Factors Affecting Mammary Tumor Incidence in Chlorotriazine-treated Female Rats: Hormonal Properties, Dosage, and Animal Strain

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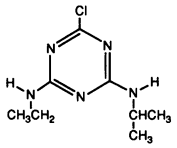
Chlorotriazines are widely used in agriculture as broadleaf herbicides. The compounds specifically inhibit photosynthesis, and, as such, display little interaction with animal systems. However, a 24-month feeding study with atrazine (ATR) revealed a significant dose-related increase of mammary tumors in female Sprague-Dawley (SD) rats. Because numerous studies indicated that ATR had a low mutagenic and oncogenic potential, it was decided to test a hypothesis that the herbicide possessed endocrine activity. Among tests for estrogenic action, oral dosing of ATR up to 300 mg/kg did not stimulate uterine weight of ovariectomized rats. However, ATR administration did reduce estrogen-stimulated uterine weight gain. Further evidence of inhibition came from measures of [^{3}H]-thymidine incorporation into uterine DNA of ATR-treated immature rats. Again, no intrinsic estrogenic activity was observed up to a 300-mg/kg dose. *In vitro*, ATR competed poorly against estradiol binding to cytosolic receptors, with an approximate IC_{50} of 10^{-5} M. Atrazine administration to SD and Fischer-344 (F-344) rats for 12 months, up to 400 ppm in food, was correlated with significant alterations of estrous cycling activity; but there was a divergent strain response. SD rats showed an increased number of days in vaginal estrus, increased plasma estradiol, and decreased plasma progesterone by 9 to 12 months of treatment. F-344 rats rdid not demonstrate treatment-related affects. A study of ultrastructure in the hypothalamic arcuate nucleus of female SD rats that were fed diaminochlorotriazine (DACT), an ATR metabolite, suggested that age-associated glial pathology was enhanced by treatment. It is proposed that antiestrogenic actions of ATR are able to disrupt critical hormone-mediated functions at very high dose levels. In SD rats, this disruption delays ovulation, maintains estrogen secretion from ovarian follicles, and produces a hormonal milieu more conducive to mammary tumors. Aging in F-344 rats promotes higher progesterone secretion, and it is predicted that mammary tumors would not be increased in ATR-fed F-344 animals. It is also concluded that the risk of ATR-related mammary tumors in humans would be quite low because of the unique response of the SD rat estrous cycle, the nature of stimulated mammary tumors in rats, and the extremely high dose levels and concentrations required for ATR to express activity *in vivo* and *in vitro*. —Environ Health Perspect 102(Suppl 11):29–36 (1994)

Key words: atrazine, Sprague-Dawley rats, Fischer-344 rats, estrous cycles, mammary tumors, estrogen receptors, antiestrogens, estrogen levels, progesterone levels

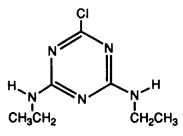
Introduction

Atrazine is a major agricultural herbicide that has been used in the United States and other countries worldwide for over 25 years, primarily for control of broadleaf and grass weed growth around corn and sorghum crops (Figure 1). The herbicide was developed and patented by CIBA-GEIGY in 1958, and since the patent expiration in 1975, has been manufactured and sold by other companies as well. Atrazine is a chlorinated member of a family of s-substituted triazines that selectively inhibit electron transport systems in plant photosynthesis (*1*). The K_i for atrazine in chloroplasts has been reported as 1.4×10^{-7} M by a mechanism believed to involve direct, competitive inhibition of an electron carrier substrate (*2*). Because of this specificity, reaction with animal systems has not been reasonably suspected, and indeed, effects at concentrations of 10^{-7}M have never been reported for test animals or humans. For example, the oral LD_{50} in rodents is approximately 3000 mg/kg (JO Kuhn, unpublished data), which calculates to 2×10^{-2} M if the substance were equally distributed in total body water. (If 1 kg of animal tissue contains 700 ml water, and the formula weight [FW] for atrazine is 216: 3000 mg/700 ml = 4286 mg/l = 19.84 mmole/l = 1.984×10^{-2} M.) Nevertheless, a concern regarding potential risk to humans exists for manufacturing employees, agricultural workers, and to the general population through crop residue and groundwater exposure.

Atrazine has been tested for mutagenic potential in more than 50 studies of gene mutation, chromosomal aberration, and



ATRAZINE



SIMAZINE

Figure 1. Chemical structures of two chlorotriazine herbicides and a common metabolite.

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primary DNA damage, and a weight-of-evidence evaluation indicates a nonmutagenic status relative to conventional health-effects-testing formats (*3*). However, a lifetime feeding study revealed a dose-related increased incidence of mammary tumor formation in atrazine-fed female Sprague-Dawley (SD) rats. Lifetime studies of male rats, or of male or female mice, did not result in increased tumors (*4*).

Mammary tumors are not an unusual finding in aged SD female rats. In four separate 24-month atrazine-feeding studies, the incidence of adenocarcinoma in vehicle-fed control female rats ranged from 17 to 40% (*5*). In a frequently cited study on the appearance of tumors following exposure to color dyes, the incidence of mammary adenocarcinoma in 35 vehicle-treated groups ranged from 0/70 to 26/62 (42%) (*6*). Thus, it appears that background noise for this parameter, in this rat strain, at old age, can be substantial and variable. Indeed, two of the four above-mentioned feeding studies of atrazine failed to demonstrate a significant increase of adenocarcinoma when compared to each experiment's own control incidence (*5*).

