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Stability of Hemoglobin and Albumin Adducts of Naphthalene Oxide, 1,2-Naphthoquinone, and 1,4-Naphthoquinone

Melissa A. Troester,* Andrew B. Lindstrom,† Suramya Waidyanatha,* Lawrence L. Kupper,‡ and Stephen M. Rappaport*,[1]

**Department of Environmental Sciences and Engineering, School of Public Health, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7400; †National Exposure Research Laboratory, U.S. Environmental Protection Agency, MD-44, Research Triangle Park, North Carolina 27711; and ‡Department of Biostatistics, School of Public Health, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7400*

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Naphthalene is an important industrial chemical, which has recently been shown to cause tumors of the respiratory tract in rodents. It is thought that one or more reactive metabolites of naphthalene, namely, naphthalene-1,2-oxide (NPO), 1,2-naphthoquinone (1,2-NPQ), and 1,4-naphthoquinone (1,4-NPQ) contribute to the tumorigenicity of this chemical. These electrophiles are all capable of covalent binding to macromolecules including DNA and proteins. The stability of cysteinyl adducts of NPO, 1,2-NPQ, and 1,4-NPQ were investigated in both hemoglobin (Hb) and albumin (Alb) of male F344 rats following a single administration of 2 different doses (400 or 800 mg naphthalene per kg body weight). To assess the stability of Alb adducts, we compared the rates of NPO-Alb turnover (half-life of approximately 2 days) and 1,2-NPQ-Alb (half-life of approximately 1 day) to the normal turnover rate of Alb in the rat (half-life = 2.5–3 days). Based on the rapid turnover of these adducts relative to Alb itself, we concluded that they were unstable. However, the stability of Alb adducts was not affected by the dose of naphthalene administered (400 or 800 mg/kg). In contrast, NPO-Hb adducts were relatively stable (rate constant of adduct instability ≤ 0.01) following a 400 mg/kg dose of naphthalene, but their stability could not be estimated following an 800 mg/kg dose due to high variability among animals. The rate constants of adduct instability obtained in this study allow application of NPO and NPQ adducts to estimate the exposure to reactive electrophilic metabolites of naphthalene in the rat. In addition, some limitations of current methods for estimating adduct instability were identified.

Key Words: **naphthalene-1,2-oxide; 1,2-naphthoquinone; 1,4-naphthoquinone; tumorigenicity; rat; hemoglobin; albumin.**

Naphthalene is an important industrial chemical, with annual use in the United States expected to reach 1.15×10^5 metric tons in 2001 (NTP, 2000). Naphthalene is also a constituent of diesel fuel and jet fuel (McDougal *et al.,* 2000), and is the simplest member of the family of polycyclic aromatic hydrocarbons that are formed as byproducts of combustion. Thus, there are many environmental sources of naphthalene, including fuels, cigarette smoke (Schmeltz *et al.,* 1976), and vehicle exhaust (Clark *et al.,* 1982).

The acute effects of human exposure to naphthalene include hemolytic anemia (Santhanakrishnan *et al.,* 1973) and cataracts (van Heyningen and Pirie, 1967). Rodents are less susceptible to naphthalene's hemolytic effects (Shopp *et al.,* 1984), but cataracts have been seen in rats exposed to naphthalene (van Heyningen, 1979). Moreover, rodents are susceptible to respiratory toxicity of naphthalene, including nasal adenomas in rats (NTP, 2000), necrosis of pulmonary bronchiolar epithelial cells in mice (Plopper *et al.,* 1992), and pulmonary alveolar/bronchiolar adenomas in female mice (Abdo *et al.,* 1992).

The species-specific toxicity of naphthalene has been attributed to differences in the site and rate of metabolism (O'Brien *et al.,* 1985), however, the ultimate toxic metabolites have not been conclusively identified. Naphthalene is initially metabolized to naphthalene-1,2-oxide (NPO) by P450 NADP(H)-dependent oxidation. Subsequent transformation of NPO yields 1-naphthol, 1,2-dihydro-1,2-dihydroxy-naphthalene, 1,2-naphthoquinone (1,2-NPQ), 1,4-naphthoquinone (1,4-NPQ), and various sulfate, glucuronide, and glutathione conjugates (NTP, 2000). Although NPO has been implicated as the principal toxic metabolite in mouse lung Clara cells (Chichester *et al.,* 1994) and rat lung (Sweeney *et al.,* 1995), 1,2-NPQ is also a major metabolite bound to cysteine residues in mouse lung Clara cells *in vitro* (Zheng *et al.,* 1997). In humans, 1,2-NPQ and 1,4-NPQ caused sister chromatid exchanges in lymphocytes and cytotoxicity to mononuclear leukocytes *in vitro*, but NPO did not demonstrate cytotoxic or genotoxic effects (Wilson *et al.,* 1996).

It is difficult to directly measure reactive electrophilic metabolites such as NPO, 1,2-NPQ and 1,4-NPQ *in vivo*, so we sought assays for protein adducts of these compounds. Our laboratory has previously investigated the use of cysteinyl adducts of hemoglobin (Hb) and albumin (Alb) to assess levels of the analogous benzene metabolites, i.e., benzene oxide (BO;

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[1] To whom correspondence should be addressed. Fax: (919) 966-0521. E-mail: stephen_rappaport@unc.edu.

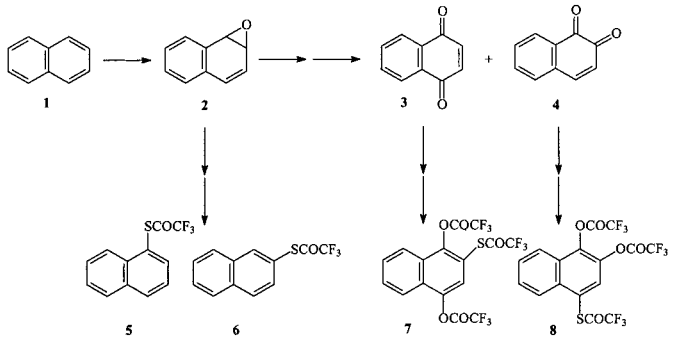

FIG. 1. Assay for measuring cysteinyl adducts formed from reaction of NPO, 1,4-NPQ, and 1,2-NPQ with Hb and Alb. Naphthalene (1) is metabolized to the electrophiles NPO (2), 1,4-NPQ (3), 1,2-NPQ (4). These electrophiles react with protein sulfhydryl groups on Hb and Alb to form adducts. Reaction of proteins with trifluoroacetic anhydride (TFAA) and methanesulfonic acid yields fluorinated derivatives NPO1-S-TFA (5), NPO2-S-TFA (6), 1,4-NPQ-S-TFA (7), and 1,2-NPQ-S-TFA (8). These derivatives can be measured using GC-MS in the negative ion chemical ionization (NICI) mode.

