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

SEP 26 1990

OFFICE OF
PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: DEET: Review of Three Mutagenicity Studies

Caswell No. 346
MRID No. 413448-01
413444-01
413443001

HED Project No. 0-0836
EPA Record No. 260702

TO: Jane Mitchell, PM Team (17)
Special Review and Re-registration Division (H7508C)

FROM: Whang Phang, Ph.D. [signature] 9/14/90
Pharmacologist
HFAS/Tox. Branch II/ HED (H7509C)

THROUGH: K. Clark Swentzel, Section Head [signature] 9/14/90
and
Marcia van Gemert, Ph.D. [signature] 9/14/90
Branch Chief
HFAS/Tox. Branch II/ HED (H7509C)

Toxicology Branch II has been requested to review three mutagenicity studies on Deet. These studies were evaluated Dynamac Corp. and approved by Toxicology Branch II. The data evaluation report of each study are attached. The conclusion for each study is summarized below:

1). San, R.H.C. and Schadly, M.B., Salmonella/mammalian-microsome plate incorporation mutagenicity assay (Ames Test) with a confirmatory assay. Microbiological Associates, Inc.; Study No. T8728.501014; Dec. 28, 1989 (MRID No. 413448-01).

Deet was tested over a concentration range of 28 to 8333 ug/plate with Salmonella typhimurium strains TA1535, TA1537, TA1538, TA98, or TA100 in the presence or absence of the S9 activation. The results indicated that Deet was not mutagenic in this test system. This study satisfies data requirements for a gene mutation assay.

2). Putman, D.L. and Morris, M.J., Chromosome Aberrations in

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Chinese hamster ovary (CHO) cells. Microbiological Associates, Inc.; Study No. T8728.337;Dec. 28, 1989 (MRID No. 413444-01).

In an assay using CHO cells, Deet was tested at concentrations ranged from 0.063 to 1.0 µl/ml in the absence of S9 activation and from 0.032 to 0.50 µl/ml in the presence of S9 activation. Results indicated that Deet was not clastogenic. The study satisfies the data requirements for a structural chromosomal aberration assay.

3). Curren, R.D., Unscheduled DNA synthesis assay in rat primary hepatocytes with a confirmatory assay. Microbiological Associates, Inc.; Study No. T8728.380009; Dec. 22, 1989 (MRID No. 413443-01).

Deet was tested in two independently conducted unscheduled DNA synthesis assays in primary rat hepatocytes at doses ranged from 0.003 to 0.3 µl/ml. The results showed that Deet was assayed to a cytotoxic level with no evidence of a genotoxic effect. The study fulfills the data requirements for an assay for other genotoxic effects.

CONFIDENTIAL BUSINESS INFORMATION
DOES NOT CONTAIN
NATIONAL SECURITY INFORMATION (EO 12065)

EPA No.: 68D80056
DYNAMAC No.: 308-A2
TASK No.: 3-08A2
September 7, 1990

DATA EVALUATION RECORD

DEET

Mutagenicity--Unscheduled DNA Synthesis in Primary Rat Hepatocytes

APPROVED BY:

Robert J. Weir, Ph.D.
Program Manager
Dynamac Corporation

Signature:

Date: Sept 6, 1990

Guideline Series 84: **MUTAGENICITY**

EPA No.: 68D80056
DYNAMAC No.: 308-A2
TASK No.: 3-08A2
September 7, 1990

DATA EVALUATION RECORD

DEET

Mutagenicity--Unscheduled DNA Synthesis in Primary Rat Hepatocytes

REVIEWED BY:

Nancy E. McCarroll, B.S.
Principal Reviewer
Dynamac Corporation

Signature:
Date: Sept 6, 1990

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Independent Reviewer
Dynamac Corporation

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Date: Sept 6, 1990

APPROVED BY:

Nicolas P. Hajjar, Ph.D.
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Date: Sept. 6, 1990

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Signature:
Date: 9/11/90

DATA EVALUATION RECORD

Tox. Chem. No.: 346

EPA File Symbol:

CHEMICAL: DEET.

STUDY TYPE: Unscheduled DNA synthesis in primary rat hepatocytes.

ACCESSION OR MRID NUMBER: 413443-01.

SYNONYM(S)/CAS NUMBER: N,N-Diethyl-m-toluamide.

SPONSOR: DEET Joint Venture/Chemical Specialties Manufacturers Association, Washington, DC.

TESTING FACILITY: Microbiological Associates, Inc., Rockville, MD.

TITLE OF REPORT Unscheduled DNA Synthesis Assay in Rat Primary Hepatocytes With a Confirmatory Assay.

AUTHOR: Curren, R. D.

STUDY NUMBER: T8728.380009.

REPORT ISSUED: December 22, 1989.

CONCLUSIONS - Executive Summary: Under the conditions of two independently performed unscheduled DNA synthesis (UDS) assays in primary rat hepatocytes, DEET did not induce a significant increase in mean net nuclear grain counts over a dose range of 0.003 to 0.3 µL/mL. In the first trial, cells exposed to 0.3 µL/mL could not be evaluated because of cytotoxicity; however, in the repeat assay, there was sufficient cell survival to score nuclear grains at this level. Doses ≥1.0 µL/mL were severely cytotoxic, and test material concentrations ≥3.0 µL/mL were insoluble. It was concluded, therefore, that DEET was assayed to a cytotoxic level with no evidence of a genotoxic effect. The study fulfills Guideline requirements for Category III, Other Mutagenic Mechanisms.

Study Classification: The study is acceptable.

A. MATERIALS:

1. Test Material:

Name: DEET.

Description: Laboratory: Clear, colorless liquid (at receipt); pale yellow liquid (at use).

Sponsor: Yellow liquid (Test Article Characterization Form); clear oily liquid (Material Safety Data Sheet).

Note: The report indicated that the minor differences in test material description were not considered to reflect a change in the test material from receipt to use.

Batch/Lot No.: A-1-96 (mixture of four representative production runs; see Appendix B, Protocol, CBI p. 21).

Purity: 98.301%

Contaminants: Not listed.

Solvent used: Dimethylsulfoxide (DMSO).

