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

004876

OFFICE OF
PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: N-521 PAC. Fungicide/Bactericide
EPA File Symbol 476-EEEI
(Dazomet 24% ai [tetrahydro-3,5-dimethyl-2H-1,3,5-thiadiazine-2-thione]). Paper Mill Use.

Tox Chem. No. 840
Accession No. 251207

FROM: Yiannakis M. Ioannou, Ph.D.
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THRU: Albin B. Kocialski, Ph.D., Supervisory Pharmacologist
Section VII, Toxicology Branch
Hazard Evaluation Division (TS-769C) ABK 12/18/85

and

Theodore Farber, Ph.D.
Chief, Toxicology Branch
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The Stauffer Chemical Company has submitted a request for the registration of N-521 PAC to be used as a fungicide/bactericide in the control of slime-forming fungi and bacteria. The registrant has submitted several types of acute studies on the technical material in support of this action and has also requested data waivers for all the acute studies on the formulated product. Based upon our scientific review and regulatory considerations our position is as follows:

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- The skin and eye irritation studies on the formulated product can be waived based upon the pH of the formulation (pH = 13.2; refer to 40 CFR 158.135).
- Based upon the directions for use and use of the formulated product the acute inhalation study does not appear to be required (refer to 40 CFR 158.135).
- The acute oral LD_{50} study conducted on the formulation to be registered (Dazomet 24% ai) was classified as Supplementary Data for the reasons stated in the review and therefore needs to be repeated.
- An acute dermal LD_{50} study on the formulation also needs to be conducted for registration.

Several acute studies were also submitted on the technical material, N-521, (also identified as Mylone®). These studies were classified as either supplementary data or invalid for the reasons stated in the attached reviews.

Mutagenicity studies were also submitted by the registrant on the technical material, N-521, and their Classification is shown below (also see attached reviews):

Study	Classification
Sex-linked recessive lethal (Drosophila)	Acceptable
Bone marrow cytogenetics (rat)	Acceptable
Transformation of BALB/3T3 cells, in vitro (mouse)	Unacceptable
Transformation of BALB/3T3 cells, in vitro (mouse)	Unacceptable
Mouse lymphoma test	Acceptable
Sister chromatid exchange (mouse)	Acceptable
Ames (Salmonella)	Unacceptable
Ames (Salmonella)	Unacceptable

Currently, our records indicate that the primary dermal irritation study and the primary eye irritation* study conducted on the technical material (N-521) were classified as either core-minimum or core-guideline. However, all other studies are either core-supplementary or invalid. Four out of eight mutagenicity studies were also classified as unacceptable. The Toxicology Branch is therefore requesting at this time studies to determine the acute oral, dermal, and inhalation toxicity of the technical product. Additionally, all mutagenicity studies classified as unacceptable should be repeated. The percent purity of the technical material tested in the primary irritation study (Accession No. 251207) and the primary dermal irritation study (Accession No. 241289) should also be submitted.

The adequacy of the submitted labeling will be reviewed pending the receipt of the requested studies. This applies to both the technical material and the formulated product, Dazomet 24% ai.

*the primary eye irritation study on the technical has been downgraded from core-guideline to core-supplementary pending clarification of the test material (i.e. did the 0.1 ml represent 0.1 ml of unadulterated, and undiluted technical product).

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Subject: Acute Oral Toxicity (LD_{50}) Study for N-521 Technical in Rats

Test Material: 5% Suspension of N-521 (Mylone)

Accession Number: 251207

Sponsor: Stauffer Chemical Company

Testing Facility: Hazleton Laboratories

Study Number: H-24

Testing Period: Unspecified

Report Submitted to Sponsor: January 1953

Materials and Methods:

Male albino rats were administered orally the test material in single doses of 500, 625, 750, or 1000 mg/kg. Seven unstarved animals were used per treatment. The treated animals were observed daily for 7 days for signs of toxicity and mortality. Surviving animals were sacrificed (on day 7) and gross necropsies were performed on sacrificed as well as on animals that died during the study.

Results:

The lowest dose level which resulted in clinical signs of toxicity was 625 mg/kg. Signs of toxicity at the 625, 750, and 1000 mg/kg included: exophthalmia, rapid respiration, tonic convulsions, tremors, ataxia and blood in urine and feces. Gross necropsies of animals that died and of those sacrificed at the end of the study, showed adherence of abdominal viscera to one another with marked irritation and dilatation of the gastro-intestinal tract. Incidence of pulmonary hemorrhage was also observed in dead animals.

Conclusions:

The $AOLD_{50}$ for the 5 percent suspension of N-521 technical in male albino rats was determined (by the method of Litchfield and Wilcoxon) to be 750 mg/kg with 95% C.L. of 636 and 885 mg/kg.

Classification: This study is classified as supplementary due to: insufficient number of animals used (7 males, no females); no fasting of animals prior to treatment; no weights were taken at sacrifice; animals observed for only 7 days.

Category of Toxicity: III

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Subject: Acute Oral Toxicity (LD_{50}) Study for N-521 Technical

Test Material: N-521 Technical

Accession Number: 251207

Sponsor: Stauffer Chemical Company

Testing Facility: Stauffer Chemical Company

Study Number: T-4249

Testing Period: Unspecified

Report Submitted to Sponsor: December 1974

Materials and Methods:

Female Sprague-Dawley albino rats weighing approximately 210 g were used in this study. The test material was administered to animals via a stomach tube. Five animals were treated with one of the following dose levels; 215, 464, 1000, and 2150 mg/kg. Animals were fasted 24 hours prior to treatment. Following treatment all animals were observed for signs of toxicity and mortality.

Results:

Animals administered the low dose (215 mg/kg) appeared normal during the 14-day observation period. Higher dose levels resulted in moderate to severe depression of animal activity. The symptoms of toxicity developed rapidly.

Conclusions:

The $AOLD_{50}$ of N-521 in female Sprague-Dawley albino rats was determined to be 584 mg/kg with 95% C.L. of 360 and 950 mg/kg.

Classification: This study is classified as supplementary.*

Category of Toxicity: III

*Based on the presence of only one sex tested, the lack of reported observations (i.e., onset, duration, severity of effects, kinds of effects).

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Subject: Acute Dermal LD_{50} Toxicity of Mylone® (N-521) in Rabbits

Test Material: Mylone® Technical

Accession Number: 251207

Sponsor: Stauffer Chemical Company

Testing Facility: Stauffer Chemical Company

Study Number: T-4931

Testing Period: Unspecified

Report Submitted to Sponsor: September 1974

Materials and Methods:

Two male and two female New Zealand white albino rabbits, with weights ranging from 1.8 to 2.3 kg were used in this study. Prior to treatment the hair was clipped from the abdominal area of each animal. The test material (200 mg/kg) was applied to the intact abdominal skin beneath a protective binder. The protective binder was removed 24 hours later and the treated skin was inspected for irritation. The test site was then washed with soap and water and rewrapped in a gauze binder. The animals were observed daily for 14 days.