Lindstrom *et al.*, 1998, 1999; Yeowell-O′Connell *et al.*, 1996, 1998), 1,2-benzoquinone (1,2-BQ), and 1,4-benzoquinone (1,4-BQ; Waidyanatha *et al.*, 1998). We extended these methods to measure cysteinyl adducts formed from reactions of NPO, 1,2-NPQ, and 1,4-NPQ with Hb and Alb (Fig. 1; Waidyanatha *et al.*, in press). Using this assay, we demonstrated that these Hb and Alb adducts of NPO, 1,2-NPQ, and 1,4-NPQ increased with dose following a single administration of 0–800 mg naphthalene per kg body weight in F344 rats (Waidyanatha *et al.*, in press).

Dosimetric applications of Hb and Alb adducts require that the first-order rate constants of adduct instability be estimated *in vivo* (Granath *et al.*, 1992). The stabilities of naphthalene-derived Hb and Alb adducts have been partially investigated in mice following ip administration of 400 mg/kg [^{3}H]naphthalene (Cho *et al.*, 1994). In that study, half-lives of 1.8 and 11.5 days were reported for uncharacterized radiolabeled adducts of Alb and Hb, respectively. However, because the stabilities of specific adducts can differ substantially (Troester *et al.*, 2000), we investigated the elimination of Hb and Alb adducts formed from NPO, 1,2-NPQ, and 1,4-NPQ in F344 rats. By applying existing models for estimating the first-order rate constants of adduct instability, we also assessed how well these models describe the true behavior of the adducts.

MATERIALS AND METHODS

Chemicals. Ascorbic acid and hydrochloric acid (conc.) were from Fisher Scientific (Pittsburgh, PA). Acetone and hexane were from Mallinckrodt (Paris, KY). Naphthalene was from Aldrich (Milwaukee, WI). Methoxyflurane was from Pitman-Moore (Mundelein, IL). Trifluoroacetic anhydride (TFAA), obtained from Pierce (Rockford, IL), was distilled once before use. Methanesulfonic acid was from Fluka Chemical Company (Switzerland). Human Hb and Alb, used for standard curves, were from Sigma (St. Louis, MO). 1,4-NPQ-bound N-acetyl-L-cysteine (1,4-NPQ-NAC), 1,2-NPQ-bound N-acetyl-L-cysteine (1,2-NPQ-NAC), and NPO-bound N-acetyl-L-cysteine (2 isomers: NPO1-NAC with sulfur substitution at the 1 position of naphthalene and NPO2-NAC with the sulfur substitution at the 2 position of naphthalene) and deuterium-labeled cysteinyl adducts of 1,2-NPQ, 1,4-NPQ, and NPO with Alb ([$^2H_5$]1,2-NPQ-Alb, [$^2H_5$]1,4-NPQ-Alb, [$^2H_6$]NPO-Alb) were previously prepared using materials and methods described in Waidyanatha *et al.* (in press).

Caution: TFAA reacts violently with water and should only be used to derivatize proteins that are completely dry.

Animals and blood collection. Forty-five male F344 rats (175–200 g) were obtained from Charles River Breeding Laboratories (Raleigh, NC) and were housed in polycarbonate cages on a 12-h light/dark cycle for 2 weeks before use. Food and water were provided *ad libitum.* Corn oil only (vehicle control) was administered by gavage to 3 rats. The remaining 42 rats were divided into 2 groups of 21 animals, and a single po dose of either 400 or 800 mg naphthalene per kg body weight was administered (in corn oil) to each animal by gavage. The 400 mg/kg dose was chosen to allow comparison with the experiments conducted at the same dose by Cho *et al.*, 1994. The 800 mg/kg dose was chosen to be 2-fold higher, but still well below the po LD_{50} (12.8 g/kg) of naphthalene in the rat (Papciak and Mallory, 1990). Three animals from each group were sacrificed at 1, 2, 7, 14, 21, 28, and 42 days following dosing. Rats were anesthetized with methoxyflurane and blood was removed by direct cardiac puncture into a heparinized syringe. Approximately 7–9 ml of blood were collected from each animal. Blood was stored on ice immediately, and plasma and red cell fractions were separated within 2 h.

Isolation and purification of Hb and Alb. Blood samples were centrifuged at 800 × *g* for 15 min and the plasma layer was removed by pipet. The red blood cell layer was washed 3 times with an equal volume of 0.9% saline. The buffy coat was removed by pipet and an equal volume of deionized water was added to the red blood cells. Samples were frozen at –20°C to lyse cells prior to isolation of Hb.

Hb and Alb were isolated according to the procedure in Rappaport *et al.* (Rappaport *et al.*, 1993) with modifications. Briefly, Hb was obtained from thawed, lysed red cells by centrifuging at 30,000 × *g* at 4°C for 40 min to remove the membranes. The supernatant was dialyzed (Spectra-Pore 1, 6000-8000 MWCO) against 4 × 3.5 l of deionized water at 4°C over 24 h (rather than by Sephadex chromatography). Globin was precipitated by dropwise addition of the dialysate to cold acidified acetone (0.1% hydrochloric acid by volume). The precipitate was washed with ice-cold acetone and dried to constant weight under vacuum at 37°C.

To isolate Alb, an equal volume of saturated aqueous ammonium sulfate was added to thawed pure plasma by dropwise addition. This mixture was centrifuged at 800 × *g* to remove immunoglobulins. The supernatant was dialyzed (Spectra-Pore 1, 12,000 MWCO) against 4 × 3.5 l of deionized water at 4°C over 24 h. The Alb solution was lyophilized to constant weight.

Measurement of NPO, 1,2-NPQ, and 1,4-NPQ adducts with Hb and Alb. Cysteinyl adducts of NPO, 1,2-NPQ, and 1,4-NPQ with Hb and Alb were assayed with the method of Waidyanatha *et al.* (in press). Briefly, 100 μl of a 50 mg/ml solution of Hb or 50 μl of a 50 mg/ml solution of Alb were added to 5 μg of deuterium-labeled protein bound internal standard ([$^2H_5$]1,2-NPQ, [$^2H_5$]1,4-NPQ, [$^2H_6$]NPO-Alb). Samples were then dried in a vacuum oven at 80°C for several h. To the dried proteins, 750 μl of TFAA and 20 μl of methanesulfonic acid were added and the proteins were incubated at 100°C for 40 min to produce 1,2-NPQ-S-TFA, 1,4-NPQ-S-TFA, and 2 structural isomers of NPO-S-TFA (NPO1-S-TFA and NPO2-S-TFA; see Fig. 1). The samples were then cooled to room temperature and excess TFAA was removed under a stream of nitrogen. To each sample, 1 ml of hexane was added, followed by 1 ml of 0.1 M Tris buffer (pH 7.5). The mixture was vortexed for 30 s and then centrifuged. The hexane layer was removed and washed twice with 1 ml of deionized water. Samples were then concentrated to 200 μl and transferred to vials for GC-MS analysis. Standard curves for NPO and NPQ adducts were prepared by adding various amounts of a NPO1-NAC, NPO2-NAC, 1,2-NPQ-NAC, and 1,4-NPQ-NAC to 2.5 mg portions of untreated human Alb (Sigma) or 5 mg portions of untreated human Hb (Sigma) and then performing the assay as described above for the experimental samples.

