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ortho-Toluidine Blood Protein Adducts: HPLC Analysis with Fluorescence Detection after a Single Dose in the Adult Male Rat[1]

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Hemoglobin (Hb) and albumin (Alb) adducts of the suspect human carcinogen *ortho*-toluidine (OT) were quantified in blood samples collected from rats after a single i.p. injection. Mild alkaline hydrolysis of Hb adducted with [^{14}C]OT followed by extraction with ethyl acetate resulted in recovery of 63% of the bound radioactivity. HPLC analysis revealed a single radiolabeled peak which was identified as OT by GC-MS. In subsequent experiments Hb and Alb adduct levels were determined by HPLC analysis of this cleavage product using fluorescence detection. 4-Ethylaniline was used as internal standard. The detection limit for OT was approximately 450 pg/injection or 5 pmol/mg Hb. Mean adduct levels for Hb increased rapidly over the first 4 hr with the highest (ng/mg Hb ± SD) 3.7 ± 0.5 detected 24 hr after OT administration at 50 mg/kg body wt. In contrast, adduct levels for pooled Alb samples increased from 0.7 ng/mg Alb at 2 hr to 2.5 ng/mg Alb at 4 hr, but were not detectable 24 hr after dosing. Hb adducts showed a linear relationship for OT doses of 10, 20, 40, 50, and 100 mg/kg body wt. The Hb adduct $t_{\frac{1}{2}}$ (11 days) was determined after a single 100 mg/kg OT dose. Hb adduct levels were quantifiable (1.3 ± 0.2 ng/mg Hb) by HPLC/fluorescence 28 days after 100 mg/kg OT. Although Hb and Alb adducts differ in stability, a ratio of such OT adducts may be useful in long-term industrial biomonitoring for evaluation of OT exposure.

The suspect human carcinogen, *o*-toluidine [2-aminotoluene; CAS Registry No. 95-53-4] has long been used in the chemical industry as an intermediate in the production of dyes (Hammerbacher, 1883) and is used in the production of rubber, pharmaceuticals, and pesticides (Cheever *et al.*, 1980; IARC, 1982). *o*-Toluidine has been reported to cause bladder tumors in various mammalian species (Morigami and Nisimura, 1940; Ekman and Strömbeck, 1947, 1949; Russfield *et al.*, 1973; Weisburger *et al.*, 1978; IARC, 1978) and is mutagenic in *in vitro* tests (Rosenkrantz and Poirier, 1979; Tanaka *et al.*, 1980; Zimmer *et al.*, 1980). Although human *o*-toluidine-related carcinogenicity is not certain (IARC, 1982, 1987), *o*-toluidine is considered a suspect human carcinogen (Rubino *et al.*, 1982; Ward *et al.*, 1991). As a result of those reports the National Institute for Occupational Safety and Health recently issued a warning that workers exposed to *o*-toluidine or aniline may be at increased risk for developing bladder cancer (NIOSH, 1991). The mechanism of many chemical carcinogens is thought to involve the modification of DNA by formation of adducts with DNA in genetic material through covalent binding of nucleophilic sites by electrophilic compounds or activated metabolites (Kiese, 1966; Weisburger and Weisburger, 1973; Kriek and Westra, 1979; Neumann, 1988; Mori *et al.*, 1988). A correlation between covalent binding to protein macromolecules and that of target organ DNA has been described for several animal carcinogens (Brookes and Lawley, 1964; Lutz, 1979; Cheever *et al.*, 1990), and a relationship between covalent binding and carcinogenic potency has been proposed (Brookes and Lawley, 1964; Lutz, 1979; Perera, 1987; Kugler-Steigmeier *et al.*, 1989). The covalent binding of *o*-toluidine was evaluated in a recent study by Brock and his co-workers (1990), which showed that *o*-toluidine was covalently bound to hepatic DNA, RNA, and protein. The current OSHA permissible exposure limit listed, as a time-weighted average, for *o*-toluidine is 5 ppm (NCI, 1979). Until recently, monitoring techniques used for determination of *o*-toluidine exposure have relied upon determination of *o*-toluidine levels in air (IARC, 1978; Wood and Anderson, 1975; Amoore and Hautala, 1983) or urine (Kuchenbecker, 1920; El-Bayoumy *et al.*, 1985). However, such analysis provides limited information on bladder target organ exposure. The biological half-life in rodents of parent *o*-toluidine or *o*-toluidine metabolites (Fig. 1) is relatively short (Cheever *et al.*, 1980), and these compounds may be similarly short lived in humans (IARC, 1982). Significantly, the presence of DNA-adducting metabolites of certain carcinogens in the blood has been reported for compounds metabolized primarily in

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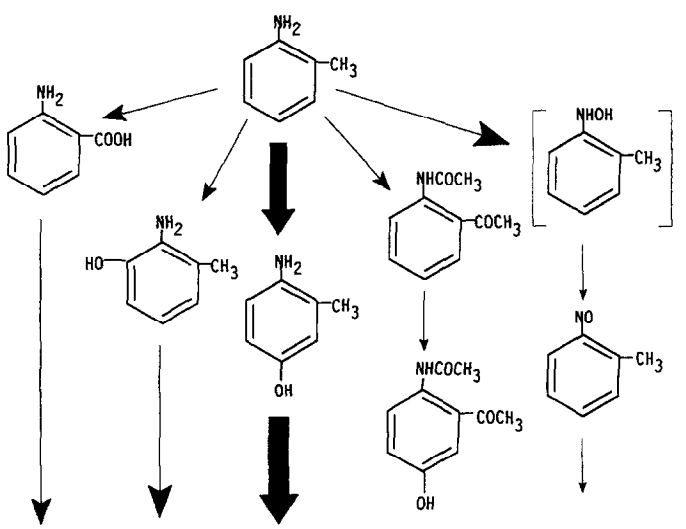
the liver (Weisburger *et al.*, 1969; Ginsberg and Atherholt, 1989). Those investigators suggested that distribution by blood after bioactivation certainly could explain tumor formation in other systemic tissues. Molecular dosimetry by measuring adducted blood proteins, as a tool for estimating internal or target organ exposure, has been proposed for biomonitoring (Osterman-Golkar *et al.*, 1976; Neumann, 1984; Ashby, 1988). Hemoglobin (Hb) binding to *o*-toluidine has been reported (Reiter and Leusser, 1952; Kiese, 1966; Weisburger and Weisburger, 1973), and it has been proposed (Fig. 2) that the adducts formed are sulfinic acid amide Hb adducts (Eyer *et al.*, 1983). Such adducts have been described for other aromatic amines (Bryant *et al.*, 1987; McMillan *et al.*, 1990a). Recent animal studies suggest that biomonitoring of *o*-toluidine can be accomplished by measurement of this Hb adduct (DeBord *et al.*, 1991). Previously, evaluation of *o*-toluidine adduct levels by gas chromatography or gas chromatography-mass spectrometry (GC-MS) have been described (Stillwell *et al.*, 1987; Birner and Neumann, 1988; Bryant *et al.*, 1988; Neumann, 1988). Those investigators reported that analysis of the cleavage product of adducted *o*-toluidine was possible after hydrolysis of the Hb. Quantification of such blood protein adducts may be of value in risk assessment by allowing a more accurate estimation of internal exposure and, if the biological half-life of the Hb adduct is sufficiently long, may indicate cumulative target dose.

The objectives of this study were to compare the effect of route of administration on *o*-toluidine–protein adduct levels in the rat and to establish the dose–response for adduct formation. Additionally, the *in vivo* stability of *o*-toluidine–protein adducts was studied to evaluate the usefullness of blood protein adducts for monitoring long-term worker exposure.



CONJUGATION AND URINARY EXCRETION

FIG. 1. Schematic of *o*-toluidine metabolism.

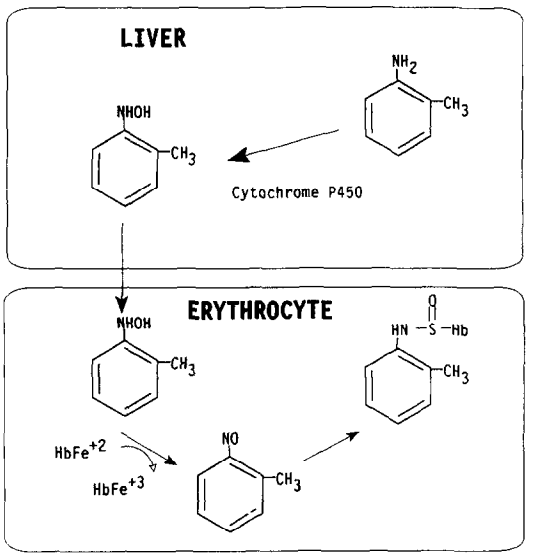


FIG. 2. Postulated formation of an *o*-toluidine–hemoglobin sulfinic acid amide adduct.

