ENDOCRINE DISRUPTION POTENTIAL OF ENDOSULFAN AND ITS SOIL TRANSFORMATION PRODUCTS

By

Janelle Ann Brieske

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ABSTRACT

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Janelle Ann Brieske

Endosulfan, known for its persistence in the environment, is the last cyclodiene pesticide in widespread use. Endosulfan (α and β isomers) and its known microbial transformation products were tested for androgen-receptor binding affinity in a cell-free *in vitro* binding assay using cytosolic prostate tissue extract from mature rats and [^{3}H]R1881 (methyltrienolone), a synthetic androgen. Endosulfan lactone was shown to inhibit binding of androgen to the androgen-receptor. The estrogenic potential of the same compounds was investigated using *in vitro* cell proliferation studies with the MCF-7 estrogen responsive human breast cancer cell line. Estrogenicity was further investigated by measuring estrogen response element/luciferase reporter gene expression in transiently transfected MCF-7 cells. Endosulfan (α and β isomers) and endosulfan diol were shown to possess estrogenic activity in both assays, with endosulfan diol exhibiting greater estrogenic activity than the parent compounds.

DEDICATION

To my parents, Dennis and Paula Brieske. Thank you for supporting me in all of my endeavors and believing in me.

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GENERAL INTRODUCTION

Use and Environmental Fate of Endosulfan

Endosulfan, 1,4,5,6,7,7-hexachloro-5-norbornene-2,3-dimethanol cyclic sulfite, was first introduced into the United States in 1954 by Farbwerke Hoechst A.G. under the registered trademark "Thiodan" (Maier-Bode, 1968). It remains the only cyclodiene insecticide registered for use in the United States. Endosulfan is now widely used throughout the world for the control of insect pests on grains, tea, fruits, vegetables, tobacco and cotton and as a wood preservative. In the United States, endosulfan is registered for use as a contact and stomach insecticide on over 60 food and nonfood crops. It is used to control over 100 different insect pests (EPA, 1980). The major domestic producer of endosulfan, FMC Corporation, was estimated to have produced 2 million pounds of the active ingredient in 1971. Total domestic production was near 3 million pounds in 1974 (Sittig, 1980) and 5,000 pounds in 1977 (HSDB, 1992). Endosulfan was not produced in the United States after 1982, but continues to be formulated and used domestically. Worldwide production in 1984 was estimated at 10,000 metric tons (22 million pounds) by the World Health Organization (WHO, 1984).

Endosulfan is produced by the Diels-Alder addition of hexachlorocyclopentadiene and cis-butene-1,4-diol in xylene,

followed by the hydrolysis of the adduct to the dialcohol. The end product is produced by treatment of the bicyclic dialcohol with thionyl chloride (Sittig, 1980). Technical grade endosulfan must contain at least 94% of two isomers, α- and β- endosulfan. The α and β isomers are present in a ratio of 7:3, respectively. The technical grade mixture may also contain up to 2% endosulfan diol and up to 1% endosulfan ether.

Chlorinated organic pesticides such as endosulfan are major contributors to global environmental contamination (Kullman and Matsumura, 1996). In a study of the global distribution of organochlorine compounds in tree bark samples, endosulfan was found in high concentrations throughout the world, including agricultural areas throughout the United States, Europe, India, the Middle East, Japan, Brazil, Australia, Taiwan, South Korea and Russia. (Simonich and Hites, 1995). Endosulfan is released directly into the atmosphere via its aerial application to crops. It may travel long distances through the air and settle on land outside of the area of application, sometimes contaminating adjacent croplands and streams (NRCC, 1975). Endosulfan is oxidized to endosulfan sulfate on plant surfaces, with levels of endosulfan diminished to less than 20% of initial levels within one week (NRCC, 1975). Although the α- and β- isomers are degraded rapidly on plant tissue, endosulfan sulfate

residues tend to be very persistent (Coleman and Dolinger, 1982).

Endosulfan is not commonly found in groundwater due to its strong sorption to soil organic matter and its low water solubility. The soil organic carbon normalized partition coefficient (K_{oc}) for endosulfan α and β are 4,000 and 20,000, respectively (Peterson and Batley, 1993). Endosulfan's sorption to sediment limits its loss from aquatic ecosystems (Peterson and Batley, 1993). Despite the fact that endosulfan is not a common groundwater contaminant, its continued use worldwide and potential for atmospheric transport contribute to widespread endosulfan contamination. Endosulfan has been found in the atmosphere, soils, sediments, surface waters, rain waters and food stuffs (USDHHS, 1993).

Endosulfan is subject to both chemical and biological degradation in the environment. In alkaline conditions such as seawater, endosulfan undergoes chemical hydrolysis to endosulfan diol (Kullman and Matsumura, 1996). Endosulfan (α and β) is degraded by a variety of soil microorganisms, resulting in the formation of several stable transformation products (Figure 1.). Martens (1976) incubated endosulfan with individual species of soil microorganisms and found that the majority of active fungi formed endosulfan sulfate as the major transformation product. Endosulfan diol was the major metabolite in incubations with soil bacteria. Katayama and

Matsumura (1993) determined that a common soil fungus, *Trichoderma harzianum*, formed endosulfan diol.

In a study of endosulfan degradation in seven different soil types, Martens (1977) determined that endosulfan sulfate was the major transformation product in aerobic incubations. The percent of endosulfan converted to endosulfan sulfate was between 30% and 60%, varying among different soils. Endosulfan diol and endosulfan lactone were minor transformation products (2.6% and 1.2%, respectively). While endosulfan is readily converted to endosulfan sulfate, the metabolite is as persistent as the parent compound and has been shown to be equally toxic (Kullman and Matsumua, 1996).

Endosulfan sulfate was also reported as the major transformation product in columns of soil flushed with N_2/CO_2 to simulate an anaerobic environment. Percent conversion was between 11 and 22% (Martens, 1977). Formation of endosulfan sulfate, an oxidation product, in the "anaerobic" column suggests that the column was partially aerated. Smaller amounts of endosulfan diol and endosulfan lactone were also observed. In water saturated soils, endosulfan diol was the major transformation product (2% to 18%) with smaller quantities of endosulfan sulfate (3% to 8%) and endosulfan hydroxyether (2% to 4%)(Martens, 1977).

Miles and Moy (1979) conducted incubations in nutrient media with a mixed culture of microorganisms obtained from a sandy loam. Each of the following compounds was incubated separately: α- and β- endosulfan and the sulfate, diol,

ether, α-hydroxyether and lactone of endosulfan. Endosulfan diol was the major transformation product in incubations of both α- and β- endosulfan with a 74% to 77% conversion. Endosulfan diol was converted to α-hydroxyether at a 28% conversion. Endosulfan α-hydroxyether was converted to endosulfan lactone (87%), which disappeared relatively quickly with a half life of 5 hours in nutrient media.

Generally, less than 1% of endosulfan is mineralized to CO_2 in soil incubations. Aerobic and anaerobic transformation products have not been clearly differentiated due to a lack of defined redox conditions in these studies. Definitive studies on the microbial transformation products of endosulfan in soils and sediments under well defined aerobic or anaerobic conditions are lacking.

Although endosulfan bioaccumulates, there is no evidence it biomagnifies in terrestrial or aquatic food chains (USDHHS, 1993). Bioaccumulation of endosulfan is evident in fish populations, with residue levels usually peaking within 1 to 2 weeks of continuous exposure (USDHHS, 1993). For aquatic organisms, bioaccumulation is measured using a bioconcentration factor, which is the ratio of a compound in an organism's tissue to the level in water. Maximum bioconcentration factors (BCFs) for endosulfan are generally less than 3,000, with tissue concentrations falling rapidly upon transfer to clean water (NRCC, 1975). BCFs vary widely

among compounds. For example, the BCF of DDT was reported to be over 60,000, whereas the BCF of 2,4-D was measured at 20 (Kenaga, 1980).

