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Review Article

An Assessment of the Developmental, Reproductive, and Neurotoxicity of Endosulfan

Marilyn H. Silva* **and Derek Gammon,**

Department of Pesticide Regulation, California Environmental Protection Agency, Sacramento, California

BACKGROUND: Endosulfan has been used for over 50 years. Although most analogs have been discontinued, endosulfan has less environmental persistence. Nevertheless, pressure groups are lobbying for a worldwide ban. The reasons are: possible rodent male reproductive toxicity, other endocrine effects and cancer; human epidemiology, and exposure studies; residues appearing in remote areas of the world, e.g., the Arctic. **METHODS**: The endosulfan toxicology database is described and risks of its use assessed. **RESULTS**: Endosulfan is an antagonist at the $GABA_A$ receptor Cl^- ionophore in mammalian CNS. Rat acute toxicity is moderate, $LD_{50} = 48$ (M) or 10 mg/kg/d (F), oral gavage; 130 (M), 70 mg/kg/d (F) dermal; $LC_{50} = 34.5\,\mu g/L$ (M), 12.6 μg/L (F), inhalation. Critical NOELs for risk assessment: acute oral (gavage) = 0.7 mg/kg/d (rabbit developmental); Subchronic oral (diet) = 1.2 mg/kg/d (rat reproduction); Chronic oral (diet) = 0.6 mg/kg/d. There were no acceptable dermal toxicity studies. The critical acute and subchronic inhalation NOELs = 0.001 mg/L, chronic inhalation = 0.0001 mg/L (estimated). Toxicity to rat sperm occurred at doses causing neurotoxicity. Endocrine effects, resulting from P450 oxygenase(s) induction, were reversible. Increased cancer, genotoxicity, or histopathology in rodents was not observed in any organ. Possible effects on brain biogenic amine levels were probably secondary. **CONCLUSIONS**: Epidemiology and rodent studies suggesting autism and male reproductive toxicity are open to other interpretations. Developmental/ reproductive toxicity or endocrine disruption occurs only at doses causing neurotoxicity. Toxicity to the fetus or young animals is not more severe than that shown by adults. *Birth Defects Res (Part B)*, 86:1–28, 2009. 2009 Wiley-Liss, Inc.

INTRODUCTION

Endosulfan (6, 7, 8, 9, 10, 10–hexachloro–1, 5, 5a, 6, 9, 9a–hexahydro–6, 9–methano–2, 4, 3–benzodioxathiepin–3–oxide), patented in 1956 (Ware, 1994), is a pesticide belonging to the chemical family of organochlorines, containing only one double bond, and it consists of two isomers (α-: 64–67%; β-: 29–32%; Maier–Bode, 1968; NRCC, 1975). Endosulfan is a contact and stomach insecticide for food and non–food crops and it is toxic to fish and other aquatic organisms (Suntio et al., 1988; Naqvi and Vaishnavi, 1993; Toledo and Jonsson, 1992). The α-isomer is more toxic to insects and mammals than the β-isomer.

Endosulfan binds to and blocks the Cl^- channel linked to the γ–amino–butyric acid ($GABA_A$)-receptor. It does not affect the GABA recognition site and so can be termed a "non-competitive GABA antagonist" (Lawrence and Casida, 1984; Abalis et al., 1986; Ffrench-Constant, 1993). Because $GABA_A$ receptors are the principal inhibitory neuroreceptors in the mammalian brain, the antagonism of GABAergic neurons within the central nervous system (CNS) causes generalized brain stimulation (Abalis et al., 1986; Cole and Casida, 1986; Gant et al., 1987; Ozoe and Matsumura, 1986). When GABA binds to its receptor ($GABA_A$), the Cl^- ion channels are opened, leading to an influx of Cl^- into neurons through an electrochemical gradient. The result is hyperpolarization of the cell membrane and inhibited neuron firing. Endosulfan prevents Cl^- from entering

Abbreviations: Ach, acetyl choline; AchE, acetyl cholinesterase; ADP, adenosine diphosphate; ATP, adenosine triphosphate; CalEPA, California Environmental Protection Agency; Cl^-, chloride; CYP, cytochrome P450; DNT, developmental neurotoxicity study; DPR, Department of Pesticide Regulation; ENEL, estimated no effect level; F, female; FIFRA, Federal Insecticide, Fungicide and Rodenticide Act; FOB, functional observational battery; FQPA, Food Quality Protection Act 1996; $GABA_A$, γ–amino–butyric $acid_A$; HDT, highest dose tested; HSD, hydroxysteroid dehydrogenase; i.p., intraperitoneal; i.v., intravenous; LC_{50}, median lethal concentration; LD_{50}, median lethal dose; LD, lactation day; LDT, lowest dose tested; LOEL, lowest observed effect level; M, male; MTD, maximally tolerated dose; NADH, nicotinamide adenine dinucleotide (reduced); NADPH, nicotinamide adenine dinucleotide phosphate; NOEL, no observed effect level; NRCC, National Research Council Canada; ppm, parts per million; PND, post natal day; PPS, preputial separation; RBC, red blood cell; RCD, risk characterization document; SB950, Birth Defects Prevention Act of 1984; s.c., subcutaneous; SF, safety factor; TAC, toxic air contaminant; USEPA, U.S. Environmental Protection Agency; UF, uncertainty factor; WHS, Worker Health and Safety Branch

The interpretations expressed are the authors' and do not necessarily reflect policies of the Department of Pesticide Regulation (DPR) of the California Environmental Protection Agency (CalEPA).

*Correspondence to: Marilyn H. Silva, Department of Pesticide Regulation, California Environmental Protection Agency, 1001 I Street, P.O. Box 4015, Sacramento, CA 95812. E-mail: msilva@cdpr.ca.gov
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neurons, thus blocking the effect of GABA binding to its $GABA_A$ receptor, resulting in uncontrolled excitation.

As one of the few organochlorines still registered for use, endosulfan has elicited worldwide concern for many reasons. The USEPA states that, "…based on environmental fate laboratory studies, terrestrial field dissipation studies, available models, monitoring studies, and published literature, it can be concluded that endosulfan is a very persistent chemical which may stay in the environment for lengthy periods of time, particularly in acid media" (USEPA, 2002). Non-governmental organizations such as Pesticide Action Network of North America (PANNA, 2008) have campaigned for a total worldwide ban of endosulfan, claiming that it is a carcinogen, teratogen, and a male reproductive toxicant. Under the Food Quality Protection Act (FQPA, 1996), for example, endosulfan was recently assessed for its potential as an endocrine disruptor because (among other reasons) it is weakly estrogenic in MCF-7 cells in vitro (Soto et al., 1994, 1995). In California, endosulfan is still widely used on many crops and there is evidence that women of childbearing age and children of all ages are exposed via diet and air (see "Complete Risk Assessment Document" in Silva, 2008). A risk assessment was conducted after a data call-in under the California Birth Defects Prevention Act (1984), which required that registrants fill data gaps for acute, subchronic, developmental, and reproductive toxicity as well as chronic effects (including cancer). Endosulfan has been recommended for listing as a toxic air contaminant (TAC) in California due to unacceptable exposure levels for infants and children (California Assembly Bill 1807, 1983).

The intent of this report is to establish whether or not there is evidence that endosulfan is an endocrine disruptor, a teratogen, or a reproductive toxicant. Detailed analyses are presented, including studies from the open literature (animal in vivo and in vitro, along with epidemiology) and registrant-submitted reports under the Federal Insecticide, Fungicide, and Rodenticide Act Guidelines (USEPA, 1997a).

MATERIALS AND METHODS

The risk assessment process involves four steps: hazard identification, dose-response assessments, exposure estimates, and risk characterization. A combination of studies in the open literature and reports submitted by the principal registrants are considered. After a review of the toxicological database, toxic effects have been identified in the laboratory animal studies, following short-term (acute), subchronic, and chronic exposure. Subsequently, after analysis of the dose-response, the lowest dose that does not cause toxicity (no-observed-effect-level: NOEL) is identified for all endosulfan exposure durations.

Additional safety factors may be required for risk assessment, following the passage of the FQPA in 1996 (FQPA, 1996). The FQPA is a law that was specifically designed to protect infants and children from the potentially harmful effects of a pesticide by mandating that the USEPA "upgrade its risk assessment process as part of the tolerance setting procedures" by requiring an explicit finding that dietary tolerances are safe for children (USEPA, 1997a,b, 1998). One of the main aspects of this law involves effects of in utero exposure and potential for endocrine-disrupting effects. These issues will be addressed in this review, with specific reference to endocrine disruption in rat, mouse, and rabbit. An extra 10-fold Safety Factor (10× SF) was allowed under FQPA, and was intended to account for pre- and post-natal developmental effects and the lack of a complete database (unless USEPA determined, based on reliable data, that a different margin would be safe).

Open literature studies performed since 1992 showed male reproductive and developmental toxicity in rats. Some of the parameters measured were not required of registrant-studies submitted under early FIFRA Guidelines prior to 1998. In 1998, FIFRA Guidelines (USEPA, 1998) for reproductive and developmental studies were revised subsequent to the FQPA and required additional endpoints to be measured such as sperm morphology, motility, and production; vaginal opening, preputial separation (PPS), and others. The USEPA required endosulfan registrants to perform a dietary DNT to evaluate both the potential for neuropathology and/or neurobehavioral toxicity to pups after exposure in utero or through milk. The DNT study also assessed the reproductive parameters that had not been included in the rat reproduction study (Edwards et al., 1984; USEPA, 2002, 2007).

The USEPA also noted a data gap for Subchronic Neurotoxicity. Since endosulfan is a neurotoxicant, the FIFRA Guideline-recommended neurobehavioral toxicity studies were performed for acute and subchronic intervals that would represent dietary exposure on an acute or seasonal basis (USEPA, 2002). In addition, endosulfan has been recommended for listing in California as a TAC, since the margin of exposure (MOE: ratio of NOEL to exposure levels, an indicator of safe limits) in infants (bystanders at field application site) is $<1{,}000$ (10× interspecies, 10× intraspecies extrapolations, 10× for TAC). According to the California Food and Agriculture Code an additional 10× SF must be added if a pesticide is listed as a TAC. Since the MOE for infant bystanders is $<1{,}000$, all effects in young animals in inhalation studies have received extra attention.

In the process of pesticide registration, studies submitted by the registrant using technical material are required under FIFRA Guidelines (as amended for the FQPA, USEPA, 1998). If a study is acceptable according to these guidelines it will be so designated for risk assessment. Information available in the open literature is usually considered to be supplemental; however, it carries equal weight under certain circumstances. For instance, studies in the open literature indicated that endosulfan might be an endocrine disruptor. Subsequently, data from USEPA-requested FIFRA Guideline studies were able to answer these concerns.

While acute, subchronic, and chronic/oncogenicity studies submitted by the registrant were not specifically designed to study developmental or reproductive effects, they were included in this review because they contain thorough descriptions of clinical signs (neurotoxicity), necropsy, weights (absolute and relative), and histopathology of all reproductive organs for both sexes. Therefore, large numbers of animals (including individual data and historical controls) could be examined for the effects of endosulfan. When such parameters were examined in studies from the open literature, it will also be indicated. The focus is on studies that are related to

reproduction, developmental stages (fetal, neonatal, pubescent, adult), endocrine disruption, and neurobehavioral toxicity occurring during the developmental stages and the eventual long-term effects on adults of both sexes. There is also a comparison of endosulfan-related effects in adults versus pups as well as males versus females. Details include, when available, vehicle, strain, and number of animals treated, treatment results, and NOEL.

RESULTS

Pharmacokinetics

The majority of endosulfan, regardless of exposure route, is excreted rapidly in feces, with virtually no retention in tissues, despite the lipophilicity of endosulfan and its primary metabolite, endosulfan sulfate (Dorough et al., 1978). After a single gavage, endosulfan administration (2 mg/kg) in female rats was assessed for metabolism and males for effects of bile duct cannulation. With bile duct cannulation, elimination in feces was decreased two-thirds at 48 hr but elimination in urine was unchanged. If the enterohepatic recirculation was a major path, then elimination in the urine would have increased and feces would have remained relatively unchanged. This suggests that metabolites passing from the liver to the intestine via the bile in the intact rat were not suitable for reabsorption and excretion via the kidney/urine. At 48 hr, oral absorption in females (urine +bile) was approximately 59.7% for α- and 39.3% for β-isomer and 13% of the α-radioactivity and 10.8% of the β-radioactivity in feces were metabolites. At 120 hr, 88% of α-[^{14}C]-endosulfan and 87% of β-[^{14}C]-endosulfan had been eliminated. Chan et al. (2005) used 5 mg/kg by gavage (1–3 doses) to male Sprague-Dawley rats to show that liver, kidney, fat, GI tract, muscle, brain, heart, lung, spleen, testis, and thyroid gland had ^{14}C activity at 1, 2, 4, and 8 hr post-dose. ^{14}C-endosulfan-derived radioactivity in blood had a distribution half- life of 31 min and a terminal elimination half-life of 193 hr. Blood concentration reached its maximum (0.36 mg/L) at 2 hr post-dosing after being rapidly absorbed from the GI tract (absorption rate constant = 3.07 hr^{-1}).

Liver microsomal P–450 was induced in both sexes of rat after single and multiple administrations of endosulfan (Fabacher et al., 1980; Siddiqui et al., 1987; Tyagi et al., 1984). Stereoselective endosulfan sulfate formation from human recombinant P450s (CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5) showed that α-endosulfan metabolism is mediated by CYP2B6, CYP3A4, and CYP3A5 and β-isomer is mediated by CYP3A4 and CYP3A5 (Lee et al., 2006). Casabar et al. (2006) and Hodgson and Rose (2008) demonstrated that α-endosulfan produced only endosulfan sulfate when it is metabolized by human liver microsomes (HLM) in vitro. They also identified P450 isoforms CYP3A4 and CYP2B6 as the primary enzymes catalyzing the metabolism of the α-endosulfan. After testing 16 different HLMs a strong correlation resulted with endosulfan sulfate formation and *S*-mephenytoin *N*-demethylase activity of CYP2B6 ($r^2 = 0.79$), and a moderate correlation with testosterone-6-β-hydroxylase activity of CYP3A4 ($r^2 = 0.54$) was observed.

Aminopyrine-N-demethylase, aniline hydroxylase, and glutathione *S*-transferase activities were induced by endosulfan in hepatic and extrahepatic tissues in the rat (Agrawal et al., 1978; Narayan et al., 1984, 1990a,b; Den Tonkelaar and Van Esch, 1974; Robacker et al., 1981; Singh and Pandey, 1989b). The proposed metabolic pathway for endosulfan in rat and sheep is shown in Figure 1 (Dorough et al. 1978; Gorbach et al., 1968; Bebe and Panemangatore, 2003; Lee et al., 2006).

Acute (LD_{50}, LC_{50}), Rat

While the acute studies are not specifically related to developmental or reproductive effects, they illustrate the gender difference in susceptibility to endosulfan neurotoxicity. Depending upon the route of exposure, females are 2–5 times more sensitive to endosulfan toxicity. There were no toxic effects on reproductive organs in any study.

Gavage. Endosulfan was administered by gavage in a single dose to Sherman rats (5/sex/dose) at 0 (ground-nut oil), 31.6, 46.4, 68.1, 100, 147, and 215 mg/kg (male: M) and at 0, 6.3, 8.0, 10, and 12.5 mg/kg (female: F) (Scholz and Weigand, 1971a,b). Beginning at 1 hr post-dosing, affected males showed depression, lacrimation, exophthalmia, labored respiration, and ataxia. Several animals (number not specified) also showed salivation, tremors, clonic- and tonic convulsions, and depressed righting, placement, and pain reflexes. Death was preceded by bloody discharge from the eyes, gasping, tonic and clonic convulsions, coma (M only) or disequilibrium, muscular tremors, and extension spasms (F only). Necropsy showed hyperemic or hemorrhagic lungs, irritation of the pyloric portion of the stomach and the small intestine, and congested kidneys and adrenals (M only) or reddening of shorter portions of the small intestine (F only). Surviving males at 100 mg/kg and below showed clinical signs for 24 hr post dosing but were normal by 48 hr. The LD_{50} was 48 mg/kg (M) and 10 mg/kg (F).

Dermal. A single endosulfan treatment was administered dermally, without occlusion, to Sherman rats (60–70/sex/dose) at unspecified dose levels (Gaines, 1969), giving LD_{50} values of 130 mg/kg (M) and 74 mg/kg (F). Dermal effects were erythema and slight desquamation. The report lacked details on body weights, actual doses tested, and clinical effects.

Inhalation. An acute, nose–only aerosol inhalation study used endosulfan in Wistar rats (5/sex/dose) at 0 (polyethylene glycol+ethanol), 0.0036, 0.0123, 0.0288, 0.0401 and 0.0658 mg/L (equivalent to 0.61 mg/kg/d in F only and 2.08, 4.87, 6.78, and 11.13 mg/kg/d in M/F) in aerosol form for 4 hr (Hollander and Weigand, 1983). Observation time periods were at 11, 149, 179, 240, 314, and 434 min and 1, 7, and 14 days post-dosing. The LC_{50} for males was 0.0345 mg/L air (5.84 mg/kg/d) and for females was 0.0126 mg/L air (2.13 mg/kg/d). Dose-related decreased body weight gain (3-d males and 14-d females post-dose), increased clinical signs (all doses: dyspnea, trembling, passivity, disturbed equilibrium, tremors, convulsions; decreased reflexes: corneal, pupillary light, righting, startle, paw, and cutaneous), as well as death and red spots on the lung were observed. The LOEL was 0.0036 mg/L (0.61 mg/kg/d, LDT in F only) based on neurotoxicity.

