DMD Fast Forward. Published on September 23, 2005 as doi:10.1124/dmd.105.005520

SATURATION TOXICOKINETICS OF THIOACETAMIDE: ROLE IN INITIATION OF LIVER INJURY

Jaya Chilakapati, Kartik Shankar, Midhun C. Korrapati, Ronald A. Hill and Harihara M. Mehendale.

Department of Toxicology (J.C., M.C.K., H.M.M.) and Division of Basic Pharmaceutical Sciences (R.A.H.), College of Pharmacy, The University of Louisiana, Monroe, Louisiana; Arkansas Children's Nutrition Center, Little Rock , Arkansas (K.S.).

(Received ------2005 ;)

Primary Laboratory of Origin: Department of Toxicology, College of Pharmacy, The University of Louisiana, Monroe, LA 71209, USA (J.C., M.C.K., H.M.M.).

a) Running Title: SATURATION OF THIOACETAMIDE BIOACTIVATION AND LIVER INJURY
CHILAKAPATI et al.

b) To whom correspondence should be addressed:

Harihara M. Mehendale, Ph. D.
Department of Toxicology
College of Pharmacy
The University of Louisiana Monroe
700 University Avenue, Sugar Hall 306
Monroe, LA 71209-0470, USA.

Phone: (318) 342-1691
Fax: (318) 342-1686
E-mail: mehendale@ulm.edu

c)

Number of Text Pages	: 35
Number of Tables	: 2
Number of Figures	: 8
Number of References	: 36
Number of Words in the Abstract	: 305
Number of Words in the Introduction	: 858
Number of Words in the Discussion	: 1354

d) **ABBREVIATIONS:** TA, Thioacetamide; TASO, thioacetamide sulfoxide; $TASO_2$, thioacetamide-S, S-dioxide; SD, Sprague Dawley; HPLC, high-performance liquid chromatography; ALT, alanine aminotransferase; SDH, sorbitol dehydrogenase; CYP2E1, cytochrome P450 2E1; [^{14}C]TA, thio[1-^{14}C]acetamide; TCA, trichloroacetic acid; AUC, area under the curve.

ABSTRACT:

Thioacetamide (TA), a potent centrilobular hepatotoxicant, undergoes a two-step bioactivation mediated by microsomal CYP2E1 to TA sulfoxide (TASO), and further to TA-S, S-dioxide ($TASO_2$), a reactive metabolite that initiates cellular necrosis. Our earlier studies showed that bioactivation-mediated liver injury of TA is not dose-proportional. The objective of this study was to examine whether increasing doses of TA lead to enzyme saturation, thereby resulting in lack of dose-response for injury: bioactivation of TA $\rightarrow$ TASO $\rightarrow$ $TASO_2$ may follow zero-order kinetics. A 12-fold dose range of TA (50, 300 and 600 mg/kg, i.p.) was injected to male SD rats. TA and TASO were quantified in plasma, liver and urine by HPLC. With increasing doses, the apparent elimination half-lives of TA and TASO increased linearly, indicating that TA bioactivation exhibits saturation kinetics. Increasing TA dose resulted in greater-than-proportional increases in plasma TA and TASO levels. The TASO/TA ratio was inversely proportional to the dose of TA. Covalent binding of ^{14}C-TA-derived radiolabel to liver macromolecules showed a less-than-dose-proportionate increase with a 12-fold higher dose. Less than dose-proportional covalent binding was confirmed in liver microsomal incubations with ^{14}C-TA. Three-fold higher excretion of TASO was seen in urine at the highest dose (600 mg/kg) compared to the lowest dose (50 mg TA /kg). Incubation of TA with rat liver microsomes and purified baculovirus-expressed rat and human CYP2E1 "Supersomes®" over a concentration range of 0.01 to 10 mM, revealed saturation of TA conversion to TASO at and above 0.05 mM TA concentration, comparable to in vivo plasma and liver levels achieved upon administration of higher doses. Calculated Km values for TA (0.1 mM) and TASO (0.6 mM) suggest that the second step of TA bioactivation is 6-fold less efficient. Collectively, the findings indicate

saturation of CYP2E1 at the first (TA to TASO) and second steps (TASO to $TASO_2$) of TA bioactivation.

The toxicokinetic profile of a compound becomes very important especially when its toxicity depends on its bioactivation to a reactive intermediate. When increasing doses of toxicants are administered, the rate of bioactivation may be limited as a result of enzyme saturation and thus production of reactive metabolites does not increase proportionately with dose (saturation, zero-order kinetics). Thioacetamide (TA) is a centrilobular hepatotoxicant, widely used as a model compound to induce acute and chronic liver disease (Mangipudy et al., 1998; Wasser and Tan, 1999; Al-Bader et al., 2000; Chu et al., 2000; Ramaiah et al., 2000). Numerous investigators use TA to study mechanisms of hepatic necrosis because of its relatively short half-life (Porter and Neal, 1978; Porter et al., 1979), well-known ability to cause acute toxicity (Mehendale et al., 1997), and because of a large window of time between its necrogenic effects and liver failure (Mehendale, 2005). Liver injury begins with bioactivation of TA to thioacetamide sulfoxide (TASO) (Step I) and further to thioacetamide-S, S-dioxide ($TASO_2$) (Step II) (Fig. 1). Thioacetamide-S, S-dioxide, an unstable, reactive metabolite, is thought to initiate necrosis by covalently binding to liver macromolecules (Hunter et al., 1977; Porter and Neal, 1978; Porter et al., 1979). The advantages of using TA as a model hepatotoxicant include high specificity for liver as a target organ, except at excessively high doses, when it can also cause marginal but transient renal injury (Barker and Smuckler, 1974), and regiospecificity for the perivenous region. When a lethal dose is administered to rats, death occurs 3.5 to 7 days after administration due to fulminant hepatic failure (Chanda and Mehendale, 1994, 1995; Mangipudy et al., 1995). This allows the luxury of a long window of time for studying the incline and decline slopes of injury and recovery by conducting time-course studies after administration of low to moderate doses (Mangipudy et al., 1995; Mangipudy et al., 1998; Mehendale, 2005).

TA is bioactivated by hepatic microsomal CYP2E1 to sulfoxide and further to $TASO_2$ (Wang et al., 2000; Ramaiah et al., 2001). CYP2E1 metabolizes a large number of low-molecular-weight compounds, many of which are industrial solvents, chemical additives, halogenated anaesthetics and drugs (Tanaka et al., 2000). Most importantly, CYP2E1 activates many xenobiotics to hepatotoxic or carcinogenic products (Lieber, 1997). Also, a wide variety of CYP2E1 substrates are known to exhibit saturation kinetics (Dekant, et al., 1995; Rappaport, et al., 2002; Lee, et al., 2000; Whysner, et al., 1996; Vittozzi, et al., 2000; Lof and Johanson., 1993; Ramsey and Young , 1978; Kreiling, et al., 1986; Kirschman, et al., 1986).

Although TA hepatotoxicity is well-characterized, and its metabolism well-studied, information on dose-response for injury and toxicokinetics is very sparse. Porter et al. (1979) reported the half-lives of TA and TASO to be 1 to 1.5 h and 1.5 to 2 h, respectively, after administration of a single dose (200 mg/kg) of TA. The extent, to which these half-lives remain consistent upon administration of lower or higher doses of TA, has not been reported.

A 12-fold dose range of TA (50 to 600 mg/kg) failed to elicit a typical dose-response relationship with respect to initial liver injury as measured by plasma ALT, SDH and histopathology (Mangipudy et al., 1995). The lethal dose (600 mg/kg) caused lower injury at 12 h than the other three doses. Injury continued to progress well after 36 h, when the compound was totally eliminated from the body (t ½ ~ 3 h). Perhaps other mechanisms that are independent of the offending toxicant such as leakage of destructive hydrolytic proteases like calpains released from dying cells or secondary inflammatory mechanisms, lead to progression of injury (Limaye et al., 2003). Liver injury of a 6-fold dose range of TA (50, 150 and 300 mg/kg) did not differ between doses during the incline or decline phases of injury. Compensatory tissue repair data revealed a dose-proportional increase over a 6-fold dose range (50 to 300 mg/kg), but

marked delay and inhibition after the highest dose (600 mg/kg). These observations run in opposition to the commonly accepted paradigm of higher doses yielding higher metabolic activation and therefore higher injury. Another study also revealed a similar lack of normal dose-response of TA liver injury in diet-restricted (DR) rats. Diet restriction, known to induce CYP2E1, substantially increases mechanism-based injury caused by TA (Ramaiah et al., 2001). Whereas initial liver injury was 6-fold higher at the low dose (50 mg/kg, i.p.), it was only 2.5-fold higher and delayed at the lethal dose of TA (600 mg/kg, i.p.) in DR rats when compared with their ad libitum cohorts (Ramaiah et al., 1998).

The objective of the present study was to test if the lack of a dose-proportional increase in liver injury observed after administration of high doses of TA is due to saturation of its bioactivation via CYP2E1 (Fig. 1). We investigated the toxicokinetics, disposition and bioactivation of TA to test our hypothesis. We report here that TA exhibits saturation toxicokinetics in the rat, and this might be at the root of absence of dose-proportional increase in bioactivation-based liver injury observed with this compound. Our in vitro findings from microsomes and purified recombinant CYP2E1 (Supersomes®) further confirm saturation kinetics of TA bioactivation.

Materials and Methods

Animals: Male Sprague Dawley (SD) rats (250-275 g) were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN) and were maintained on sawdust bedding, free of any chemical contaminants on a 12-h photoperiod in our central animal facility at 21 $\pm$ 1 ^{o}C and 50-80% relative humidity. Rats were acclimatized for a week before use in experiments. The animals had free access to water and commercial rat chow (No 7001; Teklad, Madison, WI) before and during the treatment. Animal maintenance and treatments were in accordance with the National Institutes of Health Guide for Animal Welfare, as approved by Institutional Animal Care and Use Committee.

Chemicals: Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co. (St Louis, MO). Thioacetamide was 99.7% (HPLC grade) pure. TASO was synthesized using the method described by Porter and Neal (1978). Thio [1-^{14}C] acetamide (^{14}C-TA, 5 mCi/mmol) was obtained from American Radiolabeled Chemicals Inc. (St Louis, MO).

