

Alteration of Catecholamines in Pheochromocytoma (PC12) Cells *in Vitro* by the Metabolites of Chlorotriazine Herbicide

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Received August 1, 2000; accepted September 20, 2000

The effects of four major chlorotriazine metabolites on the constitutive synthesis of the catecholamines dopamine (DA) and norepinephrine (NE) were examined, using undifferentiated PC12 cells. NE release and intracellular DA and NE concentrations were quantified, for up to 24 h after initiation of treatment with different concentrations, ranging from 0 to 400 μM , of the metabolites hydroxyatrazine (HA), 2-amino-4-chloro-6-isopropylamino-*s*-triazine (deethylchlorotriazine), 2-amino-4-chloro-6-ethylamino-*s*-triazine (deisopropylchlorotriazine), and diaminochlorotriazine (DACT). Hydroxyatrazine significantly decreased intracellular DA and NE concentrations in a dose- and time-dependent manner. This metabolite also caused a significant inhibition of NE release from the cells. In contrast, deethylchlorotriazine and deisopropylchlorotriazine significantly increased intracellular DA concentration following exposure to 50–200 μM from 12 to 24 h. Intracellular NE was significantly reduced at these same concentrations of deethylchlorotriazine at 24 h while the concentration of NE in PC12 cells exposed to deisopropylchlorotriazine was not altered at any dosage or time point measured. NE release was decreased at 18 (200 μM) and 24 h (100 and 200 μM) following exposure to deethylchlorotriazine and at 24 h (100 and 200 μM) following deisopropylchlorotriazine. DACT, at the highest concentration (160 μM), significantly increased intracellular DA and NE concentrations at 18 and 24 h. NE release was also increased at 40–160 μM at 24 h. The viability of the PC12 cells was tested using the trypan blue exclusion method. Following 18 to 24 h of treatments with HA, cell viability was reduced 10–12% at the two higher concentrations (200 and 400 μM), but, with other metabolites, the viability was reduced by only 2 to 5% at the highest concentrations. These data indicate that HA affects catecholamine synthesis and release in PC12 cells in a manner that is similar to synthesis of atrazine and simazine. On the other hand, deethylchlorotriazine and deisopropylchlorotriazine altered catecholamine synthesis in a manner similar to that observed in the rat

brain following *in vivo* exposure (i.e., increased DA and decreased NE concentration), whereas DACT appeared to produce an increase in NE release as well as in the intracellular DA and NE concentrations. Overall, these findings suggest that the catecholamine neurons may be a target for the chlorotriazines and/or their metabolites, that the metabolites produce a unique pattern of catecholamine response, and that all of the changes were seen within the same range of doses.

Key Words: chlorotriazine metabolites; hydroxyatrazine; deethylchlorotriazine; deisopropylchlorotriazine; diaminochlorotriazine; PC12 cells; dopamine; norepinephrine.

Chlorotriazine herbicides have been widely used in the United States and other countries worldwide for about 40 years (Eldridge *et al.* 1994; U.S. EPA, 1994). Specifically, these compounds block photosynthesis by inhibiting the function of the psbA gene (Worthing and Walker, 1987). The environmental fate of atrazine has been investigated extensively over the last 4 decades, and its retention and transport in soil have been reviewed in recent years (Flury, 1996; Koskinen and Clay, 1997; Ma and Selim, 1996). Atrazine is persistent in the soil (Seiler *et al.*, 1992), and has been detected in ground and surface waters at concentrations exceeding the Environmental Protection Agency's maximum contaminant level of 3 ppb (Kello, 1989).

The metabolites identified in the environment and in the urine of mammals (including humans) are presented in Figure 1. In the environment, one of the major atrazine degradation pathways is chemical hydrolysis to 6-hydroxyatrazine in the surface soil (Harris, 1967; Muir and Baker, 1978), something that is favored by low pH (5.5–6.5), elevated moisture content, high temperature, and increased organic matter levels (Ahrens, 1994; Koskinen and Clay, 1997; Ma and Selim, 1996). Microbial degradation also takes place in the soil. This process yields 6-chloro-N-[1-methyl-ethyl]-1,3,5-triazine-2,4-diamine and 6-chloro-N-ethyl-1,3,5-triazine-2,4-diamine. In addition, atrazine is also degraded by microbial enzymes to 6-hydroxyatrazine (De Souza *et al.*, 1998; Mandelbaum *et al.*, 1993). Photodegradation of atrazine can also occur in the soil. This process also produces deethylated atrazine

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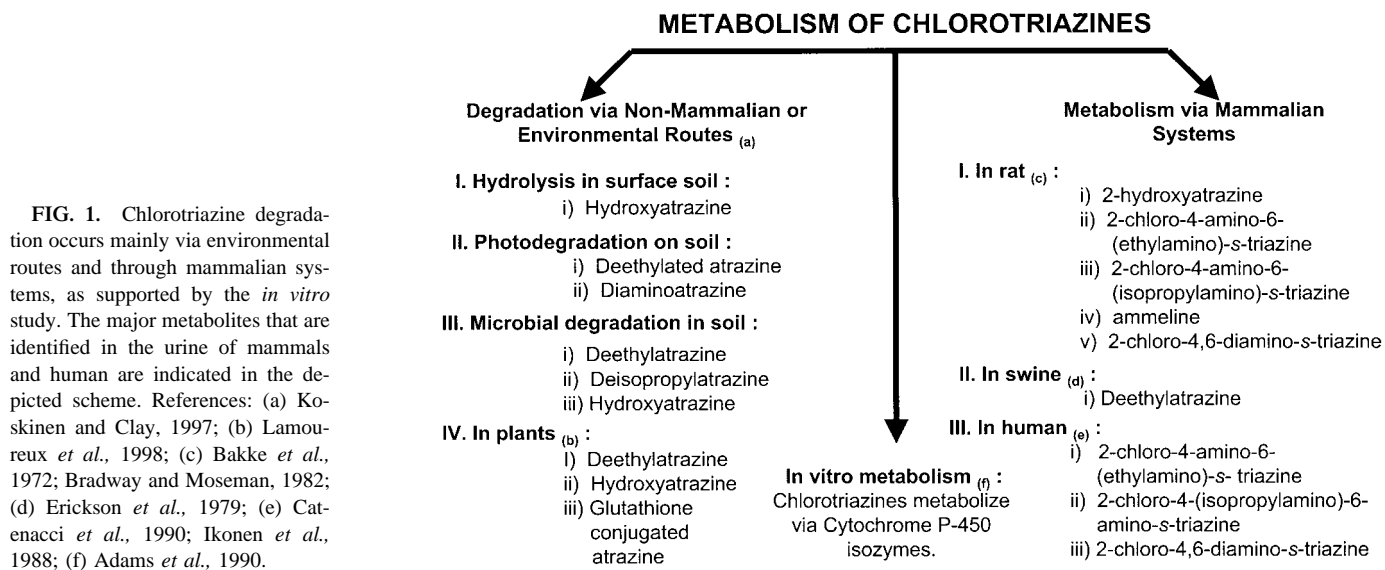


FIG. 1. Chlorotriazine degradation occurs mainly via environmental routes and through mammalian systems, as supported by the *in vitro* study. The major metabolites that are identified in the urine of mammals and human are indicated in the depicted scheme. References: (a) Koskinen and Clay, 1997; (b) Lamoureux *et al.*, 1998; (c) Bakke *et al.*, 1972; Bradway and Moseman, 1982; (d) Erickson *et al.*, 1979; (e) Catenacci *et al.*, 1990; Ikonen *et al.*, 1988; (f) Adams *et al.*, 1990.

