(2) [Reserved]

[50 FR 39321, Sept. 27, 1985, as amended at 52 FR 19069, May 20, 1987]

PART 798—HEALTH EFFECTS TESTING GUIDELINES

Subparts A–B [Reserved]

Subpart C—Subchronic Exposure

Subpart D—Chronic Exposure

Subpart E—Specific Organ/Tissue Toxicity

Subpart F—Genetic Toxicity

Subpart G—Neurotoxicity

AUTHORITY: 15 U.S.C. 2603.

SOURCE: 50 FR 39397, Sept. 27, 1985, unless otherwise noted.

Subparts A–B [Reserved]

Subpart C—Subchronic Exposure

§ 798.2250 Dermal toxicity.

(a) *Purpose.* In the assessment and evaluation of the toxic characteristics of a chemical, the determination of subchronic dermal toxicity may be carried out after initial information on toxicity has been obtained by acute testing. The subchronic dermal study has been designed to permit the determination of the no-observed-effect level and toxic effects associated with continuous or repeated exposure to a test substance for a period of 90 days. The test is not capable of determining those effects that have a long latency period for development (e.g., carcinogenicity and life shortening). It provides information on health hazards likely to arise from repeated exposure by the dermal route over a limited period of time. It will provide information on target organs, the possibilities of accumulation, and can be of use in selecting dose levels for chronic studies and for establishing safety criteria for human exposure.

(b) *Definitions.* (1) Subchronic dermal toxicity is the adverse effects occurring as a result of the repeated daily exposure of experimental animals to a chemical by dermal application for part (approximately 10 percent) of a life span.

(2) Dose in a dermal test is the amount of test substance applied to the skin (applied daily in subchronic tests). Dose is expressed as weight of the substance (g, mg) per unit weight of test animal (e.g., mg/kg).

(3) No-effect level/No-toxic-effect level/No-adverse-effect level/No-observed-effect level is the maximum dose used in a test which produces no observed adverse effects. A no-observed-effect level is expressed in terms of the weight of a test substance given daily per unit weight of test animal (mg/kg).

(4) Cumulative toxicity is the adverse effects of repeated doses occurring as a

result of prolonged action on, or increased concentration of the administered test substance or its metabolites in susceptible tissues.

(c) *Principle of the test method.* The test substance is applied daily to the skin in graduated doses to several groups of experimental animals, one dose level per unit group, for a period of 90 days. During the period of application the animals are observed daily to detect signs of toxicity. Animals which die during the test are necropsied, and at the conclusion of the test the surviving animals are sacrificed and necropsied and appropriate histopathological examinations carried out.

(d) *Limit test.* If a test at one dose level of at least 1,000 mg/kg body weight (expected human exposure may indicate the need for a higher dose level), using the procedures described for this study, produces no observable toxic effects and if toxicity would not be expected based upon data of structurally related compounds, then a full study using three dose levels might not be necessary.

(e) *Test procedures*—(1) *Animal selection*—(i) *Species and strain.* A mammalian species shall be used for testing. The rat, rabbit, or guinea pig may be used, although the albino rabbit is preferred. The albino rabbit is preferred because of its size, skin permeability, and extensive data base. Commonly used laboratory strains shall be employed. If another mammalian species is used, the tester shall provide justification/reasoning for its selection.

(ii) *Age.* Young adult animals shall be used. The following weight ranges at the start of the test are suggested in order to provide animals of a size which facilitates the conduct of the test: rats, 200 to 300 g; rabbits, 2.0 to 3.0 kg; guinea pigs, 350 to 450 g.

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

(B) The females shall be nulliparous and nonpregnant.

(iv) *Numbers.* (A) At least 20 animals (10 females and 10 males) shall be used at each dose level.

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

(2) *Control groups.* A concurrent control group is required. This group shall be an untreated or sham-treated control group 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 or cannot be made available, both untreated and vehicle control groups are required.

(3) *Satellite group.* A satellite group of 20 animals (10 animals per sex) may be treated with the high dose level for 90 days and observed for reversibility, persistence, or delayed occurrence, of toxic effects for a posttreatment period of appropriate length, normally not less than 28 days.

(4) *Dose level and dose selection.* (i) In subchronic toxicity tests, it is desirable to have a dose-response relationship as well as a no-observed-toxic-effect level. Therefore, at least 3 dose levels with a control and, where appropriate, a vehicle control (corresponding to the concentration of vehicle at the highest exposure level) shall be used. Doses should be spaced appropriately to produce test groups with a range of toxic effects. The data shall be sufficient to produce a dose-response curve.

(ii) The highest dose level should result in toxic effects but not produce severe skin irritation or an incidence of fatalities which would prevent a meaningful evaluation.

(iii) The lowest dose level should not produce any evidence of toxicity. Where there is a usable estimation of human exposure, the lowest dose level should exceed this.

(iv) Ideally, the intermediate dose level(s) should produce minimal observable toxic effects. If more than one intermediate dose is used, the dose levels should be spaced to produce a gradation of toxic effects.

(v) In the low and intermediate groups and in the controls the incidence of fatalities should be low, to permit a meaningful evaluation of the results.

(5) *Exposure conditions.* The animals are treated with test substance, ideally for at least 6 hours per day on a 7-day per week basis, for a period of 90 days. However, based primarily on practical

considerations, application on a 5-day per week basis is considered to be acceptable.

(6) *Observation period.* (i) Duration of observation shall be at least 90 days.

(ii) Animals in the satellite group scheduled for followup observations should be kept for at least 28 days further without treatment to detect recovery from, or persistence of, toxic effects.

(7) *Preparation of animal skin.* (i) Shortly before testing, fur shall be clipped from the dorsal area of the trunk of the test animals. Shaving may be employed, but it should be carried out approximately 24 hours before the test. Repeat 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.

(ii) Not less than 10 percent of the body surface area should be clear for the application of the test substance. The weight of the animal should be taken into account when deciding on the area to be cleared and on the dimensions of any covering used.

(iii) When testing solids, which may be pulverized if appropriate, the test substance should be moistened sufficiently with water or, where 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.

(8) *Application of the test substance.* (i) The test substance shall be applied uniformly over an area which is approximately 10 percent of the total body surface area. With highly toxic substances, the surface area covered may be less, but as much of the area shall be covered with as thin and uniform a film as possible.

(ii) During the exposure period, the test substance shall be held in contact with the skin with a porous gauze dressing and nonirritating tape. The test site shall 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. Restrainers may be used to prevent the ingestion of the test substance, but complete immobilization is not a recommended method.

(9) *Observation of animals.* (i) Each animal shall be observed daily, and if necessary handled to appraise its physical condition.

(ii) Additional observations shall be made daily with appropriate actions 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) Signs of toxicity shall be recorded as they are observed, including the time of onset, the degree, and duration.

(iv) Cage-side observations shall include, but not be limited to, changes in skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavior pattern.

(v) Animals shall be weighed weekly. Feed consumption shall also be determined weekly if abnormal body weight changes are observed.

(vi) At the end of the study period, all survivors in the nonsatellite treatment groups shall be sacrificed. Moribund animals shall be removed and sacrificed when noticed.

(10) *Clinical examinations.* (i) The following examinations shall be made on all animals of each sex in each group:

(A) Certain hematology determinations shall be carried out at least two times during the test period on all groups of animals including concurrent controls: After 30 days of test and just prior to terminal sacrifice at the end of the test period. Hematology determinations which are appropriate to all studies: Hematocrit, hemoglobin concentration, erythrocyte count, total and differential leukocyte count, and a measure of clotting potential such as clotting time, prothrombin time, thromboplastin time, or platelet count.

(B) Certain clinical biochemistry determinations on blood should be carried out at least two times during the test period on all groups of animals including concurrent controls: After 30 days of test and just prior to terminal sacrifice at the end of the test period. Clinical biochemistry test areas which

are considered appropriate to all studies: Electrolyte balance, carbohydrate metabolism, and liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the substance. Suggested determinations: Calcium, phosphorus, chloride, sodium, potassium, fasting glucose (with period of fasting appropriate to the species), serum glutamic pyruvic transaminase (now known as serum alanine aminotransferase), serum glutamic oxaloacetic transaminase (now known as serum aspartate aminotransferase), ornithine decarboxylase, gamma glutamyl transpeptidase, urea nitrogen, albumen blood creatinine, total bilirubin, and total serum protein measurements. 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.

(ii) The following examinations shall be made on high dose and control groups. If changes in the eyes are detected all animals should be examined.

(A) Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, shall be made prior to exposure to the test substance and at the termination of the study.

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

(11) *Gross necropsy*. (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) The liver, kidneys, adrenals, brain, and gonads shall be weighed wet, as soon as possible after dissection, to avoid drying. In addition, for the rodent, the brain; for the non-rodent, the thyroid with parathyroids also shall be weighed wet.

(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; lungs—which should be removed intact, weighed, and treated with a suitable fixative to ensure that lung structure is maintained (perfusion with the fixative is considered to be an effective procedure); nasopharyngeal tissues; brain—including sections of medulla/pons, cerebellar cortex, and cerebral cortex; pituitary; thyroid/parathyroid; thymus; trachea; heart; sternum with bone marrow; salivary glands; liver; spleen; kidneys; adrenals; pancreas; gonads; uterus; accessory genital organs (epididymis, prostate, and, if present, seminal vesicles); aorta; (skin); gall bladder (if present); esophagus; stomach; duodenum; jejunum; ileum; cecum; colon; rectum; urinary bladder; representative lymph node; (mammary gland); (thigh musculature); peripheral nerve; (eyes); (femur—including articular surface); (spinal cord at three levels—cervical, midthoracic, and lumbar); and (zymbal and exorbital lachrymal glands).

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

(i) Full histopathology on normal and treated skin and on organs and tissues, listed above, of all animals in the control and high dose groups.

(ii) All gross lesions in all animals.

(iii) Target organs in all animals.

(iv) The tissues listed in parenthesis in paragraph (e)(11)(iii) of this section, if indicated by signs of toxicity or expected target organ involvement.

(v) Lungs of animals (rodents) in the low and intermediate dose groups shall be subjected to histopathological examination for evidence of infection, since this provides a convenient assessment of the state of health of the animals.

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

(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 incidental, should be evaluated by an appropriate statistical method. Any

generally accepted statistical method may be used; the statistical methods should be selected during the design of the study.

(2) *Evaluation of results.* The findings of a subchronic dermal 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 should include the relationship between the dose of the test substance and the presence or absence, the incidence and severity, of abnormalities, including behavioral and clinical abnormalities, gross lesions, identified target organs, body weight changes, effect 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.

(3) *Test report.* In addition to the reporting requirements as specified in the EPA Good Laboratory Practice Standards under 40 CFR part 792, subpart J, the following specific information shall be reported.

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

(A) Number of animals dying.

(B) Number of animals showing signs of toxicity.

(C) Number of animals exposed.

(ii) *Individual animal data.* (A) Date of death during the study or whether animals survived to termination.

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

(C) Body weight data.

(D) Feed consumption data when collected.

(E) Hematological tests employed and all results.

(F) Clinical biochemistry tests employed and all results.

(G) Necropsy findings.

(H) Detailed description of all histopathological findings.

(I) Statistical treatment of results where appropriate.

(g) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Draize, J.H. ''Dermal toxicity,'' *Appraisal of Chemicals in Food, Drugs and Cosmetics.* The Association of Food and Drug Officials of the United States (1959, 3rd printing 1975). pp. 46–59.

(2) Fitzhugh, O.G. ''Subacute toxicity,'' *Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics.* The Association of Food and Drug Officials of the United States (1959, 3rd printing 1975). pp. 26–35.

(3) National Academy of Sciences. ''Principles and Procedures for Evaluating the Toxicity of Household Substances,'' a report prepared by the Committee for the Revision of NAS Publication 1138, under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences, Washington, DC (1977).

(4) World Health Organization. ''Part I. Environmental Health Criteria 6,''*Principles and Methods for Evaluating the Toxicity of Chemicals.* (Geneva: World Health Organization, 1978).

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19072, May 20, 1987; 53 FR 49149, Dec. 6, 1988; 54 FR 21064, May 16, 1989]

§ 798.2450 Inhalation toxicity.

(a) *Purpose.* 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 and toxic effects associated with continuous or repeated exposure to a test substance for a period of 90 days. The test is not capable of determining those effects that have a long latency period for development (e.g., carcinogenicity and life shortening). It provides 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, the possibilities of accumulation, and can be of use in selecting dose levels for chronic studies and for 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) *Definitions.* (1) 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 percent) of a life span.

(2) Aerodynamic diameter applies to the size of particles of aerosols. It is the diameter of a sphere of unit density which behaves aerodynamically as the particle of the test substance. It is used to compare particles of different size and densities and to predict where in the respiratory tract such particles may be deposited. This term is used in contrast to measured or geometric diameter which is representative of actual diameters which in themselves cannot be related to deposition within the respiratory tract.

(3) The geometric mean diameter or the median diameter is the calculated aerodynamic diameter which divides the particles of an aerosol in half based on the weight of the particles. Fifty percent of the particles by weight will be larger than the median diameter and 50 percent of the particles will be smaller than the median diameter. The median diameter describes the particle size distribution of any aerosol based on the weight and size of the particles.

(4) 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 from the trachea to the alveoli. For man, inhalable diameter is considered as 15 micrometers or less.

(5) Dose refers to an exposure level. Exposure is expressed as weight or volume of test substance per volume of air (mg/l), or as parts per million (ppm).

(6) No-effect level/No-toxic-effect level/No-adverse-effect level/No-observed-effect level is the maximum dose used in a test which produces no observed adverse effects. A no-observed-effect level is expressed in terms of weight or volume of test substance given daily per unit volume of air (mg/l or ppm).

(7) Cumulative toxicity is the adverse effects of repeated doses occuring as a result of prolonged action on, or increased concentration of the administered test substance or its metabolites in susceptible tissues.

(c) *Principle of the test method.* Several groups of experimental animals are exposed daily for a defined period to the test substance in graduated concentrations, one concentration being used per group, for a period of 90 days. During the period of administration, the animals are observed daily to detect signs of toxicity. Animals which die during the test are necropsied and at the conclusion of the test, surviving animals are sacrificed and necropsied and appropriate histopathological examinations carried out.

(d) *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 shall be employed. If another mammalian species is used, the tester shall provide justification/ reasoning for its selection.

(ii) *Age.* Young adult animals shall be used. At the commencement of the study the weight variation of animals shall not exceed ±20 percent of the mean weight for each sex.

(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 20 rodents (10 females and 10 males) shall be used for each test group. If another mammalian species is selected (e.g. dog, rabbit, or non-human primate), at least 8 animals (4 males and 4 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.

(2) *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 is used to help generate an appropriate concentration of

the substance in the atmosphere, a vehicle control group shall 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.

(3) *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 posttreatment period of appropriate length, normally not less than 28 days.

(4) *Dose levels and dose selection.* (i) In subchronic toxicity tests, it is desirable to have a concentration-response relationship as well as a no-observed-toxic-effect level. Therefore, at least 3 concentration levels with 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 lowest concentration should not produce any evidence of toxicity. Where there is a usable estimation of human exposure the lowest concentration should exceed this.

(iv) Ideally, the intermediate concentration level(s) should produce minimal observable toxic effects. If more than one intermediate concentration level is used, the concentrations should be spaced to produce a gradation of toxic effects.

(v) In the low and intermediate groups and in the controls the incidence of fatalities should be low, to permit a meaningful evaluation of the results.

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

(5) *Exposure conditions.* The animals should be exposed to the test substance, ideally for 6 hours per day on a 7-day per week basis, for a period of 90 days. However, based primarily on practical considerations, exposure on a 5-day-per-week basis for 6 hours per day is the minimum acceptable exposure period.

(6) *Observation period.* (i) Duration of observation shall be for at least 90 days.

(ii) Animals in a satellite group scheduled for followup observations should be kept for at least 28 days further without treatment to detect recovery from, or persistence of, toxic effects.

(7) *Inhalation exposure.* (i) The animals shall be tested in inhalation equipment designed to sustain a minimum dynamic air flow of 12 to 15 air changes per hour and ensure an adequate oxygen content of 19 percent and an evenly distributed exposure atmosphere. Where a chamber is used, its design should minimize crowding of the test animals and maximize their exposure to the test substance. This is best accomplished by individual caging. To ensure stability of a chamber atmosphere, the total ''volume'' of the test animals shall not exceed 5 percent of the volume of the test chamber. Oronasal or head-only exposure may be used if it is desirable to avoid concurrent exposure by the dermal or oral routes.

(ii) A dynamic inhalation system with a suitable flow control system shall be used. The rate of air flow shall be adjusted to ensure that conditions throughout the exposure chamber are essentially the same. Maintenance of slight negative pressure inside the chamber will prevent leakage of the test substance into surrounding areas.

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

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

(i) The rate of air flow shall be monitored continuously and recorded at least every 30 minutes.

(ii) 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 practicable, 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.

(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. During exposure, analysis shall be conducted as often as necessary to determine the consistency of particle size distribution.

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

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

(10) *Observation of animals.* (i) Each animal shall be observed daily and, if necessary, handled to appraise its physical condition.

(ii) Additional observations should be made daily with appropriate actions 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) Signs of toxicity shall be recorded as they are observed including the time of onset, the degree, and duration.

(iv) Cage-side observations should include, but not be limited to, changes in the skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavior pattern.

(v) Animals shall be weighed weekly. Feed consumption shall also be determined weekly if abnormal body weight changes are observed.

(vi) At the end of the study period all survivors in the nonsatellite treatment groups shall be sacrificed. Moribund animals shall be removed and sacrificed when noticed.

(11) *Clinical examinations.* (i) The following examinations shall be made on all animals of each sex in each group:

(A) Certain hematology determinations shall be carried out at least two times during the test period on all groups of animals including concurrent controls: After 30 days of test and just prior to terminal sacrifice at the end of the test period. Hematology determinations which are appropriate to all studies: Hematocrit, hemoglobin concentration, erythrocyte count, total and differential leukocyte count, and a measure of clotting potential such as clotting time, prothrombin time, thromboplastin time, or platelet count.

(B) Certain clinical biochemistry determinations on blood should be carried out at least two times during the test period on all groups of animals including concurrent controls: After 30 days of test and just prior to terminal sacrifice at the end of the test period. Clinical biochemistry test areas which are considered appropriate to all studies: Electrolyte balance, carbohydrate metabolism, and liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the substance. Suggested determinations: calcium, phosphorus, chloride, sodium, potassium, fasting glucose (with period of fasting appropriate to the species), serum glutamic-pyruvic transaminase, (now known as serum alanine aminotransferase), serum glutamic-oxaloacetic transaminase (now known as serum aspartate aminotransferase), ornithine decarboxylase, gamma glutamyl transpeptidase, urea nitrogen, albumen, blood creatinine, total bilirubin, and total serum protein measurements. 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.

(ii) The following examinations shall be made on high dose and control groups. If changes in the eyes are detected, all animals shall be examined:

(A) Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, shall be made prior to exposure to the test substance and at the termination of the study.

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

(12) *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, adrenals, brain, and gonads shall be weighed wet, as soon as possible after dissection to avoid drying. In addition, for the rodent, the brain; for the nonrodent, the thyroid with parathyroids also shall be weighed wet.

(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; lungs—which should be removed intact, weighed, and treated with a suitable fixative to ensure that lung structure is maintained (perfusion with the fixative is considered to be an effective procedure); nasopharyngeal tissues; brain—including sections of medulla/pons cerebellar cortex and cerebral cortex; pituitary; thyroid/parathyroid; thymus; trachea; heart; sternum with bone marrow; salivary glands; liver; spleen; kidneys; adrenals; pancreas; gonads; uterus; accessory genital organs (epididymis, prostate, and, if present, seminal vesicles); aorta; (skin); gall bladder (if present); esophagus; stomach; duodenum; jejunum; ileum; cecum; colon; rectum; urinary bladder; representative lymph node; (mammary gland); (thigh musculature); peripheral nerve; (eyes); (femur—including articular surface); (spinal cord at three levels—cervical, midthoracic, and lumbar); and (zymbal and exorbital lachrymal glands).

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

(i) Full histopathology on the respiratory tract and other organs and tissues, listed above, of all animals in the control and high dose groups.

(ii) All gross lesions in all animals.

(iii) Target organs in all animals.

(iv) The tissues mentioned in brackets (listed above) if indicated by signs of toxicity or target organ involvement.

(v) Lungs of animals (rodents) in the low and intermediate dose groups shall also be subjected to histopathological examination, primarily for evidence of infection since this provides a convenient assessment of the state of health of the animals.

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

(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 incidental, should be evaluated by an appropriate statistical method. Any generally accepted statistical method may be used; the statistical methods should be selected during the design of the study.

(2) *Evaluation of 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.

(3) *Test report*. In addition to the reporting requirements as specified under EPA Good Laboratory Practice Standards, 40 CFR part 792, subpart J, the following specific information shall be reported:

(i) *Test conditions*. (A) Description of exposure apparatus, including design, type, dimensions, source of air, system for generating particulates and aerosols, method of conditioning air, treatment of exhaust air, and the

method of housing animals in a test chamber.

(B) The equipment for measuring temperature, humidity, and particulate aerosol concentrations and size shall be described.

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

(A) Airflow rates through the inhalation equipment.

(B) Temperature and humidity of air.

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

(D) Actual concentration in test breathing zone.

(E) Particle size distribution (e.g., median aerodynamic diameter of particles with standard deviation from the mean).

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

(A) Number of animals dying.

(B) Number of animals showing signs of toxicity.

(C) Number of animals exposed.

(iv) *Individual animal data.* (A) Date of death during the study or whether animals survived to termination.

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

(C) Body weight data.

(D) Feed consumption data when collected.

(E) Hematological tests employed and all results.

(F) Clinical biochemistry tests employed and all results.

(G) Necropsy findings.

(H) Detailed description of all histopathological findings.

(I) Statistical treatment of results where appropriate.

(f) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Cage, J.C. ''Experimental Inhalation Toxicology,'' *Methods in Toxicology.* Ed. G.E. Paget. (Philadelphia: F.A. Davis Co. 1970, pp. 258–277.

(2) Casarett, L.J., Doull, J. ''Chapter 9.'' *Toxicology: The Basic Science of Poisons* (New York: Macmillan Publishing Co. Inc. 1975).

(3) MacFarland, H.N. ''Respiratory Toxicology,'' *Essays in Toxicology.* Ed. W.J. Hayes. Vol. 7 (New York: Academic Press, 1976) pp. 121–154.

(4) National Academy of Sciences. ''Principles and Procedures for Evaluating the Toxicity of Household Substances,'' a report prepared by the Committee for the Revision of NAS Publication 1138, under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences, Washington, DC (1977).

(5) World Health Organization. ''Part I. Environmental Health Criteria 6,'' *Principles and Methods for Evaluating the Toxicity of Chemicals.* (Geneva: World Health Organization, 1978).

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19073, May 20, 1987; 52 FR 26150, July 13, 1987; 53 FR 49150, Dec. 6, 1988; 54 FR 21064, May 16, 1989]

§ 798.2650 Oral toxicity.

(a) *Purpose.* In the assessment and evaluation of the toxic characteristics of a chemical, the determination of subchronic oral toxicity may be carried out after initial information on toxicity has been obtained by acute testing. The subchronic oral study has been designed to permit the determination of the no-observed-effect level and toxic effects associated with continuous or repeated exposure to a test substance for a period of 90 days. The test is not capable of determining those effects that have a long latency period for development (e.g., carcinogenicity and life shortening). It provides information on health hazards likely to arise from repeated exposure by the oral route over a limited period of time. It will provide information on target organs, the possibilities of accumulation, and can be of use in selecting dose levels for chronic studies and for establishing safety criteria for human exposure.

(b) *Definitions.* (1) Subchronic oral toxicity is the adverse effects occurring as a result of the repeated daily exposure of experimental animals to a chemical by the oral route for a part (approximately 10 percent) of a life span.

(2) Dose is the amount of test substance administered. Dose is expressed

as weight of test substance (g, mg) per unit weight of test animal (e.g., mg/kg), or as weight of test substance per unit weight of food or drinking water.

(3) No-effect level/No-toxic-effect level/No-adverse-effect level/No-observed-effect level is the maximum dose used in a test which produces no observed adverse effects. A no-observed-effect level is expressed in terms of the weight of a substance given daily per unit weight of test animal (mg/kg). When administered to animals in food or drinking water the no-observed-effect level is expressed as mg/kg of food or mg/ml of water.

(4) Cumulative toxicity is the adverse effects of repeated doses occurring as a result of prolonged action on, or increased concentration of, the administered test substance or its metabolites in susceptible tissue.

(c) *Principle of the test method.* The test substance is administered orally in graduated daily doses to several groups of experimental animals, one dose level per group, for a period of 90 days. During the period of administration the animals are observed daily to detect signs of toxicity. Animals which die during the period of administration are necropsied. At the conclusion of the test all animals are necropsied and histo-pathological examinations carried out.

(d) *Limit test.* If a test at one dose level of at least 1,000 mg/kg body weight (expected human exposure may indicate the need for a higher dose level), using the procedures described for this study, produces no observable toxic effects and if toxicity would not be expected based upon data of structurally related compounds, then a full study using three dose levels might 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 shall be employed. The commonly used nonrodent species is the dog, preferably of a defined breed; the beagle is frequently used. If other mammalian species are used, the tester shall provide justification/reasoning for his or her selection.

(ii) *Age*—(A) *General.* Young adult animals shall be employed. At the commencement of the study the weight variation of animals used shall not exceed ±20 percent of the mean weight for each sex.

(B) *Rodents.* Dosing shall begin as soon as possible after weaning, ideally before the rats are 6, and in any case, not more than 8 weeks old.

(C) *Non-rodent.* In the case of the dog, dosing shall commence after acclimatization, preferably at 4 to 6 months and not later than 9 months of age.

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

(B) The females shall be nulliparous and nonpregnant.

(iv) *Numbers*—(A) *Rodents.* At least 20 animals (10 females and 10 males) shall be used at each dose level.

(B) *Non-rodents.* At least eight animals (four females and four males) shall be used at each dose level.

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

(2) *Control groups.* A concurrent control group is required. This group shall be an untreated or sham-treated control group 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 or cannot be made available, both untreated and vehicle control groups are required.

(3) *Satellite group.* (Rodent) A satellite group of 20 animals (10 animals per sex) may be treated with the high dose 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.

(4) *Dose levels and dose selection.* (i) In subchronic toxicity tests, it is desirable to have a dose response relationship as well as a no-observed-toxic-effect level. Therefore, at least 3 dose levels with a control and, where appropriate, a vehicle control (corresponding to the concentration of vehicle at the highest exposure level) shall 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.

(ii) The highest dose level in rodents should result in toxic effects but not produce an incidence of fatalities which would prevent a meaningful evaluation; for non-rodents there should be no fatalities.

(iii) The lowest dose level should not produce any evidence of toxicity. Where there is a usable estimation of human exposure the lowest dose level should exceed this.

(iv) Ideally, the intermediate dose level(s) should produce minimal observable toxic effects. If more than one intermediate dose is used, the dose levels should be spaced to produce a gradation of toxic effects.

(v) For rodents, the incidence of fatalities in low and intermediate dose groups and in the controls should be low, to permit a meaningful evaluation of the results; for non-rodents, there should be no fatalities.

(5) *Exposure conditions.* The animals are dosed with the test substance ideally on a 7-day per week basis over a period of 90 days. However, based primarily on practical considerations, dosing in gavage or capsule studies on a 5-day per week basis is considered to be acceptable.

(6) *Observation period.* (i) Duration of observation shall be for at least 90 days.

(ii) Animals in the satellite group scheduled for followup observations should be kept for at least 28 days further without treatment to detect recovery from, or persistence of, toxic effects.

(7) *Administration of the test substance.* (i) The test substance may be administered in the diet or in capsules. In addition, for rodents it may also be administered by gavage or in the drinking water.

(ii) All animals shall be dosed by the same method during the entire experimental period.

(iii) Where necessary, the test substance is dissolved or suspended in a suitable vehicle. If a vehicle or diluent is needed, ideally it should not elicit important toxic effects itself nor substantially alter the chemical or toxicological properties of the test substance. It is recommended that wherever possible the usage of an aqueous solution be considered first, followed by consideration of a solution of oil and then by possible solution in other vehicles.

(iv) 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 (ppm) or a constant dose level in terms of the animals' body weight shall be used; the alternative used shall be specified.

(v) For a substance administered by gavage or capsule, the dose shall be given at approximately the same time each day, and adjusted at intervals (weekly or bi-weekly) to maintain a constant dose level in terms of animal body weight.

(8) *Observation of animals.* (i) Each animal shall be observed daily and, if necessary, handled to appraise its physical condition.

(ii) Additional observations shall be made daily with appropriate actions 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) Signs of toxicity shall be recorded as they are observed including the time of onset, degree and duration.

(iv) Cage-side observations shall include, but not be limited to, changes in skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavior pattern.

(v) Measurements shall be made weekly of feed consumption or water consumption when the test substance is administered in the feed or drinking water, respectively.

(vi) Animals shall be weighed weekly.

(vii) At the end of the 90-day period all survivors in the nonsatellite treatment groups shall be sacrificed. Moribund animals shall be removed and sacrificed when noticed.

(9) *Clinical examinations.* (i) The following examinations shall be made on all animals of each sex in each group

for rodents and all animals when non-rodents are used as test animals.

(A) Certain hematology determinations shall be carried out at least two times during the test period on all groups of animals including concurrent controls: After 30 days of test and just prior to terminal sacrifice at the end of the test period. Hematology determinations which are appropriate to all studies: Hematocrit, hemoglobin concentration, erythrocyte count, total and differential leukocyte count, and a measure of clotting potential such as clotting time, prothrombin time, thromboplastin time, or platelet count.

(B) Certain clinical biochemistry determinations on blood should be carried out at least two times during the test period on all groups of animals including concurrent controls: After 30 days of test and just prior to terminal sacrifice at the end of the test period. Clinical biochemistry test areas which are considered appropriate to all studies: Electrolyte balance, carbohydrate metabolism, and liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the substance. Suggested determinations: Calcium, phosphorus, chloride, sodium, potassium, fasting glucose (with period of fasting appropriate to the species), serum glutamic-pyruvic transaminase (now known as serum alanine aminotransferase), serum glutamic oxaloacetic transaminase (now known as serum aspartate aminotransferase), ornithine decarboxylase, gamma glutamyl transpeptidase, urea nitrogen, albumen, blood creatinine, total bilirubin, and total serum protein measurements. 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.

(ii) The following examinations shall be made on high dose and control groups. If changes in the eyes are detected, all animals should be examined.

(A) Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, shall be made prior to the administration of the test substance and at the termination of the study.

