ENVIRONMENTAL PROTECTION AGENCY

40 CFR Part 799

[OPPTS–42193; FRL–5719–5]

RIN 2070–AB76

Toxic Substances Control Act Test Guidelines

AGENCY: Environmental Protection Agency (EPA).

ACTION: Final rule.

SUMMARY: This rule establishes 11 Toxic Substances Control Act (TSCA) health effects test guidelines in the Code of Federal Regulations (CFR). Establishment of these guidelines is necessary to ensure enforceable test standards in test rules promulgated under section 4 of TSCA. Codification of this series of TSCA test guidelines does not by itself impose obligations upon any person. Obligations are only imposed when these guidelines are cross-referenced in a test rule promulgated under section 4 of TSCA.

DATES: This rule is effective on August 15, 1997.

FOR FURTHER INFORMATION CONTACT: Susan Hazen, Director, Environmental Assistance Division (7408), Office of Pollution Prevention and Toxics, Environmental Protection Agency, Rm. E-543B, 401 M St., SW., Washington, DC 20460; telephone: (202) 554–1404; TDD: (202) 554–0551; e-mail: TSCA-Hotline@epamail.epa.gov. For specific information regarding this action or related activities, please contact Roger Nelson, Chemical Control Division, OPPT; telephone: (202) 260–8163; e-mail: nelson.roger@epamail.epa.gov.

SUPPLEMENTARY INFORMATION: This final rule establishes a new series of TSCA test guidelines in the CFR.

I. Introduction

Section 4(b)(1)(B) of TSCA requires that test rules promulgated under the authority of TSCA section 4 include ''standards for the development of test data for such substance or mixture * * *.'' Test rules promulgated under TSCA section 4 must specify the standards for the development of data. Standards established in test rules for the development of data must specify how the study is to be conducted, what data will be collected, and how the data will be analyzed. The Agency has found that these specifications to a large degree can be standardized into a common set of protocols, or, as the Agency terms them, ''guidelines.'' These guidelines are organized by testing endpoint. Each test standard can modify these guidelines as needed for an individual test substance.

The Agency uses a system where standardized guidelines are organized by testing endpoint and codified in a subpart of this part. When a test rule is promulgated, the test standard specified in the test rule cross-references the guideline for the bulk of the testing requirements. In this context, the public is given notice of, and an opportunity to comment on, the guidelines as they are applied in chemical-specific test rules. This approach eliminates the need to repeat the same test specifications for each substance-specific test rule since most of the specifications for testing do not change across substances. The test specifications in a guideline can be varied, when necessary, to the specific requirements of a test rule by language in the test rule itself.

In 1985, the Agency established a set of TSCA test guidelines in 40 CFR parts 795 through 798 (50 FR 39252, September 27, 1985). These guidelines were established as standardized protocols for laboratory testing of an effect or characteristic deemed important for the evaluation of health or environmental hazards of a chemical. Standardized guidelines are necessary for the establishment of enforceable test standards in test rules promulgated under section 4 of TSCA.

The Agency has over time amended and improved these guidelines (52 FR 19072, May 20, 1987). In order to reduce the text of the CFR, the Agency deleted those guidelines which had not been cited in any test rules (60 FR 31917, June 19, 1995 (FRL–4955–2)).

II. OPPTS Harmonized Test Guidelines

EPA is undertaking a comprehensive modification, or harmonization, of its pesticides and toxics guidelines for testing of health effects, environmental effects, and chemical fate. The rationale for this harmonization is to incorporate state of the art science, and to minimize variations among the protocols contained in:

1. Test guidelines developed by the EPA Office of Pesticide Programs (OPP), which appeared in publications of the National Technical Information Service.

2. The series of TSCA test guidelines established in 1985, which are contained in 40 CFR parts 795, 796, 797, and 798.

3. Guidelines published by the Organization for Economic Cooperation and Development (OECD).

Harmonization operates as follows: EPA scientists develop guidelines (or modify existing guidelines) for specific endpoints. The new or rewritten guidelines are reviewed by other Agency experts and, in some instances, presented at domestic and international colloquia to solicit the views of recognized experts and the regulated community. The draft harmonized guidelines are made available as public drafts. A notice is published in the **Federal Register** announcing their availability and soliciting public comment.

Seven of the 11 health effects test guidelines that are being codified in subpart H of 40 CFR part 799 have their origin in this harmonization process. A notice was published in the **Federal Register** of June 20, 1996, (61 FR 31522 (FRL–5367–7)) announcing the availability of the proposed test guidelines for Series 870—Health Effects Test Guidelines and soliciting public comment. Comments were received, and a meeting of the Agency's Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP) was held on October 29 and 30, 1996. The SAP, an advisory committee consisting of scientific experts both inside and outside the U.S. Government, reviewed the guidelines and made comments. The Agency reviewed these comments in developing the harmonized health effects guidelines.

Four of the 11 guidelines (§ § 799.9510, 799.9530, 799.9538, and 799.9539) were initially developed by the OECD.

III. TSCA Test Guidelines

Harmonization has resulted in significantly improved guidelines. However, creating a single set of guidelines which can be used by both OPP, in its administration of the FIFRA and the Federal Food, Drug and Cosmetic Act (FFDCA), and the Office of Pollution Prevention and Toxics (OPPT), which administers TSCA presented certain challenges.

Under FIFRA, test guidelines are used in an interactive process between the Agency and registrants seeking registration of pesticides or food residue tolerances. Flexibility to tailor required testing to individual circumstances is critical, and the Agency has considerable discretion to determine whether submitted test results are adequate to support the requested action. Under this scheme, registrants have an intrinsic motivation to conduct well-grounded testing. Thus, pesticide testing protocols tend to have few absolute requirements specifying the details of the conduct of the testing.

By contrast the Agency is required under section 4 of TSCA to impose prescriptive test requirements by notice and comment rulemaking. Rules

promulgated under section 4 of TSCA specify classes of affected parties, usually manufacturers and processors of the chemical being specified for testing, rather than interacting with companies on an individual basis. These rulemakings typically take years to complete. Without initiating another rulemaking process, the Agency has the ability to require further testing only if the tests were not conducted in accordance with the procedures specified in the test rule. In addition, the Agency has an alternative process of negotiating TSCA testing requirements via enforceable consent agreements (ECAs), but these agreements require the consent of all the parties involved.

Under TSCA section 4 enforceable test standards, much in the conduct of these test protocols is left to the judgment of those professionals conducting the testing. EPA believes that certain provisions must be mandatory whenever the guidelines are cross-referenced in specific test rules.

Therefore, the Agency has used the OPPTS harmonized health effects test guidelines developed using the public notice and comment process described in Unit II. of this preamble as well as certain OECD guidelines to create the TSCA-specific test guidelines which are the subject of this rule. Future TSCA section 4 test rules will cross-reference part 799 guidelines rather than the older, 1985 non-harmonized guidelines in 40 CFR parts 795 through 798. The only significant difference between the TSCA test guidelines and the OPPTS harmonized test guidelines is that certain recommended procedures in the OPPTS harmonized test guidelines are made mandatory (i.e., the guideline states that they ''shall'' be carried out).

IV. Codification in 40 CFR Part 799

The Agency had originally planned not to publish the guidelines in the CFR, but to instead make the guidelines available via other means (such as the Internet) and reference the guidelines in specific test rules using the incorporation by reference procedures provided by 5 U.S.C. 552(a)(1)(E) and 1 CFR part 51. In the **Federal Register** document proposing the TSCA section 4 test rule for 21 hazardous air pollutant substances (HAPs) (61 FR 33178, 33187, June 26, 1996 (FRL–4869–1)), the Agency stated that it was considering using incorporation by reference. Subsequently, however, the Director of the Office of Federal Register advised EPA that the planned TSCA section 4 process for guideline incorporation was not eligible for incorporation by reference under 1 CFR part 51. Therefore, the Agency finds it necessary to codify a separate set of TSCA test guidelines into the CFR. As discussed in this preamble, the TSCA guidelines are essentially those resulting from the harmonization process with minor changes to promote enforceability. EPA has elected to codify these new guidelines in part 799 so as to distinguish them from the pre-harmonization guidelines in 40 CFR parts 795 through 798.

These guidelines will be placed in a new subpart H of part 799. In addition, EPA plans to reserve additional subparts of part 799 for test guidelines, so that the structure of part 799 would be as follows:

Subpart A—General Provisions

Subpart B—Specific Chemical Test Rules

Subpart C—Testing Consent Orders

Subpart D—Multichemical Test Rules

Subpart E—G [Reserved]

Subpart H—Health Effects Test Guidelines

The TSCA test guidelines currently in 40 CFR parts 795 through 798 will be retained for so long as there exist test rules whose data reimbursement periods under TSCA section 4(c) have not expired and which cross-reference the guidelines.

This table identifies the TSCA test guideline number with its comparable OPPTS harmonized test guideline public draft.

TABLE 1.—TSCA Test Guidelines Cross-Referenced to the OPPTS Harmonized Test Guidelines

Guideline title	TSCA 40 CFR section	OPPTS harmonized test guideline (public draft)
TSCA acute inhalation toxicity with histopathology	799.9135	870.1350
TSCA subchronic inhalation toxicity	799.9346	870.3465
TSCA prenatal developmental toxicity	799.9370	870.3700
TSCA reproduction and fertility effects	799.9380	870.3800
TSCA carcinogenicity	799.9420	870.4200
TSCA bacterial reverse mutation test	[1]799.9510	[1]OECD 471 and 472
TSCA in vitro mammalian cell gene mutation test	[1]799.9530	[1]OECD 476
TSCA mammalian bone marrow chromosomal aberration test	[1]799.9538	[1]OECD 475
TSCA mammalian erythrocyte micronucleus test	[1]799.9539	[1]OECD 474
TSCA neurotoxicity screening battery	799.9620	870.6200
TSCA immunotoxicity	799.9780	870.7800

[1]The four TSCA genetic toxicity testing guidelines were adopted from the OECD guideline series and not the OPPTS public drafts.

Codification of these guidelines does not itself impose any obligations on any person. Obligations are imposed only when the guidelines are cross-referenced in individual TSCA section 4 rulemakings. When cross-referenced in such test rules, the pertinent TSCA guidelines serve as test standards for only these particular section 4 rules. EPA may propose modifications to the various guidelines as they are utilized for chemical-specific test rules. In each chemical-specific test rule, the proposed test standards and any modifications thereto will be subject to public notice and comment.

V. Guideline by Guideline Discussion

In this unit is a summary of the significant changes made to the 11 harmonized guidelines proposed on June 20, 1996, which are being published in this document.

A. Section 799.9135 TSCA Acute Inhalation Toxicity with Histopathology

1. EPA dropped the requirement for a 1-hour (hr) exposure test. The Agency recognizes that such a technically difficult test would not be likely to yield useful information due to complicating factors such as biological rhythms and inapplicability to insoluble or chemically inactive particulates. Instead, EPA is requiring a 4-hr exposure point with a trigger for an 8-hr exposure point. Test sponsors have

the option to extrapolate from shorter-term exposures.

2. EPA dropped the requirement for performing histopathology in all animals and substituted a triggered approach (wherein gross pathology will be performed only when the frequency and severity of adverse effects for dosed animals are greater than those for control animals in the study).

3. EPA dropped the requirement of a breathing zone purity determination as unnecessary since the Agency now believes that standard inhalation toxicology will provide the purity measurement of the test substances.

4. EPA requires only a single control group in some circumstances. If both 4- and 8-hr exposures are being conducted in the study, then there would be a single control at the 8-hr exposure provided adequate historical control data show no changes in histopathology or bronchoalveolar lavage between controls for these test periods. If the 8-hr exposure is being performed as a result of the 4-hr trigger, there would need to be control groups for both 4- and 8-hr exposure groups.

5. EPA redefined the test exposure to 4 hrs of exposure to the target concentration as defined by an average of plus or minus 5% for gases and plus or minus 11% for particles. This redefinition establishes exposure tolerances, which better assures known test concentration than the original provision which only allowed for test exposure after the test chamber reached equilibrium.

6. EPA now distinguishes air change requirements between nose-only exposure (300 milliter (mL)/minutes (min)/animal) and whole-body exposure (at least 12 to 15 air changes per hr).

7. EPA changed its description of the respiratory histopathology requirements to ensure that inflated state and fixed pressure with infusion fixation are used to prepare the lungs for examination.

8. EPA added the requirement to specify the anatomical location where the four sections are to be taken for nasal histopathology.

B. Section 799.9346 TSCA Subchronic Inhalation Toxicity

1. EPA changed the terms used for certain weekly observations from ''motor activity'' to ''level of activity'' and from ''grip strength'' to ''altered strength'' to reinforce the point that these observations need not be automated.

2. ''Dose'' and ''dose level'' were changed to ''concentration'' and ''dosing'' was changed to ''exposure'' to reflect that this is an inhalation study.

C. Section 799.9370 TSCA Prenatal Developmental Toxicity

EPA made no significant changes to this guideline.

D. Section 799.9380 TSCA Reproduction and Fertility Effects

1. EPA added the requirement for a triggered quantitative evaluation of primordial follicles from qualitative evidence of a possible treatment-related effect. While the Agency recognizes that there are issues concerning the validity of existing methods used to screen ovarian-primordial follicle counts, the Agency believes that the necessity to identify early senescence in females outweighs these concerns. EPA considers data about the effects of chemical substances on effects such as early female senescence to be essential to protecting human health.

2. EPA reduced the requirement for taking organ weights for pups already opened for necropsy. The guideline only requires organ weight data from one randomly selected pup/sex/litter rather than the three pups specified in the public draft. The Agency believes that collection of organ weight data from one pup/sex/litter rather than three will reduce burdens without compromising the ability to detect a treatment-related effect on brain, spleen, or thymus weight. The random selection is to be made from the population of pups already opened for necropsy.

3. EPA reduced the requirement that 20 adult animals per sex per exposure group be examined for histopathology to 10 animals (randomly chosen) per sex per exposure group. This reduction was made because there would be little additional statistical value in examining more than 10 animals per sex per group. Since the guideline still requires that gross necropsy and organ weight data be collected for all parental animals and that the weighed organs be preserved, questions about interpretation of marginal histopathological effects can be resolved by evaluation of the tissues from these animals.

4. EPA dropped the requirement of histopathology of developmental anomalies observed macroscopically in F1 and F2 weanlings. Since the intent of this requirement was to confirm the nature of the lesions already identified macroscopically, the Agency believes that the added value of the information would not be worth the cost of the evaluation.

E. Section 799.9420 TSCA Carcinogenicity

1. EPA revised the guideline to allow 5-day per week dosing for both gavage and capsule administration. This change was made to eliminate the disparity between the original 7-day specification for capsules and 5 days for gavage since there was no justification for this disparity.

2. EPA changed the terms used for certain weekly observations from ''motor activity'' to ''level of activity'' and from ''grip strength'' to ''altered strength'' to reinforce the point that these observations need not be automated.

3. The requirement for the immunotoxicity screen has been deleted. The Agency agreed that the immunotoxicity screen conducted at study termination would provide little meaningful information on the potential toxicity of the chemical on the immune function system due to the geriatric changes in the animals.

4. EPA deleted the requirement for the weighing of spleens because their weight would be unacceptably variable due to the amount of blood lost during the exsanguination process. (The weighing of spleens is still a requirement in the immunotoxicity guideline).

F. Genetic Toxicity Testing

1. *Section 799.9510 TSCA Bacterial Reverse Mutation Test.*

2. *Section 799.9530 TSCA In Vitro Mammalian Cell Gene Mutation Test.*

3. *Section 799.9538 TSCA Mammalian Bone Marrow Chromosomal Aberration Test.*

4. *Section 799.9539 TSCA Mammalian Erythrocyte Micronucleus Test.*

EPA is incorporating these genetic toxicity guidelines directly from the OECD versions. The Agency made format changes in order to ensure consistency with the TSCA test guidelines format. The Agency actively participated in international discussions regarding the development of these guidelines. EPA participated in the review of the OECD drafts. EPA believes that because these OECD guidelines were developed with international scientific input through the OECD guideline development process, they provide state-of-the-art guidance which is equivalent to and more broadly accepted than that in the OPPTS harmonized test guidelines public drafts published on June 20, 1996. The process EPA used in developing the four TSCA genetic toxicity test guidelines is described in reference 5 of Unit VI. of this preamble.

G. Section 799.9620 TSCA Neurotoxicity Screening Battery

EPA made no significant changes to the public draft of this guideline although EPA made two clarifications to address SAP concerns. Clarifications to the positive control treatment were made to indicate that such testing need not be done as frequently as every 12 months. Examples were eliminated to clarify EPA's position that permanently injurious chemicals are not necessary, though EPA continues to believe that chemical exposures are appropriate

H. Section 799.9780 TSCA Immunotoxicity

1. EPA incorporated the recommendation of the SAP that the requirement for flow cytometric analysis of lymphocyte and Natural Killer (NK) cell phenotypes be eliminated. A test for the primary antibody (IgM) response to sheep red blood cell (PFC) or enzyme linked immunosorbent assay (ELISA) would still be required. The guideline now sets the required exposure time for the anti-sheep red blood cells (SRBC) assay at 28 days, thus providing information on the effects of the test material on non-specific immunity.

2. EPA adopted the SAP recommendation to delete the ''optional immunotoxicity screen'' because lymphocyte phenotyping by flow cytometry should be an option.

3. EPA added the requirement that appropriate species-specific monoclonal antibodies be used in the phenotyping assay. The Agency accepts the SAP recommendation that this will allow sufficient flexibility to allow for future advances in flow cytometry and antibody marker technology.

4. EPA adopted the SAP recommendation that a minimum of eight animals per treatment group be used in order to yield a sufficient statistical power to detect a 20% change based upon the inter-animal variation usually encountered in these assays.

5. EPA added the intraperitoneal route of exposure to the guideline in response to the SAP comment that this is an acceptable method for immunization with SRBCs.

6. EPA adopted the SAP recommendation that testing laboratories need not perform a positive control after every experiment. Instead, it is sufficient to include this control every 6 months or whenever new reagents are titrated.

VI. Public Record

The official record for this rulemaking, as well as the public version, has been established for this rulemaking under docket control number OPPTS–42193 (including comments and data submitted electronically). This record contains the basic information considered by EPA in developing this rule. EPA will supplement this record as necessary.

A public version of this record, including printed, paper versions of electronic comments, which does not include any information claimed as Confidential Business Information (CBI), is available for inspection from 12 noon to 4 p.m., Monday through Friday, except legal holidays. The public record is located in the TSCA Nonconfidential Information Center, Rm. NE-B607, 401 M St., SW., Washington, DC 20460.

The record includes the following information:

1. Public drafts of seven OPPTS harmonized health effects guidelines.

2. Four OECD genetic toxicity test guidelines.

3. References contained in TSCA health effects test guidelines promulgated in this document.

4. Final report of the FIFRA Scientific Advisory Panel meeting, held October 29–30, 1996.

5. USEPA. Memorandum, Angela Auletta to Roger Nelson. HAPs Rule: OECD Process for Update of Genetic Toxicity Test Guidelines. March 10, 1997.

VII. Regulatory Assessment Requirements

A. Waiver of Notice of Proposed Rulemaking and Delay in Effective Date

Because the test guidelines codified in this document have no substantive effect on any person without further rulemaking, and such rulemaking would be conducted under public notice and comment procedures, EPA finds that public notice and comment are unnecessary for this action. Thus, this rule may be promulgated without prior opportunity for public notice and comment, pursuant to the Administrative Procedure Act, 5 U.S.C. 553(b)(3)(B), and may be made effective immediately, without a 30-day delay, pursuant to 5 U.S.C. 553(d)(3).

B. Executive Order 12866, Executive Order 12898, and Executive Order 13045

This action is not subject to Executive Order 12866 (58 FR 51735, October 4, 1993) since, as explained in Units I. and IV. of this preamble, the guidelines are not intended to have the force and effect of law until they are cross-referenced in future test rules through public notice and comment procedures that establish those rules. For the same reason, this action is not considered under Executive Order 12898 (59 FR 7629, February 16, 1994) as having a disproportionately high and adverse human health or environmental effect on minority populations and low-income populations. In addition, the action is not subject to Executive Order 13045 ''Protection of Children From Environmental Health Risks and Safety Risk'' (62 FR 19885, April 23, 1997) since it is neither economically significant under Executive Order 12866 nor does it concern an environmental health risk or safety risk that an agency has reason to believe may disproportionately affect children.

C. Paperwork Reduction Act

This rule does not contain information collection requirements that necessitate the approval of OMB under the Paperwork Reduction Act of 1980 (44 U.S.C. 3501 *et seq.*).

D. Regulatory Flexibility Act

The guidelines codified in this document do not constitute a rule for which EPA must publish a general notice of proposed rulemaking under 5 U.S.C. 553(b). Therefore, sections 603 and 604 of the Regulatory Flexibility Act, 5 U.S.C. 603 and 604 do not apply to this action.

E. Unfunded Mandates Reform Act

Title II of the Unfunded Mandates Reform Act of 1995 (UMRA), Pub. L. 104–4, which establishes requirements for Federal agencies to assess the effects of certain regulatory actions on State, local, and tribal governments and the private sector, does not apply. This action contains neither a private sector nor an intergovernmental mandate because it does not impose an enforceable duty on anyone. Furthermore, a written statement is not required under section 202 of UMRA because section 202 only applies to rules for which a general notice of proposed rulemaking was published, and no such notice was issued for this rule.

F. Submission to Congress and the General Accounting Office

This action is not a major rule as defined by 5 U.S.C. 804(2). Pursuant to 5 U.S.C. 801(a)(1)(A), EPA has submitted a report containing this rule and other required information to the U.S. Senate, the U.S. House of Representatives, and the Comptroller General of the General Accounting Office prior to its publication in today's **Federal Register**.

List of Subjects in 40 CFR Part 799

Environmental protection, Chemicals, Hazardous substances, Health, Reporting and recordkeeping requirements.

Dated: August 7, 1997.

Lynn R. Goldman,

Assistant Administrator for Prevention, Pesticides and Toxic Substances.

Therefore, 40 CFR part 799 is amended as follows:

PART 799—[AMENDED]

1. The authority citation for part 799 continues to read as follows:

Authority: 15 U.S.C. 2603, 2611, 2625.

2. By adding a new paragraph (d) to § 799.1 to read as follows:

§ 799.1 Scope and purpose.

* * * * *

(d) This part contains certain TSCA test guidelines which are cross-referenced in the test rules contained in this part.

3. By adding and reserving subparts E through G.

4. By adding a new subpart H, consisting of § § 799.9135–799.9780, to read as follows:

Subpart H—Health Effects Test Guidelines

799.9135 TSCA acute inhalation toxicity with histopathology.
799.9346 TSCA subchronic inhalation toxicity.
799.9370 TSCA prenatal developmental toxicity.
799.9380 TSCA reproduction and fertility effects.
799.9420 TSCA carcinogenicity.
799.9510 TSCA bacterial reverse mutation test.
799.9530 TSCA in vitro mammalian cell gene mutation test.
799.9538 TSCA mammalian bone marrow chromosomal aberration test.
799.9539 TSCA mammalian erythrocyte micronucleus test.
799.9620 TSCA neurotoxicity screening battery.
799.9780 TSCA immunotoxicity.

Subpart H—Health Effects Test Guidelines

§ 799.9135 TSCA acute inhalation toxicity with histopathology.

(a) *Scope.* This section is intended to meet the testing requirements under section 4 of the Toxic Substances Control Act (TSCA). In the assessment and evaluation of the potential human health effects of chemical substances, it is appropriate to test for acute inhalation toxic effects. The goals of this test are to characterize the exposure-response relationship for sensitive endpoints following acute exposure and to characterize toxicologic response following acute high exposures. The latter is of particular concern in relation to spills and other accidental releases. This testing is designed to determine the gross pathology and histopathology resulting from acute inhalation exposure to a substance. Because toxic effects on the respiratory tract are of particular concern following inhalation exposure, several indicators of respiratory toxicity consisting of histopathology on fixed tissue and evaluation of cellular and biochemical parameters in bronchoalveolar lavage fluid should be employed. The respiratory histopathology consists of specialized techniques to preserve tissues of the respiratory tract in order to allow detailed microscopic examination to identify adverse effects of chemical substances on this organ system. The bronchoalveolar lavage is designed to be a rapid screening test to provide an early indicator of pulmonary toxicity by examining biochemical and cytologic endpoints of material from the lungs of animals exposed to potentially toxic chemical substances. These acute tests are designed to assess the relationship, if any, between the animals' exposure to the test substance and to demonstrate relationship between the animals' exposure and the incidence and severity of observed abnormalities, including gross or histopathologic lesions, body weight changes, effects on mortality, and any other toxic effects. These acute tests are not intended to provide a complete evaluation of the toxicologic effects of a substance, and additional functional and morphological evaluations may be necessary to assess completely the potential effects produced by a chemical substance. Additional tests may include longer-term exposures, or more in-depth evaluation of specific organ systems as indicated by signs of toxicity following acute exposure.

(b) *Source.* This a new section developed by the United States Environmental Protection Agency.

(c) *Definitions.* The following definitions apply to this section.

Aerodynamic diameter (d_{ae}) refers to the size of particles. It is the diameter of a sphere of unit density that behaves aerodynamically (has the same settling velocity in air) as the particle of the test substance. It is used to compare particles of different size, shape, and density, and to predict where in the respiratory tract such particles may be primarily deposited.

Exposure response is the relationship between the exposure concentration and the measured toxic response, whether expressed as a group mean (± standard deviation) in the case of a continuous variable or as incidence in the case of a quantal variable. This definiton should not preclude the exploration of other dose metrics in establishing this relationship.

Geometric standard deviation (GSD) is a dimensionless number equal to the ratio between the mass median aerodynamic diameter (MMAD) and either 84% or 16% of the diameter size distribution (e.g., MMAD = 2 μm; 84% = 4 μm; GSD = 4/2 = 2.0.) The MMAD, together with the GSD, describe the particle size distribution of an aerosol. Use of the GSD may not be valid for non-lognormally distributed aerosols. (If the size distribution deviates from the lognormal, it shall be noted).

Inhalability is the ratio of the number concentration of particles of a certain aerodynamic diameter, d_{ae}, that are inspired through the nose or mouth to the number concentration of the same d_{ae} present in the inspired volume of ambient air. In humans, inhalability can exceed 15 μm d_{ae}, whereas inhalability dramatically decreases for particles above 4 μm d_{ae} in small laboratory animals.

Lower respiratory tract consists of those structures of the respiratory tract below the larynx.

Mass geometric mean aerodynamic diameter or the mass median aerodynamic diameter (MMAD) is the calculated aerodynamic diameter that divides the particles of an aerosol (a gaseous suspension of fine liquid or solid particles) in half, based on the weight of the particles. By weight, 50% of the particles will be larger than the MMAD and 50% of the particles will be smaller than the MMAD.

Particle regional deposition is the fraction of inhaled particles that deposits in the specific region of the respiratory tract. The major mechanisms of particle deposition in the respiratory tract include impaction, sedimentation, diffusion, interception, and electrostatic precipitation. The deposition mechanism that is dominant for a given region depends on the respiratory tract architecture and ventilation rate of the species and the aerosol particle size and distribution. The respiratory tract in both humans and various experimental mammals can be divided into three regions on the basis of structure, size, and function:

(1) The extrathoracic region or upper respiratory tract that includes the nose, mouth, nasopharynx, oropharynx, laryngopharynx, and larynx.

(2) The tracheobronchial region that includes the trachea, bronchi, and

bronchioles (including the terminal bronchioles).

(3) The alveolar region that includes the respiratory bronchioles (if present in the species), alveolar ducts, alveolar sacs, and alveoli.

Respiratory effects are any adverse effects on the structure or functions of the respiratory system related to exposure to a chemical substance.

Target organ is any organ found to be a target of toxicity in the 4-hour (hr) high concentration group as a result of:

(1) The initial histopathologic examination (respiratory tract, liver, kidney, gross lesions); or

(2) The retrospective histopathologic examination of archived organs triggered by their identification as targets of toxicity in a 90-day study.

Toxic effects are any adverse changes (a change that is statistically and biologically significant) in the structure or function of an experimental animal as a result of exposure to a chemical substance.

Upper respiratory tract consists of those structures of the respiratory tract above and including the larynx.