(deethylchlorotriazine) and N-deethyl-N-demethylethyl atrazine (i.e., diaminochlorotriazine) (Ahrens, 1994; Koskinen and Clay, 1997; Ma and Selim, 1996). The microbial degradation of chlorinated products of atrazine has higher mobility and greater potential to contaminate groundwater than hydroxyatrazine (Sorenson *et al.*, 1993), which is strongly adsorbed onto surface soils (Ma and Selim, 1996). The half-lives for deethylatrazine (26 days), deisopropylchlorotriazine (17 days) and hydroxyatrazine (121 days) have been documented in soil samples from western Tennessee (Winkelman and Klaine, 1991), whereas in a sandy-till aquifer, the half-lives of these metabolites is considerably longer, ranging from 2700 to 3400 days (Levy and Chesters, 1995). No significant degradation of atrazine occurred after 270 days in aquifer slurries in Wisconsin, indicating that atrazine itself is resistant to degradation in groundwater and aquifer sediments (Rodriguez and Harkin, 1997), and persists for a long time in the environment, potentially affecting human and wildlife populations.

The metabolism of the chlorotriazines by different species has also been studied. *In vitro* studies, using livers of rat, mouse, goat, sheep, pig, rabbit and chicken, revealed that atrazine is metabolized in two phases. First, atrazine is catalyzed by cytochrome P450 to form the monodealkylated metabolites of atrazine (Adams *et al.*, 1990; Hanioka *et al.*, 1998). These metabolites are then conjugated with glutathione at the 2 position (Gudderwar and Dauterman, 1979). Atrazine itself can also be partly conjugated with glutathione at the 2 position (Adams *et al.*, 1990). The chemical structures of chlorotriazines and their major metabolites are presented in Figure 2. In the female rat, four types of urinary metabolites have been reported, including 2-hydroxyatrazine, two monodealkylated analogs [2-chloro-4-amino-6-(ethylamino)-s-triazine, 2-chloro-4-amino-6-(isopropylamino)-s-triazine], and ammeline (Bakke *et al.*, 1972). Diaminochlorotriazine was also identified in the blood of adult male rats (Bradway and Moseman, 1982). In swine, the primary urinary metabolite is deethylchloro-

triazine (Erickson *et al.*, 1979), whereas in occupationally exposed male humans, the two monodealkylated analogs (deethylchlorotriazine and deisopropylchlorotriazine) were present in urine (Catenacci *et al.*, 1990; Ikonen *et al.*, 1988).

Chlorotriazines and their metabolites have been reported to alter physiological processes. Chronic feeding studies revealed that dietary atrazine exposure (at 75 and 400 ppm in food) of Sprague-Dawley female rats caused premature reproductive senescence and an earlier onset of mammary gland tumors (Eldridge *et al.*, 1994; Pinter *et al.*, 1990; Stevens *et al.*, 1994; Thakur *et al.*, 1992; Wetzel *et al.*, 1994). Similar treatment in Fischer-344 females was without effect. It was hypothesized that this difference in response was the consequence of the different pattern of reproductive aging that is normally present in these two strains. Other studies have shown that atrazine can disrupt normal ovarian cycling in young adult female rats (Cooper *et al.*, 1996; Eldridge *et al.*, 1994, 1999). Cooper and

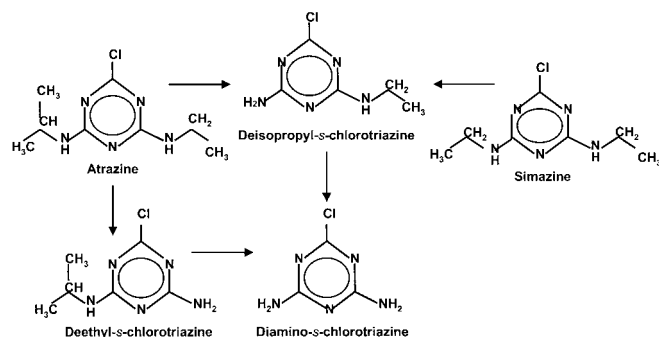


FIG. 2. Chemical structures of chlorotriazine herbicides, and the major metabolites. Atrazine, 2-chloro-4-ethylamino-6-isopropylamino-s-triazine; simazine, 2-chloro-4,6-bis(ethylamino)-s-triazine; deisopropyl-s-chlorotriazine, 2-chloro-4-ethylamino-6-amino-s-triazine; deethyl-s-chlorotriazine, 2-chloro-4-amino-6-isopropylamino-s-triazine; diamino-s-chlorotriazine, 2-chloro-4,6-diamino-s-triazine (Budavari, 1996).

colleagues have examined the mechanisms responsible for the alterations of ovarian cycling (Cooper *et al.*, 1996, 1999, 2000a) and found that atrazine causes a dose-dependent decrease in the ovulatory surge of luteinizing hormone (LH) and a concomitant suppression of prolactin secretion from the pituitaries of Sprague-Dawley and Long-Evans hooded rats (Cooper *et al.*, 2000). Atrazine was also found to cause a dose-dependent inhibition of suckling-induced prolactin release in the lactating female Wistar rat (Stoker *et al.*, 1999). Several experiments have been conducted to demonstrate that the primary site of atrazine action is within the hypothalamus (Cooper *et al.*, 2000a). It can cause a decrease in hypothalamic norepinephrine (NE) and an increase in hypothalamic dopamine (DA) concentrations (Cooper *et al.*, 1998), effects that are consistent with a decrease in LH and prolactin secretion, respectively. Numerous studies have shown that decreasing hypothalamic NE concentration will inhibit the pulsatile release of gonadotropin releasing hormone (GnRH) and the LH surge (Barracough, 1992; Ramirez *et al.*, 1984; Kalra and Kalra, 1983) and that DA inhibits prolactin secretion (Ben-Jonathan, 1985). The possibility that these catecholaminergic neurons are direct targets for atrazine and other chlorotriazines was further suggested in a recent study using the sympathetic neuronal PC12 cell line (Das *et al.*, 2000). Atrazine, simazine and cyanazine were shown to modulate the constitutive synthesis of DA and NE suggesting that the atrazine-induced reduction in hypothalamic NE could well be the result of the direct effect of atrazine or its metabolites on the CNS cells.