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

(10) *Gross necropsy.* (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, adrenals, and gonads shall be weighed wet, as soon as possible after dissection to avoid drying. In addition, for the rodent, the brain; for the non-rodent, the thyroid with parathyroids also shall be weighed wet.

(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; lungs—which should be removed intact, weighed, and treated with a suitable fixative to ensure that lung structure is maintained (perfusion with the fixative is considered to be an effective procedure); nasopharyngeal tissues; brain—including sections of medulla/pons, cerebellar cortex, and cerebral cortex; pituitary; thyroid/parathyroid; thymus; trachea; heart; sternum with bone marrow; salivary glands; liver; spleen; kidneys; adrenals; pancreas; gonads; uterus; accessory genital organs (epididymis, prostate, and, if present, seminal vesicles); aorta; (skin); gall bladder (if present); esophagus; stomach; duodenum; jejunum; ileum; cecum; colon; rectum; urinary bladder; representative lymph node; (mammary gland); (thigh musculature); peripheral nerve; (eyes); (femur—including articular surface); (spinal cord at three levels—cervical, midthoracic, and lumbar); and (zymbal and exorbital lachrymal glands); and (rodent-zymbal glands).

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

(i) Full histopathology on the organs and tissues, listed above, of all rodents in the control and high dose groups, all non-rodents, and all rodents that died or were killed during the study.

(ii) All gross lesions in all animals.

(iii) Target organs in all animals.

(iv) The tissues mentioned in brackets (listed above) if indicated by signs of toxicity of target organ involvement.

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

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

(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 incidental, should be evaluated by an appropriate statistical method. Any generally accepted statistical methods may be used; the statistical methods should be selected during the design of the study.

(2) *Evaluation of the study results.* (i) The findings of a subchronic oral toxicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of the toxic effects and the necropsy and histopathological findings. The evaluation will include the relationship between the dose of the test substance 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.

(ii) 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 EPA Good Laboratory Practice Standards, 40 CFR part 792, subpart J, the following specific information shall be reported:

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

(A) Number of animals dying.

(B) Number of animals showing signs of toxicity.

(C) Number of animals exposed.

(ii) *Individual animal data.* (A) Date of death during the study or whether animals survived to termination.

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

(C) Body weight data.

(D) Feed consumption data when collected.

(E) Hematological tests employed and all results.

(F) Clinical biochemistry tests employed and all results.

(G) Necropsy findings.

(H) Detailed description of all histopathological findings.

(I) Statistical treatment of results where appropriate.

(g) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Boyd, E.M. ''Chapter 14—Pilot Studies, 15—Uniposal Clinical Parameters, 16—Uniposal Autopsy Parameters.'' *Predictive Toxicometrics.* (Baltimore: Williams and Wilkins, 1972).

(2) Fitzhugh, O.G. ''Subacute Toxicity,'' *Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics.* The Association of Food and Drug Officials of the United States (1959, 3rd Printing 1975) pp. 26–35.

(3) Food Safety Council. ''Subchronic Toxicity Studies,'' *Proposed System for Food Safety Assessment.* (Columbia: Food Safety Council, 1978) pp. 83–96.

(4) National Academy of Sciences. ''Principles and Procedures for Evaluating the Toxicity of Household Substances,'' a report prepared by the Committee for the Revision of NAS Publication 1138, under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences, Washington, DC (1977).

(5) World Health Organization. ''Part I. Environmental Health Criteria 6,'' *Principles and Methods for Evaluating*

the Toxicity of Chemicals. (Geneva: World Health Organization, 1978).

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Subpart D—Chronic Exposure

§ 798.3260 Chronic toxicity.

(a) *Purpose*. The objective of a chronic toxicity study is to determine the effects of a substance in a mammalian species following prolonged and repeated exposure. Under the conditions of the chronic toxicity test, effects which require a long latency period or which are cumulative should become manifest. The application of this guideline should generate data on which to identify the majority of chronic effects and shall serve to define long term dose-response relationships. The design and conduct of chronic toxicity tests should allow for the detection of general toxic effects, including neurological, physiological, biochemical, and hematological effects and exposure-related morphological (pathology) effects.

(b) *Test procedures*—(1) *Animal selection*—(i) *Species and strain*. Testing should be performed with two mammalian species, one a rodent and another a non-rodent. The rat is the preferred rodent species and the dog is the preferred non-rodent species. Commonly used laboratory strains should be employed. If other mammalian species are used, the tester should provide justification/reasoning for their selection.

(ii) *Age*. (A) Dosing of rats should begin as soon as possible after weaning, ideally before the rats are 6, but in no case more than 8 weeks old.

(B) Dosing of dogs should begin between 4 and 6 months of age and in no case later than 9 months of age.

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

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

(B) The females should be nulliparous and non-pregnant.

(iv) *Numbers*. (A) For rodents, at least 40 animals (20 females and 20 males) and for non-rodents (dogs) at least 8 animals (4 females and 4 males) should be used at each dose level.

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

(C) The number of animals at the termination of the study must be adequate for a meaningful and valid statistical evaluation of chronic effects.

(2) *Control groups*. (i) A concurrent control group is suggested. This group should be an untreated or sham treated control group 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 or cannot be made available, both untreated and vehicle control groups are strongly suggested.

(ii) In special circumstances such as in inhalation studies involving aerosols or the use of an emulsifier of uncharacterized biological activity in oral studies, a concurrent negative control group should be utilized. The negative control group should be treated in the same manner as all other test animals except that this control group should not be exposed to either the test substance or any vehicle.

(3) *Dose levels and dose selections*. (i) In chronic toxicity tests, it is necessary to have a dose-response relationship as well as a no-observed-toxic-effect level. Therefore, at least three dose levels should be used in addition to the concurrent control group. Dose levels should be spaced to produce a gradation of effects.

(ii) The high dose level in rodents should elicit some signs of toxicity without causing excessive lethality; for non-rodents, there should be signs of toxicity but there should be no fatalities.

(iii) The lowest dose level should not produce any evidence of toxicity. Where there is a usable estimation of human exposure the lowest dose level should exceed this even though this dose level may result in some signs of toxicity.

(iv) Ideally, the intermediate dose level(s) should produce minimal observable toxic effects. If more than one intermediate dose is used, the dose level should be spaced to produce a gradation of toxic effects.

(v) For rodents, the incidence of fatalities in low and intermediate dose groups and in the controls should be low to permit a meaningful evaluation of the results. For non-rodents, there should be no fatalities.

(4) *Exposure conditions.* The animals are dosed with the test substance ideally on a 7-day per week basis over a period of at least 12 months. However, based primarily on practical considerations, dosing on a 5-day per week basis is considered to be acceptable.

(5) *Observation period.* Duration of observation should be for at least 12 months, and may be concurrent with or subsequent to dosing. If there is a post-exposure observation period, an interim sacrifice should be performed on no fewer than half of the animals of each sex at each dose level immediately upon termination of exposure.

(6) *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.* (A) The animals should receive the test substance in their diet, dissolved in drinking water, or given by gavage or capsule for a period of at least 12 months.

(B) If the test substance is administered in the drinking water, or mixed in the diet, exposure is continuous.

(C) For a diet mixture, the highest concentration should not exceed 5 percent.

(ii) *Dermal studies.* (A) The animals are treated by topical application with the test substance, ideally for at least 6 hours per day.

(B) Fur should be clipped from the dorsal area of the trunk of the test animals. Care must be taken to avoid abrading the skin which could alter its permeability.

(C) The test substance should be applied uniformly over a shaved area which is approximately 10 percent of the total body surface area. With highly toxic substances, the surface area covered may be less, but as much of the area should be covered with as thin and uniform a film as possible.

(D) During the exposure period, the test substance may be held if necessary, in contact with the skin with a porous gauze dressing and non-irritating tape. 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 tested with inhalation equipment designed to sustain a dynamic air flow of 12 to 15 air changes per hour, ensure an adequate oxygen content of 19 percent and an evenly distributed exposure atmosphere. Where a chamber is used, its design should minimize crowding of the test animals and maximize their exposure to the test substance. This is best accomplished by individual caging. As a general rule to ensure stability of a chamber atmosphere, the total ''volume'' of the test animals should not exceed 5 percent of the volume of the test chamber. Alternatively, oro-nasal, head-only or whole body individual chamber exposure may be used.

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

(C) Feed and water should be withheld during each daily 6 hour exposure period.

(D) A dynamic inhalation system with a suitable analytical concentration control system should be used. The rate of air flow should be adjusted to ensure that conditions throughout the equipment are essentially the same. Maintenance of slight negative pressure inside the chamber will prevent leakage of the test substance into the surrounding areas.

(7) *Observation of animals.* (i) Each animal should be handled and its physical condition appraised at least once each day.

(ii) Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study (e.g., necropsy or refrigeration of those animals found dead and

isolation or sacrific of weak or moribund animals).

(iii) Clinical signs of toxicity including suspected tumors and mortality should be recorded as they are observed, including the time of onset, the degree and duration.

(iv) Cage-side observations should include, but not be limited to, changes in skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavior pattern.

(v) Body weights should be recorded individually for all animals once a week during the first 13 weeks of the test period and at least once every 4 weeks thereafter unless signs of clinical toxicity suggest more frequent weighings to facilitate monitoring of health status.

(vi) 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.

(vii) At the end of the study period all survivors should be sacrificed. Moribund animals should be removed and sacrificed when noticed.

(8) *Physical measurements.* For inhalation studies, measurements or monitoring should be made of the following:

(i) The rate of air flow should be monitored continuously, but should be recorded at intervals of at least once every 30 minutes.

(ii) During each exposure period the actual concentrations of the test substance should be held as constant as practicable, monitored continuously and measured at least three times during the test period: at the beginning, at an intermediate time and at the end of the period.

(iii) During the development of the generating system, particle size analysis should be performed to establish the stability of aerosol concentrations. During exposure, analysis should be conducted as often as necessary to determine the consistency of particle size distribution and homogeneity of the exposure stream.

(iv) Temperature and humidity should be monitored continuously, but should be recorded at intervals of at least once every 30 minutes.

(9) *Clinical examinations.* The following examinations should be made on at least 10 rats of each sex per dose and on all non-rodents.

(i) Certain hematology determinations (e.g., hemoglobin content, packed cell volume, total red blood cells, total white blood cells, platelets, or other measures of clotting potential) should be performed at termination and should be performed at 3 months, 6 months and at approximately 6 month intervals thereafter (for studies extending beyond 12 months) on blood samples collected from all non-rodents and from 10 rats per sex of all groups. These collections should be from the same animals at each interval. If clinical observations suggest a deterioration in health of the animals during the study, a differential blood count of the affected animals should be performed. A differential blood count should be performed on samples from those animals in the highest dosage group and the controls. Differential blood counts should be performed for the next lower group(s) if there is a major discrepancy between the highest group and the controls. If hematological effects were noted in the subchronic test, hematological testing should be performed at 3, 6, 12, 18, and 24 months for a two year study and at 3, 6, and 12 months for a 1-year study.

(ii) Certain clinical biochemistry determinations on blood should be carried out at least three times during the test period: just prior to initiation of dosing (base line data), near the middle and at the end of the test period. Blood samples should be drawn for clinical chemistry measurements from all non-rodents and at least ten rodents per sex of all groups; if possible, from the same rodents at each time interval. Test areas which are considered appropriate to all studies: electrolyte balance, carbohydrate metabolism and liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the substance and signs of clinical toxicity. Suggested chemical determinations: calcium, phosphorus, chloride, sodium,

potassium, fasting glucose (with period of fasting appropriate to the species), serum glutamic-pyruvic transaminase (now known as serum alanine aminotransferase), serum glutamic oxaloacetic transaminase (now known as serum aspartate aminotransferase), ornithine decarboxylase, gamma glutamyl transpeptidase, blood urea nitrogen, albumen, blood creatinine, creatinine phosphokinase, total cholesterol, total bilirubin and total serum protein measurements. 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.

(iii) Urine samples from rodents at the same intervals as the hematological examinations under paragraph (b)(9)(i) of this section should be collected for analysis. The following determinations should be made from either individual animals or on a pooled sample/sex/group for rodents: appearance (volume and specific gravity), protein, glucose, ketones, bilirubin, occult blood (semi-quantitatively); and microscopy of sediment (semi-quantitatively).

(iv) Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, should be made prior to the administration of the test substance and at the termination of the study. If changes in eyes are detected all animals should be examined.

(10) *Gross necropsy*. (i) A complete gross examination should be performed on all animals, including those which died during the experiment or were killed in moribund conditions.

(ii) The liver, kidneys, adrenals, brain and gonads should be weighed wet, as soon as possible after dissection to avoid drying. For these organs, at least 10 rodents per sex per group and all non-rodents should be weighed.

(iii) The following organs and tissues, or representative samples thereof, should be preserved in a suitable medium for possible future histopathological examination: All gross lesions and tumors; brain—including sections of medulla/pons, cerebellar cortex, and cerebral cortex; pituitary; thyroid/parathyroid; thymus; lungs; trachea; heart; sternum and/or femur with bone marrow; salivary glands; liver; spleen; kidneys; adrenals; esophagus; stomach; duodenum; jejunum; ileum; cecum; colon; rectum; urinary bladder; representative lymph nodes; pancreas; gonads; uterus; accessory genital organs (epididymis, prostate, and, if present, seminal vesicles; female mammary gland; aorta; gall bladder (if present); skin; musculature; peripheral nerve; spinal cord at three levels—cervical, midthoracic, and lumbar; and eyes. In inhalation studies, the entire respiratory tract, including nose, pharynx, larynx, and paranasal sinuses should be examined and preserved. In dermal studies, skin from sites of skin painting should be examined and preserved.

(iv) 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 considered essential for appropriate and valid histopathological examination.

(v) If other clinical examinations are carried out, the information obtained from these procedures should be available before microscopic examination, since they may provide significant guidance to the pathologist.

(11) *Histopathology*. (i) The following histopathology should be performed:

(A) Full histopathology on the organs and tissues, listed above, of all non-rodents, of all rodents in the control and high dose groups and of all rodents 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 excessive early deaths or other problems occur in the high dose group compromising the significance of the data, the next dose level should be examined for complete histopathology.

(iii) In case the results of an experiment give evidence of substantial alteration of the animals' normal longevity or the induction of effects that might affect a toxic response, the next lower dose level should be examined fully, as described under paragraph (b)(11)(i) of this section.

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

(c) *Data and reporting*—(1) *Treatment of results.* (i) Data should 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 incidental, should be evaluated by an appropriate statistical method. Any generally accepted statistical methods may be used; the statistical methods should be selected during the design of the study.

(2) *Evaluation of study results.* (i) The findings of a chronic toxicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of the toxic effects, the necropsy and histopathological findings. The evaluation will 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 bioavailability of the test substance should be considered.

(3) *Test report.* (i) In addition to the reporting requirements as specified under 40 CFR part 792 subpart J, the following specific information should be reported:

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

(*1*) Number of animals dying.

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

(*3*) Number of animals exposed.

(B) *Individual animal data.* (*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 and water consumption data, when collected.

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

(*6*) Hematological tests employed and all results.

(*7*) Clinical biochemistry tests employed and all results.

(*8*) Necropsy findings.

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

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

(ii) In addition, for inhalation studies the following should be reported:

(A) *Test conditions.* (*1*) Description of exposure apparatus including design, type, dimensions, source of air, system for generating particulates 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 should 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*) Nominal concentration (total amount of test substance fed into the inhalation equipment divided by volume of air).

(*4*) Actual concentration in test breathing zone.

(*5*) Particle size distribution (e.g., median aerodynamic diameter of particles with standard deviation from the mean).

(d) *References.* For additional background information on this test guideline the following references should be consulted:

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

(2) D'Aguanno, W. ''Drug Safety Evaluation—Pre-Clinical Considerations,'' *Industrial Pharmacology: Neuroleptics*. Vol. I, Ed. S. Fielding and H. Lal. (Mt. Kisco: Futura Publishing Co. 1974) pp. 317–332.

(3) Fitzhugh, O.G. Third Printing: 1975. ''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 (1959, 3rd Printing 1975) pp. 36–45.

(4) Goldenthal, E.I., 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 (1959, 3rd Printing 1975) pp. 60–67.

(5) National Academy of Sciences. ''Principles and Procedures for Evaluating the Toxicity of Household Substances,'' a report prepared by the Committee for the Revision of NAS Publication 1138, under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences, Washington, DC (1977).

(6) National Center for Toxicological Research. ''Appendix B,'' *Report of Chronic Studies Task Force Committee, April 13–21, 1972*. (Rockville: National Center for Toxicological Research, 1972).

(7) Page, N.P. ''Chronic Toxicity and Carcinogenicity Guidelines,'' *Journal of Environmental Pathology and Toxicology*, 1:161–182 (1977).

(8) Schwartz, E. ''Toxicology of Neuroleptic Agents,'' *Industrial Pharmacology: Neuroleptics* Ed. S. Fielding and H. Lal. (Mt. Kisco, Futura Publishing Co., 1974) pp. 203–221.

(9) United States Pharmaceutical Manufacturers Association. *Guidelines for the Assessment of Drug and Medical Device Safety in Animals*. (1977).

(10) World Health Organization. ''Guidelines for Evaluation of Drugs for Use in Man,'' *WHO Technical Report Series No. 563*. (Geneva: World Health Organization, 1975).

(11) World Health Organization. ''Part I. Environmental Health Criteria 6,'' *Principles and Methods for Evaluating the Toxicity of Chemicals*. (Geneva: World Health Organization, 1978).

(12) World Health Organization. ''Principles for Pre-Clinical Testing of Drug Safety,'' *WHO Technical Report Series No. 341*. (Geneva: World Health Organization, 1966).

[50 FR 39397, Sept. 27, 1985, as amended at 54 FR 21064, May 16, 1989]

§ 798.3300 Oncogenicity.

(a) *Purpose*. The objective of a long-term oncogenicity study is to observe test animals for a major portion of their life span for the development of neoplastic lesions during or after exposure to various doses of a test substance by an appropriate route of administration.

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

(ii) *Age*. (A) Dosing of rodents shall begin as soon as possible after weaning, ideally before the animals are 6 weeks old, but in no case more than 8 weeks old.

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

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

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

(B) The females shall be nulliparous and non-pregnant.

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

(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) The number of animals at the termination of the study should be adequate for a meaningful and valid statistical evaluation of long term exposure. For a valid interpretation of negative results, it is essential that survival in all groups does not fall below 50 percent at the time of termination.

(2) *Control groups.* (i) A concurrent control group is required. This group shall be an untreated or sham treated control group 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 or cannot be made available, both untreated and vehicle control groups are required.

(ii) In special circumstances such as in inhalation studies involving aerosols or the use of an emulsifier of uncharacterized biological activity in oral studies, a concurrent negative control group shall be utilized. The negative control group shall be treated in the same manner as all other test animals except that this control group shall not be exposed to either the test substance or any vehicle.

(iii) The use of historical control data (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 desirable for assessing the significance of changes observed in exposed animals.

(3) *Dose levels and dose selection.* (i) For risk assessment purposes, at least 3 dose levels shall be used, in addition to the concurrent control group. Dose levels should be spaced to produce a gradation of chronic effects.

(ii) The high dose level should elicit signs of minimal toxicity without substantially altering the normal life span.

(iii) The lowest dose should not interfere with normal growth, development and longevity of the animal; and it should not otherwise cause any indication of toxicity. In general, this should not be lower than ten percent of the high dose.

(iv) The intermediate dose(s) should be established in a mid-range between the high and low doses, depending upon the toxicokinetic properties of the chemical, if known.

(v) The selection of these dose levels should be based on existing data, preferably on the results of subchronic studies.

(4) *Exposure conditions.* The animals are dosed with the test substance ideally on a 7 day per week basis over a period of at least 24 months for rats, and 18 months for mice. However, based primarily on practical considerations, dosing on a 5 day per week basis is considered to be acceptable.

(5) *Observations period.* It is necessary that the duration of an oncogenicity test comprise the majority of the normal life span of the strain of animals to be 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 test is advised.

(6) *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.* (A) The animals shall receive the test substance in their diet, dissolved in drinking water at levels that do not exceed the maximum solubility of the test chemical under testing condition.

(B) If the test substance is administered in the drinking water, or mixed in the diet, exposure shall be continuous.

(C) For a diet mixture, the highest concentration should not exceed 5 percent.

(ii) *Dermal studies.* (A) The animals are treated by topical application with the test substance, ideally for at least 6 hours per day.

(B) Fur should be clipped 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.

(C) The test substance shall be applied uniformly over a shaved area which is approximately 10 percent of

the total body surface area. With highly toxic substances, the surface area covered may be less, but as much of the area shall be covered with as thin and uniform a film as possible.

(D) During the exposure period, the test substance may be held, if necessary, in contact with the skin with a porous gauze dressing and non-irritating tape. 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 shall be tested with inhalation equipment designed to sustain a minimum dynamic air flow of 12 to 15 air changes per hour, ensure an adequate oxygen content of 19 percent and an evenly distributed exposure atmosphere. Where a chamber is used, its design should minimize crowding of the test animals and maximize their exposure to the test substance. This is best accomplished by individual caging. To ensure stability of a chamber atmosphere, the total ''volume'' of the test animals shall not exceed 5 percent of the volume of the test chamber. Alternatively, oro-nasal, head-only, or whole-body individual chamber exposure may be used.

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

(C) Feed and water shall be withheld during each daily 6-hour exposure period.

(D) A dynamic inhalation system with a suitable flow control system shall be used. The rate of air flow shall be adjusted to ensure that conditions throughout the equipment are essentially the same. Maintenance of slight negative pressure inside the chamber will prevent leakage of the test substance into the surrounding areas.

(7) *Observations of animals.* (i) Each animal shall be observed daily and if necessary should be handled to appraise its physical condition.

(ii) Additional observations shall be made daily with appropriate actions 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) Clinical signs and mortality shall be recorded for all animals. Special attention should be paid to tumor development. The day of onset, location, dimensions, appearance and progression of each grossly visible or palpable tumor shall be recorded.

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

(v) When the test substance is administered in the feed or drinking water, measurements of feed or water consumption, respectively, shall 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.

(vi) At the end of the study period all survivors are sacrificed. Moribund animals shall be removed and sacrificed when noticed.

(8) *Physical measurements.* For inhalation studies, measurements or monitoring should be made of the following:

(i) The rate of air flow shall be monitored continuously and recorded at intervals of at least once every 30 minutes.

(ii) During each exposure period the actual concentrations of the test substance shall be held as constant as practicable, monitored continuously and recorded at least three times during the test period: at the beginning, at an intermediate time and at the end of the period.

(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. During exposure, analyses shall be conducted as often as necessary to determine the consistency of particle size, distribution, and homogeneity of the exposure stream.

(iv) Temperature and humidity shall be monitored continuously, but shoud be recorded at intervals of at least once every 30 minutes.

(9) *Clinical examinations.* At 12 months, 18 months, and at sacrifice, a blood smear shall be obtained from all animals. A differential blood count shall be performed on blood smears from those animals in the highest dosage group and the controls. If these data, or data from the pathological examination indicate a need, then the 12- and 18-month blood smears from other dose levels shall also be examined. Differential blood counts shall be performed for the next lower group(s) 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.

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

(ii) The following organs and tissues or representative samples thereof, shall be preserved in a suitable medium for possible future histopathological examination: All gross lesions and tumors of all animals shall be preserved; brain—including sections of medulla/pons, cerebellar cortex and cerebral cortex; pituitary; thyroid/parathyroid; thymus; lungs; trachea; heart; spinal cord at three levels—cervical, midthoracic and lumbar; sternum and/or femur with bone marrow; salivary glands; liver; spleen; kidneys; adrenals; esophagus; stomach; duodenum; jejunum; ileum; cecum; colon; rectum; urinary bladder; representative lymph nodes; pancreas; gonads; uterus; accessory genital organs (epididymis, prostate, and, if present, seminal vesicles); mammary gland; skin; musculature; peripheral nerve; and eyes. In inhalation studies, the entire respiratory tract shall be preserved, including nasal cavity, pharynx, larynx and paranasal sinuses. In dermal studies, skin from sites of skin painting shall be examined and preserved.

(iii) 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 required for appropriate and valid histopathological examination.

(iv) If other clinical examinations are carried out, the information obtained from these procedures shall be available before microscopic examination, since they may provide significant guidance to the pathologist.

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

(A) Full histopathology on organs and tissues listed above 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.

(ii) If a significant difference is observed in hyperplastic, pre-neoplastic or neoplastic lesions between the highest dose and control groups, microscopic examination shall be made on that particular organ or tissue of all animals in the study.

(iii) If excessive early deaths or other problems occur in the high dose group, compromising the significance of the data, the next lower dose level shall be examined for complete histopathology.

(iv) In case the results of an experiment give evidence of substantial alteration of the animals' normal longevity or the induction of effects that might affect a neoplastic response, the next lower dose level shall be examined fully as described in this section.

(v) An attempt shall be made to correlate gross observations with microscopic findings.

(c) *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 incidental, shall be evaluated by an appropriate statistical method. Any generally accepted statistical method may be used; the statistical methods shall be selected during the design of the study.

(2) *Evaluation of study results.* (i) The findings of an oncogenic toxicity study

shall be evaluated in conjunction with the findings of preceding 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 bioavailability of the test substance should be considered.

(iii) In order for a negative test to be acceptable, it shall meet the following criteria: no more than 10 percent of any group is lost due to autolysis, cannibalism, or management problems; and survival in each group should be no less than 50 percent at 18 months for mice and hamsters and at 24 months for rats.

(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:

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

(*1*) Number of animals dying.

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

(*3*) Number of animals exposed.

(B) *Individual animal data.* (*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 and water consumption data, when collected.

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

(*6*) Hematological tests employed and all results.

(*7*) Clinical biochemistry tests employed and all results.

(*8*) Necropsy findings.

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

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

(*11*) Historical control data, if taken into account.

(ii) In addition, for inhalation studies the following shall be reported:

(A) *Test conditions.* (1) Description of exposure apparatus including design, type, dimensions, source of air, system for generating particulates 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 shall be described.

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

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

(*2*) Temperature and humidity of air.

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

(*4*) Actual concentration in test breathing zone.

(*5*) Particle size distribution (e.g., median aerodynamic diameter of particles with standard deviation from the mean).

(d) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Department of Health and Welfare. *The Testing of Chemicals* for Carcinogenicity, Mutagenicity, Teratogenicity. Minister of Health and Welfare. (Canada: Department of Health and Welfare, 1975).

(2) Food and Drug Administration Advisory Committee on Protocols for Safety Evaluation: Panel on Carcinogenesis. ''Report on Cancer Testing in the Safety of Food Additives and Pesticides,'' *Toxicology and Applied Pharmacology*. 20:419–438 (1971).

(3) International Union Against Cancer. ''Carcinogenicity Testing,'' *IUCC Technical Report Series*. Vol. 2., Ed. I. Berenblum. (Geneva: International Union Against Cancer, 1969).

(4) Leong, B.K.J., Laskin, S. ''Number and Species of Experimental Animals for Inhalation Carcinogenicity

Studies'' Paper presented at Conference on Target Organ Toxicity, September 1975, Cincinnati, Ohio.

(5) National Academy of Sciences. ''Principles and Procedures for Evaluating the Toxicity of Household Substances.'' A report prepared by the Committee for the Revision of NAS Publication 1138, under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences, Washington, DC (1977).

(6) National Cancer Institute. *Report of the Subtask Group on Carcinogen Testing to the Interagency Collaborative Group on Environmental Carcinogenesis*. (Bethesda: United States National Cancer Institute, 1976).

(7) National Center for Toxicological Research. ''Appendix B,'' *Report of Chronic Studies Task Force Committee*. April 13–21 (Rockville: National Center for Toxicological Research, 1972).

(8) Page, N.P. ''Chronic Toxicity and Carcinogenicity Guidelines,'' *Journal of Environmental Pathology and Toxicology*. 1:161–182 (1977).

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

(10) Sontag, J.M., Page N.P., Saffiotti, U. *Guidelines for Carcinogen Bioassay in Small Rodents*. NCI-CS-TR-1. (Bethesda: United States Cancer Institute, Division of Cancer Control and Prevention, Carcinogenesis Bioassay Program, 1976).

(11) United States Pharmaceutical Manufacturers Association. *Guidelines for the Assessment of Drug and Medical Device Safety in Animals*. (1977).

(12) World Health Organization. ''Principles for the Testing and Evaluation of Drugs for Carcinogenicity,'' *WHO Technical Report Series No. 426*. (Geneva: World Health Organization, 1969).

(13) World Health Organization. ''Part I. Environmental Health Criteria 6,'' *Principles and Methods for Evaluating the Toxicity of Chemicals*. (Geneva: World Health Organization, 1978).

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§ 798.3320 Combined chronic toxicity/oncogenicity.

(a) *Purpose*. The objective of a combined chronic toxicity/oncogenicity study is to determine the effects of a substance in a mammalian species following prolonged and repeated exposure. The application of this guideline should generate data which identify the majority of chronic and oncogenic effects and determine dose-response relationships. The design and conduct should allow for the detection of neoplastic effects and a determination of oncogenic potential as well as general toxicity, including neurological, physiological, biochemical, and hematological effects and exposure-related morphological (pathology) effects.

(b) *Test procedures*—(1) *Animal selection*—(i) *Species and strain*. Preliminary studies providing data on acute, subchronic, and metabolic responses should have been carried out to permit an appropriate choice of animals (species and strain). As discussed in other guidelines, the mouse and rat have been most widely used for assessment of oncogenic potential, while the rat and dog have been most often studied for chronic toxicity. The rat is the species of choice for combined chronic toxicity and oncogenicity studies. The provisions of this guideline are designed primarily for use with the rat as the test species. If other species are used, the tester should provide justification/reasoning for their selection. The strain selected should be susceptible to the oncogenic or toxic effect of the class of substances being tested, if known, and provided it does not have a spontaneous background too high for meaningful assessment. Commonly used laboratory strains should be employed.

(ii) *Age*. (A) Dosing of rats should begin as soon as possible after weaning, ideally before the rats are 6 weeks old, but in no case more than 8 weeks old.

(B) At commencement of the study, the weight variation of animals used

should not exceed ±20 percent of the mean weight for each sex.

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

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

(B) The females should be nulliparous and nonpregnant.

(iv) *Numbers.* (A) At least 100 rodents (50 females and 50 males) should be used at each dose level and concurrent control for those groups not intended for early sacrifice. At least 40 rodents (20 females and 20 males) should be used for satellite dose group(s) and the satellite control group. The purpose of the satellite group is to allow for the evaluation of pathology other than neoplasia.

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

(C) The number of animals at the termination of each phase of the study should be adequate for a meaningful and valid statistical evaluation of long term exposure. For a valid interpretation of negative results, it is essential that survival in all groups not fall below 50 percent at the time of termination.

