Determination of Albumin and Hemoglobin Adducts in Workers Exposed to Styrene and Styrene Oxide[1]

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Abstract

Hemoglobin and albumin adducts of the carcinogen styrene-7,8-oxide (SO) were measured in 48 workers exposed to both styrene and SO in a boat manufacturing plant. Personal exposures to both substances were measured repeatedly over the course of 1 year (styrene: 0.9–235 mg/m^3 with a mean of 64.3 mg/m^3 for 48 subjects; SO: 13.4–525 μg/m^3 with a mean of 159 μg/m^3 for 20 subjects). Cysteine and carboxylic acid adducts of SO with hemoglobin and albumin were assayed on one or more occasions for each subject. The proteins were subjected to base hydrolysis to release styrene glycol, representing carboxylic acid-bound SO, and were then treated with Raney nickel to release 1-phenylethanol and 2-phenylethanol, representing cysteine-bound SO. These three analytes were extracted, derivatized, and analyzed by gas chromatography-mass spectrometry. No evidence was found of any exposure-related increase in hemoglobin adducts. In contrast, albumin adducts were found to increase with exposures to either styrene or SO, the latter apparently being more important. This suggests that exposure to low levels of SO in the air may be important among workers in the reinforced plastics industry. Significant levels of SO adducts of albumin and hemoglobin were also detected in proteins obtained from persons without occupational exposure to styrene or to SO. This finding opens the possibility that SO is either a dietary or an environmental contaminant or is produced endogenously.

Introduction

Styrene is an important monomer widely used in the production of plastics and resins. Once absorbed into the body, styrene is metabolized via cytochrome P450, primarily through the reactive intermediate SO[3] (1, 2), which is mutagenic (3) and carcinogenic in animals (4, 5). SO is subsequently metabolized via either epoxide hydrolase to SG or glutathione *S*-transferases to glutathione conjugates, although there is apparently no evidence for glutathione conjugation in humans (1, 2). Because >85% of the absorbed dose of styrene in humans is eliminated in the urine as mandelic acid and phenylglyoxylic acid, two oxidation products of SG, it can be inferred that human hepatic metabolism of styrene proceeds almost exclusively through SO, which is subsequently detoxified by epoxide hydrolase (6).

Given the reactive nature of the metabolite SO, there has been considerable concern that exposure to styrene might produce genetic damage in workers (5, 7), particularly in the reinforced plastics industry, where exposures to styrene are the greatest (8, 9). In an attempt to better quantify tissue levels of reactive intermediates such as SO, adducts of these species with the blood proteins Hb and albumin have been used as biomarkers of exposure for electrophilic species in general (10, 11) and for SO in particular (12).

Specific protein adducts of SO have been investigated among reinforced plastics workers. These include adducts of the NH_2-terminal valine of Hb, after a modified Edman degradation (13–15), and of carboxylic acid residues of Hb, after hydrolysis to SG (16). However, results of these assays have been inconclusive. Christakopoulos *et al.* (14) measured NH_2-terminal valine adducts of SO in Hb of 7 workers and 3 controls and found statistically significant increases in adduct levels with respect to measures of exposure to styrene. On the basis of the levels of SG in blood and of urinary mandelic acid, they estimated the mean workers exposure to be in the range of 300 mg/m^3 styrene; this resulted in a mean Hb adduct level of 28 pmol/g compared to a mean value of ≤13 pmol/g in the controls. On the other hand, Brenner *et al.* (13) found no significant difference in the NH_2-terminal valine adducts of SO in Hb from 14 workers (exposed to 4–190 mg/m^3 of styrene) compared to the adduct levels in 8 controls, and Severi *et al.* (15) detected no such adducts (detection limit, 10 pmol/g globin) in 52 workers (exposed to 2.2–110 mg/m^3 of styrene). Regarding adducts of carboxylic acid residues, Sepai *et al.* (16) were unable to detect Hb adducts (detection limit, 15 pmol/g globin) in 6 workers exposed to unspecified levels of styrene.

In this paper we describe the measurement of SO adducts of Hb and serum albumin from reinforced plastics workers. Two types of adducts are measured, those of cysteinyl residues of the proteins (specifically, β-Cys 93 of Hb and Cys 34 of albumin; Ref. 17), as well as SO adducts of carboxylic acid residues. This work is part of a larger investigation of several SO biomarkers in the blood of the same workers. The other SO biomarkers include sister-chromatid exchanges and micronu-

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[3] The abbreviations used are: CV, coefficient of variation; EI, electron impact; GC, gas chromatography; Hb, hemoglobin; MS, mass spectrometry; PE, phenylethanol; NCI, negative chemical ionization; PFB, pentafluorobenzoyl; SG, styrene glycol; SIM, selected ion monitoring; SO, styrene-7,8-oxide; SO-Hb, styrene-7,8-oxide adducts of hemoglobin; SO-albumin, styrene-7,8-oxide adducts of albumin.

clei in lymphocytes (18) and DNA adducts in lymphocytes (19) and the glycophorin A gene loss mutation assay of RBCs (20). The overall aim of the research is to correlate the levels of each SO biomarker in individual workers with the corresponding personal exposures to styrene, measured over 1 year.

Although styrene is generally thought to be responsible for any genotoxicity observed among reinforced plastics workers, it was speculated almost 20 years ago that exposure to SO itself could also be important (21). SO is formed *in situ* at low levels in air (<1 mg/m^3) on reaction of styrene with either oxygen or hydroperoxides, which are used to initiate the curing of reinforced plastics (9, 22–24). Many investigators have minimized the importance of exposure to SO, *per se* (7, 22, 25, 26), because of the low levels that are generated relative to those of styrene (virtually all of which is converted to SO in humans). Nonetheless, we measured SO exposures for a subset of the worker cohort and will compare adduct levels with exposure to both styrene and SO in this analysis.

Materials and Methods

Human Subjects. The cohort consisted of 48 healthy workers of both sexes employed during 1987–1988 in a factory where boats were manufactured. Subjects were recruited with informed consent from all jobs where styrene might be present in the air, regardless of the expected intensity of exposure and regardless of smoking status, provided only that they had been employed in the current job for at least 1 year. Detailed descriptions of the cohort and the randomization of data are given by Yager *et al.* (18).

Exposure to Styrene and SO. Individual shift-long exposures to styrene were measured up to seven times over the course of 1 year at roughly 6-week intervals with personal passive monitors (18, 19). SO in air was measured using standard personal sampling methodology [National Institute for Occupational Safety and Health Analytical Method No. 303 (Cincinnati, OH) 1979]. Briefly, SO exposures of five randomly chosen subjects were measured during 6 of the 7 surveys. Air was drawn from the subject's breathing zone with a personal sampling pump at 0.2 liter/min through glass tubes containing two sections of Tenax (50 mg primary section and 25 mg back section; SKC West, Fullerton, CA). Tubes were changed midway through the work shift (after ~4 h). After sampling, the tubes were capped and stored for up to 1 week before analysis. Analysis involved desorption with 0.5 ml of ethyl acetate followed by GC with flame ionization detection. Analytes were corrected for blank values (the mean of 5 Tenax tubes for each survey that were opened, immediately capped, and stored with the experimental tubes before analysis) and for desorption efficiency, which had been determined independently for the same lot of Tenax tubes.