Results:

Mylone® did not produce any toxic effects or mortality when applied on the skin at 200 mg/kg.

Conclusions:

The $ADLD_{50}$ of Mylone® is greater than 200 mg/kg in both male and female rabbits.

Classification: This study is classified as supplementary due mainly to: use of only a single dose of 200 mg/kg instead of 2000 mg/kg required for a limit test; only four animals (two males and two females) were used for the study.

Category of Toxicity: II

Subject: Acute Inhalation (LC_{50}) Toxicity Study for Mylone® in Rats

Test Material: Mylone® Technical (N-521)

Accession Number: 251207

Sponsor: Stauffer Chemical Company

Testing Facility: Stauffer Chemical Company

Study Number: T-4931

Testing Period: Unspecified

Report Submitted to Sponsor: September 1974

Materials and Methods:

Five male and five female Sprague-Dawley albino rats weighing appriximately 200 g each were placed in a 32 liter L exposure chamber for 1 hour. The test material was generated into the chamber using a Wright Dust Feed Mechanism. The test concentrations generated were 1.6 and 1.7 mg/L/hr. The animals were observed for 14 days after exposure for signs of toxicity and mortality.

Results:

All animals exposed to Mylone® in the inhalation chamber at 1.6 to 1.7 mg/L/hr appeared normal during the 14-day observation period.

Conclusions:

The $AILC_{50}$ for Mylone® was determined to be greater than 1.7 mg/L/hr.

Classification: This study is classified as supplementary due to the following: The concentration of the test material used was very low (1.7 mg/L/hr vs 5 mg/L/hr for the limit test); time of exposure was very short (1 hour instead of 4 hours); no monitoring of air flow, aerodynamic particle size or the actual concentrations of the test substance from the breathing zone of the animals were reported.

Category of Toxicity: II

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Subject: Primary Eye Irritation Study for N-521 Technical in Rabbits

Test Material: Technical N-521 (Mylone)

Accession Number: 251207

Sponsor: Stauffer Chemical Company

Testing Facility: Hazleton Laboratories

Study Number: H-45

Testing Period: Unspecified

Report Submitted to Sponsor: December 1954

Materials and Methods:

A single application of 3.0 mg of the undiluted test material was placed in the conjunctival sac of the left eye of each animal while the untreated right eye served as control. A total of three rabbits (sex unspecified) was used for this study. Following application, the treated eyes were held closed for approximately 30 seconds and a reading was made immediately after. Additional observations for signs of eye irritation were made at 1, 4, and 24 hours posttreatment and daily thereafter until day 7 when the animals were sacrificed.

Results:

Immediately after exposure the treated eyes exhibited a very mild eye irritation characterized by slight lacrimation. The treated eyes of all animals appeared normal after 1 hour following application.

Conclusions:

A single application of 3.0 mg of N-521 to the eyes of albino rabbits produced a very mild, reversible irritation.

Classification: Supplementary*

Category of Toxicity: III

*Based on the number of animals, and the amount of test material administered.

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Subject: (1) Skin Irritation Study with N-521 (in Rabbits)
(2) Eye Irritation Study with N-521 (in Rabbits)

Test Material: N-521 Technical (Mylone)

Accession Number: 251207

Sponsor: Stauffer Chemical Comapny

Testing Facility: Western Research Center
of Stauffer Chemical Company

Laboratory Report: T-1961

Testing Period: Not given

Report Submitted to Sponsor: May 1972

Materials and Methods:

(1) Skin Irritation: The protocol followed the method of Draize (CFR Part 191.11, Chapter 1, Title 21, Federal Hazardous Substance Act). A 24-hour exposure was used.

(2) The Eye Irritation: The protocol generally followed the method of Draize (CFR Part 191.12, Chapter 1, Title 21, Federal Hazardous Substance Act). The quantity of test material placed in the eye of each animal was reported to be "10 mg or 0.1 ml." We request clarification of this statement.

Results:

(1) Skin Irritation: The primary total skin irritation score was 5.79 for both abraded and unabraded skin. The results indicated that the compound was a severe skin irritant if left in contact with the skin for 24 hours.

(2) Eye Irritation: Results reported were that no signs of irritation were observed during the 72-hour observation period.

Conclusion:

The report indicates that N-521:

1. is a severe skin irritant (PIS = 5.79)
2. is not an eye irritant

Classification: Skin irritation: Core-Guideline
Eye irritation: Core-Supplementary*

* Based on the low amount of test material used.

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Subject: Skin Irritation Study with N-521

Test Material: N-521 Technical

Accession Number: 251207

Sponsor: Stauffer Chemical Company

Testing Facility: Stauffer Chemical Company

Study Number: T-4249

Testing Period: Unspecified

Report Submitted to Sponsor: September 1973

Materials and Methods:

Primary skin irritation was determined according to the proposed FDA revision of the test for primary skin irritants published in the FEDERAL REGISTER (37 FR 244, December 19, 1972) and the proposed DOT changes in the proposed FDA protocol published in the FEDERAL REGISTER (38 FR 28, February 12, 1973). Modifications made in this test were (1) that readings were taken at 4, 24, and 48 hours after compound administration, (2) the test material, a white powder, was not moistened, (3) the skin was not abraded, and (4) irritation was determined after a 4-hour exposure.

Results:

Dermal irritation scores were zero for all time intervals for all six rabbits.

Conclusion:

The technical material is not a skin irritant under the test conditions.

Classification: Supplementary

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Subject: Primary Eye Irritation Study for N-521 Technical in Rabbits

Test Material: N-521 Technical

Accession Number: 251207

Sponsor: Stauffer Chemical Company

Testing Facility: Stauffer Chemical Company

Study Number: T-4249

Testing Period: Unspecified

Report Submitted to Sponsor: December 1974

Materials and Methods:

Six New Zealand rabbits (sex unspecified) weighing between 1.6 to 2.1 kg were used in this study. Ten milligrams of the test material were placed in one eye of each animal, and the eyes held closed for approximately 3 seconds after treatment. The untreated eye of each animal served as control. The eyes were observed at 24, 48, and 72 hours following treatment for irritation.

Results:

No irritation was seen in any of the treated or untreated eyes during the observation period of 7 days.

Conclusions:

N-521 is not an eye irritant in rabbits when given at 10 mg.

Classification: Supplementary*

Category of Toxicity: IV

*Based on the low amount of test material used.

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Subject: Mutagenicity Evaluation of N-521 Technical, Batch #149 in the Sex-Linked Recessive Lethal Test in Drosophila melanogaster

Test Material: N-521 Technical

Accession Number: 251207

Sponsor: Stauffer Chemical Company

Testing Facility: Litton Bionetics Inc.

