UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

DEC 23 1991

OFFICE OF
PESTICIDES AND TOXIC
SUBSTANCES

MEMORANDUM:

SUBJECT: Request by the registrant to reconsider the NOEL established in the Atrazine rat reproduction study.

TO: Robert Morrill
PM Team 25
Herbicide Branch,
Registration Division H7505C

FROM: Henry Spencer, Ph.D. 12/12/91
Acting Section Head
Review Section 3
Toxicology Branch I
Health Effects Division H7509C

THRU: Karl Baetcke, Ph.D. 12/18/91
Chief
Toxicology Branch I
Health Effects Division H7509C

Tox Chem No. 063
HED Project No. 1-2302

REQUESTED ACTION:

The registrant, CIBA-Geigy Corp. has requested the agency to reconsider the NOEL that was established for the atrazine rat reproduction study completed in November 1987.

BACKGROUND: CIBA-Geigy Corp. has reviewed the previously submitted rat reproduction study report and found that its own analysis was in error by the purported incorrect use of a statistical method. The result was that statistical significance was placed at 50 ppm in the study supporting a reduced body weight gain in male pups by day 21 of lactation. The registrant contends that the NOEL should be established at a level higher than the

10 ppm evaluated in the agency review.

REVIEW AND COMMENTS:

Study: rat reproduction study
Date: November , 1987
MRID: 40431301
Chemical: Atrazine, technical

The outline of the 2-generation reproduction study in the rat has been excerpted from this submission requesting reconsideration of the NOEL. The outline is added as attachment #1.

The registrant has also submitted an additional statistical analysis for this submission which has been addressed by Dr. Hugh Pettigrew , Health Effects Division statistician. The Pettigrew memo is attachment #2.

In the original agency review of the reproduction study, the reviewer found the study in general was acceptable. This reviewer also finds the study acceptibly completed, but the individual pup weight by sex for each litter to be incomplete. The registrant has however, provided an addendum of this type information which is added to the files of this study.

The main point in question appears to be whether test dose values of 50 ppm and 500 ppm of atrazine in the diet caused a significant reduction in weight gain by the F2 male pups by day 21 of lactation (weaning).

In examining effects in the study, the reviewer has first looked at possible changes which could be transferred from the parents to the newborn. Table 6.4.4 excerpted from the original report indicates that the F0 dams exhibited a significantly reduced body weight only at the 500 ppm dosage level. The same effect is noted in the F0 males in Table 6.5.1 (excerpted).

The F1 male adults also exhibited these same weight changes in the premating, time period see Table 6.13.1 as excerpted.

Gestation body weight gains in F1 females are not reduced, though the premating period showed generally lower weight gains.

This study uses the Charles River CD rat as its test subject. The CD rat produces litters that may contain from 1 to as many as 20 pups at day 0 (birth). Since the dam has a limited ability to care for all these newborn, litters are culled to a maximum of 8 pups on day 4 after birth. These are then considered to have been given

their optimum chance of survival and growth to weaning (day 21). For this reason, changes in lactational effects are usually determined from this day 4 (post culling) point.

The male F1 pups resulting from the F0 parents show a reduced body weight (reduced below controls in all dosed groups) Table 1 B (excerpted). However there is no dose response effect at day 0 or at day 4 (post culling)thus indicating a chance rearrangement in the 0 and 50 ppm groups. Again, when properly using the post culling weights for comparison of data for days 7 and 14, there is no statistically significant change at lactational day 7 or 14. Additional food intake by these pups by day 21 at the dose of 500 ppm(a dose causing weight reductions in adults) could cause the weight changes noted.

There were no changes in the body weights in the F1 female pups in Table 2B.

When examining a summary of the body weights of the F2 male pups for the period of lactation at 0, 4 (post-culling), 7, 14, and 21 days as depicted in Table 3A (attached), only at day 21 were the 50 ppm and 500 ppm dosage groups found to statistically significantly different from controls (by Healy analysis).

Confounding the evaluation, is the day 0 , 10 ppm group weight shown to be significantly different from controls. Since body weights at the higher doses of 50ppm and 500 ppm do not indicate a progression in weight loss, this lack of progression contradicts the significance of the 10 ppm effect. Further into the lactational phase the weight data for the 10 ppm group (day 4 postculling) again is unequal- being lower than all other study groups. The 50 ppm and 500 ppm group values are also both lower than the controls.

At days 7 and 14, only the 500 ppm body weight value is seen to "slip" lower than the 10 ppm and 50 ppm dosed groups, suggesting a "real" effect.

At day 21 the highest two dose levels , 50 ppm and 500 ppm, show statistically significantly reduced weight values (original Healy analysis). Note: after statistical evaluation analysis review by Dr.H.Pettigrew, the original Healy method was judged to be incorrectly used. When the appropriate methodology was used, the statistical flags were no longer present.