Blood Sampling. Blood sampling was conducted on the day after measurement of exposure to styrene and SO. Blood was collected by venous puncture into heparinized tubes from subjects at ~3-month intervals up to four times during 1 year. Samples were refrigerated for up to 6 h before processing.

Analytical Reagents. All of the reagents used for the protein adduct analyses were the same as those reported in Rappaport *et al.* (17), with the following addition. The internal standard, 4-methyl-SG, was obtained via hydrolysis of 4-methyl-SO (0.125 mol) by stirring with 0.1 M HCl (100 ml) at 21°C for 10 min. The product was extracted twice with 20 ml diethyl ether. Removal of the solvent yielded a white solid, 4-methyl-SG, which was characterized by EI-MS [*m/z* 152 ($M^{\cdot+}$), 6%; 121 (C_8H_9O), 100%; 91 (C_7H_7), 45%] and proton NMR [σ (ppm): 7.15 (C_6H_4); 5.22 and 4.69 (OH); 4.52 (CH); 3.39 (CH_2); 2.25 (CH_3)]. No contaminating peaks were observed by GC-MS.

The protein-bound internal standards, 4-methyl-SO-albumin and 4-methyl-SO-Hb, were formed by the reaction of an aqueous solution of either albumin or globin (apohemoglobin) with 4-methyl-SO, as described previously (17). The levels of adducts in the modified proteins were determined by repeatedly assaying the samples after either base hydrolysis (to release 4-methyl-SG) or reaction with Raney nickel (to release 4-methyl-2-PE), as described below for routine adduct analysis. The mean levels of carboxylic acid-bound 4-methyl-SO, with SG as the calibration standard, were found to be 39.0 nmol (SE = 1.6; n = 8) of 4-methyl-SG/mg 4-methyl-SO-albumin and 8.12 nmol (SE = 0.23; n = 8) of 4-methyl-SG/mg 4-methyl-SO-Hb. The mean levels of cysteine-bound 4-methyl-SO, with 3-phenylpropanol as the calibration standard, were found to be 1.09 nmol (SE = 0.06; n = 8) of 4-methyl-2-PE/mg 4-methyl-SO-albumin and 0.397 nmol (SE = 0.024; n = 6) of 4-methyl-2-PE/mg 4-methyl-SO-Hb. The adduct yields of 4-methyl-2-PE from both modified albumin and Hb were lower than those reported previously (17) because the reaction with Raney nickel was performed under more basic conditions required for the prior hydrolysis step that released SG (see below). However, for the Hb samples not subjected to base hydrolysis, the mean level of cysteine-bound 4-methyl-SO was found to be identical to that reported previously [*i.e.*, 0.92 nmol (SE = 0.026; n = 9) of 4-methyl-2-PE/mg 4-methyl-SO-Hb]. Although both 4-methyl-1-PE and 4-methyl-2-PE were released upon reaction of the modified proteins with Raney nickel, the estimated amount of 4-methyl-2-PE was more precise than that of 4-methyl-1-PE; consequently, 4-methyl-2-PE was used to quantitate both 1-PE and 2-PE in all analyses.

Analysis of Adducts. SO binds to protein cysteinyl residues either through the α or β carbon, producing two positional isomers, which, upon reaction with Raney nickel, give rise to 2-PE and 1-PE, respectively. SO also reacts with carboxylic acid residues to yield an ester that is readily hydrolyzed under basic conditions to give SG.

Two-thirds of the Hb samples were analyzed only for 2-PE after a procedure published previously (17), with the following modifications. First, the amounts of internal standards were greatly reduced [*i.e.*, 7.4 pmol 3-phenylpropanol (free internal standard) and 10 μg 4-methyl-SO-Hb (protein-bound internal standard)]. Second, after digesting the protein, 1 ml 0.5–1 M NaOH was added to increase the pH of the medium to 13. The use of higher pH was based on preliminary analyses suggesting that this modification increased the specificity of Raney nickel for the cysteine-bound adducts.

The remaining third of the Hb samples and all of the albumin samples were analyzed for 1-PE, 2-PE, and SG after a somewhat different procedure, which is outlined in Fig. 1. Briefly, the RBCs and plasma were separated by centrifugation and then stored at −80°C until analysis. Hb and albumin were then isolated as previously reported (17), except that the albumin samples were purified by dialysis (Spectra/Por 12,000–14,000 MW cutoff dialysis membrane; Houston, TX) against four changes of water (4 liters) over 2–3 days instead of by Sephadex chromatography. Dialysis was used to increase the protein yields above those obtained with Sephadex chromatography (17) but could have resulted in hydrolysis (and loss) of some carboxylic acid-bound SO. Purified globin (median, 22 mg; range, 20–25 mg) or albumin (median, 3.7 mg; range, 1–35 mg) in 2–4 ml water was combined with 10 μg 4-methyl-

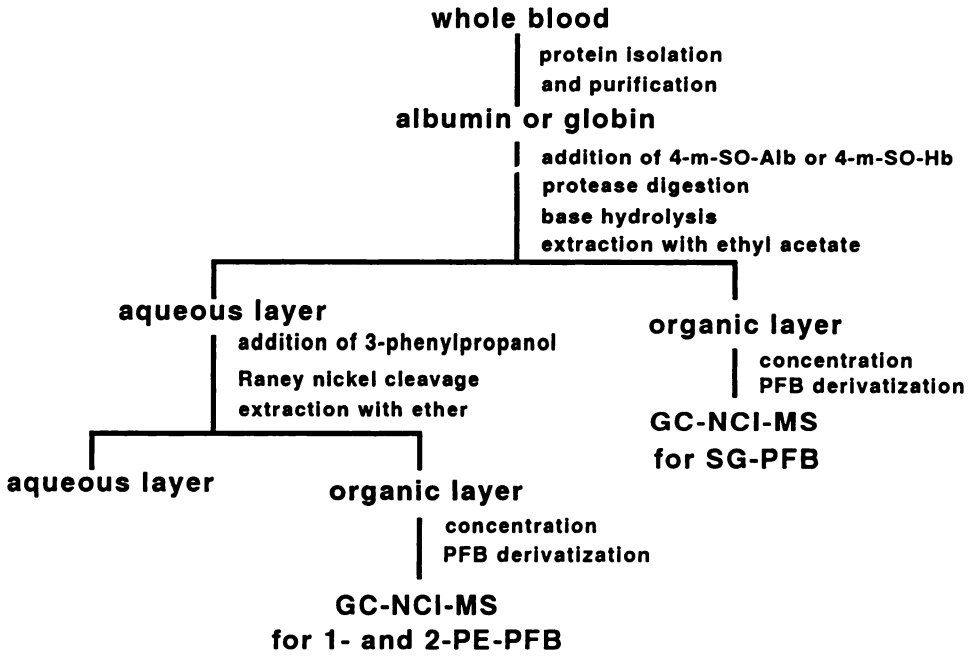

Fig. 1. Outline of the procedure for the analysis of SO-protein adducts of both carboxylic acid and cysteinyl residues.