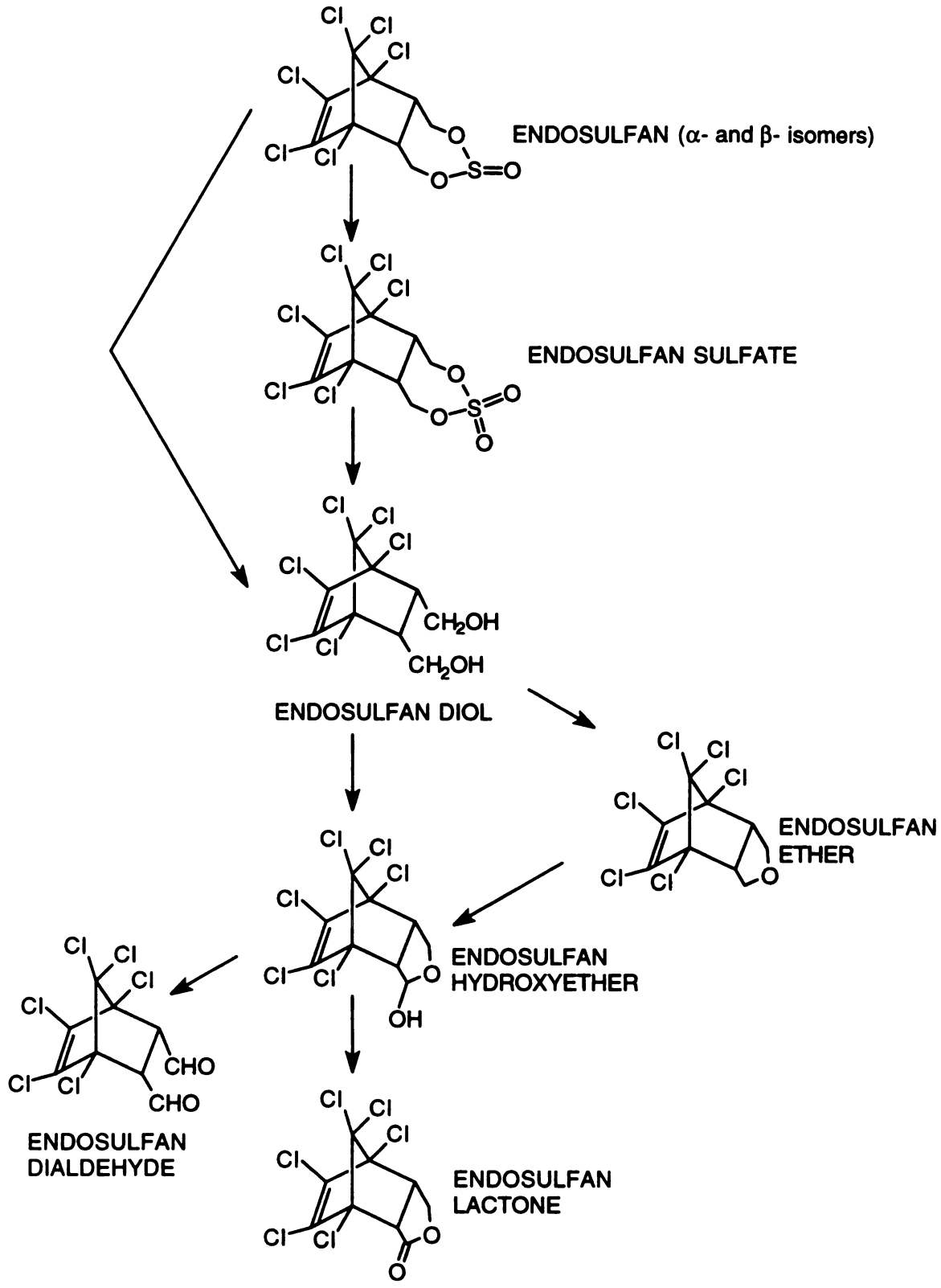

Figure 1- Microbial transformation products of endosulfan in soils. Adapted from Miles and Moy (1979).

Exposure, Metabolism and Toxicity of Endosulfan

A common route of human exposure to endosulfan is through consumption of food (USDHHS, 1993). In the United States, fruits and vegetables are the most prevalent source of exposure. Smoking tobacco treated with endosulfan and direct contact with contaminated soil are additional means of exposure. Farmers and pesticide applicators may also be exposed through dermal contact and inhalation. Acute endosulfan toxicity in humans results in adverse nervous system effects including hyperexcitability, tremors and convulsions (USDHHS, 1993).

Female rats show a greater sensitivity to endosulfan than male rats in acute toxicity studies (USDHHS, 1993). In animal studies, LD_{50} values ranged from 10 to 23 mg/kg in female rats and 40 to 125 mg/kg in male rats (Hoechst, 1990). In a four-hour nose only inhalation study, LC_{50} values were 12.6 mg/m^3 for female rats and 34.5 mg/m^3 for male rats (Hoechst, 1983). The difference in toxicity between male and female rats may relate to differences in toxicokinetics. Studies in rats and mice indicate that the α isomer of endosulfan is more toxic than the β isomer (Hoechst, 1966a, 1966b, 1975, 1990; Maier-Bode, 1968).

While many xenobiotic compounds can accumulate in fatty tissue for years, endosulfan and its metabolites are excreted in the urine or feces within a few days to a few weeks. Following exposure, endosulfan is readily metabolized to

endosulfan sulfate and endosulfan diol. These compounds can be further transformed to endosulfan lactone, hydroxyether and ether (WHO, 1984). Dorough et al. (1978) found that these nonpolar metabolites represented only a small portion of the residues found in tissues, feces and urine of exposed animals. Unidentified polar metabolites which could not be extracted made up the major portion of residues. Gupta and Ehrnebo (1979) found that almost half of the parent compound was excreted unchanged in rabbits receiving an intravenous injection of endosulfan. Metabolites including endosulfan sulfate and endosulfan diol were found in tissues and excreta following longer exposures to endosulfan (Dorough et al., 1978).

Endosulfan is metabolized in the kidneys and liver (Hoechst, 1987; Khanna et al., 1979). Increased number and size of lysosomes in the proximal convoluted tubules of the kidneys was evident in a 30-day feeding study of male rats given 34 or 68 mg/kg/day of endosulfan (Hoechst, 1987). These changes diminished significantly during the 30-day recovery period. These data indicate that endosulfan may be both metabolized and stored in the kidney. The rapid appearance of endosulfan sulfate in the liver after an intravenous exposure to endosulfan provides evidence that the liver possesses high metabolic activity in the conversion of endosulfan to endosulfan sulfate (Khanna et al., 1979). Endosulfan has been shown to induce microsomal enzyme activity, increasing liver microsomal cytochrome P-450

activity after single and multiple administrations (Siddiqui et al., 1987; Tyagi et al., 1984). Endosulfan has been shown to induce drug metabolism by increasing aminopyrine-N-demethylase and aniline hydroxylase activity (Agarwal et al., 1978).