(d) *Principle of the test method.* The test substance shall be administered to several groups of experimental animals; one concentration level and duration being used per group. Bronchoalveolar lavage shall be used to evaluate early effects on the respiratory system by examining changes in the content of the lavage fluid of the lung. At 24 hrs following exposure, the animals shall be sacrificed and necropsied, and tissue samples from the respiratory tract and other major organs will be prepared for microscopic examination. The exposure levels at which significant toxic effects on the respiratory organ system are produced are compared to those levels that produce other toxic effects. As triggered by the results of the 4-hr test, additional exposure periods of 1 hr and 8 hrs will be required to determine the effect of exposure time on the toxicity observed. A 1-hr exposure study can be elected as an option to provide data suitable for risk assessment for very short duration exposures as may occur from chemical releases. In the absence of adequate toxicological data for 1-hr exposure, the Agency will extrapolate to shorter-term exposures from the 4-hr data on the basis of concentration alone. This is a conservative method of extrapolation, consistent with general Agency methods for deriving criteria for short-term exposure from longer-term studies (a concentration x time extrapolation would result in higher concentration for a shorter duration).

(e) *Test procedures*—(1) *Animal selection*—(i) *Species.* In general, the laboratory rat and mouse should be used. Under some circumstances, other species, such as the hamster or guinea pig, may be more appropriate, and if these or other species are used, justification should be provided.

(ii) *Strain.* If rats and mice are used, the use of the F344 rat and the B6C3F1 mouse is preferred to facilitate comparison with existing data.

(iii) *Age.* Young adults shall be used. The weight variation of animals used in a test should not exceed ± 20% of the mean weight for each species.

(iv) *Sex.* Equal numbers of animals of each sex shall be used for each concentration level. The females shall be nulliparous and nonpregnant.

(v) *Health status.* Body weight and feed consumption are not sufficient indicators of the health status of animals prior to initiating an inhalation toxicity study. Prior to initiating the study, animals shall be monitored for known viral and bacterial respiratory pathogens determined by conventional microbiological assays (e.g., serology). The animals shall be free from pathogens at the start of exposure.

(2) *Number of animals.* At least five males and five females shall be used in each concentration/duration and control group. Animals shall be randomly assigned to treatment and control groups.

(3) *Control groups.* The control group shall be a sham-treated group. Except for treatment with the test substance, animals in the control group shall be handled in a manner identical to the test-group animals. Where a vehicle is used to help generate an appropriate concentration of the substance in the atmosphere, a vehicle control group shall be used. If the 4- and 8-hr exposure studies are conducted concurrently, a concurrent 8-hr sham-exposed control group may serve as the control group for both the 4-hr and the 8-hr exposure studies, provided there is adequate historical control data showing no changes in histopathology or bronchoalveolar lavage of controls exposed for 4 and 8 hrs. Similarly, if the optional 1-hr exposure study is conducted concurrently with the 4- and/or 8-hr study, the concurrent control group for those studies may also be used for the 1-hr study, provided adequate historical control data show no changes in histopathology or bronchoalveolar lavage between controls exposed for these time periods.

(4) *Concentration level and concentration selection.* For the 4-hr study, at least three concentrations shall be used in addition to the control group. Ideally, the data generated from the test should be sufficient to produce an exposure-response curve. The concentrations can either be linearly or logarithmically spaced depending on the anticipated steepness of the concentration-response curve. A rationale for concentration selection should be provided to indicate that the selected concentrations will maximally support detection of concentration-response relationship. The high concentration should be clearly toxic or a limit concentration, but should not result in an incidence of fatalities that would preclude a meaningful evaluation of the data. The lowest concentration should define a no-observed-adverse-effects level (NOAEL).

(i) *Limit concentration.* For aerosols and particles, the high concentrations need not be greater than 2 mg/L, or concentrations that cannot maintain a particle size distribution having an MMAD between 1 and 4 μm (i.e., a particle size that permits inhalability and deposition throughout the respiratory tract). For fibers, the bivariate distribution of length and diameter must ensure inhalability. For gases and vapors, the concentrations need not be greater than 50,000 ppm or 50% of the lower explosive limit, whichever is lower. If a test at an aerosol or particulate exposure of 2 mg/L (actual concentration of respirable substance) for 4 hrs or, where this is not feasible, the maximum attainable concentration, using the procedures described for this study, produces no observable toxic effects, then a full study using three concentrations will not be necessary. Similarly, if a test at a gas or vapor exposure of 50,000 ppm or 50% of the lower explosive limit, whichever is lower, produces no observable toxic effects, then a full study using three concentrations will not be necessary.

(ii) *8-Hr study and optional 1-hr study.* If the 8-hr study is triggered, three concentrations shall be tested. These concentrations should allow for the determination of an effect level and a NOAEL. If the option to perform a 1-hr study is elected, three concentrations shall be selected and tested in a similar manner.

(5) *Inhalation exposure.* Animals can be exposed to the substance by either a nose-only procedure or in a whole-body exposure chamber.

(i) *Inhalation chambers.* The animals shall be tested in inhalation equipment designed to sustain a dynamic airflow for nose-only exposures of at least 300 ml/minute/animal or an airflow for whole-body exposures of at least 12 to 15 air changes per hr and ensure an adequate oxygen content of at least 19% and an evenly distributed exposure

atmosphere. Where a whole-body chamber is used, its design shall minimize crowding by providing individual caging. As a general rule, to ensure stability of a chamber atmosphere, the total ''volume'' of the test animals should not exceed 5% of the volume of the test chamber.

(ii) *Environmental conditions.* The temperature at which the test is performed shall be maintained at 22 °C (±2 °C). Ideally, the relative humidity should be maintained between 40% and 60%, but in certain instances (e.g., tests using water as a vehicle), this may not be practical.

(iii) *Exposure periodicity.* For acute testing, the exposure design shall enable 4 hrs of exposure to the target concentrations, as defined by an average of ± 5% for gases and vapors and ± 15% for particles and aerosols. If triggered by the results of the 4-hr exposure, additional testing shall be conducted in a comparable manner using an 8-hr exposure period.

(6) *Physical measurements.* Measurements or monitoring shall be made of the following:

(i) Chemical purity of the test material shall be analyzed.

(ii) The rate of airflow shall be monitored continuously, but shall be recorded at least every 30 minutes.

(iii) The actual concentrations of the test substance shall be measured in the breathing zone. During the exposure period, the actual concentrations of the test substance shall be held as constant as practical, monitored continuously or intermittently depending on the method of analysis, and recorded at least at the beginning, at an intermediate time, and at the end of the exposure period. Well-established and published monitoring methods should be used where available. If no standard methods are available, then accuracy and precision information must be supplied.

(iv) During the development of the generating system, appropriate particle size analysis shall be performed to establish the stability of the aerosol. During exposure, analysis should be conducted as often as necessary to determine the consistency of particle size distribution. The particle size distribution shall have an MMAD between 1 and 4 µm. The particle size of hygroscopic materials shall be small enough when dry to assure that the size of the particle at saturation will still have an MMAD between 1 and 4 µm. Characterization for fibers shall include the bivariate distribution of length and diameter; this distribution must ensure inhalability.

(v) If the test substance is present in a mixture, the mass and composition of the entire mixture, as well as the principal compound, shall be measured.

(vi) Temperature and humidity shall be monitored continuously, but shall be recorded at least every 30 minutes.

(7) *Food and water during exposure period.* Food shall be withheld during exposure. Water may also be withheld in certain cases.

(8) *Observation period.* The bronchoalveolar lavage and respiratory pathology shall be conducted 24 hrs following exposure to allow expression of signs of toxicity. There is concern that some latency time will be required to allow migration of cells and macromolecules into the lungs following exposure, and that some pathology may require macromolecular synthesis or degradation before cell damage develops.

(9) *Gross pathology.* (i) All animals shall be subjected to a full gross necropsy which includes examination of orifices and the cranial, thoracic, and abdominal cavities and their contents.

(ii) At least the lungs, liver, kidneys, adrenals, brain, and gonads shall be weighed wet, as soon as possible after dissection to avoid drying.

(iii) The following organs and tissues, or representative samples thereof, shall be preserved in a suitable medium for possible future histopathological examination: All gross lesions; brain-including sections of medulla/pons; cerebellar cortex and cerebral cortex; pituitary; thyroid/parathyroid; thymus; heart; sternum with bone marrow; salivary glands; liver; spleen; kidneys; adrenals; pancreas; gonads; accessory genital organs (epididymis, prostrate, and, if present, seminal vesicles); aorta; skin; gall bladder (if present); esophagus; stomach; duodenum; jejunum; ileum; cecum; colon; rectum; urinary bladder; representative lymph nodes; thigh musculature; peripheral nerve; spinal cord at three levels cervical, midthoracic, and lumbar; and eyes. Respiratory tract tissues shall also be preserved in a suitable medium.

(10) *Histopathology.* The following histopathology shall be performed:

(i) Full histopathology shall be performed on the respiratory tract, liver and kidney of all animals in the control and high concentration groups. The histopathology of the respiratory tract is described under paragraph (e)(11) of this section.

(ii) All gross lesions which differ from controls in frequency, distribution, type, or severity in all concentration groups.

(iii) Target organs in all animals, as indicated by the observations in the high concentration group in this study. Histopathologic examination of target organs in animals at all concentration levels (rather than only to the extent necessary to define the NOAEL) can support the application of exposure-response analyses such as the benchmark concentration approach.

(iv) Archived organs identified as targets of toxicity from results of the 90-day study (if a 90-day study is required for this substance) should be elevated in high concentration animals of the 4-hr acute study to determine if they are also targets of acute toxicity.

(11) *Respiratory tract histopathology.* (i) Representative sections of the respiratory tract shall be examined histologically. These shall include the trachea, major conducting airways, alveolar region, terminal and respiratory bronchioles (if present), alveolar ducts and sacs, and interstitial tissues.

(ii) Care shall be taken that the method used to kill the animal does not result in damage to the tissues of the upper or lower respiratory tract. The lungs shall be infused with a fixative while in an inflated state of fixed pressure.

(iii) The upper respiratory tract shall be examined for histopathologic lesions. This examination shall use a minimum of four sections located as specified under paragraphs (e)(11)(iii)(A) through (e)(11)(iii)(D) of this section. An evaluation of the nasal vestibule shall be conducted. The method described by the reference under paragraph (h)(11) of this section should be given consideration. The use of additional sections shall be left to the discretion of the study pathologist, but consideration should be given to additional sections as recommended in the reference under paragraph (h)(8) of this section to ensure adequate evaluation of the entire upper respiratory tract, particularly the nasopharyngeal meatus. The following transverse sections shall be examined:

(A) Immediately posterior to the upper incisor teeth.

(B) At the incisor papilla.

(C) At the second palatal ridge.

(D) At the level of the first upper molar teeth.

(iv) The laryngeal mucosa shall be examined for histopathologic changes. Sections of the larynx to be examined include the epithelium covering the base of the epiglottis, the ventral pouch, and the medial surfaces of the vocal processes of the arytenoid cartilages.

(12) *Bronchoalveolar lavage.* (i) Animals can be exposed to the substance by either a nose-only procedure or in a whole-body exposure chamber.

(ii) Care should be taken that the method used to kill the animal results in minimum changes in the fluid of the lungs of the test animals.

(iii) At the appropriate time, the test animals shall be killed and the heart-lung including trachea removed in bloc. Alternatively, lungs can be lavaged in situ. If the study will not be compromised, one lobe of the lungs may be used for lung lavage while the other is fixed for histologic evaluation. The lungs should be lavaged using physiological saline. The lavages shall consist of two washes, each of which consists of approximately 80% (e.g., 5 ml in rats and 1 ml in mice) of the total lung volume. Additional washes merely tend to reduce the concentrations of the material collected. The lung lavage fluid shall be stored on ice at 5 °C until assayed.

(iv) The following parameters shall be determined in the lavage fluid as indicators of cellular damage in the lungs: total protein, cell count, and percent leukocytes. In addition, a phagocytosis assay shall be performed to determine macrophage activity. Assay methods described in the references under paragraphs (h)(1) and (h)(3) of this section may be used.

(13) *Combined protocol.* The tests described may be combined with any other toxicity study, as long as none of the requirements of either are violated by the combination.

(f) *Triggered testing.* If no adverse effects are seen in the 4-hr study as compared with controls, no further testing is necessary. If the 4-hr study shows positive effects in histopathology or the bronchoalveolar lavage, an 8-hr study shall be conducted. Only those tissues showing positive results in the 4-hr study must be pursued in the follow-up 8-hr study. Similarly, if the option to perform a 1-hr study is exercised, only those tissues showing positive results in the 4-hr study shall be pursued.

(g) *Data reporting and evaluation.* The final test report shall include the following information:

(1) *Description of equipment and test methods.* A description of the general design of the experiment and any equipment used shall be provided.

(i) Description of exposure apparatus, including design, type, dimensions, source of air, system for generating particles, aerosols, gasses, and vapors, method of conditioning air, treatment of exhaust air, and the method of housing animals in a test chamber.

(ii) Description of the equipment for measuring temperature, humidity, and particulate aerosol concentration and size.

(iii) Exposure data shall be tabulated and presented with mean values and measure of variability (e.g., standard deviation) and should include:

(A) Chemical purity of the test material.

(B) Airflow rates through the inhalation equipment.

(C) Temperature and humidity of air.

(D) Nominal concentration (total amount of test substance fed into the inhalation equipment divided by the volume of air).

(E) Actual concentration in test breathing zone.

(F) Particle size distribution (e.g., MMAD with GSD) and the bivariate distribution of fiber length and diameter, where appropriate.

(2) *Results*—(i) *General group animal data.* The following information shall be arranged by test group exposure level.

(A) Number of animals exposed.

(B) Number of animals dying.

(C) Number of animals showing overt signs of toxicity.

(D) Pre- and post-exposure body weight change in animals, and weight change during the observation period.

(ii) *Counts and incidence of gross alterations observed at necropsy in the test and control groups.* Data shall be tabulated to show:

(A) The number of animals used in each group and the number of animals in which any gross lesions were found.

(B) The number of animals affected by each different type of lesion, and the locations and frequency of each type of lesion.

(iii) *Counts and incidence of general histologic alterations in the test group.* Data shall be tabulated to show:

(A) The number of animals used in each group and the number of animals in which any histopathologic lesions were found.

(B) The number of animals affected by each different type of lesion, and the locations, frequency, and average grade of each type of lesion.

(iv) *Counts and incidence of respiratory histopathologic alterations by the test group.* Data shall be tabulated to show:

(A) The number of animals used in each group and the number of animals in which any histopathologic lesions were found.

(B) The number of animals affected by each different type of lesion, and the locations, frequency, and average grade of each type of lesion.

(v) *Results of the bronchoalveolar lavage study.* Data shall be tabulated to show:

(A) The amount of administered lavage fluid and recovered lavage fluid for each test animal.

(B) The magnitude of change of biochemical and cytologic indices in lavage fluids at each test concentration for each animal.

(C) Results shall be quantified as amount of constituent/mL of lavage fluid. This assumes that the amount of lavage fluid recovered is a representative sample of the total lavage fluid.

(3) *Evaluation of data.* The findings from this acute study should be evaluated in the context of preceding and/or concurrent toxicity studies and any correlated functional findings. The evaluation shall include the relationship between the concentrations of the test substance and the presence or absence, incidence, and severity of any effects. The evaluation should include appropriate statistical analyses, for example, parametric tests for continuous data and non-parametric tests for the remainder. Choice of analyses should consider tests appropriate to the experimental design, including repeated measures. The report must include concentration-response curves for the bronchoalveolar lavage and tables reporting observations at each concentration level for necropsy findings and gross, general, and respiratory system histopathology.

(h) *Reference.* For additional background information on this test guideline, the following references should be consulted. These references are available for inspection at the TSCA Nonconfidential Information Center, Rm. NE–B607, Environmental Protection Agency, 401 M St., SW., Washington, DC, 12 noon to 4 p.m., Monday through Friday, except legal holidays.

(1) Burleson, G.R., Fuller, L.B., Ménache, M.G., and Graham, J.A. Poly (I): poly (C)-enhanced alveolar peritoneal macrophage phagocytosis: Quantification by a new method utilizing fluorescent beads. *Proceedings of the Society of Experimental Biology and Medicine.* 184:468–476 (1987).

(2) Gardner, D.E., Crapo, J.D., and McClellan, R.O. (Eds.) *Toxicology of the Lung.* (Raven Press, New York, 1993) pp. i–xii, 1–30.

(3) Gilmour, G.I., and Selgrade, M.K. A comparison of the pulmonary defenses against streptococcal infection in rats and mice following O3 exposure: Differences in disease susceptibility and neutrophil recruitment. *Toxicology and Applied Pharmacology.* 123:211–218 (1993).

(4) Henderson, R.F., Benson, J.M., Hahn, F.F., Hobbs, C.H., Jones, R.K., Mauderly, J.L., McClellan, R.O., and Pickrell, J.A. New approaches for the evaluation of pulmonary toxicity: Bronchoalveolar lavage fluid analysis. *Fundamental and Applied Toxicology.* 5:451–458 (1985).

(5) Henderson, R.F. Use of bronchoalveolar lavage to detect lung damage. *Environmental Health Perspectives.* 56:115–129 (1984).

(6) Henderson, R.F., Rebar, A.H., Pickrell, J.A., and Newton, G.J. Early damage indicators in the lung. III. Biochemical and cytological response of the lung to inhaled metal salts. *Toxicology and Applied Pharmacology.* 50:123–136 (1979).

(7) McClellan, R.O. and Henderson, R.F. (Eds.) Second edition. *Concepts in Inhalation Toxicology.* (Taylor and Francis, Washington, DC, 1995) pp.i–xxiv, 1–24, 441–470.

(8) Mery, S., Gross, E.A., Joyner, D.R., Godo, M., and Morgan, K.T. Nasal Diagrams: A Tool for Recording the Distribution of Nasal Lesions in Rats and Mice. *Toxicologic Pathology.* 22:353–372 (1994).

(9) Phalen, R.F. (Ed) *Methods in Inhalation Toxicology.* (CRC Press, Boca Raton, FL, 1997) pp. i–xii, 1–12.

(10) Renne, R.A., Gideon, K.M., Miller, R.A., Mellick, P.W., and Grumbein, S.L. Histologic methods and interspecies variations in the laryngeal histology of F344/N rats and B6C3F1 mice. *Toxicology and Pathology.* 20:44–51 (1992).

(11) Young, J.T. Histopathologic examination of the rat nasal cavity. *Fundamental and Applied Toxicology.* 1:309–312 (1981).

§ 799.9346 TSCA subchronic inhalation toxicity.

(a) *Scope* This section is intended to meet the testing requirements under section 4 of TSCA. In the assessment and evaluation of the toxic characteristics of a gas, volatile substance, or aerosol/particulate, determination of subchronic inhalation toxicity may be carried out after initial information on toxicity has been obtained by acute testing. The subchronic inhalation study has been designed to permit the determination of the no-observed-effect-level (NOEL) and toxic effects associated with continuous or repeated exposure to a test substance for a period of 90 days. This study is not capable of determining those effects that have a long latency period for development (e.g., carcinogenicity and life shortening). Extrapolation from the results of this study to humans is valid only to a limited degree. It can, however, provide useful information on health hazards likely to arise from repeated exposures by the inhalation route over a limited period of time. It will provide information on target organs and the possibilities of accumulation, and can be of use in selecting concentration levels for chronic studies and establishing safety criteria for human exposure. Hazards of inhaled substances are influenced by the inherent toxicity and by physical factors such as volatility and particle size.

(b) *Source.* The source material used in developing this TSCA test guideline is the OPPTS harmonized test guideline 870.3465 (June 1996 Public Draft). This source is available at the address in paragraph (h) of this section.

(c) *Definitions.* The following definitions apply to this section.

Aerodynamic equivalent diameter is defined as the diameter of a unit density sphere having the same terminal settling velocity as the particle in question, whatever its size, shape, and density. It is used to predict where in the respiratory tract such particles may be deposited.

Concentration in a subchronic inhalation study is the amount of test substance administered via inhalation for a period of 90–days. Concentration is expressed as weight of the test substance per unit volume of air (milligrams per liter or parts per million).

Cumulative toxicity is the adverse effects of repeated exposures occurring as a result of prolonged action on, or increased concentration of the administered test substance or its metabolites in susceptible tissues.

Inhalable diameter refers to that aerodynamic diameter of a particle which is considered to be inhalable for the organism. It is used to refer to particles which are capable of being inhaled and may be deposited anywhere within the respiratory tract

Mass median aerodynamic diameter (MMAD) is the median aerodynamic diameter and along with the geometric standard deviation (GSD) is used to describe the particle size distribution of any aerosol statistically based on the weight and size of the particles. Fifty percent of the particles by weight will be smaller than the median diameter and 50% of the particles will be larger.

No-observed-effect-level (NOEL) is the maximum concentration used in a study which produces no adverse effects.

Subchronic inhalation toxicity is the adverse effects occurring as a result of the repeated daily exposure of experimental animals to a chemical by inhalation for part (approximately 10%) of a life span.

(d) *Limit test.* The exposure is at a concentration of 1 mg/L or greater (expected human exposure may indicate the need for a higher concentration), where such concentration is not possible due to physical or chemical properties of the test substance, or where the maximum attainable concentration produces no observable toxic effects. A full study using three concentrations may not be necessary.

(e) *Test procedures*—(1) *Animal selection*—(i) *Species and strain.* A mammalian species shall be used for testing. A variety of rodent species may be used, although the rat is the preferred species. Commonly used laboratory strains should be employed. If another mammalian species is used, the tester shall provide justification/reasoning for its selection.

(ii) *Age/weight.* (A) Testing should be started with young healthy animals as soon as possible after weaning and acclimatization.

(B) Exposure shall commence no later than 8 weeks of age.

(C) At the commencement of the study the weight variation of animals used shall not exceed ±20% of the mean weight for each sex.

(iii) *Sex.* (A) Equal numbers of animals of each sex shall be used at each concentration.

(B) Females shall be nulliparous and nonpregnant.

(iv) *Numbers.* (A) At least 20 rodents (10 females and 10 males) should be used for each test group. If another mammalian species is selected (e.g. dog, rabbit, or nonhuman primate), at least eight animals per group (four males and four females) shall be used.

(B) If interim sacrifices are planned, the number of animals shall be increased by the number of animals scheduled to be sacrificed before the completion of the study.

(C) To avoid bias, the use of adequate randomization procedures for the proper allocation of animals to test and control groups is required.

(D) Each animal shall be assigned a unique identification number. Dead animals, their preserved organs and tissues, and microscopic slides shall be identified by reference to the animal's unique number.

(v) *Husbandry.* (A) Animals may be group-caged by sex, but the number of animals per cage must not interfere with clear observation of each animal. The biological properties of the test substance or toxic effects (e.g., morbidity, excitability) may indicate a need for individual caging. Animals must be housed individually in inhalation chambers during exposure to aerosols.

(B) The temperature of the experimental animal rooms should be at 22 ±3 °C.

(C) The relative humidity of the experimental animal rooms should be 30–70%.

(D) Where lighting is artificial, the sequence should be 12 h light/12 h dark.

(E) Control and test animals should be fed from the same batch and lot. The feed should be analyzed to assure adequacy of nutritional requirements of the species tested and for impurities that might influence the outcome of the test. Animals should be fed and watered *ad libitum* with food replaced at least weekly. For nonrodents feeding should be at least daily and water *ad libitum*.

(F) The study should not be initiated until animals have been allowed a period of acclimatization/quarantine.

(2) *Control and test substances.* (i) Whenever it is necessary to formulate the test substance with a vehicle for aerosol generation, the vehicle ideally should not elicit toxic effects or substantially alter the chemical or toxicological properties of the test substance.

(ii) One lot of the test substance should be used, if possible throughout the duration of the study, and the research sample should be stored under conditions that maintain its purity and stability. Prior to the initiation of the study, there should be a characterization of the test substance, including the purity of the test substance and, if technically feasible, the name and quantities of unknown contaminants and impurities.

(3) *Control groups.* A concurrent control group is required. This group shall be an untreated or sham-treated control group. Except for treatment with the test substance, animals in the control group shall be handled in a manner identical to the test group animals. Where a vehicle other than water is used to generate a substance, a vehicle control group should be used. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are required.

(4) *Satellite group.* A satellite group of 20 animals (10 animals per sex) may be treated with the high concentration level for 90 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for a post-treatment period of appropriate length, normally not less than 28 days. In addition, a control group of 20 animals (10 animals of each sex) should be added to the satellite study.

(5) *Concentration levels and concentration selection.* (i) In subchronic toxicity tests, it is desirable to have a concentration-response relationship as well as a NOEL. Therefore, at least three concentration levels plus a control and, where appropriate, a vehicle control (corresponding to the concentration of vehicle at the highest exposure level) shall be used. Concentrations should be spaced appropriately to produce test groups with a range of toxic effects. The data should be sufficient to produce a concentration-response curve.

(ii) The highest concentration should result in toxic effects but not produce an incidence of fatalities which would prevent a meaningful evaluation.

(iii) The intermediate concentrations should be spaced to produce a gradation of toxic effects.

(iv) The lowest concentration should produce no evidence of toxicity.

(v) In the case of potentially explosive test substances, care should be taken to avoid generating explosive concentrations.

(6) *Administration of the test substance.* Animals should be exposed to the test substance for 6 h per day on a 7–day per week basis for a period of at least 90 days. Based primarily on practical considerations, exposure for 6 h per day on a 5–day per week basis is acceptable.

(7) *Observation period.* The animals should be observed for a period of 90 days. Animals in the satellite group (if used) scheduled for follow-up observations should be kept for at least 28 days further without treatment to assess reversibility.

(8) *Exposure specifications.* (i) The animals shall be tested in dynamic inhalation equipment designed to sustain a minimum airflow of 10 air changes per hr, an adequate oxygen content of at least 19%, and uniform conditions throughout the exposure chamber. Maintenance of slight negative pressure inside the chamber will prevent leakage of the test substance into the surrounding areas. It is not normally necessary to measure chamber oxygen concentration if airflow is adequate.

(ii) The selection of a dynamic inhalation chamber should be appropriate for the test substance and test system. Where a whole body chamber is used to expose animals to an aerosol, individual housing must be used to minimize crowding of the test animals and maximize their exposure to the test substance. To ensure stability of a chamber atmosphere, the total volume occupied by the test animals shall not exceed 5% of the volume of the test chamber. It is recommended, but not required, that nose-only or head-only exposure be used for aerosol studies in order to minimize oral exposures due to animals licking compound off their fur. Heat stress should be minimized.

(iii) The temperature at which the test is performed should be maintained at 22 ± 2 °C. The relative humidity should be maintained between 40 and 60%, but in certain instances (e.g., use of water vehicle) this may not be practicable.

(9) *Physical measurements.* Measurements or monitoring shall be made of the following:

(i) The rate of airflow shall be monitored continuously but recorded at least three times during the exposure.

(ii) The actual concentrations of the test substance shall be measured in the animal's breathing zone. During the exposure period, the actual concentrations of the test substance shall be held as constant as practicable and monitored continuously or intermittently depending on the method of analysis. Chamber concentration may be measured using gravimetric or analytical methods as appropriate. If trial run measurements are reasonably consistent (± 10% for liquid, aerosol, gas, or vapor; ± 20% for dry aerosol), then two measurements should be sufficient. If measurements are not consistent, three to four measurements should be taken. Whenever the test substance is a formulation, or it is necessary to formulate the test substance with a vehicle for aerosol generation, the analytical concentration must be reported for the total formulation, and not just for the active ingredient (AI). If, for example, a formulation contains 10% AI and 90% inerts, a chamber analytical limit concentration of 2 mg/L would consist of 0.2 mg/L of the AI. It is not necessary to analyze inert ingredients provided the mixture at the animal's breathing zone is analogous to the formulation; the grounds for this conclusion must be provided in the study report. If there is some difficulty in measuring chamber analytical concentration due to precipitation, nonhomogeneous mixtures, volatile components, or other factors, additional analyses of inert components may be necessary.

(iii) During the development of the generating system, particle size analysis shall be performed to establish the stability of aerosol concentrations with respect to particle size. The MMAD particle size range should be between 1–3 μm. The particle size of hygroscopic materials should be small enough when dry to assure that the size of the swollen particle will still be within the 1–3 μm range. Measurements of aerodynamic particle size in the animal's breathing zone should be measured during a trial run. If MMAD valves for each exposure level are within 10% of each other, then two measurements during the exposures should be sufficient. If pretest measurements are not within 10% of each other, three to four measurements should be taken.

(iv) Temperature and humidity shall be monitored continuously and recorded at least three times during an exposure.

(10) *Feed and water during exposure period.* Feed shall be withheld during exposure. Water may also be withheld during exposure.

(11) *Observation of animals.* (i) During and following exposure, observations are made and recorded systematically; individual records should be maintained for each animal. It is not always possible to observe animals during exposure in a whole-body chamber.

(ii) Observations shall be made at least once each day for morbidity and mortality. Appropriate actions should be taken to minimize loss of animals to the study (e.g., Necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals).

