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

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OFFICE OF
PESTICIDES AND TOXIC
SUBSTANCES

MEMORANDUM

SUBJECT: Copper Naphthenate, Cunapsol-5, M-Gard W-510, and Emulsifier A; Review of Acute Toxicity and Mutagenicity Studies Submitted by the Registrant under FIFRA section 6 (a) (2).

Caswell No: 245
HED Project Nos: 9-1808; 9-1989
MRID Nos: 411407-01 through 411407-10

FROM: Timothy F. McMahon, Ph.D., Toxicologist
Review Section I, Toxicology Branch II (HFAS)
Health Effects Division (H7509C)

TO: Susan Lewis PM23
Registration Division (H7505C)

THRU: Yiannakis M. Ioannou, Ph.D., Section Head
Review Section I, Toxicology Branch II (HFAS)
Health Effects Division (H7509C) 11/9/90

and

Marcia Van Gemert, Ph.D., Branch Chief
Toxicology Branch II (HFAS)
Health Effects Division (H7509C) 11/8/90

Registrant: U.S. Army Environmental Hygiene Agency
Aberdeen Proving Ground, MD

Action Requested: Review of the following Acute Toxicity and Mutagenicity studies with Copper Naphthenate, Cunapsol-5 (copper hydroxide naphthenic acid 48% a.i.), M-Gard W-510 (copper hydroxide naphthenic acid 58% a.i.), and Emulsifier A.

Recommendations: Toxicology Branch II has reviewed the Acute Toxicity and Mutagenicity studies submitted by the Registrant under FIFRA Section 6 (a) (2). A summary of the findings from these studies is found in the appended table.

Study Name	Material	Dose Levels Tested	Conclusions	Classification
Mutagenicity-Unscheduled DNA synthesis in rat hepatocytes MRID# 411407-01	copper naphthenate (purity not stated)	10, 15, and 20 µg/ml	mutagenic in test system	unacceptable
Mutagenicit Mouse Lymphoma Forward Mutation Assay MRID# 411407-02	copper naphthenate (purity not stated)	10, 25, 50, and 75 µg/ml	mutagenic in test system with metabolic activation	unacceptable
Mutagenicity-*S. Typhimurium* Mutagenicity Assay MRID# 411407-03	copper naphthenate (purity not stated)	50, 100, 200, 300 and 500 µg/plate	non-mutagenic in test system	unacceptable
Mutagenicity-*In vitro* chromosome assay in CHO cells MRID# 411407-04	copper naphthenate (purity not stated)	100, 125, 150 µg/ml (non-activated doses); 45, 60, and 75 µg/ml (activated doses)	non-activated doses not clastogenic; activated doses weakly clastogenic	unacceptable
Mutagenicity-*S. Typhimurium* Mutagenicity Assay MRID# 411407-05	copper naphthenate (purity not stated)	1-10,000 µg/plate	cytotoxic at 5000 and 10,000 µg/plate; not mutagenic in test system with or without metabolic activation	unacceptable
Mouse lymphoma forward mutation assay MRID#411407-06	copper naphthenate (purity not stated)	10-80 µg/ml (non-activated doses); 2.5-60 µg/ml (activated doses)	non-activated doses non-mutagenic; activated doses non-mutagenic	unacceptable

Study Name	Material	Dose Levels Tested	Conclusions	Classification
Mutagenicity- *In vitro* chromosome aberrations in CHO cells MRID# 411407-07	copper naphthenate (purity not stated)	25-100 µg/ml (non-activated doses); 6-40 mg/ml (activated doses)	no clastogenic effect (non-activated doses); no clastogenic effect (activated doses)	unacceptable
Mutagenicity- Unscheduled DNA synthesis in rat hepatocytes MRID# 411407-08	copper naphthenate (purity not stated)	0.1-25 µg/ml	test material inactive in test system at all doses	unacceptable

Study Type	Material	Conclusions	Toxicity Category	Classification
Acute Oral LD_{50} species: rat MRID# 411407-09	copper hydroxide naphthenic acid 48% a.i. Cunapsol 5	LD_{50} > 3000 mg/kg (males) LD_{50} > 2000 mg/kg (females)	3	supplementary
Acute Dermal LD_{50} species: rabbits MRID# 411407-09	copper naphthenate (purity not stated)	LD_{50} > 2000 mg/kg (males and females)	3	supplementary
Acute Dermal LD_{50} species: rabbit MRID# 411407-09	copper hydroxide naphthenic acid 48% a.i. Cunapsol 5	LD_{50} not determined		supplementary
Acute Inhalation LD_{50} species: rat MRID# 411407-09	copper hydroxide naphthenic acid 48% a.i. Cunapsol 5	LC_{50} > 5 mg/L (males)	4	supplementary
Dermal Sensitization species: guinea pig MRID# 411407-09	copper hydroxide naphthenic acid 48% a.i. Cunapsol 5	non-sensitizer		supplementary
Primary Dermal Irritation species: rabbit MRID# 411407-09	copper hydroxide naphthenic acid 48% a.i. Cunapsol 5	severe skin irritant	1	supplementary

Study Type	Material	Conclusions	Toxicity Category	Classification
Primary Eye Irritation species: rabbit MRID# 411407-09	copper hydroxide naphthenic acid 48% a.i. Cunapsol 5	severe eye irritant	1	supplementary
Primary Dermal Irritation species: rabbit MRID# 411407-09	copper naphthenate (purity not stated)	moderate skin irritant	3	supplementary

Study Type	Material	Conclusions	Toxicity Category	Classification
Acute Oral LD_{50} species: rat MRID# 411407-10	copper naphthenate 58% a.i. M-Gard W-510	LD_{50} not determined		supplementary
Acute Dermal LD_{50} species: rabbits MRID# 411407-10	copper naphthenate (purity not stated)	LD_{50} > 2000 mg/kg (females) LD_{50} not determined in males	3	supplementary
Acute Dermal LD_{50} species: rabbit MRID# 411407-10	copper naphthenate 58% a.i. M-Gard W-510	LD_{50} > 2000 mg/kg (males and females)	3	supplementary
Acute Inhalation LC_{50} species: rat MRID# 411407-10	copper naphthenate 58% a.i. M-Gard W-510	LC_{50} > 5 mg/L (males)	4	supplementary
Dermal Sensitization species: guinea pig MRID# 411407-10	copper naphthenate 58% a.i. M-Gard W-510	non-sensitizer		supplementary
Primary Dermal Irritation species: rabbit MRID# 411407-10	copper naphthenate (purity not stated)	moderate skin irritant	3	supplementary
Primary Dermal Irritation species: rabbit MRID# 411407-10	Emulsifier A	non-irritating	4	supplementary

Study Type	Material	Conclusions	Toxicity Category	Classification
Primary Dermal Irritation species: rabbit MRID# 411407-10	mineral spirits	slight skin irritant	3	supplementary
Primary Dermal Irritation species: rabbit MRID# 411407-10	copper naphthenate 58% a.i. M-Gard W-510	moderately irritating	3	supplementary
Primary Eye Irritation species: rabbit MRID# 411407-10	copper naphthenate 58% a.i. M-Gard W-510	non-irritating	4	supplementary

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Secondary Reviewer: Yiannakis M. Ioannou, Ph.D. 11/17/90
Section I, Toxicology Branch II (HFAS) (H7509C)

Data Evaluation Report

Study type: Acute oral-rats (81-1) Tox. Chem. No.: 245

MRID number: 411407-09

Test material: Cunapsol 5

Study number: 75-51-0497-88

Testing Facility: Department of the Army
U.S. Army Environmental Hygiene Agency
Aberdeen Proving Ground, Maryland 21010-5422

Title of report: Phase 2 Preliminary Assessment of the Relative Toxicity of Copper Naphthenate. Acute Studies: Acute Oral and Dermal Toxicity Studies.

Author(s): R.A. Angerhofer
L.M. Taylor
M.H. Weeks

Report issued: November 12, 1987

Conclusions:

Under the conditions of this study, the acute oral LD_{50} for Cunapsol 5 was determined to be > 3000 mg/kg in male rats, and > 2000 mg/kg in female rats.

Toxicity Catgory III

Core Classification: supplementary

This study does not fulfill the requirements (81-1) for an acute oral toxicity study in rats

I. MATERIALS

A. Test Material: Cunapsol 5; description: blue-green liquid having a musty odor and a pH of 11.5.

composition: copper hydroxide naphthenic acid-48%
emusifier-25%
water-27%

Lot number: 4121486

B. Test Animals: Sprague-Dawley Wistar derived rats; Source, age and weight not provided.

II. METHODS

An unstated number of male and female rats were employed in this study. Rats were given commercial laboratory chow and water ad libitum.

Cunapsol 5 was administered as the neat material by stainless steel stomach tube to male and female rats. Animals were observed for 14 days following dosing for clinical toxicity. Body weights were recorded on days 1, 3, 7, and 14 postexposure. Gross necropsy was performed on all survivors as well as those animals which died during the study.

The emulsifier ("Emulsifier B") was also tested for acute oral LD_{50} in rats under (presumably) similar conditions as for Cunapsol 5.

III. RESULTS

On page 11 of the registrant's report, it is stated that a "few" rats died during the 14 day observation period. Clinical signs were limited to lethargy and death "at relatively high dosages." Gross pathological observations on those rats which died during the observation period showed "gastric and intestinal lesions which could be attributed to the corrosive nature of the compound administered." No gross pathological findings were observed in surviving rats.

IV. CONCLUSIONS

Under the conditions of this study, the acute oral LD_{50} of Cunapsol 5 was > 3000 mg/kg in male rats, and > 2000 mg/kg in female rats. The acute oral LD_{50} for "Emulsifier B" was > 2000 mg/kg in both male and female rats.

Toxicity Category III

V. CORE CLASSIFICATION

supplementary

This study does not fulfill the requirements (81-1) for an acute oral toxicity study in rats. The following information is requested in order to upgrade this study to core minimum:

1) source, age, weight, and numbers of animals used

2) times of death and numbers of rats which died during the study

3) necropsy data on all rats

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Section I, Toxicology Branch II (HFAS) (H7509C)

Data Evaluation Report

Study type: Acute dermal-rabbits (81-2) Tox. Chem. No.: 245

MRID number: 411407-09

Test material: copper naphthenate

Study number: 75-51-0497-88

Testing Facility: Department of the Army
U.S. Army Environmental Hygiene Agency
Aberdeen Proving Ground, Maryland 21010-5422

Title of report: Phase 2 Preliminary Assessment of the Relative Toxicity of Copper Naphthenate. Acute Studies: Acute Oral and Dermal Toxicity Studies

Author(s): R.A. Angerhofer
L.M. Taylor
M.H. Weeks

Report issued: November 12, 1987

Conclusions:

Under the conditions of this study, the acute dermal LD_{50} for copper naphthenate was determined to be > 2000 mg/kg in male and female rabbits.

Toxicity Category III

Core Classification: supplementary

This study does not fulfill the requirements (81-2) for an acute dermal toxicity study in rabbits

I. MATERIALS

A. Test Material: copper naphthenate; description: a copper hydroxide-naphthenic acid reaction product; purity: not stated

B. Test Animals: New Zealand white rabbits; Source, age and weight not provided.

II. METHODS

Five male and five female rabbits were employed in this study. Rabbits. were given commercial laboratory chow and water ad libitum.

Copper naphthenate was administered as a 500 mg/ml solution in corn oil at a dose of 2000 mg/kg to the shaved backs of male and female rabbits. The treated area was occluded for 24 hours.

III. RESULTS

Dermal application of 2000 mg/kg copper naphthenate resulted in the death of 1 of 5 male rabbits, and 0 of 5 female rabbits (time of death not provided). Copper naphthenate caused slight to moderate dermal irritation, followed by scaling and sloughing of dead skin (time course not provided). Clinical signs of toxicity reported were lethargy and death. Body weights "remained stable between pretest and sacrifice."

IV. CONCLUSIONS

Under the conditions of this study, the acute dermal LD_{50} of copper naphthenate was determined to be > 2000 mg/kg in male and female rabbits.

Toxicity Category III

V. CORE CLASSIFICATION

supplementary

This study does not fulfill the requirements (81-2) for an acute dermal toxicity study in rabbits. The following information is requested in order to upgrade this study to core minimum:

1) source, age, and weight of animals used

2) statistical method for calculating LD_{50}

3) doses used in LD_{50} study

4) times of death of rabbits which died during the study

5) necropsy data on all rabbits

6) description of occlusive dressing used

7) whether animals were restrained or dressing was sufficient to prevent ingestion of test material

8) percentage of body surface over which test material was applied

9) justification for use of corn oil in dermal application of copper naphthenate

10) purity of the test material

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Data Evaluation Report

Study type: Acute dermal-rabbits (81-2) Tox. Chem. No.: 245

MRID number: 411407-10

Test material: copper naphthenate

Study number: 75-51-0497-88

Testing Facility: Department of the Army
U.S. Army Environmental Hygiene Agency
Aberdeen Proving Ground, Maryland 21010-5422

Title of report: Phase 3 Preliminary Assessment of the Relative Toxicity of Copper Naphthenate. Acute Studies: Acute Oral and Dermal Toxicity Studies

Author(s): R.A. Angerhofer
L.W. Metger

Report issued: not provided

Conclusions:

Under the conditions of this study, the acute dermal LD_{50} for copper naphthenate was > 2000 mg/kg in female rabbits. Data were not provided for determination of the acute dermal LD_{50} in male rabbits.

Toxicity Category III (for females)

Core Classification: supplementary

This study does not fulfill the requirements (81-2) for an acute dermal toxicity study in rabbits

I. MATERIALS

A. Test Material: copper naphthenate, technical; description: a blue-green amorphous solid having a musty odor and containing 9.6% copper; purity: not stated.

B. Test Animals: New Zealand white rabbits; Source, age and weight not provided.

II. METHODS

Five male and five female rabbits were employed in this study. Rabbits were given commercial laboratory chow and water ad libitum.

Copper naphthenate was administered as a 500 mg/ml solution in corn oil at a dose of 2000 mg/kg to the shaved backs of male and female rabbits. The treated area was occluded for 24 hours.

III. RESULTS

Dermal application of 2000 mg/kg copper naphthenate resulted in the death of 2 of 5 male rabbits, and 0 of 5 female rabbits. Copper naphthenate caused slight to moderate dermal irritation, followed by scaling and sloughing of dead skin (time course not provided). Clinical signs of toxicity reported were lethargy and death. Body weights "remained stable between pretest and sacrifice."

IV. CONCLUSIONS

Under the conditions of this study, the acute dermal LD_{50} for copper naphthenate was > 2000 mg/kg in female rabbits. Data were not provided for determination of the acute dermal LD_{50} in male rabbits.

Toxicity Category III (for females)

V. CORE CLASSIFICATION

supplementary

This study does not fulfill the requirements (81-2) for an acute dermal toxicity study in rabbits. The following information is requested in order to upgrade this study to core minimum:

1) source, age, weight, and numbers of animals used

2) times of death and numbers of rabbits which died during the study

3) necropsy data on all rabbits

4) description of occlusive dressing used

5) whether animals were restrained or dressing was sufficient to prevent ingestion of test material

6) percentage of body surface over which test material was applied

7) purity of test material

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Data Evaluation Report

Study type: Primary dermal irritation-rabbits (81-5) Tox. Chem. No.: 245

MRID number: 411407-10

Test material: copper naphthenate

Study number: 75-51-0497-88

Testing Facility: Department of the Army
U.S. Army Environmental Hygiene Agency
Aberdeen Proving Ground, Maryland 21010-5422

Title of report: Phase 3 Preliminary Assessment of the Relative Toxicity of Copper Naphthenate. Acute Studies: Primary Skin Irritation Studies

Author(s): R.A. Angerhofer
L.W. Metger

Report issued: not provided

Conclusions: Under the conditions of this study, copper naphthenate was determined to be moderately irritating to the skin of white rabbits.

Toxicity Category III

Core Classification: supplementary

This study does not fulfill the requirements (81-5) for a primary dermal irritation study in rabbits.

I. MATERIALS

A. Test Material: copper naphthenate, technical reaction product; description: dark blue-green amorphous solid having a musty odor and containing 9.6% copper; purity: not stated

B. Test Animals: New Zealand white rabbits; Source, age and weight not stated.

II. METHODS

Six rabbits were employed for study of the primary dermal irritation of copper naphthenate. Commercial chow and water were available ad libitum.

Initial application of copper naphthenate as the neat material resulted in staining and unreadability of the test site. Thus, copper naphthenate was applied as a 25% solution in corn oil in a volume of 0.5 ml to an intact skin site on 3 rabbits, and an abraded skin site on 3 separate rabbits. Skin sites were occluded for 24 hours. Erythema, eschar, and edema were evaluated at 24 hours, 72 hours, and 7 days at intact and abraded skin sites.

III. RESULTS

At 24 hours, 25% copper naphthenate in corn oil caused very slight to severe erythema on intact skin, while severe erythema was observed on abraded skin at 24 hours. At 72 hours, erythema was slight to severe on intact skin, and moderate to severe on abraded skin.

Edema formation on intact skin at 24 hours from application of 25% copper naphthenate in corn oil was graded as none to slight, while edema formation on abraded skin was graded as slight to moderate. At 72 hours, edema was very slight on intact skin, and very slight to moderate on abraded skin.

IV. CONCLUSIONS

Under the conditions of this study, copper naphthenate was determined to be moderately irritating to the skin of white rabbits.

Toxicity Category III

V. CORE CLASSIFICATION

supplementary

This study does not fulfill the requirements (81-5) for a primary dermal irritation study in rabbits. The following were omitted from the original study report and should be included to upgrade this study to core minimum:

1) source of animals

2) whether animals were clipped before application of test material

3) whether the test site was washed after patch removal

4) the device used to occlude the test site

5) whether animals were restrained during and/or after testing

6) a sentence explaining whether the times of observation refer to the time from the start or end of test material application

7) purity of test material

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Data Evaluation Report

Study type: Primary dermal irritation-rabbits (81-5) Tox. Chem. No.: 245

MRID number: 411407-09

Test material: copper naphthenate

Study number: 75-51-0497-88

Testing Facility: Department of the Army
U.S. Army Environmental Hygiene Agency
Aberdeen Proving Ground, Maryland 21010-5422

Title of report: Phase 2 Preliminary Assessment of the Relative Toxicity of Copper Naphthenate. Acute Studies: Primary Skin Irritation Studies

Author(s): R.A. Angerhofer
L.M. Taylor
M.H. Weeks

Report issued: November 12, 1987

Conclusions: Under the conditions of this study, copper naphthenate was determined to be moderately irritating to the skin of white rabbits.

Toxicity Category III

Core Classification: supplementary

This study does not fulfill the requirements (81-5) for a primary dermal irritation study in rabbits.

I. MATERIALS

A. Test Material: copper naphthenate, technical reaction product; description: dark blue-green amorphous solid having a musty odor. purity: not stated.

B. Test Animals: New Zealand white rabbits; Source, age and weight not stated.

II. METHODS

Six rabbits were employed for study of the primary dermal irritation of copper naphthenate. Commercial chow and water were available ad libiturn.

Copper naphthenate was applied as a 25% solution in corn oil in a volume of 0.5 ml to an intact skin site on 3 rabbits, and an abraded skin site on 3 separate rabbits. Skin sites were occluded for 24 hours. Erythema, eschar, and edema were evaluated at 24 hours, 72 hours, and 7 days at intact and abraded skin sites.

III. RESULTS

At 24 hours, copper naphthenate caused slight to moderate erythema on intact skin, and moderate to severe erythema on abraded skin. At 72 hours, erythema ranged from slight to severe on intact skin, and from slight to moderate on abraded skin.

Edema formation was very slight to slight on both intact and abraded skin at 24 hours, and very slight at 72 hours, with the exception of one rabbit, which displayed severe edema at 72 hours.

IV. CONCLUSIONS

Under the conditions of this study, copper naphthenate was determined to be moderately irritating to the skin of white rabbits.

Toxicity Category III

V. CORE CLASSIFICATION

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supplementary

This study does not fulfill the requirements (81-5) for a primary dermal irritation study in rabbits. The following were omitted from the original study report and should be included to upgrade this study to core minimum:

1) source of animals

2) whether animals were clipped before application of test material

3) whether the test site was washed after patch removal

4) the device used to occlude the test site

5) whether animals were restrained during and/or after testing

6) a sentence explaining whether the times of observation refer to the time from the start or end of test material application

7) purity of test material

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Secondary Reviewer: Yiannakis M. Ioannou, Ph.D. 11/7/90
Section I, Toxicology Branch II (HFAS) (H7509C)

Data Evaluation Report

Study type: Acute dermal-rabbits (81-2) Tox. Chem. No.: 245

MRID number: 411407-09

Test material: Cunapsol 5

Study number: 75-51-0497-88

Testing Facility: Department of the Army
U.S. Army Environmental Hygiene Agency
Aberdeen Proving Ground, Maryland 21010-5422

Title of report: Phase 2 Preliminary Assessment of the Relative Toxicity of Copper Naphthenate. Acute Studies: Acute Oral and Dermal Toxicity Studies

Author(s): R.A. Angerhofer
L.M. Taylor
M.H. Weeks

Report issued: November 12, 1987

Conclusions:

Under the conditions of this study, the acute dermal LD_{50} for Cunapsol 5 could not be determined in male and female rabbits.

Core Classification: supplementary

This study does not fulfill the requirements (81-2) for an acute dermal toxicity study in rabbits

I. MATERIALS

A. Test Material: Cunapsol 5; description: blue-green liquid having a musty odor and a pH of 11.5; purity: not stated

composition: copper hydroxide naphthenic acid-48%
emusifier-25%
water-27%

Lot number: 4121486

B. Test Animals: New Zealand white rabbits; Source, age and weight not provided.

II. METHODS

Five male and five female rabbits were employed in this study. Rabbits. were given commercial laboratory chow and water ad libitum.

Cunapsol 5 was administered as the neat material at a dose of 2000 mg/kg to the shaved backs of male and female rabbits. The treated area was occluded for 24 hours.

III. RESULTS

Dermal application of 2000 mg/kg Cunapsol 5 resulted in the death of 3 of 5 male rabbits, and 2 of 5 female rabbits (time of death not provided). Cunapsol 5 caused slight to moderate dermal irritation, followed by scaling and sloughing of dead skin (time course not provided). Clinical signs of toxicity reported were lethargy and death. Body weights "remained stable between pretest and sacrifice."

IV. CONCLUSIONS

Under the conditions of this study, the acute dermal LD_{50} of Cunapsol 5 could not be determined in male and female rabbits.

V. CORE CLASSIFICATION

supplementary

This study does not fulfill the requirements (81-2) for an acute dermal toxicity study in rabbits. The following information is requested in order to upgrade this study to core minimum:

1) source, age, and weight of animals used

2) statistical method for calculating LD_{50}

3) doses used in LD_{50} study

4) times of death of rabbits which died during the study

5) necropsy data on all rabbits

6) description of occlusive dressing used

7) whether animals were restrained or dressing was sufficient to prevent ingestion of test material

8) percentage of body surface over which test material was applied

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Secondary Reviewer: Yiannakis M. Ioannou, Ph.D. 11/9/90
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Data Evaluation Report

Study type: Acute Inhalation-rats (81-3) Tox. Chem. No.: 245

MRID number: 411407-09

Test material: Cunapsol 5

Study number: 75-51-0497-88

Testing Facility: Department of the Army
U.S. Army Environmental Hygiene Agency
Aberdeen Proving Ground, Maryland 21010-5422

Title of report: Phase 2 Preliminary Assessment of the Relative Toxicity of Copper Naphthenate. Acute Studies: Saturated Vapor Inhalation Studies

Author(s): R.A. Angerhofer
L.M. Taylor
M.H. Weeks

Report issued: November 12, 1987

Conclusions: Under the conditions of this study, the acute inhalation LC_{50} for Cunapsol 5 was > 5 mg/L in male rats. Although significant alterations in brain, liver, and kidney to body weight ratios were observed in animals exposed to Cunapsol 5 vapor generated at 100 ^{o}C, it is felt that these alterations are not of biological significance.

Toxicity Category IV

Core Classification: supplementary

This study does not fulfill the requirements (81-3) for an acute inhalation toxicity study in rats.

I. MATERIALS

A. Test Material: Cunapsol 5; description: a water emulsifiable wood preservative concentrate containing the following:

copper hydroxide naphthenic acid-48%
emulsifier-25%
water-27%

Lot number: 4121486

B. Test Animals: Male Sprague-Dawley rats; Source: not provided. weight: 72-113g.

II. METHODS

A. Atmosphere Generation:

No data on atmosphere generation were provided.

B. Exposure:

Three groups of six male rats were exposed for 8 hours to vapors of Cunapsol 5 generated at room temperature, vapors of Cunapsol 5 generated at 100 ^{o}C, or control atmosphere (temperature not specified). Rats were observed for toxic signs during exposure.

C. Post-Exposure Observations:

No post-exposure observations on clinical toxicity were provided. Body weights were recorded on post-exposure days 1, 3, 7, and 14.

III. RESULTS

A. Atmosphere generation:

Nominal chamber concentrations of Cunapsol 5 were stated as 16.6 mg/L when generated at room temperature and 40.67 mg/L when generated at 100 ^{o}C.

B. Animal observations:

As stated on page 13 of the report, inhalation of Cunapsol 5 vapors generated at

either room temperature or at 100 ^{o}C "did not produce death or outward toxic signs."
No significant body weight alterations were noted between control animals and animals exposed to Cunapsol 5 vapors generated at room temperature. Group mean post-exposure body weights of test animals exposed to Cunapsol 5 vapors generated at 100 ^{o}C were significantly lower than control on post-exposure days 1, 3, and 7. At necropsy, group mean brain to body weight ratio was significantly increased in animals exposed to Cunapsol 5 vapors generated at 100 ^{o}C, and group mean liver and kidney to body weight ratios were significantly decreased in this same group. These findings were based upon statistical analysis performed by the registrant.

IV. CONCLUSIONS

Under the conditions of this study, the acute inhalation LC_{50} for Cunapsol 5 was > 5 mg/L in male rats. Although significant alterations in brain, liver, and kidney to body weight ratios were observed in animals exposed to Cunapsol 5 vapors generated at 100 ^{o}C, it is felt that these alterations are not of biological significance.

Toxicity Category IV

V. CORE CLASSIFICATION- supplementary

This study does not fulfill the requirements (81-3) for an acute inhalation toxicity study in rats. The following information is requested in order to upgrade this study to core minimum:

1) particle size analysis

2) inhalation chamber description

3) temperature and humidity measurement data

4) explanation of statistical analysis

5) gross necropsy data

In addition to the above, it is to be noted that :

1) no female rats were used in this study

2) the weight variation of control rats was > 20% of group mean body weight

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Secondary Reviewer: Yiannakis M. Ioannou, Ph.D. 11/9/90
Section I, Toxicology Branch II (HFAS) (H7509C)

Data Evaluation Report

Study type: Dermal sensitization-guinea pigs (81-6) Tox. Chem. No.: 245

MRID number: 411407-09

Test material: Cunapsol 5

Study number: 75-51-0497-88

Testing Facility: Department of the Army
U.S. Army Environmental Hygiene Agency
Aberdeen Proving Ground, Maryland 21010-5422

Title of report: Phase 2 Preliminary Assessment of the Relative Toxicity of Copper Naphthenate. Acute Studies: Sensitization Studies

Author(s): R.A. Angerhofer
L.M. Taylor
M.H. Weeks

Report issued: November 12, 1987

Conclusions:

Under the conditions of this study, Cunapsol 5 was found to have no dermal sensitizing potential in guinea pigs.

Core Classification: supplementary

This study does not fulfill the requirements (81-6) for a dermal sensitization study in guinea pigs.

I. MATERIALS

A. Test Material: Cunapsol 5; description: a water emulsifiable wood preservative concentrate containing the following:

copper hydroxide naphthenic acid-48%
emulsifier-25%
water-27%

Lot number: 4121486

B. Positive Control Material: DNCB (presumably, 1-chloro-2,4 dinitrobenzene)

C. Test Animals: Hartley guinea pigs; Source, age, sex, and weight not provided.

II. METHODS

A. General: Ten guinea pigs were employed in this study. Guinea pigs were given commercial laboratory chow and water ad libitum.

B. Induction: A minimally irritating concentration of Cunapsol 5 was applied in a volume of 0.5ml to a patch, which was then applied three times weekly to the guinea pigs.

C. Challenge: Challenge application of test material was made after a "13 day rest period" (page 5 of report). A patch containing 0.5ml of the maximum nonirritating concentration of Cunapsol 5 was applied to the guinea pigs. Dermal irritation after challenge exposure to Cunapsol 5 was compared to initial reactions to determine the sensitizing potential of Cunapsol 5.

III. RESULTS

As stated on page 12 of the report, challenge exposure to Cunapsol 5 resulted in irritation scores that were "no greater than those for the initial application." Positive control animals showed "greatly increased" scores after challenge application.

IV. CONCLUSIONS

Under the conditions of this study, Cunapsol 5 was found to have no dermal sensitizing potential in guinea pigs.

V. CORE CLASSIFICATION- supplementary

This study does not fulfill the requirements (81-6) for a dermal sensitization study in guinea pigs. The following information is requested in order to upgrade this study to core minimum:

1) source, age, weight, and sex of animals used

2) data on dermal reactions in test and positive control guinea pigs

3) description of occlusive dressing used

4) site of application of test material

5) duration of induction period as well as duration of each application

6) data on any negative control guinea pigs used

Reviewed by: Timothy F. McMahon, Ph.D. 11/13/90
Section I, Toxicology Branch II (HFAS) (H7509C)
Secondary Reviewer: Yiannakis M. Ioannou, Ph.D. 11/13/90
Section I, Toxicology Branch II (HFAS) (H7509C)

Data Evaluation Report

Study type: Primary dermal irritation-rabbits (81-5) Tox. Chem. No.: 245

MRID number: 411407-09

Test material: Cunapsol 5

Study number: 75-51-0497-88

Testing Facility: Department of the Army
U.S. Army Environmental Hygiene Agency
Aberdeen Proving Ground, Maryland 21010-5422

Title of report: Phase 2 Preliminary Assessment of the Relative Toxicity of Copper Naphthenate. Acute Studies: Primary Skin Irritation Studies

Author(s): R.A. Angerhofer
L.M. Taylor
M.H. Weeks

Report issued: November 12, 1987

Conclusions:

Under the conditions of this study, Cunapsol 5 was determined to be severely irritating to the skin of white rabbits.

Toxicity Catgory I

Core Classification: supplementary

This study does not fulfill the requirements (81-5) for a primary dermal irritation study in rabbits.

It is to be noted pursuant to paragraph (e)(3) of Section 81-4 that data demonstrating the corrosive nature of the test substance may be submitted in lieu of a primary eye irritation study.

I. MATERIALS

A. Test Material: Cunapsol 5; description: blue-green liquid having a musty odor and a pH of 11.5.

composition: copper hydroxide naphthenic acid-48%
emusifier-25%
water-27%

Lot number: 4121486

B. Test Animals: New Zealand white rabbits; Source, age and weight not stated.

II. METHODS

Six rabbits were employed for study of the primary dermal irritation of copper naphthenate. Commercial chow and water were available ad libitum.

Cunapsol 5 was applied as a 25% solution in corn oil in a volume of 0.5 ml to an intact skin site on 3 rabbits, and an abraded skin site on 3 separate rabbits. Skin sites were occluded for 24 hours. Erythema, eschar, and edema were evaluated at 24 hours, 72 hours, and 7 days at intact and abraded skin sites.