GC/NICI-MS analysis. All GC-MS analyses were conducted in negative-ion chemical ionization (NICI) mode using a Hewlett-Packard 5890 series II plus gas chromatograph equipped with a Hewlett-Packard 5989B MS engine. A DB-5 capillary column (60 m, 0.25-mm i.d, 0.25-μm phase thickness; J & W Scientific, Inc.) was used; 2-μl samples were injected in the splitless mode with a He carrier gas flow of 1.5 ml/min. Methane (source pressure of 2 Torr) was used as the chemical ionization reagent gas.

Analysis of NPO1-S-TFA and NPO2-S-TFA. The injection port and source temperatures were 250 and 100°C, respectively. The oven temperature was held for 2 min at 75°C, then increased at 4°C/min to 160°C where it was held for 15 min, then increased at 50°C/min to 260°C where it was held for 15 min. The molecular ions of NPO1-S-TFA and NPO2-S-TFA (m/z 256) and [$^2H_6$]NPO1-S-TFA and [$^2H_6$]NPO2-S-TFA (m/z 263) were monitored using the selected ion monitoring mode as described in Waidyanatha *et al.* (in press).

Analysis of 1,2-NPQ-S-TFA and 1,4-NPQ-S-TFA. The injection port and source temperatures were 250 and 150°C, respectively. For 1,2-NPQ-Alb, 1,4-NPQ-Alb and 1,4-NPQ-Hb, the oven temperature was held for 2 min at 75°C, then increased at 6°C/min to 150°C where it was held for 28 min, then increased at 50°C/min to 260°C where it was held for 15 min. For 1,2-NPQ-Hb, the oven temperature was held for 2 min at 75°C, then increased at 6°C/min to 145°C where it was held for 35 min, then increased at 50°C/min to 260°C where it was held for 10 min. The molecular ions of 1,2-NPQ-S-TFA and 1,4-NPQ-S-TFA (m/z 383) and [$^2H_5$]1,2-NPQ-S-TFA and [$^2H_5$]1,4-NPQ-S-TFA (m/z 388) were monitored using the selected ion monitoring mode.

Statistical analysis. All statistical analyses were conducted using SAS Statistical Software (Cary, NC). Data obtained prior to peak adduct levels were excluded from linear and nonlinear regression analyses. After peak adduct levels have been obtained, it can be assumed that adduct formation does not significantly contribute to changes in adduct concentration and that the rate of change of adduct concentration is a function of adduct removal only. Standard errors were estimated for all parameters.

Estimation of rates of adduct elimination and adduct instability. Rates of elimination and instability of Alb and Hb adducts were estimated as described in Troester *et al.* (2000, 2001), respectively. We define A(t) = [RY]/[Y] as the amount of adduct RY (nmol) per g of protein (i.e., NPO1-Y, NPO2-Y, 1,2-NPQ-Y, or 1,4-NPQ-Y for Y = Hb or Alb) at some time t (d) after administration of naphthalene. Because the turnover rates of Alb and Hb follow different kinetics, they were modeled differently as described below.

Alb adducts. Following a single dose of naphthalene, Alb adducts are eliminated by first-order kinetics according to the following expression:

$$A(t) = A_0 e^{-k't} \tag{1}$$

where A_0 is the adduct concentration at t = 0 and k′ (d^{-1}) is the first-order rate constant for adduct elimination. (For a review of the derivation of this model, see Granath *et al.,* 1992.) From Equation 1, the relationship between ln[A(t)] and t is linear with intercept $\beta_0 = \ln(A_0)$ and slope $\beta_1 = -k'$, that is,

$$\ln A(t) = \ln A_0 - k't, \tag{2}$$

and k′ can be estimated by linear regression of ln A(t) on t. The rate of adduct elimination (k′) is the sum of the species-specific rate constant of Alb turnover (k_{Alb} in d^{-1}) and the rate constant of adduct instability (k in d^{-1}). That is,

$$k' = k_{Alb} + k. \tag{3}$$

For stable adducts, k = 0 and k′ is equal to k_{Alb}. Thus, adduct stability can be evaluated by comparing estimated values of k′ to known or estimated values of k_{Alb}.

Independent estimates of k′ were obtained for each Alb adduct in rats following administration of either 400 or 800 mg naphthalene per kg body weight. To determine whether k′ differed between the 2 dose groups, a 95% confidence interval for the difference between the two estimates of k′ was calculated using the multiple regression model:

$$\ln A(t) = \beta_0 + \beta_1 t + \beta_2 Z + \beta_3 Z t + E, \tag{4}$$

where E is the error component of the model, and Z is a dummy variable defined as 0 for adducts from the 400 mg/kg dose group and 1 for adducts from the 800 mg/kg dose group. Thus, the straight line models for estimating k′ at 2 different doses are incorporated into Equation 4. When Z = 0 (400 mg/kg dose),

$$\ln A(t) = \beta_0 + \beta_1 t + E, \tag{5}$$

from which $k' = -\beta_1$. When Z = 1 (800 mg/kg dose),

$$\ln A(t) = (\beta_0 + \beta_2) + (\beta_1 + \beta_3)t + E, \tag{6}$$

from which $k' = -(\beta_1 + \beta_3)$. The estimated difference between the two k′ values is therefore given by the coefficient $\hat{\beta}_3$ and the significance of this difference can be judged by the 95% confidence interval for β_3.