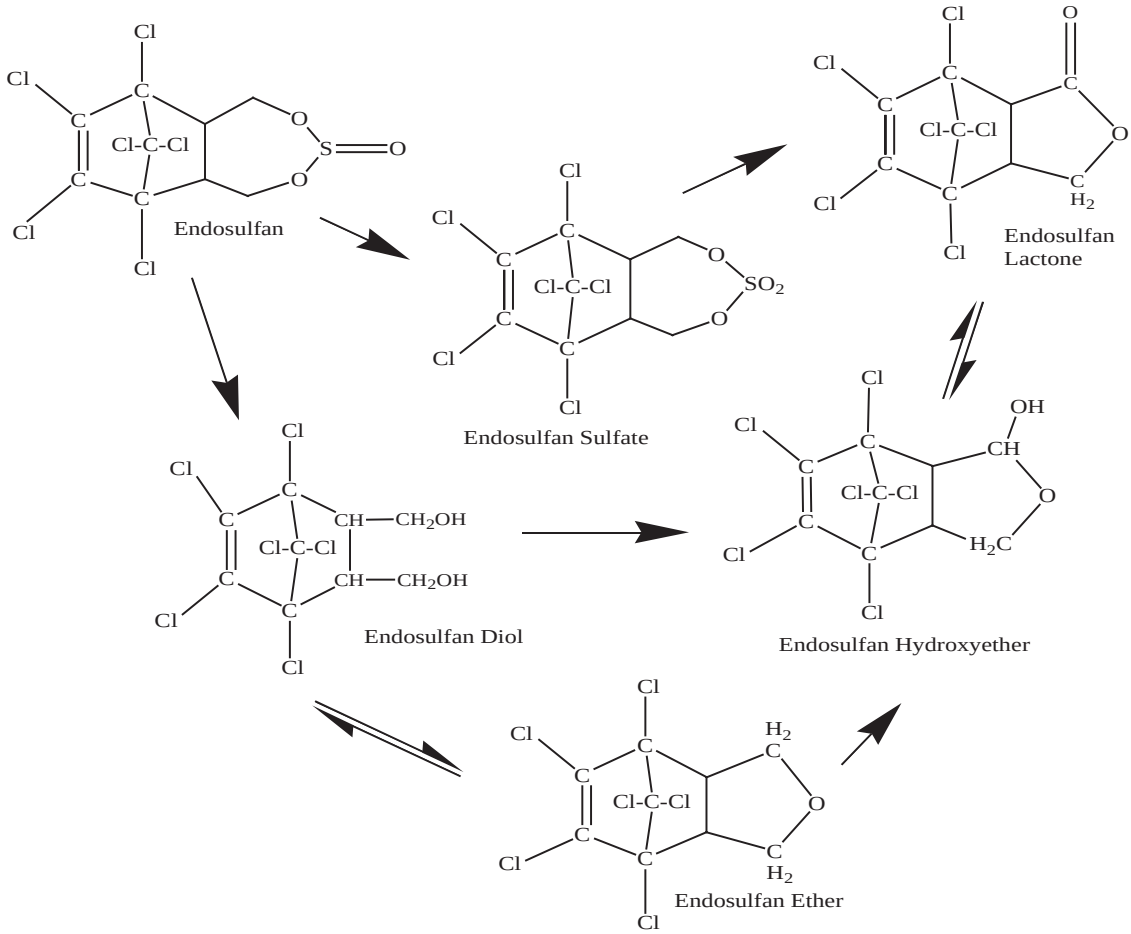

Fig. 1. Proposed Metabolic Pathway in Rat and Sheep for Endosulfan (Dorough, et al., 1978; Bebe and Panemangatore, 2003; Lee et al., 2006) Phase I reactions on endosulfan are performed with P450s: CYP2B6, CYP3A4 & CYP3A5; Phase II reaction is with GST; Other enzymes involved with endosulfan metabolism are antioxidants: SOD, GPX and CAT.

Subchronic, Rat

Dietary. CD rats (25/sex/dose+5/sex/dose recovery) were fed endosulfan in diet for 13 weeks at 0, 0.64, 1.92, 3.85, or 23.41 mg/kg/d (M) and 0.75, 2.26, 4.59, or 27.17 mg/kg/d (F) with a 4-week recovery (Barnard, et al., 1985). Clinical signs (hair loss) were seen at $\geq$4.59 mg/kg/d (F) and enlarged kidneys at $\geq$3.85 mg/kg/d (M). Both effects were reversed after a 4-week recovery period. Liver showed granular brown pigment (M) and centrilobular enlargement of hepatocytes at the HDT. Increased absolute liver weight was also observed in both sexes at the HDT. Kidney pigmentation increased at the top two doses but disappeared after the 4-week recovery. Plasma and serum biochemistry changes were also noted. Absolute epididymal weight was increased (9%) at the HDT and absolute kidney weight was increased at the two highest doses (M) and the HDT (F). Effects on absolute organ weight in the absence of effects on histology or on relative weight is not considered to be toxicologically adverse because the effects are likely due to bodyweight effects, rather than direct organ toxicity. The NOEL was established at 1.92 mg/kg/d (M) and 2.26 mg/kg/d (F).

Gavage. Albino rats (6/sex/dose) were gavaged with endosulfan at 0 (peanut oil), 0.75, 2.5, and 5.0 mg/kg/d (M) and 0.25, 0.75, and 1.5 mg/kg/d (F) for 30 days (Dikshith et al., 1984). After termination of treatment, males and females were mated to assess fertility, reproduction, and fetal development. Clinical signs (hyperexcitability, tremor, dyspnea, salivation), observed after the initial treatments, disappeared in 3–4 days. Body weights were unaffected in either sex; however, relative liver (20%), kidney (14%), and testes (100%) weights were increased at 5.0 mg/kg/d in males and relative kidney weights were decreased (13%) in females at 1.5 mg/kg/d. Blood parameters were affected (M/F: liver alkaline phosphatase, RBC, neutrophils) at high doses. There were no effects on fertility, reproduction, or fetal development in either sex. The effects in testes occurred only at the HDT where males exhibited a great deal of toxicity in the form of the effects described above. It is also possible that organ weights were increased due to induction of P450 and other enzymes at toxic doses. The NOELs were 2.5 (M) and 0.75 mg/kg/d (F) for effects on organ weights and blood.

ITRC male rats (8/dose) were treated by gavage at 0 (peanut oil), 5.0, and 10 mg/kg/d for 15 days (Gupta and Chandra, 1977). Rat body weight and body weight gain and absolute organ weights were decreased (small intestine, kidney, lungs, adrenals, testes [absolute and relative]) and 3/8 died at 10 mg/kg/d. The study was limited by the fact that animals were tested at doses exceeding a maximum tolerated dose (MTD). High mortality occurred at 10 mg/kg/d and overt testicular toxicity occurred only at this dose. There was no NOEL established and the LOEL (LDT) was 5.0 mg/kg/d, based on decreased absolute liver weight. As with the study above by Dikshith et al. (1984), effects on testicular weights appear only at the MTD or greater.

Inhalation. Endosulfan was administered by inhalation (aerosol, nose only) 21 times over 29 days (6 hr/d) to adolescent (4–6 week; 120 g M; 110 g F) Wistar rats (15/sex/dose) at 0 (air only), 0 (ethanol-polyethylene 400 [1:1]), 0.0005, 0.0010, and 0.0020 mg/L air (approximately equivalent to 0.097, 0.2, and 0.387 mg/kg/d) (Hollander and Weigand, 1984). At 29 days, 10/sex/dose were terminated and 5/sex/dose were observed for a 29-day recovery period. The NOEL was 0.2 mg/kg/d. At 0.0020 mg/L (0.387 mg/kg/d), males had clinical signs (emaciation, pale skin, squatting position, high-legged position), decreased bodyweight gain and food consumption and females had clinical chemistry effects in chloride, calcium, creatinine, and SGOT (no effects on reproductive organs in either sex). All effects were reversed by recovery day 29 except for 1 male at 0.0020 mg/L (0.387 mg/kg/d) with neurotoxicity. Five rats/sex/dose were treated 7 times (6 hr/d) over 9 days at 0.0024 mg/L (0.456 mg/kg/d) and 0.0065 mg/L (1.26 mg/kg/d). Clinical signs included neurotoxicity (M/F: disequilibrium, tremors, trembling, and sporadic tonic-clonic convulsions), death (F), and body weight (F) was decreased at both doses.

Chronic

Dietary, Rat. Crl:CD (SD) BR rats (70/sex/dose: main group) were fed endosulfan in the diet for 104 weeks at 0, 0.1, 0.3, 0.6, or 2.9 mg/kg/d (M) and 0.1, 0.4, 0.7, or 3.8 mg/kg/d (F) for tumor assessment (Ruckman et al., 1989). A satellite group (20/sex/dose), intended for blood sampling at intervals, was treated in parallel for 104 weeks. Bodyweight gain was decreased 8–18% (M/F) but there were no effects on relative testes weights. Bodyweight was not affected at any dose and while absolute testes weights decreased at the high dose, they were within historical control range. Kidney enlargement (F) and progressive glomerulonephrosis (M/F) occurred at the high dose (stated to be a common, age–related, spontaneously occurring renal disease associated with proteinuria). The chronic NOEL was 0.6 mg/kg/d in males, based on an increased incidence of aneurysms in blood vessels (which primarily affected the pancreas, mesentery, and/or liver after week 80), along with decreased male body weight gain at 2.9 mg/kg/d. The NOEL was 0.7 mg/kg/d for females.

Dietary, Mouse. Endosulfan was fed in the diet to NMRI Hoe:NMRKf (SPF71) mice (80/sex/dose) at 0, 2, 6, or 18 ppm (M: 0.28, 0.84 or 2.48 mg/kg/d; F: 0.32, 0.98, or 2.8 mg/kg/d) for 24 months (Donaubauer, 1988; Hack et al., 1995). Interim sacrifices of 10/sex/group were performed at 12 and 18 months. Males at 2.48 mg/kg/d showed a 17% decrease in body weights. In females, mortality was statistically significantly increased ($p<0.05$) at 2.8 mg/kg/d (43/60, 72%) compared with controls (33/60, 55%). Deaths began to occur in males at 45 weeks and in females at 15 weeks. Mortality occurred primarily between weeks 27 and 52 at 2.48 mg/kg/d (M) and 2.8 mg/kg/d (F). From weeks 79 through 104, there was no difference among groups for mortality. There was no specific target organ toxicity. There were no clinical signs of neurotoxicity. Bodyweight gain was decreased in males at 2.48 mg/kg/d. However, the reduction was only 5% and therefore not considered to be a noteworthy effect. At termination (104 weeks), there was no treatment-related oncogenicity. This interpretation was supported by the USEPA review of the same study (USEPA, 2001), which stated that there were no increases in incidence of any neoplastic lesion that was observed in either sex at any dose. These results were later published in the open literature (Hack et al., 1995). There were no effects on reproductive organs in either gender at any dose. The chronic NOEL was 0.84 (M) and 0.98 (F) mg/kg/d, based on increased mortality in the main group of females at 2.8 mg/kg/d.

Dietary, Dog. Endosulfan was fed in diet to Beagle dogs (6/sex/dose) at 0, 3, 10, 30, or 30/45/60 ppm (M: 0, 0.22, 0.6, 2.09, and 2.2/3.08/3.7 mg/kg/d; F: 0.19, 0.65, 1.98, and 1.95/2.78/3.57 mg/kg/d) for 1 year (Brunk, 1989). In the high-dose group, dogs were treated for 54 days at 2.2 mg/kg/d (M) and 1.95 mg/kg/d (F); for 52 days at 3.08 mg/kg/d (M) and 2.78 mg/kg/d (F) and 19–40 days at 3.7 mg/kg/d (M) and 3.57 mg/kg/d (F). One male at 2.09 mg/kg/d was killed in extremis on day 126, after 125 treatments. All high-dose dogs were sacrificed on days 146 to 147, due to an onset of extreme sensitivity to noise, frightened reactions to optical stimuli, and jerky or tonic contractions of the temporal muscles, extremities, and face, after the dose was increased to 3.7 mg/kg/d (M) and 3.57 mg/kg/d (F). One male at 2.09 mg/kg/d and one male at 3.7 mg/kg/d were terminated on days 276 and 126, respectively, due to poor condition. Both sexes showed neurotoxicity (impairment of the reflex excitability and postural reactions) at the HDT. Violent contraction of the abdominal muscles with contraction of the upper abdomen and also convulsive movement of the chaps were observed at the HDT 2.5 and 6 hr following each daily feed. Neurological symptoms, having to do with reflexes, were noted only at termination. Decreased body weights were observed in males at 2.09 mg/kg/d (−5%) and 3.7 mg/kg/d (−7%), beginning at week 44. Both genders showed a temporary decrease in percent of food consumed at $\geq$2.09 mg/kg/d (M), and $\geq$1.99 mg/kg/d (F). The NOEL was 0.6 mg/kg/d (M) and 0.65 mg/kg/d (F) based on violent contractions of the upper abdomen and convulsive movement at $\geq$2.09 mg/kg/d (M). Body weights for males and food consumption for both genders were decreased at doses of $\geq$1.98 mg/kg/d. The NOEL (0.6 mg/kg/d) from this study was used for the chronic endosulfan risk assessment. There

were no effects on reproductive organs in either gender at any dose.

Reproductive and Developmental Toxicity

Dietary (2-Generation Reproduction) Rat. Endosulfan was fed in the diet to Crl:COBS(CD)BR rats (32/sex/dose: F0 parents; 28/sex/dose: F1 parents) and each parental generation was mated twice (Edwards et al., 1984). The F1a and F2a offspring were terminated on PND 21 and for 1/sex/litter, specified organs were weighed and tissues were prepared for histology. Approximately 10 days after the weaning of the F1a and F2a pups, the F0 and F1b parental generations were remated to produce the F1b and F2b offspring. Treatment was equivalent to 0.20, 1.0, 4.99 mg/kg/d (F0 M), 0.24, 1.23, 6.18 mg/kg/d (F0 F), 0.23, 1.2, 5.72 mg/kg/d (F1b M), and 0.26, 1.32, 6.92 mg/kg/d (F1b F). Developmental endpoints were neonatal body weights, sex ratio, pup mortality, total litter loss, resorptions (early and late), gross morphology, litter size, anomalies, malformations, and others. The NOELs are expressed as mean dosages of F0 and F1b generations for males and females. There were no clinical signs of neurotoxicity. The systemic, reproductive, and pup NOELs were 1.2 mg/kg/d (M) and 1.30 mg/kg/d (F): F0 pups (F1a) had a slight decrease in mean litter weight on PND 12 (7%) and PND 21 (9.7%) and (F1b) (12%) at the high dose only. Adults (F0 F; F1 M/F) had a marginal decrease in body weight gain (F0 and F1, M and F) and food consumption (F1 M), and an increase in relative liver (F0 M, F0, and F1b F) and kidney weights (F0 and F1b M) at the high dose. Note that bodyweight decreases in pups at PND 12 to 21 are expected at the higher treatment levels. This is due to decreased palatability of treated diet or increased doses for pups as they transition from nursing to eating solid food, since both nursing and ingesting food can increase the total dose in pups. A clear-cut or monotonic dose response was not observed for pituitary, ovarian, or uterine weight effects and they were considered to be incidental. No effects were observed on neonates or pups in the absence of adult toxicity.

Gavage

Adult Male, Rat: Adult male Wistar rats (12/dose) were administered endosulfan by gavage for 7 or 15 days at 0 (groundnut oil), 2.5, 5.0, 7.5, or 10 mg/kg/d to investigate gonadal toxicity (Singh and Pandey, 1989a). After 7 or 15 days of treatment, 6/dose were terminated. Testicular cytosolic and microsomal protein (induction of xenobiotic-metabolizing enzymes) was increased 81 and 101%, respectively, at 10 mg/kg/d at 7 days, but returned to control levels by day 15. The 17–hydroxysteroid dehydrogenase percent (17–HSD%) activity was decreased at 10 mg/kg/d by 15 days. The testosterone percent (T%) in the serum was increased sporadically, at $\geq$7.5 mg/kg/d at 7 days and at 2.5 and 10 mg/kg/d by 15 days. Testicular testosterone percent (TT%) was decreased at 5 and 10 mg/kg/d at 7 days and was increased at 10 mg/kg/d by 15 days. Clinical signs of neurotoxicity were not described. Since effects were sporadic and were not dose-related in many cases, the authors performed a second experiment. Adult male Wistar rats received endosulfan by gavage at 0 (ground nut oil), 7.5, and 10 mg/kg/d (Group A = 15 days; Group B = 30 days; 6/dose/time point) or Group C at 0 and 10 mg/kg/d (6/dose) for 30 days followed by a 7-day recovery (Singh and Pandey, 1990). Results showed no changes in body weight, testicular wet weight, or cytosolic and microsomal protein contents of testis in treated rats. Serum and testicular T, 3-HSD, and 17-HSD were decreased in Group A (10 mg/kg/d) and in Group B ($\geq$7.5 mg/kg/d). Testicular T remained decreased (10 mg/kg/d) in Group C after recovery. Endosulfan affects the biosynthesis and secretion of testosterone but it has been shown in other studies within this review that systemic effects (not described in this study) would also be affected at $\geq$2.5 mg/kg/d. With the absence of a NOEL or descriptions of clinical signs, the above studies are of limited use for risk assessment.

Dams (Rat) Treated During Pregnancy, Lactation: Effects on Male Offspring: Endosulfan was administered by gavage to female Wistar rats (8/dose) at 0 (Tween 80), 1.5, and 3.0 mg/kg/d from GD 15 through LD 22 (Dalsenter et al., 1999). Male pups (15/dose/time period; 1–2 males/litter) from treated dams were examined PND 65 and 140 (puberty and adult stages, respectively). At PND 65, testis weight (absolute and relative) was increased at 1.5 and 3.0 mg/kg/d but by 140 days this was observed only at 3.0 mg/kg/d. No effects were observed on epididymal, relative seminal vesicle, or ventral prostate weights. Mean daily sperm production was decreased at PND 65 by 30% at 3.0 mg/kg/d ($p<0.05$) and 21% at 1.5 mg/kg/d ($p<0.05$) but at PND 140 the decrease was only 13% at 3.0 mg/kg/d. At PND 65 (3.0 mg/kg/d), the percentage of seminiferous tubules showing complete spermatogenesis was decreased ($p<0.05$) and observed histopathologically at puberty at 1.5 and 3.0 mg/kg/d (no longer evident at 140 d at 1.5 mg/kg/d). At PND 120, 15 males/dose were mated to virgin females and pregnancy outcome was assessed. There were no effects on fertility, litter size, % live births, birth weights, mortality, or any other parameter to F2 PND 21. Clinical signs of neurotoxicity were not described in the report.

