# 4,4′-Methylene-bis(2-chloroaniline)-DNA Adduct Analysis in Human Exfoliated Urothelial Cells by $^{32}$P-Postlabeling

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## Abstract

**4,4′-Methylene-bis(2-chloroaniline) (MOCA) is an aromatic amine used widely in industry, and human exposure to this compound is well documented. MOCA induces lung and liver tumors in rodents and urinary bladder tumors in dogs, and it is regarded as a suspect urinary bladder carcinogen in humans. In this pilot study, we investigated the occurrence of MOCA-DNA adducts in exfoliated urothelial cells of a MOCA-exposed worker by $^{32}$P-postlabeling analysis. Urine samples were collected from the worker at various times after accidental acute exposure to MOCA. DNA isolated from exfoliated urothelial cells collected from each urine sample was enzymatically digested and postlabeled with excess [$^{32}$P]ATP. Thin-layer chromatographic analysis of the labeled digests revealed the presence of a single, major DNA adduct that cochromatographed with the major *N*-hydroxy-MOCA-DNA adduct, *N*-(deoxyadenosin-8-yl)-4-amino-3-chlorobenzyl alcohol, formed *in vitro*. The MOCA-DNA adduct was detected in samples obtained between 4 and 98 h after initial exposure but not in samples collected at later times. The level of DNA adducts 4 h after exposure was determined to be 516 adducts/$10^8$ nucleotides. A 5-fold decrease in adduct level was observed 14 h later, followed by a gradual decrease over subsequent days. The results indicate that MOCA is potentially genotoxic to human urinary bladder *in vivo* and that $^{32}$P-postlabeling analysis of exfoliated urothelial cells provides a noninvasive means for biomonitoring the formation of MOCA-DNA adducts resulting from occupational exposure.**

## Introduction

MOCA[2] is a suspect human carcinogen currently used in the plastics and rubber industry as a cross-linking agent for the synthesis of polymers such as polyurethane. The presence of MOCA and MOCA derivatives in the urine of workers is well documented and indicates that a substantial amount can be absorbed and metabolized as a result of occupational exposure (1–5). While a current estimate of the number of workers exposed to MOCA is not available, a report published in 1977 estimated that between 2,100 and 33,000 U.S. workers were potentially exposed to this aromatic amine (6).

Experimental animal studies have shown that MOCA induces tumors in the liver, lung, and mammary gland of rodents (7–9) and the urinary bladder of dogs (10). The carcinogenicity of MOCA in dogs is particularly significant in that the dog is considered a reliable animal model for aromatic amine-induced urinary bladder cancer in humans (11, 12). Both dogs and humans appear to be more susceptible to the carcinogenic effects of certain arylamines, as compared to most other species examined. For example, ABP and benzidine, which are similar in structure to MOCA, induce urinary bladder tumors in both humans and dogs but not generally in rodents (13). Although the carcinogenic potential of MOCA in humans would therefore appear to be substantial, there is currently insufficient epidemiological evidence to indicate that MOCA is a human carcinogen. However, noninvasive papillary tumors of the bladder have been recently detected in two nonsmoking males out of a group of 530 MOCA-exposed workers (14). The results suggest an increased risk of bladder cancer among this group of workers and support the hypothesis that MOCA is a human bladder carcinogen.

The metabolism of MOCA has been studied *in vitro* and *in vivo* in a number of species, and the results have been summarized in a recent review (15). As with other aromatic amine carcinogens, MOCA is bioactivated by *N*-oxidation catalyzed by hepatic microsomes from a variety of species, including humans (16–18). In human liver, this reaction is catalyzed predominantly by CYP3A4 (19), whereas human hepatic CYP1A2 is primarily responsible for the *N*-oxidation of most other arylamines (20). The product of the reaction, *N*-hydroxy-MOCA, reacts with DNA *in vitro* to form a major adduct previously characterized as *N*-(deoxyadenosin-8-yl)-4-amino-3-chlorobenzyl alcohol (21, 22). This is the major DNA-adduct formed *in vivo* in rat liver (21, 22) and in dog urinary bladder epithelium[3] after the administration of MOCA, suggesting that *N*-hydroxy-MOCA is the ultimate carcinogenic form of MOCA. *In vitro*, human urothelium has been reported to metabolize MOCA to a form which

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[2] The abbreviations used are: MOCA, 4,4′-methylene-bis(2-chloroaniline); ABP, 4-aminobiphenyl; HPLC, high-performance liquid chromatography; CYP, cytochrome P-450; WBC, white blood cells; PEI, polyethyleneimine cellulose.