METHODS

Chemicals and solutions. The test compound, *o*-toluidine (99+%) was purchased from Aldrich Chemical Co. (Milwaukee, WI).[2] The *o*-toluidine[ring-^{14}C(U)], having a specific activity of approximately 30.7 mCi/mmol and a radiochemical purity of greater than 99%, was synthesized by NEN Research Products (Boston, MA). Prior to use the radiochemical purity of this compound was verified by high-performance liquid chromatography (HPLC) in conjunction with a radioactivity detector as described previously (Cheever *et al.*, 1990). The reference compound, 4-amino-3-methylphenol, was obtained from Aldrich Chemical Co. Dosing solutions were prepared by dissolving appropriate amounts of *o*-toluidine and *o*-[^{14}C]-toluidine in corn oil to give final concentrations of 10, 20, 40, 50, and 100 mg/ml (93, 187, 373, 466, and 933 μmol/ml) [sp act 16.1, 8.5, 4.6, 3.7, and 2.0 mCi/g, respectively].

Animals and doses. Male, Sprague–Dawley [Crl:CD(SD)BR outbred], cesarean-derived rats, weighing 51 to 75 g, were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA). Immediately upon receipt, these animals were placed in quarantine and maintained in an AAALAC-accredited facility throughout the course of the study. Laboratory temperatures, 22 to 25°C, and relative humidity, 45 to 50%, as well as a 12-hr light–dark cycle (light 6:00 AM to 6:00 PM) were controlled throughout the course of the studies. Animals were assigned to 23 groups of 6 rats (138 total) and provided with NIH-07 rat and mouse diet (Ziegler Brothers, Inc., Gardners, PA) and tap water *ad libitum*. At dosing the animals weighed 269 ± 47 g and were administered the appropriate dosing solution either by gavage or intraperitoneal injection (i.p.) at a constant 1-ml vol of dosing solution/kg body wt. Animals used for comparison of route of administration, time course, or dosing level were administered radioactivity in the oral and i.p. doses which averaged 40 μCi/rat. Rats within a specified group were anesthetized with pentobarbital and euthanized by exsanguination at either 4, 8, 12, 18, 24, 48, 72, 168, 336, or 672 hr after a single *o*-toluidine dose.

[2] Mention of company or product names is not to be considered an endorsement by the National Institute for Occupational Safety and Health.

Blood was obtained from the vena cava by syringe, and Hb and albumin were immediately isolated.

Isolation of hemoglobin and albumin. Blood, collected from animals treated with *o*-toluidine, was transferred immediately to EDTA-containing Vacutainers. The whole blood was centrifuged at 1500*g* for 20 min to separate erythrocytes (RBCs) and plasma. Following this, 4.5 ml of the plasma was carefully transferred to a clean 50-ml centrifuge tube for the isolation of albumin. The plasma was mixed with 45 ml of 0.2% HCl in absolute ethanol to precipitate the globulins as described by Fernandez *et al.* (1966). After mixing, the tubes were shaken at low speed at room temperature for 30 min in a shaking waterbath (Precision Scientific Co., Chicago, IL). The tubes were centrifuged at 400*g* for 30 min, and the ethanol-soluble fraction was concentrated under vacuum (approx 0.2 Torr) using a SpeedVac Model SVC 100H (Savant Instruments, Inc., Farmingdale, NY). After a 2- to 2.5-hr drying period, albumin was redissolved in 1 ml of distilled water, transferred to a 50-ml centrifuge tube, and reprecipitated with 49 ml of acetone. Following overnight refrigeration at 4°C, the albumin was repacked by centrifugation at 2000*g* for 10 min and washed three times by resuspending in acetone. The albumin was dried under a stream of nitrogen and stored at −70°C until analysis. After removal of the plasma the packed RBCs were washed three times with 10 ml of ice cold phosphate-buffered saline and repacked by centrifugation at 700*g* for 10 min. The cells were then lysed by addition of 10 ml of ice cold distilled water, and the hemolysate was centrifuged at 45,000*g* for 25 min at 4°C to eliminate cellular debris. Hb was isolated by the dropwise addition of 4 vol of cold ethanol to the lysate while mixing. After precipitation, the Hb was removed, washed three times with acetone, and dried under a stream of nitrogen (Cheever *et al.*, 1990). Hb and albumin isolated from rats dosed with *o*-toluidine were subjected to mild basic hydrolysis to allow analysis of adducted *o*-toluidine, using a modification of the procedure of Sabbioni and Neumann (1990). Initial experiments on Hb or albumin from rats dosed with 50 mg *o*-toluidine/kg body wt were conducted using hydrolysis by 1 N NaOH and 0.5% sodium lauryl sulfate at 60°C for 1, 2, 3, or 4 hr in the presence of 1 μg/ml 4-ethylaniline, as internal standard. All subsequent experiments were conducted using a 1-hr 60°C hydrolysis period, and the ratio of protein to hydrolysis media was 100 mg in 3 ml of media. Following the base-induced hydrolysis of protein adducts, the mixtures were extracted 1:1 with ethyl acetate for 24 hr. For the determination of ethyl acetate extraction efficiency (86%) ^{14}C was quantified by liquid scintillation spectrometry. The recovery of bound ^{14}C was approximately 63%. The organic phase was dried over sodium sulfate for subsequent analysis by thin-layer chromatography (TLC) and HPLC.

Thin-layer chromatography. Solvent extracts of base-hydrolyzed vehicle control hemoglobin and albumin or extracts of those proteins isolated from rats treated with *o*-toluidine were concentrated under a stream of nitrogen. A portion of this extract was reacted with pentafluoropropionic anhydride [PFPA] (El-Bayoumy *et al.*, 1986) and cochromatographed with either *o*-[^{14}C]toluidine, the pentafluoropropionamide derivative of *o*-[^{14}C]toluidine, or 4-amino-3-methylphenol on silica gel-precoated glass-backed plates (E. Merck 60F 254, 0.2-mm layer thickness). Chromatograms were developed with benzene:ethyl acetate (9:1, v/v) and extracts of base-hydrolyzed proteins were examined for the presence of any freed *o*-toluidine or *o*-toluidine metabolites. Spots corresponding to possible metabolites or reference compounds were located by observing the quenching of fluorescence activated by 254-nm radiation and by color reactions with ninhydrin. Radioactive spots were located by autoradiography using a Betascope Model 603 blot analyzer (Betagen Co., Waltham, MA). From chromatograms not visualized by chemical reactions, the silica gel-containing compounds of interest was scraped and extracted with methanol. Compounds isolated in this manner were further evaluated by HPLC or GC-MS.