III. RESULTS

Dermal irritancy of Cunapsol 5 was similar to that of the emusifier ("Emulsifier B"; appendix E and F of report). At 24 and 72 hours, severe erythema was observed in rabbits with both intact and abraded skin. Very slight to slight edema was observed at 72 hours in rabbits with intact skin, while very slight to moderate edema was observed in rabbits with abraded skin.

IV. CONCLUSIONS

Under the conditions of this study, Cunapsol 5 was determined to be severely irritating to the skin of white rabbits.

Toxicity Category I

V. CORE CLASSIFICATION - supplementary

This study does not fulfill the requirements (81-5) for a primary dermal irritation study in rabbits. The following were omitted from the original study report and should be provided to upgrade this study to core minimum:

1) source of animals

2) whether animals were clipped before application of test material

3) whether the test site was washed after patch removal

4) the device used to occlude the test site

5) whether animals were restrained during and/or after testing

6) a sentence explaining whether the times of observation refer to the time from application or removal of test material.

It is to be noted pursuant to paragraph (e)(3) of Section 81-4 that data demonstrating the corrosive nature of the test substance may be submitted in lieu of a primary eye irritation study.

Reviewed by: Timothy F. McMahon, Ph.D.
Section I, Toxicology Branch II (HFAS) (H7509C)
Secondary Reviewer: Yiannakis M. Ioannou, Ph.D. 11/7/90
Section I, Toxicology Branch II (HFAS) (H7509C)

Data Evaluation Report

Study type: Primary eye irritation-rabbits (81-4) Tox. Chem. No.: 245

MRID number: 411407-09

Test material: Cunapsol 5

Study number: 75-51-0497-88

Testing Facility: Department of the Army
U.S. Army Environmental Hygiene Agency
Aberdeen Proving Ground, Maryland 21010-5422

Title of report: Phase 2 Preliminary Assessment of the Relative Toxicity of Copper Naphthenate. Acute Studies: Eye Irritation Studies

Author(s): R.A. Angerhofer
L.M. Taylor
M.H. Weeks

Report issued: November 12, 1987

Conclusions:

Under the conditions of this study, Cunapsol 5 was determined to be severely irritating to the eyes of white rabbits.

Toxicity Catgory I

Core Classification: supplementary

This study does not fulfill the requirements (81-4) for a primary eye irritation study in rabbits.

It is to be noted pursuant to paragraph (e)(3) of Section 81-4 that data demonstrating the corrosive nature of the test substance may be submitted in lieu of a primary eye irritation study.

V. CORE CLASSIFICATION

supplementary

This study does not fulfill the requirements (81-5) for a primary dermal irritation study in rabbits. The following information is requested in order to upgrade this study to core minimum:

1) source of animals

2) data on rabbit number 2 at 72 hours not provided

3) indicate those rabbits which were subjected to eye washing after application of test material

4) provide data on scores for each subcategory of the Draize scale for each rabbit, and not just summary irritation scores.

It is to be noted pursuant to paragraph (e)(3) of Section 81-4 that data demonstrating the corrosive nature of the test substance may be submitted in lieu of a primary eye irritation study.

I. MATERIALS

A. Test Material: Cunapsol 5; description: blue-green liquid having a musty odor and a pH of 11.5.

composition: copper hydroxide naphthenic acid-48%
emusifier-25%
water-27%

Lot number: 4121486

B. Test Animals: New Zealand white rabbits; Source, age and weight not stated.

II. METHODS

Nine rabbits were employed for study of the primary eye irritation of Cunapsol 5. Commercial chow and water were available ad libitum.

Cunapsol 5 (0.1ml) was instilled into the conjunctival sac of all nine rabbits. After 20 seconds, the test material was washed out of the eyes of three rabbits with warm tap water for one minute. Irritation to the cornea, iris, and conjunctiva was evaluated 24, 48, and 72 hours after instillation of the test material. All rabbits were sacrificed after the 72hr evaluation due to the severity of the ocular reaction.

III. RESULTS

Corneal opacity, iritis, and conjunctival irritation were observed in the test eyes of all rabbits at 24, 48 , and 72 hours. From 24 through 72 hours, corneal opacity and iritis appeared to become progressively worse, as shown by irritation scores (Appendix G of report). Scores for conjunctival irritation were maximal at all times of observation.

IV. CONCLUSIONS

Under the conditions of this study, Cunapsol 5 was determined to be severely irritating to the eyes of white rabbits.

Toxicity Category I

Reviewed by: Timothy F. McMahon, Ph.D. 11/4/90
Section I, Toxicology Branch II (HFAS) (H7509C)
Secondary Reviewer: Yiannakis M. Ioannou, Ph.D. 11/7/90
Section I, Toxicology Branch II (HFAS) (H7509C)

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Data Evaluation Report

Study type: Acute oral-rats (81-1) Tox. Chem. No.: 245

MRID number: 411407-10

Test material: M-Gard W-510

Study number: 75-51-0497-88

Testing Facility: Department of the Army
U.S. Army Environmental Hygiene Agency
Aberdeen Proving Ground, Maryland 21010-5422

Title of report: Phase 3 Preliminary Assessment of the Relative Toxicity of Copper Naphthenate. Acute Studies: Acute Oral and Dermal Toxicity Studies.

Author(s): R.A. Angerhofer
L.W. Metger

Report issued: not provided

Conclusions: Under the conditions of this study, the $AOLD_{50}$ could not be determined in male and female rats.

Core Classification: supplementary

This study does not fulfill the requirements (81-1) for an acute oral toxicity study in rats

I. MATERIALS

A. Test Material: M-gard W-510; description: a water reducible wood preservative concentrate containing copper naphthenate (58%), emulsifier (10%), and hydrocarbon solvent (32%). Undiluted material was a green liquid having a musty odor and pH of 7.2

Lot number: F-17421

B. Test Animals: Sprague-Dawley Wistar derived rats; Source, age and weight not provided.

II. METHODS

Male and female rats were employed in this study for determination of the approximate lethal dose (ALD), defined as "the minimum lethal dose of a compound" (page 4 of registrant report). One animal per sex per dosage level was used to determine the ALD. Rats were given commercial laboratory chow and water ad libitum.

M-Gard W-510 was administered as the neat formulation via a stainless steel stomach tube. Animals were observed for 14 days following dosing for clinical toxicity. Body weights were recorded on days 1, 3, 7, and 14 postexposure. Gross necropsy was performed on all survivors as well as those animals which died during the study.

The components of M-Gard W-510 (Emulsifier "A", mineral spirits, and copper naphthenate) were also tested for the ALD in rats under similar conditions as for M-Gard W-510. Mineral spirits and Emulsifier "A" were adminstered undiluted, while copper naphthenate was administered as a 500mg/ml solution in corn oil.

III. RESULTS

On page 9 of the registrant's report, it is stated that a "few" rats died during the 14 day observation period. Clinical signs were limited to lethargy and death "at relatively high dosages." No clinical signs or deaths were observed from administration of either emulsifier "A" or mineral spirits.

Gross pathological observations on those rats which died during the observation period showed "gastric and intestinal lesions which could be attributed to the corrosive nature of the compound administered." No gross pathological findings were observed in surviving rats.

IV. CONCLUSIONS

Under the conditions of this study, the $AOLD_{50}$ could not be determined in male and female rats.

V. CORE CLASSIFICATION

supplementary

This study does not fulfill the requirements (81-1) for an acute oral toxicity study in rats. The following information is requested in order to upgrade this study to core minimum:

1) source, age, and weight of animals used

2) times of death and numbers of rats which died during the study

3) necropsy data on all rats

4) justification for and data on oral toxicity ($AOLD_{50}$) data in rabbits (page 4 of report)

Reviewed by: Timothy F. McMahon, Ph.D.
Section I, Toxicology Branch II (HFAS) (H7509C)
Secondary Reviewer: Yiannakis M. Ioannou, Ph.D.
Section I, Toxicology Branch II (HFAS) (H7509C)

Data Evaluation Report

Study type: Acute dermal-rabbits (81-2) Tox. Chem. No.: 245

MRID number: 411407-10

Test material: M-Gard W-510

Study number: 75-51-0497-88

Testing Facility: Department of the Army
U.S. Army Environmental Hygiene Agency
Aberdeen Proving Ground, Maryland 21010-5422

Title of report: Phase 3 Preliminary Assessment of the Relative Toxicity of Copper Naphthenate. Acute Studies: Acute Oral and Dermal Toxicity Studies

Author(s): R.A. Angerhofer
L.W. Metger

Report issued: not provided

Conclusions:

Under the conditions of this study, the acute dermal LD_{50} for M-Gard W-510 was > 2000 mg/kg in male and female rabbits.

Toxicity Category III

Core Classification: supplementary

This study does not fulfill the requirements (81-2) for an acute dermal toxicity study in rabbits.

I. MATERIALS

A. Test Material: M-gard W-510; description: a water reducible wood preservative concentrate containing copper naphthenate (58%), emulsifier (10%), and hydrocarbon solvent (32%). Undiluted material was a green liquid having a musty odor and pH of 7.2

B. Test Animals: New Zealand white rabbits; Source, age and weight not provided.

II. METHODS

Five male and five female rabbits were employed in this study. Rabbits were given commercial laboratory chow and water ad libitum.

M-Gard W-510 was administered as the neat solution at a dose of 2000 mg/kg to the shaved backs of male and female rabbits. The treated area was occluded for 24 hours. Clinical toxicity and death were monitored over a 14 day post-exposure period.

III. RESULTS

No deaths were reported from dermal application of of 2000 mg/kg M-Gard W-510. M-Gard W-510 caused slight to moderate dermal irritation, followed by scaling and sloughing of dead skin (time course not provided). No other clinical signs of toxicity were reported. Body weights "remained stable between pretest and sacrifice."

IV. CONCLUSIONS

Under the conditions of this study, the acute dermal LD_{50} for M-Gard W-510 was > 2000 mg/kg in male and female rabbits.

Toxicity Category III

V. CORE CLASSIFICATION

supplementary

This study does not fulfill the requirements (81-2) for an acute dermal toxicity study in rabbits. The following information is requested in order to upgrade this study to core minimum:

1) source, age, and weight of animals used

2) necropsy data on all rabbits

3) description of occlusive dressing used

4) whether animals were restrained or dressing was sufficient to prevent ingestion of test material

5) percentage of body surface over which test material was applied

Reviewed by: Timothy F. McMahon, Ph.D. [illegible] 11/2/90
Section I, Toxicology Branch II (HFAS) (H7509C)
Secondary Reviewer: Yiannakis M. Ioannou, Ph.D. 11/7/90
Section I, Toxicology Branch II (HFAS) (H7509C)

Data Evaluation Report

Study type: Acute Inhalation-rats (81-3) Tox. Chem. No.: 245

MRID number: 411407-10

Test material(s): M-Gard W-510, emulsifier "A", copper naphthenate

Study number: 75-51-0497-88

Testing Facility: Department of the Army
U.S. Army Environmental Hygiene Agency
Aberdeen Proving Ground, Maryland 21010-5422

Title of report: Phase 3 Preliminary Assessment of the Relative Toxicity of Copper Naphthenate. Acute Studies: Saturated Vapor Inhalation Studies

Author(s): R.A. Angerhofer
L.W. Metger

Report issued: not provided

Conclusions: Under the conditions of this study, the acute inhalation LC_{50} for M-Gard W-510 was > 5 mg/L in male rats when exposed to test material vapors generated at room temperature. Chamber concentrations of emulsifier "A" and copper naphthenate were inadequate for acute inhalation toxicity studies.

Toxicity Category IV

Core Classification: supplementary

This study does not fulfill the requirements (81-3) for an acute inhalation toxicity study in rats.

I. MATERIALS

A. Test Material(s): 1) M-gard W-510; description: a water reducible wood preservative concentrate containing copper naphthenate (58%), emulsifier (10%), and hydrocarbon solvent (32%). Undiluted material was a green liquid having a musty odor and pH of 7.2 Lot number: F-17421

2) emulsifier "A"; description: proprietary material

3)copper naphthenate; description: dark blue-green amorphous solid having a musty odor

B. Test Animals: Male Sprague-Dawley rats; Source: not provided. weights:

1) copper naphthenate: 94-120g

2) M-Gard W-510: 86-103g

3) emulsifier "A": 85-101g

II. METHODS

A. Atmosphere Generation:

No data on atmosphere generation were provided.

B. Exposure:

Rats were weighed before test article exposure. Groups of six male rats were exposed for 8 hours to vapors of each test compound generated at room temperature, vapors of each test compound generated at at 100 ^{o}C, or a control atmosphere (temperature not specified). In addition, two separate groups of six rats were exposed to vapors of M-Gard W-510 generated at 50 ^{o}C for 8 and 2 hours, respectively. Rats were observed for toxic signs during exposure.

C. Post-Exposure Observations:

No post-exposure observations on clinical toxicity were provided. Body weights were recorded on post-exposure days 1, 3, 7, and 14.

III. RESULTS

A. Atmosphere generation:

Nominal chamber concentrations of copper naphthenate were stated as less than 0.5 mg/L when generated at both room temperature and 100 °C. Chamber concentration of M-Gard W-510 generated at room temperature was stated as 5.46 mg/L, 16.75 mg/L when generated at 100 °C, and 14.48 mg/L when generated at 50°C. A two-hour exposure to M-Gard W-510 vapor generated at 50 °C produced a nominal chamber concentration of 13.67 mg/L.

Little or no vaporization was acheived with emulsifier "A".

Animal observations:

No adverse effects were noted in rats exposed to copper naphthenate vapor generated at either room temperature or 100 °C. (page 11 of registrant report). No statements were made concerning clinical toxicity from exposure to either M-Gard W-510 or emulsifier "A".

No significant body weight alterations were noted between control animals and animals exposed to copper naphthenate vapors generated at room temperature or 100 °C. Organ to body weight ratios were also unaffected in these test groups.

No significant body weight or organ to body weight changes were observed in rats exposed to M-Gard W-510 vapors generated at room temperature.

Four of six rats exposed to M-Gard W-510 vapors generated at 100°C were dead on day 1 post-exposure (page K-1 of registrant report). Five of six animals exposed to M-Gard W-510 vapors generated at 50 °C were dead on day 1 post-exposure. Two-hour exposure to M-Gard W-510 vapors generated at 50 °C did not produce any adverse effects.

IV. CONCLUSIONS

Under the conditions of this study, the acute inhalation LC_{50} for M-Gard W-510 was > 5 mg/L in male rats when exposed to test material generated at room temperature. Chamber concentrations of emulsifier "A" and copper naphthenate were inadequate for acute inhalation toxicity studies.
Toxicity Category IV

V. CORE CLASSIFICATION- supplementary

This study does not fulfill the requirements (81-3) for an acute inhalation toxicity study in rats. The following information is requested in order to upgrade this study to core minimum:

1) particle size analysis for each test compound

2) inhalation chamber description for each test compound

3) temperature and humidity measurement data for each test compound

4) explanation of statistical analyses

5) gross necropsy data

In addition to the above, it is to be noted that :

1) no female rats were used in this study

2) the nominal concentration of copper naphthenate achieved in this study (0.5 mg/L) was well below the limit dose concentration of 5 mg/L.

Reviewed by: Timothy F. McMahon, Ph.D.
Section I, Toxicology Branch II (HFAS) (H7509C)
Secondary Reviewer: Yiannakis M. Ioannou, Ph.D. 11/3/90
Section I, Toxicology Branch II (HFAS) (H7509C)

Data Evaluation Report

Study type: Dermal sensitization-guinea pigs (81-6) Tox. Chem. No.: 245

MRID number: 411407-10

Test material: M-Gard W-510

Study number: 75-51-0497-88

Testing Facility: Department of the Army
U.S. Army Environmental Hygiene Agency
Aberdeen Proving Ground, Maryland 21010-5422

Title of report: Phase 3 Preliminary Assessment of the Relative Toxicity of Copper Naphthenate. Acute Studies: Sensitization Studies

Author(s): R.A. Angerhofer
L.W. Metger

Report issued: not provided

Conclusions:

Under the conditions of this study, M-Gard W-510 was found to have no dermal sensitizing potential in guinea pigs.

Core Classification: supplementary

This study does not fulfill the requirements (81-6) for a dermal sensitization study in guinea pigs.

I. MATERIALS

A. Test Material: M-gard W-510; description: a water reducible wood preservative concentrate containing copper naphthenate (58%), emulsifier (10%), and hydrocarbon solvent (32%). Undiluted material was a green liquid having a musty odor and pH of 7.2

Lot number: F-17421

B. Positive Control Material: DNCB (dinitrochlorobenzene)

C. Test Animals: Hartley guinea pigs; Source, age, sex, and weight not provided.

II. METHODS

A. General: Ten guinea pigs were employed in this study. Guinea pigs were given commercial laboratory chow and water ad libitum.

B. Induction: A minimally irritating concentration of M-Gard W-510 (25% in corn oil) was applied in a volume of 0.5ml to a patch, which was then applied three times weekly to the guinea pigs.

C. Challenge: Challenge application of test material was made after a "13 day rest period" (page 5 of report). A patch containing 0.5ml of the maximum nonirritating concentration of M-Gard W-510 was applied to the guinea pigs. Dermal irritation after challenge exposure to M-Gard W-510 was compared to initial reactions to determine the sensitizing potential of M-Gard W-510.

III. RESULTS

As stated on page 10 of the report, challenge exposure to M-Gard W-510 resulted in irritation scores that were "no greater than those for the initial application." Positive control animals showed "greatly increased" scores after challenge application.

IV. CONCLUSIONS

Under the conditions of this study, M-Gard W-510 was found to have no dermal sensitizing potential in guinea pigs.

V. CORE CLASSIFICATION- supplementary

This study does not fulfill the requirements (81-6) for a dermal sensitization study in guinea pigs. The following information is requested in order to upgrade this study to core minimum:

1) source, age, weight, and sex of animals used

2) data on dermal reactions in test and positive control guinea pigs

3) description of occlusive dressing used

4) site of application of test material

5) duration of induction period as well as duration of each application

6) data on any negative control guinea pigs used

Reviewed by: Timothy F. McMahon, Ph.D.
Section I, Toxicology Branch II (HFAS) (H7509C)
Secondary Reviewer: Yiannakis M. Ioannou, Ph.D. YMI 11/9/90
Section I, Toxicology Branch II (HFAS) (H7509C)

Data Evaluation Report

Study type: Primary dermal irritation-rabbits (81-5) Tox. Chem. No.: 245

MRID number: 411407-10

Test material: Emulsifier "A"

Study number: 75-51-0497-88

Testing Facility: Department of the Army
U.S. Army Environmental Hygiene Agency
Aberdeen Proving Ground, Maryland 21010-5422

Title of report: Phase 3 Preliminary Assessment of the Relative Toxicity of Copper Naphthenate, (Mooney Chemicals), Acute Studies: Primary Skin Irritation Studies

Author(s): R.A. Angerhofer
L.W. Metger

Report issued: not provided

Conclusions:

Under the conditions of this study, Emulsifier "A" was determined to be non-irritating to the skin of white rabbits.

Toxicity Catgory IV

Core Classification: supplementary

This study does not fulfill the requirements (81-5) for a primary dermal irritation study in rabbits.

I. MATERIALS

A. Test Material: Emulsifier "A"; description: proprietary compound

B. Test Animals: New Zealand white rabbits; Source, age and weight not stated.

II. METHODS

Six rabbits were employed for study of the primary dermal irritation of Emulsifier "A". Commercial chow and water were available ad libitum. Animals were housed individually in wire cages.

Emulsifier "A" was applied in a volume of 0.5 ml to an intact skin site on 3 rabbits, and an abraded skin site on 3 separate rabbits. Skin sites were occluded for 24 hours. Erythema, eschar, and edema were evaluated at 24 hours, 72 hours, and 7 days at intact and abraded skin sites.

III. RESULTS

No evidence of dermal irritation from Emulsifier "A" was reported in either intact or abraded skin at 72 hours.

IV. CONCLUSIONS

Under the conditions of this study, Emulsifier "A" was non-irritating to the skin of white rabbits.

Toxicity Category IV

V. CORE CLASSIFICATION

supplementary

This study does not fulfill the requirements (81-5) for a primary dermal irritation study in rabbits. The following are requested to upgrade this study to core minimum:

1) source of animals

2) whether animals were clipped before application of test material

3) whether the test site was washed after patch removal

4) the device used to occlude the test site

5) whether animals were restrained during and/or after testing

6) a sentence explaining whether the times of observation refer to the time from the application or removal of test material.

Reviewed by: Timothy F. McMahon, Ph.D.
Section I, Toxicology Branch II (HFAS) (H7509C)
Secondary Reviewer: Yiannakis M. Ioannou, Ph.D. 11/9/90
Section I, Toxicology Branch II (HFAS) (H7509C)

Data Evaluation Report

Study type: Primary dermal irritation-rabbits (81-5) Tox. Chem. No.: 245

MRID number: 411407-10

Test material: mineral spirits

Study number: 75-51-0497-88

Testing Facility: Department of the Army
U.S. Army Environmental Hygiene Agency
Aberdeen Proving Ground, Maryland 21010-5422

Title of report: Phase 3 Preliminary Assessment of the Relative Toxicity of Copper Naphthenate, (Mooney Chemicals), Acute Studies: Primary Skin Irritation Studies

Author(s): R.A. Angerhofer
L.W. Metger

Report issued: not provided

Conclusions:

Under the conditions of this study, mineral spirits was determined to be slightly irritating to the skin of white rabbits.

Toxicity Catgory III

Core Classification: supplementary

This study does not fulfill the requirements (81-5) for a primary dermal irritation study in rabbits.

I. MATERIALS

A. Test Material: mineral spirits

B. Test Animals: New Zealand white rabbits; Source, age and weight not stated.

II. METHODS

Six rabbits were employed for study of the primary dermal irritation of mineral spirits. Commercial chow and water were available ad libitum. Animals were housed individually in wire cages.

Mineral spirits were applied in a volume of 0.5 ml to an intact skin site on 3 rabbits, and an abraded skin site on 3 separate rabbits. Skin sites were occluded for 24 hours. Erythema, eschar, and edema were evaluated at 24 hours, 72 hours, and 7 days at intact and abraded skin sites.

III. RESULTS

At 72 hours, very slight erythema and eschar formation was observed on intact skin resulting from application of mineral spirits. Erythema and eschar formation on abraded skin at 72 hours was also graded as very slight

No edema formation was observed at 72 hours from application of mineral spirits to either intact or abraded skin.

IV. CONCLUSIONS

Under the conditions of this study, mineral spirits was determined to be slightly irritating to the skin of white rabbits.

Toxicity Category III

V. CORE CLASSIFICATION

supplementary

This study does not fulfill the requirements (81-5) for a primary dermal irritation study in rabbits. The following are requested to upgrade this study to core minimum:

1) source of animals

2) whether animals were clipped before application of test material

3) whether the test site was washed after patch removal

4) the device used to occlude the test site

5) whether animals were restrained during and/or after testing

6) a sentence explaining whether the times of observation refer to the time from the application or removal of test material.

Reviewed by: Timothy F. McMahon, Ph.D. 11/6/90
Section I, Toxicology Branch II (HFAS) (H7509C)
Secondary Reviewer: Yiannakis M. Ioannou, Ph.D. 11/7/90
Section I, Toxicology Branch II (HFAS) (H7509C)

Data Evaluation Report

Study type: Primary dermal irritation-rabbits (81-5) Tox. Chem. No.: 245

MRID number: 411407-10

Test material: M-Gard W-510

Study number: 75-51-0497-88

Testing Facility: Department of the Army
U.S. Army Environmental Hygiene Agency
Aberdeen Proving Ground, Maryland 21010-5422

Title of report: Phase 3 Preliminary Assessment of the Relative Toxicity of Copper Naphthenate, (Mooney Chemicals), Acute Studies: Primary Skin Irritation Studies

Author(s): R.A. Angerhofer
L.W. Metger

Report issued: not provided

Conclusions:

Under the conditions of this study, M-Gard W-510 was determined to be moderately irritating to the skin of white rabbits.

Toxicity Catgory III

Core Classification: supplementary

This study does not fulfill the requirements (81-5) for a primary dermal irritation study in rabbits.

I. MATERIALS

A. Test Material: M-gard W-510; description: a water reducible wood preservative concentrate containing copper naphthenate (58%), emulsifier (10%), and hydrocarbon solvent (32%). Undiluted material was a green liquid having a musty odor and pH of 7.2

B. Test Animals: New Zealand white rabbits; Source, age and weight not stated.

II. METHODS

Six rabbits were employed for study of the primary dermal irritation of M-Gard W-510. Commercial chow and water were available ad libitum. Animals were housed individually in wire cages.

Application of M-Gard W-510 as neat material was not possible due to staining of the skin site. Thus, M-Gard W-510 was applied as a 25% solution in corn oil in a volume of 0.5 ml to an intact skin site on 3 rabbits, and an abraded skin site on 3 separate rabbits. Skin sites were occluded for 24 hours. Erythema, eschar, and edema were evaluated at 24 hours, 72 hours, and 7 days at intact and abraded skin sites.

III. RESULTS

At 72 hours, well-defined to moderate erythema and eschar formation was reported on intact skin from application of 25% M-gard W-510 in corn oil. Erythema and eschar ranged from very slight to severe on abraded skin at 72 hours.

Edema formation at 72 hours resulting from application of 25% M-Gard W-510 in corn oil was reported as very slight to moderate. Edema formation on abraded skin at 72 hours was reported as non-existent to very slight. No other toxicities were reported in this study.

IV. CONCLUSIONS

Under the conditions of this study, M-Gard W-510 was determined to be moderately irritating to the skin of white rabbits.

Toxicity Category III

V. CORE CLASSIFICATION

supplementary

This study does not fulfill the requirements (81-5) for a primary dermal irritation study in rabbits. The following are requested to upgrade this study to core minimum:

1) source of animals

2) whether animals were clipped before application of test material

3) whether the test site was washed after patch removal

4) the device used to occlude the test site

5) whether animals were restrained during and/or after testing

6) a sentence explaining whether the times of observation refer to the time from the application or removal of test material.

Reviewed by: Timothy F. McMahon, Ph.D.
Section I, Toxicology Branch II (HFAS) (H7509C)
Secondary Reviewer: Yiannakis M. Ioannou, Ph.D. 11/7/90
Section I, Toxicology Branch II (HFAS) (H7509C)

Data Evaluation Report

Study type: Primary eye irritation-rabbits (81-4) Tox. Chem. No.: 245

MRID number: 411407-10

Test material: M-Gard W-510

Study number: 75-51-0497-88

Testing Facility: Department of the Army
U.S. Army Environmental Hygiene Agency
Aberdeen Proving Ground, Maryland 21010-5422

Title of report: Phase 3 Preliminary Assessment of the Relative Toxicity of Copper Naphthenate. Acute Studies: Eye Irritation Studies

Author(s): R.A. Angerhofer
L.W. Metger

Report issued: not provided

Conclusions: Under the conditions of this study, M-Gard W-510 was determined to be non-irritating to the eyes of white rabbits.

Toxicity Category IV

Core Classification: supplementary

This study does not fulfill the requirements (81-4) for a primary eye irritation study in rabbits.

I. MATERIALS

A. Test Material: M-gard W-510; description: a water reducible wood preservative concentrate containing copper naphthenate (58%), emulsifier (10%), and hydrocarbon solvent (32%). Undiluted material was a green liquid having a musty odor and pH of 7.2

B. Test Animals: New Zealand white rabbits; Source, age, sex and weight not stated.

II. METHODS

Nine rabbits were employed for study of the primary eye irritation of M-Gard W-510. Commericial chow and water were available ad libitum.

A 25% solution of M-Gard W-510 in corn oil in a volume of 0.1 ml was instilled into the conjunctival sac of the eye (right or left not specified) of all nine rabbits. Test material was washed from the eyes of three rabbits after 20 seconds with warm distilled water for 1 minute. Ocular reaction was determined in all nine rabbits 24, 48, and 96 hours following application of M-Gard W-510.

III. RESULTS

No ocular irritation was observed in unwashed or washed eyes of all rabbits at any time following ocular instillation of M-Gard W-510. No other toxicities were reported for M-Gard W-510.

IV. CONCLUSIONS

Under the conditions of this study, M-Gard W-510 was determined to be non-irritating to the eyes of white rabbits.

Toxicity Category IV

V. CORE CLASSIFICATION

supplementary

This study does not fulfill the requirements (81-4) for a primary eye irritation study in rabbits. The following were omitted from the original study report and should be included to upgrade this study to core minimum:

1) source and sex of animals

2) description of the method used to score irritation (fluorescein, etc.)

3) explanation for the 7-day data in Appendix H when animals were euthanized at 96 hours.

EPA No.: 68D80056
DYNAMAC No.: 250-A
TASK No.: 2-50A
June 28, 1990

DATA EVALUATION RECORD

COPPER NAPHTHENATE

Mutagenicity--Unscheduled DNA Synthesis
in Primary Rat Hepatocytes

APPROVED BY:

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

Signature: William L. McLellan for

Date: 6-28-90

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EPA No.: 68D80056
DYNAMAC No.: 250-A
TASK No.: 2-50A
June 28, 1990

DATA EVALUATION RECORD

COPPER NAPHTHENATE

Mutagenicity--Unscheduled DNA Synthesis
in Primary Rat Hepatocytes

REVIEWED BY:

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

Signature: Nancy E. McCarroll
Date: 6-28-90

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

Signature: William L. McLellan for
Date: 6-28-90

APPROVED BY:

Roman J. Pienta, Ph.D.
Department Manager
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Signature: William L. McLellan for
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EPA Reviewer, Section I
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Signature: Stephen C. Dapson
Date: 1 November 90

Mike Ioannou, Ph.D.
EPA Section Head, Section I
Toxicology Branch II
(H-7509C)

Signature: J M Ioannou
Date: 11/2/90

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DATA EVALUATION RECORD

CHEMICAL: Copper naphthenate.

STUDY TYPE: Mutagenicity--Unscheduled DNA synthesis in primary rat hepatocytes.

MRID NUMBER: 411407-01.

TEST MATERIAL: Copper naphthenate.

SYNONYM: None listed.

SPONSOR: U.S. Army Environmental Hygiene Agency, Aberdeen Proving Ground, MD/Toxikon Corp., Woburn, MA.

TESTING FACILITY: SITEK Research Laboratories, Rockville, MD.

TITLE OF REPORT: Test for Chemical Induction of Unscheduled DNA Synthesis in Rat Primary Hepatocyte Cultures by Autoradiography.

AUTHOR: Thilagar, A.

STUDY NUMBER: 0088-5100.

REPORT ISSUED: October 25, 1988.

CONCLUSIONS/Executive Summary: Three doses of copper naphthenate (10, 15, and 20 µg/mL) were evaluated for the potential to induce unscheduled DNA synthesis (UDS) in primary rat hepatocytes. Concentrations ≥50 µg/mL were insoluble; the highest scored level (20 µg/mL) induced an ≈50% reduction in cell survival but was not genotoxic. As the dose decreased, however, increased average nuclear grain counts, cytoplasmic grain counts, mean net nuclear grain counts, and the percentage of nuclei with ≥5 grains were observed. The finding at the lowest dose (10 µg/mL) was considered "significant" by the study author. We assess, therefore, that copper naphthenate gave a presumptive positive response in this test system. The assay should be repeated to determine the reproducibility of the results. We conclude that the study is unacceptable because definitive results were not obtained, test material purity was not reported, and there were no analytical data to support actual concentration in solution.