Study Number: T-10012

Testing Period: March - May 1979

Report Submitted to Sponsor: July 1979

Materials and Methods:

The descriptions of the materials and methods used in this study were abstracted and paraphrased from the original report.

Based on preliminary toxicity studies (LD_{50} studies), 2 levels (0.025 mg/mL and 0 05 mg/mL) of N-521 were selected for use in the present study. Solvent control (Dimethylsulfoxide, 0.01 mL/mL) and positive control (Ethylmethanesulfonate, 0.015M) were also included.

Groups of 25 male and 25 female flies from the proper stock culture were allowed to mate at random in each of several bottles. Healthy male progeny from these bottles were used for dosing by parceling fifty per vial until a sufficient number for the test was collected. At least 200 treated males were used for each dose level of the test compound. The approximate, planned number of chromosomes tested and the brooding pattern employed are shown below:

CHROMOSOMES TESTED

Tests	Brood I	Brood II	Brood III	Brood IV
Low concentration	1500	1500	1500	1500
High concentration	1500	1500	1500	1500
Negative control	1500	1500	1500	1500
Positive control	100	100	100	100

PROCEDURE

Yellow-bodied males were collected within eight hours of emergence and aged one day before treatment. The flies were kept in vials (50/vial) lined with an inert glass filter paper which was saturated with 1.5 mL of 1 percent sucrose solution containing the test compound. The exposure period was 24 hours. Following a 24-hour waiting period after dosing to eliminate delayed toxic effects, the treated males were mated individually to sequential groups of three virgin Basc females. The brooding scheme employed consisted of a 2-3-3-4 day sequence which sampled spermatozoa, spermatids, spermatocytes and spermatogonia. Each treated male was assigned a unique identification number which was written onto the vial and identified the females inseminated by that male throughout the brooding sequence. In this way the progeny of each male were kept separate and the data were recorded in such a way that the origin of each tested chromosome was known. This method eliminated the possibility of false positives resulting from clusters of identical lethal mutations originating in one treated male.

The F_1 progeny of each culture was inspected to make certain the proper cross was made. The desired number of F_1 females was then pair-mated to their brothers. Where possible, an equal number of F_1 females per treated male was tested to avoid biasing the data.

In the F_2 generation, each culture vial (representing one treated X chromosome) was examined for the presence of males with yellow bodies. If this class of males was present, the culture was considered nonlethal and was discarded. If this class was absent, the vial was marked as a potential lethal and was set aside for further examination. The following criteria were applied to cultures suspected of being lethal:

> If 20 or more progeny were present and there were no yellow-bodied males, the culture was considered to carry a lethal mutation on the treated chromosome and further testing was unnecessary. The chance of missing a male carrying the treated X chromosome in a population of this size is $(1/2)^5$ or < 0.05;
>
> or
>
> If there was less than 20 progeny, or if there was one yellow-bodied male, the culture was retested by mating three of the females heterozygous for the treated and Basc chromosomes to

Basc males. The progeny of these crosses were scored for the presence of X males.

DATA ANALYSIS

When the data were compiled, the total number of X chromosomes tested equaled the sum of the lethal and nonlethal cultures. The frequency of X-linked recessive lethals was calculated as:

$$\frac{\text{number of lethals}}{\text{number of lethals + number of nonlethals}} \times 100 = \% \text{ lethal}$$

The Kastenbaum-Bowman test was used to determine the significance of the results. An increase of twice the spontaneous frequency in conjunction with a dose-response relation was considered a positive response.

Results:

In the present study 2 dose levels, 0.05 and 0.025 mg/mL, of N-521, representing 1/2 and 1/4 of the LD_{50} respectively, were used. Dimethylsulfoxide (DMSO) was used as the solvent control while ethylmethanesulfonate (EMS) was used as the positive control. The results of this study indicate that the percent of lethal mutations observed with the low (0.016%) as well as the high (0.24%) dose was comparable to that observed with the solvent control (0.22%), at all sampling times (broods) table 1. EMS, the positive control, induced statistically significantly higher lethal mutations as compared to the control. It should be noted however, that the positive control assay was not carried out concurrently with the rest of the study, and thus it cannot be directly compared with the results of this study (solvent control values from the earlier study should be included as well for comparison). It is not clear whether the same dose (0.015M) was employed in this study and if it were the same dose why the sterility in this study was so high as compared to the earlier study.

Conclusions:

Although, some individual points observed in the test groups were higher than the solvent control, the pooled frequencies of 0.14 (adjusted for clusters) and 0.24 percent lethal mutations for the low and high test doses, respectively, were not significantly different from the 0.18 percent of the solvent control. Therefore, under the conditions of this study, N-521 was found to be inactive in the production of sex-linked recessive lethals in Drosophila.

Classification: Acceptable

TABLE 1

Test Compound	Concentration	Brood	No. of Chromosomes Tested	No. Lethal	% Lethal	No. of Lethals Adjusted for Clusters	% Lethal Adjusted for Clusters
Dimethylsulfoxide (Solvent Control)	0.01 mL/mL	I	2453	10	0.41	7	0.29
		II	1821	3	0.16	3	0.76
		III	1175	1	0.09	1	0.09
		IV	1891	2	0.11	2	0.11
		Total	7340	16	0.22	13	0.18
Ethylmethane-sulfonate (Positive Control)+	0.01M	I	133	34	25.56**	Not adjusted	
		II	115	37	32.17*		
		III	121	24	19.83*		
		IV	87	16	18.39*		
		Total	456	111	24.34*		
N-521, technical (Low Dose)	0.025 mg/mL	I	2218	4	0.18	4	0.18
		II	2022	4	0.20	3	0.15
		III	874	2	0.23	2	0.23
		IV	1249	0	0	0	0
		Total	6363	10	0.16	9	0.14
N-521, technical (High Dose)	0.05 mg/mL	I	2134	6	0.28	No change	
		II	1990	5	0.25		
		III	1291	4	0.31		
		IV	966	0	0		
		Total	6391	15	0.24		

* Significant at p = 0.05 when compared to the solvent control.

+ The concurrent positive control failed to produce a sufficient number of progeny for analysis due to high sterility. The data reported here is from a study conducted in the laboratory at approximately the same time (March 5 to April 24, 1979).

00484735

Subject: Mutagenicity Evaluation of N-521 In Vivo in the Rat Bone Marrow Cytogenetic Assay

Test Material: N-521 Technical

Accession Number: 251207

Sponsor: Stauffer Chemical Company

Testing Facility: Litton Bionetics Inc.

Study Number: T-10011

Testing Period: April 1979 - June 1979

Report Submitted to Sponsor: July 1979

Materials and Methods:

The descriptions of the materials and methods used in this study were abstracted and paraphrased from the original report.