Hb adducts. Following a single dose of naphthalene, the elimination of Hb adducts can be described by mixed zero- and first-order kinetics according to:

$$A(t) = A_0\left(1 - \frac{t}{t_{er}}\right)e^{-kt}, \tag{7}$$

where t_{er} (d) represents the lifetime of the erythrocyte and k (d^{-1}) represents the rate constant of adduct instability. (For a review of the derivation of this model, see Granath *et al.*, 1992 and Troester *et al.*, 2001.) Since Equation 7 is nonlinear with respect to time, estimates of A_0 and k were obtained by nonlinear least-squares regression of adduct concentration on time (Troester *et al.*, 2001). However, preliminary inspection indicated that adduct concentrations exhibited heterogeneity of variance at the different times of sacrifice. Thus, we employed generalized least squares using a power function that models the variance of adduct concentration as a function of the mean response at each time point. (For a discussion of generalized least squares and the power function, see Davidian and Gilitinan, 1995.) Since generalized least squares is an iterative model-fitting process, we began by assuming that the variance of adduct concentration was constant across time and used nonlinear least-squares regression along with Equation 7 to estimate the parameters A_0 and k and to predict values of A(t). Then, we used the predicted value of adduct level at time t, $\hat{A}(t)$, to assign a weight ($\hat{w}_t$) to each observation (at each time t) in a second iteration according to the following power function:

$$\hat{w}_t = \frac{1}{(\hat{A}(t))^2}. \tag{8}$$

Iterations were conducted 4 times for NPO1-Hb adducts and 5 times for NPO2-Hb (with $\hat{w}_t$ recalculated using Equation 8 after each iteration); the last two iterations in each case yielded identical estimates and confidence intervals for A_0 and k.

The value of t_{er} was specified *a priori* using strain-specific estimates of t_{er} for the F344 rat (Derelanko, 1987). The estimate of t_{er} for F344 rats is associated with some uncertainty, so nonlinear regression using the approach above was conducted using the point estimate of t_{er} (66 days) for the strain, as well as both the upper and lower 95% confidence interval values for t_{er} (62 days and 70 days, respectively).

In these experiments, the concentration of Hb adducts was followed for 6 weeks following administration. As young animals increase in size, their blood volume increases proportionally and can dilute Hb adducts (Osterman-Golkar *et al.*, 1999). In our study, animals increased in body weight from 261 g (SE = 2.9 g, $n = 6$) at dosing to 301 g (SE = 3.7 g, $n = 6$) 42 days after dosing. Thus, for regression of Hb adducts via Equation 7, the body-weight (b.w.) adjusted

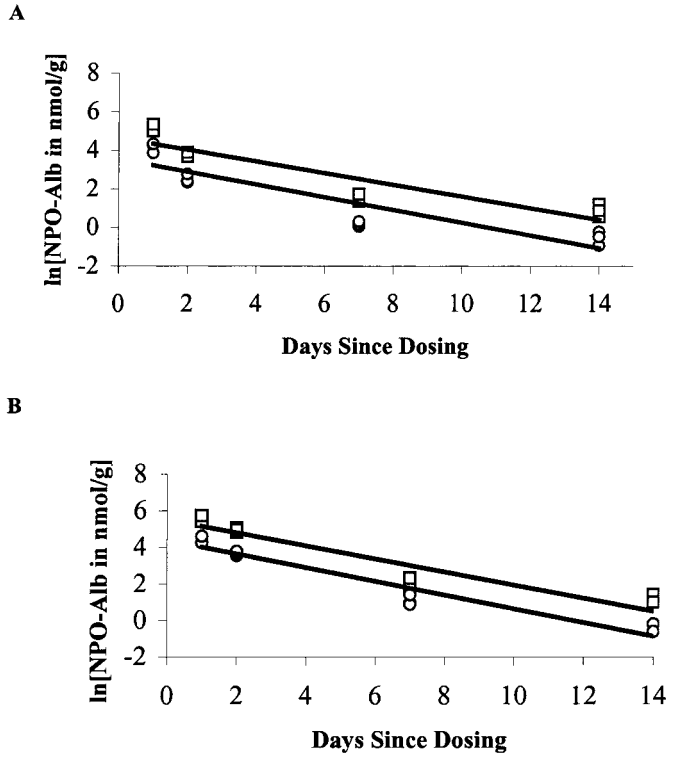

FIG. 2. Elimination of NPO1-Alb (squares) and NPO2-Alb (circles) adducts following administration of (A) 400 and (B) 800 mg naphthalene per kg of body weight. Four time points were included in the regression analysis (Equation 2) for NPO1-Alb and NPO2-Alb, with $n = 3$ for all time points except for the 14 day time point in B where $n = 2$. Lines represent the equations obtained from the least squares regressions of ln(NPO-Alb concentration) on time.

adduct level [$A(t)_{adj}$ in nmol/g] was calculated according to the following relationship (Troester *et al.*, 2001):

$$A(t)_{adj} = A(t) \cdot \frac{bw_{(sacrifice)}}{bw_{(dosing)}}. \quad (9)$$

Then, nonlinear regression of $A(t)_{adj}$ on time (d) was performed according to Equation 7 as described above.

RESULTS

Stability of Alb Adducts

The elimination of NPO-Alb, following administration of a single dose of naphthalene (400 mg/kg or 800 mg/kg) is shown in Figure 2. In both dose groups, NPO-Alb was detectable through day 14, so 4 data points (t = 1, 2, 7, 14 days) were used for regression analysis. Regression of ln A(t) on time yielded the parameter estimates summarized in Table 1. The estimated rate constant of adduct elimination (k′) ranged from 0.30–0.38 for the structural isomers of NPO-Alb (NPO1-Alb and NPO2-Alb); these rates correspond to half-lives of approximately 2 days. There was no significant difference between dose groups for estimates of k′ obtained for NPO1-Alb ($p = 0.43$) or NPO2-Alb ($p = 0.48$).

TABLE 1
Parameter Estimates for the Elimination of NPO-Alb Adducts (Equation 2)

	400 mg/kg		800 mg/kg	
	NPO1	NPO2	NPO1	NPO2
$\ln \hat{A}_0$ [a]	4.65 (0.35)	3.56 (0.39)	5.51 (0.34)	4.40 (0.24)
$\hat{k}'$ [a]	0.30 (0.04)	0.33 (0.05)	0.36 (0.05)	0.38 (0.03)
$\hat{t}_{1/2}$ [b]	2.3	2.1	1.9	1.8

[a] Estimate (SE), obtained using linear regression of ln(adduct concentration in nmol/g) on time (in days). The estimate of k′ has units d^{-1}, the calculated $t_{1/2}$ has units d.

[b] Calculated as $\hat{t}_{1/2} = \frac{\ln(2)}{\hat{k}'}$.