(2) *Control groups.* (i) A concurrent control group (50 females and 50 males) and a satellite control group (20 females and 20 males) are recommended. These groups should be untreated or sham treated control groups or, if a vehicle is used in administering the test substance, vehicle control groups. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are recommended. Animals in the satellite control group should be sacrificed at the same time the satellite test group is terminated.

(ii) In special circumstances such as inhalation studies involving aerosols or the use of an emulsifier of uncharacterized biological activity in oral studies, a concurrent negative control group should be utilized. The negative control group should be treated in the same manner as all other test animals, except that this control group should not be exposed to the test substance or any vehicle.

(iii) The use of historical control data (i.e., the incidence of tumors and other suspect lesions normally occuring under the same laboratory conditions and in the same strain of animals employed in the test) is desirable for assessing the significance of changes observed in exposed animals.

(3) *Dose levels and dose selection.* (i) For risk assessment purposes, at least three dose levels should be used, in addition to the concurrent control group. Dose levels should be spaced to produce a gradation of effects.

(ii) The highest dose level in rodents should elicit signs of toxicity without substantially altering the normal life span due to effects other than tumors.

(iii) The lowest dose level should produce no evidence of toxicity. Where there is a usable estimation of human exposure, the lowest dose level should exceed this even though this dose level may result in some signs of toxicity.

(iv) Ideally, the intermediate dose level(s) should produce minimal observable toxic effects. If more than one intermediate dose is used the dose levels should be spaced to produce a gradation of toxic effects.

(v) For rodents, the incidence of fatalities in low and intermediate dose groups and in the controls should be low to permit a meaningful evaluation of the results.

(vi) For chronic toxicological assessment, a high dose treated satellite and a concurrent control satellite group should be included in the study design. The highest dose for satellite animals should be chosen so as to produce frank toxicity, but not excessive lethality, in order to elucidate a chronic toxicological profile of the test substance. If more than one dose level is selected for satellite dose groups, the doses should be spaced to produce a gradation of toxic effects.

(4) *Exposure conditions.* The animals are dosed with the test substance ideally on a 7-day per week basis over a period of at least 24 months for rats, and 18 months for mice and hamsters, except for the animals in the satellite groups which should be dosed for 12 months.

(5) *Observation period.* It is necessary that the duration of the oncogenicity test comprise the majority of the normal life span of the animals to be used. It has been suggested that the duration of the study should be for the entire lifetime of all animals. However, a few animals may greatly exceed the average lifetime and the duration of the study may be unnecessarily extended and complicate the conduct and evaluation of the study. Rather, a finite period covering the majority of the expected life span of the strain is preferred since the probability is high that, for the great majority of chemicals, induced tumors will occur within such an observation period. The following guidelines are recommended:

(i) Generally, the termination of the study should be at 18 months for mice and hamsters and 24 months for rats; however, for certain strains of animals with greater longevity and/or low spontaneous tumor rate, termination should be at 24 months for mice and hamsters and at 30 months for rats. For longer time periods, and where any other species are used, consultation with the Agency in regard to duration of the test is advised.

(ii) However, termination of the study is acceptable when the number of survivors of the lower doses or of the control group reaches 25 percent. In the case where only the high dose group dies prematurely for obvious reasons of toxicity, this should not trigger termination of the study.

(iii) The satellite groups and the concurrent satellite control group should be retained in the study for at least 12 months. These groups should be scheduled for sacrifice for an estimation of test-substance-related pathology uncomplicated by geriatric changes.

(6) *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.* (A) The animals should receive the test substance in their diet, dissolved in drinking water, or given by gavage or capsule for a period of at least 24 months for rats and 18 months for mice and hamsters.

(B) If the test substance is administered in the drinking water, or mixed in the diet, exposure is continuous.

(C) For a diet mixture, the highest concentration should not exceed 5 percent.

(ii) *Dermal studies.* (A) The animals are treated by topical application with the test substance, ideally for at least 6 hours per day.

(B) Fur should be clipped 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.

(C) The test substance should be applied uniformly over a shaved area which is approximately 10 percent of the total body surface area. 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 possible.

(D) During the exposure period, the test substance may be held, if necessary, in contact with the skin with a porous gauze dressing and nonirritating tape. 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 tested with inhalation equipment designed to sustain a dynamic air flow of 12 to 15 air changes per hour, to ensure an adequate oxygen content of 19 percent and an evenly distributed exposure atmosphere. Where a chamber is used, its design should minimize crowding of the test animals and maximize their exposure to the test substance. This is best accomplished by individual caging. As a general rule, to ensure stability of a chamber atmosphere, the total ''volume'' of the test animals should not exceed 5 percent of the volume of the test chamber. Alternatively, oro-nasal, head only, or whole body individual chamber exposure may be used.

(B) The temperature at which the test is performed should be maintained at 22 °C (±2°). Ideally, the relative humidity should be maintained between 40 to 60 percent, but in certain instances (e.g., tests of aerosols, use of

water vehicle) this may not be practicable.

(C) Feed and water should be withheld during each daily 6-hour exposure period.

(D) A dynamic inhalation system with a suitable analytical concentration control system should be used. The rate of air flow should be adjusted to ensure that conditions throughout the equipment are essentially the same. Maintenance of slight negative pressure inside the chamber will prevent leakage of the test substance into the surrounding areas.

(7) *Observation of animals.* (i) Each animal should be handled and its physical condition appraised at least once each day.

(ii) Additional observations should be made daily with appropriate actions 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) Clinical signs and mortality should be recorded for all animals. Special attention should be paid to tumor development. The time of onset, location, dimensions, appearance and progression of each grossly visible or palpable tumor should be recorded.

(iv) Body weights should be recorded individually for all animals once a week during the first 13 weeks of the test period and at least once every 4 weeks thereafter, unless signs of clinical toxicity suggest more frequent weighings to facilitate monitoring of health status.

(v) 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.

(vi) At the end of the study period, all survivors are sacrificed. Moribund animals should be removed and sacrificed when noticed.

(8) *Physical measurements.* For inhalation studies, measurements or monitoring should be made of the following:

(i) The rate of airflow should be monitored continuously, but should be recorded at intervals of at least once every 30 minutes.

(ii) During each exposure period the actual concentrations of the test substance should be held as constant as practicable, monitored continuously and recorded at least three times during the test period: At the beginning, at an intermediate time and at the end of the period.

(iii) During the development of the generating system, particle size analysis should be performed to establish the stability of aerosol concentrations. During exposure, analyses should be conducted as often as necessary to determine the consistency of particle size distribution and homogeneity of the exposure stream.

(iv) Temperature and humidity should be monitored continuously, but should be recorded at intervals of at least once every 30 minutes.

(9) *Clinical examinations.* (i) The following examinations should be made on at least 20 rodents of each sex per dose level:

(A) Certain hematology determinations (e.g., hemoglobin content, packed cell volume, total red blood cells, total white blood cells, platelets, or other measures of clotting potential) should be performed at termination and should be performed at 3 months, 6 months and at approximately 6-month intervals thereafter (for those groups on test for longer than 12 months) on blood samples collected from 20 rodents per sex of all groups. These collections should be from the same animals at each interval. If clinical observations suggest a deterioration in health of the animals during the study, a differential blood count of the affected animals should be performed. A differential blood count should be performed on samples from animals in the highest dosage group and the controls. Differential blood counts should be performed for the next lower group(s) if there is a major discrepancy between the highest group and the controls. If hematological effects were noted in the subchronic test, hematological testing should be performed at 3, 6, 12, 18 and 24 months for a year study.

(B) Certain clinical biochemistry determinations on blood should be carried out at least three times during the

test period: Just prior to initiation of dosing (baseline data), near the middle and at the end of the test period. Blood samples should be drawn for clinical measurements from at least ten rodents per sex of all groups; if possible, from the same rodents at each time interval. Test areas which are considered appropriate to all studies: electrolyte balance, carbohydrate metabolism and liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the substance and signs of clinical toxicity. Suggested chemical determinations: Calcium, phosphorus, chloride, sodium, potassium, fasting glucose (with period of fasting appropriate to the species), serum glutamic-pyruvic transaminase (now known as serum alanine aminotransferase), serum glutamic oxaloacetic transaminase (now known as serum aspartate aminotransferase), ornithine decarboxylase, gamma glutamyl transpeptidase, blood urea nitrogen, albumen, creatinine phosphokinase, total cholesterol, total bilirubin and total serum protein measurements. 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.

(ii) The following should be performed on at least 10 rodents of each sex per dose level:

(A) Urine samples from the same rodents at the same intervals as hematological examination above, should be collected for analysis. The following determinations should be made from either individual animals or on a pooled sample/sex/group for rodents: appearance (volume and specific gravity), protein, glucose, ketones, bilirubin, occult blood (semi-quantitatively) and microscopy of sediment (semi-quantitatively).

(B) Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, should be made prior to the administration of the test substance and at the termination of the study. If changes in the eyes are detected, all animals should be examined.

(10) *Gross necropsy.* (i) A complete gross examination should be performed on all animals, including those which died during the experiment or were killed in moribund conditions.

(ii) The liver, kidneys, adrenals, brain and gonads should be weighed wet, as soon as possible after dissection to avoid drying. For these organs, at least 10 rodents per sex per group should be weighed.

(iii) The following organs and tissues, or representative samples thereof, should be preserved in a suitable medium for possible future histopathological examination: All gross lesions and tumors; brain-including sections of medulla/pons, cerebellar cortex, and cerebral cortex; pituitary; thyroid/parathyroid; thymus; lungs; trachea; heart; sternum and/or femur with bone marrow; salivary glands; liver; spleen; kidneys; adrenals; esophagus; stomach; duodenum; jejunum; ileum; cecum; colon; rectum; urinary bladder; representative lymph nodes; pancreas; gonads; uterus; accessory genital organs (epididymis, prostate, and, if present, seminal vesicles); female mammary gland; aorta; gall bladder (if present); skin; musculature; peripheral nerve; spinal cord at three levels—cervical, midthoracic, and lumbar; and eyes. In inhalation studies, the entire respiratory tract, including nose, pharynx, larynx and paranasal sinuses should be examined and preserved. In dermal studies, skin from sites of skin painting should be examined and preserved.

(iv) 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 considered essential for appropriate and valid histopathological examination.

(v) If other clinical examinations are carried out, the information obtained from these procedures should be available before microscopic examination, since they may provide significant guidance to the pathologist.

(11) *Histopathology.* (i) The following histopathology should be performed:

(A) Full histopathology on the organs and tissues, listed above, of all non-rodents, of all rodents in the control and high dose groups and of all rodents 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 excessive early deaths or other problems occur in the high dose group compromising the significance of the data, the next dose level should be examined for complete histopathology.

(iii) In case the results of the experiment give evidence of substantial alteration of the animals' normal longevity or the induction of effects that might affect a toxic response, the next lower dose level should be examined as described above.

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

(c) *Data and reporting*—(1) *Treatment of results.* (i) Data should 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 incidental, should be evaluated by an appropriate statistical method. Any generally accepted statistical methods may be used; the statistical methods should be selected during the design of the study.

(2) *Evaluation of study results.* (i) The findings of a combined chronic toxicity/oncogenicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of the toxic effects, the necropsy and histopathological findings. The evaluation will 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 should meet the following criteria: No more than 10 percent of any group is lost due to autolysis, cannibalism, or management problems; and survival in each group is no less than 50 percent at 18 months for mice and hamsters and at 24 months for rats.

(3) *Test report.* (i) In addition to the reporting requirements as specified under 40 CFR part 792, subpart J the following specific information should be reported:

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

(*1*) Number of animals dying.

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

(*3*) Number of animals exposed.

(B) *Individual animal data.* (*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 and water consumption data, when collected.

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

(*6*) Hematological tests employed and all results.

(*7*) Clinical biochemistry tests employed and all results.

(*8*) Necropsy findings.

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

(*10*) Statistical treatment of results where appropriate.

(*11*) Historical control data, if taken into account.

(ii) In addition, for inhalation studies the following should be reported:

(A) *Test conditions.* (*1*) Description of exposure apparatus including design, type, dimensions, source of air, system for generating particulates 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 should 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*) Nominal concentration (total amount of test substance fed into the inhalation equipment divided by volume of air).

(*4*) Actual concentration in test breathing zone.

(*5*) Particle size distribution (e.g. median aerodynamic diameter of particles with standard deviation from the mean).

(d) *References.* For additional background information on this test guideline the following references should be consulted:

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

(2) D'Aguanno, W. ''Drug Safety Evaluation—Pre-Clinical Considerations,'' ''*Industrial Pharmacology: Neuroleptics*. Vol. I Ed. S. Fielding and H. Lal. (Mt. Kisco, New York: Futura Publishing Co., 1974) pp. 317–332.

(3) Department of Health and Welfare. *The Testing of Chemicals for Carcinogenicity, Mutagenicity, Teratogenicity. Minister of Health and Welfare. (Canada: Department of Health and Welfare, 1975).*

(4) 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 (1959, 3rd Printing 1975). pp. 36–45.

(5) Food and Drug Administration Advisory Committee on Protocols for Safety Evaluation: Panel on Carcinogenesis. ''*Report on Cancer Testing in the Safety of Food Additives and Pesticides,*'' *Toxicology and Applied Pharmacology*. 20:419–438 (1971).

(6) 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 (1959, 3rd printing 1975) pp.60–67.

(7) International Union Against Cancer. ''Carcinogenicity Testing,'' *IUCC Technical Report Series* Vol. 2, Ed. I. Berenblum. (Geneva: International Union Against Cancer, 1969).

(8) Leong, B.K.J., and Laskin, S. ''Number and Species of Experimental Animals for Inhalation Carcinogenicity Studies,'' Paper presented at Conference on Target Organ Toxicity. September, 1975, Cincinnati, Ohio.

(9) National Academy of Sciences. ''Principles and Procedures for Evaluating the Toxicity of Household Substances,'' A report prepared by the Committee for the Revision of NAS Publication 1138, under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences, Washington, DC (1977).

(10) National Cancer Institute. *Report of the Subtask Group on Carcinogen Testing to the Interagency Collaborative Group on Environmental Carcinogenesis*. (Bethesda: United States National Cancer Institute, 1976).

(11) National Center for Toxicological. *Report of Chronic Studies Task Force Research Committee. ''Appendix B, (Rockville: National Center for Toxicological Research, 1972)).*

(12) Page, N.P. ''Chronic Toxicity and Carcinogenicity Guidelines,'' *Journal Environmental Pathology and Toxicology*. 1:161–182 (1977).

(13) Page, N.P. ''Concepts of a Bioassay Program in Environmental Carcinogenesis,'' *Advances in Modern Toxicology* Volume 3, Ed. Kraybill and Mehlman. (Washington, D.C.: Hemisphere Publishing Corp., 1977) pp. 87–171.

(14) Schwartz, E. 1974. ''Toxicology of Neuroleptic Agents,'' *Industrial Pharmacology: Neuroleptics*. Ed. S. Fielding and H. Lal. (Mt. Kisco, New York: Futura Publishing Co, 1974) pp. 203–221.

(15) Sontag, J.M., Page, N.P., and Saffiotti, U. *Guidelines for Carcinogen Bioassay in Small Rodents*. NCI-CS-TR-1 (Bethesda: United States Cancer Institute, Division of Cancer Control and Prevention, Carcinogenesis Bioassay Program, 1976).

(16) United States Pharmaceutical Manufacturers Association. *Guidelines*

for the Assessment of Drug and Medical Device Safety in Animals. (1977).

(17) World Health Organization. ''Principles for the Testing and Evaluation of Drugs for Carcinogenicity,'' *WHO Technical Report Series No. 426*. (Geneva: World Health Organization, 1969).

(18) World Health Organization. ''Guidelines for Evaluation of Drugs for Use in Man,'' *WHO Technical Report Series No. 563*. (Geneva: World Health Organization, 1975).

(19) World Health Organization. ''Part I. Environmental Health Criteria 6,'' *Principles and Methods for Evaluating the Toxicity of Chemicals*. (Geneva: World Health Organization, 1978).

(20) World Health Organization. ''Principles for Pre-Clinical Testing of Drug Safety,'' *WHO Technical Report Series No. 341*. (Geneva: World Health Organization, 1966).

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Subpart E—Specific Organ/Tissue Toxicity

§ 798.4100 Dermal sensitization.

(a) *Purpose*. In the assessment and evaluation of the toxic characteristics of a substance, determination of its potential to provoke skin sensitization reactions is important. Information derived from tests for skin sensitization serves to identify the possible hazard to a population repeatedly exposed to a test substance. While the desirability of skin sensitization testing is recognized, there are some real differences of opinion about the best method to use. The test selected should be a reliable screening procedure which should not fail to identify substances with significant allergenic potential, while at the same time avoiding false negative results.

(b) *Definitions*. (1) Skin sensitization (allergic contact dermatitis) is an immunologically mediated cutaneous reaction to a substance. In the human, the responses may be characterized by pruritis, erythema, edema, papules, vesicles, bullae, or a combination of these. In other species the reactions may differ and only erythema and edema may be seen.

(2) Induction period is a period of at least 1 week following a sensitization exposure during which a hypersensitive state is developed.

(3) Induction exposure is an experimental exposure of a subject to a test substance with the intention of inducing a hypersensitive state.

(4) Challenge exposure is an experimental exposure of a previously treated subject to a test substance following an induction period, to determine whether the subject will react in a hypersensitive manner.

(c) *Principle of the test method*. Following initial exposure(s) to a test substance, the animals are subsequently subjected, after a period of not less than 1 week, to a challenge exposure with the test substance to establish whether a hypersensitive state has been induced. Sensitization is determined by examining the reaction to the challenge exposure and comparing this reaction to that of the initial induction exposure.

(d) *Test procedures*. (1) Any of the following seven test methods is considered to be acceptable. It is realized, however, that the methods differ in their probability and degree of reaction to sensitizing substances.

(i) Freund's complete adjuvant test.

(ii) Guinea-pig maximization test.

(iii) Split adjuvant technique.

(iv) Buehler test.

(v) Open epicutaneous test.

(vi) Mauer optimization test.

(vii) Footpad technique in guinea pig.

(2) Removal of hair is by clipping, shaving, or possibly by depilation, depending on the test method used.

(3) *Animal selection*—(i) *Species and strain*. The young adult guinea pig is the preferred species. Commonly used laboratory strains should be employed. If other species are used, the tester should provide justification/reasoning for their selection.

(ii) *Number and sex*. (A) The number and sex of animals used will depend on the method employed.

(B) The females should be nulliparous and nonpregnant.

(4) *Control animals*. (i) Periodic use of a positive control substance with an acceptable level of reliability for the test system selected is recommended;

(ii) Animals may act as their own controls or groups of induced animals can be compared to groups which have received only a challenge exposure.

(5) *Dose levels.* The dose level will depend upon the method selected.

(6) *Observation of animals.* (i) Skin reactions should be graded and recorded after the challenge exposures at the time specified by the methodology selected. This is usually at 24, 48, and 72, hours. Additional notations should be made as necessary to fully describe unusual responses;

(ii) Regardless of method selected, initial and terminal body weights should be recorded.

(7) *Procedures.* The procedures to be used are those described by the methodology chosen.

(e) *Data and reporting.* (1) Data should be summarized in tabular form, showing for each individual animal the skin reaction, results of the induction exposure(s) and the challenge exposure(s) at times indicated by the method chosen. As a minimum, the erythema and edema should be graded and any unusual finding should be recorded.

(2) *Evaluation of the results.* The evaluation of results will provide information on the proportion of each group that became sensitized and the extent (slight, moderate, severe) of the sensitization reaction in each individual animal.

(3) *Test report.* In addition to the reporting requirements as specified under 40 CFR part 792, subpart J, the following specific information should be reported:

(i) A description of the method used and the commonly accepted name.

(ii) Information on the positive control study, including positive control used, method used, and time conducted.

(iii) The number and sex of the test animals.

(iv) Species and strain.

(v) Individual weights of the animals at the start of the test and at the conclusion of the test.

(vi) A brief description of the grading system.

(vii) Each reading made on each individual animal.

(f) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Buehler, E.V. ''Delayed Contact Hypersensitivity in the Guinea Pig,'' *Archives Dermatology*. 91:171 (1965).

(2) Draize, J.H. ''Dermal Toxicity,'' *Food Drug Cosmetic Law Journal*. 10:722–732 (1955).

(3) Klecak, G. ''Identification of Contact Allergens: Predictive Tests in Animals,'' *Advances in Modern Toxicology: Dermatology and Pharmacology*. Ed. F.N. Marzulli and H.I. Maibach. (Washington, D.C.: Hemisphere Publishing Corp., 1977) 4:305–339).

(4) Klecak, G., Geleick, H., Grey, J.R. ''Screening of Fragrance Materials for Allergenicity in the Guinea Pig.–1. Comparison of Four Testing Methods,'' *Journal of the Society of Cosmetic Chemists*. 28:53–64 (1977).

(5) Magnusson, B., Kligman, A.M. ''The Identification of Contact Allergens by Animal Assay,'' The Guinea Pig Maximization Test. *The Journal of Investigative Dermatology*. 52:268–276 (1973).

(6) Maguire, H.C. ''The Bioassay of Contact Allergens in the Guinea Pig'' *Journal of the Society of Cosmetic Chemists*. 24:151–162 (1973).

(7) Maurer, T., Thomann, P., Weirich, E.G., Hess, R. ''The Optimization Test in the Guinea Pig. A Method for the Predictive Evaluation of the Contact Allergenicity of Chemicals,'' *Agents and Actions*. (Basel: Birkhauser Verlag, 1975) Vol. 5/2.

(8) Maurer, T., Thomann, P., Weirich, E.G., Hess, R. ''The Optimization Test in the Guinea Pig: A Method for the Predictive Evaluation of the Contact Allergenicity of Chemicals,'' *International Congress Series Excerpta Medica No. 376*, (1975) Vol. 203.

§ 798.4350 Inhalation developmental toxicity study.

(a) *Purpose.* In the assessment and evaluation of the toxic characteristics of an inhalable material such as a gas, volatile substance, or aerosol/particulate, determination of the potential developmental toxicity is important. The inhalation developmental toxicity study is designed to provide information on the potential hazard to the unborn which may arise from exposure of the mother during pregnancy.

(b) *Definitions.* (1) Developmental toxicity is the property of a chemical that causes in utero death, structural or functional abnormalities or growth retardation during the period of development.

(2) ''Aerodynamic diameter'' applies to the behavioral size of particles of aerosols. It is the diameter of a sphere of unit density which behaves aerodynamically like the particles of the test substance. It is used to compare particles of different sizes, shapes, and densities and to predict where in the respiratory tract such particles may be deposited. This term is used in contrast to ''optical,'' ''measured'' or ''geometric'' diameters which are representation of actual diameters which in themselves cannot be related to deposition within the respiratory tract.

(3) ''Geometric mean diameter'' or ''median diameter'' is the calculated aerodynamic diameter which divides the particles of an aerosol in half based on the weight of the particles. Fifty percent of the particles by weight will be larger than the median diameter and 50 percent of the particles will be smaller than the median diameter. The median diameter and its geometeric standard deviation are used to statistically describe the particle size distribution of any aerosol based on the weight and size of the particles.

(4) ''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 from the trachea to the deep lung (the alveoli). For man, the inhalable diameter is considered here as 15 micrometers or less.

(5) ''Concentration'' refers to an exposure level. Exposure is expressed as weight or volume of test substance per volume of air (mg/1), or as parts per million (ppm).

(6) ''No-observed-effect level'' is the maximum concentration in a test which produces no observed adverse effects. A no-observed-effect level is expressed in terms of weight or volume of test substance given daily per unit volume of air.

(c) *Principle of the test method.* The test substance is administered in graduated concentrations, for at least that part of the pregnancy covering the major period of organogenesis, to several groups of pregnant experimental animals, one exposure level being used per group. Shortly before the expected date of delivery, the pregnant females are sacrificed, the uteri removed, and the contents examined for embryonic or fetal deaths, and live fetuses.

(d) *Limit test.* If a test at an exposure of 5 mg/1 (actual concentration of respirable substances) or, where this is not possible due to physical or chemical properties of the test substance, the maximum attainable concentration, produces no observable developmental toxicity, then a full study using three exposure levels might not be necessary.

(e) *Test procedures*—(1) *Animal selection*—(i) *Species and strain.* Testing shall be performed in at least two mamalian species. Commonly used species include the rat, mouse, rabbit, and hamster. If other mamalian species are used, the tester shall provide justification/reasoning for their selection. Commonly used laboratory strains shall be employed. The strain shall not have low fecundity and shall preferably be characterized for its sensitivity to developmental toxins.

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

(iii) *Sex.* Pregnant female animals shall be used at each exposure level.

(iv) *Number of animals.* At least 20 pregnant rats, mice, or hamsters or 12 pregnant rabbits are required at each exposure level. The objective is to ensure that sufficient pups are produced to permit meaningful evaluation of the potential developmental toxicity of the test substance.

(2) *Control group.* A concurrent control group shall be used. This group shall be exposed to clean, filtered air under conditions identical to those used for the group exposed to the substance of interest. In addition, a vehicle-exposed group may be necessary when the substance under study requires a vehicle for delivery. It is recommended that during preliminary range finding studies, air vs. vehicle exposure be compared. If there is no

substantial difference, air exposure itself would be an appropriate control. If vehicle and air exposure yield different results, both vehicle and air exposed control groups are recommended.

(3) *Concentration levels and concentration selection.* (i) At least three concentration levels with a control and, where appropriate, a vehicle control, shall be used.

(ii) The vehicle shall neither be developmentally toxic nor have effects on reproduction.

(iii) To select the appropriate concentration levels, a pilot or trial study may be advisable. Since pregnant animals have an increased minute ventilation as compared to non-pregnant animals, it is recommended that the trial study be conducted in pregnant animals. Similarly, since presumably the minute ventilation will vary with progression of pregnancy, the animals should be exposed during the same period of gestation as in the main study. In the trial study, the concentration producing embryonic or fetal lethalities or maternal toxicity should be determined.

(iv) Unless limited by the physical/chemical nature or biological properties of the substance, the highest concentration level shall induce some overt maternal toxicity such as reduced body weight or body weight gain, but not more than 10 percent maternal deaths.

(v) The lowest concentration level should not produce any grossly observable evidence of either maternal or developmental toxicity.

(vi) Ideally, the intermediate concentration level(s) shall produce minimal observable toxic effects. If more than one intermediate concentration is used, the concentration levels shall be spaced to produce a gradation of toxic effects.

(4) *Exposure duration.* The duration of exposure shall be at least six hours daily allowing appropriate additional time for chamber equilibrium.

(5) *Observation period.* Day 0 in the test is the day on which a vaginal plug and/or sperm are observed. The exposure period shall cover the period of major organogenesis. This may be taken as days 6 to 15 for rat and mouse, 6 to 14 for hamster, or 6 to 18 for rabbit.

(6) *Inhalation exposure.* (i)(A) The animals shall be tested in inhalation equipment designed to sustain a minimum dynamic air flow of 12 to 15 air changes per hour and ensure an adequate oxygen content of 19 percent and an evenly distributed exposure atmosphere. Where a chamber is used, its design should minimize crowding of the test animals and maximize their exposure to the test substance. This is best accomplished by individual caging. To ensure stability of a chamber atmosphere, the total ''volume'' of the test animals shall not exceed 5 percent of the volume of the test chamber.

(B) Pregnant animals shall not be subjected to beyond the minimum amount of stress. Since whole-body exposure appears to be the least stressful mode of exposure, it is the method preferred. In general oro-nasal or head-only exposure, which is sometimes used to avoid concurrent exposure by the dermal or oral routes, is not recommended because of the associated stress accompanying the restraining of the animals. However, there may be specific instances where it may be more appropriate than whole-body exposure. The tester shall provide justification/reasoning for its selection.

(ii) A dynamic inhalation system with a suitable flow control system shall be used. The rate of air flow shall be adjusted to ensure that conditions throughout the exposure chamber are essentially the same. Test material distribution should be established before animals are committed to dosing. Maintenance of slight negative pressure inside the chamber will prevent leakage of the test substance into the surrounding areas.

(iii) The temperature at which the test is performed should be maintained at 22 °C (±2°) for rodents or 20 °C (±3°) for rabbits. Ideally, the relative humidity should be maintained between 40 to 60 percent, but in certain instances (e.g., tests of aerosols, use of water vehicle) this may not be practicable.

(7) *Physical measurements.* Measurements or monitoring should be made of the following:

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

(ii) The actual concentration 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 practicable, monitored continously or intermittently depending on the method of analysis and measured at least at the beginning, at an intermediate time and at the end of the exposure period.

(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. During exposure, analysis shall be conducted as often as necessary to determine the consistency of particle size distribution.

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

(8) *Food and water during exposure period.* Food should be withheld during exposure. Water may or may not be withheld. If it is not withheld it should not come in direct contact with the test atmospheres.

(9) *Observation of animals.* (i) A gross examination shall be made at least once each day.

(ii) Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study (e.g., necropsy or refrigeration of animals found dead and isolation or sacrifice of weak or moribund animals).

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

(iv) Cage-side observations shall include, but not be limited to: Changes in skin and fur, eye and mucous membranes, as well as respiratory, autonomic and central nervous systems, somatomotor activity and behavioral pattern. Particular attention should be directed to observation of tremors, convulsions, salivation, diarrhea, lethargy, sleep, and coma.

(v) Measurements should be made weekly of food consumption for all animals in the study.

(vi) Animals shall be weighed at least weekly.

(vii) Females showing signs of abortion or premature delivery shall be sacrificed and subjected to a thorough macroscopic examination.

(10) *Gross necropsy.* (i) At the time of sacrifice or death during the study, the dam shall be examined macroscopically for any structural abnormalities or pathological changes which may have influenced the pregnancy.

(ii) Immediately after sacrifice or death, the uterus shall be removed, weighed, and the contents examined for embryonic or fetal deaths and the number of viable fetuses. Gravid uterine weights should not be obtained from dead animals if autolysis or where decomposition has occurred. The degree of resorption shall be described in order to help estimate the relative time of death.

(iii) The number of corpora lutea shall be determined for all species except mice.

(iv) The sex of the fetuses shall be determined and they shall be weighed individually, the weights recorded, and the mean fetal weight derived.

(v) Following removal, each fetus shall be examined externally.

(vi) For rats, mice and hamsters, one-third to one-half of each litter shall be prepared and examined for skeletal anomalies, and the remaining part of each litter shall be prepared and examined for soft tissue anomalies using appropriate methods.

(vii) For rabbits, each fetus shall be examined by careful dissection for visceral anomalies and then examined for skeletal anomalies.

(f) *Data and reporting*—(1) *Treatment of results.* 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 pregnant animals, the number and percentages of live fetuses and the number of fetuses with any soft tissue or skeletal abnormalities.