Toxicokinetic parameters of TA: Blood samples were collected (100 µl via retro-orbital sinus) in heparinized tubes over a time course from male SD rats treated with 50, 300 and 600 mg/kg TA intraperitoneally. Three rats were used to complete the entire time course once (n = 1). One rat was used to collect blood samples for 4 time points, overlapping one time point between rats. Three such replicate time courses were performed to achieve n = 3. Plasma was separated from heparinized blood by centrifugation for 10 min at 10,000 *g*. Plasma half-life ($t_{1/2}$) for TA and TASO were calculated using the terminal elimination constant (β), where $t_{1/2} = 0.693/\beta$. The

value of β was estimated using a semi-logarithmic plot of time versus concentration using JMP-IN statistical software (SAS Institute, Cary, NC).

In a separate set of experiments, liver samples were collected from rats after diethyl ether euthanasia at various time points after intraperitoneal TA (50, 300 and 600 mg/kg) administration. The liver samples were homogenized in saline (1 ml of saline/g liver). The liver homogenate was centrifuged and the supernatant analyzed for TA and TASO concentrations.

For urinary analysis of TA and TASO, urine was collected by housing animals in separate metabolic cages (Nalgene, Braintree, MA). Preliminary experiments confirmed that collecting urine at room temperature did not affect the stability of TA and TASO for 24 h. This was done by adding known amounts of TA and TASO to normal rat urine and storing the urine samples at either room temperature or at -20 °C. TA and TASO by HPLC at 0 and 24 hrs later and did not detect any significant differences between the two time points regardless of room temperature or cold storage. We therefore, did not find it necessary to collect the urine over dry ice. Urine was collected 12 and 24 h after treatment with TA. Standards were prepared by including known amounts of TA and TASO in plasma, liver homogenate and urine from untreated rats. Levels of TA and TASO were estimated using a reverse-phase HPLC assay as described below.

Plasma protein binding of TA: The protein unbound fraction (f_u) of TA in rat plasma was measured by the ultrafiltration method with a Centrifree (Amicon, Beverly, MA). TA was mixed with the rat plasma at 0.5, 5 and 10 mM concentrations, and incubated at 37°C for 30 min. A 100 µl aliquot of the incubate was placed in the sample reservoir of Centrifree tube in triplicate and centrifuged at 2000*g* for 30 min. After filtration, 50 µl aliquot of each filtrate and 50 µl of the incubated mixture were used to determine the concentrations of TA. The concentrations of TA

were determined by HPLC to estimate the bound concentrations (C_b), unbound concentrations (C_u), unbound fraction (f_u) and bound fraction (f_b).

HPLC-based assay for quantitation of TA and TASO: To quantitate TA and TASO in plasma, liver and urine, a reverse-phase HPLC assay was developed using 7% acetonitrile, 50 mM sodium sulfate, and 50 mM potassium phosphate buffer as the mobile phase (Shankar et al., 2003). An SPS-ODS column (5 μm, Regis Technologies, Morton Grove, IL) was used to separate the components at 1 ml/min. TA was detected by UV absorption at 212 nm, and TASO at 290 nm, using a photodiode array detector (PDA 100, Dionex Systems, Sunnyvale, CA). Retention times for TA and TASO were approximately 4.1 and 3 min, respectively.

Covalent binding of ^{14}C-TA to liver tissue: To examine covalently bound TA-reactive intermediate, livers from rats treated with ^{14}C-TA (50 and 600 mg/kg, i.p., 5 mCi/mmol, 25 μCi/rat) were flash frozen with liquid nitrogen over a time course (3 to 96 h) after TA treatment. ^{14}C-TA-derived radioactivity covalently bound to liver macromolecules was estimated using the procedure described earlier (Shankar et al., 2003). Briefly, liver tissue (200 mg) was homogenized in 1 ml of 0.9% saline, and total protein precipitated using 2 ml of 0.9 M trichloroacetic acid (TCA). Samples were centrifuged at 1000 *g* for 15 min at room temperature. The supernatant was discarded and the protein precipitate was resuspended in 3 ml of 0.6 M TCA, mixed on an agitating mixer (Thermolyne, Dubuque, IA) for 1 min and centrifuged at 1000 *g* for 3 min. After two more washings with 0.6 M TCA (3 ml per wash), the pellet was washed six times with 80% methanol (3 ml per wash). It was found that after six methanol washings no radioactivity was detected in the supernatant. The remaining pellet was dissolved in 2 ml of 1 M

sodium hydroxide (NaOH) and the ^{14}C content in an aliquot was estimated using a liquid scintillation counter (Packard Instrument Co. Meriden, CT). Total protein concentrations were estimated using Bradford's method (Bradford et al, 1976) using Bio-Rad reagent (Hercules, CA). Results are expressed as amount of TA covalently bound to protein. The linear trapezoidal rule was applied to calculate the area under the curve.

Preparation of Microsomes: Untreated rats (250 - 275 g) were euthanized under diethyl ether anesthesia. Livers were perfused in situ with ice-cold 0.9% saline, rapidly excised, blotted dry, weighed, minced and homogenized (1:5 w/v) in ice-cold Tris-acetate buffer (pH 7.4) containing 1.15% KCl. The homogenate was centrifuged at 10,000 *g* for 30 min at 4 ^{o}C. The supernatant was then centrifuged at 100,000 *g* for 60 min at 4 ^{o}C. Microsomes were resuspended by homogenization in a Potter-Elvehjem glass homogenizer, and again centrifuged at 100,000 *g*. Microsomal pellets were recovered, quick frozen with liquid nitrogen, and stored at -80 ^{o}C for later use.

Microsomal Metabolism of TA: To investigate saturation of TA metabolism to TASO in vitro, TA was incubated with hepatic microsomes. TA (0.01 mM - 10 mM) (0.1 M potassium phosphate buffer, pH 7.4) was incubated with liver microsomes (2 mg protein) and an NADPH-regenerating system (1.3 mM $NADP^{+}$, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, and 3.3 mM $MgCl_2$). Each reaction mixture had a total volume of 1 ml, and incubations were carried out in 10 ml Erlenmeyer flasks in a reciprocal metabolic shaker (Boekel Scientific, Feasterville, PA) at 37^{o}C at a speed of 50 strokes/min. Reactions were started by the addition of microsomes (2 mg of microsomal protein per ml of reaction volume) and

stopped by adding 0.2 ml 20% TCA at the end of 10 min. The reaction time and amount of protein were chosen based on previous reports (Porter and Neal, 1977) and also by conducting experiments to confirm that we were in fact in the linear range. In a separate set of experiments, reactions, wherein 0.5 mM TA was incubated with microsomes, were stopped at 10, 20 and 30 min. In another set of experiments, 0.5 mM TA was incubated with 0.5, 1, 2, 2.5 and 3 mg protein and reactions were stopped at 10 min. Reaction samples were centrifuged at 10,000 *g* for 10 min and the supernatants were assayed for TA and TASO levels by HPLC. Three kinds of controls were included- one without microsomes, one without NADPH and one without TA.

Covalent Binding of ^{14}C-TA to Liver Microsomes: ^{14}C-TA (0. 05, 0.5 and 5 mM, 0.2 µCi/reaction, Specific activity- 5 mCi/mmol) was incubated with male SD rat hepatic microsomes for 10 min following the protocol used in the preceding section. After incubation, reactions were stopped using 0.2 ml of 0.9 M trichloroacetic acid (TCA). Samples were centrifuged at 1000 *g* for 30 min at room temperature. The supernatant was discarded and the protein precipitate was resuspended in 3 ml of 0.6 M TCA, mixed on an agitating mixer (Thermolyne, Dubuque, IA) for 1 min and centrifuged at 1000 *g* for 30 min. The pellet was washed two times with 80% methanol (3 ml per wash). It was found that after two methanol washings, no radioactivity was detected in the supernatant. The remaining pellet was dissolved in 0.2 ml of 1 M sodium hydroxide (NaOH) and the ^{14}C content was estimated using a liquid scintillation spectrometer (Packard Instrument Co. Meriden, CT). Total protein concentrations were estimated using Bradford's method (Bradford et al, 1976) using Bio-Rad reagent (Hercules, CA). Results are expressed as amount of TA covalently bound to protein.

Microsomal Metabolism of TASO: To investigate depletion of TASO when compared to TA, TASO (0.01 mM- 5 mM) was incubated with hepatic microsomes. Reaction conditions were kept the same as described in the previous section and TASO levels estimated at the end of 10 min.

Metabolism of TA in Supersomes® containing cDNA-Expressed CYP2E1: To investigate saturation of TA metabolism to TASO via CYP2E1, TA was incubated with rat or human CYP2E1 Supersomes® (Gentest, Woburn, MA) containing rat or human CYP2E1+ P450 reductase + cytochrome b_5. TA (0.01 mM-10 mM) (0.1 M potassium phosphate buffer, pH 7.4) was incubated with 10 pmol of CYP2E1 and an NADPH regenerating system (1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, and 3.3 mM $MgCl_2$). Each reaction mixture had a total volume of 0.5 ml, and incubations were carried out in 10 ml Erlenmeyer flasks in a reciprocal metabolic shaker (Boekel Scientific, Feasterville, PA) at 37^{o}C at a speed of 50 strokes/min. Reactions were started by the addition of Supersomes® and stopped by adding 0.1 ml of 20% TCA at the end of 10 min. Reaction samples were centrifuged at 10,000 *g* for 10 min and the supernatants were assayed for TA and TASO levels by HPLC. Four kinds of controls were included- one without Supersomes®, one without NADPH-regenerating system and one without TA and a fourth where TA was incubated with heat-denatured Supersomes®.

Statistical analysis: Data are expressed as means $\pm$ SE. Statistical significance between two doses at the same time point was analyzed by Student's *t*-test. A one-way analysis of variance followed by Tukey's Post-Hoc test was used to determine statistical significance

between sample groups. Data were statistically analyzed using SPSS 11.0 software (SPSS Incorporated, Chicago, IL). In all cases, the criterion for statistical significance was $p \leq 0.05$.

Results

In vivo measurements of plasma TA and TASO levels:

Mean plasma concentrations of TA and its major metabolite TASO are shown in Fig. 2 A and B, respectively. Plasma TA levels peaked by 5 min after each of the three doses. TA had a half life of 16 min at the lowest dose (50 mg/kg). After administration of the higher doses, i.e. 300 and 600 mg/kg, TA levels did not decrease at a statistically significant rate from one time point to the other between 5 to 60 min and 15 to 120 min, respectively, suggesting saturation of its metabolism. Thereafter, the levels declined slowly, presumably due to conversion to TASO or excretion. Plasma TA AUC was more than dose proportional with increase in dose. The plasma clearance of TA and TASO decreased with increasing doses implying saturation of metabolism (Table 2).

