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

009726

OFFICE OF
PESTICIDES AND TOXIC
SUBSTANCES

OCT 22 1991

MEMORANDUM

SUBJECT: ID # 009001. Lindane (technical). Evaluation of a Dominant Lethal Test in rat and a Mammalian Cultured Cell Gene Mutation Assay (anaerobic conditions) to support reregistration of Lindane.

Tox. Chem. No.: 527
Shaughnessey No.: 009001

Dominant Lethal Test Project No.: 1-2397
Submission No.: S402657

Gene Mutation Assay Project No.: 1-2398
Submission No.: S402653

TO: Larry Schnaubelt, PM Team 72 Product Manager
Robert Richards, PM Team 72 Reviewer
Special Review and Reregistration Division (H7508W)

FROM: Linnea J. Hansen, Ph.D.
Toxicology Branch I, Section IV
Health Effects Division (H7509C)
Linnea J. Hansen 10/21/91

THRU: Marion P. Copley, D.V.M., D.A.B.T.
Section Head, Toxicology Branch I, Section IV
Health Effects Division (H7509C)
Marion Copley 10/17/91

CONCLUSIONS:

The two studies submitted for review for the reregistration of lindane did not satisfy the guideline requirements and are not acceptable for regulatory purposes.

Dominant Lethal Test: No evidence of increased dominant lethal effects was observed up to 10 mg/kg/day in rats; however, high dose may not have been adequate as evidenced by very small weight loss in males. There were also an insufficient number of males and insufficient pregnancies per treatment period, and no positive controls.

Core-classification: Unacceptable

Mammalian Cultured Cell Gene Mutation Assay (anaerobic conditions): No evidence of mutagenicity was observed up to cytotoxic doses of lindane (up to 50 ug/ml, non-activated conditions; up to 500 ug/ml, S-9 activated conditions) under anaerobic culture conditions. However, there was no evidence that the cells were maintained under strict anaerobic conditions and that anaerobic metabolic pathways were induced in this study.

Core-classification: Unacceptable

ACTION REQUESTED:

Toxicology Branch I received copies of two mutagenicity studies from SRRD submitted by Reed and Carnrick Research Institute (dominant lethal test, study no. 405-107; Hazleton Labs) and Centre International d'Etude du Lindane (mammalian cultured cell gene mutation, anaerobic conditions, study no. 540-VT21-b; Institute of Toxicology, Univ. of Mainz) to fulfill data requirements for reregistration of lindane.

The dominant lethal test is one of the mutagenicity tests that may satisfy the current guidelines for registration of a pesticide. The mammalian cultured cell gene mutation study reviewed here was requested by the EPA as part of a Data Call-In to determine whether anaerobic metabolites of lindane are mutagenic in this assay. The mammalian cultured cell gene mutation assay is also one of the guideline studies and a previously submitted, acceptable mammalian cell culture gene mutation assay for lindane found no evidence of mutagenicity under normal aerobic conditions.

Since OPP is currently in the process of revising its mutagenicity testing guidelines, the registrants may chose to follow either current or new guidelines until the new are operating. Under the current guidelines, the studies may be repeated and submitted for review. Under the revised guidelines, Salmonella reverse mutation, in vivo cytogenetics (rodent bone marrow: metaphase analysis or micronucleus assay), and mammalian cultured cell forward gene mutation assay (mouse lymphoma L5178Y cells, TK locus; CHO or V79 cells, HGPRT locus plus in vitro clastogenicity test; or CHO strain AS52, XGPRT locus) are required as the first tier of studies. Under the future guidelines, the dominant lethal test would not necessarily be required (unless requested if any of the first tier tests give positive results) but the anaerobic mammalian cultured cell gene mutation study would still be required as part of the separate Data Call-In.