(2) *Evaluation of results.* The findings of a developmental toxicity study shall be evaluated in terms of the observed effects and the exposure levels producing effects. It is necessary to consider the historical developmental toxicity data on the species/strain tested. A properly conducted developmental

toxicity study should provide a satisfactory estimation of a no-effect level.

(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:

(i) *Test conditions.* (A) Description of exposure apparatus including design, type, dimensions, source of air, system for generating particulates and aerosols, methods of conditioning air, and the method of housing the animals in a test chamber when this apparatus is used.

(B) The equipment for measuring temperature, humidity, and particulate aerosol concentrations and size shall be described.

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

(A) Airflow rates through the inhalation equipment.

(B) Temperature of air.

(C) Nominal concentration—total amount of test substance fed into the inhalation equipment divided by volume of air (no standard deviation).

(D) Measured total concentrations (particulate and/or gaseous phases) in test breathing zone.

(E) Particle size distribution (e.g., median aerodynamic diameter of particles with geometric standard deviation) including estimates of the percents of inhalable and non-inhalable portions for the test animals.

(iii) *Animal data.* (A) Toxic response data by concentration.

(B) Species and strain.

(C) Date of death during the study or whether animals survived to termination.

(D) Date of onset and duration of each abnormal sign and its subsequent course.

(E) Feed, body weight and uterine weight data.

(F) Pregnancy and litter data.

(G) Fetal data (live/dead, sex, soft tissue and sketetal defects, resorptions).

(g) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Department of Health and Welfare. *The Testing of Chemicals for Carcinogenicity, Mutagenicity and Teratogenicity.* Minister of Health and Welfare (Canada: Department of Health and Welfare, 1975).

(2) National Academy of Sciences. ''Principles and Procedures for Evaluating the Toxicity of Household Substances.'' A report prepared by the Committee for the Revision of NAS Publication 1138, under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences, Washington, DC (1977).

(3) World Health Organization. *Principles for the Testing of Drugs for Teratogenicity.* WHO Technical Report Series No. 364. (Geneva: World Health Organization, 1967).

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19076, May 20, 1987; 52 FR 26150, July 13, 1987; 54 FR 21064, May 16, 1989]

§ 798.4700 Reproduction and fertility effects.

(a) *Purpose.* This guideline for two-generation reproduction testing is designed to provide general information concerning the effects of a test substance on gonadal function, conception, parturition, and the growth and development of the offspring. The study may also provide information about the effects of the test substance on neonatal morbidity, mortality, and preliminary data on teratogenesis and serve as a guide for subsequent tests.

(b) *Principle of the test method.* The test substance is administered to parental (P) animals prior to their mating, during the resultant pregnancies, and through the weaning of their F_1 offspring. The substance is then administered to selected F_1 offspring during their growth into adulthood, mating, and production of an F_2 generation, up until the F_2 generation is weaned.

(c) *Test procedures*—(1) *Animal selection*—(i) *Species and strain.* The rat is the preferred species. If another mammalian species is used, the tester shall provide justification/reasoning for its selection. Strains with low fecundity shall not be used.

(ii) *Age.* Parental (P) animals shall be about 5 to 8 weeks old at the start of dosing.

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

(B) The females shall be nulliparous and non-pregnant.

(iv) *Number of animals.* Each test and control group shall contain at least 20 males and a sufficient number of females to yield at least 20 pregnant females at or near term.

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

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

(iii) If a vehicle or other additive is used to facilitate dosing, it shall not interfere significantly with absorption of the test substance or produce toxic effects.

(3) *Dose levels and dose selection.* (i) At least three dose levels and a concurrent control shall be used.

(ii) The highest dose level should induce toxicity but not high levels of mortality in the parental (P) animals.

(iii) The lowest dose level should not produce any grossly observable evidence of toxicity.

(iv) Ideally the intermediate dose level(s) should produce minimal observable toxic effects. If more than one intermediate dose is used, dose levels should be spaced to produce a gradation of toxic effects.

(4) *Exposure conditions.* The animals should be dosed with the test substance, ideally, on a 7 days per week basis.

(i) Dosing, mating, delivery, and sacrifice schedule.

(A) Daily dosing of the parental (P) males and females shall begin when they are 5 to 8 weeks old. For both sexes, dosing shall be continued for at least 10 weeks before the mating period.

(B) Dosing of P males shall continue through the 3 week mating period. At the end of the mating period, P males may be sacrificed and examined, or may be retained for possible production of a second litter. If these animals are retained for a second litter, dosing shall be continued. Dosing of the F_1 males saved for mating shall continue from the time they are weaned through the period they are mated with the F_1 females (11 weeks). F_1 males may be sacrificed after the F_1 mating period.

(C) Daily dosing of the P females shall continue through the three week mating period, pregnancy, and to the weaning of the F_1 offspring. Dosing of the F_1 females saved for mating shall continue from the time they are weaned, through the period they are mated with the F_1 males (11 weeks from the time of weaning) pregnancy, and to the weaning of the F_2 offspring.

(ii) All animals are sacrificed as scheduled.

(A) All P males should be sacrificed at the end of the 3-week mating period, or may be retained for possible production of a second litter. If these animals are retained for a second litter, dosing shall be continued.

(B) F_1 males selected for mating should be sacrificed at the end of the three week period of the F_1 mating.

(C) F_1 males and females not selected for mating should be sacrified when weaned.

(D) The P females should be sacrificed upon weaning of their F_1 offspring.

(E) F_1 dams and their F_2 offspring are sacrificed when the offspring are weaned.

(5) *Administration of the test substance*—(i) *Oral studies.* (A) It is recommended that the test substance be administered in the diet or drinking water.

(B) If administered by gavage or capsule, the dosage administered to each animal prior to mating shall be based on the individual animal's body weight and adjusted weekly. During pregnancy the dosage shall be based on the body weight at day 0 and 6 of pregnancy.

(ii) If another route of administration is used, the tester should provide justification and reasoning for its selection.

(6) *Mating procedure*—(i) *Parental.* (A) For each mating, each female shall be placed with a single male from the same dose level until pregnancy occurs or 1 week has elapsed. If mating has not occurred after 1 week, the female shall be placed with a different male.

Paired matings should be clearly identified.

(B) Those pairs that fail to mate should be evaluated to determine the cause of the apparent infertility. This may involve such procedures as additional opportunities to mate with proven fertile males or females, histological examination of the reproductive organs, and examination of the estrus or spermatogenic cycles.

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

(ii) *F_1* cross. (A) For mating the F_1 offspring, one male and one female are randomly selected at weaning from each litter for cross mating with another pup of the same dose level but different litter, to produce the F_2 generation.

(B) F_1 males and females not selected for mating are sacrificed upon weaning.

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

(iv) *Standardization of litter sizes.* (A) On day 4 after birth, the size of each litter should be adjusted by eliminating extra pups by random selection to yield, as nearly as possible, 4 males and 4 females per litter.

(B) Whenever the number of male or female pups prevents having 4 of each sex per litter, partial adjustment (for example, 5 males and 3 females) is permitted. Adjustments are not appropriate for litters of less than 8 pups.

(C) Elimination of runts only is not appropriate.

(D) Adjustments of the F_2 litters is conducted in the same manner.

(7) *Observation of animals.* (i) A gross examination shall be made at least once each day. Pertinent behavioral changes, signs of difficult or prolonged parturition, and all signs of toxicity, including mortality, shall be recorded. These observations shall be reported for each individual animal. Food consumption for all animals shall be monitored weekly except during the mating period.

(ii) The duration of gestation shall be calculated from day 0 of pregnancy.

(iii) Each litter should be examined as soon as possible after delivery for the number of pups, stillbirths, live births, sex, and the presence of gross anomalies. Live pups should be counted and litters weighed at birth or soon thereafter, and on days 4, 7, 14, and 21 after parturition.

(iv) Physical or behavioral abnormalities observed in the dams of offspring shall be recorded.

(v) P males and females shall be weighed on the first day of dosing and weekly thereafter. F_1 litters shall be weighed at birth, or soon thereafter, and on days 4, 7, 14, and 21. In all cases, litter weights shall be calculated from the weights of the individual pups.

(8) *Gross necropsy.* (i) A complete gross examination shall be performed on all adult animals, including those which died during the experiment or were killed in moribund conditions.

(ii) Special attention shall be directed to the organs of the reproductive system.

(iii) The following organs and tissues, or representative samples thereof, shall be preserved in a suitable medium for possible future histopathological examination: Vagina; uterus; ovaries; testes; epididymides; seminal vesicles; prostate, pituitary gland; and, target organ(s) when previously identified of all P and F_1 animals selected for mating.

(9) *Histopathology.* Except if carried out in other studies of comparable duration and dose levels the following histopathology shall be performed:

(i) Full histopathology on the organs listed above for all high dose, and control P_1 and F_1 animals selected for mating.

(ii) Organs demonstrating pathology in these animals shall then be examined in animals from the other dose groups.

(iii) Microscopic examination shall be made of all tissues showing gross pathological changes.

(d) *Data and reporting*—(1) *Treatment of results.* 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 pregnant, the types of change and

the percentage of animals displaying each type of change.

(2) *Evaluation of study results.* (i) An evaluation of test results, including the statistical analysis, based on the clinical findings, the gross necropsy findings, and the microscopic results shall be made and supplied. This should include an evaluation of the relationship, or lack thereof, between the animals' exposure to the test substance and the incidence and severity of all abnormalities.

(ii) 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:

(i) Toxic response data by sex and dose, including fertility, gestation, viability and lactation indices, and length of gestation.

(ii) Species and strain.

(iii) Date of death during the study or whether animals survived to termination.

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

(v) Date of observation of each abnormal sign and its subsequent course.

(vi) Body weight data for P, F_1, and F_2 animals.

(vii) Necropsy findings.

(viii) Detailed description of all histopathological findings.

(ix) Statistical treatment of results where appropriate.

(e) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Clermont, Y., Perry, B. ''Quantitative Study of the Cell Population of the Seminiferous Tubules in Immature Rats,'' *American Journal of Anatomy*. 100:241–267 (1957).

(2) Goldenthal, E.I. *Guidelines for Reproduction Studies for Safety Evaluation of Drugs for Human Use*. Drug Review Branch, Division of Toxicological Evaluation, Bureau of Science, Food and Drug Administration, Washington, DC (1966).

(3) Hasegawa, T., Hayashi, M., Ebling, F.J.G., Henderson, I.W. *Fertility and Sterility*. (New York: American Elsevier Publishing Co., Inc., 1973).

(4) Oakberg, E.F. ''Duration of Spermatogenesis in the Mouse and Timing of Stages of the Cycle of the Seminiferous Epithelium,'' *American Journal of Anatomy*. 9:507–516 (1956).

(5) Roosen-Runge, E.C. ''The Process of Spermatogenesis in Mammals,'' *Biological Review*. 37:343–377 (1962).

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19077, May 20, 1987]

§ 798.4900 Developmental toxicity study.

(a) *Purpose.* In the assessment and evaluation of the toxic characteristics of a chemical, determination of the potential developmental toxicity is important. The developmental toxicity study is designed to provide information on the potential hazard to the unborn which may arise from exposure of the mother during pregnancy.

(b) *Definitions.* (1) Developmental toxicity is the property of a chemical that causes in utero death, structural or functional abnormalities or growth retardation during the period of development.

(2) Dose is the amount of test substance administered. Dose is expressed as weight of test substance (g, mg) per unit weight of a test animal (e.g., mg/kg).

(3) No-observed-effect level is the maximum concentration in a test which produces no observed adverse effects. A no-observed-effect level is expressed in terms of weight of test substance given daily per unit weight of test animal (mg/kg)

(c) *Principle of the test method.* The test substance is administered in graduated doses for at least part of the pregnancy covering the major period of organogenesis, to several groups of pregnant experimental animals, one dose level being used per group. Shortly before the expected date of delivery, the pregnant females are sacrificed, the uteri removed, and the contents examined for embryonic or fetal deaths, and live fetuses.

(d) *Limit test.* If a test at an exposure of at least 1000 mg/kg body weight, using the procedures described for this

study, produces no observable developmental toxicity, then a full study using three dose levels might not be necessary.

(e) *Test procedures*—(1) *Animal selection*—(i) *Species and strain.* Testing shall be performed in at least 2 mammalian species. Commonly used species include the rat, mouse, rabbit, and hamster. If other mammalian species are used, the tester shall provide justification/reasoning for their selection. Commonly used laboratory strains shall be employed. The strain shall not have low fecundity and shall preferably be characterized for its sensitivity to developmental toxins.

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

(iii) *Sex.* Pregnant female animals shall be used at each dose level.

(iv) *Number of animals.* At least 20 pregnant rats, mice or hamsters or 12 pregnant rabbits are required at each dose level. The objective is to ensure that sufficient pups are produced to permit meaningful evaluation of the potential developmental toxicity of the test substance.

(2) *Control group.* A concurrent control group shall be used. This group shall be an untreated or sham treated control group, or, if a vehicle is used in administering the test substance, a vehicle control group. Except for treatment with the test substance, animals in the control group(s) shall be handled in an identical manner to test group animals.

(3) *Dose levels and dose selection.* (i) At least 3 dose levels with a control and, where appropriate, a vehicle control, shall be used.

(ii) The vehicle shall neither be developmentally toxic nor have effects on reproduction.

(iii) To select the appropriate dose levels, a pilot or trial study may be advisable. It is not always necessary to carry out a trial study in pregnant animals. Comparison of the results from a trial study in non-pregnant, and the main study in pregnant animals will demonstrate if the test substance is more toxic in pregnant animals. If a trial study is carried out in pregnant animals, the dose producing embryonic or fetal lethalities or maternal toxicity shall be determined.

(iv) Unless limited by the physical/chemical nature or biological properties of the substance, the highest dose level shall induce some overt maternal toxicity such as reduced body weight or body weight gain, but not more than 10 percent maternal deaths.

(v) The lowest dose level should not produce any grossly observable evidence of either maternal or developmental toxicity.

(vi) Ideally, the intermediate dose level(s) should produce minimal observable toxic effects. If more than one intermediate concentration is used, the concentration levels should be spaced to produce a gradation of toxic effects.

(4) *Observation period.* Day 0 in the test is the day on which a vaginal plug and/or sperm are observed. The dose period shall cover the period of major organogenesis. This may be taken as days 6 to 15 for rat and mouse, 6 to 14 for hamster, or 6 to 18 for rabbit.

(5) *Administration of test substance.* The test substance or vehicle is usually administered orally, by oral intubation unless the chemical or physical characteristics of the test substance or pattern of human exposure suggest a more appropriate route of administration. The test substance shall be administered approximately the same time each day.

(6) *Exposure conditions.* The female test animals are treated with the test substance daily throughout the appropriate treatment period. When given by gavage, the dose may be based on the weight of the females at the start of substance administration, or, alternatively, in view of the rapid weight gain which takes place during pregnancy, the animals may be weighed periodically and the dosage based on the most recent weight determination.

(7) *Observation of animals.* (i) A gross examination shall be made at least once each day.

(ii) Additional observations shall be made daily with appropriate actions 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) Signs of toxicity shall be recorded as they are observed, including

the time of onset, the degree and duration.

(iv) Cage-side observations shall include, but not be limited to: changes in skin and fur, eye and mucous membranes, as well as respiratory, autonomic and central nervous systems, somatomotor activity and behavioral pattern.

(v) Measurements should be made weekly of food consumption for all animals in the study.

(vi) Animals shall be weighed at least weekly.

(vii) Females showing signs of abortion or premature delivery shall be sacrificed and subjected to a thorough macroscopic examination.

(8) *Gross necropsy.* (i) At the time of sacrifice or death during the study, the dam shall be examined macroscopically for any structural abnormalities or pathological changes which may have influenced the pregnancy.

(ii) Immediately after sacrifice or as soon as possible after death, the uterus shall be removed and the contents 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. The weight of the gravid uterus should be recorded for dams that are sacrificed. Gravid uterine weights should not be obtained from dead animals if autolysis or decomposition has occurred.

(iii) The number of corpora lutea shall be determined for all species except mice.

(iv) The sex of the fetuses shall be determined and they shall be weighed individually, the weights recorded, and the mean fetal weight derived.

(v) Following removal, each fetus shall be examined externally.

(vi) For rats, mice and hamsters, one-third to one-half of each litter shall be prepared and examined for skeletal anomalies, and the remaining part of each litter shall be prepared and examined for soft tissue anomalies using appropriate methods.

(vii) For rabbits, each fetus shall be examined by careful dissection for visceral anomalies and then examined for skeletal anomalies.

(f) *Data and reporting*—(1) *Treatment of results.* Data shall be summarized in tablular form, showing for each test group: the number of animals at the start of the test, the number of pregnant animals, the number and percentages of live fetuses and the number of fetuses with any soft tissue or skeletal abnormalities.

(2) *Evaluation of results.* The findings of a developmental toxicity study shall be evaluated in terms of the observed effects and the exposure levels producing effects. It is necessary to consider the historical developmental toxicity data on the species/strain tested. A properly conducted developmental toxicity study should provide a satisfactory estimation of a no-effect level.

(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:

(i) Toxic response data by concentration.

(ii) Species and strain.

(iii) Date of death during the study or whether animals survived to termination.

(iv) Date of onset and duration of each abnormal sign and its subsequent course.

(v) Food, body weight and uterine weight data.

(vi) Pregnancy and litter data.

(vii) Fetal data (live/dead, sex, soft tissue and skeletal defects, resorptions).

(g) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Department of Health and Welfare. *The Testing of Chemicals for Carcinogenicity, mutagenicity and Teratogenicity.* Minister of Health and Welfare (Canada: Department of Health and Welfare, 1975).

(2) National Academy of Sciences. ''Principles and Procedures for Evaluating the Toxicity of Household Substances.'' A report prepared by the Committee for the Revision of NAS Publication 1138, under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences, Washington, DC (1977).

(3) World Health Organization. *Principles for the Testing of Drugs for Teratogenicity*. WHO Technical Report Series No. 364. (Geneva: World Health Organization, (1967).

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19077, May 20, 1987]

Subpart F—Genetic Toxicity

§ 798.5195 Mouse biochemical specific locus test.

(a) *Purpose*. The mouse biochemical specific locus test (MBSL) may be used to detect and quantitate mutations originating in the germ line of a mammalian species.

(b) *Definitions*. (1) A biochemical specific locus mutation is a genetic change resulting from a DNA lesion causing alterations in proteins that can be detected by electrophoretic methods.

(2) The germ line is comprised of the cells in the gonads of higher eukaryotes, which are the carriers of the genetic information for the species.

(c) *Reference substances*. Not applicable.

(d) *Test method*—(1) *Principle*. The principle of the MBSL is that heritable damage to the genome can be detected by electrophoretic analysis of proteins in the tissues of the progeny of mice treated with germ cell mutagens.

(2) *Description*. For technical reasons, males rather than females are generally treated with the test chemical. Treated males are then mated to untreated females to produce F1 progeny. Both blood and kidney samples are taken from progeny for electrophoretic analysis. Up to 33 loci can be examined by starch-gel electrophoresis and broad-range isoelectric focussing. Mutants are identified by variations from the normal electrophoretic pattern. Presumed mutants are bred to confirm the genetic nature of the change.

(3) *Animal selection*—(i) *Species and strain*. Mice shall be used as the test species. Although the biochemical specific locus test could be performed in a number of in bred strains, in the most frequently used cross, C57BL/6 females are mated to DBA/2 males to produce (C57BL/6 × DBA/2) F1 progeny for screening.

(ii) *Age*. Healthy, sexually-mature (at least 8 weeks old) animals shall be used for treatment and breeding.

(iii) *Number*. A decision on the minimum number of treated animals should take into account possible effects of the test chemical on the fertility of the treated animals. Other considerations should include:

(A) The production of concurrent spontaneous controls.

(B) The use of positive controls.

(C) The power of the test.

(4) *Control groups*—(i) *Concurrent controls*. An appropriate number of concurrent control loci shall be analyzed in each experiment. These should be partly derived from matings of untreated animals (from 5 to 20 percent ofthe treated matings), although some data on control loci can be taken from the study of the alleles transmitted from the untreated parent in the experimental cross. However, any laboratory which has had no prior experience with the test shall produce a spontaneous control sample of about 5,000 progeny animals and a positive control (using 100 mg/kg ethylnitrosourea) sample of at least 1,200 offspring.

(ii) *Historical controls*. Long-term, accumulated spontaneous control data (currently, 1 mutation in 1,200,000 control loci screened) are available for comparative purposes.

(5) *Test chemicals*—(i) *Vehicle*. When possible, test chemicals shall be dissolved or suspended in distilled water or buffered isotonic saline. Water-insoluble chemicals shall be dissolved or suspended in appropriate vehicles. The vehicle used shall neither interfere with the test chemical nor produce major toxic effects. Fresh preparations of the test chemical should be employed.

(ii) *Dose levels*. Usually, only one dose need be tested. This should be the maximum tolerated dose (MTD), the highest dose tolerated without toxic effects. Any temporary sterility induced due to elimination of spermatogonia at this dose must be of only moderate duration, as determined by are turn of males to fertility within 80 days after treatment. For evaluation of dose-response, it is recommended that at least two dose levels be tested.

(iii) *Route of administration.* Acceptable routes of administration include, but are not limited to, gavage, inhalation, and mixture with food or water, and intraperitoneal or intravenous injections.

(e) *Test performance*—(1) *Treatment and mating.* Male DBA/2 mice shall be treated with the test chemical and mated to virgin C57BL/6 females immediately after cessation of treatment. Each treated male shall be mated to new virgin C57BL/6 females each week. Each pairing will continue for a week until the next week's mating is to begin. This mating schedule permits sampling of all post-spermatogonial stages of germ-cell development during the first 7 weeks after exposure. Spermatogonial stem cells are studied thereafter. Repeated mating cycles should be conducted until sufficient offspring have been obtained to meet the power criterion of the assay for spermatogonial stem cells.

(2) *Examination of offspring*—(i) *Birth and weaning.* Offspring shall be examined at birth and at weaning for externally detectable changes in morphology and behavior; these could be due to dominant mutations. Such characteristics may include, but are not limited to, variations in coat color, appearance of eyes, size (in which case weighing of variant animals and littermates should be carried out), fur texture, etc. Gross changes in external form and behavior shall also be sought. Scrutiny of such visible characteristics of all animals shall be made during all subsequent manipulations of the animals.

(ii) *Tissue sampling.* Blood (about 0.1 mL) and one kidney shall be removed from progeny mice under anesthesia. Both tissues are then prepared for analysis by electrophoresis.

(iii) *Electrophoresis.* The gene products of 6 loci shall be analyzed in the blood sample by broad-range isoelectric focussing and of 27 loci in the kidney sample by starch-gel electrophoresis and enzyme-specific staining. Details on these procedures are included in paragraphs (g)(1) through (g)(3) of this section.

(iv) *Mutant identification.* Presumptive electrophoretic mutants shall be identified by variation from the normal electrophoretic banding patterns. Reruns of all variant samples shall be performed to confirm the presence of altered banding patterns. Samples from parents of progeny exhibiting banding pattern variations shall be assayed to determine whether the variant was induced by the experimental treatment or was pre-existing. All treatment-induced variants are bred to determine the genetic nature of the change.

(f) *Data and reports*—(1) *Treatment of results.* Data shall be presented in tabular form and shall permit independent analysis of cell stage-specific effects, and dose-dependent phenomena. The data shall be recorded and analyzed in such a way that clusters of identical mutations are clearly identified. The individual mutants detected shall be thoroughly described. In addition, concurrent positive control data (if employed) and spontaneous control data shall also be tabulated. These concurrent controls shall be added to, as well as compared with, the historical control data.

(2) *Statistical evaluation.* Data shall be evaluated by appropriate statistical methods.

(3) *Interpretation of results.* (i) There are several criteria for determining a positive response, one of which is a statistically significant dose-related increase in the frequency of electrophoretic mutations. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of these test points.

(ii) A test chemical which does not produce a statistically significant increase in the frequency of electrophoretic mutations over the spontaneous frequency, or a statistically significant and reproducible positive response for at least one of the test points, is considered nonmutagenic in this system, provided that the sample size is sufficient to exclude a biologically significant increase in mutation frequency.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) *Test evaluation.* (i) Positive results in the MBSL indicate that, under the

test conditions, the test chemical induces heritable gene mutations in a mammalian species.

(ii) Negative results indicate that, under the test conditions, the test chemical does not induce heritable genemutations in a mammalian species.

(5) *Test report.* In addition to the reporting requirements as specified under 40 CFR part 792, subpart J, and paragraph (h) of this section, the following specific information shall be reported:

(i) Strain, age and weight of animals used; numbers of animals of each sex in experimental and control groups.

(ii) Test chemical vehicle, doses used, rationale for dose selection, and toxicity data, if available.

(iii) Route and duration of exposure.

(iv) Mating schedule.

(v) Number of loci screened for both treated and spontaneous data.

(vi) Criteria for scoring mutants.

(vii) Number of mutants found/locus.

(viii) Loci at which mutations were found.

(ix) Use of concurrent negative and positive controls.

(x) Dose-response relationship, if applicable.

(g) *References.* For additional background information on this test guideline, the following references should be consulted:

(1) Personal communication from Susan E. Lewis, Ph.D. to Dr. Michael Cimino, U.S. EPA, OPPT, October 5, 1989.

(2) Johnson, F.M., G.T. Roberts, R.K. Sharma, F.Chasalow, R. Zweidinger, A. Morgan, R.W. Hendren, and S.E.Lewis. ''The detection of mutants in mice by electrophoresis: Results of a model induction experiment with procarbazine.'' *Genetics* 97:113–124 (1981).

(3) Johnson, F.M. and S.E. Lewis. ''Mutation rate determinations based on electrophoretic analysis of laboratory mice.'' *Mutation Research* 82:125–135 (1981a).

(4) Johnson, F.M. and S.E. Lewis. ''Electrophoretically detected germinal mutations induced by ethylnitrosourea in the mouse.'' *Proceedings of the National Academy of Sciences* 78:3138–93141 (1981b).

(5) Lewis, S.E., C. Felton, L.B. Barnett, W. Generoso, N. Cacheiro, and M.D. Shelby. ''Dominant visible and electrophoretically expressed mutations induced in male mice exposed to ethylene oxide by inhalation.'' *Environmental Mutagenesis* 8:867–872 (1986).

(h) *Additional requirements.* Testing facilities conducting the mouse biochemical specific locus test in accordance with this section shall, in addition to adhering to the provisions of §§ 792.190 and 792.195 of this chapter, obtain, adequately identify, and retain for at least 10 years, acceptable 35–mm photographs (and their negatives) of the stained isoelectric-focussing columns and the stained starch-gels obtained following analyses of blood and kidney preparations, respectively, from mutant mice, their siblings, and their parents.

[55 FR 12641, Apr. 5, 1990]

§ 798.5200 Mouse visible specific locus test.

(a) *Purpose.* The mouse visible specific locus test (MSLT) may be used to detect and quantitate mutations in the germ line of a mammalian species.

(b) *Definitions.* (1) A visible specific locus mutation is a genetic change that alters factors responsible for coat color and other visible characteristics of certain mouse strains.

(2) The germ line is the cells in the gonads of higher eukaryotes which are the carriers of the genetic information for the species.

(c) *Reference substances.* Not applicable.

(d) *Test method*—(1) *Principle.* (i) The principle of the MSLT is to cross individuals who differ with respect to the genes present at certain specific loci, so that a genetic alteration involving the standard gene at any one of these loci will produce an offspring detectably different from the standard heterozygote. The genetic change may be detectable by various means, depending on the loci chosen to be marked.

(ii) Three variations of the method currently exist for detecting newly arising point mutations in mouse germ cells:

(A) The visible specific locus test using either 5 or 7 loci.

(B) The biochemical specific locus test using up to 20 enzymes.

(C) The test for mutations at histocompatibility loci.

(iii) Of the three tests, the visible specific locus test has been most widely used in assessing genetic hazard due to environmental agents. It is the method described in this guideline.

(2) *Description.* For technical reasons, males rather than females are generally treated with the test agent. Treated males are then mated to females which are genetically homozygous for certain specific visible marker loci. Offspring are examined in the next generation for evidence that a new mutation has arisen.

(3) *Animal selection*—(i) *Species and strain.* Mice shall be used as the test species. Male mice shall be either ($C_3H \times 101)F_1$ or ($101 \times C_3H)F_1$ hybrids. Females shall be T stock virgins.

(ii) *Age.* Healthy sexually mature animals shall be used.

(iii) *Number.* A decision on the minimum number of treated animals should take into account the spontaneous variation of the biological characterization being evaluated. Other considerations should include:

(A) The use of either historical or concurrent controls.

(B) The power of the test.

(C) The minimal rate of induction required.

(D) The use of positive controls.

(E) The level of significance desired.

(iv) *Assignment to groups.* Animals shall be randomized and assigned to treatment and control groups.

(4) *Control groups*—(i) *Concurrent controls.* The use of positive or spontaneous controls is left to the discretion of the investigator. However, any laboratory which has had no prior experience with the test shall, at its first attempt, produce a negative control sample of 20,000 and a positive control, using 100 mg/kg 1-ethyl-nitrosourea, in a sample of 5,000 offspring.

(ii) *Historical controls.* Long term, accumulated spontaneous control data of 43/801,406 are available for comparative purposes.

(5) *Test chemicals*—(i) *Vehicle.* When possible, test chemicals should be dissolved or suspended in distilled water or isotonic saline buffered appropriately, if needed, for stability. Water-insoluble chemicals shall be dissolved or suspended in appropriate vehicles. The vehicle used shall neither interfere with the test compound nor produce major toxic effects. Fresh preparations of the test chemical should be employed.

(ii) *Dose levels.* Usually, only one dose level need be tested. This should be the highest dose tolerated without toxic effects, provided that any temporary sterility induced due to elimination of spermatagonia is of only moderate duration, as determined by a return of males to fertility within 80 days after treatment. For evaluation of dose-response, it is recommended that at least two dose levels be tested.

(iii) *Route of administration.* Acceptable routes of administration include gavage, inhalation, admixture with food or water, and IP or IV injections.

(e) *Test performance*—(1) *Treatment and mating.* Hybrid F_1 (C_3 H $\times$ 101 or 101 $\times$ C_3 H) male mice shall be treated with the test substance and immediately mated to virgin T stock females. Each treated male shall be mated to a fresh group of 2 to 4 virgin females each week for 7 weeks, after which he shall be returned to the first group of females and rotated through the seven sets of females repeatedly. This mating schedule generally permits sampling of all postspermatagonial stages of germ cell development during the first 7 weeks and rapid accumulation of data for exposed spermatagonial stem cells thereafter. Repeated mating cycles should be conducted until the entire spermatogonial cycle has been evaluated and enough offspring have been obtained to meet the power criterion of the assay.

(2) *Examination of offspring.* (i) Offspring may be examined at (or soon after) birth but must be examined at about 3 weeks of age at which time the numbers of mutant and nonmutant offspring in each litter shall be recorded.