Liquid chromatography. *o*-Toluidine purity and components of protein hydrolysates were analyzed using a Hewlett–Packard Model 1090M HPLC equipped with a binary DR5 solvent delivery system, variable volume sample injection module, and heated column compartment (Hewlett–Packard, Co., Waldbronn Analytical Division, Waldbronn, Germany). The HPLC column temperature was controlled at 40°C. Components were separated on a 25-cm-long × 4.6-mm-i.d. stainless-steel column packed with 10-μm Partisil 10 ODS-3 silica packing (Whatman Chemical Separations Inc., Clifton, NJ). Initially, a gradient solvent system programmed from 10% methanol in 0.05 M ammonium formate to 90% methanol over a 50-min period was used to achieve separations adequate for quantification. Components were detected initially at a 240-nm wavelength setting using a 78880A uv diode array detector module (Hewlett–Packard, Co.). Components of interest were subsequently quantified using a Model 1046A programmable fluorescence detector (Hewlett–Packard, Co.) set at ex = 237 nm, em = 345 nm. For quantitation of amines, aliquots of the extracts were mixed with 0.5 M *o*-phthalaldehyde/mercaptopropionic acid reagent [OPA] (10 mg/ml each in 0.4 N borate buffer, Hewlett–Packard, Co.) immediately prior to injection onto the column to allow for any instability of the derivative (Trippel-Schulte *et al.*, 1986; Alvarez-Coque *et al.*, 1989). Briefly, the sample injection module was programmed to take up 5 μl of 0.4 N borate buffer (pH 10.4) into the sample loop followed by 1 μl of the OPA reagent, 1 μl of distilled water, and sample. Standard solutions containing appropriate concentrations of aniline and *o*-toluidine were prepared using a 1 μg/ml internal standard solution of redistilled 4-ethylaniline. The contents of the sample loop were then mixed by cycling the sample module syringe eight times over a 50-μl volume range. After injection, radioactive components were detected using a Trace II Model 7150 radioactivity monitor (Packard Instruments, Downers Grove, IL). The derivatization was essentially complete at a 10-fold excess of reagent for the highest *o*-toluidine standard used and no additional radiolabeled peaks were detected. Subsequently, radioactive or unlabeled components were separated using a linear solvent gradient programmed from 30% methanol:0.05 M ammonium formate to 50% methanol:0.05 M ammonium formate over a 15-min period followed by a 10-min linear change to 80% methanol:0.05 M ammonium formate. Sample data were acquired for comparison of sample uv or fluorescent spectra with those of library standards acquired under the same conditions. HPLC data were processed using a HP 9000 Model 310 HPLC ChemStation (Hewlett–Packard, Co.).

Gas chromatography-mass spectroscopy. The radioactive component, isolated from protein hydrolysates of rats treated with *o*-toluidine, was analyzed using a Hewlett–Packard Model 5890A gas chromatograph, fitted with a 50-m × 0.2-mm-i.d. ultra-1 crosslinked methyl silicon (d_f = 0.33 μm) fused capillary column (Hewlett–Packard Co.) coupled by a direct capillary interface to a Model 5970A quadrupole mass spectrometer (Hewlett–Packard Co.). Helium was used as the carrier gas at a linear velocity of 38 cm/sec. Splitless injections were made onto the column with the oven temperature maintained at 75°C. The temperature was programmed from 75 to 200°C at 8°C/min. Electron impact mass spectra were acquired at an electron energy of 70 eV and at a source temperature of 200 ± 10°C. The mass spectrometer was calibrated using perfluorotributylamine as the tuning compound. These spectra were stored and processed with a Model 59970B MS ChemStation (Hewlett-Packard Co.). Mass spectrometric identifications were made by comparison of the mass spectra of radiolabeled components with those of authentic compounds. The structures of *o*-toluidine and PFPA-derivatized *o*-toluidine were investigated. For *o*-toluidine the spectrum is dominated by ions at *m/e* 106 and 107, consistent with the molecular ion and mass of that compound. Additional prominant ions at *m/e* 108 (M + 1), 79 (M + 1 − CNH_2), 73 (M + 1 − CNH_5), 66 (M − C_2NH_3), and 51 (M − C_3NH_5) were consistent with Wiley Library spectra. Examination of the spectrum for PFPA-derivatized *o*-toluidine showed a base peak at *m/e* 134 (M − C_2F_5). Additional prominant ions were noted at *m/e* 253 (M), 254 (M + 1), 119 (M − $C_2F_5CH_3$), 106 (M − C_2F_5CO), and 91 (M − C_2F_5CONH) and were consistent with library spectra. For highest sensitivity, operation of the mass spectrometer in the selected ion mode was utilized to detect the most abundant ion for *o*-toluidine either underivatized (*m/e* 106) or as the PFPA derivive (*m/e* 134).

Liquid scintillation spectrometry. For determination of *o*-[^{14}C]toluidine levels in protein hydrolysates or extracts, sample aliquots (0.01 to 0.5 ml) were solubilized by incubation for 24 hr at 60°C with 0.25 ml of methanolic

TABLE 1
Identification of Base-Hydrolyzable *o*-Toluidine Protein Adducts

Compound	RT[a]	RT[b]	R_f[c]	Color reaction
Unknown	9.456	19.399	0.407	P—Br[d,e]
Authentic *o*-toluidine	9.322	19.398	0.407	P—Br
Unknown-PFPA		23.033	0.687	
o-toluidine-PFPA		23.048	0.689	

[a] HPLC retention time (min).
[b] GC-MS retention time (min).
[c] TLC R_f value of compounds spotted on E. Merck Silica gel F60 precoated plates and run 10 cm with a benzene:ethyl acetate (9:1) mobile phase.
[d] TLC plates sprayed with ninhydrin (0.1% in isopropanol) were heated to 90°C for 10 min. Color reaction was noted after cooling and after 24 hr.
[e] P, purple; Br, brown; — indicates observation after 24 hr.

1 N NaOH (Weigel *et al.*, 1978). These solubilized samples were dissolved in 10-ml quantities of ScintiVerse II scintillation medium (Fisher Scientific Co., Fairlawn, NJ) and counted using a Model 8011 liquid scintillation spectrometer (Beckman Instruments Co., Fullerton, CA). Counting efficiencies were determined by the external standard method of Horrocks (1974).

Statistical analysis. Statistical differences between group means were determined using one-way analysis of variance using Statgraphics Version 4.0 (STSC Inc., Rockville, MD). A probability level of $p < 0.05$ was considered significant. Data was processed for determination of correlation coefficients using the HP 98820A statistical library revision B (Hewlett-Packard, Co.) installed on the HP 9000 Model 310 HPLC ChemStation. Group means ± standard deviations were plotted with linear regression lines using SigmaPlot Version 3.1 (Jandel Scientific, Sausalito, CA). Calibration curves for GC methods were tested for equality of slopes using an analysis of covariance. The model was $Y_i = M_i + C + M_iC + e_i$, $i = 1, 2$, where M is method and C is concentration. Biological half-lives were calculated by the method of Rumack and Lovejoy (1986), and adduct stability was evaluated by the method of Bergmark *et al.* (1990). The model was $[RY]/[Y] = [RY]^0/[Y] \times (1 - t/t_{er})\exp(-kt)$, where $[RY]/[Y]$ is the initial adduct level at time t, $[RY]^0/[Y]$ is the initial adduct level, k is the rate constant for elimination, and t_{er} is considered to be a 60-day lifetime for rat erythrocytes (Schalm *et al.*, 1975).

RESULTS

Identification of hemoglobin and albumin adduct cleavage products. Initial experiments showed that mild base hydrolysis at 60°C for 1 hr followed by extraction with ethyl acetate resulted in liberation of 63.1 ± 9.4% of the radioactivity associated with the Hb or albumin. Additional hydrolysis time resulted in a linear, but not statistically significant, decrease over a 3-hr period. After concentration, the extracted material was separated by TLC and a single radiolabeled component was detected by autoradiography. This single major component was cochromatographed, with or without PFPA derivatization, against appropriate *o*-toluidine or *o*-toluidine metabolites for comparison of TLC R_f value and ninhydrin color reaction (Table 1). The component, thought to be *o*-toluidine, was subsequently identified by comparison of GC-MS spectra with those of derivatized or underivatized *o*-toluidine (Figs. 3 and 4). The typical gas chromatographic trace of base-hydrolyzed rat Hb (Fig. 5) illustrates the increased sensitivity possible monitoring *m/e* 106 using the selected ion mode. Calibration curves for derivatized and underivatized *o*-toluidine were prepared using a 1-μl injection volume (Fig. 6), which indicate that significantly greater sensitivity ($p < 0.0001$) is achieved for the PFPA-derivatized *o*-toluidine. However, low recovery of samples and the time requirements encountered for sample workup of derivatized Hb extracts led to development of an automated procedure for HPLC analysis.