TASO was readily detected in plasma as early as 5 min, indicating its rapid formation. The plasma levels peaked around 60 to 90 min for the 50 mg/kg dose, whereas maximum concentrations were reached at 180 min for the 300 and 600 mg/kg doses. There was no difference in TASO concentration between the 50 and 300 mg/kg doses until 15 min after the administration and between the 300 and 600 mg/kg doses until 60 min after the administration. After reaching peak levels, TASO remained constant for 3.75 h (300 mg/kg) and 9 h (600 mg/kg), respectively, suggesting saturation of the TASO metabolism to $TASO_2$, declining rapidly thereafter. Less than dose proportional increase in peak plasma TASO levels was found with increase in dose. The metabolite to parent compound ratio was highest with the lowest dose (50 mg/kg) at all time points indicating that the rate of metabolite (TASO) production was highest at this dose (Table 1). We could thus infer that there is saturation at the first step of TA metabolism. Plasma TASO AUC was more than dose proportional with increase in dose,

suggesting saturation of further metabolism of TASO. Toxicokinetic parameters of TA and TASO are given in Table 2. Dose normalized AUC for TA and TASO showed greater values with higher doses (Table 2). With increasing doses, apparent half-lives of TA (16 $\pm$ 1 to 164 $\pm$ 7 min) and TASO (36 $\pm$ 1.6 to 247 $\pm$ 41.7 min) progressively increased (Table 2), a hallmark of zero-order kinetics. Saturable metabolism of TA as well as TASO was readily evident as the dose increased.

Plasma Protein Binding of TA: The plasma protein binding (f_b %) of TA was low (9.93 $\pm$ 1.16 %) and concentration independent for TA. The plasma protein binding values (f_b %) for TA over a 20-fold concentration range i.e., 0.5, 5 and 10 mM were 8.22 $\pm$ 3.36, 12.15 $\pm$ 4.98 and 9.41 $\pm$ 4.7, respectively. These values do not significantly differ from each other.

Liver levels of TA and TASO:

Liver TA (Fig. 2C) and TASO (Fig. 2D) levels were undetectable 8 h after treatment with 50 mg/kg. Hepatic TA levels remained constant until 6 h after the administration of 50 and 300 mg TA/kg doses (Fig. 2C), and until 3 h after the highest dose. Increases in AUC for hepatic TA and TASO were disproportionately higher with increase in dose (data not shown). TASO levels increased for the high dose only after TA levels began to decline in the plasma (Fig. 2A). Hepatic TASO levels peaked at 180 min after administration of the high (lethal) dose. After 180 min, TASO after highest dose did not differ from TASO concentrations seen after the 300 mg/kg dose until 12 h time point. With regard to the low dose, though hepatic TASO levels remained elevated until 60 min, there was a steep decline at 180 min.

Urinary excretion of TA and TASO:

Urinary excretion is represented as percentage of dose excreted in the form of unchanged TA and TASO, 12 and 24 h after TA administration (Fig. 3). Most of the urinary excretion of TA and TASO occurred by 12 h regardless of the dose as there was no significant difference between 12 and 24 h excretion. While the cumulative amount of TA excreted as a percent of dose remained similar after 12-fold increase in TA dose (50 to 600 mg/kg), excretion of TASO increased 3-fold suggesting that the metabolism of TASO is less efficient than the metabolism of TA after the higher doses. Consequently, TASO formed after the high dose is largely excreted. We have not examined the status of the sulfate conjugate and acetamide which account for the remaining urinary metabolites of TA.

Covalent binding of ^{14}C-TA to liver macromolecules:

$TASO_2$ cannot be measured as it is highly reactive and unstable. Covalent binding of ^{14}C-TA derived radiolabel to liver protein, used as an indirect method to measure the reactive intermediate $TASO_2$, showed a less than dose-proportional increase. At 50 mg TA/kg dose, covalent binding was rapid and remained relatively constant at peak level through 12 h (Fig. 4A). After 12-fold higher dose (600 mg TA/kg) covalent binding also remained constant near the peak level until 12 h, but it was considerably lower than the expected 12-fold increase (Fig. 4A). An 8-fold increase in AUC of covalent binding (Fig. 4B) was seen after the 600 mg/kg dose. This indicates saturation at the second step (formation of reactive $TASO_2$) and therefore contributing to lack of dose response for initial bioactivation-based liver injury with the lethal dose of TA (Mangipudy et al., 1995).

Covalent binding of ^{14}C-TA to liver microsomes: When the concentration of TA incubated with rat liver microsomes was increased from 0.05 to 5 mM (100-fold), covalent binding of ^{14}C-TA-derived radiolabel to rat liver microsomes increased only by 57-fold (1.42 $\pm$ 0.09 to 80.0 $\pm$ 6.5 ng/mg protein). A 10-fold increase in concentration of TA (0.05 to 0.5 mM), resulted in 7-fold increase in covalent binding (1.42 $\pm$ 0.09 to 10.11 $\pm$ 1.5 ng/mg protein). A further 10-fold increase in concentration (0.5 to 5 mM), resulted in 7.9-fold increase (10.11 $\pm$ 1.5 to 80.0 $\pm$ 6.5 ng/mg protein) in covalent binding. These results confirm the in vivo data on covalent binding of $TASO_2$ to liver tissue, which is less than dose proportional suggesting saturation of TA bioactivation to $TASO_2$.

Microsomal metabolism of increasing concentrations of TA:

TA was incubated with rat liver microsomes in standardized and optimized incubation conditions for 10 min with increasing concentrations of TA. The calculated Km and Vmax values for metabolism of TA to TASO by rat liver microsomes are 0.1 mM and 0.624 mmol/min, respectively. Increasing concentrations of TA (0.01 to 10 mM) incubated with microsomes containing NADPH regenerating system revealed a decreasing percentage of TA metabolized to TASO (Fig. 5A), which remained constant from 0.5 mM onwards. Control incubations did not produce any detectable levels of TASO. Disappearance of TA was complete at 0.01 to 0.05 mM TA concentration indicating very efficient metabolism of TA at this low and narrow range of concentrations. Only 60% of TA was detectable as TASO at these two concentrations. Because TA was not detected in these incubations after 10 min (Fig. 5B), the remaining portion of TASO must have been further metabolized to $TASO_2$, the ultimate reactive metabolite, and presumably to acetamide, a non-toxic metabolite of TA. At TA concentrations above 0.5 mM, percentage of

TA detected as TASO remained constant irrespective of the increase in TA concentration. Increasing concentration of TA from 0.5 mM to 10 mM led to progressively increasingly higher proportion of the parent compound remaining in the incubations though the same percentage of TASO was produced. This suggests that, the metabolism of TA to acetamide may also be saturated resulting in progressively greater proportion of TA remaining in these incubations (Fig. 5B).

Microsomal metabolism of TA with increasing time:

To confirm that saturation of TA metabolism to TASO observed in the microsomal incubations is indeed due to zero-order kinetics, time-dependent metabolism of TA was examined. If the enzyme saturation is due to zero-order kinetics, the product formation rate should remain constant regardless of substrate concentration, and the amount of product formed should increase in direct proportion to incubation time. In this experiment, we incubated 0.5 mM TA with rat liver microsomes under the same conditions used for the experiments described above except the length of time. The reactions were stopped at 10, 20 and 30 min. We observed increasing amounts of TASO production, and for each 10 min increment, the amount of additional TASO formed was the same (data not shown).

Microsomal metabolism of increasing concentrations of TASO:

TASO was incubated with microsomes to compare the relative efficiency of metabolism of TASO and under the same incubation conditions used earlier for TA. At the end of 10 min, 50% of TASO was left behind from the lowest concentration of TASO (0.01 mM) incubated (Fig. 6). The calculated Km and Vmax values for TASO using the substrate depletion in the rat

liver microsomal incubations are 0.62 mM and 17.47 mmol/min, respectively. This suggests that the second step of TA to its reactive metabolite $TASO_2$ is 6-fold less efficient than the first step (0.1 mM for TA→TASO). Indeed, the metabolism of TASO (Fig. 6) is less efficient than TA (Fig. 5B). It should be noted that metabolism of TASO, which includes bioactivation to $TASO_2$, was completely inhibited at 0.1 mM and higher concentrations. These findings are consistent with the sustained high plasma levels of TASO after the higher doses of TA (Fig 2B)

TA incubations with cDNA-expressed CYP2E1 Supersomes®:

These incubation experiments were aimed at two objectives: first to confirm that CYP2E1 is the major enzyme that mediates metabolism of TA; second, if metabolism of TA is saturated, it should be more readily apparent with incubations containing only CYP2E1 and should occur at lower concentrations of TA. We employed not only rat but also human CYP2E1 Supersomes® because CYP2E1 is the major enzyme known to bioactivate several commonly encountered toxicants (Ingelman-Sundberg et al., 1994; Gonzalez et al., 1991). Control incubations for rat and human Supersome experiments did not produce any detectable levels of TASO. Increasing concentrations of TA (0.01-10 mM) were incubated for 10 min with CYP2E1 (Supersomes®, Gentest) containing the NADPH-regenerating system the highest proportion of sulfoxide production i.e. approximately 70%, was observed at 0.01 mM initial TA concentration when rat CYP2E1 was employed (Fig. 7A), suggesting that CYP2E1 is the major isozyme that metabolizes TA to TASO. Saturation of sulfoxide production was evident at and above 0.5 mM TA, similar to that observed in rat liver microsomal incubations. The calculated Km and Vmax values for TA to TASO conversion with rat CYP2E1 are 5.78 mM and 1.47 mmol/min, respectively. It should be noted that metabolism of TA reached zero-order process at much lower

concentration of TA (0.05 mM) when incubated with Supersomes® as compared to microsomal incubations (Fig. 5). Presumably, the difference is due to non-specific TA binding to microsomal proteins.

When incubated with human CYP2E1 Supersomes®, the results were almost the same as the rat Supersomes® in terms of the TA concentration at which saturation was evident i.e. 0.5 mM (Fig. 8). However, the human CYP2E1 enzyme seemed to be more efficient in TASO production at 0.05 and 0.1 mM initial TA concentration (Fig. 8B) than the rat CYP2E1(Fig. 7B).

Discussion

The present data confirm that TA bioactivation is saturated at higher doses and suggest that it is likely to be the mechanism for the observed absence of a simple dose-response relationship for bioactivation-based liver injury observed with TA in previous studies (Mangipudy et al., 1995; Ramaiah et al., 1998). Thioacetamide is extensively used as a model hepatotoxicant in acute and chronic studies and thus, characterization of toxicokinetics of this compound is likely to be highly useful.