Animals

Sprague-Dawley adult male albino rats, from a random bred, closed colony were purchased from Charles River Breeding Laboratories, Inc. and used in this cytogenetic study.

Control Chemicals

Triethylenemelamine (TEM) was used as the positive control compound. The negative control consisted of the solvent or vehicle used for the test compound (water).

EXPERIMENTAL DESIGN

The animals were group housed and offered a commercial diet (Purina) and water ad libitum.

The animals were randomly assigned to 17 experimental groups of 8 each. Prior to study initiation, all animals were weighed and a mean body weight was determined for the group. Dose levels were established using this mean unless there was significant variation among individuals, in which case individual calculations were made. Animals were identified by cage number and individual eartag.

METHODOLOGY

The administration of the test compound was done by oral gavage. The vehicle water (negative control group) was also given orally while the TEM (positive control group) was given i.p.

Table 1 shows the basic design of the test. Both acute (single dose) and subchronic (5 consecutive daily doses) sequences are provided. A total of 136 rats--104 in the acute study and 32 in the subchronic study-- were used in the test as outlined in table 1.

Three hours prior to kill, the animals were injected IP with 4.0 mg/kg colchicine. At times indicated in table 1, rats were killed with CO_2 and the adhering soft tissue and epiphyses of one or both tibiae removed. The marrow was aspirated or flushed from the bone and transferred to Hank's Balanced Salt Solution (HBSS). The marrow button was collected by centrifugation and then resuspended in 0.075M KCl. The centrifugation was repeated and the pellet resuspended in Carnoy's fixative. The fixative was changed after one-half hour and the cells stored overnight at 4 °C.

Slides were prepared by dropping the cells from the fixative onto a glass slide and the film air-dried. Spreads were stained with 10 percent Giemsa at pH 6.8.

Slides were labeled with date, code, and the animal eartag numbers and scored for chromosomal aberrations. Routinely, 50 spreads were read for each animal dosed.

Results:

The present study has investigated the potential genetic activity of N-521 in rat bone marrow cells when administered as a single dose or in multiple doses at 3 dose levels. Table 1 gives the summary of the results of these assays.

The results indicate that when N-521 is given to rats either as a single dose or as a series of doses (5 consecutive daily) it did not induce significant increases in the frequency of structural aberrations in bone marrow cells. Although a higher frequency of structural aberrations was observed with the acute dose of 20 mg/kg at the 48-hour time point, the observed aberrations were simple fragments and do not constitute major biological significance. The percentage of cells with aberrations was essentially unaffected by exposure to N-521. One value, however, was significantly elevated (low single dose, 24-hour time point), but the statistical significance was mainly due to the fact that no aberrations were observed with the concurrent solvent controls.

Conclusions:

Under the conditions of the assay, N-521 was found not to be clastogenic.

Classification: Acceptable

TABLE 1

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Summary of the Cytogenetic Analysis of N-521

Treatment	Dose mg/kg	Type of Study	# of Animals Used	Time (hrs) Animals Killed After Dosing	Structural Aberrations Frequency	Total # of Cells Scored	# of Structural Aberrations	% Cells with Aberrations
Control (H_2O)	-	Acute	8	6	0.005	400	2	0.8
			8	24	-	385	0	0.0
			8	48	-	344	0	0.3
		Subchronic	8	6	0.006	362	2	1.4
Positive Control (TEM)	1.0	Acute	8	24	0.170**	100	17	12.0**
N-521	6.0	Acute	8	6	0.015	330	5	0.9
			8	24	0.003	400	1	1.8**
			8	48	0.005	400	2	1.5
		Subchronic	8	6	0.003	400	1	0.5
	20.0	Acute	8	6	0.006	330	2	0.9
			8	24	0.006	321	2	0.0
			8	48	0.017*	344	6	0.3
		Subchronic	8	6	0.003	350	1	1.4
	60.0	Acute	8	6	0.010	309	3	1.0
			8	24	-	247	0	0.0
			8	48	-	349	0	0.6
		Subchronic	8	6	-	400	0	0.5

* Significantly greater than solvent control $p < 0.05$.
** Significantly greater than solvent control $p < 0.01$.

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Subject: Mutagenicity Evaluation of Sample #100 (N-521)

Test Material: N-521 Technical

Accession Number: 251207

Sponsor: Stauffer Chemical Company

Testing Facility: Litton Bionetics Inc.

Study Number: T-6081

Testing Period: September - October 1976

Report Submitted to Sponsor: October 1976

Materials and Methods:

The descriptions of the materials and methods used in this study were abstracted and paraphrased from the original report.

A. Indicator Microorganisms

Salmonella typhimurium, strains: TA-1535 TA-98
TA-1537 TA-100
TA-1538

Saccharomyces cerevisiae, strain: D4

B. Activation System

1. Reaction Mixture

Component	Final Concentration/mL
TPN	4 u moles
Glucose-6-phosphate	5 u moles
Sodium phosphate	100 u moles
$MgCl_2$	8 u moles
KCl	33 u moles
Homogenate fraction equivalent to 25 mg of wet tissue	0.1 - 0.15 mL 9,000 x g supernatant of rat liver

2. S-9 Homogenate

A 9,000 x g supernatant was prepared from Sprague-Dawley adult male rat liver induced by Aroclor 1254 5 days prior to kill.

C. The following chemicals were used as positive controls:

1. Nonactivation: Methylnitrosoguanidine (MNNG)
2-Nitrofluorene (NF)
Quinacrine mustard (QM)

2. Activation: 2-Anthramine (ANTH)
2-Acetylaminofluorene (AAF)
8-Aminoquinoline (AMQ)

D. Solvent

Either deionized water or dimethylsulfoxide (DMSO) was used to prepare stock solutions of solid materials. All dilutions of test materials were made in either deionized water or DMSO.

E. Plate Test (Overlay Method)

Approximately 10^9 cells from an overnight culture of each indicator strain were added to separate test tubes containing 2.0 mL of molten agar supplemented with biotin and a trace of histidine. For nonactivation tests, the four dose levels of the test compound (dissolved in DMSO) were added to the contents of the appropriate tubes and poured over the surfaces of selective agar plates. In activation tests the same four dose levels of the test chemical were added to the appropriate tubes with cells. Just prior to pouring, an aliquot of reaction mixture (0.5 mL containing the 9,000 x g liver homogenate) was added to each of the activation overlay tubes, which were then mixed, and the contents poured over the surface of a minimal agar plate and allowed to solidify. The plates were incubated for 48 hours at 37 °C, and scored for the number of colonies-growing on each plate. The concentrations of all chemicals were given in the Results Section. Positive and solvent controls using both directly active positive chemicals and those that require metabolic activation were run with each assay.

