Table 2. Profile of benzo[a]pyrene (BP) metabolites produced by microsomes obtained from inner and outer adrenocortical zones*

Metabolite	Inner zone (nmoles/min × mg protein)(10^{-2})	Outer zone
3-Hydroxy-BP	51.5 ± 6.0 (62%)†	17.0 ± 1.8 (59%)
9-Hydroxy-BP	5.6 ± 0.6 (7%)	1.8 ± 0.2 (6%)
Quinones	3.0 ± 0.4 (4%)	1.5 ± 0.2 (5%)
BP-7,8-diol	8.9 ± 1.0 (11%)	3.3 ± 0.3 (11%)
BP-4,5-diol	1.6 ± 0.2 (2%)	0.7 ± 0.2 (3%)
BP-9,10-diol	12.7 ± 1.4 (15%)	4.7 ± 0.5 (16%)

* Values are expressed as means ± S.E. of five to seven experiments.

† Values in parentheses indicate percent of total metabolites.

of metabolism was virtually identical in inner and outer zone microsomes, but the rate of production of each metabolite was far greater in the inner zone. The similar pattern of metabolites produced is probably the result of similar cytochrome P-450 isozymes that metabolize BP as well as proportionately similar differences in the activities of BP hydroxylase and epoxide hydratase in the inner and outer zones. The results indicate that, although similar BP metabolites are produced by microsomes from inner and outer adrenocortical zones, the overall rate of metabolism of BP is far greater in the inner zone. Thus, the potential for toxic effects of compounds like BP, which require metabolic activation, may also be greater in the inner zone of the adrenal cortex. Further studies are now under way to examine that hypothesis.

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REFERENCES

1. J. W. DePierre and L. Ernster, *Biochim. biophys. Acta* **473**, 149 (1978).
2. H. V. Gelboin, *Physiol. Rev.* **60**, 1107 (1980).
3. D. M. Jerina and J. W. Daly, *Science* **185**, 573 (1974).
4. D. A. Pitrolo, R. C. Rumbaugh and H. D. Colby, *Drug Metab. Dispos.* **7**, 52 (1979).
5. H. D. Colby, P. B. Johnson, M. R. Pope and J. S. Zulkoski, *Biochem. Pharmac.* **31**, 639 (1982).
6. M. R. Juchau and M. G. Pedersen, *Life Sci.* **12**, 193 (1973).
7. P. K. Zachariah and M. R. Juchau, *Life Sci.* **16**, 55 (1975).
8. J. A. Long, in *Handbook of Physiology* (Eds. H. Blaschko, G. Sayers and A. D. Smith), Sect. 7, Vol. 6, p. 13. American Physiological Society, Washington, D.C. (1975).
9. P. I. Eacho and H. D. Colby, *Life Sci.* **32**, 1119 (1983).
10. K. D. Martin and V. H. Black, *Endocrinology* **112**, 573 (1983).
11. K. D. Martin and V. H. Black, *Endocrinology* **110**, 1749 (1982).
12. D. W. Nebert and H. V. Gelboin, *J. biol. Chem.* **243**, 6242 (1968).
13. G. Holder H. Yagi, W. Levin, A. Y. H. Lu and D. M. Jerina, *Biochem. biophys. Res. Commun.* **65**, 1363 (1975).
14. J. W. DePierre, M. S. Moron, K. A. M. Johannesen and L. Ernster, *Analyt. Biochem.* **63**, 470 (1975).
15. J. Van Cantfort, J. De Graeve and J. E. Gielen, *Biochem. biophys. Res. Commun.* **79**, 505 (1977).
16. F. Oesch, D. M. Jerina and J. Daly, *Biochim. biophys. Acta* **227**, 685 (1971).
17. M. O. James, J. R. Fouts and J. R. Bend, *Biochem. Pharmac.* **25**, 187 (1976).
18. S. K. Yang, J. K. Selkirk, E. V. Plotkin and H. V. Gelboin, *Cancer Res.* **35**, 3642 (1975).
19. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).

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Glutathione and glutathione *S*-transferases in the urinary bladder of different species

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The glutathione *S*-transferases (EC 2.5.1.18), a family of enzymes with broad and overlapping substrate specificities, catalyze the conjugation of a wide variety of chemically reactive, electrophilic compounds with the nucleophilic tripeptide glutathione. By preventing the interaction of these electrophiles with critical cellular macromolecules, conjugation with glutathione and the resulting formation of water-soluble, glutathione adducts represents a significant step in the detoxication and excretion of many chemical carcinogens and cytotoxicants [1].

