β-naphthylamine and 4-aminobiphenyl (Patrianakos and Hoffman 1979). Additionally, Bryant et al. (1988) detected an increase in levels of ortho-toluidine-hemoglobin adducts for smokers of black tobacco when compared with smokers of blonde tobacco. These studies suggest that quantification of ortho-toluidine-hemoglobin adducts may be applicable to occupational biomonitoring.

This study was conducted to further investigate the in vivo binding and stability of ortho-toluidine adducted to the blood proteins, hemoglobin and albumin. The utility of these proteins was further evaluated as possible biomarkers for human occupational exposure to ortho-toluidine.

Materials and methods

Chemicals and solutions. Ring-labeled [^{14}C]ortho-toluidine, which was purchased from New England Nuclear (Boston, MA), had a specific activity of 30.7 mCi/mmol and a radiochemical purity of greater than 99%. The radiochemical purity of the compound was verified by HPLC (Cheever et al. 1980). ortho-Toluidine (>99%) was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). Dosing solutions were prepared by dissolving the appropriate amount of ortho-toluidine and [^{14}C]ortho-toluidine in corn oil to give final concentrations ranging from 10 to 100 mg/g (approximate specific activity 161–213 μCi/g).

Animal and doses. Male Sprague-Dawley rats were purchased from Charles River Breeding Laboratories, Inc. (Portage, MI) and maintained in an AAALAC-accredited facility for the duration of the study. Rats weighing 269±47 g at dosing were administered a single i.p. injection of [^{14}C]ortho-toluidine at 10, 20, 40, 50, or 100 mg/kg body weight. The average radioactivity administered to each rat was 44.0 μCi. Rats were anesthetized with pentobarbital and sacrificed by exsanguination at 2, 4, 8, 18, 24, 48, or 72 h, or 7, 14, or 28 days. The effect of route of administration was evaluated in rats 24 h after administration of 100 mg/kg [^{14}C]ortho-toluidine by either oral intubation or i.p. injection. Additionally, the possible effect of enzyme induction on hemoglobin binding was studied using rats pretreated with 100 mg/kg phenobarbital i.p. for 3 days, 80 mg/kg β-naphthoflavone i.p. for 48 h, or maintained without pretreatment prior to i.p. treatment with 100 mg/kg [^{14}C]ortho-toluidine. These rats were sacrificed 24 h after treatment with [^{14}C]ortho-toluidine.

Isolation of hemoglobin. Whole blood obtained from the inferior vena cava was transferred into Vacutainers® containing EDTA, centrifuged, and the plasma layer removed. Erythrocytes were washed with ice-cold phosphate-buffered saline (pH 7.4), and lysed by the addition of 10 vol ice-cold distilled water. Hemoglobin was precipitated by dropwise addition of 4 vol cold ethanol. The precipitate was washed three times with ice-cold acetone, and dried under a stream of nitrogen. After weighing, hemoglobin was solubilized using hyamine hydroxide (Sigma Chemical Co., St Louis, MO), bleached with tert-butyl hydroperoxide, and acidified by the addition of nitric acid. Scintiverse II scintillation cocktail (Fisher Scientific Co., Fairlawn, NJ) was mixed with triplicate aliquots and bound ^{14}C was determined by liquid scintillation counting (LSC). Bound ^{14}C was assumed to be parent ortho-toluidine for the purpose of calculating the extent of protein binding.

Isolation of albumin. A modification of the method of Fernandez et al. (1966) was used to isolate albumin. Plasma from each treatment group was pooled, 10 vol 0.2% HCl/ethanol (v/v) was added, and the mixture was slowly shaken in a water bath for 30 min at room temperature. Precipitated globulins were removed by centrifugation. The supernatant containing soluble albumin was removed and dried under vacuum in a SpeedVac (Savant Model SVC 100H, Farmingdale, NY). Albumin was redissolved in water, precipitated by the addition of 10 vol ice-cold acetone, and stored overnight at 4°C. The albumin was washed three times with acetone, dried under a stream of nitrogen, and weighed. Albumin samples were dissolved in distilled water prior to the addition of scintillation cocktail. Aliquots of samples were counted in triplicate for LSC determination of bound ^{14}C. The extent of ortho-toluidine binding was calculated by assuming that the bound radioactivity was parent ortho-toluidine.