Study Classification: The study is unacceptable.

A. MATERIALS:

1. Test Material:

Name: Copper naphthenate.
Description: Black, viscous liquid.
Batch No.: P-17453
Purity: Not reported.
Contaminants: None listed.
Solvent Used: Acetone.
Other comments: The test material was stored at room temperature; solutions of the test material were prepared immediately prior to use. According to the raw data, 55.9 mg of the test material was "moderately soluble in 0.8 to 1.2 mL of acetone and doses ≥50 µg/mL precipitated in the culture medium."

2. Indicator Cells: Primary rat hepatocytes were obtained by the *in situ* perfusion of the livers of adult male Sprague-Dawley rats (200-325 g) obtained from Charles River Laboratories, Inc. 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 WME buffered with HEPES (pH 7.3) and supplemented with 2 mM L-glutamine, collagenase (100 units/mL, type I), and antibiotics. Livers were excised, cleaned of extraneous tissue, shaken in the

collagenase perfusion solution, and passed through a stainless steel sieve to release the hepatocytes.

b. Hepatocyte Harvest/Culture Preparation: Prior to treatment, the isolated hepatocytes were cultured in WME buffered with 0.01M HEPES (pH 7.3) and supplemented with 10% heat-inactivated FBS, 2mM L-glutamine, 100 units/mL penicillin, and 100 ug/mL streptomycin. Recovered cells were collected, counted, and seeded at a density of 2.5×10^5 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 incubated for ≈2 hours, washed, and refed prior to use.

4. Positive Controls: The positive control, 2-acetylaminofluorene (2AAF), was dissolved in ethanol to yield final concentrations of 2.0 and 10.0 µg/mL.

B. STUDY DESIGN:

1. Preliminary Cytotoxicity Assay: Duplicate cultures of cells, initiated from primary cultures, were exposed to 10 doses of the test material ranging from 0.05 to 500 µg/mL, the negative control (WME), or the solvent (acetone) control, for 18 to 20 hours. Following exposure, cell viability was determined by trypan blue exclusion. Based on these results, five doses were selected for the UDS assay.

2. UDS Assay:

a. Treatment/Slide Preparation: Nine prepared hepatocyte cultures (three cultures seeded into tissue culture dishes and six cultures seeded onto coverslips) were exposed for 18 hours to the selected doses of the test material, the negative (WME), the solvents (acetone for the test material and ethanol for the positive control), or the positive (2 and 10 µg/mL 2AAF) controls. The treatment medium, serum-free WME contained 10 µCi/mL [H^3]thymidine. Monolayers grown directly on dishes were used to assess cytotoxicity as described for the preliminary cytotoxicity assay.

Treated hepatocytes attached to coverslips were used for the UDS assay and were washed, swollen with 1% sodium citrate, fixed (methanol-glacial acetic acid), dried, and mounted.

b. Preparation of Autoradiographs/Grain Development: Slides were dipped into Kodak NTB emulsion, dried for 90 minutes, and stored in a refrigerator in desiccated slide boxes for 8 days. Slides were developed in Kodak D-19, fixed, stained with hematoxylin, mounted, coded, and counted.

c. Grain Counting: The nuclear grains of 300 randomly selected cells with normal morphology (50/slide) from the three highest scorable test doses, negative, solvent, and positive control groups were scored for incorporation of [^{3}H]thymidine into DNA. Net nuclear grain counts were determined by subtracting the nuclear grain count of each cell from the average cytoplasmic grain count of three nuclear-sized areas adjacent to each nucleus.

The means of net nuclear grain counts and the standard error of the means were calculated for each treatment group.

Additionally, 300 randomly selected cells were scored to determine the percentage of nuclei exhibiting S-phase DNA synthesis.

3. Evaluation Criteria:

a. Assay Validity: For the assay to be considered valid, the following criteria must be satisfied: (1) the average net nuclear grain count per cell in the negative controls (untreated and solvents) should be <5 and the percentage of cells with ≥5 net nuclear grain counts should be <20%; (2) average net nuclear grain count for the positive control should be ≥20 and at least 30% of the cells should have ≥5 net nuclear grains; (3) at least one test concentration should show a >25% reduction in growth; and (4) at least 0.2% of the negative control nuclei should show replicative DNA synthesis.

b. Positive Responses: 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 a "significant" increase in the mean net nuclear grain count over the concurrent solvent control. In the absence of a dose-related effect, a compound that showed nuclear grain counts that were "significantly" increased compared to the concurrent solvent control over two successive doses was also considered positive.

C. REPORTED RESULTS:

1. Preliminary Cytotoxicity Assay: Ten doses (0.05 to 500 µg/mL) of the test material were examined in the cytotoxicity assay.

   No cells survived exposure to test material doses ranging from 50 to 500 µg/mL. At 10 µg/mL, 73.8% of the cells survived; below this concentration the test material was not cytotoxic. Based on these findings, the five doses selected for the UDS assay were 1, 5, 10, 15, and 20 µg/mL of copper naphthenate.

2. UDS Assay: Representative results from the UDS and parallel cytotoxicity assay are presented in Table 1. Cell survival was dose related and ranged from 56.4% at the highest assayed dose (20 µg/mL) to 100% at the lowest dose (1 µg/mL). UDS activity was, therefore, scored for cultures exposed to the three highest doses (10, 15, and 20 µg/mL). Results for the slide analysis indicated no appreciable increase in UDS activity in cultures exposed to the high dose. However, as the dose decreased, the average nuclear grain count, the average cytoplasmic grain count (these values were calculated by our reviewers), the mean net nuclear grain count, and the percentage of nuclei with $\geq$5 grains increased. The highest values for these parameters were calculated for the lowest test material dose (10 µg/mL). The study author concluded "the test article caused a marginal positive response in this study."

D. REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS:

We assess that a genotoxic effect is indicated by the data from the low-dose group of copper naphthenate (10 µg/mL). However, the accompanying increase in background cytoplasmic grain counts may be related to technical problems with staining. The study author did not report test material insolubility at concentrations below 50 µg/mL; therefore, the increase in background counts cannot be attributed to test material precipitation. Since background counts were lower at the highest concentration as compared to the doses, compound insolubility probably was not a contributing factor.

We assess that the investigators should have scored lower doses to determine if the effect was dose dependent; the assay should have been repeated to determine if the effect on UDS was reproducible. We conclude, therefore, that copper naphthenate is presumptively positive in this test system; therefore, the study should be repeated to determine if the response was dose dependent and whether the increase in mean net nuclear grain counts at 10 µg/mL was valid or resulted from technical difficulties.

TABLE 1. Representative Results of the Unscheduled DNA Synthesis Rat Hepatocyte Assay with Copper Naphthenate

Treatment	Dose (µg/mL)	Cells Scored	Relative Percent Survival	Average[a] Nuclear Grain Count	Average[a] Cytoplasmic Grain Count	Mean Net Nuclear Grain Count ± S.E.	Percent Nuclei w/ ≥5 Grains
Negative Control							
Culture medium	--	300	105.1	5.11	5.49	-0.38±0.63	1.7
Solvent Control							
Acetone (test material)	--	300	100.0	4.00	4.32	-0.32±0.14	0.0
Ethanol (positive control)	--	300	100.0	6.46	6.83	-0.37±0.33	1.0
Positive Control							
2-Acetylamino-fluorene	10	300	50.0	47.83	6.96	40.87±1.79	100.0
Test Material							
Copper naphthenate	10	300	71.8	16.25	11.90	4.35±1.71	40.0
	15	300	61.5	12.85	11.48	1.37±0.45	17.6
	20	300	56.4	6.90	7.10	-0.20±0.42	1.0

[a]Values tabulated by our reviewers from the raw data.

We noted a discrepancy between doses identified on the scoring code sheet (15, 12.5, and 10 µg/mL, see Appendix B, CBI p. 26) and the reported doses. However, all other raw data entries supported the dose levels used in this study (i.e., 10, 15, and 20 µg/mL). The lack of a definitive response in conjunction with missing information on test material purity and supporting analytical data to confirm actual concentrations renders the study unacceptable.

E. QUALITY ASSURANCE MEASURES: A quality assurance statement was signed and dated October 25, 1988.

F. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 9-14. Appendix B, Appendix I of the CBI, Score Sheets, CBI p. 26.

Appendix A

Materials and Methods
(CBI pp. 9-14)

MATERIALS AND METHODS

INDICATOR CELLS

Source

Male Sprague-Dawley rats weighing approximately 200-325 g were received from Charles River Breeding Laboratories, Raleigh, North Carolina, quarantined for at least a week, and housed in the animal room according to the Standard Operating Procedures of SITEK Research Laboratories. Primary hepatocytes were obtained from these animals by in situ collagenase perfusion of the liver.

Culture Conditions

Prior to treatment, the isolated primary hepatocytes were cultured in Williams' Medium E (WME) buffered with 0.01M HEPES (pH 7.3) and supplemented with 10% heat-inactivated fetal bovine serum (HIFBS), 2mM L-glutamine, 100 units/ml penicillin and 100 ug/ml streptomycin. During the treatment period, the primary hepatocytes were cultured in WME buffered with HEPES (pH 7.3) and supplemented with 2mM L-glutamine, 100 units/ml penicillin and 100 ug/ml streptomycin. The cells were cultured in a humidified incubator at 37 ±1.0°C in an atmosphere of approximately 5% CO_2 and 95% air.

CONTROL SUBSTANCES

Positive Control

2-Acetylaminofluorene (2AAF), which induces unscheduled DNA synthesis, was used as the positive control substance. The source and purity of the 2AAF used in this study are given below.

Source: SIGMA Chemical Co. Lot #: 84F-3749

CAS Registry Number: 53-96-3

Purity: 95-97%

Solvent Controls

The following solvents used for dissolving the test article and positive control substance served as the solvent controls.

Acetone was used to prepare the test article stock solutions.

The source and lot number of the acetone used in this study are given below:

Source: Fisher Scientific Lot #: 871653

CAS Registry Number: 67-64-1

Certificate of Actual Lot Analysis

Assay	99.5%
Density (grams/ml) at 25°C	0.7852
Residue after evaporation	0.0002%
Color (APHA)	5
Water	0.5%

The positive control substance 2AAF was dissolved in ethanol. The source of the ethanol used in this study is given below:

Source: Clear Spring Distilling Co. Lot #: 18-3400, 18-1100

CAS Registry Number: 67-17-5

TEST ARTICLE

The test article Copper Naphthenate was received on July 8, 1988, and stored at room temperature. The test article was weighed, diluted with acetone, and mixed thoroughly to prepare the stock solutions. The lot number, purity and physical description of the test article were not provided by the Sponsor.

EXPERIMENTAL PROCEDURES

Preparation of Hepatocyte Cultures

The methods used for the isolation of the hepatocytes are modifications of the procedures used by G. M. Williams (1,2) and J. Bradlaw (3).

Separate hepatocyte preparations (one rat per preparation) were used for the Range Finding Test and the UDS Assay. The animal was anesthetized by inhalation of Metofane and dissected to expose the liver. Perfusion of the liver was performed as follows: A 21-gauge needle was inserted into the hepatic portal vein, and prior to initiating the perfusion, the inferior vena cava was clamped. The liver was perfused at the rate of 8-10 ml/minute for 1-2 minutes with ethylene glycol-bis-(B-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) solution [0.5mM in

Ca++-free and Mg++-free Hanks' Balanced Salt Solution (HBSS) buffered with 0.01M HEPES (pH 7.3)]. After this period, the heart was punctured and the inferior vena cava was cut below the clamp. The perfusion rate was then increased to approximately 20 ml/minute. After perfusing about 120 ml of EGTA solution, 250 ml of collagenase solution [100 units of collagenase type I/ml of serum-free WME buffered with HEPES (pH 7.3) and supplemented with 2mM L-glutamine, 100 units/ml penicillin and 100 ug/ml streptomycin] was perfused through the liver at the rate of 20 ml/minute.

The liver was then removed from the animal, trimmed of excess fat and connective tissue, and washed with ice-cold collagenase solution. The liver capsule was opened at numerous points, and the cells were removed using a stainless steel sieve. The cells were pooled, and a viable cell count was performed using the trypan blue dye exclusion method. Only cultures having more than 50% viable cells were accepted for the assay. One ml cell suspensions containing 250,000 cells were seeded into pre-conditioned 35 mm tissue culture plates containing 2 ml of culture medium. Thus for the initial seeding of hepatocytes, each culture vessel contained a total of 3 ml of medium. Cells were seeded into culture vessels without a cover glass for the cytotoxicity test and with a cover glass for the UDS Assay. The cells were seeded into nine replicate culture vessels per treatment level for the UDS Assay; six were used for evaluating cytotoxicity, and the remaining three were used for determining unscheduled DNA synthesis. Only two duplicate cultures per treatment were used in the Range Finding Test.

Test System Identification

The test cultures were labeled using an indelible pen with a code system which clearly identified the experiment number, test article, controls and concentrations. The slides were labeled similarly with pencil.

Preparation of Test Article Dosing Solutions

Immediately prior to use, Copper Naphthenate was weighed and diluted with acetone to the appropriate concentrations. Approximately 20-30 minutes elapsed between the time the test article was solubilized and the final treatment of cells. All test article and control treatments were done under UV-filtered lights to avoid possible problems of photoinactivation.

The stability of the test article under the experimental conditions was not determined by SITEK Research Laboratories.

Range Finding Test

In order to determine the appropriate test article concentrations for the UDS Assay, a Range Finding Test was performed. In the Range Finding Test, ten concentrations of Copper Naphthenate ranging from 500 ug/ml to 0.05 ug/ml were evaluated. Test article concentrations of 50 ug/ml and above formed precipitates in the culture medium and, therefore, exceeded the solubility limitation of the test article in the culture medium. Duplicate test cultures seeded approximately 2 hours earlier were used at each treatment level in the Range Finding Test. Prior to treating the cells, the cultures were washed and refed with 2 ml of WME buffered with 0.01M HEPES (pH 7.3), supplemented with 2mM L-glutamine, 100 units/ml penicillin and 100 ug/ml streptomycin. Exposure was initiated by adding 20 ul of the appropriate stock solution or solvent to the culture plates. After an 18-hour exposure period, the cells were washed with Ca^{++}-free and Mg^{++}-free phosphate buffered saline (PBS), dissociated with 0.25% trypsin, and counted for the number of viable cells using the trypan blue dye exclusion method. The average number of viable cells for the replicate cultures was determined, and Relative Cell Survival (RCS) was calculated by comparing treated to solvent control groups. Relative Toxicity was calculated by subtracting RCS from 100%.

Unscheduled DNA Synthesis Assay

The test cultures for the UDS Assay were prepared as described earlier. Three replicate cultures without cover glasses were used for the parallel cytotoxicity determination, and six replicate cultures with cover glasses were used for UDS measurements at each treatment.

In the UDS Assay, the cells were treated with either 20, 15, 10, 5.0, or 1.0 ug/ml of test article, 10 or 2.0 ug/ml of 2-AAF (positive control) or 10 ul/ml of acetone or ethanol (solvent controls). In addition, one untreated control (WME) was included in the UDS Assay. The treated cultures used for measuring UDS were also exposed to 10 uCi/ml ^{3}H-thymidine (specific activity 20 Ci/mM, New England Nuclear) for approximately 18 hours.

Following the exposure period, the parallel cytotoxicity determination was performed as described in the Range Finding Test. Relative cytotoxicity was based on RCS. The cultures for UDS measurement were washed three times in WME buffered with 0.01M HEPES (pH 7.3), 2mM L-glutamine, 100 units/ml penicillin and 100 ug/ml streptomycin, swelled in 1% sodium citrate solution, and fixed in three 15-minute changes of methanol-glacial acetic acid fixative. The cover glasses were removed, allowed to dry, and mounted cell side up on glass slides. The slides were dipped in NTB emulsion at 43-45°C, and the emulsion was

allowed to drain and dry for 90 minutes at room temperature in the dark. The slides were then stored in a refrigerator in lighttight slide boxes with a desiccant. After 8 days, the slides were developed in Kodak D-19, fixed in Kodak fixer, stained in hematoxylin stain, and mounted in Permount using #1 cover glasses.

The slides were scored "blind" in order to avoid bias on the part of the scorer. The three highest scorable test article concentrations, the 2.0 ug/ml positive control, the untreated control, and the solvent controls were coded prior to scoring. Whenever possible, 50 randomly selected nuclei (each with three background counts) were counted per culture. All grain counts were performed using an electronic colony counter (ARTEK 880) equipped with a microscope-mounted auxiliary television camera. Grain counts were done directly by using the "count" mode of the colony counter. Only cells which appeared normal and healthy were scored for UDS. Cells showing a severe cytotoxic effect, i.e., cells with constricted, irregularly shaped or very darkly stained nuclei, or cells having nuclei with a projected image of less than 4 mm^2 were not scored.

Incorporation of ^{3}H-thymidine into nuclear DNA was determined by counting the darkened grains localized over the nuclear area. The background incorporation was determined by counting at least three nucleus-size areas of cytoplasm adjacent to each nucleus. The net nuclear grain counts were determined by subtracting the average background count from the nuclear count. For each treatment, the average net nuclear grain count $\pm$ standard deviation was calculated and recorded on a summary sheet. The number of nuclei showing five or more net nuclear grain counts in each cover glass was also recorded.

In addition, 300 nuclei per culture were counted at random to determine the percentage of nuclei exhibiting S-Phase DNA synthesis.

CRITERIA FOR A VALID ASSAY

1. In the negative control (untreated and solvent controls), the average net nuclear grain count per cell should be below five and the percentage of cells with net nuclear grain counts of five or more should be less than 20%.

2. At least 30% of the cells scored in the positive control should show a net nuclear grain count of five or more and the average net nuclear grain count per cell should be 20 or more.

3. At least one of the test concentrations scored should show more than 5% reduction in RCS. This requirement should not be applied to test articles which do not show any apparent

cytotoxicity at the maximum soluble concentration or highest allowable concentration.

4. The negative control(s) (untreated and solvent controls) should exhibit at least 0.2% nuclei in replicative DNA synthesis.

EVALUATION OF TEST RESULTS

Results for the test article concentrations were considered significant if the average net nuclear grain count was increased by at least five grain counts over the concurrent solvent and/or untreated controls or more than 25% of the cells scored showed net nuclear grain count of five or more.

Positive Response

The test article is considered to have caused a positive response in this assay if:

1. The test article causes a dose-related response and at least one concentration exhibits a significant increase over its concurrent solvent control.

2. In the absence of a dose response, at least two successive concentrations exhibit a significant increase over the concurrent solvent control data.

Marginal Positive Response

The test article is considered to have caused a marginal positive response if no indication of a positive dose response is observed, but one of the test concentrations shows a significant positive response.

Negative Response

The test article is considered to have caused a negative response if no indication of a positive concentrations response is observed and none of the test concentrations show a significant positive response.

Other Considerations

The above criteria are used as guidelines in evaluating the test results. However, the Study Director may take other factors into consideration in evaluating the test results.

Appendix B

Appendix I of the CBI
Score Sheets
(CBI p. 26)

APPENDIX I

SCORE SHEETS

		Group
30A, 30B, 30C, 30D, 30E, 30F	15 ug/ml	1
25A, 25B, 25C, 25D, 25E, 25F	12.5 ug/ml	2
31A, 31B, 31C, 31D, 31E, 31F	10.0 ug/ml	3
26A, 26B, 26C, 26D, 26E, 26F	Acetone Control	4
33A, 33B, 33C, 33D, 33E, 33F	2AAF, 10 ug/ml	5
27A, 27B, 27C, 27D, 27E, 27F	Ethanol Control	6
29A, 29B, 29C, 29D, 29E, 29F	WME Control	7

EPA No.: 68D80056
DYNAMAC No.: 250-B
TASK No.: 2-50B
June 28, 1990

DATA EVALUATION RECORD

COPPER NAPHTHENATE

Mutagenicity--Mouse Lymphoma Forward Mutation Assay

APPROVED BY:

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

Signature: William S. McLellan for

Date: 6-28-90

81

EPA No.: 68D80056
DYNAMAC No.: 250-B
TASK No.: 2-50B
June 28, 1990

DATA EVALUATION RECORD

COPPER NAPHTHENATE

Mutagenicity--Mouse Lymphoma Forward Mutation Assay

REVIEWED BY:

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Signature: Nancy E. McCarroll
Date: 6-28-90

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Date: 6-28-90

APPROVED BY:

Roman J. Pienta, Ph.D.
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Signature: William L. McLellan for
Date: 6-28-90

Stephen Dapson, Ph.D.
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Toxicology Branch II
(H-7509C)

Signature: Stephen C. Dapson
Date: 1 November 1990

Mike Ioannou, Ph.D.
EPA Section Head, Section I
Toxicology Branch II
(H-7509C)

Signature: M. Ioannou
Date: 11/2/90

DATA EVALUATION RECORD

006158

CHEMICAL: Copper naphthenate.

STUDY TYPE: Mutagenicity--Mouse Lymphoma Forward Mutation Assay.

MRID NUMBER: 411407-02.

TEST MATERIAL: Copper naphthenate.

SYNONYM: None listed.

SPONSOR: U.S. Army Environmental Hygiene Agency, Aberdeen Proving Ground, MD/Toxicon Corp., Woburn, MA.

TESTING FACILITY: SITEK Research Laboratories, Rockville, MD.

TITLE OF REPORT: Evaluation of a Test Article in the L5178Y TK +/- Mouse Lymphoma Mutagenesis Assay in the Presence and Absence of Aroclor-Induced Rat Liver S-9.

AUTHORS: Kirby, P.E., Brauninger, R.M., Law, L.C., and McMurrin, W.P.

STUDY NUMBER: 0088-2400.

REPORT ISSUED: August 19, 1988.

CONCLUSIONS/Executive Summary:

Under the conditions of the mouse lymphoma forward mutation assay, nonactivated doses of copper naphthenate (10, 25, 50, and 75 µg/mL) induced an equivocal mutagenic response.

In the presence of S9 activation, a clear, dose-related response was seen at 20, 30, and 40 µg/mL; survival at these levels was 62%, 27%, and 7%, respectively. Slight but less than twofold increases were observed at lower S9-activated doses (1.0, 2.5, 5.0, and 10.0 µg/mL).

We conclude, therefore, that S9-activated copper naphthenate is mutagenic in this test system and that mutagenic activity is confined to a narrow range of reactive doses that extends into the cytotoxic range. However, the study is incomplete; no information was provided on test material purity and no analytical data to support actual concentrations used in the assay were included.

Study Classification: The study is currently unacceptable but can be upgraded if the missing study information is furnished.

A. MATERIALS:

1. Test Material:

Name:	Copper naphthenate
Description:	Viscous, black liquid
Lot #:	P-17453
Purity:	Not specified
Contaminants:	None listed
Solvent used:	Acetone

Other comments: The test material was stored at room temperature. The raw data indicated that 55.9 mg of the test material was moderately soluble in 0.8 to 1.2 mL of acetone.

2. Indicator Cells: The mouse lymphoma cell line, L5178Y ($TK^{+/-}$), clone 3.7.2C, was obtained from Dr. Donald Clive, Burroughs Wellcome, Research Triangle Park, NC. Stock cultures were cryopreserved, and a sample was checked for mycoplasma contamination.

Cultures used in the assay were grown in Fischer's Medium, supplemented with sodium pyruvate, Pluronic F68, and horse serum, and exposed to methotrexate to maintain a low background frequency of trifluorothymidine (TFT)-resistant cells.

3. S9 Fraction: The S9 fraction was derived from the livers of adult male Sprague-Dawley rats induced with Aroclor 1254. The S9 m contained the appropriate cofactors and 25% S9 liver homogenate.

4. Positive Controls: Ethylmethanesulfonate (EMS) at 0.5 and 1.0 µL/mL and 7,12-dimethylbenz(a)anthracene (DMBA) at 5.0 and 7.5 µg/mL were used as the nonactivated and S9-activated positive controls, respectively.

B. STUDY DESIGN:

1. Preliminary Cytotoxicity Assay: The preliminary cytotoxicity assay was performed with eight doses of the test material ranging from 0.1 to 500 µg/mL in both the presence and absence of S9 activation. Cells were exposed to the test material concentrations or solvent (acetone) for 4 hours. Following exposure, cells were washed, resuspended in fresh growth medium, and reincubated. Cell viability was determined 20 and 44 hours posttreatment; relative suspension growth (RSG) was calculated and used to establish a dose range for the mutation assay.

2. Mutation Assay: Cells seeded at $1x10^6$ cells/mL were exposed to the appropriate test material dose, solvent, or positive controls with or without S9 activation for 4 hours. Cells were washed, resuspended in growth medium, and reincubated for 20 and 44 hours. Daily cell counts were determined, and cells were diluted when appropriate to maintain an optimal growth rate. At the end of the expression period, cultures having an RSG of ≥10% were chosen for mutant selection.

   For mutant selection, $3x10^6$ cells were treated with 3 µg/mL of TFT and plated in triplicate in selection medium. The cloning efficiency (CE) was determined by plating 200 cells/plate (in triplicate) in cloning medium. After 10 to 12 days of incubation, TFT-resistant colonies and the total number of viable cells were counted; mutation frequencies (MFs) were calculated.

3. Evaluation Criteria:

   a. Assay Acceptability: For the assay to be considered acceptable, the following criteria must be satisfied: (1) the cloning efficiency (CE) of the solvent control should be ≥50%; (2) the background MF of the solvent control should be $<100/10^6$ cells; and (3) the MF for the positive controls should be ≥3 times higher than the solvent control.

b. Positive Response: The test material was considered positive if it induced a dose-related increase in MF that was at least twofold higher than the corresponding solvent control.

C. REPORTED RESULTS:

1. Preliminary Cytotoxicity Assay: A review of the raw data indicated that the highest nonactivated and S9-activated dose (500 µg/mL) precipitated; no cells survived treatment at this level. For the remaining nonactivated concentrations, RSG was dose related and ranged from 26% at 100 µg/mL to 104% at 0.1 µg/mL. Cytotoxicity was more severe in the presence of S9 activation; no cells were recovered at doses ≥50 µg/mL. Lower doses (0.1, 0.5, 1.0, 5.0, and 10 µg/mL) were relatively noncytotoxic. Based on these results, the five nonactivated doses selected for the mutation assay were 10, 25, 50, 75, and 100 µg/mL. Eight doses were selected for the S9-activated assay: 1.0, 2.5, 5.0, 10, 20, 30, 40, and 50 µg/mL.

2. Mutation Assay:

a. Nonactivated Test Material: Cultures exposed to the highest dose (100 µg/mL) were not cloned because of severe cytotoxicity. RSG for the remaining dose groups ranged from 21% at 75 µg/mL to 95% at 10 µg/mL (Table 1). Although MFs increased in a dose-related manner and the MF for the high dose (75 µg/mL) was twofold higher than the corresponding solvent control, total mutant colonies were either comparable to or only slightly higher than the background mutant colony count. The study authors conclude that the nonactivated test material induced an equivocal response.

b. S9-Activated Test Material: Severe cytotoxicity precluded the cloning of cells exposed to the high dose (50 µg/mL). Survival for the remaining levels was generally dose related and ranged from 7% at 40 µg/mL to ≥93% at concentrations ≤10 µg/mL (Table 1). Dose-related increases in total mutant colonies and MFs were seen over a concentration range of 10 to 40 µg/mL. The MFs were 1.2-, 2.2-, 3.8-, and 6.8-fold higher than the solvent control at 10, 20, 30, and 40 µg/mL, respectively. Lower doses (1.0, 2.5, and 5.0 µg/mL) induced slight but less than twofold increases in the MF. Our reviewers noted that the highest mutagenic effect was calculated for the 40-µg/mL dose level and

TABLE 1. Representative Results from the Mouse Lymphoma Forward Mutation Assays with Copper Naphthenate

Substance	Dose/mL	S9 Activation	Relative Percent Suspension Growth	Average Mutant Colonies	Average Viable Colonies	% Cloning Efficiency	Relative Percent Total Growth	Mutation Frequency[a] /10[6]	Increase Over Solvent[b] Control
Solvent Control									
Acetone (test material)	--	-	100	42[c]	238[c]	119	100	35	--
		+	100	52[c]	258[c]	129	100	40	--
Dimethylsulfoxide (positive control)	--	-	100	45[c]	244[c]	122	100	37	--
Acetone (positive control)	--	+	100	51[c]	242[c]	122	100	41	--
Positive Control[d]									
Ethylmethane-sulfonate	0.5 µL	-	46	300	75	38	14	800	21.6
7,12-Dimethylbenz-(a)anthracene	5.0 µg	+	73	197	206	103	62	191	4.7
Test Material									
Copper naphthenate	10 µg	-	95	51	245	123	98	42	1.2
	25 µg	-	93	45	197	99	77	46	1.2
	50 µg	-	53	47	169	84	38	56	1.5
	75 µg	-	21	42	120	60	11	70	2.0
	10 µg[e]	+	93	57	246	123	88	46	1.2
	20 µg	+	62	99	222	111	53	89	2.2
	30 µg	+	27	143	188	94	20	152	3.7
	40 µg	+	7	150	111	56	3	270	6.8

[a]Mutation Frequency (MF) = $\frac{\text{Average Mutant Colonies}}{\text{Viable Colonies}} \times 200$.

[b]Fold Increase = $\frac{\text{MF of the Test Dose}}{\text{MF of the Corresponding Solvent Control}}$.

[c]Values averaged by our reviewers.

[d]Two levels of each positive control were assayed; results for the lower dose were selected as representative.

[e]Lower S9-activated doses (1.0, 2.5, and 5.0 µg/mL) induced slight but less than twofold increases in the MF.

at this level <10% of the cells survived. From these results, the study authors concluded that S9-activated copper naphthenate was mutagenic in this test system.

D. REVIEWERS' DISCUSSION AND INTERPRETATION OF RESULTS:

We assess that the study was properly conducted and that the study authors' interpretations of the data were correct. In the nonactivated assay, copper naphthenate induced an equivocal mutagenic effect; however, in the presence of S9 activation, clear dose-related mutagenesis was demonstrated. The reported mutagenic activity of copper naphthenate was confined to a narrow dose range (20 to 40 µg/mL) that extended into the cytotoxic range. Although we conclude, in agreement with the study authors, that copper naphthenate is mutagenic in this mammalian cell assay, the study is incomplete. No information was provided on test material purity and no analytical data were furnished to confirm actual test material concentrations used in the assay. The study is, therefore, classified as unacceptable but can be upgraded if the study authors can provide the missing information.

E. QUALITY ASSURANCE MEASURES: A quality assurance statement was signed and dated August 19, 1988.

F. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 5-13.

APPENDIX A

Materials and Methods

CBI pp. 5-13.

MATERIALS AND METHODS

INDICATOR CELLS

Source

The L5178Y TK+/- Mouse Lymphoma cells, clone 3.7.2C were originally obtained from Dr. Donald Clive, Burroughs Wellcome Company, Research Triangle Park, North Carolina, on October 17, 1984. The cells were subcultured, cleansed of TK-/- cells, and cryopreserved in a large number of ampules for L5178Y TK+/- Assays. The cells used for this study were from lot number 102387. A sample of these cells was reconstituted, tested for mycoplasma contamination, and found to be free of mycoplasma.