Other comments: The test material was stored at room temperature and dilutions were prepared immediately before use. Dosing solutions

MANUFACTURING PROCESS INFORMATION IS NOT INCLUDED

were frozen after use and shipped to McLaughlin Gormley King Company for chemical analysis.

2. Indicator Cells: Primary rat hepatocytes were harvested from the livers of adult male Fischer 344 rats obtained from Charles River Laboratories, Inc., Raleigh, NC. Animals were quarantined at least 1 week prior to study initiation.

3. Cell Preparation:

   a. Hepatocyte Isolation: Each rat was anesthetized by inhalation of metofane and the livers were perfused with 0.5 mM EGTA in Hanks' buffered salt solution, pH 7.3, and serum-free Williams' Medium E (WME) containing L-glutamine (2 mM), collagenase (80 to 100 units/mL, type I), and antibiotics, buffered to pH 7.3. Livers were excised, cleaned of extraneous tissue, shaken in the collagenase perfusion solution, and either combed to release the hepatocytes or passed through a stainless-steel sieve.

   b. Hepatocyte Harvest/Culture Preparation: Recovered cells were collected, counted, and seeded at a density of 5×10^5 cells, either into preconditioned 35-mm tissue culture dishes for the cytotoxicity assay or onto coverslips in 35-mm tissue culture plates for the UDS assay. Cultures were placed in a humidified, 5% CO_2 incubator for 90 to 180 minutes, washed, and refed prior to use.

4. Positive Control: 7,12-Dimethylbenz(a)anthracene (DMBA) was dissolved in DMSO and assayed at 3 and 5 µg/mL in the preliminary cytotoxicity study and the initial UDS assay and at 3 and 10 µg/mL in the confirmation UDS assay.

B. STUDY DESIGN:

1. Preliminary Cytotoxity Assay: Duplicate cultures of cells, initiated from primary cultures, were exposed to 10 doses of the test material ranging from 0.0003 to 10 µL/mL, the negative control (WME), or the solvent control (DMSO) for 18 to 20 hours. Following exposure, aliquots of the treatment medium were removed, centrifuged, and measured for lactic acid dehydrogenase (LDH) activity. Relative cytotoxicity was assessed by subtracting the LDH activity of the media control from the LDH activity in the treated cultures and comparing the values to the amount of LDH released by exposure of high-dose cultures or solvent control cells to 1% Triton.

2. UDS Assay:

a. Treatment/Slide Preparation: Five to six prepared hepatocyte cultures (two or three cultures seeded into tissue dishes and three cultures seeded onto coverslips) were exposed for 18 to 20 hours to seven selected doses of the test material, the negative control (WME), the solvent control (DMSO), or the positive control (DMBA). Treatment medium contained 10 μCi/mL [^{3}H]thymidine. Monolayers grown directly on dishes were used to assess LDH activity as described for the cytotoxicity assay. Treated hepatocytes attached to coverslips were washed, swollen with 1% sodium citrate, fixed (ethanol-acetic acid), dried, and mounted.

b. Preparation of Autoradiographs/Grain Development: Slides were dipped into Kodak NTB emulsion, dried for 1.5 hours, and stored at 0-4°C in desiccated slide boxes for 8 to 9 days. Slides were developed in Kodak D-19, fixed, stained with hematoxylin-sodium acetate-eosin, coded, and counted.

c. Grain Counting: The nuclear grains of 150 randomly selected cells with appropriate background counts (50/slide) from each test, negative, and positive control group were scored for incorporation of tritiated thymidine into DNA. Net nuclear grain counts were determined by subtracting the average cytoplasmic grain count of three "nuclear-sized" areas adjacent to each nucleus from the nuclear grain count of each cell. Means and standard deviations were calculated for each treatment group.

3. Evaluation Criteria:

a. Assay Validity: For the assay to be considered valid, the following criteria must be satisfied: (1) the proportion of cells in repair in the negative control must be <15% and the net nuclear grain count of the solvent control must be <1; and (2) the positive control compound must induce a significant increase in the net nuclear grain count (≥5 grains/nucleus over the negative control).

b. Positive Response: The assay was considered positive if the test material induced a dose-related increase in mean net nuclear grains and one or more of the doses had an increase in the mean net nuclear grain count that was ≥5 grains/nucleus over the negative control.

In the absence of a dose-related effect, a compound that showed nuclear grain counts that were ≥5 grain/nucleus over two successive doses was also considered positive.

C. REPORTED RESULTS:

1. Preliminary Cytotoxicity Assay: Ten doses (0.0003 to 10 μL/mL) of the test material were examined in the cytotoxicity assay. The study author stated that the two highest doses (3.0 and 10.0 μL/mL) were not miscible in culture medium. As shown in Table 1, cytotoxicity, as indicated by increased LDH activity, was observed at levels ≥0.3 μL/mL; microscopic evaluation of the cultures also revealed cytotoxic effects at these levels. Below 0.3 μL/mL, DEET was not cytotoxic. Based on the findings, 0.3 μL/mL was selected as the highest concentration for the UDS assay.

2. UDS Assay: Two independent UDS assays with accompanying cytotoxicity tests were conducted with seven doses (0.0001 to 0.3 μL/mL) of the test material. In the first assay, cells exposed to 0.3 μL/mL were not scored for UDS because of severe cytotoxicity. Results from the analysis of the five groups treated with 0.003 to 0.2 μL/mL indicated no significant increase in net nuclear grain counts (Table 2). Data presented from the second trial showed that with the exception of a lessening of cytotoxicity at 0.3 μL/mL, which allowed the scoring of nuclear grains at this level, the results agreed with the earlier finding that DEET was not genotoxic in this test system.

   In both assays, the positive control, DMBA, at 3 and 5 μg/mL (first trial) and at 3 and 10 μg/mL (second trial), induced increased levels of enzymatic and UDS activity. Based on the overall results, the study author concluded that DEET was negative in this test system.

3. Analytical Delineations: Data presented from the analytical determinations of dosing solutions used in the UDS assays indicated that target concentrations of DEET prepared for the first trial ranged from a low of 72% (20-μL/mL sample) to a high of 143% (3.0-μL/mL sample) of the actual concentration. For the repeat test, all dosing solution target concentrations were within 5% of the actual concentration.