SO-Hb or 4-methyl-SO-albumin, 1 ml 0.1 M Tris-HCl buffer (pH 7.5), and protease XIV (Sigma, St. Louis, MO).

After the protein was digested at 37°C for 4–6 h (17), 1 ml 0.5–1 M NaOH was added to increase the pH of the medium to 13, and the solutions were heated to 100°C for 5 min to release SG. After being allowed to cool to room temperature, the samples were extracted twice with 5 ml ethyl acetate. The solvent was then removed under nitrogen and the analyte (SG) and internal standard (4-methyl-SG) were derivatized with 1.5 μl PFB-chloride and 3 μl pyridine in 1 ml hexane, as described previously (17), for 1-PE and 2-PE. To the aqueous layer was added 7.4 pmol 3-phenylpropanol and 150–250 mg Raney nickel (Aldrich Chemical Co., Milwaukee, WI). After extracting the cleavage products twice with 5 ml diethyl ether, the solvent was reduced under a stream of nitrogen, and the analytes (1-PE and 2-PE) and internal standards (4-methyl-2-PE and 3-phenylpropanol) were derivatized with PFB-chloride and quantified by GC-MS in the NCI mode.

GC-MS Analysis of Adducts. The derivatized samples were analyzed by GC-NCI-MS in the SIM mode using a Hewlett Packard Model 5989A MS engine coupled to a Hewlett Packard 5890 series II gas chromatograph. The column (DB-5, 30 m, 0.242 mm i.d., 1-μm phase thickness; J & W Scientific, Inc., Folsom, CA), chemical ionization reagent gas (methane, 2 torr), carrier gas (He, 1034 torr), injector port (250°C), and source (150°C) temperatures were the same as described in Rappaport *et al.* (17). The SIM ions monitored were *m/z* 316 (for SG-PFB, 1-PE-PFB, and 2-PE-PFB) and 330 (for 4-methyl-SG-PFB, 4-methyl-2-PE-PFB, and 3-phenylpropanol-PFB). These ions represent the molecular ions in each case, except for SG-PFB and 4-methyl-SG-PFB, which were monitored via fragment ions.

The oven temperature for the hydrolysis products (SG-PFB and 4-methyl-SG-PFB) was maintained at 75°C for 1 min and then increased at 50°C/min to 250°C and held for 20 min. For the remaining analytes, the oven temperature was held at 75°C for 1 min and increased at 50°C/min to 200°C and held for 11 min. Late-eluting compounds were removed by increasing the temperature to 250°C at 50°C/min and maintaining this temperature for 8 min. Injections were made in the splitless mode. Approximate retention times for the analytes were as follows: SG-PFB, 12.7 min; 4-methyl-SG-PFB, 14.7 min; 1-PE-PFB, 9.5 min; 2-PE-PFB, 10.9 min; 4-methyl-2-PE-PFB, 12.9 min; 3-phenylpropanol-PFB, 13.5 min.

Identification and Characterization of SG and 1-PE. Human albumin that had been modified *in vitro* with 300 μM SO, as described by Rappaport *et al.* (17) for the preparation of 4-methyl-SO-albumin, was assayed to confirm the identities of SG and 1-PE, which had not been previously characterized in our method. After hydrolysis of the carboxylic acid adduct (SG) or release of the cysteinyl adduct (1-PE) with Raney nickel, the organic extracts were derivatized with PFB-chloride, as described above. SG-PFB and 1-PE-PFB were characterized by GC-MS in the EI mode using a Hewlett Packard 5890 series II gas chromatograph equipped with a Hewlett Packard 5891A mass selective detector. The column characteristics and temperature programs were identical to those used for routine analysis of samples. The transfer line temperature was 280°C, and mass spectra were recorded by scanning the mass spectrometer from 50 to 650 *m/z* at an ionization energy of 70 eV. The identities of SG and 1-PE were confirmed by comparing the mass spectra obtained for SG-PFB and 1-PE-PFB from modified albumin with that of derivatized standards (Aldrich Chemical Co.).

High-Resolution GC-MS. To confirm the identity of the 2-PE background adducts, a standard, a reagent blank, and control samples of albumin and globin were analyzed by high-resolution GC-NCI-MS using a VG70–250SEQ mass spectrometer interfaced with a Hewlett Packard 5890 series II gas chromatograph. The column, injector port, and oven temperatures were identical to those described for the routine analysis of samples. Mass spectra were acquired using a resolving power of 10,000, an electron ionization energy of 150 eV, an emission current of

1.5 mA, and a source temperature of 220°C. SIM of the 2-PE-PFB molecular ion exact mass (316.0523 *m/z*) and 3-phenylpropanol-PFB exact mass (330.0679 *m/z*) used a perfluorokerosine lock mass of 330.9792 *m/z*.

Precision, Bias, and Background Levels of Adducts. The precision of the assays was estimated from analyses of control proteins that had been performed concurrently with each batch of samples. Human Hb and pooled human serum were purchased from Sigma Chemical Co. (St. Louis, MO). Control globin was isolated from Hb, and control albumin was isolated from the pooled human serum as described above. Because the control proteins had been isolated by Sigma from blood that had been pooled from many donors (Hb, 562 persons; serum, 88 persons), we regard the adduct levels in these control proteins as estimates of mean background levels in fairly large samples of (presumably unexposed) persons in the central United States.[4]

For determinations of Hb adducts, no significant differences in the observed levels were detected among batches of control globin analyzed on different days [mean level = 0.058 ± 0.017 (SE) nmol/g globin (n = 14)], based on a one-way ANOVA of the 2-PE determinations (P = 0.95). The CV for these Hb assays was estimated to be 0.330. For determinations of albumin adducts, however, significant differences of all analytes [mean levels in nmol adduct/g albumin: 2-PE, 1.18 (SE = 0.068; n = 78); 1-PE, 0.148 (SE = 0.027; n = 26); and SG, 0.862 (SE = 0.079; n = 34)] were detected by ANOVA (P < 0.05) among sets of samples of control albumin analyzed on different days. In these cases the precision was estimated from the residual errors (within-group variances) as follows: 2-PE CV, 0.397; 1-PE CV, 0.825; and SG CV, 0.534.

To remove any biases associated with differences in assays of albumin adducts conducted on different days, the level of each analyte observed in an experimental sample was adjusted by the level of the corresponding analyte measured concurrently in the control albumin as follows: adjusted level = (observed level × mean control level)/(batch control level), where "batch control level" refers to the average level of the control albumin adducts determined in a particular batch of samples, and "mean control level" refers to the overall mean levels given above for all assays of the control albumin.

Data Analysis. Exposures to styrene and SO were measured repeatedly for at least some of the subjects during the different surveys [styrene, 2 ≤ n (per subject) ≤ 7; SO, 1 ≤ n (per subject) ≤ 3]. Of those 20 workers whose exposures to SO were measured, 13 had single measurements, 4 had two measurements, and 3 had three measurements. Likewise, multiple blood samples were taken from most of the subjects [1 ≤ n (per subject) ≤ 3], and most blood samples were analyzed for protein adducts only once. Because all the albumin samples were analyzed for 1-PE, 2-PE, and SG, each adduct estimate is the mean of 1–3 determinations. However, approximately two-thirds of the Hb samples were analyzed only for 2-PE. Hence, the estimates of Hb adduct levels, as determined by measuring 1-PE and SG, usually represent only one determination for each individual.