In a later study (Dalsenter et al., 2003) mated Wistar females (27/dose) were treated with endosulfan by gavage at 0 (sunflower oil), 0.5, and 1.5 mg/kg/d from 21 days prior to mating through mating, pregnancy and lactation. Subsequently, 8 male offspring per dose were used to measure mean body weight, and relative (to body weight) testis, epididymis, seminal vesicle, and prostate weights. Eight litters/dose were the unit for determination of mean of age at testis descent and PPS. Pregnancy outcome was also assessed using 8 litters/dose for litter size, live births (%), and birth weight on PND 1 and weaning rate (%) and body weight PND 21. Male offspring (8/dose) were also selected for determination of daily sperm production, spermatid number count and sperm transit, sperm morphology, testosterone level on PND 15, 21, 33, and at termination PND140. There were no effects at any dose on the reproductive parameters measured on pups or dams. The NOEL was greater than 1.5 mg/kg/d; however, clinical signs of neurotoxicity were not described.

Sprague Dawley rats were treated with endosulfan by gavage at 0 (corn oil), 0.66, 2.0, or 6.0 mg/kg/d during GD 6–19 (Fung, 1980a,b). A retinoic acid positive control was included in the report. Clinical signs were rough coat, lethargy, flaccidity, hyperactivity, and face rubbing, observed primarily at 6.0 mg/kg/d. Dam body weights were "corrected" (body weight minus gravid uterine weight). The maternal NOEL of 2 mg/kg/d was based on significantly decreased mean body weight change (GD 0 to 20; −33%; corrected = −40%), decreased absolute body weight (GD 20 = −13%; corrected = −13%)

and increased clinical signs such as face rubbing (20/28) and lethargy (2/28) at 6 mg/kg/d. At 2.0 mg/kg/d, there was also a 14% decrease in corrected body weight gain but the "corrected" body weight gain is a final value obtained from at least 3 calculations, each introducing variations with a large potential for error. The developmental NOEL was 2 mg/kg/d, based on decreased mean fetal weights (8%), increased growth retardation, and minor skeletal malformations (sternebrae: small no. 4 and unossified) at 6.0 mg/kg/d. While misaligned sternebrae number 4 was statistically significantly increased at 0.66 and 2.0 mg/kg/d, it was not at 6.0 mg/kg/d. At 2.0 mg/kg/d, there was an increase in number and percent of resorptions, and a decreased percent in live fetuses (not observed at 6.0 mg/kg/d). There were 2/25 litters at 2.0 mg/kg/d with 6 early resorptions/litter (28.6 and 33.3%) and was not representative of overall resorptions at this dose. Kendall's Tau B correlation coefficients (Hollander and Wolfe, 1973) did not show a dose-related response from 2.0 to 6.0 mg/kg/d, and at 2.0 mg/kg/d the percent live fetuses (95.5 ± 7.5%), number of resorbed fetuses/litter (0.6 ± 1.04), and percent-resorbed fetuses (4.6 ± 7.5) were within historical control range. Since these effects were from 2 litters only and were also within historical control values, they were not considered to be treatment-related. At 6.0 mg/kg/d, however, fetuses showed a significant decrease in both body weight and length. These two measurements are fundamental measurements of fetal development stage and are, therefore, considered to be significant effects (Hughes and Tanner, 1970a,b). No malformations were reported.

Endosulfan was administered via gavage to pregnant Wistar rats (10/dose) at 0 (corn oil), 0.5, 1.0, or 2.5 mg/kg/d throughout the entire gestation period through PND 28 to investigate the effects on pups (Zhu et al., 2000). Maternal decreased body weight gain and deaths (4/10) were observed at 2.5 mg/kg/d. Although birth weights and crown-to-rump ratios were somewhat decreased at birth, these effects disappeared with time. Anogenital distances of males (PND 1, 28, and 90, respectively) were not decreased; litter size and sex ratio were not affected, there was no cryptorchidism or hypospadias in any male offspring; apoptosis in testis germ cells (examined PND 28) was not affected and there were no histological changes in these organs. Daily sperm production, epididymal sperm count, and morphology, as well as male fertility, were not affected. Results further indicate that endosulfan does not induce endocrine disruption on male offspring of rats after this extended duration of treatment.

Dams, Gavage (Rabbit): Endosulfan was administered by gavage to mated New Zealand White rabbits (3–10/dose) at 0 (corn oil), 1, 2, 4, 8, or 12 mg/kg/d during GD 6–28 (Fung, 1981a,b). All rabbits died at 8 or 12 mg/kg/d, and 2/6 died at 4 mg/kg/d. Clinical neurotoxicity (hyperactivity, opisthotonos, convulsions, and paralysis) was reported at ≥2 mg/kg/d throughout treatment. The NOEL was 1.0 mg/kg/d, based on the deaths and neurotoxicity. In the definitive study, mated New Zealand White rabbits (20/dose) were gavaged with endosulfan at 0 (corn oil), 0.3, 0.7, or 1.8 mg/kg/d during GD 6–28 (Nye, 1981). At 1.8 mg/kg/d, 6 dams were added (total = 26 dams) due to an unexpectedly high mortality. The maternal NOEL was 0.7 mg/kg/d based on increased mortality (4/20 dams died; one/d on GD 7, 10, 21, 29) and on clinical signs, which occurred during treatment: convulsions/thrashing (3/26), noisy/rapid breathing (2/26), hyperactivity (1/26), salivation (1/26), and nasal discharge (3/26) at 1.8 mg/kg/d. Deaths occurred at 1.8 mg/kg/d, beginning day 7 (4/26). Clinical signs began on the first day of dosing (GD6) at 1.8 mg/kg/d (thrashing, phonation, coughing, cyanotic), where they began on GD 18 in control (congestion/nasal congestion, 2/20) and GD 14 at 0.7 mg/kg/d (nasal congestion; 2/20). There was no developmental toxicity at any dose.

Weanling Male Rat: Sinha et al. (1997) examined the effect of endosulfan on testicular maturation with 3-week-old male Druckrey rats (5/dose) after treatment by gavage at 0 (peanut oil), 2.5, 5.0, or 10.0 mg/kg/d for 90 days (5 d/wk). Decreased sperm counts (cauda epididymis), increased sperm abnormality, decreased spermatid counts, and decreased daily sperm production were observed at all doses (≥2.5 mg/kg/d). The effects observed in the mature rats (Sinha et al., 1995) were similar to those observed in weanlings at ≥2.5 mg/kg/d (weanling effects dose-related). The authors concluded that endosulfan exposure during testicular maturation might result in disturbed spermatogenesis at sexual maturity. The LOEL for weanling and mature rats was 2.5 mg/kg/day. These studies had numerous deficiencies (uncharacterized strain of rat, no historical or positive controls, no adult male comparison group included in Sinha et al., 1997, small sample size, few animals treated/dose, no clinical observations). Sinha et al. (2001a) then used mated female Druckrey rats (3/dose), and administered endosulfan via gavage, at 0 (peanut oil), 1.0, and 2.0 mg/kg/d during GD12 (time of fetal gonad differentiation) through parturition (GD21). Neonates were weighed 5–6 hr after birth and males were weighed and fostered to untreated dams that had given birth earlier that day in order to avoid lactational exposure to endosulfan. After weaning (PND 21), 2 males/litter/dose (6 pups/dose total) were housed together until PND 100 when they were terminated, weighed, and examined for caudal epididymal sperm count, testis weight, intratesticular spermatid count, testicular marker enzymes (lactate dehydrogenase (LDH), sorbitol dehydrogenase (SDH), gamma glutamyl transpeptidase, and glucose-6-phosphate dehydrogenase). The seminal vesicles and prostate glands were also weighed. There were no treatment-related effects on body weights. Treatment at both doses caused a reduction of sperm and spermatid counts, and decreases in testis, seminal vesicle, and epididymal weights. LDH increased and SDH had decreased activities. These effects suggested that endosulfan might interfere in the process of spermatogenesis. The toxicity was dose-dependent; however, the deficiencies were as numerous as those of the initial study. Since the LOEL (1.0 mg/kg/d) for this study is lower than those achieved by adults and weanlings (Sinha et al., 1995, 1997), it would have been useful to include an adult control group for comparison.

Adult Male, Mice: Swiss albino male mice (6/dose/treatment) were gavaged with endosulfan (purity unstated) at 0 (distilled H_2O) or 3.0 mg/kg/d for 35 days (Khan and Sinha, 1996). Mean epididymal sperm counts were decreased by 80% at termination, along with an increase in abnormal sperm and abnormal sperm heads in endosulfan-treated mice. When mice were treated

concurrently with endosulfan (3.0 mg/kg/d) and vitamin C (in distilled H_2O) at 10, 20, and 40 mg/kg/d, the decrease in sperm count was less (65% at 10 mg/kg/d, 26% at 20 mg/kg/d, 22% at 40 mg/kg/d). Vitamin C also decreased the number of abnormal sperm and those with head abnormalities. There was not a NOEL achieved, clinical observations were not described, and only one dose was used.

Subcutaneous Injection, Rat

Neonatal: Endosulfan was administered subcutaneously to neonatal rats (strain not stated; PND 7, 8/sex/dose) at 0 (corn oil), 4.5, and 9.0 mg/kg/d for 15 days (Ahmad et al., 1993). After 15 days, serum testosterone (M), 17β-estradiol (F), body weights, and reproductive organ weights were measured. It was shown that decreased weights of testis, epididymus, vas deferens, prostate, seminal vesicles, ovary, oviduct, and uterus occurred at both 4.5 and 9.0 mg/kg/d. Testosterone and 17β-estradiol were also decreased at both doses for M and F, respectively. Body weights and mortality were not affected. Clinical signs, however, were not reported nor was a NOEL achieved. Results confirmed that at high doses there were effects to reproductive organs, but without clinical signs, it is not known if neurotoxicity also occurred.

In Vitro and In Vivo Effects on Steroidogenesis and Spermatogenesis

In Vitro. In an effort to understand the mechanism of endosulfan toxicity in human sperm, Turner et al. (1997) studied the ability of endosulfan to inhibit the acrosome reaction (AR: essential to fertilization). Sperm amino acid neurotransmitter receptor/Cl^- channels are integral to fertilization (Meizel, 1997). Initiation of the AR in vitro by the egg zona pellucida and by progesterone (AR initiator) involves $GABA_A$ receptor/Cl^- channels and glycine receptor/Cl^- channels, respectively (Wistrom and Meizel, 1993; Melendrez and Meizel, 1995, 1996; Shi and Roldan, 1995). As has been stated previously, endosulfan binds to and blocks the Cl^- channel linked to the $GABA_A$ receptor (Lawrence and Casida, 1984). The AR in this study was initiated in vitro in human sperm by progesterone (a physiological initiator), which activates sperm "$GABA_A$ receptor/Cl^- channels," and by glycine (a substitute for the egg zona pellucida), which activates sperm glycine receptor/Cl^- channels. At 1 nM (0.41 ng/ml or 0.41 ppb), endosulfan was shown to inhibit the AR initiated by progesterone or glycine in human sperm in vitro. The glycine-initiated AR was also inhibited at 0.1 mM endosulfan. The authors concluded that endosulfan concentrations were within the range detected in human and wildlife tissue and fluid as a result of environmental contamination. Currently, there is no link between exposure to endosulfan and human or wildlife infertility via inhibition of the AR or any other mechanism.

Primary cultures of Sertoli and germ cells were obtained from 28-day-old rats and treated in vitro with 0, 2, 20, 40, and 80 μM endosulfan for 24 and 48 hr to assess aldose reductase (AldR) and sorbitol dehydrogenase (SD) activity (Sinha et al., 2001b). There was a dose-related increase in AldR activity at ≥20 mM endosulfan after 24 and 48 hr (duration-dependent increases at ≥40 μM only). SD showed a dose-dependent decrease at ≥20 μM after 24 hr. A similar decrease in SD was observed at 48 hr, but without an accompanying duration-dependent decrease in enzyme activity. Sertoli cell–germ cell interaction is critical for the maturation of male germ cells through seminiferous epithelium to the release of mature spermatozoon into the seminiferous tubules. AldR in Sertoli cells reduces aldols to polyols (e.g., glucose to sorbitol), which are taken up by germ cells and converted to fructose and other sugars by SD but endosulfan interferes with this process. As testicular atrophy occurs, AldR increases (overcompensating), resulting in a change in the metabolism of glucose to inositol, necessary for germ cells during spermatogenesis. SD (a marker for germ cells) increases with the maturation of testis. This is because of enhanced fructose production for energy required by the testis (increases conversion of sorbitol to fructose).

In Vivo. Endosulfan was administered to adult male Wistar rats (6/group) in 6 Groups (Zhu et al., 2002). Groups 1 to 4 received 0, 2.5, 5.0, and 7.5 mg/kg/d (6/wk for 10 wk). Groups 5 and 6 received i.p. injections of vitamin C at 20 and 40 mg/kg/day with 7.5 mg/kg/d of endosulfan. Daily sperm production (DSP), sperm count, and morphology were studied after the treatments. Lipid peroxidation product (LPO) and 8-hydroxy-2′-deoxyguanosine (8-OH-2′-DG) in serum, liver, and testis homogenates were determined with enzyme-linked immunosorbent assay (ELISA). DSP and epididymal sperm count were decreased and the number of abnormal sperm was increased significantly in all treated groups. LPO and 8-OH-2′-DG in serum, liver, and testis homogenates increased at all doses. Vitamin C (antioxidant) administration protected against the endosulfan-induced sperm toxicity and oxidative damage to liver and testes. Authors concluded that oxidative damage may be a large factor in the mechanism of endosulfan reproductive toxicities at the high doses used in this study.

Wilson and LeBlanc (1998) showed that mice (M/F) treated with 7.5 mg/kg/d endosulfan for 7 days had an increase in 6α- and 16β-hydroxy-testosterone (OHT) metabolites (16β- in F only). There was a 3.3-fold increase in hydroxylation of testosterone (T) in the 16β-position. There was also a 3.6-fold increase in the rate of urinary elimination of [^{14}C]-androgen. The increase in androgen clearance was associated with a small (not statistically significant) decrease in serum T levels. T biotransformation from endosulfan exposure can result in increased elimination but homeostatic processes compensate for the effect and minimize consequences on serum hormone levels. Murono et al. (2001) confirmed that endosulfan had no effect on Leydig cells in vitro.

Estrogenicity in In Vitro and In Vivo Assays

Endosulfan showed no evidence of estrogenicity in three assays: (1) competitive binding with the mouse uterine E2 receptor, (2) transcriptional activation in HeLa cells transfected with plasmids containing an estrogen receptor as a responsive element, and (3) in the uterotropic assay in mouse.

In Vitro Assays. Endosulfan was used on mouse mammary gland organ cultures (MMOC) in vitro to test for the ability to form nodule-like alveolar lesions (NLAL) that would demonstrate an estrogenic action

(Je et al., 2005). Results showed no increases in NLAL; however, there were more alveolar buds induced than were observed in the controls. Telomerase reverse transcriptase (TERT) mRNA expression levels showed dose-related increases in upregulation and activity when endosulfan was used on MCF-7 cells (human mammary tumor cells, estrogen receptor-positive). Transient expression assays using reporter plasmids with fragments of the TERT promoter showed that this palindromic estrogen-responsive element might be responsible for the transcriptional activation by endosulfan. Despite this positive in vitro observation, there are no such endosulfan-related manifestations in in vivo studies.

Endosulfan, because of its weak estrogenic activity in vitro (ability to compete with [^{3}H]-17β-estradiol at the estrogen receptor in MCF-7 human breast cancer cells) was considered to be a potential endocrine disruptor in females (Soto et al., 1994, 1995; Andersen et al., 2002; Vanparys et al., 2006). Weak estrogenic effects (receptor binding) were induced by endosulfan with MCF-7 cells at 10 μM (Soto et al., 1994, 1995), 1 to 25 μM (Andersen et al., 2002), and 5.48 μM (Vanparys et al., 2006). In general, it is necessary to have over 1,000-fold greater concentrations of endosulfan than estradiol in vitro with MCF-7 cells in order to induce a comparable estrogenic effect. However, the relative potency of receptor binding is only one of several parameters to be considered in the translation of a pharmacological effect into a physiological response. Wade et al. (1997) tested the ability of endosulfan to induce proliferation of MCF-7 breast cancer cells (estrogen-dependent response) in vitro. Weakly stimulated MCF-7 cell proliferation (3.7-fold increase at 50 μM, dose approaching limits of solubility) was observed.

α-Endosulfan, β-endosulfan, and endosulfan sulfate were each used in a transactivation assay on Chinese hamster ovary (CHO-K1) cells at 10^{-5} M (one concentration only) to test for estrogenic and androgenic agonist/antagonist properties on 2 human estrogen receptor (hER) subtypes, hERα- and hERβ- and a human androgen receptor (hAR) in a highly sensitive in vitro assay (Kojima et al., 2004). The hERα-, hERβ-, and hAR are expression vectors for estrogenic (ERα- and ERβ-) and antiestrogenic (AR) activities that were transfected (via reporter plasmid) into the CHO cells. The α-endosulfan and endosulfan sulfate were positive in the ERα- and ERβ-transactivation assays, while β-endosulfan was active in the ERβ-transactivation assay only, indicating weak estrogenic activity. Both α- and β-endosulfan were inhibitors of androgenic transcriptional activity (endosulfan sulfate was not tested). Authors concluded that α- and β-endosulfan demonstrated both estrogenic and androgenic activities in this Tier I level in vitro test for endocrine disruption. These effects did not manifest in any of the in vivo reproduction or developmental studies, in any of the studies performed in the open literature, or in those conducted according to FIFRA Guidelines.

