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Cytotoxic and Endocrine-Disrupting Potential of Atrazine, Diazinon, Endosulfan, and Mancozeb in Adrenocortical Steroidogenic Cells of Rainbow Trout Exposed *in Vitro*

Marjolaine Bisson and Alice Hontela[1]

Département des Sciences Biologiques, Université du Québec à Montréal, Toxen Research Center, C.P. 8888, succ. Centre-Ville, Montréal, Quebec H3C 3P8 Canada

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An *in vitro* bioassay for detection and quantitative assessment of chemicals with the capacity to disrupt adrenal steroidogenesis has been developed and used to compare the cytotoxic and endocrine-disrupting potential of four pesticides. Enzymatically dispersed adrenocortical cells of rainbow trout (*Oncorhynchus mykiss*) were exposed *in vitro* to atrazine, diazinon, endosulfan, and mancozeb, and cortisol secretion in response to ACTH or dibutyryl-cAMP (dbcAMP) and cell viability were determined. The effective concentration, EC50 (concentration that inhibits cortisol secretion by 50%), the median lethal concentration, LC50 (concentration that kills 50% of the cells), and the LC50/EC50 ratio were established for the test pesticides. The pesticides were ranked as follows: EC50, endosulfan < diazinon < mancozeb < atrazine; LC50, diazinon < endosulfan < mancozeb < atrazine, with diazinon as the most cytotoxic. Endosulfan and mancozeb disrupted sites downstream of the cAMP-generating step of the cortisol synthetic pathway while atrazine seemed to act upstream from the cAMP step. The *in vitro* adrenal bioassay can be used for screening of adrenotoxicants and for mechanistic studies of adrenotoxicity. © 2002 Elsevier Science (USA)

Key Words: **adrenal; steroidogenesis; ACTH; cAMP; cortisol; atrazine; diazinon; endosulfan; mancozeb; fish.**

Recent studies demonstrated that some of the chemicals used in industrial processes, pulp and paper production, and wastewater treatments become available to aquatic species and interfere with their normal endocrine function (Van Der Kraak *et al.,* 1992; Jobling *et al.,* 1996). It has been reported that chronic environmental exposures to heavy metals, polycyclic aromatic hydrocarbons and polychlorinated biphenyls impair the capacity of fish to secrete cortisol in response to adrenocorticotropic hormone (ACTH) or to a physical confinement (Brodeur *et al.,* 1997; Girard *et al.,* 1998; Norris *et al.,* 1999). Moreover, an exposure-dependent decrease in the capacity of teleost adrenal steroidogenic cells to secrete cortisol has been documented for metals (Leblond and Hontela, 1999; Laflamme *et al.,* 2000), pesticides endosulfan and *o,p′*-DDD (Ilan and Yaron, 1980; Leblond *et al.,* 2001), and polychlorinated biphenyls (Quabius *et al.,* 1997, 2000) *in vitro*.

Cortisol, a corticosteroid hormone, is an important physiological effector of homeostasis in all vertebrates, through its effects on metabolism and immune function (Wendelaar Bonga, 1997; Hontela, 1997). In fish, cortisol also exerts antigonadal effects (Pankhurst and Van Der Kraak, 2000) and has an important role in osmoregulation (Wendelaar Bonga, 1997). Following exposure to a stressor, the hypothalamus secretes corticotropin-releasing hormone (CRH), which stimulates the pituitary to secrete ACTH. Binding of ACTH to membrane receptors of the steroidogenic cells activates a series of enzymatic reactions implicating cAMP, protein kinase A and C, using cholesterol as a substrate to the synthesis of cortisol (Patiño *et al.,* 1986; Lacroix and Hontela, 2001).

A previous *in vitro* study by Leblond and Hontela (1999) reported that, while metals disrupted adrenal steroidogenesis at numerous intracellular sites, the organochlorine pesticide *o,p′*-DDD seemed to exert its adrenotoxic effect at steps prior to the process generating cAMP. Stimulation with optimal concentrations of ACTH or dibutyryl-cAMP (dbcAMP), an analog of cAMP, could not reverse the inhibitory actions of noncytotoxic doses of metals (Cd, Zn, and Hg) on cortisol synthesis *in vitro*. In contrast, dbcAMP could restore cortisol synthesis in cells exposed to *o,p′*-DDD and counteract the effect of the pesticide. Thus, the mode of action and intracellular targets of metals and *o,p′*-DDD appeared to be different in trout adrenal steroidogenic cells. The present study was designed to investigate the effects and the mechanisms of action of four pesticides in adrenal steroidogenic cells of the rainbow trout to assess and to compare their adrenotoxic and cytotoxic potential. Little is known about the effects of pesticides on fish physiology and the adrenal function, even though these chemicals are extensively used in the environment. Atrazine, diazinon, endosulfan, and mancozeb were selected for this study, because of their

[1] To whom all correspondence should be addressed. Fax: (514) 987-4647; E-mail: hontela.alice@uqam.ca.