All measurements of a particular type were averaged by subject, and the estimated (unweighted) mean values were then used in subsequent statistical procedures using SAS-PC (SAS Institute, Cary, NC). Two-tailed significance levels were calculated.

[4] Sigma Technical Service, personal communication.

Results

Previously, we had reported a method that involved the analysis of only one of the two possible SO adducts of cysteine in blood proteins, which was detected as 2-PE (17, 27). This current analysis of albumin and Hb adducts of SO involves the quantitation of both cysteinyl adducts (detected as 1-PE and 2-PE), as well as carboxylic acid adducts (detected as SG). The identities of SG and 1-PE were confirmed by comparing the EI mass spectra obtained from *in vitro* modified albumin with those from analytical standards. The spectra for SG-PFB and 1-PE-PFB are given in Figs. 2 and 3, respectively. High-resolution MS was performed on reagent blanks and control proteins, which confirmed the identities of the background adducts (data not shown).

The ranges of exposures and adduct levels are shown in Table 1 along with estimates of the mean values of each variable. The univariate relationships between exposure to styrene and to SO and the corresponding adduct levels are depicted in Figs. 4–7 as scatter plots of the estimates of individual mean values. The relationship between the concentration of styrene and SO in air is given in Fig. 8.

As shown in Figs. 4 and 5, the estimated Hb adduct levels, whether analyzed by quantitation of 2-PE, 1-PE, or SG, displayed no apparent correlation with exposure to either styrene or to SO [the smallest P value for these correlations was P = 0.18 (2-PE *versus* SO), and in this case the regression coefficient was negative]. However, when levels of SO-albumin were plotted *versus* exposures to styrene (Fig. 6) and SO (Fig. 7), trends toward increasing adduct levels were observed in most cases. For each of the three SO-albumin adducts (measured as 1-PE, 2-PE, and SG), the linear correlation coefficient was larger for exposure to SO than for exposure to styrene, despite the fact that measurements of airborne SO were obtained from fewer than half of the subjects and typically included only one measurement per subject. Several of these linear correlation coefficients (for SO-albumin *versus* exposure) were at or near a 0.05 level of statistical significance (2-PE *versus* styrene, P = 0.017; 2-PE *versus* SO, P = 0.010; 1-PE *versus* styrene, P = 0.126; 1-PE *versus* SO, P = 0.122; SG *versus* styrene, P = 0.965; SG *versus* SO, P = 0.259).

Discussion

SO can bind to sulfhydryl groups through either the α or the β carbon to produce adducts that, after cleavage, are measured in our assay as 2-PE and 1-PE, respectively. Our previous work had focused on the more abundant α-substituted adduct (giving rise to 2-PE) because of analytical difficulties in assaying the β-substituted product (leading to 1-PE). In this study we were able to successfully quantitate both 1-PE and 2-PE because of improvements in the sensitivity of the GC-MS system. Assuming that the α and β isomers of the cysteine adduct are equally stable *in vivo*, then the ratios of the slopes of the regressions of the corresponding albumin adducts (yielding 2-PE and 1-PE, respectively) on the exposures of workers to styrene or SO suggest that SO is approximately four times more likely to react through the α carbon than through the β carbon in humans. The ratio of slopes of 2-PE/1-PE = 4.7 for styrene exposure, whereas slopes of 2-PE/1-PE = 4.0 for SO exposure. This result is somewhat higher than published results obtained *in vitro*. Pacheka *et al.* (28) incubated SO with glutathione *in vitro* and reported an α-to-β binding ratio of 1.5 for the reaction of SO with the cysteinyl residue, Yagen *et al.* (29) reacted SO with *N*-acetylcysteine, which resulted in a ratio of 1.9, and

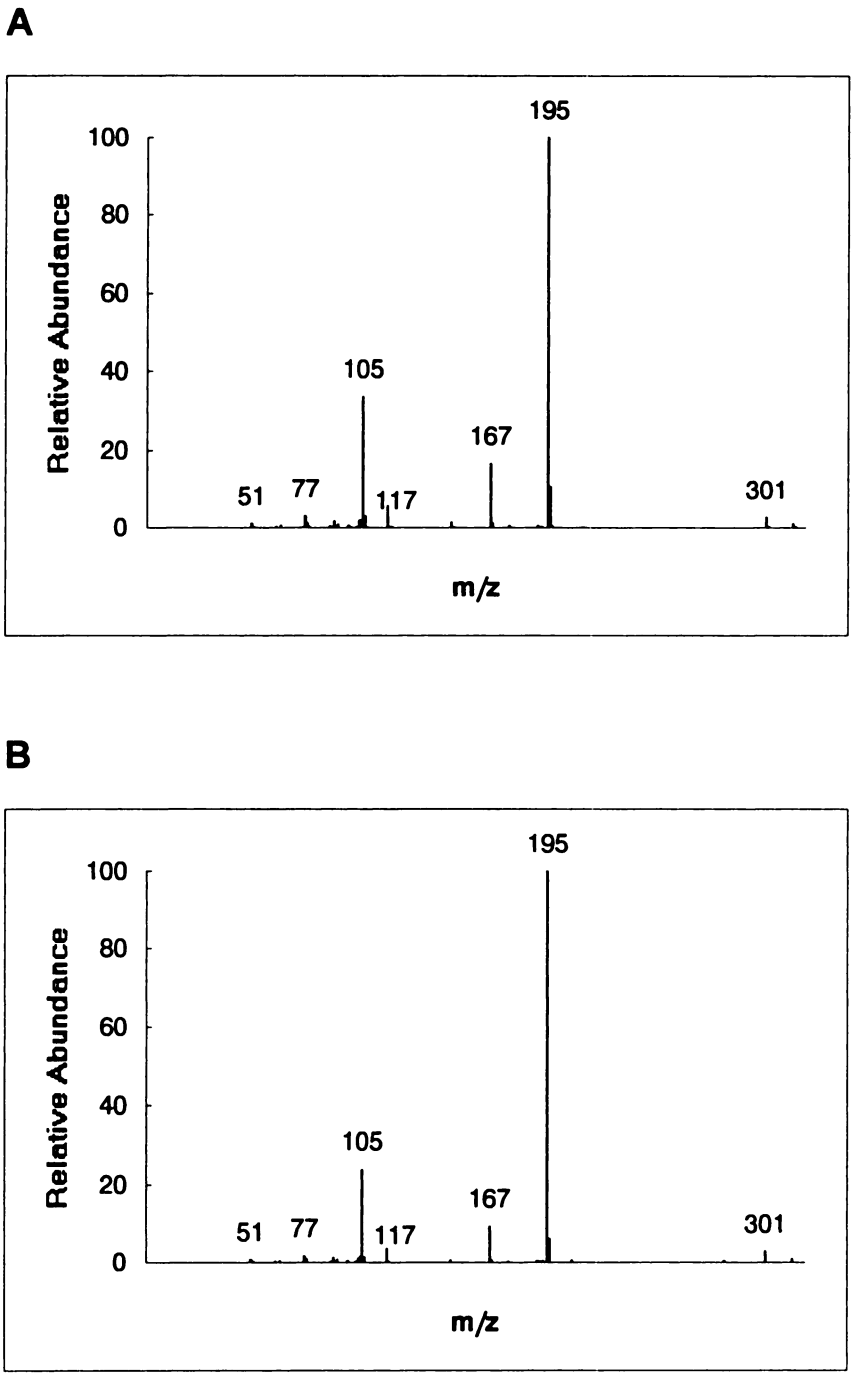

Fig. 2. Identification of SG in albumin modified *in vitro* with 300 μM SO. Comparison of the EI mass spectra of (*A*) the peak at 15.45 min with that of (*B*) a SG-PFB standard.