While acute exposure can affect various organ systems, long-term exposure primarily affects the kidneys and liver (USDHHS, 1993). Respiratory effects such as dyspnea and cyanosis were observed in cases of lethal endosulfan poisoning in humans and laboratory animals (Terziev et al., 1974). It is not clear whether endosulfan directly affects the respiratory system or if these effects are due to central nervous system toxicity. Single lethal doses in dogs caused liver congestion (FMC, 1958; Hoechst, 1970), with intermediate duration studies showing increases in liver weight in female rats (Gupta and Gupta, 1977). Ingestion of large quantities of endosulfan resulted in hemorrhage of the medullary layer of the kidneys in humans and animals (Terziev et al., 1974; FMC, 1958, 1980; Hoechst, 1970). Chronic studies in rats indicate nephrotoxic effects, such as degenerative changes in the proximal convoluted tubules (NCI, 1978). No animal studies conclusively showed that endosulfan is carcinogenic (USDHHS, 1993).

Endosulfan also appears to have the potential to cause adverse reproductive effects. Male rats given oral doses of 10 mg/kg/day of endosulfan for 15 days exhibited decreased testes weight and pronounced degenerative changes of the

seminiferous tubules (Gupta and Chandra, 1977). Male rats that consumed a time-weighted average dose of 47.6 mg endosulfan per kg per day for up to 74 weeks had testicular degeneration with necrosis of germinal cells lining the seminiferous tubules, multinucleated cells and calcium deposition resulting in aspermatogenesis (NCI, 1978). High mortality was observed at this dose, limiting the study. Exposure of male rats to 7.5 to 10 mg/kg/day of endosulfan resulted in lower gonadotrophin and testosterone plasma levels (Singh and Pandey, 1990).

Teratological effects are seen at exposure levels causing maternal toxicity. Daily oral administration of 5 or 10 mg/kg/day of endosulfan during gestational days 6 through 14 resulted in an increase in the percentage of resorptions and skeletal variations in the fetuses (Gupta et al., 1978). Dose-related maternal deaths were evident at these concentrations. Administration of doses as low as 6 mg/kg/day for two weeks prior to mating and through gestation and weaning resulted in a significant decrease in mean litter weight during lactation (Hoechst, 1982). At 6 mg/kg/day both maternal toxicity and an increase in pup mortality were evident.

Endocrine Disruption

Synthetic organic chemicals have been accumulating in the environment during the past 50 years, with annual production in excess of 300 x 10^6 tons (Schwarzenbach et al.,

1993). The increase in use of, and human exposure to, synthetic organics parallels the increasing incidence of reproductive health problems in humans and wildlife (Kelce et al., 1995; Rupa et al., 1991). Recent studies provide evidence that certain environmental contaminants cause a disruption in the endocrine system, resulting in altered reproductive function in humans and wildlife (Colborn et al., 1993). These chemicals, known as "endocrine disrupters", include certain pesticides, industrial chemicals and heavy metals. Xenobiotic-induced endocrine disruption can cause adverse health effects in fetuses, children and adults. Potential outcomes include alterations in sexual differentiation, decreased fertility and increased risk of cancers of the reproductive organs.

Certain endocrine disrupters produce their effects by mimicking or enhancing the action of a natural hormone or by inhibiting its action. Other compounds act by altering the metabolism or cellular homeostasis of an endogenous hormone. Still others may cause similar effects as a natural hormone by a mechanism other than receptor binding. Many environmental contaminants have been shown to possess estrogenic (estrogen enhancing) or antiandrogenic (androgen inhibiting) properties. Previous concern has focused primarily on estrogenic contaminants. More recently, researchers discovered that certain chemicals previously thought to be estrogenic exhibit a higher binding affinity to the androgen receptor than the estrogen receptor. The

byproducts of the fungicide Vinclozolin and p,p'-DDE (a metabolite of DDT) are examples of compounds with androgen-antagonistic effects (Kelce et al., 1995). These chemicals cause reproductive disturbances not by feminization (mediated by the estrogen receptor), but by de-masculinization (mediated by the androgen receptor).

A significant reduction in fertility, fecundity and infant survival was found among couples when husbands were directly exposed to organochlorine pesticides such as endosulfan, DDT, BHC and organophosphates in the workplace (Rupa et al., 1991). These compounds impaired spermatogenesis in rats, resulting in aspermia and reproductive incapacitance (Gray et al., 1994; Kelce et al., 1995). Prenatal exposure to the antiandrogen p,p'-DDE caused demasculinization of neonates, potentially impacting reproductive performance in adult life.

There is a recognized need to investigate estrogenic and androgenic properties of both parent compounds and stable microbial degradation products (Bitman and Cecil, 1970; Kelce et al., 1994; Kelce et al., 1995). Microbial processes have the potential to mitigate the harmful effects of a parent compound. In an study investigating the effects on toxicity of reductive dechlorination of PCBs, Mousa et al. (1996) found a reduction of inhibitory effects of PCBs on *in vitro* fertilization of mouse gametes. Quensen et al. (1998) reported a decrease in aryl hydrocarbon receptor mediated toxic potential (dioxin-like toxicity) of PCB mixtures that

had undergone microbial reductive dechlorination. However, there is also potential for an increase in the biological activity of metabolites as compared to the parent compounds. Less chlorinated PCBs (produced by anaerobic microbial dechlorination) and hydroxylated PCBs (produced by aerobic microbial transformations) have higher estrogenic activity than some more heavily chlorinated PCBs (Bitman and Cecil, 1970). While the widely used fungicide Vinclozolin binds only weakly to the androgen receptor, its metabolites M1 and M2 bind to the androgen receptor 10 and 100 times more strongly, respectively (Kelce et al., 1994). Microbial transformation products may even bind to a different receptor than their parent compound. One example is p,p'-DDE, a major metabolite of the pesticide DDT. While a form of DDT, o,p'-DDT, is weakly estrogenic, p,p'-DDE acts as a potent antiandrogen (Kelce et al., 1995). The compound inhibits androgen binding to the androgen receptor and androgen-induced transcriptional activity. Male pups exposed prenatally to p,p'-DDE exhibited reduced anogenital distance at birth and retained thoracic nipples. Treatment of weanling (21 day) male rats with 100 mg p,p'-DDE per kg per day until after puberty caused a five day delay in the onset of puberty compared to control rats. Onset of puberty was defined as the day the prepuce separated from the penis. Adult male rats treated with 200 mg per kg per day by gavage for four days had a significant reduction in weight of the seminal vesicle and ventral prostate, both of which are

androgen-dependent. Serum testosterone levels in these animals remained high (Kelce et al., 1995).

Soto et al. (1995) reported that endosulfan possesses estrogenic activity as measured by the E-screen assay for estrogenic environmental contaminants. Since endosulfan is persistent in the environment, its estrogenic activity is of particular concern. The endocrine disruption potential of endosulfan's microbial transformation products has not been previously tested, although they are known to accumulate in the environment. Arnold et al. (1996) reported that combinations of two weakly estrogenic compounds were 1000 times more potent than either chemical alone, raising concerns about exposure to mixtures of chemical contaminants. This study was later retracted when the results could not be replicated, but researchers have continued to pursue the question of synergism, which is a recognized toxicological phenomenon in certain physiological processes. The potential for synergistic interaction between endosulfan and its microbial transformation products has not previously been investigated. There is a paucity of information on the endocrine disruption potential of persistent environmental contaminants and their microbial transformation products, alone and in combination, despite a recognized potential for exposure to such mixtures of chemicals.

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CHAPTER 1

ANTIANDROGENIC POTENTIAL OF ENDOSULFAN AND ITS SOIL TRANSFORMATION PRODUCTS

INTRODUCTION

Recent studies provide evidence that certain environmental contaminants cause disruption of the endocrine system, resulting in altered reproductive function in humans and wildlife (Colborn et al., 1993). These endocrine disrupters include certain pesticides, industrial chemicals and heavy metals. Xenobiotic-induced endocrine disruption can cause adverse health effects in fetuses, children and adults. Potential outcomes include alterations in sexual differentiation, decreased fertility and increased risk of cancers of the reproductive organs.

