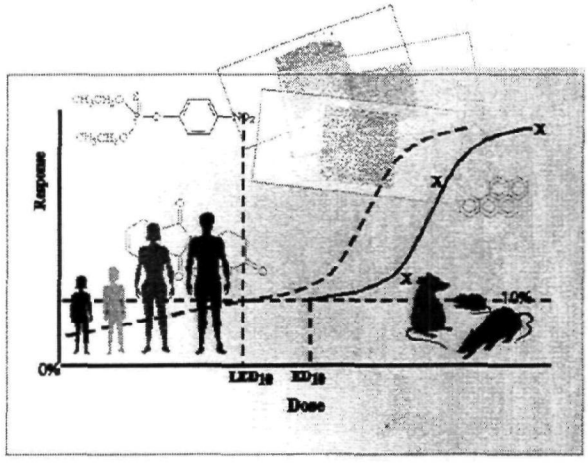
PRELIMINARY DRAFT

Hazard And Dose-Response Assessment And Characterization

Atrazine



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U.S. Environmental Protection Agency
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Health Effects Division (7509C)

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Preface

Over the last several years there has been increasing concern about the possible human health effects posed by chemicals that may alter the normal function of the endocrine system. Within the scientific community there is much debate and discussion about the extrapolation of animal findings on endocrine disruptors to predict and quantify such potential effects in humans, including children.

Agency guidance regarding endocrine perturbations in health risk assessment is limited to thyroid follicular cell carcinogens (US EPA, 1998a). Laboratory animal studies available on atrazine indicate that its mode of action in rats involves a perturbation of the neuroendocrine system that results in prolonged exposure to endogenous estrogen and prolactin. This endogenous exposure to estrogen leads to carcinogenic effects on the mammary and pituitary gland. There are also animal data available showing that there is an association between the adverse effects of atrazine on neuroendocrine control of reproductive developmental function. Given the complexity and multiplicity of effects that result from exposure to atrazine, the Office of Pesticide Programs (OPP) is at a point in its assessment of atrazine where external peer review by the FIFRA Scientific Advisory Panel (SAP) would facilitate further development and refinement of the draft health assessment document. Furthermore, very little is understood about the long term consequences that may result from prenatal and early postnatal exposures to neuroendocrine-perturbing chemicals. Thus, presenting the atrazine health assessment to the SAP at this time also allows the OPP an opportunity to obtain comments on the adequacy of the approach taken by OPP to address potential hazard to children.

The aim of the SAP review is to obtain advice and comment on the draft document on specific science issues, such as: what factors should be considered in evaluating this particular neuroendocrine mode of action?; what are the relevance and implications of this type of perturbation in humans?; what are the key biological events driving the hazard concern; and what are the potential cumulative effects and hazards on the developing brain that could result from the effects of atrazine on the function of the endocrine system? This external scientific peer review is a significant and critical step as the OPP proceeds to develop a sound and scientifically credible health risk assessment on atrazine as part of the mandate under the 1996 Food Quality Protection Act to protect public health and the environment. OPP intends to use the SAP's comments, as well as public comments that are received to further refine this draft document. Thus, the conclusions and analyses presented here within are considered preliminary.

Introduction

Over 10 years ago, atrazine was found to induce mammary gland tumors in Sprague Dawley female rats (Mayhew, 1986). Shortly afterwards, the Office of Pesticide Programs (OPP) classified atrazine as a possible human carcinogen (*Group C)* based on "limited evidence for the oncogenicity of the chemical in rats" (Hauswirth, 1988a,b). In 1988, OPP asked the FIFRA Scientific Advisory Panel (SAP) to comment on the cancer classification. The SAP agreed with OPP's classification of atrazine as a *Group C* carcinogen. The 1988 SAP also raised the possibility of a hormonal mode of action underlying atrazine's carcinogenicity (Copley, 1988). Accordingly, OPP encouraged the registrant of atrazine to pursue studies on a potential endocrine mechanism. Since that time, the registrant has completed numerous studies concerning atrazine's potential mode of carcinogenic action to explain the mammary gland tumor response found in female SD rats. The Agency's National Health and Environmental Effects Laboratory has also generated information on atrazine's neuroendocrine effects, as well as its effects on reproductive development in young rats.

The purpose of this draft document is to update and revise OPP's previous cancer assessment of atrazine by considering new information bearing on it's postulated mode of action. The draft document presents an integrative approach that uses a common neuroendocrine mode of action to evaluate the potential for both cancer and noncancer health effects (especially reproductive and developmental outcomes). This preliminary assessment also addresses how the available mode of action information influences decisions about the human hazard potential including sensitive subpopulations (*e.g.*, children). This draft document is organized into three parts, A, B, and C. Each has its own List of Contents.

- **Part A** summarizes the key conclusions on the cancer and reproductive developmental hazard potential and mode of action, and provides an integrated synthesis and characterization of the main findings:

  - *Chapter 1* provides a summary of tumor and other key data supporting the carcinogenicity of atrazine, as well as data on the reproductive developmental effects of atrazine.

- *Chapter 2* provides a technical hazard characterization and presents the mode of action analysis. The mode of action analysis is based on a framework described in the Agency's 1999 draft revisions to its guidelines for carcinogen risk assessment (US EPA, 1999a). This framework is used for judging whether the available evidence supports the mode of carcinogenic action in rats postulated for atrazine. This Chapter also discusses the common events in this mode of action which may lead to consequences on reproductive development.

- *Chapter 3* addresses what inferences can be made about the human relevance of the rat based findings on the mode of action conclusions presented in Chapter 2, and discusses whether there is special concern for children. The proposed dose-response extrapolation approach for cancer is also presented.

- **Part B** of the document (Chapters 4-9) presents a detailed carcinogenicity assessment and evaluation of the available epidemiology, toxicology, metabolism, mutagenicity, and mode of action studies on atrazine that are summarized in Chapter 1 of Part A.

- **Part C** of the document (Chapters 10-13) presents an evaluation of special reproductive/developmental studies performed on atrazine, as well as a review of available reproductive epidemiology studies.

List of Acronyms

CI	Confidence Ratio
CL	Corpea Lutea
DA	Dopamine
DACT	Diaminochlorotriazine
DMBA	Dimethylbenzanthracene
ED_{10}	Effective Dose - Central estimate on a dose associated with a 10% response adjusted for background
F-344	Fischer-344
FSH	Follicle Stimulating Hormone
GD	Gestational Day
GnRH	Gonadotrophin Releasing Hormone
HDT	Highest Dose Tested
LE	Long Evans
LED_{10}	Lower Limit on a Effective Dose - 95% Lower confidence limit on a dose associated with 10% response adjusted for background
LH	Lutenizing Hormone
LOAEL	Lowest Observed Adverse Effect Level
MTD	Maximum Tolerated Dose
NHL	non-Hodgkins Lymphoma
NOAEL	No Observed Adverse Effect Levels
OR	Odds Ratio
OVX	Ovariectomized/Ovariectomy
PCOS	Polycystic Ovarian Syndrome
PND	Postnatal Day
PoD	Point of Departure
PPS	Preputial Separation
SD	Sprague-Dawley
PRL	Prolactin
PIF	Prolactin Inhibiting Factor

Organizations

CARC	Cancer Assessment Review Committee
HED	The Office of Pesticide Program's Health Effects Division
IARC	The International Agency for Research on Cancer
MARC	Metabolism Assessment Review Committee
NTP	National Toxicology Program
SAP	Scientific Advisory Panel

PART A

Preliminary Hazard and Mode of Action Characterization

Chapter 1

1. Summary of Effects

This Chapter summarizes the data discussed in the hazard assessment portion of this document (Parts B and C). The summary forms the basis for the analysis of the mode of carcinogenic action information presented in Chapter 2 and draft OPP science policy positions on human relevance of the animal tumor findings and the classification of atrazine for human carcinogenic potential developed in Chapter 3. This Chapter also presents a summary of data on the reproductive and developmental toxicity of atrazine.

1.1 Effects Attributable to Treatment of Rats and Mice with Atrazine

Treatment of female SD rats with atrazine, but not male SD rats or Fischer 344 rats or CD-1 mice of either sex, results in neoplastic responses expressed as an increased incidence and/or an early onset of mammary carcinomas and adenomas, mammary fibroadenomas, and pituitary adenomas. Atrazine treatment of female SD rats also leads to certain non-neoplastic responses which precede and some of which may be antecedents to the neoplastic responses. A prominent effect is an attenuation of the luteinizing hormone (LH) surge that is necessary for normal reproductive cycling and a disruption of the estrous cycle. Effects on mammary tissue, namely markers of estrogen and prolactin (PRL) exposure, include increased incidences or increased severity of alveolar development, acinar development, dilated ducts, increased secretory activity, and galactoceles. Prolactin exposure is more strongly associated with the development of mammary fibroadenomas while estrogen exposure is more supportive of the development of adenomas/carcinomas. Estrogen also stimulates prolactin secreting cells and predisposes them to neoplasia. Data from short-term, high-dose studies suggest that a primary site of action of atrazine is the hypothalamus.

Results of mutagenicity assays mostly are negative. Assays designed to evaluate direct estrogenic activity of atrazine have failed to attribute exogenous estrogenic activity to atrazine treatment. Treatment with the close structural analogues, simazine and propazine, also lead to the formation of mammary tumors in female SD rats. Treatment of male SD rats or CD-1 mice of either sex with these chemicals does not result in an increased incidence of tumors at any site.

1.2 Carcinogenic Effects

Evidence from epidemiologic studies is not sufficient to establish whether atrazine may be carcinogenic to humans. Therefore, any inferences as to human carcinogenic potential must be determined from animal studies (see Part B, Chapter 4).

Table 1-1 summarizes the data on the incidence and onset of mammary adenomas/carcinomas found in carcinogenicity bioassays following administration of atrazine to female SD rats. The data generated on the formation of mammary fibroadenomas in female SD rats treated with atrazine is summarized in Table 1-2. These benign tumors are considered separate from mammary carcinomas because they are of a different cell origin than the tubular and glandular adenomas and carcinomas. Carcinomas arise from undifferentiated terminal end buds and terminal ducts of the mammary gland; fibroadenomas arise from more differentiated structures such as alveolar buds and lobules (Russo and Russo, 1996). In addition to increased incidence/early onset of mammary gland tumors, an early onset is found for pituitary adenomas. Table 1-3 summarizes data regarding the associations between atrazine treatment and the formation of pituitary adenomas.

1.2.1 Mammary Carcinomas

Treatment of female SD rats with atrazine leads to an increased incidence of mammary carcinomas and adenomas in one and two year bioassays (Mayhew, 1986; Morseth, 1998; Pettersen and Turnier, 1995). Serial sacrifice data show that atrazine treatment of female SD rats results in an early onset of mammary carcinomas (Thakur, 1991a; Pettersen and Turnier, 1995). Data on time of onset of mammary carcinomas as determined by palpation also show an early onset of mammary carcinomas (Thakur, 1992a; Morseth, 1998). The lowest dose of atrazine associated with an increased incidence in mammary carcinomas is 3.5 mg/kg/day (Mayhew *et al.*, 1986). The NOAEL in the same study was 0.5 mg/kg/day.

Table 1-1. Carcinogenicity Bioassays with Atrazine: Incidence and Onset of Mammary Adenomas/Carcinomas in Female SD Rats

Study	Duration	Tumor Incidence
Mayhew *et al.*, 1986	2-year	Dose (mg/kg/day) 0 0.5 3.5 25 50 15/88** 16/67 **27/69*** **27/68*** **45/60****
Thakur, 1991a	2-year serial sacrifice month	Dose (mg/kg/day) 0 4.23 26.63 9: 0*** 0 4 12: 0 1 2 15: 2 0 1 18: 5 2 4 24: 2 1 0
Thakur, 1992a	2-year terminal sacrifice week of onset#	Dose (mg/kg/day) 0 3.79 23.01 ≤ 52: **0/14*** 3/11 **6/18*** 53-78: 8/14 3/11 5/18 79-104: 6/14 5/11 7/18 0-104: 17/60 13/59 22/60 mean wk. onset: 78.9 72.5 65.4
Morseth, 1998	2-year week of onset#	Dose (mg/kg/day) 0 1.5 3.1 4.2 24.4 ≤52: 1/11 2/15 0/14 2/10 6/23 53-78: 5/11 6/15 7/14 6/10 7/23 79-104: 5/11 6/15 7/14 2/10 10/23 0-104: 12/80 18/80 20/79 14/80 **27/80**** mean wk. onset: 72.6 77.2 78.6 64.4 64.8
Pettersen and Turnier, 1995	1-year serial sacrifice month	Dose (mg/kg/day) 0 0.8 1.7 2.8 4.1 23.9 9: 1/10## 1/11 0/10 0/10 0/10 1/10 12: 1/25 1/24 1/25 2/25 2/24 6/25 (no tumors at 3 & 6 mo.)

* = p=≤0.05; **=p=≤0 01; at control=trend, at dose group=pairwise versus control; ***per 10 animals; #=onset as determined by first palpation of a tumor; ## incidences for adenomas and adenocarcinomas combined.

Table 1-2. Carcinogenicity Bioassays with Atrazine: Incidence and Onset of Mammary Fibroadenomas in Female SD Rats

Study	Duration	Tumor Incidence
Mayhew *et al.*, 1986	2-year	Dose (mg/kg/day) 0 0.5 3.5 25 50 20/88 24/65 21/69 21/68 20/89
Thakur, 1992a	2-year terminal sacrifice week of onset#	Dose (mg/kg/day) 0 3.79 23.01
	≤52	2/35 1/27 3/39
	53-78	16/35 15/27 18/39
	79-104	17/35 11/27 18/39
	0-104	39/60 30/59 41/60
	mean wk. onset	76.4 76.1 72.7
Thakur, 1991a	2-year serial sacrifice month	Dose (mg/kg/day) 0 4.23 26.63
	9	0 ## 0 2
	12	1 0 2
	15	2 5 1
	18	2 4 4
	24	3 3 4
Morseth, 1998	2-year week of onset#	Dose (mg/kg/day) 0 1.5 3.1 4.2 24.4
	≤52	0/15 1/18 3/26 1/26 1/22
	53-78	9/15 11/18 13/26 14/26 9/22
	79-104	6/15 6/18 10/26 11/26 12/22
	0-104	16/78 **25/79*** **34/77**** **29/78*** **25/77***
	mean wk. onset	76.1 72.4 73.7 73.3 76.3
Pettersen and Turnier, 1995	1-year month	Dose (mg/kg/day) 0 0.8 1.7 2.8 4.1 23.9
	9	1/10 0/10 0/10 0/10 1/10 1/10
	12	1/25 2/24 2/25 0/25 3/24 3/25
	(no tumors at 3 & 6 mo.)	

*p=<0.05;**p=<0.01; at control=trend, at dose group=pairwise versus control, #=Time of onset as determined by first palpation of tumor; ## = Incidence per 10 animals.

In one study, treatment of male and female F344 rats with a high dose of about 38 mg/kg/day of atrazine was reported to lead to an increased incidence of benign mammary tumors in males (Pinter *et al.*, 1990). The finding is difficult to evaluate because, among other shortcomings, no control animals survived to study termination, the study covered a lifetime and at approximately 30 months of age when the study was terminated, background mammary tumor incidence in untreated male rats would be expected to be similar to the incidence reported in the high dose group. Further, a separate study with F344 male and female rats did not show atrazine treatment induced the formation of tumors of any kind (Thakur, 1992b).

1.2.2 Mammary Fibroadenomas

With one exception (Morseth, 1998), atrazine treatment has not been shown to lead to a statistically-significant (pairwise comparisons, treatment group versus control) increased incidence of mammary fibroadenomas. The apparent increased incidence in fibroadenomas in the single study may not be treatment related because there is no dose-response trend among treatment groups over a 16-fold increase in doses; the control group incidence is low compared to historical control rates; and the incidences in atrazine treatment groups are within historical control ranges. As illustrated in Figure 1-1, data from one serial sacrifice study (Thakur, 1991a) support an association between atrazine treatment and an early onset of mammary fibroadenomas. However, an early onset of mammary fibroadenomas was not evident in the other serial sacrifice study (Pettersen and Turnier,1995). The Thakur data suggest an early onset of mammary fibroadenomas at the lowest atrazine dose administered, 4.23 mg/kg/day.

1.2.3 Pituitary Tumors

There are no increases in the incidences of pituitary tumors at the terminal sacrifice (24 month) in any of the carcinogenicity studies performed with atrazine. Because the background incidence of pituitary tumors is in the range of 80-90% at 24 months of age in SD rats, the lack of an increased incidence in pituitary tumors at terminal sacrifice may not be surprising. However, there is evidence for an earlier onset of pituitary tumors at nine and 12 months in female SD rats treated with atrazine in

one serial sacrifice study (Thakur 1991a) but not in a second 12-month study which included a nine month interim sacrifice (Pettersen and Turnier, 1995). Figure 1-1 depicts the dose-response data for the cumulative incidence of pituitary tumors over time and shows that there is an apparent early onset of pituitary tumors in the Thakur (1991a) serial sacrifice study. The information in Figure 1-1 shows that an early onset of pituitary tumors can be attributed to atrazine treatment at a dose level of 26.23 mg/kg/day. Neither an early onset nor an increased incidence of pituitary tumors is evident at an atrazine dose level of 4.23 mg/kg/day. Table 1-3 provides the incidences of pituitary tumors found at each sacrifice interval.

Figure 1-1. Cumulative Incidence of Pituitary β-Adenomas (Thakur, 1991a)

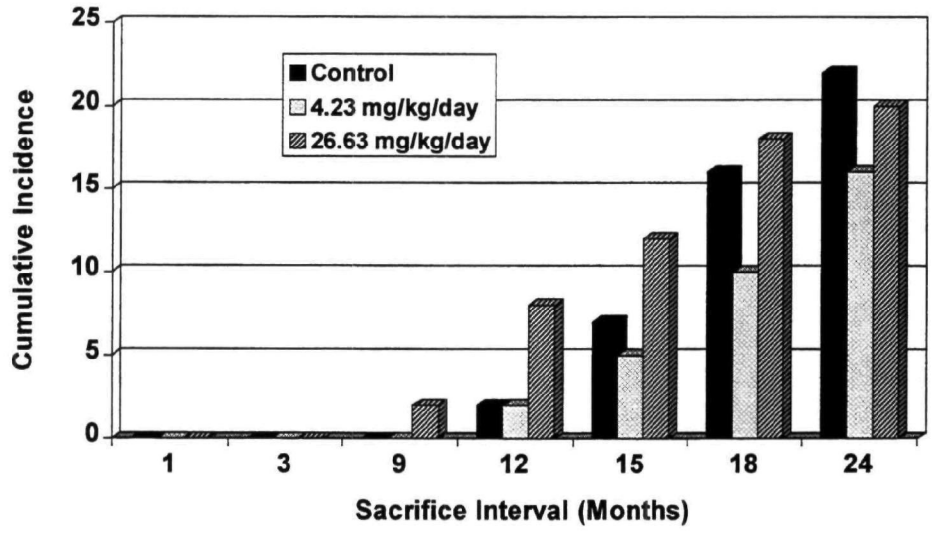


Table 1-3. Carcinogenicity Bioassay: Incidence of Pituitary Adenomas (Thakur, 1991a)

Sacrifice Time (Months)	Dose (mg/kg/day)		
	Control	4.23	26.63
9*	0**	0	2
12	2	2	6
15	5	3	4
18	9	5	6
24	6	6	2

* = No tumors at one and three months; ** = Incidence/10 Animals

1.3 Potential Antecedents to Carcinogenicity

Chronic atrazine treatment of female SD rats leads to the expression of a number of non-neoplastic neuroendocrine disruptions and of histomorphologic effects on mammary and pituitary glands. Neuroendocrine effects include attenuation of LH surges, disruption of the estrous cycles, and an increase in pituitary weights. Endocrine associated histomorphologic effects on mammary tissue include increases in the incidences of acinar/lobular development and secretory activity and severity of galactoceles, in atrazine treated animals.

As discussed in Part B, Chapter 9.3, preliminary data implicate the hypothalamic-pituitary axis as a primary site of atrazine toxicity (Cooper *et al.*, 1996, 1998; Cooper *et al.*, 2000). Atrazine appears to affect the catecholamine neurotransmitters in the hypothalamus by decreasing norepinephrine (NE) and increasing dopamine (DA) (Cooper *et al.*, 1998). The decrease in NE results in a decrease in gonadotropin releasing hormone (GnRH), with a corresponding diminution of surges of luteinizing hormone (LH). If serum LH levels do not display a proestrus afternoon surge above a critical level then ovulation does not occur, and the ovarian cycle is disrupted. The inhibition of ovulation following continued atrazine exposure leads to maintenance of a state (prolonged or constant estrus) where ovarian follicles continue to secrete estrogen. Removal of the estrogen stimulus by ovariectomy abolishes the induction of mammary tumors by atrazine treatment.

1.3.1 Attenuation of the LH Surge

Table 1-4 is a summary from a one month study on the effects of atrazine treatment on the preovulatory surge of LH in female SD rats while Table 1-5 provides a summary of the LH surge effects following six months treatment. Although LH data were collected at several time periods in addition to those shown, table entries are limited to periods when LH blood levels should be near or at baseline values (1100 hours) and the period when LH blood levels should be near or at the peak surge value (1800 hours). Thus, these time periods are appropriate points for evaluating the fold increase in serum LH compared to baseline values and for ascertaining the effects of atrazine on the preovulatory surge.

Table 1-4. LH Data (mean ± sd) from Animals Repeatedly Bled in the One-Month Study (Morseth, 1996a) (LH values given are in picograms/mL)

Dose mg/kg/day	1100 Hours	1800 Hours	Fold Increase*
0	732 ± 461	2650 ± 2389	3.6
2.5	1101 ±652	3015 ±3220	2.7
5.0	810 ±519	2717 ±2542	3.3
40	755 ±389	1450 ±857	1.9
200	514 ±503	812 ±470	1.6

*Increase = 1800 hour values (peak values) divided by the 1100 hour values (baseline values)

Table 1-5. LH Data (mean ± sd) from Animals Repeatedly Bled in the Six-Month Study (Morseth, 1996b) (doses are in LH values given are in picograms/mL)

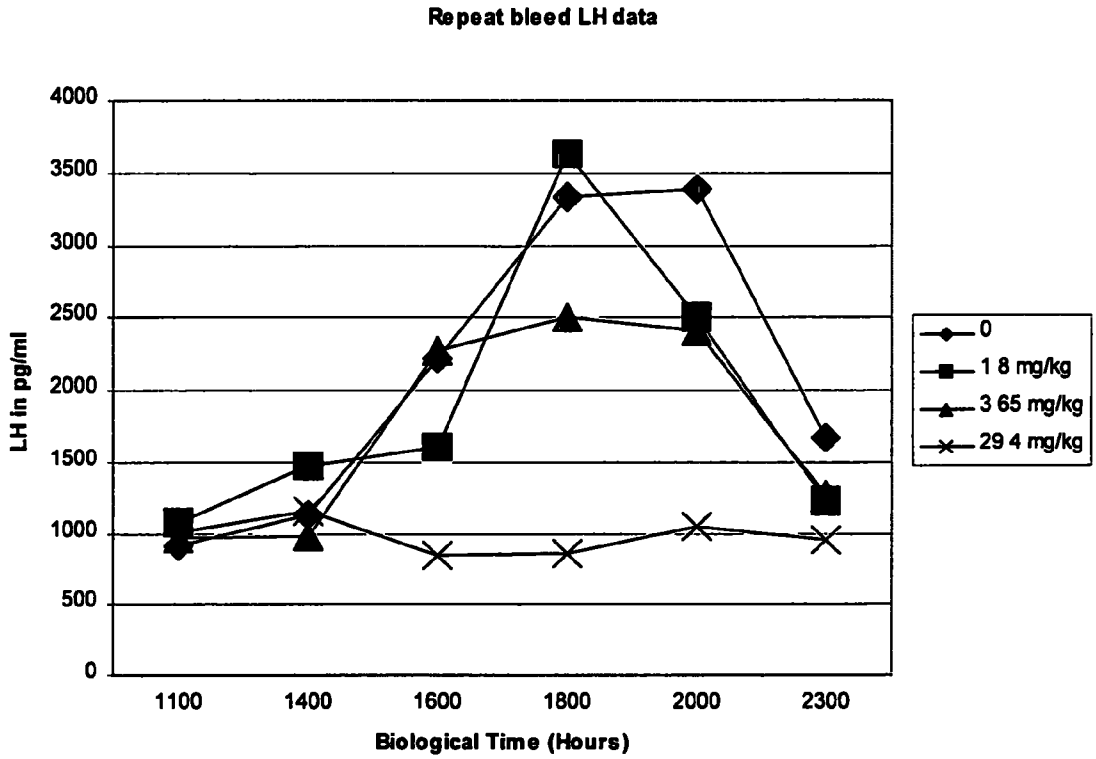
Dose mg/kg/day	1100 Hours	1800 Hours	Fold Increase*
0	909±410	3336±3138	3.7
1.8	1075±621	3631±2732	3 4
3 65	972±353	2500±1897	2.6
29 4	1005±482	858±416	<1.0

*Increase = 1800 hour values (peak values) divided by the 1100 hour values (baseline values)

As shown in Table 1-4, treatment of female SD rats with 200 mg/kg/day of atrazine for one month leads to a pronounced attenuation of the LH surge while treatment with 40 mg/kg/day suppresses the preovulatory surge to a lesser degree. Treatment with atrazine over a six month period (Table 1-5) results in effects at lower doses: an abolishment of the preovulatory surge at 29.4 mg/kg/day and an attenuation of the LH surge at 3.65 mg/kg/day. Figure 1-2 presents graphically the LH levels over the entire sampling period (1100 to 2300 hours) in the six month study. Atrazine treatment suppresses the LH surge in a time and dose dependent fashion. In other words, lower doses of atrazine require longer periods of time to produce an attenuation of the LH surge.

1
2

Figure 1-2. Effects of Atrazine Treatment on the LH Surge in Female SD Rats After Six Months of Dosing



1.3.2 Estrous Cycle Disruptions

In the normal female SD rat, approximately 20-25% of the days of the estrous cycle are spent in estrus. Atrazine treatment leads to a disruption of the normal reproductive cycle as evaluated by vaginal smears (Table 1-6) (Morseth, 1996b). As early as 13 weeks following initiation of treatment and continuing throughout the remainder of the six month study, there is a statistically-significant increase in the percentage of days spent in estrus (control - 31%; 29.4 mg/kg/day - 40%). By 21 to 22 weeks of treatment, the effect on the days in estrus is also statistically-significant in animals treated with 3.65 mg/kg/day atrazine (control - 32%, 3.65 mg/kg/day - 45%).

Table 1-6. Percentage of Days (± sd) in Estrus for SD Females Following Six-Month Exposure to Atrazine through the Diet (Morseth, 1996b)

Dose (mg/kg/day)	9-10 weeks	13-14 weeks	17-18 weeks	21-22 weeks	21- 26 weeks
0	25 ± 9.4	31 ± 22.4	34 ± 24.2	32 ± 25.4	47 ± 32.2
1.8	25 ± 4.8	28 ± 18.0	33 ± 24.7	41± 31.9	48± 35.5
3 65	26 ± 10.2	31 ± 21.1	36 ± 25.1	**45± 32.2***	54 ± 35.1
29.4	26 ± 9.3	**40 ± 27.6***	**45 ± 32.1***	**51 ± 34.8****	**63 ± 37.0***

* p≤ 0.05; ** p≤ 0.01

1.3.3 Effects on Pituitary Weights

Atrazine treatment of female SD rats leads to an early increase in pituitary weights by nine months (Table 1-7). Pituitary weights were increased by 54% over control weights at a dose level of 26.23 mg/kg/day. A less pronounced effect was observed at 4.23 mg/kg/day (25% increase over control pituitary weights) at nine months but not at other times).

Table 1-7. Effects of Atrazine Treatment on Group Mean Absolute Pituitary Weights (mg ± sd) in Female SD Rats (Thakur, 1991a)

Dose (mg/kg/day)	3 months	9 months	12 months
Control	23 ± 4	24 ± 6	37 ±20
4.23	21.2 ± 30.0 (-8%)*	30 ± 6 (+25%)	35 ± 26 (-4%)
26.23	21 ± 8 (-11%)	37 ± 8 (+54%)	42 ± 15 (+13%)

*Values in parenthesis represent percent change relative to control

1.3.4 Histomorphology of Mammary Tissue

Endocrine associated histomorphologic effects on mammary tissue found following treatment of female SD with atrazine include increases in the incidence and severity of acinar development, acinar/lobular development, secretory activity, dilated ducts with secretion, and galactoceles. Each of these effects are considered to be associated with exposure of mammary tissue to estrogen and/or prolactin (Part B, Chapter 9).

The incidences and severity of acinar development, which is primarily associated with estrogen secretion, seemed to be increased at three and nine months in both the low and high dose groups.

Secretory activity is primarily associated with prolactin exposure. At nine months, incidences of animals determined to have increased incidence and severity of secretory activity increased as a function of increasing atrazine dose-levels.

The development of dilated ducts is primarily influenced by prolactin secretion. The incidences and severity of dilated ducts (with secretion) increased markedly at the low and high dose at nine months and at the high dose at 12 months. There is also a suggestion that the incidence of lesions of ducts was increased at the high dose at three months.

The incidence and severity of galactoceles, primarily a marker of prolactin secretion, were reported to increase at both nine and 12 months in a serial sacrifice study (Thakur, 1991a). This increase is pronounced in the 26.23 mg/kg/day atrazine treatment group. The response at the 4.23 mg/kg/day does not indicate a treatment-related effect.

An examination of the individual animal data from Thakur (1991a) is quite useful in demonstrating the relationships between mammary and pituitary tumors, pituitary weights, and histomorphological indications of hormone exposure in the mammary gland. Also evident, when individual animal data from the nine month time point in this study is examined, is the early onset of these parameters. Appendix Tables 27, 28, and 29 display these parameters for each individual animal at the nine month time point in this study.

Early onset of tumors is clear from comparing the control to atrazine-treated animal data displayed in Appendix Tables 27, 28 and 29. None of the ten control animals at this time point had a mammary or pituitary tumor while five of ten and two of ten 400 ppm animals had a mammary tumor or pituitary tumor respectively. Early onset of histomorphologic markers of hormone exposure of the mammary gland is also evident. Only one of the ten control animals had a galactocele or had index weighted scores of 3 or greater for secretory activity or dilated ducts with secretion at nine months. At 400 ppm, eight of the ten animals had galactoceles and eight of the ten had weighted index scores of either three or four for secretory activity or dilated ducts with secretion.

The relationship of these parameters to each other is clear when the data from each animal at these time points is examined. For example, the one and only animal which had a galactocele in the control group, also was the only animal with a three or four weighted index score for secretory activity and dilated ducts and also had the heaviest pituitary in the group. A pituitary would be expected to be enlarged due to lactotroph hyperplasia. Lactotroph hyperplasia is associated with increased prolactin secretion; thus, the animal with the heaviest pituitary is secreting the most prolactin and this is why it is the only animal in the group with a galactocele and high scores for markers of prolactin exposure in the mammary gland. Similar examples can be found in the 70 ppm group where the two animals with the heaviest pituitaries both had galactoceles. Two other animals in this group also had a galctocele, but had pituitaries that were close to the average pituitary weight of the group. Though the pituitaries in these two animals did not weigh an exceptional amount, they were the only two animals in this group in which histopathology detected increased focal hyperplasia of the pituitary. Thus, all four animals with galactoceles (a marker of prolactin exposure) had either heavy pituitaries or focal hyperplasia of the pituitary as detected by histopathology.

1.4 Mutagenic and Estrogenic Activity

The totality of the evidence from a variety of *in vitro* and *in vivo* studies does not support a role for mutagenicity or DNA damaging potential for atrazine. A detailed evaluation of the genotoxicity studies available on atrazine, its metabolites, and structural analogues is provided in Part B, Chapter 6. Additionally, as discussed in Part B, Chapter 7, numerous studies indicate that atrazine does not have exogenous estrogenic activity.

The mutagenic compound *N*-Nitrosoatrazine (NNAT) can be formed *in vitro* when atrazine and nitrite are mixed at an acid pH. Because nitrites and atrazine can be found together in drinking water, concern has been raised about this mutagenic chemical. Although the hypothesis has been advanced that NNAT can be formed in the acid pH found in the stomach, the formation of NNAT in the stomach *in vivo* has yet to be demonstrated. Further, cancer bioassays in female Swiss mice and female Wistar rats failed to show a carcinogenic response following NNAT exposure.

1.5 Structure Activity Relationships

Like atrazine, treatment of female SD rats with simazine and propazine leads to an increased incidence and/or early onset of mammary tumors. Also like atrazine, treatment of male SD rats or CD-1 mice of either sex with simazine or propazine does not lead to an increase in tumor incidences at any site (see Part B, Chapter 8).

1.6 Doses Associated with Effects

Tables 1-8 and 1-9 list NOAELs and LOAELs for the neoplastic and non-neoplastic effects reported to be associated with treatment of female SD rats with atrazine.

1.7 Chronic, Developmental, and Reproductive Toxicity

The data summarized in sections 1.1 through 1.6 indicate that primary underlying events that lead to decreases in LH and prolactin release by the pituitary, irregular estrous cycles, and mammary and pituitary tumor formation following treatment of female SD rats with atrazine involve disruption of the hypothalamic mechanisms involved in the regulation (release) of pituitary hormone secretion. The proximal effects of atrazine that lead to these outcomes have been identified as increased dopamine levels and decreases in norepinephrine, and diminished ability to release GnRH from the hypothalamus (Cooper *et al.*, 1998). Because reproduction and development are controlled by the neuroendocrine system, there are concerns that atrazine treatment could lead to reproductive or developmental toxicity.

Table 1-8. NOAELs and LOAELs (mg/kg/day) Associated with Neoplastic Responses of Female SD Rats Treated with Atrazine

Response #	Duration of Exposure (Months)	Dose in mg/kg/day (Incidence)			Reference
		Control	NOAEL	LOAEL	
Carcinomas	24	0 (15/88)	0 5 (16/67)	3.5 (27/69)	Mayhew *et al.*, 1986
Carcinomas	24	0 (12/80)	4.2 (14/80)	24.4 (27/80**)	Morseth, 1998
Carcinomas	24	0 (17/60)	3.79 (13/59)	23.01 (22/60)	Thakur, 1992a
Carcinomas	12	0 (1/25)	4 12 (2/24)	3.9 6/25)	Pettersen & Turnier, 1995
Carcinomas	12	0 (0/14)	3.79 (3/11)	23 01 (6/18*)	Thakur, 1991a
Carcinomas	12	0 (1/11)	4.22 (2/10)	4 4 (6/23)	Morseth, 1998
Fibro-adenomas	9-15	0 (3/30)	<4.2 (5/30)	4.2 (5/30)	Thakur, 1991a
Pituitary adenomas	9-12	0 (2/20)	4.23 (2/20)	26.63 (8/20)	Thakur, 1991a

#= mammary unless otherwise specified; *p=<0.05; **p=<0.01; *** when adjusted for survival

Table 1-9. NOAELs and LOAELs (mg/kg/day) Associated with Non-Neoplastic Responses in Female SD Rats Treated with Atrazine

Response	Duration of Exposure (Months)	Dose in mg/kg/day (Response) 0	NOAEL	LOAEL	Reference
Percent days in estrus	~5	0 (32% days)	1.8 (41% days)	3.65 (45% days*)	Morseth, 1996b
LH-repeat bleed; fold increase above baseline	1	0 (3.6X)	5.0 (3 3X)	40 (1.9X)	Morseth, 1996a
LH-repeat bleed fold increase above baseline	6	0 (3 7X)	1.8 (3.3X)	3 65 (2.6X)	Morseth, 1996b
Mammary galactoceles	9	0 (10%)	4.23 (40%)	26.23	Thakur, 1991a
Mammary secretory activity[1]	9	0(24)	<4.23 (28)	4.23 (28)	McConnell, 1995
Mammary dilated ducts[1]	9	0(17)	<4.23 (24)	4.23 (24)	McConnell, 1995
Mammary acinar development[1]	3	0 (23)	<4.23 (28)	4.23 (28)	McConnell, 1995
Pituitary weights relative to control[2]	9	0	<4.23 (+25%)	4.23 (+25%)	Thakur, 1991a

*p=<0 05; **p=<0.01; when adjusted for survival; 1 - Index Score shown in parenthesis. Each grade was assigned the following values: absent=0; minimal=1; mild=2; moderately severe=3; marked=4. The sum of these values is the index score; 2 - Increase in pituitary weight relative to control shown in parenthesis.

Standard (EPA Guideline) chronic and subchronic studies conducted with atrazine do not provide insight regarding the potential of atrazine to produce lesions of reproductive organs or tissues that might lead to adverse reproductive or developmental outcomes in male or female animals. Similarly, results of developmental or reproductive toxicity guideline studies with atrazine do not show that the dam or her offspring express effects of atrazine treatment that can be associated with disruption of the hypothalamic-pituitary-ovarian axis. However, results of mode and mechanism of action studies conducted with atrazine in the adult cycling or adult, ovariectomized, estrogen-primed female rats suggest that treatment with atrazine, its structural analogues or metabolites, during other periods of the life cycle would also alter reproductive or developmental function in the dam or offspring. Special studies have been conducted that show that atrazine has reproductive and developmental effects that can be attributed to alterations in endocrine function. Summaries of the guideline and special studies are presented below. Implications of the data summaries presented are discussed in Chapter 2.

1.7.1 Chronic and Subchronic Toxicity of Atrazine

There is no clear evidence that chronic or subchronic treatment of rats or dogs with atrazine, its metabolites or structural analogues leads to effects on reproductive organs and tissue with the exception of the carcinogenicity and histomorphologic effects involving mammary tissue discussed previously. The principal effects reported in female SD rats following chronic dietary treatment with high doses of atrazine (50 mg/kg/day) include altered hematology and clinical chemistry parameters, retinal degeneration, centrolobular necrosis in the liver, rectus-femoris muscle degeneration, myeloid hyperplasia, transitional epithelial hyperplasia in the bladder and kidney, and extramedullary hematopoiesis (Mayhew *et al.*, 1986). Other effects observed in this combined chronic/carcinogenicity rat study at the high dose were histopathology findings in male rats consisting of statistically-significant increases in incidences of prostate epithelial hyperplasia and acinar hyperplasia of the mammary gland at the high dose. These effects were observed at the end of the study at which time there was increased survival in the high dose male rats compared with control male rats. Thus, the significance of the effects observed is unclear because the apparent increases may

reflect the increased number of animals that survived for 24 months at the high dose compared with the controls.

When atrazine was fed to dogs for one year, the prominent effect observed was cardiac dysfunction (O'Conner *et al.*, 1987). Chronic effects observed in a 91-week dietary study in mice were limited to hematologic alterations and decreased mean group absolute brain and kidney weights (Hazelette and Green, 1987). The only effect occasionally seen and potentially associated with endocrine alterations following subchronic or chronic treatment with atrazine, its metabolites, or structural analogues is an effect on the weight of the testes in rats and dogs. However, this effect is variable in different studies. Atrazine treatment produced no effects on the testes in a two-year rat bioassay or in a 18-month mouse bioassay. Simazine treatment resulted in a decrease in gonadal weights in males and females in a 90-day rat study. DACT did not produce effects on the gonads when administered to dogs in 90-day orone-year studies or when administered to rats in a 90-day study. G-28279, when administered to dogs for 90-days produced decreased testes weights. On the other hand, treatment with this metabolite led to increased testes weights when administered to rats for 90-days. G-30033 treatment led to increased relative testes weights when fed to rats for 90-days but produced no effects on testes weights in a 90-day dog study. The overall conclusion regarding effects on gonadal tissue is that there is no clear pattern of increased or decreased weights.

1.7.2 Developmental Toxicity of Atrazine

Results of standard (guideline) rat developmental toxicity studies with atrazine show that effects in maternal animals are confined to increased mortality and decreases in body weight gains and food consumption (Infurana, 1984; Ginkis, 1989). Fetal effects observed in the Infurana study (1984) included incomplete or delayed ossification of skull bones or other sites (NOAEL, 10 mg/kg/day and LOAEL, 70 mg/kg/day). The developmental NOAEL and LOAEL for delayed ossification in the Ginkis study (1989) were 25 and 100 mg/kg/day, respectively. Developmental effects observed in a rabbit developmental toxicity study were reduced litter sizes, increased resorptions, and delayed ossification at maternally toxic doses (appearance of blood in the cage or on the

vulva, reduced body weight gain, and reduced food consumption) (Arthur, 1984a). The NOAEL and LOAEL (developmental) in this study were 5 mg/kg/day and 75 mg/kg/day, respectively. There are no data that would suggest that the delays in ossification in fetal animals are due to disruption of the hypothalamic-pituitary-ovarian axis by atrazine and the dose-levels for producing the delays in ossification (NOAELs 5-25; LOAELs 70 - 100 mg/kg/day). Because of the limited histopathology and the lack of measurements of developmental delays (*e.g.,* vaginal opening and preputial separation) in traditional developmental studies, it is not expected that developmental effects of atrazine treatment that are associated with endocrine perturbations would be seen in the guideline rat and rabbit developmental studies.

1.7.3 Reproductive Toxicity of Atrazine

The effects on gonadal weights (both increases and decreases) occasionally observed in subchronic and chronic studies with atrazine or its metabolites were seen in multi-generation reproduction studies. In the rat multi-generation studies with atrazine, simazine, and propazine, increases were observed in relative but not absolute testes weights of adult P_0 and F_1 rats following treatment with atrazine, simazine or propazine at doses ranging from 29 to 50 mg/kg/day (Mainiero *et al.,* 1987; Epstein *et al.*, 1991; Jessup, 1979). No effect on testes weights were observed in juvenile pups. The increases in relative testes weights may be due to decreased body weights of the adult animals. As noted from the data on testes weights from subchronic and chronic studies, the significance of this finding is unclear. The multi-generation study results provided no evidence of reproductive or developmental toxicity. However, as in the case of the developmental studies performed with atrazine, the traditional, EPA Guideline studies for reproductive effects do not include observations or measurements that were selected to determine effects related to endocrine imbalances.

1.7.4 Special Studies

Several special studies have been performed with atrazine with the objective of evaluating the effects of atrazine, or its metabolites, on pregnancy maintenance and postnatal development. Table 1-10 provides a listing of key findings reported in the special studies along with NOAELs and LOAELs for the effects.

- **Pregnancy Maintenance**

When 0, 50, 100, or 200 mg/kg/day of atrazine was administered by gavage to SD, F344, Holtzman, or LE rats during GD 1-8 just prior to the diurnal prolactin surge or just prior to the nocturnal surge of prolactin, a small but significant decline in mean number of implantation sites was seen only in Fischer-344 rats at the two highest doses. Holtzman rats alone showed an increase in postimplantation loss at the two top doses (Cummings *et al.*, submitted). Serum LH levels were significantly decreased in Holtzman, or LE-hooded rats treated with 100 mg/kg, at 200 mg/kg/day in F344 rats, but at no dose in SD rats. A decrease in serum progesterone levels was seen only in Holtzman rats treated with 200 mg/kg.

In a series of experiments assessing the effect of atrazine on pregnancy maintenance in the female rat by Narotsky *et al.*, (submitted, 1999), atrazine was administered by gavage to F344, SD, and LE rats during GD 6-10. The F344 strain was the most sensitive to atrazine's effects on pregnancy maintenance (full-litter resorption); the LE strain was the least sensitive. In F344 rats, surviving litters appeared normal; however, parturition was delayed. In SD rats, full-litter resorptions were also observed, but at higher dose levels: parturition was delayed at the same dose levels as for F344 rats. In contrast, the LE hooded strain showed full-litter resorption at the same dose level as SD rats, but there were no effects on parturition.

- **Reproductive and Postnatal Effects**

In a study examining the effect of atrazine on pubertal development, young Wistar rats were treated by gavage with atrazine (12.5, 25, 50, 100 or 200 mg/kg/day) during PND 22-41 (Laws *et al.*,submitted; Laws *et al.*, 2000). Vaginal opening was significantly delayed (three or four days) by 50 and 100 mg/kg respectively. The 200 mg/kg per day treatment with atrazine for the same period delayed vaginal opening by more than seven days in 18 of 32 females. When vaginal opening did occur, irregular cycles were observed in the 50 and 100 mg/kg dose groups during the ensuing two weeks. Vaginal opening occurred shortly after dosing was stopped in the 200 mg/kg dose group, and these females also demonstrated irregular estrous cycles for the next two weeks. All animals returned to regular estrous cycles by PND 70.

In a study evaluating the pubertal development in the male, weanling Wistar rats were dosed with atrazine during PND 23-53 (Stoker *et al.*,2000a; Stoker *et al.*, submitted). The significant finding from this study was that atrazine delayed preputial separation. The LOAEL for delay in preputial separation was <12.5 mg/kg/day. No consistent effect on serum prolactin and testosterone concentrations was observed, but the serum levels of these two hormones in animals of this age fluctuate widely making significant difference difficult to identify. However, there was a significant dose-related decrease in serum LH on PND 53 ($r = -0.92$. $P < 0.0024$).

Other studies have shown that reproductive tissues in the offspring can also be affected if the dam is treated during lactation. Suckling-induced PRL release was measured in Wistar dams treated with atrazine by gavage, twice daily with 0, 6.25, 12.5, 25, or 50 mg/kg (the total daily dose was 13, 25, 50 or 100 mg/kg/day) during PND 1-4 (Cooper *et al.*, 2000). Serum PRL in dams was measured on PND 3. A significant rise in serum prolactin release was noted in all control dams within 10 minutes of the initiation of suckling. The 25 and 50 mg/kg/day treatment with atrazine inhibited prolactin release in 40% or 60% of the dams, respectively; the daily dose of 100 mg/kg inhibited this measure in all dams. In this same study, the effect of postnatal atrazine on the incidence and severity of inflammation of the lateral prostate of the offspring was examined in adult males at 90 and 120 days. While no effect was noted at 90 days of age, at 120 days, both the incidence and severity of prostate inflammation was shown to increase in those offspring of atrazine-treated dams (50 or 100 mg/kg/day). Combined treatment of dams with ovine prolactin (oPRL) and atrazine on PND 1 - 4 reduced the incidence of inflammation observed at 120 days, indicating that this increase in inflammation seen after atrazine alone resulted from the suppression of prolactin in the dam. These data demonstrate that atrazine suppresses suckling-induced prolactin release and that this suppression results in lateral prostate inflammation in the offspring. The critical period for this effect is PND1-9. It should be noted that vaginal opening was delayed in the offspring of these dams (Stoker *et al.*, submitted). Whether this effect is also related to changes in prolactin secretion in the dam remains to be determined.

Table 1-10. NOAELs/LOAELs (mg/kg/day) for Reproductive and Developmental Effects Following Treatment of Dams or Offspring of Several Rat Strains with Atrazine or its Metabolites[1]

Response and exposure period	F344	SD	Wistar	LE	Holtz-man	Ref.
Decrease in mean number of implantation sites- GD 1-8	50/100	>200	NA*	>200	> 200	Cummings *et al.*, submitted
Delayed parturition-GD 6-10	50/100	50/100	N A.	>200	N.A.	Narotsky *et al.*, submitted; 1999
Full litter resorptions-GD 1-8 or 6-10						
atrazine	25/50	100/200	N.A.	100/200	50/100	Cummings *et al.*, submitted, Narotsky *et al.*, submitted; 1999
DACT	<67/67	N.A.	N.A.	N.A.	N.A.	
DEAT	<87/87	N.A.	N.A.	N.A.	N.A.	
DIAT	> 80	N.A.	N.A.	N.A	N.A.	
OHA	<275/275	N.A.	N.A	N.A	N.A.	
Reduction in serum LH-GD 1-8	100/200	> 200	N.A.	50/100	50/100	Cummings *et al.*, submitted;
Decreased prolactin release- PND 1-4 (dams)	N.A.	N A.	13/25	N.A.	N.A.	Stoker *et al.*, 1999
Increased incidence of prostatitis-PND 1-4	N.A.	N.A.	13/25	N.A.	N.A	Stoker *et al*, 1999
Increased incidence and severity of prostatitis-PND 1-4	N.A.	N.A.	25/50	N.A.	N.A.	Stoker *et al.*, 1999
Delayed vaginal opening-PND 22-41	N.A.	N.A.	25/50	N A.	N.A.	Laws *et al.*, submitted; 2000
Delayed preputial separation-PND 23-53	N.A	N.A	<13/13	N.A.	N.A.	Stoker *et al*, submitted; 2000a

[1]Data are for atrazine unless otherwise noted; * not available

Chapter 2

2. Hazard Characterization And Mode of Action Analysis

This Chapter presents information characterizing the neoplastic and non-neoplastic effects reported from studies conducted with atrazine and considers them in the context of an analytical framework for evaluating a postulated mode of action as described in the proposed revisions to the guidelines for carcinogen risk assessment (EPA, 1999). The framework is used to judge how well the available data support a mode of action postulated for a carcinogenic agent. This Chapter draws on the information summarized in the preceding Chapter. Complete details on the carcinogenicity and chronic toxicity of atrazine are presented in Part B of this document. This Chapter also evaluates the neuroendocrine effects of atrazine on the development and function of the reproductive system. The details of these studies can be found in Part C.

2.1 Human Cancer Studies

Several epidemiologic studies have examined cancers among populations with exposures relevant to the assessment of atrazine, especially among farmers or farm residents (see Part B, Chapter 4 for details). Most are case control studies, although there are ecologic investigations and also a worker mortality study of workers directly employed in the manufacture of triazines. Studies examining the association of triazine exposure with colon cancer, leukemia, multiple myeloma, soft tissue sarcomas, and Hodgkins disease failed to find firm associations. The pooled results of three separate case-referent studies investigating atrazine exposure in the development of non-Hodgkins lymphoma (NHL) concluded that there was essentially no risk of NHL attributable to farm use of atrazine. A mortality study of workers in two triazine manufacturing plants did not find any significant excesses of deaths for any disease category. There were, however, two cases of NHL in plant workers - one of whom was relatively young (31 years). These two cases do not provide evidence of an association between atrazine exposure and NHL, but do indicate that further follow-up of workers in these triazine manufacturing plants is desirable.

Associations between triazine exposure and cancer for three hormone-responsive cancers--ovary, breast and prostate cancer has been reported. Although suggestive, these associations should not be considered as conclusive evidence of a correlation between triazine exposure and these tumor types. The studies that showed possible relationships between these tumor types and triazine exposure should be interpreted with caution because of limitations, such as misclassification of subjects, use of surrogate data for exposure, or concurrent exposure to other potentially carcinogenic compounds.

To summarize, there is suggestive evidence of a possible association of triazine exposure and NHL, prostate, breast and ovarian cancers. This evidence does not show a direct cause and effect relationship between atrazine or triazine exposures and carcinogenicity because of confounding factors and limitations in the available studies. The available evidence emphasize the need for further epidemiologic research into the association of these tumor types with atrazine exposure.

2.2 Carcinogenicity in Female SD Rats

There were dose-related increases in the incidence of mammary tumors (adenomas, adenocarcinomas, and carcinosarcomas combined) in female Sprague-Dawley (SD) rats in the seminal carcinogenicity test performed with atrazine (Mayhew *et al.*, 1986). No dose-related increases in tumor responses were observed in male SD rats. Results of subsequent bioassays, some of which included serial and/or one year sacrifices, confirmed that the predominant response observed following testing of atrazine in female SD rats is an increase in the incidence and/or early onset of mammary adenomas/carcinomas. Although less compelling, there is evidence that there is decreased latency for the formation of mammary fibroadenomas and pituitary adenomas (Thakur, 1991a and 1992a; Petersen and Turnier, 1995) and an increased incidence of mammary fibroadenomas (Morseth, 1998). An increased tumor incidence is not found at any other site in female SD rats, or at any site in male SD rats, or in either sex of Fischer 344 rats and CD-1 mice (Mayhew *et al.*, 1986; Hazelette and Green, 1987; Thakur, 1992a,b). Mammary tumors were reported in one study in male Fischer 344 rats that involved lifetime treatment with atrazine (Pinter *et al.*, 1990), but the finding is difficult to evaluate in light of the experimental design and shortcomings of the study. Furthermore, this finding is

in conflict with the results of a conventional 24-month carcinogenicity study with F344 male rats that showed no increases in mammary tumors (Thakur, 1992b). The closely related structural analogues to atrazine, simazine and propazine, also produce mammary tumors in the female SD rat but no other tumors of any type in the female SD rat and no tumors of any kind in the male SD rat or in CD-1 mice of either sex.

2.3 Postulated Mode of Carcinogenic Action

Before presenting the postulated mode of action for atrazine, it is instructive to consider aspects of the normal reproductive biology of the female SD rat and its relevance to tumor formation.

2.3.1 Reproductive Aging in Rats

With advancing age, the female Sprague-Dawley, as most strains of rats, normally undergoes a transition from regular ovarian cycles to an acylic pattern of "persistent" or "constant" estrus (Cooper and Walker, 1979; Also, see Part B, Chapter 9.1). Typically, this transition occurs prior to one year of age and is related to a disruption in both the timing and amplitude of the preovulatory surge of lutenizing hormone (Cooper *et al.*, 1980). As a result of this inability to achieve ovulation, the ovaries of the constant estrous female may contain many large follicles *(i.e.,* polyfollicular ovaries) but no corpora lutea (Huang and Meites, 1975). These follicles continue to secrete estradiol, while progesterone secretion is minimal (Huang *et al.,* 1978). This pattern of hormone secretion has been shown to facilitate the development of mammary gland tumors in aging rats and in young females in which a constant estrus has been induced (Nandi *et al.,* 1995; Russo *et al.,* 1990; Cutts and Noble, 1964; Meites, 1972). The inability to achieve an ovulatory surge of LH is the result of changes in the ability of the hypothalamus to achieve the proper release of GnRH. Changes in norepinephrine concentration occur prior to the onset of the loss of regular ovarian cyclicity (Wise *et al.,* 1997; Wise *et al.,* 1999). Conversely, treatment with CNS acting compounds such as the catecholaminergic precursor, L-dopa, will result in a reinitiation of regular cycles (Quadri *et al.,* 1973). Similarly, the age at which regular estrous cycles are disrupted can be extended if the female is placed on a diet containing L-tyrosine (*i.e.,* the amino acid precursor of L-dopa).

Persistent or constant vaginal estrus, the accompanying pattern of persistent estradiol secretion and no progesterone, also leads to an increase in pituitary weights, development of pituitary hyperplasia, and formation of pituitary adenomas in the aged female rat (Blankenstein *et al.*, 1984; McConnell, 1989a; Nelson *et al.*, 1980; Meites, 1980; McConnell, 1989b). The majority of the pituitary adenomas seen in the aged female SD have been found to originate from lacotrophs (*i.e.*, prolactin-secreting cells of the anterior pituitary) (Sandusky *et al.*, 1988). The increased number of prolactin-secreting cells results in an increased serum level of prolactin and extended or prolonged exposure of mammary tissue to higher than normal levels of prolactin. As indicated above, dietary supplementation with L-dopa and L-tyrosine (precursors to catecholamine synthesis in the central nervous system) delays reproductive aging as evidenced by maintained LH surges, normal reproductive cycling, and delayed onset of mammary gland tumor formation in treated animals compared to controls of the same age. No female Long-Evans (LE) rat developed mammary tumors by 21 months of age when fed a diet supplemented with L-tyrosine compared with a mammary tumor incidence of 67% in control (no supplement) animals (Cooper and Walker, 1979). Restored vaginal cycling is also found when aged female rats are administered L-dopa and L-tyrosine. Ovariectomy also reduces exposure of mammary tissue to estrogen and reduces or eliminates mammary tumor formation.

In summary, reproductive aging in the female rat appears to result from a disruption of hypothalamic neurotransmitter and neuropeptide (primarily noradrenergic) regulation of GnRH, and subsequently LH secretion. Importantly, the normal age-related disruption of regular cycling can be modified by pharmaceutical treatment or dietary supplementation. Finally, the resultant endorine milieu of enhanced or unopposed estrogen and prolactin secretion, provides an environment that is conducive to the development of mammary gland and pituitary tumors.

2.3.2 Atrazine Effects Relevant to Carcinogenicity

It is postulated that the carcinogenicity of atrazine is a consequence of the disruption of the normal secretory activity of the hypothalamic-pituitary-ovarian axis. Atrazine exposure adds to the formation of mammary tumors by inducing a sequence of events which intersects, at some point, with the normal reproductive aging pathway. The point of intersection appears to be the attenuation of the proestrous afternoon LH surge. Both *in vivo* and *in vitro* experiments demonstrate that atrazine exposure does not directly affect the pituitary (Cooper *et al.*, 2000) and that a decreased ability of the hypothalamus to release GnRH is likely the cause of the attenuated LH surge in the atrazine exposed SD female. Finally, pituitary weight and histomorphologic data in the mammary gland demonstrate that continued estrogen secretion also stimulates prolactin secretion by the pituitary. Again, ongoing secretion of estrogen and prolactin create an endocrinological milieu conducive to mammary gland and pituitary gland cell proliferation and eventual tumor development.