(iii) A careful clinical examination shall be made at least once weekly. Observations should be detailed and carefully recorded, preferably using explicitly defined scales. Observations should include, but not be limited to, evaluation of skin and fur, eyes and mucous membranes, respiratory and circulatory effects, autonomic effects such as salivation, central nervous system effects, including tremors and convulsions, changes in the level of activity, gait and posture, reactivity to handling or sensory stimuli, altered strength, and stereotypes or bizarre behavior (e.g., self-mutilation, walking backwards).

(iv) Signs of toxicity should be recorded as they are observed including the time of onset, degree and duration.

(v) Individual weights of animals shall be determined shortly before the test substance is administered, and weekly thereafter.

(vi) Food consumption shall also be determined weekly if abnormal body weight changes are observed.

(vii) Moribund animals should be removed and sacrificed when noticed and the time of death should be recorded as precisely as possible.

(viii) At termination, all survivors in the treatment groups shall be sacrificed.

(12) *Clinical pathology.* Hematology and clinical chemistry examinations shall be made on all animals, including controls, of each sex in each group for rodents and all animals when nonrodents are used as test animals. For rodents, the hematology and clinical chemistry parameters should be examined once prior to initiation of exposure and at terminal sacrifice. For nonrodents, the hematology and clinical chemistry parameters should be examined once prior to initiation of exposure, at monthly intervals or midway through the test period and at termination.

(i) The recommended hematology parameters are: Hemoglobin and hematocrit concentrations, red blood cell count, white blood cell count, differential leukocyte count, platelet count, and a measure of clotting potential such as prothrombin time or thromboplastin time.

(ii) Clinical chemistry parameters which are considered appropriate to all studies are electrolyte balance, carbohydrate metabolism, and liver and kidney function. Other determinations which may be necessary for an adequate toxicological evaluation include analyses of lipids, hormones, acid/base balance, methemoglobin and cholinesterase activity. Additional clinical biochemistry may be employed where necessary to extend the investigation of observed effects.The selection of specific tests will be influenced by observations on the mode of action of the substance and signs of clinical toxicity. Suggested blood clinical chemistry determinations:

(A) Electrolytes.
(*1*) Calcium.
(*2*) Chloride.
(*3*) Magnesium.
(*4*) Inorganic phosphorus.
(*5*) Potassium.
(*6*) Sodium.
(B) Enzymes.
(*1*) Alkaline phosphatase.
(*2*) Alanine aminotransferase.
(*3*) Aspartate aminotransferase.
(*4*) Gamma glutamyl transferase.
(C) Other.
(*1*) Albumin.
(*2*) Blood creatinine.
(*3*) Blood urea nitrogen.
(*4*) Globulins.
(*5*) Glucose (fasting).
(*6*) Total bilirubin.
(*7*) Total cholesterol.
(*8*) Total serum protein.

(iii) Urinalysis is not recommended on a routine basis, but only when there is an indication based on expected or observed toxicity.

(13) *Ophthalmological examination.* Ophthalmological examinations shall be made on all animals prior to the administration of the test substance and on all high concentration and control groups at termination. If changes in the eyes are detected, all animals in the other concentration groups shall be examined.

(14) *Gross pathology.* (i) All animals shall be subjected to a full gross necropsy which includes examination of the external surface of the body, all orifices and the cranial, thoracic, and abdominal cavities and their contents.

(ii) At least the liver, kidneys, brain, and gonads shall be trimmed and weighed wet, as soon as possible after dissection to avoid drying.

(iii) The following organs and tissues, or representative samples thereof, shall be preserved in a suitable medium for possible future histopathological examination:

(A) Digestive system.
(*1*) Salivary glands.
(*2*) Esophagus.
(*3*) Stomach.
(*4*) Duodenum.
(*5*) Jejunum.
(*6*) Ileum.
(*7*) Cecum.
(*8*) Colon.
(*9*) Rectum.
(*10*) Liver.
(*11*) Pancreas.
(*12*) Gallbladder (dogs).
(B) Nervous system.
(*1*) Brain (multiple sections).
(*2*) Pituitary.
(*3*) Peripheral nerve(s).
(*4*) Spinal cord (three levels).
(*5*) Eyes (retina, optic nerve).
(C) Glandular system.
(*1*) Adrenals.
(*2*) Parathyroids.
(*3*) Thyroids.
(D) Respiratory system.
(*1*) Trachea.
(*2*) Lung.
(*3*) Pharynx.
(*4*) Larynx.
(*5*) Nose.
(E) Cardiovascular/hematopoietic system.
(*1*) Aorta (thoracic).
(*2*) Heart.
(*3*) Bone marrow.
(*4*) Lymph nodes.
(*5*) Spleen.
(*6*) Thymus.
(F) Urogenital system.
(*1*) Kidneys.
(*2*) Urinary bladder.
(*3*) Prostate.
(*4*) Testes.
(*5*) Epididymides.
(*6*) Seminal vesicle(s).
(*7*) Uterus.
(*8*) Ovaries.
(G) Other.
(*1*) Lacrimal gland.
(*2*) Mammary gland.
(*3*) Skin.
(*4*) Skeletal muscle.
(*5*) All gross lesions and masses.
(*6*) Sternum and/or femur.

(15) *Histopathology.* (i) The following histopathology shall be performed:

(A) Full histopathology on the respiratory tract and other organs and tissues, listed under paragraph (e)(15)(iii) of this section, of all animals in the control and high exposure groups

and all animals that died or were killed during the study.

(B) All gross lesions in all animals.

(C) Target organs in all animals.

(D) Lungs, liver and kidneys of all animals. Special attention to examination of the respiratory tract should be made for evidence of infection as this provides a convenient assessment of the state of health of the animals.

(E) When a satellite group is used, histopathology shall be performed on tissues and organs identified as showing effects in the treated groups.

(ii) If excessive early deaths or other problems occur in the high exposure group compromising the significance of the data, the next concentration should be examined for complete histopathology.

(iii) An attempt should be made to correlate gross observations with microscopic findings.

(iv) Tissues and organs designated for microscopic examination should be fixed in 10% buffered formalin or a recognized suitable fixative as soon as necropsy is performed and no less than 48 hrs prior to trimming. Tissues should be trimmed to a maximum thickness of 0.4 cm for processing.

(f) *Data and reporting*—(1) *Treatment of results.* (i) Data shall be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, the types of lesions, and the percentage of animals displaying each type of lesion.

(ii) All observed results (quantitative and qualitative) should be evaluated by an appropriate statistical method. Any generally accepted statistical method may be used; the statistical methods including significance criteria should be selected during the design of the study.

(2) *Evaluation of study results.* The findings of the subchronic inhalation toxicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of the observed toxic effects and the necropsy and histopathological findings. The evaluation will include the relationship between the concentration of the test substance and duration of exposure, and the presence or absence, the incidence and severity, of abnormalities, including behavioral and clinical abnormalities, gross lesions, identified target organs, body weight changes, effects on mortality and any other general or specific toxic effects. A properly conducted subchronic test should provide a satisfactory estimation of a no-effect level. It also can indicate the need for an additional longer-term study and provide information on the selection of concentrations.

(3) *Test report.* In addition to reporting requirements specified under 40 CFR part 792, subpart J, the following specific information shall be reported. Both individual and summary data should be presented.

(i) Test substance characterization shall include:

(A) Chemical identification.

(B) Lot or batch number.

(C) Physical properties.

(D) Purity/impurities.

(E) Identification and composition of any vehicle used.

(ii) Test system information shall include:

(A) Species and strain of animals used and rationale for selection if other than that recommended.

(B) Age, sex, and body weight.

(C) Test environment including cage conditions, ambient temperature, humidity, and light/dark periods.

(iii) Test procedure information shall include:

(A) Method of randomization used.

(B) Full description of experimental design and procedure.

(C) Exposure regimen including concentration levels, methods, and volume.

(D) Description of test conditions; the following exposure conditions shall be reported:

(*1*) Description of exposure apparatus including design, type, volume, source of air, system for generating aerosols, method of conditioning air, treatment of exhaust air and the method of housing the animals in a test chamber.

(*2*) The equipment for measuring temperature, humidity, and particulate aerosol concentrations and size should be described.

(E) Exposure data shall be tabulated and presented with mean values and a measure of variability (e.g., standard deviation) and include:

(*1*) Airflow rates through the inhalation equipment.

(*2*) Temperature and humidity of air.

(*3*) Actual (analytical or gravimetric) concentration in the breathing zone.

(*4*) Nominal concentration (total amount of test substance fed into the inhalation equipment divided by volume of air).

(*5*) Particle size distribution, calculated mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD).

(*6*) Explanation as to why the desired chamber concentration and/or particle size could not be achieved (if applicable) and the efforts taken to comply with this aspect of the section.

(iv) Test results information shall include:

(A) *Group animal data.* Tabulation of toxic response data by species, strain, sex and exposure level for:

(*1*) Number of animals exposed.

(*2*) Number of animals showing signs of toxicity.

(*3*) Number of animals dying.

(B) *Individual animal data.* Data should be presented as summary (group mean) as well as for individual animals.

(*1*) Time of death during the study or whether animals survived to termination.

(*2*) Time of observation of each abnormal sign and its subsequent course.

(*3*) Body weight data.

(*4*) Feed consumption data, when collected.

(*5*) Results of ophthalmological examination, when performed.

(*6*) Results of hematological tests performed. .

(*7*) Results of clinical chemistry tests performed.

(*8*) Results of urinalysis tests performed.

(*9*) Necropsy findings, including absolute and relative organ weight data.

(*10*) Detailed description of all histopathological findings.

(*11*) Statistical treatment of results, where appropriate.

(g) *Quality control.* A system shall be developed and maintained to assure and document adequate performance of laboratory staff and equipment. The study shall be conducted in compliance with 40 CFR Part 792—Good Laboratory Practice Standards.

(h) *References.* For additional background information on this test guideline, the following references should be consulted. These references are available for inspection at the TSCA Nonconfidential Information Center, Rm. NE–B607, Environmental Protection Agency, 401 M St., SW., Washington, DC, 12 noon to 4 p.m., Monday through Friday, except legal holidays.

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§ 799.9370 TSCA prenatal developmental toxicity.

(a) *Scope* This section is intended to meet the testing requirements under section 4 of TSCA. This guideline for developmental toxicity testing is designed to provide general information concerning the effects of exposure on the pregnant test animal and on the developing organism; this may include death, structural abnormalities, or altered growth and an assessment of maternal effects. For information on testing for functional deficiencies and other postnatal effects, the guidelines for the two-generation reproductive toxicity study and the developmental neurotoxicity study should be consulted.

(b) *Source.* The source material used in developing this TSCA test guideline is the OPPTS harmonized test guideline 870.3700 (February 1996 Public Draft). This source is available at the address in paragraph (h) of this section.

(c) *Good laboratory practice standards.* The study shall be conducted in compliance with 40 CFR Part 792—Good Laboratory Practice Standards.

(d) *Principle of the test method.* The test substance is administered to pregnant animals at least from implantation to one day prior to the expected day of parturition. Shortly before the expected date of delivery, the pregnant females are terminated, the uterine contents are examined, and the fetuses are processed for visceral and skeletal evaluation.

(e) *Test procedures*—(1) *Animal selection*—(i) *Species and strain.* It is recommended that testing be performed in the most relevant species, and that laboratory species and strains which are commonly used in prenatal developmental toxicity testing be employed. The preferred rodent species is the rat and the preferred non-rodent species is the rabbit.

(ii) *Age.* Young adult animals shall be used.

(iii) *Sex.* Nulliparous female animals shall be used at each dose level. Animals should be mated with males of the same species and strain, avoiding the mating of siblings, if parentage is known. Day 0 in the test is the day on which a vaginal plug and/or sperm are observed in the rodent or that insemination is performed or observed in the rabbit.

(iv) *Number of animals.* Each test and control group shall contain a sufficient number of animals to yield approximately 20 animals with implantation sites at necropsy.

(2) *Administration of test and control substances*—(i) *Dose levels and dose selection.* (A) At least three-dose levels and a concurrent control shall be used. Healthy animals shall be randomly assigned to the control and treatment groups, in a manner which results in comparable mean body weight values among all groups. The dose levels should be spaced to produce a gradation of toxic effects. Unless limited by the physical/chemical nature or biological properties of the test substance, the highest dose shall be chosen with the aim to induce some developmental and/or maternal toxicity but not death or severe suffering. In the case of maternal mortality, this should not be more than approximately 10%. The intermediate dose levels should produce minimal observable toxic effects. The lowest dose level should not produce any evidence of either maternal or developmental toxicity (i.e., the no-observed-adverse-effect level, NOAEL) or should be at or near the limit of detection for the most sensitive endpoint. Two- or four-fold intervals are frequently optimal for spacing the dose levels, and the addition of a fourth test group is often preferable to using very large intervals (e.g., more than a factor of 10) between dosages.

(B) It is desirable that additional information on metabolism and pharmacokinetics of the test substance be available to demonstrate the adequacy of the dosing regimen. This information should be available prior to testing.

(C) The highest dose tested need not exceed 1,000 mg/kg/day by oral or dermal administration, or 2 mg/L (or the maximum attainable concentration) by inhalation, unless potential human exposure data indicate the need for higher doses. If a test performed at the limit dose level, using the procedures described for this study, produces no observable toxicity and if an effect would not be expected based upon data from structurally related compounds, then a full study using three-dose levels may not be considered necessary.

(ii) *Control group.* (A) A concurrent control group shall be used. This group shall be a sham-treated control group or a vehicle-control group if a vehicle is used in administering the test substance.

(B) The vehicle control group should receive the vehicle in the highest volume used.

(C) If a vehicle or other additive is used to facilitate dosing, consideration should be given to the following characteristics: Effects on the absorption, distribution, metabolism, or retention of the test substance; effects on the chemical properties of the test substance which may alter its toxic characteristics; and effects on the food or water consumption or the nutritional status of the animals.

(iii) *Route of administration.* (A) The test substance or vehicle is usually administered orally by intubation.

(B) If another route of administration is used, for example, when the route of administration is based upon the principal route of potential human exposure, the tester shall provide justification and reasoning for its selection, and appropriate modifications may be necessary. Care should be taken to minimize stress on the maternal animals. For materials administered by inhalation, whole-body exposure is preferable to nose-only exposure due to the stress of restraint required for nose-only exposure.

(C) The test substance shall be administered at approximately the same time each day.

(D) When administered by gavage or dermal application, the dose to each animal shall be based on the most recent individual body weight determination.

(iv) *Dosing schedule.* At minimum, the test substance shall be administered daily from implantation to the day before cesarean section on the day prior to the expected day of parturition. Alternatively, if preliminary studies do not indicate a high potential for preimplantation loss, treatment may be extended to include the entire period of gestation, from fertilization to approximately 1 day prior to the expected day of termination.

(f) *Observation of animals*—(1) *Maternal.* (i) Each animal shall be observed at least once daily, considering the peak period of anticipated effects after dosing. Mortality, moribundity, pertinent behavioral changes, and all signs of overt toxicity shall be recorded at this cageside observation. In addition, thorough physical examinations shall be conducted at the same time maternal body weights are recorded.

(ii) Animals shall be weighed on day 0, at termination, and at least at 3–day intervals during the dosing period.

(iii) Food consumption shall be recorded on at least 3-day intervals, preferably on days when body weights are recorded.

(iv) (A) Females shall be terminated immediately prior to the expected day of delivery.

(B) Females showing signs of abortion or premature delivery prior to scheduled termination shall be killed

and subjected to a thorough macroscopic examination.

(v) At the time of termination or death during the study, the dam shall be examined macroscopically for any structural abnormalities or pathological changes which may have influenced the pregnancy. Evaluation of the dams during cesarean section and subsequent fetal analyses should be conducted without knowledge of treatment group in order to minimize bias.

(vi) (A) Immediately after termination or as soon as possible after death, the uteri shall be removed and the pregnancy status of the animals ascertained. Uteri that appear nongravid shall be further examined (e.g. by ammonium sulfide staining) to confirm the nonpregnant status.

(B) Each gravid uterus (with cervix) shall be weighed. Gravid uterine weights should not be obtained from dead animals if autolysis or decomposition has occurred.

(C) The number of corpora lutea shall be determined for pregnant animals.

(D) The uterine contents shall be examined for embryonic or fetal deaths and the number of viable fetuses. The degree of resorption shall be described in order to help estimate the relative time of death of the conceptus.

(2) *Fetal.* (i) The sex and body weight of each fetus shall be determined.

(ii) Each fetus shall be examined for external anomalies.

(iii) Fetuses shall be examined for skeletal and soft tissue anomalies (e.g. variations and malformations or other categories of anomalies as defined by the performing laboratory).

(A) For rodents, approximately one-half of each litter shall be prepared by standard techniques and examined for skeletal alterations, preferably bone and cartilage. The remainder shall be prepared and examined for soft tissue anomalies, using appropriate serial sectioning or gross dissection techniques. It is also acceptable to examine all fetuses by careful dissection for soft tissue anomalies followed by an examination for skeletal anomalies.

(B) For rabbits, all fetuses shall be examined for both soft tissue and skeletal alterations. The bodies of these fetuses should be evaluated by careful dissection for soft-tissue anomalies, followed by preparation and examination for skeletal anomalies. An adequate evaluation of the internal structures of the head, including the eyes, brain, nasal passages, and tongue, should be conducted for at least half of the fetuses.

(g) *Data and reporting*—(1) *Treatment of results.* Data shall be reported individually and summarized in tabular form, showing for each test group the types of change and the number of dams, fetuses, and litters displaying each type of change.

(2) *Evaluation of study results.* The following shall be provided:

(i) Maternal and fetal test results, including an evaluation of the relationship, or lack thereof, between the exposure of the animals to the test substance and the incidence and severity of all findings.

(ii) Criteria used for categorizing fetal external, soft tissue, and skeletal anomalies.

(iii) When appropriate, historical control data to enhance interpretation of study results. Historical data (on litter incidence and fetal incidence within litter), when used, should be compiled, presented, and analyzed in an appropriate and relevant manner. In order to justify its use as an analytical tool, information such as the dates of study conduct, the strain and source of the animals, and the vehicle and route of administration should be included.

(iv) Statistical analysis of the study findings should include sufficient information on the method of analysis, so that an independent reviewer/statistician can reevaluate and reconstruct the analysis. In the evaluation of study data, the litter should be considered the basic unit of analysis.

(v) In any study which demonstrates an absence of toxic effects, further investigation to establish absorption and bioavailability of the test substance should be considered.

(3) *Test report.* In addition to the reporting requirements as specified under 40 CFR part 792, subpart J, the following specific information shall be reported. Both individual and summary data should be presented.

(i) Species and strain.

(ii) Maternal toxic response data by dose, including but not limited to:

(A) The number of animals at the start of the test, the number of animals surviving, the number pregnant, and the number aborting.

(B) Day of death during the study or whether animals survived to termination.

(C) Day of observation of each abnormal clinical sign and its subsequent course.

(D) Body weight and body weight change data, including body weight change adjusted for gravid uterine weight.

(E) Food consumption and, if applicable, water consumption data.

(F) Necropsy findings, including gravid uterine weight.

(iii) Developmental endpoints by dose for litters with implants, including:

(A) Corpora lutea counts.

(B) Implantation data, number and percent of live and dead fetuses, and resorptions (early and late).

(C) Pre- and postimplantation loss calculations.

(iv) Developmental endpoints by dose for litters with live fetuses, including:

(A) Number and percent of live offspring.

(B) Sex ratio.

(C) Fetal body weight data, preferably by sex and with sexes combined.

(D) External, soft tissue, and skeletal malformation and variation data. The total number and percent of fetuses and litters with any external, soft tissue, or skeletal alteration, as well as the types and incidences of individual anomalies, should be reported.

(v) The numbers used in calculating all percentages or indices.

(vi) Adequate statistical treatment of results.

(vii) A copy of the study protocol and any amendments should be included.

(h) *References.* For additional background information on this test guideline, the following references should be consulted. These references are available for inspection at the TSCA Nonconfidential Information Center, Rm. NE–B607, Environmental Protection Agency, 401 M St., SW., Washington, DC, 12 noon to 4 p.m., Monday through Friday, except legal holidays.

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(3) Burdi, A.R. Toluidine blue-alizarin red S staining of cartilage and bone in whole-mount skeltons *in vitro. Stain Technolology.* 40:45–48 (1965).

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(5) Fritz, H. Prenatal ossification in rabbits as indicative of fetal maturity. *Teratology.* 11:313–320 (1974).

(6) Fritz, H. and Hess, R. Ossification of the rat and mouse skeleton in the perinatal period. *Teratology.* 3:331–338 (1970).

(7) Gibson, J.P. *et al.* Use of the rabbit in teratogenicity studies. *Toxicology and Applied Pharmacology.* 9:398–408 (1966).

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(18) Organisation for Economic Co-operation and Development, No. 414: Teratogenicity, Guideline for Testing of Chemicals. [C(83)44 (Final)] (1983).

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§ 799.9380 TSCA reproduction and fertility effects.

(a) *Scope.* This section is intended to meet the testing requirements under section 4 of the TSCA. This section is for two-generation reproduction testing and is designed to provide general information concerning the effects of a test substance on the integrity and performance of the male and female reproductive systems, including gonadal function, the estrous cycle, mating behavior, conception, gestation, parturition, lactation, and weaning, and on the growth and development of the offspring. The study may also provide information about the effects of the test substance on neonatal morbidity, mortality, target organs in the offspring, and preliminary data on prenatal and postnatal developmental toxicity and serve as a guide for subsequent tests. Additionally, since the study design includes *in utero* as well as postnatal exposure, this study provides the opportunity to examine the susceptibility of the immature/neonatal animal.

(b) *Source.* The source material used in developing this TSCA test guideline is the OPPTS harmonized test guideline 870.3800 (February 1996 Public Draft). This source is available at the address in paragraph (g) of this section.

(c) *Good laboratory practice standards.* The study shall be conducted in compliance with 40 CFR Part 792—Good Laboratory Practice Standards.

(d) *Principle of the test method.* The test substance is administered to parental (P) animals prior to and during their mating, during the resultant pregnancies, and through the weaning of their F1 offspring. The substance is then administered to selected F1 offspring during their growth into adulthood, mating, and production of an F2 generation, until the F2 generation is weaned.

(e) *Test procedures*—(1) *Animal selection*—(i) *Species and strain.* The rat is the most commonly used species for testing. If another mammalian species is used, the tester shall provide justification/reasoning for its selection, and appropriate modifications will be necessary. Healthy parental animals, which have been acclimated to laboratory conditions for at least 5 days and have not been subjected to previous experimental procedures, should be used. Strains of low fecundity shall not be used.

(ii) *Age.* Parental (P) animals shall be 5 to 9 weeks old at the start of dosing. The animals of all test groups should be of uniform weight, age, and parity as nearly as practicable, and should be representative of the species and strain under study.

(iii) *Sex.* (A) For an adequate assessment of fertility, both males and females shall be studied.

(B) The females shall be nulliparous and nonpregnant.

(iv) *Number of animals.* Each control group shall contain a sufficient number of mating pairs to yield approximately 20 pregnant females. Each test group shall contain a similar number of mating pairs.

(v) *Identification of animals.* Each animal shall be assigned a unique identification number. For the P generation, this should be done before dosing starts. For the F1 generation, this should be done for animals selected for mating; in addition, records indicating the litter of origin shall be maintained for all selected F1 animals.

(2) *Administration of test and control substances*—(i) *Dose levels and dose selection.* (A) At least three-dose levels and a concurrent control shall be used. Healthy animals should be randomly assigned to the control and treatment groups, in a manner which results in comparable mean body weight values among all groups. The dose levels should be spaced to produce a gradation of toxic effects. Unless limited by the physical/chemical nature or biological properties of the test substance, the highest dose should be chosen with the aim to induce some reproductive and/or systemic toxicity but not death or severe suffering. In the case of parental

mortality, this should not be more than approximately 10%. The intermediate dose levels should produce minimal observable toxic effects. The lowest dose level should not produce any evidence of either systemic or reproductive toxicity (i.e., the no-observed-adverse-effect level, NOAEL) or should be at or near the limit of detection for the most sensitive endpoint. Two- or four-fold intervals are frequently optimal for spacing the dose levels, and the addition of a fourth test group is often preferable to using very large intervals (e.g., more than a factor of 10) between dosages.

(B) It is desirable that additional information on metabolism and pharmacokinetics of the test substance be available to demonstrate the adequacy of the dosing regimen. This information should be available prior to testing.

(C) The highest dose tested should not exceed 1,000 mg/kg/day (or 20,000 ppm in the diet), unless potential human exposure data indicate the need for higher doses. If a test performed at the limit dose level, using the procedures described for this study, produces no observable toxicity and if an effect would not be expected based upon data from structurally related compounds, then a full study using three dose levels may not be considered necessary.

(ii) *Control group.* (A) A concurrent control group shall be used. This group shall be an untreated or sham treated group or a vehicle-control group if a vehicle is used in administering the test substance.

(B) If a vehicle is used in administering the test substance, the control group shall receive the vehicle in the highest volume used.

(C) If a vehicle or other additive is used to facilitate dosing, consideration should be given to the following characteristics: Effects on the absorption, distribution, metabolism, or retention of the test substance; effects on the chemical properties of the test substance which may alter its toxic characteristics; and effects on the food or water consumption or the nutritional status of the animals.

(D) If a test substance is administered in the diet and causes reduced dietary intake or utilization, the use of a pair-fed control group may be considered necessary.

(iii) *Route of administration.* (A) The test substance is usually administered by the oral route (diet, drinking water, or gavage).

(B) If administered by gavage or dermal application, the dosage administered to each animal prior to mating and during gestation and lactation shall be based on the individual animal body weight and adjusted weekly at a minimum.

(C) If another route of administration is used, for example, when the route of administration is based upon the principal route of potential human exposure, the tester should provide justification and reasoning for its selection, and appropriate modifications may be necessary. Care should be taken to minimize stress on the maternal animals and their litters during gestation and lactation.

(D) All animals should be dosed by the same method during the appropriate experimental period.

(iv) *Dosing schedule.* (A) The animals should be dosed with the test substance on a 7–days–a–week basis.

(B) Daily dosing of the parental (P) males and females shall begin when they are 5 to 9 weeks old. Daily dosing of the F1 males and females shall begin at weaning. For both sexes (P and F1), dosing shall be continued for at least 10 weeks before the mating period.

(C) Daily dosing of the P and F1 males and females shall continue until termination.

(3) *Mating procedure—(i) Parental.* (A) For each mating, each female shall be placed with a single randomly selected male from the same dose level (1:1 mating) until evidence of copulation is observed or either 3 estrous periods or 2 weeks has elapsed. Animals should be separated as soon as possible after evidence of copulation is observed. If mating has not occurred after 2 weeks or 3 estrous periods, the animals should be separated without further opportunity for mating. Mating pairs should be clearly identified in the data.

(B) Vaginal smears shall be collected daily and examined for all females during mating, until evidence of copulation is observed.

(C) Each day, the females shall be examined for presence of sperm or vaginal plugs. Day 0 of pregnancy is defined as the day a vaginal plug or sperm are found.

(ii) *F1 mating.* For mating the F1 offspring, at least one male and one female should be randomly selected from each litter for mating with another pup of the same dose level but different litter, to produce the F2 generation.

(iii) *Second mating.* In certain instances, such as poor reproductive performance in the controls, or in the event of treatment-related alterations in litter size, the adults may be remated to produce an F1b or F2b litter. If production of a second litter is deemed necessary in either generation, the dams should be remated approximately 1–2 weeks following weaning of the last F1a or F2a litter.

(iv) *Special housing.* After evidence of copulation, animals that are presumed to be pregnant shall be caged separately in delivery or maternity cages. Pregnant animals shall be provided with nesting materials when parturition is near.

(v) *Standardization of litter sizes.* (A) Animals should be allowed to litter normally and rear their offspring to weaning. Standardization of litter sizes is optional.

(B) If standardization is performed, the following procedure should be used. On day 4 after birth, the size of each litter may be adjusted by eliminating extra pups by random selection to yield, as nearly as possible, four males and four females per litter or five males and five females per litter. Selective elimination of pups, i.e. based upon body weight, is not appropriate. Whenever the number of male or female pups prevents having four (or five) of each sex per litter, partial adjustment (for example, five males and three females, or four males and six females) is acceptable. Adjustments are not appropriate for litters of eight pups or less.

(4) *Observation of animals*—(i) *Parental.* (A) Throughout the test period, each animal shall be observed at least once daily, considering the peak period of anticipated effects after dosing. Mortality, moribundity, pertinent behavioral changes, signs of difficult or prolonged parturition, and all signs of overt toxicity shall be recorded at this cageside examination. In addition, thorough physical examinations should be conducted weekly on each animal.

(B) Parental animals (P and F1) shall be weighed on the first day of dosing and weekly thereafter. Parental females (P and F1) should be weighed at a minimum on approximately gestation days 0, 7, 14, and 21, and during lactation on the same days as the weighing of litters.