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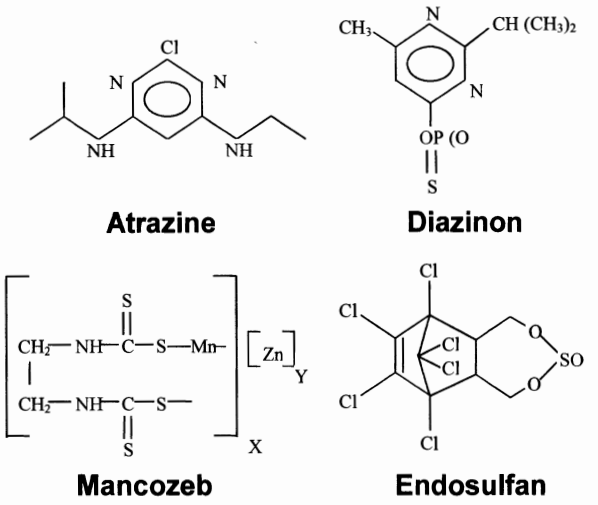

FIG. 1. Structures of the test pesticides.

different molecular structures (Fig. 1) and their presence in the aquatic environment (Table 1).

Atrazine is a commonly used herbicide reported to disrupt ovarian function by altering hypothalamic control of the pituitary and the release of luteinizing hormone and prolactin in rat (Cooper *et al.,* 2000). Appearance of mammary tumors in atrazine-treated female rats has been also documented (Eldridge *et al.,* 1999). The hypothalamo–pituitary–adrenal axis may be another target of atrazine. Atrazine depressed plasma corticosterone in larval tiger salamander (*Ambystoma tigrinum*) exposed *in vitro* (Larson *et al.,* 1998). An *in vitro* exposure of neuronal cells of adrenal medulla to atrazine decreased intracellular dopamine and norepinephrine and the release of norepinephrine by cells (Das *et al.,* 2000). The effects of atrazine on adrenal steroidogenic cells have not been investigated thus far.

Diazinon, an organophosphate, inhibits acetylcholinesterase, causing an accumulation of acetylcholine at cholinergic synapses (De Blaquière *et al.,* 2000). The effects of diazinon on the endocrine system have not been well characterized, although elevated plasma corticosterone levels were reported in mice exposed to diazinon *in utero* or neonatally via milk (Cranmer *et al.,* 1978).

Mancozeb acts as a fungicide and while little is known about the effects of mancozeb itself, many studies have focused on ethylene thiourea (ETU), its main metabolite. Following exposure to water or oxygen, mancozeb is rapidly and spontaneously degraded to ETU, reported to be a teratogen, goitrogen, and carcinogen (U.S. EPA, 1988). It can disrupt thyroid function and is causally related to thyroid cancer in animals (Steenland *et al.,* 1997). Mahadevaswami *et al.* (2000) observed an impairment in the number and the duration of estrous cycles in rats treated with mancozeb *per os,* suggesting that mancozeb acts on the hypothalamo–pituitary–ovarian axis. Tumors in the thyroid and adrenal glands have been reported in mice exposed to ETU (Chhabra *et al.,* 1992).

Endosulfan, an organochlorine still employed despite the discontinued use of many organochlorine pesticides, belongs to the chlorinated cyclodienes, which are well known blockers of neuronal $GABA_A$-gated chlorine channels (Kamijima and Casida, 2000). This neurotoxic effect probably underlies the extreme sensitivity of fish and other aquatic species to endosulfan (WHO, 1984; Naqvi and Newton, 1991; Chakravorty *et al.,* 1992). Pandey (1986) observed morphological changes of corticosteroidogenic cells from freshwater snake-headed murrel (*Ophiocephalus punctatus*) exposed *in vivo* to thiodan (trade name of endosulfan). Recently, adrenotoxic effects of endosulfan were reported in rainbow trout (Leblond *et al.,* 2001).

The aim of this study was to assess and compare the adrenal toxicity of atrazine, diazinon, endosulfan, and mancozeb *in vitro* and to elucidate their mechanisms of action using an adrenocortical cell preparation from rainbow trout. To differentiate between toxicological effects at the ACTH receptor or in the postmembrane steps downstream from generation of cAMP, secretion of cortisol was measured in response to ACTH or dbcAMP in the pesticide-exposed adrenocortical cells.