Several lines of evidence suggest that the first step of TA bioactivation (TA → TASO) is saturable. First, the ratio of TASO to TA decreased with increasing doses of TA, consistent with saturable metabolism of TA to TASO, and therefore, reduced efficiency of metabolism to TASO, the penultimate reactive metabolite, at the higher doses. Plasma protein binding of TA is not a confounding factor in this assessment. Binding of TA to plasma proteins is relatively low (9.93 $\pm$ 1.16 %) and did not differ with a 20-fold increase in TA concentration. Second, higher doses of TA resulted in more than dose-proportional increases in plasma AUC of TA. Third, an increase in dose of TA resulted in an increase in time to reach peak plasma concentration of TASO and is accompanied by an increase in apparent TA elimination half-lives. Fourth, the liver levels of TA and TASO showed a profile similar to the plasma. Fifth, results of the in vitro rat liver microsomal and rat and human supersomal incubations also revealed saturation of TA metabolism. Furthermore, bioactivation of TASO to the ultimate reactive metabolite $TASO_2$ is also saturable and less efficient than the first step. From the present study, three lines of evidence suggest the efficiency of the second step of TA bioactivation (TASO → $TASO_2$) is even lower than the first step (TA → TASO). First, after reaching peak concentrations of TASO in plasma, TASO concentration is sustained for nearly 3.8 h after 300 mg/kg dose and for 9 h after the lethal

dose (600 mg/kg). Moreover, the dose-normalized AUC of TASO increased with higher doses of TA. This is highly suggestive of saturation of TASO to $TASO_2$ conversion. Second, lower than expected increase in covalent binding of ^{14}C-TA-derived radiolabel to liver proteins was observed. The hepatotoxicity of TA is due to covalent binding of highly reactive $TASO_2$ to liver macromolecules (Miller and Miller, 1974; Hunter et al., 1977; Porter et al., 1979). In the present study, both dose- and time-dependent covalent binding of TA to liver tissue were evaluated in rats. These findings are consistent with and explain why initiation of bioactivation-mediated liver injury did not show a dose response (Mangipudy et al., 1995). This observation was further confirmed in rat liver microsomal incubations of ^{14}C-TA. Covalent binding of ^{14}C-TA-derived radiolabel to microsomal proteins was only increased 57-fold when ^{14}C-TA concentration was increased 100-fold. Third, additional in vitro microsomal incubation studies also support this conclusion. In rat liver microsomal incubations of TA and TASO, the calculated Km value for conversion of TASO to $TASO_2$ (0.62 mM) is 6-fold higher than the Km value for conversion of TA to TASO (0.1 mM) indicating that the second step of TA bioactivation is 6-fold less efficient. This is consistent with previous findings about the affinity of these compounds to microsomes. The Km value of TA metabolism is reported to be lower (0.06 mM) than that of TASO metabolism (0.4 mM) in microsomes (Porter and Neal, 1978).

In our study, we chose to measure urinary excretion of TA and TASO at 12 and 24 h at two different doses of TA based on previous studies reporting that most of the TA and TASO is eliminated in the urine by 24 h (Nygaard et al., 1954). The Nygaard study reported about 20% elimination as TA and 60% as sulfate using a single dose of 6 mg TA per rat after subcutaneous administration. We found less than 4% of the dose in the urine as the parent compound. The urinary elimination profile of TASO, the proximate reactive metabolite, showed 3-fold higher

excretion of TASO at the high dose than after the low dose. This finding is the fourth line of evidence which suggests that the metabolism of TASO is slower and more saturable than the metabolism of TA due to which more TASO is excreted unmetabolized relative to TA further supporting the above conclusion. There is a possibility that the excretion of TASO is higher at the high dose because of higher efficiency of transporters. Since there is not much known about the role of transporters in excretion of TA or TASO, this hypothesis remains speculative at this point.

In vitro studies were conducted to confirm the in vivo results of saturation of TA metabolism by CYP2E1. The range of TA concentrations chosen for these experiments included those comparable to the concentrations found in plasma and liver upon i.p. administration. The plasma TA levels far exceeded the Michaelis-Menten constant (Km=0.1 mM or 7.5 µg/ml) after i.p. administration of 300 and 600 mg/kg doses for a prolonged time. It should be recalled that plasma protein binding of TA is only 9.93 $\pm$ 1.16 % and does not change with increased concentration of TA. In hepatic microsomal incubations, at higher concentrations of TA approximating those seen in vivo, TA metabolism to TASO was saturable in contrast to its metabolism at the lower concentrations yielding around 60% conversion to TASO. The remaining percentage of TA is presumably metabolized to the non-toxic acetamide, and as $TASO_2$, the latter being covalently bound to the microsomes. One may expect that some of the TA could be lost to nonspecific binding in the microsomal incubations. Experiments wherein increasing concentrations of TASO were incubated with liver microsomes confirmed that TASO metabolism also follows saturable kinetics.

Incubations of TA with cDNA-expressed Supersomes® should be helpful in minimizing alternative effects due, for example, to nonspecific binding or metabolism by other enzymes.

These experiments are also useful to confirm that TA metabolism is mediated by CYP2E1. Moreover, Supersomes® are devoid of other proteins which would be present in liver microsomes and therefore, saturation of TA metabolism should occur at lower concentration of TA. Therefore, TA was incubated with rat liver specific cDNA-expressed CYP2E1 Supersomes®. In these incubations, no TA was detected at the end of 10 min incubations containing 0.01 mM TA, and at 50-fold higher concentration, the enzyme was saturated. It is apparent that CYP2E1 catalyzes at least 75% conversion of TA to TASO at 0.01 mM. Since CYP2E1 is a toxicologically significant enzyme because it bioactivates many drugs, industrial solvents, and carcinogens, it is of direct relevance to humans, we also incubated TA with the human c-DNA-expressed CYP2E1 enzyme Supersomes®. The results confirm that saturation and zero-order process occur at TA concentrations of 0.5 mM and upwards similar to the rat Supersomes®. At lower concentrations of 0.01 and 0.05 mM, 60% of TA was converted to TASO. The decision to employ only CYP2E1 Supersomes® in these experiments is based on the previous work establishing that CYP2E1 is the primary enzyme that mediates TA bioactivation (Wang et al., 2000). Other isoforms of CYP450 such as CYP1A1 and CYP2B1/2 are unlikely to be involved in its bioactivation: El-Hawari and Plaa, (1983) reported that neither induction by 3-methyl cholanthrene (CYP1A1) nor by phenobarbital (CYP2B1/2) increased liver injury of TA. Though FMO has been implicated in the bioactivation of TA, it was not found to be involved in the bioactivation of TA (Wang et al., 2000; Ramaiah et al., 2001). However, the potential contribution of these enzymes in the metabolism of TA has not been studied in Supersomes® or pure isozymes.

In conclusion, our study shows that TA exhibits dose-dependent metabolism. Our results reveal saturation of CYP2E1-catalyzed TA metabolism to TASO and metabolism of TASO to

$TASO_2$ in vivo as well as in vitro. The results of toxicokinetic studies, enzyme kinetic studies and covalent binding experiments suggest lower efficiency at the second step, which is the rate-limiting step. In conclusion, the present study has revealed that TA bioactivation follows saturable kinetics. Metabolic saturation is thus responsible for lack of dose-response for liver injury observed with TA.

References

Al-Bader A, Mathew TC, Abul H, Al-Sayer H, Singal PK, and Dashti HM (2000) Cholangiocarcinoma and liver cirrhosis in relation to changes due to thioacetamide. *Mol Cell Biochem* **208:**1-10.

Barker EA and Smuckler EA (1974) Non hepatic thioacetamide injury II. The morphological features of proximal renal tubular injury. *Amer J Pathol* **74:**575-590.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:**248-254.

Chanda S and Mehendale M (1994) Role of nutritional fatty acid and L-carnitine in the final outcome of thioacetamide hepatotoxicity. *FASEB J* **8:**1061-1068.

Chanda S and Mehendale HM (1995) Nutritional impact on the final outcome of liver injury inflicted by model hepatotoxicants: effect of glucose loading. *FASEB J* **9:**240-245.

Chu CJ, Lee FY, Wang SS, Chang FY, Lin HC, Wu SL, Chan CC, Tsai YT, and Lee SD (2000) Establishment of an animal model of hepatic encephalopathy. *Zhonghua Yi Xue Za Zhi* **63:**263-269.

Dekant W, Assmann M, and Urban G (1995) The role of cytochrome P450 2E1 in the species-dependent biotransformation of 1,2-dichloro-1,1,2-trifluoroethane in rats and mice. *Toxicol Appl Pharmacol* **135:**200-207.

El-Hawari AM and Plaa GL (1983) Potentiation of thioacetamide-induced hepatotoxicity in alloxan- and streptozotocin-diabetic rats. *Toxicol Lett.* **17:**293-300.

Gonzalez FJ, Ueno T, Umeno M, Song BJ, Veech RL and Gelboin HV (1991) Microsomal ethanol oxidizing system: transcriptional and posttranscriptional regulation of cytochrome P450, CYP2E1 *Alcohol Alcohol Suppl* **1:**97-101.

Hunter AL, Holsher MA, and Neal RA (1977) Thioacetamide- induced hepatic necrosis. I. Involvement of the mixed-function oxidase-enzyme system. *J Pharmacol Ther* **200:**439-448.

Ingelman-Sundberg M, Ronis MJ, Lindros KO, Eliasson E, and Zhukov A (1994) Ethanol-inducible cytochrome P4502E1: regulation, enzymology and molecular biology. *Alcohol Alcohol Suppl* **2:**131-139.

Kirschman JC, Brown NM, Coots RH, and Morgareidge K (1986) Review of investigations of dichloromethane metabolism and subchronic oral toxicity as the basis for the design of chronic oral studies in rats and mice. *Food Chem Toxicol* **24:**943-949.

Kreiling R, Laib RJ, Filser JG, and Bolt HM (1986) Species differences in butadiene metabolism between mice and rats evaluated by inhalation pharmacokinetics. *Arch Toxicol* **58:**235-238.

Lee KM, Muralidhara S, White CA, and Bruckner JV (2000) Mechanisms of the dose-dependent kinetics of trichloroethylene: oral bolus dosing of rats. *Toxicol Appl Pharmacol* **164:**55-64.