High-performance liquid chromatography fluorescence analysis. A syringe programmable sequence for automated OPA derivatization of samples for injection onto the HPLC column was evaluated for *o*-toluidine analysis (Fig. 7). This procedure, coupled with fluorescence detection, allowed savings of much sample preparation time prior to analysis of Hb hydrolysates. The HPLC fluorescence calibration curve, prepared using a 5-μl injection, is shown in Fig. 8. Although the sensitivity of the HPLC procedure is lower than that noted previously for the GC-MS technique, the possibility of larger injection volumes appears to offer added flexibility. Subsequent analysis of extracts of hydrolyzed Hb

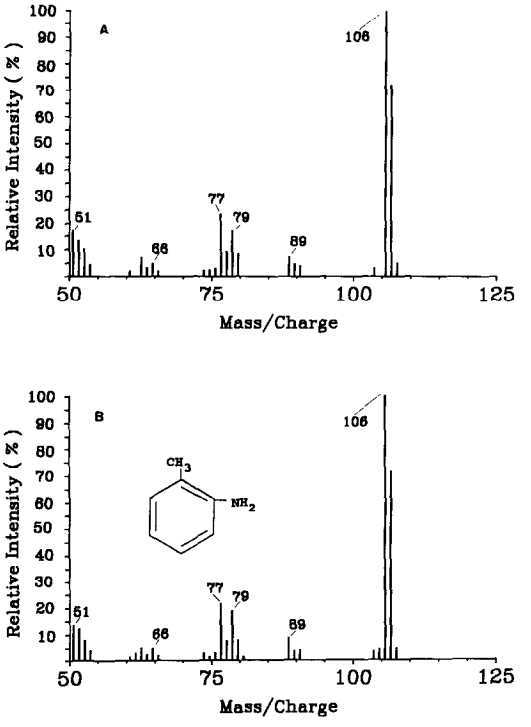


FIG. 3. Identification of *o*-toluidine from the ethyl acetate extract of base-hydrolyzed hemoglobin isolated from *o*-toluidine-treated rats. The mass spectrum of the cleavage product (A) is compared with the spectrum of authentic *o*-toluidine standard (B).

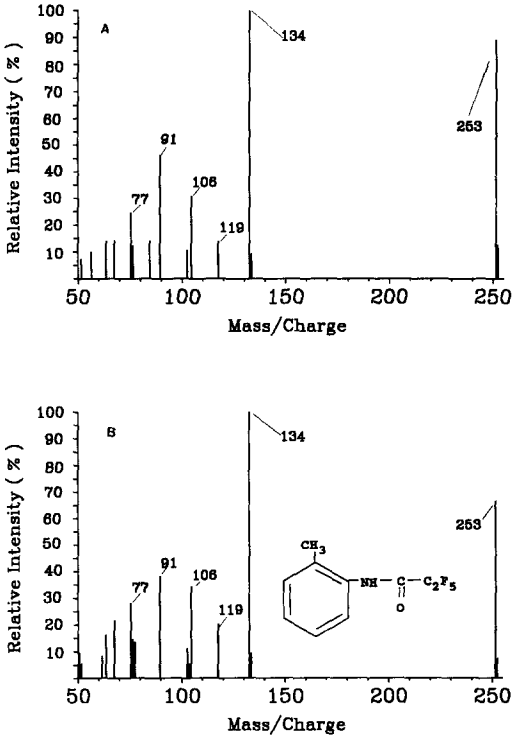


FIG. 4. Pentafluoropropionic anhydride derivative of *o*-toluidine from the ethyl acetate extract of base-hydrolyzed hemoglobin isolated from *o*-toluidine-treated rats. The mass-spectrum of the PFPA-derivatized cleavage product (A) is compared with the spectrum of PFPA-derivatized *o*-toluidine standard (B).

showed that the detection limit for *o*-toluidine after a single i.p. injection at 100 mg/kg body wt, approximately 10% of the reported LD50 value (Smyth, 1931), utilizing the HPLC procedure was approximately 450 pg/injection or 5 pmol/mg Hb. Additionally, comparison of these extracts by both HPLC and GC-MS indicates that no statistically significant difference ($p > 0.05$) was noted for those techniques. Subsequent experiments were conducted using the HPLC procedure with fluorescence detection.

Animal studies. The formation of Hb adducts was tested after either oral (p.o.) or i.p. administration of a single injection of *o*-toluidine at 50 mg/kg body wt. Hb adduct levels, determined after basic hydrolysis, showed that adduct formation was significantly higher after i.p. injection (3.5 ± 1.6 ng/mg Hb) than after p.o. administration (0.4 ± 0.6 ng/mg Hb). The levels of base-hydrolyzable *o*-toluidine adducts were evaluated for two blood proteins, Hb and albumin. Subsequent studies were conducted using i.p. injection to optimize adduct formation. After administration of a single 50 mg/kg dose of *o*-toluidine, the adduct, measured as the *o*-toluidine cleavage product, appears to accumulate more in association with Hb than with the albumin. For these studies, mean adduct levels for Hb increased rapidly over the first 4 hr with the highest 3.7 ± 0.5 ng/mg Hb detected 24 hr after administration of the test compound. In contrast, adduct levels for pooled albumin samples increased from 0.7 ng/mg albumin at 2 hr to 2.5 ng/mg albumin at 4 hr, but were not detectable 24 hr after dosing (Fig. 9). Experiments conducted to examine long-term elimination of Hb adduct levels show that these were relatively stable during the 24- to 72-hr period (Fig. 10). A linear relationship between administered dose and 4-hr Hb adduct formation in rats was noted (Fig. 11). The highest Hb adduct levels detected in these studies, 7.5 ± 0.9 ng/mg Hb, was measured in blood taken after i.p. dosing with *o*-toluidine at 100 mg/kg body wt. Additional studies were conducted to evaluate the stability of the Hb adduct in the rat by measuring levels present in blood after serial termination of rats over a 28-day period. The Hb adduct half-life ($t_{\frac{1}{2}}$ = 11 days) was determined after a single 100 mg/kg *o*-toluidine dose (Fig. 12). Hb adduct levels in those experiments continued to be detectable by HPLC/flu-

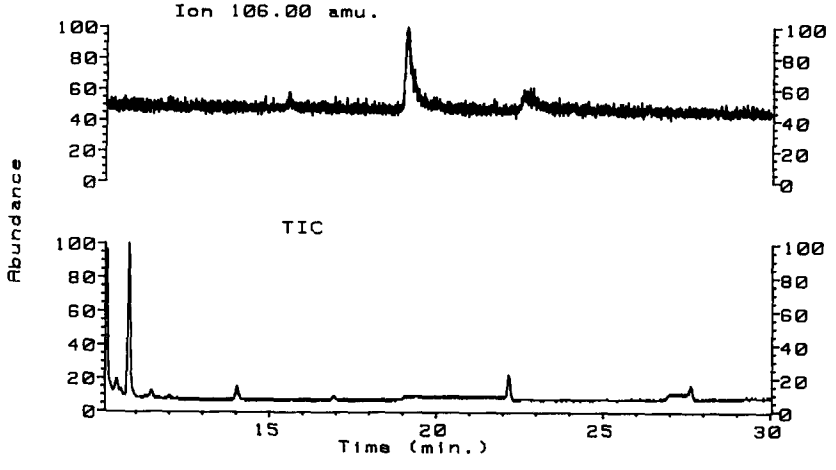


FIG. 5. Gas chromatogram (lower) of the extract after mild base hydrolysis of *o*-toluidine–hemoglobin adduct of *o*-toluidine-treated rats using a mass spectrometer as detector in the total ion mode. The upper trace shows peak for cleavage product in the same extract in the selected ion mode.

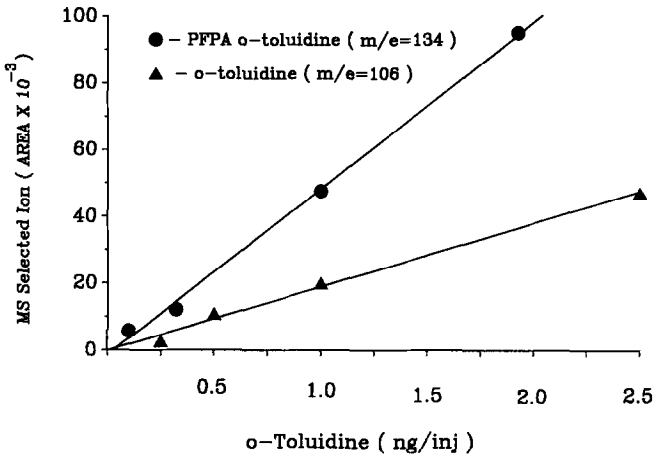


FIG. 6. Comparison of GC-MS selected ion calibration curves for underivatized and pentafluoropropionic anhydride (PFPA)-derivatized *o*-toluidine (r^2 = 0.999).

orescence 28 days after i.p. administration of *o*-toluidine at 100 mg/kg. Quantifiable amounts, 1.3 ± 0.2 ng/mg Hb, were determined for the 28-day timepoint. The rate constant, k, for the hydrolysis of the *o*-toluidine–Hb adducts has been estimated from this experiment to be $2.2(\pm 0.3) \times 10^{-3}$ h^{-1}, a value consistent with that of *N*-ethyl-*N*-nitrosourea–Hb adduct elimination reported by Bergmark *et al.* (1990).