MATERIALS AND METHODS

Experimental animals. Juvenile rainbow trout (*Oncorhynchus mykiss*), body wt 100–150 g, were purchased from a commercial supplier (Pisciculture des Laurentides, Quebec, Canada) and acclimated to the laboratory conditions for 2 weeks before use in the experiments. Fish were maintained in 600-L flow-through tanks supplied with oxygenated water at a rate of 3.8 liter/min at 15–17°C, water hardness 70 mg/liter $CaCO_3$, photoperiod at 12L:12D. They were fed daily with commercial trout food at the manufacturer's recommended rate (10 g/kg of fish).

Preparation of adrenocortical cell suspensions. Fish were anesthetized with MS 222 (0.1 g/liter of water, ICN Biomedicals), bled from caudal blood vessels with a 1-cc heparinized syringe, and perfused through the caudal vein with 30 ml of saline (0.7%) in order to remove as much blood as possible. The head kidneys where the adrenal tissue is located were then dissected and cut into 1-mm^3 fragments in minimum essential medium (MEM, Sigma), supplemented with 5 g/liter bovine serum albumin and 2.2 g/liter $NaHCO_3$, pH 7.4. Fragments were washed twice with MEM to remove red blood cells and were digested in collagenase/dispase mixture (Boehringer Mannheim), as previously described (Leblond and Hontela, 1999). After 60 min of digestion at room temperature, cell suspensions were centrifuged at 1000 rpm (300*g*) for 5 min (15°C), to remove the supernatant containing the enzymes. The pellet was resuspended in MEM, cells counted with a hemacytometer, and adjusted to 75×10^6 cells/ml.

Exposure to pesticides. The cells from each adrenal tissue were plated in a 96-well culture microplate (150 μl of 75×10^6 cells/ml per well) and preincubated for 2 h at 15°C, to reach the basal cortisol secretion (less than 1 ng/ml, the limit of detection of the radioimmmunoassay). Each fish represented one replicate ($n = 1$) of the various *in vitro* treatments; the number of fish used in each experiment with an individual pesticide was 5–10. After preincubation and centrifugation at 1000 rpm for 3 min, the supernatant was removed to eliminate cortisol secreted following the handling of the fish and of the adrenal tissue. The cells were either resuspended in 150 μl of Ringer solution (control wells) or in 150 μl of Ringer solution containing various concentrations of the tested pesticide. Each microplate contained control wells (without pesticide)

TABLE 1
Chemical and Toxicological Characteristics of Atrazine, Diazinon, Endosulfan, and Mancozeb

Name	Family	Action	Use	Guidelines for aquatic life (μg/L)	Residues in water (Québec, Ontario) (μg/L)	LC50 at 96 h in rainbow trout (mg/L)
Atrazine MW 215.69	Triazine	Herbicide	Corn, asparagus, sugarcane, forestery	2	2.5 to 20.7[a] <2 to 16[c] <0.03 to 9[d]	4.5–8.8[b]
Diazinon MW 304.34	Organophosphate	Insecticide	Fruit and vegetables, seed, tobacco, forestery, domestic use	0.003	<0.2 to 5.7[a] 1.9 to 28.3[a] 0.78[e] 0.02[d]	2.6–3.2[b]
Endosulfan ($\alpha + \beta = 2 + 1$) MW 406.93	Organochlorine	Insecticide	Cotton, tea, coffee, cereals, rice, fruit, vegetables, tobacco, strawberry	0.02	0.02 to 0.069[a] 0.53[e]	1.5[f]
Mancozeb MW 266.31	Ethylene bisdithiocarbamate	Fungicide	Potatoes, tomatoes, apple and pear trees, roses	Not available	*<1 to 2.3[g,h,i] *1.3[d,i]	1.9

[a] Forrest and Caux (1989).
[b] Worthing (1987).
[c] Environnement et Faune du Québec (1995).
[d] Giroux (1998a).
[e] Harris *et al.* (1998).
[f] Ferrando *et al.* (1991).
[g] Giroux (1998b).
[h] Environnement et Faune du Québec (1998).
[i] Residues of ETU (the main metabolite of mancozeb).

and exposed wells (with pesticide), controls were pipetted and incubated always at the same time as the exposed wells. The following chemicals were used: atrazine (99% pure), diazinon (98.8% pure), endosulfan (99% pure), and mancozeb (80% pure), all purchased from Riedel deHaën (Germany). The pesticides were dissolved in dimethylsulfoxide (DMSO, Sigma), diluted in Ringer solution 5% v/v, and then added to the wells. Ringer solution contained NaCl 6.5 g/liter, $CaCl_2$ 0.2 g/liter, KCl 0.2 g/liter, $NaHCO_3$ 0.2 g/liter, dextrose 1.0 g/liter, and Hepes 5.95 g/liter (adjusted to pH 7.5). Blanks with DMSO only were used and no effects on viability or cortisol secretion were detected (data not shown). Control and exposed wells were incubated for 60 min at 15°C and were centrifuged at 1000 rpm for 3 min. The supernatants were removed and frozen at −20°C, until analysis for LDH (Boerhinger Mannheim) to estimate cell viability, as described previously (Leblond *et al.,* 2001). The cell pellets were resuspended in 150 μl of Ringer solution and centrifuged to wash the pesticides from the cells.

