

Cytotoxicity of 1,2-epoxynaphthalene Is Correlated with Protein Binding and *in Situ* Glutathione Depletion in Cytochrome P4501A1 Expressing Sf-21 Cells

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Naphthalene is metabolized by several cytochrome P-450 (CYP) monooxygenases to 1,2-epoxynaphthalene. However, the subsequent interactions of the epoxide with macromolecules in the cells, and the significance of these interactions to cellular injury, are not well characterized. Additionally, CYP1A1, which can metabolize naphthalene to 1,2-epoxynaphthalene, may be induced by a number of xenobiotics. Yet, the *in situ* interaction between naphthalene and CYP1A1 alone, without the influence of other xenobiotic metabolizing enzymes, has not been examined. Using a model eukaryotic expression system capable of over-expressing recombinant CYP1A1, we found that naphthalene was toxic to cells expressing CYP1A1 in a dose- (LC50: 0.3 mM) and time-dependent (LT50: 12 h) manner. Naphthalene treatment of CYP1A1-expressing cells resulted in a 47% decrease in cellular glutathione (GSH) levels. Pretreatment with ethyl ester GSH, a GSH analog, protected CYP1A1-expressing cells such that viability was 30% greater than for cells treated with naphthalene alone. Cytotoxicity was strongly correlated (r^2 : 0.96) with covalent binding of cellular proteins. Alkaline permethylation techniques showed that cysteinyl-SH groups of cellular proteins are a nucleophilic target of the epoxide metabolite. These results suggest that, in the absence of other pathways, naphthalene is modified by CYP1A1 to 1,2-epoxynaphthalene, which subsequently binds cellular sulfhydryl groups on proteins and GSH.

Key Words: 1,2-epoxynaphthalene; naphthalene; glutathione; ethyl ester glutathione; CYP1A1; Sf-21; alkaline permethylation.

Naphthalene, a bicyclic, aromatic hydrocarbon, is an environmental contaminant. The major constituents of polyaromatics in ambient air are naphthalene derivatives (Arey *et al.*, 1987; Gupta *et al.*, 1996) formed during the combustion of gasoline (Nishioka *et al.*, 1982), coal (Bieniek, 1994; Mumford *et al.*, 1987), and tobacco (Schmeltz *et al.*, 1976). Aromatic petroleum refinery streams and coal tar feedstocks produce hundreds of millions of pounds annually (Kirk and Othmer, 1981). Naphthalene is an important industrial precursor and is

used in some pesticide formulations (Hill *et al.*, 1995; Witschi and Last, 1996). In mammals, naphthalene can be metabolized by various cytochrome P450 (CYP) monooxygenases, including 1A1, 1A2, 1B1, 3A7, 3A5 (Juchau *et al.*, 1998), 2E1 (Wilson *et al.*, 1996), 2F2 (Buckpitt, 1995), and 2B4 (Van Winkle *et al.*, 1996). Naphthalene-induced toxicities following metabolic activation by CYP monooxygenases have included selective cell necrosis (Cho *et al.*, 1994a), lung and kidney lesions (Buckpitt *et al.*, 1995, 1982; Buckpitt and Warren, 1983; Griffin *et al.*, 1983), and toxicity to mononuclear leukocytes and human lymphocytes (Wilson *et al.*, 1996), among others. One route of naphthalene metabolism is epoxidation. 1,2-Epoxynaphthalene is proposed to bind covalently to nucleophilic amino acid residues of cellular proteins and thus cause cytotoxicity (Zheng *et al.*, 1997). However, the subsequent interactions of the electrophilic metabolites with macromolecules in the cells, and the importance of these interactions in cellular injury are not well characterized.

It has also been proposed that conjugation with glutathione (GSH) is a detoxification route for some naphthalene metabolites (Chichester *et al.*, 1994; Iverson *et al.*, 1995). In these cases, the ability of a cell to detoxify an electrophilic metabolite, along with the propensity to form it in the first place, will govern the degree of cytotoxicity. Thus, the relatively low concentrations of GSH in pulmonary tissue (Forkert, 1997), and subsequently lessened ability to detoxify, may contribute to cytotoxicity. Some early work describes the formation of caracants in rabbits after naphthalene administration and the mercapturic acid of naphthalene was found in the urine of rabbits and rats (Bourne and Young, 1934; Young, 1947). Indeed, Boyland and Sims suggest as much, though they looked at 1,2-epoxy-1,2,3,4-tetrahydroxynaphthalene, as it was more readily available (Booth *et al.*, 1960; Boyland and Sims, 1960).

Inasmuch as toxicity appears to be concentrated in the lung, work on the toxicity of naphthalene metabolites has often emphasized specific CYP monooxygenases found in the lung, such as CYP2E1 (Wilson *et al.*, 1996), CYP2F (Buckpitt *et al.*,

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1995), and CYP2B (Van Winkle *et al.*, 1996). CYP1A1 is not constitutively expressed in lung tissue (McLemore *et al.*, 1990) nor in the liver (Omiecinski *et al.*, 1990; Warner *et al.*, 1998), hence, very little work has been done on its bioactivation of naphthalene to toxic metabolites. However, CYP1A1 is known to be induced by cigarette smoke (McLemore *et al.*, 1990), TCDD (Stephen *et al.*, 1997), phenobarbital, *N*-benzylimidazole (Papac and Franklin, 1988), various pesticides including carbaryl, cypermethrin, diflubenzuron, and tetrachlorvinphos (Delescluse *et al.*, 1998), and various common food chemicals including flavones, indoles, and methylated xanthines (Juchau *et al.*, 1998). Cigarette smoke not only induces CYP1A1 but also contains naphthalene, so it is likely that smokers are exposed to CYP1A1-generated metabolites of naphthalene. The purpose of this study was to examine the cytotoxicity of CYP1A1-dependent metabolites of naphthalene.

Our study investigated naphthalene toxicity in a time- and dose-dependent manner, to insect cells expressing CYP1A1. We examined intracellular GSH levels after naphthalene intoxication and the protective effects of GSH and ethyl ester GSH, as well as the deleterious effects of GSH depletion. Using alkaline permethylation, we investigated the covalent binding of 1,2-epoxynaphthalene and its correlation with cytotoxicity.

MATERIALS AND METHODS

Chemicals and Instruments

Chemical reagents and solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI). Hemin, immunochemicals, ethyl ester glutathione (ethyl ester GSH), GSH, diethylmaleate (DEM), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and 1-chloro-2,4-dinitrobenzene (CDNB) were obtained from Sigma Chemical Co. (St. Louis, MO). [¹⁴C]-Naphthalene was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). BCA reagent was purchased from Pierce Chemical Co. (Rockford, IL). 2-Methylthionaphthalene (compound 1), 2-ethylthionaphthalene (compound 2), and dimethoxymethylthionaphthalenes (compound 3) were synthesized as previously described (Zheng *et al.*, 1997). High performance liquid chromatography (HPLC) analysis and purification of synthetic chemicals were performed using a Varian 9010 solvent delivery system equipped with a Varian 9050 UV-VIS detector and a reverse phase C₁₈ chromatography column (250 × 4.6 mm) (Vydac, Hesperia, CA). Proton nuclear magnetic resonance (¹H-NMR) spectra were obtained from a QE-300 spectrometer (General Electric, 300 MHz). Synthetic chemicals and alkaline permethylated samples were analyzed on a Hewlett-Packard gas chromatography-mass spectrometer (GC-MS) system [HP 5890 Series II gas chromatograph with a 30 m DB-5 capillary column (J & W Scientific, Folsom, CA), HP 5971A mass-selective detector, and HP 59940A MS ChemStation (HP-UX series) controller] using 70 eV electron ionization. A linear temperature program from 100°C to 250°C at 10°C/min was used. A Perkin-Elmer fluorimeter was used to measure CYP1A1 activity with ethoxyresorufin as a model substrate (Burke and Mayer, 1974). Optical density (OD) for viability studies, GSH measurements, and protein concentrations were read with a V_{max} microplate reader (Molecular Devices, Menlo Park, CA).