In the present study, we used undifferentiated PC12 cells to examine the effect of four primary metabolites of atrazine on catecholamine metabolism in an attempt to determine whether any of these compounds would modify catecholamine synthesis and release, to characterize the pattern of change present, and to establish the relative potencies for such effects. The metabolites selected are shown in Figure 2. Recent studies in our laboratory indicate that several of these metabolites, administered *in vivo*, affect ovarian cyclicality (Cooper *et al.*, 2000b), producing effects consistent with those of atrazine. PC12 cells have been well characterized and are very similar to the sympathetic neurons in mammals, and provide an *in vitro* alternative testing model (Greene and Tischler, 1976, 1982) to examine whether the major chlorotriazine metabolites alter the constitutive synthesis of dopamine and norepinephrine.

MATERIALS AND METHODS

Chemicals/reagents. Specific chemicals/reagents include Dulbecco's Modified Essential Medium (D-MEM) with L-glutamine and high glucose and D-MEM/F-12 medium, fetal bovine serum (FBS), horse serum (HS), 1 M HEPES buffer, 7.5% (w/v) NaHCO₃ solution, and other tissue-culture related products were purchased from Gibco BRL, Life Technologies, Grand Island, NY. Rat tail collagen type VII, bovine serum albumin (BSA) initial fraction, and L-ascorbic acid, were obtained from Sigma Chemical Co., St. Louis, MO. Tissue-culture flasks, cell-culture clusters, and all other tissue-culture related plastic wares were purchased from Corning Costar Corp., Cambridge, MA.

Hydroxyatrazine (HA), 2-amino-4-chloro-6-isopropylamino-*s*-triazine (deethylchlorotriazine), 2-amino-4-chloro-6-ethylamino-*s*-triazine (deisopropylchlorotriazine), and diaminochlorotriazine (DACT) were generously provided by Novartis Crop Protection, Inc., Greensboro, NC, and their purity was 97.1%, 98.2%, 94.5%, and 95.7% respectively. The composition of about 2–5% of impurities in these compounds is not known. HPLC-grade water previously filtered and deionized through a Milli-Q Water System, Millipore Corporation, Bedford, MA, was used to prepare all buffers. All 4 chlorotriazine metabolites, HA, deethylchlorotriazine, deisopropylchlorotriazine, and DACT were dissolved in 100% ethyl alcohol as stock solutions at 15–30 mM concentration and used after a serial dilution in culture medium. A comparable concentration of ethyl alcohol (maximum 26.8 μ l/ml of media or less) was added into each corresponding control in all experiments.

PC12 cell culture. PC12 cells were originally generated and described in detail by Greene and Tischler (1976) and generously provided by Dr. Tim Shafer (Neurotoxicology Division, NHEERL, U.S. EPA, RTP, NC). They were grown in 75 cm², 0.2 mm vented cap tissue culture flasks containing D-MEM; 4,500 mg/l D-glucose, 7.5% fetal bovine serum (FBS), 7.5% horse serum (HS), 2 mM L-Glutamine, 2 mM HEPES buffer and 44 mM NaHCO₃ at 37°C under an atmosphere of 5% CO₂ plus 95% air. The culture medium was replenished at 4-day intervals based on the doubling time of PC12 cells (Greene and Tischler, 1976). Previous experiments in our lab have shown that the catecholamine synthesis in the PC12 cells can be stimulated or inhibited by a number of compounds (Das *et al.*, 2000). All experiments were performed by using the same stock of cells in a passage number of 9-to-11, to keep inter-experimental variability at a minimum. Cells were plated in 24-well rat collagen pre-coated cell culture plate, containing 0.5 ml complete D-MEM culture medium in each well, at a density of 2.5×10^5 cells/well, and incubated for 2 h. Then the D-MEM culture medium was replaced with D-MEM/F-12 medium supplemented with 2 mM L-glutamine, 2 mM HEPES buffer, 2.5 g/l BSA, 44 mM NaHCO₃ solution, and 1 mM L-ascorbic acid (added immediately before use), and incubated for ~12 h. The medium was then replaced with either fresh complete D-MEM/F-12 medium plus different concentrations of vehicle or HA, deethylchlorotriazine, deisopropylchlorotriazine, or DACT along with a standard concentration of a stimulatory (e.g., forskolin) or inhibitory (e.g., fusaric acid) compound (as positive or negative internal controls). Cells were incubated for the specified time periods as indicated.

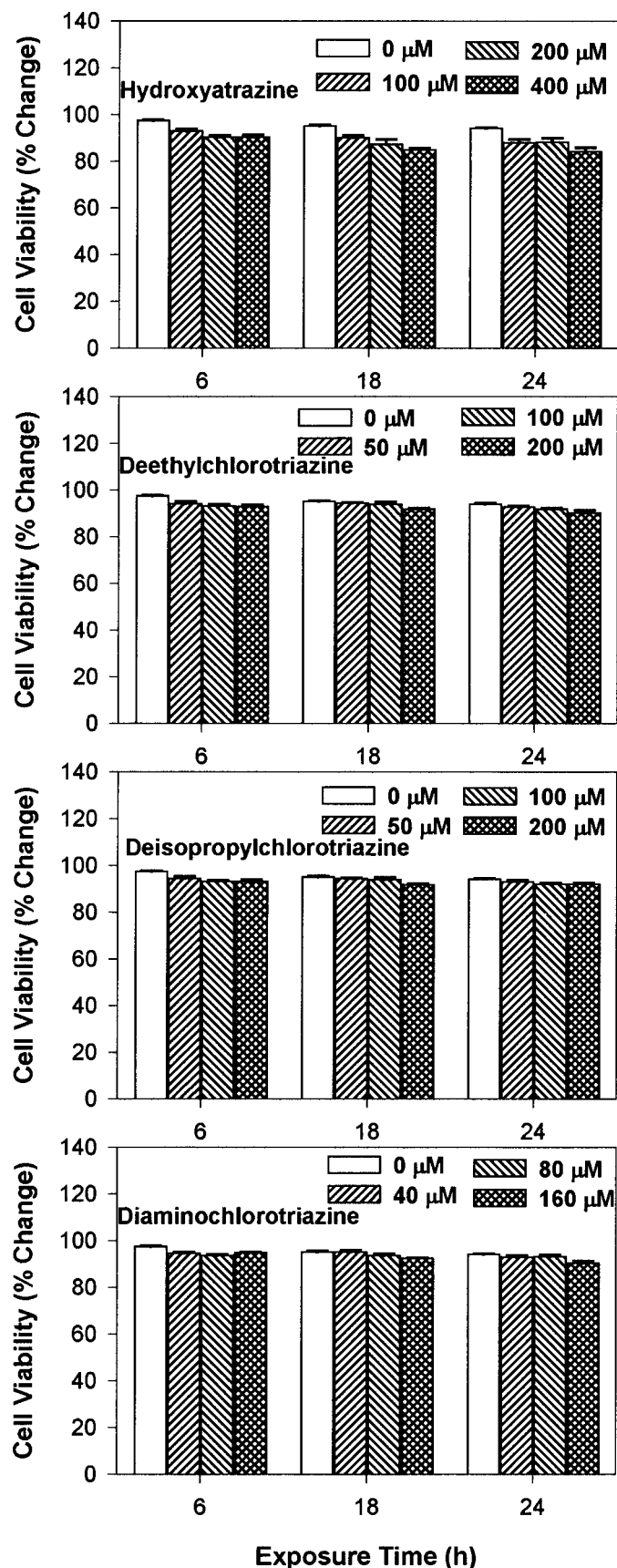
Quantification of Dopamine and Norepinephrine