Hemminki (30) reported that the reaction of SO with free cysteine *in vitro* gave a ratio of approximately 2.

Previous studies of the kinetics of the reaction of SO with the cysteinyl residues of human albumin and Hb *in vitro* showed that the second order rate constant for human albumin was 13 times that of Hb, indicating that the intrinsic reactivity of SO was much greater toward serum albumin than Hb in humans (17). However, the steady-state levels of Hb adducts should be approximately two times greater than those of albumin, assuming that exposure is constant and that SO adducts are stable. Because the life span of human RBCs (and hence of Hb) is 120–126 days (31) and the half-life of human albumin is approximately 20 days (32), then, based on calculations from Granath *et al.* (33), the steady-state Hb adduct levels should be 60-fold higher than the daily adduct increment, whereas steady-state albumin adduct levels should be 30-fold higher. Thus, we anticipated that levels of cysteinyl adducts in albumin from exposed workers would be approximately seven times higher than the corresponding Hb adducts. Because our results indicated that SO-albumin adducts of cysteinyl residues were

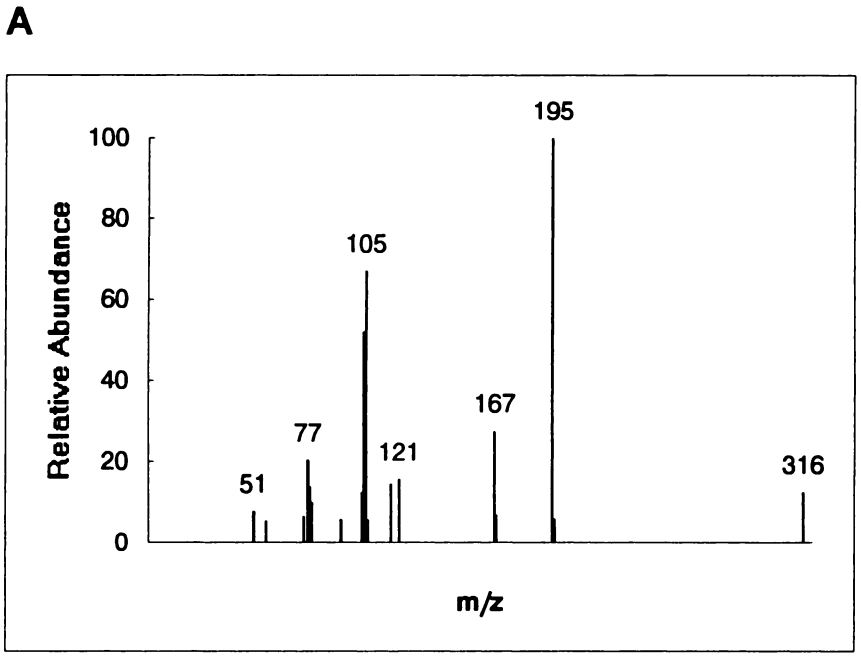

Fig. 3. Identification of 1-PE in albumin isolated from blood modified *in vitro* with 300 μM SO. Comparison of the EI mass spectra of (*A*) the peak at 12 min with that of (*B*) a 1-PE-PFB standard.

22-fold more abundant than the corresponding SO-Hb adducts (based on 2-PE), factors other than those involving the intrinsic reactivities and elimination kinetics seem to have been involved.

Because the precision of the assays of 2-PE in albumin (CV = 0.397) and in Hb (CV = 0.303) are comparable, differential assay-related errors cannot explain the observed ratio of albumin to Hb adducts. Another possibility is that significant adduction of albumin took place within the hepatocytes of the workers after conversion of styrene to SO by cytochrome P450 enzymes, rather than in the blood *per se*. Although significant intrahepatic formation of albumin adducts was not observed in rats (17), Korn *et al.* (34) estimated that the SO concentration in blood is 10–20 times greater in rats than in humans exposed to a given concentration of styrene, suggesting that much less SO is released from human hepatocytes than from rat hepatocytes. We speculate that such a large reduction in systemic bioavailability of SO (after hepatic conversion of

Table 1 Summary statistics for exposures and levels of adducts

Variable	Units[a]	No. Subjects	Measure/Subjects	Range[b]	Mean	SE
Styrene exposure	mg/m^3	48	2–7	0.9–235	64.3	10.3
SO exposure	μg/m^3	20	1–3	13.4–525	159	25.0
SO-Hb (1-PE)	nmol/g protein	40	1	0.02–0.45	0.084	0.014
SO-Hb (2-PE)	nmol/g protein	48	1–3	0.03–0.16	0.078	0.003
SO-Hb (SG)	nmol/g protein	41	1–2	0.09–4.8	0.481	0.132
SO-albumin (1-PE)	nmol/g protein	48	1–3	0.02–1.8	0.290	0.038
SO-albumin (2-PE)	nmol/g protein	48	1–3	0.24–3.7	1.68	0.116
SO-albumin (SG)	nmol/g protein	48	1–3	0.1–6.3	1.80	0.191

[a] 4.3 mg/m^3 styrene = 1 ppm and 4.9 μg/m^3 SO = 1 ppb.
[b] Range of estimated mean values among the subjects.

styrene to SO) in humans could increase the importance of even small amounts of hepatic SO-albumin to the total pool of SO-albumin in human blood and thereby account for the increased levels of SO-albumin relative to SO-Hb that we observed.

Clearly, more work is needed to understand the reason that we can detect exposure-related increases in SO-albumin but not those of SO-Hb among workers in the reinforced plastics industry. The question is perplexing in light of previous reports that levels of NH_2-terminal valine adducts of Hb were correlated with styrene exposure among highly exposed workers in the reinforced plactics industry (styrene exposures in the range of 300 mg/m^3) (14) but apparently not among workers with more modest levels of exposure [Brenner *et al.* (13) (mean styrene exposure 74 mg/m^3) and Severi *et al.* (15) (mean styrene exposure 31 mg/m^3)], which are in the range of our own study (styrene exposure ~64.3 mg/m^3). Regardless of the reason for the discrepancy between adducts of SO with albumin and Hb, we note that albumin adducts of other environmental contaminants that produce reactive epoxides after cytochrome P450 metabolism, notably those of aflatoxin B_1 (35) and of benzene (36), have been correlated with human exposures, whereas the corresponding Hb adducts have not.