(ii) Nonmutant progeny should be discarded. Mutant progeny shall be subjected to genetic tests for verification.

(f) *Data and report*—(1) *Treatment of results.* Data shall be presented in tabular form and shall permit independent analysis of cell stage specific effects

and dose dependent phenomena. The data shall be recorded and analyzed in such a way that clusters of identical mutations are clearly identified. The individual mutants detected shall be thoroughly described. In addition, concurrent positive and negative control data, if they are available, shall be tabulated so that it is possible to differentiate between concurrent (when available) and long-term accumulated mutation frequencies.

(2) *Statistical evaluation.* Data shall be evaluated by appropriate statistical methods.

(3) *Interpretation of results.* (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of specific locus mutations. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of specific locus mutations or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) *Test evaluation.* (i) Positive results in the MSLT indicate that under the test conditions the test substance induces heritable gene mutations in the test species.

(ii) Negative results indicate that under the test conditions the test substance does not induce heritable gene mutations in the test species.

(5) *Test report.* In addition to the reporting requirements as specified under 40 CFR part 792, subpart J, and paragraph (h) of this section, the following specific information shall be reported:

(i) Strain, age and weight of animals used, number of animals of each sex in experimental and control groups.

(ii) Test chemical vehicle, doses used and rationale for dose selection, toxicity data.

(iii) Route and duration of exposure.

(iv) Mating schedule.

(v) Time of examination for mutant progeny.

(vi) Criteria for scoring mutants.

(vii) Use of concurrent or negative controls.

(viii) Dose response relationship, if applicable.

(g) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Russell, L.B., Shelby, P.B., von Halle, E., Sheridan, W., Valcovic, L. The mouse specific locus test with agents other than radiations: interpretation of data and recommendations for future work: A report of the U.S. EPA's Gene-Tox Program,'' *Mutation Research,* 86:329–354 (1981).

(2) [Reserved]

(h) *Additional requirements.* Testing facilities conducting the mouse visible specific locus test in accordance with this section shall, in addition to adhering to the provisions of §§ 792.190 and 792.195 of this chapter, obtain, and retain for at least 10 years, acceptable 35–mm color photographs (and their negatives) demonstrating the visible mutations observed in mutant animals and the lack of such mutations in their siblings and parents.

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19078, May 20, 1987; 55 FR 12643, Apr. 5, 1990]

§ 798.5265 The salmonella typhimurium reverse mutation assay.

(a) *Purpose.* The *Salmonella typhimurium* histidine (his) reversion system is a microbial assay which measures $his^{-} \rightarrow his^{=}$ reversion induced by chemicals which cause base changes or frameshift mutations in the genome of this organism.

(b) *Definitions.* (1) A reverse mutation assay in *Salmonella typhimurium* detects mutation in a gene of a histidine requiring strain to produce a histidine independent strain of this organism.

(2) Base pair mutagens are agents which cause a base change in the DNA. In a reversion assay, this change may occur at the site of the original mutation or at a second site in the chromosome.

(3) Frameshift mutagens are agents which cause the addition or deletion of

single or multiple base pairs in the DNA molecule.

(c) *Reference substances.* These may include, but need not be limited to, sodium azide, 2-nitrofluorene, 9-aminoacridine, 2-aminoanthracene, congo red, benzopurpurin 4B, trypan blue or direct blue 1.

(d) *Test method*—(1) *Principle.* Bacteria are exposed to test chemical with and without a metabolic activation system and plated onto minimal medium. After a suitable period of incubation, revertant colonies are counted and compared to the number of spontaneous revertants in an untreated and/or vehicle control culture.

(2) *Description.* Several methods for performing the test have been described. Among those used are:

(i) The direct plate incorporation method.

(ii) The preincubation method.

(iii) The azo-reduction method.

The procedures described here are for the direct plate incorporation method and the azo-reduction method.

(3) *Strain selection*—(i) *Designation.* At the present time four strains, TA 1535, TA 1537, TA 98 and TA 100 should be used. The use of other strains in addition to these four is left to the discretion of the investigator.

(ii) *Preparation and storage.* Recognized methods of stock culture preparation and storage should be used. The requirement of histidine for growth should be demonstrated for each strain. Other phenotypic characteristics should be checked using such methods as crystal violet sensitivity and resistance to ampicillin. Spontaneous reversion frequency should be in the range expected either as reported in the literature or as established in the laboratory by historical control values.

(iii) *Bacterial growth.* Fresh cultures of bacteria should be grown up to the late exponential or early stationary phase of growth (approximately 10^8–10^9 cells per ml).

(4) *Metabolic activation.* Bacteria should be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system. For the direct plate incorporation method, the most commonly used system is a cofactor supplemented postmitochondrial fraction prepared from the livers of rodents treated with enzyme inducing agents such as Aroclor 1254. For the azo-reduction method, a cofactor supplemented postmitochondrial fraction prepared from the livers of untreated hamsters is preferred. For this method, the cofactor supplement should contain flavin mononucleotide, exogenous glucose 6-phosphate dehydrogenase, NADH and excess of glucose-6-phosphate.

(5) *Control groups*—(i) *Concurrent controls.* Concurrent positive and negative (untreated and/or vehicle) controls shall be included in each experiment. Positive controls shall ensure both strain responsiveness and efficacy of the metabolic activation system.

(ii) *Strain specific positive controls.* Strain specific positive controls shall be included in the assay. Examples of strain specific positive controls are as follows:

(A) Strain TA 1535, TA 100, sodium azide.

(B) TA 98, 2-nitrofluorene.

(C) TA 1537, 9-aminoacridine.

(iii) *Positive controls to ensure the efficacy of the activation system.* The positive control reference substance for tests including a metabolic activation system should be selected on the basis of the type of activation system used in the test. 2-Aminoanthracene is an example of a positive control compound in plate-incorporation tests using postmitochondrial fractions from the livers of rodents treated with enzyme inducing agents such as Aroclor-1254. Congo red is an example of a positive control compound in the azo-reduction method. Other positive control reference substances may be used.

(iv) *Class-specific positive controls.* The azo-reduction method should include positive controls from the same class of compounds as the test agent wherever possible.

(6) *Test chemicals*—(i) *Vehicle.* Test chemicals and positive control reference substances should be dissolved or suspended in an appropriate vehicle and then further diluted in vehicle for use in the assay.

(ii) *Exposure concentrations.* (A) The test should initially be performed over a broad range of concentrations. Among the criteria to be taken into consideration for determining the

upper limits of test chemical concentration are cytotoxicity and solubility. Cytotoxicity of the test chemical may be altered in the presence of metabolic activation systems. Toxicity may be evidenced by a reduction in the number of spontaneous revertants, a clearing of the background lawn or by the degree of survival of treated cultures. Relatively insoluble compounds should be tested up to the limits of solubility. For freely soluble nontoxic chemicals, the upper test chemical concentration should be determined on a case by case basis.

(B) Generally, a maximum of 5 mg/plate for pure substances is considered acceptable. At least 5 different amounts of test substance shall be tested with adequate intervals between test points.

(C) When appropriate, a single positive response shall be confirmed by testing over a narrow range of concentrations.

(e) *Test performance*—(1) *Direct plate incorporation method.* For this test without metabolic activation, test chemica1 and 0.1 m1 of a fresh bacterial culture should be added to 2.0 ml of overlay agar. For tests with metabolic activation, 0.5 ml of activation mixture containing an adequate amount of postmitochondrial fraction should be added to the agar overlay after the addition of test chemical and bacteria. Contents of each tube shall be mixed and poured over the surface of a selective agar plate. Overlay agar shall be allowed to solidify before incubation. At the end of the incubation period, revertant colonies per plate shall be counted.

(2) *Azo-reduction method.* (i) For this test with metabolic activation, 0.5 ml of S–9 mix containing 150 ul of S–9 and 0.1 ml of bacterial culture should be added to a test tube kept on ice. One-tenth milliliter of chemical should be added, and the tubes should be incubated with shaking at 30 °C for 30 min. At the end of the incubation period, 2.0 ml of agar should be added to each tube, the contents mixed and poured over the surface of a selective agar plate. Overlay agar shall be allowed to solidify before incubation. At the end of the incubation period, revertant colonies per plate shall be counted.

(ii) For tests without metabolic activation, 0.5 ml of buffer should be used in place of the 0.5 ml of S–9 mix. All other procedures shall be the same as those used for the test with metabolic activation.

(3) *Other methods.* Other methods may also be appropriate.

(4) *Media.* An appropriate selective medium with an adequate overlay agar shall be used.

(5) *Incubation conditions.* All plates within a given experiment shall be incubated for the same time period. This incubation period shall be for 48–72 hours at 37 °C.

(6) *Number of cultures.* All plating should be done at least in triplicate.

(f) *Data and report*—(1) *Treatment of results.* Data shall be presented as number of revertant colonies per plate for each replicate and dose. The numbers of revertant colonies on both negative (untreated and/or vehicle) and positive control plates shall also be presented. Individual plate counts, the mean number of revertant colonies per plate and standard deviation shall be presented for test chemical and positive and negative (untreated and/or vehicle) controls.

(2) *Statistical evaluation.* Data should be evaluated by appropriate statistical methods.

(3) *Interpretation of results.* (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of revertants. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test substance concentrations.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of revertants or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) *Test evaluation.* (i) Positive results from the *S. typhimurium* reverse mutation assay indicate that, under the test conditions, the test substance induces

point mutations by base changes or frameshifts in the genome of this organism.

(ii) Negative results indicate that under the test conditions the test substance is not mutagenic in *S. typhimurium*.

(5) *Test report*. In addition to the reporting recommendations as specified under 40 CFR part 792, subpart J, the following specific information shall be reported:

(i) Bacterial strain used.

(ii) Metabolic activation system used (source, amount and cofactor); details of preparations of S–9 mix.

(iii) Dose levels and rationale for selection of dose.

(iv) Positive and negative controls.

(v) Individual plate counts, mean number of revertant colonies per plate, standard deviation.

(vi) Dose-response relationship, if applicable.

(g) *References*. For additional background information on this test guideline the following references should be consulted:

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

(2) de Serres, F.J., Shelby, M.D. ''The *Salmonella* mutagenicity assay: recommendations,'' *Science* 203:563–565 (1979).

(3) Prival, M.J., Mitchell, V.D. ''Analysis of a method for testing azo dyes for mutagenic activity in *Salmonella typhimurium* in the presence of flavin mononucleotide and hamster liver S–9,'' *Mutation Research* 97:103–116 (1982).

(4) Vogel, H.J., Bonner, D.M. ''Acetylornithinase of *E. coli:* partial purification and some properties,'' *Journal of Biological Chemistry*. 218:97–106 (1956).

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19078, May 20, 1987]

§ 798.5275 Sex-linked recessive lethal test in drosophila melanogaster.

(a) *Purpose*. The sex-linked recessive lethal (SLRL) test using *Drosophila melanogaster* detects the occurrence of mutations, both point mutations and small deletions, in the germ line of the insect. This test is a forward mutation assay capable of screening for mutations at about 800 loci on the X-chromosome. This represents about 80 percent of all X-chromosome loci. The X-chromosome represents approximately one-fifth of the entire haploid genome.

(b) *Definitions*. (1) Lethal mutation is a change in the genome which, when expressed, causes death to the carrier.

(2) Recessive mutation is a change in the genome which is expressed in the homozygous or hemizygous condition.

(3) Sex-Linked genes are present on the sex (X or Y) chromosomes. Sex-linked genes in the context of this guideline refer only to those located on the X-chromosome.

(c) *Reference substances*. These may include, but need not be limited to, ethyl methanesulfonate or N-nitrosodimethylamine.

(d) *Test method*—(1) *Principle*. Mutations in the X-chromosome of *D. melanogaster* are phenotypically expressed in males carrying the mutant gene. When the mutation is lethal in the hemizygous condition, its presence is inferred from the absence of one class of male offspring out of the two that are normally produced by a heterozygous female. The SLRL test takes advantage of these facts by means of specially marked and arranged chromosomes.

(2) *Description*. Wild-type males are treated and mated to appropriate females. Female offspring are mated individually to their brothers, and in the next generation the progeny from each separate dose are scored for phenotypically wild-type males. Absence of these males indicates that a sex-linked recessive lethal mutation has occurred in a germ cell of the P_1 male.

(3) *Drosophila stocks*. Males of a well-defined wild type stock and females of the Muller-5 stock may be used. Other appropriately marked female stocks with multiple inverted X-chromosomes may also be used.

(4) *Control groups*—(i) *Concurrent controls*. Concurrent positive and negative (vehicle) controls shall be included in each experiment.

(ii) *Positive controls.* Examples of positive controls include ethyl methanesulfonate and N-nitroso-dimethylamine.

(iii) *Other positive controls.* Other positive control reference substances may be used.

(iv) *Negative controls.* Negative (vehicle) controls shall be included. The size of the negative (vehicle) control group shall be determined by the availability of appropriate laboratory historical control data.

(5) *Test chemicals*—(i) *Vehicle.* Test chemicals should be dissolved in water. Compounds which are insoluble in water may be dissolved or suspended in appropriate vehicles (e.g., a mixture of ethanol and Tween-60 or 80) and then diluted in water or saline prior to administration. Dimethylsulfoxide should br avoided as a vehicle.

(ii) *Dose levels.* For the initial assessment of mutagenicity, it is sufficient to test a single dose of the test substance for screening purposes. This dose should be the maximum tolerated dose, or that which produces some indication of toxicity, or shall be the highest dose attainable. For dose-response purposes, at least three additional dose levels should be used.

(iii) *Route of administration.* Exposure may be oral, by injection or by exposure to gases or vapors. Feeding of the test compound may be done in sugar solution. When necessary, substances may be dissolved in 0.7 percent NaCl solution and injected into the thorax or abdomen.

(e) *Test performance*—(1) *Treatment and mating.* Wild-type males (3 to 5 days old) shall be treated with the test substance and mated individually to an appropriate number of virgin females from the Muller-5 stock or females from another appropriately marked (with multiply-inverted X-chromosomes) stock. The females shall be replaced with fresh virgins every 2 to 3 days to cover the entire germ cell cycle. The offspring of these females are scored for lethal effects corresponding to the effects on mature sperm, mid or late stage spermatids, early spermatids, spermatocytes and spermatogonia at the time of treatment.

(2) *$F_1$1 matings.* Heterozygous F_1 females from the above crosses shall be allowed to mate individually (i.e., one female per vial) with their brothers. In the F_2 generation, each culture shall be scored for the absence of wild-type males. If a culture appears to have arisen from an F_1 female carrying a lethal in the parental X-chromosome (i.e., no males with the treated chromosome are observed), daughters of that female with the same genotype shall be tested to ascertain if the lethality is repeated in the next generation.

(3) *Number of matings.* (i) The test should be designed with a predetermined sensitivity and power. The number of flies in each group should reflect these defined parameters. The spontaneous mutant frequency observed in the appropriate control group will strongly influence the number of treated chromosomes that must be analysed to detect substances which show mutation rates close to those of the controls.

(ii) Test results should be confirmed in a separate experiment.

(f) *Data and report*—(1) *Treatment of results.* Data shall be tabulated to show the number of chromosomes tested, the number of nonfertile males and the number of lethal chromosomes at each exposure concentration and for each mating period for each male treated. Numbers of clusters of different size per male shall be reported.

(2) *Statistical evaluation.* Data shall be evaluated by appropriate statistical techniques.

(3) *Interpretation of results.* (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of sex-lined recessive lethals. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of sex-linked recessive lethals or a statistically significant and reproducible positive response at any one of the test points is considered non-mutagenic in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) *Test evaluation.* (i) Positive results in the SLRL test in *D. melanogaster* indicate that under the test conditions the test agent causes mutations in germ cells of this insect.

(ii) Negative results indicate that under the test conditions the test substance is not mutagenic in *D. melanogaster*.

(5) *Test report.* In addition to the reporting recommendations as specified under 40 CFR part 792, subpart J the following specific information shall be reported.

(i) *Drosophila* stock used in the assay, age of insects, number of males treated, number of sterile males, number of F_2 cultures established, number of F_2 cultures without progeny.

(ii) Test chemical vehicle, treatment and sampling schedule, exposure levels, toxicity data, negative (vehicle) and positive controls, if appropriate.

(iii) Criteria for scoring lethals.

(iv) Number of chromosomes tested, number of chromosomes scored, number of chromosomes carrying a lethal mutation.

(v) Historical control data, if available.

(vi) Dose-response relationship, if applicable.

(g) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Sobels, F.H., Vogel, E. ''The capacity of *Drosophila* for detecting relevant genetic damage,'' *Mutation Research* 41:95–106 (1976).

(2) Wurgler F.E., Sobels F.H., Vogel E. ''*Drosophila* as assay system for detecting genetic changes,'' *Handbook of mutagenicity test procedures.* Eds. Kilbey, B.J., Legator, M., Nichols, W., Ramel, C., (Amsterdam: Elsevier/North Holland Biomedical Press, 1977) pp. 335–373.

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19079, May 20, 1987]

§ 798.5300 Detection of gene mutations in somatic cells in culture.

(a) *Purpose.* Mammalian cell culture systems may be used to detect mutations induced by chemical substances. Widely used cell lines include L5178Y mouse lymphoma cells and the CHO and V–79 lines of Chinese hamster cells. In these cell lines the most commonly used systems measure mutation at the thymidine kinase (TK), hypoxanthine-guanine-phosphoribosyl transferase (HPRT) and $Na^{=}/K^{=}$ ATPase loci. The TK and HPRT mutational systems detect base pair mutations, frameshift mutations, and small deletions; the $Na^{=}/K^{=}$ ATPase system detects base pair mutations only.

(b) *Definitions.* (1) A forward mutation assay detects a gene mutation from the parental type to the mutant form which gives rise to a change in an enzymatic or functional protein.

(2) Base pair mutagens are agents which cause a base change in the DNA.

(3) Frameshift mutagens are agents which cause the addition or deletion of single or multiple base pairs in the DNA molecule.

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

(c) *Reference substances.* These may include, but need not be limited to, ethyl methanesulfonate, N-nitroso-dimethylamine, 2-acetylaminofluorene, 7,12-dimethylbenzanthracene or hycanthone.

(d) *Test method*—(1) *Principle.* Cells are exposed to 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. Cells deficient in thymidine kinase (TK) due to the forward mutation $TK^{=} \rightarrow TK^{-}$ are resistant to the cytotoxic effects of pyrimidine analogues such as bromodeoxyuridine (BrdU), fluorodeoxyuridine (FdU) or trifluorothymidine (TFT). The deficiency of the ''salvage'' enzyme thymidine kinase means that these antimetabolites are not incorporated into cellular nucleotides and the nucleotides needed for cellular metabolism are obtained solely from *de novo* synthesis. However, in the presence of thymidine kinase, BrdU, FdU or TFT are incorporated into the nucleotides,

resulting in inhibition of cellular metabolism and cytotoxicity. Thus mutant cells are able to proliferate in the presence of BrdU, FdU or TFT whereas normal cells, which contain thymidine kinase, are not. Similarly cells deficient in HPRT are selected by resistance to 8-azaguanine (AG) or 6-thioguanine (TG) and cells with altered $Na^{=}/K^{=}$ ATPase are selected by resistance to ouabain.

(2) *Description.* Cells in suspension or monolayer culture are exposed to the test substance, both with and without metabolic activation, for a defined period of time. Cytotoxicity is determined by measuring the colony forming ability or growth rate of the cultures after the treatment period. The treated cultures are maintained in growth medium for a sufficient period of time—characteristic of each selected locus—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. After a suitable incubation time, cell colonies are counted. The number of mutant colonies in selective medium is adjusted by the number of colonies in nonselective medium to derive the mutant frequency.

(3) *Cells*—(i) *Type of cells used in the assay.* A variety of cell lines are available for use in this assay including subclones of L5178Y, CHO cells or V–79 cells. Cell types used in this assay should have a demonstrated sensitivity to chemical mutagens, a high cloning efficiency and a low spontaneous mutation frequency. Cells should be checked for *Mycoplasma* contamination and may be periodically checked for karyotype stability.

(ii) *Cell growth and maintenance.* Appropriate culture media and incubation conditions (culture vessels, CO_2 concentrations, temperature and humidity) shall be used.

(4) *Metabolic activation.* Cells shall be exposed to test substance both in the presence and absence of an appropriate metabolic activation system.

(5) *Control groups.* Positive and negative (untreated and/or vehicle) controls shall be included in each experiment. When metabolic activation is used, the positive control substance shall be known to require such activation.

(6) *Test chemicals*—(i) *Vehicle.* Test substances may be prepared in culture media or dissolved or suspended in appropriate vehicles prior to treatment of the cells. The final concentration of the vehicle shall not interfere with cell viability or growth rate. Treatment vessels should be chosen to ensure that there is no visible interaction, such as etching, between the solvent, the test chemical, and the vessel.

(ii) *Exposure concentrations.* (A) 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 number of cells per culture is based on the expected background mutant frequency; a general guide is to use a number which is 10 times the inverse of this frequency.

(B) Several concentrations (usually at least 4) of the test substance shall be used. Generally, these shall yield a concentration-related toxic effect. The highest concentration shall produce a low level of survival (approximately 10 percent), and the survival in the lowest concentration shall approximate the negative control. Cytotoxicity shall be determined after treatment with the test substance both in the presence and in the absence of an exogenous metabolic activation system. Relatively insoluble substances should be tested up to their limit of solubility under culture conditions. For freely-soluble nontoxic substances the highest concentration used should be determined on a case-by-case basis.

(e) *Test performance.* (1) Cells shall be exposed to the test substance both with and without exogenous metabolic activation. Exposure shall be for a suitable period of time, in most cases 1 to 5 hours is effective; exposure time may be extended over one or more cell cycles.

(2) At the end of the exposure period, cells shall be washed and cultured to determine viability and to allow for expression of the mutant phenotype.

(3) At the end of the expression period, which shall be sufficient to allow

near optimal phenotypic expression of induced mutants, cells should be grown in medium with and without selective agent(s) for determination of number of mutants and cloning efficiency, respectively.

(4) Results shall be confirmed in an independent experiment. When appropriate, a single positive response should be confirmed by testing over a narrow range of concentrations.

(f) *Data and report*—(1) *Treatment of results.* Data shall be presented in tabular form. Individual colony counts for the treated and control groups shall be presented for both mutation induction and survival. Survival and cloning efficiencies shall be given as a percentage of the controls. Mutant frequency shall be expressed as number of mutants per number of surviving cells.

(2) *Statistical evaluation.* Data should be evaluated by appropriate statistical methods.

(3) *Interpretation of results.* (i) There are several criteria for determining a positive result, one of which is a statistically significant concentration-related increase in the mutant frequency. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test substance concentrations.

(ii) A test substance which does not produce either a statistically significant concentration-related increase in the mutant frequency or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) *Test evaluation.* (i) Positive results for an in vitro mammalian cell gene mutation test indicate that, under the test conditions, a substance induces gene mutations in the cultured mammalian cells used.

(ii) Negative results indicate that, under the test conditions, the test substance does not induce gene mutations in the cultured mammalian cells used.

(5) *Test report.* In addition to the reporting recommendations as specified under 40 CFR part 792, subpart J the following specific information shall be reported:

(i) Cell type used, number of cell cultures, methods used for maintenance of cell cultures.

(ii) Rationale for selection of concentrations and number of cultures.

(iii) Test conditions: composition of media, CO_2 concentration, concentration of test substance, vehicle, incubation temperature, incubation time, duration of treatment, cell density during treatment, type of metabolic activation system, positive and negative controls, length of expression period (including number of cells seeded and subculture and feeding schedules, if appropriate), selective agent(s).

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

(v) Dose-response relationship, where possible.

(g) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Amacher, D.E., Paillet, S.C., Ray, V. ''Point mutations at the thymidine kinase locus in L5178Y mouse lymphoma cells. I. Application to genetic toxicology testing,'' *Mutation Research,* 64:391–406 (1979).

(2) Amacher, D.E., Paillet, S.C., Turner, G.N., Ray, V.A. Salsburg, V.A. ''Point mutations at the thymidine kinase locus in L5178Y mouse lymphoma cells. II. Test validation and interpretation,'' *Mutation Research,* 72:447–474 (1980).

(3) Bradley, M.O., Bhuyan B., Francis, M.C., Langenback, R., Peterson, A., Huberman, E. ''Mutagenesis by chemical agents in V–79 Chinese hamster cells: a review and analysis of the literature: a report of the Gene-Tox Program,'' *Mutation Research,* 87:81–142 (1981).

(4) Clive, D., Johnson, K.O., Spector, J.F.S., Batson, A.G., Brown, M.M. ''Validation and characterization of the L5178Y $TK^{+/-}$ mouse lymphoma mutagen assay system,'' *Mutation Research,* 59:61–108 (1979).

(5) Clive, D., Spector, J.F.S. ''Laboratory procedures for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells,'' *Mutation Research,* 31:17–29 (1975).

(6) Hsie, A.W., Casciano, D.A., Couch, D.B., Krahn, D.F., O'Neill, J.P., Whitfield, B.L. ''The use of Chinese hamster ovary cells to quantify specific locus mutation and to determine mutagenicity of chemicals: a report of the U.S. EPA's Gene-Tox Program,'' *Mutation Research*, 86:193–214 (1981).

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19079, May 20, 1987]

§ 798.5375 In vitro mammalian cytogenetics.

(a) *Purpose*. The in vitro cytogenetics test is a mutagenicity test system for the detection of chromosomal aberrations in cultured mammalian cells. Chromosomal aberrations may be either structural or numerical. However, because cytogenetic assays are usually designed to analyse cells at their first post-treatment mitosis and numerical aberrations require at least one cell division to be visualized, this type of aberration is generally not observed in a routine cytogenetics assay. Structural aberrations may be of two types, chromosome or chromatid.

(b) *Definitions*. (1) Chromosome-type aberrations are changes which result from damage expressed in both sister chromatids at the same time.

(2) Chromatid-type aberrations are damage expressed as breakage of single chromatids or breakage and/or reunion between chromatids.

(c) *Reference substances*. Not applicable.

(d) *Test method*—(1) *Principle*. In vitro cytogenetics assays may employ cultures of established cell lines, cell strains or primary cell cultures. Cell cultures are exposed to the test substance both with and without metabolic activation. Following exposure of cell cultures to test substances, they are treated with a spindle inhibitor (e.g., colchicine or Colcemid#) to arrest cells in a metaphase-like stage of mitosis (c-metaphase). Cells are then harvested and chromosome preparations made. Preparations are stained and metaphase cells are analyzed for chromosomal aberrations.

(2) *Description*. Cell cultures are exposed to test compounds and harvested at various intervals after treatment. Prior to harvesting, cells are treated with a spindle inhibitor (e.g., colchicine or Colcemid#) to accumulate cells in c-metaphase. Chromosome preparations from cells are made, stained and scored for chromosomal aberrations.

(3) *Cells*—(i) *Type of cells used in the assay*. There are a variety of cell lines or primary cell cultures, including human cells, which may be used in the assay. Established cell lines and strains should be checked for *Mycoplasma* contamination and may be periodically checked for karyotype stability.

(ii) *Cell growth and maintenance*. Appropriate culture media, and incubation conditions (culture vessels CO_2 concentrations, temperature and humidity) shall be used.

(4) *Metabolic activation*. Cells shall be exposed to test substance both in the presence and absence of an appropriate metabolic activation system.

(5) *Control groups*. Positive and negative (untreated and/or vehicle) controls both with and without metabolic activation shall be included in each experiment. When metabolic activation is used, the positive control substance shall be known to require such activation.

(6) *Test chemicals*—(i) *Vehicle*. Test substances may be prepared in culture media or dissolved or suspended in appropriate vehicles prior to treatment of the cells. Final concentration of the vehicle shall not interfere with cell viability or growth rate. Treatment vessels should be chosen to ensure that there is no visible interaction, such as etching, between the solvent, the test chemical, and the vessel.

(ii) *Exposure concentrations*. Multiple concentrations of the test substance over a range adequate to define the response should be tested. Generally the highest test substance concentrations tested with and without metabolic activation should show evidence of cytotoxicity or reduced mitotic activity. Relatively insoluble substances should be tested up to the limit of solubility. For freely soluble nontoxic chemicals, the upper test chemical concentration should be determined on a case by case basis.

(e) *Test performance*—(1) *Established cell lines and strains*. Prior to use in the assay, cells should be generated from

stock cultures, seeded in culture vessels at the appropriate density and incubated at 37 °C.

(2) *Human lymphocyte cultures.* Heparinized or acid-citrate-dextrose whole blood should be added to culture medium containing a mitogen, e.g., phytohemagglutinin (PHA) and incubated at 37 °C. White cells sedimented by gravity (buffy coat) or lymphocytes which have been purified on a density gradient may also be utilized.

(3) *Treatment with test substance.* For established cell lines and strains, cells in the exponential phase of growth shall be treated with test substances in the presence and absence of an exogenous metabolic activation system. Mitogen-stimulated human lymphocyte cultures may be treated with the test substance in a similar manner.

(4) *Number of cultures.* At least two independent cultures shall be used for each experimental point.

(5) *Culture harvest time.* (i) For established cell lines and strains, multiple harvest times are recommended. However, for screening purposes, a single harvest time may be appropriate. If the test chemical changes the cell cycle length, the fixation intervals should be changed accordingly. If a single harvest time is selected, supporting data for the harvest time should be presented in such a study.

(ii) For human lymphocyte cultures, the substance to be tested may be added to the cultures at various times after mitogen stimulation so that there is a single harvest time after the initiation of the cell culture. Alternatively, a single treatment may be followed by multiple harvest times. Harvest time should be extended for those chemicals which induce an apparent cell cycle delay. Because the population of human lymphocytes is only partially synchronized, a single treatment, at, or close to, the time when metaphase stages first appear in the culture will include cells in all phases of the division cycle. Therefore, a single harvest at the time of second mitosis may be carried out for screening purposes.

(iii) Cell cultures shall be treated with a spindle inhibitor, (e.g., colchicine or Colcemid®), 1 or 2 hours prior to harvesting. Each culture shall be harvested and processed separately for the preparation of chromosomes.

(6) *Chromosome preparation.* Chromosome preparation involves hypotonic treatment of the cells, fixation and staining.

(7) *Analysis.* Slides shall be coded before analysis. In human lymphocytes, only cells containing 46 centromeres shall be analyzed. In established cell lines and strains, only metaphases containing ±2 centromeres of the modal number shall be analyzed. Uniform criteria for scoring aberrations shall be used.

(8) *Confirmatory tests.* When appropriate, a single positive response shall be confirmed by testing over a narrow range of concentrations.