Treatment of cells. A similar treatment paradigm was followed with the metabolites as reported earlier for atrazine, simazine and cyanazine (Das *et al.*, 2000). Briefly, PC12 cells were exposed to HA (0.0, 6.25, 12.5, 25.0, 50.0, 100.0, 200.0, or 400.0 μ M) for 3, 6, 12, 18, or 24 h; deethylchlorotriazine and deisopropylchlorotriazine (0.0, 12.5, 25.0, 50.0, 100.0, or 200.0 μ M) and DACT (0, 10, 20, 40, 80, or 160 μ M), for 6, 12, 18, or 24 h. These concentrations are equivalent to 1.23–78.88 ppm for HA, 2.34–37.4 ppm for deethylchlorotriazine, 2.16–34.6 ppm for deisopropylchlorotriazine, and 1.45–55.29 ppm for DACT. These values were selected for comparison with concentrations of parent atrazine, simazine, and cyanazine shown to inhibit or stimulate the constitutive synthesis and release of catecholamines in PC12 cells (Das *et al.*, 2000). The experiments were duplicated.

Collection of culture medium and cells. Following the specified treatment period, both culture medium and cells devoid of medium, were harvested in chilled HPLC mobile-phase buffer containing 4% acetonitrile, 115 mM Na₂HPO₄, 0.19 mM EDTA, 3 mM 1-heptanesulfonic acid sodium salt in HPLC-grade water, previously filtered and deionized through a Milli-Q Water System, and stored immediately at –20°C until assayed for DA and NE by HPLC.

Cell Viability Following Treatment

Trypan blue exclusion assay. Prior to examining changes in catecholamine release or intracellular concentration, the viability of the cells exposed to the different concentrations of the metabolites was examined. Following



treatment with different concentrations of the 4 chlorotriazine metabolites for the specified time periods (i.e., HA [0, 100.0, 200.0, and 400.0 μM], deethylchlorotriazine and deisopropylchlorotriazine [0, 50.0, 100.0, and 200.0 μM], and DACT [0, 40, 80, and 160 μM], for 6, 18, and 24 h), the cells were harvested to be evaluated for viability as cell suspensions in isotonic PBS solution. Trypan blue exclusion assay was used to determine viability of cells using a hemocytometer. The numbers of viable/live cells in suspension were counted by trypan blue (0.4% in PBS) exclusion in a hemocytometer and routinely contained 97–98% viable/live cells at the time of each experiment.

Quantification of DA and NE concentrations by HPLC with ECD. At each of the specified time points following exposure, the concentrations of DA and NE released into the medium and in the cell suspensions (i.e., intracellular contents) were determined by HPLC and electrochemical detection (ECD) as described previously (Goldman *et al.*, 1994) using a mobile phase buffer containing 4% acetonitrile. Prior to the assay the medium was diluted in HPLC mobile phase buffer and centrifuged at 13,000 rpm for 3 min. to remove suspended cells or any cell debris. To determine intracellular DA and NE, the cells in HPLC mobile-phase buffer were sonicated briefly (10 s) using a Fisher Model 300 sonic dismembrator and centrifuged at 13,000 rpm at 4°C for 15 min. The supernatants were further diluted in HPLC mobile phase buffer and used for the subsequent determination of DA and NE concentrations. The concentration of DA in the medium was very low or undetectable.

Statistical analysis of data. Data are expressed as mean \pm SEM and analyzed by 2-way ANOVA (using Sigma Plot Software, and GraphPad InStat, GraphPad Software, San Diego, CA; and SAS, SAS Institute Inc., Cary, NC). The Bonferroni Multiple Comparison Test was performed to evaluate the level of significance between control and the specific treatment groups. The level of significance for all statistical test was set at $\alpha = 0.05$. All comparisons were made with concomitantly run controls so that inter-assay variance would not confound the results.

RESULTS

Effect of Chlorotriazine Metabolites on Cell Viability

The viability of cells in the HA-, deethylatrazine-, deisopropylatrazine-, and DACT-treated groups and their corresponding controls were expressed as the % change, and data are summarized from a representative experiment (Fig. 3). A 10–12% decrease in the viability was observed in PC12 cells treated with the higher concentrations of HA (200 and 400 μM) at 18 and 24 h. Deethylchlorotriazine, deisopropylchlorotriazine, and DACT only slightly reduced (2–5%) the viability following exposure to higher concentrations at longer time points. Thus, higher concentrations of HA may have had an impact on the catecholamines in these experiments; lower concentrations (6.25–100 μM HA) were effective in altering

FIG. 3. Viable cells from 6–24 h (% change) following treatment with hydroxyatrazine (HA), 2-amino-4-chloro-6-isopropylamino-*s*-triazine (deethylatrazine), 2-amino-4-chloro-6-ethylamino-*s*-triazine (deisopropylatrazine), and diaminochlorotriazine (DACT). PC12 cell cultures were exposed to HA with 0 μM , 100 μM , 200 μM , and 400 μM concentrations for the indicated time points. Similarly, PC12 cell cultures were treated respectively with deethylatrazine and deisopropylatrazine 0 μM , 50 μM , 100 μM , and 200 μM concentrations, respectively. They were also exposed to DACT with 0 μM , 40 μM , 80 μM , and 160 μM concentrations for the indicated time points. Each bar is the % change value of the mean of 6 cultures of a representative experiment. Data were duplicated in the subsequent experiment.