[3] D. Segerbäck, G. T. Talaska, K. R. Kaderlik, K. L. Dooley, and F. F. Kadlubar, manuscript in preparation.

reacts with DNA (23); however, neither the bioactivation mechanism nor the chemical nature of the DNA-adducts is known.

At present there is no information on DNA adduct formation in humans exposed to MOCA. Fortunately, with recent advances in analytical biochemistry, it has become possible to measure the formation of DNA adducts in humans resulting from exposure to xenobiotics in the workplace, diet, and cigarette smoke (24). By far the most widely used tool for detecting this form of DNA damage is the $^{32}$P-postlabeling technique developed by Gupta and Randerath (25, 26). $^{32}$P-Postlabeling has been used previously to demonstrate elevated levels of xenobiotic-DNA adducts in WBC of aluminum plant workers (27), iron foundry workers (28, 29), and coke oven workers (30). Unfortunately, the application of this method to routine human biomonitoring has been limited to the analysis of those tissues which can be obtained by noninvasive means, such as WBC, skin cells, and oral mucosal cells, and generally not for internal tissues such as lung, liver, and pancreas. However, in two recent studies, Talaska *et al.* used the postlabeling method to detect and quantify ABP-DNA adducts in exfoliated urothelial cells obtained from dogs treated with ABP (31) and from human tobacco smokers (32). This suggested that exfoliated urothelial cells could serve as an important source of DNA for analyzing carcinogen-DNA adducts formed in human urinary bladder.

In the study presented here, we have investigated MOCA-DNA adduct formation in exfoliated urothelial cells of a worker acutely exposed to MOCA. Using the postlabeling method, a DNA adduct was detected which cochromatographed with the authentic major MOCA-DNA adduct prepared *in vitro*, identified previously as *N*-(deoxyadenosin-8-yl)-4-amino-3-chlorobenzyl alcohol (22). Quantitative measurements indicated relatively high adduct levels only 4 h after the acute MOCA exposure followed by a dramatic decrease over the next 17 h. The results provide evidence that MOCA causes DNA damage in the human urinary bladder *in vivo* and that a noninvasive quantitative assessment of this damage is possible using the $^{32}$P-postlabeling method.

## Materials and Methods

**Materials.** MOCA was obtained from Pfaltz and Bauer, Inc. (Waterbury, CT). [2,2',6,6'-$^3$H]MOCA (210 mCi/mmol) was obtained from Chemsyn Science Laboratories (Lenexa, KS). [$^3$H]*N*-Hydroxy-MOCA was synthesized according to the procedure of Butler *et al.* (17). The following reagents were purchased from the sources indicated: cloned T4 polynucleotide kinase (U.S. Biochemical Corp., Cleveland, OH); phenol (Ameresco, Solon, OH); micrococcal nuclease, phosphodiesterase II (Type I), ribonuclease T1, ribonuclease A (Type III-A), proteinase K, nuclease P1, apyrase (Grade I), alkaline phosphatase (Type III-S), phosphodiesterase I (Type II), calf thymus DNA (Type I), and deoxyribonuclease I (Type II) (Sigma Chemical Co., St. Louis, MO); XAR-5 X-ray film (Eastman Kodak, Dallas, TX); 5-$\mu$m-pore size cellulose nitrite filter (no. 7195004; Whatman Laboratory Products, Inc., Clifton, NJ). Macherey-Nagel (no. 801053) PEI-cellulose plates (Alltech Associates, Inc., Deerfield, IL) were used for D1 and D2 chromatography, and E. Merck (no. 5504-7) PEI-cellulose plates (Alltech Associates, Inc.) were used for all other chromatography. [$^{32}$P]ATP was prepared enzymatically as described previously (25). [$^{32}$P]$H_3PO_4$ was purchased from ICN Radiochemicals (Irvine, CA).

**Preparation of *N*-Hydroxy-MOCA-modified DNA Standard.** Calf thymus DNA (5 mg/ml) was reacted with [$^3$H] *N*-hydroxy-MOCA (0.35 mM, 164 mCi/mmol) in 10 mM potassium citrate buffer (pH 5.0) containing 0.1 mM EDTA for 24 h at 37°C. The reaction mixture was then diluted with an equal volume of the same buffer and extracted 3 times with an equal volume of *n*-butanol followed by 3 extractions with an equal volume of phenol. Next, 1/50 volume of 5 M NaCl was added to the aqueous phase, followed by 5 volumes of ice-cold ethanol to precipitate DNA. The DNA was then dried under argon and redissolved in a minimum volume of water. NaCl was added as before, and the DNA was reprecipitated with 5 volumes of ice-cold ethanol:acetone (1:1). The DNA was then washed 2 times with 70% ice-cold ethanol, dried under argon, and redissolved in 5 mM Bis-Tris buffer (pH 7.0).