Culture Conditions

The L5178Y TK+/- Mouse Lymphoma cells were cultured in F$_1$eP (Fischer's Medium for Leukemic Cells of Mice supplemented with Pluronic F68, sodium pyruvate and heat-inactivated horse serum (10%)) in 50 ml disposable centrifuge tubes at 37 $\pm$1.0°C on a roller drum rotating at approximately 25 rpm. Each culture was gassed with 5% CO_2 and 95% air prior to placement on the roller drum. Each culture was sampled daily to determine cell concentrations, and if the cell concentrations were greater than 0.3×10^6 cells/ml, the cultures were adjusted to 0.3×10^6 cells/ml.

Stock Cultures

Stock cultures were grown in F$_1$-P in T-75 plastic tissue culture flasks on a shaker rotating at approximately 125 rpm. The cultures were monitored and adjusted when necessary to maintain them in log phase growth. For the Assay, subcultures were cleansed of TK-/- cells by exposure to THMG (thymidine, hypoxanthine, methotrexate and glycine) for 24 hours and grown in THG (THMG less methotrexate) for an additional 24 hours.

CONTROL SUBSTANCES

Positive Controls

Ethyl methanesulfonate (EMS), which induces mutation at the TK locus without metabolic activation, was used at 1.0 and 0.5 ul/ml in the non-activated system. The source and lot number of the EMS used in this study are given below.

Source: Kodak Lot No.: A11E

7,12-Dimethylbenz(a)anthracene (DMBA), which causes mutation at the TK locus with metabolic activation, was used at 7.5 and 5.0 ug/ml in the activated system. The source and lot number of the DMBA used in this study are given below.

Source: Kodak Lot No.: C13A

Solvent Controls

The test article dosing solutions were prepared by dissolving the test article in acetone and performing a serial dilution.

The positive control substance, DMBA, also was dissolved in acetone to make the stock solutions. The source and purity of the acetone batch used in this study are given below:

Source: Fisher Scientific Co. Lot No.: 871653

CAS Registry Number: 67 64-1

Certificate of Actual Lot Analysis:

Appearance (clear, colorless liquid)	
Density (grams/ml) at 25°C	0.7852
Residue after evaporation	0.0002%
Color (APHA)	5
Water	0.5%

Dimethyl sulfoxide (DMSO) was used to dissolve the positive control EMS. The source and purity of the DMSO batch used in this study are given below:

Source: Fisher Scientific Co. Lot No.: 864016

CAS Registry Number: 67-68-5

Certificate of Actual Lot Analysis:

Appearance (clear, colorless liquid)	
Density (grams/ml) at 25°C	1.095
Freezing point	18.0°C
Residue after evaporation	0.002%
Color (APHA)	10
Water	0.05%

TEST ARTICLE

The test article Copper Napthenate was received on July 8, 1988, and stored at room temperature. The test article was diluted in acetone to prepare the dosing solutions.

EXPERIMENTAL PROCEDURES

Determination of Solubility of Test Article

A solubility test was performed using deionized, distilled water, DMSO, acetone and ethanol. Based on the results of the solubility test, acetone was selected for use in preparing the dosing solutions.

Preparation of Test Cultures

The L5178Y TK+/- stock cultures were maintained in log phase growth until used in the Assay. From the stock cultures a pool of cells was prepared at a concentration of $1x10^6$ cells/ml in 50% conditioned F$_1$P and 50% fresh F P. 6 ml of the cell preparation was dispensed to 50 ml disposable centrifuge tubes resulting in $6x10^6$ cells/tube. Each tube was gassed with 5% CO_2 and 95% air, sealed, and placed on a shaker to keep the cells suspended until treated.

Preparation of Metabolic Activation System

The metabolic activation system consisted of Aroclor-induced rat liver homogenate (S-9 fraction) and the cofactor pool. The S-9 fraction was prepared in 0.25M sucrose from Aroclor 1254-induced male Sprague-Dawley rats.

Approximately 1 gram of rat liver was used to make 3 ml of buffered S-9 fraction. Immediately prior to treatment, the S-9 fraction was mixed with the cofactor pool to obtain the S-9 cofactor mixture which was kept in wet ice until used. The S-9 cofactor mixture was prepared in the following proportion per ml of S-9 mix.

6.0 mg	NADP
11.25 mg	DL-Isocitric Acid
0.25 ml	S-9 Homogenate
0.75 ml	F P

The S-9 homogenate was added after the other components had been combined, mixed, the pH adjusted to approximately 6.8, and filter sterilized through a 0.45 uM filter.

Test System Identification

All test cultures were labeled with an indelible pen with a code system which clearly identified the experiment number, activation system, test article, controls and concentrations.

Preparation of Test Article Dosing Solutions

An aliquot of the test article was weighed out, and a fixed volume of acetone was added to obtain the desired test article concentration. A serial dilution was performed using acetone to prepare the remaining dosing solutions.

The stability of the test article under the experimental conditions was not determined by SITEK Research Laboratories.

Range Finding Test

In order to determine the test article concentrations that would produce 0-100% cytotoxicity, a Range Finding Test was performed. The test article was tested with and without S-9 activation at concentrations of 500, 100, 50, 10, 5.0, 1.0, 0.5 and 0.1 ug/ml. A suspension of fine particles was observed in the cultures that were treated with a concentration of 500 ug/ml.

Treatment was performed by adding 4 ml of $F_{12}P$ or 4 ml of S-9 mix to the culture and then adding the appropriate volume of test article/vehicle mixture. Two control cultures were run simultaneously with the treated cultures. The cultures were gassed with approximately 5% CO_2 and 95% air and incubated at 37°C on a roller drum apparatus rotating at 25 ±2 rpm.

After a 4-hour exposure period, the cells were pelleted by centrifuging them at approximately 1000 rpm for 10 minutes, and the test article was removed by pouring off the supernatant. Two rinses in 10 ml of $F_{12}P$ were performed, after which the cells were resuspended in 20 ml of $F_{12}P$, gassed with approximately 5% CO_2 and 95% air, and incubated at 37°C on a roller drum rotating at 25 ±2 rpm. At approximately 20 hours and 44 hours post treatment, 1 ml samples were removed from each culture to determine the cell population density of each. The 1 ml sample was placed in a vial containing 19 ml of 0.1% trypsin. The vials were incubated for 10 minutes at 37°C, after which they were placed on an automatic cell counter. Three counts were made, and the average count, corrected for coincidence, was used to determine the concentration of cells per ml for each culture. After the determination of cell numbers at 20 hours post treatment, each culture having greater than 0.3×10^6 cells/ml was adjusted to 0.3×10^6 cells/ml.

This was performed by retaining the volume of culture that would result in a final concentration of 0.3×10^6 cells/ml when fresh medium was added to it to yield a combined final volume of 20 ml.

The Suspension Growth (SG) of each culture was determined using the following formula:

$$SG = \frac{\text{Day 1 Conc.}}{0.3\times10^{6}\ \text{Cells/ml}} \times \frac{\text{Day 2 Cell Conc.}}{\text{Day 1 Adjusted Cell Conc.}}$$

The Relative Suspension Growth (RSG) of each of the test article-treated cultures was determined by calculating its growth relative to the corresponding solvent control cultures' average SG.

$$RSG = \frac{\text{SG of Treated Culture}}{\text{Average SG of Solvent Controls}} \times 100$$

Based on the results of the Range Finding Test, the test article was tested in the Assay at doses ranging from 175 to 10 ug/ml in the non-activated system and from 50 to 1.0 ug/ml in the presence of S-9 activation mix.

Mutation Assay

The test article was solubilized and a serial dilution was performed as previously described. Cultures containing 6 ml of cells at a concentration of 1×10^{6} cells/ml were prepared. These cultures contained 50% conditioned $F_{10}P$ (supernatant from the stock cultures) and 50% fresh F_0P. Immediately prior to adding the appropriate aliquots of the test article dosing solution, 4 ml of either F_0P medium or 4 ml of S-9 mix was added to each culture depending on whether or not it was to be treated in conjunction with the exogenous metabolic activation system. Immediately after addition of the test article, the culture was gassed with approximately 5% CO_2 and 95% air. After all of the cultures had been treated, they were placed on a roller drum apparatus and rotated at a speed of 25 $\pm$2 rpm in an environment of 37°C. Two solvent control cultures were included in each treatment group, i.e., with activation and without activation. In addition, two sets of cultures were treated with positive control chemicals. As indicated earlier, EMS was the positive control chemical for the non-activated portion of the Assay, and DMBA was the positive control chemical for the S-9 activated portion of the Assay.

After a 4-hour exposure period, the cells were pelleted by centrifugation at approximately 1000 rpm for 10 minutes, and the test article was removed by pouring off the supernatant. Two rinses in 10 ml of $F_{10}P$ were performed. The final rinse was followed by resuspension in 20 ml $F_{10}P$, gassing with approximately 5% CO_2 and 95% air, and incubation at 37°C on a roller drum apparatus set at 25 $\pm$2 rpm. Approximately 20 hours and 44 hours post treatment, 1 ml samples were removed from each

culture to determine the cell population density of each. The 1 ml sample was placed in a vial containing 19 ml of 0.1% trypsin. The vials were incubated for 10 minutes at 37°C, after which they were placed on an automatic cell counter. Three counts were made, and the average count, corrected for coincidence, was used to determine the concentration of cells per ml for each culture. After the determination of cell numbers, each culture having a population greater than 0.3×10^6 cells/ml was adjusted to 0.3×10^6 cells/ml. At the 20-hour point the final volume after adjustment was 20 ml. For the 44-hour point the final volume was 10 ml.

Cloning for Mutants and Viability

After the 2-day expression period, cultures were selected for cloning based on their SG. Only cultures having a Relative Suspension Growth of approximately 10% or greater were cloned.

For each culture selected for cloning, 200 ml of cloning medium (CM) was prepared. The CM was made by combining the following ingredients in the indicated proportions for each 100 ml of CM.

F₀P	70.75 ml
Horse Serum	20.0 ml
Sodium Pyruvate (Stock)	0.05 ml
Purified Agar (4% Solution)	8.75 ml

For each culture selected for cloning, 100 ml of CM was dispensed into a flask designated for the addition of the restrictive agent trifluorothymidine (TFT) and therefore the growth of TK-/- cells only, and 100 ml was dispensed into a flask designated as a Viable Count (VC) flask. The CM in the VC flask was used to culture an aliquot of cells from each culture cloned to approximate the percentage of viable cells in each culture.

The cloning process was as follows:

1. Each TFT and VC flask received 100 ml of CM and each was placed in a shaker incubator set at approximately 125 rpm and 37°C.

2. The cultures designated for cloning were centrifuged at a speed of approximately 1000 rpm for 10 minutes. 8 ml of the supernatant was aspirated and discarded. The cells were resuspended in the remaining volume of supernatant and then added to the appropriate TFT flask. Each TFT flask contained 3×10^6 cells. Each flask was replaced on the shaker incubator (125 rpm, 37°C).

3. After each TFT flask had shaken for at least 15 minutes, a 1 ml aliquot was removed from each, a 1:10 and 1:5 serial dilution was performed, and a 1 ml aliquot of the last dilution was added to the appropriate VC flask. Each VC flask contained approximately 600 cells.

4. After the completion of cell addition to each VC flask, an aliquot of TFT was added to each TFT flask. The concentration of TFT in the culture was approximately 3 ug/ml. Both TFT and VC flasks were replaced on the shaker incubator (125 ±2 rpm, 37°C).

5. After at least 15 minutes of mixing, the contents of each flask were dispensed in equal aliquots into three plates. The plates were chilled for 20 minutes in a 4°C environment and then placed in an incubator at 37°C in an atmosphere of approximately 5% CO_2 and 95% air for 10-12 days.

Enumeration of Colonies

After completion of the incubation period, the number of colonies per TFT and VC plate was determined. The colony numbers were determined by counting them with an automatic colony counter. The raw counts were increased by a correction factor determined for SITEK's colony counter (#000027). The correction factor is:

$$\text{Corrected Counts} = \frac{(\text{Raw Count} - 0.6499)}{0.8423}$$

Determination of Mutant Frequency and Induced Mutant Frequency

The Mutant Frequency (MF) of each culture that was successfully cloned was determined as a function of viable cells forming colonies. The calculation was performed as follows:

$$\text{MF Per } 10^6 \text{ Viable Cells} = \frac{\text{Average No. Mutants Per Plate}}{\text{Average No. of Colonies in the Corresponding VC Plates}} \times 200$$

The Induced Mutant Frequency (IMF) was calculated by using the following formula:

$$\text{IMF} = \text{MF of Treated Cultures} - \text{Average MF of Solvent Control Cultures}$$

Determination of Relative Suspension Growth, Relative Cloning Efficiency and Total Growth

Relative Suspension Growth

The S[illegible] and R[illegible]G of each culture was determined by performing the calculations described earlier.

Relative Cloning Efficiency

The Relative Cloning Efficiency (RCE) was determined for each culture by using the following formula:

$$RCE = \frac{\text{Average VC Count of Treated Culture}}{\text{Average VC Count of Solvent Controls}} \times 100$$

Total Growth

The Total Growth (TG) of a culture was calculated as follows:

$$TG = \frac{RSG \times RCE}{100}$$

The TG was calculated for each test article-treated culture that was successfully cloned.

Criteria For a Valid Assay

The following criteria were used in evaluating the acceptability of the Assay.

Solvent Control Cultures

1. The average Cloning Efficiency of the solvent control cultures should be 50% or higher.

2. The average MF of the solvent control cultures should be less than 100 per 10^6 viable cells.

Positive Controls

The results for the positive control cultures should be considered acceptable if:

1. The treated cultures have MF's that are three times or greater than the average of their solvent control cultures.

2. Their solvent controls have an average Cloning Efficiency of 50% or greater.

Evaluation of Test Results

The following criteria were used as guidelines in evaluating the results of the Assay for a negative, positive or equivocal response. Since it is impossible to write criteria that would apply to every configuration of data generated by the Assay, the Study Director is responsible for the ultimate decision in the evaluation of the results.

Criteria for a Negative Response

1. All of the cultures exhibiting TG of 10% and greater have MF's that are less than twice that of the mean MF of the corresponding solvent control cultures, and

2. There is no evidence of a dose-dependent response.

Criteria for a Positive Response

A response is considered positive if at least one culture has an MF that is two times or more greater than the average MF of the corresponding solvent control cultures and the response is dose dependent.

Criteria for an Equivocal Response

A response is considered equivocal if it does not fulfill the criteria of either a negative or a positive response, and/or the Study Director does not consider the response to be either positive or negative.

ARCHIVES

All raw data, information pertinent to this study and study report(s) will be maintained in SITEK Research Laboratories archives.

EPA No.: 68D80056
DYNAMAC No.: 250-C
TASK No.: 2-50C
June 28, 1990

00158

DATA EVALUATION RECORD

COPPER NAPHTHENATE

Mutagenicity--*Salmonella typhimurium* Mammalian Microsome Mutagenicity Assay

APPROVED BY:

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

Signature: William L McLellan for
Date: 6/27/90

EPA No.: 68D80056
DYNAMAC No.: 250-C
TASK No.: 2-50C
June 28, 1990

000156

DATA EVALUATION RECORD

COPPER NAPHTHENATE

Mutagenicity--Salmonella typhimurium Mammalian Microsome Mutagenicity Assay

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DATA EVALUATION RECORD

006158

CHEMICAL: Copper naphthenate.

STUDY TYPE: Mutagenicity--Salmonella typhimurium mammalian microsome mutagenicity assay.

ACCESSION NUMBER: 411407-03.

TEST MATERIAL: Copper naphthenate.

SYNONYMS/CAS NO. None listed.

SPONSOR: U.S. Army Environmental Hygiene Agency, Aberdeen Proving Ground, MD.

TESTING FACILITY: Toxicon Corp., Woburn, MA.

TITLE OF REPORT: Ames Bacterial/Microsomal Plate Incorporation Assay.

AUTHOR(S): Desai, L. S.

STUDY NUMBER: 88G-0096.

REPORT ISSUED: June 4, 1988.

CONCLUSION(S)/Executive Summary: Five concentrations of copper naphthenate (50 to 500 µg/plate) were evaluated in the Salmonella typhimurium mammalian microsome mutagenicity assay. Preliminary cytotoxicity results indicated that doses >500 µg/plate were cytotoxic (≈50% reduction in survival) in S. typhimurium TA100. Results of the mutation assay indicated that the five assayed levels (+/-S9) were neither cytotoxic nor mutagenic in S. typhimurium TA1535, TA1537, TA1538, TA98, or TA100. We conclude, therefore, that copper naphthenate was assayed to a subcytotoxic level with no evidence of a mutagenic effect. The study is, however, incomplete; no information on test material purity nor analytical data to support actual test material concentrations used in the assay were reported.

Study Classification: The study is currently unacceptable but can be upgraded if the identified missing information is furnished by the study author.

A. MATERIALS:

1. Test Material:

Name:	Copper naphthenate
Description:	Black, semisolid
Lot No.:	P-17453
Purity:	Not specified
Contaminants:	None listed
Solvent used:	Dimethylsulfoxide (DMSO).
Other comments:	The test material was stored at room temperature; the report indicated that the test material, dissolved in DMSO, was green in color.

2. Control Materials:
Negative: DMSO
Solvent/final concentration: 15 to 150 µg/plate
Positive: Nonactivation:
Sodium azide 10 µg/plate TA100, TA1535
2-Nitrofluorene 10 µg/plate TA98, TA1538
9-Aminoacridine 50 µg/plate TA1537

Activation:
2-Aminoanthracene 2.5 µg/plate all strains.

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). The S9 fraction (lot No. R356) was obtained from Microbiological Associates, Rockville, MD. The S9 mix contained the appropriate cofactors and 10% S9 liver homogenate.

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. Cytotoxicity Assay: Five doses of the test material (500, 1000, 2000, 3000, and 5000 µg/plate) were evaluated in the nonactivated cytotoxicity assay with S. typhimurium strain TA100.

b. Mutation Assays: The five doses selected for the nonactivated and S9-activated mutation assay were 50, 100, 200, 300, and 500 µg/plate.

B. TEST PERFORMANCE:

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

2. Preliminary Cytotoxicity Assay: The nonactivated preliminary cytotoxicity assay was conducted with five doses of the test material ranging from 500 to 5000 µg/plate using S. typhimurium TA100; duplicate plates were prepared for each dose level. The test material caused ≈50% or greater reduction in mutant colonies of TA100 at doses ≥1000 µg/plate; the lowest assayed dose (500 µg/plate) was not cytotoxic.

3. Mutagenicity Assay: Based on the above findings, five doses ranging from 50 to 500 µg/plate were assayed in the presence and absence of S9 activation. The nonactivated or S9-activated test material was not cytotoxic at any assayed level (Table 1). No appreciable increase in mutant colonies of any strain accompanied exposure to the five selected doses of copper naphthenate either in the presence or absence of S9 activation. It was noted, however, that the S9-activated solvent control counts for S. typhimurium TA1537, TA98, and TA100 were elevated but within the acceptable spontaneous-revertant ranges for these strains. Results for the nonactivated and S9-activated positive-control cultures showed that the sensitivity of the test system to detect a mutagenic response was adequately demonstrated.

The study author concluded that copper naphthenate was not mutagenic in this test system.

TABLE 1. Representative Results of the Salmonella typhimurium Mutagenicity Assay with Copper Naphthenate

Substance	S9 Activation	Dose (µg/plate)	Revertants per Plate of Bacterial Tester Strain[a] TA1535	TA1537	TA1538	TA98	TA100
Solvent Control							
Dimethyl sulfoxide	-	--	27 ± 2.1	17 ± 0.2	28 ± 2.6	68 ± 3.1	200 ± 9.7
	+	--	32 ± 1.5	25 ± 4.6	33 ± 2.5	72 ± 3.0	229 ± 3.1
Positive Control							
Sodium azide	-	10	278 ± 19.1	--	--	--	746 ± 3.6
2-Nitro-fluorene	-	10	--	--	313 ± 17.1	1050 ± 53.6	--
9-Aminoacridine	-	50	--	227 ± 0.2	--	--	--
2-Anthramine	+	2.5	257 ± 2.1	215 ± 5.0	509 ± 8.5	563 ± 6.4	895 ± 4.5
Test Material							
Copper naphthenate	-	500[b]	31 ± 1.5	20 ± 6.0	28 ± 4.5	57 ± 3.8	208 ± 6.4
	+	500	27 ± 3.2	26 ± 2.6	37 ± 2.0	68 ± 3.2	252 ± 11.0

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

[b]Results for lower doses (50, 100, 200, and 300 µg/plate +/-S9) did not suggest a mutagenic response.

4. Reviewers' Discussion/Conclusions: We assess that the study was properly conducted and that the author's interpretation of the data was correct. None of the assayed doses of the test material were cytotoxic or mutagenic in either the nonactivated or S9-activated test. Based on the finding of the preliminary assay showing that doses >500 μg/plate were cytotoxic, we conclude that copper naphthenate was assayed to a subcytotoxic dose with no indication of a mutagenic response. However, the study is incomplete because the purity of the test material was not reported and analytical data to support actual test concentrations used in the assay were not provided.

5. Quality Assurance Measures: A quality assurance statement was signed and dated June 4, 1988.

6. CBI Appendix: Appendix A, Materials and Methods, CBI pp. 1-5.

APPENDIX A

Materials and Methods
CBI pp. 1-5.

FEB 2 0 1985

AMES BACTERIAL/MICROSOMAL PLATE INCORPORATION ASSAY

411407-C 3

1.0 OBJECTIVE

This assay evaluates the mutagenic potential of a test article via its ability to induce back mutations at selected loci of five strain of Salmonella typhimurium in the presence and absence of an exogenous mammalian activation system.

2.0 SUMMARY

The results of the Ames Plate Incorporation Assay indicate that under the conditions specified in this report, the test article, Copper Naphthenate, did not cause a positive increase in the number of histidine revertant colonies and is considered non-mutagenic.

3.0 MANAGEMENT OF THE STUDY

3.1 Sponsor

Name and Address: U.S. Army Environmental Hygiene Agency
Building E-2100
Aberdeen Proving Ground, MD 21010-5422

Project Officer: Leroy Hetker

3.2 Testing Laboratory Toxikon Corporation
225 Wildwood Avenue
Woburn, MA 01801

3.3 Toxikon Corporation's Supervisory Personnel Assigned to the Study:

Study Director: Laxman S. Desai, D.Sc.
Study Supervisor: Asha Raghupathy, M.S.
Quality Assurance: Nancy DiGiulio, B.S.

4.0 COMPLIANCE

The present study conformed to all applicable laws and regulations. Specific regulatory requirements include EPA Good Laboratory Practice Regulations (GLP, 40 CFR, Part 792, 1983). The study reference is the TSCA Final Rules, 40 CFR, Part 798, Subpart F, 1985.

5.0 TEST SUBSTANCE

The following information should be supplied by the Sponsor wherever applicable; it does not apply to confidential information. The Sponsor is responsible for all test article characterization data as specified in 40 CFR, Part 792, Part 1983.

Description of the Test Substance: Copper Naphthenate
Chemical/Common/Trade Name: N/A
Molecular Formula: N/A
Composition/Purity: N/A
Physical State: Semi-Solid
Color: Black when solid, green when dissolved in DMSO
Density: N/A
pH: N/A

Specific Gravity: N/A
Stability: N/A
Solubility: Dissolves in DMSO
Hygroscopic Properties: N/A
Boiling Point: N/A
Flash Point: N/A
Lot/Batch #: P-17453
Quantity: 148 Grams
Source: Army-USAAPGISA
Safety Precautions: Standard Laboratory Safety Precautions Apply

5.1 The test article was stored at room temperature and in the same container in which it was received at Toxikon.

6.0 THE TEST SYSTEM

The tester strains used were the Salmonella typhimurium histidine auxotrophs TA98, TA100, TA1535, TA1537, and TA1538 as described by Ames et al, 1975. Tester strains currently in use at Toxikon were received directly from Dr. Bruce Ames, Department of Biochemistry, University of California, Berkeley.

GENOTYPE OF THE TA STRAINS USED FOR MUTAGEN TESTING

Strain	Gene Affected	Repair	LPS	Rfactor	Mutation
TA98	His D	uvr B	rfa	pkM101	frame shift
TA100	His G	uvr B	rfa	pkM101	base pair
TA1535	His G	uvr B	rfa	-	base pair
TA1537	His C	uvr B	rfa	-	frame shift
TA1538	His D	uvr B	rfa	-	frame shift

Each of the tester strains contain, in addition to a mutation in the histidine operon, two additional mutations which enhance their sensitivity to some mutagenic compounds. The rfa wall mutation induces a loss of an enzyme responsible for the synthesis of part of the lipopolysaccharide layer of the cell wall, thereby increasing the permeability of the cell to certain classes of chemicals (e.g. those containing large ring moieties) that would otherwise be excluded by a normal intact cell wall.

The second mutation is a deletion in the uvrB gene which results in a deficient DNA repair system. This deficiency enhances the sensitivity of the strains to some mutagens. Since the uvrB deletion extends through the bio gene, all the tester strains containing this deletion also require biotin for growth. The second mutation is a deletion in the uvrB gene which results in a deficient DNA repair system. This deficiency enhances the sensitivity of the strains to some mutagens. Since the uvrB deletion extends through the bio gene, all of the tester strains containing this deletion also require biotin for growth.

Finally, strains TA98 and TA100 also contain the pKM101 plasmid (carrying the R factor) which further increases the sensitivity of these two strains to some mutagens.

TA98, TA1537, and TA1538 are reverted from histidine dependence (auxoatrophy) to histidine independence (protrophy) by frame shift

mutations. TA100 and TA1535 are reverted by mutagens that cause base-pair substitutions.

006158

6.1 Maintenance of Tester Strains
Indicator strains were kept at 4° C on minimal medium plates supplemented with biotin, histidine, and ampicillin (25 ug/ml) for TA-98 and TA-100, to ensure stable maintenance of plasmid pKM101. New stock culture plates were made as needed from the frozen stock. Single colony reisolates were checked for their genotypic characteristics (his, rfa, uvrB, bio) and for the presence of the plasmid.

7.0 MEDIA
For daily use, each strain was cultured in Oxoid #2 Broth (nutrient broth) for approximately 16 hours at 37°C for a final concentration of 10^8 to 10^9 cells/ml.

The minimal media plates for the selection of histidine revertants consisted of the Vogel Bonner Medium E supplemented with 2% glucose and 1.5% Scott bactoagar. The overlay agar contained the following per 100 ml volume; 0.6 grams of purified agar, 10 ml of 0.5mM L-Histidine, 0.5mM Biotin and 0.5 grams NaCl.

8.0 ACTIVATION SYSTEM
The S9 homogenate was obtained commercially and used as the activation system. The 9000 x g supernatant was prepared from adult male rat liver induced by Aroclor 1254. The S9 mix contained the following components.

Components	Concentration per ml S9 Mix
NADP (sodium salt)	4 umoles
D-Glucose-6-phosphate	5 umoles
$MgCl_2$	8 umoles
KCl	33 umoles
Sodium Phosphate	100 umoles
Rat Liver Homogenate	100 ulliters

9.0 EXPERIMENTAL DESIGN

9.1 Dosage Selection
Doses for the actual assay were selected based on the preliminary toxicity test with the strain TA-100. For the actual assay, doses were selected with the highest dose exhibiting $\leq$ 90 percent toxicity. The assay was run in triplicate in the presence and absence of metabolic activation.

9.2 Plate Incorporation Test
To sterile test tubes placed in a 43-45°C waterbath, the following were added in order:

a. 2.0 ml of overlay agar
b. 0.015-0.150 ml of the test material to yield the appropriate dose
c. 0.1 ml to 0.2 ml of indicator organism
d. 0.50 ml of 0.2M phosphate buffer, pH 7.4

The mixture was gently mixed and poured on the minimal agar plates. After the overlay had set, the plates were incubated at 37°C $\pm$ 2°C for

approximately 48-72 hours. The number of his+ revertant colonies growing on the plates were counted and recorded.

Activation
The activation assay was run concurrently with the non-activation assay. The only difference was the addition of approximately 0.5 ml of the S9 fraction in place of the phosphate buffer.

Control Compounds
a. Negative Control- The solvent for the test material was tested at the single concentration equal to the maximum volume used to administer the highest dose of the test material. The solvent control was employed for each indicator strain and was used in the absence and presence of the S9 mix. All dilutions of the test article were made using this solvent.

b. Positive Control - Strain specific positive controls and positive controls to ensure the efficacy of the S9 mixture were assayed concurrently with the test material. The positive controls utilized in the assay were:

Activation	Chemical	Solvent	Concentration	Strain
-	Sodium azide	Water	10 ug/plate	TA-1535, TA-100
-	2-Nitrofluorene	DMSO	10 ug/plate	TA-1538, TA-98
-	9-Aminoacridine	Ethanol	50 ug/plate	TA-1537
+	2-Amino-anthracene	DMSO	2.5 ug/plate	All Strains

10.0 EVALUATION CRITERIA
No statistical analysis was used in evaluating the data. The plate test data of direct revertant colony counts was obtained from a set of selective agar plates seeded with populations of mutant cells. Because of the test material and the cells are incubated in the overlay for 48 hours and there is some cell division, spontaneous revertant control plate readings are also taken into account in the final analysis of the data.

a. Evaluation
Because most of the procedures used to evaluate the mutagenicity of this test are semiquantitative, the criteria used to determine the positive effects of a test material are based on historical data. The test results are interpreted based on the following criteria.

1. Strains TA-1535, TA-1538, TA-1537
If the solvent control value is within the normal range, a test material producing a positive response equal to three times the solvent control value is considered mutagenic.

2. Strains TA-98 and TA-100
If the solvent control value is within the normal range, the test material producing a positive dose response over three concentrations, with the highest increase equal to twice the solvent control value is considered mutagenic. Occasionally, a doubling is not necessary for TA-100 if a clear dose-related pattern is observed over several concentrations.

The following ranges of revertants for solvent controls are generally considered acceptable:

TA-1538	10-35
TA-1535	8-30
TA-1537	4-30
TA-98	20-75
TA-100	80-250

b. Dose Response Phenomena
The demonstration of dose-related increases in revertant counts is an important criterion in establishing mutagenicity. Since several doses were utilized in the actual assay, a dose response would normally be seen with a mutagenic test material. Additional testing would be performed over a narrower dose range if the mutagenic test material failed to exhibit a dose-response in the initial assay.

c. Reproducibility
If a test material produces a response in a single test which cannot be reproduced in additional runs, the initial positive test data lose significance.

d. Control Tests
Positive control assays consisted of direct-acting mutagens and mutagens requiring metabolic biotransformation. Negative controls consistad of the test material solvent in the overlay agar, together with other essential components. The negative control plates for each strain gives a reference point to which the test date were compared. The positive control essay was conducted to demonstrate that the test system was functional with known mutagens.

11.0 RESULTS
The test article , Copper Naphthenate (USAAPGISA), was dissolved in DMSO. The dose finding study (Table I) indicated that the test article was toxic at doses 1-5 mg/plate whereas the 0.5 mg/plate showed no toxicity. Based on these results the actual assay consisted of 5 different doses ranging from 0.05 mg to 0.5 mg/plate of test article.

The assay also included spontaneous revertants, positive control, and a vehicle control which served as the negative control. The control experiments indicated acceptable results demonstrating the validity of the test. The test article at the doses tested exhibited similar results to vehicle control therefore, the test article is considered non-mutagenic according to the procedure employed in this assay.