Statistical Analyses

Student's *t*-test was used to determine differences in treated and control groups. Differences were considered statistically significant at *p* ≤ 0.05. LC50

and LT50 (time for 50% loss of viability) were determined by regression analysis. All results are mean ± standard deviation.

Toxicity and Binding Studies

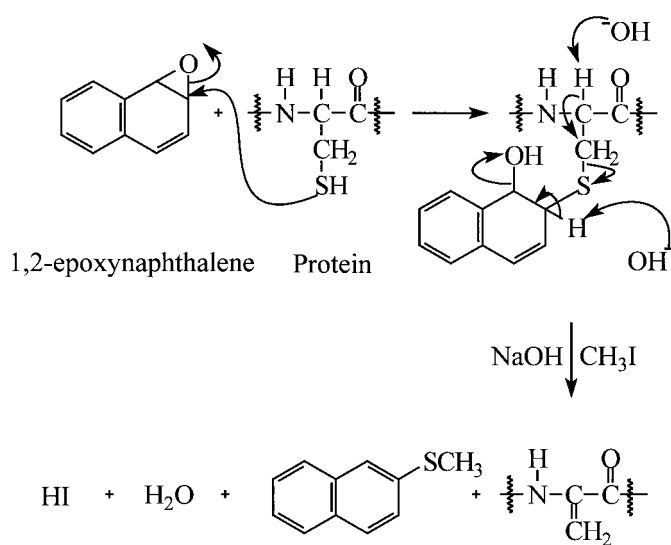
Sf-21 cells were infected with the rat CYP1A1/yeast reductase fusion baculovirus or Lac Z baculovirus as control (constructed as previously described [Grant *et al.*, 1996]) in complete medium (96% EX-Cell 401 with L-glutamine, JRH Biosciences (Lenexa, KS); 3% heat-inactivated fetal bovine serum, Intergen (Purchase, NY); and 1% Pen/Strep antibiotics, (Sigma, St. Louis, MO) at a multiplicity of infection of 0.1. One-h post-infection, 0.75 μg/ml of hemin was added to each spinner flask. At 48-h post-infection, 25 mL of cells were aliquoted into 50 mL spinner flasks, and various concentrations of naphthalene (0 mM–1.5 mM) were added. At various times post-administration, aliquots were taken and viability assays were performed as described previously (Grant *et al.*, 1996). Briefly, cells were incubated for 2 h with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). The cells were lysed with 0.5 ml lysing solution (250 mg sodium dodecyl sulfate (SDS) in 1:1 dimethyl formamide: water, pH4.5) over 12 h in the dark to dissolve the insoluble formazan product. Aliquots were then transferred to 96-well plates and the MTT hydrolysis product quantified at 560 nm. The remainder of the cells were centrifuged at 2000 rpm for 10 min, washed with sodium phosphate buffer (0.1 M, pH 7.4) (PBS) 3 times, then lysed in 0.1% SDS. The lysate was dialyzed (3000 mw cutoff) in 0.1% SDS for 36 h at 4°C. In a separate experiment, cells were incubated with 0.5 mM [¹⁴C]-naphthalene (1.5 × 10⁵ dpm/μl) for 24 h. Protein was precipitated with methanol, and the pellet washed 6 times with methanol. Aliquots of both pellet and wash were taken and radioactivity quantified by liquid scintillation counter. For those experiments in which the effects of GSH on toxicity were examined, cells were pretreated for 2 h either with 5-mM GSH, 5-mM ethyl ester GSH, or 0.125-mM diethylmaleate (DEM). The experiments were then performed as described above. Protein concentrations were measured using BCA reagent with bovine serum albumin as a standard. Glutathione levels were measured as free sulfhydryls using DTNB (Ellman, 1959) and verified in several samples by HPLC, as described (Fahey and Newton, 1987). GST levels were measured using CDNB, as described (Grant *et al.*, 1989).

Alkaline Permethylation of Sf-21 Cell Proteins

Alkaline permethylation of protein samples were conducted as described previously by Slaughter *et al.* (1993), and Slaughter and Hanzlik (1991). This method generates methylthionaphthalene derivatives that are structurally dependent on the nature of the naphthalene reactive metabolites modifying cysteine residues (Scheme 1). Briefly, the dialyzed protein samples (4.0 mL) were placed in culture tubes and mixed with 4.0 mL of 8 M NaOH, followed by addition of 1.0 mL of CH₃I. The tubes were purged with nitrogen (the protein samples, NaOH solution, and CH₃I had been pre-purged with nitrogen), sealed with Teflon-lined caps, and heated at 80°C for 4 h. Following the reaction, the mixture was cooled to room temperature, spiked with ethylthionaphthalene (compound 2) as an internal standard, and extracted with pentane. The pentane extracts were dehydrated with anhydrous Na₂SO₄, concentrated by distillation, and analyzed by GC-MS.

GC-MS Analysis

Pentane extracts obtained from alkaline permethylated protein samples were analyzed in selected ion monitoring (SIM) mode to enhance sensitivity and specificity. Molecular ions at *m/z* 174, 188, and 234, representing the molecular weight of compounds 1, 2, and 3, respectively, were selected to monitor methylthionaphthalene derivatives. Structural identification of methylthionaphthalene derivatives, formed in the alkaline permethylation of the protein samples, was conducted by mass matching and comparison of the relative retention times of peaks observed in the reconstructed ion chromatograph with those of the synthetic authentic standards, to the internal standard. In addition, following the first-round analysis, the remaining sample was consecutively



2-methylthionaphthalene

SCHEME 1

spiked with synthetic authentic standards. The resulting spiked sample was reanalyzed by GC-MS in the same SIM mode, for further structural confirmation. Quantification was performed by integration of peak area followed by standardization in consideration of mass response factors and alkaline permethylation yields. The area of peak 2 (compound 2) was used as a quantitative internal standard.

Electrophoresis and Immunoblotting.

Sf-21 cell proteins were resolved by SDS-polyacrylamide gel electrophoresis (Mini-Protean II, Bio-Rad) as described (Laemmli, 1970) using 3.3% stacking and 12% resolving gels. Proteins were transferred to polyvinylidene difluoride (PVDF) microporous membranes (Immobilon-P transfer membranes, Millipore) by an electroblotter (LKB Novablot electrophoretic transfer kit). After 3 h, transferred (43 mA) blots were blocked by shaking overnight in 5% non-fat dry milk in phosphate buffered saline Tween (PBST) buffer. The blotted polyvinylidene difluoride membranes were incubated for 1 h with a

1/2000 dilution of primary anti-1,2-epoxynaphthalene mercapturate rabbit antiserum (Marco *et al.*, 1993) in PBST buffer with 3% non-fat milk. The immunoblots were incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (1/4,000 in PBST buffer, Sigma). The blotted protein bands were detected by chemiluminescence using ECL Western blotting kits (Amersham International plc, England). Protein staining was performed using Coomassie blue.

