Albumin Adducts of Benzene Oxide and 1,4-Benzoquinone as Measures of Human Benzene Metabolism

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ABSTRACT

Albumin adducts of benzene oxide (BO-Alb) and 1,4-benzoquinone (1,4-BQ-Alb) were investigated among 134 workers exposed to benzene and 51 unexposed controls in Tianjin, China. Concentrations of both adducts increased with benzene exposure (range = 0.07–46.6 parts/million [ppm]; median = 3.55 ppm) and with urinary cotinine. Adduct levels were less than proportional to benzene exposure, suggesting saturable CYP 2E1 metabolism of benzene. Because the transition from linear to saturable metabolism began at ~1 ppm, the common assumption of linear kinetics at much higher benzene exposures could lead to substantial underestimation of leukemia risks. Adduct levels were generally lower in older workers, indicating that CYP 2E1 metabolism diminished with age, at ~2%/year of life. The ratio of 1,4-BQ-Alb:BO-Alb decreased with age and coexposure to toluene, and increased with alcohol consumption. This indicates that factors affecting CYP 2E1 metabolism exerted a greater role on production of 1,4-BQ than BO, presumably because of the second oxidation step from phenol to hydroquinone. The adduct ratio was also positively associated with urinary cotinine, suggesting that both benzene and hydroquinone from cigarette smoke affected adduct levels. Results of a limited time course study of 11 subjects indicated moderate chemical instability of 1,4-BQ-Alb (half life = 13.5 days compared with 21 days for normal Alb turnover), whereas no evidence of instability of BO-Alb was observed. This study illustrates that Alb adducts can be used to investigate the dispositions of reactive metabolites of procarcinogens in humans, provided that exposures are adequately characterized in the month preceding blood collection.

INTRODUCTION

Benzene is a volatile constituent of petroleum, its derivatives, and combustion products, and has important industrial uses (1); an estimated 13.6 million metric tons of benzene were produced in 1992 (2). Clearly recognized as a carcinogen in mammals, benzene causes hematotoxicity and leukemia in humans (3–5). Surprisingly, the mechanism by which benzene causes human diseases is unknown, despite 135 years of investigation (6). One reason for this is the complex metabolism of benzene, which gives rise to many electrophilic intermediates capable of binding with macromolecules, notably, BO, 1,2-BQ, 1,4-BQ, and the muconaldehydes. In addition, some metabolites generate reactive oxygen species (3, 6, 7).

As shown in Fig. 1, benzene is initially oxidized by cytochrome P450s (primarily CYP 2E1; Ref. 8) to BO (9, 10), which exists in equilibrium with its valence tautomer oxepin (11). Other benzene metabolites are derived from BO, notably phenol (from spontaneous rearrangement), catechol (from epoxide hydrolase-mediated hydrolysis), hydroquinone (from a second CYP 2E1 oxidation of phenol), and the muconaldehydes (presumably from a cytochrome P450-dependent oxidation of oxepin followed by ring opening; Ref. 6). Animal experiments implicate hydroquinone as a major contributor to the toxic effects of benzene after oxidation to 1,4-BQ in vivo (12, 13). The quantitative relationships between human benzene exposure and the electrophilic intermediates are fragmentary at best, resting largely on occupational studies of urinary metabolites and extrapolation from animals (5, 14, 15).

Because of our poor understanding of human benzene metabolism and the mechanism(s) of toxicity, recent efforts have turned to biomarkers to shed light on the relevant connections (e.g., Ref. 16). Because reactive metabolites are short lived, these studies have relied on Hb and Alb adducts to gain insight into the dispositions of the electrophilic intermediates, a strategy first suggested by Ehrenberg et al. and Osterman-Golkar et al. (17, 18). Stable Alb and Hb adducts have mean residence times in exposed persons of 1 and 4 months, respectively (19). Unlike short-lived urinary metabolites, Alb and Hb adducts damp the variability in exposure from day to day thereby illuminating connections between biomarkers and exposure (20, 21).

Studies of protein adducts in benzene-exposed humans have focused primarily on cysteinyl binding of BO and 1,4-BQ with serum Alb (hereafter designated BO-Alb and 1,4-BQ-Alb, respectively) among heavily exposed workers. Bechtold et al. (22) first observed an association between BO-Alb and benzene exposure (range: 4–23 ppm) among 12 workers. Yeowell-O’Connell et al. (23, 24) subsequently reported exposure-related increases in BO-Alb, 1,4-BQ-Alb, and the Hb adduct of BO among 43 workers (range: 1.7–328 ppm), and detected background adducts (of unknown origin) among 44 unexposed subjects. Reanalysis of a subset of these data suggested that production of BO-Alb began to saturate at benzene exposures >10 ppm (25). In the current investigation we report levels of Alb adducts of BO and 1,4-BQ among 134 benzene-exposed workers (range: 0.7–46.6 ppm) and 51 unexposed subjects in Tianjin, China.