Stimulation of cortisol secretion. After centrifugation, all the pellets were resuspended in 150 μl of MEM containing 1 IU/ml ACTH (Porcine adrenocorticotropin, ACTH 1–39, Sigma) or 2.0 mM dbcAMP (N^6,2′-*O*-dibutyryl adenosine 3′,5′-cyclic monophosphate, Sigma). The wells stimulated with ACTH and dbcAMP were incubated for 60 and 120 min respectively, as described and validated in previous studies with this cell preparation (Leblond *et al.,* 2001). Following the incubation, the microplate was centrifuged and supernatants were removed and frozen at −20°C, until cortisol RIA (ICN Biomedicals).

Cortisol secretion by the cells (and also cell viability) was expressed as a percentage of the control from each individual fish (cells incubated without pesticide exposure and stimulated with either ACTH or dbcAMP). Control wells for each fish must be included in this cell preparation since the variation in cortisol secretion between cells from different fish exposed to the same concentration of pesticide is less than 10% when expressed as a percentage of controls, while the variation in the absolute values of cortisol secretion between fish is relatively large due to differences in the dissection of the head kidney, in handling of the tissue, and possibly in the number of steroidogenic cells between fish. Cortisol secretion of ACTH-stimulated controls was about 25 ± 13 ng/ml (mean ± SEM); cortisol secretion by unstimulated cells was near the detection limit of the radioimmunoassay (1 ng/ml) and could not be used as control. Viability, expressed as a percentage, was estimated by subtracting lactate dehydrogenase (LDH) values in the supernatants from the maximum LDH extracted after exposure of cells to deionized water, as described by Leblond *et al.* (2001).

Data analysis. Data were transformed, when necessary, to respect normality. Differences between the controls and the cells exposed to pesticides were compared by one-way analysis of variance and Dunnett's method (α = 0.05). The differences between ACTH- and dbcAMP-stimulated cortisol secretion were tested with Student's t test (α = 0.05).

RESULTS

Dose-dependent effects on cortisol secretion and cell viability. Viability and cortisol secretion in response to ACTH and dbcAMP of trout adrenocortical cells exposed to selected pesticides *in vitro* are shown in Figs. 2–5. Except for atrazine, all the tested pesticides had dose-dependent effects on the secretory capacity and viability of the adrenocortical cells. A significant decrease of cortisol secretion and of viability, compared to unexposed controls stimulated with ACTH or dbcAMP (100%), was observed at a concentration of 500 μM of diazinon, with an undetectable secretory response to ACTH or dbcAMP and a viability of about 20% (Fig. 2). Concentrations of 50 μM and less of diazinon had no significant effects

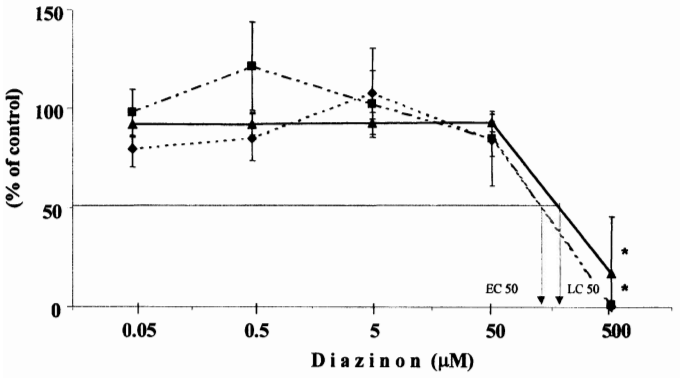

FIG. 2. ACTH- and dbcAMP-stimulated cortisol secretion and viability (percentage of control, mean ± SEM) of adrenocortical cells following *in vitro* exposure to diazinon. *Significant difference from controls not exposed to diazinon (100%) for cortisol secretion stimulated with 1.0 IU/ml ACTH (◆) or with 2.0 mM dbcAMP (■) and cell viability (▲). Statistical significance was evaluated by Dunnett's test ($p < 0.05$). The number of replicates is eight or nine for each concentration and parameter.

on cortisol secretion or viability. There were no significant differences between ACTH- and dbcAMP-stimulated cortisol secretion.

The responses of adrenocortical cells to endosulfan showed a similar pattern to the one observed with diazinon (Fig. 3). The secretory response was undetectable at 500 μM, and the viability was significantly impaired (<50%). However, at 50 μM of endosulfan, the ACTH- or dbcAMP-stimulated cortisol secretion was still significantly impaired, whereas the viability was approaching that of control (87.5%).