RESULTS

All results are mean \pm standard deviation.

Toxicity of Naphthalene

Sf-21 cells expressing CYP1A1 (150 ± 15 pmol/min/mg protein) or Lac Z were incubated with various concentrations of naphthalene. Cell viability was measured using MTT. The toxicity of naphthalene to CYP1A1 expressing Sf-21 cells was dose dependent ($LC_{50} = 0.3 \pm 0.04$ mM), while Lac Z expressing cells were unaffected (Fig. 1A). The 24-h time dependence ($LT_{50} = 12 \pm 1$ h, time for 50% loss of viability) of 1.5 mM naphthalene toxicity to CYP1A1 expressing Sf-21 cells is described by Figure 1B. LC_{50} and LT_{50} were determined by regression analysis. Because of the lack of toxicity to Lac Z-expressing cells, it appears that naphthalene toxicity in this system requires the presence of CYP1A1.

Glutathione Levels

Sf-21 cells expressing CYP1A1 were incubated with 1.5 mM naphthalene and assayed over 24 h. GSH levels dropped significantly over time from 90 ± 3.1 μ g GSH/mg protein to 42 ± 1.8 μ g GSH/mg protein as measured by DTNB conjugation. This represents a 47% decrease in GSH levels from cells incubated with a vehicle control. Additionally, CYP1A1 expressing cells incubated with 0.125 mM DEM or naphthalene and 0.125 mM DEM showed a similar decrease in GSH levels (Fig. 2). These data suggest that 1,2-epoxynaphthalene may bind GSH. GSH levels were verified by HPLC and found

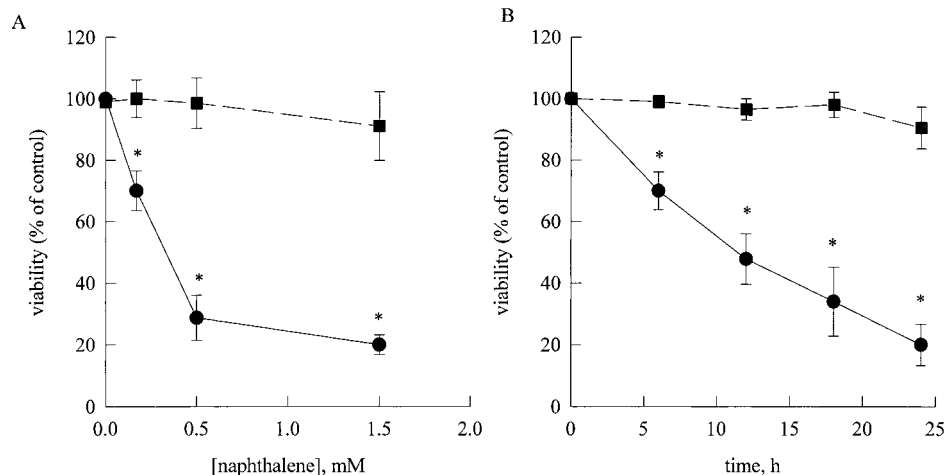


FIG. 1. Dose- and time-dependent toxicity of naphthalene. The toxicity of naphthalene to CYP1A1 expressing Sf-21 cells increases with concentration at 24 h after administration (A). Cytotoxicity is time-dependent with 1.5 mM naphthalene (B). Naphthalene was not significantly toxic to Lac Z-expressing cells. Each point represents mean and vertical bars and standard deviations ($n \geq 3$). Significant differences between CYP1A1 (circle) and Lac Z (square) expressing cells are indicated; $*(p < 0.01)$.

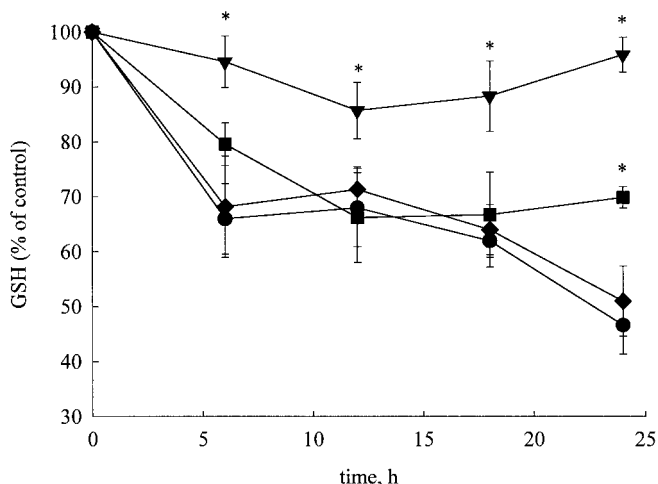


FIG. 2. Effects of naphthalene and DEM on GSH levels. Sf-21 cells expressing CYP1A1 were treated with either 1.5 mM naphthalene (circle), a vehicle control (triangle), 0.125 mM DEM (square), or 0.125 mM DEM and 1.5 mM naphthalene (diamond). Intracellular GSH in the cells treated with 1.5 mM naphthalene decreased to 47% of its original level ($90 \pm 3.1 \mu\text{g}$ GSH/mg protein), while intercellular GSH in cells treated with the vehicle control did not significantly change. GSH levels in the media ($21 \pm 8.1 \mu\text{g}$ GSH/mg protein) also did not change significantly over time (data not shown). Each point represents mean and vertical bars are standard deviations ($n \geq 3$). Significant differences from naphthalene treated cells are indicated, $*(p \leq 0.05)$.

to be $82.0 \pm 3.1\%$ of that measured by DTNB. The percentage values indicated here reflect actual GSH levels. To assess the relevance of GSH depletion, cells expressing either CYP1A1 or Lac Z were preincubated with GSH, ethyl ester GSH, DEM, or media as a vehicle control, for 2 h, followed by exposure to various concentrations of naphthalene for 24 h. Cell viability was measured using MTT. Both ethyl ester GSH and GSH decreased cytotoxicity, but ethyl ester GSH provided more potent protection, increasing cell viability by 30% at the highest naphthalene concentration. Conversely, DEM increased the potency of naphthalene toxicity to CYP1A1 expressing cells, decreasing cell viability by 50% at low naphthalene concentrations (Fig. 3). Thus, it appears that GSH is involved in protecting the cells from the toxicity of 1,2-epoxynaphthalene. Using CDNB conjugation, we were unable to detect any glutathione transferases in whole Sf-21 cell homogenate, whether the cells were expressing CYP1A1 or Lac Z. This is under conditions where we could easily see conjugation of $0.03 \mu\text{mol}/\text{min}/\text{mg}$ protein; thus there appeared to be little or no glutathione transferase activity.

Analysis of Naphthalene Covalent Adducts

Aliquots from both the dose and time-course experiments described above were independently dialyzed and derivatized by alkaline permethylation. This method generates methylthionaphthalene derivatives that are structurally dependent on the nature of the naphthalene reactive metabolites modifying cys-

teine residues (Scheme 1). The derivatives were analyzed by GC-MS and compared to authentic standards; quantification was achieved by reference to an internal standard, ethylthionaphthalene (compound 2). Only CYP1A1-expressing cells yielded a dominant peak with a mass of m/z 174 and a retention time of approximately 9.75 min, corresponding to 2-methylthionaphthalene (compound 1) (Fig. 4A). This indicates that CYP1A1 metabolizes naphthalene to 1,2-epoxynaphthalene, and this metabolite covalently binds cellular sulfhydryl groups. In the absence of CYP1A1-expressing cells, protein adducts were not detected. The lack of 3,4-dimethoxymethylthionaphthalene (compound 3), for example, indicates that neither the 1,2- nor the 1,4-naphthoquinone are made in appreciable amounts in this system.