*N*-Hydroxy-MOCA-modified DNA was then hydrolyzed essentially as described previously (22). DNA was incubated with DNase I (0.2 mg/mg DNA) in 5 mM Bis-Tris buffer (pH 7.0) containing 3 mM $MgCl_2$ for 3 h at 37°C, followed by incubation with nuclease P1 (20 $\mu$g/mg DNA) and phosphodiesterase I (0.08 units/mg DNA) for 1.5 h, and finally by treatment with alkaline phosphatase (2.0 units/mg DNA) for 16 h. The DNA hydrolysate was then extracted 2 times with an equal volume of *n*-butanol. The *n*-butanol phases, containing nucleoside adducts, were combined and concentrated to dryness under vacuum. The residue was dissolved in a minimum volume of 30% methanol and analyzed by HPLC using a $\mu$Bondapak-$C_{18}$ analytical column (3.9 mm × 300 mm) and the following methanol-$H_2O$ linear solvent gradient system: 20 to 100% methanol in 20 min followed by 100% methanol for 10 min. A single major radioactive peak eluting with the same retention time as authentic *N*-(deoxyadenosin-8-yl)-4-amino-3-chlorobenzyl alcohol was quantified by scintillation counting and used to calculate the corresponding adduct level in the *N*-hydroxy-MOCA-modified DNA standard.

**Urine Collection and Isolation of Exfoliated Cells.** Urine was collected from a 30-year-old male worker at approximately 4, 18, 24, 44, 49, 73, 95, 138, 145, 215, 354, 399, 407, and 430 h after accidental acute exposure to molten MOCA. Details of the accident are described elsewhere (33). Urine samples were frozen at −20°C after adding glycerol to a final concentration of 20%. Urothelial cells were collected by filtering the thawed, cold urine through a cellulose nitrate filter (5-$\mu$m pore size). The isolated cells contained on the filter were then transported on dry ice from Ohio to Arkansas for further analysis.

**DNA Isolation.** Isolation of DNA was carried out essentially as described by Talaska *et al.* (31), with minor modifications. The urothelial cells were removed from the filters by washing with 30–40 ml of sucrose buffer (0.25 M sucrose, 25 mM KCl, 1.8 mM $CaCl_2$, 50 mM Tris-HCl, pH 7.5). The sucrose buffer was centrifuged at 600 × *g* for 10 min; and the pellet, containing cells, was washed 4–5 times with the same buffer to remove contaminating crystals. The washed cells were then resuspended in 1 ml of 20 mM Tris-HCl buffer (pH 7.4)

containing 1% sodium dodecyl sulfate and homogenized using a motor-driven glass-Teflon homogenizer. The homogenate was then incubated at 37°C with RNase A (22 units) and RNase T1 (1.5 units) for 1 h, followed by proteinase K (1.2 units) for 30 min. Each sample was extracted in sequence with 1 volume of each of the following: phenol; phenol:chloroform:isoamyl alcohol (24:24:1); and chloroform:isoamyl alcohol (24:1). Afterwards, 0.1 volume of 4.5 M LiCl and 30 μg of glycogen were added, and the samples were chilled on ice for 15 min. DNA was precipitated by adding 2 volumes of ice-cold ethanol and then collected by centrifugation at 6000 × *g*. DNA was washed 2 times with 70% ice-cold ethanol and one time with ice-cold acetone:ethanol (1:1), dried under argon, and then dissolved in 50 μl of 1.5 mM NaCl–0.1 mM sodium citrate buffer (pH 7.0).

**$^{32}$P-Postlabeling Analysis.** The amount of DNA in each sample was determined by $^{32}$P-postlabeling followed by analysis of the labeled normal nucleotides. Thus, 4 μl of each sample were incubated with 0.05 units of micrococcal nuclease and 0.005 units of spleen phosphodiesterase in 20 mM sodium succinate buffer (pH 6.0) and 10 mM $CaCl_2$ for 3 h at 37°C. The DNA digest was then postlabeled under standard conditions using excess ATP (26). After the reaction, the presence of excess ATP was confirmed by thin-layer chromatographic analysis of a small aliquot of the reaction mixture removed before the addition of apyrase (34). The labeled digest was then diluted 100-fold with 5 mM Bicine buffer (pH 9.6) and subjected to normal nucleotide analysis as previously described (34). The amount of DNA in each sample was calculated by multiplying the amounts (cpm) of normal nucleotides by a conversion factor established by postlabeling known amounts of calf thymus DNA.