Steroid hormones, which regulate the endocrine system, exert their biological effects through binding with specific intracellular receptors. This binding activates the receptor to function as a transcription factor capable of inducing expression of genes. Xenobiotics with agonistic or antagonistic activity will bind to the receptor in a similar manner, but with different outcomes. Hormone agonists mimic the biological actions of the native hormone, while antagonists interfere with the biological functions of the native hormone. Examples are estrogen mimics such as

o,p'-DDT and certain PCB congeners (Soto et al., 1995) and antiandrogens such as p,p'-DDE (Kelce et al., 1995).

Recent research has emphasized the need to investigate endocrine disruptive activity of both parent compounds and stable microbial degradation products (Bitman and Cecil, 1970; Kelce et al., 1994; Kelce et al., 1995). Microbial processes have the potential to mitigate the harmful effects of a parent compound. In a study investigating the effects on toxicity of reductive dechlorination of PCBs, Mousa et al. (1996) found a reduction of inhibitory effects of PCBs on *in vitro* fertilization of mouse gametes. Quensen et al. (1998) reported a decrease in aryl hydrocarbon receptor mediated toxic potential (dioxin-like toxicity) of PCB mixtures that had undergone microbial reductive dechlorination. However, there is also potential for an increase in the biological activity or toxicity of metabolites as compared to the parent compounds. Less chlorinated PCBs (produced by anaerobic microbial dechlorination) and hydroxylated PCBs (produced by aerobic microbial transformations) have higher estrogenic activity than some more heavily chlorinated PCBs (Bitman and Cecil, 1970). While the widely used fungicide Vinclozolin binds only weakly to the androgen receptor, its metabolites M1 and M2 bind to the androgen receptor 10 and 100 times more strongly, respectively (Kelce et al., 1994). Microbial transformation products may even bind to a different receptor than their parent compound. One example is p,p'-DDE, a major metabolite of the pesticide DDT. While one isomer of DDT,

o,p'-DDT, is weakly estrogenic, p,p'-DDE acts as a potent antiandrogen (Kelce et al., 1995). The compound inhibits androgen binding to the androgen receptor, androgen-induced transcriptional activity, and androgen action in developing, pubertal and adult male rats. There is a paucity of information on the endocrine disruption potential of persistent environmental contaminants and their microbial transformation products, alone and in combination, despite a recognized potential for exposure to such mixtures of chemicals.

The endocrine disruptive potential of endosulfan, the only cyclodiene insecticide currently registered for use in the United States, is of particular concern due to its persistence and toxicity. Chlorinated organic pesticides such as endosulfan are major contributors to global environmental contamination (Kullman and Matsumura, 1996). In a study of the global distribution of organochlorine compounds in tree bark samples, endosulfan was found in high concentrations throughout the world, including agricultural areas throughout the United States, Europe, India, the Middle East, Japan, Brazil, Australia, Taiwan, South Korea and Russia. (Simonich and Hites, 1995). Consumption of endosulfan by rats was shown to produce testicular atrophy (Gupta and Gupta, 1979) and lower gonadotrophin and testosterone plasma levels (Singh and Pandey, 1990). Endosulfan has been shown to possess estrogenic activity

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Endosulfan (α and β) is degraded by a variety of soil microorganisms, resulting in the formation of several stable transformation products (Martens, 1977; Miles and Moy, 1979). In soil incubations, very little endosulfan is mineralized to CO_2, typically less than 1% and never more than 5%. Aerobic and anaerobic transformation products have not been clearly differentiated due to a lack of defined redox conditions in soil incubation studies. For example, endosulfan sulfate was reportedly formed as the major product both in soil columns flushed with air to create aerobic conditions (30 to 60% conversion of applied endosulfan) and in those flushed with N_2/CO_2 to create "anaerobic" conditions (11 to 20% conversion). The formation of endosulfan sulfate, an oxidation product, under anaerobic conditions suggests that the column was partially aerated. In the aerobic and "anaerobic" soil columns, endosulfan diol and endosulfan lactone were also observed (Martens, 1977). In flooded soils, endosulfan diol was the major transformation product, (up to 18%) with smaller quantities of endosulfan sulfate (3% to 8%) and endosulfan hydroxyether (2% to 4%) formed. In incubations of endosulfan in liquid culture inoculated with microorganisms eluted from soils, endosulfan diol was the major product (about 75% of applied endosulfan). Endosulfan diol was converted to endosulfan α-hydroxyether with a 28%

conversion. This product was then converted to endosulfan lactone (87%), which disappeared relatively quickly with a half life of 5 hours in nutrient media (Miles and Moy, 1979). Again, the oxidation status of the incubation was not clearly established. The degradation of endosulfan by *Phanerochaete chrysosporium* resulted in the formation of similar products (endosulfan -sulfate, -diol, and -hydroxyether) and an unknown metabolite tentatively identified as endosulfan dialdehyde (Kullman and Matsumura, 1996). Fungal metabolism was by two divergent pathways, one oxidative leading to the sulfate, and another hydrolytic leading to the diol and hydroxyether.

The major objective in this study was to assess the potential of endosulfan and its microbial transformation products to cause androgen-mediated endocrine disruption, alone and in combination. The binding of endosulfan (α and β isomers) and its metabolites to the androgen receptor was investigated using an *in vitro* binding assay with cytosolic extract from rat ventral prostate and [^{3}H]R1881 (methyltrienolone, a synthetic androgen). This compound was used because it does not bind to the testosterone-estradiol binding globulin/androgen binding protein (Bonne and Raynaud, 1975), which would most likely be present in prostate extracts. The rat can be used as a model organism for humans since rat and human androgen receptors have identical DNA binding and steroid-binding domain sequences (Lubahn et al., 1988).

To evaluate the biological significance of the *in vitro* binding study, the androgen responsive human prostatic cancer cell line, LNCaP, was used to assess the androgenic activity of endosulfan and its microbial transformation products (Horoszewicz et al., 1983). Growth assays were performed to determine whether endosulfan and its transformation products induce or inhibit LNCaP cell proliferation. Compounds acting as androgen antagonists compete for androgen-receptor binding sites. In LNCaP cells incubated with both R1881 and an antiandrogen, antiandrogens may counteract the proliferative effects of R1881.

MATERIALS AND METHODS

Chemicals

The following pesticides and metabolites were obtained from Chem Service (Westchester, PA) and used without further purification: endosulfan α (99.5% pure), endosulfan β (98.0% pure), endosulfan sulfate (98% pure), endosulfan diol (99.0% pure), endosulfan ether (99.5% pure) and endosulfan lactone (99.5% pure). Hydroxyflutamide was a gift from Schering-Plough Corp. (Dr. Rudolph Neri; Kenilworth, NJ). Radioinert R1881 and [17α-methyl-^{3}H]-methyltrienolone (R1881; 86 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Hydroxyapatite was obtained from Bio-Rad (Richmond, CA). Phenylmethylsulfonyl fluoride was purchased from Cal BioChem (La Jolla, CA). Cell culture media was purchased from Gibco Life Technologies (Grand Island, NY). All other chemicals were obtained from Sigma (St. Louis, MO) and were of the highest purity available. Solutions of endosulfan, its transformation products, hydroxyflutamide and flutamide were prepared in ethanol.