Mammary tumors in albino rats are strongly hormone dependent. It has been known for many years that the presence of estrogens and/or prolactin promotes tumor growth, while inhibition or removal of either hormone results in tumor regression (*7–9*). It is likely that the appearance of mammary tumors in senescent female SD rats is at least partially a result of lifetime exposure to an endocrine environment that promotes tumorigenesis, and that most, if not all animals would eventually demonstrate the lesions if they survived. An interesting finding from the lifetime atrazine-feeding study was that the incidence of mammary tumors was not universal and peaked near 50% in the highest dosed animals. Many well-known carcinogens in mammary tissue, such as dimethylbenzanthracene (DMBA) and *N*-methylnitrosourea (NMU), typically induce tumors in 100% of the subjects, and in a matter of weeks following a single dosing (*10,11*).

These observations suggested that an administered agent that merely enhanced tumor incidence from approximately one-fourth to one-half of the animals might not be a direct carcinogen as much as a modulator of endogenous hormone secretions, serving to stimulate an earlier appearance in a greater number of subjects. In effect, atrazine could be promoting a premature senescence of reproductive and mammary tissues. Therefore, studies were undertaken to determine whether chlorotriazines possess endocrine-related bioactivities, and whether chronic administration to test animals could induce senescencelike changes in hypothalamic-pituitary-ovarian function.

Estrogen–Antagonist Properties of Atrazine

Despite the fact that lifetime feeding of atrazine had not been associated with mammary tumors in mice or in male rats, it was nevertheless possible that chlorotriazines could possess a significant estrogenic bioactivity that could promote tumor growth in female SD rats. Consideration of this possibility was appropriate to further research because a number of other chlorinated organic molecules (whose chemical structures are not similar to estrogens) have demonstrated estrogenic activity, and some are indeed active pesticides (*12,13*). For example, the insecticide dichlorodiphenyltrichloroethane (DDT) was extensively studied and found to stimulate immature rat uterine weight gain (*14*) and total RNA and glycogen content (*15*), to competitively inhibit estrogen binding to uterine receptors (*16*), to translocate the estrogen receptor complex into the nucleus (*17*), and to promote uterine ornithine decarboxylase in uterine tissue (*18*) and progesterone receptor synthesis in rat hypothalamic and pituitary tissue (*19*). Methoxychlor, which is closely related in structure to that of DDT, possesses much the same hormonally active properties as DDT after metabolism to more active forms (*18,20,21*). Yet another chlorinated insecticide, chlordecone (Kepone), whose structure is quite distinct from DDT analogs, as well as estrogens, also has been reported to compete with estrogen binding to its receptor in rat uterus (*22,23*), rat hypothalamus (*24*) and chick oviduct (*25*). Chlordecone stimulated rat uterine weight (*22,23*), synthesis of progesterone receptor (*23*), secretion of pituitary prolactin and inhibition of luteinizing hormone (LH) (*26*), masculinization of the hypothalamus of neonatal female rat pups (*27*), and albumin synthesis in treated chickens (*25*), all of which are recognized biological properties of estrogens. In addition, other investigators have studied chlorotriazine interactions with male reproductive and endocrine function and have found inhibition of androgen metabolism and androgen receptor binding in rat pituitary and prostate (*28,29*). Therefore, it was considered a critical first step in our research to determine the possibility of estrogen expression by atrazine.

Tests of Uterine Weight Stimulation

Two experiments were conducted on ovariectomized adult female rats. In one study, the animals were given atrazine by gavage, in a vehicle of carboxymethylcellulose (CMC) and water, at doses of 0 (vehicle only), 20, 100, or 300 mg/kg/day. After 3 days of treatment, the animals were sacrificed and uteri weighed. As shown in Figure 2, uterine wet weight was not increased over control levels by atrazine doses as high as 300 mg/kg (10% of the LD_{50}). An identical result has been observed following treatment with simazine or DACT, a common animal metabolite of both herbicides (*30*). When another set of ovariectomized rats was administered atrazine by gavage and concomitantly injected sc with 2 μg estradiol daily, an inhibition of uterine weight was observed. The level of estrogen-induced stimulation was reduced 13.6 and 23.2%, respectively, by dosing with 100 and 300 mg/kg atrazine. Similar results have been obtained after dosing with simazine and with DACT, and at the same dose levels (*30*). Thus, atrazine demonstrated estrogen antagonism, and only at a high dose, with no sign of agonist activity.

These experiments, although fairly simple in design, produced three important findings. First, they suggested that chlorotriazines do not possess the estrogen bioactivity so frequently demonstrated for DDT, chlordecone, and other chlorinated pesticides described above. For example, 20 mg/kg *o,p'*-DDT was reported to stimulate immature rat uterine weight 84% by 18 hr postinjection; 120 mg/kg stimulated uter-