MATERIALS AND METHODS

Subject Recruitment and Sample Collection. All of the procedures related to recruitment of subjects, and collection and analysis of human samples were carried out following approval of local Human Investigations Committees at the institutions conducting this study. Subjects (n = 185) were recruited with informed consent from five factories in Tianjin, China. Exposed subjects (n = 134) worked in a glue factory (n = 25), a shoe factory (n = 81), or a sporting-goods factory.
(n = 28). None of the subjects were respirators. Control subjects (n = 51) were recruited in nearby food-processing (n = 25) and flour factories (n = 26) where there were no known uses of solvents. Details regarding the recruitment of subjects and descriptions of workplaces are noted.4

Full-shift exposures to benzene, toluene, and xylene were monitored with personal monitors.5 Among exposed subjects, 540 exposures were measured weekly during 4–5 weeks before blood collection, with 1–6 measurements/worker (111 subjects had 4 measurements). Exposures were monitored for all of the control subjects only on the day when blood samples were collected; because all of these measurements were below the limit of detection for benzene (0.02 ppm) control subjects were assigned an exposure of 0 for statistical purposes.

Peripheral blood was obtained from all of the subjects at the end of the work shift in 5-ml vacutainer tubes containing citrate. To examine the inter- and intrapersonal variation of Alb adducts, repeated blood samples were obtained from 11 exposed subjects (6 men and 5 women) from the glue factory on three consecutive Mondays (1 subject provided two samples). Another 11 exposed subjects (4 men and 7 women) from the glue factory provided samples for a limited time course study with blood collection after work on Friday and on the mornings of the following Saturday, Sunday, and Monday (0, 16, 40, and 64 h after exposure). (Blood was collected three times from these subjects). Urine was collected for measurement of metabolites and cotinine (cotu) as described previously (26).

During recruitment, subjects provided information about current and past exposures, as well as demographic factors, by interview. As shown in Table 1, demographic characteristics were similar among exposed and unexposed subjects in regard to gender and age. The percentage of smokers was slightly greater among exposed subjects (31% versus 20% for controls) and was highly correlated with gender, with 49 of the 52 smokers being male (94%).

**Measurements of Air Samples and Cotu.** Personal air samplers were analyzed for benzene, toluene, and xylene as described by Qu et al.6 Levels of xylene were very low (mean = 0.5 ppm; range: 0–6 ppm) and were not used for statistical analyses. Cotu, a metric for smoking intensity, was measured in urine by RIA as described previously (27, 28); pre- and postshift values were averaged for each subject before statistical analysis. Cotu levels were adjusted for urinary creatinine, measured as described in Refs. 27, 28; cotu was not measured in 1 subject (n = 184).

**Blood Processing and Quality Assurance.** After collection, blood samples were kept at 4°C for up to 3 h during transport to a local laboratory. After centrifugation at 900 × g for 10 min, plasma was separated from blood cells, aliquoted into sterile tubes, randomly assigned unique codes, and stored at −20°C for a few weeks. For quality assurance purposes, 5-ml plasma samples were obtained from an additional 10 Chinese control subjects; these samples were pooled, divided into 50 tubes, assigned pseudo-subject codes, and processed along with the experimental samples. Plasma samples were shipped to the United States and subsequently stored at −80°C before laboratory analysis.

One sample was lost during processing, so plasma samples from a total of 184 exposed and control subjects were assayed for Alb adducts. To estimate individual sources of variation in the adduct measurements, selected plasma specimens were chosen at random for replicate assays (46 specimens for BO-Alb and 71 for 1,4-BQ-Alb), and selected assays were chosen at random for replicate gas-chromatographic injections (86 assays for BO-Alb and 60 for 1,4-BQ-Alb).

Adducts were measured without knowledge of individual subjects or exposure status. After assay results were reported, subject identification codes, exposure data, levels of cotu, and demographic factors were provided to the investigators.

**Analysis of Alb Adducts.** Alb was isolated from plasma as described in Lindstrom et al. (29). Cysteinyl BO-Alb and 1,4-BQ-Alb were measured by GC-MS using the assay described by Waidyanatha et al. (30) with slight modifications (24). The corresponding Alb adduct of 1,2-BQ was also detected but could not be reliably quantified because of the lack of an appropriate internal standard. We have observed that 1,2-BQ-Alb is quite unstable under the conditions of the assay.

**Statistical Analyses.** Statistical procedures used SAS software (SAS Institute, Cary, NC) with a P of 0.05 to define statistical significance. Median levels of exposures and adducts, aggregated by exposure status and/or gender, were compared with the Wilcoxon two sample test.

