

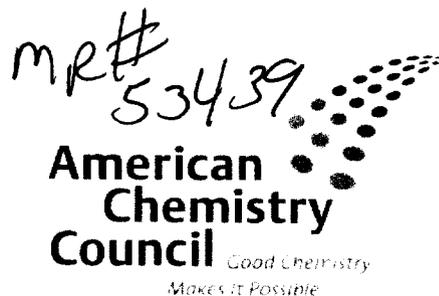
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December 11, 2001



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U.S. Environmental Protection Agency
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Re: Toxic Substances Control Act Section 8(e)

Dear Madam or Sir:

The Propylene Oxide/Propylene Glycol Panel (Panel) of the American Chemistry Council, on behalf of its member companies¹, submits this letter pursuant to Section 8(e) of the Toxic Substances Control Act, to inform EPA of information the Panel has received on a study measuring biomarkers in a group of workers reportedly exposed to propylene oxide (PO, CAS No. 75-66-9). The Panel and its member companies have not made a determination as to whether a significant risk of injury to health or the environment is actually presented by the findings.

The attached information is a manuscript entitled *Analysis of DNA- and hemoglobin adducts and sister chromatid exchanges in a human population occupationally exposed to propylene oxide – pilot study*. The manuscript was received by the Panel from one of the manuscript's authors who advised that the manuscript has been accepted for publication in the journal *Cancer Epidemiology Biomarkers and Prevention*.

The manuscript reports the study findings on biomarkers for a group of 8 workers reportedly exposed to PO at a manufacturing facility in China and 8 non-exposed persons apparently from a local occupational health center. The DNA adduct findings of this study were reported previously by the Panel to EPA on July 11, 2000 (8EHQ-0796-13687-Supplement) after these results had been received at an earlier meeting presentation. The frequencies of sister chromatid exchanges (SCE) in the workers are first reported in the manuscript. The study found

¹ Panel members include The Dow Chemical Company, Huntsman Petrochemical Corporation, and Lyondell Chemical Company.



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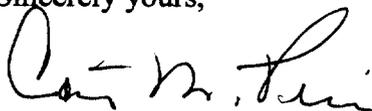
p. 2 of 2

that the exposed workers exhibited an increase in the mean SCE frequency compared to the control group.

This is the first study of which the Panel is aware to report on SCE in PO-exposed workers. The significance of the findings of this study, however, can not presently be discerned. The study was a pilot evaluation and the sample set was very small, comprising of only 8 workers each for control and exposed sample groups. Only minimal exposure information was provided with regards to other substances that may have contributed to the findings. The study's control group was noted to have SCE frequencies lower than have normally been observed. SCE are also not chemical-specific effects and the mechanisms whereby they are formed and their potential relation to human disease are not currently understood.

If you require further information on this matter, please contact Dr. Anne LeHuray, Manager, Propylene Oxide/Propylene Glycol Panel, at (703) 741-5630 or anne_lehuray@americanchemistry.com.

Sincerely yours,



Courtney M. Price
Vice President, CHEMSTAR

Enclosure

cc: Propylene Oxide/Propylene Glycol Panel (w/attachment)

Analysis of DNA- and hemoglobin adducts and sister chromatid exchanges in a human population occupationally exposed to propylene oxide - a pilot study

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Running title: Biomarkers of propylene oxide exposure

Propylene oxide, a simple alkylating agent used in the chemical industry, is weakly genotoxic and induces nasal cavity tumors in rodents upon inhalation at high air concentrations. DNA adducts, hemoglobin adducts and sister chromatid exchanges (SCE) were analyzed as biomarkers of exposure in a group of 8 propylene oxide-exposed workers and 8 non-exposed subjects. 1-(2-Hydroxypropyl)adenine in DNA of white blood cells was analyzed using a hypersensitive ^{32}P -postlabeling assay. 2-Hydroxypropylvaline in hemoglobin was measured using gas chromatography/tandem mass spectrometry. Air measurements indicated propylene oxide levels in the range of 1 to 7 ppm. All three biomarkers showed significantly increased levels in the exposed workers. 1-(2-Hydroxypropyl)adenine was recorded in 7 of the exposed workers (mean $0.66 \text{ mol}/10^9 \text{ mol nucleotides}$) but was not detected in any of the control subjects. 2-Hydroxypropylvaline was found in all subjects (means of 2.7 and $0.006 \text{ pmol}/\text{mg globin}$ in exposed workers and controls, respectively). The average frequencies of SCE were 3.70/cell in exposed workers and 2.00/cell in controls, respectively. DNA and hemoglobin adducts were correlated ($r = 0.887$) as well as DNA adducts and SCE ($r = 0.792$) and hemoglobin adducts and SCE ($r = 0.762$). The present study is the first demonstrating propylene oxide-DNA adducts in human individuals. It is also the first study indicating cytogenetic effects in humans from propylene oxide exposure although confounding effects from other sources can not be excluded.