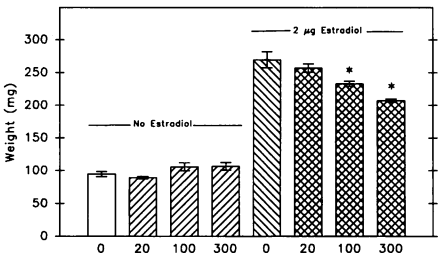

Figure 2. Uterine weight responses of ovariectomized rats that were fed atrazine by gavage. Animals were dosed for 2 days, injected once with estradiol (E_2) on the second day and uteri were dissected and weighed 24 hr later. Histograms represent means ± standard error of the mean (SEM) of six animals per dose. When no E_2 was given, uterine weights were not different from vehicle-gavaged control. When one E_2 injection was given, a significant (1-way ANOVA, $p = 0.0001$) dose-related suppression was seen. Asterisks (*) indicate group means significantly different from mean uterine weight of controls given E_2 without atrazine (Bonferroni's *post-hoc* test, both $p < 0.02$).

ine weight more than 4-fold (*15*). Second, a significant antagonist property of atrazine was observed, suggesting that the herbicides may indeed possess an endocrine-related activity of interest. Third, effects were observed only at very high doses of administration (i.e., 100 mg/kg and above). The appearance of mammary tumors also has been associated only with feeding levels above 70 ppm atrazine, which was calculated by CIBA-GEIGY to be about 5 mg/kg bw.

Inhibition of Thymidine Incorporation into Uterine DNA

Following demonstration that atrazine could retard estrogen-stimulated uterine weight growth, it was decided to examine this property in a more specific way, as in a testing situation, uterine weight increase could be impacted by numerous nonendocrine factors. Indeed, the animals dosed with atrazine in the uterine weight study had lost total body weight in a dose-related fashion. Therefore, a measure of uterine DNA synthesis was taken, because it occurs prior to tissue growth, and because increased synthesis has been shown to correlate highly to the amount of bioactive estrogen present (*31*). In this study, intact, immature female rats were purchased before weaning and used at 23 days of age. Each animal was gavaged once daily for 2 days with atrazine in CMC vehicle at doses from 0 to 300 mg/kg. On the second day, each animal also received 0.15 µg estradiol sc in saline. Animals were sacrificed 24 hr after the estradiol injection, and the uteri were dissected. Using a protocol of Stormshak et al. (*32*), tissues were sliced and incubated with [^{3}H]-thymidine for 60 min at 37°C. DNA was extracted from the incubated slices, quantified, and counted by liquid scintillation of a sample.

As shown in Figure 3, incorporation rate was significantly inhibited in animals administered 50, 100, or 300 mg/kg atrazine, but not inhibited in animals given 1 or 10 mg/kg. Furthermore, animals given 300 mg/kg atrazine without the estradiol injection showed an incorporation rate lower than that of estrogen-injected animals given 300 mg/kg, or than that of the vehicle-treated controls. This result indicated that in the same dose range which inhibited uterine weight growth atrazine also reduced an estrogen-specific early step—namely, DNA synthesis. Additional studies with simazine or the metabolite DACT have produced identical results, (*30*) suggesting that the active principle for inhibition of estrogen activity may be a didealkylated metabolite rather than the herbicide itself.

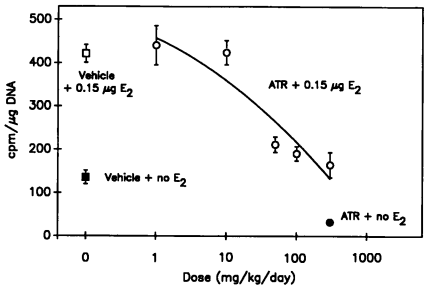

Figure 3. Incorporation (cpm/µg DNA) of [^{3}H]-thymidine into DNA extracted from uteri of rats that were administered atrazine by gavage. A total of 114 animals were used, at 23 days of age; each symbol represents the mean ± standard error of mean (SEM) of 7 to 26 animals per treatment group. A significant (1-way ANOVA, $p < 0.0001$) dose-related inhibition was observed; mean values at 1 and 10 mg/kg were not different from the mean value of animals given estradiol alone; mean values at 50, 100, and 300 mg/kg atrazine were significantly lower than the mean for the group given E_2 alone (all $p < 0.01$, Bonferroni's *post hoc* test). In addition, the mean incorporation rate in a group given atrazine alone (no E_2) was significantly lower than the mean of untreated control animals ($p < 0.05$), Bonferroni's *post hoc* test). See text and Tennant et al. (*30*) for additional details of the study.

Atrazine Interactions with the Estrogen Receptor

Following demonstration that atrazine administration was antagonistic to estrogen expression in a rat uterine model, it was necessary to determine whether this antagonism could be exerted through competitive suppression of estrogen binding to its receptor (ER). It is generally accepted that expression of many steroid hormone actions begins with binding of ligand to an intracellular protein, which then associates with the genome of the target cell to initiate transcription of specific messenger RNAs. In the case of estrogens, binding to ER is a crucial first step for many agonists and antagonists, and indeed, the above-cited agonist effects of many chlorinated pesticides have been associated with binding of the chemicals to ER (*16,22–24*).

Uterine tissue from adult SD rats was dissected, homogenized in Tris–EDTA–glycerol buffer, and cytosol was prepared (*33*) and incubated at 4°C overnight with [^{3}H]-estradiol plus or minus atrazine or unlabeled estradiol. Under these conditions it was not possible to demonstrate any competition for estradiol binding to ER in the presence of atrazine, at concentrations up to 10^{-4} M (*34*). In no case could competition be observed under any condition of concomitant incubation of steroid and herbicide, or under conditions leading to equilibrium. However, competition was demonstrable under a nonequilibrium condition (Figure 4) and as described below.

