



MANUFACTURING CHEMISTS ASSOCIATION

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Handwritten:
E. J. Huggins
5/1/75

November 21, 1974

To: Technical Task Group on Vinyl Chloride *Res*

Subject: Test Protocols for Consumer Product Safety
Commission Studies on Vinyl Chloride

Gentlemen:

Distributed herewith are copies of the subject test protocols, and Dr. Hehir's letter of November 15, 1974, soliciting review and comments. Please note the December 15 deadline for their receipt. You are free to make your comments directly to Dr. Hehir, or if you wish, MCA can forward them without identification of the sources.

Sincerely,

Kenneth D. Johnson, Ph.D.
Technical Project Manager
Vinyl Chloride Research

KDJ/mb

Enclosures

cc: Mr. A. W. Barnes
D. P. Duffield, M.D.
Dr. Tiziano Garlanda

RSV 0001124

CONSUMER PRODUCT SAFETY COMMISSION
WASHINGTON, D.C. 20207

NOV 1974

November 15, 1974

Dr. Kenneth D. Johnson
Manufacturing Chemist's Association
1825 Connecticut Avenue N.W.
Washington, D. C. 20009

Dear Dr. Johnson:

The Consumer Product Safety Commission is about to initiate its studies on vinyl chloride. The testing protocols (copy attached) were developed by the Bureau of Biomedical Science, CPSC, and the technical staff of Edgewood Arsenal. I am submitting them for your information, review, and comment.

If you have any comments or questions about the protocols we would like to receive them in writing before December 15, 1974.

Thank you for your cooperation in this matter.

Sincerely,



Robert M. Hehir
Director
Bureau of Biomedical Science
5401 Westbard Avenue
Bethesda, Maryland 20207

Enclosure

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FOR INTERNAL USE

TEST PROTOCOL FOR TOXICOLOGICAL
TESTING OF VINYL CHLORIDE MONOMER

CONSUMER PRODUCT SAFETY COMMISSION

1 NOVEMBER 1974

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It is recognized that even though a chemical alters the structure of chromosomes, it in itself may not constitute a mutagenic effect but a cell killing effect. Therefore, following a conversation with Dr. Bruno Papirmeister of Edgewood Arsenal (he is also a member of the Environmental Mutagen Panel) we decided to investigate the possibility of performing tests specifically designed to detect effects on VC on genes. The two tests that Dr. Papirmeister mentioned were: 1) the use of the Ames strains of bacteria to shed light on either an alteration of base pairing on DNA or an effect on the repair systems of DNA by VC, and 2) the use of a mouse lymphoma cell line to determine the effect of VC on a mammalian cell system. The specifics of these tests would be elaborated upon further by Dr. Papirmeister pending acceptance and funding.

Daily observations will be made on all animals for toxic signs and weekly body weights will be made.

STUDY II. REPRODUCTION STUDIES

A. Teratology Screening Program in Rats

1. The following experimental procedure is proposed as a model protocol for testing compounds for possible teratogenic effects.

2. In-Life Methods:

a. Cohabitation: Females selected for mating will be placed with male rats on day 0 in a 2:1 ratio. They will remain with the male for four days (length of rat estrus cycle) at which time the males will be removed.

b. Assignment to Group: Each cage of females will be divided randomly into six groups explained in the following experimental design.

EXPERIMENTAL DESIGN

Group	Compound	Dose	Number of Mated Females	Remarks
I		Control	20	Fetuses to be examined
II		Low	20	for visceral and skeletal
III		High	20	malformations at Day 20.
IV		Control	10	Females allowed to whelp
V		Low	10	and fetuses examined grossly,
VI		High	10	sexed, weighed and crown-rump lengths measured.

Dosage: Daily dosage will be by aerosol, whenever practical, from day 6 through day 17 of gestation. Other routes of administration will be used when the physical properties of the compound warrants such exposure.

Gross Necropsy: Scheduled sacrifices, moribund animals, and spontaneous deaths.

Dams which Abort or Deliver Early: Sacrifice on day clear signs of abortion or early delivery are observed earlier than 19 days discarded, abnormal appearing fetuses obtained earlier than 19 days fixed in 10% buffered neutral formalin, and those obtained day 19 or later cleared and stained.

3. Post-Mortum Methods: All surviving females in Groups I, II, and III.

Sacrifice: Day 20 of pregnancy

Sacrifice Agent: Ether

Examination of Reproductive System:

Uterus: (number for each horn)

Live fetuses

Dead fetuses

Implantation sites

Ovaries: (number of corpora lutea of pregnancy per ovary)

Examination of Fetuses:

All fetuses: External examination for malformations.