Guideline Series 84: **MUTAGENICITY**

Reviewed by: Linnea J. Hansen, Ph.D. 9/24/91
Section IV, Tox Branch I (H7509C)
Secondary reviewer: Irving Mauer, Ph.D. 09/23/91 10/11/91
Tox Branch I (H7509C)

DATA EVALUATION REPORT

CHEMICAL: Lindane TOX. CHEM. NO.: 527

SHAUGHNESSEY NO.: 009001

STUDY TYPE: Dominant Lethal Test in Rodent

MRID NUMBER: 62657

SYNONYMS/CAS No.: Gamma benzene hexachloride, Forlin, Gamaphex, Gammex, Isotox, Lindagam, Lin-O-Sol, Novigam, Silvanol, gamma BHC, gamma HCH; CAS # 58-89-9

SPONSOR: Reed and Carnrick Research Institute

TESTING FACILITY: Hazleton Laboratories America, Inc.

TITLE OF REPORT: Dominant Lethal Study in Rats

AUTHORS: Frederick E. Reno and Medford (only last name given)

STUDY NUMBER: 405-107

REPORT ISSUED: November 12, 1976

CONCLUSION(S) - Executive Summary:

Doses administered: 0, 1, 3 and 10 mg/kg body wt/day, administered in corn oil to male rats by subcutaneous injection; five consecutive daily doses per week for ten weeks. Very slight weight loss (less than 4%; not statistically significant) observed in males at mid and high doses.

No evidence of increased dominant lethal effects related to compound administration. Increase in early deaths among Week 1 females not dose related, not repeatable and also in part due to low values in controls and to low pregnancy rates.

Study deficiencies: No positive controls, no purity value for lindane (although lot number is specified), very small weight

losses used as criteria for toxicity (and no evidence of target organ toxicity, eg slightly reduced fertility), no ages of animals given, insufficient number of males tested and low pregnancy rates (insufficient pregnancies per treatment periods) for test groups. This study was therefore not conducted satisfactorily and is not acceptable for regulatory purposes.

Classification: Unacceptable

A signed Quality Assurance Statement was not included.

MATERIALS

1. Test Material: Lindane. White granular material
   Lot # 36346
   Purity: not specified
   Contaminants: not specified (no CBI appendix)

2. Control Materials:
   Negative (vehicle): corn oil
   administered: 1 ml/kg body wt, subcutaneous injection
   Positive: None

3. Test Animals: Species Rat Strain Sprague-Dawley Age not given
   Weights of males and females not specified.
   Source: Charles River Laboratories, Wilmington, MA

5. Test compound concentrations used: 1, 3 and 10 mg/kg body wt.

B. TEST PERFORMANCE

1. Dose Level Determination:

   No preliminary tests were performed to determine dose level and the rationale for the level chosen was not provided, although slight weight loss was observed in mid and high dose treated males.

2. Treatment:

   10 male rats were assigned to each of 4 groups (control and 3 test compound doses). Test solutions were administered daily by subcutaneous injection as described above (A-5) for 5 days per week for a total of 10 weeks. Animals were observed daily for mortality and clinical symptoms. Body weights of males were recorded weekly.

3. Mating:

Immediately after the last treatment males were housed with 2 virgin females per male for 1 week. After the first week females were removed and two new females per male mated for the second week. Evidence of mating was confirmed by vaginal smears.

4. Caesarian Procedures:

Females were sacrificed by chloroform euthanasia either 14 days following mating evidence or, lacking mating evidence, 14 days after removal from males. Uteri of sacrificed females were examined for live and dead implantations. Any abnormalities observed were recorded. Males were also sacrificed and a gross pathological exam performed.

5. Statistical Analysis:

The chi-squared method was used to analyze pregnancy rate, implantation efficiency, incidence of dead implants and incidence of live implants. Mean body weight gains of treated males were compared to controls by the F-test (analysis of variance) and the Student's T-test. Variance was stabilized when necessary by modification of the Student's t test (t') and Cochran's approximation.

C. RESULTS:

1. Clinical Symptoms and Mortality:

No clinical symptoms related to treatment were observed in any test animals. A few incidental findings that were unrelated to treatment included rough fur coats, hunched posture, urine stains, chromodacryorrhea and soft feces. A small nodule was observed on the dorsal surface of a mid-dose and a control male during the course of treatment.

2. Body Weight Gain:

Body weight gains for males were slightly lower in mid and high dose animals than controls after Week 2. These differences were not statistically significant and means of treated animal weights were less than 4% lower than control weights. Many of the body weight values were illegible due to poor photocopying (see Appendix).