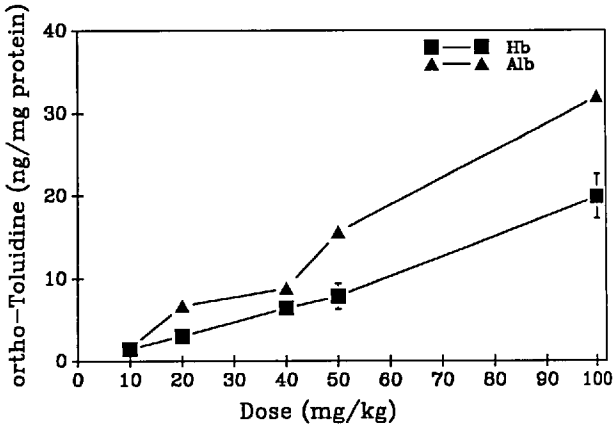

Fig. 1. Effect of dose on ortho-toluidine binding to hemoglobin (*Hb*) and albumin (*Alb*). Rats were administered 10, 20, 40, 50, or 100 mg/kg [^{14}C]ortho-toluidine i.p. and sacrificed at 4 h. ortho-Toluidine binding was determined by radioactivity and based on the assumption that all associated radioactivity was parent ortho-toluidine. Values represent means ± standard deviations ($n \geq 5$)

Statistical analysis. Descriptive statistics and one-way analysis of variance was performed using Statgraphics, version 4.0 (STSC, Inc., Rockville, MD). Group means with standard deviations and linear regression lines were plotted using Sigma-Plot™, version 3.1 (Jandel Scientific, Corte Madera, CA). Biological half-lives were calculated by the method of Rumak and Lovejoy (1986).

Results

Dose effect study

The levels of hemoglobin and albumin binding resulting from a single i.p. injection of ortho-toluidine was evaluated for rats given 10, 20, 40, 50, or 100 mg/kg [^{14}C]ortho-toluidine (Fig. 1). After 4 h, a significant amount of the administered radioactivity was bound to albumin for the 10 mg/kg treatment group (1.6 ng ortho-toluidine/mg albumin). The maximum albumin binding level was observed in rats administered 100 mg/kg [^{14}C]ortho-toluidine (32.0 ng ortho-toluidine/mg albumin). A slight decrease in albumin binding was noted in rats treated with 40 mg/kg ortho-toluidine. This decrease was likely due to experimental variation. [^{14}C]ortho-Toluidine-hemoglobin binding was dose dependent and continued to increase in a linear fashion over the range of doses tested. Peak ortho-toluidine-hemoglobin binding of 23.0±5.1 ng ortho-toluidine/mg hemoglobin was observed in the 100 mg/kg treatment group. The ortho-toluidine-hemoglobin binding index (HBI) [binding (mmol/mol hemoglobin): dose (mmol/kg)] equalled 51.8 and was calculated from the total radioactivity bound (Birner and Neumann 1988).

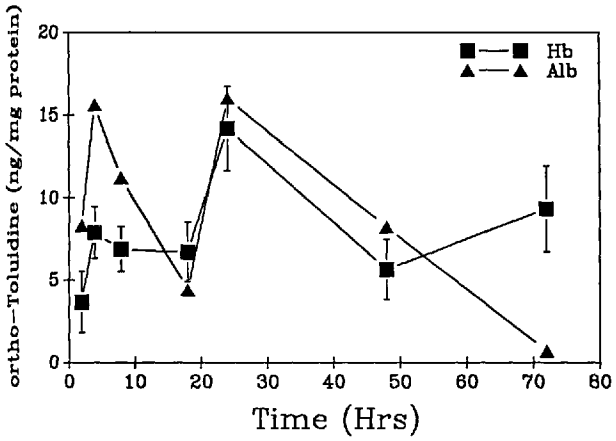

Fig. 2. Effect of time on ortho-toluidine binding to hemoglobin (*Hb*) and albumin (*Alb*). Rats were administered 50 mg/kg [^{14}C]ortho-toluidine i.p. and sacrificed at 2, 4, 8, 18, 24, 48, or 72 h. ortho-Toluidine binding was determined by radioactivity and based on the assumption that all associated radioactivity was parent ortho-toluidine. Values represent means ± standard deviations ($n \geq 6$)