DISCUSSION

The formation of tumors by industrial exposure to aromatic amines has been recognized for nearly a century (Rehn, 1895). Recently, Rubino *et al.* (1982) suggested that *o*-toluidine, a simple aniline derivative, may induce human bladder tumors. The formation of such tumors in the rat has been well documented for *o*-toluidine (IARC, 1987), but the related isomers, *meta*- or *para*-toluidine, showed only marginal tumorigenic effects (Weisburger *et al.*, 1978; U.S. EPA, 1984). The results of the current study indicate that acute exposure of rats to *o*-toluidine at 50 mg/kg body wt by the p.o. route or to doses ranging from 10 to 100 mg/kg body wt i.p. resulted in significant covalent binding with blood proteins. Previously, *o*-toluidine-related production of gene mutations *in vitro* was investigated with somewhat inconclusive results. Using the *Salmonella typhimurium* test system with rat liver S9 mix Rosenkranz and Poirier (1979) reported that *o*-toluidine showed positive mutagenic effects using 0.5 μg/ml *o*-toluidine when incubated with the S9 mixture. Zimmer *et al.* (1980), however, reported negative *in vitro* mutagenicity for *o*-toluidine. Studies conducted to evaluate genotoxicity, using the alkaline elution assay to measure DNA damage in V-79 cells exposed to *o*-toluidine, were negative, but the concentrations tested were not specified (Tanaka *et al.*, 1980). Urinary metabolites showed significant mutagenicity when urinary extracts from rats given *o*-toluidine were tested *in vitro* (Tanaka *et al.*, 1980). Those investigators suggested that a metabolite may be important in the process of mutagenesis, but did not predict the chemical species responsible. Evaluation of possible *o*-toluidine metabolites showed that *N*-hydroxy-*o*-toluidine was genotoxic in the *in vitro Salmonella*/microsomal mutagenicity assay (Hecht *et al.*, 1979; Gupta *et al.*, 1987). The *N*-hydroxy derivatives of aromatic amines have long been suspected as the proximate carcinogens (Weisburger and Weisburger, 1973), but evidence of *in vivo* formation of *N*-hydroxy-*o*-toluidine has not been forthcoming. *o*-Toluidine metabolism has been studied (Hildebrandt, 1903; Williams, 1947; Beckett and Belanger, 1976; Cheever *et al.*, 1980; Son *et al.*, 1980) to both understand the mechanism of *o*-toluidine carcino-

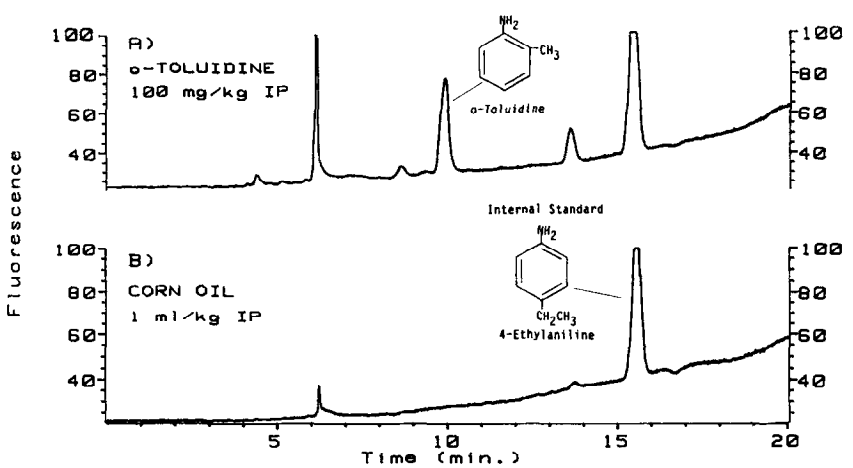


FIG. 7. Typical HPLC chromatogram (A) showing peak for cleavage product after mild base hydrolysis of *o*-toluidine–hemoglobin adduct. Rats were terminated 24 hr after i.p. administration of a single 100 mg/kg (933 μmol/kg) dose of *o*-toluidine. The fluorescence trace for control hemoglobin hydrolysate is shown for comparison (B). The retention time for the cleavage product is consistent with that of authentic *o*-toluidine.

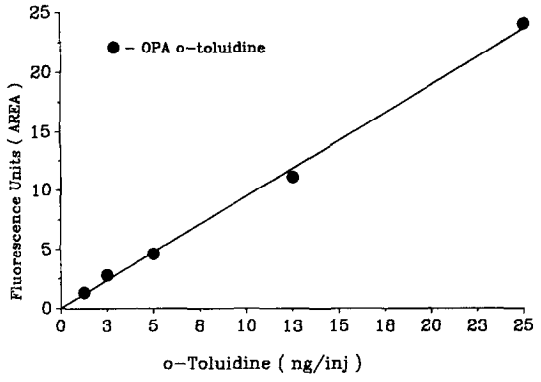


FIG. 8. HPLC fluorescence calibration curve (ex = 237, em = 345) for a 5-μl injection of *o*-toluidine after reaction with *o*-phthaldehyde (r^2 = 0.998).

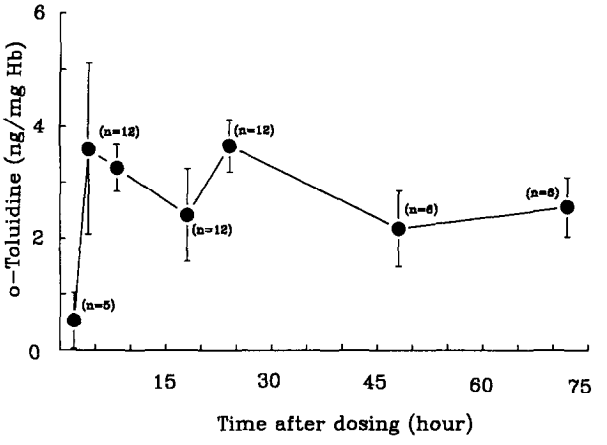


FIG. 10. Formation time of levels of base-hydrolyzable hemoglobin adducts in rats terminated after i.p. administration of a single 50 mg/kg body wt (466 μmol/kg) dose of *o*-toluidine. Values are means ± SD.

genicity and to develop appropriate biomonitoring procedures. These investigators have reported metabolic pathways consistent with those considered essential for aromatic amine carcinogenicity: *N*-acetylation, *N*-oxidation and hydroxylation, and ring oxidation (Fig. 1). Significantly, Son *et al.* (1980), after administering *o*-[*methyl*-^{14}C]toluidine to rats, noted two minor metabolites in rat urine, *o*-nitrosotoluene and 2,2'-dimethylazoxybenzene, which are thought to be derived from *N*-hydroxy-*o*-toluidine and provide evidence that it is formed. Related N-hydroxylated aromatic amines have been reported to react *in vitro* with DNA (Silk *et al.*, 1989), nucleosides (Jacobson *et al.*, 1988), or protein macromolecules (Kadlubar *et al.*, 1976; Ringe *et al.*, 1988; Segerbäck *et al.*, 1989). The degree of DNA covalent binding resulting from chemical exposure, thought to be an initial step in tumor formation, for certain chemicals has been related to Hb binding by the chemicals or reactive metabolites (Segerbäck, 1983; Cheever *et al.*, 1988). Although no report of *in vivo* *o*-toluidine–DNA adduct formation was noted in the literature, recent experiments have detected *o*-toluidine–Hb adducts in both rats and man. Protein adduct formation by *o*-toluidine was recently reported for rat Hb by Birner and Neumann (1988). These investigators estimated that the 24-hr Hb binding index [binding index = (mmol bound/mmol Hb)/mmol dose/kg body wt] was 4.0 for rats treated with 0.6 mmol/kg of *o*-toluidine. The aromatic amine metabolites, hydroxylamine and aminophenol, are thought to react with Hb (Kiese, 1966; Weisburger and Weisburger, 1973). However, reaction of the *N*-hydroxy metabolite of 3,4-dichloroaniline, a related aromatic amine, with Hb *in vitro* was reported to be an order of magnitude greater than that of the ring-hydroxylated metabolite (McMillan *et al.*, 1990b). In the current study some adduct formation by 4-amino-3-methylphenol, the principal *o*-toluidine metabolite, cannot