3. Pregnancy Frequency:

There did not appear to be a treatment-related effect on

pregnancy rate in this study. Pregnancy rates are shown below in Table 1 as % of all pregnant females/number mated. During Week 1, pregnancy rates were very low for all groups (45-60%). Values for Week 2 were higher but still relatively low (65-85%). No statistically significant differences were observed between controls and treated groups during either week.

TABLE 1: MEAN PREGNANCY RATE[1]

Dose (mg/kg)	Pregnancy Rate (%) Week 1	Week 2
0 mg/kg	60.0	70.0
1 mg/kg	45.0	85.0
3 mg/kg	50.0	65.0
10 mg/kg	50.0	80.0

[1] Taken from Table 1 of study

Table 2 below presents the number of pregnant females per test group per week. The numbers here are insufficient to allow meaningful analysis of dominant lethal effects.

TABLE 2: TOTAL NUMBER OF PREGNANT DAMS/WEEK[1]

Dose (mg/kg)	Week 1	Week 2
0 mg/kg	12	14
1 mg/kg	9	17
3 mg/kg	10	13
10 mg/kg	10	16

[1] Taken from Appendix B of the study

4. Caesarian Data:

Total implantations/pregnancy:
Total implantations per pregnancy are shown below in Table 3. No treatment-related effect on total implantations/pregnancy were observed. Values ranged from a low of 89.6 in Group 4, Week 2 to 97.4 in Group 4, Week 4 and Group 2, Week 3.

TABLE 3: MEAN IMPLANTATION EFFICIENCY[1]

Dose (mg/kg)	Implantation Efficiency (%) Week 1	Week 2
0 mg/kg	93.6	91.2
1 mg/kg	94.1	90.3
3 mg/kg	89.8	97.4
10 mg/kg	97.4	89.6

[1] Taken from Table 1 of study

Dead Implant Incidence:

The incidence of dead implants per total implants is shown below in Table 4. Treated animals in Week 1 showed some increase over controls.

TABLE 4: INCIDENCE OF DEAD IMPLANTS

Dose (mg/kg)	Incidence of Dead Implants (%) Week 1	Week 2
0 mg/kg	3.1	9.1
1 mg/kg	9.4*	10.3
3 mg/kg	6.4	9.8
10 mg/kg	6.6	9.8

[1] Taken from Table 1 of study
* Statistically significant ($p < 0.05$)

D. REVIEWER'S DISCUSSION/CONCLUSIONS:

The authors concluded from the results of this study that Lindane did not cause an increase in dead implants in rats under the conditions of this study. Increases in dead implants during Week 1 were not dose-related and not repeatable during Week 2. The control value was also somewhat low during Week 1 relative to Week 2 and to the test compound groups, and numbers of pregnant females were generally low.

There are a number of deficiencies in this study:
1) No positive control was done.
2) Very small weight loss (< 4%, not statistically significant) used as criteria for toxicity. Also no evidence of toxicity to target organ (seminiferous tubules).
3) The purity of the test compound was not specified (although lot # was given).

7

4) Animal age during the study was not given.
5) Insufficient numbers of pregnant dams were available for meaningful evaluation. In some of the test groups (including controls), a high percentage (up to 45-50%) of the females did not become pregnant, compromising the number of fetuses available for evaluation per treatment period. More males should have been tested to increase the numbers of litters.
6) No rationale given for subcutaneous administration and 5 day dosing regime.

The copy of the study received for review was barely legible to illegible in places and made evaluation of the data, body weight in particular, difficult.

The deficiencies listed above are sufficient to invalidate this study. It is not considered to have been properly conducted and is not acceptable for regulatory purposes.

