and those that received [4-$^3$H]precocene I alone. However, at 2, 4 and 8 hr after dosage, prior treatment with piperonyl butoxide markedly lowered the covalent binding of the label to liver proteins compared to control (Fig. 5a). A similar effect was observed for the covalent

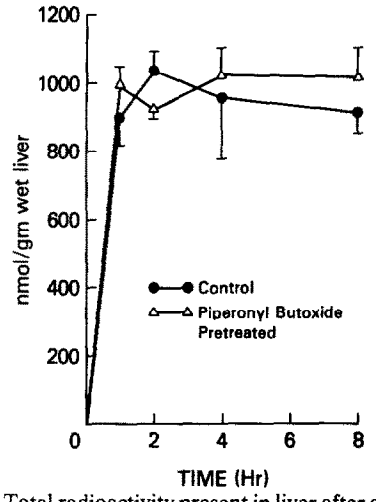


Fig. 4. Total radioactivity present in liver after a single dose of 400 mg/kg body weight of [4-$^3$H]precocene I to control and piperonyl butoxide pretreated rats. Values are expressed as mean ± SD (N = 4).

pretreated rats, 4 hr after administration of precocene I, liver GSH levels were 33% of the levels observed 1 hr after dosage. Eight hours after dosage, the liver GSH levels of rats treated with precocene I alone were 0.8 μmole GSH/g wet liver, whereas the hepatic GSH levels of rats that received both piperonyl butoxide and precocene I were 1.5 μmoles GSH/g wet liver. Thus, prior treatment with piperonyl butoxide was unable to prevent completely the depletion of liver GSH, although the amount of depletion was significantly less than that observed with rats treated with precocene I alone.

## DISCUSSION

Earlier studies have demonstrated that precocene I is a hepatotoxin causing centrilobular necrosis of liver when administered to rats [5]. The major *in vitro* microsomal metabolites of precocene I are the *cis* and *trans* 3,4-diols, which are thought to arise by spontaneous hydrolysis of the enzymatically formed precocene I 3,4-oxide [5]. The present report constitutes the first study of the *in vivo* hepatic metabolism of precocene I. The depletion of hepatic GSH

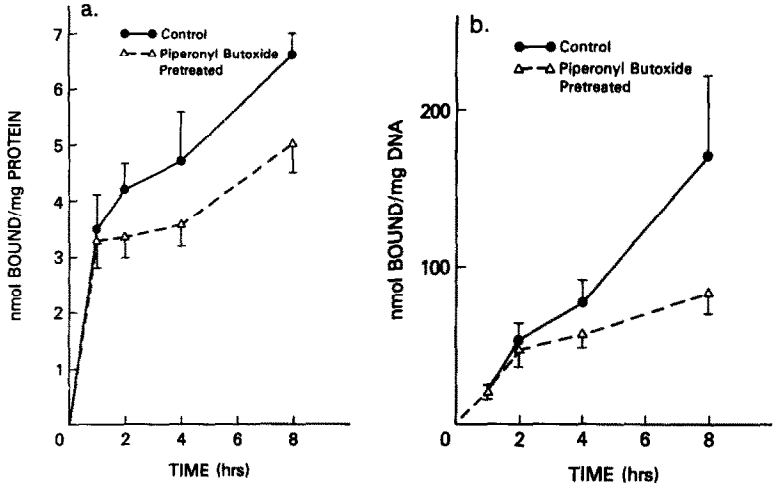


Fig. 5. (a) Covalent binding to liver proteins and (b) covalent binding to DNA after a single i.p. dose of 400 mg/kg body weight [4-$^3$H]precocene I in control and piperonyl butoxide pretreated rats. Values are expressed as mean ± SD (N = 4).

binding of label derived from [4-$^3$H]precocene I to liver DNA (Fig. 5b).