In Vivo Physiological Compensation in Mammalian Females. Endosulfan does not have an estrogenic effect in Sprague-Dawley female rats according to Wade et al. (1997). Female pups (18 days old, 10/group) were treated i.p. with 3 mg/kg/d endosulfan for 3 days to test for estrogenic potency in uterine assays. This was measured by the displacement (competitive binding) of [^{3}H]-estradiol ([^{3}H]-E) and progesterone, quantity of estrogen receptors (ER), progesterone receptors (PR), and peroxidase activity in uterus. The positive control was diethylstilbesterol, which functioned as expected, and the negative control was corn oil. Immature rats in a uterotrophic bioassay are advantageous because they have little circulating estrogen, so that changes in total circulating estrogen ([^{3}H]-E) due to endosulfan interference can be measured. Results showed that after treatment there was no increase in relative or absolute uterine weight (% of body weight), peroxidase induction in uterus, body weight or relative liver weight (% body weight). There were no effects on pituitary weight, or hormones in pituitary homogenate (follicle-stimulating hormone, luteinizing hormone, thyroid-stimulating hormone, prolactin, growth hormone) or circulating thyroid hormone (thyroxine). These results concur with previous findings of Shelby et al. (1996) where no increase in uterine effect or estrogenic effects in vivo were observed in a mouse uterotropic assay. Immature female mice were injected with endosulfan for 3 days (i.p. at 3.0 mg/kg/d). Uterine weights were determined on the 4th day. No estrogenic effects were observed.

Endosulfan was administered by gavage at 1.5, 3, 6, and 9 mg/kg/d to normal hemicastrated virgin Swiss albino mice (80–120 days old; 10/dose) for 15 days to study effects on ovarian functions (Hiremath and Kaliwal, 2002). Control females (sham operated; hemicastrated) were treated with corn oil. The right ovaries were removed on day 1 and were used as internal controls for individual animals. The total cycles, diestrus and estrus were decreased at ≥6 mg/kg/d. Total metestrus, relative ovarian weight (% of body weight), and day-16 left ovary weights were decreased at ≥3 mg/kg/d. There was a decrease in healthy follicles at ≥6 mg/kg/d and an increase in total atretic follicles that were of medium size at ≥3 mg/kg/d. Normally, ovarian hypertrophy will occur when an animal is hemicastrated but endosulfan inhibited this effect at ≥6 mg/kg/d. These effects indicate an interruption of the hypothalamic-pituitary-gonadal axis (no effects on mortality, body weight, uterus, kidneys, liver, adrenals, thymus, or thyroid). Clinical signs were not described and the NOEL was 1.5 mg/kg/d (similar to the NOELs for rat developmental and reproduction studies).

In a subsequent study, by Hiremath and Kaliwal (2003), 5 groups of female Swiss albino mice (10/group) were treated by gavage as follows: Group 1: Sham ovariectomized [(sham OVX)+olive oil]; Group 2: [OVX+olive oil]; Group 3: [OVX+endosulfan (ES = 4 mg/kg/d)]; Group 4: [OVX+17β-estradiol (17βE = 5 μg)]; Group 5: [OVX+17β-E = 5 μg+ES = 4 mg/kg/d) for 30 days. Results showed that there were no endosulfan-related effects on duration of vaginal cornification, estrus, diestrus, body weight change, relative uterine or liver weights and uterine protein, glycogen, or total lipids. The authors concluded that there was no estrogenic or antiestrogenic activity by endosulfan observed. The NOEL was greater than 4 mg/kg/d.

The modulation of estrogen-dependent genes by endosulfan in the rat has been recently described (Varayoud et al., 2008). Female Wistar rats (90 days old; 6–8/dose) were ovariectomized (OVX) and treated via subcutaneous injection at 0 (sesame oil), 17β-estradiol

(with a uterotrophic dose of 0.02 mg/kg/d or a non-uterotrophic dose of 0.0002 mg/kg/d), or with endosulfan at 0.006, 0.06, 0.6, and 6 mg/kg/d for 3 days. Animals were terminated 24 hr post-dosing, then uteri were dissected from each rat (and weighed) and the expression of mRNA for three genes was quantified by RT-PCR: complement factor-3 (C3), estrogen receptor alpha (ERα), and progesterone receptor (PR). Also quantified were the distributions of ERα and PR receptor proteins in luminal epithelium, glandular epithelium, and stroma of the uterus. The authors claimed that endosulfan acted like the non-uterotrophic E2 dose. However, an analysis of the data presented does not support this conclusion. For example, for the expression of C3 mRNA, the highest dose of endosulfan was the same as the control whereas all the lower doses were the same as the non-uterotrophic E2 dose; all doses of endosulfan gave the same inhibition of ERα mRNA as either dose of E2; all doses of endosulfan gave the same inhibition of PR mRNA as the non-uterotrophic E2 dose, significantly below control and the higher dose of E2. Endosulfan also gave rise to flat dose-response curves for ERα protein and PR protein expression. Moreover, the protein distribution was generally similar to control for endosulfan. The data presented do not provide a compelling case for endosulfan having a significant effect on any of these endocrine targets.

Neurotoxicity, Rat

The FIFRA Guideline neurotoxicity studies (acute, subchronic, and DNT) performed in rat were all negative for the neuropathology and neurobehavioral parameters examined. Neurotransmitter studies described below show that learning and memory impairment in adult rats occurred at 6.0 mg/kg/d (Lakshmana and Raju, 1994) and at 2.0 mg/kg/d (Paul et al., 1994), and footshock fighting behavior was increased in rat pups at 1.0 mg/kg/d (Zaidi et al., 1985; Seth et al., 1986). While some studies show effects on neurotransmitters, the results are preliminary and the studies were lacking in design (detailed below). It is well established that the toxicity of endosulfan is due to the disruption of the GABA-regulated chloride ionophore (Lawerence and Casida, 1984).

Gavage (Neonate or Prepubescent). Effect of endosulfan on the concentrations of neurotransmitters in various regions of the Wistar rat brain was examined (Lakshmana and Raju, 1994). Rat pups (6 rats/dose/sacrifice time; gender not specified) were treated by gavage with endosulfan at 0 (peanut oil) and 6 mg/kg/d during PND 2–25 before sacrifice at PND 10 or 25. The dose was selected because in a previous study in this laboratory no effects were observed in rats at $\leq$5.0 mg/kg/d (Garg et al., 1980). The effects on noradrenaline (NA), dopamine (DA), and serotonin (5-HT) were assessed in olfactory bulb (OB), hippocampus (HI), visual cortex (VC), brainstem (BS), and cerebellum (CB) PND 10 and 25. Performance in operant conditioning for solid food reward was assessed in 25-day-old rats. The activity of ChE was also estimated in the same parts of the brain. Results showed increased NA in OB (12%, $p<0.01$) and BS (10%, $p<0.05$) at PND 10 and in HI (20%, $p<0.001$) and CB (12%, $p<0.05$) on PND 25. DA was decreased in HI at PND 10 (42%, $p<0.001$) and 25 (45%, $p<0.001$). Serotonin was increased in OB (12%, $p<0.05$), HI (41%, $p<0.001$), VC (30%, $p<0.01$), and BS (15%, $p<0.01$) at PND10, but at PND 25 levels were decreased in BS (20% $p<0.05$) and CB (31% $p<0.01$). There were no treatment-related effects on ChE. The monoaminergic systems in developing rat brain were affected by endosulfan (in the absence of body or brain weight effects), but only at a toxic dose. Chronic endosulfan by gavage affected performance in the operant learning paradigm by increasing time to learn and retain acquired knowledge of a task. Because only one dose of endosulfan was used and the study also lacked a positive control, it is difficult to establish the toxicological significance of any of these changes in transmitter levels.

Preadolescent male rats (n = 10–12; 60–70 g) were treated with 0 (water+traganth powder) and 2 mg/kg/d endosulfan by gavage for 90 days (Paul et al., 1994). Results showed inhibition of pole-climbing escape response to electric shock (unconditioned) and learning and avoidance response to buzzer (conditioned) memory processes. The decreased pole climbing may have resulted from a suppressed motivation, according to the authors. Endosulfan increased 5-HT concentrations in the cerebrum (165%) and midbrain (347%) regions. Protein and ChE activity in the brain were unaffected. Spontaneous motor activity was increased on days 75–90 but coordination on the rota-rod apparatus was not affected. The 5-HT increase resulted in decreased escape (learning) and avoidance acquisition (memory impairment). When p-chlorophenylalanine (PCPA), a 5-HT depletory, was used on endosulfan-treated rats, only learning (not memory) was restored. Food consumption and body weight gains (both measured over 15-day periods) were decreased from day 15–30 (food) and day 30 (body weight). There was no rota-rod (neuromuscular) coordination impairment and none of the animals experienced tremors or convulsions. Because only one dose of endosulfan was used and the study also lacked a positive control, it is difficult to establish the toxicological significance of any of these changes in transmitter levels.

Endosulfan was administered by gavage to pregnant Sprague-Dawley rats (4/dose) at 0 (sesame oil), 0.61, and 6.12 mg/kg/d from GD1 through PND 21 (Cabaleiro et al., 2008). These are 100 and 1,000 × the acceptable daily intake (ADI). The male pups (10/group) were sacrificed at PND 15, 30, and 60. Maternal effects included a dose-dependent reduction in body weight at the end of gestation, along with a reduction in litter size at 6.12 mg/kg/d. Dam body weight was similar to controls at delivery and weaning. Male pup body weight was also reduced at 6.12 mg/kg/d at PND 15, 21, and 30 but not at PND 60. Prefrontal cortex from pups was obtained PND 15, 30, and 60 and assayed for aspartate, glutamate, glutamine, GABA, taurine, dopamine (DA), 3,4-dihydroxyphenylacetic acid:dopamine (DOPAC:DA) ratio, homovanilic acid/dopamine (HVA:DA) ratio, 5-HT and 5-hydroxyindole acetic acid:serotonin (5-HIAA/5-HT) ratio. For the amino acids, all (4) were elevated by endosulfan at 6.12 mg/kg/d at PND 15 and at PND 30. At PND 60, however, there was a decrease at both doses, for aspartate, glutamate, glutamine, and GABA. The meaning of these apparent increases and decreases at PND 30 and PND 60 is unclear and, moreover, it is possible that all of these points, treated and control, are

part of a single data set and, therefore, are not related to endosulfan treatment, i.e., are not biologically relevant. For taurine, dopamine, DOPAC, HVA, and 5-HT concentrations, in various brain regions, there were differences between dosed and control rats. However, there was an absence of a clear or consistent dose/response relationship at any of the times considered (PND 15, 30, and 60). Other unusual Dose/Response relationships were found for 5-HT at PND 30 where the increase was greater at 0.61 mg/kg/d than at 6.12 mg/kg/d. At PND 60, the increases were 0.61 (N.S.) and 6.12 mg/kg/d ($p<0.05$), compared with controls. The 5-HIAA/5-HT ratios were also reported: at PND 15, there was a dose-dependent reduction (N.S.), at PND 30, there was a non-dose-dependent reduction, and at PND 60, there was a dose-dependent reduction ($p<0.05$ at 6.12 mg/kg/d). A major deficiency in this study is that there were no positive (or historical) control data for any of the measured endpoints; thus the magnitude of the effects have unknown relevance and it is unclear whether endosulfan causes dose-related increases or decreases in transmitter levels in the prefrontal cortex or striatum in the neonatal male rat that are toxicologically relevant, i.e., adverse. Another deficiency that limits the use of this study for risk assessment is the lack of description of clinical signs, primarily at the high dose. Surely, endosulfan itself would be expected to cause clinical signs at this dose, based on other studies.

Gavage (Adult). Endosulfan was administered by oral gavage in a single dose to fasted adult Wistar rats (10/sex/dose) at 0 (2% starch mucilage), 6.25, 12.5, 25, 50, or 100 mg/kg (M), and 0, 0.75, 1.5, 3, 6 or 12 mg/kg (F) followed by a 15-day observation (Bury, 1997). The neurotoxicological screening (Functional Observational Battery and motor activity; FOB and MA) was performed 7 days prior to treatment initiation, 8 hr post-dosing (time of peak effect), and at 7 and 14 days post-dosing. Three weeks post-dosing, controls (10/sex) and 5/sex for all other doses (except 4/sex at 100 mg/kg) were terminated for neuropathological examination. The NOEL was 12.5 mg/kg (M) and 1.5 mg/kg (F), based on clinical signs (mortality, tonic/clonic convulsions, coarse tremor, uncoordinated gait, salivation, stupor, prone position, fright reaction, squatting posture, stilted gait, irregular respiration, straddled hind limbs, decreased spontaneous activity, panting, bristled coat, flanks drawn in, and narrowed palpebral fissure) at $\geq$25 mg/kg (M) and at $\geq$3 mg/kg (F), lasting for 1 day. There were no treatment-related effects on neuropathology or neurotoxicological screens.

Intraperitoneal (i.p.) Neonate/Pup. Neonatal albino rats (1 day old, 4/sex/dose/time point, strain not stated) were treated with endosulfan i.p. at 0 (40% polypropylene glycol), 0.5 and 1.0 mg/kg/d for 3 or 5 weeks, followed by an 8-day recovery (Zaidi et al., 1985). There was a statistically significantly increased ^{3}H-5HT binding to frontal cortical membrane at 5 weeks (1.0 mg/kg/d). This may have been due to increased maximum binding sites or alterations in the receptor affinity. At 1.0 mg/kg/d (after 5 weeks of treatment), there was an increase in fighting behavior induced by endosulfan treatment that was reversed when the 5-HT-blocker, methysergide, was administered. The NOEL was 0.5 mg/kg/d. Neurotoxicity was not reversed after the 8-day recovery period. Data were of limited use in the enlightenment of the endosulfan mechanism in the CNS because of numerous deficiencies (unnamed rat strain, purity of dosing material not stated, no adult comparison group, no clinical signs of neurotoxicity reported, no clinical sign data, no positive controls) but a NOEL was achieved for rat pups (0.5 mg/kg/d) in this and the following study.

Seth et al. (1986) treated pregnant female rats (ITRC breeding colony) with endosulfan i.p. (purity unknown) in the following groups to examine the effects on dams and pups (in utero and post-natally). Gestational Exposure Dams (5/group) received treatment (3 mg/kg/d) or vehicle (40% propylene glycol) only during gestation as follows: (1) Vehicle dams with natural pups; (2) Treated dams with their treated pups; (3) Treated dams foster nursed with control pups; (4) Control dams foster nursed with treated (in utero) pups. Pups were culled to 8 pups per dam. Gestational-Lactational Dams (5) were treated at 3 mg/kg/d throughout gestation and lactation up to 3 weeks of post partum (PP) age for their pups (culled to 4/sex/litter). Lactational Exposure Dams (5) were treated with 3 mg/kg/d from PPD for 1 to 2 or 3 weeks. Neonatal Exposure Pups (4/dose; M/F) received endosulfan at 0, 0.5, and 1.0 (i.p.) for 5 days per week up to 2, 3, or 5 weeks old. Adult Exposure males (8/dose; 8 wk old) were given endosulfan i.p. at 1 mg/kg/d (1 day) or 3 mg/kg for 15 or 30 days. At termination, brains were excised and examined in high-affinity binding assays with synaptic membrane preparations from several brain regions (corpus striatum, frontal cortex, and cerebellum). Effects of endosulfan treatment on receptor binding in brain were compared in adults and pups (gestation, lactation, and growth) using labeled ligands. Receptor binding for dopamine, acetylcholine (ACh; muscarinic), benzodiazepine, serotonin, and GABA was tested with ^{3}H-spiroperidol, ^{3}H-quinuclidinyl benzilate (QNB), ^{3}H-diazepam, ^{3}H-5HT, and ^{3}H-muscimol, respectively. It was shown that ^{3}H-spiroperidol binding (dopamine) was increased in pups that had received 3 mg/kg/d endosulfan (Gestational Exposure) throughout gestation ($p<0.05$ at 2, 3. and 5 weeks post-partum) and in pups treated in utero (3 mg/kg/day) but foster-nursed to control dams ($p<0.05$ weeks 2 and 3). Gestation-Lactation Exposed pups (4/sex/dose; 3 mg/kg/d) had an increase in ^{3}H-spiroperidol binding at weeks 2, 3, and 5. Neonatal Exposure at 0.5 mg/kg/d showed no effects from day 1 to 5 weeks (4/sex; either sex) but at 1.0 mg/kg/d at 5 weeks there was a slight increase in 5-HT and benzodiazepine and a decrease in dopamine binding. Foot-shock fighting behavior in neonatally exposed pups was examined in 10 control and 10 treated (randomly selected) and was increased in pups treated to 5 weeks of age at 1.0 mg/kg/d. These changes were observed 8 days after cessation of treatment. Adults treated at 3 mg/kg/d for 15–30 days had increased ^{3}H-5-HT binding along with increased footshock fighting (continuing 8 days post-treatment). Developing rats appeared to have increased sensitivity to endosulfan but adults were not tested at 1.0 mg/kg/d for 5 weeks, so it is not known whether or not toxicity would be comparable, especially since toxicity at 1.0 mg/kg/day was only observed at 5 weeks. Adults were tested only at 3.0 mg/kg/d for a shorter period of time (2–4 weeks). There were no

effects on ACh in any group. Despite the same deficiencies as the Zaidi et al. (1985) study, a pup NOEL 0.5 mg/kg/d was also achieved in this study.