APPENDIX

- 9 -

Appendix [illegible]

Individual and Mean Body Weight and Weight Gain Values (grams)
Dominant Lethal Study of Lindane in Rats

Group mg/kg/day	Animal No.	Week of Treatment 0	1	2	3	4	5	6	7	8	9	10	Weight Gain
1 (0)	26611	256	302	343	380	396	429	456	467	497	521	[illegible]	[illegible]
	26612	279	319	369	[illegible]	431	473	500	510	542	570	[illegible]	[illegible]
	26613	297	335	373	[illegible]	435	440	486	500	525	552	[illegible]	[illegible]
	26614	267	292	319	[illegible]	384	[illegible]	459	454	481	572	517	[illegible]
	26615	278	[illegible]	361	394	414	451	491	502	[illegible]	551	[illegible]	[illegible]
	26616	283	317	[illegible]	384	408	435	466	476	[illegible]	519	[illegible]	[illegible]
	26617	277	307	[illegible]	[illegible]	413	452	475	496	[illegible]	517	[illegible]	[illegible]
	26618	263	289	329	[illegible]	[illegible]	[illegible]	427	433	452	[illegible]	[illegible]	[illegible]
	26619	259	307	339	[illegible]	391	422	448	459	487	504	[illegible]	[illegible]
	26620	289	314	[illegible]	[illegible]	423	448	472	491	519	539	[illegible]	[illegible]
	Total	2767	3105	3462	[illegible]	[illegible]	[illegible]	4680	4870	[illegible]	5250	[illegible]	[illegible]
	N	10	10	10	10	10	10	10	10	10	10	[illegible]	10
	Mean	276.7	310.5	346.2	[illegible]	[illegible]	[illegible]	[illegible]	489.0	[illegible]	[illegible]	[illegible]	[illegible]
	S.D.	[illegible]	[illegible]	17.7	[illegible]	[illegible]	22.0	22.6	[illegible]	27.2	30.6	[illegible]	[illegible]
2 (1)	26631	256	265	[illegible]	[illegible]	[illegible]	426	451	477	505	[illegible]	[illegible]	[illegible]
	26632	279	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	486	507	[illegible]	[illegible]	[illegible]	[illegible]
	26633	285	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	490	[illegible]	[illegible]	565	[illegible]	[illegible]
	26634	266	293	[illegible]	[illegible]	[illegible]	391	410	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	26635	275	320	[illegible]	[illegible]	[illegible]	[illegible]	500	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	26636	243	327	365	[illegible]	[illegible]	[illegible]	492	503	[illegible]	[illegible]	[illegible]	[illegible]
	26637	277	318	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	461	[illegible]	[illegible]	[illegible]	[illegible]
	26638	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	26639	259	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	26640	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	510	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	Total	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	4870	[illegible]	[illegible]	[illegible]	[illegible]
	N	10	10	10	[illegible]	[illegible]	[illegible]	10	10	10	10	10	[illegible]
	Mean	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	S.D.	9.8	17.4	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	31.0	[illegible]	[illegible]	[illegible]	[illegible]
3 (3)	26641	256	282	305	332	356	[illegible]	392	413	425	[illegible]	470	[illegible]
	26642	279	318	356	[illegible]	[illegible]	[illegible]	479	499	511	[illegible]	572	[illegible]
	26643	297	332	379	[illegible]	[illegible]	[illegible]	425	417	[illegible]	[illegible]	[illegible]	[illegible]
	26644	265	333	359	[illegible]	[illegible]	[illegible]	462	491	521	535	561	[illegible]
	26645	275	317	355	[illegible]	423	[illegible]	459	515	[illegible]	547	[illegible]	[illegible]
	26646	282	324	362	438	438	487	506	514	546	[illegible]	[illegible]	[illegible]
	26647	277	307	331	[illegible]	[illegible]	426	436	451	478	537	[illegible]	[illegible]
	26648	273	299	335	[illegible]	[illegible]	422	[illegible]	[illegible]	[illegible]	512	[illegible]	[illegible]
	26649	249	334	346	[illegible]	[illegible]	432	463	485	510	531	[illegible]	[illegible]
	26650	287	336	329	[illegible]	356	429	439	459	452	[illegible]	[illegible]	[illegible]
	Total	2767	3076	3387	3733	[illegible]	4299	4529	4711	[illegible]	5181	[illegible]	[illegible]
	N	10	10	10	10	10	10	10	10	10	10	10	10
	Mean	276.7	307.6	338.7	373.3	[illegible]	429.9	452.9	471.1	[illegible]	518.0	[illegible]	[illegible]
	S.D.	9.9	11.9	16.9	22.5	[illegible]	33.1	33.2	36.3	43.4	45.0	39.4	[illegible]
4 (10)	26651	255	287	317	343	379	410	443	468	486	[illegible]	527	[illegible]
	26652	279	306	343	378	[illegible]	430	[illegible]	458	483	[illegible]	525	[illegible]
	26653	269	321	355	[illegible]	[illegible]	471	[illegible]	485	514	433	[illegible]	[illegible]
	26654	267	[illegible]	[illegible]	333	[illegible]	385	419	426	448	[illegible]	[illegible]	[illegible]
	26655	272	309	342	363	[illegible]	429	452	472	497	[illegible]	510	[illegible]
	26656	285	336	378	410	[illegible]	403	478	499	514	536	[illegible]	[illegible]
	26657	277	303	339	364	353	410	436	460	452	471	[illegible]	[illegible]
	26658	268	312	355	375	424	451	452	500	529	541	563	[illegible]
	26659[a]	268	303	326	336	370	404	433	[illegible]	467	533		
	26660	282	323	363	394	410	439	460	469	500	510	526	[illegible]
	Total	2747	3082	3413	3752	3936	4293	4530	4668	4892	5396	4707	[illegible]
	N	10	10	10	10	10	10	10	10	10	10	9	9
	Mean	[illegible]	308.2	341.0	[illegible]	393.6	[illegible]	[illegible]	466.8	489.2	[illegible]	[illegible]	[illegible]
	S.D.	10.7	15.9	21.3	22.2	[illegible]	26.8	25.5	23.9	26.9	26.5	[illegible]	[illegible]

