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4,4′-Methylenebis(2-chloroaniline) (MOCA): The Effect of Multiple Oral Administration, Route, and Phenobarbital Induction on Macromolecular Adduct Formation in the Rat

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4,4′-Methylenebis(2-chloroaniline) (MOCA): The Effect of Multiple Oral Administration, Route, and Phenobarbital Induction on Macromolecular Adduct Formation in the Rat. CHEEVER, K. L., DEBORD, D. G., AND SWEARENGIN, T. F. (1991). *Fundam. Appl. Toxicol.* **16,** 71–80. The effect of multiple oral administration of MOCA, a suspect human carcinogen, was studied in the adult male rat. As many as 28 consecutive daily doses of [^{14}C]MOCA at 28.1 μmol/kg body wt (5 μCi/day) were administered and rats were euthanized at weekly intervals for 7 weeks. MOCA adduct formation for globin and serum albumin was evaluated by determination of [^{14}C]MOCA covalent binding. The covalent binding associated with globin showed a linear increase over the 28-day exposure period with 342 fmol/mg globin 24 hr after the final dose. More extensive covalent binding was detected for albumin with 443 fmol/mg albumin after the final dose, but increases were not linear. After cessation of dosing, the albumin adduct levels decreased rapidly ($t_{1/2}$ = 4.6 days) in relation to globin adduct levels ($t_{1/2}$ = 16.1 days). The MOCA–globin adduct $t_{1/2}$ is consistent with that determined after a single 281 μmol/kg oral dose of MOCA. Significant differences related to route of administration were detected for 24-hr globin covalent binding with ip > po > dermal. Distribution of undifferentiated [^{14}C]MOCA was highest in the liver at 24 hr with tissue levels for liver > kidney > lung > spleen > testes > urinary bladder. Induction of cytochrome P450 enzymes by administration of phenobarbital (100 mg/kg/day/3 days) resulted in a significant ($p < 0.05$) increase in MOCA–globin adduct formation detected with 33.5 pmol/mg globin for induced rats versus 13.6 pmol/mg globin for control rats. Although MOCA–globin and albumin adducts show differing stability, quantification of such MOCA adducts may be useful for long-term industrial biomonitoring of MOCA.

The curing agent 4,4′-methylenebis(2-chloraniline) (MOCA; 3,3′-dichloro-4,4′-methylenedianiline; CAS Registry No. 101-14-4) has been widely used in the plastics industry (Henning, 1973; NIOSH, 1977, 1978; Versar, Inc., 1982). MOCA is carcinogenic in a variety of laboratory animals (Russfield *et al.,* 1975; Stula *et al.,* 1975, 1977; Kommineni *et al.,* 1979) and mutagenic in *in vitro* tests (Hesbert *et al.,* 1985; Mori *et al.,* 1988; Kugler-Steigmeier *et al.,* 1989; Benigni *et al.,* 1989; Wu *et al.,* 1989). Although human MOCA-related carcinogenicity is not certain (Henning, 1973; Hosein and Van Roosmalen, 1978), MOCA is considered a suspect human carcinogen (Bertazzi, 1975; Ward and Halperin, 1987; Hendry *et al.,* 1988). The mechanism of the chemical carcinogenesis is thought to involve the formation of chemical adducts in genetic material through covalent binding of nucleophilic sites by electrophilic compounds or activated metabolites (Kriek and Westra, 1979; Mori *et al.,* 1988). Although the relationship of covalent binding to protein macromolecules to that of target organ DNA is not fully understood, a correlation between such binding