The Alb adducts of the NPQs were less stable than those of NPO. The turnover of NPQ-Alb is depicted in Figure 3. In both dose groups, 1,2-NPQ-Alb concentrations were not significantly different from the controls on day 14 ($p = 0.43$ and $p =$

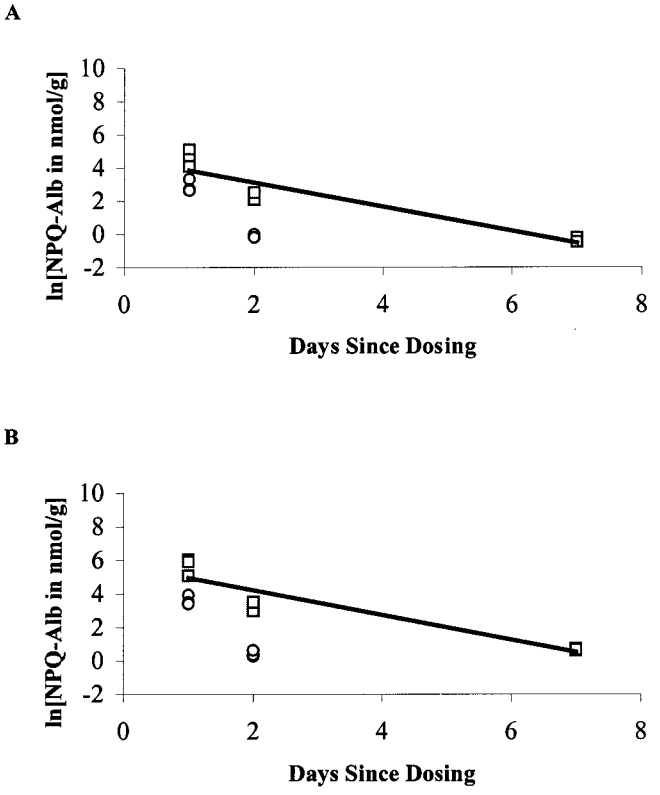

FIG. 3. Elimination of 1,2-NPQ-Alb (squares) and 1,4-NPQ-Alb (circles) adducts following administration of (A) 400 and (B) 800 mg naphthalene per kg of body weight to male F344 rats. Three time points were included in the regression analysis (Equation 2) for 1,2-NPQ-Alb, with $n = 3$ at each time point. Lines represent the equations obtained from the least squares regressions of ln(1,2-NPQ-Alb concentration) on time.

TABLE 2
Parameter Estimates for the Elimination of 1,2-NPQ-Alb Adducts (Equation 2)

	400 mg/kg	800 mg/kg
$\ln \hat{A}_0$ [a]	4.57 (0.43)	5.69 (0.36)
$\hat{k}'$ [a]	0.73 (0.10)	0.74 (0.22)
$\hat{t}_{1/2}$ [b]	0.95	0.94

[a] Estimate (SE), obtained using linear regression of ln(adduct concentration in nmol/g) on time (in days). The estimate of k′ has units d^{-1}, the calculated $t_{1/2}$ has units d.

[b] Calculated as $\hat{t}_{1/2} = \frac{\ln(2)}{\hat{k}'}$.

0.14 for 400 mg/kg and 800 mg/kg dose groups, respectively). Thus, linear regression of ln A(t) on time was conducted using data from only 3 time points (t = 1, 2, and 7 days). The estimates of k′ (0.73–0.74 d^{-1}) obtained for 1,2-NPQ-Alb did not differ significantly by dose group ($p = 0.95$) with a half-life of less than 1 day in rats (Table 2). As shown in Figure 3, levels of 1,4-NPQ-Alb were indistinguishable from controls by day 7, so there were insufficient time points to estimate k′ for this adduct. However, based upon the levels of 1,4-NPQ-Alb at days 1 and 2 after dosing (Table 3), the half-life of 1,4-NPQ-Alb would be much less than 1 day.

Stability of Hb Adducts

The estimates $\hat{A}_0$ and $\hat{k}$ from Equation 7 are shown in Table 4 for body-weight adjusted NPO1-Hb and NPO2-Hb, following administration of 400 mg/kg naphthalene. For NPO1-Hb, the estimates of k were small and positive throughout the range of t_{er} for the F344 rat. All 95% confidence intervals for k excluded 0, providing statistical evidence of adduct instability. Conversely, there was no statistical evidence of adduct instability for NPO2-Hb, since the value of $\hat{k}$ ranged from negative (given t_{er} = 62, 66 d) to positive (given t_{er} = 70 d), with all 95% confidence intervals including 0. (A negative estimate of k would suggest that adducts decay more slowly than would be expected from simple protein turnover. However, these negative estimates of k for NPO2-Hb were extremely small and not significantly different from 0.) Figure 4 illustrates the body weight-adjusted NPO1-Hb and NPO2-Hb concentrations for each animal, the corresponding fitted curves (Equation 7), and a hypothetical curve that assumes stable adducts (k = 0).

Estimates of NPO-Hb adduct instability were not obtained following administration of 800 mg/kg naphthalene because the variability among animals at each time point was extremely large and a trend of adduct decay was not discernable. Likewise, rates of instability could not be estimated for 1,2-NPQ-Hb and 1,4-NPQ-Hb due to the low concentrations and rapid disappearance of these adducts at both doses (Table 5). In the 400 mg/kg dose group, the measured concentrations of 1,2-NPQ-Hb and 1,4-NPQ-Hb were significantly different from controls only on day 1 ($p = 0.03$ and 0.003, respectively). In the 800 mg/kg dose group, the measured concentrations of 1,2-NPQ-Hb were significantly different from controls only on days 1 ($p = 0.002$) and 2 ($p = 0.02$) and 1,4-NPQ-Hb was significantly different from controls only on day 1 ($p = 0.01$).

TABLE 3
Concentration of 1,4-NPQ-Alb at Time t Following Administration of Naphthalene to Male F344 Rats

	400 mg/kg	800 mg/kg
Mean adduct concentration (nmol/g), t = 1 day	19.0 (4.5)	38.4 (6.7)
Mean adduct concentration (nmol/g), t = 2 days	0.9 (0.05)	1.6 (0.2)

Note. Values are mean (SE), $n = 3$ for all groups.

TABLE 4
Estimated Parameters from Weighted Nonlinear Regression (Equation 7) of Body-Weight Adjusted NPO1-Hb and NPO2-Hb Concentration on Time following Administration of 400 mg/kg Naphthalene to F344 Rats

t_{er} (d)	$\hat{A}_0$ (nmol/g)	$\hat{k} \times 10^3$ (d^{-1})
NPO1-Hb		
70	455 (388, 523)	11.9 (5.5, 18.3)
66	450 (383, 517)	9.6 (3.2, 16.0)
62	442 (375, 509)	6.7 (0.2, 13.2)
NPO2-Hb		
70	77.7 (63.2, 92.2)	1.0 (–7.0, 9.0)
66	76.8 (62.5, 91.0)	–1.3 (-9.2, 6.7)
62	75.5 (61.6, 89.5)	–4.1 (–12.1, 3.8)

Note. t_{er}, lifetime of erythrocyte, $\hat{A}_0$, estimated adduct concentration at t = 0, $\hat{k}$, estimated rate constant of Hb adduct instability. Parameter estimate (approximate 95% confidence limits).

