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Detection of styrene oxide–DNA adducts by ^{32}P-postlabeling

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***In vitro* reaction of DNA with styrene-7,8-oxide (styrene oxide) produced five adducts, as determined by ^{32}P-postlabeling. When styrene oxide was reacted *in vitro* with deoxyribonucleotides, five adducts were observed from 2′-deoxyguanosine-3′-monophosphate, two from 2′-deoxyadenosine-3′-monophosphate, none from 2′-deoxythymidine-3-monophosphate or 2′-deoxycytidine-3′-monophosphate. Chromatographic comparison of the adducts formed in DNA with those formed with the deoxyribonucleotides suggests that deoxyguanosine is the primary site of DNA modification. Treatment of 9L cells with 1 mM styrene oxide resulted in the formation of several DNA adducts as detected by the postlabeling procedure. Our results indicate that ^{32}P-postlabeling can be used to investigate DNA adducts formed by styrene oxide.**

Introduction

Styrene is a commercially important chemical which is essential to the production of reinforced plastics (1). Occupational exposure occurs primarily through inhalation of airborne vapor (1). Following inhalation, styrene is absorbed in the blood and metabolized to styrene-7,8-oxide (styrene oxide*) primarily via P450 oxidation in the liver (1). Styrene oxide is mutagenic in both prokaryotic (2,3) and eukaryotic test systems (4), and induces chromosome aberrations and sister chromatid exchanges in human lymphocytes *in vitro* (5,6). Styrene oxide is carcinogenic in rodents (7–9).

The potential molecular mechanisms for the genotoxicity of styrene oxide have been investigated through reactions of styrene oxide with either nucleosides (10,11) or DNA (12). In DNA reacted *in vitro* with styrene oxide, the sites of alkylation were found to be at the N^2 and N-7 positions of guanine with modifications at the N-7 position being predominate (12). These results with styrene oxide are consistent with reactions of other aryl alkylating agents with nucleosides and DNA (13–15). N-7 guanine adducts of styrene oxide have also been detected in DNA isolated from the liver after *in vivo* treatment of mice with styrene (16).

Recently, several laboratories have developed analytical methods to detect and quantitate DNA modifications in human populations exposed to or treated with genotoxic agents. Immunoassays have been used to quantitate benzopyrene adducts in DNA isolated from lymphocytes of coke and foundry workers (17,18) and *cis* platin modifications in DNA of patients undergoing chemotherapy (19,20). The ^{32}P-postlabeling procedure developed by Randerath and co-workers has been applied to detect DNA adducts associated with smoking (21,22).

The purpose of this investigation was to apply the ^{32}P-postlabeling procedure to detect the formation of styrene oxide–DNA adducts. The results indicate that the postlabeling procedure can be used to quantitate styrene oxide–DNA adducts and that guanine is the principal site of modification in DNA, and in 9L cells treated with styrene oxide.

Materials and methods

In vitro reactions

Half a milligram each of calf thymus DNA, 2′-deoxyguanosine-3′-monophosphate (3′-dGp*), 2′-deoxyadenosine-3′-monophosphate (3′-dAp), 2′-deoxycytidine-3′-monophosphate (3′-dCp) or 2′-deoxythymidine-3′-monophosphate (3′-dTp) was dissolved in 1 ml of 10 mM Tris–HCl, pH 7.5. To this was added 1–30 μl of styrene oxide (Aldrich 97%), and the mixture was incubated at 37°C for ~15 h. The mixture was extracted twice with 1 ml of diethyl ether to remove the unreacted styrene oxide.

Treatment of cells

9L cells (1.5×10^6) were placed into 100-mm dishes containing 10 ml of Eagles minimum essential medium, 10% fetal calf serum and gentamycin (50 μg/ml). Styrene oxide diluted 1/10 with ethanol was added to the cultures to give a final concentration of 1 mM styrene oxide. The control cells received only ethanol. The cultures were treated for 24 h and the cells were then collected by scraping. The DNA was isolated as previously described (23).