Serum GPT levels were monitored over a period of 8 hr after dosage with [4-$^3$H]precocene I in both the piperonyl butoxide pretreated groups and the groups of rats that received [4-$^3$H]precocene I alone (Fig. 6a). Serum GPT levels were highly elevated 8 hr after dosage in animals that received precocene I alone. Prior treatment with piperonyl butoxide blocked this elevation; the serum GPT levels were not different from rats that received vehicle alone. Administration of piperonyl butoxide alone had no effect on serum GPT levels.

Liver GSH levels were also measured in the abovementioned groups of rats over the time period examined (Fig. 6b). Four hours after dosage with precocene I alone, liver GSH was 12.5% of that observed 1 hr after dosage. In the piperonyl butoxide

after administration of precocene I, which is both time and dose dependent, indicates the probable *in vivo* formation of reactive electrophilic metabolites. GSH is known to act as an endogenous nucleophile that scavenges reactive electrophilic metabolites, thus affording protection against toxicity that might be manifested by these reactive species [10, 11]. If indeed such reactive species were formed, they would be liable to attack the nucleophilic centers of cellular macromolecules and covalently bind to them. Thus, when [4-$^3$H]precocene I was administered to male Sprague–Dawley rats, considerable covalent binding of the label to both proteins and DNA was observed in the liver. The covalent binding of the label to proteins and DNA was both time and dose dependent.

The role of metabolic activation in the observed covalent binding of the label to cellular macro-

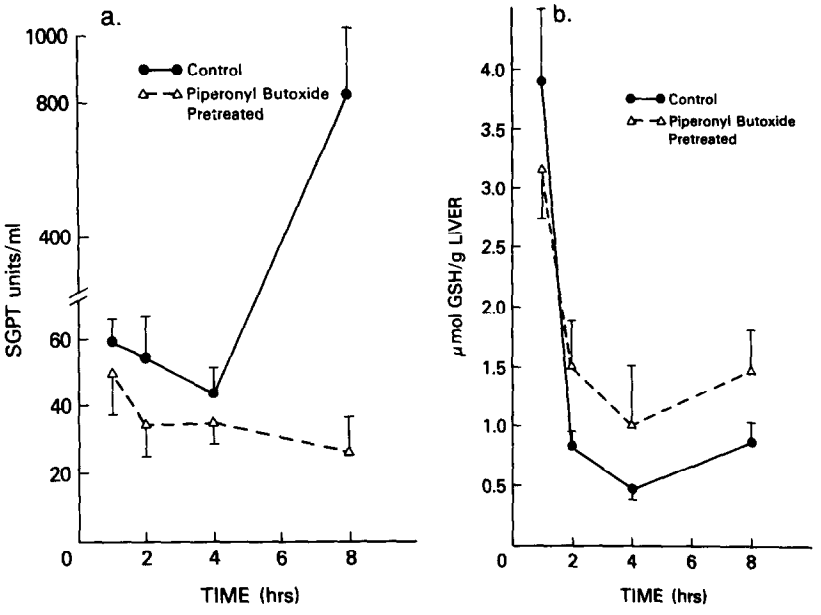


Fig. 6. (a) Serum GPT levels and (b) liver GSH levels following a single dose of 400 mg/kg body weight of precocene I in control and piperonyl butoxide pretreated rats. Values are mean ± SD (N = 4).

molecules was investigated. Prior administration of the mixed-function oxidase inhibitor piperonyl butoxide did not alter the distribution of the label to the liver. The total radioactivity (bound and unbound) present in the liver was not significantly different with or without piperonyl butoxide pretreatment over a period of 1–8 hr after administration of [4-$^3$H]precocene I. However, the mixed-function oxidase inhibitor significantly inhibited the proportion of the label bound covalently to both proteins and DNA in the liver (Fig. 5, a and b).