DISCUSSION

The kinetic theory of Alb adducts assumes that both stable and unstable adducts are turned over according to first-order processes (Equation 1). However, nonlinearity was observed in the relationship between ln A(t) and time for all Alb adducts measured in this study. This is in contrast to our previous study of benzene-derived Alb adducts formed from the cysteinyl reactions of BO and 1,4-BQ with Alb (BO-Alb and 1,4-BQ-Alb, respectively), where no lack of fit was observed from the linear first-order model (Troester *et al.*, 2000). Many previous studies have also observed linear first-order decay of Alb adducts (Cho *et al.*, 1994; DeBord *et al.*, 1992; Sabbioni *et al.*, 1987; Viau *et al.*, 1993), although one study of the stability of pentachlorophenol adducts on Alb did report what appeared to be biphasic decay of Alb adducts (Waidyanatha *et al.*, 1996). This suggests that the elimination of some Alb adducts may involve a more complicated mechanism than permitted by

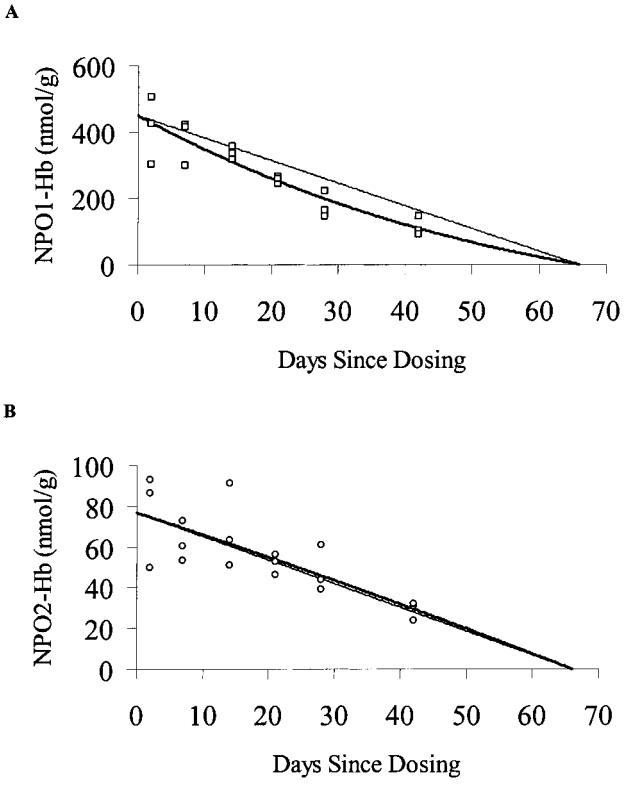

FIG. 4. Elimination of (A) NPO1-Hb and (B) NPO2-Hb adducts following administration of 400 mg naphthalene per kg body weight to male F344 rats. Each data point represents the body-weight adjusted concentration of adducts (nmol/g Hb) for a single animal. In A, the heavy line represents the fitted nonlinear model (Equation 7) where t_{er} = 66 days, $\hat{k} = 9.6 \times 10^{-3}$ d^{-1}, and $\hat{A}_0$ = 449.6 nmol/g; the lighter line represents a theoretical linear, zero-order (in k) model where t_{er} = 66 days, k = 0 d^{-1}, and A_0 = 449.6 nmol/g. In B, the heavy line represents the fitted nonlinear model (Equation 7) where t_{er} = 66 days, $\hat{k} = -1.3 \times 10^{-3}$ d^{-1}, and $\hat{A}_0$ = 76.8 nmol/g; the lighter line represents a theoretical linear, zero-order (in k) model where t_{er} = 66 days, k = 0 d^{-1}, and A_0 = 76.8 nmol/g.

current theory. Justification for an alternative model would require more time points than were available in this study, so we estimated first-order rate constants of Alb adduct instability, recognizing that this could introduce some biases. From Figures 2 and 3 it appears that these biases would not be great.

The first-order rate constants (k′) for adduct turnover indicate that NPO-Alb is more stable than NPQ-Alb. This is consistent with results from a similar stability study involving cysteinyl Hb and Alb adducts of BO and 1,4-BQ (only the mono-S-substituted adduct was measured), where BO-Alb was more stable than 1,4-BQ-Alb (Troester *et al.*, 2000). In that study, we speculated that glutathione or other protein sulfhydryls react with the mono-S-substituted 1,4-BQ-Alb to form a multi-S-substituted adduct, accounting for the apparent instability. (Previous studies have shown that chemical oxidation of hydroquinone [HQ] in the presence of glutathione results in multi-S-substituted conjugates [Eckert *et al.*, 1990, Lau *et al.*, 1988], and multi-S-substituted conjugates of HQ have been identified as *in vivo* metabolites of HQ [Hill *et al.*, 1993].)

However, if reactivity of the quinones were solely responsible for the instability of quinone-protein adducts, we would expect NPQ adducts to be more stable than the corresponding BQ adducts. That is, Michael addition occurs at the β position of an α, β-unsaturated ketone due to a partial positive charge at that position, which diminishes as the number of aromatic rings increases because of delocalization of electron density. The oxidation potentials of NPQs are also expected to be decreased relative to BQs, due to greater delocalization of the unpaired electron in the *ortho*-semiquinone radical associated with the larger ring system (McCoull *et al.*, 1999). Previous studies have shown that the rate of nucleophilic addition is slower with the *ortho*-quinone of benzo[a]pyrene than with 1,2-NPQ (Murty and Penning, 1992). To the contrary, we observed a turnover rate for 1,2-NPQ-Alb (Table 2) more than twice that of 1,4-BQ-Alb (half-life of 2.5 days [Troester *et al.*, 2000]), and the elimination of 1,4-NPQ-Alb (Fig. 3) appears to be even faster. At present, very little is understood about how structural characteristics of bound metabolites influence the stability of protein adducts.

The NPO-Alb adducts also appeared to be less stable than BO-Alb adducts ($\hat{k}' = 0.225$ d^{-1}, $\hat{t}_{1/2} = 3.1$ d [Troester *et al.*, 2000]). NPO-Alb adducts were turned over with first-order rate constants that ranged from 0.30 to 0.38 d^{-1} (Table 1). Assuming that the BO-Alb represents a stable adduct, then $\hat{k}_{Alb} = 0.23$ d^{-1} in the particular F344 rats used in our studies, from which we estimate $0.07 \leq \hat{k} \leq 0.15$ d^{-1} for NPO-Alb (Equation 3).

