UNITED STATES ENVIRONMENTAL PROTECTION AGENCY Caswell 623A

DATE: January 23, 1981

SUBJECT: Oryzalin, TB/HED review of rat reproduction, rat dominant lethal, and bacterial mutagenic studies submitted in support of PP No. 6F1859, Acc. No. 241742.

FROM: Mary L. Quaife, Ph.D., TB/HED (TS-769) MLQ. 1/23/81

TO: Mr. R. Taylor, PM
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THRU: Mr. William Burnam, Acting Chief
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PP No. 6F1859
EPA Nos. 1471-96 and 112

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CONCLUSIONS:

1. The three-generation, three-litter rat reproduction study on oryzalin is inadequate. Petitioner is asked to provide a table of incidence - and time of onset - of each adverse eye effect seen grossly, and a separate tabulation of histopathologic eye findings made, in those rats.

2. The bacterial mutagenic studies are judged adequate.

3. The rat dominant-lethal (mutagenic) study on oryzalin is not acceptable.

SUMMARY:

1. Three new TOX studies are reviewed in detail (in the following nine pages), which were carried out on oryzalin and related compounds.

2. In a three-generation, three-litter rat reproduction study on 99.0% pure oryzalin, there was statistically ($p < 0.05$) significant growth depression, including pre-weaning, at 2,250 ppm in the diet. The was no effect (at 250 ppm) and borderline effect (at 750 ppm), respectively, on growth. (We note that the oryzalin [redacted] The study is CORE-supplementary, pending resolution of eye-effects' question (which is noted in Conclusion # 1, above).

3. Under conditions of testing by modified Ames test (Cancer Res. 39, 682-93 (1979)), oryzalin of unstated purity (lot 090-310-146) was not mutagenic at levels tested between 0.1 and 1,000 micrograms per milliliter.

4. Under the same conditions of testing as in Summary # 3 (above), [redacted] mutagenic at levels tested between 0.1 and 1,000 micrograms per milliliter.

5. Under the same conditions of testing as # 3, [redacted] positive in strains TA 100 and TA 1538. In a standard Ames-technic study (using TA 100) of concentration vs. reversion frequency, this compound was shown to be a mutagen. (It is used in closed system in oryzalin synthesis, according to letter in this submission (1/30/80).)

6. In a rat dominant lethal test, 5 g/kg BW would be NOEL; except that, because of serious deficiencies in study design (cf. p. 8, this memo), study is judged unacceptable with CORE-rating as supplementary.

NEW TOX STUDIES:

1. "A multi-generation study with compound 67019 in the rat, studies R-1226, R-327, and R-647," by J. L. Carter, N. V. Owen, and E. R. Adams, Toxicology Division, Lilly Research Labs., Greenfield, Indiana, January, 1980, submitted, 1/30/80, Accession No. 241742.

Test substance. Oryzalin (compound 67019; 3,5-dinitro-N^4,N^4-di-n-propylsulfanilamide; Surflan), X-28607 (Soydeco lot 86343, batch 343, 99% pure, Appendix C) was used. We note that in Appendix C to this volume, purity of 99.0% is affirmed. Also, on p. 65 of this volume (Acc. No. 241742), it is stated, "The lot of material used in these tests was also used in chronic/oncogenic tests with this chemical. In conjunction with the latter tests, analyses of the lot were performed to determine the content of [redacted] at the date corresponding to the second month of the F_1 generation growth phase and....monthly, thereafter."

It is important to note that technical oryzalin is described as ca. 96% pure (e.g., cf. this appendix C); whereas this compound is said to be 99% pure. Also - cf. above quotation - the test compound is said to be the same lot of material as used in chronic/oncogenicity tests of oryzalin.

Procedure. Groups of 25 M and 25 F, 5-week-old Fischer 344 rats, Harlan Industries, Cumberland, Indiana, received the test compound in the diet at 0, 250, 750, or 2,250 ppm for 59, 73, and 63 days during the respective growth phases of F_0, F_1, and F_2 parental generations. Rats were continued on their respective diets until death or purposeful killing. Rats were mated and females allowed to deliver and raise offspring to 21 days of age. Representative offspring were selected and maintained on test diets for growth and mating phases. F_3 progeny were killed as weanlings. F_0 and F_1 parents were killed after beginning of growth phase of their offspring. The F_2 parents were killed after the F_3 progeny were killed.