(f) *Data and report*—(1) *Treatment of results.* Data shall be presented in a tabular form. Different types of structural chromosomal aberrations shall be listed with their numbers and frequencies for experimental and control groups. Data should be evaluated by appropriate statistical methods. Gaps or achromatic lesions are recorded separately and not included in the total aberration frequency.

(2) *Statistical evaluation.* Data should be evaluated by appropriate statistical methods.

(3) *Interpretation of results.* (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of structural chromosomal aberrations. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test substance concentrations.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of structural chromosomal aberrations or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) *Test evaluation.* (i) Positive results in the in vitro cytogenetics assay indicate that under the test conditions the test substance induces chromosomal

aberrations in cultured mammalian somatic cells.

(ii) Negative results indicate that under the test conditions the test substance does not induce chromosomal aberrations in cultured mammalian somatic cells.

(5) *Test report.* In addition to the reporting recommendations as specified under 40 CFR part 792, subpart J the following specific information shall be reported:

(i) Cells used, density and passage number at time of treatment, number of cell cultures.

(ii) Methods used for maintenance of cell cultures including medium, temperature and CO_2 concentration.

(iii) Test chemical vehicle, concentration and rationale for the selection of the concentrations used in the assay, duration of treatment.

(iv) Details of both the protocol used to prepare the metabolic activation system and of its use in the assay.

(v) Identity of spindle inhibitor, its concentration and duration of treatment.

(vi) Date of cell harvest.

(vii) Positive and negative controls.

(viii) Methods used for preparation of slides for microscopic examination.

(ix) Number of metaphases analysed.

(x) Mitotic index where applicable.

(xi) Criteria for scoring aberrations.

(xii) Type and number of aberrations, given separately for each treated and control culture, total number of aberrations per group; frequency distribution of number of chromosomes in established cell lines and strains.

(xiii) Dose-response relationship, if applicable.

(g) *References.* For additional background information on this test guideline the following references should be consulted.

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

(2) Evans, H.J. ''Cytological methods for detecting chemical mutagens,'' *Chemical mutagens, principles and methods for their detection,* Vol. 4, Ed. A. Hollaender (New York, London: Plenum Press, 1976) pp. 1–29.

(3) Howard, P.N., Bloom, A.D., Krooth, R.S. ''Chromosomal aberrations induced by N-methyl-N′-nitro-N-nitrosoguanidine in mammalian cells,'' *In Vitro* 7:359–365 (1972).

(4) Ishidate, M. Jr., Odashima, S. ''Chromosome tests with 134 compounds on Chinese hamster cells in vitro: A screening for chemical carcinogens,'' *Mutation Research,* 48:337–354 (1975).

(5) Preston, R.J., Au, W., Bender, M.A., Brewen, J.G., Carrano, A.V., Heddle, J.A., McFee, A.F., Wolff, S., Wassom, J.S., ''Mammalian in vivo and in vitro cytogenetic assays: A report of the Gene-tox Program,'' *Mutation Research,* 87:143–188 (1981).

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19079, May 20, 1987]

§ 798.5385 In vivo mammalian bone marrow cytogenetics tests: Chromosomal analysis.

(a) *Purpose.* The *in vivo* bone marrow cytogenetic test is a mutagenicity test for the detection of structural chromosomal aberrations. Chromosomal aberrations are generally evaluated in first post-treatment mitoses. With the majority of chemical mutagens, induced aberrations are of the chromatid type but chromosome type aberrations also occur.

(b) *Definitions.* (1) Chromosome-type aberrations are changes which result from damage expressed in both sister chromatids at the same time.

(2) Chromatid-type aberrations are damage expressed as breakage of single chromatids or breakage and/or reunion between chromatids.

(c) *Reference substances.* Not applicable.

(d) *Test method*—(1) *Principle.* Animals are exposed to test chemicals by appropriate routes and are sacrificed at sequential intervals. Chromosome preparations are made from bone marrow cells. The stained preparations are examined and metaphase cells are scored for chromosomal aberrations.

(2) *Description.* The method employs bone marrow of laboratory rodents which have been exposed to test chemicals. Prior to sacrifice, animals are further treated with a spindle inhibitor,

(e.g., colchicine or Colcemid®) to arrest the cells in c-metaphase. Chromosome preparations from the cells are stained and scored for chromosomal aberrations.

(3) *Animal selection*—(i) *Species and strain.* Any appropriate mammalian species may be used. Examples of commonly used rodent species are rats, mice, and hamsters.

(ii) *Age.* Healthy young adult animals shall be used.

(iii) *Number and sex.* At least five female and five male animals per experimental and control group shall be used. Thus, 10 animals would be sacrificed per time per group treated with the test compound if several test times after treatment are included in the experimental schedule. The use of a single sex or smaller number of animals should be justified.

(iv) *Assignment to groups.* Animals shall be randomized and assigned to treatment and control groups.

(4) *Control groups*—(1) *Concurrent controls.* (i) Concurrent positive and negative (vehicle) controls shall be included in the assay.

(ii) *Positive controls.* A single dose positive control showing a significant response at any one time point is adequate. A compound known to produce chromosomal aberrations in vivo shall be employed as the positive control.

(5) *Test chemicals*—(i) *Vehicle.* When possible, test chemicals shall be dissolved in isotonic saline or distilled water. Water insoluble chemicals may be dissolved or suspended in appropriate vehicles. The vehicles used shall neither interfere with the test chemical nor produce toxic effects. Fresh preparations of the test compound should be employed.

(ii) *Dose levels.* For an initial assessment, one dose of the test substance may be used, the dose being the maximum tolerated dose (to a maximum of 5,000 mg/kg) or that producing some indication of cytotoxicity (e.g., partial inhibition of mitosis) or shall be the highest dose attainable (to a maximum of 5,000 mg/kg). Additional dose levels may be used. For determination of dose-response, at least three dose levels should be used.

(iii) *Route of administration.* The usual routes are oral or by intraperitoneal injection. Other routes may be appropriate.

(iv) *Treatment schedule.* In general, test substances should be administered once only. However, based on toxicological information a repeated treatment schedule may be employed.

(e) *Test performance*—(1) *Generally the test may be performed in two assays.* (i) Animals should be treated with the test substance once at the selected dose(s). Samples should be taken at three times after treatment. For rodents, the central sampling interval is 24 hours. Since cell cycle kinetics can be influenced by the test substance, one earlier and one later sampling interval adequately spaced within the range of 6 to 48 hours shall be applied. Where the additional dose levels are tested in a subsequent experiment, samples shall be taken at the predetermined most sensitive interval or, if this is not established, at the central sampling time. If the most sensitive interval is known and documented with data, only this one time point shall be sampled.

(ii) If a repeated treatment schedule is used at the selected dose(s), samples shall be taken 6 and 24 hours after the last treatment; other sampling times may be used if justified. Where the additional dose levels are tested in a subsequent experiment, samples shall be taken at the predetermined most sensitive interval or, if this is not established, at 6 hours after the last treatment.

(2) *Administration of spindle inhibitor.* Prior to sacrifice, animals shall be injected IP with an appropriate dose of a spindle inhibitor (e.g., colchicine or Colcemid®) to arrest cells in c-metaphase.

(3) *Preparation of slides.* Immediately after sacrifice, the bone marrow shall be obtained, exposed to hypotonic solution, and fixed. The cells shall then be spread on slides and stained. Chromosome preparations shall be made following standard procedures.

(4) *Analysis.* The number of cells to be analyzed per animal should be based upon the number of animals used, the negative control frequency, the predetermined sensitivity, and the power chosen for the test. Slides shall be coded before microscopic analysis.

(f) *Data and report*—(1) *Treatment of results.* Data should be presented in tabular form for both cells and animals. Different types of structural chromosomal aberrations should be listed with their numbers and a mean frequency per cell for each animal in all treated and control groups. Gaps (achromatic lesions) should be recorded separately and not included in the total abberration frequency. Differences among animals within each group should be considered before making comparisons between treated and control groups.

(2) *Statistical evaluation.* Data should be evaluated by appropriate statistical methods.

(3) *Interpretation of results.* (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of structual chromosomal aberrations or abnormal metaphase figures. Another criterion may be based upon detection of a reproducible and statistically significant positive response for a least one of the test points.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of chromosomal aberrations or abnormal metaphase figures or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) *Test evaluation.* (i) Positive results in the *in vivo* bone marrow cytogenetics assay indicate that under the test conditions the test substance induces chromosomal aberrations in the bone marrow of the test species.

(ii) Negative results indicate that under the test conditions, the test substance does not induce chromosomal aberrations in the bone marrow of the test species.

(5) *Test report.* In addition to the reporting recommendations as specified under 40 CFR part 792, subpart J the following specific information shall be reported:

(i) Species, strain, age, weight, number and sex of animals in each treatment and control group.

(ii) Test chemical vehicle, dose levels used, rationale for dose selection.

(iii) Route of administration, treatment and sampling schedules, toxicity data, negative and positive controls.

(iv) Identity of spindle-inhibitor, its concentration and duration of treatment.

(v) Details of the protocol used for chromosome preparation, number of cells scored per animal, type and number of aberrations given separately for each treated and control animal.

(vi) Mitotic index, where applicable.

(vii) Criteria for scoring aberrations.

(viii) Number and frequency of aberrant cells per animal in each treatment and control groups.

(ix) Total number of aberrations per group.

(x) Number of cells with aberrations per group.

(xi) Dose-response relationship, if applicable.

(g) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Adler, I.D., Ramarao, G., Epstein, S.S. ''In vivo cytogenetic effects of trimethyl-phosphate and of TEPA on bone marrow cells of male rats,'' *Mutation Research,* 13:263–273 (1971).

(2) Evans, H.J. ''Cytological methods for detecting chemical mutagens,'' *Chemical Mutagens: Principles and Methods for Their Detection,* Vol. 4. Ed. A. Hollaender (New York and London: Plenum Press, 1976) pp. 1–29.

(3) Kilian, J.D., Moreland, F.E. Benge, M.C., Legator, M.S., Whorton, E.B. Jr. ''A collaborative study to measure intralaboratory variation with the in vivo bone morrow metaphase procedure,'' *Handbook of mutagenicity test procedures.* Eds. Kilby, B.J., Legator, M. Nichols, C., Ramel, D., (Amsterdam: Elsevier/North Holland Biomedical Press, 1977) 243–260.

(4) Preston, J.R., Au, W., Bender, M.A., Brewen, J.G., Carrano, A.V. Heddle, J.A., McFee, A.F., Wolff, S., Wassom, J. ''Mammalian in vivo and vitro cytogenetics assays: Report of

the Gene-Tox Program,'' *Mutation Research, 87:143–188 (1981).*

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19080, May 20, 1987]

§ 798.5395 In vivo mammalian bone marrow cytogenetics tests: Micronucleus assay.

(a) *Purpose.* The micronucleus test is a mammalian *in vivo* test which detects damage of the chromosomes or mitotic apparatus by chemicals. Polychromatic erythrocytes in the bone marrow of rodents are used in this assay. When the erythroblast develops into an erythrocyte the main nucleus is extruded and may leave a micronucleus in the cytoplasm. The visualization of micronuclei is facilitated in these cells because they lack a nucleus. Micronuclei form under normal conditions. The assay is based on an increase in the frequency of micronucleated polychromatic erythrocytes in bone marrow of treated animals.

(b) *Definition.* Micronuclei are small particles consisting of acentric fragments of chromosomes or entire chromosomes, which lag behind at anaphase of cell division. After telophase, these fragments may not be included in the nuclei of daughter cells and form single or multiple micronuclei in the cytoplasm.

(c) *Reference substances.* Not applicable.

(d) *Test method*—(1) *Principle.* (i) Animals are exposed to test substance by an appropriate route. They are sacrificed, the bone marrow extracted and smear preparations made and stained. Polychromatic erythrocytes are scored for micronuclei under the microscope.

(ii) Micronuclei may also be detected in other test systems:

(A) Tissue culture.

(B) Plants.

(C) Blood smears.

(D) Fetal tissues.

(E) Meiotic cells.

(F) Hepatic cells.

(iii) The present guideline is based on the mammalian bone marrow assay.

(2) *Description.* The method employs bone marrow of laboratory mammals which are exposed to test substances.

(3) *Animal selection*—(i) *Species and strain.* Mice are recommended. However, any appropriate mammalian species may be used.

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

(iii) *Number and sex.* At least five female and five male animals per experimental and control group shall be used. Thus, 10 animals would be sacrificed per time per group if several test times after treatment were included in the experimental schedule. The use of a single sex or a smaller number of animals should be justified.

(iv) *Assignment to groups.* Animals shall be randomized and assigned to treatment and control groups.

(4) *Control groups*—(i) *Concurrent controls.* Concurrent positive and negative (vehicle) controls shall be included in each assay.

(ii) *Positive controls.* A compound known to produce micronuclei *in vivo* shall be employed as the positive control.

(5) *Test chemicals*—(i) *Vehicle.* When appropriate for the route of administration, solid and liquid test substances should be dissolved or suspended in distilled water or isotonic saline. Water insoluble chemicals may be dissolved or suspended in appropriate vehicles. The vehicle used shall neither interfere with the test compound nor produce toxic effects. Fresh preparations of the test compound should be employed.

(ii) *Dose levels.* For an initial assessment, one dose of the test substance may be used, the dose being the maximum tolerated dose (to a maximum of 5,000 mg/kg) or that producing some indication of cytotoxicity, e.g., a change in the ratio of polychromatic to normochromatic erythrocytes. Additional dose levels may be used. For determination of dose response, at least three dose levels shall be used.

(iii) *Route of administration.* The usual routes of administration are IP or oral. Other routes may be appropriate.

(iv) *Treatment schedule.* Test substances should generally be administered only once. However, based upon toxicological information a repeated treatment schedule may be employed.

(e) *Test performance*—(1) *Treatment and sampling times.* (i) Animals shall be treated with the test substance once at the highest tolerated dose. Sampling

times should coincide with the maximum responses of the assay which varies with the test substance. Therefore, using the highest dose, bone marrow samples should be taken at least three times, starting not earlier than 12 hours after treatment, with appropriate intervals following the first sample but not extending beyond 72 hours. When other doses are used sampling shall be at the maximum sensitive period, or, if that is not known, approximately 24 hours after treatment. Other appropriate sampling times may be used in addition. If the most sensitive interval is known and documented with data, only this one time point need be sampled.

(ii) If a repeated treatment schedule is used, samples shall be taken at least three times, starting not earlier than 12 hours after the last treatment and at appropriate intervals following the first sample, but not extending beyond 72 hours.

(iii) Bone marrow shall be obtained immediately after sacrifice. Cells shall be prepared, put on slides, spread as a smear and stained.

(2) *Analysis.* Slides shall be coded before microscopic analysis. At least 1,000 polychromatic erythrocytes per animal shall be scored for the incidence of micronuclei. The ratio of polychromatic to normochromatic erythrocytes should be determined for each animal by counting a total of 200 erythrocytes. To ensure consistency with OECD and other guidelines, 1,000 polychromatic erythrocytes are recommended. Additional information may be obtained by scoring normochromatic erythrocytes for micronuclei.

(f) *Data and report*—(1) *Treatment of results.* Criteria for scoring micronuclei shall be given. Individual data shall be presented in a tabular form including positive and negative (vehicle) controls and experimental groups. The number of polychromatic erythrocytes scored, the number of micronucleated polychromatic erythrocytes, the percentage of micronucleated cells, the number of micronucleated normochromatic erythrocytes, and, if applicable, the percentage of micronucleated erythrocytes and the ratio of normochromatic to polychromatic erythrocytes shall be listed separately for each experimental and control animal. Absolute numbers shall be included if percentages are reported.

(2) *Statistical evaluation.* Data should be evaluated by appropriate statistical methods.

(3) *Interpretation of results.* (i) There are several criteria for determining a positive response, one of which is a statistically significant dose-related increase in the number of micronucleated polychromatic erythrocytes. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test substance concentrations.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of micronucleated polychromatic erythrocytes or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) *Test evaluation.* (i) The results of the micronucleus test provide information on the ability of a chemical to induce micronuclei in polychromatic erythrocytes of the test species under the conditions of the test. This damage may have been the result of chromosomal damage or damage to the mitotic apparatus.

(ii) Negative results indicate that under the test conditions the test substance does not produce micronuclei in the bone marrow of the test species.

(5) *Test report.* In addition to the reporting recommendations as specified under 40 CFR part 792, subpart J, the following specific information shall be reported:

(i) Species, strain, age, weight, number and sex of animals in each treatment and control group.

(ii) Test chemical vehicle, dose levels used, rationale for dose selection.

(iii) Rationale for and description of treatment and sampling schedules, toxicity data, negative and positive controls.

(iv) Details of the protocol used for slide preparation.

(v) Criteria for identifying micronucleated erythrocytes.

(vi) Dose-response relationship, if applicable.

(g) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Cihak, R. ''Evaluation of benzidine by the micronucleus test,'' *Mutation Research*, 67: 383–384 (1979).

(2) Cole, R.J., Taylor, N., Cole, J., Arlett, C.F. ''Short-term tests for transplacentally active carcinogens. 1. Micronucleus formation in fetal and maternal mouse erythroblasts,'' *Mutation Research*, 80: 141–157 (1981).

(3) Kliesch, U., Danford, N., Adler, I.D. ''Micronucleus test and bone-marrow chromosome analysis. A comparison of 2 methods in vivo for evaluating chemically induced chromosomal alterations,'' *Mutation Research*, 80: 321–332 (1981).

(4) Matter, B., Schmid, W. ''Trenimon-induced chromosomal damage in bone-marrow cells of six mammalian species, evaluated by the micronucleus test,'' *Mutation Research*, 12: 417–425 (1971).

(5) Schmid, W. ''The micronucleus test,'' *Mutation Research, 31:9–15 (1975).*

(6) Schmid, W. ''The micronucleus test for cytogenetic analysis,'' *Chemical Mutagens, Principles and Methods for their Detection.* Vol. 4 Hollaender A, (Ed. A ed. (New York and London: Plenum Press, (1976) pp. 31–53.

(7) Heddle, J.A., Hite, M., Kurkhart, B., Mavournin, K., MacGregor, J.T., Newell, G.W., Salamone, M.F. ''The induction of micronuclei as a measure of genotoxicity. A report of the U.S. Environmental Protection Agency Gene-Tox Program,'' *Mutation Research*, 123: 61–118 (1983).

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19080, May 20, 1987; 52 FR 26150, July 13, 1987; 52 FR 34654, Sept. 14, 1987]

§ 798.5450 Rodent dominant lethal assay.

(a) *Purpose.* Dominant lethal (DL) effects cause embryonic or fetal death. Induction of a dominant lethal event after exposure to a chemical substance indicates that the substance has affected germinal tissue of the test species. Dominant lethals are generally accepted to be the result of chromosomal damage (structural and numerical anomalies) but gene mutations and toxic effects cannot be excluded.

(b) *Definition.* A dominant lethal mutation is one occurring in a germ cell which does not cause dysfunction of the gamete, but which is lethal to the fertilized egg or developing embryo.

(c) *Reference substances.* These may include, but need not be limited to, triethylenemelamine, cyclophosphamide or ethyl methanesulfonate.

(d) *Test method*—(1) *Principle.* Generally, male animals are exposed to the test substance and mated to untreated virgin females. The various germ cell stages can be tested separately by the use of sequential mating intervals. The females are sacrificed after an appropriate period of time and the contents of the uteri are examined to determine the numbers of implants and live and dead embryos. The calculation of the dominant lethal effect is based on comparison of the live implants per female in the treated group to the live implants per female in the control group. The increase of dead implants per female in the treated group over the dead implants per female in the control group reflects the post-implantation loss. The post-implantation loss is calculated by determining the ratio of dead to total implants from the treated group compared to the ratio of dead to total implants from the control group. Pre-implantation loss can be estimated on the basis of corpora lutea counts or by comparing the total implants per female in treated and control groups.

(2) *Description.* (i) Several treatment schedules are available. The most widely used requires single administration of the test substance. Other treatment schedules, such as treatment on five consecutive days, may be used if justified by the investigator.

(ii) Individual males are mated sequentially to virgin females at appropriate intervals. The number of matings following treatment is governed by the treatment schedule and should ensure that germ cell maturation is adequately covered. Females are sacrificed in the second half of pregnancy and the uterine contents examined to determine the total number

of implants and the number of live and dead embryos.

(3) *Animal selection*—(i) *Species.* Rats or mice are generally used as the test species. Strains with low background dominant lethality, high pregnancy frequency and high implant numbers are recommended.

(ii) *Age.* Healthy, sexually mature animals shall be used.

(iii) *Number.* An adequate number of animals shall be used taking into account the spontaneous variation of the biological characteristics being evaluated. The number chosen should be based on the predetermined sensitivity of detection and power of significance. For example, in a typical experiment, the number of males in each group shall be sufficient to provide between 30 and 50 pregnant females per mating interval.

(iv) *Assignment to groups.* Animals shall be randomized and assigned to treatment and control groups.

(4) *Control groups*—(i) *Concurrent controls.* Generally concurrent positive and negative (vehicle) controls shall be included in each experiment. When acceptable positive control results are available from experiments conducted recently (within the last 12 months) in the same laboratory these results can be used instead of a concurrent positive control.

(ii) *Positive controls.* Positive control substances shall be used at a dose which demonstrates the test sensitivity.

(5) *Test chemicals*—(i) *Vehicle.* When possible, test substances shall be dissolved or suspended in isotonic saline or distilled water. Water-insoluble chemicals may be dissolved or suspended in appropriate vehicles. The vehicle used shall neither interfere with the test chemical nor produce toxic effects. Fresh preparations of the test chemical should be employed.

(ii) *Dose levels.* Normally, three dose levels shall be used. The highest dose shall produce signs of toxicity (e.g., slightly reduced fertility and slightly reduced body weight). However, in an initial assessment of dominant lethality a single high dose may be sufficient. Nontoxic substances shall be tested at 5g/kg or, if this is not practicable, then as the highest dose attainable.

(iii) *Route of administration.* The usual routes of administration are oral or by IP injection. Other routes may be appropriate.

(e) *Test performance.* (1) Individual males are mated sequentially at appropriate predetermined intervals to one or two virgin females. Females should be left with the males for at least the duration of one estrus cycle or alternatively until mating has occurred as determined by the presence of sperm in the vagina or by the presence of a vaginal plug.

(2) The number of matings following treatment should be governed by the treatment schedule and should ensure that germ cell maturation is adequately covered.

(3) Females should be sacrificed in the second half of pregnancy and uterine contents examined to determine the number of implants and live and dead embryos. The ovaries may be examined to determine the number of corpora lutea.

(f) *Data and report*—(1) *Treatment of results.* Data shall be tabulated to show the number of males, the number of pregnant females, and the number of nonpregnant females. Results of each mating, including the identity of each male and female, shall be reported individually. For each female, the dose level and week of mating and the frequencies of live implants and of dead implants shall be enumerated. If the data are recorded as early and late deaths, the tables shall make that clear. If preimplantation loss is estimated, it shall be reported. Preimplantation loss can be calculated as the difference between the number of corpora lutea and the number of implants or as a reduction in the average number of implants per female in comparison with control matings.

(2) *Statistical evaluation.* Data shall be evaluated by appropriate statistical methods. Differences among animals within the control and treatment groups shall be considered before making comparisons between treated and control groups.

(3) *Interpretation of results.* (i) There are several criteria for determining a

positive result, one of which is a statistically significant dose-related increase in the number of dominant lethals. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of dominant lethals or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) *Test evaluation.* (i) A positive DL assay suggests that under the test conditions the test substance may be genotoxic in the germ cells of the treated sex of the test species.

(ii) A negative result suggests that under the conditions of the test the test substance may not be genotoxic in the germ cells of the treated sex of the test species.

(5) *Test report.* In addition to the reporting recommendations as specified under 40 CFR part 792, subpart J the following specific information shall be reported:

(i) Species, strain, age and weights of animals used, number of animals of each sex in experimental and control groups.

(ii) Test substance, vehicle used, dose levels and rationale for dosage selection, negative (vehicle) and positive controls, experimental observations, including signs of toxicity.

(iii) Route and duration of exposure.

(iv) Mating schedule.

(v) Methods used to determine that mating has occurred (where applicable).

(vi) Criteria for scoring dominant lethals including the number of early and late embryonic deaths.

(vii) Dose-response relationship, if applicable.

(g) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Brewen, J.G., Payne, H.S., Jones, K.P., Preston, R.J. ''Studies on chemically induced dominant lethality. I. The cytogenetic basis of MMS-induced dominant lethality in post-meiotic germ cells'' *Mutation Research*, 33:239–250 (1975).

(2) Ehling, U.H., Machemer, L., Buselmaier, E., Dycka, D., Frohberg, H., Kratochvilova, J., Lang, R., Lorke, D., Muller, D., Pheh, J., Rohrborn, G., Roll, R., Schulze-Schencking, M., Wiemann, H. ''Standard protocol for the dominant lethal test on male mice. Set up by the Work Group ''Dominant lethal mutations of the ad hoc Committee Chemogenetics,'' *Archives of Toxicology*, 39:173–185 (1978).

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19081, May 20, 1987]

§ 798.5460 Rodent heritable translocation assays.

(a) *Purpose.* This test detects transmitted chromosomal damage which manifests as balanced reciprocal translocations in progeny descended from parental males treated with chemical mutagens.

(b) *Definitions.* (1) A heritable translocation is one in which distal segments of nonhomologous chromosomes are involved in a reciprocal exchange.

(2) Diakinesis and metaphase I are stages of meiotic prophase scored cytologically for the presence of multivalent chromosome association characteristic of translocation carriers.

(c) *Reference substances.* Not applicable.

(d) *Test method*—(1) *Principle.* When a balanced reciprocal translocation is induced in a parental male germ cell, the resulting progeny is translocation heterozygote.

(i) *Basis for fertility screening.* Male translocation heterozygotes may be completely sterile. This class consists of two types of translocations:

(A) Translocations between non-homologous chromosomes in which at least one of the breaks occurs close to one end of a chromosome.

(B) Those that carry multiple translocations. The majority of male translocation heterozygotes are semisterile—they carry one or (rarely) two translocations. The degree of semisterility is dependent upon the proportions of balanced and unbalanced

(duplication-deficiency) gametes produced in the ejaculate as a function of meiotic segregation. Balanced and unbalanced sperm are equally capable of fertilizing an egg. Balanced sperm lead to viable progeny. Unbalanced sperm result in early embryonic lethality.

(ii) *Basis for cytological screening.* The great majority of male translocation heterozygotes can be identified cytologically through analysis of diakinesis metaphase I spermatocytes. Translocation heterozygotes are characterized by the presence of multivalent chromosome association such as a ring or chain of four chromosomes held together by chiasmata in paired homologous regions. Some translocation carriers can be identified by the presence of extra long and/or extra short chromosomes in spermatogonial and somatic cell metaphase preparations.

(2) *Description.* Essentially, two methods have been used to screen for translocation heterozygosity; one method uses a mating sequence to identify sterile and semisterile males followed by cytological examination of suspect male individuals; the other method deletes the mating sequence altogether and all F_1 male progeny are examined cytologically for presence of translocation. In the former approach, the mating sequence serves as a screen which eliminates most fully fertile animals for cytological confirmation as translocation heterozygotes.

(3) *Animal selection*—(i) *Species.* The mouse is the species generally used, and is recommended.

(ii) *Age.* Healthy sexually mature animals shall be used.

(iii) *Number.* (A) The number of male animals necessary is determined by the following factors:

(*1*) The use of either historical or concurrent controls.

(*2*) The power of the test.

(*3*) The minimal rate of induction required.

(*4*) Whether positive controls are used.

(*5*) The level of significance desired.

(B) [Reserved]

(iv) *Assignment to groups.* Animals shall be randomized and assigned to treatment and control groups.

(4) *Control groups*—(i) *Concurrent controls.* No concurrent positive or negative (vehicle) controls are recommended as routine parts of the heritable translocation assay. However, investigators not experienced in performing translocation testing shall include a substance known to produce translocations in the assay as a positive control reference chemical.

(ii) *Historical controls.* At the present time, historical control data must be used in tests for significance. When statistically reliable historical controls are not available, negative (vehicle) controls shall be used.

(5) *Test chemicals*—(i) *Vehicle.* When appropriate for the route of administration, solid and liquid test substances should be dissolved or suspended in distilled water or isotonic saline. Water-insoluble chemicals may be dissolved or suspended in appropriate vehicles. The vehicle used shall neither interfere with the test chemical nor produce toxic effects. Fresh preparations of the test chemical should be employed.

(ii) *Dose levels.* At least two dose levels shall be used. The highest dose level shall result in toxic effects (which shall not produce an incidence of fatalities which would prevent a meaningful evaluation) or shall be the highest dose attainable or 5g/kg body weight.

(iii) *Route of administration.* Acceptable routes of administration include oral, inhalation, admixture with food or water, and IP or IV injection.

(e) *Test performance*—(1) *Treatment and mating.* The animals shall be dosed with the test substances 7 days per week over a period of 35 days. After treatment, each male shall be caged with 2 untreated females for a period of 1 week. At the end of 1 week, females shall be separated from males and caged individually. When females give birth, the day of birth, litter size, and sex of progeny shall be recorded. All male progeny should be weaned, and all female progeny should be discarded.

(2) *Testing for translocation heterozygosity.* When males are sexually mature, testing for translocation heterozygosity shall begin. One of two methods shall be used; the first method involves mating, determining those F_1 progeny which are sterile or

semisterile and subsequent cytological analysis of suspect progeny; the other method does not involve mating and determining sterility or semisterility; all progeny are examined cytologically.

(i) *Determination of sterility or semisterility*—(A) *Conventional method.* Females are mated, usually three females for each male, and each female is killed at midpregnancy. Living and dead implantations are counted. Criteria for determining normal and semisterile males are usually established for each new strain because the number of dead implantations varies considerably among strains.

(B) *Sequential method.* Males to be tested are caged individually with females and the majority of the presumably normal males are identified on the basis of a predetermined size of 1 or 2 litters. Breeding pens are examined daily on weekdays beginning 18 days after pairing. Young are discarded immediately after they are scored. Males that sire a litter whose size is the same as or greater than the minimum set for a translocation-free condition are discarded with their litter. If the litter size is smaller than the predetermined number, a second litter is produced with the same rule applying. Males that cannot be classified as normal after production of a second litter are tested further by the conventional method or by cytological confirmation of translocation.

(ii) *Cytological analysis.* For cytological analysis of suspected semisteriles, the air-drying technique is used. Observation of at least 2 diakinesis-metaphase 1 cells with mutivalent association constitutes the required evidence for the presence of a translocation. Sterile males are examined by one of two methods, those with testes of normal size and sperm in the epididymis are examined by the same techniques used for semisteriles. Animals with small testes are examined by squash preparations or, alternatively, by examination of mitotic metaphase preparations. If squash preparations do not yield diakinesis-metaphase 1 cells, analysis of spermatogonia or bone marrow for the presence of unusually long or short chromosomes should be performed.