Females of the F-344 rat strain have a rather low background incidence of mammary tumors. In contrast to SD, LE, and Wistar females, this strain goes through a different pathway for reproductive senescence. F-344 females age through a process termed repeated pseudopregnancy, a condition where there are normal LH surges and ovulation occurs but continued secretion of progesterone by corpus lutea leads to a vaginal cytology indicative of diestrous. Mammary tumors are not induced by atrazine in F344 female rats. It would seem that the differences in reproductive aging between the F-344 and SD strains influence their sensitivity and response to atrazine administration.

Figure 2-1 illustrates the postulated mode of action of atrazine in female SD rats on the activity of the hypothalmic-pituitary-ovarian axis and the development of mammary and to some extent pituitary neoplasms. Effects associated with atrazine treatment on the activity of this axis are:

1. Atrazine exposure affects - either directly or indirectly - the hypothalamus, leading to a decreased secretion of hypothalamic norepinephrine (NE) (Cooper 1998)[1].

2. Hypothalamic NE normally modulates the release of gonadotropin releasing hormone (GnRH) from the hypothalamus. Decreased NE levels result in decreased release of GnRH from the hypothalamus (Cooper, 1998).

3. GnRH is the hormone responsible for inducing the pituitary gland to release luteinizing hormone (LH). A decreased GnRH level leads to an attenuated LH release (Cooper *et al.*, 2000, Morseth, 1996a, b).

4. LH normally provides a signal to the ovaries promoting ovulation. Below some critical level, the decreased serum levels of LH are insufficient to stimulate ovulation.

5. Estrogen from ovarian follicles normally provides a feed back to the hypothalamus to stimulate a pituitary LH surge which promotes ovulation. Following atrazine exposure, there is insufficient GnRH to stimulate ovulation. Under the tonic secretion of LH and FSH, the ovarian follicles persist and continue to secrete estradiol. In turn, under the continued stimulation of estradiol, the pituitary lactotrophs become hypertrophied and secrete increasing amounts of prolactin.

[1]Cooper (1998) has also shown that acute atrazine treatment results in an increase in hypothalamic dopamine which in turn results in a decrease in pituitary prolactin. This acute effect is not expected to be associated with neoplasia but has potential reproductive consequences under certain circumstances.

6. Estrogen acts on the mammary gland increasing the risk of mammary tumors, especially carcinomas and adenomas.

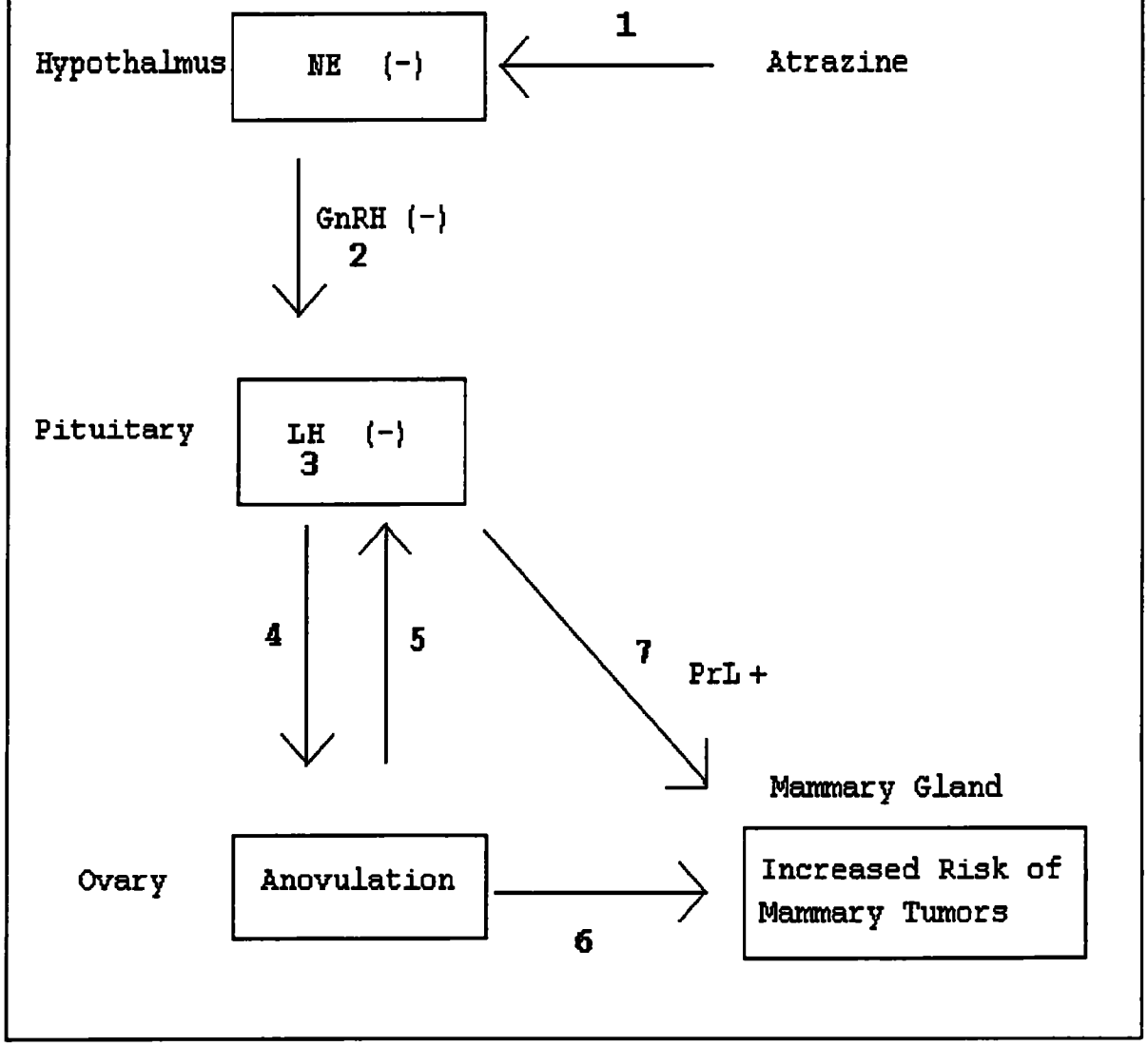
7. Prolactin derived from the hyperplastic lactotrophs (prolactin secreting cells) described in step 5 also acts on the mammary gland (in concert with estrogen) to increase the risk of mammary tumors, particularly fibroadenomas.

8. Tumor formation by atrazine does not appear to involve direct mutagenic effects nor does atrazine act as a direct estrogen agonist.

2.4 Evaluation of the Postulated Mode of Carcinogenic Action

In this section, the evidence linking the formation of mammary and pituitary tumors in female SD rats with disruption of biochemical activities in the hypothalamic-pituitary-ovarian axis is examined. These sections also examine the evidence supporting or refuting the postulated mode of action described in Figure 2-1 as the causal mode of action associated with the carcinogenicity of atrazine in female SD rats.

Figure 2-1. Postulated Effects of Atrazine Treatment on the Hypothalamic-Pituitary-Ovarian Axis



1
2
3
4

2.4.1 Key Events

Data showing that the hypothalamus appears to be a primary initial site of action for atrazine primarily come from short-term, high dose studies conducted in Long Evans (LE) females[2] by the EPA's National Health and Effects Research Laboratory (Cooper *et al.*, 1998; Cooper, 2000). These studies provide evidence that atrazine affects hypothalamic catecholamine levels. A decrease in NE results in a decrease in gonadotropin releasing hormone (GnRH), with a corresponding diminution of pituitary surges of luteinizing hormone (LH). These *in vivo* observations are further supported by *in vitro* studies using pheochromocytoma cells. In this cell line, both dopamine and norepinephrine are synthesized constitutively. Das *et al.* (2000, in press) have shown that catecholamine synthesis is suppressed, in a dose dependent manner, following exposure to atrazine. Evidence for a hypothalamic site of action for the neuroendocrine disrupting effects of atrazine include the following observations:

- the pulsatile release of GnRH from the hypothalamus is impaired in the female rat following atrazine exposure (Cooper *et al.*, 1998)

- the atrazine-induced suppression of LH secretion can be reversed following treatment with synthetic GnRH (Cooper *et al.*, 2000)

- there is a dramatic increase in the hypothalamic concentration of GnRH following exposure to atrazine demonstrating that release (and not synthesis) of GnRH is impaired (Ford *et al.*, 2000)

[2]Some rat strains (LE, Wistar and SD included) undergo a similar reproductive aging process which is characterized by the appearance of persistent (or constant) estrus by approximately one year of age and under similar neuroendocrine events. Thus, the LE female rat is considered to be a valid model for evaluating atrazine's mode of action resulting in mammary tumors in SD females.

- related to these effects on GnRH release, a hypothalamic site of action also appears to be responsible for the inhibition of prolactin release as the atrazine-induced suppression of prolactin secretion is not observed if the pituitary is removed from its normal location (within the sella turcica, beneath the hypothalamus) and placed beneath the kidney capsule (Cooper *et al.*, 2000).

Suppression of the LH surge in female SD rats is considered to be a necessary precursor for the development of atrazine-induced mammary gland tumors. This is because LH blood levels must reach a sufficient magnitude to induce ovulation and to maintain normal reproductive cycles. When atrazine reduces LH output to the critical point where there is not enough to trigger ovulation, a physiological state results which is characterized by prolonged or persistent estrous. This state leads to continued stimulation of mammary tissue by estrogen. Evidence for an attenuation of the LH surge and an early onset of prolonged and/or persistent estrus is provided in several studies (Morseth 1996a,b; Thakur 1991a; Eldridge *et al.*, 1993a). Removal of the estrogen stimulus by ovariectomy completely abolishes the formation of mammary tumors following chronic administration of atrazine (Morseth, 1998). Estrogen has been strongly implicated in mammary gland cell proliferation and the enhancement of neoplastic transformation in rodents and humans (for review see Russo and Russo, 1996; Nandi, 1996).

The attenuation of LH surges and disruption of the normal reproductive cycles in female SD and Long-Evans hooded rats treated with atrazine mirrors prominent features of the normal reproductive aging process in these strains. This process features a diminution of LH blood levels, a failure to ovulate, and a state of persistent estrus.

Prolonged estrogen secretion resulting from atrazine treatment appears to lead to other consequences. There is evidence that sustained exposure of the pituitary gland to estrogen leads to an increase in pituitary weights, pituitary hyperplasia, development of lesions characteristic of prolactin secretion, and the formation of pituitary adenomas (Thakur, 1991a; McConnell, 1995). The sustained secretion of prolactin is believed to play a role in the development of mammary tumors, in general, but a more prominent role in the development of mammary fibroadenomas (Welsch, 1985).

2.4.2 Correlation of Effects and Dose

There is a strong association between dose levels of atrazine that lead to an early onset and increased incidences of mammary tumors and doses that produce biochemical perturbations that have been linked to reproductive aging *(i.e.*, suppression of LH surges and prolonged or persistent estrus). Table 2-1, lists the lowest dose (LOAEL) which elicited each of the effects associated with atrazine treatment. Tables 1-8 and 1-9, Chapter 1, may be referred to by the reader for NOAELs and LOAELs of all data on tumor and non-neoplastic effects.

A dose of 3.5 mg/kg/day and above that leads to an early onset and/or increased incidence of mammary carcinomas in female SD rats also leads to attenuation of LH secretion. Examination of Figure 1-2, Chapter 1, indicates that administration of atrazine at a dose level of 3.65 mg/kg/day results in a diminution of the LH surge. This is the same dose that results in estrous cycle perturbations. At a dose level of 29.4 mg/kg/day, the LH surge is completely suppressed. If attenuation of the LH surge were indeed a key event in mammary and pituitary tumor formation, then doses that result in an attenuation of the LH surge would be expected to result in an increased incidence or early onset of these tumors. Doses of 4.2 and 24.4 mg/kg/day resulted in an early onset of mammary carcinomas (Morseth, 1998). Doses of 3.79 and 23.01 mg/kg/day resulted in an early onset of mammary carcinomas in another study (Thakur, 1992a). The evidence for an early onset of mammary fibroadenomas and pituitary tumors is less strong as these effects were only seen in one study (Thakur, 1991a).

Table 2-1. LOAELs for Tumor Formation and Non-Neoplastic Effects in Female SD Rats

Effect/Time of Observation	LOAEL (mg/kg/day)	Reference
LH-repeat bleed; increase above baseline (6 months)	3.65	Morseth, 1996b
Prolonged days in estrus (6 months)	3.65	Morseth, 1996b
Mammary carcinomas - decreased latency (12 months)	3.79	Thakur, 1992a
Mammary carcinomas - increased incidence (24 months)	3.5	Mayhew *et al.*, 1986
Mammary galactoceles (9 months)	4.23	Thakur, 1991a
Increased pituitary weights (9 months)	4.23	Thakur, 1991a
Pituitary adenomas - decreased latency (9 months)	26.23	Thakur, 1991a
Mammary fibroadenomas - decreased latency (15 months)	4.23	Thakur, 1991a

There is also a correlation between time spent in estrus and tumor formation. The data from the 1998 Morseth study, as described in Thakur (1999), shows that there is a statistically-significant correlation between percent days spent in estrus during both the one to 46 week and 17 to 26 week time intervals, and an increased mammary carcinomas incidence. Moreover, examination of the animals in this study, where there was an especially early tumor onset (prior to 52 weeks), showed that there was an unusually long period of time spent in estrus. Five of six female SD rats that developed mammary carcinomas by 52 weeks spent >70 % of the days in estrus between weeks 17 to 26.

As discussed previously, estrogen stimulation of the pituitary gland is believed to cause an increase in the secretion of prolactin, a hormone closely associated with the development of mammary tumors, especially fibroadenomas. There is histomorphologic evidence (*e.g.*, acinar development, dilated ducts, and increases in the incidence and severity of galactoceles) of an early onset of increased prolactin secretion at 4.23 mg/kg/day (McConnell, 1995). It is biologically plausible that this early exposure to prolactin may contribute to the early onset of mammary fibroadenomas as seen in Thakur (1991a). There is also an early onset of increased pituitary weights in this study. Absolute pituitary weights at 4.23 mg/kg/day are increased by 25% at nine months. The increase is likely due to the mitogenic effect on pituitary lactotrophs of estrogen derived from unovulated follicles. The larger pituitaries would be expected to secrete increased amounts of prolactin. This is indicated by the early onset of prolactin-dependent histomorphologic parameters and the early onset of mammary fibroadenomas.

The lowest atrazine dose showing effects on LH, the pituitary gland, and the estrous cycle is somewhere between 3 and 4 mg/kg/day. The LH surge attenuation occurred at 3.65 mg/kg/day, but did not occur at 1.8 mg/kg/day. The estrous cycle alterations occurred at 3.1 and 3.65 mg/kg/day in two separate studies. In these studies, the estrous cycle alteration did not occur at 1.5 and 1.8 mg/kg/day. There is only one study on serum estradiol levels. Although this study shows an early onset of increased estradiol levels at 4.23 mg/kg/day, a clear dose effect level is uncertain due to variability in the data and the lack of confirmatory data at other timepoints in the same study (*e.g.*, six months). The main factor is that estrogen secretion is prolonged during persistent estrus which results in continuous stimulation of the mammary gland.

2.4.3 Temporal Association of Effects

Data from chronic studies in female SD rats administered atrazine consistently show that there is an early onset of mammary tumors. This is what would be expected if atrazine accelerated the reproductive aging process. Therefore, it is anticipated that precursor events to mammary tumors would have their onset in atrazine treated females before that of untreated SD females undergoing normal reproductive aging. The temporal pattern of effects found following atrazine treatment are summarized in Figure 2-2.

In untreated aging female SD rats, prolonged days in estrus begin as early as nine months and shortly thereafter they enter into persistent estrus. Extended days in estrus, a key event associated with the formation of mammary tumors begins earlier in rats treated with atrazine than in controls. An increased number of days in estrus begins as early as 3.5 or 5.5 months in females administered 29.4 mg/kg/day or 3.65 mg/kg/day of atrazine, respectively (Morseth, 1996b). These data were confirmed in a separate study which showed that by 3.3 months SD females exposed to 24.4 mg/kg/day were spending approximately 26% more days in estrus than control animals (Thakur, 1999). Dietary administration of 3.65 mg/kg/day of atrazine leads to attenuation of the proestrus afternoon LH surge after as little as six months of atrazine exposure. Thus, exposure to atrazine decreases the onset time of attenuated LH surge and persistent estrus. These effects have been identified as the precursor events in the pathway towards mammary tumors in rats. In keeping with these findings, animals receiving 4.23 mg/kg/day (lowest dose tested) manifest an early onset of histomorphologic changes in mammary tissue (*e.g.,* increased incidences and severity of acinar formation, secretory activity, and galactoceles) following six to nine months of treatment of female SD rats with atrazine (McConnell, 1995). These changes are primarily indications of exposure of mammary tissue to prolactin and estrogen. This broad time line illustrates the sequence of events that occur prior to tumor development (as well as the associated effective dose levels for the response).

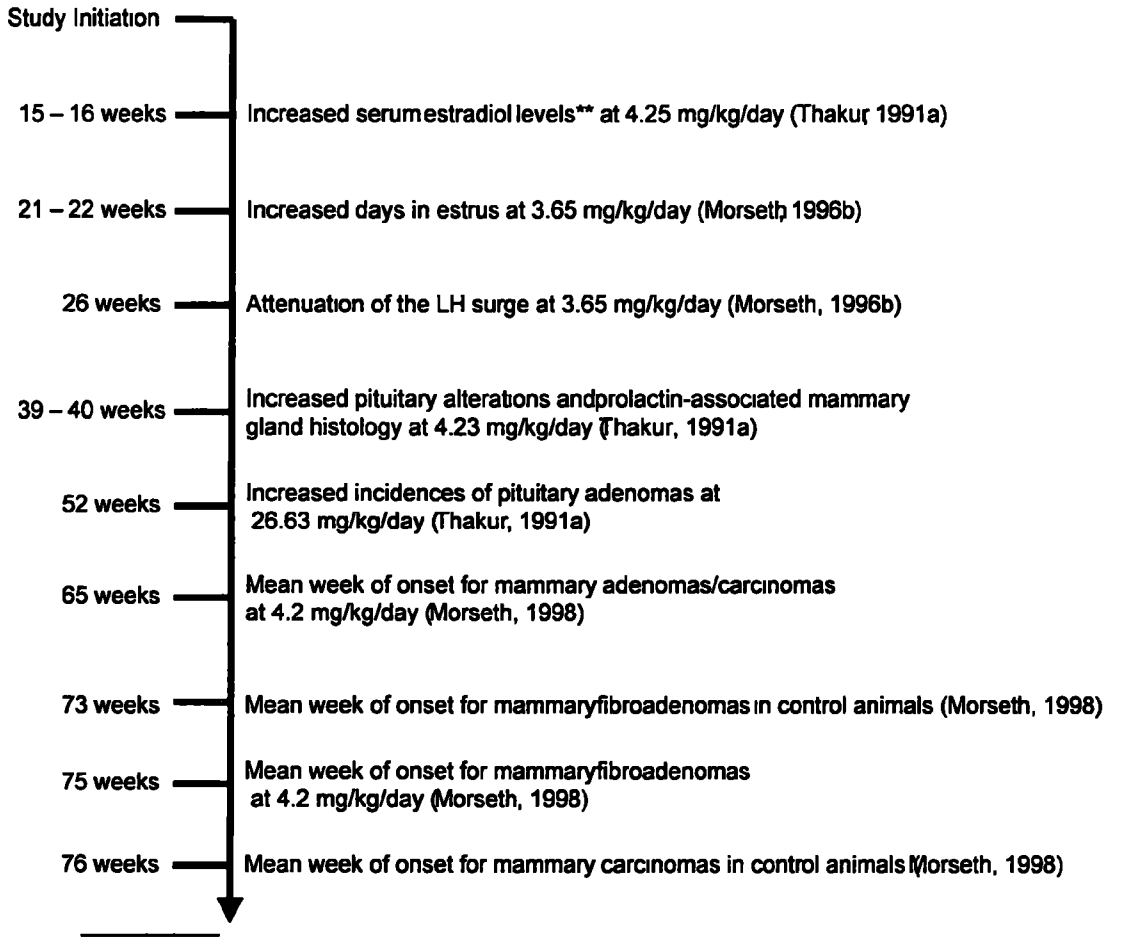
2.4.4 Biological Plausibility and Coherence of the Database

The process of normal reproductive senescence in the female SD rat has been implicated in creating a hormonal milieu conducive to mammary tumor and pituitary tumor formation, including:

- Attenuation of the pre-ovulatory LH surge;
- Increased days in estrus; and
- Prolonged exposure to endogenous estrogens and prolactin.

The events listed above have been well described in the open literature as normal and expected events in the reproductive aging of the female SD rat (Cooper and Walker, 1979; Lu, 1994; Mobbs, 1996; Smith and Conn, 1983; Zuo, 1996). Prolonged exposure to endogenous estrogens has been generally accepted as a major contributor to the high spontaneous mammary and pituitary tumor rates seen in the SD female (Welsch, 1987; Cooper, 1983; Cutts and Noble, 1964). Prolonged exposure of mammary tissue to prolactin, which results from the estrogen-induced pituitary tumors, also has been well established as a contributor to mammary carcinogenesis in the normally aging female SD (Welsch, 1970a; Welsch, 1970b; Meites, 1971; Goya *et al.*,1990).

Figure 2-2. Temporal Pattern of Atrazine Effects*



*Time when effects are first noted was dictated by study design; **Only one study available

The biologic plausibility for the mode of action proposed for atrazine-induced mammary and pituitary carcinogenicity lies in the observation that atrazine exposure has been shown to induce an earlier onset of all three of the events outlined above: attenuation of the pre-ovulatory LH surge; increased days in estrus; and prolonged exposure to endogenous estrogen. Because this sequence of events has been generally accepted as leading to mammary and pituitary carcinogenesis in the normally aging SD female, one can reasonably expect that atrazine administration would lead to the same events, including tumors, only at earlier times than in normally aging females.

Atrazine dose levels that lead to attenuation of the LH surge also are associated with disruption of the estrous cycle and an early development or increased incidence of mammary and pituitary gland tumors. One study provides histomorphologic evidence that an early onset of pituitary tumors and mammary fibroadenomas may be explained by prolonged secretion of estrogen by the anovulatory female rat, stimulation of the pituitary to undergo cell proliferation, and increased prolactin secretion by the estrogen-stimulated pituitary gland. The formation of both mammary carcinomas and mammary fibroadenomas are influenced by prolonged exposure of the mammary gland to follicle - derived estrogen and pituitary-derived prolactin. Thus, in several respects, the effects of atrazine treatment mirror biochemical alterations that have been attributed to the onset of reproductive aging and spontaneous tumor formation in the female SD rat. The mode of action proposed to account for the tumor responses in female SD rats treated with atrazine is biologically plausible because the major key biological and biochemical events shown to be altered by atrazine treatment are the same ones that have been identified as contributors to the formation of mammary and pituitary tumors in aging female SD rats.

2.4.5 Other Modes of Action

- **Mutagenicity**

Because cancer is the result of a series of genetic defects in genes controlling cell growth, division, and differentiation, an initial and prominent question to be examined is whether atrazine (or an atrazine metabolite) interacts directly with, and mutates DNA. The totality of evidence for atrazine, including data on several metabolites of atrazine and close structural analogues, does not support a mutagenic potential for atrazine, and indicates that a direct DNA reactive, mutagenic mode of action is unlikely to be an influence on atrazine tumor development. The genetic toxicology database for atrazine shows consistent negative responses in bacterial tests and inconsistent positive responses across other phylogenetic lines (which are typically weak, found at high doses, or cannot be reproduced). No subset of data points clearly establishes a direct DNA reactive mode of action for atrazine associated with the carcinogenicity.

- **Estrogen Agonistic Action**

The available evidence from *in vivo* and *in vitro* studies indicates that atrazine does not bind to the estrogen receptor or possess direct estrogenic activity. Under equilibrium conditions, atrazine does not compete with estradiol for binding to SD rat estrogen receptors (Tennant *et al.*, 1994b). Atrazine treatment does not induce changes in estrogen-responsive tissues (*e.g.*, increased uterine weight, increased uterine cell proliferation, uterine peroxidase activity and uterine progesterone receptors) in ovariectomized SD rats. Atrazine does not affect basal or estradiol induced cell proliferation in a human breast cancer cell line (MCF-7) (Safe *et al.*, 1995). Atrazine does not have agonist or antagonist action against estradiol induced luciferase activity in MCF-7 cells transfected with a Gal4-regulated human estrogen receptor chimera, thus showing failure to bind to the estrogen receptor (Conner *et al.*, 1996). Although estrogen binding was found for atrazine (Tennant *et al.*, 1994b), it was demonstrated only under

conditions that favored binding and at very high doses of atrazine relative to those that induced mammary tumors in SD females. DACT (a metabolite of atrazine) and simazine (analogue of atrazine) also do not appear to bind to the estrogen receptor (Tennant *et al.*, 1994b; Connor *et al.*, 1996).

- **Other Endocrine Imbalances**

Data presented in abstract form indicates that atrazine depresses hypothalamic norepinephrine (NE) levels and increases hypothalamic dopamine (DA) levels (Cooper, 1998). NE levels correlate directly with hypothalamic GnRH release (*i.e.*, increased NE leads to increased GnRH release) while DA levels are inversely related to pituitary prolactin release (*i.e.*, increased DA levels leads to decreased prolactin secretion). Thus, a decrease in hypothalamic GnRH secretion and a decrease in pituitary prolactin secretion might be expected from alterations of these neurotransmitters.

Chronic exposure to doses of atrazine as low as ~4 mg/kg/day leads to elevated prolactin secretion, as indicated by histomorphologic markers, presumably because of estrogen-induced pituitary lactroph hyperplasia in the anovluatory female. Prolonged exposure to serum prolactin contributes to mammary gland carcinogenesis in the rat (Welsch, 1985) because of its proliferative effect on the mammary gland tissue.

The pituitary does not appear to be a direct target of atrazine toxicity. When pituitary hormone secretion is removed from the influence of CNS hypothalamic factors (*i.e.*, by placing pituitary grafts under the kidney capsule) in LE females, there is no effect on prolactin release when animals are exposed to a dose of atrazine that suppresses the prolactin surge (Cooper, 2000). The atrazine-induced attenuation of the LH surge can be reversed by intravenous exposure to exogenous GnRH (Cooper, 2000). This implies that the pituitary is functional and the deficit responsible for LH surge attenuation is a hypothalamic insufficiency of GnRH release. *In vitro* studies provide additional support that effects on

the LH and prolactin surges are not due to a direct pituitary response to atrazine exposure (Cooper, 2000). No differences in either LH or prolactin release were found from the pituitaries of untreated females exposed to atrazine *in vitro*. These three lines of evidence indicate that the effect of atrazine on the LH surge (and the high-dose effect on the prolactin surge) involves a disruption of the GnRH pulse from the hypothalamus, rather than a direct effect on the pituitary.

There is some evidence that atrazine may enhance estrogenic activity by stimulating aromatase activity. Aromatase is an enzyme that converts androgens to estrogens. Treatment of human adrenocortical cells *in vitro* with atrazine has been shown to stimulate aromatase activity (Sanderson *et al.*, 2000). Crain *et al.* (1997) have also shown that atrazine treatment of male hatchling alligators leads to an increase in aromatase activity. Although an increase in aromatase activity would be consistent with dose-response increases in estradiol and estrone and decreases in testicular testosterone noted in a study that examined the effects of atrazine on pubertal development, the doses that resulted in effects on these hormones were well above doses that led to reproductive developmental effects (Stoker *et al.*, submitted; 2000a). It is plausible that enhanced aromatase activity may have some influence on the development of mammary tumors in SD female rats but whether or not enhanced aromatase activity is a significant contribution to the carcinogenicity, or other effects, of atrazine remains to be determined.

No other modes of action, apart from disruption of the hypothalamus-pituitary-ovarian axis, are plausible biochemical processes that could account for the early onset and increased incidence of mammary and pituitary gland tumors in female SD rats.

2.4.6 Uncertainties and Limitations

Despite numerous studies directed at understanding the mode of action for the carcinogenicity of atrazine, several uncertainties remain. In themselves they do not discount the postulated mode of action. Although the available data show that attenuation of the LH surge and disruption of the estrous cycle occur before mammary tissue and pituitary gland tumor formation, precise dose and time correlations are not available for each of the key events due to differences in study design and dose selection. Serum LH values are highly variable within dose groups, which makes it very difficult to determine accurately biologically relevant doses that are associated with effects.

There is some concern that the lack of direct effects on the pituitary was established using ectopic pituitaries and using prolactin secretion as a marker of LH secretion. There also is a lack of robust data on blood prolactin measures and serum estradiol measurements. Because prolactin measurements are not available from chronic studies, confirmation of the role of the hormone in the formation of the histomorphologic changes in mammary tissue is not possible. Histomorphologic markers are, however, generally viewed as valid indicators of prolactin secretion.

Stop-dose studies to demonstrate that induced toxicological processes leading to cancer are reversible are limited but this deficiency is offset, once again, by the lack of effects in ovariectomized female SD rats.

Finally, the initial interaction between atrazine and the rat brain has not been established, albeit effects on hypothalamic catecholamine neurotransmitter levels have been shown.

2.4.7 Preliminary Conclusions on the Postulated Mode of Carcinogenic Action

Support for atrazine's mode of action comes from several lines of evidence.

- Atrazine's induced LH and cyclicity effects have been shown in two different laboratories and in two different strains of rats (LE and SD);
- A strong correlation has been shown for atrazine induced persistent estrus and induction of mammary tumors;
- Generally, there is a strong temporal and dose-response correlation between tumor formation and precursor effects; precise correlations are not possible due to differences in study designs and dose selection;
- Although robust data on estrogen and prolactin levels are not available, ovariectomized SD rats treated with atrazine do not develop tumors, thus demonstrating the role of ovarian estrogen in atrazine's mode of action;
- A strong correlation was demonstrated between increased pituitary weights and histomorphological markers of prolactin exposure in the mammary gland, thus supporting the role of prolonged estrogen and prolactin exposure in tumor development; and
- Despite the lack of precise effective dose levels (LOAELs), data from multiple hormonal and carcinogenicity studies show that no effects of atrazine treatment are observed at a dose level between 0.5 and 1.8 mg/kg/day.

The absence of data on the detailed steps in the hypothalamus, would provide insights regarding the mechanism of action of atrazine. However, as stated in the proposed Guidelines for Carcinogen Risk Assessment (USEPA 1999), mode of action is contrasted with mechanism of action which implies a more detailed molecular description of events than does mode of action. The focus of a mode of action analysis is on a sequence of key events which lead to cancer formation and whether data are sufficient to establish a cause and effect relationship between key events and cancer. This guidance was followed in reaching the conclusion stated below.

Given the overall strengths, consistency, and specificity of the evidence, it is concluded that it is biologically plausible that treatment of female SD rats with atrazine leads to an increased incidence and/or decreased latency in the formation of mammary adenomas, carcinomas, fibroadenomas, and pituitary adenomas through a mode of action involving disruption of the hypothalamic-pituitary-ovarian axis. Disruption of the axis leads to suppression of LH surges, prolonged days spent in estrus, and exposure of mammary tissue and the pituitary gland to estrogen for a extended period. Exposure of the pituitary gland to estrogen stimulates the secretion of prolactin. Exposure of the mammary tissue to estrogen and prolactin and the pituitary gland to estrogen creates an endogenous endocrine milieu conducive to cell proliferation and tumor formation. The available data do not support a role for direct mutagenic or direct estrogenic activity for effects attributed to atrazine treatment.

2.5 Reproductive and Developmental Toxicity

The natural progression from prepubertal to postpubertal status is dependent upon the normal function of the hypothalamic-pituitary-gonadal axis. Likewise, many of the same hypothalamic mechanisms controlling pituitary function and the pituitary hormones themselves (especially LH and prolactin) play a key role in pubertal development. For example, it has been shown that an increased turnover rate in hypothalamic GnRH, NE and DA precedes the gonadal development (Matsumoto *et al.*, 1986; Ojeda, 1986).

At the time of puberty (*e.g.*, vaginal opening and first ovulation) the CNS and pituitary respond to increased concentrations of estradiol in a positive feedback fashion culminating in the first LH surge (Ojeda and Urbanski, 1994).

These processes are not specific to the rat. Inhibition of GnRH release in neonatal rhesus monkeys suppresses gonadatrophin secretion and testosterone production; this effect is associated at the time of puberty with compromised testicular growth and testosterone secretion (Plant, 1994). This same author postulated that there is a coupling between a rise in circulating LH and FSH concentrations and the transition into puberty that is a general characteristic of sexual maturation in higher primates. Thus, given that atrazine treatment of rats suppresses GnRH, LH, and prolactin release, there is a concern for potential adverse reproductive and developmental effects of atrazine in maternal animals and their offspring.

Adverse reproductive or developmental consequences have been identified following treatment of different strains of pregnant rats or neonates with atrazine or its metabolites. As noted in Chapter 1, Section 1.7, this evidence does not come from results of EPA guideline studies but from results of special studies conducted with atrazine or its metabolites. The results of these studies show that atrazine or its metabolites produce effects in pregnant, neonatal, or young adult SD, F344, Wistar, Holtzman, or LE rats that may be associated with disruption of the hypothalamic-pituitary axis. The developmental/reproductive effects observed in these studies include reductions in implantation sites, failure to maintain pregnancy, attenuation of suckling-induced prolactin release and the development of prostatitis, delayed vaginal opening, and delayed preputial separation. Table 2-2 provides a listing of the lowest NOAELs and LOAELs reported for these effects. NOAELs and LOAELS for effects on prolactin and LH

release following acute or short-term repeat dosing treatment of rats with atrazine are also provided in order to allow comparisons of developmental/reproductive NOAELs/LOAELs with the NOAEL/LOAELs that result in disruption of neuroendocrine parameters.

Treatment of young Wistar rats with atrazine during PND 22-41 delays vaginal opening three or four days and produces irregular cycles (Laws *et al.*, submitted; 2000). Treatment of weanling male Wistar rats with atrazine during PND 23-53 leads to delays in preputial separation (Stoker *et al.*, submitted; 2000a). No consistent effects on serum progesterone or LH were observed in this study but variability in hormonal levels in animals of the age studied makes comparisons with control animals difficult.

In addition to affecting the onset of puberty, the offspring of dams exposed to atrazine have also been found to be affected adversely. Administration of atrazine to dams during PND 1-4 inhibits suckling-induced prolactin release in the dams and leads to lateral prostate inflammation in the offspring. The effect on the prostate is reversible if the offspring are treated with ovine prolactin, which provides evidence that prolactin has a role in the development of prostatitis. Also, the incidence and severity of lateral prostate inflammation correlates with decreases in serum levels of prolactin. The effects on the prostate of offspring of dams treated with atrazine and the delays in pubertal developmental observed when young rats are treated with atrazine are associated with the endocrine imbalances that have been identified as critical events in the neuroendocrine mode of action attributed to the carcinogenic activity of atrazine.

As stated earlier, atrazine may also affect pregnancy maintenance in the rat. The full-litter resorptions reported following atrazine exposure on GD 6-10 (roughly coinciding with the LH-dependent period of pregnancy) are consistent with a neuroendocrine mode of action (Narotsky *et al.*, submitted). Although this effect was observed at maternally toxic doses (as defined by a decrease in body weight), treatment after the LH-dependent period caused a similar degree of maternal toxicity, but had no effect on pregnancy maintenance. Hormone measurements on GD 9 (following treatment on GD 1-8) did not show a consistent pattern across strains for prolactin, estradiol, or progesterone. However, for the Holtzman strain, the only strain of four tested to show full-litter resorptions following treatment on GD 1-8, there were reductions in serum

progesterone and LH; although not proof, these data are consistent with an LH-mediated mechanism of pregnancy loss. In contrast to treatment on GD 6-10, exposure on GD 1-8 did not cause full-litter resorption in F344 or SD rats. An explanation for the lack of effect following exposure on GD 1-8, remains unclear, but may be related to the truncated dosing regimen within the LH-dependent period, or examination of the litter on GD 9, possibly prior to the actual time of pregnancy loss.

Because pubertal development is under neuroendocrine control, it may be expected that administration of atrazine to young rats leads to delays in vaginal opening or preputial separation. The dose levels that led to delays in vaginal opening also produced irregular ovarian cycles in offspring, which supports a role for disruption of neuroendocrine control in young animals treated with atrazine or its metabolites. The reductions in implantation sites and the full-litter absorptions reported following treatment of dams with atrazine during the LH-dependent phase of pregnancy are also consistent with an effect on neuroendocrine control but other modes of action can not be discounted (*e.g.*, general toxicity at high-dose levels).

There are uncertainties, in particular, regarding the dose-response data on preputial separation (PPS). A statistically-significant effect was reported at a dose-level of 13 mg/kg/day (PPS ~42 days in controls and PPS ~ 44 days at 13 mg/kg/day, the lowest dose tested). It should be noted that this dose has a significance of $p \leq 0.05$. The next higher dose of 25 mg/kg/day approached statistical significance but did not achieve significance (*i.e.*, $p = 0.07$). Statistical-significance ($p \leq 0.05$) was achieved at the next three dose levels (50, 100, or 150 mg/kg/day). At 200 mg/kg/day there was a statistically-significant effect of delayed preputial separation (~42 days in controls and ~45 days in the high-dose rats). There was a significant dose-related decrease in LH; however, no statistically-significant effects were observed for testosterone or prolactin concentrations. The variability in levels of these hormones in young rats should be considered before much weight is placed on these data.

In summary, reproductive and developmental effects in various strains of rats that are associated with atrazine treatment include preimplantation and postimplantation losses, prostatitis in adult male offspring of treated lactating females, delays in vaginal opening and preputial separation, and disruption of the estrous cycle in young females. A reduction in prolactin release in nursing dams is strongly associated with the development of prostatitis in male adult offspring. Decreases in serum LH or prolactin were not observed to occur at dose-levels that led to delays in vaginal opening (50 mg/kg/day) and preputial separation (13 mg/kg/day) in the same study but it is presumed that the variability in levels of these hormones in juvenile animals preclude obtaining definitive data. On the other hand, a separate study using dams showed that a daily dose of ~13 mg/kg/day was sufficient to depress serum levels of prolactin in the lactating dam. To the extent that decreased prolactin levels can serve as a marker for effects on neuroendocrine control, there is a linkage between pubertal development and an effect on the hypothalamic-pituitary axis.

Table 2-2. Lowest NOAELs/ LOAELs (mg/kg/day) for Reproductive and Developmental Effects Following Short-term (1-30 Days) Treatment of Rats During Various Stages of the Reproductive Cycle with Atrazine or its Metabolites

Response	Exposure Period	Rat Strain	NOAEL/LOAEL	Reference
Preimplantation loss-nocturnal dosing only	GD 1-8	F344	50/100	Cummings *et al.*, submitted
Postimplantation loss-diurnal and nocturnal dosing	GD 6-10	Holtzman	50/100	Cummings *et al.*, submitted
Dams prolactin release decreased	PND 1-4	Wistar	13/25	Stoker *et al.*, 1999
Increased incidence of prostatitis in offspring	PND 1-4	Wistar	13/25	Stoker *et al.*, 1999
Increased incidence and severity of prostatitis in offspring	PND 1-4	Wistar	25/50	Stoker *et al.*, 1999
Delayed vaginal opening	PND 22-41	Wistar	25/50	Laws *et al.*, submitted; 2000
Delayed preputial separation	PND 23-53	Wistar	<13/13	Stoker *et al.*, submitted; 2000a
Attenuation of LH surge	Adult females - single dose 3 daily doses 21 daily doses 21 daily doses 30 daily doses Dams- GD 1-8	 LE LE LE SD SD LE & Holtzman	 200/300 <50/50 <75/75 <75/75 5/40 50/100	Cooper *et al.*, 2000; Morseth, 1996a; Cummings *et al*, submitted
Attenuation of prolactin release	Adult females - single dose 3 daily doses 21 daily doses 21 daily doses	 LE LE LE SD	 200/300 serum <50/50 pituitary <75/75 pituitary <75/75 pituitary	Cooper *et al.*, 2000
Disruption of estrous cycle	PND 22-41	Wistar	25/50	Laws *et al*, submitted; 2000

Chapter 3

3. Science Policy Considerations: Human Relevance, Children's Health Concerns, and Dose-Response Analysis

This Chapter evaluates and characterizes the human relevance of the rat toxicological findings of atrazine and postulated mode of action. This analysis focuses on the question of whether the mode of action found to be operative in rats is also operative in humans and whether any human subpoulations or life stage are apt to qualitatively respond to the mode of action differently than the general population. The key questions and rationales are presented in addressing the issue of human relevance. Also, based on the mode of action understanding, a dose-response extrapolation approach is proposed for atrazine.

3.1 Human Relevance

3.1.1 Potential Neuroendocrine Disruption

As discussed in Chapter 2, there are data supporting an understanding of how atrazine induces tumor development in the rat. Briefly, the mode of carcinogenic action underlying mammary and pituitary gland tumor formation in female SD rats involves a lack of adequate secretion of pituitary LH to stimulate ovulation, the development of persistent estrus, and prolonged stimulation of the mammary and pituitary glands by estrogen and prolactin. These hormones promote cell proliferation and predispose cells to become neoplastic. Other endocrinopathies found in the rat (*e.g.*, delayed puberty, prostatitis) are also associated with the neuroendocrine effects of atrazine on pituitary function (*i.e.*, secretion of LH and/or prolactin).

There is clear evidence (discussed in Chapter 2) that atrazine alters hypothalamic GnRH release in rats. There are some data that show that atrazine diminishes NE in the rat hypothalamus as a initial or early site of action which in turn leads to diminished GnRH release. Atrazine also increases dopamine levels which can result in a diminished pituitary prolactin secretion. Therefore, a key question to address is whether this neuroendocrine mode of action at the level of the hypothalamus may be operative in humans. In both humans and rats, hypothalamic GnRH controls pituitary hormone secretion (*e.g.*, LH, prolactin). The hypothalamic-pituitary axis is involved in the development of the reproductive system, and its maintenance and functioning in adulthood. Additionally, reproductive hormones modulate the function of numerous other metabolic processes (*i.e.*, bone formation, and immune, CNS and cardiovascular functions) (Cooper *et al.*, 1986, Plant, 1994). Given that the primary site of atrazine's effect on GnRH secretion in the rat is at the level of the hypothalamus, it is important to address the questions below:

Question. Is there evidence in primates including humans of central neural modulation of GnRH secretion by the hypothalamus? Is this central mechanism conserved across species?

Although GnRH secretion is influenced by a number of factors in primates and humans (such as circulating steroids), and the precise control mechanisms remain to be fully understood, the prevailing view is a central neural control system is involved in governing GnRH release (as reviewed by Marshall and Eagleson, 1999; Plant, 1994). For example, there have been studies in both rats and primates showing that CNS-altering drugs (*e.g.*, opiates) can alter the menstrual cycle or pubertal development (see review by Plant, 1994; Ojeda, 1986). Further, there is evidence that endogenous opioids are involved in GnRH/LH secretion in primates (Ferin and Van de Wiele, 1984), indicating that GnRH neurons are modulated by other hypothalamic neural inputs like in the rat. Therefore, if atrazine affected the hypothalamic GnRH in humans like in the rat, it is plausible to assume that this neuroendocrine mode of action would apply to humans.

3.1.2 Potential Human Health Consequences Associated with Altered GnRH/Pituitary Function

Given that the rat neuroendocrine mode of action may be operative in humans, it is important to address:

Question: What neuroendocrinopathies may result in humans if exposed to atrazine?

Atrazine interferes with the CNS control of pituitary-ovarian function and leads to irregular cycles and inhibition of ovulation in SD and LE rats. In humans and primates reproductive function/ovarian cycling is also influenced by the hypothalamic GnRH (Goldfien and Monroe, 1997; Plant 1994; Nishihara *et al.*, 1992; Terasawa and Nyberg, 1997). Therefore, a potential consequence in humans is disrupted or irregular menstrual cycles which can lead to gynecological problems such as diminished fertility, prolonged menses or excessive bleeding.

It is important to evaluate what is understood about the role of altered GnRH secretion in human ovulatory disorders. Also, a further evaluation of human anovulatory conditions may give some clues as to potential downstream endocrine effects and other health consequences. Hypothalamic amenorrhea (HA) is one model of disrupted cyclicity. HA is a manifestation of a variety of disorders associated with emotional stress, heavy exercise, self-imposed weight loss and oral contraceptive use and occurs in the absence of pathology in the pituitary and ovaries (Reame *et al.*, 1985). HA has been found to represent a spectrum of disordered GnRH secretion (presumably low frequency and variable or low amplitude pulses) that can vary over time (Perkins *et al.*, 1999). Clinically, persons fail to ovulate, as in atrazine treated SD rats. HA is characterized by normal to moderately low serum estrogen and normal to low serum LH. When serum LH is lowered, the cause appears to be a reduction in hypothalamic GnRH secretion (Perkins *et al.*, 1999). These manifestations of HA are similar to those seen with atrazine treated SD and LE rats: decreased hypothalamic GnRH, decreased pituitary LH, and failure to ovulate. These observations suggest that certain of the manifestations may be the same in humans and rats if atrazine affects the

hypothalamic neurons in similar ways. In addition to the gynecological problems associated with disrupted ovarian cycling, HA patients can suffer other health consequences. For example, they can be at an increase risk of osteoporosis later in life given that these women are estrogen deficient, and thus can experience significant losses in bone density. Women who are hypoestrogenic may also suffer from vasomotor symptoms, urogenital atrophy, cardiovascular disease, and possibly diminished cognitive and memory functions (Wren, 1997).

Polycystic ovary syndrome (PCOS) is another model of anovulation, which occurs in 6% to 8% of premenopausal women (Marshall and Eagleson, 1999). PCOS is often characterized by irregular menstrual cycles or amenorrhea, infertility, obesity, and ovaries that are polycystic with many unovulated follicles in various stages of development and atresia. Hirsutism is associated with PCOS (Schildkraut *et al.*, 1996; Hershlag and Peterson, 1996). Some of the other manifestations of PCOS are very different from that seen in atrazine treated rats. There commonly is an increase in LH secretion from the pituitary and increased synthesis of androgens (hyperandrogenism) and their conversion to estrogens. This can result in unopposed exposure to estrogen. The mechanism underlying the excess ovarian androgen secretion is unknown but may be multifactorial, and include abnormalities of steroidogenesis, effects of hyperinsulinemia, and abnormal gonadotropin secretion in stimulating ovarian steroidogenesis (Ehrmann *et al.*, 1995; Utiger, 1996; Marshall and Eagleson, 1999). PCOS is not an exact model for evaluating the consequences of atrazine exposure in humans, other than in some cases it is associated with abnormal GnRH secretion (with presumably high frequency-low amplitude pulses), anovulation, and unopposed exposure to estrogen.

As discussed in Chapter 2, atrazine has also been shown to increase the hypothalamic neurotransmitter, dopamine, which in turn results in a decrease of pituitary prolactin secretion in female rats. In both rats and humans, prolactin is one of the hormones involved in lactogenesis. It is the suckling action of the neonate that stimulates prolactin secretion, and thus the maintenance of milk production. Therefore, in humans, diminished production and secretion of milk could result if atrazine were to affect hypothalamic dopamine and suppress prolactin as in the rat. Given that the initial sucking induced prolactin response is relatively robust, atrazine exposure would not be anticipated to impact the initiation of lactation, but could potentially impact the ability to sustain milk production with continuous exposure.

Therefore, there is support from the primate literature that atrazine's neuroendocrine mode of action (CNS perturbation of GnRH secretion) may apply to humans. Human ovulatory disorders can be associated with aberrant hypothalamic GnRH pulses. These conditions indicate that altered hypothalamic GnRH secretion can broadly affect an individual's functional status, and thus lead to a variety of clinically important health consequences. These human conditions, HA and PCOS, do not prove but raise the possibility that if atrazine produced effects on hypothalamic GnRH in the human, like that seen in atrazine-treated rats, adverse health effects may ensue. The potential ability of atrazine to affect dopamine and prolactin in humans must also be considered. Below, the potential human cancer risk associated with this neuroendocrine mode of action is discussed.

3.1.3 Potential Cancer Risk Associated with Altered GnRH/Pituitary Function

It is standard Agency practice to assume that chemically induced tumors in animals have human relevance unless there are data to the contrary (US EPA 1999a). Target organ concordance is not necessarily a prerequisite for evaluation of the implications or relevance of animal tumor findings for humans. Even if there is a mode of carcinogenic action understanding for the rodent tumor findings, site concordance may or may not be expected. In the case of atrazine, there is an increased incidence and early onset of mammary gland tumors in female SD rats. As discussed below, it does not seem plausible that humans would be at an increase risk for breast cancer given that atrazine would potentially reduce the cumulative number of normal ovarian cycles (*i.e.*, one of the risk factors for humans). In fact, the neuroendocrine mode of action for atrazine raises the possibility of tumor development at other hormone-responsive site.

In assessing potential human risk, human data are generally preferable over animal data when of good quality, and should be given greater weight in the hazard characterization of an agent. Therefore, an obvious question to address is:

> **Question:** Are there data in humans to determine the human cancer potential and neuroendocrine mode of action for atrazine?

As summarized in Chapter 1 (and discussed in detail in Part B-Chapter 4), there is suggestive evidence of a possible association of triazine exposure and cancer occurrence for three hormone-responsive cancers--ovary, breast and prostate cancer. However, these associations should not be considered as conclusive evidence of an association of triazine exposure with these tumor types. There are no human or primate studies that directly examine the potential for atrazine to induce endocrine effects as have been described in the SD or LE rat special studies.

One important aspect of atrazine's postulated mode of carcinogenic action involves components in common with the reproductive aging process in SD female rats. It is well recognized that in SD female rats, as well as in other strains of rats such as LE and Wistar, reproductive aging is due to failure of hypothalamic-pituitary-gonadal function resulting in the normally aging female spending an increased percentage of days of their ovarian cycle in estrus *(i.e.*, constant estrus) (discussed in detail in Part B-Chapter 9.1). Therefore, an aging female SD rat experiences a dampening of the preovulatory pituitary LH surge which results in prolonged exposure to estrogen. In contrast, the prevailing view for humans is that reproductive aging results from a depletion of follicles from the ovary (*i.e.*, atresia). However, the potential that an age-associated loss of the hypothalamic control of GnRH secretion may contribute to significant changes in menstrual function during the perimenopausal period in women can not be discounted (*e.g.*, Wise *et al.*, 1996; 1999).

Nevertheless, to the extent that the carcinogenic effects of atrazine in SD rats are intimately tied to an interaction between effects of the chemical and the normal aging process in rats, then there may be questions as to the applicability of the carcinogenic effects to humans.

Question: Can atrazine lead to cancer through a process not involving reproductive aging; and can the neuroendocrine effects of atrazine alone set up a milieu favorable to the development of cancer in humans?

In addressing the above questions, it is important to note that of the key events identified in Figure 2-1 based on laboratory *in vitro* and *in vivo* data, atrazine's initial site of action appears to be at the level of the hypothalamus (*i.e.*, effects on hypothalamic catecholamine and GnRH levels). As discussed above, CNS control of hypothalamic GnRH is similar in primates and humans, and human conditions of anovulation, which can be associated with aberrant GnRH release, lead to a variety of health consequences. It is important to look at the cancer risk associated with the human ovulatory conditions discussed above.

HA has not been found to be associated with a cancer risk based on epidemiologic studies, although it is clearly associated with other health consequences as discussed above. Because of the prevalence of PCOS in the population, several epidemiologic studies have assessed its role in breast cancer. One showed a significantly increased risk, but only in the postmenopausal period (Coulam *et al.*, 1983); the remaining three failed to show breast cancer increases (Gammon and Thompson, 1990; Anderson *et al.*, 1997; Pierpoint *et al.*, 1998). Such findings have been interpreted as lending little support for PCOS being a risk factor for breast cancer (Solomon, 1999). A small number of patients, however, have enough estrogen to maintain the endometrium, which has the potential to become hyperplastic over time (Mansfield and Emans, 1989; Schachter and Shoham, 1994); endometrial hyperplasia is a risk factor for endometrial cancer (Rose, 1996). Case reports suggest that PCOS may predispose women to endometrial cancer at an early age, in contrast to this cancer's usual occurrence with advancing age (Jafari *et al.*, 1978; Dahlgren *et al.*, 1991). A statistically-significant increase in relative risk for endometrial cancer was noted among documented PCOS patients (Coulam *et al.*, 1983). Information linking PCOS to ovarian cancer is less well developed. One study of epithelial ovarian cancer showed a statistically-significant increase of persons with PCOS (Schildkraut *et al.*, 1996), while another did not (Coulam *et al.*, 1983).

The human conditions of anovulation only supply inferential information concerning potential cancer risks. These human disease models do not prove a potential cancer risk associated with atrazine's neuroendocrine mode of action. But on the other hand, they do not allow one to discount the possibility that if atrazine produced effects on hypothalamic GnRH like is seen in atrazine treated SD rats, disrupted cyclicity may result in an endocrine environment that may be conducive to tumor development at hormone-responsive sites. Mammary gland site concordance with SD rats should not be expected (as discussed further below), but the mode of action responsible for the rat tumors and information on PCOS raise the possibility of other endocrine sites (*i.e.*, endometrial and ovarian). Also, conditions of human anovulation disorders suggest that atrazine exposure alone may produce an endocrine imbalance that may be conducive to tumor development. Given that hypothalamic GnRH control of the preovulatory pituitary LH surge is similar in rats and primates, it seems possible that this process could be independent of the reproductive aging pattern as seen in rats.

3.1.4 Breast Cancer

Given that atrazine induced mammary gland tumors in SD rats, it is important to evaluate what is understood about endocrine influences for breast cancer.

- Estrogen seems to be an important influence in breast cancer development, as indirect indicators of estrogen stimulation are known risk factors for the disease: early age of menarche, late onset of menopause and nulliparity. However, it seems that the cumulative number of regular and not irregular ovarian cycles is the important input into breast cancer development (Henderson *et al.*, 1988; den Tonkelaar and de Waard, 1996). Consistent with this, regular exercise is associated with reduction in breast cancer risk, possibly by reducing the number of normal ovulatory cycles as is seen in hypothalamic amenorrhea (Bernstein *et al.*, 1994).

- Prolactin plays a role in mammary gland carcinogenesis in rodents, but its importance in human breast cancer development is not at all established. Prolactin together with estrogen, stimulates the human breast tissue during lactation. Unlike rats where there are significant changes in prolactin levels throughout the ovarian cycle, there is little modification during the human menstrual cycle (Goldfien and Monroe, 1997). Rats and humans do show circadian variations in prolactin. Two prospective studies among postmenopausal women have found increases in breast cancer with elevated prolactin levels, although only one was statistically-significant; retrospective studies of premenopausal women have been variable in their outcomes (Wang *et al.*, 1992; Hankinson *et al.*, 1999).

Many different pharmaceuticals induce hyperprolactinemia, including the raulwolfia drugs used to treat hypertension, tricyclic antidepressants, antipsychotic phenothiazines and methyldopa, the drug used to treat Parkinson's disease. A number of epidemiologic studies have been conducted with the raulwolfia derivatives. Some have noted no increase in breast cancer risk while others have indicated rather limited increases in postmenopausal women (Shapiro *et al.*, 1984; Williams *et al.*, 1978). It has been argued that agents which produce about a 50% increase in prolactin levels may account for the small increase in cancer risk in some of the studies (Ross *et al.*, 1984). One investigation showed that with dosing for at least 10 years or with initiation of dosing at least 10 years prior to diagnosis, significant risk ratios of about four were found (Stanford *et al.*, 1986). Antidepressants also lead to increases in prolactin levels. The relationship between their use and breast cancer have led to differing outcomes (CoHerchio *et al.*, 2000; Kelly *et al.*, 1998; Wallace *et al.*, 1982). There has been less investigation of other psychiatric drugs that produce hyperprolactinemia and its association with increases in breast cancer risk. An investigation of all 9156 schizophrenic patients in Denmark that had there first hospital admission between 1970-1987 showed no indication of increase in breast cancer risk (Mortensen, 1994), in keeping with other studies. More work is needed to probe these relationships.

Interestingly, mammary gland and breast cancers have receptors for prolactin, and studies show that prolactin mRNA and the hormone itself are synthesized by tumor cells (Clevenger *et al.*, 1995; Mershon *et al.*, 1995). It has been hypothesized that the local formation of prolactin may serve autocrine or paracrine functions within the mammary gland (Ben-Jonathan *et al.*, 1996; Vondehaar, 1999). These observations reopen the question of the role of prolactin in human breast cancer development. As of yet, the regulation and effects of locally synthesized prolactin on the breast have not been determined.