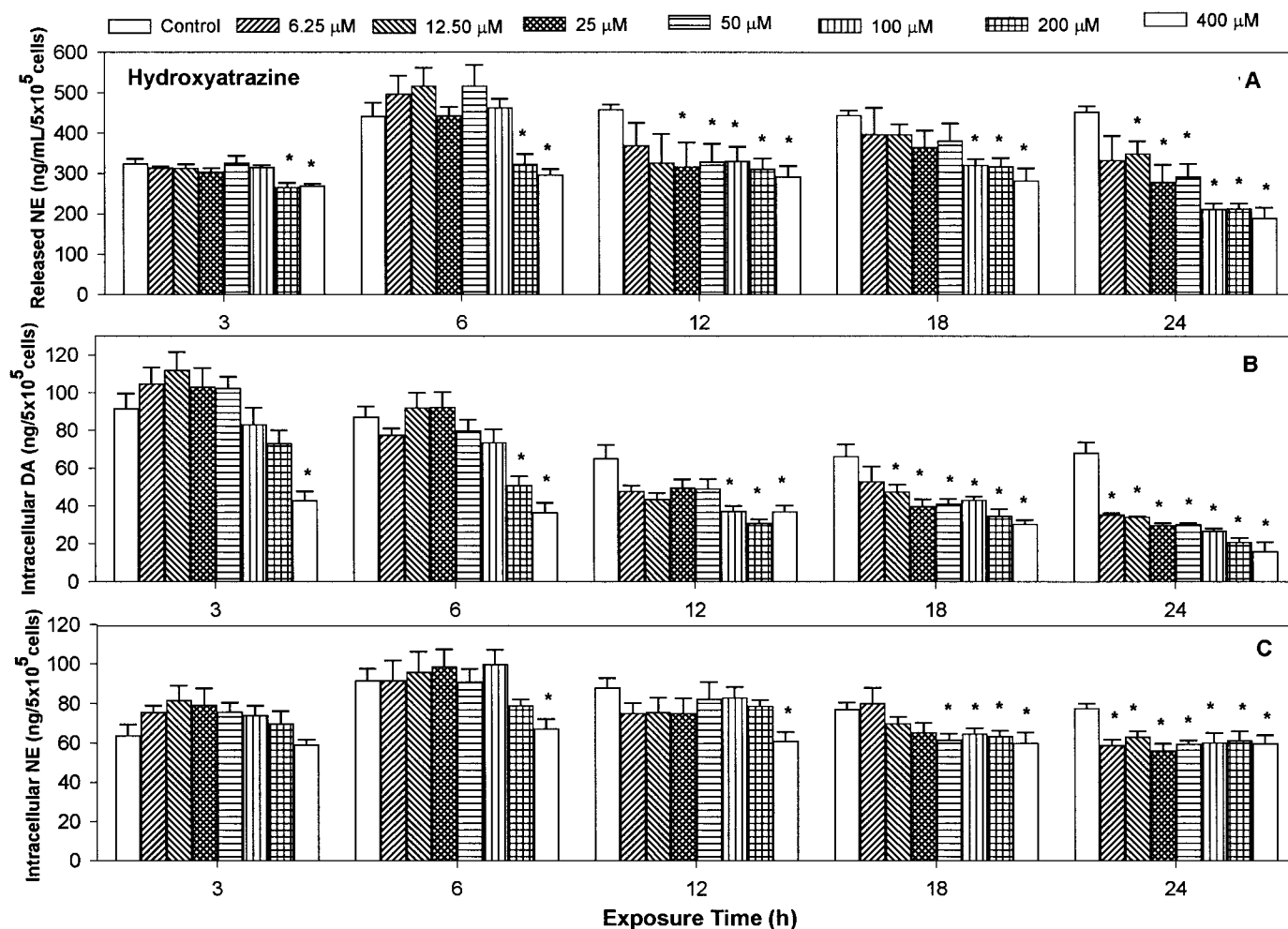


FIG. 4. Time course of released NE and intracellular DA and NE concentrations following exposure to hydroxyatrazine (HA). PC12 cell cultures were exposed to HA with 0 μM , 6.25 μM , 12.5 μM , 25 μM , 50 μM , 100 μM , 200 μM , and 400 μM concentrations for the indicated time points. A represents the amount of NE released into the medium, while B and C represent the intracellular DA and NE levels, respectively. Each bar is the value of the mean \pm SEM of ≥ 12 cultures. Data were duplicated in the subsequent experiment; * indicates the significance level ($p < 0.05$) at each time point when compared to the respective control.

the catecholamine levels when viability was not altered. The effectiveness of deethylchlorotriazine, deisopropylchlorotriazine, and DACT in altering catecholamines was not related to an effect on cell viability.

Effect of Hydroxyatrazine on the Intracellular DA and NE Concentrations and NE Release

The intracellular concentrations of DA and NE and the level of NE in the medium following exposure to hydroxyatrazine (HA) are shown in Figure 4. At each time point except at 3 h, there was a dose- and time-dependent decrease in the intracellular DA concentration. Similarly, intracellular NE concentration in these cells was reduced by exposure to 50 μM to 400 μM HA at 18 through 24 h. Norepinephrine release was also suppressed in a dose- and time-dependent manner. HA (200–400 μM) decreased the amount of NE released into the me-

dium at 3 and 6 h, while 25–400 μM HA significantly reduced the release at 12 through 24 h.

Effect of Deethylchlorotriazine and Deisopropylchlorotriazine on the Intracellular DA and NE Concentrations and NE Release

Exposure to deethylchlorotriazine and deisopropylchlorotriazine resulted in a change in catecholamine synthesis that was different from that observed following HA. As shown in Figure 5, deethylchlorotriazine increased the concentration of intracellular DA following 100–200 μM at 12 h, and 50–200 μM at 18 through 24 h. A similar pattern of increased intracellular DA concentration was observed following exposure to deisopropylchlorotriazine at 200 μM at 6 h, 100–200 μM at both 12 and 18 h, and with 50–200 μM at 24 h (Fig. 6). Intracellular NE concentration was unchanged following ex-