I.P. Adult. Male rats (ITRC breeding colony, strain not specified; 8/dose/timepoint) were treated with an i.p. endosulfan dose of 0 (40% propylene glycol), 1 and 3 mg/kg/d (single dose), or 0 and 3 mg/kg/d for 15 or 30 days (Agrawal et al., 1983). There were no effects at either dose after a single treatment; however, there was increased binding of ^{3}H–serotonin (5-HT) to frontal cortical membranes at 3 mg/kg/d after 30 days. There appeared to be an increase in affinity of the receptor, with unchanged number of receptor sites. After 30 days at 3 mg/kg/d, endosulfan induced aggressive behavior (foot-shock induced fighting behavior) that was blocked by methysergide (5-HT blocker). The NOELs were 1.0 mg/kg/d (single dose) and 3.0 mg/kg/d (treatment for 3 weeks). There were numerous deficiencies in this study (unnamed strain of rat and purity of dosing material, no clinical signs reported) that limit the use of this study for risk assessment purposes. The major design flaw was the biased selection of animals that the authors described: "Pairs of rats which would exhibit at least one fighting episode in one minute were selected for the aggressive behavior study. This preliminary screening to exclude 'non-responders' was done in all the pairs of rats selected for the present study."

Intravenous Injection (i.v.). Endosulfan competitively inhibited the binding of [^{35}S]-*t*-butylbicyclophosphorothionate (TBPS) that binds to the $GABA_A$ receptor-Cl^--ionophore in albino rat brain in vitro (Lawrence and Casida, 1984). Males were treated with i.v. endosulfan LD_{50} doses of α- and β-endosulfan and endosulfan sulfate (76, 240, and 76 mg/kg, respectively). The compounds were potent, competitive, and stereospecific inhibitors of TBPS binding to brain-specific sites. TBPS binding site is hypothesized to be the same site on the $GABA_A$ receptor ionophore where endosulfan also binds. These data provide the first example of a $GABA_A$ ionophore antagonist with sufficient affinity to remain at the specific site during receptor preparation and assay in a mammalian system, after in vivo treatment with endosulfan.

I.P. and Intracerebrovascular Injection (In Vitro). Subsequent work (Abalis et al., 1986) showed that endosulfan inhibited in vitro $GABA_A$-induced Cl^- influx in rat brain membrane microsacs. Cole and Casida (1986) used brain microsacs from male Swiss-Webster mice treated i.p. or via intracerebrovascular injection (i.c.v.) with endosulfan. Therefore, the magnitude of the [^{35}S]-TBPS binding site inhibition correlated with the severity of the poisoning signs. This supported the hypothesis that the acute toxicity of endosulfan is due to the disruption of the GABA–regulated chloride ionophore.

Dietary (Developmental Neurotoxicity, DNT). Endosulfan was fed in diet to mated female Wistar rats (30/dose) at 0, 50, 150, or 400 ppm (0, 3.74, 10.8, and 29.8 mg/kg/d) from GD 6 through LD 21 (Gilmore et al., 2006). The concentration of endosulfan in the dietary preparations was adjusted to the expected food consumption during lactation to maintain a constant level of test material consumption. Offspring from 23 litters in the control, 3.74 and 10.8 mg/kg/d groups, and pups from 21 litters at 29.8 mg/kg/d were assessed neurologically up to PND 75 in the FOB, in motor activity (MA), auditory startle response, passive avoidance learning and memory, and water maze learning and memory assessments. The motility, numbers, and morphology of sperm from male pups were evaluated. Neuropathologic examination and morphometric analysis of selected neurological tissues from the pups were performed. The mean body weight of the dams was decreased in a dose-related manner during gestation and this decrease persisted through lactation with the mean body weight of the dams at 10.8 and 29.8 mg/kg/d significantly lower than controls through LD 7. The mean food consumption was likewise affected for all of the treatment groups during gestation. The report stated that the decrease in food consumption, while transitional, was likely due to palatability. The mean body weights of the pups in all of the treatment groups during lactation were decreased but there was no treatment-related effect on fetal gestation time, live births, viability, or lactation indices. PPS was marginally delayed (4–5%) in males at 10.8 and 29.8 mg/kg/d (0 = 44.9 d; 3.74 mg/kg/d = 44.8 d; 10.8 mg/kg/d = 47.1 d; 29.8 mg/kg/d = 46.8 d; $p < 0.05$). There were no effects on time to vaginal opening or sperm motility, count, and morphology. No treatment-related effects were noted in the FOB for either the dams or the pups. Pup MA assessment, the auditory startle response, passive avoidance learning and memory, and water maze learning and memory assessments were unaffected by treatment. No neuropathological lesions were noted in either the 21-day-old pups or the 75-day-old adults. Morphometric analysis of the brain of these animals did not demonstrate any treatment-related effects. The maternal NOEL was <3.74 mg/kg/d, based upon lower mean body weights (5–6%) and lower food consumption (12%) at ≥3.74 mg/kg/d. While these decreases are marginal, they are dose-related and therefore considered potential adverse effects. The developmental NOEL was also <3.74 mg/kg/d based upon the lower mean body weights (at LDT = 8% PND 11 only) of the offspring at all doses. At the 3.74 mg/kg/d, the decreased body weight was later reversed. Reversal did not occur at the higher doses. Body weight gain for pups was also decreased on PND 11 at ≥3.74 mg/kg/d; however, this effect was reversed at the LDT for the later time-points measured. The neurotoxicity NOEL was 29.8 mg/kg/d, based upon the lack of neurological effects in the offspring at the highest dose tested.

Dietary Subchronic, (Adult). Endosulfan was fed in diet to Wistar Crl:WI[Gx/BRL/Han]IGS BR rats (12/sex/dose) at 0, 40, 225, and 600 ppm (0, 2.11, 13.7, and 37.2 mg/kg/d-M; 0, 2.88, 16.6, and 45.5 mg/kg/d-F) for 13 weeks to test for neurobehavioral, motor, and other systemic toxicity (Sheets et al., 2004). Of the 12/sex/dose treated and examined for neurotoxicity, 6/sex/dose were also examined for histopathology. One female had clonic convulsions week 1 of exposure and died week 8 and 3 females had red nasal stain at 45.5 mg/kg/d. Females had decreased body weights on day 7 (only) at ≥16.6 mg/kg/d, which was possibly due to palatability. Food consumption was decreased only week 1 in females at ≥16.6 mg/kg/d and in males at 37.2 mg/kg/d. Plasma cholinesterase activity was decreased in females at ≥16.6 mg/kg/d. Absolute and relative kidney and liver weights were increased in both sexes at ≥mid-dose. The kidneys of both sexes at all doses had an amorphous brown-to-yellow pigment in the cytoplasm of the proximal convoluted tubular epithelium, with pigment

occasionally present in the lumen of proximal tubules. The intensity of coloration was dose-related and the pigmentation was absent in the controls. The NOELs for neurotoxicity were 37.2 mg/kg/d (M) and 16.6 mg/kg/d (F). There were no treatment-related effects on FOB or MA in either sex.

Human Epidemiology

Children. An epidemiological study was performed to assess potential effects of aerial spraying of endosulfan on sexual maturation in children (Saiyed et al., 2003). Endosulfan was the only pesticide that had been used (sprayed 2–3 times/year for 20 year) on cashew nut plantations located on hilltops in villages in northern Kerala, India. Later reports have contested this, stating that other pesticides were also used in the area, including the control site, and Saiyed et al. (2003) tested only for endosulfan (Abraham, 2004; Indulkar, 2004). The village school children were exposed to endosulfan via air, water (runoff from irrigation), and soil. Control children (comparable status) were from a village 20 km away without a history of aerial endosulfan spraying. Male children (study $n = 117$; controls $n = 90$) aged 10–19 years were to receive an examination for sexual maturity rating SMR (pubic hair, testes, and penis), a blood test to assess testosterone (T), luteinizing hormone (LH), follicle-stimulating hormone (FSH), and endosulfan residues (α-, β-, and sulfate). Non-participation in the SMR was 57% for the study and 33% for controls; however, in the 43% ($n = 50$) and 77% ($n = 68$), respectively, that did participate there was a statistically significant decrease in SMR for pubic hair, testicle, and penis development with regard to R^2, intercept (b_0), age (b_1), and aerial exposure to endosulfan (AEE b_3: Score = $[b_0+b_{1\text{age}}]\ b_{2\text{AEE}}$). Blood samples on test males ($n = 67$) had lower than expected T levels and LH in blood, compared to the controls ($n = 46$). Main study males had higher than expected LH levels, compared to controls when a regression analysis was performed (LH+endosulfan $R^2 = 0.5150$; control $R^2 = 0.1826$). Endosulfan (α-, β-, and sulfate), as well as total endosulfan, was increased in the study males ($n = 70$), compared to controls ($n = 45$). The authors concluded that a follow-up should be performed on the children to assess possible long-term effects and a repeat study should also be performed with a larger sample size to validate the study findings. Results were acknowledged by the authors to be preliminary.

Critiques of the study by Saiyed et al. (2003) were presented by Indulkar (2004) and Abraham (2004) who countered some of the claims. For instance, many pesticides (in addition to endosulfan) had been used for decades in the area where it was claimed only endosulfan had been sprayed. This was also true of the so-called control area that was stated to be free of pesticide exposure. In addition to the small sample size and only 1 blood sample/subject, the normal biological range for SMR or hormone levels in that population was not described. Serum hormone levels are variable, even throughout the day, and if a range of normal is not described then, without a reference, data interpretation is not possible. Also, the levels of endosulfan, again as described by Saiyed et al. (2003), were ≥ 0.03 ppb in water and ≥ 0.3 ppb in pond sediments, well below the levels in serum samples (Saiyed et al., 2003: Total endosulfan control = 1.37 ± 0.40 ppb and study group = 7.47 ± 1.19 ppb, $p < 0.001$). Given the fact that endosulfan is rapidly cleared from the body and does not bioaccumulate, it is unknown why the serum levels were much greater than those in the environment. ''The endosulfan residue levels Saiyed et al. (2003) reported in blood samples were 1,000 times higher than those reported in water samples. Based on the physicochemical properties of endosulfan, such a finding is highly unlikely.'' (Indulkar, 2004). He also noted that the water levels reported in the study were below the maximum recommended by the USEPA. According to Abraham, authors did not mention that only 105 g a.i./acre/year was aerially applied where the permissible seasonal application rates elsewhere (e.g., United States) 1,000 g a.i./acre/year is often exceeded. Finally, Abraham (2004) indicated that the interval between annual aerial applications was 11 months, at 5–6 months ahead of monsoon rain and that the known half-life of endosulfan in the conditions in India is 30 ± 5 days. These criticisms and the preliminary aspect of the data limit the usefulness of the Saiyed et al. (2003) study for the purpose of risk assessment.

Adult Women. Women of reproductive age and children living in southern Spain had fatty tissues, placenta, umbilical cord serum, and human milk examined to assess the distribution of endosulfan and metabolites in fatty and non-fatty tissues and fluids (Cerrillo et al., 2005). The adult women of reproductive age ($n = 149$, mean age 44 years, range 33–57) had adipose tissue removed during surgery for different reasons and placentas and cord blood were sampled from 200 women at term deliveries (mean age = 29 years, range 17–43 years). Breast milk from 23 healthy women (age 17–23 years; chosen at random from the 200 women donating placentas) was sampled. Results of adipose tissue assessments showed that endosulfan ether was the most frequently found residue (49.6% of samples) where endosulfan sulfate was the most abundant (mean = 16.16 ng/g fat). In placenta, endosulfan sulfate was the most frequently detected residue (67.5%), and endosulfan lactone was in greatest abundance (15.62 ng/g fat). In cord blood, endosulfan diol was the most frequently detected (81% of samples), and the most abundant (13.23 ng/g fat). In milk, endosulfan ether was found in 100% of samples, and β-endosulfan was most abundant (10.70 ng/g fat). It was shown in previous studies that endosulfan is transmitted from mother to child via milk (Campoy et al., 2001) and that endosulfan residues were found in 40 and 30% of adipose tissue samples from children living in Murcia and Granada (Southern Spain), respectively (Olea et al., 1999).

Fetuses. It was proposed that pesticide exposure (including endosulfan and dicofol) to pregnant women living near agricultural applications induced neurotoxicity in fetuses when exposure occurred during gestation weeks 1 through 8 (period of central nervous embryogenesis) (Roberts et al., 2007). Exposure to unknown levels of endosulfan and dicofol was proposed to result in an increased incidence in autism spectrum disorder (ASD). The study authors concluded that there were many design flaws (no knowledge of diet, duration of exposure, if the selected population was actually exposed to these pesticides, and possible misclassification of both the exposure and outcome: see USEPA, 2007, and

Erdreich and Morimoto, 2007, for detailed analyses of Roberts et al., 2007) and there is no ''cause and effect'' between endosulfan and ASD. Currently, the cause of ASD is not known. Indications were that an association was not determined but that the hypothesis ''requires further study.'' These data are notable but not useful for the purpose of risk assessment.

Issues Related to Sensitive Populations

FQPA

Under FQPA, a law that was specifically designed to protect infants and children from the potentially harmful effects of a pesticide, a DNT study was required. Based on the results of this DNT study, as well as recent studies in the open literature, the USEPA submitted a revised Risk Evaluation Decision for endosulfan (USEPA, 2007). They propose to use an FQPA Safety Factor (SF) of 1 × ''…since there were no residual uncertainties for pre- and/or post-natal toxicity.'' California DPR was concerned about the effects of endosulfan after human in utero exposure (endocrine disruption, ED) and exposure in air to infant and child bystanders at pesticide application sites. Routes of endosulfan exposure (inhalation, diet, gavage, i.p.) were compared among the sensitive populations. The sensitive populations were those displaying measurable effects at the lowest doses of endosulfan exposure or treatment.

Concerns Generated From the FIFRA Guideline Studies (Table 1)

The USEPA required that sperm count, motility, and morphology be examined in young adult male rats in the DNT study (Gilmore et al., 2006) since sperm parameters were not previously examined in the two-generation reproduction study (Edwards et al., 1984; this parameter is now recommended under FIFRA Guidelines for reproduction studies). In the DNT study, no effects were observed in sperm count, sperm mobility, or sperm morphology at any dose but there was a possible delay in PPS at 29.8 mg/kg/d (PND 46.8, $p < 0.05$ vs. control PND 44.9). Pup body weights at 29.8 mg/kg/d (HDT) were decreased by 10% at PND 21 ($p < 0.01$), and remained statistically significantly decreased through PND 70 at HDT. The delayed PPS was likely secondary to pup toxicity experienced at 29.8 mg/kg/d. This study was performed according to current FIFRA Guidelines and included historical controls and individual data. Note that in the rat subchronic neurotoxicity study, no neurobehavioral or neuropathological effects were observed, nor were there effects on reproductive organs, neurotoxic, or endocrine effects in either sex (Sheets et al., 2004).

In the 104-week chronic rat study (Ruckman et al., 1989), absolute testis weight appeared to decrease at 2.9 mg/kg/d (HDT). This effect was a concern of USEPA (2002), under the FQPA that endosulfan had the potential to be a reproductive toxicant. However, since both the testis weights and body weights were within historical control range, the observation may have been incidental (not toxicologically relevant). In any event, the NOEL for this study was 0.6 mg/kg/d, and would therefore be protective for any effects occurring at higher doses.

Pituitary and uterine weight effects were observed in the 2-generation rat reproduction dietary study. The increased relative pituitary weights in F1a pups (not F1b pups) and the increased uterine weights in F2a pups (not F2b) were seen only at the HDT (6.2 mg/kg/d). Neither effect was consistent between or among generations. Because this is more than 10 times higher than the chronic dog NOEL of 0.6 mg/kg/d, the critical NOEL used in the risk assessment for endosulfan, these effects in pituitary and uterus are considered of limited toxicological relevance.

In none of the studies performed via inhalation was there an effect on the male or female reproductive organs. The animals treated were very young (4–6 weeks of age) and still maturing, and, therefore, endosulfan was not considered to be a greater risk in young animals of either sex than in adults for reproductive effects following inhalation exposure.

Data show that endosulfan is not toxic to the developmental, reproductive, and endocrine systems in the absence of neurotoxic effects, which occur at lower doses (Table 1).

Concerns Generated From Open Literature Reports on Endosulfan-Induced Toxicity to the Male Rat Reproductive System (Table 1)

The results of Sinha et al. (1995, 1997, 2001a) generated concerns due to numerous effects to the reproductive systems of males (adults, fetuses, neonates, weaning, pubescent, Table 1). The data were not useful for the purpose of risk assessment because of several major flaws associated with study designs and methods. These include the use of Druckrey rats (seldom used strain), lack of consistency among studies for sperm parameters, lack of historical controls for Druckrey rats, small test groups (3–5 rats), no clinical observations, no individual data, positive controls, or concurrent comparison groups for adults and pups (Table 1).