Lieber CS (1997) Cytochrome P-4502E1: its physiological and pathological role. *Physiol Rev* **77:**517-544.

Limaye PB, Apte UM, Shankar K, Bucci TJ, Warbritton A, and Mehendale HM (2003) Calpain released from dying hepatocytes mediates progression of acute liver injury induced by model hepatotoxicants. *Toxicol Appl Pharmacol* **191:**211-226.

Lof A and Johanson G (1993) Dose-dependent kinetics of inhaled styrene in man. *IARC Sci Publ* **127:**89-99.

Mangipudy RS, Chanda S, and Mehendale HM (1995) Tissue repair response as a function of dose in Thioacetamide hepatotoxicity. *Environ Health Perspect* **103:**260-267.

Mangipudy RS, Rao PS, Andrews A, Bucci TJ, Witzmann FA, and Mehendale HM (1998) Dose dependent modulation of cell death: Apoptosis versus necrosis in thioacetamide hepatotoxicity. *Internat J Toxicol* **17:**193-211.

Mehendale HM, Mangipudy RS, and Chanda S (1997) Thioacetamide, in *Comprehensive toxicology, Vol. IX: Hepatic and Gastrointestinal Toxicology*. pp 483-491. Elsevier, New York.

Mehendale HM (2005) Tissue repair: an important determinant of final outcome of toxicant-induced injury. *Toxicol Pathol* **33:**1-10.

Miller EC and Miller JA (1974) *Molecular Biology of Cancer* (H. Busch, ed.), p. 377. Academic Press, New York.

Nygaard O, Eldjarn L, and Nakken KF (1954) Studies on the metabolism of thioacetamide-S^{35} in the intact rat. *Cancer Res* **14:**625-628.

Porter WR and Neal RA (1978) Metabolism of thioacetamide and thioacetamide-S-oxide by rat liver microsomes. *Drug Metab Dispos* **6:**379-388.

Porter WR, Gudzinowcz MJ, and Neal RA (1979) Thioacetamide-induced hepatic necrosis. II. Pharmacokinetics of thioacetamide and thioacetamide-S-oxide in the rat. *J Pharmacol Exp Ther* **208:**386-391.

Ramaiah SK, Apte UM, and Mehendale HM (2001) Cytochrome P4502E1 induction increases thioacetamide liver injury in diet-restricted rats. *Drug Metab Dispos* **29:**1088-1095.

Ramaiah SK, Apte UM, and Mehendale HM (2000) Diet restriction as a protective mechanism in noncancer toxicity outcomes. A review. *Internat J Toxicol* **19:**413-429.

Ramaiah SK, Soni MG, Bucci TJ, and Mehendale HM (1998) Diet restriction enhances compensatory liver tissue repair and survival following administration of lethal doses of thioacetamide. *Toxicol Appl Pharmacol* **150:**12-21.

Ramsey JC and Young JD (1978) Pharmacokinetics of inhaled styrene in rats and humans**.** *Scand J Work Environ Health* **4:**84-91.

Rappaport SM, Yeowell-O'Connell K, Smith MT, Dosemeci M, Hayes RB, Zhang L, Li G, Yin S, and Rothman N (2002) Non-linear production of benzene oxide-albumin adducts with human exposure to benzene. *J Chromatogr B Analyt Technol Biomed Life Sci* **778:**367-374.

Shankar K, Vaidya VS, Wang T, Bucci TJ, and Mehendale HM (2003) Streptozotocin-induced diabetic mice are resistant to lethal effects of thioacetamide hepatotoxicity. *Toxicol Appl Pharmacol* **188:**122-134.

Tanaka E, Terada M, and Misawa S (2000) Cytochrome P450 2E1: its clinical and toxicological role. *J Clin Pharm Ther* **25:**165-175.

Vittozzi L, Gemma S, Sbraccia M, and Testai E (2000) Comparative characterization of CHCl(3) metabolism and toxicokinetics in rodent strains differently susceptible to chloroform-induced carcinogenicity. *Environ Toxicol Pharmacol* **8:**103-110.

Wang T, Shankar K, Ronis MJJ, and Mehendale HM (2000) Potentiation of thioacetamide liver injury in diabetic rats is due to CYP2E1. *J Pharmacol Exp Ther* **294:**473-479.

Wasser S and Tan CE (1999) Experimental models of hepatic fibrosis in the rat. *Ann Acad Med Singapore* **28:**109-111.

Whysner J, Conaway CC, Verna L, and Williams GM (1996) Vinyl chloride mechanistic data and risk assessment: DNA reactivity and cross-species quantitative risk extrapolation. *Pharmacol Ther* **71:**7-28.

Footnotes

Send reprint requests to:

Dr. Harihara M. Mehendale

Department of Toxicology

College of Pharmacy

The University of Louisiana at Monroe

700 University Avenue

Monroe, LA 71209-0470

USA.

E-mail: mehendale@ulm.edu

These studies were supported by the National Institutes of Environmental Health Sciences grant ES-09870 and the corresponding author was partly supported by the Louisiana Board of Regents Support Fund through The University of Louisiana Kitty DeGree Endowed Chair in Toxicology.

Legends for Figures

Fig. 1. *Thioacetamide (TA) metabolism at high doses is subject to saturation kinetics.* TA metabolism to TASO, mediated by CYP2E1, follows saturation kinetics. Further conversion to the reactive intermediate by CYP2E1 also involves saturation, resulting in disproportionate increase in covalent binding of reactive intermediate and therefore lower than expected bioactivation-based liver injury.

Fig. 2. *Plasma and hepatic concentrations of thioacetamide (TA) and thioacetamide sulfoxide (TASO) as a function of TA dose*. Male Sprague Dawley rats were treated with 50, 300 or 600 mg/kg of TA via i.p. injection in saline vehicle, and plasma was collected via serial blood sampling over a time course of 0 to 1040 min. Plasma TA **(A)** and TASO levels **(B)** were measured by reverse-phase HPLC. See Materials and Methods for detailed sample collection and HPLC procedure. Liver samples were collected at various time points after treatment with 50, 300 and 600 mg/kg TA. Hepatic TA **(C)** and TASO levels **(D)** were measured. Results are expressed as means $\pm$ SE (n=3).

Fig. 3. *Urinary elimination of TA and TASO.* Male Sprague Dawley rats were treated with 50 and 600 mg TA/kg and placed in metabolic cages (Nalgene). Urine was collected at 12 and 24 h after administration. Urinary TA and TASO concentrations were determined using reverse-phase HPLC. The values are expressed as the percent of dose excreted as TA or TASO by 12 and 24 h. Results are expressed as means $\pm$ SE (n=3). # Significantly different from TA conc. at the same dose. * Significantly different from TASO conc. at 50 mg/kg dose. $P \leq 0.05$.

Fig. 4. *Effect of dose on covalent binding of [^{14}C-TA] in TA treated rats measured over a time course.* Rats were treated with [^{14}C-TA] (50 and 600 mg/kg, i.p., 5 mCi/mmol, 25 μCi/rat). Livers were excised and flash frozen at 3, 6, 12, 16, 48, 72 and 96 h. Results are expressed as amount of TA covalently bound to protein (4A) and AUC from 0-96 h (4B). Details as under Materials and Methods. Results are expressed as means $\pm$ SE (n=3). # Significantly different from 50 mg/kg. $P \leq 0.05$.

Fig. 5. *Microsomal metabolism of TA to TASO.* Microsomes from untreated rats were incubated with TA in the presence of an NADPH-regenerating system over a range of concentrations (0.01 mM-10 mM). The reactions were carried out for 10 min. TA and TASO were measured by HPLC after stopping the reaction. The results are expressed as percentage conversion to TASO **(A)** and percentage TA remaining **(B).** Details as under Materials and Methods. Results expressed as means $\pm$ SE (n=3). **(A)** # Significantly different from 0.01 mM. **(B)** # Significantly different from 0.5 mM. * Significantly different from 10 mM. $P \leq 0.05$.

Fig. 6. *Microsomal metabolism of TASO.* Microsomes from untreated rats were incubated with TASO in the presence of an NADPH-regenerating system over a range of concentrations (0.01 mM-5 mM). The reactions were carried out for 10 min. TASO was measured by HPLC after stopping the reaction. The results are expressed as percentage TASO remaining. Details as under Materials and Methods. Results expressed as means $\pm$ SE (n=3). # Significantly different from 0.01 mM. $P \leq 0.05$.

Fig. 7. *Metabolism of TA to TASO in Rat CYP2E1* Supersomes®. TA was incubated with 10 pmol of CYP2E1 (Rat Supersomes®, Gentest) in the presence of an NADPH-regenerating system over a range of concentrations (0.01 mM-10 mM). The reactions were carried out for 10 min. TA and TASO were measured by HPLC, after stopping the reaction. The results are expressed as percentage conversion to TASO **(A)** and percentage TA remaining **(B)**. Details as under Materials and Methods. Results expressed as means $\pm$ SE (n=3). **(A)** # Significantly different from 0.01 mM. ! Significantly different from 0.05 mM. * Significantly different from 0.1 mM. **(B)** # Significantly different from 0.05 mM. ! Significantly different from 0.1 mM. $P \leq 0.05$.

Fig. 8. *Metabolism of TA to TASO in Human CYP2E1* Supersomes®. TA was incubated with 10 pmol of CYP2E1 (Human Supersomes®, Gentest) in the presence of an NADPH regenerating system over a range of concentrations (0.01 mM-10 mM). The reactions were carried out for 10 min. TA and TASO were measured by HPLC, after stopping the reaction. The results are expressed as percentage conversion to TASO **(A)** and percentage TA remaining **(B)**. Details as under Materials and Methods. Results expressed as means $\pm$ SE (n=3). **(A)** # Significantly different from 0.01 mM. ! Significantly different from 0.1 mM. **(B)** # Significantly different from 0.05 mM. ! Significantly different from 0.1 mM. $P \leq 0.05$.

DMD Fast Forward. Published on September 23, 2005 as DOI: 10.1124/dmd.105.005520
This article has not been copyedited and formatted. The final version may differ from this version.

TABLE 1

Ratio of TASO: TA in plasma after administration of TA at 12- fold dose range

Time (in min)	Dose of thioacetamide (mg/kg)		
	50	300	600
5	0.15 ± 0.03	0.03 ± 0.01	0.02 ± 0.004
15	0.3 ± 0.05	0.08 ± 0.02	0.02 ± 0.003
30	0.62 ± 0.03	0.16 ± 0.01	0.04 ± 0.003
45	1.24 ± 0.14	0.21 ± 0.01	0.06 ± 0.002
60	7.41 ± 0.89	0.22 ± 0.07	0.08 + 0.01

Note: Plasma TASO/TA values are represented for each time point. Plasma TA and TASO values were obtained from the same experiments as presented in **Fig. 2. A** & **B**. Results are expressed as means ± SE (n=3).