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and carcinogenic potency has been proposed (Brooks and Lawley, 1964; Lutz, 1979; Kugler-Steigmeier *et al.*, 1989). Biomonitoring techniques used for determination of MOCA exposure have previously relied upon determination of urinary MOCA or MOCA metabolite levels (Nieminen *et al.*, 1983; Skarping and Renman, 1983; Gristwood *et al.*, 1984; Groth *et al.*, 1984; Thomas and Wilson, 1984; Castegnaro *et al.*, 1985; Ducos *et al.*, 1985; Trippel-Shulte *et al.*, 1986; Cocker *et al.*, 1990), procedures which provide little information on bladder or liver target organ exposure (Fig. 1). More recently, the transport of DNA-adducting metabolites by blood has been reported for certain carcinogens (Ginsberg and Atherholt, 1989), and molecular dosimetry using blood proteins has been proposed for biomonitoring. Work on the formation of adducts with hemoglobin (Hb) (Segerback *et al.*, 1989; Cheever *et al.*, 1990) or tissue DNA (Silk *et al.*, 1989; Cheever *et al.*, 1990) by MOCA suggested that the quantification of blood protein or DNA adducts may be of value in risk assessment by allowing a more accurate estimation of the cumulative bladder or liver target dose. Additionally, recent work (Morton *et al.*, 1988; Butler *et al.*, 1989) indicates that activation of MOCA may be susceptible to chemical induction.

The objective of this study was to quantitate tissue macromolecular adduct formation after induction of drug metabolizing enzymes or long-term oral administration of MOCA, a model aromatic amine, in the rat. The availability of such techniques may allow use of adducts as time–integral indices for the evaluation of worker exposure and risk assessment.

METHODS

Chemicals and solutions. The test compound, MOCA, was provided by the Anderson Development Co. (Adrian, MI).[1] The MOCA was recrystallized from methanol–water and dried over anhydrous H_2SO_4 prior to analysis for chemical purity (>99.5%) by high-pressure liquid chromatography (HPLC) as described previously (Cheever *et al.*, 1990). [*Methylene*-^{14}C]MOCA, having a specific activity of approximately 50 mCi/mmol and a radiochemical purity of greater than 99%, was obtained from Chemsyn Science Laboratories (Lenexa, KS). The radiochemical purity of this compound was verified by HPLC in conjunction with a radioactivity detector prior to use. The reference compound, *N*-hydroxy-4,4'-3,3'-dichlorodiphenylmethane (*N*-hydroxy-MOCA, 95%), was obtained from Chemsyn Science Laboratories.

A dosing solution was prepared for the long-term oral studies by dissolving appropriate amounts of MOCA and [^{14}C]MOCA in corn oil to give a final concentration of

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

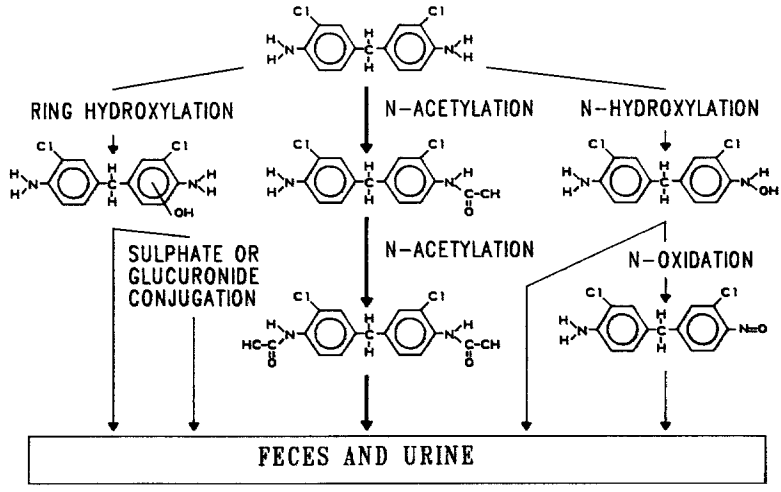

FIG. 1. Proposed scheme of 4,4'-Methylenebis(2-chloroaniline) (MOCA) metabolism and excretion.

5.6 μmol/ml [sp act, 8.5 μCi/ml]. A second dosing solution was prepared for comparison of oral and ip administration by dissolving MOCA and [^{14}C]MOCA in corn oil to give a final concentration of 56 μmol/ml [sp act, 84.8 μCi/ml]. Additional unlabeled dosing solutions were prepared by dissolving appropriate amounts of MOCA in corn oil to give final concentrations of 746.7 and 44.8 μmol/ml.