Receptor Binding Assay

Male Sprague Dawley rats of 120 to 150 days of age were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Rats were castrated at Harlan Sprague Dawley prior to air shipping. Castration was necessary because cytosolic

androgen receptors in the prostate tissue are below detection limits in intact males (Kelce et al., 1994). Rats were euthanized with CO_2 (initially mixed with O_2) 24 hours after castration. Prostate tissue was harvested and immediately immersed in ice-cold TEDG buffer (5 ml/g tissue, pH 7.4), consisting of 10 mM Tris, 1.5 mM EDTA, 10% glycerol (v/v), and 1 mM each of dithiothreitol, phenylmethylsulfonyl fluoride, and sodium molybdate. Buffer was prepared from concentrated stock solutions immediately before prostate tissue was harvested. Stock solutions of Tris, EDTA and sodium molybdate were prepared in water and stored at 4° C. A stock solution of phenylmethylsulfonyl fluoride was prepared in ethanol and stored at 4° C. Dithiothreitol was added directly to the TEDG buffer. The tissue was minced with scissors into 1-2 mm^3 pieces and homogenized with a Polytron tissue homogenizer. The extract was centrifuged at 30,000 x g (4° C) for 30 min. The supernatant, containing unoccupied cytosolic androgen receptors, was collected for use in the binding assays. Aliquots were frozen at -70° C and thawed on ice prior to use. The capacity for [^{3}H]R1881 binding to the receptor is retained after freezing and thawing one, two or three times (Kelce et al., 1994)

Cytosolic extract was incubated in 12 x 75 mm tubes with [^{3}H]R1881 and varying concentrations of test compound for 20 hours at 4°C (Kelce et al., 1994). The original 1.0 mCi/ml stock of [^{3}H]R1881 was diluted with ethanol to make a 1 x 10^{-7}M solution. Unlabelled R1881 was dissolved in ethanol and

diluted to make a 5 x 10^{-5}M solution. Both solutions were stored in amber-colored vials under N_2. Each total binding incubation contained 300 µl of cytosolic extract and 3 nM [^{3}H]R1881, with or without increasing concentrations of endosulfan, one of the microbial transformation products, or hydroxyflutamide (an antiandrogen used as a positive control). Binary combination tubes contained two test compounds. Parallel incubations containing a 500-fold molar excess of radioinert R1881 were used to determine nonspecific binding.

When the incubation was complete, portions of the cytosolic extract (100 µl) were removed and added in duplicate to 15 ml centrifuge tubes, each containing 0.5 ml of a 60% slurry of hydroxyapatite in Tris (pH 7.4). Tubes were kept in an ice-water bath throughout the procedure. The centrifuge tubes were vortexed every five minutes for 20 minutes. Tubes were centrifuged at 400 x g (4° C) for 2 to 3 minutes, then returned to the ice-water bath. The supernatant in each tube was aspirated and discarded. To each tube, 2 ml of ice-cold Tris was added and the tube was vortexed. Tubes were centrifuged at 400 x g for 2-3 minutes (4° C). The supernatant was decanted, with the remaining liquid blotted using absorbent "diaper" paper. This was repeated three additional times. Following the final decantation, 2 ml of ethanol was added to each tube. Tubes were vortexed every five minutes for 15 minutes and

centrifuged at 400 x g for 10 minutes. The supernatant from each tube was poured into its respective labeled 20 ml scintillation vial. To each tube, 14 ml of scintillation cocktail was added and bound hormone was measured by scintillation counting. Specific binding was determined by subtracting nonspecific binding from total binding in each set of parallel tubes.

LNCaP Cell Proliferation Assay

LNCaP-FG cells are of human origin and were obtained from American Type Culture Association (Rockville, MD). LNCaP cells were grown in RPMI 1640 containing 10% fetal bovine serum at 37°C in a humidified atmosphere of 95% ambient air and 5% CO_2. Passages 26 to 31 were used in the proliferation assays. Cells were plated in Corning 24-well dishes at a density of 20,000 cells/well. After cells were allowed to attach to the surface of the wells, media was aspirated and fresh basal media (RPMI 1640 with 5% charcoal stripped calf serum) was added to each well. On day one of the cell proliferation assay, wells were aspirated and basal media containing a specific concentration of a test compound was added.

The MTT assay was performed on day five. This test assesses the activity of living cells via mitochondrial dehydrogenase activity. To each well, 100 µl of 3[4,5-dimethylthizol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT, 5

media and the cells were incubated for three hours at 37°C in 95% ambient air and 5% CO_2. This assay relies on cleavage of the tetrazolium ring by mitochondrial dehydrogenases of cells, forming insoluble purple MTT formazan crystals. After the incubation, each well was aspirated and 1 ml of 0.04 N HCl in isopropanol with was added to extract the purple color. After 15 minutes incubation at room temperature, absorbance was measured at 570 nm using a Shimadzu UV-1201 uv-visible spectrophotometer.

RESULTS

Androgen Binding to the Androgen Receptor

Endosulfan lactone competitively reduced binding of androgen to the androgen-receptor, with an inhibitor concentration necessary for 50% inhibition (IC_{50}) of 450 μM (Table 1, Figure 2). Endosulfan α and endosulfan sulfate showed slight inhibition of binding at high concentrations (1 mM) (Table 2), while endosulfan β, endosulfan ether and endosulfan alcohol showed no activity in this assay. Hydroxyflutamide, a known inhibitor, had an IC_{50} of 1.7 μM in our laboratory (Table 1, Figure 2). Preliminary experiments testing binary combinations of these compounds suggest additive effects but no strong synergistic activity.

Androgen-Responsive Cell Proliferation

The LNCaP androgen responsive cell line exhibited a clear dose-related growth response in the presence of methyltrienolone. No effects of endosulfan and its soil transformation products were evident in studies assessing both androgenicity and antiandrogenicity. Hydroxyflutamide, an antiandrogenic compound used as a positive control, also failed to exert an effect. A mutation in the LNCaP cell line's androgen receptor steroid-binding domain has been reported (Veldscholt et al., 1990). This mutation was found

line's androgen receptor steroid-binding domain has been reported (Veldscholt et al., 1990). This mutation was found to alter androgen receptor binding affinity for certain compounds. A suitable androgen-responsive cell line without a steroid-binding domain mutation was not available.

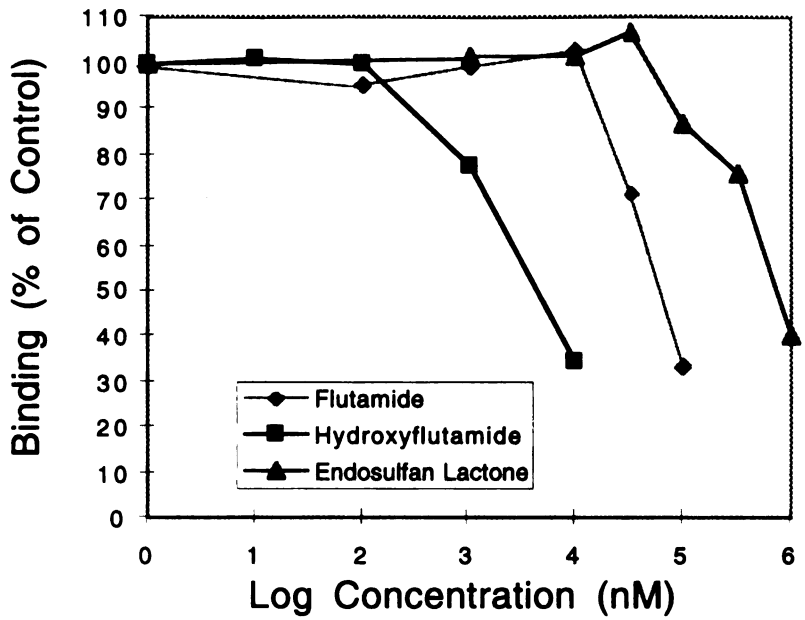

Figure 2- Androgen Receptor Binding of [^{3}H]R1881 in the Presence of Increasing Concentrations of Inhibitors