**Table 1** Demographic characteristics of exposed and control subjects

<table>
<thead>
<tr>
<th>Status</th>
<th>Sex</th>
<th>No. (%)</th>
<th>Median age (range)</th>
<th>No. smokers (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exposed</td>
<td>Male</td>
<td>66 (49)</td>
<td>37 (18–52)</td>
<td>39 (59)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>68 (51)</td>
<td>38 (29–47)</td>
<td>3 (4.4)</td>
</tr>
<tr>
<td>Exposed</td>
<td>All</td>
<td>134 (100)</td>
<td>37.5 (18–52)</td>
<td>42 (31)</td>
</tr>
<tr>
<td>Control</td>
<td>Male</td>
<td>24 (47)</td>
<td>28 (19–43)</td>
<td>10 (42)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>27 (53)</td>
<td>38 (28–46)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Control</td>
<td>All</td>
<td>51 (100)</td>
<td>34.0 (19–46)</td>
<td>10 (20)</td>
</tr>
</tbody>
</table>

ANOVA estimates of within- and between-subject variance components of benzene exposures were estimated with the following random effects model:

\[
\ln(X_{ij}) = \mu_i + \beta_i + \epsilon_{ij},
\]

where \(\ln(X_{ij})\) is the natural logarithm of benzene exposure for the \(i\)th subject on the \(j\)th day, \(\mu_i\) is the fixed population mean (logged) exposure, \(\beta_i\) is the random subject effect and \(\epsilon_{ij}\) is the random error term. It is assumed that the \(\beta_i\) and \(\epsilon_{ij}\) are normally distributed with means of zero and variances of \(\sigma^2_{\beta}\) and \(\sigma^2_{\epsilon}\), representing the within- and between-subject components of variance, respectively (31). Sources of variation of adduct levels were estimated according to the following random-effects model:

\[
\ln(A_{ijkl}) = \mu_k + \alpha_i + \beta_{ij} + \gamma_{ij} + \epsilon_{ijkl},
\]

where \(\ln(A_{ijkl})\) represents the logged level of adduct A for the \(i\)th subject, the \(j\)th exposure, the \(k\)th subject-specific exposure to benzene and toluene, and the \(l\)th adduct. Because we assume underlying lognormal distributions of exposures (\(X_{ij}\)) and adduct levels (\(A_{ijkl}\)), the fold ranges representing 95% of the random-subject effects were estimated as 
\[
\hat{R}_{95\%} = \exp(3.92/\sqrt{\sigma^2_{\beta} + \sigma^2_{\epsilon}}),
\]

and the CV of the assay was estimated as 
\[
CV_{assay} = \left\{\exp(\sigma^2_{\epsilon}/\sigma^2_{\beta}) - 1\right\}^{1/2}.
\]

Multiple linear regression was used to investigate effects of exposure and covariates on the subject-specific (logged) adduct level. If replicate GC-MS injections and/or assays had been performed, adduct levels were averaged first by injection and then by assay before statistical analysis. For the 11 subjects with multiple blood specimens over a weekend, only the adduct level at the end of the work shift (time = 0 h) was used; however, for the 11 subjects with blood specimens collected on 3 consecutive Mondays, the logged GM was used. The subject-specific GM levels of the two adducts (pmol/g Alb) and their difference, representing \([1,4\text{-BQ-Alb}]/[\text{BO-Alb}]\), were regressed on the corresponding subject-specific exposures to benzene and toluene. Because control subjects had been assigned an exposure of zero, the natural logarithm of \([1/\text{subject-specific GM exposure, ppm}]\) was used to represent exposures to benzene (benexpo) and toluene (tolexpo; Refs. 33, 34). The following covariates were investigated: age (y), sex (female = 0, male = 1), cotu (\(\mu\)g/g creatinine), self-reported number of alcoholic drinks/d (drnkday), cumexp (ppm-y), and dummy variables for factory. Initial models included all of the independent variables plus plausible two-way interactions. Separate analyses were conducted among control subjects.

Backwards elimination was used with retention of variables at a significance level of 0.05. Collinearity was investigated. Residual analysis involved visual inspection, normality plots, studentized residuals, leverage and Cook’s distance. Based on these criteria, 1 subject was excluded from the regression of \([\text{InBO-Alb}]/\text{Alb}\) among controls. Stratified analyses were performed for significant interactions, using tertiles of age and/or cotu and three categories of benzene exposure [zero (controls), \(<3.55\) ppm, and \(\geq3.55\) ppm].

### RESULTS

#### Summary Statistics.

Median values and ranges of exposure to benzene, toluene, and cumexp are summarized in Table 2. Among exposed subjects, the median concentrations were 3.55 ppm for benzene (range: 0.07–46.6 ppm) and 17.4 ppm for toluene (range: 0.41–193 ppm). Differences in median exposures between male and female workers were not significant. The median estimated cumulative exposure was 51.1 ppm-y (range: 6.12–623 ppm-y) and was not significantly different between male and female subjects.