When cytosol was incubated with atrazine for 30 min at room temperature, then cooled in ice, and radiolabeled estradiol was added, a clear inhibition of steroid binding was observed. Figure 4 shows the effect of preincubation with atrazine at 0.1 mM, but inhibition was demonstrable with simazine and DACT as well, and at lower concentrations (*34*). Approximate IC_{50} calculations were made for all three compounds in the range of 10^{-5} M, versus an IC_{50} of 10^{-10}M for estradiol. Although this concentration of chlorotriazine might appear unreasonably large for expression of relevant biological action, the level is in line with the *in vivo* dosing range of our studies described earlier: 300 mg/kg, distributed equally in 700 ml of total body water, is approximately 2×10^{-3} M. An intracellular concentration of 10^{-5} M for a small lipophilic molecule such as atrazine, or a metabolite in a test animal continuously fed 500 ppm for a lifetime does not seem unreasonable.

Scatchard analysis was conducted by incubation of radiolabeled estradiol and ER in the presence of unlabeled estradiol or atrazine (Figure 5). As before, the results were obtainable only by preincubation of competitor for 30 min at 25°C, followed by 60 min of [^{3}H]-estradiol at 4°C. By comparison with the Scatchard plot for excess unlabeled estradiol, the plot for atrazine displacement had both a lower slope and a slightly reduced B_{max}, suggest-

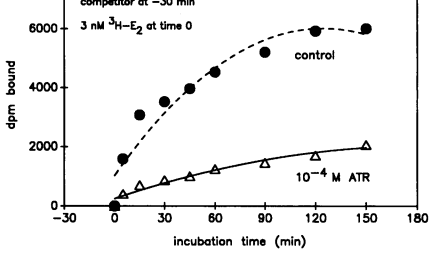

Figure 4. Competition of atrazine against [^{3}H]-estradiol binding to uterine cytosol. Tissue was prepared by homogenization of intact rat uteri and centrifugation at 105,000*g* for 60 min. Note that cytosol was incubated with 0.1 mM atrazine for 30 min at 25°C, then put on ice, prior to addition of radioligand. Each point represents the mean of triplicate tubes; incubations were stopped at 8 points between 5 and 150 min after addition of tracer. Additional details of the methodology appear in the text and Tennant et al. (*34*).

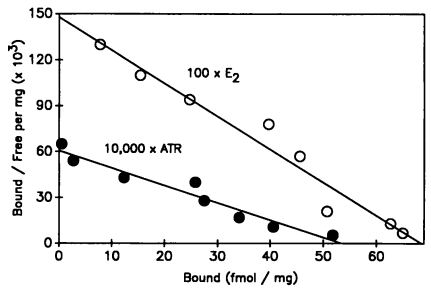

Figure 5. Scatchard plots of [^{3}H]-estradiol binding to uterine cytosol in the presence of 100-fold molar excess unlabeled E_2 (○) or 10,000-fold molar excess unlabeled atrazine (●). Cytosol preparation and incubations conducted as described in text and in the legend to Figure 4. It should be emphasized that competition or a Scatchard plot could be generated for atrazine only if the herbicide were incubated with cytosol for 30 min at 25°C prior to addition of radiolabeled E_2 at 4°C. Furthermore, no competition appeared under any condition with 100-fold excess unlabeled atrazine. For the E_2 plot: K_D = 0.46 nM, B_{max}= 68.5 fmole/mg protein, r = 0.979. For the atrazine plot: K_D = 0.88 nM, B_{max} = 53.4 fmole/mg protein, r = 0.971.

ing the possibility of some competitive and noncompetitive inhibition by atrazine.

Thus, atrazine was able to interact with ER binding of estrogen, but the nature of the interactions appeared weak and occurred only at very high concentrations. Under equilibrium conditions, no competition of atrazine was evident, suggesting that the herbicide might be able to interfere only if it were present at elevated levels and prior to exposure of the target cells to estrogens. In fact, such conditions were present during both of our studies on uterine weight stimulation and thymidine incorporation described earlier, and the condition could theoretically exist during certain periods of diestrus in a continuously fed, cycling female rat, when ovarian estrogen secretion is at an ebb.

The results of all of the studies described to this point suggest further that a threshold for estrogen antagonist expression by atrazine in rats is found in the dosage range of 50 to 100 mg/kg for oral administration, and a concentration range 10^{-5} M for interaction with uterine estrogen receptors. In all of our studies on several chlorotriazine compounds, whether by oral dosing or by incubations *in vitro*, we have never observed any actions to occur at levels lower than the 10^{-5}M range. These observations are particularly relevant when considering human risk because they suggest that, insofar as these endocrine actions are concerned avoidance of exposure to levels in excess of 50 mg/kg/day, or of intracellular concentrations greater than 10^{-5}M should provide an environment free of related adverse effects.