Results:

In the present study, a wide range of concentrations of the test compound was used (0.1 , 1.0, 10, 100, and 500 ug/plate) with 6 different tester strains of microorganisms with and without metabolic activation. The results show that in both systems (with or without metabolic activation) the test compound did not result in significantly higher number of revertants at any concentration tested as compared to the solvent control. The higher concentration of the test compound (100 and 500 ug per plate) resulted in lower number of revertants in certain tester strains) in both assays

(with and without metabolic activation) as compared to the solvent controls (table 1). A significantly higher number of revertants per plate was obtained in the positive controls as compared to the solvent controls (table 1).

Conclusions:

The test compound sample #100 (N-521), did not demonstrate mutagenic activity in any of the assays conducted and is considered to be nonmutagenic under the experimental conditions employed in this study. However, the concentrations of the test material used were not high enough to determine the mutagenic potential of this compound (5,000 ug/plate should have been tested for conclusive evidence of positive or negative mutagenicity).

Classification: Unacceptable

00048836

004876

Subject: Mutagenicity Evaluation of N-521 in Salmonella typhimurium

Test Material: N-521 (pure 100% ai)

Accession Number: 251207

Sponsor: Stauffer Chemical Company

Testing Facility: Stauffer Chemical Company

Study Number: T-10044

Testing Period: July, 1979 - March 1980

Report Submitted to Sponsor: June 1980

Materials and Methods:

The descriptions of the materials and methods used in this study were abstracted and paraphrased from the original report.

A. Indicator Microorganisms

Salmonella typhimurium, strains: TA-1535, TA-1537, TA-1538, TA-98, and TA-100 used as the indicator organism.

B. Metabolic Activation System

1. Reaction Mixture

Component	Final Concentration/ml
NADP	4 mM
Glucose-6-phosphate	5 mM
Sodium phosphate (buffer)	100 mM
$MgCl_2$	8 mM
KCl	34 mM
Homogenate S-9 fraction	100 uL/mL

C. Positive Control Chemicals: Anthramine, 5 ug/plate was used as the positive control in all Aroclor and phenobarbital-induced livers in activated systems, with all tester strains. Positive controls used in nonactivated systems were: Sodium azide at 10 ug/plate for TA-1535 and TA-100, 9-aminoacridine at 5 ug/plate for TA-1537 and 2-nitrofluorene of 10 ug/plate for TA-1538 and TA-98.

D. Test Material Concentrations

Dose levels used in the nonactivated assay ranged from 3.7 to 300 ug/plate. For assays with metabolic activation dose levels ranged from 37 to 1000 ug/plate (Aroclor induced rat liver) or 12.3 to 1000 ug/plate for all other assays.

E. Solvent/Vehicle

Dimethylsulfoxide (DMSO) was used to prepare stock solutions of the test substance. All dilutions of stock solutions were made in DMSO.

Experimental Design

F. Plate Test (Agar Incorporation) Protocol No. IVT-7.

Approximately 10^9 cells from an overnight culture of each indicator strain growing in nutrient broth were added to separate test tubes containing 2.0 mL of molten agar supplemented with biotin and a trace of histidine. In addition, each tube received the designated concentration of the test substance and either 0.5 mL of phosphate buffer (nonactivation) or 0.5 mL of a reaction mixture containing the 9,000 x g liver homogenate supernatant (S-9 activation assay). The contents of the tubes were mixed and then poured onto the surface of selective agar plates and allowed to solidify. The plates were incubated for approximately 48 hours at 37 °C and scored for the number of colonies growing on each plate. At least 5 dose levels of the test substance and positive and negative controls (solvent/vehicle and medium) were used in each assay.

G. Liver S-9 Preparation (9000 x g) Supernatant

Sprague-Dawley male rats or B6C3F$_1$ male mice purchased from the Charles ~~River~~ Breeding Laboratories were used as the source of hepatic S-9.

Aroclor 1254 (500 mg/kg) was administered intraperitoneally (i.p.) by a single injection in corn oil to rats weighing approximately 200 grams or mice weighing approximately 20 grams 5 days prior to their sacrifice. Animals were deprived of food on the evening before sacrifice. Phenobarbital was injected i.p. at 80 mg/kg in corn oil to rats and mice once a day for 5 consecutive days prior to sacrifice. At sacrifice the livers from 7 to 10 rats or 20 to 50 mice were removed and transferred to sterile, ice cold 0.15M KCl. They were washed with 0.15M KCl, weighed, minced, and diluted with 3 mL of 0.15M KCl per

gram (wet weight) of liver. These fragments were homogenized and the homogenate from all animals was pooled prior to being centrifuged for 20 minutes at 9000 x g at 4 °C. After centrifugation the S-9 supernatant fractions was transferred by pipetting to storage vials. These were immediately frozen and retained at approximately -80 °C until used.

Recording and Presenting Data

The numbers of colonies on each plate were counted on a New Brunswick Biotran II Automated Colony Counter and presented as revertants per plate for each indicator strain employed in the assay. The positive and solvent/vehicle controls are provided as reference points.

Results:

The present study employed 5 tester strains of Salmonella typhimurium, several concentrations of the test compound (ranging from 3.7 to 1000 ug/plate) and different positive controls. The studies were carried out with or without metabolic activation, with rat or mouse liver S-9 fractions induced with Aroclor or phenobarbital. The present results indicate that either medium control (S-9 homogenate) or solvent/vehicle control gave similar numbers in background revertants. Positive controls resulted in all cases in statistically significant increase in revertants/plates as compared to vehicle controls. The test compound did not, in any test (with or without activation, mouse or rat, Aroclor or phenobarbital induced) increase the number of revertants in any of the tester strains. In all cases, the highest concentration used (1000 ug/plate) resulted in lower numbers of revertants compared to controls. Lower number of revertants were observed with doses 300 and 333.3 ug/plate mainly with TA-100 tester strain.

Conclusions:

N-521, is reported as not mutagenic in the Ames Salmonella microsome assay either directly or in the presence of a metabolic activation system containing either Aroclor or phenobarbital induced mouse or rat S-9 livers.

Classification:

The present study is classified as Unacceptable mainly for 2 reasons: (a) the dose levels used were not high enough (5000 ug/plate required for conclusive results); (b) inappropriate positive controls were used in most assays.

004876

Subject: Mutagenicity Evaluation of N-521 in an In Vitro Cytogenetic Assay Measuring Sister Chromatid Exchange and Chromosome Aberrations

Test Material: N-521 Technical

Accession Number: 251207

Sponsor: Stauffer Chemical Company

Testing Facility: Litton Bionetics, Inc.

Study Number: T-6410

Testing Period: January - February 1979

Report Submitted to Sponsor: March 1979

Materials and Methods:

The descriptions of the materials and methods used in this study were abstracted and paraphrased from the original report.