TABLE 5
Concentrations of 1,2-NPQ-Hb and 1,4-NPQ-Hb at Time t following Administration of 400 or 800 mg Naphthalene per kg Body Weight to F344 Rats

	400 mg/kg		800 mg/kg	
Time (days)	1,2-NPQ-Hb	1,4-NPQ-Hb	1,2-NPQ-Hb	1,4-NPQ-Hb
1	2.4 (0.5)*	1.4 (0.05)*	6.1 (0.8)*	2.1 (0.2)*
2	1.1 (0.4)	0.7 (0.3)	2.1 (0.4)*	0.9 (0.1)

Note. Mean adduct concentration in nmol/g (SE). Control levels (mean ± SE) were 0.8 ± 0.06 nmol/g and 0.9 ± 0.05 nmol/g.
* Significantly different from control levels ($p < 0.05$).

Interestingly, this range of $\hat{k}$ for NPO-Alb is not consistent with values predicted for NPO-Hb (Table 4), which indicated no evidence of instability *in vivo*. This is not the first study to demonstrate a lack of agreement between the stability of Hb and Alb adducts (e.g. *ortho*-toluidine [DeBord *et al.*, 1992], benzo[a]pyrenediolepoxide [Viau *et al.*, 1993], and naphthalene [Cho *et al.*, 1994]), although these previous studies did not characterize metabolite- and amino acid-specific adducts.

To our knowledge, the dose-dependence of protein adduct stability has not been tested previously. In this study, the dose of naphthalene did not appear to have a significant effect on the stability of naphthalene-derived Alb adducts. Statistical comparisons of the rate constants obtained for NPO-Alb, 1,2-NPQ-Alb, and 1,4-NPQ-Alb at 2 different doses (400 mg/kg and 800 mg/kg) demonstrated no significant difference in adduct stability.

NPO1-Hb adducts were only slightly unstable and NPO2-Hb adducts were stable following administration of 400 mg/kg naphthalene. However, the stability of NPO-Hb could not be investigated following an 800 mg/kg dose of naphthalene due to high variability in adduct concentration among animals at each time point. We suspect that the variability that is apparent in Hb adduct concentration, but not Alb adduct concentration, is a result of the greater toxicity of naphthalene on the hematopoeitic (site of Hb production) system relative to the hepatic system (site of Alb production). Hematopoietic effects are common following administration of a wide variety of chemicals due to the involvement of several organs (including bone marrow, thymus, lymph nodes, spleen, liver, stomach, intestines, and kidney among others) in the production of red blood cells. Toxicity to any of these organs could alter the concentration of Hb or the lifetime of the red blood cell (t_{er}), thereby increasing variability in adduct levels. In fact, naphthalene has been shown to cause hemolytic anemia in humans, though rodents may be less susceptible to these effects (NTP, 2000). Therefore, in spite of our inability to document instability of NPO-Hb following an 800 mg/kg dose, we suspect that these adducts will not be useful indicators of exposure at doses of naphthalene that induce acute toxicity.

It was once believed that only stable Hb and Alb adducts were useful for exposure assessment. More recently, relationships between unstable adducts and blood concentrations of electrophiles have been thoroughly described and equations have been published that require estimates of k to calculate the mean daily blood concentration of electrophile (Granath *et al.*, 1992, Troester *et al.*, 2001). These equations account for differences in k_{Alb} and t_{er} between species, but the validity of these equations ultimately depends upon the assumption that k is similar across species. Direct estimates of k in humans are seldom available. In spite of the instability observed for some of the naphthalene-derived adducts, these adducts are still more persistent than the reactive electrophiles from which they are formed and are useful in estimating the systemic doses of these metabolites.

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REFERENCES

Abdo, K. M., Eustis, S. L., McDonald, M., Jokinen, M. P., Adkins, B., Jr., and Haseman, J. K. (1992). Naphthalene: A respiratory tract toxicant and carcinogen for mice. *Inhalat. Toxicol.* **4,** 393–409.

Chichester, C. H., Philpot, R. M., Weir, A. J., Buckpitt, A. R., and Plopper, C. G. (1991). Characterization of the cytochrome P-450 monooxygenase system in nonciliated bronchiolar epithelial (Clara) cells isolated from mouse lung. *Am. J. Respir. Cell Mol. Biol.* **4,** 179–186.

Cho, M., Jedrychoswki, R., Hammock, B., and Buckpitt, A. (1994). Reactive naphthalene metabolite binding to hemoglobin and albumin. *Fundam. Appl. Toxicol.* **22,** 26–33.

Clark, C., Henderson, T., Royer, R., Brooks, A., McClellan, R., Marshall, W., and Naman, T. (1982). Mutagenicity of diesel exhaust particulate extracts: Influence of fuel composition in two diesel engines. *Fundam. Appl. Toxicol.* **2,** 38–43.

Davidian, M., and Gilitinan, D. M. (1995). Nonlinear regression models for individual data. In *Nonlinear Models for Repeated Measurement Data* (D. M. Gilitinan, Ed.), pp. 17–62. CRC Press, Boca Raton, FL.

DeBord, D. G., Swearengin, T. F., Cheever, K. L., Booth-Jones, A. D., and Wissinger, L. A. (1992). Binding characteristics of *ortho*-toluidine to rat hemoglobin and albumin. *Arch. Toxicol.* **66,** 231–236.

Derelanko, M. J. (1987). Determination of erythrocyte life span in F-344, Wistar, and Sprague-Dawley Rats using a modification of the [^{3}H]diisopropylfluorophosphate ([^{3}H]DFP) method. *Fundam. Appl. Toxicol.* **9,** 271–276.

Eckert, K. G., Eyer, P., Sonnenbichler, J., and Zetl, I. (1990). Activation and detoxification of aminophenols. III. Synthesis and structural elucidation of various glutathione addition products to 1,4-benzoquinone. *Xenobiotica* **20,** 351–361.

Granath, F., Ehrenberg, L., and Törnqvist, M. (1992). Degree of alkylation of macromolecules *in vivo* from variable exposure. *Mutat. Res.* **284,** 297–306.

Hill, B. A., Kleiner, H. E., Ryan, E. A., Dulik, D. M., Monks, T. J., and Lau, S. S. (1993). Identification of multi-S-substituted conjugates of hydroquinone by HPLC-coulometric electrode array analysis and mass spectroscopy. *Chem. Res. Toxicol.* **6,** 459–469.

Lau, S. S., Hill, B. A., Highet, R. J., and Monks, T. J. (1988). Sequential oxidation and glutathione addition to 1,4-benzoquinone: Correlation of toxicity with increased glutathione substitution. *Mol. Pharmacol.* **34,** 829–836.