Prior treatment with piperonyl butoxide also partially prevented the depletion of liver GSH; the amount of GSH in the liver of piperonyl butoxide pretreated rats was twice that of rats administered with precocene I alone (Fig. 3b). Although piperonyl butoxide pretreatment afforded only partial protection against depletion of hepatic GSH by precocene I, it was able to protect totally against the hepatotoxic effects of precocene I. The serum levels of GPT in rats administered precocene I alone were elevated markedly, but the enzyme levels in piperonyl butoxide pretreated rats were not significantly different from rats receiving vehicle alone (Fig. 6a). Thus, prior administration of the mixed-function oxidase inhibitor lowers the covalent binding to cellular macromolecules, partially prevents the depletion of hepatic GSH, and totally alleviates the hepatotoxic effects of precocene I, suggesting the initial role of mixed-function oxidase in these events.

The above results are consistent with the view that the hepatotoxicity of precocene I is mediated by reactive metabolite(s) generated by the mixed-function oxidases in the liver. Covalent binding of the radiolabel to tissue macromolecules after *in vivo* administration of a radiolabeled toxic compound can serve as a useful indicator of metabolism of the parent compound to reactive metabolite by the specific target organ [12]. However, the use of *in vivo* pretreatment with inhibitors such as piperonyl butoxide is essential to establish the relationship between metabolism and production of reactive metabolites, as well as the relationship of the reactive metabolite(s) to the production of toxicity. The present results, while not necessarily implying a direct causative role for macromolecular alkylation in the cellular toxicity for precocene I, nevertheless indicate that the compound in relatively high doses is capable of producing metabolism-related severe hepatic injury.

The hepatotoxic response to precocene I shows an exceedingly sharp threshold phenomenon in relationship to the dose of the compound administered. This may indicate that the liver is normally well protected against subtoxic doses of precocene I by hepatic GSH. Only at doses of precocene I that cause extensive depletion of GSH (presumably through conjugation with electrophilic metabolites of precocene I) does hepatotoxicity occur. The striking effect of piperonyl butoxide in preventing the hepatotoxicity, while only more moderately inhibiting the precocene I-related GSH depletion, is also a likely reflection in this marked threshold phenomenon for precocene I toxicity. Any future assessment of toxicological implications of precocene I should obviously take into account not only the toxic potential of the compound but also the physiological protective mechanisms that may be available and which may or may not be adequate under the given conditions.

## REFERENCES

1. W. S. Bowers, T. Ohta, J. S. Cleere and P. A. Marsella, *Science* **193**, 542 (1976).
2. G. T. Brooks, G. E. Pratt and R. C. Jennings, *Nature, Lond.* **281**, 570 (1979).
3. G. E. Pratt, R. C. Jennings, G. F. Hamnett and G. T. Brooks, *Nature, Lond.* **284**, 320 (1980).
4. N. Nordquist, D. R. Thakker, H. Yagi, R. E. Lehr, A. W. Wood, W. Levin, A. H. Conney and D. M. Jerina, in *Molecular Basis of Environmental Toxicity* (Ed. R. S. Bhatnagar), p. 329. Ann Arbor Science Publishers, Ann Arbor, MI (1980).
5. R. A. Halpin, K. P. Vyas, S. F. El-Nagar and D. M. Jerina, *Chem. Biol. Interact.* **48**, 297 (1984).
6. S. Reitman and S. Frankel, *Am. J. clin. Path.* **28**, 56 (1957).
7. T. P. M. Akerboom and H. Sies, in *Methods in Enzymology* (Ed. W. B. Jakoby), p. 372. Academic Press, New York (1981).
8. V. Ravindranath, M. G. McMenamin, J. H. Dees and M. R. Boyd, *Toxic. appl. Pharmac.*, **85**, 78 (1986).
9. C. W. Dunnett, *J. Am. statist. Ass.* **50**, 1096 (1955).
10. J. R. Mitchell, D. J. Jollow, W. Z. Potter, J. R. Gillette and B. B. Brodie, *J. Pharmac. exp. Ther.* **187**, 211 (1973).
11. E. M. Kosower in *Glutathione: Metabolism and Function* (Eds. I. M. Arias and W. B. Jakoby), p. 1. Raven Press, New York (1976).
12. M. R. Boyd, L. T. Burka and B. J. Wilson, *Toxic. appl. Pharmac.* **32**, 147 (1975).