TABLE 2

Toxicokinetic Parameters Estimated for TA and TASO following ip Administration of 50, 300 and 600 mg/kg doses of TA

Parameter	Dose of thioacetamide (mg/kg)					
	50		300		600	
	TA	TASO	TA	TASO	TA	TASO
T_{max} (min.)	5	90	5	180	5	180
C_{max} (μg/ml)	30.5 ± 2.7	19.6 ± 1.12	198 ± 27.3	50.2 ± 2.42	586.8 ± 31.29	77 ± 2.9
AUC (μg.min/ml)	802 ± 9	2037 ± 71	26509 ± 1421.6	15443± 1631	141460± 23434	53198± 3399
AUC/dose	16 ± 2	40.7 ± 1.4	88.4 ± 4.7	51.5 ± 5.4	235.8 ± 39.1	88.7 ± 5.7
Apparent $t_{½}$ (min)	16 ± 1	36 ± 1.2	74.5 ± 4	81.4 ± 16.7	241.36± 21.6	247 ± 41.7
Plasma Clearance (l/kg.hr)	1.63 ± 0.21	0.82 ± 0.06	0.30 ± 0.01	0.15 ± 0.04	0.14 ± 0.01	0.04 ± 0.01

Note: Plasma TA and TASO concentrations were determined using reversed-phase HPLC. Male Sprague Dawley rats were treated with 50, 300 or 600 mg TA/kg via i.p. injection in saline vehicle, and plasma was collected via serial blood sampling over a time course of 0 to 1040 min. See Materials and Methods for detailed sample collection and HPLC procedure. Results are expressed as means ± SE (n=3).

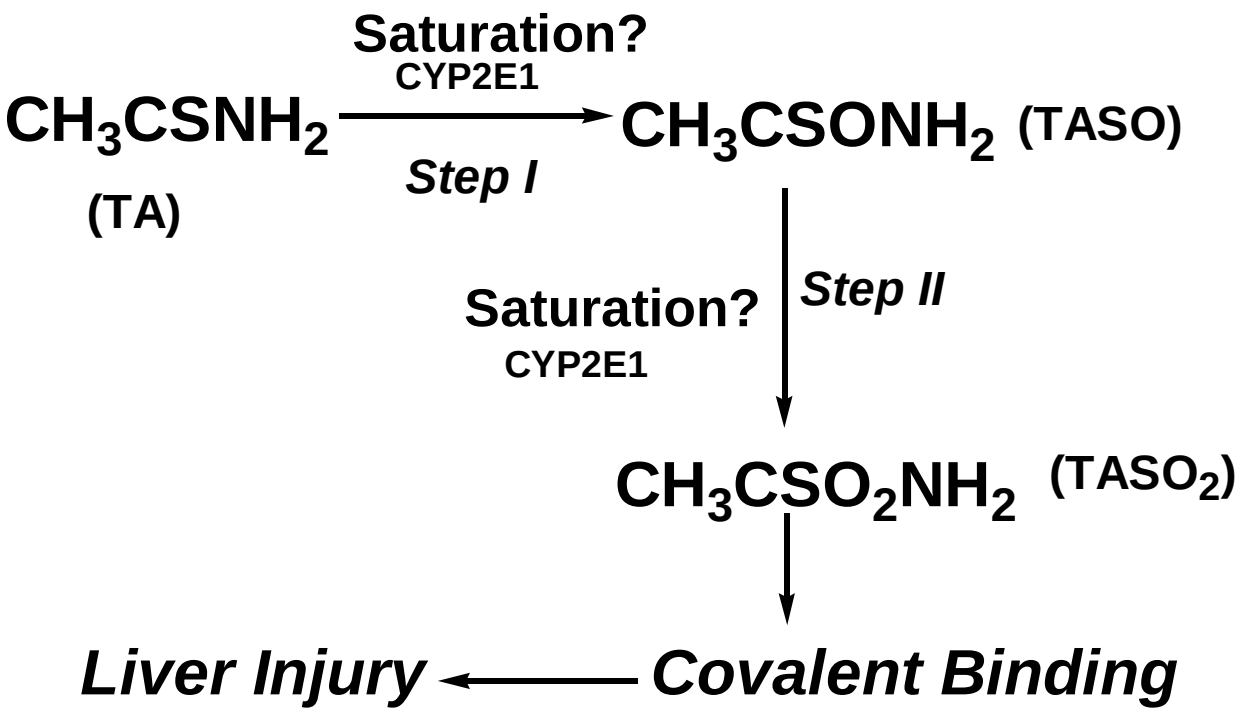

Fig. 1.

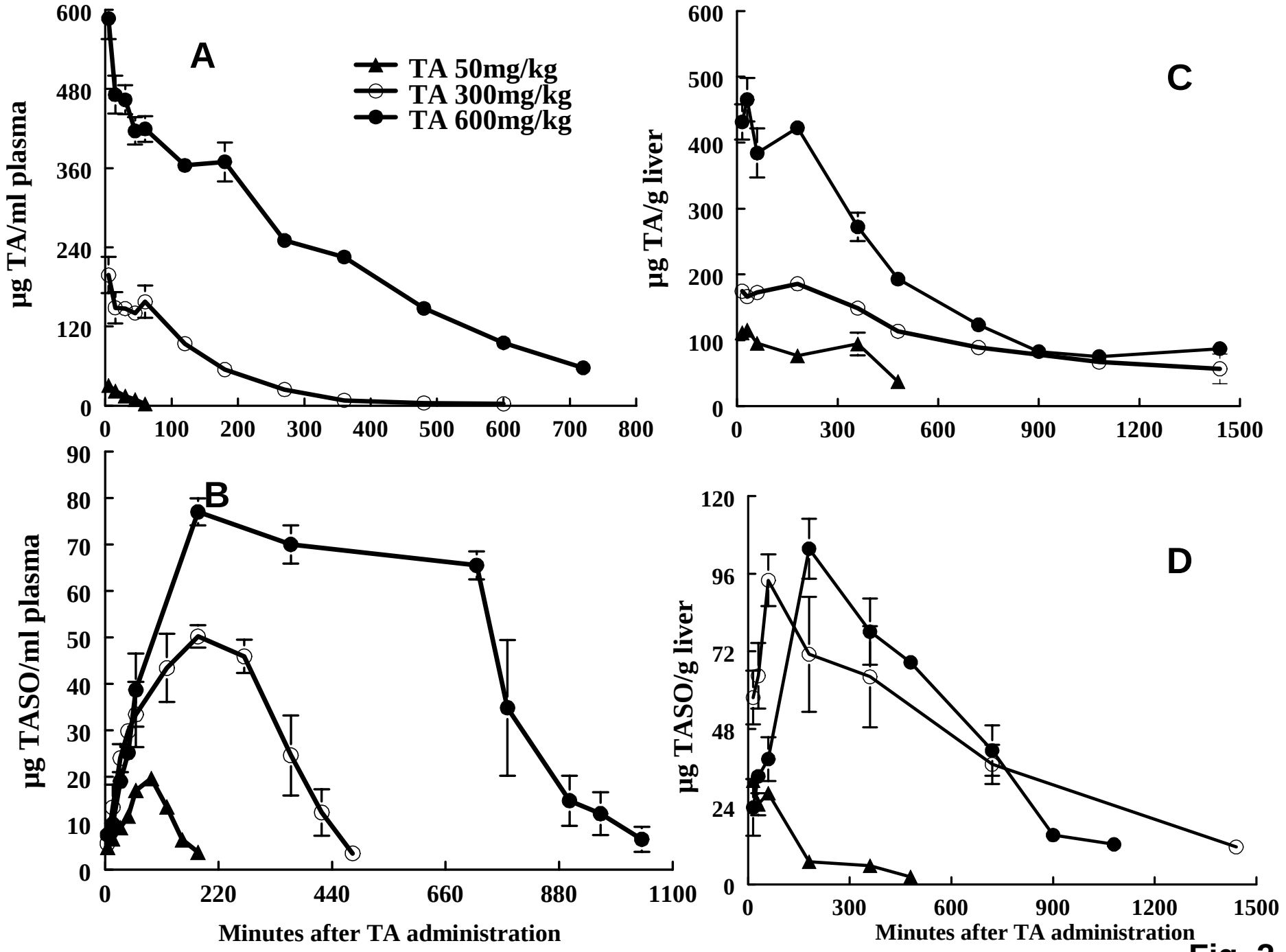

Fig. 2.