3.1.5 Cancer Classification

In the past, OPP had classified atrazine as a Group C, possible human carcinogen based on an increased incidence of combined mammary carcinomas/adenomas and fibroadenomas in female SD rats, in accordance with the 1986 cancer risk assessment guidelines. Recently, the OPP Cancer Assessment Review Committee (CARC) proposed that atrazine should be classified as a **likely human carcinogen** in accordance with the draft 1999 revisions to the cancer risk assessment guidelines (*i.e.*, US EPA, 1999a). The basis of this current proposal is as follows:

- Consistent findings in female SD rats of an increased incidence and early onset of mammary gland carcinomas/adenomas in several studies, and suggestive evidence of an early onset of pituitary adenomas and mammary fibroadenomas;

- Mode of action evidence that indicates hypothalamic disruption of GnRH control of pituitary function by atrazine, and critical reductions in LH and resultant anovulation; and

- Similarity in humans and rats for CNS control of pituitary function.

Therefore, if atrazine affected hypothalamic GnRH as in the rat, this opens the possibility that an endocrine imbalance may result which could lead to several different health consequences including cancer at hormone responsive tissues.

3.2 Potential Health Effects of Atrazine in Children

3.2.1 Reproductive/Developmental Hazard

The data summarized in Chapter 2 indicates that the primary underlying process that leads to mammary and atrazine involves disruption of the hypothalamic pituitary-gonadal-axis. pituitary gland tumor development in female SD rats following treatment with This axis is also involved in reproductive development. Therefore, as summarized in Chapter 1.7, it is not surprising that atrazine treatment also results in adverse reproductive and developmental outcomes in special studies using several different strains of rats *(i.e.,* F344, SD, Wistar, LE, Holtzman). These outcomes include interruption of regular ovarian cycling, decreased suckling induced prolactin release and increased incidence and severity of prostatitis, and delays in vaginal opening and preputial separation.

Rat and human reproductive development and puberty are under similar hypothalamic-pituitary control, especially LH and prolactin (Matsumoto *et al.*, 1986, Ojeda, 1986). After the first trimester in humans, fetal LH and FSH are used to complete genital maturation (Hsing, 1997). There is an appreciable release of LH commencing at parturition that extends until four to six months of postnatal life. Thereafter, LH is suppressed until puberty begins. There is a re-awakening of the hypothalamic-pituitary-gonadal axis at puberty. The exact mechanism underlying this pubertal LH release is unknown. For male sexual development, LH is required to stimulate the Leydig cells for testosterone production, and androgens are responsible for the outward signs of pubertal development. LH and FSH are required to begin ovarian activation, follicle growth, and steroid production in female sexual development. Estrogen secreted from the ovary triggers breast growth and other body changes. Some adolescent patients with delayed puberty display low levels of LH and/or FSH (Styne 1997; Kulin 1996). Therefore, there is concern that if children were exposed to atrazine and if it affected the hypothalamic-pituitary-gonadal axis and the pituitary LH and PRL releases as in rats, there is the potential for delayed puberty or altered pubertal growth in both female and male adolescents. Delayed puberty is

not without health consequences. For example, girls with delayed menarche show a higher incidence of scoliosis, stress fractures, and osteopenia than do girls with normal time of menarche (Goldfien and Monroe, 1997). Additionally, abnormal puberty may result in problems manifested later in life (*e.g.,* osteoporosis (Styne, 1997).

Exposure to atrazine in lactating dams (Wistar rats) suppresses suckling-induced prolactin release which eventually results in hyperprolactinemia and prostatitis in the lateral prostate in young adult offspring. It is reasonable to assume that this suppression of pituitary prolactin secretion in the dam is due to atrazine's effect on hypothalamic catecholamine levels (*i.e.,* dopamine). Prolactin does play a role in the development and maintenance of the human prostate. Critical periods for developmental exposures and the hormonal involvement in the induction of prostatitis remain unknown in humans. In humans, nonbacterial prostatitis of undefined etiology is an important clinical problem that has been associated with infertility (Meares, 1998; Huaijin *et al.*, 1998). There is a suggestion in the literature that chronic proliferative inflammation in the prostate may be a precursor event to prostatic carcinogenesis (De Marzo *et al.,* 2000; Leav *et al.,* 1999). It should be acknowledged that the relevance of effects in the rat prostate as a human model has been debated. However, Because the dorsal and lateral prostate of the rat are considered to be the most homologous to the human prostate (Price, 1963), the increase in inflammation observed in young male rat offspring should not be discounted.

In summary, because of the similarity between rats and humans of the influence of hypothalamic GnRH on the growth and morphogenesis of the reproductive system, the concern is raised about the potential health effects due to early life exposure to atrazine, some of which may not be manifested until later in life.

3.2.2 Cancer Hazard

As stated in the July 1999 Draft revisions to the EPA's cancer risk assessment guidelines, when information is developed to show a mode of carcinogenic action that is expected to be relevant to adults, an evaluation needs to be made as to whether this mode of action is relevant to children. When there is no cancer information on children *per se*, a "cogent biological rationale needs to be developed regarding whether the mode of action is applicable to children." In the case of atrazine, although there are no animal data directly evaluating its neoplastic potential from pre- and postnatal exposures *per se*, there is information indicating that atrazine can affect the hypothalamic-pituitary axis and cyclicity in young animals. So reliance is placed upon both data concerning the neuroendocrine effects in young animals as well as using biological arguments to evaluate children's cancer concern.

If atrazine were to produce neuroendocrine effects in humans like it does in SD rats, projections can be made as to potential consequences in children, using what is understood about the key events described for its postulated mode of action. Components of the neuroendocrine system develop during fetal life, with varying manifestations at different times. As discussed above, the preovulatory LH surge controlling ovulation does not happen until puberty. Considering the purported mode of atrazine action involving attenuation of the preovulatory LH surge and disruption of ovarian cycling as a critical event, it is reasonable to assume that this mode of action may also be operative in children from puberty onward. Furthermore, the rodent cancer bioassays on atrazine as well as the accompanying LH/cyclicity mode of action studies used young pubertal rats (six to eight weeks of age). Thus, there is a potential cancer concern for children as a result of exposure during puberty and continued over a lifetime. The rat studies on decreased suckling induced prolactin release and increased incidence and severity of prostatitis in male offspring, however, raise the question of whether prepubertal exposure may lead to a potential prostate cancer risk later in adult life. At this time there is no indication of such an outcome, however, conventional cancer testing may not screen for such potential. Further study would be needed to determine whether there is or is not any hazard capability.

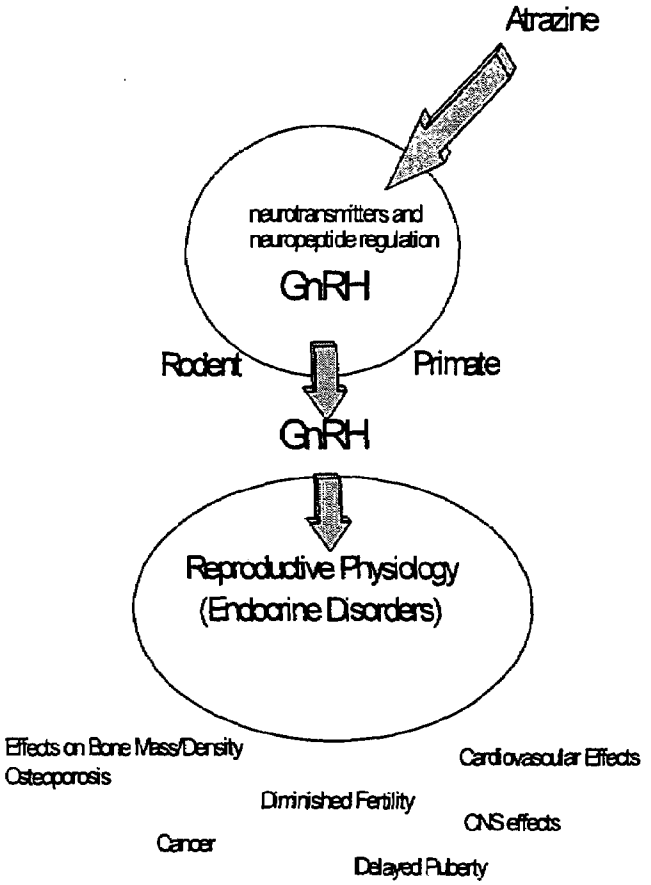
3.2.3 Summary of Children's Health Concern

Rat studies using atrazine treatment *in utero* or during early life demonstrate a wide spectrum of endocrinopathies (*e.g.*, delayed puberty, disrupted cycling, prostatitis, reproductive organ weight changes, hyperprolactemia) associated with the disruption of the neuroendocrine control of pituitary function. There are numerous studies in the literature indicating that altered neuroendocrine status in children lead to a variety of health outcomes. Furthermore, as discussed previously, CNS-GnRH control of reproductive development is similar in primates and rats. Thus, the rat studies on atrazine raise concern for the susceptibility of the fetus and young child if exposed to atrazine. The consequence in children due to this neuroendocrine mode of action would depend on the developmental stage of exposure and the duration of exposure. For example, prepubertal exposures would most likely result in developmental effects, and postpubertal exposure may result in a variety of health consequences including cancer. There is no direct information on cancer responses following pre- or postnatal exposure.

3.3 Summary of Atrazine Human Hazard Potential

As shown in Figure 3-1, atrazine operates via a neuroendocrine mode of action that alters hypothalamic GnRH and pituitary LH and PRL secretions. It is recognized that across species and even among different strains of a species endocrinological interactions can differ significantly (Neumann *et al.*, 1996). However, atrazine's central neuroendocrine mode of action is likely to be operative in humans given that in both rats and primates a central neural control influences GnRH and pituitary function. The variety of endocrinopathies found in the atrazine treated rats (*e.g.*, mammary and pituitary gland tumors, delayed puberty, disrupted cyclicity, prostatitis in young rats) raise concern about the potential human health consequences that may ensue from this neuroendocrine perturbation, including adverse reproductive and developmental outcomes or delayed acquisition of normal reproductive potentialities. This neuroendocrine mechanism also raises concern for potential cancer risk in humans.

Figure 3-1. Atrazine's Neuroendocrine Mode of



3.4 Dose-Response Analysis

In 1988, the U.S. EPA presented a dose-response assessment of atrazine (Hauswirth 1988a; US EPA 1988). That assessment used the female SD mammary tumor incidence from the study by Mayhew *et al.* (1986) and the linearized multistage (LMS) model to estimate an oral slope factor and a unit risk of 2.22×10^{-1} $[mg/kg/day]^{-1}$. The current dose-response analysis considers the mode of action data as discussed in Chapter 2. Additionally, the two-step approach to dose response assessment as described in the proposed revisions to U.S. EPA Guidelines for Carcinogen Risk Assessment (US EPA, 1999a) are utilized in this dose-response analysis. This two-step process distinguishes between the observed range of empirical data and the range of extrapolation.

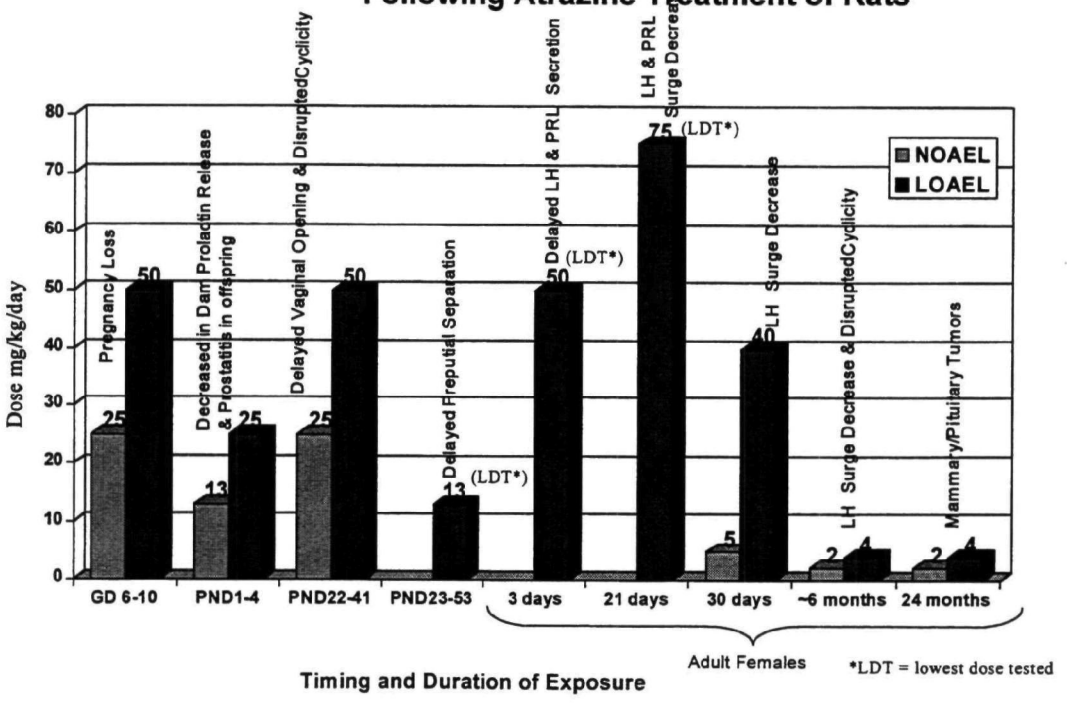
The weight of evidence does not support mutagenicity nor direct estrogenicity as components of atrazine's mode of carcinogenic action. As discussed in Chapter 2, the weight of evidence supports a conclusion that atrazine acts to cause mammary and pituitary gland tumors in female Sprague-Dawley rats by causing a attenuation of the preovulatory surge of LH which results in anovulation and an endocrine milieu that is conducive to tumor development. The critical event, the attenuation of the LH surge, is consistent with a nonlinear phenomenon in that there is a dose of atrazine that does not affect the LH surge or disrupt cyclicity. Therefore, it is proposed that dose-response assessment should proceed by a margin of exposure analysis.

An increased incidence and/or early onset of mammary and pituitary gland tumors in the rat is only one endocrinopathy found after atrazine treatment. The reproductive and developmental consequences (*e.g.,* disrupted cyclicity, delayed puberty, prostatitis in male offspring) that are found after atrazine treatment are of equal concern. These reproductive/developmental effects also originate from the effects of atrazine on the hypothalamic control of pituitary function through its interference with hypothalamic catecholamines and GnRH neurotransmitters. Thus, given the commonality in the mode of action, it is recommended that a point of departure for dose-response extrapolation be based on the most sensitive effects associated with atrazine's neuroendocrine mode of action.

3.4.1 Selecting a Point of Departure

Margin of exposure analysis begins with selection of a point of departure (POD) considered to represent the lowest reliable endpoint in the range of observation, being either tumor incidence, a key endocrine related effect or data on a proximal event that is an integral part of the mode of action process. Figure 3-2, provides an overview of the NOAELs/LOAELs for key endocrine related effects of atrazine from various studies in rats at different life stages and for different treatment durations. As discussed in Chapter 1 and Part C, the NOAELs for the effects of atrazine on pregnancy, pubertal onset and prostatitis are, for the most part, at or above 25 mg/kg/day. The exceptions are:

Figure 3-2. Key Endocrine-Related Effects Following Atrazine Treatment of Rats



- The NOAEL for delay of preputial separation in Wistar rats is not clear as a significant delay was seen at ~13 mg/kg/day (albeit near statistical significance; p = 0.07), but not at the next highest dose of 25 mg/kg/day; and

- The NOAEL for dam decreased prolactin release and resultant prostatitis in male offspring is ~13 mg/kg/day.

Dose-response data from long term repeat dosing studies are lacking for the effects on hypothalamic catecholamines and GnRH, *i.e.,* atrazine's initial site of action. However, it is the pulsatile GnRH secretion from the hypothalamus that determines the pituitary LH secretion (a critical event in atrazine's mode of action). Therefore, it is assumed that effects on LH secretion are a mirror of effects on GnRH secretion and that data on the serum LH are reasonable surrogate measures of the GNRH secretion. There are more data over different doses and time points for the attenuation of the LH surge. Thus, this is an appropriate POD. As illustrated in Figure 3-2, the NOAELs/LOAELs for the LH data are compared to the other key end points (mammary gland tumors, increased days in estrus, delayed puberty, suppression of suckling-induced prolactin and resultant prostatitis) that result from this neuroendocrine mode of action (also see Tables 1.9, 1.10, 2.1, and 2.2).

Selecting the NOAELs for the attenuation of the LH surge to determine a point of departure rather than from curve-fitting in the observable range of LH data is done here because it is not known over just what level of attenuation of the LH surge is necessary in order to produce clinically relevant effects. As discussed in the draft 1999 guidelines for carcinogen risk assessment, "the observed range of data may be represented by a NOAEL/LOAEL procedure when a margin of exposure analysis is chosen as the default procedure for nonlinear dose-response extrapolation" (US EPA,1999a).

As the treatment duration is increased, the dose that is needed to attenuate the preovulatory LH surge decreases. For example, it takes an extremely high dose of a single day of dosing of atrazine to attenuate the LH surge (*i.e.*, NOAEL = 200; LOAEL = 300mg/kg; Cooper *et al.*, 1999). However, with longer durations of dosing, much lower doses of atrazine can attenuate the LH surge (*i.e.*, NOAEL = ~2 mg/kg; LOAEL = ~4 mg/kg after six months of dosing; Morseth, 1996b). As shown in Figure 3-1, several types of reproductive/developmental effects can arise in postnatal rats following a few days of dosing up to several weeks of dosing with atrazine (*e.g.*, delayed puberty, prostatitis, increased days in estrus). As depicted in Table 1-10, NOAELs for these reproductive effects range from13 mg/kg/day up to 100 mg/kg/day.

With respect to effects that result from longer durations, LOAELs for precursor events associated with carcinogenesis (increased days in estrus, attenuation of the LH surge) and tumors consistently ranged between ~3 to 4 mg/kg/day. Likewise, NOAELs for various parameters were ~2 mg/kg/day or higher in all cases except one. In the Mayhew (1986) study, a significant tumor increase was noted at ~4 mg/kg/day but not at the lowest dose tested, 0.5 mg/kg/day. Based on consideration of the all the bioassay studies in SD rats and the repeat dose LH studies, as well as consideration of the dose spread in the Mayhew (1986) bioassay, LOAELs for carcinogenic, LH, and cyclicity effects tended to be approximately 4 mg/kg/day and NOAELs tended to be ~2 mg/kg/day. Clearly, there is a correspondence of doses that lead to tumor formation and doses that produce effects on LH levels and cyclicity. Thus, the point of departure for chronic effects is the dose of 1.8 mg/kg/day which is the NOAEL for attenuation of the proestrus afternoon LH surge in Morseth (1996b).

3.4.2 Point of Departure Using LED_{10} From The Tumor Data

Although data based on the attenuation of the LH surge is the preferred POD, for comparative purposes PODs based on the modeling of tumor data to derived $LED_{10's}$ are also presented. The most appropriate study to use in selecting a point of departure for tumors is Morseth, 1998. Five bioassays using the SD rat are available which examine tumor incidence and early onset. One of these studies (Petterson and Turnier, 1995) is a one year study and is not deemed appropriate for that reason. Another study (Thakur, 1991a) is not considered because only two dose groups were used and the study employed many serial sacrifices which resulted in a very small "n" value by the later timepoints in the study. A third bioassay (Thakur, 1992a) used only two dose groups. The two remaining studies, Mayhew (1986) and Morseth (1998), which employed four dose groups, both may be considered for use in selecting a point of departure. LED_{10s} for both of these studies are presented in Table 3-3. And ranged from ~2 to 3 mg/kg/day for mammary gland carcinomas and adenomas combined. These values represent equivalent human doses.[3] The NOAELs/LOAELs for mammary gland tumors are 0.5 and 3.5 mg/kg/day; and 4.2 and 24.4 from the Mayhew and Morseth studies, respectively. It should be noted that Morseth (1998) provides time to tumor information and used contemporary criteria for pathological evaluations. Also, Morseth (1998) had accompanying estrus cycling data.

The NOAEL for the LH surge attenuation and the LED_{10} for carcinomas and adenomas from Morseth (1998) are 1.8 mg/kg/day *(i.e.*, 0.48 mg/kg/day in human equivalents. Therefore, a POD based on the NOAEL for attenuation of the LH surge is comparable to a POD based on tumor response.

[3]Conversion to human equivalents performed by multiplying the rat dose in mg/kg/day by 0.266.

Table 3-2. LED_{10}s in Human Equivalents (And Revised Q_1*)

Study	Mammary Gland Tumors	LED_{10} (mg/kg/day)
Mayhew, 1986	Combined adenomas, carcinomas, and adenosarcomas	2.1
Mayhew, 1986	Fibroadenomas	3.0
Morseth, 1998	Combined adenomas and carcinomas	1.8
Morseth, 1998	Fibroadenomas	3.5
Morseth, 1998	Incidence of combined carcinomas and adenomas	($Q_1^* = 1.12 \times 10^{-1}$ mg/kg/day)

Data in this table from US EPA, 1999b and 1999d.

Table 3-2, also provides a revised Q* estimate for comparison purposes only. Given the mode of action understanding for atrazine, the nonlinear extrapolation approach is preferred over the linear default approach. The linear extrapolation is not supported by the mode of action data.

3.5 Summary and Conclusions on the Proposed OPP Science Policy Positions: Mode of action, Human Relevance, Children's Health Concerns, and Dose-Response Extrapolation

Listed below are the proposed science policy conclusions regarding the postulated mode of carcinogenic action in SD female rats. The relevance of the rat reproductive/developmental studies and the female SD rat tumor findings their mode of action to humans, including concerns for children. Recommendations are also made for the dose-response approach that should be considered in the cancer risk assessment.

3.5.1 Postulated Rat Tumor Mode of Action

Members of the pesticide program Cancer Assessment Review Committee (CARC) reviewed information on atrazine bearing on the formation of mammary and pituitary tumors in female SD rats. The CARC concluded that the increased incidence and early onset of mammary gland carcinomas and adenomas were well supported by several rat bioassay studies. The evidence for an early onset of mammary fibroadenomas and pituitary adenomas was considered to be suggestive.

Based on the *Mode of Action Framework Analysis* presented in Chapter 2, judgments were made on three considerations underpinning the mode of action of these tumors. The Committee agreed that:

- Atrazine does not have a significant mutagenic component to its mode of action;
- Direct atrazine binding to the estrogen receptor is not an influence on tumor development; and
- The neuroendocrine mode of action for the mammary and pituitary tumors is "biologically plausible" and is supported overall by the weight of the evidence.

As discussed in Chapter 2, there are several strengths of the mode of action proposal. For example, atrazine' s induced LH and cyclicity effects have been shown in two different laboratories and in two different strains of rats (LE and SD). Furthermore, there is a strong correlation has been shown for atrazine induced persistent estrus and induction of mammary tumors. Generally, there is a strong temporal and dose-response correlation between tumor formation and precursor effects. Ovariectomized SD rats treated with atrazine do not develop tumors, thus demonstrating the role of ovarian estrogen in atrazine's mode of action. Finally, a strong correlation was demonstrated between increased pituitary weights and histomorphological markers of prolactin exposure in the mammary gland, thus supporting the role of prolonged estrogen and prolactin exposure in tumor development. Although significant amounts of

data have been developed to demonstrate how atrazine may produce mammary and pituitary tumors in SD rats, there are uncertainties or limitations in the available data base (as discussed in Chapter 2.4.5). It should be emphasized that the uncertainties or limitations in the data in themselves do not discount the postulated mode of action, and that the strengths of the data provides compelling evidence in support of the postulated mode of action. However, the uncertainties/weaknesses in the data should be should be considered in the final risk characterization.

3.5.2 Relevance of Rat Mode of Action to Humans and Carcinogenicity Classification

It is proposed that **the postulated mode of action is assumed as being relevant to human cancer potential given that a primary initial site of action in rat involves the CNS control of pituitary function**. It is EPA science policy that animal tumor responses are presumed to be indicative of human cancer potential unless there is substantive information to the contrary. This default is intended to be public health protective and departure from this default must have a strong accompanying scientific basis. OPP views the differences between reproductive aging in humans and rats as an insufficient scientific basis to depart from the default. Therefore, if atrazine were to act on the hypothalamus of humans as in the rat and caused CNS alterations which influence endocrine function on physiological processes including ovarian cycling, there is the potential for various adverse health outcomes, including cancer.

The OPP Cancer Assessment Review Committee proposed that atrazine should be classified as a **likely human carcinogen** (US EPA, 1999a).

3.5.3 Children's Hazard

Data are available from animal studies on atrazine to assess potential effects in children that may be associated with its neuroendocrine mode of action. **Based on the endocrinopathies found in postnatal rats, it is reasonable to assume that children would potentially be susceptible to atrazine's neuroendocrine mode of action which may lead to a variety of health consequences** (See section 3.2). How atrazine's neuroendocrine mode of action is manifested depends on the life stage exposed as well as the duration and level of exposure. Data following prepubertal exposures in rats demonstrate adverse developmental effects including delay in puberty and prostatitis. In reference to the mammary tumors in rats and their mode of action, a cogent biological rationale informs that situation. LH secretion is quiescent until puberty. Therefore, it is not expected that atrazine would pose a cancer hazard following prepubertal exposure. However, starting with exposures at puberty, cancer hazard may be evident. As with adult exposures, certain endocrine responsive sites in the female may be at risk for cancer development.

3.5.4 Dose-Response

Based on atrazine's mode of carcinogenic action, a nonlinear dose-response extrapolation approach is the preferred approach for quantifying the cancer risk. A cancer hazard in adults resulting from infant and children exposure to atrazine cannot be ruled out. Infants and children, however, would not be expected to demonstrate a unique susceptibility to tumors induced by this mode of action, with the possible exception of an increased postpubertal risk of tumors. In order to assure adequate protection of all susceptible subpopulations (*i.e.*, women and children) for both cancer and noncancer effects for potential exposures throughout their lifetime, **it is recommended that the health risk assessment be performed utilizing the most sensitive endpoint associated with atrazine's neuroendocrine mode of action**. A NOAEL of ~2 mg/kg bw/day, based on attenuation of the LH surge following six months of atrazine treatment, is recommended as the point of departure for the health risk assessment using the MOE approach. For continuous exposures, this NOAEL is viewed as appropriate given atrazine's neuroendocrine mode of action which potentially leads to a variety of health consequences including cancer, and is viewed protective of all populations (including women and children).

3.6 Other Reviews

There have been a number of reviews on the carcinogenicity of atrazine by other organizations:

- Draft report of the Cornell University Program on Breast Cancer and Environmental Risk Factors in New York State (Snedeker and Clark, 1999);.
- The International Agency for Research on Cancer (IARC, 1999);
- The National Registration Authority for Agricultural and Veterinary Chemicals of Australia (NRA, 1997);
- The United Kingdom - in a report to the European Commission (United Kingdom Pesticide Directorate, 1996);

- A report published by a U.S. consulting group under contract to the Triazine Network (a national coalition of grower organizations and individuals) (Cantox, 2000); and

- A consensus report of a scientific panel commissioned by Novartis Crop Protection (Consensus Panel, 2000).

It should be noted that the current EPA draft atrazine assessment has generally reached similar conclusions with the above reviews on several issues concerning the carcinogenicity of atrazine (see Table 3-3). There appears to be consensus that mutagenicity and direct binding to the estrogen receptor do not play a significant role in atrazine's carcinogenic action in SD rats (IARC, 1999; Snedeker and Clark, 1999; Cantox 2000; Consensus Panel 2000; NRA, 1997; United Kingdom Pesticide Directorate, 1996). Further, these reviews have also concluded that an endocrine mode of carcinogenic action in SD rats is biologically plausible and is supported by the evidence. Although there is general agreement about support for a mode of action, there are different views on the role of accelerated reproductive senescence in the SD rat tumor response. For example, the United Kingdom Pesticide Directorate (1996) states that the reproductive aging hypothesis is not adequately proven, but that the tumors do appear to be caused by a "disturbance of endogenous hormone levels." Also, Snedeker and Clark (1999), concluded that there were inconsistencies or lack of data on certain hormonal measures (such prolactin and estradiol) which did not lead support to the premature reproductive aging hypothesis, but "there is evidence that it can affect hormones along the hypothalamic pituitary gonadal axis."

Although there is general agreement among different organizations, there are differences in the conclusions regarding human relevance and cancer classification. Snedeker and Clark (1999) concluded that atrazine is a "possible breast carcinogen." This document concludes that site concordance should not be assumed and that the potential exists for cancer at other hormone-responsive sites (*e.g.*, endometrium). Several other organizations including IARC (1999) concluded that the mode of carcinogenic action in SD rats in not relevant to humans. EPA/OPP may have had more data on the mode of action than these reviews, particularly on the hypothalamus as a primary site of action (Cooper *et al.*, 2000; Das *et al.*, 2000). But more importantly, these analyses considered

the disruption of hypothalamic control by atrazine in a broader sense leading to several neuroendocrinopathies (*e.g.*, delayed puberty, prostatitis, mammary gland tumors) in the rat, rather than focusing on the reproductive aging process and induction of mammary gland tumors in rats. Unlike these reviews, this analyses evaluated the neuroendocrine controls of pituitary function in rodents and primates, including humans, and concluded that there is a potential for carcinogenic effects independent of reproductive aging, and that primates may have some aging components in common with rat. Also, the LH response were not limited to SD rats also found in LE rats. The reproductive effects were also found in other strains such as Wistar rats.

Table 3-3. Other Reviews on the Carcinogenicity of Atrazine*

	Mutagenic	Direct Estrogenicity	Mode of Carcinogenic Action	Human Cancer Concern
EPA/OPP (This Draft)	No	No	Support	"Likely human carcinogen"
Snedeker and Clark (1999)	"	"	Some Support	"Possible breast carcinogen"
IARC (1999)	"	"	Support	"Not relevant" (Group 3)
Cantox (2000)	"	"	Support	'Not likely to be carcinogenic"
Consensus Panel (2000)	"	"	Support	"Not relevant"
NRA (1997)	"	"	Support	"Not considered to be relevant"
United Kingdom Pesticide Directorate (1996)	"	"	Support	"A strong case for non-classification"

Part B

Hazard Assessment and Review of Available Studies

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Chapter 4

4 Human Epidemiological Studies

Several epidemiologic studies have examined cancers among populations with exposures relevant to the assessment of atrazine, especially among farmers or farm residents (Table 4-1). Most are case control studies, although others include ecologic investigations and a worker mortality study associated with triazine manufacturing.

4.1 Colon Cancer

Associations between herbicide use by farmers and colon cancer in Kansas was investigated in a case-control study (Hoar *et al.*, 1985). Starting with 57 cases of colon cancer and 948 controls, the odds ratio (OR) for the subset using triazine herbicides was 1.4 (95% C.I. 0.2-7.9). The sample size in this group was very small with only two cancer cases with confirmed exposure to triazines and 43 controls. The study author stated that the data did not support an association between colon cancer and herbicide exposure

An ecologic study of ecodistricts in Canada compared triazine exposures and cancer incidences (Van Leeuwen *et al.*, 1999). Association of triazine exposure with several cancers, including colon, were examined. Significant negative associations were found in both sexes ($p = 0.041$ in females and 0.006 in males).

4.2 Non-Hodgkins Lymphoma

Zahm *et al.* (1993b) pooled results of three case-referent studies conducted in three midwestern states that investigated atrazine exposure in the development of non-Hodgkins lymphoma (NHL). Starting with 993 males with NHL and 2918 controls, persons were queried as to their pesticide exposures. An OR = 1.4 (95% CI 1.1-1.8) was found for atrazine use and NHL. However, when adjustments were made for use of 2,4-dichloroacetic acid and organophosphate use, the OR = 1.2 (0.9-1.7). The authors concluded that there was essentially no risk of NHL attributable to farm use of atrazine.

NHL was investigated among women who lived or worked on a farm in eastern Nebraska (Zahm *et al.*, 1993a). The OR for those who reported that they lived on a farm where atrazine was used was 1.4 (95% CI 0.6 - 3.0) with 11 cases and 31 controls. For those women who reported having personally used atrazine the OR = 2.2 (0.1 - 31.5) with only one case and two controls. Cyanazine was also investigated and the OR for NHL and those who reported using cyanazine was 1.3 (0.3 - 4.5) with four cases and 12 controls. The study author noted that there were too few subjects in any of these analyses to adequately assess associations.

Correlations were made between pesticide use (1993) and NHL incidence (1988-1992) among California counties (Mills, 1998). There were negative correlations for white males and females and Hispanic males; the correlation was positive for Hispanic females (0.12), but it was not statistically-significant.

An ecologic study of ecodistricts in Canada compared triazine exposures and cancer incidences (Van Leeuwen *et al.*, 1999). Association of triazine exposure with several cancers, including NHL, were examined. No association was found in females and a negative association was found in males.

A mortality study of workers in two triazine manufacturing plants that was supplied to EPA (Delzell and Sathiakumar, 1996) did not find any significant excesses of deaths for any disease category. There were, however, two cases of NHL in plant workers - one of whom was relatively young (31 years). These two cases do not provide evidence of an association between atrazine exposure and NHL, but do indicate that further follow-up of workers in these triazine manufacturing plants would be helpful.

4.3 Soft Tissue Sarcoma

A population-based case-control study of soft-tissue sarcoma (STS) in Kansas demonstrated that there was no increased risk among farmers (Hoar *et al.*, 1986). The lack of association persisted when years of herbicide use or frequency of herbicide use were considered. Analyses examining atrazine specifically were not conducted.

A study previously described above (Mills, 1998) examined associations between pesticide use and STS. Positive correlations were not noted for either males or females for atrazine use and STS.

4.4 Other Hematologic Cancers: Hodgkins Disease, Leukemia, Multiple Myeloma

Hoar *et al.* (1986) examined associations between herbicide use and Hodgkins disease (HD). An OR for atrazine exposure and HD was not reported in the study, but for herbicide use in general the OR was 0.9 (95% CI 0.5 - 1.5). The study author did not consider herbicide exposure to be associated with HD.

Association of pesticide exposure (including atrazine and cyanazine) to leukemia was investigated in a population-based case-control study of adult white men in Iowa and Minnesota (Brown *et al.*, 1990). The OR in those who reported mixing, loading and applying atrazine or cyanazine was 1.0 (0.6 - 1.5) for atrazine and 0.9 (0.5 - 1.6) for cyanazine.

Mills (1998) also examined associations between pesticide use and leukemia. Positive correlations were not noted for either Hispanic or white males or females for atrazine use and leukemia.

Triazine exposure and multiple myeloma in Iowa farmers was investigated in a case-control study by Burnmeister (1990). The OR for triazine use and multiple myeloma was 1.29 and was not significant.

4.5 Ovarian Cancer

A case-control study of epithelial ovarian cancer was conducted in woman between the ages of 20 and 69 who lived in a province in Italy where triazine herbicides are used in farming (Donna *et al.*, 1989). A relative risk (RR) of 2.7 (95% CI 1.0 - 6.9) was found for subjects who reported that they definitely had been exposed to triazine herbicides; the sample size in this subgroup was seven cases and seven controls. The authors considered that there was some risk of ovarian cancer among women who were exposed to triazines.

An ecologic study of ecodistricts in Canada compared triazine exposures and cancer incidences (Van Leeuwen *et al.*, 1999). Association of triazine exposure with several cancers, including ovarian, were examined. No association between atrazine exposure and ovarian cancer was found.

4.6 Breast Cancer

An ecologic study of counties in Kentucky compared measures of triazine exposures (ground and surface water measurements, and acres of land planted with corn and triazine application rates) with state cancer incidences (Kettles *et al.*, 1997). For the years 1993-1994, the OR = 1.14 (95% CI 1.08 - 1.19) for counties with medium triazine exposure, compared with OR = 1.2 (1.13 - 1.28) for high exposure counties. Although only slightly greater than 1.0, these OR's were still statistically-significant ($p<0.0001$).

4.7 Prostate Cancer

Pounds of atrazine applied in California counties during the calendar year 1993 were compared with state cancer incidences (Mills, 1998). A statistically-significant correlation coefficient of 0.67 was obtained for blacks and prostate cancer. The correlation coefficients for whites, Asians and Hispanics were not statistically significant.

4.8 Stomach Cancer

An ecologic study of ecodistricts in Canada compared triazine exposures and cancer incidences (Van Leeuwen *et al.*, 1999). Association of triazine exposure with several cancers, including stomach, were examined. A significant positive association was found in both sexes ($p = 0.242$ in females and 0.046 in males).

4.9 Summary

Colon cancer does not appear to be associated with triazine exposure as suggested by a non-significant OR of 1.4 in a single study (Hoar, 1985). The sample size in this study for triazine use was, however, very small. An ecologic study found negative associations between atrazine exposure and colon cancer in both sexes (Van Leeuwen *et al.*, 1999).

Soft tissue sarcoma does not appear to be associated with atrazine exposure. The correlation coefficient from Mills, 1998 was not significant and the OR from Hoar *et al.* (1986) was 0.9. Hodgkins disease does not appear to associated with atrazine as indicated by an OR of 0.9 in Hoar *et al.* (1986).

Two studies examined an association of atrazine to leukemia. One found an OR of 1.0 (Brown, *et al.*, 1990) and the other found a correlation coefficient which was not significant (Mills, 1998). Leukemia does not appear to be associated with atrazine exposure.

Triazine exposure and multiple myeloma do not appear to be associated. The OR in a single study was 1.29 (Burnmeister, 1990). This OR was not significant.

The results in regards to non-Hodgkins lymphoma (NHL) are mixed, but overall indicative of a lack of association of triazines with NHL. One study found a significant association with an OR of 2.5 (Hoar, *et al.*, 1985). When the data from this study is pooled with data from two other studies, a much lower OR of 1.4 is found (Zahm, 1993b). The Zahm, 1993b study represents the pooled data from three separate studies. Thus, the sample size is quite large -- 130 cases and 249 controls. The positive Hoar *et al.*, 1985 study, by comparison, had 14 cases and 43 controls. An OR of 2.2 was found for women who had reported using atrazine (Zahm, 1993a). The sample size in this study was very small with only one case and two controls. A fourth study failed to find any positive correlations for either Hispanics or white males or females for atrazine use and NHL (Mills, 1998). The low OR in the pooled study with a large sample size, combined with the lack of positive correlations in Mills, 1998, indicates that atrazine has not yet been clearly shown to be associated with atrazine. Further research in this area is desirable though given the positive association seen in Hoar, *et al.*, 1985 and the previously described incidence of two cases of NHL in workers employed at triazine manufacturing plants.

The most clear associations between atrazine and cancer occurs for ovary, breast and prostate cancer. Interestingly, all three of these cancers are known to be hormone-responsive. These associations should not be considered as conclusive evidence of an association of atrazine with these tumor types though.

Two of the associations (breast and prostate cancer) were found in ecologic studies. Ecologic studies contain inherent limitations and causal effects can not be found from ecologic studies. The primary limitation of an ecologic study is that chemical exposure can not be confirmed. Exposure in epidemiologic studies can sometimes be uncertain. For example, interview-based studies rely on a persons memory to determine exposure and a recall bias may be evident. But in ecologic studies exposure is most uncertain. In ecologic studies the researcher has no idea at all if the persons who contracted cancer had any exposure at all to the chemical in question. The researcher only knows that the person lived in a county in which the chemical was used or lived in a county which had chemical contamination of the water supplies.

The association with ovarian cancer seen in Donna, *et al.*, 1989, is also weakened by confounding variables. The most dramatic weakness in this study is the small sample size in the "defiantly exposed" group. This group consists of only seven cases and seven controls. Furthermore, close examination of this group reveals that it may be even smaller. A description of the exposure of the seven women in the "defiantly exposed" group is included as an appendix to the study. Examination of the descriptions in the appendix show that three out of the seven did not actually recall exposures to triazines at all. Rather, these three noted only that they had worked in fields where herbicides were used, but that they could not recall the names of the herbicides. The small sample size limits of the defiantly exposed group weakens the conclusions from this study.

4.10 Conclusions

The results of the human epidemiology studies do not provide clear evidence of an association between triazines and cancer. Some of the studies, particularly those in which hormone-responsive cancers such as breast, ovary and prostate, were examined, are suggestive of a possible association. There is also suggestive evidence of a possible association of triazine exposure and NHL. Further epidemiologic research is needed - especially in the area of hormone-responsive cancers.

Table 4-1. Odds Ratios (OR), Risk Ratio (RR) or Correlation Coefficient

Study	Cancer	Risk Measure or Correlation
Hoar *et al.*, 1985	Colon	*Triazines*: OR = 1 4 95% CI= 0.2 - 7.9
Hoar *et al.*, 1986	Non-Hodgkins lymphoma[1]	*Triazines.* OR = 2.5 95% CI= 1.2 - 5 4
Donna *et al.*, 1989	Ovary	*Triazines.* RR = 2.7 95% CI= 1.0 - 6.9
Brown *et al.*, 1990	Leukemia	*Atrazine.* OR = 1.0 95% CI= 0.6 - 1.5 *Cyanazine.* OR = 0.9 95% CI= 0.5 - 1.6
Burmeister, 1990	Multiple myeloma	*Triazines*: OR = 1.3 95% CI not given
Zahm *et al.*, 1993a	Non-Hodgkins lymphoma in women	*Use on farm*: OR = 1.4 95% CI= 0 6 - 3.0 *Personal use*: OR = 2.2 95% CI= 0.1 - 31.5
Zahm *et al.*, 1993b	Non-Hodgkins lymphoma in men	*Atrazine.* OR = 1.4 95% CI= 1.1 - 1.8
Kettles *et al* , 1997	Breast	*Triazine exposure* *Medium:* OR = 1.14 95% CI = 1.08 - 1.19 *High*: OR = 1.2 95% CI = 1.13-1.28
Mills, 1998	Prostate[2]	Statistically-significant correlation = 0.67 between atrazine exposure and prostate cancer in blacks, but not in whites, Hispanics and Asians
Van Leuwen *et al.*, 1999	Stomach[3]	Female p= +0.242 Male p = +0.046

[1]Soft tissue sarcoma and Hodgkins disease were also investigated and determined, by the study author, not to have a significant association with atrazine exposure.

[2]Leukemia, non-Hodgkins lymphoma, soft tissue, brain and testis cancer were examined, but a significant correlation was seen only with prostate cancer.

[3] Bladder, colon, brain, NHL and ovary cancer were also examined, but either no association or a negative association was seen in each case.

Chapter 5

5 Chronic Rodent Bioassay Studies

The carcinogenicity of atrazine in the female Sprague-Dawley (SD) rat has been confirmed in several two-year bioassays. These studies show that atrazine exposure results in an increased incidence and an early onset of mammary tumors in female SD rats (Mayhew *et al.*, 1986; Thakur, 1991a[1]; Thakur, 1992a; Morseth, 1998; Pettersen and Turnier, 1995). No tumor response is seen in SD male rats, however.

A two-year bioassay in both sexes of the mouse was negative for carcinogenicity, as were two-year bioassays in male and female F-344 rats (Hazeltte and Green, 1987; Thakur, 1991b; Thakur, 1992b).

Table 5-1 displays summaries of the mammary tumor incidence and onset in all the rodent bioassays that have been submitted to the Agency and also a study from the open literature (Pinter *et al.*, 1990). Additional details concerning these studies can be found in the discussion that follows. Appendix Table 1 also summarizes in further detail the results from the studies performed in the SD rat.

Table 5-2 displays summaries of pituitary adenoma incidences in all the rodent bioassays that have been submitted to the Agency. Pituitary tumor onset is difficult to determine as pituitary tumors are not palpable as are mammary tumors. However, in a serial sacrifice study, an early onset of pituitary tumors can be discerned in female SD rats (Thakur, 1991a). Only female pituitary tumor incidence is displayed in Table 5-2.

[1]Data from the studies referred to here as Thakur, 1991a, Thakur, 1991b, Thakur 1992a and Thakur 1992b, have been published in the open literature as Wetzel *et al.* 1994.

Table 5-1. Summary of Female Mammary Tumor Incidence in Two- and One-Year Rodent Bioassays Using Atrazine

Study	Species/ Strain	Duration	Mammary Tumor Incidence	Mammary Tumor Onset
Mayhew *et al*, 1986	Rat/SD	2 year	Statistically-significant increase in female carcinomas at 3 5 mg/kg/day when adjusted for survival	Not determined in this study
Thakur, 1991a	Rat/SD	2- year with serial sacrifices	A significant positive trend for fibroadenomas is seen	The percentage of carcinomas occurring in the first year of the study was 0 in controls, 33% at 4 23 mg/kg/day, and 50% at 26 23 mg/kg/day
Thakur, 1992a	Rat/SD	2- year	No statistically-significant increases in female fibroadenomas or carcinomas seen at either 3 79 or 24 01 mg/kg/day	The percentage of carcinomas and adenomas occurring in the first year of the study in controls was 0% while at 3 79 mg/kg and 23.01 mg/kg/day 27 3 and 33.3% of the carcinomas appeared in the first year of the study
Morseth, 1998	Rat/SD, both OVX and intact	2-year	No tumors seen in OVX animals Carcinoma, and fibroadenoma incidences at 3 1 mg/kg/day are increased two-fold over control values in intact animals	The mean week of onset for carcinomas and adenomas in controls was 72.6 while the mean week of onset for the 1.5, 3 1, 4 2 and 24 4 mg/kg/day groups was 77 2, 78.6, 64 4 and 64 8
Pettersen and Turnier, 1995	Rat/SD	1-year	six carcinomas/adenomas and four fibroadenomas are seen at the 23.9 mg/kg/day group compared to one carcinoma and two fibroadenomas in the control group.	The increased incidence of tumors at one year indicates an earlier onset.
Hazelette and Green, 1987	Mouse/CD-1	91 weeks	No increase in any tumor in either sex with exposures up to 386 mg/kg/day for males and 483 mg/kg/day for females	Not altered in Atrazine exposed animals
Thakur, 1991b	Rat/F-344	2- year with serial sacrifices	No increase in any tumor in either sex with exposures up to 34 mg/kg/day in both	Not altered in Atrazine exposed animals
Thakur, 1992b	Rat/F-344	2-year	No increase in tumors of any kind in either sex with exposures up to 20 mg/kg/day for males and 26 mg/kg/day for females	Not altered in Atrazine exposed animals
Pinter *et al*, 1990	Rat/F-344	Lifetime	Statistically-significant increase in male benign mammary tumors	Increased survival in dose groups versus controls resulted in delayed time of onset

Table 5-2. Summary of Female Pituitary Adenoma Incidence in Two- and One -Year Rodent Bioassays Using Atrazine

Study	Species/ Strain	Duration	Pituitary Adenoma Incidence by Dose Group (doses in mg/kg/day)
Mayhew *et al.*, 1986	Rat/SD	2 year	Control= 47/68 (69%); 0 5 = 41/63 (65%); 3.5 = 49/68 (72%); 25= 47/65 (72%); 50= 35/63 (56%)
Thakur, 1991a	Rat/SD	2- year with serial sacrifices	Control = 22/70 (31%); 4 23 = 16/70 (23%); 26.23 = 20/70 (29%)
Thakur, 1992a	Rat/SD	2- year	Control = 43/58 (74%); 3.79 = 45/58 (78%); 23.01 = 46/60 (77%)
Morseth, 1998	Rat/SD, both OVX and intact	2- year	*OVX* - Control = 42/80 (53%); 1.5= 39/80 (49%); 3.1 = 35/80 (44%), 4.2 = 42/80 (53%); 24.4 = 41/80 (51%) *Intact* - Control = 56/80 (70%); 1.5= 60/80 (75%); 3.1 = 52/80 (65%); 4.2 = 56/80 (70%); 24.4 = 54/80 (68%)
Petersen and Turnier, 1995	Rat/SD	1-year	Control= 2/55 (4%); 0 8 = 5/55 (9%); 1.7 =6/55 (11%); 2.8 = 4/55 (7%); 4.1 = 1/55 (2%); 23.9 =5/55 (9%)
Hazelette and Green, 1987	Mouse/CD-1	91 weeks	Control= 0/60; 1.6 = 0/60; 47.4 = 0/60; 246.9 = 3/60 (5%); 482.7 = 0/60
Thakur, 1991b	Rat/F-344	2- year with serial sacrifices	Control= 9/67 (13%), 0.68 = 6/69 (9%); 4 82 = 7/65 (11%), 14.05 = 5/66 (8%); 34.33 = 5/67 (7%)
Thakur, 1992b	Rat/F-344	2-year	Control = 22/60 (37%), 0.49 = 26/60 (43%), 3.43 = 20/58 (34%); 9.87= 19/59 (32%), 20.17 = 13/59 (22%)
Pinter *et al.*, 1990	Rat/F-344	Lifetime	Control = 32/41 (78%); 18.75 = 23/43 (53%); 37.5 = 35/50 (70%)

5.1 Mayhew *et al.*, 1986

The initial study that raised concerns about the possible carcinogenic effects of atrazine exposure was a carcinogenicity study conducted in male and female Sprague-Dawley rats at dietary dose levels of 0, 10, 70, 500 or 1000 ppm (0, 0.5, 3.5, 25 or 50 mg/kg/day). The Maximum Tolerated Dose (MTD) was likely exceeded in this study at the 1000 ppm dose in females. Mortality was significantly increased from 49% mortality at 104 weeks in the controls to 75% mortality at 104 weeks in females of the 1000 ppm group (see Table 5-4). Terminal body weight was also significantly decreased in 1000 ppm females in this study. There was a 27.2% decrease in group mean body weight ($p<0.01$) in the 1000 ppm females compared to controls. Male survival in the 1000 ppm group was significantly increased at 1000 ppm compared to controls, but body weight was significantly decreased compared to controls - 18.7% less than controls at 104 weeks. Based on the decreased body weight and increased mortality seen in females, and the decreased body weight seen in males, 1000 ppm is deemed to exceed the MTD of Atrazine in this strain of rats. The second-highest dose in this study, 500 ppm, likely is very close to the MTD. Male body weight at this dose is reduced 8.2% compared to controls at 104 weeks while male survival is not significantly altered compared to controls. Female survival is not significantly altered at this dose compared to controls, but body weight is reduced by 18.9% ($p<0.05$) compared to controls at 104 weeks. Based on the lack of significant effect in males seen at the 500 ppm dose and the uncertain effect seen in females (lack of a significant increase in mortality with a significant decrease in body weight) it seems likely that 500 ppm is very close to the MTD for atrazine in this strain of rat.

The conclusions drawn about the MTD of atrazine are important given that 1000 ppm exceeds the MTD and 500 ppm is assumed to be very close to the MTD. Thus, subsequent two-year carcinogenicity studies have used a dose of 400 ppm as the high dose to have the high dose be slightly below the MTD.

The mammary tumor incidences seen in this study are reported below in Table 5-3 and mortality is shown in Table 5-4.

Table 5-3. Mammary Tumor Incidence in the Mayhew Study (as determined by US EPA, 1988)

Tumor Type	Dose (mg/kg/day)				
	Control	0.5	3.5	25	50
adenocarcinomas/ carcinosarcomas combined	15/88 17% 0.000**	16/67 24% 0.39	27/69 39% 0.024*	27/68 40% 0.019*	45/60 51% 0.000**
adenomas and fibroadenomas combined	20/88 23% 0 446	24/65 37% 0.110	21/69 30% 0.373	21/68 31% 0 373	20/89 22% 0.468

NOTE: Significance for the trend is indicated at control. Significance of pairwise comparison *vs.* controls is noted at dose group.

Incidence values are number of tumor bearing animals over number of animals at risk

*p< 0.05; **p<0.01 as indicated by Peto Prevalence Test

Table 5-4. Mortality in the Mayhew Study (as determined by US EPA, 1988)

	Dose (mg/kg/day)				
	Control	0.5	3.5	25	50
Mortality at terminal sacrifice	34/59 49%**	39/70 56%	40/70 57%	44/70 63%	52/69 75%**

NOTE: Significance for the trend is indicated at control. Significance of pairwise comparison noted at dose group. Statistical test used are cox's or Generalized Krushkal-Wallis.

**p<0.01

A type of statistical analysis that examines tumor incidence and mortality is the Peto Prevalence test. The results of this test are displayed in Table 5-3. The results of this test showed that for the 70, 500 and 1000 ppm groups there was a statistically-significant (SS) pairwise increase in incidence of mammary adenocarcinomas and carcinosarcomas combined at 70, 500 and 1000 ppm, and that there was a dose-related trend for these tumors that was also SS ($p < 0.01$). The study authors of the Mayhew report also conducted Cox-Tarone and Gehan-Breslow tests to examine tumor incidence in light of the decrease mortality in the females. The results from these tests were similar to the results from the Peto test.

5.2 Thakur Studies

These are four studies - two using the SD strain and two using F-344 strain. These studies consisted of both terminal (all animals sacrificed after two-years exposure) and serial sacrifice (10 animals per group sacrificed at varying timepoints) protocols. The studies using the SD strain are discussed below while the studies with the F-344 strain are discussed in section 5.6 Appendix Table 2 displays summaries of the study design for these studies.

5.2.1 Serial Sacrifice Protocol (Thakur, 1991a)

Seventy SD female rats (no males were used) were exposed through the diet to doses of atrazine (97%) at 0, 70 and 400 ppm (0, 4.23 and 26.23 mg/kg/day) for two years. Ten females per dose were sacrificed at one, three, nine, 12, 15, 18 and 24 months.

Mortality was increased in a dose-dependent manner. There were five unscheduled deaths in the control group, six in the 70 ppm group, and eight at 400 ppm. Using the Gehan-Breslow test there was a statistically-significant (SS) negative trend for survival (survival decreased as the dose increased). Another statistical test - the Cox-Tarone test - did not indicate a significant trend in either direction. A statistically-significant reduction in body weights were found at several timepoints in the 400 ppm group compared to controls.

The results of this study is significant in regards to pituitary tumors. Because pituitary tumors cannot be detected by palpation, a serial sacrifice study is the most appropriate way to determine onset for pituitary tumors. Pituitary tumor incidences by timepoint are displayed in Table 5-5, below. The hormonal basis for early onset of pituitary tumors is discussed in section 9.2.6

Table 5-5. Pituitary β-Adenoma Incidences by Timepoint in Thakur Serial Sac, 1991a

Sacrifice time (mo.)	Control	4.23 mg/kg/day	26.63 mg/kg/day
1	0	0	0
3	0	0	0
9	0	0	2
12	2	2	6
15	5	3	4
18	9	5	6
24	6	6	2
0-12	2	2	8
0-24	22	16	20

NOTE: Ten animals in each group. Unscheduled sacrifice animals are included in this table

5.2.2 Terminal Sacrifice Protocol (Thakur, 1992a)

Sixty SD females (no males were used) were exposed through the diet to doses of atrazine of 0, 70 and 400 ppm (0, 3.79 and 23.01 mg/kg/day) for two years (Appendix Table 2 summarizes the protocol used for the Thakur studies with both SD and F-344 rats). Mortality was high in the controls and increased in a dose-related manner. Mortality was 48% in controls, 58% in the 70 ppm group, and 63% in the 400 ppm group. Two survival tests were contradictory in determining whether or not this increase in mortality was significant. Analysis with the Gehan-Breslow test showed a negative trend in survival with dose (increased mortality with increasing dose) while a Cox-Tarone test found that the increases in mortality were not significant. Group mean body weights were significantly decreased, compared to controls, at the 400 ppm group as early as four weeks and remained significantly decreased up to, and including, week 76. Body weight gains were significantly decreased for the period from study initiation to week 76. Both absolute body weight at week 104 and body weight gain from week five to 104 of the 400 ppm group, were lower than controls, but not statistically-significantly. Group mean food consumption in the 400 ppm group was decreased compared to controls for the first 13 weeks of the study. After 13 weeks though, there was no significant difference. The only finding at gross necropsy that may have been related to compound exposure was an increase in enlarged spleens in the 400 ppm dose group. The control and 70 ppm groups were observed to have five and three animals with enlarged spleens, respectively, while the 400 ppm group had 15. This finding was not observed in the Mayhew study, in the Thakur terminal sacrifice study, or in any other bioassays that followed the Thakur series of studies.

Histopathology revealed that mammary and pituitary neoplasms were a common occurrence. Table 5-6 displays mammary tumor incidence data by tumor type. There was not a statistically-significant increase in fibroadenomas or carcinomas at the doses tested, compared to controls. This is true whether or not mortality is taken into account through Cox or Gehan-Breslow tests. Table 5-6 displays the p values calculated by the study authors for mammary tumor incidences.