(C) During the premating and gestation periods, food consumption shall be measured weekly at a minimum. Water consumption should be measured weekly at a minimum if the test substance is administered in the water.

(D) Estrous cycle length and normality should be evaluated by vaginal smears for all P and F1 females during a minimum of 3 weeks prior to mating and throughout cohabitation; care should be taken to prevent the induction of pseudopregnancy.

(E) For all P and F1 males at termination, sperm from one testis and one epididymis shall be collected for

enumeration of homogenization-resistant spermatids and cauda epididymal sperm reserves, respectively. In addition, sperm from the cauda epididymis (or vas deferens) should be collected for evaluation of sperm motility and sperm morphology.

(*1*) The total number of homogenization-resistant testicular sperm and cauda epididymal sperm should be enumerated. The method described in the reference under paragraph (g)(8) of this section may be used. Cauda sperm reserves can be derived from the concentration and volume of sperm in the suspension used to complete the qualitative evaluations, and the number of sperm recovered by subsequent mincing and/or homogenizing of the remaining cauda tissue. Enumeration in only control and high-dose P and F1 males may be performed unless treatment-related effects are observed; in that case, the lower dose groups should also be evaluated.

(*2*) An evaluation of epididymal (or vas deferens) sperm motility should be performed. Sperm should be recovered while minimizing damage (the evaluation techniques as described in the reference under paragraph (g)(8) of this section may be used), and the percentage of progressively motile sperm should be determined either subjectively or objectively. For objective evaluations, an acceptable counting chamber of sufficient depth can be used to effectively combine the assessment of motility with sperm count and sperm morphology. When computer-assisted motion analysis is performed, the derivation of progressive motility relies on user-defined thresholds for average path velocity and straightness or linear index. If samples are videotaped, or images otherwise recorded, at the time of necropsy, subsequent analysis of only control and high-dose P and F1 males may be performed unless treatment-related effects are observed; in that case, the lower dose groups should also be evaluated. In the absence of a video or digital image, all samples in all treatment groups should be analyzed at necropsy.

(*3*) A morphological evaluation of an epididymal (or vas deferens) sperm sample shall be performed. Sperm (at least 200 per sample) should be examined as fixed, wet preparations (the techniques for such examinations is described in the references under paragraphs (g)(4) and (g)(8) of this section may be used) and classified as either normal (both head and midpiece/tail appear normal) or abnormal. Examples of morphologic sperm abnormalities would include fusion, isolated heads, and misshapen heads and/or tails. Evaluation of only control and high-dose P and F1 males may be performed unless treatment-related effects are observed; in that case, the lower dose groups should also be evaluated.

(ii) *Offspring.* (A) Each litter should be examined as soon as possible after delivery (lactation day 0) to establish the number and sex of pups, stillbirths, live births, and the presence of gross anomalies. Pups found dead on day 0 should be examined for possible defects and cause of death.

(B) Live pups should be counted, sexed, and weighed individually at birth, or soon thereafter, at least on days 4, 7, 14, and 21 of lactation, at the time of vaginal patency or balanopreputial separation, and at termination.

(C) The age of vaginal opening and preputial separation should be determined for F1 weanlings selected for mating. If there is a treatment-related effect in F1 sex ratio or sexual maturation, anogenital distance should be measured on day 0 for all F2 pups.

(5) *Termination schedule.* (i) All P and F1 adult males and females should be terminated when they are no longer needed for assessment of reproductive effects.

(ii) F1 offspring not selected for mating and all F2 offspring should be terminated at comparable ages after weaning.

(6) *Gross necropsy.* (i) At the time of termination or death during the study, all parental animals (P and F1) and when litter size permits at least three pups per sex per litter from the unselected F1 weanlings and the F2 weanlings shall be examined macroscopically for any structural abnormalities or pathological changes. Special attention shall be paid to the organs of the reproductive system.

(ii) Dead pups or pups that are terminated in a moribund condition should be examined for possible defects and/or cause of death.

(iii) At the time of necropsy, a vaginal smear should be examined to determine the stage of the estrous cycle. The uteri of all cohabited females should be examined, in a manner which does not compromise histopathological evaluation, for the presence and number of implantation sites.

(7) *Organ weights.* (i) At the time of termination, the following organs of all P and F1 parental animals shall be weighed:

(A) Uterus (with oviducts and cervix), ovaries.

(B) Testes, epididymides (total weights for both and cauda weight for either one or both), seminal vesicles (with coagulating glands and their fluids), and prostate.

(C) Brain, pituitary, liver, kidneys, adrenal glands, spleen, and known target organs.

(ii) For F1 and F2 weanlings that are examined macroscopically, the following organs shall be weighed for one randomly selected pup per sex per litter.

(A) Brain.

(B) Spleen and thymus.

(8) *Tissue preservation.* The following organs and tissues, or representative samples thereof, shall be fixed and stored in a suitable medium for histopathological examination.

(i) For the parental (P and F1) animals:

(A) Vagina, uterus with oviducts, cervix, and ovaries.

(B) One testis (preserved in Bouins fixative or comparable preservative), one epididymis, seminal vesicles, prostate, and coagulating gland.

(C) Pituitary and adrenal glands.

(D) Target organs, when previously identified, from all P and F1 animals selected for mating.

(E) Grossly abnormal tissue.

(ii) For F1 and F2 weanlings selected for macroscopic examination: Grossly abnormal tissue and target organs, when known.

(9) *Histopathology*—(i) *Parental animals.* Full histopathology of the organs listed under paragraph (e)(8)(i) of this section shall be performed for ten randomly chosen high dose and control P and F1 animals per sex, for those animals that were selected for mating. Organs demonstrating treatment-related changes shall also be examined for the remainder of the high-dose and control animals and for all parental animals in the low- and mid-dose groups. Additionally, reproductive organs of the low- and mid-dose animals suspected of reduced fertility, e.g., those that failed to mate, conceive, sire, or deliver healthy offspring, or for which estrous cyclicity or sperm number, motility, or morphology were affected, shall be subjected to histopathological evaluation. Besides gross lesions such as atrophy or tumors, testicular histopathological examination should be conducted in order to to identify treatment-related effects such as retained spermatids, missing germ cell layers or types, multinucleated giant cells, or sloughing of spermatogenic cells into the lumen. Examination of the intact epididymis should include the caput, corpus, and cauda, which can be accomplished by evaluation of a longitudinal section, and should be conducted in order to identify such lesions as sperm granulomas, leukocytic

infiltration (inflammation), aberrant cell types within the lumen, or the absence of clear cells in the cauda epididymal epithelium. The postlactational ovary should contain primordial and growing follicles as well as the large corpora lutea of lactation. Histopathological examination should detect qualitative depletion of the primordial follicle population. A quantitative evaluation of primordial follicles should be conducted for all F1 females if any of the following treatment-related findings were observed:

(A) Reductions in ovarian weight and abnormal ovarian histopathology findings, e.g., follicular cysts or qualitative evidence of a reduced population of primordial follicles.

(B) Abnormal estrous cyclicity and female infertility.

(C) Depletion of testicular spermatid counts in F1 males and evidence of germ cell depletion in testicular histopathology evaluations.

(ii) *Examination of ovarian sections.* If a quantitative evaluation is performed, ten ovarian sections shall be taken at least 100 µm apart from the inner third of each ovary. Examination should include enumeration of the total number of primordial and antral follicles from these 20 sections (the technique for this histological assessment as described in the reference under paragraph (g)(2) of this section may be used) for comparison with control ovaries.

(iii) *Weanlings.* For F1 and F2 weanlings, histopathological examination of treatment-related abnormalities noted at macroscopic examination should be considered, if such evaluation were deemed appropriate and would contribute to the interpretation of the study data.

(f) *Data and reporting*—(1) *Treatment of results.* Data shall be reported individually and summarized in tabular form, showing for each test group the types of change and the number of animals displaying each type of change.

(2) *Evaluation of study results.* (i) An evaluation of test results, including the statistical analysis, shall be provided. This should include an evaluation of the relationship, or lack thereof, between the exposure of the animals to the test substance and the incidence and severity of all abnormalities.

(ii) When appropriate, historical control data should be used to enhance interpretation of study results. Historical data, when used, should be compiled, presented, and analyzed in an appropriate and relevant manner. In order to justify its use as an analytical tool, information such as the dates of study conduct, the strain and source of the animals, and the vehicle and route of administration should be included.

(iii) Statistical analysis of the study findings should include sufficient information on the method of analysis, so that an independent reviewer/statistician can reevaluate and reconstruct the analysis.

(iv) In any study which demonstrates an absence of toxic effects, further investigation to establish absorption and bioavailability of the test substance should be considered.

(3) *Test report.* In addition to the reporting requirements as specified under 40 CFR part 792, subpart J, the following specific information shall be reported. Both individual and summary data should be presented.

(i) Species and strain.

(ii) Toxic response data by sex and dose, including indices of mating, fertility, gestation, birth, viability, and lactation; offspring sex ratio; precoital interval, including the number of days until mating and the number of estrous periods until mating; and duration of gestation calculated from day 0 of pregnancy. The report should provide the numbers used in calculating all indices.

(iii) Day (week) of death during the study or whether animals survived to termination; date (age) of litter termination.

(iv) Toxic or other effects on reproduction, offspring, or postnatal growth.

(v) Developmental milestone data (mean age of vaginal opening and preputial separation, and mean anogenital distance, when measured).

(vi) Number of P and F1 females cycling normally and mean estrous cycle length.

(vii) Day (week) of observation of each abnormal sign and its subsequent course.

(viii) Body weight and body weight change data by sex for P, F1, and F2 animals.

(ix) Food (and water, if applicable) consumption, food efficiency (body weight gain per gram of food consumed), and test material consumption for P and F1 animals, except for the period of cohabitation.

(x) Total cauda epididymal sperm number, homogenization-resistant testis spermatid number, number and percent of progressively motile sperm, number and percent of morphologically normal sperm, and number and percent of sperm with each identified anomaly.

(xi) Stage of the estrous cycle at the time of termination for P and F1 parental females.

(xii) Necropsy findings.

(xiii) Implantation data and postimplantation loss calculations for P and F1 parental females.

(xiv) Absolute and adjusted organ weight data.

(xv) Detailed description of all histopathological findings.

(xvi) Adequate statistical treatment of results.

(xvii) A copy of the study protocol and any amendments should be included.

(g) *References.* For additional backgound information on this test guideline, the following references should be consulted. These references are available for inspection at the TSCA Nonconfidential Information Center, Rm. NE–B607, Environmental Protection Agency, 401 M St., SW., Washington, DC, 12 noon to 4 p.m., Monday through Friday, except legal holidays.

(1) Gray, L.E. *et al.* A dose-response analysis of methoxychlor-induced alterations of reproductive development and function in the rat. *Fundamental and Applied Toxicology.* 12:92–108 (1989).

(2) Heindel, J.J. *et al.* Ed. Hirshfield, A.N. Histological assessment of ovarian follicle number in mice as a screen of ovarian toxicity. *Growth Factors and the Ovary* (Plenum, NY, 1989) pp. 421–426.

(3) Korenbrot, C.C. *et al.* Preputial separation as an external sign of pubertal development in the male rat. *Biology of Reproduction.* 17:298–303 (1977).

(4) Linder, R.E. *et al.* Endpoints of spermatoxicity in the rat after short duration exposures to fourteen reproductive toxicants. *Reproductive Toxicology.* 6:491–505 (1992).

(5) Manson, J.M. and Kang, Y.J. Ed. Hayes, A.W. Test methods for assessing female reproductive and developmental toxicology. *Principles and Methods of Toxicology* (Raven, NY, 1989).

(6) Organisation for Economic Co-operation and Development, No. 416: Two Generation Reproduction Toxicity Study, Guidelines for Testing of Chemicals. [C(83)44 (Final)] (1983).

(7) Pederson, T. and Peters, H. Proposal for classification of oocytes and follicles in the mouse ovary. *Journal of Reproduction and Fertility.* 17:555–557 (1988).

(8) Seed, J., Chapin, R.E. E.D. Clegg, L.A. Dostal, R.H. Foote, M.E. Hurtt, G.R. Klinefelter, S.L. Makris, S.D. Perreault, S. Schrader, D. Seyler, R. Sprando, K.A. Treinen, D.N.R. Veeramachaneni, and Wise, L.D. Methods for assessing sperm motility, morphology, and counts in the rat, rabbit, and dog: a consensus report. *Reproductive Toxicology.* 10(3):237–244 (1996).

(9) Smith, B.J. *et al.* Comparison of random and serial sections in assessment of ovarian toxicity. *Reproductive Toxicology.* 5:379–383 (1991).

(10) Thomas, J.A. Eds. M.O. Amdur, J. Doull, and C.D. Klaassen. Toxic responses of the reproductive system. *Casarett and Doull's Toxicology* (Pergamon, NY, 1991).

(11) Working, P.K. and Hurtt, M. Computerized videomicrographic analysis of rat sperm motility. *Journal of Andrology.* 8:330–337 (1987).

(12) Zenick, H. *et al.* Ed. Hayes, A.W. Assessment of male reproductive toxicity: a risk assessment approach. *Principles and Methods of Toxicology* (Raven, NY, 1994).

§ 799.9420 TSCA carcinogenicity.

(a) *Scope.* This section is intended to meet the testing requirements under section 4 of TSCA. The objective of a long-term carcinogenicity study is to observe test animals for a major portion of their life span for development of neoplastic lesions during or after exposure to various doses of a test substance by an appropriate route of administration.

(b) *Source.* The source material used in developing this TSCA test guideline is the OPPTS harmonized test guideline 870.4200 (June 1996 Public Draft). This source is available at the address in paragraph (g) of this section.

(c) *Definitions.* The following definitions apply to this section.

Carcinogenicity is the development of neoplastic lesions as a result of the repeated daily exposure of experimental animals to a chemical by the oral, dermal, or inhalation routes of exposure.

Cumulative toxicity is the adverse effects of repeated dose occurring as a result of prolonged action on, or increased concentration of, the administered test substance or its metabolites in susceptible tissues.

Dose in a carcinogenicity study is the amount of test substance administered via the oral, dermal or inhalation routes for a period of up to 24 months. Dose is expressed as weight of the test substance (grams, milligrams) per unit body weight of test animal (milligram per kilogram), or as weight of the test substance in parts per million (ppm) in food or drinking water. When exposed via inhalation, dose is expressed as weight of the test substance per unit volume of air (milligrams per liter) or as parts per million.

Target organ is any organ of a test animal showing evidence of an effect induced by a test substance.

(d) *Test procedures*—(1) *Animal selection*—(i) *Species and strain.* Testing shall be performed on two mammalian species. Rats and mice are the species of choice because of their relatively short life spans, limited cost of maintenance, widespread use in pharmacological and toxicological studies, susceptibility to tumor induction, and the availability of inbred or sufficiently characterized strains. Commonly used laboratory strains shall be used. If other mammalian species are used, the tester shall provide justification/reasoning for their selection.

(ii) *Age/weight.* (A) Testing shall be started with young healthy animals as soon as possible after weaning and acclimatization.

(B) Dosing should generally begin no later than 8 weeks of age.

(C) At commencement of the study, the weight variation of animals used shall not exceed ±20% of the mean weight for each sex.

(D) Studies using prenatal or neonatal animals may be recommended under special conditions.

(iii) *Sex.* (A) Equal numbers of animals of each sex shall be used at each dose level.

(B) Females shall be nulliparous and nonpregnant.

(iv) *Numbers.* (A) At least 100 rodents (50 males and 50 females) shall be used at each dose level and concurrent control group.

(B) If interim sacrifices are planned, the number shall be increased by the number of animals scheduled to be sacrificed during the course of the study.

(C) For a meaningful and valid statistical evaluation of long term exposure and for a valid interpretation of negative results, the number of animals in any group should not fall below 50% at 15 months in mice and 18 months in rats. Survival in any group should not fall below 25% at 18 months in mice and 24 months in rats.

(D) The use of adequate randomization procedures for the proper allocation of animals to test and control groups is required to avoid bias.

(E) Each animal shall be assigned a unique identification number. Dead animals, their preserved organs and tissues, and microscopic slides shall be identified by reference to the unique numbers assigned.

(v) *Husbandry.* (A) Animals may be group-caged by sex, but the number of animals per cage must not interfere with clear observation of each animal. The biological properties of the test substance or toxic effects (e.g., morbidity, excitability) may indicate a need for individual caging. Animals should be housed individually in dermal studies and during exposure in inhalation studies.

(B) The temperature of the experimental animal rooms should be at 22±3 °C.

(C) The relative humidity of the experimental animal rooms should be 30 to 70%.

(D) Where lighting is artificial, the sequence should be 12 h light/12 h dark.

(E) Control and test animals should be fed from the same batch and lot. The feed should be analyzed to assure uniform distribution and adequacy of nutritional requirements of the species tested and for impurities that might influence the outcome of the test. Animals should be fed and watered ad libitum with food replaced at least weekly.

(F) The study should not be initiated until animals have been allowed a period of acclimatization/quarantine to environmental conditions, nor should animals from outside sources be placed on test without an adequate period of quarantine.

(2) *Control and test substances.* (i) Where necessary, the test substance is dissolved or suspended in a suitable vehicle. If a vehicle or diluent is needed, it should not elicit toxic effects itself. It is recommended that wherever possible the use of an aqueous solution be considered first, followed by consideration of solution in oil, and finally solution in other vehicles.

(ii) One lot of the test substance should be used, if possible, throughout the duration of the study, and the research sample should be stored under conditions that maintain its purity and stability. Prior to the initiation of the study, there should be a characterization of the test substance, including the purity of the test compound, and, if possible, the name and quantities of contaminants and impurities.

(iii) If the test or control substance is to be incorporated into feed or another vehicle, the period during which the test substance is stable in such a mixture should be determined prior to the initiation of the study. Its homogeneity and concentration should be determined prior to the initiation of the study and periodically during the study. Statistically randomized samples of the mixture should be analyzed to ensure that proper mixing, formulation, and storage procedures are being followed, and that the appropriate concentration of the test or control substance is contained in the mixture.

(3) *Control groups.* A concurrent control group (50 males and 50 females) is required. This group shall be

untreated or if a vehicle is used in administering the test substance, a vehicle control group. If the toxic properties of the vehicle are not known, both untreated and vehicle control groups are required.

(4) *Dose levels and dose selection.* (i) For risk assessment purposes, at least three dose levels shall be used, in addition to the concurrent control group. Dose levels should be spaced to produce a gradation of effects. A rationale for the doses selected must be provided.

(ii) The highest dose level should elicit signs of toxicity without substantially altering the normal life span due to effects other than tumors. The highest dose should be determined based on the findings from a 90–day study to ensure that the dose used is adequate to asses the carcinogenic potential of the test substance. Thus, the selection of the highest dose to be tested is dependent upon changes observed in several toxicological parameters in subchronic studies. The highest dose tested need not exceed 1,000 mg/kg/day.

(iii) The intermediate-dose level should be spaced to produce a gradation of toxic effects.

(iv) The lowest dose level should produce no evidence of toxicity.

(v) For skin carcinogenicity studies, when toxicity to the skin is a determining factor, the highest dose selected should not destroy the functional integrity of the skin, the intermediate dose should be a minimally irritating dose, and the low dose should be the highest nonirritating dose.

(vi) The criteria for selecting the dose levels for skin carcinogenicity studies, based on gross and histopathologic dermal lesions, are as follows:

(A) Gross criteria for reaching the high dose:

(*1*) Erythema (moderate).

(*2*) Scaling.

(*3*) Edema (mild).

(*4*) Alopecia.

(*5*) Thickening.

(B) Histologic criteria for reaching the high dose:

(*1*) Epidermal hyperplasia.

(*2*) Epidermal hyperkeratosis.

(*3*) Epidermal parakeratosis.

(*4*) Adnexal atrophy/hyperplasia.

(*5*) Fibrosis.

(*6*) Spongiosis (minimal-mild).

(*7*) Epidermal edema (minimal-mild).

(*8*) Dermal edema (minimal-moderate).

(*9*) Inflammation (moderate).

(C) Gross criteria for exceeding the high dose:

(*1*) Ulcers, fissures.

(*2*) Exudate/crust (eschar).

(*3*) nonviable (dead) tissues.

(*4*) Anything leading to destruction of the functional integrity of the epidermis (e.g., caking, fissuring, open sores, eschar).

(D) Histologic criteria for exceeding the high dose:

(*1*) Crust (interfollicular and follicular).

(*2*) Microulcer.

(*3*) Degeneration/necrosis (mild to moderate).

(*4*) Epidermal edema (moderate to marked).

(*5*) Dermal edema (marked).

(*6*) Inflammation (marked).

(5) *Administration of the test substance.* The three main routes of administration are oral, dermal, and inhalation. The choice of the route of administration depends upon the physical and chemical characteristics of the test substance and the form typifying exposure in humans.

(i) *Oral studies.* If the test substance is administered by gavage, the animals are dosed with the test substance on a 7–day per week basis for a period of at least 18 months for mice and hamsters and 24 months for rats. However, based primarily on practical considerations, dosing by gavage or via a capsule on a 5–day per week basis is acceptable. If the test substance is administered in the drinking water or mixed in the diet, then exposure should be on a 7–day per week basis.

(ii) *Dermal studies.* (A) The animals should be treated with the test substance for at least 6 h/day on a 7–day per week basis for a period of at least 18 months for mice and hamsters and 24 months for rats. However, based primarily on practical considerations, application on a 5–day per week basis is acceptable. Dosing should be conducted at approximately the same time each day.

(B) Fur should be clipped weekly from the dorsal area of the trunk of the test animals. Care should be taken to avoid abrading the skin which could alter its permeability. A minimum of 24 hrs should be allowed for the skin to recover before the next dosing of the animal.

(C) The test substance shall be applied uniformly over a shaved area which is approximately 10% of the total body surface area. In order to dose approximately 10% of the body surface, the area starting at the scapulae (shoulders) to the wing of the ileum (hipbone) and half way down the flank on each side of the animal should be shaved. The volume of application should be kept constant and should not exceed 100 μL for the mouse and 300 μL for the rat; different concentrations of the test solution should be prepared for different dose levels. With highly toxic substances, the surface area covered may be less, but as much of the area as possible should be covered with as thin and uniform a film as practical. The test material is not removed after application.

(D) During the exposure period, the application site should not be covered when mice or hamsters are the species of choice. For rats, the test substance may be held in contact with the skin with a porous gauze dressing and nonirritating tape if necessary. The test site should be further covered in a suitable manner to retain the gauze dressing and test substance and ensure that the animals cannot ingest the test substance.

(iii) *Inhalation studies.* (A) The animals should be exposed to the test substance for 6 h/day on a 7–day per week basis, for a period of at least 18 months in mice and 24 months in rats. However, based primarily on practical considerations, exposure for 6 h/day on a 5–day per week basis is acceptable.

(B) The animals shall be tested in dynamic inhalation equipment designed to sustain a minimum air flow of 10 air changes per hr, an adequate oxygen content of at least 19%, and uniform conditions throughout the exposure chamber. Maintenance of slight negative pressure inside the chamber will prevent leakage of the test substance into surrounding areas.

(C) The selection of a dynamic inhalation chamber should be appropriate for the test substance and test system. Where a whole body chamber is used to expose animals to an aerosol, individual housing must be used to minimize crowding of the test animals and maximize their exposure to the test substance. To ensure stability of a chamber atmosphere, the total volume occupied by the test animals shall not exceed 5% of the volume of the test chamber. It is recommended, but not required, that nose-only or head-only exposure be used for aerosol studies in order to minimize oral exposures due to animals licking compound off their fur. Heat stress to the animals should be minimized.

(D) The temperature at which the test is performed should be maintained at 22±2 °C. The relative humidity should be maintained between 40 to 60%, but in certain instances (e.g., tests of aerosols, use of water vehicle) this may not be practicable.

(E) The rate of air flow shall be monitored continuously but recorded at least three times during exposure.

(F) Temperature and humidity shall be monitored continuously but should be recorded at least every 30 minutes.

(G) The actual concentrations of the test substance shall be measured in the breathing zone. During the exposure period, the actual concentrations of the test substance should be held as constant as practicable, monitored continuously or intermittently depending on the method of analysis. Chamber concentrations may be measured using gravimetric or analytical methods as appropriate. If trial run measurements are reasonably consistent (± 10% for liquid aerosol, gas, or dry aerosol), the two measurements should be sufficient. If measurements are not consistent, then three to four measurements should be taken.

(H) During the development of the generating system, particle size analysis shall be performed to establish the stability of aerosol concentrations with respect to particle size. Measurement of aerodynamic particle size in the animals's breathing zone should be measured during a trial run. If median aerodynamic diameter (MMAD) values for each exposure level are within 10% of each other, then two measurements during the exposures should be sufficient. If pretest measurements are not within 10% of each other, three to four measurements should be taken. The MMAD particle size range should be between 1–3 μm. The particle size of hygroscopic materials should be small enough to allow pulmonary deposition once the particles swell in the moist environment of the respiratory tract.

(I) Feed shall be withheld during exposure. Water may also be withheld during exposure.

(6) *Observation period.* It is necessary that the duration of the carcinogenicity study comprise the majority of the normal life span of the strain of animals used. This time period shall not be less than 24 months for rats and 18 months for mice, and ordinarily not longer than 30 months for rats and 24 months for mice. For longer time periods, and where any other species are used, consultation with the Agency in regard to the duration of the study is advised.

(7) *Observation of animals.* (i) Observations shall be made at least once each day for morbidity and mortality. Appropriate actions should be taken to minimize loss of animals from the study (e.g., necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals).

(ii) A careful clinical examination shall be made at least once weekly. Observations should be detailed and carefully recorded, preferably using explicitly defined scales. Observations should include, but not be limited to, evaluation of skin and fur, eyes and mucous membranes, respiratory and circulatory effects, autonomic effects such as salivation, central nervous system effects, including tremors and convulsions, changes in the level of activity, gait and posture, reactivity to handling or sensory stimuli, altered strength and stereotypes or bizarre behavior (e.g., self-mutilation, walking backwards).

(iii) Body weights shall be recorded individually for all animals; once a week during the first 13 weeks of the study and at least once every 4 weeks, thereafter, unless signs of clinical toxicity suggest more frequent weighing to facilitate monitoring of health status.

(iv) When the test substance is administered in the feed or drinking water, measurements of feed or water consumption, respectively, should be determined weekly during the first 13 weeks of the study and then at approximately monthly intervals unless health status or body weight changes dictate otherwise.

(v) Moribund animals shall be removed and sacrificed when noticed and the time of death should be recorded as precisely as possible. At the end of the study period, all survivors shall be sacrificed.

(8) *Clinical pathology.* At 12 months, 18 months, and at terminal sacrifice, a blood smear shall be obtained from all animals. A differential blood count should be performed on blood smears from those animals in the highest dosage group and the controls from the terminal sacrifice. If these data, or data from the pathological examination indicate a need, then the 12– and 18–month blood smears should also be examined. Differential blood counts should be performed for the next lower groups if there is a major discrepancy between the highest group and the controls. If clinical observations suggest a deterioration in health of the animals during the study, a differential blood count of the affected animals shall be performed.

(9) *Gross necropsy.* (i) A complete gross examination shall be performed on all animals, including those that died during the experiment or were killed in a moribund condition.

(ii) The liver, lungs, kidneys, brain, and gonads should be trimmed and weighed wet as soon as possible after dissection to avoid drying. The organs should be weighed from interim sacrifice animals as well as from at least 10 animals per sex per group at terminal sacrifice.

(iii) The following organs and tissues, or representative samples thereof, shall be preserved in a suitable medium for possible future histopathological examination.

(A) Digestive system.
(*1*) Salivary glands.
(*2*) Esophagus.
(*3*) Stomach.
(*4*) Duodenum.
(*5*) Jejunum.
(*6*) Ileum.
(*7*) Cecum.
(*8*) Colon.
(*9*) Rectum.
(*10*) Liver.
(*11*) Pancreas.
(*12*) Gallbladder (mice).
(*13*) Bile duct (rat).
(B) Nervous system.
(*1*) Brain (multiple sections).
(*2*) Pituitary.
(*3*) Peripheral nerves.
(*4*) Spinal cord (three levels).
(*5*) Eyes (retina, optic nerve).
(C) Glandular system.
(*1*) Adrenals.
(*2*) Parathyroids.
(*3*) Thyroids.
(D) Respiratory system.
(*1*) Trachea.
(*2*) Lung.
(*3*) Pharynx.
(*4*) Larynx.
(*5*) Nose (inhalation studies only).
(E) Cardiovascular/hematopoietic system.
(*1*) Aorta (thoracic).
(*2*) Heart.
(*3*) Bone marrow.
(*4*) Lymph nodes.
(*5*) Spleen.
(*6*) Thymus.
(F) Urogenital system.
(*1*) Kidneys.
(*2*) Urinary bladder.
(*3*) Prostate.
(*4*) Testes/epididymides.
(*5*) Seminal vesicles.
(*6*) Uterus.
(*7*) Ovaries.
(G) Other.
(*1*) Lacrimal gland.
(*2*) Mammary gland.
(*3*) Skin.
(*4*) Skeletal muscle.
(*5*) All gross lesions and masses.
(*6*) Sternum and/or femur.