Based on the above evaluation by Dr. Pettigrew, the Toxicology Branch does not consider the 50 ppm LEL previously determined as the appropriate value for this study .

COMMENT ON NEW LEL.

The evaluation of a lactation effect by inspecting only body wt. gain reduction at day 21 of weaning provides for the possible confounding of real lactational effects of the chemical. The 21 day parameter end-point is the end result of both parent chemical and metabolite intake via the maternal milk supply and food intake from the food source of the dam.

Most young animals eat a greater quantity of food on a g/kg bwt. basis than do older animals of the same strain. Since young in the lactational phase begin ingesting food from the supply for the dam during the early part of the last week (14-21 days), changes in the neonate body weights from 1.increased food intake and 2.milk source of test chemical, would not represent a lactational effect per se.

In the present case the body weights of the F2 male pups in the 10 ppm and 50 ppm groups at day 7 and day 14 are essentially the same even though they are less than the control group value. This reviewer maintains that this time point (7 and 14 day) using the day 4 post culling) data as a control should be the end point on which to base a lactational effect. The added week of dual intake , especially at 500 ppm is expected to further increase the body wts reduction changes already seen.

The additional wt. changes are not seen at the lowest two dose levels (10 ppm and 50 ppm) while the only change noted by this reviewer is that of a consistent ,though not statistically significant, fall in F2 male pup body wts at 500 ppm when compared to the post culling control and the other dose levels group data.

The 500 ppm dose is therefore considered to be an LEL, and 50 ppm is established as the NOEL.

CONCLUSIONS:

1. The statistical methodology of Healy was considered to be inappropriate by statistical standards of today.

2. Toxicology Branch considers the NOEL of 50 ppm to be established for the rat reproduction study. The LEL is based on the reduced body wt in F2 male pups at 500 ppm, at the 7 and 14 day time periods, though the data show no statistical significance.

3. Lactational effects are chosen in this study to limit effects to the maternal milk supply and not the combined effects with the maternal food source also.

4. This reevaluation is contingent upon the approval of the RFD group.

TK Review dated 12/23/91

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Pages 6 through 12 are not included.

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UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

DEC 10 1991

OFFICE OF
PESTICIDES AND TOXIC

Subject: Atrazine Technical, Supplemental Information for the Two-generation Reproduction Study in Rats

From: Hugh M. Pettigrew, Ph.D., Statistician
Science Support and Special Review Section
Science Analysis and Coordination Branch
Health Effects Division (H7509C)

To: Henry Spencer, Ph.D., Acting Section Head
Section III, Toxicology Branch I
Health Effects Division (H7509C)

Thru: Kerry Dearfield, Ph.D., Acting Head
Science Support and Special Review Section
Science Analysis and Coordination Branch
Health Effects Division (H7509C)

Summary

At your request, I have reviewed the document entitled Atrazine Technical, Supplemental Information for the Two-generation Reproduction Study in Rats, completed on August 15, 1991, submitted by Ciba-Geigy Corporation. This document presents a review of the relevant data from the study as well as additional statistical information in support of the Registrant's contention that the reproductive NOEL based on the study is at least 50 ppm.

Based on a review of the material submitted, this reviewer believes that the revised Healy analysis and the covariance analyses provide the correct analysis of the data as far as determining which differences are statistically significant.

Background

The Registrant has submitted the original and revised "Healy analyses" for the F_1 and F_2 Male and Female pup body weights. This method of analysis was proposed by M. J. R. Healy , who observed that "experiments using animal litters as experimental units usually require a weighted analysis to allow for variations in litter size" and described "methods for assessing appropriate weights" (Journal of the Royal Statistical Society (Series C), Applied Statistics, Volume 21, No. 2, 1972, pp.155-159.) A copy of this reference is attached.

The Registrant contends that a review of the original Healy analysis procedure determined that pairwise comparisons were done inappropriately, in that paired comparisons should not have been conducted in the absence of a statistically significant F-statistic. The correct procedure, as the Registrant points out, is to carry out pairwise comparisons only if the F-test is significant, thereby controlling the Type I (false-positive) error rate. Therefore the revised Healy analysis should be considered correct as far as determining which differences are statistically significant.

An alternative method of adjusting for differences in litter size is to apply the analysis of covariance, using litter size at day zero as the covariate. The covariance analyses submitted by the Registrant were run using the SAS statistical analysis system. This method of analysis further reduces the number of apparently statistically significant results originally reported.

It should be pointed out that these remarks apply only to the screening of the data to determine <u>statistical</u> significance. The data must still be examined for <u>biologically</u> meaningful results.