Table 5-6. Female Mammary Gland Tumor Incidences in the SD Terminal Sacrifice Protocol (Thakur, 1992a) (calculated using Cox-Tarone and Gehan-Breslow tests)

	Dose (mg/kg/day)		
	Control	**3.79**	**23.01**
Fibroadenoma	39/60 (65%)	30/59 (51%)	41/60 (68.3%)
p value			
Cox-Tarone		0.9141	0.1070
Gehan- Breslow		0.6401	0.2114
Carcinoma	17/60 (28%)	13/59 (22%)	22/60 (33.6%)
p value			
Cox-Tarone		0.8316	0.1590
Gehan- Breslow		0.7613	0.0810

5.3 Morseth, 1998

Atrazine (97.1%) was administered to 800 female Sprague-Dawley rats. The rats were divided into two groups of 400 each. One group was ovarectomized (OVX) while the other was left intact. Atrazine was mixed with the diet at dose levels of 0 (control) 25, 50, 70 and 400 ppm (0, 1.5, 3.1, 4.2, 24.4 mg/kg/day for intact animals and 0, 1.2, 2.5, 3.5, and 20.9 mg/kg/day for OVX animals) for two years. There were 80 females at each dose level - 20 for a 12-month sacrifice and 60 for a 24-month sacrifice.

The trend for survival was statistically-significantly (SS) decreased in the dosed groups compared to the controls. Survival was as follows: 43.3% in controls; 31.7% - 25 ppm; 28.8% - 50 ppm; 31.6% - 70 ppm; 21.7% 400 ppm. Body weight was SS reduced in the first half of the study in the 400 ppm group (other groups were not significantly altered), but by the end of the study body weights were similar to control values.

Neoplastic histopathological findings were mostly limited to the pituitary and the mammary gland. Neither intact nor OVX dosed animals showed an increase in pituitary tumors compared to their respective controls, but intact animals did show a 20-30% greater incidence of pituitary adenomas compared to OVX animals.

There were few mammary tumors in the interim sacrifice animals, which is not surprising given that these animals were sacrificed after only one-year. Excluding the interim sacrifice and looking only at those animals that were sacrificed at 24 months and those that died prematurely, there was an increase in mammary tumor incidence at all intact dose groups compared to controls. Looking at carcinomas alone incidence values are: 18.3%; 36.7%; 33.9%; 20%; and 41.7% for the control, 25, 50, 70 and 400 ppm dose groups respectively. Fibroadenomas alone were: 26.6%; 40%; 52.5%; 45%; and 40% for the control, 25, 50, 70 and 400 ppm dose groups respectively.

Table 5-7 below displays a statistical analysis of mammary tumor incidence in intact animals using a Peto's Prevalence Test. Incidence values shown below differ from those described in the paragraph above because interim sacrifice animals are included in the analysis shown in Table 5-8 (USEPA, 1999c). A different survival-adjusted statistical analysis (Cox-Tarone) conducted by the study author showed significant pairwise increases at 3.1 mg/kg/day for fibroadenomas compared to control, but did not show significant pairwise comparison to control for the 1.5 mg/kg/day group.

Table 5-7. Mammary Gland Tumor Incidence in Intact Animals in Morseth, 1998 Study

	Dose (mg/kg/day)				
	Controls	**1.5**	**3.1**	**4.2**	**24.4**
Fibroadenoma	16/78 (21%) 0.233	25/79 (32%) 0 030*	34/77 (44%) 0.000**	29/78 (37%) 0 014*	25/77 (32%) 0.014*
Carcinoma	12/80 (15%) 0.002**	18/80 (22%) 0.112	20/79 (25%) 0.067	14/80 (18%) 0.395	27/80 (34%) 0.007**
Adenoma	0/28	0/24	1/20 (5%)	0/21	0/15

NOTE: Significance for the trend is indicated at control. Significance of pairwise comparison noted at dose group. Incidence values are number of tumor bearing animals over number of animals at risk.

*p<0.05; **p<0.01

Not a single mammary tumor of any sort was seen in any OVX animal. The lack of mammary tumors in OVX animals provides evidence indicates that an intact ovary is mandatory for mammary tumorogenesis in the SD female. The results found in OVX animals will be discussed more fully in section 7.3. Bi-weekly estrous cycle measurements were also made in this study. The results of these measurements are discussed below under section 9.2 Estrous Cycle.

5.4 Pettersen and Turnier, 1995

This study exposed female SD rats (55 per group) through the diet to doses of atrazine of 0, 15, 30, 50, 70, or 400 ppm (0, 0.8, 1.7, 2.8, 4.1, or 23.9 mg/kg/day). This study was a serial sacrifice protocol in which 10 animals in each group were sacrificed at 3, six, and nine months and the remaining 25 animals were sacrificed at one year following initiation of dosing. Dosing appeared to be adequate, as body weights in the last 10 months of the study were reduced 8 to 12% in the 400 ppm animals compared to controls. Body weight gains over the last 10 months of the study were reduced 11 to 18% in the 400 ppm group compared to controls. There were no differences in survival among dose groups in this study. No mammary tumors were seen in the three- and six-month sacrifices. There was a significant positive dose-related trend in mammary tumors as well as a significant increase in mammary tumors between control and 400 ppm animals using a pairwise comparison. Table 5-8 displays tumor incidences from this study.

Table 5-8. Number Of Animals With Mammary Tumors In The Pettersen and Turnier, 1995 Study

	Dose(mg/kg/day)					
	0	**0.8**	**1.7**	**2.8**	**4.1**	**23.9**
3 and 6 months	0/20 had tumors	0/20 had tumors	0/20 had tumors	0/20 had tumors	0/20 had tumors	0/20 had tumors
9 month	F=1/10	C=1[1] no other tumors	0/10 had tumors	0/10 had tumors	F=1/10	C=1/10 F=1/10
12 month (Terminal Sac)	C=1/25 F=1/25 A=0/25	C=1/24 F=2/24 A=0/24	C=0/25 F=2/25 A=1/25	C=1/25 F=0/25 A=1/25	C=1/24 F=3/24 A=1/24	C=5/25 F=3/25 A=1/25
Total	C/A=1/55 F=2/55	C/A=2/55 F=2/55	C/A=1/55 F=2/55	C/A=2/55 F=0/55	C/A=2/55 F=4/55	C/A=7/55 F=4/55

NOTE: Numerator is the number of animals with tumors, denominator is the number of animals examined

C=adenocarcinoma; F= fibroadenoma; A = adenoma

[1]This tumor occurred in an animal scheduled to be sacrificed at 12 months but found dead on study day 218.

5.5 Hazelette and Green, 1987

Atrazine (purity not given) was administered to CD-1 mice through the diet to 59-60 animals/sex/dose, at dose levels of 0,10,300,1500 and 3000 ppm (male/female mean daily dose 0/0, 1.4/1.6, 38.4/47.9, 194.0/246.9, 385.7/482.7 mg/kg/day)for 91 weeks. The doses given were adequate as indicated by toxic effects, such as a decrease in mean body weight gain of both sexes (23.5%/11%, M/F) and an increase in cardiac thrombi in the females, are seen at both 1500 and 3000 ppm, while no dose-related toxic effects are seen at 10 and 300 ppm. There was also an increase in mortality ($p < 0.05$) in 3000 ppm females, but not males, with only 25% of the females surviving versus 39-43% of the females surviving in the other female dose groups. At the doses tested, there was not a treatment-related increase in tumor incidence when compared to controls.

5.6 F-344 Two-Year Bioassays

5.6.1 Serial Sacrifice Protocol (Thakur, 1991b)

Seventy F-344 rats (females only) per dose were exposed ad libitum to diet that had been mixed with atrazine (97.1%) to the appropriate doses of 0 (negative control), 10, 70, 200 and 400 ppm (0, 0.68, 4.82, 14.05, 34.33 mg/kg/day). Ten animals per dose group were sacrificed after approximately one, three, nine, 12, 15, and 18 months exposure to the test article.

There was not an increase in mortality due to compound exposure, and there was no increased incidence of clinical signs in dosed animals compared to controls. The doses tested appeared to be sufficiently high because there was a decreased absolute body weight and body weight gain in the 400 ppm group compared to the controls. Group mean absolute body weight in the 400 ppm group compared to controls was also significantly decreased compared to controls at several time points though the final mean body weight was not significantly decreased compared to controls. The final group mean body weight for the 400 ppm group was 6.6% less than the mean control value. During the course of the study the 400 ppm animals gained an average of 116.7 gm compared to the weight gain in the control group of 133.3 gm (14% less than controls). This difference in body weight gain was statistically-significant at a p value of 0.05. There was not an increase in mammary tumors or any other type of tumor at any dose group.

5.6.2 Terminal Sacrifice Protocol (Thakur, 1992b)

Sixty F-344 rats per sex per dose were fed technical grade atrazine through the diet at doses of 0 (negative control), 10, 70, 200 and 400 ppm (0, 0.49, 3.43, 9.87 and 20.17 mg/kg/day for males and 0, 0.61, 4.35, 12.71, and 26.18 mg/kg/day for females) for two years. Mortality in either sex was not affected by treatment. Male control mortality was 30% while male mortality in the 400 ppm group was 32%. Mortality in the other male dose groups was slightly lower than controls, ranging from 22 to 25%. Female mortality was 22% in the controls and 27% in the 400 ppm group. Female mortality in the other dose groups ranged from 17 to 25%. Body weights and body weight gains were adversely affected by compound exposure, especially at the 400 ppm dose in each sex. Mean group body weights were statistically-significantly reduced versus controls at the four, 13, 24, 52, 76 and 104 week timepoint in the both the male and female 400 ppm group. Percent body weight reductions ranged from 5.1 to 9.3% in the males and 5.3 to 6.4% in the females. Percent body weight gains were also significantly decreased in both sexes of the 400 ppm group for all the time periods examined - 0-4, 0-13, 0-24, 0-52, 0-76 and 0-104 weeks. The range of percent reductions compared to controls was 11.3 to 15.9% in males and 10.7 to 17.4% in females. The reduction in percent body weight gain, compared to controls, in males for the entire study (weeks 0-104) was 11.3% and for females it was 11.6%. Mean group food consumption was significantly decreased (4.8% versus controls) for the 0-104 week period in 400 ppm males, but was not significantly decreased in females. There were no findings at gross necropsy that could be attributed to compound exposure and organ weights were not altered between control and dosed animals.

The incidence of mammary gland fibroadenomas was increased in dosed females compared to controls. This increase was not statistically-significant. Even at its highest dose level the percentage of animals with fibroadenomas was below the historical level for the laboratory where the Thakur studies were conducted. There were no increases in mammary tumors of any type in dosed males versus controls. Two out of the 55 control males examined at histopathology were found to have a mammary tumor (both were fibroadenomas). Only one out of 54 males in the 10 ppm group and one out of 58 in the 400 ppm group were found to have mammary tumors (one fibroadenoma and one carcinoma) while not males in the 70 and 200 ppm group had a mammary tumor of any type. Therefore, dosing with atrazine did not increase mammary tumor incidence in F-344 males.

Table 5-9. Female Mammary Tumor Incidence In F-344 Terminal Sacrifice Protocol In The Thakur Terminal Sacrifice Study (1992b)

	Dose (mg/kg/day)					
	Control	**0.5**	**3.4**	**9.9**	**20.2**	**Historical Control**
Fibroadenoma	2/60 (3.3%)	5/60 (8.3%)	5/60 (8.3%)	7/60 (11.7%)	6/59 (10.2)	Mean = 14.9% Range= 3-23%
Unadjusted p value	0.2514	0.2198	0.2195	0.0815	0 1295	
Carcinoma	2/60 (3.3%)	0/60 (0%)	2/60 (3.3%)	3/60 (5%)	2/59 (3.4%)	Mean = 3.8% Range= 2-15%
Unadjusted p value	0.4640	0.2479	0.0907	0.5000	0.6843	

NOTE: Historical control data from Hazelton Labs, 1984

5.7 Pinter *et al.*, 1990

The Pinter *et al.* study exposed Fischer-344 rats of both sexes to atrazine (98.9%) that was mixed in the diet at concentrations of 0, 500 and 1000 ppm. The control groups started with 56 males and 50 females; the 375/500 ppm group started with 55 males and 53 females; and the 750/1000 ppm group started with 53 males and 55 females. Unlike most carcinogenicity assays where surviving animals are sacrificed after approximately 104 weeks on study, in this study animals were allowed to live out their natural life span, except for four males and six females that were sacrificed moribund. Table 5-10 displays the mammary tumor incidence in males in Pinter *et al.*, 1990.

Table 5-10. Mammary Tumors In Males in Pinter *et al.*, 1990 [1]

	Control	375 ppm	750 ppm
Total number of tumor-bearing males	1/48	1/51	8/53
Total number of benign tumors	1/48	1/51	9/53**
Total number of malignant tumors	0/48	0/51	1/53

[1]Data from Table 2 in Pinter *et al.*, 1990.

**p<0.01 using Fisher Exact test comparing high dose group to low dose group.

5.8 Pinter *et al.*, 1990

The Pinter *et al.* study exposed Fischer-344 rats of both sexes to atrazine (98.9%) that was mixed in the diet at concentrations of 0, 500 and 1000 ppm (the 500 and 1000 ppm doses were reduced to 375 and 500 ppm after eight weeks of treatment due to toxicity). The control groups started with 56 males and 50 females; the 375/500 ppm group started with 55 males and 53 females; and the 750/1000 ppm group started with 53 males and 55 females. Unlike most carcinogenicity assays where surviving animals are sacrificed after approximately 104 weeks on study, in this study animals were allowed to live out their natural life span, except for four males and six females that were sacrificed moribund. Table 5-10 displays the mammary tumor incidence in males in Pinter *et al.*, 1990.

Table 5-10. Mammary Tumors In Males in Pinter *et al.*, 1990[1]

	Control	375 ppm	750 ppm
Total number of tumor-bearing males	1/48	1/51	8/53
Total number of benign tumors	1/48	1/51	9/53**
Total number of malignant tumors	0/48	0/51	1/53

[1]Data from Table 2 in Pinter *et al.*, 1990.

**p<0.01 using Fisher Exact test comparing high dose group to low dose group.

Mammary gland tumors in the dosed females in this study were not altered in incidence compared to controls. Mammary gland tumors in dosed males were altered. The incidence of benign tumors (adenomas, fibroadenomas, and fibromas) was one tumor in 48 animals in controls, 1/51 in the 375 ppm group and 9/53 in the 750 ppm group. The study authors performed a statistical analysis to determine if this increase in tumors was significant. The authors found that when a pairwise comparison was done between the 750 ppm group and the 375 ppm group there was a significant increase at 750 ppm ($p<0.01$). Generally dose groups are compared to controls to determine changes in tumor incidence following dosing. The study authors choose not to perform a pairwise comparison to controls in this case, however, because the animals in the control group died much sooner than the animals in either dose group. The last control male died before study week 120; the last 375 ppm male died between week 120 and 130; and the last 750 ppm male died between weeks 130 and 140. The differences in survival in this study confound the results. The study authors state: "The tumors in the high-dose group appeared later in time than those in the control or low-dose group." The one tumor in the control group occurred at week 111 and the sole tumor in the 375 ppm group occurred at 119 weeks. By contrast, the average mean time of tumor appearance in the 750 ppm group was 121.3 week ± 15.4 weeks. The increase in tumors seen in the older animals could be due to the exposure to atrazine or could also be due to the simple fact that these were old animals.

Mammary tumors in F-344 males have been shown to increase in incidence in untreated males as they age (Solleveld, 1984). Table 5-11 displays mammary tumor incidences in untreated, aged F-344 males. Table 5-11shows that a difference of only a few months can greatly increase the incidence of mammary tumors as the rats age. Tumor incidences double as the rats age from 98-110 weeks to 111-123 weeks. Incidences double again as the rats age from 111-123 weeks to 124 to 136 weeks.

Table 5-11. Mammary Gland Fibroadenomas in Male F-344 Rats by 12 Week Time Periods (Solleveld, 1984)

	98-110 Weeks	111-123 Weeks	124-136 Weeks	>137 Weeks
Mammary Fibroadenoma	3/77 (4%)	13/143 (9%)	27/148 (18%)	22/95 (23%)

The study authors seem to realize the possible relationship between the age of the high-dose males and their high tumor incidences. The authors cite Solleveld (1984) in an attempt to show that, even given the increased tumor incidence in aged males, the atrazine exposed males had increased tumor incidences. Pinter *et al.*, (1990) notes:

> "The incidence of benign mammary gland tumors in male F344 rats was reported to be 2.2% for 110 to 16 weeks; in the life span studies (more than 116 weeks), however, 13.4% of the male animals had benign mammary gland tumors [Solleveld, 1984 is cited]. In our study, 16.9% of the high-dose, males had benign mammary tumors."

The 2.2% the authors of the Pinter *et al.* study refer to in the above quote is historical control data from untreated males in several National Toxicology Program two-year bioassays. The 13.4% is historical control data for mammary tumors in untreated males greater than 116 weeks in age in life-span studies. The difference between 2.2% mammary tumor incidence at 116 weeks in the two-year bioassays and the 13.4% in the life span studies again emphasizes the dramatic increases in tumors that occur as the animals age beyond approximately two years of age.

The study authors seem to believe that atrazine exposure is inducing the additional tumors between 13.4% and 16.9%. However, the study author's Table 2 on page 537 of their publication shows that only eight of 53 (15%, not 16.9%) males had a mammary tumor of any sort. The origin of the 16.9% value is unknown. The true difference - according to the data the study authors present in their Table 2 - is between the 13.4% and 15%. Additionally, there was one incidence of an adenocarcinoma in the high-dose males. The Pinter *et al.* publication does not include any description of which male had this carcinoma but if this tumor occurred in an animal that did not also have a benign tumor then the number of animals with benign tumors drops to seven out of 53 -- 13.2% -- almost identical to the 13.4% cited by the study authors from the Solleveld (1984) paper.

It is concluded that the authors of this study have not made a case that the increase in male benign mammary tumors is due to atrazine exposure. The tumors appearing in the high-dose males do not appear to be found at a rate any higher than what would be expected for F-344 males of a comparable age.

5.9 Summary and Discussion of the Two-Year Bioassay Studies

Increased incidences of mammary fibroadenomas or carcinomas were seen in three out of four separate two-year bioassay studies using Sprague-Dawley rats.

Atrazine exposure in a two-year bioassay using CD-1 mice did not result in increased incidences of mammary tumors in either sex, despite the compound being given to the mice at doses that resulted in decreases in body weight gain of 23.5% in females and 11% in males and a significant increase in mortality in females.

Atrazine exposure in two separate two-year bioassays using the F-344 strain of rats also did not result in increased incidences of mammary tumors. One of the bioassays used rats of both sexes (terminal sacrifice protocol) and did not see increases in mammary tumors in either sex, while the other study employed only females (serial sacrifice protocol) without seeing an increased incidence of mammary tumors. Dosing was adequate in both studies as indicated by the 14% decrease in female body weight gain the 400 ppm dose group compared to controls (serial sacrifice protocol) and the 11.3% (♂) and 11.6% (♀) reductions in percent body weight gain at 400 ppm compared to controls in the terminal sacrifice study. Doses of atrazine that produced equivalent, or even less than, reductions in body weight in the two-year bioassays with the SD strain reductions) produced mammary tumors. For example, in Thakur, 1992a (the terminal sacrifice protocol), female body weights gains were reduced 12 to13% at 104 weeks in the 400 ppm group; in Mayhew *et al.* (1986) female body weight in the 70 ppm group were reduced only 3.25, yet this was still sufficient to produce mammary tumors.

A study by Pinter *et al.* (1990) also showed a lack of carcinogenic effect for atrazine in F-344 females. This study did find an increase in benign mammary tumors in F-344 males when high-dose males were compared to low-dose males. However, this study was a life-span study rather than a two-year bioassay and the males of the high-dose group (in which the increases in benign mammary tumors were seen) survived significantly longer than males in the controls or low-dose group. Examination of the tumor incidence in the high-dose males from this study suggest that mammary tumor incidences were most likely comparable to what would be expected for males of this strain and age.

Chapter 6

6 Genotoxicity Studies

An important question to address in the hazard assessment is whether atrazine also has the potential to be DNA reactive and act as an initiator within the context of the multistage model of carcinogenesis. Thus, following initiation (*i.e.*, a mammary gland cell acquires a mutation that results in unregulated proliferation), tumor promotion (*i.e.*, clonal expansion of the genetically altered cell) in the mammary tissue would then be hormonally-mediated. The most desirable data to address this issue would be information on genetic alterations in the relevant target tissue. This information is not available for atrazine. There are a large number of studies using standard genotoxicity assays to evaluate the mutagenic potential of atrazine.

Atrazine has been examined for its ability to induce mutations in microorganisms, insect, and plants, and to induce chromosomal aberrations *in vitro* and *in vivo* in both mammalian and nonmammalian organisms. Additionally, atrazine has been tested in other assays using endpoints that are indicative of DNA damage, but are not measures of mutation *per se* (*e.g.*, genetic recombination, sister chromatid exchanges, DNA strand breakage, and unscheduled DNA synthesis).

Although more than 50 studies are available on atrazine, some findings reported in the literature are presented in insufficient detail for evaluation or results are inconclusive due to study design problems. Furthermore, the results on atrazine are inconsistent even within the same test system and genetic endpoint evaluated. Thus, in evaluating the mutagenic potential it is important to take a weight- of- evidence approach that considers the overall response patterns or trends for mutation, chromosomal damage, and other indicators of DNA damage. In looking at the overall trends in the database, it is important to consider the type of end point evaluated and the test system/organism used. More emphasis is placed on end points that are direct measures of mutation and chromosomal aberrations rather than indicators of DNA damage (*e.g.*, sister chromatid exchanges, DNA strand breaks). Also, results from mammalian systems are emphasized more than results from assays using nonmammalian organisms. Likewise, mammalian *in vivo* data are preferred over data from *in vitro* tests.

As summarized in Appendix Table 5 Most of the mutagenicity studies on atrazine have been reported as negative. The majority of these negative results come from mutation studies in bacteria. Beyond the bacteria results, the response profile for atrazine is heterogenous and closer to a split between negatives and positives. Nevertheless, the response patterns or trends for mutation and chromosomal damage tend to be more convincing for the number and type of negative responses found after atrazine treatment than for the positive data, which typically were weak, observed at high treatment concentrations of atrazine, or were not repeatable. Data on several metabolites of atrazine and its close structural analogues (propazine and simazine) do not support a mutagenic potential for these compounds. Therefore, the totality of evidence does not support a mutagenic potential for atrazine, and indicates that a direct DNA reactive/mutagenic mode of action is unlikely to be an influence of atrazine on mammary gland tumor development (or at any other site). A discussion of the literature supporting this conclusion follows.

6.1 Mutation Studies

As summarized in Appendix Table 5, most studies on atrazine for mutation induction are bacteria tests with a few assays in yeast, fungi and in the fruit fly *Drosophila melanogaster*. There is no compelling evidence for mutation induction as a mode of carcinogenic action for atrazine given the consistent negative responses in bacterial tests, and the inconsistent positive responses across other phylogenetic lines (where responses tended to be weak, found at high doses, and/or were not reproducible).

When atrazine was evaluated in the Ames assay (with a variety of *Salmonella typhimurium* tester strains) by several different laboratories, it was consistently negative even when a mammalian liver metabolic activation system was incorporated (Seiler, 1973; Poole and Simmon *et al.*, 1977; Lusby *et al.*, 1979; Bartsch *et al.*, 1980; Sumner *et al.*, 1984; Deparde, 1986; Kappas,1988; Mersch-Sundermann *et al.*, 1988; Zeiger *et al.*, 1988 1992; Ruiz and Marzin, 1997).

There are no acceptable mutation studies in: Butler and Hoagland, 1989; Anderson *et al.*, 1972; Morichetti *et al.* 1992, mammalian systems. Although Adler (1980) reported a negative result for a gene mutation test (HPRT assay) in V79 cells, this paper does not contain sufficient detail to allow an independent assessment of the finding.

Tests in yeast and fungi have yielded heterogenous results. For example, mutation induction was reported in *Schizosaccharomyces pombe* with or without plant cell activation (Mathias, 1987) and in *Aspergillus nidulans* only with mammalian cell activation (Benigni *et al.*, 1979). When atrazine was evaluated in *Saccharomyces cerevisiae* without exogenous activation, a negative result was reported in one paper (Emnova *et al.*, 1987), while a weak positive finding was observed by Morichetti *et al.*, 1992. The reported positives are mostly found at high doses of atrazine. Furthermore, gene conversion and mitotic recombination, which are indicators of DNA damage, were not increased in yeast and fungi exposed to atrazine (de Bertoldi *et al.*, 1980; Emnova *et al.*, 1987; Kappas, 1988), except when plant cell activation was incorporated into the assay (Plewa and Gentile, 1976). Because these lower eucaryotic assays have intrinsic rates of positive responses that occur sporadically, this conflicting database in fungi and yeast must be interpreted carefully in the context of the weight-of- evidence and results from other organisms.

Microbial systems (*Salmonella, E. coli*, yeast) have been used as indicators of mutational damage after atrazine treatment in host-mediated assays (Adler, 1980; Simmon *et al.*, 1977). The mouse host-mediated assays on atrazine have yielded mix results, with *Salmonella* (injected intraperitoneally) being negative and *E. coli* and yeast (injected into the mouse testes) as positive. However, because of the variability in cell recovery, these assays are not viewed as reliable indicators of mutagenicity.

Some information is available in insects. In general, positive results in *Drosophila* were reported for somatic mutation in the spot wing test (Tripathy *et al.*, 1993; Torres *et al.*, 1992) or in the sex-linked recessive lethal assay (Tripathy *et al.*, 1993; Murnik and Nash, 1977) under certain conditions (larval feeding or at high doses). Murnik and Nash (1977) tested atrazine, simazine and cyanazine in the *Drosophila* sex linked recessive lethal assay. The authors further concluded that, "these triazine herbicides may be weak mutagens," and that "Much larger experiments are needed to determine with confidence the mutagenic potential of the herbicides." The results reported by Murnik and Nash (1977) were considered inconclusive by an expert Gene-Tox panel because of the inadequate sample size used and possible variability confounding the interpretation (Lee *et al.*, 1983).

6.2 Chromosome Aberration Studies

Several studies are available for the induction of chromosome aberrations in mammal systems using both *in vitro* and *in vivo* assays. Although the *in vitro* results have been conflicting, the majority of available *in vivo* data indicate that atrazine is not clastogenic (chromosome breaking), particularly in the bone marrow or in germ cells of the mouse. The few positive findings found *in vitro* tests are likely the result of cellular toxicity or stress and not a direct DNA mechanism of action. It should be noted that *in vitro* cytogenetic assays tend to have a relatively high frequency of sporadic positive responses that are usually associated with toxicity or other nonmutagenic events (*e.g.*, high osmolality, low pH) (Brusick *et al.*, 1998).

6.2.1 *In Vitro* Assays

Atrazine did not produce chromosomal aberrations in Chinese hamster cells (Ishidate, 1988). A marginal increase in chromosome aberrations (less than a doubling in the response over background, and may be within the variation of background) was reported in human peripheral blood lymphocytes up to 1.0 μg/mL (Meisner *et al.*, 1992; Meisner *et al.*, 1993). By contrast, Lioi *et al.*, 1998, reported a large increase in the incidence of chromosome aberrations in human blood peripheral blood lymphocytes was reported at a similar dose (Lioi *et al.*, 1998). It should be noted that agents that are mutagenic/clastogenic generally induce sister chromatid exchanges (SCE's) at lower doses. Thus, in the study by Lioi *et al.* (1998), it is unusual that such a strong dose-related response for chromosome aberrations was accompanied by only a marginal increase in SCE's (which was not dose-related) over the same concentration range (5 to 51 μM). Other studies using human peripheral blood lymphocytes, found atrazine to be negative up to a concentration of 10 μg/mL (Dunkelberg *et al.*, 1994) or up to 50 μg/mL (Kligerman *et al.*, 2000a) for SCE induction.

The lack of a clear SCE response suggests that the Lioi *et al.* positive findings for chromosome aberrations may reflect cellular toxicity or stress. Lioi *et al.* state that their positive chromosomal aberration response for atrazine "...indicated an induction of a pro-oxidant state of the cells as an initial response to pesticide exposure." given the increase found for glucose 6-phosphate dehydrogenase activity in exposed cells. Positive results were reported using flow cytometry methods in Chinese hamster ovary cells (Rayburn and Biradar, 1995.) This study is flawed and considered inconclusive because the method of cell lysis and staining of the nuclei used by the authors may have introduced artifacts. Flow cytometry analysis (which essentially measures the distribution of DNA between cells undergoing mitosis) is not as reliable as direct cell analysis by microscopy for evaluating clastogenicity. Many factors can alter the flow cytometry results, such as cleanliness of the machine, flow rate, cell number, air bubbles, incomplete cytolysis, incomplete RNA'ase digestion and sample preparation, thus making it different to interpret induced genetic changes by a chemical versus induced artifacts due to study conduct.

EPA NHEERL conducted an *in vitro* cytogenetic study on atrazine (as well as simazine and cyanazine) to resolve the contradictory cytogenetic findings reported in the literature on human peripheral blood lymphocytes. No induction of chromosomal aberration or SCE's was found in human peripheral blood lymphocytes after exposure to atrazine up to a dose of 50 μg/mL (Kligerman, *et al.*, 2000a).

6.2.2 *In Vivo* Assays

Atrazine administered in drinking water at 20 μg/mL (20 ppm), was found to be negative in a 30- and 90-day mouse ($B6C3F_1$ males and females) study using metaphase analysis to evaluate the incidence of chromosomal aberrations (Meisner *et al.*, 1992; Roloff *et al.*, 1992). It should be noted that bone marrow cytogenetic evaluations with out chromosome painting are insensitive for chronic studies. Atrazine tested negative in a mouse (Tif:MAGf) bone marrow assay evaluating micronuclei induction (Ceresa, 1988a). The study by Ceresa (1988a) consisted of two parts. In the first phase, both sexes of mice were dosed with a single gastric intubation of 2250 mg/kg atrazine in carboxymethyl cellulose, with animal sacrifices at 16, 24 or 48 hours following treatment. In the second phase of the study, both sexes of mice were treated with a single dose of atrazine at 562.5, 1175 or 2250 mg/kg with bone marrow cells harvested 24 hours post-treatment.

More recently, Gebel *et al.* (1997) examined a variety of herbicides, including atrazine in the mouse bone marrow micronuclei assay. In this study, NMRI mice of both sexes were gavaged with several doses of atrazine dissolved in corn oil up to 1750 mg/kg, and 48 hours later the animals were sacrificed. The results from this study showed that atrazine only induced a small increased in micronuclei in female mice at a dose of 1400 mg/kg that is approximately 80% of the LD_{50}. It should be noted that this is a very high dose of atrazine because at the next higher dose (1750 mg/kg), half the animals died (*i.e.*, it was the LD_{50}). The study authors state that *"(A)trazine... revealed significant aneugenic/clastogenic activities in the micronucleus test in vivo in female NMRI mice. However, these results only could be achieved in female animals at doses near to the maximum tolerated dose. Thus, an in vivo genotoxic potential for ... Atrazine seems questionable."* Although Adler (1980) cites a mouse bone marrow cytogenetic study in which atrazine was given by oral gavage and was found to be positive for clastogenetic effects at 2000 mg/kg (also a very high dose), no further details were provided to evaluate the acceptability of this finding. It should be noted that EPA's NHEERL found both atrazine and simazine to be negative for micronuclei induction in mice (Kligerman, *et al.*, 2000b).

Atrazine has been evaluated in dominant lethal assays for germ cell chromosomal damage. In a negative study by Hertner (1993), male mice were exposed via oral gavage up to 2400 mg/kg bw of atrazine. Males were mated sequentially with untreated virgin females at different days to allow evaluation of exposed male gametes at various germ cell stages of development. There were no significant increases for resorptions or dead fetuses at any dose. Although Adler (1980) cites a dominant lethal study in the mouse in which atrazine given by oral gavage caused an increase in dominant lethal mutations at 1500 and 2000 mg/kg, the lack of details precludes an independent assessment of the study.

6.3 Other Indicators of DNA Damage or Mutagen Exposure

Atrazine has been negative for the induction of unscheduled DNA synthesis (UDS) in rat hepatocyte cultures (Hertner, 1992; Puri and Muller, 1984). In the study by Hertner (1992), there was no evidence of UDS when hepatocytes from adult male Tif:RAIf rats were exposed *in vitro* to atrazine at several concentrations up to 1670 μg/mL for 16 to 18 hours (139 μg/mL was a precipitating concentration). In agreement, atrazine exposure did not induce UDS in the study by Puri and Muller (1984), which also used primary rat hepatocytes from adult male Tif:RAIf rats that were exposed to several concentrations of atrazine for five hours up to 150 µg/mL, where precipitation of the test article occurred.

Ribas *et al.* (1995) used the single cell-gel electrophoresis assay (SCGE, or the comet assay) to examine DNA strand breakage in human lymphocytes treated *in vitro* with several concentrations up to 200 µg/mL of atrazine for four hours both with and without S9 rat liver activation. In this study, atrazine was found to cause a marginal increase in alkaline labile sites only in the absence of mammalian liver S9 activation. The study authors refer to the atrazine results as a "weak positive" and noted: "The extent of DNA migration showed that only in cultures treated without S9 fraction there was a slight but significant increase and this took place only when the concentrations were high (100 and 200 µg/mL)." The weak positive findings by Ribas *et al.* were similar to the weak effect reported by another laboratory using the DNA alkaline elution assay to detect DNA strand breakage in stomach, kidney, and liver of Sprague-Dawley female rats treated with a single oral dose of 875 mg/kg of atrazine or with 350 mg/kg of atrazine given five or 15 successive days (Pino *et al.*, 1988). Another study

using an excision repair assay to evaluate atrazine for DNA damage in human lymphocytes up to a dose of 100 µg/mL, reported negative results without activation (Surrallés *et al.*, 1995). Although a positive finding was reported in another comet assay evaluating DNA damage in the tadpole, *Rana catesbeiana* (Clements *et al.*, 1997), it is uncertain whether this finding can be attributed to atrazine because the study was conducted with commercial formulation (Aatrex) that was of low atrazine purity (43%). It should be noted that Roundup was reported as positive in this study. The active ingredient in Roundup, glyphosate, is nonmutagenic when tested in standard genotoxicity assays (Flowers and Kier, 1978; Li, 1983; Shirasu *et al.*, 1978). Atrazine was also found to negative in an SOS chromotest (Ruiz and Marzin, 1997).

6.4 Mutagenicity Studies in Plants

Plant assays have yielded mix results. The induction of mutations but not chromosome aberrations have been observed in *Zea mays* (Morgun *et al.*, 1982). Conflicting results are reported in *Hordeum vulgare* for both mutation and chromosome aberrations (Wuu and Grant, 1966; Stroev, 1968; Muller *et al.*, 1972). The induction of chromosome aberrations is found when a high enough dose is evaluated in *Vicia faba* (Wuu and Grant, 1967). Although the metabolism in plants is qualitatively similar to that in mammalian, quantitative differences may exist in certain plant systems that may allow for expression of mutagenicity.

6.5 Metabolites of Atrazine

Metabolism of the triazine herbicides, atrazine, simazine and propazine, in mammalian species results primarily in chloro-s-triazine metabolites (Simoneux, 1995). The major pathway for metabolism of the triazine herbicides in plants is hydroxylation (Simoneux, 1995). The major plant metabolite is hydroxy-atrazine. Several standard mutagenicity studies on hydroxy-atrazine and a variety of these chloro-metabolites (Diaminochlortriazine, G-28279, and G-30033) have been consistently negative for mutagenicity, and thus do not appear to exhibit a mutagenic potential (summarized in Appendix Table 4).

Though not truly a metabolite of atrazine, the mutagenic potential of *N*-nitrosoatrazine is also discussed.

6.5.1 Diaminochlortriazine metabolite (DACT – 6-chloro-1,3,5-triazine-2,4-diamine; didealkyl atrazine)

DACT (the structure is shown in Figure 8-1) was negative in the *Salmonella*/Ames assay when evaluated up to the limit concentration of 5000 µg per plate in tester strains TA 98, TA100, TA1535, and TA1537 with and without metabolic activation from the S9 fraction of Aroclor-treated rats (Deparde and Karimi, 1987). In an UDS assay using isolated human fibroblasts, DACT was also negative up to 600 µg/mL (which exceeded the solubility limit of 400 µg/mL) (Meyer, 1987).

6.5.2 G-28279 metabolite - 6-chloro-N-ethyl-1,3,5-triazine-2,4-diamine; deisopropyl atrazine

G-28279 (structure is shown in Figure 8-1) was tested up to a concentration 5000 µg per plate and found to be negative in the *Salmonella*/Ames assay when evaluated in tester strains TA 98, TA100, TA1535, and TA1537 with and without metabolic activation from the S9 fraction of Aroclor-treated rats (Deparde, 1990). G-28279 was also negative for inducing UDS in exposed hepatocytes from adult male Tif:RAlf rats when tested up to a cytotoxic dose (800 µg/mL) (Gelnick, 1991a). G-28279 was tested at the maximum tolerated dose of 480 mg/kg without inducing an increase in micronuclei in bone marrow cells of exposed adult Tif:MAGf mice of both sexes (Ogorek, 1991a).

6.5.3 G-30033 metabolite - 6-chloro-N-(1-Methyl ethyl)-1,3,5-triazine-2,4-diamine; deethyl atrazine

G-30033 (structure is shown in Figure 8-1) was tested up to a concentration 5000 µg per plate and found to be negative in the *Salmonella*/Ames assay when evaluated in tester strains TA 98, TA100, TA1535, and TA1537 with and without metabolic activation from the S9 fraction of Aroclor-treated rats (Deparde, 1989). G-30033 was also negative for inducing UDS in exposed hepatocytes from adult male Tif:RAlf rats when tested up to a cytotoxic dose (1000 μg/mL) (Gelnick, 1991b). G-30033 was tested at the maximum tolerated dose of 480 mg/kg without inducing an increase in micronuclei in bone marrow cells of exposed adult Tif:MAGf mice of both sexes (Ogorek, 1991b).

6.5.4 Hydroxy-atrazine, G-34048

Hydroxy-atrazine (structure is shown in Figure 8-1) was negative when evaluated at concentrations up to 5000 μg/plate in Salmonella tester strains TA 98, TA100, TA1535 and TA1537 (Deparde, 1988). Tests were conducted in the presence and absence of mammalian metabolic activation S9 fraction of Tif:RAlf rats treated with Aroclor 1254. When hepatocytes from adult male Tif:RAlf rats were exposed to hydroxy-atrazine at concentrations up to 1500 μg/mL (precipitation seen at doses ≥12.5 μg/mL), no increase in UDS was found (Hertner, 1988). Hydroxy-atrazine was negative in a UDS assay in which human fibroblast cells were exposed *in vitro* to concentrations up to 1500 μg/mL under nonactivating conditions only (Meyer, 1988). *In vivo*, hydroxy-atrazine was tested up to the limit dose of 5000 mg/kg without inducing an increase in micronuclei in bone marrow cells of exposed adult Tif:MAGf mice (Ceresa, 1988c).

6.5.5 *N*-Nitrosoatrazine

N-Nitrosoatrazine (NNAT) can be formed *in vitro* when atrazine and nitrite are mixed at an acid pH (Wolfe, *et al.*, 1976). Because nitrites and atrazine can be found together in drinking water, the hypothesis has been advanced that NNAT can be formed in the acid pH found in the stomach. The formation of NNAT in the stomach *in vivo* has yet to be demonstrated.

The genotoxicity of NNAT has been tested in the Ames assay, V-79 mutation assay, newt micronucleus test, and a clastogenicity assay using human lymphocytes.

Results of a modified Ames assay (available only as an abstract) showed NNAT to cause an increase in revertants in the TA 100 and 98 strains with hamster S9 fraction at 525 μg/plate and 1 mg/plate, respectively (Weisenberger *et al.*, 1987). The study authors considered these results to be "mildly mutagenic." This abstract also noted that atrazine was tested and found to be nonmutagenic.

A V79 assay (available only as an abstract) was considered by the study investigators to produce results that indicated that NNAT was "strongly mutagenic" (Weisenberger *et al.*, 1988). This abstract also noted that atrazine was tested and found to be nonmutagenic.

A micronucleus assay using peripheral red blood cells (RBC) from newt larvae resulted in an increase micronuclei at doses of 7.5 and 15 ppm after a 12 day exposure while no increase in micronuclei was seen at 3.75 ppm (Haridon, 1993).

A clastogenicity assay in lymphocytes from normal human volunteers found that NNAT at doses as low as 0.0001 µg/mL produced significant elevations of chromosome break frequency and percent of cells with aberrations. A significant elevation of the mitotic index was not observed at 0.0001 µg/mL. Mitotic index was significantly increased at the next highest dose of 0.001 µg/mL (Meisner, *et al.*, 1993).

6.6 Close Structural Analogues: *Simazine and Propazine*

As discussed in Chapter 8, atrazine is an s-triazine pesticide and is closely related to simazine and propazine as 2-chloro-4,6-bis-(alkyamino)-s-triazines. To further explore the mutagenicity of atrazine, the available databases on propazine and simazine were also evaluated. The available studies are predominantly negative, and thus do not provide convincing evidence of a mutagenic potential for simazine or propazine. Although there were some positives reported they tended to be marginal, found at very high concentrations or were not reproducible.

6.6.1 Simazine

As summarized is in Appendix Table 6 simazine has been evaluated in bacterial for mutation including various *Salmonella* tester strains and found to be negative by several different laboratories even when metabolic activation was incorporated into the assay (Mersch-Sundermann *et al.*, 1988; Simmon *et al.*, 1977; Jones *et al.*, 1984; Seiler, 1973; Lasinski, Kapeghian, and Green, 1987; Fahrig, 1974). Simazine was negative for the induction of gene mutation and gene conversion in *S. cerevisiae* (Fahrig, 1974; Siebert and Lemperle, 1974; Simmon *et al.*, 1977; Jones *et al.*, 1984; Emnova *et al.*, 1987). Like atrazine, conflicting results were reported in plant and insect studies. Only one mammalian *in vitro* gene mutation assay was found for simazine that reported a weak positive in the presence of metabolic activation (Jones *et al.*, 1984). This study is considered inconclusive by an expert Gene-Tox panel (Mitchell *et al.*, 1997). Simazine is also negative at concentrations up to the solubility limit in the primary rat hepatocyte UDS assay (Hertner, 1992).

Most *in vitro* cytogenetic assays on simazine are inconclusive due to study design problems, but negative results have been reported for the induction of SCE's and chromosomal aberrations. To resolve the inconclusive cytogenetic literature, EPA NHEERL recently evaluated simazine in human peripheral blood lymphocytes with *in vitro* exposures up to 37.5 μg/mL (a dose at the limit of toxicity and solubility) and found no induction of either chromosomal aberrations or SCE's (Kligerman *et al.*, 2000b). *In vivo*, simazine was tested up to the limit dose of 5000 mg/kg without inducing an increase in micronuclei in the bone marrow of exposed adult Tif:MAGf mice of both sexes (Ceresa, 1988b). EPA's NHEERL found simazine to be negative when evaluated by the mouse micronucleus test (Kligerman, *et al.*, 2000b).

6.6.2 Propazine

The available genotoxicity studies on propazine are summarized in Appendix Table 7. Propazine has been negative in bacterial mutagenicity assays (Kappas, 1988; Shirasu, 1975). Propazine has also been evaluated *in vitro* for the induction of gene mutation (at the HPRT locus) in Chinese hamster lung cells (V79) under both activating and nonactivating conditions (Ciba-Geigy, 1986). Studies without metabolic activation exposed V79 cells for 21 hours up to 1000 µg/mL of atrazine. Studies with metabolic activation exposed V79 cells for five hours up to 2000 µg/mL of atrazine along with the S9 fraction from Arochlor-treated male rats. In the experiment without S9 activation, a weak positive response (dose-related) was found at concentrations 400 µg/l. An equivocal response that was not dose-related was seen in the experiment with S9 activation.

Propazine was negative for UDS when tested up to the solubility limit in (62.5 µg/mL) exposed hepatocytes from adult, male TiF:RAlf rats (Puri, 1984). Propazine was reported to be negative for chromosome aberrations in Chinese hamster cells *in vitro* (Ishidate, *et al.*, 1981; Ishidate 1983),and negative for the induction of micronuclei when tested up to 5000 mg/kg in adult female Chinese hamsters (Ciba-Geigy, 1984).

6.7 Summary and Discussion of Mutagenicity Data

The genetic toxicology database for atrazine shows consistent negative results for bacterial mutation assays. Beyond the bacterial tests, the database is heterogeneous and contains conflicting test responses. Reported positive responses tended to be weak and found at high doses of atrazine. No subset of data points clearly establishes a direct DNA reactive mode of action for atrazine associated with the carcinogenicity. Although the DNA damaging potential of atrazine can not be entirely dismissed in Drosophila and plants, these finding may be the result of species specific metabolism. Although some positive findings were reported for clastogenicity in mammalian systems, these responses were in conflict with other studies using the same assay approach and may be associated with toxicity. Data on several metabolites of atrazine and its close structural analogues (propazine and simazine) do not support a mutagenic potential for these chemicals.

In summary, the totality of evidence does not support a mutagenic potential for atrazine, and indicates that a direct DNA reactive/mutagenic mode of action is unlikely to be a component of atrazine-induced mammary gland neoplasia (or on tumor development at any other site).

N-nitrosoatrazine has been shown to be mutagenic in four different types of mutagenicity assays. However, the chemical reaction which generates NNAT has never been demonstrated to occur *in vivo*, and cancer bioassays in female Swiss mice and female Wistar rats failed to show a carcinogenic response following NNAT exposure (Weisenberger,1990 - abstract).

In conclusion, exposure to atrazine is not likely to pose a mutagenic hazard to humans, especially at lower exposure levels experienced by humans.

Chapter 7

7 Estrogenic Activity

Several studies employing both *in vitro* and *in vivo* assays are available concerning the potential of atrazine to act as an estrogen agonist *(i.e.*, mimic the effects of estradiol exposure). Some of the assays also test the ability of atrazine to function as a progesterone mimic. The results of these studies together provide evidence that atrazine does not have direct estrogenic effects. These studies are discussed below and their results are also summarized in Table 7-1.

7.1 *In Vivo* Assays

Tennant *et al.* conducted a study known as the uterotrophic response assay. This is an accepted procedure for measuring estrogenic activity (Korach and McLachlan, 1995). As an *in vivo* assay, it incorporates aspects such as metabolism, serum binding and pharmacokinetics. Exposure to an estrogenic agent increases the weight of the uterus by causing cellular proliferation of endometrial cells, leading to an increase in thickness of the uterine endometrium. Rats or mice are exposed to the suspected estrogenic agent for three or four days, sacrificed, and their uterine weight is compared to that of the control animals. Immature or OVX animals are used to decrease interference from endogenous estrogens.

In this study, OVX Sprague-Dawley rats were treated for three days with 20, 200 or 300 mg/kg of atrazine. An increase in uterine weights over controls was not found.

Tennant *et al.* (1994a) also evaluated atrazine for uterine thymidine incorporation. This test measures an increase in uterine cell proliferation. Rather than measuring proliferation of uterine cells by weighing the uterus, this test measures the incorporation of thymidine into cellular DNA prior to cell division.

In this assay, female Sprague-Dawley rats were fed radiolabeled thymidine in their diet and then exposed to 1, 10, 50, 100 or 300 mg/kg/day atrazine for three days. The atrazine-treated animals had less thymidine incorporation into uterine cells than did control rats.

Table 7-1. *In vitro* and *in vivo* Hormonal Studies with Atrazine

Study Type	Dose/Duration	Results
In vivo Uterine bioassay in OVX SD females[1]	Atrazine and DACT at 0, 20, 200 or 300 mg/kg for three days. Simazine at 100 and 300 mg/kg/day for three days	*An increase in uterine weight indicates estrogenic activity* **+ control** - estradiol at 2 µg stimulated uterine weight gain; **Atrazine**/DACT/Simazine - uterine weights in dose groups were similar to control weights
In vivo Progesterone receptor binding assay in OVX SD females [1]	Atrazine at 0, 50 or 300 mg/kg for three days followed by 5 µg/kg E2	*An increase in the ability of progesterone receptor (PR) to bind its agonist indicates estrogenic activity* **+ control** - estradiol at 2 µg increased PR binding of agonist; **Atrazine** - 300 mg/kg/day decreased the ability of PR to bind a PR agonist; **DACT** - 300 mg/kg/day decreased the ability of PR to bind a PR agonist; **Simazine** - 300 mg/kg/day decreased the ability of PR to bind a PR agonist
In vitro Uterine Thymidine Incorporation assay[1]	Atrazine at 0, 1.0, 10, 50, 100 or 300 mg/kg for three days followed by 0 15 µg of E2	*An increase in uterine thymidine incorporation indicates estrogenic activity* **+ control** - estradiol at 0 15 µg increased uterine thymidine incorporation; **Atrazine** - 300 mg/kg/day decreased uterine thymidine incorporation; **DACT** - 300 mg/kg/day decreased uterine thymidine incorporation; **Simazine** - 300 mg/kg/day decreased uterine thymidine incorporation
In vitro MCF-7 Cell Proliferation Assay[2]	Atrazine/simazine at 0 01, 0 1, 1.0, 10 and 100 µM for 11 days	*An increase in the proliferation of MCF-7 cells indicates estrogenic activity* **+ control** - 1 nM estradiol induced a two-fold increase in cell number; **Atrazine/simazine** - no increase in cell numbers was seen at any dose of these compounds
In vitro Gel-shift assay for PR-PRE complex[2]	Atrazine/simazine at 1 µM	*An increase in the retardation through an electrophoretic gel of a complex consisting of PR + PR agonist + PR DNA binding region, indicates estrogenic activity* **+ control** - 1 nM estradiol resulted in a large increase in the retardation of the complex through a gel; **Atrazine/simazine** -Movement of the complex through the gel was not altered by these compounds

In vitro Luciferase reporter gene assays in MCF-7 cells[2]	Atrazine/simazine at 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M	*An increase in luciferase activity indicates estrogenic activity* **+ control** - estradiol at doses as low as 10^{-12} M resulted in increases in luciferase activity **Atrazine/simazine** - no increase in luciferase activity was seen with either compound at any dose
In vitro Estrogen receptor competitive binding assay using uteri from rats that *had not* previously been exposed to triazines[3]	Atrazine/simazine/DACT at 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} for equilibrium conditions A variety of experiments were also run in which conditions favored triazine binding to the estrogen receptor. These were termed disequilibrium conditions.	*Displacement of estrogen from its receptor indicates estrogenic activity* No displacement of estradiol from its receptor was observed at any dose with any of the triazine compounds under equilibrium conditions Under conditions that favor triazine binding to the estrogen receptor (disequilibrium conditions) triazine will displace estrogen from its receptor.
In vitro Estrogen receptor competitive binding assay using uteri from rats *previously exposed* to triazines[3]	Atrazine/simazine/DACT at 50 and 300 mg/kg/day for two days to OVX SD females	*Displacement of estrogen from its receptor indicates estrogenic activity* At 50 mg/kg/day displacement of estrogen from it receptor was not significantly altered with any of the triazine compounds. At 300 mg/kg/day there was a significant decrease in estrogen binding with all three triazine compounds.
In vitro Yeast assay using transfected human estrogen receptor[4]	Both maximal (1.0x 10^{-9} M, in this system) and submaximal (2.5 x 10^{-9} M) concentrations of atrazine, and the chloratrazine metabolites alone and in varying combinations were tested	*An increase in β-galactosidase activity indicates binding of atrazine to the estrogen receptor* Nether atrazine alone, nor any of the many combinations of atrazine with the atrazine metabolites caused an increase in β-galactosidase activity.
In vitro Estrogen receptor mediated growth in yeast[5]	Atrazine and simazine at 10 µM	*Growth of the yeast indicates estrogenic activity* **+ control** - Yeast colonies exposed to estradiol at 1nM proliferated **Atrazine/simazine** - Yeast colonies exposed to atrazine and simazine did not proliferate.
In vivo Uterine peroxidase assay using uteri from female SD rats	Atrazine and simazine at 50, 150, and 300 mg/kg/day were exposed female SD rat through gavage for three days	*Uterine peroxidase activity indicates estrogenicity* **+ control**- estradiol at 10 µg/day results in a 10-fold increase in uterine peroxidase activity. **Atrazine/simazine** - neither compound at any dose increased uterine peroxidase activity.

[1]Tennant *et al.*, 1994a.; [2]Safe *et al* , 1995.; [3]Tennat *et al.*, 1994b.; [4]Graumann *et al.*, 1999; [5]Conner *et al.*, 1996.

7.2 *In Vitro* Assays

Safe *et al.* (1995) used MCF-7 cells, a human breast cancer cell line that proliferates best in the presence of estrogen, to evaluate the potential estrogenic activity of atrazine. In this method, cells are grown in culture medium that has been charcoal-filtered to ensure that it is free of estrogens. The assay is conducted by adding the suspected estrogenic compound to the cell's medium and measuring cell proliferation, usually by counting the cells.

MCF-7 cells exposed to 10 μM atrazine did not show increased proliferation over control cells in this study.

Tennant *et al.* (1994b) evaluated the ability of atrazine, simazine and diaminochlorotriazine (DACT, a metabolite of both atrazine and simazine) to bind to the rat uterine estrogen receptor. A uterine cytosol extract was prepared from female Sprague-Dawley rats. These preparations are expected to be rich in estrogen receptors. The uterine cytosol was incubated with radiolabeled estrogen and one of the test chemicals. After an appropriate incubation time, estrogen bound to its receptor was separated from unbound estrogen. High levels of unbound estrogen would indicate that one of the test compounds was competing with estrogen for binding to estrogen receptors.

Neither atrazine, simazine, nor DACT treatment was able to compete with estrogen for binding to the estrogen receptor. No competitive binding was apparent under conditions of equilibrium. Only when excessive amounts of triazines were used (10,000-fold molar excess), was a slight competitive binding observed. Atrazine, simazine and DACT were not considered, under the conditions of this study, to effectively compete with estrogen for binding to the estrogen receptor.

Tennant *et al.* (1994b) also used an assay approach similar to the one described above with the exception that the triazines were administered by oral gavage, and uterine slices were incubated with radiolabeled estrogen instead of cytosol extracts. The advantage of this method compared to the test without *in vivo* preincubation is that exposure to the triazine compounds is done *in vivo*.

In this study OVX female Sprague-Dawley rats were exposed to 50 or 300 mg/kg/day of either atrazine, simazine, or DACT for two days. The animals were sacrificed, uterine slices were prepared, and incubated with radiolabeled estrogen. Dosing at 300 mg/kg/day statistically reduced estrogen binding by 33% with atrazine, 39% with simazine and 24% with DACT. Dosing at 50 mg/kg/day reduced estrogen binding by 18% with atrazine, 21% with simazine and 13% with DACT, but values were not statistically-significant. At high doses, triazine compounds are able to bind to rat uterine estrogen receptors *in vivo*.

Conner *et al.* (1996) used the reporter gene assays to evaluate atrazine. In these experiments two constructs---a Gal4-HEGO chimeric receptor and a GAL4-regulated promoter containing five ERE's and the luciferase gene--were placed into the MCF-7 cell line. The luciferase gene is a reporter gene whose product can be easily measured because it emits light when cells are exposed to a compound that binds the estrogen receptor.

Treatment of these MCF-7 cells with estrogen in the nM range produced large increases in luciferase activity compared to controls. However, treatment with atrazine or simazine at doses as high as 10 µM failed to produce any increases in luciferase activity compared to controls. These experiments show that, in this system, atrazine and simazine fail to bind to the estrogen receptor.

Conner *et al.* (1996) evaluated atrazine estrogen-dependent growth yeast strain. The PL3 yeast strain requires for growth a medium supplemented with the amino acids histidine and leucine, and the pyrimidine base uracil. If these yeast cells were transformed so as to contain the human estrogen receptor, then estrogen could take the place of uracil and allow growth of this strain on histidine and leucine-containing medium. ER-positive PL3 cells in media supplemented with histidine, leucine and estrogen will show growth after only one day of culture and continue to proliferate for at least five days.

ER-positive PL3 cells supplemented with histidine, leucine and either 10 µM atrazine or 10 µM simazine show no proliferation even after five days of culture. This study demonstrates that, in a yeast cell expressing human ER, neither atrazine nor simazine have estrogenic effects.

Bradlow *et al.* (1995) and Safe and McDougal (1998) examined the 16α to 2-hydroxyestrone metabolite ratios after atrazine treatment. Estradiol forms many metabolites. It has been hypothesized by some investigators that the ratio of the 16 metabolites to the 2-hydroxyestrone (2-OHE) metabolites may be a factor in mammary carcinogenesis. It is believed that a high 16α to 2-OHE ratio may be correlated with mammary carcinogenesis (Telang *et al.*, 1997). Nevertheless, others question whether this ratio is truly predictive.