A. Indicator Cells

The cells used in this study were derived from the Fischer mouse lymphoma cell line, L5178Y. The cells are heterozygous for a specific autosomal mutation at the TK locus and are bromodeoxyuridine (BrdU) sensitive. The cells were maintained in Fischer's medium for leukemia cells of mice with 10 percent horse serum and sodium pyruvate.

B. Control Compounds

The test compound was soluble in medium, so the medium described above was used as the negative control.

Ethylmethanesulfonate (EMS), which induces mutation by base-pair substitution, was dissolved in culture medium and used as the positive control for the nonactivation studies at a final concentration of 0.5 uL/mL.

Dimethylnitrosamine (DMN), which induces mutation by base-pair substitution and requires metabolic biotransformation by microsomal enzymes, was used as the positive control for the activation studies at a final concentration of 0.3 uL/mL.

EXPERIMENTAL DESIGN

Toxicity

The effect of each chemical on the survival of the indicator cells was determined by exposing the cells to a wide range of chemical concentrations in complete growth medium in a preliminary cytotoxicity test. Toxicity was measured as loss in growth potential of cells induced by a 4-hour exposure to the chemical followed by a 24-hour expression period in growth medium. A minimum of 4 concentrations was selected from the concentration range by using as the highest dose a level that showed a reduction in growth potential. For the final assay at least 3 lower doses, including levels which were below the toxic range, were added. Those compounds that were relatively nontoxic to cells growing in suspension were tested at concentrations of up to 5 mg/mL when solubility permitted. Toxicity produced by chemical treatment was also monitored during the main (cytogenetic) experiment.

C. Assays

1. Nonactivation

Prior to each treatment, cells were cleansed of spontaneous TK-/- by growing them in medium containing thymidine, hypoxanthine, methotrexate, and glycine (THMG). (This medium permits the survival of only those cells that produce TK, and can therefore utilize the exogenous thymidine from the medium.) The test compound was added to the cleansed cells in growth medium at the predetermined doses for 4 hours at 37 °C. The incubation period was terminated by washing the cells twice with growth medium. Fresh growth medium was then added together with BrdU (final concentration 20 uM) and cells were incubated for an additional 20 hours or two cell cycles. Colchicine (final concentration 5×10^{-5} mg/mL) was present during the final 3 hours of culture to arrest cells in metaphase. Cells were harvested by centrifugation, resuspended in 0.075M KCl hypotonic solution, and then washed 3 times in methanol/acetic acid (3:1) before being dropped onto slides and air dried.

2. Activation Assay

The activation assay differs from the nonactivation assay in the following manner only. S-9 was added to 10 mL growth medium containing appropriate

cofactors and the desired number of cleansed cells. After adding the test compound, the flask was incubated with agitation for 4 hours at 37 °C. The incubation period was terminated by washing the cells twice with growth medium.

3. Staining for SCE and Chromosome Aberration

Cells were stained for sister chromatid differentiation and subsequent SCE evaluation according to a modification of the Giemsa method of Korenberg and Freedlander. Cells to be scored for chromosome aberrations were stained with Giemsa.

Preparation of 9,000 x g Supernatant

Male, ramdom bred rats (Fischer 344) pretreated with Aroclor 1254 were killed (by cranial blow) and the livers were immediately dissected from the animals and placed in ice-cold 0.25 M sucrose buffered with Sodium Phosphate at pH 7.4. The livers were washed twice with fresh buffered sucrose and completely homogenized. The homogenate was centrifuged for 20 min at 9,000 x g and the supernatant was stored at -80 °C until used in the activation system. This preparation was added to culture medium along with the appropriate cofactors in the concentrations described below.

Component	Final Concentration/mL
NADP (sodium salt)	2.4 mg
Isocitric acid	4.5 mg
Homogenate S-9 fraction	10 uL

Scoring

The average number of sister chromatid exchanges per cell and per chromosome were determined and compared with values for the negative control compound to determine significance. Similar comparisons were made from the chromosome aberration data.

Results:

In the present study, the test compound N-521, was evaluated for its ability to induce chromosome aberrations and sister chromatid exchanges (SCE) in the presence or absence of metabolic activation. The test compound was used in 5 different dose levels (1.56, 3.13, 6.25, 12.50, and 25.00 ug/mL) and positive, negative and solvent controls were included in the assay. The results show that as far as chromosome aberrations are concerned no dose

response relationship was observed when no activation was used (table 1). The lowest dose, (1.56 ug/mL) resulted in a significant increase in chromosome aberrations as compared to solvent control. The middle dcse, 6.25 ug/mL, also elevated the frequency of cells with aberrations while in the other doses (3.13, 17.50, and 25.00 ug/mL) the frequency of aberrations was comparable to solvent control. In the presence of metabolic activation, the test compound induced statistically significant increases ($p < 0.05$) in the frequency of cells with aberrations at 3 dose levels (1.56, 6.25, and 25.00 ug/mL with 4, 5, and 5 cells respectively), table 1. N-521 induced SCE's only at the highest dose level tested (25.00 ug/mL) without metabolic activation (14.1 as compared to 10.3 SCE/cell in control). With metabolic activation, the number of SCEs/cell induced by the test chemical was comparable to those recorded in solvent control (table 2).

Conclusions:

Due to its high toxicity, N-521 was tested in very low dose levels in this study. At these low dose levels, N-521 was found to be weakly clastogenic inducing chromosome aberrations particularly when used directly. At the highest dose level, N-521 also increases the frequency of SCE's in the absence of activation.

Classification: Acceptable

TABLE 1

Chromosome Aberrations Induced *In Vitro* by N-521

Treatment	Concentration	Number of Cells[1] with Aberrations	
		Without Activation	With Activation
Solvent Control	0.1 mL/tube	1	0
Negative Control	-	0	2
Positive Control	0.3 ul/mL	15**	18**
N-521	1.56 ug/mL	7*	4*
	3.13 ug/mL	1	2
	6.25 ug/mL	6	5*
	12.50 ug/mL	0	2
	25.00 ug/mL	2	5*

[1] Total of 50 cells examined/treatment.
* Significantly greater than solvent control, $p < 0.05$.
** Significantly greater than solvent control, $p < 0.01$.

TABLE 2

Sister Chromatid Exchange (SCE) Frequencies in Cells Exposed to N-521

Treatment	Concentration	Sister Chromatid Exchanges			
		Without Activation		With Activation	
		Total No. SCE	SCE/Cell	Total No. SCE	SCE/Cell
Solvent Control	0.1 mL/tube	97	10.3	144	15.3
Negative Control	-	84	9.0	116	12.4
Positive Control	0.3 ul/mL	570*	59.4**	424*	43.6*
N-521	1.56 ug/mL	82	8.5	121	13.0
	3.13 ug/mL	90	9.4	132	13.9
	6.25 ug/mL	103	11.0	112	11.9
	12.50 ug/mL	92	10.1	110	11.8
	25.00 ug/mL	133	14.1*	116	11.8

* Significantly greater than solvent control, $p < 0.05$.
** Significantly greater than solvent control, $p < 0.01$.