The remainder of each sample was digested with micrococcal nuclease (0.235 units/μg DNA) and spleen phosphodiesterase (0.025 units/μg DNA) under the same conditions as above, followed by incubation with nuclease P1 (0.1 μg/0.2 μg DNA) under standard conditions (34). The DNA digest was postlabeled with excess carrier-free [$^{32}$P]ATP (300 μCi) under the same conditions as above, and the presence of excess ATP was confirmed as before. The postlabeled mixture was then spotted on a 10 × 20 cm Macherey-Nagel PEI-cellulose plate to which a wick had been previously attached along the 10-cm edge. After the plate was developed overnight, in the long direction (D1), with 1.7 M sodium phosphate buffer (pH 6.0), it was cut 15 cm from the bottom (13.5 cm above the origin), and the upper portion including the wick was discarded. The lower portion of the plate was soaked twice in water for 5 min and air dried. The plate was then turned 90° and developed with the same solvent in the short direction (D2). After washing and drying, as before, the plate was then analyzed by autoradiography to locate the adduct spot. The adduct, which typically moves 5 cm above the origin after D1 and moves 2 cm after D2, was then contact-transferred (D3) (35) to a 10 × 10 cm E. Merck PEI-cellulose plate by development with 8.5 M urea and 3.6 M lithium formate buffer (pH 3.5). After washing and drying, the plate was developed with 7.45 M urea, 0.8 M LiCl, and 0.5 M Tris-HCl (pH 8.0) in a direction 90° to that of D3 and then analyzed by autoradiography.

The major MOCA-DNA adduct was identified by cochromatography with the *N*-hydroxy-MOCA-DNA adduct standard. Adduct levels were calculated according to the following equations:

$$\frac{\text{nmole adduct}}{\mu\text{g DNA}} = \frac{\text{cpm adduct}}{\mu\text{g DNA}} \times \frac{\text{nmole adduct standard}}{\text{cpm adduct standard}}$$

and

$$\text{RAL} = \frac{\text{nmole adduct}}{\text{nmol nucleotides}} = \frac{\text{nmole adduct}}{\mu\text{g DNA}} \times \frac{\mu\text{g DNA}}{3.3 \text{ nmole nucleotides}}$$

where the ratio of nmoles adduct standard to cpm adduct standard is a conversion factor, established by postlabeling known amounts of the standard *N*-hydroxy-MOCA-modified DNA. The adduct level of the standard was determined by measuring $^{3}$H associated with the adduct nucleoside after HPLC (described above) and liquid scintillation counting. It should be noted that this calculation assumes that the adducts from each sample, including the standard, are labeled to the same extent and that adduct recoveries are the same.

## Results

**Isolation of DNA from Exfoliated Urothelial Cells of a Worker Exposed to MOCA.** Exfoliated urothelial cell DNA was successfully isolated from 14 separate urine samples collected from a worker at various times after acute exposure to MOCA. Microscopic examination of the isolated cells confirmed the presence of exfoliated cells without bacterial contamination. The amount of DNA in each sample was determined by $^{32}$P-postlabeling followed by normal nucleotide analysis as described in "Materials and Methods." An autoradiogram of the normal nucleotides is shown in Fig. 1. The average amount of DNA recovered from the samples was 264 ng, with a range between 85 and 773 ng. Most of samples (11 of 14) fell within a range between 85 and 239 ng. Relatively large amounts of DNA, 477, 543, and 773 ng, were isolated from 3 samples that were obtained 18, 49, and 73 h, respectively, after exposure.

**Preparation of [$^{3}$H]*N*-Hydroxy-MOCA-DNA and Analysis by $^{32}$P-Postlabeling.** The reaction between *N*-hydroxy-MOCA and DNA, *in vitro*, has previously been shown to form a major adduct, characterized by Silk *et al.* (21) and by Segerbäck and Kadlubar (22) as *N*-(deoxyadenosin-8-yl)-4-amino-3-chlorobenzyl alcohol and a minor adduct that appears to be *N*-(deoxyadenosine-8-yl)-4-amino-3-chlorotoluene (22). In this study, *N*-hydroxy-MOCA-modified DNA was digested to deoxyribonucleosides and extracted with *n*-butanol. After extraction, 93% of the original radioactivity was present in the *n*-butanol fraction; and HPLC analysis confirmed the presence of two radioactive peaks with retention times identical to those of the major and minor adducts found previously (22) (data not shown). It was found that 75% of the radioactivity associated with the *N*-hydroxy-MOCA-DNA sample corresponded to the major adduct, and its binding level was calculated accordingly. The *N*-hydroxy-MOCA-DNA sample was then used as a postlabeling