Bradlow *et al.* (1995), exposed MCF-7 cells to several different chemicals including atrazine. MCF-7 cells were placed in medium containing [16α-^{3}H] estradiol (to determine 16α metabolite levels) and [2-^{3}H] estradiol (to determine 2-OHE metabolite levels) and atrazine at 1×10^{-5} M. Incubation was for 48 hours following which measurements of the various metabolites were made. The 16α to 2-OHE ratio in the atrazine exposed cells was approximately seven to nine times higher than the negative control and three to four times higher than ratios seen with the known mammary carcinogen dimethyl benzanthracene (DMBA).

Safe and McDougal (1998) exposed MCF-7 cells to several different chemicals including atrazine. Exposure to atrazine was at 1×10^{-5} M for 48 hours followed by another 48 hours of atrazine plus [16α-^{3}H]estradiol or 48 hours of atrazine plus radiolabeled [2-^{3}H]estradiol. The ratio of 16αmetabolites to 2-OHE metabolites was not increased following exposure to atrazine (in fact it was somewhat decreased compared to negative controls).

The results with atrazine in this assay have been contradictory; one group of investigators (Bradlow *et al.*, 1995) reported an increased ratio, supportive of potential carcinogenicity, while another group (Safe and McDougal, 1998) could not confirm these results.

Tran *et al.* (1996) used a yeast strain transfected with the human estrogen receptor to evaluate atrazine. A yeast cell line was transfected with the human estrogen receptor linked to the *lacZ* gene. This system is similar to that used by Conner *et al.* described above; the major exceptions were that the cells are yeast rather than MCF-7, and the reporter system is β-galactosidase rather than luciferase. To examine the role of different domains of the estrogen receptor, these investigators also transfected a gene encoding for the human estrogen receptor minus the first 179 amino acids.

Atrazine, cyanazine, simazine and the DACT metabolite were used in concentrations ranging from 207 nM to 2075 nM for atrazine, simazine and DACT, and up to 10,000 nM for cyanazine. All exposures included estradiol at either 20 or 0.5 nM (referred to the by the study authors as maximal and sub-maximal concentrations, respectively). With both the full and the truncated receptor, no concentration of triazine, in combination with either 0.5 or 20 nM or estradiol, resulted in β-galactosidase activity greater than what was seen with estradiol alone.

The experiments done with the complete estrogen receptor plus 0.5 nM estradiol (but not 20 nM) showed that all four triazines resulted in less β-galactosidase activity than with estradiol alone. Studies with the truncated receptor showed that at all triazine doses with both 0.5 and 20 nM estradiol, β-galactosidase activity was not altered compared to estradiol alone. Under none of the conditions tested did any of the triazines have estrogenic activity. Anti-estrogenic activity was seen, but only with the submaximal concentration and not the maximal concentration of estradiol. Since, the anti-estrogenic activity was not seen in the experiments done with the truncated receptor, it can be assumed that the amino-terminus of the estrogen receptor is responsible for the anti-estrogenic activity.

Experiments by Graumann *et al.* (1999) used also employed yeast that had been transfected with DNA coding for the human estrogen receptor linked to a β-galactosidase expression system. Only the full receptor was used.

Yeast were exposed to atrazine or atrazine plus the atrazine metabolites Desethyatrazine (DE; 6-chloro-N-ethyl-1,3,5-triazine-2,4-diamine) and Desisopropylatrazine (DI; 6-chloro-N-(1-Methyl ethyl)-1,3,5-triazine-2,4-diamine) over a variety of concentrations and combinations. Atrazine alone, atrazine plus DE, atrazine plus DI and all three together were used. Like the Tran *et al.* study, both maximal (1.0×10^{-9} M, in this system) and submaximal (2.5×10^{-9} M) estradiol concentrations, along with varying concentrations and combination of triazines, were used.

In agreement with the data of Tran *et al.* (1996), no concentration or combination of triazines at either maximal or submaximal conditions resulted in an increase in β-galactosidase activity. In contrast to the Tran paper, no concentration or combination of triazines at either maximal or submaximal conditions resulted in a decrease in β-galactosidase activity either.

The Tran and Graumann papers, using similar systems, found that none of the triazine compounds tested possessed intrinsic estrogenic activity. The results in regards to anti-estrogenic activity are contradictory with one study (Tran) finding anti-estrogenic activity and one study (Graumann *et al.*,1999) not finding such activity.

A study has been published which examines the upregulation of aromatase activity by atrazine. Aromatase - also known as cytochrome P450 19 (CYP19) - is the enzyme responsible for the conversion of androgens to estrogens (specifically, the catalysis of androstenedione to estrone and testostrone to estradiol). An upregulation of CYP19 activity would be expected to result in increased serum estrogen levels as an increased amount of androgens are converted to estrogens. An increase in CYP19 activity has been observed following atrazine treatment in a human adrenocortical carcinoma cell line - H295R (Sanderson *et al.*, 2000).

7.3 Special Carcinogenicity Bioassay Study (Morseth, 1998)

As discussed in Chapter 5, in a two-year oncogenicity study (Morseth, 1998), groups of estradiol-implanted OVX and intact female SD rats were exposed to 97.1% atrazine at doses of 0, 25, 50, 70 and 400 ppm. The primary purpose of this study was to examine the effect of ovariectomy on mammary tumor development in the SD rat following atrazine exposure. This study also provides information concerning the potential estrogenic activity of atrazine in relation to mammary carcinogenesis.

If atrazine induces mammary tumors by acting directly on mammary epithelium, then OVX animals might be expected to develop mammary tumors as do intact animals. Removal of the ovaries of an animal should not have affected the ability of atrazine to affect mammary epithelial cells and commence carcinogenesis. However, the difference in mammary tumor incidence between OVX and intact animals was striking. Not a single OVX animal in any dose group developed a mammary tumor of any type. In contrast, among intact animals, 38.3% of controls and 68.3% of the 400 ppm group developed mammary neoplasia.

Overall, the results from this study, showing a complete lack of mammary tumors in OVX animals, provides further evidence that atrazine does not induce mammary tumors by binding to and activating mammary tissue estrogen receptors. This evidence is also consistent with the conclusion that mutagenicity is not a key component of atrazine's carcinogenicity.

7.4 Noncancer Effects Relevant to Estrogenic Activity

A variety of bioassays using atrazine have been submitted to the Agency. Many of these assays measure parameters that might be expected to be altered were atrazine acting as an estrogenic agent. This section examines subchronic dog and rat studies; chronic dog studies; multi-generation reproduction studies in the rat; and developmental toxicity studies in the rat and rabbit, for alterations in parameters that may be indicative of a possible estrogenic effect.

Alterations in the chronic dog and subchronic rat and dog studies that were considered to possibly indicate an estrogenic effect were: changes in testes or prostate weights in males; changes in ovarian or uterine weight in females; histopathology findings in the testes (including seminiferous tubules) or prostate of males; histopathology findings in the ovaries, uterus, vagina or mammary gland of females.

Data in the multigeneration studies that were examined included: parental testes, ovary and uterine weights; parental histopathology findings in the testes or prostate of males; histopathology findings in the ovaries, uterus, vagina or mammary gland of female parents; malformations or variations of the testes and prostate of male offspring and the ovaries, uterus, vagina or mammary gland of female offspring; and, the ability of male offspring to impregnate females and the ability of females to become pregnant and deliver healthy pups.

In the developmental toxicity studies, data from visceral examinations were examined for malformations or variations of the gonads in offspring. In addition, the ratio of male to female offspring was examined. Maternal organ weights and histopathology were not determined in these studies (which were conducted under the Subdivision F guidelines). Thus, maternal organ weight and histopathology data are not available to analyze for potential estrogenic effects.

In addition to examining data from the above mentioned atrazine bioassays, data from bioassays with simazine and the atrazine/simazine metabolites DACT, G-28279, and G-30033 were also scrutinized for potential estrogenic effects.

7.4.1 Subchronic Dog Studies

Subchronic studies with atrazine and propazine in the dog are not available, but subchronic dog studies using simazine, G-28279 and G-30033 are available. Prostate and uterine weights were determined in the G-28279 and G-30033 studies, but not in the simazine or DACT studies.

Simazine. Testes weights in male Beagle dogs were decreased (46% decrease in absolute testes weight and 27% decrease in testes weight relative to body weight, compared to controls) in male Beagle dogs exposed to the high dose of 133.6 mg/kg/day of simazine. Ovary weights in females were not significantly altered at any dose including the high dose tested of 136 mg/kg/day. No increases in histopathology findings in either the male testes or prostate or the female ovaries, uterus, vagina or mammary gland were observed in dose groups compared to controls (Tai *et al.*, 1985a)

G-28279. Testes weights in male Beagle dogs exposed to the two highest doses tested in this study of 18.9 or 33.4 mg/kg/day were decreased. Compared to controls absolute testes weight decreased 31.4% at 33.4 mg/kg/day and testes weight relative to brain weight was decreased 36.7%. The decreases compared to controls in the 18.9 mg/kg/day group were 22.85 and 25.3% for absolute testes weight and testes weight relative to brain weight. Prostate weight in these two dose groups were also reduced compared to controls. The percent reductions at the high dose were 60.2% and 62.6% for absolute weight and prostate weight relative to brain weight. The reductions in the 18.9 mg/kg/day group were 51.55 and 51.9% for absolute weight and for prostate weight relative to brain weight. Ovary or uterus weights in the females of any dose group were not altered compared to controls. No increases in histopathology findings in either the male testes or prostate or the female ovaries, uterus, vagina or mammary gland were observed in dose groups compared to controls (Thompson *et al.*, 1992).

G-30033. Male testes and prostate weights were not significantly altered in Beagle dogs exposed to a high dose 28.85 mg/kg/day. Females ovary weights were not altered in females exposed to a high dose of 32.18 mg/kg/day. Both absolute uterine weights and uterine weight relative to brain weight were significantly decreased in the high-dose females. However, these changes were deemed by the study reviewer to likely be secondary to body weight loss rather than a direct effect of G-30033 exposure. No increases in histopathology findings in either the male testes or prostate or the female ovaries, uterus, vagina or mammary gland were observed in dose groups compared to controls (Rudzki *et al.*, 1992).

DACT. Male and female Beagle dogs used in this study showed no significant alteration in testes or ovary weight up to the high doses of 24.1 and 32.7 mg/kg/day for males and females respectively. There was no increase in histopathology findings in the female ovary, uterus, vagina or mammary gland in dose groups compared to controls. There was, however, an increased incidence of hypospermia and hypospermatogenesis in all four of the 24.1 mg/kg/day males, while no animal in any of the other group, including the controls, displayed these findings (Thompson *et al.*, 1990).

7.4.2 Subchronic Rat Studies

Subchronic studies with atrazine and propazine in the rat are not available, but subchronic rat studies using simazine, G-28279 and G-30033 are available.

Simazine. Absolute testes weights were decreased (15.4%) in male SD rats exposed to the high dose of 276 mg/kg/day of atrazine while testes weights relative to body weight were not altered compared to controls. Absolute ovary weights in the females exposed to either 142 or 276 mg/kg/day were reduced by 20% and 40%, respectively, while relative ovary weights were not altered compared to controls. No increases in histopathology findings in either the males testes or prostate or the female ovaries, uterus, vagina or mammary gland were observed in any dose groups compared to controls (Tai *et al.*, 1985b).

G-28279. Male SD rats showed increased testes weights relative to body weight at the high dose tested (34.9 mg/kg/day) in this study. Relative testes weights were increased 139% compared to controls, but absolute testes weights at this dose were within 5% of control values. Ovary weights were not significantly altered at any dose. No increases in histopathology findings in either the testes or prostate or the ovaries, uterus, vagina or mammary gland were observed in any dose groups compared to controls (Schneider, 1992).

G-30033. Testes and ovary weights of the RAlf rats used in this study were not significantly altered even at the high does of 35.1 for males and 38 mg/kg/day for females. No increases in histopathology findings in either the males testes or prostate or the female ovaries, uterus, vagina or mammary gland were observed in dose groups compared to controls (Gerspach, 1991).

DACT. Sprague-Dawley rats were exposed to doses of DACT of up to 34.1 mg/kg/day for males and 40.2 mg/kg/day for females. Testes weights relative to body weight were increased 22% at 34.1 mg/kg/day and absolute testes weights were increased 6.6% compared to controls. No significant alterations in ovary weights were noted at any dose. No increases in histopathology findings in either the male testes or prostate or the female ovaries, uterus, vagina or mammary gland were observed (Pettersen *et al.*, 1991).

7.4.3 Chronic Dog Studies

Chronic (12-month) dog studies with atrazine DACT, and simazine are available. Chronic dog studies with propazine, G-28279 and G-30033 are not available.

Atrazine. The Beagle dogs used in this study showed no significant alteration in testes or ovary weights up to the high dose used of 33.65 and 33.8 mg/kg/day for males and females, respectively. There were no histopathology findings in the testes, prostate, ovaries, uterus, vagina or mammary gland (O'Connor *et al.*, 1987).

Simazine. Doses of up to approximately 43 mg/kg/day did not alter either absolute or relative testes or ovary weights in beagle dogs exposed to simazine for one year. No increases in histopathology findings in either the male testes or prostate or the female ovaries, uterus, vagina or mammary gland were observed in dose groups compared to controls.

DACT. The Beagle dogs used in this study showed no significant alteration in testes or ovary weights up to the high dose used of 24.1 and 32.7 mg/kg/day for males and females, respectively. There was no increased incidence of histopathology findings in the female ovary, uterus, vagina or mammary gland in dose groups compared to controls. There was an increased incidence of hypospermia and hypospermatogenesis in two of the four 24.1 mg/kg/day males, but not in any other group, including the controls (Thompson *et al.*, 1990).

7.4.4 Multi-Generation Reproduction Studies

Atrazine. Sprague-Dawley rats were exposed to atrazine at concentrations up to a high dose of 39 mg/kg/day for males and 42.8 mg/kg/day for females. Testes weight relative to body weight was significantly increased in both parental generations at the high dose (11.1% increase in the F_0 generation and 11.1% increase in the F_1 generation). Although slightly decreased compared to controls, absolute testes weights were within 10% of control values. Ovary weights were not significantly altered compared to controls. There was no increased incidence of histopathology findings in the testes, prostate, uterus, vagina or ovaries (histopathology was not done on the mammary gland) at any dose in either generation of parental animals, compared to controls. There were no necropsy or histopathology findings in the male offspring of atrazine-treated dams that would indicate excessive maternal exposure to estrogens (Mainiero *et al.*, 1987).

Simazine. Sprague-Dawley rats were exposed to simazine at concentrations up to a high dose of 28.89 mg/kg/day for males and 34.96 mg/kg/day for females. Testes weight relative to body weight was significantly increased in both parental generations at the high dose (11.5% increase in the F_0 generation and 20% increase in the F_1 generation). Absolute testes weights were within 10% of control values for both parental generations at this dose. Ovary weights relative to body weights were significantly increased in the F_1 parental generation only at the high dose. Absolute ovary weights in both parental generations at all doses, were not significantly altered compared to controls. There was not an increased incidence of histopathology findings in the testes, prostate, uterus, vagina or ovaries (histopathology was not done on the mammary gland) at any dose in either generation of parental animals, compared to controls. There were no necropsy or histopathology findings in the male offspring of atrazine-treated dams that would indicate excessive maternal exposure to estrogens (Epstein *et al.*, 1991).

Propazine. Sprague-Dawley rats were exposed to doses of up to 50 mg/kg/day of propazine in this three-generation study. F_0 paternal testes weights relative to body weight, at the high dose only, were significantly increased (15.6%) compared to controls. Absolute testes weights at this dose (and every other dose) in this generation were within 5% of control values. Testes weight relative to body weight in the F_1 generation was not significantly altered though it was increased 16.6% in the high-dose groups compared to controls. Absolute testes weight in all dose groups was within 5% of control values. Testes weights relative to body weights were significantly increased (22%) compared to controls in the high-dose of the F_2 generation parents. Absolute testes weights were within 5% of control values for all other dose groups tests in this generation. Ovary weights in the first two generations were not significantly different, either absolutely or relative to body weight, than control ovary weights. Ovary weights in the F_2 generation parents were significantly decreased (27.6%) at the mid-dose of 5 mg/kg/day. The ovary weights at the other two doses in this generation were higher than the ovary weights at 5 mg/kg/day and were within 10% of control values. There was not an increased

incidence of histopathology findings in the testes, prostate, uterus, vagina or ovaries (histopathology was not done on the mammary gland) at any dose in any generation of parental animals, compared to controls. There were no necropsy or histopathology findings in the male offspring of atrazine-treated dams that would indicate excessive maternal exposure to estrogens (Jessup, 1979).

7.4.5 Rat Developmental Toxicity Studies

Atrazine. Pregnant Sprague-Dawley rats were exposed to up to 100 mg/kg/day of atrazine from days six to 15 of their pregnancies. There were no findings, in the visceral examinations, of any malformations or variations in the gonads at any dose. The sex ratios in all the dose groups were similar to the control sex ratio (Ginkis, 1991).

Simazine. Pregnant Sprague-Dawley rats were exposed to up to 600 mg/kg/day of simazine from days six to 15 of their pregnancies. There were no findings, in the visceral examinations, of any malformations or variations in the gonads at any dose. The sex ratios in all the dose groups were similar to the control sex ratio (Mainiero *et al.*, 1986).

Propazine. Pregnant Sprague-Dawley rats were exposed to up to 600 mg/kg/day of propazine from days six to 15 of their pregnancies. There were no findings, in the visceral examinations, of any malformations or variations in the gonads at any dose. The sex ratios in all the dose groups were similar to the control sex ratio (Fritz, 1976).

DACT. Pregnant Sprague-Dawley rats were exposed to up to 150 mg/kg/day of DACT from days six to 15 of their pregnancies. There were no findings, in the visceral examinations, of any malformations or variations in the gonads at any dose. The sex ratios in all the dose groups were similar to the control sex ratio (Hummal *et al.*, 1989).

G-30033. Pregnant Tif:RAI rats were exposed to up to 100 mg/kg/day of G-30033 from days six to 15 of their pregnancies. There were no findings, in the visceral examinations, of any malformations or variations in the gonads at any dose. The sex ratios in all the dose groups were similar to the control sex ratio (Gerspach, 1991).

G-28279. Pregnant Tif:RAI rats were exposed to up to 100 mg/kg/day of G-28279 from days six to 15 of their pregnancies. There were no findings, in the visceral examinations, of any malformations or variations in the gonads at any dose. The sex ratios in all the dose groups were similar to the control sex ratio (Marty, 1992).

7.4.6 Rabbit Developmental Toxicity Studies

Atrazine. Pregnant New Zealand White rabbits were exposed to up to 75 mg/kg/day of atrazine from days seven to 19 of their pregnancies. There were no findings, in the visceral examinations, of any malformations or variations in the gonads at any dose. The sex ratios in all the dose groups were similar to the control sex ratio (Arthur, 1984a).

Simazine. Pregnant New Zealand White rabbits were exposed to up to 200 mg/kg/day of simazine from days seven to 19 of their pregnancies. There were no findings, in the visceral examinations, of any malformations or variations in the gonads at any dose. The sex ratios in all the dose groups were similar to the control sex ratio (Arthur, 1984b).

Propazine. Pregnant New Zealand White rabbits were exposed to up to 50 mg/kg/day of atrazine from days seven to 19 of their pregnancies. One male offspring in the mid-dose group of 10 mg/kg/day was found to have absent testes. Otherwise, there were no findings, in the visceral examinations, of any malformations or variations in the gonads at any dose. The sex ratios in all the dose groups were similar to the control sex ratio (Knapp, 1995).

The bioassays described above provide little evidence of an estrogenic effect. Dosing in utero with estrogenic agents has been associated with findings such as cryptochidism, hypospadias and anorchism (Daston *et al.*, 1997; Danzo, 1998). Developmental toxicity studies in both the rat and rabbit and multigeneration reproduction studies in the rat, failed to show an increased incidence of these, or any other, abnormalities of the gonads. Multigeneration reproduction studies in the rat did not indicate that male offspring of atrazine-treated dams had any difficulties impregnating females although decreased fertility in males exposed to increased levels of estrogen or estrogen mimicking compounds in utero, has been described (Daston *et al.*, 1997; Danzo, 1998). Developmental toxicity studies in both the rat and rabbit and multigeneration reproduction studies in the rat, failed to show any alterations in sex ratios compared to controls. Ample evidence is available that estrogen exposure can result in germ cell depletion in the seminiferous tubules within a few weeks of commencement of estrogen administration (Blanco-Rodríguez and Martínez-García, 1996).

Subchronic rat and dog studies and chronic dog studies failed to show any histopathology alterations in the seminiferous tubules or in any part of the testes, or in the prostate. Excessive exposure to estrogen induces proliferation of the uterine endometrium. Subchronic rat and dog studies and chronic dog studies failed to show any histopathology alterations in the uterus, and, in the studies where uterine weights were determined, no alteration in uterine weights in response to triazine exposure were noted either. Excessive estrogen exposure might also be expected to result in alterations in the vagina - such as vaginal hyperplasia and vaginal wall thickening, or changes in the ovaries - such as an increase in ovarian stromal cells or enlarged ovaries. Subchronic rat and dog studies and chronic dog studies failed to show any histopathology alterations in the vagina or ovaries following exposure to the triazines. Ovarian weights were also not affected by triazine exposure.

The only potential endocrine alteration that is consistently seen in these bioassays is an alteration in either relative or absolute testes weight. This alteration is, however, somewhat variable. In seven rat studies and one dog study testes weights were increased; in two rat studies weight were decreased; in three dog studies and one rat study testes weights were not altered. Thus, in a total of 14 studies, testes weight was increased in eight studies, decreased in two, and not altered in four. Excessive estrogen stimulation would be expected to decrease testes weight.

The studies described above do not indicate an estrogenic effect of atrazine, simazine, propazine, DACT, G-30033, or G-28279. All the parameters examined for possible estrogenic effects were unaltered save one- testes weight. The testes weight alterations were variable, and the majority of the time they were altered, they were increased when one would expect that they would be decreased.

7.5 Overall Conclusions of Estrogenic Activity Data

Multiple studies that examine the estrogenic activity of atrazine, simazine and DACT have been reviewed by the Agency. Most, but not all, studies indicate that these chemicals do not possess estrogenic activity. Utertrophic response assays, uterine thymidine incorporation assays, MCF-7 cell proliferation assays, luciferase reporter gene assays in MCF-7 cells, *in vivo* progesterone receptor binding assays, uterine peroxidase assays, and estrogen receptor mediated growth in were all negative for estrogenic or progesterone activity of the triazine herbicides.

The few that yielded positive results were performed under conditions that favored an estrogenic effect of atrazine, or were performed at very high dose levels relative to the doses that induce mammary tumors in SD females.

A study examining atrazine's effects on aromatase activity indicated an upregulation of aromatase *in vitro* activity following atrazine treatment. The role this finding may play in bringing about an increase in serum estrogen *in vivo* is unclear as this study was conducted in a cell line and assays such as utertrophic response assays, and uterine thymidine incorporation assays, conducted *in vivo*, were negative.

An examination of the data from several bioassays (chronic, subchronic, developmental, and reproductive studies) employing atrazine, simazine, propazine and the major atrazine metabolites did not provide any evidence of an estrogenic effect resulting from exposure to these compounds.

Chapter 8

8 Structure Activity Relationship

Atrazine belongs to a class of compounds known as triazines in reference to the triazine ring structure that they contain. Several compounds containing triazine rings are presently registered for use in the U.S. as pesticides. Many of these pesticides (but not all) will have nitrogen atoms attached to carbon two and four. Compounds with this moiety are more appropriately referred to as "amino-s-triazines." These triazine-containing chemicals can be divided into a several classes of compounds: sulfonylurea-triazine compounds (that do not have the nitrogen at C2 and 4); alkyamino, alkythio-triazines, alkoxy-triazines and chloro-triazines. Atrazine is a chlorotriazine. The distinction between the chemical classes lies in the groups attached to the R1 position of the triazine ring (C6). Chloro-triazines will have a chlorine atom at the R1 position. The alkyamnio-triazines will have an alkyamnio moiety at R1 and the alkoxy will have an hydroxyl moiety at R1. Alkythio compounds will have an alkythio group at R1. Sulfonylurea compounds will have, at R1, a sulfonated urea moiety to which another structure, frequently a benzene ring, is attached. The structures of atrazine and several atrazine metabolites are shown in Figure 8-1. The structures of the amino-s-triazine ring itself and of chlosulfuron, a representative example of a sulfonylurea compound, are shown in Figures 8-2 and 8-3. The structures of the chloro-triazine pesticides simazine and propazine are shown in Figure 8-4.

Two-year bioassay studies using female Sprague-Dawley rats have been performed on several of the triazine compounds. As shown in Tables 8-1 and 8-2, (Only those studies that used Sprague-Dawley or CD rats, were submitted to EPA as guideline studies, and have undergone an EPA review are included in these tables) bioassay results demonstrate that the triazine ring structure, in and of itself, is not carcinogenic for mammary tumors in the female Sprague-Dawley rat (Spencer, 1991). Not shown in Tables 8-1 and 8-2 are the results from five two-year bioassays using sulfonyurea compounds. Four out of the five studies with sulfonyurea compounds were negative for carcinogenicity. The fact that four out of five sulfonyurea and three out of four alkyl amino, alkoxy, or alkythio-triazine compounds failed to induce tumors, including mammary tumors, in SD rats indicates that simply containing a triazine ring is not sufficient to render a compound carcinogenic. Chlorine at the R1 position seems to promote an increased carcinogenic potential. For example, all four of the chlorotriazine compounds are able to induce mammary tumors in female Sprague-Dawley rats. The

importance of a chlorine in the R1 position is further demonstrated by the lack of carcinogenicity seen with hydroxyatrazine (2-hydroxy-4-ethyl amino-6-isopropyl amino-s-triazine) (Chow and Hart, 1995). Hydroxyatrazine is a major plant metabolite of atrazine that differs from atrazine only in that the chlorine at R1 is replaced by a hydroxyl-group.

Table 8-1. Results of Two-Year Bioassays with Alkylamino, alkoxy, and alkythio-triazine Compounds

Chemical	Species/ Strain	Results	Reference
Cyromazine R1 - NH_2	Rat- Sprague-Dawley	Negative for oncogenicity in doses up to 3000 ppm	Blair, *et al.*, 1981
Prometryn R1- SCH_3	Rat- Sprague-Dawley	Negative for oncogenicity in doses up to 1500 ppm	Chau, *et al.*,1991
Terbutryn R1-SCH_3	Rat- CD®BR	Positive for female mammary tumors at 3000 ppm	Ciba-Geigy, 1980
Prometon R1- OCH_3	Rat- Sprague-Dawley	Negative for oncogenicity in doses up to 1000 ppm	O'Conner, *et al.*, 1988

Table 8-2. Results of Two-Year Bioassays with Chloro-triazine Compounds

Chemical	Species/ Strain	Results	Reference
Atrazine R1- Cl	Rat- Sprague-Dawley	Positive for female mammary tumors at 70 ppm	Mayhew, *et al.*, 1986
Simazine R1- Cl	Rat- Sprague-Dawley	Positive for female mammary tumors at 100 ppm	McCormick, *et al.*, 1988
Propazine R1- Cl	Rat- Sprague-Dawley	Positive for female mammary tumors at 3 ppm	Jessup, 1980a
Cyanazine R1- Cl	Rat- Sprague-Dawley	Positive for female mammary tumors at 5 ppm	Bogdanffy, 1990

The lack of carcinogenicity of the hydroxyatrazine metabolite is further supported by decisions reached by the HED Metabolism Committee (MARC) which concluded in a September 29, 1995 meeting that: "For atrazine, the residues of concern for cancer dietary risk are parent and chloro metabolites" (US EPA, 1995).

Figure 8-1. Structures of Atrazine and Major Metabolites

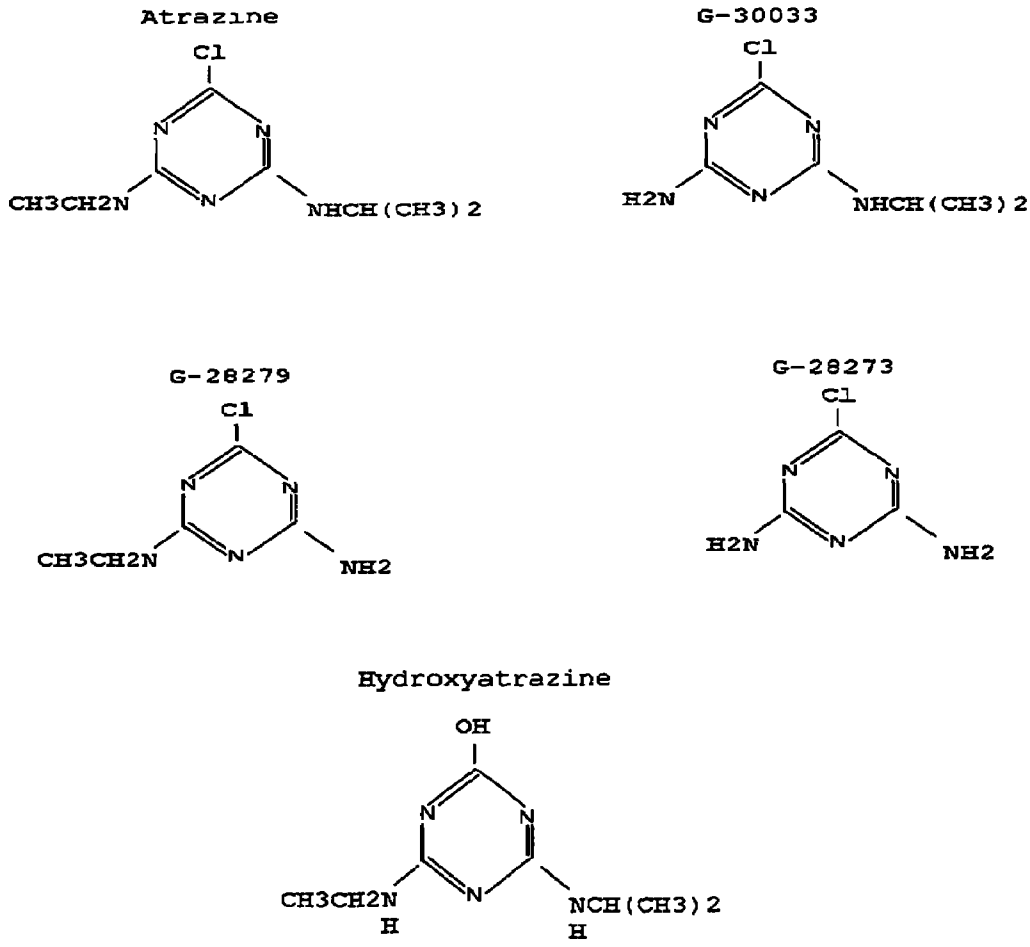
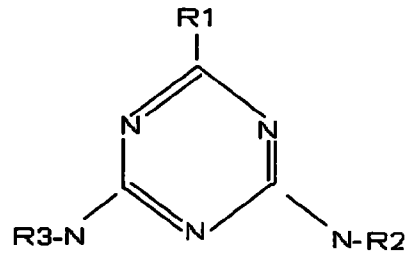
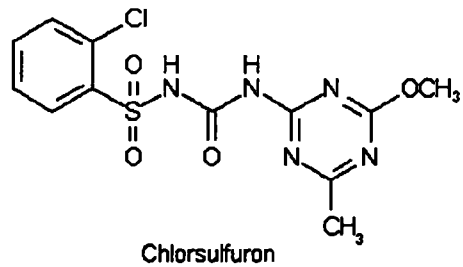


Figure 8-2. Structure of the Amino-s-Triazine Ring



Amino-s-Triazine Ring

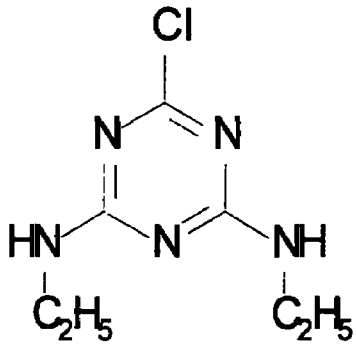
Figure 8-3. Structure of Chlorsulfuron



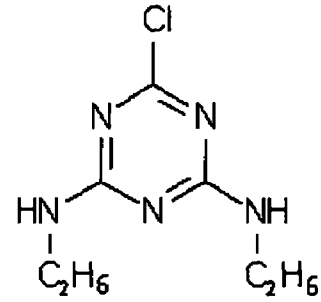
Chlorsulfuron

Figure 8-4. Structures of Simazine and Propazine

Simazine



Propazine



Of special interest when looking at structural analogues of atrazine is the functional similarity between those compounds most similar to atrazine (simazine, propazine and cyanazine) in regards to carcinogenicity. Like atrazine, all three of these compounds are positive for mammary tumors in the female Sprague-Dawley rat (see Table 8-2) and all three have been tested in two-year mouse bioassays and have been found to be negative for carcinogenicity (Hazelette and Green, 1988; Jessup, 1980b; Gellatly, 1981). Like atrazine, genotoxicity studies with simazine and propazine do not support a mutagenic potential for these compounds (see Chapter 6 - *Structural Analogs of atrazine* of this document). Mutagenicity studies with cyanazine have yielded mixed results, although the presence of the cyano group in cyanazine confounds comparison of this compound with the three other chloro-s-triazines relative to mutagenicity.

Chapter 9

9 Hormonal and Estrus Cyclicity Studies

The four previously described two-year bioassays in the SD rat clearly demonstrated that atrazine may increase mammary tumor incidence or decrease latency in the SD female. Atrazine exposure may also decrease pituitary adenoma latency in the SD female, but does not appear to alter pituitary tumor incidence. Reviews of numerous mutagenicity studies indicates that DNA damage does not appear to be contributing to these carcinogenic effects. Furthermore, results indicate that atrazine does not appear to be acting as a xenoestrogen to induce mammary tumors. The two-year bioassays with the CD-1 mouse and the F-344 rat demonstrated that there are strain/species differences in the carcinogenic effects of atrazine.

The lack of a mutagenic or exogenous estrogenic effect of atrazine, combined with the known hormonal dependence of rat mammary tumors, suggests that a perturbation of an endogenous hormonal mechanism may be responsible for the increase in mammary tumors and decreased latency of pituitary adenomas seen following atrazine exposure in the SD rat.

Mammary tumors and pituitary adenomas in the SD female are both very common occurrences. The historical control data shown in Table 9-1 demonstrates the high background tumor incidence rate for these tumor types in the female SD rat. The background tumor incidence rates of mammary tumors in SD males is <2% - much lower than in females (Lang, 1992; McMartin *et al.*, 1992). Pituitary adenomas, on the other hand, are very common in the SD male. Spontaneous pituitary adenoma rates are approximately 60% in SD males (Lang, 1992; McMartin *et al.*, 1992).

Table 9-1. Mammary Tumor and Pituitary Adenoma Historical Control Incidence Data For Sprague-Dawley Females At 24 Months

Source	Mammary Fibroadenoma	Mammary Carcinoma	Pituitary Adenoma
Pooled Charles River[1] mammary tissues from 1250 female rats in 19 studies examined	= 31.4% Range= 13 7-49.0%	= 17.68% Range= 7.1-31.4%	= 72.1% Range= 31.4-88.8%
Pooled Ciba-Geigy, Summit, N.J.[2] mammary tissues from 585 female rats in nine studies examined	= 31.3% Range= 20-43 3%	= 16.8% Range= 6.730%	=84.7% Range= 79.7-90%

[1]Lang, 1992
[2]McMartin *et al.*, 1992

It has been hypothesized that the effect of atrazine exposure is to decrease the latency of mammary cancer in the SD female rat (Stevens, 1994).

> "...it has been hypothesized that the lifetime administration of s-triazines to female Sprague-Dawley rats produces an endocrine-mediated imbalance, which causes precocious age-related changes, possibly resulting in the earlier onset or increased incidence of mammary tumors."

Implicit in this hypothesis is the belief that the high mammary tumor incidences seen in control SD females are due to the reproductive aging process in that strain. It is reasonable to assume the same mode of action proposed for mammary tumors (induction of an early onset of a state resembling reproductive aging and its associated hormonal imbalance) is also responsible for the early onset of pituitary tumors.

Pituitary tumors in the SD female are also known to be age-related (Blankenstein *et al.*, 1984; McComb *et al.*, 1984; Sandusky *et al.*, 1988). Pituitary tumors in the female rodent are known to be, at least in part, estrogen-dependent, and increased exposure to estrogens is associated with increased incidences of pituitary adenomas, hyperplasia and increased pituitary weights (Blankenstein *et al.*, 1984; McConnell, 1989a; Nelson *et al.*, 1980; Meites, 1981; McConnell, 1989b).

9.1 Rat Reproductive Aging Process

Mammary tumors are recognized as a common and expected occurrence in female SD rats, and appear to be increased primarily in aging animals. The age-related nature of the mammary tumors in female SD rats has been commented on by various authors (Cooper, 1983; Cutts and Noble, 1964). The age-related nature of the mammary tumors in SD females is illustrated by the mammary tumor incidences in control animals in the two-year oncogenicity studies described in this document. The great majority (75%) of the mammary tumors in control rats in the Thakur study (1991a) occurred when the rats were greater than one-year old. The other Thakur study (1992a) also had 75% of the mammary tumors in controls appearing after one year of age. A third two-year oncogenicity study found that 82% of the mammary tumors in control animals occurred when the animals were greater than one-year old (Morseth, 1998).

Understanding the hypothesis that the mechanism of reproductive aging in the female Sprague-Dawley leads to a high incidence of mammary tumors in females of that strain necessitates understanding the processes of reproductive aging in SD rats that are believed to lead to increased incidences of mammary tumors. At this point, a brief review and summary of these processes in the SD rat are presented. For comparisons sake, the reproductive aging process in the F-344 rat is also presented.

9.1.1 Sprague-Dawley

9.1.1.1 Alterations at the Ovary And Vagina

For reasons that are not yet completely understood, an aging female SD rat experiences a dampening of the preovulatory pituitary gonadotropin (luteinizing hormone, LH) surge (Zou, 1996; Cooper and Walker, 1979; Huang *et al.*, 1978). The preovulatory LH surge is responsible for inducing ovulation and, when the amplitude of the surge falls below a critical threshold, there is failure to ovulate. The ovaries of females with subthreshold LH surges will have reduced numbers of, or no, functional corpea lutea (CL), an increased number of secondary and antral follicles, and an increased number of follicles undergoing atresia (Smith and Conn, 1983; Cooper *et al.*, 1996; Huang and Meites, 1975). The increase in numbers of unruptured follicles results in prolonged exposure to

moderately elevated levels of serum estrogens as these follicles continue to secrete estrogen. Eventually, the unovulated follicles do undergo the process of atresia. Thus, the ovaries of aging SD rats will display increased numbers of atretic follicles. In the aging SD rat, each successive wave of follicles that undergoes atresia is replaced by a new crop that will again sustain the serum estradiol levels at a moderately elevated level.

Corpea lutea form from follicles that have ovulated and undergo the process of "lutenization" in which the granulosa cells of the follicle begin to secrete progesterone instead of estradiol. Because follicle ovulation is reduced in aging SD females, CL numbers will be reduced.

The vaginal smears of aging SD rats will consist primarily of cornified epithelial cells reflecting the tonic level of estradiol in the serum and the absence of, or low levels of, progesterone. The presence of vaginal cornification day after day as a result of the aging process is termed "constant estrus."

These changes typically begin to take place in a normally aging rat at approximately nine months of age. A typical estrous cycle in a young SD rat (< nine months in age) is four or five days in length. The first two days are diestrus, the third is proestrus and the fourth is estrus. Five-day cycles typically have an extra day of diestrus. During the aging process, cycles first become irregular and then the majority of the females transition into constant estrus. In the final months of the females life, she may become completely acyclic. Vaginal smears performed on animals at this time will indicate that the animals are in a state of extended or persistent diestrus.

The above described pattern should be considered a general rule only. The exact age at which these changes take place and their exact order may vary with the individual rat or with separate colonies of rats (LeFevere and McClintock, 1988).

9.1.1.2 Alterations at the Pituitary

Age-associated pituitary alterations have been well-described in the SD female. Pituitary weight, pituitary hyperplasia and pituitary adenomas have all been reported to be increased in aged female rats that undergo constant estrus as their primary mode of reproductive aging (SD and Long-Evans, for example) (Cónsole *et al.*, 1997; McComb *et al.*, 1984). A proliferation of the cells of the anterior pituitary that secrete prolactin (*i.e.*, lactotrophs) has been shown to be responsible for much of the increase in pituitary weight and hyperplasia seen in the aged female SD. The great majority of the pituitary adenomas seen in the aging female SD have also been found to have originated from lactotrophs (Sandusky *et al.*, 1988).

An increase in lacotroph number might be expected to result in increased serum prolactin levels in the aging rat as these cells continue to secrete prolactin. Increased serum prolactin levels are seen in aged SD females, and these increased serum prolactin levels have been correlated with increased pituitary weight, increased pituitary hyperplasia, and increased incidence of pituitary adenomas (Baird *et al.*, 1990; McComb *et al.*, 1986; van Putten *et al.*, 1988).

The alterations seen at the pituitary are believed to be due to the prolonged exposure to moderately elevated levels of serum estrogens that occur following anovulation (Nelson *et al.*, 1980; Goya *et al.*, 1990; McConnell, 1989b). Serum estrogen is known to be mitogenic to pituitary lactotrophs, and the prolonged exposure to moderately elevated serum estradiol levels likely mediates the increases in pituitary weight, hyperplasia and adenomas seen in the aging SD female. Additionally, estrogen appears to damage tuberoinfundibular neurons in the hypothalmus to inhibit production of prolactin inhibiting factor (PIF) (Sarkar *et al.*, 1982). PIF inhibits the production by the pituitary of prolactin. Thus, inhibition of its production by estrogen would have the effect of increasing prolactin production by the pituitary.

9.1.2 Fischer-344

9.1.2.1 Alterations at the Ovary and Vagina

Like the young-adult Sprague-Dawley female, the young-adult female F-344 typically displays regular four- to five-day estrous cycles. However, unlike the SD female, the ability of the aging F-344 rat to obtain an ovulatory LH surge is not compromised. Also, unlike the SD female that develops a pattern of constant estrus within the first year of life, the regular ovarian cycles present in the F-344 give way to a pattern of repetitive pseudopregnancies (Huang *et al.,* 1978; McConnell, 1989a; Estes, 1982). In this condition, ovulation occurs periodically and the newly formed CL are maintained for extended periods (10 to16 days). There are two distinct changes in the pattern of hormone secretion that are present in the pseudopregnant female - increases in serum prolactin and serum progesterone. First, there are two, daily prolactin peaks that occur just before lights on and just before lights out. In response to the elevated prolactin levels, the ovarian CL are maintained. The functional CL are responsible for the elevated levels of progesterone present in these animals. In addition to CL, the ovaries of an aging F-344 will contain a moderate number of antral and secondary follicles, and a moderate number of atretic follicles. Again, these findings are in contrast to the SD female that will contain few, if any CL, many secondary and antral follicles, and many atretic follicles. Also, in contrast to the constant estrus female that is characterized by uninterrupted cornification (typical of the aging SD rat), the vaginal cytology of the pseudopregnant female is primarily leukocytic. This vaginal smear pattern is similar to that seen during the diestrus period of the estrous cycle and this pattern may be referred to as "persistent diestrus," although the term "repetitive pseudopregnancy" is also used, and is the more descriptive term. As was the case with the SD, in the final months of the F-344 females life, she may become completely acyclic. The same disclaimer about variability in SD rat reproductive aging process applies to the F-344. The description above can only be seen as a generality.

9.1.2.2 Alterations at the Pituitary

The alterations seen in the pituitary of the female SD are not seen in the aging F-344 rat. The incidence of pituitary adenomas in female F-344 rats at two-years of age is much less than the incidence of pituitary adenomas in female SD rats at two-years of age (Sandusky *et al.*, 1988). Increases in pituitary weights and pituitary hyperplasia are also not commonly seen in the aging F-344 female.

9.1.3 Summary of the Reproductive Aging Process in SD and F-344 Rats

Characteristics of reproductive aging in the female SD have been throughly studied (Cooper, 1983; Cooper *et al.*, 1996; LeFevere and McClintock, 1988; Meites *et al.*, 1980). The appeal of hypothesizing that these events may result in mammary carcinogenicity comes from the prolonged exposure to estrogen and prolactin that results from these events. As noted above -- the increased days spent in estrus in the aging SD female appears to be related to the attenuated LH surge, which results in numerous unovulated ovarian follicles constantly producing estrogen. The prolonged exposure to estrogen acts at the pituitary to stimulate increased prolactin production.

The role of prolonged or inappropriate exposure to estrogen and prolactin in mammary carcinogenicity in rats has been well-established (Nandi *et al.*, 1995; Russo *et al.*, 1990; Cutts and Noble, 1964; Meites, 1972). It is reasonable to hypothesize that the prolonged exposure to estrogen and prolactin that results from the normal reproductive aging process in SD females may be responsible for the high levels of mammary tumors normally seen in this strain. This hypothesis was first advanced as long ago as 1966 (Durbin *et al.*, 1966).

The mode of reproductive aging in the F-344 rat is different from that of the SD female. Reproductive aging in the F-344 does not result in prolonged estrogen exposure. Prolactin levels are increased in the aging F-344, as are progesterone levels.

A summary of the different modes of reproductive aging in the SD, and F-334 is shown in Figure 9-1.

Figure 9-1. Summary of the Reproductive Aging Process in Different Rat Strains

F-344

- Normal cycle is a four to five days with 25% of the time spent in estrus, 25% spent in proestrus and 50% spent in diestrus;
- Reproductive aging becomes evident at approximately 12 months;
- Reproductive aging is characterized by increased prolactin surges that leads to maintenance of the corpea lutea;
- There is an increase in the days spent in diestrus, and increased exposure to progesterone.
- In very aged animals, acyclicity is common

Sprague-Dawley, Long Evans, Wistar[1]

- Normal cycle is a four to five day cycle with 25% of the time spent in estrus, 25% spent in proestrus and 50% spent in diestrus;
- Reproductive aging becomes evident at approximately nine to 12 months,
- Reproductive aging is characterized by decreased gonadotropin surges that leads to maintenance of primary, secondary and antral ovarian follicles;
- An irregular cycling pattern develops followed by an increase in the days spent in estrus, and prolonged exposure to estrogen;
- Pituitary alterations such as increase in pituitary weight, increases in pituitary hyperplasia and pituitary -adenomas become common as the animal ages;
- Acyclicity develops in the final months of life
- Normal cycling → irregular cycles → prolonged estrus → acyclicity
  (begins around 5-6 weeks old) *(occurs in the last few months of life ≥ 21 months of age)*

[1]There may be temporal differences between and among strains

9.1.4 Strain Differences in Reproductive Aging and Mammary Tumors

Evidence supporting the hypothesis that the normal reproductive aging process in the SD female leads to mammary tumors in this strain can be found by comparing the hormonal environment associated with reproductive aging and mammary tumor historical control values in the F-344 to the SD. Direct experimental evidence implicating the reproductive aging process of the SD is also available and is discussed below.

9.1.4.1 The Different Hormonal Environments of the SD and F-344 Rats During Reproductive Aging

The hormonal environment of the normally aging SD female is one of increased exposure to both estrogen and prolactin. The hormonal environment of the normally aging F-344 rat is one of increased exposure to prolactin and progesterone.

Increased or prolonged exposure to estrogen or prolactin is implicated in mammary carcinogenesis. Female rats that have been implanted subcutaneously with pellets of estrogen have very high incidences of mammary tumors and an earlier onset of those tumors than would be expected (Cooper, 1983; Cutts and Noble, 1964). Animals that have had pituitaries implanted under their renal capsule[2] or have been given lesions in the arcuate nucleus-median eminence of the hypothalmus[3] also have high incidences and earlier onset of mammary tumors (Welsch *et al.*, 1970a and 1970b). In contrast, increased or prolonged exposure to progesterone does not increase the incidence or decrease the onset of mammary tumors in the rat (Welsch, 1987).

[2]Implantation of pituitaries under the renal capsule results in high levels of prolactin secretion from the implanted pituitaries

[3]Lesions in this area of the brain result in production of high levels of pituitary prolactin.

Increased exposure to either estrogen or prolactin increases mammary tumor risk in the rat, while increased exposure to progesterone - in the absence of increased exposure to either estrogen or prolactin - does not increase mammary tumor risk. Progesterone can, in fact, be anti-carcinogenic. The ratio of estrogen to progesterone is an important aspect to consider when examining mammary carcinogenesis. Progesterone has been shown to "oppose" estrogen. This means that high levels of progesterone can counteract high levels of estrogen and reduce the increased risk of mammary cancer seen with high serum estrogen levels. The molecular mechanism by which progesterone opposes estrogen's actions appears to be by down-regulation of mammary, hypothalamic and pituitary estrogen receptors (Mauvais-Jarvis *et al.*, 1987; Libertun *et al.*, 1979; Cho *et al.*, 1993; Brann *et al.*, 1988).

9.1.4.2 Different Mammary Tumor Historical Control Values in the SD and the F-344

The Thakur studies described above show the low incidence of mammary tumors in control F-344 females. Charles River Laboratories has reported relatively low historical control rates for female F-344 mammary carcinomas (1.5%) and fibroadenomas (12%) while control rates for other types of mammary tumors are even lower (Lang, 1990). A recent report of the spontaneous neoplasms seen in the National Toxicology Program (NTP) two-year bioassays confirms a low mammary carcinoma rate for F-344 females (3.1%), but finds that the fibroadenoma incidence rates are now 41.2% after having increased from 28% in 1985 (Haseman *et al.*, 1998). Hazelton Labs reports the historical control incidence of F-344 female mammary carcinomas (adenocarcinomas and carcinomas combined) as 3.8% and the rate of fibroadenomas as 14.9% (Hazelton Labs, 1994). The values for background mammary tumors in the F-344 are much lower than in the SD (with the exception of adenocarcinomas in the NTP studies). These differences in background mammary tumor incidences could be due the different mechanisms of reproductive aging.

Historical control data from rat strains other than the SD and F-344 confirms that animals whose predominant mode of reproductive aging is constant estrus have higher background mammary tumor incidences than F-344. Table 9-2 displays the mode of aging in several strains of rats and the historical control incidence of mammary tumors in each strain.

Table 9-2. Relationship of Reproductive Aging and Mammary Tumor Incidence In Various Rodent Strains

Strain/ Species	Reproductive Aging	Spontaneous Mammary Tumor Incidence	References
SD/rat	Constant estrus	~30% fibroadenoma ~12% carcinoma	Aging – Estes, 1982 Tumors – Lang, 1992
Wistar/rat	Constant estrus	~25% fibroadenoma ~13% carcinoma	Aging – Mora *et al.*, 1994a; Mora *et al.*,1994b Tumors – Walsh and Poteracki, 1994
Long-Evans Hooded/rat	Constant estrus	~50% with either fibroadenoma or carcinoma	Aging – Estes, 1982 Tumors – Cooper, 1983
F-344/rat	Pseudopregnancy	~12% fibroadenoma ~2% carcinoma	Aging – Estes, 1982 Tumors – Lang, 1990

9.1.5 Studies in Which Premature Aging Was Artificially Delayed or Induced and How Mammary Tumor Incidences Were Affected

Experimental evidence is available that examines the relationship between reproductive aging and mammary carcinogenesis.

9.1.5.1 Mammary Tumor Onset Is Delayed in Rats Fed a Diet Supplemented with L-tyrosine

Supplementing the diet of the female Long-Evans rat with the amino acid L-tyrosine will delay the onset of constant estrus (Cooper and Walker, 1979). These studies showed that approximately 95% of the female Long-Evans rats on regular diets will be in constant estrus by 13 months of age while 60% of the females fed L-tyrosine will still be cycling normally at 16 months of age. Mammary tumor onset in the Long-Evans females fed L-tyrosine supplemented diets was also delayed. Mammary tumor incidence in control LE females was 67% by 19 to 21 months of age. No female fed an L-tyrosine supplemented diet had a mammary tumor at 19 to 21 months. Even at 25 months of age, L-tyrosine supplemented animals tumor incidence rates are only 25%.

The implication from these findings is that the delay in the appearance of mammary tumors is due to the delay in the onset of constant estrus (*i.e.*, the delay in reproductive aging).

9.1.5.2 Mammary Tumor Onset Is Accelerated in Females Receiving High Levels of Estrogen Through Implants

The hormonal state of constant estrus rodents is one of moderately elevated levels of serum estrogen and low levels of serum progesterone (Huang *et al.*, 1978). Ovariectomizing female rodents and then exposing them to a chronically high level of estrogen through subcutaneous implants of silastic tubing containing estrogen will mimic the hormonal state seen in constant estrus females and these females will develop mammary tumors much earlier than control animals (Cooper, 1983). The mammary tumor incidence rate at 12 months for Long-Evans females who were OVX at four months of age and given implants is 100% while the control rate is less than 5%.

9.1.5.3 Mammary Tumor Onset Is Accelerated in Females Whose Reproductive Aging Has Been Accelerated by Exposure to Constant Light

Rats exposed to constant light (rather than a light cycle such as 12 hours light/12 hours dark or 14 hours light/10 hours dark) show an earlier onset of constant estrus. Such rats show an increased incidence and earlier onset (latency was decreased by 34% compared to animals on light/dark cycles) of mammary tumors- primarily adenocarcinomas (Molina *et al.*, 1981).

9.1.6 The Correlation Between Increased Days in Estrus and Mammary Tumors in a Two-Year Bioassay

Estrous cycle evaluations were performed in a two-year bioassay using SD females (tumor data from this study - Morseth, 1998 - is discussed in section 5.3 while estrous cycle data is discussed below under section 9.2.3 – Results of Estrous Cycle Measurements in the SD rat - Thakur, 1999). These evaluations showed that the percent of total days spent in estrus was significantly higher in animals with mammary tumors than in those without. Tumor latency was also reduced in animals that spent a longer time in estrus.

9.1.7 Summary and Discussion of the Hypothesis that Mammary Tumors Are Induced by the Reproductive Aging Process

The hypothesis that the mode of reproductive aging in some strains of rat (including the SD) may contribute to mammary tumor formation has received attention for many years. The hypothesis is well established in that:

- Female SD rats undergo a mode of reproductive aging that results in extended periods of estrus;
- These periods of extended estrus result in prolonged exposure to moderately elevated serum estrogen derived from unovulated ovarian follicles;
- Serum estrogen acts at the pituitary to increase prolactin secretion;
- Increased exposure to estrogen and prolactin increases the risk of mammary carcinogenicity in rats.

The connection between the mode of reproductive aging and mammary carcinogenicity can be confirmed by examining rodent strains in that reproductive aging involves induction of a primarily constant estrus state. The estrous cycles of the SD, Wistar, and Long-Evans strain of rat all progress through a state of constant estrus as the animals age and all these strains have high background incidences of mammary tumors. The estrous cycle of the aging F-344 strain of rat is primarily one of pseudopregnancy and background mammary tumor incidences in the F-344 rat are low.

Experimental evidence showing the relationship between reproductive aging and mammary carcinogenicity is provided by studies in which the reproductive aging process in rats was either delayed or accelerated resulting in mammary tumor onset being delayed or accelerated. Delaying the onset of reproductive aging by feeding rats diet supplemented with L-tyrosine delayed the onset of mammary tumors. Accelerating the reproductive aging process by creating a hormonal state that mimics the hormonal state of reproductive aging results in mammary tumors appearing at a much earlier age. Accelerating the reproductive aging process by exposure to constant light also accelerates the onset of mammary tumors.

9.2 The Hypothesis that Atrazine Exposure Induces an Early Onset of Attenuated LH Surge, Increased Days In Estrus, and Prolonged Exposure to Estradiol

It is postulated that atrazine induces an early onset or increased incidence of mammary tumors by inducing an early occurrence of an attenuated LH surge, increased days in estrus, and prolonged exposure to estrogen and prolactin. The evidence testing this hypothesis can be found in six areas:

- Examination of the time-to-tumor in atrazine exposed rats;
- Examination of serum estradiol levels in atrazine exposed rats;
- Examination of alterations in the ovary (early onset of decreased CL, increased antral, secondary and atretic follicles) and vagina (early onset of increased days in estrus) of atrazine exposed rats;
- Examination of the pre-ovulatory LH surge in atrazine exposed rats.
- Examination of pituitary alterations in atrazine-exposed rats.
- Examination of mammary gland alterations that are indicative of prolactin exposure in atrazine-exposed rats.

9.2.1 Time-to-Tumor

Examination of the onset time of mammary tumors in female SD rats in the two-year bioassays (excluding the Mayhew study that is not amenable to this type of analysis) shows that atrazine exposure did induce an earlier onset of carcinomas (Tables 9-3 and 9-4). An early onset of fibroadenomas was less evident, but nevertheless, there also appeared to be an early of onset of this tumor type. Onset time was determined in these studies by examining the histopathology data and the clinical observations and correlating the appearance, by palpation, of each mass that was subsequently confirmed by histopathology to be a mammary tumor. Analysis of the onset time for mammary tumors in the two-year bioassays with structurally related s-triazines simazine and propazine, are also considered (Tables 9-5 and 9-6). Additional data concerning onset time can be obtained from a one-year study submitted by the registrant (Pettersen and Turnier, 1995).