Rats were housed individually in stainless steel cages, except that they were housed in pairs during the two-week mating trials. Temperature was 77 $\pm$ 2° F., with relative humidity of 45%, minimum, and light-dark cycle of 12 hrs. Each rat was numbered (but not the litters) and ear-punched for dose. A standard natural food mash ration, prepared every 2 to 4 weeks, was used. It and Greenfield City water were available ad lib. New test diets were made, each two weeks.

At the start of the growth phase of each parent generation, samples of test diet at each level were assayed for oryzalin content, as were one-week-old samples. Rats were examined, daily, during the growth phases and, more carefully, once each week. During the growth phase, all rats were weighed, weekly, and food consumption was determined then. During the reproduction phase, females were weighed on the date of separation from the male and on the day the pups were 21 days old; food consumption was not measured.

Records were made of the date of parturition; the number of live and stillborn in each litter; and the number, weight, and condition of the survivors on days 1, 7, 14, and 21 after the delivery. Progeny were weighed as litters on days 1, 7, and 14 and, individually - and sex was determined - on day 21.

Parents that died were submitted for a gross autopsy. Offspring that died up through 21 days of age were examined grossly with particular attention given to the heart. In the F_0 and F_1 parental generations, two weanlings per sex per control litter and one pup per sex per litter in test groups were given a gross internal examination - with particular attention paid to the heart. All surviving F_3 pups were examined grossly, internally (heart morphology). The following organs from one pup per sex per litter (F_3) were fixed; prepared for histological examination; and examined by a pathologist: Spleen, lymph node, skeletal muscle, lung, heart, salivary gland, liver, pancreas, stomach, duodenum, ileum, jejunum, colon, kidney, urinary bladder, prostate, testis, uterus, ovary, skin, mammary gland, adrenal, thyroid, and thymus.

In each generation, adult females which failed to deliver were killed and examined for evidence of pregnancy. After offspring in each generation were examined, males and females which had been pregnant were given terminal eye and physical examinations and were killed.

Mean values of the individual "parameters" were calculated for each dose-group and generation. An indicator of overall reproductive performance, the mean number of offspring raised to weaning age per pregnant female, was determined.

The following reproduction indices were calculated:

Fertility - proportion of females that were pregnant.
Gestation Survival - proportion of newborn pups that were alive.
Survival - proportion of offspring that survived 1, 7, 14, or 21 days.

Statistical Analyses. Mean body weight gain was analyzed according to Dunnett (New tables for multiple comparisons with a control. Biometrics 20, 482-92 (1964)). The mean litter weight was used as the independent sampling unit (Weil, C. S., Selection of the valid number of sampling units and a consideration of their combination in toxicological studies involving reproduction, teratogenesis, or carcinogenesis. Fd. Cosmet. Toxicol. 8, 177-82 (1970)). Mean progeny weight on day 21 was analyzed statistically by the method of Dunnett. However, since multiple comparisons were made at 21 days over three generations, the critical value of "t" used in the analyses was a Bonferroni "t" (Miller, R. P., Simultaneous statistical inference, McGraw-Hill, New York, N.Y., pp. 67-70, 1966) with $p \leq 0.05$.

Results.

Chem. assay results. [redacted] is reported for five monthly intervals, August - December, 1977 - analyses done on "lot X-28607" of oryzalin; this time-interval corresponds to "second month of F_1 growth phase" through end of that generation study. Values are [redacted] in consecutive assays.

Diet assay results are judged satisfactory, i.e., within ca. ±10% of theoretical; except that only half of theoretical content of oryzalin was found in the "aged-one-week" sample of low-dose (250-ppm) test diet fed to F_1 rats. This is judged not to affect the validity of the overall study.

Physical effects. No overt signs of toxicity are said to have been seen due to exposure to oryzalin. Rats did show yellow-orange or discolored urine and stained perianal fur or tail, due to the color of the compound. Four parent rats died, apparently, due to unrelated causes. Many rats suffered from abnormal eye conditions, notably those of F_1 generation, diagnosed as conjunctivitis; at mating-time, only a mid-dose rat (#266) and a high-dose rat (#307) showed unilateral corneal opacity. Rats of F_2 generation which showed exophthalmus, opacity, dark purple color, or atrophy are said to have not showed substantive tissue alteration on microscopic examination of the eyes.