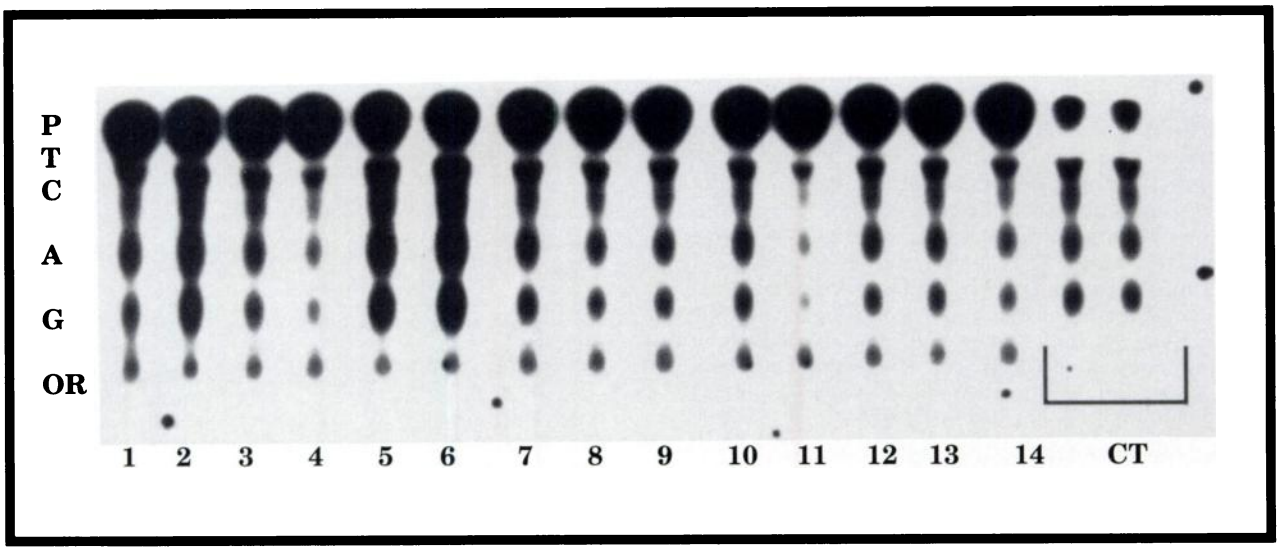


*Fig. 1.* Autoradiogram of normal nucleotides from $^{32}$P-postlabeled DNA. DNA isolated from exfoliated urothelial cells of a worker at timed intervals after acute exposure to MOCA (*1-14, left to right,* earlier to later samples); calf thymus DNA (*CT*); inorganic phosphate (*P*); and 3′,5′-bisphosphates of thymidine, deoxycytidine, deoxyadenosine, and deoxyguanosine (*T, C, A,* and *G,* respectively). *OR,* origin.

standard to detect and quantify the major adduct in DNA isolated from human exfoliated urothelial cells. Preliminary postlabeling experiments indicated that the major and minor MOCA-DNA adducts had very similar chromatographic properties and could only be partially resolved. In addition, our previous work (22) revealed that the major adduct was resistant to nuclease P1 digestion while the minor adduct was sensitive to nuclease P1. Thus, in order to avoid problems with poor chromatographic separation between the minor and major adducts and to optimize detection of the major adduct, DNA samples were digested with nuclease P1 prior to postlabeling.

Unlike other DNA adducts derived from aromatic amines, the major MOCA adduct is not retained at the origin of the PEI-cellulose plate during typical D1 chromatographic conditions (0.9 to 1.7 M sodium phosphate buffer, pH 6.0). As shown in Fig. 2, the major adduct migrates substantially, typically 5 cm above the origin, during the initial chromatographic step. Following chromatography in the second direction (D2), the adduct moves away from the interfering background and can be located after autoradiography for the subsequent contact transfer step (D3).[4]

A comparison of adduct levels determined by $^{3}$H/HPLC analysis, as described above, with that determined by $^{32}$P-postlabeling indicated that the latter method gave values 30% of that determined by measuring $^{3}$H. This indicates that the postlabeling method described in this study is underestimating adduct levels by 70%. This is apparently due in large part to incomplete adduct recovery, since the contact transfer efficiency was only 50%.