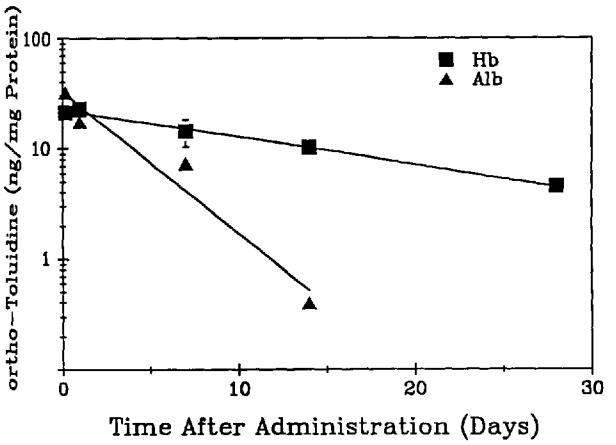

Fig. 3. Comparison of log-linear regression plots for ^{14}C-labeled hemoglobin (*Hb*) and albumin (*Alb*) of rats administered 100 mg/kg [^{14}C]ortho-toluidine i.p. and sacrificed at 4 or 24 h or 7, 14, or 28 days. ortho-Toluidine binding was determined by radioactivity and based on the assumption that all associated radioactivity was parent ortho-toluidine. Values represent means ± standard deviations ($n \geq 6$)

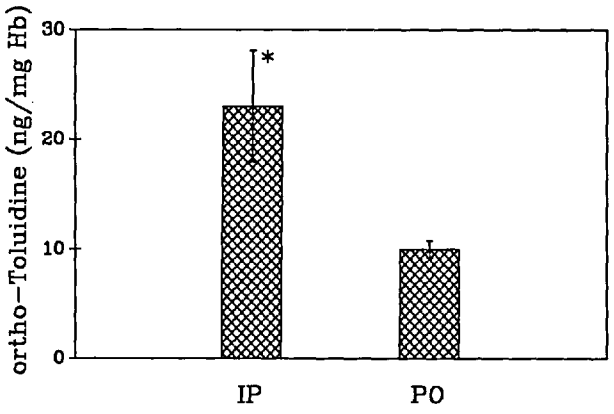

Fig. 4. Comparison of the effect of route of administration on formation of ortho-toluidine-hemoglobin adducts. Rats were administered 100 mg/kg [^{14}C]ortho-toluidine by i.p. injection (IP) or oral intubation (PO). ortho-Toluidine binding was determined by radioactivity and based on the assumption that all associated radioactivity was parent ortho-toluidine. Values represent mean ± standard deviations ($n = 6$). * Statistically significant difference ($p < 0.0001$)

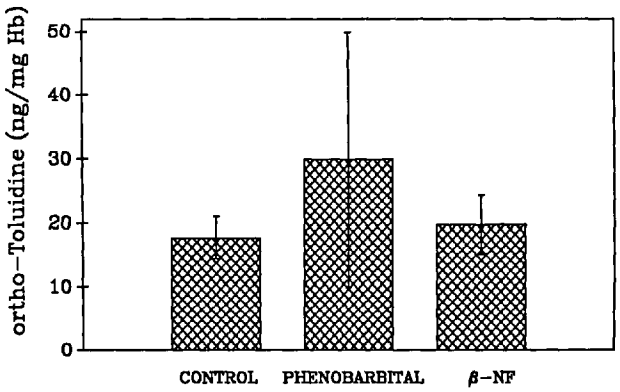

Fig. 5. Effects of enzymatic induction on ortho-toluidine binding to hemoglobin. Rats were pretreated with either phenobarbital, β-naphthoflavone (β-NF), or without pretreatment prior to administration of 100 mg/kg [^{14}C]ortho-toluidine. ortho-Toluidine binding was determined by radioactivity and based on the assumption that all associated radioactivity was parent ortho-toluidine. Values represent means ± standard deviations ($n = 6$)

Time course study

A time course study was conducted to determine ortho-toluidine binding to albumin and hemoglobin in rats after administration of a single 50 mg/kg dose of [^{14}C]ortho-toluidine (Fig. 2). The ortho-toluidine-albumin binding was highest after 4 h (15.6 ng ortho-toluidine/mg albumin), whereas peak ortho-toluidine-hemoglobin binding was detected at 24 h after treatment (14.2 ± 2.6 ng ortho-toluidine/mg hemoglobin). Decreased levels of ortho-toluidine bound to albumin was detected 8 and 18 h after treatment relative to levels present after either 4 or 24 h. Elimination of [^{14}C]ortho-toluidine bound to albumin was relatively rapid. At 72 h after administration, essentially no radioactivity was associated with pooled albumin. However, a significant amount of the bound radioactivity was still associated with hemoglobin at the 72-h time point (9.3 ± 2.6 ng ortho-toluidine/mg hemoglobin).