In the first two studies, male adults (90 days old treated for 70 days) and pups (3 weeks old treated for 90 days) achieved LOELs of 2.5 mg/kg/d for male reproduction parameters such as increased sperm abnormality, decreased sperm and spermatid counts, and decreased daily sperm production (Sinha et al., 1995, 1997). The third study by Sinha et al. (2001a) followed pups from dams that had been treated with endosulfan (GD 12-birth) and had a LOEL of 1.0 mg/kg/d for effects in pups at PND 100. Based on the latter LOEL, it may appear that male fetuses are more sensitive to endosulfan than male adults; however, (unlike the pups) adult males were never tested at doses lower than 2.5 mg/kg/d and so the actual sensitivity levels were not tested between the age groups. With small sample sizes ($n = 3$–5) and without historical controls, it is unknown whether ''effects'' observed at the LOELs were within a historical control range or were truly effects of treatment. When sample sizes are so small for parameters such as sperm count (where the potential for broad variation occurs) and studies are not repeated, then the data cannot reasonably be interpreted or relied upon for risk assessment purposes. Verification of results in open literature studies is dependent on reproducibility. The only conclusion that can be drawn from these studies is that adult males show effects at 2.5 mg/kg/d that are

Table 1
Reproduction Effects in Young Males Versus Adults (M/F) After Endosulfan Exposure

Author	Strain of animal source[a]	Treatment duration	No./dose	LOEL/NOEL (mg/kg/d)[c]
Effects in rats				
Sinha et al. (1995)	Druckrey[b] (ITRC India)	90-d-old M+70d treatment	5 M	LOEL = 2.5 (LDT) M Repro
Sinha et al. (1997)	Druckrey[b] (ITRC India)	3-week-old M pups; 90d, 5d/wk	5 M	LOEL = 2.5 (LDT) M Repro
Sinha et al. (2001a)	Druckrey[b] (ITRC India)	GD12-birth, x-fostered to PND 100	3 dams	LOEL = 1.0 (LDT) M Repro
Dikshith et al. (1984)	Wistar[b] (ITRC, India)	Adult M gavage 30 d prior to mating	5 M/10F	Repro NOEL >5.0 M; 1.5 F NOEL = 2.5 M; 0.75 F
Dalsenter et al. (1999)	Wistar[b] (FURGS, Brazil)	Dams GD15–LD22; obs PND 65, 140; mate F1 M to virgin F PND 120; F2 obs PND 21[d]	8 Dams F1 8 litters F2	Repro M Pup LOEL d65 = 1.5 Repro M NOEL d120 & 140 >1.5 Dam NOEL = 1.5
Dalsenter et al. (2003)	Wistar[b] (FURGS, Brazil)	21-d premating-weaning Observed PND140	8 M	Repro Male NOEL >1.5 Dam & M Pup NOEL >1.5
Zhu et al. (2000)	Wistar[b] (Chinese stock, China)	Gestation-PND28	10 M	Repro Dam & M Pup NOEL >2.5 Dam NOEL = 1.0[f]
Gilmore et al. (2006)[g,h]	Wistar Crl:WI(Han)[b] (CR, NC)	GD6-LD21; observations PND 21 (sperm)	23 litters; 4 M/litter	Repro Dam & M Pup NOEL > 29.8 Dam & Pup LOEL < 3.74 (LDT)[e]
Edwards et al. (1984)[g,h]	Crl:COBS(CD)BR[b] (CR, UK)	2 Generation; 2 litters/generation	28–30 M/F	Repro Dam & Pup NOEL >5.9 Adult Pup NOEL = 1.2
Fung (1980a,b)[g,h]	CD SD[b] (CR, MI)	GD6–19	28 dams	Developmental Fetal NOEL = 2.0 Dam NOEL = 2.0
Effects observed in rabbits				
Nye (1981)[g,h]	NZ W (HU, WI) Rabbit	GD6–28	26 dams	Developmental Fetal NOEL >1.8 Dam NOEL = 0.7

LD = lactation day; GD = gestation day; PND = postnatal day; M = male; F = female; obs = observations; LDT = lowest dose tested; HDT = highest dose tested; NZ W=New Zealand White rabbits; Repro=reproductive Grey, *italics* = Studies where clinical observations for neurotoxicity were not reported. Grey, bold = Studies where NOELs for reproduction or developmental effects exceed the HDT and/or systemic NOEL.

[a]Abbreviations for animals and their animal sources: ITRC = Industrial Toxicology Research Centre, Lucknow, India; Federal University of Rio Grande do Sul, Brazil; HU = Hoppers Unlimited; NZ W = New Zealand White rabbits; CR = Charles River Laboratories: WI = Wisconsin, MI = Michigan; UK = United Kingdom; NC = North Carolina.

[b]Rat strains.

[c]Reproductive effects may include: sperm count, sperm production, sperm motility, sperm morphology, testes weights, epididymal weights, fertility, mating, litter, or offspring effects.

[d]F1 males were examined PND 65 and 140 (8 dams treated/dose); F1 PND 120 15 males/dose mated to virgin females. Pregnancy outcome and F2 pup observed to PND 21.

[e]The short-term decrease in body weight in pups at the LDT only was likely due to decreased palatability of diet as pups transitioned from nursing to treated diet prior to weaning. Pups at the higher doses did not recover from the decreased body weight effect.

[f]Four out of ten dams died and there were clinical signs of neurotoxicity in dams at 2.5 mg/kg/d.

[g]Studies performed according to Good Laboratory Standards in Brazil, the United Kingdom, or the United States.

[h]FIFRA Guideline studies.

similar to effects observed in male pups at 1.0 and 2.5 mg/kg/d.

Due to the numerous deficiencies in study design, in addition to the complete lack of follow-up studies to confirm findings (e.g., lack of data reproduction), the studies of Sinha et al. were not useful in the process of risk assessment.

Studies Examining the Possibility of Increased Sensitivity to Endocrine Disruption in Males After Treatment With Endosulfan (Table 1)

Two studies from the laboratory of Dalsenter et al. (1999, 2003) investigated the effects of endosulfan on Wistar F1 male fetuses and neonates after gavage treatment of dams GD 12 through PND 22. In addition, these F1 males were mated on PND 120 to assess possible effects on fertility and reproduction and for effects on F2 litters. Maternal body weights were statistically significantly decreased during pregnancy at 3.0 mg/kg/d (maternal weights not measured after PND 1). Male pups had decreased daily sperm production and decreased ''percent seminiferous tubules with complete spermatogenesis'' at PND 65 at 1.5 and 3.0 mg/kg/d (pups not assessed on a per litter basis). On PND 140 when all the pups had reached maturity, the effects to the male reproductive system were observed only at 3.0 mg/kg/d (HDT), where maternal toxicity had also been observed (Maternal NOEL = 1.5 mg/kg/d). There were no effects on reproduction for the males mated on PND 120. A possible explanation for the slight effects observed on the reproductive parameters at PND 65 is that rat pup reproductive systems are still in a stage of growth and development that will not reach maturity until PND 70 to 108 or more (Krinke, 2000; Zemunik et al., 2003; Ekwall et al., 1984; Korenbrot et al., 1977). Three to 8–10 weeks is the weaning, adolescent to adult developmental period (dependent on rate of development of each individual; Krinke, 2000; Zemunik et al., 2003; Ekwall et al., 1984; Korenbrot et al., 1977). ''False'' positives for daily sperm production and percent seminiferous tubules with complete spermatogenesis can be recorded when treated rat pups experience a normal variation in individual developmental rates. Historical controls provided by a given conducting laboratory, along with large numbers of treated animals and positive controls (such as are required in FIFRA Guideline studies), provide the breadth of information needed to lower the possibility of data misinterpretation. Pup body weights were decreased by 15% on PND 21 and by 6.1% on PND 65 at 3.0 mg/kg/d (no effect at 1.5 mg/kg/d at PND 1, 21, 65 or 140). On PND 21, the mortality rate (%) was slightly higher (2.5% at 3.0 mg/kg/d vs. 1.1% for control) at 3.0 mg/kg/d and this may have resulted from maternal toxicity. The effects observed in male offspring at 3.0 mg/kg/d appear to be toxicologically significant and possibly related to maternal toxicity. It can be concluded that treatment of dams GD 12 to PND 22 provides a NOEL for reproductive effects in adult males (1.5 mg/kg/d) equal to that of treated dams (body weight decrease at 3.0 mg/kg/d). Three strengths of the Dalsenter et al. (1999) study design are larger sample size (15/dose), use of two time-points for assessment of effects, and use of a well-characterized strain of rat (Wistar) where historical controls are available. The results of this study support the NOEL of 1.2 mg/kg/d (body weight and other systemic effects in adults and pups) obtained from dietary treatment of Sprague-Dawley rats for 2 generations with 2 litters per generation (Edwards et al., 1984).

Dalsenter et al. (2003) continued their initial work in another study where dams were treated by gavage with endosulfan at 0 (sunflower oil), 0.5, and 1.5 mg/kg/d from 21 days prior to mating through lactation in order to test the effect of endosulfan on fetuses, neonates and pups (Table 1). Male pups were examined on PND 15, 21, 33, and 140. Advantages of this study are numbers of animals treated (15/dose), multiple timepoints for assessment of results, use of a well-characterized strain of rat (Wistar), and assessment of results based on the litter as a treatment unit. No maternal toxicity was observed and there were no effects on sperm production; sperm count in cauda epididymis, sperm transit, sperm morphology, or serum testosterone at 1.5 mg/kg/d (HDT). The males mated on PND 120 (15/dose) to untreated females resulted in no reproductive effects in the dams, or offspring. These data support the previous results of Dalsenter et al. (1999) as well as the 2-generation reproductive toxicity study of Edwards et al. (1984; NOEL = 1.2 mg/kg/d).

Zhu et al. (2000) administered endosulfan by gavage to pregnant Wistar rats from GD 0 through PND 28 to investigate the effects of endosulfan on fetuses and pups at doses of 0 (vegetable oil), 0.5, 1.0, and 2.5 mg/kg/d. The NOEL is 1.0 mg/kg/d (neurotoxicity, death, decreased body weight) and is comparable to the rat reproduction NOEL based on systemic effects other than developmental or reproductive effects (Edwards et al., 1984). In the dietary developmental neurotoxicity study (FIFRA Guideline; Gilmore et al., 2006), there were no effects on Wistar male pups on reproduction parameters (litter data, sperm count, morphology, or motility) at any dose (NOEL >29.8 mg/kg/d). The LDT effect in dams (23 litters) and pups was decreased body weight and not reproductive or neurotoxicity (Dam and pup NOEL <3.74 mg/kg/d). Dikshith et al. (1984) showed that males treated with endosulfan via gavage for 30 days prior to mating had no reproductive effects at doses of 5.0 mg/kg/d. There were also no effects to the F1 litters. However, neurotoxicity was observed in dams at 0.75 mg/kg/d and in males at 2.5 mg/kg/d. Other studies such as those performed by Fung (1980a,b) showed that rat pups (visceral and skeletal anomalies) were affected at doses that were either identical to or greater than those causing effects in dams. This was also observed with rabbits (Fung, 1981a,b; Nye, 1981; FIFRA Guideline studies).

Although many of the open literature reports have deficiencies, as a body of evidence they support the findings of the DNT study and the two-generation rat reproduction study where the NOELs were based on body weight reductions and other systemic effects in adults and pups or where male reproduction was only affected at higher doses (Edwards et al., 1984; FIFRA Guideline). The 2-generation reproduction study covers all time periods from 8–10 weeks of age (pre-mating), mating, gestation, lactation, weaning, growth, to adulthood, then reproduction for a 2nd generation through weaning of the F2 pups. In none of these treatment periods was there evidence of effects on reproduction, or reproductive organs (weights, histopathology, gross

Table 2
Variability of Control Sperm Count Measurements by Laboratory and Method

Author[a]	Method	Sperm no. Cauda Epididymus $\times 10^6$/ml testes homogenate	Age at measurement
Sinha et al. (1995)	Robb[b]	752 ± 21 (Druckrey)	PND 160 ($n = 5$)
Sinha et al. (1997)	Robb[b]	58 ± 2 (Druckrey)	PND 111 ($n = 5$)
Sinha et al. (2001a)	Robb[b]	179 ± 3 (Druckrey)	PND 100 ($n = 6$)
Dalsenter et al. (1999)	Robb[b]	PND 65: 506 ± 94 (Wistar)	PND 65 ($n = 15$)
		PND 140: 467 ± 91	PND 140 ($n = 15$)
Dalsenter et al. (2003)	Robb[b]	287 ± 12 (Wistar)	PND 140 ($n = 8$)
Gilmore et al. (2006)	IVOS[b]	320 ± 107 (Wistar)	PND 21 ($n = 10$)
Zhu et al. (2000)	Robb[b]	685 ± 233 (Wistar)	PND 90 ($n = 15$)

No historical controls available for Druckrey rats. IVOS = Hamilton Thorne Integrated Visual Operating System, Beverly, MA (2005).
[a]All studies performed by gavage with the exception of Gilmore et al. (2006) (dietary).
[b]Robb et al. (1978).

observations, function) in either sex at any time point. There were 28–30 animals/sex/dose and individual data were provided, along with historical controls. The USEPA (2002) required a DNT study be performed to assess endosulfan effects on developmental neurobehavioral toxicity and sperm. In addition to the negative findings for effects on sperm in the DNT study, all male reproductive tissues (testes, seminiferous tubules, vas deferens, prostate, epididymis) from the FIFRA Guideline combined rat, subchronic rat (diet, dermal, inhalation), chronic dog, and mouse oncogenicity, involving hundreds of animals, were examined grossly, histopathologically, and were weighed at necropsy. There were no treatment-related effects observed in reproductive organs of either sex at any dose.

The above studies support a body of evidence demonstrating that fetuses, neonates, and pups are affected at doses similar to those affecting dams and adults via dietary or gavage treatment. Therefore, based on the weight-of evidence (Table 1), endosulfan would not be considered a selective developmental or reproductive toxicant or an endocrine disruptor. Effects in dietary studies were on body weights, whereas gavage treatment incurred primarily neurotoxicity. Although there were no individual data or historical controls in either study by Dalsenter, the test results were mutually confirming. In both studies, Wistar rats were used and historical controls are available. The results of both supported the critical reproduction dietary NOEL (1.2 mg/kg/d) selected by DPR, USEPA (USEPA, 2007) and the World Health Organization (WHO, 1984, 2006) for risk assessment. Due to the reproducibility of the Dalsenter studies, they are useful support for reproduction parameters in the endosulfan risk assessment.

Inconsistencies Leading to Problems With Data Interpretation (Tables 2 and 3)

With the exception of the studies by Sinha et al. (1995, 1997, 2001a) with inconsistent male results and numerous deficiencies, adult female rat endpoints were affected at either equal to or lower doses than those affecting the fetuses, neonates, pups, and adult males.

Sperm Counts (Table 2)

The same method of sperm count was used (Robb et al., 1978), but Druckrey rats (Sinha et al., 2001a) had a 38% lower control value than Wistar rats (Dalsenter et al., 2003). Within the Druckrey rat studies, the control sperm count varied appreciably (752 ± 21 million sperm/ml, Sinha et al., 1995; 58 ± 2.21 million sperm/ml, Sinha et al., 1997; 179 ± 2.21 million sperm/ml; Sinha et al., 2001a). The sampling of sperm did not appear to be standardized. It would have been informative to include historical controls for Druckrey rats, or at least to have tested more than 3–5 animals per dose. These inconsistencies render the data of limited value for risk assessment purposes.

Sperm Effects, Dam Body Weights, and Pup Body and Testes Weights (Table 3)

In Table 3, dam, pup, and pup testes weights, as well as effects on sperm parameters, are compared in an attempt to find a possible relationship among them. Potentially, a decrease in dam body weight could cause a decrease in pup weights and thus also a decrease in absolute testes weights. These developmental delays might influence sperm parameters. If effects were directly on the male reproductive system, then they could occur in absence of dam or pup body weight effects. Data in Table 3 do not indicate that endosulfan has a direct effect on the male reproductive tract. Most studies showed no effects on testis (gross or histopathology) at doses below those that also induce effects in dams or at doses below those selected for risk assessment (see Table 6 for all critical NOELs). For example, Sinha et al. (2001a) showed *decreased* absolute and relative testis weight at 2.0 mg/kg/d, and sperm effects at <1.0 mg/kg/d; conversely, Dalsenter et al. (1999) showed *increased* relative testis weight (absolute unaffected) plus sperm effects at a similar dose (≥ 1.5 mg/kg/d; Note that sperm/testis effects remained at PND 140 at 3.0 mg/kg/d only in the Dalsenter study). Between these studies, the treatments (Sinha: GD 12–21; Dalsenter: GD 15–22) were similar but the subsequent ages of pups at termination were different (Sinha: PND 100; Dalsenter: PND 65 and 140). Dalsenter bracketed a developmental sequence (adolescence PND 65 to adult PND 140) so that effects observed in the sperm and testes at 1.5 mg/kg/d on PND 65 were observed only at 3.0 mg/kg/d by PND 140. However, there were significant differences between these two studies that cannot be discounted. For example, rat strain (Druckrey vs. Wistar), vehicle (peanut oil vs.

Table 3
Comparison of Effects on Dam Body Weight, Pup Body Weight, Sperm and Testis Weight

Strain (reference)[a]	Dam Bwt[c,d,e]	Pup Bwt[c]	Testis Wt[c,d,e]	Sperm effects[f]	LOEL/NOEL (mg/kg/d)
Druckrey[(1)b]	Not reported	c. No effect	c. No effect d. No effect	PND 160: (+)[f]	LOEL = 2.5
Druckrey[(2)b]	Not reported	c. No effect	c. No effect d. No effect	PND 110: (+)[f]	LOEL = 2.5
Druckrey[(3)b]	c. No effect[i]	c. No effect	c. ↓ HDT e. ↓ HDT	PND 100 (+)[f]	LOEL = 1.0
Wistar[(4)b,g]	c. ↓ HDT	c. ↓ HDT	PND65: c, e. No effect d. No effect PND140 c, e. No effect; d. at HDT only	PND65 (+)[f] L-HDT[j] PND140: (+) HDT	F1 mating[g] NOEL > 3.0 Pup LOEL d65 = 1.5 Pup NOEL d140 > 3.0 HDT
Wistar[(5)b]	c. No effect[i]	c. No effect	c: No effect d: No effect	PND140: (−)	Dam & M NOEL > 1.5 HDT
Crl:COBS (CD)BR[(6)b]	F0 c. No effect e. ↓ HDT F1 c. No effect e. No effect	c. F1a, F1b: ↓ Litter wt HDT	c: No effect d: No effect	F0 (F1a,b) & F1b (F2a,b): (−)[h]	Adult & pup NOEL = 1.2
Wistar Crl:WI(Han)[(7)b]	c. ↓ GD 13–20 ↓ LD 0–7	c. ↓ PND 4–21	Not weighed	PND 75: (−)	Dam & pup LOEL = 3.74
Wistar[(8)b]	e. ↓ HDT[i]	c. No effect	d. No effect	PND 90: (−)	Dam NOEL = 1.0 Male pup NOEL > 2.5

PND = post natal day; M = male; F = female; HDT = highest dose tested; L-HDT = 1.5 and 3.0 mg/kg/d – 2 doses tested in this study; F0 and F1 = 1st and 2nd parental generations; F1a,b & F2a,b = offspring from 2 matings (a & b)/generation; NA = not applicable; wt = weight; bwt = body weight. (+), (−) = Positive and negative for sperm effects, respectively. Grey, *italics* = Studies where clinical observations for neurotoxicity were not reported. Grey, Bold = Studies showing that the NOELs for systemic effects are less than or equal to NOELs for reproduction effects in male pups. Other studies (e.g., References 1–3), the systemic effects for dams or adult females were not described.