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-acredited 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 were controlled throughout the course of the studies. Animals were assigned to 13 groups of five rats and provided with NIH-07 rat and mouse diet (Ziegler Brothers, Inc., Gardners, PA) and tap water *ad libitum*. For the induction studies, rats were injected with sodium phenobarbital (PB) at 100 mg/kg body wt daily for 3 days prior to dosing with MOCA. The animals used for the long-term study weighed 190–220 g at initiation of dosing and were administered [^{14}C]MOCA at 28.1 μmol/kg body wt daily for up to 28 days either by gavage at a constant 5-ml volume of dosing solution/kg body wt. Animals used for comparison of route of administration or induction were 45 days old on the day of treatment, and the average administered radioactivity in the 280 μmol/kg body wt oral and ip doses was 80 μCi/rat. Rats within a specified time group were anesthetized with pentobarbital and terminated by exsanguination weekly during the 4-week treatment period and during a 3-week recovery period for the long-term study or 24 hr after a single MOCA dose. Blood was obtained by cardiac puncture, and tissues were immediately frozen in liquid nitrogen and stored at −70°C prior to analysis.

Isolation of globin and albumin. Blood collected after treatment with MOCA was transferred immediately to EDTA-containing Vacutainers. A 3-ml aliquot of the whole blood was mixed at room temperature with 5 ml of phosphate-buffered saline (PBS), pH 7.4 and carefully layered onto 3 ml of Histopaque-1077 (Sigma Chemical Co., St. Louis, MO). The lymphocytes and serum were separated by centrifugation at 400*g* for 30 min. Following this, lymphocytes were transferred to a clean centrifuge tube, washed, with PBS, and stored at −70°C until time of analysis. After removal of the lymphocytes, the packed erythrocytes (RBCs) were washed three times with 10 ml of ice-cold PBS and repacked by centrifugation at 400*g* for 30 min. The cells were then lysed by addition of 20 ml of ice-cold distilled water, and the hemolysate was centrifuged at 25,000*g* for 25 min at 4°C to eliminate cellular debris. Globin was isolated by the dropwise addition of 10 ml of hemolysate to 50 ml of ice-cold 1% HCl in acetone. After standing, the globin precipitate was removed and dried under vacuum as described previously (Cheever *et al.*, 1990). Serum albumin was isolated after removal of the globulins. Globulins were precipitated by the addition of 9 ml of serum to 100 ml of 0.2% HCl in absolute ethanol. The mixture was stirred during addition and maintained at 37°C throughout a 30-min incubation period as described by Fernandez *et al.* (1966). After centrifugation at 400*g* for 30 min, the soluble albumin fraction was decanted and dried under vacuum. Proteins were further processed for evaluation of covalent binding as described by Martin and Gardner (1987). The globin and albumin powders were dried under a stream of nitrogen and weighed, and the bound ^{14}C was quantified by liquid scintillation spectrometry (LSC). Globin and albumin isolated from rats dosed with unlabeled MOCA at 3734 μmol/kg body wt were subjected to acid hydrolysis to allow analysis of adducted MOCA using a modification of the procedure of Shugart and Matsunami (1985). Weighed amounts of the proteins were dissolved in 5 ml of water and 50 μl of 12 N HCl was added. After mixing, the solutions were incubated in sealed glass vials at 80°C for 4 hr to release any MOCA–protein adducts. Following the acid-induced hydrolysis of proteins, the mixtures were continuously extracted with methylene dichloride for 24 hr. Similar hydrolysis and extraction of proteins treated with radiolabeling showed that approximately 40% of the radioactivity was extracted. The organic phase was dried over sodium sulfate and concentrated under a stream of nitrogen for subsequent analysis by thin-layer chromatography (TLC) and HPLC.

MOCA tissue distribution and covalent binding. In a separate experiment, the levels of undifferentiated radioactivity, representing [^{14}C]MOCA and MOCA metabolites, were determined for certain tissues (liver, lung, kidney, lymphocyte, brain, kidney, testis, spleen, and urinary bladder) for five rats 24 hr after administration of [^{14}C]MOCA at 28.1 μmol/kg body wt by gavage. The rats were anesthetized with pentobarbital and terminated by exsanguination. The tissues were immediately weighed and homogenized with 2 vol of cold 0.1 M Tris/0.01 M EDTA (pH 7.7) using a Polytron PCU-1 homogenizer equipped with a PC-10 probe (Kinematica GmbH, Luzern, Switzerland). Aliquots of tissue homogenates were digested and ^{14}C levels were analyzed by LSC. Covalent binding levels for [^{14}C]MOCA were subsequently determined for these tissues as previously described.