Long-Term Effects of Atrazine on Estrous Cycling and Reproductive Hormone Secretion of Female Rats

While research focusing on elucidation of mechanisms for an estrogen antagonist action by atrazine remains an important goal, it also becomes necessary to consider how an enhanced incidence of mammary tumors in senescence could derive from chronic administration of a weak antiestrogen to test animals. Preliminary results from studies conducted by CIBA-GEIGY indicated that female rats, when fed atrazine at doses of 100 ppm and greater, displayed abnormal and irregular estrous cycling patterns. Our laboratory conducted a 2-week study of SD female rats that were administered atrazine by gavage at 100 and 300 mg/kg/day (*35*). Results showed that estrous cycling became significantly lengthened and less regular in atrazine-treated animals, with additional days of estrus. The experimental design was repeated in F-344 rats, which also displayed fewer abnormal estrous cycles with atrazine dosing. When occurring, the additional days of the F-344 estrous cycles were in metestrus/diestrus rather than in estrus. Because SD female rats are known to enter transient periods of constant estrus with advancing age, while F-344 animals have a greater tendency to increased days in pseudopregnancy with aging (*36,37*), it was proposed that a similar divergence of strain response might be occurring with chronic atrazine dosing. In concurrent, long-term atrazine feeding studies (one used SD female rats, the other F-344 females), estrous cycling patterns were monitored and blood samples were collected throughout the studies. As this manuscript was being prepared, data had been analyzed through the first year of a 2-year dosing schedule, and those results are presented here. Additional results from these studies have also been published (*38*).

Estrous Cycling and Vaginal Cytology Characteristics

Three groups of 70 SD female rats were fed 0, 70, or 400 ppm atrazine; five groups of F-344 animals were fed 0, 10, 70, 200, or 400 ppm atrazine. A set of 10 animals of each strain and feeding level was sacrificed at 1, 3, 9, or 12 months of feeding and trunk blood was collected. Vaginal smears were prepared and read daily for 14 to 20 days prior to sacrifice, and animals were sacrificed on the day of proestrus, when possible.

SD female rats showed a significant trend in an increased number of estrus days both with aging and dosing ($p < 0.01$), while F-344 rats did not (Figure 6). In particular, SD rats that were fed atrazine for 9 months at 400 ppm demonstrated nearly twice as many days in vaginal estrus, on average, as their cohorts of the same age that were fed vehicle, or than SD rats that were fed the same 400 ppm dose for 3 months. By 12 months of feeding, the vehicle-control rate of estrus had risen to the level seen with 9 months of 400 ppm feeding, but the level in 12 months, 400-ppm animals had continued to rise. By the 12th month, the occurrence of estrus in SD rats had now risen from about one per five-cycle day to nearly three per 5 days, while in F-344 females, the occurrence of estrus held steady with dosing.

This finding was substantiated by additional analyses of the types of epithelial cells on the vaginal smear slides (data not presented here). Results showed that the density of cornified epithelial cells, which represents overall estrogen bioactivity in the animal, rose steadily in direct relation to dose and advancing age in SD rats; in

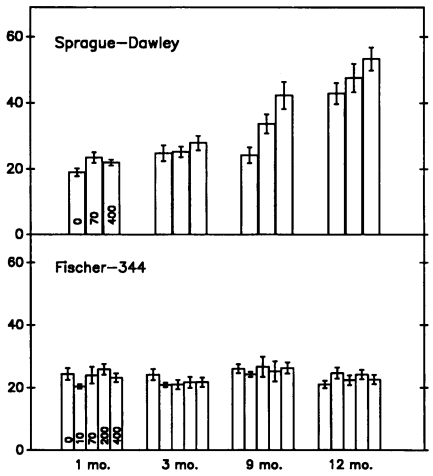

Figure 6. Percent of total estrous cycle days judged as estrus in two rat strains fed atrazine continuously for 12 months. Histograms represent mean ± SEM of 10 animals on whom vaginal smears were prepared for 15 to 20 consecutive days at each sampling time. Doses, in ppm, are indicated by numbers inside the histograms for 1 month/dosing. For the SD strain, a two-way ANOVA revealed significant effects of dosage (p = 0.026), time ($p < 0.0001$) and a dose × time interaction (p = 0.012). Within the 9- and 12-month sampling times a significant effect of dose was observed (p = 0.002, p = 0.039, respectively). No significant overall effects of dosage or time were observed for this parameter in Fischer-344 rats.

F-344 animals, cornified cell density was elevated only at 12 months of treatment, and then equally for all dosing groups. The density of nucleated epithelial cells, another vaginal cell type that correlates more closely to overall progesterone bioactivity, was unchanged with dose or time in the SD animals, and was increased with time only in treated F-344 subjects (no dosing effects). Thus, it was apparent that atrazine dosing had altered estrous cycling activity in a way that caused increased estrogen production in SD but not in F-344 rats. The SD animals appeared to have an age-related trend in that direction, which became advanced or accelerated by atrazine feeding.

Estradiol and Progesterone Levels in Atrazine-fed Female Rats

Plasma samples obtained at sacrifice from the sets of 10 animals in each treatment group were analyzed for estradiol and progesterone by radioimmunoassay (RIA) procedures. As predicted from examination of vaginal cytology, responses of the two rat strains were quite dissimilar (Figure 7).

In SD animals, plasma estradiol demonstrated a marked dose-related increase, particularly at 400 ppm ($p < 0.01$), and F-344 animals also displayed a rise of plasma-estradiol levels in relation to increasing age, but not to dosing. Plasma progesterone levels decreased with advancing age in SD rats and increased with age in F-344 animals. Plasma-estradiol and progesterone changes at 12 months of age were particularly notable in both strains when each was compared to levels at 9 months of age. However, neither strain showed a significant trend to change plasma–progesterone as a function of dose. Plasma–prolactin was not elevated in either strain in relation to age or treatment dose (data not shown), which supports a conclusion that prolactin, which can stimulate mammary tumor growth in rats (*7–9*), was not a likely factor in tumorigenesis associated with chlorotriazine administration.