[a](1) Sinha et al. (1995); (2) Sinha et al. (1997); (3) Sinha et al. (2001a); (4) Dalsenter et al. (1999); (5) Dalsenter et al. (2003); (6) Edwards et al. (1984); (7) Gilmore et al. (2006); (8) Zhu et al. (2000).

[b]Rat strains.

[c]c = Absolute weights.

[d]d = Relative weights.

[e]e = Body weight gain.

[f]Sperm observations may include: epididymal sperm count, sperm production, sperm motility, sperm morphology, spermatid count, abnormal sperm, and others.

[g]F1 males were examined PND 65 and 140 (8 dams treated/dose) after dam treatment GD 15-PND 21; F1 at PND 120 15 males/dose mated to virgin females. Pregnancy outcome and F2 pup observed to PND 21 resulted in no reproduction effects.

[h]No effects on sperm or testes or other male reproductive organs after examination by histopathology.

[i]Summary only, no data.

[j]L-HDT = Dose response for effects at low and high doses (1.5 and 3.0 mg/kg/d, respectively).

Tween 80), and, most importantly, number of animals per dose (3 vs. 15). Because the sample size in the Sinha study was so small, the dosing material was of lesser purity, the strain of rat is uncharacterized, the study was not reproduced, and there were no positive controls or historical controls, it cannot be determined whether the effects in Sinha et al. (2001a) are real or are false positives, with no toxicological significance. The reversal in sperm and testis effects at 1.5 mg/kg/d (Dalsenter et al., 1999) could be due to differences in individual rates of pup development that disappeared when all pups were fully mature. When Dalsenter et al. (2003) repeated the study with an extended treatment period (21 days premating to weaning), no effects were observed on sperm parameters, or dam, pup, or testis weights at 1.5 mg/kg/d (HDT). Zhu et al. (2000) showed results similar to Dalsenter et al. (2003). Dams were treated throughout gestation to PND 28 and although the dam body weights were decreased at the high dose (25 mg/kg/d), there were no effects on sperm parameters, pup or testes weights, or any other measured parameters in males. Zhu et al. (2000), Dalsenter et al. (2003), Edwards et al. (1984), and Gilmore et al. (2006), in contrast to Sinha, showed no lasting effects on pups after in utero and perinatal treatment with endosulfan. The Sinha et al. (1995, 1997) studies performed in adults (1995) and weanlings (1997) were not interpretable because of the deficiencies described above for Sinha et al. (2001a) and because the studies had no context for comparison. When requested, the laboratory of Sinha was not able to provide positive or historical control data for dam, pup, or testes weights or for sperm parameters for Druckrey rats and they stated that they no longer use those rats (personal communication). In 2 cases (Edwards et al., 1984; Gilmore et al., 2006), the dams had decreased body weights or body weight gain and, in turn, the litter weights were decreased at the same doses. Testes weights were not measured in Gilmore et al. (2006), but even with reduced dam and pup weights in Edwards et al. (1984) for the F0 adults and the F1a and F1b pups (highest dose tested: HDT), there were no effects on testes weights (or weights/histopathology in any other reproductive organs) in any generation (2 generations with 2 matings/gen). Most importantly, the effects occurred in the male reproductive tract at toxic doses (3.0 mg/kg/d) when for the purposes of risk assessment, the oral acute NOEL is 0.7 mg/kg/d, subchronic NOEL is 1.2 mg/kg/d, and chronic NOEL is 0.6 mg/kg/d. While the acute NOEL is usually the highest, in the case of endosulfan, the rabbit, in the developmental toxicity study, was the most sensitive species, providing a low NOEL. All of these NOELs are well below doses where effects are observed in the male reproductive system in studies that appeared to be well conducted. The effects observed do not indicate that endosulfan is a direct reproductive or developmental toxicant, nor do they indicate that it is an endocrine disruptor.

Other Endocrine Effects

Endosulfan was administered by gavage at 1.5, 3, 6, and 9 mg/kg/d to normal hemicastrated virgin Swiss albino mice (80–120 days old; 10/dose) for 15 days to study effects on ovarian function (Hiremath and Kaliwal, 2002). The NOEL for subchronic effects (decreased cycles, metestrus, relative ovarian weight, decreased healthy follicles, increased atretic follicles) was 1.5 mg/kg/d. Endosulfan administered by gavage to 5 groups of female Swiss albino mice (10/group) and treated as follows: Sham ovariectomized (sham OVX)+ olive oil, OVX+olive oil; OVX+4 mg/kg/d endosulfan (ES), OVX+17β-estradiol (5 μg) and OVX+17β-estradiol (5 μg)+ES (4 mg/kg/day) for 30 days (Hiremath and Kaliwal, 2003) showed no effects on duration of vaginal cornification, estrus, diestrus, body weight, relative uterine or liver weights and uterine protein, glycogen or total lipids. The NOEL was 4 mg/kg/d. There has been no association between endosulfan exposure and breast/mammary gland cancer or with any cancer in open literature or FIFRA-Guideline studies.

Effects on Sperm From High Doses of Endosulfan Administered by Gavage

Endosulfan was positive in the chromosomal aberration test for mouse sperm. This occurred only at highly toxic gavage doses of 32 and 42 mg/kg/d for 5 days (Usha Rani and Reddy, 1986). Some animals that survived showed chromosomal aberrations in sperm. Other cytogenetic tests were performed to assess effects of endosulfan on rat spermatogonia and spermatocytes (Dikshith and Datta, 1977; Dikshith, et al., 1978). Male rats were gavaged with endosulfan up to 55 mg/kg/d for 5 or 10 days but showed no increase in chromosomal aberrations or mitotic indices in spermatogonial cells at any dose or in either treatment period (Dikshith and Datta, 1977). Subsequently, male albino rats were gavaged with endosulfan at 11.6 mg/kg/day for 30 days, but showed no chromosome breaks (% comparison) in spermatogonial cells, compared to control (Dikshith et al., 1978). Metaphases in spermatogonial cells were decreased only at highly toxic doses (11.6 mg/kg/day). Adult male rats treated at high doses of endosulfan (e.g., 7.5 mg/kg/d, Wilson and LeBlanc, 1998; 10 mg/kg/d, Srivastava et al., 1991) and rat testicular cells tested in vitro (Turner et al., 1997; Sinha et al., 2001b) have implicated endosulfan in the disruption of sperm development and hormonal function. However, selection of low critical NOELs (0.7 to 1.2 mg/kg/d) for risk assessment would protect for chromosomal damage or other effects to sperm observed only at high doses.

Endocrine Disruption and the FQPA Safety Factor

Uncertainty associated with data gaps was addressed with the submission of the USEPA-requested studies (subchronic neurotoxicity and DNT studies, both dietary) since (with the exception of occupational exposure) endosulfan exposure is primarily through diet. The concern for endosulfan-induced adverse developmental effects in male offspring in utero or via milk was alleviated by the DNT study. No effects to F1 sperm parameters or neurotoxicity occurred in the DNT study at doses up to 29.8 mg/kg/d. There was no quantitative or qualitative evidence of increased susceptibility to fetuses, neonates, or adolescents, following in utero or neonatal exposures, of rats or rabbits to endosulfan during gestation or throughout reproduction cycles. The USEPA does not maintain the need for an FQPA uncertainty factor (FQPA = 1 ×; USEPA, 2007).

Table 4
Comparison of Rat and Rabbit Pups/Adults for Neurotoxic Effects Following Endosulfan Exposure

Author	Strain of animal and source	Duration	Vehicle	No./dose	Neurotoxicity NOEL (mg/kg/d)[d]
Effects observed in rats					
Agrawal et al. (1983)	Albino ITRC Endosulfan purity?	Adult M 15 or 30 d	i.p. 40% polypropylene glycol	Adult 8-10 M	NOEL? 3.0 Only dose tested: neurotransmitter binding; footshock fighting behavior in animals selected for aggressive behavior
Seth et al. (1986)	Rat strain? Endosulfan purity?	Dam: G or G & Lac; Adult M: 15 or 30d; M/F pup PND1 to 2, 3 or 5 wks	i.p. 40% polypropylene glycol	Adult: 8 M 5 F Pup: 4 M/F	M/F pup NOEL = 0.5 neurotransmitter binding; footshock fighting behavior (both at 5 weeks only) at 1.0 (HDT). Adult M/F NOEL/LOEL? 3.0 only dose tested; no clinical observations reported.
Zaidi et al. (1985)	Albino ITRC; Endosulfan purity?	M/F PND1 for 3–5 weeks+8 d recovery	i.p. 40% polypropylene glycol	4/sex	M/F pup NOEL = 0.5 neurotransmitter binding; footshock fighting behavior
Dikshith et al. (1984)	Wistar[b] (ITRC, India)	Adult M gavage 30d prior to mating	Peanut oil 98% pure	5M/10F	Adult M/F NOEL = 0.75 M and 0.25 F
Fung (1980a,b)[e]	CD SD[b] (CR, MI)	GD6–19	Corn oil	28 dams	Dam NOEL = 2.0 Fetal NOEL ≥6.0 (Fetuses affected developmentally by maternal toxicity)
Edwards et al. (1984)[e]	Crl:COBS(CD)BR[b] (CR in UK)	2 Generation; 2 litters/ generation	Diet	28–30M/F	Adult, pup NOEL ≥5.92 (HDT)
Gilmore et al. (2006)[e]	Wistar Crl:WI(Han)[b] (CR, NC)	GD6–LD21; Obs PND 21 (sperm)	Diet	23 litters; 4 M/litter	Dam, pup NOEL ≥29.8 (HDT)
Zhu et al., (2000)	Wistar[b] (Chinese stock, China)	Gestation-PND28	Corn oil	10 M	Male pup NOEL ≥2.5
Effects observed in rabbits					
Nye (1981)[e]	NZ W (HU, WI)	GD6–28	Corn oil	26 dams	Dam NOEL = 0.7 Fetal NOEL ≥1.8
The inhalation noel for subchronic exposure = 0.001 mg/L (0.2 mg/kg/d) neurotoxicity[c]					

Abbreviations: LD = lactation day; Lac = lactation; G = gestation; GD = gestation day; PND = post natal day; M = male; F = female; obs = observations; HDT = highest dose tested. Grey, bold = Studies where NOELs for neurotoxic effects ≥ the highest dose tested and/or NOEL systemic (not reproductive) effects.
[a]Abbreviations for animals and the sources: ITRC = Industrial Toxicology Research Centre, Lucknow, India; Federal University of Rio Grande do Sul, Brazil; HU = Hoppers Unlimited; NZ W = New Zealand White rabbits; CR = Charles River Laboratories; WI = Wisconsin, MI = Michigan,; UK = United Kingdom; NC = North Carolina.
[b]Rat strains.
[c]The inhalation NOEL is presented to indicate that a low NOEL for males and females was used for inhalation (nose only aerosol) when compared to all other routes of exposure (Hollander et al., 1984). Adult male Sprague-Dawley rat whole body exposure aerosol inhalation NOEL = 2.0 mg/kg/day (3 hr/day; 5 days/week; 30 days; Bebe and Panemangalore, 2003), a higher NOEL by that route even though whole body exposure (the more likely scenario) might produce a much lower NOEL.
[d]The NOELs for neurotoxicity are primarily due to clinical signs of neurotoxicity, including death unless otherwise indicated.
[e]Studies performed according to Good Laboratory Practices (USA).

Issues Related to Neurotoxicity in Sensitive Populations (Table 4)

Effects in Adult Versus Young Rats After Intraperitoneal Endosulfan Administration. Effects observed by Agrawal et al. (1983), Zaidi et al. (1985), and Seth et al. (1986) raised concerns about possible increased neurotoxicity in young rats compared with adults after i.p. administration of endosulfan. Adults, young pups, pregnant dams, and neonates were treated i.p. for various lengths of time. The discussion below points out the numerous deficiencies in these studies that render them of limited use for risk assessment purposes. The studies were designed to determine the mechanism of action of endosulfan in the brain of adult and neonatal rats. However, the results from two laboratories, published in the early 1980s, showed that cyclodiene insecticides, such as endosulfan, were potent antagonists at the $GABA_A$ chloride channel. Lawrence and Casida (1984) and Casida and Lawrence (1985) demonstrated this mechanism in several assay systems, with different radioligands. Casida showed a correlation between [^{35}S]TBPS receptor binding inhibition potency and toxicity, in rodents, for a series of cyclodienes, including endosulfan.

Agrawal et al. (1983) (Table 4). Agrawal et al. (1983) have shown that endosulfan does not have direct effects on [^{3}H]-5HT binding in rat brain, at 0.1 to 100 μM, in vitro. The effects observed, after i.p. in vivo dosing, include a slight, equivocal stimulation of binding after 30 days of dosing at 3 mg/kg/d (but not at 1.0 mg/kg/d for 30 days). There was no effect on [^{3}H]-5HT binding in rat cortex, in vitro, thus any possible effects of endosulfan on binding are likely to be indirect. In vivo dosing with endosulfan for 30 days at 3 mg/kg/d had a marginal effect on [^{3}H]-5HT binding, measured in vitro and the Scatchard plots suggest little, if any, difference in [^{3}H]-5HT binding in control and endosulfan-dosed rats. While the authors claim no change in B_{max} with a decrease in K_d (affinity increased) with endosulfan, the Scatchard plot suggests that the changes, if any, in both parameters are similar. No error bars are given. The (6) points in the mid-range (40–100 p.mol/g protein) show little differences between control and endosulfan-treated rats. The conclusion was that "It is also possible that endosulfan may have affected other neuronal systems which regulate the activity of serotonergic systems." The $GABA_A$-chloride ionophore is blocked potently and competitively (measured using ^{3}H-PTX and ^{35}S-TBPS) by endosulfan. Another major deficiency is that there was no assessment of clinical signs of neurotoxicity or clinical observations and therefore a clear picture of "effects" cannot be formed. Also, due to the difficulty of interpretation of effects measured in vitro after in vivo dosing, this study is of limited use for endosulfan risk assessment.

Zaidi et al. (1985) (Table 4). In this report, some possible effects of endosulfan were assessed on 5-HT receptors in rat pups starting PND 1. Results showed that 1 μM endosulfan had no effect on [^{3}H]5HT binding in rat frontal cortex, in vitro. I.p. treatment of rat pups at 0.5 mg/kg/d for 5 d/week for 3 weeks had no effect on [^{3}H]5HT binding, but 1 mg/kg/d for 5 weeks resulted in increased binding. Scatchard analysis indicated an increase in affinity (Kd ↓) with no change in B_{max}. However, there are too few data points (5) to make any of these claims with respect to the Scatchard analysis. Furthermore, the nature of the endosulfan-dosing regimen (in vivo) makes it very difficult to demonstrate any effects in a subsequent in vitro equilibrium, radioligand-binding assay (Bondy, 1982). Such experiments might only work if endosulfan were acting as a potent, irreversible binding agent that reacted (chemically) and specifically with the receptor. This is hard to imagine for a relatively stable molecule such as endosulfan.

A behavioral correlate for the receptor binding was an assay involving aggression in rats after foot shock. This increased after endosulfan at 1 mg/kg/d but not at 0.5 mg/kg/d and this effect appeared to be blocked by the 5HT antagonist methysergide. However, as recognized by the Zaidi et al. (1985), "many mechanisms are known to be involved in footshock induced aggressive behavior." Methysergide did not block this effect in undosed, control rats. Therefore, although endosulfan may interact with serotonin systems in some way, there was no indication of a direct involvement, thereby adding little to an understanding of the mechanism of action of endosulfan. The pups were also tested at a point in development (birth to 5 weeks) where the xenobiotic metabolizing enzymes, the brain and systemic functions are still developing and, therefore, the results cannot be directly compared to those obtained for adults (acknowledged by the authors). It would have been more useful for data interpretation to assess the pups when fully mature in order to determine the possibility of lasting effects after treatment during neonatal/adolescent periods. The conclusions are also questionable because there were no clinical signs described and the positive control methysergide was not effective with controls ("…the failure of methylsergide to block the foot-shock induced fighting behavior in control rats indicates that serotonin alone is not responsible for such type of aggression." Barr et al., 1979).

Seth et al. (1986) (Table 4). A similar in vivo dosing regimen for endosulfan was employed in this study as in Zaidi et al. (1985) except pregnant rats were dosed at 3 mg/kg/d i.p. for 15 or 30 days and rat pups at 0.5 or 1 mg/kg/d for 10, 15, or 25 days from PND 1. Brain regions were isolated and subjected to receptor binding assays in vitro, using 5 radioligands (Bondy, 1982). These were agonists/antagonists for DA, acetylcholine muscarinic (ACh_m), benzodiazepine (BZD), γ-amino butyric acid ($GABA_A$), and 5HT. No clinical signs were reported, DA receptor binding with endosulfan was decreased (Kd ↑ B_{max} ↓), 5HT and BZD binding were increased. There were no effects on ACh and $GABA_A$. Due to this study design, it is difficult to draw conclusions from this work since no pups tested at 0.5 and 1.0 mg/k/d were also later tested at adulthood. Without this control, it is not known if the effects of endosulfan exposure were permanent, transitional, or artifactual effects. None of the (5) receptors studied would be expected to be a primary target for endosulfan. This would require a labeled $GABA_A$ Cl^- channel antagonist (Table 4).

Difference in Sensitivity Between Male and Female Adults in Acute Studies (Table 5)

In Table 5 are listed the LD/LC_{50} (gavage, dermal, aerosol, and i.p.), NOEL and LOELs in rat and mouse. Note that in rat, the females are always more sensitive and have lower LD/LC_{50} values than males and that neurotoxicity is the primary effect regardless of route.