**$^{32}$P-Postlabeling Analysis of Exfoliated Urothelial DNA Isolated from a Worker after Acute Exposure to MOCA.** Exfoliated urothelial cell DNA isolated from a worker after accidental acute exposure to MOCA was subjected to $^{32}$P-postlabeling analysis. An autoradiogram of $^{32}$P-labeled DNA 18 h after exposure is shown in Fig. 3*A*. The results indicate the presence of a DNA adduct with chromatographic properties identical to that of the major MOCA-DNA adduct standard, shown in Fig. 3C. As shown in Fig. 3*B*, this adduct could not be detected after 17 days, indicating that the adduct was exposure related.

Quantitative measurements of MOCA-DNA adducts at various times after exposure are shown in Fig. 4. Adduct levels were relatively high, at 516 adducts/$10^{8}$ nucleotides, only 4 h after exposure. This was followed by a 5-fold decrease 14 h later. Over the next 3 days, only a gradual decrease was observed. At 98 h after exposure, the adduct level was 6/$10^{8}$ nucleotides, which is approximately 1% of the peak level, 4 h after exposure. The MOCA-DNA adduct could not be detected in samples collected at later times, indicating that the adduct level had decreased below the lower limit of detection (*vide infra*).

## Discussion

In this study, we have demonstrated that acute exposure of a worker to MOCA resulted in the rapid and extensive formation of MOCA-DNA adducts in urothelial cells exfoliated into the urine. The results suggest that MOCA may be genotoxic to the human urinary bladder and support strongly the hypothesis that MOCA is a potential human urinary bladder carcinogen.

The subject in this study was a 30-year-old male worker who was accidentally sprayed with molten MOCA over much of his upper body (33). Using the $^{32}$P-

[4] It was found that the chromatographic behavior of the major adduct during the D1 and D2 steps depended on the brand of PEI-cellulose plate that was used. A comparison between E. Merck and Macherey-Nagel plates indicated that only the latter brand gave optimal chromatographic results. With E. Merck plates, the adduct migrated close to the top of the plate and appeared distorted and diffuse.

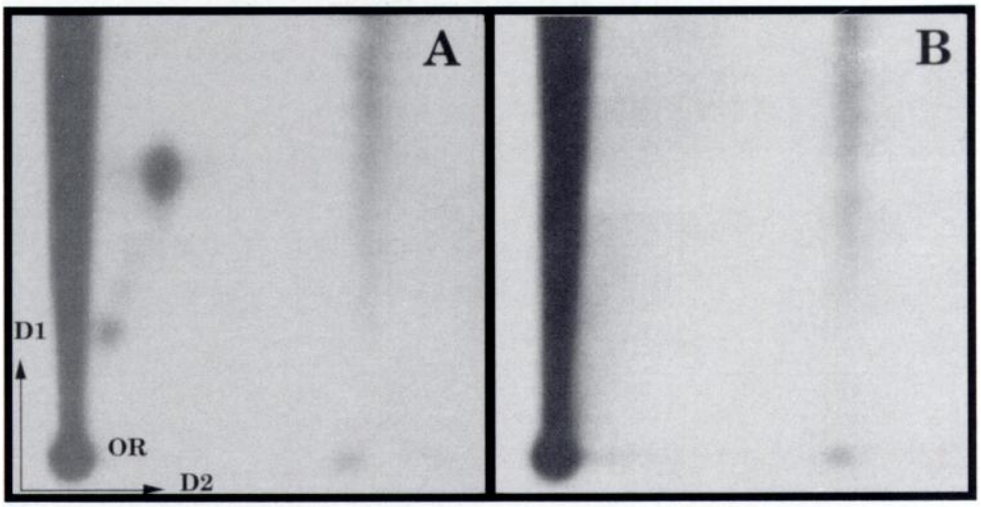


*Fig. 2.* Autoradiogram of $^{32}$P-postlabeled DNA after D1 and D2 chromatography. *A*, calf thymus DNA modified *in vitro* with *N*-hydroxy-MOCA; *B*, unmodified calf thymus DNA. *OR*, origin.

postlabeling method, a DNA adduct was detected in exfoliated urothelial cells isolated from the individual's urine after the acute MOCA exposure. The DNA adduct exhibited chromatographic properties identical to that of the authentic major MOCA-DNA adduct prepared *in vitro* by reacting *N*-hydroxy-MOCA with DNA (Fig. 3) and to that of the major adduct observed in bladder epithelium of dogs treated with MOCA (data not shown). The adduct level reached a peak within 4 h after the accident and decreased with time until it could no longer be detected (Fig. 4). This indicated that formation of the DNA adduct correlated with the time of the accident and that the decrease in adduct level correlated with the cessation of MOCA exposure. These data provide strong evidence that the adduct detected in this study is the major MOCA-DNA adduct, which has been previously characterized as *N*-(deoxyadenosin-8-yl)-4-amino-3-chlorobenzyl alcohol (21, 22).