^{32}P-postlabeling

The ^{32}P-postlabeling procedure used was as described by Randerath *et al.* (24). One microgram of purified DNA was enzymatically digested to 2′-deoxyribonucleotide 3′-monophosphates (3′-dNps) in 20 mM sodium succinate, 8 mM $CaCl_2$, pH 6 buffer at 37°C for 2 h with 2 μg of micrococcal nuclease (16 018 U/mg, Worthington Biochemicals), and 2 μg of spleen phosphodiesterase (10.8 U/mg, Worthington Biochemicals) in a final volume of 10–15 μl. Aliquots (2.5–3 μl) of enzymatically digested DNA (0.17 μg), 3′-dGp, 3′-dAp, 3′-dCp or 3′-dTp reacted with styrene oxide were converted into ^{32}P-labeled 2′-deoxyribonucleotide-3′-5′-bisphosphates (3′,5′-dpNps) by incubation of the 3′-dNps with 100 μCi of [^{32}P]ATP (3000 Ci/mmol, Amersham) in 10 μl, 3 μl of ATP (600 μM) and 1.5 μl of T_4 kinase (2 U/μl, Bethesda Research Laboratories) in 4 μl of kinase buffer [40 mm *N,N*-bis(2-hydroxyethyl)glycine (bicine), 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.1 mM spermidine, pH 9.0] at 37°C for 30 min. Potato apyrase (3 μl) (200 U/ml, Sigma) was added and incubated for 30 min. The final volume was ~25 μl.

Purification of styrene oxide adducts

Four samples of 20 μl each of ^{32}P-labeled 3′,5′-dpNps were applied to a 10 × 10 cm reverse-phase TLC plate (Whatman KC-18), as described by Randerath (24). A paper wick was attached to the top of the TLC plate. The TLC plate was developed overnight in 0.4 M ammonium formate, pH 6. The following day, 1.2-cm square chips containing the origin were placed in contact with PEI cellulose plates (10 × 10 cm, Brinkman). The adducts were transferred from the reverse-phase chip to a 10 × 10 cm polyethyleneimine (PEI) plate as follows. The reverse phase chip was attached to the PEI plate with a plastic clip at 2 cm from the bottom and 2 cm from the left edge of the PEI plate. The adducts were transferred to the PEI plate by developing the plate in *n*-propanol/H_2O (1/1) containing 1% nonidet P-40 for 60 min at 50°C (24). The plates were subsequently washed for 7 min with H_2O, followed by a wash (10 min) in 0.15 M ammonium formate, pH 3.5. The plates were air dried, paper wicks were attached, and the

Abbreviations: Styrene oxide, styrene-7,8-oxide; 3′-dGp, 2′-deoxyguanosine-3′-monophosphate; 3′-dAp, 2′-deoxyadenosine-3′-monophosphate; 3′-dCp, 2′-deoxycytidine-3′-monophosphate; 3′-dTp, 2′-deoxythymidine-3′-monophosphate; 3′-dNp, 2′-deoxyribonucleotide 3′-monophosphate; 3′,5′-dpNp, 2′-deoxyribonucleotide-3′-5′-bisphosphate; bicine, *N,N*-bis(2-hydroxyethyl)glycine; PEI, polyethyleneimine.

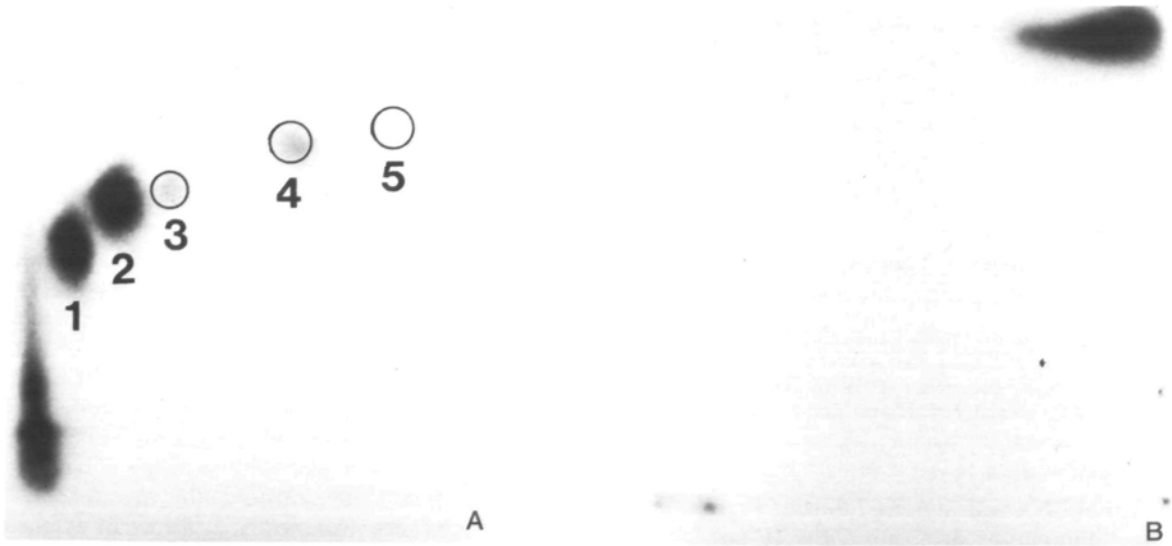

Fig. 1. (**A**) Autoradiogram of ^{32}P-postlabeled calf thymus DNA reacted with 5 μl of styrene oxide *in vitro* and chromatographed as described in text. (**B**) Autoradiogram of ^{32}P-postlabeled calf thymus DNA control. For these autoradiograms the films were exposed at −70°C for 3 h.