[a]Group 4 animal 26659 was found dead during Week 9 and [illegible] replaced by [illegible] which had been treated concurrently with this group of animals.

008726

Guideline Series 84: **MUTAGENICITY**

Reviewed by: Linnea J. Hansen, Ph.D. Linnea J. Hansen 10/15/91
Section II, Tox Branch I (H7509C)
Secondary reviewer: Irving Mauer, Ph.D. 10/11/91 mr 10/15/91
Tox Branch I (H7509C)

DATA EVALUATION REPORT

CHEMICAL: Lindane TOX. CHEM. NO.: 527

SHAUGHNESSEY NO.: 009001

STUDY TYPE: Mammalian cells in culture gene mutation assay in hamster v79 cells

MRID NUMBER: 144500

SYNONYMS/CAS NO.: Gamma BHC, gamma HCH, Forlin, Gamaphex, Gammex, Isotox, Lin-0-Sol, Novigam, Lindagam, Silvanol; CAS # 58-89-9

SPONSOR: Centre International d'Etude du Lindane (C.I.E.L.), Brussels, Belgium; c/o Dr. F. Pistel, Celamerck, D-6507 Ingelheim am Rhein, FRG

TESTING FACILITY: Institute of Toxicology, University of Mainz, Obere Zahlbacher Strasse 67, D-6500 Mainz, FRG

TITLE OF REPORT: Mammalian Cell (V79) Mutagenicity Test on Lindane using Anaerobic Exposure Conditions

AUTHOR(S): Dr. H.R. Glatt

STUDY NUMBER(S): 540-VT21-b

REPORT ISSUED: October 18, 1985

CONCLUSION(S) - Executive Summary:

Doses tested: Direct (non-activated) mutagenicity - 2.5, 5, 10, 25, 50, 70, 100 and 150 ug/ml; Indirect (S9-activated) mutagenicity - 5, 10, 25, 50, 100, 250 and 500 ug/ml.

No evidence of gene mutation in hamster V79 cells exposed under aerobic and anaerobic conditions up to cytotoxic doses of lindane (above 50 ug/ml in direct mutagenicity tests; above 500 ug/ml in indirect mutagenicity tests) were observed.

Study deficiencies: No experimental verification that

10

anaerobic conditions were established and maintained and that anaerobic metabolic pathways were induced in cells (positive controls for anaerobic mutation rate did not show increased mutation frequency compared to aerobic cultures, culture media not pre-equilibrated with N_2/CO_2, compound added immediately following N_2/CO_2 addition without allowing equilibration time for anaerobic conditions), no monitoring of pH of culture medium during 72 hr incubations, no statistical evaluation of data.
Core Classification: Unacceptable. This study is not acceptable for regulatory purposes.