Growth and food consumption. There was no effect on either body-weight gain or food consumption in F_0 rats. However, their weanling offspring in the high-dose group weighed less than corresponding controls and showed significant ($p \leq 0.05$) decrease in body-weight gain during post-weaning growth; this was true of both males and females. Mid-dose F_1 rats weighed less as weanlings and during post-weaning growth, but not significantly so. Food consumption of F_1 high-dose rats was somewhat less than that of corresponding controls (ca. 2 g/day). High-dose F_2 weanlings weighed significantly less than controls ($p < 0.05$), and they weighed less throughout the duration of the growth period; although the rate of weight increase was not affected. Food consumption of these rats was only ca. 1 g/day less. F_3 pups at the high dose weighed less than controls, also.

Indices of reproduction performance. Fertility indices for all female control and test groups, throughout three generations, varied from 84 to 96% in apparent random fashion. In like manner, gestation survival indices were all either 99 or 100%. Survival indices, calculated at 1, 7, 14, or 21 days, were very close for control or test rat pups on any given day within a given generation. For all generations, dose-levels, and time-intervals at which determined, the survival indices showed extremes of 80 to 99%, inclusive. Thus, the test compound did not affect any of these indices of reproduction performance.

Litter-size, gestation-length, and sex distribution. Live-born litter-sizes, mean values, varied from 8.5 ± 0.6 to 10.1 ± 0.4 for all test and control groups in the three generations. Similarly, mean gestation-lengths varied from 22.0 to 22.5 days. Mean values for one per cent of males in litters, similarly, varied from 41 to 58%, apparently at random. For the generations, dose-levels, and time-intervals at which determined, the mean litter-size, gestation-length, and sex distribution showed no apparent effects of exposure to oryzalin. (Although live-born litter-sizes are cited here, preceding paragraph notes that up to 99% of rats were born alive.)

Progeny observation and examinations. A total of 2,556 offspring was delivered in the study. In addition to yellow-stained fur and eye conditions, described above, some runts, pups with ringtail, and those with short or no tail were observed; incidence is said unrelated to oryzalin dose. Of 47 pups that died by 21 days of age which were examined (some were cannabalized and could not be), one high-dose F_2 pup had multiple craniofacial defects and dwarf-like; this is not attributed to oryzalin exposure, due to its singular occurrence. We concur. All surviving (226) F_1 pups given a gross examination appeared normal. Two of 204 F_2 pups had excessive fluid around one ovary; the others were normal. Of all 711 surviving F_3 pups examined, few had abnormal findings. Histopathologic findings on 45 control and 128 treated F_3 pups examined were confined to mild interstitial pneumonia in one control rat. All this is affirmed by the Senior Pathologist, James L. Carter, D.V.M., Ph.D.

Discussion. In this three-generation, three-litter reproduction study, oryzalin did not affect reproduction indices, litter size, gestation length, or sex distribution of progeny. At 2,250 ppm, both pre- and post-weaning growth or body weights of F_1 and/or F_2 rats were decreased significantly ($p < 0.05$), and F_3 weanlings weighed less than corresponding controls. At 750 ppm, we judge, there was borderline growth suppression. At 250 ppm, there was no effect on growth. (Growth of F_0 rats was not affected by any dietary level of oryzalin.)

A potential effect on rats exposed to oryzalin in utero and pre-weaning (as well as later) is noted, which needs clarification. In all test groups and prevalent in F_1 and F_2 rats, squinted or closed eyes, exudate from the eyes, exophthalmus, dark purple eyes, hair loss around the eyes, cloudy eyes, and eye opacities were seen. Petitioner gives (p. 71 of the study report) sparse information on their occurrence and judges them not related to the test compound.

Petitioner is asked to provide a table of incidence - and time of onset - of all adverse eye effects seen grossly, and a separate tabulation of histopathologic eye findings, for all rats of the reproduction study.

Conclusions.

1. Oryzalin, 99.0% pure [redacted] by assay), did not affect reproduction indices that were measured; litter size; length of the gestation period; or the sex distribution of the progeny.

2. Oryzalin had a significant ($p < 0.05$) effect or borderline effect (at 2,250 and 750 ppm, respectively) on pre-weaning and post-weaning growth of rats; it had no effect on growth at 250 ppm.

3. A potential adverse effect of oryzalin on eyes of rats exposed in utero and later is shown, regarding which clarification is asked of the Petitioner (please cf. third paragraph, this page).

CORE-rating - Supplementary, upgradable to Minimum on resolution of eye-effects' question.

NOEL - Not determined, pending such resolution.