This interesting difference in the stability of bound radioactivity was further evaluated after administration of 100 mg/kg [^{14}C]ortho-toluidine to rats (Fig. 37). At this dose ortho-toluidine binding was similar to that noted for 50 mg/kg with peak albumin binding at 4 h, while ortho-toluidine-hemoglobin binding peaked at 24 h and was more persistent. Radioactivity associated with pooled albumin samples decreased rapidly, and by 14 days post-treatment had essentially been eliminated. However, for hemoglobin a significant portion of the bound radioactivity was retained and approximately 20% of the original amount was detectable after 28 days (4.6 ng ortho-toluidine/mg hemoglobin). Biological half-lives for albumin and hemoglobin were calculated to be 2.6 and 12.3 days, respectively.

Route of administration study

The routes of administration of ortho-toluidine in the rat was found to have a significant effect on the resulting ortho-toluidine-hemoglobin binding (Fig. 4). After 24 h, rats treated by i.p. injection had an approximately two-fold greater ortho-toluidine-hemoglobin level of binding than rats treated for the same time by oral intubation (23.0 ± 5.1 ng ortho-toluidine/mg hemoglobin after i.p. injection versus 10.0 ± 0.8 ng ortho-toluidine/mg hemoglobin after oral intubation; $p < 0.0001$).

Induction study

Subsequent evaluation of possible effects of metabolism on ortho-toluidine-hemoglobin binding, which may reflect bioactivation of aromatic amines to carcinogens, was investigated using phenobarbital and β-naphthoflavone (Fig. 5). A trend toward increased ortho-toluidine-hemoglobin binding was observed for rats pretreated with phenobarbital in comparison with either rats pretreated with β-naphthoflavone or with rats which received no pretreatment. This increase, however, was not statistically significant ($p < 0.05$).

Discussion

Results of the current study show that ortho-toluidine binds extensively in the rat to both hemoglobin and albumin. Similar hemoglobin binding was previously demonstrated for a single time point by Birner and Neumann (1988); however, the stability of the adduct, considered important for biomonitoring was not evaluated. Binding characteristics of [^{14}C]ortho-toluidine to rat hemoglobin and albumin have thus been determined. Significant [^{14}C]ortho-toluidine binding to albumin occurs, but is rapidly eliminated. After 4 h, binding levels for albumin were almost twice that of hemoglobin binding at the 50 mg/kg treatment group. Conversely, ortho-toluidine binding to hemoglobin accumulated more slowly than noted for albumin, but persisted for as long as 28 days. Decreases in hemoglobin and albumin binding noted at 8 and 18 h were repeatable and may be due to redistribution of [^{14}C]ortho-toluidine from blood to cells or tissues. The biological half-life of ortho-toluidine-albumin was determined to be 2.6 days. This corresponds to the 2–3 day half-life reported for rat albumin (Skipper and Tannenbaum 1990).

The half-life for ortho-toluidine-hemoglobin binding was calculated to be 12.3 days. This is less than the 23–34 day half-life of the rat erythrocyte (Schalm et al. 1975). This difference may be due to increased red blood cell fragility or the removal of ortho-toluidine from the hemoglobin moiety. For the current study the 24 h HBI was calculated to be 51.8. This value is approximately 10-fold greater than the previously reported HBI of 4.0 ± 0.65 for ortho-toluidine (Birner and Neumann 1988). However, several differences between the two studies may explain this discrepancy. Birner and Neumann administered ortho-toluidine by oral intubation to rats of a different strain and sex. Results from our study show a significant decrease in binding when ortho-toluidine is administered by the oral route in comparison with i.p. administration. Additionally, the HBI calculated for the current study is based on total bound radioactivity, while Birner and Neumann appear to have based their calculations for the HBI on radioactivity hydrolyzed off the hemoglobin. Current work indicates that as much as 40% of the bound ^{14}C may be resistant to hydrolysis (Cheever et al. 1992).

The route of administration also affected the level of ortho-toluidine-hemoglobin binding. Hemoglobin isolated from rats treated by the i.p. route had over twice the amount of bound ortho-toluidine than hemoglobin isolated from rats treated orally. This difference was somewhat surprising, since the absorption of ortho-toluidine from the rat gut is efficient (>90%). (Cheever et al. 1980). The observed decrease in ortho-toluidine binding to hemoglobin after oral administration suggests that increased hepatic macromolecular binding may have occurred due to bioactivation of ortho-toluidine to reactive metabolites, a process which could be affected by the rate of uptake.