Median values and ranges of Alb adducts, cotu, and drnkday are summarized in Table 3 for subjects aggregated by exposure status and gender. For BO-Alb, the median levels were higher among exposed workers (2370 pmol/g for females and 393 pmol/g for males) than controls (176 pmol/g for females and 170 pmol/g for males; \(P < 0.0001\) for both comparisons). However, no significant differences were observed between males and females of the same exposure status. For 1,4-BQ-Alb, median levels were also higher among exposed workers (2370 pmol/g for females and 2700 pmol/g for males) than among controls (874 pmol/g for females and 1380 pmol/g for males; \(P < 0.001\) for both comparisons), and were also higher for males than females in both exposed and control workers (\(P < 0.05\) for exposed and \(P < 0.01\) for controls).

Median levels of cotu were higher among males than females (619 versus 18.7 \(\mu\)g/g creatinine for exposed workers and 642 versus 15.5 \(\mu\)g/g creatinine for controls; \(P < 0.0001\) for both comparisons) but did not differ significantly by gender.
exposure status. Self-reported alcohol consumption (drnkday) ranged from 0 to 7 drinks/d for exposed males and 0–3 drinks/day for exposed females.

Sources of Random Variation. Estimates of the variance components for random effects in Models 1 and 2 are summarized in Table 4. Regarding benzene exposure, the estimated between-subject variance component ($\sigma^2_{\text{benexpo}} = 2.42, R^2_{0.95} = 445$-fold) accounted for 83% of the total variation, whereas the within-subject variance component ($\sigma^2_{\text{within}} = 0.495$) accounted for 17%. Regarding adduct levels, the between-subject variance component accounted for 86–94% of the total variation with $\sigma^2_{\text{Ben-Alb}} = 0.523 (R^2_{0.95} = 17$-fold) and $\sigma^2_{\text{1,4-BQ-Alb}} = 0.651$ for 1,4-BQ-Alb ($R^2_{0.95} = 24$-fold). The remaining 6–14% of the total variation was distributed among specimens ($\sigma^2_{\text{specimen}}$), assays ($\sigma^2_{\text{assay}}$), and GC-MS injections ($\sigma^2_{\text{injection}}$). As indicated by $CV_{\text{assay}}$, assay precision was 30% for Ben-Alb and 20% for 1,4-BQ-Alb, values quite similar to CVs estimated more conventionally from the 50 quality assurance specimens (26% for Ben-Alb and 17% for 1,4-BQ-Alb).

Given an estimated half-life of 21 days for human serum Alb (36, 37), the values of $\sigma^2_{\text{Ben-Alb}}$ in Table 4 probably underestimate the true specimen-related variance components because of autocorrelation of adduct levels measured on three consecutive Mondays. Nonetheless, the small values of $\sigma^2_{\text{Ben-Alb}}$ tend to confirm the expectation that levels of Ben-Alb and 1,4-BQ-Alb respond to benzene exposure over weeks to months rather than transient exposures from day to day (19).

Multiple Linear Regression. Scatter plots of adduct levels versus exposures are shown in Fig. 2. For both adducts, increasing trends are seen with exposure, and significant background levels are apparent when benexpo = 0. The lines in the plots represent the least squares solutions given by ln[BO-Alb] = 5.165 + 0.494(benexpo) and ln[1,4-BQ-Alb] = 6.847 + 0.535(benexpo; n = 184).

The final multivariate model for ln[BO-Alb] is summarized in Table 5 ($R^2 = 0.659$). Significant effects were observed for benexpo, age, and benexpo*age. After stratification by age the estimated slope of ln[BO-Alb] on benexpo diminished from 0.555 in the youngest tertile (mean = 25.9 y) to 0.418 in the oldest tertile (mean = 42.9 year; $P > 0.05$). After stratification by benzene exposure the estimated slope of ln[BO-Alb] on age diminished from 9.38 × 10⁻³ for controls to −26.8 × 10⁻³ for workers exposed ≥3.55 ppm ($P < 0.05$). This indicates that, among exposed workers, older subjects had lower levels of BO-Alb at a given benzene exposure.

Results of regression analyses of ln[1,4-BQ-Alb] are shown in Table 6 ($R^2 = 0.692$). Significant effects were observed for the following variables: benexpo, age, cotu, and benexpo*cotu. The results point to lower levels of 1,4-BQ-Alb among older workers and to higher levels among smokers at a given benzene exposure. From stratified analyses, the estimated slope of ln[1,4-BQ-Alb] on benexpo diminished from 0.576 in the lowest tertile of cotu (mean = 5.48 μg/g creatinine) to 0.367 in the highest tertile (mean = 1750 μg/g creatinine; $P < 0.05$). Likewise, the estimated slope of ln[BO-Alb] on cotu diminished from 2.60 × 10⁻⁴ for controls to −7.86 × 10⁻⁵ for workers exposed ≥3.55 ppm ($P < 0.05$).