Thin-layer chromatography. Solvent extracts of acid-hydrolyzed vehicle control globin and albumin or extracts of those proteins isolated from rats treated with MOCA were cochromatographed with [^{14}C]MOCA or *N*-hydroxy-MOCA on silica gel-precoated mylar-backed plates (E. Merck 60F 254, 0.2-mm layer thickness). Chromatograms were developed with either benzene:ethyl acetate (9:1 v/v) or toluene:ethyl alcohol (20:1 v/v). The latter solvent system was used in the examination of extracts of acid-hydrolyzed proteins for the presence of any freed MOCA or *N*-hydroxy MOCA. Spots corresponding to possible MOCA, MOCA metabolites, or reference compounds were located by observing the quenching of fluorescence activated by 254 nm radiation and by color re-

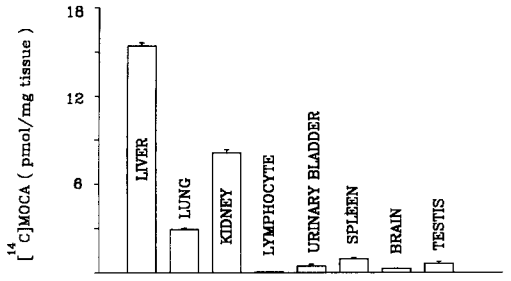

FIG. 2. MOCA tissue distribution. Comparison of undifferentiated ^{14}C organ and blood cell levels for rats terminated 24 hr after oral administration of a single 281 μmol/kg dose of 4,4′-methylenebis(2-chloroaniline) (MOCA). Values are means ± SE, $n = 5$.

actions with ninhydrin. For experiments conducted using radioactivity, 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 were scraped and eluted with methanol. Compounds isolated in this manner were further evaluated by HPLC.

Liquid chromatography. MOCA 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, FRG). The HPLC column temperature was controlled at 40°C. Components were separated on a 25-cm-long × 7.6-mm-i.d. stainless-steel column packed with 5 μm LC-C18-DB silica packing (Supelco, Inc., Bellefonte, PA). 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 and quantified using a Model 1040M uv diode array detector and a Model 1046A programmable fluorescence detector (Hewlett–Packard Co.), at 254 nm or ex = 280 nm, em = 391 wavelength settings, respectively. Radioactive components were determined using a Trace II Model 7150 radioactivity monitor (Packard Instruments, Downers Grove, IL). Subsequently radioactive or unlabeled components were separated isocratically using 70% methanol in 0.05 M ammonium formate for acquisition and 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.).

Liquid scintillation spectrometry. For determination of tissue ^{14}C levels, sample aliquots (0.01 to 0.5 ml) were solubilized in triplicate by incubation for 24 hr at 60°C with 0.25 ml of methanolic 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. Data were processed using the HP 98820A Statistical Library Revision B (Hewlett–Packard Co., Fort Collins, CO) installed on the HP 9000 Model 310 HPLC ChemStation. A probability level of $p < 0.05$ was considered significant. Linear regression lines were determined and fit through the data for each plot using Sigma-Plot Version 3.1 (Jandel Scientific, Sausalito, CA). Biological half-lives were calculated by the method of Rumack and Lovejoy (1986).

RESULTS

Tissue distribution of [^{14}C]MOCA. The radioactivity present in tissues, 24 hr after a single po injection of 281 μmol/kg [^{14}C]MOCA, was highest in the rat liver with 15.3 pmol/mg liver. The kidney concentration was second highest with 7.6 pmol/mg kidney, approximately half that found in liver. Levels of the radioactivity present in the tissues tested were liver > kidney > lung > spleen > urinary bladder > testis > brain > lymphocyte (Fig. 2). These values could represent unadducted MOCA or MOCA metabolites. However, previous work has shown that the radioactivity present in liver or various liver subcellular fractions 24 hr after administration of [^{14}C]MOCA is approximately 69% bound (Cheever *et al.*, 1990).