Arylamine bladder carcinogens such as 2-naphthylamine are metabolized by hepatic cytochrome P-450 monooxygenases to *N*-hydroxy-2-naphthylamine which is further conjugated with glucuronic acid. The glucuronide then serves as a transport form for the proximate carcinogen which is generated at the slightly acidic pH of the urine

[2, 3]. The demonstration that cultured rat, hamster and human bladder epithelial cells can metabolize polycyclic aromatic hydrocarbons, aflatoxin B_1 and *N*-nitrosamines [4–6] in conjunction with recent measurements of cytochrome P-450 in bovine bladder microsomes [7] suggests, however, that hepatic metabolism may not be requisite for the formation of the ultimate carcinogenic electrophiles from a variety of compounds. In addition, the findings that rat and rabbit bladder epithelial cells have significant prostaglandin synthetase activity [8] and that this enzyme mediates the metabolic activation of urinary bladder carcinogens such as benzidine [9] and *N*-[4-(5-nitro-2-furyl)-2-thiozolyl]formamide (FANFT) [10] further support the view that electrophiles generated directly in the bladder epithelium may be important in bladder carcinogenesis.

The pathologic outcome of the formation of highly electrophilic compounds in a particular organ often depends upon the relative rates of activation and detoxication. Because a number of recent studies have shown that glutathione conjugation occurs with reactive metabolites formed from proximate bladder carcinogens such as 2-naphthylamine [11], *N*-acetyl-2-aminofluorene [12] and several of the polycyclic aromatic hydrocarbons [13], we have examined the levels of reduced glutathione and the activity of glutathione *S*-transferases in bladder epithelium of a number of different species. Furthermore, we have examined the ability of various agents known to induce xenobiotic metabolizing enzymes in other organs to alter the activity of bladder glutathione *S*-transferases.

Materials and methods

Animals. Female, New Zealand rabbits, weighing 2–3 kg, were obtained from the Mission Laboratories, Rosemead, CA. Female mongrel dogs (35 lb) were obtained from the San Bernadino animal shelter, San Bernadino, CA. Male Wistar rats (200–400 g) were purchased from the Hilltop Breeding Laboratories, Chatsworth, CA. Hartley guinea pigs (male, 300 g), Golden Syrian hamsters (male, 70–85 g), and male and female Sprague–Dawley rats were from the Charles River Breeding Laboratories, Wilmington, MA. All rodents were housed on either sanicell or on hardwood bedding and were given food and water *ad lib.*

Human subjects. Surgical specimens were obtained at the City of Hope Medical Center from three patients with their consent and the approval of the Human Subjects Review Committee. All human tissue specimens were determined by pathology to be stage A, grade 2–3 transitional cell carcinomas of the bladder.

Chemicals. Reduced glutathione was purchased from Calbiochem–Behring, La Jolla, CA. 1-Chloro-2,4-dinitrobenzene (CDNB), from Eastman Kodak, Rochester, NY, was recrystallized from ethanol before use. The following chemicals were obtained from the suppliers indicated: *o*-phthaldehyde (OPT), Sigma Chemical Co., St. Louis, MO; Arochlor 1254, Analabs, North Haven, CT; phenobarbital, J. T. Baker, Phillipsburg, NJ; and diethyl maleate, Aldrich Chemical Co., Milwaukee, WI.

Animal treatments. All dose solutions were prepared such that 5 ml was administered intraperitoneally per kg body weight. Phenobarbital (50 mg/kg, dissolved in saline) was administered twice daily for 5 days and the rats were killed 24 h after the last dose. Arochlor 1254 (107 mg/kg) was dissolved in corn oil and administered 48 hr before sacrifice. Corn oil solutions of diethyl maleate (600 μl/kg) were given 1.5 hr before sacrifice. Control animals received the appropriate vehicle only.

