UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
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OFFICE OF
RESEARCH AND DEVELOPMENT

MEMORANDUM:

SUBJECT: Review of Atrazine Rat Studies

FROM: Thomas M. Crisp, Ph.D., Thomas M. Crisp
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TO: Dr. Melba Morrow
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As requested in Dr. Elaine Z. Francis's memorandum of March 6, 1997, to Dr. Karen Hammerstrom, ACD for NCEA, please find below my review and comments to the various studies on Atrazine, submitted to OPP. Please note that this review is only for volumes 3 through 7 of 41, the only ones submitted attached to the memorandum of March 6th. I assume that volumes 1 and 2 have been reviewed earlier or did not require my evaluation, since they were not in the package delivered to me.

Review of Volumes 3 and 4 entitled: "Evaluation of the Luteinizing Hormone (LH) Surge in Atrazine-Exposed Female Sprague-Dawley Rats - 6 Month Report"

The purpose of this study was an attempt to elucidate a hormonally-mediated mechanism underlying the development of mammary gland tumors in Sprague-Dawley female rats exposed to Atrazine. To this end, the investigators evaluated the effects of Atrazine in a subchronic (6 month) dietary (feeding) study. Endpoints observed included LH and prolactin surges in ovariectomized, estrogen-treated rats that had been exposed to 0, 25, 50 or 400 ppm Atrazine for 26 weeks and estrous cyclicity as verified by vaginal smear cytology at selected intervals during the course of Atrazine treatment.

Introduction: Unfortunately, the authors failed to provide the rationale for the "estrogen provocation test" to determine functionality of the hypothalamic regulation of LH and prolactin surges in the Introduction (pg 12). This is a surprising oversight on the part of the investigators, because it points to a possible site of action for this herbicide and provides credence to a potential mechanism for endocrine disruption and mammary gland tumor initiation/promotion.

Methods: In general, this section lack details. This reviewer has some reservation as to

the method of recording vaginal smear data from air-dried specimens (pg 17), but the method may be adequate in the hands of experienced investigators. At least this reviewer is giving these investigators the benefit of the doubt. Data (particularly prolactin determinations) obtained from serial bleeds over a short term are questionable because of hemorrhagic stress (pg 19). RIA methods for LH, prolactin and estradiol are not provided, along with validation of the assays in the laboratory of the investigators. This reviewer does have some questions and concerns about the execution of the assays. First, why were the gonadotropins measured in plasma rather than serum (pg 19)? What was the amount of heparin in collection tubes and was this volume subtracted from the volume of plasma used in calculating concentrations of LH and prolactin? Does heparin interfere in the assays? If not, how was this determined to be the case? What was the range for the standard curves for each assay? What was the volume of plasma used in the assays? What was the intra- and interassay % error? How were the kits employed validated in the laboratory of the investigator? It is surprising why estradiol was not measured in serum during the course of the exposure period prior to ovariectomy to document that estradiol levels were indeed elevated in Atrazine-treated animals. If the working hypothesis for mammary gland tumor development in the Sprague-Dawley female rat is due in part to precocious and extended elevated serum estrogens following Atrazine exposure, it would seem appropriate, if not essential, to make these determinations. Another appropriate study would be to evaluate mammary gland tumor development in ovariectomized, Atrazine-treated Sprague-Dawley and another less sensitive strain of rat. Under these circumstances, one would suspect that Atrazine-induced ovarian estrogen production would be eliminated and mammary gland tumor development in the Sprague-Dawley rat would be curtailed.

Results: The reference by Cooper, et al., 1996, on pg 25 does not match that in the reference section (pg 33). Methods for determining fraction of interval in diestrus and estrus for results shown in test tables 3 and 4 respectively are not spelled out clearly (pg 26 and 27). How were these determined? Estradiol concentrations should have been placed in the text. One has to look in the Appendix (pgs 339-343) for the estradiol measurements. There is an error on pg 28. The heading lists prolactin data, but refer to baseline LH values.

Discussion and Conclusion: The authors make some point that a threshold and NOEL could be demonstrated at the 25 ppm and 50 ppm doses for Atrazine effects (pgs 30-33). This is not correct since the data in test tables 3 and 4 (pg 26 and 27) show a significant difference from controls at 50 ppm for weeks 21-22.

References: There are a number of references in this section that are not mentioned in the text.