Subject: Mutagenicity Evaluation of N-521 in Mouse Lymphoma Multiple Endpoint Test

Test Material: N-521 Technical

Accession Number: 251207

Sponsor: Stauffer Chemical Company

Testing Facility: Stauffer Chemical Company

Study Number: T-10136

Testing Period: August 1979 - March 1980

Report Submitted to Sponsor: November 1980

Materials and Methods:

The descriptions of the materials and methods used in this study were abstracted and paraphrased from the original report.

The cell line L5178Y (TK+/-) used in this assay was derived from the 3.7.2 clone of Fischer L5178Y cells provided by Dr. Donald Clive.

Frozen stocks were maintained in liquid nitrogen. Laboratory cultures were periodically checked for mycoplasma contamination. The cultures were routinely exposed to methotrexate, which selects against spontaneously arising TK-/- cells, to maintain a low background frequency of trifluorothymidine (TFT) resistant cells.

Growth medium consisted of RPMI 1640, horse serum (10% by volume), glutamine (2mM), penicillin-streptomycin, sodium pyruvate (22 mg/mL), and pluronic (50 mg/mL). Treatment medium was growth medium with serum reduced to 5 percent, cloning medium was growth medium with the omission of pluronic, and addition of noble agar to a final concentration of approximately 0.35 percent. Selective medium was cloning medium with 4 ug/mL TFT.

Treatment

Approximately 12 x 10^6 cells were exposed to 5 to 6 doses of test substance, solvent/vehicle, medium, or positive control substances, for 4 hours in a total volume of 20 mL. At the end of the exposure time, the substance was removed and the cells were resuspended in 40 mL of medium. Half of the cells were used for the mutation assay and half for the cytogenetic assay, as described below.

The assay was run both without and with the inclusion of a metabolic activation system.

Mutation Assay

The procedure used was adapted from the assay reported by Clive, et al., 1975. At the end of treatment, cells were incubated with agitation for 48 hours. Cultures which have reached a density of 4×10^5 cells/mL or greater 24 hours after treatment were reduced to 3×10^5 cells/mL to allow maximum growth, recovery, and expression of induced TK-/- phenotype. At 48 hours the cells were cloned in soft agar: 3×10^6 cells in selective medium for TFT resistant cells, and 600 cells in nonselective medium for viable counts (VC) or number of cells capable of cloning. The mutation frequency is the number of mutant cells per 10^6 viable cells.

The plates were incubated in a humidified 5 percent CO_2 37 °C incubator for 9 to 11 days.

Cytogenetic Assay

The procedure used was adapted from the assay reported by Lebowitz, et al., 1977. After treatment the cells were resuspended in growth medium and 10^{-4} mM BrdU. Cells were harvested, exposed to hypotonic medium, and fixed in Carnoy's fixative. The cell suspension was distributed on slides and stained either with Giemsa (for aberrations) or with Hoechst stain and exposed to a black light (for sister chromatid exchange) and Giemsa.

(1) The first 50 well spread metaphases were counted and scored for aberrations. A mitotic index was recorded by counting 500 cells randomly in groups of 100, recording the number of cells in mitosis.

(2) Fifteen to twenty cells per dose were scored for sister chromatid exchanges.

Liver S-9 Preparation (9000 x g) Supernatant

Sprague-Dawley male rats purchased from the Charles River Breeding Laboratories were used as the source of hepatic S-9.

Aroclor 1254 (500 mg/kg) was administered intraperitoneally (i.p.) by a single injection in corn oil to rats weighing approximately 200 grams five days prior to their sacrifice. Animals were deprived of food on the evening before sacrifice.

At sacrifice the livers from 7 to 10 rats were removed and transferred to sterile, ice cold 0.15M KCl. They were washed with 0.15M KCl, weighed, minced, and diluted with 3 mL of 0.15M KCl per gram (wet weight) of liver. These fragments were homogenized and the homogenate from all animals was pooled prior to being centrifuged for 20 minutes at 9000 x g at 4 °C. After centrifugation the S-9 supernatant fraction was transferred by pipetting to storage vials and immediately stored at approximately -80 °C until used.

Metabolic Activation System:

One mL of S-9 homogenate, 240 ug NADP and 450 ug isocitrate were added in a final treatment volume of 20 mL.

Control Substances:

Medium (S-9), solvent vehicle and positive control (EMS) were included in all assays as reference points.

Results:

N-521 was evaluated for genetic activity in the L5178Y mouse lymphoma multiple endpoint test. The test chemical was found to increase mutation frequency at the thymidine kinase locus. Table 1 summarizes the results of 2 trials and it shows that all dose levels in one trial and levels greater than 1 ug/mL in the other trial have induced gene mutations statistically significantly higher than the solvent control. Mutations were observed only when N-521 was used directly (without activation).

In the cytogenetic assays, a statistically significant increase of structural chromosome aberrations occurred at dose levels of 4 and 5 ug/mL when N-521 was used without activation (table 1). At lower dose levels there were increases in numerical aberrations. Endoreduplication, a rare spontaneous aberration, was observed in most of the dose levels. No significant increase in sister chromatid exchanges (SCE's) was observed when N-521 was assayed either with or without activation (tables 1 and 2).

Conclusions:

When assayed directly, (in the absence of metabolic activation) N-521 was found to be mutagenic at the thymidine kinase locus and clastogenic in the mouse lymphoma multiple endpoint test. No genetic activity was observed when N-521 was assayed in the presence of an activation system.

Classification: Acceptable

TABLE 1

Mouse Lymphoma Multiple Endpoint Test, Direct Assay

Treatment	Concentration	Gene Mutations Mutant Frequency x 10^{-6} Trial A	B	# of Cells with Structural Aberrations[1] A	B	SCE/Cell Trial A	B
Solvent Control	—	46	43	0	0	9.9	10.2
Positive Control (EMS)	0.5 uL/mL	634	309	6*	3*	45.0*	46.8*
N-521	0.6 ug/mL	32	131	-	1	-	11.9
	0.8 ug/mL	58	145	1	0	10.6	10.6
	1.0 ug/mL	114	131	-	0	10.0	10.5
	2.0 ug/mL	70	87	-	1	13.5	9.9
	4.0 ug/mL	158	109	3**	4**	13.0	9.8
	5.0 ug/mL			4*		10.0	

[1] Fifty cells scored/trial
* Significantly greater than solvent control $p < 0.01$.
** Significantly greater than solvent control $p < 0.05$.