Table 1- Inhibitor Concentrations Required for 50% Androgen Displacement (IC_{50} Values)

Test Compound	Concentration (μM)
Endosulfan α	> 1,000
Endosulfan β	> 1,000
Endosulfan Alcohol	> 1,000
Endosulfan Ether	> 1,000
Endosulfan Lactone	450
Endosulfan Sulfate	> 1,000
Hydroxyflutamide	1.7
Flutamide	60

Table 2- Androgen Receptor Binding (% Control) in Incubations with 3 nM [^{3}H]R1881 and 1,000 µM Test Compound

Endosulfan α	75.6
Endosulfan β	113.0
Endsulfan Alcohol	101.1
Endosulfan Ether	106.4
Endosulfan Lactone	37.7
Endosulfan Sulfate	85.2

DISCUSSION

The finding that endosulfan lactone, a transformation product of an estrogenic parent compound, binds to the androgen receptor is interesting in light of recent studies showing that a compound and its transformation products may bind to different steroid hormone receptors (Kelce et al., 1995). The *in vitro* binding assay does not fully predict biological activity since androgen-receptor binding was measured in a cell free system. This assay alone cannot determine if a compound which binds to the androgen receptor acts as an antiandrogen or as an androgen, yet animal studies indicate administration of endosulfan results in demasculinization, suggesting antiandrogenic activity. Studies showing that endosulfan induces testicular atrophy (Gupta and Gupta, 1979) and lowered gonadotrophin and testosterone plasma levels (Singh and Pandey, 1990) indicate that endosulfan and its metabolites in the body elicit estrogenic and possibly antiandrogenic effects.

Metabolism studies show that endosulfan lactone forms in the body (WHO, 1984). Endosulfan is readily metabolized to endosulfan sulfate and endosulfan diol, which can be further transformed to endosulfan lactone, hydroxyether and ether (WHO, 1984). Significant amounts of endosulfan are excreted without metabolism (Gupta and Ehrnebo, 1979), and it is likely that the parent compound acts directly on the estrogen receptor. Whether other byproducts of endosulfan exert

estrogenic or antiandrogenic effects *in vivo* remains to be determined. Endosulfan lactone, like many other endocrine disruptors, must be present in concentrations orders of magnitude higher than natural androgens to compete for androgen receptor binding sites. Further investigation is necessary to determine the potential impact on health of humans and wildlife. Endosulfan lactone is a minor soil transformation product, with 1.2% of endosulfan converted to endosulfan lactone in aerobic incubations (Martens, 1977). Miles and Moy (1979) found that endosulfan lactone disappeared rapidly from sterile aqueous media and media inoculated with microorganisms obtained from a sandy loam soil. Endosulfan lactone had a half life of 5 hours under both conditions. The degradation was most likely catalyzed by metal salts in the media, since the half life of endosulfan lactone is 30 hours in distilled water (Miles and Moy, 1979).

The question that remains is whether compounds with weak endocrine disruption activity could combine to exert an appreciable effect. Arnold et al. (1996) reported that binary pairs of weakly estrogenic compounds synergized, exerting an estrogenic effect up to 1600 times greater than either individual compound at the same concentration. Other researchers could not replicate these results and the paper was retracted. While the *in vitro* binding study showed no evidence of synergistic activity between endosulfan and its soil transformation products, an additive response was

detected. It remains plausible that combinations of compounds with weak endocrine disruptive activity have the potential to cause adverse reproductive or developmental effects due to additivity.

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CHAPTER 2

ESTROGENIC POTENTIAL OF ENDOSULFAN AND ITS SOIL TRANSFORMATION PRODUCTS

INTRODUCTION

Recent studies provide evidence that certain environmental contaminants cause disruption of the endocrine system, resulting in altered reproductive function in humans and wildlife (Colborn et al., 1993). These endocrine disruptors include certain pesticides, industrial chemicals and heavy metals. Xenobiotic-induced endocrine disruption can cause adverse health effects in fetuses, children and adults. Potential outcomes include alterations in sexual differentiation, decreased fertility and increased risk of cancers of the reproductive organs.

Steroid hormones, which regulate the endocrine system, exert their biological effects through binding with specific intracellular receptors. This binding activates the receptor to function as a transcription factor capable of inducing expression of genes. Xenobiotics with agonistic or antagonistic activity will bind to the receptor in a similar manner, but with different outcomes. Hormone agonists mimic the biological actions of the native hormone, while antagonists interfere with the biological functions of the native hormone. Examples are estrogen mimics such as

o,p'-DDT and certain PCB congeners (Soto et al., 1995) and antiandrogens such as p,p'-DDE (Kelce et al., 1995).

Recent research has emphasized the need to investigate endocrine disruptive activity of both parent compounds and stable microbial degradation products (Bitman and Cecil, 1970; Kelce et al., 1994; Kelce et al., 1995). Microbial processes have the potential to mitigate the harmful effects of a parent compound. In a study investigating the effects on toxicity of reductive dechlorination of PCBs, Mousa et al. (1996) found a reduction of inhibitory effects of PCBs on *in vitro* fertilization of mouse gametes. Quensen et al. (1997) reported a decrease in aryl hydrocarbon receptor mediated toxic potential (dioxin-like toxicity) of PCB mixtures that had undergone microbial reductive dechlorination. However, there is also potential for an increase in the biological activity or toxicity of metabolites as compared to the parent compounds. Less chlorinated PCBs (produced by anaerobic microbial dechlorination) and hydroxylated PCBs (produced by aerobic microbial transformations) have higher estrogenic activity than some more heavily chlorinated PCBs (Bitman and Cecil, 1970). While the widely used fungicide Vinclozolin binds only weakly to the androgen receptor, its metabolites M1 and M2 bind to the androgen receptor 10 and 100 times more strongly, respectively (Kelce et al., 1994). Microbial transformation products may even bind to a different receptor than their parent compound. One example is p,p'-DDE, a major metabolite of the pesticide DDT. While one isomer of DDT,

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An important tool for studying a compound's potential to disrupt physiological processes without the expensive use of animals is *in vitro* cell culture techniques. The estrogen responsive human breast cancer cell line, MCF-7, was used to assess the estrogenic activity of endosulfan and its microbial transformation products (Katzenellenbogen et al., 1987). Estrogens are trophic hormones involved in cell growth. Compounds acting as estrogen-mimics stimulate MCF-7 cell proliferation in the absence of estradiol. Growth assays were used to determine whether endosulfan and its microbial transformation products induce cell proliferation through estrogenic activity.

The expression of certain genes is mediated through the interaction of a hormone with its receptor. Interaction with the hormone allows the receptor to bind to a specific DNA

sequence, known as a hormone responsive element. Estrogens, both synthetic and natural, exert their physiological effects through binding with the nuclear estrogen receptor (ER) that functions as a nuclear transcription factor upon ligand binding and activation. Thus, estrogens may modulate expression of genes that contain recognition sequences (or response elements) for the activated estrogen receptor. A number of cellular genes such as cathepsin D and pS2 are known to be induced by estrogens (Rochefort, 1995).

Cell based bioassays can be developed to examine estrogenic activity of chemicals through the use of genetic engineering technology. In this technique, the estrogen response element (ERE) sequences are placed upstream of reporter genes, such as luciferase, which contain a promoter element. A plasmid is then constructed and plasmid DNA is transfected into cell lines that express ER. Cells are treated with estrogenic agents and the level of expression of the reporter gene is assayed following a short exposure. Estrogenicity of the test compounds is proportional to reporter gene activity. The ability of endosulfan and its microbial transformation products to induce or interfere with gene transcription was investigated in MCF-7 cells transfected with the estrogen response element and luciferase reporter gene.