Approximately 2/3 of fetuses: Examine skeletons for anomalies and ossification variations after alizarin red staining.

Approximately 1/3 of fetuses: Examine Bouin's fixed fetuses under 5X and 20X magnification for neural and visceral defects after serially sectioning by the slicing technique of Wilson.

4. Post Partum Examination: All females that whelp in groups IV, V, and VI.

Examination of fetuses: External examination for malformations, and external sex determination. Weigh and measure crown-rump distance (from the frontal-parietal suture to base of tail at 24 hours post partum).

STUDY I. LIFETIME CANCER STUDY IN RATS AND MICE

A. Protocol

Respective groups of 90 male and 90 female rats and mice will be exposed once to concentration levels of 50, 500, 5000, and 50,000 ppm of vinyl chloride for one hour. Twenty males and 20 females of each species will be sacrificed before exposure to establish blood chemistry and pathology base lines and 90 males and 90 females of each species will serve as negative experimental controls. No positive controls will be carried. The exposure chamber used for testing will be precalibrated for each exposure level.^{1/} Airflow will be regulated, producing stable, dynamic exposure conditions. Following calibration of the chamber, animals will be exposed to the required concentration. Continuous sampling will be performed during each exposure to determine the concentration of airborne VC.

The exposure and sacrifice schedules area as follows:

SACRIFICE SCHEDULES

Time of Sacrifice (Months)	Exposure Concentration (ppm)									
	Control		50		500		5000		50,000	
	M	F	M	F	M	F	M	F	M	F
0	20	20								
8	20	20	20	20	20	20	20	20	20	20
16	20	20	20	20	20	20	20	20	20	20
24	50	50	50	50	50	50	50	50	50	50
TOTALS	110	110	90	90	90	90	90	90	90	90

A complete gross pathological examination will be performed on each negative control and exposed animal. Light microscopic examination will be made of the following tissues: Lung and trachea, heart, liver, stomach, small intestine, large intestine, spleen, kidneys and bladder, bone marrow (sternum), adrenals, pancreas and duodenum, brain (2 sections), pituitary, spinal cord (cervical), skin, thyroid, uterus-ovaries, eye, muscle, and bone femur.

Particular emphasis will be placed on examination of brain, lung and liver tissues and the Zymbal gland in the rodent ear.

1/ NOTE: Appropriate methods of dispersion will be used. NCI and OSHA regulations will be observed throughout.¹⁰⁴

Blood Chemistry

This phase of the study will be limited to certain blood chemistry values related to liver damage to include γ -glutamic transpeptidase, alkaline phosphatase, serum glutamic pyruvic transaminase, ornithine carbamyl transferase, direct and indirect bilirubin. Mice and rats will be divided into control and test at the 5000 exposure level initially for one hour. Blood samples will be taken at one day, two days, four days, seven days, 14 days and 21 days, from 240 rats and 240 mice. Samples from rats will be run individually, those from mice pooled from three animals--totaling 840 analyses. Statistical analyses for significant differences will be performed at the t .05 level for each parameter measured. If positive results are found, the experiment will be repeated at the 500 ppm level for one hour. If no effects are found then multiple exposures will be considered.

Liver Microsomal Enzymes

As per agreement with Dr. Andrew Ulsamer, CPSC, the liver microsomal enzyme study on levels of NADPH cytochrome reductase, cytochrome P-450 and aminopyrine demethylase activities will be done on an intra-laboratory basis on selected animals as a pilot study. The animals chosen will be those showing the greatest blood enzyme changes. If positive results are found these will be incorporated into the assays performed by Edgewood. Microsomes for these assays can be isolated by Edgewood by standard procedures. Ten males and 10 females will receive a single dose of 400 μ l CCl₄/100 g body by stomach intubation in mineral oil (Glencle, Biochem Pharm. 23 1697, 1972).

Metabolic Fate of Vinyl Chloride

¹⁴C labeled vinyl chloride will be injected (subcutaneously) into 10 rats (five males and five females) at sufficient levels for easy detection. The rats will be housed individually in Delmar glass metabolism cages. The amount of labeled vinyl chloride excreted in urine, feces and expired air, over a 72 hour period will be monitored. ¹⁴C counting will be done using a Packard Liquid Scintillation Counter. An equal number of control rats will be run for baseline count. In addition, individual tissue, including liver, brain, blood, and kidneys, will be run. If a tissue stored is well labeled, individual protein, lipid and nucleic acids will be isolated and counted. This last test can be done by CPSC.