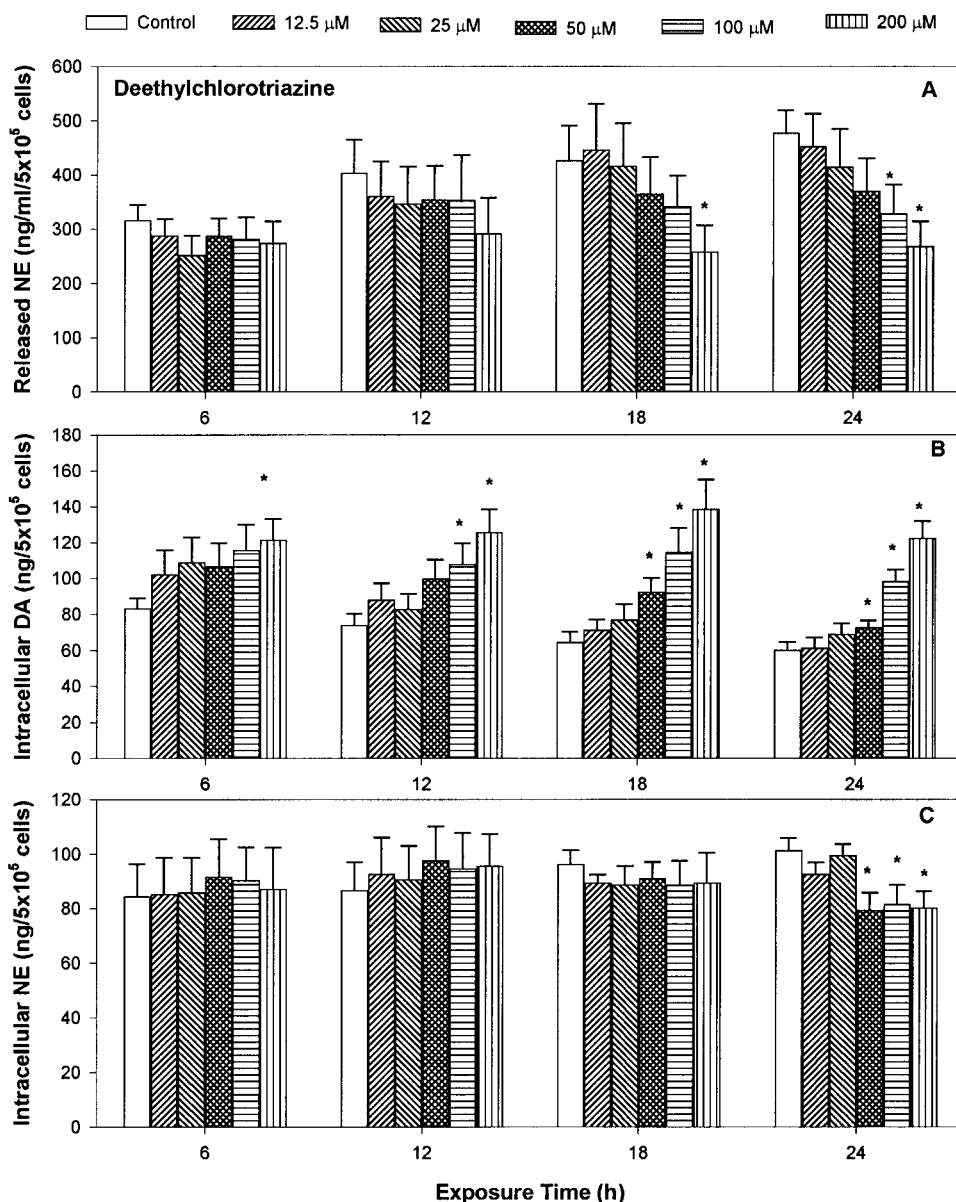


FIG. 5. Time course of released NE and intracellular DA and NE levels following exposure to 2-amino-4-chloro-6-isopropylamino-*s*-triazine (deethylatrazine). PC12 cell cultures were exposed to deethylatrazine with 0 μ M, 12.5 μ M, 25 μ M, 50 μ M, 100 μ M, and 200 μ M concentrations for the indicated time points. A represents the amount of NE released into the medium, while B and C represent the intracellular DA and NE levels, respectively. Each bar is the value of mean \pm SEM of \geq 12 cultures. Data were duplicated in subsequent experiment; * indicates the significance level ($p < 0.05$) at each time point, as compared to the respective control.

posure to both deethylchlorotriazine and deisopropylchlorotriazine at all concentrations through 18 h of exposure (Figs. 5 and 6). However, at 24 h, deethylchlorotriazine (50–200 μ M) significantly reduced the intracellular NE concentration. NE release was also decreased by both deethylchlorotriazine and deisopropylchlorotriazine at 18 h (for deethylchlorotriazine) and 24 h (for both deethylchlorotriazine and deisopropylchlorotriazine) with doses of 100–200 μ M (Figs. 5 and 6).

Effect of Diaminochlorotriazine on the Intracellular DA and NE Concentrations and NE Release

Intracellular DA and NE concentrations and released NE concentrations following exposure to DACT are shown in Figure 7. The intracellular concentrations of DA and NE sig-

nificantly increased following exposure to 160 μ M DACT at 18 through 24 h, while NE release was significantly increased following exposure to 40–160 μ M DACT after 24 h of treatment.

DISCUSSION

The results of these experiments are important for several reasons:

- The present results demonstrate that all four of the chlorotriazine metabolites tested in this study can modify catecholamine synthesis in PC12 cells.
- The pattern of changes observed following exposure was dependent on the metabolites tested.