(f) *Data and report*—(1) *Treatment of results.* (i) Data shall be presented in tabular form and shall include the number of animals at risk, the germ cell stage treated, the number of partial steriles and semisteriles (if the fertility test is used), the number of cytogenetically confirmed translocation heterozygotes (if the fertility test is used, report the number of confirmed steriles and confirmed partial steriles), the translocation rate, and either the standard error of the rate or the upper 95 percent confidence limit on the rate.

(ii) These data shall be presented for both treated and control groups. Historical or concurrent controls shall be specified, as well as the randomization procedure used for concurrent controls.

(2) *Statistical evaluation.* Data shall be evaluated by appropriate statistical methods.

(3) *Interpretation of results.* (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of heritable translocations. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of heritable translocations or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) *Test evaluation.* (i) Positive results in the heritable translocation assay indicate that under the test conditions the test substance causes heritable chromosomal damage in the test species.

(ii) Negative results indicate that under the test conditions the test substance does not cause heritable chromosomal damage in the test species.

(5) *Test report.* In addition to the reporting recommendations as specified under 40 CFR part 792, subpart J, the following specific information shall be reported:

(i) Species, strain, age, weight and number of animals of each sex in each group.

(ii) Test chemical vehicle, route and schedule of administration, toxicity data.

(iii) Dosing regimen, doses tested and rationale for dosage selection.

(iv) Mating schedule, number of females mated to each male.

(v) The use of historical or concurrent controls.

(vi) Screening procedure including the decision criteria used and the method by which they were determined.

(vii) Dose-response relationship, if applicable.

(g) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Generoso, W.M., Bishop, J.B., Goslee, D.G., Newell, G.W., Sheu, G-J, von Halle, E. ''Heritable translocation test in mice,'' *Mutation Research*, 76:191–215 (1980).

(2) [Reserved]

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19081, May 20, 1987]

§ 798.5500 Differential growth inhibition of repair proficient and repair deficient bacteria: "Bacterial DNA damage or repair tests."

(a) *Purpose.* Bacterial DNA damage or repair tests measure DNA damage which is expressed as differential cell killing or growth inhibition of repair deficient bacteria in a set of repair proficient and deficient strains. These tests do not measure mutagenic events *per se*. They are used as an indication of the interaction of a chemical with genetic material implying the potential for genotoxicity.

(b) *Definition.* Test for differential growth inhibition of repair proficient and repair deficient bacteria measure differences in chemically induced cell killing between wild-type strains with full repair capacity and mutant strains deficient in one or more of the enzymes which govern repair of damaged DNA.

(c) *Reference substances.* These may include, but need not be limited to, chloramphenicol or methyl methanesulfonate.

(d) *Test method*—(1) *Principle.* The tests detect agents that interact with cellular DNA to produce growth inhibition or killing. This interaction is recognized by specific cellular repair systems. The assays are based upon the use of paired bacterial strains that differ by the presence of absence of specific DNA repair genes. The response is expressed in the preferential inhibition of growth or the preferential killing of the DNA repair deficient strain since it is incapable of removing certain chemical lesions from its DNA.

(2) *Description.* Several methods for performing the test have been described. Those described here are:

(i) Tests performed on solid medium (diffusion tests).

(ii) Tests performed in liquid culture (suspension tests).

(3) *Strain selection*—(i) *Designation.* At the present time, *Escherichia coli polA* (W3110/p3478) or *Bacillus subtilis rec* (H17/M45) pairs are recommended. Other pairs may be utilized when appropriate.

(ii) *Preparation and storage.* Stock culture preparation and storage, growth requirements, method of strain identification and demonstration of appropriate phenotypic requirements should be performed using good microbiological techniques and should be documented.

(4) *Bacterial growth.* Good microbiological techniques should be used to grow fresh cultures of bacteria. The phase of growth and cell density should be documented and should be adequate for the experimental design.

(5) *Metabolic activation.* Bacteria should 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 postmitochondrial fraction prepared from the livers of rodents treated with enzyme inducing agents. The use of other species, tissues or techniques may also be appropriate.

(6) *Control groups*—(i) *Concurrent controls.* Concurrent positive, negative, and vehicle controls should be included in each assay.

(ii) *Negative controls.* The negative control should show nonpreferential growth inhibition (i.e., should affect

both strains equally). Chloramphenicol is an example of a negative control.

(iii) *Genotype specific controls.* Examples of genotype specific positive controls are methyl methanesulfonate for *polA* strains and mitomycin C for *rec* strains.

(iv) *Positive controls to ensure the efficacy of the activation system.* The positive control reference substance for tests including a metabolic activation system should be selected on the basis of the type of activation system used in the test.

(v) *Other positive controls.* Other positive control reference substances may be used.

(7) *Test chemicals*—(i) *Vehicle.* Test chemicals and positive and negative control reference substances should be dissolved in an appropriate vehicle and then further diluted in vehicle for use in the assay.

(ii) *Exposure concentrations.* The test should initially be performed over a broad range of concentrations. Among the criteria to be taken into consideration for determining the upper limits of test chemical concentration are cytotoxicity and solubility. Cytotoxicity of the test chemical may be altered in the presence of metabolic activation systems. For freely soluble nontoxic chemicals, the upper test chemical concentration should be determined on a case by case basis. Because results are expressed as diameters of zones of growth inhibition in the diffusion test, it is most important that the amounts of chemical on the disc (or in the wells) are exact replicates. When appropriate, a positive response should be confirmed by testing over a narrow range of concentrations.

(e) *Test performance*—(1) *Diffusion assay*—(i) *Disc diffusion assays.* Disc diffusion assays. may be performed in two ways:

(A) A single strain of bacteria may be added to an agar overlay or spread on the surface of the agar and the test chemical placed on a filter disc on the surface of the agar or;

(B) DNA repair proficient and DNA repair deficient bacteria may be streaked in a line on the surface of the agar of the same plate and a disc saturated with test chemical placed on the surface of the agar in contact with the streaks.

(ii) *Well diffusion assays.* In well diffusion assays, bacteria may be either added to the agar overlay or spread onto the surface of the agar. A solution of the test chemical is then placed into a well in the agar.

(2) *Suspension assays.* (i) A bacterial suspension may be exposed to the test chemical and the number of surviving bacteria determined (as colony-forming units) either as a function of time of treatment or as a function of the concentration of test agent.

(ii) Nonturbid suspensions of bacteria may be exposed to serial dilutions of the test agent and a minimal inhibitory concentration for each strain determined, as evidenced by the presence or absence of visible growth after a period of incubation.

(iii) Paired bacterial suspensions (usually with some initial turbidity) may be treated with a single dose of the chemical. Positive results are indicated by a differential inhibition in the rate of increase of turbidity of the paired cultures.

(3) *Number of cultures.* When using a plate diffusion procedure, at least two independent plates should be used at each dilution. In liquid suspension assays, at least two independent specimens for determination of the number of viable cells should be plated.

(4) *Incubation conditions.* All plates in a given test should be incubated for the same time period. This incubation period should be for 18 to 24 hrs at 37 °C.

(f) *Data and report*—(1) *Treatment of results*—(i) *Diffusion assays.* Results should be expressed in diameters of zones of growth inhibition in millimeters or as areas derived therefrom as mm^2. Dose-response data, if available, should be presented using the same units.

(ii) *Liquid suspension assays.* (A) Survival data can be presented as dose responses, preferably as percentage of survivors or fractional survival of each strain or as a relative survival (ratio) of the two strains.

(B) Results can also be expressed as the concentrations required to effect a predetermined survival rate (e.g., D_{37}, the dose permitting 37 percent survival). These data are derived from the

survival curve. The concentration should be expressed as weight per volume, as moles, or as molarity.

(C) Similarly, results can be expressed as minimal inhibitory concentration or as minimal lethal dose. The former is determined by the absence of visible growth in liquid medium and the latter is determined by plating dilutions onto semisolid media.

(iii) In all tests, concentrations must be given as the final concentrations during the treatment. Raw data, prior to transformation, should be provided. These should include actual quantities measured, e.g., neat numbers. For measurement of diffusion, the diameters of the discs and/or well should be indicated and the measurements should indicate whether the diameter of the discs and/or well was subtracted. Moreover, mention should be made as to whether the test chemical gave a sharp, diffuse, or double-zone of growth inhibition. If it is the latter, the investigator should indicate whether the inner or the outer zone was measured.

(iv) Viability data should be given as the actual plate counts with an indication of the dilution used and the volume plated or as derived titers (cells per ml). Transformed data alone in the absence of experimental data are not acceptable (i.e, ratios, differences, survival fraction).

(2) *Statistical evaluation.* Data should be evaluated by appropriate statistical methods.

(3) *Interpretation of results.* (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related preferential inhibition or killing of the repair deficient strain. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points.

(ii) A test substance which does not produce either a statistically significant dose-related preferential inhibition or killing of the repair deficient strain or a statistically significant and reproducible positive response at any one of the test points is considered not to interact with the genetic material of the organisms used in assay.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) *Test evaluation.* DNA damage tests in bacteria do not measure DNA repair *per se* nor do they measure mutations. They measure DNA damage which is expressed as cell killing or growth inhibition. A positive result in a DNA damage test in the absence of a positive result in another system is difficult to evaluate in the absence of a better data base.

(5) *Test report.* In addition to the reporting recommendations as specified under 40 CFR part 792, subpart J the following specific information should be reported:

(i) Bacterial strains used.

(ii) Phase of bacterial cell growth at time of use in the assay.

(iii) Media composition.

(iv) Details of both the protocol used to prepare the metabolic activation system and its use in the assay.

(v) Treatment protocol, including doses used and rationale for dose selection, positive and negative controls.

(vi) Method used for determination of degree of cell kill.

(vii) Dose-response relationship, if applicable.

(g) *References.* For additional background information on this test guideline the following references should be consulted:

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

(2) Kada, T., Sadie, Y., Tutikawa, K. ''In vitro and host-mediated ''rec-assay'' procedures for screening chemical mutagens; and phloxine, a mutagenic red dye detected,'' *Mutation Research,* 16:165–174 (1972).

(3) Leifer, Z., Kada, T., Mandel, M., Zeiger, E., Stafford, R., Rosenkranz, H.S. ''An evaluation of bacterial DNA repair tests for predicting genotoxicity and carcinogenicity: A report of the U.S. EPA's Gene-Tox Program,'' *Mutation Research,* 87:211–297 (1981).

(4) Slater, E.E., Anderson, M.D., Rosenkranz, H.S. ''Rapid detection of mutagens and carcinogens.'' *Cancer Research,* 31:970–973 (1971).

§ 798.5955 Heritable translocation test in drosophila melanogaster.

(a) *Purpose.* The heritable translocation test in *Drosophila* measures the induction of chromosomal translocations in germ cells of insects. Stocks carrying genetic markers on two or more chromosomes are used to follow the assortment of chromosomes in meiosis. The F_1 male progeny of treated parents are individually mated to females and the F_2 progeny phenotypes are scored. The observed spectrum of phenotypes is used to determine the presence or absence of a translocation. This is usually indicated by a lack of independent assortment of genes on different chromosomes.

(b) *Definitions*—(1) Chromosome mutations are chromosomal changes resulting from breakage and reunion of chromosomes. Chromosomal mutations are also produced through nondisjunction of chromosomes during cell division.

(2) Reciprocal translocations are chromosomal translocations resulting from reciprocal exchanges between two or more chromosomes.

(3) Heritable translocations are reciprocal translocations transmitted from parent to the succeeding progeny.

(c) *Reference substances.* These may include, but need not be limited to, ethyl methanesulfonate or N-dimethylnitrosamine.

(d) *Test method*—(1) *Principle.* The method is based on the principle that balanced reciprocal chromosomal translocations can be induced by chemicals in the germ cells of treated flies and that these translocations are detected in the F_2 progeny using genetic markers (mutations). Different mutations may be used as genetic markers and two or more of the four chromosomes may be genetically marked for inclusion in this test.

(2) *Description.* Wild-type males are treated with chemical and bred with females of known genetic markers. The F_1 males are collected and individually bred with virgin females of the female parental stock. The resulting F_2 progeny are scored. Putative translocation carriers are confirmed with an F_3 cross.

(i) *Illustrative example.* The following example serves to illustrate the method. Males carrying genes for red eye color on chromosomes II and III are bred with females of white eye color carrying alleles for brown (bw) on the second chromosome and scarlet (st) and pink (pp) on the third chromosome. The F_1 male progeny are bred with virgin females of the female parental stock and the resulting F_2 progeny are examined for eye color phenotypes. If there is no translocation in the F_1 male, then the resulting F_2 progeny will have four eye color phenotypes: red, white, orange, and brown. If the F_1 male carries a translocation between chromosomes II and III, only red and white eye phenotypes are obtained in the F_2 generation. This happens because the F_1 translocation heterozygote produces two balanced (carrying either the parental or the translocated configuration of markers) and two unbalanced gametes. The unbalanced gametes (carrying one normal and one translocated chromosome) are unable to develop into normal individuals in the F_2 generation.

(ii) [Reserved]

(3) *Drosophila stocks.* Wild-type males and females of the genotype bw:st:pp (white eyes) may be used in the heritable translocation test. Other appropriately marked *Drosophila* stocks may also be used.

(4) *Control groups.* (i) Concurrent positive and negative (vehicle) controls should be included in each experiment.

(ii) Negative (vehicle) controls should be included. The size of the negative (vehicle) control group should be determined by the availability of appropriate laboratory historical control data.

(iii) If the historical control data are of sufficient numbers, concurrent controls may not be necessary.

(5) *Test chemicals*—(i) *Vehicle.* Test chemicals should be dissolved in water. Compounds which are insoluble in water may be dissolved or suspended in appropriate vehicles (e.g., a mixture of ethanol and Tween-60 or 80), and then diluted in water or saline prior to administration. Dimethylsulfoxide should be avoided as a vehicle.

(ii) *Dose levels.* For the initial assessment of mutagenicity, it may be sufficient to test a single dose of the test substance. This dose should be the maximum tolerated dose or that which

produces some indication of toxicity. If the test is being used to verify mutagenic activity, at least two additional exposure levels should be used.

(iii) *Route of administration.* Exposure may be oral, by injection or by exposure to gases or vapours. Feeding of the test compound may be done in sugar solution. When necessary, substances may be dissolved in 0.7 percent NaCl solution and injected into the thorax or abdomen.

(e) *Test performance*—(1) *P1 mating.* (i) In the primary screen of a chemical, it is enough to sample one germ cell stage, either mature sperm or spermatids (for indirect acting mutagens). Other stages may be sampled if needed, i.e., when mature germ cells give a positive result and data from earlier germ cells are needed for the purpose of risk assessment. Thus, the treated males may be mated only once for a period of 3 days to sample sperm or transferred every 2 to 3 days to cover the entire germ cell cycle.

(ii) Mass matings may be performed because the control rate for translocations in the available literature is very low (near 0) and clustered events are extremely rare. Mated females may be aged for 2 weeks in order to recover an enhanced incidence of translocation due to the storage effect. The females are then allowed to lay eggs and F_1 males are collected for test mating.

(2) *F_1 mating.* F_1 males should be bred with virgin females of the parental female stock. Since each F_1 male represents one treated gamete of the male parent, the F_1 males have to be mated individually to virgin females. Each F_1 male should be mated to three females to ensure sufficient progeny.

(3) *Scoring the F_2 generation.* F_2 cultures (each representing 1 F_1 male tested) should be scored for the presence or absence of phenotype variations (linkage of markers) from the expected types. The test should be designed with a predetermined sensitivity and power. The number of flies in each group should reflect these defined parameters. The spontaneous mutant frequency observed in the appropriate control group will strongly influence the number of treated chromosomes that must be analyzed to detect substances which show mutation rates close to those of the controls. A positive test should be confirmed by F_3 mating trials.

(4) *Number of replicate experiments.* Replicate experiments are usually performed for each dose of the compound tested. If a chemical is a potent inducer of translocations, one experiment may be sufficient. Otherwise two or three replicate experiments should be done.

(f) *Data and report*—(1) *Treatment of results.* Data should be tabulated to show the number of translocations and the number of fertile F_1 males at each exposure for each germ cell stage sampled.

(2) *Statistical evaluation.* Data should be evaluated by appropriate statistical methods.

(3) *Interpretation of results.* (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of heritable translocations. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of heritable translocations or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) *Test evaluation.* (i) Positive results in the heritable translocation test in *Drosophila* indicate that under the test conditions the test substance causes chromosome damage in germ cells of this insect.

(ii) Negative results indicate that under the test conditions the test substance does not cause chromosomal damage in *D. melanogaster*.

(5) *Test report.* In addition to the reporting recommendations as specified under 40 CFR part 792, subpart J, the following specific information should be reported:

(i) *Drosophila* stock used in the assay, age of insects, number of males treated, number of F_2 cultures established, number of replicate experiments.

(ii) Test chemical vehicle, treatment and mating schedule, exposure levels, toxicity data, dose and route of exposure.

(iii) Positive and negative (vehicle) controls.

(iv) Historical control data, if available.

(v) Number of chromosomes scored.

(vi) Criteria for scoring mutant chromosomes.

(vii) Dose-response relationship, if applicable.

(g) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Wurgler, F.E., Sobels, F.H., Vogel, E. ''*Drosophila* as assay system for detecting genetic changes,'' *Handbook of mutagenicity test procedures*. Eds. Kilby, B.J., Legator, M., Nichols, W., Ramel, C. (Amsterdam: Elsevier/North Holland Biomedical Press, 1979) pp. 335–374.

(2) [Reserved]

Subpart G—Neurotoxicity

§ 798.6050 Functional observational battery.

(a) *Purpose.* In the assessment and evaluation of the potential human health effects of substances, it may be necessary to test for neurotoxic effects. Substances that have been observed to cause neurotoxic signs (e.g., convulsions, tremors, ataxia) in other toxicity tests, as well as those having a structural similarity to known neurotoxicants, should be evaluated for neurotoxicity. The functional observational battery is a noninvasive procedure designed to detect gross functional deficits in young adults resulting from exposure to chemicals and to better quantify neurotoxic effects detected in other studies. This battery of tests is not intended to provide a detailed evaluation of neurotoxicity. It is designed to be used in conjunction with neuropathologic evaluation and/or general toxicity testing. Additional functional tests may be necessary to assess completely the neurotoxic potential of a chemical.

(b) *Definitions.* (1) Neurotoxicity is any adverse effect on the structure or function of the central and/or peripheral nervous system related to exposure to a chemical substance.

(2) A toxic effect is an adverse change in the structure or function of an experimental animal as a result of exposure to a chemical substance.

(c) *Principle of the test method.* The material is administered by an appropriate route to laboratory rodents. The animals are observed under carefully standardized conditions with sufficient frequency to ensure the detection of behavioral and/or neurologic abnormalities, if present. Various functions that could be affected by neurotoxicants are assessed during each observation period.

(d) *Test procedures*—(1) *Animal selection*—(i) *Species and strain.* The laboratory rat or mouse is recommended. Although information will generally be lacking, whenever possible the choice of species should take into consideration such factors as the comparative metabolism of the chemical and species sensitivity to the toxic effects of the test substance, as evidenced by the results of other studies. The potential for combined studies should also be considered. Standard strains should be used.

(ii) *Age.* Young adult animals (at least 42 days old for the rat or mouse) shall be used.

(iii) *Sex.* (A) Equal numbers of animals of each sex are required for each dose level.

(B) The females shall be nulliparous and nonpregnant.

(2) *Number of animals.* At least eight animals of each sex should be used at each dose level and should be designated for behavioral testing. If interim sacrifices are planned, the number should be increased by the number of animals scheduled to be sacrificed before the end of the study. Animals shall be randomly assigned to treatment and control groups.

(3) *Control groups.* (i) A concurrent (''sham'' exposure or 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.

(ii) Concurrent or historic data from the laboratory performing the testing

shall provide evidence of the ability of the procedures used to detect major neurotoxic endpoints such as limb weakness or paralysis (e.g., acrylamide), CNS stimulation (e.g., β, β′-iminodiproprionitrile) autonomatic signs (e.g., physostigmine).

(iii) A satellite group may be treated with the high dose level for the duration of exposure and observed for reversibility, persistence, or delayed occurrence of toxic effects for a post-treatment period of appropriate duration, normally not less than 28 days.

(4) *Dose levels and dose selection.* At least 3 doses, equally spaced on a log scale (e.g., ½ log units) over a range of at least 1 log unit shall be used in addition to a zero dose or vehicle administration. The data should be sufficient to produce a dose-effect curve.

(i) The highest dose shall produce (A) clear behavioral effects or (B) life-threatening toxicity.

(ii) The data from the lower doses must show either (A) graded dose-dependent effects at 2 dose levels or (B) no effects at 2 dose levels, respectively.

(5) *Duration and frequency of exposure.* The duration and frequency of exposure will be specified in the test rule.

(6) *Route of exposure.* The test substance shall be administered by the route specified in the test rule. This route will usually be the one most closely approximating the expected route of human exposure. The exposure potocol shall conform to that outlined in the appropriate acute or subchronic toxicity study guideline under subpart B or subpart C of this part.

(7) *Combined protocol.* Subjects used for other toxicity studies may be used if none of the requirements of either study are violated by the combination.

(8) *Study conduct.* (i) All animals in a given study should be observed carefully by trained technicians who are blind with respect to the animals' treatments. Standard procedures to minimize observer variability shall be followed. 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 inter-observer reliability is required. All animals should be observed prior to initiation of exposure. Subsequent observations should be made with sufficent frequency to ensure the detection of behavioral and/or neurologic abnormalities, if present. At minimum, observations at 1 hour, 6 hours, 24 hours, 7 days, and 14 days and monthly thereafter are recommended. In a subchronic study, subsequent to the first exposure all observations should be made before the daily exposure. The animals should 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 function should be used. The development of objective quantitative measures of the observational endpoints specified is encouraged.

(ii) The following is a minimal list of observations that shall be noted:

(A) Any unusual responses with respect to body position, activity level, coordination of movement, and gait.

(B) Any unusual or bizarre behavior including, but not limited to, headflicking, head searching, compulsive biting or licking, self-mutilation, circling, and walking backwards.

(C) The presence of:

(*1*) Convulsions.

(*2*) Tremors.

(*3*) Increased levels of lacrimation and/or red-colored tears.

(*4*) Increased levels of salivation.

(*5*) Piloerection.

(*6*) Pupillary dilation or constriction.

(*7*) Unusual respiration (shallow, labored, dyspneic, gasping, and retching) and/or mouth breathing.

(*8*) Diarrhea.

(*9*) Excessive or diminished urination.

(*10*) Vocalization.

(D) Forelimb/hindlimb grip strength. The procedure described by Meyer et al. (1979), under paragraph (f)(9) of this section is recommended.

(E) Sensory function. A simple assessment of sensory function (vision, audition, pain perception) shall be

made. Marshall et al. (1971) under paragraph (f)(8) of this section have described a neurologic exam for this purpose; these procedures are also discussed by Deuel (1977), under paragraph (f)(4) of this section. Irwin (1968) under paragraph (f)(7) of this section described a number of reflex tests intended to detect gross sensory deficits, including the visual placing response, Preyer reflex, and tail pinch. Many procedures have been developed for assessing pain perception (e.g., Ankier, 1974 under paragraph (f)(1) of this section; D'Amour and Smith 1941 under paragraph (f)(3) of this section; Evans 1971 under paragraph (f)(6) of this section).

(e) *Data reporting and evaluation.* In addition to the reporting requirements specified under 40 CFR part 792 subpart J the final test report must include the following information.

(1) *Description of system and test methods.* (i) A detailed description of the procedures used to standardize observation, including the arena and operational definitions for scoring observations.

(ii) Positive control data from the laboratory performing the test that demonstrate the sensitivity of the procedures being used. Historic data may be used if all aspects of the experimental protocol are the same, including personnel.

(2) *Results.* The following information must be arranged by test group dose level.

(i) In tabular form, data for each animal must 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).

(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 percentage of animals showing each abnormal sign at each observation time.

(D) The mean and standard deviation for each continuous endpoint at each observation time.

(3) *Evaluation of data.* The findings of a functional observational battery should be evaluated in the context of preceding and/or concurrent toxicity studies and any correlative 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 should include appropriate statistical analyses. Choice of analyses should consider tests appropriate to the experimental design and needed adjustments for multiple comparisons.

(f) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Ankier, S.I. ''New hot plate tests to quantify antinociceptic and narcotic antagonist activities,'' *European Journal of Pharmacology*, 27: 1–4 (1974).

(2) Coughenour, L.L., McLean, J.R. and Parker, R.B. ''A new device for the rapid measurement of impaired motor function in mice,'' *Pharmacology, Biochemistry and Behavior, 6:* 351–353 (1977).

(3) D'Amour, F.E., Smith, D.L. ''A method for determining loss of pain sensation,'' *Journal of Pharmacology and Experimental Therapeutics, 72:* 74–79 (1941).

(4) Deuel, R.K. ''Determining sensory deficits in animals,'' *Methods in Psychobiology* Ed. Myers R.D. (New York: Academic Press, 1977) pp. 99–125.

(5) Edwards, P.M., Parker, V.H. ''A simple, sensitive and objective method for early assessment of acrylamide neuropathy in rats,'' *Toxicology and Applied Pharmacology, 40:* 589–591 (1977).

(6) Evans, W.O. ''A new technique for the investigation of some analgesic drugs on reflexive behavior in the rat,'' *Psychopharmacologia, 2:* 318–325 (1961).

(7) Irwin, S. ''Comprehensive observational assessment: Ia. A systematic quantitative procedure for assessing the behavioral and physiologic state of the mouse,'' *Psychopharmacologia, 13:* 222–257 (1968).

(8) Marshall, J.F., Turner, B.H., Teitlbaum, P. ''Sensory neglect produced by lateral hypothalamic damage,'' *Science, 174:* 523–525 (1971).

(9) Meyer, O.A., Tilson, H.A., Byrd, W.C., Riley, M.T. ''A method for the

routine assessment of fore- and hindlimb grip strength of rats and mice,'' *Neurobehavioral Toxicology, 1:* 233–236 (1979).

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19082, May 20, 1987]

§ 798.6200 Motor activity.

(a) *Purpose*—(1) *General.* In the assessment and evaluation of the toxic characteristics of a substance, determination of the effects of administration of the substance on motor activity is useful when neurotoxicity is suspected.

(2) *Acute Motor Activity Test.* The purpose of the acute motor activity test is to examine changes in motor activity occurring over a range of acute exposure levels. These changes may then be evaluated in the context of changes occurring in other organ systems. This test is an initial step in determining the potential of a substance to produce acute neurotoxicity and may be used to screen members of a class of substances for known neurotoxicity, and/or to establish a dosage regimen prior to the initiation of subchronic neurotoxicity testing.

(3) *Subchronic Motor Activity Test.* The purpose of the subchronic motor activity test is to determine whether the repeated administration of a suspected neurotoxicant results in changes in motor activity. These changes may be evaluated in the context of changes occurring in other organ systems. This test is an initial step in determining the potential of a substance to produce subchronic neurotoxicity.

(b) *Definitions.* (1) Neurotoxicity is the adverse effect on the structure or function of the central and/or peripheral nervous system related to exposure to a chemical substance.

(2) Motor activity is any movement of the experimental animal.

(3) A toxic effect is an adverse change in the structure or function of an experimental animal as a result of exposure to a chemical substance.

(c) *Principle of the test method.* The test substance is administered to several groups of experimental animals, one dose being used per group. Measurements of motor activity are made. The exposure levels at which significant changes in motor activity are produced are compared to those levels which produce toxic effects not originating in the central and/or peripheral nervous system.

(d) *Test procedures*—(1) *Animal selection*—(i) *Species and strain.* Testing shall be performed in a laboratory rat or mouse. The choice of species should take into consideration such factors as the comparative metabolism of the chemical and species sensitivity to the toxic effects of the test substance, as evidenced by the results of other studies, the potential for combined studies, and the availability of other toxicity data for the species.

(ii) *Age.* Young adult animals (at least 42 days old for rat or mouse) should be used.

(iii) *Sex.* (A) Equal numbers of animals of each sex are required for each dose level for the motor activity test.

(B) The females shall be nulliparous and nonpregnant.

(2) *Number of animals.* Animals shall be randomly assigned to test and control groups. Each test or control group must be designed to contain a sufficient number of animals at the completion of the study to detect a 40 percent change in activity of the test groups relative to the control group with 90 percent power at the 5 percent level. For most designs, calculations can be made according to Dixon and Massey (1957) under paragraph (f)(1) of this section, Neter and Wasserman (1974) under paragraph (f)(5) of this section, Sokal and Rohlf (1969) under paragraph (f)(9) of this section, or Jensen (1972) under paragraph (f)(3) of this section.

(3) *Control groups.* (i) A concurrent control group is required. This group must be an untreated group, 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 or cannot be made available, both untreated and vehicle control group are required.

(ii) Positive control data are required to demonstrate the sensitivity and reliability of the activity measuring device and testing procedure. These data should demonstrate the ability to detect increases or decreases in activity and to generate a dose-effect curve or its equivalent using three values of the dose or equivalent independent variable. A single administration of the

dose (or equivalent) is sufficient. It is recommended that chemical exposure be used to collect positive control data. 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.

(iii) A satellite group may be treated with the high dose 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.

(4) *Dose levels and dose selection.* At least 3 doses, equally spaced on a log scale (e.g., ½ log units) over a range of at least 1 log unit shall be used in addition to a zero dose or vehicle administration. The data should be sufficient to produce a dose-effect curve.

(i) The highest dose shall produce (A) clear effects on motor activity or (B) life-threatening toxicity.

(ii) The data from the lower doses must show either (A) graded dose-dependent effects at 2 dose levels or (B) no effects at 2 dose levels, respectively.

(5) *Duration of testing.* The duration of exposure will be specified in the test rule.

(6) *Route of administration.* The test substance shall be administered by the method specified in the test rule. This will usually be the route most closely approximating the route of human exposure. The exposure protocol shall conform to that outlined in the appropriate acute or subchronic toxicity study guideline.

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

(8) *Study conduct*—(i) *General.* Motor activity must 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 decreases nor so high as to preclude increases. Each device shall be tested by standard procedure 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 percent of the session for most treatments and animals. All sessions should have the same duration. Treatment groups shall be counter-balanced 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 home cage or novel test cage and environmental distractions. Tests shall be executed by an appropriately trained individual.

(ii) *Acute.* Testing shall be timed to include the time of peak signs.

(iii) *Subchronic.* All animals shall be tested prior to initiation of exposure and at 30 ±2, 60 ±2 and 90 ±2 days during the exposure period. Testing shall occur prior to the daily exposure. Animals shall be weighed on each test day and at least once weekly during the exposure period.