Mutagenicity Testing of Vinyl Chloride at Edgewood Arsenal (See Appendix I)

Concerning the testing of vinyl chloride (VC) for chromosomal aberrations, 10 rats per concentration (highest first) would be randomly selected for bone marrow study and testes would be processed from the males of this group for effects on the spermatocyte chromosomes.

5. Post-natal Evaluation (Offspring of Groups IV, V, VI) During Lactation Litter Observations:

Pups observed: Daily record presence of dead pups.

Pups counted: Days 0-4 (daily), 14 and 21.

Pups sexed and weighed: Males and females for each litter on days 4, 7, 14, and 21.

Dead pups: Examine externally, necropsy to maximum extent possible, check for presence of milk and weigh only if found dead on days 4, 7, 14, and 21.

Death of entire litter prior to day 21: Sacrifice dam, save mammary tissue in buffered neutral formalin. Subsequent histological processing and microscopic examination of mammary tissue will be performed only with the understanding that lactation can be classified as present or absent, but not evaluated quantitatively or as to sufficiency to support pups.

Dead or Moribund Dams: Post mortum examinations of dams. Sacrifice surviving pups, and use same procedure as for dead pups.

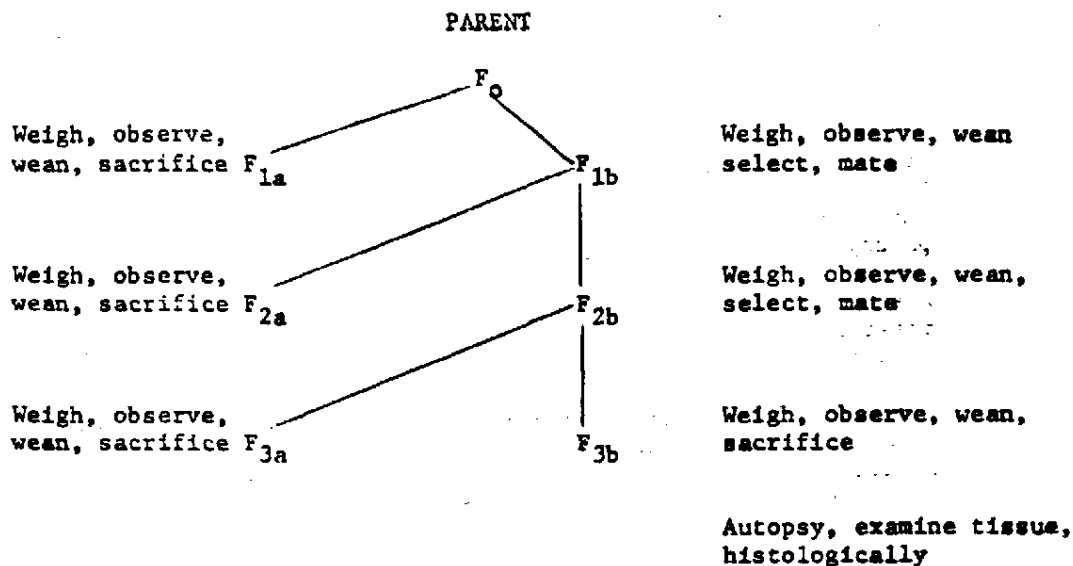
6. Post-Weaning Evaluation (Offspring of Groups IV, V, VI)

Wean date: Day 21 of lactation.

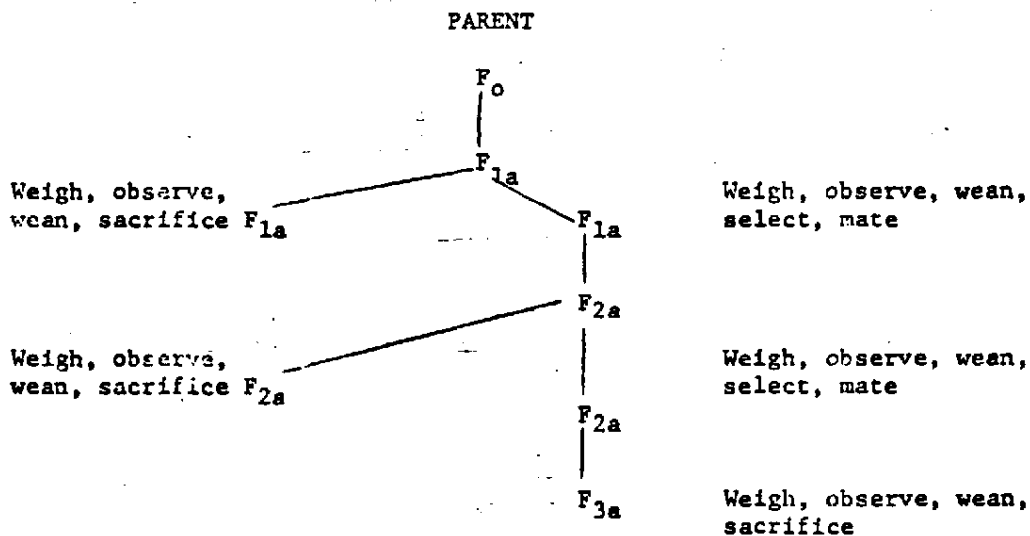
Post-weaning observation: 10 males and 10 females F₁ offspring randomly selected from each group will be held until two years of age for carcinogenic evaluation.