Table 9-3. Time to Mammary Tumor in the Female SD Rat- Thakur (1992a) Terminal Sacrifice Protocol

	Dose (mg/kg/day) and Tumor Type					
	0 Fib	**3.79 Fib**	**23.01 Fib**	**0 Carc**	**3.79 Carc**	**23.01 Carc**
x̄ wk of appear	76.4	76.1	72 7	78 9	72.5	65.4
≤52wk	2/35 (5.7%)	1/27 (3.7%)	3/39 (7.7%)	0/14 (0%)	3/11 (27.3%)	6/18 (33.3%)
p-value	0.379	0.598	0.552	0.024*	0.072	0.021*
53-78 wk	16/35 (45.7%)	15/27 (55.6%)	18/39 (46.2%)	8/14 (57.1%)	3/11 (27.3%)	5/18 (27.8%)
p-value	0.414	0.304	0.578	0.099	0.138	0.094
79-104 wk	17/35 (48.6%)	11/27 (40.7%)	18/39 (46.2%)	6/14 (42.9%)	5/11 (45.5%)	7/18 (38.9%)
p-value	0.15	0.361	0.510	0.423	0.608	0.553

Notes: The dose shown is in mg/kg/day.

Fib= fibroadenomas. Carc= carcinomas and adenomas combined.

*$p \leq 0.05$

Table 9-4. Time to Mammary Tumor in the Two-year Morseth (1988) Study

	Dose (mg/kg/day) and Tumor Type									
	0 Fib	1.5 Fib	3.1 Fib	4.2 Fib	24.4 Fib	0 Carc	1.5 Carc	3.1 Carc	4.2 Carc	24.4 Carc
≥wk of appear	76.1	72 4	73.7	73.3	76.3	72.6	77 2	78.6	64.4	64.8
≤52wk	0/15 (0%)	1/18 (5.6%)	3/26 (11 5%)	1/26 (3.8%)	1/22 (4.5%)	1/11 (9 1%)	2/15 (13.3%)	0/14 (0%)	2/10 (20 0%)	6/23 (26.1%)
p-value	0.549N	0.546	0.244	0 634	0.595	0.047*	0.619	0.440N	0.462	0.252
53-78 wk	9/15 (60.0%)	11/18 (61.1%)	13/26 (50 0%)	14/26 (53 8%)	9/22 (40 9%)	5/11 (45.5%)	6/15 (40.0%)	7/14 (50.0%)	6/10 (60.0%)	7/23 (30.4%)
p-value	0.104N	0 614	0.386N	0.479N	0.211N	0.110N	0 548	0 570	0 410	0.315
79-104 wk	6/15 (40.0%)	6/18 (33 3%)	10/26 (38.5%)	11/26 (42.3%)	12/22 (54.5%)	5/11 (45.5%)	7/15 (46.7%)	7/14 (50 0%)	2/10 (20.0%)	10/23 (43 5%)
p-value	0.092	0.486N	0 590N	0.575	0.297	0.526	0.632	0.570	0.221N	0.600

NOTEs. The dose shown is in mg/kg/day.

Fib= fibroadenomas. Carc= carcinomas and adenomas combined

Trend for the dose is shown in the control column. "N" indicates that the trend is negative, otherwise trend is positive.
*p≤0 05

Table 9-5. Time to Mammary Tumor with Simazine in SD rats (McCormick *et al.*, 1988)

	Dose (mg/kg/day) and Tumor Type							
	0 Fib	0.52 Fib	5.3 Fib	63.1 Fib	0 Carc	0.52 Carc	5.3 Carc	63.1 Carc
wk of appear	86	81.7	89.6	68.8	83 4	80.7	82.1	65.9
≤52wk	1/23 (4.3%)	1/28 (3.6%)	0/18 (0%)	6/42 (14.3%)	2/18 (11.1%)	3/17 (17.6%)	2/20 (10.0%)	13/44 (29.5%)
p-value	0.029*	0.704	0.561	0.212	0.026*	0.472	0.656	0.110
53-78 wk	6/23 (26.1%)	7/28 (25.0%)	3/18 (16.7%)	26/32 (61 9%)	3/18 (16.6%)	4/17 (23.5%)	4/20 (20.0%)	18/44 (40.9%)
p-value	0.000**	0.590	0.370	0.006**	0.013*	0.466	0.563	0.059
79-104 wk	16/23 (69.6%)	20/28 (71.4%)	15/18 (83.3%)	10/42 (23.8%)	13/18 (72.2%)	10/17 (58.8%)	14/20 (70.0%)	13/44 (29.5%)
p-value	0.000**N	0.563	0.260	0 000** N	0.000** N	0.316 N	0.583 N	0.003** N

NOTEs: The dose shown is in mg/kg/day.

Fib= fibroadenomas. Carc= carcinomas and adenomas combined

Trend for the dose is shown in the control column. "N" indicates that the trend is negative; otherwise trend is positive.

*p≤0.05, **p≤0.01.

Table 9-6. Time to Mammary Tumor with Propazine in SD rats (Jessup, 1980a)

	Dose (mg/kg/day) and tumor type							
	0 Fib	0.2 Fib	6.4 Fib	68 Fib	0 Carc	0.2 Carc	6.4 Carc	68 Carc
wk of appearance	84	80.6	77.6	75.7	90.2	77.8	85.2	77.28
≤52wk	1/21 (4.8%)	0/21 (0%)	1/17 (5.9%)	0/19 (0%)	0/7 (0%)	0/15 (0%)	0/13 (0%)	2/25 (8.0%)
p-value	0.406	0.500N	0 701	0.525N	0.170	1.000	1.000	0.605
53-78 wk	4/21 (19.0%)	10/21 (47.6%)	8/17 (47.1%)	13/19 (68.4%)	2/7 (28.6%)	8/15 (53.3%)	4/13 (30.8%)	11/25 (44.0%)
p-value	0.005**	0.050	0.067	0.002**	0.430	0.268	0.664	0.389
79-104 wk	16/21 (76.2%)	11/21 (52.4%)	8/17 (47.1%)	6/19 (31.6%)	5/7 (71.4%)	7/15 (46.7%)	9/13 (69.2%)	12/25 (48.0%)
p-value	0.010*N	0.099N	0.065N	0.006** N	0.220N	0.268N	0.664N	0.254N

NOTEs: The dose shown is in mg/kg/day.

Fib= fibroadenomas. Carc= carcinomas and adenomas combined.

Trend for the dose is shown in the control column. "N" indicates that the trend is negative; otherwise trend is positive.

*p≤0.05; **p≤0.01.

9.2.1.1 Thakur, 1992a

The mean week of fibroadenoma onset in controls in this study was 76.4 weeks. The mean week of fibroadenoma onset in the 3.79 mg/kg/day and 23.01 mg/kg/day groups was 76.1 and 72.7, respectively. The mean week of onset for carcinomas was 78.9 in controls while the mean week of onset for carcinomas and adenomas in the 3.79 mg/kg/day and 23.01 mg/kg/day groups was 72.5 and 65.4.

The percentage of carcinomas and adenomas occurring in the first year of the study in controls was 0% while at 3.79 mg/kg/day and 23.01 mg/kg/day 27.3 and 33.3% of the carcinomas appeared in the first year of the study.

9.2.1.2 Thakur, 1991a

The percentage of fibroadenomas occurring in the first year of the study in the controls was 16.7. At 4.23 mg/kg/day and 26.23 mg/kg/day the percentage was 0 and 20%.

The percentage of carcinomas occurring in the first year of the study was 0 in controls and 33% at 4.23 mg/kg/day and 50% at 26.23 mg/kg/day.

9.2.1.3 Morseth, 1998

The mean week of fibroadenoma onset in controls in this study was 76.1 weeks. The mean onset in the 1.5, 3.1, 4.2 and 24.4 mg/kg/day groups was 72.4, 73.7, 73.3 and 76.3 weeks, respectively. The mean week of onset for carcinomas and adenomas in controls was 72.6 while the mean week of onset for the 1.5, 3.1, 4.2 and 24.4 mg/kg/day groups was 77.2, 78.6, 64.4 and 64.8, respectively.

The percentage of fibroadenomas in the control group that occurred in the first year of the study was 0. The percentage of fibroadenomas that occurred in the first year of the study in the 1.5, 3.1, 4.2 and 24.4 mg/kg/day groups was 5.6, 11.5, 3.8 and 4.5, respectively. The percentage of carcinomas and adenomas that occurred in the first year of the study was 9.1 and the percentage that occurred in the 1.5, 3.1, 4.2 and 24.4 mg/kg/day groups was 13.3, 0, 20, 26.1, respectively.

There was an increased mammary tumor incidence at the 53 week interim sacrifice, which also indicates and earlier tumor onset. There were no fibroadenomas at the 53 week sacrifice in the 20 females of the control group, but in the dose groups the fibroadenoma rates were 1/20, 2/19, 2/20, 1/20 in the 1.5, 3.1, 4.2 and 24.4 mg/kg/day groups, respectively.

9.2.1.4 Pettersen and Turnier, 1995

The percentage of animals with fibroadenomas in the controls was 5.9 and the percentage that occurred at the 0.8, 1.7, 2.8, 4.1 and 23.9 mg/kg/day dose groups was 5.9, 5.9, 0, 8.8, and 8.8, respectively. The percentage of animals with carcinomas and adenomas in the control group was 2.9 and the percentage that occurred at the 0.8, 1.7, 2.8, 4.1 and 23.9 mg/kg/day dose groups was 2.9, 2.9, 5.9, 5.9 and 14.7, respectively.

9.2.1.5 McCormick *et al.*, 1988 (simazine)

The mean week of fibroadenoma onset in controls in this study was 86 weeks. The mean onset in the 0.52, 5.3, and 63.1 mg/kg/day groups was 81.7, 89.6 and 68.8 weeks, respectively. The mean week of onset for carcinomas and adenomas was 83.4 for controls and 80.7, 82.1 and 65.9 for the 0.52, 5.3, and 63.1 mg/kg/day groups, respectively.

The percentage of fibroadenomas occurring in the first year of the study in controls was 4.3 and the percentage of the 0.52, 5.3, and 63.1 mg/kg/day groups occurring in the first year of the study was 3.6, 0, and 14.3, respectively. The percentage of carcinomas occurring in the first year of the study in controls was 11.1 and the percentage occurring in the dose groups was 17.6, 10 and 29.5, respectively.

9.2.1.6 Jessup, 1980a (propazine)

The mean week of fibroadenoma onset in controls in this study was 84 weeks. The mean fibroadenoma onset in the 0.2, 6.4 and 68 mg/kg/day groups was 80.6, 77.6 and 75.7 weeks, respectively. The mean week of carcinoma onset in controls was 90.2 and the mean week of onset for the 0.2, 6.4 and 68 mg/kg/day groups was 77.8, 85.2 and 77.3, respectively.

The percentage of fibroadenomas occurring in the first year of the study was 4.8 and the percentage of fibroadenomas occurring in the first year in the 0.2, 6.4 and 68 mg/kg/day group was 0, 5.9, and 0, respectively. The percentage of carcinomas that occurred in the first year of the study in controls was 0 and the percentage occurring in the dose groups was 0, 0, and eight, respectively.

9.2.2 Conclusions Of The Time-to-Tumor Data

The data from four two-year and one-year bioassays demonstrate that there is a decreased time-to-tumor for mammary tumors in the female SD rat following atrazine exposure. The earlier onset is more evident for carcinomas and adenomas than for the fibroadenomas. The mean week of carcinoma onset drops with atrazine exposure in both of the two-year studies with atrazine for which mean week of tumor onset is applied and also drops with exposure in the two-year bioassays with simazine and propazine.

The percentage of carcinomas occurring in the first year of the study is increased with exposure in three two-year bioassays with atrazine and is also increased in a one-year bioassay. Both the simazine and propazine studies showed an increase in percent of carcinomas and fibroadenomas occurring in the first year in dosed animals compared to controls.

9.2.3 Alterations in the Ovary and Vagina

9.2.3.1 Sprague-Dawley

Aging SD rats normally undergo alterations in their estrous cycles. These alterations are described above under the section 9.1 and are summarized in Figure 9.1. In brief, the alterations in a normally aging SD female rat are an increase in the percentage of days of the estrous cycle spent in estrus from 25% prior to nine months old to >40% after nine months old. The increase in days spent in estrus occurs at the expense of days spent in diestrus and proestrus.

Eldridge *et al.* (1993a) examined estrous cycle alterations in female SD rats in response to atrazine exposure. Care should be taken when reading this document not to confuse the study that is referred to as Thakur, 1991a and the study referred to as Eldridge, 1993a. The Thakur and Eldridge studies are, in fact, the same studies. The hormone and estrous cycle evaluation are referred to in this document as Eldridge, 1993a while the animal necropsy and histopathology portions of this study is referred to as Thakur, 1991a.

Histomorphologic evaluation of the ovaries and other tissues (including the vagina and mammary gland) from the Thakur/Eldridge studies was performed and is referred to as McConnell, 1995 in this document. Only the histomorphologic evaluation of the ovaries and the vagina are discussed in this section of the document. Discussed under section 9.2.5 is the histomorphic evaluation of the mammary gland of atrazine-exposed rats.

Therefore, the three citations -- Thakur, 1991a; Eldridge, 1993a; and McConnell, 1995 -- refer to different analysis performed on the same group of SD or F-344 rats as part of the same study.

Additionally, estrous cycles were evaluated in one month, six month and two-year studies -- Morseth, 1996a, 1996b, 1998). The protocol and measurements for estrous cycles in these studies are described below.

9.2.3.2 Fischer-344

Aging F-344 rats normally undergo alterations in their estrous cycles. These alterations are described above and are summarized in Figure 9.1. In brief, a state of pseudopregnancy or persistent diestrus becomes a common occurrence in F-344 rats as they progress beyond approximately nine months of age. The ovaries of animals in persistent diestrus contain CL, a moderate number of secondary and antral follicles, and moderate numbers of atretic follicles.

The same study that examined serum hormone levels in F-344 (Eldridge *et al.*, 1993b) also examined estrous cycle alterations in response to atrazine exposure. Doses and sacrifice schedules for these two studies were previously described. Histomorphologic evaluation of the ovaries and other tissues (including the vagina, mammary gland, and pituitary) from the Thakur/Eldridge studies was performed and is referred to as McConnell, 1995 in this document.

9.2.3.3 Protocol For Examination of the Ovaries and Estrous Cycle Measurements

Thakur Studies. Two separate studies were conducted: one with SD females and one with F-344 females. In both studies, 10 animals per dose group were sacrificed after approximately one, three, nine, 12, 15, and 18 months of exposure to atrazine. Two weeks before scheduled sacrifice daily vaginal smears were performed. The smears were examined for the presence of keratinized epithelium, nucleated epithelium and leucocytes. The presence of well-defined keratinized cells was taken to be indicative of estrus. The presence of leucocytes in the vaginal smears indicated diestrus and the presence of moderate- to- dense nucleated epithelium and moderate cornified epithelium was taken to be indicative of proestrus. Following the 14 days of smear collection animals were sacrificed at their next proestrus day. Thus, some animals were sacrificed on their scheduled sacrifice date (on day 15 after vaginal smears were begun) while others were sacrificed in the week following their scheduled sacrifice date (on days 16 to 21 following initiation of vaginal smears, depending on when their next proestrus phase occurred). Any animals that had not had a proestrus phase by day 21 after initiation of vaginal smears was sacrificed on day 21. Surviving animals were sacrificed at 24 months, again following the procedure described above whereby each animal is sacrificed on a proestrus phase, if possible.

Results of the estrous cycle evaluations were examined at: the dose groups at each individual timepoint compared to controls; for trend within dose at each individual timepoint; and the effect of treatment over time. These are the same parameters for which the hormone measurements were analyzed.

The ovaries of all females in these studies (both the SD and F-344 studies) were examined by standard histomorphologic techniques for several parameters including (but not limited to): absence of CL; reduced number of CL; presence of secondary and antral follicles; presence of atretic follicles.

The parameters of absence of CL and reduced number of CL were graded as simply being "present" or "absent." The parameters of secondary, antral follicles, and atretic follicles were graded on a scale of zero to five with zero indicating the parameter was absent and one to five indicating the parameter was present - the higher the number the more of the follicles were present.

Morseth, 1996a (one-month study). Vaginal smears were performed after seven days of treatment and continued daily for three weeks. The criteria for evaluation and classification of vaginal smears was similar to that followed in the Thakur studies.

Morseth, 1996b (six-month study). Vaginal smears were performed on the first day of treatment and continued daily for 14 consecutive days every four weeks. Thus, an animal was smeared in cycles of two weeks smearing followed by two weeks non-smearing. This cycle continued throughout the study. The criteria for evaluation and classification of vaginal smears was similar to that followed in the Thakur studies.

Morseth, 1998 (two-year study). Estrous cycles evaluations were performed on all intact females in this study (80 per dose group). Vaginal smears were performed for two consecutive weeks every two weeks starting on study week one. Thus, animals had two weeks of smears followed by two weeks without smears. The criteria for evaluation and classification of vaginal smears was similar to that followed in the Thakur studies. Estrous cycle data for the first 46 weeks of the study have been analyzed.

9.2.3.4 Results of Ovarian Histomorphology and Estrous Cycle Measurements in the SD Rat (McConnell, 1995 and Eldridge, 1993a)

The only study that performed histomorphologic examination of the ovaries of atrazine-treated SD rats was the Thakur, 1991a study. The histomorphologic data are presented in McConnell, 1995 while the vaginal smear data (for determination of phase in estrous cycle) are presented in Eldridge, 1993a.

Ovarian Histomorphologic Examination (McConnell, 1995). An early onset of anovulation is seen as early as three months following atrazine exposure. After three months of exposure the number of control animals with an absence of CL was zero of ten while the number of treated animals with no CL was one of ten at the 4.23 mg/kg/day group and two of ten in the 26.23 mg/kg/day group. This small increase in animals with no CL indicates that incidence of anovulation was increased in the dose groups. The fact that no CL were seen at all indicates that ovulation had not even occurred in the recent past, as even the CL from previous cycles in which ovulation did occur had regressed (*i.e.*, become corpus albicans). The number of animals at three months with reduced numbers of CL was increased from two in the control group to three at 4.23 mg/kg/day and three at 26.23 mg/kg/day. Reduced CL number also indicates anovulation, but in these animals ovulation likely did occur in the recent past as CL of some type were present. The antral follicle group mean graded index (antral

follicle score on the above described scale of zero to five divided by the number of animals in the group) was 1.2 for control, 2.1 for 4.23 mg/kg/day and 2.2 for 26.23 mg/kg/day. This increase in antral follicles again indicates anovulation.

Evidence of early onset of anovulation was also seen at nine months. After nine months of exposure the number of control animals with an absence of CL was six of ten while the number of treated animals with no CL was seven of ten at the 4.23 mg/kg/day group and ten of ten in the 26.23 mg/kg/day group. The antral follicle group mean graded index was 2.6 for control, 3.1 for 4.23 mg/kg/day and 3.8 for 26.23 mg/kg/day.

By 12 months of exposure the ovarian histomorphology indicated that nearly all the animals in all dose groups were not ovulating.

Estrous Cycle Evaluations (Eldridge, 1993a). Appendix Table 8 displays the results of the estrous cycle analyses done with the SD rat in Eldridge, 1993a.

The estrous cycle results seen in this study are what would be expected in the SD strain of rat. The percent days spent in estrus in control animals increases as the animals age at the expense of days spent in diestrus, and proestrus. Linear regression analysis indicated that the decrease in diestrus and proestrus and the increase in estrus over the period of one to18 months was statistically- significant at $p<0.01$. The % days in both diestrus and proestrus are also decreased in a statistically-significant manner ($p<0.01$) in both dose groups for the period from one to 18 months. Both dose groups showed a significant ($p<0.01$) trend toward an increased % days spent in estrus from months one to 18. Such an increase was seen in controls, is to be expected, and did not appear to be altered by exposure to atrazine.

The percent days of the cycle spent in estrus was dramatically increased in dosed animals compared to controls at nine, 12 and 18 months. Females in the 4.23 and 26.23 mg/kg/day groups at nine months spent an average of 34.3 and 44.8% of their days in estrus, respectively ($p<0.05$ at 70 and $p<0.01$ at 400 ppm). This is compared to 24% spent in estrus at the controls. The dose-related trend, as determined using a Terpstra-Jonckbeere Trend Test, showed a significant increase in % days spent in estrus ($p<0.01$). At 12 months there was also a dose-related trend ($p<0.05$) but the increases in dosed groups compared to controls as determined by ANOVA were not significant at either dose.

The percent days in proestrus is similar in dosed animals compared to controls for all timepoints. There is a significant trend ($p<0.01$) in both dose groups for a decrease in % days spent in proestrus over time. This decrease was seen in controls also and is to be expected. Atrazine exposure did not appear to effect this trend.

The percent days spent in diestrus was significantly decreased in dose groups compared to controls at nine, 12 and 18 months. Females at nine months spent a mean of 44.8% of the days of the estrous cycle in diestrus. Dosed females spent 36.2% ($p<0.05$ compared to control) for the 70 ppm dose and 25.9% ($p<0.01$ compared to control) of the days in diestrus. The dose-related trend at nine months was significant at $p<0.01$. At 12 months there was also a dose-related trend that was significant at $p<0.01$. The 4.23 mg/kg/day group, at 12 months, was decreased 14% compared to control, but this was not statistically-significant. The 24.23 mg/kg/day group at 12 months was decreased 28% compared to controls and this was significant ($p<0.05$). The trend at 18 months was decreased in a dose-related manner ($p<0.01$) while both the 4.23 and 24.23 mg/kg/day groups compared to controls by ANOVA were also statistically-significantly decreased ($p<0.05$, for both).

To summarize the estrous cycle data from this study:

- When analyzed for effect over time it was seen that animals in both dose groups and the controls exhibited an increase in % days in estrus and a decrease in % days in di- and proestrus as the study progressed. These alterations are to be expected in an aging SD female rat and exposure to atrazine did not appear to affect these parameters compared to controls.

- When analyzed for effect of dose it was seen that there was a significant increase, compared to controls, in percent days spent in estrus at nine, 12 and 18 months in both dose groups. The percent days spent in diestrus significantly decreased in both dose groups compared to controls.

9.2.3.5 Results of Estrous Cycle Measurements in the SD Rat (Morseth, 1996a and 1996b)

These studies examined the effect of atrazine on the estrous cycle and on plasma concentrations of the hormones LH and prolactin. The hormone measurement data from these studies will be discussed below. Preovulatory LH levels, and the estrous cycle evaluations will be discussed here. The studies were of one month (Morseth, 1996a) and six months (Morseth, 1996b) in duration.

One-Month Study. Female SD rats were exposed to 0, 2.5, 5, 40 and 200 mg/kg/day technical grade atrazine for 28 to 31 days. Ninety females per dose group were used. Dosing was by gavage once a day, at approximately the same time each day.

The results of the smears indicated an effect of atrazine exposure on vaginal cycling. Atrazine exposure induced a dose-dependent increase in the number of animals having irregular cycles. The nature of the irregular cycles was both an increase in estrous cycle blocks (two consecutive days in estrus) and an increase in diestrus blocks (four consecutive days in diestrus). The effects of atrazine on the estrous cycle in this study are most pronounced at 40 and 200 mg/kg/day with a statistically-significant increase in females displaying diestrus blocks at both 40 and 200 mg/kg/day and a statistically-significant increase in females displaying estrus blocks at 200 mg/kg/day only (p 0.05 for both diestrus and estrus blocks using pairwise comparison). These data are shown in Appendix Table 9.

Six-Month Study. Female SD rats were exposed, through the diet, to 0, 1.8, 3.65 and 29.4 mg/kg/day technical grade atrazine for six months. Ninety females per dose group were used.

A statistically-significant increase in percent days in estrus was evident as early as 3.5 months into the study in the high dose group, and in the mid-dose group by 5.5 months into the study. The low-dose group never experienced statistically-significant alterations in their estrus cycles. These data are shown in Appendix Table 10.

9.2.3.6 Results of Estrous Cycle Measurements in the SD Rat (Morseth, 1998)

The estrous cycle evaluations performed in this two-year bioassay demonstrate that atrazine-treated females display increased days in estrus sooner than control animals. All animals, irrespective of dose group, spent a normal amount of time in estrus (approximately 25% of the days spent in estrus) for the first 10 weeks of the study. By the 13 to 14 week measurement period though, the atrazine-treated animals began to display more days spent in estrus. These increases were most evident in animals of the high-dose group while, for most timepoints, the other three dose groups showed only marginal increases in percent days in estrus. The differences between the control and dose groups were most evident at week 25 to 26 where controls spent 53.7% of the days in estrus compared to 63.8, 59.7, 55.4, and 72.1% of the days in estrus for 1.5, 3.1, 4.2, and 24.4 mg/kg/day groups. Appendix Table 11 displays the percent days in estrus by dose group for all the time periods up to 46 weeks. Animals dosed with atrazine also showed an increase in the likelihood of having an estrus block of seven days during one of the three measurement periods in the 17-26 week interval. Again, this effect was most evident at the high-dose group, but was also seen at the other dose groups. Appendix Table 12 displays these data.

In addition to examining the amount of time spent in estrus by atrazine-treated animals compared to control animals, this study also examined the relationship between amount of time spent in estrus and mammary tumor incidence. There was a clear relationship between the amount of time spent in estrus and mammary tumor onset. These data are shown in Appendix Tables 13 and 14.

9.2.3.7 Results of Ovarian Histomorphology and Estrous Cycle Measurements in the F-344 Rat (McConnell, 1995)

The only study that performed histomorphologic examination of the ovaries of atrazine-treated Fischer rats was the Thakur, 1991b study. The histomorphologic evaluation data is presented as McConnell, 1995 while the vaginal smear data (for determination of phase in estrous cycle) is presented as Eldridge, 1993b. HED believes that the estrous cycle evaluation (vaginal smears) reported in Eldridge, 1993b are unreliable. Thus, these data are not reported. The histomorphology data from McConnell, 1995 are instead used to determine stage of the estrous cycle.

Ovarian Histomorphologic Examination in the F-344 (McConnell, 1995). The great majority of F-344 rats in all groups, both control and dose groups, maintained CL throughout most of the study. Only at the final, 24-month, timepoint were there dramatic decreases in CL numbers. The atrazine-treated animals at this timepoint did not show decreases in CL numbers any more severe than the control animals. The reduction in CL numbers at this late timepoint appears to be a consequence of a natural progression of the animals from persistent diestrus into acyclicity. All animals in all dose groups maintained moderate numbers of secondary, antral and atretic follicles throughout the study - including the 24-month timepoint.

Histomorphology to Determine Stage of the Estrous Cycle in the F-344 (McConnell, 1995). All animals in all dose groups appeared to maintain normal cycles through the first 12 months of the study. At the 15-month timepoint approximately half the animals in all dose groups (five of ten in control; three of ten at 0.68 mg/kg/day; 6 of 11 at 4.82 mg/kg/day; five of ten at 14.05 mg/kg/day, and five of ten at 34.33 mg/kg/day) were in a state of extended diestrus. The state of extended diestrus was indicated by the presence of increased vaginal mucification -- which is indicative of extended diestrus (McConnell, 1989a). At the 18-month timepoint approximately 70 to 80% of the animals in all dose groups displayed increased vaginal mucification. The persistent diestrus (or pseudopregnancy) that is characteristic in an aging F-344 was evident by 15 months and was quite common by 18 months. At 24 months the incidence of vaginal mucification was still high, but the previously mentioned reduction in animals with CL indicated that a progression towards acyclicity was occurring in the animals in this study.

9.2.4 Summary And Discussion From The Ovarian Histomorphology and Estrous Cycle Measurements In F-344 and SD Strains

Over the duration of the study, both strains of rat exhibited ovarian histomorphology and estrous cycles that would be expected for those strains. The F-344 maintained CL throughout most the 24-month study and showed an increase in days spent in diestrus (as indicated by vaginal mucification) at the post-12 month timepoints (McConnell, 1995). The SD showed decreased numbers (and frequently, a complete absence of) CL, increases in secondary, antral and atretic follicles, and an increase in percentage of days in estrus (as indicated by the vaginal smears) as the study progressed (Eldridge, 1993b; McConnell, 1995).

Atrazine exposure in the F-344 did not seem to alter estrous cycles; atrazine exposure in the SD increased the number of estrus and diestrus blocks after as little as one month of exposure (Morseth, 1996a). Three months of exposure increased the percentage of days spent in estrus and decreased the percentage of days spent in diestrus, but not significantly so (Thakur, 1991a). By nine months of atrazine exposure the percentage of days spent in estrus were significantly increased compared to controls and the percentage of days spent in diestrus was significantly decreased. The increase in estrus and diestrus blocks after one month of atrazine exposure indicates that these females were cycling irregularly. The increased days spent in estrus at three and nine months indicates that the atrazine-exposed animals in this study were entering constant estrus sooner than the control animals.

As noted above, reproductive aging is manifested in the estrous cycle first as irregular cycling, then as constant estrus, and finally as acyclicity/persistent diestrus. Atrazine exposure was able to induce irregular cycling in the SD females after only one month of exposure - when the animals in this study were about three months of age.

The Thakur, 1991a; Eldridge, 1993a, and the two shorter duration Morseth studies indicate that atrazine exposure can alter estrous cyclicity in the female SD rat. Atrazine exposure did not seem to effect estrous cyclicity in the F-344 rat (McConnell, 1995).

The estrous cycle data from the Morseth two-year study (described in Thakur, 1999) confirmed the findings from Eldridge (1993a) and the shorter duration Morseth studies (Morseth 1996a and 1996b). In this study atrazine exposure resulted in SD females spending an increased amount of time in estrus earlier than control animals. The high-dose group (26.23 mg/kg/day) females in the Thakur study showed a group mean of 44.8% of the days in estrus at nine months. The control animals reached this level of days in estrus, but not until 12 months when their group mean days in estrus was 42.6%. The high-dose (24.4 mg/kg/day) females in the Morseth, 1998 study had a group mean of 47.8% of the days in estrus during weeks 17 to 18. The control animals reached this level of days in estrus, but not until weeks 21 to 22 when their group mean days in estrus was 45.6%. All four studies together provide strong evidence that atrazine can disrupt estrous cycles in the SD female and can lead to an early onset of increased percent days in estrus compared to control animals.

The Morseth (1998) study examines correlations between days in estrus and mammary tumor incidence and onset and demonstrates that increased days in estrus early in the life of an animal decreases the time to onset of mammary tumors. This Morseth study also demonstrated that even animals who are not spending increased time in estrus during the early period of life, will show an increased risk of mammary cancer when chronically fed atrazine.

9.2.5 Serum Estradiol and Prolactin Levels

Given the postulated mode of action, it is important that one examine serum estradiol and prolactin levels and confirm that they are, in fact, altered following exposure to atrazine.

Serum hormone levels of estradiol (as well as progesterone, prolactin and corticosterone) in atrazine exposed SD female rats were examined in Eldridge, 1993a. Serum hormone levels of these four hormones were examined in F-344 females in Eldridge, 1993b.

Serum corticosterone was measured as an indicator of stress while estradiol, prolactin, and progesterone were measured because of their roles (estrogen and prolactin as promoters of and progesterone as a possible inhibitor of) mammary carcinogenesis.

Eldridge 1993a and 1993b are discussed below. The histomorphological evaluation of the mammary gland in atrazine-exposed SD rats is also discussed below (McConnell, 1995)

9.2.5.1 Protocol and Rationale for Hormone Measurement

Doses and sacrifice schedules for these two studies were previously described. The protocol and measurements for hormone levels in these studies are described below.

Blood was collected from the trunk of all animals at scheduled sacrifice. Two weeks prior to each scheduled sacrifice vaginal smears were performed. Animals were sacrificed during proestrus, if possible. If, after 21 days of vaginal smears, an animal was not in proestrus, then this animal was sacrificed, irrespective of what stage of the estrous cycle the animal was in. Blood samples were used for the determination of serum estradiol, progesterone, prolactin, and corticosterone. The hormones estradiol, progesterone, and prolactin all are important in the regulation and maintenance of normal reproductive functioning in the rat and could potentially play roles in mammary tumor pathogenesis. Corticosterone measurements were taken to test the hypothesis that dosing the animals with atrazine produces stress (and thus an elevation of serum corticosterone levels) that may play a role in mammary gland neoplasia.

Radioimmunoassay techniques were used to measure levels of each hormone. Standard curves were constructed and sample values were compared to the standard curve. The results of the measurements were examined for alterations in:

- each individual timepoint compared to controls;
- trend within dose, and;
- effect of treatment over time.

9.2.5.2 Results of Hormone Measurements -- F-344 (Eldridge, *et al.*, 1993b)

The results of the hormone measurements did not reveal any consistently statistically-significant alterations in serum hormone levels compared to controls for any of the hormones tested. There were occasional significant alterations such as significantly decreased ($p < 0.05$) progesterone levels in the 4.82 mg/kg/day dose and corticosterone levels in the 34.33 mg/kg/day group at the 12-month timepoint compared to controls; significant negative trends in estradiol levels at the 12-month timepoint, progesterone levels at the 12- and 18-month timepoints, and corticosterone levels at the 12- and 15-month timepoints, and prolactin levels displayed a significant positive trend at the three-month timepoint only. Careful consideration of these alterations indicated that they did not appear to be related to atrazine exposure.

There were alterations in serum hormone levels that were seen in control as well as treated rats. These were: decreases in estradiol levels in the later half of the study; significantly increasing progesterone levels from one to18 months. Decreased estradiol and increased progesterone are expected in rats undergoing a reproductive aging process involving pseudopregnancy (Huang *et al.*, 1978). Exposure to atrazine did not alter the age-related changes in estradiol or progesterone levels.

An increase in prolactin levels might be expected in an aging rat undergoing pseudopregnancy. However, consistent increases in serum prolactin levels were not seen.

9.2.5.3 Results of Hormone Measurements - SD (Eldridge *et al.*, 1993a)

Appendix Table 15 displays the results of the hormone measurements in the SD females.

Serum progesterone and corticosterone levels did not show any significant dose-related alterations compared to controls. Serum prolactin levels in dosed groups did not show any significant dose-related alterations with the exception of the 26.23 mg/kg/day group that did however, show a negative trend ($p<0.01$) over the nine to 18 month period and a positive trend at nine months. These alterations in serum prolactin likely were related to compound exposure.

Serum estradiol levels in the control rats in this study showed a positive trend (levels increased as time increased) over months one through nine. Exposure to atrazine did not alter this trend. The 4.23 and 26.23 mg/kg/day dose groups also showed a significantly positive trend from months one through nine. These trends are expected as constant estrus would be expected to begin to set in as these animals approach nine months of age. Examination of the pairwise comparisons at three months indicates that treated animals had an early onset of increased serum estradiol levels compared to controls. At three months control estradiol levels were 3.5 ng/mL, 70 ppm levels were 11.2, and 400 ppm levels were 16.2 ng/mL. The increase at 70 ppm was significant at $p<0.05$, the increase at 400 ppm was significant at $p<0.01$ and the trend, determined using a Terpstra-Jonckbeere Trend Test, was positive at $p<0.05$. At nine months control and 4.23 mg/kg/day group estradiol levels were similar, but the 26.23 mg/kg/day group compared to controls was increased 44%. At nine months estradiol levels were elevated compared to control, but not significantly so.

Prolactin levels were not altered either as a result of atrazine exposure or as result of aging in this study.

9.2.5.4 Results of the Histomorphologic Evaluation of the Mammary Gland - SD (McConnell, 1995)

The mammary gland is clearly a hormone-responsive tissue. Various tissues in the mammary gland contain receptors for the hormones estrogen, progesterone and prolactin and exposure to these hormones effects on these tissues. A detailed histomorphologic analysis of mammary gland (and other) tissues from the rats in the Thakur, 1991a study was performed. This histomorphologic analysis is referred to in this document as McConnell, 1995.

The mammary glands were examined for these alterations:

- Acinar development - indicative primarily of estradiol exposure
- Acinar/lobular development- indicative of both prolactin and progesterone exposure
- Secretory activity - indicative of prolactin, and to a lesser extent, estrogen and progesterone exposure
- Dilated ducts with secretion - indicative of prolactin, and to a lesser extent, progesterone exposure
- Galactocele (milk cyst) - indicative of prolactin, and to a lesser extent, progesterone exposure

The alterations in the tissues of the acinar region are indicative of estrogen exposure (ductal epithelial hyperplasia and acinar development). The alterations related to milk production are primarily prolactin-dependant (secretory activity, dilated ducts with secretion, galactocele).

Appendix Table 16 displays the results of the histomorphologic analysis of the mammary glands at months one, three, nine and 12 in the SD rats from Thakur, 1991a. Values at 15, 18 and 24 months are not shown as these values are similar to control values. The index weighted scores of several of the above listed parameters are shown in this table. An index weighted score assigns a numerical value to the severity of the finding assigned by the pathologist. The higher the index weighted score, the more severe was the finding in that group.

Increased prolactin exposure in the rat is associated with formation of galactoceles (milk cysts). Galactocele incidence and severity in this study are shown in Appendix Table17. The results of the histomorphologic analysis are described below:

Acinar Development. An early onset of increased exposure to estrogen is indicated by examination of the column of Appendix Table 16 labeled "Acinar Development." The index scores at the one-month timepoint are slightly higher in the dose groups than in the control, but a dose-response relationship was not seen. At the three-month timepoint the index scores are again increased over control. The increase is at this timepoint is dose-related though with the high dose being more severe than the low dose. At both nine and 12 months the index score again indicate more severe acinar development in the dose groups compared to the controls with the increase in severity being especially obvious at the high dose.

Acinar/Lobular Development. An early onset of this parameter is evident. The one- and three-month timepoints have index scores in the dose groups that are similar to the control index scores. The dose group index scores are clearly increased compared to controls at nine and 12 month timepoints though. This indicates that the atrazine-treated animals were exposed to elevated levels of prolactin at an earlier timepoint than the control animals.

Secretory Activity. The index scores for secretory activity also demonstrate an early onset of increased prolactin exposure in the dose groups compared to the controls. Index scores at one and three months in the dose groups were similar to control values, while index scores in both dose groups were clearly elevated compared to controls at the nine- and 12-month timepoints.

Dilated Ducts with Secretion. Index scores at one and three months in the dose groups were similar to control values. The index scores for the 4.23 mg/kg/day group compared to controls were only slightly elevated. The index scores for the 26.23 mg/kg/day group were greatly elevated compared to controls

Galactocele Incidence and Severity. No galactoceles were observed in any group at the one- and three-month timepoints. At nine and 12 months galactoceles in the dose groups were increased in both number and severity. By 15 months galactocele incidence and severity were similar between control and dose groups.

9.2.6 Summary And Discussion Of The Hormone Measurements and Histomorphologic Alterations In F-344 And SD Strains

Serum corticosterone levels were not altered in F-344 or SD female rats by atrazine exposure. Serum corticosterone levels reflect stress and the lack of any alteration in these levels indicates that the dosing, and more importantly, the regular vaginal lavages, were not causing the animals in these studies undue stress that may have compromised the results of these studies.

Atrazine exposure in the F-344 females did not alter serum hormone levels of estradiol, progesterone or prolactin. Both control and dose groups did see decreases in serum estradiol levels for the latter half of the study and a generally increasing level of progesterone for the one to 18 month period. These changes in estradiol and progesterone are to be expected in animals undergoing reproductive aging through pseudopregnancy. What was not seen, but would be expected in an animal undergoing reproductive aging through pseudopregnancy, were increases in serum prolactin levels.

Accurate serum prolactin levels from the rat can be difficult to obtain because many different factors can cause dramatic alterations in serum prolactin levels. For example, simple inadvertent stimulation of a female rats nipples can induce large increases in serum prolactin (Freeman, 1981). Prolactin levels are also very sensitive to stress. A rat in pseudopregnancy is especially difficult to obtain accurate serum prolactin measurements from as these animals will display twice-daily prolactin surges. Prolactin measurements from these animals will vary dramatically depending on whether or not a measurement is taken during a surge, and, if taken during a surge, at what point in the surge. Accurate prolactin measurements in a young, unmated rat, are easier to obtain as these animals have relatively static prolactin levels except for have one, or possibly two, prolactin surges every four days (a proestrus afternoon surge and, sometimes, a smaller surge on estrus) (Freeman, 1981; Butcher *et al.*, 1974).

Despite these difficulties, it is still surprising that increased prolactin levels were not measured in this study in the aging animals.

Atrazine exposure in the SD females did not alter serum progesterone levels or serum prolactin levels in dose groups compared to controls. The serum estradiol levels in the SD rat were dramatically altered by atrazine exposure and deserve special attention. Atrazine exposure resulted in an early exposure to high levels of estrogen. The levels of serum estrogen in the dosed groups of 70 and 400 ppm females at three months were 11.2 ± 12.6 and 16.2± 13 ng/mL compared to only 3.5 ± 6.4 ng/mL in the control females at this time point. While the standard deviations in these groups are large the increases are three to over four-fold, and the increases are statistically-significant using a pairwise comparison. The atrazine exposed groups had higher serum estrogen levels than controls at the three-month timepoint. There was also a positive dose-related trend over the first nine months of the study in estradiol levels. Exposure to such high levels of estrogen this early in the rats life is not normal. Exposure to these levels of estrogen at nine months, as can be seen from Appendix Table 15, is normal. The early exposure to these high estrogen levels may be leading to an earlier onset of mammary tumors.

As was the case with the F-344, increased prolactin levels would be expected as a consequence of the normal aging process in the SD; yet increases in serum prolactin were not seen in this study. Old SD females with pituitary adenomas can have serum prolactin levels that are approximately 13 times higher than in young SD females (Sarkar *et al.*, 1982). An increase in all the groups (both control and atrazine-treated) would be expected as the pituitaries increased in size and pituitary adenomas became common. By the 18- and 24-month timepoints the majority of the animals in all groups had pituitary adenomas; yet serum prolactin levels at these two timepoints were similar to control values. Increases in serum prolactin levels have been seen in numerous studies in the published literature and are accepted to be a normal part of the aging process in the SD female and other rats that undergo constant estrus as the predominant mode of reproductive aging (Cónsole *et al.*, 1997; McComb *et al.*, 1984; Sandusky *et al.*, 1988; Baird *et al.*, 1990; McComb *et al.*, 1986; van Putten *et al.*, 1988).

The fact that measured serum prolactin levels were not increased with age in either strain must be considered a weakness in this study, despite the difficulties of such measurements. This is especially true in the SD study where very large increases in serum prolactin levels should have been evident.

Because of the lack of increased serum prolactin (as determined by direct serum measurements) in the aged animals in Eldridge, 1993a, the histomorphologic data from this study was examined especially closely for any signs of increased prolactin exposure.

The incidences and severity of all four parameters that indicate increased exposure to prolactin (acinar/lobular development, secretory activity, dilated ducts with secretion, galactoceles) were increased in atrazine-treated groups compared to controls at the nine and 12-month timepoints. Index scores for values before nine months and after 12 months were similar among dose groups. The increased index scores at nine and 12 months in dose groups compared to controls indicates an early onset of increased prolactin exposure in the dose groups compared to the control. With time, the normal aging process proceeds in the control animals and by 15 months the control animals have "equalized" or "caught up" with the dose groups. Thus, index scores are similar for the timepoints after 12 months. Index scores are similar for the timepoints prior to nine months because increases in prolactin require first that estrogen be increased. The increased estrogen then acts at the pituitary to induce lactotroph hyperplasia that results in increased prolactin levels and, finally, increased incidence and severity of these mammary gland findings. Appendix Table 15 shows increases in serum estradiol at three months in the dose groups compared to controls while Appendix table 16 shows a dose-related increase in acinar development (primarily an estrogen-dependent effect) at three months in the dose groups compared to control groups. The increased serum estrogen levels seen at three months are affecting the pituitary between three and by nine months the pituitary lactotrophs are proliferating and producing prolactin such that by nine months prolactin-dependent alterations at the mammary gland are evident in the dosed animals.

9.2.7 Preovulatory LH Levels

The effect of aging on the preovulatory luteinizing hormone (LH) surge, has been briefly discussed previously in this document. As in humans, ovulation is triggered in rats by a sudden and dramatic increase (a surge) in serum LH levels. The attenuation of the preovulatory LH surge in aging female rat strains, including the SD, has been well-described (Lu *et al.*, 1979, Cooper *et al.*, 1980). One- and six-month studies examining the effect of atrazine exposure on the preovulatory LH surge are available (Morseth, 1996a; Morseth, 1996b; Minnema, 2000). These data show that atrazine exposures as short as one month can dramatically attenuate the pre ovulatory LH surge. Plasma prolactin levels were also determined in the Morseth, 1996a study and results of this analysis were reported. Plasma prolactin concentrations have been shown to undergo a preovulatory surge in rats similar to the LH surge (Butcher *et al.*, 1974). However, this document will not go into a detailed discussion of the effect of atrazine on the plasma prolactin surge simply because the role of this event in female rat inducing ovulation are not as well described as a the LH surge.

9.2.7.1 Protocol for LH Surge Measurement -- One-Month Study (Morseth, 1996a)

Female SD rats were exposed to 0, 2.5, 5, 40 and 200 mg/kg/day technical grade atrazine for 28 to 31 days. Ninety females per dose group were used. Dosing was by gavage once a day, at approximately the same time each day. After 28 to 31 days of atrazine exposure the animals were OVX. Vaginal smears were performed from days seven to the day prior to OVX to determine the animals cycling patterns. Estradiol implants were placed in each animal seven days following OVX and the animals were sacrificed three days later. Thus, OVX occurred 10 days prior to sacrifice and estradiol implants occurred three days prior to sacrifice. This protocol of OVX followed by estradiol implantation, followed by sacrifice has been previously used by other investigators to induce an LH surge in SD female rats (Legan *et al.*, 1975).

Ovarectomization of the animals followed by implantation of estradiol implants was done in an attempt to synchronize the estrous cycles of the animals so that the LH surge in each animal would occur at approximately the same time. This allows for comparison of the surge between dose groups and also points the investigator to a specific time at which the LH surge should be occurring. With this information the animals may be bled and serum LH levels measured. Animals were bled for serum hormone measurements at six different timepoints spread over 12 hours. The first two time points were 1100 and 1400 in biologic time (biologic time being time in the light cycle - biologic time 1200 is mid-point of the light cycle or noon of the light cycle). These first two timepoints are baseline. The other timepoints for serum measurement are biologic time 1600, 1800, 2000, and 2300. The peak of the LH surge would be expected to occur in the late afternoon of the light cycle (around 1800 biologic time). This equates to the late afternoon of proestrus in a normally cycling rat. By 2300 biologic time the LH surge would be expected to be over and LH values should return to baseline levels.

Out of the 90 animals in each group 10 were "repeat bleed." These animals were bled at each timepoint from the jugular vein for the first four bleedings, through the ocular venous plexus for the fifth bleed and the trunk for the last timepoint. The remaining 80 animals in each dose group were sacrificed, and trunk blood was collected according to the schedule shown in Appendix Table 18.

9.2.7.2 Results of LH Surge Measurements -- One-Month Study

The means and standard deviations for serum LH measurements from this study are shown in Appendix Table 19.

Non-repeat Bleed. Plasma LH values of the 200 mg/kg/group were significantly decreased at 1600 and 1800 compared to controls at that timepoint (specific p value not given). There were also non-significant decreases in plasma LH levels, compared to controls, in the 5 and 40 mg/kg group (45.4% and 36.8%, respectively). There was not a great increase in the 200 mg/kg group in the magnitude of the peak response over its own baseline value. Control mean baseline (1100 and 1400 hours) values are 998 and 1122 pg/mL compared to a peak value of 5138 pg/mL at 1800: approximately a five -fold increase. The baseline values for the 200 mg/kg group are similar to controls - 873 and 1099 pg/mL. However, the peak value in the 200 mg/kg group is only 2752 pg/mL: an increase of only 2.5-fold.

Repeat Bleed. Peak values compared to controls in the 200 mg/kg group were, as in the non-repeat bleed set, significantly decreased. The peak control value (1800 hours) was 2650 pg/mL while the peak 200 mg/kg value was 812 pg/mL (1800 hours). The 40 mg/kg group was decreased compared to controls (1450 pg/mL) but not significantly so. There was little increase in the 200 mg/kg group in the magnitude of the response over its own baseline value. Control baseline values were 732 and 786 pg/mL compared to peak values of 2650 pg/mL: approximately a 3.5-fold increase. Peak values in the 200 mg/kg group were increased only about 45% over baseline values (812 vs. 514 and 453 pg/mL).

9.2.7.3 Summary of the Plasma LH Measurements from the One-Month Study

The plasma LH values seen in the control animals in both the repeat and non-repeat bleed data indicate that one month of atrazine exposure dramatically attenuates the preovulatory LH surge in the high-dose group of 200 mg/kg/day. This effect is only statistically-significant in the high-dose group of 200 mg/kg, but it can be seen in the 40 mg/kg/day group also.

The results seen in this study are confirmed by a separate 28-day using similar protocols and identical doses (Minnema, 2000). The LH surge, in this study, was statistically significantly attenuated at both the 40 and 200 mg/kg/day doses.

9.2.7.4 Protocol for LH Surge Measurement -- Six-Month Study (Morseth, 1996b[4])

The protocol for this study was very similar to the protocol for the one-month study. The main differences were:

- duration of atrazine exposure (26 weeks);
- route of exposure (through the diet);
- and, dose levels (25, 50 and 400 ppm - 1.8, 3.65, and 29.44 mg/kg/day).

Other than these differences, the six-month study was conducted much the same as the one-month study. Animals were OVX 10 days prior to sacrifice and implanted with estradiol implants three days prior to sacrifice. Ninety females per group were used in a sacrifice schedule identical to that used in the one-month study (shown in Appendix Table 18). Blood collection and plasma hormone measurements were performed identical to the methods used in the one-month study.

[4]Data from the study referred to here as Morseth 1996b has been published in the open literature as Eldridge *et al.*, 1999.

9.2.7.5 Results of LH Surge Measurements -- Six-Month Study

The means and standard deviations for serum LH measurements from this study are shown in Appendix Table 20.

Non-repeat Bleed. Plasma LH values of the 400 ppm group were significantly decreased at 1400, 1800, and 2000 hours compared to controls at those timepoints (specific p value not given). There was not a large increase in the 200 mg/kg group in the magnitude of the response over its own baseline value (specific p value not given). Control mean baseline (1100 and 1400 hours) values are 1900 and 2326 pg/mL compared to a peak value of 3458 pg/mL at 1800: approximately a 1.6 -fold increase. The baseline values for the 200 mg/kg group are slightly less than controls - 1863 and 1420 pg/mL. However, the values at 1600 and 1800 in the 200 mg/kg group are only 1913 and 1356 pg/mL. The average of the 1100 and 1440 hour and 1600 and 1800 hour in the 200 mg/kg groups are essentially the same - 1641 and 1634 pg/mL.

Appendix Table 20 displays the baseline values, peak LH values and % increase of peak values over baseline. Examination of this table shows that at the high-dose group of 29.4 mg/kg/day there is clearly a decrease in the strength of the LH surge. At this dose level the surge does not seem to be occurring at all.

Repeat Bleed. Baseline values for plasma LH are similar among controls and all dose groups. There is a statistically-significant decrease at 1600, 1800, 2000, and 2300 in the 29.4 mg/kg/group compared to controls. The 50 ppm (3.65 mg/kg/day) group had a decrease at 1800 (25%) compared to controls. Compared to its own baseline values, the LH values in the 29.4 mg/kg group were not altered. Values at 1600 and 1800 are actually slightly lower than baseline values. Appendix Table 20 displays the baseline values, peak LH values and % increase of peak values over baseline. Appendix Figure 1 displays a line graph of the results from the repeat bleed group of this study.

9.2.7.6 Summary of the Plasma LH Measurements from the Six-Month Study

An attenuation of the LH surge at the high dose of 400 ppm (29.4 mg/kg/day) is clear. Examination of the data from Appendix Table 20 shows that plasma LH values for both the repeat and non-repeat bleeds at this dose remain essentially flat over the six timepoints.

The other dose groups do not appear to be as affected by atrazine exposure. The non-repeat bleed data for the dose groups is very similar to controls. There is a decrease in the strength of the LH surge at the mid-dose of 50 ppm (3.65 mg/kg/day), but the magnitude of this decrease is not large and given the variability inherent in this assay (as indicated by the large standard deviations), it is difficult to draw firm conclusions based on this decrease.

9.2.8 Summary And Discussion Of The LH Surge Studies

In the non-repeat bleed set of the one-month study the baseline values for the 200 mg/kg/day group are similar to controls. However, the peak value in the 200 mg/kg/day group is increased only 2.5 fold – much less than the five-fold increase seen in control peak values versus control baseline values. The results from the repeat bleed set for the one-month study are even more indicative of an attenuated LH surge. Baseline values for both control and 200 mg/kg/day groups are, again, similar. The peak LH values are much less in the 200 mg/kg group compared to the control though. Peak control LH values in the repeat bleed set were increased approximately 3.5-fold over baseline. Peak values in the 200 mg/kg group were increased only about 45% over baseline values.

The results seen the six-month study in the 29.4 mg/kg/day group also indicate an attenuated LH surge. Both the repeat and non-repeat data sets show plasma LH levels that are flat over time. Control LH values at their peak are about 67% above baseline values for the non repeat bleed data set and about 226% above baseline values in the repeat bleed set. Peak values in the 29.4 mg/kg group are 17% (non-repeat bleed) and 20% (repeat bleed) lower than baseline values though.

A major factor to consider when drawing conclusions from this study is, as indicated above, the large standard deviations in the data. Examination of the data shown in Tables 20 and 21 shows that the standard deviations are quite large and, in fact, sometimes exceed the means. An extreme amount of variability is to be expected with this type of data. Variability can be expected between rats in both timing and magnitude of the LH surge. Although the animals were synchronized by photoperiod and by OVX, there will still be variability in timing of the surge among rats in a dose group. It is hoped that all the rats will have their plasma LH levels be at their peak at 1800 hours when they are sacrificed and blood is collected. Clearly, however, this will not be the case. Some rats will experience a peak LH surge prior to 1800 hours and some after. The magnitude of the LH surge peak will also vary among rats. This is due largely to the differential rate at which the animals reproductive systems age. The variability of reproductive aging among female rats in a particular strain has been well described (Cooper *et al.*, 1986; Lu *et al.*, 1994; LeFevere and McClintock, 1988). Because the LH surge is attenuated as part of the female SD rats reproductive aging process, the variability in rate of reproductive aging means that animals of the same chronological age will have LH surges of varying magnitude.

Even given the variability inherent in the LH measurements in this type of study, there are some conclusions that can be reached with confidence. There is little doubt that in the one-month study at 200 mg/kg/day and in the six-month study at 29.4 mg/kg/day, there is an attenuation of the LH surge. Appendix Figure 1 displays a line graph of the mean plasma LH levels in the repeat bleed group from the six-month study displayed without standard deviation or standard error bars. The decreased LH surge at 3.65 mg/kg/day in the six-month study is less apparent but may be considered biologically-significant.

9.3 The Site of Action for Atrazine Attenuation of the LH Surge

Experiments have been conducted examining the mechanisms underlying the attenuation of the LH surge produced by atrazine. The focus of these experiments has been the target site of action in the brain for atrazine. The LH for the proestrus afternoon LH surge comes from the anterior pituitary and the release of LH from the pituitary is controlled by gonadotropin releasing hormone (GnRH), which is produced in the hypothalamus. Thus, atrazine could be altering the LH surge by acting at either the hypothalamus (and affecting GnRH - the signal to release LH) or by acting at the pituitary and directly affecting its ability to secrete LH.

Experiments examining the effects of atrazine exposure on the hypothalamus and pituitary were conducted at the Reproductive Toxicology Division of National Health and Environmental Effects Research Laboratories (Cooper *et al.*, 1998, Cooper *et al.*, 2000, Das *et al.*, 1999, Das *et al.*, submitted). These studies, through the results of both *in vivo* and *in vitro* experiments, demonstrate that atrazine appears to be attenuating the LH surge by acting on the hypothalamus, rather than directly affecting the pituitary.

An *in vivo* experiment using the Long-Evans (LE) strain of rat, administered GnRH through a cardiac catheter to OVX atrazine-treated females to see if GnRH exposure could reverse the atrazine-induced attenuation of the LH surge. Females, in this study, given atrazine only showed an attenuation of the LH surge -- which was expected. Females given atrazine plus 50 ng/rat of GnRH, did not display an attenuated LH surge. This provides evidence that atrazine is affecting the ability of the hypothalamus to release GnRH.