TABLE 1. Representative Results of the Preliminary Cytotoxicity Assay with DEET: Lactate Dehydrogenase (LDH) Activity

Treatment	Dose (μL/mL)	Average[a] LDH Activity (Units/L)	Corrected[b] LDH Activity (Units/L)	Relative Percent[c] Cytotoxicity
Negative Control				
Culture medium	--	68.0	-53 5	-13
Solvent Control				
Dimethylsulfoxide	10	121.5	0.0	0
Dimethylsulfoxide +1% Triton	10	523.5	402.0	100
Test Material Control				
DEET +1% Triton	10	477.0	355.5	88
Test Material				
DEET	0.3[d]	378.5	257.0	64[e]
	1.0	536.0	414.5	103[e]
	3.0[f]	517.0	395.5	98[e]
	10.0[f]	462.0	340.5	85[e]

[a]Average of two samples.

[b]Corrected LDH = Average LDH of Test Groups - Solvent Control LDH.

[c]Relative Percent Cytotoxicity = $\frac{\text{Corrected LDH of Test Groups}}{\text{Corrected LDH of 10 } \mu\text{L/mL DMSO + 1\% Triton}} \times 100.$

[d]Lower doses (0.1, 0.03, 0.01, 0.003, 0.001, and 0.0003 μL/mL) were not cytotoxic.

[e]Cytotoxic effects observed on the monolayer cultures at these levels.

[f]Reported to be immiscible in tissue culture medium.

TABLE 2. Representative Results of the Unscheduled DNA Synthesis Rat Hepatocyte Assays with DEET

Treatment	Dose/mL	Cytotoxicity: Average[a] Lactate Dehydrogenase Activity (Units/L)	Cytotoxicity: Corrected[b] LDH Activity (Units/L)	Cytotoxicity: Percent[c] Cytotoxicity	UDS Activity: Number of Cells Scored	UDS Activity: Mean Net Nuclear Grain Count Grains ± Standard Deviations	UDS Activity: Percent Cells with >5 Net Nuclear Grains
Negative Control							
Culture medium	--[d]	74.5	-9.0	-2	150	-1.7 ± 2.5	0
	--[e]	53.7	-23.3	-4	150	-1.4 ± 1.4	0
Solvent Control							
Dimethylsulfoxide	10 μl[d]	83.5	0.0	0	150	-2.8 ± 2.8	0
	10 μl[e]	82.0	0.0	0	150	-1.1 ± 1.5	0
Dimethylsulfoxide + 1% Triton	10 μl[d]	511.3	428.0	100	150	--	--
	10 μl[e]	616.0	534.0	100	150	--	--
Positive Control[f]							
7,12-Dimethylbenz(a)anthracene	3 μg[d]	175.5	92.0	21	150	20.7 ± 6.7*	100
	3 μg[e]	134.0	52.0	10	150	6.8 ± 4.2*	67
Test Material							
DEET	0.2 μl[d,g]	142.5	59.0	14	150	-0.5 ± 2.2	1
	0.3 μl[d]	366.0	282.5	66	0[h]	--	--
	0.2 μl[e,g]	193.3	111.3	21	150	1.3 ± 2.0	3
	0.3μl[e]	270.7	188.7	35	150	0.6 ± 2.9	5

[a]Average of two samples in the first trial and three samples in the repeat trial.

[b]Corrected LDH = Average LDH of Test Group - Solvent Control LDH.

[c]Percent Cytotoxicity = $\frac{\text{Corrected LDH of Test Groups}}{\text{Corrected LDH of Solvent Control Cultures Exposed to 1\% Triton}} \times 100.$

[d]Results for the first trial.

[e]Results for the repeat trial.

[f]Two levels were assayed in each trial; the lowest level was selected as representative.

[g]Results for lower doses (0.1, 0.03, and 0.01 μg/mL in both trials and 0.003 μg/mL in the first trial) did not indicate a genotoxic effect.

[h]Too cytotoxic to evaluate UDS activity.

*Conforms to the reporting laboratory's criteria for a positive response.

D. REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS: We assess that the study was well conducted and that the author correctly interpreted the data. The lack of agreement for the high-dose (0.3 µL/mL) cytotoxicity data between the first and repeat trials was probably related to minor procedural differences rather than a dosing error, since analytical determinations indicated that the actual concentration of DEET in the high dose from the first assay was ≈20% less than in the high-dose solution used in the subsequent trial. We do not, however, consider that this concentration difference compromised the outcome of the study.

Although the study author gave no reason for increasing the concentration of DMBA in the repeat assay, the findings at all assayed concentrations (3, 5, or 10 µg/mL) clearly demonstrated the sensitivity of the test system to detect a genotoxic response. We conclude, therefore, that DEET was assayed over an appropriate range of test material doses with no indication of a genotoxic effect.

E. QUALITY ASSURANCE MEASURES: A quality assurance statement was signed and dated December 28 1989.

F. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 9-12; Appendix B, Protocol, CBI pp. 21-30.

APPENDIX A

Materials and Methods
(CBI pp. 9-12)

80301 DEET RIN 8220-92

Page_____ is not included in this copy.

Pages 14-24 are not included.

The material not included contains the following type of information:

____ Identity of product inert ingredients.

____ Identity of product impurities.

____ Description of the product manufacturing process.

____ Description of quality control procedures.

____ Identity of the source of product ingredients.

____ Sales or other commercial/financial information.

____ A draft product label.

____ The product confidential statement of formula.

____ Information about a pending registration action.

✓ FIFRA registration data.

____ The document is a duplicate of page(s) ________.

____ The document is not responsive to the request.

The information not included is generally considered confidential by product registrants. If you have any questions, please contact the individual who prepared the response to your request.