12.0 REFERENCES
40 CFR, Part 798, Subpart F, Section 798.5265, 1985.

13.0 VERIFICATION DATA

Date of Sample: 04/12/88
Date of Test Initiation: 05/09/88
Date of Test Completion: 05/16/88
Date of Study Report: 05/17/88

EPA No.: 68D80056
DYNAMAC No.: 250-D
TASK No.: 2-50D
June 28, 1990

DATA EVALUATION RECORD

COPPER NAPHTHENATE

Mutagenicity--In Vitro Chromosome Assay with
Chinese Hamster Ovary (CHO) Cells

APPROVED BY:

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

Signature: William L. McLellan for

Date: June 27, 1990

EPA No.: 68D80056
DYNAMAC No.: 250-D
TASK No.: 2-50D
June 28, 1990

DATA EVALUATION RECORD

COPPER NAPHTHENATE

Mutagenicity--*In Vitro* Chromosome Assay with
Chinese Hamster Ovary (CHO) Cells

REVIEWED BY:

Nancy E. McCarroll, B.S.
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Date: 6-28-90

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Dynamac Corporation

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Stephen Dapson, Ph.D.
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Toxicology Branch II
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Signature: Stephen C. Dapson
Date: 1 November 1990

Mike Ioannou, Ph.D.
EPA Section Head, Section I
Toxicology Branch II
(H-7509C)

Signature: M. Ioannou
Date: 11/7/90

DATA EVALUATION RECORD

004158

CHEMICAL: Copper naphthenate.

STUDY TYPE: Mutagenicity--In vitro chromosome assay in Chinese hamster ovary (CHO) cells.

MRID NUMBER: 411407-04.

TEST MATERIAL: Copper naphthenate.

SYNONYM: None listed.

SPONSOR: U.S. Army Environmental Hygiene Agency, Aberdeen Proving Ground, MD/Toxikon Corp., Woburn, MA.

TESTING FACILITY: SITEK Research Laboratories, Rockville, MD.

TITLE OF REPORT: Test for Chemical Induction of Chromosomal Aberration in Cultured Chinese Hamster Ovary (CHO) Cells With and Without Metabolic Activation.

AUTHOR: Thilagar, A.

STUDY NUMBER: 0088-3110.

REPORT ISSUED: September 15, 1988.

CONCLUSIONS/Executive Summary:

Three nonactivated (100, 125, and 150 µg/mL) and three S9-activated (45, 60, and 75 µg/mL) doses of copper naphthenate were evaluated for cytogenetic effects in Chinese hamster ovary (CHO) cells.

The nonactivated doses were not clastogenic. Although nonsignificant increases in the percentage of cells with aberrations were seen at the three S9-activated doses, borderline significant ($p = 0.063$) effects were observed at the mid-dose level. Similarly, rare complex aberrations (dicentrics, triradials, and quadriradials) were scored from cultures exposed to all S9-activated levels. The data indicate weak clastogenic activity; however, we are unable to reach definitive conclusions because the effect was not dose dependent and the results were not confirmed. In addition, the purity of the test material was not reported, and no analytical data were provided to verify the actual concentrations used in the assay. Copper naphthenate is, therefore, classified as presumptively positive in this test system.

Study Classification: The lack of a definitive result and the lack of complete information on the test material makes this study unacceptable. The study should be repeated to determine if these findings are reproducible. We further recommend that the missing test material information be submitted with the findings of the repeat study.

A. MATERIALS:

1. Test Material:

Name:	Copper naphthenate
Description:	Black, viscous liquid
Batch No.:	P-17453
Purity:	Not reported
Contaminants:	None listed
Solvent Used:	Acetone

Other comments: The test material was stored at room temperature; solutions of the test material were prepared immediately prior to use. According to the raw data, 27.4 mg of the test material was "soluble in 0.1 mL of acetone and doses ≥250 µg/mL precipitated in the culture medium."

2. Cell Line: The Chinese hamster ovary cells (CHO-K_1) used in this assay were obtained from the American Type Culture Collection, Rockville, MD. Prior to use, the CHO cells were grown for 18 to 24 hours in Ham's F-12 medium supplemented with 10% fetal calf serum, L-glutamine, and antibiotics.

3. S9 Fraction: The S9 fraction was derived from the livers of adult male Sprague-Dawley rats induced with Aroclor 1254. The S9 mix contained the appropriate cofactors and 15 µL/mL of the S9 fraction.

4. Positive Controls: Triethylenemelamine (TEM) at 0.5 µg/mL was used as the nonactivated positive control; cyclophosphamide (CP) at 30 µg/mL served as the S9-activated positive control.

B. STUDY DESIGN:

1. Preliminary Cytotoxicity Assay: Prepared cultures, seeded at $5x10^5$ cells/flask, were exposed without S9 activation to 9 test material doses (1.0 to 1000 µg/mL) and with S9 activation to 10 doses (0.5 to 500 µg/mL); untreated and solvent control cultures were included, and duplicate cultures were prepared for each treatment group. In the nonactivated system, cells were exposed for 16 hours to the test material, washed, trypsinized, and counted to determine survival. In the S9-activated system, cultures were treated for 2 hours, washed, refed, and reincubated for 14 hours. After incubation, cells were washed, trypsinized, and counted. Based on the preliminary cytotoxicity results, doses were selected for the cytogenetic assay.

2. Cytogenetic Assay:

a. Treatment: Prepared cultures (4 replicates/group), seeded at $5x10^5$ cells, were exposed to seven nonactivated (20 to 150 µg/mL) and six S9-activated (5.0 to 75 µg/mL) test material doses, the solvent control (acetone), or the positive controls (0.5 µg/mL TEM -S9 and 30 µg/mL CP +S9). Two of the four replicate cultures from each group were used for the parallel cytotoxicity test and were processed as described for the preliminary cytotoxicity assay. The remaining cultures were used for metaphase collection and analysis.

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 to 3 hours. Under S9-activated conditions, cells were exposed for 2 hours, washed, refed culture medium, and incubated for an additional 14 hours. Colcemid was added 2 to 3 hours before cell harvest.

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

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

c. Statistical Methods: The data were evaluated for statistical significance at a p value of 0.05 by Chi-square analysis.

3. Evaluation Criteria:

a. Assay Validity: The assay was considered valid if (1) the percent of cells with aberrations in the negative control group did not exceed 6%; (2) ≥25% of the cells scored in the positive control groups showed chromosome aberrations; and (3) at least one of the test material doses caused a ≥25% reduction in the Relative Cell Growth (RCG).

b. Positive Responses: The test material was considered positive if it caused a significant and dose-related increase in the number of cells with aberrations relative to the solvent control. In the absence of a dose response, if two consecutive doses induced a significant increase in the number of cells with aberrations, the test material was also considered positive.

C. REPORTED RESULTS:

1. Preliminary Cytotoxicity Assay: Results of the preliminary cytotoxicity assay indicated that no cells survived exposure to the three highest nonactivated (250, 500, and 1000 µg/mL) or two highest S9-activated (250 and 500 µg/mL) doses. Relative survival for the remaining nonactivated doses ranged from 65% at 100 µg/mL to 95% at 1.0 µg/mL. Relative survival at 100 µg/mL +S9 was 15%; survival for doses <100 µg/mL +S9 ranged from 66% at 50 µg/mL to 87% at 0.5 µg/mL. Based on these results, the doses selected for the cytogenetic assay were 20, 35, 50, 75, 100, 125, and 150 µg/mL-S9 and 5, 15, 30, 45, 60, and 75 µg/mL +S9.

2. Cytogenetic Assay: As shown in Table 1, 72% of the cells survived exposure to the highest nonactivated dose (150 µg/mL). Metaphases were, therefore, scored from the 100-, 125-, and 150-µg/mL dose groups. There was no significant increase in the percentage of cells with

TABLE 1. Representative Results of the CHO Cell *in vitro* Cytogenetic Assay with Copper Naphthenate

Substance	Dose (µg/mL)	S9 Activation	Relative Percent Survival	No. of Cells Scored	% Cells with Aberrations	Aberrations per Cell	Biologically Significant Aberration[a] (No./Type)
Negative Control							
Untreated Cells	--	-	113	100	2.0	0.04	1TF; 3IF
		+	101	100	2.0	0.02	1R; 1QR
Solvent Control							
Acetone	--	-	100	100	2.0	0.02	2IF
		+	100	100	2.0	0.02	1TB; 1IF
Positive Control							
Triethylenemelamine	0.5	-	66	100	45.0*	0.73	10TB; 11IB; 5TF; 26IF; 2R; 6QR; 12TR; 1CR
Cyclophosphamide	30.0	+	85	100	47.0*	0.81	12TB; 13IB; 6TF; 23IF; 3D; 1R; 10QR; 10TR; 3CR
Test Material							
Copper naphthenate	150[b]	-	72	100	2.0	0.02	1TB; 1IF
	45	+	85	100	8.0	0.10	5TB; 3IF; 1QR; 1TR
	60	+	73	100	9.0	0.09	3TB; 2IF; 1D; 2QR; 1TR
	75	+	51	100	5.0	0.07	1TB; 2IF; 3QR; 1TR

[a]Abbreviations used:

TB - Chromatid Break; IF - Isochromatid Fragment; TR - Triradial
IB - Isochromatid Break; R - Ring Chromosome; D - Dicentric
TF - Chromatid Fragment; QR - Quadriradial; CR - Complex Interchanges

[b]Results for lower nonactivated doses (100 and 125 µg/mL) did not indicate a clastogenic response.

*Significantly higher than the untreated control ($p < 0.01$) by Chi-square tests.

aberrations in metaphases scored for the selected nonactivated doses. In the presence of S9 activation, the percent survival for cultures exposed to the three highest doses of copper naphthenate was ≥51%. Metaphase analysis for these dose groups showed slight but not significant increases in chromosome aberrations. However, the results for the mid-dose group approached borderline significance ($p = 0.063$). Our reviewers noted the presence of rare complex aberrations (triradials, dicentrics, and quadriradials) at the three scored doses of the test material. Furthermore, it was noted that one ring and one quadriradial were scored in the S9-activated, untreated control cultures; none were seen in the solvent-treated cultures.

Based on these findings, the study author concluded, "Under the conditions of this study and according to the criteria set for evaluating the results, copper naphthenate does not induce chromosome aberrations in cultured Chinese hamster ovary cells."

D. REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS:

We assess that the nonactivated test material was assayed to an appropriate concentration and did not induce a clastogenic response. We further assess, in contrast to the study author, that no definitive conclusions can be reached for S9-activated copper naphthenate for the following reasons:

1. The number of cells with aberrations increased as the dose decreased. A similar reverse-dose trend was noted in an unscheduled DNA synthesis primary rat hepatocyte assay conducted with the identical batch number of the test material (see Data Evaluation Record 250-A). Additionally, findings from the S9-activated phase of a mouse lymphoma assay (see Data Evaluation Record 250-B) clearly demonstrated a mutagenic effect.

2. The presence of rare complex aberrations at all S9-activated doses, as well as the borderline significant response at the mid-dose level (60 µg/mL), suggests weak clastogenic activity. However, this finding is difficult to interpret because complex aberrations were also seen in the S9-activated medium control group.

Based on the above considerations, we conclude that copper naphthenate is a presumptive clastogenic material. The assay should be repeated to determine whether the response is reproducible. Additionally, test material purity information was not provided, and no analytical data to support actual concentrations used in the assay were included in the final report or raw data.

E. QUALITY ASSURANCE MEASURES: A quality assurance statement was signed and dated September 15, 1988.

F. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 9-17.

APPENDIX A

MATERIALS AND METHODS
(CBI pp. 9-17)

MATERIALS AND METHODS

INDICATOR CELLS

Source

The Chinese hamster ovary (CHO) cell line used in this study was originally obtained from the American Type Culture Collection. The cell line ATCC No. CCL 61, CHO K-I was cloned, and a subclone suited for the requirements of this assay was selected.

Culture Conditions

The CHO cells were routinely cultured in Ham's F-12 nutrient medium supplemented with 10% heat-inactivated fetal bovine serum (HIFBS), 2mM of L-glutamine, 100 units/ml of penicillin and 100 ug/ml of streptomycin at 37 ±1.0°C in a CO_2 incubator (approximately 5% CO_2 and 95% air) in T-75 plastic tissue culture flasks. The cells were routinely subcultured just before confluency using 0.05% trypsin.

Stock Cultures

The CHO cells were propagated in antibiotic-free medium to obtain a sufficient number of cells for freezing a large number of stock ampules. The cells were cryopreserved in Ham's F-12 nutrient medium supplemented with 10% HIFBS and 8% dimethyl sulfoxide (DMSO) and stored in the vapor phase of liquid nitrogen. Prior to using the stock cultures for the test, representative ampules were tested for contaminating microorganisms, including mycoplasma, and found free of the above. Cell cultures initiated from the stock ampules and maintained by subculturing for less than a month were used in the assays.

CONTROL SUBSTANCES

Positive Controls

Triethylenemelamine (TEM, CAS Registry Number 51-18-3), which causes chromosome aberrations without metabolic activation, was used at 0.5 ug/ml in the non-activated system. The source and lot number of the TEM used in this study are given below.

Source: Polysciences Lot No.: 45272

Cyclophosphamide (CP, CAS Registry Number 6055-19-2), which requires metabolic activation, was used at 30 ug/ml in the activated system. The source and lot number of the CP used in this study are given below.

Source: SIGMA Chemical Co. Lot No.: 114F-0393

The stability of TEM and CP was not determined by SITEK Research Laboratories under the experimental conditions.

Solvent Controls

Acetone was used to dissolve the test article. The source and purity of the acetone used in this study are given below.

Source: Fisher Scientific Co. Lot No.: 871653

CAS Registry Number: 67-64-1

Certificate of Actual Lot Analysis

Assay	99.5%
Density (grams/ml) at 25°C	0.7852
Residue after evaporation	0.0002%
Color (APHA)	5
Water	0.5%

The culture medium (serum-free Ham's F-12 medium) was used as the solvent for the positive control articles.

TEST ARTICLE

The test article Copper Napthenate was received on July 8, 1988, and stored at room temperature. The test article was dissolved and diluted in acetone to prepare the dosing solutions.

EXPERIMENTAL PROCEDURES

Solubility Test

In order to determine the proper vehicle for delivering the test article to the test system, a solubility test was performed. The solubility of the test article in DMSO, acetone and ethanol was tested.

Metabolic Activation System

The metabolic activation system consisted of Aroclor-induced rat liver homogenate (S-9 fraction) and "CORE" in the proportion of 1:4. The "CORE" was prepared by dissolving 24 mg of nicotinamide-adenine dinucleotide phosphate (NADP) and 45 mg of DL-isocitric acid per ml in deionized distilled water. The solution was filter sterilized and kept frozen at -60 to -170°C. Sprague-Dawley rats were induced by Aroclor 1254 at a final concentration of 500 mg of Aroclor per kilogram of rat weight. The livers from induced rats were combined with 3 ml of 0.15M KCl per gram of liver and homogenized. The homogenate was centrifuged at 9000xg for 10 minutes, and the supernatant (S-9 fraction) was collected and frozen at -60 to -170°C.

Immediately before treatment with the test article, the S-9 fraction was combined with the "CORE" in the proportion of 1:4 and added to the medium at 7.5 ml per 100 ml. The final S-9 containing medium contained 1.44 mg/ml of NADP, 2.7 mg/ml of DL-isocitric acid and 15 ul/ml of rat liver homogenate.

Test System Identification

All test cultures were labeled with an indelible pen with a code system which clearly identified the experiment number, activation system, test article concentrations and controls. Slides were labeled with pencil with the test article number, test system, and code numbers for the concentrations tested.

Preparation of Test Article Dosing Solutions

The test article was weighed, dissolved and diluted in acetone to appropriate concentrations prior to use. Approximately 20-30 minutes elapsed between the time the test article was solubilized and the final treatment of the cells. All test article and control treatments were done under UV-filtered lights to avoid possible problems of photoinactivation.

The stability of the test article under the experimental conditions was not determined by SITEK Research Laboratories.

Preparation of Test Cultures

The CHO stock cultures were grown in Ham's F-12 nutrient medium supplemented with 10%, heat-inactivated fetal bovine serum (HIFBS), 2mM L-glutamine, 100 units/ml penicillin and 100 ug/ml streptomycin. Stock cultures growing in T-75 cm^2 tissue culture flasks were harvested and used to prepare the test cultures for the Assay. The culture medium from the T-75 cm^2 flasks was discarded, and the cells were washed with Ca^{++}-free and Mg^{++}-free phosphate buffered saline (PBS). The

cells were then dissociated by adding 2.0 ml of 0.05% trypsin to each flask. The flasks were incubated at 37 ±1.0°C until the cells dissociated. 5 ml of complete culture medium was then added to each of the stock culture flasks, the cell suspensions were pooled, and the trypsin was removed by centrifugation. The cells were resuspended in fresh complete culture medium, and an aliquot of the cell suspension was diluted to the appropriate concentration and counted using a cell counter. Based on the cell counts, a separate cell suspension with $1x10^5$ cells/ml was prepared to seed the test flasks. An appropriate number of T-25 cm^2 tissue culture flasks was seeded with 5 ml of cell suspension to obtain test culture flasks with $5x10^5$ cells/flask. The flasks were incubated at 37 ±1.0°C in a humidified incubator in an atmosphere of approximately 5% CO_2 and 95% air for 18-24 hours.

Range Finding Test

In order to determine the test article concentrations that would produce 0-100% cytotoxicity, a Range Finding Test was performed. The test article was weighed, and a serial dilution was prepared in acetone. The test article was tested at concentrations of 1000, 500, 250, 100, 50, 25, 10, 5.0, and 1.0 ug/ml in the non-activated system and at 500, 250, 100, 50, 25, 10, 5.0, 2.5, 1.0, and 0.5 ug/ml in the activated system. The test article was observed to form precipitates at test concentrations of 250 ug/ml and above.

Test cultures which were seeded approximately 18-24 hours earlier were used in the Range Finding Test. Two replicate cultures were used at each dose level.

In the non-activated system, the culture medium was removed, and 5 ml of fresh medium supplemented with 10% HIFBS was added to each of the culture flasks. The cells were then exposed to the test article for 16 hours.

After the exposure period, the medium was removed from the flasks, and the cells were washed with PBS. 2.0 ml of 0.05% trypsin was added to two cultures at each dose level. The flasks were incubated until the cells were dissociated. An aliquot of the cell suspension was diluted and counted in an electronic cell counter. The number of cells per flask was calculated for each dose level, and the Relative Cell Growth (RCG) was determined using the following formula:

$$RCG = \frac{\text{No. Cells in Test Flask}}{\text{No. Cells in Solvent Flask}} \times 100$$

In the activated system, the medium was removed, and 5 ml of serum-free medium containing S-9 mix was added to each of the culture flasks prior to treatment. The cells were exposed to the test article for 2 hours by adding appropriate volumes of test article stock solutions to the culture medium. After the exposure period, the cells were washed with PBS, refed with culture medium containing 10% HIFBS, and allowed to grow for approximately 14 hours at 37 ±1.0°C in an atmosphere of approximately 5% CO_2 and 95% air. After the incubation period, the cells were washed with PBS, trypsinized, and the cells were counted to determine the RCG.

Chromosome Aberration Assay

The Chromosome Aberration Assay was run with a concurrent Parallel Toxicity Test which was performed as in the case of the Range Finding Test, but with the same treatment procedures as the Chrcmosome Aberration Assay.

The test cultures were prepared as described for the Range Finding Test. Four replicate cultures were treated at each dose level; two were used for cytotoxicity determination in the Parallel Toxicity Test, and the other two were used in the evaluation of induced chromosome aberrations.

The cells were treated with seven doses of the test article ranging from 150 to 20 ug/ml in the non-activated system and at six dose levels ranging from 75 to 5.0 ug/ml in the activated system. One dose of the positive control and appropriate solvent controls were included in both the non-activated and activated systems.

Parallel toxicity determinations were done as described in the Range Finding Test. The Chromosome Aberration Assay was performed as described below.

In the non-activated system, the cells were treated in complete medium with 10% HIFBS. The cultures were treated for 16 hours. After the exposure period, the cells were washed with PBS and refed with complete medium containing 0.1 ug/ml of Colcemid. After 2-3 hours of Colcemid treatment, the dividing cells were harvested by mitotic shake off, swelled in hypotonic KCl, and fixed in Carnoy's fixative (3:1 of methanol and acetic acid).

In the activated system, the cells were treated in serum-free medium containing the S-9 mixture. Treatment was terminated after 2 hours of exposure to the test article. The cultures were allowed to grow for another 14 hours in complete medium with 10% serum, after which the cells were treated with 0.1 ug/ml of Colcemid for 2-3 hours before the dividing cells were harvested and fixed. The fixed cells were kept at 0-4°C for at least 12 hours. The cells were then collected by centrifugation, resuspended in a small volume of fresh fixative, and dropped on microslides to prepare chromosome spreads. The slides were air dried, stained in Giemsa stain, and mounted in Permount using #1 cover glasses.

The slides were scored "blind" in order to avoid bias on the part of the scorer. The three highest test doses, along with one positive control, one solvent control (acetone) and an untreated control, were scored from the non-activated and activated systems. Only cells showing 19 $\pm$2 chromosomes were scored, and the Vernier readings of the cells with chromosome aberration(s) were recorded. A total of 50 cells per culture flask was scored and recorded on the data sheet.

The types of chromosome aberrations scored and the corresponding abbreviations used are given below (1,2):

tg - Chromatid gap - an achromatic region of a width not greater than that of the single chromatid, occurring anywhere along the length of either of the two chromatids of a chromosome.

Isg - Isochromatid gap - same as above, but occurring in both the chromatids of the same chromosome at the same locus.

tb - Chromatid break - an achromatic region of a width greater than that of a single chromatid occurring along the length of either of the chromatids of a chromosome, or a chromatid fragment lying adjacent to but not aligned along the axis of either of the two chromatids.

Isb - Isochromatid break - same as above but occurring in both chromatids of the same chromosome at the same locus.

tf - Chromatid fragment - a piece of chromatid without a centromeric region, but not appearing in connection with any chromosome.

Isf - Isochromatid fragment - same as above but appearing in pairs.

d - Dicentric chromosome - chromosome with two centromeres.

r - Ring chromosome - chromatids or chromosomes with the two ends joined together to form a ring, with or without a centromere.

qr - Quadriradial - simple interchanges occurring between chromatids of two chromosomes and resulting in four-armed configurations.

tr - Triradial - simple interchanges occurring between one chromosome and chromatids of another chromosome, resulting in three-armed configurations.

Cr - Complex interchanges - multiarmed configurations resulting from breakage and reunion of two or more chromosomes.

pu - Pulverization - extreme fragmentation of chromosomes and individual chromosomes no longer recognizable.

sd - Severely damaged cell - cell with ten or more aberrations.

e - Endoreduplication - multiples of two chromatids connected by a centromere.

The slides were decoded after scoring, the chromosome aberration data from the score sheets were consolidated on a Summary Table, and the number of aberrations per cell and the percentage of cells with one or more aberrations for each dose level were calculated. The chromosome aberration data from the two harvests were analyzed separately. Chromatid gaps, chromosome gaps and endoreduplication were scored, but they were not included in calculating the percentage of cells with aberrations and the number of aberrations per cell. Of the remaining aberrations, each aberration scored was counted as one, except pulverization (pu) and severely damaged cell (sd). Each of these two aberrations was considered equal to ten aberrations in calculating the number of aberrations per cell. For statistical analysis, the percentage of aberrant cells at each dose was compared against its concurrent solvent control or the historic solvent control wherever the number was 2% or less.

CRITERIA FOR A VALID ASSAY

1. In the negative control, the number of cells with aberrations (excluding gaps and endoreduplication) should not exceed 6%.

2. At least 25% of the cells scored in the positive control should show one or more chromosome aberrations.

3. At least one of the test doses scored should show more than 25% reduction in the RCG. This requirement should not be applied to test articles where no apparent toxicity could be achieved at the maximum soluble dose or highest allowable dose.

EVALUATION OF TEST RESULTS

Statistical Analysis

The number of cells with aberrations at each treatment level was analyzed for statistical significance, in comparison with the solvent control, using a Chi-square analysis. Significance was determined at the 95% confidence level ($P<0.05$).

Positive Response

The test article is considered to have caused a positive response in this assay if:

1. The test article shows a positive dose-response trend and a statistically significant increase over that of the concurrent solvent controls in the number of cells with aberrations at one or more dose levels.

2. In the event there is no positive dose-response trend, an increase in the number of cells with chromosome aberrations at two consecutive test doses should be statistically significant at the 95% confidence level in a Chi-square analysis.

Equivocal Response

The test article is considered to have caused an equivocal response if:

1. One of the test doses shows a statistically significant increase in the number of cells with aberrations without an accompanying positive dose-response trend.

2. The test article shows a statistically significant dose-response trend, but none of the dose levels shows a significant increase in the number of cells with aberrations.

Negative Response

The test article is considered to have caused a negative response if no indication of a positive dose response is observed and none of the test doses shows a statistically significant increase in the percentage of aberrant cells.

Other Considerations

The above criteria will be used as guidelines in evaluating the test results. However, the Study Director may take other factors into consideration in evaluating the test results.

ARCHIVES

All original raw data, the final report and stained slides are archived at SITEK Research Laboratories, 12111 Parklawn Drive, Rockville, Maryland, 20852.

REFERENCES

1. Evans, H. J. and M. L. O'Riordan. Human peripheral blood lymphocytes for the analysis of chromosome aberrations in mutagen tests. Mut. Res., 31:135-148, 1975.

2. Savage, R. K. John. Classification and relationships of induced chromosomal structural changes. J. Med. Genetics., 12:103-122, 1975.

EPA No.: 68D80056
DYNAMAC No.: 250-E
TASK No.: 2-50E
June 28, 1990

DATA EVALUATION RECORD

COPPER NAPHTHENATE

Mutagenicity--*Salmonella typhimurium* Mammalian Microsome Mutagenicity Assay

APPROVED BY:

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

Signature: William L. McMillan for

Date: 6-28-90

EPA No.: 68D80056
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DATA EVALUATION RECORD

COPPER NAPHTHENATE

Mutagenicity--*Salmonella typhimurium* Mammalian Microsome Mutagenicity Assay

REVIEWED BY:

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Mike Ioannou, Ph.D.
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Toxicology Branch II
(H-7509C)

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Date: 11/2/90

DATA EVALUATION RECORD

CHEMICAL: Copper naphthenate.

STUDY TYPE: Mutagenicity--Salmonella typhimurium mammalian microsome mutagenicity assay.

ACCESSION NUMBER: 411407-05.

TEST MATERIAL: Copper naphthenate.

SYNONYMS/CAS NO. None listed.

SPONSOR: U.S. Army Environmental Hygiene Agency, Aberdeen Proving Grounds, MD.

TESTING FACILITY: Litton Bionetics, Inc., Kensington, MD.

TITLE OF REPORT: Mutagenicity Evaluation of Copper Naphthenate 5/8/85 (Chapman Chemical) in the Ames Salmonella/Microsome Plate Test.

AUTHOR(S): Jagannath, D.R. and Myhr, B.C.

STUDY NUMBER(S): 20988.

REPORT ISSUED: July 1985.

CONCLUSION(S) - EXECUTIVE SUMMARY: Eight concentrations of copper naphthenate (1 to 10,000 µg/plate) were evaluated in the Salmonella typhimurium mammalian microsome mutagenicity assay. The highest assayed dose was cytotoxic to all strains both with and without S9 activation. Cytotoxicity was also apparent at the 5000-µg/plate level +/-S9 in the majority of strains. Although the data did not indicate that the test material was mutagenic in S. typhimurium TA1535, TA1537, TA1538, TA98, or TA100, the use of single plates at each assayed dose of copper naphthenate is not a scientifically sound practice. In addition to this major study flaw, no information on test material purity, lot number of the sample used in the assay, or analytical data to verify actual concentrations

the data are insufficient to support the classification of copper naphthenate as nonmutagenic in this test system.

Study Classification: The study is unacceptable.

A. MATERIALS:

1. Test Material:

Name:	Copper naphthenate
Description:	Blue solid (Note: Reporting laboratory's Safety Office indicated that the test material was a green paste.)
Lot No.:	Not specified
Purity:	Not specified
Contaminants:	None listed
Solvent used:	Acetone
Other comments:	Storage conditions were not reported. Raw data indicated that 200 mg/mL of the test material formed a clear blue solution in acetone.

2. Control Materials:
Negative: Acetone
Solvent/final concentration: 0.025 to 0.15 mL/plate
Positive: Nonactivation:
Sodium azide 10 µg/plate TA100, TA1535
2-Nitrofluorene 10 µg/plate TA98, TA1538
9-Aminoacridine 50 µg/plate TA1537
Other:

Activation:
2-Aminoanthracene 2.5 µg/plate all strains.

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). The S9 fraction (lot No. 05039) was obtained from an unspecified commercial source. The S9 mix contained the following components:

Component	Concentration per Milliliter S9 Mix
NADP (sodium salt)	4 µmoles
D-glucose-6-phosphate	5 µmoles
$MgCl_2$	8 µmoles
KCl	33 µmoles
Sodium phosphate buffer pH 7.4	100 µmoles
S9 fraction	100 µliters

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:

Cytotoxicity Assay: Fourteen doses of the test material ranging from 1.22 to 10,000 µg/plate were evaluated in the nonactivated cytotoxicity assay with S. typhimurium strain TA100.

Mutation Assays: The eight doses selected for the nonactivated and S9-activated mutation assay were 1, 10, 100. 500, 1,000, 2,500, 5,000, and 10,000 µg/plate

B. TEST PERFORMANCE:

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

2. Preliminary Cytotoxicity Assay: The nonactivated preliminary cytotoxicity assay was conducted with 14 doses of the test material ranging from 1.22 to 10,000 µg/plate using S. typhimurium TA100; single plates were prepared for each dose level. The test material at doses ≥2500 µg/plate was cytotoxic as indicated either by the presence of "micro colonies" of TA100 at 5,000 and 10,000 µg/plate or by the reduction in the revertant colony count at 2,500 µg/plate.

3. Mutagenicity Assay: Based on the above findings, eight doses ranging from 1 to 10,000 µg/plate were assayed in the presence and absence of S9 activation. Single plates were prepared for each test material dose; however, positive and solvent control groups were assayed in duplicate. The highest nonactivated and S9-activated dose was severely cytotoxic in all strains. With the exception of strain TA100, 5000 µg/plate +/-S9 was also cytotoxic (Table 1). Reduced revertant colony counts were also noted for strains TA1535, TA1537, TA1538, and TA98 at 2500 µg/plate +/-S9; however, we are unable to determine if these counts represent compound cytotoxicity or normal plating variation since only single cultures were assayed.

TABLE 1. Representative Results of the Salmonella typhimurium Mutagenicity Assay with Copper Naphthenate

Substance	S9 Activation	Dose (µg/plate)	Revertants per Plate of Bacterial Tester Strain[a] TA1535	TA1537	TA1538	TA98	TA100
Solvent Control[b]							
Acetone	-	--	25	11	11	41	166
	+	--	13	10	23	50	139
Positive Control[b]							
Sodium azide	-	10	1479	--	--	--	1407
2-Nitro-fluorene	-	10	--	--	1075	1003	--
9-Aminoacridine	-	50	--	208	--	--	--
2-Anthramine	+	2.5	336	558	2064	2456	2701
Test Material							
Copper naphthenate	-	1000[c]	24	8	8	40	152
	-	2500	12	4	6	16	163
	-	5000[d]	6	1	3	4	154
	+	1000[c]	14	7	14	24	144
	+	2500	11	5	5	22	161
	+	5000[d]	9	0	0	12	160

[a]Duplicate plates were prepared for the solvent and positive control groups; single plates were assayed for each test material dose group.
[b]Average values calculated by our reviewers.
[c]Values for lower doses (1, 10, 100, and 500 µg/plate +/-S9) did not suggest a mutagenic effect.
[d]The highest assayed dose (10,000 µg/plate +/-S9) was severely cytotoxic in all strains.