Figures: The peak surges of LH (1800 hrs) given in figure 1 are approximately 1/10 th of the circulating concentrations of hormone in the normal cycling adult rat. While these values may be valid for this particular animal model, they are not representative of the normal cycling female rat. It would be informative if the authors would indicate the number of determinations for each time point, along with SEM error bars for each determination. Since figure 2 data are from serial bleeds, this reviewer has some reservation about the usefulness of the information. There is an error in the "y" axis of the bar graph of figure 3. I think the concentration for

prolactin should be ng/ml, not pg/ml - a error of 1000 fold.

I have done some spot checking of some of the calculations and for the most part find them accurate. For example, pg 50, the body weight means and standard deviations for groups 1 and 4 at week 27 are correct. It would be helpful to provide some information for the reader in a footnote legend for Table 5, that the numbers given refer to number of rats, along with some level of statistical significance. In spot checking for accuracy, the numbers in the table conform to the raw data in Appendix 6, but do not agree entirely with data provided in figure 4. The standard deviations and errors of the mean concentrations for LH, prolactin and estradiol in limited spot checking are accurate, but are quite high (e.g., Table 6A, pg 73). This fact is a cause for concern in assessing the significance of this study.

In summary, this reviewer finds the data interesting and suggestive that Atrazine is able to disrupt the estrous cycle by attenuating or delaying the LH surge response to estradiol implants in ovariectomized Sprague-Dawley female rats. Furthermore, the data would suggest that Atrazine promotes the development of prolong periods of estrus during a course of treatment for 26 weeks. A LOAEL of 50 ppm can be demonstrated at the 21-22 weeks interval. Without knowing serum estrogen levels prior to ovariectomy and the physiological status of the hypothalamic-pituitary axis, however, the data presented here are insufficient in themselves to demonstrate a convincing mechanism for Atrazine-induction of mammary gland tumors in Sprague-Dawley female rats.

Review of Volumes 5 and 6 entitled: "Chronic(12/24 month) Study in Rats with Atrazine Technical Supplement to EPA Guideline NO. 83-1"

The purpose of this chronic dietary feeding study was to determine the incidence and onset of mammary gland tumors in control and Atrazine-treated ovariectomized and intact female Sprague-Dawley rats. Dose levels were 0, 25, 50, 70 and 400 ppm in feed, with exposure starting at 8 weeks of age and terminating after 52 weeks.

Methods: This reviewer was not clear about the diets fed to control and Atrazine-treated rats. Was the control PMI certified diet the same feed (minus Atrazine) as the Atrazine-containing feed with respect to chemical composition, nutrient quality, size, texture, palatability? Also, it was not clear to this reviewer why the inguinal mammary gland was taken for histopathology and not the thoracic (which is the dominant and most susceptible one for mammary gland neoplasia). It is also unclear why Group 5 body weight values at week one presented statistical problems.

Results: On pg 22, the investigators speak of palpable masses in the 400 ppm intact group. Were they referring to mammary gland or other tumors? Was the size recorded along with number of tumors per animal? No mention of these criteria were presented in this section. On pg 23, the authors say that significant body weight changes took place among various groups but were considered spurious and unrelated to treatment without further explanation. What is the evidence for this? On pg 25, there is an upward surge in food consumption at the 25 th week interval with the only explanation of an "8 day food consumption interval". This needs

clarification. Text Table 3 on pg 26 presents the mean compound consumption for the ovariectomized and intact female rats. However, no statistical treatment is mentioned for the obvious fact that every dosed, intact group consumed more Atrazine than the ovariectomized groups. Furthermore and paradoxically, the data demonstrate that ovariectomized rats consumed more food than the intact rats as evidenced by the mean body weights of the two groups (figure 2, pg 24). Were these facts analyzed statistically? If not, why not? Clearly, these observations may explain, in part, why the ovariectomized groups "faired" better than the intact group and thus provide an alternative explanation for the outcome other than a hormonal mode of action. This issue needs exploration, analysis and statistical evaluation.

Discussion and Conclusion: The authors allege a threshold for mammary gland tumors in this "12 month chronic", dietary feeding study using Atrazine-treated intact female Sprague-Dawley rats based upon the observation that only 4 and 6 rats in the 70 and 400 ppm groups exhibited mammary gland neoplasia, respectively, with reduced numbers in lower exposed groups. This allegation is not warranted, since the time frame for the study is only 12 months and perhaps of insufficient latency for mammary gland tumor expression. Clearly, early ovariectomy prior to Atrazine exposure appears to have a protective function in mammary gland neoplasia after 12 months and it is reasonable to assume that this is afforded by reduced serum estrogens, a known risk factor in human and animal mammary gland cancers. Nevertheless, this reviewer thinks it premature to make such assumptions after only 12 months. A full two year study seems justified.