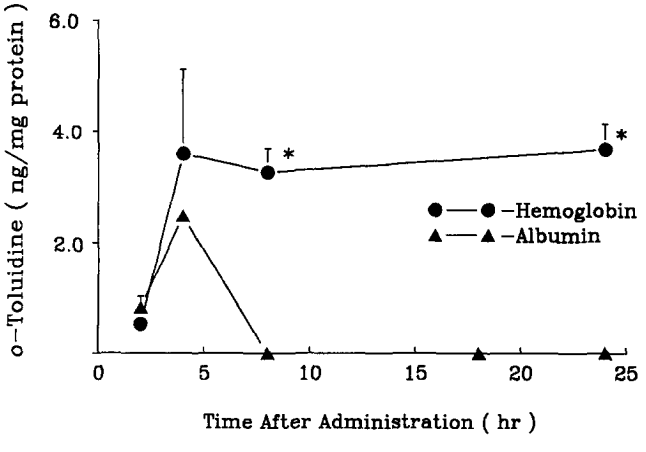


FIG. 9. Comparison of levels base-hydrolyzable hemoglobin and albumin adducts formed in rats terminated after i.p. administration of a single 50 mg/kg body wt (466 μmol/kg) dose of *o*-toluidine. Values are means ± SD, n = 6. *Statistically greater than detected for corresponding albumin samples ($p < 0.05$).

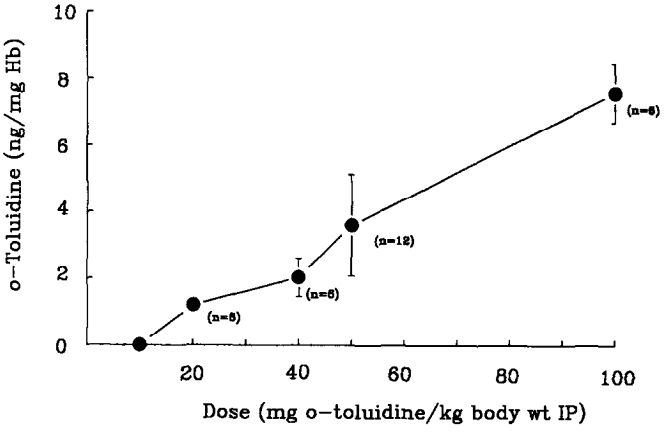


FIG. 11. Dose response for hemoglobin adduct levels in rats terminated 4 hr after i.p. administration of a single dose of *o*-toluidine. Values are means ± SD.

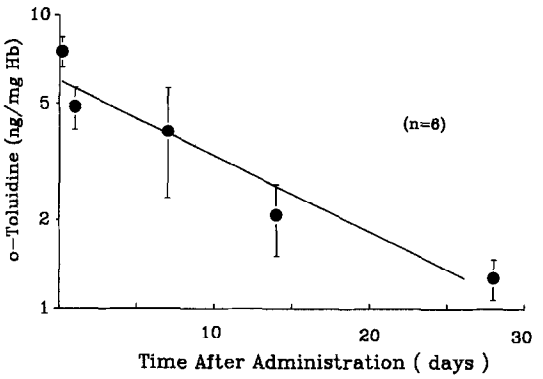


FIG. 12. Elimination of base-hydrolyzable hemoglobin adducts in rats terminated after i.p. administration of a single 100 mg/kg body wt (933 μmol/kg) dose of *o*-toluidine. Values are means ± SD.

be ruled out since the recovery of bound radioactivity was not complete after alkaline hydrolysis. A single quantifiable product, identified as *o*-toluidine, was detected after hydrolysis of isolated Hb, and may indicate binding by the *N*-hydroxy metabolite. Analysis of this hydrolysis cleavage product by HPLC with fluorescence detection was accomplished using an automated precolumn derivatization, a relatively rapid procedure which gave results consistent with GC-MS selected ion mode analysis. The single dose *o*-toluidine–Hb adduct formation *in vivo* was, after 4-hr i.p. administration, approximately 10-fold greater than after p.o. administration: an effect which may be related to increased liver concentrations of the compound—possibly resulting in increased activation. Detectable levels in Hb were noted 29 days after administration of 100 mg/kg of *o*-toluidine i.p. However, the portion of albumin-adducted *o*-toluidine which could be recovered by alkaline hydrolysis decreased rapidly after 4 hr. This apparent difference in stability between the two *o*-toluidine–protein adducts may be related to differences in their binding affinities or the relatively long (60 day) mean lifespan reported for rat erythrocytes (Schalm *et al.*, 1975) in comparison with the reported 2- to 3-day turnover for rat albumin (Skipper and Tannenbaum, 1990). Independently, Birner and Neumann (1988) and Stillwell *et al.* (1987) reported that detection of the *o*-toluidine–Hb cleavage product was possible and suggested that measuring those Hb adducts would be useful in biomonitoring of *o*-toluidine exposure. In the present study analysis of the *in vivo* *o*-toluidine–Hb binding by HPLC showed linear increases with increasing i.p. doses of *o*-toluidine. However, for isolated albumin no cleaved *o*-toluidine was detected by HPLC/fluorescence after alkaline hydrolysis of albumin from timepoints later than 4 hr after dosing even though levels of bound *o*-[^{14}C]toluidine were high. Formation of a second albumin adduct by reaction with 4-amino-3-methylphenol could explain that observation. McMillan *et al.* (1990b) indicated that binding characteristics for metabolites of a related aromatic amine with Hb differ widely. The *in vitro* potency of the *N*-hydroxy compound, *N*-hydroxy-3,4-dichloroaniline, was reported to be an order of magnitude greater than that of the ring-hydroxylated compound, 6-hydroxy-3,4-dichloroaniline. Additional studies to define the *in vivo* binding characteristics for *o*-toluidine are planned, but the results of this study suggest that albumin may not be useful for routine biomonitoring. However, a ratio of the Hb to albumin adduct levels could be useful for evaluation of recent exposure. The HPLC method may not be adequate for differentiating between *o*-toluidine levels reported for smokers and nonsmokers (Stillwell *et al.*, 1987; Bryant *et al.*, 1988). However, it is anticipated that the procedure will prove to be valuable in the detection of either acute or chronic exposure to *o*-toluidine. In the current study *o*-toluidine–Hb adducts were quantifiable, and the sensitivity of this procedure can be increased significantly by concentrating extracts of the hydrolyzed Hb.

In summary, the results of this study show that alkaline hydrolysis of *o*-toluidine blood macromolecular adducts released a single cleavage product identified as the parent compound. HPLC analysis of this compound detected the cleavage product as many as 29 days after a single *o*-toluidine injection. In the rat the Hb biological half-life for adducted *o*-toluidine detectable by this procedure is far greater than that of albumin, suggesting that quantification of the Hb adducts may provide a more valuable index of cumulative long-term individual exposure.

REFERENCES

Alvarez-Coque, M. C. G., Hernández, M. J. M., Camañas, and Fernández, C. M. (1989). Formation and instability of *o*-phthalaldehyde derivatives of amino acids. *Anal. Biochem.* **178,** 1–7.

Amoore, J. E., and Hautala, E. (1983). Odor as an aid to chemical safety: Odor thresholds compared with threshold limit values and volatilities for 214 industrial chemicals in air and water dilution. *J. Appl. Toxicol.* **3,** 279–281.

Ashby, J. (1988). Comparison of techniques for monitoring human exposure to genotoxic chemicals. *Mutat. Res.* **204,** 543–551.

Beckett, A. H., and Belanger, P. M. (1976). Metabolic N-oxidation of secondary and primary amines as a route to ring hydroxylation, to various *n*-oxygenated products, and to dealkylation of secondary amines. *Biochem. Pharmacol.* **25,** 211–214.

Bergmark, E., Belew, M., and Osterman-Golkar, S. (1990). Separation and enrichment of alkylated globin chains as a means of improving the sensitivity of hemoglobin adduct measurements. *Acta Chem. Scand.* **44,** 630–635.