No appreciable increase in revertant colonies of any strain was noted at any test dose either in the presence or absence of S9 activation. By contrast, data presented for the positive control groups indicated that all strains responded to the mutagenic action of the appropriate nonactivated or S9-activated positive controls.

The study author concluded that copper naphthenate was not mutagenic in this test system.

4. Reviewers' Discussion/Conclusions: Copper naphthenate was assayed over an appropriate concentration range and there was no evidence of a mutagenic response under nonactivated or S9-activated conditions. We assess, however, that the study is technically unacceptable. The use of single plates at each dose of the test material both with and without S9 activation is not an acceptable practice. Replicates are needed to provide evidence of reproducible results and an indication of mutant colony count variability; otherwise an evaluation of test material results is not possible. Additionally, the report lacked information on test material purity, the lot number of the test sample, and analytical data to support actual concentrations used in the assay.

5. Quality Assurance: A quality assurance statement was signed and dated July 16, 1985.

6. CBI Appendix: Appendix A, Materials and Methods, CBI pp 5-13.

APPENDIX A

Materials and Methods, CBI pp 5-13

AMES SALMONELLA/MICROSOME PLATE ASSAY

1. OBJECTIVE

The objective of this study was to evaluate a test material for mutagenic activity in a bacterial assay with and without a mammalian S9 activation system.

2. RATIONALE

The Salmonella typhimurium strains used at LBI are all histidine auxotrophs by virtue of mutations in the histidine operon. When these histidine-dependent cells are grown in a minimal media petri plate containing a trace of histidine, only those cells that revert to histidine independence (his+) are able to form colonies. The trace amount of histidine allows all the plated bacteria to undergo a few divisions; this growth is essential for mutagenesis to occur. The his+ revertants are easily scored as colonies against the slight background growth. The spontaneous mutation frequency of each strain is relatively constant, but when a mutagen is added to the agar the mutation frequency is increased 2- to 100-fold. Cells which grow to form colonies on the minimal media petri plates are therefore assumed to have reverted, either spontaneously or by the action of a test substance to his+ genotype.

3. MATERIALS

A. Indicator Microorganisms

The Salmonella typhimurium strains used in this assay were obtained from Dr. Bruce Ames, University of California at Berkeley (Ames et al., 1975). The following strains are used:

Strain Designation	Gene Affected	Additional Mutations: Repair	LPS	R Factor	Mutation Type Detected
TA-1535	his G	Δ uvr B	rfa	-	Base-pair substitution
TA-1537	his C	Δ uvr B	rfa	-	Frameshift
TA-1538	his D	Δ uvr B	rfa	-	Frameshift
TA-98	his D	Δ uvr B	rfa	pKM101	Frameshift
TA-100	his G	Δ uvr B	rfa	pKM101	Base-pair substitution

3. MATERIALS (continued)

The aforementioned strains have, in addition to the mutation in the histidine operon, a mutation (rfa-) that leads to defective lipopolysaccharide coat, a deletion that covers genes involved in the synthesis of vitamin biotin (bio-) and in the repair of ultraviolet (uv) - induced DNA damage (uvrB-). The rfa- mutation makes the strains more permeable to many large molecules. The uvrB- mutation decreases repair of some types of chemically or physically damaged DNA and thereby enhances the strain's sensitivity to some mutagenic agents. The resistant transfer factor plasmid (R factor) pKM101 in TA-98 and TA-100 is believed to cause an increase in error-prone DNA repair that leads to many more mutations for a given dose of most mutagens (McCann, et al., 1975). In addition, plasmid pKM101 confers resistance to the antibiotic ampicillin, which is a convenient marker to detect the presence of plasmid in the cells.

The indicator strains were kept at 4°C on minimal medium plates supplemented with a trace of biotin, an excess of histidine, and ampicillin (25 ug/ml) for TA-98 and TA-100, to ensure stable maintenance of plasmid pKM101. New stock culture plates are made as often as necessary from frozen master cultures or from single colony reisolates that were checked for their genotypic characteristics (his, rfa, uvrB, bio) and for the presence of the plasmid.

B. Media

For daily use, an inoculum from stock culture plates is grown overnight at 37°C in Oxoid Media #2 (nutrient broth) and used in the mutagenicity test. The minimal media plates for the selection of histidine revertants consisted of the Vogel Bonner Medium E (Vogel and Bonner, 1956) with 2% glucose and 1.5% bactoagar. The overlay agar contained the following per 100 ml volume; 0.6 gms of purified agar, 10 ml of 0.5mM L-Histidine-0.5mM Biotin and 0.5 g NaCl according to the method of Ames, et al. (1975).

C. Activation System

(1) S9 Homogenate

A 9,000 x g supernatant prepared from Sprague-Dawley adult male rat liver induced by Aroclor 1254 (described by Ames et. al., 1975) was purchased commercially and used in this assay.

3. MATERIALS (continued)

(2) S9 Mix

Components	Concentration per Milliliter S9 Mix
NADP (sodium salt)	4 µmoles
D-glucose-6-phosphate	5 µmoles
$MgCl_2$	8 µmoles
KCl	33 µmoles
Sodium phosphate buffer pH 7.4	100 µmoles
Organ homogenate from rat liver (S9 fraction)	100 µliters

4. EXPERIMENTAL DESIGN

A. Dosage Selection

Doses were selected for the actual assay based on a preliminary toxicity test with the strain TA-100. Fourteen doses using two-fold dilutions from 10,000 µg per plate for solids and 150 µl per plate for liquids were used in this dose selection assay. For the actual assay, at least six doses were selected with the highest dose exhibiting 100% toxicity. Nontoxic chemicals were tested up to 10 mg per plate for solids and 150 ul per plate for liquids.

B. Toxicity Studies

To a sterile test tube containing 2.0 ml of overlay agar (placed in a 43°-45°C water bath) the following were added:

- 0.025 - 0.150 ml of a solution of the test material to give the appropriate dose.
- 0.2 ml of an overnight culture.
- 0.5 ml of 0.2M phosphate buffer, pH 7.4.

This mixture was swirled gently and then poured on to minimal agar plates (see 3B, Media). After the overlay agar had set, the plates were incubated at 37°C for approximately 2 days. The number of colonies growing on the plates were counted and recorded.

A reduction in the number of revertants, appearance of microcolonies, or clearing of the background lawn on the test material treated plates as compared to the solvent control plates were considered as indications of toxicity by the test material.

4. EXPERIMENTAL DESIGN (continued)

B. Toxicity Studies (continued)

If the doses are specified by the sponsor, the toxicity studies as outlined in 4:B, will not be performed on the test material and the assays are run using the specified doses. These doses will be reported in the interpretation of results and in the results tables.

C. Mutagenicity Testing

The procedure used is based on the paper published by Ames et. al., 1975) and was performed as follows:

(1) Nonactivation Assay

To a sterile test tube placed in a 43°C-45°C water bath the following were added in order:

(a) 2.00 ml of overlay agar (see 3B. Media).

(b) 0.025 - 0.15 ml of a solution of the test chemical to yield the appropriate dose.

(c) 0.2 ml of indicator organism.

(d) 0.50 ml of 0.2M phosphate buffer, pH 7.4.

This mixture was swirled gently and then poured onto minimal agar plates (see 3B, Media). After the top agar had set, the plates were incubated at 37°C ± 2° for approximately 2 days. The number of his+ revertant colonies growing on the plates were counted and recorded.

(2) Activation Assay

The activation assay was run concurrently with the nonactivation assay. The only difference was the addition of 0.5 ml of S9 mix (see 3C:2, Activation System) in place of 0.5 ml of phosphate buffer that was added in nonactivation assays. All other details are similar to the procedure for nonactivation assays.

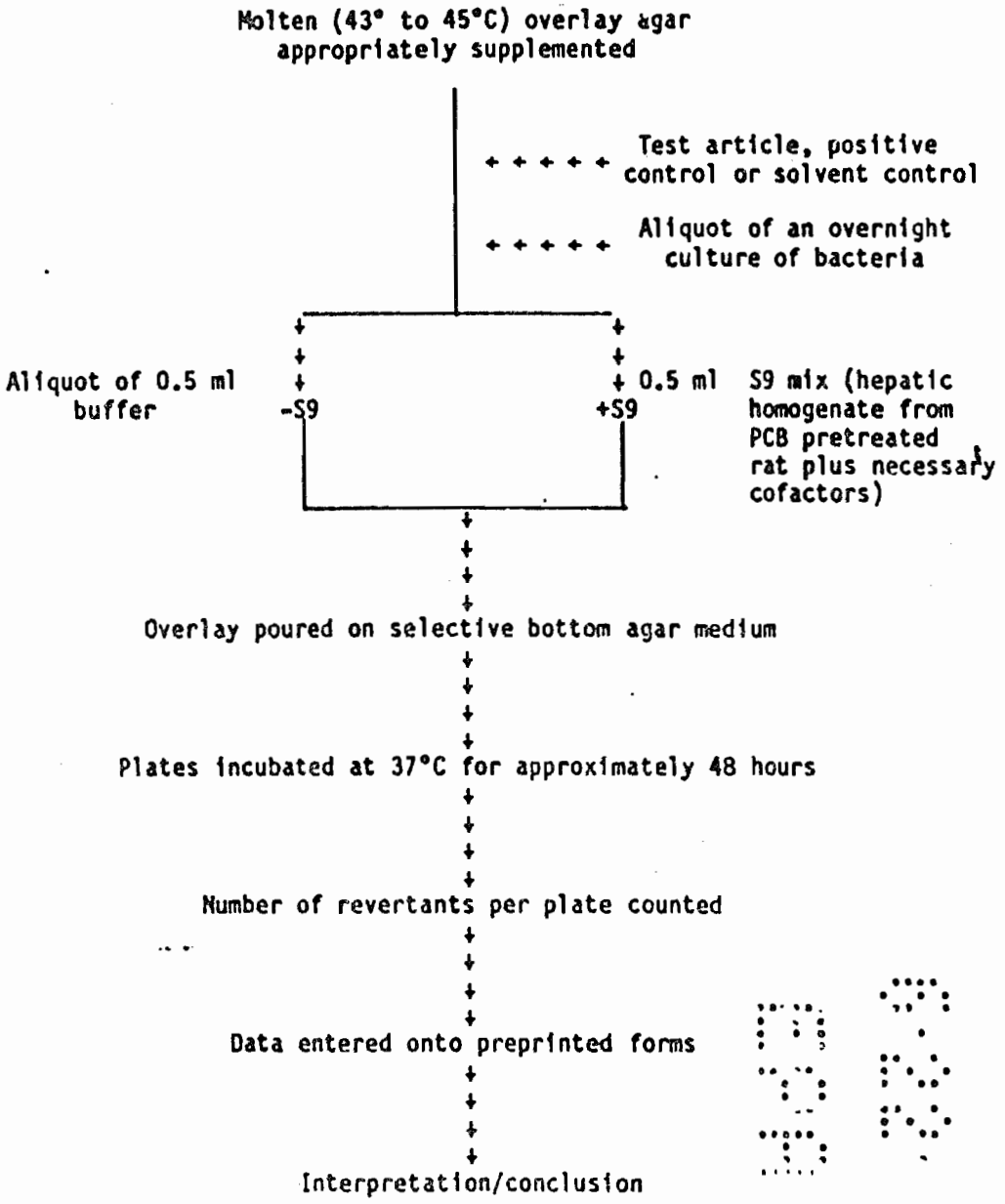
A detailed flow diagram for the plate incorporation assay is provided in Figure 1.

D. Control Compounds

(1) A negative control, consisting of the solvent used for the test material, was assayed concurrently with the test material. The solvent control was employed for each indicator strain and was used in the absence and presence of S9 mix.

FIGURE 1

REVERSE MUTATION ASSAY
(Agar Incorporation Method)



4. EXPERIMENTAL DESIGN (continued)

D. Control Compounds (continued)

(1) Negative Control Article

The solvent was tested at a single concentration equal to the maximum volume used to administer the highest dose of the test article or at the concentration indicated in the Results Table(s) of this report. The solvent used to prepare the stock solution of the test article is given in the Results Section of this report. All dilutions of the test article were made using this solvent.

(2) Positive Control Articles

Strain specific positive controls and positive controls to ensure the efficacy of the S9 mixture were assayed concurrently with the test material. The following positive controls were employed in the assays:

Assay	Chemical	Solvent	Concentrations per Plate (µg)	Salmonella Strains
Nonactivation	Sodium azide (SA)	Water	10.0	TA-1535, TA-100
	2-Nitrofluorene (NF)	Dimethyl-sulfoxide	10.0	TA-1538, TA-98
	9-aminoacridine (9-AA)	Ethanol	50.0	TA-1537
Activation	2-anthramine	Dimethyl-sulfoxide	2.5	For all strains

5. EVALUATION CRITERIA

Statistical methods are not currently used, and evaluation is based on the criteria included in this protocol.

Plate test data consist of direct revertant colony counts obtained from a set of selective agar plates seeded with populations of mutant cells suspended in a semisolid overlay. Because the test material and the cells are incubated in the overlay for approximately 2 days and a few cell divisions occur during the incubation period, the test is semiquantitative in nature. Although these features reduce the quantitation of result, they provide certain advantages not contained in a quantitative suspension test:

5. EVALUATION CRITERIA (continued)

- The small number of cell divisions permits potential mutagens to act in replicating DNA, which is often more sensitive than nonreplicating DNA.

- The combined incubation of the test material and the cells in the overlay permits constant exposure of the indicator cells for approximately 2 days.

A. Evaluation Criteria for Ames Assay

Because the procedures used to evaluate the mutagenicity of the test material were semiquantitative, the criteria used to determine positive effects were inherently subjective and were based primarily on a historical data base. Most data sets were evaluated using the following criteria:

(1) Strains TA-1535, TA-1537 and TA-1538

Data sets will be evaluated as positive if a dose response is observed over a minimum of three test concentrations and the increase in revertants is equal to or greater than three times the solvent control value at the peak of the dose response. The solvent control value should be within the normal range for evaluating the results (see also 5B: Dose response phenomena).

(2) Strains TA-98 and TA-100

Data sets will be evaluated as positive if a dose response is observed over a minimum of three test concentrations and the increase in revertants achieves a doubling of the solvent control value at the peak of the dose response. The solvent control value should be within the normal range for evaluating the results (see also 5B: Dose response phenomena).

The following ranges of revertants for solvent controls are generally considered acceptable:

TA-1535:	8-30
TA-1537:	4-30
TA-1538:	10-35
TA-98:	20-75
TA-100:	80-250

(3) Pattern

Because TA-1535 and TA-100 are both derived from the same parental strain (G-46) and because TA-1538 and TA-98 are both derived from the same parental strain (D3052), to some extent there is a built-in redundancy in the microbial assay. In general, the two strains of a set respond to the same mutagen and such a pattern is sought. Generally, if a strain responds to a mutagen in nonactivation tests, it will do so in activation tests. Occasionally, exceptions to this pattern may be seen.

5. EVALUATION CRITERIA (continued)

B. Dose-Response Phenomena

The demonstration of dose-related increases in revertant counts is an important criterion in establishing mutagenicity. Since we employ several doses in the actual assay, a dose response would normally be seen with a mutagenic test material. Additional tests may be performed over a narrower dose range if the mutagenic test material fails to exhibit a dose-response in the initial assay. However, occasionally it is difficult to generate a dose-response and the test material will be evaluated based on the available data.

C. Reproducibility

If a test material produces a response in a single test which cannot be reproduced in additional runs, the initial positive test data lose significance.

D. Control Tests

Positive and negative control assays are conducted with each experiment and consist of direct-acting mutagens for nonactivation assays and mutagens requiring metabolic biotransformation in activation assays. Negative controls consist of the test material solvent in the overlay agar, together with the other essential components. The negative control plate for each strain gives a reference point to which the test data are compared. The positive control assay is conducted to demonstrate that the test systems are functional with known mutagens.

REFERENCES

1. Ames, B.N., McCann, J. and Yamasaki, E.: Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutation Res., 31:347-364, 1975.

2. McCann, J., Springarn, N.E., Kobori, J. and Ames, B.N.: Detection of carcinogens as mutagens: Bacterial tester strains with R factor plasmids. Proc. Nat. Acad. Sci. USA, 72:979-983, 1975.

3. Vogel, H.J. and Bonner, D.M.: Acetylornithinase of E. coli; partial purification and some properties. J. Biol. Chem., 218:97-106, 1956.

EPA No.: 68D80056
DYNAMAC No.: 250-F
TASK No.: 2-50F
June 28, 1990

DATA EVALUATION RECORD

COPPER NAPHTHENATE

Mutagenicity--Mouse Lymphoma Forward Mutation Assay

APPROVED BY:

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

Signature: William L. McLellan for

Date: June 28, 1990

EPA No.: 68D80056
DYNAMAC No.: 250-F
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DATA EVALUATION RECORD

COPPER NAPHTHENATE

Mutagenicity--Mouse Lymphoma Forward Mutation Assay

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DATA EVALUATION RECORD

CHEMICAL: Copper naphthenate.

STUDY TYPE: Mouse lymphoma forward mutation assay.

MRID NUMBER: 411407-06.

TEST MATERIAL: Copper naphthenate.

SYNONYMS/CAS NO.: None listed.

SPONSOR: U.S. Army Environmental Hygiene Agency, Aberdeen Proving Ground, MD.

TESTING FACILITY: Litton Bionetics, Inc., Kensington, MD.

TITLE OF REPORT: Mutagenicity of Copper Naphthenate in a Mouse Lymphoma Mutation Assay.

AUTHORS: Cifone, M.A. and Myhr, B.C.

STUDY NUMBER: 20989.

REPORT ISSUED: November 1985.

CONCLUSIONS/Executive Summary:

Copper naphthenate was evaluated for the potential to cause forward gene mutations in mouse lymphoma L5178Y ($TK^{+/-}$) cells in both the presence and absence of S9 activation. The results indicated that six nonactivated doses (10 to 80 µg/mL) were not mutagenic; doses

>80 µg/mL were severely cytotoxic. In the presence of S9 activation, severe cytotoxicity was apparent at concentrations >60 µg/mL. No evidence of a mutagenic effect was observed at the six treatment levels (2.5, 20, 30, 40, 50, or 60 µg/mL) selected for expression of forward mutations at the thymidine kinase locus. Although the assay was performed in a technically acceptable manner, there is concern regarding the validity of the results. The negative findings of this assay conflict with the results of an independently performed mouse lymphoma assay.[1]

In the latter assay, it was concluded that S9-activated copper naphthenate induced a clear dose-related mutagenic effect that was confined to a narrow range of concentrations (20, 30, and 40 µg/mL). Additionally, the concentration of S9 in the S9 cofactor mix (25%) was ≈2.5 times higher than the level used in the currently reviewed assay (with the exception of the 10% S9, the concentration of other components in the S9 mix were not listed).

It was also noted that both studies used comparable dose ranges of copper naphthenate and the assessment of cytotoxicity was generally in agreement. However, results from the mutation assay, the physical description of copper naphthenate, the solvent, and the S9 concentrations used in these assays were different (see Section D, Reviewer's Discussion and Interpretation of Results). Furthermore, neither study provided information on test material purity or analytical data to verify actual concentrations used in the assays. Based on the above considerations, both studies were classified as unacceptable; more important, however, we are not able to determine which results are valid. It is, nevertheless, conceivable that while copper naphthenate was soluble in both acetone and DMSO, mutagenic impurities that may have been present in the sample dissolved in DMSO were highly soluble in this solvent.

Study Classification: The study is unacceptable. It is recommended that the assay be repeated. It is considered prudent that the repeat test use both ethanol and acetone as the test material solvents, and include varying concentrations of S9 in the metabolic reaction mixture to determine if these differences between the studies affected the outcome of the results.

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[1]DER 250B; Kirby et al. Evaluation of a Test Article in the L5178Y TK$^{+/-}$ Mouse Lymphoma Mutagenesis Assay in the Presence and Absence of Aroclor-Induced Rat Liver S9; unpublished Study No. 0088-2400, prepared for the U.S. Environmental Hygiene Agency, Aberdeen Proving Grounds, MD/Toxicon, Woburn, MA, by SITEK Research Laboratories, Rockville, MD; dated August 19, 1988; MRID No. 411407-02.

A. MATERIALS:

1. Test Material:

Name:	Copper naphthenate (Chapman Chemical, 5/8/85)
Description:	Green solid
Lot No.:	Not specified
Purity:	Not specified
Contaminants:	None listed
Solvent used:	Absolute ethanol (ETOH)
Other comments:	Storage conditions were not reported. The test material formed an opaque blue-green solution in ETOH at a concentration of 100 mg/mL. The test material at concentrations up to 125 µg/mL appeared soluble in culture medium (RPMI 1640 medium); higher levels (250 to 1000 µg/mL) formed a blue-green precipitate in RPMI 1640. Stock solutions were prepared immediately prior to use.

2. Indicator Cells: The mouse lymphoma cell line, L5178Y ($TK^{+/-}$), clone 3.7.2, was derived from the Fischer L5178Y cell line and obtained from Dr. Donald Clive, Burroughs Wellcome, Research Triangle Park, NC. Stock cultures were maintained in liquid nitrogen. Cultures were periodically checked for mycoplasma contamination and exposed to methotrexate to maintain a low background frequency of trifluorothymidine (TFT)-resistant cells.

3. S9 Fraction: The S9 fraction (Batch No. R-232) was commercially prepared (source not reported) and was derived from the livers of adult male rats (strain not specified) induced with Aroclor 1254. The S9 mix contained the 10% S9 microsome fraction (see Mouse Lymphoma Assay raw data, CBI p. 1) and unreported concentrations of NADP and isocitric acid.

4. Positive Controls: Ethylmethanesulfonate (EMS) at 0.25 to 0.5 µL/mL and 3-methylcholanthrene (3-MCA) at 1.0 to 4.0 µg/mL were used as the nonactivated and S9-activated positive controls, respectively.

5. Media/Growth Conditions: Cells were grown in RPMI 1640 medium supplemented with 10% horse serum, L-glutamine, sodium pyruvate, antibiotics, and pluronic solution. Cloning medium was growth medium with the addition of agar (0.35%); selection medium was cloning medium containing 3 µg/mL TFT. All cultures were maintained in a humidified incubator at 37°C in 5% carbon dioxide.

B. STUDY DESIGN:

1. Preliminary Cytotoxicity Assay: The preliminary cytotoxicity assay was performed with a "wide range" of test material doses, starting with either the highest soluble concentration or a maximum applied dose of 5000 µg/mL; lower doses were separated by twofold dilutions. Cells (density not reported) were exposed to the test material concentrations for 4 hours either in the presence or absence of S9 activation. Following exposure, cells were washed and resuspended in fresh growth medium, and cell viability was determined the next day. Relative toxicity was calculated and used to establish a dose range for the mutation assay that included doses yielding 0 to 90% cytotoxicity.

2. Mutation Assay: Cells seeded at $6x10^6$ cells/tube were exposed to the appropriate test material doses, solvent, or positive controls with or without S9 activation for 4 hours. Cells were washed, resuspended in growth medium, and reincubated for 2 days. Daily cell counts were determined, and cells were diluted when appropriate to maintain an optimal growth rate. At the end of the expression period, at least five doses were chosen for mutant selection.

   For mutant selection, $1x10^6$ cells were seeded into triplicate selection medium plates. The cloning efficiency (CE) was determined by plating 200 cells/plate (in triplicate) in cloning medium. After 10 to 14 days of incubation, TFT-resistant colonies and the total number of viable cells were counted; mutation frequencies (MF) were calculated.

3. Evaluation Criteria:

   a. Assay Acceptability: For the assay to be considered acceptable, the following criteria must be satisfied: (1) the CE of the solvent control should be between 60 and 130%; (2) the average suspension growth of the solvent control value must be at least $8x10^5$; (3) the background MF of the solvent control should range from $10x10^{-6}$ to $100x10^{-6}$; (4) positive control values must be within the reporting laboratory's historical range; (5) the test material must be assayed to a dose that reduces the relative growth to 10 to 20%; and (6) the test material MF can be evaluated only if the relative CE is $\geq$10% and the total number of viable clones exceeds 60.

b. Positive Response: The test material was considered positive if it induced a dose-related increase in the MF that was at least 150% of the concurrent background frequency (solvent control) plus $10x10^{-6}$.

C. REPORTED RESULTS:

1. Preliminary Cytotoxicity Assay: Data were not reported from the preliminary cytotoxicity assay; the authors stated, however, that test material doses ≥250 µg/mL were insoluble in tissue culture medium. The authors further indicated that 125 µg/mL of the test material both with and without S9 activation was lethal. Based on these results, the highest dose selected for the nonactivated and S9-activated mutation assays was 100 µg/mL.

2. Mutation Assay: In the nonactivated assays, the highest dose (100 µg/mL) was severely cytotoxic. Six doses (10, 30, 40, 50, 60, and 80 µg/mL) were cloned for mutant selection. Percent relative suspension growth was dose related and ranged from 18.6% at 80 µg/mL to 97.8% at 10 µg/mL. No appreciable increases in total mutant colonies or MFs were observed in cultures exposed to the six nonactivated doses of copper naphthenate. Similarly, the S9-activated test material (2.5, 20, 30, 40, 50, and 60 µg/mL) induced a dose-related cytotoxic effect, which ranged from 18.1% relative suspension growth at 60 µg/mL to >100% survival at 2.5 µg/mL, but was not mutagenic. By contrast to the negative results for the test material, the positive controls (0.25 and 0.40 µL/mL EMS -S9 and 2.5 and 4.0 µg/mL 3-MCA +S9) induced clear mutagenic responses. Based on these findings, the study authors concluded that copper naphthenate was not mutagenic in the mouse lymphoma forward mutation assay. Representative results from the nonactivated and S9-activated assays are presented in Table 1.

D. REVIEWERS' DISCUSSION AND INTERPRETATION OF RESULTS:

We assess that the study was properly conducted and that the study authors' interpretation of the data was correct. Under both nonactivated and S9-activated conditions, copper naphthenate was assayed to cytotoxic levels with no indication of a mutagenic effect. It was noted, however, that the results of this mouse lymphoma assay were not in agreement with the findings of a subsequent mouse lymphoma assay performed at

TABLE 1. Representative Results from the Mouse Lymphoma Forward Mutation Assay with Copper Naphthenate

Substance	Dose/mL	S9 Activation	Relative Percent Suspension Growth[a]	Total Mutant Colonies	Total Viable Colonies	% Cloning Efficiency	Relative Percent Total Growth	Mutation Frequency[b] x 10^{-6}
Solvent Control								
Ethanol	--	-	100.0	128	589	98.1	100.0	43.6
	--	+	100.0	148	621	103.5	100.0	47.6
Positive Control[c]								
Ethylmethanesulfonate	0.25 µL	-	76.4	1070	450	75.0	58.4	475.5*
3-Methylcholanthrene	2.5 µg	+	80.0	726	220	36.7	28.4	660.0*
Test Material								
Copper naphthenate	80 µg[d]	-	18.6	137	573	97.3	18.1	47.8
	60 µg[d]	+	18.1	150	437	70.4	12.7	68.6

[a]Relative Percent Suspension Growth = $\frac{\text{Suspension Growth (test group)}}{\text{Suspension Growth (solvent control)}} \times 100.$

[b]Mutation Frequency (MF) = $\frac{\text{Total Mutant Colonies}}{\text{Total Viable Colonies}} \times 2\times10^{-4}.$

[c]The positive controls were assayed at two dose levels; results from the lowest dose were selected as representative.

[d]Results for lower doses (10, 30, 40, 50, or 60 µg/mL -S9 or 2.5, 20, 30, 40, or 50 µg/mL +S9) did not indicate a mutagenic effect.

*Fulfills the reporting laboratory's criterion for a positive response (i.e., 150% of the concurrent solvent control MF + 10×10^{-6}): >75.4×10^{-6} -S9 or 81.4×10^{-6} +S9.

another laboratory (see DER 250B; Kirby et al.; August 19, 1988; MRID No. 411407-02). The study conducted by Kirby et al. indicated that comparable dose ranges of copper naphthenate (10 to 100 µg/mL -S9 and 1 to 50 µg/mL +S9) induced cytotoxic effects (i.e., <20% cell survival at doses ≥75 µg/mL -S9 and at doses ≥40 µg/mL +S9), an equivocal mutagenic effect under nonactivated conditions, and a dose-dependent mutagenic response over a narrow S9-activated concentration range (20 to 40 µg/mL). Other differences noted in these studies were the physical description of the test material (green solid in the currently reviewed assay and a viscous black liquid in the study of Kirby et al.), the solvents (absolute ethanol in the currently reviewed assay in contrast to acetone in the Kirby et al. study), and the concentration of S9 in the S9-cofactor mix (10% in the reviewed study; 25% in the study of Kirby et al.). Neither study provided information on the purity of copper raphthenate; therefore, the impact of test material purity on these conflicting results cannot be assessed. Both studies were performed in an acceptable manner. However, because of the lack of agreement between independently performed assay results, the physical differences among test samples, and the use of different solvents and different concentrations of S9, we are unable to determine which of the two assays represents a valid assessment of the potential mutagenic activity of copper naphthenate. It is, nevertheless, conceivable, that while copper naphthenate was soluble in both acetone and DMSO, mutagenic impurities that may have been present in the sample dissolved in DMSO were highly soluble in this solvent.

E. Quality Assurance Measures: A quality assurance statement was signed and dated November 24, 1985.

F. CBI Appendix, Appendix A, Materials and Methods, CBI pp. 3-11.

APPENDIX A

CBI Appendix, Materials and Methods
(CBI pp. 3-11)

VII. STUDY DATES:

A. Initiation Date: July 30, 1985

B. Completion Date: October 30, 1985

VIII. SUPERVISORY PERSONNEL:

A. Study Director: Maria A. Cifone, Ph.D.

B. Laboratory Supervisor: Violeta V. Balinas

IX. MATERIALS:

A. Indicator Cells

The mouse lymphoma cell line, L5178Y $TK^{+}/-$ - 3.7.2C, used in this assay was derived from the Fischer L5178Y line of Dr. Donald Clive. Stocks are maintained in liquid nitrogen and laboratory cultures are periodically checked for the absence of mycoplasma contamination by culturing methods. To reduce the negative control frequency (spontaneous frequency) of TK-/- mutants to as low a level as possible, cell cultures are exposed to conditions which select against the TK-/- phenotype (exposure to methotrexate) and are then returned to normal growth medium for three to eight days before use.

B. Media

The cells are maintained in RPMI 1640 medium supplemented with pluronic solution, L-glutamine, sodium pyruvate, antibiotics, and horse serum (10% by volume). Cloning medium consists of the preceding growth medium with the addition of agar to a final concentration of 0.35% to achieve a semisolid state. Selection medium is cloning medium containing 3 µg/ml of TFT.

C. Control Compounds

1. Negative Control Articles

In all cases, the negative control article is the solvent used in the assay. Three solvent controls are included in each assay. In the place of the test material in the solvent controls, an equivalent volume of the solvent of choice is included in the assay mixture (the final concentration of organic solvent in the growth medium is no more than 1%). For test substances assayed with activation, the solvent controls include the activation mixture.