Assay of glutathione and glutathione S-transferase activity. To avoid fluctuations in glutathione level due to diurinal variations, all animals were killed within a 4-hr time period. Rodents were killed by either cervical dislocation or decapitation. Dogs were killed by a lethal injection of pentobarbital. Bladders were rapidly removed, placed in ice-cold 150 mM saline, everted and rinsed in the same buffer. Mucosa was dissected from the dog bladder. The tissue was sonicated in 2.5 to 5.0 ml of 5 mM EDTA at 0–4°. Eight sonications (Heat Systems Cell Disruptor equipped with a standard microtip), each at 5-sec duration and a setting of 50 W, were used to release the epithelial glutathione and glutathione *S*-transferases. In preliminary experiments, rat bladders were sonicated for periods of time ranging from 1 × 5 sec to 64 × 5 sec. The supernatant fraction was assayed for glutathione *S*-transferase activity, and the remaining bladder was fixed in neutral buffered formalin, embedded in paraffin, and 5–6 μm sections were stained with hematoxylin and eosin and examined by light microscopy. An 8 × 5 sec sonication time resulted in optimal specific activities of the glutathione *S*-transferases and in little or no detectable removal of the submucosa. At longer sonication times, a decrease in the specific activity of the glutathione *S*-transferase and partial removal of cells of the submucosa were observed. The sonicated bladder was centrifuged at 9000 *g* for 30 min, and the resulting supernatant fraction was used for the assay of glutathione *S*-transferase.

Rat liver glutathione *S*-transferase activity was measured using 105,000 *g* supernatant fraction prepared by standard techniques [14].

Glutathione *S*-transferase activity was assayed with 1-chloro-2,4-dinitrobenzene at 25° according to the method of Habig *et al.* [15]. All assays were performed on a Cary 210 dual beam spectrophotometer. Enzyme protein content was determined by the method of Lowry *et al.* [16], and results are expressed as nmoles per min per mg.

After an aliquot was removed for protein determination, an equal volume of 8% H_3PO_4 was added to a 1- to 2-ml aliquot of the sonicate to precipitate the protein, and the samples were centrifuged at 9000 *g* for 30 min. Reduced glutathione was assayed by the OPT-fluorescence coupling procedure of Cohn and Lyle [17]. Fluorophore formation was conducted at pH 8, and the fluorescence intensity was read on an Aminco Bowman Spectrophotofluorimeter using excitation and emission wavelengths of 350 and 420 nm respectively. Standard curves, prepared using reduced glutathione, were linear in the range of sample values (5–30 μg glutathione/ml). To be certain that the method of tissue preparation described here was not causing significant oxidation of reduced glutathione, both oxidized and reduced glutathione were assayed in rat bladders by the high pressure liquid chromatographic procedure of Reed *et al.* [18]. In all eight samples studied, oxidized glutathione levels were either undetectable or were less than 0.5% of the levels of reduced glutathione.

Results and discussion

Substantial species differences were observed in bladder epithelial glutathione *S*-transferase activities with 1-chloro-2,4-dinitrobenzene (Table 1). The specific activity of rabbit bladder glutathione *S*-transferase was the highest of any species tested and was 10- to 20-fold higher than the activity in the rat, dog or in human biopsy specimens. Although species comparisons in the relative sensitivity to various bladder carcinogens are difficult because different doses, routes of administration and protocols were used in the reported studies, 2-naphthylamine-induced bladder tumors have been shown to occur in the dog and human (species with low glutathione *S*-transferase activity) but have not been demonstrated in the hamster or rabbit (species with high glutathione *S*-transferase activity) (see Ref. 19 for a review of bioassays of chemicals causing bladder tumors in different species). This positive correlation is of interest because 2-naphthylamine has been shown to form glutathione adducts [11]. Such correlations must be interpreted with considerable caution, however, because differences in sensitivity to a particular carcinogenic agent will depend upon the relative toxicokinetics in a given species as well as DNA replication and repair rates. Nevertheless, the data

in Table 1 do indicate that bladder epithelium has the capacity to form glutathione adducts. In addition to the marked species differences in bladder epithelial glutathione *S*-transferase activity, moderate differences in activity between neoplastic and adjacent "normal" bladder epithelium of the human were noted. Similar variations between tumor and nontumor tissue in the rates of glucuronide and sulfate conjugation have been demonstrated in human lung and colon by Cohen *et al.* [20, 21]. Whether these differences in the rate of xenobiotic conjugation can be exploited in the design of tumor cell selective cytotoxicants remains to be explored.

Differences in the reduced glutathione levels in the bladder of the various species tested were considerably less pronounced than the differences in glutathione *S*-transferase activities and did not appear to correlate with the specific activity of the *S*-transferase enzymes. Recent studies with the hepatotoxicant acetaminophen indicate that the extent of tissue injury by overdoses of this analgesic may depend not only on the absolute tissue level of glutathione but on the rate of resynthesis of this tripeptide [22]. Thus, while the quantities of glutathione detected in the bladder are not nearly as high as the liver, under ordinary circumstances they may be sufficient to provide adequate protection from electrophiles, especially if the rate of synthesis of this tripeptide is high in bladder epithelium. The marked depletion of bladder epithelial glutathione by diethyl maleate (Table 2) shows that the levels of this nucleophile in the bladder can be altered by agents that form glutathione adducts and that treatment with diethyl maleate might offer a useful experimental approach to determining whether glutathione plays a significant role in the detoxication of urinary cytotoxicants like FANFT.