The biphasic decrease in adduct levels, characterized by an early dramatic loss followed by a longer period in which adduct levels decreased only slightly, is qualitatively similar to the results obtained in animal studies when carcinogen-DNA adduct persistence is measured (36). However, in this study, the initial decrease in adduct levels occurred in hours instead of days, suggesting that the biological process responsible for adduct loss is not related to adduct repair. Instead, the dramatic decrease in adducts probably indicates the preferential elimination of urothelial cells which are damaged as a result of the acute MOCA exposure.

In a previous study by Osorio *et al.* (33), urinary MOCA levels were measured in this same individual after the acute MOCA exposure, and it was estimated that the half-life for urinary MOCA was 23 h. A comparison between MOCA-DNA adduct levels and urinary MOCA levels as a function of time after the exposure indicates

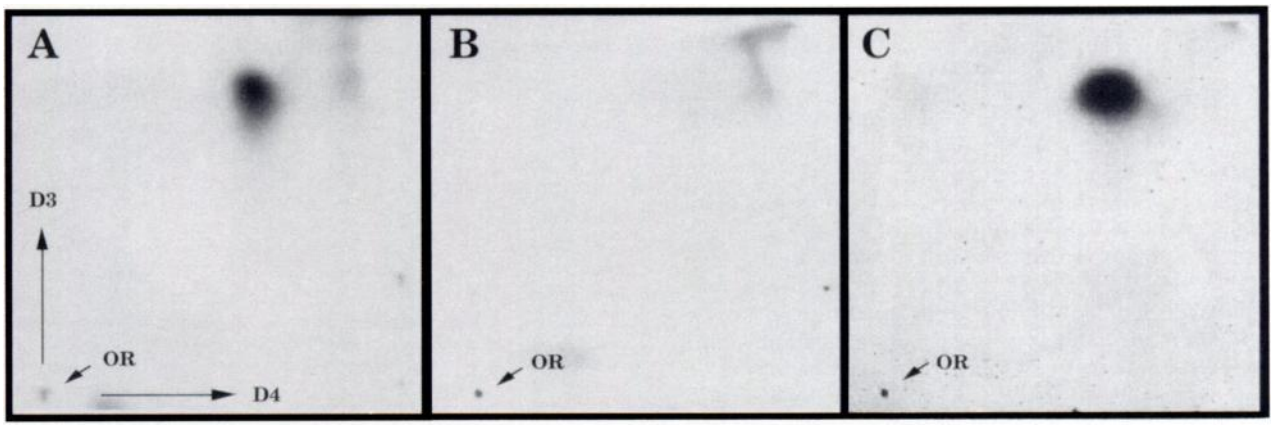


*Fig. 3.* Autoradiograms of $^{32}$P-postlabeled DNA after D1–D4 chromatography. DNA from exfoliated urothelial cells of a worker, 18 h (*A*) and 17 days (*B*) after acute exposure to MOCA; *C*, DNA modified *in vitro* with *N*-hydroxy-MOCA. *OR*, origin.

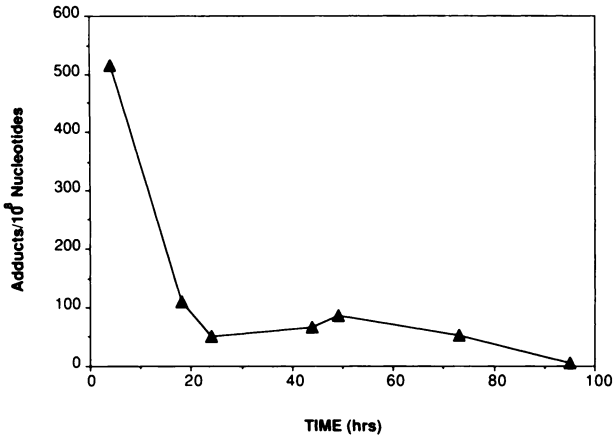


*Fig. 4.* MOCA-DNA adduct levels in exfoliated urothelial cells of a worker at various times after acute exposure to MOCA.

that in each case, beginning after a peak at 4 h, a substantial decrease occurs over a 20-h period, followed by a gradual loss during subsequent days. A major difference, however, was that the change in urinary MOCA levels was much less dramatic than that of DNA adducts during the first 24 h (50% *versus* 90% decrease). After 4 days, urinary MOCA levels and MOCA-DNA adduct levels were 6% and 1% of the 4 h peak level, respectively. Thus, there appears to be only a rough correlation between urinary MOCA levels and MOCA-DNA adduct levels in exfoliated urothelial cells.