Binding of Naphthalene Metabolites to Cellular Proteins

Protein from CYP1A1- or Lac Z-expressing Sf-21 cell homogenate was incubated with various concentrations of naphthalene. The proteins were separated on a 12% SDS-polyacrylamide gel, transferred to PVDF microporous membranes, and detected with antibodies raised against 1,2-epoxynaphtha-

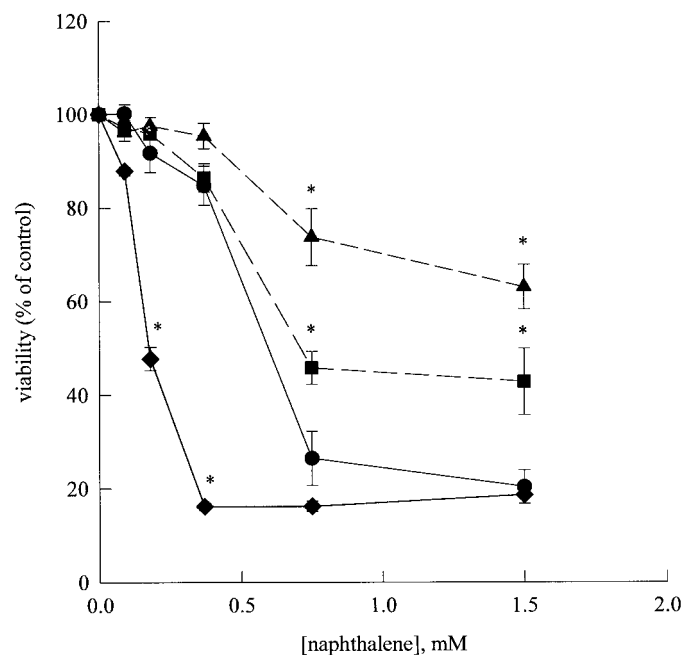


FIG. 3. Effects of ethyl ester GSH, GSH, and DEM on cell viability. CYP1A1-expressing cells were pretreated with either 5 mM ethyl ester GSH (triangle), 5 mM GSH (square), 0.125 mM DEM (diamond), or a vehicle control (circle) 2 h before 24-h incubation with naphthalene. Both ethyl ester GSH and GSH protected CYP1A1-expressing cells from the toxicity of high concentrations of naphthalene, but ethyl ester GSH was 35% more protective than GSH and 50% more protective than the vehicle control at 1.5 mM naphthalene. Conversely, DEM decreased cell viability by 50% at low naphthalene concentrations. Each point represents mean and vertical bars are standard deviations ($n \geq 3$). Significant differences from the vehicle control are indicated, $*(p < 0.05)$.

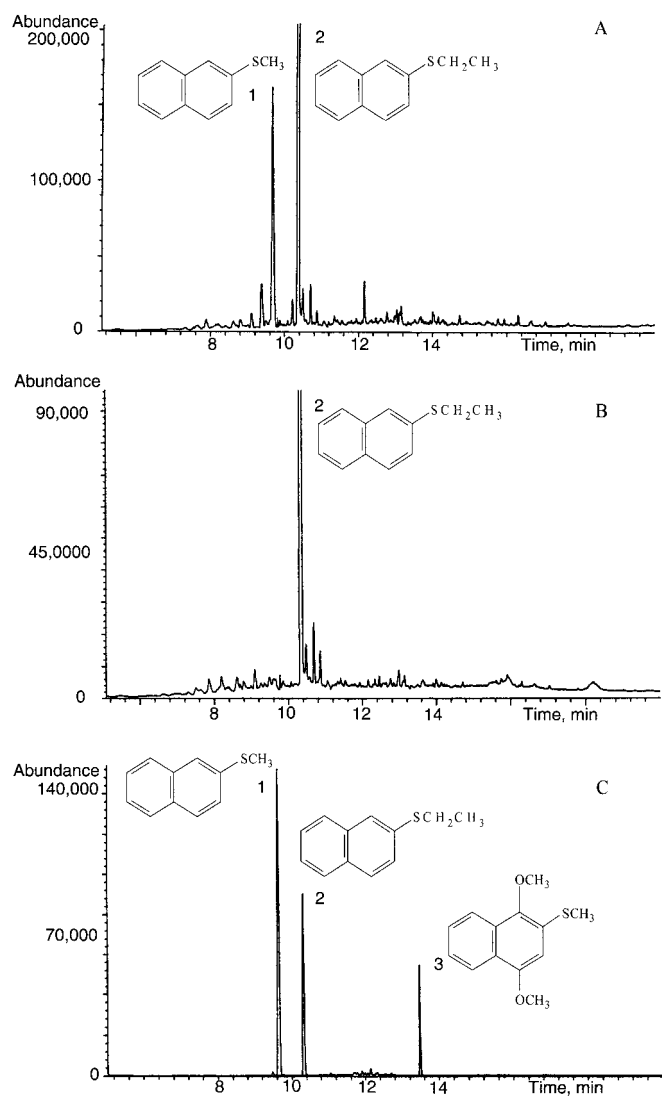


FIG. 4. GC-MS selected ion chromatograms of independently derivatized dialysate of CYP1A1 expressing cells (A) and Lac Z-expressing cells (B) incubated with 1.5 mM naphthalene for 24 h. C shows the authentic standards. Ion currents at m/z 174,188, and 234 were continuously monitored for each GC-MS run. Only the CYP1A1-expressing samples contained a fragment with a molecular weight of 174 g/mol, corresponding to compound 1, peak 1. Both sets of cells were spiked with compound 2 (peak 2), as an internal standard. Compound 3 (peak 3) was not present; it had a molecular weight of 234 g/mol and a retention time of approximately 13.5 min.

lene-derived mercapturic acid (Marco *et al.*, 1993) (Fig. 6). For the higher concentrations of naphthalene, bands at 43, 55, and 58 kDa were observed. The 58 kDa band reacted strongly with the anti-epoxynaphthalene mercapturate antibody. The Lac Z homogenate incubated with naphthalene and CYP1A1 homogenate incubated with lower concentrations of naphthalene showed no reaction to the antibody. Additionally, none of the lanes showed a band at 120 kDa, the molecular weight of the CYP1A1/yeast reductase fusion protein, or at 116 kDa, the molecular weight of Lac Z. This suggests that naphthalene is

metabolized by CYP1A1 but does not covalently bind to the fusion protein or to the Lac Z control protein. The Coomassie-stained gel shows bands around 120 kDa and additionally a strong band around 60 kDa, the molecular weight of bovine serum albumin, which is present in the cell media (Fig. 7).

A similar study performed with antibodies raised to protein-bound quinone metabolites of naphthalene (Zheng *et al.*, 1997) failed to detect any protein binding. Thus, the immuno-chemical information supports the GC-MS data that all protein binding is due to the reactive epoxide metabolite.