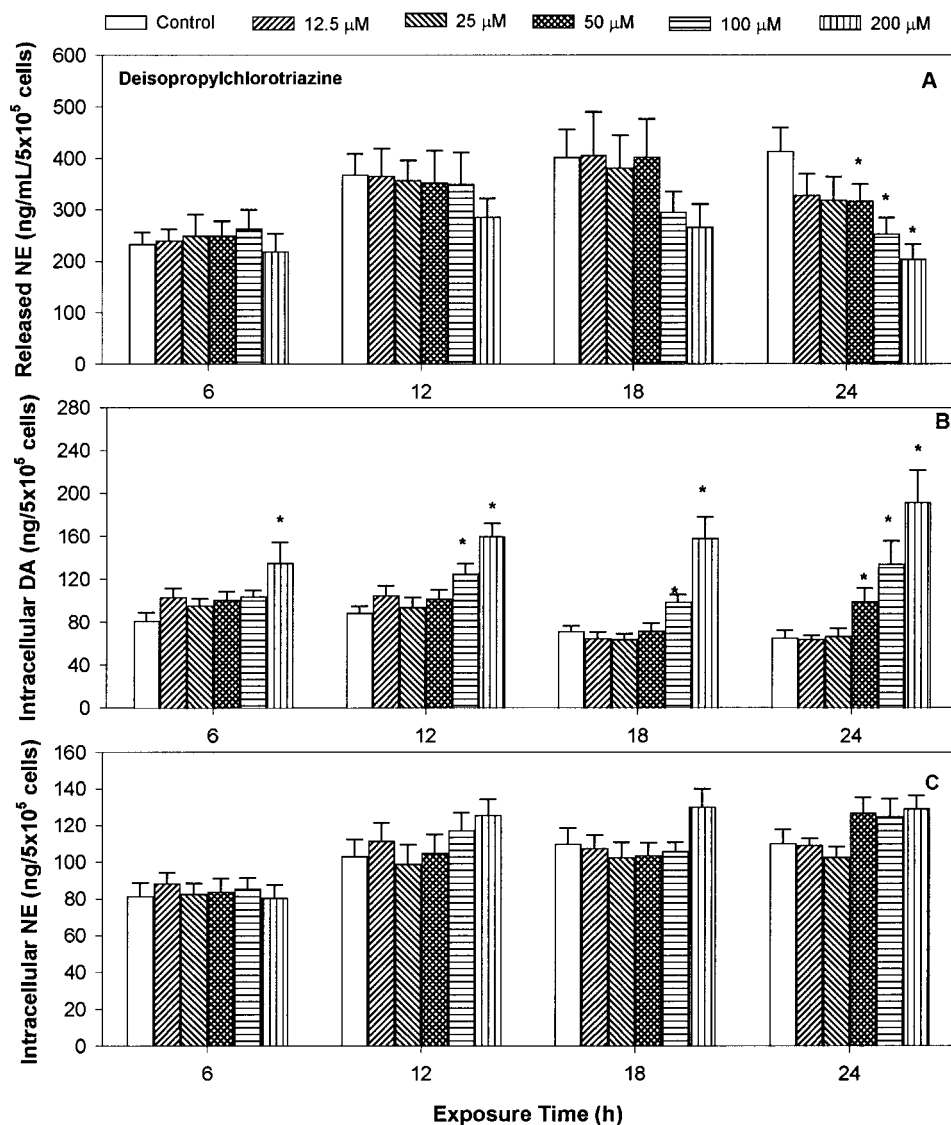


FIG. 6. Time course of released NE and intracellular DA and NE levels following exposure to 2-amino-4-chloro-6-ethylamino-*s*-triazine (deisopropylatrazine). PC12 cell cultures were exposed to deisopropylatrazine with 0 μM , 12.5 μM , 25 μM , 50 μM , 100 μM , and 200 μM concentrations for the indicated time points. A represents the amount of NE released into the medium, while B and C represent the intracellular DA and NE levels, respectively. Each bar is the value of the mean \pm SEM of ≥ 2 cultures. Data were duplicated in the subsequent experiment; * indicates the significance level ($p < 0.05$) at each time point, as compared to the respective control.

- Although some differences were noted in the concentration of the metabolites necessary to alter catecholamine metabolism, these differences were all within the same range of doses.

The different pattern of catecholamine metabolism observed after chlorotriazine exposure may provide some insight into which metabolites may be important enough to be involved in the reported *in vivo* responses. Specifically, hydroxyatrazine (HA) significantly decreased intracellular DA and NE levels, and in turn lowered NE release at the longer time points. This change in catecholamine concentrations is similar to that previously reported following exposure to the parent chlorotriazines, atrazine and simazine, using this same PC12 cell system (Das *et al.*, 2000). The range of HA concentrations required to alter catecholamine synthesis and release was also consistent with the range of atrazine and simazine concentrations reported

to effectively alter catecholamine metabolism. It should be noted that HA, at the higher concentrations (200 and 400 μM), altered the viability of the PC12 cells. However, the range of doses (6.25–100 μM) that was effective in significantly reducing intracellular DA and NE concentrations and NE release was well below these apparent toxic levels. The fact that HA produced changes in catecholamine synthesis and NE release similar to those observed following atrazine exposure might be expected, since PC12 cells express a high level of cytochrome b5 and NADH:cytochrome P450 reductase (Mapoles *et al.*, 1993), which may metabolize atrazine primarily to HA in culture.

Another pattern of response was seen following DACT exposure, in which all these measures were either not different ($<40 \mu\text{M}$, 6 and 12 h) or increased (40–160 μM , 18 and 24 h). It is of interest that Sanderson *et al.* (2000), in evaluating the

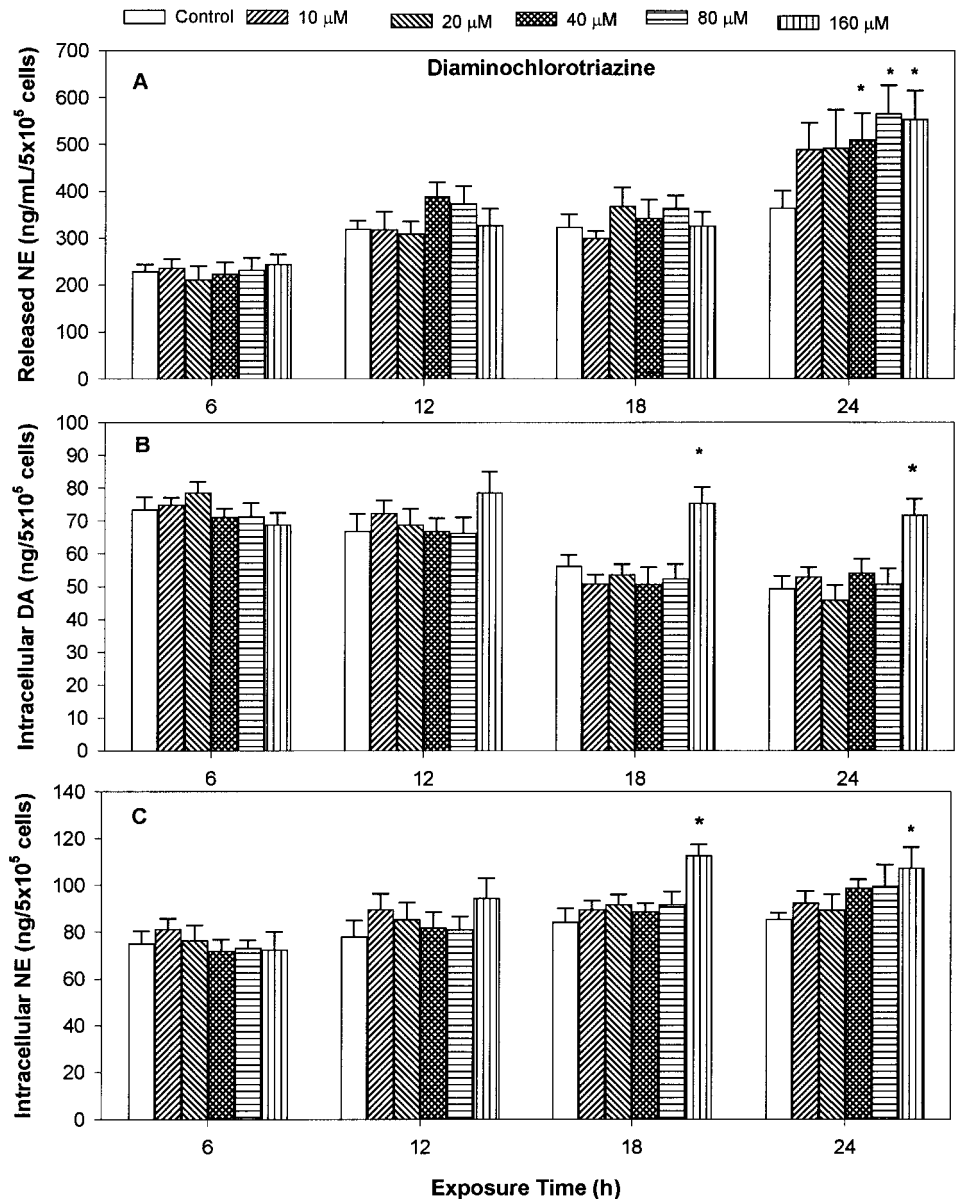


FIG. 7. Time course of released NE and intracellular DA and NE levels following exposure to diaminochlorotriazine (DACT). PC12 cell cultures were exposed to DACT with 0 μ M, 10 μ M, 20 μ M, 40 μ M, 80 μ M, and 160 μ M concentrations for the indicated time points. A represents the amount of NE released into the medium, while B and C represent the intracellular DA and NE levels, respectively. Each bar is the value of the mean \pm SEM of \geq 12 cultures, where experiments duplicated; * indicates the significance level ($p < 0.05$) at each time point, as compared to the respective control.

ability of chlorotriazines to induce aromatase activity in human adrenocortical carcinoma H295R cells, also found that the response of H295R cells to deisopropylchlorotriazine and deethylchlorotriazine was similar, while that to DACT was different (Sanderson *et al.*, 2000). The two other chlorinated metabolites of atrazine and simazine, deethylchlorotriazine and deisopropylchlorotriazine, induced a third pattern of change. Both induced an increase in intracellular DA concentration and decreased intracellular NE and NE release. It is interesting that this pattern of response is similar to that observed *in vivo* following atrazine exposure (Cooper *et al.*, 1998; see Table 1).