Introduction

Propylene oxide (PO) is a common industrial chemical mainly used for production of propylene glycols, polyether polyols and propylene glycol ethers. The Swedish NGV/TWA for PO is 2 ppm and in the US the OSHA PEL/TWA is 20 ppm and the ACGIH TLV/TWA is 2 ppm. PO is weakly mutagenic, clastogenic and induces nasal cavity tumors in rodents upon inhalation at high doses (1,2).

PO is a direct alkylating agent introducing 2-hydroxypropyl (HP) groups upon reactions with nucleophilic sites in DNA and proteins (2-4) and such adducts could therefore be used to monitor PO exposures (5). The major adduct formed in DNA following *in vitro* reaction of PO is 7-HP-guanine, followed by the adducts 3-HP- and 1-HP-adenine and 3-HP-cytosine (3,6). These adducts are chemically unstable: 7-HP-guanine and 3-HP-adenine will depurinate forming apurinic sites and 1-HP-adenine and 3-HP-cytosine will spontaneously convert to N^6 -HP-adenine and 3-HP-uracil, respectively (3,6). PO adducts have recently been analyzed in rats exposed to PO by inhalation. 7-HP-guanine was analyzed by gas chromatography/mass spectrometry (GC/MS) and by ^{32}P -postlabeling (7,8); the minor adducts has only been detected using the latter assay (6). Significant repair of either 7-HP-guanine or 1-HP-adenine was not seen in those animal studies. The level of 1-HP-adenine corresponded to about 2% of 7-HP-guanine, but the recovery in the postlabeling assay exceeded that of 7-HP-guanine (50% vs 12%). In addition, 1-HP-adenine was chemically more stable ($t_{1/2} = 9.2$ days for rearrangement to an N^6 adduct compared to $t_{1/2} = 5$ days for depurination of 7-HP-guanine (6)). It

was therefore suggested that this minor adduct could be an alternative for monitoring PO exposures (6).

Hemoglobin is the protein most commonly used for monitoring exposures to chemical carcinogens. PO adducts in hemoglobin are chemically stable, with the same life span as the erythrocytes themselves (about 4 months in humans). Hemoglobin adducts have been measured in humans following occupational exposure to PO (5,9). DNA adducts from this compound have not been determined in human populations this far. Chromosomal aberrations, micronuclei and unscheduled DNA synthesis have been analyzed in a human population exposed to PO (10-12). An effect on unscheduled DNA synthesis was observed, but the lack of a control population made it impossible to establish an effect of exposure for the cytogenetic data. The aim of the present study was therefore to measure both DNA- and hemoglobin adducts and sister chromatid exchanges (SCE) in a small group of workers occupationally exposed to PO.

Materials and methods

Sample collection

Five to ten ml of venous blood was collected from 8 PO-exposed workers at a PO-producing plant and from an equal number of control subjects at an institute of occupational health in the Liaoning province, Peoples Republic of China. The mean age was 29 (range 26-34) for the exposed group and 31

(range 26-33) for the control subjects. The individuals in the exposed group had worked 7-10 years in the factory, with at least 2 years with the present duties. There was an equal distribution of sexes and there were 2 smokers in each group. PO air samples were collected on activated charcoal tubes for 20-60 min and analyzed by gas chromatography. Historical exposure measurements suggest that the levels were about 10 ppm or less. Stationary air measurements recorded one day prior to blood sample collection revealed the highest levels in the packing area (3.7-6.9 ppm). In some common rooms and in the area of polymerization the levels were 0.9-1.7 ppm. The workers spend 1-1.5 hr per day in a packing area, transferring PO from larger reservoirs through pipes to containers of about 100 liters, and the rest of the 8-hr working day in the common rooms not performing work duties related to PO or other exposures.

Blood samples were collected in 2 heparinized glass tubes. One tube was frozen directly and used for later isolation of white blood cell DNA (7). The other tube was used for setting up the cell culture for cytogenetic analysis and for isolation of globin.

Adduct analyses

1-HP-adenine was analyzed by ^{32}P -postlabeling as described (6). Postlabeled samples were mixed with the synthetically prepared standard 1-HP-5'-dAMP and separated by HPLC. The standard was detected by UV, the peak collected and pooled from 4 runs, each based on 10 μg of DNA from the same

person. The pooled sample was heated in 0.1 M NaOH (80°C, 30 min) and reanalyzed by HPLC with on-line UV and radioisotope detection. This treatment quantitatively converted 1-HP-5'-dAMP to *N*⁶-HP-5'-dAMP. A DNA sample modified *in vitro* with ¹⁴C-labelled PO was used as an external standard to determine the recovery of 1-HP-adenine in the postlabeling assay (6). When analyzing this DNA a recovery through the whole postlabeling procedure of 50% was obtained. The detection limit (after correcting for recovery) was 0.1 adduct per 10⁹ nucleotides. All human samples were coded before analysis and each sample was analyzed at least twice.