The time-dependent toxicity of cells intoxicated with 1.5 mM naphthalene was strongly correlated (r^2 : 0.85) with covalent binding (Fig. 5). Their maxima are reached at 16 h. Additionally, covalent binding was highly correlated (r^2 : 0.96) with the dose dependent toxicity of naphthalene (data not shown). Additionally, radioactive binding studies with 0.5 mM naphthalene revealed that $3 \pm 0.2\%$ of naphthalene is covalently bound in cells expressing CYP1A1, while no naphthalene is bound in cells expressing Lac Z. This 3% covalent binding equates to approximately 20 nmol of naphthalene equivalent bound per mg of non-dialyzable cellular protein. This value correlates well with the 28 nmol/mg naphthalene bound using the alkaline permethylation method.

DISCUSSION

We demonstrated in this study that toxicity to CYP1A1-expressing cells is dose- and time-dependent and that naphtha-

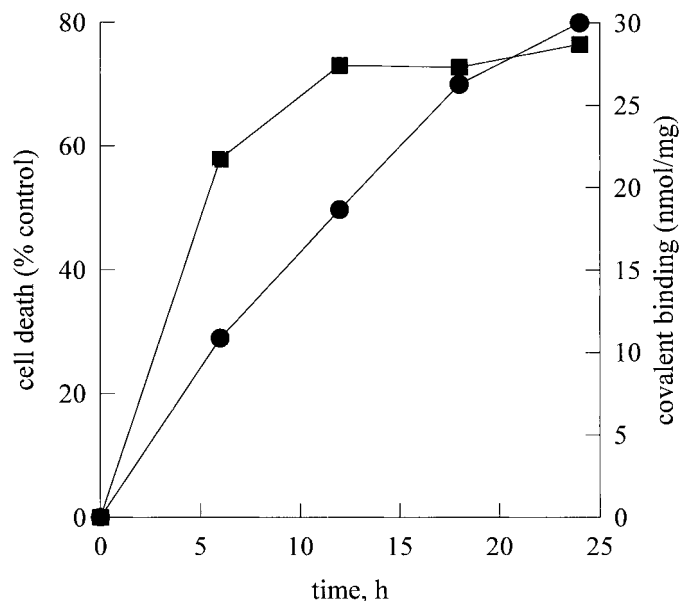


FIG. 5. Time-dependent correlation of covalent binding and cytotoxicity in naphthalene-incubated, CYP1A1-expressing Sf-21 cells. Covalent binding (square) was strongly correlated with cytotoxicity (circle) when CYP1A1-expressing cells were incubated with 1.5 mM naphthalene and aliquots taken over time. Covalent binding at 24 h accounted for $3 \pm 0.2\%$ of total administered naphthalene, which corresponds to the covalent binding seen using alkaline permethylation.



FIG. 6. Western blot of homogenates of CYP1A1- or Lac Z-expressing Sf-21 cells incubated with various concentrations of naphthalene for 24 h. From left, CYP1A1-expressing Sf-21 cells incubated with 1.5 mM (lane 1), 0.5 mM (lane 2), 0.17 mM (lane 3), 0.08 mM (lane 4), Lac Z-expressing Sf-21 cells incubated with 1.5 mM (lane 5) naphthalene. The band at 58 kDa in lanes 1 and 2 reacted strongly with the anti-naphthalene mercapturate antibody. Otherwise, identical Western blots with an antibody that detects thioether-linked quinones (Zheng *et al.*, 1997) failed to detect any proteins (data not shown).

lene is not toxic to Sf-21 cells not expressing this enzyme; thus, CYP1A1 bioactivates naphthalene to a toxic epoxide metabolite in this system. Additionally, the depletion of GSH over time in CYP1A1 cells challenged with naphthalene, the ability of ethyl ester GSH to protect CYP1A1-expressing cells from naphthalene-induced toxicity, and the increased toxicity in CYP1A1-expressing cells treated with DEM and naphthalene suggest that GSH plays a crucial role in cellular protection.

Because of the likelihood of CYP1A1 induction in humans and their potential exposure to naphthalene, we decided to examine the interaction of CYP1A1 and naphthalene without the confounding factors of other xenobiotic metabolizing enzymes. The Sf-21 cell-baculovirus system is ideal for that. Sf-21 cells are noctuid ovarian cells and baculoviruses are insect-specific viruses; thus, toxicity, covalent binding, and cellular co-factors can be assessed *in situ*, but the difficulties of working with whole animals, or mammalian cell culture, are removed. The transgenic baculovirus assay system has the disadvantages inherent to any cell-based system. The cells must be transiently transfected and there is a narrow window of time (24–48 h) in which *in situ* toxicity assays may be done. This is also a very simple model for a very complex system—a whole animal. However, when teasing apart the effects of particular metabolizing enzymes, this can be an advantage. These cells have very low levels of many endogenous enzymes involved in xenobiotic metabolism, including undetectable, or very low levels of, CYP monooxygenases, epoxide hydrolases (Grant *et al.*, 1996), glucuronosyl transferases (Nguyen and Tukey, 1997), and esterases (Charles *et al.*, 1996), among others. Thus, effects can be attributed solely to the reaction of the enzyme and the compound of interest. The baculovirus expression system is widely used in the pharmaceutical industry to express human CYP monooxygenases to generate possible metabolites of xenobiotics (Gonzalez, F.J. *et al.*, 1991). Grant *et al.* (1996) extended the concept of expression of CYP monooxygenases to the development of a simple system for examining geno- and cytotoxicity of reactive metabolites generated by CYP monooxygenases and other enzymes.

Others have seen the depletion of GSH in the presence of naphthalene metabolites such as the epoxide (Buckpitt, A. *et*

al., 1992), especially in the presence of glutathione transferases (Chichester *et al.*, 1994), and the quinone (Iverson, *et al.*, 1995; Murty and Penning, 1992). However, the absence of GSH depletion by lower concentrations of the epoxide in human lymphocytes has been reported as well (Wilson *et al.*, 1996), and cytotoxicity and GSH depletion have been attributed solely to the quinone metabolite (Wilson *et al.*, 1996). This confusion is compounded by the multiple enzymes and isozymes present in most cell types. Addition of GSH has been shown to protect liver microsomes from some of the toxicity of naphthalene metabolites (Tingle *et al.*, 1993); however, GSH cannot usually cross cell membranes, whereas ethyl ester GSH can (Nishida *et al.*, 1996). The levels of protection we saw with ethyl ester glutathione in Sf-21 cells were comparable to those seen by Tingle *et al.* (1993) using ethyl ester GSH in a microsomal system. We did see some protection by GSH, which is presumably occurring outside the cell. These data suggest that GSH protects the cells from the epoxide metabolite and depletion occurs in the absence of other clearance mechanisms, as