IX. MATERIALS: (continued)

C. Control Compounds (continued)

2. Positive Controls

Ethylmethane sulfonate (EMS) is highly mutagenic via alkylation of cellular DNA and is used at 0.25 to 0.5 µl/ml as a positive control for nonactivation studies.

3-Methylcholanthrene (MCA) requires metabolic activation by microsomal enzymes to become mutagenic and is used at 1.0 to 4.0 µg/ml as a positive control for assays performed with activation.

X. EXPERIMENTAL DESIGN:

A. Solvent Selection

The solvent of choice for Copper Naphthenate in this assay is absolute ethanol. Glass tubes and pipets are used for all test chemical stock solutions. Test chemical stocks are not stored but are prepared fresh for each mutagenesis experiment. Unless specified by the client, the test material is assayed at a pH between 7.0 and 8.0. Neutralization with HCl or NaOH is performed if necessary to maintain the desired pH range. Mouse lymphoma cultures and plates are labeled according to SOP No. 509.

B. Dose Selection

After the selection of a suitable solvent, a preliminary cytotoxicity experiment is performed to establish an appropriate concentration range for the mutation experiment. This study is performed both with and without S9 activation since substantial shifts in toxicity often occur for the two test conditions. A wide range of test article concentrations is tested for cytotoxicity, starting with the highest soluble concentration or a maximum applied dose of 5 mg/ml (or 5 µl/ml) for watersoluble articles or 1 mg/ml (or 1 µl/ml) for articles in organic solvents and followed by two-fold dilution steps. After an exposure time of 4 hours at 37°C, the cells are resuspended in growth medium and incubated for 24 hours, using procedures identical to those for the mutation experiments. A cell count is determined after the 24-hour period to measure the reduction in cell growth relative to the solvent control cell cultures. Usually 7 to 12 doses are then selected for the mutation experiments, using the following criteria:

X. EXPERIMENTAL DESIGN: (Continued)

B. Dose Selection (continued)

- Concentrations are chosen in appropriate steps to cover a toxicity range from little or no survival to 80-100% growth compared to the solvent control, or

- If little or no toxicity is observed and solubility is maintained, the mutation experiment is routinely initiated with a maximum concentration of 5 mg/ml (or 5 µl/ml). If insolubility occurs at concentrations less than 5 mg/ml (or 5 µl/ml) then the maximum concentration is 1 mg/ml (or 1 µl/ml) or the limits of solubility, whichever is highest.

- For insoluble test materials, a range of concentrations that is practical is assayed.

Although seven (or more) doses may be selected to initiate a mutation experiment, the objective is to carry five doses through the entire experiment. This procedure compensates for daily variations in cellular toxicity and helps to ensure the choice of at least four 4 doses appropriately spaced in the relative growth range of approximately 10-100%.

C. Mutagenicity Testing

1. Nonactivation Assay

The assay procedure used is based on reports by Clive and Spector (1975) and Clive, et al. (1979). The cells for the experiment are obtained from logarithmically growing laboratory stock cultures and are seeded into a series of tubes at 6×10^6 cells per tube. The volume during treatment with the test chemical and throughout the expression period is 10 ml per tube and 20 ml per tube respectively. The dosed tubes are placed in a 37±2°C shaker incubator for an exposure period of 4 hours. Afterwards, the cells are washed twice, resuspended in growth medium and returned to the incubator.

The appearance of the treated cultures (precipitate formation, oil separation, pH change) is recorded at the time of treatment; any changes over the four-hour treatment period are also noted.

X. EXPERIMENTAL DESIGN: (Continued)

C. Mutagenicity Testing (continued)

1. Nonactivation Assay (continued)

A standard expression period of two days is used to allow recovery, growth and expression of the TK-/- phenotype. Cell densities are determined on Day One (about 24 hours after treatment) and are adjusted to $3x10^5$ cells/ml to maintain optimal growth rates. If the cells fail to multiply to a density of $4x10^5$/ml, the cultures are returned to the incubator without being diluted. On Day Two, cell counts are again determined, and appropriate cultures are selected for cloning and mutant selection.

Five doses are usually selected for mutant analysis to include a wide range of toxic action so that moderately toxic treatments (approximately 50% relative growth or greater) and highly toxic treatments (approximately 10-20% relative growth) are represented, if possible. Tubes with cell densities less than approximately $3x10^5$ cells/ml are not considered for analysis.

Each tube culture selected for analysis is sampled to obtain cells for exposure to the selection agent and to determine the cloning efficiency of the population. A total sample size of $3x10^6$ cells is suspended in selection medium to select for mutants. This sample is distributed into three 100 mm dishes so that each dish contains approximately $1x10^6$ cells. The cloning efficiency is determined by serially diluting the sample and seeding each of three dishes with approximately 200 cells in cloning medium. All of the dishes are placed in a 37±2°C incubator with approximately 5% CO_2/humidified air for colony development. After 10 to 14 days in the incubator, the colonies are counted with an electronic colony counter.

The mutant frequency is calculated by dividing the total number of colonies in each set of three mutant selection dishes by the total count in the set of three viable count dishes and multiplying by $2x10^{-4}$. If one corresponding dish in either set is lost to contamination or another cause, the colony count on the missing dish is determined by a proportion equation based on weights of the three selection dishes and the colony counts in the two acceptable dishes. A mutant frequency calculated in this manner is footnoted to indicate the possibility of a spurious variation.

X. EXPERIMENTAL DESIGN: (Continued)

C. Mutagenicity Testing (continued)

1. Nonactivation Assay (continued)

The measurement of the toxicity of each treatment is the relative suspension growth of the cells over the two-day expression period multiplied by the cloning efficiency, relative to the average solvent control. Although not strictly a measure of cell survival, this parameter (called percent relative growth) provides a measure of the effectiveness of treatment and is used as the basis for selecting doses for any necessary repeat trials.

2. Activation Assay

The activation assay is often run concurrently with the nonactivation assay; however, it is an independent assay performed with its own set of solvent and positive controls. The two assays are identical except for the addition of the S9 fraction of rat liver homogenate and necessary cofactors (CORE) during the four-hour treatment period. The 10 ml volume during treatment includes this S9 activation mix which is prepared just prior to use and kept on ice. CORE consists of nicotinamide adenine dinucleotide phosphate (NADP, the sodium salt) and isocitric acid. The S9 homogenate is commercially prepared and consists of the 9000 x g supernatant prepared from Aroclor 1254-induced adult male rat livers. Each batch of S9 is checked for sterility and assayed for AHH activity and protein content. Normal activation of the MCA positive control treatment must be achieved in the mouse lymphoma assay before accepting a particular batch for test article screening.

D. Assay Acceptance Criteria

An assay will normally be considered acceptable for evaluation of the test results only if all of the criteria given below are satisfied. The activation and nonactivation portions of the mutation assays are usually performed concurrently, but each portion is in fact an independent assay with its own positive and negative controls. The activation or nonactivation assays will be repeated independently, as needed, to satisfy the acceptance and evaluation criteria.

X. EXPERIMENTAL DESIGN: (Continued)

D. Assay Acceptance Criteria (continued)

1. The average absolute cloning efficiency of the negative controls (average of the solvent controls) should be between 60% and 130%. A value greater than 100% is possible because of errors in cell counts (usually ± 10%) and cell division during unavoidable delays between the counting and cloning of many cell cultures. Cloning efficiencies below 60% do not necessarily indicate substandard culture conditions or unhealthy cells. Assay variables can lead to artificially low cloning efficiencies in the range of 50% to 60% and still yield internally consistent and valid results. Assays with cloning efficiencies in this range are conditionally acceptable and dependent on the scientific judgement of the study director. All assays below 50% cloning efficiency are unacceptable.

2. The minimum acceptable value for the suspension growth of the average negative control (average of solvent and untreated control values) for two days is 8.0. Lower values will render an assay unacceptable for evaluation because of the high frequency of unreliable measurements for both the induced mutant frequency and toxicity of a given treatment.

3. The background mutant frequency (average frequency of the solvent controls) is calculated separately for concurrent activation and nonactivation assays, even though the same population of cells is used for each assay. The activation negative controls contain the S9 activation mix and typically have a somewhat higher mutant frequency than the nonactivation negative controls. For both conditions, the normal range of background frequencies for assays performed with different cell stocks is 10×10^{-6} to 100×10^{-6}. Assays with backgrounds outside this range are not necessarily invalid but will not be used as primary evidence for the evaluation of a test material. These assays can provide supporting evidence.

4. A positive control is included with each assay to provide confidence in the procedures used to detect mutagenic activity. The minimum acceptable mutant frequency induced by 0.3 µl/ml EMS (nonactivation assay) is 200×10^{-6}; for 2.5 µg/ml MCA (activation assay) the minimum is 200×10^{-6}. An assay is acceptable in the absence of a positive control (loss due to contamination or technical error) only if the test article clearly shows mutagenic activity as described in the evaluation criteria. If the test article appears to have no, or only weak, mutagenic activity, an acceptable assay must have a positive control mutant frequency above the minimum.

X. EXPERIMENTAL DESIGN: (Continued)

D. Assay Acceptance Criteria (continued)

5. For test articles with little or no mutagenic activity, an assay should include applied concentrations that reduce the relative growth to 10 to 20% of the average solvent control or reach the maximum applied concentrations given in the evaluation criteria. The relative growth represents a calculation of survival that is based on both relative suspension growth during the expression period and relative cloning efficiency at the time of plating. Because of the fact that mutant frequencies increase as a function of lethality, an attempt to obtain treatments in the range of 10 to 20% relative growth must be made for an assay to be considered conclusive. This requirement is waived if a dose increment of about 1.5 or less causes excessive toxicity. There is no maximum toxicity requirement for test articles which clearly show mutagenic activity.

6. An experimental mutant frequency will be considered acceptable for evaluation only if the relative cloning efficiency is 10% or greater and the total number of viable clones exceeds about 60. These limits avoid problems with the statistical distribution of colonies that can be scored among dishes.

7. Mutant frequencies are normally derived from sets of three dishes for both the mutant colony count and the viable colony count. In order to allow for contamination losses, an acceptable mutant frequency can be calculated from a minimum of two dishes per set if the colony numbers in the two dishes differ by no more than about three-fold.

8. The mutant frequencies for five treated cultures are normally determined in each assay. A required number of different concentrations cannot be explicitly stated, although a minimum of three analyzed cultures is considered necessary under the most favorable test conditions to accept a single assay for evaluation of the test material.

E. Assay Evaluation Criteria

Mutation assays are initiated by exposing cell cultures to a range of concentrations of test material that is expected, on the basis of preliminary toxicity studies, to span the cellular responses from no observed toxicity to growth to complete lethality within 24 hours of treatment. Then five dose levels are usually selected for completion of the mutation assay. The doses are selected to cover a range of toxicities to growth with emphasis on the most toxic doses. An assay may need to be repeated with different concentrations in order to properly evaluate a test material.

X. EXPERIMENTAL DESIGN: (Continued)

E. Assay Evaluation Criteria (continued)

The minimum criterion considered necessary to demonstrate mutagenesis for any given treatment will be a mutant frequency that is at least 150% of the concurrent background frequency plus 10×10^{-6}. The background frequency is defined as the average mutant frequency of the solvent negative controls. The minimum increase is based on extensive experience which indicates that assay variability increases with higher backgrounds and the calculated minimum increase as defined above is often a repeatable result; statistical analysis for the confidence limits is not yet available.

The observation of a mutant frequency that meets the minimum criterion for a single treated culture within a range of assayed concentrations is not sufficient evidence to evaluate a test material as a mutagen. The following test results must be obtained to reach this conclusion for either activation or nonactivation conditions:

- A dose-related or toxicity-related increase in mutant frequency should be observed. It is desirable to obtain this relation for at least three doses, but this depends on the concentration steps chosen for the assay and the toxicity at which mutagenic activity appears.

- If an increase of about two times the minimum criterion or greater is observed for a single dose near the highest testable toxicity, as defined in the Assay Acceptance Criteria, the test material will be considered mutagenic. Smaller increases at a single dose near the highest testable toxicity will require confirmation by a repeat assay.

- For some test materials, the correlation between toxicity and applied concentration is poor. The proportion of the applied material that effectively interacts with the cells to cause genetic alterations is not always repeatable or under control. Conversely, measurable changes in frequency of induced mutants may occur with concentration changes that cause only small changes in observable toxicity. Therefore, either parameter, applied concentration or toxicity (percent relative growth), can be used to establish whether the mutagenic activity is related to an increase in effective treatment. A negative correlation with dose is acceptable only if a positive correlation with toxicity exists. An apparent increase in mutagenic activity as a function of decreasing toxicity is not acceptable evidence for mutagenicity.

- Treatments that induce less than 10% relative growth are included in the assay, but are not used as primary evidence for mutagenicity as it relates to risk assessment.

X. EXPERIMENTAL DESIGN: (Continued)

E. Assay Evaluation Criteria (continued)

A test article is evaluated as nonmutagenic in a single assay only if the minimum increase in mutant frequency is not observed for: 1) a range of applied concentrations that extends to toxicity causing 10 to 20% relative growth or 2) in the case of relatively nontoxic materials, a range of applied concentrations routinely extending to the maximum of 5 mg/ml (or 5 µl/ml) unless limited by solubility.

The ASSAY ACCEPTANCE AND EVALUATION CRITERIA are presented to acquaint the sponsor with the considerations used by the Study Director to determine assay validity and the mutagenic activity of the test material. This presentation may not encompass all test situations, and the Study Director may use other criteria, especially when data from several repeat assays are available, to arrive at a conclusion. The report will provide the reasoning involved when departures from the above descriptions occur.

XI. INTERPRETATION OF RESULTS:

The test material, Copper Naphthenate, formed an opaque blue-green liquid in absolute ethanol at 100 mg/ml. Just prior to each assay, stock solutions were prepared by performing serial dilutions in absolute ethanol. The cytotoxicity and mutation assays were then initiated by performing final 1:100 dilutions of the stocks into medium containing the cells. The test material appeared soluble in the assay medium up to 125 µg/ml but treatments from 250 µg/ml to 1000 µg/ml were cloudy and contained a blue-green precipitate. In the preliminary cytotoxicity assay assay, the test material was lethal with and without metabolic activation at 125 µg/ml. The mutation assays were therefore initiated with treatments from 0.625 µg/ml to 100 µg/ml in an attempt to obtain a wide range of toxicities for analysis.

One trial of the mutation assay was performed and the results are shown in Table 1.

Under nonactivation conditions (Table 1), the test material was excessively toxic at 100 µg/ml (data not shown). Six treatments from 10 µg/ml to 80 µg/ml were therefore chosen for mutant analysis and a good range of toxicities were induced (percent relative growths, 83.5% to 18.1%). None of the assayed treatments induced a mutant frequency that exceeded the minimum criterion for mutagenesis of 75.5×10^{-6}. The test material was therefore considered nonmutagenic without activation in this assay.

EPA No.: 68D80056
DYNAMAC No.: 250-G
TASK No.: 2-50G
June 28, 1990

DATA EVALUATION RECORD

COPPER NAPHTHENATE

Mutagenicity--*In vitro* Chromosome Aberrations
in Chinese Hamster Ovary Cells

APPROVED BY:

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

Signature: William L. McFadden for

Date: 6-28-90

EPA No.: 68D80056
DYNAMAC No.: 250-G
TASK No.: 2-50G
June 28, 1990

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DATA EVALUATION RECORD

COPPER NAPHTHENATE

Mutagenicity--*In vitro* Chromosome Aberrations
in Chinese Hamster Ovary Cells

REVIEWED BY:

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DATA EVALUATION RECORD

Guid 158

CHEMICAL: Copper naphthenate.

STUDY TYPE: Mutagenicity--In vitro chromosome aberrations in Chinese hamster ovary cells.

MRID NUMBER: 411407-07.

TEST MATERIAL: Copper naphthenate.

SYNONYMS: None listed.

SPONSOR: U.S. Army Environmental Hygiene Agency, Aberdeen Proving Ground, MD.

TESTING FACILITY: Litton Bionetics, Inc., Kensington, MD.

TITLE OF REPORT: Evaluation of Copper Naphthenate 5/8/85 in an In Vitro Cytogenetic Assay Measuring Chromosomal Aberration Frequencies in Chinese Hamster Ovary (CHO) Cells.

AUTHORS: Ivett, J.L. and Myhr, B.C.

STUDY NUMBER: 20990.

REPORT ISSUED: November 25, 1985.

CONCLUSIONS/Executive Summary: Copper naphthenate was assayed without metabolic activation over a dose range of 25 to 100 µg/mL. Cytotoxicity was apparent at the highest assayed dose, but no evidence of a clastogenic effect was seen in Chinese hamster ovary cells harvested at 10 hours. In the presence of S9 activation doses of the test material ranging from 6 to 40 µg/mL were not clastogenic following a 10-hour cell harvest. The highest assayed dose, however, was cytotoxic. The S9-activated test material (30 to 60 µg/mL) was also evaluated for chromosome damage following a delayed 20-hour cell harvest. Results indicated that copper naphthenate at doses ≥40 µg/mL +S9 induced cytotoxic effects but was not clastogenic. However, the study did not contain information on test material purity, the lot number of the assay sample was not reported, and analytical data to support actual concentrations in solution were not provided.

Study Classification: The study is unacceptable but can be upgraded if the missing test material information is provided.

A. MATERIALS:

1. Test Material:

Name:	Copper naphthenate
Description:	Dark green solid
Batch No.:	Not reported
Purity:	Not reported
Contaminants:	None listed
Solvent Used:	Ethanol (ETOH)

Other comments: Storage conditions were not provided. Solubility studies were conducted with water, dimethylsulfoxide, ETOH, and acetone; based on these results, ETOH was selected as the solvent of choice. The report indicated that the test material formed a green solution in ETOH at 253 mg/mL after 15 minutes in a 37°C water bath and formed a fine precipitate in culture medium (McCoy's 5a medium) at a concentration of 500 µg/mL. The initial range-finding assay, therefore, was conducted with doses of the test material ranging from 0.0167 to 500 µg/mL.

2. Cells Line: The Chinese hamster ovary cells (CHO-WBL) used in this assay were originally obtained from Dr. Sheldon Wolff, University of California, San Francisco, CA. The CHO cells were grown in McCoy's 5a medium, supplemented with 10% fetal calf serum, for 24 hours prior to use.

3. S9 Fraction: The S9 fraction was derived from the livers of male rats induced with Aroclor 1254. The S9 reaction mixture contained 15 µL/mL rat liver S9.

4. Positive Controls: The following clastogenic agents were included in the assay.

Positive Control	Dose (µg/mL)	S9 Activation	Assay
Mitomycin C (MMC)	0.25	-	Preliminary Cytotoxicity Assay
Cyclophosphamide (CP)	20.0	+	Preliminary Cytotoxicity Assay
MMC	0.5, 1.0	-	Cytogenetic Assay; 10-hour harvest
CP	12.5, 17.5	+	Cytogenetic Assay; 10-hour harvest
CP	25.0, 50.0	+	Cytogenetic Assay; 20-hour harvest

NOTE: Cells exposed to only one of the two doses of each positive control used in the nonactivated and S9-activated cytogenetic assays were analyzed for chromosome aberrations.

B. STUDY DESIGN:

1. Preliminary Cytotoxicity Assay: Prepared cell cultures, seeded at 0.3×10^6 cells/flask, were exposed with or without S9 activation to half-log dilutions of the test material ranging from 0.0167 to 500 µg/mL, the solvent control, or the positive controls.

   In the nonactivated system, cells were treated for 2 hours; BrdU (10 µM) was added to the cultures, and incubation was continued for an additional 23 hours. Cell monolayers were washed, refed with fresh complete medium containing BrdU, and reincubated in the presence of 0.1 µg/mL colcemid for 2.5 hours. In the S9-activated system, cultures were exposed for 2 hours without FCS. After exposure, cells were washed twice, refed with complete medium containing BrdU (10 µM), and reincubated for 23 hours. Colcemid was added, and cultures were incubated for an additional 2.5 hours.

   After incubation, monolayers were visually evaluated for confluency, and metaphase cells were collected by mitotic shake off. Cells were swollen in a hypotonic 0.075 M solution of potassium chloride and washed three times in fixative (methanol:acetic acid, 3:1), and slides were prepared. Estimation of cell-cycle delay was accomplished

by staining the cells with the modified fluorescent-plus-Giemsa techniques of Perry and Wolff[1] and Goto et al.[2] One hundred cells from each culture were examined for the percentage of first division (M_1), between first and second (M_{1+}) divisions, and beyond second ($>M_2$) division metaphase cells.

2. Cytogenetics Assay:

   a. Treatment: Prepared cultures (in duplicate) were exposed to the appropriate test material doses, the negative control, or the positive controls. In the nonactivated system, cells were dosed for 7.25 hours. Cultures were washed, refed medium containing colcemid, and reincubated for approximately 2.5 hours. Under S9-activated conditions, cells were exposed for 2 hours, washed, refed culture medium, and incubated for 7.9 or 16.9 hours. Colcemid was added 2.5 hours before the cultures were harvested.

      Metaphase cells were collected and fixed. Slides were stained and coded.

   b. Metaphase Analysis: One hundred morphologically normal cells per culture were scored for chromosome aberrations. At least 25 cells were scored from one of each positive control culture.

3. Statistical Methods: The data were evaluated for statistical significance at $p \leq 0.05$ by Fisher's Exact test. Negative and solvent control values were statistically evaluated; results were pooled if no significant differences were observed.

4. Evaluation Criteria: No criteria to establish assay validity or a positive response were presented. The biological significance of the results was evaluated relative to the overall chromosome aberration frequencies, percentage of cells with aberrations, percentage of cells with >1 aberration, dose response, and the types of aberrations observed.

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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.

[2]Goto, K., Maeda, S., Kano, Y., and Sugiyama, T. Factors involved in differential Giemsa-staining of sister chromatids. Chromosoma (1978) 66: 351-359.

C. REPORTED RESULTS:

1. Preliminary Cytotoxicity Assay: The cytotoxicity assay was conducted with test doses ranging from 0.0167 to 500 µg/mL separated by half-log dilutions, in the presence or absence of S9 activation.

   a. Without S9 Activation: No cells survived treatment with the two highest assayed doses (167 and 500 µg/mL); at 50 µg/mL, monolayer confluency was slightly reduced (75% of the control); however, there was no effect on cell-cycle kinetics (Table 1). Doses selected for the nonactivated cytogenetic assay ranged from 5 to 100 µg/mL with a "regular" 10-hour cell harvest.

   b. With S9 Activation: Cytotoxicity was complete (100%) at the two highest S9-activated doses. Cell-cycle delay with adverse effects on the confluency and the appearance of the monolayer were apparent at 50 µg/mL. Below this level, monolayer confluency and progression through the cell cycle were essentially normal (Table 1). Based on these data, a dose range of 4 to 40 µg/mL was selected for evaluation in the 10-hour cell harvest assay, and a dose range of 40 to 80 µg/mL was assayed with a delayed 20-hour harvest time.

2. Cytogenetic Assay:

   a. Nonactivated Test Material: The report stated that a reduction of mitotic cells and the presence of cellular debris were observed in cells harvested from cultures exposed to the highest nonactivated dose (100 µg/mL). Metaphases were examined from the 25-, 50-, 75-, and 100-µg/mL dose levels. No significant increases in the number of aberrations per cell, percent cells with aberrations, or the percent cells with >1 aberration were seen (Table 2).

   Our reviewers noted the occurrence of chromosome-type aberrations (dicentrics) at all scored nonactivated levels. In the absence of a clear increase in chromatid-type aberrations, however, this finding cannot be interpreted as evidence of potential clastogenic activity.

   b. S9-Activated Test Material: At the 10-hour harvest interval, no cells were recovered from the 40-µg/mL dose level. Analysis of metaphases from cultures

TABLE 1. Results from the Preliminary Test for Delay of Cell-Cycle Progression with Copper Naphthenate

Substance	Dose/ mL	S9 Activation	% Cells[a] M_1	% Cells[a] M_{1*}	% Cells[a] $>M_2$	Monolayer Confluency (%)
Negative Control						
Culture Medium	--	-	2	1	97	100
	--	+	2	5	93	100
Solvent Control						
Ethanol	10.0 µL	-	1	9	90	100
		+	-	2	98	100
Positive Control						
Mitomycin C	0.25 µg	-	96	4	-	88
Cyclophosphamide	20.0 µg	+	14	70	16	86
Test Material						
Copper naphthenate	50.0 µg[bc]	-	-	11	89	75
	16.7 µg	+	-	7	93	86
	50.0 µg[b]	+	24	76	-	29

[a]Percent cells in first division (M_1), between first and second (M_{1*}) divisions, or beyond second division ($>M_2$).

[b]Higher doses (167 and 500 µg/mL) with or without S9 activation were completely cytotoxic.

[c]Results for lower doses (5.0 and 16.7 µg/mL -S9 or 5.0 µg/mL +S9) showed no appreciable effect on monolayer confluency or cell-cycle kinetics.

Substance	Dose/mL	Harvest Time (Hours)	S9 Activation	No. of Cells Scored	No. of Aberrations per Cell	% Cells with Aberrations	% Cells with >1 Aberrations	Biologically Significant Aberrations[a] No./Type
Pooled Negative Control								
McCoy's 5a Medium/Ethanol	--	10	-	200	>0.04	3.0	2.5	1TR; 5PU; 1AB
	--;	10	+	200	0.02	2.0	0.0	--
	--;	20	+	200	0.02	1.5	0.0	1AF; 2D
Positive Control								
Mitomycin C	1.0 µg	10	-	25	0.48	36.0*	8.0	3TB; 4TR; 4QR; 1RF
Cyclophosphamide	25.0 µg	10	+	25	>0.56	44.0*	16.0*	1TB; 5TR; 2QR; 1SB; 3AF; 1D; 1PU
	12.5 µg	20	+	25	0.84	60.0*	20.0*	4TB; 4TR; 2QR; 2CR; 1SB; 1AF; 1R; 3ID; 3CI
Test Material								
Copper Naphthenate	100 µg[b]	10	-	200	>0.03	3.0	1.0	1TB; 1QR; 2D; 1PU; 1P+
	20 µg[c]	10	+	200	>0.05	5.0	0.5	5TB; 2AF; 2D; 1PU
	30 µg[d]	20	+	200	0.03	2.0	0.5	4D; 1R

[a]Abbreviations used:
TB - Chromatid break
TR - Triradial
QR - Quadriradial
CR - Complex rearrangement
SB - Chromosome break
AF - Acentric fragment
D - Dicentric
R - Ring
RF - Ring with acentric fragment
PU - Pulverized chromosome (spread containing 1 fragment or pulverized cell)
P+ - Pulverized chromosomes (spread containing ≥2 fragmented chromosomes)
ID - Interstitial deletion
CI - Chromosome intrachange
AB - Abnormal monocentric chromosome

[b]Highest nonactivated dose scored; signs of compound cytotoxicity were apparent at this level (i.e., reduced mitotic cells and cellular debris). Results for lower nonactivated doses (25, 50, and 75 µg/mL) did not indicate a clastogenic effect.

[c]Highest S9-activated dose scored; no cells survived exposure to 40 µg/mL +S9. Results for lower levels (6 and 8 µg/mL) did not indicate a clastogenic response.

[d]Three trials were performed; the first two trials were aborted because of fungal contamination. Results from the third trial indicated that no cells survived treatment with higher doses (40, 50, or 60 µg/mL).

*Significantly higher than the pooled negative control values ($p < 0.01$) as determined by Fisher's Exact test.

treated with 6, 8, or 20 µg/mL of the test material did not reveal any significant differences among treatment and pooled negative control groups (Table 2). The report stated that while the delayed 20-hour harvest assay was repeated because of fungal contamination, evidence of severe cytotoxicity at 40, 60, and 80 µg/mL prompted the use of lower doses (30, 40, 50, and 60 µg/mL) in the third assay. Due to cytotoxicity at levels ≥40 µg/mL, metaphases were examined only at the low dose. Results presented in Table 2 indicated that exposure to 30 µg/mL +S9 did not induce significant increases in chromosome aberrations.

Under the nonactivated conditions and the S9-activated conditions, which included a 10-hour and 20-hour cell harvest, the sensitivity of the test system to detect the clastogenic activity of the appropriate positive control was clearly demonstrated. From the results, the study authors concluded that copper naphthenate was not clastogenic in this mammalian cell cytogenetic assay.

REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS:

We assess that the study was properly conducted and that the authors interpreted the data correctly. In both the presence and absence of S9 activation, copper naphthenate was assayed up to cytotoxic levels with no indication of a clastogenic effect. By contrast, both the nonactivated and S9-activated positive controls induced significant ($p \leq 0.01$) increases in chromosome aberrations, indicating that the sensitivity of the assay to detect a clastogenic response was adequate.

The study is, however, incomplete. No information on test material purity, the lot number of the assay sample, or analytical data to support actual concentrations used in the study was provided. We conclude, therefore, that while the study was technically sound, the lack of required test material information renders the study unacceptable. However, if the study authors provide the missing test material information, the study can be upgraded.

QUALITY ASSURANCE MEASURES: A quality assurance statement was signed and dated November 15, 1985.

CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 2-6.

APPENDIX A

Materials and Methods
(CBI pp. 2-6)

In Vitro Chromosomal Aberration Assay with Copper Naphthenate 5/8/85

I. SPONSOR: U.S. Army

II. MATERIAL TESTED

A. Client's Identification: Copper Naphthenate 5/8/85

B. Genetics Assay No.: 8022

C. Date Received: May 17, 1985

D. Physical Description: Dark green solid

III. TYPE OF ASSAY: In Vitro Cytogenetic Assay Measuring Chromosomal Aberration Frequencies in Chinese Hamster Ovary (CHO) Cells

IV. PROTOCOL NO.: 437, Edition 10

V. STUDY DATES:

A. Initiation Date: June 24, 1985

B. Completion Date: September 6, 1985

VI. SUPERVISORY PERSONNEL:

A. Study Director: James L. Ivett, Ph.D.

B. Laboratory Supervisor: Carol S. Spicer

VII. OBJECTIVE:

The objective of this in vitro assay was to evaluate the ability of Copper Naphthenate 5/8/85 to induce chromosomal aberrations in Chinese hamster ovary (CHO) cells, with and without metabolic activation.

VIII. EXPERIMENTAL DESIGN:

In this assay a rangefinding study was first conducted 1) to determine the dose range to be used in the chromosomal aberrations assay and 2) to determine the optimal time of harvest of the dosed cells such that primarily metaphase cells which were in the first metaphase since exposure to the test article would be analyzed for chromosomal aberrations. The doses used in the rangefinding assay ranged from 16.7 ng/ml of the test article solution through 500 µg/ml in a half-log series. The highest three surviving doses from both the nonactivation and activation trials were then analyzed for toxicity (visual observations of monolayer confluence) and cell cycle kinetics. The doses and harvest times to be used in the nonactivation and activation assays were then determined.

VIII. EXPERIMENTAL DESIGN: (Continued)

In the chromosomal aberrations assays duplicate cultures were used at each dose level. Single cultures were used for the negative control, solvent control and at each of two doses of the positive control. Chromosomal aberrations were analyzed from the four highest doses from which results could be obtained and from only one of the positive control doses.

IX. MATERIALS AND METHODS:

A. General

The Chinese hamster ovary cells (CHO-WBL) used in this assay were from a permanent cell line and were originally obtained from the laboratory of Dr. S. Wolff, University of California, San Francisco. The cells have since been recloned to maintain karyotypic stability. The CHO cells were grown in McCoy's 5a which was supplemented with 10% fetal calf serum (FCS), L-glutamine, penicillin and streptomycin.