The effects on cortisol secretion and on viability were even more distinct for mancozeb (Fig. 4). A significant impairment of cortisol secretion was observed, without a significant reduction of viability, at a concentration of 500 μM. Even at 50 μM

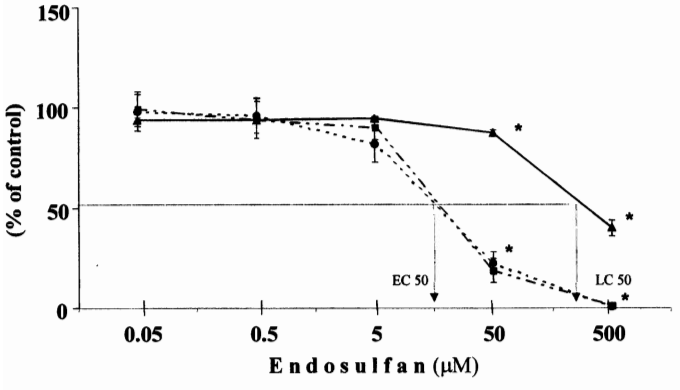

FIG. 3. ACTH- and dbcAMP-stimulated cortisol secretion and viability (percentage of control, mean ± SEM) of adrenocortical cells following *in vitro* exposure to endosulfan. *Significant difference from controls not exposed to endosulfan (100%) for cortisol secretion stimulated with 1.0 IU/ml ACTH (◆) or with 2.0 mM dbcAMP (■) and cell viability (▲). Statistical significance was evaluated by Dunnett's test ($p < 0.05$). The number of replicates is 8–10 for each concentration and parameter. (Data modified from Leblond *et al.*, 2001).

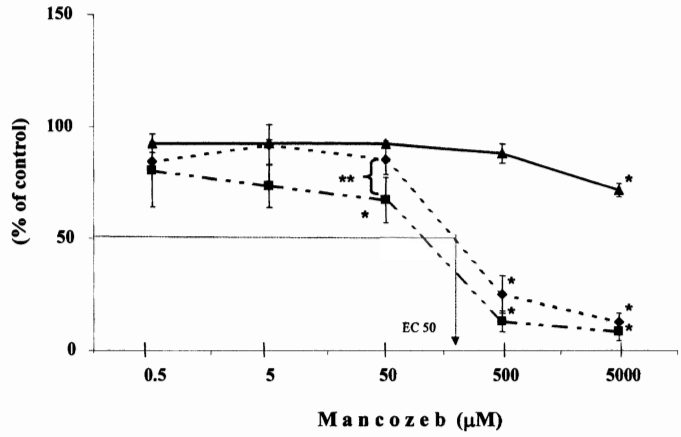

FIG. 4. ACTH- and dbcAMP-stimulated cortisol secretion and viability (percentage of control, mean ± SEM) of adrenocortical cells following *in vitro* exposure to mancozeb. *Significant difference from controls not exposed to mancozeb (100%) for cortisol secretion stimulated with 1.0 IU/ml ACTH (◆) or with 2.0 mM dbcAMP (■) and cell viability (▲). **Significant difference between cortisol secretion stimulated with ACTH and dbcAMP. Statistical significance was evaluated by Dunnett's test ($p < 0.05$) and Student's t test ($p < 0.05$). The number of replicates is five or six for each concentration and parameter.

of mancozeb, a significant reduction of cortisol secretion was observed with dbcAMP-stimulated cortisol secretion while viability was not reduced.

The responses of adrenocortical cells to atrazine are shown in Fig. 5. Effects on viability were not detectable even at the highest concentration tested in the present study (500 μM). A significant increase of cortisol secretion was observed at 500 μM of atrazine when cells were stimulated with ACTH but not with dbcAMP. However, a significant impairment of ACTH-stimulated cortisol secretion was observed at concentrations of atrazine between 5 and 0.005 μM. Stimulation with dbcAMP restored cortisol secretion to the levels of control (unexposed to atrazine) cells in wells exposed to atrazine at concentrations of 5 to 0.005 μM. A concentration < 0.005 μM of atrazine (NOAEL) did not have a detectable effect on viability or cortisol secretion.