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Animal Litters as Experimental Units

By M. J. R. Healy

M.R.C. Clinical Research Centre

Summary

Experiments using animal litters as experimental units usually require a weighted analysis to allow for variation in litter size. This paper describes methods of assessing appropriate weights for fully randomized and for randomized block experiments.

Keywords: ANIMAL EXPERIMENTS; VARIANCE COMPONENTS; WEIGHTED ANALYSIS OF VARIANCE

1. Introduction

In several areas of research, it is required to assess the effects of treatments applied to female animals by measurements made on their offspring. This leads to the use of whole families or litters as experimental units, and because the litters will usually be of different sizes the units will have differing accuracies. A weighted analysis is thus appropriate and the problem of estimating the weights arises.

The problem exists because of the presence of between-litter as well as within-litter variability. The situation may be illustrated by considering the two extreme situations. In the first there is no between-litter variability, the division into litters is essentially arbitrary and the best estimate of the mean for a set of litters is the mean of all the observed values, or, in other terms, the weighted mean of litter means using litter sizes as the weights. At the other extreme all the values in a single litter are equal. There is now no within-litter variability and no extra information is provided by any animal in a litter after the first. The best estimate of the mean of a set of litters is now obtained by giving the litter means equal weight, irrespective of litter sizes. In realistic situations, an intermediate system of weighting is appropriate, with weights increasing with the litter size but not as fast.

To get further, we assume that a reading can be expressed as the sum of three parts:

$$y = \mu + \epsilon_1 + \epsilon_2, \tag{1}$$

where μ is an overall mean value which will depend upon the treatment applied, ϵ_1 is a random term, with mean zero and variance σ_1^2, which is common to all readings within a particular litter and ϵ_2 is an independent random term with mean zero and variance σ_2^2. The variance of the mean of a litter of n animals is then $\sigma_1^2 + \sigma_2^2/n$ and its appropriate weight is the reciprocal of this. Putting σ_1^2 or σ_2^2 equal to 0, we obtain the two extreme situations described above.

2. Fully Randomized Designs

When treatments are applied to females at random, the analysis is quite straightforward. First a three-level analysis of variance is constructed with rows for Treatments, Litters within treatments and Within litters. The mean squares from

15

this analysis are used to estimate σ_1^2 and σ_2^2 and finally weighted treatment means can be calculated and compared. The first two steps form a standard type of hierarchical analysis of variance and an appropriate computer program may be available, but the procedure will be described here with desk calculation in mind.

It will be simplest to illustrate the technique by analysing an actual example. Table 1 relates to an experiment in which female mice were exposed to live virus, killed virus or a control solution, the effects being measured by weighing the placentas of the offspring. The full data are rather bulky and Table 1 contains the litter sizes, litter totals and means and within-litter sums of squares. From these figures, it is easy

TABLE 1

Placental weights of offspring of treated mice: litter sizes, litter total and means (g) and within-litter sums of squares

Treatment	n	Σx	$\bar{x}$	*Sums of squares*
Live virus	10	5·5	0·5500	0·0250
	3	1·3	0·4333	0·0067
	12	6·3	0·5250	0·1625
	17	8·8	0·5176	0·1847
	13	8·8	0·6769	0·1031
	7	3·0	0·4286	0·0143
	11	5·0	0·4545	0·0273
	6	3·9	0·6500	0·0150
	3	1·7	0·5667	0·0067
	13	8·2	0·6308	0·1277
	13	8·8	0·6769	0·1231
	108	61·3		0·7961
Dead virus	11	8·7	0·7909	0·1891
	11	8·0	0·7273	0·1218
	11	9·6	0·8727	0·2618
	16	11·4	0·7125	0·1975
	14	8·6	0·6143	0·0771
	11	7·3	0·6636	0·0655
	11	6·0	0·5455	0·0673
	85	59·6		0·9801
Controls	12	9·1	0·7583	0·1692
	7	6·2	0·8857	0·2286
	14	9·1	0·6500	0·1750
	1	1·2	1·2000	0
	4	2·8	0·7000	0·0600
	38	28·4		0·6328
	231	149·3		2·4090

to derive the analysis of variance in Table 2, which demonstrates clearly that there is considerable litter-to-litter variability in placental weight.

TABLE 2

Analysis of variance of data from Table 1

	d.f.	*Sums of squares*	*Mean squares*
Treatments	2	1·3132	0·6566
Litters within treatments	20	2·0924	0·1046
Within litters	208	2·4090	0·0116

The variances σ_1^2 and σ_2^2 can be estimated using the mean squares from the analysis of variance. σ_2^2 is in fact estimated by the mean square within litters, 0·0116. The expectation of the mean square between litters is $k\sigma_1^2+\sigma_2^2$ where k is a kind of average litter size whose evaluation is described by Gower (1962) and Gates and Shiue (1962). Specializing their formulae to the case in hand, we can obtain k in the following steps:

1. For each treatment, sum the squares of the litter sizes and divide by the total number of offspring—for the controls, this gives:

$$(12^2+7^2+14^2+1^2+4^2)/38 = 10{\cdot}68.$$

2. Subtract these quantities from the overall total number of animals.
3. Divide the result by the degrees of freedom for litters within treatments.