The formation of the major MOCA-DNA adduct in human urothelial cells provides indirect evidence for the formation of *N*-hydroxy-MOCA *in vivo*. *N*-Hydroxy-MOCA is likely to be the ultimate carcinogenic form of MOCA, since it can react directly with DNA without further derivatization. The major site of formation of *N*-hydroxy-MOCA is the liver; and in human hepatic tissue, CYP3A4 is the primary enzyme responsible for this reaction (19). Studies with human and dog hepatic microsomes have shown that *N*-hydroxy derivatives of arylamine carcinogens can be further metabolized by UDP-glucuronosyltransferase(s) to form the corresponding *N*-hydroxy arylamine *N*-glucuronides (37, 38); and in whole animals, these conjugates are transported to the target tissue, urinary bladder (39–41). In the mildly acidic environment of urinary bladder, the glucuronides are susceptible to hydrolysis to reform the *N*-hydroxy arylamine (37, 40). Alternatively, as in the case of *N*-hydroxy-ABP, a substantial amount of the free, unconjugated form can be transported directly to the urinary bladder (41). Thus, by comparison with these studies, a plausable biochemical mechanism for the formation of MOCA-DNA adducts in humans could involve the formation of *N*-hydroxy-MOCA in the liver and transport to the urinary bladder as either the free unconjugated form or as the *N*-glucuronide conjugate, where upon deconjugation, the reformed *N*-hydroxy-MOCA aglycone can react directly with epithelial cell DNA to form a DNA adduct. Recent studies indicate that human liver microsomes catalyze the *N*-glucuronidation of *N*-hydroxy-MOCA at rates comparable to that of the *N*-hydroxy derivatives of 2-naphthylamine and ABP,[5] providing further support for this hypothesis.

During the period in which MOCA-DNA adducts could be detected, urinary MOCA levels ranged from 1700 ppb (at 4 h) to 109 ppb (at 99 h) (33). Although MOCA levels at the upper end of this range are not expected in workers exposed to MOCA under normal, routine working conditions, a number of occupational biomonitoring studies have measured urinary MOCA at levels which approximate the lower end of this range. For example, in a 1971 study reported by Linch *et al.* (1), average concentrations of urinary MOCA in 31 American workers ranged from 160 to 1260 ppb, and in a second study conducted between 1982 and 1984, average urinary levels among 39 workers from three different French factories ranged from 18 to 236 ppb (2). In addition, 9.2% of 3323 urine samples obtained from 54 U.S. companies, between 1980 and 1983, had MOCA levels greater than 100 ppb (3). Thus, by comparison with these studies and assuming a roughly linear correlation between urinary MOCA levels and MOCA-DNA adduct levels, workers with urinary MOCA levels in the 100 ppm range may have DNA adduct levels approximating the lower limit of sensitivity of our $^{32}$P-postlabeling assay, which is approximately 5 adducts/$10^8$ nucleotides.

The single most important factor which limited the sensitivity of this method was the relatively small amount of DNA analyzed (average, 0.23 $\mu$g). Since the nuclease P1 enhancement procedure is used, as much as 10 to 30 $\mu$g of DNA can potentially be postlabeled without substantial interfering background. Therefore, postlabeling larger amounts of DNA may increase the sensitivity of detection and enhance the potential utility of this method for routine biomonitoring.

Prior to this study, occupational biomonitoring of human MOCA exposure has been limited to measurements of urinary MOCA and MOCA metabolites. Although these measurements can provide valuable information on the extent of MOCA absorption as well as the nature of metabolic end products, they provide no information on DNA damage that can potentially occur as a result of MOCA exposure. In this regard, the postlabeling method described here provides a noninvasive means to measure DNA damage induced by MOCA exposure: *i.e.*, the formation of MOCA-DNA adducts. More importantly, this method analyzes DNA adducts formed in the suspect target tissue, the urinary bladder; and it could be an important tool for assessing the potential cancer risk associated with occupational MOCA exposure. Thus, the scope of future biomonitoring studies involving MOCA-exposed workers should be broadened to provide an assessment of both urinary MOCA and MOCA-DNA adducts in exfoliated urothelial cells.

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# 4,4'-Methylene-bis(2-chloroaniline)-DNA adduct analysis in human exfoliated urothelial cells by 32P-postlabeling.

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