Pathology Report: This report is based solely upon the first 20 out of 80 rats per group after 52 weeks of treatment. Clearly, more tumors were found in the intact groups than in the ovariectomized groups, where no mammary gland tumors were found. The authors conclude that Atrazine had no direct proliferative effect on the mammary gland. This is an unwarranted assumption, since these investigators failed to include any specific proliferation assays (e.g.., DNA labeling index) into their experimental protocol. What they should have said was that there was no evidence to indicate that Atrazine contributed to mammary gland neoplasia (as verified by gross and histopathologic observation) in the ovariectomized Sprague-Dawley female rat after 52 weeks of treatment. The current report summary (pg 7) says that there was no effect of treatment on mammary tumor incidence at feeding levels < or = 70 ppm. Yet, text table 2 (pg 32) shows 4 out of 20 rats examined exhibited mammary gland neoplasia in the 70 ppm group. Furthermore, the authors report (pg 33) that the elevated incidence of mammary gland tumors in the 400 ppm intact group was not statistically significant. This finding seems to contradict an earlier study by some of the same authors, who showed a significant increase in mammary gland incidence from controls at feeding levels of 400 ppm in Sprague-Dawley female rats (Wetzel, LT, et al., Chronic effects of Atrazine on estrus and mammary tumor formation in female Sprague-Dawley and Fischer 344 rats, J Toxicol Environ Health 43: 169-182, 1994). How do the authors reconcile this discrepancy?

I have looked quickly over some of the data provided in the Appendices, but have not scrutinized or spot checked the calculations.

Review of Volume 7 entitled: "Evaluation of a Hormonal Mode of Action for Mammary Carcinogenesis of the Chlorotriazine Herbicides: Second Consensus Panel Report"

This summary presents some of the conclusions by a panel of scientists that convene in October of 1996 and is based upon new data dealing with the possible mechanism for mammary gland tumor development in the Atrazine-treated female Sprague-Dawley rat. This reviewer thinks that some of the conclusions lack validity. The third of the six numbered conclusions states that Atrazine neither stimulated mammary tumor growth nor increased proliferative activity in mammary tissue. This conclusion cannot be substantiated, since the studies did not include growth or proliferative assays into their experimental design. The fifth conclusion states that the precocious appearance of Atrazine-induced episodes of persistent estrus was associated with prolonged estrogen secretion and ovulation failure. This too is an assumption since the investigators failed to determine estradiol secretion levels during the course of Atrazine exposure and did not assess ovulation by observing oviductal oocytes or formation of newly-formed corpora lutea in these studies. At least these endpoints were not mentioned in these reports. In the first "bullet" conclusion, the panel refers to the fact that the chloro-s-triazines are not genotoxic, but provide no references to support the statement. Again, in the second bullet , the panel members state that the mammary tumor response is the result of failed ovulation. This is not justified by the data provided, since ovulation wasn't measured. The third bullet conclusion again states that the mammary tumor response is associated with prolonged estrogen levels and ovulation failure, but these processes were not specifically measured. Finally, the fifth bullet states that the available data "firmly establishes a relationship between Atrazine exposure and mammary tumors with the disruption of the hormonal milieu. Since estradiol was not measured, this can only be an assumption. Finally, the panel recommends that the chloro-s-triazines be regulated with a MOE approach. This is not justified since the other triazines (Simazine and Propazine) were not studied in the current report.

In a quick and dirty dose response arithmetic plot using the investigators supplied data, I plotted dose against the number of female rats exhibiting persistent estrus at 13 and 14 weeks. I essentially got a straight line from the low dose of 25 ppm to the high dose of 400 ppm. With this linearity, a NOAEL could not be defined. If this observation is confirmed and holds up for the other time points, it seems inappropriate to assume a threshold.

Taken together, this reviewer thinks that there are a sufficient number of unresolved questions that require answers before postulating a "unifying" and scientifically-credible mechanism of action for mammary gland neoplasia in the Atrazine-treated Sprague-Dawley female rat.

cc/
Elaine Z. Francis (8104)
Karen Hammerstrom (8601)

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