Effect of route or phenobarbital induction on in vivo covalent binding. The covalent binding of [^{14}C]MOCA was evaluated for liver, whole blood, and isolated globin 24 hr after administration of a single 281 μmol/kg dose of MOCA. The compound was administered to groups of rats by either the po or ip route. A third group was pretreated with PB (100 mg/kg/day/3 days) to test for induction of enzymatic activation and given MOCA (281 μmol/kg body wt ip) 24 hr after the third dose of PB. The radioactivity present in the globin, a potentially useful marker for MOCA exposure, was 13.6 pmol/mg blood for the ip exposures and 7.9 pmol/mg globin for the oral

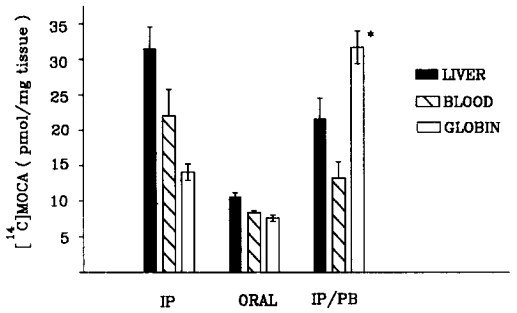

FIG. 3. MOCA covalent binding. Comparison of bound ^{14}C levels determined for liver, blood, and isolated globin in rats (with or without phenobarbital pretreatment 100 mg/kg/3 day ip). Rats were terminated 24 hr after oral administration of a single 281 μmol/kg dose of [^{14}C]MOCA. Values are means ± SE, $n = 5$. *Statistically greater than corresponding globin binding levels for naive rats ($p < 0.05$).

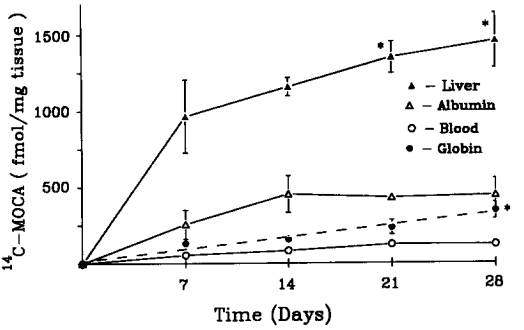

FIG. 4. MOCA covalent binding. Solid lines show accumulation of undifferentiated ^{14}C in liver (▲) or whole blood (○), and dotted lines show covalently bound ^{14}C for globin (●) or albumin (△). Rats were terminated after as many as 28 consecutive 28.1 μmol/kg daily doses of [^{14}C]MOCA. Values are means ± SE, $n = 5$. *Statistically greater than detected in corresponding tissues after 1 week of continuous dosing ($p < 0.05$).

exposures at termination. The covalently bound radioactivity present in both blood and liver was greater after ip than after po administration. A statistically significant ($p < 0.05$) increase in the ^{14}C binding, 33.5 pmol/mg globin, was noted for PB-pretreated animals on comparison with unpretreated animals (Fig. 3).

Multiple oral administration of [^{14}-C]-MOCA. The effect of long-term dosing of MOCA on accumulation of [^{14}C]MOCA in the liver and blood or the covalent binding levels in globin and albumin was evaluated in the rat. The radioactivity present in the livers of animals given as many as 28 consecutive oral doses of 28.1 μmol/kg body wt was determined. The radioactivity appeared to accumulate more rapidly in liver than in the blood with 1455 fmol/mg liver and 122 fmol/mg blood detectable after 29 days. Covalent binding associated with the globin showed a linear increase over the 28-day exposure with as much as 342 fmol/mg globin appearing 24 hr after the final MOCA dose. More extensive covalent binding was detected for albumin (443 fmol/mg albumin), an additional protein biomarker (Segerback *et al.*, 1989) after the final dose, but the increases were not linear with time (Fig. 4). After cessation of the 28-day MOCA dosing period the disappearance of ^{14}C present in liver, whole blood, globin, and albumin was monitored for an additional 21 days. The albumin binding levels decreased rapidly ($t_{1/2} = 4.6$ days) in relation to globin levels ($t_{1/2} = 16.1$ days), but was still detectable 21 days after the last dose of MOCA (16.7 fmol/mg albumin). Significantly higher globin binding levels, 131.6 fmol/mg globin, were detected at that timepoint (Fig. 5). The biological half-lives for the bound ^{14}C in tissues