Assuming that all adduction of albumin takes place in the blood and that the SO-albumin adducts are stable *in vivo*, then it is possible to estimate the average blood concentration of SO (which we designate as $\overline{[SO]}$) which would be expected, in a typical worker from this cohort, on the basis of the measurements of SO-albumin. Using a relationship developed by Ehrenberg and Törnqvist (37), $\overline{[SO]}$ can be related to adduct levels as follows:

$$\overline{[SO]} = \frac{[SO - albumin]_{ss} \cdot k_e}{[albumin] \cdot k_{SO-Alb}} \quad (1)$$

where $k_e = 1.4 \times 10^{-3}$ h^{-1} is the first-order elimination rate constant for human serum albumin (32), [albumin] = 24.3 mg/ml = 3.5×10^{-4} M is the concentration of albumin in blood (31), and k_{SO-Alb} = 32 liter (mol albumin)$^{-1}$ h^{-1} is the second-order rate constant for the reaction of SO with albumin in whole blood [based on measurements of 2-PE in human blood *in vitro* by Rappaport *et al.* (17)]. The quantity [SO − albumin]$_{ss}$ = 1.68 nmol/g albumin = 4.03×10^{-8} M represents the steady-state adduct level estimated by the mean value of 2-PE in our sample of workers. Thus, we find that $\overline{[SO]}$ = 4.70 $\times 10^{-9}$ M = 0.57 μg/liter blood for a typical worker in this cohort.

Only three studies have been published in which SO was measured directly in the blood of reinforced plastics workers (all measurements were made during the work shift). Löf *et al.* (38) measured SO in the blood of 10 workers (mean styrene exposure, 94.9 mg/m^3) and reported that most of the levels were at the detection limit (2.4 μg/liter); one worker was at twice this level (4.8 μg/liter). Assuming that a typical value for these workers would be 2.4 μg/liter during the work shift, then the corresponding value of $\overline{[SO]}$ = 2.4 μg/liter × 8 h exposure/24 h × 5 days exposure/7 days = 0.57 μg/liter. Christakopoulos *et al.* (14) measured SO in 7 workers (styrene exposure estimated at 300 mg/m^3 based on urinary mandelic acid) and found a mean level of 11 μg/liter, from which we estimate $\overline{[SO]}$ = 2.6 μg/liter. Finally, Korn *et al.* (34) measured SO in 13 workers (range of styrene exposures, 42–310 mg/m^3); we used their data to estimate a mean SO level of 2.2 μg/liter from which the estimated $\overline{[SO]}$ = 0.52 μg/liter. These few direct estimates of $\overline{[SO]}$ are certainly consistent with our indirect estimate of 0.57 μg/liter and lend credibility to the use of cysteinyl SO-albumin adducts as dosimeters for SO among reinforced plastics workers.

Although Fig. 7*C* points to a trend toward increased carboxylic adducts of SO-albumin with exposure to SO, the strength of the correlation is much weaker than that for the corresponding cysteinyl adducts (Fig. 7, *A* and *B*). Because the precision of our assay for SG was in the range of those for 1-PE and 2-PE, differential assay errors are probably not the primary reason for this discrepancy. A more likely explanation relates to the possible instability of carboxylic acid adducts, as indicated by the behavior of ethyl esters of Hb from mice after injection of *N*-ethyl-*N*-nitrosourea (39). We also note that no evidence of a trend toward higher levels of carboxylic acid adducts of either albumin or Hb were observed with increasing styrene exposure (Figs. 4*C* and 6*C*), consistent with observations of others who attempted to correlate SO-Hb adducts (measured as SG) with exposures of reinforced plastics workers (16).

The presence of significant background levels of SO-Hb and SO-albumin in this and other studies raises interesting questions regarding the origin(s) of these adducts. [Note that the following background levels have been reported: Christakopoulos *et al.* (14), ≤13 pmol/g; Bergmark *et al.* (39), unspecified background detected; Brenner *et al.* (13), controls had adduct levels one-fifth the levels of the exposed; and Sepai *et al.* (16), 38.9 pmol/g in control human Hb, although exposed subjects were not above the 15 pmol/g detection limit.] Because the controls in our study consisted of human globin and albumin that had been extracted from pooled samples of blood by Sigma Chemical Co., the levels of adducts therein can be viewed as representative of persons in the central United States. Because these persons are unlikely to have been occupationally exposed to styrene or to SO, it is relevant to consider the likelihood that styrene or SO might have arisen from other environmental and/or dietary sources.

Styrene is a ubiquitous contaminant, arising from natural,

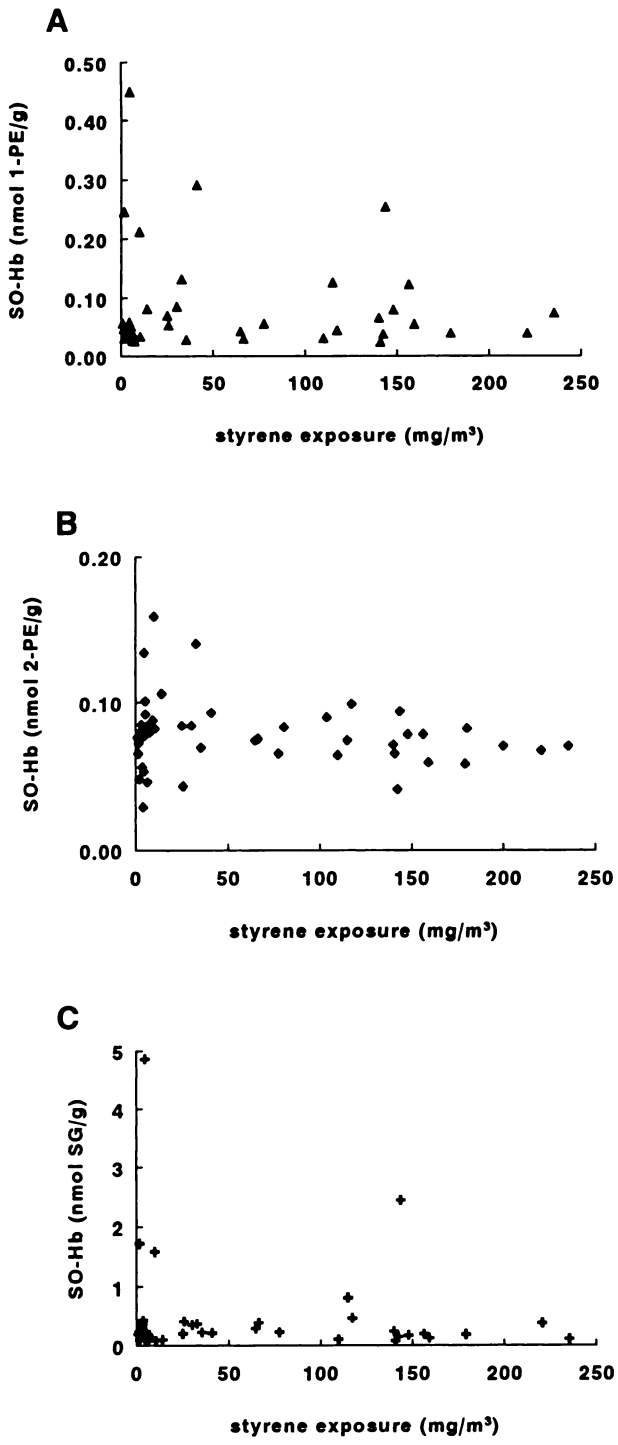

Fig. 4. Scatter plots showing relationships between SO-Hb and exposure to styrene: (*A*) cysteinyl adduct measured as 1-PE, (*B*) cysteinyl adduct measured as 2-PE, (*C*) carboxylic acid adduct measured as SG.