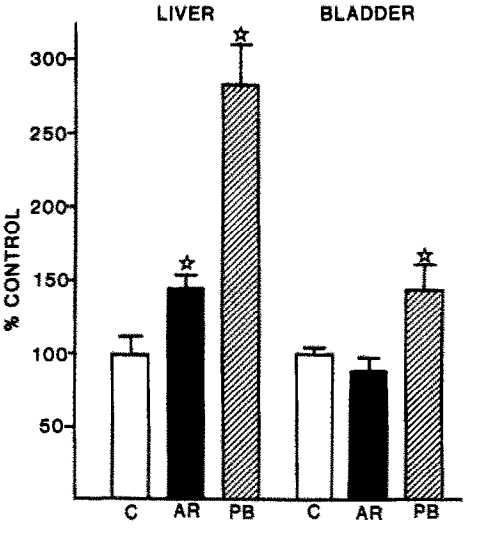

Fig. 1. Effect of Arochlor 1254 (AR) or phenobarbital (PB) pretreatment on hepatic and bladder epithelial glutathione *S*-transferase. Values are the mean ± S.E. for four rats. Stars indicate a significant difference from control ($P < 0.05$), Student's two-tailed *t*-test.

Table 1. Species differences in glutathione and glutathione *S*-transferase activities in the urinary bladder

Species/Strain	Glutathione *S*-Transferase*	Glutathione†	N
Rabbit/New Zealand	2130 ± 370	4.9 ± 0.4	3
Guinea pig/Hartley	870 ± 47	5.1 ± 0.9	5
Hamster/Golden Syrian	452 ± 62	8.6 ± 0.8	5
Rat/Sprague–Dawley	194 ± 25	13.4 ± 2.9	13
Rat/Wistar	90 ± 9	5.4 ± 0.5	3
Dog/Mongrel	107 (91–122)‡	0.8 (0.6–0.9)	2
Human resection/Biopsy	122 ± 20§		3
	81 ± 14‖		3

* Values are nmoles CDNB conjugate/min/mg protein and are expressed as mean ± S.E.

† Values are μg reduced glutathione/mg protein and are expressed as mean ± S.E.

‡ Values in parentheses are the range.

§ Neoplastic tissue.

‖ Adjacent tissue (pathologically without tumor).

Table 2. Effect of treatment with diethyl maleate on reduced glutathione levels in rat bladder epithelium and liver

	Glutathione*			
Treatment	Bladder epithelium	% Control	Liver	% Control
Corn oil	7.1 ± 0.5		1.90 ± 0.13	
Diethyl maleate	2.5 ± 0.1†	34	0.28 ± 0.01†	15

* Glutathione levels are reported as μg/mg protein for bladder and mg/g wet tissue weight for liver. Values are the mean ± S.E.M. for three animals.

† Significantly different from control ($P < 0.05$), Student's two-tailed *t*-test.

To explore the possibility that exposure to environmental agents known to induce xenobiotic metabolizing enzymes could alter the specific activities of detoxication enzymes in the bladder epithelium, groups of four rats each were treated with phenobarbital or Arochlor 1254 at doses previously shown to result in significant increases in hepatic glutathione *S*-transferase activities [23–25]. The data in Fig. 1 indicate that treatment with either Arochlor 1254 or with phenobarbital markedly increased glutathione *S*-transferase activity in rat liver. In the bladder epithelium, phenobarbital increased the specific activity of glutathione *S*-transferases while treatment with Arochlor had no significant effect. The data in Fig. 1 and Table 2 suggest that a variety of environmental agents are capable of altering the relative ability of the bladder epithelium to detoxify electrophilic carcinogenic or cytotoxic agents.

In summary, the results of this work indicate that bladder epithelial cells are fully capable of catalyzing the metabolic deactivation of proximate carcinogens through conjugation with glutathione. Moreover, glutathione and the glutathione transferases may very well play a role in modulating the cytotoxic/mutagenic actions of electrophiles formed in other tissues and excreted in the urine. In addition, these studies have demonstrated that reduced glutathione levels and the specific activity of the glutathione *S*-transferases may be altered by other environmental agents and suggest that further investigations to understand those factors which may predispose certain individuals to the deleterious effects of bladder carcinogens may prove fruitful.