An *in vitro* experiment using perfused anterior pituitaries removed from untreated female LE, showed that atrazine could not directly affect the ability of the anterior pituitary to secrete LH. Pituitaries perfused *in vitro* were able to produce an LH surge when primed with estradiol. Adding 100 µM atrazine to the perfusion system did not affect the ability of the pituitaries to produce an LH surge following estradiol priming.

These two experiments taken together provide evidence that atrazine inhibits the proestrus afternoon LH surge through an action on the hypothalamus rather than a direct action on the anterior pituitary. Specifically, it appears that atrazine may somehow inhibit the hypothalamic secretion of GnRH.

Data from the Cooper lab indicates that a decrease in hypothalamic norepinephrine levels may be responsible for the reduced capacity of the hypothalamus to secrete GnRH (Cooper *et al.,* 1999a). In these studies, exposure of LE rats to a three-day exposure of 50, 100, 200 and 300 mg/mL/day of atrazine resulted in significant depressions of hypothalamic norepinephrine levels at all dose levels. This study is supported by *in vitro* studies using triazines and PC12 cells that showed that exposure through the medium of 50, 100 and 200 µM atrazine resulted in dramatic decreases in norepinephrine release at all dose levels starting as early as six hours following the start of exposure and continuing for up to 48 hours following exposure (Das, *et al.* , 1999).

Disruption of GABAergic neurotransmission by atrazine may also play a role in the decrease in GnRH release seen following atrazine exposure. Gamma-aminobutyric acid type a receptors ($GABA_A$) are known to play a crucial role in GnRH release. *In vitro*, atrazine (and cyanazine) have been shown to disrupt agonist binding to the $GABA_A$ receptor in cortex from male Long-Evans rats (Schafer, *et al.*, 1999). Such disruption could contribute to the decreased hypothalmic GnRH release seen following atrazine exposure.

9.4 The Data Examining the Association Between Atrazine Exposure and An Attenuated Proestrus Afternoon LH Surge, Increased Days and Estrus and a Prolonged Exposure to an Elevated Level of Estradiol

This document presents several studies examining an association between atrazine exposure and an early onset of alterations in the above described parameters - estrous cycle, serum estradiol levels; serum LH levels. A study is also available examining the correlation between estrous cycles and mammary tumor incidence/onset. The time of tumor onset is also examined in several studies in an attempt to confirm that normally occurring events (mammary tumor induction) are, in fact, occurring earlier following atrazine exposure.

The results indicate that atrazine exposure does appear to result in an early onset of these parameters. Increased days in estrus, increased serum estradiol levels, attenuated LH surge, and onset of mammary tumors all occur earlier in atrazine-treated females than they do in untreated females.

9.4.1 Atrazine Exposure Results in an Earlier Onset of Increased Days in Estrus

One-month of atrazine exposure at 40 mg/kg/day induced estrus blocks. Longer term studies revealed that atrazine exposure at lower levels also has the ability to increase days in estrus if the exposure is of long enough duration. A dose of 3.65 mg/kg/day in a six-month study was able to induce an increase in days spent in estrus over controls as early as 5.5 months into the study. A dose of 4.23 mg/kg/day in a two-year study (the low-dose tested in this particular study) induced an increased percentage of days spent in estrus at nine months -- an event that is not seen in control SD females in this study until about 12 months into the study. A separate two-year study showed that as little as 1.5 mg/kg/day could induce an increase in percent days spent in estrus. This increase was marginal and was not statistically-significant though. Furthermore, the increase in days in estrus seen at this dose was apparently not of the magnitude to increase a female rats risk of mammary cancer as there was not an increase in mammary tumor incidence or decrease in mammary tumor onset at this dose in this study. The next highest dose in the same study (3.1 mg/kg/day) also resulted in an increase in days in estrus. The increase in days estrus seen in this study was slightly greater than the increase seen at 1.5 mg/kg/day, but was still not statistically-significant. It was apparently enough to cause an increase an animals risk for mammary cancer as mammary cancer incidences at this dose were increased about two-fold over concurrent control values.

9.4.2 Atrazine Exposure Results in an Earlier Onset of Increased Serum Estradiol Levels

Atrazine exposure of only three months resulted in an increase in serum estradiol levels in SD females. Levels equivalent to those seen at three months were not seen in control animals in this study until nine months. Because there was no intermediate timepoint between three and nine months in this study it is difficult to determine just when the control animals achieved serum estradiol levels equivalent to those seen in the dosed animals at three months. Clearly, though, the dosed animals had higher serum estradiol at three months than the controls did -- indicating that this parameter of reproductive aging was achieved earlier in the dosed rat than in the control rat.

9.4.3 Atrazine Exposure Results in an Earlier Onset of Attenuated LH Surges

Atrazine exposure at high levels (40 and 200 mg/kg/day) was able to attenuate the preovulatory LH surge after only one month of exposure. Exposure to atrazine at lower levels (29.4 mg/kg/day) significantly weakened the LH surge after six months exposure. The lowest dose of atrazine that was able to induce a weakening of the LH surge was 3.65 mg/kg/day, but this weakening was not statistically-significant. It is not clear exactly when a normally aging animal would be expected to experience an weakened LH surge, but the studies described here showed that the control animals did not experience a weakened LH surge when the concurrently run dosed animals did.

9.4.4 Atrazine Exposure Results in an Earlier Tumor Onset

Tumor onset times were consistently decreased following atrazine exposure. The decrease in tumor onset times implies that the process of tumor formation is occurring at an earlier chronological age due to atrazine exposure. Tumor incidence rate were not always increased following atrazine exposure- they were not increased in Thakur, 1992a and only the trend for fibroadenomas was increased in Thakur, 1991a. Tumor onset times were decreased in every study in which they were examined - Thakur, 1991a and 1992a; and Morseth, 1998.

9.5 Pituitary Adenomas

9.5.1 Onset of Pituitary Alterations Following Atrazine Exposure

Table 5-5 provides evidence that the latency period of pituitary adenomas is decreased following atrazine exposure. Pituitary adenomas are known to be age-related in the rodent. The time to onset of other age-related pituitary alterations may also be decreased in atrazine exposed SD females. As described above under section 9.1, pituitary weights and incidences of pituitary hyperplasia are also increased in the untreated female SD rat with age. There is evidence that an early onset of increased pituitary weights and an early onset of pituitary hyperplasia may also be occurring in response to atrazine exposure. Appendix Tables 21 and 22 display absolute and relative (to body weight) pituitary weights from Thakur, 1991a. Appendix Table 23 displays absolute and relative (to body) pituitary weight from SD females exposed to atrazine for six months in Morseth, 1996b.

Three months of exposure did not result in an increase in either absolute or relative- to-body pituitary weights in the dose groups compared to controls. By nine months of exposure there was a clear dose-related increase in both absolute and relative pituitary weights. The effect was still evident at 12 months, but only at the high dose and the effect was less severe at the high dose at 12 months compared to nine months. Pituitary weights at 15, 18 and 24 months are comparable in dose groups compared to controls. The incidence of pituitary focal hyperplasia (as recorded in the histopathology records of this study) was marginally increased in dose groups compared to the control at nine months. There was only one incidence of this histology finding in the control group (it was assigned the grade "slight" by the examining pathologist) while there were two incidences at 4.23 mg/kg/day (both "slight") and two incidences at 26.23 mg/kg/day (one "minimal" and one "slight"). The early onset of increased pituitary weights following atrazine exposure seen in Thakur, 1991a is confirmed in Morseth, 1996b. Both absolute and relative pituitary weights increases are >20% at 29.4 mg/kg/day after six months of exposure.

9.5.2 Role of Early Onset of Pituitary Alterations in Mammary Carcinogenesis

The pituitary alterations - adenomas, hyperplasia and increased pituitary weight - all result in increased serum prolactin levels (Baird *et al.*, 1990; McComb *et al.*, 1986; van Putten *et al.*, 1988). The association between increased prolactin exposure and mammary tumors has been well-described (Meites, 1972; Meites, 1981; Russo *et al.*, 1990). Likewise, an association between pituitary alterations of the types mentioned above and mammary tumors had been well-described (Blankenstein *et al.*, 1984; McConnell, 1989a; Goya *et al.*, 1990). The studies described in this document also provide evidence of an association between these pituitary alterations and mammary tumors. Appendix Table 24 shows that majority of SD females with mammary tumors also had pituitary adenomas. Females without mammary tumors also had high incidences of pituitary adenomas, but the incidences were generally lower than for animals with mammary tumors. Appendix Tables 25a and b show that pituitary weights in females with mammary tumors were higher than pituitary weights in females without mammary tumors. The mean absolute pituitary weight in animals with mammary tumors in Morseth, 1998 was 46% greater than the mean pituitary weight in animals that did not have mammary tumors. The difference was even more apparent in the Thakur, 1992a study where the mean absolute pituitary weight in females with mammary tumors was 94% greater than in those without mammary tumors.

9.5.3 Pathogenesis of Pituitary Alterations

As has been previously discussed, estrogen is mitogenic to the pituitary lactotrophs of the rodent. It is therefore biologically plausible that the same increase in anovulation and accompanying prolonged exposure to serum estrogens that is believed to contribute to mammary carcinogenesis following atrazine exposure would also contribute to pituitary hyperplasia and neoplasia.

If an increase in serum estrogens leads to the afore-mentioned pituitary alterations then it would be reasonable to expect that increases in serum estrogen would precede the pituitary alterations. Indeed, increases in serum estradiol are seen as early as three months following the

initiation of atrazine exposure (Appendix Table 15). Dose-related increases in acinar development (a histomorphologic alteration highly dependent on estrogen) are found following three months of exposure while dose-related increases of galactoceles, secretory activity and other histomorphologic alterations indicating prolactin exposure are not seen until nine months of exposure (Appendix Tables 16 and 17).

If estrogens derived from unovulated follicles contribute to pituitary alterations then OVX animals would be expected to have lower incidences of these pituitary alterations. The OVX animals in Morseth, 1998 did have lower incidences of pituitary adenomas, but not as much less as might be expected. As previously noted, ovariectomy was able to reduce mammary tumor incidences from about 50% having some sort of mammary tumor to zero percent having any sort of mammary tumor. The effect of ovariectomy on the pituitary adenoma rate was much less pronounced. Ovariectomy dropped the pituitary adenoma rate from about 70% at terminal sacrifice for the intact animals to about 50% at terminal sacrifice in the OVX animals. Interestingly, though ovariectomy had only mild impact on pituitary tumor incidences, ovariectomy was able to dramatically reduce pituitary weights at the end of the study. Absolute pituitary weights at terminal sacrifice in the OVX animals were only about a quarter the value of the intact animals. Relative pituitary weights were only 35% the weight in OVX compared to intact animals. Appendix Table 26 displays these data. The dramatic decrease in pituitary weight in conjunction with only a mild decrease in pituitary adenoma incidence may be explained by an earlier onset of the pituitary tumors in the intact animals compared to the OVX. The data in Appendix Table 26 provides evidence of an early onset of pituitary adenomas in intact animals versus OVX when it shows a 6% incidence of pituitary adenomas in the interim sacrifice OVX animals compared to a 17% incidence in interim sacrifice intact animals. The data in Appendix Table 26 also shows that OVX animals had a much reduced incidence of "enlarged" pituitaries compared to intact animals. The decreased incidence of enlarged pituitaries and the reduced weight of the pituitaries may indicate that, though many OVX animals still got pituitary tumors, these tumors occurred later in life and thus, by the time the animals were sacrificed, had not had as much time to grow and were thus of a smaller size.

A serial sacrifice study comparing OVX and intact animals would be useful in determining if pituitary tumor onset is delayed in OVX animals versus intact animals. While HED is not interested in pituitary tumor onset in OVX animals *per se*; the fact that pituitary tumors are not more dramatically decreased in OVX animals raises some doubts about a mode of action for atrazine-mediated pituitary tumors that depends on prolonged exposure to follicular-derived estrogen. Were the same mode of action to apply to pituitary tumors that applies to mammary tumors then one would expect pituitary tumors to behave like mammary tumors when the ovaries are removed. That is, one would expect pituitary tumor rates to drop to zero, or close to zero, following OVX.

9.5.4 Summary and Conclusion for Pituitary Alterations

There is ample evidence in the open literature that exposure to follicular-derived estrogen in CE rats leads to an increased incidence of prolactin-secreting pituitary adenomas and increased pituitary weight or focal hyperplasia. Being that atrazine exposure seems to result in an early onset of constant estrus and increased estradiol exposure, one would expect that these pituitary alterations would also show an early onset following atrazine exposure. Pituitary weight data from Thakur, 1991a and Morseth, 1996 both show an early onset of increased pituitary weight following atrazine exposure. Pituitary adenoma data from Thakur, 1991a shows that there is an early onset of pituitary adenomas following atrazine exposure.

Further research is desirable into why ovariectomy does not reduce pituitary tumor incidence to the same extent as it does mammary tumors incidence despite there apparently having the mode of action.

Part C

Hazard Assessment and Review of Available Studies

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Part B of this document described a variety of experiments demonstrating an effect of atrazine on the pituitary-hypothalamic-ovarian axis of the rodent. These alterations would be expected to result in the establishment of a hormonal environment conducive to the development of mammary and pituitary tumors (*i.e.*, prolonged exposure to endogenous serum estrogens and serum prolactin).These neuroendocrine perturbations might also be expected to have effects other than tumorgenicity.

The suppression of LH release from the pituitary (described in Part B, section 9.2.7) might be expected to affect pregnancy in the rat. Alterations in LH release from the anterior pituitary might also be expected to affect pubertal development. Atrazine exposure has also been shown to alter serum prolactin levels following acute, high-dose exposure (Cooper *et al.*, 2000; these data are not discussed in Part B). Alterations of serum prolactin could affect pregnancy and pubertal development as well as having other potentially adverse effects -- specifically an increased risk of prostate inflammation in adults male rats whose mothers were exposed to atrazine while nursing.

Data describing atrazine effects on serum prolactin and possible effects of LH suppression and serum prolactin alterations on pregnancy, pubertal development, and prostatitis are described below. Most of these data are derived from the labs of Dr. Ralph Cooper at EPA. Also described is a study by Dr. Barry Zirkin of Johns Hopkins University examining atrazine effects on pubertal development in male rats. Understanding the data described below requires some background knowledge of the role LH and prolactin play in pregnancy and pubertal development in the rodent.

Brief backgrounds of the roles of prolactin and LH in pregnancy and pubertal development, followed by a discussion of the data showing atrazine's effects on these parameters, constitute the bulk of this chapter. The remainder of Part C is a brief discussion of a trio of open literature epidemiology studies that investigate potential associations of reproductive anomalies with atrazine exposure, and a discussion of a trio of studies from the lab of Dr. Jasna Kniwald in Yugoslavia that examine possible effects of atrazine on testosterone metabolism in the male rat.

The discussion of the testosterone metabolism studies immediately follows. A discussion of the epidemiology studies is next followed by a discussion of atrazine's effects on pregnancy, puberty and prostatitis.

10. Possible Effects of Atrazine Exposure on Testosterone Metabolism

The steroid hormone testosterone is converted into 5α-reduced metabolites by tissues *in vivo*. These 5α-reduced metabolites are primarily 5α-dihydrotestostrone (DHT), 3α-diol and androstenedione. Tissues such as the prostate, seminal vesicles, hypothalamus and pituitary contain 5α-reductase enzymes and are primarily responsible for reducing testosterone to these compounds. The enzymes responsible for these conversions are 5α-reductase, 3α-HSD and 17β-HSD. The 5α-reduced metabolites (particularly DHT) are able to bind the testosterone receptor, and testosterone receptor-5α-reduced metabolite complexes are believed to be more stable than testosterone-receptor complexes. The rat prostate also has receptors specific for DHT.

Three published papers have examined the effect of atrazine on testosterone metabolism. The first of these papers examined the effects of atrazine exposure with either *in utero* or *in utero* plus early postnatal exposure (Kniewald *et al.*, 1987). Female Fischer-344 rats were treated by s.c. injection once a day during their entire pregnancy with 16.6 mg/kg/day of atrazine or deethyatrazine (DET) and sacrificed at PND 28; or were treated once daily by s.c. injection with the same dose of both compounds during both pregnancy and lactation and were sacrificed at 21 days postnatal. Activity of pituitary reductase enzymes, and DHT and estradiol receptor binding sites in the prostate and uterus were determined in all animals.

A second study exposed young adult male Fischer-344 rats to either 60 or 120 mg/kg/day atrazine or DET by gavage for seven days (Babic-Gojmerac *et al.*, 1989). On the eighth day animals were sacrificed, and the anterior pituitary and hypothalamus were excised and activity of reductase enzymes responsible for testosterone metabolism were measured. *In vitro* studies were also conducted in which the anterior pituitary and hypothalamus were removed from young adult males and exposed to atrazine and DET *in vitro*. The activity of reductase enzymes was then determined.

The third study also used young-adult male Fischer-344 rats and dosed them by gavage with 120 mg/kg/day of atrazine for seven days (Simic *et al.*, 1991). On the eighth day the animals were sacrificed and DHT-receptor complexes in the prostate were measured.

These studies indicate that testosterone metabolism is not greatly altered if animals are treated only *in utero*. Testosterone metabolism can be significantly altered if male and females are treated *in utero* and during lactation or if males are treated as young adults. The studies indicate that atrazine and DET are able to inhibit the conversion of testosterone to its more active reduced forms. The inhibition of metabolism of testosterone to its more active forms by atrazine indicates an anti-androgenic effect of atrazine which may result in adverse consequences.

11. Epidemiology

11.1 IUGR in Iowa Communities

The incidence of intrauterine growth retardation (IUGR) in Iowa was investigated in an ecologic study (Munger *et al.*, 1997). The definition of IUGR can vary considerably (Seeds, 1984). IUGR can, generally speaking, be defined as low birth weight. Infants that are delivered full-term are usually defined as displaying IUGR if they weigh less than 2,500 grams at birth. For infants that are delivered prior to full term, birth weight below the tenth percentile for gestational age is frequently used to define IUGR.

IUGR was defined in the Munger study as birth weight below the 10^{th} percentile for gestational age as defined by California standards for non-Hispanic whites. The Munger study was a an ecologic study in which estimated exposures to pesticides through drinking water were compared to birth weights in an attempt to determine associations between the two. Data on levels of pesticide contamination in drinking water were obtained from the 1986 to 1987 statewide municipal water survey of Iowa which included data from 856 municipal water sources across the state. Birth weight data were obtained from birth certificate data obtained from the Iowa Department of Public Health. Factors such as maternal smoking, maternal education, quality of prenatal care, geographic region and community size were evaluated in an attempt to account for potentially confounding data.

An association between IUGR and maternal exposure to drinking water from the Rathburn system was found. The Rathburn system is a community drinking water system that obtains its water from the Rathburn reservoir in southern Iowa and serves several communities and counties in southern Iowa. The 1986 to 1987 drinking water survey found the Rathburn system to have elevated levels of herbicide contamination with the most notable contaminant being atrazine. The mean atrazine contaminant level in the Rathburn system 2.2 µg/L compared to a mean atrazine contaminant level of 0.6 µg/L in all other Iowa surface water suppliers. The rate of IUGR incidence from 1984 to 1990 in communities served by the Rathburn system was 11.2% compared to 6.4% for all other surface water suppliers in Iowa.

Regression models of IUGR showed that atrazine had the best fit (*i.e.*, the best positive association between drinking water contaminant levels and IUGR incidence) of all the contaminants examined in the study. The study authors note: "Atrazine had the best fit in the regression models of IUGR, but independent effects of other herbicides, which are intercorrelated, cannot be ruled out."

Although useful, the Munger study should be, because it is an ecologic study, regarded as a preliminary study which needs to be verified by more detailed epidemiologic studies. The deficiencies of ecologic studies are discussed in Part B section 4.9 and the study authors of the Munger study state:

> "Because of the limitations of the ecologic design of this study, including aggregate rather than individual measures of exposure and limited ability to control for confounding factors related to source of drinking water and risk of IUGR, a causal relationship between any specific water contaminant and risk of IUGR cannot be inferred."

11.2 Birth defects in Rural Minnesota

A study published in the open literature examined the possibility that offspring of pesticide appliers may display higher incidences of birth defects (Garry *et al.*, 1996). This study used information from the Minnesota Department of Agriculture (MDA) to identify persons in Minnesota who were certified to apply restricted use pesticides in 1991. This applicator information was linked to birth data supplied by the Minnesota Center for Health Statistics of the Minnesota Department of Health to determine birth defect rates among pesticide applicators in Minnesota. The birth data were also compared to pesticide use data supplied by MDA to examine associations between birth defects and quantitative pesticide use for both pesticide applicators and the general population.

Minnesota pesticide applicators did have significantly ($p<0.001$) more children born with a birth anomaly than did the general population of Minnesota. However, when birth data from pesticide applicators from one of three crop growing regions are compared to birth data from the general population from the same region, a statistically-significant difference ($p<0.02$) is seen only in one of the three regions. Use of any specific pesticide was not implicated in the increases in birth defects seen in pesticide appliers *vs.* the general population.

When use of specific pesticides was examined by comparing pesticide use by county cluster to birth defects in that cluster, the authors noted that use of chlorophenoxy herbicide and fungicides seemed to have the strongest association with birth defects. The results for atrazine use did not show a clear association of atrazine use with birth anomalies. There was a significant increase in birth defects when counties with atrazine use levels of >100,000 lbs AI/county cluster are compared to county clusters with <100,000 lbs AI, but if clusters with use levels of >25,000 lbs AI are compared to clusters with <25,000 lbs AI/cluster then a significant association is not seen.

11.3 Male Pesticide Exposure and Pregnancy Outcome

To examine the potential effects of paternal pesticide exposure on pregnancy outcome, farm activity in male farmers in Ontario, Canada was evaluated for the three months prior to conception through conception (Savitz *et al.*, 1997). Male farmers were surveyed about their farming activities for the past five years to obtain information about their activities during the above-described time around conception. Pregnancy outcomes were determined by questionnaires completed by the farm couples. Farm activities by the males were compared to pregnancy outcome to determine potential relationships of farm activities (specifically mixing, loading or applying pesticides) to pregnancy outcomes (specifically the risk of preterm delivery, miscarriage, small for gestational age [SGA] and sex ratio). If the males had used pesticides in the time period around conception, then the specific pesticides used were reported.

An increased risk of miscarriage was not associated with atrazine use as a crop herbicide (adjusted odds ratio [AOR] 1.5) or atrazine use as a yard herbicide (AOR = 1.2). An increased risk of SGA was not associated with atrazine or cyanazine use as a crop herbicide (AOR 0.5 and 0.8 respectively) or with atrazine use as a yard herbicide (AOR= 0.5). Sex ratio data were not separated out into exposures to specific chemicals but, since the AORs for proportion of male births for all farm activities involving chemicals ranged from 0.8 to 1.1, it is apparent that there was not an increased risk of alterations in sex ratio associated with use of any pesticide in males.

There was, however, an increased risk of preterm delivery associated with atrazine use by males around the time of conception. The AOR for use of atrazine as a crop herbicide was 2.4 (95% CI 0.8 to 7.0) and the AOR for use of atrazine as a yard herbicide was 4.9 (95% CI 1.6-15).

11.4 Summary and Conclusions

An association of exposure to atrazine-contaminated water and IUGR was seen, but because of the limitations of this study (*i.e.*, an ecologic study) the study authors conclude that the results are, "a preliminary finding that needs to be verified by more detailed epidemiologic studies." An association of atrazine use and birth defects in Minnesota communities was also seen, but the association was inconsistent. The inconsistencies in the data weaken the positive finding seen and the study authors do not dwell on the positive finding noted for atrazine. Male atrazine use around the time of conception failed to show an association for three of the four reproductive parameters examined (miscarriage, SGA and sex ratio). A positive association was seen for preterm delivery though. There is little biologic plausibility in associating a parameter such as preterm delivery with male chemical exposure. The effect of male chemical exposures on this endpoint has not been extensively studied and, as the study authors note: "Maternal characteristics, particularly reproductive and medical, are most strongly associated with preterm delivery."

The data provided by these three epidemiology studies do not provide clear evidence of an association between atrazine exposure and reproductive anomalies.

12. Background for Pregnancy, Pubertal and Prostatitis Papers

12.1 Role of Prolactin and LH in Pregnancy

Progesterone, acting at the uterus, is essential to maintain pregnancies in mammals. The major source of this vital progesterone in all mammals is the corpus luteum (CL). All mammals require progesterone throughout pregnancy from fertilization to parturition.

The sole source of progesterone during pregnancy in the rat is the CL. During early pregnancy (from implantation to approximately GD seven in the rat) the CL is maintained by prolactin derived from the anterior pituitary (Terkel, 1988). During mid-gestation (from GD 7 to 10) the CL is maintained by lutenizing hormone (LH) (Rothchild, 1981; Terkel, 1988). After GD 10, prolactin-like compounds produced by the placenta (*i.e.*, placental lactogens) function to maintain the CL throughout the remainder of the pregnancy (Gibori, *et al.*, 1988; Linzner and Fisher, 1999).

The source of progesterone in pregnancy in humans is the CL for about the first six weeks of pregnancy. At approximately the sixth week of pregnancy, humans display a "luteal-placental shift" in which the placenta assumes the responsibility of progesterone secretion and the CL becomes quiescent (Stouffer, *et al.*, 1989).

Thus, all mammals require progesterone throughout pregnancy, but in the rat the only source of this progesterone is the CL whereas in the humans the CL is the initial source, and the placenta supplies progesterone for the greater part of the pregnancy. Maintenance of the CL in the rat is accomplished by prolactin, LH and placental lactogens. Pituitary-derived prolactin plays the primary role in maintaining the CL in early pregnancy; LH is primarily responsible for maintaining the CL in mid-gestation; and, placental lactogens maintain the CL during late gestation.

12.2 Role of Prolactin and LH in Pubertal Development

12.2.1 Female

Pubertal development in the female rat has been well-characterized (Ojeda, 1980; Ojeda, 1983). The onset of puberty in the female is a transitional period that culminates with the initiation of cyclic surges of luteinizing hormone (LH) from the pituitary that stimulate ovulation. Vaginal opening generally coincides with the first ovulation and occurs at 32 or 33 days of age in the female rat. The hormonal changes which induce the first ovulation are similar in many respects to the hormonal changes which induce all other ovulations in rodents. The sequence of hormonal changes preceding the first ovulation is as follows:

1. Serum estradiol levels increase followed by;

2. A dramatic increase (surge) in serum luteinizing hormone (LH);

3. Serum prolactin levels dramatically increase concomitant with the LH surge.

Exposure to atrazine has been shown to attenuate the proestrus LH and prolactin surges in Long-Evans and Sprague-Dawley rats. Since both of these hormones are important for normal pubertal development, it is reasonable to hypothesize that atrazine may affect the onset of puberty in the female rodent. Atrazine's attenuation of the proestrus LH surge is described in detail in Part B sections 9.2.7 and 9.2.8. Atrazine's attenuation of prolactin release is described below in section 13.1.

In addition, reports that atrazine can reduce hypothalamic norepinephrine concentrations (Cooper *et al.*, 1998) and that intravenous injections of GnRH restore the estrogen-induced secretion of LH in ovariectomized, atrazine-treated female rats (Cooper *et al.*, 2000) suggest that possible effects on neurotransmitters and their regulation of pituitary hormone synthesis/secretion could also alter the onset of puberty.

Thus, to examine the effects of atrazine on female pubertal development, a study was conducted using the "Research Protocol for the Assessment of Pubertal Development and Thyroid Function in Juvenile Female Rats" (U.S. EPA, 1998b; Goldman *et al.*, 2000).

12.2.2 Male

The onset of puberty in the male rat involves a complex interplay of several hormones including LH, FSH, testosterone and prolactin (Nazian and Mahesh, 1980; Piacsek and Goodspeed, 1978). It has been shown that an increased turnover rate in hypothalamic GnRH, NE and DA precedes the dramatic increase in testosterone (Matsumoto *et al.*, 1986) prior to the onset of puberty. LH stimulates testosterone secretion by the Leydig cells. At the same time, LH secretion varies only slightly as puberty approaches. However, there is an increased sensitivity of the testes to LH prior to puberty, due to other hormonal influences, such as increased prolactin secretion, that facilitate an upregulation of LH receptors (Kamberi *et al.*, 1980; Odell *et al.*, 1973; Vihko *et al.*, 1991). In contrast, there is a higher threshold for the gonadotropin/gonadal steroid feedback mechanism in the adult male (Gupta *et al.*, 1975; Nazian and Mahesh, 1980) as compared to the immature male, making the immature male more sensitive to the feedback of testosterone. As this feedback sensitivity decreases, the hypothalamic-pituitary unit becomes more effective at stimulating testicular development, because there is less inhibition of gonadotropins by testosterone.

Development of the size of the penis and cornification of the epithelium of the prepuce and preputial separation in immature rats are regulated by androgens (Marshall, 1966). A decrease in testosterone during the juvenile period can delay preputial separation (Lyons *et al.*, 1942) and reduce the size of the androgen-dependent tissues, such as the ventral prostate and seminal vesicles. Normally, testosterone levels rise gradually from PND 20 to 40, and abruptly double by PND 50 (Matsumoto *et al.*, 1986; Monosson *et al.*, 1999). Atrazine exposure has been shown to alter LH and prolactin secretion in female rats. An effect on LH and prolactin secretion in immature male rats, and thus on pubertal onset, may also be possible.

To examine the effects of atrazine on male pubertal development, a study was conducted using the "Research Protocol for the Assessment of Pubertal Development and Thyroid function in Juvenile Male Rats" (U.S. EPA, 1998b).

12.3 Role of Prolactin in Prostatitis

Hyperprolactinemia prior to puberty in male rats has been shown to lead to lateral prostate inflammation in young adult rats (Stoker *et al.*, 2000b). One possible cause of hyperprolactinemia in immature male rats is a deficiency in milk-derived prolactin. Milk-derived prolactin plays a critical role in the development of the tuberoinfundibular dopaminergic neurons (TIDA) of the hypothalamus of a developing rat (Shyr, *et al.*, 1986). The TIDA neurons function to inhibit prolactin secretion from the anterior pituitary. Organization and development of these neurons occurs mainly during the first postnatal week in the rat (Ojeda and McCann, 1974).

Thus, if developing rats do not receive a sufficient amount of prolactin from their mothers milk during the first week after birth, the TIDA neurons will not develop properly and may not be able to sufficiently provide an inhibitory check to prolactin secretion in the adult animal. The resultant hyperprolactinemia is associated with development of prostatitis in the adult.

13. Data

13.1 Atrazine Effects on Prolactin

Similar to the preovulatory LH surge that is described in Part B section 9.1.1. of this document, rodents also display a preovulatory prolactin surge (Blank, 1986). Studies demonstrating atrazine-associated effects on this prolactin surge are presented in Cooper, *et al.*, 2000 (other data from this publication relating to the site of action of atrazine-associated LH alterations are described in Part B section 9.3 of this document).

The prolactin studies described in Cooper *et al.*, 2000, use adult Long-Evans (LE) and Sprague-Dawley (SD) female rats which were ovariectomized (OVX) and given estrogen-containing implants. Three days later they were dosed by gavage with a single dose of 97.1% atrazine suspended in carboxymethylcellulose at dose levels of 0, 50, 100, 200, or 300 mg/kg (this protocol of OVX, estradiol implantation, and sacrifice three days later, is an established model of the LH and prolactin surges and was also used in the LH surge experiments described in Part B section 9.2.7). Separate groups of LE and SD females were OVX, implanted with estradiol pellets, and given daily doses at the same dose levels as the single-exposure animals for the three days leading up to sacrifice. Lastly, other LE and SD females were OVX, dosed daily for 21 days at dose levels of 0, 75, 150 or 300 mg/kg/day, implanted with estrogen pellets on day 21 and then sacrificed three days later.

Thus, atrazine exposures consisted of: single exposures; three-day exposures; and,21-day exposures. Following sacrifice, blood was collected and serum prolactin was measured.

A single exposure in the SD females resulted in no statistically-significant differences between controls and any of the dose groups. The LE rats showed statistically-significant decreases in serum prolactin levels in the high-dose group of 300 mg/kg only.

Three days of atrazine exposure resulted in statistically-significant attenuation of the prolactin surge in SD females at 300 mg/kg/day, but not at any other dose. The prolactin surge in LE rats under this exposure protocol was significantly attenuated at 100, 200 and 300 mg/kg/day and was delayed at 50 mg/kg/day.

The 21-day exposure resulted in significantly lower prolactin levels in both strain at the 150 and 300 mg/kg/day doses, but at 75 mg/kg/day prolactin levels were not significantly different from control levels.

These data clearly demonstrate that atrazine has the ability to suppress estrogen-induced prolactin surges. Further data described in this paper demonstrate that, as was the case for the atrazine-associated LH surge attenuation, the effect of atrazine on prolactin secretion does not appear to be due to a direct effect on the pituitary. Rather, the hypothalamus appears to be the site of action for this effect of atrazine exposure.

13.2 Atrazine Effects on Pregnancy

13.2.1 Implantation and Early Pregnancy

Cummings *et al.*, 2000 (submitted) examined the effects of atrazine on implantation and early pregnancy in several strains of rats. Technical grade atrazine (ATR) of 97.1% purity was administered daily by gavage to rats during GD 1 to 8 (day 0 = sperm +). Dose levels included 0, 50, 100, and 200 mg/kg/day of ATR. Rats were divided into groups such that half were dosed at 2 p.m. (just prior to the diurnal prolactin surge of early pregnancy) and half were dosed at 2 a.m. (just prior to the nocturnal surge of prolactin). Within each time interval group, four strains of rats were each tested at each of the four dose levels listed above. Rat strains used were Holtzman, Fischer-344, Sprague-Dawley, and Long-Evans hooded. Clinical signs of toxicity consisted of decreased mean body weight at necropsy in the 200 mg/kg groups. Necropsies were performed on GD 9 of pregnancy. A small but significant decline in mean number of implantation sites was seen at 100 mg/kg in Fischer-344 rats (nocturnal dosing interval) and Sprague-Dawley rats (diurnal dosing interval), as well as at 200 mg/kg in Holtzman rats (nocturnal dosing). Holtzman rats alone showed both an increase in resorptions and a decrease in serum progesterone (at 200 mg/kg) as well as a decrease in serum LH at the same dose. Long-Evans and Fischer-344 rats also exhibited a decrease in serum LH in the 200 mg/kg dose group.

Summary/Conclusion

The LOAEL is 100 mg/kg for the effect on implantation and 200 mg/kg for all other parameters.

The NOEL is 50 mg/kg for the effect on implantation and 100 mg/kg for all other parameters.

13.2.2 Pregnancy Maintenance: Strain Comparisons of Sensitivity to Atrazine-Induced Pregnancy Loss in Rats

In a series of developmental toxicity studies (Narotsky *et al.*, 1999, Narotsky *et al.*, submitted) technical grade atrazine (97.1%) was administered by gavage, in 1% methylcellulose, to F344, Sprague-Dawley, and Long-Evans hooded rats at 0, 25, 50, 100, or 200 mg/kg/day on GD 6 to 10. This time frame was selected because it coincides with the LH-dependent period of pregnancy. In preliminary work, the authors identified this period of pregnancy as the most sensitive to the effects of atrazine. Using 200 mg/kg atrazine (by gavage), they found full-litter resorptions in 20 of 30 dams dosed from GD 6 to 10 while the same treatment was without effect in nine dams dosed from GD 11 to 15. Based on this information, the following study compared potential strain differences in response to atrazine. The dams were allowed to deliver and their litters were examined on PND's one and six. The F344 strain was the most sensitive to atrazine's effects on pregnancy maintenance; the Long-Evans strain was the least sensitive. In the F344 rats, maternal toxicity (weight loss, piloerection) and developmental toxicity (full-litter resorption, *i.e.*, pregnancy loss) were observed at ≥50 mg/kg. Among surviving litters, increased prenatal mortality was observed at 200 mg/kg, and parturition was delayed at 100 mg/kg. In Sprague-Dawley rats, similar effects were observed, albeit at different dose levels; maternal weight loss was noted at ≥25 mg/kg, full-litter resorption was observed only at 200 mg/kg, and delayed parturition was seen at ≥100 mg/kg. In contrast, the Long-Evans hooded strain showed maternal weight loss at ≥100 mg/kg and full-litter resorption at 200 mg/kg, but no effects on parturition.

Similar experiments conducted using the atrazine metabolites desethylatrazine, desisipropyl atrazine, diaminochlorotriazine and hydroxyatrazine, demonstrated that these metabolites were of equal or lesser potency than parent atrazine.

Summary/Conclusion

The maternal and developmental NOAELs and LOAELs for each strain are tabulated below.

	F344	Sprague-Dawley	Long -Evans
Maternal NOAEL	25 mg/kg	--	50 mg/kg
Maternal LOAEL	50 mg/kg	25 mg/kg	100 mg/kg
Developmental NOAEL	25 mg/kg	50 mg/kg	100 mg/kg
Developmental LOAEL	50 mg/kg	100 mg/kg	200 mg/kg

13.3 Atrazine Effects on Pubertal Development

13.3.1 Female

A recently completed study (Laws *et al.*, 2000, Laws *et al.* submitted) evaluated the effects of atrazine on pubertal development in the female Wistar rat. Atrazine (97.1%) was administered by oral gavage (in a suspension of 1% methyl cellulose) to 165 female Wistar rats, 15 or 30 rats/dose, at dose levels of 0, 12.5, 25, 50, 100 or 200 mg/kg/day, from PND 22 through 41. To evaluate the effects of lower body weight gain during treatment, a pair-fed group (n=15) was included where the food intake of each pair-fed rat was dependent upon the amount consumed by its respective mate in the ATR 200 mg/kg/day group. Half of the rats were killed on PND 41 and liver, kidney, adrenal, ovary, uterus and pituitary weights were collected. Estrous cyclicity was evaluated in the remaining females by monitoring changes in vaginal epithelial cells from vaginal opening through PND 70.

The mean body weights ± SEM for all treatment groups were equal on PND 22. Body weight on PND 41 was unaltered by 12.5, 25, 50 and 100 mg/kg/day, but was significantly reduced by 11% in the 200 mg/kg/day and 9% in the pair-fed groups. As compared with the control, the total gain in body weight during the 20-day treatment period was reduced to 17% and 15% in the ATR 200 mg/kg/day and pair-fed groups, respectively.

Vaginal opening was significantly delayed 2.3, 3.9 and 7.1 days following exposure to 50, 100 and 200 mg ATR/kg, respectively. In addition, vaginal opening did not occur in 18/31 females in the highest ATR dose group by the end of the dosing period. Body weight at the time of vaginal opening was significantly increased in the 50, 100 and 200 mg/kg/day groups as compared with the controls (as would be expected due to the increase in age at vaginal opening). However, no significant difference in the age or body weight at the time of vaginal opening was observed between the control and the pair-fed groups.

Irregular estrous cycles (*e.g.*, increased number of days of diestrus) were observed between the time of vaginal opening and PND 41 in females exposed to 50 and 100 mg/kg/day, but returned to normal by the end of the 30-day exposure period. Once dosing was discontinued, vaginal opening occurred in all females in the 200 ATR group within four to five days. The estrous cycles in the ATR 200 females were irregular during the first 15-day interval following vaginal opening, but also returned to regular four to five day cycles by PND 70.

Summary/Conclusion

Atrazine exposure delayed vaginal opening and altered estrous cycles in female Wistar rats following oral exposure during PND 22 to 41. The LOAEL for vaginal opening is 50 mg/kg/day and the NOAEL is 25 mg/kg/day under the conditions of this protocol. The LOAEL for estrous cycle alteration is 50 mg/kg/day and the NOAEL is 25 mg/kg/day under the conditions of these assays. The effect on the estrous cycle is reversible as indicated by the fact that normal estrous cycles resumed in all females by the end of the 30-day post-exposure period. Data from this study are consistent with an effect on the central nervous system and subsequent alterations in hormonal control during pubertal development.

13.3.2 Male

Work from two separate laboratories has examined the effect of atrazine exposure on puberty onset in male rats. Data from the lab of Dr. Ralph Cooper (*et al.*, 2000) will be described first followed by data from the lab of Dr. Barry Zirkin (Trentacoste *et al.*, 2000).

Stoker *et al.*, 2000a, and Stoker *et al.*, submitted undertook a series of experiments using the weanling male Wistar rat. Animals were treated from PND day 23 to 53 with atrazine. Atrazine (97.1%) was administered by daily gavage to male Wistar rats of similar body weight at doses of 12.5, 25, 50, 100, 150 and 200 mg/kg/day. Six rats per dose were used at 12.5, 25, and 150 mg/kg/day and 20 to 24 per dose were used at 50, 100 and 200 mg/kg/day. An additional ten rats were pair-fed to match the food intake of the 200 mg/kg/day rats. Parameters measured were: body weights; prostate, seminal vesicle, epididymis and testes weights; preputial separation (PPS); and serum testosterone, estradiol, estrone, LH and prolactin levels. Organ weights and hormone measures were taken on day 53. Body weights and preputial separation were determined daily fro PND 23 to 53.

Body weights in the 200 mg/kg/day group were significantly decreased from day 43 to 53 compared to controls. There were no significant alterations in body weight in any other dose group. Testes weights (neither absolute nor relative to body weight) were not altered in any dose group. Absolute epidydimal and seminal vesicle weights were significantly decreased in the 200 mg/kg/day group and the pair-fed group. When adjusted for body weight, the seminal vesicles were still significantly reduced but the epididymis were not. Lateral prostate weights were not altered by atrazine treatment, but ventral prostate weights, both absolute and relative to body weight, were significantly decreased in all dose groups from 50 to 200 mg/kg/day. Serum hormone levels, for the most part, were not significantly altered by treatment with atrazine. There was, however, a statistically-significant increase in serum estrone and estradiol levels at 200 mg/kg/day compared to controls.

The major effect of atrazine on the male rats in this study was a delay in preputial separation. Preputial separation, which occurred on about day 42 in the controls in this study, was delayed by 2.3, 1.7, 1.7, 1.7 and 3 days in the 12.5, 50, 100, 150 and 200 mg/kg/day groups. The pair-fed animals displayed delays of two days compared to controls. A significant delay was not seen at the 25 mg/kg/day dose with the mean day of preputial separation being 43 days.

Trenatcoste, *et al.,* 2000 dosed male Sprague-Dawely rats by gavage from PND 22 to 47. Nine to twelve animals per dose level were dosed at 1, 2.5, 5, 10, 25, 50, 100 or 200 mg/kg/day of atrazine (96.1%). A separate study termed a "food deprivation study" was also conducted. In this study animals were dosed at 100 mg/kg/day and the amount of food consumed on a daily basis was measured. A second group of rats was vehicle-treated and fed the average daily intake of food consumed by the atrazine-treated group while a third group was vehicle-treated and fed *ad lib*. Parameters measured in both studies were body weights, serum and intratesticular (interstitial fluid) testosterone levels, serum LH levels, testes, epididymis, ventral prostate and seminal vesicle weights. Unlike the above described Stoker *et al.*, 2000a and Stoker *et al.*, submitted study, PPS was not measured in this study.

Body weights were significantly reduced in the 100 and 200 mg/kg/day dose groups compared to controls. Serum and intratesticular testosterone levels at 100 and 200 mg/kg/day were significantly reduced. Serum LH reduced 17 and 20% at he 100 and 200 mg/kg/day groups, respectively. Only the 200 mg/kg/day decrease in LH concentration was significant. Significant reductions in seminal vesicle and ventral prostate weight were seen at 100 and 200 mg/kg/day. Testes and epididymis weights were not significantly altered at any dose. Body weights, organ weights and hormone levels were not significantly altered at any dose from one to 50 mg/kg/day.

The food deprivation study also showed significant reductions in body weight, serum and interstitial testosterone, serum LH, ventral prostate weight, and seminal vesicle weight in rats treated with 100 mg/kg/day atrazine compared to vehicle-treated controls fed *ad lib*. The pair-fed, food deprived rats (the vehicle-treated rats fed the average daily intake of the 100 mg/kg/day rats) also showed significant decreases in serum and interstitial testosterone, serum LH, ventral prostate weight, and seminal vesicle weight compared to animals fed *ad lib*. The reductions in body weight, serum LH, and ventral prostate and seminal vesicle weights were almost identical between the 100 mg/kg/day and the pair-fed, food-deprived rats.

13.4 Atrazine Effects on Prostate

As described above, atrazine has been shown to depress the secretion of prolactin. Section 3.3. above describes the role of milk-derived prolactin in development of the TIDA neurons in the neonatal rat hypothalmus, and the resulting hyperprolactinemia followed by lateral prostatitis that is the consequence of incomplete development of these neurons. To summarize these points: without early lactational exposure to PRL, TIDA neuronal growth is impaired and elevated PRL levels are present in the prepubertal male. Hyperprolactinemia in the adult male rat has been implicated in the development of prostatitis.

Thus, early lactational exposure of dams to agents that suppress suckling-induced PRL release (possibly atrazine) could lead to a disruption in TIDA development in the suckling male offspring, followed by altered PRL regulation and subsequent hyperprolactinemia and prostatitis in these male offspring.

To test the hypothesis that atrazine exposure of dams during lactation could initiate the above-described sequence of events, Cooper *et al.*, 1999, measured suckling-induced PRL release in Wistar dams treated with atrazine (by gavage, twice daily on PND 1 to 4 at 0, 6.25, 12.5, 25, and 50 mg/kg) or the dopamine receptor agonist bromocriptine (BROM, s.c., twice daily at 0.052, 0.104, 0.208 and 0.417 mg/kg). BROM is known to suppress PRL release. Serum PRL was measured on PND 3 using a serial sampling technique and indwelling cardiac catheters.

A significant rise in serum PRL release was noted in all control females within 10 minutes of the initiation of suckling. Fifty mg/kg ATR inhibited suckling-induced PRL release in all females, whereas 25 and 12.5 mg/kg ATR inhibited this measure in some dams and had no discernible effect in others. The 6.25 mg/kg dose of ATR was without effect. BROM also inhibited suckling-induced PRL release at the two highest doses.

To examine the effect of postnatal ATR and BROM on the incidence and severity of inflammation (INF) of the lateral prostate of the offspring, adult males were examined at 90 and 120 days. While no effect was noted at 90 days of age, at 120 days both the incidence and severity of prostate inflammation was increased in those offspring of ATR-treated dams (25 and 50 mg/kg). The 12.5 mg/kg ATR and the two highest doses of BROM increased the incidence, but not severity, of prostatitis. Combined treatment of ovine prolactin (oPRL) and 25 or 50 mg/kg ATR on PND 1 to 4 reduced the incidence of inflammation observed at 120 days, indicating that this increase in INF seen after ATR alone resulted from the suppression of PRL in the dam. Testing to determine whether or not there is a critical period for these effects revealed that the critical period for this effect is PND 1 to 9.

13.5 Summary/Conclusion

These data demonstrate that ATR suppresses suckling-induced PRL release and that this suppression results in an increase in lateral prostate inflammation in the offspring and that the critical period for this effect is PND 1 to 9.

13.5.1 Summary

The NOAELs for the above-described effects on pregnancy, pubertal onset and prostatitis are, for the most part, at or above 25 mg/kg/day. The exceptions are:

- A NOAEL for maternal effects in the pregnancy maintenance studies, in the SD rat strain only, was not found and the LOAEL is 25 mg/kg/day;
- The NOAEL for delay of pubertal onset in males is not clear as a significant delay was seen at 12.5 mg/kg/day, but not at the next highest dose of 25 mg/kg/day;
- The NOAEL for prostatitis is 12.5 mg/kg/day.

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Appendices

Appendix Table 1: Summary of the Atrazine Two Year and One Year Bioassays Using the SD Strain of Rat

Study	Duration	Doses Tested	Female Mammary Fibroadenoma Incidence (doses are in ppm)	Fibroadenoma P Values Adjusted for Survival (Trend is indicated at control)	Female Mammary Carcinoma Incidence (doses are in ppm)	Carcinoma P Values Adjusted for Survival (Trend is indicated at control)
Mayhew, *et al.*, 1986	2 year	0, 10, 70 , 500 or 1000 ppm (0, 0.5, 3.5, 25 or 50 mg/kg/day)	0= 23%; 10= 37%; 70= 30%; 500= 31%; 1000=22 %	0= 0.446; 10=0.110, 70= 0 373 ; 500= 0.373; 1000= 0.468	0= 17%; 10=24 %; 70=39 %; 500= 40%; 1000=51 %	0=0.00 ; 10=0.39; 70= 0 024 ; 500= 0.019; 1000= 0.000
Thakur, 1991a	2- year with serial sacrifices	0, 70 and 400 ppm (0, 4.23 and 26.23 mg/kg/day)	0= 11.6%; 70= 17.9%; 400= 18.8%	0=0.484 ; 70=0.213; 400=0.084[2]	0=13% ; 70=6%, 400=15.9%	0=0 092 ; 70=0 254; 400=0.619[2]
Thakur, 1992a	2- year	0, 70 and 400 ppm (0, 3.79 and 23.01 mg/kg/day)	0= 65%; 70=51 %; 400= 68.3%	0=not meaningful ; 70= 0 914; 400=0.107[2]	0=28% ; 70=22%; 400=33.6%	0= not meaningful; 70=0.832; 400=0 159[2]
Morseth, 1998[1]	2 year	0, 25, 50, 70 and 400 ppm (0, 1.5, 3.1, 4.2, 24.4 mg/kg/day)	0= 21%; 25= 32%; 50=44 % 70=37 %; 400=32%	0=0.23; 25=0.03; 50=0.00; 70=0.014;400=0.014	0= 15% ; 25=22% 50=25% ; 70=18% ; 400= 34%	0=0.002; 25=0.112; 50=0.067; 70=0.395; 400=0.007

Study	Duration	Doses Tested	Female Mammary Fibroadenoma Incidence (doses are in ppm)	Fibroadenoma P Values Adjusted for Survival (Trend is indicated at control)	Female Mammary Carcinoma Incidence (doses are in ppm)	Carcinoma P Values Adjusted for Survival (Trend is indicated at control)
Pettersen, and Turnier, 1995[3]	1 year	0, 15, 30, 50, 70, or 400 ppm (0, 0.8, 1.7, 2 8, 4.1, or 23 9 mg/kg/day)	0= 5.9%; 15= 5.9% 30= 5.9%; 50=0 %; 70= 11.3%; 400= 11 9%	Survival was similar between groups. Thus, a survival adjusted analysis is not meaingful.	0= 2.9%; 15=2.9 % 30=2 9%; 50= 5 9%; 70=2.9%; 400= 17.1%	Survival was similar between groups. Thus, a survival adjusted analysis is not meaingful.

[1]This study employed both ovarectomized and intact animals. However, no ovarectomized animal was found to have a mammary tumor Thus, results shown are for intact animals only.

[2] Based on Cox-Tarone .

[3] Incidence rates are based on 9 and 12 month timepoints only

Appendix Table 2: The Thakur Study Design

Type of study	Sacrifice	Strain	Sex	Doses	MRID
Serial Sacrifice	at 1, 3, 9, 12, 15, 18 and 24 months	one study with SD one study with F-344	Females only in both strains	0, 10, 70, 200 and 400 ppm for F-344 0, 70 and 400 ppm for SD	42085001 -SD 42146101 - F-344
Terminal Sacrifice	after 24 months	one study with SD one study with F-344	Females only for SD both sexes for F-344	0, 10, 70, 200 and 400 ppm for F-344 0, 70 and 400 ppm for SD	42204401 - SD 42227001 - F-344
Estrous cycle and hormone evaluations	serial sacrifices	one study with SD one study with F-344	Females only	all doses	42743903 - F-344 42743902 - SD

Appendix Table 3: Tumor Incidence by timepoints in Thakur, 1991a. Values shown are the number of rats with that type of mammary tumor. Mammary tumors found in animals which died an unscheduled death are included in the data for the timepoint which immediately followed the animals death.

	Control	4.23 mg/kg/day	26.63 mg/kg/day
1 month	no mammary tumors of any type	no mammary tumors of any type	no mammary tumors of any type
3 month	no mammary tumors of any type	no mammary tumors of any type	no mammary tumors of any type
9 month	no mammary tumors of any type	no mammary tumors of any type	Fibroadenomas= 2 Carcinomas=4
12 month	Fibroadenomas= 1 Carcinomas=0	Fibroadenomas=0 Carcinomas=1	Fibroadenomas= 2 Carcinomas=2
15 month	Fibroadenomas= 2 Carcinomas= 2	Fibroadenomas=5 Carcinomas=0	Fibroadenomas=1 Carcinomas=1
18 month	Fibroadenomas=2 Carcinomas=5	Fibroadenomas=4 Carcinomas=2	Fibroadenomas=4 Carcinomas=4
24 month	Fibroadenomas=3 Carcinomas=2	Fibroadenomas=3 Carcinomas=1	Fibroadenomas=4 Carcinomas=0
0-12 month total	Fibroadenomas= 1 Carcinomas= 0	Fibroadenomas= 0 Carcinomas= 1	Fibroadenomas= 4 Carcinomas= 6
0-24 month total	Fibroadenomas= 8 Carcinomas= 9	Fibroadenomas= 12 Carcinomas= 4	Fibroadenomas= 13 Carcinomas= 11

Appendix Table 4: All 20 Guideline studies with Atrazine, Propazine, Simazine or Atrazine mammalian metabolites that have been submitted and found acceptable by HED

Chemical	Study Type	Endpoint	Cell type/Species	Results	MRID
Atrazine	Ames Test	Gene Mutation	TA 98, 100, 1535, 1537 and 1538	Negative when tested with and w/o activation up to the limit dose of 5000 µg/plate	0060642
Atrazine	Ames Test	Gene Mutation	TA 98, 100, 1535 and 1537	Negative when tested with and w/o activation up to the limit dose of 5000 µg/plate	40246601
Atrazine	Unscheduled DNA Synthesis	DNA Damage	Isolated rat hepatocytes strain- Tif:RAlf	Negative when tested up to the solubilty limit	00161790/ 40246602
Atrazine	Unscheduled DNA Synthesis	DNA Damage	Isolated rat hepatocytes strain- Tif:RAlf	Negative when tested up to the solubilty limit	42547105
Atrazine	Micronucleus Assay	Clastogenicity (Chromosomal aberrations)	Mouse Strain-Tif:MAGf	Negative up to a dose causing death in the mouse	40722301
Atrazine	Dominant Lethal Assay	Genotoxicity to germinal tissue (effects which cause embry. or fetal death)	Mouse Strain-Tif:MAGf	Negative when tested in doses which induced toxicity	42637003

Appendix Table 4 cont.

Chemical	Study Type	Endpoint	Cell type/Species	Results	MRID
Simazine	Ames Test	Gene Mutation	TA 98, 100, 1535, 1537, and 1538	Negative both with and w/o activation when tested up to the solubility limit	40614406
Simazine	Unscheduled DNA Synthesis	DNA Damage	Isolated rat hepatocytes strain- Tif:RAlf	Negative when tested up to the solubilty limit	4144902
Simazine	Micronucleus Assay	Clastogenicity (Chromosomal aberrations)	Mouse strain-Tif:MAGf	Negative up to the limit dose of 5000 mg/kg	41442901
Propazine	In vitro mammalian cell gene mutation assay	Gene Mutation	V79 cell line - Chinese Hamster, fibroblast - like	Positive dose-related response (5-23x background) w/o activation at 800 and 1000 µg/ml Weak (5x background and non-dose related) mutagenic response with activation at 2000 µg/ml	0016322
Propazine	Unscheduled DNA Synthesis	DNA Damage	Isolated rat hepatocytes strain- Tif:RAlf	Negative when tested up to the solubilty limit	00150623
Propazine	Micronucleus Assay	Clastogenicity (Chromosomal aberrations)	Female Chinese Hamsters	Negative up to the limit dose of 5000 mg/kg	00150622
DACT	Ames Test	Gene Mutation	TA 98, 100, 1535, and 1537	Negative with and w/o activation up to the limit dose of 5000 µg/plate	40722302
DACT	Unscheduled DNA Synthesis	DNA Damage	CRL 1521 cell line - human fibroblast - like	Negative without activation only when tested up to solubilty limits	40722303
G-28279	Ames Test	Gene Mutation	TA 98, 100, 1535, and 1537	Negative when tested with and w/o activation up to the limit dose of 5000 µg/plate	43049101
G-28279	Unscheduled DNA Synthesis	DNA Damage	Isolated rat hepatocytes strain- Tif:RAlf	Negative up the cytotoxic dose of 800 µg/ml	43049105

Appendix Table 4 cont.

