MEASUREMENT OF STYRENE-OXIDE CYSTEINE ADDUCTS IN HEMOGLOBIN BY SELECTIVE CATALYTIC REDUCTION

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Alkylating agents, either direct-acting or generated *in vivo*, form an important class of environmental mutagens and carcinogens. These agents are characterized by their ability to bind covalently to nucleophilic sites in proteins and DNA. Due to the long life time of red blood cells and the stability of protein adducts, Ehrenberg and coworkers first (Calleman 1978; Osterman-Golkar 1976) proposed the use of hemoglobin (Hb) as a dosimeter for alkylation *in vivo*.

Since then, several methods have been devised to detect hemoglobin adducts (Bryant 1987; Calleman 1978; Farmer 1980; Neumann 1984). Here, we report an alternative method that is sensitive, specific and relatively simple. It is based on the well known desulfurization by Raney nickel (Ra-Ni) (Pettit 1962; Pizey 1974), in which the carbon-sulphur bond formed by the alkylating agent and cysteine is selectively cleaved and at least one new carbon-hydrogen bond is formed. Pachecka et al. (Pachecka 1979) demonstrated that when styrene-7,8-oxide-glutathione adducts were refluxed with Ra-Ni in ethanol, 1-phenylethanol (1-PE) and 2-phenylethanol (2-PE) were produced. This reaction between cysteine-bound styrene oxide and Ra-Ni is shown in Figure 1. We utilized this reaction to determine the amount of cysteine-bound styrene-7,8-oxide (SO) which reacted with Hb *in vitro* and *in vivo*.

MATERIALS AND METHODS

C-14 labeled SO was obtained from Amersham; it was diluted to a specific activity of 1.5 mCi/mmol with unlabeled SO before use. Styrene and SO were purchased from Aldrich and used without further purification. Human blood was incubated with SO *in vitro* at 37°C for 2 hr. In the *in vivo* experiment, Male Sprague-Dawley rats weighing 300g were given a single i.p. injection of styrene in corn oil. Blood was collected from each animal by cardiac puncture 20 hr after dosing. Globin (Gb), from both human and rat blood, was prepared by the following procedure. After lysing the red blood cells with water and spinning down the cell debris, the Hb solution was dialysed exhaustively at 5°C against distilled water. Then, the Hb solution was added dropwise to a cold 0.1% HCl, acetone solution to precipitate Gb (Anson 1930). The Gb was isolated by filtration, washed with acetone and hexane, and dried to constant weight in a desiccator. The radioactivity associated with the Gb samples was determined by liquid scintillation counting. The Gb was denatured by heating to 80°C

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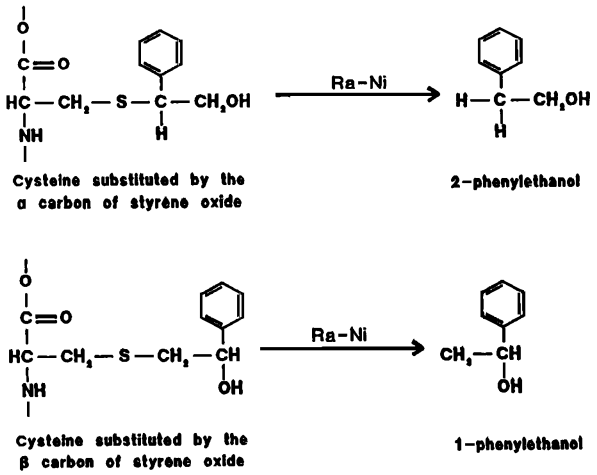

Figure 1. Cleavage of styrene-oxide-cysteine by Ra-Ni.

for 25 min and then digested with Protease XXV (from Sigma, about 7% W/W of Gb) at 37°C for 6 hr. After adjusting the pH to 12, the solution was spiked with an internal standard (100 ng of 3-phenyl-1-propanol). The sample was then shaken with Ra-Ni (50% slurry, from Aldrich, 5-6 g per g of Gb) at 5°C for 40 min to cleave the cysteine adduct. Upon completion of the reaction, the solution was extracted twice with 6 ml of ethyl ether. The ether fractions were combined, washed twice with 0.1 M HCl and then reduced almost to dryness under a stream of nitrogen. To derivatize the products of the Ra-Ni reaction, 0.5 ml of hexane, 3 ul of pyridine and 1.5 ul of pentafluorobenzyl chloride (PFB-chloride, from Aldrich) were added to the residue and the solution was warmed to 50°C for 20 min. After cooling to room temperature, the sample was dried under a stream of nitrogen, and the remaining residue was dissolved in 0.5 ml of 85% methanol and extracted with 0.5 ml hexane (Bogaert, 1978). A portion of this solution was injected into a Varian 3700 gas chromatograph equipped with an electron-capture detector and a capillary column (15-m x 0.32-mm i.d. DB-5, 1.0 um film thickness from J and W. Scientific). The injection volume was 2 ul with a split ratio of 20 to 1. The carrier gas was helium with a linear velocity of 29 cm/sec. Analysis was performed isothermally at 180°C.