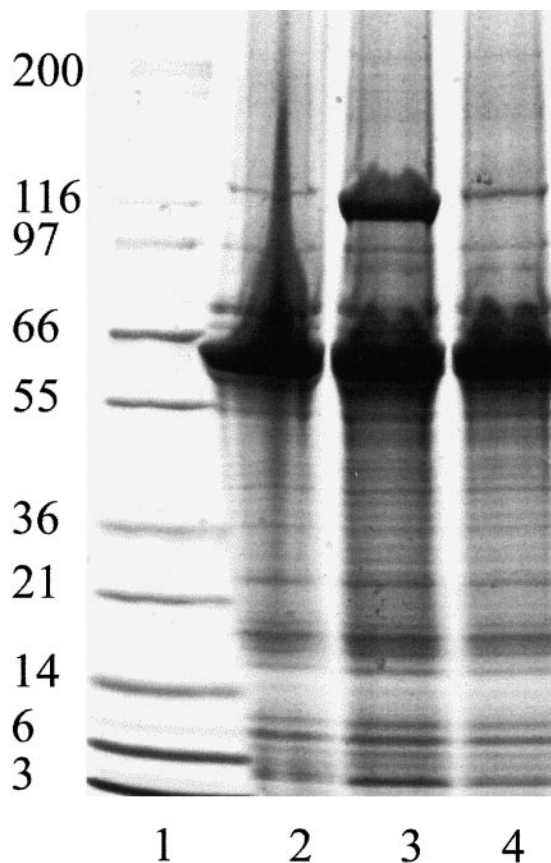
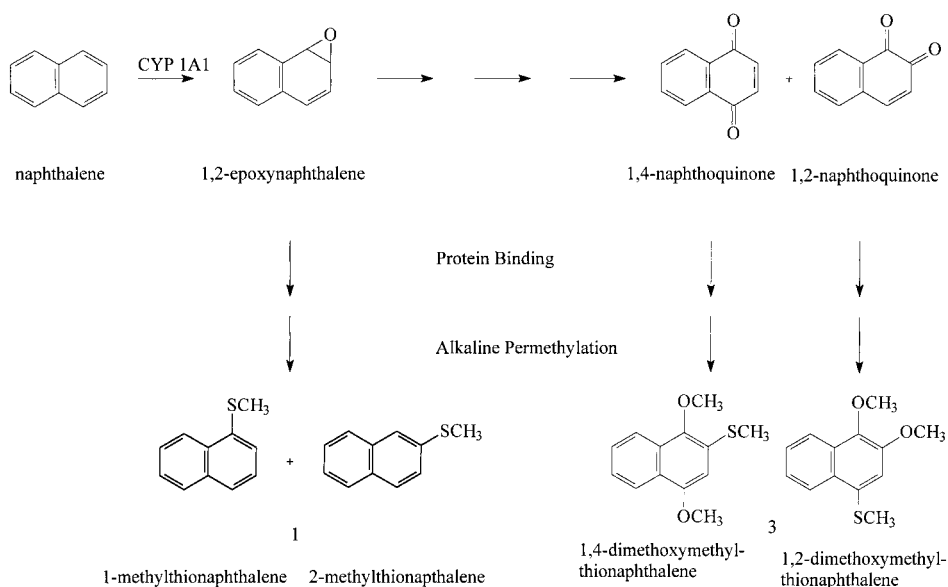


FIG. 7. SDS-PAGE of homogenates of CYP1A1 or Lac Z-expressing Sf-21 cells incubated with or without 0.5 mM naphthalene for 24 h. From left, molecular weight marker (lane 1), CYP1A1-expressing cells incubated with vehicle control (lane 2), Lac Z-expressing cells incubated with 0.5 mM naphthalene (lane 3), CYP1A1-expressing cells incubated with 0.5 mM naphthalene (lane 4). Lac Z migrates at 116 kDa; the CYP1A1-NADPH reductase fusion protein migrates at 120 kDa.



SCHEME 2

may occur in pulmonary tissue. These data are supported by the fact that GSH depletion by DEM increased the potency of naphthalene toxicity to CYP1A1-expressing cells.

Metabolite structure was elucidated from studies of protein-bound naphthalene. This method, alkaline permethylation, detects epoxide and quinone-derived metabolites of aromatic systems covalently bound to sulfur nucleophiles of proteins (Slaughter and Hanzlik, 1991; Slaughter *et al.*, 1993; Zheng *et al.*, 1997). Alkaline permethylation of naphthalene epoxide-cysteine protein adducts would generate 2-methylthionaphthalene (compound 1) (Scheme 2), whereas, alkaline permethylation of quinone-cysteine protein adducts should produce dimethoxymethylthionaphthalenes (compound 3) resulting from either the 1,2- or the 1,4-naphthoquinone alkylation of cysteine residues of proteins (Scheme 2). The results of the alkaline permethylation of the dialysate of CYP1A1-expressing cells incubated with naphthalene show the presence of 2-methylthionaphthalene (compound 1), from the reaction of 1,2-epoxynaphthalene with a cysteine residue (see Scheme 1). Because compound 1 was not present in the alkaline permethylated dialysate of the Lac Z-expressing cells incubated with naphthalene, it must be a metabolite of CYP1A1. Additionally, compounds such as the dimethoxymethylthionaphthalenes, which would result from the alkaline permethylation of the naphthoquinones covalently bound to the sulfur of a cysteine residue, were not found under conditions where ng levels of compound could easily be distinguished from background.

In this system, thiol linked adducts of naphthalene epoxide are thought to be the major covalent adducts. The alkaline permethylation only detects thiol ether-linked protein adducts and estimates of covalent binding based on ^{14}C experiments are quantitatively very similar to results from GC-MS, as shown in the results section. In addition, adducts are detected on Western blot to naphthalene adducts using antibodies highly

selective for thiol adducts of naphthalene 1,2-oxide. Antibodies sensitive for other forms of protein-bound naphthalene did not detect protein adducts from this system.

Covalent binding was highly correlated with both dose- and time-dependent toxicity. The Lac Z-expressing cells showed no evidence of covalent binding of any naphthalene metabolites to cysteine residues, nor did they show any toxicity due to naphthalene at or up to 1 mM. This result further emphasizes that the toxicity of naphthalene in this system is due to enzymatic activation by CYP1A1, followed by protein binding. The dose-dependent binding and toxicity curves displayed a threshold effect. This was also seen in the Western blot. This is what one would expect to see if GSH were protective in this system.

It is not clear to what protein naphthalene is covalently bound. The data from the SDS-PAGE and Western blot suggest that it might simply be bound to albumin. However, the antibody used recognizes the mercapturic acid metabolite of naphthalene but does not cross-react or reacts very weakly (<5%) with a variety of other naphthalene derivatives (Marco *et al.*, 1993). Most importantly, 1,2-epoxynaphthalene does not detectably react with albumin (Cho *et al.*, 1994b). It is notable, however, that recent work in mammalian systems has detected acetaminophen-binding proteins at 44 kDa, identified as a subunit of glutamine synthetase (Bulera *et al.*, 1995), and at 54 kDa, identified as aldehyde dehydrogenase (Landin *et al.*, 1996). However, 58 kDa and 56 kDa proteins were most strongly bound by acetaminophen and have been identified as acetaminophen-binding protein and selenium-binding protein (Bartolone *et al.*, 1992; Cohen *et al.*, 1997; Pumford *et al.*, 1992).

Zheng *et al.* (1997) found both dimethoxymethylthionaphthalenes and methylthionaphthalene after alkaline permethylation of the dialysate of Clara cells incubated with naphthalene, indicating the presence of both the 1,2-naphthoquinone and

1,2-epoxynaphthalene as reactive metabolites. Clara cells are known to express multiple CYP monooxygenases (Buckpitt *et al.*, 1995). Since we saw only saw a derivative of the epoxide metabolite, we propose that the naphthoquinone does not spontaneously generate under these conditions; enzymes needed for its production were absent and it was not formed by CYP1A1.

These data lead us to speculate that 1,2-epoxynaphthalene is short-lived due to reaction with water, soluble epoxide hydrolase, and other enzymes. When enzyme systems are present that generate longer-lived quinone metabolites, Michael reactions with quinones predominate in cytotoxicity and in depleting GSH. Thus, we have used the baculovirus-Sf-21 cell system to isolate continuous *in situ* production of 1,2-epoxynaphthalene from other reactions, and demonstrate that the epoxide itself is capable of cytotoxicity and GSH depletion. This further validates the baculovirus-Sf-21 cell system.