The effect of metabolic induction on ortho-toluidine-hemoglobin binding was investigated in vivo. It has long been recognized that bioactivation of aromatic amines is required for production of cancer in the rat (Stillwell et al. 1987). N-hydroxylation of aromatic amines, primarily by hepatic monooxygenases, results in metabolites which react with target organ DNA or proteins (Bryant et al. 1987; Silk et al. 1989). Additionally, upon entering the erythrocyte, hydroxylamines are oxidized by the heme moiety to form nitroso compounds which are thought to react with amino acid sulfhydryl groups, resulting in stable sulfinamide adducts (Skipper and Tannenbaum 1987). An increase in the metabolism of aromatic amines has previously been reported to occur after pretreatment of rats with either β-naphthoflavone or phenobarbital (Kato 1986; Butler et al. 1989). The role played by metabolic induction in the formation of ortho-toluidine-hemoglobin adducts remains unclear, since results from this study showed that metabolic induction had no statistically significant effect on ortho-toluidine-hemoglobin formation. In contrast with these results significant increases in macromolecular binding after P450 enzyme induction have been reported for other related aromatic amines (Butler et al. 1989; Cheever et al. 1991). A possible explanation for this discrepancy is an unexpectedly large rat-to-rat variability in the current study which was noted for the phenobarbital pretreated animals; even though the livers of those animals showed significant morphological changes consistent with induction.

The analysis of human blood protein adducts as biomarkers in measurement of aromatic amine exposure may prove to be very valuable. Several aromatic amines, including ortho-toluidine, 4-aminobiphenyl, and 2-naphthylamine have been shown to bind to the hemoglobin of cigarette smokers (Stillwell et al. 1987; Bryant et al. 1988). Application of hemoglobin adducts for use as biomarkers has a major advantage over other possible biomonitoring techniques, such as measurement of urinary metabolites, since quantification of hemoglobin adducts is less dependent upon the time between exposure and sample collec-

tion. Determination of blood protein adducts for the estimation of the ortho-toluidine internal dose after an acute exposure in humans may be more useful than utilizing urinary levels, particularly if humans eliminate ortho-toluidine as quickly as has been reported for rats, (92% of a 50 mg/kg dose via the urine within 24 h) (Cheever et al. 1980). Lewalter and Korallus (1985) analyzed urine and hemoglobin collected from workers who were exposed to the related aromatic amines, aniline and p-chloroaniline, in an industrial accident. Urinary levels of these compounds were not detectable after 24 h; however, hemoglobin adducts levels peaked after 24 h and persisted for up to 7 days. For occupations in which chronic exposure to aromatic amines could occur, steady-state levels of hemoglobin adducts may be attained which would indicate continued exposure over the 60–120 day lifespan of a human erythrocyte. Estimation of target organ exposure (i.e. DNA) to ortho-toluidine by measurement of hemoglobin adduct formation may be possible, since such relationships have been observed for ethylene oxide (Osterman-Golkar et al. 1983) and the aromatic amine, 2-acetylaminofluorene (Pereira and Chang 1981). Provided binding follows first order kinetics, it may be possible to predict DNA adduct formation. This relationship between binding of ortho-toluidine to hemoglobin and DNA is currently being investigated.

One of the difficulties in assessing albumin adducts as biomarkers is the difference in half-life between rat and human albumin, 2–3 days versus 20–25 days, respectively (Skipper and Tannenbaum 1990). For some compounds such as aflatoxin B_1, the carcinogenic metabolites bind preferentially to serum albumin (Sabbioni et al. 1987). In this study it was also shown that aromatic amines bind to albumin. However, it is presently unclear the utility of albumin adducts for biomonitoring studies. Sabbioni and Neumann (1990) were unable to hydrolyze 4,4-methylene bis(2-chloroaniline) off of albumin from rats sacrificed 24 h after treatment. In additional studies from this laboratory, ortho-toluidine could be hydrolyzed off of albumin from rats sacrificed 2 or 4 h after treatment, but not 24 h after treatment (Cheever et al. 1992). This suggests that either formation of a secondary non-base hydrolyzable adduct is formed, or that albumin adducts may be prevented from hydrolyzing due to protein configuration. Albumin may be a valuable biomarker for aromatic amines provided other methodology can be developed for detection of these adducts.

In summary, results from this study demonstrate that ortho-toluidine binds to both albumin and hemoglobin and that a linear dose relationship exists for hemoglobin. Additionally, the biological half-lives for the protein adducts are several times that reported for elimination of ortho-toluidine or its metabolites via the urine, thus, providing evidence that these proteins may be valuable biomarkers of exposure to ortho-toluidine in the occupational setting.

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