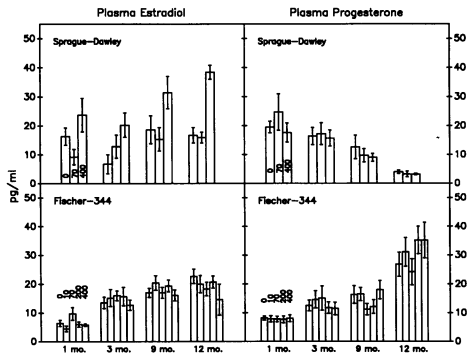

Figure 7. Plasma estradiol (E_2) and progesterone (P_4) in two rat strains fed atrazine continuously for 12 months. Samples were collected at sacrifice on a day of vaginal proestrus, when possible. Hormones were measured by solid-phase radioimmunoassays (Diagnostic Products Corporation, Los Angeles, CA). For plasma E_2 in Sprague-Dawley (SD) rats, a significant effect of dosage ($p = 0.033$) but not duration was observed; in Fischer-344 (F-344) rats, a significant effect of duration ($p = 0.009$) but not dosage was observed. For plasma P_4, a significant overall decrease with duration was observed for SD rats ($p = 0.016$), and a significant increase was observed for F-344 animals ($p = 0.026$). No treatment-related effects were observed for either hormone in either strain; no dosage–time interactions were observed.

Overall, the effect of long-term atrazine dosing appeared to be a dose- and age-related increase in estrogen levels relative to progesterone, in SD rats. In F-344 rats, however, estrogen levels held steady, or even fell relative to progesterone, and only in the more aged subjects. No significant treatment effects appeared in the F-344 animals. These overall results represent a substantial strain-related divergent response to oral feeding of identical amounts of atrazine, and both responses appeared similar to those normally demonstrated by each strain with advancing age (*36,37*). The most noteworthy aspect of these divergent responses is that the SD rats, which developed an increased internal estrogen environment relative to progesterone, were the strain in which mammary tumors also appeared. F-344 animals developed an internal environment that favored progesterone relative to estrogen. Progesterone is known to reduce or retard mammary tumor formation in rodents (*7–9*), and this suggests a mechanism whereby F-344 rats would respond to atrazine feeding with no enhancement of mammary tumor development.

A second noteworthy overall result was the relative absence of responses in endocrine secretion and control of estrous cycling through the first 3 months of treatment. Our earlier 2-week studies had produced significant changes in both estrous cycling and reproductive steroid levels, but these experiments were conducted by gavage of 100 and 300 mg/kg once a day, which exceeded the maximum tolerated dose. The chronic, 1-year feeding studies were conducted by mixing atrazine into the diet, up to 400 ppm, and the animals were not experimentally handled except for preparation of vaginal smears. However, even these high levels of administration (up to 0.04% of total food intake) resulted in measurable changes of the parameters studied only after a significant lag time; perhaps a substantial accumulation of agent is necessary or the metabolic alteration of atrazine may be an important step. As described earlier, DACT, the didealkylated metabolite (Figure 1), contains estrogen antagonist activity at a similar dose to that of atrazine itself (*30*).

The chronic 1-year feeding studies demonstrated that ovulatory cycling is disrupted in animals fed atrazine. It is difficult to couple this finding mechanistically with our previously observed estrogen–antagonist properties, given our present knowledge. It might be concluded that atrazine (or a metabolite) inhibits an estrogen-dependent process necessary for prompt ovulation and regular estrous cycling, but such a specific process is not easily identifiable. The neuroendocrine control of ovulation is a delicate balance of steroids, pituitary gonadotropins, hypothalamic neuropeptides, and central neurotransmitters, plus environmental factors. The precise reason for strain-related divergence of estrous cycling patterns in aging SD versus F-344 rats is also not known, so it remains to be elucidated how an antiestrogenic action on the part of the herbicide can be translated into the same final result through different target sites.

Ultrastructural Changes in Arcuate Nucleus with Chlorotriazine Feeding

The rat hypothalamus controls ovulation through secretion of regulatory hormones that control release of pituitary gonadotropins, which in turn stimulate ovarian function. One of the focal hypothalamic areas of reproductive control is the arcuate nucleus: lesions or isolation of this nucleus in female SD rats causes the arrest of estrus cycles in the estrus (*36*), and it has long been proposed that reproductive aging in female SD rats begins with deterioration of function at this site (*36*). Some years ago, ultrastructural examination of arcuate nucleus tissue from aging SD female rats revealed the presence of dendritic and axonal degeneration, and astrocytic and microglial reaction (*39*). This picture of neuropathological change had actually been observed earlier in estrogen-treated young female rats, which also had developed a persistent vaginal estrus (*40*) and inability to generate preovulatory LH

surges (*41*). It was suggested that continual estrogen exposure advances the aging process by inducing pathologic change in the hypothalamus. We investigated by electron microscopy the possible role of chlorotriazine administration through examination of hypothalami from female rats that were fed DACT (*42*).