Lindstrom, A. B., Yeowell-O'Connell, K., Waidyanatha, S., McDonald, T. A., Golding, B. T., and Rappaport, S. M. (1998). Formation of hemoglobin and albumin adducts of benzene oxide in mouse, rat, and human blood. *Chem. Res. Toxicol.* **11,** 302–310.

Lindstrom, A. B., Yeowell-O'Connell, K., Waidyanatha, S., McDonald, T. A., and Rappaport, S. M. (1999). Investigation of benzene oxide in bone marrow and other tissues of F344 rats following metabolism of benzene *in vitro* and *in vivo*. *Chem. Biol. Interact.* **122,** 41–58.

McCoull, K. D., Rindgen, D., Blair, I. A., and Penning, T. M. (1999). Synthesis and characterization of polycyclic aromatic hydrocarbon o-quinone depurinating N7-guanine adducts. *Chem. Res. Toxicol.* **12,** 237–246.

McDougal, J. N., Pollard, D. L., Weisman, W., Garrett, C. M., and Miller, T. E. (2000). Assessment of skin absorption and penetration of JP-8 jet fuel and its components. *Toxicol. Sci.* **55,** 247–255.

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.

NTP (2000). *NTP Technical Report on the Toxicology and Carcinogenesis Studies of Naphthalene in F344/N Rats (Inhalation Studies)*. National Toxicology Program. NIH Publication No. 01–4434.

O'Brien, K. A., Smith, L. L., and Cohen, G. M. (1985). Differences in naphthalene-induced toxicity in the mouse and rat. *Chem. Biol. Interact.* **55,** 109–122.

Osterman-Golkar, S., Perez, H. L., Csanady, G. A., Kessler, W., and Filser, J. G. (1999). Methods for monitoring of propylene oxide exposure in Fischer 344 rats. *Toxicology* **134,** 1–8.

Papciak R. J., and Mallory, V. T. (1990). Acute toxicological evaluation of naphthalene. *J. Am. Coll. Toxicol. B Acute Toxic. Data* **1,** 17–19.

Plopper, C. G., Suverkropp, C., Morin, D., Nishio, S., and Buckpitt, A. (1992). Relationship of cytochrome P-450 activity to Clara cell cytotoxicity. I. Histopathologic comparison of the respiratory tract of mice, rats and hamsters after parenteral administration of naphthalene. *J. Pharmacol. Exp. Ther.* **261,** 353–363.

Rappaport, S. M., Ting, D., Jin, Z., Yeowell-O'Connell, K., Waidyanatha, S., and McDonald, T. (1993). Application of Raney nickel to measure adducts of styrene oxide with hemoglobin and albumin. *Chem. Res. Toxicol.* **6,** 238–244.

Sabbioni, G., Skipper, P. L., Buchi, G., and Tannenbaum, S. R. (1987). Isolation and characterization of the major serum albumin adduct formed by aflatoxin B1 *in vivo* in rats. *Carcinogenesis* **8,** 819–824.

Santhanakrishnan, B. R., Ranganathan, G., and Raju, V. B. (1973). Naphthalene induced haemolytic anaemia with haemoglobinuria. *Indian J. Pediatr.* **40,** 195–197.

Schmeltz, I., Tosk, J., and Hoffmann, D. (1976). Formation and determination of naphthalenes in cigarette smoke. *Anal. Chem.* **48,** 645–650.

Shopp, G. M., White, K. L., Jr., Holsapple, M. P., Barnes, D. W., Duke, S. S., Anderson, A. C., Condie, L. W., Jr., Hayes, J. R., and Borzelleca, J. F. (1984). Naphthalene toxicity in CD-1 mice: General toxicology and immunotoxicology. *Fundam. Appl. Toxicol.* **4,** 406–419.

Sweeney, L. M., Shuler, M. L., Babish, J. G., and Ghanem, A. (1995). A cell culture analogue of rodent physiology: Application to naphthalene toxicology. *Toxicol. in Vitro* **9,** 307–316.

Troester, M. A., Kupper, L. L., and Rappaport, S. M. (2001). Comparison of non-linear and linear models for estimating haemoglobin adduct stability. *Biomarkers* **6,** 251–261.

Troester, M. A., Lindstrom, A. B., Kupper, L. L., Waidyanatha, S., and Rappaport, S. M. (2000). Stability of hemoglobin and albumin adducts of benzene oxide and 1,4- benzoquinone after administration of benzene to F344 rats. *Toxicol. Sci.* **54,** 88–94.

van Heyningen, R. (1979). Naphthalene cataract in rats and rabbits: A resume. *Exp. Eye Res.* **28,** 435–439.

van Heyningen, R., and Pirie, A. (1967). The metabolism of naphthalene and its toxic effects on the eye. *Biochem. J.* **102,** 842–852.

Viau, C., Mercier, M., and Blondin, O. (1993). Measurement of hemoglobin and albumin adducts of benzo[a]pyrenediolepoxide and their rate of elimination in the female Sprague-Dawley rat. *Arch. Toxicol.* **67,** 468–472.

Waidyanatha, S., Troester, M. A., Lindstrom, A. B., and Rappaport, S. M. (in press). Measurement of hemoglobin and albumin adducts of naphthalene-1,2-oxide, 1,2-naphthoquinone, and 1,4-naphthoquinone after administration of naphthalene to F344 rats. *Chem. Biol. Interact.*

Waidyanatha, S., Lin, P. H., and Rappaport, S. (1996). Characterization of chlorinated adducts of hemoglobin and albumin following administration of pentachlorophenol to rats. *Chem. Res. Toxicol.* **9,** 647–653.

Waidyanatha, S., Yeowell-O'Connell, K., and Rappaport, S. M. (1998) A new assay for albumin and hemoglobin adducts of 1,2- and 1,4-benzoquinones. *Chem. Biol. Interact.* **115,** 117–139.

Wilson, A. S., Davis, C. D., Williams, D. P., Buckpitt, A. R., Pirmohamed, M., and Park, B. K. (1996). Characterisation of the toxic metabolite(s) of naphthalene. *Toxicology* **114,** 233–242.

Yeowell-O'Connell, K., McDonald, T. A., and Rappaport, S. M. (1996). Analysis of hemoglobin adducts of benzene oxide by gas chromatography-mass spectrometry. *Anal. Biochem.* **237,** 49–55.

Yeowell-O'Connell, K., Rothman, N., Smith, M. T., Hayes, R. B., Li, G., Waidyanatha, S., Dosemeci, M., Zhang, L., Yin, S., Titenko-Holland, N., and Rappaport, S. M. (1998). Hemoglobin and albumin adducts of benzene oxide among workers exposed to high levels of benzene. *Carcinogenesis* **19,** 1565–1571.

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.