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DOES NOT CONTAIN
NATIONAL SECURITY INFORMATION (EO 12065)

EPA No.: 68D80056
DYNAMAC No.: 308-A1
TASK No.: 3-08A1
September 7, 1990

DATA EVALUATION RECORD

DEET

Mutagenicity--Salmonella typhimurium/Mammalian Microsome Mutagenicity Assay

APPROVED BY:

Robert J. Weir, Ph.D.
Program Manager
Dynamac Corporation

Signature:

Date: Sept. 6, 1990

Guideline Series 84: **MUTAGENICITY**

EPA No.: 68D80056
DYNAMAC No.: 308-A1
TASK No.: 3-08A1
September 7, 1990

DATA EVALUATION RECORD

DEET

Mutagenicity--Salmonella typhimurium/Mammalian Microsome Mutagenicity Assay

REVIEWED BY:

Nancy E. McCarroll, B.S.
Principal Reviewer
Dynamac Corporation

Signature: [signature]
Date: Sept 6, 1990

I. Cecil Felkner, Ph.D.
Independent Reviewer
Dynamac Corporation

Signature: [signature]
Date: Sept 6, 1990

APPROVED BY:

Nicolas P. Hajjar, Ph.D.
Department Manager
Dynamac Corporation

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Date: Sept. 6, 1990

John H. S. Chen, D.V.M.
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Whang Phang, Ph.D.
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EPA Section Head, Section II
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Signature: [signature]
Date: 9/11/90

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DATA EVALUATION RECORD

Tox. Chem. No.:

EPA File Symbol:

CHEMICAL: DEET.

STUDY TYPE: Salmonella/mammalian activation gene mutation assay.

ACCESSION OR MRID NUMBER: 413448-01.

SYNONYM/CAS NUMBER: N,N-Diethyl-m-toluamide.

SPONSOR: DEET Joint Venture/Chemical Specialties Manufacturers Association, Washington, DC.

TESTING FACILITY: Microbiological Associates, Inc., Rockville, MD.

TITLE OF REPORT: Salmonella/Mammalian-Microsome Plate Incorporation Mutagenicity Assay (Ames Test) With a Confirmatory Assay.

AUTHORS: San, R. H. C. and Schadly, M. B.

STUDY NUMBER: T8728.501014.

REPORT ISSUED: December 28, 1989.

CONCLUSIONS - Executive Summary: DEET, over a concentration range of 28 to 8333 µg/plate -S9, was not mutagenic in *Salmonella typhimurium* strains TA1535, TA1537, TA1538, TA98, or TA100. The highest nonactivated dose was cytotoxic in all strains. Under S9 activation, the same dose range was not mutagenic. However, DEET at concentrations ≥833 µg/plate induced a cytotoxic effect in strain TA1538, which prompted the evaluation of lower doses (2.8 to 833 µg/plate +S9) with this strain. These results also showed that DEET was not mutagenic. The overall findings were confirmed in an independently performed assay. We assess that an appropriate range of test material doses were evaluated and that DEET was not mutagenic in this test system. Therefore, we conclude that the study fulfills the Guideline requirements for Category I, Gene Mutations.

Study Classification: The study is acceptable.

A. MATERIALS:

1. Test Material:

Name: DEET

Description: Laboratory: Clear, colorless liquid (at receipt); pale yellow liquid (at use).

Sponsor: Yellow liquid (Test Article Characterization Form); clear oily liquid (Material Safety Data Sheet)

Note: The report indicated that the minor differences in test material description were not considered to reflect a change in the test material from receipt to use.

Batch/Lot No.: A-1-96 (Mixture of four representative production runs. See Appendix A, Protocol, CBI p. 54.)

Purity: 98.301%

Contaminants: Not listed.

Solvent used: Dimethylsulfoxide (DMSO).

Other comments: The test material was stored at room temperature and dilutions were prepared immediately before use. Dosing solutions were frozen after use and shipped to McLaughlin King Co. for analytical determinations.

2. Control Materials:

Negative: DMSO.

Solvent/final concentration: 50 µL/plate

Positive: Nonactivation:

Sodium azide	1.0	µg/plate TA100, TA1535
2-Nitrofluorene	1.0	µg/plate TA98, TA1538
ICR-191	2.0	µg/plate TA1537

Other:

Activation: 2-Aminoanthracene (2-anthramine) 0.5 µg/plate all strains.

MANUFACTURING PROCESS INFORMATION IS NOT INCLUDED

3. Activation: S9 derived from

X Aroclor 1254	X induced	X rat	X liver
___ phenobarbital	___ noninduced	___ mouse	___ lung
___ none		___ hamster	___ other
___ other		___ other	

If other, describe below. Describe S9 composition (if purchased, give details).

S9 mix composition:

H_2O	0.56 mL
1.00 M NaH_2PO_4/K_2HPO_4 (pH 7.4)	0.10 mL
0.05 M Glucose-6-phosphate	0.10 mL
0.04 M NADP	0.10 mL
0.20 M $MgCl_2$/0.825 M KCl	0.04 mL
S9	0.10 mL
TOTAL	1.00 mL

4. Test Organism Used: S. typhimurium strains

___ TA97 x TA98 x TA100 ___ TA102 ___ TA104
x TA1535 x TA1537 x TA1538; list any others:

Test organisms were properly maintained: YES.
Checked for appropriate genetic markers (rfa mutation, R factor): YES.

5. Test Compound Concentrations Used:

a. Preliminary cytotoxicity assay: Ten doses (8.3, 28, 55, 83, 278, 555, 833, 2778, 5555, and 8333 µg/plate) were evaluated with or without S9 activation in S. typhimurium strain TA100. Single plates were used per dose per condition.

b. Initial mutation assays: Six nonactivated doses (28, 84, 278, 833, 2778, and 8333 µg/plate) and six S9-activated doses (278, 555, 833, 2778, 5555, and 8333 µg/plate) were assayed in all tester strains. A repeat S9-activated assay was performed with strain TA1538; the doses used in the repeat assay were 2.8, 8.3, 28, 84, 278, and 833 µg/plate.

c. Confirmatory mutation assays: Six doses with and without S9 activation (28 to 8333 µg/plate) were assayed in all tester strains except TA1538. The S9-activated dose range for this strain was 2.8 to 833 µg/plate.

B. TEST PERFORMANCE:

1. Type of Salmonella Assay: __x__ Standard plate test
_____ Pre-incubation (___) minutes
_____ "Prival" modification
_____ Spot test
_____ Other (describe).

a. Protocol:

1) Plating procedures: In general, similar procedures were used for the preliminary cytotoxicity and the mutation assays.