Toxicological characteristics of diazinon, endosulfan, mancozeb, and atrazine. The concentration that kills 50% of adrenocortical cells (LC50) and the concentration that impairs 50% of ACTH-stimulated cortisol secretion (EC50) *in vitro* were determined for the pesticides investigated in the present study (Table 2). These two toxicological parameters were used to rank the adrenotoxicity of the tested pesticides, according to their cytotoxicity (LC50): diazinon < endosulfan < mancozeb < atrazine, and their endocrine toxicity (EC50): endosulfan < diazinon < mancozeb < atrazine. The ratio LC50 to EC50 was used to compare the specificity of the toxicant to impair cortisol secretion by trout adrenocortical cells. The higher the ratio, the higher was the capacity of the pesticide to impair cortisol secretion without a loss of cell viability. The

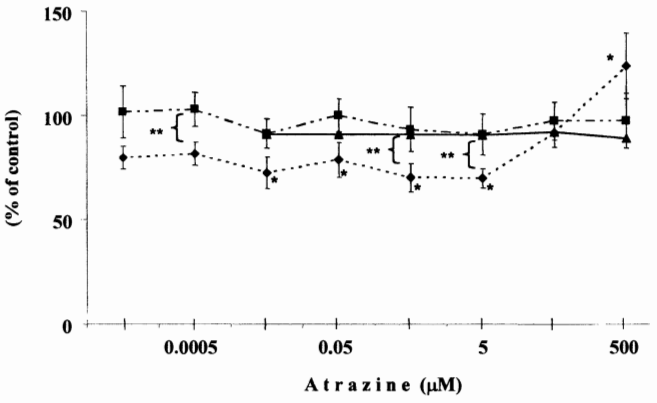

FIG. 5. ACTH- and dbcAMP-stimulated cortisol secretion and viability (percentage of control, mean ± SEM) of adrenocortical cells following *in vitro* exposure to atrazine. *Significant difference from controls not exposed to atrazine (100%) for cortisol secretion stimulated with 1.0 IU/ml ACTH (◆) or with 2.0 mM dbcAMP (■). **Significant difference between cortisol secretion stimulated with ACTH and dbcAMP; ▲, viability. Statistical significance was evaluated by Dunnett's test ($p < 0.05$) and Student's t test ($p < 0.05$). The number of replicates is five to eight for ACTH and dbcAMP and three to six for viability.

TABLE 2
Adrenotoxicity (LC50, EC50, and LC50/EC50) of Xenobiotics Tested in Rainbow Trout Adrenocortical Cells *in Vitro*

Toxicant	Viability LC50 (μM)	ACTH-stimulated cortisol production EC50 (μM)	LC50/EC50
Atrazine[a]	>50,000	>50,000	—
$CdCl_2$	10,800	168	64.29
$ZnCl_2$	22,800	355	64.22
Mancozeb	>5,000	312	>16.04
Endosulfan	405	38	10.66
CH_3HgCl	1,140	116	9.83
$HgCl_2$	199	22.3	8.92
o,p'-DDD	385	130	2.96
Diazinon	305	233	1.31

[a] Toxicants investigated in the present study are indicated by italics. Toxicants investigated by Leblond and Hontela (1999) using the same *in vitro* system are also included in the table. EC50, concentration that impairs 50% of cortisol secretion *in vitro*; LC50, concentration that kills 50% of the adrenocortical cells *in vitro*. The adrenal specificity of these xenobiotics is evaluated with the ratio of LC50 to EC50.

pesticides were ranked as follows according to their LC50/EC50: mancozeb > endosulfan > diazinon. The ratio LC50/EC50 for mancozeb was 16, therefore, a concentration about 16-fold higher was required to kill 50% of cells than the concentration required to impair 50% of cortisol secretion. In contrast, the ratio for diazinon was about 1; its endocrine toxicity being almost the same as its cytotoxicity. The LC50 and EC50 could not be determined for atrazine, since concentrations higher than 500 μM (highest concentration tested) were at the limit of solubility.

DISCUSSION

Fish are susceptible to pesticides dissolved in water and can be exposed to these chemicals through gills and skin and by contaminated food. Although acute toxicity (LC50 or LD50) has been assessed for numerous pesticides (Worthing, 1987; Ferrando *et al.*, 1991; Table 1), there are virtually no data characterizing endocrine toxicity of pesticides in teleost fish or other aquatic species. The effects and the mechanisms of action of atrazine, diazinon, endosulfan, and mancozeb on cortisol secretion were investigated *in vitro* in this study. The capacity of trout adrenocortical cells to respond to ACTH and dbcAMP following pesticide exposure *in vitro* was evaluated to identify the intracellular site of action of each pesticide. The adrenotoxicity patterns were characterized with dose–responses and important differences in the mode of action were identified for the first time in the trout model (Fig. 6).