A signed Quality Assurance Statement was present.

A. MATERIALS

1. Test Material: Lindane, technical. White powder, stored at -20° C in dark; stable to light, heat and dark. Batch # 84044/074 (Celamerck, Ingelheim, Germany) Purity 99.9%
Contaminants: not listed (no CBI Appendix included)
Solvent used: DMSO (60 ul)

2. Control Materials:

Negative: 1) DMSO - solvent for aristolochic acid, BPDE, MNNG, DNP
2) acetone:triethylamine, 1000 v/v - solvent for DBPA
Solvent/final concentration: DMSO/60 ul; acetone:triethylamine/60 ul

Positive:
Non-activated:
1) Aristolochic acid, 1, 2, 5, 10, 20, 50, 100 ug/ml: compound has increased mutagenicity under anaerobic conditions in bacteria
2) Anti-benzo(a)pyrene-7,8-diol 9,10-oxide (BPDE), 0.1 ug/ml
3) 1,8 dinitropyrene (DNP), 0.5, 1.0, 2.0 ug/ml: compound has increased mutagenicity under anaerobic conditions in bacteria, poorly mutagenic in mammalian cells
4) N-methyl-N'-nitrosoguanidine (MNNG), 0.5, 1.0 ug/ml
Activated:
1) Benzo(a)pyrene (BP), 50 ug/ml
2) Dimethylnitrosoamine (DMN), 1000 ug/ml

3. Activation: S9 derived from
_X_ Arochlor 1254 _X_ induced _X_ mouse _X_ liver

S9 Mix Composition: Livers from treated mice were

11

homogenized in 3 volumes of ice cold sterile PBS-HEPES (pH and molarity of Hepes not specified) and centrifuged at 9000 x g for 10 minutes. The S9 was stored at -70° C until needed and immediately prior to use a cofactor solution containing 197 mM glucose-6-phosphate, 28 mM NADP, 26 mM NADH and 11 mM NADPH in PBS-HEPES was added 1:3 (v:v) to S9.

4. Test Cells: mammalian cells in culture:
Chinese Hamster V79 cells (derived from lung)

Cells were properly maintained and were routinely checked for Mycoplasma contamination; however, there was no mention of assessment of karyotype stability or cleansing against high spontaneous background.

5. Locus Examined:

__X__ hypoxanthine-guanine-phosphoribosyl transferase (HGPRT)
Selection agent
and concentration: __7 ug/ml__ 6-thioguanine (6-GT)

6. Test compound concentrations used:
Non-activated conditions: 2.5, 5, 10, 25, 50, 70, 100 and 150 ug/ml (concentrations used varied among experiments - see Results and Data Tables)

Activated conditions: 5, 10, 25, 50, 100, 250 and 500 ug/ml

B. TEST PERFORMANCE

1. Cell treatment:
a. Cells exposed to test compound for:
__72__ hours (non-activated) __2__ hours (activated)

b. Cells exposed to positive controls for:
__72__ hours (non-activated) __2__ hours (activated)

c. Cells exposed to negative and/or solvent controls for:
__72__ hours (non-activated) __2__ hours (activated)

d. After washing, cells cultured for __8__ days (expression period) before cell selection

e. After expression, cells cultured for __10-11__ days in selection medium to determine numbers of mutants and for __7-9__ days without selection medium to determine cloning efficiency.

2. Protocol: Several experiments were performed for this study and are outlined below:

12

a) Cytotoxicity Assay: Preliminary cytotoxicity assays were performed to assess cytotoxicity to V79 cells of 1) anaerobic conditions and 2) lindane under anaerobic conditions in the absence and presence of S9. The first experiment evaluated the effect of increasing anaerobic exposure time on cell cloning efficiency. Four cultures per time point were plated at 150 cells in 5 ml DME/5% FCS, allowed to attach, flushed with N_2 and 5% CO_2 (except for the aerobic control) and incubated for 2.5, 3.5, 8.7, 18.7, 24, 48 and 72 hr. Aerobic conditions were reestablished and medium replaced. Cells were maintained for 8 days, fixed and colonies counted counted.