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REFERENCES

1. L. F. Chasseaud, *Adv. Cancer Res.* **29**, 175 (1979).
2. F. F. Kadlubar, J. A. Miller and E. C. Miller, *Cancer Res.* **38**, 3628 (1978).
3. F. F. Kadlubar, L. E. Unruh, T. J. Flammang, D. Sparks, H. K. Mitchum and G. J. Mulder, *Chem. Biol. Interact.* **33**, 129 (1981).
4. H. Autrup, R. C. Grafstrom, B. Christensen and J. Kieler, *Carcinogenesis* **2**, 763 (1981).
5. R. Langenbach, L. Malick and S. Nesnow, *J. natn. Cancer Inst.* **66**, 913 (1981).
6. B. P. Moore, R. M. Hicks, M. A. Knowles and S. Redgrave, *Cancer Res.* **42**, 642 (1982).
7. J. M. Poupko, J. L. Radomski and W. L. Hearn, *Cancer Res.* **41**, 1306 (1981).
8. W. W. Brown, T. V. Zenser and B. B. Davis, *Am. J. Physiol.* **239**, 1435 (1980).
9. T. V. Zenser, M. B. Mattammal, H. J. Armbrecht and B. B. Davis, *Cancer Res.* **40**, 2839 (1980).
10. S. M. Cohen, T. V. Zenser, G. Murasaki, S. Fukushima, M. B. Mattammal, N. S. Rapp and B. B. Davis, *Cancer Res.* **41**, 3355 (1981).
11. M. Unger, O. Anderson and J. Clausen, *Cancer Res.* **37**, 1264 (1977).
12. G. J. Mulder, L. E. Unruh, F. E. Evans, B. Ketterer and F. F. Kadlubar, *Chem. Biol. Interact.* **39**, 111 (1982).
13. S. Hesse, B. Jernstrom, M. Martinez, P. Moldeus, L. Christodoulides and B. Ketterer, *Carcinogenesis* **3**, 757 (1982).
14. P. Mazel, in *Fundamentals of Drug Metabolism and Disposition* (Eds. B. N. LaDu, G. Mandell and E. L. Way), p. 527. Williams & Wilkins, Baltimore (1971).
15. W. H. Habig, M. J. Pabst and W. B. Jacoby, *J. biol. Chem.* **249**, 7130 (1974).
16. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
17. V. H. Cohn and J. Lyle, *Analyt. Biochem.* **14**, 434 (1966).
18. D. J. Reed, J. R. Babson, P. W. Beatty, A. E. Brodie, W. W. Ellis and D. W. Potter, *Analyt. Biochem.* **106**, 55 (1980).
19. D. B. Clayson and E. H. Cooper, *Adv. Cancer Res.* **13**, 271 (1970).
20. G. M. Cohen, E. M. Gibby and R. Mehta, *Nature, Lond.* **291**, 662 (1981).
21. G. M. Cohen, R. C. Grafstrom, E. M. Gibby, L. Smith, H. Autrup and C. C. Harris, *Cancer Res.* **43**, 312 (1983).
22. B. H. Lauterburg, Y. Vaishnav, W. G. Stillwell and J. R. Mitchell, *J. Pharmac. exp. Ther.* **213**, 54 (1980).
23. M. G. Parkki, J. Marniemi and H. Viano, *J. Tox. environ. Hlth.* **3**, 903 (1977).
24. A. J. Baars, M. Jansen and D. D. Breimer, *Biochem. Pharmac.* **27**, 2487 (1978).
25. T. N. Thompson, J. B. Watkins, Z. Gregus and C. D. Klaussen, *Toxic. appl. Pharmac.* **66**, 400 (1982).

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Effect of maternal pethidine administration on neonatal brain cyclic AMP levels and ornithine decarboxylase activities

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Pethidine is widely used for the relief of pain during labor [1, 2]. After intramuscular or intravenous administration, the drug is readily transferred from the mother to the fetus [3]. This results in a depressive effect on the ventilation of newborns and in longer-lasting adverse effects on the neuromuscular physiology and behavior of the offspring [4].

Despite its wide use in obstetrics, the information available on the effects of the drug on the developing tissues in infants is rather limited.

Ornithine decarboxylase (EC 4.1.1.17, ODC), which is the rate-limiting enzyme in the polyamine biosynthetic pathway, appears to be associated with rapid cell growth [5]. It has been shown [6] that both fetal and neonatal rat