STUDY III. MULTI-GENERATION REPRODUCTION (See attached Bibliography, Appendix II)

The three generation reproduction study was described in the "Procedures for Appraisal of the Toxicity of Chemicals in Foods" by the Staff of the Division of Pharmacology, Food and Drug Administration in 1949.^{1,2,3} The same agency further described this test in 1955 and 1959.^{4,5} The report of the Science Advisory Committee to the President of the United States 1963⁶, recommended that a multi-generation test be used in toxicity studies on pesticides. The multi-generation test was described by Fitzhugh.¹ The general plan for the multi-generation test is as follows:



The three generation test has been modified for our screening procedures. The F_{1a} litter is split into two groups and utilized in the following general plan.



TEST PROTOCOL

EXPERIMENTAL DESIGN

GROUP	COMPOUND	DOSE	NO. OF MALES	NO. OF FEMALES
I		Control	15	15
II		Low	15	15
III		High	15	15

a - Extra males and females are started on treatment to give greater assurance that 10 and 10 respectively will survive until the mating period.

1. General Methods

Assignment to Group: Prior to treatment the males and females are randomly assigned to groups.

Dosage: Males should begin treatment at least 60-days, and females at least 15 days prior to mating to ensure at mating that all gametes have developed under full influence of the compound.

Frequency: Daily until mating.

Route of Administration: Inhalation of aerosol.

Physical Observations: Daily for signs of toxic effects and mortality.

2. Cohabitation

Number of Animals: Ten males and ten females per group are selected for mating.

Procedure: One male and one female are caged together for mating. They are allowed to remain together until the 21st day of lactation.

3. Gestation

Dead or Moribund Dams: Examine uterine contents and count implantation sites.

Pre-Delivery Preparation: Examine daily for signs of parturition.

4. Lactation

Day 0 of Lactation: Day on which all pups have been delivered.

Litter Observations:

Pups Observed: Daily, record presence of dead pups.

Pups sexed and weighed: Days 0, 4, 7, 14, and 21.

Dead pups: Examine externally, necropsy to maximum extent possible, check for presence of milk.

Death of Entire Litter Prior to Day 21: Sacrifice dam, save mammary tissue for microscopic examination.

5. Weaning

All pups will be weaned on Day 21 of lactation. Forty-five males and 45 females will be selected randomly to be used as parents of the F₂ generation. They will be mated at 12 weeks of age. The same procedure as outlined above in 3, 4, and 5 will be followed through the F₃ generation.

6. Autopsy Procedures

Ten males and ten females from each dose group and control group will be sacrificed at day 21 of gestation. This procedure will be followed with each of the three generations. A gross necropsy will be performed to evaluate any anomalies or gross pathological changes present in the offspring.

7. Carcinogenic Evaluation of F₀ Breeders

The F₀ breeders will be held until they are 24 months of age for observation of the development of tumors. They will be autopsied at 24 months of age. A complete autopsy, both gross and microscopic, will be performed at this time.

APPENDIX I
MUTAGENICITY TESTING OF VINYL CHLORIDE

Detection of DNA-damaging and Mutagenic Chemical Compounds In Vitro.

A. Repair Test:

This test utilizes six strains of E. coli which differ significantly in their ability to repair damage to their DNA. The test is performed as follows:

Small, known amounts of the test compound (in solution or in neat form) are added to small filter disks which are then placed in the center of petri dishes which have been seeded with the indicator bacteria. The six test strains utilized include the three repair-capable strains of E. coli (polA+, hcr+, and rec+) and three incapable strains are compared. A test compound is deemed to have DNA-damaging properties if the ratio of the diameter of growth inhibition for repair-incapable strains/diameter of growth inhibition for repair-capable strains is significantly greater than one.