Diazinon decreased both ACTH- and dbcAMP-stimulated cortisol secretion and viability below 20% of control at 500 μM and, since the decrease of cortisol secretion closely followed the decrease of viability, the reduction of cortisol secretion may be attributed to a cytotoxic effect of diazinon. The adrenotoxic pattern of diazinon in trout adrenal cells can therefore be represented by the dose–response in Fig. 6A for a chemical with a LC50/EC50 ratio of 1. This pattern shows that the endocrine toxicity of this type of chemical is almost the same as its cytotoxicity. Cytotoxic effects at similar concentrations of diazinon were also observed in retina cells in the

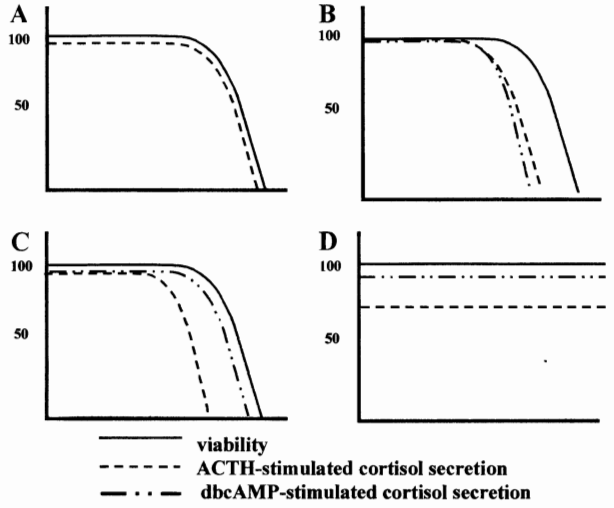

FIG. 6. Patterns of adrenotoxicity according to the LC50/EC50 ratio of model toxicants tested *in vitro* using the trout adrenocortical cell suspensions. (A) Toxicant with LC50/EC50 = 1 (cytotoxic; e.g., diazinon), (B) toxicant with LC50/EC50 > 10 (endocrine-specific action with effects downstream from cAMP step; e.g., endosulfan, mancozeb), (C) toxicant with LC50/EC50 > 10 (endocrine-specific action with effects upstream from cAMP step), and (D) toxicant with undetectable LC50 and EC50 (endocrine-specific action with effects upstream from cAMP step; e.g., atrazine).

embryonic teleost medaka *Oryzias latipes* (Hamm *et al.,* 1998) and hepatoma cell line derived from the top minnow *Poeciliopsis lucida* (Babich *et al.,* 1991). In a study with rat adrenal cells, Civen and Brown (1974) reported an inhibition of ACTH- and cAMP-stimulated corticosterone secretion at 1×10^{-3} M of diazinon. However, when cells were stimulated with pregnenolone, a substrate for corticosterone synthesis, corticosterone secretion was restored, suggesting that diazinon was not cytotoxic to the rat adrenal cells at the endocrine-disrupting dose. Diazinon may interfere at steps between cAMP generation and formation of pregnenolone by cholesterol side-chain cleavage. Other pesticides including Round-up target the StAR protein, known to rate limit the transfer of cholesterol from the outer to the inner mitochondrial membrane prior to pregnenolone in mammalian steroidogenic cells (Walsh *et al.,* 2000). Mammalian adrenal cells appear less sensitive than fish adrenal cells to diazinon, since the dose that killed more than 20% of cells in fish in the present study had no effect in mammals (Civen and Brown, 1974). The difference in vulnerability to diazinon between rat and piscine adrenal cells demonstrates the importance of species-specific bioassays for toxicological assessment and the difficulty of extrapolation between species.

The response of the adrenocortical cells to endosulfan, an organochlorine pesticide, was similar to diazinon. Endosulfan significantly impaired cortisol secretion with only a slight increase of cell mortality (12.5%) at 50 μM. Neither ACTH nor dbcAMP could restore cortisol secretion at this concentration of endosulfan, similar to a previous study (Leblond *et al.,* 2001). This suggests that, in trout adrenocortical cells, endosulfan could disrupt cortisol synthesis at multiple steps, including downstream from the step of cAMP generation. In another model, Randall *et al.* (1990) provided evidence that endosulfan affects the generation of cAMP or steps above in mouse hepatocytes since the addition of dbcAMP prevented the inhibition of the gap junctional intercellular communication by endosulfan. These data suggest that the mechanism of action of endosulfan may be different in different cells. The adrenotoxic pattern of endosulfan in trout adrenal cells is represented in Fig. 6B. A ratio of LC50/EC50 of about 10 suggests that endosulfan has a higher endocrine toxicity than cytotoxicity since the concentration required to kill 50% of the cells is about 10 times higher than the concentration that disrupts 50% of cortisol secretion.

The dose–response to mancozeb was similar to endosulfan but with even lower cytotoxicity (Fig. 6B). Thus, mancozeb is another pesticide with the capacity to disrupt the normal secretory response of adrenal cortical cells, without exerting cytotoxic effects. Mancozeb is known to cause apoptosis in the human breast cancer line MCF-1 at 0.34×10^{-4} M and 1.88×10^{-4} *in vitro* during 24- and 48-h exposures (Lin and Garry, 2000). Cell mortality was not significant in the present study with mancozeb, but the exposure time was only 1 h and the mechanism of apoptosis may require 60 to 90 min (depending on dose, duration, and cell type) to promote DNA fragmentation leading to a loss of viability (Corcoran *et al.,* 1994; Reader *et al.,* 1999). Stimulation with dbcAMP could not restore cortisol secretion in cells exposed to mancozeb, suggesting that mancozeb acts downstream from cAMP generation (Fig. 6B). The pattern represented in Fig. 6C could be attributed to an adrenotoxicant (e.g., *o,p'*-DDD; Leblond and Hontela, 1999) with a similar dose-dependent response to mancozeb, but with a restoration of the cortisol secretion following stimulation with dbcAMP. However, this pattern was not observed in the present study.