As shown in the example in Figure 8, astrocyte inclusion granules increased in number with aging (20 versus 48 weeks), and granule number increased even further in 48-week-old rats that were fed 1000 ppm DACT beginning at 20 weeks of age. By group analysis, astrocyte granule-density of 48-week-old untreated controls increased 56% over the 20-week level ($p < 0.01$), while density of DACT-fed, 48-week-old animals was increased another 48% over age-matched controls ($p < 0.05$). This increase of glial reactivity in the same direction as normal aging suggests that the arcuate nucleus of the DACT-treated animals had aged at a faster rate than normal. Because we have already established that SD female rats fed chlorotriazines produced significantly more estrogen (Figure 7), it seems possible that atrazine-fed female rats developed irregularity of cycling and episodes of constant estrus because of estrogen toxicity in the hypothalamus.

It should also be mentioned that arcuate nucleus lesions induced by high doses of estradiol administered to young rats were substantially blocked if the animals were ovariectomized (*43*). In addition, constant low-dose estrogen administration, even to ovariectomized rats, enhances arcuate nucleus gliosis (*44*). These findings suggest that "toxic" events early in life can have persistent effects, if the subjects continue to secrete estrogen, and that continual estrogen secretion is equally important for more complete neuropathology to develop. This would support a concept that, in long-term, atrazine-treated SD rats, the early effects of dosing may be vitally important in initiating a chain of events that culminate in an increased incidence of mammary tumors. Endogenous hormone levels are likely to be lower during the time of actual tumor growth, so the environment in senescence, while possibly involved in tumorigenesis, nevertheless may be secondary to events earlier in life.

Summary and Conclusions

When presented with the observations that SD female rats that were fed a substantial quantity of atrazine daily for a lifetime developed a significant (but not ubiquitous) increase of mammary cancer, that other rodent strains and species did not form these tumors after atrazine feeding, and that numerous tests of mutagenicity were negative, it became logical to propose that the herbicide could be exerting a hormonelike or endocrine-modulatory action in the test animals. Other compounds, particularly chlorinated pesticides, are known to be estrogenic, and although atrazine structure does not resemble that of estrogens, the possibility of a direct interaction seemed reasonable.

It was found that atrazine, when present at very high levels *in vivo* or *in vitro*, can exert an apparent antiestrogenic effect. A variety of test modalities, including uterine weight, thymidine incorporation into DNA, and estrogen receptor binding, suggest that atrazine, as well as simazine and the common metabolite DACT, can inhibit or reduce estrogen-stimulated activity in target areas. None of the tests suggested that chlorotriazines have estrogen agonist activity, or a hormone-stimulatory activity of any kind. To our knowledge, no other chemical has been shown to possess this constellation of actions.

When administered by gavage or in food, atrazine induced significant disruptions of normal estrous cycling in two different rat strains, and the respective responses also were different. A hypothesized view of these alterations is illustrated in Figure 9. Atrazine-treated SD animals appeared to delay the succeeding ovulation, as indicated by the increased number of days in vaginal estrus. The response was likely due to arrested development of mature ovarian follicles which, when combined with plasma-hormone data, suggests that the treated animals existed in an elevated estrogen environment.

It is significant that the respective rat strains each undergo alterations of estrous cycling with aging, but we propose that atrazine administration resulted in an advancement or enhancement of the reproductive aging process in SD rats. The study of hypothalamic ultrastructure provides supporting evidence for this conclusion. The specific mechanism by which atrazine is able to accomplish this effect may lie in understanding the nature of reproductive aging in the respective strains. It is possible that the herbicide is able to inhibit or reduce to a critical degree some function(s) involved with the ovulatory cycle that are estrogen-dependent. In SD rats, the essential result would be an augmented estrogen

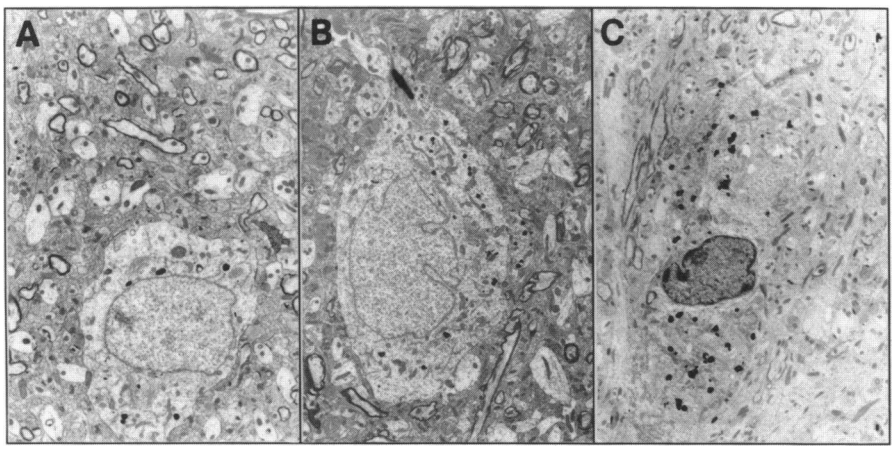

Figure 8. Representative sections showing astrocytes in the arcuate nucleus of female rats that were fed lab chow containing vehicle at ages 20 weeks (*A*) or 48 weeks (*B*), or 1000 ppm diaminochloroatrazine (DACT) until age 48 weeks (*C*). Feeding was begun at 20 weeks of age. Note the increase of astrocytic granules with advancing age (*A* compared to *B*), and the additional increase in a DACT-treated animal (*B* compared to *C*). See text and Tennant et al. (*42*) for further discussion of group results. Magnification ×1950.