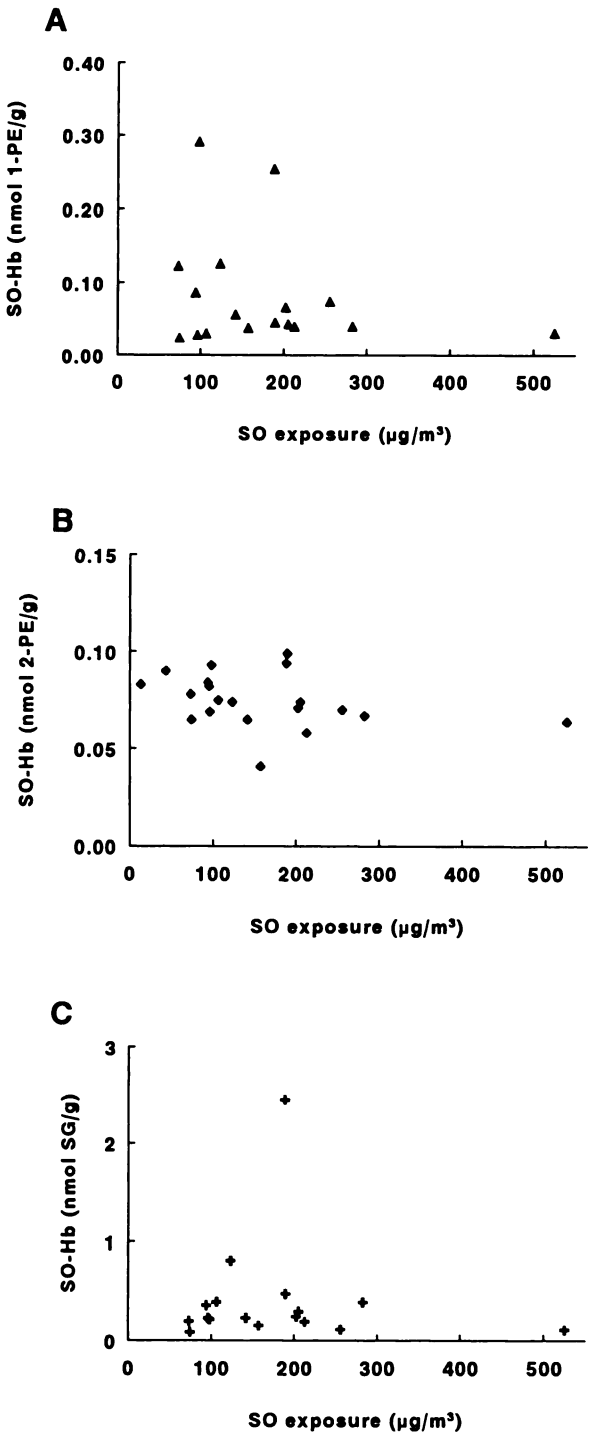

Fig. 5. Scatter plots showing relationships between SO-Hb and exposure to SO: (*A*) cysteinyl adduct measured as 1-PE, (*B*) cysteinyl adduct measured as 2-PE, (*C*) carboxylic acid adduct measured as SG.

as well as anthropogenic, sources, including cigarette smoke, automobile exhaust, carpet outgassing, and food stored in polystyrene containers (40, 41). Consequently, styrene has been detected at low levels in ambient indoor and outdoor air (42, 43), as well as in the blood (44) and adipose tissue (45) of unexposed individuals. However, because our results and those of others (46) indicate that styrene is a fairly inefficient producer of adducts in humans (Figs. 4 and 6), only exposures to very high levels of styrene (several hundred mg/day) could have resulted in the background levels detected. For example,

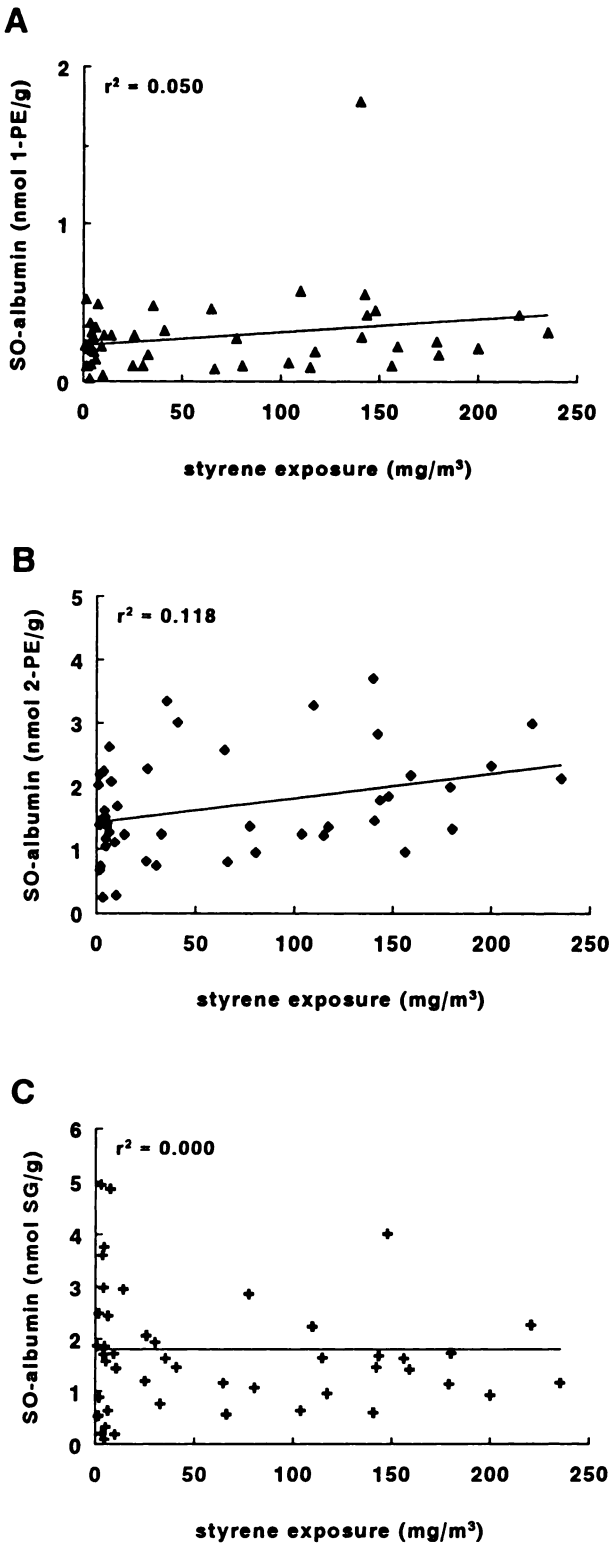

Fig. 6. Scatter plots showing relationships between SO-albumin and exposure to styrene: (*A*) cysteinyl adduct measured as 1-PE, (*B*) cysteinyl adduct measured as 2-PE, (*C*) carboxylic acid adduct measured as SG.