plates were developed in 1.8 M lithium formate and 4.25 M urea, pH 3.5, from bottom to top of the plate to ~1 cm onto the wick. The plates were washed twice (7 min) in H_2O, air dried and then developed at a right angle to the previous direction of development in 0.36 M LiCl, 0.22 M Tris−HCl, 3.8 M urea, pH 8.0, to ~2 cm on the wick. They were washed twice with H_2O, and then developed in the same direction to ~2 cm onto the wick with 1.7 M Na_2HPO_4, pH 6.0. The plate was washed for 5 min in H_2O. The adducts were located by autoradiography using Kodak XAR-5 film and a DuPont Chronex-Lightning Plus intensifying screen. The film was exposed at −70°C for 3 h for the *in vitro* reactions, and for 17 h for the cell samples.

Detection of normal nucleotides

Of the ^{32}P-labeled sample 1 μl was applied to a PEI cellulose plate (20 × 20 cm) that had been washed with 0.1 M ammonium formate pH 3.5 for 10 min and air dried. The chromatogram was developed in the first dimension with 1.5 M ammonium formate, pH 3.5, and washed with 0.01 Tris−base buffer for 10 min, followed by a second wash in H_2O for 5 min. It was then developed at a right angle to the previous direction of development in 0.3 M $(NH_4)_2SO_4$, pH 6, and air dried. The labeled nucleotides was detected by autoradiography for 30 min at room temperature. They were collected by scraping, and radioactivity determined by liquid scintillation counting.

Calculation of adduct frequency

The radioactive spots on the PEI cellulose sheets detected by autoradiography were scraped into liquid scintillation vials containing 5 ml of scintillation cocktail (Safety Solve, Research Products Inc.) and radioactivity was determined by liquid scintillation counting. Regions adjacent to the radioactive spots were also scraped, placed in scintillation vials and counted for background determination. The counting efficiency for ^{32}P was determined to be 0.760 with Safety Solve as the scintillation cocktail, and 0.306 for Cerenkov counting. The background counts were subtracted from those of the styrene-oxide adducts. The frequency of each styrene-oxide adducts was calculated as:

$$\frac{\text{c.p.m. in styrene oxide adduct}}{\text{c.p.m. in normal nucleotides + c.p.m. in styrene-oxide adduct}}$$

Results

Reaction of calf-thymus DNA with 5 μl styrene oxide produced five adducts, as detected by the ^{32}P-postlabeling procedure (Figure 1A). Control samples showed no radioactive spots when postlabeled using the same conditions as the styrene-oxide-treated sample (Figure 1B). Two major adducts, adducts 1 and 2, accounted for ~88% of the total modification (Table I). Adduct 3 was 8% of the modification, and adducts 4 and 5 ~3% each. For these studies, the overall level of DNA modification by 5 μl styrene oxide was $\sim 4.4 \times 10^{-4}$ adducts/nucleotide. The extent of DNA modification was dependent on styrene oxide dose from 1 to 5 μl. At higher concentrations of styrene oxide (10−30 μl) the level of modification plateaued. The extent of formation of each adduct as a percentage of the total was independent of the styrene-oxide concentration. The stabilities of the DNA adducts (at −20°C) were tested by repeatedly analyzing the samples over a 3-month interval. The relative amount of each adduct and the overall extent of DNA modification was independent of storage time.

Table I. Reactions of styrene oxide[a] *in vitro*

Adduct	3′-dGp[b]	DNA[c]
1	0.458	0.461 ± 0.074
2	0.451	0.414 ± 0.035
3	0.021	0.077 ± 0.035
4	0 023	0.037 ± 0.027
5	0.025	0.031 ± 0.022

[a]Values expressed as fractions of total.
[b]Average of two determinations.
[c]Mean and SD of 11 determinations.