Chemical	Study Type	Endpoint	Cell type/Species	Results	MRID
G-28279	Micronucleus Assay	Clastogenicity (Chromosomal aberrations)	Mouse strain-Tif:MAGf	Negative up to the maximum tolerated dose of 480 mg/kg	43093103
G-30033	Ames Test	Gene Mutation	TA 98, 100, 1535, and 1537	Negative when tested with and w/o activation up to the limit dose of 5000 µg/plate	43093102
G-30033	Unscheduled DNA Synthesis	DNA Damage	Isolated rat hepatocytes strain- Tif.RAlf	Negative in doses up to the cytotoxic dose of 1000 µg/ml	43093106
G-30033	Micronucleus Assay	Clastogenicity (Chromosomal aberrations)	Mouse strain-Tif:MAGf	Negative up to the maximum tolerated dose of 480 mg/kg	43903104

Appendix Table 5: Database for the Genotoxicity of Atrazine*

Test system	Results[a] Without Exogenous Metabolic Activation	With Exogenous Metabolic Activation	Dose[b] (LED or HID)	Reference**
MUTATION				
Bacteriophage T4, forward mutation	-	NT	20 ug/plate	Andersen *et al.* (1972)
Bacteriophage, reverse mutation	-	NT	1000 ug/plate	Andersen *et al. (1972)*
Salmonella typhimunum, forward mutation, 6AG^R	-	-	250 ug/ml	Adler (1980)
Salmonella typhimurium TA100, TA98, TA1535, TA1537, TA1538 reverse mutation	-	-	5000 ug/plate	Poole & Simmon (1977; DER)
Salmonella typhimurium TA100, TA98, TA1535 reverse mutation	-	-	100 ug/plate	Lusby *et al* (1979)
Salmonella typhimurium TA100, TA98 reverse mutation	NT	-	1100 ug/plate	Bartsch *et al. (1980)*
Salmonella typhimurium TA100, TM677 reverse mutation	NT	-[c]	30000 ug/plate	Sumner *et al* (1984)
Salmonella typhimunum TA100, TA98, TA97, TA1535, TA1537, TA1539 reverse mutation	-	-	1000 ug/plate	Kappas(1988)
Salmonella typhimurium TA100, reverse mutation	NT	+[c]	NG	Means *et al* (1988)
Salmonella typhimurium TA100, TA98, TA97, TA102 reverse mutation	-	-	1000 ug/plate	Mersch-Sundermann *et al* (1988)
Salmonella typhimurium TA100, TA98, TA97, TA1535, TA1537, TA1538 reverse mutation	-	-	1000 ug/plate	Zeiger *et al*, (1988)
Salmonella typhimurium TA100, TA98, TA97 reverse mutation	-	NT	2000 ug/plate	Butler & Hoagland (1989)
Salmonella typhimurium TA100, TA98, TA102, TA1535, TA1537 reverse mutation	-	-	1000 ug/plate	Ruiz & Marzin (1997)
Salmonella typhimunum TA100, TA98, TA1535, TA1537 reverse mutation	-	-	5000 ug/plate	Deparde (1986; DER)
Salmonella typhimurium TA100, TA98 reverse mutation	-	-	1000 ug/plate	Morichetti *et al.* (1992)
Salmonella typhimurium TA1530, TA1531, TA1532, TA1534, *his* G45 reverse mutation (spot test)	-	NT	NG	Seiler (1973)
Salmonella typhimunum, (eight unidentified strains) reverse mutation	-	NT	NG	Andersen *et al (972)*
Salmonella typhimurium, (strains not identified) reverse mutation	-	-	NG	Adler (1980)
Escherichia coli, forward mutation, AMP^R	-	-	430 ug/plate	Adler, (1980)
Saccharomyces cerevisiae, reverse mutation (stationary phase cells)	-	NT	75600 ug/ml	Morichetti *et al.* (1992)
Saccharomyces cerevisiae, reverse mutation (logarithmic phase cells)	(+)	NT	2160 ug/ml	Morichetti *et. al.* (1992)
Saccharomyces cerevisiae, forward mutation	-	NT	50 ug/ml	Emnova *et al* (1987)
Schizosaccharomyces pombe, reverse mutation	+	NT	17.5 ug/ml	Mathias (1987)
Schizosaccharomyces pombe, reverse mutation	+	+[c]	70 ug/ml	Mathias (1987)
Aspergillus nidulans, forward mutation	-	+	2500 ug/ml	Benigni *et al.* (1979)
Gene mutation, Chinese hamster lung V79 cells, *hprt* locus	-	-[e]	2000 ug/ml	Adler (1980)
Host-mediated assay, *Escherichia* coli Amp^R in mouse		+	100 po x 1	Adler (1980)
Drosophila melanogaster, somatic mutation	+		1000 ug/g feed	Torres *et al* (1992)
Drosophila melanogaster, somatic mutation	+		200 ug/g feed	Tripathy *et al.* (1993)
Drosophila melanogaster, sex-linked recessive lethal mutation	(+), I^G		100 ug/g feed	Murnik & Nash (1977)
Drosophila melanogaster, sex-linked recessive lethal mutation	-		2000 ug/g feed	Adler (1980)
Drosophila melanogaster, sex-linked recessive lethal mutation	(+), I^E		200 ug/g feed	Tripathy *et al.* (1993)
Drosophila melanogaster, dominant lethal mutation	(+) I^G,		100 ug/g feed	Murnik & Nash (1977)
Drosophila melanogaster, sex-linked recessive lethal mutation			10,000 ug/g feed	Njagi *et al.* (1980)

Table 5 (cont)

Test system	Results[a] Without Exogenous Metabolic Activation	With Exogenous Metabolic Activation	Dose[b] (LED or HID)	Reference
CHROMOSOME ABERRATIONS--IN VITRO				
Chromosomal aberrations, Chinese hamster CHO cells *in vitro*	-	-	2000 ug/ml	Adler (1980)
Chromosomal aberrations, human lymphocytes *in vitro*	+	NT	0.15 ug/ml	Lioi *et al* (1998)
Chromosomal aberrations, Chinese hamster CHO cells *in vitro*	-	NT	250 ug/ml	Ishidate (1988)
Chromosomal aberrations, human lymphocytes *in vitro*	-	NT	50 ug/ml	Kligerman *et al* (1999)
Chromosomal aberrations, human lymphocytes *in vitro*	(+)	NT	0.1 ug/ml	Meisner *et al.* (1992)
Chromosomal aberrations, human lymphocytes *in vitro*	(+)	NT	1 0 ug/ml	Meisner *et al* (1993)
CHROMOSOME ABERRATIONS-IN VIVO				
Micronucleus formation, Tif.MAGf female mouse bone-marrow cells *in vivo*	-		2250mg/kg po x 1	Ceresa (1988a; DER)
Micronucleus formation, Tif.-MAGf male mouse bone-marrow cells *in vivo*	-		2250mg/kg po x 1	Ceresa (1988a; DER)
Micronucleus formation, NMRI female mouse bone-marrow cells *in vivo*	(+)		1400mg/kg: po x 1	Gebel *et al* (1997)
Micronucleus formation, NMRI male mouse bone-marrow cells *in vivo*			1750mg/kg po x 1	Gebel *et al* (1997)
Micronucleus formation, female mouse bone-marrow cells *in vivo*	-		500mg/kg ip x 2	Kligerman *et al* (1999)
Chromosome aberrations, mouse bone-marrow cells *in vivo*			20 ppm dw	Meisner *et al* (1992)
OTHER INDICATORS OF DNA DAMAGE				
Eschenchia coli PQ37	-	-	1000 *ug/ml*	Ruiz & Marzin *(1997)*
Saccharomyes cerevisiae, gene conversion	-	+c	10 *ug/ml*	Plewa and Gentile *(1976)*
Saccharomyes cerevisiae, gene conversion	-	-	2000 *ug/ml*	Adler (1980)
Saccharomyes cerevisiae, gene conversion			4000 *ug/ml*	de Bertoldi *et al (1980)*
Saccharomyes cerevisiae, gene conversion (stationary phase cells)	+	NT	64800 *ug/ml*	Morichetti *et al. (1992)*
Saccharomyes cerevisiae, gene conversion (logarithmic phase cells)	-	NT	540 *ug/ml*	Morichetti *et al.* (1992)
Saccharomyes cerevisiae, mitotic recombination	-	NT	50 *ug/ml*	Emnova *et al.* (1987)
Aspergillus nidulans, gene conversion	-	NT	8000 ug/ml	de Bertoldi *et al* (1980)
Aspergillus nidulans, mitotic recombination	-	+	NG	Adler (1980)
Aspergillus nidulans, mitotic recombination	-	-	1000 ug/ml	Kappas(1988)
DNA damage, human lymphocytes in vitro	(+)	-	100 ug/ml	Ribas *et al*, (1995)
Unscheduled DNA synthesis, human EUE cells *in vitro*	-	-[e]	650 ug/ml	Adler (1980)
Unscheduled DNA synthesis, rat primary hepatocytes *in vitro*	-	NT	139 ug/ml	Hertner (1992; DER)
Unscheduled DNA synthesis, rat primary hepatocytes *in vitro*	-	NT	150 ug/ml	Puri & Muller (1984; DER)
DNA strand breaks, rat stomach, liver and kidney *in vivo*	(+)		875mg/kg po x 1	Pino *et al* (1988)
DNA strand breaks, rat stomach, liver and kidney *in vivo*	(+)		350mg/kg po x 15	Pino, *et al* (1988)
DNA strand breaks, rat lung *in vivo*	(+)		875mg/kg po x 1	Pino *et al* (1988)
DNA strand breaks, rat lung *in vivo*	(+)		350mg/kg po x 15	Pino *et al* (1988)
Rana catesbeiana tadpoles, DNA damage	+[d, f][E]		4 8 mg/kg	Clements *et al* (1997)
Sister chromatid exchanges, human lymphocytes *in vitro*	-,[G]	NT	NG	Ghiazza *et al* (1984)
Sister chromatid exchanges, human lymphocytes *in vitro*	-	-	10 ug/ml	Dunkelberg *et al* (1994)
Sister chromatid exchanges, human lymphocytes *in vitro*	-	NT	50 ug/ml	Kligerman *et al* (19 99)
Sister chromatid exchanges, human lymphocytes *in vitro*	(+)	NT	0 1 ug/ml	Lioi *et al* (1998)

Sister chromatid exchange, Chinese hamster CHO cells *in vitro*	-	-	2000 ug/ml	Adler (1980)
DNA repair exclusive of unscheduled DNA synthesis, human lymphocytes in vitro	-	NT	25 ug/ml	Surralles *et al.* (1995)

Table 5. cont.

Test system	Results[a] Without Exogenous Metabolic Activation	With Exogenous Metabolic Activation	Dose[b] (LED or HID)	Reference
PLANT TESTS				
Hordeum vulgare, mutation	+	NT	1000 mg/kg	Wuu & Grant (1966)
Hordeum vulgare, mutation	-	NT	200 mg/kg	Stroev (1968)
Zea mays, mutation	+	NT	200 mg/kg	Morgun *et al.* (1982)
Zea mays, mutation	+	NT	NG	Plewa *et al* (1984)
Nicotiana tabacum, mutation		-	NT	NG Briza (1999)
Tradescantia *paludosa*, micronucleus formation	-	NT	200 mg/kg	Ma et al, (1984)
Hordeum vulgare, chromosomal aberrations	+	NT	500 mg/ kg spray	Wuu & Grant (1967 a)
Hordeum Vulgare, chromosomal aberrations	-	NT	2000 mg/kg	Muller *et* 471 (1972)
Vicia faba, chromosomal aberrations	+	NT	400 mg/kg	Wu & Grant (1967b)
Vicia faba, chromosomal aberrations	-	NT	200 mg/kg	Khudoley *et* al, (1997)
Sorghum sp., chromosomal aberrations	+	NT	NG[d]	Liang & Liang (1972)
Sorghum sp., chromosomal aberrations	-	NT	NG	Muller *et* al. (1972)
Sorghum sp., chromosomal aberrations	+	NT	NG	Lee *et al.* (1974)
Nigella damascena, chromosomal aberrations	-	NT	320 mg/kg	Mathias (1987)
Nigella damascena, chromosomal aberrations	+	NT	40[d] mg/kg	Mathias (1987)
Zea mays, chromosomal aberrations	-	NT	200 mg/kg	Morgun *et al.* (1992)
ANEUPLOIDY[f]				
Aspergillus nidulans	-	+	2000 ug/ml	Benigni *et al* (1979)
Neurospora crassa,	+	NT	NG	Griffiths (1979)
Drosophila melanogaster	+		100 ug/g feed	Murnik & Nash (1977)
GERM CELL EFFECTS				
Dominant lethal effects mouse (all germ cell stages)	-		2400mg/kg PO x 1	Hertner (1993; DER)
Dominant lethal effects mouse spermatids	+		1500mg/kg po x 1	Adler (1980)
Sperm morphology, mouse	-		600mg/kg ip x 4	Osterloh

* This Table was adopted and updated from Dearfield et al., 1993

** DER, data entry record-study was submitted by registrant and considered acceptable guideline study after review by EPA's Office of Pesticide Program

[a] +, positive; (+) weakly positive; -, negative; IG, determined to be inconclusive finding by the GeneTox Panel; IE, determine to be an inconclusive finding by EPA review; NT, not tested

[b] LED, lowest effective dose, HID, highest ineffective does; in vitro test, mg/kg bw/day; NG, not given; po, oral (gavage or gastric intubation); dw, drinking water; d , days; ip, intraperitoneal

[c] Tested extracts of atrazine-treated Zea *mays*

[d] Commercial pesticide

[e]Positive with potato microsomes at doses up to 3 mM

[f] Aneuploidy, chromosome loss or gain is not typically associated with a DNA reactive mutagenic mechanism but usually involves disruption of spindle formation or chromosomal segreagation

Table 6. Database for the Genotoxicity of Simazine

Test system	Results[a]		Dose b (LED or HID)	Reference
	With Exogenous Metabolic Activation	Without Exogenous Metabolic Activation		
MUTATION				
Escherichia coli PQ37, SOS chromotest	NT	-	NG	Mersch-Sundermann *et al* (1988)
Salmonella typhimurium TA1978/TA1538 and SL525/SL4700 differential toxicity	-	NT	2000 ug/disc	USEPA (1984)
Bacillus subtilis rec strains, differential toxicity	-	NT	1000 ug/disc	Kuroda *et al* (1992)
Salmonella typhimurium TA100, TA98, TA1535, TA1537, TA1538, reverse mutation	NT	-	NG	Simmon *et al* (1977)
Salmonella typhimurium TA100, TA98, reverse mutation	-	-	5000 ug/plate	USEPA (1984)
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, reverse mutation	-	-	1000 ug/plate	USEPA(1977)
Salmonella typhimurium TA100, reverse mutation	NT	+[c]	NG	Means *et al* (1988)
Salmonella typhimurium TA100, TA102, TA97, reverse mutation	-	-	1000 ug/plate	Mersch-Sundermann *et al* (1988)
Salmonella typhimurium TA1530, TA1531, TA1532, TA1534, G46, reverse mutation (spot test)	-	NT	NG	Sieler (1973)
Salmonella typhimurium, (eight unidentified strains) reverse mutation	-	NT	NG	Andersen *et al* (1972)
Escherichia coli, forward mutation	-	NT	NG	Fahrig (1974)
Escherichia coli WP2 uvr, reverse mutation	-	-	1000 ug/plate	USEPA (1984)
Serratia marcescens, reverse mutation	-	NT	NG	Fahrig (1974)
OTHER INDICATIONS OF DNA DAMAGE				
Saccharomyces cerevisiae, gene conversion	-	NT	NG	Fahrig (1974)
Saccharomyces cerevisiae, gene conversion	-	NT	1000[d]	Siebert & Lemperle (1974)
Saccharomyces cerevisiae D3, homozygosis by recombination	-	-	50000	USEPA (1977)
Saccharomyces cerevisiae D7, mitotic recombination	-	-	25000	USEPA (1984)
Saccharomyces cerevisiae D7, reverse mutation	-	-	25000	USEPA (1984)
Saccharomyces cerevisiae D7, gene conversion	-	-	25000	USEPA (1984)
Saccharomyces cerevisiae, reverse mutation	-	NT	5	Emnova et al. (1987)
Drosophila melanogaster, somatic mutation	+		2000 ug/g feed	Tripathy *et al* (1995)
Drosophila melanogaster, sex-linked recessive lethal mutation			10 ng/fly inj	Benes & Sram (1969)
Drosophila melanogaster, sex-linked recessive lethal mutation	+		6 ng/fly inj	Murnik & Nash (1977)
Drosophila melanogaster, sex-linked recessive lethal mutation			6000 ug/g feed	Murnik & Nash (1977)
Drosophila melanogaster, sex-linked recessive lethal mutation	+		2000 ug/g feed	Tripathy *et al* (1995)
Drosophila melanogaster, dominant lethal test	+		6000 ug/g feed	Murnik & Nash (1977)
Drosophila melanogaster, aneuploidy	-		6000 ug/g feed	Murnik & Nash (1977)
Gene mutation, mouse lymphoma L5178Y cells *in vitro, tk locus in vitro*	-	*(+)I*	*300*	Jones et al. (1984)
CHROMOSOME ABERRATION				
Chromosomal aberrations, Chinese hamster ovary CHO cells *in vitro*	- NT	0 01	Biradar & Rayburn (1995)	
Chromosomal aberrations, human lymphocytes *in vitro*	- NT	37.5	Kligerman et al (1999)	
Micronucleus formation, mouse bone-marrow and peripheral blood cells *in vivo*	NT	500 po x 2	USEPA (1984)	
Micronucleus formation, both sexes,mouse bone-marrow *in vivo*	- NT	5000 po x 1	Ceresa (1988a)	

Table 6 (cont)

Test system	Results[a]		Dose[b] (LED or HID)	Reference
	Without Exogenous Metabolic Activation	With Exogenous Metabolic Activation		
OTHER INDICATORS OF DNA DAMAGE				
Sister chromatid exchange, Chinese hamster ovary CHO cells *in vitro*	-	NT	1700	USEPA (1984)
Sister chromatid exchange, Chinese hamster lung V79 cells *in vitro*	-	NT	2	Kuroda *et al* (1992)
Sister chromatid exchange, human lymphocytes *in vitro*	-	NT	37.5	Kligerman et al (1999)
Sister chromatid exchange, human lymphocytes *in vitro*	(+), I	NT	NG	Ghiazza *et al* (1984)
Sister chromatid exchange, human lymphocytes *in vitro*			*10*	Dunkelberg *et al* (1994)
Unscheduled DNA synthesis, human lung WI 38 fibroblasts *in vitro*	-		*200*	USEPA (1984)
Unscheduled DNA synthesis, rat primary heptocytes	-	NT		Hertner (1992)
PLANT ASSAYS				
Hordeum vulgare, mutation	+	NT	1000	Wuu & Grant (1966)
Hordeum vulgare, mutation		NT	200	Stroev (1968a)
Rizobium meliloti, mutation		NT	5000	Kaszubiak (1968)
Zea mays, chlorophyll mutation	+	NT	200	Morgun et al. (1982)
Zea mays, mutation	+	NT	NG	Plewa et al (19 84)
Fragaria ananassa, mutation	+	NT	2	Malone & Dix (1990)
Tradescantia paludosa, micronuclei		NT	200	Ma et al (1984)
Hordeum vulgare, chromosomal aberrations	+	NT	500	Wuu & Grant (1966)
Hordeum vulgare, chromosomal aberrations	+	NT	500 spray	Wuu & Grant (1967a)
Hordeum vulgare, chromosomal aberrations	(+)	NT	500	Stroev (1968b)
Hordeum vulgare, chromosomal aberrations	(+)	NT	500[c]	Kahlon (1980)
Vicia faba, chromosomal aberrations	+	NT	200[c]	Wuu & Grant (I 967b)
Vicia faba, chromosomal aberrations	+	NT	5	Hakeem & Shehab (1974)
Vicia faba, chromosomal aberrations	(+)	NT	1000	de Kergommeaux *et al* (1983)
Allium cepa, chromosomal aberrations	+	NT	20	Chubutia & Ugulava (1973)
Crepis capillaris, chromosomal aberrations	+	NT	1000	Voskanyan & Avakyan (1984)
ANEUPLOIDY TESTS				
Neurospara crassa, aneuploidy	-	NT	NG	Griffiths (1979)

a +, positive; (+), weakly positive, -, negative; NT, not tested

b LED, lowest effective dose; HID, highest ineffective dose; unless otherwise stated, in-vitro test, ug/mL; in-vivo test, mg/kg bw/day; NG, not given; inj, injection, po, oral

[c]Tested with extracts of simazine-treated *Zea mays*

[d]Commercial pesticide tested

Table 7. Database for the Genotoxicity of Propazine

Test system	Results[a]		Dose[b]	Reference**
Without Exogenous Metabolic Activation	With Exogenous Metabolic Activation	(LED or HID)		
MUTATION				
Bacteriophage, forward mutation	-	NT	100 ug/plate	Andersen *et al* (1972)
Bacteriophage, reverse mutation	-	NT	2000 ug/plate	Andersen *et al* (1972)
Salmonella typhimurium TA100,TA98,TA1535,TA1537,TA1538 reverse mutation	-	-	5000 ug/plate	Kappas(1988)
Gene mutation, Chinese hamster lung V79 cells, *hprt* locus	(+)	-	400	Ciba-Geigy (1986; DER)
CHROMOSOME ABERRATIONS--IN VITRO				
Chromosomal aberrations, Chinese hamster CHO cells *in vitro*	-	-	3000 ug/ml	Ishidate (1983)
CHROMOSOME ABERRATIONS--IN VIVO				
Micronucleus formation, Hamsters *in vivo*	-		50000 po x 1	Ciba-Geigy (1984; DER)
OTHER INDICATORS OF DNA DAMAGE				
Unscheduled DNA synthesis, rat primary hepatocytes -	NT	62.5	Puri (1984)	
Aspergillus nidulans, crossing over	-	-	800	Kappas (1988)

**DER, data entry record-study was submitted by registrant and considered acceptable guideline study after review by EPA's Office of Pesticide Program.

[a] +, positive; (+), weakly positive, -, negative, I^G, determined to be an inconclusive finding by the GeneTox Panel; I^E, determined to be an inconclusive finding by EPA review; NT, not tested

[b] LED, lowest effective dose; HID, highest ineffective dose; in-vivo test, mg/kg bw/day, NG, not given; po, oral (gavage or gastric intubation); dw, drinking water, d, days, ip, intraperitoneal

Appendix Table 8: Data from Eldridge, 1993a. DI=Diestrus; PR=Proestrus; ES=Estrus

% days in stages of estrous cycle in SD rats						
mg/kg/day▾	3 months	9 months	12 months	15 months	18 months	24 months
0	DI= 46.1±7.1 PR= 29.5 ±4 6 ES= 24 8±7 7	DI= 44 8@-- ±7.6 PR= 30.9 ±5 7 ES= 24.2@++ ±7.6	DI= 31.1@-- ±5.3 PR= 26.0±7 9 ES= 42 6@+ ±10 1	DI= 36.7±15.1 PR= 19.2±8.2 ES= 44.4±12 2	DI= 31.0 @--±5 5 PR= 24.4@-±4 1 ES= 44.9@++ ±5.7	DI= 31.6 ±22 2 PR= 21.0±5.7 ES= 47.8±18 9
4.23	DI= 42 6±7.4 PR=32.5 ±7.6 ES= 25 2±4.9	DI= 36.2*±7.9 PR= 29.4±6.5 ES= 34.4*±9.0	DI= 26.9±8.5 PR= 26.1±8.4 ES= 47.2±13.7	DI= 32 8±14.0 PR= 24.6±8.7 ES= 42.7±12.6	DI= 23 0*±9.0 PR= 20.1±6.4 ES= 57.2*±12.5	DI= 37 0±32.8 PR= 13.3±8.1 ES= 50 0±27.3
26.23	DI= 42.0±6.7 PR= 30.2±6.3 ES= 27.8±7.6	DI= 25.9**±9.1 PR= 29.4±6 2 ES= 44.8**±11.6	DI= 22.4*±7.5 PR= 24.6±7.3 ES= 53.3±11.2	DI= 28 0±11.3 PR= 22.4±5.1 ES= 49.6±12.2	DI= 25.5*±17.3 PR= 18.7±7.9 ES= 55.9*±20.7	DI= 55.0±9.9 PR= 21.5±10.6 ES= 24.0±0 0

@+ Dose-related trend is statistically significant, in positive direction, at $p \leq 0.05$
@++ Dose-related trend is statistically significant, in positive direction, at $p \leq 0.01$
@- Dose-related trend is statistically significant, in negative direction, at $p \leq 0.05$
@-- Dose-related trend is statistically significant, in negative direction, at $p \leq 0.01$
* Statistically Significant at $p < 0.05$
** Statistically signficant at $p<0.01$

Appendix Table 9. Data from Morseth, 1996a. Data from One Month study exposing SD females through the diet

Days In Estrus vs. Time					
Dose ▸	0 mg/kg/day	2.5 mg/kg/day	5 mg/kg/day	40 mg/kg/day	200 mg/kg/day
# normally cycling animals	67 (74.4%)	66 (73.3%)	65 (72.2%)	50 (55.6%)	33 (36.7%)
# animals with diestrus blocks	21 (23.3%)	20 (22.2%)	21 (23 3%)	36 (40%)	51 (56 7%)
# animals with estrus blocks	3 (3.3%)	3 (3.3%)	4 (4.4%)	6 (6.6%)	11 (12.2%)

Appendix Table 10 : Data from Morseth, S., 1996b. Six month study exposing SD females through the diet

% Days in Estrus or Diestrus							
Dose (mg/ kg/day)▪	1-2 weeks	5-6 weeks	9-10 weeks	13-14 weeks	17-18 weeks	21-22 weeks	25-26 weeks
0	DI= 58 ± 9.2 ES=22 ± 5.2	DI= 55 ± 8.7 ES= 23 ± 5.1	DI= 54 ± 7.5 ES= 25 ± 9.4	DI= 49 ± 17 2 ES= 31 ± 22.4	DI= 47 ± 18.1 ES= 34 ± 24.2	DI= 51 ± 22 3 ES= 32 ± 25 4	DI= 40 ± 25.7 ES= 47 ± 32.2
1.8	DI= 57 ± 10 2 ES= 22 ± 5 6	DI= 55 ± 7.6 ES= 23 ± 4.5	DI= 54 ± 7.5 ES= 25 ± 4.8	DI= 53 ± 15.1 ES= 28 ± 18	DI= 49 ± 19.4 ES= 33 ± 24.7	DI= 43 ± 24.6 ES= 41 ± 31.9	DI= 42 ± 29.6 ES= 48 ± 35 5
3.65	DI= 56 ± 10.2 ES= 22 ± 5.4	DI= 53 ± 10 9 ES= 25 ± 10	DI= 51 ± 8.7 ES= 26 ± 10.2	DI= 49 ± 16 ES= 31 ± 21.1	DI= 47 ± 18.8 ES= 36 ± 25 1	**DI= 39 ± 23.9**** **ES= 45 ± 32.2***	DI= 34 ± 27 3 ES= 54 ± 35.1
29.4	DI= 61 ± 11.5 ES= 21 ± 7	DI= 55 ± 10 ES= 24 ± 7.4	DI= 52 ± 10 ES= 26 ± 9.3	**DI= 44 ± 21.6** **ES= 40 ± 27.6***	**DI= 41 ± 25 2** **ES= 45 ± 32.1***	**DI= 37 ± 27.7**** **ES= 51 ± 34.8****	**DI= 29 ± 30.2*** **ES= 63 ± 37.0***

* p≤ 0 05; ** p≤ 0 01

Appendix Table 11: Data from Thakur, 1999

Days in Estrus vs. Time					
Dose▸ Study Weeks	Control	1.5 mg/kg/day	3.1 mg/kg/day	4.2 mg/kg/day	24.4 mg/kg/day
1-14	26 12 ± 0.71	28 57 ± 0.86	26.38±0.87	26.45±0.72	28.91± 0.97
17-26	45 79 ± 2.05	50.28 ± 2.05	48.67 ± 2.25	48 34 ± 2 1	61.3 ± 2 3
29-38	77 22 ± 2 13	74 63 ± 2 2	71.4 ± 2 29	67.4 ± 2.36	80.75 ± 2 02
41-46	81.51 ± 2.44	75.77 ± 2.73	70.8 ± 2.81	73.41 ± 2 84	83.74 ± 2.24

Appendix Table 12: Data from Thakur, 1999

Percent of Animals with Estrus Blocks of at least 7 days					
Dose ▸ Study Weeks ▾	Control	1.5 mg/kg/day	3 1 mg/kg/day	4.2 mg/kg/day	24.4 mg/kg/day
17-18	17.5	15	21.52	17.5	26 35
21-22	22.78	28.75	31.65	33.74	50.63
25-26	30.38	36.25	36.71	33.75	50.63

Appendix Table 13: Data from Thakur, 1999

Percent Days in Estrus During Weeks 1-46 and Tumor Response for all Dose Groups Combined			
	No Tumor	Fibroadenoma	Carcinoma
Mean percent days in estrus	50.869	55 275#	60 346*
Standard Error	1.27	1.074	1.596
N	217	128	91

p=0.0341 compared to animals with no tumor
*p=0 0000 compared to animals with no tumor

Appendix Table 14: Data from Thakur, 1999

Percent Days in Estrus During Weeks 17-26 and Tumor Response for all Dose Groups Combined			
	No Tumor	Fibroadenoma	Carcinoma
Mean percent days in estrus	48.077	52.223	60.803*
Standard Error	1.864	2 489	2 995
N	216	128	91

*p=0.0003 compared to animals with no tumor

Appendix Table 15: Data from Eldridge, 1993a. E=Estradiol; PROG=Progesterone; PRL= Prolactin

Serum hormone levels in SD rats (E in pg/ml; PROG and PRL in ng/ml)						
mg/kg/day*	3 months	9 months	12 months	15 months	18 months	24 months
0	E= 3.5±6.4 PROG= 15.6 ±7 9 PRL= N/A	E= 22 6±20.6 PROG= 11 6±11.9 PRL= 17.8 ±12.4 &	E= 13 1±10.6 PROG= 4.0±1.5 PRL= 13.2±2.9	E= 17.3±12.8 PROG= 14.2 ±19 PRL= 16.1±15.2	E= 3 7 ±3.6 PROG= 19.6 ±29.3 PRL= 20.8 ±8.8	E= 2.1±3.3 PROG= 2 8±1.2 PRL=20.3 ±4 9
4.23	E= 11.2±12.6* PROG= 16.5±10.7 PRL= N/A	E= 20.7±26.1 PROG= 8.2±6.6 PRL= 24.3±10.4	E= 12.5±21.6 PROG= 6.9±11.4 PRL= 11 9±6.8	E= 18.8±18 PROG= 4.1±3 PRL= 11.2±7 6	E= 16.1±21.6 PROG= 11.7*±28 7 PRL= 17±6.3	E= 3.4±5.3 PROG= 13.3±22 1 PRL= 14.2± 6.4
26.23	E= 16.2±13* PROG= 14.3±7 3 PRL= N/A	E= 31.2±28 1 PROG= 7 4±4 1 PRL= 45.8±20**	E= 11.7±7.5 PROG= 3 2±1.4 PRL= 15±3 6	E= 18.4±7.6 PROG= 20±24.5 PRL= 15±11	E= 5 6±7.1 PROG= 4.4±4.9 PRL= 17.5±8.7	E= 0.9± 0 9 PROG= 3.9±0.6 PRL= 13 5±1.1

& Dose-related trend is statistically significant, in positive direction, at $p \leq 0.05$

@ Dose-related trend is statistically significant, in negative direction, at $p \leq 0.05$

* Statistically Significant at $p < 0.05$

** Statistically Significant at $p < 0.01$

N/A - samples from the 1 and 3 month timepoints were not available for analysis because these samples were inavertatly hydrolyzed.

Appendix Table 16: Histomorphology Analysis in the SD, McConnell, 1995

Values shown below are Index Weighted Scores at 1, 3, 9 and 12 months into the study.

Index Weighted Score[1] at 1, 3, 9 and 12 months				
Finding▸ Dose▾	Acinar Development (Estrogen)	Acinar/Lobular Development (Prolactin)	Secretory Activity (Prolactin)	Dilated Ducts with Secretion (Prolactin)
Control	1=15 3= 20 9= 28 12= 31	1= 9 3= 22 9= 23 12= 25	1= 15 3= 17 9= 24 12= 31	1= 9 3= 12 9= 17 12= 23
4.23 mg/kg/day	1= 22 3= 24 9= 33 12= 33	1= 10 3= 17 9= 28 12= 30	1= 14 3= 14 9= 28 12= 36	1= 12 3= 11 9= 24 12= 25
26 23 mg/kg/day	1= 21 3= 27 9= 45 12= 41	1= 12 3= 16 9= 42 12= 36	1= 12 3= 17 9= 46 12= 39	1= 9 3= 15 9= 45 12= 41

1 The index weighted score is calculated as such: the severity of the findings, as determined by the examining pathologist, is converted to a numerical value and the numerical values for each group are summed. The score of absent= 1; minimal =2; mild=3; moderate=4 and marked=5.

Appendix Table 17: Galactocele Incidence and Severity in the SD Female, Thakur, 1991a

Finding▸ Dose▾	One and Three month	Nine Months	12 Months	15 Months
Control	None at either timepoint	1 (slight)[1]	5 (4- minimal; 1 moderate)	7 (2- slight, 1- moderate, 1- moderatly severe; 3- severe
4.23 mg/kg/day	None at either timepoint	4 (2-minimal;1-slight; 1-moderate)	5 (1-minimal; 3-slight; 1- moderately severe)	7 (3 - minimal,4- slight)
26 23 mg/kg/day	None at either timepoint	8 (2-minimal; 1- moderate; 5 moderately severe)	10 (5-slight; 4-moderate; 1- moderately severe)	9 (2- minimal; 3- slight; 2- moderate, 1- moderatly severe; 1- severe)

1 The scores are: minimal, slight; moderate; moderately severe and marked

Appendix Table 18: Data from Morseth, 1996a and 1996b*

Timepoints for measurement of LH surge in both the one and six month studies				
Biologic time	# animals for non-repeat bleed	# animals for repeat bleed	Expected state of serum LH levels	In a normally cycling rat this is equivalent to:
1100	10	10	baseline	proestrus morning
1400	15	10	baseline	early afternoon proestrus
1600	15	10	LH surge	mid- afternoon proestrus
1800	15	10	LH surge	Late afternoon proestrus
2000	15	10	LH surge	Proestrus evening
2300	10	10	baseline	Proestrus evening

* There were 90 females in each group: 10 + 15 + 15 + 15 + 15 + 10 non-repeat bleed animals = 80 animals plus the 10 repeat bleed animals equals 90 animals per group.

Appendix Table 19: Data from Morseth, 1996a . Doses are in mg/kg/day. LH values given are in picograms/ml.

LH data from the one-month study						
Biologic Time▸	1100	1400	1600	1800	2000	2300
mean and SD *nonrepeat bleed*	0= 998 ± 614 2.5= 943 ± 614 5.0= 1140 ±715 40= 1219 ±467 200= 873 ±656	0= 1122 ± 564 2.5= 1171 ±802 5.0= 882 ±926 40= 1125 ±795 200= 1099 ±863	0= 3315 ± 2684 2.5= 20951 ±1315 5.0= 3099 ±2521 40= 3518 ±4514 200= 1685 ±2962	0= 5138 ± 4403 2.5= 4489 ±4345 5.0= 2804 ±13 40= 3246 ±1981 200= 2752 ±3137	0= 2242 ± 1850 2.5= 1118 ±412 5 0= 1554 ±14 40= 1740 ±1157 200= 1853 ±1138	0= 761 ± 288 2.5= 486 ±138 5.0= 508 ±317 40= 689 ±373 200= 1126 ±81645
mean and SD *repeat bleed*	0= 732 ± 461 2 5= 1101 ±652 5.0= 810 ±519 40= 755 ±389 200= 514 ±503	0= 786 ± 557 2.5= 2222 ±1220 5.0= 1678 ±1602 40= 1037 ±829 200= 453 ±313	0= 1301 ± 1031 2.5= 3029 ±2383 5 0= 4971 ±5047 40= 1137 ±629 200= 552 ±311	0= 2650 ± 2389 2.5= 3015 ±3220 5 0= 2717 ±25 40= 1450 ±857 200= 812 ±470	0= 2606 ± 2076 2.5= 1731±1447 5 0= 2954 ±3515 40= 1477 ±1296 200= 1140 ±328	0= 1671 ± 674 2 5= 1475 ± 456 5 0= 1431±345 40= 1362 ±329 200= 1080 ±30142

Appendix Table 20: Data from Morseth, 1996b. Doses are in mg/kg/day. LH values given are in picograms/ml.

LH data from the six-month study						
Biologic Time▸	1100	1400	1600	1800	2000	2300
mean and SD *nonrepeat bleed*	0= 1900 ±775 1 8= 1816 ±543 3.65= 1581 ±791 29.4= 1863 ±788	0= 2326 ±1082 1.8= 1606 ±926 3.65= 1799 ±933 29.4= 1420 ±622	0= 2669 ±1464 1.8= 2507 ±1008 3.65= 2463 ±1201 29 4= 1913 ±799	0= 3458 ±2310 1.8= 3235 ±2751 3.65= 3175 ±1685 29.4= 1356 ±760	0= 2327 ±1668 1.8= 2249 ±1498 3.65= 1899 ±752 29.4= 1308 ±477	0= 1178 ±337 1.8= 1258 ±428 3.65= 1063 ±383 400= 1129 ±350
mean and SD *repeat bleed*	0= 909 ±410 1 8= 1075 ±621 3.65= 972 ±353 29 4= 1005 ±482	0=1136 ±554 1.8= 1468 ±977 3.65= 984 ±466 29.4= 1155 ±620	0= 2213 ±2562 1.8= 1603 ±682 3 65= 2277 ±1470 29.4= 850 ±352	0= 3336 ±3138 1 8= 3631 ±2732 3.65= 2500 ±1897 29.4= 858 ±416	0= 3388 ±3344 1.8= 2510 ±1138 3.65= 2409 ±1525 29.4= 1042 ±627	0= 1672 ±426 1.8= 1229 ±492 3.65= 1271 ±559 400= 953 ±549

Appendix Table 21: Group Mean Absolute Pituitary weights by Timepoints, Thakur, 1991a
Weights are in mg.

Absolute Pituitary Weights			
Dose (mg/kg/day) ▾	3 months	9 months	12 months
Control	x̄= 23.0 SD= 4.2	x̄= 24 0 SD= 6 4	x̄= 37 0 SD= 19.9
4 23	x̄= 21.2 (-8%)[1] SD= 3.0	x̄= 29.9 (+25%) SD= 6.1	x̄= 35.4 (-4%) SD= 26.4
26.23	x̄= 20 5 (-11%) SD= 8.0	x̄= 37.0 (+54%) SD= 7.9	x̄= 41.8 (+13%) SD= 14.5

1 Values in parenthesis represent percent change relative to control

Appendix Table 22: Group Mean Pituitary Weights Relative to Body Weight, Thakur, 1991a
Values represent pituitary weight as a percentage of body weight

Relative Pituitary Weights			
Dose (mg/kg/day) ▾	3 months	9 months	12 months
Control	$\bar{x}$= 0.00697 SD= 0.0012	$\bar{x}$= 0.00607 SD= 0.00163	$\bar{x}$= 0.00985 SD= 0.0062
4.23	$\bar{x}$= 0.00668 (-4%)[1] SD= 0.00123	$\bar{x}$= 0.00765 (+26%) SD= 0.00187	$\bar{x}$= 0.00830 (-16%) SD= 0 00559
26 23	$\bar{x}$= 0.00677 (-3%) SD= 0.00249	$\bar{x}$= 0.00967 (+59%) SD= 0.00245	$\bar{x}$= 0.01239 (+26%) SD= 0 00572

1 Values in parenthesis represent percent change relative to control

Appendix Table 23: Group Mean Pituitary Weights: Absolute and Relative to Body Weight, Morseth, 1996b
Values represent pituitary weight as a percentage of body weight

Absolute and Relative Pituitary Weights				
Dose (mg/kg/day) ▸	Control	1.8	3.65	29.4
Absolute	$\bar{x}$= 23 0 SD= 5.0	$\bar{x}$= 24.0 (+4%) SD= 5.0	$\bar{x}$= 24.0 (+4%) SD= 5.0	$\bar{x}$= 28.0 (+22%) SD= 8.0
Relative	$\bar{x}$= 0.0075 SD= 0.0017	$\bar{x}$= 0.0078 (+4%) SD= 0.0015	$\bar{x}$= 0.0081(+8%) SD=0.0015	$\bar{x}$= 0.0096 (+28%) SD= 0 003

1 Values in parenthesis represent percent change relative to control

Appendix Table 24: Association of Pituitary Adenomas/Hyperplasia and Mammary Tumors

Study▸	Thakur, 1992a	Morseth, 1998
Animals *Without* Mammary Tumors	86% of the animals had either pituitary adenoma or hyperplasia	62% of the animals had either pituitary adenoma or hyperplasia
Animals *With* Mammary Tumors	86% of the animals had either pituitary adenoma or hyperplasia	93% of the animals had either pituitary adenoma or hyperplasia

Appendix Table 25a: Association of Absolute Pituitary Weight with Mammary Tumors

Absolute Pituitary Weight		
Study▸	Thakur, 1992a	Morseth, 1998
Animals *Without* Mammary Tumors	$\bar{x}$ = 0.0742 SD= 0.0823	$\bar{x}$ = 0.117 SD= 0.076
Animals *With* Mammary Tumors	$\bar{x}$ = 0.144 (94%)[1] SD= 0 109	$\bar{x}$ = 0.171 (46%) SD= 0.119

1 Value in parenthesis represents percent increase over pituitary weight of animals without tumors

Appendix Table 25b: Association of Relative Pituitary Weight with Mammary Tumors

Relative Pituitary Weight		
Study▸	Thakur, 1992a	Morseth, 1998
Animals *Without* Mammary Tumors	$\bar{x}$ = 0.021 SD= 0.024	$\bar{x}$ = 0.0321 SD= 0.029
Animals *With* Mammary Tumors	$\bar{x}$ = 0.0379 (80%)[1] SD= 0.0307	$\bar{x}$ = 0.053 (65%) SD= 0.068

1 Value in parenthesis represents percent increase over pituitary weight of animals without tumors

Appendix Table 26: Pituitary Tumor and Focal Hyperplasia Incidenes and Piutiatry Weights In OVX *vs* Intact Animals, Morseth, 1998

Pituitary Tumor and Enlarged Pituitary Incidence and Pituitary Weight[1]		
	OVX	Intact
β-Adenoma (12 months)	x = 6%	x = 17%
β-Adenoma (24 months)	x = 50 2%	x = 69.8%
Absolute Pituitary Wt. in grams (12 months)	x = 0.020	x = 0.0314
Absolute Pituitary Wt in grams (24 months)	x = 0.051	x = 0 184
Relative Pituitary Wt. as a % of body wt. (12 months)	x = 0.0037	x = 0.00832
Relative Pituitary Wt as a % of body wt (24 months)	x = 0.0116	x = 0.0331
Enlarged Pituitary	x = 34%	x = 86%

1 Values shown represent means for all the OVX or intact animals combined, regardless of dose group. Mean values between dose groups were very similar.

Appendix Table 27: Control individual animal data from the nine month timepoint of the two-year serial sacrifice study,Thakur 1991a

Control animals at 9 months							
Animal#	Mammary tumor	Pituitary Alteration	Pituitary wt. (abs. in mg)	Galactocele	Acinar/lobular Development	Secretory Activity	Dilated Duct with Secretion
B94752	none	Foc. Hy. -slight	26	none	1	1	-
B94753	none	none	30	none	2	2	1
B94754	none	none	24	none	-	1	-
B94755	none	none	27	none	1	2	1
B94756	none	none	25	none	1	1	-
B94757	none	none	16	none	2	-	-
B94758	none	none	19	none	2	2	1
B94759	none	none	33	slight	2	3	4
B94760	none	none	26	none	2	2	-
B94761	none	none	14	none	-	-	-

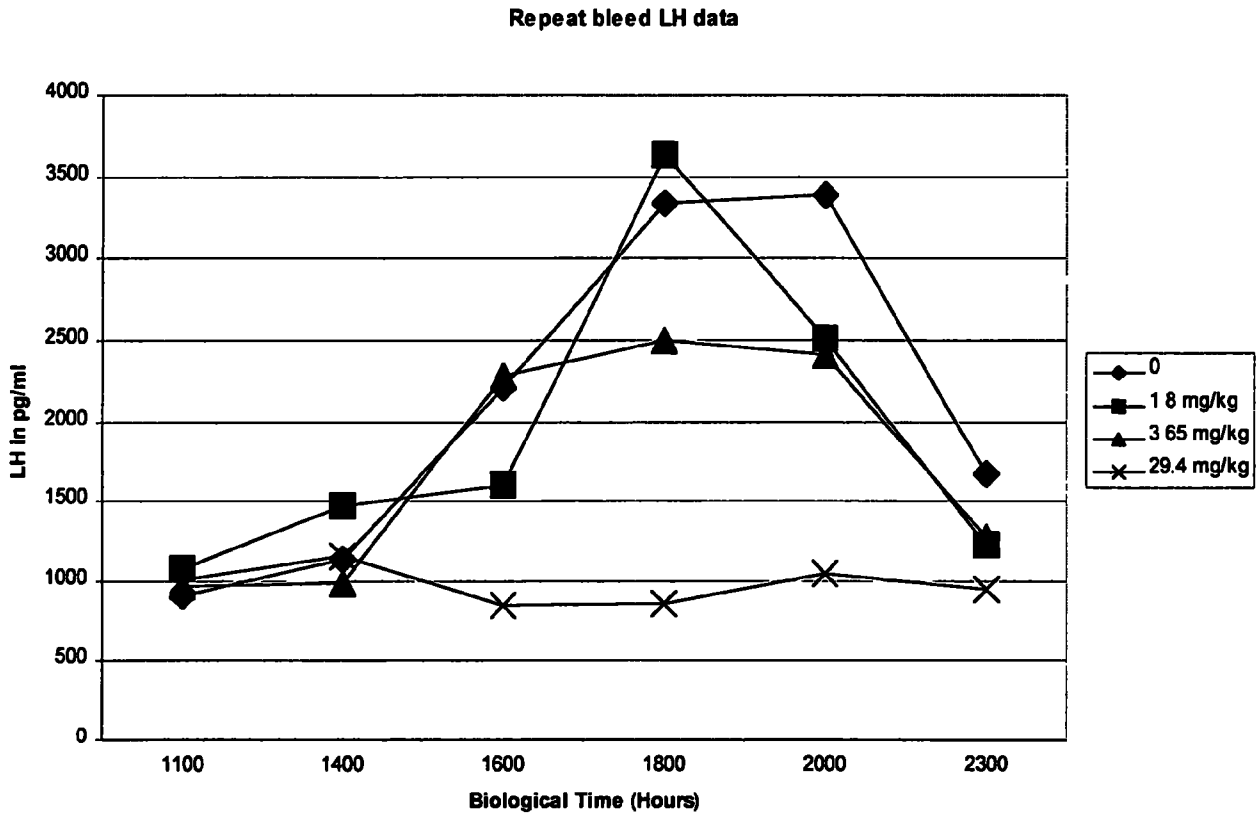
Appendix Table 28: Low dose (70 ppm, 4.23 mg/kg/day) individual animal data from the nine month timepoint of the two-year serial sacrifice study, Thakur 1991a.

70 ppm animals at 9 months							
Animal#	Mammary tumor	Pituitary Alteration	Pituitary wt. (abs. in mg)	Galactocele	Acinar/lobular Development	Secretory Activity	Dilated Duct with Secretion
B94822	none	none	28	none	2	2	1
B94823	none	Foc. Hy -slight	28	minimal	1	1	-
B94824	none	none	41	moderate	3	4	4
B94825	none	none	29	none	1	2	-
B94826	none	none	21	none	2	-	-
B94827	none	none	27	none	2	2	2
B94828	none	none	37	minimal	2	2	3
B94829	none	none	35	none	2	2	2
B94830	none	Foc. Hy. -slight	29	slight	3	3	2
B94831	none	none	24	none	-	-	-

Appendix Table 29: High dose (400 ppm, 26.23 mg/kg/day) individual animal data from the nine month timepoint of the two-year serial sacrifice study, Thakur 1991a.

400 ppm animals at 9 months							
Animal#	Mammary tumor	Pituitary Alteration	Pituitary wt. (abs. in mg)	Galactocele	Acinar/lobular Development	Secretory Activity	Dilated Duct with Secretion
B94892	carcinoma	none	36	moderate	2	3	4
B94893	none	none	36	moderately severe	4	4	4
B94894	fibroadenoma	none	38	moderately severe	3	4	4
B94895	none	none	23	none	4	4	4
B94896	none	none	37	severe	4	4	4
B94897	carcinoma	Focal hy. - mimimal	36	moderately severe	4	4	4
B94898	carcinoma	adenoma	55	minimal	2	3	2
B94899	none	Focal hy.- slight	41	moderately severe	4	4	4
B94900	none	none	33	moderately severe	4	4	4
B94901	fibroadenoma	adenoma	35	none	1	2	1

Appendix Figure 1: Mean plasma LH levels from the repeat bleed group of the 6-month study (Morseth, 1996b)



	Name	Source	Tolerances	SF Decision	SF List
1	1,12-DODECANEDIOL DIMETHACRYLATE POLYMER	other	1	none	
2	1,6-HEXANEDIOL DIMETHYACRYLATE POLYMER	other	1	none	
3	12-HYDROXYSTEARIC ACID-- POLYETHYLENE GLYCOL	other	1	none	
4	2-BUTENEDIOIC ACID (Z)-, POLYMER WITH ETHENOL	other	1	none	
5	A-(P-NONYLPHENYL)W-HYDROSYPOLY(OXYPROPYLENE	other	1	none	
6	A-{P-1,1,3,3- TETRAMETHYLBUTYL)PHENYL} POLY(OXYPROPYLENE	other	1	none	
7	A-BUTYL-W- HYDROXYPOLY(OXYPROPYLENE BLOCK POLYMER	other	1	none	
8	ACETIC ACID ETHENYL ESTER, POLYMER WITH ETHENOL	other	1	none	
9	ACRYLAMIDE POTASSIUM ACRYLATE--ACRYLIC ACID COPOLYMER	other	1	none	
10	ACRYLIC ACID--SODIUM ACRYLATE--SODIUM-2-METHYLPROPANE	other	1	none	
11	ACRYLIC ACID--STEARYL METHACRYLATE COPOLYMER	other	2	none	
12	ACRYLONITRILE--STYRENE--HYDROXYJPROPYL METHACRYLATE	other	1	none	
13	A-HYDRO-W-HYDROXPOLY(OXYPROPYLENE)	other	2	none	
14	A-HYDRO-W-HYDROXYPOLY(OXYETHYLENE)	other	1	none	
15	ALKYL (C12-C20) METHACRYLATE- METHACRYLIC ACID COPOLYM	other	1	none	
16	CELLULOSE ACETATE	other	1	none	
17	CINNAMALDEHYDE	other	1	none	
18	ETHYLENE	other	26	none	
19	ETHYLENE GLYCOL DIMETHACRYLATE POLYMER	other	1	none	
20	ETHYLENE GLYCOL DIMETHYACRYLATE--LAURYL METHACRYLATE	other	1	none	
21	GIBBERELLIC ACID	other	37	none	
22	HYDROXYETHYL CELLULOSE	other	1	none	
23	HYDROXYPROPYL CELLULOSE	other	1	none	
24	HYDROXYPROPYL METHYLCELLULOSE	other	1	none	
25	LAURYL METHACRYLATE-- 1,6- HEXANEDIOL DIMETHACRYLATE	other	1	none	
26	MALEIC ACID MONOBUTYL ESTER-- VINYL METHYL ETHER COPOL	other	2	none	
27	MALEIC ACID MONOISOPROPYL ESTER-VINYL METHYL ETHER	other	2	none	
28	MALEIC ANHYDRIDE-- METHYL VINYL ETHER, COPOLYMER	other	1	none	
29	METHYL METHACRYLATE- 2-SULFOETHYL METHACRYLATE	other	1	none	
30	METHYL METHACRYLATE-- METHACRYLIC ACID-- MONOMETHOXY	other	1	none	
31	METHYL VINYL ETHER--MALEIC ACID COPOLYMER	other	2	none	
32	METHYLCELLULOSE	other	2	none	
33	OCTADECANOIC ACID, 12-HYDROXY-, HOMOPOLYMER OCTADEC	other	1	none	
34	PELARGONIC ACID	other	2	none	
35	PHOSPHINE RESULTING FROM USE O	other	1	none	
36	POLY (VINYL PYRROLIDONE)	other	2	none	
37	POLY(OXYETHYLENE/OXYPROPYLENE) MONOALKYL(C6-C10)	other	1	none	
38	POLY(OXYPROPYLENE) BLOCK POLYMER WITH POLY(OXYETHYLE	other	2	none	
39	POLY(PHENYLHEXYLUREA), CROSS-LINKED, MINIMUM	other	1	none	
40	POLY(VINYLPYRROLIDONE-1- HEXADECENE)	other	1	none	
41	POLY(VINYLPYRROLIDONE-1-EICOSENE	other	1	none	
42	POLYACRYLIC ACID	other	1	none	
43	POLYETHYLENE	other	2	none	
44	POLYETHYLENE GLYCOL--POLYISOBUTENYL ANHYDRIDE--TALL	other	1	none	
45	POLYETHYLENE, OXIDIZED	other	1	none	
46	POLYMETHYLENE POLYPHENYLISOCYANATE, POLYMER	other	1	none	
47	POLYPROPYLENE	other	1	none	
48	POLYSTYRENE	other	2	none	
49	POLYTETRAFLUOROETHYLENE	other	1	none	
50	POLYVINYL ACETATE	other	1	none	
51	POLYVINYL ACETATE--POLYVINYL ALCOHOL COPOLMER	other	1	none	
52	POLYVINYL ALCOHOL	other	2	none	
53	POLYVINYL CHLORIDE	other	1	none	
54	RED PEPPER	other	1	none	

55	SODIUM CARBOXYMETHYLCELLULOSE	other	1	none
56	STEARYL METHACRYLATE--1,6- HEXANEDIOL DIMETHACRYLATE	other	1	none
57	STYRENE-2-ETHYLHEXYL ACRYLATE- GLYCIDYL METHACRYLATE	other	1	none
58	TRICHODERMA VIRIDE SENSU BISBY	other	13	none
59	VINYL ACETATE--ALLYL ACETATE-- MONOMETHYL MALEATE	other	1	none
60	VINYL ACETATE--ETHYLENE COPOLYMER	other	1	none
61	VINYL ACETATE--VINYL ALCOHOL ALKYL LACTONE COPOLYMER	other	1	none
62	VINYL ALCOHOL--DISODIUM ITACONATE COPOLYMER	other	1	none
63	VINYL ALCOHOL--VINYL ACETATE-- MONOMETHYL MALEATE	other	1	none
64	VINYL PYRROLIDONE DIMETHYLAMINOETHYLMETHACRYLATE	other	2	none
65	BEAUVARIA BASSIANA	RD	16	none
66	BENSULFURONMETHYL	RD	2	none
67	CARBON DISULFIDE	RD	4	none
68	CLOPYRALID (Dichloropyridineca	RD	39	none
69	COPPER CARBONATE	RD	2	none
70	COPPER HYDROXIDE	RD	1	none
71	COPPER LINOLEATE	RD		none
72	COPPER OLEATE	RD	1	none
73	COPPER OXYCHLORIDE	RD	1	none
74	COPPER SULFATE	RD	1	none
75	CRYOLITE	RD	36	none
76	CYFLUTHRIN	RD	4	none
77	DICHLOROPYRIDINECARBOXYLIC ACI	RD	5	none
78	FENOXAPROPETHYL	RD	4	none
79	FENVALERATE	RD	67	none
80	GLYPHOSATE AND ITS METABOLITES	RD	129	none
81	Hydroprene	RD	1	none
82	LAMBDACYHALOTHRIN	RD	8	none
83	TRALOMETHRIN	RD	2	none
84	BACILLUS THURINGIENSIS	RED	2	none
85	BACILLUS THURINGIENSIS CRYLAB	RED	1	none
86	BACILLUS THURINGIENSIS VARIETY	RED	1	none
87	BROMACIL	RED	2	none
88	BROMOXYNIL	RED	90	none
89	BUTRALIN	RED	6	none
90	Carbamyl-2,4,5-trichlorobenzoi	RED	38	none
91	COLLETOTRICHUM GLOEOSPORIOIDES	RED	2	none
92	DICHLOBENIL	RED	50	none
93	DIPHENYLAMINE	RED	3	none
94	HYDRAMETHYLNON	RED	4	none
95	METRIBUZIN	RED	57	none
96	PARAQUAT DICHLORIDE	RED	116	none
97	TERBACIL	RED	32	none
98	THIOBENCARB	RED	25	none
99	TRICLOPYR	RED	34	none
100	ZINC PHOSPHIDE	RED	6	none