To tubes containing 2.5-mL volumes of molten top agar containing 0.5 mM biotin and 0.5 mM histidine, 100 µL of an overnight broth culture of the appropriate tester strain and 50 µL of the appropriate test material dose, solvent, or positive controls were added. For the S9-activated test, 0.5 mL of the S9 cofactor mix was added to tubes containing 2.0 mL of top agar; tester strains and test and control solutions were added as described. The contents of the tubes were mixed, poured over Vogel-Bonner minimal medium E, and incubated at 37°C for 48 hours. At the end of incubation, plates either were immediately scored for revertant colonies or were refrigerated and subsequently counted with an automatic colony counter. Means and standard deviations were determined for the mutation assay.

2) Sterility controls:

A sterility test was performed on the highest dose of the test material, and 0.5 mL of the S9 mix as described for the mutation assay.

3) Evaluation criteria:

a) Assay validity: The assay was considered valid if the following criteria were met: (1) the presence of the appropriate genetic markers was verified for each strain; (2) the spontaneous revertants of each strain fell within the reporting laboratory's acceptable ranges; (3) cell densities were ≥0.6 x 10^9 cells/mL; and (4) all positive controls caused at least a 3-fold increase in revertants per plate compared with the respective solvent control.

b) Positive response: The test material was considered positive if it caused ≥2-fold increase in mean revertant colonies of at least one tester strain and the increase was accompanied by a dose-response to increasing concentrations of the test material.

2. Preliminary Assay: Ten doses ranging from 8.3 to 8333 µg/plate +/-S9 were assayed for cytotoxic effects on strain TA100. The study authors stated: "Due to the change in volume upon mixing, the resulting stock was 166.7 mg/mL, rather than the target concentration 200 mg/mL."

Results of the cytotoxicity assay indicated that in the absence of S9 activation, reduced revertant colonies and a slightly reduced background lawn of growth were observed at 5555 and 8333 µg/plate; the highest S9-activated dose caused a slight reduction in TA100 revertants. Therefore, the dose ranges selected for the mutation assay were 28 to 8333 µg/plate -S9 and 278 to 8333 µg/plate +S9.

3. Mutation Assays: The study author stated that the first mutation assay with DEET was not completed because test material dilutions were not saved for analytical determinations. The nonactivated phase of the second assay was not evaluated because of excessive cytotoxicity in the solvent control and test groups. The presented results are, therefore, from the successfully completed assays.

As shown in Table 1, colony counts for all strains were reduced at 8333 µg/plate -S9. For the remaining nonactivated concentrations, there was no clear indication of cytotoxicity and the test material did not induce a

TABLE 1. Representative Results of the *Salmonella typhimurium* Mutagenicity Assay with DEET

Substance	S9 Activation	Dose (µg/plate)	Revertants per Plate of Bacterial Tester Strain[a]				
			TA1535	TA1537	TA1538	TA98	TA100
Solvent Control							
Dimethylsulfoxide	-	--	8 ± 2	8 ± 2	6 ± 3	27 ± 3	119 ± 6
	+	--	8 ± 4	7 ± 4	10 ± 3[b]	15 ± 2	108 ± 4
Positive Control							
Sodium azide	-	1.0	353 ± 12	--	--	--	475 ± 31
2-Nitrofluorene	-	1.0	--	--	393 ± 38	246 ± 15	--
ICR-191	-	2.0	--	115 ± 6	--	--	--
2-Anthramine	+	0.5	55 ± 3	45 ± 5	577 ± 96[b]	551 ± 8	807 ± 55
Test Material							
DEET	-	833[c]	12 ± 1	2 ± 2	3 ± 4	24 ± 2	113 ± 7
	-	2778	8 ± 4	4 ± 2	2 ± 3	17 ± 2	108 ± 2
	-	8333	5 ± 3	4 ± --[a]	2 ± 2	5 ± 5	65 ± 8[d]
	+	833[e]	10 ± 5	4 ± 1	8 ± 3[b]	13 ± 5	104 ± 13
	+	2778	6 ± 2	5 ± 1	ND	14 ± 2	96 ± 11
	+	5000	8 ± 3	2 ± 2	ND	10 ± 1	84 ± 18
	+	8333	6 ± 3	3 ± 2	ND	8 ± 2	79 ± 7

[a]Means and standard deviations of counts from triplicate plates except for strain TA1537 at 8333 µg/plate -S9; because of contamination only one plate was counted.

[b]Repeat S9-activated assay with strain TA1538 because of cytotoxicity at levels ≥833 µg/plate.

[c]Results for lower doses (28, 84, and 278 µg/plate) did not indicate a mutagenic response.

[d]Reduction in the background lawn of growth.

[e]Results for lower doses (278 and 555 µg/plate in strains TA1535, TA1537, TA98, and TA100 and 2.8, 8.3, 28, 84, and 278 µg/plate in strain TA1538) did not indicate a mutagenic response.

37

mutagenic response. Under S9 activation, there was no definitive evidence of cytotoxicity in strain TA1535, TA1537, TA98, or TA100. Similarly, there was no increase in reversion to histidine prototrophy of these strains. However, reduced colony counts and/or adverse effects on the background lawn of growth were apparent for strain TA1538 at doses ≥833 µg/plate. Accordingly, a repeat S9-activated trial was conducted with this strain; results show that DEET, over a dose range of 2.8 to 833 µg/plate, was neither cytotoxic nor mutagenic in TA1538.

Findings from the confirmation assay (Table 2) were in general agreement with the earlier results indicating that the highest nonactivated dose was cytotoxic in all strains, and that the test material was not mutagenic either with or without S9 activation. Although the test material results were negative, the sensitivity of the test system to detect mutagenesis was adequately demonstrated by the response of each tester strain to the appropriate nonactivated or S9-activated positive control in both assays. Based on the overall results, the study authors concluded that DEET was not mutagenic in this test system.

4. Analytical Determinations of Dosing Solutions: Chemical analyses were performed on the six test material dilutions used in the S9-activated portion of the first completed assay, the eight solutions used in the nonactivated and repeated S9-activated test with TA1538, and the eight solutions used in the confirmation assay. The data indicated that DEET concentrations in all dosing solutions were within 10% of the respective theoretical concentration.