3′-dGp was reacted *in vitro* with 5 μl of styrene oxide. Five adducts were again detected by the postlabeling procedure. With longer exposure of the film a weak sixth adduct was detected (Figure 2A). Adducts 1 and 2 accounted for ~91% of the reaction of styrene oxide with 3′-dGp, and adducts 3, 4 and 5 accounted about equally for the remaining modifications. The relative mobilities of the adducts on the PEI plates produced by reaction of styrene oxide with 3′-dGp were similar to the corresponding DNA adducts. To investigate this in more detail the reaction of styrene oxide with DNA and 3′-dGp were repeated. The samples were ^{32}P-postlabeled individually and then chromatographed both individually and as a mixture. Autoradiographic analysis of both individual samples and the mixture were similar to those shown in Figures 1 and 2A. These results further suggest that the products of reaction between styrene oxide, DNA and 3′-dGp are similar.

Reaction of 3′-dAp with 5 μl of styrene oxide produced two adducts, as detected by the postlabeling procedure (Figure 2B). Comparison of the relative mobilities on the PEI plates with the

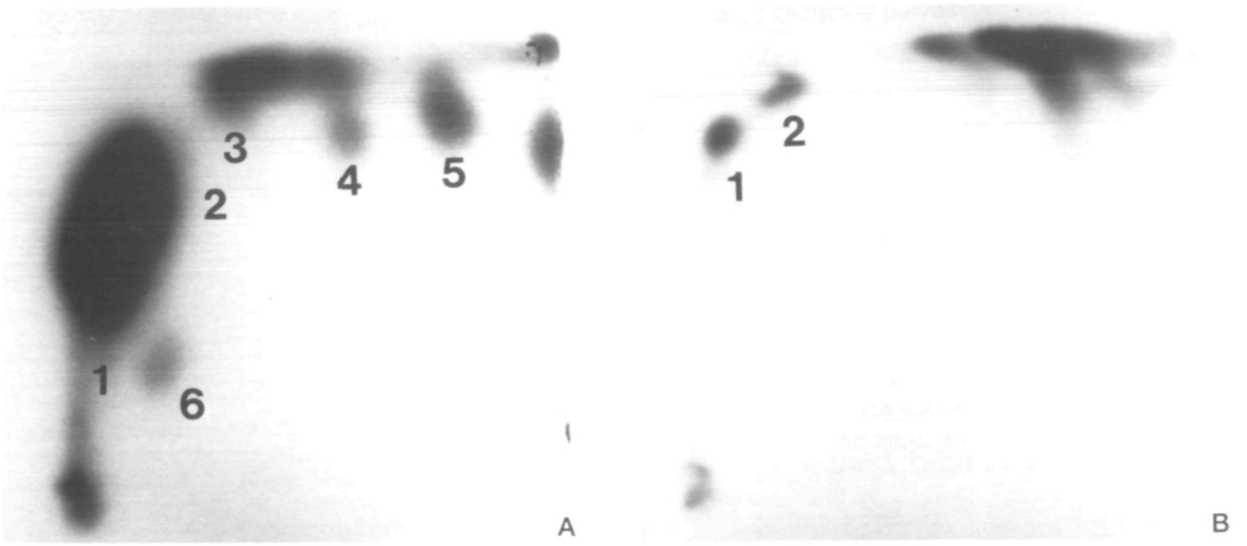

Fig. 2. Autoradiograms of ^{32}P-postlabeled 3′-dGp (**A**) and 3′-dAp (**B**) reacted with 5 μl styrene oxide *in vitro*. For these autoradiograms the films were exposed at −70°C for 3 h.

styrene oxide–DNA adducts suggests that they were not produced in detectable quantities in the reaction of styrene oxide with DNA. Reaction of styrene oxide (5 μl) with 3′-dCp and 3′-dTp produced no adducts as detected by the postlabeling procedure.

9L cells were treated with 1 mM styrene oxide for 24 h. The overall extent of DNA modification in 9L cells by styrene oxide was 19.5×10^{-6} adducts/nucleotide. Comparison of the styrene oxide reaction with DNA *in vitro* (Figure 1A) and 9L cells (Figure 3) suggests that similar DNA adducts are formed in both cells and DNA reacted with styrene oxide.

Discussion

We have investigated the reaction of styrene oxide with DNA using the ^{32}P-postlabeling method developed by Randerath and co-workers (24). Reaction of styrene oxide with DNA and with 3′-dGp produced five adducts. The similar mobilities of the two sets of adducts via TLC indicate that guanine is the principal base for DNA modification by styrene oxide. Our results of reaction of styrene oxide with DNA are in good agreement with those of Savela *et al.* (12), who reacted [^{3}H]styrene oxide with DNA and observed that guanine modifications accounted for 85% of the total (the remaining products were not identified).