(iv) In inhalation studies, the entire respiratory tract, including nose, pharynx, larynx, and paranasal sinuses should be examined and preserved. In dermal studies, skin from treated and adjacent control skin sites should be examined and preserved.

(v) Inflation of lungs and urinary bladder with a fixative is the optimal method for preservation of these tissues. The proper inflation and fixation of the lungs in inhalation studies is essential for appropriate and valid histopathological examination.

(vi) Information from clinical pathology, and other in-life data should be considered before microscopic examination, since they may provide significant guidance to the pathologist.

(10) *Histopathology.* (i) The following histopathology shall be performed:

(A) Full histopathology on the organs and tissues under paragraph (d)(9) (iii) of this section of all animals in the control and high dose groups and all animals that died or were killed during the study.

(B) All gross lesions in all animals.

(C) Target organs in all animals.

(D) Lungs, liver, and kidneys of all animals. Special attention to examination of the lungs of rodents should be made for evidence of infection since this provides an assessment of the state of health of the animals.

(ii) If the results show substantial alteration of the animal's normal life span, the induction of effects that might affect a neoplastic response, or other effects that might compromise the significance of the data, the next lower dose levels shall be examined as described under paragraph (d)(11)(i) of this section.

(iii) An attempt should be made to correlate gross observations with microscopic findings.

(iv) Tissues and organs designated for microscopic examination should be fixed in 10% buffered formalin or a recognized suitable fixative as soon as necropsy is performed and no less than 48 hrs prior to trimming. Tissues should be trimmed to a maximum thickness of 0.4 cm for processing.

(e) *Data and reporting*—(1) *Treatment of results.* (i) Data shall be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, the types of lesions, and the percentage of animals displaying each type of lesion.

(ii) All observed results (quantitative and qualitative) shall be evaluated by an appropriate statistical method. Any generally accepted statistical methods may be used; the statistical methods including significance criteria shall be selected during the design of the study.

(2) *Evaluation of study results.* (i) The findings of a carcinogenicity study should be evaluated in conjunction with the findings of previous studies and considered in terms of the toxic effects, the necropsy and histopathological findings. The evaluation shall include the relationship between the dose of the test substance and the presence, incidence, and severity of abnormalities (including behavioral and clinical abnormalities), gross lesions, identified target organs, body weight changes, effects on mortality, and any other general or specific toxic effects.

(ii) In any study which demonstrates an absence of toxic effects, further investigation to establish absorption and bioavailablity of the test substance should be considered.

(iii) In order for a negative test to be acceptable, it must meet the following criteria: No more than 10% of any group is lost due to autolysis, cannibalism, or management problems; and survival in each group is no less than 50% at 15 months for mice and 18 months for rats. Survival should not fall below 25% at 18 months for mice and 24 months for rats.

(iv) The use of historical control data from an appropriate time period from the same testing laboratory (i.e., the incidence of tumors and other suspect lesions normally occurring under the same laboratory conditions and in the same strain of animals employed in the test) is helpful for assessing the significance of changes observed in the current study.

(3) *Test report.* (i) In addition to the reporting requirements as specified under 40 CFR part 792, subpart J, the following specific information shall be reported. Both individual and summary data should be presented.

(A) Test substance characterization should include:

(*1*) Chemical identification.

(*2*) Lot or batch number.

(*3*) Physical properties.

(*4*) Purity/impurities.

(*5*) Identification and composition of any vehicle used.

(B) Test system should contain data on:

(*1*) Species and strain of animals used and rationale for selection if other than that recommended.

(*2*) Age including body weight data and sex

(*3*) Test environment including cage conditions, ambient temperature, humidity, and light/dark periods.

(C) Test procedure should include the following data:

(*1*) Method of randomization used.

(*2*) Full description of experimental design and procedure.

(*3*) Dose regimen including levels, methods, and volume.

(4) *Test results*—(i) *Group animal data.* Tabulation of toxic response data by species, strain, sex, and exposure level for:

(A) Number of animals exposed.

(B) Number of animals showing signs of toxicity.

(C) Number of animals dying.

(ii) *Individual animal data.* Data should be presented as summary (group mean) as well as for individual animals.

(A) Time of death during the study or whether animals survived to termination.

(B) Time of observation of each abnormal sign and its subsequent course.

(C) Body weight data.

(D) Feed and water consumption data, when collected.

(E) Results of clinical pathology and immunotoxicity screen when performed.

(F) Necropsy findings including absolute/relative organ weight data.

(G) Detailed description of all histopathological findings.

(H) Statistical treatment of results where appropriate.

(I) Historical control data.

(iii) *Inhalation studies.* In addition, for inhalation studies the following shall be reported:

(A) *Test conditions.* The following exposure conditions shall be reported.

(*1*) Description of exposure apparatus including design, type, dimensions, source of air, system for generating particulate and aerosols, method of conditioning air, treatment of exhaust air and the method of housing the animals in a test chamber.

(*2*) The equipment for measuring temperature, humidity, and particulate aerosol concentrations and size should be described.

(B) *Exposure data.* These shall be tabulated and presented with mean values and a measure of variability (e.g. standard deviation) and should include:

(*1*) Airflow rates through the inhalation equipment.

(*2*) Temperature and humidity of air.

(*3*) Actual (analytical or gravimetric) concentration in the breathing zone.

(*4*) Nominal concentration (total amount of test substance fed into the inhalation equipment divided by volume of air).

(*5*) Particle size distribution, calculated MMAD and geometric standard deviation (GSD).

(*6*) Explanation as to why the desired chamber concentration and/or particle size could not be achieved (if applicable) and the efforts taken to comply with this aspect of the sections.

(f) *Quality assurance.* A system shall be developed and maintained to assure and document adequate performance of laboratory staff and equipment. The study shall be conducted in compliance with 40 CFR Part 792—Good Laboratory Practice Standards.

(g) *References.* For additional background information on this test guideline, the following references should be consulted. These references are available for inspection at the TSCA Nonconfidential Information Center,

Rm. NE–B607, Environmental Protection Agency, 401 M St., SW., Washington, DC, 12 noon to 4 p.m., Monday through Friday, except legal holidays.

(1) Benitz, K.F. Ed. Paget, G.E. Measurement of Chronic Toxicity. *Methods of Toxicology* (Blackwell, Oxford, 1970) pp. 82–131.

(2) Fitzhugh, O.G. Chronic Oral Toxicity, Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics. The Association of Food and Drug Officials of the United States. pp. 36–45 (1959, 3rd Printing 1975).

(3) Goldenthal, E.I. and D'Aguanno, W. Evaluation of Drugs, Appraisal of the Safety of Chemicals in Foods, Drugs, and Cosmetics. The Association of Food and Drug Officials of the United States. pp. 60–67 (1959, 3rd Printing 1975).

(4) Organisation for Economic Co-operation and Development. Guidelines for Testing of Chemicals, Section 4–Health Effects, Part 451 Carcinogenicity Studies (Paris, 1981).

(5) Page, N.P. Chronic Toxicity and Carcinogenicity Guidelines. *Journal of Environmental Pathology and Toxicology.* 11:161–182 (1977).

(6) Page, N.P. Eds. Kraybill and Mehlman. Concepts of a Bioassay Program in Environmental Carcinogenesis. Vol.3. *Advances in Modern Toxicology* (Hemisphere, Washington, DC., 1977) pp. 87–171.

(7) Sontag, J.M. *et al.* Guidelines for Carcinogen Bioassay in Small Rodents. NCI-CS-TR-1 United States Cancer Institute, Division of Cancer Control and Prevention, Carcinogenesis Bioassay Program (Bethesda, MD).

§ 799.9510 TSCA bacterial reverse mutation test.

(a) *Scope.* This section is intended to meet the testing requirements under section 4 of TSCA.

(1) The bacterial reverse mutation test uses amino-acid requiring strains of *Salmonella typhimurium* and *Escherichia coli* to detect point mutations, which involve substitution, addition or deletion of one or a few DNA base pairs. The principle of this bacterial reverse mutation test is that it detects mutations which revert mutations present in the test strains and restore the functional capability of the bacteria to synthesize an essential amino acid. The revertant bacteria are detected by their ability to grow in the absence of the amino acid required by the parent test strain.

(2) Point mutations are the cause of many human genetic diseases and there is substantial evidence that point mutations in oncogenes and tumor suppressor genes of somatic cells are involved in tumor formation in humans and experimental animals. The bacterial reverse mutation test is rapid, inexpensive and relatively easy to perform. Many of the test strains have several features that make them more sensitive for the detection of mutations, including responsive DNA sequences at the reversion sites, increased cell permeability to large molecules and elimination of DNA repair systems or enhancement of error-prone DNA repair processes. The specificity of the test strains can provide some useful information on the types of mutations that are induced by genotoxic agents. A very large data base of results for a wide variety of structures is available for bacterial reverse mutation tests and well-established methodologies have been developed for testing chemicals with different physico-chemical properties, including volatile compounds.

(b) *Source.* The source material used in developing this TSCA test guideline are the OECD replacement guidelines for 471 and 472 (February 1997). This source is available at the address in paragraph (g) of this section.

(c) *Definitions.* The following definitions apply to this section:

A reverse mutation test in either *Salmonella typhimurium* or *Escherichia coli* detects mutation in an amino-acid requiring strain (histidine or tryptophan, respectively) to produce a strain independent of an outside supply of amino-acid.

Base pair substitution mutagens are agents that cause a base change in DNA. In a reversion test this change may occur at the site of the original mutation, or at a second site in the bacterial genome.

Frameshift mutagens are agents that cause the addition or deletion of one or more base pairs in the DNA, thus changing the reading frame in the RNA

(d) *Initial considerations.* (1) The bacterial reverse mutation test utilizes prokaryotic cells, which differ from mammalian cells in such factors as uptake, metabolism, chromosome structure and DNA repair processes. Tests conducted *in vitro* generally require the use of an exogenous source of metabolic activation. *In vitro* metabolic activation systems cannot mimic entirely the mammalian *in vivo* conditions. The test therefore does not provide direct information on the mutagenic and carcinogenic potency of a substance in mammals.

(2) The bacterial reverse mutation test is commonly employed as an initial screen for genotoxic activity and, in particular, for point mutation-inducing activity. An extensive data base has demonstrated that many chemicals that are positive in this test also exhibit mutagenic activity in other tests. There are examples of mutagenic agents which are not detected by this test; reasons for these shortcomings can be ascribed to the specific nature of the endpoint detected, differences in metabolic activation, or differences in bioavailability. On the other hand, factors which enhance the sensitivity of the bacterial reverse mutation test can lead to an overestimation of mutagenic activity.

(3) The bacterial reverse mutation test may not be appropriate for the evaluation of certain classes of chemicals, for example highly bactericidal compounds (e.g. certain antibiotics) and those which are thought (or known) to interfere specifically with the mammalian cell replication system (e.g. some topoisomerase inhibitors and some nucleoside analogues). In such cases, mammalian mutation tests may be more appropriate.

(4) Although many compounds that are positive in this test are mammalian carcinogens, the correlation is not absolute. It is dependent on chemical class and there are carcinogens that are not detected by this test because they act through other, non-genotoxic mechanisms or mechanisms absent in bacterial cells.

(e) *Test method*—(1) *Principle.* (i) Suspensions of bacterial cells are exposed to the test substance in the presence and in the absence of an exogenous metabolic activation system. In the plate incorporation method, these suspensions are mixed with an overlay agar and plated immediately onto minimal medium. In the preincubation method, the treatment mixture is incubated and then mixed with an overlay agar before plating onto minimal medium. For both techniques, after 2 or 3 days of incubation, revertant colonies are counted and compared to the number of spontaneous revertant colonies on solvent control plates.

(ii) Several procedures for performing the bacterial reverse mutation test have been described. Among those commonly used are the plate incorporation method, the preincubation method, the fluctuation method, and the suspension method. Suggestions for modifications for the testing of gases or vapors are described in the reference in paragraph (g)(12) of this section.

(iii) The procedures described in this section pertain primarily to the plate incorporation and preincubation methods. Either of them is acceptable for conducting experiments both with and without metabolic activation. Some compounds may be detected more

efficiently using the preincubation method. These compounds belong to chemical classes that include short chain aliphatic nitrosamines, divalent metals, aldehydes, azo-dyes and diazo compounds, pyrollizidine alkaloids, allyl compounds and nitro compounds. It is also recognized that certain classes of mutagens are not always detected using standard procedures such as the plate incorporation method or preincubation method. These should be regarded as ''special cases'' and it is strongly recommended that alternative procedures should be used for their detection. The following ''special cases'' could be identified (together with examples of procedures that could be used for their detection): azo-dyes and diazo compounds (alterative procedures are described in the references in paragraphs (g)(3), (g)(5), (g)(6), and (g)(13) of this section), gases and volatile chemicals (alterative procedures are described in the references in paragraphs (g)(12), (g)(14), (g)(15), and (g)(16) of this section), and glycosides (alterative procedures are described in the references in paragraphs (g)(17) and (g)(18) of this section). A deviation from the standard procedure needs to be scientifically justified.

(2) *Description*—(i) *Preparations*—(A) *Bacteria.* (*1*) Fresh cultures of bacteria should be grown up to the late exponential or early stationary phase of growth (approximately 10^9 cells per ml). Cultures in late stationary phase should not be used. The cultures used in the experiment shall contain a high titre of viable bacteria. The titre may be demonstrated either from historical control data on growth curves, or in each assay through the determination of viable cell numbers by a plating experiment.

(*2*) The culture temperature shall be 37°C.

(*3*) At least five strains of bacteria should be used. These should include four strains of *S. typhimurium* (TA1535; TA1537 or TA97a or TA97; TA98; and TA100) that have been shown to be reliable and reproducibly responsive between laboratories. These four *S. typhimurium* strains have GC base pairs at the primary reversion site and it is known that they may not detect certain oxidizing mutagens, cross-linking agents, and hydrazines. Such substances may be detected by *E.coli* WP2 strains or *S. typhimurium* TA102 (see paragraph (g)(19) of this section) which have an AT base pair at the primary reversion site. Therefore the recommended combination of strains is:

(*i*) *S. typhimurium* TA1535.

(*ii*) *S. typhimurium* TA1537 or TA97 or TA97a.

(*iii*) *S. typhimurium* TA98.

(*iv*) *S. typhimurium* TA100.

(*v*) *E. coli* WP2 *uvrA*, or *E. coli* WP2 *uvrA* (pKM101), or *S. typhimurium* TA102. In order to detect cross-linking mutagens it may be preferable to include TA102 or to add a DNA repair-proficient strain of *E.coli* [e.g. *E.coli* WP2 or *E.coli* WP2 (pKM101).]

(*4*) Established procedures for stock culture preparation, marker verification and storage should be used. The amino-acid requirement for growth should be demonstrated for each frozen stock culture preparation (histidine for *S. typhimurium* strains, and tryptophan for *E. coli* strains). Other phenotypic characteristics should be similarly checked, namely: the presence or absence of R-factor plasmids where appropriate [i.e. ampicillin resistance in strains TA98, TA100 and TA97a or TA97, WP2 *uvrA* and WP2 *uvrA* (pKM101), and ampicillin + tetracycline resistance in strain TA102]; the presence of characteristic mutations (i.e. rfa mutation in *S. typhimurium* through sensitivity to crystal violet, and *uvrA* mutation in *E. coli* or *uvrB* mutation in *S. typhimurium*, through sensitivity to ultra-violet light). The strains should also yield spontaneous revertant colony plate counts within the frequency ranges expected from the laboratory's historical control data and preferably within the range reported in the literature.

(B) *Medium.* An appropriate minimal agar (e.g. containing Vogel-Bonner minimal medium E and glucose) and an overlay agar containing histidine and biotin or tryptophan, to allow for a few cell divisions, shall be used. The procedures described in the references under paragraphs (g)(1), (g)(2), and (g)(9) of this section may be used for this analysis.

(C) *Metabolic activation.* Bacteria shall be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system. The most commonly used system is a cofactor-supplemented post-mitochondrial fraction (S9) prepared from the livers of rodents treated with enzyme-inducing agents such as Aroclor 1254 (the system described in the references under paragraphs (g)(1) and (g)(2) of this section may be used) or a combination of phenobarbitone and β-naphthoflavone (the system described in the references under paragraphs (g)(18), (g)(20), and (g)(21) of this section may be used). The post-mitochondrial fraction is usually used at concentrations in the range from 5 to 30% v/v in the S9-mix. The choice and condition of a metabolic activation system may depend upon the class of chemical being tested. In some cases it may be appropriate to utilize more than one concentration of post-mitochondrial fraction. For azo-dyes and diazo-compounds, using a reductive metabolic activation system may be more appropriate (the system described in the references under paragraphs (g)(6) and (g)(13) of this section may be used).

(D) *Test substance/preparation.* Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted if appropriate prior to treatment of the bacteria. Liquid test substances may be added directly to the test systems and/or diluted prior to treatment. Fresh preparations should be employed unless stability data demonstrate the acceptability of storage.

(ii) *Test conditions*—(A) *Solvent/vehicle.* The solvent/vehicle shall not be suspected of chemical reaction with the test substance and shall be compatible with the survival of the bacteria and the S9 activity (see paragraph (g)(22) of this section). If other than well-known solvent/vehicles are used, their inclusion should be supported by data indicating their compatibility. It is recommended that wherever possible, the use of an aqueous solvent/vehicle be considered first. When testing water-unstable substances, the organic solvents used should be free of water.

(B) *Exposure concentrations.* (*1*) Amongst the criteria to be taken into consideration when determining the highest amount of test substance to be used are cytotoxicity and solubility in the final treatment mixture. It may be useful to determine toxicity and insolubility in a preliminary experiment. Cytotoxicity may be detected by a reduction in the number of revertant colonies, a clearing or diminution of the background lawn, or the degree of survival of treated cultures. The cytotoxicity of a substance may be altered in the presence of metabolic activation systems. Insolubility should be assessed as precipitation in the final mixture under the actual test conditions and evident to the unaided eye. The recommended maximum test concentration for soluble non-cytotoxic substances is 5 mg/plate or 5 µl/plate. For non-cytotoxic substances that are not soluble at 5mg/plate or 5µl/plate, one or more concentrations tested should be insoluble in the final treatment mixture. Test substances that are cytotoxic already below 5mg/plate or 5µl/plate should be tested up to a cytotoxic concentration. The precipitate should not interfere with the scoring.

(*2*) At least five different analyzable concentrations of the test substance shall be used with approximately half

log (i.e. $\sqrt{10}$) intervals between test points for an initial experiment. Smaller intervals may be appropriate when a concentration-response is being investigated.

(*3*) Testing above the concentration of 5 mg/plate or 5µl/plate may be considered when evaluating substances containing substantial amounts of potentially mutagenic impurities.

(C) *Controls.* (*1*) Concurrent strain-specific positive and negative (solvent or vehicle) controls, both with and without metabolic activation, shall be included in each assay. Positive control concentrations that demonstrate the effective performance of each assay should be selected.

(*2*)(*i*) For assays employing a metabolic activation system, the positive control reference substance(s) should be selected on the basis of the type of bacteria strains used. The following chemicals are examples of suitable positive controls for assays with metabolic activation:

Chemical	CAS No.
9,10-Dimethylanthracene	[CAS no. 781–43–1]
7,12-Dimethylbenzanthracene	[CAS no. 57–97–6]
Congo Red (for the reductive metabolic activation method)	[CAS no. 573–58–0]
Benzo(a)pyrene	[CAS no. 50–32–8]
Cyclophosphamide (monohydrate)	[CAS no. 50–18–0]
	[CAS no. 6055–19–2]
2-Aminoanthracene	[CAS no. 613–13–8]

(*ii*) 2-Aminoanthracene should not be used as the sole indicator of the efficacy of the S9-mix. If 2-aminoanthracene is used, each batch of S9 should also be characterized with a mutagen that requires metabolic activation by microsomal enzymes, e.g., benzo(a)pyrene, dimethylbenzanthracene.

(*3*) For assays performed without metabolic activation system, examples of strain-specific positive controls are:

Chemical	CAS No.	Strain
(a) Sodium azide	[CAS no. 26628–22–8]	TA1535 and TA100
(b) 2-Nitrofluorene	[CAS no. 607–57–8]	TA 98
(c) 9-Aminoacridine or ICR 191	[CAS no. 90–45–9] or [CAS no. 17070–45–0]	TA1537, TA97 and TA97a
(d) Cumene hydroperoxide	[CAS no. 80–15–9]	TA102
(e) Mitomycin C	[CAS no. 50–07–7]	WP2 *uvrA* and TA102
(f) N-Ethyl-N-nitro-N-nitrosoguanidine or 4-nitroquinoline 1-oxide	[CAS no. 70–25–7] or [CAS no. 56–57–5]	WP2, WP2 *uvrA* and WP2 *uvrA* (pKM101)
(g) Furylfuramide (AF-2)	[CAS no. 3688–53–7]	Plasmid-containing strains

(*4*) Other appropriate positive control reference substances may be used. The use of chemical class-related positive control chemicals may be considered, when available.

(*5*) Negative controls, consisting of solvent or vehicle alone, without test substance, and otherwise treated in the same way as the treatment groups, shall be included. In addition, untreated controls should also be used unless there are historical control data demonstrating that no deleterious or mutagenic effects are induced by the chosen solvent.

(3) *Procedure*—(i) *Treatment with test substance.* (A) For the plate incorporation method, without metabolic activation, usually 0.05 ml or 0.1 ml of the test solutions, 0.1 ml of fresh bacterial culture (containing approximately 10^8 viable cells) and 0.5 ml of sterile buffer are mixed with 2.0 ml of overlay agar. For the assay with metabolic activation, usually 0.5 ml of metabolic activation mixture containing an adequate amount of post-mitochondrial fraction (in the range from 5 to 30% v/v in the metabolic activation mixture) are mixed with the overlay agar (2.0 ml), together with the bacteria and test substance/test solution. The contents of each tube are mixed and poured over the surface of a minimal agar plate. The overlay agar is allowed to solidify before incubation.

(B) For the preincubation method the test substance/test solution is preincubated with the test strain (containing approximately 10^8 viable cells) and sterile buffer or the metabolic activation system (0.5 ml) usually for 20 min. or more at 30–37 °C prior to mixing with the overlay agar and pouring onto the surface of a minimal agar plate. Usually, 0.05 or 0.1 ml of test substance/test solution, 0.1 ml of bacteria, and 0.5 ml of S9-mix or sterile buffer, are mixed with 2.0 ml of overlay agar. Tubes should be aerated during pre-incubation by using a shaker.

(C) For an adequate estimate of variation, triplicate plating should be used at each dose level. The use of duplicate plating is acceptable when scientifically justified. The occasional loss of a plate does not necessarily invalidate the assay.

(D) Gaseous or volatile substances should be tested by appropriate methods, such as in sealed vessels (methods described in the references under paragraphs (g)(12), (g)(14), (g)(15), and (g)(16) of this section may be used).

(ii) *Incubation.* All plates in a given assay shall be incubated at 37 °C for 48–72 hrs. After the incubation period, the number of revertant colonies per plate is counted.

(f) *Data and reporting*—(1) *Treatment of results.* (i) Data shall be presented as the number of revertant colonies per plate. The number of revertant colonies on both negative (solvent control, and

untreated control if used) and positive control plates shall also be given.

(ii) Individual plate counts, the mean number of revertant colonies per plate and the standard deviation shall be presented for the test substance and positive and negative (untreated and/or solvent) controls.

(iii) There is no requirement for verification of a clear positive response. Equivocal results shall be clarified by further testing preferably using a modification of experimental conditions. Negative results need to be confirmed on a case-by-case basis. In those cases where confirmation of negative results is not considered necessary, justification should be provided. Modification of study parameters to extend the range of conditions assessed should be considered in follow-up experiments. Study parameters that might be modified include the concentration spacing, the method of treatment (plate incorporation or liquid preincubation), and metabolic activation conditions.

(2) *Evaluation and interpretation of results.* (i) There are several criteria for determining a positive result, such as a concentration-related increase over the range tested and/or a reproducible increase at one or more concentrations in the number of revertant colonies per plate in at least one strain with or without metabolic activation system. Biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results. However, statistical significance should not be the only determining factor for a positive response.

(ii) A test substance for which the results do not meet the criteria described under paragraph (f)(2)(i) of this section is considered non-mutagenic in this test

(iii) Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance. Results may remain equivocal or questionable regardless of the number of times the experiment is repeated.

(iv) Positive results from the bacterial reverse mutation test indicate that a substance induces point mutations by base substitutions or frameshifts in the genome of either *Salmonella typhimurium* and/or *Escherichia coli*. Negative results indicate that under the test conditions, the test substance is not mutagenic in the tested species.

(3) *Test report.* In addition to the reporting requirements as specified under 40 CFR part 792, subpart J, the following specific information shall be reported. Both individual and summary data should be presented.

(i) Test substance:

(A) Identification data and CAS no., if known.

(B) Physical nature and purity.

(C) Physicochemical properties relevant to the conduct of the study.

(D) Stability of the test substance, if known.

(ii) Solvent/vehicle:

(A) Justification for choice of solvent/vehicle.

(B) Solubility and stability of the test substance in solvent/vehicle, if known.

(iii) Strains:

(A) Strains used.

(B) Number of cells per culture.

(C) Strain characteristics.

(iv) Test conditions:

(A) Amount of test substance per plate (mg/plate or ml/plate) with rationale for selection of dose and number of plates per concentration.

(B) Media used.

(C) Type and composition of metabolic activation system, including acceptability criteria.

(D) Treatment procedures.

(v) Results:

(A) Signs of toxicity.

(B) Signs of precipitation.

(C) Individual plate counts.

(D) The mean number of revertant colonies per plate and standard deviation.

(E) Dose-response relationship, where possible.

(F) Statistical analyses, if any.

(G) Concurrent negative (solvent/vehicle) and positive control data, with ranges, means and standard deviations.

(H) Historical negative (solvent/vehicle) and positive control data, with e.g. ranges, means and standard deviations.

(vi) Discussion of the results.

(vii) Conclusion.

(g) *References.* For additional background information on this test guideline, the following references should be consulted. These references are available for inspection at the TSCA Nonconfidential Information Center, Rm. NE–B607, Environmental Protection Agency, 401 M St., SW., Washington, DC, 12 noon to 4 p.m., Monday through Friday, except legal holidays.

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§ 799.9530 TSCA in vitro mammalian cell gene mutation test.

(a) *Scope.* This section is intended to meet the testing requirements under section 4 of TSCA. The *in vitro* mammalian cell gene mutation test can be used to detect gene mutations induced by chemical substances. Suitable cell lines include L5178Y mouse lymphoma cells, the CHO, AS52 and V79 lines of Chinese hamster cells, and TK6 human lymphoblastoid cells under paragraph (g)(1) of this section. In these cell lines the most commonly-used genetic endpoints measure mutation at thymidine kinase (TK) and hypoxanthine-guanine phosphoribosyl transferase (HPRT), and a transgene of xanthine-guanine phosphoribosyl transferase (XPRT). The TK, HPRT and XPRT mutation tests detect different spectra of genetic events. The autosomal location of TK and XPRT may allow the detection of genetic events (e.g. large deletions) not detected at the HPRT locus on X-chromosomes (For a discussion see the references in paragraphs (g)(2), (g)(3), (g)(4),(g)(5), and (g)(6) of this section).

(b) *Source.* The source material used in developing this TSCA test guideline is the OECD guideline 476 (February 1997). This source is available at the address in paragraph (g) of this section.

(c) *Definitions.* The following definitions apply to this section:

Base pair substitution mutagens are substances which cause substitution of one or several base pairs in the DNA.

Forward mutation is a gene mutation from the parental type to the mutant form which gives rise to an alteration or a loss of the enzymatic activity or the function of the encoded protein.

Frameshift mutagens are substances which cause the addition or deletion of single or multiple base pairs in the DNA molecule.

Mutant frequency is the number of mutant cells observed divided by the number of viable cells.

Phenotypic expression time is a period during which unaltered gene products are depleted from newly mutated cells.

Relative suspension growth is an increase in cell number over the expression period relative to the negative control.

Relative total growth is an increase in cell number over time compared to a control population of cells; calculated as the product of suspension growth relative to the negative control times cloning efficiency relative to negative control.

Survival is the cloning efficiency of the treated cells when plated at the end of the treatment period; survival is usually expressed in relation to the survival of the control cell population.

