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

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OFFICE OF
PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: Response to previous Toxicology Branch comments on a rabbit teratology, microbial, and dominant lethal studies. EPA Reg. No. 33773-1. Tox. Chem. No. 116A

TO: John H. Lee
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THRU: Jane Harris, Ph. D., Section Head JH 4/30/85
Review Section 6
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Hazard Evaluation Division (TS 769)

FROM: Roger Gardner, Toxicologist
Review Section 6 Roger Gardner 4-30-85
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Actions requested

Comments on the response to previous Toxicology Branch reviews of the following studies:

1. A rabbit teratology study
2. A Salmonella typhimurium mutagenicity assay (Ames test)
3. A dominant lethal assay in rats.

Recommendations and Conclusions

1. Although the rabbit teratology study follows standards published in 1965, it should be regarded as limited or supplementary. The study is not invalid (see Sections II. and III. A.).

2. Results of the microbial assays indicate that bronopol is not mutagenic in Salmonella typhimurium (see Section III. B.).

3. The dominant lethal study in mice is acceptable and suggests that bronopol may have an effect on germ cells in males.

I. Background

A. Previous Toxicology Branch comments

A previous Toxicology Branch review (Gardner, 1984) stated:

> The additional individual animal data submitted for the rabbit teratology study indicate that only 10, 9, 7, and 9 rabbits were used in the control, low, mid, and high dose groups, respectively. The reported decrease in body weight gain is also of questionable toxicological significance...

The previous review further commented on the microbial mutagenicity assays as follows:

> ..., the studies are not acceptable since replicate plate counts used to determine reported means were not included. In addition, only one dose was used for the semiquantitative assay.

With respect to the dominant lethal assay, the previous review stated:

> ...the test mice were not mated over a period long enough to fully evaluate spermatogenesis. The authors noted a decrease in the "implantation rate" during the second and third mating weeks for the 100 mg/kg dosed group. A decreased implantation rate was also observed in the females mated with males given the 10 mg/kg dose i. p. during the fourth mating week. These decreases were attributed by the investigators to the decrease in pregnancy rate which they associated with the occurrence of overt toxicity (indicated by mortality) in test animals in that group...

B. The Boots Company, PLC Response

A letter dated February 18, 1985, from the Boots Company contains the responses to the comments described in section I. above.

With respect to the rabbit teratology study, the letter states:

> ..., at 10 mg/kg daily,...there was a treatment-related slight effect on bodyweight...based on the following criteria.

i) Three rabbits given 10 mg/kg daily actually lost weight during the treatment period; all other rabbits on test gained weight.

ii) The mean weight gain for the 10 mg/kg daily group was significantly lower than for the control group ($P<0.05$).

iii) The mean bodyweight for the 10 mg/kg daily group on days 10 and 13 was significantly lower than the equivalent control value ($P<0.05$), and the mean values on days 16 and 17 were very close to significance.

The number of pregnant animals per group is a little lower than would normally be expected by present-days standards...The study is considered valid because there was maternal toxicity at the highest dose level but no indication of a teratogenic response. This judgement was able to be substantiated by the fact that there was a sufficient number of pregnant does in the groups to permit statistical analysis of the data. Indeed, at the time that the study was conducted (March, 1965), the only appropriate guideline was the President's Science Advisory Committee on the Use of Pesticides; this advised that '...numbers of pregnant animals and offspring must be adequate for statistical significance'.

The missing mutagenicity data was provided as an attachment to the letter and is discussed in Section II., below (see Appendix also).

The response to comments on a dominant lethal assay were as follows:

...The philosophy of testing only meiotic and post-meiotic stages (i. e., weeks 1 to 4 after dosing male mice) because no clastogen has been shown to produce exclusively pre-meiotic effects...The positive control data obtained with METEPA in this study clearly show that dominant lethality peaks at about the second week of mating and is falling by the fourth week. Consequently, it is extremely unlikely that the treatment regimen would have failed to detect a mutagenic effect.

Comment was also made about the lower number of implantations...It is noteworthy that none of the

> bronopol-treated groups showed any increase in the number of intrauterine deaths and, as clastogenic effects are typically manifest by dead implants in this assay, it is unlikely that a pre-implantation loss is a sequal to any genetic effects of bronopol. If indeed there was a treatment-related response on litter size, it was almost certainly a consequence of non-genetic toxic effects of bronopol.

II. Discussion

A. Teratology study in rabbits

In addition to comments quoted in Section I above, the previous review also stated:

> ...at day 17 of gestation (last day of dosing), the group mean maternal body weights, as determined from the reported individual weights, were 3.930, 4.097, 4.100, and 4.077 kg for the control, low, mid, and high dose groups, respectively. The body weight gain effect is minor when compared with body weights themselves, and such a comparison implies that the significance of the decrease in body weight gain is not necessarily (toxicologi-cal)...