ACTH-stimulated cortisol secretion was inhibited by atrazine whereas dbcAMP-stimulated secretion was not. The restoration of cortisol secretion in atrazine-exposed cells after dbcAMP stimulation suggests that atrazine acts upstream from the cAMP step. These results could be represented by the adrenotoxicity pattern in Fig. 6D. The viability was not significantly decreased even at the highest concentration tested (500 μM). Similarly, Das *et al.* (2000) observed no significant reduction in the viability of Pheochromocytoma PC12 adrenal medulla cells following 6 h of *in vitro* exposure to 200 μM of atrazine. The mechanism mediating the increase of cortisol secretion observed in the present study at the highest concentration of atrazine is presently not understood. However, this increase required ACTH stimulation because cortisol secretion was not detectable with unstimulated adrenal cells exposed to atrazine (data not shown). Sanderson *et al.* (2000) demonstrated that an *in vitro* exposure to 30×10^{-6} M of atrazine for 24 h increased the aromatase induction of CYPmRNA responsible for cortisol production. Moreover, Messner *et al.* (1979) reported an inhibition of phosphodiesterase by 10×10^{-4} to 40×10^{-4} M of atrazine. Phophodiesterase is known to break down cAMP and its inhibition may result in excess hormone production and secretion. Atrazine could also impair protein kinase C, which inhibits cortisol secretion in adrenocortical cells in rainbow trout (Lacroix and Hontela, 2001). The mechanisms of action of atrazine in the adrenal cells have not been elucidated thus far.

Important differences in LC50 (concentration that kills 50% of adrenocortical cells) and EC50 (the concentration that inhibits 50% of cortisol secretion) of pesticides tested in the present study were revealed. Adrenocortical cells were more sensitive to diazinon and endosulfan than mancozeb and atrazine. The ranking of LC50 in trout adrenocortical cells *in vitro* (diazinon < endosulfan < mancozeb < atrazine) was different from the ranking of LC50 at 96 h of trout exposed *in vivo* to the pesticides: endosulfan < mancozeb < diazinon < atrazine (Table 1). The difference in the ranking of *in vitro* and *in vivo* LC50 may be attributed to the time of exposure, biotransformation of the parent compound, and organs other than the adrenals being more sensitive to the pesticide. EC50 could not be determined for atrazine; however, a significant, although small (27.56%) inhibition of cortisol was observed at a very low concentration (0.005 μM). The EC50s were in the

same order of magnitude for the other three pesticides tested, and, compared to heavy metals and *o,p'*-DDD (Table 2), endosulfan and $HgCl_2$ had the highest endocrine toxicity whereas mancozeb and $ZnCl_2$ had the least endocrine toxicity (Table 2). Although the adrenal bioassay validated in the present study is useful for quantitative comparison of adrenotoxic potential of test xenobiotics, extrapolation of the results from this acute exposure (60 min) assay to environmental exposures of fish in the wild (Table 2) remains difficult, since data on tissue residues of pesticides in fish exposed in the environment are scarce (Naqvi and Vaishnavi, 1993). However, laboratory bioaccumulation studies indicate that diazinon, atrazine, and endosulfan accumulate rapidly in liver, kidney, and muscle of exposed fish (Görge and Nagel, 1990; Tsuda *et al.,* 1990; Sancho *et al.,* 1994), suggesting an exposure route for the adrenal tissue as well.

The LC50/EC50 ratio is an important parameter that can be used to compare endocrine toxicity and cytotoxicity of various xenobiotics. Mancozeb, with the highest ratio, was identified as the pesticide with the highest endocrine-disrupting potential in the trout adrenal cells, whereas diazinon, with a ratio of about one, was characterized as a cytotoxic chemical, with a low specific endocrine-disrupting potential. This study provided new adrenotoxicity data for four pesticides currently used for pest control. Different patterns of adrenotoxicity were established for the pesticides by comparing the cytotoxicity and endocrine-disrupting potential in the adrenal cell. Such an approach could facilite comparison of endocrine toxicants in other toxicological studies. Future studies are required to identify the intracellular sites of action of the pesticides, to determine whether the effects are reversible, whether longer exposures to lower concentrations have comparable effects, and whether biotransformation of the parent compound influences its adrenal toxicity.

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