The multiple reaction products of styrene oxide with 3′-dGp agrees with the study of Hemminki and Hesso (11), who observed that styrene oxide attacked guanosine at the N-7, N^2, and O^6 positions of guanine. Analysis of the autoradiograms showed that, in both DNA and 3′-dGp, adducts 1 and 2, and 4 and 5 had similar mobilities on the PEI plates. In addition, the relative extents of formation were similar. Since styrene oxide can react at either the alpha or beta position to form structural isomers, adducts 1 and 2 and 4 and 5 may be pairs of isomers.

The N-7 position of guanine has been shown to be the principal site of modification for styrene oxide in both DNA and guanosine; N-7 adducts accounted for 82% of the total DNA modification (11,12). In our experiments adducts 1 and 2 represented 90% of the reaction of styrene oxide with either DNA or 3′-dGp. However, several studies have shown that N-7 aryl and alkyl guanosine derivatives in DNA and nucleosides are very susceptible to both chemical depurination and imidazole ring opening (25–30). HPLC analysis of styrene oxide reacted with deoxyguanosine has shown that the principal reaction product is a depurinated N-7 adduct (unpublished observation). Depurinated adducts will not be detected by the postlabeling procedure. Therefore, if adducts 1 and 2 are derived from N-7 adducts, they have either resisted depurination or are ring-opened products. Further work is required for their structural identification.

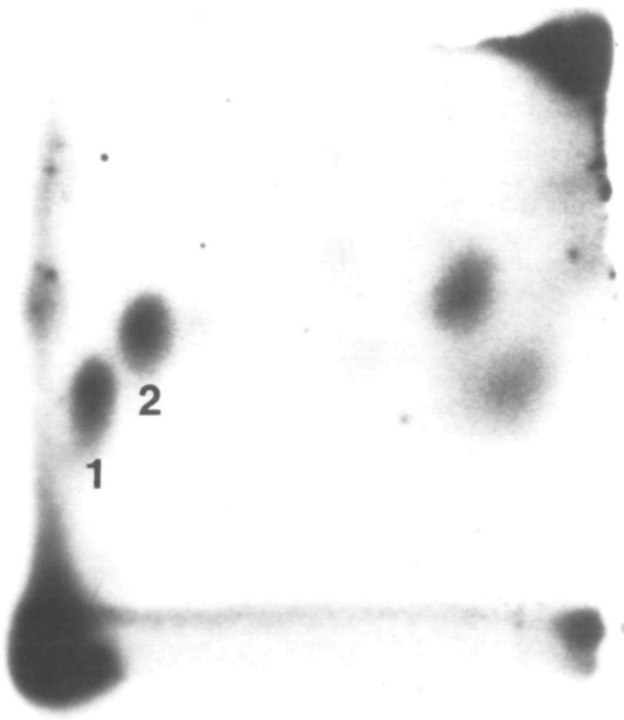

Fig. 3. Autoradiogram of ^{32}P-postlabeled DNA isolated from 9L cells treated *in vitro* with 1 mM styrene oxide. For these autoradiograms the films were exposed at −70°C for 17 h.

Reaction of 3′-dAp with styrene oxide produced two adducts as detected by the postlabeling procedure. These results are also

consistent with previous studies (12) which have shown that styrene oxide attacks both the N-1 and N^6 positions of deoxyadenosine. However, comparison of the autoradiograms of the reaction products of styrene oxide with 3′-dAp and DNA indicate that the styrene oxide-3′-dAp products were not produced in DNA. Reaction of styrene oxide with 3′-dCp and 3′-dTp did not produce any products as determined by the postlabeling procedure. These results are in contrast to those of Savela *et al.* (12), who reported alkylation at the N^4, N-3, and O^2 positions of deoxycytidine, and at the N-3 position of thymidine. Further studies are required to reconcile this difference in results.

Treatment of 9L cells with 1 mM styrene oxide produced several DNA adducts as detected by the ^{32}P-postlabeling assay; adducts 1 and 2 once again were the principal products detected. The concentrations of styrene oxide used here are similar to those which have been shown to induce sister chromatid exchanges and chromosome aberrations in human lymphocytes *in vitro* (5,6); this indicates that the postlabeling method allows styrene oxide adducts to be detected at biologically relevant doses.

This investigation has clearly shown that the postlabeling method can be applied to detect the formation of styrene oxide–DNA adducts. Currently, the greatest occupational exposure to styrene occurs during the production of reinforced plastics (1). Several investigations (31–35) have shown increases in chromosome aberrations in workers exposed to styrene. We are currently applying the postlabeling method to DNA isolated from lymphocytes of such workers in an effort to determine if styrene oxide–DNA adducts result from occupational exposures to styrene.

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