The process is illustrated in Table 3. Using this value of k and the estimate of σ_2^2, we can now estimate σ_1^2 from the mean square for litters within treatments as 0·0095.

TABLE 3

Variance component estimation from Table 1

(Litter size)²/number of animals:	live virus	11·70
	dead virus	12·44
	controls	10·68
$k = (231-34{\cdot}82)/20$		
$= 9{\cdot}809$		
Estimated variance components:	$s_2^2 = 0{\cdot}0116$	
	$s_1^2 = (0{\cdot}1046-0{\cdot}0116)/9{\cdot}809$	
	$= 0{\cdot}0095$	

At this point it is desirable to tabulate the estimated weights, $1/(s_1^2+s_2^2/n)$, as shown in Table 4. These weights may then be applied to the litter means in Table 1 to obtain weighted treatment means and the weighted sum of squares for litters within treatments, and hence the error variance per unit weight, s^2. This process is illustrated in Table 5. The difference between the dead virus group and the controls is $0{\cdot}096 \pm 0{\cdot}072$, where the standard error is calculated as $\sqrt{\{s^2 \times (1/670+1/410)\}}$; if we accept the non-significance of this difference, we may combine these two

treatments and compare their mean with that of the live virus group, obtaining $0{\cdot}7400 - 0{\cdot}5579 = 0{\cdot}182 \pm 0{\cdot}050$, a clearly significant difference.

The effect of the between-litter variability is seen in Table 4, where it is apparent that a litter of ten animals is only worth twice as much as a litter of one animal. Although it is usually more costly to raise more litters than to measure more offspring,

TABLE 4

Estimated weights for different litter sizes

n	*Weight*	*n*	*Weight*
1	47	10	94
2	65	11	95
3	75	12	96
4	81	13	96
5	85	14	97
6	87	15	97
7	89	16	98
8	91	17	98
9	93		

TABLE 5

Weighted treatment means from Table 1

	Mean	*Weight*	*Sums of squares within treatments*	*S.E.*
Live virus	0·5579	997	7·9098	±0·036
Dead virus	0·7036	670	6·8018	±0·044
Controls	0·7994	410	11·3330	±0·056
		$s^2 = 26{\cdot}0446/20$		
		$= 1{\cdot}3022$		

it is also more rewarding, and not much information is lost if (say) half the animals of the larger litters are discarded, provided this is done strictly at random.

The significance of each of the differences in our example is obvious in general terms. If a precise level of significance is required, a *t*-test should be used with degrees of freedom equal to those for litters within treatments. Such a test ignores uncertainties in the weights, which themselves depend upon the estimated variance components; insofar as small changes in the weights scarcely affect the weighted means, these uncertainties should not be important in an experiment large enough to give useful results. The uncertainties will usually be mainly associated with the between-litter component σ_1^2 and so with the litters within treatment mean square—this is another reason for increasing the number of litters as far as possible rather than merely the total number of offspring.

3. Structured Designs

The situation is rather more complicated when the mother animals are arranged (as would normally be desirable) in a randomized block design. In the basic model (formula (1)), μ now depends both on the treatment and on the block of the design, while ϵ_1 is now a random interaction term. This is technically a mixed model and the differing numbers in the litters render it unbalanced. The estimation of variance components under these conditions has been much discussed (see, for example, Searle, 1968, and the discussion and references therein). The between-litter component can be estimated from the interaction (or residual) and within-litter mean squares after the fashion of Table 3, but the recommended technique (Henderson's method 3) involves a non-orthogonal analysis of variance and the evaluation of the factor k is quite complex. A simpler method is available in the usual case in which each block-by-treatment combination is represented by a single litter. An initial analysis is made of the cell means, giving each one equal weight—this involves simply the textbook analysis of a randomized block experiment. The residual mean square in this analysis has an expected value of $\sigma_1^2+\sigma_2^2\{\sum(1/n)/N\}$, where n is the number of animals in a litter and N the total number of litters. An estimate of σ_2^2 can be had from a second analysis of variance estimating Between-cell and Within-cell mean squares, and hence an estimate of σ_1^2 obtained. The appropriate weight can now be estimated for each cell of the table and a final (non-orthogonal) analysis made to estimate the treatment means.

Acknowledgement

I am grateful to Dr C. Coid for letting me use his data as an illustrative example.

References

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