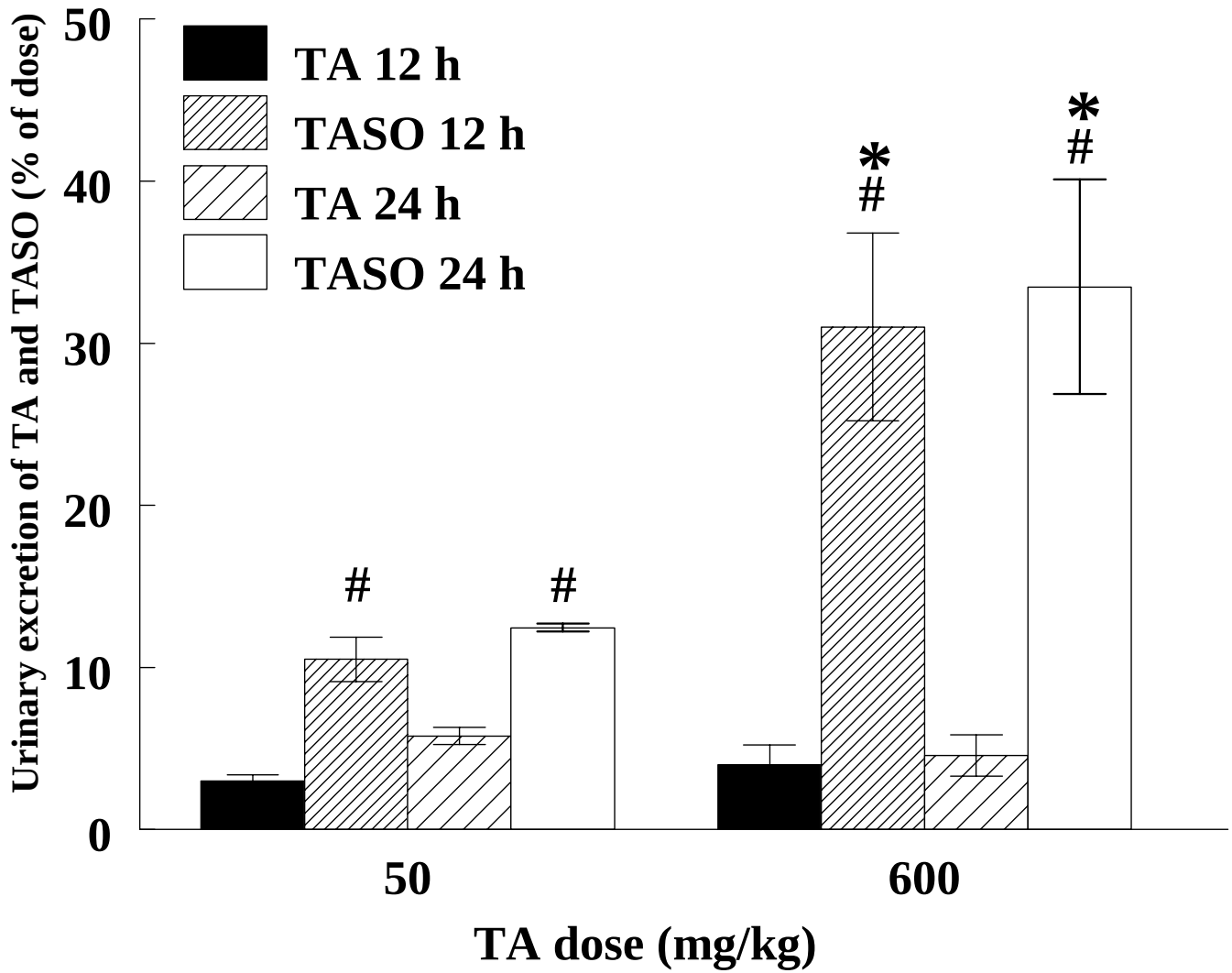

Fig. 3.

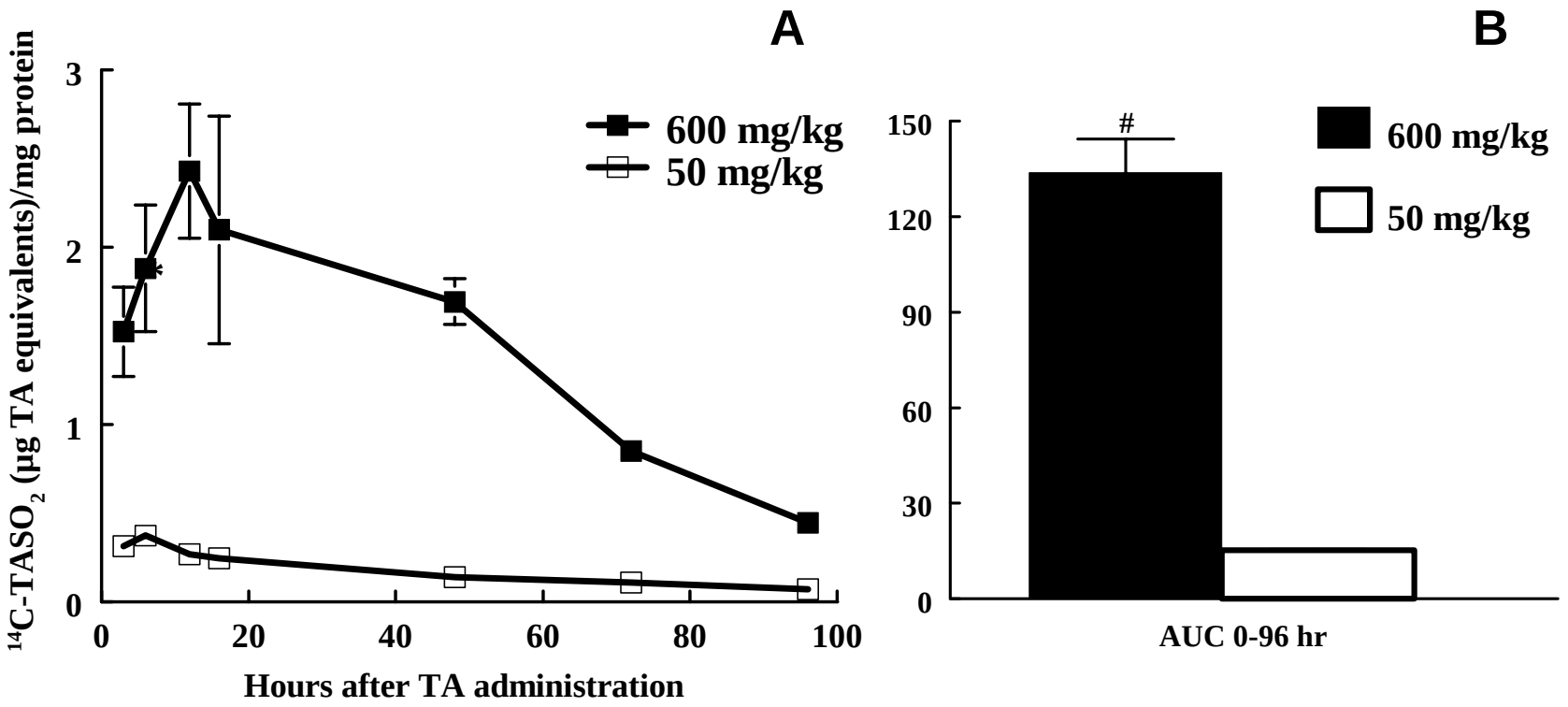

Fig. 4.

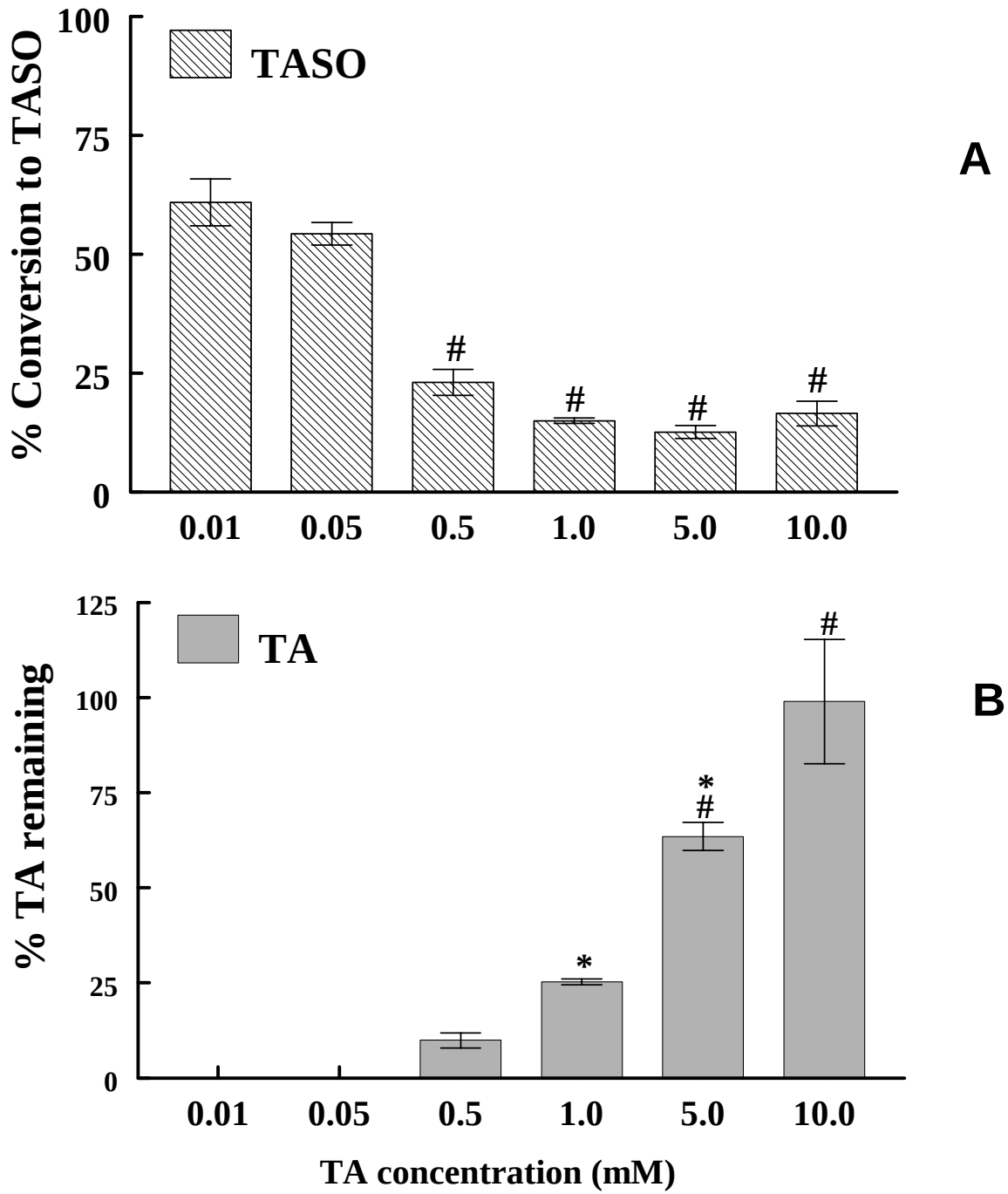

Fig. 5.