5. Reviewer's Discussion/Conclusions: We assess that the study authors' interpretation of the data was correct. The test material was assayed to a nonactivated level that was cytotoxic in all strains. Although the S9-activated test material was cytotoxic only in strain TA1538, the high dose (8333 µg/plate) was considered adequate for evaluation of noncytotoxic compounds in this test system. We conclude, therefore, that DEET was tested over an appropriate dose range with no indication of a mutagenic effect.

6. Was the test performed under GLP? YES . (A quality assurance statement was signed and dated January 3, 1990.)

7. CBI Appendix: Appendix A, Protocol, CBI pp. 54-64; CBI Appendix B, Materials and Methods, CBI pp. 10-17.

TABLE 2. Representative Results of the Confirmatory *Salmonella typhimurium* Mutagenicity Assays with DEET

Substance	S9 Activation	Dose (µg/plate)	Revertants per Plate of Bacterial Tester Strain[a]				
			TA1535	TA1537	TA1538	TA98	TA100
Solvent Control							
Dimethyl sulfoxide	-	--	7 ± 3	3 ± 1	5 ± 3	14 ± 1	98 ± 6
	+	--	8 ± 4	8 ± 3	12 ± 4	19 ± 3	125 ± 1
Positive Control							
Sodium azide	-	1.0	574 ± 19	--	--	--	740 ± 25
2-Nitrofluorene	-	1.0	--	--	487 ± 37	289 ± 22	--
ICR-191	-	2.0	--	21 ± 4	--	--	--
2-Anthramine	+	0.5	36 ± 7	40 ± 6	432 ± 20	383 ± 20	560 ± 51
Test Material							
DEET	-	833[b]	8 ± 2	3 ± 3	4 ± 3	9 ± 3	92 ± 2
	-	2778	10 ± 2	4 ± 1	2 ± 1	10 ± 2	92 ± 14
	-	8333	4 ± 1	2 ± 1	1 ± 1	7 ± 1	58 ± 9
	+	833[c]	12 ± 2	5 ± 3	7 ± 2	19 ± 2	111 ± 3
	+	2778	8 ± 1	3 ± 2	ND	15 ± 6	109 ± 13
	+	8333	5 ± 2	2 ± 1	ND	7 ± 2	83 ± 5

[a]Means and standard deviations of counts from triplicate plates.

[b]Results for lower doses (28, 84, and 278 µg/plate) did not indicate a mutagenic effect.

[c]Results for lower doses (28, 84, and 278 µg/plate with strains TA1535, TA1537, TA98, and TA100 and 2.8, 8.3, 28, 84, and 278 µg/plate with strain TA1538) did not indicate a mutagenic effect.

APPENDIX A

Protocol
(CBI pp. 54-64)

80301 DEET RIN 8220-92

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Pages 41-60 are not included.

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- ____ Description of the product manufacturing process.
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- ____ Identity of the source of product ingredients.
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EPA No.: 68D80056
DYNAMAC No.: 308-A3
TASK No.: 3-08A3
September 7, 1990

DATA EVALUATION RECORD

DEET

Mutagenicity--Mammalian Cells in Culture Cytogenetic Assay in Chinese Hamster Ovary (CHO) Cells

APPROVED BY:

Robert J. Weir, Ph.D.
Program Manager
Dynamac Corporation

Signature: [signature]

Date: Sept 6, 1990

Guideline Series 84: **MUTAGENICITY**

EPA No.: 68D80056
DYNAMAC No.: 308-A3
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September 7, 1990

DATA EVALUATION RECORD

DEET

Mutagenicity--Mammalian Cells in Culture Cytogenetic Assay in Chinese Hamster Ovary (CHO) Cells

REVIEWED BY:

Nancy E. McCarroll, B.S. Principal Reviewer Dynamac Corporation	Signature: Nancy E. McCarroll Date: Sept 6 1990
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K. Clark Swentzel EPA Section Head, Section II Toxicology Branch II (H-7509C)	Signature: K Clark Swentzel Date: 9/11/90

DATA EVALUATION RECORD

Tox. Chem. No.: 346

EPA File Symbol:

CHEMICAL: DEET.

STUDY TYPE: Mammalian cells in culture cytogenetic assay in Chinese hamster ovary (CHO) cells.

ACCESSION OR MRID NUMBER: 413444-01.

SYNONYM(S)/CAS NUMBER: N,N-Diethyl-m-toluamide.

SPONSOR: DEET Joint Venture/Chemical Specialties Manufacturers Association, Washington, DC.

TESTING FACILITY: Microbiological Associates, Inc., Rockville, MD.

TITLE OF REPORT: Chromosome Aberrations in Chinese Hamster Ovary (CHO) Cells.

AUTHORS: Putman, D. L. and Morris, M. J.

STUDY NUMBER: T8728.337.

REPORT ISSUED: December 28, 1989.

CONCLUSIONS - Executive Summary: Four nonactivated (0.125, 0.25, 0.50, and 1.0 µL/mL) and four S9-activated (0.063, 0.125, 0.25, and 0.50 µL/mL) doses of DEET were evaluated for cytotoxic effects in Chinese hamster ovary (CHO) cells. Doses ≥1.5 µL/mL +/-S9 were cytotoxic. Because of cell cycle delay at 0.5 µL/mL -S9, an 18-hour cell harvest was performed for the nonactivated phase of testing; a normal 10-hour cell harvest was used for the S9-activated assay. Results indicated that DEET was not clastogenic at any assayed level, either with or without S9 activation in a well-controlled study. We conclude, therefore, that the study satisfies Guideline requirements for Category II, Structural Chromosome Aberrations.

Study Classification: The study is acceptable.

A. MATERIALS:

1. Test Material:

Name: DEET.

Description: Laboratory: clear, colorless liquid (at receipt); pale yellow liquid and/or light, yellow liquid (at use).

Sponsor: Yellow liquid (Test Article Characterization Form).

Clear, oily liquid (Material Safety Data Sheet).

Note: The report indicated that the minor differences in test material description were not considered to reflect a change in the test material from receipt to use.