Viability is the cloning efficiency of the treated cells at the time of plating in selective conditions after the expression period.

(d) *Initial considerations.* (1) In the *in vitro* mammalian cell gene mutation test, cultures of established cell lines or cell strains can be used. The cells used are selected on the basis of growth ability in culture and stability of the spontaneous mutation frequency. Tests conducted *in vitro* generally require the use of an exogenous source of metabolic activation. This metabolic activation system cannot mimic entirely the mammalian *in vivo* conditions. Care should be taken to avoid conditions which would lead to results not reflecting intrinsic mutagenicity. Positive results which do not reflect intrinsic mutagenicity may arise from changes in pH, osmolality or high levels of cytotoxicity.

(2) This test is used to screen for possible mammalian mutagens and carcinogens. Many compounds that are positive in this test are mammalian carcinogens; however, there is not a perfect correlation between this test and carcinogenicity. Correlation is dependent on chemical class and there is increasing evidence that there are carcinogens that are not detected by this test because they appear to act through other, non-genotoxic mechanisms or mechanisms absent in bacterial cells.

(e) *Test method*—(1) *Principle.* (i) Cells deficient in thymidine kinase (TK) due to the mutation $TK^{+/-} \rightarrow TK^{-/-}$ are resistant to the cytotoxic effects of the pyrimidine analogue trifluorothymidine (TFT). Thymidine kinase proficient cells are sensitive to TFT, which causes the inhibition of cellular metabolism and halts further cell division. Thus mutant cells are able to proliferate in the presence of TFT, whereas normal cells, which contain thymidine kinase, are not. Similarly, cells deficient in HPRT or XPRT are selected by resistance to 6-thioguanine (TG) or 8-azaguanine (AG). The properties of the test substance should be considered carefully if a base analogue or a compound related to the selective agent is tested in any of the mammalian cell gene mutation tests. For example, any suspected selective

toxicity by the test substance for mutant and non-mutant cells should be investigated. Thus, performance of the selection system/agent shall be confirmed when testing chemicals structurally related to the selective agent.

(ii) Cells in suspension or monolayer culture shall be exposed to the test substance, both with and without metabolic activation, for a suitable period of time and subcultured to determine cytotoxicity and to allow phenotypic expression prior to mutant selection. Cytotoxicity is usually determined by measuring the relative cloning efficiency (survival) or relative total growth of the cultures after the treatment period. The treated cultures shall be maintained in growth medium for a sufficient period of time, characteristic of each selected locus and cell type, to allow near-optimal phenotypic expression of induced mutations. Mutant frequency is determined by seeding known numbers of cells in medium containing the selective agent to detect mutant cells, and in medium without selective agent to determine the cloning efficiency (viability). After a suitable incubation time, colonies shall be counted. The mutant frequency is derived from the number of mutant colonies in selective medium and the number of colonies in non-selective medium.

(2) *Description*—(i) *Preparations*—(A) *Cells.* (*1*) A variety of cell types are available for use in this test including subclones of L5178Y, CHO, CHO-AS52, V79, or TK6 cells. Cell types used in this test should have a demonstrated sensitivity to chemical mutagens, a high cloning efficiency and a stable spontaneous mutant frequency. Cells should be checked for mycoplasma contamination and should not be used if contaminated.

(*2*) The test should be designed to have a predetermined sensitivity and power. The number of cells, cultures, and concentrations of test substance used should reflect these defined parameters. The parameters discussed in the reference under paragraph (g)(13) of this section may be used. The minimal number of viable cells surviving treatment and used at each stage in the test should be based on the spontaneous mutation frequency. A general guide is to use a cell number which is at least ten times the inverse of the spontaneous mutation frequency. However, it is recommended to utilize at least 10^6 cells. Adequate historical data on the cell system used should be available to indicate consistent performance of the test.

(B) *Media and culture conditions.* Appropriate culture media and incubation conditions (culture vessels, temperature, CO_2 concentration and humidity) shall be used. Media should be chosen according to the selective systems and cell type used in the test. It is particularly important that culture conditions should be chosen that ensure optimal growth of cells during the expression period and colony forming ability of both mutant and non-mutant cells.

(C) *Preparation of cultures.* Cells are propagated from stock cultures, seeded in culture medium and incubated at 37°C. Prior to use in this test, cultures may need to be cleansed of pre-existing mutant cells.

(D) *Metabolic activation.* Cells shall be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system. The most commonly used system is a co-factor-supplemented post-mitochondrial fraction (S9) prepared from the livers of rodents treated with enzyme-inducing agents such as Aroclor 1254 or a combination of phenobarbitone and β-naphthoflavone. The post-mitochondrial fraction is usually used at concentrations in the range from 1–10% v/v in the final test medium. The choice and condition of a metabolic activation system may depend upon the class of chemical being tested. In some cases it may be appropriate to utilize more than one concentration of post-mitochondrial fraction. A number of developments, including the construction of genetically engineered cell lines expressing specific activating enzymes, may provide the potential for endogenous activation. The choice of the cell lines used should be scientifically justified (e.g. by the relevance of the cytochrome P450 isoenzyme to the metabolism of the test substance).

(E) *Test substance/preparations.* Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted if appropriate prior to treatment of the cells. Liquid test substances may be added directly to the test systems and/or diluted prior to treatment. Fresh preparations should be employed unless stability data demonstrate the acceptability of storage.

(ii) *Test conditions*—(A) *Solvent/vehicle.* The solvent/vehicle shall not be suspected of chemical reaction with the test substance and shall be compatible with the survival of the cells and the S9 activity. If other than well-known solvent/vehicles are used, their inclusion should be supported by data indicating their compatibility. It is recommended that wherever possible, the use of an aqueous solvent/vehicle be considered first. When testing water-unstable substances, the organic solvents used should be free of water. Water can be removed by adding a molecular sieve.

(B) *Exposure concentrations.* (*1*) Among the criteria to be considered when determining the highest concentration are cytotoxicity and solubility in the test system and changes in pH or osmolality.

(*2*) Cytotoxicity should be determined with and without metabolic activation in the main experiment using an appropriate indicator of cell integrity and growth, such as relative cloning efficiency (survival) or relative total growth. It may be useful to determine cytotoxicity and solubility in a preliminary experiment.

(*3*) At least four analyzable concentrations shall be used. Where there is cytotoxicity, these concentrations shall cover a range from the maximum to little or no toxicity; this will usually mean that the concentration levels should be separated by no more than a factor between 2 and $\sqrt{10}$. If the maximum concentration is based on cytotoxicity then it shall result in approximately 10–20% but not less than 10% relative survival (relative cloning efficiency) or relative total growth. For relatively non-cytotoxic compounds the maximum concentration should be 5 mg/ml, 5 µl/ml, or 0.01 M, whichever is the lowest.

(*4*) Relatively insoluble substances should be tested up to or beyond their limit of solubility under culture conditions. Evidence of insolubility should be determined in the final treatment medium to which cells are exposed. It may be useful to assess solubility at the beginning and end of the treatment, as solubility can change during the course of exposure in the test system due to presence of cells, S9, serum etc. Insolubility can be detected by using the unaided eye. The precipitate should not interfere with the scoring.

(C) *Controls.* (*1*) Concurrent positive and negative (solvent or vehicle) controls both with and without metabolic activation shall be included in each experiment. When metabolic activation is used the positive control chemical shall be one that requires activation to give a mutagenic response.

(*2*) Examples of positive control substances include:

Metabolic Activation condition	Locus	Chemical	CAS No.
Absence of exogenous metabolic activation	HPRT	Ethylmethanesulfonate	[CAS no. 62–50–0]
		Ethylnitrosourea	[CAS no. 759–73–9]
	TK (small and large colonies).	Methylmethanesulfonate	[CAS no. 66–27–3]
	XPRT	Ethylmethanesulfonate	[CAS no. 62–50–0]
		Ethylnitrosourea	[CAS no. 759–73–9]
Presence of exogenous metabolic activation.	HPRT	3-Methylcholanthrene	[CAS no. 56–49–5]
		N-Nitrosodimethylamine	[CAS no. 62–75–9]
		7,12-Dimethylbenzanthracene	[CAS no. 57–97–6]
	TK (small and large colonies).	Cyclophosphamide (monohydrate)	[CAS no. 50–18–0] [CAS no. 6055–19–2]
		Benzo(a)pyrene	[CAS no. 50–32–8]
		3-Methylcholanthrene	[CAS no. 56–49–5]
	XPRT	N-Nitrosodimethylamine (for high levels of S-9)	[CAS no. 62–75–9]
		Benzo(a)pyrene	[CAS no. 50–32–8]

(*3*) Other appropriate positive control reference substances may be used, e.g., if a laboratory has a historical data base on 5-Bromo 2′-deoxyuridine [CAS No. 59–14–3], this reference substance could be used as well. The use of chemical class-related positive control chemicals may be considered, when available.

(*4*) Negative controls, consisting of solvent or vehicle alone in the treatment medium, and treated in the same way as the treatment groups shall be included. In addition, untreated controls should also be used unless there are historical control data demonstrating that no deleterious or mutagenic effects are induced by the chosen solvent.

(3) *Procedure*—(i) *Treatment with test substance.* (A) Proliferating cells shall be exposed to the test substance both with and without metabolic activation. Exposure shall be for a suitable period of time (usually 3 to 6 hrs is effective). Exposure time may be extended over one or more cell cycles.

(B) Either duplicate or single treated cultures may be used at each concentration tested. When single cultures are used, the number of concentrations should be increased to ensure an adequate number of cultures for analysis (e.g. at least eight analyzsable concentrations). Duplicate negative (solvent) control cultures should be used.

(C) Gaseous or volatile substances should be tested by appropriate methods, such as in sealed culture vessels. Methods described in the references under paragraphs (g)(20) and (g)(21) of this section may be used.

(ii) *Measurement of survival, viability, and mutant frequency.* (A) At the end of the exposure period, cells shall be washed and cultured to determine survival and to allow for expression of the mutant phenotype. Measurement of cytotoxicity by determining the relative cloning efficiency (survival) or relative total growth of the cultures is usually initiated after the treatment period.

(B) Each locus has a defined minimum time requirement to allow near optimal phenotypic expression of newly induced mutants (HPRT and XPRT require at least 6–8 days, and TK at least 2 days). Cells are grown in medium with and without selective agent(s) for determination of numbers of mutants and cloning efficiency, respectively. The measurement of viability (used to calculate mutant frequency) is initiated at the end of the expression time by plating in non-selective medium.

(C) If the test substance is positive in the L5178Y $TK^{+}/^{-}$ test, colony sizing should be performed on at least one of the test cultures (the highest positive concentration) and on the negative and positive controls. If the test substance is negative in the L5178Y $TK^{+}/^{-}$ test, colony sizing should be performed on the negative and positive controls. In studies using TK6$TK^{+}/^{-}$, colony sizing may also be performed.

(f) *Data and reporting*—(1) *Treatment of results.* (i) Data shall include cytotoxicity and viability determination, colony counts and mutant frequencies for the treated and control cultures. In the case of a positive response in the L5178Y $TK^{+}/^{-}$ test, colonies are scored using the criteria of small and large colonies on at least one concentration of the test substance (highest positive concentration) and on the negative and positive control. The molecular and cytogenetic nature of both large and small colony mutants has been explored in detail and is discussed in the references under paragraphs (g)(22) and (g)(23) of this section. In the $TK^{+}/^{-}$ test, colonies are scored using the criteria of normal growth (large) and slow growth (small) colonies (a scoring system similar to the one described in the reference under paragraph (g)(24) of this section may be used). Mutant cells that have suffered the most extensive genetic damage have prolonged doubling times and thus form small colonies. This damage typically ranges in scale from the losses of the entire gene to karyotypically visible chromosome aberrations. The induction of small colony mutants has been associated with chemicals that induce gross chromosome aberrations. Less seriously affected mutant cells grow at rates similar to the parental cells and form large colonies.

(ii) Survival (relative cloning efficiencies) or relative total growth shall be given. Mutant frequency shall be expressed as number of mutant cells per number of surviving cells.

(iii) Individual culture data shall be provided. Additionally, all data shall be summarized in tabular form.

(iv) There is no requirement for verification of a clear positive response. Equivocal results shall be clarified by further testing preferably using a modification of experimental conditions. Negative results need to be confirmed on a case-by-case basis. In those cases where confirmation of negative results is not considered necessary, justification should be provided. Modification of study parameters to extend the range of conditions assessed should be considered in follow-up experiments for either equivocal or negative results. Study parameters that might be modified include the concentration

spacing, and the metabolic activation conditions.

(2) *Evaluation and interpretation of results.* (i) There are several criteria for determining a positive result, such as a concentration-related, or a reproducible increase in mutant frequency. Biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results. Statistical significance should not be the only determining factor for a positive response.

(ii) A test substance, for which the results do not meet the criteria described in paragraph (f)(2)(i) of this section is considered non-mutagenic in this system.

(iii) Although most studies will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance. Results may remain equivocal or questionable regardless of the number of times the experiment is repeated.

(iv) Positive results for an *in vitro* mammalian cell gene mutation test indicate that the test substance induces gene mutations in the cultured mammalian cells used. A positive concentration-response that is reproducible is most meaningful. Negative results indicate that, under the test conditions, the test substance does not induce gene mutations in the cultured mammalian cells used.

(3) *Test report.* The test report shall include the following information:

(i) Test substance:

(A) Identification data and CAS no., if known.

(B) Physical nature and purity.

(C) Physicochemical properties relevant to the conduct of the study.

(D) Stability of the test substance.

(ii) Solvent/vehicle:

(A) Justification for choice of vehicle/solvent.

(B) Solubility and stability of the test substance in solvent/vehicle, if known.

(iii) Cells:

(A) Type and source of cells.

(B) Number of cell cultures.

(C) Number of cell passages, if applicable.

(D) Methods for maintenance of cell cultures, if applicable.

(E) Absence of mycoplasma.

(iv) Test conditions:

(A) Rationale for selection of concentrations and number of cell cultures including e.g., cytotoxicity data and solubility limitations, if available.

(B) Composition of media, CO_2 concentration.

(C) Concentration of test substance.

(D) Volume of vehicle and test substance added.

(E) Incubation temperature.

(F) Incubation time.

(G) Duration of treatment.

(H) Cell density during treatment.

(I) Type and composition of metabolic activation system including acceptability criteria.

(J) Positive and negative controls.

(K) Length of expression period (including number of cells seeded, and subcultures and feeding schedules, if appropriate).

(L) Selective agent(s).

(M) Criteria for considering tests as positive, negative or equivocal.

(N) Methods used to enumerate numbers of viable and mutant cells.

(O) Definition of colonies of which size and type are considered (including criteria for ''small'' and ''large'' colonies, as appropriate).

(v) Results:

(A) Signs of toxicity.

(B) Signs of precipitation.

(C) Data on pH and osmolality during the exposure to the test substance, if determined.

(D) Colony size if scored for at least negative and positive controls.

(E) Laboratory's adequacy to detect small colony mutants with the L5178Y $TK^{+}/-$ system, where appropriate.

(F) Dose-response relationship, where possible.

(G) Statistical analyses, if any.

(H) Concurrent negative (solvent/vehicle) and positive control data.

(I) Historical negative (solvent/vehicle) and positive control data with ranges, means, and standard deviations.

(J) Mutant frequency.

(vi) Discussion of the results.

(vii) Conclusion.

(g) *References.* For additional background information on this test guideline, the following references should be consulted. These references are available for inspection at the TSCA Nonconfidential Information Center, Rm. NE–B607, Environmental Protection Agency, 401 M St., SW., Washington, DC, 12 noon to 4 p.m., Monday through Friday, except legal holidays.

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§ 799.9538 TSCA mammalian bone marrow chromosomal aberration test.

(a) *Scope.* This section is intended to meet the testing requirements under section 4 of TSCA. The mammalian bone marrow chromosomal aberration test is used for the detection of structural chromosome aberrations induced by test compounds in bone marrow cells of animals, usually rodents. Structural chromosome aberrations may be of two types, chromosome or chromatid. An increase in polyploidy may indicate that a chemical has the potential to induce numerical aberrations. With the majority of chemical mutagens, induced aberrations are of the chromatid-type, but chromosome-type aberrations also occur. Chromosome mutations and related events are the cause of many human genetic diseases and there is substantial evidence that chromosome mutations and related events causing alterations in oncogenes and tumor suppressor genes are involved in cancer in humans and experimental systems.

(b) *Source.* The source material used in developing this TSCA test guideline is the OECD guideline 475 (February 1997). This source is available at the address in paragraph (g) of this section.

(c) *Definitions.* The following definitions apply to this section:

Chromatid-type aberration is structural chromosome damage expressed as breakage of single chromatids or breakage and reunion between chromatids.

Chromosome-type aberration is structural chromosome damage expressed as breakage, or breakage and reunion, of both chromatids at an identical site.

Endoreduplication is a process in which after an S period of DNA replication, the nucleus does not go into mitosis but starts another S period. The result is chromosomes with 2,4,8,...chromatids.

Gap is an achromatic lesion smaller than the width of one chromatid, and with minimum misalignment of the chromatids.

Numerical aberration is a change in the number of chromosomes from the normal number characteristic of the animals utilized.

Polyploidy is a multiple of the haploid chromosome number (n) other than the diploid number (i.e., 3n, 4n and so on).

Structural aberration is a change in chromosome structure detectable by microscopic examination of the metaphase stage of cell division, observed as deletions and fragments, intrachanges or interchanges.

(d) *Initial considerations.* (1) Rodents are routinely used in this test. Bone marrow is the target tissue in this test, since it is a highly vascularised tissue, and it contains a population of rapidly cycling cells that can be readily isolated and processed. Other species and target tissues are not the subject of this section.

(2) This chromosome aberration test is especially relevant to assessing mutagenic hazard in that it allows consideration of factors of *in vivo* metabolism, pharmacokinetics and DNA-repair processes although these may vary among species and among tissues. An *in vivo* test is also useful for further investigation of a mutagenic effect detected by an *in vitro* test.

(3) If there is evidence that the test substance, or a reactive metabolite, will not reach the target tissue, it is not appropriate to use this test.

(e) *Test method*—(1) *Principle.* Animals are exposed to the test substance by an appropriate route of exposure and are sacrificed at appropriate times after treatment. Prior to sacrifice, animals are treated with a metaphase-arresting agent (e.g., colchicine or Colcemid®). Chromosome preparations are then made from the bone marrow cells and stained, and metaphase cells are analyzed for chromosome aberrations.

(2) *Description*—(i) *Preparations*—(A) *Selection of animal species.* Rats, mice and Chinese hamsters are commonly used, although any appropriate mammalian species may be used. Commonly used laboratory strains of young healthy adult animals should be employed. At the commencement of the study, the weight variation of animals should be minimal and not exceed ± 20% of the mean weight of each sex.

(B) *Housing and feeding conditions.* The temperature in the experimental animal room should be 22°C (± 3°C).

Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning, the aim should be 50–60%. Lighting should be artificial, the sequence being 12 hrs light, 12 hrs dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. The choice of diet may be influenced by the need to ensure a suitable admixture of a test substance when administered by this method. Animals may be housed individually, or be caged in small groups of the same sex.

(C) *Preparation of the animals.* Healthy young adult animals shall be randomly assigned to the control and treatment groups. Cages should be arranged in such a way that possible effects due to cage placement are minimized. The animals are identified uniquely. The animals are acclimated to the laboratory conditions for at least 5 days.

(D) *Preparation of doses.* Solid test substances shall be dissolved or suspended in appropriate solvents or vehicles and diluted, as appropriate, prior to dosing of the animals. Liquid test substances may be dosed directly or diluted prior to dosing. Fresh preparations of the test substance should be employed unless stability data demonstrate the acceptability of storage.

(ii) *Test conditions*—(A) *Solvent/vehicle.* The solvent/vehicle shall not produce toxic effects at the dose levels used, and shall not be suspected of chemical reaction with the test substance. If other than well-known solvents/vehicles are used, their inclusion should be supported with data indicating their compatibility. It is recommended that wherever possible, the use of an aqueous solvent/vehicle should be considered first.

(B) *Controls.* (*1*) Concurrent positive and negative (solvent/vehicle) controls shall be included for each sex in each test. Except for treatment with the test substance, animals in the control groups should be handled in an identical manner to the animals in the treated groups.

(*2*) Positive controls shall produce structural chromosome aberrations *in vivo* at exposure levels expected to give a detectable increase over background. Positive control doses should be chosen so that the effects are clear but do not immediately reveal the identity of the coded slides to the reader. It is acceptable that the positive control be administered by a route different from the test substance and sampled at only a single time. The use of chemical class related positive control chemicals may be considered, when available. Examples of positive control substances include:

Chemical	CAS No.
Triethylenemelamine	[CAS no. 51–18–3]
Ethyl methanesulphonate	[CAS no. 62–50–0]
Ethyl nitrosourea	[CAS no. 759–73–9]
Mitomycin C	[CAS no. 50–07–7]
Cyclophosphamide (monohydrate)	[CAS no. 50–18–0] [CAS no. 6055–19–2]

(*3*) Negative controls, treated with solvent or vehicle alone, and otherwise treated in the same way as the treatment groups, shall be included for every sampling time, unless acceptable inter-animal variability and frequencies of cells with chromosome aberrations are available from historical control data. If single sampling is applied for negative controls, the most appropriate time is the first sampling time. In the absence of historical or published control data demonstrating that no deleterious or mutagenic effects are induced by the chosen solvent/vehicle, untreated controls shall be used .

(3) *Procedure*—(i) *Number and sex of animals.* Each treated and control group shall include at least 5 analyzable animals per sex. If at the time of the study there are data available from studies in the same species and using the same route of exposure that demonstrate that there are no substantial differences in toxicity between sexes, then testing in a single sex will be sufficient. Where human exposure to chemicals may be sex-specific, as for example with some pharmaceutical agents, the test should be performed with animals of the appropriate sex.

(ii) *Treatment schedule.* (A) Test substances are preferably administered as a single treatment. Test substances may also be administered as a split dose, i.e. two treatments on the same day separated by no more than a few hrs, to facilitate administering a large volume of material. Other dose regimens should be scientifically justified.

(B) Samples shall be taken at two separate times following treatment on one day. For rodents, the first sampling interval is 1.5 normal cell cycle length (the latter being normally 12–18 hr) following treatment. Since the time required for uptake and metabolism of the test substance as well as its effect on cell cycle kinetics can affect the optimum time for chromosome aberration detection, a later sample collection 24 hr after the first sample time is recommended. If dose regimens of more than one day are used, one sampling time at 1.5 normal cell cycle lengths after the final treatment should be used.

(C) Prior to sacrifice, animals shall be injected intraperitoneally with an appropriate dose of a metaphase arresting agent (e.g. Colcemid® or colchicine). Animals are sampled at an appropriate interval thereafter. For mice this interval is approximately 3–5 hrs; for Chinese hamsters this interval is approximately 4–5 hrs. Cells shall be harvested from the bone marrow and analyzed from chromosome aberrations.

(iii) *Dose levels.* If a range finding study is performed because there are no suitable data available, it shall be performed in the same laboratory, using the same species, strain, sex, and treatment regimen to be used in the main study (an approach to dose selection is presented in the reference under paragraph (g)(5) of this section). If there is toxicity, three dose levels shall be used for the first sampling time. These dose levels shall cover a range from the maximum to little or no toxicity. At the later sampling time only the highest dose needs to be used. The highest dose is defined as the dose producing signs of toxicity such that higher dose levels, based on the same dosing regimen, would be expected to produce lethality. Substances with specific biological activities at low non-toxic doses (such as hormones and mitogens) may be exceptions to the

dose-setting criteria and should be evaluated on a case-by-case basis. The highest dose may also be defined as a dose that produces some indication of toxicity in the bone marrow (e.g. greater than 50% reduction in mitotic index).

(iv) *Limit test.* If a test at one dose level of at least 2,000 mg/kg body weight using a single treatment, or as two treatments on the same day, produces no observable toxic effects, and if genotoxicity would not be expected based on data from structurally related compounds, then a full study using three dose levels may not be considered necessary. For studies of a longer duration, the limit dose is 2,000 mg/kg/body weight/day for treatment up to 14 days, and 1,000 mg/kg/body weight/day for treatment longer than 14 days. Expected human exposure may indicate the need for a higher dose level to be used in the limit test.

(v) *Administration of doses.* The test substance is usually administered by gavage using a stomach tube or a suitable intubation cannula, or by intraperitoneal injection. Other routes of exposure may be acceptable where they can be justified. The maximum volume of liquid that can be administered by gavage or injection at one time depends on the size of the test animal. The volume should not exceed 2 ml/100g body weight. The use of volumes higher than these must be justified. Except for irritating or corrosive substances which will normally reveal exacerbated effects with higher concentrations, variability in test volume should be minimized by adjusting the concentration to ensure a constant volume at all dose levels.

(vi) *Chromosome preparation.* Immediately after sacrifice, bone marrow shall be obtained, exposed to hypotonic solution and fixed. The cells shall be then spread on slides and stained.

(vii) *Analysis.* (A) The mitotic index should be determined as a measure of cytotoxicity in at least 1,000 cells per animal for all treated animals (including positive controls) and untreated negative control animals.

(B) At least 100 cells should be analyzed for each animal. This number could be reduced when high numbers of aberrations are observed. All slides, including those of positive and negative controls, shall be independently coded before microscopic analysis. Since slide preparation procedures often result in the breakage of a proportion of metaphases with loss of chromosomes, the cells scored should therefore contain a number of centromeres equal to the number $2n \pm 2$.

(f) *Data and reporting*—(1) *Treatment of results.* Individual animal data shall be presented in tabular form. The experimental unit is the animal. For each animal the number of cells scored, the number of aberrations per cell and the percentage of cells with structural chromosome aberration(s) shall be evaluated. Different types of structural chromosome aberrations shall be listed with their numbers and frequencies for treated and control groups. Gaps shall be recorded separately and reported but generally not included in the total aberration frequency. If there is no evidence for a difference in response between the sexes, the data may be combined for statistical analysis.

(2) *Evaluation and interpretation of results.* (i) There are several criteria for determining a positive result, such as a dose-related increase in the relative number of cells with chromosome aberrations or a clear increase in the number of cells with aberrations in a single dose group at a single sampling time. Biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results (some statistical methods are described in the reference under paragraph (g)(6) of this section). Statistical significance should not be the only determining factor for a positive response. Equivocal results should be clarified by further testing preferably using a modification of experimental conditions.

(ii) An increase in polyploidy may indicate that the test substance has the potential to induce numerical chromosome aberrations. An increase in endoreduplication may indicate that the test substance has the potential to inhibit cell cycle progression. This phenomenon is described in the references under paragraphs (g)(7) and (g)(8) of this section.

(iii) A test substance for which the results do not meet the criteria described in paragraph (f)(2)(i) of this section is considered non-mutagenic in this test.

(iv) Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgment about the activity of the test substance. Results may remain equivocal or questionable regardless of the number of experiments performed.

(v) Positive results from the *in vivo* chromosome aberration test indicate that a substance induces chromosome aberrations in the bone marrow of the species tested. Negative results indicate that, under the test conditions, the test substance does not induce chromosome aberrations in the bone marrow of the species tested.

(vi) The likelihood that the test substance or its metabolites reach the general circulation or specifically the target tissue (e.g., systemic toxicity) should be discussed.

(3) *Test report.* The test report shall include the following information:

(i) Test substance:

(A) Identification data and CAS No., if known.

(B) Physical nature and purity.

(C) Physicochemical properties relevant to the conduct of the study.

(D) Stability of the test substance, if known.

(ii) Solvent/vehicle:

(A) Justification for choice of vehicle.

(B) Solubility and stability of the test substance in solvent/vehicle, if known.

(iii) Test animals:

(A) Species/strain used.

(B) Number, age and sex of animals.

(C) Source, housing conditions, diet, etc.

(D) Individual weight of the animals at the start of the test, including body weight range, mean and standard deviation for each group.

(iv) Test conditions:

(A) Positive and negative (vehicle/solvent) controls.

(B) Data from range-finding study, if conducted.

(C) Rationale for dose level selection.

(D) Details of test substance preparation.

(E) Details of the administration of the test substance.

(F) Rationale for route of administration.

(G) Methods for verifying that the test substance reached the general circulation or target tissue, if applicable.

(H) Conversion from diet/drinking water test substance concentration parts per million (ppm) to the actual dose (mg/kg body weight/day), if applicable.

(I) Details of food and water quality.

(J) Detailed description of treatment and sampling schedules.

(K) Methods for measurement of toxicity.

(L) Identity of metaphase arresting substance, its concentration and duration of treatment.

(M) Methods of slide preparation.

(N) Criteria for scoring aberrations.

(O) Number of cells analyzed per animal.

(P) Criteria for considering studies as positive, negative or equivocal.