The statement that numbers of pregnant animals and offspring should be adequate for statistical significance may not be appropriate. A teratology study makes use of several types of observations for which sample sizes of 7 to 10 are not sufficient for statistical significance within the context of variability generally found in rabbits.

Although the study was done according to standards of 20 years ago, it must still be regarded as limited or supplementary. It is not considered, as the letter suggests, to be invalid.

B. Mutagenicity (Bacterial assays)

The previous Toxicology Branch review (Gardner, 1985) apparently misinterpreted punctuation in the tables reporting results of

the microbial assays (See resubmitted table in Appendix I and compare with the table on page I-16 in Appendix II below).

Although only two replicate plates were used per dose level, the absence of growth at the 200 and 1000 ug/plate doses and the intended use of bronopol as a bactericide indicates that the test substance is unlikely to be mutagenic in bacteria.

C. Dominant lethal assay

As the Boots Company letter suggests, the pre-implantation losses are indicative of other non-genetic toxicity, and evaluations during weeks 1 to 4 following dosing will detect meiotic and post-meiotic effects. However, dominant lethal studies are also considered useful in detecting effects on spermatogenesis. On that basis, a protocol which mates treated males over a period long enough to cover the spermatogenic cycle is recommended. Since the pre-implantation losses suggest a potential for germ cell effects, the dominant lethal study can be considered acceptable.

D. Data gaps

As stated in the previous review, the Toxicology Branch reserves comment on the need for additional data until exposure data associated with the proposed textile and industrial uses become available and have been reviewed by the Exposure Assessment Branch.

References

Gardner, R., Memorandum dated December 3, 1984. To: John H. Lee, Registration Division. Subject: Review of acute toxicity studies on Bronacide 10A and additional information on previously submitted studies. EPA Reg. Nos. 47374-E and 33753R. Tox. Chem. No. 116A) stated:

APPENDIX

Resubmitted mutagenicity data table and
previous Data Evaluation Record
(see Section II. B.)

TABLE 1

Diffusion head tests for in vitro mutagenicity using base-pair substitution and frameshift detecting microbial indicators

Compounds tested as 1% solutions in water unless otherwise indicated

Compound	*Escherichia coli*				*Salmonella typhimurium*				
	WP2	UvrA	CM561	CM611	G46	TA1535	TA1536	TA1537	TA1538
Bronopol	–	–	–	–	–	–	–	–	–
N-methyl-N'-nitro-N-nitrosoguanidine (in DMSO)	++	++	+	+	+++	+++	ND	ND	ND
9-aminoacridine hydrochloride	ND	ND	ND	ND	ND	ND	–	+	–
2,7-diaminofluorene (in DMSO)	ND	ND	ND	ND	ND	ND	–	–	+
Water	–	–	–	–	–	–	–	–	–
Dimethyl sulphoxide (DMSO)	–	–	–	–	–	–	–	–	–

Approximate numbers of colonies in revertant zone:

+++ = More than 1000
++ = More than 100
+ = 50-100
± = Less than 50
– = No revertant zone
ND = Not done

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TABLE 2

In vitro semi-quantitative assays for mutagenicity using Salmonella typhimurium indicator strains, with and without the addition of a rat liver microsomal fraction (S-9)

Compounds in water tested at 1000 ug/plate unless otherwise indicated

Compound	Revertant colonies per plate								
	with microsomes				without microsomes				
	TA1535	TA1536	TA1537	TA1538	G46	TA1535	TA1536	TA1537	TA1538
Bronopol 40 ug	10,5	0,0	6,5	25,18	0,0	0,0	0,0	0,0	0,0
N-methyl-N'-nitro-N-nitrosoguanidine (in DMSO)	5000+	ND	ND	ND	5000+	5000+	ND	ND	ND
9-aminoacridine hydrochloride 200 ug	ND	0,0	1000+	40,35	ND	ND	0,0	1000+	20,22
2-aminofluorene (in DMSO)	ND	0,0	8,10	200+	ND	ND	0,0	10,4	29,30
Water	4,9	0,0	9,8	12,20	4,5	11,12	0,0	12,14	32,40
Dimethyl sulphoxide (DMSO)	5,7	0,0	8,3	6,8	3,7	12,18	0,0	8,4	6,5

ND = Not done

004431

TABLE 3

Host-mediated assay in mice; Salmonella typhimurium strain TA1530 as indicator organism