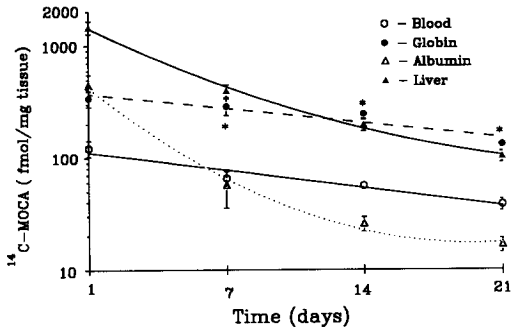

FIG. 5. Solid lines show elimination of undifferentiated ^{14}C from liver (▲) or whole blood (○), and dotted lines show covalently bound ^{14}C for globin (●) or albumin (△). Rats were terminated at 7-day intervals after administration of 28 consecutive 28.1 μmol/kg daily doses of [^{14}C]MOCA. Values are means ± SE, $n = 5$. *Statistically greater than corresponding albumin binding levels ($p < 0.05$).

tested after long-term MOCA dosing were globin > blood > liver > albumin (Table 1).

TLC and HPLC analysis of globin and albumin adduct cheavage products. Initial experiments showed that the radioactivity associated with the globin precipitate was not released upon repeated aqueous and organic washes. However, mild acid hydrolysis followed by extraction with methylene dichloride resulted in the liberation of radioactivity. After concentration, the extracted material was separated by TLC and a single radiolabeled component was detected by autoradiography. After isolation of this component, subsequent HPLC analysis revealed a single radiolabeled peak with a retention time consistent with that of MOCA. Further, to show that detection of nonradiolabeled material associated with this peak was possible by HPLC coupled with uv or fluorescence spectrophotometry detection after acid hydrolysis of protein, a group of rats was treated with MOCA at 3734 μmol/kg body wt, a relatively high dosing level. After isolation and acid hydrolysis of globin or albumin, the identity of the single major peak was confirmed by comparison with authentic MOCA TLC R_f value and ninhydrin color reaction (Table 2) as well as by HPLC R_t and library spectra (Fig. 6). An HPLC uv standard curve was prepared for MOCA, and the MOCA adduct cleavage product was quantified (Table 3).

TABLE 1

BIOLOGICAL HALF-LIVES OF 4,4'-METHYLENEBIS-(2-CHLOROANILINE) (MOCA)

Tissue	Biological half-life (days)[a] oral administration
Globin	16.1
Albumin	4.6
Whole blood	13.3
Liver	5.5

[a] Comparison of ^{14}C biological half-lives calculated for ^{14}C-labeled liver or whole blood and for covalently bound ^{14}C-labeled globin or albumin in rats terminated after cessation of 28-day MOCA administration at 28.1 μmol/kg. Biological half-lives were calculated by the method of Rumack and Lovejoy (1986).

TABLE 2

IDENTIFICATION OF ACID-HYDROLYZABLE 4,4'-METHYLENEBIS(2-CHLOROANILINE) (MOCA) ADDUCTS OF GLOBIN OR ALBUMIN

Compound	RT[a]	R_f[b]	Color reaction
Unknown	20.595	0.589	P----Br[c,d]
Authentic MOCA standard	20.537	0.585	P----Br

[a] HPLC retention time (min). Cleavage products isolated from hydrolyzed protein were separated isocratically on a reverse phase column using 70% methanol in 0.05 M ammonium formate.

[b] 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.

[c] 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.

[d] P, purple; Br, brown; ---- indicates observation after 24 hr.