(v) Results:

(A) Signs of toxicity.

(B) Mitotic index.

(C) Type and number of aberrations, given separately for each animal.

(D) Total number of aberrations per group with means and standard deviations.

(E) Number of cells with aberrations per group with means and standard deviations.

(F) Changes in ploidy, if seen.

(G) Dose-response relationship, where possible.

(H) Statistical analyses, if any.

(I) Concurrent negative control data.

(J) Historical negative control data with ranges, means and standard deviations.

(K) Concurrent positive control data.

(vi) Discussion of the results.

(vii) Conclusion.

(g) *References.* For additional background information on this test guideline, the following references should be consulted. These references are available for inspection at the TSCA Nonconfidential Information Center, Rm. NE–B607, Environmental Protection Agency, 401 M St., SW., Washington, DC, 12 noon to 4 p.m., Monday through Friday, except legal holidays.

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(8) Huang, Y., Change, C., and Trosko, J. E. Aphidicolin-Induced Endoreduplication in Chinese Hamster Cells. *Cancer Research.* 43, 1362–1364 (1983).

§ 799.9539 TSCA mammalian erythrocyte micronucleus test.

(a) *Scope.* This section is intended to meet the testing requirements under section 4 of TSCA.

(1) The mammalian erythrocyte micronucleus test is used for the detection of damage induced by the test substance to the chromosomes or the mitotic apparatus of erythroblasts by analysis of erythrocytes as sampled in bone marrow and/or peripheral blood cells of animals, usually rodents.

(2) The purpose of the micronucleus test is to identify substances that cause cytogenetic damage which results in the formation of micronuclei containing lagging chromosome fragments or whole chromosomes.

(3) When a bone marrow erythroblast develops into a polychromatic erythrocyte, the main nucleus is extruded; any micronucleus that has been formed may remain behind in the otherwise anucleated cytoplasm. Visualization of micronuclei is facilitated in these cells because they lack a main nucleus. An increase in the frequency of micronucleated polychromatic erythrocytes in treated animals is an indication of induced chromosome damage.

(b) *Source.* The source material used in developing this TSCA test guideline is the OECD guideline 474 (February 1997). This source is available at the address in paragraph (g) of this section.

(c) *Definitions.* The following definitions apply to this section:

Centromere (kinetochore) is a region of a chromosome with which spindle fibers are associated during cell division, allowing orderly movement of daughter chromosomes to the poles of the daughter cells.

Micronuclei are small nuclei, separate from and additional to the main nuclei of cells, produced during telophase of mitosis (meiosis) by lagging chromosome fragments or whole chromosomes.

Normochromatic erythrocyte is a mature erythrocyte that lacks ribosomes and can be distinguished from immature, polychromatic erythrocytes by stains selective for ribosomes.

Polychromatic erythrocyte is a immature erythrocyte, in an intermediate stage of development, that still contains ribosomes and therefore can be distinguished from mature, normochromatic erythrocytes by stains selective for ribosomes.

(d) *Initial considerations.* (1) The bone marrow of rodents is routinely used in this test since polychromatic erythrocytes are produced in that tissue. The measurement of micronucleated immature (polychromatic) erythrocytes in peripheral blood is equally acceptable in any species in which the inability of the spleen to remove micronucleated erythrocytes has been demonstrated, or which has shown an adequate sensitivity to detect agents that cause structural or numerical chromosome aberrations. Micronuclei can be distinguished by a number of criteria. These include identification of the presence or absence of a kinetochore or centromeric DNA in the micronuclei. The frequency of micronucleated immature (polychromatic) erythrocytes is the principal endpoint. The number of mature (normochromatic) erythrocytes in the peripheral blood that contain micronuclei among a given number of mature erythrocytes can also be used as the endpoint of the assay when animals are treated continuously for 4 weeks or more. This mammalian *in vivo* micronucleus test is especially relevant to assessing mutagenic hazard in that it allows consideration of factors of *in vivo* metabolism, pharmacokinetics and DNA-repair processes although these may vary among species, among tissues and among genetic endpoints. An *in vivo* assay is also useful for further investigation of a mutagenic effect detected by an *in vitro* system.

(2) If there is evidence that the test substance, or a reactive metabolite, will not reach the target tissue, it is not appropriate to use this test.

(e) *Test method*—(1) *Principle.* Animals are exposed to the test substance by an appropriate route. If bone marrow is used, the animals are sacrificed at appropriate times after treatment, the bone marrow extracted, and preparations made and stained (test techniques described in the references under paragraphs (g)(1), (g)(2), and (g)(3) of this section may be used). When peripheral blood is used, the blood is collected at appropriate times after treatment and smear preparations are

made and stained (the test techniques described in the references under paragraphs (g)(3), (g)(4), (g)(5), and (g)(6) of this section may be used). For studies with peripheral blood, as little time as possible should elapse between the last exposure and cell harvest. Preparations are analyzed for the presence of micronuclei.

(2) *Description*—(i) *Preparations*—(A) *Selection of animal species.* Mice or rats are recommended if bone marrow is used, although any appropriate mammalian species may be used. When peripheral blood is used, mice are recommended. However, any appropriate mammalian species may be used provided it is a species in which the spleen does not remove micronucleated erythrocytes or a species which has shown an adequate sensitivity to detect agents that cause structural or numerical chromosome aberrations. Commonly used laboratory strains of young healthy animals should be employed. At the commencement of the study, the weight variation of animals should be minimal and not exceed ± 20% of the mean weight of each sex.

(B) *Housing and feeding conditions.* The temperature in the experimental animal room should be 22°C (± 3°C). Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning, the aim should be 50–60%. Lighting should be artificial, the sequence being 12 hrs light, 12 hrs dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. The choice of diet may be influenced by the need to ensure a suitable admixture of a test substance when administered by this route. Animals may be housed individually, or caged in small groups of the same sex.

(C) *Preparation of the animals.* Healthy young adult animals shall be randomly assigned to the control and treatment groups. The animals are identified uniquely. The animals are acclimated to the laboratory conditions for at least 5 days. Cages should be arranged in such a way that possible effects due to cage placement are minimized.

(D) *Preparation of doses.* Solid test substances shall be dissolved or suspended in appropriate solvents or vehicles and diluted, if appropriate, prior to dosing of the animals. Liquid test substances may be dosed directly or diluted prior to dosing. Fresh preparations of the test substance should be employed unless stability data demonstrate the acceptability of storage.

(ii) *Test conditions*—(A) *Solvent/vehicle.* The solvent/vehicle should not produce toxic effects at the dose levels used, and should not be suspected of chemical reaction with the test substance. If other than well-known solvents/vehicles are used, their inclusion shall be supported with reference data indicating their compatibility. It is recommended that wherever possible, the use of an aqueous solvent/vehicle should be considered first.

(B) *Controls.* (*1*) Concurrent positive and negative (solvent/vehicle) controls shall be included for each sex in each test. Except for treatment with the test substance, animals in the control groups should be handled in an identical manner to animals of the treatment groups.

(*2*) Positive controls shall produce micronuclei *in vivo* at exposure levels expected to give a detectable increase over background. Positive control doses should be chosen so that the effects are clear but do not immediately reveal the identity of the coded slides to the reader. It is acceptable that the positive control be administered by a route different from the test substance and sampled at only a single time. In addition, the use of chemical class-related positive control chemicals may be considered, when available. Examples of positive control substances include:

Chemical	CAS No.
Ethyl methanesulphonate	[CAS no. 62–50–0]
Ethyl nitrosourea	[CAS no. 759–73–9]
Mitomycin C	[CAS no. 50–07–7]
Cyclophosphamide (monohydrate)	[CAS no. 50–18–0]
	[CAS no. 6055–19–2]
Triethylenemelamine	[CAS no. 51–18–3]

(*3*) Negative controls, treated with solvent or vehicle alone, and otherwise treated in the same way as the treatment groups shall be included for every sampling time, unless acceptable inter-animal variability and frequencies of cells with micronuclei are demonstrated by historical control data. If single sampling is applied for negative controls, the most appropriate time is the first sampling time. In addition, untreated controls should also be used unless there are historical or published control data demonstrating that no deleterious or mutagenic effects are induced by the chosen solvent/vehicle.

(*4*) If peripheral blood is used, a pre-treatment sample may also be acceptable as a concurrent negative control, but only in the short peripheral blood studies (e.g., one to three treatment(s)) when the resulting data are in the expected range for the historical control.

(3) *Procedure*—(i) *Number and sex of animals.* Each treated and control group shall include at least 5 analyzable animals per sex (techniques described in the reference under paragraph (g)(7) of this section may be used). If at the time of the study there are data available from studies in the same species and using the same route of exposure that demonstrate that there are no substantial differences between sexes in toxicity, then testing in a single sex will be sufficient. Where human exposure to chemicals may be sex-specific, as for example with some pharmaceutical agents, the test should be performed with animals of the appropriate sex.

(ii) *Treatment schedule.* (A) No standard treatment schedule (i.e. one, two, or more treatments at 24 h intervals) can be recommended. The samples from extended dose regimens are acceptable as long as a positive effect has been demonstrated for this study or, for a negative study, as long as toxicity has been demonstrated or the limit dose has been used, and dosing continued until the time of sampling. Test substances may also be administered as a split dose, i.e., two treatments on the same day separated by no more than a few hrs, to facilitate administering a large volume of material.

(B) The test may be performed in two ways:

(*1*) Animals shall be treated with the test substance once. Samples of bone marrow shall be taken at least twice, starting not earlier than 24 hrs after treatment, but not extending beyond 48 hrs after treatment with appropriate interval(s) between samples. The use of sampling times earlier than 24 hrs after treatment should be justified. Samples of peripheral blood shall be taken at least twice, starting not earlier than 36 hrs after treatment, with appropriate intervals following the first sample, but not extending beyond 72 hrs. When a positive response is recognized at one sampling time, additional sampling is not required.

(*2*) If two or more daily treatments are used (e.g. two or more treatments at 24 hr intervals), samples shall be collected once between 18 and 24 hrs following the final treatment for the bone marrow and once between 36 and 48 hrs following the final treatment for the peripheral blood (techniques described in the reference under paragraph (g)(8) of this section may be used).

(C) Other sampling times may be used in addition, when relevant.

(iii) *Dose levels.* If a range finding study is performed because there are no suitable data available, it should be performed in the same laboratory, using the same species, strain, sex, and treatment regimen to be used in the main study (guidance on dose setting is provided in the reference in paragraph (g)(9) of this section). If there is toxicity, three dose levels shall be used for the first sampling time. These dose levels shall cover a range from the maximum to little or no toxicity. At the later sampling time only the highest dose needs to be used. The highest dose is defined as the dose producing signs of toxicity such that higher dose levels, based on the same dosing regimen, would be expected to produce lethality. Substances with specific biological activities at low non-toxic doses (such as hormones and mitogens) may be exceptions to the dose-setting criteria and should be evaluated on a case-by-case basis. The highest dose may also be defined as a dose that produces some indication of toxicity in the bone marrow (e.g. a reduction in the proportion of immature erythrocytes among total erythrocytes in the bone marrow or peripheral blood).

(iv) *Limit test.* If a test at one dose level of at least 2,000 mg/kg body weight using a single treatment, or as two treatments on the same day, produces no observable toxic effects, and if genotoxicity would not be expected based upon data from structurally related substances, then a full study using three dose levels may not be considered necessary. For studies of a longer duration, the limit dose is 2,000 mg/kg/body weight/day for treatment up to 14 days, and 1,000 mg/kg/body weight/day for treatment longer than 14 days. Expected human exposure may indicate the need for a higher dose level to be used in the limit test.

(v) *Administration of doses.* The test substance is usually administered by gavage using a stomach tube or a suitable intubation cannula, or by intraperitoneal injection. Other routes of exposure may be acceptable where they can be justified. The maximum volume of liquid that can be administered by gavage or injection at one time depends on the size of the test animal. The volume should not exceed 2 ml/100g body weight. The use of volumes higher than these must be justified. Except for irritating or corrosive substances which will normally reveal exacerbated effects with higher concentrations, variability in test volume should be minimized by adjusting the concentration to ensure a constant volume at all dose levels.

(vi) *Bone marrow/blood preparation.* Bone marrow cells shall be obtained from the femurs or tibias immediately following sacrifice. Cells shall be removed from femurs or tibias, prepared and stained using established methods. Peripheral blood is obtained from the tail vein or other appropriate blood vessel. Blood cells are immediately stained supravitally (the test techniques described in the references under paragraphs (g)(4), (g)(5), and (g)(6) of this section may be used) or smear preparations are made and then stained. The use of a DNA specific stain (e.g. acridine orange (techniques described in the reference under paragraph (g)(10) of this section may be used) or Hoechst 33258 plus pyronin-Y) can eliminate some of the artifacts associated with using a non-DNA specific stain. This advantage does not preclude the use of conventional stains (e.g., Giemsa). Additional systems (e.g. cellulose columns to remove nucleated cells (the test techniques described in the references under paragraph (g)(12) of this section may be used)) can also be used provided that these systems have been shown to adequately work for micronucleus preparation in the laboratory.

(vii) *Analysis.* The proportion of immature among total (immature + mature) erythrocytes is determined for each animal by counting a total of at least 200 erythrocytes for bone marrow and 1,000 erythrocytes for peripheral blood (techniques described in the reference under paragraph (g)(13) of this section maybe used). All slides, including those of positive and negative controls, shall be independently coded before microscopic analysis. At least 2,000 immature erythrocytes per animal shall be scored for the incidence of micronucleated immature erythrocytes. Additional information may be obtained by scoring mature erythrocytes for micronuclei. When analyzing slides, the proportion of immature erythrocytes among total erythrocytes should not be less than 20% of the control value. When animals are treated continuously for 4 weeks or more, at least 2,000 mature erythrocytes per animal can also be scored for the incidence of micronuclei. Systems for automated analysis (image analysis) and cell suspensions (flow cytometry) are acceptable alternatives to manual evaluation if appropriately justified and validated.

(f) *Data and reporting*—(1) *Treatment of results.* Individual animal data shall be presented in tabular form. The experimental unit is the animal. The number of immature erythrocytes scored, the number of micronucleated immature erythrocytes, and the number of immature among total erythrocytes shall be listed separately for each animal analyzed. When animals are treated continuously for 4 weeks or more, the data on mature erythrocytes should also be given if it is collected. The proportion of immature among total erythrocytes and, if considered applicable, the percentage of micronucleated erythrocytes shall be given for each animal. If there is no evidence for a difference in response between the sexes, the data from both sexes may be combined for statistical analysis.

(2) *Evaluation and interpretation of results.* (i) There are several criteria for determining a positive result, such as a dose-related increase in the number of micronucleated cells or a clear increase in the number of micronucleated cells in a single dose group at a single sampling time. Biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results (the test techniques described in the references paragraphs (g)(14) and (g)(15) of this section may be used). Statistical significance should not be the only determining factor for a positive response. Equivocal results should be clarified by further testing preferably using a modification of experimental conditions.

(ii) A test substance for which the results do not meet the criteria described is considered non-mutagenic in this test.

(iii) Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance. Results, may remain equivocal or questionable regardless of the number of times the experiment is repeated. Positive results in the micronucleus test indicate that a substance induces micronuclei which are the result of chromosomal damage or damage to the mitotic apparatus in the erythroblasts of the test species. Negative results indicate that, under the test conditions, the test substance does not produce micronuclei in the immature erythrocytes of the test species.

(iv) The likelihood that the test substance or its metabolites reach the general circulation or specifically the target tissue (e.g. systemic toxicity) should be discussed.

(3) *Test report.* In addition to the reporting requirements as specified under 40 CFR part 792, subpart J, the following specific information shall be reported. Both individual and summary data should be presented.

(i) Test substance:

(A) Identification data and CAS no., if known.

(B) Physical nature and purity.

(C) Physiochemical properties relevant to the conduct of the study.

(D) Stability of the test substance, if known.

(ii) Solvent/vehicle:

(A) Justification for choice of vehicle.

(B) Solubility and stability of the test substance in the solvent/vehicle, if known.

(iii) Test animals:

(A) Species/strain used.

(B) Number, age, and sex of animals.

(C) Source, housing conditions, diet, etc.

(D) Individual weight of the animals at the start of the test, including body weight range, mean and standard deviation for each group.

(iv) Test conditions:

(A) Positive and negative (vehicle/solvent) control data.

(B) Data from range-finding study, if conducted.

(C) Rationale for dose level selection.

(D) Details of test substance preparation.

(E) Details of the administration of the test substance.

(F) Rationale for route of administration.

(G) Methods for verifying that the test substance reached the general circulation or target tissue, if applicable.

(H) Conversion from diet/drinking water test substance concentration parts per million (ppm) to the actual dose (mg/kg body weight/day), if applicable.

(I) Details of food and water quality.

(J) Detailed description of treatment and sampling schedules.

(K) Methods of slide preparation.

(L) Methods for measurement of toxicity.

(M) Criteria for scoring micronucleated immature erythrocytes.

(N) Number of cells analyzed per animal.

(O) Criteria for considering studies as positive, negative or equivocal.

(v) Results:

(A) Signs of toxicity.

(B) Proportion of immature erythrocytes among total erythrocytes.

(C) Number of micronucleated immature erythrocytes, given separately for each animal.

(D) Mean ± standard deviation of micronucleated immature erythrocytes per group.

(E) Dose-response relationship, where possible.

(F) Statistical analyses and method applied.

(G) Concurrent and historical negative control data.

(H) Concurrent positive control data.

(vi) Discussion of the results.

(vii) Conclusion.

(g) *References.* For additional background information on this test guideline, the following references should be consulted. These references are available for inspection at the TSCA Nonconfidential Information Center, Rm. NE–B607, Environmental Protection Agency, 401 M St., SW., Washington, DC, 12 noon to 4 p.m., Monday through Friday, except legal holidays.

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(10) Hayashi, M., Sofuni, T., and Ishidate, Jr., M. An Application of Acridine Orange Fluorescent Staining to the Micronucleus Test. *Mutation Research*. 120, 241–247 (1983).

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(13) Gollapudi, B. and McFadden, L.G. Sample size for the estimation of polychromatic to normochromatic eruthrocyte ratio in the bone marrow micronucleus test. *Mutation Research*. 347, 97–99 (1995).

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§ 799.9620 TSCA neurotoxicity screening battery.

(a) *Scope.* This section is intended to meet the testing requirements under section 4 of TSCA. This neurotoxicity screening battery consists of a functional observational battery, motor activity, and neuropathology. The functional observational battery consists of noninvasive procedures designed to detect gross functional deficits in animals and to better quantify behavioral or neurological effects detected in other studies. The motor activity test uses an automated device that measures the level of activity of an individual animal. The neuropathological techniques are designed to provide data to detect and characterize histopathological changes in the central and peripheral nervous system. This battery is designed to be used in conjunction with general toxicity studies and changes should be evaluated in the context of both the concordance between functional neurological and neuropatholgical effects, and with respect to any other toxicological effects seen. This test battery is not intended to provide a complete evaluation of neurotoxicity, and additional functional and morphological evaluation may be necessary to assess completely the neurotoxic potential of a chemical.

(b) *Source.* The source material used in developing this TSCA test guideline is the OPPTS harmonized test guideline 870.6200 (June 1996 Public Draft). This source is available at the address in paragraph (g) of this section.

(c) *Definitions.* The following definitions apply to this section.

ED is effective dose.

Motor activity is any movement of the experimental animal.

Neurotoxicity is any adverse effect on the structure or function of the nervous system related to exposure to a chemical substance.

Toxic effect is an adverse change in the structure or function of an experimental animal as a result of exposure to a chemical substance.

(d) *Principle of the test method.* The test substance is administered to several groups of experimental animals, one dose being used per group. The animals are observed under carefully standardized conditions with sufficient frequency to ensure the detection and quantification of behavioral and/or neurologic abnormalities, if present. Various functions that could be affected by neurotoxicants are assessed during each observation period. Measurements of motor activity of individual animals are made in an automated device. The animals are perfused and tissue samples from the nervous system are prepared for microscopic examination. The exposure levels at which significant neurotoxic effects are produced are compared to one another and to those levels that produce other toxic effects.

(e) *Test procedures*—(1) *Animal selection*—(i) *Species.* In general, the laboratory rat should be used. Under some circumstances, other species, such as the mouse or the dog, may be more appropriate, although not all of the battery may be adaptable to other species.

(ii) *Age.* Young adults (at least 42 days old for rats) shall be used.

(iii) *Sex.* Both males and females shall be used. Females shall be nulliparous and nonpregnant.

(2) *Number of animals.* At least 10 males and 10 females should be used in each dose and control group for behavioral testing. At least five males and five females should be used in each dose and control group for terminal neuropathology. If interim neuropathological evaluations are planned, the number should be increased by the number of animals scheduled to be perfused before the end of the study. Animals shall be randomly assigned to treatment and control groups.

(3) *Control groups.* (i) A concurrent (vehicle) control group is required. Subjects shall be treated in the same way as for an exposure group except that administration of the test substance is omitted. If the vehicle used has known or potential toxic properties, both untreated or saline treated and vehicle control groups are required.

(ii) Positive control data from the laboratory performing the testing shall provide evidence of the ability of the observational methods used to detect major neurotoxic endpoints including limb weakness or paralysis, tremor, and autonomic signs. Positive control data are also required to demonstrate the sensitivity and reliability of the activity-measuring device and testing procedures. These data should demonstrate the ability to detect chemically induced increases and decreases in activity. Positive control groups exhibiting central nervous system pathology and peripheral nervous system pathology are also required. Separate groups for peripheral and central neuropathology are acceptable (e.g. acrylamide and trimethyl tin). Positive control data shall be collected at the time of the test study unless the laboratory can demonstrate the adequacy of historical data for this purpose, i.e. by the approach outlined in this section.

(4) *Dose level and dose selection.* At least three doses shall be used in addition to the vehicle control group. The data should be sufficient to produce a dose-effect curve. The Agency strongly encourage the use of equally spaced doses and a rationale for dose selection that will maximally support detection of dose-effect relations. For acute studies, dose selection may be made relative to the establishment of a benchmark dose (BD). That is, doses may be specified as successive fractions, e.g. 0.5, 0.25, ...n of the BD. The BD itself may be estimated as the highest nonlethal dose as determined in a preliminary range-finding lethality study. A variety of test methodologies may be used for this purpose, and the method chosen may influence subsequent dose selection. The goal is to use a dose level that is sufficient to be judged a limit dose, or clearly toxic.

(i) *Acute studies.* The high dose need not be greater than 2 g/kg. Otherwise, the high dose should result in significant neurotoxic effects or other clearly toxic effects, but not result in an incidence of fatalities that would preclude a meaningful evaluation of the

data. This dose may be estimated by a BD procedure as described under paragraph (e)(4) of this section, with the middle and low dose levels chosen as fractions of the BD dose. The lowest dose should produce minimal effect, e.g. an ED10, or alternatively, no effects.

(ii) *Subchronic and chronic studies.* The high dose need not be greater than 1 g/kg. Otherwise, the high dose level should result in significant neurotoxic effects or other clearly toxic effects, but not produce an incidence of fatalities that would prevent a meaningful evaluation of the data. The middle and low doses should be fractions of the high dose. The lowest dose should produce minimal effects, e.g. an ED10, or alternatively, no effects.

(5) *Route of exposure.* Selection of route may be based on several criteria including, the most likely route of human exposure, bioavailability, the likelihood of observing effects, practical difficulties, and the likelihood of producing nonspecific effects. For many materials, it should be recognized that more than one route of exposure may be important and that these criteria may conflict with one another. Initially only one route is required for screening for neurotoxicity. The route that best meets these criteria should be selected. Dietary feeding will generally be acceptable for repeated exposures studies.

(6) *Combined protocol.* The tests described in this screening battery may be combined with any other toxicity study, as long as none of the requirements of either are violated by the combination.

(7) *Study conduct*—(i) *Time of testing.* All animals shall be weighed on each test day and at least weekly during the exposure period.

(A) *Acute studies.* At a minimum, for acute studies observations and activity testing shall be made before the initiation of exposure, at the estimated time of peak effect within 8 hrs of dosing, and at 7 and 14 days after dosing. Estimation of times of peak effect may be made by dosing pairs of rats across a range of doses and making regular observations of gait and arousal.

(B) *Subchronic and chronic studies.* In a subchronic study, at a minimum, observations and activity measurements shall be made before the initiation of exposure and before the daily exposure, or for feeding studies at the same time of day, during the 4th, 8th, and 13th weeks of exposure. In chronic studies, at a minimum, observations and activity measurements shall be made before the initiation of exposure and before the daily exposure, or for feeding studies at the same time of day, every 3 months.

(ii) *Functional observational battery*—(A) *General conduct.* All animals in a given study shall be observed carefully by trained observers who are unaware of the animals' treatment, using standardized procedures to minimize observer variability. Where possible, it is advisable that the same observer be used to evaluate the animals in a given study. If this is not possible, some demonstration of interobserver reliability is required. The animals shall be removed from the home cage to a standard arena for observation. Effort should be made to ensure that variations in the test conditions are minimal and are not systematically related to treatment. Among the variables that can affect behavior are sound level, temperature, humidity, lighting, odors, time of day, and environmental distractions. Explicit, operationally defined scales for each measure of the battery are to be used. The development of objective quantitative measures of the observational end-points specified is encouraged. Examples of observational procedures using defined protocols may be found in the references under paragraphs (g)(5), (g)(6), and (g)(9) of this section. The functional observational battery shall include a thorough description of the subject's appearance, behavior, and functional integrity. This shall be assessed through observations in the home cage and while the rat is moving freely in an open field, and through manipulative tests. Testing should proceed from the least to the most interactive with the subject. Scoring criteria, or explicitly defined scales, should be developed for those measures which involve subjective ranking.

(B) *List of measures.* The functional observational battery shall include the following list of measures:

(*1*) Assessment of signs of autonomic function, including but not limited to:

(*i*) Ranking of the degree of lacrimation and salivation, with a range of severity scores from none to severe.

(*ii*) Presence or absence of piloerection and exophthalmus.

(*iii*) Ranking or count of urination and defecation, including polyuria and diarrhea. This is most easily conducted during the open field assessment.

(*iv*) Pupillary function such as constriction of the pupil in response to light or a measure of pupil size.

(*v*) Degree of palpebral closure, e.g., ptosis.

(*2*) Description, incidence, and severity of any convulsions, tremors, or abnormal motor movements, both in the home cage and the open field.

(*3*) Ranking of the subject's reactivity to general stimuli such as removal from the cage or handling, with a range of severity scores from no reaction to hyperreactivity.

(*4*) Ranking of the subject's general level of activity during observations of the unperturbed subject in the open field, with a range of severity scores from unresponsive to hyperactive.

(*5*) Descriptions and incidence of posture and gait abnormalities observed in the home cage and open field.

(*6*) Ranking of any gait abnormalities, with a range of severity scores from none to severe.

(*7*) Forelimb and hindlimb grip strength measured using an objective procedure (the procedure described in the reference under paragraph (g)(8) of this section may be used).

(*8*) Quantitative measure of landing foot splay (the procedure described in the reference under paragraph (g)(3) of this section may be used).

(*9*) Sensorimotor responses to stimuli of different modalities will be used to detect gross sensory deficits. Pain perception may be assessed by a ranking or measure of the reaction to a tail-pinch, tail-flick, or hot-plate. The response to a sudden sound, e.g., click or snap, may be used to assess audition.

(*10*) Body weight.

(*11*) Description and incidence of any unusual or abnormal behaviors, excessive or repetitive actions (stereotypies), emaciation, dehydration, hypotonia or hypertonia, altered fur appearance, red or crusty deposits around the eyes, nose, or mouth, and any other observations that may facilitate interpretation of the data.

(C) *Additional measures.* Other measures may also be included and the development and validation of new tests is encouraged. Further information on the neurobehavioral integrity of the subject may be provided by:

(*1*) Count of rearing activity on the open field.

(*2*) Ranking of righting ability.

(*3*) Body temperature.

(*4*) Excessive or spontaneous vocalizations.

(*5*) Alterations in rate and ease of respiration, e.g., rales or dyspnea.

(*6*) Sensorimotor responses to visual or proprioceptive stimuli.

(iii) *Motor activity.* Motor activity shall be monitored by an automated activity recording apparatus. The device used must be capable of detecting both increases and decreases in activity, i.e., baseline activity as measured by the device must not be so low as to preclude detection of decreases nor so high as to preclude detection of increases in activity. Each device shall be tested by standard procedures to ensure, to the extent possible, reliability of operation

across devices and across days for any one device. In addition, treatment groups must be balanced across devices. Each animal shall be tested individually. The test session shall be long enough for motor activity to approach asymptotic levels by the last 20% of the session for nontreated control animals. All sessions shall have the same duration. Treatment groups shall be counterbalanced across test times. Effort should be made to ensure that variations in the test conditions are minimal and are not systematically related to treatment. Among the variables which can affect motor activity are sound level, size and shape of the test cage, temperature, relative humidity, lighting conditions, odors, use of the home cage or a novel test cage, and environmental distractions.