The positive control agents which were used in the assays were Mitomycin C (MMC) for the nonactivation series and cyclophosphamide (CP) in the metabolic activation series. In the rangefinding assay doses of MMC (250 ng/ml) and CP (20 µg/ml) were selected which would slow the rate of cellular proliferation. In the chromosomal aberration assays two concentrations of MMC (500 ng/ml and 1 µg/ml) and CP (12.5 µg/ml and 17.5 µg/ml, 10 hour harvest or 25 µg/ml and 50 µg/ml, 20 hour harvest) were used to induce chromosomal aberrations in the CHO cells although only one dose was actually analyzed in each of the aberration assays.

In the nonactivation assays the cells were exposed to the test article continuously until approximately 2.5 hours prior to the harvest of the cells. At that time the test article was washed from the cells and the cells were treated with colcemid for the remainder of the culture period to accumulate cells in metaphase.

In the metabolic activation assays the CHO cells were exposed to the test article for two hours in the presence of a rat liver S9 reaction mixture (S9 15 µl/ml, NADP 1.5 mg/ml, Isocitric acid 2.7 mg/ml). The S9 fraction was derived from the liver of male rats which had been previously treated with Aroclor 1254 to induce the mixed function oxidase enzymes which are capable of metabolizing chemicals to more active forms. At the end of the test article exposure interval the test article and the S9 activation mixture were washed from the cells. The cells were then recultured with complete culture medium and incubated for the appropriate interval of time. Colcemid was added to the cultures approximately 2.5 hours before the termination of the cultures.

IX. MATERIALS AND METHODS: (Continued)

Prior to the harvest of the cultures visual observations of toxicity were made. These observations included an assessment of the percent confluence of the cell monolayer within the culture flasks. The cultures were also evaluated for the presence of mitotic (large rounded cells) or dead cells floating in the medium. Only flasks from the highest four to six surviving doses from which metaphase cells for analysis were expected were harvested. The metaphase cells were collected by mitotic shake-off and were treated with 0.075 M KCl hypotonic solution. The cultures were then fixed with an absolute methanol:glacial acetic acid (3:1) fixative and were washed several times before air-dried slides were prepared.

B. Rangefinding Assays

In the nonactivation rangefinding assay the cultures were set up approximately 24 hours prior to treatment by seeding 0.3×10^6 cells per 25 cm^2 flask into 5 ml of complete McCoy's 5a medium. The cultures were then dosed with the test article as has been described in the General Methods section. However, in the nonactivation trial 5-bromo-2'-deoxyuridine (BrdUrd) was added at a final concentration of 10 µM approximately 2 hours after the initial exposure of the cells to the test article and the cultures were reincubated for an additional 23 hours. The test article was then washed from the cells with phosphate buffered saline and fresh complete medium with BrdUrd (10 µM) and colcemid (0.1 µg/ml) was added. The slides were then harvested as previously described and were differentially stained for the analysis of cell cycle delay using a modified fluorescent-plus-Giemsa (FPG) technique (after Perry and Wolff, 1974, Goto, et.al., 1978). The slides were stained for 10 minutes with Hoechst 33258 (5 µg/ml) in a pH 6.8 phosphate buffer, were mounted in the same buffer and were exposed at 55° - 60°C to "blacklight" from two 15 Watt fluorescent bulbs for the amount of time required for the differentiation of the BrdUrd incorporated sister chromatids (about 3-5 minutes). The slides were then stained in a 5% Giemsa solution for 5 to 20 minutes and were air-dried and coverslipped using Depex mounting medium.

In the rangefinding assay with metabolic activation the CHO cells were incubated at 37°C for 2 hours in the presence of the test article and the S9 reaction mixture in the growth medium without FCS. After the exposure period the cells were washed twice with buffered saline. Complete McCoy's 5a medium with 10 µM BrdUrd was then added to the cultures which were then incubated for an additional 23 hours when 0.1 µg/ml colcemid was added to collect metaphase cells. The cultures were then harvested and slides were prepared and stained as was described for the nonactivation rangefinding assay.

One hundred consecutive metaphase plates were assessed for the number of cell cycles through which the cells had progressed while in the presence of BrdUrd.

IX. MATERIALS AND METHODS: (Continued)

C. Chromosomal Aberrations Assays

One day after culture initiation, the CHO cells to be used in the nonactivation trial were treated with the test article at predetermined doses for 7.25 hours. The cultures were then washed with buffered saline. Complete McCoy's 5a medium containing 0.1 µg/ml colcemid was placed back onto the cells. Two and one half hours later the cells were harvested and air dried slides were made. The slides were then stained in pH 6.8 buffered 5% Giemsa solution for the analysis of chromosomal aberrations.

The cultures that were treated under the conditions of metabolic activation were incubated at 37°C for two hours in the presence of the test article and the S9 reaction mixture in McCoy's 5a medium without FCS. After the two hour exposure period the cells were washed twice with buffered saline and the cells were refed with complete McCoy's 5a medium. The cells were incubated for an additional 7.9 hours or 16.9 hours with 0.1 µg/ml Colcemid present during the last 2.5 hours of incubation. The metaphase cells were then harvested and prepared for cytogenetic analysis.

One hundred cells from each duplicate culture at four dose levels of the test article and from each of the negative and solvent control cultures were analyzed for chromosomal aberrations. From one of the positive control cultures at least 25 cells were scored for chromosomal aberrations. For control of bias, all slides except for the positive controls were coded prior to analysis and were scored "blind". The microscope stage location was recorded on the data sheets for those cells in which aberrations were noted.

The following factors were taken into account in the evaluation of the chromosomal aberrations data:

i. The overall chromosomal aberration frequencies.

ii. The percentage of cells with any aberrations.

iii. The percentage of cells with more than one aberration.

iv. Any evidence for increasing amounts of damage with increasing dose, i.e. a positive dose response.

v. The estimated number of breaks involved in the production of the different types of aberrations which were observed, i.e., complex aberrations may have more significance than simple breaks.

IX. MATERIALS AND METHODS: (Continued)

Chromatid and isochromatid gaps if observed were noted in the raw data. They were not, however, tabulated in the tables or considered in the evaluation of the ability of the test article to induce chromosomal aberrations since they may not represent true chromosomal breaks.

Those cells with pulverized chromosomes also received special attention when the data were tabulated. Cells classified as PU, P+ or PC were considered to contain one aberration for statistical purposes, however, a ">" is placed before the total number of aberrations per cell in the tables to indicate that the value is a minimum number. A cell classified as "GT" is considered to contain 10 aberrations for statistical purposes but a ">" is also included in the tables for this classification to indicate that it is a minimum number.

Statistical analysis employed the Fisher's Exact Test with an adjustment for multiple comparisons to compare the percentage of cells with aberrations in each treatment group with the results from the pooled solvent and negative controls (the solvent and negative controls were statistically evaluated for similarity prior to the pooled evaluation). Test article significance was established where $p<0.05$. All factors as stated previously were taken into account and the final evaluation of the test article was based upon scientific judgement.

X. RESULTS:

A. Solubility and Dose Determination

Solubility of the test article was determined for a concurrently conducted LBI protocol 401. In that evaluation, the test article was insoluble in water and DMSO at 100 mg/ml but soluble in acetone at 200 mg/ml. Solubility was evaluated for the aberrations assay in ethanol and acetone. In ethanol a green solution resulted at 253 mg/ml after approximately 15 minutes in a 37°C water bath. When this stock was diluted 1:100 in culture medium for an attempted final concentration of 2.5 mg/ml, the test article formed a green precipitate which adherred to the plastic tube. Serial dilutions of this stock were performed and subsequently diluted 1:100 into culture medium. At a diluted concentration of 500 µg/ml a fine precipitate formed which remained in an even supension. In acetone the test article formed a suspension of oily globules at 498 mg/ml and no further evaluation of this solvent was performed. Ethanol was the solvent of choice with a top stock of 50 mg/ml. A dose range of 16.7 ng/ml through 500 µg/ml was tested in the rangefinding assay.

B. Rangefinding Assay Without Metabolic Activation

In the rangefinding assay without metabolic activation there was complete toxicity at 167 µg/ml and 500 µg/ml. Cell cycle kinetics were analyzed at 5.0 µg/ml through 50 µg/ml (Table 1). The test article caused no significant cell cycle delay and a regular harvest was selected for the nonactivation aberrations assay. A dose range of 5.0 µg/ml through 100 µg/ml was tested.

EPA No.: 68D80056
DYNAMAC No.: 250-H
TASK No.: 2-50H
June 28, 1990

DATA EVALUATION RECORD

COPPER NAPHTHENATE

Mutagenicity--Unscheduled DNA Synthesis in
Primary Rat Hepatocytes

APPROVED BY:

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

Signature: William L. McLellan for

Date: June 27, 1990

EPA No.: 68D80056
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DATA EVALUATION RECORD

COPPER NAPHTHENATE

Mutagenicity--Unscheduled DNA Synthesis in
Primary Rat Hepatocytes

REVIEWED BY:

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Principal Reviewer
Dynamac Corporation

Signature: Nancy E. McCarroll
Date: 6-28-90

I. Cecil Felkner, Ph.D.
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DATA EVALUATION RECORD

006156

HEMICAL: Copper naphthenate.

TUDY TYPE: Mutagenicity--Unscheduled DNA synthesis in primary at hepatocytes.

RID/ACCESSION NUMBER: 411407-08.

EST MATERIAL: Copper naphthenate.

YNONYMS/CAS NO.: None listed.

PONSOR: U.S. Army Environmental Hygiene Agency, Aberdeen Proving round, MD.

ESTING FACILITY: Litton Bionetics, Inc., Kensington, MD.

ITLE OF REPORT: Evaluation of Copper Naphthenate in the Rat rimary Hepatocyte Unscheduled DNA Synthesis Assay.

UTHORS: Cifone, M.A. and Myhr, B.C.

TUDY NUMBER: 20991.

EPORT ISSUED: November 1985.

CONCLUSIONS/Executive Summary: Under the conditions of the assay, seven doses of copper naphthenate, ranging from 0.1 to 25 µg/mL did not induce an appreciable increase in the net nuclear grain counts of treated rat hepatocytes. Cytotoxicity was clearly demonstrated at concentrations ≥25 µg/mL. It is concluded, therefore, that the test material is inactive in the primary rat hepatocyte unscheduled DNA synthesis (UDS) assay. However, the lack of information on test material purity, the lot number of the assay sample, and supporting analytical data to confirm actual test material concentrations in solution preclude full acceptance of the study.

Study Classification: The study is currently unacceptable but can be upgraded if the missing test material information can be supplied by the sponsor.

I. MATERIALS:

1. Test Material:

Name:	Copper naphthenate
Description:	Green solid
Batch No.:	Not reported
Purity:	Not reported
Contaminants:	None listed
Solvent used:	Absolute ethanol (ETOH)

Other comments: Storage conditions were not provided. The report indicated that the test material formed a blue-green solution in ETOH at 100 mg/mL and was soluble in culture medium (Williams' Medium E, WME) up to 100 µg/mL. Concentrations ranging from 250 to 1000 µg/mL formed a "blue-green gel" in WME. Solutions used in the assay were prepared immediately prior to use.

2. Indicator Cells: Primary rat hepatocytes were obtained by the in situ perfusion of the liver of an adult male Fischer 344 rat (150-300 g) purchased from Charles River Breeding Laboratories, Inc.

3. Cell Preparation:

a. Perfusion Technique: The liver was perfused with Hanks' balanced salts solution containing 0.5 mM EGTA and Hepes buffer, pH 7.0, for 4 minutes and with 50-100 u/mL collagenase in WME for 10 minutes. The liver was excised, removed to a culture dish containing WME and collagenase, and mechanically dispersed to release the hepatocytes.

b. Hepatocyte Harvest/Culture Preparation: Recovered cells were centrifuged, resuspended in WME containing serum and dexamethasone, counted, and aliquoted (0.5×10^6 cells/3 mL WME) onto plastic coverslips. The cultures were placed in a humidified, 37°C, 5% CO_2 incubator for a 1.5- to 2-hour attachment period. Unattached cells were removed; viable cells were refed and established as monolayer cultures.

4. Positive Control: To demonstrate assay sensitivity to detect UDS, 0.05 µg/mL 2-acetylaminofluorene (2-AAF) was included as the positive control chemical.

). STUDY DESIGN:

1. Dose Selection: Initially, 15 concentrations of the test material were assayed (1000-0.025 µg/mL in dilutions of approximately twofold steps). When the viability estimate was obtained (20-24 hours after treatment initiation), at least six of these doses were chosen for analysis of nuclear labeling, starting with the highest dose that resulted in a sufficient number of cells with intact morphologies and proceeding to successively lower doses.

2. UDS Assay:

a. Treatment: Five replicate, monolayer cultures were exposed to the selected doses of the test material, negative control (ETOH), or positive control (2-AAF, 0.05 µg/mL) for 18-19 hours in WME containing 1 µCi/mL [^{3}H]thymidine. Treated monolayers were washed twice with WME; two of the five replicates for each treatment group were used to determine cytotoxicity. These cultures were refed, reincubated, and monitored for cytotoxicity at 20-24 hours posttreatment by trypan blue exclusion.

b. UDS Slide Preparation: The remaining cultures were washed with medium containing 1 mM thymidine. Treated hepatocytes, attached to coverslips, were exposed to 1% sodium citrate for 8-10 minutes, fixed in acetic acid:ethanol (1:3), dried, and mounted.

c. Preparation of Autoradiographs/Grain Development: Slides were coated with Kodak NTB2 emulsion, dried for 7-10 days at 4°C in light-tight desiccated boxes, developed in Kodak D-19, fixed, stained with Williams' modified hematoxylin and eosin, coded, and counted.

d. Grain Counting: The nuclear grains of 150 morphologically normal cells for each test dose and negative and positive controls were counted microscopically. Net nuclear grain counts were determined by subtracting the nuclear grain counts of each cell from the average cytoplasmic grain count of three nuclear-sized areas adjacent to each nucleus.

3. Evaluation Criteria:

a. Assay Validity: For the assay to be considered valid, the following criteria must be satisfied: (1) hepatocytes recovered from the perfusion step and monolayer cultures used for the assay must show ≥70% viability; (2) the solvent controls should have net nuclear grain counts of ≤2; (3) the positive control must demonstrate the sensitivity of the test system to detect UDS; (4) data must be obtained from at least two replicate cultures/dose; and (5) the highest dose must show cytotoxicity, the limit of solubility, or reach the maximum recommended dose for this assay (5000 µg/mL).

b. Positive Response: The assay was considered positive if (1) an increase in the mean nuclear grain count was ≥6 grains/nucleus over the negative control value, (2) the percent of nuclei with ≥6 grains exceeded 10 percent of the negative control population, or (3) the percent of nuclei with ≥20 grains was ≥2% of the examined population.

REPORTED RESULTS:

1. UDS Assay: Based on the raw data, the cytotoxicity assessment indicated that doses ≥50 µg/mL were completely cytotoxic. Percent survival for the remaining doses ranged from 21.8% at 25 µg/mL to 93.2% at 0.25 µg/mL; survival was not determined for the lower levels. Seven doses (0.1. 0.25, 0.5, 1.0, 2.5, 10.0, and 25.0 µg/mL) were, therefore scored for UDS activity. As shown in Table 1, nuclear grain counts for the selected doses were slightly higher than the solvent control value but did not approach the minimum increase (≥6 grains/nucleus over the negative control) required to conclude a positive effect.

By contrast, the positive control, 0.05 µg 2-AAF/mL, induced marked increases in UDS grains per nucleus, the percent nuclei with ≥6 grains, and the percent of nuclei with ≥20 grains.

TABLE 1. Representative Results of the Unscheduled DNA Synthesis in Rat Hepatocyte Assay with Copper Naphthenate

eatment	Dose	Cells Scored	Percent Survival[a] (21 Hours)	Average Nuclear Grain Count[b]	Average Percent Nuclei w/ ≥6 Grains	Average Percent Nuclei w/ ≥20 Grains
nt Control						
anol	1%	150	100.0	0.18	0.0	0.0
ive Control						
cetylamino-uorene	0.05 µg/mL	150	91.2	6.22[b]	44.0[c]	2.0[c]
Material						
per naphthenate	10 µg/mL[d]	150	58.9	0.33	0.0	0.0
	25 µg/mL[e]	150	21.8	0.28	0.0	0.0

rvival = $\frac{\text{No. of viable cells/unit area test dose}}{\text{No. of viable cells/unit area solvent control}} \times 100.$

age of net nuclear grain counts on triplicate coverslips.

ilis reporting laboratory's criteria for a positive effect.

lts for lower concentrations (0.10, 0.25, 0.5, 1.0, and 2.5 µg/mL) did not indicate a genotoxic effect.

est dose scored; higher concentrations (50, 100, 250, 500, and 1000 µg/mL), as indicated by the raw data, completely cytotoxic.

Based on the overall results, the study authors concluded, "The test material, copper naphthenate, did not induce significant changes in the nuclear labeling of primary rat hepatocytes for an applied concentration range of 25 µg/mL to 0.10 µg/mL."

EVIEWERS' DISCUSSION/INTERPRETATION OF STUDY RESULTS:

e assess that the study was conducted properly and that the uthors' interpretation of the data was correct. None of the oses induced an appreciable increase in UDS. The cytotoxic ffect demonstrated at doses ≥25 µg/mL indicated that the test ubstance entered the hepatocytes and that the lack of response as not due to the inability of the test material to penetrate he cell wall. The study adequately demonstrated both the olubility limits and cytotoxicity of the test material. imilarly, the ability of the test system to detect UDS was learly shown by the findings with the positive control (2-AAF, .05 µg/mL). We conclude, therefore, that test material, opper naphthenate, failed to induce a genotoxic response in well-controlled study. However, the lack of information on est material purity, the lot number of the assayed sample, and upporting data to confirm actual test material concentrations n solution renders the study unacceptable.

UALITY ASSURANCE MEASURES: A quality assurance statement was igned and dated November 25, 1985.

BI APPENDIX: Appendix A, Materials and Methods, CBI pp. 3-9.

APPENDIX A

Materials and Methods
CBI pp. 3-9

V. TYPE OF ASSAY: Primary Rat Hepatocyte Unscheduled DNA Synthesis Assay

VI. PROTOCOL NUMBER: 447, Edition 6

VII. STUDY DATES:

A. Initiation Date: June 25, 1985

B. Completion Date: September 20, 1985

VIII. SUPERVISORY PERSONNEL:

A. Study Director: Maria A. Cifone, Ph.D.

B. Laboratory Supervisor: Maria McKeon

IX. MATERIALS:

A. Indicator Cells

The indicator cells for this assay were hepatocytes obtained from an adult male Fischer 344 rat (150-300 g), which was purchased from Charles River Breeding Laboratories, Inc. The animals scheduled for this assay were fed Purina Certified Rodent Chow (Formula 5002)* and water ad libitum. One animal, identified by cage card, was used for the assay after a minimum quarantine period of five days.

The cells were obtained by perfusion of the liver in situ with a collagenase solution (see Section 4C). Monolayer cultures were established on plastic coverslips in culture dishes and were used the same day for initiation of the UDS assay. All cultures were maintained as monolayers at 37±2°C in a humidified atmosphere containing approximately 5% CO_2.

B. Medium

The cell cultures were established in Williams' Medium E supplemented with 5% fetal bovine serum, 2mM L-glutamine, 2.4µM dexamethasone, 90 U/ml penicillin, 90 µg/ml streptomycin sulfate, and 140 µg/ml gentamicin. After the establishment period, the dexamethasone and serum components were removed. This latter culture medium is referred to simply as WME.

C. Controls

1. Negative control

A negative control consisting of assay procedures performed on cells exposed only to the test material solvent was performed. If the test material was not soluble in water, a stock solution in an organic solvent (normally dimethylsulfoxide; DMSO) was prepared; the final concentration of solvent in the growth medium was 1% in the treated cultures and the negative (solvent) control.

IX. MATERIALS: (Continued)

C. Controls (continued)

2. Positive control

The positive control compound is known to induce UDS in rat hepatocyte primary cell cultures. 2-Acetyl aminofluorene (2-AAF) at 2.24×10^{-7}M (0.05 µg/ml) was used as the positive control.

X. EXPERIMENTAL DESIGN:

A. Dosing Procedure

The test material was dissolved, if possible, at the highest desired concentration in WME containing 1% serum, and lower concentrations were then prepared by serial dilution with WME plus 1% serum. If the test material was incompletely soluble in WME, dimethylsulfoxide (DMSO) was investigated as the solvent. A solution in DMSO is serially diluted with DMSO and each stock is then diluted 1:100 (or greater) into WME plus 1% serum to obtain the final desired concentrations of test material. If incomplete solubility was obtained in both WME and DMSO, the chosen vehicle was one giving a higher solubility and/or the best dispersion characteristics. Fresh preparations of test material in the vehicle were used for the biological testing. Treatments were initiated by replacing the medium on the cell cultures with WME (1% serum) containing the test material at the desired concentrations.

B. Dose Selection

The dose selection procedure was an integral part of the UDS assay in order to select appropriate doses for a particular, fresh primary culture of hepatocytes. A range of 15 concentrations was applied initially to the cells, starting at approximately 1000 µg/ml (or 1000 nanoliters/ml) and diluting in approximately two-fold steps to about 0.025 µg/ml (or 0.025 nanoliters/ml). A viable cell count (trypan blue exclusion) was then obtained about 20-24 hours after initiation of the treatments. At least 6 concentrations were chosen for analysis of nuclear labeling, starting with the highest dose that resulted in a sufficient number of survivors with intact morphologies and proceeding to successively lower doses. If the test material was freely soluble and relatively non-toxic, a maximum concentration of 5 mg/ml (or 5 microliters/ml) or another limit specified by the Sponsor was tested.

X. EXPERIMENTAL DESIGN: (Continued)

C. UDS Assay

This assay was based on the procedures described by Williams (1977, 1980). The hepatocytes were obtained by perfusion of livers in situ for about 4 minutes with Hanks' balanced salts (Ca^{++} - Mg^{++}-free) containing 0.5 mM ethyleneglycol-bis (β-aminoethyl ether)-N, N-tetraacetic acid (EGTA) and HEPES buffer at pH 7.0. Then WME with 50-100 units/ml of collagenase was perfused through the liver for about 10 minutes. The hepatocytes were obtained by mechanical dispersion of excised liver tissue in a culture dish containing the WME culture medium and collagenase. Clumps of cellular tissue and debris were removed either by filtering the suspension through sterile cheesecloth or by allowing the clumps to settle to the bottom of a tube. The filtrate or decantate was centrifuged and the cell pellet resuspended in WME containing 5% serum and 2.4 μM dexamethasone. After obtaining a viable cell count, a series of 35-mm culture dishes (each containing a 25-mm round, plastic coverslip) was inoculated with approximately 0.5 x 10^6 viable cells in 3 ml of WME plus dexamethasone and 5% serum per dish.

An attachment period of 1.5 to 2 hours at 37±2°C in a humidified atmosphere containing about 5% CO_2 was used to establish the cell cultures. Unattached cells were then removed and the cultures were refed with WME. The UDS assay was initiated within 3 hours by replacing the media in the culture dishes with 2.5 ml WME containing 1% fetal bovine serum, 1 μCi/ml ^{3}H-thymidine, and the test material at the desired concentration. Each treatment, including the positive and negative controls, was performed on five cultures, two of which were used for cytotoxicity measurements. After treatment for 18-19 hours, the UDS assay was terminated by washing the cell monolayers twice with WME. Three of the cultures from each treatment were washed with WME containing 1 mM thymidine and were further processed as described below. The other two cultures used to monitor the toxicity of each treatment were refed with WME and returned to the incubator. At 20-24 hours after the initiation of the treatments, viable cell counts (trypan blue exclusion) were determined to estimate cell survival relative to the negative control.

The nuclei in the labeled cells were swollen by placement of the coverslips in 1% sodium citrate for 8-10 minutes, and then the cells were fixed in acetic acid:ethanol (1:3) and dried for at least 24 hours. The coverslips were mounted on glass slides (cells up), dipped in an emulsion of Kodak NTB2, and dried. The coated slides were stored for 7-10 days at 4°C in light-tight boxes containing packets of Drierite. The emulsions were then developed in D19, fixed and stained with Williams' modified hematoxylin and eosin procedure.

X. EXPERIMENTAL DESIGN: (Continued)

C. UDS Assay (continued)

The cells were examined microscopically at approximately 1500 x magnification under oil immersion and the field was displayed on the video screen of an automatic counter. UDS was measured by counting nuclear grains and subtracting the average number of grains in three nuclear-sized areas adjacent to each nucleus (background count). This value is referred to as the net nuclear grain count. The coverslips were coded to prevent bias in grain counting.

The net nuclear grain count was determined for 50 randomly selected cells on each coverslip. Only nuclei with normal morphologies were scored, and any occasional nuclei blackened by grains too numerous to count were excluded as cells in which replicative DNA synthesis occurred rather than repair synthesis. If the actual count for any nucleus was less than zero (i.e., cytoplasmic count was greater than nuclear count), a net value of zero was used in the calculation of the mean value. The mean net nuclear grain count was determined from the triplicate coverslips (150 total nuclei) for each treatment condition. Occasionally, a coverslip is recounted at a later date or by a different technician. Since a different cell population will generally be scored, the average count for 50 cells was used in the calculation of the mean for the triplicate treatment.

D. Assay Acceptance Criteria

An assay normally is considered acceptable for evaluation of the test results only if all of the criteria listed below are satisfied. This listing may not encompass all test situations, so the Study Director must exercise scientific judgement in modifying the criteria or considering other causes that might affect assay reliability and acceptance.

1. The viability of the hepatocytes collected from the perfusion process normally exceeds 70%. A variety of factors can affect cell yield and viability, so values below 70% are not uncommon nor necessarily detrimental. A lower limit for acceptability is set at 50% in order to avoid the possible use of a damaged, unrepresentative sample of cells.

2. The viability of the monolayer cell cultures used for the assay treatments must be 70% or greater. Normally, the viability of attached cells is about 90%.

X. EXPERIMENTAL DESIGN: (Continued)

D. Assay Acceptance Criteria (continued)

3. The number of viable cells in the negative (or solvent) control cultures should remain reasonably stable over the experimental time period because rapidly declining (dying) cultures may not respond in a representative manner to the test material treatments. Therefore the average number of viable cells in the negative (or solvent) control cultures must not be less than 50% of the cell number at the beginning of the treatment period.

4. The labeling in the negative (or solvent) control cultures must not exceed an average of 2 net grains/nucleus, or 10% of the cells with 6 or more grains, or 1% of the cells with 20 or more grains. The attainment of any one of these criteria will invalidate the assay. Normally, the control mean net nuclear grain count is about 0.4 to 0.9, approximately 1% of the nuclei have 6 or more net grains, and none of the nuclei have 20 or more net grains.

5. The positive control is used to demonstrate that the cell population employed was responsive and the methodology was adequate for the detection of UDS. For test materials causing weak or no UDS activity, the average response to the positive control treatments must exceed all three criteria used to indicate UDS (see Section 6). For test materials clearly causing a dose-related UDS activity, an assay will be acceptable in the absence of a positive control lost for technical reasons. Typical responses to treatments with 0.05 µg/ml of 2-AAF are as follows: 16±7 mean net nuclear grain count, 87±17% of the nuclei having 6 or more net grains, and 31±23% of the nuclei having 20 or more net grains.

6. Grain count data obtained for a given treatment is acceptable as part of the evaluation if obtained from at least two replicate cultures and at least 50 cells per culture.

7. A minimum of 6 dose levels will be analyzed for nuclear grain counts. Repeat trials need only augment the number of analyzed dose levels in the first trial to achieve a total of 6 different concentrations.

8. The highest analyzed dose must approach an excessive toxicity (defined by cell morphologies unsuitable for counting grains) or result in test material insolubility, or reach the highest applicable dose of 5000 µg/ml (or 5000 nl/ml).

X. EXPERIMENTAL DESIGN: (Continued)

E. Assay Evaluation Criteria

Several criteria have been established which, if met, provide a basis for evaluation of a test material as active in the UDS assay. These criteria are formulated on the basis of published results and laboratory experience and are used in lieu of a statistical treatment, at this time, to indicate a positive response. While the criteria are arbitrary guidelines that may not be applicable to all assays and may need revision as the data base increases, they represent a reasonable approach to the evaluation of a test material.

The test material is considered active in the UDS assay at applied concentrations that cause:

(1) An increase in the mean net nuclear grain count to at least six grains per nucleus after subtraction of the concurrent negative control value, and/or

(2) An increase in the percent of nuclei having 6 or more net grains to at least 10% of the analyzed population after subtraction of the concurrent negative control value, and/or

(3) The percent of nuclei with 20 or more grains to reach or exceed 2% of the analyzed population.

Generally, if the first condition is satisfied, the second and often the third condition will also be met. However, satisfaction of only the second or third condition can also indicate UDS activity. Different DNA-damaging agents can give a variety of nuclear labeling patterns, and weak agents may strongly affect only a small minority of the cells. Therefore, all three of the above conditions are considered in an evaluation.

A dose-related increase in UDS for at least two consecutive applied concentrations is also desirable to evaluate a test material as active in this assay. In some cases, UDS can increase with dose and then decrease to near-zero with successively higher doses. If this behavior is associated with increased toxicity, the test material can be evaluated as active. If an isolated increase occurs for a treatment far removed from the toxic doses, the UDS will be considered spurious.

X. EXPERIMENTAL DESIGN: (Continued)

E. Assay Evaluation Criteria (continued)

The test material is considered inactive in this assay if none of the above conditions are met and if the assay includes the maximum applied dose or other doses that are shown to be toxic by the survival measurements. If little or no toxicity is demonstrated for any of the applied doses and the test material remains soluble in the culture medium, the assay may be considered inconclusive and may be repeated with higher doses after consultation with the Sponsor.

The positive control nuclear labeling is not used as a reference point to estimate mutagenic or carcinogenic risk associated with the UDS activity of the test material. UDS elicited by test agents in this assay is probably more dependent on the type of DNA damage inflicted and the available repair mechanisms than on the potency of the test agent as a mutagen or carcinogen. Some forms of DNA damage are repaired without the incorporation of new nucleic acids. Thus, the positive controls are used to demonstrate that the cell population employed was responsive and the methodology was adequate for the detection of UDS.

XI. INTERPRETATION OF RESULTS:

The test material, Copper Naphthenate, formed a blue-green solution in absolute ethanol at 100 mg/ml. Just prior to the assay, a solution was prepared and serially diluted with absolute ethanol to obtain a series of stock concentrations. The test concentrations were then prepared by making 1:100 dilutions of these stocks into WME medium containing 1% fetal bovine serum. Fifteen test concentrations ranging from 0.025 µg/ml to 1000 µg/ml were thereby prepared. Following preparation of the test concentrations, the treatments were initiated by replacing the media on the cultures with the media containing the different concentrations of test material. The test material appeared soluble in the assay medium up to 100 µg/ml but a blue-green gel was present at the air/media interface at concentrations from 250 µg/ml to 1000 µg/ml.

One trial of the UDS assay was performed and the results are shown in Table 1.

The hepatocytes for the UDS assay were collected at approximately 80.5% viability (determined by trypan blue exclusion), and about 84.0% of the viable cells attached to the culture dishes during the 1.5-hour settling period. The treatments were initiated approximately 2.5 to 3 hours later with cell monolayers that were about 89.8% viable. After an additional 21 hours in culture (which encompassed the 18-hour treatment period), the average viable cell count in the solvent control