B. Mutation Test:

The mutagenic activity of a chemical will be examined in Salmonella typhimurium according to procedures described by Bruce Ames coworkers^{1,2}. A set of tester strains harboring specific alterations in the histidine locus is utilized and the reverse mutation frequency to histidine independence is determined. The sensitivity of the assay is greatly enhanced by introduction of the mutations into these tester strains - elimination of excision repair capability and

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loss of the cell wall lipopolysaccharide which allows more ready penetration of chemicals across the cell membrane. Since some compounds require metabolic activation to express their mutagenic potential, additional tests will be performed with test compounds which are incubated with liver homogenates supplemented with appropriate cofactors. Analysis of mutagenic activity will include an examination of dose-response curves and a comparison with known mutagens (e.g. alkylating agents, frameshift mutagens, etc.).

Detection of Mutagens by a Host-Mediated Assay Employing the L5178Y Murine Leukemia^{3,4}

Studies involving somatic mammalian cells employ the murine lymphoblast L5178Y. These tumor cells require an exogenous supply of the usually non-essential amino acid asparagine for growth. The cell's capacity to grow in an asparagine deficient environment is the biochemical-genetic marker which discloses a mutation occurring after treatment of the host animal with a suspected mutagen.

The cells are implanted in the peritoneal cavity of BDF1 male mice, in which they grow as an ascites tumor. The mouse host provides the milieu in which an injected chemical compound can undergo activation, detoxification, storage or excretion. Such biotransformation is a very important consideration if the tests are to have application to effects on man. We have established methods for the large-scale production and cloning of the lymphoblast cells, and have developed suitable treatment schedules and routes of administration for the drugs and compounds. The cells have a doubling time of approximately 12 hours whether grown in the mouse peritoneal cavity or in the cell culture. The spontaneous mutation

frequency at the asparagine locus was found to be 1-5 mutated cells per million, a value similar to other known mutation frequencies. Mice bearing the lymphoblast ascites cells are treated 4 days after inoculation by an alternate route with the chemical in question and after 3 additional days of growth the cells are harvested and subcultured in vitro in the presence and absence of asparagine. The mutation frequency is the average of three values for each dose and is expressed as the number of colonies formed in the absence of asparagine per million viable cells, the indication of mutagenic potency being provided by a dose-response curve. Comparison of the mutation frequencies induced in vivo and in vitro exposures provides a measure of the effects of host metabolism. Thus, it is not necessary to identify and study separately all of the possible metabolites of a chemical in order to obtain evidence of mutagenic activity in a compound. In addition changes in activity attributable to different routes and/or schedules of administration are readily detected by the test.

Cytogenetic Effects in Somatic Cells

The host-mediated assay employing mammalian cells has the advantage over the corresponding bacterial assay in that it permits simultaneous analysis of induced chromosomal aberrations. In a typical protocol both the L5178Y and bone marrow cells harvested from animals 24 hours after exposure. Cells of each kind are incubated in appropriate media for 4 hours and slides are prepared from the resulting culture. For each condition, at least 200 metaphase cells are analyzed for chromosomal

anomalies. The aberrations scored include the presence and proportion of breaks, gaps, deletions, or fragmentations, and whether fragments are of the chromated or isochromated type. The presence of exchanges, rings, dicentrics, and the proportion of cells with multiple anomalies are also ascertained. Complete karyotypes and model numbers for the L5178Y/ASN and mouse bone marrow cells have been determined and the presence of a marker chromosome in the former has been noted. To date, the cytogenetic effects of several known mutagens and unknowns have been investigated and the validity and sensitivity of the assay have been confirmed.

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APPENDIX II

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VINYL CHLORIDE TEST SCHEDULE - FY 75 - FY 78

Test Phase	FY 75 (QTR)				FY 76 (QTR)				FY 77 (QTR)				FY 78 (QTR)			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
I. Lifetime cancer study in rats and mice																
Biochemical determinations																
Metabolic fate of vinyl chloride																
Detection of DNA damaging & mutagenic chemical compounds <u>in vitro</u>																
Host mediated assay																
Cytogenetic effects																
II. Teratology																
III. Multigeneration																

Note: 1. Accomplished by Edgewood Arsenal or Consumer Product Safety Commission
2. With cancer evaluation
3. Two month exposure

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