2. "A Dominant lethal study with compound 67019 (oryzalin) in the rat," Study R-149, by J. L. Carter, N. V. Owen, and E. R. Adams, Lilly Res. Labs., Greenfield, Ind., November, 1979, Acc. No. 241742.

Test compound. Oryzalin, 3,5-dinitro-N^4, N^4-di-n-propylsulfanilamide, Lot X-28607, 99.0% pure. By assay (of January, 1979), [redacted]

Procedure. Groups of 10 M adult Wistar-derived rats each received a single dose (by gavage) of 0 (vehicle) or 5 g/kg body weight oryzalin as a 25% (w/v) suspension in 10% (w/v) aqueous acacia solution. Dose volume was 20 ml/kg.

Three days after dosing, each male was mated with an untreated, virgin female for one week. The female was removed to a separate cage and a new female introduced. The cycle was repeated for a total of 8 consecutive weeks.

Males were weighed when dosed (mean ± S.E. = 428 ± 7 g); after one and two weeks, and at end of the study. Females were weighed at mating (mean ± S.E. = 191 ± 1 g); on gestation day 0 (if positive for copulatory plug); and on day killed.

Males were watched for evidence of toxicity for 4 days after dosing.

Females were killed on gestation day 20 (or 18 or 19 to avoid a week-end). Females without plug were killed, two weeks after separation from the male. All females received a gross internal examination. Numbers of corpora lutea in the ovaries and numbers and location of fetuses and early and late resorptions in the uterus were determined. Fetuses were examined for viability.

After 8 weeks, males were examined externally and killed.

For each set of 10 females, for each week, mean numbers of: Pregnant females; live fetuses/litter; resorption sites; implantation sites; and corpora lutea were obtained.

Following reproduction indices, calculated (each week) for each set of 10 females, were obtained:

Gestation survival - proportion of fetuses alive.

Resorption - proportion of implanted conceptuses that were resorbed.

Implantation - proportion of implantations of corpora lutea.

Implantation occurrence and resorption incidence were analyzed statistically; since dominant lethal mutation increases pre-implantation loss and/or post-implantation death. The male is taken as the experimental

unit. (Cf. Weil, C. S. Selection of the valid number of sampling units and a consideration of their combination in toxicologic studies involving reproduction, teratogenesis, or carcinogenesis. Fd. Cosmet. Toxicol., 8, 177-82 (1970) for choice of the male as sampling unit used here.)

Data for weeks 1 and 2, 3 and 4, 5 and 6, and 7 and 8 were pooled. This is justified in that TEM, a known dominant lethal agent, showed positive effects during the first four weeks. (Cf. Bateman, A. J., "Induction of dominant lethal mutations in rats and mice with triethylenemelamine (TEM). Genet. Res. Camb. 1,381-92 (1960).)

Then, for each male, for each pair of weeks, the resorption and implantation indices (as percentages) were determined; an arcsin square root of the percentage transformation was conducted (to normalize distribution); an "F" value was determined by one-way analyis of variance; and this was compared to a Bonferroni "F" to determine statistical significance of any difference ($p \leq 0.05$) between control and oryzalin-treated males. (Cf. Miller, R. P. Simultaneous Statistical Inference. McGraw-Hill, New York, N.Y., pp. 67-70, 1966.)

Results.

Mortality. There were no deaths.

Toxicity. There were no overt toxic signs in males.

Body weights. The oryzalin did not affect mean body weights of males.

Maternal findings. Eleven females/of 160 had hydronephrosis. Two controls had vaginal and excess uterine bleeding. One test female had purulent material in a uterine horn.

Reproduction findings and reproduction indices. Five control females and three test females were not pregnant; of these, two control and three test females had been mated during the first mating week. All control males sired at least six litters and all test males, at least seven.

Treatment did not affect means of live litter-size (9.5 to 11.0); corpora lutea (10.9 to 12.9); resorption sites (0.2 to 1.4); implantation sites (9.8 to 12.3); resorption indices (0.02 to 0.11); or implantation indices (0.85 to 1.03) for the the eight mating weeks. All 1,580 fetuses were alive and grossly normal. All gestation survival indices are 1.00.

Statistical evaluation. There were no statistically significant differences (i.e., $p \leq 0.05$) for implantation occurrence or resorption incidence in any of the four (2-week) periods. This shows that there was no increase in pre-implantation losses or post-implantation deaths due to oryzalin treatment in this study.

Conclusion. As tested, oryzalin given to male rats did not show dominant lethal effects.