Among control subjects, as shown in Table 7, age and cotu explained 21% of the variability for ln[BO-Alb] and 50% for ln[1,4-BQ-Alb]. The estimated coefficient for age was positive for ln[BO-Alb] (0.016) and negative for ln[1,4-BQ-Alb] (−0.019). The estimated coefficient for cotu was about twice as large for ln[1,4-BQ-Alb; 2.42 × 10⁻⁴] as for ln[BO-Alb; 1.44 × 10⁻⁴].

The model of ln[1,4-BQ-Alb]/BO-Alb, summarized in Table 8 ($R^2 = 0.154$), showed significant effects of benexpo ($P = 0.037$), tolexpo ($P = 0.031$), age ($P = 0.046$), drnkday ($P = 0.024$), and cotu ($P = 0.0002$). The adduct ratio was positively related to benexpo, drnkday, and cotu, and inversely related to tolexpo and age.

Mixed Models of Time Course Data. Logged adduct levels were investigated as a function of time (0–64 h after exposure) among 11 persons over a weekend without exposure to benzene. Mixed models yielded a significant coefficient for ln[1,4-BQ-Alb], of −0.0512 d⁻¹ (95% CI: −0.0802, −0.0222; $P = 0.0015$) but not for ln[BO-Alb] ($P = 0.513$). No significant effects were identified for age, sex, and cotu.

Table 4 Random sources of variation of benzene exposure and adduct levels

<table>
<thead>
<tr>
<th>Variable</th>
<th>Source</th>
<th>Degrees of freedom</th>
<th>Variance component</th>
<th>Percentage of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene Subject</td>
<td>133</td>
<td>$\sigma^2_B$ = 2.42</td>
<td>83.0</td>
<td></td>
</tr>
<tr>
<td>Exposure Error</td>
<td>406</td>
<td>$\sigma^2_{\text{Error}}$ = 0.495</td>
<td>17.0</td>
<td></td>
</tr>
<tr>
<td>BO-Alb Subject</td>
<td>185</td>
<td>$\sigma^2_{\text{Subject}}$ = 0.523</td>
<td>85.7</td>
<td></td>
</tr>
<tr>
<td>Specimen</td>
<td>40</td>
<td>$\sigma^2_{\text{Specimen}}$ = 0.008</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>Assay</td>
<td>45</td>
<td>$\sigma^2_{\text{Assay}}$ = 0.073</td>
<td>12.0</td>
<td></td>
</tr>
<tr>
<td>Injection</td>
<td>85</td>
<td>$\sigma^2_{\text{Injection}}$ = 0.014</td>
<td>2.32</td>
<td></td>
</tr>
<tr>
<td>1,4-BQ-Alb Subject</td>
<td>185</td>
<td>$\sigma^2_{\text{Subject}}$ = 0.651</td>
<td>93.6</td>
<td></td>
</tr>
<tr>
<td>Specimen</td>
<td>40</td>
<td>$\sigma^2_{\text{Specimen}}$ = 0.026</td>
<td>3.71</td>
<td></td>
</tr>
<tr>
<td>Assay</td>
<td>67</td>
<td>$\sigma^2_{\text{Assay}}$ = 0.013</td>
<td>1.90</td>
<td></td>
</tr>
<tr>
<td>Injection</td>
<td>61</td>
<td>$\sigma^2_{\text{Injection}}$ = 0.005</td>
<td>0.76</td>
<td></td>
</tr>
</tbody>
</table>

* Refers to random effects Model 1 for (logged) exposure measurements and Model 2 for (logged) adduct levels.
Table 6 Final regression model for ln(1,4-BQ-Alb) ($R^2 = 0.692$)

For each subject, ln(1,4-BQ-Alb) represents the natural logarithm of the level of the Alb adduct of 1,4-benzoquinone (pmol/g Alb); age is the age (y); benexpo = ln[1 + GM], where GM represents the geometric mean benzene exposure (ppm)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Parameter estimate</th>
<th>95% CI</th>
<th>$P$</th>
<th>$\Delta R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>7.393</td>
<td>7.076, 7.710</td>
<td>$&lt; 0.0001$</td>
<td>0.209</td>
</tr>
<tr>
<td>benexpo</td>
<td>0.572</td>
<td>0.511, 0.634</td>
<td>$&lt; 0.0001$</td>
<td>0.603</td>
</tr>
<tr>
<td>age</td>
<td>$-0.019$</td>
<td>$-0.028$, $-0.011$</td>
<td>$&lt; 0.0001$</td>
<td>0.031</td>
</tr>
<tr>
<td>cotu</td>
<td>$2.56 \times 10^{-4}$</td>
<td>$1.60 \times 10^{-4}$, $3.52 \times 10^{-4}$</td>
<td>$&lt; 0.0001$</td>
<td>0.045</td>
</tr>
<tr>
<td>benexpo*cotu</td>
<td>$-8.89 \times 10^{-5}$</td>
<td>$-5.12 \times 10^{-5}$, $-2.59 \times 10^{-5}$</td>
<td>0.006</td>
<td>0.013</td>
</tr>
</tbody>
</table>