In conclusion, humans are exposed to naphthalene, which is metabolized by many CYP monooxygenases. In mice, selective pulmonary lesions appear to lead to death following naphthalene exposure (Buckpitt *et al.*, 1995; Cho *et al.*, 1994a). However, we do not know the primary intracellular target of naphthalene nor how this binding initiates a series of events leading to cell death. While constitutively expressed CYP monooxygenases are capable of metabolizing naphthalene, induction of CYP1A1 in exposed individuals may enhance risk. Therefore, using a baculovirus expression system, we have shown that CYP1A1 expression dramatically increases cell death, glutathione depletion, and the formation of covalent adducts of naphthalene linked to protein through a thioether in position 2. The toxicity and covalent binding of naphthalene adducts is especially important when considering the exposure of smokers with induced lung CYP1A1 exposed to naphthalene in cigarette smoke.

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REFERENCES

- Arey, J., Zielinska, B., Atkinson, T., and Winer, A. (1987). Polycyclic aromatic hydrocarbon and nitroarene concentrations in ambient air during a wintertime high NO_x episode in the Los Angeles basin. *Atmos. Environ.* **21**, 1437–1444.
- Bartolone, J. B., Birge, R. B., Bulera, S. J., Bruno, M. K., Nishanian, E. V., Cohen, S. D., and Khairallah, E. A. (1992). Purification, antibody production, and partial amino acid sequence of the 58-kDa acetaminophen-binding liver proteins. *Toxicol. Appl. Pharmacol.* **113**, 19–29.
- Bieniek, G. (1994). The presence of 1-naphthol in the urine of industrial workers exposed to naphthalene. *Occup. Environ. Med.* **51**, 357–359.
- Booth, J., Boyland, E., Sato, T., and Sims, P. (1960). Metabolism of polycyclic compounds: 17. The reaction of 1,2-dihydronaphthalene and 1,2-epoxy-1,2,3,4-tetrahydronaphthalene with glutathione catalyzed by tissue preparations. *Biochem. J.* **77**, 182–186.
- Bourne, M. C., and Young, L. (1934). The metabolism of naphthalene in rabbits. *Biochem. J.* **28**, 803–808.
- Boyland, E., and Sims, P. (1960). Metabolism of polycyclic compounds: 16. The metabolism of 1,2-dihydronaphthalene and 1,2-epoxy-1,2,3,4-tetrahydronaphthalene. *Biochem. J.* **77**, 175–181.
- Buckpitt, A., Buonarati, M., Avey, L. B., Chang, A. M., Morin, D., and Plopper, C. G. (1992). Relationship of cytochrome P450 activity to Clara cell cytotoxicity: II. Comparison of stereoselectivity of naphthalene epoxidation in lung and nasal mucosa of mouse, hamster, rat, and rhesus monkey. *J. Pharmacol. Exp. Ther.* **261**, 364–72.
- Buckpitt, A., Chang, A. M., Weir, A., Van Winkle, L., Duan, X., Philpot, R., and Plopper, C. (1995). Relationship of cytochrome P450 activity to Clara cell cytotoxicity: IV. Metabolism of naphthalene and naphthalene oxide in microdissected airways from mice, rats, and hamsters. *Mol. Pharmacol.* **47**, 74–81.
- Buckpitt, A. R., Smart, G., and Baker, B. (1982). Pulmonary bronchiolar damage of naphthalene administered by inhalation. *Fed. Proc.* **41**, 7997–7999.
- Buckpitt, A. R., and Warren, D. L. (1983). Evidence for hepatic formation, export, and covalent binding of reactive naphthalene metabolites in extra-hepatic tissues *in vivo*. *J. Pharmacol. Exp. Ther.* **226**, 8–16.
- Bulera, S. J., Birge, R. B., Cohen, S. D., and Khairallah, E. A. (1995). Identification of the mouse liver 44-kDa acetaminophen-binding protein as a subunit of glutamine synthetase. *Toxicol. Appl. Pharmacol.* **134**, 313–320.
- Burke, M. D., and 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.
- Charles, J.-P., Wojtasek, H., Lentz, A. J., Thomas, B. A., Bonning, B. C., Palli, S. R., Parker, A. G., Dorman, G., Hammock, B. D., Prestwich, G. D., and Riddiford, L. M. (1996). Purification and reassessment of ligand binding by the recombinant, putative juvenile hormone receptor of the tobacco hornworm, *Manduca sexta*. *Arch. Insect Biochem. Physiol.* **31**, 371–393.
- Chichester, C. H., Buckpitt, A. R., Chang, A., and Plopper, C. G. (1994). Metabolism and cytotoxicity of naphthalene and its metabolites in isolated murine Clara cells. *Mol. Pharmacol.* **45**, 664–672.
- Cho, M., Chichester, C., Morin, D., Plopper, C., and Buckpitt, A. (1994a). Covalent interactions of reactive naphthalene metabolites with proteins. *J. Pharmacol. Exp. Ther.* **269**, 881–889.
- Cho, M., Jedrychowski, R., Hammock, B., and Buckpitt, A. (1994b). Reactive naphthalene metabolite binding to hemoglobin and albumin. *Fundam. Appl. Toxicol.* **22**, 26–33.
- Cohen, S. D., Pumford, N. R., Khairallah, E. A., Boekelheide, K., Pohl, L. R., Amouzadeh, H. R., and Hinson, J. A. (1997). Selective protein covalent binding and target organ toxicity. *Toxicol. Appl. Pharmacol.* **143**, 1–12.
- Delescluse, C., Ledirac, N., de Sousa, G., Pralavorio, M., Lesca, P., and Rahmani, R. (1998). Cytotoxic effects and induction of cytochromes P450 1A1/2 by insecticides, in hepatic or epidermal cells: Binding capability to the Ah receptor. *Toxicol. Lett.* **96**, 33–39.
- Ellman, G. L. (1959). Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **82**, 70–77.
- Fahey, R., and Newton, G. (1987). Determination of low-molecular-weight thiols using monobromobimane fluorescent labeling and high performance liquid chromatography. *Methods Enzymol.* **143**, 85–96.
- Forkert, P.G. (1997). Conjugation of glutathione with the reactive metabolites of 1, 1-dichloroethylene in murine lung and liver. *Microsc. Res. Tech.* **36**, 234–242.
- Gonzalez, F. J., Kimura, S., Tamura, S., and Gelboin, H. V. (1991). Expression