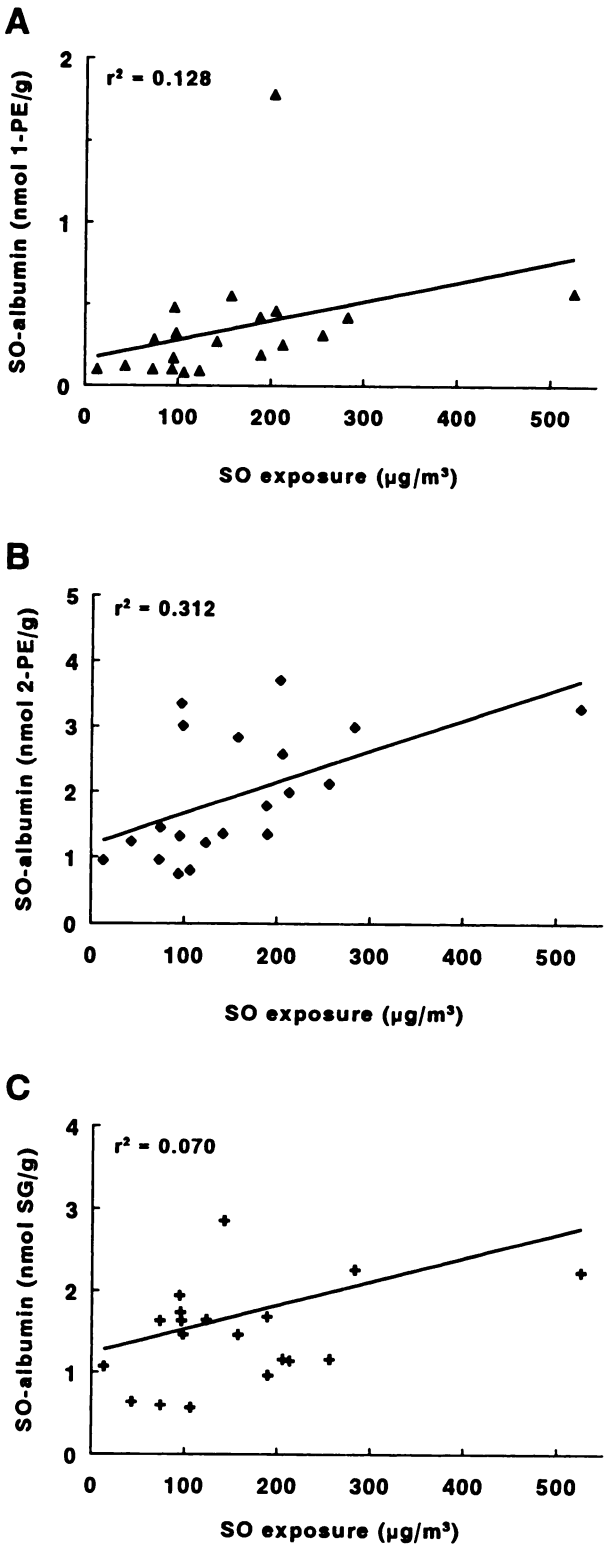

Fig. 7. Scatter plots showing relationships between SO-albumin and exposure to SO: (*A*) cysteinyl adduct measured as 1-PE, (*B*) cysteinyl adduct measured as 2-PE, (*C*) carboxylic acid adduct measured as SG.

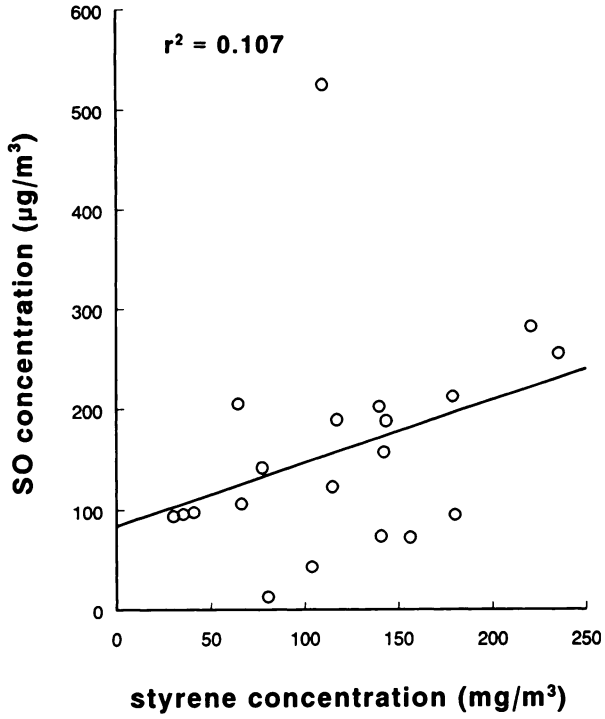

Fig. 8. Scatter plot showing relationship between exposure to styrene and exposure to SO.

given an average styrene exposure of 64.3 mg/m^3, a respiratory ventilation rate of 1 m^3/h (for a healthy worker at light/moderate exercise), and a respiratory retention for styrene of 0.94 (47), a typical worker in our study would have retained 483 mg of styrene in an 8-h work day. Because environmental exposures tend to be very small [hundreds of µg/day at most (42)] compared to occupational exposures, they are unlikely to have contributed significantly to the background levels of adducts.

On the other hand, our results suggest that SO is a more potent producer of albumin adducts than styrene *in vivo* (Figs. 6 and 7) and is, therefore, a more likely source of background adducts. Although we could find no information regarding nonoccupational exposures to SO, we note that SO has been detected in distillates of dried tobacco (48), and could, therefore, be a constituent of certain plant products in the diet. It is also possible that the background adducts originated from SO (or possibly from another electrophilic species that produces the same adduct) that was formed either endogeneously or as a byproduct of metabolism. In other studies, significant levels of background adducts of simple electrophilic species have been linked to such dietary and endogeneous sources (49–51), and we see no reason why a simple epoxide like SO could not be similarly formed *in vivo*. We also note that Christakopoulos *et al.* (14) reported that two of four controls had detectable levels of SO in their blood (2.4 µg/liter, the detection limit), although Korn *et al.* (34) could not detect SO in any of three controls (detection limit, 0.9 µg/liter).

Perhaps our most interesting finding is the observation that SO adducts of albumin appear to be more strongly correlated with exposure to SO (Fig. 7) than to styrene (Fig. 6). Indeed, when multiple linear regressions were performed for the 20 subjects with measurements to both airborne styrene and SO, levels of SO-albumin (measured as 2-PE) were significantly correlated with exposure to SO but not with exposure to styrene. Furthermore, as shown in Fig. 8, exposure to SO ($P < 0.05$) was weakly (and positively) correlated with exposure to styrene ($P = 0.16$), opening the possibility that the observed correlation of SO-albumin with styrene exposure among the full cohort was due to coexposure to SO. More work is needed to understand the relative roles that coexposures to styrene and SO play in the production of protein adducts, as well as of other biomarkers of genotoxic effects.

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