Table 7 Final regression models for adducts among control subjects

For each subject, ln(BO-Alb) and ln(1,4-BQ-Alb) represent the natural logarithms of the levels of the Alb adducts of benzene oxide and 1,4-benzoquinone, respectively (pmol/g Alb); age is the age (y).

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Independent Variable</th>
<th>Parameter estimate</th>
<th>95% CI</th>
<th>$P$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ln(BO-Alb)</td>
<td>Intercept</td>
<td>4.536</td>
<td>4.064, 5.009</td>
<td>$&lt; 0.0001$</td>
<td>0.209</td>
</tr>
<tr>
<td></td>
<td>age</td>
<td>0.016</td>
<td>0.003, 0.030</td>
<td>0.021</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(benexpo = $0.552$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>cotu</td>
<td>$1.44 \times 10^{-4}$</td>
<td>$4.93 \times 10^{-5}$</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(cotu = $0.185$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ln(1,4-BQ-Alb)</td>
<td>Intercept</td>
<td>7.431</td>
<td>6.984, 7.877</td>
<td>$&lt; 0.0001$</td>
<td>0.499</td>
</tr>
<tr>
<td></td>
<td>age</td>
<td>$-0.019$</td>
<td>$-0.036$, $-0.006$</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(benexpo = $0.156$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>cotu</td>
<td>$2.42 \times 10^{-4}$</td>
<td>$1.57 \times 10^{-4}$</td>
<td>$&lt; 0.0001$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(cotu = $0.343$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 8 Final regression model for ln(1,4-BQ-Alb/BO-Alb) ($R^2 = 0.154$)

For each subject, 1,4-BQ-Alb and ln(BO-Alb) represent the natural logarithms of the levels of the Alb adduct of 1,4-benzoquinone and BO-Alb (pmol/g Alb); age is the age (y).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Parameter estimate</th>
<th>95% CI</th>
<th>$P$</th>
<th>$\Delta R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>2.024</td>
<td>1.659, 2.388</td>
<td>$&lt; 0.0001$</td>
<td>0.007</td>
</tr>
<tr>
<td>benexpo</td>
<td>0.080</td>
<td>0.005, 0.156</td>
<td>0.037</td>
<td>0.007</td>
</tr>
<tr>
<td>tolexpo</td>
<td>$-0.071$</td>
<td>$-0.135$, $-0.007$</td>
<td>0.031</td>
<td>0.018</td>
</tr>
<tr>
<td>age</td>
<td>$-0.011$</td>
<td>$-0.021$, $0.133 \times 10^{-4}$</td>
<td>0.046</td>
<td>0.012</td>
</tr>
<tr>
<td>denkday</td>
<td>0.073</td>
<td>0.010, 0.136</td>
<td>0.024</td>
<td>0.046</td>
</tr>
<tr>
<td>cotu</td>
<td>$1.35 \times 10^{-4}$</td>
<td>$6.58 \times 10^{-5}$, $2.05 \times 10^{-4}$</td>
<td>0.0002</td>
<td>0.071</td>
</tr>
</tbody>
</table>

DISCUSSION

Our results confirm previous findings that levels of BO-Alb and 1,4-BQ-Alb increased with benzene exposure (22–24). These trends were easily discerned at a median benzene exposure of 3.55 ppm (Fig. 2), attributable in part to the wide range of exposures (0–46.6 ppm) as well as the modest contributions of random errors associated with Alb specimens and assays (Table 4). Because we observed no gender-related differences, our results do not support Brown et al. (38) who predicted that women would metabolize more benzene than men; however, because 49 of the 52 smokers in our study were male we cannot rule out possible confounding by smoking.

Supralinear Production of Adducts. Previous analyses of BO-Alb in a sample of 30 Chinese workers exposed to benzene from 0.2 to 302 ppm suggested supralinear production of adducts, commencing in the range of 10 ppm (25). That conjecture was based on a straight-line slope of the regression of ln(BO-Alb) on ln(benzene exposure) that was significantly less than 1 (21). Using similar reasoning we report supralinear production of both BO-Alb and 1,4-BQ-Alb in a much larger sample of workers exposed to generally lower levels of benzene, given log-space slopes that were significantly less than 1 (Fig. 2; BO-Alb: slope = 0.494 with upper 95% confidence limit = 0.550; 1,4-BO-Alb: slope = 0.535 with upper 95% confidence limit = 0.599). We infer from this that adduct levels were less than proportional to benzene exposure over the range of 0.07 to 46.6 ppm.