Batch/Lot No.: A-1-96 (mixture of four representative production runs; see Appendix A, Protocol, CBI p. 22).

Purity: 98.301%

Contaminants: Not listed.

Solvent used: Dimethylsulfoxide (DMSO).

Other comments: The test material was stored at room temperature, protected from light. Dosing solutions were frozen after use and shipped to McLaughlin Gormley King Co. for analytical determinations.

2. Control Materials:

Negative: McCoy's 5A medium supplemented with 10% fetal bovine serum, glutamine, and antibiotics.

Solvent/final concentration: DMSO/1%.

Positive: Nonactivation (concentrations, solvent): Triethylenemelamine (TEM) was prepared in distilled water at a final concentration of a 5 µg/mL.
Activation (concentrations, solvent): Cyclophosphamide (CP) was prepared in distilled water at a final concentration of 50 µg/mL.

MANUFACTURING PROCESS INFORMATION IS NOT INCLUDED

3. Activation: S9 derived from

X Aroclor 1254	X induced	X rat	X liver
___ phenobarbital	___ noninduced	___ mouse	___ lung
___ none		___ hamster	___ other
___ other		___ other	

If other, describe below. Describe S9 composition (if purchased, give details). Prior to use, each batch of S9 was assayed for its ability to metabolize 2-anthramine and 7,12-dimethylbenz(a)anthracene to mutagenic forms using Salmonella typhimurium TA100.

The composition of the S9 mix per mL of growth medium was as follows:

NADP	1.4 mg
Isocitric acid	2.7 mg
S-9	15 µL

4. Test Compound Concentrations Used:

a. Preliminary cytotoxicity assay: Nine doses (0.0005, 0.0015, 0.005, 0.015, 0.05, 0.15, 0.5, 1.5, and 5 µL/mL) were evaluated with and without S9 activation.

b. Nonactivated conditions: Five doses (0.063, 0.125, 0.25, 0.5, and 1 µL/mL) were assayed with a delayed 18-hour harvest time.

c. S9-activated conditions: Five doses (0.032, 0.063, 0.125, 0.25, and 0.5 µL/mL) were assayed with a normal 10-hour harvest time.

5. Test Cells: CHO-K_1 cells were obtained from the American Type Culture Collection, Rockville, MD. Prior to use, the CHO cells were grown for 16 to 24 hours in McCoy's 5a medium.

Properly maintained: Yes.

Cell line or strain periodically checked for Mycoplasma contamination? Not reported.

Cell line or strain periodically check for karyotype stability? Not reported.

B. TEST PERFORMANCE:

1. Cell Treatments:

a. Cells exposed to test compound for:
_16_ hours (nonactivated) _2_ hours (activated)

b. Cells exposed to positive controls for:
_16_ hours (nonactivated) _2_ hours (activated)

c. Cells exposed to negative and/or solvent controls for:
_16_ hours (nonactivated) _2_ hours (activated)

2. Protocol:

a. Preliminary assay: Prepared cultures, seeded at 5 x 10^5 cells/flask, were exposed with or without S9 activation to nine half-log dilutions of the test material (0.0005 to 5 μl/mL) and the solvent control (DMSO).

In the nonactivated system, cells were exposed for 6 hours to the test material; 50 μL of 1 mM BrdU were added to the cultures 2 hours after initiation. At the conclusion of the 6-hour treatment, cells were washed, refed fresh medium containing 0.01 mM BrdU and incubated for a total of 24 hours. In the S9-activated system, cultures were treated for 2 hours. After exposure, cells were washed, refed with complete medium containing BrdU (0.01 mM), and reincubated for 24 hours.

Two hours prior to the end of incubation, a final concentration of 0.1 μg/mL colcemid was added to each culture. Metaphases were harvested, fixed, and stained with the modified fluorescent-plus-Giemsa technique of Perry and Wolff.[1] One hundred cells from each dose group were examined for the percentage of first division (M_1), second division (M_2), and third division (M_3) metaphases. Mitotic indices were determined by counting the number of mitotic cells in a population of 500 scored cells. Based on these results, dose selection and harvest times for the cytogenetic assay were established.

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[1] Perry, P., and Wolff, S. New Giemsa method for the differential staining of sister chromatids. Nature (1974) 251: 156-158.

b. Cytogenetic assay:

1) Treatment: Prepared cultures (in duplicate), seeded at 5 x 10^5 cells, were exposed to the selected test material doses, the negative control (culture medium), the solvent control (DMSO), or the positive controls (TEM -S9 or CP +S9).

In the nonactivated assay, cells were dosed for 16 hours. Cultures were washed, refed medium containing 0.1 μg/mL colcemid, and reincubated for 2 hours. Under S9-activated conditions, cells were exposed for 2 hours, washed, refed culture medium, and incubated for an additional 8 hours. Colcemid was added 2 hours before the cultures were harvested.

Metaphase cells were collected and fixed. Slides were stained with 5% Giemsa and coded.

2) Metaphase analysis: One hundred metaphase cells per group (50/culture) were scored for chromosome aberrations. Chromatid and chromosome gaps were counted, but not included, in the final analysis. Mitotic indices were calculated.

3) Statistical methods: The data were evaluated for statistical significance at p values of 0.05 and 0.01 by the Fisher's Exact test.

4) Evaluation criteria:

a) Assay validity: The assay was considered valid if the percent of cells with aberrations in the untreated and solvent controls did not exceed 6% and the number of cells with aberrations in the positive control was significantly higher ($p \leq 0.05$) than in the solvent control.

b) Positive response: The test material was considered positive if it caused a significant and dose-related increase in the percentage of cells with aberrations relative to the solvent control.

C. REPORTED RESULTS:

1. Preliminary Cytotoxicity Assay: The report indicated that the highest dose (5 μg/mL) was only partially soluble; all other concentrations of test material were soluble. The high concentration had no adverse effects on the osmotic pressure of the tissue culture medium. The study authors further indicated that no mitotic cells were recovered from cultures exposed to 1.5 or 5 μL/mL +/- S9.