Table 5
Female Compared With Male Sensitivity in Acute Studies

Species	Exposure	Endpoints	Dose mg/kg/d
Rat M/F	Gavage	LD_{50} study: death, clinical signs of neurotoxicity, stomach and GI irritation; kidney congestion, lung, and adrenal effects; Scholz and Weigand (1971a,b)	LD_{50} M 48 F 10 LOEL: M 31.6 F 6.3
Rat M/F	Gavage+15d observation	Acute dietary neurotoxicity: death, convulsions (F) 8 hours postdosing; no clinical signs (M/F) after 1 day (Bury, 1997)	NOEL: M 12.5 F 1.5
Rat	Gavage	LD_{50} study: death (Gaines, 1969)	LD_{50} M 43 F 8
Rat	Dermal	LD_{50} study: death (Gaines, 1969)	LD_{50} M 130 F 74
Rat (M/F)	Aerosol, nose only (4 hr)	LC_{50} study: clinical signs neurotoxicity all doses. (Hollander and Weigand 1983)	LC_{50} M 5.8; 34.5 μg/L F 2.1; 12.6 μg/L LOEL: F 0.61
Rat (M)	i.p. (95% ethanol)	LD_{50} study: clinical signs of neurotoxicity, death (Gupta and Gupta, 1979)	LD_{50} M 8.0
Mouse (M/F)	i.p. (95% ethanol)	LD_{50} study: clinical signs of neurotoxicity, death (Gupta and Gupta, 1979)	LD_{50} M 6.9 F 7.5

M = male; F = female.

The i.p. LD_{50} (8.0 mg/kg) and nose-only aerosol LC_{50} (5.8 mg/kg) for males are comparable values; therefore, it is reasonable to suspect that doses at which effects occur in acute studies via these routes might also be similar. This is because both routes allow a direct entry of endosulfan into the bloodstream and then to the brain where neurotoxic effects are rapidly observed. In the mouse, both males and females appeared to be equally susceptible to endosulfan.

Considerations for Risk Assessment: Neuro/Neurobehavioral, Reproductive, and Developmental Toxicity and Endocrine Disruption (Table 6)

Although endosulfan is neurotoxic (all ages, M/F) and effects to the male rat reproductive system have been reported, for regulatory purposes, evidence shows that the doses causing endocrine disruption are in excess of doses causing neurotoxicity. The magnitude of effects needs also to be considered and, for regulatory purposes, this is done on a case-by-case basis. Usually, a 10% body weight decrement in adult animals is a deciding line over which body weight decrease is considered an adverse effect. In pups, however, a 4–5% body weight decrease is considered to be adverse. For example, decreased pup body weights and body weight gains were observed at all doses in the DNT study (Gilmore et al., 2006) on PND 11 and in the interval PND 4–11, respectively. At the LDT, the pup weights recovered by PND 21. In dams at the LDT, body weights were less affected, but they were statistically significantly decreased (LDT; GD 13 = 5%, $p < 0.05$; GD 20 = 6%, $p < 0.05$). It is not known whether the body weight effects observed at the low dose in pups was due to maternal toxicity, or lack of palatability of milk and subsequently food palatability, or if it was due to a direct treatment effect. This question could not be answered without further study, so even though the magnitude of effects was different for dams and pups, for the purposes of risk assessment, both dams and pups had body weight effects at the low dose and, therefore, a LOEL was determined based on this. Any dose that would not be toxic to the dams would also not be toxic to the pups and vice versa. In this case, even at the low dose for pups (3.74 mg/kg/d) the effects were reversed later in the lactation period. The USEPA used the dietary DNT study in their subchronic dermal risk assessment (route-to-route extrapolation), since an acceptable dermal study was not available (USEPA, 2004, 2007, Table 6). There was no NOEL established in this study; the USEPA used a 3× uncertainty factor (UF) to establish an approximate value of (3.74 mg/kg/d) (3 × UF) = 1.25 mg/kg/d for the NOEL. This was based on (1) the use of pup body weight decreases as the most sensitive endpoint for the most sensitive occupational population (F) for regulatory purposes, (2) the pup body weight decreases occurred only during lactation (not at PND 0) and the pup weights recovered at weaning (PND 22). This effect was also observed in pups in the dietary 2-generation rat reproduction study where the NOEL was 1.2 mg/kg/d. For the endosulfan risk assessment, DPR used the NOEL from the rat reproduction study (1.2 mg/kg/d) for subchronic dermal and oral exposure (Silva, 2008; Table 6). The value of the NOELs is virtually equal between the USEPA and DPR for dermal and oral

Table 6
Comparison of Critical No-Observed-Effect Levels (NOELs) and Endpoints for Risk Characterization Between the Department of Pesticide Regulation and U.S. Environmental Protection Agency

Exposure	1. DPR NOEL	2. USEPA NOEL	Effects/endpoints (Refs.)
Acute Oral/dermal: general population, including infants and children	Gavage: developmental, rabbit[a] = 0.7 mg/kg/d	Oral gavage, rat NOAEL = 1.5 mg/kg/d[d] FQPA = 1 × [a]	1. Abortions, death, convulsions, neurotoxicity post-dose[1] 2. ↑ Convulsions (F) 8 hr post-dose [2]
Acute (1–30 d) and subchronic (1–6 mos) inhalation[b]	Nose only aerosol (21d), rat NOEL = 0.001 mg/L; 0.2 mg/kg/d[b]	Nose only aerosol (21d), rat NOAEL = 0.001 mg/L; 0.2 mg/kg/d[d]	1. and 2. ↓ body weight gain and lymphocyte counts (M); ↑creatinine values (F) [3]
Subchronic oral/dermal: short (1–30d) and intermediate (1–6 mos)	Dietary, reproduction, rat NOEL = 1.2 mg/kg/d	Dietary DNT rat: LOAEL = 3.7 mg/k/d	1. ↑ Kidney and liver weights; ↓ food consumption and body weight gains[4] 2. ↓ Pup weight[5]
Chronic oral/dermal: general population, including infants and children	Dietary, dog (l year) 0.6 mg/kg/d	Dietary, combined (chronic/oncogenicity), rat: NOAEL = 0.6 mg/kg/d[d] FQPA = 1 × [a]	1. Premature deaths, neurotoxicity; ↓ body wt gain and food consumption[6] 2. ↓ Body wt gain, ↑ marked progressive glomerulonephrosis and blood vessel aneurysms (M)[7]
Chronic inhalation (> 6 months)	21-d inhalation, rat for chronic inhalation[c] ENEL[c] 0.02 mg/kg/d	Long-term inhalation exposure is not expected for endosulfan	1. ↓ Body wt gain and lymphocyte counts in (M); ↑creatinine (F)[3]
Cancer	Group E: evidence of non-carcinogenicity for humans.		Q* Not calculated. Chronic combined study (rats) and carcinogenicity study (mice) resulted in no ↑ frequency of tumors.

(Refs) 1. Nye (1981); 2. Bury (1997); 3. Hollander et al. (1984); 4. Edwards et al. (1984); 5. Gilmore et al. (2006); 6. Brunk (1989); 7. Ruckman et al. (1989).
[a]Food Quality Protection Act (FQPA, 1996); Safety Factor = 1 × (USEPA, 2007) for dietary exposure to infants and children.
[b]The subchronic inhalation NOEL of 0.2 mg/kg/day was used by DPR for both the acute and subchronic NOEL, since an acceptable acute inhalation NOEL was not achieved. Thus, the acute NOEL was actually lower than might be expected, since acute NOELs are usually higher than subchronic.
[c]A 10 × UF is added to the subchronic inhalation NOEL to extrapolate to obtain a chronic inhalation "Estimated No Effect Level" (ENEL) = (Subchronic Inhalation NOEL) ÷ UF 10 × extrapolation from subchronic to chronic exposure.
[d]Values also used in the preliminary risk assessment for endosulfan by Health Canada (2007).

subchronic risk assessment. Both NOELs were based on developmental or reproductive studies based on effects on offspring, even though effects were observed in adults at the same dose (LOEL = 3.74 mg/kg/d).

While there are few conclusions (mechanistic or otherwise) that can be drawn from the neurotransmitter studies of Agrawal et al. (1983), Zaidi et al. (1985), and Seth et al. (1986), an i.p. NOEL for young rats (0.5 mg/kg/d) was obtained and can be compared to the inhalation NOEL also obtained from young rats 0.0010 mg/L (0.2 mg/kg/d). Unfortunately, there were no direct comparisons of adult versus pup with the same doses and dosing periods within the same study (or even between studies) so that no conclusions could be made relating to possible age-related effects. Male rats in the Agrawal study were specifically selected for aggressive behavior, presenting a selection bias and preventing comparison with results in adult males to those of Seth et al. (1986). Agrawal et al. (1983) used only one dose (3.0 mg/kg/d) in the repeated dose assay (15 or 30 d) to test for neurotransmitter binding and footshock fighting behavior, thereby preventing a dose/age/effects comparison to the results in young animals obtained in the Seth or Zaidi studies. In the studies by Zaidi et al. (1985) and Seth et al. (1986), effects were seen in pups for footshock fighting behavior and neurotransmitter binding but only at 5 weeks at 1.0 mg/kg/d. No adults in any study were treated at the lower doses (0.5 and 1.0 mg/kg/d) for the same treatment periods as the pups. However, both studies confirmed footshock fighting behavior and neurotransmitter binding in pups at 1.0 mg/kg/d. Subchronic i.p. NOEL for pups was 0.5 mg/kg/d (bearing in mind that clinical signs of neurotoxicity were not described in any of the studies, nor was dosing material purity provided). These studies can also be used to support gavage and dietary data showing that rat pups and adults have similar sensitivity to endosulfan when it is administered on a subchronic basis. Ultimately, though, the major limitations to these studies render them to be of limited use for risk assessment. In the definitive inhalation study, effects were observed in 4–6-week-old rats beginning at 9 days (clinical signs of neurotoxicity) and rats were treated 21 times via nose-only inhalation over 29 days. The pups in the Seth and Zaidi studies showed no effects prior to 5 weeks. Other studies shown in Table 4, where endosulfan was administered by gavage or by diet, show that fetuses, neonates, pups, and young adults only show neurotoxicity or other systemic effects at doses that are equal to or greater than the doses affecting adults. Therefore, the critical inhalation NOEL of 0.0010 mg/L (0.2 mg/kg/d) was appropriate for subchronic exposures for the purpose of risk assessment. The study, performed in young animals according to peer-reviewed guidelines, was used for risk assessment for pups, dams, adult males, fetuses, and neonates based on the previously described data.

There was no developmental or reproductive inhalation study performed to evaluate inhalation effects on fetuses, neonates and pups. In California, this has become an issue because endosulfan has been recommended for listing as a TAC and infant bystanders at application sites comprise a sensitive ''at-risk'' group. All data from the acceptable subchronic rat inhalation study indicated that preadolescent/young adults (age 4–6 weeks of age) show neurotoxicity, body weight decrease, and blood effects in the absence of histopathological effects to any reproductive organs in either sex. There is no direct route-to-route extrapolation for oral to inhalation studies but studies by Seth et al. (1986) and Zaidi et al. (1985) had i.p. NOELs of 0.5 mg/kg/d in adolescent rats based on neurotoxicity. This is about 2.5 times higher than inhalation NOEL of 0.0010 mg/L (0.2 mg/kg/d) but the impact of the first pass to the liver is unknown in either exposure route. It might be supposed with inhalation exposure that endosulfan would enter the circulation and go to the brain, where i.p. injection delivers endosulfan where it might be absorbed and pass into the portal vein more readily, based on vicinity. The NOEL is 0.7 mg/kg/d in the rabbit developmental study and the three routes of entry (inhalation, i.p., and oral) present NOELs that are very similar and based on neurotoxic effects. In none of these studies were there effects on reproductive organs, nor did endosulfan have endocrine-disrupting effects in developing animals. The results of the DNT also support this conclusion.

USEPA Safety Factors for FQPA

Even though the preponderance of non-occupational endosulfan exposure is dietary, the USEPA has recommended that the FQPA SF not be retained (USEPA, 2007). The database uncertainties have been satisfied and ''there are no residual uncertainties for pre and/or post-natal toxicity.'' The final decision about the FQPA SF for endosulfan ultimately falls with the USEPA; however, it appears the decision is based upon the weight of evidence that endosulfan is not an endocrine disruptor or neurotoxin in fetuses, neonates, and pups at doses lower than those that induce effects in adults. The values used for the endosulfan risk assessment generated by the California DPR and also by the USEPA are presented in Table 6 for the purpose of comparison (see Silva, 2008, and USEPA, 2000, 2002, 2007, for full risk assessment documents.).The critical NOEL (lowest NOEL in an acceptable study for a given interval) in the most sensitive species was selected for oral acute, subchronic, and chronic exposures. The acute oral NOEL (0.7 mg/kg/d) was obtained from a developmental study in pregnant rabbit (Nye, 1981). The subchronic oral NOEL (1.2 mg/kg/d) was from a reproduction study in rat (Edwards et al., 1984) and the chronic oral NOEL was from a chronic dog study (NOEL = 0.57 mg/kg/d; Brunk, 1989). Critical inhalation NOELs were obtained from a rat inhalation study, where the subchronic NOEL (0.001 mg/L; 0.2 mg/kg/d; Hollander et al., 1984) was used for both the acute and subchronic exposure durations. The critical chronic inhalation NOEL was extrapolated from the subchronic NOEL by dividing a $10\times$ factor to provide a chronic ''estimated no effect level'' (ENEL = 0.001 mg/L $\div 10\times$ = 0.0001 mg/L; or $0.2 \div 10 = 0.02$ mg/kg/d). Below are the significant studies evaluated to obtain critical NOELs.

SUMMARY AND DISCUSSION

Endosulfan, like other organochlorines such as DDT, dicofol, methoxychlor, and others, many of which have been banned in the United States, is primarily a neurotoxicant (Plimmer and Gammon, 2003). However,

because endosulfan has weak estrogenic properties (Soto et al., 1995, 1996) and because of results obtained in studies that were unacceptable for regulatory purposes (Sinha et al., 1995, 1997, 2001a), it has been labeled a developmental and reproductive toxicant and an endocrine disruptor (PANNA, 2008). The risk assessment concern, of course, is that these presumptive effects resulting from endosulfan exposure during critical developmental stages (in utero, or to infants and children) will result in endocrine disruption and subsequent neurotoxicity, developmental or reproductive adverse effects that are irreversible. The data presented in this review, after suitable analysis, do not support the case that endosulfan is a developmental or reproductive toxicant or an endocrine disruptor. The weight of evidence is that, while some studies showed effects that raised concerns, the results of studies that included peer-reviewed protocols, both positive and negative controls (such as FIFRA Guideline studies), and had results that were reproduced, did not support these concerns. Animals treated in utero, as neonates or as pups (or throughout all developmental stages), do not experience effects at doses lower than those inducing toxicity in adults. Unfortunately, unlike the FIFRA-Guideline studies, many of the literature reports contain inadequate numbers of replicates to demonstrate statistically significant effects. Moreover, negative and positive controls are usually inadequate in these literature studies.

There is ample evidence that endosulfan can be acutely poisonous to humans through accidental and intentional exposure as documented in California by Beauvais (2008), in the United States (USEPA, 2002), Canada (Health Canada, 2007), and throughout the world (WHO, 1998) and the effect observed is generally neurotoxicity. Endosulfan is highly toxic to wildlife such as fish. Usually fish (e.g., yellow tetra: *hyphessobrycon bifasciatus*) are killed as a result of endosulfan discharge into rivers, as well as application to wetlands at rates recommended for pest control (Jonsson and Toledo, 1993). Endosulfan has been considered by some to be a Persistent Organic Pollutant (IPEN, 2005) throughout the world because it has been detected in Europe in lakes in the Alps, Pyrenees, and Caledonian Mountains (Carrera et al., 2002). It has also been detected in the Arctic (eastern Russia and western Canada) in the air, rain, snow, ice and fine particulate, water ways of lakes, river, sea, sediment, soil, plants, and amphibia (Fan, 2008; Usha and Harikrishnan, 2005). For these reasons, mitigation to protect humans and the environment may be necessary.

Mathematical models have been developed to explain and predict biomagnification of organic pollutants (Kelly et al., 2007). Among persistent organic pollutants, endosulfan has a relatively low (<5) $\log K_{ow}$ of 3.7, meaning that it has a low tendency to bioaccumulate in water-respiring organisms. However, endosulfan has a high $\log K_{OA}$ (octanol/air partition coefficient) of 7.1, meaning that it will have a high tendency to bioaccumulate in air-respiring organisms. Movement of endosulfan through air may, therefore, explain its occurrence thousands of miles away from its use sites.

On the molecular level, the work of Casabar et al. (2006) and Hodgson and Rose (2008) demonstrated that α-endosulfan induced P450 enzymes (CYP2B6 and CYP3A4) and that these isoforms are the enzymes that also correlate with testosterone 6β-hydroxylase activity in human hepatocytes. Using individual P450 isozymes, it was also shown that CYP2B6 and CYP3A4 were responsible for the majority of endosulfan metabolism. Endosulfan induction of CYP2B6 and CYP3A4 can result in decreased testosterone levels as has been shown with rat (Singh and Pandey, 1989b; Ahmad et al., 1993), and mouse (Wilson and LeBlanc, 1998) after exposure to endosulfan at high doses. The results of Saiyed et al. (2003) suggest that exposure to high doses of endosulfan may affect serum testosterone levels in young boys. The CYP2B6 and CYP3A4 characterization assays demonstrate that endosulfan could be used as a probe for their catalytic activity in vitro (Casabar et al., 2006). These assays also demonstrate that affecting endogenous P450 isozymes, used normally in the metabolism of endogenous steroid hormone substrates (e.g., testosterone-6-β-hydroxylase activity of CYP3A4), may lead to unforeseen endocrine consequences of xenobiotics.

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