Compound	Frequency and dose mg/kg	Mutation Frequency (Mf)	Mutation Index (Mf test/Mf control)
Bronopol, oral in water	6 x 50	0.5×10^{-8}	Less than 1.0
	6 x 25	0.7×10^{-8}	Less than 1.0
	6 x 12.5	0.7×10^{-8}	Less than 1.0
Dimethyl nitrosoamine i.m. in saline	2 x 330	21.5×10^{-8}	23.9
Control		0.9×10^{-8}	1.0

DATA EVALUATION RECORD

Citation: Everest, R. P. September 12, 1974. Mutagenicity testing by means of in vitro microbial test, the host mediated assay, and the dominant lethal assay in mice. Unpublished report. Submitted by Inolex Chemical Company. EPA Acc. No. 247199. Resubmitted under EPA Acc. No. 252632.

Materials and Methods

Test substances: Technical grade bronopol was used. Reference mutagens used in this study included 9-aminoacridine, 5-bromouracil, proflavin sulfate, B-naphthylene, MNNG, benz(a)pyrene, 2-aminofluorene, 2,7-diaminofluorene, cyclophosphamide, hydrazine, captan, B-propiolactone, EMS, MMS, METEPA, trimethyl phosphate, quinacrine, and dimethyl sulfoxice.

Test species: Salmonella typhimurium strains TA1535, TA1536, TA1537, and TA1538 were used. Samples of each bacterial strain were grown overnight at 37°C in nutrient broth, and subsequently centrifuged and resuspended in 0.9% saline. One-half ml of each culture was added to 10 ml molten saline agar (45° C) along with a sterile solution of 0.5 ml histidine and biotin (1 mM/ml). This molten agar mixture was layered over Vogel-Bonner minimal agar in 2 ml aliquots.

In assays with metabolic activation, a 0.4 ml aliquot of the test strain in saline (see previous paragraph) was mixet with 0.4 ml of the test substance or reference mutagen solution. This mixture was added to 1.5 ml of the S9 mix (see below), and that mixrure was added to 6 ml molten agar. Two ml aliquots of the agar mixture were layered onto Vogel-Bonner plates as described above.

Esherichia coli strains WP2, UvrA, CM561, and CM611 were used along with the S. typhimurium strains mentioned above in spot tests. The E. coli are grown in nutrient broth overnight and resuspended in phophate buffer (0.67 M, pH 7.0) as described above for S. typhimurium. A 0.5 ml aliquot of inoculum is mixed with 9 ml molton salt agar (0.6% agar, 0.6% NaCl) which is layered onto broth supplemented Davis-Mingoli minimal agar plates. The necessary tryptophan is provided in the nutrient broth supplement.

Preparation of Rat Liver S9 mix: Male Boots Wistar arts were given water containing 0.1% phenobarbitone ad libidum. After one week the animals were sacrificed. The livers were

removed, homogenized 0.15 M KCl, and centrifuged at 9000 x g. The supernatant was decanted and retained. One part of the liver 9000 xg supernatant was mixed with nine parts of a cofactor solution to make the S9 mix. The cofactor solution was made up of 70 ml phosphate buffer (100mM, pH 7.4) containing 31 mg TPN, 18 mg Glucose-6-phosphate, 16 mg $MgCl_0$ and 25 mg KCl. Seven ml of the cofactor solution was added to 3 ml of the

Experimental procedure---spot tests: The test substance as a 0.1% solution in water in fishspine beads was placed on each agar plate for diffusion into the medium. These plates were incubated for 3 days at 37° C after inoculation with tester strains. The known mutagens were evaluated as 1% solutions in water or DMSO.

Experimental procedure---semi-quantitative assay: For these assays 0.5 ml of the test solution was added to the molten agar layered onto the Vogel-Bonner plates (see above). The Bronopol solution tested contained 40 ug/plate. All test solutions were evaluated for sterility, and a vehicle control and negative control were assayed. Each test solution was evaluated on 3 plates.

Experimental procedure---host mediated assay: Test species: The tester strain of S. typhimurium used in this assay was TA1530. Female OLAC mice were used as the host.

Groups of 5 female mice were given 6 daily doses of 12.5, 25, or 50 mg test substance per kg body weight in water by oral intubation. On the seventh day the animals received the same dose along with an intraperitoneal injection of the test strain of bacteria. A fourth group received a doses of 330 mg dimethyl nitrosamine by intramuscular injection on the day before i. p. injection of the bacteria. A second dose of the positive control substance was administered on the day of the bacterial injection.

Three hours after injection of the bacteria, the organisms were recovered by intraperitoneal aspiration, and triplicate plates were made for determination of the number of organisms recovered as well as the number of revertants. The plating procedures were the same as those used for the non-activated assays described above.