These results provide insight for analyzing or evaluating any *in vivo* effect of either chlorotriazines or their metabolites. Both *in vivo* and *in vitro* studies, using rat and human tissues,

have shown that atrazine is metabolized primarily to the three chlorinated metabolites (Catenacci *et al.*, 1990; Hanioka *et al.*, 1999; Ikonen *et al.*, 1988; Lang *et al.*, 1997), while the hydroxylated metabolite is considered as a minor one (Hanioka *et al.*, 1998). Hanioka, using an anti-rat CYP2C11, demonstrated that the major pathway for this metabolic process was cytochrome P450. Conjugation to glutathione was also shown to play a minor part in atrazine phase-II metabolism (Adams *et al.*, 1990). Thus, the differences between the effect of the hydroxylated vs. chlorinated chlorotriazine metabolites may reflect the ability of the PC12 cells to produce primarily the hydroxylated form, whereas *in vivo*, the chlorinated metabolites are predominant.

Data from the present study demonstrate that the common

TABLE 1
Summary of Changes in Catecholamines following Exposure to Chlorotriazines and Their Metabolites

Monoamines	In vitro						In vivo
	Atrazine	Simazine	DECT	DICT	DACT	HA	Atrazine
Intracellular DA	↓	↓	↑	↑	↑	↓	↑
Intracellular NE	↓	↓	↓	↓	↑	↓	↓
NE release	↓	↓	↓	↓	↑	↓	↓

Note. Represents the pattern of changes in the intracellular DA and NE, and NE release following atrazine, simazine, and their metabolites like deethylchlorotriazine (DECT), deisopropylchlorotriazine (DICT), diaminochlorotriazine (DACT), and hydroxyatrazine (HA) both *in vitro* and *in vivo*. Original data reported in the present study and by Cooper *et al.*, 1998; Das *et al.*, 2000. ↑ increase, ↓ decrease.

core-structure of the chlorotriazines and the metabolites is essential for retaining the ability to alter the catecholamine concentrations in PC12 cells. Since the hydroxylated and chlorinated metabolites tested in the present study express different pharmacological effects, it is suggested that the attached side chains are the determining factor in how catecholamine synthesis is affected (i.e., either inhibition or stimulation) in PC12 cells. Although the precise impact of the chlorotriazine mediates on catecholamines *in vivo* remains to be determined, these observations would indicate that chlorotriazine-induced changes *in vivo* could be a key part of the neuroendocrine cascade responsible for altered reproductive development and altered reproductive processes in adulthood. A number of reports suggest that atrazine exposure *in utero* and/or in adult life causes altered developmental and adult reproductive processes (Cooper *et al.*, 1996, 1999, 2000; Cummings *et al.*, 2000; Eldridge *et al.*, 1994, 1999; Infurna *et al.*, 1988; Laws *et al.*, 2000; Stevens *et al.*, 1994; Stoker *et al.*, 1999, 2000; Tennant *et al.*, 1994; Wetzel *et al.*, 1994).

In addition, *in vivo* studies provide evidence that atrazine is primarily metabolized to deethyl- and deisopropylatrazine within 2–3 h of administration (Catenacci *et al.*, 1990; Ikonen *et al.*, 1988; Timchalk *et al.*, 1990). Our present findings also suggest that the effect of the chlorotriazines and their metabolites on reproductive function is additive. Both parent chlorotriazines and their metabolites are persistent in the environment (half-lives, 244 days) (Frank and Siron, 1985; Parmeggiani, 1983; Santolucito and Nauman, 1992; Solomon *et al.*, 1996), even though degradation by atzABC catabolic genes in atrazine-degrading bacteria can occur (De Souza *et al.*, 1998; Mandelbaum *et al.*, 1993).

In summary, like the chlorotriazines, the metabolites tested in this study are also capable of interacting with the sympathetic neuronal PC12 cells to alter the constitutive synthesis and release of catecholamines. Based on the LOEL of atrazine (12.5 μ M) required to alter catecholamine synthesis in PC12 cells identified previously (Das *et al.*, 2000), the present data indicate that the HA is more potent and deethyl- and deisopropylchlorotriazine are less potent than atrazine. Combined, our studies indicate that effective change in catecholamine synthe-

sis and release occur with all chlorotriazines, with HA being the most potent and deethylchlorotriazine, deisopropylchlorotriazine, and diaminochlorotriazine the least (HA > atrazine > simazine > deethylchlorotriazine > deisopropylchlorotriazine > diaminochlorotriazine). The effect of HA exposure on catecholamine synthesis and release was very similar to that after atrazine and simazine exposure in these same cells (Das *et al.*, 2000). In contrast, the observed effects following deethyl- and deisopropylchlorotriazine, but not the DACT exposure, is more like that observed *in vivo* after atrazine exposure (i.e., increased DA and decreased NE concentrations). Thus the pattern of metabolism may determine the particular effect on catecholamine synthesis and release. The primary focus of this study was to compare the pattern of responses between the parent chlorotriazines with that of their major metabolites in altering catecholamine synthesis and release in PC12 cells, which is a critical first step in characterizing the chlorotriazines and eventually determining their mechanism of action. Whether the cellular mechanism(s) of action for these effects on catecholamines is at the level of synthetic enzymes, intracellular processes, or the cell membrane remains to be determined.

ACKNOWLEDGMENTS

This study has been funded wholly or in part by the U.S. Environmental Protection Agency. The authors acknowledge the assistance of Connie A. Meacham, Neurotoxicology Division, and Christy Lambright, Reproductive Toxicology Division, NHEERL, U.S. EPA, in PC12 cell culture. The authors also thank Dr. Susan C. Laws, Endocrinology Branch, Reproductive Toxicology Division, and Dr. William R. Mundy, Cellular and Molecular Toxicology Branch, Neurotoxicology Division, NHEERL, U.S. EPA, for their expert comments on the manuscript.

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