CORE Rating: Supplementary

Apparent NOEL 5 g/kg BW, only level tested.

1. Too small a dose of oryzalin was administered (5 g/kg = LD0, according to report); and an LD5 (= ca. 1/5 of the LD50) is used, usually.

2. The three-day delay between dosing and mating should not have been imposed.

3. A positive control was not included to show sensitivity of the system.

4. The 99.0% pure oryzalin test sample used does not appear to be typical of oryzalin technical (which is ca. 96% pure, according to some sources).

5. Two female rats per male/week are suggested as the correct number; only one was used here.

6. Use of three to four dose-levels, one to cause overt, non-reproduction toxicity, is suggested.

Note: Regarding the above comments (1-6), please see (1) memos of Drs. L. Anderson (11/9/79) and A. Gross (3/26/80) of TB/HED and (2) suggested protocol for dominant lethal mutagenicity test given in "Testing of chemicals for carcinogenicity, mutagenicity, and teratogenicity," Health and Welfare, Canada, p. 85.

3.
Bacterial Mutagenic Studies: The effects of Lilly No. 67019, oryzalin; No. 30545 [redacted] upon bacterial systems known to detect mutagenic events, by C. Z Thompson and R. E. McMahon, Lilly Res. Labs., Greenfield, Indiana, December, 1979, Acc. No. 241742.

Test substances. Oryzalin, lot # 090-310-146; Lilly No. 30545, lot # 069-254-263; and Lilly No. 56277, lot # P69605, were used.

Procedure. Reference is "Assay of 855 Test Chemicals in Ten Tester Strains Using a New Modification of the Ames Test for Bacterial Mutagens," by R. E. McMahon. J. C. Cline, and C. Z. Thompson. Cancer Research 39, 682-93 (March, 1979)

The procedure allows testing in ten tester strains over a 10,000-fold concentration gradient both with and without metabolic activation (inclusion of polychlorinated biphenyl-induced rat liver supernatant). Five bacterial strains, D3052, TA 1538, C3076, and TA 1537, are considered as frame-shift mutants; five, G46, TA 1535, TA 100, WP2, and WP2uvrA⁻, are presumed to detect base substitution mutagens.

Data obtained can show (a) the activity spectrum in tester strains; (b) identification as either frame-shift or base substitution mutagens; (c) minimal concentration at which auxotroph growth is inhibited, and (d) mutagenic potency in terms of minimal concentration at which mutagenicity is observed.

In all tests, negative and positive controls (2-acetylaminofluorene and streptozotocin) were used.

Results. Oryzalin was not mutagenic, as tested, at between 0.1 and 1,000 micrograms per milliliter. Positive controls gave expected results.

Compound 30545 gave a negative response at levels tested, between 0.1 and 1,000 micrograms per milliliter, Two positives gave expected positive results, streptozotocin without microsomal activation and 2-acetylaminofluorene with activation.

Compound 56277 gave a positive response to strain TA 100 and appeared to be "weakly" active to strain TA 1538. Accompanying positive controls gave expected results. A concentration vs. reversion frequency study was performed in strain TA 100 (using standard Ames procedure). Reversion rate increased with increasing concentration of test compound. At one milligram per plate, 56277's reversion rate was three times the spontaneous rate.

Conclusions.

1. Under the conditions of this modified Ames test procedure, oryzalin is not mutagenic.

2. Under the conditions of this modified test procedure, Ames, Lilly compound No. 30545 [redacted] is not mutagenic.

3. Under the conditions of this modified test procedure, Lilly compound No. 56277 [redacted] has "weak" bacterial mutagenic properties - confirmed with expanded Ames-technic study with one strain, TA 100.*

CORE rating is judged by this reviewer to be Minimum.

We judge the study adequate to support requirement for Ames' type testing for mutagenicity of these three substances.

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* Mutagenic potential of compound No. 56277 in Strain TA 100

Micrograms of # 56277/plate	Revertant colonies per plate A/			Average	Net revertant rate
	1	2	3		
0	263	300	278	280	0
3.9	280	309	325	305	25
7.8	325	290	297	304	24
15.6	358	308	334	333	53
31.25	375	382	368	375	95
62.5	407	410	415	411	131
125	474	454	444	457	177
250	654	622	694	657	377
500	903	973	958	945	665
1,000	1,171	1,122	1,045	1,113	833
2,000	1,175	1,078	996	1,083	803

A/ Triplicate plates were run at each concentration.