We attribute the supralinear production of adducts to saturable metabolism of benzene (leading to BO) and of phenol (leading to hydroquinone), both of which are substrates for human and rodent CYP 2E1 (8, 39–41). These processes are governed by Michaelis-Menten kinetics, where the transition from first-order (linear) to zero-order kinetics produces a supralinear relationship with exposure. Because benzene is a high-affinity substrate for CYP 2E1, its metabolism is perfusion limited, and this kinetic transition can be abrupt (42). Thus, the proportions of benzene converted to toxic metabolites can vary greatly among persons exposed to benzene in the range of transition.

Given the general lack of relevant human data, there is great uncertainty as to where the transition from linear to saturated metabolism occurs for benzene. Applications of physiologically based pharmacokinetic models predicted linear metabolism in humans exposed below 10 ppm of benzene (43) and saturation above 100 ppm (44). Our results indicate a lower point of transition, with deviations from linearity beginning at ~1 ppm. This is illustrated in Fig. 3, which presents the data and predictions in natural space. Clearly, adduct production was well within the saturable range at 10 ppm. By comparing the fitted curves with the dashed lines, representing expected low-dose linearity, we predict deviations from linear metabolism of −10% at 1 ppm, −50% at 10 ppm, and −62% at 20 ppm.

Because quantitative risk assessments for benzene have assumed linear increases in leukemia risk, extending to exposures much greater than 10 ppm (e.g., Ref. 45), our results suggest that the true
risks can be substantially larger than currently predicted. Indeed, our data support the findings of Hayes et al. (46) who reported excess leukemia risks among Chinese workers exposed below 10 ppm of benzene and the appearance of a supralinear exposure-response curve.

**Adducts of 1,4-BQ versus BO.** The estimated low-dose linear slope for 1,4-BQ-Alb [569 pmol (g Alb)^{-1}/ppm benzene] is about six times greater than that for BO-Alb [94.9 pmol (g Alb)^{-1}/ppm benzene; see Fig. 3]. This indicates that cysteinyl Alb residues react much more readily with 1,4-BQ than with BO, as has been observed with microsomal rat proteins in vitro (47). Yet, because benzene undergoes two CYP 2E1 oxidations en route to 1,4-BQ, the effects of saturable metabolism should be more pronounced for 1,4-BQ-Alb than for BO-Alb. To investigate this notion, we modeled ln(1,4-BQ-Alb/BO-Alb) with the idea that factors reducing metabolism would lead to negative effects, whereas those enhancing or bypassing metabolism would lead to positive effects. The final model identified several significant effects, most of which were in the postulated direction (Table 8). The positive association of ln(1,4-BQ-Alb/BO-Alb) with daily alcohol consumption (drnkday) points to the well-documented induction of CYP 2E1 by ethanol (39, 40, 48, 49). Because benzene and toluene are competitive CYP 2E1 inhibitors of phenol metabolism (reviewed in Ref. 50), we anticipated that ln(1,4-BQ-Alb/BO-Alb) would be negatively associated with benexpo and tolexpo; this was the case for toluene but not for benzene. The negative coefficient for age suggests that metabolism of phenol was hampered more than that of benzene among older workers (discussed below), whereas the positive coefficient for cotu points to the combined smoking contributions of hydroquinone and benzene to 1,4-BQ-Alb.

**Effects of Age.** Age was significantly associated with 1,4-BQ-Alb, BO-Alb, and their ratio (Tables 5–8). For 1,4-BQ-Alb, older workers had lower exposure-adjusted adduct levels regardless of exposure status. However, for BO-Alb the interaction of age and benexpo resulted in different effects depending on the exposure category. The coefficient for age was positive among controls and became increasingly negative for workers exposed below and at or above 3.55 ppm. Because control workers were not exposed to measurable levels of benzene, this suggests that older workers metabolized benzene and phenol at lower rates than younger workers. Guided by the estimated coefficient for age of 0.019 year^{-1} (Table 6), we infer a 2% reduction in metabolism of benzene to 1,4-BQ/y of life. Thus, we predict a ~2-fold difference in 1,4-BQ-Alb production, given an age range of 18–52 y in our study.