- of mammalian cytochromes P450 using baculovirus. *Methods Enzymol.* **206**, 93–99.
- Grant, D. F., Bender, D. M. and Hammock, B. D. (1989). Quantitative kinetic assays for glutathione S-transferases and general esterase in individual mosquitoes using an EIA reader. *Insect Biochem.* **19**, 741–751.
- Grant, D. F., Greene, J. F., Pinot, F., Borhan, B., Moghaddam, M. F., Hammock, B. D., McCutchen, B., Ohkawa, H., Luo, G., and Guenther, T. M. (1996). Development of an *in situ* toxicity assay system using recombinant baculoviruses. *Biochem. Pharmacol.* **51**, 503–515.
- Griffin, K. A., Johnson, C. B., Breger, R. K., and Franklin, R. B. (1983). Toxicity of 2-methylnaphthalene with observations on the hepatic and pulmonary formation of isomeric dihydrodiols in DBA/2J mice. *Toxicologist* **26**, 213–230.
- Gupta, P., Harger, W. P., and Arey, J. (1996). The contribution of nitro- and methylnitronaphthalenes to the vapor-phase mutagenicity of ambient air samples. *Atmos. Environ.* **30**, 3157–3166.
- Hill, R. H., Jr., Head, S. L., Baker, S., Gregg, M., Shealy, D. B., Bailey, S. L., Williams, C. C., Sampson, E. J., and Needham, L. L. (1995). Pesticide residues in urine of adults living in the United States: Reference range concentrations. *Environ. Res.* **71**, 99–108.
- Iverson, S. L., Hu, L. Q., Vukomanovic, V., and Bolton, J. L. (1995). The influence of the *p*-alkyl substituent on the isomerization of *o*-quinones to *p*-quinone methides: Potential bioactivation mechanism for catechols. *Chem. Res. Toxicol.* **8**, 537–544.
- Juchau, M. R., Boutelet-Bochan, H., and Huang, Y. (1998). Cytochrome-P450-dependent biotransformation of xenobiotics in human and rodent embryonic tissues. *Drug Metab. Rev.* **30**, 541–568.
- Kirk, R. and Othmer, D. (1981). Naphthalene. In *Kirk-Othmer Encyclopedia of Chemical Technology*, pp. 698–719. Wiley, New York.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembling of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Landin, J. S., Cohen, S. D., and Khairallah, E. A. (1996). Identification of a 54-kDa mitochondrial acetaminophen-binding protein as aldehyde dehydrogenase. *Toxicol. Appl. Pharmacol.* **141**, 299–307.
- Marco, M. P., Nasiri, M., Kurth, M. J., and Hammock, B. D. (1993). Enzyme-linked immunosorbent assay for the specific detection of the mercapturic acid metabolites of naphthalene. *Chem. Res. Toxicol.* **6**, 284–293.
- McLemore, T. L., Adelberg, S., and Liu, M. C. (1990). Expression of CYP1A1 gene in patients with lung cancer: Evidence for cigarette smoke-induced gene expression in normal lung tissue and for altered gene regulation in primary pulmonary carcinomas. *J. Natl. Cancer Inst.* **82**, 1333–1339.
- Mumford, J. L., Harris, D. B., Williams, K., Chuang, J. C., and Cooke, M. (1987). Indoor air sampling and mutagenicity studies of emissions from unvented coal combustion. *Environ. Sci. Technol.* **21**, 308–311.
- Murty, V. S., and Penning, T. M. (1992). Polycyclic aromatic hydrocarbon (PAH) ortho-quinone conjugate chemistry: Kinetics of thiol addition to PAH ortho-quinones and structures of thioether adducts of naphthalene-1,2-dione. *Chem. Biol. Interact.* **84**, 169–188.
- Nguyen, N., and Tukey, R. (1997). Baculovirus-directed expression of rabbit UDP-glucuronosyltransferases in *Spodoptera frugiperda* cells. *Drug Metab. Disp.* **25**, 745–749.
- Nishida, K., Ohta, Y., Ito, M., Nagamura, Y., Kitahara, S., Fujii, K., and Ishiguro, I. (1996). Conversion of gamma-glutamylcysteinylethyl ester to glutathione in rat hepatocytes. *Biochim. Biophys. Acta* **1313**, 47–53.
- Nishioka, M. C., Petersen, B. A., and Lewtas, J. (1982). Comparison of nitroaromatic content and direct-acting mutagenicity of diesel emissions. In *Polynuclear Aromatic Hydrocarbons: Physical and Biological Chemistry*. (M. Cooke, A. J. Dennis, and G. L. Fisher, Eds.), pp. 603–613. Battelle Press, Columbus, OH.
- Omiecinski, C. J., Redlich, C. A., and Costa, P. (1990). Induction and developmental expression of cytochrome P4501A1 messenger RNA in rat and human tissues: Detection by the polymerase chain reaction. *Cancer* **50**, 4315–4321.
- Papac, D. I., and Franklin, M. R. (1988). N-Benzylimidazole, a high magnitude inducer of rat hepatic cytochrome P-450 exhibiting both polycyclic aromatic hydrocarbon- and phenobarbital-type induction of phase I and phase II drug-metabolizing enzymes. *Drug. Metab. Disp.* **16**, 259–264.
- Pumford, N. R., Martin, B. M., and Hinson, J. A. (1992). A metabolite of acetaminophen covalently binds to the 56 kDa selenium-binding protein. *Biochem. Biophys. Res. Commun.* **182**, 1348–1355.
- Schmeltz, I., Tosk, J., and Hoffman, D. (1976). Formation and determination of naphthalenes in cigarette smoke. *Anal. Chem.* **48**, 645–650.
- Slaughter, D. E., and Hanzlik, R. P. (1991). Identification of epoxide- and quinone-derived bromobenzene adducts to protein sulfur nucleophiles. *Chem. Res. Toxicol.* **4**, 349–359.
- Slaughter, D. E., Zheng, J., Harriman, S., and Hanzlik, R. P. (1993). Identification of covalent adducts to protein sulfur nucleophiles by alkaline permethylation. *Anal. Biochem.* **208**, 288–295.
- Stephen, F. D., Drahusuk, A. T., and Olson, J. R. (1997). Cytochrome P450 1A1 induction in rat lymphoid tissues following *in vivo* and *in vitro* exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin requires protein kinase C. *Toxicology* **124**, 39–51.
- Tingle, M. D., Pirmohamed, M., Templeton, E., Wilson, A. S., Madden, S., Kitteringham, N. R., and Park, B. K. (1993). An investigation of the formation of cytotoxic, genotoxic, protein-reactive, and stable metabolites from naphthalene by human liver microsomes. *Biochem. Pharmacol.* **46**, 1529–1538.
- Van Winkle, L. S., Buckpitt, A. R., and Plopper, C. G. (1996). Maintenance of differentiated murine Clara cells in microdissected airway cultures. *Am. J. Respir. Cell. Mol. Biol.* **14**, 586–598.
- Warner, M., Hellmold, H., Magnusson, M., Rylander, T., Hedlund, E., and Gustafsson, J. (1998). Extrahepatic cytochrome P450: Role in *in situ* toxicity and cell-specific hormone sensitivity. *Arch. Toxicol.* **20**(Suppl.), 455–463.
- Wilson, A. S., Davis, C. D., Williams, D. P., Buckpitt, A. R., Pirmohamed, M., and Park, B. K. (1996). Characterization of the toxic metabolite(s) of naphthalene [published erratum appears in *Toxicology* 1997 **120**(1), 75]. *Toxicology* **114**, 233–242.
- Witschi, H. R., and Last, J. A. (1996). Toxic responses of the respiratory system. In *Casarett & Doull's Toxicology: The Basic Science of Poisons* (C. D. Klaassen, Ed.), pp. 443–462. McGraw-Hill, New York.
- Young, L. (1947). The metabolic conversion of naphthalene to 1,2-dihydronaphthalene-1,2-diol. *Biochem. J.* **41**, 417–422.
- Zheng, J., Cho, M., Jones, A. D., and Hammock, B. D. (1997). Evidence of quinone metabolites of naphthalene covalently bound to sulfur nucleophiles of proteins of murine Clara cells after exposure to naphthalene. *Chem. Res. Toxicol.* **10**, 1008–1014.