(iv) *Neuropathology: Collection, processing and examination of tissue samples.* To provide for adequate sampling as well as optimal preservation of cellular integrity for the detection of neuropathological alterations, tissue shall be prepared for histological analysis using in situ perfusion and paraffin and/or plastic embedding procedures. Paraffin embedding is acceptable for tissue samples from the central nervous system. Plastic embedding of tissue samples from the central nervous system is encouraged, when feasible. Plastic embedding is required for tissue samples from the peripheral nervous system. Subject to professional judgment and the type of neuropathological alterations observed, it is recommended that additional methods, such as glial fibrillary acidic protein (GFAP) immunohistochemistry and/or methods known as Bodian's or Bielchowsky's silver methods be used in conjunction with more standard stains to determine the lowest dose level at which neuropathological alterations are observed. When new or existing data provide evidence of structural alterations it is recommended that the GFAP immunoassay also be considered. A description of this technique can be found in the reference under paragraph (g)(10) of this section.

(A) *Fixation and processing of tissue.* The nervous system shall be fixed by in situ perfusion with an appropriate aldehyde fixative. Any gross abnormalities should be noted. Tissue samples taken should adequately represent all major regions of the nervous system. The tissue samples should be postfixed and processed according to standardized published histological protocols (protocols described in the references under paragraphs (g)(1), (g)(2), or (g)(11) of this section may be used). Tissue blocks and slides should be appropriately identified when stored. Histological sections should be stained for hematoxylin and eosin (H&E), or a comparable stain according to standard published protocols (some of these protocols are described in the references under paragraphs (g)(1) and (g)(11) of this section).

(B) *Qualitative examination.* Representative histological sections from the tissue samples should be examined microscopically by an appropriately trained pathologist for evidence of neuropathological alterations. The nervous system shall be thoroughly examined for evidence of any treatment-related neuropathological alterations. Particular attention should be paid to regions known to be sensitive to neurotoxic insult or those regions likely to be affected based on the results of functional tests. Such treatment-related neuropathological alterations should be clearly distinguished from artifacts resulting from influences other than exposure to the test substance. A stepwise examination of tissue samples is recommended. In such a stepwise examination, sections from the high dose group are first compared with those of the control group. If no neuropathological alterations are observed in samples from the high dose group, subsequent analysis is not required. If neuropathological alterations are observed in samples from the high dose group, samples from the intermediate and low dose groups are then examined sequentially.

(C) *Subjective diagnosis.* If any evidence of neuropathological alterations is found in the qualitative examination, then a subjective diagnosis shall be performed for the purpose of evaluating dose-response relationships. All regions of the nervous system exhibiting any evidence of neuropathological changes should be included in this analysis. Sections from all dose groups from each region will be coded and examined in randomized order without knowledge of the code. The frequency of each type and severity of each lesion will be recorded. After all samples from all dose groups including all regions have been rated, the code will be broken and statistical analysis performed to evaluate dose-response relationships. For each type of dose-related lesion observed, examples of different degrees of severity should be described. Photomicrographs of typical examples of treatment-related regions are recommended to augment these descriptions. These examples will also serve to illustrate a rating scale, such as 1+, 2+, and 3+ for the degree of severity ranging from very slight to very extensive.

(f) *Data reporting and evaluation.* The final test report shall include the following information:

(1) *Description of equipment and test methods.* A description of the general design of the experiment and any equipment used shall be provided. This shall include a short justification explaining any decisions involving professional judgment.

(i) A detailed description of the procedures used to standardize observations, including the arena and scoring criteria.

(ii) Positive control data from the laboratory performing the test that demonstrate the sensitivity of the procedures being used. Historical data may be used if all essential aspects of the experimental protocol are the same. Historical control data can be critical in the interpretation of study findings. The Agency encourages submission of such data to facilitate the rapid and complete review of the significance of effects seen.

(2) *Results.* The following information shall be arranged by test group dose level.

(i) In tabular form, data for each animal shall be provided showing:

(A) Its identification number.

(B) Its body weight and score on each sign at each observation time, the time and cause of death (if appropriate), total session activity counts, and intrasession subtotals for each day measured.

(ii) Summary data for each group must include:

(A) The number of animals at the start of the test.

(B) The number of animals showing each observation score at each observation time.

(C) The mean and standard deviation for each continuous endpoint at each observation time.

(D) Results of statistical analyses for each measure, where appropriate.

(iii) All neuropathological observations shall be recorded and arranged by test groups. This data may be presented in the following recommended format:

(A) *Description of lesions for each animal.* For each animal, data must be submitted showing its identification (animal number, sex, treatment, dose, and duration), a list of structures examined as well as the locations, nature, frequency, and severity of lesions. Inclusion of photomicrographs is strongly recommended for demonstrating typical examples of the type and severity of the neuropathological alterations observed. Any diagnoses derived from

neurological signs and lesions including naturally occurring diseases or conditions, should be recorded.

(B) *Counts and incidence of neuropathological alterations by test group.* Data should be tabulated to show:

(*1*) The number of animals used in each group and the number of animals in which any lesion was found.

(*2*) The number of animals affected by each different type of lesion, the locations, frequency, and average grade of each type of lesion.

(3) *Evaluation of data.* The findings from the screening battery should be evaluated in the context of preceding and/or concurrent toxicity studies and any correlated functional and histopathological findings. The evaluation shall include the relationship between the doses of the test substance and the presence or absence, incidence and severity, of any neurotoxic effects. The evaluation shall include appropriate statistical analyses, for example, parametric tests for continuous data and nonparametric tests for the remainder. Choice of analyses should consider tests appropriate to the experimental design, including repeated measures. There may be many acceptable ways to analyze data.

(g) *References.* For additional background information on this test guideline, the following references should be consulted. These references are available for inspection at the TSCA Nonconfidential Information Center, Rm. NE–B607, Environmental Protection Agency, 401 M St., SW., Washington, DC, 12 noon to 4 p.m., Monday through Friday, except legal holidays.

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§ 799.9780 TSCA immunotoxicity.

(a) *Scope.* This section is intended to meet the testing requirements under section 4 of TSCA. This section is intended to provide information on suppression of the immune system which might occur as a result of repeated exposure to a test chemical. While some information on potential immunotoxic effects may be obtained from hematology, lymphoid organ weights and histopathology (usually done as part of routine toxicity testing), there are data which demonstrate that these endpoints alone are not sufficient to predict immunotoxicity (Luster *et al.*, 1992, 1993 see paragraphs (j)(8) and (j)(9) of this section). Therefore, the tests described in this section are intended to be used along with data from routine toxicity testing, to provide more accurate information on risk to the immune system. The tests in this section do not represent a comprehensive assessment of immune function.

(b) *Source.* The source material used in developing this TSCA test guideline is the OPPTS harmonized test guideline 870.7800 (June 1996 Public Draft). This source is available at the address in paragraph (j) of this section.

(c) *Definitions.* The following definitions apply to this section.

Antibodies or immunoglobulins (Ig) are part of a large family of glycoprotein molecules. They are produced by B cells in response to antigens, and bind specifically to the eliciting antigen. The different classes of immunoglobulins involved in immunity are IgG, IgA, IgM, IgD, and IgE. Antibodies are found in extracellular fluids, such as serum, saliva, milk, and lymph. Most antibody responses are T cell-dependent, that is, functional T and B lymphocytes, as well as antigen-presenting cells (usually macrophages), are required for the production of antibodies.

Cluster of differentiation (CD) refers to molecules expressed on the cell surface. These molecules are useful as distinct CD molecules are found on different populations of cells of the immune system. Antibodies against these cell surface markers (e.g., CD4, CD8) are used to identify and quantitate different cell populations.

Immunotoxicity refers to the ability of a test substance to suppress immune responses that could enhance the risk of infectious or neoplastic disease, or to induce inappropriate stimulation of the immune system, thus contributing to allergic or autoimmune disease. This section only addresses potential immune suppression.

Natural Killer (NK) cells are large granular lymphocytes which nonspecifically lyse cells bearing tumor or viral antigens. NK cells are up-regulated soon after infection by certain microorganisms, and are thought to represent the first line of defense against viruses and tumors.

T and B cells are lymphocytes which are activated in response to specific antigens (foreign substances, usually proteins). B cells produce antigen-specific antibodies (see the definition for ''antibodies or immunoglobulins''), and subpopulations of T cells are frequently needed to provide help for the antibody response. Other types of T cell participate in the direct destruction of cells expressing specific foreign

(tumor or infectious agent) antigens on the cell surface.

(d) *Principles of the test methods.* (1) In order to obtain data on the functional responsiveness of major components of the immune system to a T cell dependent antigen, sheep red blood cells (SRBC), rats and/or mice[1] shall be exposed to the test and control substances for at least 28 days.[2] The animals shall be immunized by intravenous or intraperitoneal injection of SRBCs approximately 4 days (depending on the strain of animal) prior to the end of the exposure. At the end of the exposure period, either the plaque forming cell (PFC) assay or an enzyme linked immunosorbent assay (ELISA) shall be performed to determine the effects of the test substance on the splenic anti-SRBC (IgM) response or serum anti-SRBC IgM levels, respectively.

(2) In the event the test substance produces significant suppression of the anti-SRBC response, expression of phenotypic markers for major lymphocyte populations (total T and total B), and T cell subpopulations (T helpers (CD^4) and T cytotoxic/suppressors (CD^8)), as assessed by flow cytometry, may be performed to determine the effects of the test substance on either splenic or peripheral-blood lymphocyte populations and T cell subpopulations. When this study is performed, the appropriate monoclonal antibodies for the species being tested should be used. If the test substance has no significant effect on the anti-SRBC assay, a functional test for NK cells may be performed to test for a chemical's effect on non-specific immunity.[3] For tests performed using cells or sera from blood (ELISA or flow cytometry), it is not necessary to destroy the animals, since immunization with SRBCs at 28 days is not expected to markedly affect the results of other assays included in subchronic or longer-term studies (these tests are discussed in the reference under paragraph (j)(7) of this section). The necessity to perform either a quantitative analysis of the effects of a chemical on the numbers of cells in major lymphocyte populations and T Cell subpopulations by flow cytometry, or a splenic NK cell activity assay to assess the effects of the test compound on non-specific immunity shall be determined on a case-by-case basis, depending upon the outcome of the anti-SRBC assay.

(e) *Limit test.* If a test at one dose level of at least 1,000 mg/kg body weight (or 2 mg/L for inhalation route of exposure) using the procedures described for this study produces no observable toxic effects or if toxic effects would not be expected based upon data of structurally related compounds, then a full study using three dose levels might not be necessary. Expected human exposure may indicate the need for a higher dose level.

(f) *Test procedures*—(1) *Animal selection*—(i) *Species and strain.* These tests are intended for use in rats and/or mice. Commonly used laboratory strains shall be employed.[4] All test animals shall be free of pathogens, internal and external parasites. Females shall be nulliparous and nonpregnant. The species, strain, and source of the animals shall be identified.

(ii) *Age/weight.* (A) Young, healthy animals shall be employed. At the commencement of the study, the weight variation of the animals used shall not exceed ± 20% of the mean weight for each sex.

(B) Dosing shall begin when the test animals are between 6 and 8 weeks old.

(iii) *Sex.* Either sex may be used in the study; if one sex is known or believed to be more sensitive to the test compound, then that sex shall be used.

(iv) *Numbers.* (A) At least eight animals shall be included in each dose and control group. The number of animals tested shall yield sufficient statistical power to detect a 20% change based upon the interanimal variation which may be encountered in these assays.

(B) To avoid bias, the use of adequate randomization procedures for the proper allocation of animals to test and control groups is required.

(C) Each animal shall be assigned a unique identification number. Dead animals, their preserved organs and tissues, and microscopic slides shall be identified by reference to the animal's unique number.

(v) *Husbandry.* (A) Animals may be group-caged by sex, but the number of animals per cage shall not interfere with clear observation of each animal. The biological properties of the test substance or toxic effects (e.g., morbidity, excitability) may indicate a need for individual caging.

(B) The temperature of the experimental animal rooms shall be at 22 ± 3°C.

(C) The relative humidity of the experimental animal rooms shall be between 30 and 70%.

(D) Where lighting is artificial, the sequence shall be 12 hrs light, 12 hrs dark.

(E) Control and test animals shall be maintained on the same type of bedding and receive feed from the same lot. The feed shall be analyzed to assure adequacy of nutritional requirements of the species tested and for impurities that might influence the outcome of the test. Rodents shall be fed and watered *ad libitum* with food replaced at least weekly.

(F) The study shall not be initiated until the animals have been allowed an adequate period of acclimatization or quarantine to environmental conditions. The period of acclimatization shall be at least 1 week in duration.

(2) *Control and test substances.* (i) The test substance shall be dissolved or suspended in a suitable vehicle. Ideally, if a vehicle or diluent is needed, it shall not elicit toxic effects or substantially alter the chemical or toxicological properties of the test substance. It is recommended that an aqueous solution should be used. If solubility is a problem a solution in oil may be used. Other vehicles may be considered, but only as a last resort.

(ii) One lot of the test substance shall be used, if possible, throughout the duration of the study, and the research sample shall be stored under conditions that maintain its purity and stability. Prior to the initiation of the study, there shall be a characterization of the test substance, including the purity of the test compound and if technically feasible, the name and quantities of any known contaminants and impurities.

(iii) If the test or positive control substance is to be incorporated into feed or another vehicle, the period during which the test substance is stable in such a mixture shall be determined prior to the initiation of the study. Its homogeneity and concentration shall also be determined prior to the initiation of the study and periodically during the study. Statistically randomized samples of the mixture shall be analyzed to ensure that proper mixing, formulation, and storage procedures are being followed, and that the appropriate concentration of the test

[1] If absorption/distribution/metabolism/excretion (ADME) data are similar between species, then either rats or mice may be used for the test compound in question. If such data are lacking, both species should be used.

[2] Because there is a fairly rapid turnover of many of the cells in the immune system, 28 days is considered sufficient for the purposes of the anti-SRBC tests.

[3] When these optional tests are included, the phenotypic or NK cell analyses may be performed at 28 days of exposure, or at a later timepoint if ADME data suggest that a longer exposure is more appropriate.

[4] The study director shall be aware of strain differences in response to SRBC. For example, if the $B_6C_3F_1$ hybrid mouse is used in the PFC assay, a response of 800–1,000 PFC/10^6 spleen cells in control mice should be the minimally acceptable PFC response.

or control substance is contained in the mixture.

(3) *Control groups.* (i) A concurrent, vehicle-treated control group is required.

(ii) A separate untreated control group is required if the toxicity of the vehicle is unknown.

(iii) A positive control group with a known immunosuppressant (e.g., cyclophosphamide) shall be included in the study. A group of at least eight animals shall be given the immunosuppressive chemical.

(4) *Dose levels.* (i) In repeated-dose toxicity tests, it is desirable to have a dose-response relationship and a no observed immunotoxic effect level. Therefore, at least three dose levels and a negative control shall be used, unless a limit test is performed as specified under paragraph (e) of this section.

(ii) The highest dose level shall not produce significant stress, malnutrition, or fatalities, but ideally should produce some measurable sign of general toxicity (e.g., a 10% loss of body weight).

(iii) The lowest dose level ideally shall not produce any evidence of immunotoxicity.

(5) *Administration of the test substance.* (i) The test substance, vehicle, or positive control substance shall be administered for at least 28 days for the anti-SRBC assay. The route of administration of the test material will usually be oral; however, this shall be determined by the likely route of occupational or indoor exposure. Therefore, under certain conditions, the dermal or inhalation route of exposure may be more relevant for the study. All animals shall be dosed by the same method during the entire experimental period.

(ii) If the test substance is administered by gavage, the animals are dosed with the test substance ideally on a 7-days-per-week basis. However, based primarily on practical considerations, dosing by gavage on a 5-days-per-week basis shall be acceptable. If the test substance is administered in the drinking water, or mixed directly into the diet, then exposure shall be on a 7-days-per-week basis.

(A) For substances of low toxicity, it is important to ensure that when administered in the diet, the quantities of the test substance involved do not interfere with normal nutrition. When the test substance is administered in the diet, either a constant dietary concentration in parts per million (ppm) or a constant dose level in terms of the animal's body weight shall be used; the alternative used should be specified.

(B) For a substance administered by gavage, the dose shall be given at approximately the same time each day, and adjusted at intervals (weekly for mice, twice per week for rats) to maintain a constant dose level in terms of the animal's body weight.

(iii) If the test substance is administered dermally, use paragraphs (f)(5)(iii)(A) through (f)(5)(iii)(D) of this section.

(A) *Dose levels and dose selection.* (*1*) In this test, it is desirable to determine a dose-response relationship as well as a NOEL. Therefore, at least three dose levels plus a control and, where appropriate, a vehicle control (corresponding to the concentration of vehicle at the highest dose level) group should be used. Doses should be spaced appropriately to produce test groups with a range of toxic effects. The data should be sufficient to produce a dose-response curve.

(*2*) The highest dose level should elicit signs of toxicity but not produce severe skin irritation or an incidence of fatality which would prevent a meaningful evaluation. If application of the test substance produces severe skin irritation, the concentration may be reduced, although this may result in a reduction in, or absence of, other toxic effects at the high dose level. If the skin has been badly damaged early in the study, it may be necessary to terminate the study and undertake a new one at lower concentrations.

(*3*) The intermediate dose levels should be spaced to produce a gradation of toxic effects.

(*4*) The lowest dose level should not produce any evidence of toxic effects.

(B) *Preparation of animal skin.* Shortly before testing, fur should be clipped from not less than 10% of the body surface area for application of the test substance. In order to dose approximately 10% of the body surface, the area starting at the scapulae (shoulders) to the wing of the ileum (hipbone) and half-way down the flank on each side of the animal should be shaved. Shaving should be carried out approximately 24 hrs before dosing. Repeated clipping or shaving is usually needed at approximately weekly intervals. When clipping or shaving the fur, care should be taken to avoid abrading the skin which could alter its permeability.

(C) *Preparation of test substance.* (*1*) Liquid test substances are generally used undiluted, except as indicated in paragraph (f)(5)(iii)(A)*(2)* of this section.

(*2*) Solids should be pulverized when possible. The substance should be moistened sufficiently with water or, when necessary, a suitable vehicle to ensure good contact with the skin. When a vehicle is used, the influence of the vehicle on toxicity of, and penetration of the skin by, the test substance should be taken into account.

(*3*) The volume of application should be kept constant, e.g. less than 300 <greek-m≤L for the rat; different concentrations of test solution should be prepared for different dose levels.

(D) *Administration of test substance.* (*1*) The duration of exposure should be at least for 90 days.

(*2*) The animals should be treated with test substance for at least 6 hrs/day on a 7-day per week basis. However, based on practical considerations, application on a 5-day per week basis is acceptable. Dosing should be conducted at approximately the same time each day.

(*3*) The test substance should be applied uniformly over the treatment site.

(*4*) The surface area covered may be less for highly toxic substances. As much of the area should be covered with as thin and uniform a film as possible.

(*5*) During the exposure period, the test substance should be held in contact with the skin with a porous gauze dressing. The test site should be further covered with nonirritating tape to retain the gauze dressing and the test substance and to ensure that the animals cannot ingest the test substance. Restrainers may be used to prevent the ingestion of the test substance, but complete immobilization is not recommended.

(iv) If the test substance is administered by the inhalation route, use the procedures under paragraphs (e)(2), (e)(3), (e)(6), (e)(8), (e)(9), and (e)(10) of 40 CFR 799.9346. The exposure time for the anti-SRBC test shall be at least 28 days.

(6) *Observation period.* Duration of the observation period shall be at least 28 days.

(7) *Observation of animals.* (i) Observations shall be made at least once each day for morbidity and mortality. Appropriate actions shall be taken to minimize loss of animals to the study (e.g., necropsy of those animals found dead and isolation or euthanasia of weak or moribund animals).

(ii) A careful clinical examination shall be made at least once a week. Observations shall be detailed and carefully recorded, preferably using explicitly defined scales. Observations shall include, but not be limited to: evaluation of skin and fur, eyes and mucous membranes; respiratory and circulatory effects; autonomic effects, such as salivation; central nervous system effects, including tremors and convulsions, changes in the level of

motor activity, gait and posture, reactivity to handling or sensory stimuli, grip strength, and stereotypes or bizarre behavior (e.g., self-mutilation, walking backwards).

(iii) Signs of toxicity shall be recorded as they are observed, including the time of onset, degree and duration.

(iv) Food and water consumption shall be determined weekly.

(v) Animals shall be weighed immediately prior to dosing, weekly (twice per week for rats) thereafter, and just prior to euthanasia.

(vi) Any moribund animals shall be removed and euthanized when first noticed. Necropsies shall be conducted on all moribund animals, and on all animals that die during the study.

(vii) The spleen and thymus shall be weighed in all animals at the end of the study.

(g) *Immunotoxicity tests*—(1) *Functional tests.* Either a splenic PFC assay or an ELISA shall be used to determine the response to antigen administration.

(i) *Antibody plaque-forming cell (PFC) assay.* If the antibody PFC assay is performed, the criteria listed under paragraphs (g)(1)(i)(A) through (g)(1)(i)(F) of this section shall be adhered to. Assays described in the references under paragraphs (j)(2) and (j)(4) of this section may be used.

(A) The T cell-dependent antigen, SRBC, shall be injected intravenously or intraperitoneally, usually at 24 days after the first dosing with the test substance.[5] Although the optimum response time is usually 4 days after immunization, some strains of test animal may deviate from this time point. The strain to be used shall be evaluated for the optimum day for PFC formation after immunization.

(B) The activity of each new batch of complement shall be determined. For any given study, the SRBCs shall be from a single sheep, or pool of sheep, for which the shelf life and dose for optimum response has been determined.

(C) Modifications of the PFC assay described in paragraph (g)(1)(i) of this section exist and may prove useful; however, the complete citation shall be made for the method used, any modifications to the method shall be reported, and the source and, where appropriate, the activity or purity of important reagents shall be given. Justification or rationale shall be provided for each protocol modification. Discussions of modifications of the PFC assay are available in the references under paragraphs (j)(5),(j)(6), and (j)(10) of this section

(D) Samples shall be randomized and shall be coded for PFC analysis, so that the analyst is unaware of the treatment group of each sample examined.

(E) Spleen cell viability shall be determined.

(F) The numbers of IgM PFC per spleen, and the number of IgM PFC per 10^6 spleen cells shall be reported.

(ii) *Immunoglobulin quantification.* As an alternative to a PFC assay, the effects of the test substance on the antibody response to antigen may be determined by an Enzyme-Linked Immunosorbent Assay (ELISA). Comparison between the PFC and ELISA assays for immunotoxicity assessment are discussed in the references under paragraphs (j)(5), (j)(6), and (j)(10) of this section. Test animals shall be immunized with SRBCs as for the PFC assay. IgM titers in the serum of each test animal shall be determined (usually 4 days after immunization). As with the PFC assay, the optimum dose of SRBCs and optimum time for collection of the sera shall be determined for the species and strain of animal to be tested. Several methods are described in the reference under paragraph (j)(11) of this section).

(iii) *Natural killer (NK) cell activity.* The methods described in the reference under paragraph (j)(3) of this section may be used to demonstrate the effects of at least 28 days of exposure to a test substance on spontaneous cytotoxic activity. In this assay, splenocytes from treated and untreated test animals are incubated with ^{51}Cr-labeled YAC-1 lymphoma cells. The amount of radiolabel released from the target cells after incubation with the effector cells for four hrs is used as a measure of NK cytolysis. The following points shall be adhered to when using the NK cell assay:

(A) Assay controls shall be included to account for spontaneous release of radiolabel from target cells in the absence of effector cells, and also for the determination of total release of radiolabel.

(B) Target cells other than YAC-1 lymphoma cells may be appropriate for use in the assay. In all cases, target cell viability shall be determined.

(C) Modifications of the protocol exist that may prove useful. However, complete citation shall be made to the method used. Modifications shall be reported, and where appropriate, the source, activity, and/or purity of the reagents should be given. Justification or rationale shall be provided for each protocol modification.

(2) *Enumeration of splenic or peripheral blood total B cells, total T cells, and T cell subpopulations.* The phenotypic analysis of total B cell, total T cell, and T cell subpopulations from the spleen or peripheral blood by flow cytometry should be performed after at least 28 days of dosing; this may be performed at a later timepoint, if ADME data suggest that a longer exposure is more appropriate. If an exposure period longer than 28 days is used, then these tests may be performed in conjunction with subchronic (ninety day oral, dermal, or inhalation) toxicity studies, when these studies are required. Methods described in the references under paragraphs (j)(1) and (j)(5) of this section may be used.

(h) *Data and reporting*—(1) *Treatment of results*—(i) Data shall be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing effects, the types of effects and the percentage of animals displaying each type of effect.

(ii) All observed results, quantitative and incidental, shall be evaluated by an appropriate statistical method. Any generally accepted statistical methods may be used; the statistical methods including significance criteria shall be selected during the design of the study.

(2) *Evaluation of study results.* The findings of an immunotoxicity study shall be evaluated in conjunction with the findings of preceding studies and considered in terms of other toxic effects. The evaluation shall include the relationship between the dose of the test substance and the presence or absence, and the incidence and severity of abnormalities, including behavioral and clinical abnormalities, gross lesions, identified target organs, body weight changes, effects on mortality and any other general or specific toxic effects. A properly conducted test shall provide a satisfactory estimation of a no-observed-effect level. It may indicate the need for an additional study and provide information on the selection of dose levels.

(3) *Test report.* In addition to the reporting requirements as specified under 40 CFR part 792, subpart J, the following specific information shall be reported. Both individual and summary data should be presented.

(i) The test substance characterization shall include:

(A) Chemical identification.

(B) Lot or batch number.

(C) Physical properties.

(D) Purity/impurities.

(E) Identification and composition of any vehicle used.

[5] If the SRBCs are administered by the intraperitoneal route, the study director should be aware that a low percentage of animals may not respond because the antigen was accidentally injected into the intestinal tract.

(ii) The test system shall contain data on:

(A) Species, strain, and rationale for selection of animal species, if other than that recommended.

(B) Age, body weight data, and sex.

(C) Test environment including cage conditions, ambient temperature, humidity, and light/dark periods.

(D) When inhalation is the route of exposure, a description of the exposure equipment and data shall be included as follows:

(1) Description of test conditions; the following exposure conditions shall be reported:

(*i*) Description of exposure apparatus including design, type, volume, source of air, system for generating aerosols, method of conditioning air, treatment of exhaust air and the method of housing the animals in a test chamber.

(*ii*) The equipment for measuring temperature, humidity, and particulate aerosol concentrations and size should be described.

(2) Exposure data shall be tabulated and presented with mean values and a measure of variability (e.g., standard deviation) and include:

(*i*) Airflow rates through the inhalation equipment.

(*ii*) Temperature and humidity of air.

(*iii*) Actual (analytical or gravimetric) concentration in the breathing zone.

(*iv*) Nominal concentration (total amount of test substance fed into the inhalation equipment divided by volume of air).

(*v*) Particle size distribution, calculated mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD).

(*vi*) Explanation as to why the desired chamber concentration and/or particle size could not be achieved (if applicable) and the efforts taken to comply with this aspect of the section.

(E) Identification of animal diet.

(iii) The test procedure shall include the following data:

(A) Method of randomization used.

(B) Full description of experimental design and procedure.

(C) Dose regimen including levels, methods, and volume.

(iv) Test results should include the following data:

(A) Group animal toxic response data shall be tabulated by species, strain, sex, and exposure level for:

(*1*) Number of animals exposed.

(*2*) Number of animals showing signs of toxicity.

(*3*) Number of animals dying.

(B) Individual animal data shall be presented, as well as summary (group mean data).

(C) Date of death during the study or whether animals survived to termination.

(D) Date of observation of each abnormal sign and its subsequent course.

(E) Absolute and relative spleen and thymus weight data.

(F) Feed and water consumption data, when collected.

(G) Results of immunotoxicity tests.

(H) Necropsy findings of animals that were found moribund and euthanized or died during the study.

(I) Statistical treatment of results, where appropriate.

(i) *Quality control.* A system shall be developed and maintained to assure and document adequate performance of laboratory staff and equipment. The study shall be conducted in compliance with the 40 CFR Part 792—Good Laboratory Practice.

(j) *References.* For additional background information on this test guideline, the following references should be consulted. These references are available for inspection at the TSCA Nonconfidential Information Center, Rm. NE–B607, Environmental Protection Agency, 401 M St., SW., Washington, DC, 12 noon to 4 p.m., Monday through Friday, except legal holidays.

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