Although we are unaware of previous evidence that age reduced metabolism of benzene (or phenol), investigations of CYP 2E1 metabolism of acetaminophen produced ambiguous results, with some reporting slower metabolism among older persons and others finding no effect (reviewed in Refs. 51, 52). Because previous studies had fewer subjects, including those with frank liver diseases, our results arguably provide the strongest evidence that age reduces CYP 2E1 activity among healthy persons. As to a possible mechanism, George et al. (53) reported significant reduction in microsomal protein content of CYP 2E1 in livers from 71 subjects, with an estimated reduction of 0.5%/y of life.

**Effects of Smoking.** Yeowell-O’Connell et al. (24) reported that cotu was positively correlated with 1,4-BQ-Alb but not BO-Alb among controls from Shanghai, China. Because cigarette smoke is the major source of benzene exposure outside the workplace (54), we expected to find associations between cotu and adducts of both BO and 1,4-BQ among control workers. Strong associations were observed between ln(1,4-BQ-Alb) and cotu among all of the subjects (Table 6) and controls (Table 7), and between ln(BO-Alb) and cotu among controls (Table 7). Because hydroquinone is a constituent of cigarette smoke as well as benzene (55–58), the greater effect of smoking on 1,4-BQ-Alb is reasonable. On the basis of the coefficients in Table 7, smoking appears to produce two times more 1,4-BQ-Alb than BO-Alb.

**Background Adducts.** The relatively high levels of BO-Alb and 1,4-BQ-Alb among unexposed subjects also confirm earlier observations (23, 24, 59). The GM levels of BO-Alb and 1,4-BQ-Alb among nonsmoking controls (n = 41) were 167 and 958 pmol/g Alb, respectively. On the basis of the low-dose linear slopes shown in Fig. 3, these background adducts correspond to occupational benzene exposures (8 h/day and 5 days/week) of 1.76 and 1.68 ppm or to continuous exposures of 0.418 and 0.400 ppm, respectively. Because environmental concentrations of benzene in the United States reportedly ranged from 0.002 to 0.010 ppm (54), it seems unlikely that benzene contributed appreciably to this background of adducts. Indeed, continuous exposure at 0.015 ppm would account for only 4% of the background adducts in these subjects.

Evidence also indicates that the unquantified background sources of BO-Alb and 1,4-BQ-Alb were different. That is, among controls, BO-Alb and 1,4-BQ-Alb were not significantly correlated (r = 0.106; P = 0.458 for logged values), and age was positively associated with BO-Alb but negatively associated with 1,4-BQ-Alb (Table 7). We suspect that the background of 1,4-BQ-Alb arose from the many endogenous and dietary sources of phenol and hydroquinone (discussed in Ref. 24). However, the origin(s) of BO-Alb is problematic because there are no known sources of BO other than benzene. Clearly, more research into these background adducts is warranted.

**Stability of Alb Adducts.** We could find only one reference to the stability of Alb adducts in humans (60). In that investigation, Alb adducts of methylhexahydrophthalic anhydride were stable, being eliminated with Alb turnover at rate k_{Alb} (0.033 d^{-1}; half-time = 21 days; Refs. 36, 37). After mixed model analysis of Alb adducts from 11 subjects monitored over a weekend, we found no evidence of loss of BO-Alb (P = 0.513) but saw significant loss of 1,4-BQ-Alb at a rate of 0.0512 d^{-1} (half-time = 13.5 days). Because the observed elimination rate of 1,4-BQ-Alb was significantly > k_{Alb} = 0.033 d^{-1}, we infer that 1,4-BQ-Alb was moderately unstable. Furthermore, the estimated rate constant for adduct instability was k = 0.0512 − 0.033 = 0.018 d^{-1}, which is considerably smaller than the value of 0.053 d^{-1} estimated in F344 rats dosed with benzene (35). Thus, we have evidence that the stability of 1,4-BQ-Alb varies substantially between humans and rats. Because 1,4-BQ-Alb is retained in the reactive quinone form, we attribute this instability to additional sulfhydryl substitution reactions leading to the formation of multi-S-substituted adducts (30, 61, 62).

We offer the following conclusions from this study of 184 subjects exposed to 0–46.6 ppm of benzene. There is strong evidence that CYP 2E1 metabolism of benzene begins to saturate with occupational exposure in the range of 1 ppm. If regulators continue to assume that the exposure-response relationship for benzene is linear at much higher exposures, substantial underestimation of the risk of leukemia could occur. The effect of metabolic saturation is more pronounced for 1,4-BQ than for BO, presumably because of a second CYP 2E1 oxidation step. Metabolism of benzene and phenol diminish with age at an overall rate of ~2%/y of life. Among persons not occupationally exposed to benzene, cigarette smoking is a significant source of both BO and 1,4-BQ. The Alb adduct of 1,4-BQ is moderately unstable in humans.
whereas that of BO shows no evidence of instability. Finally, we conclude that Alb adducts are well suited for investigations of human metabolism of procarcinogens, provided that exposures are adequately characterized during the month before blood collection.

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