DISCUSSION

Carcinogenicity associated with aromatic amines has been recognized for nearly a century (Rehn, 1895). MOCA is a substituted model aromatic amine which has been reported to induce liver hepatocellular carcinomas, mammary carcinomas, lung adenocarcinomas, and zymbal gland tumors in rats and urinary bladder tumors in dogs, following administration by the oral route (NIOSH, 1978). The results of the current study indicate that acute exposure of rats to MOCA at 281 μmol/kg body wt by both oral and ip routes or long-term exposure at 28.1 μmol/kg/day by the oral route resulted in significant covalent binding with blood proteins. Extensive evidence of genetic effects of MOCA has been accumulated (Ward *et al.*, 1987). MOCA has been shown to produce gene mutations *in vitro* using the *Salmonella typhimurium* test system with rat liver S9 mix (Messelson and Russell, 1977; Hesbert *et al.*, 1985; Wu *et al.*, 1989; Kugler-Steigmeier *et al.*, 1989). The *in vitro* genotoxicity of MOCA has also been reported by Mori *et al.* (1988) who used rat hepatocytes

to evaluate DNA repair. Additionally, recent experiments have detected the formation of MOCA–DNA adducts by rat liver *in vitro* (Silk *et al.*, 1989; Kugler-Steigmeier *et al.*, 1989) or *in vivo* (Silk *et al.*, 1989; Segerback *et al.*, 1989; Cheever *et al.*, 1990). Similarly, MOCA–DNA covalent binding has been investigated for both dog (Shivapurkar *et al.*, 1987; Segerback *et al.*, 1989) and human bladder cells (Shivapurkar *et al.*, 1987). Protein adduct formation by MOCA was recently reported for rat globin and serum albumin by Segerback and his co-workers (1989). These investigators estimated that the percentage of a single MOCA dose bound to blood proteins in the rat was 0.05% to globin and 0.5% to albumin, but they did not specify the dosing level. In contrast to the single ring major adduct reported for DNA (Silk *et al.*, 1989), Segerback indicated that for proteins the bound material involves an intact MOCA ring system, a finding consistent with the postulated reaction of protein with N-hydroxylated MOCA metabolites. Segerback and his co-workers suggested that MOCA–albumin adducts would be useful in biomonitoring of MOCA exposure.

In the present study the 28-day *in vivo* MOCA–albumin binding was significantly higher than the MOCA–globin binding after repeated oral administration of the compound. However, the rate of accumulation of globin adducts was linear, whereas the formation of MOCA–albumin adducts appeared to have reached a plateau after 14 days of exposure. The formation of MOCA–globin adducts appears to be 7- to 12-fold lower after daily administration of 28.1 μmol/kg doses for 10 days than after a single 281 μmol/kg dose of MOCA. The biological half-life for those ad-

TABLE 3

COMPARISON OF GLOBIN OR ALBUMIN 4,4′-METHYLENEBIS(2-CHLOROANILINE) (MOCA) ACID-HYDROLYZABLE ADDUCTS

Tissue	ng MOCA/mg tissue[a]	Percentage of dose
Globin	33.40	1.1×10^{-6}
Albumin	15.54	1.2×10^{-6}

[a] Rats were terminated 24 hr after oral administration of a single 3734 μmol/kg dose of unlabeled MOCA and covalent adducts liberated by mild acid hydrolysis. The cleavage product, MOCA, was quantified by HPLC uv spectroscopy using a reverse phase column. Components were separated isocratically using 70% methanol in 0.05 M ammonium formate.