Birner, G., and Neumann, H.-G. (1988). Biomonitoring of aromatic amines. II. Hemoglobin binding of some monocyclic aromatic amines. *Arch. Toxicol.* **62,** 110–115.

Brock, W. J., Hundley, S. G., and Lieder, P. H. (1990). Hepatic macromolecular binding and tissue distribution of *ortho*- and *para*-toluidine in rats. *Toxicol. Lett.* **54,** 317–325.

Brookes, P., and Lawley, P. D. (1964). Reaction of some mutagenic and carcinogenic compounds with nucleic acids. *J. Cell. Comp. Physiol.* **64,** 111–128.

Bryant, M. S., Skipper, P. L., Tannenbaum, S. R., and Maclure, M. (1987). Hemoglobin adducts of 4-aminobiphenyl in smokers and nonsmokers. *Cancer Res.* **47,** 602–608.

Bryant, M. S., Vineis, P., Skipper, P. L., and Tannenbaum, S. R. (1988). Hemoglobin adducts of aromatic amines: Associations with smoking status and type of tobacco. *Proc. Natl. Acad. Sci. USA* **85,** 9788–9791.

Cheever, K. L., Richards, D. E., and Plotnick, H. B. (1980). Metabolism of *ortho*-, *meta*-, and *para*-toluidine in the adult male rat. *Toxicol. Appl. Pharmacol.* **56,** 361–369.

Cheever, K. L., Richards, D. E., Weigel, W. W., Begley, K. B., DeBord, D. G., Swearengin, T. F., and Savage, R. E., Jr. (1990). 4,4′-Methylene-bis(2-chloroaniline) [MOCA]: Comparison of macromolecular adduct formation after oral or dermal administration in the rat. *Fundam. Appl. Toxicol.* **14,** 273–283.

Cheever, K. L., Richards, D. E., Weigel, W. W., Begley, K. B., Savage, R. E., Jr., and Daniel, F. B. (1988). Macromolecular adduct formation by 4,4′-methylene-bis(2-chloroaniline) (MOCA) in the adult male rat. *Scand. J. Work Environ. Health* **14,** 57–59.

DeBord, G., Cheever, K., Weigel, W., Dankovic, D., Swearengin, T., Booth-Jones, A., Wissinger, L. (1991). Binding of *o*-toluidine to rat hemoglobin and albumin. *Proc. Am. Assoc. Cancer Res.* **32,** 97.

Ehrenberg, L., Hiesche, K. D., Osterman-Golkar, S., and Wennberg, I. (1974). Evaluation of genetic risks of alkylating agents: Tissue doses in the mouse from air contaminated with ethylene oxide. *Mutat. Res.* **24,** 83–103.

Ekman, B., and Strömbeck, J. P. (1947). Demonstration of tumorigenic decomposition products of 2,3′-azotoluene. *Acta Physiol. Scand.* **14,** 44–50.

Ekman, B., and Strömbeck, J. P. (1949). The effect of some split products of 2,3′-azotoluene on the urinary bladder in the rat and their excretion on various diets. *Acta Pathol. Microbiol. Scand.* **26,** 447–467.

El-Bayoumy, K., Donahue, J. M., Hecht, S. S., and Hoffmann, D. (1986). Identification and quantitative determination of aniline and toluidines in human urine. *Cancer Res.* **46,** 6064–6067.

Eyer, P., Lierheimer, E., and Strosar, M. (1983). Site and mechanism of covalent binding of 4-dimethylaminophenol to human hemoglobin, and its implications to the functional properties. *Mol. Pharmacol.* **23,** 282–290.

Fernandez, A., Sobel, C., and Goldenberg, H. (1966). An improved method of determination of serum albumin and globulin. *Clin. Chem.* **12,** 194–205.

Ginsberg, G. L., and Atherholt, T. B. (1989). Transport of DNA-adducting metabolites in mouse serum following benzo[*a*]pyrene administration. *Carcinogenesis (London)* **10,** 673–679.

Gupta, R. L., Gupta, A. K., Pathak, D. P., and Juneja, T. R. (1987). Mutagenic studies of *ortho*-toluidine and its potential metabolites. *Ind. J. Exp. Biol.* **25,** 618–622.

Hammerbacher, F. (1883). Ueber die Bildung von Aetherschwefelsäuren. *Pfugers Arch. Gesamte Physiol. Menschen Tiere* **33,** 94–102.

Hecht, S. S., El-Bayoumi, K., Tulley, L., and LaVole, E. (1979). Structure-mutagenicity relationships of *N*-oxidized derivatives of aniline, *o*-toluidine, 2′-methyl-4-aminobiphenyl, and 3,2′-dimethyl-4-aminobiphenyl. *J. Med. Chem.* **22,** 981–987.

Hendry, W. F., Blandy, J. P., Glashan, R. W., Hall, R. R., and Wallace, D. M. A. (1988). Occupational bladder cancer: A guide for clinicians. *Br. J. Urol.* **61,** 183–191.

Hildebrandt, H. (1903). Über das Verhalten halogensubstituierter Toluole und der Amidobenzosauren im Organismus. *Beitr. Chem. Physiol. Pathol.* **3,** 364–372.

Horrocks, D. L. (1974). *Applications of Liquid Scintillation Counting,* pp. 72–74. Academic Press, New York.

IARC (1978). *Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man: Some Aromatic Amines and Related Nitro Compounds—Hair Dyes, Coloring Agents and Miscellaneous Industrial Chemicals,* Vol. 16, pp. 349–366. WHO, IARC, Lyon.

IARC (1982). *Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man: Some Aromatic Amines, Anthraquinones and Nitroso Compounds, and Inorganic Fluorides Used in Drinking-Water and Dental Preparations,* Vol. 27, pp. 155–175. WHO, IARC, Lyon.

IARC (1987). *Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man: Overall Evaluation of Carcinogenicity: An Updating of IARC Monographs,* Vols. 1 to 42, Suppl. 7, pp. 362–363. WHO, IARC, Lyon.

Jacobson, M. D., Shapiro, R., Underwood, G. R., Broyde, S., Verna, L., and Hingerty, B. E. (1988). Synthesis and conformation of a dinucleoside monophosphate modified by aniline. *Chem. Res. Toxicol.* **1,** 152–159.

Kadlubar, F. F., Miller, J. A., and Miller, E. C. (1976). Microsomal *N*-oxidation of the hepatocarcinogen *N*-methyl-4-aminoazobenzene and the reactivity of *N*-hydroxy-*N*-methyl-4-aminoazobenzene. *Cancer Res.* **36,** 1196–1208.

Kiese, M. (1966). The biochemical production of ferrihemoglobin forming derivatives from aromatic amines, and mechanisms of ferrihemoglobin formation. *Pharmacol. Rev.* **18,** 1091–1161.

Kriek, E., and Westra, J. G. (1979). Metabolic activation of aromatic amines and amides and interactions with nucleic acids. In *Chemical Carcinogens and DNA* (P. L. Grover, Ed.), Vol. 2, pp. 1–28. CRC Press, Boca Raton, FL.

Kuchenbecker, A. (1920). Über den Nachweis aromatischer Amidoverbindungen im Harn. *Zentralbl. Gewerbehyg.* **8,** 68–72.

Kugler-Steigmeier, M. E., Friederich, U., Lutz, W. K., Maier, P., and Schlatter, C. (1989). Genotoxicity of aniline derivatives in various short-term tests. *Mutat. Res.* **211,** 279–289.

Lutz, W. K. (1979). In vitro covalent binding of organic chemicals to DNA as a quantitative indicator in the process of chemical carcinogenesis. *Mutat. Res.* **65,** 289–356.

McManus, M. E. (1989). The role of cytochromes P-450 and *N*-acetyl transferase in the carcinogenicity of aromatic amines and amides. *Clin. Exp. Pharmacol. Physiol.* **16,** 491–495.

McMillan, D. C., Freeman, J. P., and Hinson, J. A. (1990a). Metabolism of the arylamide herbicide propanil. I. Microsomal metabolism and *in vitro* methemoglobinemia. *Toxicol. Appl. Pharmacol.* **103,** 90–101.

McMillan, D. C., McRae, T. A., and Hinson, J. A. (1990b). Propanil-induced methemoglobinemia and hemoglobin binding in the rat. *Toxicol. Appl. Pharmacol.* **105,** 503–507.