MATERIALS AND METHODS

Chemicals

The following pesticides and metabolites were obtained from Chem Service (Westchester, PA) and used without further purification: endosulfan α (99.5% pure), endosulfan β (98.0% pure), endosulfan sulfate (98.0% pure), endosulfan diol (99.0% pure), endosulfan ether (99.5% pure) and endosulfan lactone (99.5% pure). Cell culture media was purchased from Gibco Life Technologies (Grand Island, NY). The luciferase assay kit was obtained from Promega Corporation (Madison, WI). The Galacto-Light Plus kit was purchased from Tropix (Bedford, MA). All other chemicals were obtained from Sigma (St. Louis, MO) and were of the highest purity available. Solutions of endosulfan, its transformation products, o,p'-DDT and estradiol were prepared in ethanol.

MCF-7 Cell Proliferation Assay

MCF-7 cells were grown in MEM-α media supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 95% ambient air and 5% CO_2. Six days before treatment, media was removed and replaced with MEM (without phenol red) with 5% charcoal stripped calf serum. The hormone free culture was necessary to render the cells estrogen responsive. One day before treatment, cells were plated in 24-well dishes at a density of 10,000 cells/well. On the day of treatment, test

compounds (endosulfan compounds, estradiol or o,p'-DDT at varying concentrations) dissolved in ethanol were added to test tubes containing phenol red free MEM with 5% charcoal-stripped calf-serum media. Binary combination tubes contained two different test compounds. Final concentration of ethanol was 0.1% and did not affect cell viability or growth. Media was aspirated from each well and 1 ml of fresh media containing a specific test compound or mixture of test compounds was added.

The MTT assay was performed on day five. This test assesses the activity of living cells via mitochondrial dehydrogenase activity. To each well, 100 µl of 3[4,5-dimethylthizol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT, 5 mg/ml in distilled water), was added to the tissue culture media and the cells were incubated for three hours at 37°C in 95% ambient air, 5% CO_2. This assay relies on cleavage of the tetrazolium ring by mitochondrial dehydrogenases of cells, forming insoluble purple MTT formazan crystals. After the incubation, each well was aspirated and 1 ml of 0.04 N HCl in isopropanol with was added to extract the purple color. After 15 minutes incubation at room temperature, absorbance was measured at 570 nm using a Shimadzu UV-1201 uv-visible spectrophotometer.

Estrogen Response Element Gene Expression Assay

MCF-7 cells were plated at a density of 100,000 cells/well in 24-well dishes. The following day, cells were cotransfected with the ERE-luciferase and β-galactosidase plasmids (10:1). The plasmid PT109, containing three vitellogenin estrogen response elements upstream of the luciferase reporter gene and thymidylate kinase (TK) promoter, was a gift from Dr. Craig V. Jordan at Northwestern University (Chicago, IL). The CMV-β-galactosidase plasmid was provided by Dr. Robert M. Bigsby, Department of Obstetrics and Gynecology, Indiana University (Indianapolis, IN). Transfection was performed by diluting 41 µl (24 µg DNA) of 3x ERE plasmid and 2.4 µl (2.4 µg DNA) of CMV β-gal plasmid with opti-MEM for a final volume of 400 µl. A 30 µl (1 µg/µl) aliquot of Lipofectamine was diluted to 400 µl with opti-MEM (a special medium suitable for transfections). The two solutions were gently mixed and allowed to stand at room temperature for 30 minutes to facilitate incorporation of the plasmid DNA into the lipid vesicle. Cells were rinsed with MEM-α containing no antibiotics or serum. The transfection mix was diluted to 24.5 ml with opti-MEM. This solution was dispensed to each well at a volume of 0.5 ml/well. Cells were transfected overnight. The transfection media was

removed and 1 ml MEM-α was added to each well. Cells were treated with test compounds (endosulfan compounds and o,p'-DDT: 10^{-5}M, estradiol: 10^{-10}M) the following day. After a 24 hour incubation, cells were lysed in 100 µl of the reporter lysis buffer and stored at -70°C.

Lysates were thawed and the luciferase and β-galactosidase assays were performed. Luciferase assay buffer (100 µl) from the luciferase assay kit was added to each assay tube followed by the addition of 20 µl of cell lysate. The light output was measured immediately using a LB-9501 Berthold Luminometer. Three replicates were used for each treatment.

The β-galactosidase assay measures the expression of β-galactosidase in mammalian cells transfected with an expression vector. The expression of β-galactosidase is used to normalize the activity of the luciferase reporter gene in transient transfection experiments. The Galacton substrate was diluted 100-fold with Galacto-Light Reaction buffer. A portion (100 µl) of the reaction buffer was dispensed into each of the assay tubes. An aliquot (20 µl) of the cell lysate of each sample was added to each tube and incubated for 30 minutes at room temperature. Light Emission Accelerator solution from the assay kit was added to each

tube (100 µl), mixed and immediately placed in the LB-9501 Berthold Luminometer. A 20 second light emission measurement was taken. Luciferase activity of each lysate was normalized to 100,000 light units of β-galactosidase activity.

RESULTS

MCF-7 Cell Proliferation

Endosulfan (α and β isomers) was found to be estrogenic in the MCF-7 cell proliferation assay (Figure 3). Both endosulfan α and endosulfan β promoted estrogen-responsive cell proliferation in the MCF-7 cells in a dose related manner (Figure 4). The microbial degradation and hydrolysis product endosulfan diol also exhibited estrogenic properties similar to o,p'-DDT. Endosulfan diol and o,p'-DDT caused slightly greater cell proliferation than endosulfan (α and β isomers). Binary compounds did not exert a synergistic effect.

Estrogen Response Element Gene Expression

Endosulfan diol and o,p'-DDT caused the greatest estrogen response element gene expression, followed by endosulfan α and β isomers, as measured by the luciferase reporter gene activity in a preliminary experiment (Figure 5). All other transformation products tested showed slight estrogenic activity above the control, but less than that of endosulfan and endosulfan diol. This test is extremely sensitive and can detect estrogenic activity not apparent in the MCF-7 cell proliferation assay. Binary combinations of

compounds were found to have additive effects but no synergistic activity.

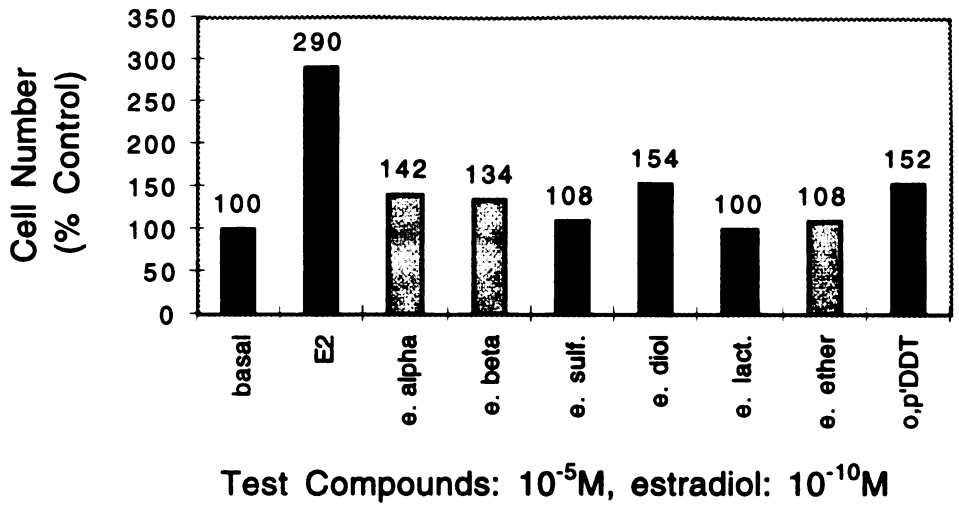

Figure 3- MCF-7 Cell Proliferation in the Presence of Endosulfan Compounds and o,p'-DDT