Experimental procedure---dominant lethal assay: Groups of 10 male mice were given single daily oral doses of 20 or 100 mg test substance per kg body weight. Doses were administered for 6 consecutive days prior to mating. Female mice received

no test substance. Each male was housed with 3 females for one week, and the females were maintained for 14 days beyond the midpoint of the mating period. At that time they were sacrificed and examined. The three females were replaced each week for the four consecutive weeks following dosing.

Gross necropsies were performed on the females to determine the pregnancy rate, number of live implants, and the number of dead implants.

Reported Results

Spot test: According to the report, a 1% aqueous solution of bronopol was not mutagenic in the E. coli or S. typhimurium strains tested. The positive control substance (MNNG) was mutagenic under the test conditions.

Semi-quantitative assay: The average number of revertants observed on the replicate plates for each compound were reported as follows:

Compound	G46	TA1535	TA1536	TA1537	TA1538
		With S-9 mix			
Bronopol	-	10.5	0	6.5	25.18
MNNG	-	>5000	-	-	-
9-aminoacridine HCl	-	-	0	>1000	40.35
2-aminoflourene	-	-	0	8.1	>200
Water	-	4.9	0	9.8	12.20
DMSO	-	5.7	0	8.3	6.8
		Without S-9 mix			
Bronopol	0	0	0	0	0
MNNG	>5000	>5000	-	-	-
9-aminoacridine HCl	-	-	0	>1000	20.22
2-aminoflourene	-	-	0	10.4	29.30
water	4.5	11.12	0	12.14	32.40
DMSO	3.7	12.18	0	8.4	6.5

Host mediated assay: The reported mutation frequencies are as follows:

Dose (mg/kg)	Frequency
0	0.9×10^{-8}
12.5	0.5×10^{-9}
25	0.7×10^{-8}
50	0.7×10^{-8}
330*	21.5×10^{-8}

*Positive control (dimethyl nitrosamine)

Dominant lethal assay: Cummulative mortality in treated males was reported as follows:

Mating week	Intraperitoneal		Oral doses (mg/kg)		
	10	METEPA*	0	20	100
1	0	0	0	0	1
2	0	0	0	0	4
3	1	0	0	0	4
4	1	0	0	0	4

*Positive control (25 mg/kg, i.p.)

The pregnancy rate (%) was reported as follows:

Mating week	Intraperitoneal		Oral doses (mg/kg)		
	10	METEPA*	0	20	100
1	33	83	70	67	44
2	45	67	57	73	39
3	48	70	55	80	50
4	44	56	56	76	56

The mean number of live implants per dam were reported as follows:

Mating week	Intraperitoneal		Oral doses (mg/kg)		
	10	METEPA*	0	20	100
1	9.2	7.4	9.4	9.8	8.5
2	8.1	4.2	9.6	8.4	6.7
3	9.9	6.9	9.4	9.3	6.0
4	8.7	8.7	10.4	10.6	9.6

The mean number of dead implants was reported as follows:

Mating week	Intraperitoneal 10	Intraperitoneal METEPA*	Oral doses (mg/kg) 0	Oral doses (mg/kg) 20	Oral doses (mg/kg) 100
1	0.7	3.5	0.6	1.0	0.5
2	1.2	3.9	0.5	0.9	0.4
3	0.3	3.1	1.0	0.5	0.2
4	0.2	1.2	0.7	0.7	0.5

The mean total implantations per dam were reported as follows:

Mating week	Intraperitoneal 10	Intraperitoneal METEPA*	Oral doses (mg/kg) 0	Oral doses (mg/kg) 20	Oral doses (mg/kg) 100
1	9.9	10.9	10.0	10.8	9.0
2	9.3	8.1	10.1	9.3	7.1
3	10.2	10.0	10.4	9.8	6.2
4	8.9	9.9	10.0	11.2	10.1

Discussion and Conclusions

The bacterial assays indicated that, under test conditions, the test substance did not cause mutations. However, the studies are not acceptable since replicate plate counts used to determine reported means were not included. In addition, only one dose was used for the semiquantitative assay.

The dominant lethal assay is also unacceptable because the test mice were not mated over a period long enough to fully evaluate spermatogenesis. The authors noted a decrease in the "implantation rate" during the second and third mating weeks for the 100 mg/kg dosed group. A decreased implantation rate was also observed in the females mated with males given the 10 mg/kg dose i. p. during the fourth mating week. These decreases were attributed by the investigators to the decrease in pregnancy rate which they associated with the occurrence of overt toxicity (indicated by mortality) in test animals in that group (see Reported Results section above).