Mori, H., Yoshimi, N., Sugie, S., Iwata, H., Kawai, K., Mashizu, N., and Shimizu, H. (1988). Genotoxicity of epoxy resin hardeners in the hepatocyte primary culture/DNA repair test. *Mutat. Res.* **204,** 683–688.

Morigami, S., and Nisimura, I. (1940). Experimental studies on aniline bladder tumors. *Gann* **34,** 146–147.

Morton, K. C., Mei-Sie, L., Siedlik, P., and Chapman, R. (1988). Metabolism of 4,4′-methylene-bis-2-chloroaniline (MOCA) by rats *in vivo* and formation of *N*-hydroxy MOCA by rat and human microsomes. *Carcinogenesis (London)* **9,** 731–739.

NCI, National Cancer Institute (1979). *Bioassay of o-Toluidine Hydrochloride for Possible Carcinogenicity,* DHEW Publication No. (NIH) 79-1709. U.S. Government Printing Office, Washington DC.

Neumann, H.-G. (1984). Analysis of hemoglobin as a dose monitor for alkylating and arylating agents. *Arch. Toxicol.* **56,** 1–6.

Neumann, H.-G. (1988). Biomonitoring of aromatic amines and alkylating agents by measuring hemoglobin adducts. *Int. Arch. Occup. Environ. Health* **60,** 151–155.

NIOSH, National Institute for Occupational Safety and Health (1991). *Preventing Bladder Cancer from Exposure to o-Toluidine and Aniline,* DHHS (NIOSH) Publication No. 90-116. Cincinnati, OH.

Osterman-Golkar, S., Ehrenberg, L., Segerbäck, D., and Hällström, I. (1976). Evaluation of genetic risks of alkylating agents. II. Haemoglobin as a dose monitor. *Mutat. Res.* **34,** 1–10.

Perera, F. P. (1987). The significance of DNA and protein adducts in human biomonitoring studies. *Mutat. Res.* **205,** 255–269.

Rehn, L. (1895). Blasengeschwulste bei Fuchsin Arbeitern. *Arch. Klin. Chir.* **50,** 588.

Reiter, M., and Leusser, K. (1952). Einfluß der Methylsubstitution auf die Hämiglobin- (Methämoglobin-) Bildung und die Allgemeintoxizität des Anilins. *Arch. Exp. Pathol. Pharmakol.* **214,** 158–164.

Ringe, D., Turesky, R. J., Skipper, P. L., and Tannenbaum, S. R. (1988). Structure of the single stable hemoglobin adduct formed by 4-aminobiphenyl in vivo. *Chem. Res. Toxicol.* **1,** 22–24.

Rosenkranz, H. S., and Poirier, L. A. (1979). Evaluation of the mutagenicity and DNA-modifying activity of carcinogens and noncarcinogens in microbial systems. *J. Natl. Cancer Inst.* **62,** 873–892.

Rubino, G. F., Scansetti, G., Piolatto, G., and Pira, E. (1982). The carcinogenic effect of aromatic amines: An epidemiological study on the role of *o*-toluidine and 4,4′-methylene bis(2-methylaniline) in inducing bladder cancer in man. *Environ. Res.* **27,** 241–254.

Rumack, B. H., and Lovejoy, F. H., Jr. (1986). Clinical toxicology. In *Toxicology: The Basic Science of Poisons* (C. D. Klaassen, M. O. Amdur, and J. D. Doull, Eds.), third ed., pp. 879–881. MacMillan Co., New York.

Russfield, A. B., Homburger, F., Weisburger, E. K., and Weisburger, J. H. (1973). Further studies on carcinogenicity of environmental chemicals including simple aromatic amines. *Toxicol. Appl. Pharmacol.* **25,** 446.

Sabbioni, G., and Neumann, H.-G. (1990). Quantification of haemoglobin binding of 4,4′-methylenebis(2-chloroaniline) (MOCA) in rats. *Arch. Toxicol.* **64,** 451–458.

Schalm, O. W., Jain, N. C., and Carroll, E. J. (1975). Erythrocytes: Their production, function and destruction. In *Veterinary Hematology.* 3rd Edition, pp. 356–404. Lea and Febiger, Philadelphia, PA.

Segerbäck, D. (1983). Alkylation of DNA and hemoglobin in the mouse following exposure to ethene and ethene oxide. *Chem. Biol. Interact.* **45,** 139–151.

Segerbäck, D., Kadlubar, F. F., and Talaska, G. (1989). Macromolecular adducts of 4,4′-methylene-bis(2-chloroaniline) [MOCA] and their application to biomonitoring. *Proc. Am. Assoc. Cancer Res.* **30,** 120.

Silk, N. A., Lay, J. O., Jr., and Martin, C. N. (1989). Covalent binding of 4,4′-methylenebis(2-chloroaniline) (MOCA) to rat liver DNA *in vivo* and of its N-hydroxylated derivative to DNA *in vitro. Biochem. Pharmacol.* **38,** 279–287.

Skipper, P. L., and Tannenbaum, S. R. (1990). Protein adducts in the molecular dosimetry of chemical carcinogens. *Carcinogenesis* **11,** 507–518.

Smyth, H. F. (1931). The toxicity of certain benzene derivatives and related compounds. *J. Ind. Hyg.* **13,** 87–96.

Son, O. S., Everett, D. W., and Fiala, E. S. (1980). Metabolism of *o*-[*methyl*-^{14}C]toluidine in the F344 rat. *Xenobiotica* **10,** 457–468.

Stillwell, W. G., Bryant, M. S., and Wishnok, J. S. (1987). GC/MS analysis of biological important aromatic amines. Application to human dosimetry. *Biomed. Environ. Mass Spectrom.* **14,** 221–227.

Tanaka, K.-I., Marui, S., and Toshiko, M. (1980). Mutagenicity of extracts of urine from rats treated with aromatic amines. *Mutat. Res.* **79,** 173–176.

Trippel-Schulte, P., Zeiske, J., and Kettrup, A. (1986). Trace analysis of selected benzidine and diaminodiphenylmethane derivatives in urine by means of liquid chromatography using precolumn sample preconcentration, UV and electrochemical detection. *Chromatographica* **22,** 138–146.

U.S. EPA, U.S. Environmental Protection Agency (1984). *Health and Environmental Effects Profile for Toluidines,* U.S. EPA Publication No. 600/X-84/151. Washington, DC.

Ward, E., Carpenter, A., Markowitz, S., Roberts, D., and Halprin (1991). Excess bladder cancer in workers exposed to ortho-toluidine and aniline. *J. Natl. Cancer Inst.* **83,** 501–506.

Weigel, W. W., Plotnick, H. B., and Conner, W. L. (1978). Tissue distribution and excretion of ^{14}C-epichlorohydrin in male and female rats. *Res. Commun. Chem. Pathol. Pharmacol.* **20,** 275–287.

Weisburger, J. H., Grantham, P. H., and Weisburger, E. K. (1969). The transport of chemical carcinogens by blood: Metabolites of *N*-2-fluorenylacetamide and *N*-hydroxy-*N*-2-fluorenylacetamide as a function of time. In *Physico-Chemical Mechanisms of Carcinogenesis* (E. D. Bergmann and B. Pullman, Eds.), Proc. Israel Acid. Sci. Humanities, pp. 262–283.

Weisburger, E. K., Russfield, A. B., Homburger, F., Weisburger, J. H., Boger, E., Van Dongen, C. G., and Chu, K. C. (1978). Testing of twenty-one environmental aromatic amines or derivatives for long-term toxicity or carcinogenicity. *J. Environ. Pathol. Toxicol.* **2,** 325–356.

Weisburger, J. H., and Weisburger, E. K. (1973). Biochemical formation and pharmacological, toxicological, and pathological properties of hydroxylamines and hydroxamic acids. *Pharmacol. Rev.* **25,** 1–66.

Williams, R. T. (1947). *Detoxication Mechanisms,* p. 138. Chapman Hall, London.

Wood, G. O., and Anderson, R. G. (1975). Personal air sampling for vapors of aniline compounds. *Am. Ind. Hygiene Assoc. J.* **25,** 538–548.

Zimmer, D., Mazurek, J., Petzold, G., and Bhuyan, B. K. (1980). Bacterial mutagenicity and mammalian cell DNA damage by several substituted anilines. *Mutat. Res.* **77,** 317–326.