N-(2-Hydroxypropyl)valine (HP-valine) was measured using the N-alkyl Edman method as previously described (13). All samples were coded before analysis. Hemoglobin was only obtained from 6 of the 8 control individuals. Each sample was analyzed once. Previous studies have demonstrated a good reproducibility of the method with a standard deviation of less than 5% of the mean.

Cytogenetic analysis

Each culture was set up by adding 0.5 ml whole blood into 5 ml culture medium containing F10 medium (Gibco), 20% foetal calf serum (heat inactivated, Gibco, Breda, The Netherlands), heparin (32 U/ml, Sigma, St. Louis, MO, USA), l-glutamine (1.65 mM, Gibco), antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin) and phytohaemagglutinin (0.3 mg/ml, Murex, Dartford, UK). 5-Bromo-2'-deoxyuridine (BrdU, 10 µM, Sigma) was

added and the cultures were incubated for 72 h at 37°C in a 95% humidified incubator with 5% CO₂. Air dried metaphase preparations were made in a routine way.

Sister chromatid differentiation using fluorescence plus Giemsa staining was not successful indicating that the level of incorporated BrdU was low. Therefore, an anti-BrdU antibody was used for detecting SCEs. The slides were denatured for 1 min in 0.07 M NaOH and dehydrated in an ethanol series (70, 90, 100%). Visualization of chromatids incorporated with BrdU was accomplished with anti-BrdU mouse antibody (Boehringer, Mannheim, Germany) and Alexa fluor 488 anti mouse antibody (Molecular Probes, Eugene, OR, USA) as described (14). Samples were coded prior to scoring.

Results

After separation of the alkaline-treated samples by HPLC a radioactivity peak showing co-chromatography with the UV peak of *N*⁶-HP-adenine was detected in 7 out of 8 exposed workers, but not in any of the controls. Some representative HPLC radiograms are shown in figure 1. The adduct levels ranged from undetectable to 1.0 mol (mean 0.66 ± 0.34 mol) per 10^9 mol normal nucleotides. For statistical calculations, an adduct level of 0.05 per 10^9 (50% of the detection limit) was used for samples with undetectable levels. The adduct levels in samples from PO workers were significantly higher than those of the controls (Mann-Whitney two sample test, $p = 0.0012$). The adduct HP-valine was found in all human samples; the levels ranged from 0.005 to

0.008 pmol per mg of globin among the controls and from 0.13 to 4.92 (mean 2.69 ± 1.52) pmol per mg of globin among the exposed workers. The effect of exposure was highly significant ($p = 0.0018$). The mean SCE frequency was 3.70 ± 2.11 for the exposed workers and 2.00 ± 0.52 for the control group. The difference was significant, $p = 0.011$). Since the two smokers in the control group were the individuals with the highest SCE frequency the difference between exposed and controls was increased if excluding smokers ($p = 0.0038$). The correlation between levels of 1-HP-adenine and HP-valine was strong ($r = 0.887$) and highly significant ($p < 0.0001$) (Fig. 2A). There was also a relatively strong correlation between levels of 1-HP-adenine and SCE frequencies ($r = 0.792$; $p = 0.00026$) and between HP-valine and SCEs ($r = 0.766$; $p = 0.0014$) (Fig. 2B), respectively. The two control persons for which HP-valine was not analyzed (and therefore not included in the correlation analysis) had both 1.96 SCE per cell, i.e. very close to the mean of the unexposed group. Excluding smokers the correlation between SCE and HP-adenine was 0.851 ($p = 0.00044$) and the correlation between SCE and HP-valine was 0.757 ($p = 0.011$). Excluding the control subjects the correlation between 1-HP-adenine and HP-valine was 0.713 ($p = 0.047$).

Discussion

We had earlier developed and applied a very sensitive method for detection of adducts of PO at 1-adenine (6). The same method has also been used for analysis of 1-adenine adducts in a human population exposed to butadiene

(15). The main reasons for the high sensitivity were the high recovery of the adduct in the ^{32}P -postlabeling assay (about 50% for PO) and the procedure used for analysis. Analysis of 1-HP-adenine was difficult because several potentially interfering unknown radioactivity peaks eluted very close to the standard. However, by collecting the 1-HP-adenine peak and converting 1-HP-adenine to N^6 -HP-adenine sensitivity and specificity was increased. With this procedure it was possible to analyze a greater amount of DNA (40 μg vs 10 μg) and 1 adduct per 10^{10} nucleotides could be reliably detected.