The second cytotoxicity experiment tested increasing concentrations of lindane in the absence and presence of S9. Ten concentrations of lindane from 2.5-500 ug/ml were added and cultures plated in triplicate as described above were incubated anaerobically for 72 hr in the absence of S9 and 2 hr in the presence of S9 1:13 in PBS - Hepes (to minimize cytotoxicity).

b) Mutagenicity of directly active compound under aerobic and anaerobic exposure conditions: Aristolochic acid, a compound known to show enhanced mutagenicity in bacteria under anaerobic anaerobic conditions, was tested in V79 cells under aerobic and anaerobic conditions. 1.5×10^6 cells were seeded in 30 ml DME/5% FCS in 800 ml flasks. 6 flasks were used for each control and test compound concentration. Two days after seeding, cells were treated with DMSO or acetone:triethylamine solvent controls) at 60 ul/ml, aristolochic acid or positive control at the appropriate concentrations. Cultures were flushed with N_2 plus 5% CO_2 and incubated for 3 days. Following the incubation, aerobic conditions were reestablished and cells were passaged and replated at 3 and 6 $\times 10^6$ cells. Cells were assayed for mutation frequency as described in Section B-1.

c) Mutagenicity of lindane in absence and presence of S9 activation: Several experiments testing mutagenicity of lindane on V79 cells under aerobic or anaerobic conditions were performed in the absence and presence of S9 and positive controls. Tests were performed essentially as described above except for S9 activated cultures, which were incubated 2 hr in PBS-Hepes instead of culture media.

3. Criterial for positive mutagenicity test:

The study authors claimed that no satisfactory statistical method was available for analysis of this kind of experiment. The criteria for determination of positive and negative results were as follows: A result was considered

negative if mutation frequency at each treatment concentration increased less than 2-fold or less than 10 x 10^{-6}. A result was considered positive if the mutation frequency increased 5-fold or more over solvent control and was at least 40 x 10^{-6} (for a minimum of 10^6 cells). Values falling in between these criteria were repeated and then were considered positive if both experiments gave at least 2-fold higher frequency than solvent controls and were at least 10 x 10^{-6}.

C. RESULTS:

1. Preliminary cytotoxicity assay:

Results of the cytotoxicity assay of anaerobic conditions on V79 cells are shown in Table 1 of the Appendix, taken directly from the study. Cloning efficiencies for cells exposed to anaerobic conditions for 2.5 - 72 hr were not adversely affected relative to controls. Absolute cloning efficiencies varied from 81% - 90%.

The second cytotoxicity experiment evaluated cloning efficiency of cells treated with lindane under anaerobic conditions in the absence or presence of S9 mix. Cells incubated 72 hr in the absence of S9 showed marked toxicity when treated with more than 10 ug/ml lindane. Cells incubated with 5 and 10 ug/ml lindane had 55% and 50% cloning efficiency, respectively, compared to 70% for controls. Cells incubated 2 hr in the presence of S9 showed marked toxicity at doses of lindane above 150 ug/ml. Cloning efficiencies ranged from 47 - 73% up to 150 ug/ml and did not appear to decrease in a dose-related manner within this dose range.

2. Mutagenicity assay:

Results from the mutagenicity assays are presented in Tables 2-4 of the Appendix, taken directly from the study.

Effect of anaerobic conditions on mutation frequency of aristolochic acid: Table 2 in the Appendix presents data from the "positive control" for aerobic and anaerobic mutation frequencies. Appropriate dose range was first determined to be 1 - 20 ug/ml (Experiment M52), followed by a second experiment testing this range (Experiment M 58). A dose-related increase in mutation frequency was observed under aerobic and anaerobic contitions but mutation frequencies were not higher in anaerobic incubations. Cloning efficiencies ranged from 41% -81% and did not decrease with increased dose in the non-cytotoxic dose range.