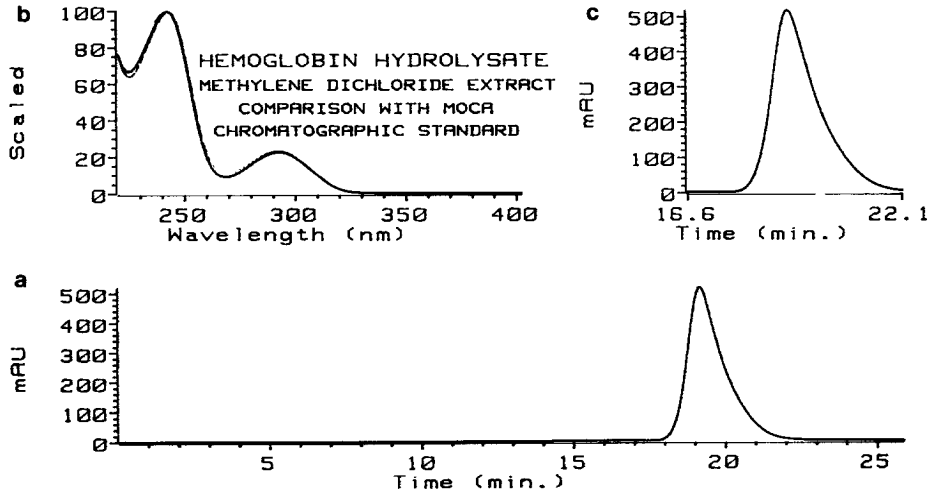

FIG. 6. (a) HPLC chromatogram showing peak for cleavage product after mild acid hydrolysis of MOCA–hemoglobin adduct. Rats were terminated 24 hr after oral administration of a single 3734 μmol/kg dose of unlabeled MOCA. The uv spectrum of the single major peak is shown in (b) and is identical with the overlay of a reference MOCA library spectrum acquired under the same chromatographic conditions. The retention time for this peak, shown enlarged in (c), is consistent with authentic MOCA.

ducts, however, formed after continuous 28-day exposure (28.1 μmol/kg/day) is consistent with that reported previously for a single MOCA dose at 281 μmol/kg body wt (Cheever *et al.*, 1990). Significant route-related differences in the order of [^{14}C]MOCA tissue levels and covalent MOCA binding have been detected in the rat with 24-hr MOCA tissue levels ip > po > dermal, a route considered to be a significant source of exposure (Chin *et al.*, 1983; Manis *et al.*, 1984), after a single administration. Covalent MOCA binding to tissue macromolecules has previously been shown to be approximately 100-fold less after dermal MOCA exposure than after oral administration (Cheever *et al.*, 1990).

For both the development of biomonitoring techniques and the determination of potential carcinogenicity, the study of MOCA metabolism has long been of interest (Glowinski *et al.*, 1978; Farmer *et al.*, 1981; Tobes *et al.*, 1983; Manis and Braselton, 1984; Yoneyama and Matsumura, 1984; Hesbert *et al.*, 1985; Manis and Braselton, 1986; Chin *et al.*, 1983; Cocker *et al.*, 1990). Pathways of MOCA metabolism consistent with those considered essential for the carcinogenicity reported for other arylamines include N-acetylation, N-oxidation and hydroxylation, and ring oxidation (Fig. 1). Maples *et al.* (1990) suggested that the mechanism of this amine–hemoglobin adduct formation may involve production of thiyl free radicals *in vivo*. Significantly, Morton *et al.* (1988) recently reported similar rates for the formation of *N*-hydroxy-MOCA by human and rat liver microsomes *in vitro*. These investigators suggested that the N-hydroxylation of MOCA, considered to be an activation step related to macromolecular adduct formation and possible carcinogenesis, was mediated by the cytochrome P450 enzyme system. Induction of the rats by phenobarbital, but not 3-methylcholanthrene, prior to isolation of microsomes resulted in an increased rate of hydroxylation. Later, Butler and her co-workers (1989), utilizing purified rat liver cytochrome P450 monooxygenases, showed that MOCA activation was preferentially catalyzed by the phenobarbital-inducible enzymes, cytochrome $P450_{PB\text{-}B}$ and cytochrome $P450_{PB\text{-}D}$. The current *in vivo* study shows that phenobarbital induction in the rat results in a significantly higher level of MOCA binding to blood protein, a finding which supports the work of those investigators.

Studies of related aromatic amines by Land *et al.* (1989) and McManus (1989) indicate that polymorphisms in the expression of N-hydroxylation by human tissue may, along with acetylation ability, be a critical factor in their susceptibility to cancer. The current study suggests that possible chemical induction and individual variations in MOCA activation catalyzed by the cytochrome P450 enzymes may be an important consideration in cancer risk assessment.

In summary, the results of this study show that enzymatic induction by phenobarbital resulted in a significant increase in covalent binding of ^{14}C to globin after administration of [^{14}C]MOCA. After hydrolysis the macromolecular adduct cleavage product was shown to be the parent compound. In the rat the globin biological half-life for adducted [^{14}C]MOCA is far greater than that of albumin, suggesting that quantification of the globin–MOCA adducts may provide a more valuable index of cumulative long-term industrial exposure.

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