Furthermore, by performing the conversion to an N^6 -adenine adduct the evidence for correct adduct identification was strengthened. This rearrangement also occurs spontaneously (3,6), but we found, contrary to Solomon and co-workers (3), that the rate at physiological conditions was rather slow in PO-modified DNA (half-life 9.2 days) (6). An additional support for a slow rate is the finding that following 4 weeks of chronic exposure of rats to PO only some 20% of 1-HP-adenine had rearranged to N^6 -HP-adenine (6).

The two step procedure used for analysis together with the finding that all except one of the PO-exposed workers and none of the control subjects had detectable adduct levels support the contention that the adduct analyzed was formed as a consequence of PO exposure and that it was indeed 1-HP-adenine. The exposed worker who showed no detectable HP-adenine had also a considerably lower level of HP-valine than the rest of the exposed workers (see Fig. 2).

HP-valine could be detected in all samples, but the levels in control subjects were about 600 times lower than in PO workers. Assuming the same ratio for 1-HP-adenine one would expect the level of this adduct in controls to

be on the order of 100 times below detection limit of the postlabeling assay. Still, background levels of HP-valine were above the detection limit of the assay, showing the superior sensitivity of hemoglobin adduct measurements compared to the most sensitive assay for DNA adduct analysis. HP-valine in PO-exposed workers has been analyzed by Boogard *et al.* (9). The adduct levels per unit dose were close to those reported here. We found an average adduct level of 2.7 pmol/mg globin in the 8 PO plant workers and the average air concentration of PO was estimated to 2.3 ppm during working hours. Boogard *et al.* (9) found an adduct increment of 2.55/pmol per g globin per ppmh, corresponding to an accumulated adduct level of 2.1 pmol/mg globin for an occupational exposure to 2.3 ppm. The low background level of HP-valine shows that contributions from non-occupational sources are minor. The origin of HP-valine in the control subjects is unknown but propene is a likely candidate chemical. This simple alkene, which is metabolically converted to propylene oxide (16), is present at ppb levels in air contaminated with tobacco smoke or automobile exhausts (17). There were only two smokers in the unexposed group and their smoking habits are unknown. The levels of HP-valine of the smokers were similar to those of the non-smokers, indicating that tobacco smoking is not the major contributing factor to the found background levels.

Induction of SCE following PO exposure has so far only been studied in cells *in vitro* and in experimental animals (18-21). PO induced SCE in cellular systems and in the mouse, but not in the cynomolgus monkey (18). The SCE frequencies found in the control group of this study were lower than normally observed (22). Possibly, the low base line frequency, as well as the low

induced frequencies of SCEs, were due to the low incorporation of BrdU which could be detected only by immunological techniques. It has been shown that frequencies of SCEs are directly correlated to the level of incorporated BrdU (14). Excluding smokers strengthened the effect of PO exposure and only marginally changed the correlation between SCE and Hb- or DNA adducts.

Poly(propylene glycol) monobutyl ether was the only other chemical reported to be handled by the workers. This compound has to our knowledge not been tested, but judged from testing of related substances it is not expected to be genotoxic. It is therefore likely that the observed increase in SCE occurred as a result of PO exposure, but confounding effects from genotoxic impurities of the polymer or other unknown sources cannot be excluded.

Bearing in mind that DNA- and hemoglobin adducts and SCE integrate exposure for different time intervals, the good correlation between all three endpoints was somewhat unexpected. One possible explanation is that the exposure levels had not varied much during the last months. Although the present study was of small scale, the presence of a DNA adduct not previously seen in humans was demonstrated as was the usefulness of the studied endpoints for monitoring PO exposures at levels at or below current TLVs.

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Fig. 1. HPLC separation of ^{32}P -postlabeled DNA from white blood cells of (A), a control person and (B) and (C), propylene oxide-exposed workers. The position of N^6 -HP-5'-dAMP is indicated with an arrow. "—" radioactivity, "—" UV.

The HPLC system was first run isochratically with 100% of 0.5 M ammonium formate containing 20 mM phosphoric acid (pH 4.6) for 5 min, followed by a linear gradient to 14% methanol over 40 min, isochratically with 15% methanol for 20 min and finally a linear gradient to 100% of methanol over 15 min.

Fig. 2. Correlation between (A), levels of hemoglobin and DNA adducts and (B), between frequencies of sister chromatid exchanges and levels of hemoglobin adducts. Data for unexposed individuals are indicated by a horizontal arrow and for smokers by a vertical arrow.