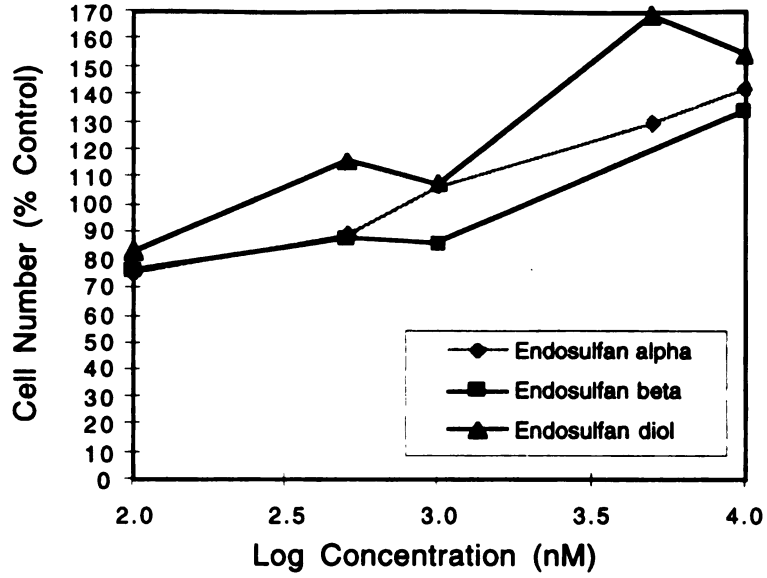

Figure 4- MCF-7 Cell Proliferation in the Presence of Increasing Concentrations of Endosulfan and Endosulfan Diol

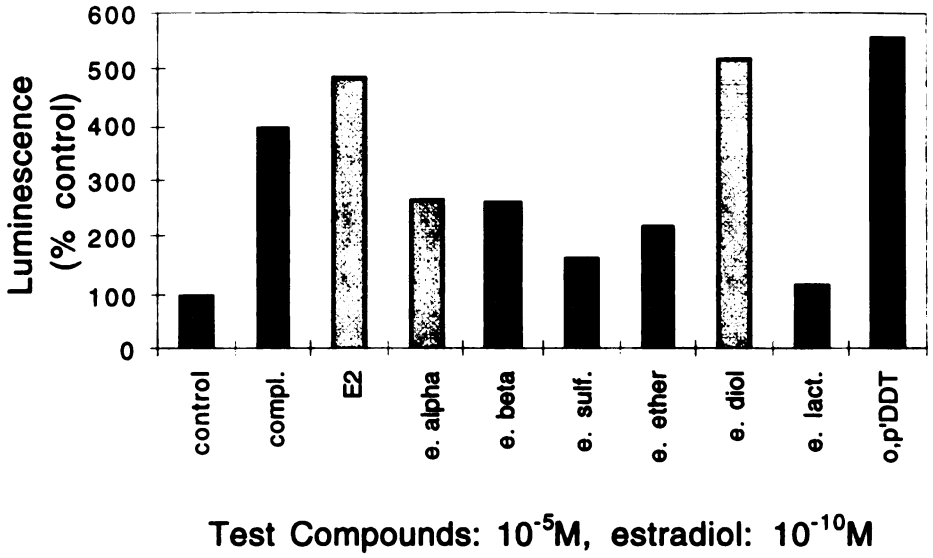

Figure 5- Estrogen Response Element Gene Expression in the Presence of Endosulfan Compounds and o,p'-DDT

DISCUSSION

The estrogenic activity of endosulfan α, endosulfan β, endosulfan diol and o,p'-DDT (used as a positive control) as measured by estrogen response element gene expression correlated with the compounds' potential to cause estrogen-responsive cell proliferation in MCF-7 cells. Other transformation products (i.e. endosulfan -sulfate and -ether), not active in the cell proliferation assay were found to exert effects on gene expression in the luciferase assay. This finding may indicate the potential of these compounds to modulate gene expression without causing an increase in cell proliferation. These compounds may have the potential to cause nonproliferative changes in cells. It is likely, however, that these compounds have very limited activity which can be detected by the more sensitive luciferase assay.

The finding that endosulfan diol is estrogenic is particularly significant since it is a major degradation product of endosulfan in flooded soils (Martens, 1977) and in incubations in aqueous nutrient media (Miles and Moy, 1979). Endosulfan diol is also the major fungal degradation product (Martens, 1976). The presence of endosulfan diol as a stable transformation product in soils warrants concern about its effects on humans and wildlife. In addition to its occurrence in soils, endosulfan diol is also commonly found in waters (Peterson and Batley, 1993). In a study measuring

endosulfan compounds in lagoons and irrigation channels, endosulfan (α and β) and endosulfan sulfate were found to be more prevalent in sediment than water (Peterson and Batley, 1993). In contrast, endosulfan diol was found in substantial concentrations in water. Endosulfan diol represented 50% of total endosulfan compounds measured in water, but only 3% on particulates (Peterson and Batley, 1993). The significant presence of endosulfan diol in water may result in greater exposure of aquatic organisms to endosulfan diol than to other endosulfan compounds.

Whereas endosulfan is known to be highly toxic to fish, endosulfan diol is considered non-toxic (Peterson and Batley, 1993). Although endosulfan diol may not elicit an acute toxic responses, it may have the potential to cause serious reproductive outcomes. Further research is needed to determine the potential of endosulfan compounds to effect endocrine system functioning in exposed human and wildlife populations. Since fish, reptiles, amphibians and birds possess different estrogen receptor structures than mammals, they may respond differently to endosulfan compounds.

A significant finding of the study was that while endosulfan and some of its transformation products were estrogenic, endosulfan lactone may possess androgen disruptive effects as indicated by its ability to bind to the androgen receptor. The biological significance is that exposure to endosulfan could cause endocrine disruption of both estrogen dependent as well as androgen dependent

systems. Such an effect could have significant impact on the reproductive health of both males and females.

Endosulfan diol and lactone can both form in the body as well as in soil and aquatic ecosystems. Following exposure, endosulfan is readily metabolized to endosulfan sulfate and endosulfan diol. These compounds can be further transformed to endosulfan lactone, hydroxyether and ether (WHO, 1984). A significant amount of endosulfan passes through the system unchanged. Gupta and Ehrnebo (1979) found that almost half of the parent compound was excreted unchanged in rabbits receiving an intravenous injection of endosulfan. Metabolites including endosulfan sulfate and endosulfan diol were found in tissues and excreta following longer exposures to endosulfan (Dorough et al., 1978).

Clearly, a parent compound and its transformation products possess distinct toxicological properties. This study reinforces the need to investigate endocrine disruption potential of both parent compounds and their stable metabolites. Transformation of environmental contaminants has the potential to mitigate a parent compound's toxicity (Mousa et al., 1996; Quensen et al., 1998). However, this work and that of Kelce et al. (1995) and Soto et al. (1995) provide compelling evidence that transformation may result in increased biological activity, including endocrine disruptive effects. Furthermore, a parent compound and its transformation products may bind to different steroid hormone

receptors or cause toxicological effects by different mechanisms altogether.

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