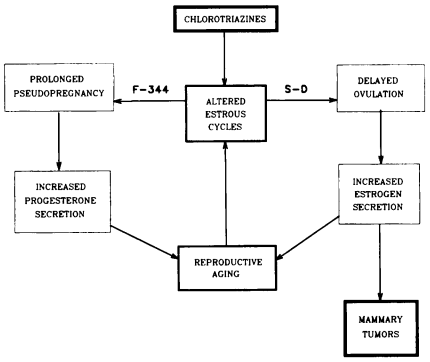

Figure 9. Proposed mechanism by which atrazine administration could interact with rat estrous cycling control to support an environment-enhancing mammary tumor formation. Note the divergent responses of the Sprague-Dawley (SD) versus Fischer-344 strains.

environment and stimulation of mammary tumor growth later in life; F-344 rats, with augmented progesterone secretion, would not be as likely to develop the tumors. Our basic conclusion, therefore, is that atrazine administration does not actually induce new tumors, but rather that a normal rate of tumor appearance is shifted to an earlier time in life through manipulated hormone secretion.

The critical time for stimulation of tumor formation may not be during old age, but the midage of 9 to 18 months. After this time, ovarian steroid output falls precipitously and a state of constant diestrus ensues. Indeed, the often-studied, tumor-inducing agents such as DMBA and NMU lose effectiveness when administered past 90 days of age (*8–11*): Therefore, if mammary tumor growth in senescence may be promoted by a hormone environment that existed earlier in life, the effect of an administered agent during the first half of life may be most critical. There are, of course, other possible reasons why mammary tumors appear in treated animals, among them being direct alterations of steroid hormone synthesis, metabolism, or clearance. The herbicides could also exert a variety of nonendocrine actions working in concert with hormone alterations.

Possible Significance for Human Risk

It is always difficult, and perhaps not even appropriate, to attempt to interpret results from animal research in direct terms of risk assessment for humans. However, the present studies have yielded a number of results that might be useful to those concerned with safety and hazards of agricultural chemicals. We observed a variety of effects that pointed to disruption of reproductive cycling and hormonal activity in atrazine-treated rats, but the required doses had a definite threshold in the range of 50 to 100 mg/kg/day and 0.01 mM. Numerous doses and concentrations below these levels did not elicit responses, and one may conclude that human exposure below the same levels would be similarly ineffective. On the basis of our findings, it is extremely likely that exposure to trace amounts of atrazine would yield no effect whatever on the parameters tested. It should be emphasized that the estrous cycling changes we observed did not appear until after several months of continuous exposure, suggesting that occasional exposure even to high atrazine levels might be tolerated easily. An increased rate of mammary tumor formation resulting from our proposed mechanism would likely require a continuously altered hormone environment, which in the case of humans is many years.

Atrazine is metabolized in mammals principally by dealkylation of the amino groups (Figure 1), and we have observed basically similar biochemical effects of one principal metabolite, DACT, as with atrazine itself. Studies in humans have shown that metabolism proceeds in much the same manner as in rodents (*45*), except that SD rats tend to sequester metabolites in their erythrocytes (*46*). This results in a two-compartment model of elimination of the metabolites, and a slower rate of clearance from rats than humans (IWF Davidson, unpublished personal communication). Therefore, one might surmise that the rat model is more sensitive than other species, including humans, to the hormone-related effects we observed, because metabolism and clearance in rats would proceed at a slower rate.

Additionally, human menstrual function is quite distinct from the rat estrous cycle, particularly in relation to the mode of aging in each species. Rodents and many other mammalian orders lose control of estrous function by means of neuroendocrine (neurotransmitter/neurophysiologic) failure, so that factors which disrupt or damage proper neuroendocrine activity could be viewed as contributing to the aging process. The response, as we observed in SD rats, is a delay of ovulation, persistent mature ovarian follicles, and enhanced estrogen output during mid age. Reproductive aging in women seems to involve primarily the declining population of ovarian follicles rather than neuroendocrine disturbance. Indeed, recent studies of the events of primate ovulation appear to implicate a reduced role for critical neuroendocrine control of the process (*47*). This would suggest that human females in particular would be less susceptible than rodent females to an agent that disrupts neuroendocrine control of ovulation. Furthermore, menstrual cycles of middle-aged women are characterized by decreased, not increased, estrogen secretion (*48*).

Finally, one should consider what is known about mammary cancer in women as compared to rodents. The types of tumors observed in senescent atrazine-fed rats were quite different from those seen following treatment with DMBA and other tumorigenic materials (*5*). Indeed, the pathologic picture of atrazine-associated tumors was not different from that seen in age-matched controls; there were simply more tumors and more rats with tumors. Tumors that are directly inducible in rats can be maintained rather easily by estrogens and/or prolactin secretion, as previously described. Tumors in women are not so completely correlated to endogenous hormone exposure; approximately one-half of human mammary tumors are not estrogen-responsive because they lack the intracellular estrogen receptor necessary for expression of hormone action (*9*). Mammary cancer in women more indirectly correlates to hormone exposure by virtue of reports that, for example, women who first bear children at a younger age seem to be at reduced risk (*9*). Overall, environmental influences seem to affect the appearance of human cancer less than do inheritance and familial history. Therefore, one may conclude that a significant risk of mammary cancer in humans who are exposed to atrazine and chlorotriazine herbicides, while theoretically possible from a variety of sources that are not the subject of our studies, is not supported by the present experiments.

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