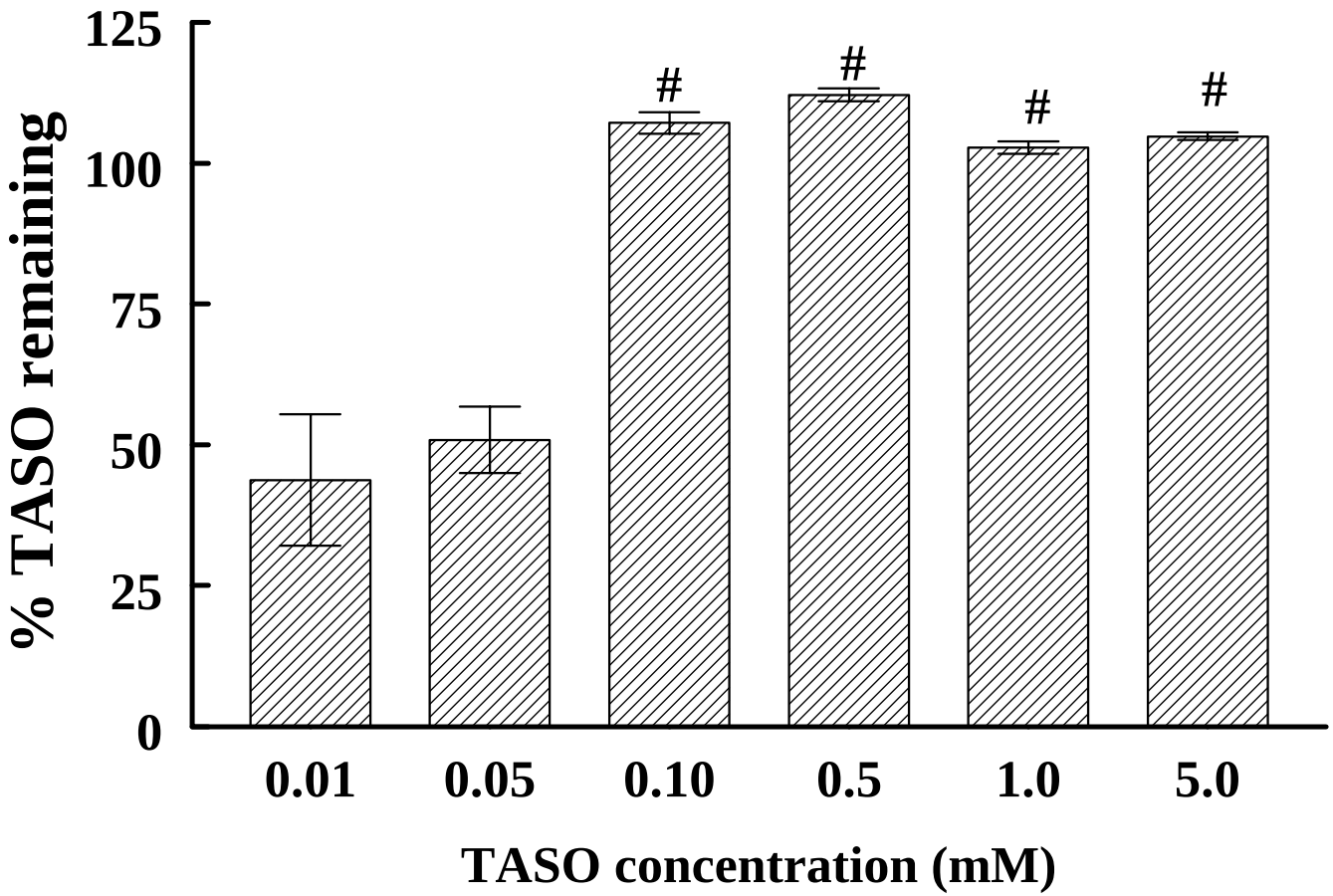

Fig. 6.

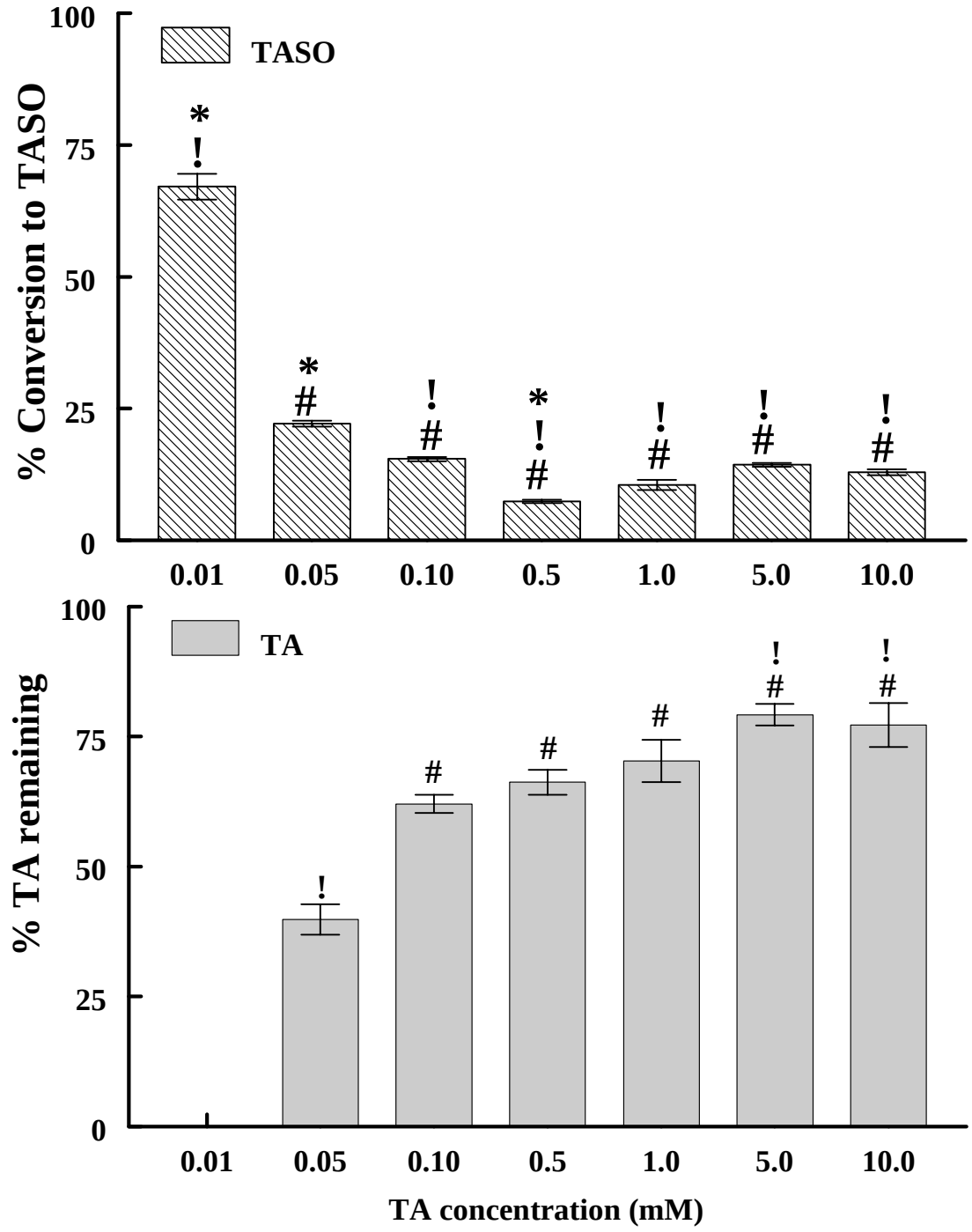

Fig. 7.

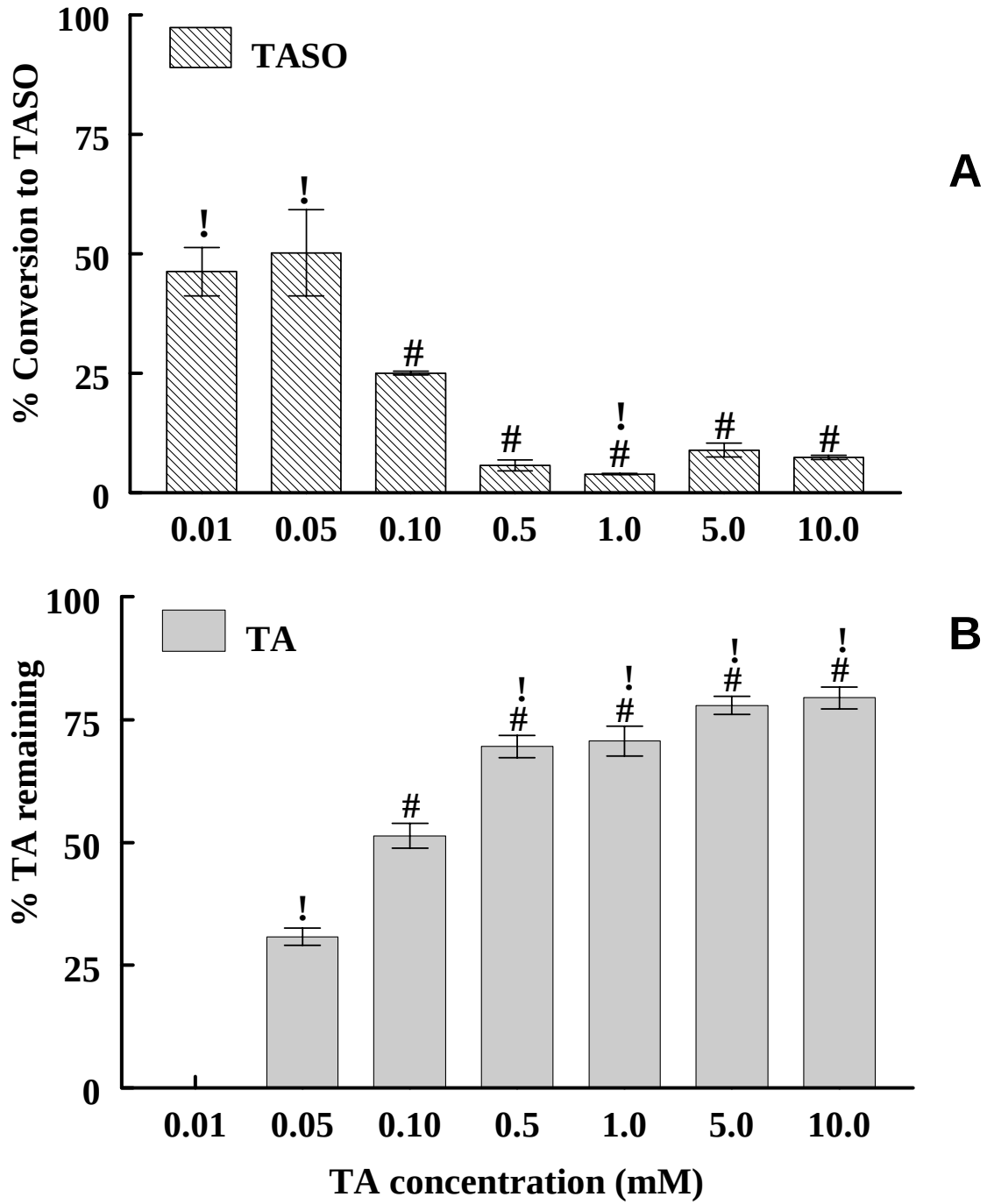

Fig. 8.