Direct mutagenicity tests of lindane on V79 cells:

Results of direct mutagenicity tests are shown in Table 3 of the Appendix. Several separate experiments were performed. Experiment 1 (M67) tested mutagenicity of lindane under anaerobic conditions at 7 concentrations between 5 - 150 ug/ml. Doses above 50 ug/ml were too cytotoxic to analyze. Lindane at 5 ug/ml gave an increased mutation frequency of 30.4×10^{-6}, compared to an average of 5.6×10^{-6} for controls (5.4-fold increase over solvent controls), but mutation frequencies at higher doses (10, 25, 50 ug/ml) were no different than solvent control values. BPDE gave a positive mutagenic response. 1,8 dinitropyrene produced a very marginal increase in mutation frequency as expected, but there was no dose-response and no difference between aerobic and anaerobic mutation frequencies.

Based on these results, lindane was tested again in Experiments 2 - 4 (M 72, 76, 95) at concentrations between 2.5 - 10 ug/ml. Mutation frequencies for lindane at 5 ug/ml in Experiment 2 were similarly increased but solvent control values were also elevated in this experiment (mean 13.8). Lindane did not cause increased mutation frequency in V79 cells at any concentration in Experiments 3 and 4. The study authors concluded that since the increase observed initially was not reproducible, lindane did not increase the mutation frequency in V79 cells under either anaerobic or aerobic culture conditions.

S9 activated mutagenicity tests of lindane of V79 cells: Results of these experiments are shown in Table 4 of the Appendix. Four experiments (M68, 73, 97 and 105) tested lindane between 5 and 500 ug/ml. Cloning efficiencies varied between 21% - 119% (excepting a few non-viable cultures) and did not appear to be related to dose. Lindane did not appear to be mutagenic at any dose in these experiments. Experiment 2 showed slightly increased mutation frequency at 25 and 100 ug/ml (42.1 and 37.7×10^{-6}, respectively) but these were not reproducible in any of the other experiments. DMN and BP-treated cultures showed enhanced mutation frequencies that varied from experiment to experiment.

C. DISCUSSION/CONCLUSIONS:

There was no evidence in this study that lindane was mutagenic to V79 cells under anaerobic conditions. However, a number of experimental deficiencies were noted:

1) Establishment of anaerobic conditions. The study authors need to characterize their anaerobic culture system more thoroughly prior to initiation of mutagenicity tests. There was insufficient evidence to prove that anaerobic conditions

were maintained in the cell cultures during incubations and no evidence of induction of anaerobic metabolic pathways in the cells. The author did not state clearly that culture media were deaerated prior to incubation and there was also no equilibration period allowed between addition of N_2/CO_2 and addition of test compound. This is particularly important for the S9 incubation, which was only 2 hr long. Cultures treated with aristolochic acid, a compound meant to serve as a positive control under anaerobic conditions, did not show any difference between aerated and deaerated cultures. It has been previously shown that aristolochic acid has enhanced mutagenicity in bacteria under anaerobic conditions, but its use here as a positive control is questionable since it has not been demonstrated in mammalian cells.

2) Conditions of S9 incubation. Cells treated with S9 were incubated in the presence of PBS-Hepes instead of culture media minus serum. This introduced another variable into the interpretation of the data. It is also not clear how completely anaerobic conditions could be established and whether sufficient anaerobic metabolism could proceed within the two-hour incubation. No positive or negative controls were incubated in the absence of S9 in the S9-activated experiments to determine integrity of S9 preparations.

3) Cell culture: Plating dilutions were quite variable, indicating growth rates for the cells were not the same under various conditions. There was also no mention of pH monitoring of culture medium during the 72 hr incubations.

4) Statistical analyses. Although criteria for positive and negative results were established in the methods section of the study, no statistical significance (or standard deviations) were calculated for the data and the rationale for criteria used to determine positive and negative results was not clearly explained. Current guidelines require data analysis to include statistical treatment.

While this study supports previous results that lindane does not cause increased mutation frequency under normal, aerobic conditions in V79 cells, it does not provide sufficient evidence that lindane is metabolized anaerobically in these cultures and that the anerobic metabolites are not mutagenic. Because of the above deficiencies, the study is graded unacceptable and is not acceptable for regulatory purposes.

HANSEN/PC-1/LINDANE-MGM/0006/LINDANE\PROJ# 1-2398/9-22-91