RESULTS AND DISCUSSION

Our interest in monitoring the bioavailable dose of SO received by humans exposed to styrene led us to seek a simple method for monitoring SO-Gb adducts. In developing such a method we were intrigued with Pacheka's use of Ra-Ni to cleave SO-cysteine adducts from glutathione conjugates (Pachecka 1979). We applied a modification of that procedure to release adducts from Gb which had been derived from either human blood, modified with styrene oxide *in vitro*, or blood from rats to which styrene had been administered *in vivo*. We observed in both cases that reaction of Gb with Ra-Ni yielded 2-PE, one product which would be expected to result from cleavage of SO-cysteine.

Analysis of Human Blood Reacted with Styrene Oxide In Vitro

5-ml portions of fresh human whole blood were reacted *in vitro* with SO at concentrations ranging between 27 and 318 uM. Applying the Ra-Ni method to the Gb samples, a linear dose-response relationship was observed as shown in Figure 2. The amount of 2-PE detected was directly proportional to the amount of styrene oxide

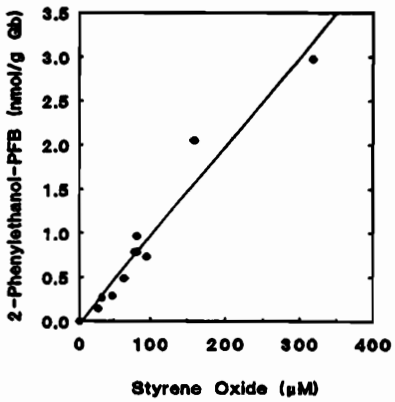

Figure 2. Dose-response curve showing globin adducts in human whole blood modified with styrene-oxide *in vitro*. (Corrected for 81.6% recovery).

added, with a slope of 9.83×10^{-3} nmol 2-PE/g Gb/uM styrene oxide (r=0.97). The detection limit of 2-PE-PFB was estimated to be 0.04 nmol/sample; assuming 5ml of blood per sample.

Analysis of Blood From Rats Treated with Styrene In Vivo

Since styrene is metabolized to SO in many animal species including humans (Leibman 1975; Ohtsuji 1971), we wished to demonstrate that this method can detect SO formed *in vivo*. Male Sprague-Dawley rats were treated with 0, 0.5, 1, 2 and 3 mmol styrene/kg body weight by i.p. injection. Upon analysis of the Gb isolated from these animals, a linear dose-response curve was observed (Figure 3), with a slope of 2.30 nmol 2-PE/g Gb/mmol styrene/kg body weight (r=0.90).

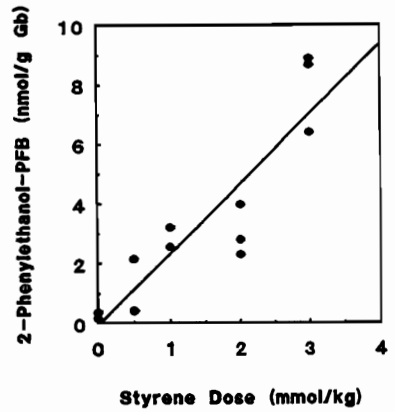

Figure 3. Dose-response curve showing globin adducts in rats following a single i.p. dose of styrene. (Corrected for 81.6% recovery).

Proportion of Cysteine Adducts

Although we showed that Ra-Ni could be successfully applied to cleave styrene-oxide adducts from Gb; our work indicated that only about 6% of the total binding was actually recovered as isomers of phenylethanol (Table 1) following the Ra-Ni reaction. This suggests either that the Ra-Ni is rather inefficient at cleaving cysteine adducts from Gb or (more likely) that other nucleophilic sites on the Gb chain account for the bulk of reaction with SO. Further experimentation is required to resolve this question.

Table 1. Styrene oxide-globin adducts detected by the Ra-Ni method* (Estimated mean ± std. dev. and number of replicates for each group)

Styrene-Oxide Conc. (uM)	% of total adducts detectable by the Ra-Ni method
33	5.8 ± 0.6 (2)
50	4.8 (1)
82	6.1 ± 0.9 (3)
Total	5.8 ± 0.8 (6)

* Corrected for 81.6% recovery and assuming 60% of styrene oxide-cysteine adducts are alpha-substituted (Pachecka, 1979). Total adducts were determined by counting Gb which had been reacted with 14C-labeled styrene oxide.

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