TABLE 2

Mouse Lymphoma Multiple Endpoint Test, Metabolic Activation Assay

Treatment	Concentration	Gene Mutations Mutant Frequency $x.10^{-6}$ Trial A	Number of Cells With Structural Aberrations Trial A	B	SCE/Cell Trial A	B
Solvent Control	-	42	1	0	10.5	9.2
Positive Control (DMN)	0.3 uL/mL	729*	14*	13**	62*	57.7*
N-521	4 ug/mL	31	-	0	-	9.3
	6 ug/mL	27	1	0	10.6	-
	8 ug/mL	30	1	0	11.7	9.3
	10 ug/mL	37	1	0	12.0	7.3
	20 ug/mL	42	1	0	11.8	7.8

* Significantly greater than solvent control $p < 0.01$.
** Significantly greater than solvent control $p < 0.05$.

Subject: Mutagenicity Evaluation of N-521 in the in vitro Transformation of BALB/3T3 Cells Assay

Test Material: N-521 Technical

Accession Number: 251207

Sponsor: Stauffer Chemical Company

Testing Facility: Litton Bionetics, Inc.

Study Number: T-6412

Testing Period: April 1978

Report Submitted to Sponsor: June 1978

Materials and Methods:

The descriptions of the materials and methods used in this study were abstracted and paraphrased from the original report.

Cells (source not specified) were grown in Eagle's minimal essential medium (ENEM) supplemented with 10 percent fetal calf serum (FCS). Approximately 10^4 cells were seeded into a 25 cm^2 flask and incubated for 24 hr in EMEM to permit attachment. After the cells were attached, the control and test chemicals were added to the appropriate plates. Eight to ten replicates per concentration level were prepared. 3-Methylcholanthrene (MCA) at 5 ug/mL was used as a positive control, and the test compound solvent (or vehicle) was used as an untreated control. A minimum of 4 concentrations of the test substance was examined in the evaluation. Dose levels tested were: 0.078, 0.156, 0.312, 0.625, or 1.25 ug/mL. The selection of dose levels was based on preliminary cytotoxicity tests measuring the relative cloning efficiency of 3T3 cells over a wide range of chemical concentrations. Chemical exposure was for 72 hr at 37 °C in 5 percent CO_2. No metabolic activation system was implemented in this study.

Incubation

After exposing the cells with the control and test chemicals, the flasks were washed free of the compound and replenished with fresh (EMEM) medium containing 5 percent FCS. The flasks were then incubated an additional 3 to 4 weeks with twice weekly medium changes. Flasks were monitored daily for cell integrity and the formation of foci.

Scoring

After incubation (expression period), the medium was aspirated from the cell layer and the cells were washed with buffered saline. The plates were stained with Giemsa, washed, air-dried, and examined for darkly stained foci. All potential foci (type not specified) were examined microscopically. The results are presented as the number of foci per set of replicate plates at each concentration and a mean value was calculated.

Results:

In the present study, N-521 was used at 5 different concentrations (0.078, 0.156, 0.312, 0.625, and 1.250 ug/mL) and each concentration was incubated with BALB/3T3 test cells for 72 hours at 37 °C. These concentrations did not result in any significant cell toxicity. Higher dose levels (2.5 and 5.0 ug/mL) used in a preliminary toxicity test have shown significant cytotoxicity (data not reported by authors).

The results of the morphological transformation assay were considered negative by the authors. None of the test concentrations increased the number of transformed clones per dish and they were comparable to the results obtained from the solvent (DMSO) control. The chemical 3-methylcholanthrene, used in this assay as a positive control, induced a significant increase compared to the negative (DMSO) control (approximately 3-fold higher) in the number of transformed foci per dish.

Conclusions:

Under the conditions of this study, N-521 did not induce morphologic transformation in BALB/3T3 cells.

Classification:

The present study is classified as Unacceptable mainly due to the failure of the authors to carry out a metabolic activation assay.

Subject: Evaluation of N-521 in the Morphological Transformation of BALB/3T3 Cells

Test Material: N-521 Technical

Accession Number: 251207

Sponsor: Stauffer Chemical Company

Testing Facility: Stauffer Chemical Company

Study Number: T-10137

Testing Period: September 1979 to April 1980

Report Submitted to Sponsor: December 1980

Materials and Methods:

The descriptions of the materials and methods used in this study were abstracted and paraphrased from the original report.

The 1-1 subclone of a clone A-31 of BALB/3T3 mouse cells was obtained from Dr. Takeo Kakunaga of the NCI. Stocks were maintained in liquid nitrogen and laboratory cultures were checked periodically to ensure the absence of mycoplasma contamination.

Cultures were grown and passaged in Eagle's Minimal Essential Medium (EMEM) supplemented with 10 percent fetal bovine serum, glutamine (2 mM), penicillin (100 units/mL), and streptomycin (100 ug/mL).

Control Substances

Medimum (EMEM), solvent/vehicle (DMSO), and positive controls (3-methylcholanthrene) were included in transformation assay procedures as reference points.

Experiment Design

a. Dose Selection - Toxicity Test

The toxicity test was used to select doses for the transformation assay. Ten dose levels were chosen, starting with a maximum final concentration of 3 mg/mL and decreasing in threefold dilution steps. Three culture dishes were used per dose. Dishes were seeded approximately 24 hours before dosing with 200 cells per dish. Cells were exposed to the test substance for three days, then washed and incubated in growth medium for an additional 6 to 10 days. When the colonies were of good size, the

plates were fixed, stained, and counted. A relative survival for each dose was obtained by comparing the number of colonies surviving treatment to the number of colonies in solvent/vehicle control dishes.

b. Transformation Assay (Without Metabolic Activation)

Cells were plated at a density of 1 x 10^4 cells/T-25 flask approximately 24 hours prior to treatment. Fifteen flasks were treated for each of the five doses of the test substance, a positive control, and two negative (solvent/vehicle and medium) controls. After a 3-day exposure period, the cells were washed and refed. Flasks were refed twice weekly for 4 to 6 weeks. The plates were then fixed, stained and counted for the number of foci/flask.

Results:

N-521 was evaluated for its ability to induce morphological transformation of BALB/3T3 cells in the absence of metabolic activation systems. In an initial toxicity range finding assay, N-521 was used at dose levels ranging from 0.15 ug to 3,000 ug/mL. As judged by the reduction in clonal survival, all dose levels used resulted in some degree of toxicity to the cells. Hence a dose range of 0.025 to 0.4 ug/mL was selected for the transformation assay. The results of this assay show that none of the doses used resulted in transformed foci.

Conclusions:

N-521 has no tranforming activity under the conditions of the BALB/3T3 morphological transformations assay in the absence of metabolic activation.

Classification: Unacceptable