(e) *Data reporting and evaluation.* In addition to the reporting requirements specified under 40 CFR part 792, subpart J the final test report must include the following information:

(1) *Description of system and test methods.* (i) Positive control data from the laboratory performing the test which demonstrate the sensitivity of the procedure being used.

(ii) Procedures for calibrating and assuring the equivalence of devices and balancing treatment groups.

(2) *Results.* The following information must be arranged by test group (dose level).

(i) In tabular form, data must be provided showing for each animal:

(A) Its identification number.

(B) Body weight, total session activity counts, and intrasession subtotals for each date measured.

(ii) Group summary data should also be reported.

(3) *Evaluation of data.* An evaluation of the test results (including statistical analysis comparing total activity

counts at the end of exposure of treatment vs control animals must be made and supplied. This submission must include dose-effect curves for motor activity expressed as activity counts.

(f) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Dixon, W.J., Massey, E.J. *Introduction to Statistical Analysis* 2nd Ed. (New York: McGraw-Hill, 1957).

(2) Finger, F.W. ''Measuring behavioral activity,'' *Methods in Psychobiology* Vol. 2. Ed. R.D. Myers (New York: Academic, 1972) pp. 1–19.

(3) Jensen, D.R. ''Some simultaneous multivariate procedures using Hotelling's T^2 Statistics,'' *Biometrics*, 28:39–53 (1972).

(4) Kinnard, E.J. and Watzman, N. ''Techniques utilized in the evaluation of psychotropic drugs on animals activity,'' Journal of Pharmaceutical Sciences, 55:995–1012 (1966).

(5) Neter, J. and Wasserman, W. *Applied Linear Statistical Models.* Homewood, Richard D. Irwin, Inc., 1974.

(6) Reiter, L.E. ''Use of activity measures in behavioral toxicology,'' *Environmental Health Perspectives*, 26:9–20 (1978).

(7) Reiter, L.W. and MacPhail, R.C. ''Motor Activity: A survey of methods with potential use in toxicity testing,'' *Neurobehavioral Toxicology*, 1: Suppl. 1, 53–66 (1979).

(8) Robbins, T.W. ''A critique of the methods available for the measurement of spontaneous motor activity,'' *Handbook of Psychopharmacology*. Vol. 7. Eds. Iversen, L.L., Iversen, D.S., Snyder, S.H. (New York: Plenum, 1977) pp. 37–82.

(9) Sokal, R.P. and Rohlf, E.J. *Biometry*. (San Francisco: W.H. Freeman and Co., 1969).

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19082, May 20, 1987]

§ 798.6400 Neuropathology.

(a) *Purpose.* The techniques in this guideline are designed to develop data on morphologic changes in the nervous system for chemical substances and mixtures subject to such testing under the Toxic Substances Control Act. The data will detect and characterize morphologic changes, if and when they occur, and determine a no-effect level for such changes. Neuropathological evaluation should be complemented by other neurotoxicity studies, e.g. behavioral and neurophysiological studies. Neuropathological evaluation may be done following acute, subchronic or chronic exposure.

(b) *Definition.* Neurotoxicity or a neurotoxic effect is an adverse change in the structure or function of the nervous system following exposure to a chemical agent.

(c) *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 sacrificed and tissues in the nervous system are examined grossly and prepared for microscopic examination. Starting with the highest dosage level, tissues are examined under the light microscope for morphologic changes, until a no effect level is determined. In cases where light microscopy has revealed neuropathology, the no effect level may be confirmed by electron microscopy.

(d) *Test procedure*—(1) *Animal selection*—(i) *Species and strain.* Testing shall be performed in the species being used in other tests for neurotoxicity. This will generally be the laboratory rat. The choice of species shall take into consideration such factors as the comparative metabolism of the chemical and species sensitivity to the toxic effects of the test substance, as evidenced by the results of other studies, the potential for combined studies, and the availability of other toxicity data for the species.

(ii) *Age.* Animals shall be young adults (150–200 gm for rats) at the start of exposure.

(iii) *Sex.* Both sexes shall be used unless it is demonstrated that one sex is refractory to the effects.

(2) *Number of animals.* A minimum of six animals per group shall be used. The tissues from each animal shall be examined separately. It is recomse (iv)mended that ten animals per group be used.

(3) *Control groups.* (i) A concurrent control group(s) is (are) required. This group must be an untreated control group or, if a vehicle is used in administering the test substance, a vehicle

control group. If the vehicle used has a known or potential toxic property, both untreated and vehicle control groups are required.

(ii) A satellite group of animals may be treated with the high 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.

(4) *Dose levels and dose selection.* At least 3 doses, equally spaced on a log scale (e.g., ½ log units) over a range of at least 1 log unit shall be used in addition to a zero dose or vehicle administration. The data should be sufficient to produce a dose-effect curve.

(i) The highest dose shall produce (A) clear behavioral effects or (B) life-threatening toxicity.

(ii) The data from the lower doses must show either (A) graded dose-dependent effects at two dose levels or (B) no effects at two dose levels, respectively.

(5) *Duration of testing.* The exposure duration will be specified in the test rule. This will generally be 90 days exposure.

(6) *Route of administration.* The test substance shall be administered by a route specified in the test rule. This will generally be the route most closely approximating the route of human exposure. The exposure protocol shall conform to that outlined in the appropriate acute or subchronic toxicity guideline.

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

(8) *Study conduct*—(i) *Observation of animals.* All toxicological (e.g., weight loss) and neurological signs (e.g., motor disturbance) shall be recorded frequently enough to observe any abnormality, and not less than weekly.

(ii) *Sacrifice of animals*—(A) *General.* The goal of the techniques outlined for sacrifice of animals and preparation of tissues is preservation of tissues morphology to simulate the living state of the cell.

(B) *Perfusion technique.* Animals shall be perfused *in situ* by a generally recognized technique. For fixation suitable for light or electronic microscopy, saline solution followed by buffered 2.5 percent glutaraldehyde or buffered 4.0 percent paraformaldehyde, is recommended. While some minor modifications or variations in procedures are used in different laboratories, a detailed and standard procedure for vascular perfusion may be found in the text by Zeman and Innes (1963) under paragraph (f)(7) of this section, Hayat (1970) under paragraph (f)(3) of this section, and by Spencer and Schaumburg (1980) under paragraph (f)(6) of this section. A more sophisticated technique is described by Palay and Chan-Palay (1974) under paragraph (f)(4) of this section.

(C) *Removal of brain and cord.* After perfusion, the bonystructure (cranium and vertebral column) shall be exposed. Animals shall then be stored in fixative-filled bags at 4 °C for 8-12 hours. The cranium and vertebral column shall be removed carefully by trained technicians without physical damage of the brain and cord. Detailed dissection procedures may be found in the text by Palay and Chan-Palay (1974) under paragraph (f)(4) of this section. After removal, simple measurement of the size (length and width) and weight of the whole brain (cerebrum, cerebellum, pons-medulla) shall be made. Any abnormal coloration or discoloration of the brain and cord shall also be noted and recorded.

(D) *Sampling.* Unless a given test rule specifies otherwise, cross-sections of the following areas shall be examined: The forebrain, the center of the cerebrum, the midbrain, the cerebellum and pons, and the medulla oblongata; the spinal cord at cervical and lumbar swelling (C_3–C_6 and L_1–L_4); Gasserian ganglia, dorsal root ganglia (C_3–C_6, L_1–L_4), dorsal and ventral root fibers (C_3–C_6, L^1–L_4), proximal sciatic nerve (midthigh and sciatic notch), sural nerve (at knee), and tibial nerve (at knee). Other sites and tissue elements (e.g., gastrocnemius muscle) should be examined if deemed necessary. Any observable gross changes shall be recorded.

(iii) *Specimen storage.* Tissue samples from both the central and peripheral nervous system shall be further immersion fixed and stored in appropriate fixative (e.g., 10 percent buffered formalin

for light microscopy; 2.5 percent buffered gluteraldehyde or 4.0 percent buffered paraformaldehyde for electron microscopy) for future examination. The volume of fixative versus the volume of tissues in a specimen jar shall be no less than 25:1. All stored tissues shall be washed with buffer for at least 2 hours prior to further tissue processing.

(iv) *Histopathology examination.* (A) *Fixation.* Tissue specimens stored in 10 percent buffered formalin may be used for this purpose. All tissues must be immersion fixed in fixative for at least 48 hours prior to further tissue processing.

(B) *Dehydration.* All tissue specimens shall be washed for at least 1 hour with water or buffer, prior to dehydration. (A longer washing time is needed if the specimens have been stored in fixative for a prolonged period of time.) Dehydration can be performed with increasing concentration of graded ethanols up to absolute alcohol.

(C) *Clearing and embedding.* After dehydration, tissue specimens shall be cleared with xylene and embedded in paraffin or paraplast. Multiple tissue specimens (e.g. brain, cord, ganglia) may be embedded together in one single block for sectioning. All tissue blocks shall be labelled showing at least the experiment number, animal number, and specimens embedded.

(D) *Sectioning.* Tissue sections, 5 to 6 microns in thickness, shall be prepared from the tissue blocks and mounted on standard glass slides. It is recommended that several additional sections be made from each block at this time for possible future needs for special stainings. All tissue blocks and slides shall be filed and stored in properly labeled files or boxes.

(E) *Histopathological techniques.* Although the information available for a given chemical substance may dictate test-rule specific changes, the following general testing sequence is proposed for gathering histopathological data:

(*1*) *General staining.* A general staining procedure shall be performed on all tissue specimens in the highest treatment group. Hematoxylin and eosin (H&E) shall be used for this purpose. The staining shall be differentiated properly to achieve bluish nuclei with pinkish background.

(*2*) *Special stains.* Based on the results of the general staining, selected sites and cellular components shall be further evaluated by the use of specific techniques. If H&E screening does not provide such information, a battery of stains shall be used to assess the following components in all appropriate required samples: neuronal body (e.g., Einarson's gallocyanin), axon (e.g., Bodian), myelin sheath (e.g., Kluver's Luxol Fast Blue) and neurofibrils (e.g., Bielchosky). In addition, peripheral nerve fiber teasing shall be used. Detailed staining methodology is available in standard histotechnological manuals such as AFIP (1968) under paragraph (f)(1) of this section, Ralis et al. (1973) under paragraph (f)(5) of this section, and Chang (1979) under paragraph (f)(2) of this section. The nerve fiber teasing technique is discussed in Spencer and Schaumberg (1980) under paragraph (f)(6) of this section. A section of normal tissue shall be included in each staining to assure that adequate staining has occurred. Any changes shall be noted and representative photographs shall be taken. If a lesion(s) is observed, the special techniques shall be repeated in the next lower treatment group until no further lesion is detectable.

(*3*) *Alternative technique.* If the anatomical locus of expected neuro-pathology is well-defined, epoxy-embedded sections stained with toluidine blue may be used for small sized tissue samples. This technique obviates the need for special stains for cellular components. Detailed methodology is available in Spencer and Schaumberg (1980) under paragraph (f)(6) of this section.

(*4*) *Electron microscopy.* Based on the results of light microscopic evaluation, specific tissue sites which reveal a lesion(s) shall be further evaluated by electron microscopy in the highest treatment group which does not reveal any light microscopic lesion. If a lesion is observed, the next lower treatment group shall be evaluated until no significant lesion is found. Detailed methodology is available in Hayat (1970) under paragraph (f)(3) of this section.

(F) *Examination—(1) General.* All stained microscopic slides shall be examined with a standard research microscope. Examples of cellular alterations (e.g., neuronal vacuolation, degeneration, and necrosis) and tissue changes (e.g., gliosis, leukocytic infiltration, and cystic formation) shall be recorded and photographed.

(*2*) *Electron microscopy.* Since the size of the tissue samples that can be examined is very small, at least 3 to 4 tissue blocks from each sampling site must be examined. Tissue sections must be examined with a transmission electron microscope. Three main categories of structural changes must be considered:

(*i*) *Neuronal body.* The shape and position of the nucleus and nucleolus as well as any change in the chromatin patterns shall be noted. Within the neuronal cytoplasm, cytoplasmic organelles such as mitochondria, lysosomes, neurotubules, neurofilaments, microfilaments, endoplasmic reticulum and polyribosomes (Nissl substance), Golgi complex, and secretory granules shall be examined.

(*ii*) *Neuronal processes.* The structural integrity or alterations of dendrites, axons (myelinated and unmyelinated), myelin sheaths, and synapses shall be noted.

(*iii*) *Supporting cells.* Attention must also be paid to the number and structural integrity of the neuroglial elements (oligodendrocytes, astrocytes, and microglia) of the central nervous system, and the Schwann cells, satellite cells, and capsule cells of the peripheral nervous system. Any changes in the endothelial cells and ependymal lining cells shall also be noted whenever possible. The nature, severity, and frequency of each type of lesion in each specimen must be recorded. Representative lesions must be photographed and labeled appropriately.

(e) *Data collection, reporting, and evaluation.* In addition to information meeting the requirements stated under 40 CFR part 792 subpart J, the following specific information shall be reported:

(1) *Description of test system and test methods.* A description of the general design of the experiment shall be provided. This shall include a short justification explaining any decisions where professional judgment is involved such as fixation technique and choice of stains.

(2) *Results.* All observations shall be recorded and arranged by test groups. This data may be presented in the following recommended format:

(i) *Description of signs and lesions for each animal.* For each animal, data must be submitted showing its identification (animal number, treatment, dose, duration), neurologic signs, location(s) nature of, frequency, and severity of lesion(s). A commonly-used scale such as 1 + , 2 + , 3 + , and 4 + for degree of severity ranging from very slight to extensive may be used. Any diagnoses derived from neurologic signs and lesions including naturally occurring diseases or conditions, should also be recorded.

(ii) *Counts and incidence of lesions, by test group.* Data shall be tabulated to show:

(A) The number of animals used in each group, the number of animals displaying specific neurologic signs, and the number of animals in which any lesion was found;

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

(iii) *Evaluation of data.* (A) An evaluation of the data based on gross necropsy findings and microscopic pathology observations shall be made and supplied. The evaluation shall include the relationship, if any, between the animal's exposure to the test substance and the frequency and severity of the lesions observed.

(B) The evaluation of dose-response, if existent, for various groups shall be given, and a description of statistical method must be presented. The evaluation of neuropathology data should include, where applicable, an assessment in conjunction with other neurotoxicity studies performed (eg. electrophysiological, behavioral, neurochemical).

(f) *References.* For additional background information on this test guideline the following references should be consulted:

(1) AFIP. *Manual of Histologic Staining Methods*. (New York: McGraw-Hill (1968).

(2) Chang, L.W. *A Color Atlas and Manual for Applied Histochemistry*. (Springfield, IL: Charles C. Thomas, 1979).

(3) Hayat, M.A. ''Vol. 1. Biological applications,'' *Principles and techniques of electron microscopy*. (New York: Van Nostrand Reinhold, 1970)

(4) Palay S.L., Chan-Palay, V. *Cerebellar Cortex: Cytology and Organization*. (New York: Springer-Verlag, 1974).

(5) Ralis, H.M., Beesley, R.A., Ralis, Z.A. *Techniques in Neurohistology*. (London: Butterworths, 1973).

(6) Spencer, P.S., Schaumburg, H.H. (eds). *Experimental and Clinical Neurotoxicology*. (Baltimore: Williams and Wilkins, 1980).

(7) Zeman, W., JRM Innes, J.R.M. *Craigie's Neuroanatomy of the Rat*. (New York: Academic, 1963).

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§ 798.6500 Schedule-controlled operant behavior.

(a) *Purpose*. (1) In the assessment and evaluation of the potential human health effects of substances, it may be necessary to test for functional neurotoxic effects. Substances that have been observed to produce neurotoxic signs in other toxicity studies (e.g. CNS depression or stimulation), as well as substances with a structural similarity to known neurotoxicants should be evaluated for these effects.

(2) This guideline defines procedures for conducting studies of schedule-controlled operant behavior, one way of evaluating functional neurotoxic effects (Dews, 1972 under paragraph (f)(1) of this section; NAS 1975, 1977, 1982 under paragraphs (f)(4), (5) and (6) of this section). Our purpose is to evaluate the effects of acute and repeated exposures on the rate and pattern of responding under schedules of reinforcement. Operant behavior tests may be used to evaluate many other aspects of behavior (Laties, 1978 under paragraph (f)(3) of this section). Additional tests may be necessary to completely assess the behavioral effects of any substance. Behavioral evaluation should be used in conjunction with neuropathologic evaluation and the evaluation of other toxic effects.

(b) *Definitions*—(1) *Neurotoxicity*. Neurotoxicity or a neurotoxic effect is an adverse change in the structure or function of the nervous system following exposure to a chemical agent. Behavioral toxicity is an adverse change in the functioning of the organism with respect to its environment following exposure to a chemical agent.

(2) *Operant, operant behavior, operant conditioning*. An operant is a class of behavioral responses which change or operates on the environment in the same way. Operant behavior is further distinguished as behavior which is modified by its consequences. Operant conditioning is the experimental procedure used to modify some class of behavior by reinforcement or punishment.

(3) *Schedule of reinforcement*. A schedule of reinforcement specifies the relation between behavioral responses and the delivery of reinforcers, such as food or water (Ferster and Skinner, 1957 under paragraph (f)(2) of this section). For example, a fixed ratio (FR) schedule requires a fixed number of responses to produce a reinforcer (e.g. FR 30). On a fixed interval (FI) schedule, the first response after a fixed period of time is reinforced (e.g. FI 5 minutes).

(c) *Principle of the test method*. Experimental animals are trained to perform under a schedule of reinforcement and measurements of their operant behavior are made. Several doses of the test substance are then administered according to the experimental design (between groups or within subjects) and the duration of exposure (acute or repeated). Measurements of the operant behavior are repeated. A descriptive and statistical evaluation of the data is made to evaluate the nature and extent of any changes in behavior in relation to exposures to the test substance. Comparisons are made between any exposures that influence the behavior and exposures that have neuropathological effects or effects on other targets of the chemical.

(d) *Test procedures*—(1) *Experimental design*. These test procedures may be used to evaluate the behavior of experimental animals receiving either acute

or repeated exposures. For acute exposure studies, either within-subject or between groups, experimental designs may be used. For repeated exposure studies, between groups designs should be used, but within subject comparisons (pre-exposure and post-exposure) are recommended and encouraged.

(2) *Animal selection*—(i) *Species.* (A) For most studies, the laboratory mouse or rat is recommended. Standard strains should be used.

(B) Under some circumstances other species may be recommended.

(ii) *Age.* Experimental animals should be young adults. Rats or mice should be at least 14 and 6 weeks old, respectively, prior to exposure.

(iii) *Sex.* (A) Approximately equal numbers of male and female animals are required for each dose level and control group.

(B) Virgin females should be used.

(iv) *Experimental history.* Animals should be experimentally and chemically naive.

(3) *Number of animals.* Six to twelve animals should be exposed to each level of the test substance and/or control procedure. If post exposure effects are examined, a separate group, 6 to 12 additional animals not sacrificed for pathology, will required in subchronic studies.

(4) *Control groups*—(i) *Untreated controls.* A concurrent ''sham'' exposure or vehicle control group or session (according to the design of the study) is required. The subjects should be treated similarly except that administration of the test substance is omitted.

(ii) *Positive controls.* Positive control data is required to demonstrate that the experimental procedures, under the specific conditions in the testing laboratory, are sensitive to substances known to affect operant behavior. Both increases and decreases in response rate should be demonstrated. Data based on acute exposures will be adequate. Data should be collected according to the same experimental design as that proposed for the test substance. Historical data on the procedure collected in the same species and under the same conditions in the testing laboratory may be acceptable, but the presentation of concurrent control data is strongly encouraged since it provides evidence that the test has remained sensitive.

(5) *Dose levels and dose selection.* At least 3 doses, equally spaced over a log scale (e.g., 10, 30, 100), over a range of at least 1 log unit shall be used in addition to a zero dose or vehicle administration. The data should be sufficient to produce a dose-effect curve.

(i) The highest dose shall produce: (A) Clear behavioral effects; or (B) life-threatening toxicity.

(ii) The data from the lower doses must show either: (A) Graded dose-dependent effects at 2 dose levels; or (B) no effects at 2 dose levels, respectively.

(6) *Duration of exposure.* The duration and frequency of exposure will be specified in the test rule.

(7) *Route of Administration.* The route of administration will also be specified in the test rule and will usually be identical to one of the anticipated or actual routes of human exposure. For some chemicals, another route (e.g. parenteral) may be justified. The exposure protocol should conform to that outlined in the appropriate acute or subchronic toxicity study guideline under subpart B or subpart C of this part.

(8) *Study conduct*—(i) *Apparatus.* Behavioral responses and the delivery of reinforcers shall be controlled and monitored by automated equipment located so that its operation does not provide unintended cues or otherwise interfere with the ongoing behavior. Individual chambers should be sound attenuated to prevent disruptions of behavior by external noise. The response manipulanda, feeders, and any stimulus devices should be tested before each session; these devices should periodically be calibrated.

(ii) *Chamber assignment.* Concurrent treatment groups should be balanced across chambers. Each subject should be tested in the chamber to which it is initially assigned.

(iii) *Deprivation and training.* (A) If a nonpreferred positive reinforcer is used, all subjects should be deprived of food until they reach a fixed percentage (e.g. 80 to 90 percent, commonly) of their ad libitum body weight or for a fixed period (e.g., 18 hours) prior to training. Deprivation should be kept constant throughout the study.

(B) Subjects must be trained until they display demonstrable stability in performance across days prior to exposure. One simple and useful criterion is a minimum number of sessions on the schedule and no systematic trend during the 5 days before exposure.

(C) Cumulative records of cumulative responding over time for each animal should be presented to demonstrate that the pattern of responding is representative of that generated by the schedule of reinforcement.

(iv) *Time, frequency, and duration of testing*—(A) *Time of testing.* All experimental animals should be tested at the same time of day and with respect to the time of exposure. For acute studies, testing should be performed when effects are estimated to peak, usually shortly after exposure. For subchronic studies, subjects should be tested prior to daily exposure in order to assess cumulative effects.

(B) *Frequency of testing.* The maintenance of stable operant behavior normally will require regular and frequent (e.g., 5 days a week) testing sessions. Animals should be weighed on each test day.

(C) *Duration of testing.* (*1*) Experimental sessions should be long enough to reasonably see the effects of exposure, but brief enough to be practical. Under most circumstances, a session length of 30–40 minutes should be adequate.

(*2*) If the nature or duration of effects following cessation of repeated exposure are a concern, animals from the high dose group should be tested following exposure for a suitable period of time.

(v) *Schedule selection.* The schedule of reinforcement chosen should generate response rates that may increase or decrease as a function of exposure. Many schedules of reinforcement can do this: a single schedule maintaining a moderate response rate; fixed-interval schedules, which engender a variety of response rates in each interval; or multiple schedules, where different components may maintain high and low response rates.

(e) *Data reporting and evaluation.* In addition to the reporting requirements specified under 40 CFR part 792, subpart J the final test report should contain the following information:

(1) *Description of system, test methods, experimental design, and control data.* (i) A description of the experimental chamber, programming equipment, data collection devices, and environmental conditions.

(ii) A description of the experimental design including counterbalancing procedures, and the stability criterion.

(iii) A description and statistical evaluation of positive control and other control data, including standard measures of central tendency, variability, coefficient of variation of response rates, and the slope of the dose-effect curve.

(2) *Results.* (i) Data for each animal should be arranged by test group in tabular form including the animal identification number, body weight, pre-exposure rate of responding, changes in response rate produced by the chemical, and group data for the same variables, including standard measures of central tendency, variability and coefficient of variation.

(ii) A description and statistical evaluation of the test results: With particular reference to the overall statistical procedures (e.g., parametric or nonparametric) dose-effect curve, and calculation of slope. Presentation of calculations is encouraged.

(f) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Dews, P.B. ''Assessing the Effects of Drugs,'' *Methods in Psychobiology*, Vol. 2, Ed., R.D. Myers (New York: Academic Press, 1972) 83–124.

(2) Ferster, C.B. Skinner, B.F. Schedules of Reinforcement. (New York: Appleton-Century-Crofts, 1957).

(3) Laties, V.G. ''How Operant Conditioning can Contribute to Behavioral Toxicology,'' Environmental Health Perspectives, 28: 29–35 (1978).

(4) National Academy of Science. *Principles for Evaluating Chemicals in the Environment.* (Washington, DC: National Academy of Sciences, 1975).

(5) National Academy of Science. *Principles and Procedures for Evaluating the Toxicity of Household Substances.* (Washington, DC: National Academy of Sciences, 1977).

(6) National Academy of Science. ''Strategies to determine needs and priorities for toxicity testing,'' Appendix 3B. *Reference Protocol Guidelines For Neurobehavioral Toxicity Tests*. 2: 123–129 (1982).

§ 798.6560 Subchronic delayed neurotoxicity of organophosphorus substances.

(a) *Purpose*. In the assessment and evaluation of the toxic characteristics of organophosphorus substances the determination of subchronic delayed neurotoxicity may be carried out, usually after initial information on delayed neurotoxicity has been obtained by acute testing or by the demonstration of inhibition and aging of neurotoxic esterase in hen neural tissue. The subchronic delayed neurotoxicity test provides information on possible health hazards likely to arise from repeated exposures over a limited period of time. It will provide information on dose response and can provide an estimate of a non-effect level which can be of use for establishing safety criteria for exposure.

(b) *Definitions*. Subchronic delayed neurotoxicity is a prolonged, delayed-onset locomoter ataxia resulting from repeated daily administration of the test substance.

(c) *Principle of the test method*. Multiple dose levels of the test substance are administered orally to domestic hens (*Gallus gallus domesticus*) for 90 days. The animals are observed at least daily for behavioral abnormalities, locomotor ataxia and paralysis. Histopathological examination of selected neural tissues is undertaken at the termination of the test period.

(d) *Test procedures*—(1) *Animal selection*. The adult domestic laying hen, aged 8 to 14 months, is recommended. Standard size breeds and strains should be employed.

(2) *Number of animals*. Ten hens should be used for each treatment and control group.

(3) *Control group*—(i) *General*. A concurrent control group should be used. This group should be treated in a manner identical to the treated group, except that administration of the test substance is omitted.

(ii) *Reference substances*. If a positive control is used, a substance which is known to produce delayed neurotoxicity should be employed. Examples of such substances are triorthocresyl phosphate (TOCP) and leptophos.

(4) *Housing and feeding conditions*. Cages or enclosures which are large enough to permit free mobility of the hens and easy observation of gait should be used. Where the lighting is artificial, the sequence should be 12 hours light, 12 hours dark. Appropriate diets should be administered as well as an unlimited supply of drinking water.

(5) *Dose levels*. At least three dose levels should be used in addition to the control group(s). The highest dose level should result in toxic effects, preferably delayed neurotoxicity, but not produce an incidence of fatalities which would prevent a meaningful evaluation. The lowest dose level should not produce any evidence of toxicity.

(6) *Route of administration*. Oral dosing each day for at least 5 days per week should be carried out, preferably by gavage or administration of gelatine capsules.

(7) *Study conduct*—(i) *General*. Healthy young adult hens free from interfering viral diseases and medication and without abnormalities of gait should be acclimatized to the laboratory conditions for at least 5 days prior to randomization and assignment to treatment and control groups. The test or control substance should be administered and observations begun. All hens should be carefully observed at least once daily throughout the test period. Signs of toxicity should be recorded, including the time of onset, degree and duration. Observations should include, but not be limited to, behavioral abnormality, locomotor ataxia and paralysis. At least once a week the hens should be taken outside the cages and subjected to a period of forced motor activity, such as ladder climbing, in order to enhance the observation of minimal responses. The hens should be weighed weekly. Any moribund hens should be removed and sacrificed.

(ii) *Pathology*—(A) *Gross necropsy*. In the presence of clinical signs of delayed

neurotoxicity useful information may be provided by gross necropsy.

(B) *Histopathology*. Tissues from all animals should be fixed *in situ*, using perfusion techniques. Sections should include medulla oblongata, spinal cord and peripheral nerves. The spinal cord sections should be taken from the upper cervical bulb, the mid-thoracic and lumbosacral regions. Sections of the proximal region of the tibial nerve and its branches and of the sciatic nerve should be taken. Sections should be stained with appropriate myelin and axon-specific stains. Microscopic examination should be carried out on all hens in the control and high-dose groups. Microscopic examination should also be carried out on hens in the low and intermediate dose groups when there is evidence of effects in the high-dose group.

(e) *Data reporting and evaluation*—(1) *Test report*. In addition to the reporting requirements specified under 40 CFR part 792, subpart J the final test report must include the following information:

(i) Toxic response data by group with a description of clinical manifestations of nervous system damage; where a grading system is used the criteria should be defined.

(ii) For each animal, time of death during the study or whether it survived to termination.

(iii) The day of observation of each abnormal sign and its subsequent course.

(iv) Body weight data.

(v) Necropsy findings for each animal, when performed.

(vi) A detailed description of all histopathological findings.

(vii) Statistical treatment of results, where appropriate.

(2) *Treatment of results*. (i) Data may 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 or effects, the types of lesions or effects and the percentage of animals displaying each type of lesion or effect.

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

(3) *Evaluation of results*. The findings of a subchronic delayed neurotoxicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of the incidence and severity of observed neurotoxic effects and any other observed effects and histopathological findings in the treated and control groups. A properly conducted subchronic test should provide a satisfactory estimation of a no-effect level based on lack of clinical signs and histopathological changes.

(f) *References*. For additional background information on this test guideline the following references should be consulted:

(1) Abou-Donia, M.B. ''Organophosphorus ester-induced delayed neurotoxicity'' Annual Review of Pharmacology and Toxicology, 21:511–548 (1981).

(2) Abou-Donia, M.B., Pressing, S.H. ''Delayed neurotoxicity from continuous low-dose oral administration of leptophos to hens.'' *Toxicology and Applied Pharmacology*, 38:595–608 (1976).

(3) Baron, R.L. (ed). ''Pesticide Induced Delayed Neurotoxicity,'' Proceedings of a Conference, February 19–20, 1976, Washington, DC. U.S. Environmental Protection Agency. EPA Report No. 600/1–76–025, Washington, DC (1976).

(4) Cavanaugh, J.B. ''Peripheral neuropathy caused by chemical agents'' *Critical Reviews of Toxicity*, 2:365–417 CRC Press, Inc. (1973).

(5) Johannsen, F.R., Wright, P.L., Gordon, D.E., Levinskas, G.L., Radue, R.W., Graham, P.R. ''Evaluation of delayed neurotoxicity and dose-response relationship of phosphate esters in the adult hen,'' *Toxicology and Applied Pharmacology*, 41:291–304 (1977).

(6) Johnson, M.K. ''Organophosphorus esters causing delayed neurotoxic effects: mechanism of action and structure/activity studies,'' *Archives of Toxicology*, 34:259–288 (1975).