   As shown in Table 1, reduced mitotic indices (MIs) were seen following exposure to nonactivated 0.15 and 0.5 μL/mL DEET. Cell cycle delay was apparent at 0.5 μL/mL, and although slight effects on cell cycle kinetics were observed at doses ≥0.005 μL/mL, the data did not clearly suggest adverse effects on cell cycling. In the presence of S9 activation, reduced MIs were reported at the 0.15- and 0.5-μL/mL dose levels; however, there was no evidence of cell cycle delay. Based on the results, five levels (0.063 to 1 μL/mL) were assayed without S9 activation using an 18-hour cell harvest, and five doses (0.032 to 0.5 μL/mL) were tested with S9 activation using a 10-hour cell harvest.

2. Cytogenetic Assay: Representative results of the cytogenetic assay performed with DEET are presented in Table 2. A dose-related decrease in the MI was observed in the nonactivated phase of testing; MIs ranged from 0.6 at 1.0 μL/mL to 4.9 at 0.125 μL/mL. The study authors also stated that 1.0 μL/mL -S9 had slight cytotoxic effects on the monolayer. In the presence of S9 activation, the MI for the high dose (0.5 μL/mL) was slightly reduced compared to the negative and solvent control value. For the remaining S9-activated dose groups, MIs were generally comparable to the solvent control group. The nonactivated or the S9-activated test levels did not cause an appreciable increase in the percentage of cells with aberrations or the number of aberrations per cell (Table 2). By contrast, both the nonactivated and S9-activated positive controls (TEM and CP) induced significant ($p \leq 0.01$) increases in the percentage of cells with aberrations.

   Based on the results, the study authors concluded that DEET was negative in the CHO cell cytogenetic assay.

3. Analytical Determinations: Data presented from the analytical determinations of test material dilutions prepared for the cytogenetic assay indicated that target concentrations were within 10% of the actual concentration.

TABLE 1. Representative Results from the Preliminary Cytotoxicity Assay with DEET

Substance	Dose (µL/mL)	S9 Activation	% Cells[a] M1	M2	M3	Mitotic[b] Index
Solvent Control						
Dimethylsulfoxide	--	-	2	98	0	3.2
	--	+	4	96	0	5.2
Test Material						
DEET	0.005[c]	-	13	87	0	4.0
	0.015	-	15	85	0	3.4
	0.05	-	14	86	0	3.4
	0.15	-	17	82	1	2.8
	0.5[d]	-	39	61	0	2.0
	0.05[c]	+	12	88	0	5.0
	0.15	+	0	100	0	3.0
	0.5[d]	+	2	98	0	1.8

[a] Percent cells in first (M_1), second (M_2), or third (M_3) division.

[b] Number of metaphase cells per 500 cells scored.

[c] Lower doses (0.0015 and 0.0005 µL/mL -S9 and 0.015, 0.005, 0.0015, and 0.0005 µL/mL +S9) had no appreciable cytotoxic effects.

[d] No mitotic cells were recovered from cultures exposed to 1.5 or 5.0 µL/mL (+/- S9).

TABLE 2. Representative Results of the CHO Cell *in vitro* Cytogenetic Assay with DEET Following an 18-Hour Nonactivated and a 10-Hour S9-Activated Cell Harvest

Substance	Dose	Harvest Time (hours)	S9 Activation	No. of Cells Scored	Mitotic Index (%)	Cells with Aberrations (%)	Aberrations per Cell ± Standard Deviations	Biologically Significant Aberrations[a] (No./Type)
Negative Control								
Untreated cells	--	18	-	100	5.5	2	0.05 ± 0.411	1TB; 4SB
	--	10	+	100	7.6	1	0.02 ± 0.200	2SB
Solvent Control								
Dimethylsulfoxide	1%	18	-	100	5.0	2	0.02 ± 0.141	1TE; 1D
	1%	10	+	100	9.9	2	0.02 ± 0.141	2D
Positive Control								
Triethylene-melamine	0.5 µg	18	-	100	2.9	29*	0.39 ± 0.695	22TB; 12TE; 5SB
Cyclophosphamide	50.0 µg	10	+	100	2.0	10*	0.19 ± 1.032	5TB; 1TE; 1SB; 2D; 1SD
Test Material								
DEET	0.5 µL[b]	18	-	100	2.3	0	0.00 ± 0.000	--
	1.0 µL	18	-	100	0.6	1	0.01 ± 0.100	1TB
	0.25 µL[b]	10	+	100	9.4	4	0.040 ± 0.197	1SB; 2D; 1R
	0.50 µL	10	+	100	6.4	1	0.010 ± 0.100	1TB

[a]Abbreviations Used:

TB - Chromatid break; TE - Chromatid exchange; SB - Chromosome break; D - Dicentric chromosome; R - Ring; SD - Severely damaged (e.g., pulverized chromosome or cell with ≥10 aberrations).

[b]Lower dose groups (0.25 and 0.125 µL/mL -S9 and 0.125 and 0.063 µL +S9) were not significantly higher than the appropriate solvent control group.

*Significantly ($p \leq 0.01$) higher than the solvent control by Fisher's Exact test.

4. Reviewer's Discussion/Conclusions: We assess that the study was properly conducted and that the study authors' interpretation of the data was correct. In both the nonactivated and S9-activated cytogenetic assays, DEET was assayed to cytotoxic levels but failed to induce a clastogenic effect. Although several chromosome-type aberrations (1 chromosome break, 2 dicentrics, and 1 ring) were scored at 0.25 µL/mL +S9, in the absence of chromatid-type aberrations, this result is insufficient as an indicator of a clastogenic effect.

   The sensitivity of the test system to detect clastogenesis was adequately demonstrated by the significant ($p \leq 0.01$) increase in the percentage of cells with aberrations in the positive control groups. We conclude, therefore, that DEET was assayed over an appropriate concentration range both in the presence and absence of S9 activation with no evidence of a clastogenic effect in a well-controlled study.

5. Was test performed under GLPs? YES. (A quality assurance statement was signed and dated, December 28, 1989.)

6. CBI Appendix: Appendix A, Materials and Methods, CBI pp. 9-13; Appendix B, Study Protocol, CBI pp. 22-29.

APPENDIX A

Materials and Methods
(CBI pp. 9-13)

80301 DEET RIN 8220-92

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